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RESEARCH PAPER

The Impact of Glycosylation on the Pharmacokinetics of a TNFR2:Fc Fusion Protein Expressed in Glycoengineered *Pichia Pastoris*

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ABSTRACT

Purpose *P. pastoris* has previously been genetically engineered to generate strains that are capable of producing mammalian-like glycoforms. Our objective was to investigate the correlation between sialic acid content and pharmacokinetic properties of recombinant TNFR2:Fc fusion proteins generated in glycoengineered *P. pastoris* strains.

Methods TNFR2:Fc fusion proteins were generated with varying degrees of sialic acid content. The pharmacokinetic properties of these proteins were assessed by intravenous and subcutaneous routes of administration in rats. The binding of these variants to FcRn were also evaluated for possible correlations between *in vitro* binding and *in vivo* PK.

Results The pharmacokinetic profiles of recombinant TNFR2:Fc produced in *P. pastoris* demonstrated a direct positive correlation between the extent of glycoprotein sialylation and *in vivo* pharmacokinetic properties. Furthermore, recombinant TNFR2:Fc produced in glycoengineered *Pichia*, with a similar sialic acid content to CHO-produced etanercept, demonstrated similar *in vivo* pharmacokinetic properties to the commercial material. *In vitro* surface plasmon resonance FcRn binding at pH6.0 showed an inverse relationship between sialic acid content and receptor binding affinity, with the higher affinity binders having poorer *in vivo* PK profiles.

Conclusions Sialic acid content is a critical attribute for modulating the pharmacokinetics of recombinant TNFR2:Fc produced in glycoengineered *P. pastoris*.

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ABBREVIATIONS

ASGPR	asialoglycoprotein receptor
AUC	area under the curve
BR3-Fc	B cell-activating factor receptor 3 (BR3) Fc fusion protein
CHO	Chinese hamster ovary
Fc	crystallizable fragment of antibody
FcRn	neonatal Fc receptor
GlcNAc	n-acetylglucosamine
IgG1	immunoglobulin G subclass 1
IMID	immune-mediated inflammatory disease
IV	intravenous
LFA3TIP	lymphocyte function-associated molecule 3 Fc fusion protein
PK	pharmacokinetics
rhEPO	recombinant human erythropoietin
SC	subcutaneous
TNF	tumor necrosis factor
TNFR2	tumor necrosis factor receptor 2
TSA	total sialic acid content

INTRODUCTION

Immune-mediated inflammatory diseases (IMID) are believed to result from the dysregulation of inflammatory pathways driven by cytokines, such as tumor necrosis factor (TNF). The development of TNF inhibitors has clinically demonstrated the relationship between TNF and IMID (1). As such TNF inhibitors are at the forefront in the treatment rheumatoid arthritis, psoriasis, psoratic arthritis, ankylosing spondylitis and Crohn's disease. Blocking the interaction between TNFα and its receptor has been the target for a

number of biologics, including monoclonal antibodies and Fc-fusion molecules (2). Etanercept (brand name Enbrel®) was an early entrant in the group of anti-TNF sequestrants that has shown efficacy in the management of several of the above listed IMID. Enbrel® achieved blockbuster status soon after its launch reaching sales of~$6.6 billion USD worldwide in 2009 (3).

TNF-α, one of the key players in the inflammatory response, occurs naturally as a homotrimer. Likewise, in the active state, the TNF receptor is presented on the cell surface as a trimer. However, the extracellular domain of the receptor can be shed from the cell surface to generate a soluble form of the receptor, which attenuates TNF activity. This was the basis of research performed by Mohler *et al.*, who demonstrated that a soluble dimeric form of TNFR was efficient at antagonizing TNF activity (4). This dimeric TNFR form involved the fusion of the TNFR2 ectodomain to the IgG1 Fc domain, thus generating an immunoadhesin termed etanercept (TNFR2:Fc). The complexity of this molecule is schematically represented in Fig. 1a. The TNFR2 ectodomain is composed of several subdomains. At the N-terminus of the ectodomain are four cysteine-rich subdomains which are involved in the binding of TNF. Adjacent to these domains is a stem region, which is present in the membrane-bound receptor, connecting the cysteine-rich subdomains to the transmembrane domain. Together the cysteine-rich domain and the stem region form the ectodomain. In etanercept, two of these ectodomains are fused to the Fc domain of IgG1 to form a homodimeric fusion protein. The complexity of this molecule is increased greatly by the presence of extensive N- and O-linked glycosylation, especially on the TNFR2 portion of the molecule. Specifically, each monomer possesses three N-linked glycosylation sites, one in the CH2 domain of the Fc and two located in the fourth cysteine-rich domain of the TNFR2 ectodomain, thus giving a total of six N-linked sites in the intact dimer. The O-glycosylation status of TNFR2:Fc is less well-defined due to the lack of predictable glycosylation consensus sites for occupancy and the potential for heterogenous mucin-type glycoforms. However, limited characterization has indicated that several O-glycosylation sites are present within a specific peptide located in the stem region of the TNFR2 ectodomain (5). Together both N- and O-linked glycans significantly add to the final molecular mass of the molecule. Furthermore, since TNFR2:Fc is typically produced in mammalian cell lines, both the N- and O-linked glycans

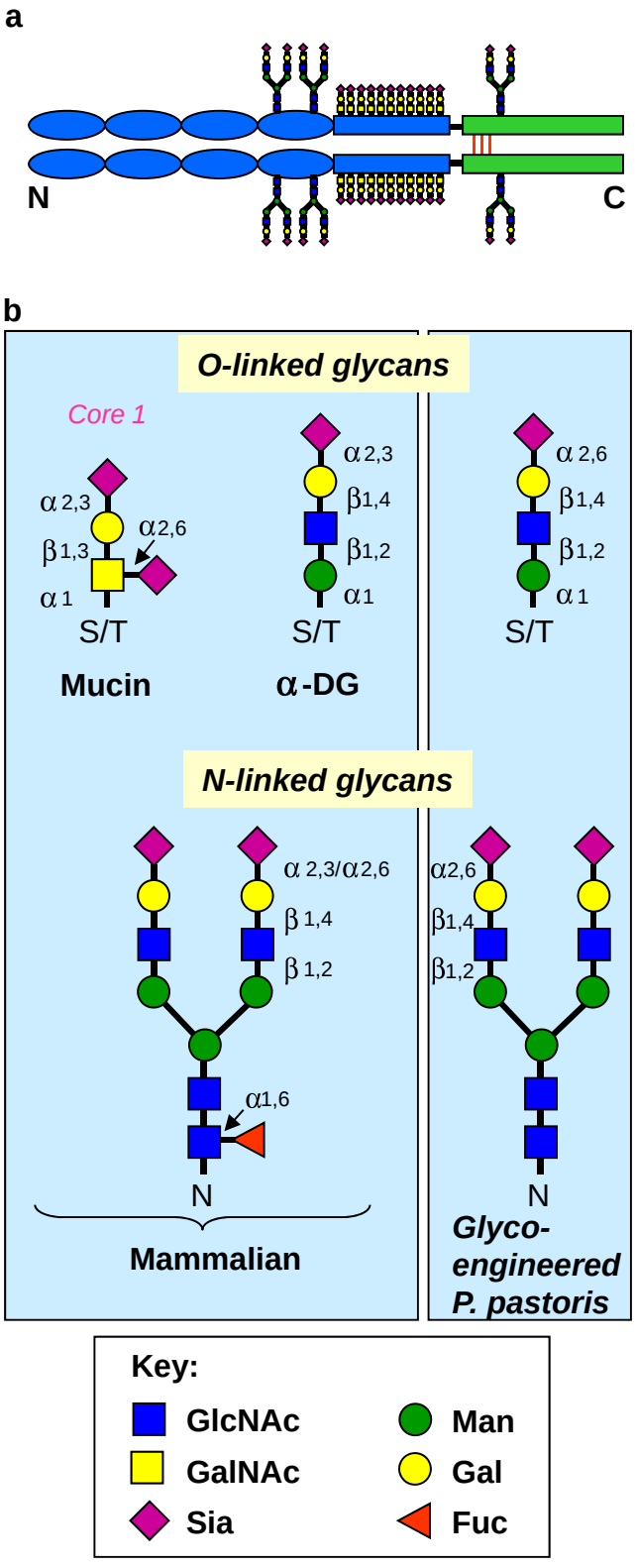

Fig. 1 Schematic diagrams illustrating TNFR2:Fc and comparative glycosylation states. (**a**) TNFR2:Fc is a dimeric fusion protein, possessing two N-terminal ectodomains of the TNFR2 receptor (*blue*) and a C-terminal Fc domain of IgG1 (*green*). Interchain disulfide bonds of the Fc domain create a homodimer. The three N-linked glycosylation sites present on each fusion protein monomer are represented in a similar depiction to the detailed glycan illustration in (**b**). The TNFR2 domain is highly O-glycosylated, with glycan sites being concentrated within the stem region of this ectodomain fragment. O-Glycans situated on the molecule are represented in a similar depiction to the detailed glycan illustration in (**b**). (**b**) For comparative purposes the N- and O-linked glycans present on mammalian and glycoengineered *P. pastoris* glycoproteins are illustrated.

can possess an extensive number of terminal sialic acid residues (Fig. 1b). The presence of this charged monosaccharide not only affects the physicochemical properties of the molecule but also serves a significant role in biological function, including half-life, receptor-mediated interactions and bioavailability.

A feature of the Fc domain of etanercept is that it has the ability to interact with the FcRn receptor and potentially increase half-life of the molecule. Many biologically active proteins and peptides have very short half-lives. Typically, in the case of IgG, the *in vivo* half-life is enhanced *via* the FcRn mediated recycling mechanism (6). Likewise, the half-life of an Fc-fusion protein can be extended because the Fc domain potentially binds to FcRn, which rescues the fusion protein from degradation in the endosomes. In addition to half-life extension, the Fc domain can provide the fusion protein with other benefits, such as, by enhancing expression and secretion, providing a facile purification strategy using Protein-A chromatography, and improving solubility and stability.

Typically, therapeutic glycoproteins are made using mammalian cell lines, with CHO cell lines being the most widely utilized. There are several shortcomings with mammalian cell culture, including production cost, requirement for complex media, viral contamination and the introduction of non-human glycoforms (including N-glycolylneuraminic acid) that have encouraged researchers to look for alternative systems. The ability of yeast and filamentous fungi to grow in defined media in the absence of animal-derived growth factors, relative ease of scale-up, and the high yields of secreted protein, have made them attractive platforms for therapeutic protein development (7). Although the initial stages of human and yeast N-linked glycosylation pathways in the endoplasmic reticulum are similar, subsequent modifications in the Golgi differ significantly. To this end, scientists at GlycoFi (a wholly owned subsidiary of Merck & Co., Inc.) have genetically engineered the yeast *Pichia pastoris* to produce proteins with human-like N-linked glycans, as depicted in Fig. 1b (8–12). More recently, we have engineered *Pichia* to produce proteins with mammalian-like O-linked glycans terminating in sialic acid (Hamilton & Nett, manuscript in preparation). Subsequently, therapeutic glycoproteins can now be generated using the *Pichia* expression platform with both mammalian-like N- and/or O-linked glycoproteins terminating in sialic acid. One novel feature of this expression platform is that the glycan profile of the recombinant glycoprotein can be tailored by using a strain that has been engineered with a defined glycosylation pathway. It is known that the sialic acid content of a glycoprotein can play a significant role in its serum half-life. For example, the sialic acid content of erythropoietin is a critical attribute for its half-life (13–15). Using the *Pichia* expression system, with defined sialylation pathways, we can now assess the relationship between sialic acid content and the pharmacokinetic properties of more complex molecules, including etanercept. In a separate investigation, TNFR2:Fc has been expressed in glycoengineered *Pichia* strains to produce glycoproteins possessing varying levels of sialic acid content (Hamilton & Stadheim 2012; Gomathinayagam & Li 2012, manuscripts in preparation). Here, we assessed the pharmacokinetic properties of a subset of these molecules and benchmarked them to a commercial source of etanercept.

MATERIALS AND METHODS

Reagents

A commercial etanercept (Enbrel®) was purchased from a Pharmacy. A yeast expression vector containing a DNA sequence coding for etanercept with the same amino acid sequence as Enbrel® was constructed. Etanercept preparations with controlled glycosylation profiles were prepared from glycoengineered *Pichia pastoris* host strains based on the methods described in (11) (Hamilton & Stadheim 2012; Hamilton & Nett 2012, manuscripts in preparation). Etanercept with terminal sialylation on N-linked glycans and to various degrees on O-linked glycans were generated. Specifically, the *Pichia* produced Forms 1, 3, and 5A of TNFR2:Fc were isolated from strains YGLY10299, YGLY12680 and YGLY14252 as described in Hamilton *et al.* (Hamilton & Nett 2012, manuscript in preparation). Table I presents the glycan characterization of these variants.

Purification and Characterization of TNFR2:Fc Glycoproteins

Recombinant TNFR2:Fc produced in *Pichia* was purified and formulated as described in Gomathinayagam *et al.* (manuscript in preparation). Briefly, recombinant TNFR2:Fc Forms 1, 3 and 5A were purified initially through affinity chromatography employing MabSelect™. For Forms 1 and 3, the MabSelect pool samples were subsequently purified employing macro-prep Ceramic Hydroxyapatite Type I 40 μm chromatography in a bound and elute mode to remove aggregated forms of TNFR2:Fc protein. For Form 5A, highly sialylated N- and O-glycan containing TNFR2:Fc protein was separated as a flow-through and wash-unbound fraction on macro-prep Ceramic Hydroxyapatite chromatography. Subsequently all three *Pichia* produced TNFR2:Fc variants were purified employing Hydrophobic Interaction Chromatography (Phenyl Sepharose™ 6 FF) followed by Cation Exchange Chromatography (SP sepharose FF). The three variants were formulated in the same formulation buffer as commercial Enbrel®, filter sterilized

Table I Glycan Composition of Etanercept Glycovariants

	N-Glycosylation (%)				O-Glycosylation (%)					TSA (mol/mol)
	Neutral	Mono-sialylated	Hybrid sialylated	Bi-sialylated	Man1	Man2	ManGlcNAc	ManGlcNAcGal	ManGlcNAcGalSia	
Form 1	43.3	28.5	16	12.2	96	4	NA	NA	NA	3
Form 3	44.6	33.1	10.9	11.4	15	3	23	30	29	10
Form 5A	8.9	22	6.5	62.6	15	11	0	7	67	21
Enbrel®	33	44.9 (A1 & A1F)	ND	22.1 (A2 & A2F)	NA	NA	NA	NA	NA	25

N- and O-linked glycans were released from the proteins and quantitated as described in the Materials and Methods section. *NA* not applicable; *ND* not detected

using 0.2 μm PES (PolyEtherSulfone) membrane filters and stored @4°C.

The N- and O-linked glycans were released from the TNFR2:Fc and analyzed. Briefly, for N-linked glycan analysis, the glycans were released from TNFR2:Fc by treatment with PNGase F, labeled with 2-aminobenzamide (2-AB) and analyzed by HPLC as described previously (11). For O-glycan analysis, the O-glycans were released from TNFR2:Fc by β-elimination under alkaline conditions, processed and analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described previously (16). Detailed characterization of the released O-glycans was performed by *in vitro* enzymatic digestions using neuraminidase, galactosidase and hexosaminidase as per the manufacturer's recommendation (New England BioLabs, Ipswich, MA) and as outlined by Hamilton *et al.* (Hamilton & Nett 2012, manuscript in preparation). The total sialic acid content of each TNFR2:Fc variant was determined using acid hydrolysis to release the sialic acid, followed by measuring the mole ratio of sialic acid per mole of protein using HPAEC-PAD, as described by Hamilton *et al.* (Hamilton & Nett 2012, manuscript in preparation).

In Vivo Dosing

Jugular vein pre-cannulated male Sprague–Dawley rats (10–12 weeks age, weight between 275 and 350 g) were purchased from Charles River Laboratories (Wilmington, MA). Rats were dosed at 1 mg/kg either subcutaneously at lower back or intravenously through jugular vein. Serum samples were prepared *via* tail vein at 0, 4, 24, 48, 72, 96, 120, 144, 168 and 192 h post dosing for SC administration. The serum sample collection time points following IV were 0, 0.25, 1, 6, 24, 48, 72, 96, 120, 144, 168 and 192 h. The *in vivo* work was performed according to Merck IACUC guidelines.

Immunoassay for Antibody Detection

A qualified immunoassay was developed to detect TNFR2:Fc in rat serum using the microfluidic, Gyrolab® Bioaffy workstation (Gyros, Uppsala, Sweden). Briefly, a biotinylated anti-TNFR2 mouse monoclonal antibody (R&D Systems, Minneapolis, MN) was used as the capture antibody and an anti-human Fc rabbit monoclonal (Epitomics, Burlingame, CA) was used as the detection antibody after labeling with ALEXA 647 fluorescent dye (Invitrogen, Life Technologies, Grand Island, NY). TNFR2:Fc concentrations were interpolated from standard curves, generated using known amounts of either Enbrel® or *Pichia* produced material. The assay range was established with spiked quality control samples and the lower limit of quantitation was 0.8 ng/ml in 100% rat serum. The entire immunoassay was automated using the Gyrolab® Bioaffy workstation and Bioaffy 200 compact disks (Gyros, Uppsala, Sweden).

FcRn Binding Assay

A Biacore T-100 instrument (GE Healthcare Biosciences, Pittsburgh, PA) was used to determine etanercept : FcRn binding kinetics. The extracellular domain of human FcRn (both α and β chain) was expressed using a Baculovirus system and purified *via* a 6xHis tag. FcRn was immobilized onto a Biacore CM5 biosensor chip *via* amine coupling at densities ~200 RU (17). The kinetics experiments were conducted at 25°C using PBSP (50 mM $NaPO_4$, 150 mM NaCl and 0.05% (v/v) polysorbate 20, pH 6.0) as running buffer with a flow rate of 30 μL/min. Etanercept was diluted from a stock with pH 6.0 running buffer to 25, 50 and 100 nM. The etanercepts were allowed to bind FcRn for 3 min, followed by 2 min of dissociation. Two 30 s pulses of PBSP, pH 7.5 were used to regenerate the chip. To determine K_D at pH 6.0, the data from all concentrations was used simultaneously to fit a two-state reaction model found in the Biacore T-100 Evaluation software.

PK and Statistical Analysis

Key pharmacokinetic parameters of interest were calculated for each animal using non-compartmental analysis of serum mAb concentration-time data. (WinNolin Enterprise Version

5.01, Pharsight Corp, Mountain View, CA). One-way ANOVA was used for the comparative evaluation. A p value of <0.05 is considered statistically significant.

RESULTS

Analytical Characterization of TNFR2:Fc Expressed in Glycoengineered Yeast

Three forms of TNFR2:Fc glycovariants were generated using the glycoengineered *Pichia pastoris* expression platform. Form 1 was produced by the glycoengineered strain YGLY10299 and was described as an O-mannose reduced form. As indicated in Table I, this material possessed N-linked glycans with terminal sialic acid but the O-linked glycans were characteristic *Pichia* glycans that had been reduced to primarily a single mannose structure, and as such did not possess sialic acid. Form 3 material was referred to as the O-sialylated form and was produced by the glycoengineered strain YGLY12680. This form possessed sialic acid on both the N- and O-linked glycans (Table I). Form 5A material was similar to the Form 3 material except that through strain selection and purification enrichment, enhanced sialylation was obtained on both the N- and O-linked glycans (Table I). For comparative purposes the N-glycan profile of Enbrel® was analyzed and is reported in Table I. Due to the complexity of the O-glycan profile on this mammalian-derived product, and the lack of defined glycan standards, the O-glycan profile was not determined. To assess the total sialic acid (TSA) content of the three *Pichia* produced forms and Enbrel®, sialic acid was released, quantitated by High pH Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using Dionex and reported as moles of sialic acid per mole of protein, as described in Hamilton *et al.* (Hamilton & Nett 2012, manuscript in preparation). As reported in Table I, Form 5A had a TSA content of 21 mol/mol which was comparable to that of Enbrel®, which was 23 mol/mol. Form 3, which was O-sialylated like Form 5A, had a lower TSA of 10, while the O-mannose reduced Form 1, which only possessed sialic acid on its N-linked glycans, had a TSA of 3 mol/mol (Table I).

Two-dimensional electrophoretic analysis of the three *Pichia*-produced and the CHO-produced materials showed that sialylation greatly affected the PI of the molecule (Fig. 2). Furthermore, although the glycosylation data reported in Table I represented the average glycosylation profile for each etanercept preparation, it is apparent from Fig. 2 that within each preparation there was a population of molecules with a defined range of sialylation. Form 1, which lacked sialylation on O-linked glycans, had the highest PI range. The *Pichia* produced form with the enhanced sialylation, Form 5A, had a low PI range, similar to that of Enbrel®. Each of these three preparations of etanercept demonstrated the presence of a similar number of sialylated species, as observed on the 2D gel (Fig. 2). By comparison, Form 3 appeared to have a broader range of sialylated species residing in the middle of the PI range. As expected, those samples that had the lowest PI range were those that had higher sialic acid contents, and as such, higher TSA values as listed in Table I. Going forward in this report samples will be discussed in terms of their TSA values, which represent the average sialic acid content of each sample, and does not necessarily reflect the number of individual sialylated species present.

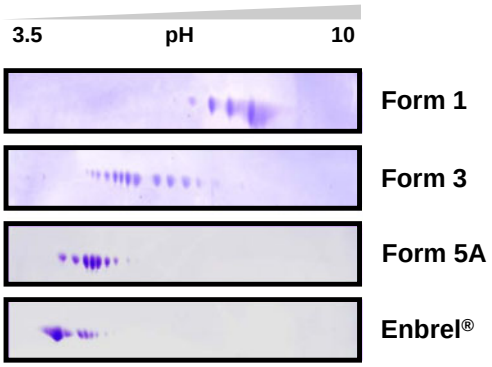

Fig. 2 Iso-electric focusing of TNFR2:Fc produced in representative glycoengineered *P. pastoris* strains compared to that of commercial Enbrel®.

Enhanced Sialylation is Critical for the Pharmacokinetics of Glycoengineered Yeast Produced Etanercept Molecules

The pharmacokinetics of Forms 1, 3 and 5A were compared with Enbrel® in rats following subcutaneous (SC) or intravenous (IV) administrations. As shown in Fig. 3a and Table II, Form 1 was cleared rapidly and did not have typical absorption profiles associated with SC administration, resulting in approximately a 600-fold lower systemic exposure (AUC_{last}) in comparison to Enbrel®. Form 3 exhibited a typical SC administration absorption profile but still showed approximately a 5-fold lower systemic exposure than that of Enbrel®. By comparison, the systemic exposure of Form 5A was not distinguishable from that of Enbrel®. Analysis of the C_{max} values for Forms 1 and 3 following SC administration indicated that these were approximately 115- and 4-fold lower, respectively, when compared to Enbrel®. By contrast, the C_{max} of Form 5A was similar to that of Enbrel®. The Form 1 exhibited an extremely short T_{max} of 4 h. Like the other parameters measured following SC administration, the T_{max} of Form 5A was similar to that of Enbrel®.

To determine the relative contribution of absorption and clearance to the PK profiles, an IV pharmacokinetic study was conducted (Fig. 3b and Table II). The results showed

that Form 1 had a faster rate of clearance in the alpha phase, a typical feature for glycan-associated receptor clearance, such as in the case of mannose-mediated clearance (18). As seen in the concentration time profile, and calculated from the drug concentrations (data not shown), the initial concentrations (15 min after IV dosing) of Form 1 and Form 3 were similar to each other but each was about 15% lower than that of Enbrel®. Six hours after dosing, the concentration difference was extended to about 165-fold lower for Form 1- and 3-fold lower for Form 3 in comparison to Enbrel®. The serum concentration of Form 5A remained similar to that of Enbrel® throughout the study. The clearance (CL) for Form 1 and Form 3 were about 34- and 3-fold faster, respectively, than that of Enbrel®, while again that of Form 5A was similar to that of the Enbrel®. While the half-life of Form 5A was similar to that of Enbrel®, Form 1 or Form 3 exhibited a significantly shorter half-life. The SC bioavailability for Form 1 was dramatically lower (>10-fold) than, consistent with its rapid CL, while that for Form 3 was similar to those for Form 5A and Enbrel®. The results from SC and IV administration suggested that clearance, and possibly also absorption, contributed to the poor PK properties of Forms 1 and 3. A major difference in the four forms of TNFR2:Fc assessed in this study was the extent of sialylation, as mentioned above. When the AUC_{last} and C_{max} of these variants following SC administration were plotted against their TSA content, a strong correlation between TSA and these key PK parameters was apparent (Fig. 4). These results suggest that the extent of sialylation is critical for the pharmacokinetics of etanercept.

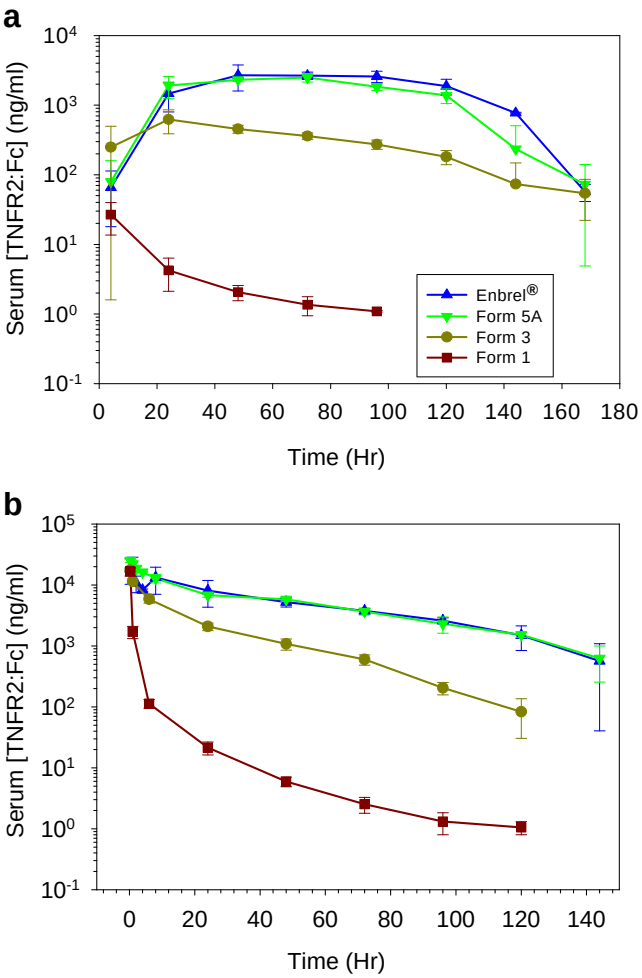

Fig. 3 Serum drug concentration time curves of etanercept glycovariants following (**a**) SC or (**b**) IV administrations. Sprague–Dawley rats (*male*) were dosed either SC or IV at 1 mg/kg with Form 1, Form 3, Form 5A or commercial TNFR2:Fc (Enbrel®). Serum samples were collected and TNFR2:Fc concentrations determined by an immunoassay. Data were expressed as mean ± SD. *N*=5 for Form 1, Form 3 and Form 5A; *N*=10 for Enbrel®.

Table II Key PK Parameters of Etanercept Glycovariants and Enbrel® Following SC and IV Administration

	Form 1	Form 3	Form 5A	Enbrel®
IV				
$t_{1/2}$ (hr)	20.6 ± 3.4*	19.9 ± 2.7*	26 ± 7.7	30 ± 8.8
AUC_{last} (hr*ng/mL)	19668 ± 2608*	201014 ± 22195*	714920 ± 43371	714667 ± 208323
CL (mL/hr/kg)	51.4 ± 6.4*	5.0 ± 0.6*	1.4 ± 0.1	1.5 ± 0.5
SC				
C_{max} (ng/mL)	26 ± 13*	773 ± 333*	2542 ± 314	2982 ± 365
T_{max} (hr)	4 ± 0*	34 ± 26	21 ± 0.4	21 ± 0.3
AUC_{last} (hr*ng/mL)	461 ± 243*	59672 ± 16489*	241793 ± 30828	275043 ± 35174
Bioavailability (%)	3 ± 1*	31 ± 7	34 ± 4	38.5 ± 5

Sprague–Dawley rats (male) were dosed either IV or SC at 1 mg/kg with Form 1, Form 3, Form 5A or Enbrel®. Serum samples were collected and TNFR2:Fc concentrations determined by an immunoassay. Data were expressed as mean ± SD. *N*=5 for Form 1, Form 3 and Form 5A; *N*=10 for Enbrel®. * $p<0.05$ when compared to Enbrel®

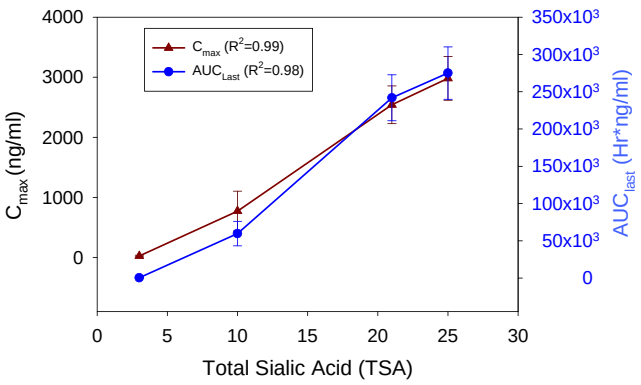

Fig. 4 Correlations between TSA and AUC_{last} and C_{max}. C_{max} and AUC_{last} from Table II (SC administrations) were plotted against total sialic acid contents (TSA) from Table I.

TNFR2:Fc with Lower Sialylation Showed Higher Binding Affinity to Human FcRn

FcRn is considered to be the critical regulator for the long serum residence time for IgG and IgG Fc-containing molecules (19). To investigate if the reduced half-lives of the lower sialylated variants were due to reduced FcRn binding, the pH 6.0 binding affinities (KD) of the four etanercept molecules were measured by surface plasmon resonance using Biacore. Since the binding affinity of Enbrel® to FcRn has previously been well characterized by Biacore (19), the data obtained in the current study compared the binding of each of the *Pichia* produced etanercept molecules to that of the commercial control in two separate experiments. One experiment compared Form 1 and Form 3 to Enbrel®, while the second compared the Form 5A to Enbrel®. As can be seen from Fig. 5a, Form 1 bound to human FcRn the strongest, with its relative KD being 70% lower than that of commercial Enbrel®. The binding of Form 3 was 30% lower than that of the commercial control material, hence also demonstrating stronger binding to FcRn than Enbrel®. As shown in Fig. 5b, the Form 5A exhibited similar FcRn binding intensity to that of the commercial control, with relative affinity being similar. As such, this *in vitro* data indicated that the reduced half-lives of the lower sialylated variants did not correlate with reduced FcRn binding. In fact, we observed an inverse relationship between the extent of sialylation of the *Pichia* produced material and their binding to recombinant human FcRn, as assessed by surface plasmon resonance at pH 6.0 using Biacore.

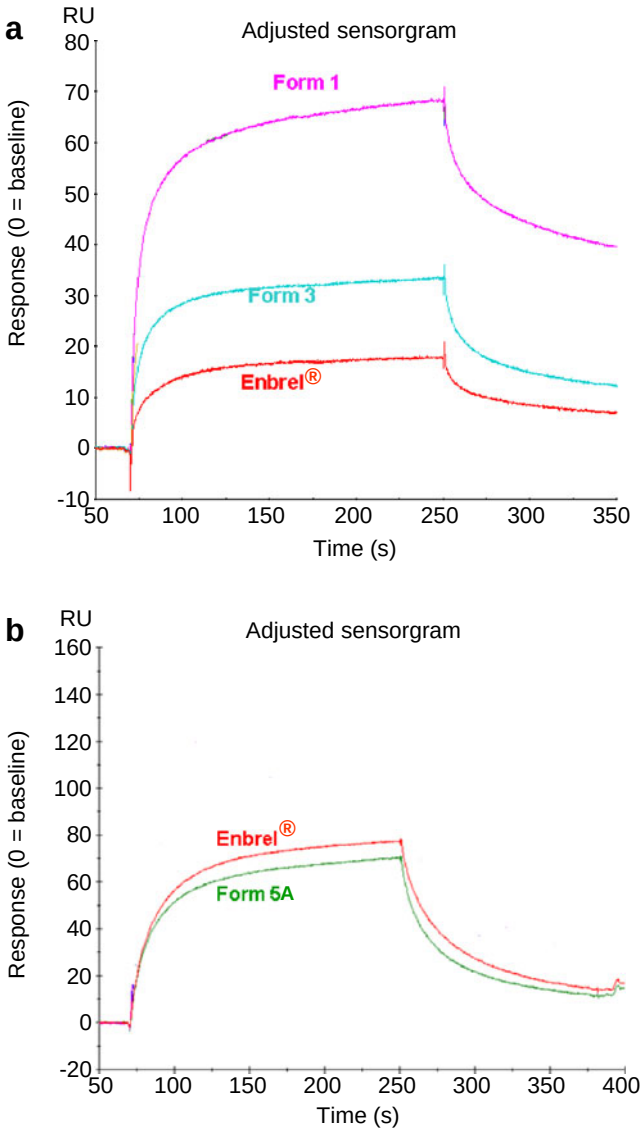

Fig. 5 Comparative *in vitro* Biacore analysis between *Pichia* produced material and commercial Enbrel®. (**a**) Experiment 1, comparison of Form 1, Form 3 and Enbrel®. (**b**) Experiment 2, comparison of Form 5A and Enbrel®.

DISCUSSION

A total of seven Fc-fusion products have been approved for treating various diseases with many more in development (20). Led by etanercept, Fc-fusion products represent an important class of biologics in the treatment of a variety of illnesses. The circulating half-lives of most peptides or proteins is typically very short, being a matter of minutes or few hours, and as such many approaches have been taken by the therapeutic protein industry to overcome this short-coming (21). One such approach is the fusion of these molecules to an Fc domain, which can extend their half-lives greatly. This increase in half-life may not only be a consequence of increased size of the molecule but also due to recycling of the molecule by the FcRn mechanism. Romiplostim, a Fc-TPO mimetic peptide fusion, exhibited 16- to 24-fold faster clearance in FcRn knock-out mice than in normal mice, demonstrating the critical role of the Fc to FcRn interaction in the PK of this Fc-fusion product (22). Typically, Fc-fusion products have shorter half-lives than complete antibodies. To investigate the reason for these differences, Suzuki *et al.* compared the pH 6.0 binding affinity of a number of Fc-fusion products to those of full IgG mAb products (19). In this study they demonstrated that the binding affinity of Fc-fusion proteins to FcRn was significantly reduced in comparison to intact IgG. In one example, the authors demonstrated that the binding to FcRn by the Fc domain of etanercept could be increased through removal of the TNFR2 ectodomain by papain digestion. Thus the authors suggested that the Fc-fusion partner, the TNFR2 ectodomain, influenced the structural environment of the FcRn binding region, thus leading to a reduced association between the Fc domain and the FcRn. In addition to the peptide structure of the TNFR2 ectodomain influencing the FcRn binding region of the Fc domain, it is feasible that

due to the significant post-translational modifications of the molecule, that glycosylation may also affect FcRn binding. Here, we show that through glycan engineering, the binding of etanercept to the FcRn can be modulated. Interestingly, those forms produced containing a lower TSA content, bound more efficiently to the FcRn. In contrast, Form 5A, possessing the highest TSA content, bound the weakest to the receptor under the conditions of the *in vitro* Biacore assay used. Conversely, the *in vivo* PK parameters demonstrated a direct correlation between the extent of sialylation and *in vivo* pharmacokinetics. Recently, it has been shown that binding affinity at pH 6.0 alone does not necessarily translate to a slower clearance of IgG molecule; other factors such as the dissociation at neutral pH (7.4) also plays an important role (17,23). The combination of high affinity to FcRn at pH 6.0, and the faster and more complete dissociation at pH 7.4, correlate to a slower *in vivo* clearance of IgG molecules (17,23). Since the dissociation at pH 7.4 was not measured for Enbrel® and the *Pichia* produced material, the contribution of the unfavorable pH 7.4 dissociation to Form 1's poor PK property cannot be completely excluded. Interestingly, other data obtained by comparing the interactions between the binding of humanized IgG1 variants to mouse and cynomolgus monkey FcRns, and how these correlated to the pharmacokinetics observed in a mouse model, suggested that understanding *in vivo* pharmacokinetics may involve considering multiple factors in tandem, and not just FcRn association and dissociation properties (24). Furthermore, in this same study, and in other studies (25,26), differences in the cross-species binding of FcRn to different antibody subtypes and to antibodies from different host organisms have been identified. However, human IgG1 has previously been demonstrated to interact with the rat FcRn (27), and as such, the cross-species interaction present in the current *in vivo* study is thought to be representative of native FcRn:Fc binding. In summary, the data presented in this report does indicate that while FcRn recycling may play a dominant role in the half-lives of other Fc-possessing molecules, this is not the overriding factor for etanercept half-life. More so, in the current study we have demonstrated that the glycosylation state of the ectodomain can define the *in vivo* PK profile of this molecule.

Comparing the glycosylation profiles of the various *Pichia* produced etanercept molecules indicated differences in their terminal glycan structures, which may be responsible for their differing *in vivo* pharmacokinetics. Form 1 had a better binding to FcRn than that of Enbrel®, and yet this molecule was cleared so fast that the absorption phase was not apparent following SC administration. Form 1 was unique, in that its O-linked glycans were composed primarily of individual O-linked mannose residues, which may have been accessible for binding to mannose receptors located in the liver and skin. This would be consistent with the IV pharmacokinetic properties observed with this form of etanercept having a rapid serum drug concentration decline in the alpha phase. Further glycoengineering of the yeast host cell resulted in the production of etanercept Form 3. Through the introduction of the enzyme protein O-linked-mannose β-1,2-N-acetylglucosaminyltransferase 1 (POMGnT1) terminal O-mannose content was reduced to 15%, while terminal N-acetylglucosamine (GlcNAc, 23%), galactose (30%) and sialic acid (29%) were introduced. With this form the PK properties were greatly improved but were still not comparable to Enbrel®. Comparison of the N-linked glycan profiles from Form 1 and Form 3 indicated that there was no significant difference with these glycans. As such, the resulting improvement in the PK attributes were a direct consequence of O-glycan engineering. The fact that the PK profile of Form 3 was still not similar to those of Enbrel®, suggested that the current glycan complement may also have some liabilities. To this end, it is known that terminal galactose can bind to asialoglycoprotein receptors (ASGPR) in the liver and that terminal GlcNAc can engage mannose receptors (28,29). Therefore, even though we have added terminal sialic acid to a population of the Form 3 material, the presence of terminal galactose and GlcNAc may explain the poorer PK properties when compared to Enbrel®. In contrast to both Form 1 and Form3, Form 5A demonstrated comparable PK properties to that of commercial Enbrel®. Through strain selection and purification enrichment, sialylation levels on this *Pichia* produced material were comparable to those on Enbrel®. As a consequence, the levels of terminal galactose and GlcNAc were reduced, which would reduce clearance by the ASGPR and mannose receptors, respectively.

As a consequence of enhancing the sialylation of *Pichia* produced etanercept, not only did the terminal glycan profile change but the molecule also increased in size due to the addition of the extra saccharide residues and attained a lower pI, which may have improved solubility. Both of these features could also contribute to the enhanced PK properties of the Form 5A etanercept. Irrespective of which specific characteristic of enhanced sialylation is responsible for the improved pharmacokinetic profile of Form 5A, it is apparent that increased TSA results in a more efficacious molecule. Likewise, this observation has been observed with other therapeutic proteins. For example, in the case of recombinant human erythropoietin (rhEPO), one of the most extensively studied therapeutic glycoproteins. As reviewed by Egrie and Browne (30), hyperglycosylation of rhEPO by increasing the number of glycosylation sites resulted in enhanced sialic acid content on the molecule. In turn, this produced an EPO therapeutic that had significantly enhanced biological activity and serum half-life. Interestingly, this modification did result in reduced binding to the EPO receptor. In the current report, the binding of the

Pichia produced etanercept to TNF has not been discussed, but *in vitro* cell based assays were performed and demonstrated that no significant difference in TNF binding was observed when compared to commercial Enbrel® (Hamilton & Stadheim 2012, manuscript in preparation). Another example that enhanced sialylation improves the pharmacokinetics of a therapeutic protein has been reported for LFA3TIP (31). This molecule is a fusion of the lymphocyte function-associated molecule 3 and the Fc-domain of IgG1. When LFA3TIP produced in murine NS0 myeloma cell lines was compared to the same molecule produced in CHO, a significant reduction in half-life of the molecule was observed (31). Glycan analysis indicated that the material produced in CHO cells had higher levels of sialylation. Furthermore, not only can the glycan profile of a therapeutic protein be modulated by using different expression systems, but changes in the manufacturing process can also alter sialylation levels. In the case of B cell-activating factor receptor 3 (BR3)-Fc, changes in the manufacturing process have been shown to have a direct relationship between PK and the sialic acid content of the molecule (32). BR3-Fc molecules with high sialic acid content exhibited much higher systemic exposure following IV administration, when compared to those molecules possessing lower sialylation levels or when desialylated. The rapid clearance of low sialic acid containing or desialylated BR3-Fc was attributed to the exposed galactose, which bound to the ASGPRs and was subsequently degraded in the liver in nonparenchymal cells. Comparing the glycosylation of etanercept, rhEPO, LFA3-TIP and BR3-Fc the location of the sialic acid on either N- or O-linked sites does not seem as important as its overall presence. Specifically rhEPO has up to 5 N-linked and 1 O-linked site; LFA3-Fc has 8 potential N-linked sites; while both BR3-Fc and etanercept have predominant O-linked glycosylations.

In conclusion, using the humanized *Pichia* expression platform we have generated a number of forms of etanercept with varying extents of sialylation. Assessment of these forms indicated that there was a direct correlation between *in vivo* half-life and sialic acid content. Low sialic acid content resulted in low bioavailability and short half-life, while high sialic acid content increased both bioavailability and half-life. Furthermore, to benchmark the pharmacokinetic properties of the *Pichia* produced material, it was compared to Enbrel®. The data clearly demonstrated that when the sialic acid content was similar between the *Pichia* produced material and commercial Enbrel®, that the pharmacokinetic properties were very comparable in rats, and potentially in humans. As such, this study demonstrates that the *Pichia* expression platform can be utilized to generate complex recombinant glycoproteins, with pharmacokinetic properties that are comparable to those produced by traditional commercial mammalian expression systems.

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