Engineered Thio-Trastuzumab-DM1 Conjugate with an Improved Therapeutic Index to Target Human Epidermal Growth Factor Receptor 2–Positive Breast Cancer

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Abstract

Purpose: Antibody drug conjugates (ADCs) combine the ideal properties of both antibodies and cytotoxic drugs by targeting potent drugs to the antigen-expressing tumor cells, thereby enhancing their anti-tumor activity. Successful ADC development for a given target antigen depends on optimization of antibody selection, linker stability, cytotoxic drug potency, and mode of linker-drug conjugation to the antibody. Here, we systematically examined the in vitro potency as well as in vivo preclinical efficacy and safety profiles of a heterogeneous preparation of conventional trastuzumab-mcc-DM1 (TMAb-mcc-DM1) ADC with that of a homogeneous engineered thio-trastuzumab-mpeo-DM1 (thioTMAb-mpeo-DM1) conjugate.

Experimental Design and Results: To generate thioTMAb-mpeo-DM1, one drug maytansinoid 1 (DM1) molecule was conjugated to an engineered cysteine residue at Ala114 (Kabat numbering) on each trastuzumab-heavy chain, resulting in two DM1 molecules per antibody. ThioTMAb-mpeo-DM1 retained similar in vitro anti-cell proliferation activity and human epidermal growth factor receptor 2 (HER2) binding properties to that of the conventional ADC. Furthermore, it showed improved efficacy over the conventional ADC at DM1-equivalent doses (μg/m²) and retained efficacy at equivalent antibody doses (mg/kg). An improved safety profile of >2-fold was observed in a short-term target-independent rat safety study. In cynomolgus monkey safety studies, thioTMAb-mpeo-DM1 was tolerated at higher antibody doses (up to 48 mg/kg or 6,000 μgD M1/m²) compared with the conventional ADC that had dose-limiting toxicity at 30 mg/kg (6,000 μg DM1/m²).

Conclusions: The engineered thioTMAb-mpeo-DM1 with broadened therapeutic index represents a promising antibody drug conjugate for future clinical development of HER2-positive targeted breast cancer therapies. Clin Cancer Res; 16(19); 4769–78. ©2010 AACR.

Antibody-targeted chemotherapy is designed for selective delivery of cytotoxic drugs to tumor cells by linking them to monoclonal antibodies, thereby enhancing antibody therapeutic activity while minimizing the systemic effects of a cytotoxic drug. Antibody drug conjugates (ADCs) are composed of three key elements that can influence the therapeutic index: antibody, linker, and drug. Successful ADC development therefore involves optimization of a matrix of parameters concerning antibody selection, cytotoxic drug potency, and linker stability properties (1–3). In addition, the choice of target antigen (expression in cancer versus normal tissues) and the ability to internalize the antigen-antibody complex are crucial as they determine tumor cell-specific delivery of cytotoxic drug (3). Currently, highly potent cytotoxic drugs (maytansinoids, auristatins, anthracyclins, duocarmycins, etc.) with diverse mechanisms of action are being conjugated to antibodies, and their therapeutic activities are being evaluated in preclinical and clinical studies (4–13). To date, one ADC, anti-CD33-calicheamicin conjugate (gemtuzumab ozogamicin or Mylotarg), is approved in the United States for the treatment of acute myeloid leukemia (14).

Drug maytansinoid 1 (DM1), a potent antimitotic inhibitor, is a maytansine derivative that belongs to ansamitocin group of natural products (15, 16). Several maytansinoid antibody conjugates are in various stages of clinical development, including IMGN242 (anti-Muc1), IMGN901 (anti-CD56), MLN2704 (anti-PSMA), and...
Human epidermal growth factor receptor 2 (HER2)/ErbB2 is the target of the therapeutic agents trastuzumab and lapatinib, which are used to treat HER2-positive breast cancer. However, tumors in some patients often relapse after trastuzumab or lapatinib treatments due to altered signaling mechanisms downstream or parallel to the HER2 signaling pathway. To circumvent these problems, we recently developed a trastuzumab-mcc-DM1 (TMAB-mcc-DM1), an armed antibody drug conjugate to overcome trastuzumab-resistant tumor growth in HER2-positive breast cancer patients. In the current study, we made further significant progress by developing a homogeneous engineered thio-trastuzumab-mpeo-DM1 (thioTMAB-mpeo-DM1) conjugate that displays improved safety over a conventional TMab-mcc-DM1 with an equivalent efficacy. Thus, our studies support the argument that engineered thioTMAB-mpeo-DM1 in combination with a noncleavable linker has a better therapeutic index than TMAB-mcc-DM1 and represents a promising antibody drug conjugate for future clinical development in combating HER2-positive breast cancer.

We recently reported a THIOMAB technology platform that produces a more homogeneous preparation of antibody drug conjugates (19). Compared with a conventional monoethyl auristatin E (MMAE)-conjugated anti-MUC16 ADC, site-specific conjugation of MMAE to an anti-MUC16 THIOMAB improved the overall tolerability in rats and monkeys in acute and short-term toxicity studies. Specifically, MUC16 THIOMAB (TDC) exhibited less liver and bone marrow toxicity in rats and monkeys compared with that of an anti-MUC16 ADC. Moreover, the TDC retained efficacy despite having a reduced drug load (two drugs per antibody) relative to a conventional ADC (average of 3-4 drugs per antibody; ref. 19). Consequently, we aimed to test whether the THIOMAB platform can be applied to cytotoxic drugs other than auristatin derivatives. In the studies presented herein, we designed a THIOMAB version of trastuzumab (thio-trastuzumab) to prepare homogenously conjugated TMAB-mcc-DM1. DM1 was conjugated to thio-trastuzumab using a nonreducible bis-maleimido-trioxyethylene glycol (BMPEO) linker. The resulting THIOMAB drug conjugate (thioTMAB-mpeo-DM1) was shown to have two DM1 drugs per antibody conjugated selectively to the engineered cysteine residues, whereas the conventional TMab-mcc-DM1 ADC was a mixture of conjugates with heterogeneous species having various drug to antibody ratios (0-7), with an average of 3.5 drugs/antibody. DM1 is conjugated to several different lysine residues in the conventional antibody format conjugation shown. Analysis of data from both preclinical efficacy and safety studies indicate that thioTMAB-mpeo-DM1 may have an improved therapeutic index over conventional TMab-mcc-DM1 ADC.

Materials and Methods

Synthesis and purification of mpeo-DM1

BMPEO (72 mg, 0.02 mmol) was added to a two-neck 14/20 50-mL flask and dissolved in 4 mL of solvent mixture containing dimethyl formamide, acetonitrile, and water (2:1:1). The flask was gently purged with nitrogen gas for 15 minutes. The solution was cooled to 0°C in an ice/water bath. L-DM-1 (30 mg, 0.04 mmol) was dissolved in 1 mL of dimethyl formamide. The solution was added dropwise in the 50-mL flask and the reaction was monitored by analytical high performance liquid chromatography (HPLC) method using ONYX Monolithic C18 (5 μm) reverse phase chromatography (3 mL/minute flow-rate, 5-50% acetonitrile gradient in water containing 0.05% trifluoroacetic acid as a mobile phase). Upon completion of the reaction, the reaction mixture was brought to room temperature (approx. 20°C) and filtered through a 0.45-μm Nylon Whatman filter-disc. The crude sample was applied to a preparative HPLC to purify monomeric mpeo-DM1 (100 mL/minute flow-rate, 5-60% acetonitrile gradient in water containing 0.05% trifluoroacetic acid). Typical yield after HPLC purification was about 50% to 70% of starting DM1 material.
Antibody drug conjugates

The trastuzumab and TMAb-mcc-DM1 used in this study were prepared as described earlier (19). Construction and production of the THIOMAB variant of trastuzumab was reported previously (19). Briefly, a cysteine residue was engineered at Ala114 position (Kabat numbering) of trastuzumab-heavy chain to produce its THIOMAB variant. The thio-trastuzumab-MPEO-DM1 conjugate (thioTMAb-mpeo-DM1) was produced and the drug to antibody ratio (DAR) of ADCs was determined by liquid chromatography/mass spectrometry (LC/MS) analysis as described earlier (19).

Flow cytometric analysis and cell viability assays

The SK-BR-3 cell line used in this study, a cell line high in human epidermal growth factor receptor 2 (HER2) expression with over 1.5 million copies per cell, was obtained from the American Type Culture Collection, and cells were cultured in Ham's F-12 high glucose (50:50) supplemented with 10% heat-inactivated fetal bovine serum and 2 mmol/L L-glutamine (all from Invitrogen Corp.). SK-BR-3 cells (100,000 cells per sample) were incubated on ice with trastuzumab, thio-trastuzumab, TMAb-mcc-DM1, or thioTMAb-mpeo-DM1 at various concentrations in the range of 0 to 1,600 ng/mL in fluorescence activated cell sorting (FACS) buffer (PBS buffer containing 1% bovine serum albumin) for 1 hour. Cells were washed with FACS buffer and incubated with phycoerythrin-labeled goat anti-human Fc secondary antibody (1:3,000 dilution) on ice for 1 hour. All samples were washed using FACS buffer and fixed with 2% paraformaldehyde followed by FACS analysis using a BD FACSCalibur system (BD Biosciences). Cell viability assays in the presence of TMAb-mcc-DM1 and thioTMAb-mpeo-DM1 (0-10 μg/mL) were carried out in SK-BR-3 cells in a 96-well format as described previously (10).

In vivo efficacy studies

The Fo5 mouse mammary tumor model was employed to evaluate the in vivo efficacy of TMAb-mcc-DM1 and thioTMAb-mpeo-DM1 (single-dose i.v. injections) as described previously (10). The Fo5 model is a transgenic mouse model in which the human HER2 gene is overexpressed in mammary epithelium under transcriptional regulation of the murine mammary tumor virus promoter (MMTV-HER2). The HER2 overexpression causes spontaneous development of a mammary tumor. The mammary tumor of one of these founder animals [founder 5 (Fo5)] has been propagated in subsequent generations of FVB mice by serial transplantation of tumor fragments (~2 x 2 mm in size). All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Rat and cynomolgus monkey toxicity studies

The safety profile of thioTMAb-mpeo-DM1 and TMAb-mcc-DM1 was evaluated in naïve Sprague-Dawley and Crl:CD(SD) rats (Charles River Laboratories) and cynomolgus monkeys (Macaca fascicularis, Prime Resources Development Bio Services Limited, Hong Kong and Pacific Forest Resources). Use of animals and the study protocols were approved by the Institutional Animal Use and Care Committee at Genentech, Inc., and Charles River Laboratories (Reno, NV) for the rat and monkey studies, respectively. Young female rats (75-80 grams) were given a single i.v. bolus, tail-vein injection of vehicle, TMAb-mcc-DM1 (4,222 μg/m², 47 mg ADC/kg), or thioTMAb-mpeo-DM1 (2,221, 4,222, or 6,000 μg DM1/m²; 47, 89, or 127 mg TDC/kg; 5 animals/ADC or TDC group or 3 animals/vehicle group) on study day 1. Toxicologic assessments included evaluation of body weights (predose on study day 1 and daily thereafter), daily clinical observations, standard serum chemistry and hematology (study days 5 and 12), total trastuzumab antibody and antibody-drug conjugate levels (study day 12), and macroscopic and microscopic pathology. Animals were euthanized and necropsy was done on study day 12. At necropsy, organs were weighed and tissues were collected. Tissues were processed routinely to H&E sections and were evaluated by a board-certified pathologist.

Young female monkeys (2.4-3.4 kg) were given a single, slow bolus i.v. injection (saphenous vein as the preferred site of injection) of vehicle, TMAb-mcc-DM1 (6,000 μg/m², 30 mg/kg), or thioTMAb-mpeo-DM1 (6,000 or 8,000 μg DM1/m²; 48 or 65 mg/kg; 4 animals/group) on study days 1 and 22. Toxicologic assessments included evaluation of body weights (weekly), clinical observations (at least daily), standard serum chemistry [twice prestudy, days 4, 8, 15, 22 (predose), 25, 29, and 44], hematology [twice prestudy, days 4, 8, 15, 22 (predose), 25, 29, and 44], and coagulation parameters [twice prestudy, days 8, 22 (predose), and 44], and macroscopic and microscopic pathology. Animals were euthanized and necropsy was done on study day 44. At necropsy, organs were weighed and tissues were collected. Tissues were processed routinely to H&E sections and were examined by a board-certified pathologist.

Pharmacokinetic studies

Ten female Sprague-Dawley rats (75-80 g) in two groups (n = 5 rats/group) were either given a single i.v. dose of 47 mg/kg (4,222 μg DM1/m²) TMAb-mcc-DM1, or 47 mg/kg (2,221 μg DM1/m²) thioTMAb-mpeo-DM1. Eight female cynomolgus monkeys in two groups (n = 4 monkeys/group) were given two i.v. doses, three weeks apart, of either 30 mg/kg (6,000 μg/m²) TMAb-mcc-DM1, or 48 mg/kg (6,000 μg/m²) thioTMAb-mpeo-DM1. The conjugate antibody concentrations of TMAb-mcc-DM1 or thioTMAb-mpeo-DM1 in serum were determined as described previously (10). The assay limit of quantitation was 164 ng/mL in rat serum and 66 ng/mL in cynomolgus monkey serum. The serum samples collected from monkey pharmacokinetic studies were the same as used in monkey toxicity studies, described above. The plasma concentration-time data for each animal were analyzed using a noncompartmental analysis method (Model 201, WinNonlIn Pro, v.5.2; Pharsight Corporation).
Pharmacokinetic parameters were determined for each animal, and means ± SD were reported.

Results

Engineered thioTMAb-mpeo-DM1 conjugate is homogeneous with two DM1 drugs per antibody

We had reported previously that anti-MUC16-vc-MMAE TDC displayed a uniform DAR distribution with 1-DAR or 2-DAR species and all MMAE drugs were attached to a single conjugation site via an engineered cysteine residue (A114C, Kabat numbering; ref. 19). In contrast, the conventional anti-MUC16-vc-MMAE ADC contained a heterogeneous mixture of various DAR species with MMAE conjugated to various light- and heavy-chain cysteine residues generated from reduced interchain disulfide bonds. To test whether engineered thioTMAb-mpeo-DM1 conjugates would show similar homogeneity in drug conjugation, we first chemically coupled a BMPEO linker to DM1-SH and purified it by reversed-phase chromatography. BMPEO-DM1 was conjugated to the engineered cysteine residue on the thio-trastuzumab (Fig. 1). The conjugation process for the conventional TMAb-mcc-DM1, in which the maytansine drug is covalently linked to ε-amino group of lysine residues, was described previously (10). We analyzed the DAR
distribution for both conventional and engineered DM1 conjugates by LC/MS analysis. As shown in Fig. 2, thioT-MAb-mpeo-DM1 had approximately 90% 2-DAR species with an average DAR of 1.8. In contrast, DAR species from 0 to 7 were observed with TMAb-mcc-DM1, which had an overall average of 3.3 (Fig. 2A and B).

**Engineered thioTMAb-mpeo-DM1 displayed equal in vitro activity and in vivo efficacy at comparable antibody doses of conventional ADC**

We tested thioTMAb-mpeo-DM1 and TMAb-mcc-DM1 conjugates for their ability to bind to HER2-expressing SK-BR-3 cells using flow cytometry analysis. Both conjugates were able to bind to these cells similar to that of unconjugated trastuzumab and thio-trastuzumab (Fig. 2C and Supplementary Fig. S1). It was previously shown that TMAb-mcc-DM1 displayed potent cell-killing activity by inducing mitotic cell-cycle arrest, thereby causing apoptotic cell death and cellular lysis in HER2-expressing cell lines, whereas HER2-negative cells were unaffected (10). ThioTMAb-mpeo-DM1 also showed a similar cell-killing activity in SK-BR-3 cells; as expected unconjugated thio-trastuzumab or trastuzumab alone showed only modest anti–cell proliferative activity in a 3-day short-term assay (Fig. 2D). We also tested the in vitro potency of Lys-mcc-DM1 and Cys-mpeo-DM1, the anticipated active metabolites of TMAb-mcc-DM1 and thioTMAb-mpeo-DM1, respectively. Unlike free DM1, both metabolites showed poor cell-killing activity, indicating that they display similar cell impermeable properties (Supplementary Fig. S2).

The in vivo efficacy of TMAb-mcc-DM1 and thioTMAb-mpeo-DM1 conjugates was tested in the MMTV-HER2 Fo5 trastuzumab-resistant mammary tumor model. MMTV-HER2 Fo5 tumor explants were implanted into the no. 2/3 mammary fat pad of CRL nu/nu mice. When tumors reached an average volume of 180 mm³, the mice were treated with LP32 (2 mg/kg) or placebo (0.9% saline) s.c. weekly for 3 weeks.

![Fig. 2. ThioTMAb-mpeo-DM1 displayed more homogenous drug attachment to antibody compared with TMAb-mcc-DM1 (A and B) and both conjugates showed similar HER2 binding to (C) and in vitro cell killing properties against (D) the HER2-amplified SK-BR-3 breast cancer cell line. A, LC/MS profile for TMAb-mcc-DM1; L, linker without drug conjugated to antibody. B, LC/MS profile for thioTMAb-mpeo-DM1. C, conjugated (TMAb-mcc-DM1 and thioTMAb-mpeo-DM1) or unconjugated trastuzumab (trastuzumab and thio-trastuzumab) variants (0.1 μg/mL) were tested for their ability to bind to SK-BR-3 cells by FACS analysis. D, in vitro cell proliferation assay was done with trastuzumab, thio-trastuzumab, TMAb-mcc-DM1, and thioTMAb-mpeo-DM1.](image-url)
were the randomized and then given a single i.v. dose of DM1 conjugates on study day 0. As seen earlier, TMAb-mcc-DM1 showed partial inhibition in tumor growth at 10 mg/kg, which equates to a DM1 dose of 560 μg/m². ThioTMAb-mpeo-DM1 had comparable activity at the same antibody concentration, but at lower DM1 dose of 330 μg/m² (Fig. 3A; ref. 10). At an equivalent DM1 dose, thioTMAb-mpeo-DM1 (approximately 535 μg DM1/m²; 18 mg TDC/kg) was more efficacious than TMAb-mcc-DM1 (approximately 540 μg DM1/m²; 10 mg/kg). We also tested efficacy at three different dose levels (5, 10, and 15 mg/kg). Both conjugates showed comparable tumor inhibition at all matched antibody doses and the minor differences observed were not statistically significant (Fig. 3C). The control anti-IL8-mcc-DM1 (15 mg/kg) or trastuzumab alone (15 mg/kg) were previously shown as not having any detectable activity in the MMTV-HER2 Fo5 trastuzumab-resistant mammary tumor model (10).

**Improved nonclinical safety with thioTMAb-mpeo-DM1 compared with a conventional TMAb-mcc-DM1 conjugate**

The anti-MUC16-vc-MMAE THIOMAB drug conjugate showed a 4-fold improvement on a mg/kg basis with respect to bone marrow and liver toxicities compared with a conventional anti-MUC16-vc-MMAE conjugate in short-term toxicity studies (19). To determine whether a THIOMAB format would similarly improve the tolerability of DM1 conjugates, we compared the safety profiles of thioTMAb-mpeo-DM1 and TMAb-mcc-DM1 in Sprague-Dawley rats and cynomolgus monkeys. Trastuzumab binds to cynomolgus monkey (Kd, 0.72 nmol/L) and human HER2 (Kd, 0.65 nmol/L) with similar affinity, but does not bind to rat ErbB2 (HER2; ref. 10). However, because we observed that the toxicities of antibody-DM1 conjugates are similar to those of DM1 alone and are not directly related to antigen binding, both rats and monkeys are considered appropriate nonclinical safety models regardless of species-dependent affinity differences for HER2 binding.

In rats, a single i.v. dose of TMAb-mcc-DM1 (4,222 μg DM1/m², 47 mg ADC/kg) was generally well tolerated and resulted in transient body weight loss and/or decreased body weight gain (Fig. 4A). Elevations in serum aspartate aminotransferase (AST; Fig. 4B) and alanine aminotransferase (ALT; Supplementary Fig. S1A) were observed 5 days postdose and returned to levels comparable with those of vehicle-treated rats by study day 12. Consistent with minimal to mild liver injury, these changes correlated with microscopic hepatic findings (Supplementary Table S1A). In addition, administration of TMAb-mcc-DM1 (4,222 μg DM1/m², 47 mg ADC/kg) produced minimal to mild hematologic changes. As shown in Fig. 4C, reduced platelet counts were noted 5 days postdose followed by a compensatory rebound on study day 12. Increased neutrophil counts on study days 5 and 12 were also observed (Supplementary Fig. S1B).
In contrast, thioTMAb-mpeo-DM1 administration at equivalent mg/kg doses of antibody (2,221 μg/m², 47 mg TDC/kg) or μg/m² DM1 (4,222 μg DM1/m², 89 mg TDC/kg) did not result in the same degree of weight loss, reduced platelet counts, or elevations in AST and ALT in rats (Fig. 4A-C, Supplementary Fig. S1A). Effects on body weight and liver enzyme elevations similar to that of TMAb-mcc-DM1 were observed at a higher dose (6,000 μg DM1/m², 127 mg TDC/kg) of thioTMAb-mpeo-DM1 (Fig. 4A and B), consistent with a better safety

### Figure 4

**Engineered thioTMAb-mpeo-DM1 shows lower toxicity than conventional thioTMAb-mpeo-DM1 in rat and cynomolgus monkey safety studies.** Normal Sprague Dawley rats (n = 5 animals/group except n = 3 animals/vehicle group, single i.v. dose on day 1) and monkeys (n = 4 animals/group, two i.v. doses on study days 1 and 21) were given TMAb-mcc-DM1 or thioTMAb-mpeo-DM1 at the indicated dose levels. A, rats were weighed daily after dosing, and changes in body weight over time relative to study day 1 are plotted. Blood was drawn from rats on study days 5 and 12 for clinical chemistry (B, serum AST levels) and hematology (C, platelet counts). Clinical chemistry (D, serum AST levels) and hematology (E, platelet counts) parameters from monkeys are shown up to study day 44.

**Rat toxicity studies:** Fig 4A, 4B, 4C

- TMAb-mcc-DM1 (TMAb-ADC) : 47 mg/kg, 4,222 μg/m²
- ThioTMAb-mpeo-DM1 (TMAb-TDC1) : 47 mg/kg, 2,221 μg/m²
- ThioTMAb-mpeo-DM1 (TMAb-TDC2) : 89 mg/kg, 4,222 μg/m²
- ThioTMAb-mpeo-DM1 (TMAb-TDC3) : 127 mg/kg, 6,000 μg/m²

**Cynomolgus monkey toxicity studies:** Fig 4D, 4E

- TMAb-mcc-DM1 (TMAb-ADC) : 30 mg/kg, 6,000 μg/m²
- ThioTMAb-mpeo-DM1 (TMAb-TDC4) : 48 mg/kg, 6,000 μg/m²
- ThioTMAb-mpeo-DM1 (TMAb-TDC5) : 65 mg/kg, 8,000 μg/m²
Similarly, smaller increases in neutrophil counts were observed with all dose levels of thioTMAb-mpeo-DM1 compared with TMAb-mcc-DM1 (Supplementary Fig. S1B). At all doses levels of thioTMAb-mpeo-DM1, platelet counts were comparable with vehicle-treated rats (Fig. 4C). Additional histologic findings in which there were no apparent differences in severity at comparable dose levels of TMAb-mcc-DM1 and thioTMAb-mpeo-DM1 are shown in Supplementary Table S1A.

Intravenous administration of two doses (3 weeks apart) of both thioTMAb-mpeo-DM1 and TMAb-mcc-DM1 to monkeys was also generally well tolerated with no negative effects on body weight (data not shown). Similar to rats, hepatic and hematologic toxicities were noted suggesting that these changes were not antigen mediated. Elevations in AST (Fig. 4D), and to a lesser extent ALT (Supplementary Fig. S1C), were observed with both thioTMAb-mpeo-DM1 and TMAb-mcc-DM1 seven days after dosing on study days 8 and 29. As was observed in rats, however, thioTMAb-mpeo-DM1 was better tolerated than TMAb-mcc-DM1 in that the extent of the AST and ALT elevations were lower at an equivalent dose of thioTMAb-mpeo-DM1 compared with TMAb-mcc-DM1 (Fig. 4D and Supplementary Fig. S1C). Hepatocyte degeneration was only seen with thioTMAb-mpeo-DM1 at the 8,000 μg DM1/m2 (65 mg TDC/kg) dose (Supplementary Table S1B). Reduced platelet counts were prominent in monkeys given TMAb-mcc-DM1 at 6,000 μg DM1/m2 (30 mg ADC/kg) 7 days postdose. This reduction seemed less severe in monkeys given thioTMAb-mpeo-DM1 at doses of 6,000 (48 mg TDC/kg) and 8,000 μg DM1/m2 (65 mg TDC/kg; Fig. 4E). Interestingly, although increased neutrophil counts were observed with TMAb-mcc-DM1 on study day 4, slightly decreased neutrophil counts were noted with thioTMAb-mpeo-DM1 at doses of 6,000 and 8,000 μg DM1/m2 on study day 8 (Supplementary Fig. S1D). The reason for this difference, which may be reflective of different time response relationships between these conjugates, is not known, but myeloid hypercellularity, likely in response to the decrease in neutrophils, was also observed in these groups (Supplementary Table S1B). Additional histologic findings in monkeys that were comparable between animals dosed with thioTMAb-mpeo-DM1 and TMAb-mcc-DM1 are shown in Supplementary Table S1B.

**Both conventional TMAb-mcc-DM1 and engineered thioTMAb-mpeo-DM1 showed similar pharmacokinetic properties**

To understand whether the improved safety profile is due to differences in clearance between the engineered thioTMAb-mpeo-DM1 and TMAb-mcc-DM1, the pharmacokinetic properties of both TMAb-mcc-DM1 and thioTMAb-mpeo-DM1 in rats and cynomolgus monkeys were tested. As described above, trastuzumab does not cross-react with rat ErbB2 (HER2) but does bind to the

### Table 1. Mean ± SD observed and dose-normalized pharmacokinetic parameters for trastuzumab conjugates in cynomolgus monkeys

<table>
<thead>
<tr>
<th>Actual dose (mg/kg)</th>
<th>Antibody drug conjugate</th>
<th>$C_{\text{max}}$ (μg/mL)</th>
<th>$AUC_{0-21}$ (day × μg/mL)</th>
<th>DN $C_{\text{max}}$ (μg/mL)</th>
<th>DN $AUC_{0-21}$ (day × μg/mL)</th>
<th>Clearance (mL/day/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>TMAb-mcc-DM1</td>
<td>736 ± 117</td>
<td>2,310 ± 65.7</td>
<td>24.5 ± 3.90</td>
<td>77.0 ± 2.19</td>
<td>12.3 ± 0.499</td>
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<tr>
<td>48</td>
<td>ThioTMAb-mpeo-DM1</td>
<td>1,160 ± 247</td>
<td>4,530 ± 652</td>
<td>24.2 ± 5.15</td>
<td>94.4 ± 13.6</td>
<td>10.0 ± 1.65</td>
</tr>
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![Fig. 5. Both TMAb-mcc-DM1 and thioTMAb-mpeo-DM1 display similar pharmacokinetic properties in cynomolgus monkeys (A) and in rats (B). Conjugated antibody concentration versus time profile following i.v. administration to female Sprague Dawley rats on study day 1 or to cynomolgus monkeys on study days 1 and 21. Symbols, mean serum concentrations determined from 4 to 5 animals at each time point; error bars, SD of the mean concentrations at each time point.](image)
cynomolgus monkey ortholog (10). As expected, following i.v. administration of either conjugate to cynomolgus monkeys, the antibody serum concentrations attained their maximum levels immediately following administration; subsequently, the concentrations declined over time as shown in Fig. 5A. The noncompartmental pharmacokinetic parameters for both conjugates are tabulated in Table 1. On a protein dose-normalized basis, \(C_{\text{max}}\) (maximum observed antibody drug conjugate concentration in serum) and AUC\(_{0-21}\) (area under the serum antibody drug conjugate concentration versus time curve from 0–21 days following the first dose) were similar for TMAb-mcc-DM1 and thioTMAb-mpeo-DM1, suggesting similar pharmacokinetics for both ADC and TDC. A similar trend is also seen in rat pharmacokinetic properties at equivalent doses (approximately 47 mg/kg) with both conjugates.

**Discussion**

The successful development of ADCs was made possible by the significant amount of progress made over the past 20 years in optimizing several critical parameters such as target selection, use of fully human/humanized antibodies, linker stability, potent cytotoxic drugs, and ADC internalization to obtain an ideal ADC for clinical use (1–3). However, chemical conjugation occurs through covalent modification of lysine or cysteine residues, in the latter case following reduction of interchain disulfide bonds. Consequently, the conventional antibody-drug conjugates are chemically heterogeneous. (20, 21). We recently described an engineered anti-MUC16-vc-MMAE TDC and showed that this TDC had uniform drug loading with an improved preclinical safety profile compared with its conventional ADC (19). The results from the present study broaden the scope of the TDC format to a new class of cytotoxic drug (DM1) and a different target antigen (HER2). Engineered thioTMAb-mpeo-DM1 contained two DM1 drugs per antibody conjugated at single engineered cysteine residue on each heavy chain, similar to that reported with anti-MUC16-vc-MMAE TDC. ThioTMAb-mpeo-DM1 retained equivalent efficacy in HER2-expressing cell lines as the anti-MUC16-vc-MMAE TDC, suggesting similar pharmacokinetics for both ADC and TDC. A similar trend is also seen in rat pharmacokinetic properties at equivalent doses (approximately 47 mg/kg) with both conjugates.

we observed up to 2-fold improvement in the preclinical therapeutic index with a DM1-derived TDC. It is important to note that TMAb-mcc-DM1 ADC is already tolerated at higher doses, 30 mg ADC/kg (6,000 μg DM1/m²) in monkeys compared with anti-MUC16-vc-MMAE ADC that tolerates only up to 5.9 mg/kg ADC (19). The combined data presented herein, as well as those reported previously for anti-MUC16-vc-MMAE ADC and TDC, suggest that although anti-HER2 and anti-MUC16 drug-conjugates target distinct cell surface proteins with different linkers and cytotoxic drugs, the mode of toxicity for ADCs is predominantly target antigen independent (19). The clinically observed therapeutic index for TMAb-mcc-DM1 ADC is perhaps due in part to usage of a stable noncleavable linker because it was shown preclinically by Phillips et al. that TMAb-mcc-DM1 with a MCC linker is more tolerable compared with a less stable cleavable and reducible SPP linker (10).

The mechanisms for increased therapeutic index with the anti-MUC16 TDC may differ from that observed for thioTMAb-mpeo-DM1. For the thio anti-MUC16-vc-MMAE conjugate, we proposed that the cleavable linker-based MMAE TDCs achieved improvement in safety by two modes of action: (a) improving linker stability by partially protecting the conjugation site, thereby limiting drug loss from the antibody in circulation; and (b) elimination of high drug-load ADC species (4-8 DAR species) from the TDC produced in conventional ADC preparations. However, linker stability does not seem to be appreciably different between thioTMAb-mpeo-DM1 and TMAb-mcc-DM1. The exposure-based therapeutic index, represented by the plasma conjugate exposure associated with toxicity divided by that associated with efficacy, was larger for the thioTMAb-mpeo-DM1 TDC compared with ADC by 1.6- to 2.0-fold based on \(C_{\text{max}}\) and AUC, respectively. This improvement was driven primarily by an improvement in tolerability associated with the TDC, i.e., conjugate exposure associated with toxicity was greater for the ADC at 48 mg/kg than for ADC at 30 mg/kg. The exposure associated with efficacy was similar for the TDC and ADC (data not shown). In light of the observation that the pharmacokinetic properties of the two trastuzumb conjugates are similar, it is unlikely that the apparent increase in therapeutic index with thioTMAb-mpeo-DM1 conjugate is due to the hydrophilicity of the mpeo linker. Thus, the improvement in the nonclinical safety profile of thioTMAb-mpeo-DM1 is likely due to the elimination of high DAR species or drug loss from high DAR species.

HER2 targeted breast cancer therapy is well established clinically, with the approval of trastuzumab (22, 23) by the U.S. Food and Drug Administration in 1998 and the more recent approval of lapatinib, a dual-tyrosine kinase inhibitor (24). Despite the significant progress being made to battle cancer with these targeted therapies, tumors in some patients often relapse due to altered signaling mechanisms downstream or parallel to the HER2 signaling pathway. Thus, an armed antibody-based
therapy would potentially overcome the resistance to trastuzumab- or lapatinib-based therapies, providing the target is still expressed. The use of a stable, noncleavable linker in TMAB-mcc-DM1 results in an ability to overcome trastuzumab-resistant tumor growth in preclinical efficacy models and is better tolerated than TMAB-DM1 conjugates with reducible linkers in preclinical safety studies (Fig. 4 and ref. 10). Recent phase I clinical data with TMAB-mcc-DM1 further confirmed that at maximum tolerated dose (3.6 mg/kg every 3 weeks in HER2-positive breast cancer patients), the TMAB-mcc-DM1 conjugate showed substantial clinical activity (clinical benefit rate of 73%) with mild and reversible toxicity (25). The data presented for the engineered TDC in combination with a stable noncleavable linker in this study represent a promising antibody drug conjugate for future clinical development in the further advancement of combating HER2-positive breast cancer.

References


Disclosure of Potential Conflicts of Interest

All authors are Genentech/Roche employees and shareholders of Roche Stock programs. E. Ha was an employee of Genentech during the course of this work, but recently left.

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