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Design of a Turn-on Fluorescence Assay for the Identification and Application of Improved ADC Linkers

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Abstract

Antibody-drug conjugates (ADCs) are a class of drug currently used for the targeted treatment of cancer. The prototypical linker used for such ADCs is the Val-Cit-PABC linker due to its rapid cleavage rate by the lysosomal enzyme cathepsin B as well as its stability in human plasma. However, recent studies have shown this system to be unstable in the presence of various enzymes such as carboxylesterases and neutrophil elastases. To mitigate this issue, we have designed a peptide library that can be readily screened in order to identify linker sequences that are still rapidly cleaved by lysosomal enzymes but are stable in human and mouse plasma. In short, the library was designed to utilize a turn-on fluorescence assay made possible by the fluorophore AMC (7-Amino-4-methylcoumarin). AMC is known to be non-fluorescent when the 7-amino group is bound as an amide but is highly fluorescent upon cleavage of the amide bond. Therefore, AMC can be employed as a fluorescent probe for rapid determination of amide bond cleavage – specifically that of ADC linkers. Lysosomal ADC processing relies on cleavage of the amide bond between the linker and the cytotoxic payload, and therefore the turn-on fluorescence assay provides a simple method for determining whether particular peptide linkers are susceptible to such cleavage. Due to its poor nucleophilicity, the AMC was attached to a single amino acid and subsequently coupled to variable tripeptide linkers. All individual compounds were purified and characterized by LCMS resulting in 130 linkers for screenings. We will report the results of the linker stability and plasma stability studies focusing on linkers that have the best potential for incorporation in ADC designs.

Turn-on Fluorescence with 7-Amino-4-methylcoumarin (AMC)

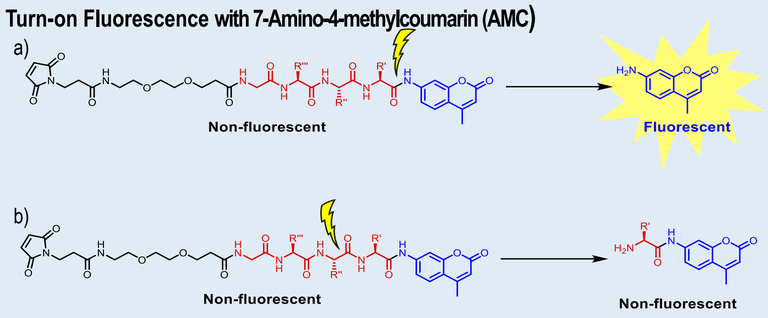
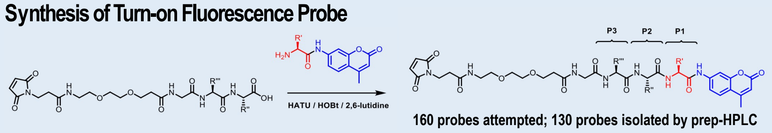


Figure 1. Turn-on fluorescence with AMC (blue) and tetrapeptide linker (red). AMC known to be non-fluorescent when the 7-amino group is bound as an amide. a) Upon cleavage of the P1-P1' amide bond, fluorescence is observed. b) Cleavage of any other peptide bond would not result in fluorescence.

Synthesis of Turn-on Fluorescence Probe



160 probes attempted; 130 probes isolated by prep-HPLC

Figure 2. The peptide library was designed as follows by WuxiApptech: 3-Maleimidopropionic acid-PEG2-GLY-P_3-P_2, where P_3 and P_2 are variable amino acids representing a mixture of polar and nonpolar amino acids of various sizes. Due to the poor nucleophilicity of the AMC, it cannot be directly coupled to the tripeptide. Instead, the AMC is attached to a single amino acid (P_1) and subsequently coupled to the tripeptide linker. Eight amino acid-AMC molecules were each coupled to roughly twenty tripeptide linkers, resulting in a library of ~160 peptides. The preparation of the library required the optimization of various coupling reactions. All individual compounds were purified by preparative high-performance liquid chromatography (HPLC) and were characterized by liquid chromatography-mass spectroscopy. (LC-MS). 130 peptide-AMC conjugates had > 70% purity and were used for further studies.

Synthesis of Antibody-AMC Conjugates

130-membered library was prepared

Figure 3. HER2-Tras antibody (18.6 mg/mL, 1 equiv.) was reduced with TCEP (5.0 mM, 10 equiv.) overnight at 37°C. This reaction was then buffer exchanged with PBS using a sephadex column. In a 96-well plate-based format, the above reaction (0.10 mg, 1 equiv.) was combined with the peptide-AMC conjugate (500 µM, 5 equiv.) and enough PBS to make a final volume of 100 µL per well and allowed to sit at room temperature for 2 hours. Above reaction underwent 4 rounds of centrifuge filtration (1500xg, 10 min) using a 96-well filter plate. Final volume of antibody-AMC conjugate HER2-Tras-3-Mal-AEEA-Gly-P3-P2-P1-AMC was made to be 200 µL with PBS. HER2-Tras and peptide-AMC conjugate concentration were quantified using two standard curves, respectively. DAR of antibody-AMC conjugates were calculated based off of the concentrations obtained from the standard curves. The concentration (µM) of peptide-AMC conjugate was divided by the concentration of HER2-Tras to yield the DAR. Using the protein TQD-MS method, the DAR of a few antibody-AMC conjugates was confirmed.

Turn-on Fluorescence Assay Scheme

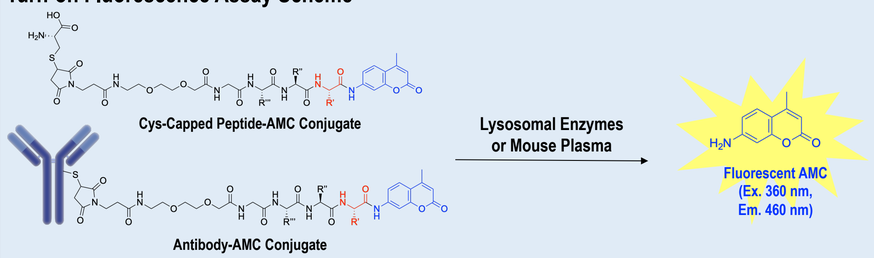


Figure 4. A cysteine residue was added to the N-terminal maleimide of the peptide-AMC conjugate to prevent the maleimide from reacting with the active-site cysteine present in many lysosomal proteases. Peptide-AMC and antibody-AMC conjugates screened for stability in lysosomal enzymes (tritosomes) and mouse plasma. AMC fluorescence monitored over time.

Turn-on Fluorescence Assay Set-Up

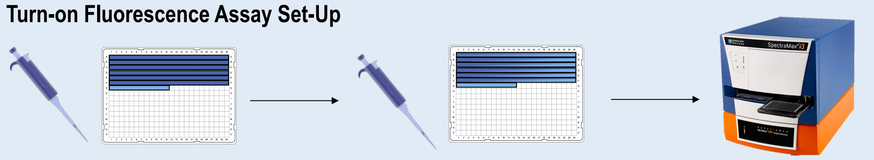


Figure 5. This is a high throughput assay performed in a 384-well plate. Cysteine is added to the peptide-AMC conjugate to quench the reactivity of the maleimide. Then the enzyme is added to the peptide-AMC conjugate or antibody-AMC conjugate and their fluorescence is measured over time in the plate reader at Ex. 360 nm Em. 460 nm. AMC release is quantified using a standard curve run in the plate at the same time.

Lysosomal and Serum Stability of Peptide-AMC Conjugates and Antibody-AMC Conjugates

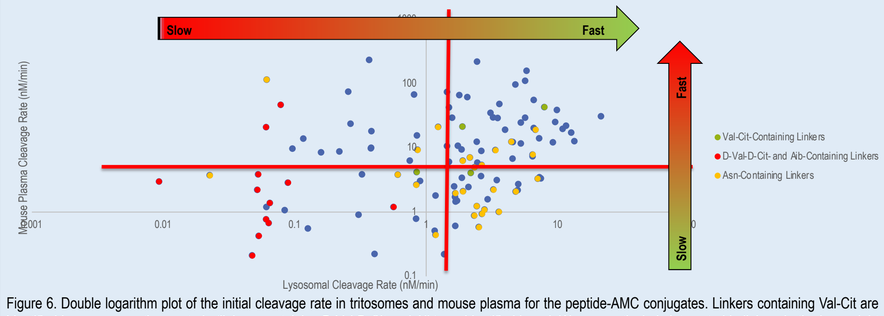


Figure 6. Double logarithm plot of the initial cleavage rate in tritosomes and mouse plasma for the peptide-AMC conjugates. Linkers containing Val-Cit are identified in green, negative controls (linkers containing D-Val-D-Cit and Aib) are identified in red. We are particularly interested in linkers in the lower right quadrant of the plot because these linkers are highly cleaved in the lysosome and relatively stable in mouse plasma.

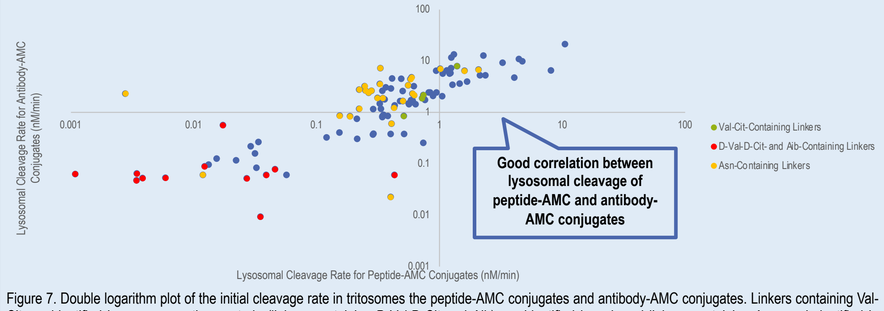


Figure 7. Double logarithm plot of the initial cleavage rate in tritosomes the peptide-AMC conjugates and antibody-AMC conjugates. Linkers containing Val-Cit are identified in green, negative controls (linkers containing D-Val-D-Cit and Aib) are identified in red, and linkers containing Asn are indentified in orange. The linear trend of the plot suggests that the lysosomal cleavage of the AFCs is consistent with that of the linkers.

Antibody-AMC Conjugate Linker Sequences

Table 1. Initial Lysosomal and Mouse Plasma Cleavage Rates for Antibody-AMC Conjugates

Linker Sequence (P_3-P_2-P_1-AMC)	Initital Cleavage Rate (nM/min)		Linker Sequence (P_3-P_2-P_1-AMC)	Initital Cleavage Rate (nM/min)	
	Tritosomes	Mouse Plasma		Tritosomes	Mouse Plasma
Gln-Phe-Asn-AMC	7.24	6.62E-06	Ser-Cit-Asn-AMC	1.90	3.25E-05
Gln-Asn-Leu-AMC	7.03	2.08E-06	Gly-Val-Gly-AMC	1.72	6.96E-06
Ser-Asn-Leu-AMC	4.79	2.96E-07	Ser-Cit-Ser-AMC	1.67	3.90E-06
Ser-Asn-Asn-AMC	3.56	7.08E-06	Ala-Ala-Cit-AMC	1.66	6.95E-06
Gln-Phe-Ser-AMC	2.93	2.18E-06	Gly-Cit-Ala-AMC	1.36	1.23E-05
Gln-Asn-Asn-AMC	2.65	9.05E-06	D-Val-D-Cit-Ser-AMC	0.06	1.93E-05
Ser-Asn-Ser-AMC	2.32	6.05E-06	Pro-Aib-Gly-AMC	0.05	8.66E-06

We suspect linkers highlighted in purple (P_3-Asn-P_1-AMC) to be cleaved by legumain, an asparaginyl endopeptidase that is overexpressed in a variety of cancers and is known to be present in the lysosome.

Conclusions

ADC linker stability was successfully evaluated through the use of a turn-on fluorescence assay with AMC. Lysosomal and mouse serum stability screenings identified a set of linkers with improved stability compared to the prototypical Val-Cit-PABC. While linkers with Val-Cit in the P3 and P2 as opposed to the typical P2 and P1 position doesn't make it a direct comparator, there are still plenty of linkers identified which had more rapid cleavage in tritosomes and higher stability in mouse plasma compared to those containing Val-Cit. More than half of these exemplar linkers were ones that contained asparagine: Ala-Ala-Cit-AMC, Gly-Val-Gly-AMC, Gly-Cit-Ala-AMC, Ser-Asn-Asn-AMC, Ser-Asn-Leu-AMC, Ser-Asn,Ser-AMC, Ser-Cit-Asn-AMC, Ser-Cit-Ser-AMC, Gln-Asn-Asn-AMC, Gln-Phe-Ser-AMC, and Gln-Trp-Asn-AMC. This is consistent with previous reports from our lab which identified a series of Asn-containing linkers as being highly cleaved in the lysosome and stable in mouse serum. The identification of linkers that are more stable than Val-Cit-PABC provides an opportunity for an exciting new generation of ADC linkers.

Further Study of Linker Stability in Cells

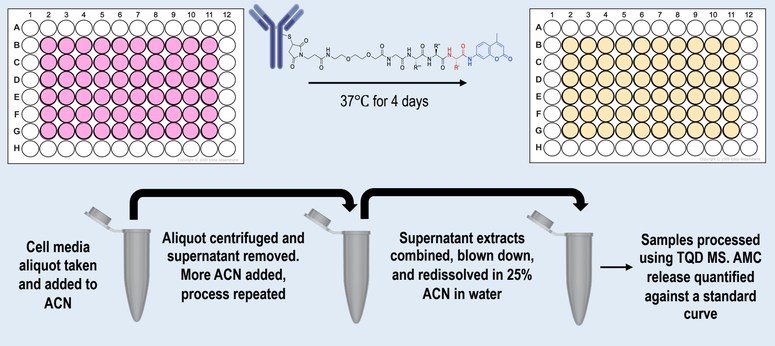


Figure 7. SKBR3 cells were cultured in three 96-well plates with RPMI-1640 medium with 10% fetal bovine serum. 15 µL of antibody-AMC conjugate was added to each well (n = 130). Cells incubated at 37°C with 5% CO2 for 4 days. After 4 days, 80 µL of cell media was removed from each well and added to 240 µL ACN. Mixture was vortexed, spun down, and 250 µL of the supernatant was removed. This process was repeated. Two 250 µL extracts were combined, dried, and redissolved in 80 µL of 25% ACN in water. Quantification of AMC released from AFCs in cells was performed using Waters Acquity UPLC (H-class) with Xevo TQD (triple quadrupole) mass spectrometer.

Acknowledgements

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