Influence of improved FcRn binding on the subcutaneous bioavailability of monoclonal antibodies in cynomolgus monkeys

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Engineering monoclonal antibodies (mAbs) with improved binding to the neonatal Fc receptor (FcRn) is a strategy that can extend their in vivo half-life and slow their systemic clearance. Published reports have predominantly characterized the pharmacokinetics of mAbs after intravenous administration. Recently, studies in mice suggest FcRn may also play a role in affecting the subcutaneous bioavailability of mAbs. Herein, we examined whether five mAbs engineered with the T250Q/M428L Fc mutations that improved their FcRn interactions, and subsequently their in vivo pharmacokinetics after intravenous administration, had improved subcutaneous bioavailability compared with their wild-type counterparts in cynomolgus monkeys. Similar to the intravenous administration findings, the pharmacokinetic profiles of our variant mAbs after subcutaneous injection showed improved half-life or clearance. In contrast, a clear effect was not observed on the subcutaneous bioavailability. We expect that while FcRn may play a role in determining mAb subcutaneous bioavailability, multiple biopharmaceutical and physiological factors are likely to influence the success of engineering strategies aimed at targeting this pathway for improving bioavailability.

In many clinical indications, such as those requiring chronic treatment, it is desirable that the antibody therapeutic can be self-administered for the convenience of patients. While subcutaneous delivery is the most common alternate parenteral route, dose, dose volume, antibody biophysical characteristics and formulation in combination with less than absolute bioavailability are all substantial challenges to successful development. The factors contributing to the reduced bioavailability of some mAbs are not well understood, although it has been speculated that antibody degradation due to differences in proteolytic stability may be the predominant process. Protection of IgGs from proteolysis within the subcutaneous space may provide a means to increase their bioavailability and make this delivery route a more viable option. Recent studies have provided some evidence that FcRn plays a role in mAb bioavailability after subcutaneous injection. The strongest proof comes from a study in FcRn

mAbs have become increasingly important therapeutic choices in a wide array of indications, including cancer, inflammation, bone and autoimmune diseases. The compelling efficacy of these biomedicines has spurred advancements in mAb engineering to optimize their pharmaceutical properties. Many of these engineering approaches have focused on improving the pharmacokinetic/pharmacodynamic properties leading to reduced dose or dose frequency. Over the last decade, one of the more intense areas of study for modulating the pharmacokinetic properties of mAbs has involved the interaction of the Fc region with FcRn. FcRn functions through binding immunoglobulin G (IgG) in the acidic (pH ~ 6) environment of the endosome and recycling them back into circulation upon exposure to physiological pH (pH ~ 7.4). IgG that is not bound to FcRn within endosomes undergoes proteolytic degradation in lysosomes. The proportion of IgG processed through the recycling vs. degradative pathways is believed to be important in determining the half-life of an IgG in the circulation. There is substantial evidence that the half-life of a mAb can be affected by mutating residues in the CH2 and CH3 regions that improve FcRn binding properties. These reports have provided evidence that specific mutations (T250Q/M428L, M428L, M252Y/S254T/T256E, M428L/N434S, N434A) to a humanized IgG1, IgG4 or IgG2 molecule can result in ~2- to ~4-fold longer in vivo elimination phase half-life in either cynomolgus or rhesus monkeys.
deficient mice (~80% vs. ~28%, respectively). 

Supportive evidence for the role of FcRn in affecting bioavailability also was provided in a study suggesting decreased subcutaneous bioavailability of an engineered variant mAb that had lost the pH-dependency of binding to murine FcRn. These observations make similar engineering approaches to alter this receptor interaction a potential path for improving the subcutaneous bioavailability of therapeutic antibodies.

While the findings in mice suggest that eliminating or substantially decreasing the influence of the FcRn pathway leads to a negative impact on antibody subcutaneous bioavailability, it is not entirely clear that the relation of engineered improvements in FcRn-IgG binding to enhanced intravenous pharmacokinetics can be extended to increasing subcutaneous bioavailability. To date, there are no published reports testing this hypothesis directly. It has been speculated that FcRn may be performing multiple functions in the subcutaneous space such as actively transporting IgG across the vascular endothelium (from the interstitial fluid into the systemic circulation) or protecting IgG from pre-systemic catabolism via its recycling capability. Alternatively, it is possible that the poor bioavailability of IgG observed in FcRn knockout mice is not a direct effect, but more likely the result of ‘non-specific’ factors that influence physiology, such as low IgG/albumin concentrations in these animals or the influence of low protein levels on osmotic pressure within the subcutaneous space of the knockout animals. Currently, the mechanism(s) by which FcRn may influence bioavailability have not been experimentally defined. It is also known that the interaction of IgG with murine FcRn can have different characteristics than that with primate or human FcRn. 

This in itself makes it important to translate observations made in rodent systems to that of a higher species such as primate. With these points in mind, we designed the present study to extend the initial observations in mice to primate, to evaluate the impact of engineered improvements in FcRn binding affinity on subcutaneous bioavailability and to test the robustness of the outcome by studying these changes on the Fc of multiple antibodies.

A panel of five IgG4 antibodies was engineered with the T250Q/M428L Fc mutations previously shown by other investigators to have improved FcRn binding affinity and improved pharmacokinetic properties after intravenous administration. The five mAbs, which have identical sequences in the CH1, CH2, CH3, hinge regions but vary in heavy and light chain variable regions, were evaluated after intravenous and subcutaneous administration to cynomolgus monkeys. The antibodies were directed toward soluble peripheral targets that are present at insignificant concentrations in normal healthy primates to avoid the complication of nonlinear pharmacokinetics driven by target-mediated clearance.

In the studies reported here, each cynomolgus monkey was administered a solution formulation containing all five wild-type molecules or five T250Q/M428L IgG4 variants via an intravenous or a subcutaneous injection. This approach was taken in order to provide a judicious use of primates for these studies. In addition, due to practical considerations and the potential for eliciting an immune response, the bioavailability study was executed in a parallel, not crossover, design.

Several studies were conducted to evaluate the effect of the T250Q/M428L mutations on the biophysical properties of the five mAbs. One month long solution storage studies at 4°C, room temperature and 37°C showed the variants mAbs had no enhanced degradation and aggregation or changes in antigen binding affinity relative to their respective wild-type antibody counterparts (data not shown), indicating no significant differences in proteolytic sensitivity or aggregation properties were introduced by the T250Q/M428L Fc mutations. Evaluation of the isoelectric point (pI) of each IgG showed the T250Q/M428L mutations did not change the charge of the molecules (data not shown). In addition, at the concentrations used for the study (1 mg/mL/antibody), the stability of the dose formulations were evaluated. In these studies, neither soluble nor insoluble antibody aggregates were observed (data not shown), indicating the heterogeneity of the solutions did not negatively affect the solubility of the individual antibody components. At the doses studied, it is very unlikely that we would observe an influence of the mixture on the pharmacokinetics of any of the individual components, particularly in regard to potential competition at the receptor. This is supported by the similarity in pharmacokinetic parameters and subcutaneous bioavailability observed when one of the antibodies and the counterpart variant were studied independently and compared with the results obtained when dosed in the mixture (data not shown). Importantly, in the course of this study, no obvious instances of an anti-drug antibody response that would confound the interpretation of the pharmacokinetic results were observed.

After intravenous administration (Fig. 1), the five T250Q/M428L Fc variant antibodies exhibited improved peripheral kinetics characterized by a mean increase in exposure of ~1.6-fold (range 1.2- to 2.9-fold), a mean decrease in clearance of ~1.8-fold (range of 1.2- to 3.3-fold) and a prolonged mean half-life of ~1.9-fold (range of 0.9- to 2.6-fold) relative to their respective wild-type mAb (Table 1). The improvements in the intravenous pharmacokinetics of each variant were expected given the T250Q/M428L mutations had increased FcRn affinity at pH 6.0 and showed no direct binding to receptor at pH 7.4 in vitro (Sup. Table 1); properties which have been shown by us and others to be important for improving mAb in vivo kinetics. The improved kinetic properties of the five T250Q/M428L variant IgGs were also observed after subcutaneous administration (Fig. 2) with enhanced mean exposures of ~1.8-fold (range of 0.9- to 3.8-fold), slowed mean clearance of ~1.8-fold (range of 0.9- to 4.0-fold) and increased mean elimination half-life of ~2.6-fold (range of 1.7- to 4.2-fold) within each mAb platform (Table 2). The subcutaneously administered T250Q/M428L variant IgGs also displayed the same rank order of effect on pharmacokinetic parameters as observed following intravenous injection (Tables 1 and 2). In contrast, there was little to no consistent effect of FcRn binding affinity on the absolute subcutaneous bioavailability of the variant mAbs.
compared with the wild-type counterpart (Table 2). Across the five mAbs studied, we saw a range of effects from slight improvement (10–12%), to no effect, to a decrease (20%) in the mean bioavailability of the variants. Although there may have been a subtle trend for the wild-type mAbs having the lowest mean bioavailability to be modestly benefited (Table 2), this was not statistically meaningful. Compartmental pharmacokinetic analysis of the absorption rate constant ($k_a$) (Table 2) confirmed what appeared to be a prolonged $T_{\max}$ (Table 2) for the variants relative to their wild-type mAbs. The changes in $k_a$ for the antibodies ranged from ~1.4- to 2.1-fold, with the exception of mAb C1 which displayed a marginal (~1.1-fold) increase in $k_a$. The most pronounced effects on $k_a$ were for mAbs B1 and D1 (Table 2). With respect to this observation, we found the magnitude of the effect on absorption rate constant trended reasonably with increased in vitro binding affinity (Tables 2 and S2) and slowed rate of complex dissociation at pH 6.0 (data not shown). While the relevance of this observation is unclear, it does suggest that there may be an interaction of the variant mAbs with FcRn at the subcutaneous site, although we cannot rule out some other property of the molecules that may hinder their absorption from the site. If the observation is mediated via FcRn, in the end, it does not result in enhanced bioavailability in the case of the mAbs we evaluated.

Figure 1. Increased FcRn binding affinity slows the systemic clearance of five mAbs. (A–E) Pharmacokinetic profiles of the five wild-type (Δ) and five T250Q/M428L variant (●) mAbs variant antibodies after intravenous to male cynomolgus monkeys. A1, B1, C1, D1 and E1 denote the five mAbs tested. Doses were administered as a single intravenous injection of a solution formulation containing all five wild-type molecules or five T250Q/M428L IgG4 variants for a total of a 5 mg/kg dose (1 mg/kg of each mAb). Serum concentrations were determined using a validated antigen capture ELISA for each mAb. The five mAb backbones (as either wild-type IgG or T250Q/M428L variant mAbs) were selective for a single antigen and showed no cross-reactivity/nonspecific binding to non-cognate antigens in the ELISAs when examined individually or as a mixture of the five wild-type or five T250Q/M428L variant IgGs. Data are the mean ± SD of three animals/timepoint.
Table 1. Pharmacokinetic parameters for five humanized wild-type mAbs and T250Q/M428L variants in cynomolgus monkeys after intravenous administration.\(^{15,16}\)

| mAb      | \(C_{\text{max}}\) (mg/mL) | \(\text{AUC}_{0-\infty}\) (mg•h/mL) | CL (mL/h/kg) | \(t_{1/2}\) (h) |
|----------|-----------------------------|-----------------------------------|--------------|-----------------|
| A1 Wild type | 27 ± 2                       | 4264 ± 1242                      | 0.25 ± 0.06  | 328 ± 162       |
| A1 T250Q/M428L | 22 ± 5                       | 5069 ± 1063                      | 0.20 ± 0.05  | 285 ± 59        |
| B1 Wild type  | 41 ± 5                       | 5705 ± 2622                      | 0.21 ± 0.11  | 268 ± 50        |
| B1 T250Q/M428L | 30 ± 4                       | 7850 ± 1080                      | 0.13 ± 0.02  | 336 ± 146       |
| C1 Wild type  | 25 ± 5                       | 2843 ± 192                       | 0.35 ± 0.02  | 126 ± 17        |
| C1 T250Q/M428L | 17 ± 2                       | 3324 ± 507                       | 0.31 ± 0.04  | 263 ± 59        |
| D1 Wild type  | 28 ± 5                       | 1905 ± 453                       | 0.55 ± 0.14  | 126 ± 46        |
| D1 T250Q/M428L | 24 ± 0.6                     | 2910 ± 587                       | 0.35 ± 0.08  | 278 ± 107       |
| E1 Wild type  | 18 ± 4                       | 1347 ± 640                       | 0.88 ± 0.44  | 94 ± 51         |
| E1 T250Q/M428L | 23 ± 3                       | 3863 ± 849                       | 0.27 ± 0.06  | 246 ± 115       |

Note: *Serum concentrations determined using validated antigen capture ELISAs. *Data are the mean ± SD of the pharmacokinetic parameters determined from three monkeys per group. †Determined from non-compartmental pharmacokinetic analyses. \(C_{\text{max}}\), maximal observed serum concentration; \(\text{AUC}_{0-\infty}\), area under the serum concentration curve from zero to infinity; Concentration; CL, clearance; \(t_{1/2}\), elimination half-life.

In summary, our data do not support the hypothesis that the interaction of FcRn with IgG can be effectively engineered to enhance subcutaneous bioavailability. Our results do not exclude the role of FcRn in modulating bioavailability, which has been implicated based on studies in FcRn knockout mice.\(^{15,16}\) The observation that the absorption rate constant from the subcutaneous space had a relationship to FcRn binding affinity, in fact, would be consistent with a role for FcRn. There are several plausible explanations for our results in the context of the FcRn knockout mouse data. It is entirely possible that while FcRn contributes to bioavailability, the magnitude of the contribution is less than what is apparent when the system is entirely eliminated as in a knockout animal. In this respect, engineered improvements in the FcRn interaction may appear invisible to other biochemical/biophysical properties or physiological processes that govern absorption from the site.\(^{15,16,19}\) In particular, several studies have demonstrated that the anatomic site of a subcutaneous injection can influence the bioavailability and rate of absorption of protein drugs,\(^{19}\) thus further examination of different injection sites with Fc mutant mAbs is important for future work. The preponderance/contribution of the FcRn interaction in the subcutaneous space could also possibly be dissected by studying the relative bioavailability of a mAb engineered to lose complete pH-dependent receptor binding, as in the Abedeg\(^{1,20}\) interaction, which has been previously described. Along the same lines, it may be that engineered improvements only become apparent when the wild-type antibody has particularly poor bioavailability characteristics. Our observations do not imply that benefit would not be observed with other Fc mutations or mAb frameworks, but certainly challenges the concept and invites additional study of this mechanism. Such a strategy would best be considered on a case by case basis depending on the particular liabilities in the therapeutic mAb, such as formulatability/solubility and dose. Overall, our results strongly suggest that multiple biopharmaceutical and physiological factors are likely to influence the success of engineering strategies aimed at targeting this pathway for improving bioavailability.

Cell Culture

293EBNA cells were maintained at 37°C under 5–8% CO\(_2\) conditions in Dulbecco’s modified Eagle’s medium/F-12 (Gibco) supplemented with 20 mM HEPES (Gibco), 5 mg/mL nucellin (Eli Lilly and Company), 0.4 mg/mL tropolone (Sigma Aldrich), 0.075% (w/v) F68 (Gibco) and 50 mg/mL Geneticin® (Sigma Aldrich).

Construction, Expression and Purification of Recombinant Proteins

The antibody variant was derived from a humanized IgG4 Fc variant library created using a Kunkel-based strategy\(^{21}\) described earlier in reference 9 and 17. The T250Q/M28L variant was obtained by site-directed mutagenesis of the wild-type humanized IgG4 clone using the QuikChange method (Stratagene) and confirmed by DNA sequencing. The wild-type and T250Q/M428L variants IgGs were expressed in 293EBNA cells and purified from concentrated culture supernatants using Protein A Sepharose (GE Healthcare) affinity chromatography followed by size exclusion chromatography methods described previously in reference 9 and 17. Recombinant, soluble cynomolgus monkey FcRn (Fc-FcRn) was expressed in 293EBNA cells transfected with the plasmids encoding for the soluble portion aFcRn and b,m and the protein was purified as previously described in reference 9 and 17.

mAb:FcRn Binding Affinity Measurements with Surface Plasmon Resonance (BIACore)

The interaction of the wild-type and T250Q/M428L variant IgGs with recombinant, immobilized cynomolgus monkey FcRn was monitored by surface plasmon resonance (SPR) detection using a BIACore 2000 instrument (Biacore Inc.,) and characterized as previously described in reference 15. The binding data were obtained by subtracting the signal of flow...
cell 1 from the other two flow cells. Kinetic binding constants were determined through global fits of the average of two data sets collected on separate days using BIAevaluation, version 3.1. The kinetics (association and dissociation rates) were simultaneously fit to a heterogeneous binding model to determine the equilibrium dissociation constant ($K_D$) value for each FcRn:IgG interaction. The data curves for each phase of the sensorgrams had low residuals and $\chi^2$ values.

Figure 2. Improved peripheral pharmacokinetics do not translate to greater in vivo subcutaneous bioavailability for five mAbs. (A–E) Pharmacokinetic profiles of the five wild-type (Δ) and five T250Q/M428L variant (●) mAbs after subcutaneous administration to male cynomolgus monkeys. A1, B1, C1, D1 and E1 denote the five mAbs tested. Doses were administered as a single subcutaneous injection of a solution formulation containing all five wild-type molecules or five T250Q/M428L IgG4 variants for a total of a 5 mg/kg dose (1 mg/kg of each mAb). Data are the mean ± SD of three animals/timepoint.
Cynomolgus Monkey Pharmacokinetic Study

A cynomolgus monkey pharmacokinetic study was performed to study the pharmacokinetics of five T250Q/M428L Fc variants IgG4s and their wild-type mAb counterparts. In the study, 12 male cynomolgus monkeys (2.8–3.8 kg) were assigned to one of four study groups and each animal received a single intravenous or subcutaneous dose of a mixture of the five wild-type or five T250Q/M428L Fc variant IgGs dissolved in PBS (pH 7.4) at 1.0 mg/kg. The subcutaneous dose was administered to the mid-scapular region on the back. The single dose parallel study vs. a crossover design was used to evaluate the pharmacokinetics/bioavailability of the wild-type and T250Q/M428L variant IgGs in cynomolgus monkeys in lieu of the limitations [long elimination half-life of mAbs (~6 to ~20 d) and the potential to elicit anti-drug antibodies (ADA) with repeat injections] associated with the evaluation of mAb kinetics within a single animal after multiple administrations. Blood samples were collected from the femoral vein prior to dosing and at 6, 12, 24, 48, 72, 96, 120, 168, 240, 336, 384, 456, 528, 600, 648 and 696 h after administration of the dose. In addition, blood samples were also collected 1 h after administration to animals that received an intravenous dose. All blood samples were allowed to clot at ambient temperature prior to centrifugation to obtain serum.

Bioanalytical Assays and Pharmacokinetic Data Analysis

Concentrations of the wild-type and T250Q/M428L variant IgGs in cynomolgus monkey serum were determined using one of five validated antigen capture ELISAs for each of the five mAb backbones. The five mAb backbones (as either wild-type IgG or T250Q/M428L mutants) were selective for a single antigen and showed no cross reactivity/nonspecific binding to non-cognate antigens in the ELISAs when examined individually or as a mixture of the five wild-type or five T250Q/M428L variant IgGs.

The wild-type and T250Q/M428L variant IgG standards were prepared in cynomolgus monkey serum using a standard curve range of 0.78 to 50 ng/mL for mAbs A1 and B1 and 1.56 to 100 ng/mL for mAbs C1, D1 and E1. The lower limit of quantitation (LLOQ) was defined as 2 ng/mL for mAbs A1 and B1 and 4 ng/mL for mAbs C1, D1 and E1. Pharmacokinetic parameters reported from a non-compartmental analysis were calculated using the WinNonlin Professional software package (Pharsight Corporation, Mountain View, CA). Serum concentration-time data were calculated using a model-independent approach based on the statistical moment theory. The parameters calculated included the area under the curve (AUC_{0-\infty}), clearance (CL), volume of distribution (V_{ss}) and elimination half-life (t_{1/2}). A compartmental pharmacokinetic analysis was also employed, to further explore potential effects of the Fc mutations on absorption rate. A standard two compartment model, with 1st-order absorption from the subcutaneous site was implemented using a population pharmacokinetic modeling approach in NONMEM VII. Intravenous and subcutaneous data were fit simultaneously for each antibody, and first order absorption rate constants were compared for the wild-type mAbs and T250Q/M428L mutants.

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Note

Supplemental material can be found at: www.landesbioscience.com/journals/mabs/article/19364/
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