Targeting Drug Conjugates to the Tumor Microenvironment: Probody Drug Conjugates

Jack Lin and Jason Sagert

Abstract The tolerability and ultimately efficacy of ADCs are limited by 2 major issues: (1) antigen expression that is too low on tumors, resulting in insufficient toxin delivery to the tumor, especially within the confines of the clinical MTD established by linker/payload-driven off-target toxicity and (2) too much antigen expression on normal healthy tissues, resulting in on-target but off-tumor toxicity. In this chapter, we will review strategies for making antibody prodrugs that have been or could be used to selectively deliver drug to a tumor compared to normal tissues. These technologies have the potential to lower on-target, off-tumor toxicities and enable better efficacy of ADCs due to better target selection and the delivery of higher concentrations of drug to tumors.

Keywords Ab drug conjugate (ADC) · Linker/payload · Linker/toxin · Toxicity · Mask · MMP9 · pH · Probody · Protease · Tumor microenvironment

Introduction

Antibody drug conjugates (ADCs) harness the specificity of antibodies to deliver potent cytotoxic drugs to malignant cells. Conceptually, ADCs widen the therapeutic window of potent cytotoxic drugs that would have been too toxic to deliver on their own without the targeting provided by the antibody. The promise of ADCs has been validated by the FDA approvals of gemtuzumab ozogamicin (Mylotarg) for acute myelogenous leukemia in 2000 [1], brentuximab vedotin (Adcetris) for Hodgkin lymphoma in 2011 [2], ado-trastuzumab emtansine (T-DM1, Kadcyla) for Her2+ metastatic breast cancer in 2013 [3], and more recently, inotuzumab

J. Lin (✉) · J. Sagert
CytomX Therapeutics, Inc., South San Francisco, CA, USA
e-mail: mkavanaugh@cytomx.com

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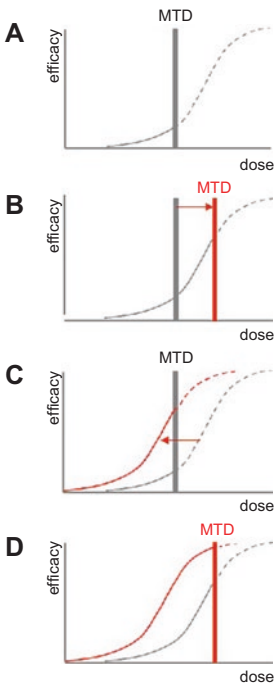


	Features	Examples
A	A typical ADC where off-target toxicity determines the MTD	Roche Her2 ADC (Kadcyla™)
B	Solving off-target toxicity with next generation linker/payload to permit higher dosing.	Mersana Her2 ADC Zymeworks Her2 ADC Ambrx Her2 ADC and several other Her2 ADCs
C	Solving on-target (off-tumor) toxicity to target best tumor antigens for maximal payload delivery.	CytomX CD166 PDC (CX-2009)
D	Combining technologies for maximal efficacy	

Fig. 1 Conceptual framework for the state of current and future ADCs. The horizontal axis denotes ADC dose and the vertical axis denotes the corresponding antitumor response. MTD, or the maximum tolerated dose, is the highest dose where the probability of encountering a dose-limiting toxicity equals the pre-specified target level (usually less than 30%). Solid lines depict dose-efficacy curves below the MTD and dashed lines depict dose-efficacy curves above the MTD. The intersect of the MTD line and the dose-efficacy curve represents the magnitude of the theoretical antitumor efficacy, which could be improved by increasing the MTD (panel **b**), shifting the dose-efficacy curve to the left (panel **c**), or a combination of both (panel **d**). See text for details

ozogamicin for B-cell precursor ALL in 2017. However, the enthusiasm and optimism for ADCs has been tamed by a string of setbacks in the clinic. Gemtuzumab ozogamicin was withdrawn from the market voluntarily in 2010 [4], only to be re-introduced in 2017. A number of ADCs in clinical development were halted due to excessive toxicity or the inability to dose to sufficient levels to impart strong efficacy signals ([5, 6]). There are now abundant data that identify two main causes of ADC failures in the clinic: (1) antigen expression that is too low on tumors, resulting in insufficient toxin delivery to the tumor, especially within the confines of the clinical MTD established by linker/payload-driven off-target toxicity and (2) too much antigen expression on normal healthy tissues, resulting in on-target but off-tumor toxicity.

Solving the issue of off-target toxicity, which are toxicities resulting from the linker/payload irrespective of the antibody target, has been one of the most active areas of ADC research (Fig. 1a, b). The strongest data that suggest off-target

toxicities are a major obstacle in ADC development comes from surveys of auristatin-based and maytansine-based ADCs in clinical development [5–7]. Despite the diversity of targets investigated in the clinic, the maximum tolerated dose (MTD) of ADCs with the two commonly used payloads, the auristatin MMAE and the maytansinoid DM4, are mostly in the 3 mg/kg and 5 mg/kg range, respectively [7]. Further evidence that linker/payload-driven off-target toxicities dictate the clinical MTD is that the dose-limiting toxicities (DLT) of most ADCs are more consistent with those of the free toxin, such as myelosuppression and peripheral neuropathy, than with those expected from the antibody alone [5]. For example, a DLT for the anti-Her2 antibody trastuzumab is cardiotoxicity that is thought to be an on-target toxicity derived from Her2 expression in the heart. In contrast, the DLT for T-DM1, the mcc-DM1-conjugated version of trastuzumab, is reversible thrombocytopenia that is thought to be an off-target toxicity from the linker/payload [8]. These data point to reducing linker/payload-driven off-target toxicity as a way to improve the clinical MTD and boost ADC efficacy. A surrogate marker that has been used for predicting off-target toxicity, albeit still unproven given the limited data, is the use pharmacokinetic (PK) properties of ADCs. Greater circulating half-life and exposure correlate with less off-target toxicity and better tolerability. Since ADC linker/payloads are largely hydrophobic, numerous strategies have been proposed to increase the solubility of ADCs and thereby improve their PK properties. These approaches include limiting the number of linker/payloads per antibody molecule with site-specific conjugation (e.g. conjugation to engineered cysteines, non-natural amino acids, or specific sequence motifs) and improving the solubility of the linker-payloads (e.g. PEGylated, quaternary ammonium, or beta-glucuronic acid linkers). These efforts are described in more detail in a recent review [9] and in other chapters of this book.

In contrast to addressing the linker/payload-driven off-target toxicities of ADCs, a complementary approach is to increase the potency of the ADC within the existing confines of the linker/payload-driven MTD (Fig. 1c). One approach is to use more potent linker/payloads (e.g. DNA-alkylating toxins and ADCs with high drug-antibody ratios, or DAR) that could conceivably target lower-expressing tumor antigens; however, given that many of these potent next-generation linker/toxins are also accompanied by a reduction in MTD, it remains to be seen whether there will be an increased therapeutic window. Another approach is to redefine the ADC target space and target tumor antigens that would yield a more potent effect. Current ADCs are severely hampered by the availability of suitable tumor antigens that have all of the desired features: high expression in tumors to drive high uptake of the drug, high differential expression between tumor and normal tissues to avoid on-target toxicity, efficient internalization to deliver maximal toxin to the tumor cells, homogeneously expression on all tumor cells to reduce the likelihood of drug resistance, and sufficient prevalence in different tumors to warrant its development. The dearth of suitable ADC targets, especially for solid tumors, is exemplified by the large number of ADCs targeting the Her2 antigen and competing to be the best T-DM1 “biobetter” drug. In contrast, a few attempts to target high prevalence and high expression tumor antigens have resulted in on-target off-tumor toxicities, including ADCs targeting the Lewis-Y

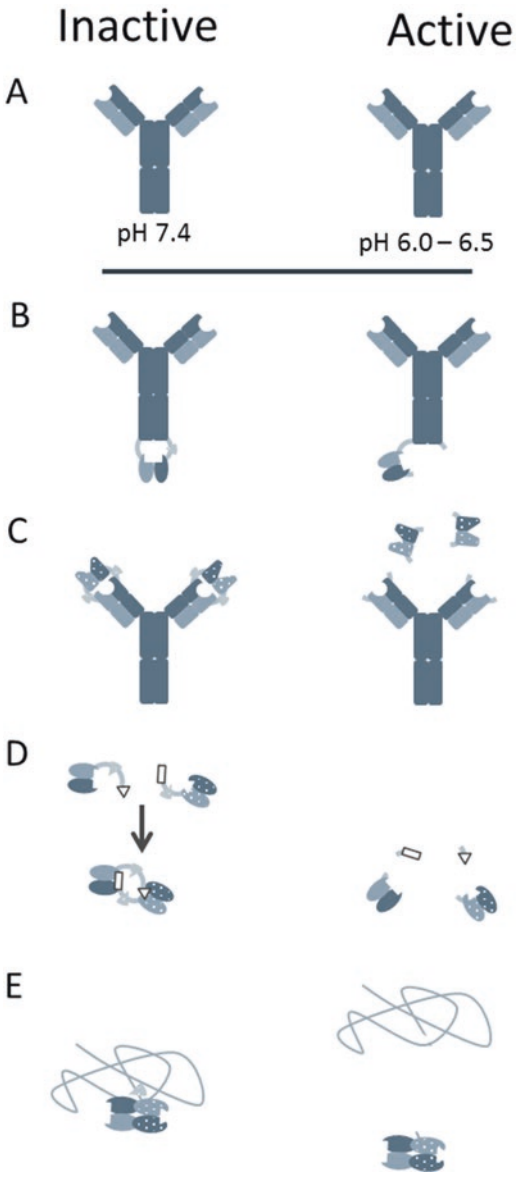


Fig. 2 Diagram of various antibody and antibody fragment formats that may localize to tumors. The left column shows the less active format and the right column shows the more active format of (**a**) pH dependent binding, (**b**) trivalent antibodies, (**c**) dual variable domain antibodies, (**d**) cross-masking antibodies, and (**e**) ProTIA formats

antigen [10], CD44v6 [11], and EphA2 [12]. One proposed solution to safely expand the addressable target space for ADCs to more desirable antigens is the use of antibody drug conjugates that are designed to be preferentially active in the tumor microenvironment, and thus spare normal tissues.

In this chapter, we will review two general strategies for making antibody prodrugs that have been or could be used to selectively deliver drug to a tumor: differential pH-sensitivity and protease activatability (Fig. 2). As discussed below,

these strategies exploit two common attributes of the tumor microenvironment that differ from normal tissues: the slightly acidic pH in the tumor (Fig. 2a) and the dysregulated proteolytic tumor milieu (Fig. 2b–e).

Acidic Tumor Microenvironment

Various imaging modalities have confirmed that tumor microenvironments are slightly more acidic than normal tissues [13, 14]. Two interrelated mechanisms contribute to the acidic tumor microenvironment: hypoxia and tumor metabolism [15]. Hypoxia, from inadequate vascularization in tumors, leads to the induction of hypoxia inducible factor 1 (HIF1α) that in turn upregulates the expression of carbonic anhydrase IX, glucose transporters, and glycolytic enzymes. This permits tumor cells to adopt different metabolic processes than normal cells. Normal cells utilize glycolysis under anaerobic conditions and mitochondrial oxidative phosphorylation under aerobic conditions to maximize the generation of ATP per glucose molecule. In contrast, tumor cells preferentially depend on glycolysis even under aerobic conditions, a phenomenon known as aerobic glycolysis or the "Warburg effect" (reviewed in [16, 17], and [18]). The reliance on aerobic glycolysis ultimately results in the accumulation of lactic acid as a byproduct which contributes to the tumor environment being more acidic than that of normal tissues.

Not surprisingly, the pH differential of the tumor microenvironment has been exploited as a way to engineer antibody drug conjugates that bind preferentially under these conditions. The concept of engineering pH-dependent conditional binding proteins by the incorporation of "histidine switches" was first demonstrated for cytokines [19] and subsequently in antibodies [20, 21]. Of the 20 naturally occurring amino acids, histidine is the preferred choice for this approach because its pKa confers the ability to ionize and de-ionize its side chains around physiological pH. With an antibody library enriched with histidine residues within the complementarity determining regions and with an appropriate screening strategy, one could identify antibodies that preferentially engage target either under acidic pH or at neutral pH conditions. The first pH-dependent antibodies were engineered for lower affinity at the pH of the endosome (5.5–6.0) compared to the pH of plasma (7.4) in order to decrease target-mediated degradation and promote antibody recycling from the endosomal compartment, thereby increasing circulating antibody half-life [20, 21].

To take advantage of the acidic tumor microenvironment, the opposite pH-switch is required: antibodies that bind with higher affinity at the acidic pH 6.0 compared to neutral pH 7.4. This arrangement would have an antibody that preferentially engages its targets in the acidic tumor microenvironment, while sparing normal tissues under neutral pH conditions. For an ADC, the tighter binding at the lower pH may facilitate binding in the endosomal/lysosomal compartment and target-mediated degradation of the ADC, enhancing the release of toxin payload. Halozyme and BioAtla have exploited the pH differential between normal tissue vs. tumor microenvironment to engineer an auristatin-based ADC that binds to EGFR with an

approximately tenfold stronger affinity under acidic pH 6.0–6.5 than under neutral pH 7.4 [22]. The goal is to preferentially target EGFR-expressing tumor cells in the acidic tumor environment while sparing other EGFR-expressing healthy tissues such as skin under neutral pH conditions. This ADC induced tumor regressions in cetuximab-resistant mouse xenograft models at 15 mg/kg and was well tolerated at 8 mg/kg in cynomolgus monkeys.

Potential challenges with designing pH-sensitive antibody drug conjugates are (1) the biophysical limitations on designing a highly tumor-selective targeting antibody given the relatively small pH difference (pH 6–6.5 in tumors vs. pH 7.4 in normal tissues), with possibly an even smaller pH differential in micro-metastatic tumor lesions that may be well vascularized; (2) balancing the trade-off between the need for optimal antibody sequence with the need for incorporation of histidines; and (3) the need for having the flexibility to fine-tune the desired affinity-differential between tumor vs. normal tissue.

Proteolytic Tumor Microenvironment

Proteolysis is a highly regulated process under normal physiological conditions. Many proteases work in series as part of proteolytic cascades with large amplification effects (e.g. coagulation and complement pathways); therefore, an aberrant proteolytic event could trigger devastating consequences if not for the intricate network of protease activators and inhibitors required to maintain proteolytic homeostasis. Consequently, dysregulated proteolytic activity is often the hallmark of many pathophysiological conditions, and protease inhibitors have been successfully approved to treat a number of indications including hypertension, thrombosis, viral infection, and inflammation [23].

Dysregulated extracellular proteolytic activity is also an important hallmark of most human cancers because it is required to maintain key elements of the transformed phenotype, including growth, invasion, and metastasis [24, 25]. Of the more than 500 human proteases, examples identified to be involved in cancer include serine proteases such as the type II transmembrane serine proteases [26] and urokinase plasminogen activator (uPA) systems [27]; metalloproteases such as MMPs [24] and ADAMs [28]; and cysteine proteases such as cysteine cathepsins [29]. While the importance of proteases in maintaining a proteolytic pro-tumorigenic environment is widely established, no inhibitors to extracellular proteases have been successful in treating solid tumors to date. Especially notable have been the multiple unsuccessful attempts to target extracellular MMPs with broad spectrum small molecule inhibitors (reviewed in [30]), and highly specific allosteric antibodies [31, 32]. This likely reflects the difficulty of effectively inhibiting a wide spectrum of different proteases necessary to deliver a therapeutic effect while avoiding toxicity.

Instead of neutralizing these tumor-associated proteases for direct therapeutic effect, an alternate approach is to exploit this unique proteolytic milieu in the tumor microenvironment to better target therapy to tumors. This rich proteolytic environment could be used to preferentially activate antibodies and other protein-based therapeutic agents in the tumor while sparing normal healthy tissues. Like the pH-sensitive antibodies

described above, there are also general protein engineering trade-offs associated with the entire class of protease-activatable antibodies. Some common concerns include (1) the risk of immunogenicity from additional sequence extensions from the antibody scaffold, (2) the possibility that the proteolytic milieu in mouse xenograft tumors might not adequately model those in human tumors, and (3) the identification of suitable protease substrates that are efficiently cleaved in the tumor microenvironment but not within normal tissues. These issues will be monitored as this class of protease-activatable therapeutics advance into the clinical setting. We outline below some of the different protease-activatable antibody formats that have been described.

Protease Activatable Antibody Formats

Several protease-activatable antibody- or antibody fragment-based platforms have been described in the literature or are in preclinical development. The Probody platform has been extensively used to selectively target the activity of antibody drug conjugates to tumors and will therefore be described in detail. It is not the goal of this chapter to provide a comprehensive review of protease activatable antibody formats. However, several examples of formats that could potentially be applied to ADCs will be highlighted.

Activatable Trivalent Antibodies

Metz et al. [33] describe an engineered antibody in which a disulfide-stabilized Fv (dsFv) is expressed on the C-terminus of the heavy chain. In this design, the dsFv is sterically inhibited from binding its target antigen by the Fc portion of the antibody. If a protease site is introduced between one of the Fc and dsFv portions of the protein, cleavage would result in the ability of the dsFv to swivel open and become competent to bind to its target (Fig. 2b). To demonstrate the potential of this approach, a cMET dsFv was engineered onto an anti-Her3 IgG. If substrates for MMP2, MMP9, or uPA were incorporated into one arm of the construct, the resulting protein's affinity for cMET could be increased by cleavage with the respective enzyme. Using this approach, the authors were able to demonstrate an approximately 1000-fold difference in affinity between the precursor and activated molecules in vitro. While these trivalent antibodies wouldn't provide a strict on/off switch because of the binding capabilities of the IgG portion, one could imagine that this approach could result in increased tumor targeting as a result of the enhanced avidity that would be restricted to the tumor microenvironment.

Activatable Dual Variable Domain Antibodies

Similar to the activatable trivalent antibody approach, Onuoha [34] engineered an activatable dual variable domain (aDVD) antibody on two different anti-TNF-α antibodies (adalimumab and infliximab). This was achieved by linking the variable

domains of an anti-ICAM to the N-terminus of the anti-TNF- α antibody via an MMP9 substrate/linker. In this format, the ICAM variable domain retains the ability to bind ICAM while effectively blocking the ability of adalimumab or infliximab to bind TNF-α. Upon removal of the ICAM variable domains by treatment with MMP9, the TNF- α binding was restored to that of the parental anti-TNF- α antibody. A diagram of this approach is shown in Fig. 2c. This method was capable of producing a greater than 1000-fold difference in K_D between the cleaved and uncleaved aDVDs, as measured by SPR in vitro. As with the trivalent approach, tumor protease-driven targeting could be achieved by the tumor-specific enhancement of affinity for the target.

Cross-masking Antibodies

The cross-masking antibody approach involves attaching the cognate antigen epitope of one antibody via a protease substrate-containing linker to a second antibody or antibody fragment and vice versa (Fig. 2d). Donaldson, et al. [35] demonstrated in vitro proof of concept for this approach using scFvs based on two anti-EGFR antibodies, cetuximab and matuzumab. The epitope used was a portion of soluble EGFR domain III with point mutations introduced to reduce the potential for intramolecular binding of the EGFR fragment. The individual constructs were purified, mixed together allowing the assembly of the cross-masked heterodimeric complex, followed by removal of monomer and misassembled complexes by chromatography. The authors showed that the binding of the heterodimeric complex to sEGFRvIII was significantly attenuated as compared to the MMP9-treated complex.

XTEN Platform

The XTEN platform was originally described by Amunix as a way to extend the in vivo half-life of biologics and small molecules (reviewed in [36]). The XTEN polypeptides consist of polymers of the amino acids alanine, glycine, glutamic acid, proline, serine, and threonine. These were selected for their solubility and lack of potential immunogenicity and propensity to aggregate. The original XTEN polypeptide was 864 amino acids long but XTEN polypeptides of different lengths and compositions have been subsequently evaluated. Importantly, various chemical functionalities can be engineered into XTEN peptides enabling the conjugation of different classes of molecules through various chemistries. Recently Amunix has engineered T-cell bispecifics conjugated to XTEN peptides (referred to as "XTENylation") via a protease linker and is referred to as Protease Triggered Immune Activators or ProTIA (Fig. 2e). These molecules are proposed to selectively target activity to tumors in several different ways, including preferred extravasation due to leaky tumor vasculature and removal of the XTEN polypeptide by tumor specific proteases (www.amunix.com).

Probody™ Therapeutics

The most advanced protease-activatable antibody drug conjugates are based on Probody therapeutics. Probody therapeutics are a novel class of recombinant antibody-based therapeutics that target antibody activity to the tumor by taking advantage of the dysregulation of proteases in diseased tissues. The key components are two peptide sequences encoded on the N-terminus of the light chain of antibodies collectively called the Prodomain (Fig. 3a). The first sequence is a "masking" peptide which physically blocks the ability of the antibody to bind antigen. This sequence is connected to the rest of the light chain by a second peptide sequence designed to be preferentially cleaved by proteases with increased activity in tumors. The addition of the Prodomain results in a molecule with significantly reduced affinity for its target antigen which, upon exposure to proteases, recovers the parental antibody binding affinity (Fig. 3b).

A Probody therapeutic based on the anti-EGFR antibody cetuximab was used to demonstrate the ability of the Probody technology to expand the therapeutic window of an antibody therapy [37]. Using in vivo imaging in mouse xenograft models, it was shown that the protease substrate-containing EGFR Probody therapeutic localized to the xenograft tumor and could achieve efficacy comparable to that of the naked EGFR antibody in tumor xenograft models. In contrast, a masked Probody therapeutic lacking a protease substrate showed reduced localization to xenograft tumors and no significant efficacy in tumor models. These data show that the substrate-containing anti-EGFR Probody therapeutic is capable of being activated and binding to its target antigen in the xenograft tumor microenvironment in a protease dependent manner. Desnoyers, et al. also showed that, in cynomolgus monkeys, the EGFR Probody therapeutic remained largely intact in circulation, had increased exposure due to avoidance of target-mediated drug disposition (TMDD), and reduced the dose limiting skin toxicity associated with cetuximab. It was estimated that the safety factor of the Probody therapeutic was increased over that of the antibody by between 3- to 15-fold. Taken together, the mouse and cynomolgus data demonstrate that the Probody approach is capable of expanding the therapeutic window of an antibody therapy.

Probody Drug Conjugates

The potential of Probody Drug Conjugates (PDC) to widen the therapeutic index for highly expressed targets has been proposed previously [38] and preclinical data for PDCs targeting the highly expressed antigens CD166 and CD71 have been reported [39, 40]. Here we will describe two examples of Probody Drug Conjugates. The first is an anti-Jagged PDC for which efficacy and on-target toxicity can be measured within the same in vivo mouse model system. The second example is a family of anti-CD166 PDCs that show how the interplay between mask strength, substrate choice, and efficacy can be used to fine-tune a PDC. Finally, we show that an anti-CD166 PDC that has similar efficacy as the corresponding ADC in mouse, is

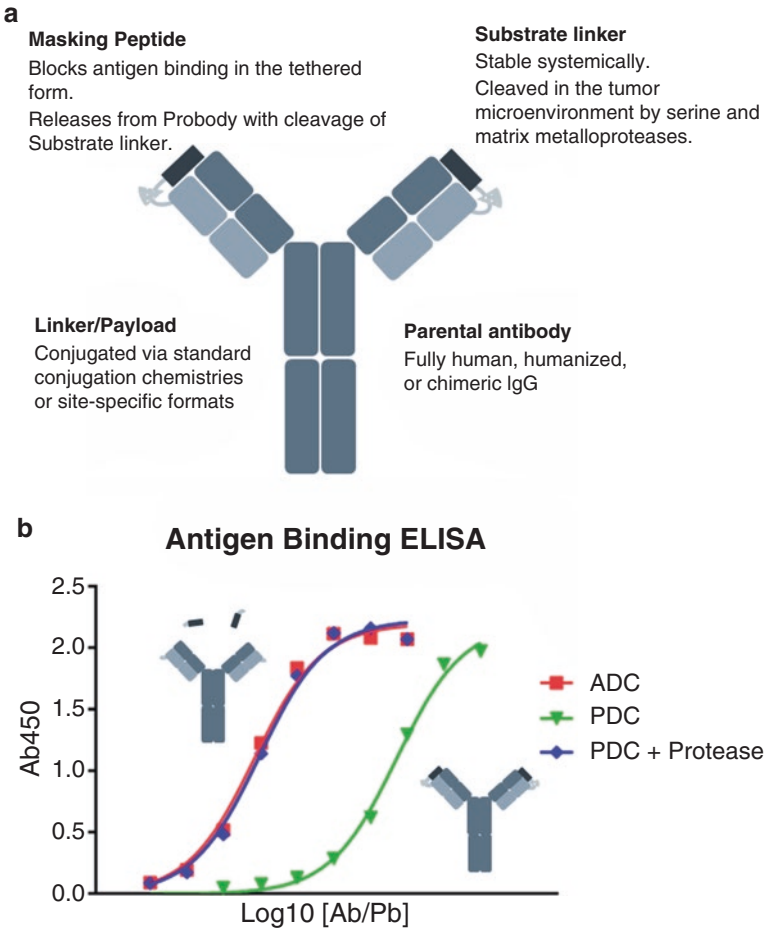


Fig. 3 The Probody Platform. (**a**) Probody drug conjugate components include a parental antibody; the Prodomain, which is comprised of a masking peptide linked to the N-terminus of the light chain of the parental antibody via a protease substrate; and finally the linker/toxin. (**b**) In their inactive form, PDCs have reduced binding for their antigen and upon activation by proteases, recover the binding equivalent to that of the parental antibody

physically stable in circulation in a nonhuman primate, and avoids the TMDD observed with the ADC, suggesting that the PDC remains functionally masked in circulation. A CD166-targeting PDC is currently being evaluated in a Phase 1 trial.

Anti-Jagged Probody Drug Conjugates

The Notch ligands Jagged 1 and Jagged 2 are attractive therapeutic targets because of the importance of the Notch pathway in cancer and tumor initiating cells [41]. We developed an antibody that binds both human and rodent Jagged 1 and 2 Notch ligands

with similar affinity and inhibits their interaction with the Notch receptors. In mice, the antibody shows on-target toxicity evidenced by significant body weight loss, hair loss and elevated serum plasma thymic stromal lymphopoietin (TSLP), consistent with what has been previously reported for Notch pathway inhibition by gamma secretase inhibitors [42] and in conditional Notch knockout animals [43]. In general, the toxicities elicited by the antibody are dose dependent and most severe at dose levels greater than 10 mg/kg. It has previously been shown that a Probody therapeutic derived from this antibody is active as monotherapy and in combination with chemotherapy in a preclinical model of pancreatic cancer [44]. The anti-Jagged Probody therapeutic dosed at 20 mg/kg results in toxicities that are mild and comparable to the 5 mg/kg dose of the antibody, demonstrating an approximately fourfold safety advantage on a dose basis for the Probody therapeutic compared to the antibody.

An anti-Jagged ADC generated from this antibody using the linker-toxin combination SPDB-DM4 shows potent in vitro cytotoxicity in several cell lines and in vivo anti-tumor activity in several xenograft models, for example the HCC1806 subcutaneous tumor xenograft model in SCID mice [45]. Tumor bearing mice were dosed on day 1 and 8 with 10 mg/kg of either the SPDB-DM4 isotype control (Isotype), anti-Jagged antibody (Ab), anti-Jagged SPDB-DM4 (ADC), or the anti-Jagged Probody SPDB-DM4 (PDC) and subsequently monitored for tumor growth and body weight change. By day 30, the Isotype-DM4 control and anti-Jagged antibody groups had similar mean tumor volumes of 863 ± 136 (average ± SEM) and 852 ± 100 mm^3, respectively (Fig. 4a). All animals in the ADC and PDC treated groups showed tumor regressions by day 9 of the study and mean tumor volumes of 13.1 ± 1.2 and 20.3 ± 3.6 mm^3, respectively, at day 30. The antibody and ADC treated animals both showed weight loss, with weights of 87 ± 5 and 82 ± 5 percent, respectively, of their starting weight at day 20 (Fig. 4b). In contrast, both the isotype-DM4 and PDC treated animals showed undetectable weight loss. As expected, the observed weight loss in the ADC treated animals was similar to that observed for the non-conjugated antibody treated group, suggesting that the toxicity was due to target (Jagged) inhibition rather than to the conjugated toxin. These results demonstrate that the PDC is capable of antitumor activity comparable to the ADC but with significantly less on-target toxicity when measured in the same animals.

Anti-Jagged Probody Therapeutic Pharmacokinetics in Non-tumor Bearing Mice

To demonstrate that the anti-Jagged Probody therapeutic is stably masked in circulation and avoids binding target in normal tissues, we conducted a 14-day single dose pharmacokinetic study in non-tumor bearing mice comparing the non-conjugated antibody with the Probody therapeutic. The PK curves and the calculated pharmacokinetic values are summarized in Fig. 5. The anti-Jagged antibody and Probody therapeutic had comparable Cmax values at 35 and 45 ug/ml, respectively.

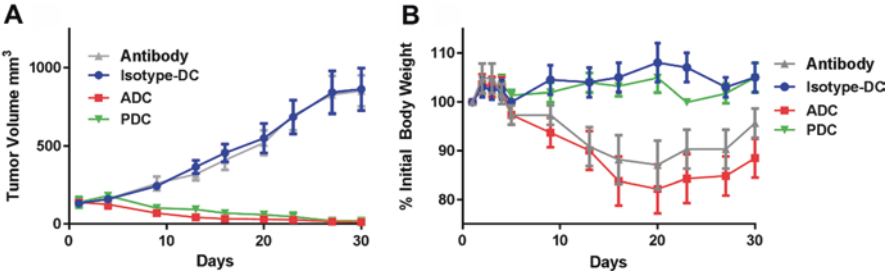


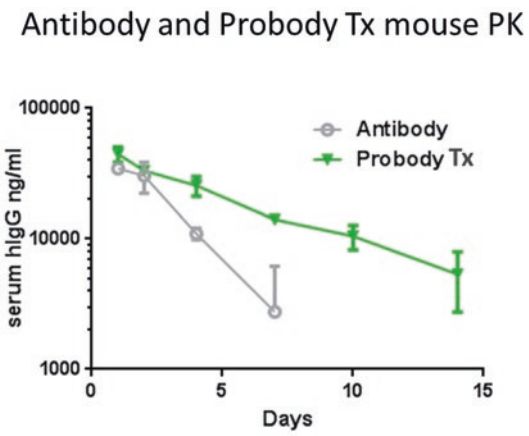
Fig. 4 Anti-Jagged ADC and PDC in the HCC1806 tumor xenograft model in SCID mice. (**a**) Tumor growth curves showing the average ± SEM tumor volumes for HCC1806 xenograft-bearing mice treated with the anti-Jagged antibody (antibody), isotype drug conjugate (isotype-DC), anti-Jagged drug conjugate (ADC), and Probody drug conjugate (PDC). (**b**) Average of percent of initial body weight ± SEM for the same animals with HCC1806 tumors in panel (**a**). All test articles were dosed at 10 mg/kg on day 1 and day 8 and each group consisted of 8 mice

The anti-Jagged antibody was more rapidly cleared to below the lower limit of detection of the assay by day 10, while the anti-Jagged Probody therapeutic showed significantly increased serum half-life (4.6 vs 1.0 days) with the Probody therapeutic concentration remaining above 4 μg/mL at day 14. The increased half-life and greater systemic exposure is consistent with the avoidance of target mediated drug disposition by the masked Probody therapeutic.

The pharmacokinetic and in vivo efficacy and safety data for the anti-jagged Probody therapeutic in preclinical studies support two main conclusions. First, the extended half-life of the PDC and lack of weight loss in PDC-treated animals compared to the ADC demonstrates that the PDC avoids target binding in healthy tissues and, therefore, on-target toxicities. Second, the PDC is capable of antitumor activity comparable to the ADC.

CD166 Probody Drug Conjugates

A second example of an attractive target for Probody drug conjugates is activated leukocyte cell adhesion molecule (ALCAM), also known as CD166. CD166 is reported to be a cell adhesion molecule expressed on many cell types including activated leukocytes, neurons, and epithelial cells. Although CD166 has been identified as a ligand for the CD6 receptor, which is expressed on T lymphocytes and implicated in T cell proliferation and activation [46], its biological functions and the consequences of its inhibition are not understood. CD166 is also highly and homogenously overexpressed in many types of cancer at high prevalence among patients. The high tumor expression and broad normal tissue expression make CD166 an example of an attractive ADC target that would be difficult to develop with traditional ADC technology, but can be addressed by Probody drug conjugates. We developed a panel of anti-CD166 Probody drug conjugates with different masks and substrates and evaluated their efficacy in a xenograft model to identify the preferred PDC design.



Article	Half life (Days)	Cmax (ug/mL)	AUClast (day*ug/mL)
Antibody	1.0	35	129
Probody Tx	4.6	45	278

Fig. 5 Total human IgG plasma levels and calculated PK parameters in nude mice dosed with either 5 mg/kg of anti-Jagged antibody (Antibody) or anti-Jagged Probody therapeutic (Probody Tx)

CD166 Probody and PDC Characterization

A humanized anti-CD166 antibody that has equivalent affinity for human and cynomolgus monkey CD166 was developed. When conjugated to SPDB-DM4, the ADC is potently cytotoxic in vitro across a large panel of human cancer cell lines [40]. A panel of CD166 Probody therapeutics was developed in which the strength of the masking peptide was varied as measured by binding to HCC1806 cells, referred to as "Low", "Medium", and "High" masked Probody therapeutics (Fig. 6a). When conjugated to SPDB-DM4, and in the absence of protease activity, all but the Low masked PDC protects against target-dependent cytotoxicity in vitro. Although the Low masked PDC does show reduced cytotoxicity as compared to the ADC, it is not completely masked and does show some level of on-target activity as compared to that of the isotype control. Upon protease treatment to remove the mask, all the activated PDCs demonstrated the same cytotoxicity as the ADC (Fig. 6b).

In Vivo Efficacy of CD166 PDCs

To determine the preferred mask/substrate combination for CD166 PDC efficacy, a panel of anti-CD166 Probody SPDB-DM4 drug conjugates was assessed in the H292 xenograft model. Besides varying the mask strength, the "Medium" mask was

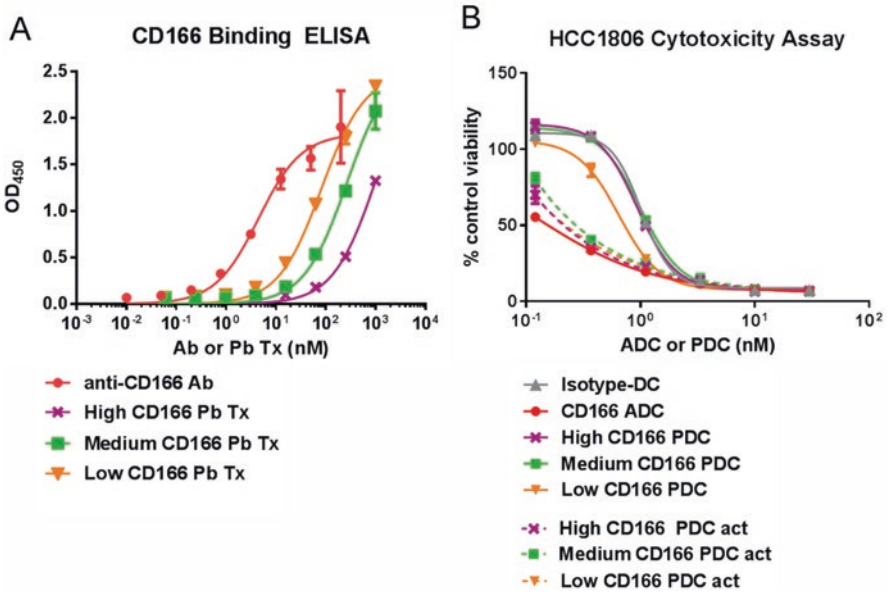


Fig. 6 ELISA binding curves (**a**) for the anti-CD166 antibody (anti-CD166 Ab) and three anti-CD166 Probody therapeutics (Pb Tx) with different masking strengths: High-CD166 Pb Tx, Medium-CD166 Pb Tx, and Low-CD166 Pb Tx. Cytotoxicity assay results (**b**) for anti-CD166 drug conjugate (CD166 ADC) and Probody drug conjugates (High-CD166 PDC, Medium-CD166 PDC, and Low-CD166 PDC) show a range of masking strengths. The High and Medium masked PDCs have similar cytotoxicity as the Isotype-DC while the Low masked PDC shows some level of on-target cytotoxicity. Each data point shows the average ± SD. When activated with a protease (act), all of the PDCs recovered the activity of the CD166 ADC. All Probody therapeutics and PDCs described here contain protease substrate 1 (see text)

evaluated with two different protease substrates, referred to as Substrate 1 (Sub1) and Substrate 2 (Sub2). Both substrates are capable of being cleaved by MMP and serine proteases, however, the substrates differ in their kinetic reactivity, with Sub2 generally being more reactive and cleavable than Sub1. The "Medium" mask was chosen to compare the two substrates as it was sufficient to avoid on-target toxicities in the vitro cytotoxicity assay.

Figure 7a shows the efficacy of the isotype control, the Low-, Medium-, and High-masked Sub1 CD166 PDCs, and the parental ADC. As might be expected, the High masked PDC showed the least efficacy and the Low masked PDC showed the most efficacy in the H292 model. Using the less cleavable Sub1, none of the PDCs achieved equivalent efficacy as the ADC. Within the same study, CD166 PDCs comprising Sub1 or Sub2 with the Medium mask were compared as described above (Fig. 7b). In this configuration, PDCs containing the more cleavable Sub2, but not the less cleavable Sub1, were capable of achieving tumor regressions similar to that of the ADC. These data together demonstrate that the activity of a PDC can be modulated by varying both the mask strength and sub-

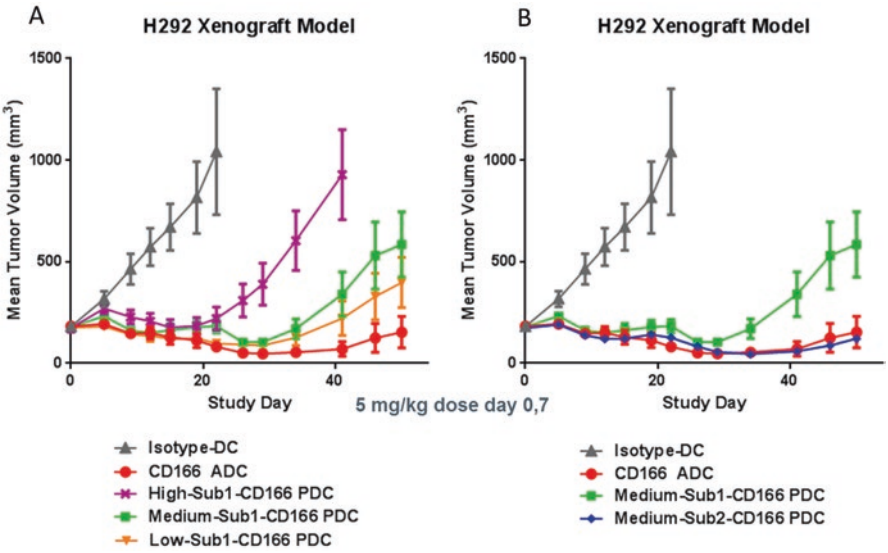


Fig. 7 H292 xenograft tumor bearing mice treated with anti-CD166 PDCs with High, Medium, and Low masks (**a**) and comparing substrates Sub1 and Sub2 in PDCs having the Medium mask (**b**). Each data point shows the average ± SEM tumor volume for each group (N = 8). Efficacy was inversely proportional with the masking strength (**a**) with the Low masked PDC showing the greatest efficacy. While the Medium masked PDC with Substrate 1 (Sub1) did not achieve efficacy similar to that of the CD166 ADC, the Medium masked PDC with Substrate 2 (Sub2) showed efficacy comparable to that of the ADC

strate composition, and that a PDC can be selected that can achieve efficacy similar to that of the unmasked ADC.

Stability of CD166 PDCs in NHP

As described above, if a PDC is sufficiently masked and the substrate is sufficiently stable in circulation to avoid binding to target in normal tissues, it would be expected that the PDC would show prolonged half-life and increased serum exposure as compared to the parental ADC. The pharmacokinetics of the two most efficacious CD166 PDCs (Low mask with the less cleavable substrate "Low-Sub1" and Medium mask with the more cleavable substrate "Medium-Sub2") and the ADC were evaluated at 5 mg/kg in non-human primate (NHP) cynomolgus monkeys. As expected, the PDCs show slower clearance than the ADC (Fig. 8). Further, as in tumor xenograft models, the PK in monkeys can be tuned by modulating the two key components of a Probody therapeutic, the mask and substrate.

Using PDCs targeting CD166, we have shown that a preferred mask/substrate pair can be identified for a PDC targeting an antigen that is expressed on both tumor and normal tissues. Using a xenograft model, we demonstrated that that

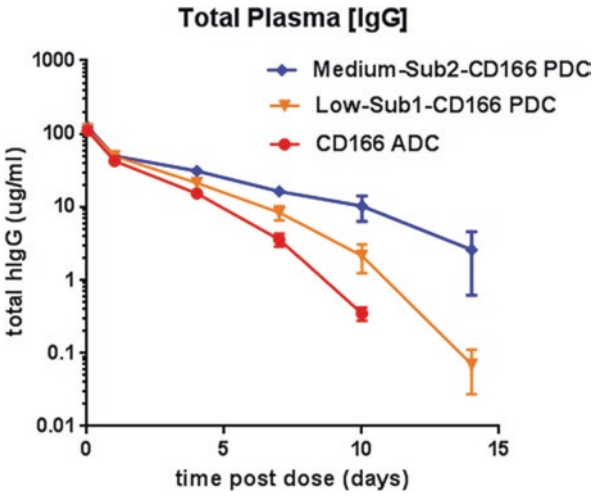


Fig. 8 Plasma human IgG concentrations measured in cynomolgus monkeys (n = 2 per test article) treated at single dose of 5 mg/kg with the Medium-Sub2 CD166 PDC, the Low-Sub1 CD166 PDC, or the anti-CD166 ADC. The average ± SD is shown for each time point. Both PDCs showed extended half-life and exposure compared to the ADC with the Medium-Sub2-PDC showing the largest increase

PDC has similar anti-tumor activity and superior PK compared to that of the parental ADC.

Summary/Future Perspectives

The development of new approaches to address the problems of on-target and off-target toxicities has generated a renewed sense of optimism in the ADC field. After a drought of ADC approvals in the past several years, there are multiple ADCs in pivotal trials for various solid and hematological cancer indications, and new ADC technologies are also being tested in early clinical trials. In the near future, it is possible that a combination of technologies may be needed to achieve the widest therapeutic window and realize the vision of ADCs replacing traditional chemotherapy as the backbone of oncology care.

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