Bispecific Antibodies Produced via Chemical Site-Specific Conjugation Technology: AJICAP Second Generation

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Abstract

Bispecific antibodies are biotherapeutics that amalgamate the specificities of two distinct antibodies into one molecule. Bispecific antibodies can be utilized in a broad range of diagnostic and therapeutic applications; however, their engineering requires genetic modification and remains time-consuming. Therefore, in this study, we used AJICAP second-generation technology, which drives the production of site-specific antibody-drug conjugates in a practical and robust manner, without genetic modification requirements, to generate bispecific antibodies. Using haloketone chemistry as an alternative to maleimide chemistry, which carries reaction risks, we successfully produced site-specific antibody conjugates. Pharmacokinetic studies revealed that the haloketone-based antibody conjugate was stable in the rat plasma. The resultant bispecific antibodies were rigorously evaluated, and surface plasmon resonance measurements and flow cytometry analyses confirmed that antigen binding remained intact. Additionally, the affinity for the neonatal Fc receptor (FcRn) was retained after conjugation. Further cytotoxicity evaluation emphasized the pronounced activity of the generated bi-specific antibodies. These preliminary findings highlight the potential of AJICAP second-generation technology in BisAb production. This novel approach introduces a fully chemical, site-specific strategy capable of producing bispecific antibodies, heralding a new era in the field of biotherapeutics.

Keywords: bispecific antibodies, antibody-drug conjugates, AJICAP, site-specific conjugation technology, maleimide alternatives, haloketone

Introduction

Bispecific antibodies (BisAbs) are genetically engineered antibodies that simultaneously bind to two different epitopes [1, 2 3]. Currently, there are more than 100 BisAb formats [4]. Their dual specificity allows for BisAbs to be used in a broad range of applications. BisAbs are used to redirect effector cells, which enables them to deliver T cells, natural killer (NK) cells, and other cells directly to tumor cells. Blicyto [5], a BisAb construct approved in 2017, employs the redirection mechanism. It recognizes and enables the redirection of CD3-positive T cells and eliminates CD19-positive acute lymphoblastic leukemia cells. BisAbs can be broadly distinguished by the absence or presence of an Fc portion. BisAbs without an Fc region, such as Blicyto, lack Fc effector functions, such as antibody-dependent cellular cytotoxicity, antibody-dependent cell phagocytosis, complement-dependent cytotoxicity, and FcRn recycling [2, 6]. By contrast, BisAbs with Fc regions exhibit Fc-mediated functions. BisAbs with Fc regions can be classified into symmetrical or asymmetrical structures. BisAbs can be utilized in a broad range of diagnostic and therapeutic applications; however, BisAbs engineering remains time consuming, especially when establishing cell culture conditions [7]. In addition, many factors, such as the balance of affinity between different antigen recognition regions, the distance between antigen recognition regions, and the linker structure [8] affect BisAb

development. Antibody engineering methods often require time-consuming studies to introduce their tag molecules [6], and although tag-free enzymatic methods have recently been established, challenges, such as reaction rate and enzyme removal, still remain [9]. Recently, chemical BisAbs engineering methods have gained considerable attention [10 2 3]. However, these published methods are mainly used for research purposes and are therefore not scalable or robust.

Therefore, we applied our preoperative technology, utilizing Fc-affinity peptide reagents, to generate a novel format for BisAb production [11]. This technology, termed "AJICAP second generation," allows for a non-engineering preparation of site-specific antibody-drug conjugates (ADCs) (Figure 1). This unique strategy produced two site-specific ADCs with two different conjugation sites (Lys248 and Lys288). In vivo biological studies have shown that these site-specific ADCs have a wider therapeutic window than conventional ADCs produced using the stochastic conjugation method[11]. Furthermore, process development toward gram-scale ADC preparation was completed, indicating that this chemical conjugation technology is a practical approach for manufacturing site-specific antibody conjugates [12].

In this study, we aimed to evaluate a novel application of this site-specific conjugation technology in the development of bispecific antibodies.

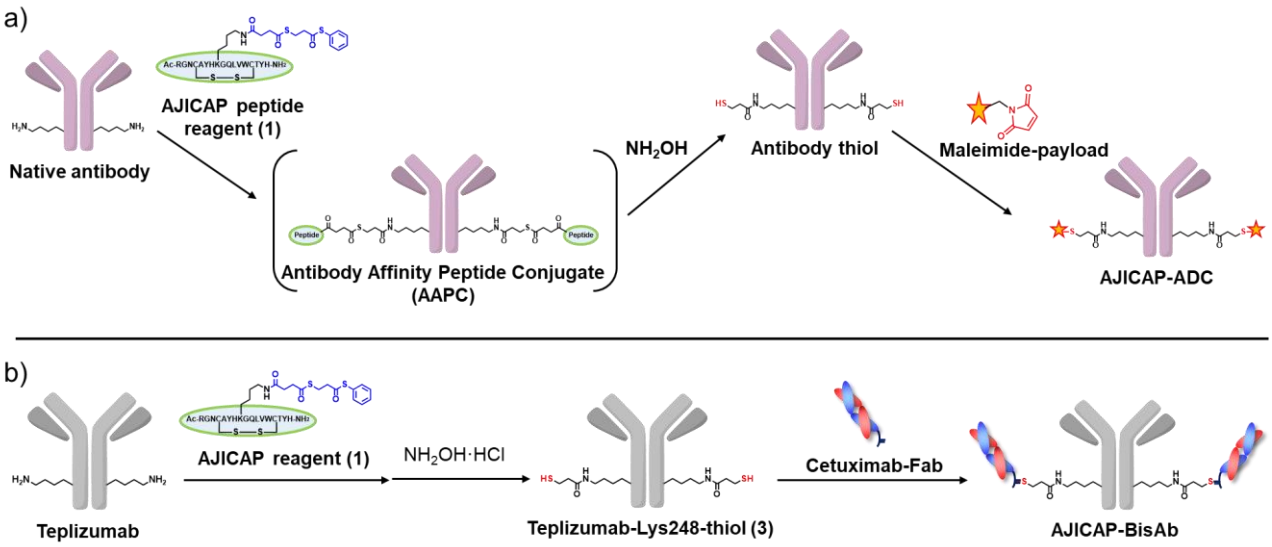

Figure 1. Overview of AJICAP second generation technology.

a) reaction sequence and b) illustration of BisAb production

Results and Discussion

In previous studies, our research group successfully enhanced the therapeutic index of site-specific ADCs[11]. The stability of these ADCs was confirmed by rat PK studies, and the newly formed thiol-maleimide bond, specifically linked to the Lys248 position of the antibody Fc region, was stable. In vivo ADC stability depends on the conjugation site [14]. Our previous results suggest that the antibody-Lys248-thiol is a promising site for the production of stable antibody conjugates. Therefore, in this study, we aimed to expand the application of antibody-Lys248-thiol by applying it to BisAb production. That is, using antibody-Lys248-thiol, we aimed to generate a novel format for BisAb format

production We selected a BisAb consisting of an anti-CD3 antibody and an anti-EGFR Fab fragment, both of which were previously evaluated in vitro [15].

As we aimed to generate a site-specific BisAb using a chemical conjugation approach, two primary considerations were approached systematically. First, we focused on developing a reliable method, such as a cross-linker compound, to facilitate the conjugation of the antibody Lys248-thiol with a second targeting entity, such as an antibody fragment.

The maleimide group, often employed as the chemical handle of payload linkers for ADCs, is difficult to install onto antibody fragments because of its tendency towards hydrolysis during the modification process. Therefore, we selected click chemistry, a well-established chemical conjugation approach distinct from thiol-maleimide, to link antibodies and antibody fragments. However, a crosslinker capable of converting Lys248-thiol to cycloalkyne is required.

Maleimide- dibenzocyclooctyne (DBCO) reagents, which are readily available and commonly used cross-linkers, possess potential risks associated with the thiol-maleimide linkage [16]. This risk arises from the possibility of a retro Michael reaction that could trigger unwanted payload detachment. Several alternatives to maleimides have been

proposed to address this issue. Haloamides are one of the most frequently employed substitutes in the haloamide group [10]. Nucleophilic substitution of the thiol group with this group is irreversible and can result in a newly formed stable linkage. However, the reactivity of the haloamide group is lower than that of maleimide; consequently, extending reaction time or increasing reaction temperatures. Several alternative groups have been recently reported [10, 17, 18]. However, an optimal reagent that balances thiol reactivity, stability, and synthesizability is yet to be established. With these considerations in mind, we employed α-haloketones.

α-haloketones have remarkable reactivity (enabled by the potential for selective transformation when combined with different reagents), which makes them ideal foundational components for the synthesis of a diverse array of compound classes [19]. Despite their confirmed utility, α-haloketones are rarely utilized as cross-linking agents. Thus, we evaluated α-haloketone as a cross-linking reagent in the production of BisAbs. We designed an α-bromoketone-DBCO reagent and assessed its synthesizability, reactivity, and in vivo stability (Figure 2). 4-(2-bromoacetyl) benzoic acid, an affordable commercial reagent [20], can be transformed into an α-bromoketone-DBCO cross-linker through a single-step amidation with DBCO-amine (Figure S1 in the Supporting Information (SI)).

126 To evaluate conjugation with an antibody incorporating a thiol, we used trastuzumab-
127 Lys248-thiol [21] as a model compound. The conjugation efficiency of α-bromoketone-
128 DBCO was similar to that of maleimide-DBCO. DBCO coupling was performed at 20
129 deg.C for an hour, leading to an enhanced conversion. A click reaction with azide-PEG3-
130 MMAE [22] was then performed, resulting in a site-specific ADC with a drug to antibody
131 ratio (DAR) of 1.8 (Figure S2). Pharmacokinetic studies, conducted in rats, were
132 performed to evaluate the stability of the resulting ADCs. Assessment of total antibody
133 levels in the trastuzumab-MMAE ADCs revealed that the ADCs had a half-life
134 comparable to that of trastuzumab, indicating that the trastuzumab-MMAE ADC was
135 highly stable during blood circulation. These findings suggest that the α-bromoketone
136 reagent is a promising alternative to maleimide and has the potential to facilitate the
137 creation of a broad spectrum of antibody conjugates.

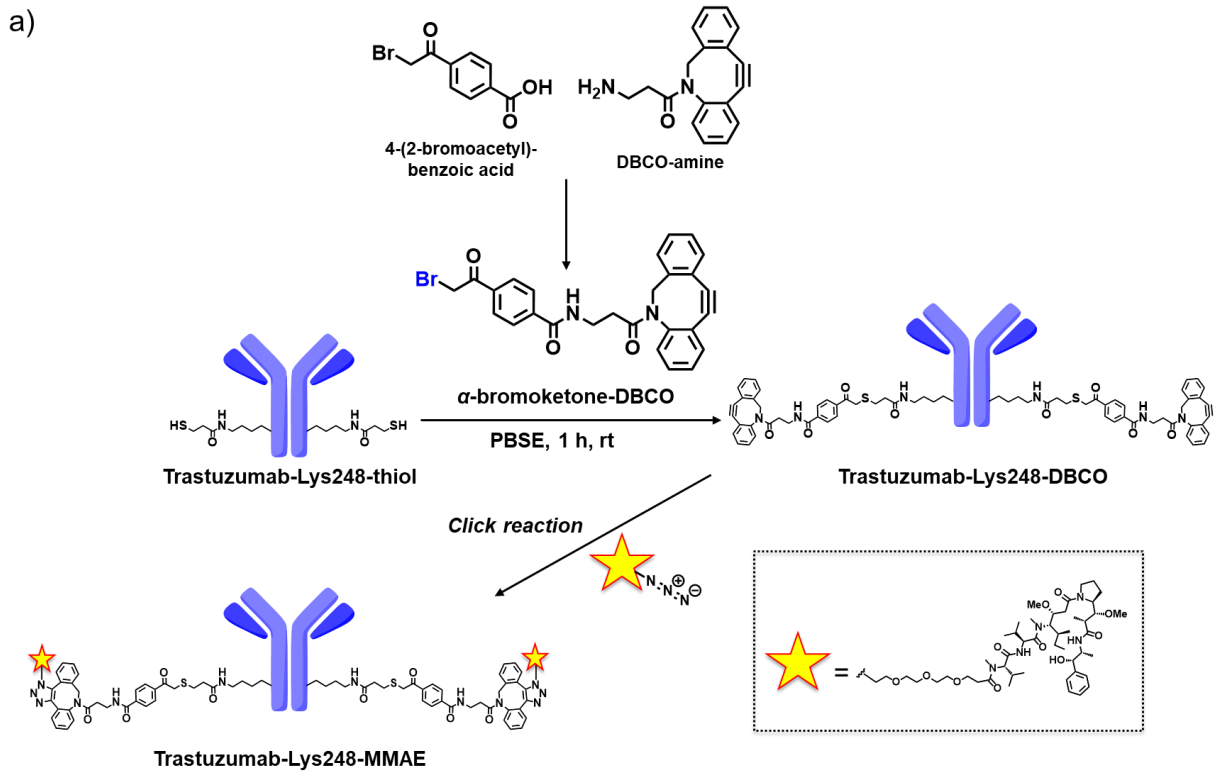

Figure 2. Evaluation of the α-bromoketone reagent as a maleimide cross-linker alternative.

a) α-bromoketone-based ADC synthesis and b) Rat PK study, plasma concentration of total mAb (black line) and total ADC (blue line) of trastuzumab-Lys248-MMAE

144 Second, we focused on the need for two distinct site-specific modification technologies:
145 one for the modification of the first antibody and the other for the modification of the
146 second affinity molecule. AJICAP second-generation technology [11] is suitable for full
147 antibody modification (in this case, modification of the first antibody). However, it is
148 incompatible with the modification of an antibody fragment (for modification of the
149 secondary antibody), owing to the absence of the Fc region.

150 To modify the antibody fragment in a site-specific manner, we chose Thiobridge
151 modification technology [23] (Figure 3). This advanced chemical conjugation technology
152 can crosslink two thiol groups possessing two leaving groups using a designated reagent.
153 Enzymatic digestion of the anti-EGFR antibody cetuximab [24] provided a Fab fragment
154 containing two free sulfhydryl groups. Sequential Thiobridge modification successfully
155 introduced an azide group into the cetuximab-Fab in a site-specific manner. These
156 reactions were monitored using quadrupole time-of-flight mass spectrometry (Q-TOF
157 MS) (Figure S3).

158 The click reaction between the DBCO-modified teplizumab and cetuximab-fab-azide
159 yielded AJICAP-BisAb. The unreacted cetuximab Fab-azide was successfully removed
160 via size-exclusion chromatography (SEC) purification. Subsequently, the resulting
161 conjugate was analyzed using Q-TOF MS (Figure S4 in SI) [25] and reversed-phase high-

performance liquid chromatography (RP-HPLC) (Figure 5S in SI)[26]. Notably, RP-HPLC analysis under non-reducing conditions proved to be efficient for UV-based analysis, while hydrophobic interaction chromatography (HIC) HPLC was unable to separate DAR effectively [27]. It is generally accepted that UV-based analysis provides more accurate DARs than Q-TOF MS, which can be influenced by ionization efficiency [28]. RP-HPLC analysis serves as a potential alternative method for the analysis of complex antibody conjugates, as exemplified in this study using AJICAP-BisAb.

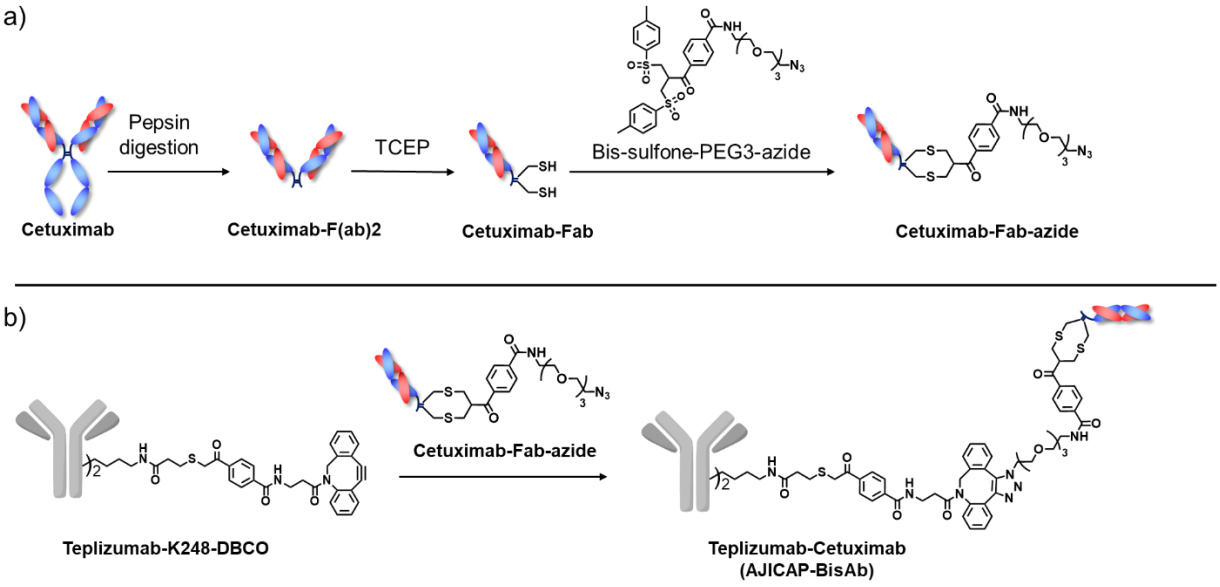

Figure 3. AJICAP-BisAb production.

a) cetuximab-Fab-azide preparation by Thiobrigdge and b) click reaction to produce AJICAP-BisAb

The resulting AJICAP BisAbs were evaluated in vitro. The antigen-binding ability of BisAbs was confirmed using two different methods: surface plasmon resonance (SPR)

(Table 1) and flow cytometry (Figure 4) [29]. The binding affinities of AJICAP-BisAb to human CD3 and EGFR were determined by flowing the analyte (AJICAP-BisAb) onto a Sensor CM5 chip immobilized with human CD3ε/δ heterodimer or EGFR ectodomain[30]. The binding affinity was then deduced from the dissociation constant (K_D), calculated using the BIA evaluation software (Cytiva) [31]. Similarly, the binding affinities of teplizumab to CD3ε/δ and cetuximab to EGFR were analyzed. As expected, AJICAP-BisAb bound to CD3ε/δ without affecting the affinity of parental teplizumab and exhibited significant EGFR affinity. To confirm the dual-binding activity of AJICAP-BisAb, we performed flow cytometry on EGFR-positive cells (A431) and CD3-expressing Jurkat cells. AJICAP-BisAbs bound to A431 and Jurkat T cells to the same extent as the parental antibodies (Figure 4).

The use of AJICAP conjugation technology resulted in Fc region modification, specifically at Lys248. Thus, binding affinity to FcRn could be reduced, owing to the steric hindrance caused by the payload linker located proximal to the FcRn binding site. However, in 2021, our research team found that AJICAP-ADC (with its conjugation site at Lys248 and payload MMAE) maintained FcRn binding affinity after conjugation [13]. These observations were consistent with expectations based on AJICAP-ADC plasma stability, as determined in the rat pharmacokinetic study. In the current study, we

193 evaluated the FcRn-binding affinity of AJICAP-BisAb (Table 1). The bispecific antibody
194 construct AJICAP-BisAb and its unmodified counterpart teplizumab exhibited analogous
195 binding kinetics when interacting with immobilized FcRn in advanced SPR analytical
196 evaluation. This kinematic parallelism is conceivable when the molecular architectures
197 of the two entities are considered (Figure S6). The SPR results strongly suggest that
198 AJICAP-BisAb and FcRn converged to form a durable molecular ensemble. MD
199 simulations were performed to provide detailed structural analysis of these observations.
200 Starting with a validated and structurally optimized model of the AJICAP-BisAbs-FcRn
201 complex, a rigorous molecular dynamics evaluation was performed using the DESMOND
202 simulation framework at a physiological temperature of 310 K and a time window of 50
203 ns. Analysis of the trajectories between 30 and 50 ns revealed consistent root mean square
204 deviation values, demonstrating the stability of the molecular complex (Figure S6a).
205 Notably, FcRn remained consistently anchored to the BisAbs, preventing spontaneous
206 dissociation events. For more detailed insights into the prevalent molecular
207 configurations during this phase, representative structural motifs were derived using a
208 clustering approach (Figure S6b). Notably, Lys248 represents an acceptable site for FcRn
209 binding, effectively bypassing the steric hindrance introduced by the conjugation of larger
210 molecules, such as cetuximab-Fab.

Table 1. Binding kinetics against CD3 (Jurkat) and human FcRn by SPR assay.

Entry	Analyte	Ligand	K_D (M)	k_a(1/Ms)	k_d(1/s)
1	Teplizumab	CD3ε/δ	7.3×10^{-9}	8.9×10^{6}	6.5×10^{-2}
2	AJICAP-BisAb	CD3ε/δ	9.5×10^{-9}	4.0×10^{6}	3.9×10^{-3}
3	Cetuximab	EGFR	2.1×10^{-9}	9.3×10^{5}	1.9×10^{-3}
4	AJICAP-BisAb	EGFR	1.7×10^{-9}	1.6×10^{6}	2.7×10^{-3}
5	Teplizumab	FcRn (pH 6.0)	2.9×10^{-8}	1.1×10^{6}	3.3×10^{-2}
6	AJICAP-BisAb	FcRn (pH 6.0)	5.4×10^{-8}	7.0×10^{4}	3.8×10^{-3}

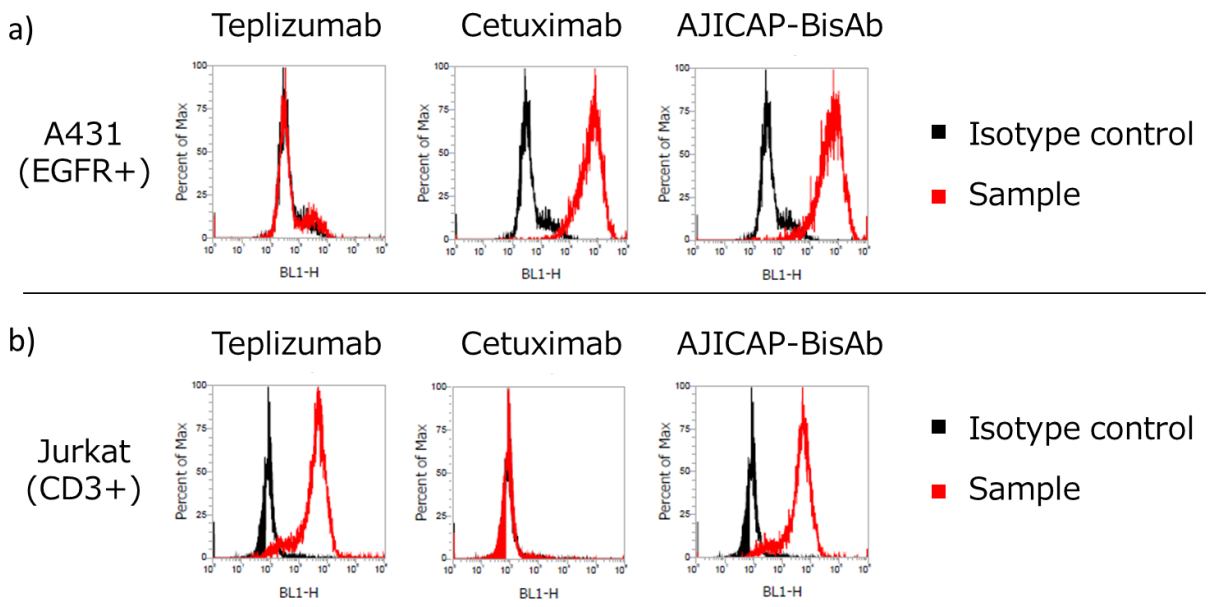

Figure 4. Representative flow cytometry graphs showing antibody binding affinity. Binding of teplizumab, cetuximab, and AJICAP-BisAb to EGFR-expressing A431 (a) and CD3-expressing Jurkat cells (b)

Finally, *in vitro* cell-based assays were performed to assess AJICAP-BisAb activity (Fig. 5). Three different cell lines were used: HT29 (human colorectal adenocarcinoma), which overexpresses the EGFR antigen; DiFi (human colorectal cancer cell line), which overexpresses the EGFR antigen; and SW-620 (human colon cancer cell line), which shows low EGFR antigen expression [15]. These cells were co-cultured with an effector-target cell ratio of 5:1. Using an in vitro cytotoxic assay, we found that AJICAP-BisAb delivers T cells to tumor cells overexpressing the target antigen, exhibiting significant killing activity. However, no activity was observed in cells with low target antigen expression. These results suggest that AJICAP-BisAb is a valuable therapeutic tool for treating a broad range of cancers that express the EGFR target antigen.

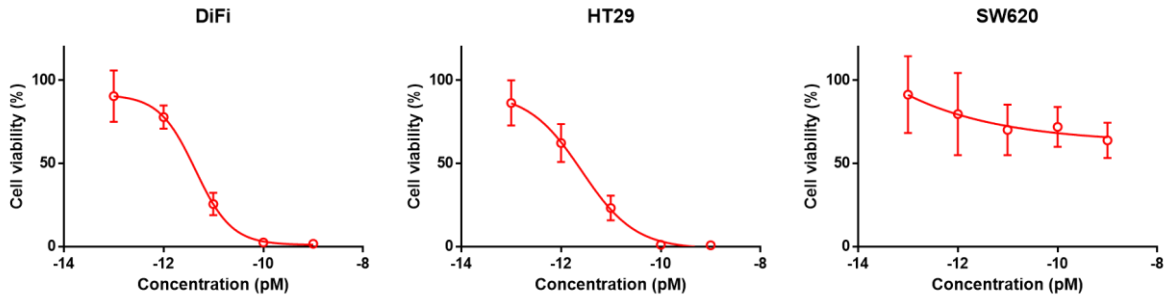

Figure 5. In vitro cytotoxic activities of AJICAP-BisAb against DiFi (human colorectal cancer cell line with EGFR antigen overexpression), HT29 (human colorectal adenocarcinoma cell line with EGFR antigen overexpression), and SW-620 (human colon cancer cell line with low EGFR antigen expression).

Although considerable progress has been made in establishing a stable and reliable synthetic method for BisAb production, this study had limitations, and several challenges still remain. Comprehensive process development, including the identification of critical process parameters and establishment of normal operating ranges, remains incomplete in this study. Ongoing efforts are devoted to further evaluate the experimental design [32], physical stability, and in vivo biological activity and optimize chromatographic separation. We are also investigating the potential application of the experimental process in continuous mode production [33].

Conclusion

In this study, we effectively chemically synthesized a BisAb via AJICAP conjugation. We identified α-bromoketone as an alternative stable linker. Using this linker, we

introduced DBCO into the teplizumab antibody and an azide group into the Fab region of cetuximab in a separate step. This facilitates the synthesis of BisAbs via a click reaction.

The resulting BisAb successfully recognized two antigens, CD3 and EGFR. In addition, BisAb retained its desired T-cell engagement activity.

High-throughput screening is essential for BisAb development. Chemical methods, such as those presented in this study, offer flexibility in adjusting factors, such as the spacer length, binding site, and number of Fab regions. Site-specific bispecific antibody generation, realized by AJICAP second generation, represents a revolutionary technique for modulating bispecific antibodies. These molecules, which are truly innovative in the field of new modalities, pave the way for cutting-edge advances in targeted therapeutics.

EXPERIMENTAL PROCEDURES

Reagents and Cells

Cetuximab (Erbitux), a human monoclonal IgG1 antibody, was purchased from Midwinter. Human IgG1 teplizumab (Tzield®) was purchased from GenScript (Tokyo, Japan). Peptide reagent 1 was prepared as previously reported [11]. Azide-PEG3-MMAE [22] was purchased from NJ Biopharmaceuticals, LLC (USA). All other chemical reagents were purchased from Sigma-Aldrich (USA).

Human A431 and Jurkat cell lines were obtained from RIKEN BioResource Research Center. CRC cell lines (DiFi, HT-29, and SW620) were purchased from and prepared as previously reported [15].

Preparation of α-haloketone-DBCO

DBCO-Amine (CAS No. 1255942-06-3, 217.4 mg), DCC (184.3 mg), and HOBt (23.3 mg) were added to a solution of 4-(2-bromoacetyl) benzoic acid (193.0 mg) in CH_2Cl_2 (9 mL) for 1 h at room temperature. The mother liquor was obtained via filtration and was concentrated and subjected to column purification (Hexane:AcOEt = 1:1). Each fraction was confirmed via thin layer chromatography, and the fraction containing the target product was concentrated and dried in vacuo to obtain a 341.9 mg solid. 1H NMR (400 MHz, CD3OD) δ 8.03 (d, J = 8.4 Hz, 2H), 7.68 (d, J = 8.4 Hz, 3H), 7.50−7.29 (m, 7H), 7.12−7.10 (m, 1H), 5.16 (d, J = 14.0 Hz, 1H), 4.68 (brs, 2H), 3.71 (d, J = 14.0 Hz, 1H), 3.50−3.44 (m, 1H), 3.39−3.34 (m, 1H), 2.59−2.52 (m, 1H), 2.31−2.24 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, $CDCl_3$ = 77.00) δ 192.9, 173.3, 168.7, 152.6, 149.4, 140.0, 137.6, 133.5, 130.4, 130.0 (2C), 129.9, 129.7, 129.2, 128.9, 128.7 (2C), 128.2, 126.5, 124.3, 123.7, 115.6, 108.9, 56.6, 37.5, 35.3, 32.2.; MS (ESI) m / z: 501 [M + H].

Experimental procedure for teplizumab-Lys248-thiol

Teplizumab was converted to teplizumab-Lys248-thiol as previously described [11].

Experimental procedure for teplizumab-Lys248-DBCO

Six equivalents of α-haloketone-DBCO (**6b**) (20 mM in DMA) was added to teplizumab-Lys248-thiol (5 mg/mL, PBSE buffer, pH 7.4). The mixture was incubated at 25 °C for 1 h. Following incubation, the reaction mixture was purified using a Centripure P50 desalting column and eluted with PBSE buffer (pH 7.4) to obtain teplizumab-Lys248-DBCO.

Papain digestion

Papain digestion of cetuximab was performed as previously described. [24]

Azide installation

Twenty equivalents of Tris(2-carboxyethyl)phosphine hydrochloride (20 mM in PBS buffer) was added to papain-digested cetuximab-F(ab)2 (5 mg/mL, PBSE buffer, pH 7.4), and the mixture was incubated at 37 °C for 1 h. Following incubation, the reaction mixture was purified using a Centripure P50 desalting column and eluted with PBSE buffer (pH 7.4). Ten equivalents of bis-sulfone-PEG3-azide (CAS No:1802908-01-5, 20 mM DMA,

Axis Pharm) was added to the eluent, and the solution was incubated at room temperature for 1 h. Following incubation, the reaction mixture was purified using a Centripure P50 desalting column, and cetuximab-Fab-azide was obtained via PBSE buffer (pH 7.4) elution.

Production of the antibody-Fab conjugate

Using the click reaction, DBCO and an azide group were introduced into the teplizumab-Lys248 antibody and the Fab region, respectively, as previously described. [11]

Instruments and analytical methods

ADC concentration and recovery were measured using a Solo-VPE system with the Slope Spectroscopy method. [21]

Q-TOF MS analysis was performed as previously reported. [21]

Hydrophobic interaction chromatography-HPLC analysis was performed as previously reported [21].

RP-HPLC analysis of intact BisAbs was performed using Sepax Proteomix RP-1000 (5 µm 2.1 × 50 mm column), as previously reported [26].

316 SEC-HPLC analysis of ADCs (**5**) was performed using Waters ACQUITY UPLC Protein

317 BEH SEC column (200 Å, 4.6 × 300 mm, 1.7 μm), as previously reported[33].

318 **Surface plasmon resonance**

319 The Sensor SA chip, Biotin CAPture Kit, and HBS-EP buffer (10 mM HEPES, 150 mM

320 NaCl, 3 mM EDTA, and 0.005% Tween-20) were obtained from Cytiva (Uppsala,

321 Sweden). Biotinylated Human CD3 epsilon&CD3 delta Heterodimer Protein,

322 Fc,His,Avitag and Fc,Flag,Avitag was purchased from ACRO Biosystems (Newark,

323 USA). EGFR ectodomain-Fc was purchased from R&D Systems (Minneapolis, MN,

324 USA) and biotinylated using a Biotin Labeling Kit -NH_2 (Dojindo, Kumamoto, Japan).

325 Biotinylated FcRn was purchased from Immunitrack (Copenhagen, Denmark).

326 Binding affinities of antibodies was measured using a Biacore T-200 SPR system (Cytiva,

327 Uppsala, Sweden); a Biotin CAPture Kit was used for CD3 ε / δ ; and EGFR and SA chip

328 were used for FcRn. Antibodies were injected at five concentrations (0.63-50 nM) and a

329 flow rate of 30 μL/min using a single-cycle kinetics method. Binding constants were

330 quantified by analyzing the bivalent binding model using BIA Evaluation software

331 (Cytiva, Uppsala, Sweden).

332 **Flow cytometry**

For flow cytometry, approximately 100,000 cells were placed in a buffer (phosphate buffer saline with 0.2% FBS) and blocked with 2% Human TruStain FcX (Biolegend) for 10 min on ice. Next, cells were incubated with 20 μg/mL AJICAP-BisAb, teplizumab, or cetuximab or isotype control antibody (Ultra-LEAF™ Purified Human IgG1 Isotype Control Recombinant Antibody, Biolegend) for 20 min on ice. The cells were washed twice with buffer and incubated for 20 min on ice with a goat anti-human IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (Thermo Fisher Scientific). The cells were washed twice, resuspended in 500 μL suspension buffer, and analyzed using Attune NxT flow cytometer (Thermo Fisher Scientific).

Molecular Structure analysis

PyMOL (Ver 2.5.4, Schrödinger, Inc.) and Desmond (Ver 2022-2, Schrödinger, Inc.) were used to construct the model structure and MD simulations [13]. Cetuzimab-Fab was constructed using AlphaFold-2 [34 35], and a cross-linker consisting of a PEG spacer and α-haloketone-DBCO was attached to the two Cys residues of Fab. The Fc-FcRn complex was constructed using PDB4n0u[36] as the template, and the Fc portion was mutated to match that of teplizumab. Finally, the AJICAP-BisAb-FcRn complex model structure was constructed by superposing the Lys248 of the Fc-FcRn complex with Lys of cetuximab-Fab. The AJICAP-BisAb-FcRn complex model structure was structurally

stabilized, and molecular dynamics simulations were performed using DESMOND at 310 K for 50 ns.

In-vitro study

Indirect T cell activity was analyzed by measuring the cell-killing efficacy of conditioned medium from a DiFi/T cell co-culture, HT29/T cell co-culture, or SW-620/T cell co-culture, as previously reported [15].

Rat PK study

Rat PK study (ELISA method) was performed as previously reported [21].

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