Drug Targeting of Erythropoietin Across the Primate Blood-Brain Barrier with an IgG Molecular Trojan Horse

Ruben J. Boado

Eric Ka-Wai Hui

Jeff Zhiqiang Lu

William M. Pardridge

ArmaGen Technologies, Inc. (R.J.B., E.K.-W.H., J.Z.L.) Santa Monica, California, and

Department of Medicine (R.J.B., W.M.P.), UCLA, Los Angeles, California

,

Running title: EPO Drug Targeting to Brain

Address correspondence to:

Dr. William M. Pardridge

UCLA Warren Hall 13-164

900 Veteran Ave.

Los Angeles, CA 90024

Ph: (310) 825-8858

Fax: (310) 206-5163

Email: wpardridge@mednet.ucla.edu

Text pages: 29

Tables: 5

Figures: 8

References: 26

Abstract: 247 words

Introduction: 461 words

Discussion: 1628 words

Abbreviations: BBB, blood-brain barrier; MAb, monoclonal antibody; EPO, erythropoietin; PD, Parkinson's disease; HIR, human insulin receptor; HIRMAb, engineered MAb against the HIR; ID, injected dose; HC, heavy chain; LC, light chain; TV, tandem vector; SFM, serum free medium; MTX, methotrexate; CHO, Chinese hamster ovary; RT, room temperature; TCA, trichloroacetic acid; VD, volume of distribution: PK, pharmacokinetics; PS, permeability-surface area; AUC, area under the curve; AA, amino acid; ECD, extracellular domain

Section assignment: Neuropharmacology

2

Abstract

Erythropoietin (EPO) is a neurotrophic factor that could be developed as a new drug for brain disorders. However, EPO does not cross the blood-brain barrier (BBB). In the present study, human EPO was re-engineered by fusion to the carboxyl terminus of the heavy chain of a chimeric monoclonal antibody (MAb) to the human insulin receptor (HIR). The HIRMAb acts as a molecular Trojan horse to ferry the EPO into brain via receptor-mediated transport on the endogenous BBB insulin receptor. The HIRMAb-EPO fusion protein was immunoreactive with antibodies to both human IgG and EPO. The HIRMAb-EPO fusion protein bound with high affinity to the extracellular domain of both the HIR, $ED_{50} = 0.21 \pm 0.05$ nM, and the EPO receptor, $ED_{50} = 0.30 \pm 0.01$ nM, and activated thymidine incorporation into human TF-1 cells with an ED_{50} of 0.1 nM. Differentially radiolabeled EPO and the HIRMAb-EPO fusion protein were injected intravenously in the adult Rhesus monkey. Whereas EPO did not cross the primate BBB, the HIRMAb-EPO fusion protein was rapidly transported into brain, at levels that produce pharmacologic elevations in brain EPO at small systemic doses. The HIRMAb fusion protein selectively targeted the brain relative to peripheral organs. In conclusion, a novel IgG-EPO fusion protein has been engineered, expressed, and shown to be bi-functional with retention of high affinity binding to both the insulin and EPO receptors. The IgG-EPO fusion protein represents a new class of EPO neurotherapeutics that has been specifically re-engineered to penetrate the human BBB.

Introduction

Erythropoietin (EPO) is a neurotrophic factor made in brain, which activates EPO receptors (EPOR) in brain (Sakanaka et al, 1998). EPO could potentially be developed as a new drug for the treatment of acute brain conditions, such as stroke or traumatic brain injury, or chronic neurodegeneration, such as Parkinson's disease (PD) (Sakanaka et al, 1998; Grasso et al, 2007; Xue et al, 2007). The brain drug development of EPO is limited by the lack of transport of large molecule pharmaceuticals across the brain capillary endothelial wall, which forms the blood-brain barrier (BBB) in vivo. Similar to other large molecule drugs, EPO does not cross the BBB in the absence of BBB disruption (Lietaud et al, 2008). Neurotrophic factors such as EPO can be re-engineered to cross the BBB as fusion proteins with BBB molecular Trojan horses (Pardridge, 2008). A molecular Trojan horse is an endogenous peptide, or peptidomimetic monoclonal antibody (MAb), that crosses the BBB via receptor-mediated transport on an endogenous BBB receptor. The most potent BBB molecular Trojan horse is a MAb to the human insulin receptor (HIR) (Pardridge et al, 1995). Genetically engineered forms of the HIRMAb have been produced and both chimeric and humanized HIRMAb's bind the human BBB insulin receptor in vitro, and cross the Rhesus monkey BBB in vivo (Boado et al, 2007a). HIRMAb-neurotrophin fusion proteins have been engineered for brain derived neurotrophic factor (BDNF) (Boado et al, 2007b) and glial derived neurotrophic factor (GDNF) (Boado et al, 2008). The HIRMAb-BDNF fusion protein induces neuroprotection in human neural cells, and crosses the BBB of the adult Rhesus monkey in vivo (Boado et al, 2007b). The HIRMAb-GDNF fusion protein activates the c-ret kinase in human neural cells, and induces

4

neuroprotection in regional brain ischemia (Boado et al, 2008), and exhibits no toxicity, effects on blood glucose, or dose-related immune reactions in primate toxicology studies (Pardridge and Boado, 2009).

The present studies describe the re-engineering of human EPO as an IgG fusion protein to enable receptor-mediated transport of EPO across the human BBB. This work describes the genetic engineering, expression, and characterization of a HIRMAb-EPO fusion protein. Mature human EPO was fused to the carboxyl terminus of the heavy chain of the chimeric HIRMAb, as depicted in Supplemental Figure 1. The HIRMAb-EPO fusion protein was expressed in COS cells transiently, and in stably transfected Chinese hamster ovary (CHO) cells, and affinity purified from serum free medium. The bi-functional properties of the HIRMAb-EPO fusion protein were examined with respect to dual binding both to the HIR and to the human EPOR. The biological activity of the HIRMAb-EPO fusion protein was confirmed with a bio-assay using human TF-1 cells. The differential transport of recombinant EPO and the HIRMAb-EPO fusion protein across the BBB in vivo was examined in the Rhesus monkey.

Methods

Engineering of HIRMAb-EPO fusion protein tandem expression vector. The

human EPO cDNA encoding for amino acids Ala^{28}-Arg^{193} (accession # NP_000790),

and excluding the 27 amino acid signal peptide, was synthesized at Retrogen (San

Diego, CA). The EPO artificial gene has a StuI site on the 5'-end followed by 'CA' to

maintain the open reading frame and to introduce a Ser-Ser-Ser linker between the CH3

region of the HIRMAb heavy chain (HC) and the amino terminus of the EPO minus the

signal peptide. The 3'-end of the EPO cDNA was engineered with a StuI site

immediately after the stop codon, TGA. An internal StuI site in the EPO cDNA was

removed by use of an alternative codon for Glu^{186} in the design of the synthetic EPO

gene. The EPO cDNA was obtained from Retrogen subcloned into the pCR-Blunt vector

(Invitrogen, San Diego, CA). The 515 nt EPO cDNA sequence was confirmed by bi-

directional DNA sequencing.

The EPO cDNA was released from the pCR vector with StuI and the ~500 bp

EPO cDNA fragment was isolated by agarose gel electrophoresis using the Qiagen gel

extraction kit (Valencia, CA). The EPO cDNA was inserted into the pHIRMAb eukaryotic

tandem expression plasmid, which has been described previously (Boado et al, 2007b),

at the HpaI site, and this expression plasmid was designated pHIRMAb-EPO. The

pHIRMAb tandem vector (TV) encodes the light chain (LC) of the chimeric HIRMAb, the

HC of the chimeric HIRMAb, dihydrofolate reductase (DHFR), and the neomycin

resistance gene. The latter genes allowed for selection of high producing host cell lines

with methotrexate and selection with G418, respectively. The entire open reading

frames for the LC, HC and DHFR expression cassettes of the pHIRMAb-EPO plasmid

were confirmed by bi-directional DNA sequencing performed at Eurofins MWG Operon (Huntsville, AL) using custom sequencing oligodeoxynucleotides synthesized at Midland Certified Reagent Co. (Midland, TX).

Transient expression of HIRMAb-EPO fusion protein in COS cells. COS cells were transfected with the pHIRMAb-EPO TV using Lipofectamine 2000, with a ratio of 1:2.5 ug DNA:uL Lipofectamine. Following transfection, the cells were cultured in serum free VP-SFM (Invitrogen, Carlsbad, CA). The conditioned serum free medium was collected at 3 and 7 days. Transgene expression and fusion protein secretion to the medium was assayed by measurement of human IgG in the conditioned medium. Human IgG ELISA was performed in Immulon 2 high binding plates (Dynex Tech., Chantilly, VA) with COS cell conditioned medium, as described previously (Boado et al, 2007b).

Production of stably transfected CHO line. The TV was linearized and DG44 CHO cells were electroporated, followed by selection in hypoxanthine-thymine deficient medium and amplification with graded increases in methotrexate (MTX) up to 80 nM in serum free medium (SFM). 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 10-30 mg/L in shake flasks at a cell density of 1-2 million cells/mL. The CHO cells were propagated in 1 L bottles until 2.4L of conditioned SFM was collected. The medium was ultra-filtered with a 0.2 um Sartopore-2 sterile-filter unit (Sartorius Stedim Biotech, Goettingen, Germany) prior to protein A chromatography.

Protein A chromatography. Serum free medium conditioned by either transiently transfected COS cells or stably transfected CHO cells was filtered and applied to a 25 mL protein A Sepharose 4 Fast Flow (GE Life Sciences, Chicago, IL) column equilibrated in 25 mM Tris/25 mM NaCl/5 mM EDTA/pH=7.1. Following application of the sample, the column was washed with 25 mM Tris/1 M NaCl/5 mM EDTA/pH=7.1, and the fusion protein was eluted with 0.1 M sodium acetate/pH=3.7. The acid eluate was pooled, Tris was added to 0.05 M, NaCl was added to 0.15 M, the pH was increased to pH=6.0, and the solution was stored sterile-filtered at 4C.

SDS-PAGE and Western blotting. The homogeneity of protein A purified fusion protein was evaluated with a reducing 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomasie Blue staining. For Western blotting, immunoreactivity was tested with a primary rabbit antibody to human EPO (R&D Systems, Minneapolis, MN) or a primary goat antibody against human IgG heavy and light chains (Vector Labs, Burlingame, CA). Human recombinant EPO was purchased from R&D Systems.

Size exclusion chromatography. Size exclusion chromatography (SEC) high performance liquid chromatography of the protein A purified HIRMAb-EPO fusion protein was performed with two 7.8 mm x 30 cm TSK-GEL G3000SW$_{XL}$ columns (Tosoh Bioscience, Tokyo, Japan) in series, under isocratic conditions at a flow rate of 0.5 ml/min with Perkin-Elmer Series 200 pump. The absorbance at 280 nm was detected with a Shimadzu SPD-10A UV-VIS detector and a Shimadzu CR-8 chart recorder. The elution of molecular weight (MW) standards (GE Healthcare, Buckinghamshire, UK),

8

blue dextran-2000, aldolase, and ovalbumin was measured under the same elution conditions.

HIR receptor assay. The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined with an ELISA using the lectin affinity purified HIR ECD. CHO cells permanently transfected with the HIR ECD were grown in serum free media (SFM), and the HIR ECD was purified with a wheat germ agglutinin affinity column, as previously described (Coloma et al, 2000). The HIR ECD (0.2 ug/well) was plated on Immulon 2 high binding 96-well plates, and the binding of the chimeric HIRMAb, or the HIRMAb-EPO fusion protein was detected with a biotinylated goat anti-human IgG (H+L) antibody (0.3 ug/well), and the ABC Elite detection system (Vector Labs). The concentration that caused 50% binding to the HIR ECD, the ED50, was determined by non-linear regression analysis.

EPO receptor assay. Binding of the HIRMAb-EPO fusion protein to recombinant human EPOR was evaluated using a fusion protein of human IgG Fc and the ECD of recombinant human EPOR, which was obtained from R&D Systems, and plated in 96-well plates overnight at 0.2 ug/well. Wells were blocked with Tris buffered saline (TBS) and 1% bovine serum albumin. Various concentrations of either the HIRMAb-EPO fusion protein, or human IgG1, were plated for 2 hours at room temperature (RT). Following aspiration, the wells were washed with TBS/0.05% Tween-20 (TBST), a conjugate of alkaline phosphatase and a goat anti-human kappa light chain antibody (Sigma Chemical Co., St. Louis, MO) was plated and detection at 405 nm was performed with an ELISA plate reader after color development with para-nitrophenylphosphate (Sigma Chemical Co.).The concentration that caused 50%

9

binding to the EPOR ECD, the ED50, was determined by non-linear regression analysis.

TF-1 bio-assay. Human TF-1 cells obtained from the American Type Culture Collection (ATCC, Manassas, VA, CRL 2003) were cultured in RPMI-1640 medium with 10% fetal bovine serum and 2 ng/mL human recombinant granulocyte-macrophage colony stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ) (Kitamura et al, 1989). Cells were plated in 96-well plates at 400,000 cells/well, and cultured overnight in medium containing no GM-CSF. The following day, the HIRMAb-EPO fusion protein was added followed by incubation for 44 hours. The medium was then supplemented with 3H-thymidine (Perkin-Elmer, Boston, MA) at a final concentration of 0.5 uCi/well. The wells were incubated at 37C for 4 hours, and intracellular radioactivity was determined following washing of the cells in a Cell Harvester (Millipore, Billerica, MA) over glass fiber/C filters under vacuum. The filter was washed 3 times with cold 10% trichloroacetic acid (TCA), and the cell lysate was solubilized in 1 N NaOH. Radioactivity was determined with a Perkin Elmer liquid scintillation spectrometer, and cell protein was determined with the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL). The cell radioactivity was divided by the thymidine specific activity (6.7 uCi/nmol), and thymidine incorporation was expressed as fmol/mg protein.

Radio-labeling of proteins. [^{125}I]-Bolton-Hunter reagent was purchased from American Radiolabeled Chemicals (St. Louis, MO). Human recombinant EPO (#286-EP) was purchased from R&D Systems (Minneapolis, MN), and shown to be homogenous by SDS-PAGE. The EPO (6 ug) was radio-labeled with 2 mCi of fresh Bolton-Hunter reagent to a specific activity of 67 uCi/ug and a trichloroacetic acid (TCA)

precipitability of >99% following purification with a 1.0x28 cm column of Sephadex G-25 and elution with 0.01 M NaH2PO4/0.15 M NaCl/pH=7.4/0.05% Tween-20 (PBST). The TCA precipitation of the labeled EPO remained >99% at 24 hours after iodination, and the EPO was administered to the primate within 24 hrs of radio-labeling. [³H]-N-succinimidyl propionate (NSP) was purchased from American Radiolabeled Chemicals. The HIRMAb-EPO fusion protein was radio-labeled with fresh NSP to a specific activity of 2.9 uCi/ug and a TCA precipitability of 96% following purification with a 1.0x28 cm column of Sephadex G-25 and elution with 0.02 M MES/0.15 M NaCl/pH=6.0/0.05% Tween-20 (MBST), where MES=4-Morpholineethanesulfonic acid. The solution was buffer exchanged with MBST/0.1% bovine serum albumin, and an Ultra-15 microconcentrator (Millipore, Bedford, MA), which increased the TCA precipitability to 99%. The ³H-labeled HIRMAb-EPO fusion protein was labeled in advance of the primate study and stored at -70C.

EPO radio-receptor assay. The retention of high affinity EPOR binding by the [¹²⁵I]-EPO following radiolabeling with the Bolton-Hunter reagent was examined with a radio-receptor assay. A mouse anti-human IgG1 Fc antibody (Invitrogen/Zymed) was plated overnight to capture a Fc fusion protein of the human EPOR ECD (R&D Systems). The wells were washed with PBS, followed by the addition of 100 uL/well of a co-mixture of [¹²⁵I]-EPO at a concentration of 0.01 uCi/well (0.15 ng/well) and various concentrations of unlabeled human EPO (R&D Systems, #286-EP), followed by a 3 hour incubation at room temperature. The wells were emptied by aspiration, washed with cold PBS, and 250 uL/well of 1 N NaOH was added, followed by heating at 60C for 30 min. Radioactivity was counted in Ultima Gold (Perkin Elmer, Downers Grove, IL) in

a Perkin Elmer Tricarb 2100TR liquid scintillation counter, and the fractional binding per well was computed. The half-saturation constant, K_D, of EPO binding to the EPOR was determined by non-linear regression analysis.

Primate brain uptake and capillary depletion analysis. An adult female Rhesus monkey, 5.6 kg, was obtained from Covance (Alice, TX). The animal was injected intravenously (IV) with 2132 uCi of [^{3}H]-HIRMAb-EPO fusion protein, 330 uCi of [^{125}I]-EPO in 3.0 mL by bolus injection over 30 seconds in the left femoral vein. The injection dose (ID) of the HIRMAb-EPO fusion protein was 130 ug/kg, and the ID for EPO was 0.9 ug/kg. The animal was initially anesthetized with intramuscular ketamine, and anesthesia was maintained by 1% isoflurane by inhalation. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Following intravenous drug administration, femoral venous plasma was obtained at 1, 2.5, 5, 15, 30, 60, and 120 min for determination of ^{3}H and ^{125}I radioactivity. The animal was euthanized at 120 min after drug injection, and samples of major organs (heart, liver, spleen, lung, skeletal muscle, kidney, and omental fat) were removed, weighed, and processed for determination of radioactivity. The cranium was opened and the brain was removed. Samples of frontal cortical gray matter, frontal cortical white matter, cerebellar gray matter, and cerebellar white matter were removed for radioactivity determination.

Samples (~2 gram) of frontal cortex were removed for capillary depletion analysis, as described previously (Triguero et al, 1990). The brain was homogenized in 8 mL cold PBS in a tissue grinder. The homogenate was supplemented with 9.4 mL

cold 40% dextran (70 kDa, Sigma Chemical Co.), and an aliquot of the homogenate

was taken for radioactivity measurement. The homogenate was centrifuged at 3200 g

at 4C for 10 min in a fixed angle rotor. The brain microvasculature quantitatively

sediments as the pellet (Triguero et al, 1990), and the post-vascular supernatant is a

measure of capillary depleted brain parenchyma. The vascular pellet and supernatant

were counted for ^{3}H and ^{125}I radioactivity in parallel with the homogenate. The volume

of distribution (VD) was determined for each of the 3 fractions from the ratio of total ^{125}I

or ^{3}H radioactivity in the fraction divided by the total ^{125}I or ^{3}H radioactivity in the 120

min terminal plasma.

Plasma and tissue samples were analyzed for ^{125}I radioactivity with a gamma

counter (Wizard 1470, Perkin Elmer), and were analyzed for ^{3}H radioactivity with a liquid

scintillation counter (Tricarb 2100TR, Perkin Elmer, Downers Grove, IL). The ^{125}I

isotope emits radiation that is detected in the ^{3}H channel (0-12 keV) of the liquid

scintillation counter (LSC). Therefore, quench curves were produced using chloroform

as the quench agent to compute the efficiency of counting of ^{125}I in the ^{3}H window, as

described previously (Boado and Pardridge, 2009). All samples for ^{3}H counting were

solubilized in Soluene-350 and counted in the LSC in Opti-Fluor O (Perkin Elmer).

Pharmacokinetics and organ PS product. The ^{3}H or ^{125}I radioactivity in

plasma, DPM/mL, was converted to % injected dose (ID)/mL, and the %ID/mL was fit to

a mono- or bi-exponential equation. The intercepts (A1, A2) and the slopes (k1, k2)

were used to compute the median residence time (MRT), the central volume of

distribution (Vc), the steady state volume of distribution (Vss), the area under the

plasma concentration curve (AUC) at 120 min, the steady state AUC (AUCss), and the

systemic clearance (CL), as described previously (Pardridge, et al, 1995). Non-linear regression analysis used the AR subroutine of the BMDP Statistical Software (Statistical Solutions Ltd, Cork, Ireland). Data were weighted by $1/(\%ID/mL)^2$.

The organ clearance (μL/min/g), also called the permeability-surface area (PS) product, is computed from the terminal organ uptake (%ID/g) and the 120 min plasma AUC (%IDmin/mL) as follows:

$$\text{organ PS product} = [(\%ID/g)/AUC]*1000$$

Gel filtration chromatography of primate plasma. Primate plasma removed at 120 min after IV injection of the [^{3}H]-HIRMAb-EPO fusion protein and [^{125}I]-EPO was analyzed with gel filtration chromatography using a 1 x 30 cm Superose 6HR column (GE Life Sciences) and a Perkin-Elmer Series 200 pump. The absorbance at 280 nm was detected with a Shimadzu SPD-10A UV-VIS detector and a Shimadzu CR-8 chart recorder. The elution volume volume of blue dextran-2000 was used as a measure of the column void volume. The injection sample was comprised of 50 uL of plasma diluted to 200 uL with PBS containing 0.05% Tween-20 (PBST). The column was eluted at 0.25 mL/min in PBST. Fractions were counted for ^{3}H and ^{125}I as described above.

Results

DNA sequencing of the pHIRMAb-EPO plasmid encompassed 9,036 nucleotides (nt), which spanned the expression cassettes for the heavy chain fusion gene, the light chain gene, and the DHFR gene. The fusion heavy chain expression cassette included a 1,986 nt open reading frame, which encoded for a 631 amino acid (AA) protein, comprised of a 19 AA IgG signal peptide, the 443 AA HIRMAb HC, a 3 AA linker (Ser-Ser-Ser), and the 166 AA human EPO minus the signal peptide, which was 100% identical to the AA sequence from Ala^{28} to Arg^{193} of human EPO (NP_000790). The predicted molecular weight of the heavy chain fusion protein, minus glycosylation, is 67,226 Da, with a predicted isoelectric point (pI) of 8.75. The light chain was comprised of 234 AA, which included a 20 AA signal peptide, a 108 AA variable region of the light chain of the HIRMAb light chain, and a 106 AA human kappa light chain constant (C)-region. The predicted molecular weight of the light chain is 23,398 Da with a predicted isoelectric point (pI) of 5.45.

Lipofection of COS cells with the pHIRMAb-EPO TV resulted in high medium human IgG levels (Table 1), as determined with a human Fc specific ELISA. The HIRMAb-EPO fusion protein was purified by protein A affinity chromatography. Following SDS-PAGE and Coomasie blue staining, the size of the light chain (LC) is the same for both the HIRMAb and the HIRMAb-EPO fusion protein (Supplemental Figure 2). The size of the heavy chain (HC) of the fusion protein is about 35 kDa larger than the HC of the HIRMAb (Supplemental Figure 2). On Western blotting, the LC of either the HIRMAb or the HIRMAb-EPO fusion protein react equally with a primary antibody directed against the human IgG (H+L), as shown in Supplemental Figure 3A. The size

of the HC of the fusion protein is about 35 kDa larger than the size of the HC of the

HIRMAb on Western blots using either the anti-human IgG primary antibody

(Supplemental Figure 3A) or the anti-human EPO primary antibody (Supplemental

Figure 3B). The anti- EPO primary antibody reacts with the HC of the fusion protein,

and with recombinant EPO, but does not react with the HIRMAb (Supplemental Figure

3B). The HIRMAb-EPO fusion protein eluted as a single peak, with <1% aggregation on

SEC HPLC (Supplemental Figure 4).

The affinity of the fusion protein for the HIR extracellular domain (ECD) was

determined with a ligand binding assay using lectin affinity purified HIR ECD (Methods).

There is comparable binding of either the chimeric HIRMAb or the HIRMAb-EPO fusion

protein to the HIR ECD with ED50 of 0.20±0.03 nM and 0.21±0.05 nM, respectively

(Figure 1).

The affinity of the HIRMAb-EPO fusion protein for the ECD of the human EPOR

was measured with an ELISA (Methods). There was no binding of human IgG1κ to the

EPOR, whereas saturable binding of the HIRMAb-EPO fusion protein was observed

(Figure 2). The affinity of the HIRMAb- EPO fusion protein for the EPOR was high, with

an ED50 of 0.30 ±0.01 nM (Figure 2). The biologic activity of the HIRMAb-EPO fusion

protein was evaluated with a bio-assay in human TF-1 cells. Thymidine incorporation

into TF-1 cells was increased via a saturable mechanism by the HIRMAb-EPO fusion

protein, with an ED50 of 0.1 nM (Figure 3).

CHO cells stably transfected with the TV encoding the HIRMAb-EPO fusion

protein were subjected to limited dilutional cloning and a high producing CHO line was

isolated, and the fusion protein was purified by protein A affinity chromatography. The

results of the SDS-PAGE, Western blotting, SEC HPLC, and HIR and EPO receptor binding assays were identical to the results obtained for the COS-derived fusion protein. The CHO-derived HIRMAb-EPO fusion protein was used for the primate brain uptake study.

The HIRMAb-EPO fusion protein was radiolabeled with [^{3}H] and the recombinant human EPO was radiolabeled with [^{125}I]. The retention of biological activity of the EPO following radio-labeling with the [^{125}I]-Bolton-Hunter reagent was confirmed with a radio-receptor assay. The design of the radio-receptor assay is shown in Figure 4A. Binding of the [^{125}I]-EPO to the EPOR is displaced by unlabeled EPO with a KD of 0.17 ± 0.09 nM (Figure 4B). This assay shows the high affinity binding of EPO to the EPOR is retained following radio-labeling with the Bolton-Hunter reagent.

The [^{3}H]-HIRMAb-EPO fusion protein and the [^{125}I]-EPO were co-injected IV into an adult Rhesus monkey. The clearance of the plasma radioactivity is shown in Figure 5A for the [^{125}I]-EPO and the [^{3}H]-HIRMAb-EPO fusion protein, and these data show that the HIRMAb-EPO fusion protein is removed from plasma much faster than is EPO. The plasma radioactivity that was precipitable with TCA is >99% for [^{125}I]-EPO at all time points (Figure 5B). The plasma radioactivity that is TCA precipitable for the [^{3}H]-HIRMAb-EPO fusion protein is >99% through 30 min, is 97% at 60 min, and is 93% at 120 min after administration (Figure 5B). The plasma clearance profiles (Figure 5A) were fit to a bi-exponential function for the HIRMAb-EPO fusion protein, and to a mono-exponential function for EPO, for estimation of the PK parameters, which are shown in Table 2 for each protein. The estimated plasma AUCss for EPO is 13-fold greater than the plasma AUCss for the HIRMAb-EPO fusion protein (Table 2). The uptake of the

17

proteins by brain and peripheral organs was measured as a % ID/100 gram tissue, and these values are given in Table 3. The brain volume of distribution (VD) of the proteins was measured with the capillary depletion method and the VD values for the homogenate, the vascular pellet, and the post-vascular supernatant are shown in Table 4. The radioactivity in the post-vascular supernatant represents intact fusion protein as the TCA precipitability of the post-vascular supernatant is 91 ± 1% (Table 4).

The BBB PS products for the HIRMAb-EPO fusion protein and recombinant EPO were computed from the 2 hour plasma AUC (Table 2) and the brain uptake (Table 3), and the PS products are given in Figure 6C. Figure 6 also displays the 2 hour plasma AUC, the %ID/100g, and the BBB PS product for EPO and the HIRMAb-EPO fusion protein compared to the same parameters for a vascular space marker, human IgG1.The brain uptake (%ID/100g) and the BBB PS product for EPO are not significantly different from the same values for the IgG1 brain plasma volume marker. The PS products for peripheral organs were similarly computed for the HIRMAb-EPO fusion protein and recombinant EPO, and these data are given in Table 5. The ratio of the PS product for the HIRMAb-EPO fusion protein, relative to the PS product for the recombinant EPO, in each organ is plotted in Figure 7.

The metabolic stability of the [^{3}H]-HIRMAb-EPO fusion protein and [^{125}I]-EPO in the primate plasma at 120 min after IV injection was verified by gel filtration chromatography. The elution of the [^{3}H]-HIRMAb-EPO fusion protein and [^{125}I]-EPO is shown in panels A and B, respectively, of Figure 8. The elution volumes of blue dextran-2000 (void volume) and IgG are shown by the arrows in Figure 8A. The peak elution of the [^{3}H]-HIRMAb-EPO fusion protein and the [^{125}I]-EPO was fraction 25 and 29,

respectively (Figure 8), and this correlated with the elution peaks of the [^{3}H]-HIRMAb-

EPO fusion protein and [^{125}I]-EPO standards.

Discussion

An IgG-EPO fusion protein could be engineered by fusion of the EPO to either the amino terminus or the carboxyl terminus of either the heavy chain (HC) or light chain (LC) of the IgG, such as the HIRMAb. However, only 1 of these conformations constrains the EPO in a dimeric configuration, and that is the fusion of the EPO to the carboxyl terminus of the HC, as shown in Supplemental Figure 1. EPO dimers may be more active than EPO monomers (Sytkowski et al, 1999). EPO binds as a monomer to a dimer of EPORs (Syed et al, 1998). The findings in this study show that fusion of the EPO to the carboxyl terminus of the IgG heavy chain allows for the retention of EPO biological activity, and the HIRMAb-EPO fusion protein binds the EPOR and activates EPO biological activity in the low nM range (Figures 2-3). The ED50 of HIRMAb-EPO fusion protein binding to the human EPOR, 0.30 ± 0.01 nM, is comparable to the KD of EPO binding to the EPOR (Elliott et al, 2008). High affinity binding of the EPO moiety of the HIRMAb-EPO fusion protein is consistent with observations that the amino terminal portion of EPO is not involved in binding to the EPOR (Syed et al, 1998). With respect to HIRMAb-EPO fusion protein binding to the HIR, fusion of EPO to the carboxyl terminus of the IgG HC leaves free the amino terminal portions of the IgG chains, which bind to the HIR (Supplemental Figure 1). The present studies show the HIRMAb-EPO fusion protein binds to the HIR with an affinity equal to the original HIRMAb (Figure 1).

The EPO receptor (EPOR) and EPO are both expressed in brain (Sakanaka et al, 1998). EPO has the same characteristics as other neurotrophic factors, as EPO is neuroprotective in neural cells exposed to cytokines, such as tumor necrosis factor (TNF)-α (Pregi et al, 2009), or toxins such as the Abeta amyloid peptide (Ma et al,

2009). The EPOR that mediates neuroprotection in brain is the same classical EPOR expressed in peripheral tissues (Um et al, 2007). EPO is neuroprotective in transient forebrain ischemia, following the direct intra-cerebral injection of the peptide (Sakanaka et al, 1998), and peripheral EPO is neuroprotective only when the BBB is disrupted (Catania et al, 2002). The importance of the BBB in EPO action within the brain was also demonstrated in a model of experimental Parkinson's disease (PD). Bypassing the BBB with the intra-cerebral injection of EPO in rats with experimental PD caused neuroprotection of the nigra-striatal tract in vivo (Xue et al, 2007). However, the peripheral administration of EPO in experimental PD was not neuroprotective (Xue et al, 2007), owing to the lack of EPO transport across the BBB. The lack of EPO transport across the non-disrupted BBB has been confirmed with measurements of immunoreactive EPO in rat brain (Lieutaud et al, 2008). No increase in brain EPO is measureable following the IV administration of large doses, 5000 U/kg, of EPO when the BBB is not disrupted (Lieutaud et al, 2008). Although the peripheral administration of large doses of EPO does not elevate EPO in brain tissue, there is an increase in the EPO concentration in cerebrospinal fluid (CSF) (Ehrenreich et al, 2002). However, drug penetration into the CSF is an index of blood-CSF barrier permeability, not BBB permeability. CSF is a filtrate of plasma, and all proteins in plasma distribute into CSF, inversely related to the molecular size of the protein (Reiber and Felgenhauer, 1987). Therefore, the detection of a peptide in CSF is not a measure of BBB transport of the peptide.

The present study examined [^{125}I]-EPO transport across the BBB in the adult Rhesus monkey. The BBB transport, as reflected by the BBB PS product, of [^{125}I]-EPO

is no different from the same parameters for a brain blood volume marker, human IgG1 (Figure 6C), which indicates that EPO does not cross the BBB. In order for peripheral EPO to penetrate the brain across the BBB, it would be necessary for blood-borne EPO to access an EPOR-mediated transport system on the luminal membrane of the brain capillary endothelium, which forms the BBB in vivo. The absence of EPO transport across the BBB in vivo indicates there is no EPOR on the luminal membrane of the BBB. Evidence has been reported for EPOR immunoreactivity at the brain microvasculature, but the receptor is located in a discontinuous pattern on the abluminal side of the endothelium (Brines et al, 2000). These characteristics are typical of receptor expression on the astrocyte foot process that invests the brain capillary.

The re-engineering of EPO as an IgG fusion protein with the HIRMAb molecular Trojan horse produces a brain penetrating form of EPO. The brain uptake of the HIRMAb-EPO fusion protein, 2.1% of injected dose/100 gram brain, is high relative to the brain uptake of a molecule confined to the brain plasma volume, such as EPO or human IgG1 (Figure 6B). Brain uptake is expressed per 100 gram brain, because the brain weight in the adult Rhesus monkey is 100 grams. The HIRMAb-EPO fusion protein penetrates the BBB and enters brain parenchyma, as demonstrated by the capillary depletion method. The brain VD of the HIRMAb-EPO fusion protein in the post-vascular supernatant is 60% of the total brain homogenate VD (Table 4), which indicates the majority of the fusion protein bound by the BBB insulin receptor has penetrated brain parenchyma by 2 hours after IV administration.

Fusion of EPO to the HIRMAb selectively targets EPO to the brain compared to insulin receptor-rich peripheral organs, as demonstrated by the ratio of the organ PS

product for the fusion protein, relative to the organ PS product for EPO (Figure 7). The

PS product reflects transport across the BBB, and not sequestration within the brain

plasma volume. The PS product for the brain plasma volume marker, human IgG1,

should be subtracted from the PS product for both EPO and the HIRMAb-EPO fusion

protein. However, since the BBB PS product of the IgG1 and EPO are not significantly

different (Figure 6C), the net PS product for EPO is zero, and a ratio of PS products

could not be calculated. Therefore, the PS product ratio for the HIRMAb-EPO fusion

protein and EPO for brain shown in Figure 7 is a minimal estimate of the increased

penetration of the BBB by the fusion protein as compared to EPO. The PS product ratio

for most peripheral organs is near unity for insulin receptor-rich organs such as skeletal

muscle, heart, and fat (Figure 7).

The brain uptake and pharmacokinetics analysis reported here for the primate

allows for initial dosing considerations with the HIRMAb-EPO fusion protein. Although

EPO is expressed in brain (Sakanaka et al, 1998), the concentration of EPO in the

control brain, or CSF, is too low to detect quantitatively by ELISA (Koehne et al, 2002).

In those cases where EPO is detectable in human CSF, the EPO concentration is very

low, 0.1 pM (Koehne et al, 2002). The concentration of EPO in plasma is also low, 2-5

pM (Elliott et al, 2008). In peripheral tissue, the concentration of EPO that causes a 50%

increase in pharmacological effect is 12 pM (Elliott et al, 2004), which is equal to 0.4

ng/mL, given an EPO molecular weight of 35,000 Da. Based on the brain uptake of the

HIRMAb-EPO fusion protein, 2.1% ID/100 gram brain (Figure 6B), the peripheral

injection of a very low dose of the fusion protein, 1 ug/kg, in a 5 kg primate would

produce a brain concentration of 1 ng/gram brain, which is a therapeutic concentration of EPO.

The effect of HIRMAb-EPO administration on hematopoiesis in peripheral tissues is not an issue in the treatment of acute conditions of the brain such as ischemia or trauma with the HIRMAb-EPO fusion protein. However, in chronic treatment of neurodegeneration with the HIRMAb-EPO fusion protein, it is necessary to obtain therapeutic effects in the brain without significant effects on hematopoiesis. This is likely to be the case given the very different pharmacokinetic (PK) profiles of EPO and the HIRMAb-EPO fusion protein (Figure 5A). Fusion of EPO to the HIRMAb reduces the plasma AUC of EPO 13-fold, from 486 ± 59 %IDmin/mL to 37.5 ± 4.6 %IDmin/mL (Table 2). The markedly different PK profile for the HIRMAb-EPO fusion protein, as compared to EPO, could limit the pharmacologic properties of the fusion protein in peripheral tissues. A peripheral injection of 1 ug/kg of the HIRMAb-EPO fusion protein would be equivalent to a dose of 20 units/kg, since EPO comprises about 20% of the amino acid content of the fusion protein (Results). A dose of 20 units/kg approximates a sub-therapeutic dose of EPO with respect to hematopoiesis. However, the effect of EPO on the mass of red cells, which persist for about 120 days, is primarily a function of the plasma AUC of EPO (Elliott et al, 2008). EPO variants that are cleared from plasma slowly have a greater effect on hematocrit. Conversely, EPO variants that are rapidly cleared from plasma have a diminished effect on hematocrit (Elliott et al, 2004; 2008). Fusion of EPO to the HIRMAb results in a 12-fold reduction in plasma AUC (Table 2). Therefore, doses of the HIRMAb-EPO fusion protein that induce neuroprotection in brain may have minimal effects on hematopoiesis in peripheral tissues.

In conclusion, these studies show it is possible to re-engineer EPO as a HIRMAb fusion protein so that circulating EPO penetrates the BBB via receptor-mediated transport on the BBB insulin receptor. A brain-penetrating form of EPO may cause pharmacologic effects within the brain following peripheral administration, even when the BBB is not disrupted. Further drug development of the HIRMAb-EPO fusion protein will require investigations into the pharmacodynamics as well as the potential toxicity and immunogenicity of this novel fusion protein.

Acknowledgements

References

Boado RJ, Pardridge WM (2009) Comparison of blood-brain barrier transport of glial-derived neurotrophic factor (GDNF) and an IgG-GDNF fusion protein in the Rhesus monkey. *Drug Metab Disp* **37**: 2299-2304.

Boado RJ, Zhang Y, Zhang Y, Pardridge WM (2007a) Humanization of anti-human insulin receptor antibody for drug targeting across the human blood-brain barrier. *Biotechnol Bioeng* **96**: 381-391.

Boado RJ, Zhang Y, Zhang Y, Pardridge WM (2007b) 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* **97**:1376-1386.

Boado RJ, Zhang Y, Zhang Y, Wang Y, Pardridge WM (2008) GDNF fusion protein for targeted-drug delivery across the human blood-brain barrier. *Biotechnol Bioeng* **100**: 387-396.

Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanerolle NC, Cerami C, Itri LM, Cerami A (2000) Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proc Natl Acad Sci USA* **97**: 10526-10531.

Catania MA, Marciano MC, Parisi A, Sturiale A, Buemi M, Grasso G (2002) Erythropoietin prevents cognition impairment induced by transient brain ischemia in gerbils. *Eur J Pharmacol* **437**: 147-150.

Coloma MJ, Lee HJ, Kurihara A, Landaw EM, Boado RJ, Morrison SL, 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.

Ehrenreich H, Hasselblatt M, Dembowski C, Cepek L, Lewczuk P, Stiefel M, Rustenbeck HH, Breiter N, Jacob S, Knerlich F, Bohn M, Poser W, Ruther E, Kochen M, Gefeller O, Gleiter C, Wessel TC, De Ryck M, Itri L, Prange H, Cerami A, Brines M, Siren AL (2002) Erythropoietin therapy for acute stroke is both safe and beneficial. *Mol Med* **8**: 495-505.

Elliott S, Egrie J, Browne J, Lorenzini T, Busse L, Rogers N, Ponting I (2004) Control of rHuEPO biological activity: the role of carbohydrate. *Exp Hematol* **32**: 1146-1155.

Elliott S, Pham E, Macdougall IC (2008) Erythropoietins: a common mechanism of action. *Exp Hematol* **36**: 1573-1584.

Grasso G, Sfacteria A, Meli F, Fodale V, Buemi M, Iacopino DG (2007) Neuroprotection by erythropoietin administration after experimental traumatic brain injury. *Brain Res* **1182**: 99-105.

Kitamura T, Tange T, Terasawa T, Chiba S, Kuwaki T, Miyagawa K, Piao YF, Miyazono K, Urabe A, Takaku F (1989) Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *J Cell Physiol* **140**: 323-334.

Koehne P, Hochhaus F, Felderhoff-Mueser U, Ring-Mrozik E, Obladen M, Buhrer C (2002) Vascular endothelial growth factor and erythropoietin concentrations in cerebrospinal fluid of children with hydrocephalus. *Child's Nerv Syst* **18**: 137-141.

Lieutaud T, Andrews PJD, Rhodes JKJ, Williamson R (2008) Characterization of the pharmacokinetics of human recombinant erythropoietin in blood and brain when

administered immediately after lateral fluid percussion brain injury and its

pharmacodynamic effects on IL-1β and MIP-2 in rats. *J Neurotrauma* **25**: 1179-

1185.

Ma R, Xiong N, Huang C, Tang Q, Hu B, Xiang J, Li G (2009) Erythropoietin protects

PC12 cells from beta-amyloid(25-35)-induced apoptosis via PI3K/Akt signaling

pathway. *Neuropharmacology* **56**: 1027-1034.

Pardridge WM, Boado RJ (2009) Pharmacokinetics and safety in Rhesus monkeys of a

monoclonal antibody-GDNF fusion protein for targeted blood-brain barrier

delivery. *Pharm Res* **10**: 2227-2236.

Pardridge WM, Kang YS, Buciak JL, Yang J (1995) 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* **12**: 807-816.

Pardridge WM (2008) Re-engineering biopharmaceuticals for delivery to brain with

molecular Trojan horses. *Bioconjug Chem* **19**: 1327-1338.

Pregi N, Wenker S, Vittori D, Leiros CP, Nesse A (2009) TNF-alpha-induced apoptosis

is prevented by erythropoietin treatment on SH-SY5Y cells. *Exp Cell Res* **315**:

419-431.

Reiber H, Felgenhauer K (1987) Protein transfer at the blood cerebrospinal fluid barrier

and the quantitation of the humoral immune response within the central nervous

system. *Clin Chim Acta* **163**: 319-328.

Sakanaka M, Wen TC, Matsuda S, Masuda S, Morishita E, Nagao M, Sasaki R (1998) In vivo evidence that erythropoietin protects neurons from ischemic damage. *Proc Natl Acad Sci USA* **95**: 4635-4640.

Syed RS, Reid SW, Li C, Cheetham JC, Aoki KH, Liu B, Zhan H, Osslund TD, Chirino AJ, Zhang J, Finer-Moore J, Elliot S, Sitney K, Katz BA, Matthews DJ, Wendoloski JJ, Egrie J, Stroud RM (1998) Efficiency of signalling through cytokine receptors depends critically on receptor orientation. *Nature* **395**: 511-516.

Sytkowski AJ, Lunn ED, Risinger MA, Davis KL (1999) An erythropoietin fusion protein comprised of identical repeating domains exhibits enhanced biological properties. *J Biol Chem* **274**: 24773-24778.

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

Um M, Gross AW, Lodish HF (2007) A "classical" homodimeric erythropoietin receptor is essential for the antiapoptotic effects of erythropoietin on differentiated neuroblastoma SH-SY5Y and pheochromocytoma PC-12 cells. *Cell Signal* **19**: 634-645.

Xue YQ, Zhao LR, Guo WP, Duan WM (2007) Intrastriatal administration of erythropoietin protects dopaminergic neurons and improves neurobehavioral outcome in a rat model of Parkinson's disease. *Neuroscience* **146**: 1245-1258.

Footnotes

Address correspondence to: Dr. William M. Pardridge, UCLA Warren Hall 131-164,

900 Veteran Ave., Los Angeles, CA 90024. E-mail:

wpardridge@mednet.ucla.edu

Legends to Figures

Figure 1. Binding of either the chimeric HIRMAb or the HIRMAb-EPO fusion protein to the HIR extracellular domain (ECD) is saturable. The ED50 of HIRMAb-EPO binding to the HIR ECD is comparable to the ED50 of the binding of the chimeric HIRMAb. Data are mean ± SE (n=3 dishes/point).

Figure 2. Binding of either human IgG1κ or the HIRMAb-EPO fusion protein to the EPO receptor (EPOR) extracellular domain (ECD) is detected by ELISA. There is no binding to the EPOR by the human IgG1κ. Data are mean ± SE (n=3 dishes/point).

Figure 3. EPO bio-assay in human TF-1 cells shows a saturable increase in thymidine incorporation into the cells in response to graded increases in medium concentration of HIRMAb-EPO fusion protein. Data are mean ± SE (n=4 dishes/point).

Figure 4. (A) Outline of radio-receptor assay for measurement of the binding of Bolton-Hunter reagent-labeled [^{125}I]-EPO to the EPOR. A mouse anti-human (MAH) IgG1 Fc was plated, which bound the Fc region of a Fc fusion of the EPOR ECD. The EPOR binds to the [^{125}I]-EPO, which is displaced by the addition of unlabeled EPO. (B) The saturable binding was analyzed by a non-linear regression analysis to yield the concentration, K_D, which produced 50% inhibition of [^{125}I]-EPO binding to the EPOR.
.

Figure 5. (A) The plasma concentration of [^{125}I]-EPO and [^{3}H]-HIRMAb-EPO fusion protein is plotted vs the time after a single intravenous injection of the proteins in the

31

adult Rhesus monkey. Data are expressed as % injected dose (I.D.)/mL. (B) The % of plasma radioactivity that is precipitable by 10% trichloroacetic acid (TCA) is plotted vs. the time after injection for both proteins. Data are mean ± SE of 3 replicates from a single primate.

Figure 6. The plasma area under the concentration curve or AUC (A), the brain uptake or % injected dose (ID) per 100 gram brain (B), and the BBB permeability-surface area (PS) product (C), are plotted for EPO, for the HIRMAb-EPO fusion protein, and for a brain plasma volume marker, human IgG1 (hIgG1). The IgG1 data are from Boado and Pardridge (2009). All measurements were made at 2 hours after intravenous administration of the protein in the Rhesus monkey. Data are mean ± SE of 3 replicates from a single primate.

Figure 7. Ratio of the organ PS product for the HIRMAb-EPO fusion protein, relative to the organ PS product for EPO, is plotted for each organ. Data are mean ± SE of 3 replicates from a single primate.

Figure 8. Elution profile of [^{3}H]-HIRMAb-EPO fusion protein (A) and [^{125}I]-EPO (B) in primate plasma removed at 120 min after IV injection and separated with a Superose 6HR gel filtration column. In panel A, the first and second arrows represent the elution volume of blue dextran-2000 and IgG, respectively.

Table 1. Secretion of immunoreactive human HIRMAb-EPO fusion protein to medium of

transfected COS cells

Days	Medium IgG (ng/mL)	
	Lipofectamine 2000 only	pHIRMAb-EPO TV
3	<3	1,825 ± 80
7	<3	6,424 ± 596

Mean ± SE (n=3 dishes).

Table 2. Pharmacokinetic parameters

parameter	units	[^{125}I]-EPO	[^{3}H]-HIRMAb-EPO
A1	%ID/mL	0.400 ± 0.002	0.251 ± 0.021
A2	%ID/mL	--	0.174 ± 0.015
k1	min-1	0.00083 ± 0.00010	0.110 ± 0.025
k2	min-1	--	0.0049 ± 0.0010
MRT	min	1208 ± 151	191 ± 37
Vc	mL/kg	44 ± 1	42 ± 2
Vss	mL/kg	--	91 ± 7
AUC$|^{120}$	%IDmin/mL	46.0 ± 0.2	18.0 ± 0.4
AUCss	%IDmin/mL	486 ± 59	37.5 ± 4.6
CL	mL/min/kg	0.037 ± 0.004	0.44 ± 0.05

Estimated from the plasma clearance data in Figure 5A.

Table 3. Organ uptake of [^{125}I]-EPO and [^{3}H]-HIRMAb-EPO in the Rhesus monkey

organ	[^{125}I]-EPO	[^{3}H]-HIRMAb-EPO
frontal gray	0.34 ± 0.02	2.1 ± 0.1
frontal white	0.17 ± 0.02	1.6 ± 0.1
cerebellar gray	0.34 ± 0.01	1.6 ± 0.1
cerebellar white	0.21 ± 0.02	1.2 ± 0.1
heart	2.2 ± 0.19	1.4 ± 0.2
liver	6.9± 0.3	17.8 ± 6.3
spleen	6.0 ± 0.1	15.9 ± 4.6
lung	9.5 ± 0.7	4.5 ± 0.6
skeletal muscle	0.39 ± 0.01	0.17 ± 0.03
fat	0.41 ± 0.05	0.26 ± 0.01
kidney	11.1± 0.2	5.2 ± 0.4

Data are % ID/100 grams; mean ± SE of 3 replicates from a single primate.

Table 4. Capillary depletion analysis of HIRMAb-EPO and EPO distribution in

brain

Parameter	EPO	HIRMAb-EPO
Homogenate VD	7.7 ± 0.7	260 ± 11
Post-vascular supernatant VD	6.3 ± 0.2	156 ± 8
Brain capillary pellet VD	0.22 ± 0.03	32 ± 9
TCA precipitation (%)	n.m.	91 ± 1

VD=volume of distribution (uL/g); TCA=trichloroacetic acid; n.m.=not measured. Mean ±

SE of 3 replicates from a single primate.

Table 5. Organ PS products for EPO and HIRMAb-EPO fusion protein

organ	PS product (µL/min/g)	
	EPO	HIRMAb-EPO
heart	0.49 ± 0.02	0.78 ± 0.11
liver	1.5 ± 0.1	9.9 ± 3.5
spleen	1.3 ± 0.1	8.9 ± 2.5
lung	2.1 ± 0.1	2.5 ± 0.3
skeletal muscle	0.086 ± 0.002	0.094 ± 0.019
fat	0.089 ± 0.010	0.14 ± 0.01
kidney	2.4 ± 0.1	2.9 ± 0.2

Data are mean ± SE of 3 replicates from a single primate.

HIRMAb-EPO
$ED_{50} = 45.4 \pm 12.8$ ng/mL
$(0.21 \pm 0.05$ nM$)$

HIRMAb
$ED_{50} = 29.3 \pm 5.0$ ng/mL
$(0.20 \pm 0.03$ nM$)$

A_{492}

ng/mL

Figure 1

HIRMAb-EPO
ED50 = 63.3 ± 3.3 ng/mL
(0.30 ± 0.01 nM)

hIgG1κ

Figure 2

ED50 = 23 ng/mL (0.10 nM)

Figure 3

A

EPO -[^{125}I]
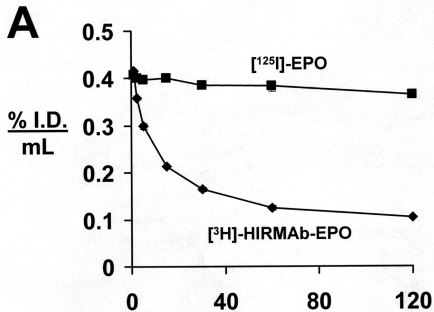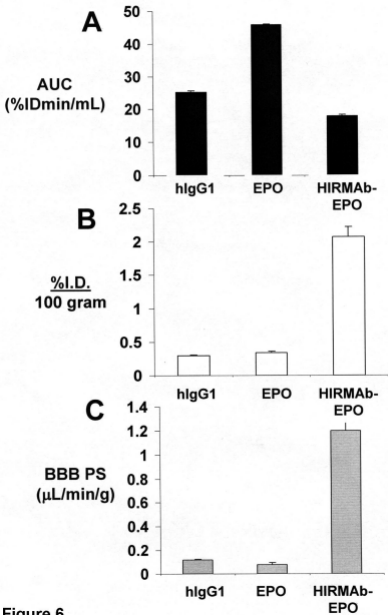
EPOR
ECD

Fc

MAH-Fc

B

[^{125}I]-EPO
bound

$K_D= 0.17 \pm 0.09$ nM

EPO (nM)

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8