Balancing charge in the complementarity-determining regions of humanized mAbs without affecting pI reduces non-specific binding and improves the pharmacokinetics

Amita Datta-Mannan1,*, Arunkumar Thangaraju1,*, Donmienne Leung2, Ying Tang2, Derrick R Witcher3, Jirong Lu3, and Victor J Wroblewski1,*

1Department of Drug Disposition Development/Commercialization; Lilly Research Laboratories; Eli Lilly and Company Corporate Center, Indianapolis, IN USA; 2Department of Biotechnology Discovery Research; Applied Molecular Evolution; Eli Lilly and Company; San Diego, CA USA; 3Department of Biotechnology Discovery Research; Lilly Research Laboratories; Eli Lilly and Company Corporate Center, Indianapolis, IN USA

*These authors contributed equally to this work.

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Abbreviations: IgGs, immunoglobulins; CDR, complementarity-determining region; FcRn, neonatal Fc receptor; K_D, equilibrium dissociation constant; ELISA, enzyme-linked immunosorbent assay; KDa, kilodalton; SPR, surface plasmon resonance; SD, standard deviation; TCA, trichloroacetic acid; 125I, Iodine 125; AUC, area under the curve; IV, intravenous; pl, isoelectric point; PK, pharmacokinetics; TMDD, target-mediated drug disposition; HBSS, Hank’s balanced salt saline solution

Lowering the isoelectric point (pI) through engineering the variable region or framework of an IgG can improve its exposure and half-life via a reduction in clearance mediated through non-specific interactions. As such, net charge is a potentially important property to consider in developing therapeutic IgG molecules having favorable pharmaceutical characteristics. Frequently, it may not be possible to shift the pI of monoclonal antibodies (mAbs) dramatically without the introduction of other liabilities such as increased off-target interactions or reduced on-target binding properties. In this report, we explored the influence of more subtle modifications of molecular charge on the in vivo properties of an IgG1 and IgG4 monoclonal antibody. Molecular surface modeling was used to direct residue substitutions in the complementarity-determining regions (CDRs) to disrupt positive charge patch regions, resulting in a reduction in net positive charge without affecting the overall pI of the mAbs. The effect of balancing the net positive charge on non-specific binding was more significant for the IgG4 versus the IgG1 molecule that we examined. This differential effect was connected to the degree of influence on cellular degradation in vitro and in vivo clearance, distribution and metabolism in mice. In the more extreme case of the IgG4, balancing the charge yielded an ~7-fold improvement in peripheral exposure, as well as significantly reduced tissue catabolism and subsequent excretion of proteolyzed products in urine. Balancing charge on the IgG1 molecule had a more subtle influence on non-specific binding and yielded only a modest alteration in clearance, distribution and elimination. These results suggest that balancing CDR charge without affecting the pI can lead to improved mAb pharmacokinetics, the magnitude of which is likely dependent on the relative influence of charge imbalance and other factors affecting the molecule’s disposition.

Introduction

Monoclonal antibodies (mAbs) represent an important class of therapeutics used in a wide array of disease states.1 Advances in antibody engineering have been invaluable to the success of these biomolecules, enabling humanization, potency and specificity optimization and improvement in their pharmaceutical properties.2 Along with these approaches, mAb engineering to improve pharmacokinetic (PK) properties is also an important consideration during the development of IgG biotherapeutics, allowing optimization of dose, frequency and therapeutic efficacy.3 It is generally well accepted that there are 2 broad mechanisms of IgG clearance: 1) target- (or antigen-) mediated drug disposition (TMDD), and 2) non-specific elimination.3,4 Non-specific clearance refers to the pinocytic uptake of IgGs into cells and subsequent partitioning to the recycling pathway based on FcRn-dependent interactions or...
the degradative (lysosomal) route.\textsuperscript{5} Fc engineering technologies to improve the FcRn binding properties of mAbs have been extensively studied and have shown some success in reducing non-specific clearance.\textsuperscript{6-8} The magnitude of PK improvement derived from IgG-FcRn engineering has been difficult to predict, which may be related to differences in the contribution of non-FcRn-mediated clearance that occurs aside from TMDD.\textsuperscript{11} With regards to this, several recent studies have also implicated modulation of the molecular charge via protein engineering in the variable or constant domain regions as an approach to reduce non-specific IgG clearance.\textsuperscript{9,10,12,13} Both Igawa et al. and Li and coworkers have demonstrated that lowering the isoelectric point (pI) by \~1 unit or more (overall range of 6.1 to 9.2 across studies) through engineering either the variable region or the mAb framework, respectively, slows IgG clearance via a non-FcRn dependent mechanism.\textsuperscript{9,11} These studies suggest reducing the overall net positive molecular charge improves mAb PK via decreased non-specific cellular interactions that, in part, may enhance intracellular IgG uptake or rate of degradation.

From a pragmatic perspective, making frank charge-based changes to a molecule that result in significant beneficial pI shifts, such as in the case of an alternate framework or multiple variable region mutations, is often not possible without loss of target binding activity that occurs due to replacement of critical binding residues or improper CDR conformation.\textsuperscript{12} Even in instances where large beneficial pI changes retain equivalent ability to engage target, multiple residue substitutions in the variable region sequence or constant domains can have a substantial negative effect on the pharmaceutical properties (thermal and chemical stability, solubility, viscosity and homogeneity) and the manufacturability of the molecule. Given these considerations, the goal of our work was to more broadly evaluate the influence of subtle modifications of molecular charge as an alternate strategy for improving the PK properties of IgGs. Along these lines, Sampei et al., found modulating a positive charge patch with the addition of an acidic residue on an anti-FIxa antibody resulted in decreased clearance of the molecule, but did not report whether pI changes were also observed.\textsuperscript{13}

We studied 2 humanized mAbs, an IgG1 (hIgG1) and an IgG4 (hIgG4), that showed unexpectedly rapid clearance in mice. The clearance findings could not be attributed to poor/aberrant FcRn binding properties or target-mediated clearance in either case. The observations and magnitude of effect were attributed to the degree of non-specific cellular binding via solvent-exposed positive charge patches within the CDRs. The effect of balancing the net positive charge on non-specific binding was more significant for the IgG4 vs. the IgG1 molecule, and was gained without a change in the pI of either mAb. This differential effect was connected to the degree of influence on cellular degradation in vitro and in vivo clearance, distribution and metabolism in mice. In the more extreme case of the IgG4, balancing the charge yielded an \~7-fold improvement in peripheral exposure, significantly reduced tissue catabolism and subsequent excretion of the proteolyzed products into urine. Balancing charge on the IgG1 molecule had a more subtle influence on non-specific binding and yielded only a modest alteration in clearance (\~1.4-fold), distribution and elimination in mice. These results support the concept that rational balancing of CDR charge distribution without affecting the pI is a feasible approach to improve IgG PK properties. The consideration of the effect of local charge patch imbalance on the in vivo properties of IgGs may enable development and further engineering of mAbs having PK liabilities tied to this mechanism.

### Results

#### Characterizing the biophysical and FcRn binding properties of the 4 IgGs

Following the construction of our CDR charge-modified IgGs, we characterized a number of properties in vitro that have been shown to have an influence on mAb and protein clearance in vivo,\textsuperscript{2,8} and compared these to each construct’s parental mAb characteristics. The pI values were determined using capillary isoelectric focusing. Our results indicated no major differences in the pI of molecules when compared within each platform (<0.3) (Table 1). A and B had pI values of 8.8 and 9.1, respectively, (Table 1) while C and D had pI values of 9.2 and 9.5, respectively (Table 1).

The $T_{m}$ of the mAbs was determined using differential scanning calorimetry (DSC). No notable differences in $T_{m}$ values were observed in the CH2, CH3 or Fab regions within each platform (Table 1). Hydrophobic interaction chromatography (HIC), showed no association of any of the IgGs in our study to the HIC column (data not shown). The binding affinities of the 4 IgG molecules with immobilized mouse FcRn was measured using previously reported surface plasmon resonance (SPR) approaches.\textsuperscript{14,15} The binding affinity ($K_D$) of the parent IgGs (B

### Description of the IgG molecules

Our study included 2 pairs of humanized IgG molecules with different isotypes, hIgG1 mAbs A and B and hIgG4 mAbs C and D, developed against 2 different undisclosed targets. Molecular models for Fv regions of parental molecules B and D were constructed using the AntibodyModeler module in MOE2012.10, followed by energy minimizations using AMBER99 Force-field. We examined the surface electrostatic-potential of the 2 models using PyMOL and we noticed the striking positively charged areas in the antigen binding regions (Fig. 1). A and C were engineered from B and D, respectively, by making minimal residue substitutions within their CDRs to balance their respective positive charge patches without significant affinity changes. Surface modeling showed that a significant positive stretch in D was dramatically disrupted by 3 residue changes in heavy chain Arg -> Gly, Thr -> Arg and Ser -> Glu, while a single Arg -> Leu mutation in light chain further reduced another positive charge patch (Fig. 1B). On the other hand, A has 3 mutations, including the addition of 2 negatively charged residues Thr -> Asp in heavy chain and Gly -> Asp in light chain, which balanced the charge distribution (Fig. 1A). In our studies, we used these 4 molecules as tools to better understand/characterize the effects of local charges within solvent-exposed regions on the non-specific binding and degradation of IgGs both in vitro and in vivo.
and D) were comparable to their respective mAbs engineered to have reduced solvent-exposed charge patches (A and C) (Table 1). No direct binding to mouse FcRn at pH 7.4 was detected for any of the 4 IgGs (data not shown).

**Interaction of the IgGs with heparin and cells**

Since the IgGs showed similar biophysical and FcRn binding properties, we proceeded to examine these molecules in assays that would differentiate the mAbs based on their CDR charge patch differences. Given the positive charge of the CDRs was modulated, we first examined the interaction of the 4 mAbs with heparin because of its inherent net negative charge and because it is found in abundance on vascular endothelia. Greater binding to heparin-coated plates was observed for both the parental IgGs (B and D) relative to their respective CDR charge patch-modified mAbs (A and C) (Fig. 2). A very strong interaction of D with heparin was observed at concentrations as low as 1.56 µg/ml (Fig. 2B). In contrast, interaction of C (i.e., the CDR charge balanced product of D) with heparin was only discernable at concentrations greater than 25 µg/mL (Fig. 2B). With regards to the other mAb pair, the non-specific binding of B to heparin was greater when compared to A, but these interactions were much weaker than the C and D pair and only discernible at concentrations above 12.5 µg/ml (Fig. 2A).

It is well established that cell membranes are composed of negatively charged components such as heparin, chondroitin and sialic acid. As an orthogonal approach to characterize the charge-based binding of our molecules, we measured the interactions of the 4 IgGs with cultured adherent HEK293 cells. We chose HEK293 cells because they are of human origin and are well behaved, they have been used routinely in cell-based studies and they have been used for assessing non-specific binding previously by others. In addition, HEK293 cells do not express the targets for the IgGs used in our study (data not shown).

**Consistent with the results obtained for the heparin-coated plate binding experiments, we observed greater interaction of both the unbalanced CDR charge patch-containing parental IgGs (B and D) relative to their respective CDR charge patch balanced mAbs (A and C) with HEK293 cells (Fig. 3). Similar to the heparin binding observations, the difference in the interaction with HEK293 cells was more evident for D and C (Figs. 2 and 3). The difference in binding of C and D to the HEK293 cells was observed at much lower concentrations (as low as 1.56 µg/ml, Fig. 3B) than A and B (pronounced at concentrations greater than 6.25 µg/ml, Fig. 3A). It should be noted that differences in detection reagent between assays for IgG1 (A and B) and IgG4 (C and D) in both the heparin and HEK293 binding assays, prevents quantitative comparison of absolute binding across the 2 antibody isotype pairs.**

Table 1. Properties of antibodies A, B, C and D

| Molecule | Mouse Target | Tm (°C) |
|----------|--------------|---------|
|          | FcRn Kd (nM) | Binding Kd (nM) | pi | C<sub>2</sub> | C<sub>4</sub> | Fab |
| A        | 476          | 1.1      | 8.8 | 67 | 83 | 70 |
| B        | 614          | 2.3      | 9.1 | 71 | 83 | 72 |
| C        | 45           | 1.8      | 9.2 | 69 | 72 | 76 |
| D        | 56           | 2.4      | 9.5 | 69 | 73 | 76 |
surface association as a result of the charge-based interactions led to increased mAb uptake by pinocytosis and proteolysis inside cells. We performed mAb degradation experiments by incubating $^{125}$I-labeled IgGs in HEK293 cells or primary mouse liver cells similar to others. For our degradation experiments, we employed the property of permeability (non-residualization) of free $^{125}$I across cell membranes. Intracellular catabolism of $^{125}$I-labeled IgGs releases free $^{125}$I that escapes into the cell medium. The amount of free $^{125}$I representing the concentration of degraded IgG in media at various times following incubation of $^{125}$I-labeled IgGs in cells was measured as trichloroacetic acid (TCA)-soluble radioactivity. As shown in Figures 4 and 5, degradation of the IgGs increased over time. Approximately 3.1-fold greater ($P < 0.001$) degradation was observed for D compared to C after 24 hours of incubation time with HEK293 cells (Fig. 4B). Approximately 4.6- to 5.9-fold ($P < 0.01$) greater degradation was observed for D compared to C in HEK293 cells for time intervals longer than 24 hours and up to 96 hours post-treatment (Fig. 4B). Similarly in mouse primary liver cells, ~4.5- to 6.3-fold greater ($P < 0.001$) degradation of D was observed relative to C (Fig. 5B). Comparable amounts of degradation were observed for A and B for up to 72 hours in HEK293 and primary mouse liver cells (Figs. 4A and 5A). However, 96 hours post-treatment, the degradation of B was ~1.4 fold ($P < 0.05$) greater than the degradation of A in mouse primary liver cells (Fig. 5A). The amount of degradation observed for each molecule was, in general, aligned with the degree of non-specific cell surface interaction of the molecules (Fig. 3). It is unlikely any degradation occurred in the extracellular milieu since there was no evidence of degradation when the molecules were incubated with used culture medium at 37$^\circ$C (data not shown). Additionally, when the mAbs were incubated with HEK293 cells at 4$^\circ$C to reduce fluid phase pinocytosis, binding in the anticipated rank order was observed (D > C and B > A) but there was no measurable degradation (data not shown), which suggests proteolysis occurs inside the cells.

In an effort to characterize the relationship of our in vitro cell binding/degradation findings with the in vivo disposition of the IgGs, we examined the peripheral blood kinetics and the tissue and urinary clearance of the molecules in mice. We used mice because our IgGs either did not cross-react with the murine versions of their targets or because there were insignificant concentrations of the antigens present in mice to influence our clearance observations. In our study, we administered a single intravenous (IV) injection of $^{125}$I-labeled versions of the IgGs to CD-1 mice and measured plasma, liver and kidney concentrations of the IgGs, as well as the proteolyzed fractions in the urine.

Consistent with the significant cellular non-specific binding observed for D, the peripheral clearance of D was very rapid compared to C (Fig. 6B). The concentrations of C and D after 5 minutes post dose reported as a percentage of injected dose per unit gram of plasma (%ID/g) were 70.7 ± 7.7 and 25.0 ± 5.2 %ID/g, respectively. The plasma elimination of D was much greater over the entire time course with the area under the curve (AUC) of D only 15% of that observed with C (Fig. 6 and Table 2). The plasma concentrations for A and B also declined in a biphasic manner (Fig. 6A). Consistent with the slightly improved non-specific binding, the plasma concentrations of A tended to be greater than for B over the time course studied with overall exposure to A approximately 30% higher than for B (Fig. 6). In a comparison of the plasma exposures across IgG isotypes, we observed a ~1.3 fold greater plasma AUC for B compared to C, consistent with the apparently greater non-specific binding of C versus B (Table 2). In separate murine PK studies, the relative plasma exposure data for all the unlabeled compounds were comparable to $^{125}$I-labeled compounds, suggesting that radiolabeling did not change the PK behavior of the compounds and indicating the radiolabel findings are reasonably representative of the unlabeled mAbs (data not shown).

Since there was no evidence of binding to blood components after ex vivo incubation with murine blood (data not shown), the differences in the range of plasma concentrations 5 minutes after dose administration were likely related to differential distribution into or binding to other organs. Therefore, to explore the
biodistribution of our IgGs in tissues, the concentration of the $^{125}$I-labeled molecules was measured in 2 highly perfused organs (liver and kidney) at 6, 24, 72 and 168 hours post dose. Figure 7 shows the liver concentrations of the mAbs reported as a percentage of injected dose per unit gram of tissue (%ID/g). We observed comparable amounts of A (charge balance engineered CDR) and B (charge unbalanced parent) in liver for all times post dose (Fig. 7A). Initially, ~6 hours post dose, the concentrations of A and B in liver were ~6% ID/g (Fig. 7A). Radioactivity in liver declined similarly over time for both molecules to a value of ~2% ID/g after 168 hours post dose. Consistent with the more rapid removal from the circulation, the liver concentration of D (charge unbalanced parent) was greater than C (charge balance engineered CDR) for all times tested (Fig. 7B). At ~6 hours post dose, the concentration of D was 38.8 ± 16.2% ID/g compared to 16.6 ± 6% ID/g for C (Fig. 7B). Both compounds were eliminated from the liver over the remaining time course, with ~1.3% and 15.1% ID/g remaining at 168 hours post dose for C and D, respectively. Of all the IgGs studied, the liver concentrations of D was the greatest, with an ~2- to 6-fold greater distribution in the liver compared to other IgGs initially after 6 hours post dose and an ~6- to 12-fold greater distribution than other IgGs following 168 hour post dose (Fig. 7A and B). Although C exhibited ~2.6-fold greater concentrations in the liver than A or B initially at ~6 hours post dose, the concentrations of C were not different from that of A or B at later time intervals.

Similar to the liver, the distribution of A (charge balance-engineered CDR) and B (charge unbalanced parent) in kidneys were comparable to one another (Figs. 7A and 8A). The concentrations of A and B in kidney were ~6.5- to 9- %ID/g at ~6 hours post dose, and declined over the 168 hour time course (Fig. 8A). At ~6 hours post dose, the concentrations of C (charge balance-engineered CDR) and D hlgG4 (charge unbalanced parent) were 7.5 ± 0.5 and 14.2 ± 5.2% ID/g, respectively (Fig. 8B). The concentrations of C and D also declined over the 168 hour time course. While the concentration of D in kidney was the greatest of all the IgGs tested at 6 hours post dose, the concentrations of C were not different from that of A or B at later time intervals. Over time, the concentration of IgG was less than 1% ID/g. In vivo cellular catabolism of IgG molecules releases free $^{125}$I molecules or small proteolytic fragments (<3 kDa) that escape into plasma and get rapidly excreted in urine. Characterization of elimination of proteolyzed IgGs in urine In vivo cellular catabolism of IgG molecules releases free $^{125}$I molecules or small proteolytic fragments (<3 kDa) that escape into plasma and get rapidly excreted in urine. Overall, in terms of the AUC values (h*%ID/g in Table 2) for the IgGs in each of the compartments, it is apparent that most of the differences between the parental D molecule and the CDR charge balanced C construct are due to the rapid peripheral clearance of D from the plasma/blood and association of the compound with liver (Table 2). When the compartment (i.e., organ) weights are taken into consideration and the data are converted to a percentage of injected dose (%ID) at 5 minutes post dose, D and C show ~25% and 71% ID, respectively, in the plasma (based on an ~1 mL plasma volume) whereas, at 6 hours post dose D and C show ~60% and 26% ID in the liver (based on an ~1.5 g liver weight). In the case of A and B, it is apparent from the tissue data (Figs. 7 and 8) that there were no striking differences from a %ID perspective at any time point in the liver or kidney when the organ weights are taken in account. However, the overall higher AUC values observed in the plasma/blood for the charge balanced A relative to the parental B (Table 2 and Fig. 6) are consistent with the slightly improved non-specific binding of A compared with B, and thus a higher %ID of A is in the peripheral circulation.

Figure 4. Comparison of degradation of (A) $^{125}$I-A and $^{125}$I-B (B) $^{125}$I-C and $^{125}$I-D in HEK293 cells.

Figure 5. Comparison of degradation of (A) $^{125}$I-A and $^{125}$I-B (B) $^{125}$I-C and $^{125}$I-D in mouse primary liver cells.
degraded IgG in urine was observed as early as 8 hours post dose and continued over the 168 hour time course. We observed comparable amounts of catabolized A and B in urine (Fig. 9A), although there was a trend for marginally (~1.1 fold) greater excretion of A over 168 hours (Fig. 9A). For the IgG4 pair of molecules, we observed ~1.7 fold greater amounts of catabolized D compared to catabolized C in urine as early as 24 hours post dose, which continued over the 168 hour collection period (Fig. 9B). Overall, the data suggests that compounds with high non-specific binding are more rapidly degraded and consequently their proteolyzed products are excreted to a greater extent in urine.

### Discussion

Previous studies have suggested that mAbs having basic isoelectric points (pI) can show aberrantly rapid clearance, presumably mediated by positive charge interactions.21,26 Lowering the pI of the mAbs has been shown to modulate this effect, yielding mAbs with more “typical” PK properties.9,11 Both Igawa et al. and Li and coworkers demonstrated that engineering either the variable region or the mAb framework to reduce the pI by ~1 unit or more (overall range of 6.1 to 9.2 across studies) reduces IgG clearance.9,11 Engineering of their mAbs to have pI values in the range of ~6 to 7 improved the exposure of their IgGs by ~2 to 4-fold, with the clearance of the improved mAbs ranging from ~0.1 to 0.2 mL/hr/kg across the studies.9,11 In our studies with B and D, deliberately lowering their overall pI values was not possible without some loss of target binding or compromise of their developability properties and as a result, this parameter could not be tested directly (data not shown). Given this scenario, we decided to investigate the influence of balancing regions of positive charge patches in mAb CDRs (without affecting the pI) on the PK, distribution and elimination in mice. Results from previous studies with pI variants suggested a reasonable mechanism to explain the rapid clearance of mAbs with high pIs in which increased fluid phase endocytosis and degradation is driven by increased cell surface binding.2,5,9,18 However, data directly supporting this hypothesis/mechanism was not provided. As such, we also studied the relation of cell surface interaction/non-specific binding to degradation rates in vitro, and attempted to connect this to tissue distribution and metabolism/elimination in vivo.

We evaluated 2 humanized IgGs, B and D, which displayed unusually rapid clearance in mice (Fig. 6 and Table 2) and other species (data not shown) during their initial development. The two IgGs (B and D) were constructed on different isotype backbones (IgG1 and IgG4, respectively). The rapid clearance of these molecules was not a consequence of target interactions (TMDD) since the mAbs either showed no cross-reactivity with the murine versions of their targets or because there were insignificant concentrations of the antigens present to influence the clearance. The poor PK of B and D was also unrelated to aberrant FcRn binding as both mAbs show receptor binding affinities at pH 6 in the range reported for molecules with well-behaved kinetics, as well as no direct FcRn interactions at neutral pH (Table 1).8 In addition, both mAbs showed reasonable thermal stability (Tm), no differences in hydrophobicity as measured by HIC, good solubility and low aggregation potential (Table 1 or data not shown). The only unusual aspect for these mAbs was the observation of increased interactions with heparin (Fig. 2). Since this biophysical characterization data suggested that charge-based interactions likely play a role in the fast clearance of B and D, we evaluated the influence of CDR charge balancing on this observation.

Although B and D both show poor baseline PK, relative to B, D showed significantly more rapid clearance (Fig. 6 and Table 2). We clearly demonstrate here that our strategy of rationally balancing CDR charge without affecting pI improves the exposure of D within the same range (~7-fold) as previous reports9,11 in which the pI was reduced by at least 1 unit, even though the baseline pI (~9) of D is ~2 to 3 units higher than that reported for molecules with improved kinetics by both Igawa et al. and Li and coworkers.7,13 Surface modeling of D suggests the more rapid clearance is likely due to the larger solvent-exposed continuous stretch of positive residues, which is not found in B (Fig. 1). The larger degree of heparin and cell binding observed for D also suggests the involvement of large continuous stretch of positive residues in the clearance of the molecule (Figs. 2B and 3B). Surface modeling of B shows a moderate positive charge patch region. In contrast, only a slight improvement (~1.4-fold) in exposure was observed when we engineered B to make A. The larger improvement in exposure for C relative to D versus A

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### Table 2. Tissue AUC_{6-168} (hr*%ID/g) for the IgGs

| Compounds | Plasma | Blood | Liver | Kidney |
|-----------|--------|-------|-------|--------|
| A         | 3979   | 1747  | 481   | 535    |
| B         | 2894   | 1127  | 406   | 565    |
| C         | 2193   | 948   | 528   | 505    |
| D         | 324    | 143   | 3405  | 587    |
relative to B is consistent with a greater negative influence of the positive charge stretch in the CDR on the baseline PK of D. The increased heparin and cell-based non-specific binding observed for D supports the idea that, mechanistically, the positive charge patch within D plays a larger role in the clearance of this molecule (Figs. 2B and 3B). Swapping a positive residue (arginine) with a neutral amino acid (glycine) and including a negatively charged glutamate (in place of a serine) appears to aid in breaking up the positive charge stretch in D hlgG4 (Fig. 1B). In the case of B hlgG1, both single residue substitutions within the moderately sized positive charge patch, as well as a swap of a negatively charged residue proximal to the patch, were made. Substituting in negative charge (aspartate) within the moderate positive charge patch creates a more neutral environment, whereas adding more negative charge distal to the positive patch balances the overall positive charge overall within the CDR and generates A without affecting the pI (Fig. 1A).

It is not entirely clear whether the different approaches used to balance the positive charge CDR changes in B and D may, in part, also explain the differential improvements in exposure. Thus, it may be possible to further optimize A and C through additional charge balancing or through some other approach. Retrospectively, we have observed this charge imbalance in several other cases, including mAbs from discovery clones and engineered variants, where clusters of positively charged residues were introduced on the antigen-binding surface. Similar to our observations with the molecules herein, rapid clearance was observed for these other antibodies in vivo (data not shown). Furthermore, since multiple factors in addition to charge-based non-specific binding affect mAb PK, the balance of all these factors may influence each platform to different degrees, making a direct comparison of a single isolated parameter across the platforms challenging.

Given antibody PK is influenced by a number of specific and non-specific factors, delineating mechanisms that affect mAb PK can be challenging, and often requires a number of in vitro and in vivo approaches to tease apart these aspects for further optimization on a case-by-case basis. Sampeii et al. recently reported modulating a positive charge patch with the addition of an acidic residue on an anti-FIXa antibody decreased the clearance of the molecule, but did not report whether pI changes were also observed, making it difficult to compare in the context of our findings.15 The molecules engineered by Igawa et al. and Li and coworkers to improve PK through lowering the pI had clearance values consistent with pure non-specific mAb clearance7,13 (~0.1 to ~0.2 mL/hr/kg), while our engineered molecules still had estimated clearance values in the range of ~0.6 to 1 mL/hr/kg. The higher clearance range of our improved mAbs suggest additional charge balancing may be needed in our molecules or perhaps that factors totally unrelated to charge also negatively affect clearance of the mAbs we studied. It is difficult to fully preclude some unintended ‘specific’ off-target binding may have contributed to our clearance observations. Other investigators have demonstrated that unintended ‘specific’ off-target binding to blood cells or serum/plasma protein explained rapid clearance for a few mAbs22-24 however, these mechanisms do not appear to contribute to our observations. Both the plasma:blood ratios as measured by radioactivity (Table 2) and the lack of the effect of sample processing to serum or plasma (data not shown) on recovered concentrations suggest that specific off-target binding to serum proteins is an unlikely explanation for the fast clearance. Thus, in this report, we attempted to correlate in vitro cell-based binding/degradation assays with in vivo biodistribution/elimination in order to characterize the mechanism(s) behind PK improvements observed through balancing CDR charge.

Previous studies with pI variants have suggested that the slower clearance of mAbs with reduced net negative charge potentially results from several mechanisms: 1) decreased fluid phase endocytosis because of poorer cell binding due to charge repulsion with the negatively charged extracellular membrane2,5,9,18 or 2) decreased lysosomal degradation rates9,11. Our cell-based studies and tissue disposition/excretion experiments are consistent with these proposed mechanisms. The reduced clearance of A and C (relative to B and D, respectively)
is likely a consequence of both these mechanisms, but to variable degrees. In particular, the increased peripheral clearance of D correlates well with the high degree of in vitro cell binding and subsequent degradation. From a conceptual perspective, D binds vascular endothelia non-specifically to a greater extent than C due to the positive charge stretch in the CDR. As a result, the greater degree/stronger of association of D with membrane components leads to the mAb's increased cellular uptake into recycling endosomes, but likely results in reduction of the ability of the FcRn to effectively salvage D. Consequently, intracellular trafficking partitions D away from the recycling pathway and toward lysosomal degradation. Thus, for D, our data suggest the increased magnitude of pinocytic binding/cellular uptake is responsible for the mAb's subsequent greater degradation within lysosomes.

In contrast, the reduction in the net positive charge via charge balancing in the CDRs of C reduces the magnitude of the molecule's non-specific binding to cells (endothelia) and decreases its uptake into tissue. In a similar respect, the weaker interactions of C with membrane components may allow more efficient salvage/recycling via the FcRn mechanism, and consequently improved PK properties compared with D. The very strong interaction of D appears to be a key factor in the findings and the observed in vivo benefits of CDR charge balancing. In this regard, while B certainly demonstrates increased non-specific cell interactions, the magnitude of this effect is clearly more subtle and occurs at higher concentrations compared to the IgG4 isotype pair. This seems to translate to only slight trends in increased tissue concentrations of B relative to A (CDR charge balanced-engineered molecule) and very subtle differences in degradation and elimination in vivo. Both our cell-based findings and in vivo data suggest that, although B has greater non-specific binding related to its CDR charge patch, which facilitates enhanced cellular uptake both in vitro and in vivo, the magnitude and strength of the interaction of B with membrane components appears to drive only slight differences relative to the CDR charge balanced A. This seems to make sense if one considers both the preponderance of the interaction at the cell surface and reduced potential influence on intracellular degradation pathways as discussed above.

In summary, the findings in this report suggest there are many nuances around applying antibody engineering to improve mAb PK properties. Applying a rationally-based approach to modify regions with high positive charge with minimal residue changes can affect the in vivo performance of a mAb. Since additional characteristics of the mAb (stability, biophysical properties, FcRn binding) can influence disposition and elimination, it will be interesting to investigate the relative roles of these mechanisms to ultimately design/engineer molecules with increased therapeutic value.

### Materials and Methods

#### Cell culture for protein expression

293EBNA cells were maintained at 37°C under 5–8% CO₂ conditions in Dulbecco’s modified Eagle’s medium/F-12 (Gibco) supplemented with 20 mM HEPES (Gibco), 5 μg/mL nucellin (Eli Lilly and Company), 0.4 μg/mL tropolone (Sigma Aldrich), 0.075% (w/v) F68 (Gibco) and 50 μg/mL Geneticin (Sigma Aldrich).

#### Construction, expression and purification of recombinant proteins

The Fab regions were obtained from humanized libraries from antibody discovery at Eli Lilly & Co. These were cloned into DNA expression vectors containing either human IgG1 or IgG4 backbone using standard molecular biology approaches and confirmed by DNA sequencing. All the IgGs were expressed in 293EBNA cells and purified from culture supernatants using Protein-A Sepharose (GE Healthcare) affinity chromatography followed by size exclusion chromatography methods. Recombinant soluble mouse FcRn was expressed in 293EBNA cells transfected with plasmids encoding for the soluble portion of αFcRn and B₂-microglobulin, and the protein was purified as described previously.  

#### Evaluation of the FcRn binding affinity

The interaction of the A, B, C and D with recombinant, immobilized mouse FcRn (mFcRn) was monitored by SPR detection using a BIACORE 3000 instrument (GE Healthcare) as described previously. Briefly, recombinant soluble mFcRn was immobilized to flow cell 2 of a CM5 sensor chip using amine coupling chemistry (GE Healthcare). The FcRn immobilization surface density was approximately 300 RU. The first flow cell was used as a blank control surface lacking FcRn. All binding experiments were performed with compounds dissolved in running buffer [phosphate-buffered saline (PBS) with 0.005% Tween 20, pH 6 or PBS with 0.005% Tween 20, pH 7.4] and the samples were run at a flow rate of 100 μL/min for 30 seconds with a dissociation time of 10 minutes. PBS (pH 7.4) was used as dissociation buffer. PBS with 0.005% Tween 20, pH 6 was used as running buffer for the experiments performed to determine the affinity of IgGs to mFcRn. A concentration range of

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*Figure 9. Cumulative excretion of proteolyzed species of (A) 
^{125}I-A and 
^{125}I-B (B) 
^{125}I-C and 
^{125}I-D in urine following 8, 24, 48, 72, 96, 120, 144 or 168 hours post dose.*
0.00316 μM to 3.16 μM of each of the IgGs was used to estimate the association and dissociation constants. The binding data were obtained by subtracting the signal of flow cell 1 (blank flow cell not coupled with FcRn) from flow cell 2. Kinetic (association and dissociation) data were then simultaneously fit to a heterogeneous binding model for IgG-FcRn interactions (BLAevaluation, Ver. 4.1). The data curves for binding and dissociation phases of the sensorgrams for the IgGs at pH 6.0 had low residuals and low χ² values. The mean of K_D values accounting for the greatest fraction of binding from 2 independent experiments were reported.

**Evaluation of mAb isoelectric points (pIs)**

The pIs of the mAbs were determined by capillary isoelectric focusing (cIEF), PA 800 plus Pharmaceutical Analysis System (Beckman Coulter). Samples were prepared by mixing 5–10 μg sample with 200 μL of 3 M urea-cIEF gel, 12.0 μL of Phama-lyte 3–10 (GE Healthcare), 20.0 μL of cathodic stabilizer (500 mM arginine), 2.0 μL of anodic stabilizer (200 mM amidodiacetic acid), 2.0 μL of each of 5 pI markers (pI 10.0, 9.5, 7.0, 5.5, and 4.1), and vortexing for 15 seconds. The electrophoresis and data collection were performed using a focusing step voltage 25 kvolts for 15 minutes, a chemical mobilization step voltage 30 kvolts for 30 minutes, a UV detection at a wavelength 280 nm and a data collection rate of 2 hertz. The cartridge temperature was 20°C and the sample storage temperature was 10°C. The data were analyzed by using 32 Karat program (Beckman Coulter).

**Evaluation of thermal stability (T_m)**

Thermal stability of samples was measured using a TA Instruments NanoDSC equipped with an autosampler. Samples diluted to 0.5 mg/mL in PBS were heated from 20 to 110°C at a rate of 1°C/min under 45 psi of pressure. Sample scans were buffer blank subtracted, converted to molar heat capacity, and fit to a 2-state model with 3 transitions representing the CH₂, CH₃ and Fab domain unfolding to obtain T_m.

**Binding to heparin-coated plates**

Heparin binding plates (BD Biosciences, Catalog no 354676) were coated overnight with 10 μg/ml of heparin (Sigma, Catalog no H3149). The plates were washed 4 times with 1X TBS Tween wash buffer (Teknova, Catalog no T0310) and blocked with PBS-casen blocking buffer (Pierce, Catalog no 37528). The plates were washed again and incubated with 1.56–100 μg/ml of A, B, C or D in blocking buffer at room temperature for 2 hours. The plates were washed and horseradish peroxidase conjugated mouse anti-human IgG (Southern Biotech, Catalog no. 9040-05) was added at a dilution of 1:5000 in HBSS+50 mM HEPES+1% ovalbumin (Sigma-Aldrich, Catalog no. A5503) (pH 7.4) at 4°C on ice for 2 hours. Following incubation, the plates were washed. Horseradish peroxidase-conjugated mouse anti-human IgG (Southern Biotech) was added at a dilution of 1:5000 in HBSS+50 mM HEPES+1% ovalbumin (pH7.4) to the plates on ice and incubated at 4°C on ice for 1 hour. The plates were washed and freshly prepared TMB substrate was added to develop the color. The reaction is stopped by adding stop solution and the plates were read in a Spectramax plus microplate reader.

**Radiolabeling of compounds with Iodine ¹²⁵I**

Approximately 2 -2.5 mg of A, B, C or D in PBS were labeled with ¹²⁵I-Nal (Perkin Elmer) using Pierce iodination beads (Thermoscientific, Catalog no 28665) or Pierce pre-coated iodination tubes (Thermoscientific, Catalog no 28601) following the manufacturer’s protocol to obtain a specific activity of ~0.07 μCi/μg or ~0.5 μCi/μg for use in in vivo plasma/tissue distribution studies or in vitro degradation. The labeled proteins were purified with a PD10 desalting column (GE healthcare, catalog no 17-0851-01) and stored at 4°C. The radiochemical concentration was determined by gamma counting using Wallac 1470 automated gamma counter (Perkin Elmer). The purity of the labeled proteins were determined by TCA precipitation or a size-exclusion high performance liquid chromatography with a gamma detector (Agilent). The percentage of free ¹²⁵I was less than 3% in all preparations.

**Determination of non-specific binding and degradation by cells**

- HEK293 cells (human embryonic kidney cells) were obtained from American Type Culture Collection and maintained in growth medium (MEM medium (Thermoscientific, Catalog no. SH30564.01) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin). Mouse primary liver cells were isolated from CD1a male mice in a similar fashion as described previously and maintained in growth medium (William’s E medium containing 10% heat-inactivated FBS, 5 μg/ml insulin, 5μg/ml transferrin, 5 gm/ml sodium selenite, 10 ng/ml dexamethansone, 2 mM L-glutamine and 50 μg/ml gentamicin) until used in degradation experiments.

For the non-specific binding assays, HEK293 cells were plated in 96-well plates at a density of ~75000 cells and grown to confluence for 2 to 3 d. On the day of the experiment, the medium in the wells were removed and the cells were incubated 1.56–100 μg/ml of A hIgG1, B hIgG1, C hIgG4 or D hIgG4 in HBSS+50 mM HEPES+1% ovalbumin (Sigma-Aldrich, Catalog no. A5503) (pH 7.4) at 4°C on ice for 2 hours. Following incubation, the plates were washed. Horseradish peroxidase-conjugated mouse anti-human IgG (Southern Biotech) was added at a dilution of 1:5000 in HBSS+50 mM HEPES+1% ovalbumin (pH7.4) to the plates on ice and incubated at 4°C on ice for 1 hour. The plates were washed and freshly prepared TMB substrate was added to develop the color. The reaction is stopped by adding stop solution and the plates were read in a Spectramax plus microplate reader.

For the degradation assays, HEK293 cells or mouse primary liver cells were plated in growth medium in 6-well tissue culture plates at a density of 357,000–500,000 cells/well. After 24 or 72 hours following plating, the growth medium in the wells were aspirated and fresh growth medium containing approximately 3 mM of ¹²⁵I-A, ¹²⁵I-B, ¹²⁵I-C or ¹²⁵I-D was added, and the plates were then incubated in a 37°C CO₂ humidified incubator. An aliquot (~100 μl) of the medium on top of the cells was taken in a sterile manner once in every 24 hours following treatment up to 96 hours. The aliquot of medium was precipitated using 15% trichloroacetic acid and the radioactivity associated...
with the pellet and supernatant were counted in a gamma counter. The trichloroacetic acid soluble radioactivity (supernatant) is represented as a percentage of total radioactivity (pellet + supernatant).

Radiolabel mAb mouse pharmacokinetic and tissue distribution studies

PK studies were conducted in male CD-1 mice (21–33 g) (Harlan, Indianapolis, IN) that were 5 weeks of age at the initiation of treatment. 0.1 ml of 5 mg/mL sodium iodide (NaI) was administered via intraperitoneal injection at a 72, 48, 24 and 0.5 hours prior to dose administration and at 1 hour post dose till the end of the study. Water containing 20 mM NaI was provided ad libitum to block excess uptake of free iodide into thyroid. A single IV dose of each of the 125I-radiolabelled compounds dissolved in PBS (pH 7.4) were administered as a bolus injection via lateral tail vein at a dose level of 3.6 mg/kg (0.26–0.27 mCi/kg 125I activity, ~7 μCi per mouse). At various time intervals (i.e., 0.083, 6, 24, 48, 72 and 168 h) blood were collected from 3 animals per treatment group for each time point into tubes containing potassium EDTA as coagulant and processed to plasma. Urine was collected from 3 animals per treatment group after 8 and 24 hours post dose and then once every 24 hours till the end of the study. At various time intervals (i.e., 6, 24, 72 and 168 h), 3 animals from each group were sacrificed. Liver and kidney were collected and weighed in polypropylene tubes. 125I radiotactive counts from blood, plasma, liver, kidney and urine samples was measured in a Wizard 1480 or Wizard 2470 automatic counter (Perkin Elmer). The radioactive counts measured per gram (ml) of each sample (blood, plasma, liver and kidney) or the total radioactive counts in urine per animal was normalized to the injected dose (ID). Phoenix (Pharsight, Version 6.3) was used to estimate the area under the curve for all the radiolabelled compounds in plasma, liver and kidney by performing non-compartmental analysis.

Statistical analysis

Significant values (P values) were calculated using either two-tailed pairwise Student’s t-test. Data with a P value < 0.05 were considered significant.

Disclosure of Potential Conflicts of Interest

At the time of the manuscript preparation all authors in this report were employees of Eli Lilly and Company, Indianapolis, IN. AT is currently employed at Hospira Healthcare India Pvt Ltd, Chennai, India. The authors do not have any conflict of interest or financial disclosure to report.

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