Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2022**; 27(8): 234
https://doi.org/10.31083/j.fbl2708234

Original Research

Biological Evaluation of Maytansinoid-Based Site-Specific Antibody-Drug Conjugate Produced by Fully Chemical Conjugation Approach: AJICAP®

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Academic Editor: Graham Pawelec
Submitted: 31 May 2022 Revised: 4 July 2022 Accepted: 21 July 2022 Published: 5 August 2022

Abstract

Background: Trastuzumab-emtansine (T-DM1, commercial name: Kadcyla) is well-known antibody-drug conjugate (ADC) and was first approved for human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer. This molecular format consisting of trastuzumab and maytansinoid payload (emtansine) is very simple, however, T-DM1 has wide heterogeneity due to non-specific conjugation, lowering its therapeutic index (TI). **Methods**: To overcome this issue during the chemical modification of the random conjugation approach to generate T-DM1, we developed a novel chemical conjugation technology termed "AJICAP®" for modification of antibodies in site-specific manner by IgG Fc-affinity peptide based reagents. **Results**: In this study, we compared site-specific maytansinoid-based ADCs synthesized by AJICAP and T-DM1 in rat safety studies. The results indicated an increase in the maximum tolerated dose, demonstrating an expansion of the AJICAP-ADC therapeutic index compared with that of commercially available T-DM1. Gram scale preparation of this AJICAP-ADC and the initial stability study are also described. **Conclusions**: Trastuzumab-AJICAP-maytansinoid produced by this unique chemical conjugation methodology showed higher stability and tolerability than commercially available T-DM1.

Keywords: antibody drug conjugate; maytansinoid; toxicology study; site-specific conjugation; AJICAP

1. Introduction

Trastuzumab-emtansine (T-DM1, commercial name: Kadcyla) is a maytansinoid-based antibody-drug conjugate (ADC), which was first approved for patients with human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer [1]. Since its success in 2013, more than 10 ADCs been introduced into the market [2,3].

ADCs have becoming an innovative cancer medicines that consist of monoclonal antibodies (mAbs) and cytotoxic payloads via chemical linkage. This molecular concept is simple; however, most ADCs currently in the market have wide heterogeneity owing to non-specific conjugation. These nonspecific conjugations are classified into two main categories: using reduced interchain native cysteine and using native lysine. The "interchain-break" cysteine-based methodology is the major manufacturing approach to produce "semi-random" ADC including mainly eight drug-to-antibody ratio (DAR) species (DAR = 0, 2, 4, 6, 8) [4]. This conjugation provides six ADCs (Adcetris, Polivy, Padcev, Blenrep, Zynlonta and Tivdak) approved by U.S. Food and Drug Administration (FDA). Native lysine conjugation is a traditional conjugation technology using activated ester groups (such as NHS) [5]. These high reactive compounds enable to react with exposed lysine residue to form stable covalent bond between antibodies and drug-linkers. This methodology produced three FDA-approved ADCs (Mylotarg, Kadcyla and Besponsa). T-DM1 (commercially name: Kadcyla) is produced by natural conjugation with available lysine residues, thereby resulting in a heterogeneous distribution of high potent payload-linker over multiple ADC sites; this process could lead to a clinically insufficient and narrow therapeutic index (TI) [6].

In addition to limited *in vivo* profile, the heterogeneity of T-DM1 could be problematic from a chemical manufacturing and control point-of-view. Some HPLC analyses, which are the most common approaches for determining the DAR, fail to provide clear chromatographic results [7,8]. To overcome ADC heterogeneity, several site-specific conjugation methods to produce more homogeneous ADCs have been developed [9]. Thus, our research group conceived a proprietary technology utilizing an Fc-affinity peptide, which can install a bi-orthogonal group onto antibodies in a site-selective manner, thereby enabling the site-specific ADCs syntheses (Fig. 1) [10,11].

This platform, termed "AJICAP", has been advantageous in several studies. First, its proof of concept was re-

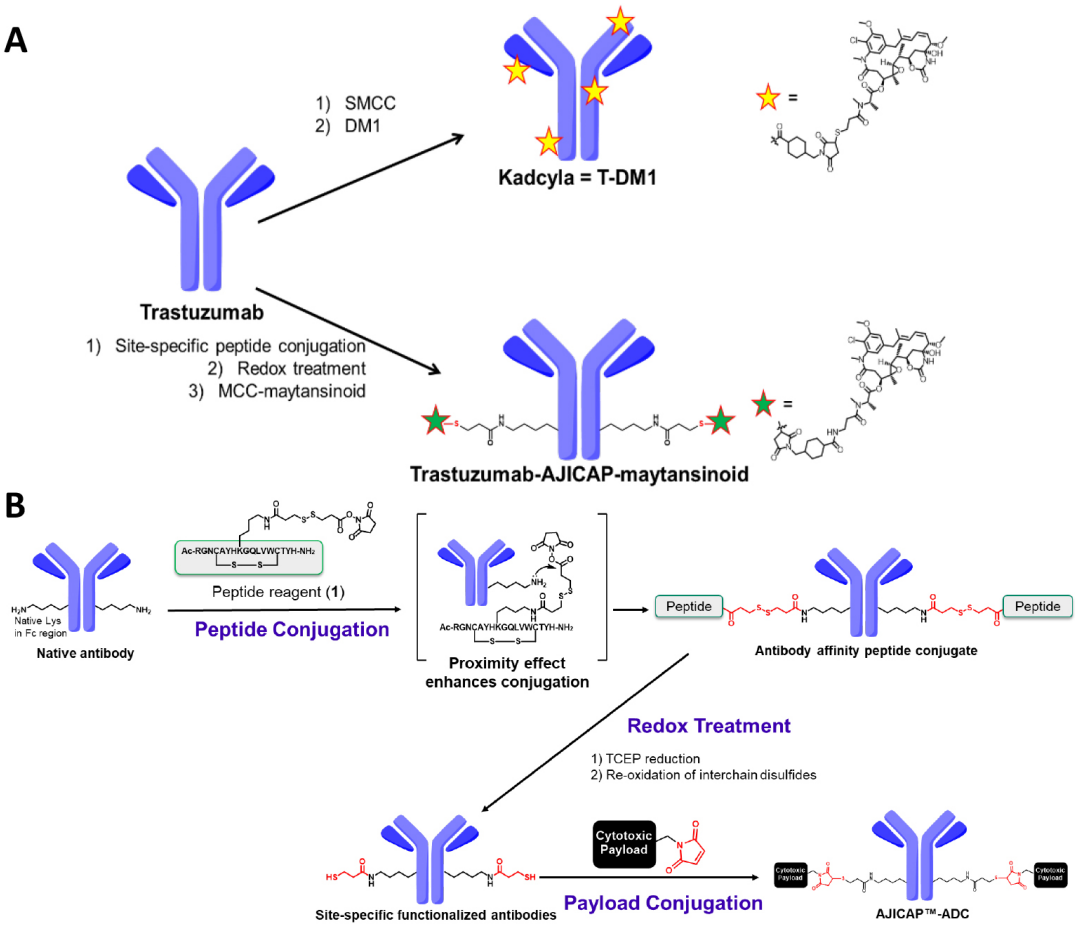


Fig. 1. Summary of the conjugation chemistry for ADC production. (A) *Upper*: Synthesis of T-DM1 by traditional native lysine conjugation; *Lower*: AJICAP site-specific conjugation to synthesize trastuzumab-AJICAP-maytansinoid. T-DMI, Trastuzumab-emtansine; ADC, antibody-drug conjugate. (B) Reaction scheme of AJICAP technology.

ported in 2019, showing highly efficient and versatile conjugation applied to several mAb subtypes such as IgG1, IgG2, and IgG4 [10]. Peptide mapping analysis of the resulting homogeneous ADC confirmed its conjugation site-occupancy at Lys 248 in the antibody Fc region [12]. The larger scale ADC preparation using a glass reactor vial based on the good manufacturing practice strategy indicated the high productivity and robustness of the AJICAP technology [13]. In addition, site-specific AJICAP-ADCs have been used as a demonstration tool to develop novel analytical approaches [14].

In contrast to the numerous synthetic/analytical reports on AJICAP-ADCs, there have been limited studies on their biological profile. Furthermore, no studies have performed a direct comparison between AJICAP-ADC and commercially available ADCs.

In order to provide a TI comparison between AJICAP-ADCs and traditional ADCs in the market, we decided to demonstrate the rat toxicology study of trastuzumab-AJICAP-maytansinoid (DAR = 2) as compared to commercially available T-DM1 (DAR = 3.4). The combination of this safety study and a previously reported efficacy study enables comparison of the TI, thereby resulting in a wide enhancement of TI by AJICAP technology [15]. To supply a sufficient quantity of trastuzumab-AJICAP-maytansinoid, gram-scale ADC preparation was also performed, thus, supporting the robustness of the previously established conjugation process [13]. To adapt the AJICAP method of conjugation, a cysteine-reactive maytansinoid (MCC-maytansinoid, Fig. 1) was synthesized as previously reported [16]. The stability of trastuzumab-AJICAP-maytansinoid was assessed to determine its appropriate storage conditions [17,18]. Overall, the results described herein support the previously reported advantages of AJICAP conjugation platform enabling site-specific ADC production.

2. Materials and Methods

2.1 Materials

Human IgG1 trastuzumab (Herceptin®) was purchased from Roche Pharmaceutical Company (Basel Switzerland). MCC-maytansinoid (catalog no: TCRS-1262) was purchased from Abzena (San Diego, CA, USA).

PNGase F and GlycoBuffer 2 were purchased from New England Biolabs (Ipswich, Massachusetts, UK). All other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 ADC Concentration

The SoloVPE spectroscopy system (C Technologies, Inc. Bridgewater, NJ, USA) provided ADC concentration.

2.3 Synthesis of Thiol-Modified Trastuzumab

Site-specific thiol intermediate was produced by a previously established procedure [13].

2.4 Synthesis of AJICAP-ADC: Payload Conjugation

To a solution of site-specific thiol intermediate (6.8 mg/mL, 1.63 g) in a PBSE buffer (50 mM phosphate buffered saline (PBS), 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4), dimethylformamide (DMF) (15 mL) and a 10-mM DMF solution of MCC-maytansinoid (10 eq., 11.7 mL) were added to form a mixture, which was then incubated at 20 °C. After 2 h, a small amount of the reaction mixture (0.5 mL) was sampled for the in-process control (IPC) analysis. Subsequently, the reaction mixture was quenched with an excess amount of a 50-mM aqueous solution of *N*-acetyl cysteine (NAC) and then incubated at 25 °C for 15 min. This reaction mixture was purified with a tangential flow filtration (TFF) system using a Sartocon Slice 200 Eco Hydrosart membrane (30 kDa; Sartorius) and diafiltration (DF) buffer as the conjugation buffer at an antibody concentration of 20 mg/mL. Next, buffer exchange of this solution was performed using a TFF system with a Sartocon Slice 200 Eco Hydrosart membrane (30 kDa; Sartorius) and DF buffer as the formulation buffer (20 mM histidine containing 5% trehalose, pH 5.2) at an antibody concentration of 5.7 mg/mL. Trastuzumab-AJICAP®-maytansinoid 4 (1.67 g, 98% yield) was then afforded in the formulation buffer.

2.5 Deglycosylated Pre-Treatment for Q-TOF Analysis

Trastuzumab-AJICAP-maytansinoid (50 μL; 1 mg/mL in 50 mM Tris-HCl buffer, pH 7.6), PNGase F (2 μL; 500,000 units/mL), 10 μL of GlycoBuffer 2 (10×), and 48 μL of water were incubated at 37 °C for 16 h. Then 5% v/v aqueous solution of trifluoroacetic acid as quencher was added to this reaction mixture and incubated at 37 °C for 30 min. The buffer exchange and sample concentration were carried out using 25 mM ammonium bicarbonate buffer with centrifugal concentrator (Vivaspin 500, 10,000 MWCO, Polyethersulfone (PES)).

2.6 Q-TOF MS Analysis

Q-TOF MS analysis was performed using a PLRPS 1000 Å 8 μm column (Agilent) connected to an Agilent 1260 HPLC system, as well as with an Agilent 6550 Q-TOF system containing a binary gradient pump, temperature-controlled column compartment, autosampler, and diode array detector. The system ran at 0.3 mL/min at 80 °C using mobile phase A (MPA: 0.1% formic acid (FA), 0.01% TFA, 2% ACN, and 98% water) and mobile phase B (MPB: 0.1% FA, 0.01% TFA, and 99.9% acetonitrile). The absorbance was estimated at 280 nm using a reference wavelength of 450 nm. Intact or deglycosylated ADC (1 mg/mL, 5 μL) was injected into the system and eluted over a 15-min run consisting of a 2-min isocratic hold at 20% MPB, a 15-min linear gradient from 20% to 95% MPB, a 2-min wash using 95% MPB, and a 3-min re-equilibration at 20% MPB. Data acquisition was performed using MassHunter BioConfirm software (Agilent) to analyze the intact MS spectra for intact deconvolution, a mass range of 100,000–180,000 and a limited m/z range of 1000–5000 were set for standardization. The DAR was determined using DAR Calculator software (Agilent).

2.7 Extracted Ion Chromatogram (EIC) Analysis

The EIC analysis was performed using Q-TOF MS system with m/z ratio of 1103.4 (M+).

2.8 Pre-Treatment for RP-HPLC Analysis

The reductive pre-treatment was performed as previously reported [19].

2.9 RP-HPLC Analysis for Reduced ADCs

RP-HPLC analysis was performed according to a previously reported study [19].

2.10 Hydrophobic Interaction Chromatography (HIC)-HPLC Analysis

HIC HPLC analysis was performed according to a previously reported study [20].

2.11 Stability Assessment

Three sets of standard solutions of trastuzumab-AJICAP-maytansinoid (4) were prepared in a formulation buffer. All the samples were stored at various temperatures (–80, –20, 4, 25, and 37 °C) for 4 weeks. Aggregation was then analyzed using size-exclusion chromatography (SEC)-HPLC.

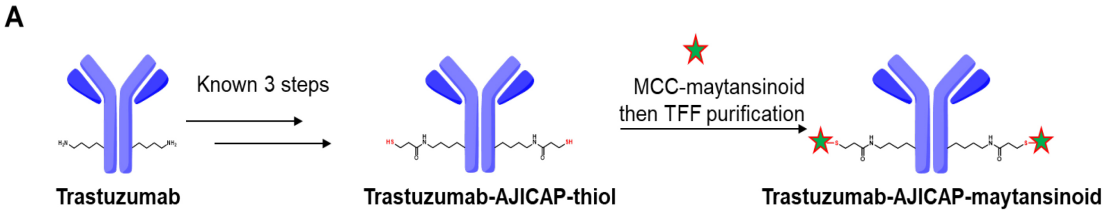
2.12 SEC Analysis of ADCs

Aggregation analysis using SEC was performed as previously reported [20].

2.13 Rat Safety Study

2.13.1 Animal Experiments

Sixty 8-week-old female Sprague-Dawley rats (Charles River Japan, Tokyo, Japan) were provided *ad libitum* access to a standard diet (Oriental Yeast, Tokyo, Japan) and water. Following the acclimatization period (1 week), the animals were stratified by body weight and randomly assigned to the following groups: (1) control



B

DAR by Q-TOF MS (Intact)	1.9
DAR by Q-TOF MS (Deglycosylated)	1.8
DAR by HIC-HPLC	1.9
DAR by RP-HPLC (reduced)	1.8
Aggregation by SEC	4.2%

C

Storage temp.	T-DM1	Trastuzumab-AJICAP-maytansinoid
-80 deg. C	0.9%	4.7%
-20 deg. C	0.9%	4.9%
4 deg. C	1.2%	5.0%
20 deg. C	9.4%	6.6%
37deg. C	29.1%	19.5%

Fig. 2. Synthesis and analysis of trastuzumab-AJICAP-maytansinoid. (A) Synthetic scheme of the gram-scale ADC preparation. (B) Analytical summary of resulting ADC. (C) Aggregation comparison of T-DM1 (literature data) and trastuzumab-AJICAP-maytansinoid (tested data) at different temperatures after 4 weeks of storage. T-DMI, Trastuzumab-emtansine.

group, treated with the vehicle (histidine buffer); (2) two T-DM1 groups, treated with 20 mg/kg and 60 mg/kg; and (3) three trastuzumab-AJICAP-maytansinoid (4) groups, treated with 20 mg/kg, 60 mg/kg, and 120 mg/kg. Each group consisted of five animals for blood chemistry test as well as five animals for clinical signs and body weight measurements. All rats were administered once through intravenous injection via the caudal vein using a butterfly needle, a 30-mL polypropylene syringe, and a syringe pump (Pump 11 Elite, Harvard Apparatus). The experimental procedures were approved by the institutional ethics committee.

2.13.2 Clinical Observations

The animals were observed once daily for any clinical signs. Individual body weights were measured on Days 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19, 20, and 21; the first day of administration was defined as Day 0.

2.13.3 Blood Chemistry Test

Blood chemistry parameters were evaluated 2 d after administration. The animals were fasted overnight and anesthetized with isoflurane prior to collecting blood from the caudal vena cava. Heparin-anticoagulated plasma samples were evaluated using an automated clinical chemistry analyzer (TBA-120FR; Toshiba Medical Systems, Inc., Tokyo, Japan) to determine aspartate (AST) and alanine aminotransferase (ALT) levels. EDTA-anticoagulated blood samples were evaluated using an automated hematology analyzer (ADVIA2120, Siemens Healthineers AG., Munich, Germany) to determine platelet (PLT) counts.

3. Results and Discussions

3.1 Gram-Scale Preparation and Analysis of Trastuzumab-AJICAP-Maytansinoid

Using a well-established previously described approach [13], a site-specific thiol-modified mAb, the intermediate ADC precursor, was prepared from trastuzumab, which is the same antibody as that in T-DM1 upon completion of a gram-scale synthesis (Fig. 2). The final step in ADC preparation is the conjugation of this thiol intermediate with MCC-maytansinoid (Fig. 2A). This reaction completion was detected by IPC analysis using HIC HPLC, as per a previous good laboratory practice (GLP) material preparation [13]. Purification to remove residual drug linkers was performed in a straightforward manner using tangential flow filtration (TFF), which is a commonly used technique that can be applied to kilogram-scale ADC manufacturing [21,22]. Compared to MC-VC-MMAE, which was previously used for preparing AJICAP-ADC, TFF could clear MCC-maytansinoid from the ADC composition. This can be attributed to the hydrophobicity difference of each drug linker. MC-VC-MMAE is relatively more hydrophobic than MCC-maytansinoid [23], thus, requiring careful monitoring of its clearance level. The removal rate of highly potent MCC-maytansinoid drug-linker was calculated by the extracted ion chromatogram (EIC) analysis, which showed that the rate was supposed to be near the detection limit in this analysis (data not shown).

Three different analytical methods were used to determine the DAR of trastuzumab-AJICAP-maytansinoid (Fig. 2B). Q-TOF MS (Intact ADC: **Supplementary Fig. 1**, deglycosylated ADC: **Supplementary Fig. 2**), RP-HPLC (**Supplementary Fig. 3**) and HIC-HPLC (**Supplementary Fig. 4**) analyses revealed that this ADC has a high DAR value (1.8–1.9). The DAR results were slightly dif-

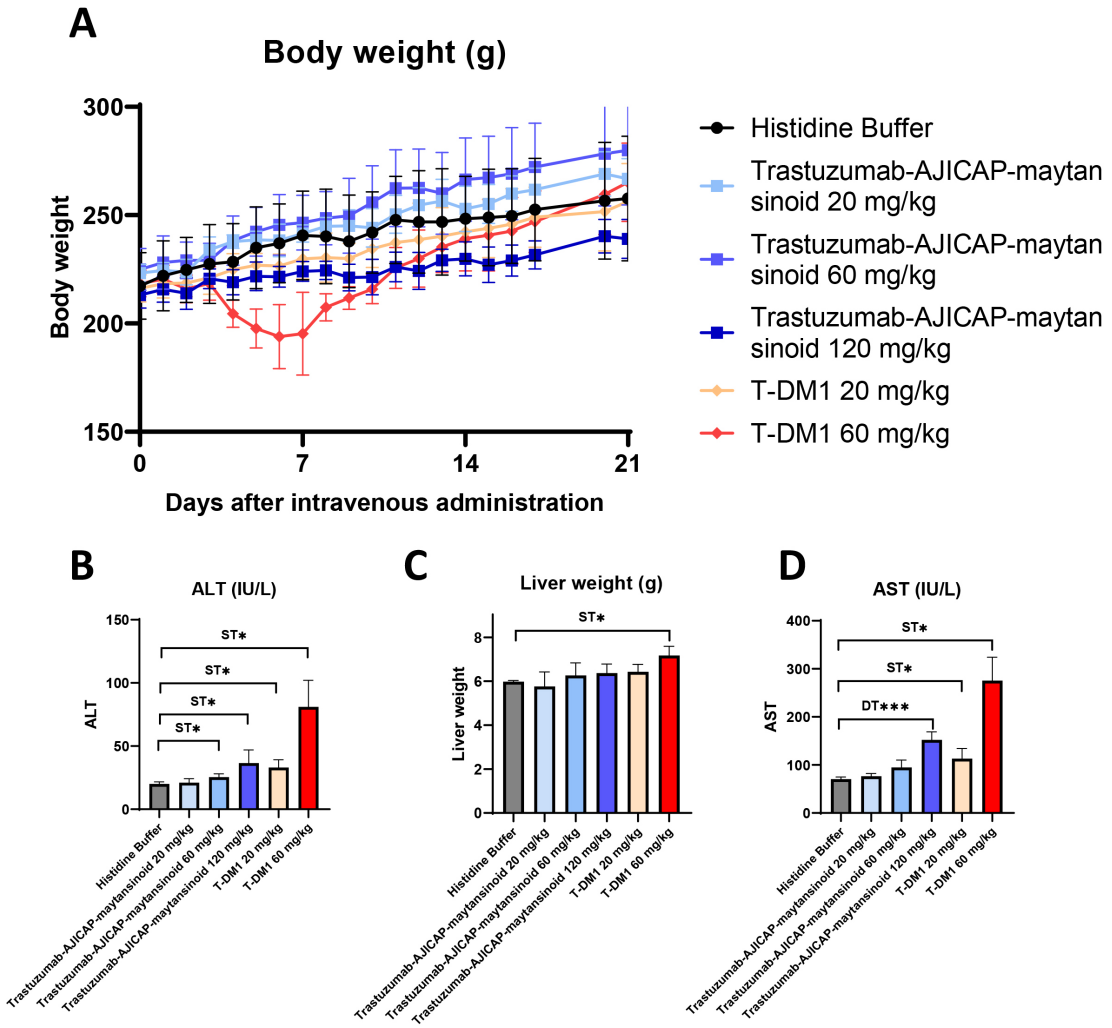


Fig. 3. Rat acute toxicity study to determine the MTD of ADCs. (A) Body weight change (N = 5 female SD rats). (B) Liver weight in rat serum at 2 days after administration (N = 5 female SD rats. (C) AST in rat serum at 2 d after administration (N = 5 female SD rats). (D) ALT levels in rat serum at 2 d after administration (N = 5 female SD rats). MTD, maximum tolerated dose; ADCs, antibody-drug conjugates; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Significantly different from Histidine Buffer: * $p < 0.05$, *** $p < 0.001$. DT, Dunnett test (two-side); ST, Steel test (two-side).

ferent among the three methods; however, this observation was also reported in several previous studies [24–26]. Aggregation percentage was within acceptance level (**Supplementary Fig. 5**).

Next, a thermal stability study of trastuzumab-AJICAP-maytansinoid was examined to estimate the suitable formulation and storage conditions towards forthcoming manufacturing processes (Fig. 2C). Several previous studies, including ours, had reported stability assessments results of T-DM1 in peer-reviewed literature; and we followed the same procedures for one of the processes applied to trastuzumab-AJICAP-maytansinoid [13,16–18].

T-DM1 was found to be relatively stable at lower temperatures (<4 °C) [11,15]. At room temperature or higher, the aggregation percentage increased significantly; however, in the current study, trastuzumab-AJICAP-maytansinoid showed more resistance to high temperature than T-DM1. As described above, T-DM1 has a heterogeneous mixture of possibly more than 80 species [27], some of which may be unstable and prone to aggregation. In contrast, AJICAP-ADC is a low heterogeneous ADC; its conjugation position is known to be stable under thermal/blood circulation. This high stability of trastuzumab-AJICAP-maytansinoid reflects the stable linkage/conjugation site produced by the AJICAP technology.

3.2 Toxicology Study in Rats

A rat acute toxicity study was performed for the MTD determination of the ADCs (Fig. 3). Mortality or severe toxicity was observed with T-DM1 at a dose of 40 mg/kg; however, no toxicity was observed with AJICAP-ADC even at a dose of 120 mg/kg (Fig. 3A). Decreases in body weight or PLT levels and increased AST levels were not observed at doses of up to 120 mg/kg

Table 1. Therapeutic index comparison of T-DM1 and trastuzumab-AJICAP-maytansinoid.

ADC	Minimum effective dose (MED)	Maximum tolerated dose (MTD)
T-DM1 (Kadcyla)	<5 mg/kg	>20 mg/kg
Trastuzumab-AJICAP-maytansinoid	<5 mg/kg	>120 mg/kg

trastuzumab-AJICAP-maytansinoid and 20 mg/kg T-DM1. Additionally, Liver weight did not change significantly at a 120 mg/kg. Thus, the MTD of trastuzumab-AJICAP-maytansinoid and T-DM1 was estimated to be at least 120 and 20 mg/kg, respectively. Neutrophil, reticulocyte, lymphocyte and platelet count data supported these estimations, considering the mode of action of maytansinoids (**Supplementary Fig. 6**) [28,29]. An exploratory rat safety toxicology study showed that the rats showed better tolerance to trastuzumab-AJICAP-maytansinoid than to T-DM1. Given the limited dosing in our study, the MTD of trastuzumab-AJICAP-maytansinoid was estimated to be at least 120 mg/kg, whereas the MTD of T-DM1 was estimated to be at least 20 mg/kg. This initial safety analysis indicated that the conjugation approach generated a stable ADC, although further biological/analytical studies are currently ongoing, such as PK. In previous report [15], a rat PK study showed that the trastuzumab-AJICAP-MMAE ADC is stable in blood circulation compared to native cysteine-based stochastic ADC. The measured levels of total antibody from trastuzumab-AJICAP-MMAE indicated a half-life similar to that expected for parent trastuzumab. Furthermore, *in vitro* binding assay using biolayer interferometry method concluded that trastuzumab-AJICAP-maytansinoid does not affect FcRn interactions [30]. These results suggest that site-specific conjugated ADCs at Lys248 result in a superior profile with regard to both safety and *in vivo* stability compared to the approved ADC, T-DM1.

Furthermore, in a previous study, we reported that the minimum effective dose (MED) of both ADCs was 5 mg/kg based on the NCI-N87 xenograft study. Thus, the TI of trastuzumab-AJICAP-maytansinoid was estimated to be qualitatively greater than that of T-DM1, considering their respective MTDs and MEDs (Table 1).

4. Conclusions

In conclusion, we applied a full chemical conjugation technology, AJICAP, to produce a maytansinoid-based site-specific ADC. The scale-down manufacturing process using TFF purification effectively removed the residual drug linkers from the final ADC composition, thus, producing DAR of nearly 2.0 ADCs without including a significant level of aggregates. The resulting site-specific ADCs showed sufficient long-term stability under different storage conditions.

Furthermore, the comparative toxicology study between trastuzumab-AJICAP-maytansinoid and commercially available T-DM1 indicated that ADCs produced by this unique chemical conjugation methodology enables therapeutic index expansion. Therefore, this platform conjugation technology has a strong potential to produce next-generation ADCs with high homogeneity and significant *in vivo* stability.

Abbreviations

T-DMI, Trastuzumab-emtansine; ADCs, antibody-drug conjugates; HER2, human epidermal growth factor receptor 2; TI, therapeutic index; DAR, drug-to-antibody ratio; DMF, dimethylformamide; MPA, mobile phase A; MPB, mobile phase B; FA, formic acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet; TFF, tangential flow filtration; EIC, extracted ion chromatogram; Q-TOF MS, Quadrupole time-of-flight mass spectrometry; MED, minimum effective dose; MTD, maximum tolerated dose.

Author Contributions

TS—Animal study, Validation, Formal analysis, Data curation and Writing—original draft preparation. KY—Conceptualization and ADC preparation. YO—Animal study, Formal analysis, Data curation. TF—Resource, Stability assessment and ADC preparation. TN, AN and YK—Methodology and Validation. BAM—Supervision, Writing—review and editing. YM—Conceptualization, Resources, ADC preparation, Writing—original draft preparation, review and editing. TO—Conceptualization, Funding acquisition, Supervision, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

The authors wish to express the gratitude to colleagues from Ajinomoto Co., Inc., as follows: Natsuki Shkida, Reiko Yuji, Kazutoshi Takahashi, Kunio Nakata and Kazutaka Shimbo for useful opinions on ADC analysis; Akira Chiba for support and encouragement during manuscript preparation.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest. YM and BAM are serving as the Guest editors of this journal. We

declare that YM and BAM had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to GP.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2708234.

References

[1] LoRusso PM, Weiss D, Guardino E, Girish S, Sliwkowski MX. Trastuzumab Emtansine: A Unique Antibody-Drug Conjugate in Development for Human Epidermal Growth Factor Receptor 2–Positive Cancer. Clinical Cancer Research. 2011; 17: 6437–6447.

[2] De Cecco M, Galbraith DN, McDermott LL. What makes a good antibody–drug conjugate? Expert Opinion on Biological Therapy. 2021; 21: 841–847.

[3] Sheyi R, Albericio F. Linkers: An assurance for controlled delivery of antibody-drug conjugate. Pharmaceutics. 2022; 14: 396.

[4] Doronina SO, Toki BE, Torgov MY. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. Nature Biotechnology. 2003; 21: 778–84.

[5] Kato K, Hamaguchi Y, Fukui H. Development of the Procedure for Enzyme-Labelling and Solid phases in Enzymoimmunoassay. Rinsho Kagaku Shinpojiumu. 1977; 16: 10–14.

[6] Matsuda Y, Mendelsohn BA. An overview of process development for antibody-drug conjugates produced by chemical conjugation technology. Expert Opinion on Biological Therapy. 2021; 21: 963–975.

[7] Matsuda Y, Mendelsohn BA. Recent Advances in Drug–Antibody Ratio Determination of Antibody–Drug Conjugates. Chemical and Pharmaceutical Bulletin. 2021; 69: 976–983.

[8] Matsuda Y, Leung M, Tawfiq Z, Fujii T, Mendelsohn BA. In-situ Reverse Phased HPLC analysis of intact antibody-drug conjugates. Analytical Sciences. 2021; 37: 1171–1176.

[9] Wu K, Yu C, Lee C, Zuo C, Ball ZT, Xiao H. Precision Modification of Native Antibodies. Bioconjugate Chemistry. 2021; 32: 1947–1959.

[10] Yamada K, Shikida N, Shimbo K, Ito Y, Khedri Z, Matsuda Y, *et al*. AJICAP: Affinity Peptide Mediated Regiodivergent Functionalization of Native Antibodies. Angewandte Chemie International Edition. 2019; 58: 5592–5597.

[11] Matsuda Y, Leung M, Okuzumi T, Mendelsohn B. A purification strategy utilizing hydrophobic interaction chromatography to obtain homogeneous species from a site-specific antibody drug conjugate produced by AJICAP first generation. Antibodies (Basel). 2020; 9: 16.

[12] Matsuda Y, Malinao M, Robles V, Song J, Yamada K, Mendelsohn BA. Proof of site-specificity of antibody-drug conjugates produced by chemical conjugation technology: AJICAP first generation. Journal of Chromatography B. 2020; 1140: 121981.

[13] Matsuda Y, Clancy C, Tawfiq Z, Robles V, Mendelsohn BA. Good Manufacturing Practice Strategy for Antibody–Drug Conjugate Synthesis Using Site-Specific Chemical Conjugation: first-Generation AJICAP. ACS Omega. 2019; 4: 20564–20570.

[14] Matsuda Y, Kliman M, Mendelsohn BA. Application of Native Ion Exchange Mass Spectrometry to Intact and Subunit Analysis of Site-Specific Antibody–Drug Conjugates Produced by AJICAP first Generation Technology. Journal of the American Society for Mass Spectrometry. 2020; 31: 1706–1712.

[15] Matsuda Y, Seki T, Yamada K, Ooba Y, Takahashi K, Fujii T, *et al*. Chemical Site-Specific Conjugation Platform to Improve the Pharmacokinetics and Therapeutic Index of Antibody–Drug Conjugates. Molecular Pharmaceutics. 2021; 18: 4058–4066.

[16] Fujii T, Reiling C, Quinn C, Kliman M, Mendelsohn BA, Matsuda Y. Physical characteristics comparison between maytansinoid-based and auristatin-based antibody-drug conjugates. Exploration of Targeted Anti-Tumor Therapy. 2021; 2: 576–585.

[17] Mohamed HE, Mohamed AA, Al-Ghobashy MA, Fathalla FA, Abbas SS. Stability assessment of antibody-drug conjugate Trastuzumab emtansine in comparison to parent monoclonal antibody using orthogonal testing protocol. Journal of Pharmaceutical and Biomedical Analysis. 2018; 150: 268–277.

[18] Duerr C, Friess W. Antibody-drug conjugates- stability and formulation. European Journal of Pharmaceutics and Biopharmaceutics. 2019; 139: 168–176.

[19] Matsuda Y, Tawfiq Z, Leung M, Mendelsohn BA. Insight into Temperature Dependency and Design of Experiments towards Process Development for Cysteine-Based Antibody-Drug Conjugates. ChemistrySelect. 2020; 5: 8435–8439.

[20] Tawfiq Z, Matsuda Y, Alfonso MJ, Clancy C, Robles V, Leung M, *et al*. Analytical Comparison of Antibody-drug Conjugates Based on Good Manufacturing Practice Strategies. Analytical Sciences. 2020; 36: 871–875.

[21] Jackson DY. Processes for Constructing Homogeneous Antibody Drug Conjugates. Organic Process Research and Development. 2016; 20: 852–866.

[22] Matsuda Y. Current approaches for the purification of antibody–drug conjugates. Journal of Separation Science. 2022; 45: 27–37.

[23] Nadkarni DV, Jiang Q, Friese O, Bazhina N, Meng H, Guo J, *et al*. Process Development and Structural Characterization of an Anti-Notch 3 Antibody–Drug Conjugate. Organic Process Research and Development. 2018; 22: 286–295.

[24] Xu Y, Jiang G, Tran C, Li X, Heibeck TH, Masikat MR, *et al*. RP-HPLC DAR Characterization of Site-Specific Antibody Drug Conjugates Produced in a Cell-Free Expression System. Organic Process Research and Development. 2016; 20: 1034–1043.

[25] Källsten M, Hartmann R, Artemenko K, Lind SB, Lehmann F, Bergquist J. Qualitative analysis of antibody–drug conjugates (ADCs): an experimental comparison of analytical techniques of cysteine-linked ADCs. The Analyst. 2018; 143: 5487–5496.

[26] F. Debaene, A. Boeuf, E. Wagner-Rousset, O. Colas, D. Ayoub, N. Corvaïa, A. Van Dorsselaer, A. Beck and S. Cianférani, Analytical Chemistry. 2014; 86: 10674–10683.

[27] Yamazaki S, Shikida N, Takahashi K, Matsuda Y, Inoue K, Shimbo K, *et al*. Lipoate-acid ligase a modification of native antibody: Synthesis and conjugation site analysis. Bioorganic and Medicinal Chemistry Letters. 2021; 51: 128360.

[28] Poon KA, Flagella K, Beyer J, Tibbitts J, Kaur S, Saad O, *et al*. Preclinical safety profile of trastuzumab emtansine (T-DM1): Mechanism of action of its cytotoxic component retained with improved tolerability. Toxicology and Applied Pharmacology. 2013; 273: 298–313.

[29] Lambert JM, Chari RVJ. Ado-trastuzumab Emtansine (T-DM1): an Antibody–Drug Conjugate (ADC) for her2-Positive Breast Cancer. Journal of Medicinal Chemistry. 2014; 57: 6949–6964.

[30] Matsuda Y, Chakrabarti A, Takahashi K, Yamada K, Nakata K, Okuzumi T, *et al*. Chromatographic analysis of site-specific antibody-drug conjugates produced by AJICAP first-generation technology using a recombinant FcγIIIa receptor-ligand affinity column. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences. 2021; 1177: 122753.