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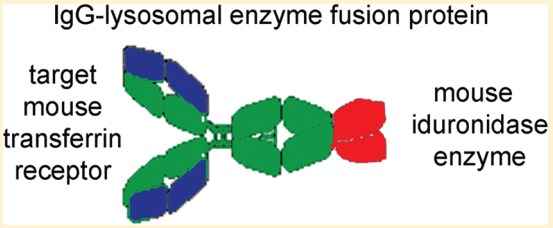
Reversal of Lysosomal Storage in Brain of Adult MPS-I Mice with Intravenous Trojan Horse-Iduronidase Fusion Protein

Ruben J. Boado,[†,‡] Eric Ka-Wai Hui,[‡] Jeff Zhiqiang Lu,[‡] Qing-Hui Zhou,[†] and William M. Pardridge*[,†]

[†]Department of Medicine, UCLA, Los Angeles, California 90024, United States

[‡]ArmaGen Technologies, Inc., Santa Monica, California 90401, United States

ABSTRACT: A mouse model of mucopolysaccharidosis (MPS) type I, which is null for the lysosomal enzyme, α-L-iduronidase (IDUA), is treated with intravenous, receptor-mediated enzyme replacement therapy of the brain. Murine IDUA, which does not cross the blood−brain barrier, is re-engineered for targeting to the brain as an IgG-enzyme fusion protein. The amino terminus of mature IDUA is fused to the carboxyl terminus of the heavy chain of a chimeric monoclonal antibody (mAb) against the murine transferrin receptor (TfR), and this fusion protein is designated cTfRMAb-IDUA. The cTfRMAb part of the fusion protein acts as a molecular Trojan horse to ferry the fused IDUA across the BBB and neuronal cell membrane via transport on the TfR. The IDUA enzyme activity of the fusion protein, 776 ± 79 units/μg protein, is comparable to recombinant IDUA. MPSI null mice, 6−8 months of age, were treated iv twice a week for 8 weeks with either saline or 1 mg/kg cTfRMAb-IDUA. The glycosoaminoglycan levels in liver, spleen, heart, and kidney were reduced by >95%, 80%, 36%, and 20%, respectively. Lysosomal inclusion bodies in the brain were quantitated from semithin sections stained with *o*-toluidine blue and normalized per 100 nucleoli per brain section. Treatment of the MPSI mice with the cTfRMAb-IDUA reduced intracellular lysosomal inclusion bodies by 73% in brain, as compared to the MPSI mice treated with saline. In conclusion, the reversal of pre-existing neural pathology in the brain of MPSI mice is possible with receptor-mediated enzyme replacement therapy of the brain.



KEYWORDS: blood−brain barrier, drug targeting, lysosomal enzyme, transferrin receptor

INTRODUCTION

There are over 40 lysosomal storage disorders,[1] and the principal therapy of these disorders is enzyme replacement therapy (ERT) with the recombinant lysosomal enzyme.[2] However, about 75% of the lysosomal storage disorders affect the central nervous system (CNS),[3] and recombinant enzymes are large molecule pharmaceutics that do not cross the blood−brain barrier (BBB).[4] Consequently, ERT does not treat the brain or spinal cord.[5]

Mucopolysaccharidosis (MPS) type I, or Hurler's syndrome, is caused by genetic mutations in the gene encoding the lysosomal enzyme, α-L-iduronidase (IDUA).[1] The CNS is affected in Hurler's syndrome, and recombinant IDUA is not effective in treating the brain.[4] Recombinant IDUA has been administered by intracerebroventricular (ICV) injection. However, the ICV route delivers drug primarily to the meningeal surface of the brain.[6] The MPSI lesions in the CNS are global, and an effective therapeutic strategy requires enzyme delivery to all parts of brain.

It is possible to deliver IDUA to all regions of brain via the transvascular route across the BBB. Although IDUA does not cross the BBB, the enzyme can be re-engineered to penetrate the brain via receptor-mediated transport across the BBB using molecular Trojan horse (MTH) technology.[7] A BBB molecular Trojan horse is a peptide or peptidomimetic monoclonal antibody (mAb) that crosses the BBB via transport on an endogenous peptide transport system within the BBB, such as the insulin receptor or transferrin receptor (TfR). The most potent MTH is a genetically engineered mAb against the human insulin receptor (HIR), and a HIRMAb-IDUA fusion protein has been engineered.[8] The HIRMAb-IDUA fusion protein penetrates the BBB in the Rhesus monkey, and 1% of the injected dose (ID) is taken by the primate brain.[8] Repeat dosing of primates with intravenous HIRMAb-IDUA shows the IgG-enzyme fusion protein has a favorable safety profile.[9]

The IDUA null mouse is an animal model of MPSI.[10] However, this mouse model cannot be treated with the HIRMAb-IDUA fusion protein, because the HIRMAb does not cross-react with the insulin receptor in species other than humans and the Rhesus monkey.[11] There is no known mAb against the mouse insulin receptor that could be used as a BBB MTH. Therefore, a surrogate MTH for the mouse has been developed, which is a chimeric mAb against the mouse TfR designated cTfRMAb.[12] The cTfRMAb is composed of heavy chain and light chain constant regions from mouse IgG1κ, and from variable regions derived from a rat IgG against the mouse TfR. The cTfRMAb rapidly penetrates the brain in the mouse, and cTfRMAb fusion proteins have been engineered; the uptake is >3% ID/g brain following intravenous (iv) administration.[13] Therefore, the purpose of the present study was to genetically engineer a cTfRMAb-IDUA fusion protein, and to treat adult MPSI mice with twice a week iv injections of the fusion protein. The goal of the study was to cause a reduction in glycosoaminoglycan (GAG) levels in peripheral tissues, and to reduce the

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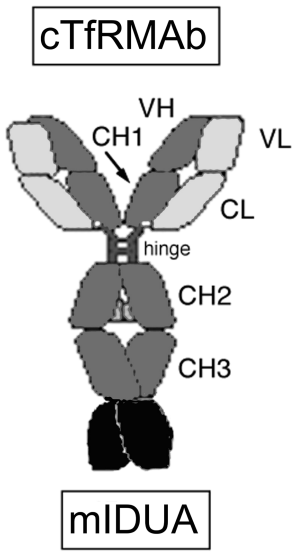

Figure 1. The cTfRMAb-IDUA fusion protein is formed by fusion of the amino terminus of the mature murine IDUA to the carboxyl terminus of the CH3 region of the heavy chain of the chimeric TfRMAb. The fusion protein is a bifunctional molecule: the fusion protein binds the mouse TfR, at the BBB, to mediate transport into the mouse brain, and expresses IDUA enzyme activity.

lysosomal storage products in the brain of the treated MPSI mice. Since repeat administration of human IDUA in mice may exacerbate an immune response, the present study first clones the murine IDUA cDNA. The fusion gene encoding the cTfRMAb-IDUA fusion protein is then constructed from the cTfRMAb and mouse IDUA genes. The amino terminus of the mature murine IDUA was fused to the carboxyl terminus of the heavy chain of the cTfRMAb (Figure 1).

EXPERIMENTAL SECTION

Engineering of pcTfRMAb-mIDUA Fusion Protein Tandem Expression Vector. The murine IDUA cDNA encoding for amino acids Glu^{26}–Ser^{643} (accession # NP_032351), and excluding the 25 AA signal peptide, was cloned by PCR using RNA derived from mouse lymphoid cells and custom oligodeoxynucleotides (ODN). The sequence of the forward 23-mer ODN is 5′-CTGAGTCACCGTACCTGGTGCGT-3′, and the sequence of the reverse 24-mer ODN is 5′-TCATGAGGCAGGGACATCCAGGTA-3′. The 1.9 kb murine IDUA was excised from an agarose gel and inserted at the *Hpa*I site of a universal tandem vector (TV) described previously,[13] to generate the cTfRMAb-IDUA fusion protein expression plasmid DNA, designated pcTfRMAb-mIDUA. The sequence of the PCR ODNs was designed to maintain the open reading frame and to introduce a Ser-Ser linker between the CH3 region of the cTfRMAb heavy chain (HC) and the amino terminus of the murine IDUA minus the enzyme signal peptide. The 3′-end of the IDUA cDNA included the stop codon, TGA. The pcTfRMAb-mIDUA tandem vector encodes the light chain (LC) of the chimeric TfRMAb, the fusion protein of the chimeric TfRMAb HC and murine IDUA, dihydrofolate reductase (DHFR), and the neomycin resistance gene. The latter genes allowed for selection of high producing host cell lines with methotrexate and G418, respectively. The entire open reading frames for the LC, HC-IDUA and DHFR expression cassettes of the pcTfRMAb-mIDUA plasmid were confirmed by bidirectional DNA sequencing performed at Eurofins MWG Operon (Huntsville, AL) using custom sequencing oligodeoxynucleotides synthesized at Midland Certified Reagent Co. (Midland, TX).

Transient Expression of pcTfRMAb-mIDUA in COS Cells. COS cells were lipofected with the pcTfRMAb-mIDUA TV using Lipofectamine 2000, with a ratio of 1:2.5 μg of DNA:μL of Lipofectamine. Following transfection, the cells were cultured in 6-well cluster dishes in serum free VP-SFM (Invitrogen, Carlsbad, CA). The conditioned medium (CM) was collected at 3 and 7 days. Transgene expression and fusion protein secretion to the medium was assayed by measurement of mouse IgG in the conditioned medium. Mouse IgG ELISA was performed in Immulon 2 high binding plates (Dynex Tech., Chantilly, VA) with COS cell conditioned medium. For large scale production of the COS-derived cTfRMAb-IDUA fusion protein, the lipofection was scaled up, and lipofected cells were plated in 10xT500 flasks. The serum free CM was collected at 3 and 7 days and pooled and ultrafiltered with a Sartopore-2 (0.45 + 0.22 um) filter (Sartorius Stedim North America, Bohemia, NY).

Protein G Chromatography. Serum free medium conditioned by transfected COS cells was filtered and applied to a 25 mL protein G Sepharose 4 Fast Flow (GE Life Sciences, Chicago, IL) column equilibrated in 25 mM Tris/25 mM NaCl/5 mM EDTA/pH = 7.1. Following application of the sample, the column was washed with 25 mM Tris/1 M NaCl/5 mM EDTA/pH = 7.1, and the fusion protein was eluted with 0.1 M glycine/pH = 2.8. The acid eluate was pooled, Tris was added to raise the pH to 5.5, and NaCl was added to 0.15 M. The volume of the pool was concentrated to 2 mL with an Ultra-15 centrifugal filter unit with a 100,000 molecular weight cutoff (Millipore, Billerica, MA), and the solution was stored sterile-filtered at either 4 °C or −70 °C. The protein content of the final drug product was measured with the bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL).

SDS−PAGE and Western Blotting. The homogeneity of protein G purified fusion protein was evaluated with a reducing 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE), followed by Coomassie Blue staining. For Western blotting after reducing SDS−PAGE, immunoreactivity was tested with a primary goat antibody against mouse IgG heavy and light chains, 0.2 μg/mL (Bethyl Laboratories, Montogomery, TX), and 0.15 μg/mL of a biotinylated horse anti-goat IgG secondary antibody (Vector Laboratories, Burlingame, CA). For the IDUA Western blotting, the primary antiserum was a 1:5000 dilution of a rabbit antiserum custom prepared against human recombinant IDUA by Prosci, Inc. (Poway, CA), and the secondary antibody was 0.3 μg/mL biotinylated goat anti-rabbit IgG (Vector Laboratories).

Transferrin Radioreceptor Assay. Binding of the cTfRMAb-IDUA fusion protein to the mouse TfR was measured with a radio-receptor assay (RRA) using the [^{125}I]-labeled 8D3 rat mAb to the mouse TfR as the binding ligand. The 8D3 TfRMAb was purified from ascites with a protein G affinity column, and the IgG was radiolabeled with [^{125}I]-Bolton−Hunter reagent (American Radiolabeled Chemicals, St. Louis, MO) to a specific activity of 10.3 μCi/μg and a trichloroacetic acid (TCA) precipitability of >99%. Mouse 3T3 fibroblasts were plated on collagen coated BD Biocoat 24-well dishes (Becton-Dickinson, Franklin Lakes, NJ), and the binding assay was performed at 4 °C for 3 h as described previously.[12] The [^{125}I]-8D3 TfRMAb was added at a tracer concentration of 0.4 nM, and increasing concentrations of either unlabeled

8D3 TfRMAb, to measure the K_D of binding, or the cTfRMAb-IDUA fusion protein, to measure the K_I of binding, were comixed with the labeled 8D3 TfRMAb. The 8D3 self-inhibition study allowed for computation of the dissociation constant, K_D, and the maximal binding, B_{max}, and the cTfRMAb-IDUA fusion protein cross-inhibition study allowed for determination of the K_I of binding to the mouse TfR. Binding curves were fit by nonlinear regression analysis, as described previously.[12]

IDUA Enzyme Assay. The IDUA enzyme activity was measured for the purified cTfRMAb-IDUA fusion protein, for mouse plasma, and for mouse organ homogenates with a fluorometric assay, as described previously,[8] using 4-methylumbelliferyl α-L-iduronide, which was purchased from Glycosynth, Ltd. (Cheshire, England). This substrate is hydrolyzed to 4-methylumbelliferone (4-MU) by IDUA, and the 4-MU is detected fluorometrically with a Farrand filter fluorometer using an emission wavelength of 450 nm and an excitation wavelength of 365 nm. A standard curve was constructed with known amounts of 4-MU (Sigma-Aldrich, St. Louis, MO). The assay was performed at 37 °C at pH = 3.5 for 60 min, and was terminated by the addition of 1 mL of 0.5 M glycine (pH = 10.3), where 1 unit =1 nmol/h. In the case of the assay of IDUA enzyme activity for homogenates of brain, heart, and kidney, the duration of the enzyme assay was extended to 6 h.

IDUA Enzyme Activity in Mouse Fibroblasts. Mouse 3T3 fibroblasts were grown on collagen coated 6-well cluster dishes to confluency. The cells were washed, and the cells were then exposed to serum free medium containing 6 μg/mL cTfRMAb-IDUA fusion protein. After a 2 h incubation at 37 °C, the medium was aspirated, the cells were washed 3 times with serum free medium, and fresh medium with 10% calf serum and no fusion protein was added to the cells, which were further incubated up to 96 h at 37 °C. Monolayers were harvested at 2, 24, 48, 72, and 96 h later, washed extensively, and cell lysates were prepared in 5 mM sodium acetate/pH = 4.0/0.2% Triton X-100. Intracellular IDUA enzyme activity was measured with the fluorometric assay, and cell protein was measured with the BCA assay.

MPSI Mice. *Acute Administration of Fusion Protein.* MPSI heterozygote and null mice were generously provided by Prof. Elizabeth Neufeld. The cTfRMAb-IDUA fusion protein was administered as an acute, single injection in both MPSI heterozygote and MPSI null mice. In the null mouse study, 4 mice were administered saline and 4 mice were administered 1 mg/kg cTfRMAb-IDUA fusion protein via the tail vein in volumes of 100 μL. The mice were euthanized 60 min later, and the brain (cerebrum, cerebellum) and peripheral organs (liver, spleen, heart, kidney), as well as terminal serum, were harvested for IDUA enzyme activity. Enzyme activity was expressed as nmol/h/mg protein for organs, or nmol/h/mL for serum.

Heterozygote mice (3 males) were 4 months of age and were used for a single injection pharmacokinetics study. The mice were injected with 75 μL/mouse (150 μg/mouse of the cTfRMAb-IDUA fusion protein) by tail vein injection with a 29g needle. Blood for collection of heparinized plasma was sampled from the orbital vein at the following time points after injection: 0.25, 2, 5, and 15, 30, and 60 min. The plasma was stored at −70 °C. For measurement of IDUA enzyme activity with the fluorometric assay, the plasma samples were diluted 1:100 to 1:10 with 0.01 M Tris/0.15 M NaCl/pH = 7.4 (TBS). The plasma IDUA enzyme activity, in nmol/h/mL, was converted to % injected dose (ID)/mL, based on the specific activity of the fusion protein, and the % ID/mL was fit to a biexponential equation,

$$\%\mathrm{ID}/\mathrm{mL} = A_1\, e^{-k_1 t} + A_2\, e^{-k_2 t}$$

The intercepts (A_1, A_2) and the slopes (k_1, k_2) were used to compute the median residence time (MRT), the central volume of distribution (V_c), the steady state volume of distribution (V_{ss}), the area under the plasma concentration curve (AUC), and the systemic clearance (CL), as described previously.[11] Nonlinear regression analysis used the AR subroutine of the BMDP Statistical Software (Statistical Solutions Ltd., Cork, Ireland). Data were weighted by $1/(\%\ \mathrm{ID/mL})^2$.

Chronic Administration of Fusion Protein. MPSI null mice (5 males, 5 females), 6−8 months of age were used for a 2 month treatment study. Mice were treated twice a week for 8 consecutive weeks with either saline or the cTfRMAb-IDUA fusion protein (1 mg/kg per dose) by tail vein injection. At the end of the treatment study, mice were euthanized and terminal serum was collected for immunity ELISA, organ GAG levels by colorimetric assay, and brain microscopy. At the fifth drug injection, the mice developed symptoms of immune reactions with decreased locomoter activity in the cage following injection of the cTfRMAb-IDUA fusion protein, and this was scored as follows: +3, severe response that clears in 60 min; +2, moderate response that clears in 30 min; +1, mild response that clears in 10 min. Mice with a score of +2 or +3 were treated with 10−20 mg/kg of diphenydramine intraperitoneal (ip).

Tissue GAG Levels. Mouse organs (liver, spleen, kidney, heart, brain) were removed at euthanasia, and the tissue (100 mg) was suspended in 0.6 mL of lysis buffer (0.2% Triton X-100 in 0.01 M Na_2HPO_4, pH = 5.8) and homogenized with a Polytron homogenizer for 5 s, followed by 3 freeze/thaw cycles. The homogenate was centrifuged at 10000*g* for 5 min at 4 °C, and the supernatant was transferred to new tubes for IDUA enzyme activity, BCA protein assay, and GAG measurement. GAG levels were quantified with the Blyscan colorimetric sulfated GAG assay kit (Biocolor Ltd., U.K.). Data are reported as μg of GAG per mg of tissue protein.

Brain Microscopy. The brain was removed and immersion fixed in 4% paraformaldehyde/2% glutaraldehyde in 0.1 M Na_2HPO_4/pH = 7.4 for 48 h at 4 °C, and postfixed in 1% osmium tetroxide. The cortex of the fixed brain was embedded in Epon, and 1 μm sections were prepared and stained with *o*-toluidine blue. The sections were examined with light microscopy at a 100× magnification (40× objective). The number of multivacuolated brain cells was counted per every 100 brain cell nucleoli. A total of 300 nucleoli per brain were counted for each mouse. The vacuoles in a GAG-laden brain cell were multiple and contiguous. Only brain cells with at least 3 contiguous vacuoles were scored. All scoring of brain cells was performed in a blinded fashion. For electron microscopy, ultrathin sections were transferred with a diamond knife to 300-mesh copper grids and stained with uranyl acetate and Sato's lead stain. Electron micrographs were photographed at 6000× magnification with a Phillips 208S electron microscope at 80 kV.

Immunity ELISA. The presence of anti-cTfRMAb-IDUA fusion protein antibodies in mouse serum was measured with a 2-site sandwich ELISA, using the cTfRMAb-IDUA fusion protein as the capture reagent and biotinylated cTfRMAb-IDUA fusion protein as the detector reagent. Alternatively, the cTfRMAb, mouse IgG1k, or the rat 8D3 mAb against the mouse TfR was used as the capture reagent, as the cTfRMAb was

engineered from the variable regions of the 8D3 mAb and the constant regions of the mouse IgG1 heavy chain and the mouse kappa light chain. The mouse serum was diluted in PBS. The capture reagent was plated overnight at 4 °C in 96-wells in 100 μL (250 ng)/well in 0.05 M NaHCO3/8.3. The wells were blocked with PBS containing 1% bovine serum albumin (PBSB), followed by the addition of 100 μL/well of the diluted mouse serum. After a 60 min incubation at 37 °C, the wells were washed with PBSB, and the wells were incubated with biotinylated cTfRMAb-IDUA fusion protein (50 ng/well) for 60 min. The wells were washed with PBSB, followed by incubation with 100 μL (500 ng/well) of a streptavidin−peroxidase conjugate (#SA-5004, Vector Laboratories) for 30 min at room temperature (RT). The wells were washed with PBSB, and 100 μL/well of *o*-phenylenediamine/H_2O_2 developing solution (#P5412, Sigma Chemical Co, St. Louis, MO) was added for a 15 min incubation in the dark at RT. The reaction was stopped by the addition of 100 μL/well of 1 M HCl, followed by the measurement of absorbance at 492 and 650 nm. The A_{650} was subtracted from the A_{492}. The ($A_{492}-A_{650}$) for the PBSB blank was then subtracted from the ($A_{492}-A_{650}$) for the sample. Mouse serum samples were screened with the immunity ELISA at 1:50 dilutions in PBS using the cTfRMAb-IDUA fusion protein as the capture reagent. For subsequent studies, serum was pooled from the 2 fusion protein-treated mice that reacted strongest in the ELISA at 1:50 dilution. This pool was then diluted 1:50, 1:300, 1:1000, or 1:3000 in PBS, and the dilution curves were compared with 3 different capture reagents: the cTfRMAb, the hybridoma-derived rat 8D3 mAb against the mouse TfR, or mouse IgG1k, which is the isotype control for the constant region comprising the cTfRMAb.

The cTfRMAb-IDUA fusion protein was biotinylated as described previously,[14] using sulfo-biotin-LC-LC-*N*-hydroxysuccinimide, where LC = long chain (#21338, Pierce Chemical Co.). The biotinylation of the cTfRMAb-IDUA fusion protein was confirmed by SDS−PAGE and Western blotting, where the blot was probed with avidin and biotinylated peroxidase. The non-biotinylated cTfRMAb-IDUA fusion protein gave no reaction in the Western blot, whereas the biotinylated fusion protein was strongly visualized at the appropriate molecular size for both heavy chain and light chain.

Statistical analysis was performed with Student's *t* test.

RESULTS

DNA sequencing of the pcTfRMAb-mIDUA plasmid encompassed 10,486 nucleotides (nt), which spanned the expression cassettes for the heavy chain fusion gene, the light chain gene, and the DHFR gene. The fusion heavy chain expression cassette included a 3,246 nt open reading frame, which encoded for a 1,081 AA protein, composed of a 19 AA IgG signal peptide, the 118 AA TfRMAb heavy chain variable region (VH), the 324 mouse IgG1 constant region, a 2 AA linker (Ser-Ser), and the 618 AA mouse IDUA minus the signal peptide, which was 100% identical to the AA sequence from Glu^{26}−Ser^{643} of mouse IDUA (NP_032351). The predicted molecular weight of the heavy chain fusion protein, minus glycosylation, is 118,556 Da, with a predicted isoelectric point (p*I*) of 6.21. The light chain was composed of 234 AA, which included a 20 AA signal peptide, a 108 AA TfRMAb variable region of the light chain (VL), and a 106 AA mouse kappa light chain constant region. The predicted molecular weight of the light chain is 23,554 Da with a predicted p*I* of 5.73. The predicted molecular weight of the cTfRMAb-IDUA heterotetrameric protein, without glycosylation, is 284,220 Da.

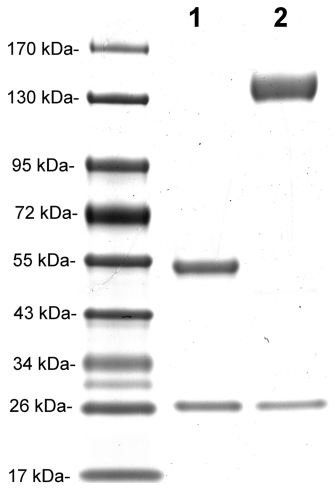

Figure 2. Reducing SDS−PAGE and Coomassie blue staining of protein G affinity purified cTfRMAb (lane 1) and the cTfRMAb-IDUA fusion protein (lane 2). Both are purified to homogeneity and are composed of a heavy chain and a light chain.

Lipofection of COS cells with the pcTfRMAb-mIDUA resulted in secretion to the medium of mouse IgG, as determined with a mouse Fc specific ELISA. The concentration of mouse IgG in the medium at 3 days following lipofection with Lipfectamine 2000 alone or with the pcTfRMAb-mIDUA plasmid was <3 and 628 ± 43 ng/mL, respectively. The cTfRMAb-IDUA fusion protein was purified by protein G affinity chromatography, and the purity was assessed with reducing SDS−PAGE and Coomassie blue staining. The size of the light chain (LC) is the same for both the cTfRMAb and the cTfRMAb-IDUA fusion protein (Figure 2). The size of the heavy chain (HC) of the fusion protein is larger than the size of the HC of the cTfRMAb, owing to fusion of the murine IDUA (Figure 2). Based on the migration in reducing SDS−PAGE of the molecular weight standards, the calculated molecular weight of the LC of the cTfRMAb or the cTfRMAb-IDUA fusion protein is 24 kDa; the calculated molecular weights of the HC of the cTfRMAb and the cTfRMAb-IDUA fusion protein are 59 and 135 kDa, respectively. On Western blotting, the LC of either the cTfRMAb or the cTfRMAb-IDUA fusion protein reacts equally with a primary antibody directed against the mouse IgG (H+L), as shown in Figure 3A. The size of the HC of the fusion protein is about 75 kDa larger than the size of the HC of the cTfRMAb on Western blots using either the anti-human IgG primary antibody (Figure 3A) or the anti-IDUA primary antibody (Figure 3B). The anti-IDUA primary antibody reacts with the HC only of the cTfRMAb-IDUA fusion protein, but does not react with either chain of the cTfRMAb (Figure 3B).

The 8D3 TfRMAb bound with high affinity to the mouse TfR expressed on the plasma membrane of mouse 3T3 fibroblasts, and the K_D and B_{max} of binding were 1.8 ± 0.5 nM and 0.64 ± 0.12 pmol/mg protein, respectively (Figure 4A). The K_I of cTfRMAb-IDUA fusion protein inhibition of binding of [^{125}I]-8D3 was 0.67 ± 0.17 nM (Figure 4B). Mouse 3T3 fibroblasts were exposed to 6 μg/mL (18 nM) of the cTfRMAb-IDUA fusion protein for 2 h, and then washed, and the decay in intracellular IDUA enzyme activity over 96 h was measured (Table 1). Intracellular IDUA enzyme activity in mouse cells persisted for days, and decayed with a half-time of 67 h (Table 1).

The IDUA enzyme specific activity of the cTfRMAb-IDUA fusion protein is 776 ± 79 units/μg protein. The cTfRMAb-IDUA fusion protein was injected iv into heterozygote MPSI mice, and the plasma IDUA enzyme activity is shown in Figure 5. These data were fit to a biexponential function (Experimental Section) to yield the pharmacokinetic parameters listed in Table 2. MPSI null mice were injected iv with either saline or the cTfRMAb-IDUA fusion protein, and serum and organ IDUA enzyme activity was measured 60 min after injection. The organ IDUA enzyme activity was not detectable in the saline injected null mice. However, IDUA enzyme activity was increased to 1−5 nmol/h/mg protein in brain and peripheral organs, including liver, spleen, heart, and kidney (Table 3).

The mean body weight of the MPSI mice treated with saline at the beginning and end of the 8 week treatment was 29.1 and 29.8 g, respectively. The mean body weight of the MPSI mice treated with the cTfRMAb-IDUA fusion protein at the beginning and end of the 8 week treatment was 27.8 and 27.2 g, respectively. One of the saline treated mice died during the course of the study. No fusion protein treated mice died during the 8 week treatment period. At the fifth dose of cTfRMAb-IDUA fusion protein, the MPSI null mice developed symptoms of immune reaction with a mean score of +2 (Experimental Section). Therefore, the mice were pretreated with 20 mg/kg diphenhydramine (DPH) at the sixth dose, which reduced the mean immune reaction score to +1. The DPH dose was reduced to 10 mg/kg for the seventh and eighth doses, and the mean immune reaction score was 0.2 and 0, respectively. From the eighth to the terminal 16th dose, no DPH pretreatment was required and no mouse showed symptoms of immune reaction. The terminal mouse serum was tested with the immunity ELISA, and the design of the assay is outlined in Figure 6A. No immune reactions were detected in the MPSI mice treated with saline, and a variable

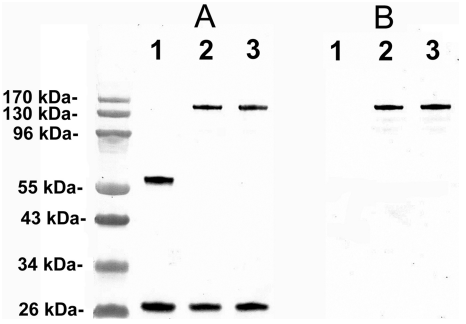

Figure 3. Western blot with either anti-mouse IgG primary antibody (A) or an anti-IDUA primary antibody (B). The immunoreactivity of the cTfRMAb-IDUA fusion protein is compared to that of the cTfRMAb. Both the cTfRMAb-IDUA fusion protein and the cTfRMAb have identical light chains on the anti-IgG Western. The cTfRMAb-IDUA fusion heavy chain reacts with both the anti-IgG and the anti-IDUA antibody, whereas the cTfRMAb heavy chain only reacts with the anti-IgG antibody. The size of the cTfRMAb-IDUA fusion heavy chain is about 75 kDa larger than the size of the heavy chain of the cTfRMAb, owing to the fusion of the 75 kDa murine IDUA to the 60 kDa cTfRMAb heavy chain. Lane 1: cTfRMAb. Lanes 2 and 3: different lots of the COS-derived cTfRMAb-IDUA fusion protein.

Table 1. IDUA Enzyme Activity in Mouse 3T3 Fibroblasts Exposed to cTfRMAb-IDUA Fusion Protein[a]

time postexposure (h)	IDUA enzyme act. (nmol/h/mg protein)
2	510 ± 40
24	405 ± 54
48	376 ± 30
72	295 ± 21
96	178 ± 66

[a] Mean ± SE (n = 4 dishes per time point). Cells were exposed to 6 μg/mL cTfRMAb-IDUA fusion protein for 2 h, followed by removal of the fusion protein from the medium for the next 96 h. The basal IDUA enzyme activity in the untreated cells was <25 nmol/h/mg protein.

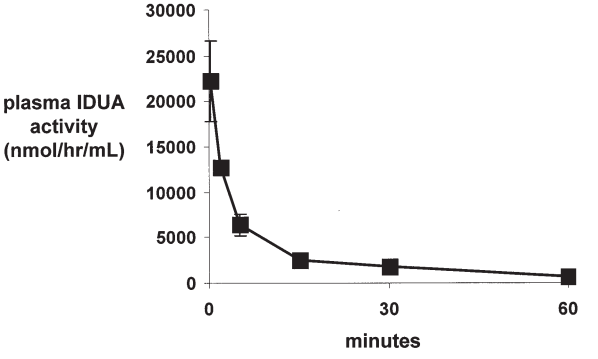

Figure 5. Plasma IDUA enzyme activity in heterozygote MPSI mice following the iv injection of 150 μg/mouse of cTfRMAb-IDUA fusion protein. Data are mean ± SE (3 mice/point).

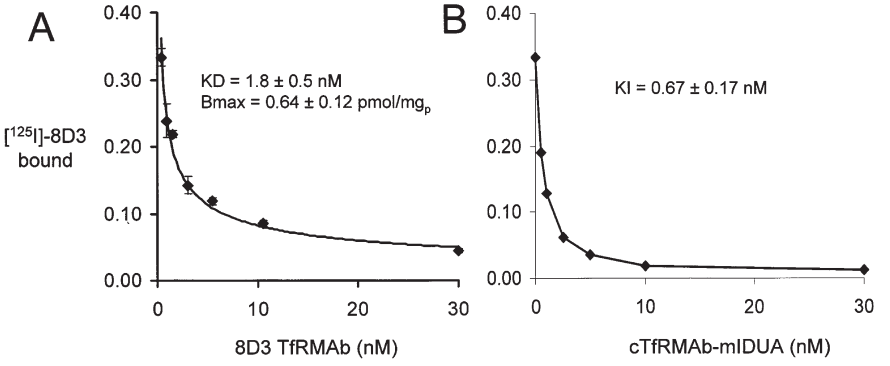

Figure 4. Radioreceptor assay of the mouse TfR uses mouse fibroblasts as the source of the mouse TfR and [^{125}I]-8D3 as the binding ligand. Binding is displaced by unlabeled 8D3 mAb (A) or the cTfRMAb-IDUA fusion protein (B). The K_D and B_{max} were determined from the 8D3 self-inhibition curve (A), and the K_I of fusion protein binding to the mouse TfR was computed from the cross-inhibition curve (B).

immune response was detected at 1:50 dilutions of the sera from the MPSI mice treated with fusion protein (Figure 6B). The sera from the 2 MPSI mice with the highest reaction in the ELISA at a 1:50 dilution (Figure 6B) were pooled, and serial dilutions of this pool were tested with 4 different capture reagents. The reactivity in the ELISA was strongest with the cTfRMAb antibody or the rat 8D3 antibody, it was intermediate with the cTfRMAb-IDUA fusion protein, and no reaction was observed when mouse IgG1κ was plated as the capture reagent (Figure 6C).

The MPSI mice were euthanized after the 8 weeks of twice/week treatment, and liver, spleen, kidney, heart, and brain were removed and homogenized for measurement of organ GAG levels. Fusion protein treatment caused significant decreases in GAG levels in liver, spleen, and heart, and GAG levels in liver were undetectable in the MPSI mice treated with the cTfRMAb-IDUA fusion protein (Table 4). There was no reduction in GAG levels in the brain, as these were low in the untreated MPSI mouse (Table 4).

Light microscopy of *o*-toluidine blue 1 μm semithin sections of mouse brain shows large perivascular multivacuolated lesions in the saline treated mouse (Figure 7A), which are reduced in size in the fusion protein treated mouse (Figure 7B). The multivacuolated intracellular lesion in brain cells of the saline treated mouse (Figure 7C) are fewer in the brain of the fusion protein treated mouse (Figure 7D). The number of multivacuolated brain cells, excluding the perivascular lesion, was blindly scored per 100 brain cell nuclei. This analysis showed a 73% reduction of multivacuolated cells in the MPSI mice treated with cTfRMAb-IDUA fusion protein, as compared to the saline treated MPSI mice (Table 5). Electron microscopy showed the large perivascular lysosomal inclusion bodies

Table 2. Pharmacokinetic Parameters of cTfRMAb-IDUA Fusion Protein Clearance from Blood in the MPSI Heterozygote Mouse[a]

parameter	units	cTfRMAb-IDUA
A_1	% ID/mL	27.2 ± 2.1
A_2	% ID/mL	5.5 ± 0.5
k_1	min^{-1}	0.393 ± 0.041
k_2	min^{-1}	0.029 ± 0.002
MRT	min	26 ± 2
V_c	mL/kg	94 ± 6
V_{ss}	mL/kg	303 ± 18
AUC (60 min)	% ID·min/mL	224 ± 6
AUC_{ss}	% ID·min/mL	257 ± 7
CL	mL/min/kg	11.8 ± 0.3

[a] Estimated from the plasma clearance data in Figure 5.

Table 3. Organ IDUA Enzyme Activity in MPSI Null Mice at 60 Min after Iv Administration of cTfRMAb-IDUA Fusion Protein (1 mg/kg)[a]

	IDUA enzyme act. (nmol/h/mg protein)	
organ	saline	cTfRMAb-IDUA
liver	<0.2	4.31 ± 0.26
spleen	<0.1	5.35 ± 0.38
cerebrum	<0.05	0.96 ± 0.26
cerebellum	<0.05	0.89 ± 0.23
heart	<0.05	1.01 ± 0.03
kidney	<0.05	0.76 ± 0.10
serum	<0.1	0.79 ± 0.17

[a] Mean ± SE (n = 4 mice per each group). Serum IDUA enzyme activity is expressed per mg serum protein, and is equal to 57 ± 12 nmol/h/mL serum.

Table 4. Organ GAG Concentrations in MPSI Mice[a]

	organ GAG (μg/mg protein)	
organ	saline	cTfRMAb-IDUA fusion protein
liver	77.8 ± 7.4	<2.5***
spleen	49.6 ± 11.6	9.9 ± 3.1**
kidney	46.6 ± 6.2	37.2 ± 3.0
heart	35.1 ± 3.8	22.6 ± 3.2*
brain	1.4 ± 0.2	1.2 ± 0.3

[a] Data (mean ± SE) are terminal organ assays at the end of the 8 week study of MPSI mice treated with either saline or 1 mg/kg of the cTfRMAb-IDUA fusion protein. ***p < 0.001. **p < 0.01. *p < 0.05.

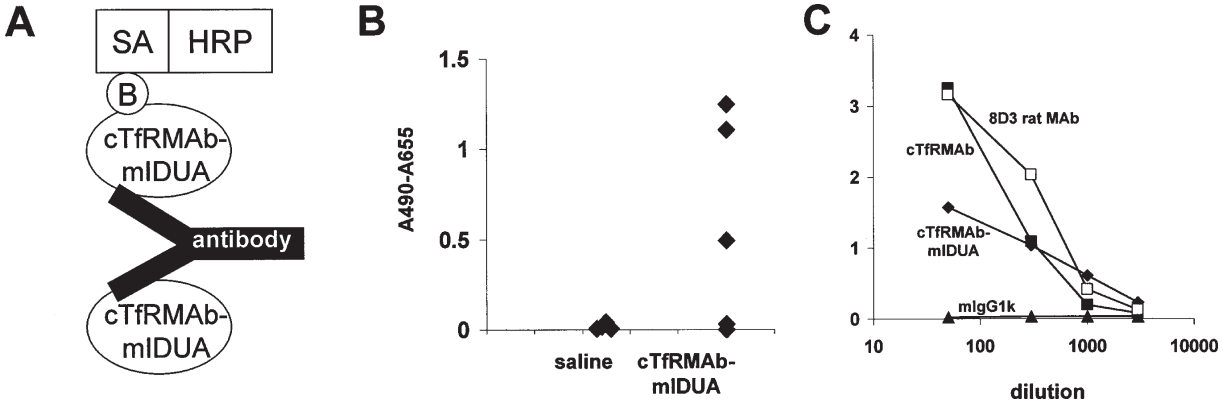

Figure 6. (A) Structure of the 2-site ELISA for detection of antibodies against the cTfRMAb-IDUA fusion protein. The cTfRMAb-IDUA fusion protein is used as the capture reagent, and the biotinylated cTfRMAb-IDUA fusion protein is used as the detector reagent, along with a complex of streptavidin (SA) and horseradish peroxidase (HRP); the biotin moiety is designated "B". (B) Absorbance at 1:50 dilutions of MPSI mouse serum taken at the end of the 8-week treatment study for mice in either the saline or the cTfRMAb-IDUA fusion protein treatment groups. The capture reagent is the cTfRMAb-IDUA fusion protein. (C) Absorbance at 1:50, 1:300, 1:1000, and 1:3000 dilutions of a pool of terminal plasma from the 2 mice in the cTfRMAb-IDUA fusion protein treatment group that reacted the highest in the screen at 1:50 dilution (panel B). The capture reagent in this experiment is the cTfRMAb-IDUA fusion protein, the cTfRMAb antibody, the rat 8D3 antibody, or mouse IgG1κ.

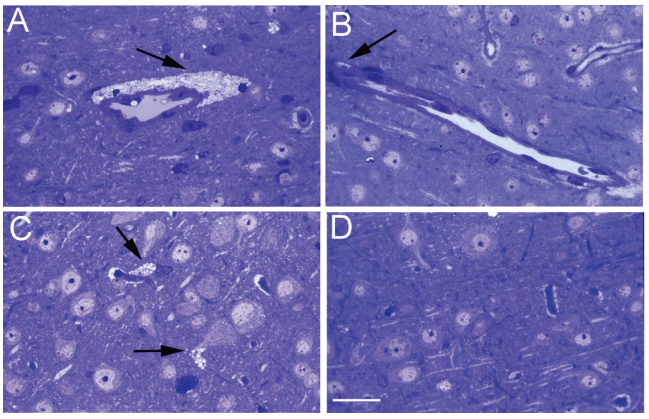

Figure 7. *o*-Toluidine blue stained semithin sections of MPSI mouse brain treated with either saline (A, C) or the cTfRMAb-IDUA fusion protein (B, D). Large perivascular multivacuolated lesion in the saline treated animal (arrow, panel A) is reduced in size in the cTfRMAb-IDUA fusion protein treated mouse (arrow, panel B). Multivaculoated intracellular inclusion bodies are more abundant in the brain of the saline treated mouse (arrows, panel C), as compared to the brain of the fusion protein treated mouse (panel D). Magnification bar = 36 μm.

Table 5. Lysosomal Storage-Laden Cells in Brain in MPSI Mice[a]

treatment	no. of multivacuolated brain cells per 100 brain cell nucleoli
saline	18.5 ± 1.1
cTfRMAb-IDUA fusion protein	5.0 ± 1.6***

[a] Cell counts (mean ± SE) were determined at the end of the 8 week study of MPSI mice treated with either saline or 1 mg/kg of the cTfRMAb-IDUA fusion protein. ***$p < 0.005$.

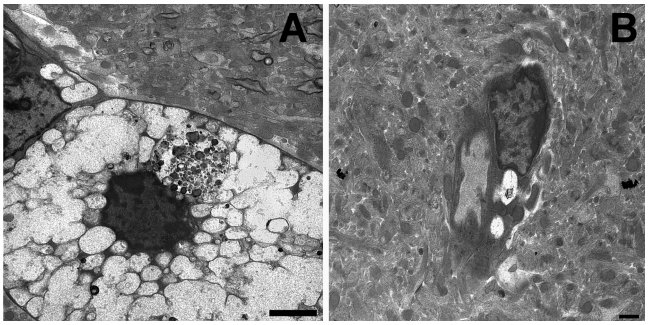

Figure 8. Electron microscopy of MPSI mouse brain treated with saline (A) or the cTfRMAb-IDUA fusion protein (B). Large perivascular lysosomal inclusion bodies seen in the brain of the saline treated mouse (A) are diminished in the fusion protein treated mouse (B). Magnification bars in panels A and B are 2 and 0.5 μm, respectively.

in brain of the saline treated mouse (Figure 8A), which were diminished in the cTfRMAb-IDUA fusion protein treated mouse (Figure 8B).

DISCUSSION

The results of this study are consistent with the following conclusions. First, a new IgG-enzyme fusion protein, the cTfRMAb-IDUA fusion protein, has been engineered and expressed. The fusion protein is bifunctional and both binds the mouse TfR (Figure 4), to mediate transport across the BBB and delivery to target cells in the mouse (Table 1), and expresses IDUA lysosomal enzyme activity (Figure 5). Second, the cTfRMAb-IDUA fusion protein is rapidly cleared from blood following iv injection (Figure 5), and distributes to the extravascular compartment (Table 2), resulting in an increase in IDUA enzyme activity in brain and peripheral organs (Table 3). Third, chronic administration of the cTfRMAb-IDUA fusion protein, at a dose of 1 mg/kg, given twice a week for 8 weeks, reduces GAG levels in peripheral organs (Table 4), and lysosomal inclusion bodies in brain (Figure 7, Table 5) in aged 6−8 month old MPSI mice. Fourth, the chronic administration of the cTfRMAb-IDUA fusion protein to MPSI mice causes an immune response, which is relatively low titer (Figure 6), and does not limit chronic therapy of the MPSI mice (Results).

The cTfRMAb-IDUA fusion protein was produced by fusion of murine IDUA to a chimeric TfRMAb derived from rat IgG variable regions and mouse IgG constant regions.[12] The IDUA part of the fusion protein is the murine form of the enzyme. The amino acid sequence of murine IDUA (NP_032351) is 80% identical to the amino acid sequence of human IDUA (NP_000194), excluding the 9 amino acid carboxyl terminal peptide that is present in the human IDUA, but not the murine IDUA.[15] The IDUA part of the fusion protein is fused to the carboxyl terminus of the HC of the cTfRMAb (Figures 2 and 3). This design places the IDUA in a dimeric configuration (Figure 1). The IDUA enzyme activity of the fusion protein was preserved, which is consistent with prior observations that IDUA exists as a dimer.[16] The IDUA enzyme specific activity of the fusion protein, 776 ± 79 units/μg protein (Results), is comparable to the enzyme activity of recombinant human IDUA.[6] Similarly, the IDUA enzyme activity is retained following fusion of human IDUA to the genetically engineered HIRMAb.[8,9] The HIRMAb-IDUA fusion protein is taken up by MPSI human fibroblasts, is localized to the lysosomal compartment as shown by confocal microscopy, and reduces GAG levels in MPSI fibroblasts.[8] The HIR is also expressed on the neuronal plasma membrane, in addition to the BBB.[17] Similarly, the TfR is also expressed on the neuronal cell membrane in addition to the BBB.[18] Therefore, fusion of the IDUA to the targeting BBB transport vector, the HIRMAb in humans or the cTfRMAb in mice, enables distribution of the fused IDUA from the blood compartment to the intraneuronal compartment in brain.

The cTfRMAb-IDUA fusion protein is rapidly removed from the blood compartment following intravenous injection in the mouse (Figure 5). The rapid availability of the fusion protein to the extravascular compartments of the body is demonstrated by the PK analysis. The systemic volume of distribution, V_{ss}, is much larger than the central volume, V_c (Table 2), which indicates the cTfRMAb-IDUA fusion protein rapidly exits the plasma compartment and is taken up by tissues. Penetration of the fusion protein into the organ compartments is also shown by the increased organ IDUA enzyme activity, as compared to the corresponding IDUA activity in serum, in the MPSI null mice following intravenous administration (Table 3).

The extent to which IDUA enzyme activity is increased in the brain following cTfRMAb-IDUA fusion protein administration is demonstrated by calculation of the brain:serum IDUA activity ratio, which is 122% (Table 3). In contrast, the brain:plasma enzyme activity ratio following the iv injection of 10 mg/kg of iduronate 2-sulfatase (IDS) in the IDS null mouse is 0.016.[19] A brain:plasma ratio of 1.6% is equal to the brain blood volume, and indicative of a lack of enzyme transport across the BBB. The brain:serum IDUA

activity ratio, following administration of the cTfRMAb-IDUA fusion protein, is 76-fold higher than the brain:plasma IDS enzyme activity following iv administration of the enzyme alone. The IDUA enzyme activity in brain following fusion protein treatment, 0.9−1.0 μmol/h/mg protein (Table 3), approximates the IDUA enzyme activity in normal mouse brain, which is 3.2 μmol/h/mg protein.[20]

The distribution of the cTfRMAb-IDUA fusion protein into peripheral tissues such as liver, spleen, and heart causes a decrease in the elevated GAG levels in these organs in adult MPSI mice following chronic treatment (Table 4). The reductions in tissue GAG caused by chronic treatment with the fusion protein range from pronounced for liver (>95%) and spleen (80%) to moderate for heart (36%) and kidney (20%) (Table 4). There is no reduction in the GAG level in brain of the MPSI mouse (Table 4), because the brain GAG concentration is not elevated in the MPSI mouse.[20,21]

There are extensive lysosomal inclusion bodies in brain of the MPSI mouse (Figure 7A,C, Figure 8A). The cellular lesion is a multivacuolated intracellular inclusion body, which is pronounced in the perivascular area (Figure 7A, Figure 8A). The multivacuolated lesion in the intracellular compartment of brain cells (Figure 7C) is smaller in size as compared to the perivascular lesion (Figure 7A). The number of parenchymal brain cells that harbor multivacuolated lesions were scored per 100 brain nucleoli (Experimental Section). Treatment of 6−8 month old MPSI null mice with twice a week intravenous cTfRMAb-IDUA fusion protein causes a 73% reduction in the number of brain cells with these multivacuolated lesions (Table 5). Therapy was not initiated in this study until the MPSI mice were 6−8 months old, which is near the time when these mice expire.[22] Therefore, pre-existing lysosomal storage products in brain in the MPSI mouse can be reversed even when enzyme replacement therapy is not initiated until the mice are 6−8 months of age, provided the IDUA is re-engineered to cross the BBB.

A concern in chronic ERT is the development of an immune reaction against the recombinant lysosomal enzyme. If the immune response is directed against the mannose 6-phosphate (M6P) moiety of IDUA, the antibody response may neutralize cellular uptake of the enzyme via the M6P receptor.[23] In the case of the cTfRMAb-IDUA fusion protein, anti-M6P antibodies would not affect enzyme delivery in vivo via the TfR. In the present study, mice developed clinical signs of immune reactions at the fifth injection of the fusion protein, and these symptoms were eliminated with diphenhydramine pretreatment (Results). However, the mice quickly developed tolerance to the fusion protein treatment and no diphenhydramine treatment was required, and no signs of immune reaction were observed, after the eighth dose of fusion protein (Results). The re-engineering of a lysosomal enzyme as an IgG fusion protein may be a preferred form of the enzyme therapeutic. The constant region of IgG contains certain amino acid sequences, called Tregitopes, which induce immune tolerance.[24] The titer against the cTfRMAb-IDUA fusion protein was measured with an immunity ELISA (Figure 6A). The absorbance at a dilution of 1:50 that was >1.0 was detected in 2 of the 5 MPSI null mice treated with fusion protein (Figure 6B). The sera from these 2 mice were pooled, and a dilution curve showed there was no significant immune response at serum dilutions greater than 1:1000 (Figure 6C). These findings indicate the immune response against the cTfRMAb-IDUA fusion protein following 8 weeks of chronic treatment was of low titer and did not limit therapy.

In conclusion, these studies describe the re-engineering of IDUA as an IgG fusion protein for targeted brain delivery, and the reduction of lysosomal inclusion bodies in the brain, and GAGs in peripheral organs in a mouse model of MPSI. The IgG part of the fusion protein is a TfRMAb, which mediates delivery of the IDUA across both the BBB and at the neuronal cell membrane. These studies provide the rationale for treatment of the brain of humans with MPSI with IgG-IDUA fusion proteins that enable the receptor-mediated transport of the lysosomal enzyme across membrane barriers in the brain from the blood.

AUTHOR INFORMATION

Corresponding Author

*UCLA Warren Hall 13-164, 900 Veteran Ave., Los Angeles, CA 90024. Phone: (310) 825-8858. Fax: (310) 206-5163. E-mail: wpardridge@mednet.ucla.edu.

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