Review Article

Theme: Critical Considerations for Design and Development of Antibody Drug Conjugates

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Antibody Drug Conjugates: Nonclinical Safety Considerations

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Received 26 February 2015; accepted 14 May 2015; published online 30 May 2015

Abstract. Antibody drug conjugates (ADCs) are biopharmaceutical molecules consisting of a cytotoxic small molecule covalently linked to a targeted protein carrier *via* a stable cleavable or noncleavable linker. The process of conjugation yields a highly complex molecule with biochemical properties that are distinct from those of the unconjugated components. The impact of these biochemical differences on the safety and pharmacokinetic (PK) profile of the conjugate must be considered when determining the types of nonclinical safety studies required to support clinical development of ADCs. The hybrid nature of ADCs highlights the need for a science-based approach to safety assessment that incorporates relevant aspects of small and large molecule testing paradigms. This thinking is reflected in current regulatory guidelines, where sections pertaining to conjugates allow for a flexible approach to nonclinical safety testing. The aim of this article is to review regulatory expectations regarding early assessment of nonclinical safety considerations and discuss how recent advances in our understanding of ADC-mediated toxicity can be used to guide the types of nonclinical safety studies needed to support ADC clinical development. The review will also explore nonclinical testing strategies that can be used to streamline ADC development by assessing the safety and efficacy of next generation ADC constructs using a rodent screen approach.

KEY WORDS: antibody drug conjugates; regulatory guidance; safety assessment; therapeutic index

INTRODUCTION

Antibody drug conjugates (ADCs) are emerging as a promising class of biopharmaceutical anti-cancer agents following the recent approvals of Kadcyla® and Adcetris® (1). From the nonclinical safety perspective, ADCs present unique challenges to standard toxicology testing due to the complex nature of the conjugate, which consists of both small and large molecule components. Specifically, the process of conjugating a highly potent cytotoxic small molecule (warhead) to a highly targeted monoclonal antibody (mAb) yields a hybrid molecule with a toxicity profile that is distinct from that of the individual components. This necessitates a science-driven approach to safety assessment that considers the unique biochemical properties of the conjugate when determining the types of nonclinical studies needed to support first in human trials with ADCs. The need for an adaptive approach is reflected in current regulatory guidelines—International Conference on Harmonization (ICH)S6(R1) and

ICH S9—where sections pertaining to conjugates allow for a flexible approach to nonclinical safety testing (2,3). The primary aim of this article is to review regulatory expectations regarding early assessment of nonclinical safety considerations and discuss how recent advances in our understanding of ADC behavior and mechanisms of toxicity can be used to guide the types of nonclinical safety studies needed to support ADC clinical development. The topics discussed in this review reflect a science-based approach to the interpretation of existing relevant regulatory guidelines and available literature and is not meant to provide a blueprint for nonclinical safety assessment of ADCs. Additional details pertaining to the design and conduct of nonclinical programs to support clinical development of ADCs can be found in a recent comprehensive industry white paper on this topic (4).

In addition to the nonclinical safety evaluation of clinical candidate ADCs, there is also a growing need within the industry to evaluate the safety of rapidly evolving ADC technologies early in the discovery process. Recent engineering efforts to improve the therapeutic index (TI) of next generation ADCs have resulted in an explosion of new technologies with the potential to significantly impact safety and pharmacokinetic (PK). Thus as a secondary aim, this

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paper will discuss screening strategies that can be used to streamline safety evaluation of novel ADC technologies.

UNIQUE CHARACTERISTICS OF ADCS THAT IMPACT SAFETY

ADCs combine three components to create a complex molecule consisting of a potent cytotoxic molecule chemically linked to a tumor-targeting monoclonal antibody *via* a cleavable or noncleavable linker. As described above, the conjugated form of the antibody has unique biochemical properties that significantly alter the safety profile of the conjugate compared to the individual components (Table I).

One of the unique characteristics of ADCs is the PK profile; conjugation significantly impacts half-life, clearance, elimination, and biodistribution of the unconjugated components (5). Elimination of unconjugated antibodies involves catabolic breakdown and/or target-mediated clearance pathways while small molecule warheads are typically cleared through hepatic and/or renal pathways (6). In contrast, elimination of an ADC involves properties of both large and small molecules. The conjugate must first be broken down by catabolism to yield ADC catabolites or undergo deconjugation to yield naked antibody and warhead components (5). Once broken down, the antibody is degraded into amino acids for recycling while the warhead undergoes renal and/or hepatic-mediated clearance. This difference in elimination also impacts half-life of the ADC. While the half-life of an unconjugated monoclonal antibody typically ranges from 1 to 3 weeks (7), the half-life of an ADC is generally only 2 to 5 days (8). This effect on half-life is thought to be related to the overall stability of the conjugate (9). This is supported by the observation that trastuzumab conjugated to DM1 *via* a cleavable linker was cleared approximately twofold faster than trastuzumab employing a more stable noncleavable linker (10). The impact of conjugation on the PK profile of the warhead is even greater; conjugation can extend the half-life of a warhead from hours to days (5,11). Biodistribution of the unconjugated components is also significantly impacted by conjugation (5). Antibodies are largely restricted to plasma space with slow diffusion into tissues (12,13) while small molecule warheads generally have a much higher volume of distribution depending on chemical properties of the molecule. The conjugation process limits warhead to antibody-restricted plasma space and target-expressing cells (14,15). This change in biodistribution can change the toxicity profile of the warhead by altering distribution of the cytotoxic

agent to tissues in which antibodies are known to accumulate—*i.e.*, liver, lungs, kidney, and skin—and to normal tissues expressing the tumor target (16,17).

Another key difference between ADCs and therapeutic mAbs is the antibody itself. While both ADCs and therapeutic mAbs display high selectivity for the target, antibodies used as part of an ADC are typically selected for their ability to undergo rapid internalization in order to elicit tumor cell-specific killing (18). Therapeutic mAbs, however, are selected based on inherent effector function, *i.e.*, blocking/agonist and/or immune-modulating activity (19).

MECHANISMS OF TOXICITY

The concept of on and off-target toxicity—*i.e.*, target-dependent and target-independent toxicity—is central to our understanding of ADC-mediated toxicity (Table II) and should be considered when deciding on the types of nonclinical studies that will provide the best dataset to evaluate ADC safety.

On-Target Toxicity

The ideal ADC target is highly expressed on tumor cells and minimally expressed or absent on normal cells. However, in reality, nearly all target proteins have some degree of normal tissue expression, which can be a major obstacle to ADC development (20). A well-known example of on-target toxicity is bivatuzumab mertansine, an anti-human CD44v6 antibody conjugated to DM1 (21). Immunohistochemistry (IHC) studies demonstrated CD44v6 staining in keratinocytes and epithelial cells in the cornea and tonsils (22). The initial phase 1 trial with bivatuzumab mertansine was halted following observations of dose-dependent skin toxicity, ending in a fatal case of toxic epidermal necrolysis (21). This finding—characterized by keratinocyte apoptosis and desquamation—was thought to be related to CD44v6 expression in the skin; other DM1 ADCs have not shown similar toxicities in the clinic (23). However, the potential for on-target toxicity can be difficult to predict based on expression patterns alone (Table III). In the case of Kadcyla® (T-DM1), the major dose-limiting toxicities observed in clinical trials were thrombocytopenia and elevated liver enzymes despite significant target expression on cardiac myocytes and epithelial cells in GI tract, lung, skin, and breast (40–42). It is clear from this example that on-target toxicity is not driven solely by target expression and that other factors—*e.g.*, proliferative/

Table I. Unique Characteristics of ADCs vs. Unconjugated Components

	ADCs	Warhead	Antibodies
Half-life	Intermediate half-life	Rapid half-life	Prolonged half-life
Clearance	Catabolism/target-mediated clearance of mAb; hepatic and/or renal elimination of warhead	Hepatic and/or renal elimination	Catabolism/target-mediated clearance
Biodistribution	Plasma volume	Variable	Plasma volume
Dosing schedule	Intermittent	Intermittent	Maintain steady state
Target interaction	High affinity; rapid internalization	N/A	High affinity; blocking or agonist activity

ADCs Antibody drug conjugates, N/A not available

Table II. Mechanisms of ADC Toxicity

	Mechanism(s)	Safety assessment
On-target	Binding/internalization of ADC in target-expressing normal cells	<i>In vivo</i> toxicity must be evaluated in crossreactive species; requires understanding of normal tissue target expression
Off-target	Instability of conjugate; nonspecific uptake into normal cells (<i>i.e.</i> , Fcγ receptors, FcRn binding, pinocytosis, <i>etc.</i>); nonspecific binding of antibody to normal cells	Off-target toxicity can be evaluated in non cross-reactive species

Table III. Examples of ADC-Mediated On- and Off-Target Toxicity in the Clinic

ADC	Target	Warhead	Normal tissue expression	Dose-limiting toxicities
AGS-16M8F	ENPP3	MMAF	GI tract (glandular cells), lung (respiratory epithelial cells), kidney tubules	Thrombocytopenia ^(24, 25)
ASG-5ME	SLC44A4	MMAE	Apical expression in polarized cells in kidney and GI tract	Neutropenia; GI toxicity; neuropathy ⁽²⁶⁾
AVE9633	CD33	DM4	BM hematopoietic cells lymphocytes, GI tract (epithelial and glandular)	Hepatotoxicity ⁽²⁷⁾
BT-062	CD138	DM4	B cells, epithelial cells in GI tract, liver, skin, kidney, pancreas, and prostate	Hand/foot syndrome; mucositis ^(28, 29)
CDX-011	GPNMB	MMAE	Melanocytes, osteoblasts, retinal epithelial cells	Rash (hand/foot and TEN); neutropenia; peripheral neuropathy ⁽³⁰⁾
CMC544	CD22	Calicheamicin	B cells	Thrombocytopenia, lymphopenia, neutropenia ⁽³¹⁾
DCDT2980S	CD22	MMAE	B cells, GI tract (glandular cells)	Neutropenia; sensory neuropathy ⁽³²⁾
IMGN242	MUC1 (CanAg)	DM4	Mutated form of MUC1 on tumor	Ocular toxicity (keratitis) ⁽³³⁾
IMGN388	αv integrin	DM4	GI tract (epithelial cells), bile duct, melanocytes, kidney tubules/glomeruli	GI toxicity, headache, confusion ⁽³⁴⁾
IMGN901	CD56	DM1	Peripheral nerves, neurons (cerebellum), lung (epithelial cells), glandular cells in GI, thyroid, and adrenal	Peripheral neuropathy ⁽³⁵⁾
PSMA ADC	PSMA	MMAE	Kidney (proximal tubules) and prostate	Neutropenia, peripheral neuropathy ⁽³⁶⁾
SAR3419	CD19	DM4	B cells	Ocular toxicity (keratitis) ⁽³⁷⁾
SGN-35	CD30	MMAE	Activated lymphocytes	Neutropenia; thrombocytopenia; elevated liver enzymes ⁽³⁸⁾
SGN-75	CD70	MMAF	B cells and some T cells	Neutropenia ⁽³⁹⁾
T-DM1	HER2	DM1	Cardiac myocytes, epithelial cells in GI tract, lung, skin, and breast	Thrombocytopenia, elevated liver enzymes ⁽⁴⁵⁻⁴⁷⁾

Dose-limiting toxicities coded blue indicate findings in organs that express target (*i.e.*, on-target); those coded red indicate findings in organs/cells that do not express target (*i.e.*, off-target)

BM bone marrow, LFT liver function tests, GI gastrointestinal

regenerative potential of target cell/organ, mechanism/potency of warhead, and accessibility of the ADC to the target cell—could play a role.

Off-Target Toxicity

Off-target toxicity refers to adverse findings in organs/cells that do not express target. These toxicities are generally related to the safety profile of the free warhead and are common across a wide range of target antigens (referenced in Tables III and IV). Off-target toxicity is a major cause of dose-limiting toxicity (DLT) for ADCs in clinical development (43,44). The maximum tolerated dose (MTD) for most ADCs conjugated to microtubule inhibitors has ranged from 2 to 5 mg/kg irrespective of target expression (7). Therefore, there is a great desire to improve TI of next generation ADCs, which can only be accomplished through greater understanding of the underlying mechanisms of off-target toxicity. The two major mechanisms of off-target toxicity are thought to be related to (1) conjugate stability and (2) nonspecific uptake into normal cells.

Stability

Instability of the conjugate—*i.e.*, inappropriate release of the warhead outside target cells—leads to off-target toxicity by increasing systemic exposure to free warhead. Two of the major factors contributing to ADC stability are (1) choice of linker and (2) conjugation chemistry used to attach the warhead to the antibody (45).

Linker instability has been cited as a primary cause of failure of first-generation ADCs—including Mylotarg®, the first marketed ADC (46). The ideal linker should be sufficiently stable to deliver the ADC to the tumor site, yet labile enough to rapidly release the warhead inside the cell. Current-generation linkers are classified as either cleavable or noncleavable (45). The majority of cleavable linkers are designed to be selectively cleaved by enzymes in a low-pH environment such as a lysosome (47). This approach has been clinically validated by Adcetris®, which uses a cleavable dipeptide linker that undergoes cathepsin B cleavage within the lysosome (48). Noncleavable linkers must undergo

catabolic breakdown within the cell to release warhead. While these linkers have generally shown improved safety profiles compared to cleavable linkers, the overall impact on TI is variable as noncleavable ADCs often have less activity in tumor types with heterogenous target expression that require bystander effect (49). Presently, the relationship between linker choice/tumor type/efficacy/safety is not well understood; the optimal linker-warhead combination must be empirically tested for each ADC.

Another factor impacting stability is the type of conjugation chemistry used to attach the warhead linker to the mAb. The most common approach to conjugation has been to nonselectively attach drugs to cysteine or lysine residues in the antibody *via* a reactive maleimide group (50). This process, also known as stochastic conjugation, produces a heterogenous mixture of ADCs with drug-antibody ratios (DARs) ranging from 0 to 8 (51). Heterogeneous DAR is thought to limit the TI of stochastic ADCs. Experiments with purified DAR species have demonstrated that higher DAR ADCs are more toxic than lower DAR ADCs at equivalent doses of antibody (52). This effect is thought to be related to faster clearance of higher DAR species. Recent advances in conjugation chemistry have led to the production of ADCs with homogeneous drug loading and improved stability (53). These technologies use nonnatural amino acids, engineered cysteines, or transglutaminases to attach the warhead linker to specific sites on the antibody (54,55). The first published data using site-specific conjugation—*i.e.*, THIOMAB technology—to generate homogenous DAR2 conjugates demonstrated significant improvements in TI compared to stochastically conjugated DAR4 ADCs (56). These site-specific ADCs were less toxic in rats and monkeys while maintaining equivalent activity in tumor xenograft models. The improvements in TI were associated with slower clearance of the ADC and decreased deconjugation secondary to maleimide exchange (57). The first known ADC using site-specific technology in the clinic is SGN-CD33A, an anti-CD33 antibody conjugated to highly potent pyrrolbenzodiazepine dimers *via* engineered cysteines (58). However, until clinical safety data is available, it remains unknown whether the improved TI with site-specific technology will translate to the clinic.

Table IV. Common Dose-Limiting Toxicities of ADCs

Dose-limiting toxicity	Warhead				
	MMAF	MMAE	Calich	DM1	DM4
Neutropenia	✓	✓	✓	✓	
Thrombocytopenia	✓	✓	✓	✓	
Peripheral neuropathy		✓		✓	
Elevated liver enzymes		✓		✓	✓
Grade 3/4 skin rash		✓	✓		
Ocular toxicity					✓
Grade 3/4 GI toxicity		✓			✓
Generalized symptoms				✓	✓
Headache, confusion, fatigue					
Lymphopenia			✓		

Common dose-limiting toxicities identified from ADCs listed in Table III

MMAF monomethyl auristatin phenylalanine, MMAE monomethyl auristatin E, Calich calicheamicin, DM1 maytansine DM1, DM4 maytansine DM4

Target-Independent Uptake into Normal Cells

While target-independent uptake of ADCs into normal cells is thought to be a major component of off-target toxicity, this is the least understood mechanism of ADC-mediated toxicity. Antibodies are known to be taken up by normal cells in an antigen-independent manner by various mechanisms—including Fc receptor-mediated uptake (e.g., mannose receptors, FcRn, and FcγR receptors), nonspecific endocytosis, and/or uptake of ADC catabolites (40,59). Theoretically, nonspecific uptake could lead to intracellular trafficking of the ADC through endosomal or lysosomal pathways, followed by release of the cytotoxic warhead inside normal cells. However, there is very little data to assess the impact of antigen-independent uptake on the overall off-target toxicity profile of ADCs. The most extensive data set to support this hypothesis is derived from a research study conducted to investigate the mechanism of T-DM1-mediated thrombocytopenia (60). This study was initiated following observations of a high incidence of dose-limiting thrombocytopenia in T-DM1 patients despite lack of target expression on platelets or platelet precursors (42,61). Preliminary *in vitro* studies with platelet-rich plasma showed that T-DM1 does not have a direct effect on platelets. However, follow-up studies with megakaryocyte cultures demonstrated surface binding and internalization of Alexa488-conjugated T-DM1 in platelet precursors. FcRγIIB appears to play a role in nonspecific uptake as preincubation with an anti-CD32 mAb decreased internalization of Alexa488-conjugated T-DM1 by approximately twofold. However, Fcγ receptor blocking experiments did not completely prevent T-DM1 uptake by megakaryocytes, demonstrating that there are other nonFcγR mechanisms involved (60). From a safety assessment standpoint, more work is needed to understand normal cell biodistribution and how engineering changes could impact the off-target safety profile of ADCs.

CURRENT REGULATORY EXPECTATIONS FOR ADCS

There are no regulatory guidelines that specifically address nonclinical safety testing of ADCs. While a Q&A subsection pertaining to ADCs is planned for ICHS9, current guidance is limited to small subsections within ICHS6(R1) and S9 (2). The major principles addressed in these documents are species selection, stability (*in vitro* and *in vivo*), and relative importance of testing the conjugated vs. unconjugated material (outlined in Table V).

Species Selection

The principles of species selection are outlined in ICHS6(R1) (3). In general, species selection for a conjugated antibody should use the same approach as an unconjugated antibody—i.e., toxicity studies should only be conducted in relevant species with antibody cross-reactivity. As with most therapeutic mAbs, the high-affinity interaction between the ADC and its target usually precludes rodent cross-reactivity. In the situation where the rodent is not a relevant species, safety evaluation can be limited to a single nonrodent species—typically nonhuman primates due to high homology with the human target. In the case where the ADC does bind

target in both rodents and nonrodents, toxicology studies must be conducted in two species (note 2). One aspect of species selection that is not covered by current guidelines is the utility of safety data generated in a nonrelevant species. For unconjugated mAbs, studies conducted in nonrelevant species—i.e., no target cross-reactivity—are considered misleading and are discouraged (3). This is due to the fact that most toxicities associated with therapeutic mAbs are related to excessive modulation of the primary pharmacological target (62). Therefore, in the absence of target engagement, toxicity studies with biopharmaceuticals do not produce any meaningful findings. In this scenario, other approaches—such as the minimum anticipated biological effect level—must be used to set the start dose (63). However, for ADCs, this argument does not hold true; the presence of a highly potent cytotoxic warhead produces significant toxicities that are unrelated to target binding (Table III). As a result, a toxicology conducted in a non cross-reactive species could provide relevant information as to the anticipated off-target toxicities and potentially be used to inform the clinical trial design and start dose (64).

Stability

As discussed previously, linker stability is one of the most important factors contributing to ADC safety (45). This is reflected in ICHS6(R1) and S9 guidelines, which state that stability and/or metabolic stability of ADCs should be addressed in human as well as test species plasma (2,3). While scientific best practices for evaluating ADC stability have yet to be established, *in vitro* stability is typically assessed by determining loss of drug-to-antibody ratio (DAR) in human and test species plasma at 37°C for 3 to 7 days duration (57). To date, specifications for ADC stability have not been established; therefore, from a regulatory perspective, the primary objective of these studies is to establish that the data obtained from repeat dose toxicology studies is representative of the potential human situation.

In addition to *in vitro* assays, ADC stability must be further evaluated *in vivo* by measuring plasma levels of ADCs and their unconjugated components in test species. While ICHS9 clearly indicates that toxicokinetic evaluation of ADCs should assess both the conjugated and the unconjugated compound(s), it does not specify which of the individual components (i.e., total mAb, warhead, and/or linker) must be measured (2). This suggests that regulators could potentially accept PK data from less than three analytes (e.g., ADC and warhead only) in the good laboratory practice (GLP) toxicology studies. The decision to assay for multiple unconjugated components is further complicated by the fact that it is very challenging from a resource perspective to develop and validate multiple bioanalytical assays early in nonclinical development. However, bioanalytical difficulties aside, *in vivo* stability is best evaluated by measuring at least total ADC, total mAb (conjugated and unconjugated), and warhead (cleavable ADC) ± linker (noncleavable ADC) in toxicology studies. This enables calculation of the deconjugation rate by comparing the concentration of total mAb to ADC in plasma (59). In addition, assaying for free warhead ± linker can further enhance understanding of

Table V. Regulatory Guidance Pertaining to Safety Assessment of Conjugated Products

	ICH6(R1) ^a	ICH9 ^b
Species selection	Test ADC in relevant species	Not addressed
ADC stability	Assess in animal and human plasma	Assess in animal and human plasma
ADC toxicity	Full safety evaluation	Full safety evaluation
Warhead toxicity	Treat as an NME ^c	"Limited" evaluation
Toxicokinetics	Not addressed	Assess conjugated and unconjugated forms

ICH International Conference on Harmonization, ADC antibody drug conjugates

^aSection 2.1 and note 2 of ICH6(R1)

^bSection 4.1 of ICH9

^cIf novel, treat as new molecular entity (NME). If previously tested, separate evaluation not necessary

conjugate instability by determining relative exposure to conjugated vs. free warhead. Therefore, the decision as to which components to should be included in the toxicokinetic analysis requires a weighted evaluation of the information needed to understand the nonclinical safety profile of ADCs.

Relative Importance of Testing Conjugated vs. Unconjugated Material

The only other principle specifically pertaining to the nonclinical safety assessment of ADCs in current guidelines is the relative importance of testing the conjugate vs. the unconjugated components in GLP toxicology studies. ICH6(R1) and ICH9 are both clear that the ADC should be the primary focus of the nonclinical safety evaluation. In addition, both guidelines agree that when the cytotoxic agent has been previously tested and there is a sufficient body of scientific information available, separate evaluation of the unconjugated warhead is not warranted (2,3). Where the two guidelines differ—is in regard to the safety evaluation of the unconjugated components (*i.e.*, unconjugated mAb and warhead). ICH6(R1) advises that nonclinical safety assessment of novel cytotoxic warheads should follow the approach used for novel molecular entities (NME), as per ICH9 recommendations. In contrast, ICH9 states that toxicity testing of unconjugated components can have a more "limited" evaluation. This difference is significant. As it reads, following ICH6(R1) would entail a full nonclinical toxicology package for a novel warhead that includes GLP toxicology studies in two species, safety pharmacology, genotoxicity assessment, and development and reproductive toxicology studies. From a regulatory perspective, it would seem likely that the recommendations in ICH9 should outweigh those in ICH6(R1) if the ADC is intended for use in patients with advanced cancer. This assumption is supported by marketing applications for Kadcyla® and Adcetris®, which did not contain a full nonclinical toxicology dataset for either of their warheads (65,66). Following ICH9 enables a flexible, science-based approach to safety testing of novel warheads that considers the unique properties of ADCs in the design of the nonclinical toxicology program.

The minimal data package of nonclinical safety studies needed to support an investigational new drug (IND) application for an ADC is outlined in Table VI.

Safety Assessment of the Conjugate

From a regulatory perspective, a GLP toxicology study with the ADC in a relevant species yields the primary data set used to inform the clinical start dose and identify potential human risks (2). As outlined in Table I of ICH 9, the duration of this study should be based on the intended dosing clinical schedule as outlined in (2). In general, a single-dose toxicity study with a 21-day observation period should be sufficient to support an every 3-week (Q3W) clinical dosing schedule unless there is a cause for concern (*e.g.*, cumulative toxicity after repeated dosing). Safety pharmacology of the conjugate should also be assessed by incorporating safety pharmacology endpoints into the pivotal GLP study. Regulatory guidelines are also clear that a thorough assessment of ADC stability should be completed prior to IND submission (2).

Safety Assessment of Novel Warheads

The main purpose of characterizing the toxicity profile of the warhead is to evaluate the worst case scenario in the event that the molecule is fully unstable. Under these circumstances, a single-dose toxicology study in a rodent is expected to characterize the safety profile of the unconjugated warhead. However, in reality, it is more likely that the warhead will undergo slow release from the conjugate by target-mediated clearance and/or instability of the linker with altered biodistribution than undergo immediate release (5). While it would be ideal to conduct a toxicology study that mimics free warhead exposure following administration of the ADC, this would be near impossible to achieve from a practical standpoint. Therefore, the most relevant safety data is derived from toxicology studies with the conjugate. In addition, toxicology studies to evaluate the warhead alone can be limited to a single rodent species (64), as potent cytotoxics have such strong primary pharmacological activity that there are usually minimal species differences in the toxicity profile of these agents (67,68). To complete the safety evaluation, the nonclinical package must also include a genotoxicity assessment of the novel warhead at the time of BLA submission.

Consideration must also be given to the need to conduct absorption, distribution, metabolism, and elimination (ADME) studies on novel cytotoxic warheads. While these studies are not required at the time of IND submission as per ICH9, it is often advantageous to conduct a small screening study to evaluate efflux substrate potential and metabolic stability of novel warheads. These types of studies can minimize the risk of

metabolic liabilities that could impact clinical development further down the line. As such, the decision to conduct ADME studies for novel warheads prior to first-in-human trials should be considered on a case-by-case basis.

Safety Assessment of the Monoclonal Antibody

Nonclinical safety evaluation of the unconjugated antibody is generally unwarranted. In theory, these studies could help evaluate the role of target biology in the safety profile of the conjugate. However, in practice, these studies are unlikely to yield decision-making data. As discussed earlier, mAbs used in ADCs are generally not selected for inherent effector function and are rather selected for their ability to undergo rapid internalization (18). In addition, ADCs are generally dosed on an intermittent schedule to allow for recovery from cytotoxic effects (19). In this scenario—*i.e.*, a rapidly internalized mAb dosed on a Q3W schedule—a toxicology study with the unconjugated mAb is unlikely to produce significant toxicity. Therefore, given that most mAbs are only cross-reactive in nonhuman primates (NHP), toxicology studies with the unconjugated antibody should generally be avoided unless there is a specific concern.

The studies outlined in Table VI will generate the safety data required to inform patients and clinicians about potential human safety risks of novel ADCs; however, they are not designed to address mechanistic questions related to ADC toxicity—*e.g.*, “what impact does conjugation site, DAR, warhead mechanism of action, and/or target expression have on safety?” These types of questions are best addressed by investigative studies that can be applied across a company’s ADC development platform and used to develop next-generation ADCs with improved TI.

SAFETY STRATEGIES TO ADVANCE ADC DEVELOPMENT

Early Assessment of On-Target Toxicity

The major obstacle to early assessment of on-target toxicity is lack of antibody cross-reactivity to the target in rodents. As described above, NHP are usually the sole relevant species for mAbs. However, due to ethical and financial considerations, NHP toxicology studies are typically conducted later in the discovery process, *i.e.*, after lead

candidate selection. This leads to a great deal of uncertainty as the molecule progresses through the pipeline. Current mitigation strategies to de-risk on-target toxicity early in the discovery process are highly reliant on immunohistochemistry (IHC). This often produces a “bottleneck” as IHC protocol optimization is time-consuming and slides must be read by a trained pathologist. In addition, as described above, it is difficult to predict on-target toxicities based on target expression alone (Table III). Other key factors such as—vital nature of organ/cell type, regenerative potential of cell, anatomical location (membrane *vs.* cytoplasm, apical *vs.* basal), mechanism of action of warhead, and differential expression of target in tumor *vs.* normal cells—should also be considered. For example, safety assessment of on-target toxicity should always take into account the warhead’s mechanism of action. Microtubule inhibitors—*e.g.*, DM1, DM4, MMAE, and MMAF—block mitosis of rapidly dividing cells by reversibly binding to microtubules (69). The reversible nature of this binding generally minimizes cytotoxicity to slowly dividing cells. Therefore, the proliferative index of the cell should be considered when evaluating the potential safety risks of a novel target—*i.e.*, if the normal cell is a slow proliferator, it is less of a concern for on-target toxicity. This assumption is supported by existing nonclinical and clinical data demonstrating that the safety profile of ADCs is largely driven by the mechanism of action of the warhead (Table IV) (40,70). It is unclear whether this relationship will hold true for novel warheads. There is a push within the field to use more potent warheads—*e.g.*, DNA inhibitors—that target cancer stem cells. These agents, which target both slowly and rapidly dividing cells (71), have greater potential for unpredictable on-target toxicity. For example, use of a DNA-damaging warhead significantly increased on-target toxicity in LGR5-expressing intestinal stem cells compared to a microtubule-inhibiting warhead (25). This potential for greater on-target toxicity necessitates use of more stringent criteria when selecting targets for highly potent ADCs as compared to first generation ADCs. Given the subjective nature of the current strategy, there is a clear need to develop better techniques to understand the impact of target expression in normal tissues. While rodent cross-reactive antibodies would enable early *in vivo* safety testing, generation of these molecules is often not feasible due to species differences in target homology (72). Other strategies would be to generate a

Table VI. Minimal Nonclinical Safety Package Required to Support IND and BLA Applications for ADCs

Study	Molecule	Study duration	IND	BLA
GLP toxicology in relevant species (incl. safety pharmacology endpoints)	ADC	Single or repeat dose ^a 3 months	✓	✓
GLP toxicology study in rat ^b	Warhead	Single dose	✓	
<i>In vitro</i> plasma stability	ADC	N/A	✓	
Tissue cross-reactivity	ADC	N/A	✓	
Genotoxicity battery ^b	Warhead			✓
Embryofetal development	ADC			✓ ^c

ADC antibody drug conjugates, BLA biologics license application, IND investigational new drug, GLP good laboratory practice

^a Refer to ICHS9 for additional details on study duration required to support IND applications

^b If warhead has been previously tested and sufficient body of scientific information is available, a separate evaluation is not warranted

^c If warhead is genotoxic and targets rapidly dividing cells, an embryofetal development toxicology study is not warranted (refer to ICHS9)

rodent surrogate or to conduct a small scale NHP study to evaluate potential on-target toxicity early in development. However, these approaches involve ethical and practical considerations that must be weighed accordingly.

Early Assessment of Novel ADC Technologies

The biggest promise of ADCs is the potential to improve the TI of highly potent cytotoxic warheads using a targeted approach to increase tumor cell delivery and decrease systemic exposure. However, despite recent advances in the field, TI remains a key challenge to ADC development. There are many ongoing efforts to use protein and chemical engineering to further improve the stability and TI of ADCs. While these efforts are gaining momentum from an engineering perspective, the inability to predict the impact of these new technologies—*i.e.*, novel conjugation chemistries, linkers, and warheads—on the safety and efficacy of ADCs continues to limit ADC development. Therefore, it is crucial to develop a strategic approach to candidate selection that enables rapid safety screening of new technologies early in the discovery process.

One such strategy is to use a rodent screening model to evaluate the impact of new technologies on the safety of novel ADC constructs. For the most part, new technologies—*e.g.*, novel conjugation chemistries, linkers, and/or protein carriers—are being developed with the intention to increase TI by improving off-target toxicity. Therefore, safety assessment does not require use of a cross-reactive species as the findings are target-independent. Using a rodent screening model offers a cost-effective approach to rapid screening of the *in vivo* impact of novel technologies. An example of this approach is highlighted in a recent publication where a single-dose rat toxicology study was used to evaluate the impact of different conjugation sites on the safety and PK of a site-specific ADC (55). For the most part, these studies should be conducted in parallel to *in vivo* efficacy studies to assess the overall impact on TI. While this is just one possible strategy that can be considered, increased reliance on rodent models can speed up development by reducing test article demand/study costs.

CONCLUSION

Regulatory guidelines specify that a full nonclinical safety evaluation of the conjugate should be conducted to support first-in-human clinical trials with ADCs. The pivotal GLP toxicology studies should be conducted in a relevant species to evaluate potential on- and off-target safety risks. This study should include toxicokinetic analysis of both the conjugated and unconjugated components to assess the impact of stability on safety. There should also be an understanding of target expression, which can be derived from the GLP TCR study or available IHC data. Safety of evaluation of the warhead is more open to interpretation but should minimally include a single-dose rodent study. These data should be sufficient to set the clinical start dose and inform the clinical monitoring plan.

While these studies are required from the regulatory perspective, it is clear that the industry must continue to develop better safety strategies to drive the ADC field forward. Clinical development of ADCs continues to be

limited by toxicity. Solving this problem can only be accomplished by filling in the gaps in our understanding of the mechanisms of ADC toxicity. One way to do so would be to employ a safety screening strategy to conduct early *in vitro* and *in vivo* mechanistic studies that are distinct from regulatory toxicology studies. While these studies are not required, they can (1) improve our understanding of the underlying mechanisms of toxicity, (2) accelerate ADC development, and (3) guide engineering efforts to improve TI of future ADCs. These efforts will in turn benefit both industry and patients as they will increase the chances of clinical success with next generation ADCs.

ACKNOWLEDGMENTS

The authors would like to thank Marlon Rebelatto, Xiang-Qing Yu, Andrew Pierce, Molly Reed, Shameen Afif-Rider, Joerg Bluemel, Christopher Frantz, Jay Harper, Song Ren, and Keith Steele.

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