# From Incubation to Identification: A Systematic Approach for Profiling Antibody-Drug Conjugate *in vitro* Catabolism

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## Introduction

Characterizing catabolic stability and identifying degradation products of ADCs in *in vitro* incubation systems are critical steps in early drug development. Our integrated *in vitro* platform bridged ADC chemistry, enzymatic metabolism, and preclinical outcomes, offer a robust tool to de-risk ADC development through linker-specific metabolic profiling. From a regulatory perspective, robust *in vitro* data strengthens IND submissions by addressing guidelines from agencies such as the FDA and EMA, which emphasize the need for thorough characterization of ADC stability and metabolic fate.

## Methods

- Prepared the following incubation buffer (2 mM TCEP in 50 mM sodium acetate, pH 5.0). Diluted the lysosomal enzymes in buffer such that the final concentration is 0.2 mg/mL for liver lysosome, 1 mg/mL for liver S9 fraction and to 1.11 U/mL for Cathepsin B Enzyme. The final ado-Trastuzumab Emtansine / Disitamab Vedotin concentration in the incubation mixture is 0.5 mg/mL.
- High Resolution Mass spectrometer capable of data dependent MS/MS analysis, with data processing software (Compound Discoverer, Thermo Fisher Scientific).

## Conclusion

- These studies provide insights into linker-payload stability, potential release catabolites identification, and the formation of catabolites that may influence efficacy or safety.
- By employing *in vitro* models—such as cathepsin B enzyme, liver lysosomes, liver S9 fraction, researchers can predict *in vivo* linker cleavage sites and catabolic behavior, assess species-specific differences, and mitigate risks associated with off-target toxicity or premature payload release.

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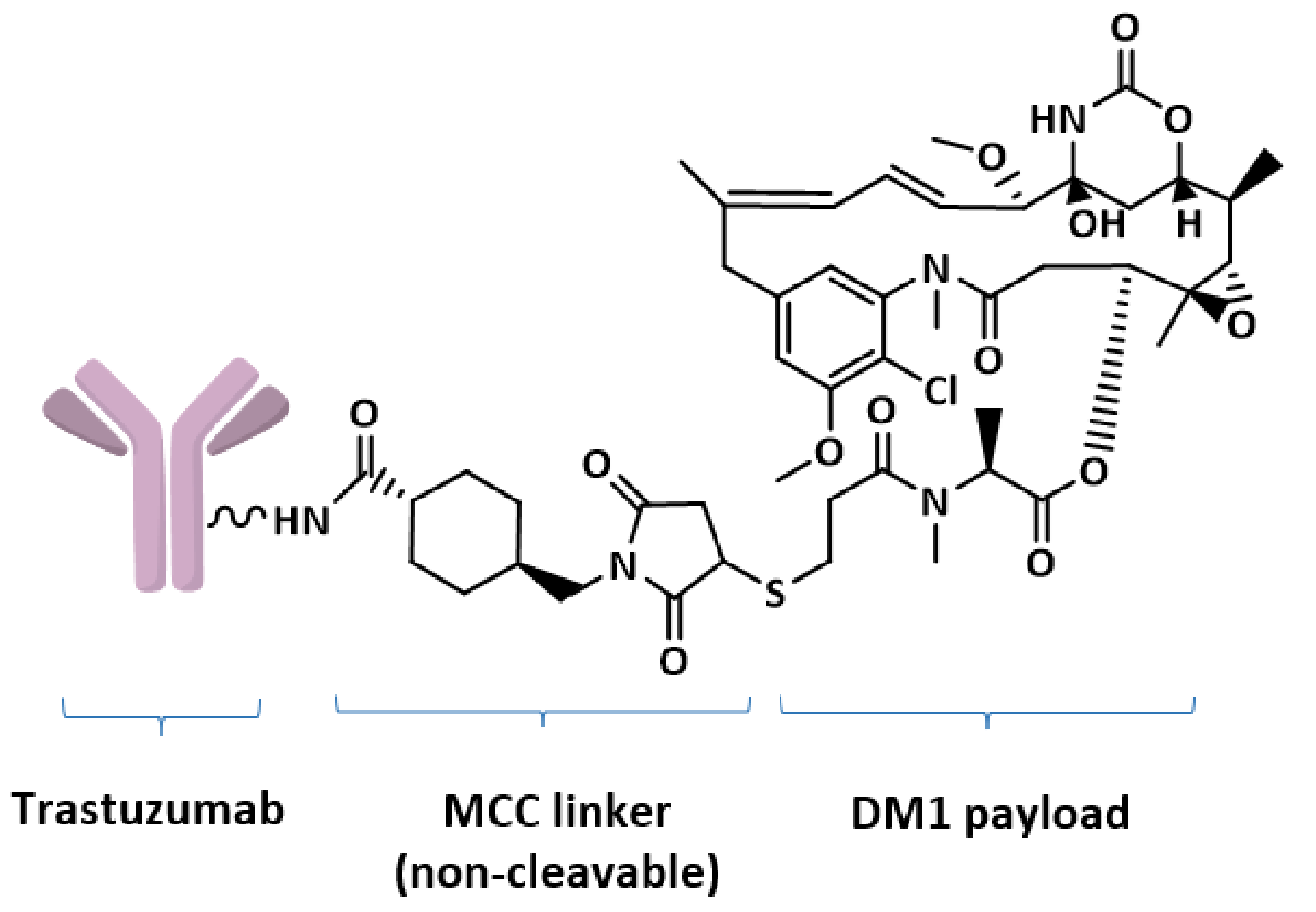
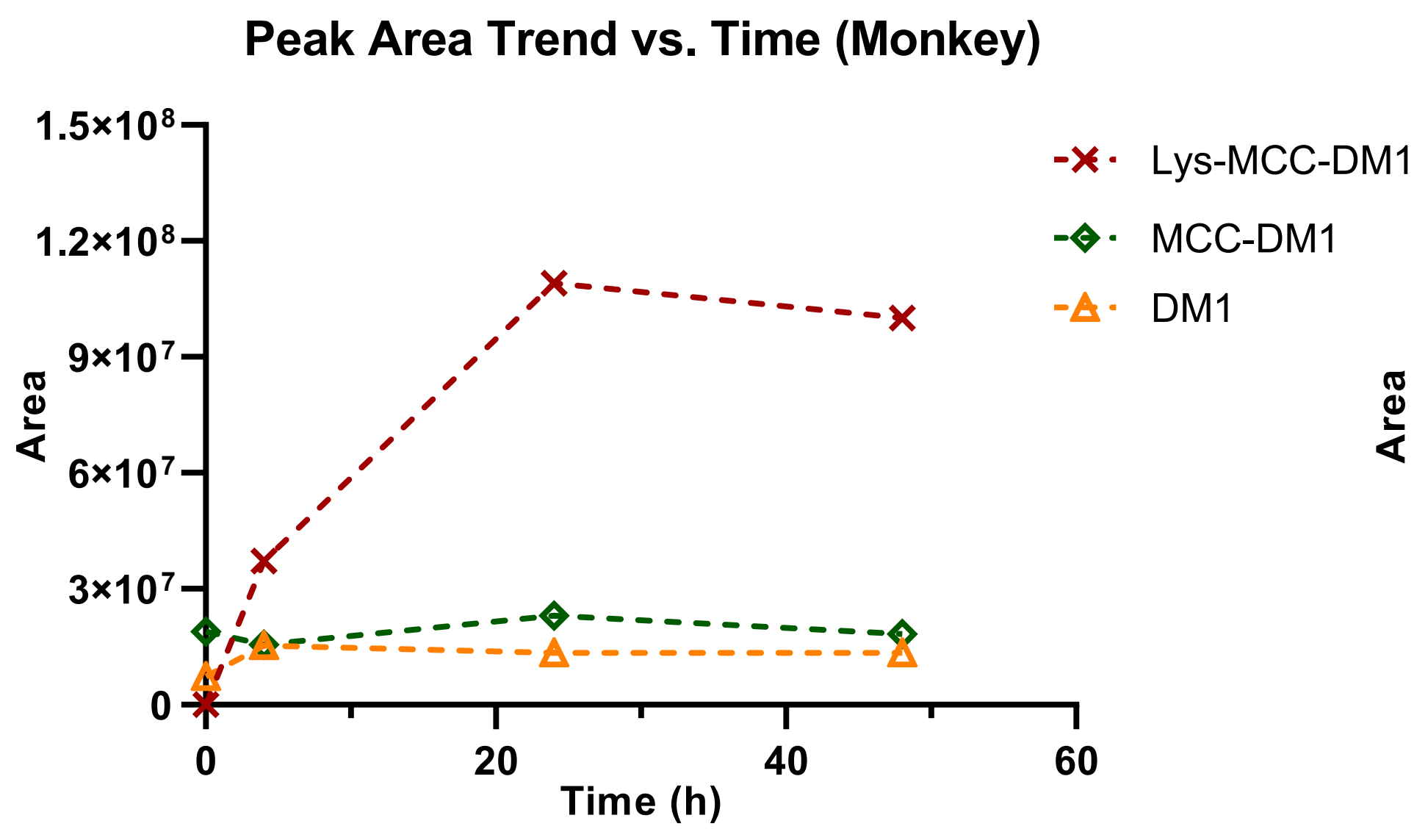
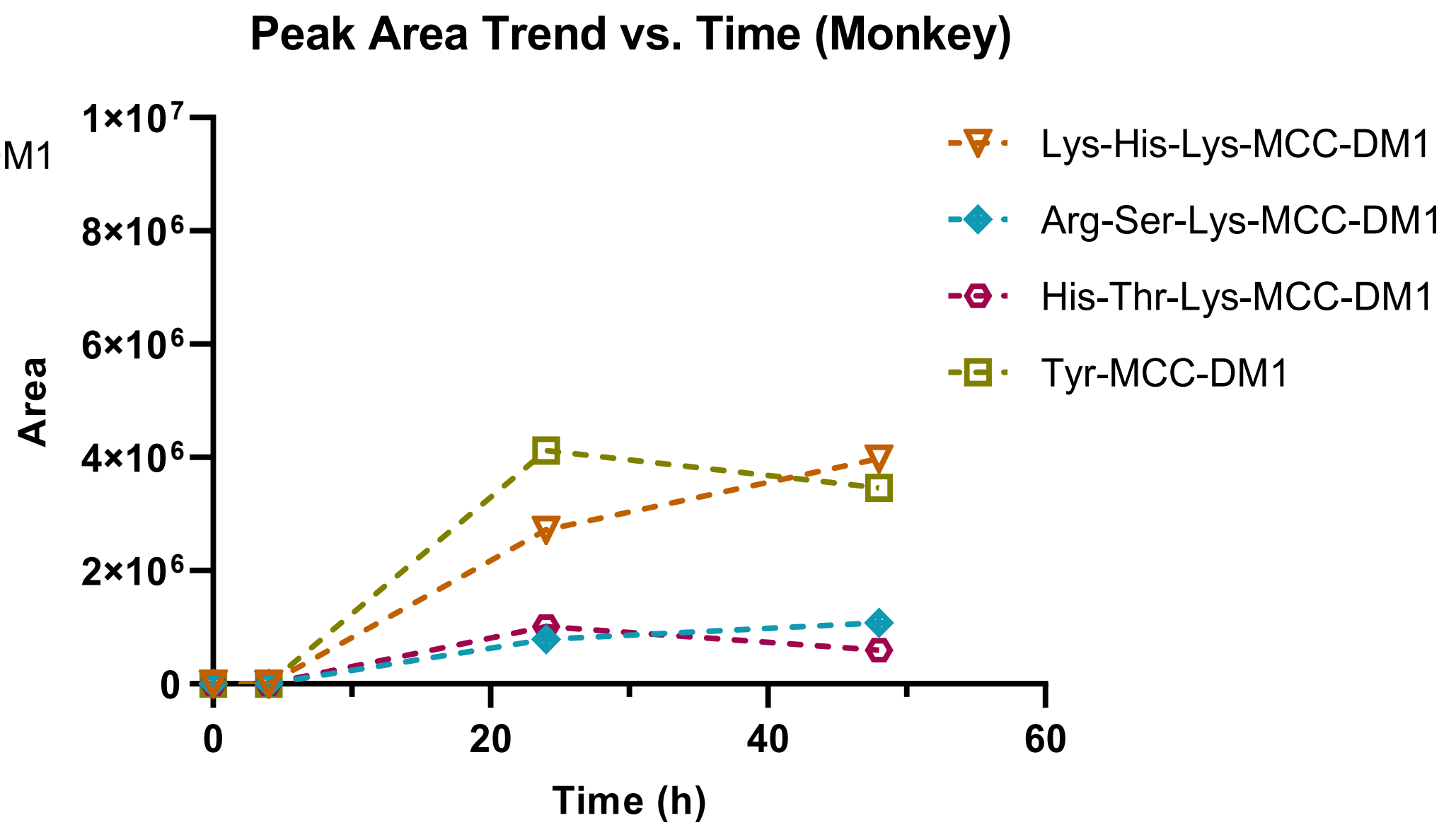
## Catabolites Information of Ado-Trastuzumab Emtansine (Kadcyla) in *in vitro* Liver Lysosomal Incubation

| Peak No. | RT(min) | Catabolite(s) | Theoretical mass *(m/z)* | Peak area (%) | | |
|---|---|---|---|---|---|---|
| | | | | Mouse @ 48hr | Monkey @ 48hr | Human @ 48hr |
| C1 | 6.73 | Lys-His-Lys-MCC-DM1 | 684.81919(2+) | 0.15% | 1.21% | 0.72% |
| C2 | 6.86 | Lys-His-Lys-MCC-DM1 | 684.81919(2+) | 0.17% | 1.64% | 1.48% |
| C3 | 7.14 | Arg-Ser-Lys-MCC-DM1 | 673.80883(2+) | - | 0.17% | 0.07% |
| C4 | 7.3 | Arg-Ser-Lys-MCC-DM1 | 673.80883(2+) | - | 0.60% | 1.18% |
| C5 | 7.23 | His-Thr-Lys-MCC-DM1 | 671.29555(2+) | 0.10% | 0.04% | 0.16% |
| C6 | 7.39 | His-Thr-Lys-MCC-DM1 | 671.29555(2+) | 0.06% | 0.39% | 0.12% |
| **C7** | **8.04** | **Lys-MCC-DM1** | **1103.47724(1+)** | **31.38%** | **39.93%** | **39.42%** |
| **C8** | **8.21** | **Lys-MCC-DM1** | **1103.47724(1+)** | **25.28%** | **31.79%** | **30.48%** |
| C9 | 8.88 | Tyr-MCC-DM1 | 1138.44561(1+) | 0.09% | 1.21% | 1.36% |
| C10 | 9 | Tyr-MCC-DM1 | 1138.44561(1+) | 0.01% | 1.26% | 1.43% |
| C11 | 9.86 | MCC-DM1 | 975.38228(1+) | 23.01% | 7.14% | 5.63% |
| C12 | 9.96 | MCC-DM1 | 975.38228(1+) | 9.49% | 5.91% | 3.75% |
| C13 | 10.46 | DM1 | 738.28217(1+) | 10.33% | 9.57% | 14.04% |







**Catabolite Identification through High Resolution Mass Spectrometry.** Total of 13 payload containing catabolites were detected after the background subtraction processing. Amino acid residues from Trastuzumab antibody was found connected to the non-cleavable linker MCC and payload DM1. Payload related catabolites C7 and C8 (a pair of Lys-MCC-DM1) were the major catabolites in lysosome incubation.

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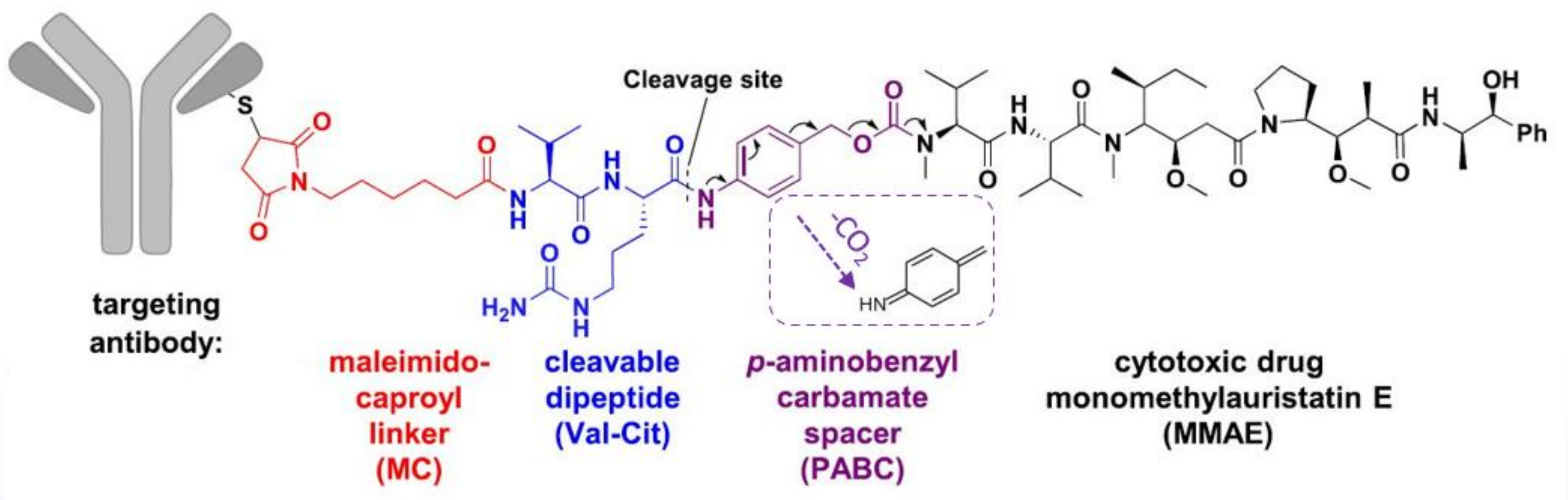
## Analyzing the Disitamab Vedotin Incubation Sample in Cathepsin B System by HRMS

| Catabolite (s) | Theoretical mass (m/z) | MS peak area of MMAE | | | |
|---|---|---|---|---|---|
| | | 0h | 1h | 2h | 4h |
| MMAE | 718.51133(1+) | 3.32E+07 | 2.70E+10 | 2.78E+10 | 2.95E+10 |



**Validating the cleavage and the cleavage site through High Resolution Mass Spectrometry.** The linker is cysteine conjugated with a maleimide caproyl and a cleavable dipeptide (Val-Cit) linker. There is a self-immolative P-aminobenzyl carbamate spacer (PABC) connecting the Val-Cit linker and Cytotoxic drug monomethyl auristatin E (MMAE). Cathepsin B, a lysosomal protease overexpressed in many tumors. The valine-citrulline (VC) linker is relatively stable and can only be cleaved by cathepsins when Disitamab Vedotin are endocytosed into lysosomes, resulting in the release of payloads to kill target cancer cells. The VC linker was completely cleaved within 1 hour and released payload MMAE to reached a plateau. This targeted approach exemplified how cleavable linkers enhance the efficacy and safety of anticancer therapies. Similar protease-cleavable linkers are used in other ADCs, the cathepsin B assay can help scientists to validate the cleavage and the cleavage site and to confirm the linker's stability in circulation and specificity in the tumor microenvironment.

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