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Blood-Brain Barrier Molecular Trojan Horse Enables Imaging of Brain Uptake of Radioiodinated Recombinant Protein in the Rhesus Monkey

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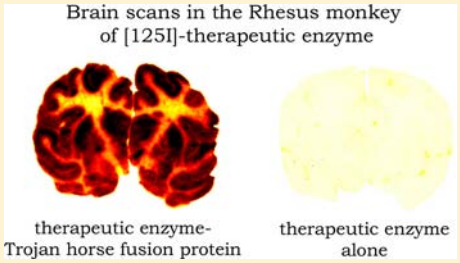
ABSTRACT: Recombinant proteins are large molecule drugs that do not cross the blood-brain barrier (BBB). However, BBB-penetration of protein therapeutics is enabled by re-engineering the recombinant protein as IgG fusion proteins. The IgG domain is a monoclonal antibody (mAb) against an endogenous BBB receptor-mediated transport system, such as the human insulin receptor (HIR), and acts as a molecular Trojan horse to ferry the fused protein across the BBB. In the present study, a recombinant lysosomal enzyme, iduronate 2-sulfatase (IDS), is fused to the HIRMAb, and BBB penetration of the IDS alone vs the HIRMAb-IDS fusion protein is compared in the Rhesus monkey. Recombinant IDS and the HIRMAb-IDS fusion protein were radiolabeled with indirect iodination with the [^{125}I]-Bolton-Hunter reagent and with direct iodination with Iodogen/[^{125}I]-idodine. IDS and the HIRMAb-IDS fusion protein have comparable plasma pharmacokinetics and uptake by peripheral organs. IDS does not cross the BBB. The HIRMAb-IDS fusion protein crosses the BBB and the brain uptake is 1% of injected dose/brain. Brain imaging shows HIRMAb-IDS penetration to all parts of brain, and immunoprecipitation of brain radioactivity shows intact fusion protein in brain. The use of BBB molecular Trojan horses enables brain imaging of recombinant proteins that are re-engineered for BBB transport.



INTRODUCTION

Brain imaging of the uptake from blood of recombinant protein therapeutics is possible with external imaging such as positron emission tomography, providing the therapeutic protein is enabled to cross the blood-brain barrier (BBB). Proteins may be re-engineered for BBB transport with molecular Trojan horse technology that produces fusion proteins of an IgG and the protein therapeutic. The IgG domain is a monoclonal antibody (mAb) against an endogenous BBB receptor transporter, such as the human insulin receptor (HIR). The HIRMAb binds the BBB insulin receptor, and this triggers transport across the BBB via the endogenous insulin receptor.[1] The HIRMAb acts as a molecular Trojan horse and ferries the fused therapeutic protein across the BBB.[2]

The model therapeutic protein used in this study is the lysosomal enzyme, iduronate 2-sulfatase (IDS).[3] Children with Mucopolysaccharidosis (MPS)-II carry mutations in the IDS gene, and are treated with enzyme replacement therapy and recombinant human IDS (idursulfase, Elaprase).[4] However, most children born with MPS-II have serious involvement of the central nervous system.[5] Enzyme replacement therapy does not treat the brain in MPS-II,[6] and this is attributed to the lack of transport across the BBB of IDS, a large molecule therapeutic. However, the BBB transport of radiolabeled human recombinant IDS has not been previously measured, and one report suggests that blood-borne IDS does penetrate the BBB, based on measurements of whole brain IDS enzyme activity.[7]

Recombinant IDS has been re-engineered to enable BBB penetration using the BBB molecular Trojan horse technology.[8,9] An IgG-IDS enzyme fusion protein is produced wherein the IgG domain of the fusion protein is a receptor-specific mAb that targets an endogenous BBB receptor such as the insulin receptor or the transferrin receptor. The receptor-specific mAb's are species specific.[2] For BBB delivery in humans, a genetically engineered HIRMAb comprises the IgG domain of the IgG-IDS fusion protein.[8,9] The HIRMAb is biologically active in Old World primates such as the Rhesus monkey, but is not active in New World primates[1] or lower animals including rodents.[10]

The purpose of this study was twofold. First, the BBB transport of IDS and the HIRMAb-IDS fusion protein was compared in the Rhesus monkey following radiolabeling of each protein with ^{125}I. Since the results of this investigation could provide support for future neuro-imaging in humans with positron emission tomography and [^{124}I]-labeled proteins, a second objective of this study was to examine 2 methods for the radio-iodination of IDS and the HIRMAb-IDS fusion protein, iodination with ^{125}I and Iodogen, and iodination with the [^{125}I]-Bolton-Hunter reagent. The Iodogen method is direct iodination of surface tyrosine residues, and is an oxidative process that can potentially damage the protein. The Bolton-Hunter reagent is indirect iodination, as the ^{125}I-Bolton-Hunter

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reagent covalently links to surface lysine residues via a nonoxidative reaction.

EXPERIMENTAL PROCEDURES

Recombinant Proteins. Recombinant human IDS (idursulfase, Elaprase) was obtained from the UCLA Pharmacy. The HIRMAb-IDS fusion protein, also designated AGT-182, was produced from stably transfected Chinese hamster ovary cells as previously described.[9] The HIRMAb-IDS fusion protein was purified by protein A affinity chromatography, cation exchange chromatography, anion exchange chromatography, and nanofiltration. The final Drug Product met acceptance criteria for identity, purity, potency, sterility, and safety. The IDS enzyme specific activity of Elaprase and the HIRMAb-IDS fusion protein was measured with a 2-step fluorometric enzyme assay with 4-methylumbelliferyl α-L-iduronide-2-sulfate as substrate as previously described.[8,9] One unit is defined as nmol per hour at 37 °C.

Radio-Iodination. The IDS and the HIRMAb-IDS fusion protein were custom radiolabeled at American Radiolabeled Chemicals, Inc. (St. Louis, MO) using either the Iodogen method or the Bolton-Hunter method. For the direct Iodogen method, the proteins were labeled with ^{125}I and Iodogen as described previously for IDS iodination.[11] IDS (1 nmol), or the HIRMAb-IDS fusion protein (1 nmol), was added to tubes precoated with 50 ug Iodogen (Pierce/Thermo-Scientific, Rockville, IL), and 74 MBq (2.0 mCi) of ^{125}I was added with incubation at room temperature for 20 min. The reaction was terminated by the addition of 50 μL of 20 mg/mL sodium metabisulfite. The labeled protein was purified with a 1 × 28 cm column of Sephadex G25 with elution in either 0.14 M NaCl/20 mM Na_2HPO_4/0.022% Tween-20/pH = 6.0 (PBST) for IDS, or 0.14 M NaCl/10 mM sodium acetate/0.001% Tween-80/pH = 6.0 (ABST) for the HIRMAb-IDS fusion protein. The trichloroacetic acid (TCA) precipitability of the protein was 98% and the specific activity was 0.3−1.0 MBq/μg (8−26 μCi/μg). For the indirect Bolton-Hunter method, 74 MBq (2.0 mCi) of ^{125}I-Bolton-Hunter reagent (monoiodinated) was transferred to a new tube and the benzene solvent was evaporated. The IDS (2 nmol) or the HIRMAb-IDS fusion protein (2 nmol) was added in 250 μL of PBST or ABST, respectively, followed by capping and incubation at RT for 45 min. The reaction was terminated by the addition of 25 μL of 1 M glycine. The labeled protein was purified with the same G25 columns as used for the Iodogen method to a TCA precipitability of 99% and a specific activity of 0.15−0.56 MBq/μg (4−15 μCi/μg). Following radiolabeling, the proteins were transferred a 5 mL glass vial with a 20 mm stopper and aluminum seal, frozen on dry ice, and shipped on dry ice overnight to the animal testing facility, MPI Research, Inc., Mattawan, MI, for next day intravenous injection in the Rhesus monkey.

Rhesus Monkey Pharmacokinetics and Brain and Organ Uptake. All experimental animal procedures were conducted according to Animal Welfare Act and the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and study protocols were approved by the Animal Research Committee at MPI Research, Inc. (Mattawan, MI). A total of 4 male Rhesus monkeys, weighing 6.1−6.6 kg, were investigated at MPI Research. The animal was administered 48 MBq (1300 μCi) of [^{125}I]-IDS, or [^{125}I]-HIRMAb-IDS fusion protein by intravenous (IV) bolus injection over 30 s in the left femoral vein. The injection dose (ID) ranged from 12 to 55 ug/kg. The animal was anesthetized with intramuscular ketamine. Following intravenous drug administration, femoral venous plasma was obtained at 2, 5, 15, 30, 60, 90, and 120 min for determination of total plasma [^{125}I] radioactivity (DPM/mL) and plasma radioactivity that is precipitated by 10% cold TCA. The plasma area under the concentration curve (AUC) for any given time after IV injection was determined with the trapezoid rule for both the TCA-precipitable and the TCA-soluble radioactivity in plasma. The animal was euthanized at 120 min after IV administration, and samples of major organs (heart, liver, spleen, lung, skeletal muscle, and omental fat) were removed, weighed, and processed for determination of radioactivity. The cranium was opened and the brain was removed. The mean and standard deviation (SD) for plasma samples was determined from replicates of 3 and for organ samples in replicates of 9.

The brain was prepared for film autoradiography and ex vivo brain imaging with the preparation of coronal sections through the forebrain, the midbrain, and the hindbrain. The brain was removed and cut into 8 mm coronal slabs with a custom Rhesus monkey matrix (ASI Instruments, Warren, MI), and the slabs were frozen in powdered dry ice, wrapped in aluminum foil, and shipped overnight on dry ice.

Samples (~2 g) of frontal cortex were removed for capillary depletion analysis to confirm transport of the protein across the BBB. The capillary depletion method separates the vascular tissue in brain from the postvascular compartment as described previously.[12] Based on measurements of the specific activity of brain capillary-specific enzymes, such as γ-glutamyl transpeptidase or alkaline phosphatase, the postvascular supernatant is >95% depleted of brain vasculature.[12] The vascular pellet and postvascular supernatant were counted for ^{125}I radioactivity in parallel with the homogenate. The volume of distribution (VD) was determined for each of the fractions from the ratio of total [^{125}I] radioactivity in the brain fraction (DPM/gram brain) divided by the total [^{125}I] radioactivity in the 120 min terminal plasma (DPM/μL plasma). The percent of radioactivity in the postvascular supernatant that was precipitable with 10% cold TCA was determined.

The TCA precipitable [^{125}I] radioactivity in plasma, DPM/mL, was converted to % injected dose (ID)/mL, and the %ID/mL was fit to a biexponential equation,

$$\%\mathrm{ID/mL} = \mathrm{A1}e^{-k1t} + \mathrm{A2}e^{-k2t}$$

Pharmacokinetic parameters were computed as described previously.[1,9]

The BBB permeability-surface area (PS) product, which has units of μL/min/gram, was computed as reviewed by Bickel[13]

$$\mathrm{PS\ product} = [(\mathrm{VD} - \mathrm{Vo})]\mathrm{Cp(T)}/\mathrm{AUC}(t)$$

where VD is the terminal brain/plasma ratio or volume of distribution (VD), Vo is the brain plasma volume, Cp(T) is the terminal plasma concentration, and AUC(t) is the plasma area under the concentration curve over the 120 min circulation period. The brain plasma volume in the Rhesus monkey, 20 ± 6 uL/gram, was determined with a [^{3}H]-labeled human IgG1 isotype control antibody that does not recognize a BBB receptor.[14] If the brain VD ≤ Vo, then the PS product = 0, which is indicative of a lack of penetration of the BBB.[13]

Brain Film Autoradiography. The frozen coronal slabs were cut to 20 μm frozen sections with a cryostat at −15 °C; the sections were air-dried and exposed to Kodak Biomax MR X-ray film (Carestream Health, Rochester, MN) for 7 or 10

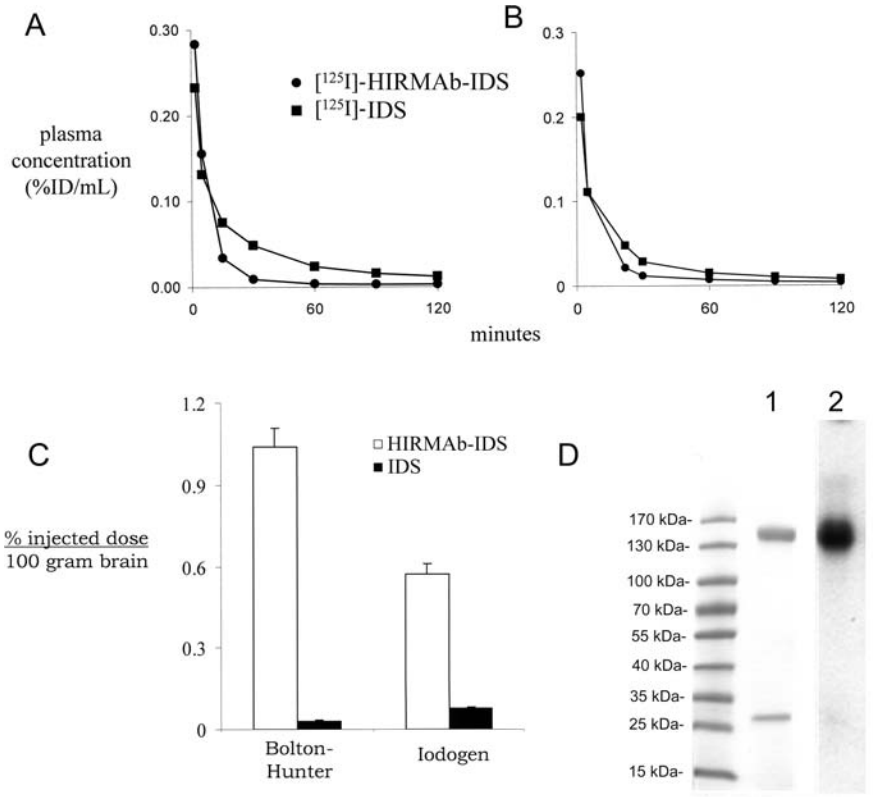

Figure 1. Plasma concentration profile of TCA-precipitable radioactivity after IV injection of either the HIRMAb-IDS fusion protein (circles) or IDS (squares) radiolabeled with either [^{125}I]-Bolton-Hunter reagent (A) or [^{125}I]-iodine and Iodogen (B). Plasma concentration is expressed as percent of injected dose (ID)/mL. (C) Brain uptake, expressed as %ID/100 g brain, for the HIRMAb-IDS fusion protein (open bars) and IDS (closed bars) radiolabeled with either [^{125}I]-Bolton-Hunter reagent (left) or [^{125}I]-iodine and Iodogen (right) at 2 h after IV administration. The data shown are for the frontal cortex gray matter. (D) (Lane 1) Migration of heavy chain and light chain of the HIRMAb-IDS fusion protein reference standard following reducing SDS-PAGE and dye staining. Molecular weight marker migration is shown on the left panel. (Lane 2) Film autoradiogram of SDS-PAGE gel separation of immunoprecipitate of radioactivity of brain removed 2 h after the IV injection of Bolton-Hunter labeled HIRMAb-IDS fusion protein. The 160 kDa heavy chain band immunoprecipitate migrates at the same molecular size as the HIRMAb-IDS fusion protein heavy chain reference standard. The light chain, which is more than 6-fold smaller than the HIRMAb-IDS fusion protein heavy chain, is barely visible as a 26 kDa band.

days followed by X-ray film development. The films were scanned and the image was saved in Photoshop, and colorized with NIH Image software. The section processing and X-ray film exposure and scanning conditions were identical for all 4 primate studies. The distribution of fusion protein in gray matter and white matter was quantified with NIH ImageJ v 1.47 analysis of the film autoradiograms for the Bolton-Hunter labeled HIRMAb-IDS fusion protein. The integrated density, which is the product of the scanned area and the mean gray area, of the grayscale image was determined for 4 regions each in gray matter and white matter of forebrain, midbrain, and hindbrain, and the mean ± SE was determined for each region.

Immunoprecipitation. For the primate injected with Bolton-Hunter labeled HIRMAb-IDS fusion protein, an immunopreciptation study was performed. To 1 g of primate brain collected at 120 min after IV injection, a 10-fold volume of lysis buffer (0.02 M Tris/0.15 M NaCl/1% NP-40/0.5% sodium deoxycholate/pH-7.0) was added followed by homogenization on a Polytron homogenizer (10 000 rpm, 20 s). The homogenate was centrifuged at 8000*g* for 10 min at 4 °C, and the supernatant was transferred to a new tube, to which was added 100 μg of protein A-agarose (Pierce Thermo Scientific #20333). The mixture was shaken overnight at 4 °C, and microfuged for 10 min at 4 °C. The immunoprecipitate pellet was washed with 10 mM Tris/0.5 M NaCl/pH = 7.0 (TBHS) twice. The final pellet was solubilized with sodium dodecyl sulfate (SDS) sample buffer, boiled, centrifuged, and spotted to a 4−15% gradient gel (BioRad, Richmon, CA), and SDS polyacrylamide gel electrophoresis (PAGE) was performed at constant voltage. An aliquot of the HIRMAb-IDS fusion protein standard was also applied to the gel, and detected by GelCode Blue staining (Pierce Thermo Scientific). Following SDS-PAGE, the gel was dried and exposed to Biomax X-ray film for 2 days, developed, dried, and scanned into Adobe Photoshop. A parallel stained image of the HIRMAb-IDS fusion protein standard was also scanned for comparison of the migration of the HIRMAb-IDS fusion protein standard and the immunoprecipitated labeled protein in brain.

RESULTS

Protein Characterization and IDS Enzyme Activity. The purity of both the IDS and the HIRMAb-IDS fusion protein was confirmed by reducing SDS-PAGE, and the identity of both proteins was confirmed by IDS Western blotting as described previously.[9] On reducing SDS-PAGE, IDS migrates as a single species of 106 kDa, whereas the HIRMAb-IDS fusion protein migrates as a 160 kDa heavy chain and a 26 kDa light chain. The enzyme activity of IDS is 271 ± 55 units/μg protein, and the IDS enzyme activity of the HIRMAb-IDS fusion protein is 246 ± 24 units/μg protein. The IDS enzyme

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Table 1. Pharmacokinetic Parameters for [^{125}I]-HIRMAb-IDS Fusion Protein and [^{125}I]-IDS[a]

		Bolton-Hunter		Iodogen	
parameter	units	HIRMAb-IDS	IDS	HIRMAb-IDS	IDS
A1	%ID/mL	0.353 ± 0.038	0.184 ± 0.049	0.253 ± 0.047	0.202 ± 0.035
A2	%ID/mL	0.0072 ± 0.0015	0.061 ± 0.018	0.013 ± 0.005	0.035 ± 0.0071
$k1$	min^{-1}	0.165 ± 0.012	0.134 ± 0.058	0.154 ± 0.031	0.162 ± 0.036
$k2$	min^{-1}	0.0093 ± 0.0023	0.0143 ± 0.0033	0.0109 ± 0.0039	0.0130 ± 0.0024
$T_{1/2}{}^{1}$	min	4.2 ± 0.3	5.2 ± 2.2	4.5 ± 0.9	4.3 ± 0.9
$T_{1/2}{}^{2}$	min	75 ± 19	48 ± 11	64 ± 23	53 ± 10
MRT	min	33 ± 9	54 ± 9	43 ± 14	55 ± 8
Vc	mL/kg	45 ± 4	67 ± 14	58 ± 10	63 ± 9
Vss	mL/kg	186 ± 46	159 ± 24	231 ± 67	205 ± 28
$AUCl^{120}$	%ID·min/mL	2.67 ± 0.15	4.86 ± 0.32	2.54 ± 0.22	3.40 ± 0.17
AUCss	%ID·min/mL	2.92 ± 0.16	5.62 ± 0.38	2.87 ± 0.27	3.97 ± 0.22
CL	mL/min/kg	5.6 ± 0.3	2.9 ± 0.2	5.4 ± 0.5	3.8 ± 0.2
Body weight	kg	6.1	6.1	6.5	6.6

[a]Parameters computed from the plasma profile of TCA-precipitable radioactivity (Figure 1A, B).

activity was not affected by either the Iodogen or Bolton-Hunter radiolabeling procedures. After radiolabeling, IDS migrated on SDS-PAGE as a single species of 106 kDa.

Plasma Pharmacokinetics. The time course of TCA-precipitable plasma radioactivity, which is expressed as %ID/mL, is shown in Figures 1A and 1B for the Bolton-Hunter and Iodogen labeled proteins, respectively. The plasma profile of TCA-precipitable radioactivity was fit to a two-exponential equation to yield the pharmacokinetic (PK) parameters shown in Table 1. The HIRMAb-IDS fusion protein and IDS are both rapidly cleared from plasma with a systemic volume of distribution (Vss) on average that is 3-fold greater than the central volume of distribution (Vc) (Table 1). The clearance rate for both proteins is high and ranges from 2.9 ± 0.2 to 5.6 ± 0.3 mL/min/kg, and the clearance rate for the HIRMAb-IDS fusion protein is about twice the clearance rate for IDS (Table 1).

Plasma Stability. The percent of plasma radioactivity that was precipitable by TCA decreased with time in proportion to the rate of clearance of the protein from plasma. The % TCA precipitability decreased from 98 ± 1% at 2 min to 23 ± 1% at 120 min after IV injection of the Bolton-Hunter labeled HIRMAb-IDS fusion protein. The % TCA precipitability decreased from 94 ± 1% at 2 min to 55 ± 1% at 120 min after IV injection of the Bolton-Hunter labeled IDS. The % TCA precipitability decreased from 97 ± 1% at 2 min to 14 ± 1% at 120 min after IV injection of the Iodogen labeled HIRMAb-IDS fusion protein. The % TCA precipitability decreased from 95 ± 1% at 2 min to 25 ± 1% at 120 min after IV injection of the Iodogen labeled IDS. The AUC for the TCA-soluble radioactivity and TCA-precipitable radioactivity in plasma was computed with the trapezoid rule and is shown in Figure 2. The AUC of the TCA-soluble radioactivity reflects the amount of low molecular weight radioactive metabolites in plasma. The plasma AUC of radioactive metabolites produced from the Iodogen-labeled protein increases with time more than twice as fast as the plasma AUC of radioactive metabolites produced from the Bolton-Hunter-labeled protein (Figure 2). The 2 h AUC of plasma TCA-soluble radioactivity is 37% of the plasma AUC of TCA-precipitable radioactivity for either the HIRMAb-IDS fusion protein (Figure 2A) or IDS (Figure 2C) radiolabeled with the Bolton-Hunter reagent, whereas the 2 h AUC of plasma TCA-soluble radioactivity is 82% of the plasma AUC of TCA-precipitable radioactivity for either the

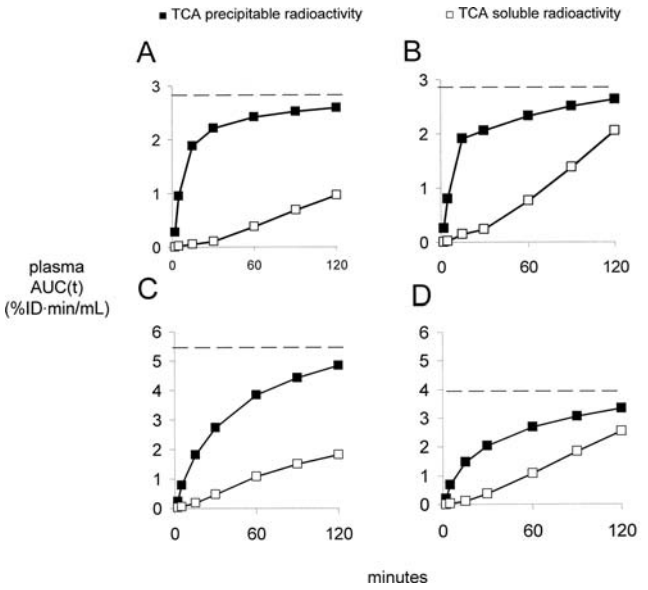

Figure 2. Plasma AUC at 2−120 min after IV injection of either [^{125}I]-HIRMAb-IDS fusion protein (A, B) or [^{125}I]-IDS (C, D). The protein is labeled with either Bolton-Hunter reagent (A, C) or Iodogen (B, D). The plasma AUC of TCA precipitable and TCA soluble radioactivity is shown in closed squares and open squares, respectively. The dashed horizontal line in each panel represents the mean AUCss, which is the maximal plasma AUC for the TCA-precipitable radioactivity. The AUCss values are given in Table 1

HIRMAb-IDS fusion protein (Figure 2B) or IDS (Figure 2D) radiolabeled with Iodogen.

Peripheral Organ Uptake of the HIRMAb-IDS Fusion Protein and IDS. The HIRMAb-IDS fusion protein and IDS are cleared to a comparable degree by peripheral organs (lung, skeletal muscle, fat, heart, kidney, spleen, and liver) (Table 2). The organs fall into 3 categories: (a) high uptake, e.g., liver and spleen, (b) moderate uptake, e.g., heart and lung, and (c) low uptake, e.g., skeletal muscle and fat (Table 2). The high renal clearance, particularly for the Bolton-Hunter reagent, likely reflects kidney clearance of low molecular weight metabolites.

Brain Uptake of HIRMAb-IDS Fusion Protein and IDS. The brain uptake is expressed as percent of injected dose (ID) per 100 g brain, since the weight of the brain in the rhesus monkey is 100 g.[1] The brain uptake in frontal cortex gray

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Table 2. Organ Uptake for [^{125}I]-HIRMAb-IDS Fusion Protein and [^{125}I]-IDS in the Rhesus Monkey[a]

	Bolton Hunter			Iodogen		
	organ uptake (%ID/100 g)			organ uptake (%ID/100 g)		
organ	HIRMAb-IDS	IDS	HIRMAb-IDS: IDS ratio	HIRMAb-IDS	IDS	HIRMAb-IDS: IDS ratio
frontal cortex white matter	0.99 ± 0.19	0.026 ± 0.007	38	0.525 ± 0.069	0.074 ± 0.009	7.1
lung	2.97 ± 0.13	3.35 ± 0.19	0.89	5.68 ± 0.37	2.56 ± 0.05	2.2
skeletal muscle	0.23 ± 0.05	0.20 ± 0.05	1.2	0.61 ± 0.12	0.43 ± 0.04	1.4
fat	0.25 ± 0.02	0.27 ± 0.03	0.93	0.40 ± 0.05	1.02 ± 0.32	0.39
heart	1.32 ± 0.11	1.11 ± 0.20	1.2	2.44 ± 0.15	1.06 ± 0.09	2.3
kidney	14.0 ± 0.69	20.97 ± 1.54	0.67	3.45 ± 0.16	3.18 ± 0.10	1.1
spleen	19.24 ± 0.24	12.36 ± 0.16	1.6	15.65 ± 1.22	5.90 ± 0.26	2.6
liver	33.23 ± 4.52	27.76 ± 0.66	1.2	19.69 ± 0.69	6.75 ± 0.26	2.9

[a]Data are mean ± SD.

Table 3. Plasma and Brain TCA Precipitability and Brain Fraction Volume of Distribution (VD) Determined with Capillary Depletion Analysis[a]

		TCA precipitability (%)			VD (μL/gram)	
molecule	plasma or brain fraction	Bolton-Hunter	Iodogen	brain capillary depletion fraction	Bolton-Hunter	Iodogen
HIRMAb-IDS fusion protein	plasma	23 ± 1	14 ± 1	brain homogenate	833 ± 103	183 ± 14
	brain postvascular supernatant	97 ± 1	73 ± 2	postvascular supernatant	510 ± 156	124 ± 18
				vascular pellet	124 ± 55	17 ± 3
IDS	plasma	55 ± 1	26 ± 1	brain homogenate	9.3 ± 1.6	22 ± 2
	brain postvascular supernatant	57 ± 14	33 ± 1	postvascular supernatant	7.2 ± 0.8	19 ± 1
				vascular pellet	0.2 ± 0.1	0.3 ± 0.1

[a]Mean ± SD. The fusion protein was administered by IV injection, and plasma and brain measurements made 120 min following injection. The homogenate VD for the [^{3}H]-human IgG1 isotype control antibody, a marker of the brain plasma volume, is 20 ± 6 μL/gram.[14]

matter of Bolton-Hunter labeled HIRMAb-IDS fusion protein and Bolton-Hunter labeled IDS is 1.04 ± 0.07% ID/brain and 0.030 ± 0.004% ID/brain, respectively (Figure 1C). Comparable levels of uptake are observed for frontal cortex white matter that is obtained by gross dissection (Table 2). The ratio of brain uptake of the HIRMAb-IDS fusion protein, relative to IDS, is 34-fold for the Bolton-Hunter labeled proteins. The brain uptake of the HIRMAb-IDS fusion protein after Iodogen labeling is reduced to 0.57 ± 0.04% ID/brain, and the brain uptake of IDS after Iodogen labeling is elevated to 0.079 ± 0.003% ID/brain (Figure 1C). The ratio of brain uptake of the HIRMAb-IDS fusion protein, relative to IDS, is 7-fold for the Iodogen labeled proteins. The increased brain uptake of radioactivity following IV administration of Iodogen labeled IDS is shown by the capillary depletion method to be due solely to the brain uptake of TCA soluble radiolabeled metabolites (Table 3).

The BBB PS product is 3.5 ± 0.4 μL/min/g for the HIRMAb-IDS fusion protein that is labeled with the Bolton-Hunter reagent, and is 1.8 ± 0.1 μL/min/g for the HIRMAb-IDS fusion protein that is labeled with Iodogen. The BBB PS product for IDS is zero, as the brain VD is not significantly different from the brain plasma volume (Table 3).

Capillary Depletion Method. The low uptake of radioactivity by brain following IV injection of [^{125}I]-IDS (Figure 1C) reflects only distribution of this enzyme in the cerebral plasma volume and not actual uptake into the brain. This is shown by the capillary depletion method, which reports the brain uptake as a volume of distribution (VD). The homogenate VD for IDS in the brain is 9.3 ± 1.6 μL/gram, for the Bolton-Hunter labeled enzyme, and is 22 ± 2 μL/gram for the Iodogen labeled enzyme, and these VD volumes are no greater than the brain plasma volume (Vo), 20 ± 6 μL/gram, for a human IgG1 brain plasma volume marker in the Rhesus monkey (Table 3).

The homogenate VD for the HIRMAb-IDS fusion protein is 833 ± 103 μL/gram for the Bolton-Hunter method and is 182 ± 14 μL/gram for the Iodogen method, and both VD values are many-fold greater than the plasma volume of brain (Table 3). The VD of the HIRMAb-IDS fusion protein in the postvascular supernatant is 61−68% of the homogenate VD (Table 3), which indicates that two-thirds of the HIRMAb-IDS fusion protein in brain has moved beyond the microvasculature and penetrated the brain parenchyma. The distribution of radioactivity into the postvascular supernatant after injection of IDS is due solely to metabolites, as is shown by an analysis of the TCA precipitability of the postvascular radioactivity (Table 3). The TCA precipitability of the radioactivity in the postvascular supernatant is 97 ± 1% and 73 ± 2%, for the Bolton-Hunter labeled HIRMAb-IDS fusion protein and the Iodogen labeled HIRMAb-IDS fusion protein, respectively (Table 3). These values are high compared to the TCA precipitability of the 120 min plasma, which is 23 ± 1% and 14 ± 1%, for the Bolton-Hunter labeled HIRMAb-IDS fusion protein and the Iodogen labeled HIRMAb-IDS fusion protein, respectively (Table 3). The ratio of brain/plasma TCA precipitability is 4.2 and 5.2 for the Bolton-Hunter labeled HIRMAb-IDS fusion protein and the Iodogen labeled HIRMAb-IDS fusion protein, respectively. In contrast, the TCA precipitability for IDS is identical for the postvascular supernatant and the 120 min plasma for either radiolabeling method (Table 3).

Immunoprecipitation. The high percent TCA precipitability, 97 ± 1%, in the postvascular supernatant after IV injection of the Bolton-Hunter labeled HIRMAb-IDS fusion protein indicates that the radioactivity in brain reflects brain

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uptake of the intact the HIRMAb-IDS fusion protein. This was confirmed by immunoprecipitation of the radioactivity in the brain removed 2 h after the IV injection of the Bolton-Hunter labeled the HIRMAb-IDS fusion protein (Figure 1D). The only radioactive species in the brain corresponds to the heavy chain of the HIRMAb-IDS fusion protein. There is no lower molecular weight band on the gel, which would be produced if the IDS enzyme was cleaved from the fusion protein in the peripheral compartment.

Brain Scanning. The film autoradiograms of forebrain, midbrain, and hindbrain/cerebellum of Rhesus monkey brain removed 2 h after the IV injection of either the HIRMAb-IDS fusion protein (left panel) or IDS (right panel) labeled with [^{125}I]-Bolton Hunter reagent is shown in Figure 3. Radio-

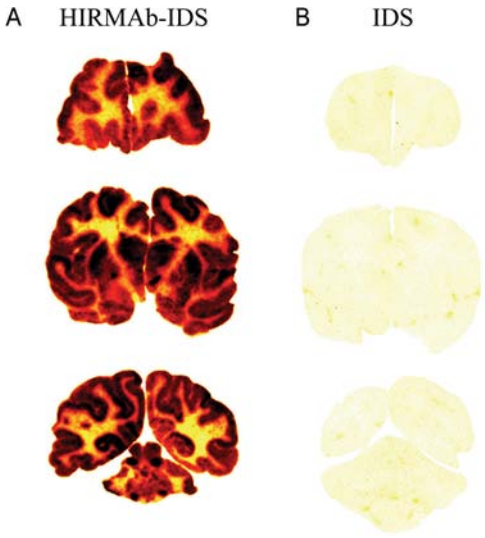

Figure 3. Film autoradiogram of 20 μm sections of rhesus monkey brain removed 120 min after IV injection of the HIRMAb-IDS fusion protein (left panel) or IDS (right panel). The forebrain section is on the top, the midbrain section is in the middle, and the hindbrain section with cerebellum is on the bottom. Scans produced after labeling of the HIRMAb-IDS fusion protein or IDS with [^{125}I]-Bolton-Hunter. X-ray films exposed for 7 days.

labeling the proteins with [^{125}I] and Iodogen, as compared to the Bolton-Hunter reagent, produces brain scans with reduced radioactivity in brain for the HIRMAb-IDS fusion protein (Figure 4A) and increased radioactivity for IDS (Figure 4B), which correlates with the measurement of total brain radioactivity shown in Figure 1C. The integrated density in gray matter of forebrain, midbrain, and hindbrain was 256 ± 15, 431 ± 75, and 302 ± 26 in gray matter, and was 82 ± 6, 137 ± 8, and 93 ± 5 in white matter, respectively, for the Bolton-Hunter labeled HIRMAb-IDS fusion protein. The overall ratio of integrated density in gray matter/white matter was 3.17 ± 0.03.

■ DISCUSSION

The results of this investigation are consistent with the following conclusions. First, the HIRMAb-IDS fusion protein and IDS are rapidly cleared from plasma with comparable pharmacokinetic parameters (Figure 1, Table 1), and have comparable uptake by peripheral organs (Table 2). Second, IDS does not cross the BBB (Figures 1C and 3; Table 3). Third, the HIRMAb-IDS fusion protein rapidly penetrates the

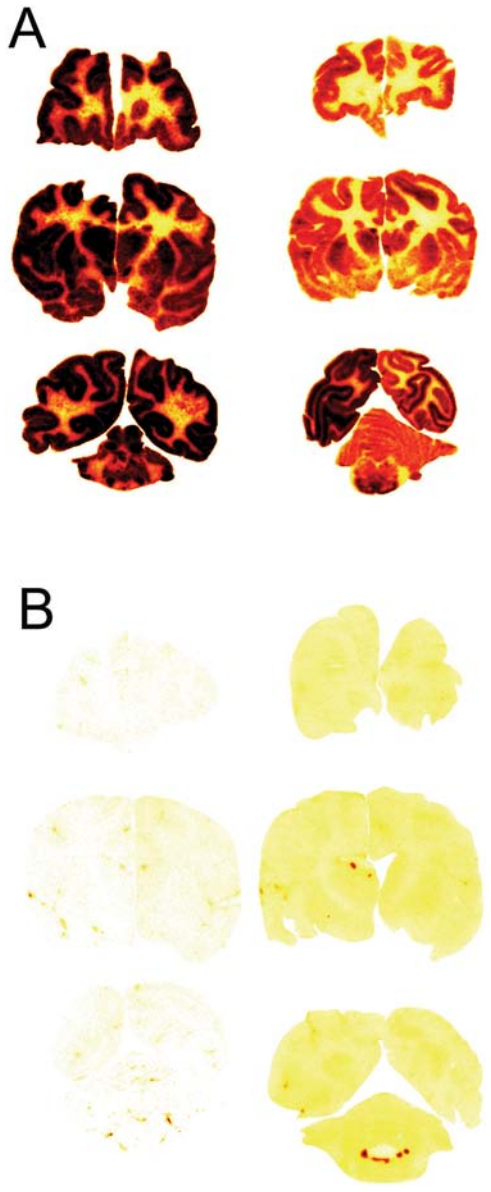

Figure 4. Film autoradiogram of 20 μm sections of rhesus monkey brain removed 120 min after IV injection of the HIRMAb-IDS fusion protein (A) or IDS (B). The forebrain section is on the top, the midbrain section is in the middle, and the hindbrain section with cerebellum is on the bottom. Scans produced after labeling of the HIRMAb-IDS fusion protein or IDS with [^{125}I]-Bolton-Hunter (left panels) or [^{125}I]-iodine and Iodogen (right panels). X-ray films exposed for 10 days.

BBB with a brain uptake of 1.0% ID/brain (Figure 1C). The enhanced brain uptake is observed in all parts of the brain (Figure 3), and the capillary depletion method shows the majority of the HIRMAb-IDS fusion protein taken up by brain has penetrated the BBB and entered brain parenchyma (Table 3). Fourth, both the HIRMAb-IDS fusion protein and IDS are rapidly degraded by peripheral tissues, which generate a time-dependent increase in TCA-soluble radiolabeled metabolites in the plasma compartment (Figure 2). The production of the TCA-soluble radiolabeled metabolites is twice as great with Iodogen-labeled proteins as compared to Bolton-Hunter-labeled proteins (Figure 2). Fifth, once in brain, the Bolton-Hunter labeled HIRMAb-IDS fusion protein is metabolically

stable, as demonstrated by the high TCA precipitability of brain radioactivity, 97 ± 1% (Table 3), and the immuno-precipitation study (Figure 1D). Sixth, the Bolton-Hunter method is preferred over the Iodogen method for imaging the brain uptake of radio-iodinated proteins. The Bolton-Hunter method gives a 5-fold higher signal/noise ratio, because of a higher signal due to higher rate of BBB penetration (Figures 1C and 4A), and a lower noise due to reduced brain uptake of radiolabeled metabolites (Figures 1C and 4B).

The rate of plasma clearance of Bolton-Hunter labeled IDS in the Rhesus monkey, 2.9 ± 0.2 mL/min/kg (Table 1), is comparable to the systemic clearance of immunoreactive IDS in the mouse, 1.3 mL/min/kg.[15] The brain uptake of immunoreactive IDS in the mouse is 0.013% ID/brain, which is comparable to the brain uptake of Bolton-Hunter labeled IDS in the monkey, 0.030 ± 0.004% ID/brain (Figure 1C). In another study, the brain uptake of IDS in the mouse was measured with an IDS enzyme assay, and the ratio of brain/plasma IDS enzyme activity was 0.015,[7] which is comparable to the brain uptake of immunoreactive IDS in the mouse,[15] or the brain uptake of radiolabeled IDS in the monkey (Figure 1C). In all these cases, the low level of brain uptake of IDS is not physiologically significant, because these low levels reflect only IDS distribution within the plasma compartment of brain. This can be shown by comparing the brain VD of IDS to the brain plasma volume, which is 20 ± 6 μL/gram in the monkey (Table 3). Any IDS found in the brain, whether the protein is measured by immunoreactivity, enzyme activity, or radioactivity, is sequestered within the plasma volume of brain, and has not penetrated the BBB. The IDS enzyme is a glycoprotein and 1−2 mannose 6-phosphate moieties are incorporated in the enzyme. The rapid uptake by peripheral tissues of mannose-6-phosphorylated lysosomal enzymes such as IDS is mediated via the 250 kDa cation independent mannose 6-phosphate receptor, or CI-MPR.[16] The CI-MPR also binds insulin-like growth factor (IGF)-2 with high affinity. However, the high affinity binding site of IGF2 at the human BBB corresponds to a 135 kDa variant of the IGF-1 receptor, and not to the 250 kDa CI-MPR.[17] The lack of IDS penetration of the BBB suggests the CI-MPR is not present on the BBB, at least in the adult, and this is corroborated by affinity cross-linking studies of IGF-2 and human brain capillaries.[17]

The IDS lysosomal enzyme can be delivered across the BBB following the re-engineering of the enzyme as an IgG-IDS fusion protein, where the IgG domain penetrates the BBB via receptor-mediated transport on the endogenous insulin receptor. The human BBB expresses the insulin receptor,[18] and the BBB insulin receptor mediates the brain uptake of circulating insulin.[19] The HIRMAb-IDS fusion protein is formed by fusion of human IDS to the carboxyl terminus of each heavy chain of the genetically engineered HIRMAb.[8,9] The difference in primate brain uptake of the HIRMAb-IDS fusion protein and IDS is illustrated by the brain scan of the Rhesus monkey injected IV with either the HIRMAb-IDS fusion protein or IDS (Figure 3). The brain uptake of the HIRMAb-IDS fusion protein, 1.0% ID/brain (Figure 1C), in the rhesus monkey is comparable to the brain uptake of fallypride, a lipid soluble small molecule.[20] The film autoradiography shows the brain uptake of the HIRMAb-IDS fusion protein is higher in gray matter as compared to white matter (Figure 3, left panel), and scanning densitometry indicates the uptake ratio for gray matter relative to white matter is 3.2 (Results). The higher uptake in gray matter reflects the 3-fold higher vascular density in gray matter as compared to white matter.[1] However, the ratio of brain uptake of gray matter, relative to white matter, measured by direct radioactivity determinations of tissue obtained by gross dissection, is closer to unity (Table 2). The lower gray matter/white matter uptake ratio determined with direct radioactivity determinations (Table 2) is most likely due to admixture of the gray and white matter in the brain samples obtained by gross dissection.

The brain uptake of the HIRMAb-IDS fusion protein and IDS is 1.04 ± 0.07%ID/brain and 0.031 ± 0.004%ID/brain (Figure 1C), respectively, following labeling of the proteins with the Bolton-Hunter reagent, which is a ratio of 34. Following radiolabeling of the proteins with Iodogen, the brain uptake of the HIRMAb-IDS fusion protein and IDS is 0.57 ± 0.04%ID/brain and 0.079 ± 0.003%ID/brain (Figure 1C), respectively, which is a ratio of 7. Therefore, the ratio of brain uptake of the 2 proteins decreases 5-fold when the Bolton-Hunter method is replaced by the Iodogen method. This ratio falls 5-fold due to a 2-fold reduction in brain uptake of the HIRMAb-IDS fusion protein (Figures 1C and 4A) and a 2.5-fold increase in brain uptake of radioactivity following labeling of IDS with Iodogen (Figures 1C and 4B). The reduction in brain uptake of the HIRMAb-IDS fusion protein is due to a 2-fold reduction in the BBB PS product for the HIRMAb-IDS fusion protein following Iodogen labeling (Results). Iodogen labeling is an oxidative process that damages proteins and can have adverse effects on mAb binding to the target antigen. The binding of a mAb to a tumor associated antigen was abolished following Iodogen labeling but was retained following Bolton-Hunter labeling.[21] The binding of a mAb to vascular endothelial growth factor was reduced 10-fold following Iodogen labeling, but was retained following Bolton-Hunter labeling.[22] The 2.5-fold increase in brain radioactivity following administration of Iodogen-labeled IDS, as compared to Bolton-Hunter labeled IDS (Figure 1C), is associated with a >100% increase in the rate of formation in plasma of TCA-soluble metabolites produced with the Iodogen labeling method (Figure 2). The TCA-soluble metabolites that are formed following the intravenous administration of proteins labeled by oxidative radio-iodination are iodo-tyrosine and iodide.[23] The Iodogen method incorporates the ^{125}I isotope on tyrosine residues of the protein, and the [^{125}I]-iodotyrosine formed by protein degradation may undergo further deiodination, which results in the formation of [^{125}I]-iodide. Iodotyrosine is a neutral amino acid that has high affinity for the large neutral amino acid transporter type 1, LAT1.[24] The LAT1 neutral amino acid transporter is highly expressed at the BBB,[25] and provides for BBB penetration of circulating [^{125}I]-iodotyrosine. In contrast, the Bolton-Hunter method conjugates the ε-amino group of lysine residues of the protein with an iodo-4-hydroxy-benzene propionyl group, and this TCA-soluble, low molecular weight metabolite does not cross the BBB. This is shown by the high TCA precipitability of brain radioactivity at 2 h after IV administration of the Bolton-Hunter labeled the HIRMAb-IDS fusion protein (Table 3). The TCA-precipitable brain radioactivity is high, 97 ± 1%, despite the low TCA-precipitable plasma radioactivity at 2 h after administration, 23 ± 1% (Table 3). These observations indicate that brain radioactivity following administration of Bolton-Hunter reagent represents only BBB penetration of intact the HIRMAb-IDS fusion protein, and not radiolabeled metabolites, which is corroborated by the immuno-precipitation study (Figure 1D). The lower level of TCA-precipitable radioactivity in brain, 73 ± 2%,

at 2 h after administration of Iodogen-labeled HIRMAb-IDS fusion protein (Table 3) is consistent with the brain uptake of TCA soluble, low molecular weight metabolites from plasma.

The appearance in plasma of TCA-soluble, low molecular weight metabolites is shown in Figure 2, which plots the plasma AUC with time for both the intact fusion protein, represented by the AUC of the TCA-precipitable radioactivity, and the plasma AUC of the low molecular weight metabolites, represented by the AUC of the TCA-soluble radioactivity. The plasma AUC is the input function of any neuro-imaging or brain uptake study of a radiolabeled test article. In the case of either the HIRMAb-IDS fusion protein or IDS, the test article is subject to rapid degradation in the periphery, which results in the release to plasma of low molecular weight metabolites. The plasma AUC of the TCA-precipitable radioactivity plateaus between 1 and 2 h for either the HIRMAb-IDS fusion protein or IDS, as the AUC(t) reaches the maximal value, which is the AUCss (dashed horizontal line in Figure 2). However, the AUC of the TCA soluble radioactivity increases with time, such that an inflection point is reached, wherein the plasma AUC of the metabolite exceeds the plasma AUC of the test article. This inflection point can be estimated by extrapolation of the curves in Figure 2, and is estimated to be 5 and 8 h for Bolton-Hunter labeled HIRMAb-IDS fusion protein and IDS, respectively, but only 2.5 and 3 h for Iodogen labeled HIRMAb-IDS fusion protein and IDS, respectively. Therefore, if the distribution of Iodogen labeled HIRMAb-IDS fusion protein or IDS in vivo is followed for a time period longer than 2.5−3 h, then the primary input function of plasma radioactivity is the low molecular weight metabolite, and not the intact protein test article.

The 2 h brain scan obtained with Bolton-Hunter labeled HIRMAb-IDS fusion protein demonstrates a global distribution of the fusion protein to all parts of brain following IV administration (Figure 3). The capillary depletion method shows the majority of the HIRMAb-IDS fusion protein in brain has distributed to the postvascular parenchyma of brain. These results were recently corroborated by emulsion autoradiography of a [^{125}I]-Bolton-Hunter labeled HIRMAb-arylsulfatase A (ASA) fusion protein.[26] The light microscopic emulsion autoradiography showed the HIRMAb-ASA fusion protein distributes throughout the brain water space.[26] The brain TCA precipitability after administration of the HIRMAb-ASA fusion protein was also >95%,[26] which indicates the only radioactivity observed in the brain is the intact fusion protein, when the protein is labeled with the Bolton-Hunter reagent.

In summary, the present study demonstrates that the re-engineering of a protein therapeutic such as IDS with the Trojan horse technology can deliver therapeutic levels of medically important enzymes across the blood-brain barrier. For future brain imaging studies of recombinant proteins with high metabolic clearance rates, such as the HIRMAb-IDS fusion protein or IDS, this study also demonstrates that the Bolton-Hunter reagent is the preferred method of radio-iodination of the protein.

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Notes

The authors declare the following competing financial interests: Drs. Boado, Hui, and Lu are employees of, and Dr. Pardridge is a consultant of, ArmaGen Technologies, Inc.

ABBREVIATIONS

ASA, arylsulfatase A; AUC, area under the plasma concentration curve; BBB, blood-brain barrier; CI-MPR, cation independent MPR; CL, systemic clearance rate; Cp, plasma concentration; HIR, human insulin receptor; HIRMAb, mAb against HIR; HIRMAb-ASA, fusion protein of HIRMAb and ASA; HIRMAb-IDS, fusion protein of HIRMAb and IDS; ID, injected dose; IDS, iduronate 2-sulfatase; IGF, insulin-like growth factor; LAT1, large neutral amino acid transporter type 1; mAb, monoclonal antibody; MPR, mannose 6-phosphate receptor; MPS, mucopolysaccharidosis; MPS-II, MPS Type II; MRT, mean plasma residence time; PK, pharmacokinetics; PS, permeability-surface area product; TCA, trichloroacetic acid; Vc, central volume of distribution; VD, volume of distribution; Vo, brain plasma volume of distribution; Vss, steady state body volume of distribution

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