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Optimization of ADC linkers: Design and Evaluation of a FRET-based ADC Linker-Library

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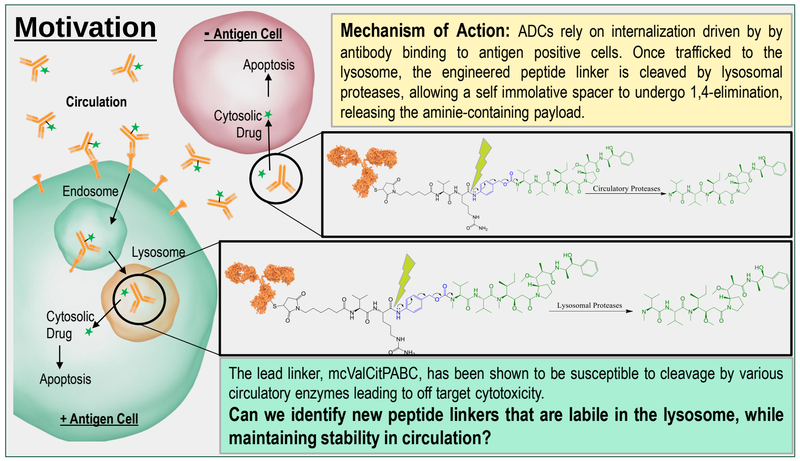
Abstract

Antibody drug-conjugate (ADC) technology is a rapidly evolving modality for targeted drug delivery. Much of the success of ADCs is due to technical advances made in the design of the peptide linkage between the antibody and the therapeutic payload.[1] This linkage has been optimized to be stable in circulation while maintaining a rapid release mechanism that is specific to lysosomal conditions. However, some ADCs have exhibited stability problems due to high solvent exposure of the linker-payload to the surrounding environment which can result in premature cleavage of the linkage by endogenous proteases in circulation, thus leading to off-target toxicity.[2] The goal of this project is to develop a new generation of proteolytically cleavable linkers that offer increased stability to extracellular enzymes. This investigation into the stability of various peptides linkages utilizes a high-throughput assay involving a fluorophore (Fluorescein) and quencher (Rhodamine) pair attached to opposing ends of a tripeptide linker, simulating the payload and antibody respectively. Through Förster resonance energy transfer (FRET), we can observe cleavage through the evolution of a fluorescein signal over time. Seventy-five distinct peptide-linked FRET pairs were synthesized and purified through preparative HPLC. Herein, we will describe the results of these studies and will report several peptide sequences that appear to offer improved properties as compared to the traditional ValCitPABC linker system.

Motivation

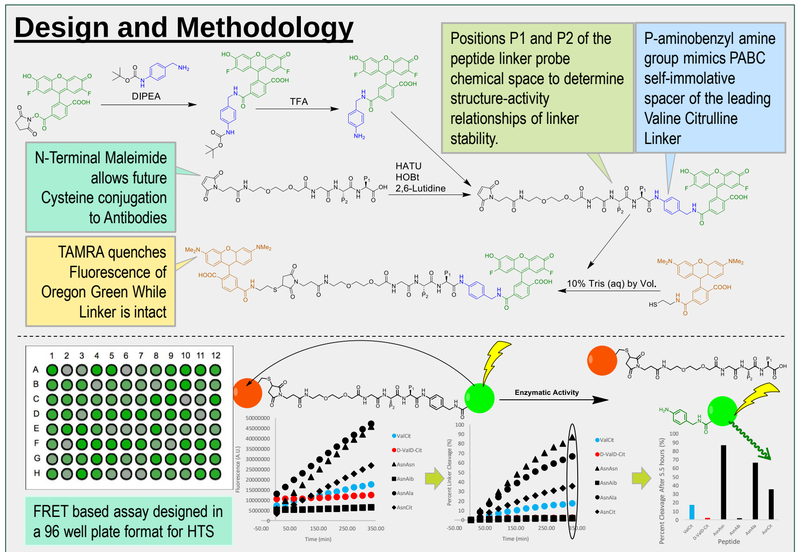


Mechanism of Action: ADCs rely on internalization driven by by antibody binding to antigen positive cells. Once trafficked to the lysosome, the engineered peptide linker is cleaved by lysosomal proteases, allowing a self immolative spacer to undergo 1,4-elimination, releasing the aminie-containing payload.

The lead linker, mcValCitPABC, has been shown to be susceptible to cleavage by various circulatory enzymes leading to off target cytotoxicity.

Can we identify new peptide linkers that are labile in the lysosome, while maintaining stability in circulation?

Design and Methodology



Positions P1 and P2 of the peptide linker probe chemical space to determine structure-activity relationships of linker stability.

P-aminobenzyl amine group mimics PABC self-immolative spacer of the leading Valine Citrulline Linker

N-Terminal Maleimide allows future Cysteine conjugation to Antibodies

TAMRA quenches Fluorescence of Oregon Green While Linker is intact

FRET based assay designed in a 96 well plate format for HTS

Lysosomal Lability Screening

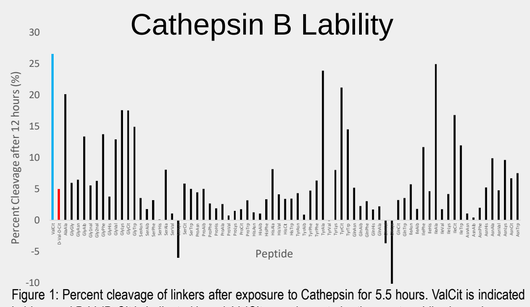


Figure 1: Percent cleavage of linkers after exposure to Cathepsin for 5.5 hours. ValCit is indicated in blue, and D-ValD-Cit is indicated in red. ValCit was shown to be the most rapidly cleaved.

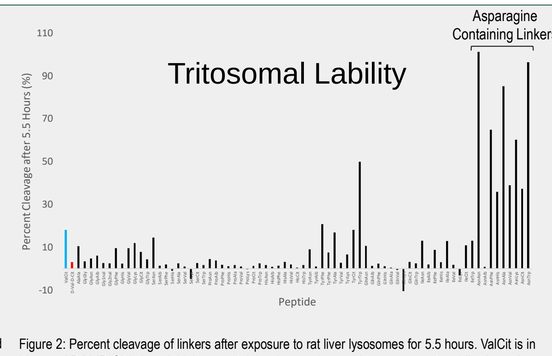


Figure 2: Percent cleavage of linkers after exposure to rat liver lysosomes for 5.5 hours. ValCit is in blue, and D-ValD-Cit is in red. Linkers containing Asparagine residues were most rapidly cleaved.

Could there be a different target for enzymatic for release?

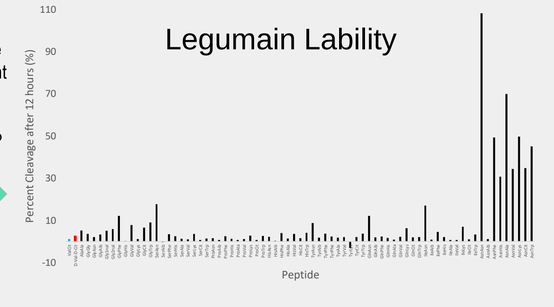


Figure 3: Percent cleavage of linkers after exposure to Legumain for 12 hours. Linkers containing Asparagine residues were most rapidly cleaved with preference to asparagine in the P2 position.

Serum Stability Screening

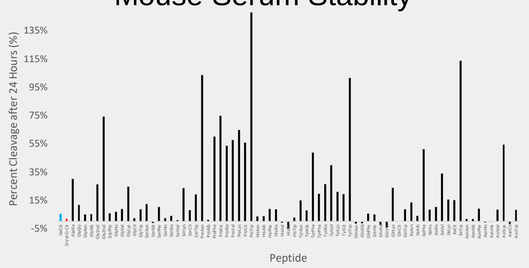


Figure 4: Percent cleavage of linkers in the presence of mouse serum for 24 hours. Proline and Tryptophan containing linkers were shown to be the most unstable.

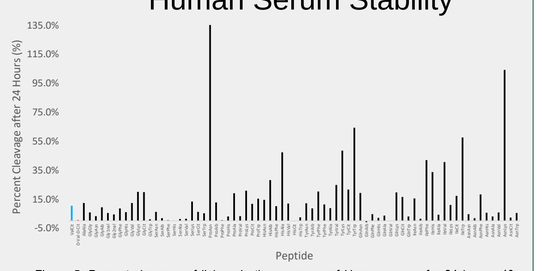
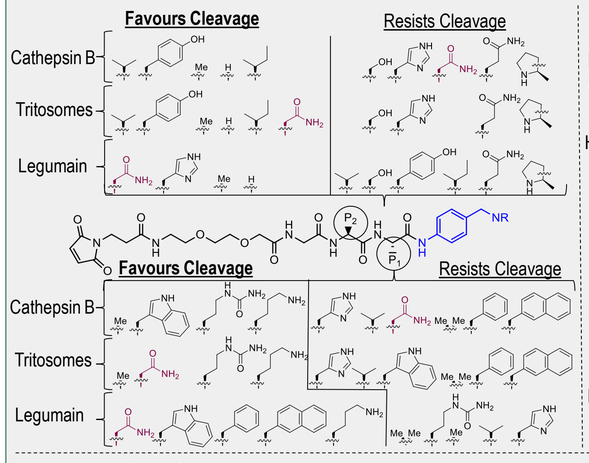
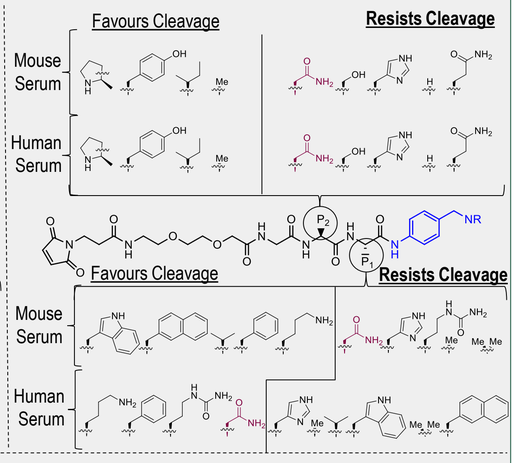


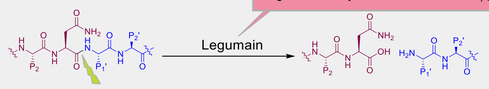
Figure 5: Percent cleavage of linkers in the presence of Human serum for 24 hours. 40 linkers were shown to be more stable than ValCit

Structure-Activity Relationship of Peptide Linkers





Legumain-Mediated Cleavage:

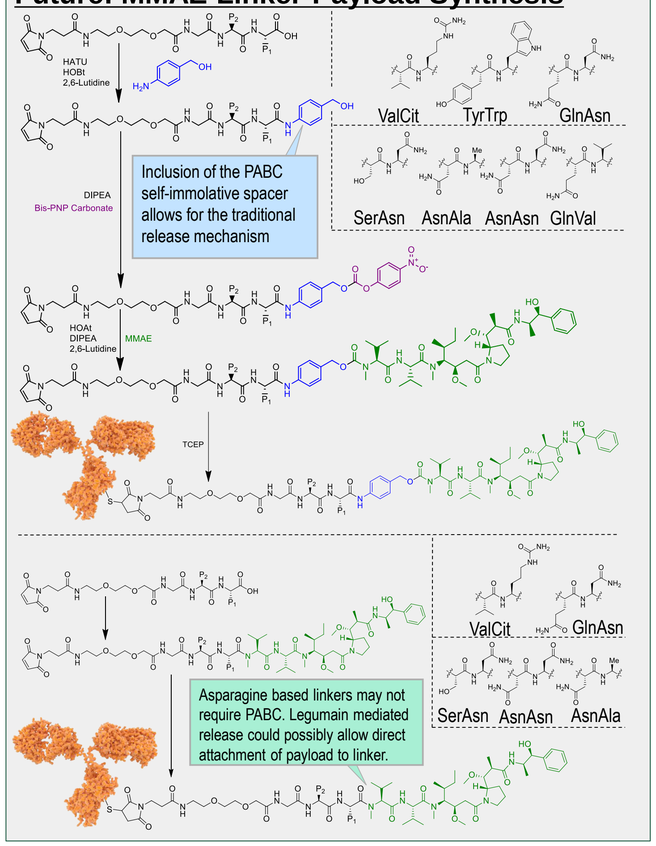


Legumain Always cleaves Asn in the P_1 position

Linkers with Asn in the "P_2" position are cleaved more readily, indicating legumain may be cleaving in a undesirable location. Since legumain cleavage is less rapid with Asn in the P_1 position, this reduction in activity may be due unfavorable binding of PABC to the P_1' position.

Can we utilize Legumain to release Payloads without the PABC spacer?

Future: MMAE Linker-Payload Synthesis



Inclusion of the PABC self-immolative spacer allows for the traditional release mechanism

Asparagine based linkers may not require PABC. Legumain mediated release could possibly allow direct attachment of payload to linker.

References and Acknowledgements:

1. Sievers, E. L.; Senter, P. D. Antibody-Drug Conjugates in Cancer Therapy. *Annu. Rev. Med.* **2013**, *64* (1), 15–29.
2. Dorywalska, M. et al. Molecular Basis of Valine-Citrulline-PABC Linker Instability in Site-Specific ADCs and Its Mitigation by Linker Design. *Mol. Cancer Ther.* **2016**, *15* (5), 958 LP – 970.

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