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Optimizing Conditions for the Conjugation of Unusual Substrates to IgG1 Antibodies Using Microbial Transglutaminase

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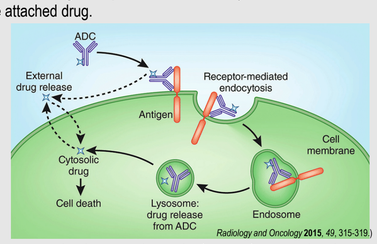
Abstract

Antibody drugs conjugates (ADCs) are an increasingly important targeted drug-delivery technology for the treatment of cancer. Site-specific ADCs are particularly advantageous due to the homogeneous nature of drug attachment, consistent drug-to-antibody ratio (DAR) and improved pharmacokinetic, efficacy and safety profile. Microbial transglutaminase is frequently used for preparation for these ADCs because it facilitates the facile formation of a specific peptide bon between alkyl amines and the amide side change of exposed glutamine residues. Previously, it was believed that the only viable substrates for transglutaminase were alkyl amines. However, we have recently found that non-amine substrates, such as hydrazines and hydrazides, are also suitable substrates for transglutaminase. These newfound substrates result in more stable products and allow for a greater diversity of drugs to be attached to ADCs. Herein, we describe the optimization of reactions conditions for the TG-mediated coupling of non-amine substrates to IgG1 antibodies.

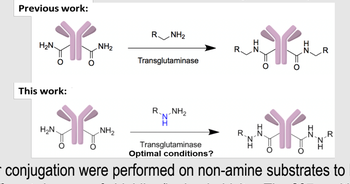
Initial conditions resulted in poor loading for many of the hydrazine and hydrazide substrates (DAR ~ 0.3). Optimization of temperature, pH, equivalents of linker payload/non-amine substrate, equivalents of transglutaminases, percentage of DMSO, and concentration of antibody. This resulted in a dramatic increase in reaction efficiency, resulting in a DAR of ~2, the maximum loading for transglutaminase-mediated site-specific conjugations. Loading of the conjugates onto a light-chain mutant of trastuzumab was evaluated using high-pressure liquid chromatography (HPLC) and mass spectrometry.

Introduction

Antibody drug conjugates (ADCs) are a targeted agents that deliver a drug only to cells that express antigens recognized by the cognate antibody. This type of delivery system is especially useful for the cytotoxic drugs since it helps to alleviate off-target and side effects. ADCs enter the cell through receptor-mediated endocytosis and are then trafficked to the lysosome, where they are degraded to release the attached drug.

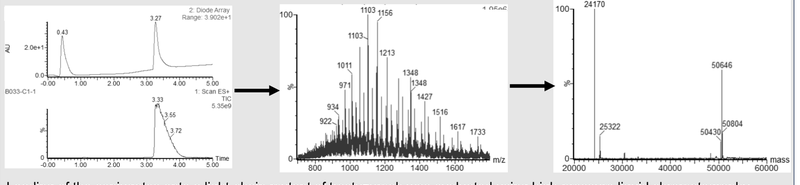


Traditionally, ADCs have been generated through stochastic (random) conjugation to exposed lysine and cysteine residues. However, in recent years most ADC development has shifted to site-specific technology in order to maximize pharmacokinetics and safety. One common method of synthesizing a site-specific ADC is via microbial transglutaminase-mediated conjugation. Previously, it was believed that the only substrates for microbial transglutaminase (mTG) were alkyl amines that resemble the side chain of lysine. We have found that hydrazines and hydrazides are also suitable substrates for mTG. Based on previously reported model studies, the resulting linkage to hydrazines and hydrazides may provide a more stable bond than alkyl amines. Moreover, use to hydrazine and hydrazide substrates allows for a larger diversity of drugs to be attached to ADCs.



Optimization of conditions for conjugation were performed on non-amine substrates to bring the DAR from ~0.3 to 2.0. Differing sites on the antibody offer different degrees of shielding/hydrophobicity. The 297 position (near the antibody glycosylation site) is particularly shielded and has proven to be an effective site in many ADCs. We therefore evaluated two permutations of this site using the optimized chemistry

Methods



Loading of the conjugates onto a light-chain mutant of trastuzumab was evaluated using high-pressure liquid chromatography (HPLC) and mass spectrometry. Food-grade mTG used is 99% inactive excipients by weight obtained from Ajinimoto. Antibodies used were a gift from Pfizer. A 20 uL sample of the antibody or ADC is treated with a reducing agent, such as TCEP, in order to separate the light and heavy chains. Both a UV and a mass spectroscopy (ES+) data was collected. The antibody ionization results in a charge envelope due to different m/z ratios that correspond to the various ionization states of the protein. Deconvolution software is used to resolve the charge envelope into a singly-charged heavy chain (~50kd) and light chain (~25kd) peak.

Optimization of Trastuzumab LC-LLQG using Thiosemicarbazide

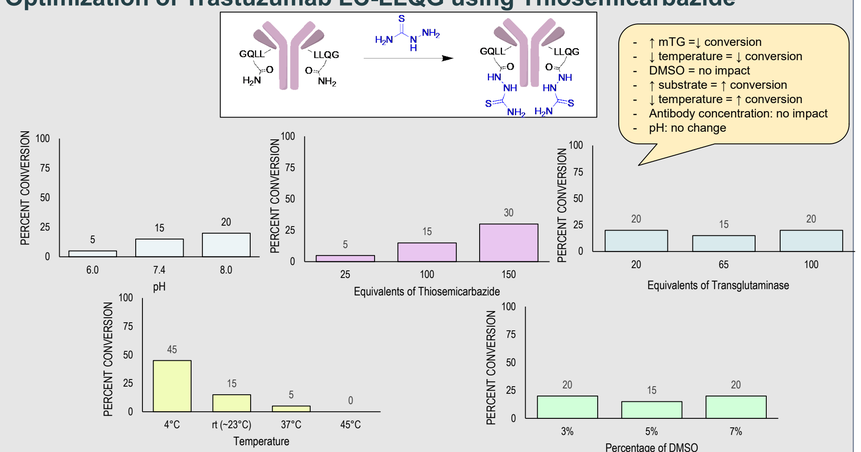


Figure 1-5. Results of optimization of using parent conditions: 1 mg/mL Tras LC-LLQG, pH 7, 100 eq thiosemicarbazide, 65 eq TG (w/w %), 5% DMSO (v/v) at room temperature for 24 hours. Loading was determined using LCMS as outlined above.

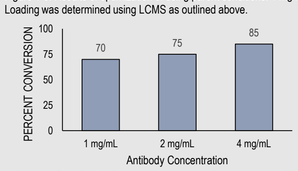


Figure 6. Results of optimization for varying antibody concentration using parent conditions: pH 7, 4° C, 300 eq thiosemicarbazide, 65 eq TG, 0% DMSO (v/v), varying antibody concentration. Loading of ADCs were determined using LCMS as outlined above.

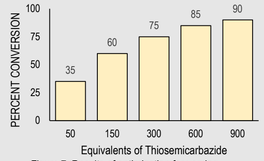


Figure 7. Results of optimization for varying thiosemicarbazide concentration using parent conditions: 1mg/mL Tras LC-LLQG, pH 7.4, 4°C, 65 eq mTG, 0% DMSO. Loading was determined by LCMS.

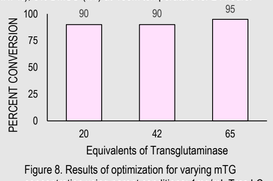


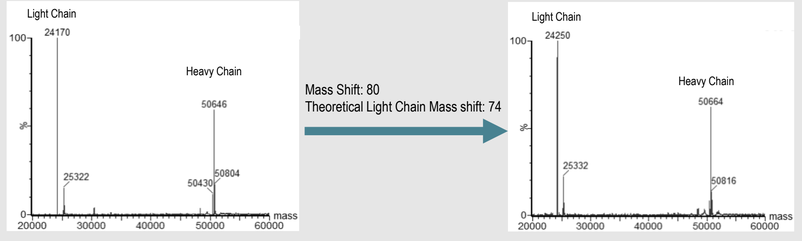
Figure 8. Results of optimization for varying mTG concentration using parent conditions: 1mg/mL Tras LC-LLQG, pH 7.4, 4° C, 900 eq TSC, 0% DMSO. mTG used is 99% inactive excipients by weight from Ajinimoto. Loading was determined by LCMS.

Optimization Conditions

Optimization Parameter	[Tras LC-Q] (mg/mL)	pH	Temp. (°C)	Wt. eq. mTG	[TSC] (μM)	Molar eq.[b] TSC	% DMSO	% Conjugation
pH	1	**6**	RT (~22°C)	65	667	50	5	5
pH	1	**7.4**	RT	65	667	50	5	15
pH	1	**8**	RT	65	667	50	5	20
Temp.	1	7.4	**45**	65	667	50	5	0
Temp.	1	7.4	**37**	65	667	50	5	5
Temp.	1	7.4	**RT**	65	667	50	5	20
Temp.	1	7.4	**4**	65	667	50	5	45
[mTG]	1	7.4	4	**20**	667	50	0	45
[mTG]	1	7.4	4	**65**	667	50	0	45
[mTG]	1	7.4	4	**130**	667	50	0	40
[mTG]	1	7.4	4	**195**	667	50	0	40
[Substrate][a]	1	7.4	4	65	**335**	**25**	0	35
[Substrate]	1	7.4	4	65	**1050**	**75**	0	60
[Substrate]	1	7.4	4	65	**2010**	**150**	0	75
[Substrate]	1	7.4	4	65	**4020**	**300**	0	85
[Substrate]	1	7.4	4	65	**6030**	**450**	0	90

[a]Substrate = TSC = Thiosemicarbazide. [b]Molar eq. TSC is relative to the number reactive Gln of Tras LC-Q (2 per antibody).

Figure 9. All optimization conditions for Trastuzumab LCQ05 and thiosemicarbazide coupling



Unloaded Loaded

Final Optimized conditions included 1 mg/mL Trastuzumab LCQ05, 900 eq thiosemicarbazide, pH 7, 65 eq mTG and 0% DMSO at 4℃ for 24 hours.

Assorted Substrates

A variety of hydrazides, hydrazines, and hydroxylamines are suitable substrates for mTG conjugation. Condition 1 was used to show which were suitable for conjugation .Conditions 2 is the improved conditions that allowed the efficient conjugation of substrates ,such as thiosemicarbazide and propanoic hydrazide, that were not amenable to conjugation under the initial conditions.

Conditions 1 consisted of 1 mg/mL Tras LC-LLQG, pH 7, 100 eq thiosemicarbazide, 6.5 eq TG (w/w %), 5% DMSO (v/v) at 22°C for 24 hours.
Condition 2 used 1 mg/mL Tras LC-LLQG, pH 7, 900 eq thiosemicarbazide, 65 eq TG (w/w %),0% DMSO (v/v) at 4°C for 24 hours.
Loading was determined using LCMS as outlined above

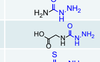
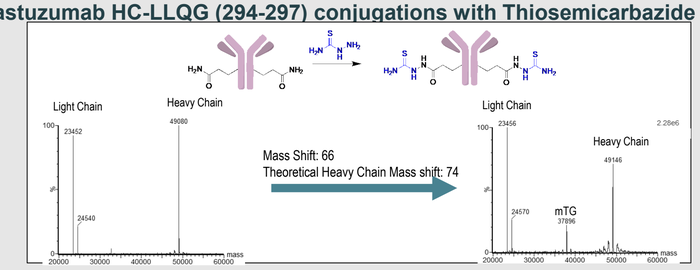
Substrate	Structure	Condition 1	Condition 2
Propanoic hydrazide		57	84
Cyanoacetohydrazide		94	ND
Benzoic hydrazide		100	ND
Semicarbazide		86	100
(Hydrazinecarbonyl)glycine		67	95
Thiosemicarbazide		16	95
p-Toluenesulfonyl hydrazide		0	10
4-hydrazinobenzoic acid		0	ND
Benzylhydrazine		77	85
o-Ethyl hydroxylamine		62	100
o-Benzylhydroxylamine		98	ND
Phenyl hydrazine		99	ND

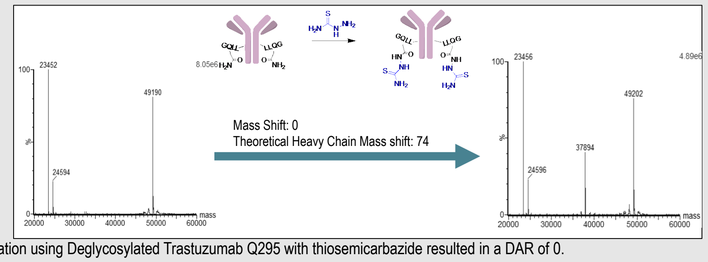
Figure 10. Comparison of optimized versus unoptimized mTG conjugations for varying substrates.

Trastuzumab HC-LLQG (294-297) conjugations with Thiosemicarbazide



Conjugation using Trastuzumab HC-LLQG (294-297) with thiosemicarbazide resulted in a DAR of 2.0.

Deglycosylated Trastuzumab Q295 conjugations with Thiosemicarbazide



Conjugation using Deglycosylated Trastuzumab Q295 with thiosemicarbazide resulted in a DAR of 0.

Conclusions

Optimization of hydrazine substrates resulted in successful conjugation for Trastuzumab LCQ05 and Trastuzumab HC-LLQG (294-297). However, conjugations using Trastuzumab Q295 were unsuccessful.

Initial couplings of Tras LCQ05-thiosemicarbazide with a maleimide resulted in moderate DAR. Future work requires optimization of the reaction with the maleimide, so hydrazine substrates can be utilized to expand the linker library for a more stable and more diverse ADCs.

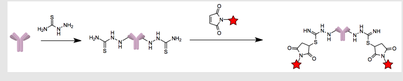


Figure 11. Reaction scheme of the coupling of Tras LCQ05 with thiosemicarbazide followed by reaction with a maleimide.

Acknowledgements

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References

1. Strop, P., Liu, S. H., Dorywalska, M., Delaria, K., Dushin, R. G., Tran, T. T., ... & Zhou, D. (2013). Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. Chemistry & biology, 20(2), 161-167.