Safety testing of monoclonal antibodies in non-human primates: Case studies highlighting their impact on human risk assessment

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Monoclonal antibodies (mAbs) are improving the quality of life for patients suffering from serious diseases due to their high specificity for their target and low potential for off-target toxicity. The toxicity of mAbs is primarily driven by their pharmacological activity, and therefore safety testing of these drugs prior to clinical testing is performed in species in which the mAb binds and engages the target to a similar extent to that anticipated in humans. For highly human-specific mAbs, this testing often requires the use of non-human primates (NHPs) as relevant species. It has been argued that the value of these NHP studies is limited because most of the adverse events can be predicted from the knowledge of the target, data from transgenic rodents or target-deficient humans, and other sources. However, many of the mAbs currently in development target novel pathways and may comprise novel scaffolds with multi-functional domains; hence, the pharmacological effects and potential safety risks are less predictable. Here, we present a total of 18 case studies, including some of these novel mAbs, with the aim of interrogating the value of NHP safety studies in human risk assessment. These studies have identified mAb candidate molecules and pharmacological pathways with severe safety risks, leading to candidate or target program termination, as well as highlighting that some pathways with theoretical safety concerns are amenable to safe modulation by mAbs. NHP studies have also informed the rational design of safer drug candidates suitable for human testing and informed human clinical trial design (route, dose and regimen, patient inclusion and exclusion criteria and safety monitoring), further protecting the safety of clinical trial participants.

**ABBREVIATIONS**

ADA, anti-drug antibody; ALT, alanine transaminase; AST, aspartate aminotransferase; BIO, Biotechnology Innovation Organization; CMV, cytomegalovirus; CRP, C-reactive protein; GLDH, glutamate dehydrogenase; DLL4, delta-like ligand 4; DRF, dose range-finding; ePPND, enhanced peri- and post-natal development; FGF19, fibroblast growth factor 19; GM-CSFRα, granulocyte-macrophage colony-stimulating factor receptor alpha; GvHD, graft-versus-host disease; HCC, hepatocellular carcinoma; IFN-γ, interferon-gamma; IL-6, -17, interleukin-6, 17; KI, knock in; KLG, keyhole limpet hemocyanin; KO, knock out; mAbs, monoclonal antibody; MLR, mixed lymphocyte reaction; MoA, mechanism of action; NCE, new chemical entity; NHPs, non-human primates; NK, natural killer; NOAEL, no effect level; NOD, non-obese diabetic; PAF, platelet-activating factor; PAP, pulmonary alveolar proteinosis; PD, pharmacodynamics; PEG, polyethylene glycol; PK, pharmacokinetics; SAA, serum amyloid A; TBA, total bile acids; TDAR, T cell-dependant antibody response; TE, thromboembolic event; TGF-β, transforming growth factor-beta; TNF, tumor necrosis factor

**INTRODUCTION**

The past 20–30 years have seen a large increase in the number of biopharmaceuticals, predominantly monoclonal antibodies (mAbs) and recombinant proteins, approved for human use. These novel therapies have established new standards of care and improved the quality of life, bringing tremendous hope to patients with severe and often refractory diseases such as cancer, autoimmune/inflammatory diseases and many other conditions. In particular, the clinical success of mAbs means that, in the coming years, more mAbs against the same or novel targets and pathways will enter clinical development or be approved for human use. Drug companies are motivated by the desire to improve the clinical efficacy achieved by first-genera-tion products (whilst maintaining and in some cases improving upon the generally well-tolerated safety profiles) and are encouraged by the growing understanding of the mechanisms of action (MoA) of protein-based therapeutics and disease aetiologies; the continuing development of bioengineering and
formulation/delivery technologies to both optimize and overcome existing biophysical, functional, immunogenic and route of administration limitations of proteins; and the ability to consider biopharmaceuticals with new specificities and functions. As a result of these major advances, mAbs and recombinant protein therapies now make up half of the drugs in the pipelines of most large biopharmaceutical companies.3

Assessing the safety of these novel large molecule drugs is a primary objective of both the sponsors developing these therapies and the regulators charged with overseeing the testing and approval process. In vitro and in vivo pharmacology and toxicology studies, coupled with exposure data, are analyzed in an integrated manner to inform the design of safe and effective dosing regimens, monitoring techniques, subject selection criteria, or stopping rules.4,5 As safety testing in animal models is a major component of overall safety assessment, sponsors and regulators recognize the importance of applying the principles of the 3Rs (Replacement, Reduction and Refinement) for protecting animals used for scientific purposes.6 This is especially important for highly human-specific mAbs, where the non-human primate (NHP) is frequently the only pharmacologically relevant species for safety assessment. Developers use rational drug design to minimize certain safety risks (e.g., removing immune-activating effector function of mAbs if not required for efficacy, avoiding cross-linking of cellular receptors) prior to selecting lead candidates for safety testing in animals. In addition, knowledge of the drug and target biology, coupled with relevant in silico, in vitro and ex vivo systems, when available, are actively utilized and in some cases can highlight the likely unacceptable toxicity of certain drugs without animal testing. In fact, it has been argued that, in cases of predictable (known MoA-based) pharmacology in vitro, in vivo studies may not be needed.7–9 While this may be true occasionally, many complex interactions that occur in vivo cannot currently be modeled in vitro. In addition, in vitro systems do not allow for an understanding of the relationship between dose, exposure, pharmacological activity and toxicity, and do not predict other effects such as local effects at the delivery site. This is also true of studies using transgenic knock-in (KI) or knock-out (KO) animals to understand a worst case scenario of modulating a target with a drug; while absence of a phenotype can support safety, if there is an adverse phenotype it is difficult to directly extrapolate these findings to a therapeutic setting where lesser degrees of target modulation are desired. For new targets and novel platforms “predictable pharmacology” may also be less predictable, as developers are limited by the existing knowledge of the target. Hence, it is acknowledged that in many cases there are no established alternatives to testing new drugs in animals. More importantly, sponsors and regulators are challenged with an increasing imperative to ensure the safety in humans.10

For biopharmaceuticals, where the vast majority of observed toxicities have been linked to the MoA and off-target effects are rare, animals studies can only be informative if pharmacologically relevant species (i.e., those in which the drug has the same biological activity as that expected in humans) are used for safety assessment, as specified within the current regulatory framework.11 If there are two pharmacologically relevant species for a clinical drug candidate (one rodent and one non-rodent) then both species are used for short-term (up to 1 month in duration) toxicology studies to initiate initial clinical trials. If the toxicological findings from these studies are similar or the findings are understood from the MoA of the product, then longer-term general toxicity studies in one species are usually considered sufficient. The rodent species should be considered unless there is a scientific rationale for using non-rodents; however, the often higher immunogenicity in rodents may preclude their use for long-term studies. In addition, prior to selection of the NHP as a relevant species, some developers assess the relevance of other non-rodent species before utilizing NHPs. In the European Union, the use of NHPs is only allowed if they are essential for the benefit of humans and no other alternative replacement methods are available.12 When the NHP is selected, it is ideally based on a strong understanding of the relevance of the pharmacology, target expression, and relative potency in this species. In addition, developers are encouraged to optimize study designs (e.g., group sizes, number of dose and recovery groups) to reduce NHP use.13–15 It is important for developers and regulators to engage and agree that use of NHPs should not be the default, but rather justified by proper rational and science-based decision-making that should both support their ethical use and better inform the safety of clinical studies. For mAbs and mAb-related products directed at foreign targets (i.e., bacterial, viral targets), a short-term safety study in one species (usually rodents) can be considered.11 When no relevant species can be identified because the biopharmaceutical does not interact with the orthologous target in any species, use of homologous molecules or transgenic rodent models can be considered.16 In some cases it is not possible to make a pharmacologically equivalent surrogate, or the biology of the target in rodents may be divergent from humans. In some of these cases, dependent on the projected benefit-risk, clinical development can be supported by in vitro studies with human cells.17–19

While these current methods of non-clinical safety assessment have been successful in screening out compounds that might cause toxicity in a substantial proportion of patients, these approaches have been less successful at predicting serious adverse effects that occur only in a relatively small proportion of patients. Some reasons given for why animal studies fail to detect these effects is that animal studies are not powered to detect rare events, and, because they are mostly conducted in healthy animals, the effects of the disease on the biological activity of test compounds is not assessed. Arguably, patients enrolled in clinical trials (at least early trials with small patient numbers) also do not reflect the full range of the population or treatment situations that occur in clinical practice. As a result, new safety issues have sometimes been identified only after medicines enter the market.

Large prospective studies to robustly assess the effectiveness of standard preclinical safety models have not been possible for various reasons, including lack of standardization of toxicity data sets, the broad array of toxicities that are assessed for in drug development, and the fact that much of the relevant data are proprietary to the innovator company. Olson et al.20 reported the findings of an industry consortium that examined a group of 150 small molecule investigational drugs that showed some form of toxicity in humans. Tamaki et al.21 evaluated 142 drugs approved in Japan, including both small molecules and
biopharmaceuticals. Both reports suggested that animal studies provided value in assessing human toxicity with overall concordance rates of 71% and 37%, respectively, although certain toxicities were poorly predicted. Recently, Van Meer concluded from a review of the approved mAbs that non-clinical safety studies in NHPs were poorly predictive of human toxicity.\textsuperscript{7} Bugelski and Martin\textsuperscript{22,23} explored the concordance between animal toxicology data and observed human adverse events for approved mAbs and IgG-Fc-based fusion proteins which revealed that, although the overall concordance of animal toxicology and clinical data was low, the direct or exaggerated immunopharmacology of mAbs were modelled well in non-clinical studies. However, the downstream sequelae of the pharmacological effects (e.g., infusion reactions, infection, cancer) were not.

These and other studies\textsuperscript{24} used a clinical adverse drug reaction (of varying nature and incidence) as a starting point, and then sought to understand if that human effect was predicted by the animal toxicity testing. Although providing important information, these analyses neither represent a complete or accurate picture of how animal studies may inform the clinical development of potential mAb therapeutics. Since only marketed drugs are included, these studies ignore the far greater numbers of drugs that failed to reach the market, or even clinical trials. Hence, they do not capture the value of animal studies in identifying drug candidate molecules and pharmacological pathways that are deemed too fraught with hazard that they not amenable for safe drug development. Nor do they highlight how animal studies have also informed the rational design of safer drug candidates suitable for humans testing, informed clinical trial design, route, dose and regimen, selection of patient populations suitable for the benefit-risk, and safety monitoring, further protecting clinical trial participants. Since these animal-related impacts occur primarily during proprietary research prior to clinical trial submissions, they often remain unpublished.

Recognizing that retrospective analyses of the utility of animal studies in predicting the safety of approved drugs do not capture the effects described above, BioSafe (a Committee of the Biotechnology Innovation Organization (BIO) specializing in the preclinical safety assessment of biopharmaceuticals) convened a task force to review current approaches to the safety assessment of mAbs, and to collect and analyze a cross-section of specific case examples from BIO member companies where NHP safety studies had meaningful and profound effects on clinical development. Seventeen case studies are summarized in Table 1; those cases for which more extensive information could be provided (BIO 1–9) are described in more detail in the next sections. The case studies were chosen to include mAbs specific for novel targets, conventional mAbs and novel mAb-related scaffolds, as well as novel routes of administration, for which the pharmacology of these molecules and targets had not been well-characterized previously, with little human precedent.

**Monoclonal antibody case studies**

**BIO-1**

BIO-1 is a human IgG4 mAb antagonist to the alpha subunit of the granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR\textsubscript{a}). It blocks the activity of GM-CSF and is currently in clinical development for rheumatoid arthritis (RA).\textsuperscript{25,26} GM-CSFR\textsubscript{a} is expressed on many cell types, including monocytes and macrophages. Its natural agonist, GM-CSF, as well as being a growth factor for hematopoietic cells, is produced by several inflammatory cell types and, through receptor activation, can mature macrophage from precursor cells and activate mature neutrophils, eosinophils, and macrophages. BIO-1 inhibits GM-CSF signaling and is expected to decrease the number of activated inflammatory cells in the synovium or other sites of inflammation.

GM-CSF is also known to serve a role in pulmonary surfactant homeostasis. The role of GM-CSF in the lung is based in part on insights from KO mice\textsuperscript{27,28} that showed accumulation of phospholipids and proteins in the lung. Additionally, there is a rare restrictive lung disorder in humans, termed pulmonary alveolar proteinosis (PAP) that is caused either by GM-CSF\textsubscript{a} mutations or anti-GM-CSF autoantibodies.\textsuperscript{29} Patients can manifest lung surfactant and protein accumulation, and in severe cases, respiratory failure. Furthermore, these anti-GM-CSF autoantibodies could reconstitute PAP in NHPs.\textsuperscript{30} Based on these data linking GM-CSF pathway blockade to lung disorders, and the unacceptable risks that pulmonary toxicity would pose for RA patients, further safety studies were undertaken.

Several independent repeat-dose toxicity studies were performed in cynomolgus monkeys,\textsuperscript{31} the only pharmacologically relevant species based on similar binding affinity and potency in functional assays using human and cynomolgus monkey granulocytes.\textsuperscript{32} Study durations ranged from 4 to 26 weeks and utilized once weekly intravenous or subcutaneous injections. Overall, the lung was identified as the most sensitive target organ. Foamy alveolar macrophages in lung were seen on studies of 11 weeks or longer, but not in studies of 4 weeks duration. The foamy appearance of macrophages reflected the intracellular accumulation of neutral lipids. This partially reversible finding was judged “non-adverse” as it reflected the normal function of macrophages (clearing foreign material) and it occurred in the absence of any necrosis or changes in lung structure or function. In a study of 26-weeks duration, lung findings included foamy macrophages, but also showed buildup of foreign material, cholesterol clefts, and granulomatous inflammation. These findings were judged “adverse” because the normal functions of macrophages were impaired, leading to accumulated debris and inflammation, which resembled human PAP. These latter findings occurred at high multiples of clinical exposure.

To more accurately characterize exposures that did not cause any lung impact, in particular exposures that did not cause accumulation of lung foamy macrophages, a lower-dose study was performed using low multiples of clinical exposure. Immunogenicity of BIO-1 led to a high rate of anti-drug antibody (ADA) and insufficient drug exposure in a majority of animals. Nevertheless, exposure was maintained in 4 animals at ~4 mg/kg/week (10 mg/week), which also represented the no-observed effect level (NOEL). Taken together, monkey studies carefully correlated exposure with response, and showed that the GM-CSFR\textsubscript{a} could be targeted safely with a mAb, without triggering adverse effects on lung or respiratory function (concerns from KO mouse and humans with anti-GM-CSFR...
| Case Study | Target; Drug Format; Indication (if disclosed) | Intended Pharmacology | Potential Safety Risk for Human | Findings in Toxicology Studies | Value of NHP Studies | Impact of NHP Data on Clinical Development |
|------------|---------------------------------------------|-----------------------|--------------------------------|-------------------------------|---------------------|------------------------------------------|
| BIO -1     | Anti-GM-CSF mAb (IgG4); Rheumatoid arthritis, other autoimmune diseases | Inhibition of the pro-inflammatory activities of macrophages | Inhibition of normal macrophage function | NHPs only relevant tox. species | Demonstrated that target with theoretical safety liabilities could be “drugged” safely | Progression to clinic |
|            |                                             |                       | Lung (inhibition of alveolar macrophage & homeostasis of surfactants) identified as target organ based on MOA, KO mice and clinical syndrome (PAP) in patients with reduced target function | Foamy macrophages and adverse buildup of foreign material, cholesterol clefts, & granulomatous inflammation due to reduced macrophage function (like human PAP). Only occurred at high multiples of clinical exposure | Allowed characterization of exposure: toxicity response to inform human dose modeling and safety margin predictions | Identified safe starting & dose escalation schemes in humans |
| BIO -2     | Anti-DLL-4 F(ab)';2; cancer Disruption of tumor angiogenesis | Inhibition of normal blood vessel structure | NHP & rodent relevant tox. species | Showed that on-target toxicities with parental IgG1 could be avoided with a short half-life F(ab)';2 | Terminated drug candidate and target abandoned. | Lower dose/exposure in Phase 2b studies; intensified pulmonary monitoring in all clinical studies; findings likely translatable to humans, poorly monitorable and could result in permanent damage to cardio-pulmonary system. |
| BIO -3     | Anti-TGFβ1 mAb (IgG4); indication not disclosed | Inhibiting TGFβ1-mediated function | Cardiovascular toxicity, skin acanthomas, immune activation and multifocal based on data from human syndromes, KO mice with adverse effects on cardiovascular and immune system inflammatory disease | NHP & rodent relevant tox. species | Identified potentially human-translatable toxicity not seen in rodent. | Progression to clinic |
|            |                                             |                       | Findings only observed in NHPs: wide array of effects (some expected) including epithelial hyperplasia in multiple tissues, enteropathy characterized by deposition of extracellular matrix with the intestinal mucosa and renal tubular inflammation and tubular injury. | Findings likely to be monitored in human studies (some expected) including epithelial hyperplasia in multiple tissues, enteropathy characterized by deposition of extracellular matrix with the intestinal mucosa and renal tubular inflammation and tubular injury. | Demonstrated that target with safety liabilities could be “drugged” safely | Identified suitable starting dose & escalation schemes in humans; changed dosing regimen from weekly to monthly to decrease risk; modified human monitoring and exclusion criteria. |
| BIO -4     | Anti-macrophage receptor; fibrosis | Inhibiting the function of pro-fibrotic macrophages | Inhibition of normal macrophage function. Osteopetrotic phenotype with short thick bones, impaired fertility /reproduction and increased hepatic enzymes (identified from KO mice) | NHP only relevant tox. species | Demonstrated that target with safety liabilities could be “drugged” safely | Progression to clinic |
|            |                                             |                       | Bone resorption, but no effects on bone density or bone architecture. No effect on reproductive endpoints; some liver enzymes markedly increased without histopath. Due to effect on Kupffer cells involved in their clearance; peri-orbital swelling with late onset & slow reversibility | Bone resorption, but no effects on bone density or bone architecture. No effect on reproductive endpoints; some liver enzymes markedly increased without histopath. Due to effect on Kupffer cells involved in their clearance; peri-orbital swelling with late onset & slow reversibility | Osteopetrotic phenotype and other effects in KO mice not observed with mAb treatment | Additional ophthalmoscopic evaluations included | Identified suitable starting dose & escalation schemes in humans. |

**Table 1. Monoclonal Antibody Case Studies.**
| **BIO-5** | Anti-cytokine mAb (IgG4): inflammatory disease | Inhibit pro-inflammatory effect of cytokine | None based on lack of toxicity in KO mice and in adult NHPs in general tox. studies. | Identified a life-threatening hazard for women and infants during the latter stages of pregnancy. Target cytokine shown to have critical role in cervical ripening not predicted from literature. KO mice nor repotted study in mice with anti-mouse cytokine mAb | Continued development in patients with autoimmune diseases, but labeling specifically warns against use in pregnancy and that women who become pregnant while receiving treatment must stop immediately |
|----------|---------------------------------------------|-----------------------------------|-----------------------------------------------------------------|-----------------------------------------------------------------|-----------------------------------------------------------------|
| **BIO-6** | Anti-CD40L Fab-PEG; Inflammatory & autoimmune conditions | Inhibition pro-inflammatory of T cell-APC interaction | Immunosuppression, infection. NHPs only relevant tox. species; rhesus sensitive to TE effects. | Confirmed in vivo screening of new drug candidates lacking Fc to circumvent human-relevant TE risk – a regulatory expectation for mAbs targeting this pathway | Allowed continuation of target program with safer lead modified to mitigate against TE risk |
| | | | mAb-induced platelet activation, TE & mortality; previous drug in class (IgG1) induced TE events in clinic leading to MI, pulmonary embolism or stroke; later shown to induce TEs in rhesus but not cyno. Fc domain required for TE effect | Expected modulation of lymphocytes and dose responsive reversible decreases in antigen-specific antibody responses following immunization | In vitro platelet activation studies alone inadequate to de-risk such a severe potential outcome | Identified suitable starting dose & escalation schemes in humans |
| | | | Block FGF-19 mediated tumor growth and invasion | No increase in TE events observed with parental IgG1. Data supplemented lack of platelet activation in vitro with human and rhesus platelets | Defined PK/PD relationship to inform pharmacologically active dose in humans | Tight monitoring for platelet effects |
| | | | Negative impact on hepatic bile metabolism (seen in KO mice and humans with reduced FGF19); bile acid diarrhea seen in FGF19-deficient humans | Significant toxicity (not observed in KO mice) at all dose levels: severe diarrhea, decreased food consumption & BW, unscheduled euthanasia after single dose; increases in AST, ALT, total bile acids- no corresponding histological changes in liver or GI tract | Termination of candidate and target |
| | | | NHP only relevant tox. species | Identified new on target effect (acute diarrhea and liver toxicity) with narrow safety window | Role in bile acid homeostasis, specifically bile transporters and effects on enterohepatic circulation, previously unknown consequence of inhibition of pathway |
| | | | | | Highlighted that further optimization of candidate required and screening in NHPs to assess anaphylaxis risk |
| **BIO-8** | Multimeric proteins (mAb-related) targeting immune cell receptors; autoimmune disease | Inhibition of auto-reactive cells and autoantibody | Cytokine release, platelet activation due to target expression on these cells; complement activation; observed in vitro with non-engineered candidates but no/low activity with candidates modified to remove these liabilities | Identified new potentially human-relevant pro-inflammatory and life-threatening safety liabilities of a novel target and novel constructs, not detected in vitro | Termination of all candidate variants |

(Continued on next page)
| Case Study | Target; Drug Format; Indication (if disclosed) | Intended Pharmacology | Potential Safety Risk for Human | Findings in Toxicology Studies | Value of NHP Studies | Impact of NHP Data on Clinical Development |
|------------|---------------------------------------------|-----------------------|---------------------------------|-----------------------------|---------------------|------------------------------------------|
| BIO-9      | Target not disclosed; belongs to JAK/STAT signaling: Transplantation | Inhibition of activation, proliferation and survival of T and NK cells | Mouse not a good translatable model since even the safest candidates with good in vitro human safety profiles and which induced no clinical symptoms in NHPs, induced anaphylactoid reactions in mice even the safest candidates with good in vitro human safety profiles and which induced no clinical symptoms in NHPs, induced anaphylactoid reactions in mice, induced anaphylactoid reactions in mice. | CMV reactivation/ opportunistic viral infections (mollivirus, adenovirus, SV40), B cell lymphoma (LCV); Reactive lymphocytosis | Better concordance of NHP data with in vitro human data compared to mouse | Allowed to highlight that alternative pathway can be activated upon antigenic stress, which can bypass the blockade of the target and reactivate the T cells | Led to the termination of the program |
| BIO-10     | Anti-BAFF IgG; SLE | Deletion of autoreactive B cells, reduction in autoantibody | NHP only relevant tox. species | Unexpected medial necrosis of the lung in cynos; not predicted by pharmacology or target expression | Identify potentially human-relevant safety liabilities of a novel mAb | Termination of drug candidate and target |
| BIO-11     | Anti-human cell membrane protein (IgG1; Fc enhanced to increase potency) | Not disclosed | NHP only relevant tox. species | Unexpected neumopoeia; further target expression showed binding to FcγR (CD16) on human neutrophils | Identify potentially human-relevant toxicity not predicted from in vitro studies | Close clinical monitoring for neutropenia in humans |
| BIO-12     | Anti-human cell surface protein IgG1 mAb and ADC | Tumor killing; pharmacology of mAb target not disclosed | NHP only relevant tox. species | Unexpected clinical pathology and histopathology effects consistent with systemic inflammatory response with naked mAb in the absence of ADAs; ADC most tox based on payload (requires mab attached for toxicity to be observed) | Identified new potentially human-relevant toxicity | Increased clinical monitoring for inflammation; Additional exclusion criteria included in clinical protocols |
| BIO-13\textsuperscript{74} | Bispecific F(ab')2; target not disclosed; ocular disease | Neutralizing target in eye | Based on intravitreal dosing, systemic exposure was low so systemic toxicity not expected | NHP & rabbit relevant species | Identified new potentially human-relevant toxicity; intravitreal route had associated risks | Termination of drug candidate; new candidates investigated |
| BIO-14     | MoAbs; IgG1; target and indication not disclosed | Not Disclosed | Cross-reactivity to a related gene product known but the impacts of relative binding to both desired and undesired targets were unknown | NHP only relevant tox. species | Allowed safety assessment of desirable on-target pharmacological effects | Termination of drug candidate; new candidates investigated |
| BIO-15\textsuperscript{75} | Anti-human cell surface membrane protein (IgG2a) | Not Disclosed | None based on known pharmacology | Undisclosed effects observed as a result of binding to both intended and unintended site | Identified new potentially human-relevant toxicity of mAb not detected in vitro | Allowed continuation of target program with safer lead without platelet phagocytosis and TCP risk |

Additional exclusion criteria included in clinical protocols.
| BIO-16 | Anti-C3b neoepitope Fab for intravitreal administration; AMD | Inhibition of complement activation in the eye | None; neutralizing complement in healthy animals should be without acute effects (potential risk of increased infection) | NHP only relevant tox. species | Anti-drug antibodies facilitated aggregation and activation of the target leading to severe consequences; Drug was expected to be anti-inflammatory. Potentially human-relevant safety liabilities if immunogenicity induced in humans | Fab and program terminated |
|---|---|---|---|---|---|---|
| BIO-17 | T-cell bispecific antibody, targeting a human cell surface protein and CD3 | Recruiting cytotoxic T cells to tumor cells expressing the target | Target expressed at low levels in normal tissues, but considered inaccessible to mAb binding | NHP only relevant tox. species | Identified new potentially human-relevant toxicity; expression of the target in iris and ciliary body not detected via IHC – expression only ascertained via qPCR after laser capture microdissection; choroid plexus and eye shown to be accessible to the mAb in vivo. | Termination of mAb |
| BIO-18 | IgG4 antibody antagonizing receptor of the tetraspan protein family | Anti-viral Tetraspanins regulate intercellular immune interaction, adhesion, migration, synapse formation. Literature indicated a potential for co-stimulation and activation of T-cell responses. KO without overt phenotype | Two monkeys in single dose PK study became moribund; data consistent with anaphylactoid shock / vascular leakage (pale, hypothermic, lateral recumbency; acidosis, hypoproteinemia, hemococoncentration, leukocytosis); edema; cytokine increases; | NHP: study helped to understand ambiguous in vitro data (cytokine release without evidence of agonistic activity) and indicated that the in vivo effects may not be entirely target-mediated. Uncovered serious acute safety concerns with uncertain but potential translation to humans. | Termination of program after generation of different IgGs from two different clones gave similar results. |
autoantibodies). Based on findings in the NHP model, human clinical trial protocols were modified to include intensified respiratory monitoring (chest X-rays, forced expiratory volume, forced vital capacity, diffusing capacity of the lung for carbon monoxide, and dyspnea scores). Phase 2b trials proceeded with doses that were approximately 3–5 times below the exposures associated with NHP foamy macrophages. 25,26,31 No treatment-related safety signals were identified.

**BIO-2**

BIO-2 is an antagonist of the delta-like ligand 4 (DLL4) pathway. DLL4, an endothelium-specific NOTCH ligand, plays a key role in vascular development. 33 During angiogenesis, luminal endothelial cells convert into “tip” cells that contribute to the development of a multicellular stalk, which then undergoes lumen formation. Inhibition of DLL4 signaling during angiogenesis disrupts the dynamic balance between tip and stalk lumen formation. Inhibition of DLL4 signaling during angiogenesis causes hypoxia, causing tumor hypoxia. 35,36 BIO-2 is an antigen-binding fragment (F(ab′)2), the product of pepsin-based digestion of a full-length anti-DLL4 parental IgG1 antibody, and inhibits DLL4 binding/signaling. BIO-2 is cross-reactive with, and has a similar affinity for, human, NHP, and rodent DLL4. DLL-4 also has a similar expression pattern in humans, NHPs and rodents. Previous preclinical studies raised safety concerns related to inhibition of DLL4; in particular, treatment of rodents and cynomolgus monkeys with the parental anti-DLL4 IgG1 resulted in liver pathology (sinusoidal dilation, hepatocellular atrophy, bile duct proliferation, and elevated liver enzymes). In addition, vascular neoplasms were observed in the skin, heart, and lungs of male rats and acute hemolytic anemia was observed in NHPs. 37 Given the interest in this pathway as it relates to cancer treatment, use of an F(ab′)2, with a shorter half-life, and so yielding incomplete or intermittent blockade of DLL4, was pursued to potentially mitigate known toxicities while maintaining efficacy. Despite amelioration of the aforementioned anti-DLL4-related toxicities associated with the IgG1 mAb, 8-week toxicity studies with the F(ab′)2 in rats and cynomolgus monkeys revealed unexpected toxicities distinct from those associated with the IgG1. Specifically, F(ab′)2 administration was associated with a dose-dependent increase in the incidence and severity of vascular changes in the heart and lung; specifically, endothelial cell proliferation on the right side of the heart, proliferative pulmonary artery mural or adventitial changes (rats only), intimal vacuolation in the pulmonary artery at the base of heart (monkeys only), and increased cellularity of the arteries in the lung (both species). 38

In addition, pulmonary mural basophilia was present in the heart and lungs of both species. After an 8-week recovery period, histopathology findings were reduced in incidence and severity; however, pulmonary arterial adventitial fibrosis was still present. The nature of these vascular changes present in the pulmonary artery and right side of the heart are likely consistent with pulmonary arterial hypertension. Histopathology changes were not accompanied by overt changes in heart rate or blood pressure in telemetry-instrumented monkeys. Another DLL-4 mAb also demonstrated dose-dependent mortality, moribundity, and clinical signs related to gastrointestinal bleeding and heart failure in cynomolgus monkeys. 39 The hypertension and heart failure observed with BIO-2 were also similar to later clinical results in trials of other anti-DLL4 mAbs, OMP21M18 40 and REGN42. 41

Overall, these studies identified a novel toxicity in cynomolgus monkeys and rodents, a previously unknown consequence of inhibiting this pathway and considered to be an on-target effect. These findings were considered to be likely translatable to humans and, importantly, were likely to be poorly monitorable and could result in permanent damage/remodeling to the cardio-pulmonary system. As a result, a decision was made to halt further development of this F(ab′)2 molecule and continue additional efforts to explore this specific NOTCH family target for cancer therapy.

**BIO-3**

BIO-3 is an IgG4 mAb antagonist of transforming growth factor beta 1 (TGFβ1), a cytokine involved in cell cycle control, regulation of early development, cell differentiation, angiogenesis, immunity, and extracellular matrix regulation that is implicated in the development of lung fibrosis. 42 The TGF superfamily is complex, and includes more than 30 mammalian isoforms, most of which are poorly understood. Since inhibition of certain isoforms of TGFβ has been shown to be beneficial in animal models of disease, BIO-3 was developed for a disease with no current treatments.

Before embarking on the project, insights into risks were gathered from varied sources including human genetic syndromes, 43 TGFβ KO mice 44–46 and human trials with a related anti-TGFβ mAb (fresolimumab), which inhibits TGFβ1, 2 and 3. 47,48 These data suggested hazards to cardiovascular and immune systems (KO mice rapidly succumb to multifocal inflammatory disease). While findings from these models raised potential safety concerns, it was impossible to discern if the degree of target inhibition needed for therapeutic intervention with a more specific antagonist to TGFβ1 would also result in safety concerns. Animal studies were thus needed to assess safety aspects.

BIO-3 was cross-reactive in both rat and cynomolgus monkeys. A 6-month repeat-dose toxicity study employed once weekly dosing and produced many adverse effects in monkeys, including epithelial hyperplasia in multiple tissues, enteropathy/deposition of extracellular matrix in the intestinal mucosa, and renal tubular inflammation as well as injury. Notably one monkey developed an epithelial tumor. These effects were not observed in rat studies. All NHP effects were plausibly linked to inhibition of TGFβ. Interestingly, cardiovascular and immune activation effects (effects seen in KO animals or other sources) were not observed in adult rats or monkeys dosed with BIO-3.

Since severe toxicities were associated with weekly dosing, additional pharmacokinetic/pharmacodynamics (PK/PD) studies were conducted that showed based on modeling that a sufficient degrees of target modulation could be maintained with once monthly dosing.
To understand if this less frequent dosing regimen could increase the margin of safety, a 9-month NHP study was performed employing once monthly dosing. This study showed pathology was limited to very mild epithelial proliferation in a specific tissue, which was considered non-adverse. Both with BIO-3 and fresolimumab, the cynomolgus studies showed that hazards seen in KO mice or human genetic mutations could be avoided through careful control of mAb dose and regimen. The animal pharmacology and toxicology data with BIO-3 supported clinical trials startup and defined a clinical dosing frequency.

Treatment with a TGF-β1 antibody in a Phase 2 study in diabetic nephropathy was not associated with clinically significant changes in safety laboratories and vital signs, there was no evidence of induction of autoimmunity, and the frequencies of various categories of adverse events were not different between the treatment and placebo groups.

**BIO-5**

BIO-5, an IgG4 mAb directed against a cytokine, is in development for inflammatory diseases. The cynomolgus monkey was the only pharmacologically relevant species for safety assessment, based on in vitro potency on downstream signaling comparable to human, in vivo efficacy in a disease model and literature about similar distribution and function of the target. By contrast, no activity of BIO-5 could be demonstrated in rodents. KO mouse studies and mice dosed with a homolog mAb did not reveal any adverse findings. General toxicity studies conducted in the adult healthy monkey with the clinical lead mAb were uneventful. Since the treated population would include women of child-bearing potential and young infants, it was necessary to assess toxicity during pregnancy. In an enhanced peri- and post-natal development (ePPND) study, pregnant monkeys were treated from gestation day 20 to parturition and infants were monitored for 9 months. BIO-5 treatment was well-tolerated during pregnancy and did not increase the normal incidence of abortions. However, in all treated pregnant animals, the duration of gestation was significantly increased, and mortality was seen in some animals at delivery without prior signs of distress. Pathologic findings suggested that the difficult deliveries (dystocia) were caused by placental retention and, in some cases, associated with significant genital blood loss. Infant losses were also seen, and judged secondary to dystocia. Infants who survived the difficult parturition developed normally, including their immune systems. None of the findings of this ePPND study were observed in KO mice nor in pregnant mice treated with an anti-mouse cytokine receptor mAb, as a consequence of a difference in the physiology of parturition between rodents and higher mammals.

Based on these findings, a deeper review of parturition literature suggested that the target could be one of many factors involved in labor-associated inflammation, cervical ripening, and then digestion of the interface between placenta and uterus in human and higher mammals; in fact, the cytokine was shown to be indispensable for normal parturition. This study showed for the first time that this factor was more critical than previously appreciated in primate parturition, and that pregnant women might also risk dystocia, hemorrhage, and infant loss if undergoing treatment with BIO-5.

In conclusion, the only appropriate species for assessing reproductive and developmental toxicity was the cynomolgus monkey. Conducting the study identified a life-threatening hazard for women and infants during the latter stages of pregnancy, one that was not predicted from prior literature, nor from mice (KO or mAb-treated). As a result of the ePPND study in monkeys, BIO-5 continued development in patients with autoimmune diseases without unexpected safety findings, but the informed consent form specifically warns against use in pregnancy and instructs that women who become pregnant while receiving BIO-5 must stop treatment immediately.

**BIO-6**

BIO-6 is an antibody Fab’ directed against CD40 ligand (CD40L or CD154) that is conjugated to polyethylene glycol (PEG). CD40L is primarily expressed on activated T-cells and,
through interactions with its receptor CD40, plays an important role in regulating interactions between T cells and other immune cells, notably B cells and antigen-presenting cells, and thus affects several important functional events thought to be involved in autoimmune disease.\(^{51}\) CD40L can also be found on other cell types, including macrophages, B cells, platelets, and non-hematopoietic cells. The biology of CD40L appears to be similar in humans, NHPs and rodents, and there is no evidence for differences in function or modes of inhibition across these species. Blockade of CD40L is efficacious in treating inflammatory and autoimmune conditions in a range of animal models. In CD40 or CD40L KO mice, there is little immunoglobulin (Ig) class switching or germinal center formation, and immune responses are severely inhibited,\(^{52,53}\) whilst human CD40L deficiency results in an inability to undergo Ig class switching and is associated with hyper-IgM syndrome.\(^{53}\)

CD40L inhibition has also shown similar effects on other pharmacological endpoints in both mice and NHPs.

The development of BIO-6 was guided by previous clinical experience with the humanized anti-CD40L IgG1 mAb, hu5c8 (BG9588, ruplizumab), which was studied in Phase 1 and Phase 2 clinical trials in numerous indications.\(^{55}\) A related full-length IgG mAb was studied in similar trials by Idec (IDEC-131; toralizumab).\(^{56}\) Although promising activity trends were noted, eight patients suffered a thromboembolic event (TE) presenting either with myocardial infarction, pulmonary embolism, or stroke, which was fatal in one patient.\(^{55}\) Although autoimmune disease patients can develop TEs at higher rates than healthy subjects, the observed incidence was much higher than expected and elevated risks were attributed to drug. Development of both hu5c8 and IDEC-131 were terminated. Prior to this, hu5c8 had shown no unexpected adverse events in toxicology studies in cynomolgus monkeys. However, a subsequent safety evaluation of hu5c8 in rhesus monkeys revealed pulmonary vascular thrombi with pulmonary arterial and arteriolar thrombosis with intimal hyperplasia. In vitro studies with human, cynomolgus and rhesus platelets showed aggregation/activation of both human and rhesus platelets with hu5c8 but not cynomolgus platelets, pointing to a translatable link between platelet activation in vitro and TE in vivo in humans and in rhesus, but not cynomolgus, monkeys. Hence, in vitro platelet studies and in vivo rhesus toxicity studies could be used as key risk assessment tools for TE prior to initiating clinical studies with anti-CD40L mAbs.

A program was undertaken to design a new, safer drug that did not cause platelet aggregation. One hypothesis for the cause of mAb-induced aggregation was that the Fc portion of the mAb might be responsible for activating platelets via binding to CD32 (FcγRIIa) on the platelet surface.\(^{57}\) To test the hypothesis, BIO-6, a PE Gyalted anti-CD40L Fab\(^{\prime}\) that lacks an Fc region and cannot bind CD32, was developed.\(^{58}\)

BIO-6, like hu5c8, has equivalent binding and potency for human, cynomolgus, and rhesus CD40L, but does not bind to rodent CD40L. BIO-6, as well as an aglycosyl version of hu5c8 that lacks FcγR binding activity, showed no evidence of human or rhesus platelet activation in vitro and no TE or vasculopathy in vivo in 3-month rhesus toxicology studies (whereas hu5c8 tested in parallel showed clear platelet activation in vitro and TE events in vivo).\(^{58}\) The only observed effects were expected modulation of lymphocytes and dose responsive reversible decreases in antigen-specific antibody responses following immunization. This PK/PD relationship was used to model pharmacologically active dosing regimens in patients. The intensively reviewed pathology in the rhesus monkey studies, as well as in vitro investigations on platelets, provided assurance for human trial safety. To date, BIO-6 has shown no evidence of TE events or other adverse events in two Phase 1 studies that would preclude further clinical studies with BIO-6. Regulators now expect to see safety demonstrated in the rhesus model prior to human studies. In vitro platelet activation studies alone are considered inadequate to de-risk such a severe potential outcome for human safety since not all cells, mediators, and physiological factors that could contribute to TE will be present in an in vitro system.

**BIO-7**

BIO-7, a humanized IgG1 directed against fibroblast growth factor 19 (FGF19), a member of the FGF family, blocks the binding to its receptor FGFFR4, and was being developed for hepatocellular carcinoma (HCC). The FGF19–FGF4 pathway stimulates hepatocyte proliferation, blocks apoptosis and promotes invasion of these cells. Mice overexpressing human FGF19 develop HCC, whilst crossing these animals with FGF4 KO mice or treatment with anti-FGF19 or anti-FGFR4 antibodies inhibits HCC.\(^{59}\)

However, FGF19 is also known to play an important physiological role in regulating (inhibiting) hepatic bile acid metabolism through repression of the gene Cyp7a1, a key rate-limiting step in the production of bile acid synthesis.\(^{60}\) The impaired regulation of bile acids by FGF19 leads to bile acid diarrhea, also known as idiopathic bile acid malabsorption.\(^{61}\) The FGF19 pathway has been studied in rodents. FGF4 KO mice have no overt abnormalities, but have a larger bile acid pool, increased excretion of bile acids, and bile acid–depleted gall bladders, indicating negative regulation of cholesterol and bile acid synthesis.\(^{62}\) However, key differences in bile acid synthesis and metabolism exist between rodents and humans, and the fact that rodents only express FGF15, an orthologue of FGF19, with a more restricted expression, limit their utility. Furthermore, cynomolgus monkeys and humans both express FGF19 in the same tissues and are similar with regard to their cholesterol and bile acid synthesis.\(^{63}\) Cynomolgus monkeys were thus chosen as the most pharmacologically relevant animal model for safety assessment because of comparable high affinity and neutralization activity of BIO-7 for both human and cynomolgus monkey FGF19.

In order to characterize the potential toxicity associated with inhibition of this pathway, a repeat-dose safety study was conducted in cynomolgus monkeys.\(^{64}\) BIO-7 was poorly tolerated at all doses from 10–100 mg/kg. Major clinical observations included severe diarrhea, decreased food consumption, and decreased body weight that resulted in the unscheduled euthanasia of all animals after only a single dose. Significant dose-dependent elevations in AST, ALT, total bile acids (TBA) and total bilirubin levels were observed in the animals. Although elevations in ALT and TBA were consistent with hepatocellular injury or dysfunction, corresponding histopathologic changes...
in the liver and gastrointestinal tract were not observed in the majority of animals, indicating that the likely underlying cause of moribundity was diarrhea and dehydration. Additional in vitro and in vivo studies showed high doses of BIO-7 had no direct cytotoxic effect on hepatocytes. BIO-7 increased Cyp7a1 expression and elevated bile acid synthesis as expected. However, this in turn altered expression of bile transporters in the liver and ileum, previously unknown biology, resulting in enhanced bile acid reflux, perturbations in enterohepatic circulation, and reduced uptake into hepatocytes. Subsequently, high concentrations of bile acids altered ileal solute concentrations, stimulated water secretion, and increased intestinal motility causing watery diarrhea.

In conclusion, these findings suggested that inhibition of FGF19 with BIO-7 de-repressed Cyp7a1 regulation and increased bile acid synthesis in vitro and in vivo as anticipated, but also altered hepatic bile acid transporter expression. As a result, elevated serum bile acids subsequently alter bile acid transporter expression in the intestine, resulting in perturbation of enterohepatic circulation and the development of diarrhea and liver toxicity. The work presented in this study was the first to demonstrate this relationship with bile acid homeostasis in a NHP as a result of on-target effects. The monkey findings were severe (compared to KO mice) and showed a very narrow safety margin in light of doses needed for therapeutic effect; additionally these findings were considered to be translatable to humans. Taken together, the decision was made to halt further development of antagonists of the FGF19 pathway.

**BIO-8**

BIO-8 is a multimeric antibody-related drug directed against a family of immune system receptors, of which some activate and some inhibit immune functions across a variety of cells types. It was in development for autoimmune diseases. There are known differences in the target receptor expression, distribution, and functions between rodents and primates (including humans). The NHP was considered to have greater pharmacological similarity to human than rodent.

Since the target receptors were expressed on leukocytes and platelets and the candidate had the potential for complement activation, in vitro human cellular studies of cytokine release, platelet and complement activation, as well as studies to assess potency (immunomodulation of disease-relevant immune cells), were conducted to optimize the safety and efficacy of the candidate series. Additionally, in vivo mouse studies were conducted with the understanding that there might be differences in pharmacologic activity based on receptor disposition or functionality. In mice there were highly variable strain-dependent safety effects, including anaphylactoid reactions, observed with all candidates, despite favorable in vitro safety profiles with human cells. Based on the human in vitro studies, three optimized lead candidates modified to reduce/remove the safety risk, but retaining varying degrees of potency (target engagement and inhibition), were selected for in vivo assessment in NHPs. Since the drugs had the potential for acute immunotoxicity, a pilot monkey study was conducted using within-animal dose escalation with a limited number of NHPs (3 per candidate). The least potent candidate was tested first (starting at low dose levels), with subsequent more potent candidates tested only once the safety had been confirmed with the previous candidate.

After the first dose, the least potent candidate showed no adverse events, whilst the second (more potent) candidate caused bruising in the absence of clinical signs; both of these candidates were administered to monkeys at higher dose levels without any additional findings. The final and most potent candidate, however, induced severe anaphylactoid reactions and bruising in all animals after the first low dose. Despite the different clinical signs, all three drug variants induced high serum levels of the inflammatory biomarkers C-reactive protein (CRP) and serum amyloid A (SAA). There were little-to-no changes in complement activation and only a slight increase in serum interleukin (IL-6). It is likely that the drugs activated liver Kupffer cells and macrophages eliciting an acute phase response. Animals were re-dosed with the third candidate, with or without a platelet-activating factor (PAF) blocker, which markedly reduced adverse symptoms, suggesting that PAF was mediating the anaphylactoid reactions, with PAF-mediated platelet activation potentially also playing a role. The third candidate was subsequently shown to stimulate PAF release from human blood cells in vitro, raising the concern that the adverse events observed in monkeys could translate to humans.

In summary, neither the acute phase response, platelet activation nor life-threatening anaphylactoid reactions were anticipated by in vitro studies with human cells. The mouse was also not a good translatable model since even the safest candidates that had good in vitro human safety profiles and which induced no clinical symptoms in NHPs, induced anaphylactoid reactions in mice. The acute phase response in NHPs was observed at low doses of even the least potent candidate, meaning that it would be very difficult to avoid such effects in humans with this series. Such pro-inflammatory responses would be unacceptable in autoimmune disease patients with an already activated immune system. Since the safety margins were considered unacceptable, all candidates were terminated before clinical trials and alternative drug formats were considered.

**BIO-9**

BIO-9 is a high affinity, antagonistic, human IgG1/λ mAb directed against a target involved in transmitting activation, proliferation, and survival signals to lymphocyte populations via the JAK/STAT signaling pathway. BIO-9-mediated blockade of lymphokine signaling results in down-modulation of lymphocytes, including T cells, natural killer (NK) cells and potentially also B cells. Genetic mutations of the target result in an immunodeficiency phenotype in man, mouse, dog, and pig, although with species differences in terms of immune cell population or function affected. Using a combination of two rat anti-mouse surrogate mAbs specific for the same target as BIO-9, the prolongation of murine islet allograft survival and reduced development of spontaneous autoimmune diabetes in the NOD mouse was demonstrated. A mouse surrogate antibody also dose-dependently prolonged mouse heterotopic heart allograft survival and prolonged survival in an allogeneic mouse graft-versus-host disease (GvHD) model. Mechanistically, the blockade of the target reduced the levels of multiple
pro-inflammatory cytokines in the GvHD model that were associated with tissue destruction secondary to T-cell activation, such as tumor necrosis factor (TNF), interferon gamma (IFN-γ) and IL-6. This data indicated the benefits of neutralizing the target of BIO-9 in conditions where multiple cytokines are involved in T cell-mediated disease pathophysiology (e.g., transplantation, GvHD).

For safety assessment, the cynomolgus monkey was considered the only pharmacologically relevant species since BIO-9 was only cross-reactive and pharmacologically active in this species. The relative affinities of BIO-9 for human and cynomolgus monkey target were similar. Further pharmacology studies in human and cynomolgus whole blood and Mixed Lymphocyte Reaction (MLR) confirmed the comparable potent inhibitory activity of BIO-9 on lymphokine signals.

BIO-9 was first evaluated in a 4-week dose-range finding (DRF) toxicity study including a single PK/PD arm in cynomolgus monkey. BIO-9 was well-tolerated and the effects observed were in line with the anticipated immunomodulation. Noteworthily, slight inflammatory and degenerative changes together with few cytomegalic cells in the heart and lungs of treated animals highlighted a viral reactivation. In a 13-week study, viral reactivations and neo-infections were the dominating themes; episodes of hyperthermia and adverse inflammatory and degenerative lesions of viral origin led to unscheduled sacrifices. As in the 4-week study, changes related to the primary pharmacology of BIO-9 (upon full receptor occupancy) included dose-related decreases in blood lymphocyte populations (T, B, and NK), decreased cellularity of the lymphoid follicles with absent or poorly developed germinal centers in the lymphoid organs and lymphoid atrophy in the thymus. Consequently, lower or completely inhibited primary T-cell-dependent antibody responses (TDAR) following immunization with tetanus toxoid or keyhole limpet hemocyanin (KLH) were observed. The most interesting, completely unexpected finding in this 13-week study was the T-cell hypercellularity (reactive lymphocytosis) observed in the lymph nodes despite maximum target engagement/occupancy and all the downstream pharmacological effects described in the blood, tissues, and consequently on the TDAR. Immunohistochemistry and immunophenotyping on the lymph nodes confirmed higher numbers of CD8+ and CD4+ T cells. These changes were unexpected given the anticipated inhibitory effect on BIO-9 on T cell development, homeostasis, proliferation, and survival. Gene expression profiling was undertaken to investigate which compensatory mechanism could possibly re-trigger an activation of the CD4+ and CD8+ T cell populations. A dose-dependent up-regulation of interferon responsive genes was evidenced, which was initially thought to be linked to viral infections. Indeed, viral products are able to activate innate immune cytoplasmic receptors to induce expression of interferon type I responses. Type I interferon signaling can then drive IFN-γ up-regulation, and therefore trigger type II interferon responses. The latter is unexpected since BIO-9 should inhibit the activation and proliferation of IFN-γ-producing CD8+ and NK cells. Shortly after the 13-week study, BIO-9 failed at prolonging renal allograft survival in cynomolgus monkey post-transplantation. The lymph nodes of the two transplanted monkeys showed T cell hypercellularity together with the up-regulation of interferon type I and type II genes, even though there were no signs of active viral infections.

In summary, this example highlights the fact that the pharmacology of BIO-9 is different between mouse and monkey, which is related to species-specificity of target biology. Upon antigenic stress (virus or graft), which was present only in monkey studies, the IFNγ signaling pathway (inhibited in mice) is induced, and this activation can bypass the blockade of the target by BIO-9. The clinical, pathological, and immunological characteristics of many infectious diseases in macaques are similar to those of humans. As far as transplantation is concerned, the monkey is a relevant model for human. Since viral (CMV) reactivation, as observed in the 13-week study, is a substantial clinical challenge in organ transplant recipients, there is the real risk that BIO-9 could activate interferon signaling in humans in response to viral infection and hence development of BIO-9 and the target program were terminated.

**Discussion**

An improved understanding of the pathways driving disease pathogenesis has in some cases led to a departure from traditional, chemically-synthesized small molecule drugs, also known as new chemical entities (NCEs), to development of large molecule protein-based therapeutics produced in living cells. These new biological entities (NBEs), such as mAbs, have higher potency and specificity for their targets, many of which are not amenable to NCE modulation, and are improving the quality of lives of millions of patients. For example, anti-TNF mAbs have an established role in the management of refractory RA, Crohn’s disease and psoriasis, and the immune checkpoint inhibitor mAbs are bringing hope to refractory cancer patients. The safety of mAbs is almost exclusively driven by the highly specific pharmacology/MoA of the drugs, and since mAbs comprise natural amino acids and have exquisite affinity and specificity for thier targets, off-target toxicity, as frequently observed with NCEs, is rare. In order to give the best chance of predicting potential adverse toxicity in humans, it is imperative that pharmacologically relevant animal species are used, that is, species in which the mAb binds to the target and has a similar pharmacological effect as that predicted in humans. Due to the highly human-specific nature of mAbs, in many cases the NHP is the only pharmacologically relevant species. In most cases, this is the cynomolgus or another macaque. Where a second species (e.g., rodent) is relevant, then ICHS6(R1) recommends longer-term studies in the rodent (and not the NHP), provided toxicity is equivalent in short-term studies. However, the often higher immunogenicity in rodents may preclude their use for longer-term studies. The importance of confirming the pharmacological relevance of the toxicology species for safety testing was highlighted by the severe adverse effects observed in humans with the anti-CD28 superagonist mAb TGN-1412, which was not predicted in NHP studies since the biology of the target had not been sufficiently characterized prior to clinical testing. Meanwhile, it is now known that CD28 is not expressed on effector memory T cells in cynomolgus monkeys as it is in humans, and hence TGN1412 does not cause widespread T-cell activation in NHPs as it does in humans.
A number of recent papers⁷–⁹, 22–24 have questioned the value of NHPs for non-clinical safety assessment of drugs. Specifically for mAbs, it was argued that almost all adverse events are highly predictable because they were either mediated by the pharmacology of the drug, which could be exaggerated by dose or exposure, or caused by immune responses to the drug.⁹ The authors argue that data from in silico and in vitro studies, as well as data from KO/KI rodents can predict the majority of adverse events with mAbs, making NHPs redundant for routine toxicology assessment. It is also argued that the immunogenicity of humanized/human mAbs in NHPs in many cases compromises a thorough safety assessment.⁷,⁸

It is indeed true that, for standard IgG-based mAbs against targets whose pharmacologic effects and safety profile have already been well-characterized in animals and humans (e.g., next-generation "me-too"), biobetter or biosimilar mAbs against soluble cytokines (e.g., TNF, IL-6, IL-17) or anti-CD20 B cell-depleting mAbs), extensive non-clinical testing programs in NHPs are unlikely to bring any added value for human risk assessment. In these cases, truncated non-clinical safety programs (fewer studies, fewer dose groups, fewer animals) with greatly reduced NHP usage should be proposed.¹⁴,⁶⁹ Indeed, alternatives to animal testing such as in vitro human cell/tissue models and the use of optimized/reduced study designs for biologics are continually being evaluated. While alternative approaches such as in silico models, tissue organoids, and organ-on-a-chip hold potential promise, the translational value of these is currently unknown, and this is especially true for biopharmaceuticals. Toxicologists should continue to question the value of general, reproductive and juvenile toxicology studies in NHPs and other species with biologics if there is no perceived value to human risk assessment (dependent on the target, exposure, pharmacological effects, and indication). Further opportunities exist for the industry to engage with regulators to revise or reduce regulatory expectations to try and achieve an optimal balance between generating scientifically relevant and potentially human translatable data whilst protecting animal well-being and promoting a reduction in animal use.

However, for mAbs against novel targets or with novel MoAs, as well as for novel mAb scaffold and structures (e.g., bi/tri-functional mAb and other constructs), where much of the pharmacology is unknown and therefore is unpredictable, assessing the safety impact of modulating the pharmacology in NHPs (provided pharmacological relevance to human has been clearly demonstrated) can be critical. The case studies highlighted herein include mAbs specific for novel targets (receptors, growth factors, and cytokines), conventional mAbs and novel mAb-related scaffolds, as well as novel routes of administration where much less is known. The pharmacology of these molecules/targets had not been well-characterized previously, with little human precedent, and so the potential adverse events were less predictable. These case studies have demonstrated that NHPs have an important role in human risk assessment of novel mAbs.

Some of the cases highlighted that, although a target/pathway had a number of safety liabilities predicted from knowledge of the target biology, in vitro data, KO mouse data and other sources, a mAb could still be administered safely to advance into clinical trials with the hope of bringing benefit to patients. In the absence of the data from animal safety testing, these drugs would not have been pursued. The animal studies were useful in defining safe starting dose levels and dose frequency, as well as informing the clinical safety monitoring programs. Although KO mice can highlight potential toxicities for further investigation, they may over-predict human toxicity since the target is completely missing in these mice and in many cases during the entire life of the animals (including during development). It is also possible that the KO animals might under-predict human risks (e.g., anti-FGF19 mAb, BIO-7), since KO animals develop without target, and may in some cases develop compensatory pathways that are not present in adult humans. As such, they may not accurately reflect the risk of administering a mAb to an adult human, where any role of the target in development will not be manifested; there are physiological differences in pathways between rodents and humans; and the mAb dosing regimen may not result in complete target blockade in all tissues all of the time. In fact, Bugelski and Martin²²,²３ showed that KO mice poorly predict the PD activity of mAbs (those approved for human use) compared to mAb administration to rodent or NHP, which did closely recapitulate the observed human pharmacology. For BIO-5, the mAb was shown to cause catastrophic effects on the pregnant mother through the inhibition of the previously unknown effect of the cytokine on parturition. This was not observed in KO mice nor in mice treated with a surrogate mAb against the mouse cytokine. Increased abortions in NHPs but not mice have previously been observed with an IL-4Ra fusion protein (inhibits IL-4) targeting a different pathway from BIO-5.⁷⁰ For BIO-9, lymphocyte activation and related toxicity was not observed in KO mice (nor in mice treated with an anti-mouse target mAb). It was only observed in NHPs upon antigenic stress (virus or graft), which induced the IFNγ signaling pathway (inhibited in mice) that bypassed the blockade of the target by BIO-9. These cases highlight the potentially different physiologies between rodents and NHPs for certain pathways.

For the anti-DLL-4 case (BIO-2), although the majority of the observed toxicities were related to the pharmacology of the mAb, NHPs and rodents were critical in the effort to identify molecules with a better therapeutic window (better balance between efficacy and safety) through PK modulation (shorter half-life and lower exposure), although ultimately new human-relevant toxicities were observed and the pathway was no longer pursued.

Other cases highlighted that some pathways, molecules and routes of administration (e.g., intravitreal for BIO-13) had safety liabilities that could be a risk to humans and the NHP data either supported termination of the drug, termination of the target program or identified specific safety and PD biomarkers for monitoring in humans. In the case of the molecules targeting immune receptors (BIO-8), in vitro studies did not highlight the pro-inflammatory and the potentially human-translatable pharmacology-driven serious safety risks associated with these molecules, which was only evident following administration to a whole animal. This case study highlighted how rational drug design, coupled with a thorough physicochemical and pharmacological in vitro and in silico characterization, should always precede hypothesis-driven confirmatory studies using as few animals as possible to meet the study objective.
Only 9 NHPs were used to assess the safety of 3 different candidate drugs and to ultimately highlight the variable but unacceptable safety risk to humans of all 3 candidates. Indeed, many other versions of BIO-8 were terminated prior to NHP work based on the human in vitro safety data.

In none of the case studies described herein did the immunogenicity of the drug compromise the overall safety assessment. Although mAbs are frequently immunogenic in NHPs, and indeed in some studies the majority of NHPs might generate ADA, it is relatively rare for the ADA response to reduce the exposure and pharmacological activity to such an extent in the majority of the animals as to compromise the safety assessment. Full human relevant exposure/PD can usually be maintained in the majority of the animals, sometimes requiring optimization of the dose levels and regimen. Adverse effects due to ADA (e.g., drug/ADA immune complex-mediated type III hypersensitivity reactions), although they are sometimes observed, are infrequent in NHP studies with proteins and rarely to an extent that the study is invalidated. It is generally accepted that the immunogenicity of biologics in animals does not predict immunogenicity in humans, and Type III hypersensitivity reactions observed in animals rarely translate to similar effects in humans. However, in some cases, the effect of ADA on the pharmacology of a mAb can provide an indication of potential safety consequences in humans if ADA were generated to the mAb. This could be ADA binding to receptor-bound mAb, causing cross-linking and altered pharmacology (e.g., agonism instead of antagonism) or cell death (e.g., by ADCC following ADA engagement of FcγR on effector cells).

BIO-16 showed ADA-mediated inflammatory responses that could potentially be induced in humans and the programs were stopped. These would not have been detected in vitro. Although the immune response is likely to be lower in humans than in animals, most biologics are immunogenic in humans to some degree, and so if ADA represents a safety risk, there is the possibility that these toxicities could be observed in humans. The intra-vitreal route was highlighted as a particular concern for ADA generation, at least for one mAb candidate (BIO-16), since it led to severe inflammation.

Despite the value of NHPs for safety assessment highlighted in these cases, it is also important to recognize the limitations of studies in NHPs. Apart from the fundamental difference in expression/biology of certain biological pathways, toxicity studies use normal animals with often low basal levels of target expression and function, target cell numbers, and baseline activation status, compared to the higher levels in disease. For example, inflammatory disease and cancer patients have increased levels of inflammatory cytokines and checkpoint inhibitor expression, respectively (with associated altered balance of activated autopathogenic T-cells and tumor-specific effector and regulatory T-cells), than those observed in non-diseased humans and animals. These differences not only present a challenge to defining the dose/response relationship in NHPs since there is often no biomarker of activity that can be used to build a human PK/PD model, but also affects the ability to assess the potential for human-relevant toxicity. In a number of the case studies, the level/function of the target in normal animals was equivalent to that in patients, perhaps increasing the predictive value of the NHPs. However, in cases where there are low levels of target expression/function in normal animals, it is important, where possible, to try to improve the translatability of the NHPs and supplement with safety and PK/PD data from efficacy studies in animal models (where target expression may be higher and more comparable to the clinical situation). Since there are very few qualified and translatable animal models in NHPs (meaning that combining safety and efficacy readouts in a single study is rarely possible), for biologics that are only cross-reactive with NHPs, rodent disease models using surrogate mAbs against the rodent target are often utilized. Currently, a combination of in vitro and in vivo pharmacology and toxicology studies in multiple test systems, sometimes with surrogate mAbs, is often required to understand the relationship between dose/exposure and pharmacology/toxicology that allows translational modeling of safe human dose levels. It is important, where possible, to develop challenging but critical in vivo activity and safety markers using a range of genomic, proteomic, and immunological assays in NHPs and humans to increase the chance of detecting early signs of toxicity and to allow comparison between non-clinical and clinical data.

Despite these limitations of toxicology studies in healthy NHPs, they have been shown to be good predictors of pharmacology in humans, and so should be able to identify the majority of potentially severe AEs induced in the majority of dosed subjects (i.e., overt pharmacology-based hazards) provided the pharmacological relevance of the NHP is confirmed. However, NHPs are poor predictors of some of the infrequent/rare downstream sequelae of biologics pharmacology, such as infusion reactions, cytokine release by certain classes of compounds, infection risk, progressive multifocal leukoencephalopathy, autoimmunity and cancer, many of which are governed by patient- (e.g., genetic variants) and disease-specific factors and other factors (e.g., co-medication) that cannot be addressed in any animal study and can only be explored in large clinical trials. However, some of these events are relatively rare and others (e.g., headache, pain, fatigue, nausea) are subjective endpoints not measurable in NHPs, and can never realistically be assessed in non-clinical studies with small numbers of animals or human cells. Hence, risk mitigation for these effects will also rely on a weight-of-evidence approach to benefit-risk assessment and careful clinical risk management based on the unique characteristics of the product, the target, and patient population to be treated.

**Conclusions**

NHP use in drug development of biopharmaceuticals is currently increasing. This is due not only to an increased number of novel multifunctional drugs in development with a perceived increased safety risk requiring rigorous assessment, but also due to conservatism on the part of toxicologists, company management and Health Authorities. This should continue to be challenged. Continuing dialogue with company management, regulatory affairs colleagues, and Health Authority reviewers should have the goal of promoting the understanding that NHP studies should not be performed as a default to satisfy a standard development and regulatory path, but should use rational, science-based decision-making in the ethical and...
scientific use of NHPs based upon the specific attributes of the product. When this is practiced, then safety assessment studies in NHPs can provide critical information for human risk assessment and safe guarding of participants in clinical trials.

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No potential conflicts of interest were disclosed.

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