Site Specific Dual Modification of Native Antibodies Via Microbial Transglutaminase

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Abstract:

Antibody-drug conjugates (ADCs) are a method of targeted drug delivery. Past research has created ADCs with a drug-to-antibody ratio of two. The aim of this research is to create a bifunctional linker that could attach two chemically distinct compounds twice. The designed linker structures were then attached to a Herceptin antibody, which targets the Her2 receptor, and tested via SDS-PAGE for addition. Results showed that a cross-linker with an ethylenedioxy spacer was the most effective linker. Using FRET, the tetrazine functional group had a rate constant of 31,333 $\frac{1}{M*s}$, while the azide functional group did not have a clear rate constant. An MTS toxicity assay showed that the antibody-drug conjugate was more toxic than the free drug. Further studies utilizing more structures with varying PEG lengths are needed to show a strong correlation between structure and conjugation efficiency.

Summary of Research:

Antibodies are naturally occurring proteins that attack specific targets, called antigens. In recent years, these antibodies have been used medicinally as antibody-drug conjugates, in which a linker molecule is attached to the antibody [1]. This allows a drug compound to attach to the linker so that it can be delivered specifically to the desired target. However, traditional antibody-drug conjugates created using the enzyme-modification method use a linker with only one functional group, and therefore can only attach one type of chemical compound [2]. Since most medicinal drug therapies use more than one chemical compound, its usefulness is very limited.

The aim of this research is to address these problems by creating a bifunctional, bioorthogonal linker molecule with tetrazine and azide functional groups. Since both groups can attach drug compounds, the linker allows for two chemically-distinct drug compounds to be attached per linker. Figure 1 presents the synthesis scheme for both linkers, which are distinguished by the spacer between the primary amine and tertiary amide functional groups. This is to test the effect of steric hindrance caused by the tetrazine and azide functional groups.

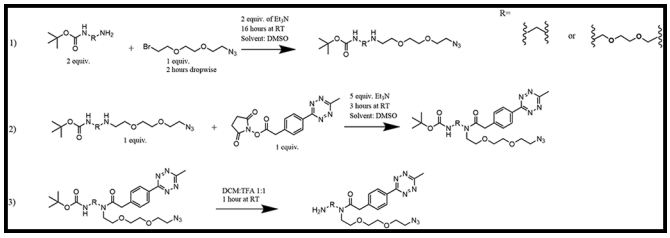

Figure 1: Synthesis scheme for both linker structures.

Once synthesized, the linkers were attached to the Herceptin antibody. This was completed by reacting the antibodies with Peptide:N-glycosidase F, which removes the native glycan at asparagine 297. The antibodies were then reacted with the desired linker and microbial transglutaminase, which attached the linkers to the antibody's conjugation site at glutamine 295.

Figure 2 gives a visual overview of this process.

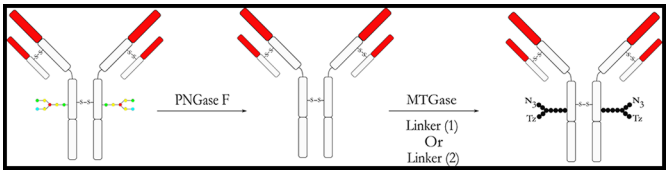

Figure 2: A general overview for the attachment of linkers. After the addition of PNGase F, the native glycan is removed at asparagine 297. The MTGase then attaches the linker to glutamine 295.

For confirmation, the antibody-linker conjugates were placed in an SDS-PAGE gel, which separates the components in solution based on molecular weight. To aid in separation, the antibody-linker conjugates were reacted with two polymers: DBCO- and TCO-modified 5,000 molecular weight polyethyleneglycol (PEG). DBCO-PEG attaches to azide functional groups, while TCO-PEG attaches to tetrazine functional groups. As control groups, two other antibody-linker conjugates were introduced to the gel: one with only an azide functional group, and one with only a tetrazine functional group. When the tags are introduced, only the DBCO will react with the azide linker, and only the TCO will react with the tetrazine linker. Both tags will react with the bifunctional linker, so an even larger shift will be observed, thereby confirming the presence of the bifunctional linker.

The kinetics of the reaction were measured using Förster resonance energy transfer. FRET uses two spectrally matched fluorophores and measures their energy transfer. Carboxyrhodamine-DBCO was used for the azide functional group, while Cy5-TCO was used for the tetrazine functional group. In this experimental setup, carboxyrhodamine-DBCO was initially attached and Cy5-TCO was introduced in solution; measuring the energy transfer allowed for a measurement for the rate of addition. In another experiment, Cy5-TCO was initially attached, and carboxyrhodamine-DBCO was introduced to measure the rate of the DBCO-Azide reaction.

Finally, a toxicity test was conducted using an MTS assay. In this assay, the antibody-drug conjugate was introduced to SKOV7 cells. While incubated at 37°C for a period of four days, the antibody-drug conjugate killed cells. Then, MTS was added, which is broken down in live cell mitochondria to produce formazan, which has an absorbance. By measuring the difference in absorbance between a control solution and the solution with antibody-drug conjugate introduced, the toxicity of the antibody-drug conjugate can be assessed.

Results and Conclusions:

Synthesis of both linkers was successful. However, only the ethylenedioxy linker conjugated to the antibody. Kinetic testing with the tetrazine functional group on the

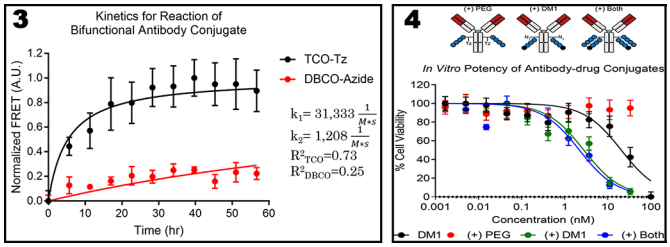

Figure 3, left: *Kinetics data for the ethylenedioxy linker. The addition of Cy5-TCO had a rate coefficient of 31,333 1/(M*s), which corresponds to literature values. The addition of carboxyrhodamine-DBCO failed, most likely due to low concentration.* ***Figure 4, right:*** *MTS toxicity assay data. Note that the antibody-drug conjugate was more toxic than the free drug.*

ethylenedioxy linker gave a rate constant of $31{,}333\,\frac{1}{M*s}$, which corresponds to an accepted literature value of $\sim 30{,}000\,\frac{1}{M*s}$ for a linker utilizing only the tetrazine functional group (Figure 3). Finally, the MTS assay showed that the antibody-drug conjugate carrying the drug compound DM1, a maintansine derivative, was more toxic than the free drug in solution. (Figure 4).

Future Work:

While the results show that an effective bifunctional linker can be synthesized and conjugated, further exploration on the effect of structure needs to be completed. Two structures are not comprehensive enough to make strong connections between structure and conjugation efficiency. More structures, with different amounts and locations of PEG spacing, should undergo the tests described above.

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References:

[1] Caddick, S.; Chudasama, V.; and Maruani, A. Recent Advances in the Construction of Antibody-Drug Conjugates. Nature Chemistry, 2016, 8, pp. 114-119.

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