Preclinical Development

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 nonreducible thioether linker to the maytansinoid antitubulin agent DM1. T-DM1 has shown 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 antibody–drug conjugates (ADC) 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 showed 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, although the ADC linker can have clear impact on the PK and the chemical nature of the catabolites formed, both linkers seem to offer the same payload delivery to the tumor.

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Introduction
An increasing number of antibody–drug conjugates (ADC) 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 immunoglobulin G (IgG1) trastuzumab linked to the maytansinoid antitubulin 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 antimitotic 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 use 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 that 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 shown that other aspects of the MOA...
(i.e., target binding, cellular uptake, and release of catalytic 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 anticancer activity in \textit{in vitro} and \textit{in vivo} efficacy, PK, and qualitative and quantitative tumor catabolism studies.

**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.

**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 subculturing tumors derived from a BT-474 variant line (courtesy of Dr. Jose Baselga). BT-474EEI tumors express approximately 0.25 \times 10^6 HER2 receptors per tumor cell and are insensitive to trastuzumab. MCF7-neo/HER2 and MCF7-neo/HER2 variants were HER2-transfected cells made at Genentech. Cells were cultured in RPMI medium supplemented with 10% heat-inactivated FBS. MCF7-neo/HER2, BT-474, and BT-474 \textit{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 \textit{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 \times 25 cm; Vydac) 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 antiglycoprotein D (gD) control antibody, 5B6, were prepared at Genentech. Antibody-[\textsuperscript{3}H]maytansinoid conjugates were prepared at ImmunoGen, Inc., as described previously (12). The ratio of linked [\textsuperscript{3}H]maytansinoid per antibody molecule (MAR) for each conjugate was as follows: T-SPP-[\textsuperscript{3}H]DM1 (3.2), T-[\textsuperscript{3}H]DM1 (4.0), 5B6-SPP-[\textsuperscript{3}H]DM1 (3.5) [SPP = N-succinimidyl 4-(2-pyridylthio)pentanoate], and hu5B6-MCC-[\textsuperscript{3}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 per well for SK-BR-3, MCF7-neo/HER2, and BT-474EEI; 200 cells per well for BT-474) in Ham’s F-12:DMEM (50:50) + 10% FBS + 2 mmol/L 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 with 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 [\textsuperscript{3}H]-labeled conjugates as described previously (12). HER2-dependent processing of the \textsuperscript{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 10^6 cells per flask. The medium was exchanged with 10 mL of RPMI containing 10% FBS and 20 to 40 nmol/L of T-[\textsuperscript{3}H]DM1 or T-SPP-[\textsuperscript{3}H]DM1. The cells were placed on ice for 2 to 3 hours or for 30 minutes at 37°C with 6% CO\textsubscript{2}. The treated cells were then washed 3 times with fresh medium and incubated at 37°C with 6% CO\textsubscript{2} with 10 mL fresh culture medium for 3 to 24 hours. 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 nonmetabolized 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 by a cell viability analyzer (Beckman Coulter). Cell volumes for the SK-BR-3, BT-474EEI, and MCF7/neu/HER2 cells were 3823, 3955, and 3648 \textmu m\textsuperscript{3}, respectively.

**Animals**

Female beige nude XID mice (Charles Rivers Laboratory) were used for all \textit{in vitro} studies. Mice were housed in a clean barrier facility in standard rodent microisolator cages.

**In vivo efficacy studies**

To allow comparison of results obtained \textit{in vitro} and \textit{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 × 10⁶) were injected into the mammary fat pad of beige nude XID mice, and tumors were grown to approximately 250 mm³. Animals were then randomized into 2 groups (n = 10 per group) and treated with a single i.v. bolus dose of 3 to 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 3,000 mm³. Tumor volumes were determined with the formula: Tumor volume (mm³) = (longer diameter × shorter diameter)² × 0.5.

In vivo catabolism studies

Mice-bearing BT-474EEI tumors (as described above) were randomized into 2 groups (n = 15 per group) and treated with a single 300 μg/kg i.v. bolus dose of T-[³H]DM1 or T-SPP-[³H]DM1 (doses based on DM1, equivalent to 10–12 mg/kg antibody). Three mice per group were sacrificed after 8 hours and 1, 2, 4, and 7 days. 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°C. Two control groups of mice (n = 3 per group) were treated with matching doses of nontargeting 5B6-MCC-[³H]DM1 or 5B6-SPP-[³H]DM1 conjugates and sacrificed after 2 days. 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).

Analytic methods

All maytansinoids were separated on an analytic C-18 column equilibrated with 20% aqueous acetonitrile (CH₃CN) containing 0.025% trifluoroacetic acid and using a linear gradient of 2% CH₃CN min⁻¹ and a flow rate of 1 mL.min⁻¹. The effluent was collected in 6 mL polypyrrole 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 minutes in a Tri-Carb 2900T liquid scintillation counter (Packard BioScience).

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

Pharmacokinetic studies

Animals (n = 20 per 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 polypyrrole collection tubes. Samples were stored at −60°C to −80°C until analysis.

ADC plasma concentration analysis

Plasma ADC concentrations were determined with an 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 naive pool approach with individual data from all animals. Data were fit to a 2-compartmental model with i.v. bolus input, first-order elimination, and macro rate constants (Model 8, WinNonlin Pro, v.5.2.1; Pharsight Corporation). 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 days 0 to 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 [area under the curve (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 nonbinding 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 nontargeting conjugates displayed little cytotoxicity. The chemical nature of the linker had little influence on the cytotoxicity of trastuzumab–DM1 conjugates toward 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 toward 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 antitumor 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 to 21 days after dosing with subsequent stasis or regrowth. 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 S1. Total trastuzumab (Tab) PK were similar for both conjugates (Supplementary Table S1), 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 ± 1.87 vs. 18.9 ± 0.29 mL/d/kg) and shorter terminal half-life (2.69 ± 0.087 vs. 5.72 ± 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 radiochromatograms 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°-MCC-[3H]DM1. The corresponding lysine-N°-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 before extraction and thus was detected as its NEM adduct in the radiograms. No maytansinoid catabolites (<0.1 pmol/10⁶ 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-hour 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 versus time (Fig. 4B). Of particular interest for understanding the antimitotic 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 maytansinoid 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 Fig. 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 hours 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 with T-SPP-DM1 (4.0 vs. 3.2) accounts for the higher maytansinoid values associated with cells treated with T-DM1 (Fig. 4B, a and b). The catabolite levels for the conjugates were divided by the total maytansinoids in the sample (acetone pellet + extract \( \sim \) conjugate bound at \( t = 0 \)) to determine the

Figure 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 (x-axis) and the CPM (y-axis). 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 versus time. A total of 1 pmol/10⁶ 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.
fraction of the conjugates processed to the observed catabolites and replotted in Fig. 4B, c. The rates of formation of catabolites from the 2 conjugates are similar when so normalized. HER2-mediated processing of these conjugates is similar in other breast carcinoma lines (Supplementary 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 days is about 3-fold greater than the concentration of T-SPP-DM1 (top), consistent with the ELISA PK in nontumor-bearing mice (Fig. 3). The plasma conjugate concentrations (ELISA) in nontumor-bearing mice (Fig. 3) when adjusted for dose, were approximately ×2 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 nonbinding control conjugates after 2 days 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 to 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). The 7 day AUC for the total maytansinoids in the tumors treated with T-DM1 was 1.5-fold higher than T-SPP-DM1.

On the basis of HPLC radiochromatograms, the catabolites of the trastuzumab conjugates in tumor tissues were identical to those identified in vitro—lysine-Nε-MCC-DM1 for T-DM1 and lysine-Nε-SPP-DM1 and DM1 for T-SPP-DM1 (Fig. 5B). The concentrations of the maytansinoid catabolites for the 2 conjugates (Fig. 5A, bottom) were very similar despite the differences in total maytansinoid levels (Fig. 5A, middle), with maximal maytansinoid catabolite concentration for both conjugates of approximately 150 nmol/L.

The lysine-Nε-MCC-DM1 catabolite observed with T-DM1 was also observed for the control 5B6-MCC-DM1 conjugate at substantially reduced levels (Fig. 6A). Similarly, the lysine-Nε-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 nontargeting 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 show 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 anticancer 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 anticancer 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 (Fig. 1; SK-BR-3, BT-474), consistent with a previous report (6). However, T-DM1 exhibited slightly greater potency in the BT-474EEI 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 hours, consistent with data reported for 125I-trastuzumab; indicating that the maytansinoid conjugation does not alter catabolism rate (21). These studies also showed that lysine-NE-MCC-DM1 is the sole T-DM1 catabolite accumulating in the tumor cells tested (Fig. 4A). The high 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 antitumor activity.

To explore the in vitro–in vivo correlation of these findings, PK, efficacy, in vivo tumor uptake, and catabolism studies were conducted with the HER2 insensitive, xenograft tumor BT474EEI. In vivo tumor catabolism data confirmed the in vitro data identifying lysine-NE-MCC-DM1 as the sole catabolic product (Fig. 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 2 times slower than T-SPP-DM1 (Supplementary Table S1), 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 as a function of plasma ADC exposure indicates similar in vivo potency (Fig. 2B).

The greater exposure of T-DM1 compared with 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 2 conjugates are similar (Fig. 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 2 conjugates are...
similar. Another possibility is that the 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\textsuperscript{ε}-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 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 maytansinoid 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 efficaciously 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 2 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. Although 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 antitumor activity in HER2-expressing cell lines and show that T-DM1 is rapidly activated by HER2-positive cancer cells to lysine-N\textsuperscript{ε}-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 with T-SPP-DM1. Sustained exposure of the lysine-N\textsuperscript{ε}-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 antitumor activity.

Disclosure of Potential Conflicts of Interest

C. Audette has an ownership interest in ImmunoGen, Inc. J. Pinkas held the role of Director (Pharmacology) in ImmunoGen, Inc.; also has ownership interest in ImmunoGen, Inc. J. Tibbitts has ownership interest (including patents) in Roche. No potential conflicts of interest were disclosed by the other authors.

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