Supporting Information:

A two-step immunocapture LC-MS-MS assay for plasma stability and payload migration assessment of cysteine-maleimide based antibody drug conjugates

Linlin Dong,[†*] Chao Li, [† ‡] Charles Locuson,[†] Susan Chen,[†] Mark Qian[†]

†Department of Drug Metabolism and Pharmacokinetics, Takeda Pharmaceuticals International, Inc., 35 Landsdowne Street, Cambridge, MA 02139

[‡]Present address: Nuventra Pharma Sciences, 2525 Meridian Parkway, Durham, NC 27713

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Supplemental Methods

Quantitation of Conjugated Payload and Total Antibody Using Single Step Immunocapture. For conjugated payload analysis, an aliquot of 10 µL each stability sample was dispensed into a 96-well Thermo Kingfisher plate with 150 µL of PBS (pH 7.4) and 0.1 mg (100 µL) target antigen coupled M-280 beads pre-added. Samples were mixed well on a shaker and allowed 40 min for ADCs to bind at room temperature. The plate was then processed with a KingFisher™ Flex magnetic particle processor as described by Xu et al.[1] Then the beads were released into 100 µL of papain activation solution. Twenty µL of 10 mg/mL activated papain solution (preactivated at 37°C for 15 min) were added to each sample well. Microwave assisted digestion was performed in the REDS at 37°C for 45 minutes. Then the sample was immediately acidified by adding 10 µL of 20% TFA. An aliquot (160 µL) of 6.25 ng/mL d8-MMAE was spiked to the sample as the internal standard (prepared in 95% acetonitrile and 0.1% formic acid in water). The plate was then vortexed for 1 min and centrifuged at 2100xg for 10 min. One µL of supernatant was injected to the LC-MS.

For total antibody analysis, the same immunocapture procedure was carried out. However, the TAK-maleimide-TPP ADC was eluted from the magnetic beads using 100 µL of 25% acetonitrile/30 mM HCl instead.[2] Following the elution, 20 µL of 1 M Tris (pH 8) was added to the eluent. Then 10 µL of 1% RapiGest and 10 µL of Lys-C (0.33 µg/µL in water) were added to each sample well. Preincubation of 30 min at 37°C was followed by 20 min microwave assisted digestion under conditions described above. The sample was then immediately acidified by

adding 10µL of 20% TFA. Twenty µL of 1 µg/mL SIL-HC peptide (prepared in 20% acetonitrile) was spiked to the sample as the internal standard followed by adding 80 µL of 50% acetonitrile then centrifuge for 10 minutes at 2100xg. Ten µL of supernatant was injected to the LC-MS.

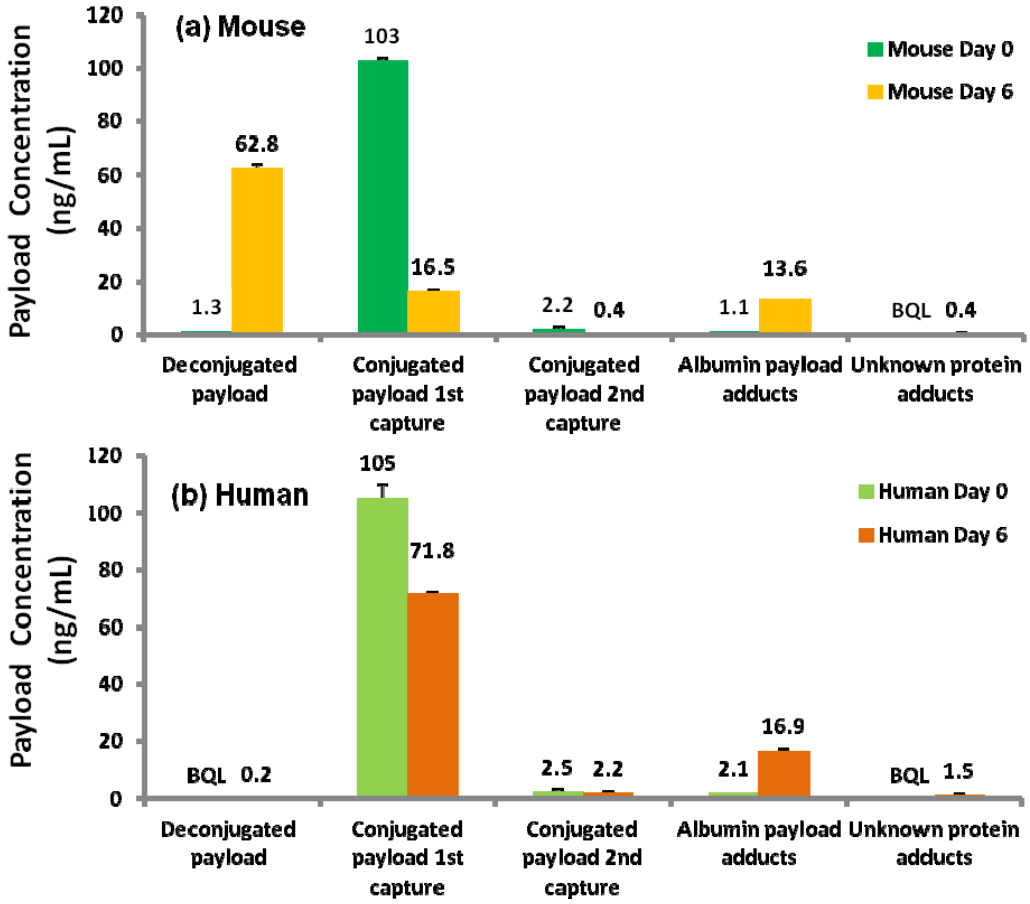
Both conjugated payload and total antibody were quantified against an ADC standard based calibration curve.

Quantitation of Deconjugated Payload and Free Payload (TPP). An aliquot of 30 µL each stability sample was first added to a 96-well extraction plate. Then 15 µL of d8-MMAE (10 ng/mL in 20% acetonitrile) was spiked to the sample as the internal standard followed by adding 160 µL acetonitrile. The plate was centrifuged for 10 minutes at 2100xg. Five µL of supernatant was injected to the LC-MS.

Liquid Chromatography and Mass Spectrometry. A Shimadzu Nexera UHPLC system (Kyoto, Japan) was interfaced to an AB Sciex 5500 QTRAP mass spectrometer (Framingham, MA). The reversed phase UHPLC separation was carried out on a Waters BEH C4 300 Å column (50x2.1 mm, 3.5 µm) with two separate gradients depending on analytes of interest. Mobile phase A (water containing 0.1% FA) and mobile phase B (acetonitrile containing 0.1% FA) were used for both gradient elutions at 0.6mL/min and at 50 ºC. For the surrogate peptide analysis, the gradient employed was as follows: 0 min, 5 % B; 0.4 min, 5 % B; 1.7 min, 90% B; 2.5 min, 90% B; and 2.51-3 min, 5% B. For TPP analysis, the gradient used was as follows: 0 min, 10 % B; 1.3 min, 50 % B; 1.5 min, 90% B; 2.3 min, 90% B; and 2.31-3 min, 10% B. LC-

MS-MS data were collected and processed using Analyst software 1.6.2 (Build 8489) provided by AB Sciex.

Figure S-1. Recovered payload concentration in each fraction of plasma stability samples (n=2) (a) mouse (b) human



Supplemental References:

(1) Xu, L.; Packer, L. E.; Qian, M. G.; Wu, J.-T. *Journal of Pharmaceutical and Biomedical Analysis* **2016**, *128*, 226-235.
(2) Xu, L.; Packer, L. E.; Li, C.; Abdul-Hadi, K.; Veiby, P. *Analytical Biochemistry* **2017**, *537*, 33-36.