Published in final edited form as:

Mol Pharm. 2020 August 03; 17(8): 2831–2839. doi:10.1021/acs.molpharmaceut.0c00231.

Eliminating Fc N-linked glycosylation and its impact on dosing consideration for a transferrin receptor antibody-erythropoietin fusion protein in mice

Joshua Yang¹, Jiahong Sun², Demi M. Castellanos¹, William M. Pardridge³, Rachita K. Sumbria^{2,4,*}

¹Henry E. Riggs School of Applied Life Sciences, Keck Graduate Institute, 535 Watson Dr, Claremont, CA, 91711, USA

²Department of Biopharmaceutical Sciences, School of Pharmacy and Health Sciences, Keck Graduate Institute, Claremont, CA 91711

³Department of Medicine, University of California, Los Angeles, CA 90095

⁴Departments of Neurology, University of California, Irvine, CA 92868

Abstract

Erythropoietin (EPO), a hematopoietic growth factor and a promising therapy for Alzheimer's disease has low permeability across the blood-brain barrier. The transferrin receptor antibody fused to EPO (TfRMAB-EPO) is a chimeric monoclonal antibody that ferries EPO into the brain via the transvascular route. However, TfRMABs have Fc-effector function-related adverse effects including reticulocyte suppression. To overcome this, we recently developed an effectorless TfRMAB-EPO fusion protein, designated TfRMAB-N292G-EPO, by eliminating the Fc N-linked glycosylation site at position 292 of the antibody heavy chain. The mutant fusion protein showed enhanced plasma clearance and dramatically reduced plasma concentrations compared with the wild-type (WT) nonmutant fusion protein. This increased clearance of the aglycosylated TfRMAB is expected to increase the injection dose of the mutant fusion protein. To provide a basis for future therapeutic uses of this IgG-neurotrophin fusion protein, the current study aimed to characterize the pharmacokinetic (PK) profile of this effectorless TfRMAB-N292G-EPO at different doses following different routes of administration in the mouse. Adult C57BL/6J male mice were injected with a single dose (3, 6, 9, or 20 mg/kg; n=3-6 per dose) of TfRMAB-N292G-EPO through either the subcutaneous (SQ) or intraperitoneal (IP) route. TfRMAB-N292G-EPO plasma concentrations were determined using an ELISA. Mice were sacrificed 24 hours after injection, and terminal blood was used for a complete blood count. Brain concentrations in the WT and mutant fusion protein treated mice were compared. We observed stark differences in the plasma PK of TfRMAB-N292G-EPO between the IP and SQ routes of administration. Dose escalation from 3-20 mg/kg increased the plasma C_{max} only 3.5-fold for the SQ route, compared with a 35-fold increase for the IP route. The plasma C_{max} was 15.0 ± 2.0 ng/mL, 21.3 ± 4.1 ng/mL, 21.3 ±

*Address correspondence to: Rachita K. Sumbria, PhD, Department of Biopharmaceutical Sciences, School of Pharmacy and Health Sciences, Keck Graduate Institute, Claremont, CA, 91711, Tel: (909) 607-0319, Fax: (909) 607-9826, rsumbria@kgi.edu.

Conflict of Interest

William Pardridge is a consultant to ArmaGen, Inc.

6.4 ng/mL and 52.8 ± 27.9 ng/mL following SQ injection, and 288 ± 47 ng/mL, 389 ± 154 ng/mL, 633 ± 194 ng/mL and $10,066 \pm 7,059$ ng/mL following IP injection for 3, 6, 9 and 20 mg/kg dose, respectively. Plasma C_{max} following the SQ route was therefore 19- to 190-fold lower compared with the IP route. This finding is consistent with a 31-fold higher apparent clearance following the SQ route compared with the IP route at the highest dose administered. Brain concentrations in the mice treated with a 3 mg/kg dose of the mutant fusion protein were lower than those in the nonmutant WT treated mice. No reticulocyte suppression was observed at the 3 mg/kg SQ dose of TfRMAb-N292G-EPO. However, reticulocyte suppression increased with an increase in dose and area under the plasma concentration–time curve (AUC) for both the IP and SQ routes. Overall, elimination of Fc N-linked glycosylation, to mitigate TfRMAb effector function side-effects, has a profound effect on the plasma exposure of TfRMAb-N292G-EPO at therapeutic as well as high doses (3–20 mg/kg). This effect is more pronounced following SQ injection. The low plasma concentrations of the mutant fusion protein following a 3 mg/kg dose resulted in negligible brain uptake. The beneficial rescue of reticulocyte reduction by the N292G mutation is a function of AUC and is negated at high doses of the N292G mutant.

Graphical Abstract

Keywords

transferrin receptor antibody; molecular Trojan horse; effectorless; Fc effector function side-effects; pharmacokinetics; erythropoietin

Introduction:

Erythropoietin (EPO), a class-1 cytokine hematopoietic growth factor mainly produced in the kidneys, offers biological benefits beyond erythropoiesis. There is mounting evidence of

the neuroprotective effects of EPO in neurodegenerative diseases.¹⁻² These neuroprotective effects include reducing inflammation, oxidative stress, and neuronal loss while elevating neurogenesis and angiogenesis.³ Besides offering neuroprotection, studies in rodent models have shown that EPO can modulate the two major pathological hallmarks of Alzheimer's disease (AD): extracellular amyloid-beta plaque accumulation and intracellular tau tangles, making it a potential treatment for AD.⁴⁻⁶ However, there are some challenges associated with utilizing EPO as a brain therapeutic.

The blood-brain barrier (BBB) acts as a selective, semi-permeable barrier that separates the brain extracellular fluid from the blood.⁷ The size and polarity of EPO prevent the macromolecule from passively diffusing across the BBB and reaching the brain parenchyma.⁸⁻¹⁰ EPO penetration of the BBB *in vivo* is minimal, and the brain uptake of EPO is no greater than that of a substance confined to the plasma volume such as IgG in the monkey,⁸ or albumin in the mouse.⁹ To ameliorate this issue, a molecular Trojan horse (MTH) can be used to shuttle EPO into the brain through a non-invasive transvascular route of administration.^{8, 11}

The MTH technology utilizes an antibody against an endogenous receptor-mediated transcytosis (RMT) system receptor fused to a therapeutic biologic, to shuttle the therapeutic biologic through the BBB.¹² The transferrin receptor-1 (TfR1) is a transcytosis receptor, which is highly expressed at the BBB.¹³⁻¹⁴ An MTH-EPO fusion protein was engineered, with the MTH domain being a rat/mouse chimeric antibody against the mouse TfR1 (TfRMAB). The TfRMAB-EPO fusion protein has two binding domains: a mouse TfR1 binding domain, TfRMAB, for brain delivery, and an EPO receptor (EPOR) binding domain, EPO, for neuroprotective effects. The TfRMAB-EPO has a high binding affinity to both the TfR1 and EPOR with low nanomolar dissociation constants (KD).^{11, 15}

A single injection of a TfRMAB comprising a human Fc-domain in mice at high doses (20-50 mg/kg) induces adverse-effects including lethargy, spastic movements, and suppression of reticulocytes.¹⁶ Similarly, our recent work showed reticulocyte suppression in mice with a single therapeutic dose (3 mg/kg) of TfRMAB-EPO comprising a mouse Fc-domain.¹⁵ These effects of TfRMAB are attributed in part to the TfRMAB Fc-effector function ensuing from the binding of TfRMABs to reticulocyte TfR and resultant antibody-dependent cell-mediated cytotoxicity.¹⁶⁻¹⁷

Administration of 'effectorless' TfRMABs comprising mutations in the Fc N-linked glycosylation partially rescues the suppression of reticulocytes in mice and eliminates this adverse effect in non-human primates.¹⁸⁻¹⁹ Studies show that such Fc N-linked glycosylation site mutations do not alter plasma clearance of a low-affinity TfRMAB in mice following a 50 mg/kg intravenous (IV) injected dose (ID),¹⁸ and do not affect the plasma clearance of a non-TfR-targeting monoclonal antibody in mice and non-human primates.²⁰⁻²¹

Based on this previous work, we recently engineered an effectorless IgG-EPO fusion protein, TfRMAB-N292G-EPO, which has a single mutation in the Fc N-linked glycosylation site with a substitution of asparagine (N) with glycine (G) at position 292.¹⁵

While this N292G mutation does not impact the binding of the IgG to the neonatal Fc receptor (FcRn), binding of the IgG to the Fc γ receptor (Fc γ R) is abolished.²² We recently reported the plasma pharmacokinetics (PK) and effects on reticulocytes of therapeutic doses (0.3-3 mg/kg) of TfRMAb-N292G-EPO following IV, intraperitoneal (IP), and subcutaneous (SQ) injection in mice.¹⁵ Compared with the nonmutant TfRMAb-EPO, mutant TfRMAb-N292G-EPO mitigated reticulocyte suppression at these doses. TfRMAb-N292G-EPO, however, showed a profound increase in plasma clearance following IV administration and a 37- to 114-fold reduction in plasma C_{max} following IP and SQ administration at therapeutic doses compared with the nonmutant TfRMAb-EPO.¹⁵ Further, the plasma PK of the TfRMAb-N292G-EPO fusion protein displayed the characteristics of non-linear PK showing a reduction in clearance with an increase in dose.¹⁵ This would mean that higher doses of the N292G mutant will be needed to achieve plasma concentrations and therefore therapeutic effects comparable to the nonmutant TfRMAb-EPO. However, it is unclear if the rescue of reticulocyte suppression can be maintained at higher doses of the N292G mutant TfRMAb-EPO.

Given the above information, this study aimed to determine if increasing the doses of the mutant TfRMAb-N292G-EPO can saturate its accelerated clearance to yield higher plasma concentrations while maintaining the rescue of reticulocyte suppression following IP and SQ administration, in mice.

Experimental Section:

TfRMAb-N292G-EPO fusion protein:

The TfRMAb-N292G-EPO fusion protein was produced by Genscript (Piscataway, NJ) in ExpiCHO cells as described previously.¹⁵ The formulation buffer of the fusion protein was 98 mM glycine, 148 mM NaCl, 28 mM Tris, 0.01% Polysorbate 80, pH=5.5.

Mouse Animal Study:

The animal studies were approved by the University of La Verne Institutional Animal Care and Use Committee. Male C57BL/6J mice (8 weeks old and weighing 22–27g) (Jackson Laboratory, Bar Harbor, ME) were provided constant access to food and water, and were maintained on a 12 h light/12 h dark cycle. The mice were randomized to either IP or SQ route of administration and received a single injection at a dose of 3, 6, 9, and 20 mg/kg of TfRMAb-N292G-EPO. The average injection volume of the TfRMAb-N292G-EPO was 100 μ L, 200 μ L, 300 μ L, and 667 μ L for the 3, 6, 9 and 20 mg/kg IP and SQ doses, respectively, all within the recommended administration volumes in a mouse.²³ The control mice were treated with an equivalent volume of saline or 3 mg/kg isotype control mouse IgG1 (Bio X Cell, West Lebanon, NH) via the SQ route. Whole blood was collected via the retro-orbital sinus at 3, 6, and 24 h following the initial injection. These sampling times were selected based on our previous work with the TfRMAb-N292G-EPO¹⁵ and other TfRMAb-based fusion proteins²⁴ showing a T_{max} of 3-6 hours following IP and SQ dosing. Plasma was collected from whole blood, and plasma concentrations of the fusion protein were measured as described previously.¹⁵ Terminal whole blood aliquot was used for a complete blood count (CBC) (Molecular Diagnostic Services, Inc., San Diego, CA).

Plasma and Brain Concentration Measurements:

The EPO receptor (EPOR) ELISA was used to determine the plasma- and brain-concentrations of the fusion proteins, as described previously.¹⁵ Perfused frozen hemi-brains of the mice treated with either the wild-type (WT) nonmutant or N292G mutant fusion protein were homogenized in 7 brain volumes (7 μ L/mg brain) of the ice-cold Tissue Protein Extraction Reagent (T-PER) (ThermoFisher Scientific, Waltham, MA) and EDTA-free Protease Inhibitor Tablet (MilliporeSigma, Burlington, MA). The brain homogenates were shaken at 4°C for 1h, centrifuged at 14,000xg for 20 min at 4°C, and the supernatant was then collected. The supernatant was concentrated using the Amicon Ultra Protein Purification and Concentration Filter Tubes (4 mL and 50 kDa cut-off; MilliporeSigma, Burlington, MA) and centrifuged at 4°C at 3000xg for 40 min. The brain concentrates were used to determine brain concentrations of the fusion proteins.

Nunc Maxisorp plate wells (Fisher Scientific, Waltham, MA) were coated with 2 μ g/mL of the EPOR/Fc fusion protein (R&D system, Minneapolis, MN) at 4°C overnight. The wells were then washed with tris-buffered saline containing 0.05% Tween-20 (TBST) and blocked with tris-buffered saline containing 1% bovine serum albumin (TBSB). Plasma samples, brain concentrates, and standards (0.03-9000 ng/well of fusion proteins) were incubated for 2 h at room temperature. After washing with TBST, wells were incubated with 100 ng/well of alkaline-phosphatase conjugated goat anti-mouse light chain (kappa) antibody (Bethyl Laboratories, Inc., Montgomery, TX) for 45 min at room temperature. The wells were incubated with P-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO) for 16-30 min in the dark, and 1.2M NaOH was added to halt the reaction. The optical density (OD) was measured at 405 nm and nonlinear regression analysis was used to determine Bmax and KD from standard curves (GraphPad Prism, San Diego, CA). TBSB blank corrected OD values were used. Plasma concentrations are reported as ng of fusion protein per mL plasma (ng/mL) and the percentage of injected dose per mL of plasma (%ID/mL). Brain concentrations in ng/g brain tissue were normalized to saline-control values.

Plasma data was analyzed using the Thermo Scientific Kinetic 5.0 (ThermoFisher Scientific, Waltham, MA) software to calculate the following PK parameters: maximum plasma concentration (Cmax), mean residence time (MRT), area under the plasma concentration-time curve (AUC), time to reach maximum plasma concentration (Tmax), clearance/bioavailability (apparent clearance), and the volume of distribution at steady-state (V_{ss}).¹⁵

In vitro Stability of the Fusion Protein:

The stability of the TfRMAb-N292G-EPO in saline and mouse plasma was determined *in vitro*. For this, the TfRMAb-N292G-EPO fusion protein (9000 ng/mL) was incubated in saline and mouse plasma at 37°C, and aliquots were collected at 0, 3, and 24 h. Concentrations of the fusion protein were determined using the EPOR ELISA described above which detects the intact fusion protein, and blank (saline and mouse plasma) corrected OD values were used. Data was presented as % of 0-hour concentration to determine changes in the concentration of the fusion protein over 24 hours, *in vitro*.

Statistical analysis:

All data are represented as mean $\pm$ SEM, and all statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA). A one-way ANOVA with Holm-Sidak's post-hoc test was used to compare more than 2 groups. Spearman correlation coefficient r was used to determine the relationship between AUC and reticulocyte count. Two-way ANOVA with repeated-measures with Holm-Sidak's post-hoc test was used to compare data with 2 independent variables (e.g. stability study). A two-tailed p-value of <0.05 was considered statistically significant.

Results:

Plasma Pharmacokinetics after SQ Injection:

Plasma concentration-time curves after SQ injections are shown in Figure 1. The C_{max} was 15.0 ± 2.0 ng/mL (0.02 ± 0.003 %ID/mL), 21.3 ± 4.1 ng/mL (0.015 ± 0.003 %ID/mL), and 21.3 ± 6.4 ng/mL (0.010 ± 0.003 %ID/mL) for the 3, 6 and 9 mg/kg SQ dose, respectively (Table 1, Figure 1A & B). The 20 mg/kg SQ dose resulted in a C_{max} of 52.8 ± 27.9 ng/mL (0.011 ± 0.006 %ID/mL), which was 2.5-fold higher than the C_{max} following the 9 mg/kg SQ dose. At 6 hours following SQ administration, the plasma concentration of TfRMAb-N292G-EPO decreased to 9.7 ± 1.2 ng/mL (0.013 ± 0.002 %ID/mL), 12.7 ± 2.1 ng/mL (0.009 ± 0.002 %ID/mL), 15.5 ± 3.9 ng/mL (0.007 ± 0.002 %ID/mL), and 27.8 ± 17.1 ng/mL (0.006 ± 0.003 %ID/mL) for the 3, 6, 9, and 20 mg/kg doses, respectively (Figure 1A & B). These plasma C_{max} values of TfRMAb-N292G-EPO for doses between 3-20 mg/kg following SQ injection are 90- to 317-fold lower than the previously reported plasma C_{max} of the nonmutant WT TfRMAb-EPO following a 3 mg/kg SQ dose (Table 1).¹⁵

Plasma Pharmacokinetics after IP Injections:

Plasma concentration-time curves after IP injections are shown in Figure 1. The C_{max} was 288 ± 47 ng/mL (0.38 ± 0.062 %ID/mL), 389 ± 154 ng/mL (0.26 ± 0.11 %ID/mL), and 633 ± 194 ng/mL (0.28 ± 0.083 %ID/mL) for the 3, 6 and 9 mg/kg dose, respectively (Table 1 & Figure 1C & D). The C_{max} following the 20 mg/kg IP dose was $10,066 \pm 7,059$ ng/mL (2 ± 1.4 %ID/mL), which was 16-fold higher than the 9 mg/kg IP dose. At 6 hours following IP administration, the plasma concentration decreased to 31.0 ± 11.9 ng/mL (0.041 ± 0.016 %ID/mL), 21.1 ± 10.1 ng/mL (0.014 ± 0.007 %ID/mL), 76.1 ± 40.9 ng/mL (0.033 ± 0.017 %ID/mL), and 568 ± 227 ng/mL (0.11 ± 0.043 %ID/mL) for the 3, 6, 9, and 20 mg/kg doses, respectively (Figure 1C & D). These plasma C_{max} values of TfRMAb-N292G-EPO for doses between 3-9 mg/kg are 2- to 5-fold lower than the previously reported plasma C_{max} of the nonmutant WT TfRMAb-EPO following a 3 mg/kg IP dose (Table 1). The plasma C_{max} of TfRMAb-N292G-EPO following a 20 mg/kg IP dose is 190-fold higher than that following SQ administration of the same dose, and is 7-fold higher than the previously reported plasma C_{max} of the nonmutant WT TfRMAb-EPO following a 3 mg/kg IP dose (Table 1).¹⁵

The time to maximum plasma concentration (T_{max}) was 180 minutes for all the groups (Table 1). The AUC(0-24h) increased with dose, with IP AUC(0-24h) being 5- to 78-fold greater than the SQ AUC(0-24h) for 3-20 mg/kg doses, respectively. Similarly, the AUC(0-

∞) increased with dose, with the IP AUC(0- ∞) being 2- to 59-fold greater than the SQ AUC(0- ∞) for 3-20 mg/kg doses, respectively. At the highest dose administered (20 mg/kg), the apparent clearance (Cl/F) was 31-fold higher following the SQ route compared with the IP route. The MRT for the SQ route was 1.5- to 9-fold higher compared with the IP route for each dose, which can be attributed to the high V_{ss} associated with the SQ route (10- to 42-fold higher than the V_{ss} at the respective IP dose) (Table 1).

Brain Fusion Protein Concentrations:

The brain concentrations of the WT nonmutant TfRMAb-EPO fusion protein were significantly higher than those of the mutant TfRMAb-N292G-EPO fusion protein following a 3 mg/kg SQ, IP and IV dose (Figure 2). The brain concentrations of the N292G mutant fusion protein following both the IP and SQ routes of administration were comparable to the background value (brain concentrations in mice treated with saline) of the EPOR ELISA assay used to detect the intact fusion protein in the brain (Figure 2).

Complete Blood Count:

No significant difference was observed in the RBC count and hemoglobin of the mice treated with either IP or SQ administration of TfRMAb-N292G-EPO compared with saline-treated mice at 24 hours after a single injection of 3, 9 and 20 mg/kg doses (Table 2). The WBC count was significantly elevated for the 9 mg/kg IP, 9 mg/kg SQ, and 20 mg/kg SQ injected mice compared to saline-treated mice by 91%, 61%, and 103%, respectively. However, no significant difference was observed in the WBC count between the isotype control mouse IgG1 and TfRMAb-N292G-EPO treated mice (Table 2).

The total reticulocytes (%) in the saline-treated mice was 5.5 ± 0.4 (Table 2), and the reticulocyte counts decreased significantly with an increasing dose following both IP and SQ routes of administration of TfRMAb-N292G-EPO compared with saline-treated mice (Table 2). Following IP injection, the total reticulocyte percentage was 2.2 ± 0.3 ($p < 0.05$), 1.6 ± 0.2 ($p < 0.001$), and 0.5 ± 0.03 ($p < 0.001$) for the 3 mg/kg, 9 mg/kg, and 20 mg/kg doses, respectively (Table 2). No significant reduction in reticulocyte counts was observed between the 3 mg/kg SQ dose compared with saline-treated mice, and the total reticulocyte percentage was 3.6 ± 1.3 , 3.1 ± 1.0 ($p < 0.05$), and 1.2 ± 0.2 ($p < 0.01$) for the 3 mg/kg, 9 mg/kg, and 20 mg/kg SQ doses, respectively (Table 2). Overall, there was a strong negative correlation ($r = -0.84$, $p < 0.0001$) between AUC and reticulocyte count (Figure 3A).

In vitro Stability of the Fusion Protein:

No significant loss of the fusion protein was observed over 24 hours at 37°C in normal saline and mouse plasma. Greater than $95 \pm 2\%$ and $99 \pm 12\%$ of the fusion protein was recovered following a 24-hour incubation in saline and mouse plasma, respectively (Figure 3B).

Discussion:

Antibodies directed against the TfR1, which is expressed at the BBB, act as vectors to shuttle neurotherapeutics into the brain across the BBB.¹⁴ Recent work has revealed Fc-

dependent effector function adverse effects associated with TfRMABs, which are partially mitigated by eliminating Fc-N-linked glycosylation.¹⁶ We recently engineered a brain-penetrable analog of EPO by fusing EPO to a high-affinity TfRMAB with an effectorless Fc-region by mutating the asparagine (N) at position 292 to glycine (G). This mutant brain-penetrating EPO fusion protein, designated TfRMAB-N292G-EPO, shows a 6- to 8-fold faster plasma clearance, 37- to 114-fold lower plasma C_{max}, and partial rescue of reticulocyte suppression compared to the WT nonmutant TfRMAB-EPO at low therapeutic doses (0.3-3 mg/kg).¹⁵ Such differences in the plasma PK between the WT and mutant TfRMAB-EPO would necessitate higher doses of the mutant fusion protein to achieve plasma concentrations and therapeutic benefits comparable to that of the WT fusion protein. The aim of the current study was, therefore, to determine if an increase in administered dose would saturate the accelerated clearance of the N292G mutant to increase its plasma exposure while maintaining the rescue in the Fc-effector function adverse effects.

For this, we examined the plasma PK of TfRMAB-N292G-EPO at doses ranging from 3 mg/kg to 20 mg/kg following IP and SQ administration in adult male C57BL/6J mice. We showed that the IP route of administration overall resulted in a higher plasma exposure (plasma C_{max} and AUC) compared to the SQ route, for all the doses studied. Despite a 7-fold dose escalation of the N292G mutant from 3 mg/kg to 20 mg/kg, plasma C_{max} increased only 3.5-fold and remained low (between 15.0 ± 2.0 ng/mL and 52.8 ± 27.9 ng/mL) following SQ administration. However, the plasma C_{max} increased 35-fold (from 288 ± 46.9 ng/mL to $10,066 \pm 7,059$ ng/mL) following IP administration as the dose was increased from 3 mg/kg to 20 mg/kg. The non-linear increase in plasma C_{max} of the mutant fusion protein following the IP and SQ injections suggests involvement of saturable processes. For e.g. saturable absorption may contribute to less than proportional increase in plasma concentrations and saturable clearance may contribute to a more than proportional increase in plasma concentrations. Such a non-linear PK behavior is not unlikely, and has been reported for other TfRMAB-based fusion proteins.^{15, 25} The extremely low plasma bioavailability of the mutant fusion protein following SQ administration becomes very evident when the plasma exposure of the mutant is compared to that of the WT nonmutant fusion protein. The plasma C_{max} of the WT nonmutant TfRMAB-EPO fusion protein following a 3 mg/kg SQ dose is $4,748 \pm 290$ ng/mL (Table 1).¹⁵ However, following a 7-fold higher 20 mg/kg dose of the mutant TfRMAB-N292G-EPO, the C_{max} is 90-fold lower at 52.8 ± 27.9 ng/mL. The plasma bioavailability following IP administration is higher than that following SQ administration of the mutant fusion protein but still lower than that of the nonmutant fusion protein. The plasma exposure of the mutant fusion protein was 2- to 5-fold lower for doses between 3-9 mg/kg, but was 7-fold higher for the 20 mg/kg dose than the plasma C_{max} after a 3 mg/kg dose of the nonmutant fusion protein (Table 1).¹⁵ The 35-fold increase in the plasma C_{max} following IP dosing with an increase in dose from 3-20 mg/kg suggests a saturation of peripheral clearance of the N292G mutant at higher doses following the IP route.

The fusion protein was stable in the mouse plasma over 24 hours, *in vitro* (Figure 3B), and the reduced bioavailability of the mutant fusion protein following SQ injection compared to IP injection may be caused by the anatomical differences in the sites of injection (Figure 3C). Both parenteral routes have different avenues for entering the systemic circulation.

Drugs administered through the IP route enter the peritoneal cavity, are absorbed via the mesenteric vasculature before being emptied into the hepatic portal vein.²⁶⁻²⁷ Lymphatic absorption from the peritoneal cavity contributes only modestly to the overall absorption process even for macromolecules.²⁸⁻²⁹ Following SQ administration, drugs are absorbed at a slower rate compared to other parenteral routes, and drugs reach the systemic circulation from the subcutis either directly by absorption into the blood capillaries underlying the skin or indirectly via the subcutis lymphatic circulation.³⁰⁻³¹ Drug molecular weight and the proportion absorbed via the lymphatics following SQ administration are linearly related, and macromolecules with a molecular mass greater than 16 kDa are absorbed primarily by the lymphatic system.³² The extent of lymphatic accumulation and catabolism after SQ administration is further dependent on the physicochemical characteristics of the molecule. Recent work showed that modification of surface glycosylation of extracellular vesicles increases lymphatic accumulation following SQ administration in mice.³³ Similarly, minor modifications to the glycan structure of EPO increased its lymphatic catabolism following SQ administration in rats.³⁴ Based on the role of lymphatic absorption and catabolism following SQ administration, and given that the mutant fusion protein is a large molecule (~200 kDa) and lacks the Fc-N-linked glycosylation,¹⁵ lymphatic catabolism of the mutant fusion protein perhaps results in the low bioavailability following SQ administration in the current study. The degradation of the fusion protein at the site of injection and in lymph nodes was however not assessed in the current study. Future studies are needed to delineate the definitive role of lymphatic catabolism in reducing plasma bioavailability of the N292G mutant after SQ injection.

The binding of the Fc-domain of a therapeutic antibody to the FcγR is important, in many cases, to elicit antibody-dependent cell-mediated toxicity.³⁵ However, for TfrMAbs that are used as delivery vectors, such an Fc-effector function is not desirable. Studies using different variants (high- and low-affinity) of human TfrMAbs with a fully active effector function report acute clinical signs and reticulocyte suppression following IV administration in mice; adverse effects that were partly rescued by eliminating the Fc-N-linked glycosylation.¹⁶ Using a high-affinity rat/mouse chimeric TfrMAb-EPO with a fully active effector function, we recently showed a similar suppression of reticulocytes 24 hours following IV, SQ, and IP administration.¹⁵ Reticulocyte suppression or alteration of other hematology indices with TfrMAb-based treatments is an acute but reversible occurrence, and long-term treatment with the high-affinity TfrMAb-EPO with a fully active effector function has no significant long-lasting hematologic adverse effects in mice.¹⁵

Nevertheless, one approach to reduce the acute suppression of reticulocytes is to use effectorless TfrMAbs, and both high- and low-affinity effectorless TfrMAbs rescue reticulocyte suppression at low doses (3-5 mg/kg).¹⁵⁻¹⁶ In the current study, we found that the rescue of reticulocyte suppression is a function of plasma exposure of the effectorless TfrMAb-EPO such that an increase in AUC of the effectorless TfrMAb-EPO increases reticulocyte suppression. The lowest AUC in the current study was observed with the 3 mg/kg SQ dose of the effectorless mutant TfrMAb-N292G-EPO fusion protein, and no significant reticulocyte suppression was observed at this dose, consistent with our previous work.¹⁵ However, despite the effector-silencing N292G mutation, the TfrMAb-N292G-EPO fusion protein significantly reduced reticulocytes as the plasma exposure increased. At the

highest dose tested (20 mg/kg), the reticulocyte suppression was comparable to that with the fully effector-functional WT TfRMAB-EPO.¹⁵ This is consistent with previous work showing only a partial rescue of reticulocyte suppression at high doses (25 mg/kg), but a complete reversal of reticulocyte suppression at low doses (5 mg/kg) of an effectorless low-affinity TfRMAB.¹⁶ Therefore, the rescue of reticulocyte suppression is lost at high doses of the effectorless mutant fusion protein.

We recently showed that both the WT and mutant TfRMAB-EPO fusion proteins bind to the TfR1 and EPOR with similar high-affinity, and result in comparable biological effect when given at the same concentration *in vitro*.¹⁵ Therefore, similar brain concentrations of the fusion proteins are expected to result in comparable therapeutic efficacy, *in vivo*. Chronic treatment with a 3 mg/kg IP and SQ dose of the WT fully effector-functional TfRMAB-EPO resulted in therapeutic effects in a transgenic mouse model of AD.³⁶⁻³⁷ With this IP and SQ dose of the WT TfRMAB-EPO, the plasma C_{max} is 1380 ng/mL and 4748 ng/mL, respectively.¹⁵ In the current study, we show that the C_{max} of the mutant fusion protein is 5-fold lower at 288 ng/mL, and 316-fold lower at 15 ng/mL following the same 3 mg/kg IP and SQ dose, respectively (Table 1). This reduction in plasma exposure of the mutant fusion protein is expected to have a significant impact on the brain concentrations of the fusion protein. Accordingly, we found a profound reduction in the brain concentrations of the mutant fusion protein compared to the WT fusion protein 24 hours following a single 3 mg/kg injection in mice, which is predicted to reduce the therapeutic efficacy of the mutant fusion protein. In fact, the brain concentrations of the mutant fusion protein were comparable to background values (brain concentrations in saline-treated mice) following IP and SQ administration (Figure 2). Notably, even at the highest SQ dose (20 mg/kg) in the current study, the plasma C_{max} was lower than the plasma C_{max} following the 3 mg/kg IP dose. Such low plasma bioavailability following the SQ route is therefore expected to result in low brain bioavailability of the mutant fusion protein even at the highest dose administered in the current study. SQ administration, which is the ideal route for repeated injections in humans, is therefore not recommended for therapeutic studies using the mutant fusion protein for doses ranging between 3-20 mg/kg. Additionally, at such high doses, the beneficial rescue of reticulocyte suppression with the mutant fusion protein is lost. Notably, the brain concentrations were measured 24 hours after injection, and the possibility that the brain concentrations of the mutant fusion protein are higher at an earlier sampling time cannot be ruled out. Taken together, the prohibitively low plasma bioavailability and negligible brain uptake of the effectorless N292G mutant compared to its WT fully effector-functional counterpart will necessitate high doses of the mutant fusion protein so that a therapeutic response similar to the WT fusion protein can be achieved. However, the beneficial rescue of reticulocyte suppression with the effectorless TfRMAB-N292G-EPO fusion protein is lost at high doses.

Another interesting observation was the elevation in the WBC counts following higher injection doses of TfRMAB-N292G-EPO. This elevation is more pronounced following the SQ route compared to the IP route. Whether this response is acute and reversible, and relates to lymphatic absorption following the SQ administration needs further investigation. However, no significant difference was observed between the WBC counts of mice treated with the isotype control mouse IgG1 and TfRMAB-N292G-EPO treated mice, and the

elevation in WBC counts in the current study is most likely associated with the IgG1 (TfRMAB) domain of the TfRMAB-N292G-EPO fusion protein.

Overall, elimination of the Fc N-linked glycosylation, to mitigate TfRMAB effector function side-effects, has a profound effect on the plasma PK and brain concentrations of TfRMAB-N292G-EPO. These effects on plasma PK were more pronounced following the SQ injection, which is also the preferred injection route for repeated dosing of biologics in humans. Plasma C_{max} following the SQ route was 19- to 190-fold lower compared with the IP route for doses between 3-20 mg/kg, illustrating the profound impact the N292G mutation can have on the choice of the route of administration and the bioavailability of the fusion protein. Further, the beneficial rescue of reticulocyte suppression by the N292G mutation is a function of AUC and is negated at high doses of the N292G mutant that are required to achieve plasma concentrations comparable to that of the WT fusion protein with a fully active effector function. These studies, therefore, shed light on important dosing considerations for future studies with effectorless TfRMAB-based fusion proteins to mitigate effector function adverse effects. The significant impact this modification may have on the dosing regimen (choice of route of administration and dose), plasma exposure and resulting brain concentrations, and resultant Fc-effector function may have to be considered before an effectorless variant of the WT TfRMAB-based fusion protein is used in therapeutic studies.

Acknowledgments

Funding: This work is supported by grants from the National Institute of Health, NIA R21AG055949 and R01AG062840, and from the Joseph H. Stahlberg Foundation to R.K.S.

Abbreviations

OD	Absorbance
AD	Alzheimer's disease
TfRMAB	Transferrin receptor antibody
AUC	Area under the plasma concentration-time curve
Asn	Asparagine
BBB	Blood-brain barrier
CHO	Chinese hamster ovary
KD	Dissociation constant
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
EPOR	Erythropoietin receptor
FcγR	Fc gamma receptor
ID	Injection dose

IP	Intraperitoneal
IV	Intravenous
B_{max}	Maximal binding
C_{max}	Maximum plasma concentration
MRT	Mean residence time
MTHs	Molecular Trojan horses
FcRn	Neonatal Fc receptor
PK	Pharmacokinetics
RMT	Receptor-mediated transcytosis
SEC	Size exclusion chromatography
SQ	Subcutaneous
TfR	Transferrin receptor
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing 0.05% Tween-20
TBSB	Tris-buffered saline containing 1% bovine serum albumin
V_{ss}	Volume of distribution at steady-state

References

1. Jelkmann W, Physiology and pharmacology of erythropoietin. *Transfus Med Hemother* 2013, 40 (5), 302–9. [PubMed: 24273483]
2. Subiros N; Del Barco DG; Coro-Antich RM, Erythropoietin: still on the neuroprotection road. *Ther Adv Neurol Disord* 2012, 5 (3), 161–73. [PubMed: 22590480]
3. Sargin D; Friedrichs H; El-Kordi A; Ehrenreich H, Erythropoietin as neuroprotective and neuroregenerative treatment strategy: comprehensive overview of 12 years of preclinical and clinical research. *Best Pract Res Clin Anaesthesiol* 2010, 24 (4), 573–94. [PubMed: 21619868]
4. Li YP; Yang GJ; Jin L; Yang HM; Chen J; Chai GS; Wang L, Erythropoietin attenuates Alzheimer-like memory impairments and pathological changes induced by amyloid beta(42) in mice. *Brain Research* 2015, 1618, 159–167. [PubMed: 26049128]
5. Sun ZK; Yang HQ; Pan J; Zhen H; Wang ZQ; Chen SD; Ding JQ, Protective effects of erythropoietin on tau phosphorylation induced by beta-amyloid. *J Neurosci Res* 2008, 86 (13), 3018–27. [PubMed: 18512763]
6. Lee ST; Chu K; Park JE; Jung KH; Jeon D; Lim JY; Lee SK; Kim M; Roh JK, Erythropoietin improves memory function with reducing endothelial dysfunction and amyloid-beta burden in Alzheimer's disease models. *J Neurochem* 2012, 120 (1), 115–24. [PubMed: 22004348]
7. Daneman R; Prat A, The blood-brain barrier. *Cold Spring Harb Perspect Biol* 2015, 7 (1), a020412. [PubMed: 25561720]
8. Boado RJ; Hui EK; Lu JZ; Pardridge WM, Drug targeting of erythropoietin across the primate blood-brain barrier with an IgG molecular Trojan horse. *J Pharmacol Exp Ther* 2010, 333 (3), 961–9. [PubMed: 20233799]

9. Banks WA; Jumbe NL; Farrell CL; Niehoff ML; Heatherington AC, Passage of erythropoietic agents across the blood-brain barrier: a comparison of human and murine erythropoietin and the analog darbepoetin alfa. *Eur J Pharmacol* 2004, 505 (1-3), 93–101. [PubMed: 15556141]
10. Zhang F; Xing J; Liou AK; Wang S; Gan Y; Luo Y; Ji X; Stetler RA; Chen J; Cao G, Enhanced Delivery of Erythropoietin Across the Blood-Brain Barrier for Neuroprotection against Ischemic Neuronal Injury. *Transl Stroke Res* 2010, 1 (2), 113–21. [PubMed: 20577577]
11. Zhou QH; Boado RJ; Lu JZ; Hui EK; Pardridge WM, Re-engineering erythropoietin as an IgG fusion protein that penetrates the blood-brain barrier in the mouse. *Mol Pharm* 2010, 7 (6), 2148–55. [PubMed: 20860349]
12. Pardridge WM, Molecular Trojan horses for blood-brain barrier drug delivery. *Curr Opin Pharmacol* 2006, 6 (5), 494–500. [PubMed: 16839816]
13. Li JY; Boado RJ; Pardridge WM, Blood-brain barrier genomics. *J Cereb Blood Flow Metab* 2001, 21 (1), 61–8. [PubMed: 11149669]
14. Pulgar VM, Transcytosis to Cross the Blood Brain Barrier, New Advancements and Challenges. *Front Neurosci* 2018, 12, 1019. [PubMed: 30686985]
15. Sun J; Boado RJ; Pardridge WM; Sumbria RK, Plasma Pharmacokinetics of High-Affinity Transferrin Receptor Antibody-Erythropoietin Fusion Protein is a Function of Effector Attenuation in Mice. *Mol Pharm* 2019.
16. Couch JA; Yu YJ; Zhang Y; Tarrant JM; Fuji RN; Meilandt WJ; Solanoy H; Tong RK; Hoyte K; Luk W; Lu Y; Gadkar K; Prabhu S; Ordonia BA; Nguyen Q; Lin Y; Lin Z; Balazs M; Searce-Levie K; Ernst JA; Dennis MS; Watts RJ, Addressing safety liabilities of TfR bispecific antibodies that cross the blood-brain barrier. *Sci Transl Med* 2013, 5 (183), 183ra57, 1-12.
17. Gómez Román VR; Murray JC; Weiner LM, Chapter 1 - Antibody-Dependent Cellular Cytotoxicity (ADCC) In *Antibody Fc*, Ackerman ME; Nimmerjahn F, Eds. Academic Press: Boston, 2014; pp 1–27.
18. Lo M; Kim HS; Tong RK; Bainbridge TW; Vernes JM; Zhang Y; Lin YL; Chung S; Dennis MS; Zuchero YJ; Watts RJ; Couch JA; Meng YG; Atwal JK; Brezski RJ; Spiess C; Ernst JA, Effector-attenuating Substitutions That Maintain Antibody Stability and Reduce Toxicity in Mice. *J Biol Chem* 2017, 292 (9), 3900–3908. [PubMed: 28077575]
19. Yu YJ; Atwal JK; Zhang Y; Tong RK; Wildsmith KR; Tan C; Bien-Ly N; Hersom M; Maloney JA; Meilandt WJ; Bumbaca D; Gadkar K; Hoyte K; Luk W; Lu Y; Ernst JA; Searce-Levie K; Couch JA; Dennis MS; Watts RJ, Therapeutic bispecific antibodies cross the blood-brain barrier in nonhuman primates. *Sci Transl Med* 2014, 6 (261), 261ra154.
20. Leabman MK; Meng YG; Kelley RF; DeForge LE; Cowan KJ; Iyer S, Effects of altered FcγR binding on antibody pharmacokinetics in cynomolgus monkeys. *MAbs* 2013, 5 (6), 896–903. [PubMed: 24492343]
21. Jacobsen FW; Stevenson R; Li C; Salimi-Moosavi H; Liu L; Wen J; Luo Q; Daris K; Buck L; Miller S; Ho SY; Wang W; Chen Q; Walker K; Wypych J; Narhi L; Gunasekaran K, Engineering an IgG Scaffold Lacking Effector Function with Optimized Developability. *J Biol Chem* 2017, 292 (5), 1865–1875. [PubMed: 27994062]
22. Monnet C; Jorieu S; Urbain R; Fournier N; Bouayadi K; De Romeuf C; Behrens CK; Fontayne A; Mondon P, Selection of IgG Variants with Increased FcRn Binding Using Random and Directed Mutagenesis: Impact on Effector Functions. *Front Immunol* 2015, 6, 39. [PubMed: 25699055]
23. Diehl KH; Hull R; Morton D; Pfister R; Rabemampianina Y; Smith D; Vidal JM; van de Vorstenbosch C; European Federation of Pharmaceutical Industries, A.; European Centre for the Validation of Alternative, M., A good practice guide to the administration of substances and removal of blood, including routes and volumes. *J Appl Toxicol* 2001, 21 (1), 15–23. [PubMed: 11180276]
24. Sumbria RK; Zhou QH; Hui EK; Lu JZ; Boado RJ; Pardridge WM, Pharmacokinetics and brain uptake of an IgG-TNF decoy receptor fusion protein following intravenous, intraperitoneal, and subcutaneous administration in mice. *Mol Pharm* 2013, 10 (4), 1425–31. [PubMed: 23410508]
25. Pardridge WM; Boado RJ; Patrick DJ; Ka-Wai Hui E; Lu JZ, Blood-Brain Barrier Transport, Plasma Pharmacokinetics, and Neuropathology Following Chronic Treatment of the Rhesus

- Monkey with a Brain Penetrating Humanized Monoclonal Antibody Against the Human Transferrin Receptor. *Mol Pharm* 2018, 15 (11), 5207–5216. [PubMed: 30226787]
26. Peters MA; Turnbow M; Buchenauer D, The distribution of methadone in the nonpregnant, pregnant and fetal rat after acute methadone treatment. *J Pharmacol Exp Ther* 1972, 181 (2), 273–8. [PubMed: 5030672]
27. Lukas G; Brindle SD; Greengard P, The route of absorption of intraperitoneally administered compounds. *J Pharmacol Exp Ther* 1971, 178 (3), 562–4. [PubMed: 5571904]
28. Barrett JS; Wahl RL; Wagner JG; Brown R; Fisher SJ, Investigations into the route of uptake and pharmacokinetics of intraperitoneally-administered monoclonal antibodies: I. Transdiaphragmatic blockade of the terminal lymphatics in the rat. *Cancer Immunol Immunother* 1990, 31 (6), 365–72. [PubMed: 2386982]
29. 5 - Intraperitoneal Drug Administration In *Techniques in the Behavioral and Neural Sciences*, Claassen V, Ed. Elsevier: 1994; Vol. 12, pp 46–58.
30. Turner PV; Brabb T; Pekow C; Vasbinder MA, Administration of substances to laboratory animals: routes of administration and factors to consider. *J Am Assoc Lab Anim Sci* 2011, 50 (5), 600–13. [PubMed: 22330705]
31. Richter WF; Bhansali SG; Morris ME, Mechanistic determinants of biotherapeutics absorption following SC administration. *AAPS J* 2012, 14 (3), 559–70. [PubMed: 22619041]
32. Supersaxo A; Hein WR; Steffen H, Effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration. *Pharm Res* 1990, 7 (2), 167–9. [PubMed: 2137911]
33. Royo F; Cossio U; Ruiz de Angulo A; Llop J; Falcon-Perez JM, Modification of the glycosylation of extracellular vesicles alters their biodistribution in mice. *Nanoscale* 2019, 11 (4), 1531–1537. [PubMed: 30623961]
34. Wang W; Chen N; Shen X; Cunningham P; Fauty S; Michel K; Wang B; Hong X; Adreani C; Nunes CN; Johnson CV; Yin KC; Groff M; Zou Y; Liu L; Hamuro L; Prueksaritanont T, Lymphatic transport and catabolism of therapeutic proteins after subcutaneous administration to rats and dogs. *Drug Metab Dispos* 2012, 40 (5), 952–62. [PubMed: 22328584]
35. Kinder M; Greenplate AR; Strohl WR; Jordan RE; Brezski RJ, An Fc engineering approach that modulates antibody-dependent cytokine release without altering cell-killing functions. *MAbs* 2015, 7 (3), 494–504. [PubMed: 25933349]
36. Chang R; Al Maghribi A; Vanderpoel V; Vasilevko V; Cribbs DH; Boado R; Pardridge WM; Sumbria RK, Brain Penetrating Bifunctional Erythropoietin-Transferrin Receptor Antibody Fusion Protein for Alzheimer's Disease. *Mol Pharm* 2018, 15 (11), 4963–4973. [PubMed: 30252487]
37. Sun J; Yang J; Whitman K; Zhu C; Cribbs DH; Boado RJ; Pardridge WM; Sumbria RK, Hematologic safety of chronic brain-penetrating erythropoietin dosing in APP/PS1 mice. *Alzheimers Dement (N Y)* 2019, 5, 627–636. [PubMed: 31660425]

Figure 1:
TfRMAb-N292G-EPO plasma concentrations after SQ (A, B) or IP (C, D) dosing represented in ng/mL (A, C) or %ID/mL (B, D). Data are shown as mean $\pm$ SEM of n = 3-6 per dose per route of administration.

Figure 2:

Brain concentrations of the WT TfRMAb-EPO and mutant TfRMAb-N292G-EPO fusion protein 24 hours following SQ, IP and IV administration of a 3 mg/kg dose in mice. The black dotted line shows the background signal in saline treated mice. The plasma data for the corresponding brain WT TfRMAb-EPO fusion protein data are published in Sun et al., 2019.¹⁵ Data are shown as mean $\pm$ SEM of $n = 3-6$ per dose per route of administration. *** $P < 0.001$ and **** $P < 0.0001$ compared to brain concentrations in mice treated with the WT TfRMAb-EPO fusion protein.

Figure 3:

Reticulocyte count is inversely correlated with AUC. The 3 mg/kg WT data is taken from Sun et al., 2019 and represents the data for the nonmutant TfRMAb-EPO.¹⁵ Spearman correlation coefficient $r = -0.84$, $p < 0.0001$ (A). No significant loss of the fusion protein in saline and mouse plasma over 24h at 37°C, *in vitro* (B). Proposed pathways for TfRMAb-N292G-EPO transit from the site of injection to systemic circulation following SQ and IP administration. The figure was created using [BioRender.com](https://www.biorender.com) (C).

Table 1:

Non-compartmental PK parameters for TTRMab-N292G-EPO in mice following IP and SQ administration
Data are presented as mean $\pm$ SEM.

Route of Administration	IP					SQ				
	TTRMab-N292G-EPO					TTRMab-N292G-EPO				
Dose (mg/kg)	3 mg/kg (n=6)	6 mg/kg (n=5)	9 mg/kg (n=6)	20 mg/kg (n=5)		3 mg/kg (n=6)	6 mg/kg (n=6)	9 mg/kg (n=6)	20 mg/kg (n=3)	
C _{max} (ng/mL)	288 $\pm$ 47	389 $\pm$ 154	633 $\pm$ 194	10,066 $\pm$ 7,059		15 $\pm$ 2.0	21 $\pm$ 4.1	21 $\pm$ 6	53 $\pm$ 28	
T _{max} (min)	180	180	180	180		180	180	180	180	
AUC(0-24h) (min)(ng/mL)	76,875 $\pm$ 15,581	85,729 $\pm$ 33,481	169,286 $\pm$ 59,306	2,249,636 $\pm$ 1,446,415		14,172 $\pm$ 1,459	15,845 $\pm$ 2,702	18,641 $\pm$ 4,801	28,887 $\pm$ 16,728	
AUC (0- ∞) (min)(ng/mL)	82,014 $\pm$ 17,383	87,597 $\pm$ 33,629	175,037 $\pm$ 61,399	2,312,403 $\pm$ 1,470,972		43,817 $\pm$ 6,813	29,564 $\pm$ 8,055	36,426 $\pm$ 8,714	39,197 $\pm$ 26,594	
MRT (min)	375 $\pm$ 43	313 $\pm$ 44	333 $\pm$ 22	431 $\pm$ 92		3,330 $\pm$ 206	1,654 $\pm$ 327	1,972 $\pm$ 275	664 $\pm$ 208	
Clearance/F (mL/min/kg)	52 $\pm$ 15	105 $\pm$ 27	108 $\pm$ 37	31 $\pm$ 13		73 $\pm$ 13	265 $\pm$ 52	319 $\pm$ 63	946 $\pm$ 642	
V _{ss} (L/kg)	17 $\pm$ 3.3	37.6 $\pm$ 13.2	35.7 $\pm$ 15.6	18 $\pm$ 11		239 $\pm$ 32	378 $\pm$ 61	599 $\pm$ 116	761 $\pm$ 622	

^{††} AUC, area under the plasma concentration-time curve; MRT, mean residence time; F, bioavailability; V_{ss}, volume of distribution at steady-state. Nonmutant TTRMab-EPO data taken from Sun et al., 2019¹⁵.

IP TTRMab-EPO (3 mg/kg; n=3): C_{max} (ng/mL): 1,380 $\pm$ 213; T_{max} (min): 180; AUC(0-24h) (min)(ng/mL): 705,390 $\pm$ 46,814; Clearance/F: 4.3 $\pm$ 0.5 (mL/min/kg)

SQ TTRMab-EPO (3 mg/kg; n=3): C_{max} (ng/mL): 4,748 $\pm$ 290; T_{max} (min): 360; AUC(0-24h) (min)(ng/mL): 4,198,080 $\pm$ 234,375; Clearance/F: 0.72 $\pm$ 0.07 (mL/min/kg)

Table 2:

Hematology indices and white blood cell count at 24 hours after a single dose of TIRMAb-N292G-EPO in mice.
Data are presented as mean $\pm$ SEM.

Route of Administration	Controls (SQ)	TIRMAb-N292G-EPO (IP)			TIRMAb-N292G-EPO (SQ)		
		Saline (n=3-7)	IgG1 (n=3)	3 mg/kg (n=3)	9 mg/kg (n=6)	20 mg/kg (n=3)	20 mg/kg (n=3)
Dose							
RBC ($10^3/\mu\text{L}$)	5.6 $\pm$ 0.3	6.7 $\pm$ 0.1	6.0 $\pm$ 0.3	5.6 $\pm$ 0.1	6.1 $\pm$ 0.4	6.0 $\pm$ 0.6	5.7 $\pm$ 0.4
HGB (g/dL)	8.5 $\pm$ 0.5	10.4 $\pm$ 0.3	9.3 $\pm$ 0.5	8.7 $\pm$ 0.2	9.6 $\pm$ 0.6	9.3 $\pm$ 1.1	8.9 $\pm$ 0.6
WBC ($10^3/\mu\text{L}$)	5.4 $\pm$ 0.7	7.8 $\pm$ 0.5	7.4 $\pm$ 0.2	** 10.3 $\pm$ 0.5	7.8 $\pm$ 0.9	* 8.7 $\pm$ 0.5	** 11.0 $\pm$ 1.8
Total Retic (%)	5.5 $\pm$ 0.4	4.5 $\pm$ 0.2	* 2.2 $\pm$ 0.3	*** \$ 1.6 $\pm$ 0.2	*** \$ 0.5 $\pm$ 0.03	3.6 $\pm$ 1.3	*** \$ 1.2 $\pm$ 0.2

* P<0.05

** P<0.01

*** P<0.001 compared to saline

\$ P<0.05 compared to IgG1

††† RBC, red blood cell; HGB, hemoglobin; WBC, White blood cell; Retic, reticulocytes

CBC was not performed at the 6 mg/kg dose.