Design and optimization of a turn-on fluorescence assay for the identification of improved ADC linkers

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Abstract
Antibody-drug conjugates (ADCs) are a class of drugs used for targeted delivery in the treatment of cancer. The prototypical linker used for such ADCs is the lysosomally cleaved Val-Cit-PABC linker. This system emerged as a result of rapid cleavage by the lysosomal enzyme cathepsin B as well as its stability in human plasma. However, recent studies have shown that this system is frequently unstable in the presence of various enzymes including neutrophil elastases and carboxylesterases. To mitigate this issue, we have designed a peptide library that can be readily screened in order to identify sequences with improved properties. In short, the library was designed to utilize a turn-on fluorescence assay—a simple assay made possible by a fluorophore, AMC (7-Amino-4-methylcoumarin), 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 fluorometric probe for rapid determination of amide bond cleavage—a crucial step in ADC development. To quantify the cleavage of the amide bond between the linker and the payload, the turn-on fluorescence assay provides a simple method for determining whether peptide linkers are susceptible to such cleavage.

Potential Applications
The turn-on fluorescence assay provides a simple method for screening linker stability in various enzymes. So far, around 150 peptide-AMC probes have been synthesized and characterized by their purity. The screening conditions for cathepsin B cleavage have been optimized, and a positive control (Val-Chit-AMC) and negative control (D-Val-D-Chit-Leu-AMC) have been identified from the initial peptide-AMC tested. This assay can be used to analyze internalization and processing of an ADC. ADCs will be prepared with AMC attached to the linker and the payload, and therefore the turn-on fluorescence assay provides a simple method for determining whether peptide linkers are susceptible to such cleavage.

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Peptide-AMC Linker Library

Screen entire library in cathepsin B, mouse and human plasma, and tritosomes

Figure 2. The peptide library was designed as follows: 3-Maleimidopropionic acid-PEG2-GLY-AA1-AA2, where AA1 and AA2 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 (AA3) 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 180 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 spectrometry (LC-MS).

Purification through Preparative HPLC-MS

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

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