Pharmacokinetic de-risking tools for selection of monoclonal antibody lead candidates

Miroslav Dostalek*, Thomayant Prueksaritanontb, and Robert F. Kellev'

aDrug Metabolism and Pharmacokinetics, Global Nonclinical Development, Shire, Lexington, MA, USA; bFaculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand; cDepartment of Drug Delivery, Genentech Inc., South San Francisco, CA, USA

ABSTRACT
Pharmacokinetic studies play an important role in all stages of drug discovery and development. Recent advancements in the tools for discovery and optimization of therapeutic proteins have created an abundance of candidates that may fulfill target product profile criteria. Implementing a set of in silico, small scale in vitro and in vivo tools can help to identify a clinical lead molecule with promising properties at the early stages of drug discovery, thus reducing the labor and cost in advancing multiple candidates toward clinical development. In this review, we describe tools that should be considered during drug discovery, and discuss approaches that could be included in the pharmacokinetic screening part of the lead candidate generation process to de-risk unexpected pharmacokinetic behaviors of Fc-based therapeutic proteins, with an emphasis on monoclonal antibodies.

Introduction

Protein therapeutics is one of the fastest growing sectors of the pharmaceutical industry. Currently, more than 75 therapeutic proteins have been approved worldwide and more than 500 are under different stages of clinical development. This expansion of therapeutic proteins is expected to continue, and sales in 2014 of $289 billion are projected to grow to $445 billion by 2019.1 Most of these therapeutic proteins are monoclonal antibodies and antibody fragments, but cytokines, growth factors, enzymes, hormones, antibody-drug conjugates are also included. They are used for treatment of a wide range of diseases, with cancer, autoimmunity and chronic inflammation being the most common.

Following the clinical success of therapeutic proteins, antibody engineering now enables the design of novel modalities based on modification of the conventional antibody format.2,3 Such engineered, Fc-based therapeutic proteins,4 which may have unnatural formats or unexpected properties, are designed to offer advantages over the conventional antibody either by improving efficacy and pharmacokinetics, allowing alternative routes of administration or production in alternative expression systems.4,5 Currently, only a few of these novel therapeutic proteins have been approved for clinical use (e.g., Kadcyla8, Removab8), but development of new formats is expected to intensify. An unwanted consequence of optimization of these novel proteins appears to be an increased incidence of candidates with unexpected pharmacokinetic properties, typified by fast elimination profiles inconsistent with those normally observed for a typical monoclonal antibody.6 Several publications illustrated challenges of unknown pharmacokinetics due to highly specific off-target binding, some of which had efficacy and safety implications. For example, Bumback et al. reported unexpectedly fast non-specific clearance, poor target tissue distribution and limited efficacy of a humanized monoclonal antibody against fibroblast growth factor receptor 4 in athymic NCr nude mice.7 Antibody recovery from mouse and rat plasma was significantly reduced compared with recovery from cynomolgus monkey or human plasma, suggesting that this could be a rodent-specific finding. This was confirmed by immunoprecipitation studies that revealed an unexpected 37 kDa band, likely derived from the rodent complement C3, as well as in vivo pharmacokinetics studies using C3 knockout mouse model that showed marked reduction in the antibody clearance. Other examples include antibodies targeting respiratory syncytial virus,8 amyloid β (Aβ),9 Factor IXA/ X,10 neuropilin receptor type 1,11 interleukin-21 receptor,12 as well as unspecified targets. The causes of the unexpected pharmacokinetic properties for the majority of molecules described in the literature, and likely many others, often remain unknown.

Although the behavior of conventional monoclonal antibodies is often predictive, which allows expectations for benchmarking to be defined; determinants of their pharmacokinetic processes are often complex and not well characterized. Many biophysical and biochemical factors have been shown to affect pharmacokinetic behavior of these proteins. These include net charge,13 local charge clusters,16,17
hydrophobicity, off-target binding, glycosylation, interactions with the antigen target, and binding to the neonatal Fc receptor (FcRn). FcRn is a membrane-associated heterodimer protein consisting of an Fc-binding α-chain and β2-microglobulin subunit that is widely expressed in various organs. Following systemic administration of Fc-based therapeutic proteins, concentration-time profiles generally show a bi-phasic pattern with a rapid distribution phase to the vascular space, followed by a slower distribution (to tissue) and elimination phase. Due to the large molecular size and polarity of therapeutic proteins, distribution is slow and depends on extravasation into tissue, distribution within the particular tissue and degradation. Therapeutic proteins return to blood via the lymphatic circulation system. They are removed from circulation via several different mechanisms, such as proteolytic degradation, non-specific endocytosis into cellular endosomal compartment followed by lysosomal degradation, Fc-gamma mediated clearance, formation of immune-complexes followed by Fc receptor- or complement-mediated clearance, and specific interaction of the complementarity-determining regions (CDR) with a target.

Because discovery and development of new drugs is cost- and labor-intensive and new discovery methods facilitate identification of a larger number of diverse therapeutic proteins that fulfill target product profile criteria, lead selection processes that minimize failure in clinical development are needed. Early elimination of therapeutic proteins with higher risk of failure can increase the success rate and speed of discovery and development. However, due to time constraints and the availability of only small quantities of each candidate at the drug discovery stage, evaluations are often limited to in silico, in vitro and small animal studies. Jarasch et al. recently reviewed the evaluation of biophysical aspects to aid in lead candidate selection. Here, we focus mainly on tools that could be used to de-risk unexpected pharmacokinetic behavior of therapeutic monoclonal antibodies.

**In silico screening tools**

Described below are multiple in silico methods of pharmacokinetic behavior of therapeutic monoclonal antibodies. Although not perfect, these methods offer a simple way to preselect candidates based on their sequence and modeled structure before more resource-intensive activities are started.

**Sequence-based in silico modeling**

Recent work demonstrated that monoclonal antibodies with identical Fc sequences but different CDR regions differ in their pharmacokinetic parameters. Sharma et al. thus presented a simple in silico tool using amino acid sequence for theoretical calculation of variable domain net charge at endosomal pH (pH = 5.5) and hydrophobicity sum for 3 CDRs (light chain CDR1, light chain CDR3, and heavy chain CDR3) to predict in vivo cynomolgus monkey non-specific clearance of IgG1-based monoclonal antibodies (prediction of human pharmacokinetics from cynomolgus monkey data reported by Hotzel et al.). The net charge was calculated by adding the contribution from all charged amino acids using the known amino acids pKa values and the Henderson-Hasselbalch equation. The hydrophobicity was calculated as \(-\frac{\sum_{i} n_{i} \cdot E_{i}}{\sum_{j} n_{j} \cdot E_{j}}\), where \(i\) and \(j\) represent the hydrophobic and hydrophilic amino acids, respectively, \(n\) represents the number of each amino acid type and \(E\) represents Eisenberg scale values.

They observed that monoclonal antibodies with hydrophobicity sum value of \(> 4.0\) or net charge \(\leq 0\) or \(\geq 6.2\) are at high risk for high non-specific clearance in cynomolgus monkey (threshold of \(> 10\) mL/day/kg for fast non-specific clearance was used by authors). On the other hand, monoclonal antibodies with hydrophobicity sum value \(\leq 4.0\) and net charge value within 0 to 6.2 should exhibit normal clearance (<10 mL/day/kg) in cynomolgus monkey. When testing 61 monoclonal antibodies with IgG1 backbones, the tool was able to predict fast non-specific clearance for 86% and normal clearance for 75% of tested antibodies. False positive data (i.e., predicted to have fast non-specific clearance by the tool but with normal clearance in vivo), however, could result in removing “good” candidate from further testing, and thus the tool should be used for prioritization when candidates with similar properties are available, rather than for candidate selection.

Bumbaca Yadav et al. reported that, after intravenous and subcutaneous administration in cynomolgus monkey, the pharmacokinetics of 2 monoclonal antibodies (humAb4D5–8 (anti-HER2) and anti-lymphotoxin α) and their engineered variants with increased and decreased net charge were consistent with the predictions from the sequence-based in silico model. All antibodies with a variable fragment (Fv) net charge \(> 6.2\) had fast non-specific clearance in cynomolgus monkey (threshold of \(> 8\) mL/day/kg was used by the authors), whereas those with Fv charge between 0 and 6.2 had expected clearance (threshold \(< 8\) mL/day/kg was used by authors). While the reason(s) for the fast clearance is unknown, the data appeared consistent with an electrostatic-based hypothesis in which the antibody with more negative charge is less attracted to the negatively charged peripheral parts of cells within tissue, and thus prone to be cleared more slowly.

Recently, Kelly et al. reported that the hydrophobicity and net charge in certain CDR regions correctly predicted faster clearance in C57BL/6 mice (threshold of \(> 20\) mL/day/kg for fast total clearance was used) for 1 of 7 (14%) and normal clearance for 5 of 9 (56%) monoclonal antibodies when testing 16 human or humanized IgG1 antibodies against multiple targets. There was no correlation between antibody isoelectric point and clearance rate, which could be due partly to the fact that the relationship between surface charge and fast nonspecific clearance (as reported by Sharma et al.) was built using cynomolgus monkey and human data. The interaction of human IgG with human and cynomolgus monkey FcRn is about 15-fold lower in affinity compared with binding to mouse FcRn.
Three dimensional structure in silico modeling

Antibody 3 dimensional (3D) in silico modeling is becoming a critical step in the antibody engineering process as it has been realized that many critical aspects of monoclonal antibodies (e.g., humanization, maturation, charge distribution, presence of residues that may affect immunogenicity, changes in stability based on different pH and temperature) cannot be predicted from simple sequence alone.7,28,29

Sampei et al. used 3D static in silico modeling for optimization of physical and chemical properties of a lead bispecific antibody (ACE910, emicizumab).26 A cluster of positively charged amino acids on the Fv surface of the lead molecule was observed via in silico 3D modeling. The authors neutralized the positive charge cluster by introducing negatively charged residues near the cluster because they observed that such a positive cluster increases non-specific binding of the molecule to extracellular matrix and increases clearance. This engineered change improved apparent clearance of the antibody by 4-fold, but did not compromise target binding.

Static models, however, do not take into account intrinsic flexibility of the antibody, and thus provide little insight into the fluctuating structure.30 The solution dynamics of the antibody are critical for its function (e.g., antigen binding, complement activation) and may also influence pharmacokinetic properties, e.g., due to intra- and inter-domain interactions as observed by Schoch et al.31 They used molecular dynamic (MD) simulations over a period of 100 ns to study the effect of net charge on IgG-FcRn interaction. They analyzed 2 IgG1 monoclonal antibodies, ustekinumab (Stelara®) with elimination half-life of 22 days32 and briakinumab (ABT874) with elimination half-life of 8–9 days.33-35 and observed that during the course of the MD simulation one of the 2 antigen-binding fragments (Fabs) of briakinumab approached FcRn and persisted in that confirmation for the rest of the simulation. In contrast, the Fabs of ustekinumab did not approach FcRn over the course of the simulation. They confirmed that the flexibility of the Fabs in the IgG-FcRn complexes allows stabilizing interaction of the Fv domain with FcRn. Such interaction could prevent efficient dissociation of the briakinumab-FcRn complex at physiologic pH and result in lysosomal degradation and fast clearance.36

Thus, 3D static and dynamic modeling have the potential to help generate more stable and developable candidates.

In vitro tools

Many in vitro tools to categorize biochemical and biophysical features of therapeutic proteins, mainly monoclonal antibodies and Fc-fusion proteins, have been reported in the literature. More specifically, these in vitro tools address binding of the proteins to: 1) non-specific components potentially leading to protein fast clearance, and 2) FcRn, known to protect IgG from catabolism, contributing to its long half-life.36 Most of these tools can be formatted in a high throughput fashion to screen large numbers of candidates, and use very small amounts of materials available during early discovery stage. Details on their applicability are described below.

Non-specific binding assays

Baculovirus particles-based ELISA assay

In 2012, Hotzel et al. described a simple enzyme-linked immunosorbent assay (ELISA)-based in vitro tool detecting non-specific binding of IgG1-based monoclonal antibodies to baculovirus (BV) particles that can identify antibodies with increased risk of unexpected clearance in both cynomolgus monkey and human.11 The tool cannot quantitatively predict non-specific clearance in cynomolgus monkey, but only differentiate between normal (< 10 mL/day/kg) and fast (> 10 mL/day/kg) clearance. BV particles were selected based on in house learning that some monoclonal antibodies bound to BV particles that did not express the target,16 which was later shown to correlate with fast clearance. BV particles were obtained by infecting 1.8 × 10⁶ Sf9 insect cells with recombinant Autographa california nucleopolyhedrovirus expressing green fluorescent protein. The authors show that monoclonal antibodies binding stronger to BV particles are at higher risk for faster clearance in cynomolgus monkey and human (CL > 5 mL/day/kg). Although the exact mechanism of BV particle-binding remains unknown, presumably the non-specific binding is likely associated with high electrostatic and hydrophobic interactions.

Using a large panel of Genentech antibodies,11 the assay was able to correctly predict normal or fast non-specific cynomolgus monkey clearance for 85% of tested monoclonal antibodies with only 3 antibodies being false negative (i.e., having faster clearance than predicted by the tool) and 3 being false positive (i.e., having slower clearance than predicted by the tool) (Spearman’s ρ = 0.53, n = 45). Hotzel et al. concluded that, mainly due to false positive data, the in vitro assay should not be considered for predicting cynomolgus monkey clearance, but rather as a screening tool to reduce the number of antibody candidates that need to be tested in cynomolgus monkey experiments.11 In addition, the assay was able to predict normal non-specific human clearance for 15 of 16 tested antibodies, with only one antibody being false negative (Spearman’s ρ = 0.83, n = 16).

Heparin binding assay and HEK293 cells

Datta-Manan et al. used simple binding to heparin-coated plates and human embryonic kidney (HEK)293 cells to address non-specific binding and differentiate monoclonal antibodies based on their CDR charge differences.37,38 Both assay formats were used to address charge-based binding because it is well established that mammalian cell membranes are formed from negatively charged components such as heparin, chondroitin and sialic acid.39,40 The authors postulated that increased non-specific binding to heparin and cells was indicative of fast non-specific clearance, and this was related to charge-based interaction between CDRs of tested antibodies and the negatively charged cell surface in vivo.

They used these tools to show that balancing the charge (reducing net positive charge) on the IgG4 molecule yielded an ~7-fold improvement in peripheral exposure and significant reduction of tissue catabolism.37 However, balancing the charge on the IgG1 molecule had an effect on non-specific binding and
also yielded an improvement in clearance, although to a much lesser extent (~1.5-fold). These results suggested that improvement in CDRs net positive charge can lead to improved pharmacokinetics. However, the authors did not find a correlation between antibody binding to heparin or HEK293 cells and in vivo pharmacokinetics, suggesting that this in vitro parameter alone cannot be used to predict in vivo pharmacokinetics. Apparently, many additional characteristics (e.g., stability, FcRn binding, biophysical properties) that can influence in vivo pharmacokinetics were also involved.

**Extracellular matrix binding assay**

Sampei et al. used binding to an extra-cellular matrix to assess physicochemical properties and thus plasma pharmacokinetics of therapeutic proteins (Method for improving physical properties of antibody, European Patent Application EP 2 662 385 A1 and United State Patent Application 20140080153). Charge- dependent binding to the negatively net charged extracellular matrix, one of the most abundant protein complexes containing glycoproteins such as collagen, fibronectin, laminin, proteoglycans, fibrin, entactin, and perlecain, has been shown to affect pharmacokinetics. The binding of a tested therapeutic protein to naturally occurring human-derived extracellular matrix immobilized onto a plate is measured using an ELISA-based technique. Using this method, the authors reported a correlation between the negatively net charged extracellular matrix binding and half-life of antibodies in wild type mouse (C57BL/6); the plasma half-lives of 3 antibodies after intravenous administration of 17.1, 6.1 and 1.9 d correlated with extracellular matrix binding values of ~1.05, 1.40 and 1.55, respectively.

The tool has been implemented to successfully reduce fast clearance of emicizumab, a bispecific monoclonal antibody targeting Factors IXa and X, based on an assumption that positive correlation (Spearman's $r = 0.72$) was observed between soluble membrane binding assay and mouse (C57BL/6) used non-specific clearance. With a threshold of 500, the tool was able to correctly predict normal or fast total clearance for 88% (14/16) of tested monoclonal antibodies, with only 1 antibody being false negative and 1 being false positive. The assay results also strongly correlated with results from a BV binding assay as reported by Hotzel et al., which reflects the fact that both assays detect similar molecular interaction between monoclonal antibodies and cell membrane protein(s).

**Polyspecificity binding assay**

Polyspecificity (also known as cross reactivity and off-target or non-specific binding) studies are generally performed by immunohistochemistry (IHC) using different cynomolgus monkey (if selected as relevant species for performing toxicology studies) and human tissues. Performing IHC assays is relatively time-consuming and resource intensive, and thus is not routinely done in the discovery phase of drug development.

Protein microarray technology allows investigation of large numbers of specific and non-specific interactions in one experiment that uses only a minimum amount of sample, and thus may be used as a high throughput screening tool. Lueking et al. reported that data from protein chips with ~400 different human proteins (Protagen, Dortmund, Germany), with ELISA-based detection, were comparable with data obtained from traditional IHC analysis. Frese et al. used Protein Panel Profiling (3P) to measure polyreactivity of commercially available therapeutic proteins for a panel of 32 test human proteins that were selected based on theoretical considerations and results from previous experiments. They demonstrated high specificity for all tested therapeutic proteins, including 4 monoclonal antibodies (adalimumab (Humira®), bevacizumab (Avastin®), rituximab (Rituxan®), ustekinumab (Stelara®)), with no significant off-target binding. Overall these results support the use of protein chip assay as an effective vitro screening tool in drug discovery.

**FcRn related assays**

**Surface plasmon resonance**

Surface plasmon resonance (SPR) spectroscopy is widely used to examine binding interactions between monoclonal antibodies and FcRn. The interactions are monitored in real time by allowing IgG to flow over FcRn immobilized on a sensor chip (mimicking what would happen in endosomal network) or allowing soluble FcRn to flow over immobilized IgG, and detecting binding by measuring changes in the progression of electromagnetic waves at the sensor surface. The distinction in assay format is important for affinity binding constant estimation because immobilized FcRn allows for an avidity contribution to binding, which results from interaction of 1 molcule of IgG with 2 molcules of FcRn (due to flexible linkage that is used for fixing FcRn on chip surface). With IgG immobilized, the binding is 1:1.

Hotzel et al. determined equilibrium dissociation for binding of 44 IgG1-based human and humanized monoclonal antibodies onto immobilized cynomolgus monkey FcRn using SPR. Although the $K_D$ values measured at pH 5.8 varied over a 7-fold range, it was not associated with a
trend in cynomolgus monkey non-specific clearance. This observation was also supported by others.\textsuperscript{15} The findings seemed to contradict earlier reports showing that increased affinity of the interaction with FcRn at pH 6.0 improved the pharmacokinetics of monoclonal antibodies in rodents,\textsuperscript{7,49} and non-human primates\textsuperscript{49-53} Given the relatively large improvement in FcRn affinity binding that lead to improved pharmacokinetics in these earlier reports (wild type IgG: $K_D = 2,400 \pm 100$ nM, $t_{1/2} = 11.4 \pm 1.5$ day; T307Q/N434S: $K_D = 241 \pm 5$ nM, $t_{1/2} = 24.9 \pm 4.7$ day\textsuperscript{48}), the authors concluded that it is not surprising that a small difference in FcRn affinity binding did not have any effect on non-specific clearance in cynomolgus monkey. In general, pharmacokinetics is not thought to be affected by a 7- to 10-fold $K_D$ difference in FcRn binding; however, one of the challenges in establishing a systematic understanding of the IgG to FcRn binding and pharmacokinetic relationship is the different technologies that have been used to measure binding interactions.\textsuperscript{40} In addition, all of the tested antibodies in the study showed rapid release from FcRn at neutral pH 7.4. This data are in line with data reported by Wang et al.,\textsuperscript{45} who did not find any correlation between the FcRn affinity binding at acidic pH ($pH = 6.0$) and in vivo pharmacokinetics using human FcRn transgenic (Tg) mice. On the other hand, apparent correlation between in vitro dissociation at neutral pH ($pH = 7.4$) and in vivo pharmacokinetics in human FcRn Tg mouse, non-human primates and human was observed.\textsuperscript{45} They proposed that in vitro FcRn dissociation at neutral pH rather than association at acidic pH should be used as a screening tool for pharmacokinetic assessment of therapeutic proteins with unmodified wild-type FcRn sequence.

**Biolayer interferometry FcRn binding assay**

A novel in vitro, high throughput screening method for predicting the FcRn-mediated aspects of half-life for human IgG1 based monoclonal antibodies was reported by Souders et al.\textsuperscript{54} In a biolayer interferometry platform, the human FcRn-loaded surface was incubated with 5 monoclonal antibodies in which the BV ELISA or heparin binding assays. The predicted half-life was within $\sim 10\%$ of that observed in clinical studies for each monoclonal antibody.

**Human FcRn affinity liquid chromatography**

Schlothauer et al. have described a novel pH-dependent human FcRn affinity liquid chromatography method for characterizing IgG and FcRn interaction. Briefly, IgG samples are analyzed on a column of immobilized FcRn withbinding at pH 5.5 and pH gradient (pH 5.5 to 8.8) elution.\textsuperscript{24} The pH gradient elution may more closely mimic physiologic conditions for the dissociation of IgG from FcRn that is captured in the SPR-based method of FcRn-IgG analysis.\textsuperscript{45,55} The enhanced avidity in the human FcRn affinity chromatography is capable of differentiating 1:2 from 1:1 IgG to FcRn binding at acidic pH.\textsuperscript{56} In addition, the human FcRn affinity liquid chromatography method seems to be able to detect weak differences in binding affinity of IgG to FcRn more readily than other in vitro tools.

Schlothauer et al. also applied the tool to differentiate and prioritize monoclonal antibodies with different Fab domains based on earlier findings that differences in FcRn binding may reflect contributions from the Fab domain to the interaction.\textsuperscript{24,56} As the FcRn rescues IgG from lysosomal degradation, such changes in the FcRn binding may result in faster than expected in vivo clearance.\textsuperscript{30} They also demonstrated that the tool is able to differentiate methionine (Met 252 or Met 428) oxidized samples, and that the level of oxidation correlates with the magnitude of the change. All the in vitro data correlated well with in vivo pharmacokinetic studies in human FcRn Tg mice (276 line, heterozygous). Similarly, Schoch et al. observed strong correlation between human FcRn column retention time and elimination half-life in human FcRn Tg mouse (276 line, heterozygous).\textsuperscript{31} They demonstrated that the charge distribution in the distal Fv domain of the antibody is involved in binding to FcRn, assuming that tightly bound Fe-Fc complexes would have fast clearance and short half-life compared with those with less tight binding (see Datta-Mannan et al. for details.\textsuperscript{38}) The authors concluded that the assay is a cost-effective tool to minimize risk of unexpected clearance, and can thus reduce the number of candidates that need to be tested in non-human primate pharmacokinetic experiments.

**In vivo tools**

While in silico tools, along with simple in vitro experimental approaches, could enable rapid screening large numbers of diverse candidates and help prioritize lead candidates, they cannot completely capture all factors that can affect in vivo pharmacokinetics. In vivo pharmacokinetic studies are usually needed in the later stages of drug discovery. Cynomolgus monkey has frequently been used as an animal model to predict human pharmacokinetics, primarily due to the reportedly high predictive accuracy of cynomolgus monkey data based on results for conventional monoclonal antibodies and Fc-fusion proteins. However, such studies involve considerable expense and may raise ethical concerns. Additionally, due to time
constraints and the availability of only small quantities of each candidate in the early discovery stage, rodent (mouse or rat) in vivo studies are generally done before those involving large animals.45,51,57

**Mouse pharmacokinetic studies**

Mice are widely used in the discovery stage of drug development to characterize pharmacokinetic properties and support efficacy studies. Mouse studies, however, are typically compromised by limited blood samples due to the low circulating blood volume of the animals.58 This limitation results in increased use of animals because a composite blood collection sampling scheme is needed to characterize pharmacokinetic properties. A serial blood microsampling technique has been demonstrated to reduce animal and material usage with improved pharmacokinetic data.59,60 As bioanalytical methods become more sensitive (e.g., GyrolabTM system, http://www.gyros.com), matrix volume requirements continue to decrease. The microsampling technique is thus feasible, and has the potential to revolutionize the way mouse pharmacokinetic, pharmacodynamic and toxicokinetic studies are conducted.

In 2005, Zhou et al. observed that the interaction of human IgG with human and cynomolgus monkey FcRn at pH 6.0 is about 15-fold lower in affinity compared with binding to mouse FcRn (370 nM vs. 24 nM).27 Such differences between mouse and human and cynomolgus monkey are highly relevant in model selection and preclinical pharmacokinetic testing of human Fc-based molecules. Thus, Tg mice expressing human FcRn have been evaluated for early assessment of in vivo pharmacokinetics. These mice lack the endogenous mouse FcRn α chain and are transgenic for the human FcRn α chain. The human transgene is under the control of the ubiquitous chicken β-actin/rabbit β-globin hybrid promoter (Tg 276 line) or native human FcRn α gene promoter (Tg 32 line).61

A significant relationship in clearance and elimination half-life values between the transgenic FcRn mouse models and cynomolgus monkey was observed for a panel of monoclonal antibodies whose target binding does not affect their clearance in either mouse or cynomolgus monkey.62 The authors demonstrated similar elimination half-lives of monoclonal antibody B21M without and with enhanced FcRn binding at pH 6.0 in Tg276 and Tg32 homozygous lines. In addition, they observed that the Tg32 homozygous line shows greater overall systemic exposure of the antibody than the Tg32 hemizygous line. The data suggested that the transgenic hemizygous Tg32 line offers value as the first screening in vivo tool and in predicting clearance in cynomolgus monkey and humans. Haraya et al. however, recommended the human FcRn Tg276 homozygous line for pharmacokinetic screening of therapeutic proteins.63 In their study, 11 marketed monoclonal antibodies and 2 Fc-fusion proteins were evaluated and the authors observed that half-life in human correlated more closely with half-life in the Tg276 homozygous line than in wild type mouse.

Avery et al. demonstrated that clearance values obtained for 27 monoclonal antibodies in human FcRn Tg mouse (Tg32 homozygous line) correlated with that obtained from clinical studies ($r^2 = 0.83$) better than wild type mouse ($r^2 = 0.61$).64 Using clearance values observed from the Tg32 homozygous line and single-species allometry scaling with an exponent of 0.93 on clearance, all the human predictions were within 2-fold of the observed human values. Their data were also in line with previously published data where the Tg276 line generally showed more rapid clearance than Tg32 line.61,62,65,66 Due to faster clearance of monoclonal antibodies in the Tg276 line, and thus the shorter time course for pharmacokinetic study, such an animal model can be used during the screening process. On the other hand, the Tg32 line is a particularly valuable tool to predict human pharmacokinetics.

Although above mentioned studies used different Tg lines, they demonstrated that pharmacokinetics from human FcRn Tg mouse strongly correlates with cynomolgus monkey and human for a panel of tested therapeutic proteins, thus highlighting the potential value of the mouse model for pharmacokinetic screening of monoclonal antibodies and Fc-fusion proteins in the preclinical stage of discovery, before pharmacokinetic studies in cynomolgus monkey. When combined with the microsampling technique, the transgenic mouse model can be used for optimizing antibody pharmacokinetic properties and prioritization of molecules for cynomolgus monkey pharmacokinetic studies.

**Cynomolgus monkey pharmacokinetic studies**

Cynomolgus monkey is widely accepted as a representative preclinical pharmacokinetic model for single-species prediction of human pharmacokinetics based on several studies using conventional monoclonal antibodies and Fc-fusion proteins. In a relatively large pharmacokinetic study in cynomolgus monkeys that evaluated 13 conventional IgG1-based monoclonal antibodies from Genentech (clearance from 2.36 to 11.5 mL/day/kg), a mean clearance value of 6.5 mL/day/kg (SD, 2.9) was reported.65 It is noteworthy that this study was conducted at doses resulting in concentrations that saturate the antigen-mediated clearance pathway, and thus only accounts for linear pharmacokinetics. Nevertheless, using cynomolgus monkey as single-species-based allometric scaling with a fixed scaling exponent of 0.85, it has been shown that monkey clearance could predict human clearance for antibodies with linear67 as well as non-linear68 pharmacokinetics with reasonable accuracy. In addition, Hotzel et al. demonstrated strong correlation between non-specific clearance values measured in cynomolgus monkey and human for 15 of 16 monoclonal antibodies ($\rho = 0.74$).11

For therapeutic proteins, it has been suggested that an exponent greater than 0.75 on non-specific clearance may provide the most accurate estimation of human clearance from non-human primate clearance.67-71 Avery et al. recently recommended a scaling exponent of 0.80 on non-human primate clearance, allowing accurate prediction of human clearance within 2-fold error for 93% of tested antibodies and 90% when including previously published data by Deng et al.,67 Dong et al.,68 and Ling et al.67,71

It is important to note, however, that the approach for scaling non-linear pharmacokinetics may succeed only if the human target pharmacology is sufficiently represented in the preclinical species on which the scaling is based.72 The allometry scaling with or without correction factors is considered an
empirical approach because it is without mechanistic understanding or molecular considerations for possible species differences.73 Recently, a more mechanistic approach using pharmacologically based pharmacokinetic modeling and target-mediated drug disposition (TMDD) models has been increasingly used.76 These new approaches allow incorporation of expression levels and turnover rates of antigens, and thus more accurately predict non-linear pharmacokinetics in human. It is not surprising that recent papers have suggested potential issues with using single species allometry scaling.1,9,11 Some of these examples clearly demonstrate that there are cases when cynomolgus monkey failed to predict human pharmacokinetics correctly, due to either under- or over-prediction.9,13 These cases also highlight the limited understanding of pharmacokinetics of therapeutic proteins and their key determinants.75

The translation of preclinical pharmacokinetic data are one of the most challenging steps in drug discovery. To improve the understanding of monoclonal antibodies pharmacokinetics and to define cynomolgus monkey in vivo pharmacokinetic characteristics needed for clinical lead selection, we summarized published data from clinical studies.18,74 Population mean and standard deviation estimates of the non-specific clearance, volumes of the central and peripheral compartments and inter-compartmental clearance were 4.5 mL/day/kg, 40 mL/kg, 45 mL/kg and 12.1 mL/day/kg, respectively. We used these estimated pharmacokinetic parameters and translated them back to define recommended parameters for cynomolgus monkey. The recommended non-specific clearance in cynomolgus monkey should be < 8 mL/day/kg (with median non-specific clearance of 6.0 mL/day/kg) for differentiating monoclonal antibodies with fast non-specific clearance and as a criterion for selection of candidate molecule for clinical development (see Fig. 1A-D for details). Such a value for non-specific clearance in cynomolgus monkey used for differentiating fast clearing antibodies is in line with that previously reported by Bumbaca Yadav et al. using preclinical data for 118 Genentech monoclonal antibodies,15 but slightly lower than reported by Hotzel et al. using preclinical data for 44 Genentech monoclonal antibodies.11 Sharma et al.,14 and Deng et al.,67 who all used 10 mL/day/kg.

Critical evaluation of the cynomolgus monkey concentration-time profile is also a key step before trying to project human pharmacokinetics. As discussed above, following systemic administration of monoclonal antibodies, concentration-time profiles generally show a bi-phasic pattern with a distribution phase (also called $\alpha$ phase), followed by a declining phase (also called $\beta$ phase) (Fig. 2A).18 At least 5 theoretical scenarios of concentration – time profile after intravenous administration of therapeutic proteins with faster than expected clearance may exist: 1) mono-exponential decline with no $\beta$-phase (Fig. 2B); 2) bi-exponential decline with pronounced rapid $\alpha$-phase and $\beta$-phase (Fig. 2C); 3) bi-exponential decline with pronounced $\alpha$-phase and expected $\beta$-phase (Fig. 2D); 4) bi-exponential decline with expected $\alpha$-phase and rapid in $\beta$-phase (Fig. 2E); and 5) multi-exponential decline, which could also be indicative of TMDD or anti-drug antibody-mediated clearance mechanism (Fig. 2F).

**Proposed screening strategy and interpretation of developability results**

Available in silico and in vitro high throughput tools allow characterization of a larger set of potential candidates before more resource-intensive activities are started. Such tools should not be used to discriminate between “developable” and “non-developable” candidates in terms of absolute values and go/no go decision, but rather to rank available candidates and to provide early indications for potential liabilities and trigger activities to address key questions in a continuous feedback loop. It

![Figure 1](http://lixoft.com). Simulated cynomolgus monkey pharmacokinetic parameters obtained from published data by Dostalek et al.18 and Dirks and Meibohm.74 Non-specific clearance (Fig. 1A), volume of distribution of the central compartment (Fig. 1B), volume of distribution of the peripheral compartment (Fig. 1C) and inter-compartmental clearance (Fig. 1D) for monoclonal antibody $X$ obtained from virtually simulated 10,000 cynomolgus monkeys after single intravenous dose of 10 mg/kg (CV of 30% was used). Virtual population of 10,000 cynomolgus monkeys was simulated by using the software Simulx (LIXOFT S.A.S., http://lixoft.com).
is noteworthy that the majority of screening tools were established by using traditional monoclonal antibodies and their high predictive accuracy may not hold true for the novel therapeutic proteins such as heterodimeric bi- or multivalent, bi- and multi-specific antibodies.

When multiple candidates with similar properties are available, a pharmacokinetic de-risking strategy should consist of a combination of tools (Fig. 3): 1) level 1 screening: simple in silico tools assessing predominantly the presence or absence of certain molecular features such as net charge and hydrophobicity of critical parts of therapeutic proteins, followed by more complex 3D modeling assessing hot spot analysis, isoelectric point, charge distribution (patch) analysis and others; 2) level 2 screening: in vitro high throughput tools addressing non-specific binding (e.g., BV ELISA, heparin binding or extracellular matrix binding assay), binding to FcRn (human FcRn liquid chromatography) and polyspecificity (e.g., protein chips); and 3) level 3 screening: in vivo simple mouse pharmacokinetic study with single rising dose, followed by cynomolgus monkey pharmacokinetic study. Prediction of human pharmacokinetics should be done with mechanistic understanding of pharmacokinetics and molecular considerations for possible species differences (Fig. 2).
Great efforts have been made to turn available in vitro and in vivo experimental data into predictive in silico tools. As in vitro and in vivo data are continuously generated, the updating of in silico models needs to be automated and fitted in the data-generation cycle to build more robust tools. However, there is still a paucity of human in vivo data in these in silico tools, and, thus, feedback from early clinical development studies to the discovery pharmacokineticists is critical in improving the early pharmacokinetic screening strategy.

Conclusions

Despite the rapid increase in knowledge of processes involved in pharmacokinetics of antibody-based therapeutic proteins, many important questions remain unanswered. Thus, comprehensive evaluation of the key factors influencing their pharmacokinetics and mechanistic studies in both preclinical and clinical settings is needed to build a battery of predictive in silico or in vitro de-risking tools. Early elimination of antibodies with higher risk of failure can increase the success rate and speed of discovery and development. As pharmacokinetics of these therapeutic proteins might be influenced by a large number of both specific and non-specific factors, using multiple high throughput screening tools may provide a more accurate picture of what the true risk for unexpected non-specific clearance might be. In addition to all the available de-risking tools, in-depth knowledge of the presence and absence of certain molecular features of the tested therapeutic protein should guide the rational selection of molecule(s) suitable for further development.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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