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Article

Chemical Site-Specific Conjugation Platform to Improve the Pharmacokinetics and Therapeutic Index of Antibody–Drug Conjugates

Yutaka Matsuda,[§] Takuya Seki,[§] Kei Yamada, Yuri Ooba, Kazutoshi Takahashi, Tomohiro Fujii, Sayaka Kawaguchi, Takahiro Narita, Akira Nakayama, Yoshiro Kitahara, Brian A. Mendelsohn,* and Tatsuya Okuzumi*

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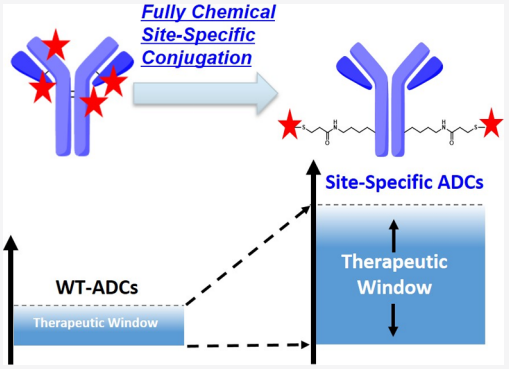
ABSTRACT: To overcome a lack of selectivity during the chemical modification of native non-engineered antibodies, we have developed a technology platform termed "AJICAP" for the site-specific chemical conjugation of antibodies through the use of a class of IgG Fc-affinity reagents. To date, a limited number of antibody–drug conjugates (ADCs) have been synthesized via this approach, and no toxicological study was reported. Herein, we describe the compatibility and robustness of AJICAP technology, which enabled the synthesis of a wide variety of ADCs. A stability assessment of a thiol-modified antibody synthesized by AJICAP technology indicated no appreciable increase in aggregation or decomposition upon prolonged storage, indicating that the unexpectedly stable thiol intermediate has a great potential intermediate for payload or linker screening or large-scale manufacturing. Payload conjugation with this stable thiol intermediate generated several AJICAP-ADCs. *In vivo* xenograft studies indicated that the AJICAP-ADCs displayed significant tumor inhibition comparable to benchmark ADC Kadcyla. Furthermore, a rat pharmacokinetic analysis and toxicology study indicated an increase in the maximum tolerated dose, demonstrating an expansion of the AJICAP-ADC therapeutic index, compared with stochastic conjugation technology. This is the first report of the therapeutic index estimation of site-specific ADCs produced by utilizing Fc affinity reagent conjugation. The described site-specific conjugation technology is a powerful platform to enable next-generation ADCs through reduced heterogeneity and enhanced therapeutic index.



KEYWORDS: *antibody–drug conjugate, ADC, site-specific conjugation, therapeutic index, chemical conjugation, AJICAP*

INTRODUCTION

More than a century ago, the German scientist Paul Ehrlich proposed the idea of a "magic bullet" drug that targets invading cells.[1] Over the last two decades, antibody–drug conjugates (ADCs) have become a potential embodiment of Ehrlich's notion. ADCs are promising cancer therapeutics consisting of recombinant monoclonal antibodies.[2−4] Although this concept may seem simple, there is a critical issue with the synthesis of traditional ADCs. Non-specific drug conjugation on naturally available amino acid residues, such as lysine and reduced interchain cysteine residues, is used to construct the chemical links between the antibodies and payloads. This stochastic conjugation results in a heterogeneous distribution of cytotoxic drugs over multiple sites on the ADCs and may lead to a clinically insufficient and narrow therapeutic index (TI).[5]

With the goal of overcoming the potentially negative impact of non-specific conjugation on the clinical use and efficacy of ADCs, some effective site-specific conjugation methods have been developed.[6] A common strategy is the engineering of a specific amino acid (or peptide moiety) into antibodies followed by selective modification using bioorthogonal chemistry for conjugation with payloads of interest. However, this protein engineering process can be costly and time consuming due to the antibody optimization required.[7]

Some enzymatic approaches have also been developed to create ADCs from antibodies and have been used to introduce biologically orthogonal functional groups at the appropriate site(s) to antibodies both with and without the need for genetic modification or the introduction of sequence tags in the antibody.[8] For example, azido-labeled antibodies have been obtained by enzymatically modifying the glycan present at

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Asn297 of the antibody Fc region to which appropriate linkers can be covalently attached via dipolar cycloaddition reactions.[9] However, there are two barriers that must be overcome to develop ADCs employing enzymatic conjugation methods: first, the establishment of large-scale enzyme production required under strict regulatory controls such as good manufacturing practice (GMP), and second, the potentially difficult optimization of the removal of the enzyme from the conjugation reaction matrix. The industry is breaking through these barriers as enzyme-produced ADCs progress through clinical development. For example, the transglutaminase enzyme can be used to conjugate antibodies to drug-linkers, and a description of the large-scale enzyme manufacturing from *E. coli* was reported.[10]

Chemical site-specific conjugation methods have emerged as an alternate method that requires neither antibody modifications through protein engineering nor complicated process development.[11] Daiichi Sankyo and AstraZeneca have launched the first site-specific ADCs onto the commercial market, which are conjugated by saturation of the reduced interchain cysteine residues using a highly loaded drug methodology.[12] While this approach using fully reduced antibodies is a solution to obtain ADCs with a nearly homogeneous drug antibody ratio (DAR), it may be of limited utility due to the requirements of their specific drug-linkers. Many drug-linkers applied in commercial and clinical ADCs are highly hydrophobic (and potent) and therefore may not be suitable for use in a high DAR ADC format.[13]

There are reports in the literature of alternative conjugation approaches employing affinity labeling, which leverage a proximity effect between an affinity compound and the antibody, usually involving ultraviolet light to initiate a covalent chemical reaction. This strategy has been applied for site-specific antibody modifications; however, there are no reports in the chemical literature describing this method for a late-stage pre-clinical or clinical stage.[11]

From these limitations of site-specific chemical conjugation, we conceived a unique strategy to use an Fc-affinity peptide for the site-specific functionalization of the biorthogonal group into antibodies. This platform, named "AJICAP", has been previously described to enable the highly efficient and versatile conjugation of IgG1, IgG2, and IgG4 antibodies.[14] The site-specificity of the resulting ADC was confirmed through peptide mapping analyses, which confirmed the desired conjugation site at Lys248 located in the antibody Fc region.[15] The scalability of AJICAP technology was confirmed by gram-scale ADC synthesis based on a GMP strategy, demonstrating that this technology is suitable for scale-up ADC manufacturing.[16,17] However, limited ADCs have been synthesized by this conjugation technology, and neither detailed analyses of the intermediate stability nor toxicology studies using animals have been reported.

Herein, we report a full investigation into the capability, robustness, and biological activity of AJICAP technology. A long-term stability assessment of site-specific thiol compounds, the precursor for AJICAP-ADCs, indicated that the thiol intermediate produced by AJICAP technology is stable for more than 1 year of storage and retained reactivity useful for the synthesis of a variety of site-specific ADCs during this time. To evaluate the drug-linker compatibility, over multiple AJICAP-ADC syntheses were performed. *In vitro* potency and *in vivo* xenograft efficacy in the HER2 + NCI-N87 model were evaluated for each AJICAP-ADC to determine the minimum effective dose (MED). A safety study in rats was also conducted to determine the maximum tolerated dose (MTD), resulting in the calculation of the TI of the AJICAP-ADCs, which was then compared to that of a traditional partial reduction cysteine-based ADC. These biological studies indicated that AJICAP-ADCs may afford an enhancement of TI over ADCs constructed via traditional cysteine conjugation technology. We conducted a pharmacokinetic (PK) experiment in rats with an AJICAP-conjugated ADC to compare the overall clearance and detected deconjugation to that of a stochastically conjugated ADC. The results of this PK study lend credence to the notion that, *ceteris paribus*, ADCs conjugated via this site-specific conjugation may afford an enhanced TI. These overall results reported herein have demonstrated that this chemical site-specific conjugation technology is a powerful platform to enable next-generation ADCs through the reduction of heterogeneity and enhancement of the TI.

MATERIALS AND METHODS

Materials. Human IgG1 trastuzumab (Herceptin) and rituximab (Rituxan) were purchased from Roche Pharmaceutical Company (Switzerland). Human IgG1 infliximab (Humira) was purchased from Sigma-Aldrich (USA). MCC-maytansinoid (Catalog No: TCRS-1262), MC-MMAF (CAS#: 863971-19-1), and MC-VA-PBD (CAS#: 1342820-51-2) were purchased from Abzena (USA). MC-VC-MMAE (CAS#: 646502-53-6) was purchased from NJ Biopharmaceuticals LLC (USA). The peptide reagent (**1**) was prepared as previously reported.[14] All other chemical reagents were acquired from Sigma-Aldrich (USA). All cell lines (HCC-1954, NCI-N87, PC-3, and SU-DUL-4 cancer cell lines) were purchased from ATCC (USA).

Instruments and Analytical Methods. ADC concentration and recovery were checked by the Slope Spectroscopy method with a Solo-VPE system.[14]

Q-TOF MS analysis was performed as previously reported.[14]

SEC-HPLC analysis was performed as previously reported.[17]

ADC Synthesis. Site-specific ADCs were synthesized according to our previous procedure.[14]

Stability Assessment. Three sets of standard solutions of trastuzumab-thiol (**3A**) were prepared in storage buffer (50 mM PBS, 10 mM EDTA, pH 7.4, 5 mg/mL). All the samples were stored at −80 °C for a period of 365 days. Analysis of the aggregation was conducted by SEC.

Molecular Modeling. The model structure of the Fc-AJICAP peptide was generated using the Protein Preparation module facilitated by Maestro Suite.[18,19] Based on the crystal structure of Fc-peptide (PDB: 6IQG),[18] Arg was mutated to Lys and then energy minimized.

***In Vitro* Cell-Based Assay.** Trastuzumab-derived ADCs (**4A**-**4D**) and trastuzumab were analyzed using the PC3, HCC-1954, and NCI-N87 cancer cell lines. Rituximab-derived ADCs (**5A**-**5D**) and trastuzumab were analyzed using the SU-DUL-4 and NCI-N87 cancer cell lines. Cells were seeded in clear, tissue culture-treated 96-well plates at approximately 2500−5000 cells per well in 100 μL of growth media (RPMI-1640 + 10% heat-inactivated fetal bovine serum) and incubated for 24 h in a humidified incubator, at 37 °C with 5% CO_2, to allow the cells to attach. Stock solutions of ADCs in 20 mM histidine buffer were diluted with growth media (RPMI-1640 + 10% heat-inactivated fetal bovine serum). The next day, 100 μL of ADCs at varying concentrations was added to each well in

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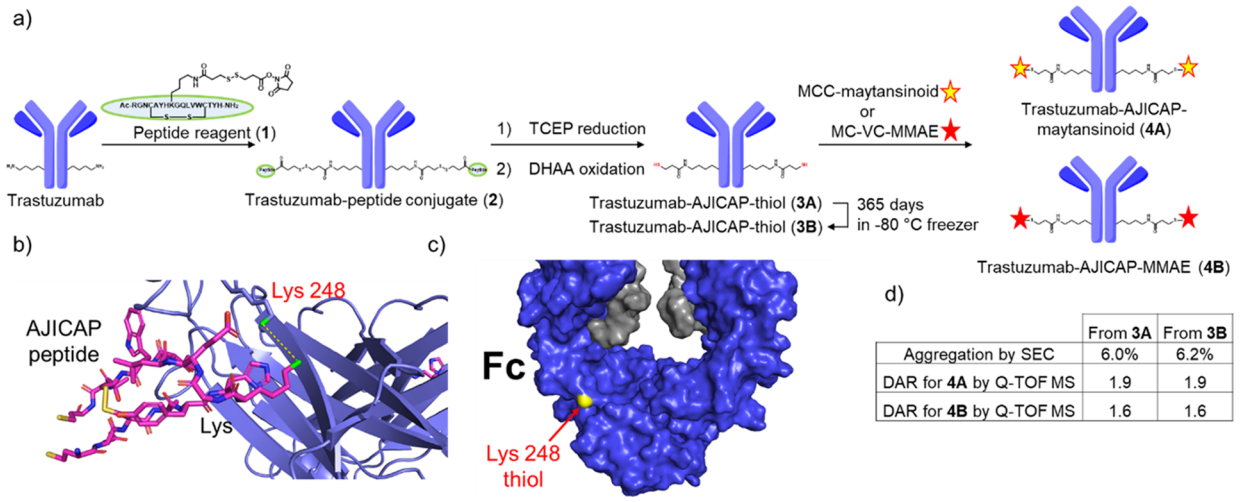

	From 3A	From 3B
Aggregation by SEC	6.0%	6.2%
DAR for 4A by Q-TOF MS	1.9	1.9
DAR for 4B by Q-TOF MS	1.6	1.6

Figure 1. Long-term stability assessment of the AJICAP-thiol intermediate. (a) Overview of the synthesis of the thiol intermediate. (b) Interaction between human IgG1 Fc and the peptide reagent (PDB: 6IQG). (c) Molecular modeling of the thiol intermediate. (d) Analytical comparison before and after storage for 365 days.

triplicate. The plates were incubated in a humidified tissue culture incubator, with 5% CO_2 at 37 °C for 5−8 days after the addition of ADCs, to measure cytotoxicity. After 5−8 days, 20 μL of premix WST-1 reagent (TaKaRa Bio) was added to each well. Plates were then incubated at 37 °C for 1−6 h. After the incubation period, the cell viability was measured using a plate reader (PerkinElmer) at 450 nm. The IC_{50} values were calculated from the cell viability data using Graph-Pad Prism Software. Data for all ADCs tested in this assay were graphed as percent survival compared with untreated control wells.

***In Vivo* Xenograft Study.** *Cells.* NCI-N87 cells (Cat #CRL-5822) were purchased through ATCC. The cells were cultured in RPMI supplemented with 10% FBS and 1% P/S in a humidified incubator at 37 °C and 5% CO_2.

Animals. NOD.CB17 homozygous mice were procured through Envigo. Mice were fed a Teklad irradiated (sterilized) mouse diet and bedded with Teklad irradiated (sterilized) corncob bedding from Envigo (Indianapolis, IN). Mice were housed in Optimice carousel sterile quarters with filtered air supply in disposable cages from Animal Care Systems, Inc. (Centennial, CO).

Implantation. On the day of implantation, the cells were trypsinized and allowed to detach from flasks. Trypsin was then neutralized with complete media, and the cells were spun at 400*g*. Media were aspirated, and the cells were resuspended in 50:50 (Cultrex: RPMI) at a concentration of 5 × 107 cells/mL. A volume of 100 μL was injected into the right hind flank of each animal (a total of 5 × 106 cells).

Study Arms and Treatments for NCI-N87. The tumor volumes were monitored, and on the first day (when mean tumor volume reached ∼120 mm^3), mice were stratified and placed into 7 or 5 treatment groups of (10) mice as outlined in Tables S1 and S2.

Rat PK Study. *Animal Experiments.* Eight 8-week-old female Sprague−Dawley rats (Charles River Japan, Tokyo, Japan) were provided with a standard diet (Oriental Yeast, Tokyo, Japan) and water ad libitum. Following the acclimatization period (1 week), the animals were stratified by body weight and randomly assigned as the AJICAP-ADC groups that were treated with 5 mg/kg and the traditional ADC group that were treated with 5 mg/kg. Each group consisted of four animals for pharmacokinetics analysis. All rats were administered once via the caudal vein with a butterfly needle for intravenous injection, a 30 mL polypropylene syringe, and a syringe pump (Pump 11 Elite, Harvard Apparatus). The experimental procedure was approved by the institutional ethics committee.

Clinical Observations. The animals were observed once daily for clinical signs. Individual body weights were measured on days 0, 7, 14, and 21, with the first day of administration defined as day 0.

Pharmacokinetics Analysis. The blood samples were collected on eight time points (immediately after administration, 6 h after administration, and 1, 2, 3, 7, 14, and 21 h after administration) via the caudal vein. The concentrations of ADC or total antibody were measured by a double sandwich ELISA method.

Rat Safety Study. *Animal Experiments.* Sixty 8-week-old female Sprague−Dawley rats (Charles River Japan, Tokyo, Japan) were provided with a standard diet (Oriental Yeast, Tokyo, Japan) and water ad libitum. Following the acclimatization period (1 week), the animals were stratified by body weight and randomly assigned as the control group that were treated with the vehicle (histidine buffer), the three trastuzumab-stochastic-MMAE(6) groups that were treated with 40, 80, and 120 mg/kg, and the two trastuzumab-AJICAP-MMAE(4B) group that were treated with 10 and 40 mg/kg. Each group consisted of five animals for the blood chemistry test and five animals for clinical signs and body weight measurement. All rats were administered once via the caudal vein with a butterfly needle for intravenous injection, a 30 mL polypropylene syringe, and a syringe pump (Pump 11 Elite, Harvard Apparatus). The experimental procedure was approved by the institutional ethics committee.

Clinical Observations. The animals were observed once daily for clinical signs. Individual body weights were measured on days 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 18, 19, and 20, with the first day of administration defined as day 0.

Blood Chemistry Test. Blood chemistry parameters were evaluated on 2 days after administration. The animals were fasted overnight and then anesthetized with isoflurane before blood was collected from the caudal vena cava. Heparin-

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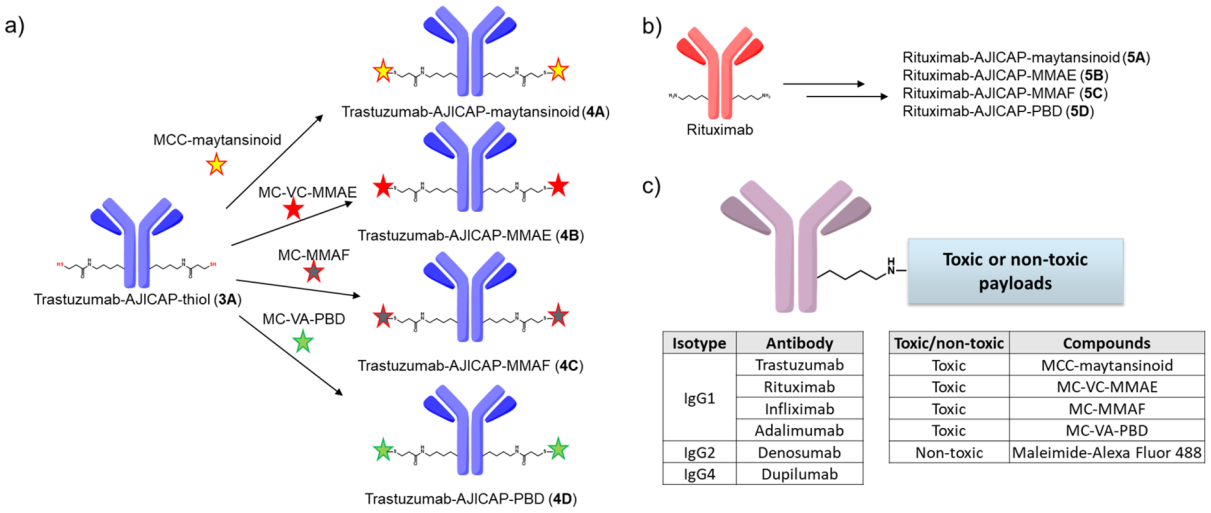

Isotype	Antibody
IgG1	Trastuzumab
	Rituximab
	Infliximab
	Adalimumab
IgG2	Denosumab
IgG4	Dupilumab

Toxic/non-toxic	Compounds
Toxic	MCC-maytansinoid
Toxic	MC-VC-MMAE
Toxic	MC-MMAF
Toxic	MC-VA-PBD
Non-toxic	Maleimide-Alexa Fluor 488

d)

	4A	4B	4C	4D	5A	5B	5C	5D
Antibody	Trastuzumab				Rituximab			
Payload	maytansinoid	MMAE	MMAF	PBD	maytansinoid	MMAE	MMAF	PBD
DAR by Q-TOF MS	1.9	1.6	1.8	1.6	1.9	1.6	1.8	1.5
Aggregation by SEC	4.2%	6.4%	5.5%	7.7%	8.7%	8.7%	8.9%	10.4%

Figure 2. Capability of AJICAP technology. (a) Syntheses of four different ADCs derived from trastuzumab. (b) Syntheses of four different ADCs derived from rituximab. (c) Compatibility with various antibodies and toxic/non-toxic payloads. (d) Summary of DAR analysis and aggregation after conjugation with various payloads.

anticoagulated plasma samples were evaluated with an automated clinical chemistry analyzer (TBA-120FR, Toshiba Medical Systems, Inc., Tokyo, Japan) to determine aspartate aminotransferase (AST). EDTA-anticoagulated blood samples were evaluated with an automated hematology analyzer (ADVIA2120, Siemens Healthineers AG., Munich, Germany) to determine the platelet count (PLT).

RESULTS

Stability Assessment of the AJICAP-Thiol Intermediate. Crucial to the establishment of this conjugation technology's breadth and robustness, the long-term stability of the ADC intermediate precursor containing site-specific free-sulfhydryls was evaluated (Figure 1). As previously reported,[14] the peptide reagent (**1**) was reacted with trastuzumab, a commonly used antibody for conjugation studies, to afford trastuzumab-peptide conjugates (**2**). Our group has already reported the completion of a gram-scale synthesis utilizing this well-established approach.[17] The proximity effect of the peptide reagent assists this conjugation to proceed in a site-specific manner due to the high affinity of the peptide toward the Fc region (Figure 1b).[18] Removal of the peptide portion of (**1**) via tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP)-mediated reduction and concomitant antibody interchain disulfide bond cleavage followed by oxidation using dehydroascorbic acid (DHAA) under mild conditions to reform the reduced interchain disulfide bridges provided the site-specifically thiol-modified trastuzumab (**3A**). All purification steps to remove impurities, including peptide-related fragments, were carried out straightforwardly utilizing tangential flow filtration, a well-known technique that enables facile scale-up and future translation to kilogram-scale ADC preparations in a manufacturing facility.[20] To examine the reactivity of the installed thiol groups, drug-linkers MCC-maytansinoid and MC-VC-MMAE (their chemical structures described in Figure S1 in the Supporting Information) were reacted with **3A** to produce two ADCs, and the respective DARs were determined by quadrupole time-of-flight mass spectrometry (Q-TOF MS). Next, an investigation of the effect of long-term storage on **3A** was conducted. Compound **3A** was stored as a frozen matrix in a −80 °C freezer for 1 year, and then the same material lots, before and after 365 days of storage, were evaluated by size exclusion chromatography (SEC) to determine the aggregation status. Conjugation efficacy of the "1-year-old" thiol-mAb (**3B**) was also confirmed by reaction to the drug-linkers to produce ADCs. Surprisingly, there was no appreciable difference between the antibodies before and after 1 year of storage in both investigations (Figures S3 and S4). An *in silico* molecular modeling study (Figure 1c and Figure S2) revealed that the thiol group in **3A** was moderately exposed.[19]

Capability of AJICAP Technology. Next, the breadth of compatibility of AJICAP technology was confirmed through 10 different ADC syntheses (Figure 2 and Figures S5−S14) including a pyrrolobenzodiazepine dimer (PBD) payload. In addition to previously reported ADC syntheses [trastuzumab-AJICAP-maytansinoid (**4A**) and trastuzumab-AJICAP MMAE (**4B**)],[14,21] MC-MMAF (for trastuzumab-AJICAP-MMAF (**4C**)) and MC-VA-PBD (for trastuzumab-AJICAP-PBD (**4D**)) (the chemical structures of these payloads are described in Figure S1) were reacted with the trastuzumab-thiol (**3A**). Conjugation with a hydrophobic PBD drug-linker required the use of propylene glycol as a co-solvent as Jeffery et al. have reported.[22] Rituximab, an antibody reported to be less stable than trastuzumab,[23] was labeled using our standard platform conjugation conditions to successfully produce ADCs **5A**−**5D** obviating protocol optimizations or modifications. To date, dozens of different antibodies among IgG1, IgG2, and IgG4 isotypes have demonstrated compatibility with this site-specific conjugation protocol, including previous published reports:

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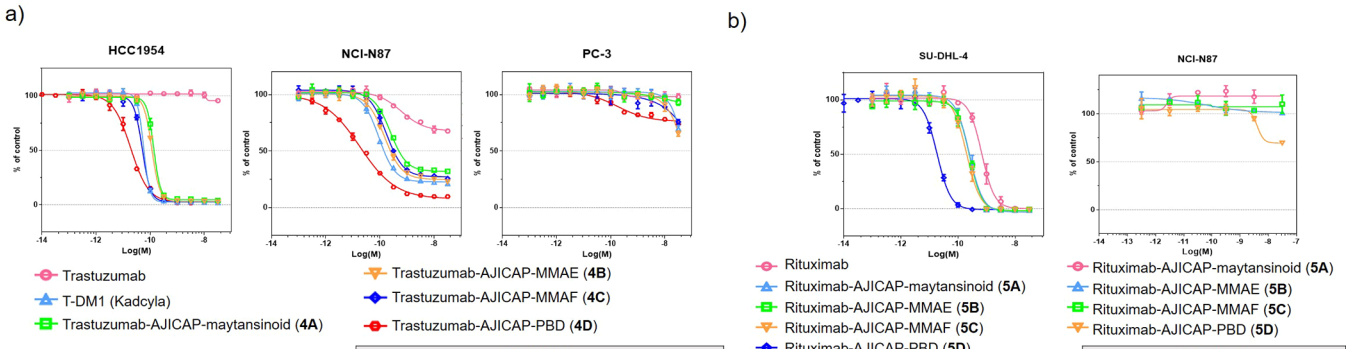

Name	Payload	DAR	IC50 (nM) HCC1954 (HER2+)	IC50 (nM) NCI-N87 (HER2+)	IC50 (nM) PC3 (HER2-)
4A	maytansinoid	1.9 (by Q-TOF MS)	0.18	0.43	>30
4B	MMAE	1.6 (by Q-TOF MS)	0.14	0.25	>30
4C	MMAF	1.6 (by Q-TOF MS)	0.044	0.24	>30
4D	PBD	1.9 (by Q-TOF MS)	0.015	0.048	>30
T-DM1	maytansinoid (DM1)	3.4 (by literature)	0.055	0.11	>30
Trastuzumab	-	-	>30	>30	>30

Name	Payload	DAR	IC50 (nM) SU-DHL-4 (CD20+)	IC50 (nM) NCI-N87 (CD20-)
5A	maytansinoid	1.9 (by Q-TOF MS)	0.16	>30
5B	MMAE	1.6 (by Q-TOF MS)	0.18	>30
5C	MMAF	1.8 (by Q-TOF MS)	0.21	>30
5D	PBD	1.5 (by Q-TOF MS)	0.01	>30
Rituximab	-	-	1.2	N.D.

Figure 3. DAR analysis and cell-based assays. (a) Summary of the *in vitro* cytotoxic activities of trastuzumab-based AJICAP-ADCs and unlabeled trastuzumab (as control). (b) Summary of the *in vitro* cytotoxic activities of rituximab-based AJICAP-ADCs and unlabeled rituximab (as control).

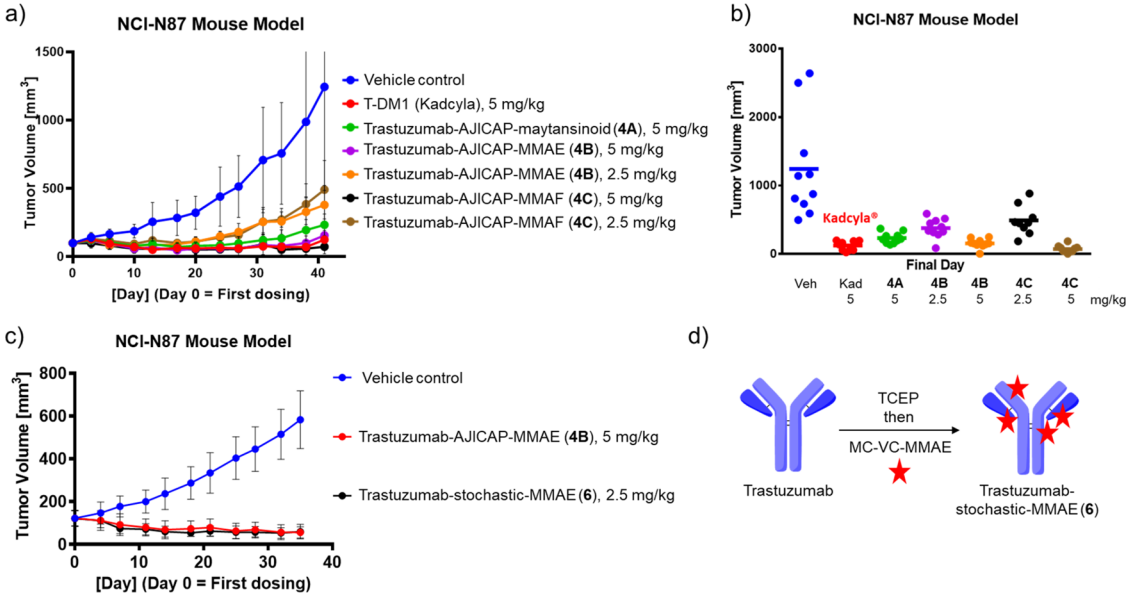

Figure 4. *In vivo* xenograft assay to determine the MED of AJICAP-ADC. (a) Anti-tumor activity of anti-HER2 AJICAP-ADCs (DAR = 2) and T-DM1 (DAR = 3.4) in the NCI-N87 xenograft tumor model. (b) ADC dose comparison plots. (c) Anti-tumor activity of anti-HER2 AJICAP-ADC (**4B**, DAR = 1.6) and stochastic cysteine-based ADC (**6**, DAR = 4.0) in the NCI-N87 xenograft tumor model. (d) Synthesis of stochastic cysteine-based ADC (**6**).

trastuzumab (IgG1), rituximab (IgG1), infliximab (IgG1); adalimumab (IgG1);[14] denoxumab (IgG2);[14] dupilumab (IgG4).[14] A fluorophore payload (Alexa Fluor) was also tested for conjugation efficiency with AJICAP-thiol intermediates (Figures S3−S23). DAR analyses of the resulting ADCs were conducted by Q-TOF MS (Figure 2d). Some variability in the DAR values between each linker-payload was observed (range 1.5−1.9), attributed to differences in the efficiency of conjugation for each drug-linker unit under unoptimized conditions; however, no significant differences in conjugation efficiency were observed between trastuzumab and rituximab employing a platform conjugation method. Aggregation levels measured after conjugation of the different linker-payloads were conducted by an SEC method that indicated that rituximab-based ADCs contained slightly more aggregates than trastuzumab-based ADCs.

***In Vitro* Cell-Based Assays.** *In vitro* cell-based assays of the ADCs were also conducted (Figure 3). To compare the cytotoxic activities of four different AJICAP-ADCs derived from trastuzumab, cell-based activity assays using HER2 antigen-overexpressing cell lines (NCI-N87: gastric cancer cell and HCC1954: breast cancer cell) and a low HER2 antigen-expressing cell line (PC-3: human prostate cancer cell) were performed (Figure 3a). For rituximab-based ADCs, both a CD20 antigen-overexpressing cell line (SU-DHL-4) and an antigen-negative expressing cell line (NCI-N87) were evaluated. Both trastuzumab-based and rituximab-based ADCs showed appreciable anti-tumor activity against the

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target antigen-overexpressing cells. In contrast, no activity against the low target antigen-expressing cells was observed.

***In Vivo* Xenograft Assay to Determine the MED of AJICAP-ADC.** To determine the MED of AJICAP-ADCs, mouse xenograft studies were evaluated (Figure 4).[24] Trastuzumab-derived AJICAP-ADCs and Kadcyla (T-DM1), a commercially available lysine-based stochastic ADC, were evaluated in the HER2-positive NCI-N87 gastric cancer xenograft model. The AJICAP-ADCs at doses of 5 mg/kg displayed significant tumor regressions comparable to Kadcyla (Figure 4a,b).

In addition to the lysine-based stochastic ADC, an AJICAP-ADC was also compared to a cysteine-based stochastic ADC modeled after a well-established established structural format in the ADC field. This stochastic ADC was synthesized from trastuzumab by TCEP-mediated partial reduction of the interchain disulfide bonds followed by conjugation with MC-VC-MMAE to a DAR of 4 in the same manner as commercially approved ADCs Adcetris, Polivy, and Padcev (Figure 4d).[25,26] The stochastic MMAE-ADC **6** was compared against the similar AJICAP-MMAE-ADC **4B** in efficacy (Figure 4c), safety (Figure 5), and PK (Figure 6) studies. The optimal DAR for

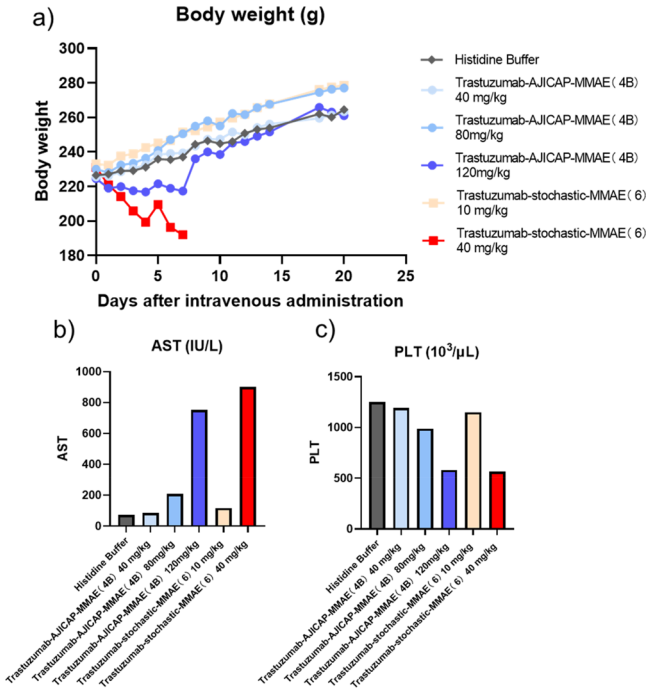

Figure 5. Rat acute toxicity study to determine the MTD of ADCs. (a) Body weight change. (N = 5 female SD rats). (b) Aspartate aminotransferase (AST) in rat serum at 2 days after administration. (N = 5 female SD rats). (c) Platelet (PLT) levels in rat serum at 2 days after administration. (N = 5 female SD rats).

an ADC depends on a large number of complex variables, including the in-part expression level of the target antigen, hydrophobicity of linker-payload, and payload class.[27,28] Stochastic ADCs produced by cysteine conjugation are a mixture of different compounds with varying DARs (DAR = 0, 2, 4, 6, and 8 are typically the major species, and there are several forms of these ADCs with regard to payload placement on the antibody). Higher DAR ADCs may show greater potency in *in vitro* and *in vivo* efficacy models; however, they may suffer greater off-target toxicities due to faster clearance via the liver.[27] On the other hand, due to reduced drug decorating the antibody, lower DAR ADCs may retain properties more similar to its parent antibody resulting in an enhanced safety profile; however, these low DAR stochastic ADC mixtures often contain significant DAR = 0 (naked antibody), which may compete for limited tumor-associated antigen and therefore result in lower *in vivo* efficacy. Site-specific ADCs created with AJICAP technology (and other site-specific methods) have a narrow degree of heterogeneity compared to stochastic DAR4 ADCs and can be more easily optimized for a desired biological outcome.

Toxicology Study in Rats. To determine the MTD of the ADCs, a rat acute toxicity study was conducted (Figure 5). Mortality or severe toxicity could be observed at doses of 120 mg/kg of trastuzumab-AJICAP-MMAE (**4B**) and 40 mg/kg of trastuzumab-stochastic-MMAE (**6**). Decreases in body weight or platelet (PLT) levels, and importantly increases in the serum aspartate aminotransferase (AST) levels, were not observed at doses of up to 80 mg/kg of trastuzumab-AJICAP-MMAE (**4B**) and 10 mg/kg of trastuzumab-stochastic-MMAE (**6**). Thus, the MTD values of trastuzumab-AJICAP-MMAE (**4B**) and trastuzumab-stochastic-MMAE (**6**) were estimated to be at least 80 and 10 mg/kg, respectively.

Plasma Pharmacokinetics in Rats. To provide a rationale for the observed expansion of the TI for our site-specific-ADCs versus traditional stochastic ADCs, an *in vivo* rat pharmacokinetics study was conducted (Figure 6). For the stochastic cysteine-based ADC, significant detachment of the payload was detected after several days (Figure 6a). In contrast, significantly less payload detachment was observed over the course of the study by comparing the curves of total mAb and the total ADC concentrations of the AJICAP-ADC (Figure 6b). Furthermore, over 21 days of the study, the total mAb clearance of the AJICAP-ADC did not change significantly.

DISCUSSION

A stable site-specifically labeled thiol intermediate is useful for at least two purposes. In terms of ADC manufacturing, a stable intermediate can be considered a "hold point" in the conjugation process, enabling the possibility of minimizing a manufacturing operation workload. In addition to this manufacturing advantage, a stable thiol-modified antibody is a convenient intermediate in a discovery setting in that it may be convergently converted to a variety of desired ADCs for early-stage ADC screening (Figure 2c). These two desirable attributes prompted us to perform a stability assessment of an AJICAP-thiol antibody intermediate. In a previous literature report, a site-specific thiol intermediate was either immediately used for payload conjugation once generated or was stored at −80 °C for later conjugation.[5,29] There are no reports specifically describing the effects of long-term storage on a site-specific sulfhydryl-labeled antibody. Furthermore, unconjugated thiol groups present in the Fc region may trigger undesired disulfide scrambling or cross-linking.[29] Recently, Tumey and co-workers synthesized a site-specific thiol modified antibody via a novel enzymatic strategy; however, the application of a carefully controlled re-oxidation step was required to avoid undesired S−S bond formation by spatially adjacent thiol groups.[29] This remarkable previous result prompted our group to conduct stability assessments and *in silico* molecular modeling analyses of installed thiol groups on Lys248 introduced by our Fc-affinity reagent. No observations

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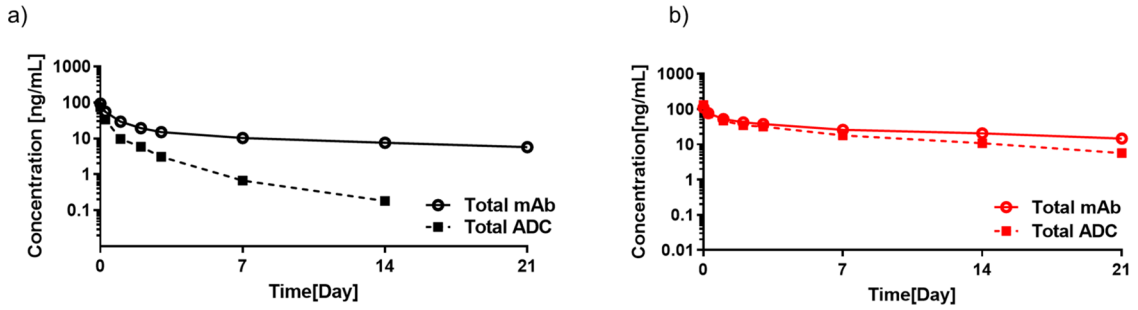

Figure 6. Plasma concentration of total mAb and total ADC in rats measured by ELISA. (a) Trastuzumab-stochastic-MMAE (**6**). (b) Trastuzumab-AJICAP-MMAE (**4B**).

of cross-linking or disulfide-bridge formation were noted post-introduction of the thiol groups at Lys248 under long-term storage conditions (Figure 1d) and multiple several freeze/thaw cycles (data not reported). This discrepancy with Tumey and co-workers' investigation[29] may be attributed to the difference in the nature of the modified sites. The thiol groups installed on Lys248 are located in a hydrophobic pocket of the Fc region (Figure 1c) and are moderately solvent exposed with the sulfhydryl groups of the AJICAP-thiol potentially sterically inhibited from reacting with other antibody thiol groups. Furthermore, the distance between two newly formed thiol groups was relatively far (over 70 Å apart, Figure S2), which was considered to avoid cross-linking to form an intramolecular disulfide bond.[31] These moderate reactivity and appropriate location of a thiol group could lead to keep aggregation percentage via intermolecular disulfide formation within the acceptance level and to avoid undesired cross-linking via intramolecular disulfide formation. This hypothesis was supported by *in silico* molecular modeling studies (Figure S2). In 2018, Kozak and co-workers reported cysteine scanning via antibody engineering to identify optimal conjugation sites balancing aggregation propensity, payload conjugation efficiency, and plasma stability.[30] In Kozak and co-workers' report, the installation of cysteine amino acids in the vicinity of the Protein A binding site resulted in either lower DARs after conjugation or poor reoxidation-step outcomes. They used immobilized antibody bound to Protein A beads for this cysteine scanning study, and the beads may have interfered with the conjugation process by steric hindrance for those cysteines introduced in the vicinity of the Protein A binding region. In our case, the Fc-affinity reagent binds to the antibody similarly to Protein A and results in the introduction of a sulfhydryl group on the side-chain of a nearby lysine residue. This newly installed moiety at Lys248 does not interfere with the Protein A binding site due to its introduction while the Fc-affinity reagent is still bound to the antibody (the affinity reagent is cleaved and removed after the covalent modification of Lys248), and antibodies conjugated in this manner retain binding to Protein A. Additionally, thiols installed at Lys248 in this manner displayed desirable reactivity (i.e., reoxidation chemistry, conjugation reactivity, and stability).

The conjugation investigation described in Figure 2 indicates compatibility of the installed AJICAP-thiol with commonly employed ADC drug-linkers. During the reaction with a relatively hydrophobic payload such as a PBD, the AJICAP-thiol reacted with the drug-linker as desired. These results indicated that AJICAP-thiol intermediates have potential application to create a wide variety of antibody-linked compounds. In addition to wide drug-linker compatibility, multiple antibodies, including IgG1, IgG2, and IgG4 subtypes, can be used for AJICAP conjugation as the Fc-affinity reagent binds similarly across these subtypes. The Fc-affinity reagent binds to an antibody Fc in a similar manner as Protein A, and the binding site of this reagent to antibodies overlaps with the binding site of the domain B of Protein A (Figure 1b). These results indicate that antibodies that can be bound to or purified with Protein A should also possess potential reactivity for site-specific conjugation mediated by the AJICAP Fc-affinity reagent.

By applying a general, unoptimized method for conjugation and purification for each ADC, we measured the resulting DAR and aggregation (Figure 2d). Notably, the aggregation present in the AJICAP-ADCs using the platform conjugation method[14] resulted in ADCs containing 90−95% monomer. Our research group has completed additional optimization studies to further streamline the conjugation reaction sequence as well as improve the characteristics of the resulting bioconjugates (these advancements will be discussed in future reports).

Cell-based assays (Figure 3) of the AJICAP-ADCs illustrated antigen-mediated target selectivity and *in vitro* cytotoxic activity reflective of the payload potency. Our previous published work[14] has demonstrated the retained affinity of AJICAP-ADCs based on trastuzumab toward HER2 measured by surface plasmon resonance (SPR), and the presently described *in vitro* assay results further supported that result. CD20-reactive rituximab-based ADCs also showed expected potency and target selectivity. These results indicated that ADCs produced by AJICAP technology have multi-antibody compatibility and the potential for use in ADC development regardless of the antibody target or isotype so long as the antibody is reactive to the Fc-affinity reagent and possesses a lysine residue at position 248.

In vivo, the HER2+ NCI-N87 xenograft model (Figure 4a−c) was utilized to demonstrate that the efficacy of trastuzumab-AJICAP-MMAE was comparable to stochastic cysteine-based ADCs. The MED of the stochastic cysteine-based ADC was determined to be 2.5 mg/kg (Figure S26). Trastuzumab-AJICAP-MMAE (DAR = 2) at 5 mg/kg showed tumor inhibition comparable to 2.5 mg/kg stochastic trastuzumab-MMAE (DAR = 4) (Figure 4c). An exploratory rat safety toxicology study showed that trastuzumab-AJICAP-MMAE (**4B**) was well tolerated compared with trastuzumab-stochastic-MMAE (**6**). Given the limited dosing of our study, the MTD of trastuzumab-AJICAP-MMAE (**4B**) was estimated to be at least 80 mg/kg, while the MTD of trastuzumab-stochastic-MMAE (**6**) was estimated to be at least 10 mg/kg. Thus, the TI of trastuzumab-AJICAP-MMAE (**4B**)

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was estimated to be qualitatively greater than trastuzumab-stochastic-MMAE (**6**), taking both the respective MTDs and MEDs into consideration.

The conjugation site (Lys248) is near to the antibody FcRn binding region, and thus, potential interference with FcRn binding has to be determined to fully characterize ADCs conjugated via this site-specific methodology. A pharmacokinetic study in rats indicated that the AJICAP-ADC maintained reasonable stability in circulation over a 3 week study (Figure 6) compared to stochastic ADC **6**. In our study, the measured levels of total antibody from AJICAP-conjugated ADC **4B** indicated a half-life similar to that expected for an unconjugated antibody (Figure 6b), and this finding was supported by a recently published *in vitro* affinity study using biolayer interferometry (BLI) that indicates that conjugation at Lys248 in this manner does not affect FcRn interactions.[32] This result and the trend of total ADC likely correspond to the increased tolerability observed for the AJICAP-ADCs and suggest that site-specific conjugation at Lys248 results in a superior profile with regard to both safety and *in vivo* stability compared to stochastic-conjugated ADCs.

In conclusion, our research group has developed a chemical conjugation technology to produce site-specific antibody−drug conjugates without the need for antibody engineering nor enzyme application. This novel methodology termed AJICAP utilizes an Fc-affinity reagent and is compatible with a wide range of antibody formats and can easily introduce two linker-payloads per antibody using native IgGs. We succeeded in creating a convenient set of thiol-labeled antibodies for use in discovery research or as a hold point in manufacturing. Furthermore, when compared against stochastically conjugated ADCs in mouse efficacy and rat PK and toxicology studies, results from ADCs generated using this chemical site-specific conjugation technique indicate a widening of the therapeutic index, which may translate into clinically relevant benefits as additional pre-clinical study data becomes available. The platform conjugation technology described herein enables rapid, site-specific, and efficient conjugation to a wide variety of native antibodies with various linker-payloads to produce next generation low-heterogeneity ADCs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.molpharmaceut.1c00473.

Details of the *in vivo* studies, figures supporting the molecular modeling, HPLC chromatograms, and Q-TOF MS analyses (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Brian A. Mendelsohn − *Ajinomoto Bio-Pharma Services, San Diego, California 92121, United States;* Present Address: Present address: Exelixis Inc., 1851 Harbor Bay Pkwy, Alameda, California 94502, United States (B.A.M.); orcid.org/0000-0002-6339-2377; Email: bmendelsohn@exelixis.com

Tatsuya Okuzumi − *Ajinomoto Co., Inc., Kawasaki-Shi, Kanagawa 210-8681, Japan;* orcid.org/0000-0001-5839-6958; Email: tatsuya_okuzumi@ajinomoto.com

Authors

Yutaka Matsuda − *Ajinomoto Co., Inc., Kawasaki-Shi, Kanagawa 210-8681, Japan; Ajinomoto Bio-Pharma Services, San Diego, California 92121, United States;* orcid.org/0000-0003-2801-4706

Takuya Seki − *Ajinomoto Co., Inc., Kawasaki-Shi, Kanagawa 210-8681, Japan*

Kei Yamada − *Ajinomoto Co., Inc., Kawasaki-Shi, Kanagawa 210-8681, Japan;* orcid.org/0000-0002-7563-0176

Yuri Ooba − *Ajinomoto Co., Inc., Kawasaki-Shi, Kanagawa 210-8681, Japan*

Kazutoshi Takahashi − *Ajinomoto Co., Inc., Kawasaki-Shi, Kanagawa 210-8681, Japan;* orcid.org/0000-0003-3275-4109

Tomohiro Fujii − *Ajinomoto Co., Inc., Kawasaki-Shi, Kanagawa 210-8681, Japan*

Sayaka Kawaguchi − *Ajinomoto Co., Inc., Kawasaki-Shi, Kanagawa 210-8681, Japan*

Takahiro Narita − *Ajinomoto Co., Inc., Kawasaki-Shi, Kanagawa 210-8681, Japan*

Akira Nakayama − *Ajinomoto Co., Inc., Kawasaki-Shi, Kanagawa 210-8681, Japan*

Yoshiro Kitahara − *Ajinomoto Co., Inc., Kawasaki-Shi, Kanagawa 210-8681, Japan*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.molpharmaceut.1c00473

Author Contributions

[§]Y.M. and T.S. contributed equally to this work.

Notes

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■ REFERENCES

(1) Strebhardt, K.; Ullrich, A. Paul Ehrlich's magic bullet concept: 100 years of progress. *Nat. Rev. Cancer.* **2008**, *8*, 473−480.

(2) Leung, D.; Wurst, J.; Liu, T.; Martinez, R.; Datta-Mannan, A.; Feng, Y. Antibody Conjugates-Recent Advances and Future Innovations. *Antibodies* **2020**, *9*, 2.

(3) Khongorzul, P.; Ling, C. J.; Khan, F. U.; Ihsan, A. U.; Zhang, J. Antibody-Drug Conjugates: A Comprehensive Review. *Mol. Cancer Res.* **2020**, *18*, 3−19.

(4) Matsuda, Y.; Mendelsohn, B. A. Recent Advances in Drug-Antibody Ratio Determination of Antibody-Drug Conjugates. *Chem. Pharm. Bull.* **2021**, DOI: 10.1248/cpb.c21-00258.

(5) Junutula, J. R.; Raab, H.; Clark, S.; Bhakta, S.; Leipold, D. D.; Weir, S.; Chen, Y.; Simpson, M.; Tsai, S. P.; Dennis, M. S.; Lu, Y.; Meng, Y. G.; Ng, C.; Yang, J.; Lee, C. C.; Duenas, E.; Gorrell, J.; Katta, V.; Kim, A.; McDorman, K.; Flagella, K.; Venook, R.; Ross, S.; Spencer, S. D.; Wong, W. L.; Lowman, H. B.; Vandlen, R.;

Sliwkowski, M. X.; Scheller, R. H.; Polakis, P.; Mallet, M. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat. Biotechnol.* **2008**, *26*, 925−932.
(6) Walsh, S. J.; Bargh, J. D.; Dannheim, F. M.; Hanby, A. R.; Seki, H.; Counsell, A. J.; Ou, X.; Fowler, E.; Ashman, N.; Takada, Y.; Isidro-Llobet, A.; Parker, J. S.; Carroll, J. S.; Spring, D. R. Site-selective modification strategies in antibody−drug conjugates. *Chem. Soc. Rev.* **2021**, *50*, 1305−1353.
(7) Kang, S. H.; Lee, C.-H. Development of Therapeutic Antibodies and Modulating the Characteristics of Therapeutic Antibodies to Maximize the Therapeutic Efficacy. *Biotechnol. Bioprocess Eng.* **2021**, *26*, 295−311.
(8) Falck, G.; Müller, K. M. Enzyme-Based Labeling Strategies for Antibody-Drug Conjugates and Antibody Mimetics. *Antibodies* **2018**, *7*, 4.
(9) Manabe, S.; Yamaguchi, Y. Antibody Glycoengineering and Homogeneous Antibody-Drug Conjugate Preparation. *Chem. Rec.* **2021**, DOI: 10.1002/tcr.202100054.
(10) Rickert, M.; Strop, P.; Lui, V.; Melton-Witt, J.; Farias, S. E.; Foletti, D.; Shelton, D.; Pons, J.; Rajpal, A. Production of soluble and active microbial transglutaminase in Escherichia coli for site-specific antibody drug conjugation. *Protein Sci.* **2016 Feb**, *25*, 442−455.
(11) Matsuda, Y.; Mendelsohn, B. A. An overview of process development for antibody-drug conjugates produced by chemical conjugation technology. *Expert. Opin. Biol. Ther.* **2021**, *21*, 963−975.
(12) Ogitani, Y.; Aida, T.; Hagihara, K.; Yamaguchi, J.; Ishii, C.; Harada, N.; Soma, M.; Okamoto, H.; Oitate, M.; Arakawa, S.; Hirai, T.; Atsumi, R.; Nakada, T.; Hayakawa, I.; Abe, Y.; Agatsuma, T. DS-8201a, A Novel HER2-Targeting ADC with a Novel DNA Topoisomerase I Inhibitor, Demonstrates a Promising Antitumor Efficacy with Differentiation from T-DM1. *Clin. Cancer. Res.* **2016**, *22*, 5097−5108.
(13) Satomaa, T.; Pynnönen, H.; Vilkman, A.; Kotiranta, T.; Pitkänen, V.; Heiskanen, A.; Herpers, B.; Price, L. S.; Helin, J.; Saarimen, J. Hydrophilic Auristatin Glycoside Payload Enables Improved Antibody-Drug Conjugate Efficacy and Biocompatibility. *Antibodies* **2018**, *7*, 15.
(14) Yamada, K.; Shikida, N.; Shimbo, K.; Ito, Y.; Khedri, Z.; Matsuda, Y.; Mendelsohn, B. A. AJICAP: Affinity Peptide Mediated Regiodivergent Functionalization of Native Antibodies. *Angew. Chem., Int. Ed.* **2019**, *58*, 5592−5597.
(15) Matsuda, Y.; Malinao, M.-C.; Robles, V.; Song, J.; Yamada, K.; Mendelsohn, B. A. Proof of Site-Specificity of Antibody-Drug Conjugates Produced by Chemical Conjugation Technology: AJICAP First Generation. *J. Chromatogr. B* **2020**, *1140*, 121981.
(16) Matsuda, Y.; Clancy, C.; Tawfiq, Z.; Robles, V.; Mendelsohn, B. A. Good Manufacturing Practice Strategy for Antibody-Drug Conjugate Synthesis Using Site-Specific Chemical Conjugation: First-Generation AJICAP. *ACS Omega* **2019**, *4*, 20564−20570.
(17) Matsuda, Y.; Yamada, K.; Okuzumi, T.; Mendelsohn, B. A. Gram-Scale Antibody-Drug Conjugate Synthesis by Site-Specific Chemical Conjugation: AJICAP First Generation. *Org. Process Res. Dev.* **2019**, *23*, 2647−2654.
(18) Kishimoto, S.; Nakashimada, Y.; Yokota, R.; Hatanaka, T.; Adachi, M.; Ito, Y. Site-Specific Chemical Conjugation of Antibodies by Using Affinity Peptide for the Development of Therapeutic Antibody Format. *Bioconjugate Chem.* **2019**, *30*, 698−702.
(19) Sastry, G. M.; Adzhigirey, M.; Day, T.; Annabhimoju, R.; Sherman, W. Protein and ligand preparation: parameters, protocols, and influence on virtual screening enrichments. *J. Comput. Aid. Mol. Des.* **2013**, *27*, 221−234.
(20) Matsuda, Y. Current Approaches for Purification of Antibody-Drug Conjugates. *J. Sep. Sci.* **2021**, DOI: 10.1002/jssc.202100575.
(21) 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* **2020**, *9*, 16.
(22) Jeffrey, S. C.; Burke, P. J.; Lyon, R. P.; Meyer, D. W.; Sussman, D.; Anderson, M.; Hunter, J. H.; Leiske, C. I.; Miyamoto, J. B.; Nicholas, N. D.; Okeley, N. M.; Sanderson, R. J.; Stone, I. J.; Zeng, W.; Gregson, S. J.; Masterson, L.; Tiberghien, A. C.; Howard, P. W.; Thurston, D. E.; Law, C. L.; Senter, P. D. A potent anti-CD70 antibody-drug conjugate combining a dimeric pyrrolobenzodiazepine drug with site-specific conjugation technology. *Bioconjugate Chem.* **2013**, *24*, 1256−1263.
(23) van Geel, R.; Wijdeven, M. A.; Heesbeen, R.; Verkade, J. M. M.; Wasiel, A. A.; van Berkel, S. S.; van Delft, F. L. Chemoenzymatic Conjugation of Toxic Payloads to the Globally Conserved N-Glycan of Native mAbs Provides Homogeneous and Highly Efficacious Antibody-Drug Conjugates. *Bioconjugate Chem.* **2015**, *26*, 2233−2242.
(24) Tawfiq, Z.; Caiazza, N. C.; Kambourakis, S.; Matsuda, Y.; Griffin, B.; Lippmeier, J. C.; et al. Synthesis and Biological Evaluation of Antibody Drug Conjugates Based on an Antibody Expression System: Conamax. *ACS Omega* **2020**, *5*, 7193−7200.
(25) Matsuda, Y.; Tawfiq, Z.; Leung, M.; Mendelsohn, B. A. Insight into Temperature Dependency and Design of Experiments towards Process Development for Cysteine-Based Antibody-Drug Conjugates. *ChemistrySelect* **2020**, *5*, 8435−8439.
(26) Tawfiq, Z.; Matsuda, Y.; Alfonso, M. J.; Robles, V.; Leung, M.; Mendelsohn, B. A. Analytical comparison of antibody-drug conjugates based on good manufacturing practice strategies. *Anal. Sci.* **2020**, 871.
(27) Sun, X.; Ponte, J. F.; Yoder, N. C.; Laleau, R.; Coccia, J.; Lanieri, L.; Qiu, Q.; Wu, R.; Hong, E.; Bogalhas, M.; et al. Effects of Drug−Antibody Ratio on Pharmacokinetics, Biodistribution, Efficacy, and Tolerability of Antibody−Maytansinoid Conjugates. *Bioconjugate Chem.* **2017**, *28*, 1371−1381.
(28) Nadkarni, D. V.; Lee, J.; Jiang, Q.; Patel, V.; Sriskanda, V.; Dutta, K.; Meyer, D. M. Impact of Drug Conjugation and Loading on Target Antigen Binding and Cytotoxicity in Cysteine Antibody−Drug Conjugates. *Mol. Pharmaceutics* **2021**, *18*, 889−897.
(29) Benjamin, S. R.; Jackson, C. P.; Fang, S.; Carlson, D. P.; Guo, Z.; Tumey, L. N. Thiolation of Q295: Site-Specific Conjugation of Hydrophobic Payloads without the Need for Genetic Engineering. *Mol. Pharmaceutics* **2019**, *16*, 2795−2807.
(30) Ohri, R.; Bhakta, S.; Fourie-O'Donohue, A.; Dela Cruz-Chuh, J.; Tsai, S. P.; Cook, R.; Wei, B.; Ng, C.; Wong, A. W.; Bos, A. B.; Farahi, F.; Bhakta, J.; Pillow, T. H.; Raab, H.; Vandlen, R.; Polakis, P.; Liu, Y.; Erickson, H.; Junutula, J. R.; Kozak, K. R. High-Throughput Cysteine Scanning To Identify Stable Antibody Conjugation Sites for Maleimide- and Disulfide-Based Linkers. *Bioconjugate Chem.* **2018**, *29*, 473−485.
(31) In our present study, the native glycochain was intact and unmodified before and after conjugation, while in Tumey's 2019 study, a deglycosylation step is employed with PNGase prior to their transglutaminase-mediated enzymatic conjugation step. Tumey and co-workers described in reference 29 that "Given the removal of the glycosylation, it is likely that the two heavy chains will partially collapse and move even closer to one another, thus enabling the ready formation of an interchain disulfide between the two thioethyl moieties."
(32) Matsuda, Y.; Chakrabarti, A.; Takahashi, K.; Yamada, K.; Nakata, K.; Okuzumi, T.; Mendelsohn, B. A. Chromatographic analysis of site-specific antibody-drug conjugates produced by AJICAP first-generation technology using a recombinant FcγIIIa receptor-ligand affinity column. *J. Chromatogr., B* **2021**, *1177*, 122753.