Insights into the Impact of Heterogeneous Glycosylation on the Pharmacokinetic Behavior of Follistatin-Fc Based Biotherapeutics

Amita Datta-Mannan, Lihua Huang, Jennifer Pereira, Benjamin Yaden, Andrew Korytko, and Johnny E. Croy

ABSTRACT

FST-ΔHBS-Fc is a follistatin (FST) based Fc fusion protein currently being developed as a novel therapy for several potential indications, including muscle wasting. Previous assessments of the pharmacokinetics and therapeutic activity of FST-ΔHBS-Fc have shown a close association of the exposure-response relationship. The current work builds upon these initial studies by investigating the glycosylation characteristics of FST-ΔHBS-Fc following recombinant expression and its impact on the pharmacokinetics in mice and Cynomolgus monkeys. The data presented indicate that FST-ΔHBS-Fc is heterogeneously glycosylated at the three putative sites in FST when recombinantly expressed in stably transfected CHO cells. Such carbohydrate heterogeneity, especially with regards to sialic acid incorporation, directly results in sugar-dependent clearance in both mice and Cynomolgus monkeys. Examination of the pharmacokinetics of FST-ΔHBS-Fc molecules containing variable sialic acid content in asialoglycoprotein receptor 1 (ASPGR-1) knockout mice support the receptor’s role as part of the clearance mechanism of the molecules. Based on the evaluation of several variably sialylated lots of material in pharmacokinetic assessments, we define specifications for average sialic acid incorporation into FST-ΔHBS-Fc that results in limited sugar-mediated clearance. Taken together, these studies highlight the importance of establishing an early understanding of the glycosylation/pharmacokinetic relationships of FST-ΔHBS-Fc which will provide a basis for future application towards optimal systemic drug delivery and dosing strategies.
INTRODUCTION

Follistatin is a regulatory glycoprotein with a myriad of biological functions in humans. (Ueno, Ling et al. 1987; Kogure, Omata et al. 1995; Kogure, Zhang et al. 1996; Fuwii, Ishikawa et al. 2005; Fumagalli, Musso et al. 2007; Kota, Handy et al. 2009) It is involved in the regulation of early fetal development and cellular differentiation, regulating follicle stimulating hormone (FSH) and luteinizing hormone (LH) release and action (Carroll, Kowash et al. 1991; Barakat, O’Connor et al. 2008; Aroua, Maugars et al. 2012), wound healing (Fumagalli, Musso et al. 2007), tissue regeneration and repair, including bone, muscle and skin (Inoue, Orimo et al. 1994; Funaba, Ogawa et al. 1996; Gajos-Michniewicz, Piastowska et al. 2010; Gajos-Michniewicz, Pawlowska et al. 2012), and limiting cancer cellular proliferation. (Ogino, Yano et al. 2008; Tumminello, Badalamenti et al. 2010; Karve, Preet et al. 2012; Ren, Chen et al. 2012; Sepporta, Tumminello et al. 2013) In all cases, these regulatory functions are conferred via its direct engagement with and potent antagonism (and in some cases agonism) of members of the TGFβ-like family of proteins. The diverse biological regulatory properties embodied by follistatin have spurred considerable interest to develop follistatin-based therapeutics to treat a wide array of potential indications. Indeed several groups have reported potent beneficial effects of follistatin administration in various indications such as, inflammation, liver repair, fibrosis, wound healing, hair regrowth and muscle disorders, including muscular dystrophy. (Kogure, Omata et al. 1995; Wankell, Munz et al. 2001; Fuwii, Ishikawa et al. 2005; Fumagalli, Musso et al. 2007; Tsuchida 2008; Zimber, Ziering et al. 2011; Datta-Mannan, Yaden et al. 2012; de Kretser, O’Hehir et al. 2012) Although, many studies indicate follistatin has the potential
to be applied to various diseases, the development of follistatin as a biotherapeutic remains a relatively unexplored frontier. This is likely due to its high degree of molecular complexity, structural properties and post-translational glycosylation which create significant challenges in optimizing the molecule’s structure-activity relationship along with its *in vivo* pharmacokinetic/pharmacodynamic (PK/PD) properties. Human follistatin is a structurally complex molecule that is comprised of 344 residues that is present in two major isoforms which result from alternative mRNA splicing: follistatin-315 (FST315) and follistatin-288 (FST288) (For a review see ref (Phillips and De Kretser 1998)). Both isoforms contain identical core regions that are formed from a series of tandemly repeated domain elements: An N-terminal domain (FST 0), followed by three successive KAZAL/EGF-like repeats units (deemed FST 1, 2 and 3) and a highly acidic C-terminal tail (Keutmann, Schneyer et al. 2004) (Figure 1). The primary sequence of follistatin is punctuated by a preponderance of 36 cysteine residues which form 18 disulfide bonds that interlink the aforementioned domain elements and that stabilize the overall protein structure. Together these various elements provide a structural scaffold which allows follistatin to interact with TGFβ-like ligands. (Thompson, Lerch et al. 2005; Cash, Rejon et al. 2009) In addition to providing a ligand binding surface, the FST 1 domain confers native heparin binding to follistatin, through the presentation of a solvent accessible heparin sulfate binding sequence (HBS) (Figure 1). (Sidis, Schneyer et al. 2005) The complexity of follistatin is further exacerbated by the presence of three putative N-linked glycosylation sites at asparagine residues in positions 95, 112 and 259 (Figure 1).
In our previous studies, we reported the first published example of the successful engineering of a FST315 variant, deemed FST-ΔHBS-Fc, which has the potential to serve as a platform for developing a parenterally administered biotherapeutic (Datta-Mannan, Yaden et al. 2012) in the treatment of diseases where muscle atrophy is implicated (Yaden, Croy et al. 2014). In these studies we fused follistatin 315 to an IgG-derived Fc and removed the protein’s intrinsic heparin binding activity to generate FST-ΔHBS-Fc. Following protein engineering, FST-ΔHBS-Fc exhibited ~1600-fold and ~90-fold improvements in exposure and half-life, respectively, in mice relative to unmodified FST315 (Datta-Mannan, Yaden et al. 2012). This molecule, in contrast to FST315, also displayed robust, dose-dependent pharmacological effects when administered subcutaneously on a weekly basis in a mouse model of muscle atrophy (Datta-Mannan, Yaden et al. 2012).

One aspect of follistatin not covered in our previous studies was the impact of N-linked glycosylation on the pharmacokinetic behavior of various FST-ΔHBS-Fc molecules. Heterogeneity in glycosylation is an important consideration when optimizing a glycoprotein for systemic therapeutic use. The main outcome of suboptimal sialylation of solvent exposed N-linked glycans in Epo or other glycoproteins (Mortensen and Huseby 1997; Bork, Horstkorte et al. 2009; Richards, Colgrave et al. 2010) leads to recognition and subsequent removal of the compound from circulation via a sugar-based interaction with a specialized subset of lectin-type receptors present on hepatic cells, called the asialoglycoprotein receptors (ASGPRs) (reviewed in (Ashwell and Harford 1982)). Given the close association of the exposure-response relationship we observed for FST-ΔHBS-Fc in our previous studies, optimization of the
pharmacokinetic properties (and by extension pharmacodynamics) of a follistatin-based therapeutic must ensure that optimal glycan character is well characterized and defined. Herein we present the influence of N-linked glycosylation on the pharmacokinetics of two FST-ΔHBS-Fc constructs (deemed MV9 and MV12). First, we found there was a strong correlation between the degree of sialylation with the clearance of the engineered FST-ΔHBS-Fc variants in both mice and Cynomolgus monkeys. Additionally, the examination of the pharmacokinetics of a subset of the FST-ΔHBS-Fc molecules in ASPGR-1 knockout mice further implicated the role of lectin-type receptors in the clearance of these molecules. Significantly, these studies highlight the molecular complexity inherent in the production of recombinant follistatin from mammalian cell culture and the dependence of in vivo pharmacokinetic properties on the extent and composition of the carbohydrates present on the molecule.
MATERIALS AND METHODS

Expression and purification of recombinant MV9 and MV12 FST-ΔHBS-Fc proteins

The MV9 and MV12 variants described within this report were expressed in stably-transfected Chinese hamster ovary cells and were generated at Eli Lilly (Indianapolis, IN, USA). The primary amino acid sequences for MV9 and MV12 are provided in the supplemental data section of this manuscript (supplemental protein sequences). Isolation of proteins from concentrated cell culture supernatants involved capture by Mab select Sepharose (GE Healthcare) affinity chromatography, followed (in certain instances) by hydrophobic interaction chromatography, and finally by preparative gel filtration chromatography. These steps generally resulted in protein purity in excess of 98%, as assessed by analytical gel filtration on a TSKG3000SWXL column.

LCMS characterization of glycosylation profiles for MV9 and MV12

LC/MS characterization of glycosylation profiles of MV9 and MV12 was conducted for immune affinity captured materials. When murine plasma samples contained at least 400 ng of either MV9 or MV12 the sample was mixed with 15 μl of magnet beads suspension immobilized anti-human IgG4 Fc antibody (4 mg antibody/10 ml the suspension) and then incubated at ambient temperature for approximately 100 min. Sample beads were then washed with 3×100 μl of 1xPBS (phosphate buffered saline) material was released by incubating with 20 μl and then 10 μl of 20 mM sodium citrate buffer, pH 3.0. Each released solution was mixed with 4 μl of 1 M tris-HCl buffer, pH 8, 0.5 ml of 50 mg/ml DTT solution and then treated with 1 μl of 0.2 mg/ml Lys-C solution at 37°C for two hours. Each digest was alkylated with 1 μl of 100 mg/ml iodoacetamide.
solution at 37°C for one hour and then mixed with 1 μl of 10% TFA in H₂O before LC/MS analysis. Each digest (approximately 5 to 10 μl) was also treated with PNGase F solution to remove N-glycosylation. Mouse plasma spiked with 1- or 20-μg/ml MV9 and MV12 was prepared and treated with the same capture and digest procedures used for the aforementioned mouse samples. For the starting materials, 20 μl aliquot of 0.5 mg/ml MV9 or MV12 solution was mixed with 80 μl of H₂O and then reduced, digested and alkylated in a similar way.

Each digest solution was analyzed by a Waters Acquity UPLC coupled to a ThermoFisher LTQ Orbitrap XL™ ETD mass spectrometer (ThermoFisher, Waltham, MA). Samples were separated on a Waters (Milford, MA) Acquity UPLC BEH C18 reversed phase column (1.0x100 mm, 1.7 μm particle size) using 0.15% formic acid (FA) in water as mobile phase A and 0.12% FA in acetonitrile as mobile phase B. The gradient program was as follows: 1% mobile phase B at a flow rate of 100 μl/min at initial and increasing to 8% B in 3.9 min, flow rate reduced to 50 μl/min in 0.1 minutes, mobile B increased to 30% in 42 minutes, mobile B increased to 90% at 1 min, at 90% and increased flow rate to 100 μL with 3 minute, back to 1% B in 0.5 min and then held at 1% B for 9.5 minutes before next injection. The eluted sample was introduced into the mass spectrometer through an ESI source operating at a positive mode with a 15000 resolution, a scan ion mass range of 250-2000, a spray voltage of 4.0 kV, a capillary temperature of 275°C, a capillary voltage of 3 V, a tube lens of 100 V and a sheath gas setting of 40 (arbitrary units).

Heparin Sepharose ELISA Binding Assay
The interaction of the MV9 and MV12 follistatin variants with heparin was measured using a plate based ELISA method. Briefly, heparin-coated binding plates (BD) were blocked in a solution of casein blocking buffer (Pierce, Rockford, IL) for one hour at room temperature. Following blocking, the plate was washed once with PBS containing 0.05% tween 20 (PBST) and then incubated with varying concentrations of FST-Fc variants in PBS for one hour at room temperature. Following incubation, plates were washed with PBST thrice in a plate washer and then incubated with a HRP-conjugated goat-polyclonal anti-human IgG in casein blocking buffer at room temperature for one hour. Plates were washed thrice and signal was developed using one-step ultra TMB-ELISA colorimetric stain, quenched in 2M H₂SO₄ and immediately read at 450nm. Subsequent data was processed using Softmax Pro 4.7 software (Molecular Devices, Sunnyvale, CA).

**SMAD Binding Element 12 (SBE12) Luciferase-based Reporter Gene Assay**

HEK293 cells stably expressing the SBE-12-luciferase system (Qiagen, Venlo, Netherlands) were seeded 50,000 to 100,000 cells/well/100 μl DMEM/F12 (Life Technologies, Carlsbad, CA) with 10% FBS into a poly-D-lysine coated 96-well plate. Following at least 16 hours incubation at 37°C, media was aspirated and replaced with 50 μl of 1% FBS-DMEM/F12. Follistatin variants were serially diluted (1:2) PBS, pH 7.4 to produce the following titration range (3000 ng/ml to 23.4 ng/ml). Each concentration was then mixed with an equal volume of 15 ng/ml of Activin A (R&D systems, Minneapolis, MN) or 45 ng/ml GDF8/myostatin (R&D systems, Minneapolis, MN and/or Eli Lilly and Company, Indianapolis, IN) and incubated at ambient temperature for 30 minutes, after which 100 μl of mixture was added to individual wells. Induction of SMAD
reporter (i.e. 100% signal) was achieved by either Activin A or GDF8/myostatin alone and negative controls (i.e. 0% background signal) was achieved by vehicle alone. Plates were incubated at 37°C for 20 hours followed by aspiration and washed once with PBS, pH 7.4. Cells in individual wells were subjected to lysis and luminescence measured using a GeniosPRO instrument (Tecan, Mannedorf, Switzerland) with substrate injection (Luciferase Reporter Gene Assay Kit, Roche, Basel, Switzerland). Values shown in the figures are representative of transfection experiments performed in triplicate. Relative luciferase units were measured and IC₅₀ curves were fitted using GraphPad Prism software (GraphPad Software, Inc, La Jolla, CA).

Murine pharmacokinetic studies

Several murine pharmacokinetic studies were conducted at a contract research organization (Covance Laboratories, Madison, WI). The conduct of the animal studies was within the established requirements of the humane animal use standards and guidelines established by Covance. Initially, prior to the conduct of N-linked glycosylation profiling, a pharmacokinetic study of FST-ΔHBS-Fc and the two FST-ΔHBS-Fc variants, MV9 and MV12, was conducted in male severe combined immunodeficient mice (SCID) (20-30 g) (Harlan, Indianapolis, IN) following a single subcutaneous dose of each molecule dissolved in PBS, pH 7.4 at a dose level of 10 mg/kg. Subsequently, following N-linked glycosylation determination, pharmacokinetic analyses for FST-ΔHBS-Fc variants characterized with 16%, 27%, 33% or 60% overall sialic acid starting content were conducted in male SCID mice (20-30 g) (Harlan, Indianapolis, IN), following a single intravenous dose via the tail vein of the variant dissolved in PBS, pH 7.4 at a dose level of 1 mg/kg. Lastly, additional
pharmacokinetic studies for FST-ΔHBS-Fc molecules containing an overall starting sialic acid content 16% and 60% were also conducted in male and female asialoglycoprotein receptor-1 (ASPGR-1) knockout mice (Jackson Laboratories, Bar Harbor, ME) mice after a single intravenous dose via the tail vein of the FST-ΔHBS-Fc variant dissolved in PBS, pH 7.4 at a dose level of 10 mg/kg. In the SCID mouse studies, blood samples were collected from three animals per treatment group per time point at 0.083 (IV only), 12 (SC only), 24, 72, 168, 252, 336 420 and 504 hours after administration. Blood samples were collected from two animals per treatment group per time point 0.083, 6, 48, 96, 168 and 336 hours post administration in the ASPGR-1 knockout mouse study. In each study, the mouse blood samples were collected by saphenous vein, tail clip, retro-orbital or cardiac puncture into tubes containing sodium EDTA as anticoagulant and processed to plasma.

*Cynomolgus monkey pharmacokinetic studies*

The Cynomolgus monkey pharmacokinetic study was conducted at a contract research organization (Covance Laboratories, Madison, WI). The conduct of the non-human primate study was within the established requirements of the humane animal use standards and guidelines established by Covance. Twelve male Cynomolgus monkeys (2- to 5-kg) were assigned to one of four study groups. Each animal received a single intravenous (IV) dose of FST-ΔHBS-Fc characterized with 16%, 27%, 33% or 60% overall starting sialic acid content dissolved in PBS, pH 7.4, at 1 mg/kg. Blood samples were collected from the femoral vein at 0.083, 1, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 288, 336, 384, 456, 528, 600 and 672 hours after
administration of the dose. Blood samples were allowed to clot at ambient temperature prior to centrifugation to obtain serum.

**Bioanalytical assays for the pharmacokinetic samples and pharmacokinetic data analysis**

Concentrations of the FST-ΔHBS-Fc variants with different overall starting sialic acid content in mouse plasma or Cynomolgus monkey serum were determined using mini-validated ELISAs for each of the compounds. Briefly, each well of an Immulon 4 microtiter plate (Thermo Electron Corporation, Waltham, MA) was coated with an anti-human follistatin IgG (R&D Systems, Minneapolis, MN) at 4°C overnight. After washing and blocking, standards for each sialic acid variant and its respective samples were added to the wells in a volume of 0.1 mL and incubated for 1 hr at room temperature. The bound FST-ΔHBS-Fc variants were detected with a HRP-conjugated mouse anti-human IgG (Southern Biotechnology Associates, Birmingham, AL). The quantitation ranges of the assays for each sialic acid variant were defined as the ability of quality control samples to demonstrate a mean recovery within a 20% coefficient of variation relative to the theoretical concentration in 20% mouse plasma or Cynomolgus monkey serum (in PBS/casein) evaluated at three concentration levels. Based on the precision and accuracy evaluated in these assays, the reportable range was 0.35 to 100 ng/mL in both Cynomolgus monkey serum and mouse plasma for each of the sialic acid variants. The lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were defined by a mean recovery within 20% of theoretical at a concentration following three freeze/thaw cycles of stability spiked samples stored at –70 °C in neat matrix for each sialic acid construct. The LLOQ and ULOQ were 0.78 ng/mL and 75
ng/mL in Cynomolgus monkey serum, respectively, for each of the sialic acid variants; whereas, the LLOQ and ULOQ in mouse plasma were 1.4 and 75 ng/mL, respectively, for all the constructs. Storage stability at –70 °C in neat matrix was established for up to 30 days for each of the variants.

Pharmacokinetic parameters were calculated using the WinNonlin Professional (Version 3.2 or 5.2) software package (Pharsight Corporation, Mountain View, CA). The pharmacokinetic parameters from the Cynomolgus monkey serum concentration-time data were calculated using a linear/log trapezoidal non-compartmental analysis model approach based on the statistical moment theory. The pharmacokinetic parameters from the murine plasma concentration-time data were calculated using a linear trapezoidal with linear interpolation non-compartmental analysis model approach which pools the data and allows the use of all the information from the sparse sampling design leveraged for the murine studies. The parameters calculated included the maximum serum concentration ($C_{\text{max}}$), area under the curve ($\text{AUC}_{0-\infty}$), clearance (CL), and elimination half-life ($t_{1/2}$) when deemed appropriate to report. Statistical significance between mean clearance and half-life values was evaluated by performing a pairwise Tukey-Kramer analysis and differences were deemed as statistically significant if p-values were less than 0.05.
RESULTS

In vitro binding properties and pharmacokinetics of the MV9 and MV12 in SCID mice

In our previously published work, we showed that fusion of FST315 to a Fc fragment of IgG and removal of its intrinsic heparin sulfate binding activity resulted in a PK/PD profile that supported parenteral delivery. (Datta-Mannan, Yaden et al. 2012) Though these changes were sufficient to optimize the PK/PD relationship, several of the other molecular characteristics of FST-ΔHBS-Fc, such as poor yields following protein purification, were found to be incompatible with continued for therapeutic development (data not shown). Subsequent engineering efforts however yielded two additional FST-ΔHBS-Fc variants, MV9 and MV12 for which these undesired properties had been resolved (data not shown). The heparin binding properties and in vitro SMAD 2/3 reporter gene assay results for both the MV9 and MV12 variants were found comparable within the limits of the assay to the parental FST-ΔHBS-Fc (Supplemental Figure 1 and Table 1, respectively).

In addition, we also performed a pharmacokinetic assessment of these two molecules in SCID mice. The average concentration-time profiles for MV9 or MV12 following a single 10 mg/kg subcutaneous administration are shown in Figure 2. Both molecules displayed a rapid distribution phase followed by a longer elimination phase characteristic of Fc fusion proteins. When compared to each other, MV12 exhibited an approximate 2-fold higher exposure (AUC) than MV9 (Table 1).
Liquid chromatography mass spectrometry (LCMS) based characterization of glycosylation properties in MV9 and MV12

Given the large degree of carbohydrate predicted to be present in MV9 and MV12, we hypothesized that the pharmacokinetic differences observed in between the variants (Figure 2) may in part be driven by differences in their overall glycosylation attributes. To better understand how glycan heterogeneity influenced the pharmacokinetic properties of MV9 and MV12, we performed an extensive LCMS-based characterization of the N-linked glycosylation profiles of the starting materials used in the aforementioned murine pharmacokinetic study. In the first experiment, we examined the sugar occupancies in MV9 and MV12 at each of the predicted sites based on primary acid amino acid sequence of the variants (Figure 1). The MS analyses indicated that each of the predicted FST N-linked sites (N95, N112, N295), as well as, the Fc site (N398) in both MV9 and MV12 contained varying carbohydrate occupancies (Supplemental Figure 2). Although varied in occupancy each N-linked site in MV9 and MV12 was observed to exhibit a unique preference for a specific carbohydrate antennary structure that was conserved across variants (Supplemental Figure 3A). Taken together these results suggest that the gross carbohydrate antennary structural preferences of the FST-ΔHBS-Fc variants are likely governed in a site-specific manner; perhaps by primary amino acid sequence and/or tertiary structures surrounding the N-linked attachment sites.

We next used MS to examine the detailed character of the carbohydrate termini present at each N-linked site within MV9 and MV12. More specifically, we calculated the ratios of average sialic acid, galactose and N-acetylglucosamine/mannose to...
oligosaccharide present in the starting materials used in the aforementioned mouse pharmacokinetic study. Examination of the N-linked glycans present in the FST region of both proteins showed that the degree of sialylation was greater in MV12, relative to MV9, resulting in an overall average of 46% more sialic acid per oligosaccharide in MV12 (Figure 3). It was also noted that in both variants the degree of sialylation at N398 was comparable and low (data not shown). Closer analysis of the asialylated species in both MV9 and MV12 showed that carbohydrate termini preferentially terminated in galactose, (Supplemental Figure 4A) followed by N-acetylglucosamine/mannose (Supplemental Figure 4B). Given the differences observed in these two proteins, we wanted to understand the impact of lot to lot variation on the glycosylation properties of FST-Fc and therefore characterized by LCMS the overall average sialylation percentage of 12 independently produced lots of MV9 and MV12 using a fixed cell culture and purification process. Unlike the conservation noted in the average antennary structures (Supplemental Figure 3A) of these molecules, the degree of site-specific sialylation (Supplemental Figure 3B) was observed to be highly varied in our expression system. These data suggest that the differential sialylation properties of MV9 and MV12 were most likely the result of lot to lot variability in their overall glycosylation properties and not the result of the differences in their overall chemical structures.

**Correlation of pharmacokinetic properties of MV9 and MV12 with specific changes in glycosylation attributes**

Given the pharmacokinetic differences highlighted in Figure 2 and Table 1 and the noted differences in the terminal structure of carbohydrate found between MV9 and
MV12, we next leveraged our MS assay to determine whether clearance rates of the two proteins could be correlated to starting average sialylation content. For this study, we collected samples of each protein from murine serum at various time points and characterized the carbohydrate structures that were present in each variant which exhibited extended circulating half-life. In an effort to address whether any bias associated with the sample extraction process prior to the MS analyses, we compared the average molar ratios of each terminal carbohydrate species to: (1) the molar amount of oligosaccharide present in the starting unprocessed samples; and (2) that of starting materials spiked into sera and then extracted. Analysis of these samples indicates that the extraction methodology did not significantly alter these ratios (data not shown). The glycosylation properties as revealed by the MS analysis of subcutaneously injected MV9 and MV12 materials recovered at 0 and 336 hours are described in Figure 4. The results show significant changes in the relative ratios of each specific terminus evaluated for both MV9 and MV12. Kinetically, these changes were punctuated by a rapid-compositional change which occurred prior to first time point measured in our study (T=12hrs), after which a slower rate of change was observed (Supplemental Figure 5A-F); this was found to be independent of the variant and N-linked site evaluated. Compositionally, we found that each FST N-linked sites in MV9 (Figure 4A) and MV12 (Figure 4B) exhibited an increased proportion of sialylated carbohydrates as circulation time progressed. Conversely, the proportion of terminal galactose and/or N-acetylglucosamine/mannose measured, decreased as a function of prolonged in vivo exposure for both variants. These changes were not limited to a specific N-linked site; as similar compositional changes with time were observed independent of attachment
Overall, a comparative analysis of the degree of change in starting vs. terminal circulating sialylated species was ~50% greater in MV9, relative to MV12, which likely reflects the preferential clearance of poorly sialylated species during prolonged circulation. One final observation was that though carbohydrates present at the Fc-position N398 were found to be largely asialylated (Supplemental Figure 2), these species were not altered to an appreciable amount during extended circulation in vivo (data not shown). This finding is consistent with other published studies on antibody-therapeutic fusions. (Jefferis 2005).

Pharmacokinetics of MV9 and MV12 sialic acid variants in SCID mice and Cynomolgus monkeys

Given the connection between the starting material's sialic acid content and the effect on its pharmacokinetic profile, we further characterized the impact of variable sialic acid content in the FST-ΔHBS-Fc variants by expressing and purifying several independent lots of MV9 and MV12, each containing differential overall average sialic acid content (quantified by LCMS analysis; Table 2). The pharmacokinetics of the MV9 molecules with varying average sialic acid content were evaluated in both SCID mice and Cynomolgus monkeys; the MV12 proteins were only examined in SCID mice. The results of these studies showed a biphasic clearance profile characterized by a rapid distribution phase followed by a longer elimination phase; consistent with the mouse studies presented above (Figure 2 and Figure 5). The average concentration versus time profiles for each MV9 and MV12 variant in SCID mice and/or Cynomolgus monkeys are shown in Figures 5 and 6, respectively. When compared to each other, the kinetic profiles of the sialic acid content variants displayed differences in both SCID...
mice and Cynomolgus monkeys. The MV9 and MV12 sialic acid variants showed mean clearance values ranging from 0.64 to 2.01 mL/h/kg in SCID mice (Table 2); whereas, in Cynomolgus monkeys, the MV9 sialic acid variants had mean clearance values ranging from 0.95 to 7.47 mL/h/kg (Table 3). Additionally, no anti-drug antibodies were detected following administration of any of the variants to Cynomolgus monkey (data not shown); anti-drug antibodies were not measured in the mouse studies given the compromised immune system background of SCID mice.

The sialic acid variants display a pattern of slowed mean clearance (CL) with increasing sialic acid content in both species (Figures 5 and 6; Tables 2 and 3). Pairwise statistical analyses of the monkey data showed statistically significant clearance differences (p value <0.05) between the following pairs: 16% and 60% variants; 16% and 33% variants; and 16% and 27% variants. Though statistically significant differences in clearance were not observed for any other pairs in monkey, the data suggests a trend for further decreasing clearance with increased average sialic acid content up to 60% (highest tested). Similar trends were observed in the murine data, but due to the nature of the sparse sampling design, no statistical analyses of the murine pharmacokinetic data were performed.

The influence of the overall average sialic acid content differences of the MV9 and MV12 variant’s elimination half-life values in SCID mice and Cynomolgus monkeys was more marginal than the effects on clearance (Tables 2 and 3). In SCID mice and Cynomolgus monkeys, the half-lives of the variants ranged from ~200 to ~300 hours and ~100 to ~200 hours, respectfully. Pairwise statistical analyses showed no statistically significant differences in half-life between the variants in monkeys. Analysis
of the relationship of the average sialic acid content with the in vivo clearance and half-life for the FST-\(\Delta\)HBS-Fc variants in both SCID mice and/or Cynomolgus monkeys show there is a reasonably direct correlation with clearance but not elimination half-life (Figure 7A-D).

**Pharmacokinetics of the MV9 sialic acid variants in ASPGR-1 Knockout Mice**

To determine whether the clearance mechanism of the FST-\(\Delta\)HBS-Fc molecules was due to interaction with a specialized subset of lectin-type receptors present on hepatic cells, we evaluated the pharmacokinetics of a pair of MV9 variants (16% and 60% average sialic acid content) in an APGR-1 knockout mouse model. The average concentration-time profiles for each MV9 variants in the ASPGR-1 knockout mice are shown in Figure 8 and Table 4. The MV9 16% and 60% average sialic acid variants showed mean clearance values of 2.3 and 1.4 mL/h/kg, respectively, in ASPGR-1 knockout mice. The elimination half-life for the two variants was not quantified due to the sparse sampling in the terminal phase of the pharmacokinetic profile.
DISCUSSION

Our previous studies investigated the PK/PD responses of various follistatin-based therapeutics for which a close relationship between these important parameters was revealed. (Datta-Mannan, Yaden et al. 2012) One critical engineering step toward developing a systemically-active follistatin-based therapeutic was achieved by fusing a heparin-binding deficient form of FST344 to the Fc of a murine and/or human IgG. (Datta-Mannan, Yaden et al. 2012) Further investigation of two FST-ΔHBS-Fc variants from this platform is disclosed in this report. Specifically, this report analyses the change in glycosylation circulation profiles of the MV9 and MV12 variants using LCMS analyses of materials collected from a head-to-head pharmacokinetic study in SCID mice (Figure 2). In these studies we found, MV12 exhibited an approximate 2-fold slower clearance relative to that of MV9 (Table 1). More striking was the fact that both molecules showed a lower than anticipated exposure (AUC) for an Fc fusion protein (Czajkowsky, Hu et al. 2012). Possible mechanisms that could account for the clearance profile differences between the therapeutic-Fcs include: (1) heparin binding, (2) target mediated clearance, (3) FcRn interactions and/or (4) anti-drug immune response. Given that both MV9 and MV12 displayed no heparin binding in vitro (Supplemental Figure 1) and that the predicted targets for follistatin (ie. TGF\(\beta\)-like family members) have low circulating levels in mice ((Wu, Chen et al. 2012) and unpublished data) it was unlikely that either heparin binding in vivo or binding to target were clearance mechanisms which could explain the pharmacokinetic differences observed. Moreover, formation of anti-drug antibodies was also unlikely to account for the observed clearance differences as these pharmacokinetic studies were performed in...
immune-compromised mice. Finally, we explored whether FcRn binding properties of these variants was involved in their rapid clearance. It is well established that the improved half-life of Fc fusion proteins is, in part, attributable to interactions of the Fc moiety with the neonatal Fc receptor (FcRn), which salvages the proteins from intracellular degradation and promotes recycling of the Fc fusion proteins back into circulation (for a review see ref (Czajkowsky, Hu et al. 2012)). Thus, it was also possible that decreased in FcRn binding properties of these variants was involved in their rapid clearance; however, comparison of the FcRn binding interactions of MV9 and MV12 showed no discernable differences (data not shown). In summary, our initial mouse pharmacokinetic data (Figure 2 and Table 1) suggested that some other biophysical (i.e. stability, charge distribution, aggregation potential, solubility) or biochemical (post-translation modifications, non-specific binding properties) dissimilarity between the variants were likely influencing their pharmacokinetics. As such we sought to better understand if glycosylation could explain the differential pharmacokinetics.

One aspect that has not been characterized in the literature is the impact of the glycosylation on the pharmacokinetic properties of follistatin-based therapeutics, either in a natively glycosylated or recombinant context. Given that native FST344 is predicted (based on primary amino acid sequence) to contain up to three N-linked glycosylation sites and in the context of the Fc platform this burden is increased to a total of eight putative sites (six in FST and two in the Fc region), we sought to better understand if glycosylation heterogeneity could explain the differential pharmacokinetic profiles of MV9 and MV12. Using a LCMS approach initially reported by (Huang, Biolsi
et al. 2005) we found that each of the putative sites in the FST regions of MV9 and MV12 were indeed occupied to varying degree with carbohydrates of heterogeneous structural character. These findings are consistent with a previous report by Hyuga et al which evaluated the glycosylation properties of recombinantly expressed and CHO-derived FST288 and FST344. (Hyuga, Itoh et al. 2004) However, one notable difference from these studies observed in our data was the observation that the non-canonical position N112, was found to be variably occupied (Supplemental Figure 2, specifically peptide 109-118).

The subsequent comparative analysis of MV9 and MV12 samples extracted from serum following prolonged in vivo circulation revealed that molecules containing carbohydrate not terminating in sialic acid were rapidly removed from circulation (Supplemental Figure 5A-F). Furthermore, a site-specific comparison of the changes in distribution of carbohydrate structure in MV9 and MV12 revealed that no single site contributed more to the clearance of poorly sialylated species; rather the rapid clearance could be attributed to changes in the average global levels of asialylated carbohydrate as a whole. This is an important distinction as it indicates that engineering to remove individual glycosylation sites would not serve to reduce the observed poor pharmacokinetic properties of FST-ΔHBS-Fc compounds. Rather, the data contends that monitoring of average sialic acid content of carbohydrates is sufficient to inform on the potential influences on the pharmacokinetic profile. To support this hypothesis, we expressed and purified and characterized the average sialic acid content of several lots of MV9 and MV12 to determine the impact of sialylation on the pharmacokinetic behavior of FST-ΔHBS-Fc compounds. Indeed, our studies of these differentially
sialylated proteins corroborated our hypothesis on the role of terminal glycosylation in affecting the pharmacokinetics of these molecules (Figures 5 and 6). In mice, both MV9 and MV12 variants containing increased initial sialic acid content were cleared more slowly from mice than variant molecules containing lower sialic acid content (Figure 5 and Table 2). We also found MV9 and MV12 sialic acid variants displayed similar kinetics \textit{in vivo} when the sialic acid content was similar. These data indicate the residue differences across MV9 and MV12 within the HBS to reduce heparin binding impacted pharmacokinetics similarly. Thus, in pharmacokinetic experiments reported herein, the exposure differences between the molecules were predominantly driven by dissimilarities in glycosylation (sialylation) and not primary amino acid differences. It is worth noting, that the examination of the pharmacokinetics of a subset of MV9 sialic acid variants in Cynomolgus monkeys showed findings similar to that in mice with clearance values increasing for constructs with lower initial sialic acid content (Figure 6 and Table 3). Additionally, within both species, the elimination half-life was similar across the sialic acid variants (Tables 2 and 3). These findings can be explained by the enrichment of optimally sialylated constructs in each population over time; thus, it is important to understand that although characterization of the average overall sialic acid content of the starting material is a useful and pragmatic approach to estimate clearance properties, each lot of protein is still heterogeneous and contains molecules with variable sialylation.

The negative impact of suboptimal sialylation on the pharmacokinetic behavior of FST-$\Delta$HBS-Fc molecules is a theme that has been explored in other glycotherapeutics and is an important consideration for future development of systemically acting
follistatin-based therapies. Specifically, several studies exploring the clearance attributes (Webster, Phipps et al. 2003; Webster, Edgington et al. 2006) of UK-279,276, a glycosylated recombinant neutrophil inhibitor factor, has strongly implicated a potential clearance mechanism that involves the binding and subsequent removal of poorly sialylated species via an avidity-driven interaction with specialized hepatic lectins, called the asialoglycoprotein receptors (ASPGR). (Weiss and Ashwell 1989; Spiess 1990; Stockert 1995) Based on the similarities in our findings with those reported for UK-279,276, we performed a limited pharmacokinetic assessment of two MV9 sialic variants containing either a low (~16%) or high (~60%) average sialic acid content in ASPGR-1 knockout mice to provide a mechanistic explanation of our findings (Figure 8 and Table 4). Indeed, in SCID mice and Cynomolgus monkeys the ~60% sialic acid variant exhibited an ~3.1-fold and ~7.5-fold slower clearance relative to the ~16% sialic acid variant, respectively; this difference in clearance was reduced to ~1.6-fold in the ASPGR-1 knockout mice. The reduced clearance difference between the variants in the knockout mice relative to SCID mice supports the notion of ASPGR as a route of clearance for FST-ΔHBS-Fc. However, it is important to note that because we used a higher dose in our pharmacokinetic evaluations of the variants in knockout mice (10 mg/kg) relative to the SCID mice (1 mg/kg), that this may have influenced our exposure findings. Therefore, a deeper understanding of exposure linearity across dose levels within the mice is needed to more fully interrogate the role of ASPGR-1 in the clearance of our molecules. It is also possible that other underdetermined attributes (i.e. charge distribution, aggregation potential, hydrophobicity and solubility differences) may influence the pharmacokinetic behavior of these molecules as has been reported.
recently for other biologics. Interestingly, we also noted for reasons unknown to us, the effect of the variable sialylation on the clearance was more pronounced in monkeys than in mice for the MV9 constructs (compare Figure 5 with Figure 6 and compare Table 2 with Table 3). The findings were not related to anti-drug antibodies affecting the clearance of the molecules in Cynomolgus monkeys (data not shown). However, the observations may be due to the ASPGR mechanism playing a greater role in the higher species relative to the rodent. It’s also possible that the physiochemical properties of the molecules lead to some differences in non-specific or unintended binding across the species, which was not assessed within the context of our current efforts. Further studies are required to determine these underlying aspects of the species differences in FST-ΔHBS-Fc pharmacokinetic behavior.

In summary, our previous (Datta-Mannan, Yaden et al. 2012) and current studies highlight the exquisite importance of understanding the interplay between heparin binding and glycosylation on the overall PK properties of follistatin-based therapies. The current studies in this report indicate that the residue differences between MV9 and MV12 within the HBS to reduce heparin binding impacted pharmacokinetics similarly and the observed PK differences between the two molecular platforms was predominantly driven by differences in glycosylation (sialylation) and not primary amino acid differences. Furthermore, these studies serve to illustrate the extreme degree of molecular complexity inherent in the production of recombinant follistatin from mammalian cell culture, and its intimate association with in vivo pharmacokinetics and by extension pharmacodynamics. Our findings strongly support the importance of careful and thorough glycosylation characterization of these recombinant proteins for a
more rich understanding of *in vivo* pharmacology data. Studies to fully determine the implications of the glycosylation profile on the PK/PD relationship are warranted to help guide the selection of the optimal sialylation profile. In addition, future studies should also be considered for thorough characterization of additional biophysical properties such as the distribution of charge and hydrophobic patches with solvent exposed region for determining attributes in follistatin-based glycotherapeutics that inform optimal systemic drug delivery and dosing strategies for the intended pharmacological application.
ACKNOWLEDGEMENTS

Amber Peariso, Victor Wroblewski and John Beals for thoughtful discussions on experimental design. Pat Eacho and Krista Schroeder for aid in performing the ASPGR-1 knockout mouse studies. Joe Berry, Joe Swartling and Jerry Kelly for expression and purification support. Robin Brown, Selina Estwick, Emmanuel Lozano and Andrea Sperry for conducting pharmacokinetic sample analyses. Stacey Lee for conducting heparin binding assays. Xuhao Yang and Yan Wang for performing SMAD binding assays. Tammy Tuley for LCMS analysis support on glycan characterization of various follistatin preparations. Carrie Croy for evaluation of editorial advice on the manuscript.
AUTHOR CONTRIBUTIONS

Participated in Research Design: AD-M, LH, BY, AK and JEC
Conducted Experiments: AD-M, LH, BY, AK, JP and JEC
Contributed New Reagents or Analytical Tools: AK, JP and JEC
Performed Data Analysis: AD-M, LH and JEC
Wrote or Contributed to the Writing of the Manuscript: AD-M, LH, BY, AK, JP and JEC
REFERENCES

Aroua, S., Maugars, G. et al. (2012). Pituitary gonadotropins FSH and LH are oppositely regulated by the activin/follistatin system in a basal teleost, the eel. Gen Comp Endocrinol 175(1): 82-91.


Mortensen, B. and N. E. Huseby (1997). Clearance of circulating gamma-glutamyltransferase by the asialoglycoprotein receptor. Enzyme forms with different


FIGURE LEGENDS

**Figure 1:** Domain architecture and location of post-translationally modified N-linked glycosylation sites in FST-ΔHBS-Fc and associated variants. N-linked glycosylation sites are designated by solid triangles with corresponding amino acid sites. The native heparin sulfate-binding site (HBS) in FST315 is located in the FST1 domain and comprises a linear stretch of amino acids 75–84. (Sidis, Schneyer et al. 2005)

**Figure 2:** The pharmacokinetics of FST-Fc and the two FST-ΔHBS-Fc variants MV9 and MV12 in SCID mice following a single 10 mg/kg subcutaneous administration of each protein. Plasma concentrations were determined using a validated ELISA for each molecule. Data represent the mean of three animals/time point for each molecule.

**Figure 3:** Comparison of the average molar ratio of carbohydrates terminating in sialic acid in the single lots of MV9 (grey bars) and MV12 (black bars). Data is represented both on a site-to-site comparison and overall global average of the three sites in the FST region of each variant. Inclusion of error bars would be undiscernible due to robustness in triplicate measurement variability and for this reason are not included.

**Figure 4:** Comparative summary of the concentrations of specific carbohydrate species terminating in defined structures in FST-ΔHBS-Fc variants MV9 and MV12 following extended in vivo circulation. Individual columns represent the molar ratio of each identified termini (sialic acid (open circles), galactose (open diamonds) and N-acetylglucosamine (open squares) of samples extracted from serum samples taken
from the SCID pharmacokinetic study. Data shown is specifically for position N112 and is representative of other sites not shown for (A) MV9 and (B) MV12.

**Figure 5:** The pharmacokinetics of the two FST-ΔHBS-Fc initial sialic acid (SA) content variants (A) MV9 and (B) MV12 in SCID mice following a single 1 mg/kg intravenous administration of each protein. Plasma concentrations were determined using a validated ELISA for each molecule. Data are the mean (+/- SD) of three animals/time point for each molecule.

**Figure 6:** The pharmacokinetics of MV9 variants with a range of initial sialic acid (SA) content in Cynomolgus monkey following a single 1 mg/kg intravenous administration of each protein. Plasma concentrations were determined using a validated ELISA for each molecule. Data are the mean (+/- SD) of three animals/time point/group for each molecule.

**Figure 7:** Relationship between the pharmacokinetic parameters (clearance and half-life) and the MV9 (open squares) and MV12 (open diamonds) initial sialic acid content variants in (A and B) SCID mice and (C and D) Cynomolgus monkeys following a single 1 mg/kg intravenous administration of each protein in both species. The mean (+/-) SD for the pharmacokinetic parameters is shown for monkey. Pairwise Tukey-Kramer analysis of mean clearance values from Cynomologous monkeys (N=3) indicate that only MV9 16% SA variant was significantly different from other SA variants tested (p<0.05). Conversely, a pairwise Tukey-Kramer analysis of the same SA variants did not indicate any significant difference in half-life values (p>0.05).
Figure 8. The pharmacokinetics of two MV9 variants with either 16% or 60% average sialic acid (SA) content in ASPGR-1 knockout mice following a single 10 mg/kg intravenous administration of each protein. Plasma concentrations were determined using a validated ELISA for each molecule. Data are the mean of two animals/time point for each molecule.
Table 1

Mean pharmacokinetic parameters FST-Fc, MV9 and MV12 in SCID mice after a single 10 mg/kg subcutaneous administration a,b,c

<table>
<thead>
<tr>
<th>Protein</th>
<th>$C_{\text{max}}$ ($\mu$g/mL)</th>
<th>$T_{\text{max}}$ (hr)</th>
<th>$\text{AUC}_{0-\text{last}}$ ($\mu$g•hr/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FST-Fc</td>
<td>11.3 (0.7)</td>
<td>24</td>
<td>1092 (38)</td>
</tr>
<tr>
<td>MV9</td>
<td>10.7 (1.2)</td>
<td>24</td>
<td>1347 (57)</td>
</tr>
<tr>
<td>MV12</td>
<td>36.5 (4.3)</td>
<td>12</td>
<td>2940 (77)</td>
</tr>
</tbody>
</table>

a Plasma concentrations determined using validated antigen capture ELISAs.
b Determined from non-compartmental pharmacokinetic analyses.
c Values in parentheses represent the standard error of the mean as calculated by the sparse sampling method used to extract pharmacokinetic parameters.

$C_{\text{max}}$, maximal observed serum concentration; $T_{\text{max}}$, time at which maximal serum concentration was observed; $\text{AUC}_{0-\text{last}}$, area under the serum concentration curve from zero to last concentration versus time point.
Table 2

Pharmacokinetic parameters FST-ΔHBS-Fc sialic acid variants in SCID mice after a single 1 mg/kg intravenous administration a,b,c,d,e.

<table>
<thead>
<tr>
<th>(FST-ΔHBS-Fc Variant)</th>
<th>$C_{\text{max}}$ (μg/mL)</th>
<th>AUC$_{0\text{-last}}$ (μg·hr/mL)</th>
<th>CL (mL/hr/kg)</th>
<th>Vss (mL/kg)</th>
<th>$t_{1/2}$ (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV9 16% SA</td>
<td>7.9 (2.1)</td>
<td>400 (23)</td>
<td>2.01</td>
<td>570</td>
<td>277</td>
</tr>
<tr>
<td>MV9 27% SA</td>
<td>12.1 (0.3)</td>
<td>546 (15)</td>
<td>1.59</td>
<td>346</td>
<td>200</td>
</tr>
<tr>
<td>MV9 33% SA</td>
<td>13.9 (1.2)</td>
<td>681 (19)</td>
<td>1.19</td>
<td>326</td>
<td>247</td>
</tr>
<tr>
<td>MV9 60% SA</td>
<td>20.3 (0.7)</td>
<td>1304 (24)</td>
<td>0.64</td>
<td>162</td>
<td>181</td>
</tr>
<tr>
<td>MV12 36% SA</td>
<td>12.5 (0.7)</td>
<td>567 (13)</td>
<td>1.49</td>
<td>350</td>
<td>194</td>
</tr>
<tr>
<td>MV12 70% SA</td>
<td>17.7 (2.5)</td>
<td>979 (69)</td>
<td>0.75</td>
<td>258</td>
<td>289</td>
</tr>
</tbody>
</table>

a Plasma concentrations determined using validated antigen capture ELISAs.

b Determined from a linear trapezoidal with linear interpolation non-compartmental analysis model.

c Values in parentheses represent the standard error of the mean as calculated by the sparse sampling analyses.

$C_{\text{max}}$, maximal observed serum concentration; AUC$_{0\text{-last}}$, area under the serum concentration curve from zero to last concentration vs. time point; CL, clearance; Vss, volume of distribution at steady state; $t_{1/2}$, elimination half-life.
Table 3

Pharmacokinetic parameters for the FST-ΔHBS-Fc sialic acid variants in Cynomolgus monkeys after a single 1 mg/kg intravenous administration a,b,c,d,e.

<table>
<thead>
<tr>
<th>FST-ΔHBS-Fc Variant</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (μg/mL)</th>
<th>AUC&lt;sub&gt;0-last&lt;/sub&gt; (μg·hr/mL)</th>
<th>CL (mL/h/kg)</th>
<th>Vss (mL/kg)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV9 16% SA</td>
<td>19.2 ± 1.0</td>
<td>139 ± 34</td>
<td>7.5 ± 2.2</td>
<td>962 ± 113</td>
<td>178 ± 51</td>
</tr>
<tr>
<td>MV9 27% SA</td>
<td>16.5 ± 6.7</td>
<td>324 ± 32</td>
<td>3.0 ± 0.4</td>
<td>481 ± 97</td>
<td>188 ± 74</td>
</tr>
<tr>
<td>MV9 33% SA</td>
<td>21.4 ± 1.8</td>
<td>347 ± 62</td>
<td>2.9 ± 0.7</td>
<td>402 ± 169</td>
<td>141 ± 64</td>
</tr>
<tr>
<td>MV9 60% SA</td>
<td>43.9 ± 5.0</td>
<td>1070 ± 174</td>
<td>1.0 ± 0.2</td>
<td>51 ± 0.1</td>
<td>98 ± 38</td>
</tr>
</tbody>
</table>

a Serum concentrations determined using validated antigen capture ELISAs.

b Data are the mean ± SD of the pharmacokinetic parameters determined from three monkeys per group.

c Determined from a linear/log trapezoidal non-compartmental analysis model approach based on the statistical moment theory.

d Pairwise Tukey-Kramer analysis of mean clearance values indicate that only MV9 16% SA variant was significantly different from other variants tested (p<0.05).

e Pairwise Tukey-Kramer analysis of mean half-life values indicate no significant difference in any of the variants tested (p>0.05).

C<sub>max</sub>, maximal observed serum concentration; AUC<sub>0-last</sub>, area under the serum concentration curve from zero to last concentration vs. time point; CL, clearance; Vss, volume of distribution at steady state; t<sub>1/2</sub>, elimination half-life.
Table 4

Pharmacokinetic parameters FST-ΔHBS-Fc sialic acid variants in ASPGR-1 knockout mice after a single 10 mg/kg intravenous administration a,b,c.

<table>
<thead>
<tr>
<th>FST-ΔHBS-Fc Variant</th>
<th>$C_{\text{max}}$ (μg/mL)</th>
<th>$\text{AUC}_{0-\text{last}}$ (μg•hr/mL)</th>
<th>$\text{AUC}_{0-\text{inf}}$ (μg•hr/mL)</th>
<th>CL (mL/hr/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV9 16% SA</td>
<td>98 (23)</td>
<td>3369 (298)</td>
<td>4343</td>
<td>2.3</td>
</tr>
<tr>
<td>MV9 60% SA</td>
<td>136 (16)</td>
<td>7131 (577)</td>
<td>7248</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*a Plasma concentrations determined using validated antigen capture ELISAs.

*b Determined from a linear trapezoidal with linear interpolation non-compartmental analysis model.

*c Values in parentheses represent the standard error of the mean as calculated by the sparse sampling analyses.

$C_{\text{max}}$, maximal observed serum concentration; $\text{AUC}_{0-\text{last}}$, area under the serum concentration curve from zero to last concentration versus time point; $\text{AUC}_{0-\text{inf}}$, area under the serum concentration curve from time zero extrapolated to infinite time; CL, clearance.
Figure 2: Graph showing the immunoreactivity (ng/ml) over time (hr) for MV12, FST-ΔHBS-Fc, and MV9. The x-axis represents time in hours (0 to 360), and the y-axis represents immunoreactivity in ng/ml. The graph illustrates a decrease in immunoreactivity over time for all three samples.
Figure 3

Average Sialic Acid Content (Mole/mole oligosaccharide)

- N95
- N112
- N259
- Average

Comparison between MV9 and MV12.
### Figure 4

#### A.

<table>
<thead>
<tr>
<th></th>
<th>Sialic Acid</th>
<th>Galactose</th>
<th>GlcNac/Man</th>
</tr>
</thead>
<tbody>
<tr>
<td>N95 T=0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N112 T=0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N259 T=0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N95 T=336hr</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N112 T=336hr</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N259 T=336hr</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

#### B.

<table>
<thead>
<tr>
<th></th>
<th>Sialic Acid</th>
<th>Galactose</th>
<th>GlcNac/Man</th>
</tr>
</thead>
<tbody>
<tr>
<td>N95 T=0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N112 T=0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N259 T=0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N95 T=336hr</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N112 T=336hr</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N259 T=336hr</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 6

Immunoreactivity (ng/ml) vs. Time (hr) for MV9 - 33% SA, MV9 - 16% SA, MV9 - 27% SA, and MV9 - 60% SA.
Figure 7

A. Clearance (ml/hr/kg)

B. Half Life (hr)

C. Clearance (ml/hr/kg) with *p<0.05

D. Half Life (hr)