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Minipig as a potential translatable model for monoclonal antibody pharmacokinetics after intravenous and subcutaneous administration

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Abbreviations: A_{abs}, amount of the drug in the absorption site depot; ATA, anti-therapeutic antibodies; F, bioavailability/fraction absorbed; β_2m, β_2-microglobulin; CL, clearance; CL_{humans}, clearance in humans; $CL_{minipigs}$, clearance in minipig; C_c, drug concentration in the central compartment; C_p, drug concentrations in the peripheral compartment; CRO, contract research organizations; ELISA, enzyme linked immunosorbent assay; FC, flow cell; FcRn, neonatal Fc receptor; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; IV, intravenous; Q, inter-compartmental clearance; pI, isoelectric point; K_a, rate of absorption; K_D, equilibrium dissociation constant; mAb, monoclonal antibody; PK, pharmacokinetic; PD, pharmacodynamic; RU, response unit; SC, subcutaneous; SPR, surface plasmon resonance; V_c, volume of distribution of the central compartment; V_p, volume of distribution of the peripheral compartment; w, scaling exponent for clearance

Subcutaneous (SC) delivery is a common route of administration for therapeutic monoclonal antibodies (mAbs) with pharmacokinetic (PK)/pharmacodynamic (PD) properties requiring long-term or frequent drug administration. An ideal in vivo preclinical model for predicting human PK following SC administration may be one in which the skin and overall physiological characteristics are similar to that of humans. In this study, the PK properties of a series of therapeutic mAbs following intravenous (IV) and SC administration in Göttingen minipigs were compared with data obtained previously from humans. The present studies demonstrated: (1) minipig is predictive of human linear clearance; (2) the SC bioavailabilities in minipigs are weakly correlated with those in human; (3) minipig mAb SC absorption rates are generally higher than those in human and (4) the SC bioavailability appears to correlate with systemic clearance in minipigs. Given the important role of the neonatal Fc-receptor (FcRn) in the PK of mAbs, the in vitro binding affinities of these IgGs against porcine, human and cynomolgus monkey FcRn were tested. The result showed comparable FcRn binding affinities across species. Further, mAbs with higher isoelectric point tended to have faster systemic clearance and lower SC bioavailability in both minipig and human. Taken together, these data lend increased support for the use of the minipig as an alternative predictive model for human IV and SC PK of mAbs.

Introduction

The primary advantages of monoclonal antibodies (mAbs) as therapeutic molecules are their target specificity and their prolonged serum persistence. During the development of mAb therapeutics, the selection of a relevant preclinical animal model is essential for the prediction of the human pharmacokinetic (PK) profile, as well as for assessing overall safety and exposure-response relationships prior to clinical studies.[1-3] Moreover, a predictive preclinical PK model is also important for understanding the impact of various molecular properties on the in vivo behavior of mAbs, thus enabling optimization of therapeutic candidates in early development.

Therapeutic mAbs are typically administered via intravenous (IV) infusion or subcutaneous (SC) injection. While the PK properties of mAbs following IV administration are well predicted by studies in non-human primates,[4-6] a reliable animal model to predict the PK of mAbs following SC administration

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has not been clearly established.[7] Relatively little is known about the mechanism of SC absorption of mAbs in different species. The major parameters that affect this process are thought to include the role of lymph and blood capillaries in systemic absorption, cross-species differences in hypodermis morphology and physiology, drug formulation, stability of the molecule, the site of injection, the depth of injection, as well as the molecular properties of the mAbs themselves.[8-11] Published data suggest that animal models are not necessarily reliable predictors of antibody SC PK in humans as there is often no apparent relationship in SC bioavailability between humans and animals.[7] Indeed, for larger biotherapeutics, absolute SC bioavailability has been reported to be higher in cynomolgus monkeys than in humans, while for biotherapeutics with molecular weights <40 kDa no clear relationship was obvious between human and animal data, both in rodents and cynomolgus monkeys.[7] Given these discrepancies, it is believed that the lack of predictability could be attributed to differences in hypodermis structure and physiology between humans and rodents or non-human primates.[12]

With the increasing ethical concerns regarding the use of primates in non-clinical testing, attention has been increasingly focused on the potential use of minipigs as non-rodent alternatives for pharmaceutical testing.[13-17] As such, minipigs are becoming more frequently used for toxicological and PK studies of small molecules.[18-20] Recently, minipigs have been used to test the immunogenicity of therapeutic proteins;[21,22] however, limited data exist for the pharmacokinetics of macromolecules in minipigs.[23-25] In particular, to the best of our knowledge, there are no studies that describe the PK of mAbs in minipigs. The combined results from previous studies have demonstrated similarities between pig/minipig and human skin and lymph architecture[26,27] that are likely key contributors to SC absorption and bioavailability of macromolecules. In addition, the thickness of the epidermis and the stratum corneum as well as the lipid composition of the stratum corneum is similar between human and pigs, which has led to the frequent use of the pig/minipig model for dermal administration.[28] The pig also has a tight link between dermis and underlying muscle, which is similar to the human situation, and these features have been attributed to the arrangement of elastic fibers in the hypodermis/subcutis, the target for SC administration.[29] The structure of the hypodermis in pigs differs from that in furred animals like rodents and cynomolgus monkeys, which have less abundant elastic fiber, and thus looser skin.[30] Similar to humans, the dermis of pigs is connected to the deep fascia via a fibrous network in the hypodermis.[29] Finally, pigs/minipigs have an easily accessible SC space with a tissue thickness similar to humans, making this species suitable to mimic the human situation for SC administration of proteins in preclinical studies. Because of these similarities, it is hypothesized that the PK of macromolecules in minipigs following SC administration may better resemble the SC PK in humans than other commonly used laboratory animals.

A robust preclinical model for predicting human PK of mAbs is also desirable to study the effects of various molecular properties of mAbs on their in vivo behaviors. Advances in antibody engineering technologies have enabled the development of a diverse class of humanized and human antibodies consisting of IgGs with such altered properties as different molecular weights, domain architectures, glycosylation patterns, electrical charge, subclasses and interactions with Fc receptors or target molecules.[31] As a result, the impact of these properties on the PK and distribution properties of IgGs is of keen interest. In particular, it has been well established that the binding of the IgG Fc domain to the neonatal Fc receptor (FcRn) plays a principal role in the long serum persistence of IgG by salvaging it from a default catabolic pathway in the vascular endothelium and bone-marrow derived cells.[32-37] Hence, altering the binding affinity of IgG to FcRn can have a significant impact on serum IgG half-life, particularly in the case where affinity is reduced.[38] In addition, previous studies also indicate that nonspecific electrostatic interactions caused by differences in cell membrane surface charge and antibody charge can affect the tissue distribution and pharmacokinetics of mAbs.[39] For instance, modification of the isoelectric point of an antibody of approximately one pI unit or more can result in significant differences in its PK properties.[39-41] Therefore, for successful non-clinical PK evaluation of mAbs, understanding the various characteristics of the mAb, such as pI, specific and non-specific binding, and FcRn affinity, can be helpful.

In this study, the PK of a series of therapeutic mAbs following IV and SC administration was assessed in Göttingen minipigs. The tested mAbs are active against soluble or membrane-bound targets with indications in oncology, inflammatory diseases or metabolic diseases. The derived PK parameters were compared with the known PK properties of these mAbs in humans. In addition, given that binding to FcRn plays an important role in the disposition and serum half-life of mAbs,[32,37] the binding affinity of these antibodies to human, pig and cynomolgus monkey FcRn was compared. Further, the impact of various characteristics of these mAbs, including FcRn binding, isoelectric point (pI) and in vitro blood cell and plasma protein binding, on their PK properties were evaluated. The goal of this study is to pave the way for further evaluation of minipig as a potential translatable model for human PK of mAbs.

Results

Pharmacokinetic study in minipigs after intravenous and subcutaneous administration. The pharmacokinetic properties of eight therapeutic human IgG antibodies were evaluated in Göttingen minipigs after IV and SC administration. The PK of one additional mAb (mAb8) was tested after IV injection only. Profiles of the mean serum/plasma mAb concentration vs. time after a single IV or SC administration for each mAb are presented in **Figure 1**. The estimated PK parameters from a compartmental analysis of both IV and SC data are summarized in **Table 1**.

Following IV injection, the serum/plasma concentration of most molecules tested exhibited a biphasic PK profile typical for mAbs, with a relatively rapid distribution phase, slow elimination phase and log-linear terminal phase with no signs of nonlinear pharmacokinetics. An exception is adalimumab, whose PK appeared to be affected by anti-therapeutic antibodies (ATA).

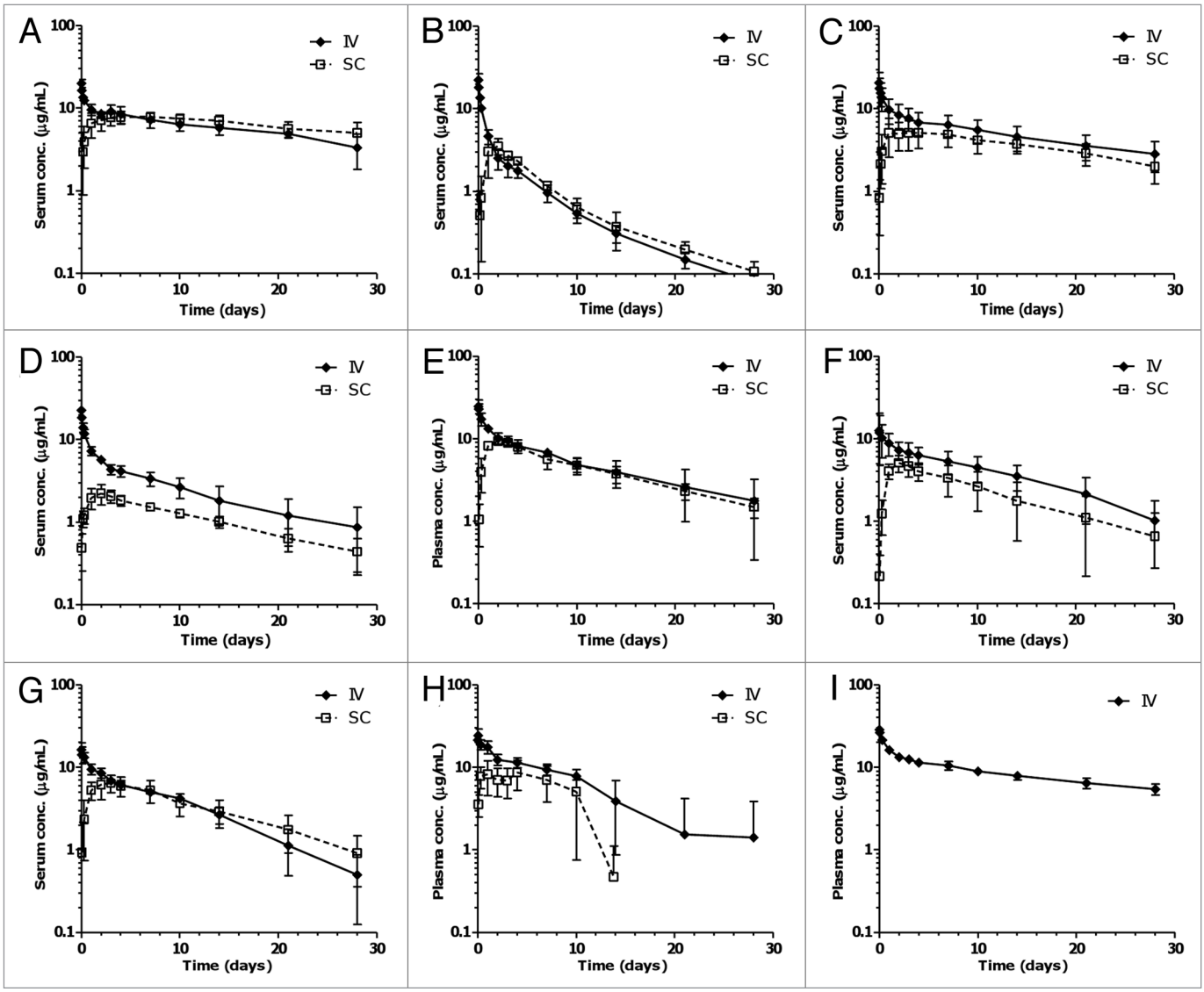

Figure 1. Dose-normalized plasma or serum concentration-time curves following intravenous (closed symbols) or subcutaneous (open symbols) administration to Göttingen minipigs of (A) mAb1, (B) mAb2, (C) mAb3, (D) mAb4, (E) mAb5, (F) mAb6, (G) mAb7, (H) adalimumab and (I) mAb8 (intravenous dosing only) (mean ± SD).

In addition, mAb7 showed a slight trend of nonlinearity in the terminal phase after IV injection. The elimination half-lives of the various mAbs were relatively long, ranging from 6.9 to 26 d. The central volume of distribution (V_c) ranged from 36 to 62 mL/kg, consistent with the expected range of plasma volumes in animals.[42] The systemic clearance (CL) was slow, ranging from 2.5 to 11 mL/day/kg for most molecules, except for mAb2 which had an unusually fast CL of 36 mL/day/kg.

Following SC injection, the antibodies were slowly absorbed into the systemic circulation with a median time to maximum concentration (T_{max}) between 1 and 4 d and with estimated rates of absorption ranging from 0.32 to 4.6 d^{-1}. With the exception of adalimumab, whose PK appears to be affected by anti-therapeutic antibodies (ATA), the elimination profiles for SC and IV administration were nearly parallel for all mAbs tested, suggesting similar systemic elimination kinetics following SC or IV injections of these mAbs. The estimated systemic bioavailability (assessed as drug fraction absorbed by compartmental modeling) following SC injection was variable among different molecules, ranging from 36 to 98%.

Comparison of minipig and human pharmacokinetic properties. *Allometric scaling of clearance.* In order to examine whether minipigs can be used to predict the clearance of mAbs in human, the correlation between the clearance rates of mAbs observed in this study and those previously obtained in human studies was examined using interspecies scaling. The results are tabulated in **Table 2**. The calculated allometric scaling exponent (w) ranged from 0.75 to 1.17, with an arithmetic mean value of 0.98 and a standard deviation of 0.16, using data from the eight mAbs in the present study. MAb2 was excluded from the calculations due to an abnormally fast clearance in minipigs, albeit normal clearance in humans. Further, analysis of linear correlation between the CL

Table 1. Minipig studies: Pharmacokinetic parameters after intravenous and subcutaneous dosing for different monoclonal antibodies (mAbs)

mAb	CL[a,c] (mL/day/kg)	Q[a,c] (mL/day/kg)	V_c[a,c] (mL/kg)	V_p[a,c] (mL/kg)	$T_{1/2\beta}$[a,d] (days)	K_a[b,c] (1/day)	F(%)[b,c,e]	T_{max}[b,f] (days)
mAb1	2.83 ± 0.60	128 ± 25	51.9 ± 2.2	54.6 ± 9.9	26.2	1.02 ± 0.33	97.5 ± 15.1	4
mAb2	36.4 ± 2.2	24.2 ± 8.9	56.4 ± 4.1	134 ± 16	6.86	0.316 ± 0.042	79.9 ± 9.4	2
mAb3	4.48 ± 0.75	72.5 ± 18.0	36.6 ± 13.5	70.9 ± 12.9	17.1	0.635 ± 0.244	65.8 ± 14.4	2
mAb4	11.1 ± 1.0	199 ± 94	47.3 ± 1.3	97.8 ± 8.3	9.29	1.61 ± 0.34	36.3 ± 2.1	2
mAb5	6.07 ± 0.97	16.0 ± 4.3	44.7 ± 3.1	37.0 ± 14.1	10.1	0.862 ± 0.129	81.2 ± 5.7	2
mAb6	8.06 ± 0.81	10.2 ± 3.1	58.5 ± 3.5	26.0 ± 4.9	7.91	0.530 ± 0.090	68.9 ± 5.5	2
mAb7	5.06 ± 0.46[g]	17.6 ± 3.0	61.6 ± 2.5	37.4 ± 3.7	n.a.[h]	0.828 ± 0.157	85.4 ± 2.6	3
mAb8	2.45 ± 0.12	27.6 ± 2.8	36.0 ± 1.1	36.4 ± 1.8	20.9	n.a.	n.a.	n.a.
adalimumab	3.48 ± 0.49	24.0 ± 18.2	55.4 ± 7.8	13.5 ± 5.1	13.8	4.61 ± 0.65	82.9 ± 8.3	1

n.a. = not available; [a]for both IV and SC animals; [b]only for SC animals; [c]mean ± standard error of the population mean parameters estimated from compartmental PK analysis; [d]mean (standard error not available since $T_{1/2\beta}$ is a derived parameter); [e]percentage of drug absorbed; [f]median observed T_{max}; [g]Nonlinear PK; only linear clearance is tabulated (nonlinear clearance parameters: V_{max}: 165 μg/day/kg, K_m: 8.27 μg/mL); [h]not reported due to nonlinear pharmacokinetics.

Table 2. Allometric scaling of clearance for various monoclonal antibodies from minipigs to humans

mAbs	Body weight (kg)	Observed CL (mL/day/kg)		Allometric scaling exponent (w)[a]
	minipigs	minipigs	humans	
mAb1	9.4	2.83	2.15[b]	0.86
mAb2	9.5	36.4	2.68[b]	n.c.
mAb3	8.2	4.48	6.09[b]	1.14
mAb4	8.4	11.1	11.5[b]	1.02
mAb5	9.1	6.07	4.5[b,c]	0.85
mAb6	8.9	8.06	4.79[b]	0.75
mAb7	8.4	5.06[d]	3.8[b]	0.86
mAb8	9.7	2.45	3.31[b]	1.15
adalimumab	10.2	3.48	4.82[e]	1.17
Mean				0.98
SD				0.16

n.c., not calculated; [a]assume human body weight of 70 kg; [b]from population PK analysis using compartmental modeling (unpublished data); [c]Nonlinear PK due to target-mediated disposition; only linear clearance is reported; [d]Nonlinear PK; only linear clearance is tabulated; [e]from den Broeder et al.[60]

observed in minipigs and humans showed a positive correlation between the two (r^2 = 0.69) (**Fig. 2A**).

Comparison of SC bioavailability and absorption rate. The available human SC bioavailability/fraction absorbed (F) and rate of absorption (K_a) data for these mAbs are summarized in **Table 3**. The correlation between human and minipig bioavailability is shown in **Figure 2B**. There is a weak correlation between minipig and human SC bioavailability (r^2 = 0.32) (**Fig. 2B**). In general, molecules with higher bioavailability in minipig tend to have higher bioavailability in human, although a few molecules showed higher bioavailability in minipigs than in humans. Out of the six mAbs with both human and minipig SC bioavailability data, one mAb had similar bioavailability in human and minipig (mAb4), one had lower bioavailability in minipig (mAb5) and the remaining four had higher bioavailability in minipig. Notably, three mAbs with the highest bioavailability in minipig (mAb1, mAb5 and adalimumab) also had the highest bioavailability in human. The SC rate of absorption is higher in minipigs than that in humans for all molecules (**Tables 1 and 3**).

Correlation between systemic clearance and bioavailability. Interestingly, there is an apparent correlation (r^2 = 0.82) between the systemic clearance and bioavailability (after excluding mAb2 which had an unusual PK in minipig) such that as clearance increases, the bioavailability tends to decrease (**Fig. 3A**). However, from the limited data available, no obvious correlation was observed in human (**Fig. 3B**).

Effect of FcRn binding, pIs and blood/plasma protein binding on pharmacokinetics. To compare the FcRn binding properties across species and to determine if differences in FcRn binding affinity could account for observed differences in PK parameters, we evaluated the binding affinity of each IgG to recombinantly-expressed human, pig and cynomolgus monkey FcRn via surface plasmon resonance (BIAcore). Purified FcRn was injected over immobilized IgGs, and the equilibrium dissociation constant (K_D) for each interaction was calculated using a simple 1:1 binding model (**Table 4**). Values for K_D are reported in nM and standard errors appear in parentheses for each value. No significant cross-species variability in the IgG-FcRn binding affinity was observed for any of the IgGs tested. In addition, binding across different IgGs was fairly consistent for a given species of FcRn. No apparent correlation was found between FcRn binding affinity and the clearance or SC bioavailability of the various mAbs in minipig or human (data not shown).

To assess the potential impact of mAb pI on PK properties, the pI values for all mAbs were obtained either experimentally by IEF or from the literature and are shown in **Table 4**. Interestingly, a clear trend was observed: mAbs with higher pI values (>~9.0) appeared to have faster clearance (after excluding mAb2 with unusually fast clearance in minipig) and lower bioavailability in both human and minipig, with the exception of mAb5 (**Fig. 4**).

To examine the effect of blood and/or plasma protein binding on PK behavior of the mAbs, in vitro binding studies were

conducted in minipig whole blood. The results demonstrate that none of the mAbs bound to blood cells (**Fig. 5A**) or plasma proteins from minipig, as determined by size-exclusion HPLC (**Fig. 5B**).

Discussion

Characterization of serum clearance, SC bioavailability and systemic absorption of mAbs in relevant animal models is crucial for designing improved formulations or drug delivery systems, as well as for the interpretation of exposure-response relationships and development of useful PK models with predictive value.[8] Preclinical experimentation remains an essential component of antibody PK testing, but there is no requirement to solely rely on traditional animal species.[43] As established models, rodents and monkeys are generally the default animal species of choice to characterize mAb PK. However, there are known differences in the skin architecture and physiology between humans and rodents or non-human primates,[12,30] and the predictability of these species for human SC PK of macromolecules has been poor.[7] The minipig is a potentially better predicative animal model given its similarity in skin physiology to human.[26-29] To date, there has been no published data on the PK of mAbs in minipig, and no systematic study to compare the applicability of minipigs and non-human primates as translatable preclinical models for this class of biotherapeutics.[44] Our present study was conducted to fill this gap.

Cross-reactivity of the respective mAb to its target has to be considered when conducting PK studies in animals, since target-mediated disposition can play a relevant role in the PK of mAbs in vivo. The cross-reactivity of the tested mAbs in minipigs was not studied, but it was not expected in this non-primate species. Also the appearance of the concentration-time curves gave no indication for a nonlinear pharmacokinetics, as they showed a log-linear elimination phase after IV administration. Thus, any potentially existing target-mediated CL pathway was saturated at the doses used in our study. An exception to this was mAb7, for which a nonlinear pharmacokinetics was evident and considered in the compartmental PK model. Thus, except for mAb7, minipig data reflect linear CL. In order to ensure an appropriate comparison between minipig and human CL data, human CL data at doses that saturated the target-mediated CL were used in the analysis for mAbs with relevant target-mediated CL in humans. The results from this study showed that, in general, the CL of mAbs in minipig is predictive of that in human, with an estimated allometric scaling exponent of 0.98 (**Fig. 2A and Table 2**) in the absence of relevant contributions from target mediated drug-disposition to the overall clearance. The inter-species scaling of the CL of mAbs have been evaluated in previous studies in reference 4, 5 and 45. These studies suggested that cynomolgus monkeys can be successfully used to predict human CL of therapeutic antibodies using an allometric scaling exponent of 0.85. The data suggest that the minipig may be used as an alternative animal model to project human CL for mAbs. It is of note, however, that these projections address only the linear clearance processes, but not target-mediated, nonlinear clearance processes,

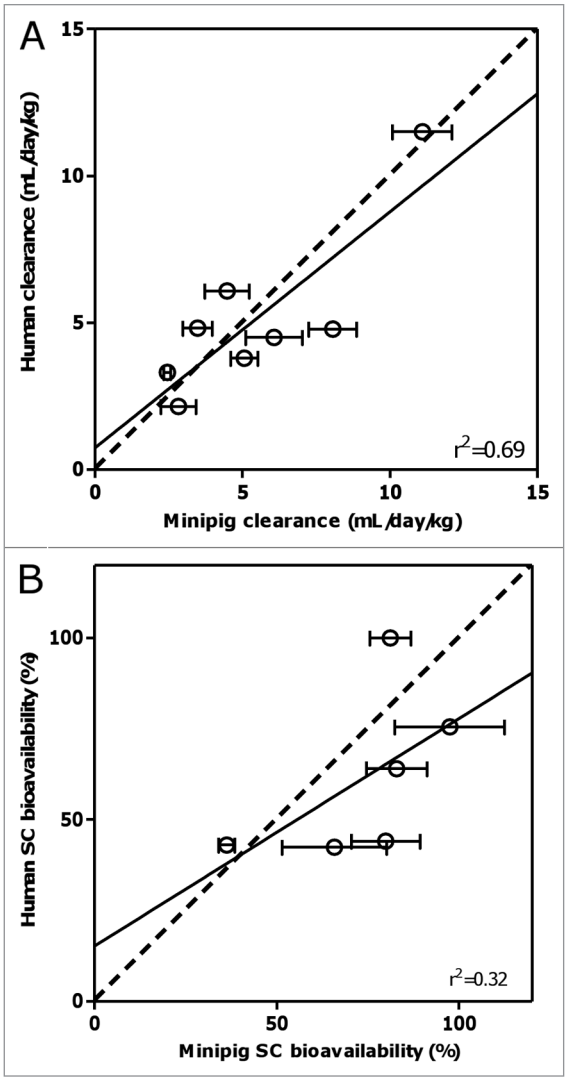

Figure 2. Correlation between minipig and human (A) clearance and (B) subcutaneous bioavailability following intravenous and subcutaneous administration of various monoclonal antibodies. Circles represent the reported mean parameter values while error bars represent the standard errors of the estimate; solid lines represent the linear regression lines (with r^2 values displayed), and dashed lines represent the hypothetical line where the minipig and human values are identical. Note: in cases when more than one bioavailability value was reported, the average of all the reported values was used for the correlation analysis. For (A), mAb2 is excluded.

which depends on the cross-reactivity of the mAb to its target in the minipig species. Allometric scaling of nonlinear CL, however, is hampered by the potential interspecies differences in target expression, turnover and affinity and has not yet been successful.[4]

Conversely, a weak correlation observed between the SC bioavailability of mAbs in minipig and human was observed (**Fig. 2B**), although it should be noted that the number of molecules available for this analysis was quite limited. Out of the six mAbs tested, one had similar bioavailability in minipig and human, one had higher bioavailability in human and four had higher bioavailability in minipig. Notably, three mAbs with the highest bioavailability in minipig (mAb1, mAb5 and adalimumab)

Table 3. Human rate of absorption and bioavailability after subcutaneous dosing for different monoclonal antibodies

mAbs	K_a (1/day)	F(%)[a]
mAb1	0.213[b]	75.6[b]
mAb2	0.251[b]	44.1[b]
mAb3	0.308[b]	42.4[b]
mAb4	0.321[b]	28–58[b,c]
mAb5	0.221[b]	100[b]
adalimumab	n.a.	64[d]

n.a.: not available; [a]percentage of drug absorbed; [b]from population PK analysis using compartmental modeling (unpublished data); [c]58% and 28% estimated for single and multiple dosing respectively (unpublished data); [d]from prescribing information.

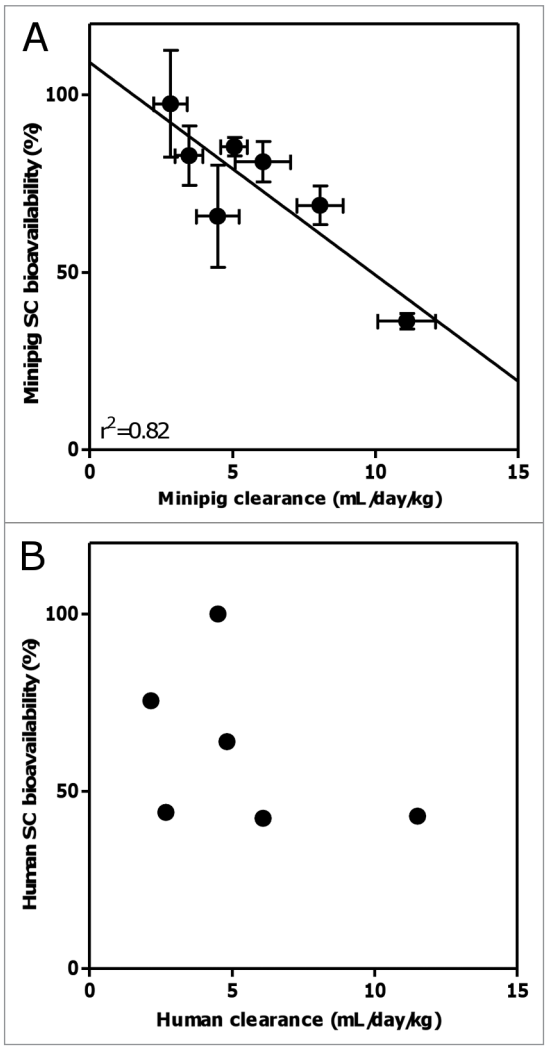

Figure 3. Correlation between clearance and subcutaneous bioavailability of the various mAbs in (A) minipig and (B) human. Black dots represent the reported mean parameter values while error bars represent the standard errors of estimate. For (A), mAb2 is excluded; solid line represents the linear regression line (with r^2 value displayed).

also had the highest bioavailability in human. Also, in the correlation of SC data from minipigs and humans, the potential role of target-mediated disposition needs to be considered. However, the chosen compartmental PK assessment and PK parameters for description of SC absorption (fraction absorbed and absorption rate constant) are independent of systemic target-mediated disposition processes. Thus, these parameters are still useful when assessed in a non-responder species, unless there is relevant target expression in the hypodermis or draining lymph vessels. The latter may lead to a relevant target-mediated 'first-pass clearance' during the absorption phase, which would reduce the fraction absorbed.

Recently published work by McDonald et al. described the SC bioavailability of therapeutic proteins between different species and showed that four out of the five marketed monoclonal antibodies reviewed had higher bioavailability in cynomolgus monkeys than in humans, with adalimumab having markedly higher bioavailability (96%) in cynomolgus monkeys.[7] Only one of the five had similar bioavailability in both species.[7] Of the mAbs discussed in this report, our in-house data suggest that two mAbs (mAb2 and mAb3) had higher SC bioavailability in cynomolgus monkeys (85% and 74%, respectively, unpublished data) than in humans. For these three antibodies (adalimumab, mAb2 and mAb3), the SC bioavailabilities measured in minipigs are closer to those obtained in humans than those obtained in cynomolgus monkeys. Hence, the results presented here provide initial evidence to suggest that minipig may be a better predictive model for human SC bioavailability of mAbs than cynomolgus monkeys although additional data are needed to confirm this. Interestingly, a clear relationship was found between clearance and SC bioavailability in minipigs when mAb2 is excluded due to its unusually fast clearance (**Fig. 3A**). This relationship suggests that the same molecular characteristics or pharmacokinetic processes may determine systemic clearance and SC absorption, the latter being reflected in the SC bioavailability.

Generally, the rate of absorption following SC administration was about 2- to 5-fold higher for most monoclonal antibodies in minipigs than in humans (**Tables 1 and 3**). This is consistent with previous studies demonstrating that the SC rate of absorption of recombinant human erythropoietin across species scales inversely with body weight.[46] Although the exact cause for the faster absorption in minipigs is not known, differences in mAb transport through the extracellular matrix of the SC space prior to lymphatic uptake[12] or differences in the lymphatic transport[8-10] may be involved. Nevertheless, it is unlikely that transport of the antibodies through lymphatic vessels is the rate-limiting step for SC absorption because the residence time in both the macro- and micro-lymphatic systems is estimated to be on the order ~1 h,[47-51] which is much shorter than the time scale of SC absorption of mAbs (on the order of days).

The serum clearance, SC bioavailability and rate of absorption of therapeutic mAbs could be affected by many factors, including, but not limited to, nonspecific binding, development of immunogenicity, target-mediated disposition, affinity to FcRn, pI, the site of SC injection and injection depth.[7] In this study, an unusually fast clearance was observed for mAb2 in minipig

despite normal clearance in human and cynomolgus monkey (data not shown). While the exact cause of the fast clearance of this mAb in the minipig is unknown, it is potentially due to non-specific binding in minipig tissues causing fast elimination of the antibody, given its clean blood/plasma protein binding profiles (**Fig. 5A and B**). The fast clearance of antibodies due to off-target binding has been previously reported in both rodents and cynomolgus monkeys.[52,53] The unexpected fast clearance of mAb2 in our study is likely a non-generalizable phenomenon specific to this particular antibody, although further monitoring of mAb PK in minipig is warranted. In addition, the PK profiles of adalimumab in minipigs indicate the presence of ATAs, consistent with findings reported in reference 21. The effect of ATA on PK of mAbs may be addressed by excluding the periods of accelerated clearance due to ATAs from the PK assessment prior to scaling, as the ATA formation against a human mAb in a laboratory animal bears no relevance to the human situation.[54] Further, target-mediated disposition can also play a major role in the clearance of mAbs in vivo. In such cases, a binding species would be more appropriate for studying the target-mediated clearance of mAbs, whereas non-binding species (such as minipig) can still be useful to investigate the linear portion of the clearance pathway. These confounding factors should be considered when using minipig as a predictive model for evaluating human clearance of mAbs.

Since FcRn protects circulating IgG from systemic elimination by recycling it away from the default catabolic pathway in vascular endothelial cells and bone marrow-derived cells,[32,37] altering the binding affinity of IgG to FcRn can have a significant impact on the PK of monoclonal antibodies.[38,55] In the study reported here, we characterized the in vitro IgG-FcRn interaction using recombinantly expressed human, pig and cynomolgus monkey FcRn in order to systematically investigate the role of FcRn in regulating the serum PK of IgG across these three species. Given the sequence identity between human and pig FcRn (~76%) and the presence of canonical intracellular trafficking motifs in the pig FcRn cytoplasmic tail (di-leucine and WXXϕ motifs),[56] it is hypothesized that the binding, trafficking and IgG salvage behavior could be similar between the pig and human/cynomolgus monkey FcRn. Indeed, our current results indicate that, as expected, the binding affinities of the human IgG molecules tested are similar across these three species (**Table 4**), thus providing additional rationale for the use of minipigs and cynomolgus monkeys as a translatable species for mAb PK. Notably, to our knowledge, this is the first controlled study to test the affinities of clinically used therapeutic mAbs to FcRn in a comparative PK study across humans, cynomolgus monkeys and pigs.

In addition to the similar binding properties of these IgGs to FcRn, no apparent correlation was observed between the FcRn binding affinity and PK of the mAbs in minipigs or human. This is consistent with recent findings that 3- to 4-fold differences in FcRn-binding affinity arising from mutations in the Fc region of a single human IgG did not result in significant PK differences in cynomolgus monkeys.[34] This lack of a correlation suggests that small differences in FcRn binding may not be the major driver for the differences in the PK of mAbs in this case, and that unexpected differences in PK among antibodies with identical constant regions may be due to non-FcRn-dependent mechanisms, such as non-specific or target-mediated clearance.[41]

Table 4. Isoelectric points (pI) and in vitro FcRn binding affinities (K_D) at pH 6 (mean and standard error)

mAbs	pI	Human FcRn K_D (nM)	Pig FcRn K_D (nM)	Cynomolgus monkey FcRn K_D (nM)
mAb1	6.1	320 (6)	288 (5)	289 (5)
mAb2	8.7	440 (7)	396 (7)	384 (4)
mAb3	9.1	380 (5)	354 (6)	355 (5)
mAb4	9.3	426 (8)	362 (6)	398 (7)
mAb5	9.4	545 (10)	486 (10)	502 (11)
mAb6	9.2	409 (9)	342 (9)	375 (6)
mAb7	8.9	382 (6)	330 (6)	347 (7)
mAb8	8.7	n.a.	n.a.	n.a.
adalimumab	8.8[a]	515 (9)	491 (9)	490 (9)

[a]from Santora et al.[61]

We next sought to determine if the PK differences in the mAbs may be due to differences in their pI values since changes in isoelectric point have been shown to affect the PK behaviors of intact antibodies.[39-41] The pI values of the mAbs used in this study varied moderately, ranging from 6.1 to 9.4 (**Table 4**). Interestingly, a clear trend was observed for most mAbs tested (with the exception of mAb5), with higher values pI (greater than ~9.0) tending to be associated with faster systemic clearance rates and lower SC bioavailabilities in both humans and minipig (**Fig. 4**). This trend is consistent with previous findings showing that increases in the pI of antibodies resulted in increased blood clearance and decreased half-life in vivo due to the nonspecific electrostatic interactions between the anionic cell membrane surface and the antibody.[39-41] While these previous observations were primarily made in rodents, our study confirmed these findings in minipigs and humans, utilizing a set of antibodies with different specificities and frameworks. In addition, we demonstrated that pI not only affects systemic clearance, but also potentially SC bioavailability. It is worth noting that in vitro binding studies showed no evidence of protein or cell binding in the minipig plasma or whole blood, respectively, for any of the antibodies tested (**Fig. 5**), suggesting that the postulated nonspecific interactions driven by mAb pI may take place primarily in tissue, or that the affinity of these interactions may be below the assay detection limit. Taken together, the current study suggests that the characteristics/processes governing systemic clearance and SC bioavailability of the mAbs tested herein are less likely to be dependent upon FcRn, plasma protein or blood cell binding and are possibly related to the electric charge of antibodies and its effect on electrostatic interactions with negatively charged cell surfaces.[39] On the other hand, the lack of a clear correlation between human clearance and SC bioavailability may be due to other factors contributing to the clearance or absorption processes, such as target-mediated disposition.

To our knowledge, this study represents the first comprehensive evaluation of the minipig as a potential translational model

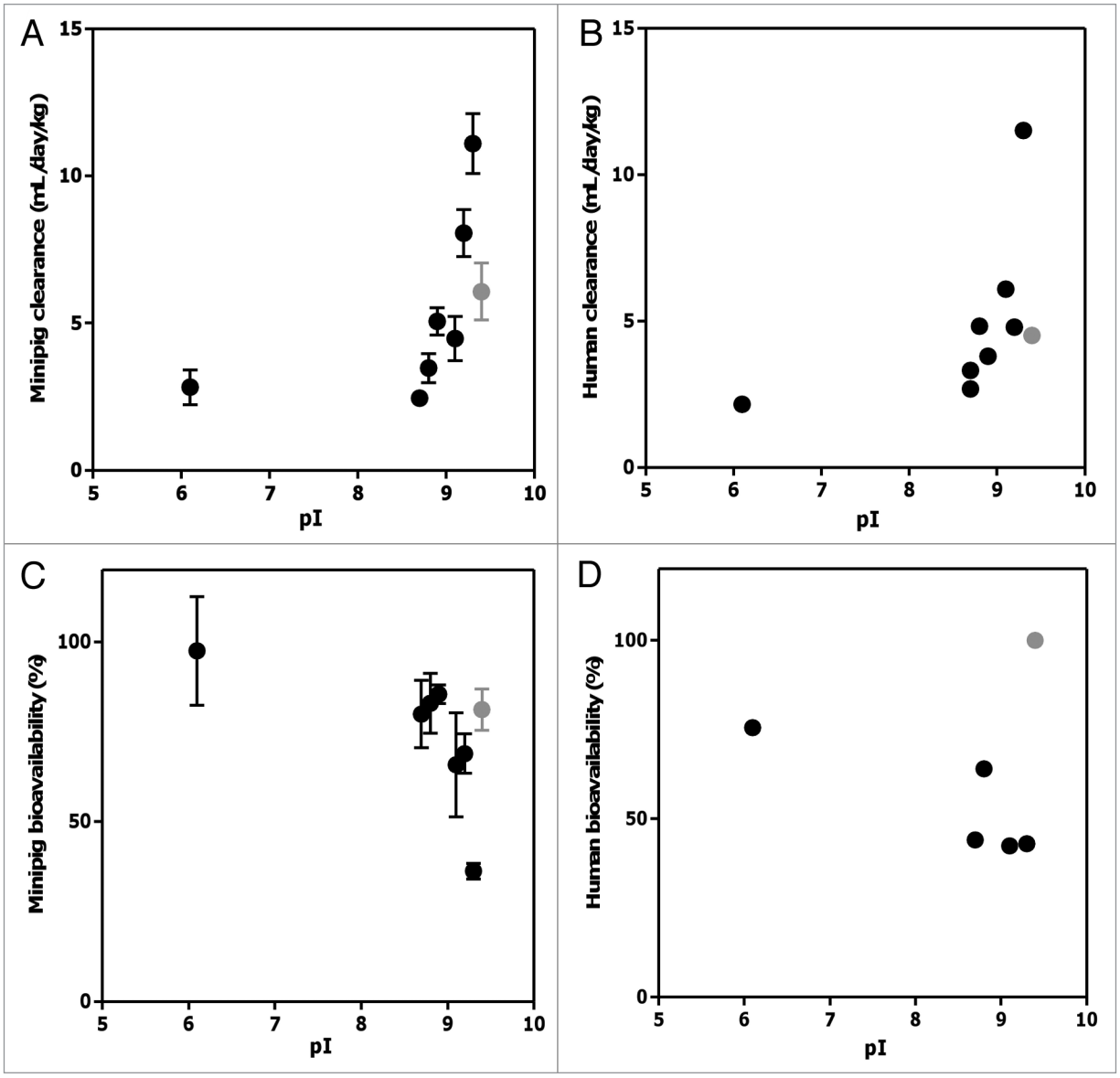

Figure 4. Correlation between pI and clearance in minipig (A) and human (B), and SC bioavailability in minipig (C) and human (D) of various monoclonal antibodies. Solid dots represent the reported mean parameter values while vertical error bars represent the standard errors of estimate. For (A), mAb2 is excluded. Grey dots represent mAb5 (outlier) whereas black dots represent all other mAbs.

for human IV and SC PK of therapeutic monoclonal antibodies. The results support the minipig as an alternative, less expensive and sufficiently predictive model over other commonly used species (e.g., cynomolgus monkeys) for evaluation of linear clearance rates and SC bioavailability of mAbs. This is further supported by the similar binding properties of human and pig FcRn for the mAbs tested herein. Moreover, both the systemic clearance and SC bioavailability of most antibodies tested appear to correlate with their pI values, suggesting the nonspecific electrostatic interactions may play a role in both processes in minipigs and humans. Taken together, this study serves as a starting point for the evaluation of the minipig as a potential alternative or better translatable model to predict IV and SC PK of mAbs in humans.

Materials and Methods

Antibodies. This study used recombinant IgG mAbs that were produced at Genentech or Hoffmann-La Roche (with the exception of adalimumab, which was produced by Abbott Pharmaceuticals and obtained from commercial sources). All non-disclosed mAbs except one were humanized IgGs, while adalimumab is a human IgG. The non-disclosed mAbs were of either IgG_1 or stabilized IgG_4 subtype, with a molecular weight around 150 kDa. Antibodies were expressed in Chinese hamster ovary cells and were purified using Protein A affinity chromatography followed by size exclusion chromatography. All materials and reagents used for this study were formulated and ensured to be pyrogen-free, either by limulus amebocyte lysate test or

material certification. Concentration was measured for all the mAb formulations, which are specified in **Table 5**.

Animals. Female and male Göttingen minipigs were purchased from Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark or Marshall BioResources, North Rose, NY. Animals were examined and weighed on the day following receipt, and were allowed to acclimate to the laboratory environment for 15–20 d prior to the first day of dosing. Prior to study initiation, animals were also trained repeatedly to be accustomed to the handling procedures for dosing and blood sampling. While on study, animals were housed individually in swine cages or housed jointly in the respective dose group. Housing and care were as specified in the applicable USA-, Danish and UK-regulations.

Intravenous and subcutaneous pharmacokinetic studies in minipigs. The PK studies of all nine IgGs were conducted in Göttingen minipig across four contract research organizations (CRO): Pipeline Biotech, Trige, Denmark; Charles Rivers Labs, Ohio, USA; Covance Laboratories, Harrogate, UK; and LAB Research, Lille Skensved, Denmark. Before each study began, the animals were quarantined and acclimated for at least 7 d. During this period, they were weighed, physically examined by a staff veterinarian and determined to be healthy at the beginning of each study. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee at each CRO. Minipigs were assigned into groups of three to five animals each, with similar average body weight in the SC and IV groups for the respective test substance. Animals received a single IV bolus dose of test article either via jugular cannula or via a catheter in the ear vein. SC dosing was done in the scapular (20 g needle) or inguinal area (27 g needle) (**Table 5**). Blood samples were collected from the femoral or jugular vein for each animal at pre-dose and multiple time points up to 28 d post-dose. Following the final blood collection on Day 28, all surviving animals were either returned to the stock population of the respective laboratory or euthanized by sodium pentobarbital injection followed by exsanguination and discarded.

Samples collection and processing. For preparation of serum samples, PK blood samples were collected into serum separator tubes (with clot activator) or into plain tubes. Samples were allowed to clot at room temperature for at least 20 min, but no longer than 1 h. The clotted samples were maintained at room temperature until centrifuged, commencing within 1 h after the collection time, at a relative centrifugal force of 2,000x g for 10 min in a refrigerated centrifuge set to maintain 4°C or, alternatively, at 3,500x g for 10 min at room temperature. The serum was separated from each of the blood samples within 20 min after centrifugation and transferred into two approximately equal aliquots of 0.5 mL each. Samples were held on dry ice until stored in a freezer set to maintain -60°C to -80°C. In the studies with adalimumab, mAb5 and mAb8, plasma was prepared using EDTA or heparin as anticoagulants.

Analysis of serum/plasma samples. Serum or plasma samples were assayed for mAbs 1 through 7 concentrations using enzyme linked immunosorbent assays (ELISA), where each analyte was captured using a recombinant human protein specific for that analyte. MAb8 and adalimumab were analyzed with a generic human IgG ELISA using an anti-human Fc antibody for both capture and detection. Minimum quantifiable concentrations for each assay were: mAb1, 65 ng/mL; mAb2, 280 ng/mL; mAb3, 81 ng/mL; mAb4, 81 ng/mL; mAb5, 391 ng/mL; mAb6, 500 ng/mL; mAb7, 20 ng/mL; mAb8, 12.5 ng/mL; and adalimumab, 12.5 ng/mL.

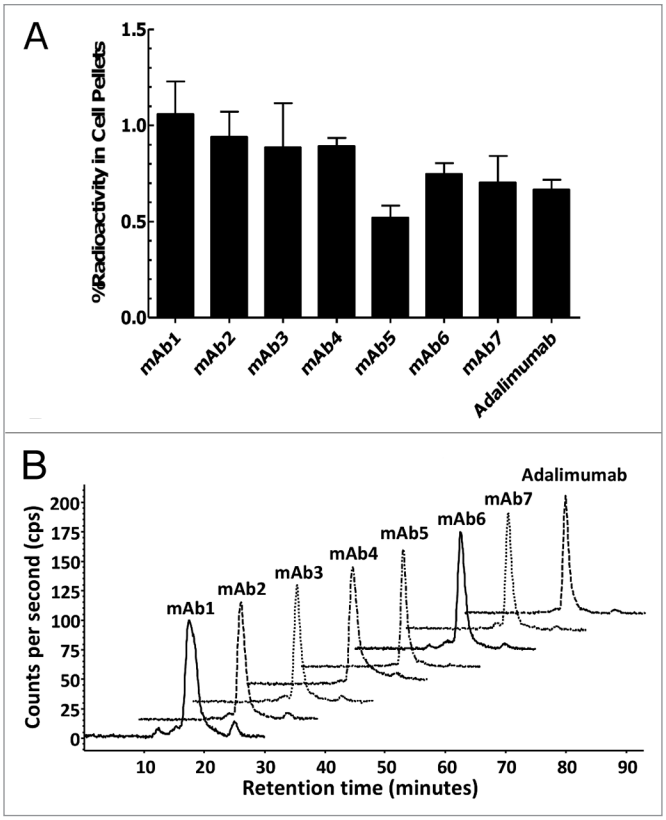

Figure 5. (A) Percent radioactivity remaining in the cell pellets following a 1 h incubation at 37°C of radioiodinated antibodies in minipig whole blood. (B) Size-exclusion HPLC radiochromatograms of [^{125}I]-antibodies in plasma isolated from minipig whole blood following 1 h incubation.

Pharmacokinetic data analysis. The serum mAb concentration-time data for all mAbs following IV or SC administration were analyzed by compartmental PK analysis using nonlinear mixed effects modeling. A two-compartmental model with first-order absorption and elimination kinetics was used to simultaneously fit the concentration-time profiles from individual animals following a single IV or SC injection for most antibodies. For mAb7, an additional nonlinear elimination term was included. The structural model is composed of two compartments with linear clearance from the first compartment ("central compartment") and linear inter-compartment exchange. Additionally, first-order absorption from an absorption site compartment was assumed in the case of SC administration. The structure model is demonstrated in:

Table 5. Experimental conditions of in vivo pharmacokinetic studies in Göttingen minipigs

mAbs	Concentration (mg/mL)	IV administration				SC administration				
		Mean body weight (kg)	Gender	n	Dose	Mean body weight (kg)	Gender	n	Dose	Administration site
mAb1	25 (IV) 125 (SC)	9.4	F	4	5 mg/kg	9.9	F	5	5 mg/kg	scapular
mAb2	150	9.5	F	5	5 mg/kg	9.7	F	5	5 mg/kg	scapular
mAb3	150	8.2	F	5	5 mg/kg	8.3	F	5	5 mg/kg	scapular
mAb4	150	8.4	F	5	5 mg/kg	8.4	F	5	5 mg/kg	scapular
mAb5	180	9.1	M	3	20 mg/kg	8.9	M	5	180 mg	inguinal area
mAb6	120	8.9	F	5	10 mg/kg	8.6	F	5	120 mg	inguinal area
mAb7	120	8.4	F	5	9 mg/kg	8.2	F	5	108 mg	inguinal area
mAb8	120	9.7	M	5	10 mg/kg	n.a.	n.a.	n.a.	n.a.	n.a.
adalimumab	50	10.2	F	3	40 mg	10.0	F	3	40 mg	inguinal area

n.a.: not applicable; [a]Excluded one animal with dosing problem.

$$\frac{dA_{abs}}{dt} = -K_a \cdot A_{abs} \qquad \text{(SC only)}$$
$$V_c \frac{dC_c}{dt} = -Q \cdot (C_c - C_p) - CL \cdot C_c + F \cdot K_a \cdot A_{abs}$$
$$V_p \frac{dC_p}{dt} = +Q \cdot (C_c - C_p) \qquad \text{Eq. 1}$$

where A_{abs} is the amount of the drug in the absorption site depot, C_c and C_p are the drug concentrations in the "central" and "peripheral" compartments, respectively. The structural model parameters included clearance (CL), volume of distribution of the central compartment (V_c) and peripheral compartment (V_p), inter-compartmental clearance (Q), first-order rate of absorption (K_a) and bioavailability or fraction absorbed (F) for subcutaneous administration. In the case of mAb7, the linear clearance term was substituted by the sum of a linear and a saturable (nonlinear) term as follows:

$$CL = CL_{lin} + \frac{V_{max}}{K_M + C_c} \qquad \text{Eq. 2}$$

Inter-individual differences of parameters were modeled by log-normal distribution. Proportional and additive error models were used for the residual errors of the observed concentration data. For mAb1–4 the modeling was performed using NONMEM (version VI; UCSF; San Francisco, CA), using the FOCE method. For mAb5–8 and adalimumab, the nonlinear mixed effect modeling software MONOLIX 3.1 [57] was used running on MATLAB 7.9.0.529 (R2009b) (The MathWorks, Inc., Natick, MA USA), with the structural model implemented in MLXTRAN. The observed time to maximum concentration (T_{max}) values were obtained directly from the observed data using WinNonlin Pro (version 5.0.1; Pharsight Corporation; Mountain View, CA USA) or ToxKin 3.5 (Entimo, Berlin, Germany).

Inter-species scaling of CL. The clearance data in minipigs ($CL_{minipigs}$) were extrapolated to clearance in humans (CL_{humans}) using the allometric equation:

$$CL_{humans} = CL_{minipigs} \cdot \left(\frac{Body\ weight_{humans}}{Body\ weight_{minipigs}} \right)^{w} \qquad \text{Eq. 3}$$

where w is the scaling exponent for clearance. Based on the observed mean $CL_{minipigs}$ and CL_{humans} and the average body weights of the minipigs in the respective studies and the typical body weight of humans (70 kg), w was calculated for each antibody using **Eq. 3**. Human CL data at doses that saturated the target-mediated CL were used in the analysis for mAbs with relevant target-mediated CL in humans.

Cloning of human, pig and cynomolgus monkey FcRn. The coding regions of the cynomolgus monkey, human and pig FcRn α-chain ectodomain and the cognate full-length β_2-microglobulin light chain (β_2m) genes were generated by gene synthesis (Blue Heron, USA). The coding regions of FcRn and recombinant β_2m were subcloned into a previously described pRK mammalian cell expression vector.[58] For expression and purification of FcRn constructs, human embryonic kidney cells 293 were transfected using FUGENE (Roche) according to the manufacturer's protocol. After 24 h of incubation with transfection complexes, cells were switched to serum-free PSO4 medium (Genentech; 1 g/L Pluronic F-68, 5.5 g/L combination nonselect medium (Life Technologies), 4.3 g/L glucose, 1.22 g/L sodium bicarbonate, 0.1 g/L gentamicin sulfate (pH 7.1); 350 milliosmolar) supplemented with 5 mg/L recombinant bovine insulin and trace elements and grown for 7 d. Cells were collected by centrifugation and soluble FcRn was purified from the culture supernatants by pH-dependent binding to human IgG-Sepharose (Amersham). Briefly, supernatants were acidified to pH 5.8 with 50 mM MES and flowed over a 4 mL hIgG-Sepharose column at ~1.5 mL/min. After washing with >10 column volumes of wash buffer (20 mM MES, 150 mM NaCl, pH 5.8), bound FcRn was eluted

with 20 mM HEPES, 150 mM NaCl, pH 8.0. Eluted FcRn was concentrated and further purified by size exclusion chromatography on a Superdex 200 column (Amersham) with PBS pH 6.0 as the running buffer. Fractions containing monomeric FcRn were pooled, and the concentration was determined on a Nanodrop 8000 spectrometer (Thermo Scientific) using the species-specific mass extinction coefficient at 280 nm.

Affinity measurements binding to human, pig and cynomolgus monkey FcRn. Binding kinetics and affinity studies were performed on purified FcRn by surface plasmon resonance (SPR) using a Biacore T-100™ instrument (GE Healthcare, Piscataway, NJ). All experiments were performed at 25°C. IgGs (5–10 μg/mL) were immobilized onto three of the individual flow cells (FC) of a Series S CM5 sensor chip (GE Healthcare), using a standard amine coupling procedure according to the manufacturer's protocol, with FC1 serving as the reference flow cell. The immobilization levels were approximately 1,000 response units (RU) per flow cell. Eight serial 3-fold dilutions of each FcRn (10 μM–1.5 nM) were prepared in running buffer (25 mM MES, 25 mM HEPES, pH 5.8, 150 mM NaCl, 0.05% Tween-20) and were injected for 60 sec at a flow rate of 50 μL/minute followed by a dissociation phase of 30 sec. Surfaces were regenerated between cycles by a single injection of running buffer at pH 8.0 (30 sec at 50 μL/min). Raw sensogram data were reduced and referenced using the Scrubber II software package (BioLogic Software, Campbell, Australia) and fit to a simple 1:1 binding model under equilibrium conditions.

In vitro blood binding studies. All antibodies were radioiodinated using the indirect iodogen addition method as previously described in reference 59. The radiolabeled proteins were purified using NAP5™ columns pre-equilibrated in PBS. The specific activities of the antibodies were in the range of 11.5 to 15.5 μCi/μg. Radioiodinated antibodies were spiked into Göttingen minipig whole blood (Bioreclamation LLC, Hicksville, NY) followed by gentle mixing. Three 0.5 mL aliquots of blood were removed and incubated for 1 h at 37°C. The aliquots were centrifuged at 12,800x g for 5 min at 4°C to separate the plasma samples from the cell pellets, after which the cell pellets were washed with 0.5 mL cold PBS. All samples were counted for radioactivity on a gamma counter (Wallac 1480 Wizard 3" EC&G Wallac; Turku, Finland).

The plasma samples were subsequently analyzed by size-exclusion high performance liquid chromatography (HPLC). Size-exclusion HPLC separation was performed on a Phenomenex™ BioSep-SEC-S 3000, 300 x 7.8 mm, 5 μm column. The mobile phase was PBS and the flow rate was 0.5 mL/min (isocratic) for 30 min. The ChemStation analog-to-digital converter was set to 25,000 units/mV, peak width 2 sec, slit 4 nM (Agilent Technologies). Iodine-125 was detected with a raytest Ramona 90 in line with a standard Agilent 1100 HPLC module system. The plasma samples were diluted 1:1 with PBS prior to loading on the column.

pI determination by isoelectric focusing (IEF). The pIs of native mAbs except mAb5 and adalimumab were determined by imaged capillary isoelectric focusing (iCIEF) using an iCE280 analyzer (ProteinSimple, Toronto, Canada). Solutions of anolyte, catholyte and pI markers were purchased from ProteinSimple. The pI of mAb5 was determined by conventional gel IEF on Immobiline™ Dry Strip pH 6–11 gel (18 cm: GE Healthcare Bio-Sciences).

Disclosure of Potential Conflicts of Interest

All authors are employees of Genentech, Inc., a member of the Roche Group or F. Hoffmann-La Roche and are Roche stockholders.

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