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Bridging Disulfides for Stable and Defined Antibody Drug Conjugates

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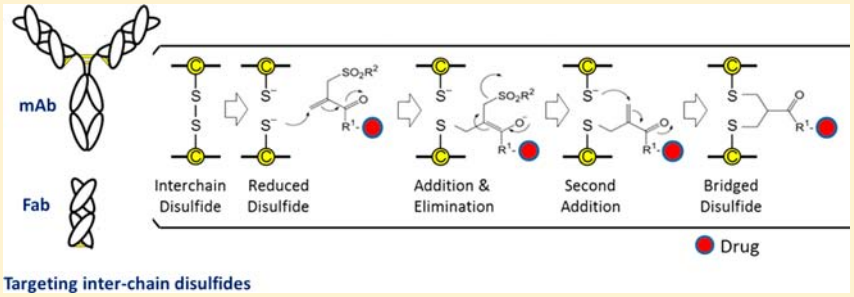

Targeting inter-chain disulfides

ABSTRACT: To improve both the homogeneity and the stability of ADCs, we have developed site-specific drug-conjugating reagents that covalently rebridge reduced disulfide bonds. The new reagents comprise a drug, a linker, and a bis-reactive conjugating moiety that is capable of undergoing reaction with *both* sulfur atoms derived from a reduced disulfide bond in antibodies and antibody fragments. A disulfide rebridging reagent comprising monomethyl auristatin E (MMAE) was prepared and conjugated to trastuzumab (TRA). A 78% conversion of antibody to ADC with a drug to antibody ratio (DAR) of 4 was achieved with no unconjugated antibody remaining. The MMAE rebridging reagent was also conjugated to the interchain disulfide of a Fab derived from proteolytic digestion of TRA, to give a homogeneous single drug conjugated product. The resulting conjugates retained antigen-binding, were stable in serum, and demonstrated potent and antigen-selective cell killing in *in vitro* and *in vivo* cancer models. Disulfide rebridging conjugation is a general approach to prepare stable ADCs, which does not require the antibody to be recombinantly re-engineered for site-specific conjugation.

INTRODUCTION

Antibody drug conjugates (ADCs) are a growing class of chemotherapeutic agents for the treatment of cancer, combining the tumor targeting properties of antibodies with the cell killing properties of potent cytotoxic drugs. Although both monoclonal antibodies (mAbs) and cytotoxic agents can be used independently to treat cancer, the covalent attachment of a drug to a targeting antibody allows selective delivery and minimizes systemic toxic side effects from exposure of the drug to healthy tissue.

The clinically established approach to conjugating cytotoxic compounds to antibodies is to use linkers that are reactive toward either the amino side chains of lysine residues or the thiol side chains of cysteines obtained from reducing interchain disulfide bonds. Conjugation to lysine residues can be achieved via reaction with reactive groups such as a succinimidyl moiety to form a stable amide bond. This approach is used for the covalent attachment of the maytansinoid derivative, DM1, to TRA to produce the FDA approved Kadcyla.[1] However, as a mAb may have more than 80 solvent-accessible lysine residues, heterogeneous mixtures with variable DAR species are produced due to the numerous sites of conjugation available.[2]

Cysteine residues are less common within a mAb and therefore provide fewer sites for attachment and with higher reactivity. An intact mAb IgG1 has four accessible disulfide bonds that can be reduced to release eight free cysteine thiols that can serve as sites for conjugation.[3−5] This strategy is used to attach MMAE to brentuximab to produce the other FDA approved ADC known as Adcetris.[5] The reaction between the mAb and the maleimide-MMAE reagent yields an ADC composed of a mixture of species with between 0 and 8 drug molecules per antibody molecule.[5,6] The formation of high drug loaded species, e.g., greater than DAR 4, is undesirable as it has been shown to lead to lower tolerability, higher plasma clearance rates, and decreased efficacy *in vivo*.[6]

Maleimide based conjugates have been shown to undergo retro-addition reactions in the presence of competing thiols.[7−9] In serum this can result in loss of the drug from the ADC by exchange reaction with the free thiol group of circulating albumin.[7,9] Monoalkylation reagents also leave the original

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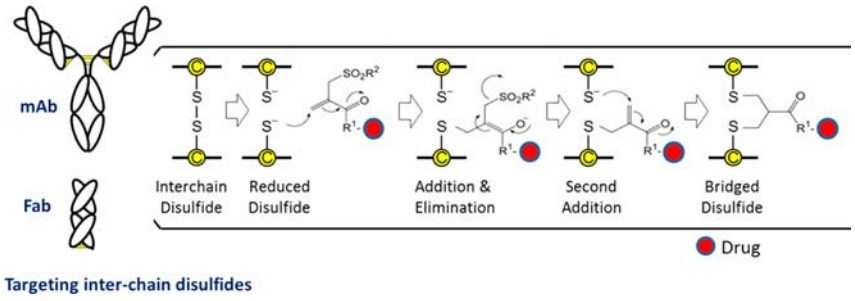

Figure 1. Conjugation of a payload to a disulfide bridge using a bis-alkylating conjugation approach involving a sequence of Michael addition and elimination reactions.

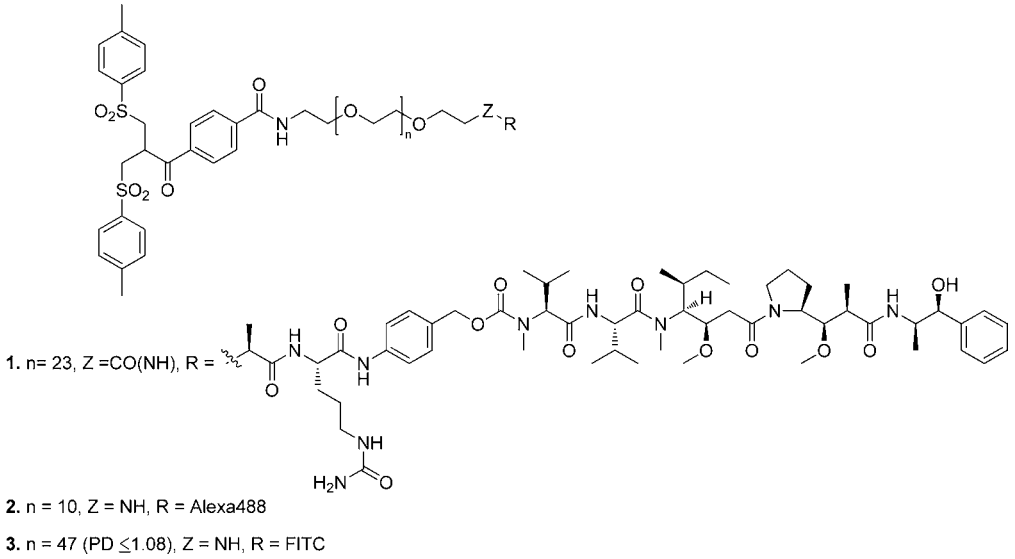

Figure 2. Chemical structures of the bis-alkylating reagents **1**, **2**, and **3** loaded with either a cytotoxic payload (MMAE) or fluorescent dyes, Alexa Fluor 488 and FITC, respectively. The fluorescent dyes are linked to the disulfide bridging linker via a noncleavable amide bond while MMAE is linked by an enzyme cleavable valine citrulline dipeptide and self-immolative *p*-aminobenzyl moiety.

disulfides unbridged, potentially introducing instability to the antibody.

One alternative to overcome a wide DAR distribution is to use mAbs with engineered cysteine residues. Antibodies known as THIOMABs have been described with an engineered and unpaired cysteine residue on each heavy or light chain designed for conjugation of a cytotoxic payload.[9−11] Each of the cysteine residues provides a site for conjugation of a thiol-reactive drug reagent. When conjugated to MMAE[10] or DM1[11] using a maleimide reagent, an ADC with a DAR close to 2 is produced. While this approach can be used to produce more homogeneous products it does not address issues associated with the instability of maleimide conjugates *in vivo*. Engineered cysteine thiols can also be capped during production such as by a disulfide forming reaction with glutathione.[10] Liberating a capped thiol to make accessible for conjugation without reducing interchain disulfides is nontrivial. In addition, the engineered cysteine residues have been shown to form triple light chain species during production.[12,13]

Another re-engineering approach is to incorporate unnatural amino acids in the antibody as sites for conjugation and avoid the use of maleimide chemistry. To improve specificity non-natural amino acids can be introduced with functionalities that enable conjugation chemistries to be used that are orthogonal to those typically used for native amino acids. ADCs have been synthesized by introducing a *p*-acetylphenylalanine residue site specifically into TRA.[14] Conjugation of a cytotoxic payload is then achieved by using an alkoxy-amine derivatized drug to form an oxime bond. Similar approaches are possible using azide/alkyne "click" chemistry.[15] Conjugation to unnatural amino acids is a promising alternative to produce homogeneous ADCs but has some limitations. Re-engineering mAbs to incorporate unnatural amino acids is complex and costly. In addition, the current methods reported for conjugation to form an oxime use a large excess of up to 30 equiv of cytotoxic reagent and have long reaction times of up to 4 days.[14] When an ADC is derived from mAbs that have previously undergone clinical development, re-engineering may not be cost-effective given that GMP manufacturing processes will already be in place.

We have developed a novel way to prepare more homogeneous and stable ADCs that does not require protein re-engineering (Figure 1). This approach uses bis-sulfone reagents that are selective for the cysteine sulfur atoms from a native disulfide (Figure 2). These reagents undergo bis-alkylation to conjugate both thiols derived from the two cysteine residues of a reduced native disulfide bond[16] such as the interchain disulfide bonds of a mAb. The reaction results in covalent rebridging of the disulfide bond via a three carbon bridge leaving the protein structurally intact. This approach has also been applied for the site-specific conjugation of poly-(ethylene glycol) (PEG) to a wide range of therapeutic proteins

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to enhance their pharmacokinetic properties while retaining their activity.[17,18]

A cytotoxic drug molecule is attached to the bis-alkylating linker via a PEG chain producing a reagent suitable for generating ADCs. The reagent can undergo reaction at each of the four interchain disulfides of a mAb if they are first reduced, to produce DAR 4 ADCs. As the disulfides are reannealed the resulting ADCs are more homogeneous and will have greater stability compared to maleimide conjugates. Here we describe the use of bis-alkylating conjugation of MMAE to TRA and a TRA derived Fab (Fab_{TRA}) to produce biologically active model drug conjugates.

RESULTS

Preparation of Drug Conjugates. Conjugation of both cysteine thiols derived from a reduced disulfide bond using the bis-alkylating approach is carried out in two steps and analogous to a standard maleimide conjugation process, namely, (1) disulfide reduction to release the two cysteine thiols followed by (2) conjugation under mild conditions. Conjugation by bis-alkylation leads to the formation of a three-carbon bridge relinking the two cysteine residues to which the drug payload is covalently attached.[18] The three-carbon bridge is small enough and sufficiently flexible not to perturb the tertiary structure of the antibody.[20]

The MMAE bis-alkylating reagent **1** was prepared with a Cathepsin B cleavable valine citrulline *p*-aminobenzyl ether linker designed to allow release of MMAE intracellularly from the ADC. A small ethylene glycol spacer of 24 repeat units and discrete mass was also incorporated into the reagent to improve the water solubility and enable more efficient conjugation. It was not possible to perform the conjugation without PEG present in the reagent due to low solubility in the conjugation media. It was also hoped that the incorporation of PEG would reduce the propensity of the resulting ADCs to aggregate and potentially improve the pharmacokinetic profile of the conjugates in the case of the Fab. A PEG size of 24 repeat units was chosen as this was one of the largest sizes of PEG available with a discrete mass.

TRA was selected as a model antibody to explore the preparation of ADCs by bis-alkylation because of well-established *in vitro* and *in vivo* models. In addition to the TRA ADC, a Fab drug conjugate (Fab_{TRA}-bisAlk-vc-MMAE) was prepared which, when analyzed by SDS-PAGE, gave supporting evidence that the bis-sulfone functionality of MMAE reagent **1** was able to rebridge an interchain disulfide. Purified Fab_{TRA} was first treated with DTT to reduce the accessible single interchain disulfide[17] and conjugation was performed after removal of residual DTT. Analysis by SDS-PAGE showed that conjugation conversion was nearly quantitative (95%) using only 2 equiv of reagent **1** with only a trace amount of reduced Fab_{TRA} remaining in the reaction mixture (Figure 3). When 1.5 equiv of reagent **1** was used, 88% conversion was observed. Unreduced Fab_{TRA} ran as a single band at 50 kDa (Lane 2), but the reduced Fab_{TRA}, after treatment with DTT, shifted in apparent molecular weight running as a single band at 25 kDa (Lane 3). This band corresponded to the V_HC_{H1} from the heavy chain and the V_LC_L from the light chain which dissociate in the presence of SDS after reduction of the disulfide and migrate together as they are the same size. In contrast, the Fab_{TRA}-bisAlk-vc-MMAE conjugate migrated as a single band at 50 kDa indicating that

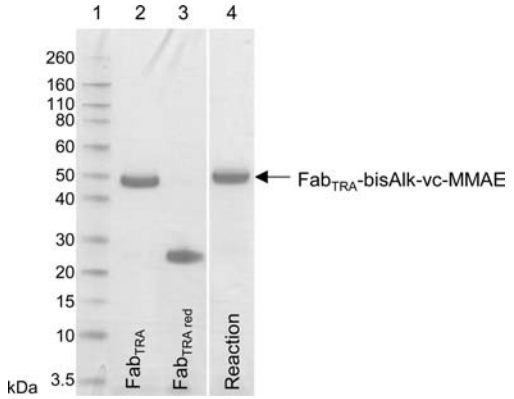

Figure 3. SDS-PAGE analysis of a conjugation reaction mixture between MMAE and Fab_{TRA} using the bis-alkylating reagent **1**. Unreduced Fab_{TRA} and the Fab_{TRA}-bisAlk-vc-MMAE conjugate each run as a single band at 50 kDa, while the reduced protein runs as a single band at around 25 kDa corresponding to the V_HC_{H1} and V_LC_{L1} chains.

the disulfide had been bridged by the bis-alkylating ADC reagent (Lane 4).

The rebridging of the Fab_{TRA} interchain disulfide bond using reagent **1** was further confirmed by running SDS-PAGE under reducing conditions (Figure 4a) with no dissociation of the heavy and light chain fragments observed as would be expected for unconjugated Fab_{TRA}. This result also demonstrated that the bis-thioether bonding to the Fab_{TRA} was more stable than the original disulfide bond as the sample survived incubation with 10 mM DTT for 30 min prior to loading on the gel, which might be expected to cause dissociation of the reagent from the Fab_{TRA} leading to heavy and light chain dissociation. Analysis of the Fab_{TRA}-bisAlk-vc-MMAE conjugate by SE-HPLC (Figure 4b) indicated high purity with no indication of any product aggregation. RP-HPLC also showed that a single product was formed with a purity greater than 95% (Figure 4c).

Conditions similar to the preparation of Fab_{TRA}-bisAlk-vc-MMAE were used to conjugate reagent **1** to TRA forming TRA-bisAlk-vc-MMAE. Although there is a total of 16 disulfide bonds in an intact IgG_1, the 12 intrachain disulfide bonds are less readily reduced and are less affected even by high concentrations of reducing agents.[21] The four accessible interchain disulfide bonds can therefore be used for conjugation under moderate reducing conditions. Analytical HIC was used to analyze the reaction mixtures, as conjugation with MMAE gave sufficient separation of the different DAR species to determine the overall DAR distribution. Varying the concentration of reductant and the stoichiometries of reactants (mAb to reagent **1**), it was possible to affect the DAR distribution. When 6 equiv of MMAE reagent **1** were added to fully reduced TRA it was possible to achieve 78% of the DAR 4 variant and no unconjugated TRA (Figure 5). Lower average DAR could also be achieved if desired by adapting reaction conditions. A sample was prepared with an average DAR of 2.8 and with 2−4 drug molecules conjugated accounting for greater than 95% of the whole sample. A minimal amount of unconjugated mAb remained (<1%).

Stability of Conjugation. It has been reported that instability of maleimide conjugates in serum results in deconjugation of the drug and subsequent cross conjugation of the drug to other thiol containing species including serum albumin.[7,9] To determine whether similar cross conjugation

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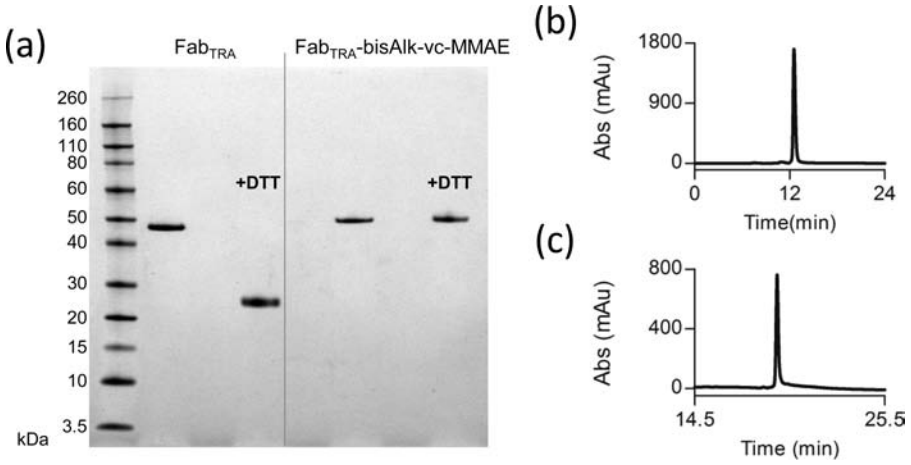

Figure 4. Analytical characterization of purified Fab$_{TRA}$-bisAlk-vc-MMAE: (a) SDS-PAGE analysis demonstrating disulfide bridging. After reduction the Fab$_{TRA}$-bisAlk-vc-MMAE conjugate remains as a single band at 50 kDa indicating that the disulfide was bridged by the ADC reagent as the interchain disulfide is no longer reducible. (b) SE-HPLC analysis and (c) RP-HPLC analysis demonstrating the purity and homogeneity of the Fab drug conjugate which elutes as single peak under both analytical techniques.

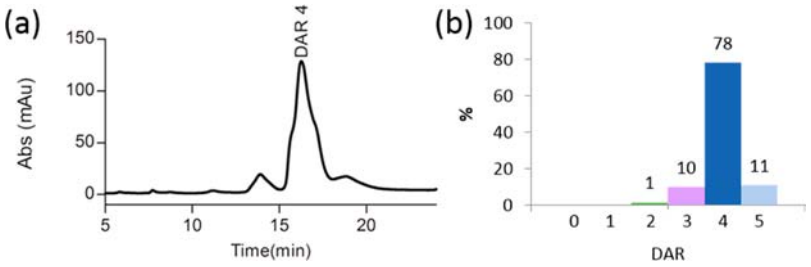

Figure 5. HIC analysis of TRA and bis-alkylating MMAE reagent **1** reaction mixture. (a) HIC chromatogram, (b) histogram showing DAR distibution based on relative peak areas from HIC chromatogram.

occurred for conjugates prepared with the bis-alkylating linker, a serum stability study was conducted using an Alexa Fluor 488 fluorescently labeled bis-alkylation reagent **2** conjugated to TRA. A maleimide functionalized Alexa Fluor 488 reagent was conjugated to TRA as a control. Both conjugates were incubated in either 50% rat or undiluted human serum for up to 96 h at 37 °C. Serum samples were analyzed by SE-HPLC with fluorescence detection to monitor the liberation of free reagent and/or formation of albumin-Alexa Fluor 488 adducts.

For the bis-alkyl TRA conjugate prepared using Alexa Fluor 488 labeled reagent **2**, the SE-HPLC chromatogram remained largely unchanged over 96 h incubation in both rat and human serum indicating that the disulfide conjugation was extremely stable to serum exposure (Figure 6). The main peak eluted at 11.0 min corresponding to the starting TRA-Alexa Fluor 488 conjugate. In contrast, incubation of the TRA maleimide Alexa Fluor 488 conjugate in both rat and human serum resulted in chromatograms with very different profiles after 96 h. The peak corresponding to the TRA maleimide conjugate at 11.0 min decreased in peak area by 47% (rat) or 24% (human) and several new peaks were observed. New peaks eluting at 11.9 and 16.3 min corresponded to an albumin adduct and deconjugated Alexa Fluor 488 dye, respectively. The albumin adduct, visible in both species' serum, indicated that deconjugation and cross conjugation to albumin from the maleimide derived TRA conjugate had occurred.

In addition to linker deconjugation, some evidence of antibody instability was also seen with the maleimide conjugates. A new early peak eluting at 8.5 min for both sera incubations indicated the formation of aggregates. A second new peak observed eluting at 13.3 min was consistent with a light chain-Alexa Fluor 488 conjugate based on comparison with molecular weight standards. This suggests that there was some dissociation of TRA-Alexa Fluor 488 conjugate over time as the heavy and light chains were not covalently linked through the interchain disulfide bonds. These signs of antibody instability were not observed for the conjugate prepared using the disulfide rebridging reagent **2**.

Experiments were also conducted to monitor the change in DAR over time for MMAE TRA conjugates incubated in an artificial serum composed of 20 mg/mL HSA in PBS. Artificial serum was used as endogenous IgGs in whole serum would overlap with the conjugate peaks in the analytical HIC method used for DAR analysis.

The drug was conjugated to TRA either using MMAE reagent **1** or maleimide-val-cit-PAB-MMAE. The reactions yielded conjugates with an average DAR of 2.3 for the bis-alkylating reagent and 3.2 for the maleimide reagent. Each conjugate at a final concentration of 1.0 mg/mL was incubated at 37 °C for up to 120 h and loss of MMAE was monitored by measuring the change over time of HIC peak areas corresponding to each DAR species. The conjugate prepared using reagent **1** showed no decrease in DAR after 120 h. In contrast, analysis of the incubation mixtures of the maleimide conjugate showed that there was a decrease in the average DAR indicating loss of MMAE with time (Figure 7). It is likely that the deconjugated MMAE cross-conjugated to the HSA present

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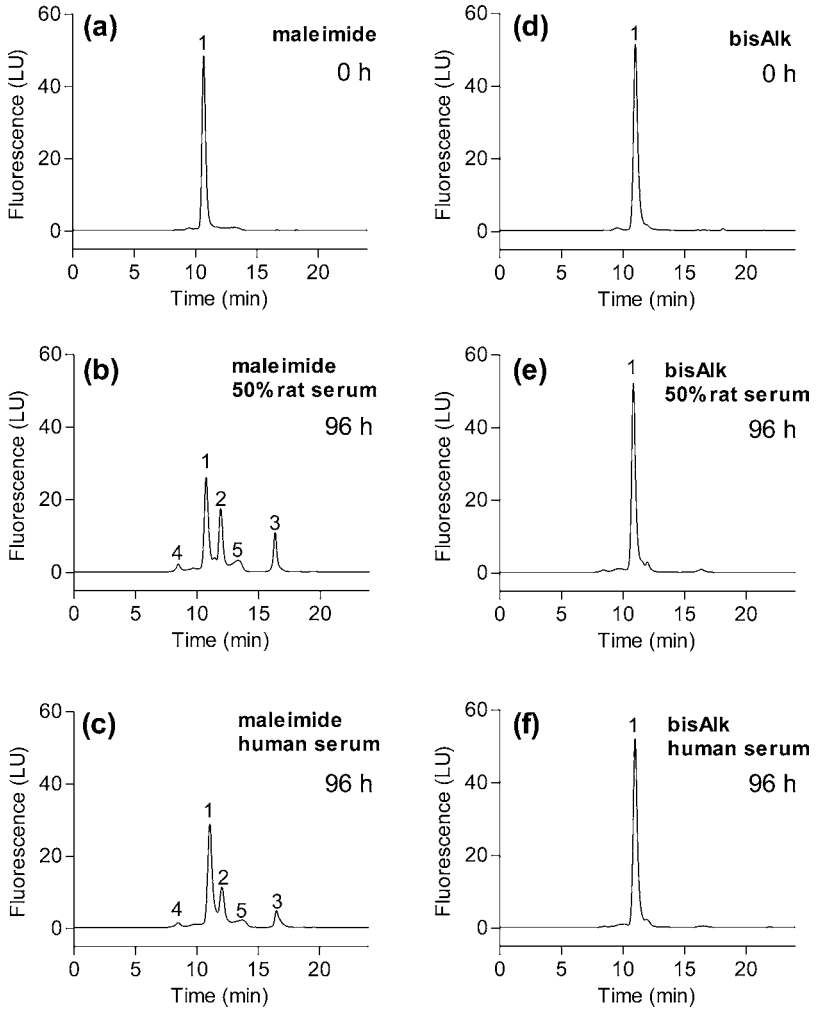

Figure 6. Serum stability of TRA-Alexa Fluor 488 conjugates prepared using either bis-alkylating or maleimide conjugation; maleimide conjugate incubated for (a) 0 h, (b) 96 h in rat serum, (c) 96 h in human serum; bis-alkylation conjugate incubated for (d) 0 h, (e) 96 h in rat serum, (f) 96 h in human serum. The peaks were identified as follows: peak 1, Trastuzumab-Alexa Fluor 488 conjugate; peak 2, albumin-Alexa Fluor 488 conjugate; peak 3, released Alexa Fluor 488; peak 4, aggregated conjugate; peak 5, light chain-Alexa Fluor 488 conjugate.

in the mixture, although this was not detected using this method.

Retained Binding Activity of Conjugates Prepared Using Bis-Alkylating Reagents. The binding of TRA and TRA MMAE conjugates to the extracellular domain of HER2 was measured by ELISA to determine whether antigen specific binding was retained after conjugation.

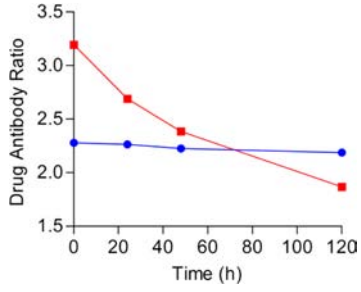

Figure 7. Change in DAR over time for ADCs incubated with HSA as measured by analytical HIC. The DAR of the maleimide based conjugate (red -■-) is seen to decrease over time while little change is observed in the TRA-bisAlk-vc-MMAE DAR (blue -●-).

Both mAb and Fab conjugates were found to have retained binding activity which was comparable to the unconjugated parent molecule (Figure 8) demonstrating that the bis-alkylating linker has a minimal impact on receptor binding.

Internalization of Conjugates. Confocal microscopy was used to monitor internalization of both TRA and Fab_{TRA} conjugates prepared using the FITC labeled bis-alkylating reagent **3**. Following incubation with SK-BR-3 cells at 37 °C for 4 h, visualization of the cells showed that both Fab and mAb conjugates had been internalized as efficiently as the unconjugated antibody (Figure 9). No internalization was observed in the A549 cell line (data not shown).

***In Vitro* Cytotoxicity of Conjugates Prepared Using the Bis-Alkylating Linker 1.** The *in vitro* potency of TRA-bisAlk-vc-MMAE (DAR 2.8) and Fab_{TRA}-bisAlk-vc-MMAE (DAR 1) conjugates was determined using the HER2-positive human breast cancer cell lines, SK-BR-3 and BT-474, and the antigen-negative cell lines, A549 and MCF-7.

Fab_{TRA}-bisAlk-vc-MMAE was found to reduce viability of SK-BR-3 cells and its activity was found to match that of free MMAE (Figure 10). No antiproliferative activity was observed for unconjugated Fab_{TRA} in the concentration range tested (data not shown). When tested in an antigen-negative cell line,

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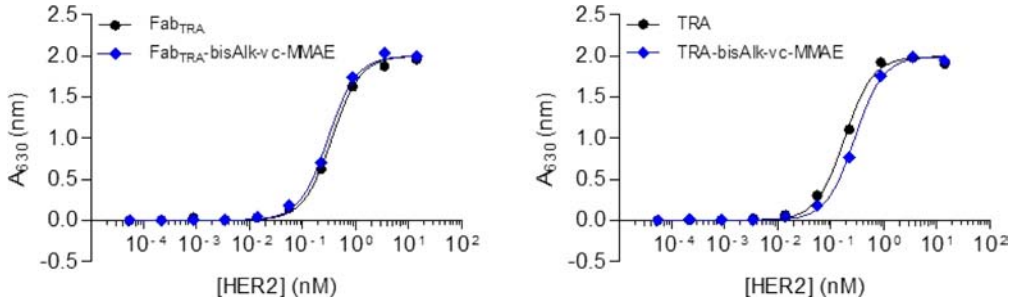

Figure 8. Antibody−drug conjugates obtained using bis-alkylating MMAE reagent **1** retained the specific antigen-binding of the naked antibody (ELISA) in both Fab and mAb formats.

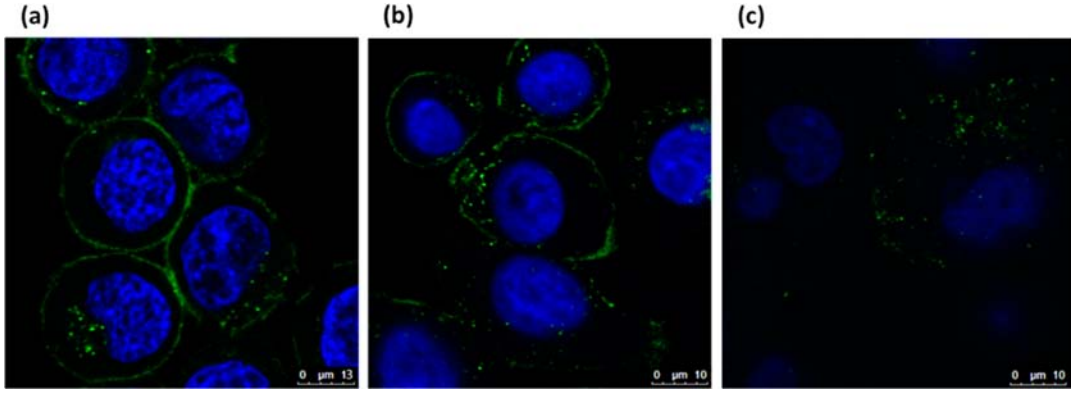

Figure 9. Antibody conjugates obtained using the disulfide bis-alkylating approach internalize into HER2-positive cells (SK-BR-3). (a) Trastuzumab detection with an anti-human mAb conjugated to Alexa Fluor 488. (b) TRA-bisAlk-FITC conjugate. (c) Fab$_{TRA}$-bisAlk-FITC conjugate.

A549, the potency of Fab$_{TRA}$-bisAlk-vc-MMAE was about 3 orders of magnitude lower than that of free MMAE.

The TRA-bisAlk-vc-MMAE conjugate was found to have potent antiproliferative activity in both antigen positive cell lines (Figure 11). In the antigen negative cell lines, the conjugate was found to decrease viability but the potency was about 3 orders of magnitude lower than that of free drug supporting antigen selective delivery of the MMAE.

***In Vivo* Efficacy of TRA and Fab$_{TRA}$ - MMAE Conjugates.** The ability of the TRA-bisAlk-vc-MMAE (DAR 2.8) and Fab$_{TRA}$-bisAlk-vc-MMAE (DAR 1) conjugates to inhibit tumor growth *in vivo* was evaluated in a BT-474 breast cancer model. The conjugates were administered to female CB.17 SCID mice bearing subcutaneous BT-474 tumor xenografts.

In the Fab$_{TRA}$-bisAlk-vc-MMAE conjugate study, mice were treated with 20 mg/kg of the Fab conjugate with dosing on alternate days over 25 days. Control groups were treated with either 20 mg/kg TRA (every fourth day), 0.3 mg/kg free MMAE (alternate days), or vehicle (alternate days). For Fab$_{TRA}$-bisAlk-vc-MMAE conjugate and free MMAE dosed groups each dose was equivalent with respect to the amount of MMAE administered.

Fab$_{TRA}$-bisAlk-vc-MMAE was found to be highly active against the BT-474 xenografts resulting in a marked delay in tumor growth (Figure 12). Treatment with Fab$_{TRA}$-bisAlk-vc-MMAE resulted in a median time to end point (TTE) of 66.3 days, corresponding to a tumor growth delay (TGD) of 48.7 days. In contrast, TRA (TTE 24.6 days) and free drug (TTE 21.8 days) each elicited only marginal TGDs of 7.0 days and 4.2 days, respectively, which did not translate to meaningful activity. Fab$_{TRA}$-bisAlk-vc-MMAE was well tolerated and fluctuations in body weight were similar between groups.

In the TRA-bisAlk-vc-MMAE study, mice were treated with 10 mg/kg, 20 mg/kg, or 30 mg/kg based on TRA with dosing every fourth day for up to 21 days. The treatment regimen was chosen based on published BT-474 xenograft studies with trastuzumab based ADCs and because the specific BT-474 model used was particularly fast-growing in comparison.

Dosing was stopped when tumor volume reached less than 10% of the day 1 mean volume so the number of doses received were 4, 5, and 6 for the 30 mg/kg, 20 mg/kg, and 10 mg/kg groups, respectively. Control groups were treated with either 20 mg/kg TRA, 0.4 mg/kg free MMAE, or vehicle on the same dosing schedule.

TRA-bisAlk-vc-MMAE was well tolerated with no dose related effect on body weight observed. Tumor growth was delayed at all three doses and the conjugate demonstrated a dose-related efficacy in the model (Figure 13). Apparent curative activity was attained at the two higher doses which each elicited a TGD of 30.2 days with 10 out of 10 tumor-free survival. The 10 mg/kg dose also resulted in the maximum TGD of 30.2 days with tumor regression but no tumor-free survival. As expected, only a marginal tumor growth delay was obtained for groups treated with TRA or free drug.

■ DISCUSSION

ADCs are a proven strategy for improving the therapeutic window of cytotoxic drugs. The high levels of toxicity associated with the administration of highly potent dolastatin[22,23] and maytansinoids[24−26] has prevented their use as therapies. Despite the limitation of these compounds as small molecule cytotoxic agents, their derivatives MMAE and DM1

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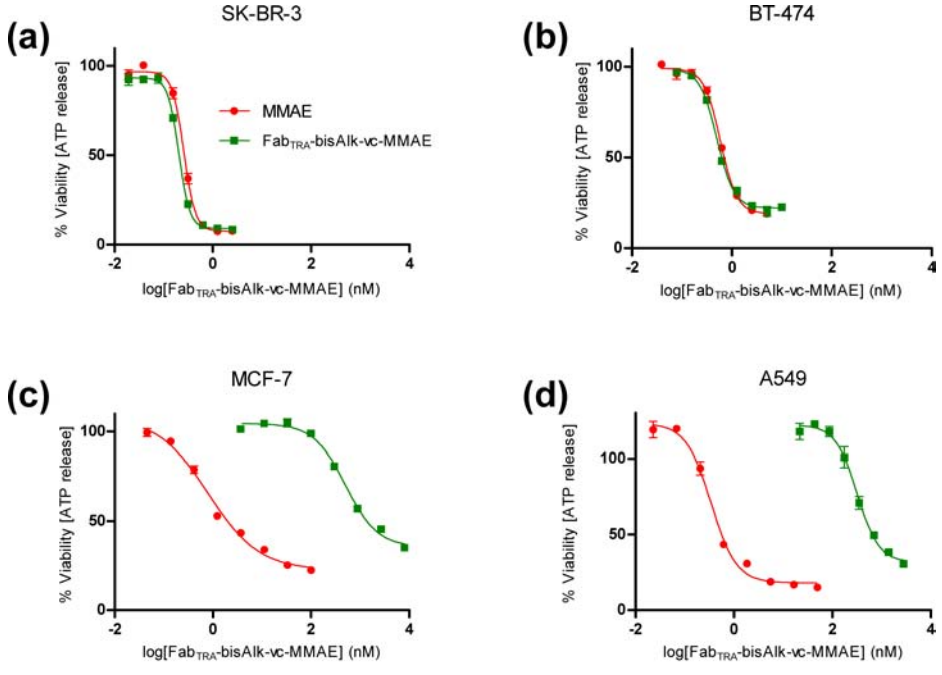


(e)

Sample	IC$_{50}$ (nM) SK-BR-3	BT-474	MCF-7	A549
MMAE	0.26	0.60	0.8	0.34
Fab$_{TRA}$-bisAlk-vc-MMAE	0.20	0.50	506	320

Figure 10. *In vitro* potency of Fab$_{TRA}$-bisAlk-vc-MMAE. (a,b) Potent antiproliferative effect is seen for both the conjugate and free drug in HER2 positive cell lines SK-BR-3 and BT-474. (c,d) In antigen negative cell lines the conjugate was found to decrease viability but the potency was two to three orders of magnitude lower than that of free drug depending on the cell line. (e) IC$_{50}$ values for free drug and conjugate in both antigen positive and negative cell lines.

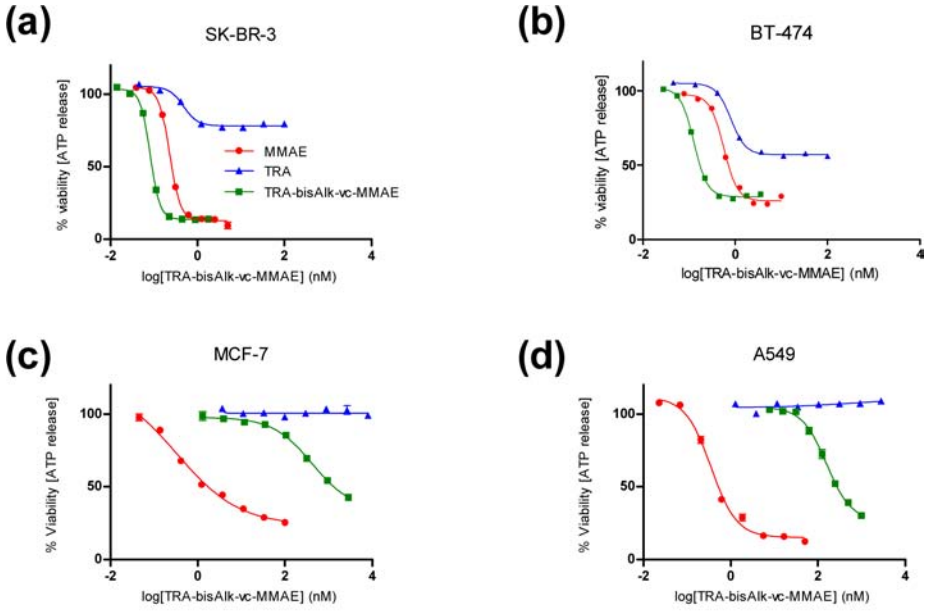
have demonstrated clinical efficacy when incorporated into the approved ADCs, Adcetris[27,28] and Kadcyla,[29] respectively.

Despite the clinical success of many ADCs in both hematological and solid tumors, they are far from the promise of a "magic bullet". Most clinical-stage ADCs have very similar maximum tolerated doses with dose limiting toxicities due to off-target toxicity. Strategies to produce ADCs with a wider therapeutic window need to focus on improving efficacy and tolerability, which is linked to having better defined ADC structures. ADCs with more than four drug molecules are more toxic, clear faster, and are less effective.[6] Although higher drug loading has been found to increase potency *in vitro*[5,30,31] this does not translate to a marked improvement of efficacy *in vivo*.[6] In experiments investigating the *in vitro* cytotoxicity of MMAE conjugates of cAC10 (brentuximab), an anti-CD30 mAb, a trend in potency was observed of DAR8 > DAR4 > DAR2.[6] However, *in vivo* the higher loaded ADC was found to have lower activity than the DAR 4 and DAR 2 species at an equivalent drug dose. This unexpected difference in potency is attributed to the different pharmacokinetic properties of the conjugates. The ADC with eight drug molecules loaded was found to clear faster than the lower DAR conjugates resulting in reduced exposure.

Current strategies for preparing ADCs via conjugation to naturally occurring amino acids produce heterogeneous mixtures with varying amounts of drug conjugated.[2,6] Maleimide conjugation of mAbs after full reduction of the interchain disulfide bonds generates ADCs with up to eight drug molecules attached.[5,6,31] A lower average DAR can be achieved after partial reduction, but this still yields a distribution of conjugates with up to 30% of the ADC bearing over four drug molecules. Each of the individual DAR species within this mixture is also likely to be a mixture of different isomers with different conjugation sites and potentially different pharmacological properties. Although problems with broad DAR distribution can be overcome by engineering new sites for conjugation (e.g., cysteine residues or non-native amino acids) the re-engineering involved may lead to increased development timelines and problems with manufacturability/scalability and ultimately increased costs.

We have shown that disulfide bridging reagents can be used as an alternative thiol conjugation method to *mono*-alkylation that results in a narrower DAR distribution and can produce an ADC with DAR 4 as the major product. The TRA-bisAlk-vc-MMAE ADC prepared contained only low amounts (<1%) of unconjugated antibody. Optimal ADCs will contain little or no unconjugated parent antibody since this can act as a competitive inhibitor, preventing binding of the ADC to the target cell.

The conjugates formed using the bis-alkylating linker were stable in serum and in the presence of high concentrations of albumin over 5 days. Maleimide conjugates by comparison were shown to be unstable with cross conjugation reactions to albumin occurring. In a study comparing conjugates with different sites of conjugation and associated differences in stability, the more stable ADCs elicited better efficacy and



(e)

Sample	IC_{50} (nM) SK-BR-3	BT-474	MCF-7	A549
MMAE	0.23	0.57	0.5	0.34
TRA-bisAlk-vc-MMAE (based on ADC concentration)	0.08	0.13	417	158

Figure 11. *In vitro* potency of TRA-bisAlk-vc-MMAE ADC (DAR 2.8). (a,b) Potent antiproliferative effect is seen for both the conjugate and free drug in HER2 positive cell lines SK-BR-3 and BT-474. (c,d) In antigen negative cell lines the conjugate was found to decrease viability but the potency was 2 to 3 orders of magnitude lower than that of free drug depending on the cell line. (e) IC_{50} values for free drug and conjugate in both antigen positive and negative cell lines.

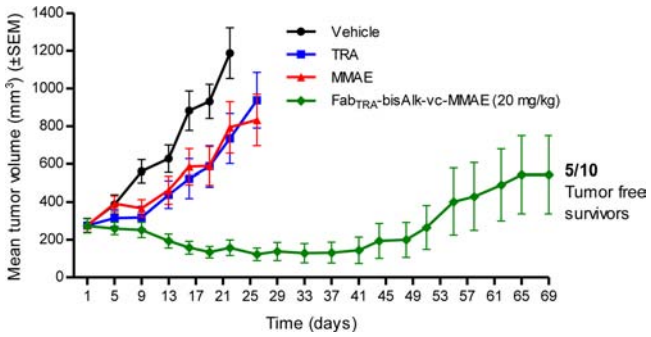


Figure 12. *In vivo* efficacy of Fab_{TRA}-bisAlk-vc-MMAE (DAR = 1).

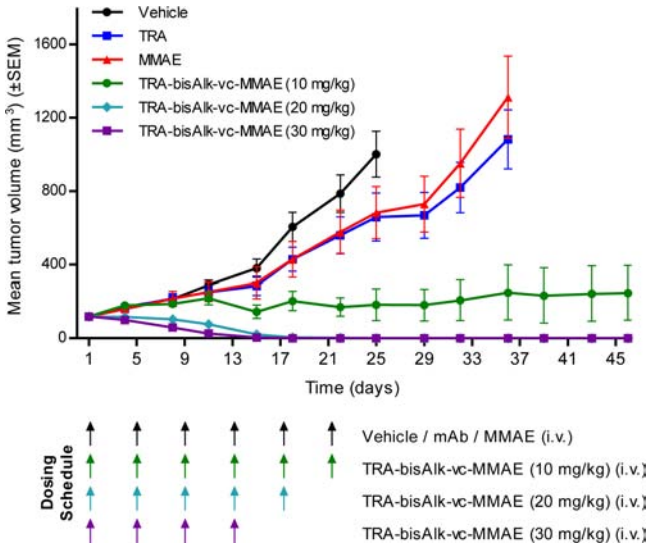


Figure 13. *In vivo* efficacy of TRA-bisAlk-vc-MMAE ADC (DAR = 2.8).

survival in *in vivo* cancer models.[9] Instability leading to deconjugation disarms the ADC resulting in increasing amounts of naked antibody which reduces efficacy, acts as a competitive inhibitor, and is also likely to have a longer half-life and thus may compromise subsequent treatment cycles.

Whole molecule IgGs may be limited in their tumor penetration due to their large size restricting diffusion and extravasion from the vasculature.[32] One approach to improve penetrability is to use smaller antibody fragments, such as domains, scFvs, minibodies, and other engineered formats.[33,34] A limitation of these smaller formats is that their circulation half-lives are too short for sufficient tumor exposure and accumulation. By contrast, whole IgGs have very long circulation half-lives due to FcRn mediated recycling. However, the longer an ADC is in circulation, the greater the chance it will bind off-target sites and degrade, which may lead to increased systemic toxicity. An optimal half-life must balance the requirements for sufficient tumor exposure and accumu-

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lation while ensuring ultimate elimination pathways limit toxicity. Many studies have shown the utility of PEG in improving the biodistribution of fragment antibodies for drug conjugate and tumor radioimaging applications, often with relatively small PEG sizes of less than 5 kDa that increase the apparent size of the fragment to above the renal filtration cutoff threshold.[35] Avoiding kidney exposure and directing clearance toward the liver, which has greater capacity for detoxification, may be beneficial in improving tolerability. With the disulfide conjugation approach described here, modifying the length of the PEG chain incorporated between the reactive bis-sulfone and the drug would confer increased half-life to a Fab conjugate as can be achieved by protein PEGylation,[38,39] allowing the optimization of the pharmacokinetic profile of the Fab for drug conjugate use.

Monovalent antibody formats may also avoid the antigen barrier which has been proposed as a factor contributing to the limited penetration of mAbs in many solid tumors due to their bivalent and high affinity binding.[36] Also, the bivalent binding of antibodies may cross-link antigens and lead to unwanted activation of signaling pathways. One example is the agonistic effect seen for DN-30 anti-Met bivalent antibody but not as a Fab or single Fab arm antibody.[37,40]

The conjugation of a cytotoxic payload via a three carbon disulfide bridge to produce a biologically active Fab drug conjugate was exemplified using a Fab derived from TRA. A Fab fragment has a single interchain disulfide that is readily reduced. After reduction of Fab_{TRA}, MMAE was successfully conjugated using the bis-alkylating reagent **1**. The resulting drug conjugate was homogeneous with respect to DAR with a single drug molecule conjugated, and was structurally intact with the disulfide bridged by the linker.

Conjugation of MMAE to a mAb or Fab using the bis-alkylating reagent approach was shown to produce effective ADCs. The conjugates retained binding and demonstrated antigen-selective *in vitro* cytotoxicity. Both mAb and Fab conjugates were also found to be well tolerated and efficacious *in vivo*. We believe bis-alkylation is a general approach to producing stable and more homogeneous ADCs that is efficient and predictable and does not rely on reengineering of the antibody for site-specific conjugation.

EXPERIMENTAL PROCEDURES

Materials. TRA (Herceptin) was produced by Roche. Maleimide-val-cit-PAB-MMAE and NH_2-val-cit-PAB-MMAE were purchased from Concortis Biosystems. Boc-NH-PEG(2 kDa)-NH_2 was purchased from Creative PEGWorks. Maleimide-C5 and all other PEG precursors were purchased from IRIS Biotech Gmbh. Alexa Fluor 488 C_5-maleimide and Alexa Fluor 488 NHS ester were purchased from Invitrogen. All other reagents and solvents were purchased from Acros Organics or Sigma-Aldrich and used as received. ^{1}H NMR data were collected using a Bruker 400 or 600 MHz spectrometer in the NMR solvent stated. Chemical shifts δ are given in ppm downfield from tetramethylsilane.

All cell lines were purchased from the American Type Culture Collection (ATCC), except for A549 which was purchased from the Health Protection Agency Culture Collection (HPACC). Cells were maintained in McCoy's 5a (SK-BR-3), DMEM:F12 (BT-474), Dulbecco's modified Eagle medium (DMEM) supplemented with 200 mM glutamine (A549), or Minimum Essential Medium (MEM) supplemented with 10 μg/mL insulin (MCF-7). All media were complemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. All media were obtained from Life Technologies. Cell lines were grown at 37 °C and 5% CO_2 in a CO_2 air-jacket incubator (Binder).

The extracellular domain of the HER2 receptor (ECD/HER2) was purchased from Stratech Scientific Ltd., UK. Nunc Maxisorp microtiter plates were purchased from Thermo Scientific. Anti 6xHis antibody conjugated to HRP was purchased from Clontech Laboratories Inc.

Preparation of Bis-Sulfone-PEG(24)-COOH. Bis-sulfone-PEG(24)-COO*t*Bu was prepared from H_2N-PEG(24)-COO*t*-Bu according to the method described previously for methoxy PEG bis-sulfone synthesis.[18,19] Removal of the *t*-butyl protecting group was achieved by stirring the bis-sulfone-PEG(24)-COO*t*Bu (0.98 g, 0.58 mmol) in 50% trifluoroacetic acid in DCM (8 mL) at room temperature for 2 h. The solvents were removed under reduced pressure, then the residue was dissolved in acetone (30 mL). The solution was filtered through a sintered glass filter to remove insoluble material, then cooled in a dry ice bath to give a white precipitate. The precipitate was collected by centrifugation (4566 g, 30 min). The resulting off-white solid was dried under vacuum (0.82 g, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.49 (s, 6H, Ar−CH_3), 2.59 (t, 2H, PEG−CH_2−COOH), 3.47−3.79 (m, PEG), 4.33 (m, 1H, SO_2−CH_2−C*H*−CH_2−SO_2-), 7.03 (s, 1H, PEG-N*H*-carbonyl), 7.36 (d, 4H, SO_2*Ar*), 7.66 (d, 2H, CO*Ar*), 7.69 (d, 4H, SO_2*Ar*), 7.81 (d, 2H, CO*Ar*).

*Synthesis of Bis-Sulfone-PEG(24)-MMAE (Reagent **1**).* HATU (14 mg, 0.036 mmol) was added to a stirred suspension containing Na_2CO_3 (4 mg, 0.036 mmol), H_2N-val-cit-PAB-MMAE (53 mg, 0.048 mmol), and bis-sulfone-PEG(24)-COOH (40 mg, 0.024 mmol) in anhydrous DCM/DMF (3:1, 1 mL) and stirred for 48 h under an argon atmosphere. The crude mixture was purified using an anion exchange column eluting with MeOH followed by column chromatography eluting with MeOH/DCM (5:95 v/v). Compound **1** was isolated as a colorless solid (46 mg, 71%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.60−0.99 (m, aliphatic side chains), 2.43 (s, Me-Ts), 3.36−3.66 (m, PEG), 7.15−7.28 (m, Ar), 7.31 (d, J = 8.3 Hz, Ar), 7.54−7.62 (m, Ar), 7.79 (d, J = 8.3 Hz, Ar).

Preparation of Boc-NH-PEG(2 kDa)-FITC. Boc-NH-PEG(2 kDa)-NH_2 (107 mg, 0.056 mmol) was dissolved in DCM (1 mL). Triethylamine (8.6 μL, 0.062 mmol) was then added followed by FITC (100 mg, 0.25 mmol) in DMF (1 mL). The resulting mixture was stirred at room temperature under an argon atmosphere for 24 h. Volatile solvents were removed by evaporation, then the product was precipitated by addition of acetone (20 mL). The precipitate was collected by centrifugation (4566 g, 30 min), then dried under vacuum to afford a yellow solid (76 mg, 59%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.40 (s, 9H, *t*-Bu), 3.20−3.28 (m, 2H, PEG), 3.39−3.44 (m, 1H), 3.47−3.51 (m, 2H), 3.52−3.72 (m, 183H, PEG), 3.73−3.87 (m, 2H), 5.20 (br s, 1H), 6.50 (dd, 2H, J = 8.6, 2.3 Hz), 6.58 (d, 2H, J = 8.6 Hz), 6.67 (d, 2H, J = 2.3 Hz), 7.03 (d, 1H, J = 8.3 Hz), 7.53−7.58 (m, 1H), 7.94 (dd, 1H, J = 8.3,1.7 Hz), 8.09 (d, 1H, J = 1.7 Hz), 9.22 (br s, 2H), 9.44 (s, 1H, NH).

Preparation of H_2N-PEG(2 kDa)-FITC. Boc-NH-PEG(2 kDa)-FITC (73 mg, 0.031 mmol) was dissolved in DCM (3.6 mL), then trifluoroacetic acid (0.4 mL) was added and the resulting mixture stirred at room temperature for 2 h. Volatiles were removed by evaporation under reduced pressure. The crude product was dissolved in acetone (20 mL). The solution

was filtered through a sintered filter to remove insoluble material, then cooled in a dry ice bath to give a yellow precipitate. The precipitate was collected by centrifugation (4566 g, 30 min), then dried under vacuum to afford a yellow solid (47 mg, 76%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.15 (s, 2H, PEG), 3.45−3.94 (m, 167H, PEG), 6.59 (dd, 2H, J = 8.6, 2.3 Hz), 6.66 (d, 2H, J = 8.6 Hz), 6.78 (d, 2H, J = 2.3 Hz), 7.07 (d, 1H, J = 8.3 Hz), 7.76 (br, 2H, NH), 7.94 (d, 1H, J = 8.3 Hz), 8.29 (s, 2H), 9.31 (br, 2H, PhO*H*).

*Preparation of Bis-Sulfone-PEG(2 kDa)-FITC (Reagent **3**).* 4-[2,2-Bis[(*p*-tolylsulfonyl)methyl]acetyl) benzoic acid-NHS ester (bis-sulfone NHS) was prepared according to a previously described method.[18] H_2N-PEG(2 kDa)-FITC (23 mg, 0.010 mmol) was dissolved in DCM (6 mL) with the bis-sulfone NHS ester (24.9 mg, 0.042 mmol) and pyridine (38 μL). The resulting mixture was stirred at room temperature under argon for 48 h. Volatiles were removed by evaporation under reduced pressure. The crude product was dissolved in acetone (20 mL). The solution was filtered through a sintered filter to remove insoluble material, then cooled in a dry ice bath to give a yellow precipitate. The precipitate was collected by centrifugation (4566 g, 30 min), then dried under vacuum to afford a yellow solid (15 mg, 60%). ^{1}H NMR (400 Hz, $CDCl_3$) δ 2.49 (s, 6H, PhCH_3), 3.11 (s, 1H, PEG), 3.45−3.94 (m, 167H, PEG), 4.35 (m, 1H, CH), 6.59 (dd, 2H, J = 8.7, 2.3 Hz), 6.67 (d, 2H, J = 8.6 Hz), 6.77 (d, 2H, J = 2.2 Hz), 7.07 (d, 1H, J = 8.3 Hz), 7.37 (d, 4H, J = 8.0 Hz), 7.66 (d, 2H, J = 8.4 Hz), 7.71 (d, 4H, J = 8.0 Hz), 7.75−7.81 (m, 2H), 7.84 (dd, 2H, J = 8.4 Hz), 7.87−7.92 (m, 1H), 8.00−8.03 (m, 1H), 8.14−8.28 (m, 2H), 9.0 (br s, 1H).

Preparation of 4-(2,2-Bis[(p-tolylsulfonyl)methyl]acetyl) Benzoic Acid HOBt Ester. Under an argon atmosphere, a stirred mixture of 4-(2,2-bis[(*p*-tolylsulfonyl)methyl]acetyl) benzoic acid (1.00 g, 2.0 mmol), prepared as described previously,[16,18] 1-hydroxybenzotriazole (0.27 g, 2.0 mmol), and anhydrous tetrahydrofuran (20 mL) were cooled using an ice bath. Neat 1,3-diisopropylcarbodiimide (313 μL, 2.0 mmol) was then added dropwise at ∼0 °C. The reaction mixture was stirred for 20 min, the ice-bath was then removed, and the reaction mixture stirred at room temperature. After 16 h, the resulting precipitate was isolated using a no. 3 sintered glass funnel and washed with tetrahydrofuran (2 × 5 mL) and dried *in vacuo*. The crude solid was suspended in methanol (15 mL) and collected by filtration after stirring for 1 h to afford the desired active HOBt ester (0.84 g, 68%) as a white solid. ^{1}H NMR (600 MHz $CDCl_3$,) δ 2.49 (s, 6H, PhCH_3), 3.52 (m, 2H, PhSO_2CH_2), 3.65 (m, 2H, PhSO_2CH_2), 4.49 (q, 1H, C*H*, J = 6.5 Hz), 7.39, 7.74 (AB dd, SO_2*Ar*, 8H, J = 8.6 Hz), 7.49 (m, 2H, Ph*H,* HOBt), 7.60 (m, 1H, Ph*H*, HOBt), 7.93, 8.31 (AB dd, CO*Ar*, 4H, J = 8.4 Hz), 8.14 (d, 1H, Ph*H*, HOBt, J = 9.2 Hz).

Preparation of Bis-Sulfone-PEG(12)-NH-Boc. Under an argon atmosphere, a mixture of 4-(2,2-bis[(*p*-tolylsulfonyl)-methyl]acetyl) benzoic acid HOBt ester (0.12 g, 194 μmol), α-amino-ω-(*t*-butyloxycarbonyl)-dodecae(ethylene glycol) (0.10 g, 155 μmol) and anhydrous chloroform (4 mL) was stirred at room temperature. After 16 h, the reaction mixture was concentrated down to 1 mL of total volume, and purified by flash silica chromatography (gradient elution 100% DCM to DCM:MeOH 10:90 v/v) to afford the desired bis-sulfone-PEG(12)-NH-Boc (0.15 g, 87%) as a white solid. ^{1}H NMR (600 MHz $CDCl_3$,) δ 1.42 (s, 9H, $(CH_3)_3$), 2.46 (s, 6H, PhCH_3), 3.28 (m, 2H, CH_2O), 3.45−3.56 (m, 4H, CH_2O), 3.58−3.68 (m, 46H, CH_2OPEG + PhSO_2CH_2), 4.28−4.35 (m, 1H, C*H*), 7.54 (br, 1H, N*H*), 7.34, 7.64 (AB dd, SO_2*Ar*, 8H, J = 8.4 Hz), and 7.61, 7.82 (AB dd, CO*Ar*, 4H, J = 8.5 Hz).

Preparation of Bis-Sulfone-PEG(12) Amine TFA Salt. The bis-sulfone-PEG(12)-NH-Boc (0.147 g, 125 μmol) was dissolved in formic acid (2 mL) and the resulting mixture was stirred at room temperature. After 16 h, the reaction was concentrated under vacuum and the residue was resuspended in water (1.5 mL), and purified by reverse-phase (C-18) chromatography (gradient elution H_2O/MeCN buffers contended 0.1% TFA, 0% to 100%) to afford the desired bis-sulfone-PEG(12) amine as a TFA salt (0.097 g, 68%). ^{1}H NMR (600 MHz $CDCl_3$,) δ 2.46 (s, 6H, PhCH_3), 3.16 (m, 2H, CH_2O), 3.48−3.52 (m, 2H, CH_2O), 3.59−3.73 (m, 46H, CH_2OPEG + PhSO_2CH_2), 3.80 (m, 2H, CH_2O), 4.28−4.34 (m, 1H, C*H*), 7.53 (br, 1H, N*H*), 7.36, 7.66 (AB dd, SO_2*Ar*, 8H, J = 8.4 Hz), and 7.64, 7.86 (AB dd, CO*Ar*, 4H, J = 8.5 Hz).

*Preparation of Bis-Sulfone-PEG(12)-Alexa Fluor 488 (Reagent **2**).* To a mixture of bis-sulfone-PEG(12) amine (0.005 g, 4.80 μmol), Alexa Fluor 488 NHS ester (0.0025 g, 3.88 μmol), and anhydrous DMF (0.5 mL) under an argon atmosphere was added sodium bicarbonate (0.0010 g, 11.9 μmol), and the reaction mixture was stirred at room temperature. After 56 h, the reaction mixture was neutralized by adding acetic acid (50 μL), purified by reverse-phase (C-18) chromatography (gradient elution H_2O/MeCN buffers contended 0.1% TFA, 0% to 100%) to afford the desired bis-sulfone-PEG(12)-Alexa Fluor 488 (0.002 g, 33%) as a red solid. Purity by HPLC at 488 nm 91.2%. ESI-MS, *m/z:* 1543.1 (10%) $[M + 1H]^+$; 772.1 (100%) $[M + 2H]^{2+}$.

Preparation of Fab_{TRA}. Digestion buffer (20 mM NaH_2PO_4 pH 7.0, 10 mM EDTA, 50 mM cysteine, 50 mL) was warmed to 37 °C. TRA (150 mg) was resuspended in ultrapure water (7.5 mL). An aliquot (5 mL, 100 mg) of the resulting solution was then diluted with the prewarmed digestion buffer (40 mL). Papain (5 mL, 1 mg/mL) was then added to the TRA solution to give a TRA:papain ratio of 20:1 (w/w). The mixture was mixed gently then incubated at 37 °C for 30 min with gentle mixing. Purification was carried out by affinity chromatography on a protein L column (Pierce) followed by hydrophobic interaction chromatography using a XK 16/10 column (GE Healthcare) packed in-house with Toyopearl, Phenyl-650S resin (Tosoh Bioscience LLC).

*Conjugation to Fab_{TRA} Using Bis-Alkylating MMAE Reagent **1**.* Conjugation of reagent **1** to Fab_{TRA} was carried out using methods described previously for conjugation of PEG to disulfide bonds using the bis-alkylating linker.[17,18]

Conjugation to TRA Using Bis-Alkylating Reagents. Conjugation of bis-alkylating MMAE reagent **1**, bis-alkylating Alexa Fluor 488 reagent **2**, or bis-alkylating FITC-reagent **3** to TRA was carried out using methods described previously for conjugation of PEG to disulfide bonds.[17,18]

Conjugation to TRA Using Maleimide-val-cit-PAB-MMAE. TRA (10 mg/mL) in 20 mM sodium phosphate pH 8.0, 150 mM NaCl, 20 mM EDTA was treated with tris(2-carboxyethyl)phosphine (TCEP, 2 mol equiv) at 40 °C for 60 min. The reductant was then removed by buffer exchange into fresh pH 8.0 phosphate buffer by gel filtration (PD-10 column, GE Healthcare). Determination of free thiols using 5,5′-dithiobis(2-nitrobenzoic acid) showed that there were approximately four SH groups per mAb. Maleimide-val-cit-PAB-MMAE (1.5 equiv per thiol) in DMSO (final concentration 5% v/v) was added and the resulting mixture incubated

at 40 °C overnight. Unreacted reagent was removed by gel filtration (PD-10, PBS).

Conjugation to TRA Using Alexa Fluor 488 C_5-Maleimide. TRA (10 mg/mL) in 20 mM sodium phosphate pH 8.0, 150 mM NaCl, 20 mM EDTA was treated with dithiothreitol (DTT, 3 mol equiv) at 40 °C for 60 min. The reductant was then removed by buffer exchange into fresh pH 8.0 phosphate buffer by gel filtration (PD-10 column, GE Healthcare). Determination of the number of free thiols present using 5,5′-dithiobis(2-nitrobenzoic acid) showed that there were approximately 3.5 SH groups per mAb. Alexa Fluor 488 C_5-maleimide (1.2 equiv per thiol) in MeCN (final concentration 5% v/v) was added and the resulting mixture incubated at 4 °C overnight. Unreacted reagent was removed by gel filtration (PD-10, PBS).

Analytical Hydrophobic Interaction Chromatography (HIC). Analytical HIC of conjugates was carried out using a TOSOH, TSKgel Butyl-NPR column (35 × 4.6 mm) connected to a Dionex Ultimate 3000RS HPLC system. The method consisted of a linear gradient from 80% buffer A (1.5 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0) to 86% buffer B (20% isopropanol (v/v) in 50 mM sodium phosphate) over 20 min at a flow rate of 0.8 mL/min. The temperature was maintained at 30 °C throughout the analysis and UV detection was carried out at 214 nm and 280 nm. For each analysis an injection of 10 μg of mAb or ADC was carried out.

Analytical Size Exclusion Chromatography (SEC). An Acquity UPLC BEH200 column (Waters) connected to a Dionex HPLC system was used for SEC. The mobile phase was PBS, pH 7.4, containing 10% (v/v) isopropanol or 15% (v/v) acetonitrile and the flow rate was kept constant at 0.25 mL/min. The column was maintained at 30 °C throughout the analysis. The analysis was carried out in a 24 min isocratic elution with UV detection at 214, 248, and 280 nm.

Alexa Fluor 488 Conjugate Serum Stability. Alexa Fluor 488-TRA conjugates were diluted with 50% rat or human serum at a concentration of 0.1 mg/mL in sterile tubes. Sodium azide was added (final concentration 1 mM), and then the mixtures were split into 4 equal aliquots. One aliquot was immediately frozen at −80 °C. The remaining samples were transferred to a CO_2 incubator and kept at 37 °C. Aliquots were removed from the incubator after 24, 48, and 96 h and transferred to a −80 °C freezer. After the final time point, mixtures were analyzed by SE-HPLC using the method described above including fluorescence detection with an excitation wavelength of 495 nm and emission wavelength of 525 nm.

ADC Stability in the Presence of Free Thiols. TRA-bisAlk-vc-MMAE was diluted to a final concentration of 1 mg/mL with PBS pH 7.4 containing human serum albumin (HSA) at a concentration of 20 mg/mL. Sodium azide was added (final concentration 1 mM), and then the mixtures were split into 4 equal aliquots. One aliquot was immediately frozen at −80 °C. The remaining samples were transferred to a CO_2 incubator and kept at 37 °C. Aliquots were removed from the freezer after 24, 48, and 120 h and transferred to a −80 °C freezer. After the final time point, mixtures were analyzed by analytical hydrophobic interaction chromatography using a method modified from the standard protocol with a ProPac 2.1 mm × 100 mm HIC-10 column (Fisher Scientific). The method consisted of a linear gradient from 100% buffer A (50 mM sodium phosphate pH 7.0, 1.5 M ammonium sulfate) to 100% buffer B (50 mM sodium phosphate pH 7.0, 20% isopropanol) in 50 min. The flow rate was 1 mL/min and the temperature was set at 30 °C. Detection was carried out by following UV absorption at 214, 248, and 280 nm.

Anti-HER2 ELISA. Maxisorp microtiter plates (96-well) were coated overnight at 4 °C with TRA, Fab_{TRA}, TRA-bisAlk-vc-MMAE, and Fab_{TRA}-bisAlk-vc-MMAE (5 μg/mL in 0.05 M carbonate/bicarbonate buffer, pH 9.6). The plates were washed three times with PBS/0.05% Tween 20 (PBS-T) and blocked with 3% BSA/PBS-T for 1 h at room temperature. Serial dilutions of ECD/HER2 were subsequently added and the plates incubated for 3 h at room temperature. After 3 washes in PBS/T, HRP-conjugated anti-6xHis antibody was added for 1 h at room temperature to detect bound ECD/HER2. Tetramethyl benzidine (TMB) substrate and stop reagents were then added in sequence, and the absorbance read at 630 nm using a Spectramax M3 microplate reader. GraphPad Prism 5 software using one site-specific binding with hill slope fit was used for analysis.

Cell Viability Assays (Cell Titer Glo). The effect of TRA-bisAlk-vc-MMAE and Fab_{TRA}-bisAlk-vc-MMAE MMAE conjugates on the viability of HER2-positive breast cancer cell lines was assessed by Cell Titer Glo luminescence assay (Promega). HER2-negative cell lines were included in the assays to confirm antigen selectivity. Cells were seeded in 96-well white opaque plates (Corning) at a density of 5 × 10^3 to 1 × 10^4/well dependent on the cell line. Cells were allowed to attach for 24 h at 37 °C and 5% CO_2 in a humidified atmosphere. Following incubation, cell culture medium was removed and serial dilutions of TRA MMAE conjugates, TRA, and free drug were added. Cells were incubated at 37 °C and 5% CO_2 for 96 h and viability in response to treatment was quantified according to the manufacturer's instructions. Briefly, Cell Titer Glo reagent was added to the wells and plates were incubated on a plate shaker for 3 min and 300 rpm. Cells were incubated for 20 min at room temperature to allow stabilization of the signal and luminescence was subsequently measured on a SpectraMax3 micro plate reader (Molecular Devices). A four-parameter curve fitting model was applied (GraphPad) to generate a dose−response curve. Response was expressed as % viability of untreated controls.

Internalization Assay. SK-BR-3 and A549 cells were seeded on poly(D-lysine) coated coverslips in 24-well plates at a density of 2 × 10^5/well or 3 × 10^4/well, respectively. Cells were allowed to attach for 24 h at 37 °C and 5% CO_2 in a humidified atmosphere. Cells were subsequently surface labeled for 30 min on ice by replacing the cell culture medium with 4 μg/mL TRA, TRA-bisAlk-FITC, or Fab_{TRA}-bisAlk-FITC in serum-free medium. Following incubation, control plates were washed with PBS and coverslips were carefully mounted on microscope slides in ProLong Gold antifade reagent (Life Technologies). The remaining plates were washed three times with serum free medium, transferred to a CO_2 air-jacket incubator, and incubated for 4 h at 37 °C and 5% CO_2 to allow internalization. Cells were subsequently washed three times with PBS and acetate buffer (pH 2.5) to remove membrane bound TRA or TRA-FITC conjugates, respectively. Samples were fixed with 4% paraformaldehyde in PBS, washed three times with PBS, and either mounted on microscope slides as described above (FITC conjugates) or permeabilized with 0.5% Triton-X100 in PBS (TRA). Binding and internalization of unconjugated TRA was detected by means of Alexa Fluor 488 conjugated goat anti-

human IgG secondary antibody (Life Technologies). Cells were imaged with a Leica SP5 laser scanning confocal microscope.

In Vivo Efficacy Studies. Xenografts were initiated in female severe combined immunodeficient mice (Fox Chase SCID, C.B-17/Icr-Prkdcscid, Charles River Laboratories) with BT-474 human breast carcinomas maintained by serial subcutaneous transplantation in SCID mice. On the day of tumor implant, each test mouse received a 1 mm^3 BT-474 tumor fragment implanted subcutaneously in the right flank, and tumor growth was monitored as the average size approached the target range. Tumors were measured in two dimensions using calipers, and volume was calculated using the formula:

$$\text{tumor volume}(mm^3) = \frac{w^2 \times l}{2}$$

where w = width and l = length, in mm, of the tumor.

The animals were fed *ad libitum* water (reverse osmosis, 1 ppm of Cl), and NIH 31 Modified and Irradiated Lab Diet. The mice were housed on irradiated Enrich-o'cobs Laboratory Animal Bedding in static microisolators on a 12 h light cycle at 20−22 °C and 40−60% humidity. On day 1 of the study, mice were randomized into treatment groups (n = 10), and dosing was initiated. All agents were administered intravenously (i.v.) into the tail vein in a dosing volume of 0.2 mL per 20 g of body weight (10 mL/kg), scaled to the body weight of the individual animal.

In Vivo Efficacy of Fab Conjugate. Mice were treated with 20 mg/kg of DAR 1 Fab_{TRA}-bisAlk-vc-MMAE with dosing on alternate days over 25 days. Control groups received either vehicle or 0.3 mg/kg free MMAE (on the same schedule as the Fab conjugate) or 20 mg/kg TRA (every fourth day for four doses).

In Vivo Efficacy of mAb Conjugate. Mice were treated with 10 mg/kg, 20 mg/kg, or 30 mg/kg DAR 2.8 TRA-bisAlk-vc-MMAE (based on TRA) with dosing every 5 days for up to 7 occasions. Dosing was stopped when the tumor volume reached less than 10% of the day 1 mean volume. Control groups were treated with either 20 mg/kg TRA, 0.4 mg/kg free MMAE, or vehicle on the same dosing schedule.

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Notes

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