Supplementary Information for:

A Case Study Comparing Heterogeneous Lysine- and Site-Specific Cysteine-Conjugated Maytansinoid Antibody-Drug Conjugates (ADCs) Illustrates Benefits of Lysine Conjugation

Nicholas C. Yoder[1,*], Chen Bai[1], Daniel Tavares[1,2], Wayne C. Widdison[1], Kathleen R. Whiteman[1,3], Alan Wilhelm[1], Sharon D. Wilhelm[1,4], Molly A. McShea[1,5], Erin K. Maloney[1], Olga Ab[1], Lintao Wang[6], Shan Jin[6,7], Hans K. Erickson[1,8], Thomas A. Keating[1], and John M. Lambert[1]

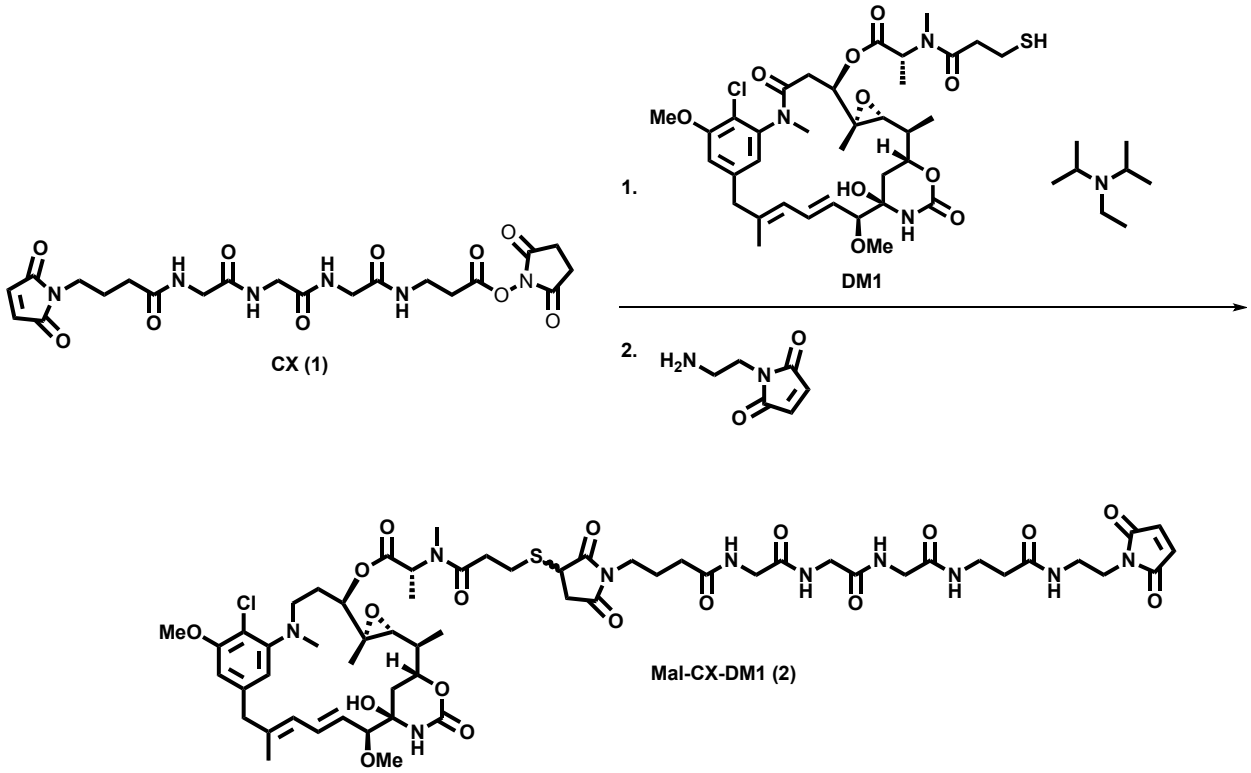
*Correspondence to: nicholas.yoder@immunogen.com

1. Science, Technology, and Translation; ImmunoGen, Inc.; 830 Winter Street, Waltham MA 02451
2. Current affiliation: Takeda Oncology, Cambridge MA
3. Current affiliation: Unum Therapeutics, Cambridge MA
4. Current affiliation: Merck Exploratory Science Center, Cambridge MA
5. Current affiliation: Morphic Therapeutics, Waltham MA
6. Technical Operations; ImmunoGen, Inc.; 830 Winter Street, Waltham MA 02451
7. Current affiliation: QLB Biotherapeutics, Boston MA
8. Current affiliation: Genentech, Inc., South San Francisco CA

General Synthetic Methods.

DM1 was manufactured at Sicor Inc. Synthesis of the core heterobifunctional CX linker (Supplementary Figure 1) has been previously reported [Singh MCT 2016]. All other reagents were obtained from Sigma-Aldrich. Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Ascend 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. NMR chemical shifts are reported in δ values relative to the utilized NMR solvent. High resolution mass spectra were acquired on a Thermo Fisher Q-Exactive mass spectrometer. HPLC purifications were performed using a Varian ProStar preparative HPLC system.

Synthesis of Mal-CX-DM1 (2).



Supplementary Figure 1. Scheme for chemical synthesis of compound **2** (Mal-CX-DM1).

A 10 mL round bottom flask equipped with a stir bar was charged with DM1 (145.4 mg, 0.197 mmol) and DMF (1 mL). CX linker (103 mg, 0.197 mmol) and *N*,*N*-diisopropylethylamine (DIPEA) (0.069 mL, 0.394 mmol) were sequentially added. The reaction was placed under an argon atmosphere and stirred for ~45 minutes at room temperature. 1-(2-aminoethylmaleimide) hydrochloride (34.6 mg, 0.197 mmol) was added directly to this crude reaction. Stirring was continued for ~1h at room temperature. Upon completion, no work-up was performed and the desired product was isolated by semi-preparative C18 HPLC using a Targa C18 10 μm, 20 x 250 mM column. The column was eluted with a gradient of acetonitrile in water containing 0.1% formic acid (see Supplementary Table S1).

Time (min)	% Acetonitrile
0	15
3.75	32
6.25	48
0:00	58
15	100
18	100
18.5	15
20	15

Supplementary Table S1. HPLC gradient used for purification of **2**.

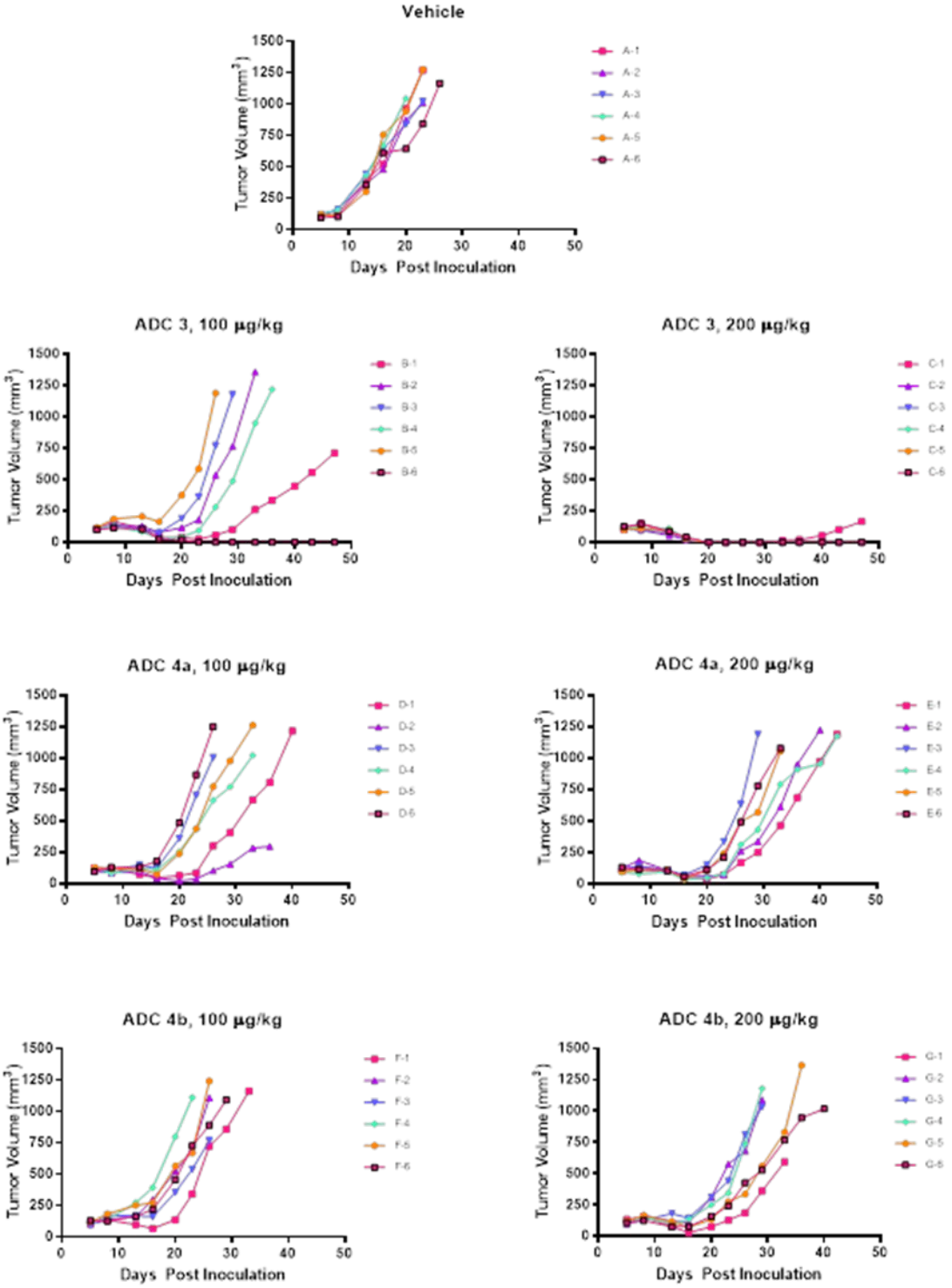
Product containing fractions were combined and concentrated *in vacuo* to give 130.2 mg of the desired Mal-CX-DM1. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 0.78 (d, *J* = 2.0 Hz, 3H), 1.12 (d, *J* = 6.4 Hz, 3H), 1.18 (d, *J* = 6.8 Hz, 3H), 1.22– 1.30 (m, 2H), 1.45 (tt, *J* = 10.2, 5.0 Hz, 2H), 1.59 (s, 3H), 1.61 – 1.72 (m, 3H), 2.04 (dt, *J* = 14.3, 2.7 Hz, 1H), 2.08 – 2.18 (m, 4H), 2.30 – 2.41 (m, 1H), 2.64 – 2.70 (m, 1H), 2.72 (s, 2H), 2.76 – 3.06 (m, 3H), 3.10 (d, *J* = 1.6 Hz, 2H), 3.14 – 3.23 (m, 4H), 3.32 – 3.55 (m, 10H), 3.64 (dd, *J* = 5.9, 3.2 Hz, 2H), 3.71 (t, *J* = 5.3 Hz, 4H), 3.86 (dd, *J* = 9.0, 3.9 Hz, 1H), 3.90 – 3.96 (m, 3H), 4.03 – 4.12 (m, 1H), 4.53 (dd, *J* = 12.0, 2.9 Hz, 1H),

5.31 (dp, *J* = 5.6, 3.9, 2.7 Hz, 1H), 5.51 – 5.60 (m, 1H), 5.75 (s, 2H), 6.50 – 6.62 (m, 3H), 6.85 (s, 1H), 7.00 (s, 2H), 7.16 (td, *J* = 7.0, 6.4, 1.8 Hz, 1H), 7.72 (t, *J* = 5.7 Hz, 1H), 7.95 (t, *J* = 6.1 Hz, 1H), 8.01 – 8.14 (m, 2H). ^{13}C NMR (101 MHz, DMSO-*d*6) δ 11.86, 13.56, 14.88, 15.55, 23.59, 32.82, 35.68, 35.75, 37.30, 37.60, 38.19, 39.51, 40.57, 42.46, 42.58, 55.37, 56.60, 57.05, 60.50, 67.22, 73.65, 80.48, 88.69, 114.45, 117.62, 128.92, 133.07, 134.97, 141.71, 151.68, 155.77, 169.00, 169.50, 169.98, 170.91, 170.98, 171.52, 175.37. HRMS (ESI) m/z: $[M+H]^+$ Calcd for $C_{58}H_{77}ClN_{10}O_{19}S$ 1285.4848; found 1285.4827

Biochemical characterization of ADCs

ADC	UV DAR	SEC DAR	Monomer (%)	Free Drug (%)	H2L2 (%)	Yield (%)
3 (Lys-conjugated M9436A-CX-DM1)	3.3	3.5	99.5	< 1	ND	91
4a (M9436A-C205-Mal-CX-DM1)	2.0	1.9	99.7	1.8	94	85
4b (M9436A-C400-Mal-CX-DM1)	1.7	2.1	99.0	< 1	93	96
4c (M9346A-C118-Mal-CX-DM1)	2.1	2.1	99.3	1.2	94	85
4d (M9346A-C239-Mal-CX-DM1)	2.0	2.1	99.6	3.5	92	87

Supplementary Table S2. Biochemical characterization of ADCs.



Supplementary Figure S2. Tumor growth curves for individual mice from the KB xenograft efficacy study depicted in Figure 7.