Foundation review: Nonclinical development of biopharmaceuticals

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The nonclinical development plan (NDP) for biotechnology-derived pharmaceuticals (biologics) as addressed in this review comprises aspects of nonclinical drug safety (toxicology and safety pharmacology), nonclinical pharmacokinetics (PKs) and nonclinical and clinical bioanalytics. A variety of bioanalytical methods are needed to describe PK behavior and to monitor immune response in laboratory animals and humans. The NDP will often differ from established programs for small molecules to account for target differences, a lack of or limited species crossreactivity and/or the limited applicability of long-term studies, as well as immunogenicity assessment. Special study designs, scientifically justified and driven by a case-by-case approach, might overcome these hurdles.

Introduction

Modern biologics are biotechnology-derived pharmaceuticals (also designated as 'biopharmaceuticals', 'biotech drugs' or 'biotherapeutics') comprising different compound classes [1] (Box 1). Chemically, biologics are mainly represented by glycoproteins comprising L-amino acids and various sugar molecules. They are mostly used for the diagnosis, prevention and treatment of serious and chronic diseases.

Monoclonal antibodies (mAbs) and rDNA-derived products make up the majority of marketed and close-to-market biologics and are the focus of this review. rDNA products comprise two-thirds of global biologics sales. MAbs are becoming more prominent and now represent the majority of biologics with more than 150 compounds (30–50% of all biologics in development) in company-sponsored clinical studies [2–6]. Antibody fragments and fusion proteins have great potential as innovative therapeutic agents with their targeted therapy approach versus functional approaches.

Both biologics and small molecules have to be proven to be pure, safe and potent within their development. Nonclinical development as addressed in this review comprises nonclinical drug safety (toxicology and safety pharmacology); nonclinical pharmacokinetics, or PKs (see Glossary) and bioanalytics for preclinical and clinical sample analysis, including immunogenicity evaluation. All these activities are prerequisites for moving into clinical development and must assess potential safety risks. As is the case for small molecules, some toxicology studies do not necessarily have to be performed before first use in humans but can be performed at later stages [7]. The nonclinical testing strategy for biologics has to be adapted and refined from experience with small molecules. Differences in the physicochemical properties and resulting differences in

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GLOSSARY

Antidrug antibodies (ADAs) ADAs bind to the protein but do not neutralize its activity; it is still able to form immune complexes.
Neutralizing ADAs (nADAs) nADAs bind to the protein and block its activity.
Nonclinical development plan (NDP) when a product has advanced to the clinic, the term 'nonclinical' is sometimes used to describe animal safety studies, and 'preclinical' is sometimes used to describe only the animal safety studies before first use in humans. However, the two terms are often used interchangeably.
Bioanalytics for biologics, several types of assay for quantification of the protein itself and its biological activity; the detection and characterization of binding ADAs and neutralizing ADAs; and, if necessary, to monitor antibodies that might have been generated in response to host cell proteins and/or other (high molecular) impurities or compound parts (e.g. PEGs).
First-in-human dose (FIH) different approaches (e.g. NOAEL- or MABEL-based) can be used to calculate FIH. All available information has to be taken into consideration (i.e. a case-by-case basis is used) to safeguard the safety of subjects in FIH studies.
No observed adverse effect level (NOAEL) the NOAEL is determined in the most sensitive and relevant species in nonclinical safety studies and adjusted with allometric factors or on the basis of PKs.
Minimal anticipated biological effect level (MABEL) the MABEL is the anticipated dose level leading to a minimal biological effect level in humans; potential differences of sensitivity for the mode of action of the drug (SMD or biologic) between humans and test animals need to be considered.
Pharmacokinetics/pharmacodynamics (PKs/PDs) studying nonclinical exposure–response relationships in conjunction with dosing information enables understanding of the clinical therapeutic dose range.
Drug–drug interaction (DDI) biologics coadministered with an SMD have limited DDI potential because there are no overlapping clearance mechanisms. DDI studies should be performed with a risk-based approach.
Polyethylen glycol (PEG) conjugation of biologics with PEG is a common method of increasing their elimination half-life by reducing their renal excretion and enzymatic degradation owing to increased steric bulk.
Antibody–drug conjugate (ADC) mAbs, usually in IgG format, conjugated to a cytotoxic drug via a chemical linker, enhancing the (limited) efficacy of 'naked' mAbs.
Target-mediated drug distribution (TMDD) specifically mAbs, which target membrane-associated antigens, might have complex, nonlinear PKs. PK, dependent on the amount of target and its turnover rate.

PKs, toxicity, immunogenicity and bioanalytics of biologics compared with small molecules are substantial [8]. Risks other than those known from our extensive experience with small-molecule pharmaceuticals determine the development program of biologics. Binding specificity and affinity often limit species crossreactivity and selection of adequate animal species. Immunogenicity as an inherent property of large molecules, like biologics, complicates the nonclinical testing strategy and needs an adapted experimental, as well as bioanalytical, testing strategy.

Recognizing the special features of biologics, the International Conference on Harmonization (ICH) S 6 guidance of 1997 introduced the 'case-by-case' approach [9]. This can be interpreted as a 'best science' approach and should also hold true for small-molecule pharmaceuticals. However, with small molecules, we can plan

BOX 1

Therapeutic biologics – compound classes

(i) Monoclonal antibodies (mAbs); (ii) recombinant protein therapeutics (rDNA products), such as replacement molecules for endogenous compounds (e.g. coagulation factors and insulin), and therapeutic cytokines (e.g. interferons and interleukins); (iii) hybrid and modified molecules (such as protein–synthetic constructs [pegylated, glycoengineered proteins]), fusion proteins (such as antibody fusion constructs), antibody fragments and biological conjugates (such as conjugated antibodies); (iv) nucleic acid molecules, such as antisense oligodeoxynucleotides; (v) vaccines directed against noninfectious disease targets; (vi) gene therapy products and therapeutically used viruses; and (vii) cell therapy products.

programs based on long-term experience and comprehensive regulatory guidance. In many cases, standard nonclinical development programs (NDPs) can be followed with predefined study protocols. For biologics, we not only have less experience but also have a highly heterogeneous compound class requiring a custom-prepared program based on the individual compound. That means not only defining appropriate safety programs but also avoiding nonrelevant studies with no or very limited predictive value.

After gaining experience with individual biologics passing through clinical development to the market, we can base our future programs on lessons learned and narrow down the small-molecule approach. In the meantime, regulatory guidance is evolving for biologics, as are published data and textbooks on preclinical safety and PK evaluation of biologics [10,7,11].

How to estimate safety risks of biologics in nonclinical development

The objectives of the nonclinical safety program for biologics are similar to those for small-molecule drugs (SMDs): to recognize potential toxicities (hazard identification and characterization, and risk assessment), to identify appropriate parameters for clinical monitoring (e.g. biomarkers) and to contribute to first-in-human dose (FIH) selection. This also includes assessing the limitations of nonclinical studies in predicting safety issues for the human situation. Nevertheless, the nonclinical program has to be designed for clinical decision-making. The program for biologics, however, is often different from SMD programs because of the nature of the therapeutic protein, its species specificity and its immunogenicity.

The ICH S 6 guidance from 1997 recognized that a biologic might be devoid of pharmacological efficacy in the standard species for toxicity testing. The guidance gives more flexibility (e.g. allows that only one species can be used for general toxicity testing when pharmacological relevance can be demonstrated in one species only) [9]. Gaining experience with individual classes of biologics (such as mAbs and rDNA products) over the past ten years, regulatory expectations have been increasing for scientifically justified and consistent nonclinical programs. An ICH expert working group is presently discussing an update of the ICH S 6 guidance via an addendum for five selected topics (species selection, study design, developmental and reproductive toxicity, carcinogenicity and immunogenicity). An European Medicines Agency (EMEA) guideline on immunogenicity assessment of biologics was adopted in 2008 [12]; further

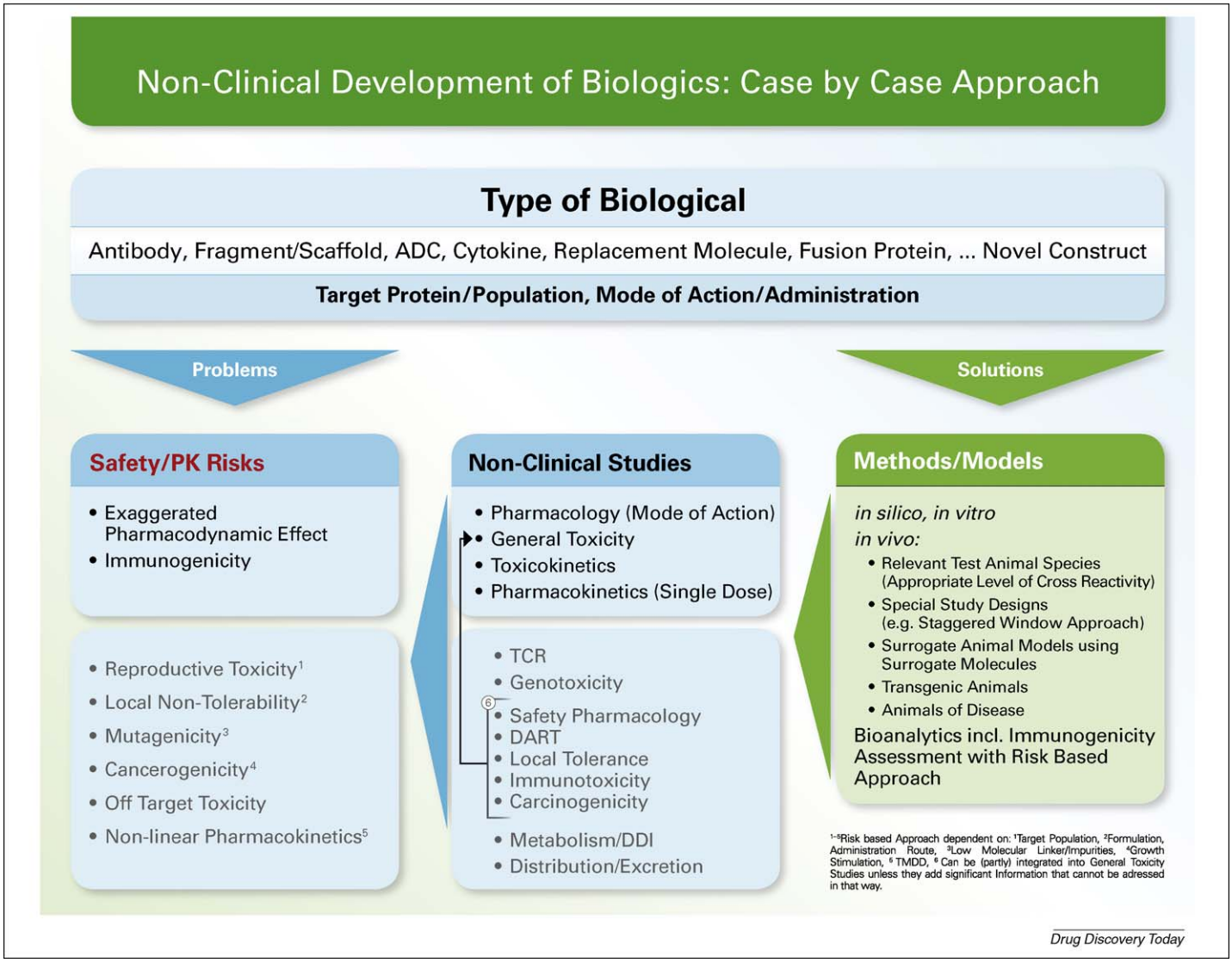

FIGURE 1

Case-by-case approach for nonclinical development of biologics. Risk-based assessment using information on molecule type and/or mechanism of action and human indication, population, or administration, as well as best science and evolving knowledge. Abbreviations: ADC, antibody–drug conjugate; TCR, tissue crossreactivity; DART, developmental and reproductive toxicity assessment; DDI, drug–drug interaction; TMDD, target-mediated drug disposition.

guidance and a concept paper are planned for 2009 [13,14]. Other guidance documents are available (e.g. describing the comparability exercise and the clinical development of biologics, as well as how to mitigate risks for FIH trials with SMDs and biologics [15–17]).

Safety risks are generally directly related to uncertainty and are reduced through knowledge and best scientific practices. However, because of the heterogeneity of this compound class (Box 1) and the different approaches needed for different types of biologics, our future NDPs must be considered largely on a case-by-case basis [18] (Fig. 1).

Species selection for nonclinical studies with biologics

Species specificity and the availability of toxicologically relevant animal models are two of the greatest challenges encountered when planning nonclinical safety programs with human(ized) proteins, such as mAbs and rDNA products in animal species. Why is species selection so important for biologics? Owing to their predominant composition of naturally occurring amino acids and carbohydrates, adverse effects observed for biologics can mostly be attributed to the compound's pharmacological activity (i.e. to exaggerated pharmacological activity or action on unwanted target cells and/or tissues) [19]. Therefore, intrinsic toxicity (i.e. no exaggerated pharmacological activity) is not of major concern. With molecular weight (MW) ranges above those of small molecules, biologics usually cause no effects owing to direct interaction with intracellular mechanisms that are not receptor mediated. The animal species selected for nonclinical safety studies, therefore, has to be proven for specificity and predictivity of its pharmacological and 'superpharmacological' response. Target expression, distribution and primary structure of the target should be known. Binding and occupancy of the target, as well as functional consequences (including cell signaling), have to be investigated. Data on the functionality of additional functional

domains in animals (e.g. the Fc receptor system for mAbs) should be known because the interaction of such domains apparently attributed to the life-threatening cytokine storm observed in the clinical development of the anti-CD28 superagonist antibody TGN-1412 [20]. Although none of the 174 biologics approved in the USA and Europe between January 1995 and June 2007 had to be withdrawn from the market for safety reasons, a more in-depth evaluation of the mode of action might have predicted some safety problems during the development phase [21].

The primary requirement for a mAb is that it crossreacts with and functionally modulates the target antigen in the test animal species in a manner comparable with that of the human antigen. This can be assessed by analyzing the sequence and structural properties of the antigen and the crucial residues in the binding region (epitope mapping) and by understanding the sequence conversation and cross-species homology, and finally, it can be proven by characterizing the affinity and functional potency [22]. Tissue crossreactivity (TCR) studies in a panel of human tissues determining the level of crossrecognition, therefore, are obligatory with targeted biologics such as mAbs. Thus, potential binding to nontarget tissues can be identified. Early TCR studies in animal tissues can be used to select the relevant preclinical test species and to obtain information on potential targets of toxicity.

Nonhuman primates (NHPs) are often more appropriate for nonclinical safety studies of biologics than rodents because of their close phylogenetic similarity to human targets. This compensates for the usually low number of animals used in primate studies (primarily for ethical reasons, but also for limited animal availability, technical challenges and costs incurred when conducting large studies with NHPs), although the lower number of animals reduces the statistical power in these studies to detect rare treatment effects [23]. Cynomolgus monkeys (*Macaca fascicularis*) are the preferred species for nonclinical testing of therapeutic biologics such as mAbs and cytokines; alternatively, rhesus monkeys *(Macaca mulatta*) can be used [24]. However, even NHP models with close sequence homology to the human target might have limited predictivity, depending on the specific compound class, as shown for antilymphocyte antibodies. They were poor predictors of cytokine release syndrome, as observed in the later clinical trial, because additional functional domains have reduced (structural or functional) homology [20]. They can be sensitive indicators of T-cell activation, proliferation and/or depletion, and even of the potential for cytokine release, however [22]. The use of chimpanzees and other great ape species is not recommended for nonclinical studies, mainly because of ethical and logistical issues [18,24]. Even with a high probability of ending up using NHPs as test animals, species selection has to be performed for each new biologics development program. There are examples of the successful use of other animal species than NHPs, even if they are not appropriate for the whole toxicology program. Rats and rabbits, for example, are preferred models for reproduction toxicology studies. Cohorts might be used to test different segments of embryofetal development to overcome hurdles owing to the emergence of neutralizing antibody formation (the 'staggered window' approach) [25].

Failure to identify suitable test animal species might require alternatives, such as transgenic models, testing corresponding animal (surrogate) biological entities (homologous proteins) and/or the use of animal models of human disease. However, the risks and benefits of these approaches have to be weighed carefully to ensure that misleading results are not generated. Nonetheless, development and validation of these approaches (still) need more time and resources than the use of traditional models. A pragmatic alternative in early phase development is the selection of a development candidate with a distinct level of crossrecognition in the wild-type test animal species (e.g. cynomolgus monkeys).

Transgenic or knockout animals have been increasingly used in the recent past for nonclinical safety programs [26]. The key is to characterize these models for antigen expression and functional integrity to compensate for the limited availability of historical test data known for traditional test animal models. This means genotyping individual animals and fully describing the resulting phenotype. Transgenic animals might express the target, but the organ expression pattern might differ from the natural expression pattern. Furthermore, they might react differently in downstream pathways and nature of physiological effects.

Homologous proteins (surrogate biologics) have to be well characterized for similarity to their human analogs, specificity and production and bioanalytical capabilities. In addition, their use would only have limited predictivity when functional homology does not exist (e.g. when a murine analog is used in a rodent model with a different biology compared with humans). Using species-homologous counterparts of the human protein can increase understanding of the physiological role of the target but does not necessarily predict the response in humans.

Animal models of the human disease (induced and spontaneous models of disease, gene knockouts and transgenic animals) might be an acceptable alternative to toxicity studies in normal animals and could be especially helpful when a target is only expressed in a disease state (such as a tumor marker) and/or when target-mediated clearance might influence the exposure [27]. Even if originally developed to evaluate the pharmacological efficacy of the compound, they might be of value, especially for biologics, owing to their exaggerated pharmacology in the toxicity testing. However, a transgenic mouse model with a single modulated target is not automatically a model of disease because most diseases are multifactorial [28]. For all these alternative models, PKs, pharmacodynamics (PDs) and immunogenicity should be assessed as were done in traditional animal models.

Dose selection for nonclinical safety and FIH studies

Besides selecting an appropriate animal model, selecting the right dose (especially the high dose) and dose regimen and frequency might be a challenge for biologics, especially for multiple-dose animal studies. Dose selection should be based on maximum observed pharmacological action and anticipated human dose. Establishing a maximum tolerated dose is preferred but not always applicable. Alternatives might be the use of maximum feasible dose or some suitable multiple (e.g. 10×) of the clinical dose, taking into account any species differences in binding affinity. The safety factor used depends on the clinical indication (life-threatening versus nonlife-threatening), the patient population (e.g. special populations, such as children), acute versus chronic treatment and other factors (e.g. concomitant medication) [18,24]. Although regulatory guidance is given for selection of FIH [9,29–31], the starting dose will finally be selected on a risk-based strategy for each individual biologic. In the case that a relationship

exists between exposure and toxicity, it provides information for computing margins and, thus, the safe starting dose in humans based on the no observed adverse effect level. Target saturation plays a key part in dose selection for biologics. Because toxicity of biologics usually arises from exaggerated pharmacological effects, when combined with a narrow therapeutic range and/or a steep dose–response relationship, the pharmacologically active dose is often a more sensitive indicator of potential toxicity for biotechnology-derived pharmaceuticals. The more complex minimal anticipated biological effect level approach is based on any biological effect and corresponding PK and PD data [17]. This approach is recommended for drugs (small molecules, as well as biologics) with additional risk factors such as limited knowledge about a (new) target and/or limited relevance, and/or a lack of a nonclinical animal model (as more often observed for biologics). Identification of potential risk factors related to target and mode of action is an essential requirement, even in early phases of development, because this might result in increased efforts compared with standard development. However, it has to be considered that a starting dose calculated by this approach could be too low and, therefore, nonactive, which is crucial when exposing, for example, seriously ill cancer patients.

Study design and types of nonclinical safety studies

Besides the challenges of species selection for the general toxicity testing of biologics (single- and repeated-dose toxicity studies), there might be even more challenges for specialty studies such as reproductive toxicity, carcinogenicity and immunotoxicity. This is because adequate exposure needed for specific treatment regimens can be hindered by the formation of antidrug antibodies (ADAs) with drug neutralization or accelerated clearance. Therefore, toxicology programs with multiple-dose administration of biologics often have less long-term treatments than SMDs. However, this does not mean that a biologic can be developed more rapidly and at lower cost in the nonclinical stage. Special study designs, the use of more sophisticated and costly animal models, longer observations periods owing to ADA response and, especially, the manufacturing complexity for proteins often result in slightly longer and more costly programs, as observed with small molecules [32].

Exogenously administered proteins (biologics) usually do not directly interact with DNA; genotoxicity testing, therefore, is commonly not deemed to be necessary. Depending on other risks inherent in the formulation (e.g. reactive small molecular linker used for modified antibodies or small molecule impurities), genetic toxicity studies might be needed as well.

Because of the special nature of biologics, the usefulness of commonly accepted safety pharmacology study designs in traditional laboratory animal species such as mouse, rat and dog is limited. The ICH S 6 guidance emphasizes investigation into undesirable pharmacological activity in appropriate species in the main organ systems (CNS, cardiovascular, respiratory and renal), either in separate studies or incorporated into toxicology studies. If a biologic is known to have direct physiological impact or if TCR studies indicate binding to, for example, heart, lung or brain tissues, a stand-alone safety pharmacology study should be considered [9,33,34]. That also applies for biologics that reach new targets and/or for which mechanism of action has not been fully characterized or understood. *In vitro* assays, such as the hERG ion channel assay, are not advisable for biologics because large molecules either might not enter the cells or are too large to bind to the ion channel.

When moving into phase II clinical development, longer term general toxicology studies in appropriate animal species, as are more or less routinely performed for small molecules, should be considered. But again, limitations arise from nonappropriate or inadequate exposure owing to antibody formation. It is generally accepted that chronic toxicity studies of biologics need not exceed a six-month duration and might even be shorter when scientifically justified [9]. In addition, the exaggerated pharmacological effects usually observed in nonclinical safety studies are generally apparent within the first month of treatment. There are also examples of when chronic studies could not be performed because of high incidence of antibody formation with neutralizing activity (nADA) (e.g. in case interferons and alemtuzumab) [23].

Safety hazards such as specific toxicity, local tolerability, reproductive toxicity and even more specific juvenile toxicity have to be addressed in nonclinical safety studies in parallel to further clinical testing and be based on the target patient population. Special study designs, such as staggered window approaches, for these studies might require treatment of parallel animal groups for distinct intervals.

Carcinogenicity testing might have to be specifically considered for biologics (e.g. for growth factors that might impact tumor promotion). In these cases, general carcinogenic effects such as initiation and/or progression must be discriminated from the pharmacological effect and human relevance has to be considered. Generally, however, *in vivo* carcinogenicity studies, as requested for small molecules, are considered inappropriate for biologics [9]. Alternative approaches might include *in vitro* cell-based assays, xenograft models and tumor-promotion models [18].

Local tolerance studies can usually be integrated into the general toxicology studies unless they add considerable information that cannot be addressed in that way. These studies could be very helpful for screening new formulations.

For many therapeutic biologics, PDs outlast PKs because of notable delays in the expression of PD effects relative to the PK profile. This might also result in delayed toxic effects. Therefore, specifically tailored study designs with longer observation periods (e.g. those including special recovery groups) are often necessary for biologics to be predictive for the human situation [35,36].

Immunogenicity studies of biologics within nonclinical drug safety

In contrast to small molecules, biologics are usually inherently immunogenic. Nowadays, most biologics derived from human protein libraries are humanized. Although the 'foreignness-based' intrinsic immunogenicity of these compounds is reduced in humans, immunogenicity is often still present in the human situation and might be even more complex. Immune responses to these compounds with identical or very similar structure to endogenous proteins are induced by a different, not fully understood mechanism that is based on breaking immune tolerance [37]. Impurities, fragments, aggregates, non-natural glycosylation or alteration of the quaternary structure caused by the production

process (change) are other major factors contributing to immunogenicity of biologics. Other treatment-related (e.g. frequency of dosing) and patient-related (e.g. disease) factors influence the incidence of immunogenic effects of a compound. Unwanted immune responses, therefore, remain a major safety concern after the administration of biologics to subjects and have to be monitored in nonclinical and clinical development.

After approval, the safety problems of biologics are often related to immunomodulatory effects and seen as infections [21]. Unintended immunomodulatory effects have been observed with active tuberculosis associated with anti-TNF alpha mAbs [38] and should be taken into consideration in the assessment of the safety of biologics. Specific immunotoxicity studies have to be performed for biologics, as well as SMDs, only on a case-by-case basis. Immunologically mediated adverse effects might be induced either primarily, as a consequence of the pharmacological activity (immunosuppression or immunostimulation), or secondarily, owing to the immunogenic potential resulting in immune complex formation, induction of hypersensitivity reactions or autoimmune reactions via immunological crossreactivity toward endogenous counterparts.

The high variability in the effects of antibodies generated after administration of biologics in the nonclinical setting and the likelihood of different effects of any antibodies generated in humans (neutralizing, non-neutralizing or even chaperonizing activity) raise doubts about routinely conducting extensive nonclinical immunogenicity studies for prediction of these effects in humans [39]. However, of important value when assessing immunogenicity in nonclinical safety studies is proof of the validity of the toxicity studies [40]. The value of these activities is not the measurement of resulting changes in the effects *per se* but in aiding in the interpretation of, for example, unclear results observed. ADA formation does not necessarily invalidate a nonclinical study. In cases in which ADAs do not neutralize drug efficacy, it is reasonable to conduct a toxicology study (dosing through). The administration of very high doses might overwhelm the available ADA or might induce tolerance [18,23]. However, ADA formation should be monitored and correlated to any toxicological observation. More extensive investigations on ADA formation might have value in comparative studies of different protein structural variants and various formulations, as well as for products resulting from changes in manufacturing processes. Although some controversy remains in industry about the merit or utility of comparative data [25], lack of immunogenicity and increased immunogenicity in a variety of species might indicate lower or higher probability of immunogenicity in humans [41]. However, it is generally accepted that animal models are currently not able to predict immunogenicity of biologics in humans. This is based on differences in how immunologically foreign a recombinant protein is viewed to be by a given species and also in differences of the immune systems. Even if a biologic proves to be antigenic in humans, as it was in animal models (and vice versa), there is little guarantee that the functional consequences of immunization will be the same [42].

It would be highly desirable in the future to estimate the risk of immunogenicity for human use in a nonclinical setting. Although *in silico* methods for the prediction of T-cell epitopes have been developed and used in the early development phases of new biologics [43], these models lack adequate validation. In addition, *in vitro* models using human functional T-cell assays can be used to evaluate lymphocyte responses in the presence of antigen. However, the limited applicability of these *in vitro* models means that information derived from such systems should be carefully interpreted and negative data do not necessarily predict lack of immunogenicity *in vivo*.

There are other *in vitro* assay types available, which should preferably be used in combination with *in silico* methods. *In vivo* methods, such as HLA transgenic mice or humanized mice, still require further validation work to demonstrate their predictive value [44,45].

PKs in nonclinical development of biologics

As with small molecules, the appropriate PK properties of a biologic can lead to improved efficacy and safety. For example, creating muteins with high stability and activity and prolonged half-life is a valuable approach to selecting the right development candidate. The chemical bioconjugation of polymers to biologics (e.g. pegylation) should be accompanied by PK investigations as well. Nonclinical PK investigations as part of the NDP are an important prerequisite for planning, accompanying (toxicokinetics) and evaluating toxicological studies.

Study design and types of nonclinical PK studies

General PK principles are equally applicable to biologics and small molecules [36]. However, traditional ADME studies in nonclinical development of small molecules are generally not applicable to biologics [8]. The nonclinical PK program for biologics is usually less extensive than for small molecules. What is the reason for this? An important feature of many biologics is that their biological activity is dependent upon the route of administration. Oral delivery of biologics is limited by barriers such as enzymatic and pH-dependent degradation in the gastrointestinal tract, low epithelial permeability and instability under formulation conditions. Therefore, biologics have an extremely low (<1%) and erratic bioavailability after oral administration. Although recent advances in oral delivery have been notable for peptide drugs such as insulin [46], this route is still limited to small biologics and still requires a considerable increase in bioavailability to make it viable for commercialization.

The lungs have been studied extensively as a delivery route for proteins because their large surface area enables rapid absorption owing to close contact between alveoli in the deep lung and the circulation. Even though the inhalation and intranasal routes of administration offer the advantages of ease of administration, delivery to a surface area rich in vascular and lymphatic networks and the bypass of first-pass metabolism, they are still not widely established on the market [36]. Biologics, therefore, are mostly administered parenterally: subcutaneously (SC), intramuscularly (IM) and intravenously (IV). IV administration is the most pharmacokinetically reliable mode of administration. This route allows complete bioavailability and rapid delivery but is less convenient than IM or SC.

The systemic absorption of biologics given SC or IM occurs via the blood capillaries or via the lymphatic system, the latter being more important with increasing MW [36]. Proteolysis during interstitial and lymphatic transit might result in reduced systemic

absorption and bioavailability. Absorption is influenced not only by MW but also by noncompound-dependent factors such as site of administration, coadministration of albumin, depth of injection, blood flow at injection site, exercise, rubbing and temperature. This should be taken into account when planning nonclinical studies, which should preferably use the same route as the human studies.

The distribution and metabolism of biologics follows that known for endogenous proteins. Distribution is usually limited to plasma and/or extracellular fluids. Large molecules (MW >30 kDa) cross blood capillaries slowly and active transport process might be included. The distribution of biologics can also be influenced by protein binding. They might bind to specific binding proteins involved in their transport and regulation. Binding proteins might modulate efficacy at the cellular level and might also affect the PKs and metabolism of protein therapeutics. The generally held beliefs that only drug not bound to plasma proteins is able to distribute throughout the body and exert a pharmacological effect or that plasma protein binding impairs the elimination of certain types of small molecules need not hold true for biologics. Specific *in vivo* binding proteins have been identified for a range of therapeutic proteins. These binding proteins might have an inhibitory or stimulatory effect on the biological activity of the therapeutic protein and might also influence PKs considerably [47,48]. However, protein-binding studies with biologics are still not performed routinely, for methodological reasons. Receptor interaction (especially receptor-mediated endocytosis) contributes substantially to the distribution behavior of biologics. Distribution to tissues is often part of the elimination process. Small measured volumes of distribution are often underestimations owing to routinely performed noncompartmental analysis and do not necessarily mean low tissue penetration.

The only more or less routinely used PK studies for biologics are single-dose PK studies in the animal species selected for the toxicological program. When needed, free and bound plasma concentrations should be determined. Biodistribution studies might be helpful to assess targeting and the major excretion organs. In this case, the radioactive labeled molecule (e.g. ^{125}Iodine label) should be available with an adequate activity. Whether the labeled molecule is still pharmacologically effective and stable has to be proven by using *in vitro* and *in vivo* studies.

Administered high molecular proteins are mostly metabolized. Sites of biologics' metabolism are the liver, kidney, blood and extravascular sites of administration. Only proteins with MW <60 kDa are filtered by the kidneys. Proteins are mostly not excreted with the urine but reabsorbed and/or metabolized by proximal tubular cells of the nephron. Biologics are degraded to small peptides and individual amino acids with pathways equally and generally understood for endogenous compounds. Hepatocytes are mainly responsible for the catabolism of biologics in the liver using carrier-mediated membrane transport, as well as endocytosis and pinocytosis for transport processes. Amino acids as metabolites of biologics are reused in the endogenous amino acid pool for the *de novo* biosynthesis of structural or functional body proteins. Interspecies variation in metabolism and metabolites, although important for small molecules, is not an issue for biologics. Classical metabolism studies, therefore, are not needed. In addition, limitations of current analytical methods to detect and distinguish protein metabolites and the putative lack of pharmacological or toxicological activity of the metabolites need to be considered. Similarly, mass balance studies usually used to determine the excretion pathways of small molecules (and their metabolites) are generally not useful for biologics [9].

However, questions about metabolism, distribution and/or excretion and related safety issues might arise when proteins are coupled to small molecules or polymers. MAbs conjugated to toxophores (antibody–drug conjugates, or ADCs) are a promising approach to enhancing the limited efficacy of many 'naked' mAbs. ADCs comprise an antibody, usually in IgG format, conjugated to a cytotoxic drug via a chemical linker [49]. In this case, information on the metabolism of the individual toxophore, which might be split from the protein in the body, has to be generated in case there are no valuable historical data available. In the case of pegylated (PEG) proteins, knowledge about the metabolic pathway of the PEG moiety can be helpful for the risk assessment of the molecule. However, the technical challenges when considering, for example, a labeled PEG molecule for this type of studies would make any experimentation on the metabolism of PEG of little value [50].

There is currently no universal drug–drug interaction (DDI) strategy available for biologics. The reason for this is that there are no overlapping clearance mechanisms of biologics and small molecules (SMDs). Because biologics are usually cleared by non-cytochrome P450 (CPY)-dependent liver metabolism, *in vitro* metabolism-based interaction studies are generally not predictive for this compound class. Very few PK interactions have been observed so far with biologics. There are some examples (e.g. interferons and interleukins) in which an impact on the CPY system has been demonstrated. However, no conclusions have been made on the clinical consequences [51,52]. Therefore, a sensible risk-based DDI assessment based on the individual molecule should be integrated into the overall development program with biologics [53]. Other potential interactions based on overlapping mechanism of action and/or alteration in target should be considered as well.

Another frequently observed PK feature of biologics elimination goes back to its target-mediated drug disposition (TMDD). Specifically, mAbs that target membrane-associated antigens might have complex, nonlinear PKs [54,55]. When antigen concentration is high, plasma half-life is short because the mAb binds to its target and is rapidly cleared from the blood. However, as the mAb accumulates, a new steady state is reached. Eventually, when the target is either totally depleted or saturated, clearance of the mAb will be at its slowest and half-life at its longest, approaching the half-life of endogenous IgG (approximately 21 days). Because of its nonlinear and time-dependent PKs, no single estimate of half-life can be reported. This behavior is not seen in nonclinical development when nondisease models with healthy animals are used for PK studies.

Special study types for PK/PD approaches and comparability exercise

The combination of PD and PK information in integrated PK/PD models is generally considered a promising tool for rationalizing and accelerating small-molecule development. They – together with the use of biomarkers – support extrapolation from animal

models to humans and from volunteers to target populations. PK/PD concepts have even more reason to be included in biologics development. PK and drug exposure do not necessarily drive PD in the same way that is often observed for small molecules, but both PK and PD can be bi-directionally interdependent, as seen for mAbs [36]. Nonclinical modeling approaches, including allometric scaling, mechanism of action and toxicological information, can be used not only for defining concentration–response relationships in animal models and for projection of human PK/PD and dosing (translation) but also for feeding back early drug discovery to find good successors when first human data are available (retranslation). Because of the similar catabolism of biologics in animal species and humans, allometric scaling can be often applied successfully, especially if there are no species-dependent interactions with target proteins [56]. For mAbs with linear elimination, simple allometric scaling from only monkey PK data has been used successfully for the prediction of human clearance (CL) and volume of distribution [57]. For FIH selection, the whole spectrum of nonclinical information has to be used, such as dose–response data of biological effects in human and animal cells and *in vivo* animal studies, information about mechanism of action and relative species sensitivity, receptor occupancy estimates, calculated exposure of targets or target cells in humans *in vivo* and the PK/PD modeling approach [31,46].

It is inevitable that during the development phase, changes occur in the manufacturing process of biologics to improve product quality and quantity. Despite continued advances in analytical methods for characterizing products, current analytical methods cannot fully predict safety and efficacy. Changes in three-dimensional structure of the product through the changed process can ultimately affect the PK profile, receptor affinity and immunogenicity.

Therefore, comparative PK studies in animals can be helpful to establish equivalence between two products. Because more than similarity in terms of absorption and bioavailability is of interest, the standard bioequivalence design might not be optimal. In fact, the risk of differences in elimination rate might be more probably, requiring the demonstration of comparability on clearance and/or half-life. The choice of single-dose design, steady-state studies or repeated determination of PK parameters with a treatment period in between should be justified. Short-term PK studies at pharmacological, rather than supra-pharmacological, doses seem to be a more effective way of comparing two products in a nonclinical study in a comparability exercise [39].

Within the discovery phase of SMDs, a wide variety of (high-throughput) assays, both *in vitro* and *in vivo*, have been established in the past 15 years to reduce the developability risks when progressing from nonclinical into clinical development [58]. Drug metabolism and PKs-related attrition risks of SMDs, such as limited oral bioavailability, CYP-dependent metabolism and interaction with SMD transporters, are usually not relevant for biologics. There are, however, other risk factors, such as immunogenicity, inappropriate half-life and complex and nonlinear PKs, which still need more specific early screening assays with toxicological and PK endpoints to determine the druggability of early biologics candidates. *In vitro* binding of IgG variants to the neonatal Fc receptor can, for example, be used for mAb engineering with improved PKs such as decreased CL [59]. Immunogenicity might influence the PK of biologics through antibody-mediated clearance. Although non-neutralizing ADAs bind to the protein but do not neutralize it, their ability to form immune complexes has been associated with decreased efficacy and accelerated clearance. Neutralizing ADAs (nADAs) can block activity, thereby altering the concentration–effect relationship. By contrast, PD effects might be prolonged in case of chaperonizing effects (i.e. constant release of drug product from previously formed immune complexes). Such effects can be monitored during multiple-dose studies, preferably by monitoring the drug's PD activity. Monitoring of (neutralizing) ADA formation should be considered for all multiple-dose toxicity studies (after single dose, only in the case of biologics with long half-lives) but only performed when valuable. Information on PKs, PDs and adverse effects is often more useful for study interpretation. When these data do not indicate any influence of (neutralizing) ADAs, their routine monitoring would not add any value. PK data after multiple doses are preferably generated as part of toxicokinetic investigations. Toxicokinetics determines systemic exposure at dosages that need to be considerably above the anticipated clinical exposure levels. The sampling regimen for analysis of ADAs should consider the presence of free circulating drug, which might considerably influence the analytical procedure. This is especially true for biologics with long half-lives (e.g. mAbs) that require very long sampling intervals apart from last dosing.

Bioanalytics of biologics in nonclinical and clinical development

Small molecules generally need a PK assay for quantification of the unchanged compound and its (major) metabolite(s). High-performance liquid chromatography (HPLC) and mass spectrometry (MS) have been highly developed as standard technologies in that field. By contrast, biologics require several types of assay for quantification of the protein itself, its biological activity and the detection and characterization of binding ADAs, neutralizing ADAs and, if necessary (especially for clinical sample analysis), assays to monitor antibodies that might have been generated in response to host cell proteins and/or other (high molecular) impurities or compound constituents (e.g. PEG moieties). This requires not only different technologies and a wider variety of assay types but also more capacity, time and logistical effort.

Assay types used in nonclinical development of biologics

When developing a PK assay strategy, the needs of the nonclinical and clinical development program have to be considered, such as sensitivity of the assay based on nonclinical and estimated clinical dose, planed combination treatments, estimated target levels, PK/PD modeling approaches and so on. Looking beyond assay technologies, sample-clean procedures routinely used for small molecules (e.g. solvent extraction and affinity chromatography) can usually not be used for biologics. Biologics are mostly analyzed without extraction or only with a crude protein precipitation step. Therefore, extensive tests of the matrix effects are required during method development. Ligand-binding assays (immunoassays) are still widely used for quantification of biologics. Innovations in MS instrumentation with much higher mass accuracy have already been applied to smaller MW biologics but still remain unavailable for routine quantification of large proteins (>10–20 kDa) in biomatrices [60].

Immunoassays require the use of a specific antigen or antibody to capture and/or detect the analyte of interest. Essential reagents such as poly- or monoclonal antibodies might be difficult to obtain in early stages of development. Furthermore, assay development often encounters challenges stemming from interfering residuals in the sample matrix. Dynamic range and linearity, limited in comparison to MS methods, are additional issues that often need to be addressed. Immunoassays are also generally less precise than methods such as MS. Heterogeneity of biologics (e.g. mixtures of differently glycosylated species and various degradation products) can raise specificity problems [61].

In addition to the PK assay (or mass assay), the activity assay (bioassay) is particularly useful for measuring the neutralizing activity of an ADA, although it was originally developed to measure product efficacy (e.g. for product release). Activity assays might be performed *in vitro* (often human-cell-line based techniques) or *in vivo* (animal models). These assays might suffer from lack of specificity owing to the potentially confounding influence of substances that modulate the biological activity of the compound of interest. They are, however, the only way to determine whether a protein is still intact and active, which cannot be measured by a 'usual' PK assay.

Immunogenicity assays strategy within biologics development

Developing and validating assays for immunogenicity assessment are the greatest challenges for bioanalytics of biologics. Immunogenicity assays should be designed to be sensitive and specific for the intended purpose of assessing risk and impact of ADAs on safety, efficacy and PKs/PDs. The development and validation of assays to measure the different types of antibodies generated in the nonclinical and clinical setting has been improved substantially in recent years. Key considerations for immunogenicity assay development and validation have been widely published [62–67] and include an EMEA guideline [12]. Generally, the strategy for antibody detection and characterization follows the three-tiered approach of screen, confirm and titer. A screening assay should be used to monitor all samples, generated for antibody screening, for binding antibodies (ADAs), followed by confirmatory and specificity assay for positively tested samples. This is typically followed by characterization of titer and/or relative concentration of the ADAs. Samples with confirmed ADAs can subsequently be tested for antidrug neutralizing activity (nADAs).

Immunogenicity assays for investigating antibody development against a protein therapeutic agent use a variety of analytical formats and detection methods, each platform having its own advantages and disadvantages. Ligand-binding assays are typically developed for ADA measurement using plate, bead or surface plasmon resonance surfaces. Of crucial importance to efficacy and toxicity (exaggerated activity) is whether the antibody can inhibit the pharmacological activity of the protein (neutralizing effect). To neutralize, an antibody must bind an epitope at or near the receptor-binding domain of the therapeutic biologic and, thus, inhibit receptor binding [37]. Neutralizing ADAs should be determined in the nonclinical, as well as clinical, setting based on a confirmed positive ADA response. The major assay platforms are often cell-based assays; alternatively, ligand-binding assay can be considered using a risk-based approach [68]. Cell-based activity assays used for product release are often adapted for use with biomatrices to measure the neutralizing activity of ADAs. These are *in vitro* procedures that utilize cells that interact with or respond to the therapeutic either directly or indirectly in a measurable manner in the presence of test sample for the detection of neutralizing ADAs [64]. Not all nonclinical studies will need to be subjected to intensive immunogenicity characterization; discrete strategies based on the assessed immunogenic risk of the drug and the assay methodology should be employed. In particular, the efforts to develop and validate nADA assays should be balanced against the value of these data for interpretation of the toxicological data [65,67]. A pragmatic approach is to collect and store samples from all nonclinical studies in which animals are exposed for more than seven days. The decision to analyze samples for immunogenicity assessment should depend on the utility of these data to meaningfully add to study interpretation when considered with other parameters (PKs, PDs and toxicity data) [28].

Last but not least, the technology available in the bioanalytical laboratory often determines the assay strategy. Reliable performance of the assay is dependent upon properly functioning analytical equipment and computer systems, as well as upon the training and dexterity of the analyst. Therefore, method validation establishes 'system-suitability', which should be maintained during the in-study phase [68].

Immunogenicity assays have to be characterized (validated) for specific performance characteristics such as screening and specificity cut point, sensitivity, selectivity, precision, robustness, stability and ruggedness suitable for the assay's intended purpose [66]. These requirements will vary depending on the use of the assay (nonclinical versus clinical), stage of product development, nature of product and target population. Applying a risk-based approach to determining the extent of immunogenicity testing in the nonclinical and clinical settings, the potential consequences of the immunogenicity need to be identified and evaluated [68,69]. Assays for measuring ADA response should be established in the nonclinical stage of development to estimate the value (predictivity) of the applied animal models. Their major goal is to understand drug exposure and efficacy within toxicokinetic and PK studies and to understand secondary adverse reactions as a consequence of immunological reactions [68]. They can also give some reasoning for highly variable drug exposure and immune-related toxicities in individual animals. Immunogenicity assays at this stage of development should answer questions about the presence of antibodies, their titer and, when needed (risk-based approach), the Ig class. All bioanalytical efforts in the nonclinical phase are the basis for having assays in place during clinical development.

Clinical studies usually need more background information to ensure efficacy and safety. The significance of the antibody response is determined by assessing the magnitude and duration of the response, and by correlation with changes in PKs and adverse events. For assays used in this stage of development (especially later than phase I), additional information on Ig isotypes might also be considered, as well as information on specificity of the antibodies and their capability to alter drug or endogenous protein activity (autoimmunity). The higher the potential of immunogenicity to adversely affect a patient's health, the more diligently immunogenicity should be examined (e.g. by more extensive and more frequent ADA testing and characterization) [66,68].

Concluding remarks

Because of the striking differences between physicochemical and resulting biological properties of biologics compared to small molecules, many of the principles of conventional nonclinical evaluation have required modification to achieve scientifically valid outcomes.

Biologics are often highly species specific in action and immunogenic in test animal species and humans. Therefore, an understanding of the biological activity of biologics is essential for reasonable safety evaluation. The overall NDP of a biologic is dependent on the specific compound class but generally differs from small-molecule development programs. There is mostly no scientific rationale for performing long-term studies over several months (e.g. carcinogenicity and chronic toxicity) for compounds that produce a strong immunological response, including neutralizing the pharmacological effect. The usually shorter treatment periods within chronic studies of biologics are more than equalized, however, by more sophisticated animal models for safety testing and increased manufacturing activities. Some key studies are usually performed during an NDP of a biologic similar to a small molecule, such as acute and multiple-dose toxicity studies, single-dose PK studies in the toxicologically relevant species and toxicokinetics. When reasonable, specific questions such as local tolerance, immunotoxicity and safety pharmacology can be integrated into the multiple-dose study designs. Special study designs such as the staggered window approach might be helpful when performing specific toxicity studies. NDP is also triggered by the target product profile and the planned final drug product labeling. Nonlinear PKs, TMDD and immunogenicity of human(ized) proteins pose extra challenges in the design and evaluation of these studies. Biologics require a spectrum of bioanalytical assays for quantification of the protein itself, its biological activity, the detection and characterization of ADAs and its neutralizing capacity. Different platforms have been developed over the past couple of years, especially for immunogenicity testing, which continues to be of great interest and concern to the industry and regulatory agencies. Although approaches for nonclinical development of biologics varied across the industry in the past, trends with regard to more standardized NDPs are emerging for biologics, driven by lessons learned from past experience and the practice that has evolved on the basis of the available regulatory guidance.

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