Characterization of \textit{in vivo} biotransformations for trastuzumab emtansine by high-resolution accurate-mass mass spectrometry

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\textbf{ABSTRACT}

Trastuzumab emtansine (T-DM1) is an antibody-drug conjugate (ADC) designed for the treatment of HER2-positive cancers. T-DM1 is composed of the humanized monoclonal antibody trastuzumab connected to a maytansine derivative cytotoxic drug, via a nonreducible thioether linker at random lysine residues, and therefore has a very complex molecular structure. It was anticipated that T-DM1 undergoes biotransformations in circulation. However, there was limited knowledge on these structural changes due to bioanalytical challenges. Here, we have investigated the \textit{in vivo} biotransformations of T-DM1 using a high-resolution accurate-mass (HR/AM) mass spectrometry approach. Three types of biotransformations were characterized for T-DM1 in circulation in tumor-bearing mice, including cysteine or glutathione adduct formation via maleimide exchange, loss of maytansinol via ester hydrolysis, as well as addition of H$_2$O via linker-drug hydrolysis. These results provide new insights into \textit{in vivo} catabolism of T-DM1.

\textbf{Introduction}

Antibody-drug conjugates (ADCs) are a fast-growing class of therapeutics, with four already approved by the US Food and Drug Administration and over 60 molecules in clinical trials for a broad range of cancer indications.\cite{1,2} ADCs are designed to deliver cytotoxic drugs specifically to tumor cells to improve therapeutic efficacy while reducing off-target toxicity.\cite{3-5} An ADC molecule consists of three components: monoclonal antibody, linker and cytotoxic drug. Therefore, ADCs have very complex molecular structures with characteristics of both large and small molecules. In addition, the conjugation reaction typically results in a heterogeneous mixture with a range of different drug-to-antibody ratios (DARS). For example, conjugation through lysine residues often leads to a distribution of DARS ranging from 0 to 9, while conjugation through reduced interchain disulfide bonds results in mainly even numbered DARS 0, 2, 4, 6 and 8.\cite{6,7}

Trastuzumab emtansine (T-DM1; Kadcyla\textsuperscript{TM}, Genentech Inc.) is an ADC approved for human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer\cite{8,9} and is under investigation for the treatment of a variety of HER2-positive cancers. T-DM1 is composed of trastuzumab, a nonreducible thioether linker (N-maleimidylmethyl)cyclohexane-1-carboxylate (MCC), and a derivative of the antimicrotubule agent maytansine (DM1) (Figure 1). Following internalization and proteolytic degradation of the antibody part of T-DM1 in lysosome, the released DM1-containing catabolites inhibit tubulin polymerization and eventually cause cell death.\cite{10-12} DM1 is conjugated to trastuzumab through random lysine residues, resulting in a mixture of DAR0 to DAR8 with an average DAR of approximately 3.5.\cite{6}

ADCs often undergo biotransformations in circulation and characterization of these structural changes will help elucidate the mechanism of ADC metabolism. So far there is very limited knowledge on T-DM1 biotransformations due to challenges in bioanalysis. In a previous report, the catabolic fate of T-DM1 has been investigated at the small molecule level and three catabolites were identified in rat plasma, including DM1, MCC-DM1 and Lysine-MCC-DM1.\cite{13,14} It is anticipated that further investigation of T-DM1 catabolites at the antibody conjugate level will provide additional insights into T-DM1 biotransformations. We had previously explored both \textit{in vivo}\cite{6} and \textit{in vitro}\cite{15} stability for T-DM1 by affinity capture liquid chromatography-mass spectrometry (LC-MS) where all DAR species containing the trastuzumab antibody were captured and analyzed. Our data showed a gradual shift from high DARS to low DARS over time \textit{in vivo} in cynomolgus monkey plasma or \textit{in vitro} in human plasma, which indicates that the higher DAR species may lose drug to form new lower DAR species. However, the structures of these new species were not identified due to insufficient mass resolution of quadrupole-time-of-flight mass spectrometry (Q-TOF MS). To address this issue, we have recently evaluated and implemented a high-resolution, accurate-mass (HR/AM) Orbitrap MS approach which enabled more comprehensive characterization of \textit{in vivo} biotransformations for site-specific THIOMAB\textsuperscript{TM} antibody-drug conjugates (TDCs).\cite{16}

Here, we used an affinity capture HR/AM Orbitrap MS strategy to investigate \textit{in vivo} biotransformations of T-DM1,
which is a more challenging molecule compared to TDCs due to its highly heterogeneous and complex structure. Three types of biotransformations and their corresponding antibody-conjugated catabolites were characterized for T-DM1 in circulation in mice bearing HER2-positive human gastric cancer xenografts. To the best of our knowledge, this is the first report demonstrating the comprehensive structural changes and catabolites of T-DM1 at the intact antibody conjugate level.

Results

**Characterization of T-DM1 using HR/AM orbitrap MS versus Q-TOF MS**

We first analyzed T-DM1 extracted from mouse plasma harvested at 1, 4 or 7 days post dose using conventional Q-TOF MS, and observed a change in DAR distribution over time (Fig. S1). However, the resolution of TOF was insufficient to characterize the newly formed species. To gain new insights into *in vivo* biotransformations of T-DM1, we further analyzed the same samples using a HR/AM Orbitrap MS approach. The overall DAR distribution patterns were consistent between TOF (Fig. S1) and Orbitrap (Figure 2) MS data. The deconvoluted mass spectra for T-DM1 dosing solution (0 hour) confirmed that T-DM1 was a complex mixture. In addition to the conjugates carrying 0–7 MCC-DM1, some minor species formed by addition of free MCC linker or glycation of the antibody were detected. Both TOF and Orbitrap data demonstrated a shift toward lower DAR species over time, indicating a gradual loss of drug in circulation. A major difference between Q-TOF and Orbitrap data is that there were many more satellite peaks adjacent to each main DAR species in Orbitrap spectra, which were well resolved due to an enhanced mass resolution. When comparing the peak profiles of T-DM1 at different time points, the number and relative abundance of these satellite peaks were observed to grow over time (Figure 2), which indicates formation of new ADC species due to structural changes of the original DAR species. On the basis of the mass differences between these new species and the original T-DM1 molecules, three types of *in vivo* biotransformations for T-DM1 have been successfully identified.

**T-DM1 undergoes loss of DM1 drug in circulation via maleimide exchange**

A detailed view of the mass range between the DAR2 (147,093.71 Da) and DAR3 (148,053.39 Da) main peaks at 7 days post dose provided insightful information on T-DM1 biotransformations, where three new species were characterized as cysteine or glutathione adducts (Figure 3). Based on the 618-Da mass difference between the 147,435.47-Da peak and the DAR3 peak, the 147,435.47-Da species was characterized as a cysteine adduct formed by loss of a DM1 drug from DAR3 followed by addition of a cysteine residue. Similarly, the data suggest that the 147,617.13-Da species was formed by loss of a DM1 and addition of a glutathione to DAR2, while the 147,670.30-Da species was formed by loss of a DM1 from the DAR3 + MCC species and addition of a cysteine residue. Maleimide exchange was the mechanism for these structural changes. The cysteine adducts have a greater abundance than the glutathione adduct (Figure 3), which is consistent with a previous observation for a disulfide-linked PBD dimer TDC. This may be attributed to the higher concentration of reduced cysteine in plasma. Examination of the rest of the peaks within this mass range is discussed in the following section. When analyzing Orbitrap data, the mass tolerance was set as 25 ppm. The theoretical and experimental mass changes for the biotransformations identified in this study are listed in Table S1.

Similar maleimide exchange-based biotransformations were observed for the other molecules carrying different numbers of linker-drug. For example, Figure S2 showed the peak profiles of cysteine and glutathione adducts within the mass range between DAR3 and DAR4 main peaks at 7 days post dose, where the 148,388.52-Da peak is consistent with a cysteine adduct (DAR4 – DM1 + cysteine) and the 148,633.92-Da peak is consistent with a glutathione adduct (DAR4 – DM1 + glutathione).

**T-DM1 undergoes loss of maytansinol in circulation via ester hydrolysis**

A second type of *in vivo* biotransformation characterized for T-DM1 was loss of maytansinol. On the basis of a mass difference of 541 Da between the peak 147,512.52 and the DAR3 main peak shown in Figure 4, the 147,512.52-Da molecule was characterized as a T-DM1 catabolite formed by loss of a maytansinol from DAR3 followed by addition of OH. Ester hydrolysis was the mechanism for this structural change. Similar biotransformation was observed for different DAR species. For example, the 148,472.91-Da peak in Figure S2 represents a catabolite where a maytansinol was cleaved from DAR4 (DAR4 – maytansinol + OH).

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**Figure 1. Structure of T-DM1.**
Figure 2. Deconvoluted mass spectra showing changes in drug-to-antibody ratio distribution for T-DM1 in circulation in mice. Mice were dosed intravenously with 5 mg/kg of T-DM1 and plasma was harvested at 1, 4 and 7 days post dose. The samples were analyzed by affinity capture LC-MS on an orbitrap instrument. Dosing solution (0 hour) was used as a control.
Figure 3. T-DM1 catabolites indicate drug loss via maleimide exchange in circulation in mice. A detailed view of the deconvoluted spectrum at 7 days post dose in the mass range 147,000–148,500 Da displayed three new peaks corresponding to cysteine or glutathione adducts formed by maleimide exchange. The modification site was indicated in the top panel of the figure. Cys represents cysteine, and GSH represents glutathione.

Figure 4. T-DM1 catabolites indicate partial drug loss via ester hydrolysis in circulation in mice. A detailed view of the deconvoluted spectrum at 7 days post dose in the mass range 147,000–148,500 Da displayed a new species formed by loss of a maytansinol followed by addition of an OH. The modification site was indicated in the top panel of the figure.
**T-DM1 is subject to a small mass increase over time in circulation due to hydrolysis of linker-drug**

When comparing the mass of T-DM1 molecules across different time points, a gradual mass increase was observed for DAR2, 3 and 4 over time. For example, an approximate 13-Da mass increase was detected for the DAR2 main peak, which changed from 147,081.00 Da at 0 hour to 147,093.71 Da at 7 days post dose. Similar mass changes were observed for DAR3 and 4. Based on the molecular structure of T-DM1 (Figure 1) and our previous experience with trastuzumab-MC-vc-MMAE ADCs, we hypothesized that the linker-drug of T-DM1 underwent hydrolysis in circulation which resulted in a gradual mass increase over time. To confirm the hypothesis, we reduced the molecules captured from mouse plasma and analyzed them by LC-MS on a Q Exactive Plus. An overview of the deconvoluted spectra in the mass range of 20–55 kDa demonstrated that three light chain species with 0–2 linker-drug (i.e., L, L + LD and L + 2LD, where L represents light chain and LD represents linker-drug) were detected (Fig. S3). Similar phenomena were also observed for the heavy chain species. Further examination of the deconvoluted spectra focusing on the L + LD species showed that this molecule underwent hydrolysis at 4 days post dose, which resulted in two newly formed 24,416.13-Da and 24,434.82-Da molecules, corresponding to L + LD + H₂O and L + LD + 2H₂O, respectively (Figure 5). In contrast, no hydrolysis was detected in naked light chain (Figure 5). The same hydrolysis species were also detected at 7 days post dose for L + LD, but not for naked light chain (data not shown). Taken together, these observations suggest that the linker-drug rather than the antibody underwent hydrolysis and that more than one site was involved, which contributed to the mass increase of T-DM1. When examining the structure of the linker-drug, we found a hydrolysis site in the well-known hydrolysis-susceptible succinimide ring in the MCC linker, which is highlighted in green in Figure 6. There might be an additional hydrolysis site in the maytansinol moiety.

We were able to assign structures for most of the ions detected in the HR/AM Orbitrap mass spectra. For example, we successfully assigned all DAR2 peaks at 7 days post dose. The bottom panel of Figure 6 shows a detailed peak assignment for all DAR2 species. In addition to the pre-existing glycation and DAR2 + MCC species, three types of in vivo biotransformations as described above were characterized for T-DM1. The sites of these biotransformations were highlighted in the top panel of Figure 6. Another example showing the peak assignment for DAR3 species was summarized in Figure S2.

**Discussion**

We previously analyzed the circulation stability of T-DM1 by affinity capture LC-MS on a Q-TOF mass spectrometer, and our data showed a gradual shift from high DAR to low DAR species over time in cynomolgus monkey plasma. The observation indicates that T-DM1 may be subject to structural changes in circulation. Although the Q-TOF data allowed estimation of the average DAR values and evaluation of in vivo stability for T-DM1, the structures of the newly formed species could not be proposed due to insufficient resolution of Q-TOF MS.

Our group recently reported two strategies to enhance the mass resolution when analyzing ADCs by affinity capture LC-MS.

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**Figure 5.** Reduced light chain molecular masses confirm hydrolysis of T-DM1 linker-drug in circulation in mice. Following analysis of intact T-DM1, the samples at 0 hour (dosing solution) and 4 days post dose were further reduced with TCEP and analyzed by HR/AM Orbitrap MS. L represents light chain, and LD represents linker-drug.
The first strategy improved mass resolution by reducing the size of ADCs using IdeS digestion. The analytes generated by this method were truncated ADCs where the Fc regions were removed. Therefore, this approach is primarily applicable for ADCs with conjugation sites located in the Fab region. The second strategy was to analyze ADCs in the intact form using HR/AM Orbitrap MS, which preserves the structural information of ADCs as much as possible with minimal treatment. This approach has broader applications, being suitable for ADCs with site-specific conjugations in both Fab and Fc regions, as well as for ADCs with random lysine conjugations. Since T-DM1 is an ADC where the DM1 drug is conjugated to the antibody through random lysine residues, we have herein investigated its biotransformations using the second strategy.

Three types of biotransformations were characterized for T-DM1 in circulation in mice. The major biotransformation was cysteine or glutathione adduct formation via maleimide exchange, which resulted in loss of DM1 drug. This finding correlated well with the observation in a previous study focusing on small molecule catabolites where DM1 was characterized as a catabolite for T-DM1 in rat plasma. Formation of free DM1 was also observed when incubating T-DM1 with mouse plasma in vitro. In this study, instead of examining small molecule catabolites, we investigated antibody-conjugated catabolites using affinity capture HR/AM Orbitrap MS, which provided new insights into T-DM1 catabolism. For example, our data indicated that both cysteine and glutathione can be added to the MCC linker after DM1 is cleaved from the ADC, thereby generating multiple antibody-conjugated catabolites. Similarly, the data also suggested that following loss of maytansinol by ester hydrolysis, a new catabolite containing trastuzumab, the MCC linker and a drug fragment was formed. Since trastuzumab is included in these antibody-conjugated catabolites, they could be specifically delivered to HER2+ tumor cells.

An interesting finding was that most of T-DM1 species underwent a gradual small mass increase over time in circulation. Based on our prior experience, we hypothesize that the small mass increase was due to hydrolysis of the ADC. When analyzing reduced T-DM1, we clearly detected hydrolysis for the linker-drug at 4 and 7 days post dose. The intact MS data indicate that the non-hydrolyzed molecule was not resolved from the corresponding hydrolyzed version and they merged into one peak. Although Orbitrap MS has superior mass resolution in comparison to conventional TOF-MS, the mass change resulting from
hydrolysis was too small to be unambiguously resolved for a ~150-kDa molecule. In this case, it is essential to further analyze the reduced samples to confirm this biotransformation.

Although the circulation biotransformations described here could potentially affect the activity for T-DM1, these structural changes were a gradual and relatively slow process. T-DM1 has in fact exhibited good efficacy with tumor regression in a HER2-positive human gastric cancer xenograft in mice (Fig. S4).

In summary, three types of in vivo biotransformations and multiple antibody-conjugated catabolites were characterized for T-DM1 using an affinity capture HR/AM Orbitrap MS approach. The information helps improve understanding of the in vivo catabolism of T-DM1.

Materials and methods

Mouse plasma samples

The mouse study was carried out in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Genentech, Inc. C.B-17 SCID mice (Charles Rivers Laboratories, Reno, NV) bearing HER2 gastric cancer xenografts were administered a single intravenous injection of T-DM1 through the tail vein. Blood samples were collected into tubes containing lithium heparin via retro-orbital bleeds and used to derive plasma. The plasma samples were stored at −70°C until the following analysis.

Affinity capture

T-DM1 was selectively extracted from plasma samples by affinity capture as described previously. Briefly, human HER2 extracellular domain (ECD) was biotinylated and immobilized onto Dynabeads M-280 Streptavidin (Invitrogen, Carlsbad, CA) in a 96-well plate, and then the ECD-bead system was used to capture T-DM1 by incubating with plasma samples for 2 hours at room temperature. The captured molecules were then washed with HBS-EP buffer (10 mM Hepes [pH 7.4], 150 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid [EDTA], 0.005% Surfactant P20) (GE Healthcare, Piscataway, NJ) and deglycosylated using N-Glycanase (Prozyme, San Leandro, CA) at 37°C overnight. After extensive washing of the beads with HBS-EP, water and 10% acetonitrile, the ADC analytes were eluted using 30% acetonitrile with 1% formic acid. A KingFisher 96 magnetic particle processor (Thermo Fisher Scientific, Waltham, MA) was used to mix, wash, gather, and transfer the paramagnetic beads in the above steps.

Separation of intact T-DM1 species by reversed phase liquid chromatography

Chromatographic desalting and separation of T-DM1 were performed on a nanoACQUITY UPLC® system (Waters Corporation, Milford, MA) equipped with a PS-DVB monolithic column (500 µm i.d. x 5 cm) (Thermo Fisher Scientific, Waltham, MA). Five microliters of the ADC analyte were loaded onto the column and separated from interference species with a gradient of mobile phase A (0.1% formic acid) and mobile phase B (acetonitrile, 0.1% formic acid) at a flow rate of 15 µL/min. The gradient was 0%–40% B in 4 min, 40% B for 3 min, and 40–100% B in 1.5 min.

Intact T-DM1 analysis by Q-TOF MS

Following chromatographic separation, the samples were analyzed on a TripleTOF 5600 mass spectrometer (AB Sciex, Concord, ON) equipped with a DuoSpray™ ion source. The instrument was operated in positive ion mode. The main parameters were set as follows: ionspray voltage floating, 5 kV; declustering potential, 250 V; collision energy, 20 V. TOF-MS spectra were extracted from the total ion chromatograms (TICs) and deconvoluted using the Bayesian Protein Reconstruct algorithm incorporated in BioAnalyst 1.5.1 software (AB Sciex, Concord, ON).

Intact T-DM1 analysis by Orbitrap MS

The T-DM1 samples were also analyzed on a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an Ion Max API source. For intact ADCs, the spectra were acquired using a Full MS method at a resolution setting of 17,500 and the key parameters were optimized as follows: spray voltage, 3.2 kV; sheath gas flow rate, 8; S-lens RF level: 100; in-source CID, 100 ev; AGC target, 3 x 10⁶; maximum injection time, 150 ms; microscans, 10. Full MS spectra were extracted from the TICs and deconvoluted using the ReSpect™ algorithm incorporated in Protein Deconvolution 4.0 software (Thermo Fisher Scientific, Bremen, Germany). The mass tolerance was set as 25 ppm.

Analysis of reduced T-DM1 by Orbitrap MS

For analysis of light and heavy chain species, the captured ADC molecules were reduced by incubation at 37°C for 40 min with 10 mM tris(2-carboxyethyl)phosphine (TCEP). Chromatographic separation of light and heavy chains was performed on a nanoACQUITY UPLC® system equipped with a PS-DVB monolithic column (200 µm i.d. x 25 cm) (Thermo Fisher Scientific, Waltham, MA) using a gradient of mobile phase A (0.1% formic acid) and mobile phase B (acetonitrile, 0.1% formic acid) at a flow rate of 3 µL/min. The gradient was 0%–25% B in 4 min, 25% B for 3 min, 25–35% B in 22 min, and 35–60% B in 3 min. Reduced ADCs were analyzed on a Q Exactive Plus mass spectrometer, and deconvolution was performed using Protein Deconvolution 4.0.

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Disclosure of Potential Conflicts of Interest

No potential conflict of interest was reported by the authors.

Abbreviations

| Abbreviation | Description                          |
|--------------|--------------------------------------|
| ADC          | antibody-drug conjugate              |
| DAR          | drug-to-antibody ratio               |
| DM1          | a derivative of maytansine           |
| ECD          | extracellular domain                 |
| HR/AM        | high-resolution accurate-mass        |
| L            | light chain                          |
| LD           | linker-drug                          |
| MCC          | (N-maleimidylmethyl)cyclohexane-1-carboxylate |
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