The effect of different linkers on target cell catabolism and pharmacokinetics/pharmacodynamics of trastuzumab maytansinoid conjugates.

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ABSTRACT

Trastuzumab emtansine (T-DM1) is an antibody-drug conjugate consisting of the anti-HER2 antibody trastuzumab linked via a non-reducible thioether linker to the maytansinoid anti-tubulin agent DM1. T-DM1 has demonstrated favorable safety and efficacy in patients with HER2-positive metastatic breast cancer. In previous animal studies, T-DM1 exhibited better pharmacokinetics (PK) and slightly more efficacy than several disulfide-linked versions. The efficacy findings are unique, as other disulfide-linked ADCs have shown greater efficacy than thioether linked designs. To explore this further, the in vitro and in vivo activity, PK, and target cell activation of T-DM1 and the disulfide-linked T-SPP-DM1 were examined. Both ADCs demonstrated high in vitro potency, with T-DM1 displaying greater potency in two of four breast cancer cell lines. In vitro target cell processing of T-DM1 and T-SPP-DM1 produced lysine-Nε-MCC-DM1, and lysine-Nε-SPP-DM1 and DM1, respectively; in vivo studies confirmed these results. The in vitro processing rates for the two conjugate to their respective catabolites were similar. In vivo, the potencies of the conjugates were similar and T-SPP-DM1 had a faster plasma clearance than T-DM1. Slower T-DM1 clearance translated to higher overall tumor concentrations (conjugate plus catabolites), but unexpectedly, similar levels of tumor catabolite. These results indicate that the ADC linker can have clear impact on the PK and the chemical nature of the catabolites formed, both linkers appear to offer the same payload delivery to the tumor.

Introduction

An increasing number of antibody-drug conjugates (ADCs) are entering clinical
trials for the treatment of cancer (1). One of the most advanced and promising, trastuzumab-emtansine (T-DM1) has shown favorable efficacy and safety in clinical trials for the treatment of patients with HER2-positive metastatic breast cancer (2-4). T-DM1 is an ADC that contains the humanized anti-HER2 IgG1 trastuzumab linked to the maytansinoid anti-tubulin agent DM1 via a thioether bond. T-DM1 retains the multiple mechanisms of action (MOA) described for trastuzumab,(5) and conjugation with DM1 confers the potential for additional cell killing activity via the delivery of potent anti-mitotic maytansinoid catabolites to targeted cancer cells. The efficacy of T-DM1 in patients who progressed on HER2–directed therapies underscores the importance of this additional activity (2-4).

T-DM1 is distinct from other clinically tested maytansinoid containing ADCs by virtue of its thioether linker. All other such ADCs in clinical testing utilize disulfide-based linkers (1). The thioether linker is considered more stable because it resists chemical or enzymatic cleavage in biological systems, in contrast to disulfide-based linkers which may be cleaved following thiol-disulfide exchange reactions. T-DM1 was found to be slightly more active in mouse models than T-SPP-DM1 and several other disulfide-linked ADCs(6). This contrasts with similar studies of other ADCs that led to the selection of disulfide-based linkers for clinical development (7-9).

The factors determining the efficacy of an ADC include pharmacokinetics (PK), tumor penetration and accumulation, target binding and cellular uptake, release of active catabolic products, and potency of the catabolic products. Previous studies have shown that the plasma concentrations of thioether-linked ADCs decrease more slowly than those of disulfide-linked ADCs, likely due to greater linker stability (6, 10); which may account
for differences in the efficacy between T-DM1 and disulfide-linked analogues. It has also been demonstrated that other aspects of the MOA (i.e., target binding, cellular uptake, and release of catabolic products) may be similar for disulfide-linked and thioether-linked ADCs (11). To develop a better understanding of the MOA of trastuzumab maytansinoid conjugates and differences in the preclinical activity between these ADCs we investigated the molecular basis for the anti-cancer activity in \textit{in vitro} and \textit{in vivo} efficacy, PK, and qualitative and quantitative tumor catabolism studies.

\textbf{Materials and Methods}

All experimental procedures conformed to the principles of the Guide for the Care and Use of Laboratory Animals and the American Physiological Society and were approved by the Institutional Animal Care and Use Committees of the respective laboratories.

\textbf{Cell lines and reagents}

The SK-BR-3 and BT-474 breast carcinoma cell lines were obtained from The American Type Culture Collection and used within 2 months of receipt. The BT-474EEI trastuzumab-resistant breast carcinoma cell line was derived at Genentech (6) by sub-culturing tumors derived from a BT-474 variant line (courtesy of Dr. Jose Baselga). BT-474EEI tumors express approximately 0.25 x 10^6 HER2 receptors per tumor cell and are insensitive to trastuzumab. MCF7-neo/HER2 are HER2-transfected cells made at Genentech. Cells were cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum. MCF7-neo/HER2, BT-474 and BT-474 in vivo-selected variant (Baselga) were genotyped and authenticated using Illumina Golden Gate single
nucleotide polymorphism testing. Although the parent cells of the BT-474EEI cells (BT-474 Baselga variant) were genotyped as BT-474, the resulting cells (EEI) after in vivo-selection have not been authenticated at this point. Porapak extraction cartridges were obtained from Waters. Ultima Gold scintillation fluid was obtained from PerkinElmer. \(N\)-ethylmaleimide (NEM) and all other chemicals were obtained from Sigma-Aldrich. The 10 mm C-18 column (0.46 x 25 cm, Vydak) was obtained from the Nest Group. The ULTRA-TURRAX T8 dispersing instrument with an S8N dispersing tool was obtained from IKA Works Inc. Trastuzumab and the humanized anti-glycoprotein D (gD) control antibody, 5B6, were prepared at Genentech. Antibody-[\(^{3}\text{H}\)]maytansinoid conjugates were prepared at ImmunoGen, Inc., as described previously (12). The ratio of linked \([^{3}\text{H}]\)maytansinoid per antibody molecule (MAR) for each conjugate was as follows: T-SPP-[\(^{3}\text{H}\)]DM1 (3.2), T-[\(^{3}\text{H}\)]DM1 (4.0), 5B6-SPP-[\(^{3}\text{H}\)]DM1 (3.5) [SPP = \(N\)-succinimidyl 4-(2-pyridyldithio)pentanoate], and hu5B6-MCC-[\(^{3}\text{H}\)]DM1 (3.6) [SMCC = \(N\)-succinimidyl 4-(\(N\)-maleimidomethyl)cyclohexane-1 carboxylate]. Unlabeled antibody-maytansinoid conjugates with similar MAR values were prepared at Genentech.

**Clonogenic assays**

Cells were plated at low density (100 cells/well for SK-BR-3, MCF7-neo/HER2 and BT-474EEI; 200 cells/well for BT-474) in Ham's F-12:DMEM (50:50) + 10% FBS + 2 mM L-glutamine and allowed to adhere overnight. Conjugates were added the next day and the cells incubated until colony formation was determined to be maximal. The medium was removed and the colonies stained with crystal violet dye (0.5% in methanol). Colonies were quantified using a GelCount™ (Oxford Optronix).
**In vitro catabolism studies**

A stable tritium label was incorporated into the C-20 methoxy group of the DM1 used to prepare the $^3$H-labeled conjugates as described previously (12). HER2-dependent processing of the $^3$H-labeled conjugates was investigated in HER2-overexpressing BT-474EEI, MCF7-neo/HER2, and SK-BR-3. Cells were plated in flat bottom T-75 cell culture flasks in cell culture medium and grown to a density of approximately $10^7$ cells per flask. The medium was exchanged with 10 mL of RPMI containing 10% FBS and 20-40 nmol/L of T-[$^3$H]DM1 or T-SPP-[$^3$H]DM1. The cells were placed on ice for 2-3 h or for 30 min at 37°C with 6 % CO$_2$. The treated cells were then washed three times with fresh medium and incubated at 37°C with 6 % CO$_2$ with 10 mL fresh culture medium for 3-24 h. The spent medium was separated and cells were harvested at selected time intervals and analyzed for catabolites following acetone extraction as described previously (11, 12). The precipitates from the acetone extractions were solubilized and analyzed for tritium by liquid scintillation counting (LSC) to assess the level of protein-bound maytansinoid associated with the cell pellet, presumed to be non-metabolized conjugate or DM1 still linked to large protein fragments.

Maytansinoid catabolites were isolated from the medium by solid phase extraction followed by reversed-phase high pressure liquid chromatography (HPLC). Media samples were applied to 6 mL porapak cartridges equilibrated with 3 mL acetone and then with 3 mL water. The cartridges were washed with 4 mL water and the maytansinoids eluted with 4 mL acetone. The eluates were evaporated and analyzed by reversed-phase HPLC and LSC as described for extracts containing the cell catabolites.
**Cell volume measurements**

Cell volumes were calculated from average cell diameters as measured using a cell viability analyzer (Beckman Coulter). Cell volumes for the SK-BR-3, BT-474EEI and MCF7/neo-HER2 cells were 3823 μm³, 3955 μm³, and 3648 μm³, respectively.

**Animals**

Female beige nude XID® mice (Charles Rivers Laboratory, Hollister, CA) were used for all *in vivo* studies. Mice were housed in a clean barrier facility in standard rodent micro-isolator cages.

**In vivo efficacy studies**

To allow comparison of results obtained *in vitro* and *in vivo*, a mouse xenograft model was selected that was suitable for both systems. Previously, the founder 5 (Fo5) mouse allograft transplant model was used for in vivo comparisons of maytansinoid ADC efficacy (6). However, this model was not suitable for these studies as it cannot be cultured *in vitro*. Thus, the BT-474EEI xenograft model, suitable for *in vitro* and in vivo investigations, was chosen (13). BT-474EEI cells (2 x 10⁷) were injected into the mammary fat pad of beige nude XID mice and tumors were grown to ~250 mm³. Animals were then randomized into nine groups (n=10/group) and treated with a single i.v. bolus dose of 3-18 mg/kg (~60-360 μg/kg based on DM1 dose) of the conjugates. Tumors were measured with calipers twice weekly for 24 days following dosing, and then weekly for 120 days or until tumors reached a volume of 3000 mm³. Tumor volumes were determined using the formula: Tumor volume (mm³) = (longer diameter X
shorter diameter)\(^2\) \times 0.5.

**In vivo catabolism studies**

Mice bearing BT-474EEI tumors (as described above) were randomized into 2 groups (n = 15/group) and treated with a single 300 \(\mu\)g/kg i.v. bolus dose of T-[\(^3\)H]DM1 or T-SPP-[\(^3\)H]DM1 (doses based on DM1, equivalent to 10-12 mg/kg antibody). Three mice per group were sacrificed after 8 h, 1 d, 2 d, 4 d, and 7 d. Immediately following sacrifice, the systemic circulation was flushed by injecting 5.0 mL PBS into the left ventricle and draining through an incision in the inferior vena cava. Whole tumor tissues were collected and frozen at -80\(^\circ\)C. Two control groups of mice (n = 3/group) were treated with matching doses of non-targeting 5B6-MCC-[\(^3\)H]DM1 or 5B6-SPP-[\(^3\)H]DM1 conjugates and sacrificed after 2d. Tumors were homogenized and analyzed for total radioactivity by solubilization and LSC, and maytansinoid catabolites by HPLC and LSC as described previously. To determine the amount of protein-free maytansinoid catabolites present in the tumors, the tumor homogenates were extracted with organic solvent and analyzed as described previously (12).

**Analytical methods**

All maytansinoids were separated on an analytical C-18 column equilibrated with 20% aqueous acetonitrile (CH\(_3\)CN) containing 0.025% trifluoroacetic acid (TFA) and using a linear gradient of 2% CH\(_3\)CN min\(^{-1}\) and a flow rate of 1 mL min\(^{-1}\). The effluent was collected in 6 mL polypropylene scintillation vials (1 mL fractions). Counts per
minute (CPM) of tritium associated with each fraction were determined by mixing each vial with 4 mL Ultima Gold liquid scintillation cocktail before counting for 5 min in a Tri-Carb 2900T liquid scintillation counter (Packard BioScience, Downer Grove, IL).

The identities of the catabolites were confirmed by liquid chromatography mass spectrometry (LCMS) by methods similar to those described previously (11).

Pharmacokinetic studies

Animals (n = 20/group) were administered a 3 mg/kg i.v. bolus dose of T-DM1 or T-SPP-DM1. At selected intervals up to 42 days following dosing, blood samples were collected on a rotational basis, with n = 4 animals per time point. Blood samples were processed for plasma by centrifugation and plasma decanted to polypropylene collection tubes. Samples were stored at -60 to -80ºC until analysis.

ADC plasma concentration analysis

Plasma ADC concentrations were determined with an enzyme-linked immunosorbent assay (ELISA) that measured any trastuzumab antibody containing one or more conjugated DM1 molecules. An anti-DM1 monoclonal antibody was coated on ELISA plates. Captured ADC was detected with biotinylated HER2 extracellular domain (HER2 ECD) followed by streptavidin-horseradish peroxidase. The limit of quantitation (LOQ) was 1.6 ng/mL. Total trastuzumab concentrations were determined as described previously (6) using an ELISA that measured any trastuzumab antibody. In brief, HER2 ECD was coated on ELISA plates. Captured total trastuzumab was detected with goat-anti-human IgG-horseradish peroxidase. The LOQ was 1.6 ng/mL.
Pharmacokinetic analysis

The PK analysis of plasma ADC ELISA concentration–time data was conducted using a naïve pool approach with individual data from all animals. Data were fit to a two-compartmental model with i.v.-bolus input, first-order elimination, and macro-rate constants (Model 8, WinNonlin® Pro, v.5.2.1; Pharsight Corporation; Mountain View, CA). Nominal sample collection times and dose concentrations were used in the data analysis.

Exposure Response

In vivo response was assessed by calculating the mean tumor volume for each animal up to Day 38 following dosing. The mean tumor volume is a sum of the measured tumor volumes in an individual animal from Day 0 to Day 38, divided by the number of sampling times. Day 38 was chosen to allow for the observation of full ADC response without substantial tumor regrowth. Evaluation of other time points did not affect the results (data not shown). To show exposure-response, the individual animal mean tumor volume value for each dose group was plotted against the estimated plasma ADC exposure (AUC) for that dose group.
Results

HER2-positive breast carcinoma cells display high sensitivity to T-DM1 and T-SPP-DM1

The cytotoxic potencies of T-DM1 and T-SPP-DM1 and the matching non-binding conjugates were compared in the trastuzumab-sensitive breast cancer cell lines SK-BR-3 and BT-474, and the trastuzumab-insensitive breast cancer cell lines BT-474EEI and MCF7-neo/HER2 using a clonogenic assay (Fig. 1). Both trastuzumab conjugates were found to be potent inhibitors of colony formation in the HER2-positive cell lines, whereas the non-targeting conjugates displayed little cytotoxicity. The chemical nature of the linker had little influence on the cytotoxicity of trastuzumab-DM1 conjugates towards BT-474 (Fig. 1A) and SK-BR-3 cells (Fig. 1B), consistent with an earlier report (6). However, the T-DM1 was more cytotoxic than T-SPP-DM1 towards BT-474EEI (Fig. 1C) and MCF7-neo/HER2 cells (Fig. 1D).

T-DM1 and T-SPP-DM1 display similar efficacy in mice bearing trastuzumab-insensitive BT-474EEI tumors

The anti-tumor activity associated with T-DM1 and T-SPP-DM1 in mice bearing the BT-474EEI tumors is shown in Fig. 2A. Following treatment with trastuzumab conjugates, tumor volume decreased in proportion to dose with maximum response at 18 mg/kg of either conjugate. Tumor regression reached its maximum approximately 10-21 days after dosing with subsequent stasis or re-growth. The exposure-response relationship for T-DM1 and T-SPP-DM1 was similar (Fig. 2B).

Pharmacokinetics of T-DM1 and T-SPP-DM1
The PK of T-DM1 and T-SPP-DM1 are shown in Fig. 3 and Supplementary Table 1. Total trastuzumab (Tab) PK were similar for both conjugates (Supplementary Table 1), indicating that conjugation with the respective linker-DM1 did not differentially affect the antibody PK behavior. T-SPP-DM1 ADC concentrations decreased more rapidly than T-DM1, with T-SPP-DM1 exhibiting a faster clearance ($40.1 \pm 1.87$ vs. $18.9 \pm 0.29$ mL/day/kg) and shorter terminal half-life ($2.69 \pm 0.087$ vs. $5.72 \pm 0.150$ days), consistent with a previous report (6). Central compartment volume of distribution was similar between the conjugates, as expected, based on the general similarity in their structure.

**Characterization of maytansinoid catabolites following in vitro exposure of breast cancer cells to T-DM1 and T-SPP-DM1**

The radio-chromatograms in Fig. 4A show the catabolites of T-[3H]DM1 and T-SPP-[3H]DM1 following treatment of the BT-474EEI cells. The sole T-[3H]DM1 catabolite at each time point was found to be lysine-$N^\epsilon$-MCC-[3H]DM1. The corresponding lysine-$N^\epsilon$-SPP-[3H]DM1 catabolite of T-SPP-[3H]DM1 was observed along with a similarly abundant DM1 catabolite. The DM1 catabolite was alkylated at its free sulfhydryl group by NEM in the suspension buffer prior to extraction and thus was detected as its NEM adduct in the radiograms. No maytansinoid catabolites (< 0.1 pmol/10^6 cells) were detected in the acetone extracts of the spent medium samples indicating that little, if any, efflux of maytansinoids from the cells had occurred over the 24 h assay.

**The catabolism rates of trastuzumab conjugates following exposure to BT-474EEI**
cells are similar

The radioactivity associated with the maytansinoid catabolites in acetone extracts (Fig. 4A) and precipitates from the acetone extractions were converted to pmol/cell and nmol/L and plotted vs. time (Fig. 4B). Of particular interest for understanding the anti-mitotic activity of T-DM1 is the concentration of its maytansinoid catabolites in the cytoplasm of the targeted cells. The molar values for the catabolites in Fig. 4B may not accurately reflect their cytoplasmic concentrations if they accumulate in subcellular compartments such as lysosomes. However, previous studies with anti-CanAg maytansioioid conjugates similar to those described here suggest that maytansinoid catabolites are retained by targeted cells through binding to their cytoplasmic tubulin target (11). Therefore, the molar values in Figure 4 likely approximate the true cytoplasmic values. The amount of maytansinoids in the precipitates (representing intact conjugates and any potential protein fragments) was found to decrease at approximately the same rate for both conjugates. This decrease was matched by a corresponding increase in the maytansinoid catabolites in the extracts, reaching maximal intracellular maytansinoid concentrations of 200 nmol/L and 300 nmol/L after 24 h for cells treated with T-SPP-DM1 and T-DM1, respectively. In separate experiments the total maytansinoids associated with the extract and the precipitate for each time point were equivalent to the amount of conjugate bound at t = 0 (data not shown) indicating that all maytansinoid catabolites had been accounted for (upper curves, Fig. 4B). Approximately 30% more T-DM1 was bound to cells after the incubation and wash steps than T-SPP-DM1. This, coupled with a higher MAR for T-DM1 compared to T-SPP-DM1 (4.0 vs. 3.2) accounts for the higher maytansinoid values associated with cells treated with T-
DM1 (Fig. 4B panels a and b). The catabolite levels for the conjugates were divided by the total maytansinoids in the sample (acetone pellet + extract − conjugate bound at t = 0) to determine the fraction of the conjugates processed to the observed catabolites and re-plotted in Fig. 4B, panel c. The rates of formation of catabolites from the two conjugates are similar when so normalized. HER2-mediated processing of these conjugates is similar in other breast carcinoma lines (Fig. S1).

**Catabolism of trastuzumab conjugates within tumor tissue**

The concentration of the conjugates in plasma (based on DM1) was calculated from the plasma radioactivity and shown in Fig. 5A. The concentration of T-DM1 after 7 d is about 3-fold greater than the concentration of T-SPP-DM1 (top panel), consistent with the ELISA PK in non-tumor bearing mice (Fig. 3). The plasma conjugate concentrations (ELISA) in non-tumor-bearing mice (Fig. 3) when adjusted for dose, were approximately 2x higher than the estimated plasma conjugate concentrations derived from the plasma radioactivity measurements in tumor bearing mice, consistent with observations from a separate study (14). The causes of these discrepancies are not known, but do not affect the interpretation of this study. The plasma concentrations of the non-binding control conjugates after 2 d were equivalent to the matched trastuzumab conjugates, as expected.

Maytansinoid concentrations in the tumors of mice treated with trastuzumab conjugates were found to reach a maximum level at about 1-2 days with peak concentrations of approximately 700 nmol/L (equivalent to 9% ID/g) or 500 nmol/L, (equivalent to 7% ID/g) for T-DM1 or T-SPP-DM1, respectively, followed by a gradual
decline (Fig. 5A middle panel). The 7 d AUC for the total maytansinoids in the tumors treated with T-DM1 was 1.5-fold higher than T-SPP-DM1.

Based on the HPLC radiochromatograms, the catabolites of the trastuzumab conjugates in tumor tissues were identical to those identified in vitro — lysine-$N^\epsilon$-MCC-DM1 for T-DM1 and lysine-$N^\epsilon$-SPP-DM1 and DM1 for T-SPP-DM1 (Fig. 5B). The concentrations of the maytansinoid catabolites for the two conjugates (Fig 5A, bottom panel) were very similar despite the differences in total maytansinoid levels (Fig. 5A, middle panel), with maximal maytansinoid catabolite concentration for both conjugates of approximately 150 nmol/L.

The lysine-$N^\epsilon$-MCC-DM1 catabolite observed with T-DM1 was also observed for the control 5B6-MCC-DM1 conjugate at substantially reduced levels (Figure 6A). Similarly, the lysine-$N^\epsilon$-SPP-DM1 and DM1 catabolites observed for T-SPP-DM1 were also observed for the 5B6-SPP-DM1 conjugate — again at reduced levels.

Concentrations of maytansinoid catabolites of the non-targeting conjugates were approximately 5-fold and 3-fold lower, respectively, than the concentrations of the catabolites of T-DM1 and T-SPP-DM1 (Fig. 6B). These results demonstrate the effect of HER2 targeting on the maytansinoid delivery of trastuzumab-DM1 conjugates.
Discussion

Interest in ADCs has grown in response to the favorable safety and efficacy associated with T-DM1 and SGN-35 — the latter, an antibody-auristatin conjugate targeting CD30-positive tumors (2, 15). Additional ADCs are in early clinical trials and more are expected to follow (1, 16). Efforts to understand the molecular basis for the anti-cancer activities of ADCs have intensified; with studies of maytansinoid and auristatin conjugates providing a mechanistic basis for their clinical activity (11, 12, 17-20).

The aims of these studies were to investigate the MOA and anti-cancer activity of T-DM1 and the role of the linker in these processes. To explore the underlying mechanisms determining trastuzumab-maytansinoid ADC activity, *in vitro* studies were conducted assessing ADC potency and the kinetics of ADC uptake into tumor cells, and the identity and accumulation of the catabolic products. Both trastuzumab conjugates were highly, and similarly, potent against HER2-expressing cell lines (Figure 1) (SK-BR-3, BT-474), consistent with a previous report (6). However, T-DM1 exhibited slightly greater potency in the BT474EEI and MCF7-neo/HER2 cell lines. It is not clear why these cell lines behave differently than the other cell lines tested.

*In vitro* tumor cell uptake and catabolism studies showed that the uptake and catabolism occurred at a comparable rate for both ADCs (Fig. 4B). This finding was expected, based on the similarity in HER2 binding affinity of the ADCs (data not shown), and the assumption that target-mediated cellular trafficking and catabolism of the ADCs are independent of linker type. The half-life of cellular catabolism of the ADCs was found to be approximately 19 h, consistent with data reported for $^{125}$I-trastuzumab;
indicating that the maytansinoid conjugation does not alter catabolism rate (21). These studies also showed that lysine-\(^{N^\epsilon}\)MCC-DM1 is the sole T-DM1 catabolite accumulating in the tumor cells tested (Fig. 4A). The high \textit{in vitro} activation rate for T-DM1 within cancer cells is consistent with its potent cytotoxicity (Fig. 1) and is likely an important factor in its anti-tumor activity.

To explore the \textit{in vitro-in vivo} correlation of these findings, PK, efficacy, \textit{in vivo} tumor uptake and catabolism studies were conducted with the HER2\(^+\), trastuzumab-insensitive, xenograft tumor BT474EEI. In vivo tumor catabolism data confirmed the \textit{in vitro} data identifying lysine-\(^{N^\epsilon}\)MCC-DM1 as the sole catabolic product (Figure 5B).

Previous nonclinical studies reported that T-DM1 displayed slightly greater efficacy than T-SPP-DM1, when compared by dose (6). It was hypothesized that the greater efficacy of T-DM1, which uses a thioether to link DM1 to the antibody, compared with T-SPP-DM1, which contains a disulfide linker, may be related to improved PK or differences in the tumor accumulation of active catabolic products. The plasma clearance of T-DM1 is approximately two times slower than T-SPP-DM1 (Supplemental Table 1), likely due to greater stability of the thioether linker. This results in greater plasma exposure (AUC) per unit dose of T-DM1, but does not result in a substantive increase in efficacy (Fig. 2A). Indeed, comparing ADC efficacy a function of plasma ADC exposure indicates similar \textit{in vivo} potency (Fig. 2B).

The greater exposure of T-DM1 compared to T-SPP-DM1 corresponds with increased total tumor maytansinoid concentrations (Fig. 5A) for T-DM1, a logical sequela based on the similarity in the factors determining tumor uptake and binding for both ADCs. However, despite differences in total tumor maytansinoid concentrations,
the tumor catabolite exposure levels for the two conjugates are similar (Figure 5A). This was not expected, as the higher total maytansinoid concentrations for T-DM1, coupled with the more residualizing nature of its catabolic product predicted higher catabolite concentrations for T-DM1. The reasons for the similarity in tumor catabolite levels are unclear. One possibility is that the primary accumulation of conjugate in the tumor driving the subsequent HER2-mediated catabolism occurs within the first day or so when the plasma concentrations for the two conjugates are similar. Another possibility is that the \textit{in vivo} cleavage of the disulfide-linked T-SPP-DM1 was more efficient than T-DM1 despite similar in vitro processing rates. For example, direct cleavage of DM1 from T-SPP-DM1 within tumor tissue via thiol-disulfide exchange reactions could increase the processing rate by complementing the lysine-$N^\varepsilon$-SPP-DM1 released via the lysosomal degradation route.

It is postulated that the cellular catabolic products of ADCs are responsible for their cytotoxic activity. The observation of similar tumor catabolite concentrations for T-DM1 and T-SPP-DM1 when both conjugates are administered at the same ADC dose coupled with the slightly greater \textit{in vitro} potency of T-DM1 in BT474EEI cells would predict greater efficacy for T-DM1; however, no substantive difference in efficacy was observed (Fig. 2). This is a unique observation as, in studies with ADCs targeting the CanAg antigen antibody maytansioiid conjugate, thioether-linked conjugates were less active than disulfide-linked conjugates, even when tumor catabolite concentrations for the thioether-linked ADC were somewhat higher than those of the disulfide-linked ADC (12), an observation attributed to bystander killing (11-13). Both the disulfide-linked and thioether-linked anti-CanAg conjugates were efficiently degraded in the lysosomes of
cancer cells to yield the corresponding lysine-linker-maytansinoid catabolites. For the thioether-linked conjugate, no further catabolism was observed. However, the catabolites of the two disulfide-linked conjugates huC242-SPP-DM1 and huC242-SPDB-DM4 were both further processed to yield catabolites capable of diffusing throughout tumor tissue, thereby enhancing the activity of the disulfide-linked conjugates through bystander killing mechanisms. While this may also apply to the trastuzumab ADCs in this study, the greater in vitro potency of T-DM1 may compensate for the bystander effect associated with T-SPP-DM1 to explain the similarity in in vivo efficacy.

In summary, the studies described herein provide a detailed understanding of mechanistic aspects of the PK, tumor uptake, and pharmacologic activity of T-DM1 and contrast those properties with a more labile disulfide linker. These studies confirm that T-DM1 and T-SPP-DM1 have potent anti-tumor activity in HER2 expressing cell lines and demonstrate that T-DM1 is rapidly activated by HER2-positive cancer cells to lysine-\(N^\varepsilon\)-MCC-DM1. Improved linker stability with T-DM1 leads to greater plasma ADC exposure and tumor uptake of total maytansinoid than T-SPP-DM1. Surprisingly, the increase in total tumor uptake with T-DM1 does not translate into greater tumor catabolite concentrations or improved efficacy, when compared to T-SPP-DM1. Sustained exposure of the lysine-\(N^\varepsilon\)-MCC-DM1 catabolite in tumors of treated mice provides a mechanistic basis for the preclinical activity of T-DM1. The efficacy and tumor targeting characteristics of T-DM1 and T-SPP-DM1 suggest that both linker formats are effective in payload delivery and anti-tumor activity.
References:


Figure Legends

**Fig. 1.** *In vitro* clonogenic survival assays of trastuzumab-DM1 conjugates in HER2-overexpressing breast cancer cell lines (IC₅₀ values (ng/mL)). Colony number was assessed after 7 (MCF7-neo/HER2) to 21 (BT-474) days of exposure to T-DM1, T-SPP-DM1, or control ADCs.

A. (a) T-SPP-DM1 = 4.0 (b) T-MCC-DM1 = 5.0

B. (a) T-SPP-DM1 = 1.8 (b) T-MCC-DM1 = 1.1

C. (a) T-SPP-DM1 = 30.0 (b) T-MCC-DM1 = 6.0

D. (a) T-SPP-DM1 = 10.0 (b) T-MCC-DM1 = 3.0

**Fig. 2. A.** Efficacy of trastuzumab-DM1 conjugates in mice bearing BT-474EEI tumors. Individual tumor volumes vs time following a single i.v. bolus dose of trastuzumab-DM1 conjugates on day 0. Solid grey lines represent individual animal tumor volumes. The green line is Lower Limit of Quantitation (8 mm³). Values below this are statistically imputed and recorded as 8 to avoid extreme negative or infinite values on log scale. The blue line is fitted control profile. **B.** Mean tumor volume measurements for each animal through day 38 versus ADC exposure (AUC) with box-plots overlaid. The horizontal line is the median of the 10 animal mean volumes in each group; the ends of the box range from the 3rd to 7th highest of the 10 means. This "interquartile range" (IQR) is an outlier-resistant estimate of the variability of each group of mean volumes. The dashed "whiskers" extend 1.5 IQR's from the ends of the box, denoting a Gaussian distribution-based estimate of statistical variability. Values beyond the whiskers are therefore considered statistically aberrant.
Fig. 3. Plasma pharmacokinetics in non-tumor bearing mice. Mean (± SD) ADC and Tab concentrations following i.v. bolus administration of 3 mg/kg of trastuzumab-DM1 conjugates.

Fig. 4. Activation of trastuzumab-DM1 conjugates in HER2-positive breast carcinoma cells. A) HPLC radiograms of the target-cell catabolites following exposure of BT474EEI cells to T-[3H]DM1 and T-SPP-[3H]DM1. Cells were harvested at the indicated time points and analyzed for maytansinoids. The chromatograms show the fraction number (abscissa) and the counts per minute (ordinate). B) Rates for the catabolism of conjugates. Panels a and b show the concentration of maytansinoid catabolites of T-DM1 (a) and T-SPP-DM1 (b) formed within cells over time. Concentrations were calculated from the radioactivities in A and plotted vs. time. 1 pmol/10^6 cells is equivalent to 253 nmol/L. The corresponding concentration of intact conjugate still associated with the cells (●) was determined from the radioactivity associated with the acetone precipitates. (▲) Total maytansinoid levels. Panel c shows the percentage of the conjugates processed at each time. The processing of T-DM1 (□) and T-SPP-DM1 (○) was calculated from a and b by dividing the catabolites of each conjugate formed at each time by the corresponding total maytansinoid levels.

Fig. 5. Tumor localization and catabolism of trastuzumab-DM1 conjugates. (A) Plasma clearance of conjugate (top panel) and accumulation of total maytansinoids (middle) and maytansinoid catabolites (bottom) in tumors following administration of a single i.v. dose.
of 200 μg/kg (based on DM1 concentration) of T-[³H]DM1 (▲), T-SPP-[³H]DM1 (○), 5B6-MCC-[³H]DM1 (□) and 5B6-SPP-[³H]DM1 (●) to mice. Concentrations of conjugate in plasma (DM1 concentration) and total maytansinoid (conjugate + catabolites) in tumor were calculated from the total radioactivity in plasma and tumors samples, respectively, expressed as “concentration”. The maytansinoid catabolite levels in the tumors were determined by the radioactivity associated with HPLC catabolites shown in (B). The counts per minute (CPM) of tritium in each effluent fraction was determined by liquid scintillation counting. The radiograms show the fraction number on the abscissa and CPM of tritium on the ordinate.

**Fig. 6.** HER2-dependence of maytansinoid delivery to tumor. (A) HPLC radiograms associated with the 2 d tumor catabolites of T-[³H]DM1, T-SPP-[³H]DM1 and the non-targeting 5B6-MCC-[³H]DM1 and 5B6-SPP-[³H]DM1 conjugate. The radiograms show the fraction number (abscissa) and counts per minute of tritium (CPM) (ordinate). (B) Concentrations of the 2 d tumor catabolites were calculated from the peaks of radioactivity in the radiograms from A.
Figure 2

A

B

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