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Research Article

Comparison of Neutralizing Antibody Assays for Receptor Binding and Enzyme Activity of the Enzyme Replacement Therapeutic Naglazyme® (Galsulfase)

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Abstract. Most patients receiving Naglazyme® (galsulfase, rhASB) enzyme replacement therapy for mucopolysaccharidosis type VI develop an antibody response. To evaluate the impact of this response, two *in vitro* neutralizing antibody (NAb) assays were developed based on the two steps of the mechanism of action. Neutralization of enzyme activity was detected by inhibition of rhASB cleavage of a fluorogenic substrate. Neutralization of receptor binding was detected by decreased binding of labeled rhASB to immobilized soluble receptor. For the enzyme activity NAb assay, serum pretreatment was required to isolate antibodies from interfering phosphate ions, with sensitivity of ≤5 μg/mL. The receptor binding NAb assay used a five-fold dilution, with sensitivity of ≤40 μg/mL. Cutpoints for percent inhibition were based on 95% confidence intervals from naïve sera. Clinical samples were similarly likely to be positive in both assays than positive for neutralization of only one step in the mechanism of action. The two NAb assays yielded complementary information about potential neutralization of rhASB. Relative estimated sensitivity between neutralization assays did not correlate with the number of positive clinical samples or patients. *In vitro* NAb assays based on a well-understood mechanism of action provide specific information about the NAb mechanism.

KEY WORDS: enzyme replacement therapy; immunogenicity; naglazyme; neutralizing antibody; MPS VI.

INTRODUCTION

All biopharmaceuticals have a risk of generating an immune response. This immunogenicity has the potential to impact safety and efficacy of the biopharmaceutical. One of the most severe safety events observed due to immunogenicity was the pure red cell aplasia seen in a small number of patients taking erythropoietin (1). Decrease in efficacy has been observed in a wide variety of products including interferon beta (2), factor VIII (3), and alglucosidase alfa (4). For most biopharmaceuticals, the immune response does not impact safety or efficacy (5). Due to the potential impact, the FDA is requiring immunogenicity evaluation for all biopharmaceutical products. A crucial component of understanding the immunogenicity is to characterize the capacity of the antibody response to neutralize the biopharmaceutical activity and any endogenous counterparts, which could impact efficacy and safety, respectively.

The goal in development of neutralizing antibody (NAb) assays is to gather relevant information about the biopharmaceutical in the context of the patient receiving treatment. As a result, NAb assays are more diverse than screening antibody assays and selection of a final assay format is dependent on the individual biopharmaceutical's mechanism of action. Recent recommendations have been published for the development of screening assays (6) and cell-based neutralizing assays (7). Although cell-based assays are preferred for some complex mechanisms of action, they are neither the only option nor most informative for all mechanisms of action. This manuscript describes the development of two cell-free NAb assays for Naglazyme® (galsulfase). As part of a post-marketing commitment and to adhere to the latest industry standards, we established two cell-free assays to detect neutralization either of enzyme activity or of receptor binding.

Lysosomal storage diseases known as mucopolysaccharidoses (MPS) are caused by a deficiency in an enzyme or combination of enzymes. The MPS diseases are characterized

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ABBREVIATIONS: 4-MU, 4-methylumbelliferone; 4-MUS, 4-methylumbelliferyl sulfate; BSA, bovine serum albumin; CIMPR, calcium-independent mannose-6-phosphate receptor; DPBS, Dulbecco's phosphate buffered solution; FDA, Food and Drug Administration; MPS VI, mucopolysaccharidosis type VI, Maroteaux–Lamy syndrome; NAb, neutralizing antibody; rhASB, recombinant human Arylsulfatase B, recombinant human *N*-acetyl-galactosamine 4-sulfatase, Naglazyme; RT, room temperature; SA-HRP, streptavidin-horseradish peroxidase; sCIMPR, soluble domain of CIMPR.

by intra-lysosomal accumulation of undegraded glycosaminoglycans, excessive urinary excretion of glycosaminoglycans, progressive physical deterioration, and premature death (8,9). MPS VI (Maroteaux–Lamy syndrome) is a lysosomal storage disease in which the affected patients lack the enzyme *N*-acetyl-galactosamine 4-sulfatase, also known as arylsulfatase B (ASB). ASB catabolizes the sulfate moiety at the nonreducing terminal of the glycosaminoglycan (GAG) dermatan sulfate (8–10). In the absence of the enzyme, the stepwise degradation of dermatan sulfate is blocked and the substrate accumulates intracellularly in the lysosome in a wide range of tissues. Rapidly progressing patients may die from disease related complications such as respiratory infection or cardiac disease as early as the teenage years.

Recombinant human ASB (rhASB, marketed as Naglazyme) has been developed as an enzyme replacement therapy for the treatment of MPS VI (11–14). The mechanism of action is based on two sequential steps, the uptake and trafficking to the lysosome and enzymatic cleavage of the accumulated GAG substrates. To evaluate the presence of NAb, assays were designed to identify neutralization of both steps of the mechanism of action-receptor binding and enzyme activity.

Lysosomal enzymes, including rhASB, possess bis-mannose 6-phosphate oligomannose glycans that bind with high affinity and specificity to the calcium-independent mannose-6-phosphate receptor (CIMPR) found on the surface of most cells (8,9). CIMPR mediates uptake of rhASB and trafficking to the lysosome, and functions *in vivo* to perform reuptake of lysosomal enzymes that have been released from cells. Antibodies that disrupt the uptake of rhASB can decrease efficacy by preventing the biopharmaceutical from reaching the site of action. If a patient has some residual enzyme, these antibodies could also inhibit reuptake and trafficking of the endogenous enzyme, which is normally scavenged by the CIMPR receptor binding. Since binding to the soluble domain of CIMPR has been demonstrated to be both necessary and sufficient for uptake and trafficking to the lysosome (15), the measurement of antibodies that inhibit receptor binding can be used as a surrogate measurement of cellular uptake and trafficking to the lysosome.

Once in the lysosome, rhASB catalyzes hydrolysis of the nonreducing terminal dermatan 4-sulfate ester (11,16). Removal of this sulfate allows the continued breakdown of dermatan sulfate by the other lysosomal enzymes. Antibodies that inhibit the enzymatic activity of rhASB can decrease efficacy by preventing this substrate hydrolysis. If a patient has some residual enzyme activity, these antibodies could also inhibit activity of the endogenous enzyme by trafficking to the lysosome with enzyme from reuptake by sCIMPR, possibly leading to increased pathology. To test the inhibition of rhASB by antibodies, the enzyme activity is measured in the presence or absence of patient antibodies. A fluorogenic sulfatase substrate was used rather than the endogenous substrate dermatan sulfate. Use of a more promiscuous small molecule substrate is feasible in the NAb assay format since no other endogenous sulfatases will be present in the assay system.

Both NAb assays were adapted from analytical chemistry procedures used for either lot release or additional characterization of rhASB. The technical challenges to develop these assays illustrate some of the unique challenges for individual NAb assays. The clinical immunogenicity data illustrated that patient populations could be subdivided based on which steps of the mechanism of action were potentially neutralized. The assay development and clinical data provide further support that scientifically justified cell-free NAb assays that are based on a well-understood mechanism of action are appropriate components of a risk-based immunogenicity program.

MATERIALS AND METHODS

Materials

Naglazyme® (rhASB) was obtained from BioMarin Pharmaceutical Inc. (Novato, CA).

Individual and pooled naïve human sera were purchased from Binding Site (San Diego, CA) and BioReclamation (Hicksville, NY).

Polyclonal sheep anti-rhASB (G192) was obtained from Covance (Denver, PA) and several polyclonal rabbit anti-rhASB (BP14, BP15, J8549, J8550) were obtained from Covance and Antibodies Inc. (Davis, CA). All antibodies were purified using Protein G affinity columns obtained from GE Healthcare (Piscataway, NJ) followed by affinity chromatography with an rhASB column made using a HiTrap NHS-activated HP column from GE Healthcare. Polyclonal rabbit anti-laronidase (BP13) was obtained from Covance, and was purified using Protein G affinity column.

EZ-Link Sulfo-NHS-LC-Biotin was purchased from Pierce (Rockford, IL). Antibody concentrations were measured using a BCA kit and biotin quantitation was performed using an EZ Biotin Quantitation kit, both from Pierce.

Immunosorp high-binding plates for the receptor binding NAb assay were acquired from Nunc (Rochester, NY). The purified soluble extracellular domain of bovine CIMPR (sCIMPR) was obtained from Dr. Peter Lobel (Piscataway, NJ) (17). Streptavidin conjugated to horseradish peroxidase (SA-HRP) was purchased from Pierce (Rockford, IL). 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was acquired from BioRad (Hercules, CA).

4-MUS fluorogenic substrate for the enzyme activity NAb assay was acquired from Sigma (St. Louis, MO). UltraLink Protein A/G resin for antibody isolation was acquired from Pierce. MultiscreenHTS HV filter plates and vacuum manifold were obtained from Millipore (Billerica, MA).

Biotin Labeling of rhASB

rhASB was buffer exchanged into 10 mM sodium phosphate, 150 mM NaCl, pH 7.8, and concentrated to 2 mg/mL. Biotin (2 mg/mL in water) was added at 2.5-fold molar excess challenge ratio and incubated with gentle rocking at RT for 1 h prior to quenching with 20–25% (*v*/*v*) 2 M glycine. The reaction was buffer exchanged into product formulation buffer, and adjusted to 1 mg/mL as measured by the BCA method. Biotin concentration was measured using the EZ Biotin Quantitation kit. Label ratios were calculated as [label]/[rhASB] and labeling efficiencies were calculated as label ratio/challenge ratio.

Receptor Binding NAb Assay

Immunosorp plates were coated with 4 μg/mL sCIMPR in 100 mM sodium carbonate, 70 mM sodium bicarbonate (pH 9.5) overnight at 4°C. Plates were washed three times with 0.1% Tween-20, 0.05% ProClin 300, DPBS (wash buffer) and blocked for 1 h at RT with 2% BSA, 0.05% Tween-20, DPBS (blocking buffer). Serum was diluted ten-fold in 2 nM biotin–rhASB in blocking buffer and incubated for 1 h at RT on an orbital shaker to allow formation of antibody and rhASB complexes. After shaking out the blocking solution from the sCIMPR coated plate, 100 μL/well of the serum dilutions in biotin–rhASB was added and incubated for 1 hr at RT with shaking. Plates were washed three times with wash buffer and a solution of 100 μL/well 25 ng/mL SA-HRP in blocking buffer was added and incubated for 30 min at RT with shaking. Plates were washed three times with wash buffer prior to addition of 100 μL/well TMB substrate. After developing color for 10–15 min at RT, reactions were stopped with 100 μL/well 2 N H_2SO_4 and absorbance read at 450 nm.

Enzyme Activity NAb Assay

Antibodies were isolated from serum using a pretreatment step. One hundred microliters of Protein A/G resin slurry was added to the Multiscreen plate and washed 2× 200 μL DPBS. The resin was resuspended in 100 μL of a two-fold dilution of serum in DPBS (50 μL of neat serum). The slurry was incubated for 1 h at RT with shaking. The resin was washed 2× 200 μL DPBS and 3× 200 μL ultrapure water. Antibodies were eluted using a 10 min incubation at RT with shaking in 200 μL 0.1 M glycine (pH 2.7). Eluate was partially neutralized to the final assay pH of 5.6 by the addition of 69 μL 1 M sodium acetate. Forty microliters of partially neutralized eluate was preincubated with 10 μL of 62.5 ng/mL rhASB, 50 mM sodium acetate, 0.05% Tween-20 (pH 5.6) at 37°C for 1 h to permit binding of antibodies to rhASB. One hundred microliters/well of pre-warmed 5 mM 4-MUS, 0.05 mg/mL BSA, 50 mM sodium acetate (pH 5.6) was added and incubated at 37°C for 20 min. The reaction was stopped by addition of 150 μL/well of 0.35 M glycine, 0.44 M carbonate (pH 9.76). Fluorescence of 4-MU was read with excitation at 366 nm and emission at 446 nm, with cutoff at 435 nm.

Sample Preparation

Samples used for development and validation of the NAb assays were composed of affinity-purified anti-rhASB antibodies spiked into naïve serum or buffer and subsequently treated in the same manner as clinical samples. Samples prepared in naïve serum contained non-specific immunoglobulin from the donor.

Sample Collection

Three clinical studies (ASB-01-04, ASB-03-05, ASB-03-06) were selected for reanalysis of serum samples (12,14). In these studies, all patients received the final therapeutic dose of weekly 4 h intravenous infusions of 1 mg/kg. Serum samples were collected at intervals of up to 12 weeks apart throughout the studies. The samples were frozen and shipped on dry ice, and subsequently stored at −70°C to −85°C. Clinical research followed the principles of the Declaration of Helsinki in 1984 from the World Medical Association. Protocols were approved by an institutional review board at each participating clinical site. Written consent was obtained from all parents or guardians before enrollment, and written assent was obtained from all patients.

RESULTS

The development and validation of both neutralizing antibody assays is presented along with clinical results to illustrate patterns of NAb immunogenicity. The receptor binding neutralizing antibody assay is presented first, the enzyme activity neutralizing antibody assay second, and the clinical results last.

Receptor Binding Neutralizing Antibody Assay

Optimization of Assay Conditions

The assay was adapted from an existing analytical method originally designed to characterize rhASB from process changes. In order to adapt this assay to measure the presence of NAb, several design aspects were altered or optimized.

The first step in the mechanism of action of rhASB is uptake of the enzyme into the cells. Since CIMPR binding is necessary and sufficient for uptake of rhASB (15), an *in vitro* receptor binding assay was implemented (Fig. 1). A major challenge in developing the assay was determining a combination of rhASB and sCIMPR that allowed measurement of small changes in binding and was similar to the cellular uptake curves in a cell line. From early experiments for rhASB characterization, a coating concentration of 4 μg/mL sCIMPR was selected, as it was the lowest coat concentration that yielded reproducible signals. Later work demonstrated that the K_d calculated from Hanes–Woolf plots (18) was of similar magnitude K_{uptake} from uptake curves in fibroblasts, confirming published work that CIMPR binding is sufficient for uptake (15) (Fig. 2). The 2 nM rhASB concentration was

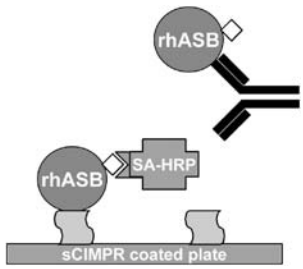

Fig. 1. Schematic of receptor binding neutralizing antibody assay. A plate-based binding assay is used. The serum sample is preincubated with biotin–rhASB prior to addition to a plate coated with sCIMPR. SA-HRP allows colorimetric detection of biotin–rhASB and sCIMPR complexes. Percent inhibition is evaluated relative to receptor binding with a pool of naïve serum

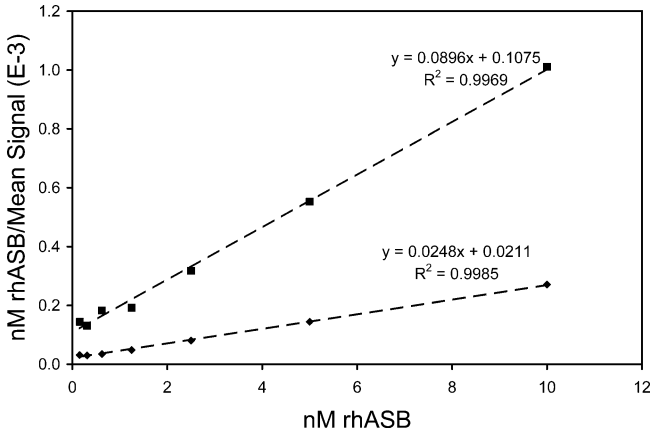

Fig. 2. Comparison of receptor binding assay with fibroblast uptake. The Hanes–Woolf plots (18) for cellular uptake (*squares*) and receptor binding (*diamonds*). K_{uptake}=1.20 nM for cellular uptake (0.1075/0.0896) and K_d=0.85 nM for receptor binding (0.0211/0.0248)

selected as twice the K_d, which was the highest signal where the binding response *versus* rhASB concentration remained relatively steep. Therefore a receptor binding NAb assay at this concentration of rhASB was anticipated to be more sensitive to changes in signal than would an assay using a higher rhASB concentration. To minimize the impact of biotin masking NAb epitopes, a low label ratio was used for the biotin–rhASB reagent, previously optimized to yield an average ratio of 1.5 biotin per rhASB (19).

Matrix Interference

In order to characterize the effect of serum on the concentration response relationship, 43–125 μg/mL affinity-purified G192 was diluted in 0–50% human serum. For each G192 concentration in 20% serum, the accuracy ranged from 90–108% relative to equivalent G192 concentration in 10% serum dilution used for testing clinical samples (Table I), indicated that up to twice the serum concentration could be incorporated with relatively moderate signal changes. The robustness of the initial ten-fold serum dilution was confirmed from small, discrete changes in preparation (data not shown). Since the 0% serum was significantly different from 10% serum, this assay is not suitable for a titration based assay unless an equal percentage of naïve serum is used in the diluent.

Table I. Receptor Binding NAb Assay Interference from Serum Matrix

Serum concentration (%)	Percent reduction with anti-rhASB (μg/mL)[a]			
	0[b]	43[b]	72[b]	125[b]
0	−8.5	−15	37	66
10	0	21	58	75
20	9.8	19	63	81
50	23.9	47	75	88

[a] Percent reduction calculated relative to 10% serum, 0 μg/mL G192 anti-rhASB
[b] Concentration of G192 anti-rhASB (μg/mL)

Assay Cut Point

The 95% confidence interval was calculated from the percent reduction of 50 naïve human sera. The small global population of patients precluded using naïve patients to establish the cut point. In the receptor binding NAb assay, a 95% confidence interval generated a reduction in rhASB binding of 8.5% (Fig. 3). This rate is predicted to generate a 5% false positive rate, which was selected as an appropriate false positive rate for this assay. Sera samples with a reduction of at least 8.5% generate a positive result.

Specificity

The affinity-purified sheep-anti-rhASB antibody G192 and rabbit-anti-rhASB BP15 were able to inhibit rhASB binding to CIMPR in a concentration-dependent manner while affinity-purified anti-laronidase (BP13) did not inhibit receptor binding at any concentration (Fig. 4). The lack of inhibition by an alternate antibody demonstrated that the assay is specific for antibodies that bind rhASB. G192 was subsequently used as the positive control due to abundant supply for subsequent clinical testing.

Sensitivity

To estimate the limit of detection, affinity-purified G192 was spiked into naïve human serum at concentrations from 35–50 μg/mL. The lowest concentration that was able to reduce binding with acceptable precision was 40 μg/mL, with four of six replicates above 8.5% reduction (Fig. 5). Due to the polyclonal nature of the antibodies, the limit of detection is represented as less than or equal to 40 μg/mL. Since only a subset of the antibodies in the polyclonal population are anticipated to inhibit receptor binding, the sensitivity using polyclonal antibodies is an upper limit of the sensitivity

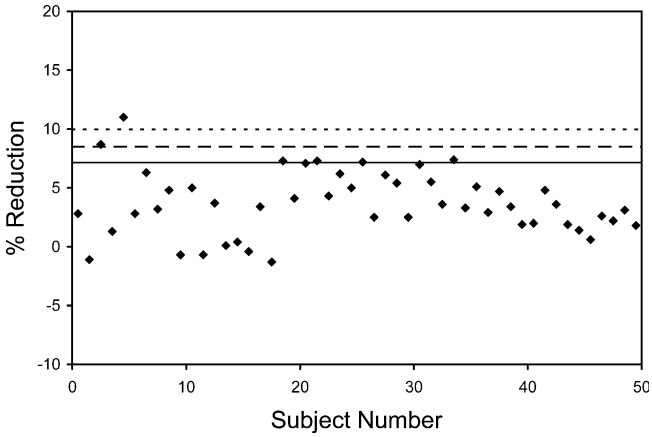

Fig. 3. Receptor binding Nab assay cutpoint determination. Fifty naïve sera were assayed to determine confidence intervals, shown for 90% (*solid line*), 95% (*dashed line*), and 99% (*dotted line*)

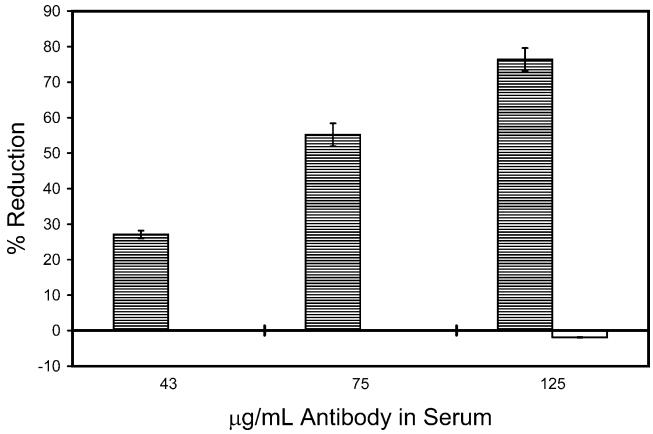

Fig. 4. Receptor binding NAb assay specificity. Inhibition of receptor binding was evaluated with increasing concentration of anti-rhASB antibody (G192—*horizontal stripes*) and anti-laronidase antibody (BP13—*white*) at 43–125 μg/mL in naïve serum

estimate. Since the variability of spiked samples was large close to the limit of detection, 9–12% reduction of receptor binding was considered borderline positive.

Of note, it was found that concentrations less than 30 μg/mL G192 led to increased binding of biotin–rhASB to sCIMPR, resulting in a negative percent reduction. This indicates that some antibodies can enhance receptor binding. This may be due to antibodies bridging two biotin–rhASB molecules so that binding of one biotin–rhASB to the plate could result in a signal from two biotin–rhASB molecules.

Tolerance of Free Drug

For the receptor binding NAb assay, affinity-purified G192 (43–125 μg/mL) was spiked in neat serum containing 0–1,000 ng/mL rhASB. With free rhASB, the receptor binding increased, sometimes resulting in a negative percent reduction (Table II). This may be due to the free rhASB binding a fraction of the neutralizing antibodies, allowing more biotin–rhASB to bind the receptor. The 43 μg/mL G192 sample was below the limit of detection even with 10 ng/mL rhASB. For higher concentrations of G192, the accuracy of measuring inhibition of receptor binding in the presence of 100 ng/mL rhASB was below the 75% accuracy relative to 0 ng/mL rhASB. This assay is not suitable for measuring receptor binding NAb from serum samples collected between initiation of infusion and 1 day after the end of infusion.

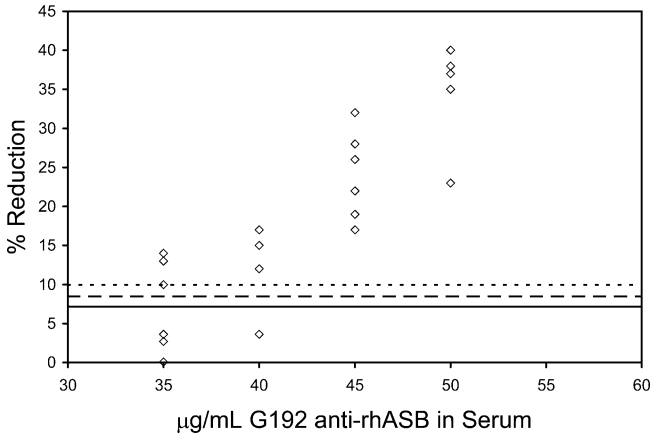

Fig. 5. Receptor binding NAb assay limit of detection. Confidence intervals are shown for 90% (*solid line*), 95% (*dashed line*), and 99% (*dotted line*). Six replicates were evaluated for 35–50 μg/mL in pooled naïve human serum

Table II. Receptor Binding NAb Assay Interference from Free Drug

Free drug (ng/mL)	Percent reduction with anti-rhASB (μg/mL)[a]			
	0[b]	43[b]	72[b]	125[b]
0	0	26	53	78
10	−3.6	−5.5	45	74
100	−3.1	−25	37	57
1,000	21.8	−93	−17	36

[a] Percent reduction calculated relative to 0 ng/mL rhASB, 0 μg/mL G192 anti-rhASB
[b] Concentration of G192 anti-rhASB (μg/mL)

The presence of free rhASB in the absence of NAb had been anticipated to generate a reduction of receptor binding of biotin–rhASB. In the absence of NAb, an increase in receptor binding inhibition for biotin–rhASB relative to 0 ng/mL rhASB was only observed for 1,000 ng/mL rhASB. This may indicate that the receptors on the plate are not saturated so the addition of unlabeled rhASB can be tolerated at least to 100 ng/mL without a decrease in biotin–rhASB binding.

Precision

Intra-assay precision was evaluated using the same sample across the entire plate. Two-way ANOVA showed that there were no row or column biases in the results ($p<0.05$).

Inter-assay precision was evaluated using three standards at 43–125 μg/mL affinity-purified G192 in serum tested across at least three independent assay dates by two analysts. The inter-assay CV% ranged from 4–20%, with greater variability seen at lower concentrations of antibody.

Enzyme Activity Neutralizing Antibody Assay

Optimization of Assay Conditions

The assay was adapted from an existing analytical method originally designed as a lot release assay for rhASB. In order to adapt this assay to measure the presence of NAb, several design aspects were altered or optimized.

The second step in the mechanism of action is rhASB cleavage of dermatan sulfate substrate. A microplate assay with fluorogenic 4-MUS substrate was implemented (Fig. 6). The fluorogenic substrate was used as a surrogate substrate since the fluorescent product yielded good sensitivity in buffer systems. The 62.5 ng/mL rhASB concentration was selected as the lowest concentration qualified for the lot release assay. The linearity of enzyme activity with further dilution of rhASB was maintained from 12.5–62.5 ng/mL

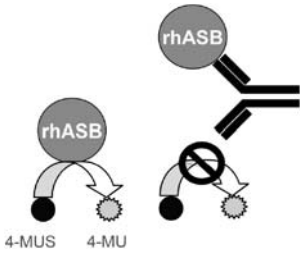

Fig. 6. Schematic of enzyme activity neutralizing antibody assay. The total antibody fraction is isolated from the Protein A/G step and preincubated with rhASB prior to addition of a fluorogenic substrate 4-MUS. Enzyme activity is measured by release of 4-MU from the cleavage of 4-MUS. Percent inhibition is evaluated relative to enzyme activity with antibodies isolated from a pool of naïve serum

(Fig. 7). Since rhASB is a sulfatase, it is sensitive to concentrations of phosphate and sulfate ions; so a major focus of development was identification of a robust method to remove interfering sulfate and phosphate ions. After comparing capacity and reproducibility of various resin and bead systems, the UltraLink Protein A/G resin was selected for implementation.

Matrix Interference

Since a pretreatment step was required for the enzyme activity NAb assay, the recovery from the pretreatment was investigated as a surrogate for matrix interference. Across nine individual sera, antibody inhibition of rhASB was within 30% of 100% recovery (Fig. 8), indicating that serum interference was effectively removed. The seven samples from one assay date clustered around 130% while the two samples from a second assay date clustered around 100%, indicating a potential bias in construction of the positive control. One individual exhibited poor recovery, indicating that this assay has the potential to generate false negative results in clinical testing.

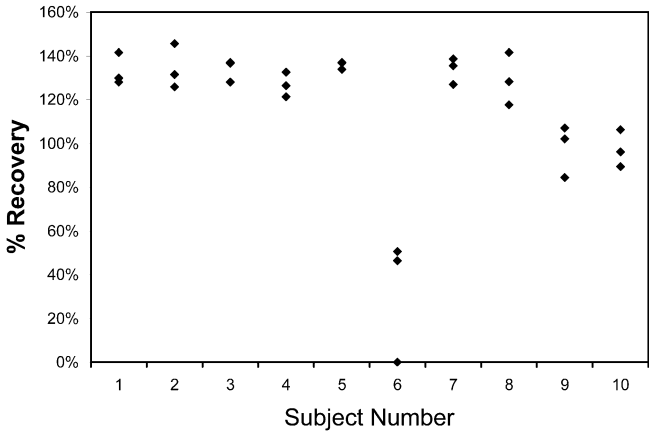

Fig. 8. Enzyme activity NAb assay recovery from individual sera. Ten individual sera were spiked with 25 μg/mL BP14. Three replicates from each sample were tested and compared to a control sample prepared in buffer to evaluate the percent recovery

Establishing Assay Cut Point

The 95% confidence interval was calculated from the percent reduction of 50 naïve human sera. The small global population of patients precluded using naïve patients to establish the cut point. The distribution of naïve samples in the enzyme activity NAb assay was more variable than in the receptor binding NAb assay (Fig. 3) and also showed a definite bias towards increased rhASB activity (Fig. 9). This wide distribution raised the concern that a 5% false positive rate would yield an unacceptably high false negative rate. To set a more appropriate cut point for the assay, a 90% confidence interval relative to the average signal from the naïve samples was used as the threshold for positive results, which corresponds to a reduction in rhASB activity of 10%. Although the false negative rate may still be high, given the variable data, further reducing the confidence interval to 80% would significantly increase the false positive rate. Since the assay does not have a confirmatory component, a 90%

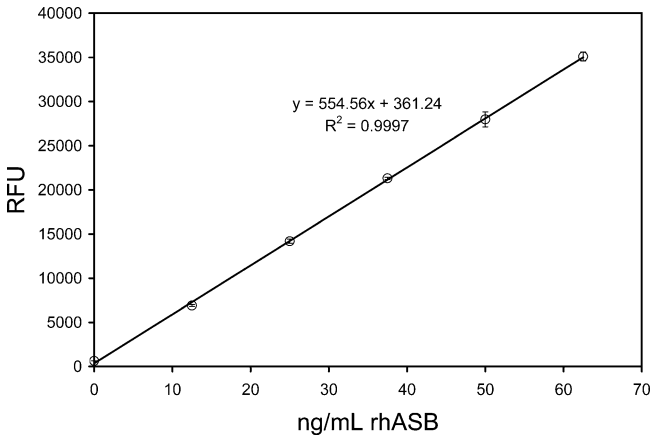

Fig. 7. Enzyme activity NAb assay concentration response curve. The RFU signal was linear for a dilution series of 0–62.5 ng/mL of rhASB in the assay system without antibodies present

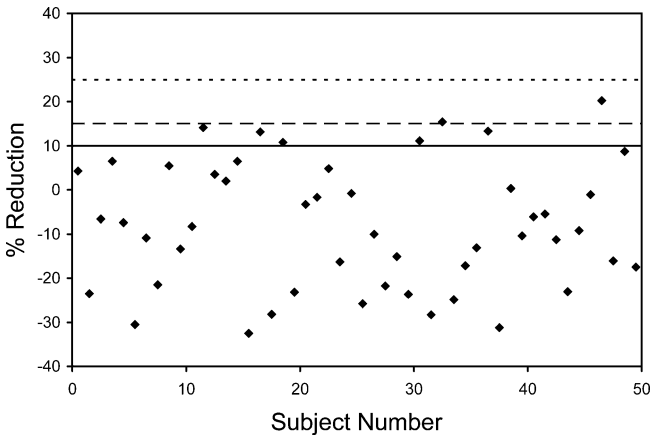

Fig. 9. Enzyme activity NAb assay cutpoint determination. Fifty naïve sera were assayed to determine confidence intervals, shown for 90% (*solid line*), 95% (*dashed line*), and 99% (*dotted line*)

confidence interval was selected as a compromise between sensitivity and specificity.

Specificity

Three of four affinity-purified anti-rhASB antibodies (BP14, J3549, J3550) were able to inhibit rhASB activity while one affinity purified anti-rhASB (G192) and one affinity purified anti-laronidase (BP13) were not able to inhibit rhASB activity at any concentration (Fig. 10). The lack of inhibition by BP13 confirms that the assay is specific for anti-rhASB antibodies. The lack of inhibition by G192 confirms that the assay is specific for antibodies that inhibit enzyme activity rather than detecting all antibodies that bind rhASB. BP14 was subsequently used as the positive control since sufficient supply was available.

Sensitivity

To establish the limit of detection, affinity-purified BP14 was spiked into naïve human serum at concentrations from 2.5–20 μg/mL in pooled naïve human serum and compared to the 90% and 95% confidence intervals. At the 90% confidence interval set as the cutpoint, BP14 at 5 μg/mL or greater was able to inhibit the activity of rhASB (Fig. 11). Since only a subset of the antibodies in the polyclonal population is anticipated to inhibit enzyme activity, the sensitivity using polyclonal antibodies is an upper limit of the sensitivity estimate.

Tolerance of Free Drug

For the enzyme activity NAb assay, affinity-purified BP14 (10–150 μg/mL) was spiked in neat serum containing 0–1,000 ng/mL rhASB. If free drug in the serum samples is retained through the antibody purification step, then this could result in higher signal in the activity assay and mask any reduction due to antibodies. When rhASB concentration was greater than 10 ng/mL, the accuracy of measuring inhibition of enzyme activity was ≤76% of the inhibition relative to no drug added (Table III). At 1,000 ng/mL rhASB in serum, the enzyme activity from retained free drug was far greater than the signal from the rhASB added to the assay. This assay is not suitable for measuring enzyme activity NAb from serum samples collected between initiation of infusion and 1 day after the end of infusion.

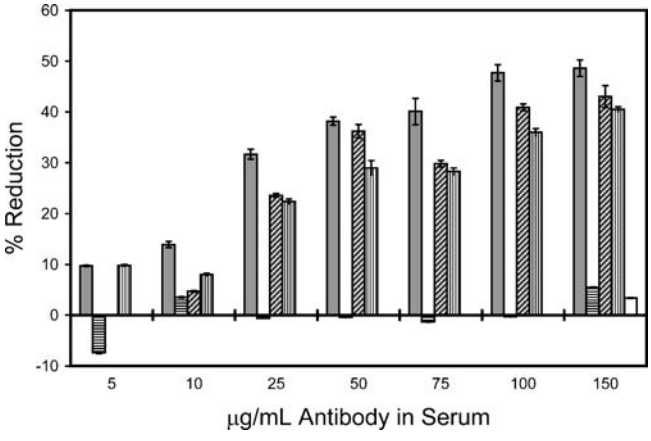

Fig. 10. Enzyme activity NAb assay specificity. Inhibition of enzyme activity was evaluated with increasing concentration of four anti-rhASB antibodies (BP14—*gray*, G192—*horizontal stripes*, J3549—*diagonal stripes*, J3550—*vertical stripes*) and one anti-laronidase antibody (BP13—*white*) at 0–150 μg/mL in naïve serum

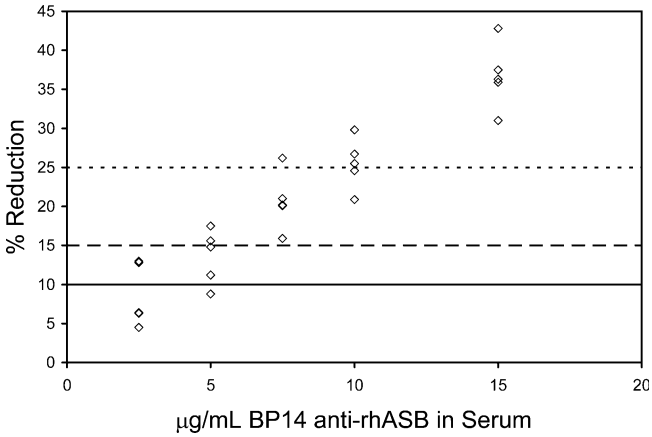

Fig. 11. Enzyme activity NAb assay limit of detection. Confidence intervals are shown for 90% (*solid line*), 95% (*dashed line*), and 99% (*dotted line*). Five replicates were evaluated for 2.5–20 μg/mL in pooled naïve human serum

Precision

Intra-assay precision was evaluated using the same sample across the entire plate. Two-way ANOVA showed that there were no row or column biases in the results ($p<0.05$).

Inter-assay precision was evaluated using four standards at 10–75 μg/mL affinity-purified BP14 in serum tested across at least four independent assay dates by two analysts. The inter-assay CV% ranged from 10–27%, with greater variability seen at lower concentrations of antibody. The higher variability in this assay is likely due to the inclusion of the pretreatment step. Since pretreatment was absolutely required, relative variability was included as one of the criteria used to select the exact pretreatment protocol.

Clinical Sample Analysis

Clinical samples that were positive from the total antibody screening assay (19) were tested in both NAb

Table III. Enzyme Activity NAb Assay Interference from Free Drug

Free drug (ng/mL)	Percent reduction with anti-rhASB (μg/mL)[a]					
	10[b]	25[b]	50[b]	75[b]	100[b]	150[b]
0	34	48	47	50	63	57
10	28	42	51	53	58	52
100	16	29	34	38	41	43
1,000	−234	−214	−53	−60	−86	−68

[a] Percent reduction calculated relative to 0 ng/mL rhASB, 0 μg/mL BP14 anti-rhASB
[b] Concentration of BP14 anti-rhASB (μg/mL)

assays. Rather than reporting positive and negative, results were characterized by level of NAb: undetected (0), borderline (1), moderate (2), or elevated (3). For the receptor binding NAb assay, the borderline range corresponded to the percent reduction that was above cutpoint but was variable in determination of the limit of detection (9–12%), the moderate range corresponded to 12–49% reduction, and the elevated range corresponded to any signal above 50% reduction. For the enzyme activity NAb assay, the borderline range corresponded to percent reduction between the 90% and 95% confidence intervals (15–25%), the moderate range corresponded to 26–40% reduction, and the elevated range corresponded to values above the plateau observed in the specificity experiments (>40%).

Over three clinical studies, 427 samples (across 48 individual patients) were positive for specific anti-rhASB antibodies and were tested in both NAb assays. Most samples (64%, 274/427) were negative in both NAb assays (Table IV). Of samples that exhibited neutralization for one step in the mechanism of action, half exhibited neutralization in both (20%, 85/427) and half neutralized in one step while leaving the other unaffected (16%, 68/427). Slightly more samples tested positive in the receptor binding NAb assay (31%, 133/427) than in the enzyme activity NAb assay (25%, 105/427). Including all results, the correlation coefficient was 0.77. Excluding the results that were negative in both assays, the correlation coefficient was 0.45.

Of the 48 patients in the clinical trials, 43 had a persistent total antibody response in the screening assay (19), so were categorized based on the presence and persistence of the two separate NAb readouts. Patients with at least one positive NAb result were considered transient or persistent depending on how many samples were positive and whether the patient had negative NAb results after the positive NAb result (Table V). Of these 43 patients, 23% (10/43) had no detectable neutralizing antibodies for either step in the mechanism of action and 23% (10/43) had persistent neutralizing antibodies for both steps in the mechanism of action. For receptor binding, the first step in the mechanism of action, 23% of patients (10/43) had a transient neutralizing response and 37% (16/43) had a persistent neutralizing response. For enzyme activity, the first step in the mechanism of action, 26% of patients (11/43) had a transient neutralizing response and 28% (12/43) had a persistent neutralizing response. The positive timepoints in the transient response in one NAb assay did not generally coincide with the timepoints in the transient or persistent detection in the second NAb assay.

Table IV. Number of Individual Samples with Neutralizing Antibodies

Level in receptor binding NAb	Level in enzyme activity Nab			
	0	1	2	3
0	274[a]	14[a]	5[a]	1[a]
1	11[a]	2[a]	5[a]	0[a]
2	33[a]	4[a]	0[a]	0[a]
3	4[a]	8[a]	33[a]	33[a]

[a] Number of individual samples (out of 427) with each particular combination of level in receptor binding NAb assay and enzyme activity NAb assay

Table V. Patterns from Patients with Neutralizing Antibodies

Pattern in receptor binding NAb	Pattern in enzyme activity NAb		
	None detected	Transient	Persistent
None detected	10[a]	6[a]	1[a]
Transient	6[a]	3[a]	1[a]
Persistent	4[a]	2[a]	10[a]

[a] Number of patients (out of 43 with persistent total antibody response) with each particular combination of detection patterns in receptor binding NAb assay and enzyme activity NAb assay

DISCUSSION

Assay Cut Points

Both NAb assays satisfied the critical design criteria. We established distributions of naïve sera, which were used to establish the cut points based on confidence intervals. Traditionally, a 95% confidence is used, which is anticipated to yield a 5% false positive rate but does not directly predict a false negative rate. The receptor binding NAb assay had a relatively tight distribution, so the 95% confidence interval was used as the cut point. The enzyme activity NAb activity had a more scattered distribution of naïve sera, leading to the concern that a 95% confidence interval would lead to an unacceptably high false negative rate. So for the enzyme activity NAb assay, the 90% confidence interval was used as the cut point.

Absence of Confirmatory Assay

Neither assay uses a confirmatory assay to verify that the inhibition of receptor binding or enzyme activity is a result of antibodies specific to rhASB. Possible confirmatory methods were contemplated but none were appropriate for implementation.

Lack of a confirmatory assay means that false positive samples based on cutpoints from the 95% and 90% confidence intervals were reported as positive. To mitigate this challenge, clinical samples were reported in levels corresponding to increasing confidence that the values are truly positive. In addition, trends in NAb responses within patients were evaluated with single or sporadic positive results given less weight than a persistent antibody response.

Sensitivity Evaluation

The nominal sensitivity was ≤5 μg/mL in the enzyme activity NAb assay and ≤40 μg/mL in the receptor binding NAb assay. Since an unknown fraction of the polyclonal antibody positive controls are neutralizing, the sensitivity can only be characterized as less than or equal to the concentration of the lowest detected positive control. If a neutralizing monoclonal antibody had been identified, the sensitivity would have been listed without the ≤ qualifier.

The sensitivity of the total antibody binding assay was 75 ng/mL (19), meaning that the upper limit of the difference in sensitivity is 67-fold for the enzyme activity NAb assay and

533-fold for the receptor binding NAb assay. Depending on the exact percentage of the polyclonal population that has neutralizing capacity, some positive samples in the total antibody assay that have neutralizing antibodies may be missed. During assay development, the impact of this limitation cannot be known, only minimized. If available, neutralizing monoclonal antibodies would allow more precise estimates of this limitation.

Although the nominal sensitivity was better for the enzyme activity NAb assay, more samples were positive in the receptor binding NAb assay. This illustrates the difficulty in measuring assay sensitivity for NAb assays. Although different polyclonal antibodies are used as positive controls in these two NAb assays, the same difficulty arises when using the same polyclonal antibody for both assays. This is clearly illustrated in examining the specificity of the enzyme activity NAb assay; no inhibition is observed for the sheep polyclonal antibody, which is the best polyclonal antibody for inhibiting receptor binding.

The ability of bound antibody to sterically hinder the interaction of rhASB with the large sCIMPR was anticipated to be greater than the ability of bound antibody to sterically hinder the interaction of rhASB with a small fluorescent substrate. For the sheep polyclonal antibody and the clinical samples, the observations agreed with this expectation. For the rabbit polyclonal antibodies, however, the antibodies tended to inhibit enzyme activity at lower concentrations than they inhibited receptor binding.

The receptor binding NAb assay had increased receptor binding of biotin–rhASB at low concentrations of antibody and in the absence of serum. The former may be due to antibodies that enhance receptor binding or bridge between two biotin–rhASB molecules. The latter may be due to nonspecific interactions of biotin–rhASB with the sCIMPR plate that were blocked by the presence of 10% serum.

The measurement of assay sensitivity is a useful tool when applied during method optimization and when comparing results from NAb assay formats based on the same step of the mechanism of action. Nominal sensitivity should not be used, however, as a primary criterion to choose between NAb assays based on different steps in the mechanism of action as this could lead to selecting a less relevant NAb assay to meet an arbitrary sensitivity goal.

Interference from Free Drug

Prior pharmacokinetic analysis showed that in patients on 24 weeks of infusion therapy, the concentration of rhASB in the plasma ranged from less than 100 ng/mL in more than 50% of patients to not greater than 663 ng/mL in any patient at the 5 hour time point (13). rhASB concentrations above 100 ng/mL are unlikely 7 days post infusion when antibody titers are measured. To characterize interference from free drug in the NAb assays, the decrease in sensitivity was measured in serum containing up to 1 μg/mL rhASB in the event that a patient has an antibody response that dramatically extends the PK curve or samples are acquired during or immediately after an infusion due to an adverse event.

The presence of free drug was only somewhat tolerated in both NAb assays. Based on the data from free drug interference, rhASB concentrations above 100 ng/mL would cause false negatives for clinical samples with NAb well above the limit of detection. Therefore, neither assay is suitable for testing samples with the possibility of elevated rhASB concentration. To ensure that false negatives are not reported, samples for testing should be acquired at least 1 day after the IV infusion, and were typically acquired 7 days after IV infusion immediately prior to the next dose.

Clinical Results

For individual sample analysis, more samples were positive for neutralizing antibodies that inhibit receptor binding than inhibit enzyme activity. In addition, more patients had persistent NAb responses that interfere with receptor binding than enzyme activity. Samples and patients that tested positive for neutralizing antibodies for one step in the mechanism of action were not necessarily positive for neutralizing antibodies for the other step. This is not in any way surprising because the two steps of the mechanism are biochemically distinct.

Because neither assay has a confirmatory assay, the patients with a transient response were identified, but categorized separate from the patients with a persistent response. The purpose of this exercise was to place proper emphasis on patients with more certain presence of neutralizing response rather than single positive results that may be false positives.

For the 43 patients, we were able to identify several distinct populations (Table V). To evaluate the potential effectiveness *versus* a cell-based assay, we performed a virtual experiment and recharacterized the patients to compress the neutralizing antibody assays into a single readout (Fig. 12). First, we assumed that all samples and patients that were identified in the two cell-free assays would have been

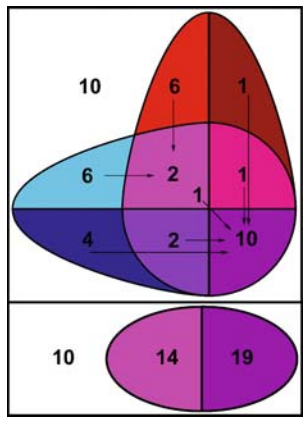

Fig. 12. Compression of neutralizing antibody assays into single readout. The upper Venn diagram illustrates the patient patterns for the two cell-free neutralizing antibody assays (from Table V). The *red ellipse* includes patients with enzyme activity neutralizing response (*bright red*: transient, *dark red*: persistent). The *blue ellipse* includes patients with receptor binding neutralizing response (*bright blue*: transient, *dark blue*: persistent). The intersections between the populations are shown in various *shades of purple*. *Arrows* designate how the data set would be compressed in a single cell-based readout as shown in the lower Venn diagram (*purple*: transient; *dark purple*: persistent)

detected in the hypothetical cell-based assay, such that a persistent response in either or both cell-free assays would yield a persistent response in a cell-based assay. Second, we identified one patient for whom the combination of nonoverlapping transient responses in both assays would have been interpreted as a persistent response in a single assay. This compressed the data into three patient populations: ten negative, 14 transient, and 19 persistent.

The potential impact was evaluated by considering the reasonable scenario that only one neutralization step impacts safety or efficacy. If neutralization of the receptor binding step is most critical, only 84% patients (16/19) that would be reported as persistent NAb positives actually have a persistent receptor binding NAb response. If neutralization of the enzyme activity step is most critical, only 63% of patients (12/19) that would be reported as persistent NAb positives actually have a persistent enzyme activity NAb response. In order to detect differences in safety or efficacy profiles for neutralization of either step in the mechanism of action, the differences would need to overcome the noise from samples that neutralized the other step.

These two assays provide complementary information about the patients' immunogenic state after treatment with Naglazyme. The range of responses allows analysis of different subpopulations of patients, such as no neutralizing antibodies detected, neutralizing antibodies against both steps in the mechanism of action, neutralizing antibodies against receptor binding, and neutralizing antibodies against enzyme activity. This discrimination between different steps of the mechanism of action can help identify which, if any, step of the mechanism of action is linked to changes in safety and efficacy.

CONCLUSIONS

Selection of an appropriate NAb assay is a critical component of an immunogenicity evaluation. Two *in vitro* NAb assays based on the two steps of the mechanism of action were developed to provide complementary data about neutralization of individual steps of the mechanism of action that may impact safety or efficacy *in vivo*. Neutralization of receptor binding would impact cellular uptake of rhASB, and may be more likely *in vivo* due to the interaction of rhASB and antibody in the extracellular space and blood. Neutralization of enzyme activity would decrease efficacy of rhASB by preventing it from cleaving substrate in the lysosome, but may be less likely as the low pH and proteolysis that are characteristic of this subcellular compartment could decrease or eliminate the interaction of rhASB and antibody, if taken up by the cell.

In a clinical immunogenicity program, the primary goal is to provide a reliable data set that provides the best possible opportunity to evaluate the impact of antibodies on safety and efficacy. For many biopharmaceuticals, the most appropriate assay format is a cell-based neutralizing antibody assay (7,20). In complex mechanisms of action, a cell-based assay may be the only option that guarantees that neutralization of any and all mechanistic steps is captured in the readout. For well-understood mechanisms of action, however, the use of a combined readout from one cell-based assay can reduce the discrimination of different patient populations relative to cell-free neutralizing antibody assays.

Two separate *in vitro* assays for neutralization of each step in the mechanism of action provide the opportunity to evaluate the impact of immunogenicity in a more comprehensive manner by reporting the results individually rather than in one combined readout in a cell-based assay. By separating the NAb evaluation into two assays, patients can be evaluated independently for the presence of antibodies capable of neutralizing each step of the mechanism of action. If neutralization of only one step in the mechanism of action impacts the safety or efficacy, that observation could be missed if a combined cell-based assay were implemented since samples that neutralized the step that impacted safety or efficacy could be confounded with some samples that only neutralize the other step and cause the statistical significance to be missed. The information from testing of clinical sera samples in both NAb assays provides clear evidence that, as expected, not all samples will have antibodies that neutralize both steps in the mechanism of action.

For well-understood mechanisms of action, cell-free neutralizing antibodies provide a distinct advantage over a cell-based neutralization assay that only provides two possible readouts, positive and negative, with no further discrimination of the mechanism of neutralization.

For enzyme-replacement therapies of lysosomal storage diseases, the use of cell-free neutralizing antibody assays is well-founded, provides valuable discrimination between different types of neutralizing antibodies, and has resulted in successful marketing applications in the US, EMEA, and additional worldwide regulatory agencies.

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