Enzymatic conjugation using branched linkers for constructing homogeneous antibody–drug conjugates with high potency†

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Antibody–drug conjugates (ADCs) are emerging therapeutic agents in the treatment of cancer, and various conjugation strategies and chemical linkers have been developed to efficiently construct ADCs. Despite previous extensive efforts for improving conjugation efficiency and ADC homogeneity, most ADC linkers developed to date load only single payloads. Branched linkers that can load multiple payload molecules have yet to be fully explored. It is logical to envisage that a multi-loading strategy allows for increase in drug-to-antibody ratio (DAR) with less chemical or enzymatic modification to the antibody structure compared to traditional linear linkers, leading to efficient ADC construction, minimal destabilization of the antibody structure, and enhanced ADC efficacy. Herein, we report that the branched linkers we designed can be quantitatively installed on an anti-HER2 monoclonal antibody by microbial transglutaminase (MTGase)-mediated conjugation without impairing its antigen binding affinity, enabling modular installation of payload molecules and construction of homogeneous ADCs with increased DARs (up to 8). An anti–HER2 antibody–monomethyl auristatin F conjugate constructed using our branched linkers showed greater in vitro cytotoxicity against HER2-expressing breast cancer cell lines than that consisting of linear linkers, demonstrating the effectiveness of the branched linker-based payload delivery. Our finding demonstrates that enzymatic ADC construction using branched linkers is a promising strategy, which may lead to innovative cancer therapeutics.

Introduction

Chemotherapy is one of the major clinical options in treatment of cancer, especially leukemia.1 Although extensive studies have led to improved chemotherapeutic regimens, severe side effects derived from off-target cytotoxicity of chemotherapeutic agents often result in the deterioration of a patient’s quality of life and discontinuation of treatment. Antibody–drug conjugates (ADCs), therapeutic monoclonal antibodies tethered to highly cytotoxic molecules (payloads) through chemical linkers, have emerged as a promising therapeutic format that can circumvent such issues in cancer chemotherapy.2–4 This molecular platform enables the selective delivery of cytotoxic payloads to target cancer cells through antibody–antigen interaction and following internalization, resulting in a broader therapeutic window compared to the use of chemotherapeutic agents alone.5 Long circulation life, preferable biodistribution and pharmacokinetic (PK) profiles of ADCs are also advantageous features from a drug development perspective. Indeed, two ADCs have been approved by the Food and Drug Administration (FDA): Adcetris®, for the treatment of CD30-positive relapsed or refractory Hodgkin’s lymphoma and systemic anaplastic large cell lymphoma;6,7 and Kadcyla®, for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer.8,9 In addition, more than 60 ADCs are in clinical trials as of 2016.4,10

Conjugation methods and chemical linkers are crucial factors determining the PK and stability profiles of ADCs.11 Traditional conjugation methods are lysine–amide coupling and cysteine–maleimide coupling, which are employed for preparing the FDA-approved Adcetris® and Kadcyla®.12,13 While simple and most frequently used, these methods yield ADCs that differ in conjugation sites and drug-to-antibody ratios (DARs). Such heterogeneous ADCs often suffer from increased clearance rates14,15 and require strictly controlled production.
to minimize DAR variation. To overcome this problem, site-specific conjugations have emerged as a means to construct homogeneous ADCs. Junutula and co-workers reported the THIOMAB technology that utilizes two cysteine residues incorporated by genetic engineering for linker conjugation to give ADCs with defined DARs. ADCs obtained by this method showed improved PK profiles and in vivo efficacy compared to heterogeneous ADCs prepared by the traditional cysteine–maleimide coupling. Since then, other methods for constructing homogeneous ADCs including cysteine rebridging, incorporation of non-natural amino acids, and (chemo) enzymatic approaches have been developed. Schibli and co-workers reported an antibody–linker conjugation method using a microbial transglutaminase (MTGase). Through MTGase-mediated transpeptidation, this method covalently attaches the payload by a strain-promoted azide–alkyne cyclization to the side chain of glutamine 295 (Q295) of the human IgG heavy chain using MTGase, followed by installation of the payload by a strain-promoted azide–alkyne cyclization to afford an ADC with a DAR of 2 (linear linker) or 4 (branched linker). MTGase, microbial transglutaminase.

Results and discussion
Assessment of cathepsin B-mediated cleavage of the branched linkers
To identify the rational design of branched ADC linkers that can release two payloads inside the target cancer cell, we synthesized a series of linear and branched fluorescent probes (Fig. 2 and Schemes S1–S4, see ESI† for synthesis details). These model linker units consisted of cathepsin B-cleavable valine–citulline (Val–Cit) with or without polyethylene glycol (PEG) spacers. The dipeptide sequence has been used in many successful ADCs including the FDA-approved ADC Adcetris. The sequence is stable in circulation but in lysosomes it undergoes cathepsin B-mediated cleavage, resulting in the intracellular release of payload.41 We also installed trytophan and 2,4-dinitrophenylethylenediamine (EdDnp) as a fluorophore/quencher(s) pair, which is commonly used in the Förster resonance energy transfer (FRET) assay. We assessed the release of EdDnp from each synthetic model linker unit in the presence of human cathepsin B (Fig. 2 and S1†). We found that the linear PEG (+) probe 2 released EdDnp more efficiently than linear PEG (−) 1. Gratifyingly, the release rate of the branched PEG (+) probe 4 was comparable to that of the linear PEG (+) probe 2. In contrast, the branched PEG (−) probe 3 showed marginal release of EdDnp. We surmise that the structural congestion of the branched probe 3 due to the lack of PEG spacers prevents cathepsin B from accessing Val–Cit moieties. These results clearly illustrate that the spacer is a crucial component for retaining the high responsiveness of the Val–Cit containing linkers to cathepsin B-mediated cleavage, especially in the branched linker format.

Synthesis and conjugation of the branched linkers
With the rational linker design in hand, we set out to construct ADCs containing branched linkers. First, we designed and synthesized branched linkers 5–7 (Fig. 3). These linkers contained (1) a lysine scaffold as a branching point, (2) PEG spacers, (3) a primary amine for MTGase-mediated antibody-linker conjugation, and (4) two azide groups as reaction handles for the following payload installation by the azide–alkyne click reaction (vide infra). We constructed these linkers by sequential amide couplings of each component (Scheme S3†). The azide and primary amine were spatially sequestered with PEG spacers to minimize the steric congestion of the linker arms. In addition, we envisaged that highly hydrophilic PEG spacers could help reduce the hydrophobicity of the ADCs to be con-
constructed, which is crucial to prevent protein aggregation. Indeed, PEG chains installed on the payload terminus or the linker reportedly prevent ADCs forming non-covalent oligomers.

Next, we performed a conjugation of the branched linkers synthesized to an anti-HER2 IgG1. In this study, we used an engineered anti-HER2 monoclonal antibody (mAb) with a mutation of the asparagine 297 of the heavy chain into alanine (N297A), which was developed by our group. This mutation allowed us to omit the removal of the N-glycan chain on the asparagine 297, a required step for MTGase-mediated antibody-linker conjugation. We attempted to install branched linkers 5–7 onto the N297A anti-HER2 mAb according to the reported protocol (linker: 80 equiv., antibody: 1.0 mg mL$^{-1}$, MTGase: 6.7 unit per mg$^{-1}$ antibody). However, the conversion rates were unsatisfactory (50–79%, entries 1–3 in Table 1 and Fig. S2 and S3†), resulting in mixtures of somewhat heterogeneous antibody–linker conjugates. The bulkiness of primary amine-containing molecules often leads to low efficiency in MTGase-mediated protein labeling. Indeed, Schibli and co-workers used a simple linear linker for ADC construction to achieve quantitative conversion. This finding made us recognize that the reaction conditions needed to be optimized to attach our bulky branched linkers to the N297A anti-HER2 mAb in an efficient manner. Thus, we screened various reaction conditions using the branched linker 5, the most reactive linker of the three. We found that the amount of MTGase did not show a significant impact on the conversion rate (entry 4). In contrast, a higher concentration of the N297A anti-HER2 mAb substantially improved the conjugation efficiency (entry 5). In addition, increasing the amount of linker 5 turned out to be effective for improving the conversion rate (entries 6 and 7).

We further examined various reaction conditions, and finally found effective conditions enabling nearly quantitative conjugation (entries 8 and 9).

Incubation of the reaction mixture at 37 °C overnight resulted in partial loss of the product probably due to protein denaturing. Thus, we decided to perform the linker conjugation in the following sections at room temperature. The...
optimal reaction conditions also enabled highly efficient conjugation of branched linkers 6 and 7 to the N297A anti-HER2 mAb (entries 10 and 11). This success demonstrates that the MTGase-based transpeptidation can efficiently conjugate even bulky linkers to antibodies under optimal conditions. In addition, this finding is encouraging because additional modifications of linker structure may be adopted to fine-tune ADC physicochemical properties and further increase DARs. Our results also indicate that the MTGase-mediated transpeptidation could be used more generally for various protein modifications.

**Payload installation by click chemistry and stability study**

Finally, we coupled the N297A anti-HER2 mAb–branched linker 5 conjugate obtained and the potent antimitotic agent monomethyl auristatin F (MMAF), a relatively hydrophilic payload frequently used in ADCs. We employed the strain-promoted azide–alkyne cycloaddition (copper-free click reaction) using the MMAF module 8 containing dibenzocyclooctyne (DBCO), PEG spacer, Val–Cit, and p-aminobenzyloxycarbonyl (PABC) (Fig. 4a). PABC was incorporated to allow for traceless release of MMAF upon cathepsin B-mediated cleavage of Val–Cit. The anti-HER2 mAb–linker 5 conjugate (4.0 mg mL\(^{-1}\)) was incubated with DBCO–MMAF 8 (1.5 equiv. per reaction site) in PBS/4% DMSO. The click reaction reached full completion within 1 h to give nearly homogeneous ADC 9 with an average DAR of 3.9 (determined based on UV traces, Fig. 4b, c, S4, and S5†). We also prepared two control ADCs in the same manner: an N297A anti-HER2 mAb–MMAF conjugate containing linear linkers (linear ADC 10, DAR: 1.9)\(^{31}\) and an N297A non-targeting IgG conjugated with MMAF through the branched linker 5 (non-targeting branched ADC 11, DAR: 3.9).

The cleavable branched linkers installed on ADC 9 were stable under physiological conditions; no significant degradation of the linkers was observed in human plasma at 37 °C after 7 days, indicating that the DAR did not significantly change during incubation (Fig. 5†). Furthermore, size-exclusion chromatography (SEC) analysis revealed that ADC 9 existed predominantly in the monomer form (Fig. 4d). These results support the validity of our linker design from a drug development perspective. It has been reported that N297Q mutated IgGs provide four conjugation sites (Q295 and Q297 per heavy chain) for the MTGase-mediated transpeptidation, enabling the installation of four linear linker–payload modules to IgGs.\(^{27,47}\) Gratifyingly, we found that the branched linker 5 could be installed on a N297Q anti-HER2 mAb. Following click conjugation with MMAF module 8 yielded a high-loading ADC with an average DAR of 7.4 (Fig. S8†). Although the obtained ADC contained a small amount of lower DAR species, its homogeneity is higher than general ADCs constructed by traditional lysine or cysteine coupling. We believe that the optimization of the branched linker structure and reaction conditions for N297Q mAb-based conjugation will allow for the construction of highly homogeneous, higher DAR ADCs using our methodology. Such an effort will be reported from our laboratory in due course.

**Evaluation of the ADCs for antigen binding and cytotoxicity**

We evaluated anti-HER2 ADCs 9 and 10 for binding affinity and specificity to the HER2 in cell-based ELISA assays using the human breast cancer cell lines SKBR-3 (HER2 positive) and MDA-MB-231 (HER2 negative) (Fig. 5 and S9†). Branched ADC 9 showed a high binding affinity to SKBR-3, comparable to those of linear ADC 10 and the parent N297A anti-HER2 mAb (K\(_D\) = 0.98, 1.12, and 0.64 nM, respectively). In contrast, non-targeting ADC 11 did not show HER-2 specific binding. None of the ADCs bound to MDA-MB-231. These results demonstrate that the branched linker-MMAF moieties within ADC 9 do not

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**Table 1** MTGase-mediated antibody–branched linker conjugation

<table>
<thead>
<tr>
<th>Entry</th>
<th>Linker [equiv.]</th>
<th>Linker</th>
<th>Antibody (mg mL(^{-1}))</th>
<th>Temperature (°C)</th>
<th>Conversion(^b) (%)</th>
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<td>7</td>
<td>6.2</td>
<td>r.t.</td>
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\(a\) MTGase (26.9 U mg\(^{-1}\) antibody). \(b\) Determined based on deconvoluted ESI-mass spectra. \(c\) After 3 h. Partial loss of the product was observed after overnight incubation.
impact the antigen recognition and specificity. As previously reported, the conjugation site Q295 in the Fc moiety is distant from the antigen recognition site in the Fab region, allowing for the MTGase-based conjugation of linker–payload components at this position without detrimental effects on the antigen binding. Our results are consistent with this observation.

To investigate how the branched linker-based conjugation influences cell killing potency, we tested ADCs 9–11 and the parent N297A anti-HER2 mAb in cell-based assays using three human cell lines with varying HER2 expression levels: SKBR-3 (HER2++), MDA-MB-453 (HER2+), and MDA-MB-231 (HER2−) (Fig. 6). Branched ADC 9 (DAR: 3.9) exerted greater potency than linear ADC 10 (DAR: 1.9) in SKBR-3 (EC50: 0.36 nM and 0.83 nM, respectively) whereas non-targeting ADC 11 and the unmodified N297A anti-HER2 mAb showed marginal cytotoxicity. The 2.3-fold difference between the EC50 values of ADCs 9 and 10 was statistically significant (confirmed by the extra sum-of-squares F test, \( p < 0.0001 \)). In addition, the maximum cell killing effect of ADC 9 (85% cell killing at 5.3 nM) was higher than that of ADC 10 (71% cell killing at 13.3 nM) with a statistically significant difference (Student’s t test, \( p < 0.005 \)). We also observed the enhancement of ADC efficacy in the moderately HER2-positive cell line MDA-MB-453; the
towards lower concentrations compared to that of linear ADC following click chemistry-based coupling of MMAF molecules HER2 mAb using MTGase under optimized conditions. The branched linkers containing a primary amine and two azide groups could be installed on Q295 of an anti-HER2 mAb using click chemistry reactions. Testing with various mAbs and payloads will reveal the applicability of our technology. In addition, testing of the prototype ADC constructed in this study for in vivo pharmacokinetics, stability, and efficacy will provide more detailed information to fine-tune the structure (e.g., length of the PEG spacers) and physicochemical properties of the branched linker–payload component toward clinically relevant ADCs. Such an effort will be of crucial importance to load more hydrophobic payloads than MMAF (e.g., monomethyl auristatin E and maytansinoids) as high-DAR ADCs containing hydrophobic payloads reportedly suffer from aggregation as well as rapid clearance.\textsuperscript{43} Along with optimization of the branched linker structure, the reported methods for masking payloads using external long PEG chains may help reduce such risks.\textsuperscript{18,44}

As a more interesting approach, installing two different reaction handles on the branched linker scaffold could enable the facile construction of ADCs loading two different payloads through click chemistry-based orthogonal couplings. Such a heterologous loading strategy will yield ADCs with dual modes of action, which is challenging to achieve with traditional linear linkers in terms of ADC homogeneity. The dual-loading ADC is an emerging format exemplified recently for combating drug-resistant tumors.\textsuperscript{40} Altogether, the enzymatic ADC construction using branched linkers is a promising strategy for developing potent ADCs, which may lead to innovative cancer therapeutics in the future.

Conflict of interest
The authors declare no competing financial interest.

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Notes and references