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Exploring the effects of linker composition on site-specifically modified antibody–drug conjugates

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ABSTRACT

In the context of antibody–drug conjugates (ADCs), noncleavable linkers provide a means to deliver cytotoxic small molecules to cell targets while reducing systemic toxicity caused by nontargeted release of the free drug. Additionally, noncleavable linkers afford an opportunity to change the chemical properties of the small molecule to improve potency or diminish affinity for multidrug transporters, thereby improving efficacy. We employed the aldehyde tag coupled with the hydrazino-*iso*-Pictet-Spengler (HIPS) ligation to generate a panel of site-specifically conjugated ADCs that varied only in the noncleavable linker portion. The ADC panel comprised antibodies carrying a maytansine payload ligated through one of five different linkers. Both the linker-maytansine constructs alone and the resulting ADC panel were characterized in a variety of in vitro and in vivo assays measuring biophysical and functional properties. We observed that slight differences in linker design affected these parameters in disparate ways, and noted that efficacy could be improved by selecting for particular attributes. These studies serve as a starting point for the exploration of more potent noncleavable linker systems.

1. Introduction

Antibody–drug conjugates (ADCs) promise to alter the landscape of anti-cancer therapeutics by targeting highly cytotoxic drug molecules directly to cancer cells. The success of currently approved ADCs has inspired a spate of research and development efforts in the area; dozens of new ADCs are in pre-clinical or clinical trials [1]. ADCs comprise a monoclonal antibody, a cytotoxic payload, and a linker that joins them together [2]. The monoclonal antibody targets the payload to cells expressing the antigen on their surface, and the cytotoxic payload kills the cells upon internalization of the ADC. The linker is literally the central component of an ADC; it contains the reactive group that governs the conjugation chemistry, and serves as a chemical spacer that physically connects the drug payload to the antibody. As such, the linker is also the most versatile aspect of the ADC. It can be modified in any number of ways to influence various drug/linker characteristics (e.g., solubility) [3,4] and ADC properties (e.g., potency, pharmacokinetics, therapeutic index, and efficacy in multidrug resistant cells) [5–11].

There are essentially two broad classes of ADC linkers; those that are chemically labile or enzymatically-cleavable, and those that are chemically stable or noncleavable [12]. Labile/cleavable linkers are designed to keep the ADC intact when in circulation but release the drug payload upon internalization by the target cell. Some cytotoxic payloads—for example, MMAE—require a cleavable linker, as they do not tolerate substitutions [13,14]. By contrast, other cytotoxic payloads—for example, maytansine—can accommodate substitutions while maintaining potency [15]. Such drugs are good substrates for the development of noncleavable linkers. By design, noncleavable linkers do not contain chemical functionalities that are readily susceptible to intracellular degradation. Therefore, after an internalized ADC is trafficked to the lysosome, the antibody moiety is proteolytically degraded into amino acids while the cytotoxic drug remains attached via the linker to an amino acid residue [16]. The retention of the linker as part of the active metabolite allows for the modulation of the overall properties of the metabolite (e.g., by altering hydrophobicity, length, and charge) in order to improve potency.

We previously reported a novel site-specific ligation chemistry that takes advantage of an aldehyde-tagged protein [17]. The aldehyde tag is a straightforward means of site-specifically functionalizing proteins for chemical modification. The genetically-encoded tag consists of a pentapeptide sequence (CXPXR) that is

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specifically recognized by formylglycine-generating enzyme (FGE) [18-20]. During protein expression in cells, the cysteine residue in the sequence is recognized by FGE and oxidized co-translationally to formylglycine. The resulting aldehyde affords a bioorthogonal chemical handle for ligation (Fig. 1). Linkers terminating in a 2-((1,2-dimethylhydrazinyl)methyl)-1*H*-indole react with the aldehyde by way of a hydrazino-*iso*-Pictet-Spengler (HIPS) reaction to form an azacarboline, resulting in a stable C–C bond joining the antibody and payload.

The aldehyde tag platform allows for site-specific conjugation that yields a highly homogenous product. Accordingly, this technology is well-suited for performing structure activity relationship studies in the context of an intact ADC. Here, we isolated linker composition as a single variable for optimization while the other ADC components—antibody backbone, cytotoxic payload, conjugation site, drug-to-antibody ratio, and conjugation chemistry—were held constant. By characterizing a panel of five drug/linkers and their corresponding conjugates, we explored the impact of small changes in linker design on ADC potency and stability.

2. Materials and methods

2.1. Linker synthesis

Synthetic routes and analytical data are provided in the Supplemental materials.

2.2. Microtubule polymerization assay

We used the Tubulin Polymerization Assay Kit (Cytoskeleton) according to the manufacturer's instructions for the fluorescence-based test. All test articles were used at 3 μM.

2.3. Direct ELISA antigen binding

Maxisorp 96-well plates (Nunc) were coated overnight at 4 °C with 1 μg/mL of human HER2-His (Sino Biological) in PBS. The plate was blocked with ELISA blocker blocking buffer (ThermoFisher), and then the αHER2 wild-type antibody and ADCs were plated in an 8-step series of 2-fold dilutions starting at 100 ng/mL. The plate was incubated, shaking, at room temperature for 2 h. After washing in PBS 0.1% Tween-20, bound analyte was detected with a donkey anti-human Fc-γ-specific horseradish peroxidase (HRP)-conjugated secondary antibody. Signals were visualized with Ultra TMB (Pierce) and quenched with 2 N H_2SO_4. Absorbance at 450 nm was determined using a Molecular Devices SpectraMax M5 plate reader and the data were analyzed using GraphPad Prism.

2.4. Bioconjugation, purification, and HPLC analytics

Humanized anti-HER2 IgG antibodies (15 mg/mL) bearing the aldehyde tag (LCTPSR) at the C-terminus of the heavy chain were conjugated to maytansine-containing drug linkers (8 mol equivalents drug:antibody) for 72 h at 37 °C in 50 mM sodium citrate, 50 mM NaCl pH 5.5 containing 0.85% DMA and 0.085% Triton X-100. Free drug was removed using tangential flow filtration. Unconjugated antibody was removed using preparative-scale hydrophobic interaction chromatography (HIC; GE Healthcare 17-5195-01) with mobile phase A: 1.0 M ammonium sulfate, 25 mM sodium phosphate pH 7.0, and mobile phase B: 25% isopropanol, 18.75 mM sodium phosphate pH 7.0. An isocratic gradient of 33% B was used to elute unconjugated material, followed by a linear gradient of 41–95% B to elute mono- and diconjugated species. To determine the DAR of the final product, ADCs were examined by analytical HIC (Tosoh #14947) with mobile phase A: 1.5 M ammonium sulfate, 25 mM sodium phosphate pH 7.0, and mobile phase B: 25% isopropanol, 18.75 mM sodium phosphate pH 7.0. To determine aggregation, samples were analyzed using analytical size exclusion chromatography (SEC; Tosoh #08541) with a mobile phase of 300 mM NaCl, 25 mM sodium phosphate pH 6.8.

2.5. In vitro cytotoxicity

The HER2-positive breast carcinoma cell line, NCI-N87, was obtained from ATCC and maintained in RPMI-1640 medium (Cellgro) supplemented with 10% fetal bovine serum (Invitrogen) and Glutamax (Invitrogen). 24 h prior to plating, cells were passaged to ensure log-phase growth. On the day of plating, 5000 cells/well were seeded onto 96-well plates in 90 μL normal growth medium supplemented with 10 IU penicillin and 10 μg/mL streptomycin (Cellgro). Cells were treated at various concentrations with 10 μL of diluted analytes, and the plates were incubated at 37 °C in an atmosphere of 5% CO_2. After 6 d, 100 μL/well of CellTiter-Glo reagent (Promega) was added, and luminescence was measured using a Molecular Devices SpectraMax M5 plate reader. GraphPad Prism software was used for data analysis, including IC_{50} calculations.

2.6. In vitro stability

ADCs were spiked into rat plasma at ~1 pmol (payload)/mL. The samples were aliquoted and stored at −80 °C until use. Aliquots were placed at 37 °C under 5% CO_2 for the indicated times and then were analyzed by ELISA to assess the anti-maytansine and anti-Fab signals. A freshly thawed aliquot was used as a reference starting value for conjugation. All analytes were measured together on one plate to enable comparisons across time points. First, analytes were

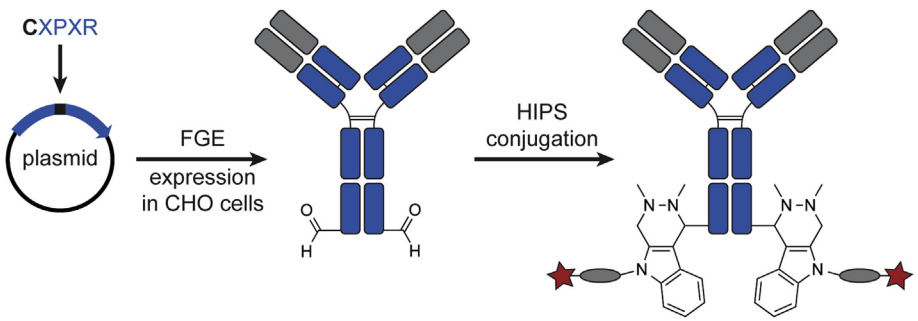

Fig. 1. The aldehyde tag coupled with HIPS ligation yields site-specifically modified antibodies. Using standard molecular biology techniques, a formylglycine-generating enzyme (FGE) recognition sequence (CXPXR) is site-specifically inserted into the backbone of the antibody. FGE co-translationally oxidizes the cysteine residue to formylglycine. The aldehyde of formylglycine can then be reacted with nucleophiles to form a stable C–C bond.

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