Stability of ADC linker payloads in sub-cellular fractions

Measurement of warhead release in common ADC linkers after incubation in lysosomes and S9 fraction

Antibody drug conjugates (ADCs) combine a cytotoxic molecule linked to a targeting antibody, and this linkage is the subject of a great deal of research and focus. Some payloads have an uncleavable linker which can only be released after complete digestion of its co-protein, however other linkers are designed to be cleaved inside cells via a variety of mechanisms. Many linkers are peptidic in nature and undergo cleavage in the lysosome after being trafficked into the cell[1]. With increasing variety in these linkers, it is important to ensure the toxic warhead is released correctly and efficiently. Though trial cleavages can be performed with isolated enzymes such as Cathepsin B, a better test is to use sub-cellular fractions that better reflect the enzymatic cocktail that is present inside cells.

Here, we outline an analysis strategy to determine the stability and cleavage of four toxin linkers from approved ADCs (Vedotin (Val-Cit), Tesirine (Val-Ala), Deruxtecan (Gly-Gly-Phe-Gly), and Mafodotin (uncleavable)) in human liver S9 fraction and human lysosome. The toxins were all reacted with N-Acetyl cysteine before incubation to reduce reactivty in their conjugation moieties. The samples were incubated in human liver lysosomes, or S9 fraction (Xenotech), at a concentration of 0.05 mg/ml for time periods up to 24 hours at 37°C. Sub-cellular fractions were also bolstered with a catabolic buffer and NADPH RapidStart (Xenotech) to ensure metabolic activity. The samples were heat inactivated after incubation for five minutes at 95°C, before removal of the protein via solvent crash. All data was collected on a SCIEX X500B Mass Spectrometer with an EXION HPLC. The instrument was operated in IDA mode to confirm species via fragmentation. The chromatography was performed with a Kinetix C18 column (1.7µm, 50x2.1mm) on a five minute gradient of water and acetonitrile both with 0.1% formic acid.

In trials, all the toxin linkers were stable at up to 24 hours in catabolic buffers lacking the sub-cellular fractions.

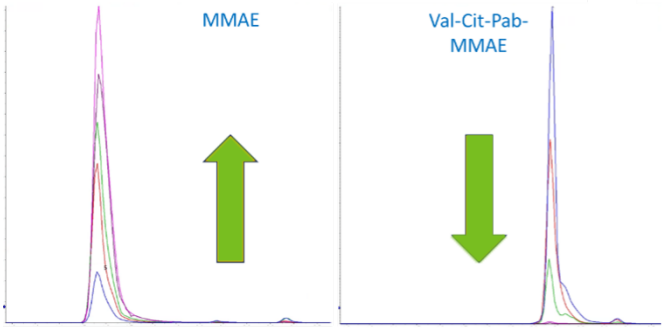

Figure 1: XIC of MMAE and vc-MMAE in human liver S9 fraction. MMAE (717 m/z) and vedotin (1316 +NAC m/z) were monitored over a 24 hour period and saw no significant drop in intensity nor the appearance of new peaks. *Blue T0 Purple T15 Red T30 Orange T60 Green T90 Dark green T120 Cyan T1440 (minutes).*

Figure 1 shows the XIC of vedotin and MMAE over 24 hours in human liver S9 fraction. We observe over time that the peak owing to the complete toxin linker disappears and a peak of free MMAE increases as the valine-citrulline linker is cleaved by proteases present in the sample. S9 contains a variety of enzymes that are present in cytosol and microsomes.

The rate of cleavage of vedotin was observed to be higher in lysosomal extract that in S9. ADCs are trafficked through the lysosomal degradation pathway, so this is likely where the cytotoxic compound would be released in cells. The three peptide linker studied, all showed near complete cleavage after 24 hours, however the deruxtecan and tesirine cleaved more slowly. Mafodotin has a non-cleavable linker and showed no proteolytic changes over this time-scale.

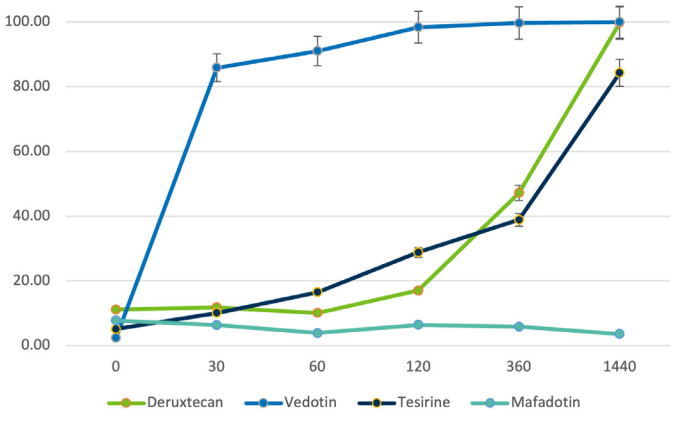

Figure 2: Rate of linker cleavages in human liver lysosomes. The peptide linkers all cleave over the time course, however the valine-citrulline linker reaches over 80% digestion within 30 minutes.

References:
1: Chalouni C, & Doll S. Journal of Experimental & Clinical Cancer Research, 37:20, 2018