Study on the Heterogeneity of T-DM1 and the Analysis of the Unconjugated Linker Structure under a Stable Conjugation Process

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ABSTRACT: Trastuzumab emtansine (T-DM1) is a target-specific anti-cancer antibody-drug conjugate (ADC). In the present study, critical quality attributes for different manufactured products, such as the drug to antibody ratio (DAR), conjugation site, and site conjugation ratio, are similar, which is contrary to the traditional view that conjugation at lysine sites is randomly assigned. To investigate this result, a series samples with different DARs were prepared. Site conjugation ratios of the 27 different conjugation sites (corresponding to 54 potential sites) were analyzed. We found that the correlation coefficients of the 26 site conjugation ratio and the DAR were \( R^2 > 0.9 \), and the remaining one was \( R^2 > 0.7 \). By comparing three batches of samples with a DAR value of \( \sim 3.3 \) in a stability study, we found that degradation rates of conjugation sites of the samples incubated at 40 °C were basically the same. These data show that the conjugation ratio and the conjugation stability of each site may remain consistent if the process parameters are stable. LC/MS/MS was used to study the unconjugated linker of the crosslink byproducts produced by a two-step method. We determined four forms of unconjugated linkers: (N-maleimidomethyl) cyclohexane-1-carboxylate (MCC) unconjugated to DM1, hydrolyzed MCC unconjugated to DM1, lys-MCC-lys, and lys-MCC-cys. We believe that the current study can provide an effective guide for the processing of ADCs, control of product quality, and reduction of side reaction products.

INTRODUCTION

Antibody drug conjugates (ADCs) have a high degree of heterogeneity. 5,6 Because conjugation can occur in several but not all of the available sites of antibodies, a particular ADC can contain multiple conjugation molecules. Therefore, an ADC is a mixture of conjugated substances, and appropriate tests are required to evaluate the heterogeneity and ensure product consistency. 3 To demonstrate the consistency of an ADC, a comprehensive method needs to be established to analyze the consistency of different batches of ADCs. It is important to assess the consistency because even tiny structural differences can significantly affect the safety and/or effectiveness of ADCs. 5 The ADCs on market are derived from different multiple batches not only in the process of clinical trials but after the ADCs come into the market. Ensuring consistency between batches enables better evaluation of the relationship of the ADCs and their clinical outcomes, thereby eliminating the efficacy or safety risks caused by quality differences among batches.

Trastuzumab emtansine (T-DM1) is an ADC for human epidermal growth factor receptor 2 (HER2)-positive breast cancer therapy. 5,6 and it was approved by FDA in 2013 with the trade name Kadcyla (ado-trastuzumab emtansine) in 2013. Adotrastuzumab emtansine employs DM1, a semisynthetic cytotoxic payload of the maytansinoid class, which is conjugated via lysine residues (its primary amine group) of the antibody. Compared to sugar chain conjugation, ADCs with site-specific properties derived from nonnatural amino acid conjugation, disulfide conjugation, and conjugation primary amine-(N-maleimidomethyl) cyclohexane-1-carboxylate (MCC)—DM1-connected ADCs possess a high heterogeneity due to rich conjugation sites of the antibody sequence. Therefore, the batch quality must be strictly and thoroughly compared and assessed because of the high heterogeneity of T-DM1.

As shown in Figure 1A, we studied the T-DM1 prepared by a two-step method. 7–9 First, the antibody was coupled with the bifunctional linker succinimidyl 4-MCC (SMCC) to obtain the intermediate T-MCC. The remaining SMCC and the derivative were then removed and DM1 was added to form a covalent bond with the other functional group of the MCC moiety of the intermediate to complete the conjugation processes. In the chemical conjugation reaction, antibodies, toxin moieties, and SMCC varied with the process parameters being fluctuated. All the process parameters such as pH, temperature, stirring speed, buffer system, bioreactor texture, and organic solvent will influence the quality of the final products. For example, it is found in the process development...
that ADCs produced in different temperatures, even with same drug to antibody ratio (DAR), vary differently to a large extent in the site conjugation ratio. Therefore, compared to the antibody, ADC heterogeneity is more complicated to evaluate. In-depth equivalence assessment need to be addressed in effective payload, conjugation sites, drug distribution, drug conjugation stability, and unconjugated linker through more mass spectrometry techniques.

SMCC is a bifunctional linker that not only acts as a bridge in T-DM1 but also causes many side reactions.\textsuperscript{10–12} LC/MS/MS has proven to be an efficient and powerful tool for studying these byproducts. Figure 2 shows the DAR that was characterized by mass spectrometry after T-DM1 deglycosylation. In addition to main peaks of the antibodies connected to a series of DM1 separated with a 957 Da difference, there is a 222 Da difference between adjacent main peaks. Chen et al.\textsuperscript{7} considered this peak as an active unreached maleate group of MCC. Carrasco-Triguero et al.\textsuperscript{13} consider that this is the reason why the active maleate group of MCC is linked to another primary amine group of lysine on the antibody to form a crosslink. The present study shows that unconjugated linkers exist in a variety of forms. In addition to the two cases described above, the active MCC maleimide group forms a maleimide-thiol conjugate with a free disulfide bond to obtain another cross-linker.\textsuperscript{14} Furthermore, maleamides may be hydrolyzed and are not able to undergo a Michael addition reaction with DM1 when hydrolyzed.\textsuperscript{15} Currently, the formation of metabolites during pharmacokinetic reactions of T-DM1 has not been clarified, and the mechanism of metabolic product formation needs to be further studied.\textsuperscript{16} As there are side reaction products of the unconjugated linker, the existence and proportion of T-DM1 need to be well estimated and strictly controlled.

In this study, we analyzed the heterogeneity of ADC batches in the aspects of the DAR, conjugated sites, drug distribution, and site-conjugation stability. By adjusting the process parameters used to prepare different samples, the relationship of the site conjugation ratio and process parameters was studied, and the unconjugated linkers were identified. This analysis is important for the production of lysine-conjugated ADCs.

**RESULTS**

**Analysis of the DAR.** The DAR, as a critical quality attribute, determines the payload of ADCs and is critical for evaluating ADC heterogeneity.\textsuperscript{17,18} Intact MS has been widely recognized as a fast and accurate method for characterizing the ADC drug load curve. In T-DM1 analysis, the pH and duration of the enzyme-cutting depeptide are the major factors of the drug distribution curve and the unconjugated linker. Figure 2A shows that when the N-glycosidase reaction condition is strictly controlled at pH 7.4 for 3.5 h, the 0–7 DM1-
Table 1. Summary of Analytical Results of MS-DAR and UV-DAR of Different Samples

|                | K-Lot1 | K-Lot2 | K-Lot3 | K-Lot4 | B-Lot1 | B-Lot2 | B-Lot3 | B-Lot4 |
|----------------|--------|--------|--------|--------|--------|--------|--------|--------|
| MS-DAR         | 3.24   | 3.30   | 3.26   | 3.29   | 3.28   | 3.21   | 3.24   | 3.29   |
| UV-DAR         | 3.48   | 3.40   | 3.40   | 3.46   | 3.45   | 3.46   | 3.39   | 3.34   |
| unconjugated linker | 5.87% | 6.78% | 6.06%  | 6.02%  | 5.36%  | 4.87%  | 4.67%  | 4.65%  |

Table 1 shows that the difference of the DAR of the four batches is less than 0.1, and the proportion of unconjugated linkers is ~5%. By using MCC as DM1 as a post translational modification (PTM), the chymotryptic peptide conjugation site containing PTM was identified by tandem mass spectrometry (MS/MS). 42 sites (corresponding to 84 sites of the complete molecule, 4 sites of N-terminal amino groups, and 80 lysine sites, Table 2) were identified with the 547 Da characteristic peak, mass error <10 ppm, no dehydration modification, and adequate collision-induced dissociation (CID) as basic requirements of conjugated peptide identification. 42 different differential sites were all identified in the samples.

Because of the incomplete signal of the unconjugated peptide at the 15 conjugation sites, only 27 different conjugation sites from four batches of samples (B-lot1-4) were counted and included in the relative standard deviation (RSD) calculation (Table 3). The RSD of LC-126K was 12.02%, and the RSD of the other 26 sites was less than 10%. This result indicates that the conjugation level of the site under the same conjugation process may also be relatively stable. To further validate the experimental results, the conjugation rate of 27 sites of four different batches of Kadcyla was analyzed (Table 3): only 5 sites had RSD > 10%: LC-103K, 25.40%; LC-126K, 54.97%; HC-208K, 12.89%; HC-320K, 16.32%; and HC-395K, 19.52%. The 10% RSD threshold was confirmed by analytical repeatability analysis. During the test, a sample was divided into four, and the four subsamples were prepared in parallel before analysis of the proportion of conjugation. It was found that even when the pretreatment and characterization analysis were the same, the differences in enzyme cutting and mass spectrometry ionization efficiency resulted in a slight difference in the proportion of conjugation at the same conjugation site. According to statistics, the deviation due to instrumental and sample pretreatment differences should not exceed 10%.

To further understand the above findings, we strictly controlled the conjugation reaction process parameters and contact materials: buffer solution (pH 6.5), antibody concentration (12.5–13 mg/mL), final N,N-dimethylacetamide (DMA) concentration of 10%, temperature (25°C), and stirring speed (120 rpm); only the proportions of SMCC and DM1 were changed, samples with DAR values of 1.10, 1.37, 1.62, 2.05, 2.08, 2.81 and 3.73 were produced with the same batch of antibody (Table 1). The conjugation sites of all samples were analyzed. The above 42 conjugation sites are confirmed for the sample with DAR 3.73. In the low-DAR sample, 35 DM1 conjugation modification sites are found. Furthermore, no different conjugation sites from the previous sample were found in the above DAR gradient samples. At the same time, we observed that with the DAR increased, the proportion of each site increased accordingly.

Three different lysine conjugation sites for the antibody N-terminal, hinge regions, and Rf region were selected for further study (Figure 3). Take the HC-225K site as an example, the conjugation ratios were 2.33, 2.94, 3.03, 4.08, 4.37, 6.19, and 8.39%, as shown in Figure 3. The data were fitted perfectly well with the corresponding DAR values of seven samples, the regression model y = 0.025x + 0.0077, R² = 0.993 (Table 4). R² represents the correlation coefficient between the MS-DAR value and the site conjugation level. The fitted curve is more linearly related when R² is closer to 1. It is generally believed that when R² > 0.8, the curve has a high linear correlation. When 0.5 < R² < 0.8, it is considered that the curve fitted is shown as a significant straight line. This result is vastly different from traditional recognition that lysine conjugation is randomly reacted. To ensure the validity of the data, the conjugation sites with incomplete unconjugated peptide signals were removed, and linear analysis was performed on 27 conjugation sites in all samples of gradient DAR.

Table 4 shows R² > 0.99 for nine sites, R² > 0.95 for a total of 26 sites, R² > 0.9 for all sites, and R² of 0.785 for only one site LC-126K, the reason why R² for site LC-126K is relatively lower is because the conjugation level on this site is lower than other sites, it may be biased because of a relatively lower coefficient of variance (CV) values. We introduce the p value to determine the significance of the regression model using regression analysis by IBM SPSS statistics 22. It is generally considered that p < 0.05 is statistically significant, and when p
| no. | Peptide                                             | observed | RT (min) | theoretical MW | mass error (ppm) |
|-----|-----------------------------------------------------|----------|----------|----------------|-----------------|
| 1   | LC-N DIQMTQSPSLSASVQGR                              | 89.02    | 89.85    | 2835.2506      | −1.8            |
| 2   | LC-39K ASQDVNTAVAVYQQSKPGK                          | 82.58    | 83.40    | 3243.5474      | −2.6            |
| 3   | LC-42K ASQDVNTAVAVYQQSKPGKAPK                       | 76.66    | 77.65    | 3025.5074      | −0.5            |
| 4   | LC-103K SGTDFDTITSLQPEDFATYQCQHYTTPFGQQGTKVEIK       | 94.37    | 93.78    | 5613.5723      | −1.8            |
| 5   | LC-107K VEIKR                                       | 78.55    | 79.61    | 1600.7734      | −0.2            |
| 7   | LC-126K TAAAPVQFIPPSDEOQLSKTGASVQCLNNFYPR            | 114.26   | 114.69   | 4681.2688      | −2.6            |
| 8   | LC-145K EAKVQMK                                     | 81.04    | 82.00    | 1844.8582      | −1.0            |
| 9   | LC-149K VQQKVDNALQSNGSEQSVTEQSDKSTYSLTSLTSLK        | 83.96    | 84.63    | 3633.6344      | −3.4            |
| 10  | LC-169K VDNALQSGNSQSVTEQSDKSTYSLTSLTSLK            | 85.37    | 86.27    | 4576.0738      | −2.8            |
| 11  | LC-183K DSPYSLSLTLTSLKADYEK                         | 97.71    | 98.36    | 4908.2543      | −1.2            |
| 12  | LC-188K ADYEKHK                                      | 69.14    | 70.24    | 2268.0812      | −1.2            |
| 13  | LC-190K HKVYACEVTHQGLLSVPVTK                        | 71.38    | 72.28    | 3097.4452      | −3.3            |
| 14  | LC-207K YADSVKG                                      | 76.97    | 78.85    | 3336.5358      | −2.1            |
| 15  | HC-N EVQLVESGGGLVQPGGSLR                             | 94.83    | 95.53    | 2838.3673      | −1.6            |
| 16  | HC-30K LSAASGFNIDTYYHWVR                             | 92.95    | 93.42    | 3194.4769      | −2.8            |
| 17  | HC-43K QAPGKGEWVAR                                  | 87.79    | 88.58    | 2268.0812      | −1.2            |
| 18  | HC-65K YADSVKG                                      | 76.87    | 77.98    | 1851.8277      | −0.7            |
| 19  | HC-76K FTISADTSKNTAYLQMSLR                           | 87.95    | 88.78    | 3217.4875      | −2.5            |
| 20  | HC-124K WGGDGFYAMIDWGGQQTLTVMVSSSKGSVPVLAPSSK        | 102.20   | 102.89   | 4908.2543      | −1.2            |
| 21  | HC-136K GPSVPNLAPSSKSTSGTAALGGLCVK                  | 94.30    | 95.20    | 3446.7113      | −2.6            |
| 22  | HC-208K DYFPEPVTVSWSNALGTVHFCAPQSLQGLYSLSVVTTPSSSGTQYICNVNHKPSNTK | 99.30    | 99.88    | 7699.6789      | −3.2            |
| 23  | HC-213K DYFPEPVTVSWSNALGTVHFCAPQSLQGLYSLSVVTTPSSSGTQYICNVNHKPSNTKVDK | 96.64    | 97.17    | 8011.8692      | −2.7            |
| 24  | HC-216K VDK                                          | 74.26    | 75.71    | 1446.6676      | −0.1            |
| 25  | HC-217K KVEPK                                        | 74.42    | 75.87    | 1556.7360      | −0.1            |
| 26  | HC-225K SCDKHHTCPCCPAPELLGGPSVFLFPKPK               | 91.80    | 92.69    | 4291.0066      | −2.7            |
| 27  | HC-249K THTCPPCAPELGGPSVFLFPK                        | 100.60   | 101.24   | 3800.8220      | −2.3            |
| 28  | HC-251K THTCPPCAPELGGPSVFLFPK                        | 99.04    | 99.60    | 4617.2384      | −2.9            |
| 29  | HC-277K TPEVCTVYDVSHEDEPKGFKFNYYDGVHELHNAK           | 92.79    | 93.11    | 4754.1760      | −2.9            |
| 30  | HC-291K FWYVYDGVHEVNAKKTPR                          | 80.13    | 81.02    | 3116.4629      | −1.9            |
| 31  | HC-293K TKPR                                        | 73.68    | 75.09    | 1457.6888      | −0.2            |
| 32  | HC-320K VSVLTVLHTQDMLNGKEYK                         | 97.78    | 98.41    | 3184.5718      | −2.5            |
| 33  | HC-323K EYCKK                                       | 73.54    | 74.92    | 1683.7088      | −0.1            |
| 34  | HC-325K CVKSNK                                      | 72.72    | 74.13    | 1691.7462      | −0.2            |
| 35  | HC-329K VSNKALPAIEK                                 | 83.92    | 84.77    | 2223.1061      | −1.4            |
| 36  | HC-337K ALPAPIETSK                                  | 88.19    | 89.95    | 2224.1265      | −1.3            |
| 37  | HC-341K TISKK                                       | 76.00    | 77.14    | 1603.7731      | 0.4             |
| 38  | HC-343K AKGQPR                                      | 73.25    | 74.72    | 1612.7843      | 0.4             |
| 39  | HC-363K EEMTKQVSLTCLVK                              | 88.57    | 89.44    | 2736.2624      | −1.3            |
| 40  | HC-395K GFYPSDIAVEWENQGPENNYKTTTPVLDSDGFFLYSK        | 98.01    | 98.72    | 5355.3998      | −3.1            |
| 41  | HC-417K LTVDKSR                                     | 78.30    | 79.46    | 1774.8375      | −0.5            |
| 42  | HC-442K WQGQNVFSVMHEALNHJYQKSLSLPG                  | 84.94    | 85.49    | 4398.9700      | −3.1            |
antibodies to crosslink the reaction, hereceptin-DM1 (H-DM1) DAR 3.12 (Table 1) was prepared by the standard crosslink process, and the MS-DAR results show that S-HLINK’s unconjugated linkers are as high as 31.83% and that they remain at ~4% for H-DM1. This shows that high temperatures have a great impact on the conjugation process. Two samples’ conjugation sites were analyzed as data shown in Table 4. The 27-site conjugation rate of H-DM1 was substituted into the corresponding linear equation, and the R² and p values are generally consistent of both the initial equation and new linear equation. The p value (Sig. P, using univariate analysis of variance by IBM SPSS statistics 22) of both slope differences is >0.5, showing no difference statistically. The MS-DAR values of the S-HLINK samples and H-DM1 were substituted into 27 initial equations, and the coefficients of variation (CV % values) of the theoretical site conjugation ratio and the actual measured values were calculated. The 26 H-DM1 sites have CV values < 10%, the 9 S-HLINK sites have CV values > 10%, and the overall deviation fluctuation is also larger for the 9 S-HLINK sites. We also adjusted the pH of the crosslink buffer system to 7.5, and a large number of unknown peaks was shown as characterized by MS of the product. It was studied that SMCC was hydrolyzed and DM1 was cleaved because of the long crosslink reaction in alkaline buffer.

The above results prove that the efficiency of the batch conjugation is consistent as long as the conjugation process is consistent and the quality of the antibody is similar. However, if the key process parameters of conjugation change, such as temperature and pH, the site conjugation rate will change. Through analysis of the conjugation ratio, the consistency between different batches of samples can be analyzed.

**Stability Study of the Conjugation Site.** Stress tests are often used to identify chemical degradation at each site of antibodies.3,21 If the quality of the ADC batches is consistent, the structural stability of each batch should be comparable. We exposed three batches of 100 g of T-DM1 (liquid form) to a high-temperature condition (40 °C) for 2 months to assess the rate of shedding at each conjugation site.

Figure 4 shows that during the incubation time of T-DM1 at high temperatures, the ultraviolet detection for drug to antibody ratio (UV-DAR) decreased and the content of free small molecules in solution increased rapidly; the trend of each antibody ratio (UV-DAR) decreased and the content of free small molecules in solution increased rapidly; the trend of each site conjugation rate will change. Through analysis of the conjugation ratio, the consistency between different batches of samples can be analyzed.

![Figure 3](image)

**Figure 3.** Linear fitting curve of MS-DAR and DM1 conjugation site levels (three different conjugation sites), taking the HC-225K site (the blue line) as an example, the conjugation ratios were 2.33, 2.94, 3.03, 4.08, 4.37, 6.19, and 8.39%.

< 0.001, the regression model is extremely significant. The results showed p > 0.001 only for the LC-126K site and p < 0.001 for the remaining sites. These sites with different conjugation ratios at different regions of the antibody sequence showed good linearity, which also suggests that the small molecule conjugation efficiency at each site under fixed process parameters is probably relatively conservative.

During the study of the DAR gradient samples, we prepared a sample S-HLINK DAR 3.54 with the conditions that reaction temperature increased and other parameters remain unchanged. At the same time, to investigate the impact of antibodies to crosslink the reaction, herceptin-DM1 (H-DM1) DAR 3.12 (Table 1) was prepared by the standard crosslink process, and the MS-DAR results show that S-HLINK’s unconjugated linkers are as high as 31.83% and that they remain at ~4% for H-DM1. This shows that high temperatures have a great impact on the conjugation process. Two samples’ conjugation sites were analyzed as data shown in Table 4. The 27-site conjugation rate of H-DM1 was substituted into the corresponding linear equation, and the R² and p values are generally consistent of both the initial equation and new linear equation. The p value (Sig. P, using univariate analysis of variance by IBM SPSS statistics 22) of both slope differences is >0.5, showing no difference statistically. The MS-DAR values of the S-HLINK samples and H-DM1 were substituted into 27 initial equations, and the coefficients of variation (CV % values) of the theoretical site conjugation ratio and the actual measured values were calculated. The 26 H-DM1 sites have CV values < 10%, the 9 S-HLINK sites have CV values > 10%, and the overall deviation fluctuation is also larger for the 9 S-HLINK sites. We also adjusted the pH of the crosslink buffer system to 7.5, and a large number of unknown peaks was shown as characterized by MS of the product. It was studied that SMCC was hydrolyzed and DM1 was cleaved because of the long crosslink reaction in alkaline buffer.

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Figure 4 shows that during the incubation time of T-DM1 at high temperatures, the ultraviolet detection for drug to antibody ratio (UV-DAR) decreased and the content of free small molecules in solution increased rapidly; the trend of each batch was basically the same. After the sample incubation at 40 °C for 10 days, intact MS detection signal was chaotic and irregular and unable to conduct MS-DAR statistical analysis. According to mass spectrum data analysis, the chaotic signal may be derived from DM1 shedding in different ways resulting in variation of modified molecules left on the lysine residues of the antibody, according to the mass spectrometry analysis.

Figure 5 shows the degradation rate at each conjugation site for 62 days determined by peptide mapping. The data shows that the degradation rate at different sites differs significantly. For example, the LC-126K site could not be detected, while HC-417K degradation was only ~28%, which demonstrates that the shedding of DM1 is also related to the solvent-accessible surface area of each conjugated site. Except the 6 sites with RSD > 10%, the RSD of the remaining 21 sites was still below the threshold of 10%. This result indicates that after the same conjugation reaction procedure, the trend of the change in antibody quality remains the same, and the physicochemical properties of the final products (T-DM1) are also equivalent if the crosslink processes are the same.
We find that while the UV-DAR decreasing rate (Figure 4A), the content increasing rate of free small molecules (Figure 4B) and the corresponding degradation rate of the conjugation site were not consistent. When incubated for 30 days at high temperature, UV-DAR was reduced by ~14%, and the free small-molecule content was less than 5%. UV-DAR decreased by ~28% when incubated for 62 days at high temperatures, whereas peptide analysis showed that the majority of the conjugation site degradation rate had exceeded 50%. We perceive that if the shedding small molecules are not complete DM1 molecules, the UV absorption of the solution will be biased because the degradation rate determined by UV-DAR is the rate of the whole sample containing degradative DM1 which cannot be removed, while peptide analysis determines the degradation rate of each site. Peptide modification analysis revealed that a large number of MCC~DM1 breaks at the ether bond, and the shedding small molecules with a molecular weight of 547 Da are characteristic fragments (Figure 6). In addition, long-term stability experiments confirmed the presence of the corresponding peptide, which suggests that the toxic moiety degradation of T-DM1 cannot be genuinely evaluated merely by qualifying small free drug content or UV-DAR. LC/MS analysis of the conjugation site ratio should be applied to determine the drug payload.

**Unconjugated Linker.** The major side product in T-DM1 prepared by a two-step method is the unconjugated linker, it is formed because the interval between the two reactions of the nucleophilic substitution reaction and subsequent DM1 Michael addition reaction is quite long, and there is an ultrafiltration exchange step to remove the remaining SMCC, there is sufficient time for a side reaction of the SMCC maleimide active group. Chen\(^7\) reports that the unconjugated linkers of the sample are all lys-MCC-lys. However, the molecular weight of six crosslink peptides increases by 222 Da, according to the literature. Liu and Chen\(^7\) identified three sites which only connected to SMCC, instead of theoretical 219 Da, according to the literature. Liu and Chen\(^7\) identified three sites which only connected to SMCC, whereas peptide analysis showed that the majority of the conjugation site degradation rate had exceeded 50%. We perceive that if the shedding small molecules are not complete DM1 molecules, the UV absorption of the solution will be biased because the degradation rate determined by UV-DAR is the rate of the whole sample containing degradative DM1 which cannot be removed, while peptide analysis determines the degradation rate of each site. Peptide modification analysis revealed that a large number of MCC~DM1 breaks at the ether bond, and the shedding small molecules with a molecular weight of 547 Da are characteristic fragments (Figure 6). In addition, long-term stability experiments confirmed the presence of the corresponding peptide, which suggests that the toxic moiety degradation of T-DM1 cannot be genuinely evaluated merely by qualifying small free drug content or UV-DAR. LC/MS analysis of the conjugation site ratio should be applied to determine the drug payload.
To find the potential cross-linkers, we modified all peptide sequences of 42 sites with DM1 conjugation and set it only bind to MCC (i.e., +219 Da). A total of 42 modified peptide sequences were introduced into the protein modification database of UNIFI MS software as a protein modification. These long, crosslink peptides were characterized by a high-energy collisional dissociation fragmentation technique. In the UNIFI software analysis, a cross-linker-modified peptide was defined as an α peptide, and the peptide identified as a protein modification was defined as a β peptide. 18 sites were identified as the lysine–MCC–lysine form and 1 lys-SMCC-cys (Table 5) by high-energy collision dissociation analysis of highly unconjugated linker peptide fragments. In an example (Figure 7), fragmentation of both α and β chains was observed in the MS/MS spectrum. Bioconjugate techniques introduced that the reaction of maleimide with thiol groups is 1000 times faster than the primary amine reaction at pH 7.0. When the first step crosslink completed, the free maleimide group of MCC can form a covalent bond with the primary amine that has a close spatial position. In addition, when the mercapto group of the nearby cystine side chain is free, MCC maleimide can rapidly form a cross-linked covalent bond with it. Using the full-length IgG (PDB ID: 1HZH) crystal structure to extract the spatial distance of these cross-linkers (Figure 8), it shows that the maximum distance of the amino acid residues at both ends of the cross-linker in the hinge region is 30 Å, and the distance between amino acid residues at both ends of the cross-linker in the nonhinge region is not more than 15 Å. In this study, the hinge area tends to be more flexible, which corresponds to that observed in previous studies.

Based on the above results, the unconjugated linker modification of Kadcyla and the candidate biosimilar are identified. Additionally, by detecting the retention time of the peptide and the m/z signal and comparing them to the identified cross-linker signals, it was found that all 19 cross-linker forms exist. In addition, both Kadcyla and the candidate biosimilar have the unconjugated linker form of +219 and +237 Da.

**DISCUSSION**

Considering the fact that only a small proportion of heterogeneous products can be used to objectively evaluate the acceptable treatment window and relationship between the efficacy and toxicity of the drugs because of the reason that toxic moieties conjugated to ADCs. Some clinical trials failed because of safety problems caused by the toxicity of toxic moieties. Accordingly, ADC developers strongly emphasize conjugation processes. However, as they solve the connectivity conundrum, scalability and other process concerns often emerge. Therefore, the structural heterogeneity analysis of

Figure 4. (A) Is degradation rate at each conjugation site calculated by UV-DAR, incubated at 40 °C over ~62 day time period. (B) Is time course displaying the free drug, incubated at 40 °C over ~30 day time period, the sample contains all forms of free drugs.

Figure 5. Degradation profile at each conjugation site (40 °C for 62 days) of B-Lot1, B-Lot2 and B-Lot3 determined by peptide mapping. Degradation rate data are shown by mean ± standard error bar.
lysine-linked ADCs is extremely important for the development of production technology. Although detection methods, such as size-exclusion chromatography, differential scanning calorimetry, capillary isoelectric focusing, and circular dichroism spectroscopy have been widely used to assess the molecular physicochemical properties in terms of thermodynamics, charge, and hydrophobicity, we do not have a clear understanding of the microscopic drug conjugation sites. It is reported that drug conjugation sites can significantly affect linker stability and antibody localization.25,26 Thus, detailed in-depth structural characterization is an important part of the quality assessment of lysine-linked ADCs. In this study, we used MS-based technology to obtain the structural information of the T-DM1 candidate drug and Kadcyla (in addition to the literature) and analyzed the relationship between the conjugation sites and the process through statistical analysis.

The conjugation sites, DM1 conjugation site ratio, and the unconjugated linker are the characteristic properties of an ADC, all of which reflect the possible distribution of conjugation sites of the toxic moieties and the side effects in the crosslink reaction process. According to the number of conjugation sites, the identified 42 sites are distributed in all samples. Regarding the proportion of conjugation sites, although the degree of ionization of different peptides is different, this is only a relative proportion. According to the statistical analysis of 27 sites, four batches of T-DM1 drug candidates have consistent conjugation ratios, except for slight differences in the conjugation ratios at one site, their conjugation ratios of the remaining sites are consistent, and the consistent chemical stability of the interbatch conjugation sites was also demonstrated in the thermal stability studies. Unconjugated linkers are the main side-reaction products during the conjugation process. For four batches of ADCs, the percentage of coupled linkers ranges from 4.3 to 5.1%. We identified four forms of unconjugated linkers, and in particular, the two forms of crosslinks that would affect the advanced structure of drug molecules. The above information shows that under the strict control of the conjugation process, each lysine site results in the same conjugation ratio and the same side reaction level, and the interbatch consistency of ADCs can be ensured.

Over time, for all lysine-linked ADC conjugation sites, the identification of lys residues as conjugation sites for small molecules increased from 47 to 90%. Thus, we believe that, unlike the traditional concept, the attachment of small molecules may be distributed over all lys residues, and the ratio of solvent exposure at each site is directly related to the efficiency of the site conjugation and shedding rate. With the development of mass spectrometry techniques, it is possible to identify small fractional conjugations at all lys sites. ADCs are an emerging technology relative to antibody drugs. However, more efforts and time are needed to overcome technical barriers. Only when industry and regulators learn from each other, a more effective platform process can be developed and applied in parallel with the regulatory framework.

**CONCLUSIONS**

In conclusion, the relationship between the conjugation sites and the manufacturing technology has been elucidated and forms of unconjugated linkers have been identified. We find that the physicochemical properties of the final products (T-DM1) are equivalent if the crosslink processes are the same and that the UV-DAR decreasing rate, the content increasing rate of free small molecules, and the corresponding degradation rate of the conjugation site were not consistent. Also, we find that the conjugation efficiency and the conjugation stability of each site remain consistent if the process parameters are stable. Our findings may provide an evidence as an effective guide for manufacturing of ADCs and thus decrease side production during reaction in addition to have a good control of the product quality.

**MATERIALS AND METHODS**

**Sample and Materials.** Kadcyla and Herceptin were purchased from Genentech Roche. Naked IgG1 mAb Trastuzumab was manufactured by Shanghai Pharmaceuticals Holding Co., Ltd. (Shanghai, China). DM1 was obtained from Shanghai Pharmaceuticals Inc. Sequencing grade-modified.
| no. | site | peptide (α) | peptide (β) | observed mass (Da) | mass error (ppm) | observed m/z | charge | observed RT (min) |
|-----|------|-------------|-------------|--------------------|-----------------|-------------|--------|------------------|
| 1   | HC225-MCC-LC-183 | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK | DSTYSLSSTTLSDKADYEK | 5661.761 | 2.4 | 944.466 | 6 | 70.33 |
| 2   | HC225-MCC-LC-190 | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK | HKVYACEVTHQGLSPVT | 5693.813 | 1.3 | 814.265 | 7 | 60.31 |
| 3   | HC225-MCC-LC-207 | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK | VYACEVTHQGLSPVTFSKFN | 5932.895 | −0.2 | 848.420 | 7 | 64.16 |
| 4   | HC225-MCC-HC-136 | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK | GPSVFLPLAPSSTSSGTAALGCLVK | 6042.032 | 0.1 | 1007.845 | 6 | 73.3 |
| 5   | HC225-MCC-HC-221 | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK | VEPKSCDK | 4515.185 | −0.1 | 753.370 | 6 | 62.31 |
| 6   | HC225-MCC-HC-225 | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK | 6887.377 | 1.6 | 861.804 | 8 | 76.81 |
| 7   | HC225-MCC-HC-249 | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK | THTCPPCPAPELGGPSVFLFPPKPK | 6397.226 | 6.8 | 914.753 | 7 | 78.48 |
| 8   | HC225-MCC-HC-323 | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK | EYKCK | 4280.081 | 2.8 | 714.186 | 6 | 62.26 |
| 9   | HC225-MCC-HC-325 | SCDKTHTCPPCPAPELLGGPSVFLFPPKPK | VSNKALPAPIEK | 4819.461 | −1.1 | 804.083 | 6 | 65.33 |
| 10  | HC249-MCC-LC-190 | THTCPPCPAPELGGPSVFLFPPKPK | HKVYACEVTHQGLSPVT | 5203.632 | 2.1 | 868.111 | 6 | 62.31 |
| 11  | HC249-MCC-HC-136 | THTCPPCPAPELGGPSVFLFPPKPK | GPSVFLPLAPSSTSSGTAALGCLVK | 5551.855 | 1.4 | 926.149 | 6 | 77.24 |
| 12  | HC249-MCC-HC-277 | THTCPPCPAPELGGPSVFLFPPKPK | TPEVTCSVTVSHEDPEVKFNWYVDGEVEHNAK | 5087.567 | 8.4 | 848.767 | 6 | 61.4 |
| 13  | HC-249-MCC-HC-395 | THTCPPCPAPELGGPSVFLFPPKPK | GEFYSDIAVEWESNGQPENNYKTTPVLDSDGFFLYSK | 7461.592 | 2.2 | 1244.438 | 6 | 83.16 |
| 14  | HC-277-MCC-HC-325 | TPEVTCSVTVSHEDPEVKFNWYVDGEVEHNAK | CKVSNK | 4751.282 | 1.4 | 792.720 | 6 | 63.05 |
| 15  | HC-277-MCC-HC-329 | TPEVTCSVTVSHEDPEVKFNWYVDGEVEHNAK | VSNKALPAPIEK | 5282.621 | −2.8 | 881.276 | 6 | 65.32 |
| 16  | LC-126-MCC-LC-183 | TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR | DSTYSLSSTTLSDKADYEK | 6022.015 | 0.9 | 1211.209 | 5 | 90.78 |
| 17  | LC207-MCC-LC-207 | VYACEVTHQGLSPVTFSKFN | VYACEVTHQGLSPVTFSKFN | 4978.429 | 0.7 | 830.578 | 6 | 50.48 |
| 18  | LC207-MCC-HC-221 | VYACEVTHQGLSPVTFSKFN | VEPKSCDK | 3560.719 | 1.1 | 712.950 | 5 | 40.43 |
| 19  | HC-Nterm-MCC-LC-134 | EVQLVESGGGLVQPSGSLR | SGTAHVCLNNFYPR | 3840.930 | −7.6 | 960.988 | 4 | 60.62 |
Trypsin and Asp-N were purchased from Promega Corporation (Madison, WI, USA).

**Sample Preparation for Intact LC/MS.** ADC samples were diluted to a concentration of 4 mg/mL in 100 μL of 1 M tris-HAc buffer (pH 7.8). Then, 2.5 μL of glycerol-free PNGase F (New England BioLabs, Ipswich, MA, USA) solution was added to each sample, followed by incubation at 37 °C for 4 h.

**Intact LC/MS.** Deglycosylated ADC was desalted using a Waters Mass PREPTM Micro Desalting VanGuardTM precolumn (2.1 × 5 mm) on a Waters ACQUITY UPLC H-Class Bio system. The mobile phase consisting of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (ACN) (phase B) was delivered at a flow rate of 0.3 mL/min using a 6 min gradient program from 5% (B) to 100% (B). The column temperature was set to 70 °C. The eluent was diverted into a Waters Xevo G2S Q-TOF mass spectrometer for analysis. The capillary voltage for the Q-TOF was set at 3 kV, and the cone voltage was set at 120 V. The source and desolvation temperatures were 150 and 350 °C, respectively.

The MS spectra were collected at 1 spectrum per second, and the Q-TOF analyzer was set to scan from m/z 200 to 4000. The mass spectra were then deconvoluted and analyzed using the UNIFI Scientific Informatics System (version 1.8) software.

**Drug-to-Antibody Ratio.** The relative MS responses for peaks observed in the resultant deconvoluted spectra were employed for DAR calculations. The first peak in the deconvoluted ADC spectrum was assigned as deconvoluted trastuzumab, D0. The following peaks were assigned as D1-7 based on an averaged mass difference of 957 Da (DM1 with linker). The second series of less abundant peaks observed were separated by an offset of approximately +222 Da. They are unconjugated linkers, assigned as D1+L to D7+L. The average DAR was calculated using eq 1, where i denotes the drug load for each mAb isoform:

$$DAR = \frac{\sum_{i=1}^{m} (\text{peak height of mAb with drug loading } i)}{\sum_{i=1}^{m} (\text{peak height of mAb with drug loading } i \text{ with unconjugated linker})} / \text{(total peak height of all species)}$$

**Sample Preparation for Peptide Mapping.** The ADC samples were denatured in 6 M guanidine chloride, (0.25 M Tris, pH 7.5). The denatured antibody solution was mixed with 500 mM DTT to a final concentration of 10 mM, incubated at 37 °C for 60 min, alkylated by adding 500 mM iodoacetamide stock solution to a final concentration of 20 mM, and incubated at room temperature in the dark for 30 min. Buffer exchange (0.1 M Tris, 2 M urea, pH 7.8) was performed using an NAP-5 column (GE Healthcare, Wilmington, MA, USA). Sequencing grade-modified lys/trypsin was added to each sample (enzyme-to-protein ratio, 1:25, w/w), and the samples were incubated at 37 °C for 4 h. The digested peptide mixture was diluted to 0.45 mM. Leucine enkephalin (LeuEnk, sequence YGGFL) was added to the mixture at the final concentration of 0.05 mM. The injection volume for each LC/MS run was 10 μL.
Peptide Mapping LC/MS. Mobile phase A was prepared with water containing 0.1% formic acid, while mobile phase B was prepared with acetonitrile with 0.1% formic acid. Peptides from protein digests were separated on an ACQUITY UPLC peptide column (2.1 × 100 mm BEH C18 column, 1.7 μm) using a 115 min linear gradient at a flow rate of 0.300 mL/min from 2 to 45% (B). The column temperature was set to 70 °C. For the data acquisition during the peptide analysis, a Xevo G2S Q-TOF mass spectrometer was operated either in MSE or Fast DDA mode. The capillary voltage for the Q-TOF was set at 3 kV, and the cone voltage was set at 60 V. The source and desolvation temperatures were 120 and 400 °C, respectively. The MS spectra were collected at a rate of 1 spectrum per second, and the Q-TOF analyzer was set to scan from m/z 50 to 2000. For the MSE mode, the instrument alternated between low-energy and high-energy scans (0.4 s per scan), which were used to generate intact peptide ions (from low-energy scans) and peptide product ions (from high-energy scans). A collision energy ramp between 25 and 65 V was used for fragmenting peptides in the high-energy scans.

Relative Site Occupancy Calculation. The raw LC/MS data for peptide analysis were processed using the UNIFI Scientific Informatics System (Version 1.8) to generate precursor masses and the associated product ion masses (charge state reduced and de-isotoped) for subsequent protein identification and quantification. The following criteria were used to identify the conjugated peptides during the current analysis: (1) mass accuracy for the matched precursors must be within 5 ppm of mass error; (2) at least three primary fragment ions must be matched for each mass-confirmed peptide; and (3) signature fragments (m/z 547.221) must correspond to the drug payload and are observed for fragmenting peptides. For peptide quantification, extracted ion chromatogram peak areas that correspond to all the charge states along with all the specified adduct ions of each peptide (e.g., sodiated adducts) were combined as a single measure to quantify the abundance of the peptide and its conjugated isomers. All the peak areas were normalized against the peaks of the spiked-in internal standards, and triplet injections were performed for each sample. The relative site occupancy from digestion was calculated as the ratio of the conjugated peptide peak area to the total peptide peak area using eq 2.

\[ \text{relative site occupancy} = \frac{\text{peak area conjugated peptide}}{\text{peak area conjugated peptide} + \text{peak area unconjugated peptide}} \]

Free Drug Analysis. The ADC samples (200 μL each) were treated with 400 μL of cold methanol and incubated for 30 min in an ice bath. The samples were then centrifuged at 13 000 rpm for 10 min, and the supernatant was subjected to further analysis using RP (ZORBAX Eclipse Plus 95 Å C18, 4.6 × 100 mm, 3.5 μm) liquid chromatography. The standard curve was generated using serial dilution of a DM1 standard stock solution. The free DM1 amount was calculated by eq 3 as follows: free DM1 % = moles of free DM1/(moles of ADC × DAR).

Protocol for Conjugation. A solution of SMCC was prepared in DMA at a concentration of approximately 20 mM, and a solution of DM1 was prepared in DMA at a concentration of approximately 10 mM. Trastuzumab at a concentration >12 mg/mL was displaced in buffer A, pH 6.5 (buffer A = 50 mM PB, 50 mM NaCl, 2 mM EDTA, pH 6.5). Based on the estimated reagent scale, DMA was slowly added, followed by a 2- to 10-fold molar excess of the SMCC solution. The conjugation reaction was carried out using approximately 10 mg/mL trastuzumab with 10% DMA in buffer A, pH 6.5, and continued for 2 h. Excess SMCC was removed by gel filtration using a Sephadex G25 desalting resin with buffer A. Under stirring, DMA was slowly added, followed by a 2- to 7-fold molar excess of the DM1 solution, such that the final content of organic cosolvent was 10%. The reaction was allowed to proceed for 3 h, and the product was purified by the G2S desalting resin.

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ABBREVIATIONS
ADC, antibody-drug conjugate; B-lot1-4, 4 batches of samples; CD, circular dichroism; CID, collision-induced dissociation; cIEF, capillary isoelectric focusing; CV, coefficient of variance; DAR, drug to antibody ratio; DM1, maytansinoid, a semi-synthetic cytotoxic payload of the maytansinoid class; DMA, N,N-dimethylacetamide; DSC, differential scanning Calorimetry; HC, Heavy chain; HCD, high-energy collisional dissociation; H-DM1, Herceptin-DM1; HER2, human epidermal growth factor receptor 2; K-lot1-4, 4 batches of Kadcyla, light chain; mAb, monoclonal antibody; MCC, (N-maleimidomethyl) cyclohexane-1-carboxylate; RSD, relative standard deviation; SEC, size-exclusion chromatography; SMCC, succinimidyl 4-((N-maleimidomethyl) cyclohexane-1-carboxylate; Sig P, significance P value; S-HLINK, the highly unconjugated linker sample; T-DM1, trastuzumab-DM1; UV-DAR, ultraviolet detection for drug to antibody ratio

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