Research Article

Pharmacokinetics of Anti-hepcidin Monoclonal Antibody Ab 12B9m and Hepcidin in Cynomolgus Monkeys

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Received 15 March 2010; accepted 9 July 2010; published online 25 August 2010

Abstract. Hepcidin is a key regulator responsible for systemic iron homeostasis. A semi-mechanistic PK model for hepcidin and a fully human anti-hepcidin monoclonal antibody (Ab 12B9m) was developed to describe their total (free + bound) serum concentration-time data after single and multiple weekly intravenous or subcutaneous doses of Ab 12B9m. The model was based on target mediated drug disposition and the IgG–FcRn interaction concepts published previously. Both total Ab 12B9m and total hepcidin exhibited nonlinear kinetics due to saturable Fc–FcRn interaction. Ab 12B9m showed a limited volume of distribution and negligible linear elimination from serum. The nonlinear elimination of Ab 12B9m was attributed to the endosomal degradation of Ab 12B9m that was not bound to the FcRn receptor. The terminal half-life, assumed to be the same for free and total serum Ab 12B9m, was estimated to be 16.5 days. The subcutaneous absorption of Ab 12B9m was described with a first-order absorption rate constant k_a of 0.0278 h^{-1} , with 86% bioavailability. The model suggested a rapid hepcidin clearance of approximately $800\text{ mL h}^{-1}\text{ kg}^{-1}$. Only the highest-tested Ab 12B9m dose of $300\text{ mg kg}^{-1}\text{ week}^{-1}$ was able to maintain free hepcidin level below the baseline during the dosing intervals. Free Ab 12B9m and free hepcidin concentrations were simulated, and their PK profiles were nonlinear as affected by their binding to each other. Additionally, the total amount of FcRn receptor involved in Ab 12B9m recycling at a given time was calculated empirically, and the temporal changes in the free FcRn levels upon Ab 12B9m administration were inferred.

KEY WORDS: FcRn; hepcidin; modeling; monkey; pharmacokinetics.

INTRODUCTION

Iron homeostasis in vertebrates is dominated by the lack of an excretory route for excess iron. Serum iron level is regulated by the rate of iron entry through the duodenal mucosa, which affects net iron absorption, and by the rate of iron release from macrophages recycling iron from aged or damaged erythrocytes (1,2). Export of iron from duodenal enterocytes and macrophages into plasma is regulated by the plasma membrane transporter ferroportin (3,4). Hepcidin, a hormone peptide of 25 amino acids synthesized by the liver, binds to ferroportin, causes ferroportin internalization and degradation, and thereby blocks the iron export (5,6). The presence of hepcidin in urine (7,8) suggests hepcidin elimination by the kidney.

Hepcidin production is increased in response to high circulating iron levels (9). Increased hepcidin levels decrease

iron release from intestinal enterocytes and iron-storage cells (e.g., macrophages) by reducing ferroportin expression on these target cells. This leads to decreased circulating iron levels, which in turn remove the stimulus for further hepcidin production. When the hepcidin level falls, the ferroportin level recovers, resulting in increased iron availability in circulation. Hepcidin is therefore the key regulator responsible for systemic iron homeostasis (10) and has been suggested to be a strategic target for iron regulation in the treatment of various iron disorders such as hyporesponsiveness to erythropoietin (11–13).

Ab 12B9m is a fully human immunoglobulin G subtype 2 (IgG₂) monoclonal antibody that binds to monkey and human hepcidin with similar affinities ($K_d \sim 1\text{ pM}$). In cynomolgus monkey studies, a significant total hepcidin accumulation was observed, suggesting a fast turnover rate for free hepcidin and/or limited renal elimination of the Ab 12B9m–hepcidin complex as compared with free hepcidin. Although the role of hepcidin in iron regulation has been elucidated in recent years, quantitative information regarding hepcidin production, elimination, and turnover rate has been lacking. In this paper, total concentrations of Ab 12B9m and hepcidin obtained after single and multiple intravenous and subcutaneous doses of Ab 12B9m were used to jointly characterize their pharmacokinetics through the development of a semi-

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mechanistic model based on target mediated drug disposition (TMDD) and saturable FcRn-mediated IgG recycling.

TMDD occurs when the time course of the concentration is influenced by the interaction between the drug and its pharmacological target (14). General pharmacokinetic models have been developed to account for drug–receptor binding, internalization, and degradation, as well as the receptor turnover (15–23). Similar principles have also been applied to drugs targeting soluble ligands (24–27).

In addition, FcRn is an endosomal salvage receptor that binds to and protects IgGs from degradation during endosomal recirculation (28,29). The influence of the FcRn on the IgG disposition has been studied using physiologically based pharmacokinetic models (30–32). In addition to FcRn-mediated disposition, the reticuloendothelial system might play a role in phagocytosis and elimination of IgGs and their immunocomplexes (33). Smaller proteins, such as hepcidin, are often filtered by the kidney glomeruli and undergo tubular reabsorption and/or elimination (34). Due to the large molecular size of monoclonal antibodies, clearance of intact Ab 12B9m and hepcidin–Ab 12B9m complex through the kidney is negligible.

Consequently, Ab 12B9m and Ab 12B9m–hepcidin complex were thought to undergo two parallel elimination processes: (1) nonspecific distribution and elimination via the reticuloendothelial system and (2) FcRn-mediated endosomal recycling and degradation. However, the relative contribution of each clearance pathway was unknown. Therefore, one objective of this study was to characterize the pharmacokinetics of hepcidin and Ab 12B9m in cynomolgus monkeys, and to assess the relative importance of the above clearance mechanisms.

METHODS

Study Design. Data available from two studies conducted by Amgen Inc. in cynomolgus monkeys were used in this analysis. The first study was a single-dose PK study, and a total of 18 naive male animals were assigned to six groups and received a single dose of Ab 12B9m at 0.5, 5, or 50 mg/kg either by intravenous (IV) or subcutaneous (SC) administration. The second study was a repeated-dose toxicokinetic study, and male and female cynomolgus monkeys were assigned to five different treatment groups ($n=5$ group⁻¹ sex⁻¹). Animals in group 1 received placebo; animals in groups 2, 3, and 4 received once weekly IV doses of Ab 12B9m at 5, 40, and 300 mg/kg, respectively; while animals in group 5 received once weekly SC doses of Ab 12B9m at 300 mg/kg for 4 weeks. Toxicokinetic data were collected from all animals up to study day 29 with intensive toxicokinetic sampling after doses on study days 1 and 22, and from two animals per group per sex during a 19-week treatment-free period up to study day 155.

All animals were treated in accordance with the USDA Animal Welfare Act (9 CFR, Parts 1, 2, and 3) and the conditions specified in the Guide for Care and Use of Laboratory Animals (ILAR publication, 1996, National Academy Press).

Pharmacokinetic Sampling and Bioanalytical Methods.

In order to measure total concentrations of hepcidin and Ab 12B9m, blood samples were collected from each animal in

polypropylene tubes containing no anticoagulant. In the single-dose study, blood samples were collected at predose, and at 0.5, 4, 24, 48, 96, 168, 336, 504, 672, 1,008, 1,344, and 1,680 h postdose. In the multiple-dose study, blood samples were collected on study days 1 and 22 at predose, and approximately 0.5 (IV only), 4, 24, 48, 72 (SC only), 96, and 168 h postdose. Trough blood samples were collected at predose on study day 15. Blood samples were also collected during the treatment-free period on study days 44, 58, 72, 86, 100, 114, 128, 142, and 155. Whole blood was set at ambient temperature for at least 30 min and then centrifuged. The resulting serum was collected and stored at -70°C until analyzed.

Total Serum Hepcidin. Total serum concentrations of hepcidin were determined with a high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method using synthetic cynomolgus monkey hepcidin as reference standard and N-terminal truncated human hepcidin (amino acids 6–25) as the internal standard (35). A fast dissociation step ($\text{pH}=1$) was conducted to release hepcidin from the Ab 12B9m–hepcidin complex, followed by solid-phase extraction on 100 μL serum. A reversed-phase HPLC separation was performed on a Varian Polaris C18-A column (7.5×2.0 mm) using a gradient elution. The mobile phase consisted of 5% methanol in water with 0.1% formic acid (solvent A) and 95% methanol in water with 0.1% formic acid (solvent B). MS/MS detection was performed on a Sciex API-4000 triple quadrupole mass spectrometer with an electrospray ionization source. MS/MS ion transitions for intact cynomolgus hepcidin (H25) and the internal standard were m/z 705.3 $\rightarrow$ 651.5 and m/z 548.9 $\rightarrow$ 693.6, respectively. The lower limit of quantification was 5 ng/mL. Over the linear range of the H25 assay (5–1,000 ng/mL), the mean inter-assay coefficient of variation was lower than 6%, and the mean percentage accuracy were within 95–105%. Two H25 metabolites with 20 and 22 amino acids (H20 and H22, respectively) as N-terminal hydrolysis products were determined. However, because their levels were insignificant (combined $<10\%$ of H25), the metabolites were not included for this modeling work.

Total Serum Ab 12B9m. The free Ab 12B9m and its H25 complex could not be separately determined due to technical difficulties (*i.e.*, complex dissociation during storage and/or enzyme-linked immunosorbent assay (ELISA)). Instead, the total serum concentrations of Ab 12B9m were determined using a validated ELISA. Microplate wells were coated with a mouse anti-Ab 12B9m monoclonal antibody as the capture antibody. Standard samples, quality controls (QC), study samples, or blank control were loaded into the wells after pretreatment at 1:100 with I-Block Buffer+5% BSA. Ab 12B9m bound to the immobilized capture antibody. A horseradish peroxidase (HRP) conjugated mouse anti-human Fc detection antibody was then added to the wells, followed by a tetramethylbenzidine (TMB) peroxide substrate solution. TMB reacted with the peroxide and in the presence of HRP produced a colorimetric signal that was proportional to the amount of Ab 12B9m bound by the capture reagent. The color development was quenched, and the intensity of the color (optical density (OD)) was measured at 450–650 nm. The conversion of OD units for study samples and QCs to corresponding concentrations was achieved through a computer software mediated comparison with a standard curve

assayed on the same plate, which was regressed according to a Logistics (Auto Estimate) regression model with a weighting factor of $1/Y$ using a 7.0.0.01 Watson data reduction package. The lower limit of quantification was 50 ng/mL. The validated range of the assay was 50 to 2,000 ng/mL, and the mean intra- and inter-assay coefficients of variation were 6% and 8%, respectively. The ELISA specificity was tested in the presence of different concentrations of monkey hepcidin, and a control human IgG that does not bind to hepcidin. Neither the control IgG nor hepcidin interfered with the assay, indicating that the assay specifically measures the total Ab 12B9m concentration.

In order to model the Ab 12B9m–H25 binding stoichiometry and target coverage, concentrations were converted to their molar equivalents. It was assumed that Ab 12B9m is bivalent, and its two Fab arms bind to two H25 molecules independently. Thus, a molecular weight of 75 kDa, instead of Ab 12B9m's molecular weight of 150 kDa (IgG2), was used to convert Ab 12B9m concentration to Fab molar equivalence. The H25 molecular weight of 2.815 kDa was used to calculate H25 molar concentrations.

Anti-Ab 12B9m Antibodies. Anti-Ab 12B9m antibodies were determined in serum samples from the repeated dose TK study using a validated Meso Scale Discovery method.

The approximate assay sensitivity was 19 ng/mL of rabbit positive control antibody in neat cynomolgus monkey serum. The assay can detect 500 ng/mL rabbit polyclonal antibodies in the presence of 50 µg/mL of Ab 12B9m in neat cynomolgus monkey serum.

Semi-mechanistic Pharmacokinetic Model. The concept of TMDD PK model has been applied to describe total serum concentrations of Ab 12B9m and H25 (Fig. 1). The free drug compartment was assigned to Ab 12B9m (Ab), and the free target compartment consisted of H25. Upon binding to H25 at a second-order rate k_{onH} , Ab 12B9m formed the complex AbH that can dissociate to form free Ab 12B9m and H25 at a first-order rate k_{offH} . Both free and bound Ab 12B9m distribute to peripheral compartments, Ab_p and AbH_p , respectively, and the same first-order distribution and elimination rate constants (k_{cp} , k_{pc} , and k) were assumed for both species. The absorption of Ab 12B9m from the subcutaneous site, Ab_{sc} , was described by a first-order process (k_a) and bioavailability (F). H25 production and elimination were characterized by a zero-order rate (k_{inH}) and a first-order rate constant (k_{tH}), respectively. Both Ab 12B9m and AbH were subjected to protective binding to the FcRn receptor. The binding was preceded by a first-order uptake (k_{up}) by cells expressing FcRn receptors, followed by the binding to the free FcRn receptors in the endosomal compartments for the Ab 12B9m (Ab_E) and the Ab 12B9m–H25 complex (AbH_E). The total number of FcRn receptors (Fc_{tot}) was assumed to be constant, and the FcRn receptor binding and dissociation constants (k_{onA} and k_{offA}) were identical for both Ab_E and AbH_E . The competition between Ab 12B9m and the endogenous IgGs for FcRn, as well as the competition between Ab 12B9m and ferroportin for H25, was neglected for simplicity. The antibodies unbound to FcRn in the endosome (*i.e.*, Ab_E and AbH_E) were degraded at a first-order rate constant (k_{deg}), whereas the antibodies bound to the FcRn receptors ($FcAb_E$ and $FcAbH_E$) were recycled back

Fig. 1. Semi-mechanistic pharmacokinetic model for H25 and Ab 12B9m in cynomolgus monkeys. Ab_{sc} represents the SC depot for Ab 12B9m SC dosing. Ab 12B9m and Ab 12B9m–H25 complex distribute into their central compartments (Ab and AbH), peripheral compartments (Ab_p and AbH_p), and endosome compartments (Ab_E and AbH_E). In endosome, Ab 12B9m and Ab 12B9m–H25 complex binds to FcRn to form complexes ($FcAb_E$ and $FcAbH_E$). The intercompartment distribution was described with a set of first-order rate constants (k_{cp} , k_{pc} , k_{up} , and k_R), and elimination from Ab and AbH follows first-order kinetics (k). It was assumed that Ab 12B9m and Ab 12B9m–H25 complex share the same parameter as listed above. H25 is produced at a constant rate (k_{inH}) and eliminated with first-order kinetics (k_{tH}). In serum, the binding between Ab 12B9m and H25 is governed by the association rate constant (k_{onH}) and the dissociation rate constant (k_{offH}); similarly, in endosome, the binding of Ab 12B9m and Ab 12B9m–H25 complex to FcRn is described by k_{onA} and k_{offA} .

to the serum compartment at a first-order rate constant (k_R). The FcRn binding in the endosomal compartments was further reduced by the assumption of the equilibrium between the dissociation and binding processes:

$$\frac{Fc \times Ab_E/V_E}{FcAb_E} = \frac{k_{offA}}{k_{onA}} = K_{DA} \text{ and} \quad (1a, b)$$

$$\frac{Fc \times AbH_E/V_E}{FcAbH_E} = \frac{k_{offA}}{k_{onA}} = K_{DA}$$

where V_E denotes the volume of the endosomal compartment, K_{DA} is the dissociation constant for the FcRn complex with Ab or AbH in the acidified endosome, and Fc is the amount of free FcRn receptors in the endosomal compartment:

$$Fc = Fc_{tot} - FcAb_E - FcAbH_E \quad (2)$$

The final PK model was described by the following system of differential equations:

$$\frac{dAb_{sc}}{dt} = -F \cdot k_a \cdot A_{sc} \quad (3)$$

$$\frac{dAb}{dt} = F \cdot k_a \cdot A_{SC} - (k + k_{up} + k_{cp}) \cdot Ab - k_{onH} \cdot H_{25} \cdot Ab / V_c + k_{offH} \cdot AbH + k_R \cdot FcAb_E + k_{pc} \cdot Ab_p \quad (4)$$

$$\frac{dAb_p}{dt} = k_{cp} \cdot Ab - k_{pc} \cdot Ab_p \quad (5)$$

$$\frac{dH_{25}}{dt} = k_{inH} - k_H \cdot H_{25} - k_{onH} \cdot H_{25} \cdot Ab / V_c + k_{offH} \cdot AbH \quad (6)$$

$$\frac{dAbH}{dt} = k_{onH} \cdot H_{25} \cdot Ab / V_c - (k_{offH} + k + k_{up} + k_{cp}) \cdot AbH + k_{pc} \cdot AbH_p + k_R \cdot FcAbH_E \quad (7)$$

$$\frac{dAbH_p}{dt} = k_{cp} \cdot AbH - k_{pc} \cdot AbH_p \quad (8)$$

$$\frac{dAb_{Etot}}{dt} = k_{up} \cdot Ab - k_{deg} \cdot Ab_E - k_R \cdot FcAb_E \quad (9)$$

$$\frac{dAbH_{Etot}}{dt} = k_{up} \cdot AbH - k_{deg} \cdot AbH_E - k_R \cdot FcAbH_E \quad (10)$$

where V_c denotes the central compartment volume of Ab 12B9m, which was assumed to be the same for H25 and the Ab 12B9m–H25 complex; Ab_{Etot} and AbH_{Etot} are the total amounts, respectively, of Ab 12B9m and Ab 12B9m–H25 in the endosomal compartments:

$$\begin{aligned} Ab_{Etot} &= Ab_E + FcAb_E \text{ and } AbH_{Etot} \\ &= AbH_E + FcAbH_E \end{aligned} \quad (11a, b)$$

The free and bound endosomal antibodies were calculated according to the equilibrium assumption (see [Appendix](#)):

$$Ab_E = \frac{Ab_{Etot} \cdot (K_{DA} \cdot V_E + Ab_E + AbH_E)}{K_{DA} \cdot V_E + Fc_{tot} + Ab_E + AbH_E} \quad (12)$$

and

$$AbH_E = \frac{AbH_{Etot} \cdot (K_{DA} \cdot V_E + Ab_E + AbH_E)}{K_{DA} \cdot V_E + Fc_{tot} + Ab_E + AbH_E} \quad (13)$$

where

$$\begin{aligned} Ab_E + AbH_E &= \frac{1}{2} (Ab_{Etot} + AbH_{Etot} - K_{DA} \cdot V_E - Fc_{tot}) \\ &+ \frac{1}{2} \sqrt{(Ab_{Etot} + AbH_{Etot} - K_{DA} \cdot V_E - Fc_{tot})^2 + 4 \cdot (Ab_{Etot} + AbH_{Etot}) \cdot K_{DA} \cdot V_E} \end{aligned} \quad (14)$$

$$FcAb_E = \frac{Fc_{tot} \times Ab_E}{K_{DA} \cdot V_E + Ab_E + AbH_E} \quad (15)$$

$$FcAbH_E = \frac{Fc_{tot} \times AbH_E}{K_{DA} \cdot V_E + Ab_E + AbH_E} \quad (16)$$

The initial conditions for the model variables were zero except for Ab_{SC} , Ab , and H_{25} . The initial values of Ab_{SC} and Ab were the first doses administered SC or IV, whereas the initial value for H_{25} was the baseline H_{25} serum amount H_{25_0} . The steady state for Eq. 6 resulted in the following baseline equation:

$$k_{inH} = k_H \cdot H_{25_0} \quad (17)$$

The linear disposition parameters for Ab 12B9m were re-parameterized in terms of clearances and volumes:

$$k = \frac{CL}{V_c} \quad (18)$$

and

$$k_{cp} = \frac{Q}{V_c} \text{ and } k_{pc} = \frac{Q}{V_p} \quad (19a, b)$$

where Q denotes the distributional clearance and V_p is the volume of the peripheral compartment. Total Ab 12B9m and total H25 serum concentrations were expressed as

$$C_{Abtot} = \frac{Ab + AbH}{V_c} \text{ and } C_{Htot} = \frac{H_{25} + AbH}{V_c} \quad (20a, b)$$

Statistical Modeling. The naive pooled data approach was used in this analysis, and data from all dosing groups were used simultaneously to fit the model parameters. The following model for variance of the residual error both for total Ab 12B9m and total H25 data was applied:

$$\text{Var}(C_{Obs}) = (a + b \cdot C_{Pred})^2 \quad (21)$$

where C_{Obs} and C_{Pred} were the observed and predicted total serum concentrations, respectively, and a and b were the

variance parameters. The maximum likelihood minimization was employed to characterize the time course of total serum H25 and Ab 12B9m concentration using ADAPT 5 (36). The same software was used in performing the model-based simulations.

Model-Based Simulations. The estimates of the PK parameters were used for simulations of free Ab 12B9m (Ab/V_c), free H25 ($H25/V_c$), and the Ab 12B9m–H25 complex (AbH/V_c) concentrations in serum following the studied dosing schedules. The steady-state peaks for free H25 were evaluated at 504 h following once weekly administration of Ab 12B9m. To explore the role of FcRn-mediated disposition, free Ab 12B9m-time profiles were simulated in the presence and absence ($Fc_{tot}=0$) of FcRn following a single IV and SC dose. Percent of the unbound FcRn was calculated as follows after multiple IV and SC doses of 300 mg/kg q.w.:

$$\frac{Fc_{tot} - FcAb_E - FcAbH_E}{Fc_{tot}} \cdot 100 \quad (22)$$

RESULTS

In total, 907 and 1,082 quantifiable total serum concentrations of Ab 12B9m and H25 from 68 animals, respectively, were used in this analysis. The incidence of immunogenicity was low after repeated dosing: 5% and 25% during the Ab 12B9m treatment period and the treatment-free period, respectively. Binding antibodies were also detected in two animals (20%) receiving control article, probably due to pre-existing anti-human antibody IgGs in the animals. The overall impact on PK was minimal. The mean terminal profile of total Ab 12B9m following 40 mg/kg weekly IV dosing showed a crossover with that of 300 mg/kg IV group. This was presumably due to a male outlier. Large variability was also observed in the terminal data in the 40 mg/kg group. Considering the general linear terminal profiles at all other dose levels in both studies, and the small sample size during the treatment-free period (two animals per sex per group), data after study day 85 (five time points in total) were excluded for the 40 mg/kg group (Fig. 2).

A PK model based on TMDD concept was initially considered to model Ab 12B9m pharmacokinetics in both serum and endosomal compartments. Due to problems with estimating the model parameters, several reductions were tested which included the absence of the peripheral compartments for Ab_p and AbH_p , and binding equilibrium assumptions both for Ab and Ab_E . The presented model performed the best with respect to the minimal value of the objective function and the precision of the parameter estimates measured by the CV%. The following constraints were imposed on the structural parameters of the PK model described by Eqs. 2–16: (1) $k_{up} = k_R$; (2) $k_{offH} = k_{DH} \cdot k_{onH}$, where k_{DH} was fixed at the experimentally determined value of 0.001 nM (data on file); (3) the parameters K_{DA} and V_E were not identifiable separately, but since they operate in the model only as a product, $K_{DA} \cdot V_E$ was estimated; (4) the Ab 12B9m linear clearance estimate (CL) was very small and was

subsequently fixed to 0, as previously suggested (32). The estimates of the remaining parameters are shown in Table I. The precisions of the estimates were reasonable ($CV\% < 26\%$) except for Q , V_p , and k_{onH} which had CV% of 141.4%, 51.6%, and 53.0%, respectively, indicating redundancy of the peripheral compartments that was tested with a negative outcome. However, the reduced model was inferior based on the Akaike information criteria.

The results of the model fit are shown graphically in Figs. 2 and 3. The PK model adequately described the total Ab 12B9m and H25 serum concentrations after a single dose. The peaks and troughs, as well as the accumulation and the terminal phase for the total Ab 12B9m serum concentrations following multiple IV and SC doses, were well captured, except that the terminal phase was slightly overestimated for the 5 mg/kg IV group. The r^2 values for the observed vs. predicted correlation for individual dose data ranged from 0.90 to 0.99.

The estimated value of the central compartment was close to the approximate serum volume of 0.04 L/kg in cynomolgus monkeys (37), suggesting drug confinement into the vascular space. The volume of the peripheral compartment constitutes only 11% of the serum compartment, indicating limited distribution of Ab 12B9m to peripheral tissues or lack of specific tissue binding. Consistent with observations by other group (32), the estimate of the linear clearance from the serum compartment (CL) was very small, and consequently, it was fixed to 0. The distributional clearance (Q) was minimal compared with the total cardiac output, suggesting limited distribution to extravascular space and explaining the difficulties in getting a precise estimation of the model parameters that describe the nonspecific distribution to peripheral compartment. Unfortunately, V_E and K_{DA} were not identifiable due to insufficient information about the binding of Ab 12B9m to the FcRn receptors in endosome. Instead, the product $K_{DA} \cdot V_E$ was estimated. This made it difficult to directly assess the endosome contribution to the distribution of Ab 12B9m and its H25 complex. The constraint $k_{up} = k_R$ was applied as in previous models of IgG pharmacokinetics for rats (32). Our estimate of k_{up} was approximately 30% lower than one reported previously (32), resulting in the half-life values for the IgG uptake and recycling of 46.2 h. The half-life for the endosomal degradation of Ab 12B9m was estimated as 33 h, approximately 70% lower than the previous estimation obtained from rats (32). It is not well understood whether the magnitude of these discrepancies is associated with the between-species differences. Only the molar amount of the total FcRn receptor in the endosomes was estimated to be 1,503 nmol/kg, a value that cannot be converted to molar concentrations without V_E . Also, this value was supposed to reflect the amount of FcRn that is involved in Ab 12B9m endosomal recirculation, and thus should not be compared with any reported FcRn expression levels.

The PK model provided estimates of the turnover of free H25 in cynomolgus monkey. The endogenous level of H25 calculated as $H25_0/V_c$ yields 9.5 nM, a value consistent with the measured H25 levels for the placebo group. The half-life for the elimination of free H25 was estimated as 2.3 min, and the calculated clearance was approximately 800 mL h^{-1} kg^{-1} (the product of k_H and V_c), which implies a very fast turnover process. The H25 synthesis rate calculated from Eq. 17 was

Fig. 2. Time course of total Ab 12B9m (*upper panels*) and H25 (*lower panels*) concentrations following repeated weekly intravenous (*left panels*) or subcutaneous (*right panels*) administrations of Ab 12B9m. The *symbols* represent individual measured concentrations, and the *solid lines* represent model predictions

$7.6 \text{ nmol kg}^{-1} \text{ h}^{-1}$. The estimate of the binding constant k_{onH} might be biased by the assumption that k_{DH} is known. The calculated value for $k_{\text{offH}} = k_{\text{onH}} \cdot k_{\text{DH}}$ was 0.000743 h^{-1} , which implies a very slow dissociation process. However, fixing k_{offH} at 0 resulted in a very short terminal half-life for free Ab 12B9m that was not considered physiologically possible.

Despite that both Ab 12B9m and H25 data were of the total serum concentrations, the PK model allowed to infer the serum concentrations of the free species. Figures 4 and 5 represent the simulated time courses for free Ab 12B9m and H25 serum concentrations after single or repeated dosing regimens applied in the studies, respectively.

The simulated free Ab 12B9m serum concentration-time profiles following a single IV dose injection exhibits a two-phase decline (Fig. 4a). This coincides with an instantaneous decrease of the simulated free H25 serum concentrations followed by a rapid return to the baseline of 9.5 nM (Fig. 4b). The initial rapid decrease of the simulated Ab 12B9m concentrations is due to binding to free H25. The extent of decline in free Ab 12B9m concentration is larger than for free H25, since H25 is rapidly produced at an estimated rate $k_{\text{inH}} = 7.6 \text{ nmol kg}^{-1} \text{ h}^{-1}$. The return times of free H25 to the baseline values (Fig. 4b) coincide with the time when the slower second phase of free Ab 12B9m decline was observed (Fig. 4a). The slopes of the terminal phases of Ab 12B9m in the log scale are equal to 0.0017 h^{-1} for all doses, which corresponds to a half-life of 16.5 days. The free Ab 12B9m serum concentration-time courses following a single

SC dose administration do not show the absorption phases at doses of 0.5 and 5 mg/kg (Fig. 4c). This can be explained as Ab 12B9m binds to H25 immediately after SC absorption into circulation. The absorption process becomes more dominant at 50 mg/kg SC, where an absorption phase can be observed with t_{max} and C_{max} of free Ab 12B9m estimated to be 22.5 h and 1,770 nM, respectively. The analogous values for the total Ab

Table I. Semi-mechanistic Pharmacokinetic Model: Estimates of the Model Parameters

Parameter	Estimate	CV%
k_a, h^{-1}	0.0278	6.0
F	0.860	2.6
$\text{CL, L kg}^{-1} \text{ h}^{-1}$	0 ^a	N/A
$V_c, \text{L/kg}$	0.0438	3.8
$V_p, \text{L/kg}$	0.00483	51.6
$Q, \text{L kg}^{-1} \text{ h}^{-1}$	0.00245	141.4
$\text{H25}_0, \text{nmol/kg}$	0.415	6.2
$k_{\text{up}}, \text{h}^{-1}$	0.0150	10.8
$k_{\text{onH}}, \text{nM}^{-1} \text{ h}^{-1}$	0.543	53.0
k_{DH}, nM	0.001 ^a	N/A
$\text{Fc}_{\text{tot}}, \text{nmol/kg}$	1503	14.9
k_H, h^{-1}	18.3	6.8
$K_{\text{DA}} \cdot V_E, \text{nmol/kg}$	312	26.3
$k_{\text{deg}}, \text{h}^{-1}$	0.0208	32.3

^a Parameter was fixed

Fig. 3. Time course of total Ab 12B9m (upper panels) and H25 (lower panels) concentrations following single intravenous (left panels) or subcutaneous (right panels) administration. The symbols represent individual measured concentrations, and the solid lines represent model predictions

12B9m serum concentrations are 65.7 h and 6,560 nM, respectively. The decreases in free H25 serum concentrations following SC administration of a single dose of Ab 12B9m are minimal for two lower doses (Fig. 4d). For the largest dose of 50 mg/kg, a H25 nadir of 0.18 nM corresponds with the $t_{\max}$ of free Ab 12B9m at 22.5 h. After the nadir, free H25 returns to the baseline in a two-phase manner. The faster phase ends at approximately 62 h, and it is followed by a slower phase. The slower phase coincides with a terminal phase for free Ab 12B9m that starts at approximately 62 h. The slopes in the log scale of the terminal phases for free Ab 12B9m are identical for the IV and SC doses. These are also equal to the slopes of the terminal phases for the Ab 12B9m–H25 complex, which achieves serum concentration levels 8,890-fold higher than serum free Ab 12B9m concentrations at the same time. Consequently, in the terminal phases, 0.01% of total Ab 12B9m is present in the free form and 99.99% is bound to H25. This is a consequence of the low value of the dissociation equilibrium constant of 1 pM.

Free Ab 12B9m and free H25 serum concentration following four weekly doses of Ab 12B9m are shown in Fig. 5. The free Ab 12B9m time profiles show negligible accumulation upon repeated doses with troughs at 0.15, 1.9, 16,000, and 1,900 nM for IV doses at 5, 40, and 300 mg/kg (Fig. 5a), and SC dose at 300 mg/kg (Fig. 5c), respectively. As shown in Fig. 5b, the time for free H25 to return to the baseline at ≤ 40 mg/kg of Ab 12B9m is less than 168 h; therefore, free H25 $C_{\max}$ concentrations at steady state are equal to the baseline value ($H25_0 = 9.5$ nM). For the larger

dose of 300 mg/kg, the times to attain 95% of $H25_0$ are 422 and 440 h for IV and SC doses, respectively, and consequently, the steady-state concentration of H25 is lower than $H25_0$ only at 300 mg/kg. The steady-state free H25 $C_{\max}$ values are 0.022 and 0.018 nM, and the free H25 nadir concentrations are 0.0033 and 0.0071 nM for IV and SC doses of 300 mg/kg, respectively. Figure 6 represents a relationship between a weekly dose of Ab 12B9m and the peak concentration of free H25 at steady-state $C_{\max,SS}$. There is a threshold dose above in which $C_{\max,SS}$ rapidly decreases from ≥ 6 to 0.1 nM. For IV administration, this decrease occurs for doses in the range 150–170 mg/kg, whereas for SC doses, this range is 130–170 mg/kg.

The PK model provides a means of assessment for the degree the FcRn protects Ab 12B9m from degradation in the endosomal compartment. Figure 7 shows simulations of the free serum Ab 12B9m concentrations with and without FcRn receptor ($Fc_{tot} = 0$). The simulated free Ab 12B9m time courses in the absence of FcRn receptor have shorter terminal half-lives than those in the presence of FcRn receptor. The terminal slopes in the log scale are identical for all doses both IV and SC and equal to 0.015 h^{-1} . The analogous slope values in the presence of FcRn are equal to 0.0017 h^{-1} , which corresponds to an 8.8-fold increase in the terminal half-life. Figure 8 represents the percentage of the free endosomal FcRn remaining following multiple-dose administration of IV and SC Ab 12B9m at 300 mg/kg. The extent of the decrease reaches 18% and 22% of the initial Fc_{tot} value for IV and SC doses, respectively. The most

Fig. 4. Simulation of free Ab 12B9m and free H25 (solid lines) as well as Ab 12B9m-H25 complex (broken lines) serum concentrations following single IV and SC administration of Ab 12B9m. Parameter values used for simulations are presented in Table I. **a** Free Ab 12B9m and Ab 12B9m-H25 complex profiles after an IV dose; **b** free H25 profiles after an IV dose; **c** free Ab 12B9m and Ab 12B9m-H25 complex profiles after a SC dose; **d** free H25 profiles after a SC dose

rapid decline in the FcRn level occurs within the first week. The percentage of free FcRn seems to reach a steady state after four doses and returns to the baseline after cessation of dosing.

DISCUSSION

The objective of this study was to characterize the pharmacokinetics of H25 and Ab 12B9m in cynomolgus monkeys based on the TMDD concept. Monoclonal antibodies are often used to capture soluble target ligands that cause disease symptoms when excessively expressed. During drug research and development, it is usually preferred to measure free drug and free target concentrations if feasible, as the free concentrations directly drive efficacy and reflect target coverage. In this case, however, free concentrations could not be accurately measured due to technical difficulties, *i.e.*, complex dissociation during sample storage and/or assay. As a result, total Ab 12B9m and total H25 concentrations were measured, and modeling was conducted to infer the serum concentration-time courses of free Ab 12B9m and free H25 from the total serum concentrations. In this situation, it is important to assess whether it is possible to predict the suppression of the free target soluble ligand based on available total drug and total ligand concentration data using a binding model. In some of the TMDD model applications, the time course of the target was inferred as no direct quantitative data of the target were available for the analysis. In some other cases, either total drug concentration and free target concentration (26), or free drug concentration and total target concentration (38) were determined. Recently, Lowe

and Gautier demonstrated that it was possible to predict the suppression of free unbound IgE from omalizumab PK data and total IgE data (39). A similar conclusion was reached by the authors in performing a model-based simulation followed by parameter estimation when free and total Ab12B9m concentrations as well as free and total H25 concentrations were available and compare these results with those obtained from only total Ab 12B9m and total H25 data (*i.e.*, excluding the free antibody and free ligand concentrations) (data not shown). Given the similarity of the model parameter estimates in presence or absence of the simulated free Ab12B9m and free H25 concentrations, the total Ab 12B9m and the total H25 PK data are informative with respect to the time course of free Ab 12B9m and free H25 concentrations, and the current model is deemed adequate to infer free hepcidin concentration-time profiles, which were consistent with the temporal profiles of serum iron elevation following different Ab 12B9m treatment (data on file).

A semi-mechanistic model with two binding processes of Ab 12B9m to H25 and FcRn receptor was developed. The Ab 12B9m binding to H25 was modeled based on a TMDD model proposed by Mager and Jusko (38), and Ab 12B9m interaction with FcRn receptor was based on the model introduced by Hansen and Balthasar (39). The FcRn-mediated Ab 12B9m disposition was deemed necessary as increased elimination of total Ab 12B9m was observed following four weekly doses at 300 mg/kg. A model with linear elimination alone adequately described PK at doses up to 50 mg/kg, but overestimated the exposure at 300 mg/kg by ~40%. Partial FcRn saturation seems possible as the total Ab 12B9m concentration following 300 mg/

Fig. 5. Simulation of time courses of free Ab 12B9m (*upper panels*) and free H25 (*lower panels*) concentrations following Ab 12B9m multiple weekly intravenous (*left panels*) and subcutaneous (*right panels*) administration. Parameter values used for simulations are presented in Table I. **a** Free Ab 12B9m and Ab 12B9m–H25 complex profiles after IV doses; **b** free H25 profiles after IV doses; **c** free Ab 12B9m and Ab 12B9m–H25 complex profiles after SC doses; **d** free H25 profiles after SC doses

kg dosing reached more than 10^5 nM, approximately ~40% of the endogenous IgG level of 2.7×10^5 nM (40). The reported model with saturable FcRn binding describes the nonlinear PK and predicted about 80% FcRn saturation for multiple IV and SC dose of 300 mg/kg q.w. as shown in Fig. 8.

Given the complexity of the system, a series of simplifying assumptions were incorporated into the model. These included identical PK parameters for free Ab 12B9m and Ab

12B9m–H25 complex, the same volume of distribution for Ab 12B9m and H25, a quasi-equilibrium assumption for binding of Ab 12B9m and FcRn receptor, equality between the endosomal uptake and recycling rate constants, and fixing the equilibrium dissociation constant for H25 at an experimentally determined value. Additionally, the constant concentration of the total FcRn receptor was assumed. Competitive interaction between Ab 12B9m and endogenous IgG for FcRn, as well as competition between ferroportin and Ab 12B9m for H25, was initially considered, but during the modeling exercise, it appeared that the contribution of these two processes was minimal to the goodness of fit and the parameter identifiability was questionable; thus, they were removed from the final model for simplicity. Such a reduced model allowed estimation of model parameters with acceptable precision. The model was further used for simulations of free Ab 12B9m and free H25 PK profiles.

The proposed PK model consisted of two clearance mechanisms of Ab 12B9m: a linear clearance from serum and a nonlinear clearance from the endosomal compartment. The estimate of the linear clearance was negligible, rendering the degradation of endosomal Ab 12B9m unbound to the FcRn receptors as a major elimination pathway. Binding of Ab 12B9m to H25 and FcRn receptor constituted nonlinear and saturable processes. Only the latter contributed to the PK of the total Ab 12B9m, whereas both processes were determinants of nonlinear PK for free Ab 12B9m, and free and total

Fig. 6. Plot of the maximum of free H25 serum concentration at steady state as a function of dose administered once a week. The *solid line* represents IV injections, and the *broken line* represents SC injections. Parameter values used for simulations are presented in Table I

H25 serum concentrations. Consistently with the protective mechanism of FcRn receptor on IgG degradation, the PK model attributed the prolonged half-life of Ab 12B9m in serum to the recycling of the Ab 12B9m–FcRn complex. A simple first-order absorption model adequately described the subcutaneous data rendering a bioavailability value of 86%.

The model also provided an estimate of the total amount of the FcRn receptor involved in Ab 12B9m recycling at a given time. However, it needs to be interpreted with caution given the various model assumptions.

The increases in concentration of total H25 relative to the baseline following administration of Ab 12B9m implied a high turnover rate for this peptide. The PK model estimated half-life (2.3 min), and synthesis rate ($7.6 \text{ nmol kg}^{-1} \text{ h}^{-1}$) of H25 confirmed this observation. The H25 clearance was approximately $800 \text{ mL h}^{-1} \text{ kg}^{-1}$, higher than the glomerular filtration rate ($125 \text{ mL h}^{-1} \text{ kg}^{-1}$) (37). This was not surprising considering that the degradation rate accounted for H25 binding to ferroportin (10,41), renal excretion (8,42), and other elimination pathways. The increases in concentration of total H25 relative to the baseline are probably not related to an increase in hepcidin production because a decrease in serum iron would have been evident and it is not (data not shown).

Simulations showed that weekly administration of the highest dose of 300 mg/kg resulted in sustained decrease in free serum H25 concentration by 50% or more, whereas at lower doses, H25 returned to the baseline values before the next dose (Fig. 5). The quantitative information about a

Fig. 7. Simulations of free Ab 12B9m serum concentrations following a single IV (**a**) and SC (**b**) dose of Ab 12B9m in the presence (solid lines) and absence (broken lines) of the binding to the FcRn receptor. Parameter values used for simulations are presented in Table I. The broken lines were generated assuming $F_{cTot}=0$

Fig. 8. Simulation of free FcRn concentrations as percent of the total FcRn concentrations upon administration of multiple IV (solid lines) and SC (broken lines) dose 300 mg/kg q.w. of Ab 12B9m. The percentage was calculated according to Eq. 22. Parameter values used for simulations are presented in Table I

steady-state free H25 level upon multiple administration of Ab 12B9m is important to understand the therapeutic effect of Ab 12B9m since free H25 binds to ferroportin and inhibits iron transport to plasma. However, up to date, quantitative and dynamic relationships between hepcidin suppression and serum iron level, as well as between changes in iron and hemoglobin levels, have not been established yet. An initial exploratory analysis (data on file) of the relationship between serum iron and inferred free hepcidin level evidences a clock-wise hysteresis, which warrants additional research to fully understand the relationship.

In summary, a PK model has been developed to describe the total Ab 12B9m and total H25 serum concentration upon single and multiple IV and SC doses of Ab 12B9m in cynomolgus monkeys. The PK model allows inference of free Ab 12B9m and free H25 serum concentrations from total serum concentrations. Free Ab 12B9m exhibits nonlinear kinetics with a terminal half-life of 16.5 days that is equal to the terminal half-life of the total Ab 12B9m. The model suggested a high turnover rate for free H25 with a production rate of $7.6 \text{ nmol kg}^{-1} \text{ h}^{-1}$ and elimination half-life of 2.3 min. The model provides a relationship between multiple doses of Ab 12B9m and steady-state serum concentration of free H25. The semi-mechanistic model could be further adapted and applied for analysis of total anti-H25 antibody pharmacokinetics in humans.

ACKNOWLEDGMENTS

This study has been sponsored by AMGEN, which was involved in the study design, data collection, analysis, interpretation, writing of the manuscript, and the decision to submit the manuscript for publication. JX, YMW, HL, MR, MM, YW, BH, and JJPR were employees of AMGEN and own stock in AMGEN when the analysis was conducted. WK received consulting fees from AMGEN. They have no other conflict of interest to declare.

The authors thank Drs. George Doellgast and Hossein Salimi-Moosavi for their support on ELISA development and optimization. The authors also thank Dr. Barbra Sasu and Dr. Andrew Chow for their scientific support.

APPENDIX

Derivation of Eqs. 9–16. The full (non-reduced) TMDD PK model as presented in Fig. 1 encompasses differential equations describing Ab_E , $FcAb_E$, AbH_E , and $FcAbH_E$ variables:

$$\frac{dAb_E}{dt} = k_{up} \cdot Ab - k_{deg} \cdot Ab_E - k_{onA} \cdot Fc \cdot Ab_E / V_E + k_{offA} \cdot FcAb_E \quad (23)$$

$$\frac{dFcAb_E}{dt} = k_{onA} \cdot Fc \cdot Ab_E / V_E - (k_{offA} + k_R) \cdot FcAb_E \quad (24)$$

$$\begin{aligned} \frac{dAbH_E}{dt} = & k_{up} \cdot AbH - k_{deg} \cdot AbH_E - k_{onA} \cdot Fc \\ & \cdot AbH_E / V_E + k_{offA} \cdot FcAbH_E \end{aligned} \quad (25)$$

$$\frac{dFcAbH_E}{dt} = k_{onA} \cdot Fc \cdot AbH_E / V_E - (k_{offA} + k_R) \cdot FcAbH_E \quad (26)$$

Let E denote the amount of free and H25 bound Ab 12B9m in the endosomal compartment:

$$E = Ab_E + AbH_E \quad (27)$$

Similarly, let FcE denote the amount of the complexes $FcRn$ - Ab 12B9m and $FcRn$ - Ab 12B9m-H25 in the endosomal compartment:

$$FcE = FcAb_E + FcAbH_E \quad (28)$$

Adding Eqs. 23 and 25, and Eqs. 24 and 26 results in the following differential equations for E and FcE :

$$\frac{dE}{dt} = k_{up} \cdot (Ab + AbH) - k_{deg} \cdot E - k_{onA} \cdot Fc \cdot E / V_E + k_{offA} \cdot FcE \quad (29)$$

$$\frac{dFcE}{dt} = k_{onA} \cdot Fc \cdot E / V_E - (k_{offA} + k_R) \cdot FcE \quad (30)$$

Consequently, the combined species E bind to the $FcRn$ receptor as if it is a single drug. The equilibrium assumptions Eq. 1a, b can be written as

$$Fc \cdot Ab_E / V_E = K_{DA} \cdot FcAb_E \text{ and } Fc \cdot AbH_E / V_E = K_{DA} \cdot FcAbH_E \quad (31a, b)$$

Adding Eq. 31a, b and dividing by FcE results in

$$\frac{Fc \cdot E / V_E}{FcE} = K_{DA} \quad (32)$$

which means that the equilibrium assumption applies also to the combined species E . Repeating the argument for the quasi-equilibrium TMDD model (43), one can conclude that

$$E = \frac{1}{2} \left(E_{tot} - Fc_{tot} - K_{DA} \cdot V_E + \sqrt{(E_{tot} - Fc_{tot} - K_{DA} \cdot V_E)^2 + 4 \cdot E_{tot} \cdot K_{DA} \cdot V_E} \right) \quad (33)$$

and

$$\frac{dE_{tot}}{dt} = k_{up} \cdot (Ab + AbH) - k_{deg} \cdot E - k_R \cdot FcE \quad (34)$$

where

$$E_{tot} = E + FcE \quad (35)$$

Notice that Eq. 33 is identical to Eq. 14, since Eq. 34 is the sum of Eqs. 9 and 10. In order to derive Eqs. 15 and 16, one needs to re-write Eq. 2 to the following form

$$Fc = Fc_{tot} - FcE \quad (36)$$

and substitute it to Eq. 31a, b. These will constitute a system of two linear equations with unknowns $FcAb_E$ and $FcAbH_E$

that can be easily solved resulting in Eqs. 15 and 16. Finally, Eqs. 11a, b and 15 imply

$$Ab_{E_{tot}} = Ab_E + \frac{Fc_{tot} \times Ab_E}{K_{DA} \cdot V_E + Ab_E + AbH_E} \quad (37)$$

Multiplication of Eq. 37 by the denominator yields

$$\begin{aligned} & Ab_{E_{tot}} \cdot (K_{DA} \cdot V_E + Ab_E + AbH_E) \\ & = Ab_E \cdot (K_{DA} \cdot V_E + Fc_{tot} + Ab_E + AbH_E) \end{aligned} \quad (38)$$

and Eq. 15 follows. Equation 16 can be derived in a similar way.

Equations 32 and 33 imply that

$$FcE = \frac{Fc_{tot} \cdot E}{K_{DA} \cdot V_E + E} \quad (39)$$

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