Structure-Activity Relationships of Antibody-Drug Conjugates: A Systematic Review of Chemistry on the Trastuzumab Scaffold

*Siddharth S. Matikonda, Ryan McLaughlin, Pradeep Shrestha, Carol Lipshultz, Martin J. Schnermann**

Chemical Biology Laboratory, NIH/NCI/CCR, 376 Boyles Street, Frederick, Maryland 21702, United States

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ABSTRACT Antibody-drug conjugates (ADCs) are a rapidly growing class of cancer therapeutics. The goal of ADCs is to overcome the low therapeutic index of conventional cytotoxic agents. However, realizing this goal has been a significant challenge. Consisting of an antibody linked to a therapeutic payload, ADCs comprise many components which can be modified, including the target, payload, linker, and bioconjugation method. Many approaches have been developed to improve the physical properties, potency, and selectivity of ADCs. The anti-HER-2 antibody trastuzumab, first approved in 1998, has emerged as an exceptional targeting agent for ADCs, as well as a broadly used platform for testing new technologies, The extensive work in this area enables the comparison of various linker strategies, payloads, drug-to-antibody ratios (DAR), and mode of attachment. In this review, these conjugates, ranging from the first clinically approved trastuzumab ADC, Kadcyla (T-DM1) to the latest variants, are described with the goal of providing a broad overview and comparison of existing and emerging conjugate technologies.

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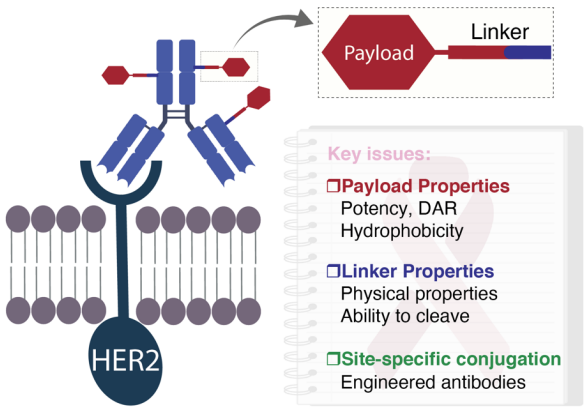

Introduction

Monoclonal antibodies (mAbs) are invaluable oncology therapeutics with over 60 approved by the FDA.[1] However, the combination of target engagement and Fc effector function is often insufficient to induce persistent tumor regression.[2] As a consequence, there have been extensive effort to augment the activity of these therapeutics.[3-4] The union of mAbs and small molecule therapeutics or antibody-drug conjugates (ADCs) has been particularly successful.[2, 5-11] This approach builds on fully intact mAbs, which have often been already applied in clinical settings in isolation, and benefits from extensive development efforts towards small molecule therapeutics. There has been remarkable progress in this area, including the approval of Mylotarg (gemtuzumab ozogamicin), first in 2000, and then Adcetris (brentuximab vedotin) and Kadcyla (Tz-DM1) in the early 2010s. Following these critical early approvals, the last decade has seen the approval of an additional 12 ADCs and many more in various stages of clinical development.[12]

An ADC has three separate components: the mAb, the drug payload, and a connecting linker. Each of these play an important role. Successful targeted drug release requires overcoming complex and, sometimes almost paradoxical, requirements. For example, hydrophobic drugs are used as payloads for ADCs because upon release they readily diffuse across cellular membranes.[13] However, ADCs with hydrophobic payloads are prone to aggregation, non-specific binding, and uptake by phagocytic cells.[14-18] Additionally, an optimal linker between the payload and antibody should be stable in circulation, but readily cleaved upon binding and uptake into target-positive cells. The clinical use of this agents has revealed several additional challenges. For example, recent studies have shown that both Kadcyla and Adcetris are prone to aggregation.[19-20] Furthermore, the maleimide linker used in these drugs can be cleaved through a retro-Michael reaction resulting in free payload or albumin-conjugated DM1 (for Kadcyla).[21-23] Lastly, it is becoming clear that many toxicities observed are target-independent and relate entirely to off-target ADC uptake and release of the payload.[24] These issues of stability and off-target release are chemical challenges in nature. Therefore, advances in bioconjugation chemistry and linker/payload design have a central role to play in future efforts to develop this therapeutic modality.

Overexpressed in almost 30% of breast cancer, as well as several additional solid tumor types, HER-2 is an archetypical tumor associated antigen.[25-26] The anti-HER2 antibody, trastuzumab (Tz), was first approved in 1998 and finds use as the first line treatment of HER2-positive patients.[27-30] Due to a significant clinical need, there have been extensive efforts to develop Tz ADCs leading to two approvals and another compound in late state development (Figure 1C).[31] Kadcyla (2013), was approved for metastatic HER2-positive breast and gastric cancer, consists of Tz linked to a tubulin targeting DM1 via a N-succinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate (MCC) linker.[2] This drug has been broadly applied, with annual revenue now exceeding one billion US dollars. Recently, Enhertu was approved for unresectable or metastatic breast cancer (for patients previously administered with at least two other anti-HER2-based regimens).[32] Enhertu consists of a potent topoisomerase inhibitor Deruxtecan conjugated via a tumor-selective stable linker to trastuzumab (Tz-) with a DAR (drug to antibody ratio) of 8.[33-35] Recent clinical data suggests it is superior to Kadcyla in low HER2 copy number cases.[36-38,39] A final agent, SYD985, uses a duocarmycin payload, seco-duocarmycin-hydroxybenzamide-azaindole (seco-DUBA), attached to trastuzumab via a val-cit-PABA linker.[40-41] This agent was granted fast-track designation by the FDA for pre-treated HER2-positive metastatic breast cancer.[42]

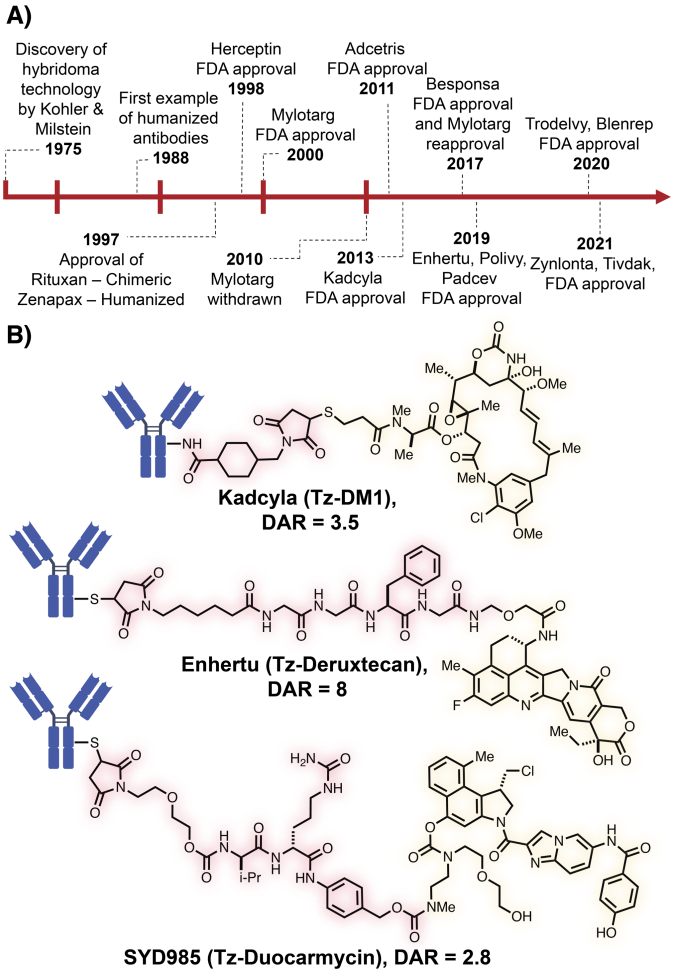

Figure 1. A) Notable milestones in ADC development. B) Chemical structures of Tz-ADCs: Kadcyla and Enhertu (FDA approved) and SYD985 (FDA fast-track designation).

The extensive literature with Tz-ADCs provides an opportunity to isolate the impact of linker and payload chemistry. We have identified literature reports of 100 Tz-ADCs spread over 75 primary papers. In the attached table (Table S1), we have summarized the chemistry used and any available *in vitro* and *in vivo* data. Using this data set, we attempt to summarize key chemical components of Tz-ADCs. Our goal is to define structure activity relationships that contribute to potent and selective *in vitro* and *in vivo* activity, and we hope this review will be a valuable tool to both established and entering practitioners.

Payload Properties

The therapeutic payload plays a central role in ADC function. In Figure 2, we summarize payloads used with Tz and key properties. These agents are typically natural products and their derivatives. Their biological targets are typically those of classical chemotherapies, tubulin, or DNA. The earliest generations of ADCs used relatively low-potency payloads (>100 nM). These ADCs typically exhibited minimal clinical efficacy despite promising initial results.[2, 43-45] Since these early studies, highly potent payloads (IC_{50}'s in the low nM to sub-nM range) have nearly invariably been pursued.[2, 46] Two classes of payloads that received significant attention are the

tubulin-targeting auristatins and maytansinoids, including in extensive efforts with Tz ADCs.[34, 47-49] These agents have been highly successful, leading to the approved ADCs mentioned above, and stimulated efforts to identify even more potent options. Extensive efforts over the past decade have focused on potent DNA damaging agents, particularly, the pyrrolobenzodiazepine (PBD) dimers and derivatives of the natural product duocarmycin. Below we highlight several insights that have emerged from these payload optimization studies, as well as other payloads that are starting to be explored.

Upon cleavage, it is reasonable to presume that hydrophobic payloads could cross the plasma membrane and distribute beyond the target cell (i.e., bystander effects), while hydrophilic payloads would be more confined to the original target cell.[50-51] This supposition has been best validated through the comparison of MMAE (non-polar) and MMAF (a charged) payloads.[52] MMAE is comparatively more membrane permeable and is more potent (lower IC_{50}) than MMAF. However, MMAF, which is more hydrophilic due to its charged carboxylic terminus, has limited passive diffusion and does not efficiently diffuse out of the cell. MMAF ADCs typically require higher tumor expression of target antigen to be effective, but are more potent than vc-MMAE ADCs when targeting internalizing antigens *in vitro.*[51] In the case of tumors with sparse and heterogenous expression of target antigen, MMAE ADCs are advantageous as the released free drug can diffuse out of the target cell and enter surrounding cells to cause bystander killing.[53]

While highly hydrophobic payloads can exhibit exceptional *in vitro* potency, their use is often associated with poor absorption, distribution, metabolism, and excretion (ADME) properties when applied *in vivo*. ADCs with hydrophobic payloads are prone to aggregation, non-specific binding, and uptake by phagocytic cells.[14-18] The covalent attachment of hydrophobic payloads increases the net hydrophobicity, while generating hydrophobic patches on the otherwise hydrophilic surface of the mAb. While these effects are directly proportional to the DAR, they depend on the site of conjugation.[54] To address these issues, an MMAE payload was modified by attachment of a glycoside leading to Tz-MMAU (Figure 2). The resulting more polar MMAU ADCs are less prone to protein aggregation resulting in lower systemic toxicity, when compared to less polar MMAE ADCs.[55] This modification reduces the hydrophobicity of MMAE and enabled the generation of higher DAR molecules (up to 8).[55] The glycoside-MMAE prodrug was stable in the bloodstream and extracellular space and released the active payload inside target cells upon hydrolysis by lysosomal glycosidases. The Tz-MMAU exhibits high *in vitro* potency (IC_{50} = 30 and 25 pM, respectively) in HCC1954 (copy number = 1×10^6) and NCI-H522 (copy number = 7×10^4) cell lines. There was evidence of bystander-activity (IC_{50} = 60 pM) in the case of Tz-MMAU in experiments involving the co-culture of Jurkat ($HER2^-$) and HCC1954 ($HER2^+$) cells. An *in vivo* study using the subcutaneous EGFR+ HSC-2 tumor xenograft model in nude mice showed the DAR=8 Tz-MMAU to be effective against 100 mm^3 tumors when dosed at 3 mg/kg (arrested tumor growth) and 10 mg/kg (complete tumor shrinkage and no recurrence for 100 days).

The potency of indolinobenzodiazepines (IGNs) and pyrrolobenzodiazepine (PBD) dimers is 100–1000-fold greater than traditional chemotherapy agents. One example, ADCT-502 is a highly potent PBD-dimer-based trastuzumab ADC (DAR = 1.7).[56-57] ADCT-502 was found to be active against HER2-positive, fluorescent in situ hybridization-negative (FISH-negative) breast cancer xenografted mice in the experimental group when dosed at only 0.2 mg/kg, compared to a marginal response seen when dosed with 30 mg/kg of Kadcyla.[56] Notably, ADCT-502 showed indirect bystander killing activity in $HER2^-$ MDA-MB-468 cells incubated with conditioned

medium from ADCT-502-treated HER2$^+$ SK-BR-3 cells. Unfortunately, ADCT-502 was discontinued in 2018 due to significant clinical toxicity in pulmonary tissue, though other trastuzumab PBDs are continuing to progress through clinical testing.[58] Another example of novel highly potent payload is SYD985, which contains the potent DUBA-prodrug (seco-DUBA). Seco-DUBA is highly active against HER2$^+$ SK-BR-3 (IC_{50} = 0.09 nM) and SK-OV-3 (IC_{50} = 0.43 nM) and the HER2$^-$ SW620 (IC_{50} = 0.09 nM) cells. Conversely, the ADC SYD985 (DAR = 2) is found to be highly active only against the HER2-positive breast and ovarian cancer with an IC_{50} of 0.22 nM and 0.44 nM, respectively.[40]

Enhertu is modified with the topoisomerase inhibitor deruxtecan, a payload capable of bystander killing. This recently approved ADC has been reported to show increased *in vivo* efficacy in NCI-N87 xenografts compared to Kadcyla, which uses a DM1 payload of similar *in vitro* potency.[59-60] Enhertu is approved to treat gastric cancer that has heterogenous expression of HER2 and was recently reported to significantly improved both progression-free and overall survival in DESTINY-Breast04 trial in patients with HER2-low metastatic breast cancer.[61,39]

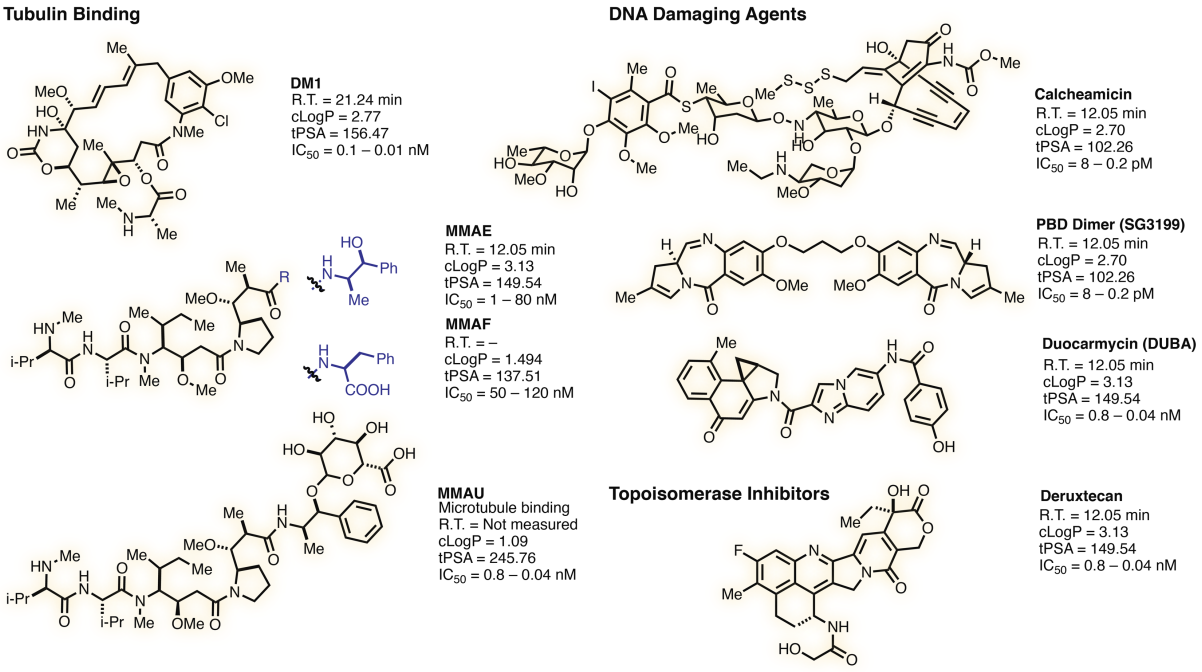

Figure 2. Examples of payloads used in ADC and their properties. R.T = retention time measured by Thurston *et al.* in their UPLC-based assay to assess the hydrophobicity of payloads.[49] cLogP and tPSA were measured using chemdraw. IC_{50} = Literature cell-growth inhibitory values across multiple reports (see Table S1).

Drug-to-Antibody ratio (DAR)

The drug-to-antibody ratio (DAR) represents the average amount of payload molecules conjugated to each antibody. Although a higher DAR would seem preferable, increasing DAR can be accompanied with significant issues.[62-63] For example, an analysis of Tz-MMAE conjugates with well-defined, yet varied DARs found that while at DAR 2 or 4, there was only 0.7% and 4.7% respectively aggregation over the course of two weeks, anything higher than that showed an

exponential increase in aggregation during the same time period.[64] The buildup of high molecular weight aggregate species is problematic as it can inhibit binding to receptor targets, can contribute to toxicity, and often alters clearance pathways.

Optimal DAR is an even greater consideration when using very hydrophobic payloads. Gieseler and coworkers released a report showing how the payload hydrophobicity, measured through total polar surface area (tPSA) and partition coefficients (LogP), correlates with sample aggregation over time.[54] These experiments were conducted at an uniform DAR of 8. The authors did not examine the effects of using varied drug-antibody ratios, but these experiments provide insight into the downsides of using high DARs with hydrophobic payloads. While not always the case, there are clear examples when increased DAR improves ADC activity. For example, a series of Tz-MMAE ADCs were generated through the use of a bis-sulfone based linker that specifically targets the reduced interchain disulfide bonds to produce stable ADCs with homogenous payload distributions.[65] This linker consists of a bis-alkylating bis-sulfone group conjugated to MMAE *via* a val-cit-PAB. It successfully attached the payload to the four interchain disulfides of Tz (Tz-bisAlk-vc-MMAE) with a narrow DAR (i.e., 4 ≅ 78%) distribution. Disulfide re-bridging with DAR 1– 4 was tested against SK-BR-3 cells as well as BT-474 xenograft tumor mouse models (Figure 3).[66] *In vitro*, the IC_{50} values were 0.12 nM, 0.07 nM, 0.05 nM, and 0.04 nM for DAR 1 – 4, respectively, compared to 0.22 nM for free MMAE. During the *in vivo* study, only DAR 4 completely ablated the tumor, while DAR 1-3 lead to DAR-dependent tumor-growth inhibition. These findings suggest that higher loading is preferable, so long as the DAR is kept low enough to prevent aggregation. A follow up study demonstrated a clear improvement in the stability of Tz-bisAlk-vc-MMAE (DAR = 4) compared to Tz-vc-MMAE (DAR = 4) over 120 h in free-thiol containing HSA.[66]

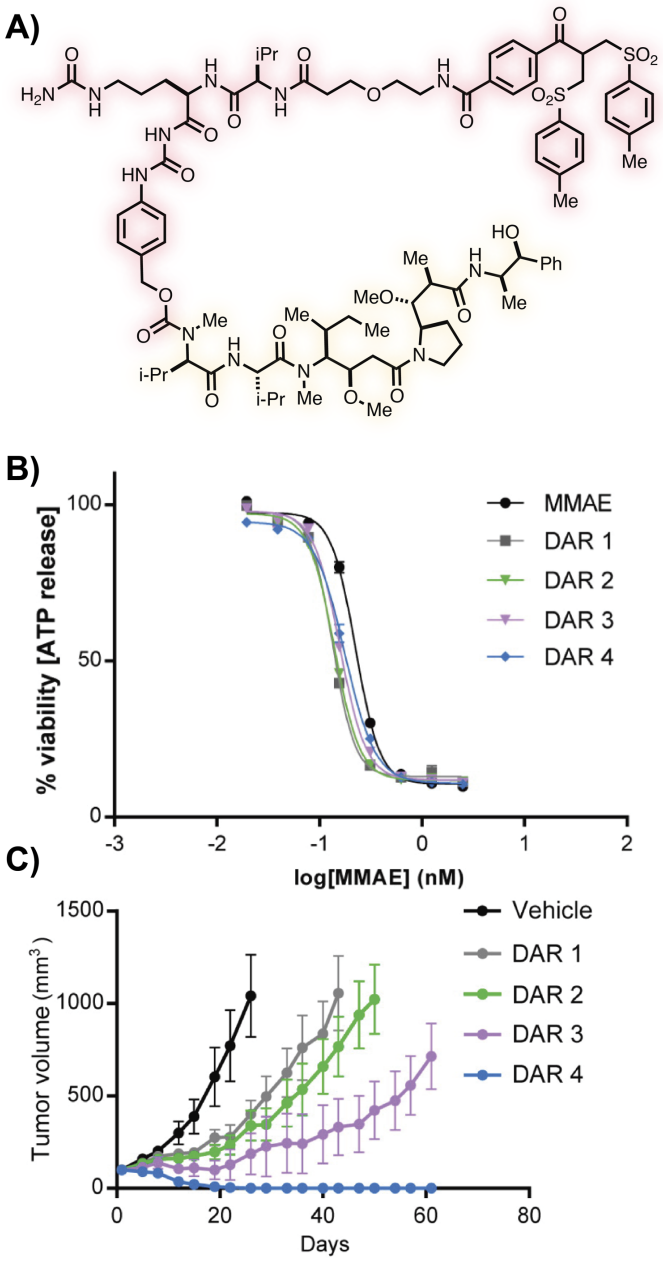

Figure 3. A) Structure of bisAlk-vc-MMAE motif and the B) *in vitro,* and C) *in vivo* evaluation of individual DAR 1-4 variants of Tz-bisAlk-vc-MMAE. Adapted from ref. 66 with permission from American Chemical Society.

A recent approach has extended the reach of high-DAR ADCs. Mersana developed Tz-dolaflexin, which enable DARs of 10–12 (Figure 4A,B).[67] Dolaflexin is based on a biodegradable, biocompatible, water-soluble polymer poly-1-hydroxymethylethylene hydroxymethylformal (PHF) polymer, termed "fleximer", capable of carrying multiple drug molecules.[68] When conjugated to Tz through a maleimide, Tz-dolaflexin is highly potent *in vivo* at a single dose of 0.67 mg/kg in mouse xenograft models and resulted in a prolonged tumor-free survival after a single 2 mg/kg dose in HER2 2+ expressing model that is insensitive to Tz-DM1. In mice, 20 and

30 mg/kg doses were well-tolerated, suggesting a therapeutic index (TI) > 40. This approach is proposed to benefit from a interesting type of bystander effect.[69] The membrane-permeable payload, auristatin F-hydroxypropylamide (AF-HPA), is capable of bystander killing. However, it undergoes amide hydrolysis to the membrane-impermeable and highly potent auristatin AF.[70] Co-culture assays with HER2-positive and HER2-negative cells were used demonstrate bystander killing. Additional biodistribution studies of the Dolaflexin-based ADCs were consistent with the proposed mechanism.[71]

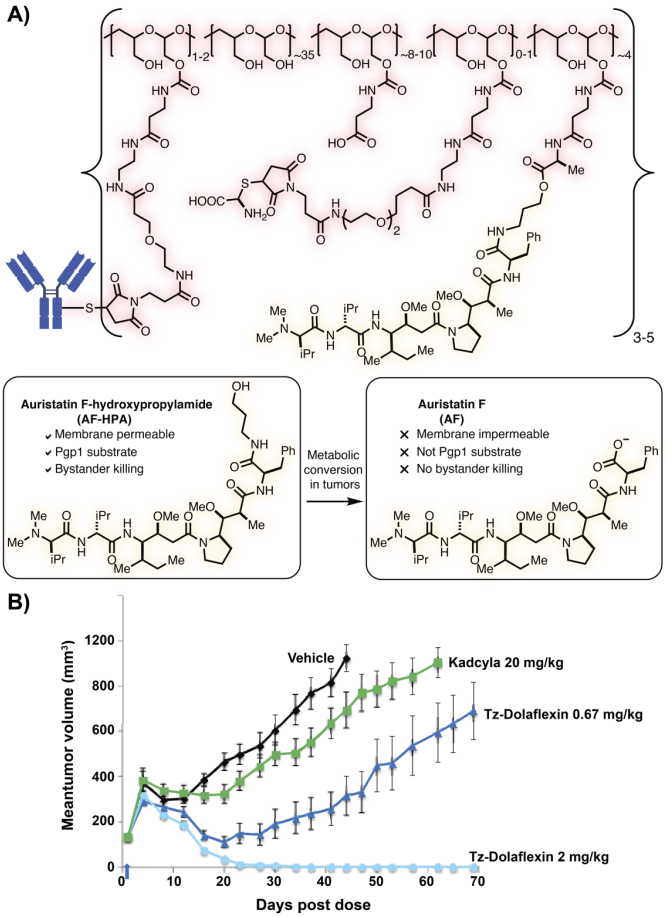

Figure 4. A) T-dolaflexin structure and drug release. B) Activity in HER2-positive JIMT-1 breast cancer xenograft. Adapted from ref. 66 with permission from American Association for Cancer Research.

In a separate example, Mersana Therapeutics further demonstrate the potential of their PHF-polymer-based Fleximer technology (Figure 6) using a vinca derivative-labelled trastuzumab ADC (DAR = 20).[68, 72] Interestingly, the antigen binding (k_d/per second) of Tz-PHF-vinca (5.1×10^{-6}) was similar to Tz (4.0×10^{-6}) in spite of appending a large polymer with 20 molecules of payload. HER2-positive specific *in vitro* potency was seen with an IC_{50} of 20.1 nM and 8.3 nM in SK-BR-3 and BT-474 cell lines, respectively. Highly efficient tumor-killing (over 60 days) was seen in NCI-N87 and BT-474 mouse xenograft models at 15.6 mg/kg and 20 mg/kg with 3 doses (1 week apart), respectively. This polymer-based approach offers an advantage over low-DAR ADCs, since it tolerates the use of moderately potent payloads and potentially expands the therapeutic window.

Linker Properties

An ideal linker must be stable while the ADC is circulating in the blood to limit off-target toxicity and enable release of the payload only at the target site.[2, 73] Not only acting to the append the payload to the antibody, linkers play an important role in the optimization of an ADC's PK/PD properties.[74-76] Published detailed linkers screens are uncommon, but those that are available provide key insights.

Linker chemistry can play a central role in activity and controlling bystander effects.[77-78] This is because with non-cleavable linkers, the drug-linker motif remains attached to at least the amino acid it was conjugated to. The resulting amino acid-linker-drug catabolites are typically too polar to exhibit bystander activity but can exhibit reduced potency compared to the corresponding ADC with a cleavable linker. However, non-cleavable linkers have proved beneficial in a few selective cases. Tz-DM1 is one such example which incorporates the non-cleavable succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (succinimidyl MCC, or SMCC) thioether moiety.[79] The design for this linker arose from stability and *in vitro* efficacy studies conducted by Sliwkowski and coworkers.[80-81] This study analyzed the effects of steric hindrance on the stability of disulfides within the linker design, comparing four different disulfide linkers as well as the non-reducible SMCC linker as seen in Figure 5A. It was observed that the Tz-MCC-DM1 conjugate and the Tz-SSNPP-DM4 conjugate, which has three methyl groups adjacent the disulfide group, had nearly identical mouse serum stabilities. After a period of seven days, 70% of the DM compounds were still conjugated to Trastuzumab. In contrast, the least hindered conjugate, Tz-SPDP-DM1, was completely cleared within 3 days. The (monomethyl) conjugate, Tz-SPP-DM1, also exhibited almost complete clearance within the seven-day period, while Tz-SSNPP-DM3 showed moderate clearance. When these conjugates were administered as single 10 mg/kg doses with MMTV-HER2 Founder 5 (Fo5) tumors, the Tz-MCC-DM1 and Tz-SSNPP-DM3 conjugates showed the greatest efficacy while the Tz-SPP-DM1 and Tz-SSNPP-DM4 conjugates produced only a slight response compared to a vehicle injection. Furthermore, weight change experiments conducted in Sprague-Dawley rats showed that the SMCC linker conjugate had virtually no difference in toxicity compared to the control injection at over twice the therapeutic dose in the tumor study, while Tz-SPP-DM1 showed a dramatic weight loss. This toxicity difference is believed to be due to lower levels of circulating free drug caused by serum cleavage with the SMCC linker compared to all other linkers.[82] Given the stability of Tz-MCC-DM1, as well as its efficacy and low toxicity *in vivo*, the SMCC linker was chosen as the optimal linker to study and continued on to further testing and eventual clinical success, as Kadcyla. In this example, the benefits of reduced toxicity, due to the non-cleavable linker, outweighed any benefits of well-controlled cleavable linker chemistry.

Another recent example of this type of linker screen used an auristatin derivative[83] This auristatin derivative was optimized by screening various linkers against HCC1954 cells *in vitro*. The derivative was then conjugated to Trastuzumab using the linkers shown in Figure 5B, with the val-cit-PABA being the only linker that provided response *in vitro* or *in vivo*. This val-cit-PABA conjugate possessed an improved IC_{50} of 0.06 nM compared to an IC_{50} of 0.39 nM as free drug. Moreover, this cleavable conjugate was effective when tested against HCC1954 implant tumors in CB17/SCID mice, showing similar efficacy to the clinically approved conjugate Tz-DM1. The authors also postulated that the carbamate-based linkers, which were theoretically cleavable, would be effective *in vitro*. However, these conjugates worked no better than the non-cleavable

amide-linked conjugates due to the poor susceptibility of the carbamate functional group to intracellular conditions.

These linker screens also highlight the importance of considering the metabolic fates of each payload and the interplay between linker and payload chemistry. In the case of DM1, previous studies have shown that the disulfide-DM1 metabolite released by lysosomal processing is rapidly cleared after exiting cells.[84] Therefore, while the payload in theory could undergo bystander killing effect, diffusion can also lower the overall drug concentration in the targeted cell while not leading to growth effects on adjacent cells. The auristatin, particularly MMAE, payloads, in contrast, are almost two orders of magnitude more hydrophobic (cLogP) and therefore exhibit excellent membrane permeability. In this case, a non-cleavable linker that traps the drug metabolites in the targeted cell would impact its inhibitory function.

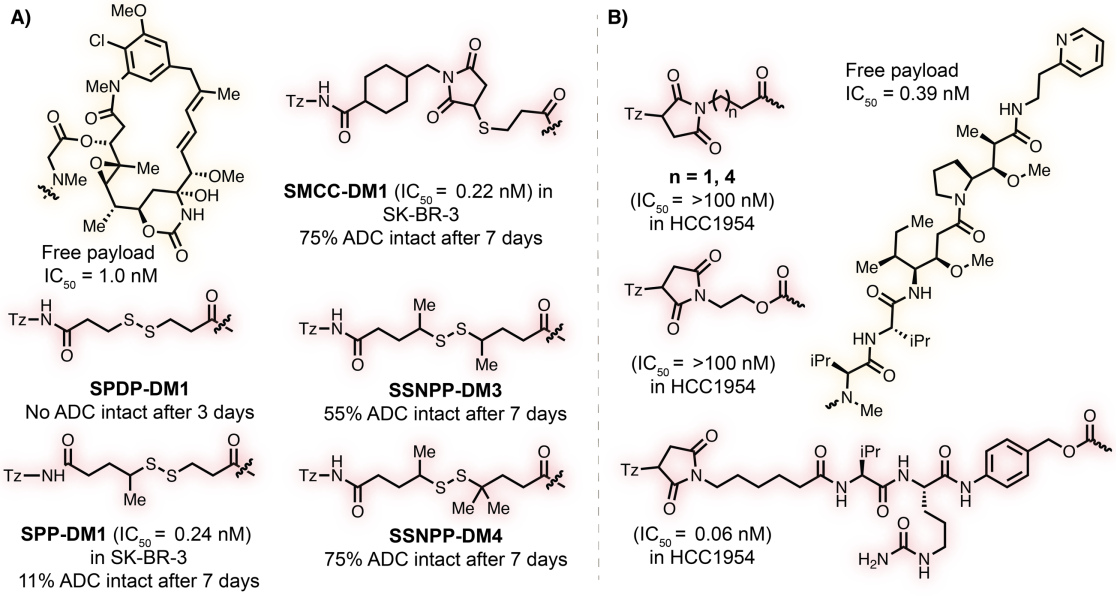

Figure 5. Linker screen to understand the impact of linker choice on the efficacy of A) DM1 and B) MMAE-based ADCs

Another recently developed trastuzumab-based ADC (CAT-01-106) is reported to reduce toxicity through linker and conjugation choices (Figure 6).[85-86] Like T-DM1, this ADC utilizes a maytansinoid payload, but it is site specifically conjugated with a non-cleavable linker. Likely due to conjugation choices, this ADC is observed as more tolerable during *in vivo* studies. Together, the linker and payload, termed RED-106, offer unique benefits, including excellent tolerability and efficacy providing a wide therapeutic window and resistance to the drug efflux pump, P-glycoprotein/MDR1.[86] CAT-01-106 (DAR 1.8) shows improved tolerability and survivability with greater anti-tumor action despite being at half the DAR of Kadcyla. In rats and cynomolgus monkeys, data indicated a therapeutic window increase of 5- to 10- fold for CAT-01-106, and 40% higher exposure levels. While these levels are still below the trastuzumab alone, the clinical trials on CAT-01-106 indicate comparable exposure levels alongside effective cytotoxicity and tolerability.[85]

Lastly, systematic linker design can be critical for enhancing the bystander killing effect. In a recent study, three ADCs were Tz-DM1, DS-8201a (Tz-Deruxtecan), and Anti-HER2-DXd were tested in co-cultures of KPL-4 and MDA-MB-468 (Figure 1, Figure 6).[87] DS-8201a and Anti-HER2-DXd both use cleavable linkers, but only the exatecan analog produced by DS-8201a is cell permeable. All three conjugates could kill KPL-4 cells due to their targeting of HER2 expressed on the membrane but could not kill the MDA-MB-468 cells, as they are HER2-negative. Interestingly, when the KPL-4 and MDA-MB-468 cells were grown together and tested with the conjugates, DS-8201a was almost as effective at killing the MDA-MB-468 cells as it was at killing the KPL-4 cells. However, neither Tz-DM1 nor Anti-HER2-DXd could kill the MDA-MB-468 cells, and instead only selectively killed the KPL-4 cells. This study emphasized the importance of linker design in leveraging the bystander killing effect of an attached payload.

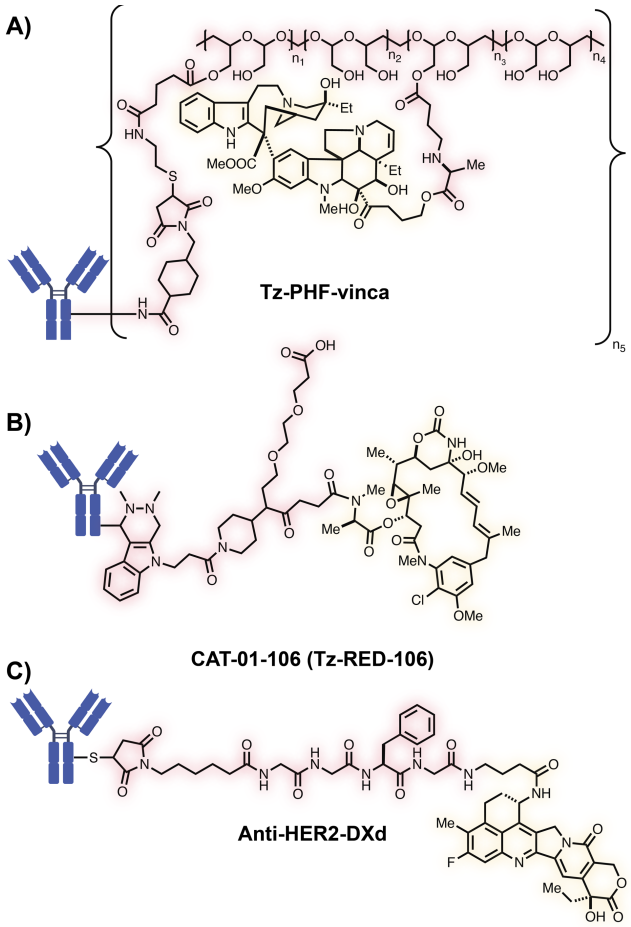

Figure 6. Chemical structure of A) Tz-PHF-vinca (DAR = 20), B) CAT-01-106 (DAR = 1.8), and C) Anti-HER2-DXd (DAR ≅ 8).

Site-specific conjugation

Conventional conjugation methods provide heterogeneous payload distribution, which can contribute to sub-optimal therapeutic indices and higher clearance rates. Additionally, the formation of higher DAR ADCs through the hinges cysteines via reduction/modification of solvent accessible (interchain) disulfide bonds can affect the antibody's structural integrity and the overall *in vivo* stability of the ADC. Additionally, despite having a seemingly non-cleavable linker, Tz-

MCC-DM1 (Kadcyla) shows a significant deconjugation through maleimide retro-Michael chemistry. These concerns have led to extensive efforts to develop site-specific antibody conjugation technology.[88-89] The most broadly employed of these was identified by Genentech and uses engineered-cysteine substitutions at defined light-and heavy-chain positions provide reactive thiol groups for uniform payload-linker conjugation.[90] These ADCs, known as THIOMABs, have a near-uniform stoichiometry of payloads per antibody molecule without disruption of interchain disulfide bonds. Unfortunately, initially-reported variants suffered high deconjugation rates as the result of the thiol-reactive linkers being positioned in a highly solvent-accessible site, resulting in maleimide exchange with reactive thiol groups in albumin.[91-92] This deconjugation issue was overcome with the development of second generation THIOMABs, which were identified through a systematic screening of possible linkage sites.[93] It was observed that accessible sites with a positively charged local amino acid environment promote hydrolysis of the succinimide ring in the linker, thereby preventing the retro-Michael addition. In related efforts, Junutula *et al*. compared three trastuzumab ADCs with engineered cysteines introduced at different positions.[94] Each engineered cysteine (LC-V205C, HC-A114C and HC-S396C) had varying levels of solvent accessibility and local charge. It was observed that conjugates derived from cysteine residues with low solvent accessibility and positive local charge were more plasma stable, suggesting that these features prevent maleimide exchange with plasma thiols. These differences in stability translated to an increase in therapeutic efficacy, *in vitro* target antigen binding, internalization, and potency.

A series of DM1 derivatives comparing both linker chemistry and site-specific conjugation were evaluated (Figure 7). Using an engineered thio-HC-A114C-Tz via a BMPEO (bismaleimidopolyethylene oxide) linker results in a site-specific (HC-A114K) Thiomab (T-Tz-MPEO-DM1).[82] The authors compared Tz-MCC-DM1 (DAR 3.3) and T-Tz-MPEO-DM1 (DAR 1.8) for their PK/PD properties and overall efficacy. The *in vitro* evaluation of both the ADC and TDC (Thiomab Drug Conjugate) of interest showed similar cell-permeability, target binding, and cell-killing against SK-BR-3 cell line. An *in vivo* analysis was conducted in the MMTV-HER2 Fo5 Tz-resistant mammary tumor model. The TDC (T-Tz-MPEO-DM1) was found to be much more efficacious than the ADC (Tz-MCC-DM1) when similar amounts of DM1 were dosed. The exposure-based therapeutic index was larger for the TDC compared with ADC by 1.6- to 2.0-fold based on C_{max} and AUC, respectively. There was an improvement in tolerability associated with the TDC as the conjugate exposure associated with toxicity was greater for the TDC at 48 mg/kg than for ADC at 30 mg/kg.

To test the role of stability, two TDCs (T-Tz-MPA-May and T-Tz-MC-May) without a reversible thioether succinimide connection between the drug and linker were compared (Figure 7).[95] Additionally, these were conjugated at the LC-V205C site of trastuzumab to minimize maleimide exchange at the linker-Tz junction. The new TDCs had increased *in vitro* stability in plasma relative to Tz-MCC-DM1 over time, which can be a result of both reduction in the deconjugation and an increased stability of the TDC due to lower DAR. The *in vitro* evaluation in the SK-BR-3 cell line showed that T-Tz-MPA-May (DAR = 1.8) and T-Tz-MC-May (DAR = 1.8) had an IC_{50} of 10.9 nM and 11.8 nM, respectively. In comparison, the IC_{50} for Tz-MCC-May (DAR = 3.5) was only 5 nM. Interestingly, incubation in mouse plasma for 168 hours showed no free DM1 in the case of T-Tz-MPA-May and 180 nM of DM1 in Tz-MCC-DM1. This stability improvement led to improve *in vivo* (HER2-positive Fo5 transplant tumor model) efficacy (at 10 mg/kg), with the stabilized T-Tz-MPA-May (DAR = 1.8) matching the efficacy of Tz-MCC-DM1 (DAR = 3.5) with half the amount of drug while showing improved efficacy over the T-Tz-MPEO-

DM1 TDC (DAR = 1.8) with the same drug load. Its noteworthy that T-Tz-MPA-May (10 mg/kg) resulted in a higher tumor growth inhibition (TGI) at 93% compared with 79% of the T-Tz-MPEO-DM1 TDC. Further improvement in efficacy could be achieved by engineering four cysteine residues into Tz. The resulting DAR = 3.9 site-specific ADCs (T-Tz-MPA-May, T-Tz-MC-May) matched the *in vitro* potency of Tz-MCC-DM1 while showing significantly better *in vivo* efficacy. Site-specific ADCs with DAR=2 are reported to demonstrate a 2-fold improvement in safety profiles over Tz-MCC-DM1with improved efficacy in Sprague-Dawley rats and cynomolgus monkeys.[82]

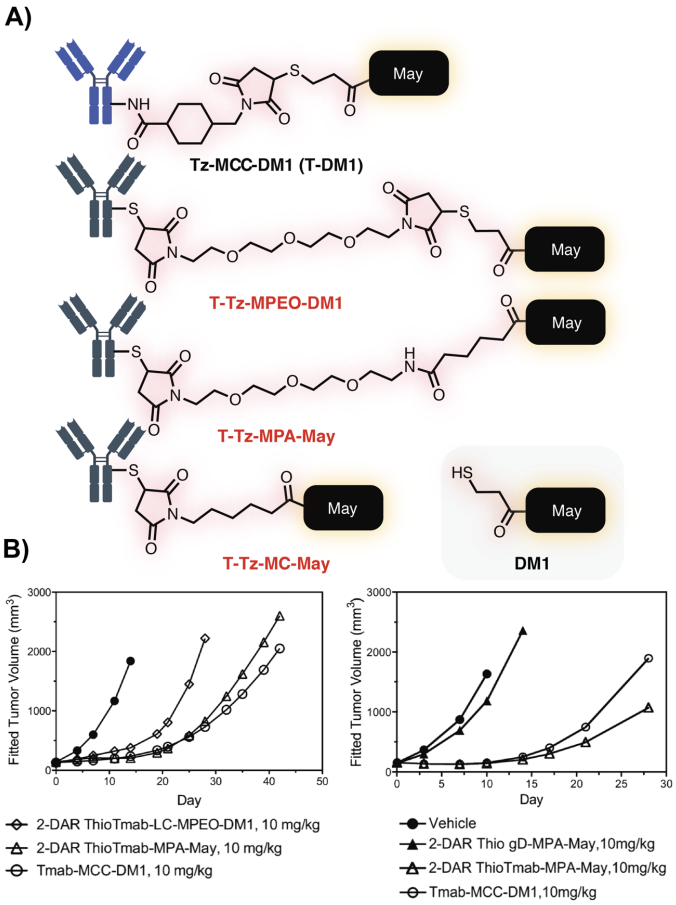

Figure 7. Comparison of the Tz-based THIOMAB TDCs. Adapted from ref. 93 with permission from American Chemical Society.

A recent study found conjugating the peptide-based payloads to mAb can impacted by the termini used. Using ADC conjugates with Dolastatin 15 (Dol15) attached at either the C- or N-terminus using a variety of cleavable and non-cleavable linkers.[96] Interestingly, neither the N-terminus conjugate utilizing the cleavable Val-Cit moiety nor the conjugate using the MCC moiety produced any significant inhibitory effect *in vitro* (IC_{50} > 500 nM). However, both non-cleavable conjugates at the C-terminus, one using the MCC moiety, and another conjugated through an amide, produced potent responses *in vitro* (IC_{50} of 0.50 nM and 0.31 nM respectively). The authors speculate that these differences in activity were due to varied metabolic products produced by each conjugate in lysosomes, with the N-terminal conjugates producing lysosome-impermeable metabolites while the C-terminal derivatives produced permeable metabolites. Analysis of cell

lysates after exposure to each conjugate identified the proposed metabolite structures, lending support to this idea.

Disulfide re-bridging methods involve the reduction of the endogenous disulfide bonds and reacting with a cysteine selective cross-linking reagent. As this approach does not require genetic engineering, it provides a straightforward, commonly used technique for site-specific antibody modification. In 2014, this was first applied using the bis-sulfone bridging method to link the MMAE through PEG_{24} spacer and Val-Cit-PABC linker to trastuzumab resulting in the DAR 4 (78%) species as the major product of the reaction.[97] Notably, the ADC displayed higher efficacy and excellent stability in the presence of human serum albumin (HSA) compared to the unconjugated trastuzumab in a xenograft model after 5 doses at 20 mg/kg. In a follow-up study by Bryant *et al*., an analogous ADC containing a shorter PEG_6 spacer was evaluated and compared to T-DM1. In a JIMT-1 mouse xenograft model it was found that while both ADCs showed comparable activities at a dosage of 5 mg/kg, the re-bridged ADC was significantly more efficacious than T-DM1 at 10 mg/kg. In related efforts, Sorrento therapeutics recently reported the first efficacy results of A166 ADC loaded with the proprietary duostatin-5 site-specifically conjugated to trastuzumab via a val-cit peptide. This ADC incorporates the site-specific disulfide re-bridging method K-Lock™ conjugation chemistry. Many cysteine-engineered antibodies require a two-step reduction–partial oxidation process to expose the reactive cysteine prior to conjugation. However, one report described the expression of a trastuzumab variant (LC-Q124C) that was isolated with a ready-to-conjugate poorly-exposed and highly reactive cysteine.[98] Modification of this residue with maleimidocaproyl-Val-Cit-PAB-MMAE produced a homogeneous ADC with a DAR of 2.

Comparison of Trastuzumab ADCs in SK-BR-3 Cell-line

Direct comparisons of the activities of various ADCs can be difficult due to the use of different cell lines and tumor models during testing. However, a commonly used HER2-positive breast cancer cell line, SK-BR-3, provides some basis for comparison given its widespread use for *in vitro* inhibition studies.[33, 40, 66, 80, 96, 99-102] Table 1 shows a variety of Trastuzumab conjugates ranked by their activity against SK-BR-3 cells We used this presentation to allow assessment of key ADC design criteria. From this data, we make several observations. First, hydrophobic payloads tend to be less effective, especially when conjugated at high DAR. This can be seen when comparing Monomethyl Auristatin E (MMAE) conjugates to those containing Dolastatin 15 (Dol15), both of which are auristatin derivatives. Dol15 is an order of magnitude more hydrophobic. When both are conjugated to Trastuzumab at DAR 4, MMAE is almost eight times more potent than Dol15. Furthermore, all the MMAE conjugates were more potent than every single Dol15 conjugate. The most hydrophilic payload, Deruxtecan, is near the top of the efficacy list possibly due to a high DAR. From a clinical standpoint, this could explain the higher response rate in patients with low HER2-positive expression. Another observation from these data is that cleavable linkers when it comes to the most potent ADCs. Of the top eight conjugates, only one, Tz-DM1, contains a non-cleavable linker. This is likely due to the ability of cleavable ADCs to release payloads more efficiently. T-DM1 is an exception because of high DM1 system clearance as previously discussed. Lastly, lower DARs tend to be favorable for activity. This is the result of second-generation ADCs utilizing very hydrophobic, aggregation-prone payloads that would reduce efficacy if conjugated at higher DAR. The notable exception is Tz-Deruxtecan, which has a hydrophilic payload that likely avoids aggregation. Remarkably, Tz-bisAlk-vc-MMAE can

achieve higher activity than most other conjugates even at low DAR and outperforms all other compared conjugates at DAR 4. Conversely, low potency payloads like cisplatin perform poorly even when conjugated at high DAR. These observations reinforce the need to employ high-potency payloads to be able to achieve efficacy, while also maintaining the physical properties required to prevent aggregation.

Table 1. Comparing the *in vitro* potency of different Tz-ADCs in SK-BR-3 cell line. clogP and tPSA computed for free drug.

Combination	Payload	DAR	clogP	tPSA	Linker	Cleaving ability	IC_{50} (nM)
Tz-bisAlk-vc-MMAE	MMAE	4	4.86	149.5	Val-Cit with Disulfide Bridging	C	0.04
Tz-Deruxtecan (DS-8201a)	Deruxtecan	7.7	-0.58	300.41	Protease-Sensitive Peptide	C	0.044
Tz-DM1	DM1	1.9	4.04	156.5	Maleimide Conjugate	NC	0.047
Tz-bisAlk-vc-MMAE	MMAE	3	4.86	149.5	Val-Cit with Disulfide Bridging	C	0.05
Tz-bisAlk-vc-MMAE	MMAE	2	4.86	149.5	Val-Cit with Disulfide Bridging	C	0.07
Tz-bisAlk-vc-MMAE	MMAE	1	4.86	149.5	Val-Cit with Disulfide Bridging	C	0.12
T-Tz-PEG_{12}-vc-PAB-MMAE	MMAE	1.8	4.86	149.5	NH-PEG12-vc-PAB	C	0.19
Tz- Duo (SYD985)	Duocarmycin	2	1.1	135.7	Peptide	C	0.22
Tz-deBouganin (T-deB)	deBouganin	1.9	–	–	–	–	0.275
Tz-Amide-C-Term-Dol15	Dol15	4	5.85	132.9	Direct Lysine conjugated	NC	0.31
Tz-MC-Dol15-C-Terminus	Dol15	5.2	5.85	132.9	Maleimide Conjugate	NC	0.50
Tz-PHF-Vinca	Vinca alkaloid	20	–	–	Polyacetal backbone/ester linkage	C	1.3
Tz-MI130004	MI130004	1.8	4.23	132.06	Extended MC linker	NC	30
Tz-MC-Dol15-N-Terminus	Dol15	3.8	5.85	132.9	Maleimide Conjugate	NC	666
Tz-MC-vc-PABC-Dol15-N-Terminus	Dol15	5.9	5.85	132.9	Maleimide w/ Cleavable Peptide	C	542
Tz-Cisplatin	Cisplatin	6.8	-1.68	53.6	Amide, Coupled from Protein	NC	21330

Conclusions

Since the first ADC was approved for clinical use almost two decades ago, this strategy has led to a series of effective cancer therapeutics. To date, twelve ADCs have been approved and many more are in various stages of clinical development.[103] Despite this progress, the optimization of new ADCs is a daunting task owing to the many variables involved. Issues of aggregation and limits of antigen-based uptake mean that it is beneficial to apply high potency payloads. The development and translation of second-generation ADCs using auristatin, maytansinoid, and exatecan conjugates has validated this premise. However, newer technology, like Mersana Therapeutic's Dolaflexin platform, illustrate the potential of strategies that apply high DAR. This could open the potential to using of less toxic, better tolerated payloads. Moreover, advancements in site-specific conjugation have transformed ADCs from being heterogeneous mixtures of conjugates with different DARs to more homogenous entities, owing to the advancement of THIOMABs and the application of disulfide bridging. Additionally, several groups have recently reported their efforts in using mAbs to build immune stimulating antibody conjugates (ISACs).

Efforts to apply these approaches have found that conjugating immunomodulating drugs (e.g. TLR agonists) to Tz can provide durable reductions to tumors that are refractory to the standard care of therapies.[104-105]

This review stresses the critical role of chemical components in building an efficient ADC. There remains a significant need for technologies to assess these factors earlier in the ADC design process. A powerful approach is to apply optical imaging to address questions of antibody targeting and linker activation.[59, 106-110] Such studies have provide significant evidence highlighting the dramatic role of payload properties on antibody targeting.[111-114] Thurber and coworkers recently reported efforts to image bystander penetration of microtubule inhibitors, DNA-damaging agents, and topoisomerase inhibitors.[107] They note that MMAE, calicheamicin D, and exatecan showing the greatest bystander killing of the agents measured. In a separate report, the authors also found that lowering the DAR during treatment with T-DM1 ADC can improve the intertumoral distribution of the ADC resulting in a better *in vivo* efficacy.[59] This approach has been extended to a clinical setting in approach that used panitumumab-IRDye800CW conjugate as a surrogate for ADC delivery. These efforts observed that co-administering the parent mAb along with the antibody conjugate significantly improved the tissue penetration and thus its therapeutic efficacy.[109]

In addition to assessing the tumor targeting of mAbs, efforts to define the role of ADC linker chemistry would benefit from new imaging approaches. Our group recently reported fluorogenic (i.e., turn-ON) probes that operate in the near-infrared (NIR) range.[115] We has used the antibody-fluorophore conjugates as a surrogate to compare a panel of several common ADC linkers across two antibodies (anti-EGFR, Cetuximab, and anti-CD-276).[116] These *in vivo* imaging studies clearly indicate that cathepsin-cleavable linkers provide higher tumor activation relative to hindered or non-hindered disulfides, at least in this context. Critically, cellular studies alone suggested similar activation across the linkers – an observation that highlights the need for *in vivo* methods that assess linker stability in complex settings.[106] Going forward, such methods have significant potential to assist in the optimization of new linkers, for example against non-internalizing antigens and emerging targets.[110, 117-119] Overall, advances to the chemical components – the linker and payload - has broad potential to lead to next-generation ADCs with improved tolerability suitable for a range of otherwise challenging tumor classes.

ASSOCIATED CONTENT

Supporting Information. A summary of the chemistry used and any available *in vitro* and *in vivo* data is listed in the attached Supporting Information document.

AUTHOR INFORMATION

Corresponding Author

*Martin J. Schnermann

Email: martin.schnermann@nih.gov

Author Contributions

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