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DELIVERY OF β-GALACTOSIDASE TO MOUSE BRAIN VIA THE BLOOD-BRAIN BARRIER TRANSFERRIN RECEPTOR

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Running Title: Targeting Enzymes Across the Blood-Brain Barrier

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Abbreviations: BBB, blood-brain barrier; TfR, transferrin receptor; MAb, monoclonal antibody; SA, streptavidin; S-SMPB, sulfosuccinimidyl-4-(p-malimidophenyl)butyrate; NHS, N-hydroxysuccinimide; BCA, bicinchoninic acid; LC, long chain; ERT, enzyme replacement therapy; HABA, 2-(4'-hydroxyazobenzene)benzoic acid; IV, intravenous; 8D3/SA, conjugate of 8D3 TfRMAb and SA; gal, β-galactosidase; ID, injected dose; HIR, human insulin receptor; GTP, γ-glutamyl transpeptidase

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ABSTRACT

Enzyme replacement therapy of lysosomal storage disorders is complicated by the lack of enzyme transport across the blood-brain barrier (BBB). The present studies evaluate the delivery of a model enzyme across the BBB following enzyme conjugation to a BBB receptor-specific monoclonal antibody (MAb). Bacterial β-galactosidase (116 kDa) was conjugated to the rat 8D3 MAb to the rat transferrin receptor (TfR) via a streptavidin (SA)-biotin linkage. The unconjugated β-galactosidase or the β-galactosidase-8D3 conjugate was injected intravenously in adult mice and enzyme activity was measured at 1 and 4 hours in brain and peripheral organs (liver, spleen, kidney, heart). Unconjugated β-galactosidase was rapidly removed from the blood compartment owing to avid uptake by liver and spleen. There was minimal uptake of the unconjugated β-galactosidase by brain. Following conjugation of the enzyme to the 8D3 TfRMAb, there was a 10-fold increase in brain uptake of the enzyme based on measurement of enzyme activity. Histochemistry of brain showed localization of the enzyme in the intra-endothelial compartment of brain following intravenous injection of the enzyme-MAb conjugate. The capillary depletion technique showed that more than 90% of the enzyme-8D3 conjugate that entered into the endothelial compartment of brain passed through the BBB to enter brain parenchyma. In conclusion, high molecular weight enzymes, such as bacterial β-galactosidase, can be conjugated to BBB targeting antibodies for effective delivery across the BBB in vivo. Fusion proteins comprised of BBB targeting antibodies and recombinant enzymes could be therapeutic in the treatment of the brain in human lysosomal storage disorders.

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INTRODUCTION

Lysosomal storage disorders are treated with recombinant enzyme replacement therapy (ERT). The majority of lysosomal storage disorders affect the brain (Cheng and Smith, 2003). A major limitation in the ERT of lysosomal storage disorders is the lack of transport of the therapeutic enzyme across the brain capillary wall, which forms the blood-brain barrier (BBB). The involvement of the central nervous system is generally severe in lysosomal storage disorders (Cheng and Smith, 2003), and it is important to develop BBB drug delivery strategies for therapeutic enzymes. Recombinant proteins as large as 40,000 Daltons have been delivered across the BBB in vivo with molecular Trojan horses that access endogenous BBB receptor-mediated transport systems (Pardridge, 2001). A peptidomimetic monoclonal antibody (MAb) to the BBB transferrin receptor (TfR) mediated the delivery of several peptides and recombinant proteins across the BBB with in vivo CNS pharmacological effects following intravenous administration (Pardridge, 2002). The recombinant protein is attached to the TfRMAb via avidin-biotin technology. In this approach, the non-transportable protein drug is mono-biotinylated in parallel with the production of a TfRMAb-streptavidin (SA) conjugate. Owing to the very high affinity of SA binding of biotin, there is instantaneous formation of the protein-TfRMAb conjugate following mixing of the mono-biotinylated drug and the TfRMAb-SA (Pardridge, 2001).

Lysosomal enzymes have a molecular weight of 50-100 kDa, and it is not clear if BBB molecular Trojan horses such as a receptor-specific MAb can deliver a protein of this size across the BBB in vivo. Molecular Trojan horses have delivered liposomes carrying genes across the BBB (Pardridge, 2002). However, to date, the largest protein delivered across the BBB with a molecular Trojan horse is pegylated brain derived neurotrophic factor, which has a molecular

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weight of about 40,000 Da (Pardridge et al, 1998). The present studies use bacterial β-galactosidase, which has a molecular weight of 116,000 Da, as a model enzyme for delivery to mouse brain. The rat 8D3 MAb to the mouse TfR, which enters brain via the BBB TfR (Lee et al, 2000), is used in the present studies to deliver enzyme to the brain.

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METHODS

Materials

Recombinant E. coli β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) with a specific activity of 700 -1200 Units/mg protein was purchased from Roche Diagnostics Corporation (Indianapolis, IN). Sulfo-N-hydroxysuccinimide (NHS)-long chain (LC)-LC-biotin, EZ biotin quantitation kit, Traut's reagent, sulfosuccinimidyl-4-(p-malimidophenyl)butyrate (S-SMPB) and the bicinchoninic acid (BCA) protein assay reagents were from Pierce (Rockford, IL). [^{3}H]-biotin (60 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA). Protein G Sepharose and Sephacryl S-300HR were obtained from Amersham Biosciences (Piscataway, NJ). Sephadex G-25, recombinant streptavidin, and dextran (60,000-90,000 Da) were obtained from Sigma (St. Louis, MO). The β-galactosidase histochemistry kit was from Invitrogen (Carlsbad, CA). The Beta-Glo β-galactosidase luminescence assay system, lysis buffer, and the β-galactosidase Enzyme Assay System were purchased from Promega (Madison, WI).

Synthesis of 8D3/SA conjugate

The rat hybridoma line secreting the 8D3 MAb to the mouse TfR was cultured on a feeder layer of mouse thymocytes and peritoneal cells in Dulbecco modified Eagle medium with 10% fetal bovine serum (Lee et al, 2000). The hybridoma cells were propagated as ascites in nude mice, and the 8D3 MAb was purified by protein G affinity chromatography. A 1:1 conjugate of the 8D3 MAb and streptavidin (SA) was prepared by stable thiol-ether linkage using 8D3 thiolated with Traut's reagent at a 40: 1 molar ratio of Traut's reagent. The SA was activated with S-SMPB at a 24:1 molar ratio, and the 8D3/SA conjugate was purified with a

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2.5x95 cm column of Sephacryl S-300HR in PBST (0.01 M Na_2HPO_4, 0.15 M NaCl, pH=7.4, 0.05% Tween-20). The elution of the 8D3/SA conjugate and unconjugated SA were monitored by adding a trace amount of [^{3}H]-biotin to the mixture prior to addition to the column. The fractions containing the 8D3/SA conjugate (Figure 1A, reaction I) were pooled and stored at –20 °C.

Mono-biotinylation of β-galactosidase and biotin quantitation

Bacterial β-galactosidase was homogeneous on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and migrated with a molecular weight (MW) of 116,00 Da (Figure 1B). The β-galactosidase was dissolved in 0.05 M $NaHCO_3$/8.5 and the protein concentration was determined with the BCA assay. The sulfo-NHS-LC-LC-biotin, 45 nmol/μl was prepared in 0.05 M $NaHCO_3$/8.5, and 19 μl of sulfo-NHS-LC-LC-biotin solution (855 nmol) was added to 5 mg (43 nmol) of β-galactosidase, which was 20: 1 molar ratio of biotin: β-galactosidase. The mixture was capped and rocked end over end for 60 min at room temperature. The sample was applied to a 0.7 X 15 cm Sephadex G-25 column, eluted with 10 ml of 0.01 M PBS/7.4 at 0.5 ml/min, and 0.5 ml fractions were collected. The 3 fractions comprising the first A280 peak were pooled, the protein concentration was determined, and the biotin-LC-LC- β-galactosidase (Figure 1A, reaction II) was stored at –20C. The enzymatic activity of β-galactosidase or biotinylated beta-gal (biotin-LC-LC-β-galactosidase) was measured with either the spectrophotometric method or the Beta-Glo luminescence assay system.

The molar ratio of sulfo-NHS-LC-LC-biotin to β-galactosidase was determined to yield 1-1.5 biotin moieties per enzyme molecule. The degree of biotinylation was determined with Pierce EZ biotin quantitation kit per the manufacturer's instructions, which monitors the binding

of 2-(4'-hydroxyazobenzene)benzoic acid (HABA) to avidin by absorbance at 500 nm with an extinction coefficient of 34 mM^{-1}. The displacement of HABA from avidin is proportional to the biotin content in the biotin-LC-LC- β-galactosidase.

The β-galactosidase/8D3 conjugate, also designated β-gal-8D3 (Figure 1, reaction III), was formed by mixing a 1:1 molar ratio of biotin-LC-LC- β-galactosidase and the 8D3/SA conjugate at 15 min at room temperature. There was no loss in β-galactosidase enzyme activity following mono-biotinylation and attachment to the 8D3/SA conjugate (Figure 1C).

Brain delivery of β-galactosidase and β-galactosidase/8D3 in adult mice

Adult female BALB/c mice weighing 20-25 g (Charles River Laboratories,Wilmington, MA) were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine intra-peritoneal. The mice were injected via the jugular vein with either unconjugated β-galactosidase or the β-gal-8D3 conjugate. In the high dose treatment, mice were administered either (a) 150 μg/mouse of unconjugated β-galactosidase, or (b) 150 μg/mouse of biotinylated β-galactosidase conjugated to 300 μg/mouse of 8D3/SA. In the low dose treatment, mice were administered either (a) 15 μg/mouse of unconjugated β-galactosidase, or (b) 15 μg/mouse of biotinylated β-galactosidase conjugated to 30 μg/mouse of 8D3/SA. The mice were sacrificed at either 1 or 4 hours after intravenous (IV) injection. The brain, liver, spleen, heart and kidney were removed, weighed and frozen on dry ice. The blood from each mouse was collected, heparinized and stored at –20C. Organs and blood were also removed from un-injected mice to determine the activity of endogenous β-galactosidase at neutral pH.

β-galactosidase enzyme activity measurements

The spectrophotometric assay for β-galactosidase enzyme activity (β-galactosidase Enzyme Assay System, Promega) was not used owing to interference in the absorbance readings by endogenous tissue pigments. Enzyme activity was measured with Promega Beta-Glo luminescence assay system. The tissue was extracted with Promega lysis buffer at a ratio of 2 ml buffer to 0.5 g tissue, followed by homogenization with a Brinkmann Polytron PT3000. The homogenate was centrifuged for 10 min at 12,000 *g*, and the supernatant was used to measure β-galactosidase activity with the Promega Beta-Glo assay solution at pH=7.6. The mixture was incubated in the dark at room temperature for 1 hour. The relative light units (RLU) were measured with a luminometer (EG&G Berthold, Oak Ridge, TN), and the RLU was converted to milliunits (mU) of enzyme activity based on a β-galactosidase standard curve. The protein content in the organ extract was measured with the BCA reagent. Organ enzyme activity was measured as: (a) mU/mg protein, (b) mU/gram organ weight, or (c) % injected dose (ID)/g organ weight. The ID was computed from the known specific activity (mU/μg) of the unconjugated β-galactosidase or the β-gal-8D3 conjugate. The endogenous β-galactosidase enzyme activity in un-injected mice was also measured in each organ.

Brain β-galactosidase histochemistry

Mice were anesthetized and injected with maximal doses of either of unconjugated β-galactosidase (300 μg/mouse) or the β-gal-8D3 conjugate (300 μg/mouse of biotin-LC-LC-β-galactosidase mixed with 600 μg/mouse of 8D3/SA conjugate) via the jugular vein. At 60 min after IV injection, the brain plasma volume was cleared with a 4 min infusion of 4 mL cold PBS into the ascending aorta at a rate of 1 mL/min, followed by a 20 min perfusion of 20 ml of

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fixative (2 % paraformaldehyde in 0.01 M PBS/7.4 with 0.5 % glutaraldehyde and 2 mM $MgCl_2$) at a rate of 1 ml/min. The brain was removed and divided into 4 coronal slabs, and the slabs were immersion-fixed in the same fixative at 4 °C for 4 hours. The tissue was washed briefly in 0.1 M phosphate-buffered water (PBW)/7.4 and then placed in 30 % sucrose/0.1 M PBS/7.4 for 24 hours at 4 °C. The brain slab was frozen in Tissue-Tek O.C.T. compound and stored at -70 °C until sectioning. Frozen section of 40 μm were prepared on an HM505 microtome (Mikron, San Diego, CA), and β-galactosidase histochemistry was performed with the Invitrogen β-galactosidase staining kit. The frozen section was fixed with 2 % formaldehyde and 0.2 % glutaraldehyde in 0.01 M PBS/7.4 for 5 min. After washing in PBS, the section was incubated in X-gal staining solution (4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02 % IGEPAL CA-630, 0.01 % sodium deoxycholate and 1 mg/ml X-gal, pH 7.4) at 37 °C overnight, where X-gal= 5-bromo-4-chloro-3-indoyl-β-D-galactoside. The pH of the incubation was maintained at 7.4 throughout the incubation. After staining with X-gal, the section was briefly washed in distilled water, mounted without counter-staining, and photographed.

A dot-blot assay was developed to determine the minimal β-galactosidase enzyme activity that could be detected with a colorimetric histochemical assay. Enzyme (100 uL) was spotted with a Biorad dot blot apparatus in a 3 mm circle to nitrocellulose filter paper in the following amounts: 68, 6.8, 0.68, 0.068, and 0.0068 mU with or without fixation of the blotted filter paper in 0.2% glutaraldehyde in 0.1 M Na_2HPO_4/7.4/2 mM $MgCl_2$ for 2 min. Enzyme activity in the filter paper was measured with the colorimetric technique (β-galactosidase Enzyme Assay System, Promega). The amount of enzyme that was barely detected by eye was >2 mU with fixation and >1 mU without fixation. A 40 micron section of mouse brain weighs

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approximately 1 mg. Therefore, it would be necessary to achieve a β-galactosidase enzyme activity >2,000 mU/g brain in order to visualize the enzyme in brain parenchyma with a colorimetric technique such as histochemistry.

Brain capillary depletion method

Mice were anesthetized and injected with the β-gal-8D3 conjugate (150 μg/mouse of biotin-LC-LC-β-galactosidase mixed with 300 μg/mouse of 8D3/SA conjugate) via the jugular vein. At 60 min after IV injection, the brain plasma volume was cleared with a 4 min infusion of 4 mL cold PBS into the ascending aorta at a rate of 1 mL/min. The brain was removed, weighed and homogenized in a cold physiological buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2O_4, and 10 mM D-glucose, pH 7.4) with a glass tissue grinder, followed by the addition of cold dextran to a final concentration of 40%. After removal of an aliquot of the homogenate, the remainder was centrifuged at 3,200 *g* for 10 min at 4°C and the supernatant was carefully separated from the capillary pellet with the capillary depletion technique described previously (Triguero et al, 1990). The homogenate, post-vascular supernatant, and the capillary pellet were solubilized in Promega Lysis buffer. The β-galactosidase enzymatic activity was measured with the Promega Beta-Glo assay system as described above and reported as mU/gram brain for each of the 3 fractions. The enzyme activity of a brain microvascular-enriched enzyme, γ-glutamyl transpeptidase (GTP), was measured with a spectrophotometric assay using L-γ-glutamyl-p-nitroanilide as a substrate (Diagnostic Chemicals Ltd., Charlottetown, PE, Canada). GTP is highly enriched at the brain microvasculature, and is also expressed in brain parenchymal cells. The volume of the microvascular endothelium in brain is ~0.1% of the total intracellular volume in brain.

Therefore, GTP enzyme activity in the post-vascular supernatant approximates the GTP enzyme activity in the starting brain homogenate; however, the ratio of GTP enzyme activity in the vascular pellet, relative to the brain homogenate, is a quantitative measure of the extent to which the post-vascular supernatant has been depleted of the microvascular component of brain (Triguero et al, 1990).

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RESULTS

Following the IV administration of the low dose (15 ug/mouse) of the unconjugated β-galactosidase, the enzyme was rapidly cleared from blood by liver, spleen, and kidney (Figure 2). The enzyme was cleared by liver and spleen after the IV administration of the high dose (150 ug/mouse) of the unconjugated β-galactosidase (Figure 3). The high dose caused minimal saturation of the uptake of the unconjugated enzyme by liver and spleen. The 60 min enzyme activity in liver was 1,144 ± 190 mU/g (7.7 ± 0.9 mU/mg protein) and 41,086 ± 8,497 mU/g (386 ± 84 mU/mg protein) after the IV injection of the low dose and high dose, respectively, of unconjugated enzyme. The 60 min enzyme activity in spleen was 3,038 ± 384 mU/g (21 ± 3 mU/mg protein) and 32,686 ± 5,777 mU/g (367 ± 68 mU/mg protein) after the IV injection of the low dose and high dose, respectively, of unconjugated enzyme. The brain uptake of the unconjugated enzyme was minimal at both the low dose (Figure 2, inset) and the high dose of enzyme (Figure 3, inset). The 60 min enzyme activity in brain was 121 ± 3 mU/g (2.0 ± 0.1 mU/mg protein) and 116 ± 26 mU/g (2.8 ± 0.6 mU/mg protein) after the IV injection of the low dose and high dose, respectively, and both values approximated the endogenous enzyme activity in the un-injected mouse brain, 85 ± 3 mU/g (1.5 ± 0.1 mU/mg protein).

Conjugation of the enzyme to the TfRMAb accelerated uptake in peripheral tissues and the largest increases in uptake were observed for liver and spleen at the low dose of enzyme (Figure 2). At the low dose, the brain uptake of β-galactosidase was increased 10-fold following conjugation to the TfRMAb (Figure 2, inset). At the high dose, the uptake of the enzyme-TfRMAb conjugate by liver and spleen showed saturation (Figure 3), whereas the brain uptake was still increased 10-fold following conjugation to the TfRMAb (Figure 3, inset).

The β-galactosidase enzyme activity was rapidly eliminated from mouse organs in vivo, and organ enzyme activity at 4 hours after an IV injection was no greater than the endogenous organ enzyme activity at the high dose injection (Table 1). The rapid clearance of β-galactosidase from blood in the adult mouse is shown in Table 2, which indicates the blood concentration of the unconjugated enzyme is <1% injected dose (ID)/mL blood at 60 minutes after IV injection.

The brain uptake of the unconjugated β-galactosidase or the β-galactosidase-TfRMAb conjugate was measured with histochemistry after treatment with maximal doses (Methods). At 60 min after an IV injection of the unconugated enzyme, there is no measurable enzyme activity in brain in either the parenchymal or capillary compartment (Figure 4C). At 60 min after an IV injection of the high dose of the β-galactosidase-TfRMAb conjugate, the enzyme product is detected by histochemistry in the capillary compartment throughout the entire brain, including cerebellum (data not shown) and a representative low magnification view is shown in Figure 4B. High magnification microscopy (Figure 4A) shows the enzyme within the microvascular endothelium; this enzyme activity is localized to the intra-endothelial compartment, and not the plasma compartment, because the brain was saline cleared prior to perfusion fixation for histochemistry (Methods). The brain vasculature was effectively cleared of enzyme as shown by the absence of vascular enzyme product following injection of the un-conjugated enzyme (Figure 4C). Histochemical product in brain parenchyma was not clearly detectable, because the brain β-galactosidase enzyme activity was less than the threshold for colorimetric detection, 2000 mU/g (Methods). However, brain parenchymal enzyme activity was demonstrated with the capillary depletion technique and a luminescence-based assay of brain β-galactosidase enzyme activity (Figure 5). More than 90% of the brain β-galactosidase enzyme activity was localized to the

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post-vascular supernatant compartment at 60 minutes following intravenous administration of the high dose of the β-galactosidase-TfRMAb conjugate (Table 3). Measurement of the activity of a brain microvascular enriched enzyme, GTP, showed that the vascular pellet GTP activity was 16-fold greater than GTP activity in the brain homogenate, indicating the post-vascular supernatant was 94% depleted of the capillary compartment (Table 3).

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DISCUSSION

The results of this study are consistent with the following conclusions. First, unconjugated β-galactosidase is rapidly cleared from blood in vivo (Table 2), owing to rapid uptake of the unconjugated enzyme by liver and spleen (Figures 2-3). Second, once inside cells, β-galactosidase is rapidly degraded in vivo such that 99% of the organ enzyme activity is lost at 4 hours after an intravenous injection (Table 1). Third, the 116 kDa β-galactosidase (Figure 1B) can be conjugated to the 8D3 TfRMAb without loss of enzyme activity (Figure 1C). Fourth, there is minimal brain uptake of the unconjugated β-galactosidase, but there is a 10-fold increase in brain uptake of enzyme following conjugation to the 8D3 TfRMAb (Table 1, Figures 2-3).

The β-galactosidase is rapidly removed from the blood due to the avid uptake of the enzyme by liver and spleen (Figures 2-3), which confirms the earlier observation of Onodera et al (1983). The blood concentration of the β-galactosidase/TfRMAb is 5- to 10-fold higher than the corresponding blood concentration of unconjugated β-galactosidase at 1-4 hours after injection (Table 2). The delayed clearance of the enzyme/TfRMAb conjugate may be related to the larger size of the enzyme/TfRMAb conjugate, as compared to the unconjugated enzyme. Following transport of the unconjugated β-galactosidase into cells, the enzyme is rapidly degraded in vivo, and the organ enzyme activity at 4 hours after intravenous injection is no greater than the endogenous enzyme activity (Table 1). In liver, the organ enzyme activity following intravenous injection of unconjugated β-galactosidase decreases 275-fold between 1 and 4 hours after administration (Table 1). This observation confirms an earlier study on the rapid degradation of bacterial β-galactosidase by mouse organs in vivo (Scherpereel et al, 2001).

In contrast to the rapid uptake of the unconjugated enzyme by peripheral tissues, the brain uptake of unconjugated β-galactosidase is nil, as shown by the absence of any change in

brain enzyme activity following injection of the low and high enzyme doses (Results). The brain delivery of β-galactosidase was accomplished in the present work by conjugating the enzyme to the TfRMAb. The bacterial β-galactosidase was conjugated to the 8D3 TfRMAb via a streptavidin-biotin linker. The 50 kDa recombinant SA was conjugated to the 8D3 TfRMAb via a stable thiol-ether linker (Figure 1A, reaction I) in parallel with the mono-biotinylation of β-galactosidase (Figure 1A, reaction II). The enzyme was mono-biotinylated with NHS-LC-LC-biotin (Methods), which positions a 14-atom spacer between the biotin and the ε-amino group of a surface lysine residue on the enzyme. This long spacer arm eliminates steric hindrance of the enzyme caused by attachment to the SA. Consequently, there is no loss of enzyme activity following attachment to the TfRMAb (Figure 1C). The mono-biotinylation of the enzyme was confirmed with the HABA assay (Methods). It is important that the enzyme is conjugated with only 1 biotin moiety. Higher degrees of biotinylation cause the formation of high molecular weight aggregates, owing to the multivalency of SA binding of biotin (Green, 1975). Such high molecular weight aggregates are rapidly removed in vivo. Conversely, attachment of the β-galactosidase to the TfRMAb via the SA-biotin linkage delayed the removal of the enzyme from blood, as the 60 min blood concentration of the β-galactosidase-TfRMAb was 5-fold greater than the corresponding blood concentration for the unconjugated enzyme (Table 2).

The molecular weight of the recombinant SA is 50 kDa and the molecular weight of the recombinant β-galactosidase is 116 kDa (Figure 1B). The combined size of the SA and the β-galactosidase is 166 kDa or approximately 10% greater than the size of the 150 kDa 8D3 TfRMAb. Despite the large size of the β-galactosidase, the 8D3 TfRMAb targets the enzyme to mouse brain in vivo (Figure 2, Inset). There is a 10-fold increase in the brain uptake of the β-galactosidase following conjugation the 8D3 TfRMAb, relative to the unconjugated enzyme

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(Figure 2, inset). When expressed per gram brain tissue, the peak β-galactosidase enzyme activity in brain was 484 ± 62 mU/g brain at 60 minutes following the IV injection of the high dose of the β-galactosidase-8D3 conjugate. This level of β-galactosidase enzyme activity in brain cannot be detected with histochemistry using colorimetric methods such as the standard X-gal technique, where a minimal enzyme activity level of 2,000 mU/g is required (Methods). It was not possible to inject even larger amounts of enzyme/MAb conjugate, because the dose used for the histochemical study in Figure 3 is a saturating concentration of the TfRMAb. The dose of 300 μg of β-galactosidase conjugated to 600 μg of 8D3/SA per mouse is equivalent to 12 mg/kg of the β-galactosidase and 24 mg/kg of the 8D3/SA conjugate, and this dose of 8D3 TfRMAb completely saturates the BBB TfR. The BBB transport of the MAb is 50% saturated at a systemic dose of 2-4 mg/kg of the 8D3 MAb (Lee et al, 2000). Although the β-galactosidase enzyme activity could not be detected in brain parenchyma with the histochemical method, the presence of the enzyme in the intra-endothelial compartment of brain could be detected following the intravenous administration of the high dose of the β-galactosidase-8D3 conjugate (Figure 3A and B). This histochemical assay demonstrates the targeting of the enzyme to the BBB compartment of brain, whereas no measurable enzyme activity was detected in the endothelial compartment following intravenous injection of the unconjugated enzyme (Figure 3C). The histochemical product in the endothelial compartment of brain was not due to entrapment of the enzyme in the blood compartment because the brain was saline cleared prior to perfusion fixation for the histochemistry (Methods). The adequacy of the saline clearance is demonstrated by the inability to detect histochemical product in the capillary compartment following injection of the unconjugated enzyme (Figure 4C).

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It is possible to detect the β-galactosidase enzyme activity in the endothelial cell of brain because this compartment has such a small volume. The intra-endothelial compartment in brain, < 1 μl/g, is about 1000-fold lower than the extra-vascular volume in brain (Pardridge, 2001). Therefore, when the enzyme-TfRMAb conjugate passes through the endothelial compartment, the enzyme activity is concentrated in the small endothelial volume, which allows for light microscopic histochemical detection. An identical intra-endothelial vascular staining pattern was reported previously following systemic administration of a TfRMAb conjugated to 5 nm gold (Bickel et al, 1994). The transport of the β-galactosidase/TfRMAb conjugate across the BBB and into brain parenchyma was demonstrated with the capillary depletion technique (Methods) as shown in Table 3. The β-galactosidase enzyme activity in the post-vascular supernatant is > 90% of the corresponding enzyme activity in the homogenate following IV injection of the β-galactosidase-TfRMAb conjugate (Table 3). Therefore, more than 90% of the β-galactosidase/8D3 conjugate that enters into the endothelial compartment passes through the BBB to enter brain parenchyma. This observation is in accord with prior work, which showed that >80% of the TfRMAb undergoes transcytosis through the BBB and into brain parenchyma within a 10-minute internal carotid artery perfusion of brain (Skarlatos et al, 1995).

The β-galactosidase enzyme activity in brain, and in other organs, returns to the endogenous level by 4 hours after an intravenous injection of the enzyme-TfRMAb conjugate (Table 1), which confirms earlier work by Scherpereel et al (2001). This rapid inactivation of either the unconjugated enzyme or of the β-galactosidase-TfRMAb conjugate contrasts with prior work showing peak β-galactosidase enzyme activity in brain is observed between 4 and 8 hours following the IV injection in mice of a fusion protein of β-galactosidase and the cationic peptide of the TAT protein of the human immunodeficiency virus (Schwarze et al, 1999).

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Conjugation of the cationic TAT peptide to a protein causes a >20-fold increase in the organ clearance of the protein in vivo (Lee and Pardridge, 2001). Therefore, a conjugate of the TAT cationic peptide and β-galactosidase might be rapidly eliminated from blood and organs such as brain in vivo.

In conclusion, these studies demonstrate the feasibility of delivering enzymes across the BBB with receptor-specific monoclonal antibodies that cross the BBB via endogenous receptor-mediated transport systems. With respect to protein targeting to the human brain, a MAb to the human insulin receptor (HIR) is 9-fold more active as a BBB drug delivery vector than is the TfRMAb (Pardridge, 2001). A genetically engineered form of the HIRMAb has been produced and is equally effective as a targeting vector as the original murine HIRMAb (Coloma et al, 2000). A fusion protein comprised of a therapeutic enzyme and a genetically engineered HIRMAb could prove to be of therapeutic value for the treatment of lysosomal storage disorder of the brain in humans.

REFERENCES

Bickel, U, Kang YS, Yoshikawa T and Pardridge WM (1994) In vivo demonstration of subcellular localization of anti-transferrin receptor monoclonal antibody-colloidal gold conjugate in brain capillary endothelium. *J Histochem Cytochem* **42**:1493-1497.

Coloma MJ, Lee HJ, Kurihara A, Landaw EM, Boado RJ, Morrison SL and Pardridge WM (2000) Transport across the primate blood-brain barrier of a genetically engineered chimeric monoclonal antibody to the human insulin receptor. *Pharm Res* **17**:266-274.

Cheng SH and Smith AE (2003) Gene therapy progress and prospects: gene therapy of lysosomal storage disorders. *Gene Ther* **10**:1275-1281.

Green NM (1975) Avidin. *Adv Protein Chem* **29**:85-133.

Hoyoux A, Jennes I, Dubois P, Genicot S, Dubail F, Francois JM, Baise E, Feller G and Gerday C (2001) Cold-adapted beta-galactosidase from the Antarctic psychrophile Pseudoalteromonas haloplanktis. *Appl Environ Microbiol* **67**:1529-1535.

Lee HJ, Engelhardt B, Lesley J, Bickel U and Pardridge WM (2000) Targeting rat anti-mouse transferrin receptor monoclonal antibodies through blood-brain barrier in mouse. *J Pharmacol Exp Ther* **292**:1048-1052.

Lee HJ and Pardridge WM (2001) Pharmacokinetics and delivery of tat and tat-protein conjugates to tissues in vivo. *Bioconjug Chem* **12**:995-999.

Onodera H, Takada G, Tada K and Desnick RJ (1983) Microautoradiographic study on the tissue localization of liposome-entrapped or unentrapped 3H-labeled beta-galactosidase injected into rats. *Tohoku J Exp Med* **140**:1-13.

Pardridge WM, Wu D, and Sakane, T. (1998) Combined use of carboxyl-directed protein pegylation and vector-mediated blood-brain barrier drug delivery system optimizes brain uptake of brain-derived neurotrophic factor following intravenous administration. *Pharm Res.* **15**: 576-582

Pardridge WM (2001) *Brain Drug Targeting; The Future of Brain Drug Development.* Cambridge University Press, Cambridge, United Kigndom.

Pardridge WM (2002) Drug and gene targeting to the brain with molecular Trojan horses. *Nat Rev Drug Discov* **1**:131-139.

Scherpereel A, Wiewrodt R, Christofidou-Solomidou M, Gervais R, Murciano JC, Albelda SM and Muzykantov VR (2001) Cell-selective intracellular delivery of a foreign enzyme to endothelium in vivo using vascular immunotargeting. *Faseb J* **15**:416-426.

Schwarze SR, Ho A, Vocero-Akbani A and Dowdy SF (1999) In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* **285**:1569-1572.

Skarlatos S, Yoshikawa T and Pardridge WM (1995) Transport of [125I]transferrin through the rat blood-brain barrier. *Brain Res* **683**:164-171.

Triguero D, Buciak J and Pardridge WM (1990) Capillary depletion method for quantification of blood-brain barrier transport of circulating peptides and plasma proteins. *J Neurochem* **54**:1882-1888.

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FIGURE LEGENDS

Figure 1. Conjugate synthesis. (A) Reaction I: Thiolation of the 8D3 MAb with Traut's reagent is performed in parallel with the activation of recombinant streptavidin (SA) with S-SMPB. The thiolated 8D3 MAb and activated SA are conjugated to form a stable thiol-ether linkage between the 8D3 MAb and SA. Reaction II: Bacterial β-galactosidase is mono-biotinylated with sulfo-NHS-LC-LC-biotin. The double LC linker provides a 14-atom spacer between the biotin moiety and the epsilon-amino group of surface lysine residues on the enzyme. Reaction III: The β-galactosidase-8D3 conjugate is formed upon mixing the mono-biotinylated β-galactosidase (β-gal-LC-LC-biotin) and the 8D3-SA conjugate. (B) SDS-PAGE of molecular weight standards (left lane) and E. coli β-galactosidase (right lane). The size of the molecular weight standards is shown in the figure. The β-galactosidase migrates at a molecular weight of 116 kDa. (C) The β-galactosidase enzyme activity is unchanged following conjugation to the 8D3 monoclonal antibody. Data are mean ± SE (n = 3), and are normalized for mg of β-galactosidase protein.

Figure 2. Low dose injection study. Percent of injected dose (ID) per gram tissue is shown for mouse liver, spleen, kidney, heart and brain (inset) at 60 min after an IV injection of a low dose (15 ug/mouse) of β-galactosidase in either the unconjugated form (closed bars) or as a conjugate with the 8D3 TfRMAb (open bars). Data are mean ± SE (n = 3). The injected dose per gram organ was computed from the specific activity of the injected enzyme or enzyme-8D3 conjugate (mU/ug) and the injected dose of enzyme (ug). The endogenous β-galactosidase enzyme activity (Table 1) was subtracted for each organ.

Figure 3. High dose injection study. Percent of injected dose (ID) per gram tissue is shown for mouse liver, spleen, kidney, heart and brain (inset) at 60 min after an IV injection of a high dose (150 ug/mouse) of β-galactosidase in either the unconjugated form (closed bars) or as a conjugate with the 8D3 TfRMAb (open bars). Data are mean ± SE (n = 3). The injected dose per gram organ was computed from the specific activity of the injected enzyme or enzyme-8D3 conjugate (mU/ug) and the injected dose of enzyme (ug). The endogenous β-galactosidase enzyme activity (Table 1) was subtracted for each organ.

Figure 4. Brain histochemistry. Mice brain was saline flushed and perfusion fixed at 60 minutes following intravenous injection of a maximal dose (300 ug/mouse) of either the β-galactosidase-8D3 conjugate (panels A and B) or the unconjugated β-galactosidase (panel C). The magnification bar in panel A is 48 microns. The magnification bar in panel B is 180 microns. The magnification of panels B and C are identical.

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Table 1. Organ enzyme activity at 1 and 4 hours after intravenous administration of high dose of either β-galactosidase (gal)/8D3 conjugate or unconjugated enzyme

Organ	β-gal-8D3 at 1 hour	β -gal at 1 hour	β-gal-8D3 at 4 hours	β -gal at 4 hours	Endogenous activity
Brain	13.9± 1.0	2.8 ± 0.6	1.6 ± 0.1	1.2 ± 0	1.5 ± 0.1
Liver	766 ± 128	386 ± 84	3.2 ± 1.1	1.4 ± 0.3	1.7 ± 0.7
Spleen	293 ± 22	367 ± 68	18.2 ± 0.8	7.1 ± 0.4	5.7 ± 0.3
Heart	46 ± 16	19 ± 4	1.1 ± 0.2	0.4 ± 0.3	0.68 ± 0.01
Kidney	108 ± 31	61 ± 8	9.0 ± 1.6	7.0 ± 0.3	6.1 ± 0.1

Units of enzyme activity are mU/mg protein, not corrected for endogenous enzyme activity. Data are means ± SE. injection dose=150 ug β-gal as either unconjugated enzyme or as a conjugate with the 8D3 TfRMAb. Specific activity of β-gal= 748 U/mg protein; specific activity of β-gal-biotin/SA-8D3= 790 U/mg protein. The endogenous activity is the β-galactosidase enzyme activity in organs from un-injected mice.

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Table 2. Blood β-galactosidase enzyme activity

Dose (ug)	Units	β-gal-8D3 at 1 hour	β -gal at 1 hour	β-gal-8D3 at 4 hours	β -gal at 4 hours	Endogenous activity
150	mU/mL	4879 ± 762	960 ± 35	493 ± 21	38 ± 9	4.3 ± 0.5
	%ID/mL	4.1 ± 0.6	0.85 ± 0.04	0.41 ± 0.01	0.034 ± 0.008	--
15	mU/mL	939 ± 46	28 ± 2	n.d.	n.d.	--
	%ID/mL			n.d.	n.d.	--

Data are means ± SE. mU/mL of enzyme activity were converted into %ID/mL based on the specific activity (mU/ug) and the injection dose (ID) of the unconjugated β-galactosidase (gal) or the β-gal-8D3 conjugate.

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Table 3. Capillary depletion study

fraction	β -galactosidase enzyme activity (mU/mg protein)	γGTP activity (mU/mg protein)
homogenate	6.9 ± 0.5	34 ± 1
post-vascular supernatant	6.7 ± 0.3	33 ± 1
vascular pellet	1.5 ± 0.03	529 ± 25

Data are means ± SE (n=3 mice). The β-galactosidase enzyme activity and the γGTP enzyme activity were measured in brain fractions removed 60 min after an intravenous injection of 150 μg/mouse of the β-galactosidase/8D3 conjugate. The brain was separated into total homogenate, post-vascular supernatant, and vascular pellet with the capillary depletion technique (Methods).

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A

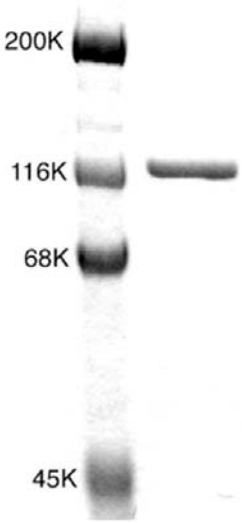
I. 8D3 MAb —Traut's reagent→ thiolated 8D3

thiolated 8D3 —S-SMPB activated SA→ 8D3-SA

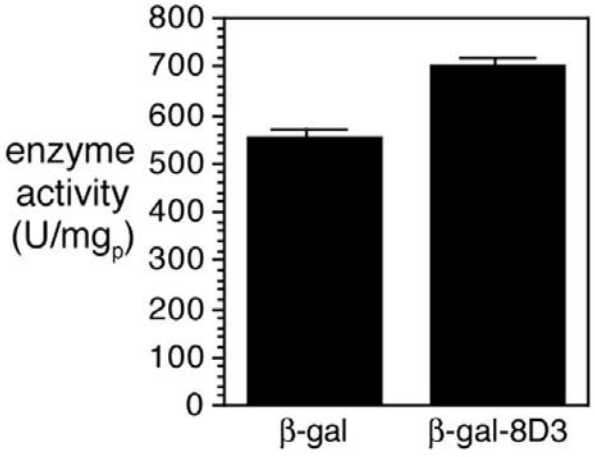
II. β-galactosidase (gal) —sulfo-NHS-LC-LC-biotin→ β-gal-LC-LC-biotin

III. β-gal-LC-LC-biotin + 8D3-SA → β-gal-8D3 conjugate

B



C



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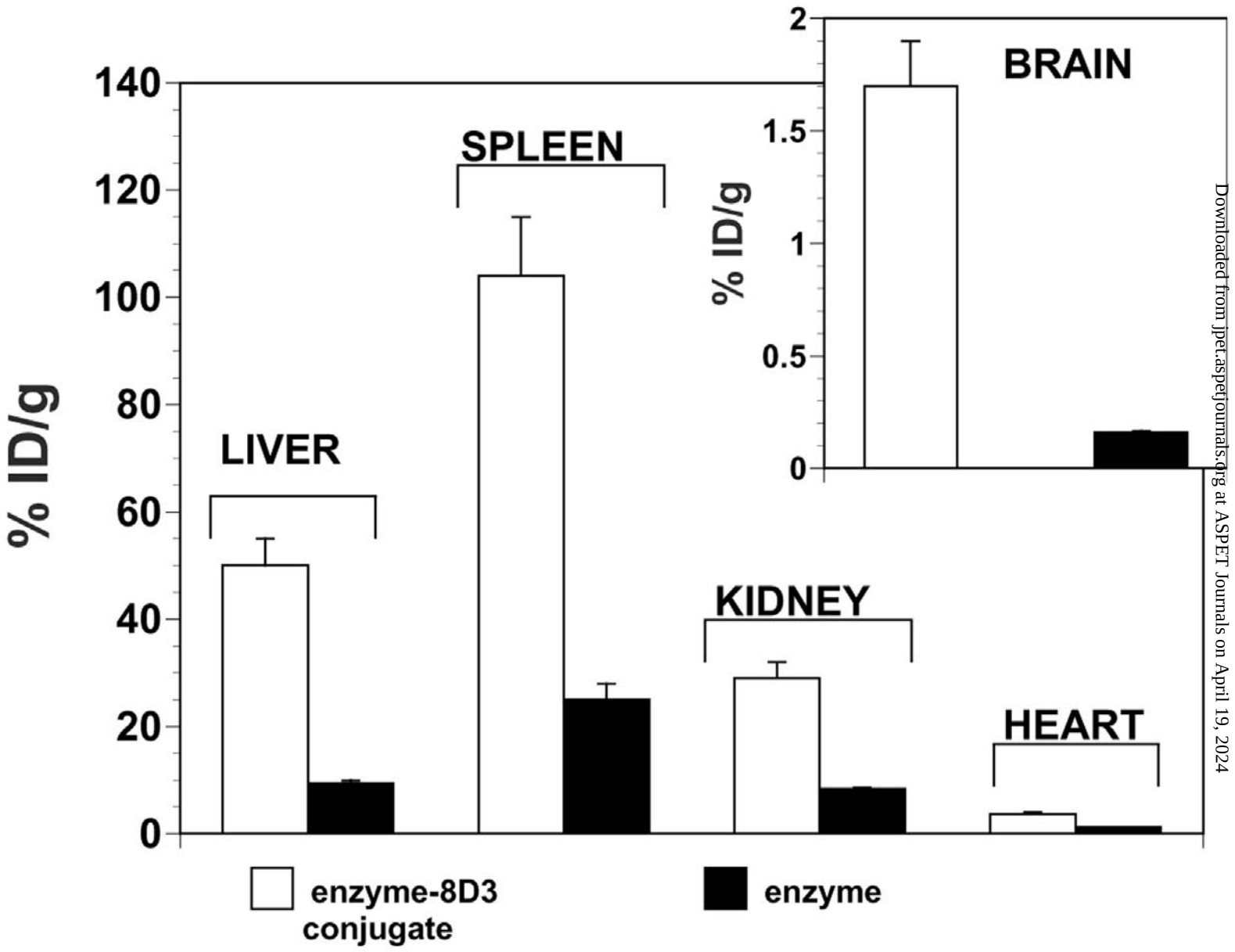

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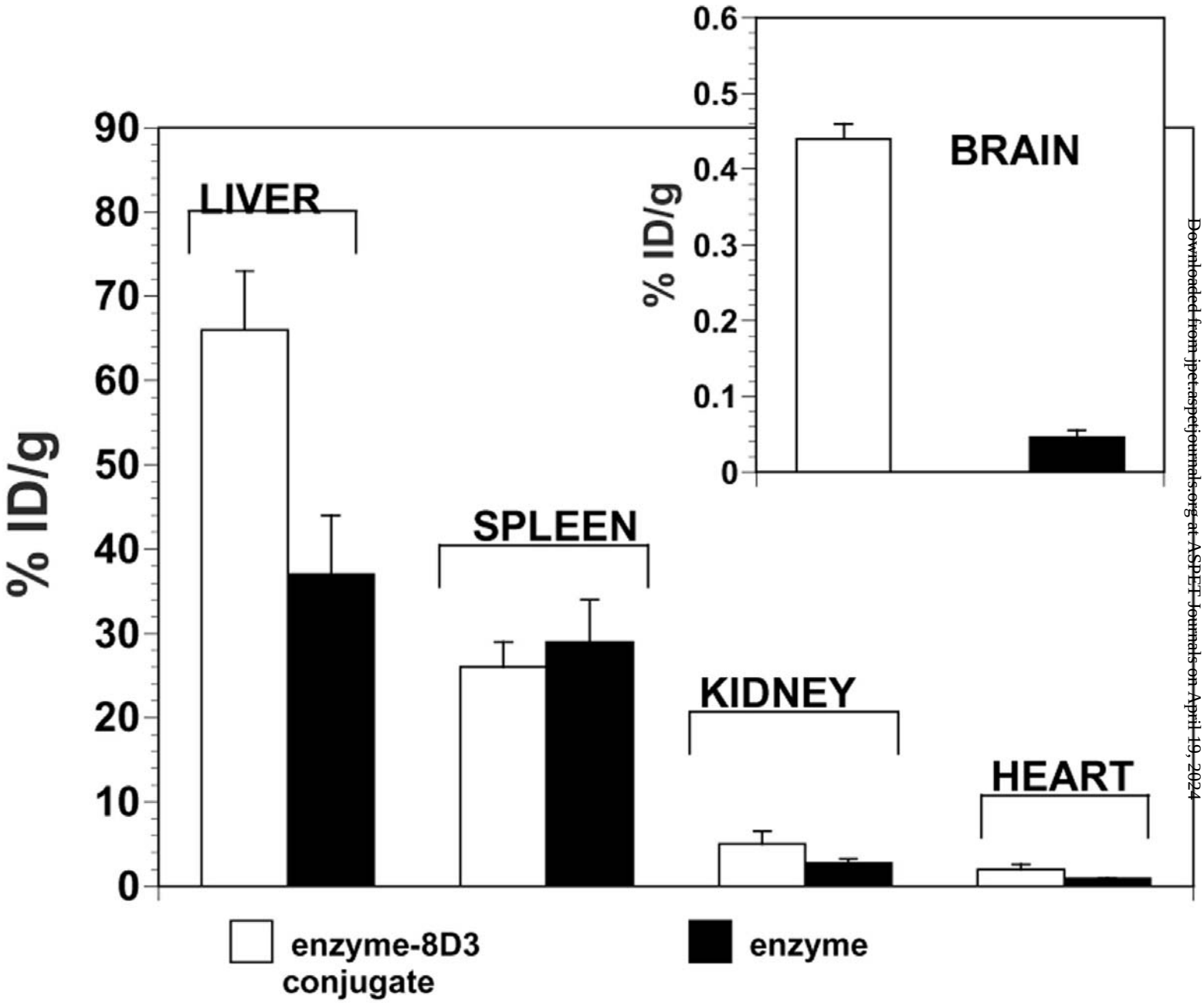

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