Novel IgG1 Cysteine Insertion Sites Enable Site-Specific Conjugation and Precise Control of Drug to Antibody Ratio

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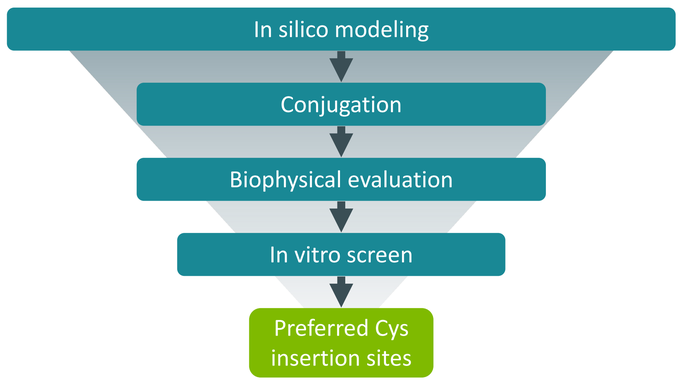
Introduction

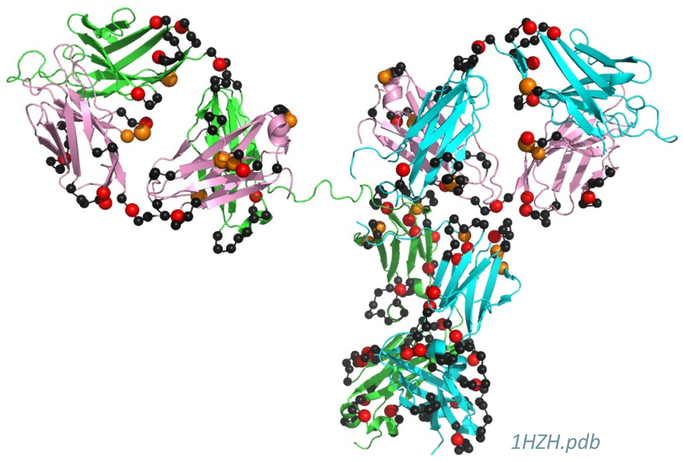
Background

- Engineering antibodies to include cysteine residue insertions allow for site-specific (SS) conjugation to thiol reactive small molecules to generate antibody drug conjugates (ADCs).[1]
- The insertion of cysteine residues into an antibody can impact important properties, such as structure, function, and expression.
- Important ADC properties including deconjugation rate, susceptibility to payload metabolism and overall hydrophobicity can be impacted by the location of the inserted cysteine residue.
- The Azymetric™ technology permits heterodimeric Fc pairing and asymmetric combination of one or more cysteine insertion sites.

Approach



Cys Insertion Sites Considered



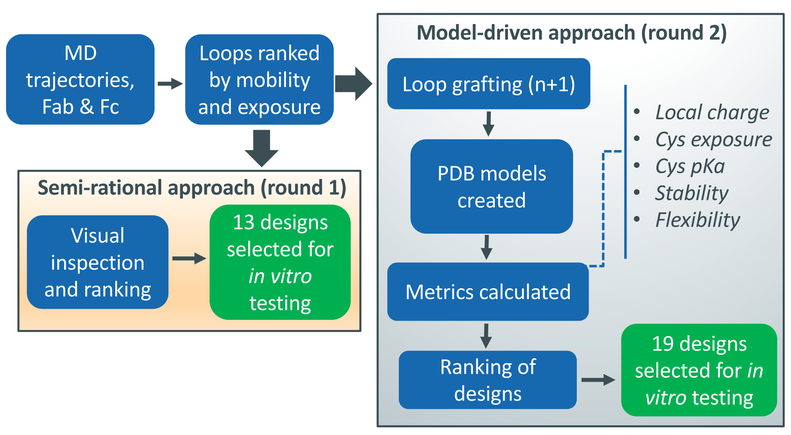
1HZH.pdb

***Black**: Cys insertion sites evaluated in silico*
Red + Orange: Cys insertion antibodies expressed and evaluated as ADCs
Orange: preferred Cys insertion sites identified

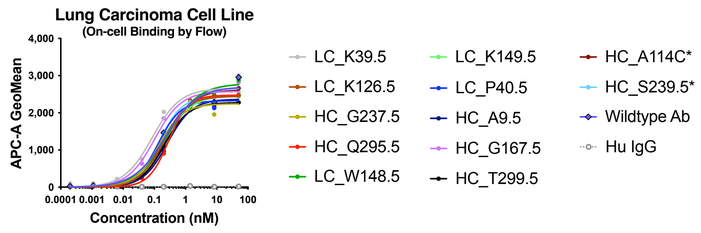
*Benchmark engineered antibodies from MedImmune[1] (C239i or S239.5, cysteine insertion between S239 and V240) and Genentech (Thiomab™ HC_A114C,[2] HC_S239C,[3] LC_K149C[4]) were also evaluated

In silico Approach Predicts Positions in Loop Regions Amenable to Cysteine Insertion

- A combination of semi-rational and model-driven in silico approaches led to the selection of 32 Cys insertion designs for expression, conjugation and characterization as ADCs

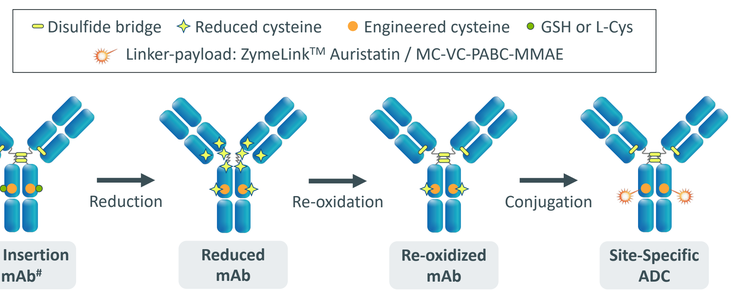


Binding is Unaffected by Cys Insertion



Robust Conjugation Protocol Affords Site-Specific ADCs

Conjugation Protocol



#Engineered cysteines are typically in oxidized form, capped with GSH or L-Cys

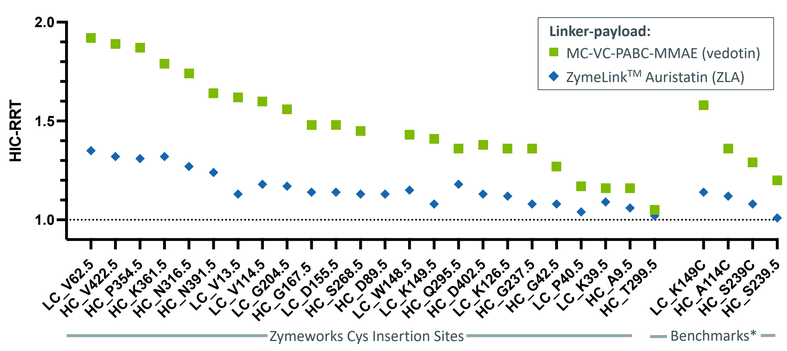
Biophysical and In Vitro Characterization Workflow

Analytical Method	Properties Assessed
Hydrophobic Interaction Chromatography (HIC)	• Relative hydrophobicity
Size Exclusion Chromatography (SEC)	• Mono-dispersity: % monomer, %HMWS, %LMWS
Mass Spectrometry	• Drug to antibody ratio (DAR) • Site of conjugation (i.e. light chain or heavy chain)
Capillary Electrophoresis Sodium Dodecyl Sulfate (CE-SDS)	• Molecular integrity • Intact disulfide bonding of interchain cysteines
On-cell Binding by Flow Cytometry	• Target binding of native antigen by antibody or ADC
In Vitro Cytotoxicity	• Potency of ADC

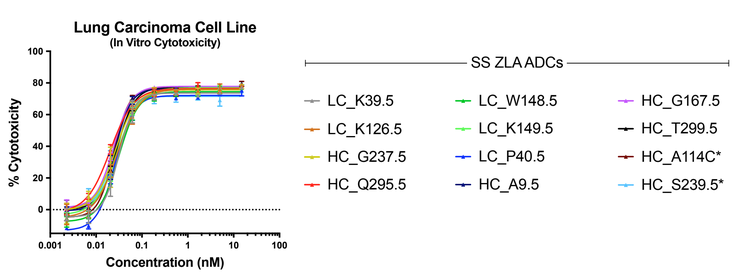
Cys Insertion Location Determines Overall Hydrophobicity of the ADC

- The conjugation of linker-payloads to an antibody leads to an increase in hydrophobicity (measured by HIC) as compared to the native antibody. It has been established that ADCs with high hydrophobicity are cleared more rapidly in vivo.
- To mitigate the effects of linker-payload conjugation on ADC hydrophobicity, we identified Cys insertion sites that mask the hydrophobicity of the linker-payload
- Below, the HIC relative retention time (RRT = RT_{DAR2}/RT_{DAR0}) of Cys insertion ADCs is compared to benchmark site-specific ADCs

Relative Hydrophobicity of Cys Insertion Site-specific ADCs

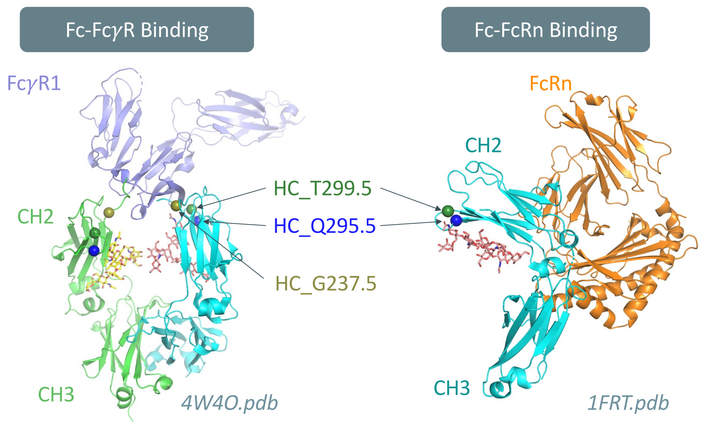


DAR 2 Cys Insertion SS ADCs Demonstrate Equivalent In Vitro Activity



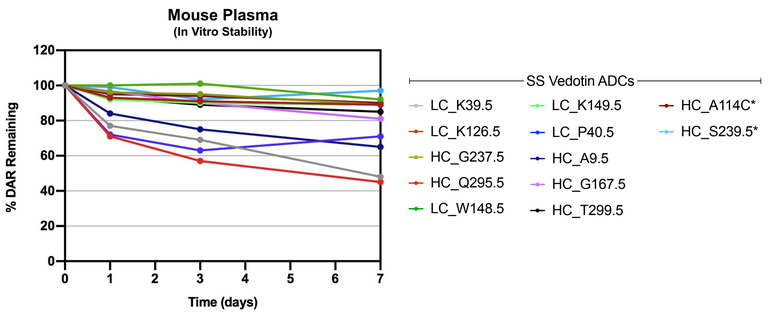
Cys Insertion in CH2 Domain Near Hinge Perturbs Antibody Effector Function

- Cys insertion at HC_G237.5 and HC_T299.5 completely abolishes FcγR binding
- Cys insertion at HC_Q295.5 leads to 3-fold reduction in FcγR binding affinity
- None of the Cys insertion sites evaluated impact FcRn binding

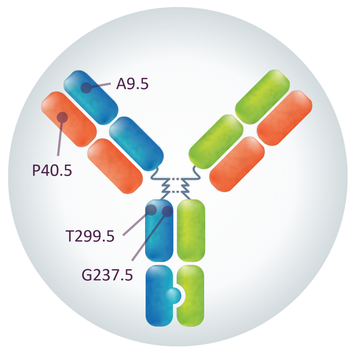


SS ADCs Derived from Cys Insertion Designs Offer a Range of Linker-payload Deconjugation Rates

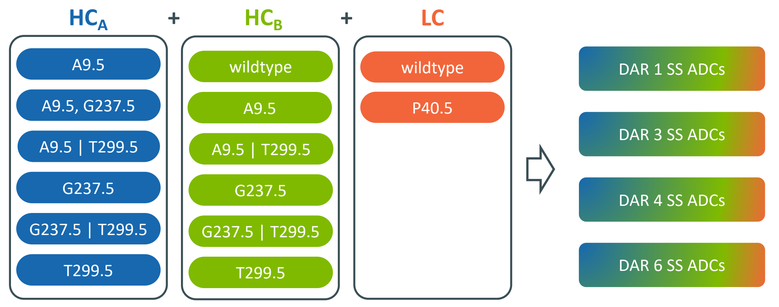
- ADCs produced by cysteine conjugation using thiol-maleimide chemistry can undergo deconjugation via a retro-Michael reaction
- The site of linker-payload attachment influences the rate of retro-Michael deconjugation



Azymetric™ Platform Enables Precise Control of Drug to Antibody Ratio

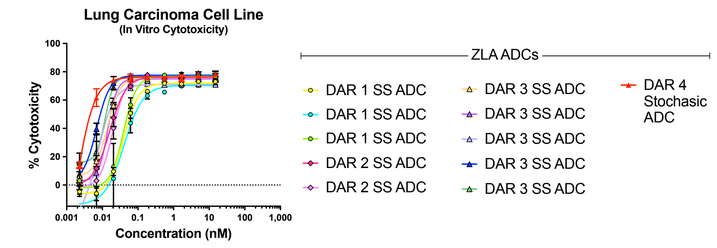


- DAR-tuned ADCs from DAR 1 to DAR 6 were produced by combining Cys insertion sites with Azymetric™ technology
- No adverse effects on antibody properties were observed from the incorporation of multiple Cys insertion sites
- SS ADCs generated from Cys insertion demonstrated favorable biophysical properties compared to similar ADCs prepared using stochastic conjugation methods



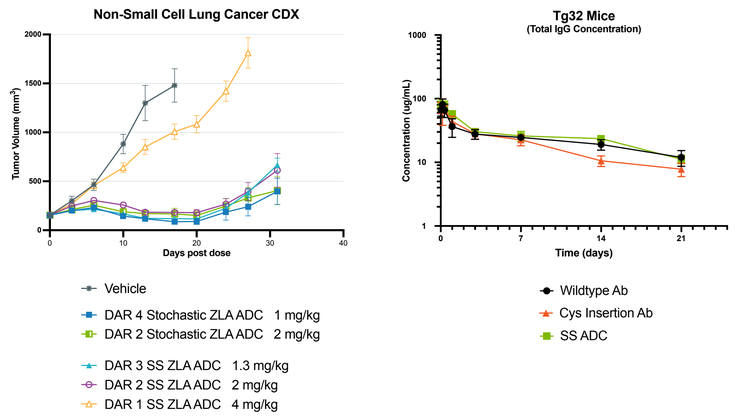
Higher DAR correlates with better preclinical activity for DAR 1-3 SS ADCs

In Vitro Potency Correlates with DAR of SS ADCs (DAR 1-3)



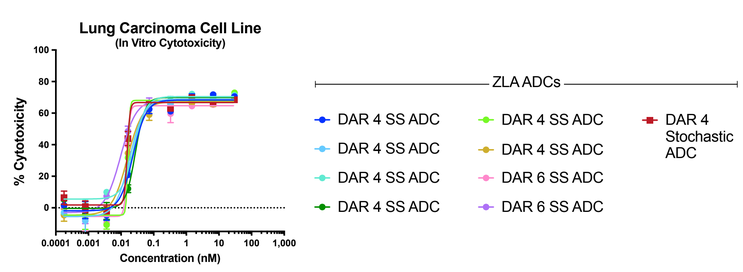
Note: DAR 4 Stochastic ADC prepared by interchain cysteine conjugation method.

SS ADCs Demonstrate Anti-tumor Activity and Antibody-like PK



Notes:
DAR 4 Stochastic ZLA ADC prepared by interchain cysteine conjugation method
DAR 2 Stochastic ZLA ADC prepared by lysine conjugation method
DAR 3 SS ZLA ADC made with Cys insertions at HC_A(G237.5, T299.5) and HC_B (T299.5)
DAR 2 SS ZLA ADC made with Cys insertions at HC_A(T299.5) and HC_B (T299.5)
DAR 1 SS ZLA ADC made with Cys insertion at HC_A(T299.5)

Combination of Cys Insertion Designs Permits the Assessment of DAR 4 and DAR 6 SS ADCs



Note: DAR 4 Stochastic ADC prepared by interchain cysteine conjugation method

Conclusions

- A combined in-silico and in vitro screening strategy was developed to identify novel Cys insertion sites for the generation of site-specific ADCs
- Cys insertion sites are compatible across different IgG1 antibodies
- A repertoire of insertion sites feature a range of FcγR binding affinities and varying degrees of protection against linker-payload deconjugation
- The Azymetric™ platform enabled the combination of multiple Cys insertion sites to produce site specific ADCs up to DAR 6
- Site specific ADCs generated from Cys insertion have favorable biophysical properties, are active in vivo and display pharmacokinetic profiles similar to wildtype antibodies

References

1. Dimasi et. al., *Molecular Pharmaceutics* **2017**, *14,* 1501-1516
2. Junutula et. al., *Nature Biotechnology* **2008**, *26,* 925-932
3. Thompson et. al., *Journal of Controlled Release* **2016**, *236,* 100-116
4. Su et. al., *Bioconjugate Chemistry* ***2018***, *29,* 1155-1167

Acknowledgement

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