

Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index

Jagath R Junutula¹, Helga Raab¹, Suzanna Clark¹, Sunil Bhakta¹, Douglas D Leipold¹, Sylvia Weir¹, Yvonne Chen¹, Michelle Simpson¹, Siao Ping Tsai¹, Mark S Dennis¹, Yanmei Lu¹, Y Gloria Meng¹, Carl Ng¹, Jihong Yang¹, Chien C Lee¹, Eileen Duenas¹, Jeffrey Gorrell¹, Viswanatham Katta¹, Amy Kim¹, Kevin McDorman^{1,2}, Kelly Flagella¹, Rayna Venook¹, Sarajane Ross¹, Susan D Spencer¹, Wai Lee Wong¹, Henry B Lowman¹, Richard Vandlen¹, Mark X Sliwowski¹, Richard H Scheller¹, Paul Polakis¹ & William Mallet¹

Antibody-drug conjugates enhance the antitumor effects of antibodies and reduce adverse systemic effects of potent cytotoxic drugs. However, conventional drug conjugation strategies yield heterogenous conjugates with relatively narrow therapeutic index (maximum tolerated dose/curative dose). Using leads from our previously described phage display-based method to predict suitable conjugation sites, we engineered cysteine substitutions at positions on light and heavy chains that provide reactive thiol groups and do not perturb immunoglobulin folding and assembly, or alter antigen binding. When conjugated to monomethyl auristatin E, an antibody against the ovarian cancer antigen MUC16 is as efficacious as a conventional conjugate in mouse xenograft models. Moreover, it is tolerated at higher doses in rats and cynomolgus monkeys than the same conjugate prepared by conventional approaches. The favorable *in vivo* properties of the near-homogenous composition of this conjugate suggest that our strategy offers a general approach to retaining the antitumor efficacy of antibody-drug conjugates, while minimizing their systemic toxicity.

Targeted therapy using monoclonal antibodies (mAbs) has revolutionized cancer treatment, with several mAbs recognizing antigens expressed on the surfaces of tumor cells already having demonstrated their clinical potential^{1,2}. As antibodies against tumor-specific antigens often lack therapeutic activity, they alternatively can be covalently linked to cytotoxic drugs. In principle, selective delivery of cytotoxic agents should reduce the systemic toxicity associated with traditional small-molecule chemotherapeutics^{3,4}.

Antibodies have been conjugated to a variety of cytotoxic drugs, including small molecules that alkylate DNA (e.g., duocarmycin and calicheamicin), disrupt microtubules (e.g., maytansinoids and auristatins) or bind DNA (e.g., anthracyclins)⁵. These antibody-drug conjugates (ADC) have displayed potent and selective killing of target tumor cells *in vitro* and in mouse tumor xenograft studies. Humanized anti-CD33 conjugated to calicheamicin (gemtuzumab ozogamicin; Mylotarg) was approved by the US Food and Drug Administration in 2000 for the treatment of acute myeloid leukemia, and several ADCs are being actively pursued to combat diverse forms of cancer^{6–19}. An adequate safety margin will be required to make ADCs a common therapeutic option for cancer.

Cytotoxic drugs are generally conjugated to antibodies either through lysine side-chain amines or through cysteine sulphydryl groups activated by reducing interchain disulfide bonds. Both of these procedures yield heterogenous products, containing a mixture of species with different molar ratios of drug to antibody, linked at

different sites, each with distinct *in vivo* pharmacokinetic, efficacy and safety profiles^{8,20}. In a study that underscored the consequences of this heterogeneity, researchers purified ADC fractions with exactly two, four or eight drugs attached to each antibody and compared these fractions for *in vivo* efficacy, tolerability in mice and pharmacokinetics⁸. The most heavily conjugated species had the lowest maximum tolerated IgG dose and most rapid clearance but did not confer a proportional increase in efficacy. This suggests that it would be desirable to selectively generate only conjugates with a moderate drug stoichiometry, perhaps two drugs per antibody. However, the purification process used in that study is not practical on the scale required for clinical testing, as a large amount of ADC would yield a relatively small amount of the desired stoichiometric fraction. Additionally, this approach still yields antibodies with disrupted interchain disulfide bonds, potentially affecting antibody stability and/or distribution *in vivo*. Finally, even a purified ADC with a uniform stoichiometry would still carry drugs conjugated to multiple sites and therefore be a complex mixture of unique entities. Each species could potentially have a distinct set of properties, and consistent batch-to-batch production would be difficult to control.

To limit the potential liabilities associated with such conjugation methods, we have engineered reactive cysteine residues at specific sites in antibodies to allow drugs to be conjugated with defined stoichiometry without disruption of interchain disulfide bonds. The excellent yields of these antibodies, named THIOMABs, and their

¹Genentech Inc., 1 DNA Way, South San Francisco, California 94080, USA. ²Present address: Division of Pathology, Charles River Preclinical Services, Nevada, 6995 Longley Lane, Reno, Nevada 89511, USA. Correspondence should be addressed to W.M. (wmallet@gene.com) or J.R.J. (jagath@gene.com).

Received 17 April; accepted 19 June; published online 20 July 2008; doi:10.1038/nbt.1480

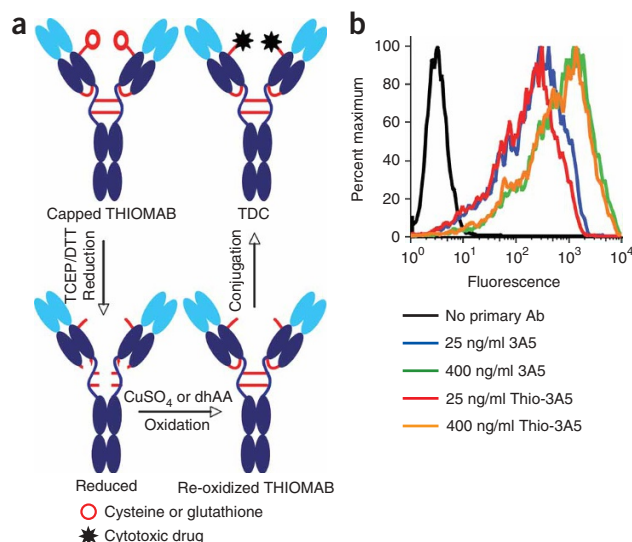


Figure 1 Characterization of THIOMABs. (a) Conjugation of cytotoxic drugs to engineered THIOMABs. Schematic representation of the reduction and oxidation process used to generate reactive THIOMABs and their conjugation to biotin or cytotoxic drugs. (b) The THIOMAB variant of anti-MUC16 3A5 retains high-affinity binding to cell-surface antigen. Humanized anti-MUC16 Thio-3A5 or conventional 3A5 antibody was incubated with OVCAR-3 cells expressing endogenous MUC16 and bound antibody detected using a fluorescent anti-human Fc secondary antibody. Flow cytometry histograms illustrate binding at saturating (400 ng/ml) and subsaturating (25 ng/ml) concentrations. At all antibody concentrations analyzed, the two anti-MUC16 variants gave equivalent binding, suggesting equivalent affinities for the antigen. Ab, antibody; TCEP, tris(2-carboxyethyl)phosphine; dhAA, dehydroascorbic acid; DTT, dithiothreitol; TDC, THIOMAB-drug conjugate.

derivatives conjugated with a drug of interest suggest the potential for scale-up to allow clinical evaluation. One such THIOMAB-drug conjugate (TDC) retains the *in vivo* efficacy of a conventional ADC and exhibits superior safety in preclinical models.

RESULTS

Engineering cysteine residues for site-specific conjugation

In seeking sites that might be substituted with cysteine residues, we focused on those not involved with antibody effector functions, such as the light and heavy chains of the constant domains of the antibody Fab region, which has no apparent role in antigen binding or in Fc-mediated effector functions²¹. We recently reported a phage display-based method (PHESELECTOR) to screen reactive cysteines on the Fab surface of the antibodies²². As applying this approach to the anti-Her2 antibody trastuzumab (Herceptin) suggested the suitability of the variants LC-V110C and HC-A114C (Kabat numbering) for site-specific labeling of Fabs²², we selected these two sites to develop a conjugation process in the context of full-length antibodies. After expression in Chinese hamster ovary (CHO) cells, our initial attempts to conjugate THIOMABs in a single step with cysteine-reactive probes, *N*-ethyl maleimide or biotinyl-3-maleimido-propionamidyl-3,6-dioxaoctanediamine (biotin-PEO-maleimide) were unsuccessful. Liquid chromatography (LC)/mass spectroscopy (MS) analysis confirmed that the engineered cysteine residues were in mixed disulfides with cysteine or glutathione, presumably formed during the fermentation process (Supplementary Fig. 1 online).

Figure 1a illustrates the conjugation scheme that was subsequently developed to covalently attach thiol-reactive probes to the engineered cysteines. First, the cysteine and glutathione adducts were removed from the THIOMABs by partial reduction followed by diafiltration. This partial reduction also disrupts interchain disulfide bonds but not intrachain disulfide bonds. The interchain disulfide bonds were allowed to reform by air oxidation or by accelerated oxidation using CuSO_4 or dehydro-ascorbic acid (dhAA), as demonstrated by non-reducing SDS-PAGE analysis (Supplementary Fig. 2a online) or by LC/MS analysis (Supplementary Fig. 3 online). After this treatment, THIOMAB variants LC-V110C and HC-A114C (in the context of two different mAbs) were then conjugated with biotin-PEO-maleimide. Biotinylation of the appropriate antibody subunit was demonstrated by probing western blots with streptavidin-horseradish peroxidase (Supplementary Fig. 2b online). Whereas 100% biotin conjugation (two moles of biotin per mAb) was observed for the HC-A114C variant, only 25–50% was seen with the LC-V110C variant (Supplementary Fig. 2b online). Papain digestion and LC/MS analysis showed that during reoxidation, some of the LC-V110 cysteines had formed an unexpected disulfide bond between the two Fab portions of the antibody and thus were unavailable for drug conjugation (Supplementary Fig. 4 online). The HC-A114C variant of various antibodies did not show this property and so was selected for further studies (Supplementary Table 1 and Supplementary Fig. 2c online).

The efficient conjugation at HC-A114C but not at LC-V110C prompted us to seek further sites for cysteine engineering. We substituted most of the available serine, alanine and valine residues (24 variants) in the light chain domain of the trastuzumab-Fab. Thio-Fab phage were isolated, biotinylated and tested for binding to the Her2 extracellular domain (ECD) and streptavidin, as described earlier²². LC-V205C, LC-S114C, LC-V110C and LC-S127C showed the highest thiol reactivity values (0.8–1.0) (Supplementary Fig. 5 online). The discrepancy between the results with the LC-V110C Fab versus the LC-V110C full-length antibody prompted us to evaluate the most promising sites in the context of the full-length

Table 1 Analytical characterization of anti-MUC16 drug conjugates

Antibody-drug conjugate	Scale	DAR	Monomer (%)	Drug conjugate species distribution (%)						
				0	1	2	3	4	6	8
ADC	1 mg–1 gm	3.10	98	12	3	42	3	32	7	1
ch3A5 TDC initial process	~50 mg	1.60	95	5	35	60	–	–	–	–
hu3A5 TDC initial process	~50 mg	1.60	98	6.4	29	64.5	–	–	–	–
hu3A5 TDC improved process	<10 g	1.97	>98	0.6	6.7	88.4	4.3	–	–	–
TDC improved process	>100 g	2.00	>98	0.3	3.3	92.1	4.3	–	–	–

Drug conjugate species distribution and drugs/antibody were quantified based on hydrophobic interaction chromatographic analysis as described in Methods. Percent of monomer was determined by size-exclusion chromatography.

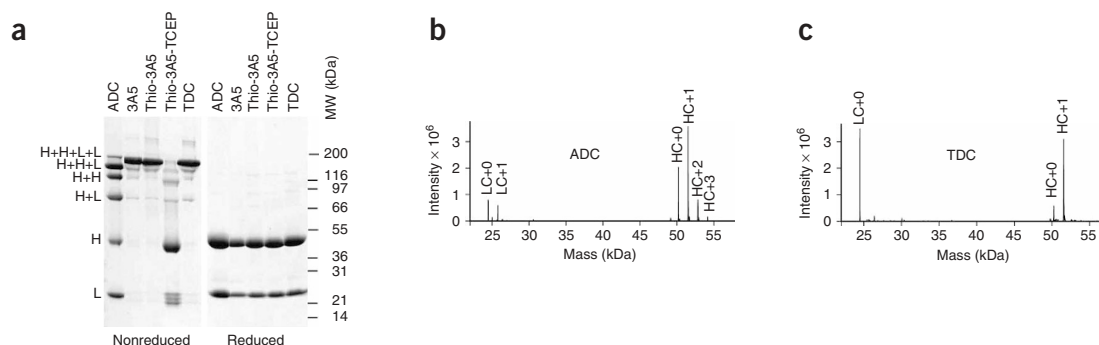


Figure 2 TH1OMAB drug conjugates retain interchain disulfide bonds with site-specific drug attachment. **(a)** SDS-PAGE analysis of antibody-drug conjugates under nonreducing and reducing conditions. The appearance of multiple species in the standard ADCs is due to loss of interchain disulfide bonds. **(b,c)** Deconvoluted mass spectra of ADC **(b)** and TDC **(c)** variants of an antibody against MUC16. Drug conjugates are deglycosylated and reduced before LC/MS analysis. ADC mass spectra displayed zero or one drug species on the light chain and zero, one, two or three drug species on the heavy chain. TDC displayed only one drug species on the heavy chain. ADC, anti-MUC16-MC-vc-PAB-MMAE; TDC, thio-anti-MUC16-MC-vc-PAB-MMAE; TH1OMAB-TCEP, TH1OMAB reduced with tris(2-carboxyethyl)phosphine (TCEP); H, heavy chain; L, light chain.

antibody. Based on current and previous PHESELECTOR assay results²², we have selected eight sites on the light chain (LC-V15C, LC-V110C, LC-S114C, LC-S121C, LC-S127C, LC-A153C, LC-S168C and LC-V205C) and four sites on the heavy chain (HC-S112C, HC-S113C, HC-S115C and HC-T116C). All of these TH1OMABs were expressed and purified along with the HC-A114C variant and unmodified trastuzumab. The purified proteins were conjugated to biotin-maleimide, and the extent of conjugation was quantified by LC/MS analysis. As 9 of the 13 TH1OMABs showed >90% conjugation efficiency, they should be as suitable for site-specific conjugation of thiol reactive probes as the HC-A114C variant described below (Supplementary Table 1 online). In this report, we focus on the properties of one TH1OMAB (anti-MUC16) and its conjugates. Studies with multiple antibody-antigen combinations have established the general utility of this strategy (data not shown).

Anti-MUC16 TH1OMAB retains antigen binding and specificity

The successful biotinylation of HC-A114C variants prompted us to test TDCs *in vitro* and *in vivo*. MUC16 is a cell-surface protein that is overexpressed in many ovarian malignancies. As we previously reported encouraging findings with conventional anti-MUC16 (monoclonal antibody 3A5) drug conjugates⁶, which were efficacious at tolerated doses *in vivo*, we chose to focus on anti-MUC16 to evaluate the potential of the TH1OMAB strategy for expanding the therapeutic index. Chimeric (ch3A5) and fully humanized (hu3A5) anti-MUC16 antibodies were engineered to have the HC-A114C mutation (Kabat numbering; equivalent to A118C in Eu numbering and A117C in sequential numbering). Generation of the chimeric immunoglobulin was described previously⁶. To produce a humanized anti-MUC16 antibody, the complementarity-determining regions (CDR) of mu3A5 were grafted into human consensus V_LKL and V_HsubgroupIII frameworks (Supplementary Methods and Supplementary Figs. 6a,b online). Relative to chimeric 3A5, the binding affinity of the 3A5 CDR-graft for MUC16 was markedly reduced (Supplementary Table 2 online). To restore binding, we introduced mutations into the CDR regions of the 3A5 graft to reconstitute appropriate CDR-framework interactions or to select more favorable CDR-antigen interactions. A library of CDR variants was displayed as Fab on phage and panned for improved interactions with the antigen. Enhanced binding was observed only in variants with substitutions in CDR-H3 (Supplementary Fig. 6c online). Four representative clones,

reformatted and expressed as IgG and assessed for MUC16 binding relative to chimeric 3A5, demonstrated that changes in CDR-H3 fully restore antigen binding (Supplementary Table 2 online). One clone was taken forward as humanized anti-MUC16.

To be useful for therapeutic development, the TH1OMAB strategy must yield an antibody with comparable or higher binding affinity and specificity for the target antigen as compared with the conventional antibody. Affinities of humanized anti-MUC16 HC-A114C TH1OMAB and conventional 3A5 IgG were compared by flow cytometry on OVCAR-3 cells (ovarian cancer cells that express endogenous MUC16), serially diluting the antibodies until a reduced shift was observed (Fig. 1b). Antibodies were in excess of cellular binding sites throughout the titration. At each concentration tested, thio-anti-MUC16 bound to OVCAR-3 cells as efficiently as conventional anti-MUC16. Surface plasmon resonance-based analyses using portions of the human MUC16 ECD also confirmed the high affinity of this TH1OMAB for this antigen ($K_D = 116$ pM). Comparison of cells with high and low or absent MUC16 expression based on RT-PCR studies revealed that this TH1OMAB binds to cells that express MUC16 but not to MUC16-negative cell lines (Supplementary Fig. 7 online). Thus, substitution at HC-A114 does not affect antigen binding.

Anti-MUC16 TH1OMAB yields nearly homogeneous conjugates

TH1OMAB 3A5 antibodies were partially reduced and reoxidized to yield two free thiol groups per antibody, then conjugated to the cytotoxic drug monomethyl auristatin E (MMAE) via the protease-labile maleimido-caproyl-valine-citrulline-para-amino-benzyloxy carbonyl (MC-vc-PAB) linker (Supplementary Fig. 8a online)^{23,24}. For simplicity, the conventional conjugate (anti-MUC16-MC-vc-PAB-MMAE) will subsequently be referred to as anti-MUC16 ADC and the TH1OMAB conjugate (thio-anti-MUC16-MC-vc-PAB-MMAE) will be referred to as anti-MUC16 TDC. Anti-MUC16 ADC contained an average drug-antibody ratio (DAR) of 3.1 (Table 1) and migrated as multiple species on nonreducing SDS-PAGE, consistent with the loss of interchain disulfide bonds through drug conjugation (Fig. 2a). In contrast, the interchain disulfides were retained in anti-MUC16 TDC, which migrated as a single major band. Hydrophobic interaction chromatography was used to resolve antibodies with different stoichiometries of drug conjugation. Anti-MUC16 TDC was modified with zero, one or two drugs (average DAR = 1.6; Table 1 and

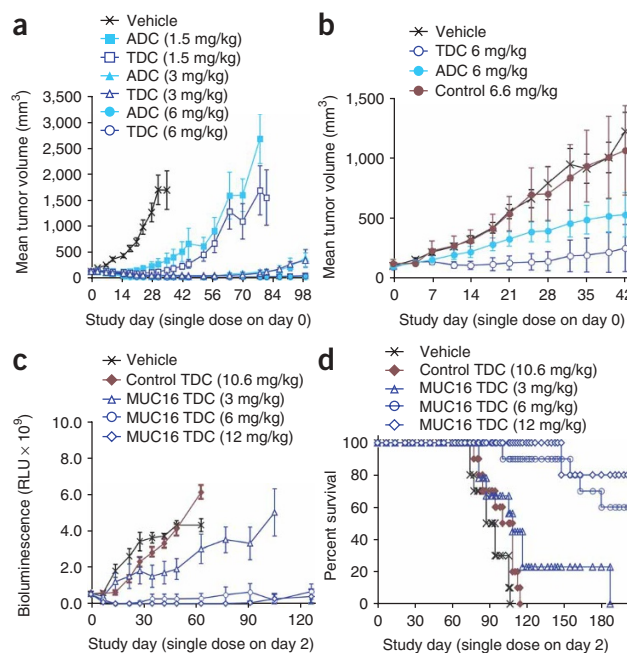


Figure 3 *In vivo* efficacy is retained with the TDC format. **(a)** Tumors derived from OVCAR-3 cells were serially transplanted into the mammary fat pads of female SCID mice, as previously reported⁶, and anti-MUC16 ADC and TDC were compared. When palpable tumors were established, mice were randomized to a mean tumor volume of ~150 mm³ in each group (ten/group; range = 100–200 mm³) and then treated intravenously once (day 0) with chimeric anti-MUC16 ADC (3.1 drugs per antibody) or TDC (1.6 drugs per antibody) at the indicated doses. Mean tumor volumes ($\pm$ s.e.m.) are plotted over time, with the ADC and TDC curves superimposed for the 3 mg/kg and 6 mg/kg groups. **(b)** Female nu/nu mice were inoculated with OVXF 1023 primary ovarian cancer tumors and anti-MUC16 ADC and TDC were compared. Chimeric anti-MUC16 ADC (3.1 drugs per antibody) or TDC (1.6 drugs per antibody) or a control ADC were dosed once as indicated. Mean tumor volumes ($\pm$ s.e.m.) are plotted over time. **(c,d)** Female SCID mice were inoculated in the peritoneal cavity with OVCAR-3/luciferase cells, as previously reported⁶. Tumor burden was assessed by bioluminescence measurement after injection of luciferin. Once bioluminescence was stable (~3 weeks after inoculation), mice were grouped on the basis of luminescence and dosed once with humanized anti-MUC16 TDC (1.6 drugs per antibody) or an irrelevant TDC (1.9 drugs per antibody) as indicated. Changes in mean bioluminescence intensities ($\pm$ s.e.m.) over time. Bioluminescence intensities plotted in terms of relative light units (RLU) **(c)**. Changes in the percentages of surviving mice over time **(d)**.

Supplementary Fig. 8b online). The absence of higher stoichiometric ratios indicates that only the engineered A114C cysteines were conjugated, as confirmed by LC/MS analysis and peptide mapping (Supplementary Figs. 9 and 10 and Supplementary Tables 3 and 4 online). In line with previous reports^{8,14}, standard 3A5 ADCs are a mixture of seven different drug stoichiometries (zero, one, two, three, four, six and eight drugs per antibody; Table 1 and Supplementary Fig. 8b online) with the possibility of conjugating to any of eight cysteines, potentially generating >100 different ADC species. LC/MS analysis showed that the linker drug was distributed to both the light (one drug) and heavy chain (one, two or three drugs) of anti-MUC16 ADC but only to the heavy chain (one drug) of the cognate TDC (Fig. 2b,c). Finally, anti-MUC16 TDC retains high affinity for the human MUC16 ECD ($K_D = 117$ pM).

Along with reduced heterogeneity, the anti-MUC16 TDC preparation lacks species that have the higher drug loads reported to not be tolerated as well in rodents⁸. Subsequent process development has yielded conjugates with almost exactly two drugs per antibody. The species carrying the two drugs on the engineered cysteines and nowhere else is by far the predominant species in the preparation (Table 1 and Supplementary Fig. 8d online).

Anti-MUC16 TDC displays comparable efficacy to ADC

We previously have reported that the conventional anti-MUC16 drug conjugates are highly efficacious in mouse OVCAR-3 xenograft models⁶. To determine if the thio-anti-MUC16 retains this property, the ADC and TDC variants of anti-MUC16 were subjected to *in vitro* cell proliferation and *in vivo* xenograft studies. *In vitro*, the ADC and TDC formats of chimeric anti-MUC16 had similar cytotoxicities, with median inhibitory concentration (IC_{50}) < 50 ng antibody/ml against OVCAR-3 cells and PC3 cells transfected with a recombinant form of MUC16 (Supplementary Fig. 11 online). The IC_{50} values of the anti-MUC16 TDC were approximately twofold higher than those of the ADC, possibly due to the twofold lower drug load of the TDC. Parental PC3 cells lacking MUC16 expression were not affected by either conjugate up to 3 μ g/ml, demonstrating the specificity of the

antiproliferative effect. The rates of internalization into OVCAR-3 cells *in vitro* were comparable, as expected, given that neither method of drug conjugation detectably influences antigen binding (data not shown).

Anti-MUC16 ADC and anti-MUC16 TDC were also compared *in vivo* using chimeric antibody conjugates and an OVCAR-3 xenograft model (Fig. 3a). Mice bearing established tumors (~150 mm³) were dosed once with either anti-MUC16 ADC or anti-MUC16 TDC over a range of dose levels. The anti-MUC16 TDC was at least as active as the ADC at each IgG dose level, providing partial efficacy at 1.5 mg/kg (MMAE doses are 35 μ g/m² for the TDC and 71 μ g/m² for the ADC) and near-complete elimination of tumors at 3 mg/kg (69 versus 141 μ g/m² MMAE) and 6 mg/kg (139 versus 283 μ g/m² MMAE). When stated in terms of MMAE dose, anti-MUC16 TDC was approximately twice as efficacious as anti-MUC16 ADC. No adverse effects of either conjugate were observed at any dose level. Anti-MUC16 TDC was also at least as active as anti-MUC16 ADC against a transplant xenograft model of ovarian cancer (Fig. 3b), and subsequent studies have demonstrated potent activity of the TDC against several tumor models (Figs. 3c,d and Supplementary Figs. 12,13 online). The study using the intraperitoneal OVCAR-3/luciferase xenograft model (Figs. 3c,d) demonstrated single-dose activity of the anti-MUC16 TDC at 3 mg/kg against a tumor growing at a more relevant anatomic site, with improved activity at higher dose levels. Our previous studies using the ADC also achieved significant efficacy but with multiple weekly dosing⁶.

Improved therapeutic index with anti-MUC16 TDCs

As noted above, the different conjugation procedures yielded anti-MUC16 ADC and TDC with different drug stoichiometries. Therefore, equivalent efficacy using the anti-MUC16 TDC is achieved with approximately one-half the dose of cytotoxic MMAE. Our preliminary studies have indicated that the toxicity of ADCs in animals is closely associated with the cytotoxic drug dose, and the adverse events are largely consistent with the safety profile of the drug itself. This suggests that the anti-MUC16 TDC may be better tolerated in animals than the ADC at equivalent mg/kg dose levels.

We evaluated the safety of the anti-MUC16 ADC and TDC in Sprague-Dawley rats and cynomolgus monkeys. Both species express

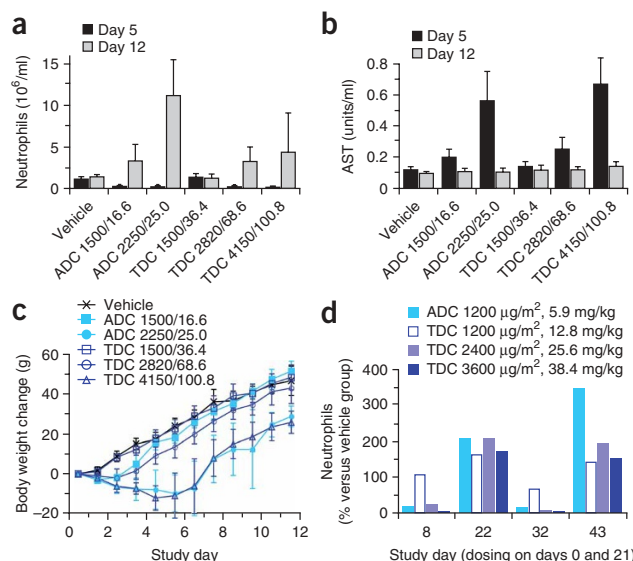


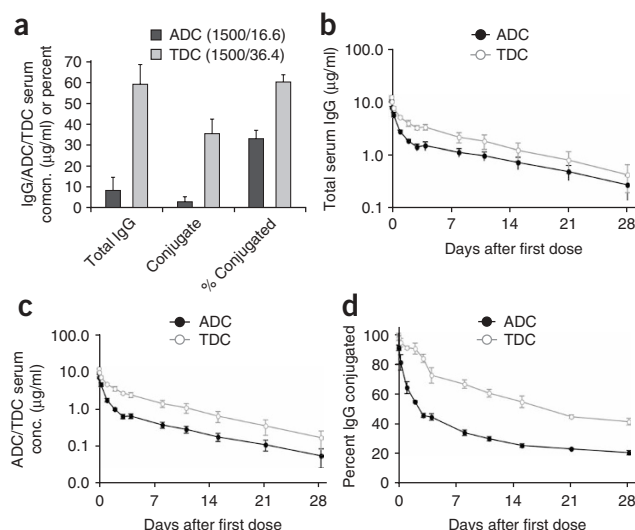
Figure 4 The TDC format is better tolerated *in vivo*. (**a–c**) Normal Sprague-Dawley rats were dosed once (day 1) with humanized anti-MUC16 ADC or TDC at the indicated dose levels. Dose levels are given in terms of $\mu\text{g}/\text{m}^2$ MMAE (the cytotoxic drug dose per body surface area, derived from the stoichiometry of conjugation) and mg/kg IgG; for example, “TDC 1500/36.4” indicates TDC dosed at 36.4 mg/kg IgG, which at 1.6 drugs per IgG corresponds to 1,500 $\mu\text{g}/\text{m}^2$ MMAE. Blood was drawn from rats at study day 5 (4 d after dosing) and day 12 (immediately before they were killed) for hematology (neutrophil counts in **a**) and serum chemistry (serum AST levels in **b**). X-axis labels for **b** apply also to **a** (treatment groups are graphed in the same order). (**c**) Rats were weighed daily after dosing and changes in body weight over time relative to day 1 plotted. (**d**) Higher doses of the TDC format are required to reduce neutrophil counts in cynomolgus monkeys. In two separate studies, female Chinese cynomolgus monkeys were dosed on days 1 and 22 with: humanized anti-MUC16 ADC (5.9 mg/kg IgG = 1,200 $\mu\text{g}/\text{m}^2$ MMAE; white bars); TDC at 12.8 mg/kg IgG (1,200 $\mu\text{g}/\text{m}^2$ MMAE; light gray bars); or TDC at 25.6 mg/kg IgG (2,400 $\mu\text{g}/\text{m}^2$ MMAE; dark gray bars); or TDC at 38.4 mg/kg IgG (3,600 $\mu\text{g}/\text{m}^2$ MMAE; black bars). Blood was drawn for hematology and serum chemistry at the indicated intervals (day 22 values are from before the second dose). Average circulating neutrophil counts were normalized to the average counts from vehicle-treated monkeys at the given time point of the same study. Note the nadir in neutrophil levels ~ 1 week after dosing, followed by a recovery to normal levels within 3 weeks.

MUC16, although the primary sequence of the cynomolgus monkey antigen is more similar to the human counterpart than the rat antigen is. In a competitive binding assay, mAb 3A5 binding to CA125 was inhibited with similar concentrations of human and monkey MUC16 ECD proteins ($\text{IC}_{50} = 0.76$ nM and 1.88 nM, respectively), but competition by the rat MUC16 ECD was much less efficient ($\text{IC}_{50} = 13.5$ nM). Both rats and cynomolgus monkeys are sensitive to antibody-MMAE conjugates. As safety studies using a related cytotoxic compound, dolastatin-10, showed mice to be relatively insensitive to this class of drug, the mouse models used in the efficacy studies were not considered useful for safety assessment²⁵. In rats, a single dose of 16.6 mg/kg anti-MUC16 ADC (1,500 $\mu\text{g}/\text{m}^2$ MMAE) produced a marked depletion of circulating neutrophils and other white blood cells at day 5 (4 d post-dose; **Fig. 4a** and data not shown), followed by a compensatory rebound at day 12. This anti-MUC16 ADC dose also led to a mild elevation in serum levels of the liver enzyme aspartate aminotransferase (AST; **Fig. 4b**) and transient weight loss (**Fig. 4c**). The AST levels were more profoundly affected by a 50% increase in dose (24.5 mg/kg ADC; 2,250 $\mu\text{g}/\text{m}^2$ MMAE); at that dose, three of six rats did not survive to the end of the study (day 12). In contrast, 36.4 mg/kg of anti-MUC16 TDC (equivalent to 1,500 $\mu\text{g}/\text{m}^2$ MMAE exposure) yielded no adverse effects, with all parameters essentially identical to vehicle-treated animals. A dose of 68.6 mg/kg anti-MUC16 TDC (2,820 $\mu\text{g}/\text{m}^2$ drug) produced toxicities

that were nearly equivalent to those observed using the anti-MUC16 ADC at one-fourth that dose. Although the highest anti-MUC16 TDC dose (100.8 mg/kg ; 4,150 $\mu\text{g}/\text{m}^2$ MMAE) led to pronounced effects (and two of six rats were killed due to excessive weight loss), the overall profile of adverse effects was quite similar to that observed at the higher anti-MUC16 ADC dose (24.5 mg/kg ADC; 2,250 $\mu\text{g}/\text{m}^2$ MMAE). The same trends were observed in a preliminary study using chimeric conjugates (**Supplementary Fig. 14** online).

The most prominent adverse event in cynomolgus monkeys dosed with anti-MUC16 ADC or TDC is a reversible decrease in neutrophils. Whereas a marked decrease was induced by anti-MUC16 ADC at a drug exposure of 1,200 $\mu\text{g}/\text{m}^2$ drug (5.9 mg/kg antibody), anti-MUC16 TDC at 1,200 $\mu\text{g}/\text{m}^2$ drug (12.8 mg/kg antibody) yielded no notable adverse events, with neutrophil counts tracking closely with sham-treated animals (**Fig. 4d**). Doubling the dose resulted in decreased neutrophil counts, which were completely reversible. An even higher dose of anti-MUC16 TDC (38.4 mg/kg ; 3,600 $\mu\text{g}/\text{m}^2$

Figure 5 In rats, a higher proportion of TDC is retained in circulation, compared with its ADC counterpart. (**a**) Serum levels of total anti-MUC16 IgG (“Total antibody”) and antibody carrying at least one cytotoxic drug (Conjugate) were measured at day 12 of the rat safety study described in **Figure 4a–c**. The percentage of antibody with at least one drug still attached (% Conjugated) was calculated from the ratio of Conjugate and Total IgG and is plotted next to the absolute values for ease of interpretation. Data for the 1,500 $\mu\text{g}/\text{m}^2$ MMAE groups are shown. (**b–d**) In a separate study, normal Sprague-Dawley rats were dosed once with 0.5 mg/kg chimeric ADC or TDC at study day 0. At the indicated intervals, blood was drawn for determination of total anti-MUC16 IgG (**b**) and anti-MUC16 carrying at least one MMAE (**c**). The fraction of drug-conjugated to total IgG was determined as the ratio of conjugated to total IgG and is plotted over time (**d**). Similar data were observed at higher dose levels.



drug) gave no marked effects beyond the neutrophil decrease, which was more pronounced than at the middle-dose level but remained reversible. Importantly, no toxicities were observed in cornea, lung, oviduct and uterus—all organs known to express MUC16. The only notable histopathologic findings were minimal-to-mild increases in bone marrow myelopoiesis and minimal-to-mild thymic lymphoid depletion, consistent with the decreases in neutrophil count and indicative of a regenerative response.

These results demonstrate that the anti-MUC16 TDC is safer than anti-MUC16 ADC in preclinical models, even when compared on the basis of cytotoxic drug dose (that is, equivalent $\mu\text{g}/\text{m}^2$). To begin to understand this, we have analyzed the kinetics of clearance of each type of conjugate in rats. At a dose of 36.4 mg/kg IgG (1,500 $\mu\text{g}/\text{m}^2$ MMAE), anti-MUC16 TDC remained in circulation at much higher levels than anti-MUC16 ADC dosed at 16.6 mg/kg IgG (1,500 $\mu\text{g}/\text{m}^2$ MMAE), even after accounting for the differences in dose levels (Fig. 5a). Nonetheless, anti-MUC16 TDC at that dose yielded no adverse effects. We observed the same results when dosing rats with chimeric ADC and TDC at a matched mg/kg dose (Supplementary Fig. 14d online). A more thorough kinetic analysis using chimeric antibodies (Fig. 5b–d) showed that the total TDC is cleared somewhat more slowly than the ADC (9.5 ± 2.9 versus 16.1 ± 3.5 ml/day/kg), and the proportion of TDC still bearing at least one drug decreased substantially more slowly than the corresponding proportion of ADC (14.1 ± 3.0 versus 41.6 ± 4.8 ml/day/kg). Interestingly, the ADC and TDC kinetics are comparable in tumor-bearing mice (Supplementary Fig. 15 online). Slower clearance of the TDC variant has consistently been observed using different antibodies, suggesting that the kinetics of the anti-MUC16 conjugates in the mouse may be an anomaly. The drug conjugate assay generates a lower signal from a TDC with exactly one drug than from a TDC with two drugs. However, the discrepancy is minor and cannot explain the different behaviors of the ADC and TDC variants. Therefore, we conclude that despite bearing fewer drugs per antibody on average, the TDC variants retain the conjugated drugs more effectively in rats than their ADC counterparts.

DISCUSSION

The unfavorable *in vivo* effects associated with heterogeneity in the drug load and sites of attachment in antibody-drug conjugates could compromise their promise as cancer therapeutics. Conjugation through lysine residues was shown to distribute to ~ 40 different sites, potentially resulting in $>10^6$ ADC species²⁰. Conjugates generated through cysteines by partial reduction of interchain-disulfide bonds also have variable stoichiometry (zero to eight drugs per antibody) and potentially yield >100 species⁸. Solvent-accessible interchain-disulfide bond cysteines have been replaced with serine to allow directed conjugation to the remaining cysteines²⁶. However, elimination of these disulfide bonds could disrupt quaternary structure of the antibody, thereby perturbing the behavior of the antibody *in vivo*, including changes in antibody effector functions^{27–29}. Our THIOMAB technology resolves the issue of conjugate heterogeneity by directing the attachment of drugs at defined sites and with near-uniform stoichiometry. Additionally, the conjugation chemistry retains all of the native immunoglobulin disulfide bonds. Cysteine engineering into antibodies for site-specific conjugation has been achieved previously but with poor yield and no potential for large-scale processing^{30,31}. Notably, site-specific conjugation to antibody-Fabs has been reported using cysteine engineering^{22,32}. However, the engineered cysteines were shown to be blocked by cysteinylolation or glutathionylation in the context of full-length antibodies (Supplementary Fig. 1 online). Our novel conjugation method reactivates the

engineered cysteines for conjugation, thus achieving site-specific antibody-drug conjugates at high yield and purity. We have observed no challenges with this conjugation strategy even up to a multi-gram scale. Also, our methodology should be amenable to a wide range of cytotoxic drugs, requiring only that the drugs be compatible with sulfhydryl-directed conjugation chemistries.

These properties alone represent a substantial advancement for product development. In addition, we have found that the anti-MUC16 TDC exhibits a markedly improved therapeutic index in preclinical animal models. When comparing matched IgG (mg/kg) dose levels, our data show equivalent efficacy of the anti-MUC16 TDC and ADC in mouse xenografts, whereas in rats the 68.6 mg/kg (2,820 $\mu\text{g}/\text{m}^2$ MMAE) dose of the TDC exerts similar toxicities as the 16.6 mg/kg (1,500 $\mu\text{g}/\text{m}^2$ MMAE) dose of the ADC. When compared in terms of MMAE exposure, the TDC is both safer and more efficacious than the ADC. Although the success of the approach has yet to be tested in humans, even a modest increase in the range of safe and efficacious doses could dramatically enhance the clinical value of the conjugate. The efficacy and toxicity of an antibody-drug conjugate could be influenced substantially by a host immune response against the conjugate. In cynomolgus monkeys, we have observed little if any immune response against the anti-MUC16 TDC and only sporadic responses against the ADC. Improved safety without loss of efficacy has been observed for several different antibodies targeting multiple tumor antigens (our unpublished results), indicating that the THIOMAB technology is a general path to an expanded therapeutic window.

We do not yet know what accounts for the improved safety of the TDC in rats and primates. The *in vivo* kinetics of the total TDC in rats are quite comparable to those of native (nonconjugated) antibodies, whereas the total ADC clears somewhat faster. When considering the kinetics of conjugated antibodies, the difference between the TDC and the ADC is more pronounced, and over time a far greater proportion of circulating TDC retains at least one drug (Fig. 5b–d). This could point to a mechanism of clearance used by a fraction of the ADC but not the TDC and leading to ADC metabolism and toxicity. For example, the relatively more highly conjugated species within the ADC preparation may clear more rapidly, thereby enriching for the unconjugated antibody that is 12% of the original preparation (Table 1). Consistent with our findings, a correlation has been shown between total antibody clearance and toxicity in mice dosed with a DAR8 ADC⁸. Therefore, it is possible that the relative safety of the TDC can be attributed at least partly to the absence of high-drug-load species. Although the ADC preparations used in the present studies did not contain high levels of antibodies with greater than four drugs attached, rats may be more sensitive than mice to more moderate drug loading. The previous work⁸ suggests that a similarly favorable profile of safety and activity might be observed with a hypothetical homogenous ADC preparation having a preponderance of a single species bearing two drugs conjugated at two sites only. ADC conjugation chemistry does not presently allow such a preparation at the scale necessary for preclinical evaluation, not to mention the scale required for clinical testing.

An alternative explanation for the pharmacokinetic data is that the MMAE may be more readily released from the ADC than from the TDC in circulation. Several mechanisms might lead to greater stability of the TDC. The engineered cysteines may be relatively ‘protected’ sites that resist proteolytic attack in circulation. We have observed that the accessibility of a cysteine residue varies depending where it is located in the antibody-Fab²². An ADC is produced by conjugating a drug at reduced hinge disulfides, one of the most accessible regions

of the antibody³³. Therefore, the ADC may be more readily processed than the TDC and release more active MMAE in circulation, thus increasing ADC toxicity in rats. Also, although the TDC and the ADC variants have equivalent affinity for FcRn (Supplementary Figs. 16,17 and Supplementary Table 5 online), it is possible that the ADC is less stable while traversing the FcRn-mediated intracellular recycling pathway.

Either mechanism (differences in antibody clearance or rate of MMAE release) could affect safety. Indeed, relatively higher levels of ADC-derived free MMAE in circulation could readily account for the adverse effects that we have observed in our preclinical safety studies. Specifically, the myelotoxicity manifested by marked neutrophil decline was also the dose-limiting toxicity in animals treated with dolastatin-10, the parent molecule of MMAE. Also, elevated serum AST results from dosing rats with dolastatin-10, ADC and TDC. The rapid clearance of highly conjugated ADC could also directly lead to toxicity within the organ of clearance and systemic release of MMAE. At present, we cannot distinguish between these two mechanisms, and both may operate.

Our preclinical safety data suggest that MMAE exposure will determine the tolerability of the anti-MUC16 TDC in the clinic. If so, one clear benefit to the TDC over the ADC would be to reduce the MMAE exposure at an efficacious dose of conjugate. In turn, the improved tolerability of the TDC could permit higher dose levels on a mg/kg antibody basis. This could be critical to producing a therapeutic benefit for patients with more challenging malignancies, such as reduced sensitivity to MMAE or relatively lower levels of MUC16 expression. Overall, the TDC conjugation strategy confers improved *in vivo* properties and may represent a decisive advancement in the development of therapeutic antibody-drug conjugates.

METHODS

Site directed mutagenesis, THIOMAB expression and purification. Cysteine mutations were introduced in antibody light or heavy chain constructs (in pRK expression vectors) using double-stranded DNA as a template by PCR-based site-directed mutagenesis as described earlier²². THIOMAB light and heavy chain constructs were transiently transfected into CHO cells, and the antibodies were purified over Protein A columns followed by ion exchange chromatography.

Conjugation. THIOMAB conjugation was performed at Genentech, Seattle Genetics or NPIL Pharma UK using methods developed at Genentech. Before conjugation of the THIOMAB to biotin or MMAE derivatized with a maleimide-containing linker, the blocking cysteine or glutathione that was present on the introduced cysteine was removed by mild reduction in PBS at 25 °C by the addition of tenfold molar excess reducing agent, TCEP or dithiothreitol (DTT) followed by diafiltration. To re-form the interchain disulfide bonds, the THIOMAB was incubated for three hours at 25 °C with CuSO₄ or with dhAA (Sigma-Aldrich) at a twofold molar excess over the reducing agent concentration. The formation of interchain disulfide bonds was monitored either by nonreducing SDS-PAGE or by denaturing reversed phase high-performance liquid chromatography (HPLC). PLRP column chromatography. The maleimide-linked labeling reagent, either biotin-PEO-maleimide (tenfold molar excess over protein) or MC-vc-PAB-MMAE (threefold molar excess over protein), was incubated with the activated THIOMAB for 1 h at 25 °C. The antibody conjugate was purified on HiTrap S column (GE Healthcare Bio-Sciences) to remove excess reagents. The number of conjugated biotin or MC-vc-PAB-MMAE molecules per mAb was quantified by LC/MS analysis. Initial biotin conjugation experiments were carried out with CuSO₄; all the cytotoxic drug conjugation experiments described in this paper were carried out with dhAA.

Mass spectrometric analysis. LC/MS analysis was performed on a TSQ Quantum Triple quadrupole mass spectrometer with extended mass range

(Thermo Electron). Samples were chromatographed on a PRLP-S, 1000 Å, microbore column (50 mm × 2.1 mm, Polymer Laboratories) heated to 75 °C. A linear gradient from 30–40% B (solvent A, 0.05% TFA in water; solvent B, 0.04% TFA in acetonitrile) was used and the eluant was directly ionized using the electrospray source. Data were collected by the Xcalibur data system and deconvolution was performed using ProMass (Novatia). Before LC/MS analysis, antibodies or drug conjugates (50 µg) were treated with PNGase F (2 units/ml; PROzyme) for 2 h at 37 °C to remove N-linked carbohydrates.

Hydrophobic interaction chromatography (HIC). Samples were injected onto a Butyl HIC NPR column (2.5 µm, 4.6 mm × 3.5 cm) (Tosoh Bioscience) and eluted with a linear gradient from 0 to 70% B at 0.8 ml/min (A, 1.5 M ammonium sulfate in 50 mM potassium phosphate, pH 7; B, 50 mM potassium phosphate pH 7, 20% isopropanol). An Agilent 1100 series HPLC system equipped with a multi-wavelength detector and Chemstation software was used to resolve and quantify antibody species with different ratios of drugs per antibody.

Flow cytometry and *in vitro* studies. OVCAR-3 cells (30,000 cells per sample) were incubated on ice with humanized conventional or thio anti-MUC16 mAb for 75 min in 1 ml total volume. Antibodies were applied at 25, 50, 100, 200 and 400 ng/ml in PBS + 1% FBS + 2 mM EDTA. After this incubation, cells were washed and then incubated with phycoerythrin-labeled goat anti-human Fc secondary antibody (1 h on ice). Cells were then washed and analyzed by flow cytometry as described previously⁶. Based on our published data, 3×10^4 OVCAR-3 cells express $\sim 1 \times 10^{10}$ binding sites for anti-MUC16 antibody 3A5. Even the lowest antibody concentration tested (25 ng or $\sim 1 \times 10^{11}$ antibodies) provides a molar excess of antibodies over binding sites. Therefore, the concentration at which binding is reduced (as detected by flow cytometry) will reflect the affinity of the antibody for MUC16.

Cell proliferation in the presence of antibody-drug conjugates was assessed in PC3/neo (MUC16-negative), PC3/MUC16 and OVCAR-3 cells in a 96-well format essentially as described previously⁶. Binding affinities of anti-MUC16 variants were determined by surface plasmon resonance and by enzyme-linked immunosorbent assays (ELISA) using conventional procedures (described in Supplementary Methods online).

***In vivo* efficacy.** Efficacy studies were performed using female C.B-17 severe combined immunodeficient (SCID) beige mice (Charles River Laboratories). All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. The OVCAR-3 mammary fat pad transplant efficacy model was employed as described previously⁶, evaluating tumor volume after a single intravenous dose. The OVCAR-3/luciferase model has also been described previously⁶.

Safety assessment. The toxicities of anti-MUC16 ADC and TDC were compared in female adolescent Sprague-Dawley rats (100–125 g) receiving a single intravenous bolus dose (day 1). Body weight was measured daily. Analyses of serum chemistry and hematology (including quantification of separate lymphocytic populations) were conducted using sera collected on days 5 and 12. A thorough histopathological assessment followed euthanasia and necropsies on day 12.

The toxicities of anti-MUC16 ADC and TDC also were evaluated in two separate studies using female cynomolgus monkeys of Chinese origin (2.6–3.0 kg) receiving two bolus doses on days 1 and 22. Animals were dosed such that each animal in a given group received the same MMAE drug dose (in µg/m²), allowing the antibody dose (in mg/kg) to vary slightly according to body weight. Each animal was observed twice daily for mortality, abnormalities, and signs of pain or distress. Body weights were measured on unfasted animals twice during the predose phase, before dosing on day 1 and weekly thereafter. Analyses of clinical chemistry, hematology and coagulation were made with blood collected twice during the predose phase and on days 4, 8, 15, 22 (predose), 25, 32 and 43. Animals were euthanized on day 43 by anesthesia with sodium pentobarbital and exsanguination, and tissues were subjected to a thorough gross pathological and histopathological evaluation.

***In vivo* kinetic analyses.** The disposition of the anti-MUC16 antibody-drug conjugates *in vivo* was analyzed by measuring the serum concentrations of

antibody and of drug conjugate. For kinetic analyses in rats (Figs. 5b–d), serum was collected at 5 min, 1 h, 6 h, 24 h, and 2, 3, 4, 8, 11, 15, 21 and 28 d after a single intravenous dose. For kinetic analyses in mice (Supplementary Fig. 15 online), serum was collected at 3 min, 1 h, 6 h, 24 h, and 2, 3, 4, 7, 11, 14 and 21 d after a single intravenous dose. Concentrations of antibody–drug conjugates bearing at least one cytotoxic drug were measured with an ELISA that used the MUC16 ECD protein for capture and anti-MMAE mouse monoclonal antibody SG2.15 (generously provided by Seattle Genetics) plus anti-mouse-Fc-horse-radish peroxidase (HRP) for detection. We have observed a decreased signal in this assay for TDC with one versus two conjugated drugs. Therefore, the levels of conjugates may be greater than what we have measured. Total ch3A5 and ch3A5 THIOMAB concentrations in serum were measured with an ELISA that used the MUC16 ECD protein for capture and anti-human-Fc HRP as the secondary antibody. This assay measures any anti-MUC16 antibody, both with and without conjugated MMAE. The assays have lower limits of quantification of 0.78 ng/ml with a minimum dilution of 1:10. The serum concentration–time data from each animal was analyzed using a two-compartment model with IV bolus input, first-order elimination and macro-rate constants (Model 8, WinNonlin Pro v.5.0.1, Pharsight Corporation). Serum from the day 12 bleeds of the rat safety study were assayed using the same formats to generate the total and conjugated antibody data shown in Figure 5a.

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGMENTS

The authors wish to thank our Genentech colleagues: Jennifer Speer for preparing trastuzumab THIOMAB DNA constructs; Mary Cole for insights into the OVCAR-3 intraperitoneal efficacy model; Elmer Wu, Darshana Patel, Mark Rowen and Anthony Delucchi for providing critical reagents; Natalia Gomez and George Dutina for large-scale transient transfection/fermentation; Fred Jacobson and Charity Bechtel for their help with analytical characterization of TDCs; and Allen Ebens for critical review of the manuscript. We thank Damon Meyer and his colleagues at Seattle Genetics for preparation of the early lots of anti-MUC16 ADC and TDC and for many helpful comments and suggestions. We thank employees of NPIL Pharma UK for their assistance with large-scale conjugations. We also thank the staff of Oncotest for conducting some of the efficacy studies described in this manuscript. Anti-MMAE mouse monoclonal antibody SG2.15 was generously provided by Seattle Genetics, Inc.

AUTHOR CONTRIBUTIONS

J.R.J. and W.M. led the overall program, designed experiments, performed *in vitro* studies, analyzed the data and wrote the manuscript. Y.C. and M.S.D. humanized the anti-MUC16 antibody. S.B. generated the anti-MUC16 THIOMAB DNA constructs and performed pilot expression studies. M.S., E.D. and J.G. performed larger-scale antibody production. H.R. established procedures for TDC conjugation and analytical characterization. C.C.L. carried out analytical characterization of TDCs. S.W., S.P.T., Y.L., Y.G.M., C.N. and J.Y. performed *in vitro* binding studies. S.C., R. Venook and S.R. performed *in vivo* efficacy studies. D.D.L. designed and analyzed pharmacokinetic studies. A.K., K.M. and K.F. designed and executed safety assessment studies. V.K., S.D.S., W.L.W., H.B.L., R. Vandlen, M.X.S., R.H.S. and P.P. provided direction and guidance for the various functional areas and assisted in writing the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturebiotechnology/>

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