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AGT-181: Expression in CHO Cells and Pharmacokinetics, Safety, and Plasma Iduronidase Enzyme Activity in Rhesus Monkeys

Ruben J. Boado^{1,2}, Eric K.-W. Hui¹, Jeff Zhiqiang Lu¹, and William M. Pardridge²

¹ ArmaGen Technologies, Inc., Santa Monica, CA 90401

² UCLA, Los Angeles, CA 90024

Abstract

Enzyme replacement therapy is not effective for the brain, owing to the lack of transport of the enzyme across the blood-brain barrier (BBB). Recombinant proteins such as the lysosomal enzyme, iduronidase, can penetrate the human BBB, following the re-engineering of the protein as an IgG fusion protein, where the IgG moiety targets an endogenous BBB transport system. The IgG acts as a molecular Trojan horse to ferry the fused protein into brain. AGT-181 is a genetically engineered fusion protein of human iduronidase and a chimeric monoclonal antibody against the human insulin receptor. Adult Rhesus monkeys were administered repeat intravenous doses of AGT-181 ranging from 0.2–20 mg/kg. Chronic AGT-181 dosing resulted in no toxicity at any dose, no changes in organ histology, no change in plasma or cerebrospinal fluid glucose, and no significant immune response. AGT-181 was rapidly removed from plasma, based on measurements of either plasma immunoreactive AGT-181 or plasma iduronidase enzyme activity. Plasma pharmacokinetics analysis showed a high systemic volume of distribution, and a clearance rate comparable to a small molecule. The safety pharmacology studies provide the basis for future drug development of AGT-181 as a new therapeutic approach to treatment of the brain in Hurler's syndrome.

Keywords

Trojan horse; blood-brain barrier; insulin receptor; primate; iduronidase

1. Introduction

There are over 40 lysosomal storage disorders (Neufeld, 1991), and about 75% affect the brain (Cheng and Smith, 2003). The standard treatment of these disorders is enzyme replacement therapy (ERT) with the recombinant enzyme (Brady and Schiffmann, 2004). However, ERT is not active in the brain (Wraith, 2001), because the enzyme is not transported across the blood-brain barrier (Miebach, 2005). Mucopolysaccharidosis (MPS) Type I, Hurler's syndrome, is caused by mutations in the gene encoding the lysosomal enzyme, α -L-iduronidase (IDUA) (Scott et al, 1991). Prior work has shown that IDUA can be made transportable across the BBB following the re-engineering of the enzyme as an IgG fusion protein, where the IgG part is a chimeric monoclonal antibody (MAb) against the human insulin receptor (HIR) (Boado et al,

To whom correspondence should be addressed: Dr. William M. Pardridge, ArmaGen Technologies, Inc., 914 Colorado Ave., Santa Monica, CA 90401, Ph: 310-917-1275, Fax: 310-917-1276, wpardridge@armagen.com.

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2008). The HIRMAb acts as a molecular Trojan horse to ferry the IDUA across the BBB and into the lysosomal compartment of target cells. The HIRMAb targets the insulin receptor only in humans and Old World primates, such as the Rhesus monkey, and is not active in other species (Pardridge et al, 1995). The HIRMAb-IDUA fusion protein is transported across the Rhesus monkey BBB in vivo at rates that enable normalization of brain IDUA enzyme activity (Boado et al, 2008).

The HIRMAb-IDUA fusion protein, designated AGT-181, was previously expressed transiently in COS cells (Boado et al, 2008). In the present work, the identical HIRMAb-IDUA fusion protein was expressed in permanently transfected Chinese hamster ovary (CHO) cells. The AGT-181 fusion protein was purified with 3 chromatographic columns, followed by nanofiltration, and formulation as a sterile liquid. The purpose of these studies was to perform an initial chronic dosing of AGT-181 in Rhesus monkeys. The histology of brain and other major organs at the end of the study, and the formation of antibodies directed against AGT-181, were examined. Study parameters included glycemic control in plasma and cerebrospinal fluid (CSF), plasma clearance of the immunoreactive HIRMAb-IDUA fusion protein, and plasma IDUA enzyme activity. These studies describe the safety profile of an IgG-enzyme fusion protein that is derived from a monoclonal antibody that targets the human insulin receptor.

2. Materials and Methods

2.1 Engineering of tandem vector and production of CHO line

The cDNA encoding the human IDUA cDNA, minus the sequence encoding the signal peptide, was fused to the carboxyl terminus of the CH3 region of the heavy chain (HC) of the chimeric HIRMAb. A tandem vector (TV) was engineered in which the expression cassettes encoding this fusion HC, as well as the HIRMAb light chain (LC), and the murine DHFR, on a single strand of DNA (Boado et al, 2007). The 3 expression cassettes spanned 7,822 nucleotides. The light chain was comprised of 234 amino acids (AA), which included a 20 AA signal peptide. The predicted molecular weight of the light chain is 23,398 Da with a predicted isoelectric point (pI) of 5.45. The fusion protein of the HIRMAb heavy chain and IDUA was comprised of 1,091 AA, which included a 19 AA signal peptide. The predicted molecular weight of the heavy chain, without glycosylation, is 118,795 Da with a predicted pI of 8.85. The domains of the fusion heavy chain include a 113 AA variable region of the heavy chain (VH) of the HIRMAb, a 330 AA human IgG1 constant (C)-region, a 2 AA linker (Ser-Ser), and the 627 AA IDUA.

The TV was linearized and DG44 CHO cells, adapted to serum free medium (SFM), were electroporated with the tandem vector, selected with G418 and hypoxanthine-thymine deficient medium, and amplified with graded increases of methotrexate (MTX) up to 80 nM. The CHO line underwent 2 successive rounds of 1 cell/well dilutional cloning, and positive clones were selected by measurement of medium human IgG concentrations by enzyme-linked immunosorbent assay (ELISA). The CHO line was stable through multiple generations, and produced medium IgG levels of 10–20 mg/L in shake flasks at a cell density of 1–2 million cells/mL in serum-free medium (SFM).

2.2 Manufacturing and analysis of fusion protein

The AGT-181 fusion protein was manufactured in a setting that could be replicated in future Good Manufacturing Practice (GMP) production for clinical trials. A 50L Wave bioreactor was seeded with the transfected CHO cells, and the medium was expanded to 22L. The bioreactor was maintained for approximately 3 weeks in perfusion mode, where approximately 20L of SFM was perfused and collected each day. The viable cell density peaked at 8 million cells/mL, and the medium IgG peaked at 140 mg/L. Approximately 250L of conditioned

medium was clarified with depth filtration, and the fusion protein was initially purified with a 1.0L column of MAb Select Xtra in a 100/500 PBG column (GE Life Sciences, Chicago, IL). The protein A purified fusion protein was then purified with cation exchange chromatography (SP Sepharose, GE), anion exchange chromatography (Q Sepharose, GE), Virosart CPV nano-filtration (Sartorius, Goettingen, Germany), and final diafiltration against 0.01 M sodium acetate/0.14 M NaCl/pH=5.5 (ABS) buffer. The yield of AGT-181 fusion protein in the final drug product was 6 grams of protein from the 22L bioreactor. CHO host protein was <1 parts per million (PPM), as determined by ELISA (Cygnus Technologies, Southport, NC); protein A was 7 PPM, as determined by ELISA (Cygnus Technologies); DNA was <0.002 PPM as determined by real time PCR using CHO cell DNA as the assay standard; and endotoxin was 0.3 EU/mg protein, as determined by the limulus amoebocyte lysate assay (Lonza Biologicals, Portsmouth, NH). The final product was a clear, colorless solution of 5.5 mg/mL, and conformed to specifications with regard to identity (human IgG and IDUA Western blotting), potency (HIR binding affinity, IDUA enzyme activity), purity [sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion high performance liquid chromatography (HPLC), and cation exchange HPLC], amino acid analysis (UCLA Core Facility, Los Angeles, CA), and glycosylation analysis (neutral monosaccharide, N-terminal oligosaccharide, Charles River, Malvern, PA). The protein was stable as a sterile liquid stored at 4°C for at least 1 year. The final concentration of the AGT-181 fusion protein that was injected into primates was confirmed by UV absorption spectrophotometry. The IDUA enzyme specific activity of the AGT-181 fusion protein was determined with a fluorometric assay described below and was 928 units/ug protein, where 1 unit=1 nmol/hr.

2.3 Primate study

Rhesus monkeys (*Macaca mulatta*) of mixed sex were used for all studies, and were housed at MPI Research, Inc. (Mattawan, MI) in stainless steel cages in a controlled environment (18 to 28° C and 30–70% relative humidity) on a 12-h light/dark cycle. Lab Diet Certified Primate Diet (PMI Nutrition International) was provided twice daily. Tap water was provided *ad libitum*. All procedures were in compliance with the *Animal Welfare Act Regulations*, and were approved by the Institutional Animal Care and Use Committee.

The animals were treated with 0, 0.2, 2, or 20 mg/kg of the AGT-181 fusion protein administered as an intravenous infusion in the saphenous vein over a 6-min period, and animals were dosed twice/week for 4 weeks for a total of 8 doses. There were 2 animals (1 male, 1 female) in each of the 4 treatment groups, for a total of 8 primates. Treatment was administered on days 1, 4, 8, 11, 15, 18, 22, and 25, followed by euthanasia on day 26.

Adult, experimentally naïve male and female Rhesus monkeys, 4.0–6.0 kg body weight, were studied. A complete physical exam, ophthalmoscopic exam, and electrocardiogram (ECG) were conducted on all animals pre-test and prior to euthanasia. Cageside observations were made twice per day, and a detailed clinical exam was performed on each animal 1 hr post-dose. Body weights were measured pre-test and twice a week during the study. Food consumption was measured daily during the study. Clinical pathologic examinations were performed pretest and prior to euthanasia, and included urinalysis, coagulation tests (prothrombin, activated partial thromboplastin time), hematologic profile (complete blood count, differential, platelets), and chemistry panel (sodium, potassium, chloride, calcium, phosphorous, total bilirubin, urea nitrogen, creatinine, total protein, albumin, globulin, cholesterol, glucose, aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase, and sorbitol dehydrogenase). Organ weights were measured and hematoxylin and eosin microscopic analysis was performed at necropsy for brain and peripheral organs (liver, lung, heart, kidney, and sciatic nerve). Brain (cerebrum, midbrain, cerebellum, medulla/pons, spinal cord) was also evaluated by glial fibrillary acid protein (GFAP) immunocytochemistry, and

by fluoro Jade B fluorescence microscopy. The primate brain was sectioned and paraffin-blocked into 9 blocks/brain, as described by Garman (2003). The brain was fixed by head-only perfusion fixation with 10% neutral buffered formalin. Blood (1 mL) was removed from the femoral vein and collected in tubes with K2-EDTA at 0, 2, 5, 30, 60 min, 2, 4, and 23 hrs after the first IV injection of the AGT-181 fusion protein, and just prior to euthanasia. Cerebrospinal fluid (CSF) was removed via the cisterna magna at 0, 3, and 23 hrs after the first IV injection of the AGT-181 fusion protein. CSF and plasma glucose were measured with a glucose oxidase enzymatic spectrophotometric method (Glucose Assay Kit, Cayman Chemical Co., Ann Arbor, MI).

2.4 IDUA enzyme activity

IDUA enzyme activity was measured in primate plasma using an enzymatic fluorometric assay and 4-methylumbelliferyl α -L-iduronide (MUBI, Glycosynth, Ltd., Cheshire, England) as the assay substrate, as described previously (Boado et al, 2008).

2.5 Measurement of immunoreactive HIRMAb-IDUA fusion protein by ELISA

The concentration of the AGT-181 fusion protein in plasma was measured with a 2-site sandwich ELISA, using the HIR ECD as the capture reagent, and an anti-IDUA antibody as the detector reagent. The assay was designed so that immunoreactivity would be measured only on the intact AGT-181 fusion protein, and requires the presence of both parts of the fusion protein, the HIRMAb moiety and the IDUA moiety, in order to measure a positive signal. A murine MAb against the HIR (ab36550, Abcam, Cambridge, MA) was plated in 96-well plates overnight at 4°C in 0.1 M NaHCO₃/8.3 (100 ng/well); this antibody binds an epitope on the HIR that is spatially removed from the HIRMAb binding site. The solution was removed by aspiration, the wells were washed with 0.01 M Tris/0.15 M NaCl/7.4 (TBS) and 100 μ L (200 ng)/well of lectin affinity purified HIR extracellular domain (ECD) was added followed by a 90 min incubation at room temperature (RT). The wells were washed with TBS/0.05% Tween-20 (TBST), and either the sample or the AGT-181 reference standard was added in 100 μ L/well followed by a 60 min incubation at RT. A 1:2000 dilution of rabbit anti-human IDUA antibody was applied in a volume of 100 μ L, followed by a 30 min incubation at RT. Following washing with TBST, a conjugate of goat anti-rabbit IgG and alkaline phosphatase (#AP-1000, Vector Labs, Burlingame, CA) was applied in a volume of 100 μ L (500 ng)/well followed by a 45 min incubation at RT. The wells were washed with TBST, and 100 μ L/well of p-nitrophenylphosphate (#P5994, Sigma Chemical Co., St. Louis, MO) was incubated in the dark at RT for 15–30 min. The color development was terminated by the addition of 100 μ L/well of 1.2M NaOH, and color development was measured with an ELISA plate reader at 405 nm. The limit of detection was 1 ng/well of AGT-181 fusion protein. The standard curve was determined with 0–1000 ng/mL solutions of AGT-181 fusion protein and was curvilinear, which was fit with a non-linear regression analysis using the AR subroutine of the BMDP Statistical Software (Statistical Solutions Ltd, Cork, Ireland). Accuracy was confirmed by spiking control Rhesus monkey serum with 10–100 ng/mL concentrations of the AGT-181 fusion protein. The intra-assay coefficient of variation (CV), and the inter-assay CV were <15%. Assay specificity was demonstrated by showing that high concentrations (10,000 ng/mL) of either the HIRMAb or human IgG1 caused no reaction in the ELISA. The rabbit polyclonal antiserum was prepared by Prosci, Inc. (Poway, CA) using recombinant human IDUA as the antigen.

2.6 Anti-HIRMAb-IDUA immune response ELISA

The presence of anti-HIRMAb-IDUA antibodies in monkey plasma was detected with a 2-site sandwich ELISA, using the AGT-181 fusion protein as the capture reagent and biotinylated AGT-181 fusion protein as the detector reagent. As a positive control, a rabbit anti-HIRMAb-

IDUA antiserum was prepared at Prosci, Inc. (Poway, CA). The rabbit anti-HIRMAb-IDUA antisera, and the respective preimmune rabbit sera, were diluted in 0.01 M NaHPO₄/0.15 M NaCl/7.4 (PBS). The AGT-181 fusion protein 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 a 1:50 dilution of Rhesus monkey plasma, or various dilutions of the rabbit anti-HIRMAb-IDUA antisera diluted in PBS. After a 60 min incubation at 37°C, the wells were washed with PBSB, and the wells were incubated with biotinylated AGT-181 fusion protein (1.25 µg/mL) 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 Labs) for a 30 min at RT. The wells were washed with PBSB, and 100 µL/well of o-phenylenediamine/H₂O₂ developing solution (#P5412, Sigma) was added for a 20 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 nm and 650 nm. The A₆₅₀ was subtracted from the A₄₉₂. The (A₄₉₂-A₆₅₀) for the PBSB blank was then subtracted from the (A₄₉₂-A₆₅₀) for the sample.

The AGT-181 fusion protein (1.0 mL of 5.5 mg/mL in ABS buffer) was biotinylated with 35 µL of 15 mg/mL sulfo biotin-LC-LC-N-hydroxysuccinimide, where LC=long chain (#21338, Pierce Chemical Co., Rockford, IL) by rocking 60 min at RT. The solution was transferred to an Ultra-15 micro-concentrator (Millipore, Boston, MA) for buffer exchange in ABS buffer, and removal of the unreacted biotin reagent. The biotinylation of the AGT-181 fusion protein was confirmed by SDS-PAGE and Western blotting, where the blot was probed with a mixture of avidin and biotinylated peroxidase. The biotinylated HIRMAb-IDUA was strongly visualized at the appropriate molecular size for both heavy chain and light chain. The effect of biotinylation on the AGT-181 fusion protein binding to the HIR ECD was measured by ELISA. The biotinylated AGT-181 fusion protein bound with high affinity to the HIR ECD with an ED₅₀ of 0.25 ± 0.03 nM. The effect of biotinylation on the IDUA enzyme activity of the AGT-181 fusion protein was measured with the enzymatic fluorometric assay, which showed no change in IDUA enzyme activity following biotinylation.

2.7 Western blotting

Immunoreactive HIRMAb-IDUA fusion protein was detected in primate plasma at 60 min after IV injection of either the 0 or 20 mg/kg dose of AGT-181 by Western blotting. Samples of plasma (1 µL) were solubilized in 100 µL of sodium dodecylsulfate (SDS) sample buffer, and 15 µL/lane was applied to a 4–12% gradient SDS-polyacrylamide gels, in parallel with AGT-181 fusion protein standard (10 ng/lane). Following blotting to nitrocellulose, the filter was probed with a rabbit polyclonal antisera against human IDUA, and binding was detected with a biotinylated goat anti-rabbit secondary antibody, as described previously (Boado et al, 2008).

2.8 Pharmacokinetics analysis

The rate of removal from plasma of the AGT-181 fusion protein was examined following acute administration to Rhesus monkeys. The serum concentration of immunoreactive AGT-181 fusion protein (ng/mL) was divided by the injection dose (ID) to compute the % ID/mL, and this data was fit to a 2-exponential equation: %ID/mL = A₁e^{-k₁t} + A₂e^{-k₂t}, A₁ and A₂ are the intercepts, and k₁ and k₂ are the slopes of each exponential function. These values were used to compute the pharmacokinetics (PK) parameters, including the mean residence time (MRT), the central volume of distribution (V_c), the extra-vascular volume of distribution (V_{ss}), the area under the concentration curve (AUC), and the systemic clearance (CL), as described previously (Pardridge and Boado, 2009). Data were weighted by 1/(%ID/mL)². The non-linear regression analysis was performed with the PAR program of the BMDP2007 Statistical Software package.

2.9 Statistical analyses

Statistical significance was determined by analysis of variance (ANOVA) and Bonferroni correction, using the 7D program of the BMDP2007 Statistical Software package.

3. Results

Administration of the study drug was associated with no deaths, no clinical findings, no change in food intake, no change in body or organ weights, no ECG changes, and no change in 40 clinical pathology blood or urine tests (Methods) for any of the treatment groups, ie, 0, 0.2, 2, or 20 mg/kg IV twice/week for 4 weeks. There were no macroscopic or microscopic changes in peripheral organs (liver, heart, kidney, lung, sciatic nerve) based on hematoxylin and eosin staining. Brain was stained with 3 methods: hematoxylin & eosin staining, GFAP immunocytochemistry, and fluoro Jade B fluorescence microscopy. The brain was examined in 10 areas: frontal lobe, occipital lobe, basal ganglia, anterior thalamus, middle thalamus, posterior thalamus, midbrain, cerebellum, medulla oblongata, and anterior spinal cord, and no significant astrogliosis, based on GFAP immunocytochemistry, or neurodegeneration, based on fluoro Jade B fluorescent microscopy, was observed. There was no change in glycemic control in any of the 4 treatment groups in plasma between 0 and 240 min after dosing (Figure 1). There was no change in CSF glucose or CSF/plasma glucose ratio at 3 hours after drug administration (Table 1). Similarly, there was no change in CSF glucose parameters at 23 hours after drug administration (data not shown). No glycosuria was detected.

A 2-site sandwich ELISA was developed for the measurement of immunoreactive AGT-181 fusion protein in plasma. The structure of the assay is shown in Figure 2A, which shows that the HIR ECD is used as the capture reagent, and an anti-IDUA antibody is used as the detector reagent. Therefore, the assay only measures intact HIRMAb-IDUA fusion protein, and does not detect the HIRMAb without the attached IDUA (Methods). The spiking of control Rhesus monkey plasma with known concentrations of the HIRMAb-IDUA reference standard yielded a linear increase in the concentration of fusion protein detected by the assay (Figure 2B). The time-dependent changes in plasma concentration of the HIRMAb-IDUA fusion protein are shown in Figure 3 following the IV injection of 0.2, 2, or 20 mg/kg of the fusion protein. The plasma IDUA enzyme activity was also measured, and changes in the plasma enzyme activity shown in Table 2 parallel the decline in plasma immunoreactive HIRMAb-IDUA fusion protein (Figure 4). The overlap of the plasma decay curve for the immunoreactive fusion protein and the IDUA enzyme activity suggests the AGT-181 fusion protein is intact during this time period, and this was confirmed by Western blotting (Figure 5). Only the ~150 kDa heavy chain of the AGT-181 fusion protein was detected in plasma with an anti-IDUA antibody, and no lower size immunoreactive species, which would be indicative of a cleaved IDUA moiety, were detected (Figure 5).

The plasma concentration curves shown in Figure 3 were fit to a 2-exponential PK model (Methods) to yield the PK parameters in Table 3. The PK profile was generally linear, as there was a linear relationship between plasma AUC and dose of HIRMAb-IDUA fusion protein (Figure 6). The fusion protein was rapidly cleared by tissues *in vivo* as the systemic clearance was 3–16 mL/kg/min (Table 3). The extravascular volume of distribution, V_{ss} , was large, 400–1000 mL/kg, and was more than 3- to 6-fold greater than the central volume of distribution, V_c (Table 3).

A 2-site sandwich ELISA was developed to measure the formation of antibodies against the AGT-181 fusion protein, and the structure of the ELISA is outlined in Figure 7A. Owing to the bivalency of antibody binding, the anti-AGT-181 antibody binds both the HIRMAb-IDUA capture reagent and the biotinylated HIRMAb-IDUA detector reagent (Figure 7A). As a positive control, a rabbit anti-HIRMAb-IDUA antiserum, and the respective pre-immune

serum, was tested for reactivity at dilutions ranging from 1:30 to 1:100,000. The pre-immune serum gives no reaction in the assay, whereas the rabbit antiserum is strongly reactive at high dilutions of the antiserum (Figure 7B). Reaction above the pre-immune level is observed at 1:30,000 dilution, and the dilution that gives 50% of the maximal titer is approximately 1:1,000 (Figure 7B). The immune signals for a 1:50 dilution of terminal primate plasma are shown in Figure 7C. Only 1 monkey showed an immune response producing an ELISA signal above a cutoff of 0.3, and there was no relationship between the low immune titer and the dose of AGT-181 (Figure 7C).

4. Discussion

The results of this study are consistent with the following conclusions. First, chronic dosing of AGT-181 has an excellent safety profile at all doses with no clinical, histologic, or laboratory findings, and no effect on glycemic control in plasma (Figure 1) or CSF (Table 1). Second, the AGT-181 fusion protein is stable in vivo, based on the parallel plasma decay curves for the immunoreactive fusion protein and plasma IDUA enzyme activity (Table 2, Figures 3 and 4), and the Western blot analysis (Figure 5). Third, the PK profile of AGT-181 is similar to that of a small molecule, as demonstrated by the rapid clearance of the fusion protein from plasma and the high systemic volume of distribution (Table 3). Fourth, there is a minimal immune response generated by repeat dosing of the AGT-181 fusion protein, based on a sandwich ELISA (Figure 7).

AGT-181 represents a re-engineering of human recombinant IDUA as an IgG-enzyme fusion protein so as to make the IDUA transportable across the human BBB (Pardridge, 2008). The AGT-181 toxicology study was performed in the Rhesus monkey, since the HIRMAb cross reacts with the insulin receptor of Old World primates, such as the Rhesus monkey, but not New World primates (Pardridge et al, 1995). A recent Tissue Cross-Reactivity (TCR) study with a fusion protein of the HIRMAb and glial derived neurotrophic factor (GDNF) showed a parallel interaction of the HIRMAb fusion protein with the insulin receptor in brain and peripheral organs in the human and Rhesus monkey (Pardridge and Boado, 2009). Toxicity arising from repeat dosing of AGT-181 in the Rhesus monkey would most likely be caused by the HIRMAb part of the fusion protein, since IDUA, a normal lysosomal enzyme, has been administered to humans chronically without limiting toxicity (Clarke et al, 2009). Toxicity caused by HIRMAb would most likely arise from agonist or antagonist interactions with the insulin receptor, causing either hypo- or hyper-glycemia. However, chronic administration of very high doses of the HIRMAb-IDUA fusion protein has no effect on glycemic control in either plasma (Figure 1) or CSF (Table 1). No specific effect on CSF glucose is expected since glucose uptake by brain is not regulated by insulin (Daniel et al, 1976). The highest dose of AGT-181 administered in this study was 20 mg/kg (Methods). This dose is >30-fold greater than the expected therapeutic dose of AGT-181 in humans, 0.6 mg/kg. As shown in prior brain uptake studies in primates (Boado et al, 2008), the administration of 0.6 mg/kg of the HIRMAb-IDUA fusion protein is projected to cause a 100% normalization of brain IDUA enzyme activity. Even lower doses would be expected to be therapeutic, since tissue clearance of lysosomal storage products occurs with <5% replacement of tissue lysosomal enzyme activity (Muenzer and Fisher, 2004). Therefore, this initial dose-finding study suggests there may be a wide safety margin for future administration of AGT-181 in humans.

The efficacy of the AGT-181 fusion protein in vivo would be lost if the peptide linkage between the HIRMAb heavy chain and the IDUA enzyme was cleaved prior to entry into the CNS. However, this linker is a short 2-amino acid sequence (serine-serine) (Boado et al, 2008), which is not generally subject to endoproteases. The stability of the circulating AGT-181 fusion protein in vivo is demonstrated by the parallel decline in HIRMAb-IDUA immunoreactivity and IDUA enzyme activity (Figure 4). Owing to the design of the sandwich ELISA used to

measure immunoreactive AGT-181 (Figure 2A), both the HIRMAb and the IDUA parts of the fusion protein must be intact in order to be detected in the assay. The stability of the AGT-181 fusion protein in blood is corroborated by the Western blot studies, which detects only the HIRMAb-IDUA fusion heavy chain in plasma, and not free IDUA (Figure 6).

The pharmacokinetics (PK) of AGT-181 clearance from plasma can be determined from either measurements of plasma immunoreactive HIRMAb-IDUA fusion protein (Figure 3) or plasma IDUA enzyme activity (Table 2), since both parameters show comparable decay curves (Figure 4). This correlation also provides evidence for the specificity of the sandwich ELISA used to measure AGT-181 in plasma (Figure 1A). The plasma PK properties of AGT-181 are comparable to a small molecule and depart dramatically from the classical PK of a typical MAb-based therapeutic. Monoclonal antibodies are cleared very slowly from plasma with mean residence times (MRT) of days or even weeks. In contrast, the MRT of AGT-181 ranges from 1–2 hours depending on the dose (Table 3). MAb drugs have systemic volumes of distribution (V_{ss}) that are comparable to the central volume of distribution (V_c). In contrast, the V_{ss} of AGT-181 is several-fold greater than the V_c (Table 3). The high V_{ss} of AGT-181 arises from the rapid egress of the fusion protein from the plasma compartment owing to receptor-mediated uptake into tissues via the insulin receptor. This rapid egress from plasma is reflected in the high rates of clearance of AGT-181 in the primate, 3–16 ml/kg/min (Table 3).

The rapid uptake of AGT-181 by peripheral tissues was also demonstrated in a prior study in the Rhesus monkey, which used 125I-labeled HIRMAb-IDUA fusion protein (Boado et al, 2008). IDUA is normally taken up by peripheral tissues via the mannose-6-phosphate receptor (M6PR) (Tsukimura et al, 2008). However, following fusion to the HIRMAb, the IDUA enters cells via the insulin receptor, not the M6PR (Boado et al, 2008). The HIRMAb-IDUA is taken up by fibroblasts from MPS-I patients, and confocal microscopy demonstrated triaging of the AGT-181 fusion protein into the lysosomal compartment of target cells, as well as a reduction in glycosaminoglycan (GAG) accumulation within the target cell (Boado et al, 2008). In parallel with the uptake of the AGT-181 fusion protein by peripheral tissues, the fusion protein also rapidly distributes into the brain in vivo, as demonstrated previously in the adult Rhesus monkey (Boado et al, 2008). Approximately 1% of the injected dose is taken up by the primate brain in vivo. Since the IDUA enzyme specific activity of the fusion protein is high, 928 units/mg protein (Methods), the high brain uptake of the AGT-181 fusion protein can cause a nearly 100% normalization of brain IDUA enzyme activity following the administration of a therapeutic dose of 0.6 mg/kg of the fusion protein (Boado et al, 2008).

Engineered IgG-enzyme fusion proteins are potentially immunogenic molecules. However, recent evidence suggests that the re-engineering of recombinant proteins as IgG fusion proteins may actually induce immune tolerance. The constant region of human IgG contains amino acid sequences, called Tregitopes, which interact with circulating T lymphocytes to induce immune tolerance (DeGroot et al, 2008). The immunogenicity of the AGT-181 in this short repeat dosing study was evaluated with the sandwich ELISA shown in Figure 7A. A rabbit antiserum against AGT-181 was produced as a positive control, and the assay shows a very high immune response at dilutions as high as 1:10,000 (Figure 7B). However, the immune response against repeat dosing of AGT-181 in the Rhesus monkey is mild, with only 1 monkey showing an ELISA signal above the cutoff at a very low dilution, 1:50, of primate plasma (Figure 7C).

In summary, these studies describe the initial toxicologic, PK, and immune response evaluations following repeat dosing of a new HIRMAb-IDUA fusion protein, AGT-181, in the adult Rhesus monkey. The drug is shown to have an excellent safety profile following a 4-week chronic dosing. Recombinant IDUA does not cross the BBB and cannot treat the brain in MPS-I. AGT-181 represents a re-engineering of IDUA so that this lysosomal enzyme is

transportable across the human BBB via receptor-mediated transport on the endogenous BBB insulin receptor. AGT-181 was engineered with the intent to treat the brain in MPS-I. If the drug development pathway of AGT-181 is successful, the BBB molecular Trojan horse technology could be applied to other lysosomal enzymes, and other recombinant proteins, to enable penetration of the human brain following non-invasive systemic administration.

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References

- Boado RJ, Zhang Y, Pardridge WM. Genetic engineering, expression, and activity of a fusion protein of a human neurotrophin and a molecular Trojan horse for delivery across the human blood-brain barrier. *Biotechnol Bioeng* 2007;97:1376–1386. [PubMed: 17286273]
- Boado RJ, Zhang Y, Xia CF, Wang Y, Pardridge WM. Genetic engineering of a lysosomal enzyme fusion protein for targeted delivery across the human blood-brain barrier. *Biotechnol Bioeng* 2008;99:475–484. [PubMed: 17680664]
- Brady RO, Schiffmann R. Enzyme-replacement therapy for metabolic storage disorders. *Lancet Neurol* 2004;3:752–756. [PubMed: 15556808]
- Cheng SH, Smith AE. Gene therapy progress and prospects: gene therapy of lysosomal storage disorders. *Gene Ther* 2003;10:1275–1281. [PubMed: 12883523]
- Clarke LA, Wraith JE, Beck M, Kolodny EH, Pastores GM, Muenzer J, Rapoport DM, Berger KI, Sidman M, Kakkis ED, Cox GF. Long-term efficacy and safety of laronidase in the treatment of mucopolysaccharidosis I. *Pediatrics* 2009;123:229–240. [PubMed: 19117887]
- Daniel PM, Love ER, Pratt OE. Insulin and the way the brain handles glucose. *J Neurochem* 1975;25:471–476. [PubMed: 1151383]
- De Groot AS, Moise L, McMurry JA, Wambre E, Van Overtvelt L, Moingeon P, Scott DW, Martin W. Activation of natural regulatory T cells by IgG Fc-derived peptide “Tregitopes”. *Blood* 2008;112:3303–3311. [PubMed: 18660382]
- Garman RH. Evaluation of large-sized brains for neurotoxic endpoints. *Toxicol Pathol* 2003;31(Suppl): 32–43. [PubMed: 12597429]
- Miebach E. Enzyme replacement therapy in mucopolysaccharidosis type I. *Acta Paediatr Suppl* 2005;94:58–60. [PubMed: 15895714]discussion 57
- Muenzer J, Fisher A. Advances in the treatment of mucopolysaccharidosis type I. *N Engl J Med* 2004;350:1932–1934. [PubMed: 15128891]
- Neufeld EF. Lysosomal storage diseases. *Annu Rev Biochem* 1991;60:257–280. [PubMed: 1883197]
- Pardridge WM. Re-engineering biopharmaceuticals for delivery to brain with molecular Trojan horses. *Bioconjug Chem* 2008;19:1327–1338. [PubMed: 18547095]
- Pardridge WM, Boado RJ. Pharmacokinetics and Safety in Rhesus Monkeys of a Monoclonal Antibody-GDNF Fusion Protein for Targeted Blood-Brain Barrier Delivery. *Pharm Res*. 2009;in press
- Pardridge WM, Kang YS, Buciak JL, Yang J. Human insulin receptor monoclonal antibody undergoes high affinity binding to human brain capillaries in vitro and rapid transcytosis through the blood-brain barrier in vivo in the primate. *Pharm Res* 1995;12:807–816. [PubMed: 7667183]
- Scott HS, Anson DS, Orsborn AM, Nelson PV, Clements PR, Morris CP, Hopwood JJ. Human alpha-L-iduronidase: cDNA isolation and expression. *Proc Natl Acad Sci U S A* 1991;88:9695–9699. [PubMed: 1946389]
- Tsukimura T, Tajima Y, Kawashima I, Fukushima T, Kanzaki T, Kanekura T, Ikekita M, Sugawara K, Suzuki T, Togawa T, Sakuraba H. Uptake of a recombinant human alpha-L-iduronidase (laronidase) by cultured fibroblasts and osteoblasts. *Biol Pharm Bull* 2008;31:1691–1695. [PubMed: 18758061]
- Wraith JE. Enzyme replacement therapy in mucopolysaccharidosis type I: progress and emerging difficulties. *J Inher Metab Dis* 2001;24:245–250. [PubMed: 11405343]

Figure 1. Plasma glucose in 4 treatment groups of Rhesus monkeys at the pre-test (0 minutes), and various times after the bolus IV injection of 0, 0.2, 2, or 20 mg/kg of the AGT-181 fusion protein. Data are mean $\pm$ S.D. (n=2 animals per group). There are no significant changes in plasma glucose among the treatment groups.

Figure 2.

(A) Structure of the 2-site ELISA used to measure the concentration of the HIRMAb-IDUA fusion protein. A mouse monoclonal antibody (mMAb) against the HIR is plated, which captures the HIR ECD derived from CHO cells. The HIRMAb part of the fusion protein binds the HIR ECD, and the IDUA part of the AGT-181 fusion protein is bound by a rabbit anti-IDUA antibody, which is bound by a conjugate of a goat anti-rabbit (GAR) antibody and alkaline phosphatase (AP). (B) Control Rhesus monkey plasma was spiked with 5 different doses of the HIRMAb-IDUA reference standard. There is a linear correlation between the observed concentration and the predicted concentration of fusion protein.

Figure 3.
Concentration of immunoreactive AGT-181 at different times after IV injection of 3 different doses, 0.2, 2, 20 mg/kg, of the fusion protein.

Figure 4. Comparison of the concentration of immunoreactive HIRMAb-IDUA fusion protein and plasma IDUA enzyme activity at different times after an IV injection of 0.2 mg/kg AGT-181. Data are expressed as a % of injected dose (ID)/mL plasma.

Figure 5.

Western blot with an anti-IDUA antiserum. Lane 1: 10 ng AGT-181 Reference Standard; lane 2: male monkey treated with vehicle; lane 3: female monkey treated with vehicle; lane 4: male monkey treated with 20 mg/kg AGT-181; lane 5: female monkey treated with 20 mg/kg AGT-181. Plasma samples were taken 60 min after IV injection of vehicle or AGT-181.

Figure 6. Linear relationship between the plasma AUC and the dose of HIRMAb-IDUA fusion protein. Data are mean $\pm$ S.D. (n=2 animals/group).

Figure 7.

(A) Structure of the 2-site ELISA for detection of antibodies against the HIRMAb-IDUA fusion protein. The HIRMAb-IDUA fusion protein is used as the capture reagent, and the biotinylated HIRMAb-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 various dilutions of a rabbit antiserum prepared against the HIRMAb-IDUA fusion protein (diamonds), and the respective pre-immune serum (squares). Data are means of duplicates that varied <10%. (C) Absorbance at 1:50 dilutions of Rhesus monkey plasma taken at day 28, following the administration of 0, 0.2, 2, or 20 mg/kg of the HIRMAb-IDUA fusion protein twice/week for 4 consecutive weeks of the study. A significant immune reaction is registered with an (A490–A655) signal >0.3.

Table 1

CSF glucose parameters at 3 hours after AGT-181 administration

Dose (mg/kg)	CSF glucose (mg/dl)	Plasma glucose (mg/dl)	CSF/plasma ratio
0	97 ± 21	97 ± 30	1.02 ± 0.10
0.2	119 ± 23	107 ± 21	1.11 ± 0.10
2	64 ± 2	73 ± 4	0.87 ± 0.02
20	85 ± 5	124 ± 29	0.70 ± 0.12

Mean ± SD (n=2 monkeys/group). No statistical differences between the control group (dose=0) and the treatment groups (dose=0.2–20 mg/kg) by ANOVA.

Table 2

Plasma IDUA enzyme activity in Rhesus monkeys

	units/mL		
minutes	0.2 mg/kg	2.0 mg/kg	20.0 mg/kg
0	34 ± 9	21 ± 11	40 ± 11
2	746 ± 294	10,506 ± 545	172,040 ± 5,503
5	312 ± 117	6,432 ± 592	115,264 ± 5,330
30	120 ± 42	4,854 ± 464	75,584 ± 977
60	81 ± 34	3,926 ± 342	65,288 ± 4,185
120	58 ± 22	3,721 ± 64	53,230 ± 745
240	25 ± 15	2,394 ± 162	33,155 ± 926
1380	27 ± 12	17 ± 5	224 ± 57

Mean ± SE (n=2 per group).

Table 3**HIRMAb-IDUA Pharmacokinetic Parameters**

Parameter	units	AGT-181 dose		
		0.2 mg/kg	2.0 mg/kg	20.0 mg/kg
A1	%ID/mL	0.099 ± 0.047	0.106 ± 0.056	0.167 ± 0.113
k1	min ⁻¹	0.441 ± 0.150	0.441 ± 0.150	0.376 ± 0.235
A2	%ID/mL	0.0136 ± 0.0019	0.0387 ± 0.0049	0.0448 ± 0.0097
k2	min ⁻¹	0.0131 ± 0.0010	0.0086 ± 0.0009	0.00771 ± 0.00161
MRT	min	63 ± 5	109 ± 11	121 ± 24
Vc	mL/kg	181 ± 66	135 ± 55	96 ± 52
Vss	mL/kg	1020 ± 125	448 ± 50	393 ± 75
AUCss	%ID·min/mL	1.26 ± 0.10	4.78 ± 0.32	6.26 ± 0.68
AUCss	ug·min/mL	12.5 ± 1.0	487 ± 32	6093 ± 662
CL	mL/kg/min	16.2 ± 1.2	4.10 ± 0.27	3.27 ± 0.36
BW	kg	4.9 ± 0.5	5.1 ± 0.9	4.9 ± 0.9
Dose	mg	0.98 ± 0.10	10.2 ± 1.8	97.5 ± 17.5
No. animals		2	2	2

Based on ELISA measurements of plasma immunoreactive HIRMAb-IDUA (Figure 3).