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REVIEW

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Key factors influencing ADME properties of therapeutic proteins: A need for ADME characterization in drug discovery and development

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ABSTRACT

Protein therapeutics represent a diverse array of biologics including antibodies, fusion proteins, and therapeutic replacement enzymes. Since their inception, they have revolutionized the treatment of a wide range of diseases including respiratory, vascular, autoimmune, inflammatory, infectious, and neurodegenerative diseases, as well as cancer. While *in vivo* pharmacokinetic, pharmacodynamic, and efficacy studies are routinely carried out for protein therapeutics, studies that identify key factors governing their absorption, distribution, metabolism, and excretion (ADME) properties have not been fully investigated. Thorough characterization and in-depth study of their ADME properties are critical in order to support drug discovery and development processes for the production of safer and more effective biotherapeutics. In this review, we discuss the main factors affecting the ADME characteristics of these large macromolecular therapies. We also give an overview of the current tools, technologies, and approaches available to investigate key factors that influence the ADME of recombinant biotherapeutic drugs, and demonstrate how ADME studies will facilitate their future development.

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Introduction

It has been nearly 40 y since biologists first learned to produce human growth hormone and insulin. The discovery of recombinant protein technology revealed the potential of proteins as therapeutic agents; a potential which has been increasingly realized throughout the intervening years.[1] What started with relatively small, native proteins has gradually expanded to include monoclonal antibodies (mAbs), cytokines, replacement enzymes and more recently, a diverse array of protein products. These protein products merge together biologic and pharmacologic elements yielding engineered antibody derivatives (e.g., nanobodies, Fabs, scFvs), antibody-drug conjugates (ADCs), fusions of therapeutic proteins with native and non-native products, and bispecific antibodies. This burgeoning diversity of protein therapeutics has resulted in a concomitant increase in the number of biologics in clinical development, with more than 400 molcules currently in clinical trials around the world. These molecules are being assessed for their potential to treat a variety of diseases, including cancer, immunological disorders, and infectious diseases.[2] Despite this promise, there is a sobering attrition rate for biologics in the clinic, with only 12% of those molecules entering the clinic and reaching the market.[3] The causes for this attrition may vary, but lack of efficacy is often identified as a major contributor.[4] Optimizing efficacy requires, among other things, sufficient drug delivery to the intended target site. Indeed, a key pillar proposed for improving the clinical success rate is the confirmation of sufficient drug exposure at the effect site.[4] To achieve this goal, one must either 1) measure effect site concentrations directly (often impractical in humans), 2) assume that drug at the effect site is in equilibrium with the blood compartment, 3) use nonclinical absorption, distribution, metabolism, and excretion (ADME) data to derive an informed estimate, or 4) employ the mechanistic mathematical models to characterize and predict the time-course of drug effects in tissues, at effect sites, and in complex with the pharmacological receptor.

Therapeutic proteins have traditionally been administered to patients intravenously, which is both inconvenient and expensive. As the popularity of these therapeutics has grown, their route of administration has increasingly shifted toward non-intravenous delivery methods. These delivery methods include inhalation and parenteral administration (subcutaneous (SC) and intramuscular) along with depot formulations facilitating sustained-release and other formulations which are thought to improve SC delivery by including helper enzymes such as hyaluronidase. In order to maximize the development and application of such approaches we must gain a better understanding of the mechanisms and determinants underlying the absorption of these high molecular weight therapeutic agents. Even for the seemingly well characterized therapeutic class of mAbs, our ability to accurately predict human bioavailability (F) and absorption kinetics remains poor.

The evolution from more native protein therapeutics (e.g., cytokines, antibodies) to biotherapeutics with more novel and complex structures including polyethylene glycol (PEG)-conjugated proteins or peptides, fusion proteins, and ADCs, has

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introduced new challenges related to the stability, catabolism, and elimination of these products. These characteristics can affect the observed pharmacology as well as the pharmacokinetics (PK) of the protein therapeutic. Regulatory agencies recognize the challenges and potential value of determining the ADME characteristics of therapeutic proteins, as evidenced by the inclusion of a section on disposition in the European Medicines Agency's Guideline on the Clinical Investigation of the Pharmacokinetics of Therapeutic Proteins.[5] While the agency acknowledges that studies of the disposition of therapeutic proteins may not be necessary, it suggests that "specific studies of the route of elimination and metabolism…and identification of metabolites in vitro should be considered and discussed on a case-by-case basis" and that active metabolites should be measured. For therapeutic proteins with non-native conformations, where reliance on well described ADME properties may not be possible, the need for dedicated ADME investigations may become paramount.

As the realm of protein therapeutics grows, both in interest and diversity, it becomes clear that a greater understanding of the ADME properties of these molecules will be critical to their design, development, and use. In this review, we discuss the relationships between protein therapeutic diversity, current knowledge, and the available tools to assess ADME properties. We hope to illustrate the benefit of utilizing these tools as a means to gain a better mechanistic understanding of the PK, pharmacodynamics (PD), and metabolism of protein therapeutics and to emphasize the importance of understanding biotherapeutic ADME as a way to drive forward the selection of successful drug candidates.

Protein therapeutic diversity and ADME

The current chemical space defining protein therapeutics is vast. These molecules come in many unique forms, with various structural components that each affect the molecule's behavior. In this section, we touch on a few components of biotherapeutic diversity with a focus on how each of these components can affect the ADME properties of the therapeutic by introducing structural diversity.

Influence of molecular mass on ADME

Perhaps the most striking characteristic of therapeutic proteins is the range in molecular weight observed in these therapeutics. While there is some uncertainty about the mass distinction between peptides and proteins, it is often stated that peptidic molecules above 3–5 kgdaltons (kDa) are defined as proteins. Using this definition, the range in mass of therapeutic proteins spans from molecules as small as calcitonin (3.5 kDa) to mAbs (150 kDa). It is important to distinguish between molecular weight and physical size, which can be represented by measurements such as the hydrodynamic radius. These two parameters tend to correlate, particularly for globular proteins, but not necessarily for proteins tethered to less structured moieties such as PEGylated proteins and other more novel constructs. Nevertheless, the mass of a therapeutic protein, regardless of its overall shape, can have dramatic effects on its absorption. We will discuss these effects as they pertain to SC absorption, as this is the most common non-intravenous route of administration.

While drug delivery technology platforms have advanced significantly, inhalation and oral administration of biotherapeutic agents remain formidable challenges for formulation scientists. As large, hydrophilic, and chemically labile molecules, most biotherapeutics are virtually excluded from traditional tablet and capsular formulations.[6] Consequently, most biologics currently on the market are injected. Unfortunately, this route of drug administration tends to incur low patient compliance and a higher cost of therapy. Despite new advancements in modern oral and pulmonary delivery devices, certain barriers still compromise the absorption of biologics through these routes. The oral route of drug delivery poses a challenge due to degradation of biotherapeutics in the gastrointestinal (GI) tract. Alternatively, the nasal cavity and lung can be effectively targeted for drug absorption, thereby avoiding the proteolytic enzymes and first-pass metabolism of the GI tract. Although similar threats to protein therapeutics (such as metabolic enzymes and macrophages) are found in the lungs, the general metabolic activities and pathways of the respiratory epithelium differ from those observed in the GI tract.[7] Furthermore, while the alveolar epithelium and capillary endothelium remain fairly impermeable to many hydrophilic substances of large molecular size, the endothelial junctions allow for the passage of larger molecules of about 4–6 nm – the size of many biotherapeutics.[8] Additionally, the mucosal lining of the pulmonary epithelium and the surfactant coating the alveoli have high concentrations of protease inhibitors, and presumably protect peptides and proteins from degradation. Nonetheless, this protection appears to be an exception rather than the rule, as membrane-associated (on epithelium and endothelium) and intracellular (in macrophages, lymphocytes, neutrophils and mast cells) proteases and peptidases can readily degrade administered peptides and proteins.[9] Although oral administration remains a challenge in the delivery of therapeutic biologics, inhalation poses a promising potential route of administration if current challenges can be overcome. Until then, the focus will remain on SC delivery for these molecules.

Relatively little is known about the mechanism of SC absorption of proteins in different species, and conflicting results have been reported on the effects of the delivery route and site of administration on protein PK.[10] Upon SC administration, therapeutic proteins are delivered to the hypodermis, a space consisting of adipose tissue separated by a fibrovascular network.[11-13] Within the hypodermis lies a network of extracellular matrix (ECM) consisting of a number of matrix proteins including collagen, proteoglycans, and glycosaminoglycans, which together confer a net negative charge to the ECM that may have an effect on the interactions of the ECM with potential therapeutic proteins. Therapeutic proteins move through the ECM via diffusion and convection thereafter entering systemic circulation through blood or lymphatic capillaries. The fraction undergoing lymphatic absorption increases with an increase in molecular size.[15,16] The diffusion velocity of smaller proteins (<15 kDa) is greater than their convection velocity allowing them to diffuse rapidly through the ECM and primarily into the blood capillaries.[13,18] The diffusion velocity of larger molecules is dramatically hindered by the mesh-like framework

of the ECM limiting their local migration.[15,16] Therefore convection is believed to be the primary mechanism responsible for antibody transport away from the SC injection site. Bulk flow of interstitial fluid (IF) drives these larger molecules toward blind-ended lymphatic capillaries which, unlike the endothelium of continuous blood capillaries, expand in response to increases in IF pressure allowing fluid to flow in but not out. Once biotherapeutics enter the lymphatic system, they eventually drain into the venous system. This passage through the lymphatic system is considerably faster than their migration through the ECM,[13] marking interstitial convection as the rate-limiting step of absorption.

An accurate description of the quantitative relationship between molecular size and the fraction absorbed by lymphatics remains elusive, particularly across species.[13] Deviations from our current models arise from studies reporting that some mid-sized proteins, ranging from 5.6–60 kDa, show little lymphatic absorption.[18] The cause of this discrepancy is not known, but may be related to species and injection site differences. What is even less understood is the effect of molecular weight on bioavailability as there appears to be little correlation between these parameters.[11,21] This lack of correlation, while not intuitive, may be attributed to a multitude of factors affecting bioavailability such as target binding, catabolism, and the administration site. As such, the major parameters affecting these processes are thought to include the roles of lymph and blood capillaries in systemic absorption, cross-species differences in hypodermis morphology and physiology, drug formulation, stability of the molecule, the site of injection, the depth of injection, as well as the molecular properties of the proteins themselves.[5,14,22-24] More work needs to be done in order to more precisely characterize the contributions of these complicating factors to the SC absorption of biologics.

Once a biotherapeutic reaches systemic circulation, mass (or size) can influence its distribution. Tissue distribution is determined by the reversible movement of molecules into and out of tissues from the circulation. Movement across the vascular barrier into the interstitial fluid of tissues can occur either by movement across the endothelial cells that make up this barrier, or between those cells. The size and charge of therapeutic proteins generally limits movement across the lipid bilayers constituting the cell membranes unless facilitated by an active transport process, such as the neonatal Fc receptor (FcRn) of IgG. The contribution of active transport to tissue distribution is an active area of study that we discuss in the context of FcRn below. Movement between cells (paracellular) is generally dependent on the nature of the endothelium lining the capillaries in that tissue. Three types of capillary endothelium have been described that exhibit distinct transport mechanisms.[25-29] Continuous non-fenestrated endothelium, found in muscle, CNS, skin, and lung, is thought to be the most resistant to transport, restricting movement to water and solutes less than 3 nm in radius. In the brain, a special case of non-fenestrated endothelium exists where tight junctions between endothelial cells act as a stringent barrier to paracellular transport.[29,30] Continuous fenestrated endothelium (renal glomeruli, synovial tissue, intestinal mucosa) is more permissive due to the presence of fenestrations between cells, but still retains a high resistance to the passage of large macromolecules (e.g., albumin) due to the presence of a non-membranous diaphragm across the opening.[27,28] Discontinuous endothelium (liver, spleen, bone marrow) has the greatest permeability.[26,29] It is characterized by large fenestrations lacking diaphragms, pores within individual cells, and a poorly formed basement membrane.[27,28]

Passive movement via convection or diffusion between the cells is thought to be the more dominant process for protein therapeutic distribution.[31] Diffusion into and out of tissues is dependent on the concentration gradient between the plasma and interstitial fluid and the surface area of the exchange area. Convection is dependent on both the surface area of exchange and the pressure gradient across the vessel. Which process is dominant for a given therapeutic protein depends primarily on size and charge, but for molecules the size of antibodies the movement is likely governed by convection.[32] For antibodies, which lie at the high end of the protein therapeutic mass scale, the extent of tissue distribution has been thoroughly studied, and there appears to be little species or molecule dependency in the non-specific tissue (i.e., not target-mediated) distribution. The estimated interstitial fluid-to-plasma ratio for antibodies and other large therapeutic proteins at steady state ranges from ~0.3-1 (derived from physiologically based PK modeling).[33,34] As described above, the ratio is thought to be tissue dependent, with tissues possessing non-fenestrated endothelium at the lower end of the scale while those tissues with fenestrated or discontinuous endothelium at the higher end of the scale. Direct measurements of the interstitial fluid concentrations of therapeutic proteins have not been reported; however, studies of interstitial fluid concentrations of albumin and IgG in skin have reported an interstitial fluid to plasma ratio (0.5–0.6) generally consistent with these predictions.[35,36] Little information has been published describing the interstitial fluid-to-plasma ratios for other proteins, but it is reasonable to expect that the same processes govern those proteins and that smaller proteins would have higher ratios. Once within the interstitial space, many of the same dynamics and influences previously described for absorption still apply (e.g.,, interactions with ECM) and the movement of protein therapeutics and other macromolecules (including PEGylated molecules) into and through the tissues is governed primarily by convection, though still dependent on mass and volume.[37,38] As described above, larger molecules have more limited distribution to the extracellular space, and distribute into and out of tissues more slowly.[33,39,40] This can have an effect on molecule choice for different disease states, as has been nicely demonstrated for tumors by several groups.[41]

Disease state can also influence vascular permeability and transport, often leading to increased permeability.[27,28] In the case of tumors, a large body of research exists on solute movement into and through tumors, which are known to have perturbed vascular function and physiologic fluid flow.[31-34,42-44] For other disease states, much less is known about the effect of changes in vascular permeability and other disease processes on the movement of protein therapeutics into and out of affected tissues. Therapeutic proteins are eliminated via several mechanisms, including proteolytic degradation in the plasma or extracellular fluid, renal filtration, and cellular uptake followed by proteolytic degradation (e.g., receptor-mediated uptake by cell surface receptor targets or Fc receptors, and fluid

phase pinocytosis). Of these, renal filtration followed by tubular uptake and degradation is the most dependent on the mass of the protein therapeutic. It has been long known that the rate of renal elimination of proteins is inversely related to size.[45] Commonly quoted dogma states that proteins above ~50 kDa are not subject to renal elimination due to the inability of such proteins to pass through the renal glomerular barrier. While useful, this maxim is not entirely true because size is only one factor governing renal excretion of proteins, with charge and tubular reabsorption playing important roles in renal clearance as well. Given the high perfusion rate of the kidney, even solutes that are inefficiently filtered can be subject to substantial excretion in the glomerular filtrate. For example, considerable evidence exists indicating that relatively large quantities of albumin pass into the glomerular filtrate with the vast majority reabsorbed in the renal tubules.[46] Renal excretion can also play a significant role in the elimination of large therapeutic proteins, as evidenced by a 30% decreased clearance of a 63 kDa peptibody fusion protein in subtotal nephrectomised rats.[47] Similar evidence exists for PEGylated peptides where the renal elimination of parent molecule (~45 kDa) or the 40 kDa PEG component was greater than 70%.[49] Readers interested in the mechanisms of renal elimination of macromolecules are directed to reviews by Harraldsson et al [48] and Maack.[45] The effect of therapeutic protein size on other routes of elimination is less well understood, but size is not likely to affect processes such as receptor-mediated endocytosis, vesicular transport, or cellular pinocytosis.

As mentioned previously, size and shape are often but not always correlated. Biotherapeutics are typically thought to have a generally globular shape. A notable exception may be PEGylated proteins, where PEG molecules (which often range from 1-50 kDa) are conjugated to a protein or peptide, usually to enhance its PK or stability.[49] The conjugation of PEG increases the mass of a protein or peptide, resulting in similar ADME effects to those described above. However, PEG has very different physiochemical characteristics than proteins, including greater flexibility, lower density, and distinct aqueous behavior. As such, the ADME characteristics of native therapeutic proteins and PEGylated proteins of a similar size may differ. This topic is discussed in greater detail below.

Influence of charge on ADME

Other physicochemical characteristics of therapeutic proteins contribute to their diversity and affect their ADME properties. The surface charge of a therapeutic protein is a property of the amino acid sequence of the protein and the pH of its surroundings. This physicochemical property is complex and heterogeneous, resulting in a mixture of charge variant species within the population of therapeutic protein product. Charge heterogeneity is typically caused by deamination, isomerization, or post-translational modification, which can cause a change in the net charge of a protein, thereby leading to the formation of acidic and basic variants.[51] Most therapeutic proteins have an isoelectric point (pI) in the range of 5-9, with most antibodies being slightly positively charged, with a pI of 7–9.[10,51] This surface charge can lead to interactions with other molecules and tissue constituents in the body, thereby affecting ADME. As described above, the interstitial space has a net negative charge, and proteins with a net positive charge may have delayed absorption following SC dosing, likely due to charge-charge interactions in the SC space.[11,13] Changes in charge can also dramatically affect distribution, as described by Hong et al.[42] In this study antibodies and antibody fragments with greater net positive charge were found to have increased renal clearance and greater tissue distribution, likely due to increased interaction with negative charges on the cell surface. Other examples are described in a review of the effects of antibody charge by Boswell et al.[50] Changes in pI of more than one pI unit can have physiologic consequences, including the increased plasma clearance and tissue retention described above. However, it is important to keep in mind that for most therapeutic proteins, the range of charge heterogeneity within the product is small, and several studies have demonstrated that such minor changes in charge have little overall impact on PK and distribution.[50,51]

The effects of charge on protein therapeutics can also be observed in relation to their altered elimination. Charge is known to affect renal filtration at the glomerulus due to negative charges on the glomerular basement membrane.[50] Thus, negatively charged molecules undergo less renal filtration than more positively charged molecules of a similar size.[48] In addition, the renal tubular epithelium, a site for reabsorption of some protein therapeutic molecules from the glomerular filtrate, has a net negative charge, which can facilitate improved reabsorption of more positively charged proteins.[50] Other recent studies from the last 2 y have tried to correlate changes in charge with the PK of antibody therapeutics. These studies also emphasize the point that balancing charge in the complementarity-determining regions (CDR), variable domain (Fv), or framework of the therapeutic antibody can lead to improved PK, the magnitude of which is likely dependent on the relative influence of charge imbalance and other factors affecting the molecule's disposition.[52-54] Li *et al.* provided direct evidence of improved antibody PK following the incorporation of a more negative charge on the variable light (VL) frameworks.[53] A variant with a pI of 8.61 exhibited rapid clearance whereas a molecule with pI of 6.10 exhibited relatively slow clearance. Both variants exhibited comparable binding to rat FcRn, but biodistribution studies showed that the high pI variant was catabolized in the liver and spleen. These results suggest antibody charge can have an effect on PK through alterations in antibody catabolism independent of FcRn-mediated recycling. However, in another study, Schoch *et al.* demonstrated that the charge distribution on the variable fragment (Fv) domain is involved in excessive FcRn binding.[54] This excessive binding prevents efficient FcRn–IgG dissociation at physiological pH, thereby reducing FcRn-dependent terminal half-lives.

In the development of therapeutic monoclonal antibodies, research and development has focused largely on the IgG1, IgG2, and IgG4 isotypes, with the IgG1 isotype remaining the most popular to date.[55] Although all IgG variants, each of these isotypes contain slight differences in their hinge and constant regions that affect their functionality and half-life in vivo. In order to identify possible ADME differences between different isotypes, Datta-Mannan *et al.* showed that the effect of balancing the CDR net positive charge on nonspecific binding was more significant for the IgG4 versus the IgG1 molecule.[52] This

differential effect was connected to the degree of influence on cellular degradation in vitro and in vivo clearance, distribution and metabolism. In the more extreme case of the IgG4 isotype, balancing the charge yielded greater than fold7- improvement in peripheral exposure, as well as significantly reduced tissue catabolism and subsequent excretion of proteolyzed products in urine. Consequently, balancing CDR charge on the IgG1 isotype had a more subtle influence on non-specific binding and yielded only a modest alteration in clearance, distribution and elimination.

Influence of glycosylation on ADME

Glycosylation is a post-translational modification of proteins by which carbohydrates (glycans) are added to specific amino acids. Such modification is a natural phenomenon associated with the production of therapeutic proteins in eukaryotic production systems. The glycosylation of a therapeutic protein can be dependent on a number of factors, including cell production system and cell culture conditions. This interesting, but highly complex, topic is outside the scope of this review and interested readers are directed to some excellent reviews on the subject.[56,57] The amount and nature of glycosylation can dramatically affect the behavior of the proteins. The most commonly described roles for glycosylation are related to receptor binding and Fc effector function in antibodies, with recombinant erythropoietin and afucosylated rituximab as illustrative examples.[58,59] However, the glycosylation profile of a therapeutic protein can substantially affect its PK and distribution. Introducing N-linked glycosylation to proteins as a strategy for improving systemic residence time (generally by increasing size or modifying binding to glycoprotein receptors) has been exemplified by darbepoetin alfa (a hyperglycosylated form of erythropoietin), follicle-stimulating hormone, and with a single chain diabody.[58,60] For antibodies and fusion proteins possessing an antibody Fc, the effect of glycosylation on PK and distribution is less clear. Human antibodies contain N-linked glycans at Asn 297 of the Fc, and some antibodies also possess N-glycosylation sites in the Fv region.[61] These glycans are typically inaccessible to cellular receptors (e.g.,, mannose or asialoglycoprotein receptors) that could facilitate binding and increased clearance. Work by Huang et al.[61] has shown that changes in the N-glycosylation of neither the Fc nor the Fv regions of an antibody affect clearance in mice. However, other studies have demonstrated that Fc glycosylation can indeed have effects on PK. By following the glycan patterns over time following mAb administration in humans, it was observed that high mannose-5 glycan forms were more rapidly eliminated compared to other glycoforms.[62] Wright et al. found that an IgG with a high mannose glycan also exhibited more rapid elimination in mice.[63] PK and distribution studies in mice of a series of Fc fusion proteins differing in their levels of O-linked sialylation showed that variants that were desialylated or with a low level of sialylation had more rapid clearance and greater tissue uptake than those with higher sialylation.[64] However, this was not thought to be mediated by the asialoglycoprotein receptor. The influence of glycosylation on tissue distribution is not well described. One would rationally assume that the increased size provided by extensive glycosylation of a small protein would affect not only its elimination but also its distribution. As the protein size increases, the proportional increase in size conferred by glycosylation is lower, which would likely result in more modest effects on distribution. For example, the glycosylation of a mAb adds only 10–15% to the mass of the protein.[65] The importance of glycosylation on the distribution of therapeutic proteins is nicely exemplified by enzyme replacement therapies for the treatment of lysosomal storage diseases. The distribution of these exogenously administered enzymes is often determined by the interaction of mannose and mannose-6-phosphate molecules on the enzyme with the respective receptors on tissues.[66] Thus, tissue distribution is determined by the relative density of these receptors on cells.[67] Unfortunately, this limits distribution of the enzyme to cells with high receptor expression (e.g., reticuloendothelial cells) and limits distribution to other affected organs that may be less highly expressing (e.g.,, kidney, lung).

Influence of diverse protein modifications on ADME: Mechanism of action enhancement and half-life extension

Researchers have explored a number of strategies to enhance or optimize the efficacy of protein therapeutics, often by modifying the protein structure, fusing a smaller protein to another protein fragment, or chemically conjugating an active peptide or chemical to a protein. These modifications typically result in either an enhancement in the pharmacologic activity or a prolongation of the drug exposure in the circulation. Two types of modifications can have a major impact on the pharmacologic and ADME characteristics of these molecules. First, conjugation of moieties that enhance or modify the pharmacology (e.g., ADCs, radioimmunoconjugates, immunotoxins) has the potential to change not only the efficacy and toxicity associated with the therapeutic protein to which they are conjugated, but also the ADME characteristics. For example, the catabolism and catabolic products of an unconjugated antibody may be of little interest due to the close similarity to endogenous antibodies. In contrast, the potentially potent catabolic products of an ADC can have pharmacologic effects that may differ from the parent ADC due to dissimilar distribution and elimination properties. While the distribution of an unconjugated antibody to tissues not expressing target may be quite benign, this may not be true for an ADC where non-specific uptake and catabolism can have serious consequences. Estimates of tissue cytotoxin levels based on the established understanding of mAb distribution may be helpful, but work by Alley et al.[68] shows that the proportion of free (and presumably active) to conjugated cytotoxin varied widely between tissues. This suggests that some tissues may be more effective in releasing or accumulating active cytotoxin. In most cases, conjugation of a small molecule cytotoxin to a mAb has been shown to have little impact on mAb clearance and distribution.[69,70] There are, however, some examples where conjugation has been shown to affect distribution resulting in adverse outcomes. An anti-Lewis Y mAb was found to have typical biodistribution in patients by scintigraphy, but upon conjugation with calicheamicin demonstrated increased clearance, decreased tumor uptake, and increased liver uptake that was associated with liver toxicity.[71,72] The effect of drug loading on clearance and toxicity was

demonstrated by the observation that highly loaded ADCs exhibited more rapid clearance and greater toxicity,[73] possibly due to perturbations in the antibody structure, resulting in scavenging by Fc-gamma receptors or inefficient neonatal Fc receptor (FcRn) recycling. For immunotoxins, i.e., protein toxins conjugated to antibodies or antibody fragments, there is less known. It was reported that conjugation of gelonin to an antibody against a tumor antigen (gp240) resulted in a nearly fold2- increase in plasma clearance of the conjugate as compared to the unconjugated antibody, in addition to uptake of the conjugate into spleen and kidney.[74] Such examples suggest that careful investigation of the effects of conjugation on distribution and clearance are important aspects in the development of ADCs.

The second type of modification is the conjugation or engineering of proteins to improve their residence time in the body. As described above, small proteins are rapidly cleared from the body and this can limit their pharmacologic efficacy. Many strategies have been used to address this problem for small proteins, and these typically fall into 2 general categories: those that increase the hydrodynamic volume (i.e., increase in size), and those that utilize FcRn-mediated recycling in addition to volume increase.[75]

Increase in size

The first category involves increasing the size of a small therapeutic protein to limit elimination by the kidney. This includes such approaches as conjugation with hydrophilic polymers (e.g.,, polyethylene glycol, polydextrans, starch), increasing the amount of glycosylation, or recombinant extension of the therapeutic protein amino acid sequence to increase its size (e.g., XTEN).[76] This discussion will focus on proteins and peptides conjugated to PEG as most of the literature describes such molecules. Molecular size has a substantial effect on therapeutic protein ADME properties, as described previously. However, a small protein conjugated with a hydrophilic polymer may not possess the same ADME properties as an unconjugated protein of a similar mass.

Differences in absorption and distribution between protein therapeutics and polymer-conjugated proteins of similar mass might be expected based on the potential differences in hydrodynamic radius. Per unit of mass, PEG is thought to possess a much greater molecular volume likely due to the extended conformation of the PEG polymer and the ability to coordinate water molecules.[41] In addition, the molecular charge of PEG and other polymers is considerably different than that of proteins, which will influence interactions with endogenous molecules in tissues. However, SC absorption of PEGylated proteins appears to occur at a similar rate and magnitude as therapeutic proteins of a similar mass.[11,49,77] A number of studies of the tissue distribution of polymer conjugated proteins have been conducted and those related to PEG have been summarized in a recent review by Baumann et al.[49] PEG size played a clear role in distribution, but the few studies that have evaluated this relationship have not identified a consistent trend.[78] The catabolism/metabolism and elimination of PEGylated and other polymer-conjugated proteins may differ from that of therapeutic proteins consisting solely of amino acids. The polymer component of these molecules is subject to different metabolic processes, and cellular or organ elimination may also differ. The metabolism of PEGs is dependent on molecular weight, with minimal metabolism being observed for PEG molecules of the size typically used for therapeutic conjugation (>5 kDa).[79] What catabolism does occur can be of the peptide or protein conjugated to the PEG.[16,80,81] Elimination of PEGylated proteins can occur via elimination of the parent molecule or via catabolism of the protein or peptide component with subsequent elimination of the PEG component. In general, renal elimination appears to be the predominant route of elimination for PEG and PEGylated proteins, with a rate that is inversely proportional to PEG or PEGylated protein size.[41,49] Interestingly, PEGylated protein clearance appears to be somewhat slower than that of a similarly sized protein. While a direct comparison was not found, the clearance of a F(ab')2 or a diFab with a mass of ~100kDa was 10–fold15- faster than that of an IgG,[36,82] whereas Fab-PEGs can have clearances with only a fold2- difference from comparable IgGs.[49,83] The reasons for this may be due to differences in hydrodynamic radius between globular proteins and linear or branched hydrophilic polymers resulting in slower renal clearance for polymer conjugated proteins.[41] A full discussion of this is beyond the scope of this review, but other reviews of this topic are available.[41,49,83,84]

Increase in size in conjunction with FcRn-mediated recycling

The second category for reducing therapeutic protein clearance leverages both an increase in size and the FcRn-mediated physiologic recycling process of IgG and albumin. This can be achieved by fusing or conjugating the therapeutic protein or peptide to an immunoglobulin Fc (e.g.,, etanercept, abatacept, romiplostim) or albumin, or by engineering the therapeutic protein with a binding site for a larger circulating native protein (e.g., albumin-binding Fabs, albumin-binding domain antibodies),[75,76] thereby enabling binding of the Fc- or albumin-binding construct to FcRn. The FcRn is a major histocompatibility complex class I-related receptor composed of an α chain and $\beta(2)$ microglobulin expressed by cells of many tissues of the body. It functions in the reduced pH of the endosome by means of pH-dependent binding (high affinity at pH 6, low affinity at pH 7.4) to albumin or IgG (via the Fc), followed by recycling and release of bound protein at the cell surface where FcRn affinity is considerably lower.[85] The addition of an FcRn binding site to a therapeutic protein will thus reduce plasma clearance of the biotherapeutic by facilitating its recycling in the endosome and preventing degradation in the lysosome. For Fc fusions or conjugates, while one might expect that the clearance should be identical to that of a native IgG, it is commonly noted that the plasma clearance of these constructs is substantially greater, possibly due to differences in binding to FcRn or increased renal clearance due to their smaller size.[75,86] By changing the size of the construct and including binding to FcRn, one might also expect differences in absorption and distribution from that seen with the unaltered protein or peptide, and this has been commonly observed.[37,87,88]

The addition of FcRn binding could have effects on absorption and distribution independent of the increase in molecular

size, and the relative role of each of these contributors remains unclear. The role of FcRn in the ADME of protein therapeutics is still an active area of research, with most of this work being conducted with antibodies. The effect of FcRn binding on absorption has proved to be controversial due to potentially conflicting data. Studies have been conducted with wild-type and FcRn knockout (KO) mice, as well as primates using antibodies with varying FcRn binding characteristics. A fold3-improvement in SC bioavailability of an IgG1 in wild-type mice vs. FcRn KO mice was described, suggesting that FcRn is important in SC absorption.[77] Using a series of anti-A β mouse IgG2a FcRn binding variants, Deng et al.[89] found similar bioavailability (F = 76–86%) for the wild type and a variant antibody with enhanced FcRn binding at pH 6 and pH 7.4; a modest increase in bioavailability (95%) for a variant with enhanced pH 6 binding (which would confer improved FcRn-mediated recycling and transcytosis); and substantially lower F (41%) for a variant with no FcRn binding, also supporting a role for FcRn in SC absorption.[90] This is consistent with a mouse study of human anti-TNF IgG1 FcRn variants that demonstrated that an antibody variant with substantially increased FcRn affinity at pH 7.4 (~25 fold) and only modestly increased affinity at pH 6 (~1.75 fold) reduced the half-life of this variant to approximately half of the value of the normal antibody. This decreased half-life is presumably due to less efficient recycling by FcRn as a result of poor release of the antibody at the cell surface.[89] Poor release of the antibody may lead to increased intracellular catabolism of the protein, increasing systemic elimination of the antibody and decreasing SC bioavailability. In support of this hypothesis, in a rat study of rituximab, SC co-administration of nonspecific IgG to saturate local FcRn resulted in decreased F of rituximab.[18] In contrast to the above findings, experiments in primates using several human IgG4 antibodies and corresponding variants with 15–60 fold increased FcRn binding at pH 6 found no difference in F between the native and FcRn variant antibodies.[91] From this work, it appears that decreases in FcRn recycling (via FcRn blocking or increased FcRn affinity at pH 7.4) can have substantial effects on F, while increases in FcRn affinity at pH 6 (as a means of attempting to increase FcRn recycling) provides little improvement in F beyond that already achieved with the native Fc.

FcRn has the potential to play several roles in therapeutic protein distribution. At the endothelial barrier of the capillaries, FcRn could recycle Fc or albumin-containing therapeutic proteins back into circulation, thereby limiting their penetration into the interstitial space. Alternatively, FcRn could facilitate transcytosis of the same molecules into the interstitial space, thereby improving distribution relative to other similarly sized proteins. The effect of FcRn binding on distribution has been studied, again with IgGs, with disparate outcomes. Using tissue/blood area under the curve (AUC) ratio (T/B ratio) as a measure of tissue distribution, the T/B ratio for a human IgG1 was higher in spleen, liver, kidney, and lung in FcRn α chain KO mice compared to wild-type mice, while the T/B ratio was higher in wild-type mice for fat, lymph node, skin and muscle.[92] In a similar study using an FcRn β(2)-microglobulin KO mouse model, tissue to plasma ratios were higher in muscle and skin in the WT mice relative to KO with little difference in lung, gut, liver, kidney, heart, and spleen.[93] Using an alternative approach, studies in normal mice using human IgG1 FcRn binding variants with fold6- greater binding affinity (pH dependency not noted) or no binding to FcRn found little or no difference in tissue/plasma AUC ratios between the variants and wild-type IgGs.[94] The reasons for the discrepancy between studies is not known, but could be attributed to the different durations of the studies, or physiologic differences between wild-type and FcRn KO mice that affect the balance between uptake and return of antibody from tissue. At this time, gaps remain in our mechanistic understanding of the effects of binding to FcRn on absorption and distribution of therapeutic proteins, but, as illustrated in the studies described above, some insights are beginning to emerge.

Our understanding of the role of FcRn in catabolism and elimination is still evolving. It is generally believed that the binding to FcRn by an Fc moiety or albumin in the endosome allows recycling or transcytosis, thus providing protection from endocytic degradation.[85] The importance of this interaction is evident from studies showing increased antibody or fusion protein clearance in animals lacking FcRn or increased clearance in normal animals of antibodies with reduced FcRn binding.[90-95] What is less well understood are the details of the underlying mechanisms for this effect, specifically the major tissues and cells involved, the efficiency of the process, and the overall impact on clearance and distribution to various tissues. Studies determining the key tissues involved in mAb catabolism are emerging and other aspects of FcRn-mediated tissue dynamics are currently under study.[93,94] A less well-studied role for FcRn in therapeutic protein elimination is related to renal elimination. FcRn is expressed in the podocytes and brush border of the proximal tubular epithelium of the kidney.[96] In the podocytes, FcRn is thought to act to clear the glomerular slits of filtered IgG by transporting it into the urine where it is presumably reabsorbed by FcRn in the proximal tubules.[97] However, this presumption may not hold as there is evidence that FcRn may act by transporting IgG from the interstitium into the urine.[96] It is clear from this discussion that the ability of a therapeutic protein to bind to FcRn can significantly affect its ADME characteristics, but that many of the dynamics and mechanisms of this interaction remain to be elucidated.

Influence of target binding on ADME

The desired property of all therapeutic proteins is binding to its therapeutic target. The specificity of this interaction is a key advantage for this class of molecules over many other therapeutic agents. The effect of target binding on the ADME properties of the therapeutic protein depends on a number of factors related to both the target and the therapeutic, including the amount, turnover rate, and location of the target and the binding affinity of the drug to the target. Little information has been reported on the potential effect of the target on the absorption of protein therapeutics. For many therapeutic proteins, movement through the SC space and transit through the lymph system will expose the drug to soluble or cell-surface target (e.g.,, TNF, IGF1R, VEGFR, CD11b). This interaction with the target could result in receptor-mediated uptake and degradation of the therapeutic protein or the formation of immune complexes

that are subsequently cleared. Both of these would result in a decrease in absorption and bioavailability. Searches for studies specifically evaluating the effect of target binding on F were not successful; however, there are examples whereby increasing F with increasing SC dose may indicate saturation of degradation or target binding at the site of administration or during the absorption process.[98]

The effects of drug target on distribution can be evident immediately after dosing where, for example, a highly abundant or rapidly generated target in plasma or blood can interact with the administered therapeutic protein, thereby limiting the apparent tissue distribution of the free drug.[99] Cell surface targets in tissue can substantially enhance uptake and retention of protein therapeutics at sites of target abundance. This is most clearly illustrated by the large body of data describing the high concentrations of antibody that can be achieved in tumors with high levels of target.[43,68,70,72,100-103] This phenomenon is not limited to tumors, as evidenced by recombinant IL-10 distribution to tissues with receptor overexpression and PK/PD evidence for other therapeutic proteins.[98,104-107]

ADME enabling-technologies and methods in drug discovery and development

In contrast to small molecule drugs, the development of in vitro and in vivo correlation tools for protein therapeutics lags behind due to the more complex nature of biotherapeutics. The lack of appropriate tools to study therapeutic exposure, metabolic or metabolic-like biotransformation, and target engagement in the vascular and tissue spaces makes ADME characterization of biologics more difficult as compared to small molecule therapeutics.[108] High throughput ADME methods that currently exist for small molecule drugs have not been as strongly pursued for antibody protein therapeutics, reflecting to a certain degree the substantially lower attrition rate and the associated lower numbers of potential clinical protein therapeutic candidates.[109] This section represents an attempt at summarizing the available tools and approaches for generating ADME data.

ADME tools and technologies

Common considerations for the ADME-related issues for therapeutic biologics include target mediated clearance, FcRn recycling for Fc-containing proteins, immunogenicity, isoform heterogeneity, and metabolic stability, especially for relatively low molecular weight proteins.[110] Therapeutic biologics generally have limited distribution in tissues. Therefore, most of the relevant ADME studies for protein therapeutics rely on in vivo evaluations, which are based on a variety of techniques aimed at following unlabeled/labeled drug/metabolite using either antibody-based or mass spectrometry (MS)-based techniques.[111]

Quantitative and analytical tools

Immunoassays, such as the Enzyme-linked immunosorbent assay (ELISA), are the most commonly used techniques for the quantification of proteins in serum due to their high sensitivity and specificity. However, such methods can become expensive and labor intensive when applied to a large number of proteins and samples.[111] Thus, higher throughput analytical approaches and instrument platforms are continuously being pursued. One example is the "generic ELISA" which uses a reagent recognizing human Fc, allowing for the detection of anti-drug antibodies and PK studies of any human Fc-containing biologics.

In recent years, advances in technology have made automated unattended screening broadly accessible, and have further facilitated the implementation of new robotic systems and approaches to increase productivity and efficiency for screening during the development of biotherapeutics. Some of the major platforms include the Gyrolab immunoassay workstation, which is currently being used for drug analyte (nanoliter) sample measurement and for PK studies 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 inter-subject variability associated with non-serial sampling needed for a mouse PK study that relies on a conventional ELISA for bioanalysis.[111]

Another technology particularly attractive for bioanalysis of proteins is MS. The 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 a potentially higher throughput rate.[111,112] Quantitative liquid chromatography–mass spectrometry (LC-MS) has also been applied to the quantitation of anti-drug antibodies in human and cynomolgus monkey serum in the presence of high circulating concentrations of the protein therapeutic.[113] The development of quantitative mass spectrometric assays has been evolving over the past decade and will continue to be the most powerful tool for ADME studies of therapeutic biologics.

Labeling techniques for ADME and imaging studies

The labeling of therapeutic biologics is an invaluable technique that has been used extensively to analyze PK and metabolism, measure absolute tissue concentrations, and facilitate imaging studies. Proteins possess unique sequences of amino acid residues, making them perfect targets for labeling. Generally, the type of label and the protein conjugation strategy must be carefully tailored to each application. Radionuclides are used extensively, but they have a short shelf-life and require special handling and disposal. Today, the 2 main methods of radiolabeling proteins are halogenation and the complexation of metallic radioisotopes. Radiohalogens (such as radioiodines) can be directly conjugated to proteins in one step, mainly via formation of a stable covalent bond. An alternative approach to the direct method is the indirect incorporation of radiohalogens into small organic molecules. These molecules contain activating prosthetic groups allowing for iodination and conjugation with proteins under mild conditions. The reader is referred to a review by Wilbur for a thorough overview of the current radioiodination methods and reagents for conjugate labeling.[114]

Some radiometallic nuclides (e.g., Tc-99m) can bind proteins directly by forming coordinate bonds with various functional groups. However, the stability of such conjugates is questionable since most proteins, including mAbs, do not

possess functional groups capable of forming stable metal bonds. One solution to this problem is to attach a bifunctional chelate that can be covalently conjugated to proteins and will chelate a radiometal, thereby forming a stable radionuclide-chelate-protein label.[115] Due to the diversity of the metallic radionuclides, many different variations of bifunctional chelators are used depending on the choice of radionuclide. The size, charge, and electron configuration of the metallic radionuclide (e.g.,, indium-111, copper-64, zirconium-89) will determine the coordination number required of a bifunctional chelator in order to accommodate the radiometal ion. A recent review by Price and Orvig provides a convenient and accessible overview of the field of radiometal chelating agents.[116]

The coupling of fluorescent moieties to proteins to create labeled protein reagents has also become a routine and important procedure in biologics. Molecules that absorb in the near-infrared (NIR) region (700–1000 nm) can be efficiently used to visualize and investigate in vivo molecular targets because most tissues generate little NIR fluorescence. The most common organic NIR fluorophores are small molecule organic dyes such as polymethines. Their physical properties, biodistribution, PK and applications for in vivo fluorescence imaging have been summarized in a recent review.[117] Often, a succinimidyl-ester functional group is attached to a fluorophore core and this functionality confers reaction specificity with primary amines to form fluorophore-antibody conjugates.

Ideally, the labeling procedures should minimally alter the properties of the molecule of interest, and the physical half-life of a radionuclide should match the biological half-life of the molecule to be labeled. However, a label may directly perturb the function of a protein, and the reaction conditions used to introduce the label may inadvertently promote undesirable changes such as oxidation, deamidation, side-chain isomerization, or aggregation.[118] The absence of gross changes in PK or molecular weight is not always sufficient characterization of labeled proteins, and binding or other functional assays are needed to assess the integrity (e.g., immunoreactivity) of a labeled probe.[119] Methods for labeling of proteins, in general, should also be rapid and give high yields. The labeled protein that is obtained should be of high specific activity and should be labeled in a manner that results in a stable attachment of the label. Optimization of reaction parameters is almost always a requisite of radiolabeling because radionuclides are generally dilute and contain many minor impurities from processing of the target material as well as from the chemicals used, which are present in more abundance than the radionuclides themselves.[114] Any label should remain coupled to its conjugate protein for the duration of the experiment and, ideally, make no difference to its behavior.[120] These are fundamental criterion in choosing an appropriate label for a particular study. The label itself will remain optically active or radioactive, and will be detected in any biodistribution images, but it no longer reveals the presence of the therapeutic protein per se. The distribution of excreted or catabolized labels can confound the biodistribution and imaging of certain tissues, especially in and around the hepatobiliary system, gut, kidneys, and urinary bladder. When imaging abdominal sites this can be a limitation and has been a major driver in the selection among labels.[120] Thus, appropriate choice of label allows tailoring of the properties of the labeled protein to the application required.

Today, several labels, including radiohalogens and radiometals, have fundamentally extended the possibilities of detection and imaging techniques and in turn caused the need for the development of chemical methods for their conjugation. In fact, the advance of available imaging instrumentation combined with well characterized labeling chemistry for antibodies and other protein therapeutics has enabled detailed in vivo, dynamic and quantitative measurements of radiolabeled and fluorescent probes with quantitative whole-body autoradiography (QWBA), and molecular imaging, including single-photon emission computed tomography (SPECT), positron emission tomography (PET), and optical imaging.

Different ADME methods and approaches

The investigation of the ADME properties of therapeutic proteins requires tools and experimental approaches that are specific to this class of agents. Before embarking on investigations of therapeutic protein ADME, it is worthwhile to consider what information is most critical for answering the questions at hand. For instance, absorption studies may be interested only in the amount of parent therapeutic protein in serum or plasma after SC administration, thus requiring a simple parent drug assay. Alternatively, a study may also be designed to investigate the mechanisms of the route of absorption (e.g., lymphatic or vascular) or the degree of catabolism occurring during this process, requiring analysis in lymph tissue of both parent therapeutic and perhaps catabolites. Distribution studies most commonly evaluate concentrations of the parent drug in tissues of interest, but can also be designed to measure concentrations of catabolic products and associated pharmacologically active products (e.g., cytotoxic drug from an ADC). In most instances, a quantitative method is required as this provides the most useful data for informative comparisons of molecules and strategies, and is also ideal for PK/PD and other modeling analyses.

When considering a strategy for investigating protein therapeutic ADME, it is worthwhile to take into consideration the following criteria:

Analyte of interest

A good place to start when planning ADME studies is to identify the critical analytes necessary to understand the ADME characteristics of the biotherapeutic. There are a number of considerations critical to study design that one should assess when determining the analyte(s) of interest. Is one interested in parent or catabolite? In many situations the analyte(s) of interest is/are determined by the format of the therapeutic protein and is likely to be the parent molecule, unless one is interested in identifying or measuring the catabolic products. If there is evidence or concern that the parent drug is not the only pharmacologically active species, the identification and ADME assessment of active catabolites may be of interest to inform PK/PD or drug design. For some situations, it is critical to understand the ADME of the pharmacologically active moieties of the therapeutic protein, and perhaps any other components

that might contribute to its behavior. For example, for an Fc-fusion it might be appropriate to ensure that the sampling and analytical method are capable of measuring both the presence of the antigen-binding domain, confirming pharmacologic activity, and the Fc domain, confirming the stability of the molecule.

For therapeutic proteins with more than one pharmacologically relevant component (e.g.,, ADCs, bispecific antibodies), it may be necessary to ensure that the methods used are capable of confirming that all of the pharmacologically active components of the molecule are intact. It may also be of interest to measure the identities and amounts of the individual active components, for example, to assess differences in ADME of the active components upon ADC catabolism. A particularly elegant example is the work of Alley et al. investigating the tissue distribution of the antibody and cytotoxin in normal and tumor tissues following dosing with an ADC.[68] This was achieved by radiolabeling each component with a separate radioisotope and using tissue sampling to obtain highly quantitative tissue concentrations. This work provided valuable insights into the differences in tissue accumulation and processing of this potent therapeutic. In some situations, the identity of the catabolic products may not be known at the time of the design of the ADME studies, and thus careful consideration of the analytical methods and study design required to ensure that all relevant catabolic products can be identified and quantitated is needed. This is nicely illustrated by the catabolism and excretion assessment conducted for ado-trastuzumab emtansine (T-DM1).[121] Beyond ADCs, there has been more interest in the biotransformation (defined as the physical alteration of a biotherapeutic due to peripheral intermediate catabolism or truncation) of therapeutic proteins containing only native amino acid, particularly as it relates to understanding molecule stability and candidate selection.[122] This strategy was used to evaluate the in vivo stability of 3 peptibody analogs; such data could be used to guide decisions about molecule design and progression.

Matrix

The matrix containing the analyte(s) of interest has a major impact on the study design and choice of analytical method. If one is interested in measuring multiple analytes in a single matrix (e.g., both the cytotoxic drug and the antibody components of an ADC), the use of separate analytical modalities for each analyte may be required. For distribution studies, the matrix is usually tissue, which poses some analytical and study design issues. Determination of the concentration of therapeutic proteins in tissue using certain methods (e.g.,, immunoassays, mass spectrometry) can be more difficult than in liquid matrices such as plasma or serum, often resulting in lower sensitivity and time-consuming method development for each tissue.[108,111,123,124] For the investigation of therapeutic protein elimination, one will likely collect urine, bile, or feces. In addition to considerations related to collection, each of these matrices has characteristics that may determine the analytical method used for detection of the analyte. Beyond analytical aspects, the choice of matrix will also determine how a study is conducted. Blood sampling for measurement of blood, plasma, or serum is quite routine. Collection of excreta (bile, urine, feces) will require specialized techniques and equipment such as metabolism cages and biliary cannulation, particularly if one is interested in understanding the mass balance of parent and catabolite elimination. Such methodologies have been in use for small molecule drugs for many years, with the details described in recent reviews.[125,126] Measurement of analytes in tissue presents the researcher with several choices related to analytical method and study design. The most commonly used strategy is a destructive approach where the sacrifice of individuals or groups of animals at specified time points is followed by quantitation of the analytes of interest in specific tissues. The investigator then has several options for analyte determination and quantification. The two most common are assaying individual tissues separately using either radiometric methods (i.e., "cut and count") or non-radiometric methods (e.g., immunoassay or MS). Alternatively, using radiolabeled material, one can employ QWBA to generate a 2-dimensional whole body image consisting of cross sections of the organs which, by exposing to x-ray film, can provide a measure of analyte in each tissue.[101,127,128] While these methods can provide excellent quantitative information, depending on the analytical method used, they have the disadvantage of preventing serial assessment of tissue distribution within individual animals and can require relatively large numbers of animals. These limitations can often be overcome by the use of non-destructive methods such as imaging, including PET, SPECT, and optical imaging. Imaging allows real-time, serial assessment of tissue concentrations, but in some situations can lack the necessary spatial resolution and sensitivity.[129,130] Ultimately, the choice of strategy and analytical method is determined by balancing availability, sensitivity, importance of serial sampling, and need for spatial resolution.

Spatial resolution

For distribution, and perhaps absorption, spatial resolution can be important in understanding the mechanistic aspects of ADME and may also inform the relationship between distribution and pharmacologic effect. Traditionally, these have been determined using radiolabeled tracers by dissection studies followed by gamma or liquid scintillation counting and autoradiography. Such an approach, however, is tedious and requires a large number of animals to ensure the reproducibility and reliability of the results. The continuing development of high-resolution PET and SPECT scanners for small animals and the availability of suitable isotopes (e.g., ^{64}Cu, ^{89}Zr, ^{111}In, ^{131}I) are providing an alternative which simplifies considerably the measurement for the kinetics and biodistribution of radiolabeled biotherapeutics. The major advantages of radionuclide-based molecular imaging techniques (SPECT and PET) are that they are very sensitive (down to the picomolar level), quantitative, and there is no tissue penetration limit. Another advantage is that SPECT has good spatial resolution (0.35mm vs. One.5mm for PET) allowing differentiation of tracer uptake on the suborgan level. It is important to keep in mind that although no single technology currently provides all the answers one would like, however, integrating different modalities into other in vivo methodologies (e.g. QWBA, microautoradiography) can enhance our quantitative understanding of intra-tissue distribution. An illustrative example is the distribution of therapeutic

proteins into the brain. The brain is a highly vascularized organ with a relatively high proportion of endothelial cells. In determining the concentration of a therapeutic protein in the brain parenchyma, it is critical to ensure that the method used for assessing distribution is capable of distinguishing endothelial uptake from parenchymal uptake. This might be accomplished by using a quantitative or semi-quantitative method with a high degree of spatial resolution capable of distinguishing signal in parenchyma from that associated with the vasculature, such as quantitative microautoradiography.[127,131,132] The importance of spatial resolution in understanding therapeutic protein distribution within tumors has been the subject of several studies, illustrating differences in tumor penetration and tumor distribution related to drug format and co-administration of antiangiogenic agents.[128]

Analytical method sensitivity

The assay sensitivity necessary for evaluating ADME is a critical factor in study design and strategy. This aspect is also highly influenced by other aspects of study design, including matrix and analyte. Ideally, the assay should be sufficiently sensitive to produce reliable quantitative measures of the desired analyte (s). In addition, it may be important for the assay to be capable of measuring analyte(s) at pharmacologically relevant concentrations, or with the necessary accuracy and precision to evaluate differences in analyte or tissue concentrations. As noted above, the matrix chosen can have an impact on the assay sensitivity, with tissue matrices often having lower sensitivities with methods such as MS and immunoassays. The planned dose will also affect the choice of assay due to limitations of sensitivity. If one is interested in characterizing dose-dependent differences in distribution to a tissue or in protein therapeutic catabolism, it may be necessary to use relatively low doses to be below the level of saturation of the target or catabolic process. This may put high demands on some analytical methods and require careful consideration of the assay strategy.

Specificity

As noted earlier, a key aspect of developing an ADME strategy is determining the analyte(s) of interest. Once this has been determined, the analytical method must be appropriately chosen to specifically measure the relevant analyte(s). For molecules as large and complex as protein biotherapeutics, this may not be straightforward. One example is that of a bispecific IgG antibody in which each arm of the antibody binds to a separate antigen and the arms are held together by disulfide bonds.[133] To assess the in vivo stability of this construct, which may be subject to in vivo arm switching, a PK study was conducted with plasma samples analyzed by a dual-binding immunoassay that allowed the investigators to confirm that both arms were simultaneously present on the measured analyte. For antibody ADCs, immunoassays have been developed that require the presence of both the cytotoxic drug and the target-binding region of the antibody, thus confirming that both pharmacologically relevant moieties are present in the sample. For radiometric methods and many imaging modalities, it is important to recognize that analyte specificity is not always assured. Most methods used to radiolabel therapeutic proteins (or to conjugate with imaging probes) rely on random conjugation to lysine or cysteine residues. Unless orthogonal methods of analysis are used (e.g.,, immunoassays, size-exclusion chromatography), simple measurement of the radiologic signal in the sample does not ensure that the measured analyte is pure and identical to the administered drug. Unexpected degradation or catabolism of the therapeutic protein can lead to radiodetection, or an imaging signal, which quantifies a mixture of products unless more specific methods of analysis are applied. A special case is ADCs where the precise quantitation of the antibody and the cytotoxic drug may require analytical methods or radiolabeling strategies specifically designed for analytes with very different physicochemical characteristics.[68,134] The use of mass spectrometry has also become more common in the quantitation of protein therapeutics.[123,135] Because of the size of most protein therapeutics and the presence of large amounts of endogenous protein in most matrices, analysis by MS requires either substantial sample preparation or the use of enzymatic digestion to facilitate accurate and specific quantitation of the analyte of interest. The details of this emerging technology are beyond the scope of this discussion, but are the subject of several other reviews.[123,136]

Animal species

The choice of species in which to conduct protein therapeutic ADME studies is dependent on a number of important factors. In many situations, a goal of the ADME investigation is to generate information that will guide decisions and inform outcomes in humans. Therefore, the animal model used for ADME studies should be chosen with some thought to the ultimate translational validity of the data. Many therapeutic proteins have distinct species cross-reactivity properties, with little or no binding to target in some animal species. If the ADME properties of interest, particularly tissue distribution and perhaps absorption, may be influenced by binding of the protein therapeutic to its target, then careful consideration should be given to using either a species to which the therapeutic binds or the use of a surrogate molecule with suitable target-binding properties. For protein therapeutic catabolism, it has been generally assumed that the catabolic products (typically small peptides and amino acids) differ little between species.[35] Thus, investigations of this qualitative nature of this process may have little dependence on species. However, the rate at which these processes occur may be considerably different between species if one accepts the notion that protein therapeutic catabolism scales between species in a similar fashion to that of metabolic rate. This would infer that therapeutic protein catabolism should occur at a greater rate per unit of body mass in smaller species.[137] A special case again applies to ADCs where the linker and cytotoxic drug may be differently catabolized between species. While this has not proved to be true for the 2 ADCs where data exists, the divergence in linker and cytotoxin chemistry between ADCs may require each ADC to be assessed individually.[121,138]

Modeling of protein therapeutics

A fundamental relationship exists between PK/PD and ADME, with the ADME properties of a molecule determining the

observed PK, and thereby impacting the PD. Currently, a comprehensive understanding of the mechanistic aspects of protein therapeutic ADME is not available, and the complex interplay between the physiology of the animal or human with the physicochemical and target binding characteristics of protein therapeutics can make it difficult to predict the resultant disposition. Common among these is the observation that PK/PD can be non-linear with dose, complicating the understanding of the dose-response and interspecies translation. This makes it difficult to determine the appropriate dose and dosing regimen for the desired therapeutic effect and to predict human PK/PD. One particularly important example in this regard is the sometimes profound effect of target binding on the PK/PD of a therapeutic protein; referred to as target-mediated drug disposition (TMDD).[149] In this example, and many others, a critical component for building a PK/PD relationship is a thorough understanding of the underlying mechanistic processes (e.g., target distribution, number, and turnover, nature of non-target binding interactions, FcRn binding), often only obtained from in vitro studies, which can be integrated with in vivo ADME data and incorporated into mathematical PK/PD models.[150-153] Such models not only provide valuable predictions of human PK or help with dose and dosing regimen prediction, but also allow the building and testing of mechanistic hypotheses which can be used to guide further in vitro and in vivo studies. Recently, highly mechanistic models of protein therapeutic disposition have started to be developed, particularly for monoclonal antibodies. These physiologically-based pharmacokinetic (PBPK) models have generally focused on the interaction between IgG and FcRn in various normal tissues and in several species, but show promise in their potential to expand to include target dynamics and the estimate of IgG concentrations in diseased tissues.[37,154-156] Further, in vitro and in silico tools have advanced in their abilities to approximate individual ADME parameters to more accurately predict the manner in which protein drug candidates are absorbed, distributed, and eliminated under in vivo conditions. For example, a modeling analysis of the effects of molecular size and binding affinity on tumor targeting was conducted to guide the design of new therapeutic protein drugs.[148] As these modeling strategies develop, it is critical to acknowledge that the models and their predictions can only be as good as the supporting data and our understanding of the complex mechanisms and characteristics associated with the ADME and pharmacology of our protein therapeutics, requiring continued advances in these areas.

Looking ahead

Over the last 15 years, the demand for basic and mechanistic ADME studies of protein therapeutics has continued to increase, not only for building the foundation of comprehensive ADME technology, but also for paving the way toward rational design of novel therapeutics. Today, more than 40 mAb and Fc-fusion therapeutics are marketed in the United States, with over 400 more in clinical development stages.[139,147] Because of the complexity of the next generation of biologics, many unmet needs in ADME research remain, and the associated scientific and technical approaches require ongoing improvement. In the past, data from conventional preclinical in vivo studies were the basis for the characterization of biologics. Physicochemical properties are seldom related to the toxicity, metabolism, PK, and PD parameters of biotherapeutics. As such, many fundamental questions remain unsolved and more investigation is needed to elucidate how ADME properties are altered by charged amino acid substitutions in the variable or constant regions of the antibody, different IgG subclasses, glycosylation, target-mediated effects, and different injection routes and sites.[10,140] Furthermore, very little is known about the role of factors influencing SC absorption and uptake into the lymphatic system and lymph nodes.[21] There is a particular need for a systematic evaluation of the molecular weight, surface charge, post-translational modifications, dose and formulation of biologics, as well as the role of FcRn, to address these many unknowns.[10] Therefore, it is believed that an appropriate use of effective and validated preclinical in vitro and in vivo ADME systems should facilitate mechanistic understanding of SC absorption and associated determinants, and aid human PK prediction of bioavailability and potential variability.[144]

ADME processes have been crucial in enhancing the possibility of the success of low molecular weight drugs, and it is expected that this will translate to novel biologics in the near future.[110] Strategies for conducting these studies will require the right set of high-quality data to help inform decisions made internally, as well as by regulatory agencies.[145] Today, improvements in tools and technologies, including MS-based techniques and imaging tools, provide high sensitivity in quantitative and high throughput bioanalytical methods that facilitate rapid assessment of the PK and metabolic profile of biologics, even when present in low levels in both blood fluids and tissues.[68,69,121,125,138] The field of ADME of biologics is still rapidly expanding with new concepts and technologies. As these tools become more readily available in drug development settings, analyses that once were prohibitively complex and expensive will be used to help scientists develop better strategies aimed at optimizing the PK/ADME profiles of biologics.[109] Despite these advances, there is great need to incorporate findings from basic research into mechanistic PK/ADME studies with the aim to facilitate intelligent design and development of next-generation biologic drugs.[77,99,146] This is an exciting time that should see the emergence of even more efficient ADME studies, using various technologies and information to expedite the evaluation of new therapeutic protein candidates

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No potential conflicts of interest were disclosed

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