Review Article

Challenges and Opportunities in Absorption, Distribution, Metabolism, and Excretion Studies of Therapeutic Biologics

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Abstract. With the advancement of biotechnology in the last two decades, optimized and novel modalities and platforms of biologic moieties have emerged rapidly in drug discovery pipelines. In addition, new technologies for delivering therapeutic biologics (*e.g.*, needle-free devices, nanoparticle complexes), as well as novel approaches for disease treatments (*e.g.*, stem cell therapy, individualized medicine), continue to be developed. While pharmacokinetic studies are routinely carried out for therapeutic biologics, experiments that elucidate underlying mechanisms for clearance and biodistribution or identify key factors that govern absorption, distribution, metabolism, and excretion (ADME) of biologics often are not thoroughly conducted. Realizing the importance of biologics as therapeutic agents, pharmaceutical industry has recently begun to move the research focus from small molecules only to a blended portfolio consisting of both small molecules and biologics. This trend brings many opportunities for scientists working in the drug disposition research field. In anticipation of these opportunities and associated challenges, this review highlights impact of ADME studies on clinical and commercial success of biologics, with a particular focus on emerging applications and technologies and linkage with mechanistic pharmacokinetic/pharmacodynamic modeling and biomarker research.

KEY WORDS: ADME; biodistribution; pharmacokinetics; therapeutic biologics.

INTRODUCTION

With recent successes in the approval and commercialization of monoclonal antibodies (mAbs), Fc proteins and peptides, drug development for therapeutic biologics has captured substantial attention. Over 20 mAbs have been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA). Since the approval of adalimumab in 2002, human or humanized mAbs are a dominant fast-growing category of targeted therapeutic agents (1). In recent years, other novel modalities and platforms of biologic moieties have emerged rapidly in drug discovery pipelines (Table I) (2–14).

For example, a wave of novel, antigen-specific antibody fragment-based constructs may soon enter clinical evaluation (4). Specifically, several types of antibody fragment technologies are currently employed: antigen-binding fragments (*e.g.*, ranibizumab); single-chain variable fragments (*e.g.*, efungumab);

recombinant proteins with size reduction achieved via removal of the domains that are non-essential for function. With the intention to increase potency and prolong half-life, a new class of peptides (or miniproteins) has been synthesized by a ring-closing metathesis reaction to make an all-hydrocarbon "staple" between successive turns of a peptide α-helix (2). It is postulated that stapling of peptides could have an improved resistance to proteolysis compared to traditional linear peptides, while achieving high cell penetration through endocytic vesicle trafficking (2). Other modalities and platforms of biologics that are at various stages of development include multifunctional biologics (3), mAb or Fc protein conjugates with exogenous active moieties (8), and biologics with a variety of half-life extension approaches (*e.g.*, albumin binding, pegylation) (13,14). Selective targeting of tumors by radiation and cytotoxic drugs conjugated to mAbs has been a topic of considerable interest and an area of continued development (8,9). This class of molecules can be illustrated by the recently approved brentuximab vedotin, a CD30-specific antibody–drug conjugate (ADC). In addition to therapeutic proteins, oligonucleotides, small interfering RNAs (siRNA), and aptamers also have been actively pursued for variety of indications (10–12). In parallel with novel modalities and platforms of biologics, new technologies for delivering therapeutic biologics (*e.g.*, needle-free devices, formulation with nanoparticles, intranasal and ocular delivery) and novel approaches to disease treatments (*e.g.*, stem cell therapy, individualized medicine) continue to be developed.

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Table I. Examples of New Modalities and Platforms for Therapeutic Biologics

Biological modalities and platforms	Examples
Synthetic peptides (2)	Hydrocarbon stapled α-helical peptide
Bi-functional biologics (3)	IL-2/IL-12 Fc fusion protein
Antibody fragments, such as antigen-binding fragment (Fab), single chain variable fragment (scFv), and immunoRNase (4,5)	Ranibizumab Efungumab Erb-hRNase
Compact antibodies, such as small modular immunopharmaceuticals (SMIP) and nanobodies (6,7)	2LM20-4 ALX-0081
Small molecule/peptide and protein conjugates, such as antibody-drug conjugate (ADC) (8,9)	Brentuximab vedotin
siRNA, oligonucleotides, and aptamers (10–12)	Mipomersen ALN-VSP02 Pegaptanib
Biologics with half-life extension approaches, such as PEGylation, HESylation, glycosalation, Fc fusion, IgG binding, enhanced FcRn binding, albumin binding or fusion (13,14)	Certolizumab pegol Ozoralizumab (ATN-103)

These advances will bring new challenges for *in vivo* disposition assessment of biologics.

Although pharmacokinetics (PK) of therapeutic biologics are routinely determined in nonclinical research and in clinical trials, in-depth assessments of absorption, distribution, metabolism, and excretion (ADME) properties and mechanisms driving those properties have not been the focus of much attention since the 1992 publication of *Protein Pharmacokinetics and Metabolism* by Ferraiolo *et al.* (15). Recently, unusual PK profiles of mAbs have been reported for a number of mAbs, such as Anti-Abeta Ab2, a humanized monoclonal antibody against amino acids 3–6 of primate amyloid beta (Abeta), and a humanized antifibroblast growth factor receptor 4 antibody (16,17). To understand possible causes for such unusual PK profiles, mechanistic-based ADME studies are often required. Other important, yet poorly understood ADME issues include the role of neonatal Fc receptor (FcRn) in absorption and tissue distribution, the relative contribution of lymphatic system to subcutaneous (SC) absorption in different species, the influence of net charge and local charge clusters on tissue distribution of therapeutic proteins, and the complex impact of glycosylation on PK profiles (17). As an increasing number of novel therapeutic biologics enter drug discovery pipelines, demands for mechanistic ADME studies of biologics will continue to grow.

Despite the great clinical and commercial success for some biologic drugs, the rate of clinical success across the industry needs to be improved. Recently, significant research efforts have been focused on understanding the correlation between PK, especially drug concentrations at the target site, and pharmacodynamics (PD) in order to improve clinical trial outcomes. As pointed out by van der Graaf, lack of clinical efficacy in phase II trials is considered as the primary reason for drug failure (18). Thus, a concept of three pillars of survival for drug development was proposed by the author, in which three key questions should be addressed before a drug candidate is selected for clinical trials: (1) Does the compound reach the target organ(s) at the concentration that is necessary for the desired target coverage? (2) Does the compound bind to the target(s) *in vivo* with the coverage required for biological activity? (3) Does the compound exert the functional modulation of the target? In order to answer these key questions, in-depth investigations of ADME

properties and relationship between ADME profiles and pharmacological effects (*e.g.*, biomarker activity) are essential in preclinical and clinical studies. The conventional systemic PK assessment strategy for therapeutic biologics needs to be expanded to cover the drug exposure information at the target site and enable the correlation of drug concentrations and *in vivo* activities by mechanistic PK/PD modeling. This review will identify the challenges related to ADME studies, provide perspectives on scientific and technical approaches to address these challenges during various stages of drug development of therapeutic biologics, and discuss emerging applications and technologies. Due to the increased emphasis on linking ADME studies with mechanistic PK/PD modeling, the recent progress in biodistribution and biomarker assay development will also be highlighted in this review.

CHALLENGES FOR MECHANISTIC ADME STUDIES OF THERAPEUTIC BIOLOGICS

Due to their large molecular size and complicated tertiary structure (Fig. 1), the ADME issues for therapeutic biologics often are different from that for small molecules. The common considerations for the ADME-related issues for therapeutic biologics are summarized in Table II, which include target-mediated clearance, the FcRn recycling for Fc-containing proteins, immunogenicity, isoform heterogeneity, and metabolic stability, especially for relatively small molecular weight (MW) proteins and peptides. Many of the challenges for the mechanistic ADME studies of therapeutic biologics stem from the lack of effective and validated *in vitro* systems. For example, the metabolic/catabolic stability of mAbs or other large therapeutic proteins cannot be easily assessed *in vitro* with liver microsomes or cultured hepatocytes, which are commonly used for small molecule studies. In contrast to prediction of oral bioavailability for small molecules, there are no reliable *in vitro* systems that can help to predict bioavailability of therapeutic biologics after SC administration. Therapeutic biologics generally have limited distribution in tissues. Thus, it is important to understand the tissue penetration mechanism and, consequently, the relationship between tissue concentration and efficacy.

In general, biologics are metabolized/catabolized into small peptide fragments or amino acids that are ready for
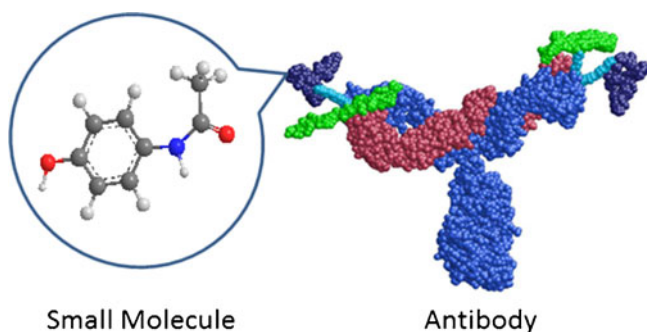
Fig. 1. Size comparison of a typical small molecule (illustrated by acetaminophen) and an IgG antibody

renal excretion or recycling into protein synthesis. The rate of metabolism for biologics is compound- or modality-dependent. On one end of the spectrum, metabolism of small peptides and recombinant human proteins with a low MW tends to be very rapid. The metabolic stability for this type of molecules can potentially be assessed *in vitro*, similar to the assays employed for small molecules. On the other end of the spectrum, metabolism of human IgG proteins is very slow, resulting in a long half-life of 7–28 days. As extensively reviewed in literature, antibody molecules with an Fc portion is protected from degradation by binding to FcRn in endothelial cells, explaining the long half-lives of these proteins (19–21). Thus, for mechanistic-based metabolism studies of biologics, validated *in vitro* systems that could maintain biologic activities (*e.g.*, receptor-mediated uptake, endocytosis, internalization, lysosomal digestion, and/or antigen presentation) for days or weeks would be extremely valuable. To this end, a 3D hollow fiber human hepatocyte bioreactor system has been developed and characterized recently for long-term metabolism and toxicity studies, although further refinement of the system is needed before it can be adapted for the *in vitro* assessment of biologics.

Another challenge involved in ADME studies of biologics is the species difference in target-binding properties and host immune response to a biologic. It is well established that the target-mediated clearance and antidrug–antibody-mediated clearance for therapeutic biologics are species-dependent. In addition, there are significant species differences in FcRn/IgG

interactions (22). Thus, common laboratory animals (*e.g.*, rodents, dogs) may not always be relevant for prediction of ADME profiles of therapeutic biologics in humans. While transgenic animals that express human targets or receptors can be useful for the qualitative assessment of potential clearance mechanisms, a more quantitative approach is needed to predict ADME profiles in humans.

Analytical assay development for therapeutic biologics is another hurdle encountered in ADME studies. Structure similarity between endogenous proteins and therapeutic biologics and the hydrophilic nature of these macromolecules make the extraction and purification of biologic drugs from *in vivo* samples very difficult. The detection limit of analytical instruments makes the direct measurement (*i.e.*, without prior purification/enrichment step, such as immunocapture) of therapeutic proteins in biologic matrices (plasma or tissue homogenates) a challenging task. Very often, enzymatic digestion may be required prior to the structure identification or quantitation of therapeutic proteins for *in vivo* samples. In these cases, the recovery of therapeutic biologics during sample treatment and the assay interference of endogenous proteins become major concerns. However, with the improvement in instrumentation and sample handling methodology, significant progress on assay throughput and detection has been made in recent years.

EMERGING TECHNOLOGIES FOR ADME STUDIES OF THERAPEUTIC BIOLOGICS

Quantitative Analytical Method Development

Conventional analytical approaches for pharmacokinetic studies of biologics heavily rely on ligand binding assays, such as the enzyme-linked immunosorbent assay (ELISA) (23,24). Advantages of ligand-binding assays include high specificity and sensitivity, ease of sample handling, and low cost. However, generating high quality and specific reagents for ELISA methods developed to quantify therapeutic biologics and antidrug antibodies (ADAs) could be a time-consuming and labor-intensive process (25,26). While pharmaceutical companies usually are willing to spend resources on lead candidates and focus on resolving complicated issues halting advancement of these candidates, analytical support for early discovery research often

Table II. Key Factors Determining ADME Profiles of Therapeutic Biologics

ADME-related considerations for biologics	Key contributing factors
Physical/chemical properties	Size, shape, charge, stability, heterogeneity in isoforms (including post-translational modifications)
Absorption mechanism	Route of administration specific issues (*e.g.*, contribution of lymphatic absorption after subcutaneous injection), formulation, injection site, subject characteristics, FcRn- and target-dependent mechanisms, physical/chemical properties
Distribution patterns	Size, shape, charge, target binding, FcRn- and target-dependent mechanisms, route of administration, formulation
Elimination pathways	Proteolysis, target-mediated clearance, nonspecific endocytosis and formation of immune-complexes followed by complement- or Fc receptor-mediated clearance, protection from catabolism *via* FcRn mechanism
Nonlinear kinetics	Saturable target mediated clearance, immunogenicity, FcRn (for very high doses or possibly for FcRn mutants)
Subject characteristics	Body weight, age, sex, disease status, prior exposure to biologics, concomitant medications
Species difference in PK profiles	Target binding affinity, FcRn/IgG interactions, immunogenicity, contribution of lymphatic absorption, off-target effects

is limited. On the other hand, the pace of generating new biotherapeutic candidates has improved dramatically due to the advancement in protein engineering and supporting purification/production technologies. Thus, the demand for supporting discovery activity, such as lead optimization, is paramount. In order to improve the throughput rate, new analytical approaches and instrument platforms are being continuously pursued. One example is the application of "generic ELISA", which uses a reagent recognizing the Fc portion of a human protein, utilized to support PK studies of human Fc containing biologics in animals. Automation with robotic systems is also highly desirable for the screening of new biologic constructs. One example is the Gyrolab immunoassay platform (Gyros, Uppsala, Sweden), which incorporates the microfluidic concept into a ligand binding assay (27). Applications of the Gyrolab immunoassay workstation for pharmacokinetic studies have been reported recently (28,29). The nanoliter sample requirement for the assay is particularly valuable for pharmacokinetic studies in mice, an animal species commonly used in early efficacy evaluations. With a reduced blood sample size of 10–20 μL for each time point, serial bleeding can be conducted in the same mouse over the time course for a PK study, minimizing the intersubject variability associated with nonserial sampling needed for a mouse PK study that relies on a conventional ELISA for bioanalysis.

As the use of biologics or macromolecules spreads to medical, cosmetic, and food sectors, interference of endogenous proteins similar to therapeutic biologics on an immunoassay developed for therapeutic proteins will become a major issue. Alternative approaches that are less liable to such interference have been actively pursued.

One technology particularly attractive for bioanalysis is mass spectrometry (MS), which has been extensively used in the drug development for small molecules (30,31). Characteristics and performances of commonly used mass spectrometers for proteins have been reviewed by Domon and Aebersold (32). Advantages of MS over ELISA include the improved selectivity between structurally similar peptides and proteins, reduced requirements for specific reagents, improved precision and accuracy, and potentially higher throughput rate (33). To improve the sensitivity and reduce the interference of peptide fragments that originate from endogenous proteins, immunoprecipitation is usually applied in the sample extraction process. This is particularly important for tissue homogenates or plasma samples with low drug concentrations; however, this additional step adds to assay complexity and requires additional reagents. Recent developments in the immunoaffinity purification on magnetic beads coated with antibodies for a rapid and efficient purification of therapeutic proteins from biologic matrices (34), and the invention of commercially available temperature-controlled tissue beaters (e.g., Precellys® with the patented cooling technique that keeps temperature at approximate 4°C during homogenization) are expected to enhance the throughput rate in sample treatment for bioanalysis.

To facilitate the mass fragment detection, isotope-labeled protein standards are often used in the protein quantitation by MS (35,36). A recent strategy called Protein Standard Absolute Quantification has been developed, in which a full-length stable-isotope-labeled protein will be synthesized as a standard marker for the therapeutic protein. During the sample analysis process, the full-length stable-isotope-labeled protein standard will be spiked into biologic fluids (e.g., plasma or urine) containing the therapeutic protein that needs to be quantified. Both the labeled protein standard and the therapeutic protein will undergo the purification and enzymatic digestion simultaneously in the same vial. Thus, it will minimize the difference in the sample treatment associated with other methods (e.g., using a stable-isotope-labeled peptide as the standard) (37).

The MS approach also has been applied to the quantitation of antidrug antibodies. Neubert et al. utilized a magnetic bead-based immunoprecipitation method followed by quantitative liquid chromatography–mass spectrometry (LC-MS) to detect ADA in human and cynomolgus serum in the presence of high-circulating concentrations of the protein therapeutic (38). It is anticipated that the development of quantitative mass spectrometric assays will be a booming area for ADME studies of therapeutic biologics.

Non-Invasive Imaging Technologies for Biodistribution and PK/PD Studies

In order to understand in vivo tissue distribution patterns and address the key "Three Pillar"-related questions described in the "INTRODUCTION" section, investigations of biodistribution and target occupancy have become the main focus of preclinical ADME studies. The application of non-invasive imaging technique in biodistribution studies is rapidly growing in both academic and industrial research. As shown in a recent report issued by the National Heart, Lung, and Blood Institute, publications on the application of molecular imaging for cardiovascular research has increased tenfold in the last 10 years (39).

Common imaging technologies used in animal biodistribution studies include optical imaging with luminescence and fluorescence molecular probes, radiotracer-based single photon emission computed tomography (SPECT) and positron emission tomography (PET) imaging, magnetic resonance imaging (MRI), ultrasound imaging, and X-ray imaging (40,41). In contrast to the conventional cut-and-count approach or whole-body autoradiography with radiolabeled biologics, imaging studies can be conducted in live animals, which provides the advantage of obtaining real-time dynamics on the biodistribution of a test article from the same animal. Information gained from in vitro human cell-based assays and in vivo animal studies with fluorescent- or radiotracer-labeled biologics can be used to guide the dose regimen design for clinical trials. However, it should be pointed out that while in-life imaging can provide an overall picture on biodistribution patterns, its sensitivity may be limited, especially for the in vivo fluorescent imaging (42). In most cases, the imaging approach is considered to be semiquantitative or qualitative. Technologies with relatively high sensitivity and selectivity, such as SPECT and PET, are more attractive for clinical studies. To further enhance the sensitivity, combination imaging systems (e.g., PET/CT, SPECT/CT, PET/MRI) have been developed (43).

While a cutting-edge imaging technique is instrumental to support novel research activities, the conventional radio-isotope probe method in combination with tissue counting or whole body autoradiography remains to be the workhorse in laboratory research for biologics. The radioisotopes most commonly used in ADME studies for therapeutic biologics include ^{18}F, ^{99m}Tc, ^{32}P, ^{14}C, ^{111}In, ^{131}I, ^{125}I, and ^{123}I. Among

that, ^{125}I is usually considered as the first choice in the labeling of biologics in preclinical biodistribution studies owing to the low cost, well-known protein iodination chemistry, and its fit-for-purpose physical properties of gamma emission and 60-day decay half-life. However, there are several concerns associated with using ^{125}I labeled proteins, such as a rapid loss of ^{125}I label for certain types of molecules and the potential for bioactivity change of biologics during the iodination procedure (44). Therefore, caution should be applied in the data interpretation of radiolabeled studies.

Structure Elucidation for *In Vivo* Degradants and Isoforms of Therapeutic Biologics

Similar to the research for small molecules, structure elucidation of major degradants of a therapeutic protein will help to identify the "metabolic soft spot" for biologics. Such information is valuable to guide the design of new constructs with improved *in vivo* stability and PK properties. In addition, structure elucidation of *in vivo* samples also helps the assessment of potential differences in the elimination kinetics of protein product isoforms, as a biologic drug is often a collection of large protein isoforms and not a single molecular entity (45). Information gained from the *in vivo* measurement of various isoforms (*i.e.*, products of a specific posttranslational modification) may guide selection or design of a new drug product with improved ADME profiles.

Identifying metabolic degradants of therapeutic biologics is a technically challenging task. Lack of an effective extraction and purification method is one of the major hurdles. Although immunoprecipitation with drug specific or "generic" (*e.g.*, anti-IgG) antibodies may help the purification process, such approach is not suitable for degradants that do not bind to capture antibodies. Therefore, separation of proteins and degradants by gel electrophoresis followed by in-gel proteolytic digestion or liquid chromatography is frequently performed.

For structure identification and characterization of degradants, biologic isoforms, and immunocomplexes in biologic matrices, MS approaches developed for proteomic research are commonly used. Two fundamental strategies for MS analysis of protein structures are the "bottom-up" and the "top-down" approaches (46–48). In the "bottom-up" approach, purified proteins or complex protein mixtures are subjected to chemical or enzymatic cleavage, and the peptide products are usually separated by chromatography followed by the tandem mass spectrometry analysis. In the "top-down" approach, intact protein ions or large protein fragments are directly subjected to the gas-phase fragmentation for MS analysis (47). Given the complexity in protein structures, a single approach may not be sufficient to provide comprehensive information for the structure identification purpose. An integrated approach has been applied to identify protein isoforms arising from various amino acid modifications (*e.g.*, acetylation, phosphorylation) and genetic variants (*e.g.*, single nucleotide polymorphic isoforms) (48). This combination strategy overcomes the major limitations of the traditional bottom-up (*e.g.*, inability to characterize multiple, unexpected protein isoforms and genetic variants) or the top-down (*e.g.*, low throughput) approach.

The structures of metabolic degradants, isoforms, or immunocomplexes are determined by a comparison of the peptide mass spectra with theoretical peptide masses calculated from a proteomic or genomic database. However, at present, the *de novo* peptide identification *via* tandem MS is a time-consuming process (49). Technical challenges include assembling, analysis, and interpretation of a large volume of data generated from fragments of a macromolecule, requiring a construction of sophisticated computation system with a comprehensive database capability. With the rapid progress in MS technology and bioinformatics, structure identification technique for *in vivo* degradants is expected to mature with a reasonable throughput rate in the future.

Recent advances in nuclear magnetic resonance (NMR) allow to use this technology to rapidly determine protein and protein–ligand structures, to efficiently screen fragment-based libraries to identify biological relevant ligand interactions, and, ultimately, to identify new therapeutic targets (50). However, the application of NMR can be limited due to the general upper weight limitation of ~25 kDa and concerns about the interference of endogenous proteins. Despite these limitations, NMR technology has significant potential in drug discovery and in the development of therapeutic biologics.

In Vitro Predictive Tools for ADME of Therapeutic Biologics

Despite the urgent need for metabolic stability screening of new biologic constructs in discovery research, there is, to date, no validated *in vitro* system that can be used for prediction of *in vivo* clearance (51). For example, optimizing the interactions between IgG and FcRn is becoming a more widespread approach to increase exposure and reduce dosing frequency of an IgG-based biologic (52). *In vitro* FcRn binding properties (*e.g.*, cell-based binding in FcRn-expressing cell lines) have been used to guide Fc engineering of new mAbs with a desirable half-life (53, 54). However, the correlation between *in vitro* binding constant (k_d) and *in vivo* clearance is not always straightforward (53). Therefore, quantitative modeling that incorporates the kinetic parameters for FcRn and Fc-containing protein interactions (*e.g.*, k_{on} and k_{off} rates at acidic and neutral pHs) and other determinants of clearance is needed to improve the *in vitro* and *in vivo* correlations, since the relative contribution of a given *in vitro* binding parameter to the overall *in vivo* clearance may differ among a series of Fc-containing proteins (17).

In vitro predictive tools for tissue penetration are also highly desirable for therapeutic biologics. In recent research on tumor uptake prediction, an *in silico* model has been proposed by Thurber *et al.* that could help the design of biologic constructs for solid tumors (55). The authors indicate that theoretical analyses of ADME profiles of a potential therapeutic biologic will provide specific guidance with respect to design of biophysical and biochemical properties of the therapeutic biologic, such as agent size, affinity, and target antigen. The current data analyses suggest that IgG-sized constructs exhibit the most favorable balance between systemic clearance and vascular extravasation, resulting in a maximal tumor uptake. Quantitative predictions of the effect of dose and binding affinity on tumor uptake and penetration can be achieved using the information on antigen expression level and metabolic half-life (56). This quantitative model has been tested in xenografted tumors in mice, and needs to be further verified in cancer patients. The use of computational design to engineer therapeutic biologics based on tissue distribution patterns and target binding properties is an attractive area of future research.

In Vitro Predictive Tools for Immunogenicity

Immunogenicity is often a major concern during the discovery and development of biotherapeutics and may affect ADME profiles in species-dependent manner (57,58). Successful prediction of immunogenicity will help to reduce the risk in drug candidate selection (59). Although immunogenicity of a novel therapeutic protein is usually assessed in animals (most commonly in monkeys) prior to human trials, the primary goal of these assessments is to support preclinical PK, pharmacology, and toxicology data interpretation. Due to species differences in a variety of immune system components involved in an immune response to a particular therapeutic biologic and differences in the "degree of foreignness" of a biologic between animals and humans, predictions of human immunogenicity based on animal data are generally not considered to be reliable, hence the value of mechanistic immunogenicity studies in animals can be restricted (60). Thus, it is desirable to use *in silico* or *in vitro* human cell-based assessments for the immunogenicity prediction, including predictions of aggregation prone regions and T and B cell epitopes. To this end, Kumar *et al.* described the importance of investigating the impact of aggregation on immunogenicity and potential coupling of T and B cell epitopes and aggregation-prone regions (61). Computational tools that can predict aggregation prone regions as well as T- and B-cell immune epitopes from protein sequence and structure have become available recently from several commercial vendors (58, 62–64). However, none of these tools have been thoroughly validated with *in vivo* human data and, therefore, the standards for prediction tools have not been established (63).

Cell-based assays using human blood are being used in combination with *in silico* approaches to predict human immune responses for a variety of therapeutic applications, including vaccines, transplantation, and, in recent years, therapeutic biologics. For example, peripheral blood mononuclear cells (PBMC)-derived T cell immunogenicity assays have been used to monitor and predict immune response and outcome in the field of transplantation (65). The assay has the capability to elicit the response from a PBMC population with low frequency of antigen-specific T cells and potentially can be used to distinguish therapeutic proteins with high and low immunogenicity potential. However, depending on assay conditions, different responses may be obtained for a given therapeutic protein in PBMCs, therefore, similar to *in silico* tools, cell-based assays need to be optimized with respect to predictive value in clinical human studies. The considerations for optimization and validation of an *in vitro* PBMC-derived T cell assay for immunogenicity prediction of biotherapeutics have been discussed by Wullner *et al.* (66). To streamline the application of pharmacogenomics to immunogenicity predictions, an individualized T cell epitope measure (iTEM) tool has been developed to estimate an individual's T cell response to a protein antigen based on HLA-binding predictions. While further refinement of the *in vitro* conditions is needed, the system is preliminarily validated for prospective iTEM predictions using data from *in vitro* and *in vivo* studies (67).

In summary, a collective *in silico* and *in vitro* approach in conjunction with *in vivo* animal models (*e.g.*, genetically modified animals designed to have some aspects of human immune systems) could be used to rank–order protein drug candidates for their immunogenic potential. However, the validation of these predictions is yet to come and will rely on the collection of comprehensive databases and likely collaboration across bio-pharmaceutical companies. Since immunogenicity impacts both the ADME profile and biological activity of biologics, advances in predictive immunogenicity tools are likely to impact the success rate in biotherapeutic drug development.

Assay Development for Biomarkers

As discussed in the "INTRODUCTION" section, bio-marker assay development is a growing area of research and stems from the need to improve the current low success rate of clinical trials, as well as by the increasing emphasis on personalized medicine and targeted therapies. In many cases, the failure of clinical trials is due to the fact that only a subset of patients expresses the responding phenotype. Pharmaceutical companies together with regulatory agencies have made significant investments in biomarker development, with the focus on translation to clinical practice and linkage of PK, PD, and disease biomarkers. Ultimately, it is important to select a "right" biomarker, *i.e.*, to identify key biomarkers related to intended indication(s) (68,69). In addition, developing appropriate biomarkers in animal disease models that can be used in translational research to support human clinical trials will be very valuable. Both *ex vivo*, *in situ*, and *in vivo* biomarkers approaches are being pursued, sometimes in parallel, as discussed below.

Common techniques used in *ex vivo* biomarker assays include immunoassays, MS, flow cytometry, polymerase chain reaction, and bioactivity assays. The assay development for biomarkers could be a time-consuming process. Hurdles to method development include the requirements on specificity and stability in complex biological matrices, wide dynamic range, and assay sensitivity. Requirements for method validation (*e.g.*, specificity, selectivity, assay acceptance criteria, stability, and reproducibility) and the utility of protein biomarkers have been reviewed by Lee (70). Targeted proteomic strategies have been applied to quantify biomarker candidates at concentrations of nanograms per milliliter or picograms per milliliter by LC-MS (71).

Recently, matrix-assisted laser desorption/ionization imaging MS, a new *in situ* molecular imaging technology, has been used in the biomarker field (72–74). The imaging MS can conduct a direct *in situ* analysis of biologics from thin tissue sections and provide important insights into biological processes because the native distributions of molecules and histological features remain intact throughout the analysis. A wide variety of molecules can be imaged, including proteins, peptides, and metabolites. From the perspectives of translational research and clinical proteomics, this method can correlate molecular readouts to histopathological changes found in disease tissues (74,75).

Quantitative or semiquantitative *in vivo* approaches for directly monitoring pharmacological activities of therapeutic biologics are becoming more widespread in translational research. For example, the putative utility and validity of electroencephalograph (EEG) as a surrogate biomarker for a number of major central nervous system disorders, including depression, schizophrenia, and pain, have been described (76). Similarities in spontaneous EEG and cortical activity potential measures from rodents to humans provide a solid

foundation for the use of EEG as a translatable biomarker. EEG monitoring can be performed in a relatively non-invasive, stress- and pain-free manner, and the identical computational algorithms can be used for EEG signal processing and analyses across lab animal species and humans. It should be emphasized that in order to make a link between biomarker data and a clinical outcome (efficacy and/or safety), biomarker data in animals and humans need to be interpreted in an integrated manner together with the corresponding PK/ADME information.

PRACTICAL APPLICATIONS

Case Studies

To demonstrate the importance and practical aspects of mechanistic ADME studies in drug discovery and development research, two case studies are presented in this review. The first case study emphasizes the importance of biodistribution assessment in early phase studies of new biologics to assist in clinical development of ADCs. The second case study illustrates the importance of integrated assessment of presystemic metabolism and absorption rate on the *in vivo* exposure of a peptide drug after SC injection with a controlled release (CR) formulation.

Case Study 1

ADCs are complex drugs that consist of potent cytotoxic drugs linked to antibodies *via* chemical linkers. The concept of ADCs has attracted extensive interests due to its promises of potential tumor-specific delivery of cytotoxic drugs, improved safety window compared to the monodrug, and long half-life associated with the antibody portion of the molecule [77].

CMD-193 is an ADC comprised of a humanized antibody directed against the Lewis Y (Le^y) antigen conjugated with calicheamicin. CMD-193 binds to the Le^y antigen, a tetrasaccharide expressed on the cell surface of many solid tumors (*e.g.*, liver cancer), *via* its antibody moiety [78]. Upon binding to its target on tumor cells, CMD-193 is internalized; the active calicheamicin moiety is released and is able to bind to the minor groove of tumor cell DNA, causing double-strand DNA breaks, the inhibition of DNA synthesis, and apoptosis.

In a phase I study, biodistribution of [111In]CMD-193 was evaluated in patients who had histologically confirmed solid malignancies displaying Le^y antigen positive. The PK and tissue distribution pattern of [111In]CMD-193, determined by whole body γ camera scans and SPECT imaging, were compared to those for [111In]Hu3S193, a parental antibody of CMD-193. Following IV infusion, biodistribution and PK of [111In]CMD-193 were found to be significantly different from that for [111In]hu3S193 [78]. In contrast to prominent tumor-specific uptake and long half-life in blood observed for [111In]hu3S193, [111In]CMD-193 had fast clearance and short half-life, rapid uptake in liver parenchyma, and lack of tumor uptake (Table III). Although the actual cause for the altered biodistribution of CMD-193 compared to the parent antibody is unknown, it is speculated that a physicochemical change induced by conjugation of the antibody with calicheamicin may lead to the altered biodistribution profile. Thus, understanding the effect of payload on the biodistribution of ADC will help to optimize the tumor uptake and improve the efficacy. In this example, biodistribution data provided a valuable content for the interpretation of the lack of efficacy observed in the clinical trial for CMD-193.

Case Study 2

For most therapeutic biologics, SC injection is preferred over the IV administration due to convenience. The SC absorption rate depends on multiple factors, including the size of a molecule and the formulation used. In an effort to develop a SC-delivered peptide therapeutic for weight loss and/or diabetes treatment, a CR formulation of an oxyntomodulin analog peptide (referred to as "Peptide A") was developed with the intent to prolong the absorption process and reduce the dosing frequency compared to that from the conventional instantaneous released (IR) formulation [79]. In rats, the SC absorption rate of peptide A was reduced by the CR formulation, as evidenced by a shift in the time (T_{max}) to reach the maximal concentration (C_{max}) from 1.3 h for the IR formulation to 10 h for the CR formulation after SC injection of 2 mg/kg. However, the overall systemic exposure, determined by the C_{max} and the area under the concentration–time curve (AUC), were decreased significantly in rats treated with the CR formulation compared to that with the IR formulation. The SC bioavailability with the CR formulation was reduced by ~40% compared to that for the IR formulation. Similar findings were observed in monkeys and humans following SC administration with both IR and CR formulations.

To understand the potential cause for the reduced systemic exposure, detailed ADME studies for peptide A were conducted in rats. Biodistribution and excretion studies with [125I] Peptide A indicated that the SC administered radioactive dose was absorbed well from the injection site and the radioactivity was almost completely recovered in urine (as free iodine and/or degradants) over a collection period of 10 days. Further studies by *in vitro* metabolic stability testing of [125I]Peptide A with fresh rat skin and *in vivo* metabolic profiling of rat plasma and skin samples collected at the SC injection site suggested that peptide A was metabolically unstable in skin and that degradation of the peptide in skin during its prolonged residence at the injection site could potentially contribute to a decrease in the SC bioavailability for the CR formulation. This example illustrates that *in vivo* PK profiles depend on the interplay of ADME properties of therapeutic biologics. Knowing the mechanisms that govern PK profiles will help rational optimization in half-life extension and/or delivery strategy.

Table III. Peak Tumor Uptake and Liver Uptake of [111In]CMD-193 and its Parental Antibody [111In]hu3S193

	[111In]CMD-193	[111In]hu3S193
Peak tumor uptake (μg/g)	0.15±0.03	2.9±1.7
Liver uptake (% injected dose)	33±6.0	7.3±1.5

Adapted from Herbertson *et al.* [78]

Hot Topics and Emerging Applications

ADME studies for biologics may bridge into multiple aspects of drug discovery and development, and the applications listed below highlight the emerging focus in the pharmaceutical industry:

1. *PK/PD modeling*: Because of the interdependency of PK, biomarkers and efficacy, mechanistic PK/PD modeling is a critical tool in the design of clinical dose regimens, as described in the previous sections. While sophisticated modeling tools and model libraries are already available or continued to be built, validation of these PK/PD models will require extensive experimental data generated from *in vitro* or *in vivo* ADME studies.

2. *Blood–brain barrier (BBB) transport*: Although BBB transport has been extensively studied for small molecules, this area has not been fully explored for therapeutic proteins. Knowledge of the mechanism of BBB transports for biologics will benefit the drug discovery for central nervous system (CNS) indications, as well as the safety assessment for therapeutic biologics. A recent approach to improve BBB penetration of therapeutic biologics is to engineer a bifunctional IgG-based fusion protein, with one arm binding to a CNS target and the second arm targeting an endogenous BBB receptor, such as the human insulin receptor (HIR) or the transferrin receptor (80). This approach can be illustrated by the brain uptake of the human insulin receptor mAb-human TNF receptor fusion protein (81). However, sufficient delivery of protein drugs to the CNS remains as a major challenge for pharmaceutical research (51).

3. *Drug–drug interactions*: As the number of approved therapeutic biologics increases, potential drug–drug interactions (DDI) between concurrently administered drugs (small molecule–biologic drugs or biologic–biologics drugs) become a concern. The importance on therapeutic protein–drug interactions and guidance that recommends how and when to evaluate such interactions have been published recently by FDA (82,83). However, preclinical tools and *in vitro* test systems for assessing drug interaction potential of therapeutic proteins are limited. Thus, DDI assessment is often evaluated as a part of clinical trials. Knowledge gaps and areas for future research have been summarized in the AAPS Workshop Report on strategies to address DDI for biologics (84). This topic will be monitored closely in the future development of therapeutic biologics. With continuous data collection and knowledge-building process, strategies and methodologies to address DDI potential for new biologics will become mature.

4. *Novel delivery systems*: With only a few exceptions, therapeutic biologics are almost exclusively administered by parenteral routes. To increase target-specific delivery, improve safety and convenience for patients, and support for individualized medication, new delivery systems are actively pursued. Some examples include polymeric microstructured arrays for potential delivery of proteins (85), needle-free injection devices for SC injection (86), and nanoparticles to protect siRNA from serum degradation (87). To assess the utility of these new drug delivery systems, PK and ADME properties of biologics delivered by novel technologies often are compared to conventional IV or SC injection. Species selection for nonclinical PK evaluations of new delivery systems can be drug-, device-, and route-dependent, ranging from rodents to monkeys and mini-pigs (88).

5. *Biosimilar/comparability assessments*: Biosimilars are a new class of drugs intended to offer comparable safety and efficacy to the reference, off-patent biologics. Biosimilars have attracted great attention with the hope that they will allow wide-spread availability of currently expensive biologic products (89). Given their structural complexity, multifaceted manufacturing process, and, as a result, challenges for predicting impact of the manufacturing process changes on immunogenicity, biosimilars are not generic alternatives *per se* and generally are not interchangeable (90). Thus, unique regulatory pathways are required for biosimilars (91,92). PK and immunogenicity assessments, together with efficacy and toxicology studies, are key components in the development of biosimilars. Traditionally, comparability assessment has been conducted during the manufacturing process of therapeutic biologics, since it is a complex process that involves continual refinement throughout product development, post-approval, and marketing. Common reasons to alter the manufacturing process include the benefits of better yields, scale-up, new technologies and increased purity, as well as new formulations or product delivery systems (93). Since the definition of biosimilars continues to evolve, comparability assessment and characterization of biologic products during the manufacturing process will remain a "hot" topic. Strategies and methodologies for the comparability assessment, including PK and ADME studies, will continue to emerge, which will bring research in this area to a new horizon.

6. *Placenta transport of therapeutic biologics*: Unlike the drug development for small molecules, placenta transport of biologic drugs is not studied extensively, likely because of the common belief that the placental transport is minimal for biologics. However, placental transport of immunoglobulins has been recognized for more than 50 years (94,95). A specific FcRn-mediated binding of IgG at the maternal surface of the placenta has been proposed as the first step in the transport mechanism by which IgG is transferred from the mother to the fetus (94,96,97). The role of the second placental barrier, the fetal capillary endothelium, is not yet clear. The expression of FcRn is known to be relatively low on fetal vessels' endothelium, suggesting possible other mechanism(s) involved in IgG transport across this placental layer (98). The challenge of drug concentration measurements for fetal tissues limits placental transport studies. Additional research is required to fully understand the mechanism of placental transfer of various biological modalities and its implications in the safety assessment of therapeutic biologics.

SUMMARY

Driven by the need for improved clinical success and by the competitive landscape in pharmaceutical industry, demands for mechanistic ADME studies of novel biologics continue to increase. Despite advancements in the science of ADME processes of biologic drugs, many fundamental questions remain unanswered. ADME studies for therapeutic biologics could be a

challenging task due to the complex nature of these novel molecules and to limited bioanalytical methods. Despite significant progress in the technologies pertaining to ADME studies of therapeutic biologics, a breakthrough in bioanalytical methodology and *in vitro* predictive tools will be required to bring the ADME science of therapeutic biologics to a new horizon. The cost for development, validation, and implementation of these new technologies is significant. Thus, research activities should focus on critical issues that impact the development of drug candidates, and mechanistic ADME studies should be carried out on a case-by-case basis. One of the key objectives of ADME studies is to link PK profiles in circulation and action site to *in vivo* responses. Therefore, advances, as well as limitations in biomarker technologies, may drive the priorities and design of PK/PD and ADME studies for therapeutic biologics. Over time, knowledge gained from in-depth ADME mechanistic studies in nonclinical and clinical settings will advance our understanding on key factors that govern the *in vivo* disposition of therapeutic biologics across various modalities. This knowledge is instrumental for optimization of disposition properties, and, ultimately, human efficacy and safety profiles of new therapeutic biologics.

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