Optimization of Tubulysin Antibody-Drug-Conjugates: A Case Study in Addressing ADC Metabolism

L. Nathan Tumey*, Carolyn A. Leverett, Beth Vetelino, Fengping Li, Brian Rago, Xiaogang Han, Frank Loganzo, Sylvia Musto, Guoyun Bai, Sai Chetan K. Sukuru, Edmund I. Graziani, Sujiet Puthenveetil, Jeffrey Casavant, Anokha Ratnayake, Kimberly Marquette, Sarah Hudson, Venkata Ramana Doppalapudi, Joseph Stock, Lioudmila Tchistiakova, Andrew J. Bessire, Tracey Clark, Judy Lucas, Christine Hosselet, Christopher J. O'Donnell, and Chakrapani Subramanyam

Supporting information

General Methods

Synthetic Experimental Procedures:

Experiments were carried out under inert atmosphere (nitrogen or argon), particularly in cases where oxygen- or moisture-sensitive reagents or intermediates were employed. Commercial solvents and reagents were used without further purification, including anhydrous solvents where appropriate (Sure-Seal™ products from the Aldrich Chemical Company, Milwaukee, Wisconsin). Mass spectrometry data is reported from either liquid chromatography-mass spectrometry (LC-MS) or atmospheric pressure chemical ionization (APCI).

NMR data acquisition and processing:

Chemical shifts for nuclear magnetic resonance (NMR) data are expressed in parts per million (ppm, δ) referenced to residual peaks from the deuterated solvents employed. In some cases, ^{13}C NMR shifts were determined by HMBC correlations and were noted as so in the tabulated form. Samples were prepared in DMSO-*d*6 with a concentration of 5 - 30 mM in 3 mm NMR tubes. NMR data were recorded at 300 K on Bruker AVANCE III 500 MHz spectrometer equipped with DCH cryoprobe. 1D ^{1}H spectra were acquired using standard pulse sequence with 32K data points, 8000 Hz sweep width, 3 s repetition time and 16 scans. 1D ^{13}C spectra were collected with 64 K data points and 2K – 8K scans. Standard 2D NMR methods were used to confirm the structures. DQF-COSY spectra were recorded with spectral width of 10 ppm, 2048 points on F2 and 256 increments on F1. Heteronuclear HSQC and HMBC spectra were obtained with spectral width of 10 ppm on F2, and 150 ppm and 220 ppm on F1 dimension, respectively.

The data matrix was 2048 ×128 and zero-filled to 2048 ×512. All data were processed using MestreNova 9.0.

Purification Methods:

Method A: Column: Phenomenex Luna C18 (2), 150 x 21.2 mm I.D., 5 µm; Mobile phase A: 0.1% formic acid in water (v/v); Mobile phase B: 0.1% formic acid in acetonitrile (v/v); Gradient: variable, increasing gradient of B in A over 10 to 20 minutes. Flow rate: 27 mL/minute. Temperature: not controlled; Detection: DAD 215 nm, 254 nm; MS (+) range 150-2000 daltons; Instrument: Waters FractionLynx.

Method B: Column: Phenomenex Luna C18 (2), 150 x 21.2 mm I.D., 5 µm; Mobile phase A: 0.02% TFA in water (v/v); Mobile phase B: 0.02 % TFA in acetonitrile (v/v); Gradient: variable, increasing gradient of B in A over 10 to 20 minutes. Flow rate: 27 mL/minute. Temperature: 45 °C; Detection: DAD 215 nm, 254 nm; MS (+) range 150-2000 daltons; Instrument: Waters FractionLynx.

Method C: Column: Waters Sunfire, C18, 19 x100 mm I.D., 5 µm; Mobile phase A: 0.05% TFA in water (v/v); Mobile phase B: 0.05% TFA in acetonitrile (v/v); Flow rate 25 mL/minute. Detection: DAD 215 nm; MS (+) range 160-1000 daltons; Instrument: Waters FractionLynx.

Analytical Protocols:

Protocol A: Column: Waters Acquity UPLC HSS T3, C18, 2.1 x 50 mm I.D., 1.7 µm; Mobile phase A: 0.1% formic acid in water (v/v); Mobile phase B: 0.1% formic acid in acetonitrile (v/v); Gradient: 5% B over 0.1 minute, 5% to 95% B over 2.5 minutes, 95% B over 0.35 minute; Flow rate: 1.25 mL/minute. Temperature: 60 °C; Detection: 200-450 nm; MS (+) range 100-2000 daltons; Injection volume: 5 µL; Instrument: Waters Acquity.

Protocol B: Column: Waters Acquity UPLC HSS T3, C18, 2.1 x 50 mm I.D., 1.7µm; Mobile phase A: 0.1% formic acid in water (v/v); Mobile phase B: 0.1% formic acid in acetonitrile (v/v); Gradient: 5% B over 0.1 minute, 5% to 95% B over 1.5 minute, 95% B over 0.35 minute; Flow rate: 1.25 mL/minute. Temperature: 60 °C; Detection: 200-450 nm; MS (+) range 100-2000 daltons; Injection volume: 5 µL; Instrument: Waters Acquity.

Protocol C: Column: Phenomenex Luna C18 (2), 150 x 3.0 mm I.D., 5 µm; Mobile phase A: 0.1% formic acid in water (v/v); Mobile phase B: 0.1% formic acid in acetonitrile (v/v); Gradient: 5% B over 1.5 minutes, 5% to 100% B over 8.5 minutes, then 100% B for 1 minute; Flow rate: 0.75 mL/minute. Temperature: 45 °C; Detection: DAD 215 nm, 254 nm; MS (+) range 150-2000 daltons; Injection volume: 10 µL; Instrument: Agilent 1200 LCMS.

Protocol D: Column: Atlantis dC18, 50 x 4.6 mm I.D., 5 µm; Mobile phase A: 0.05% TFA in water (v/v); Mobile phase B: 0.05% TFA in acetonitrile (v/v); Gradient: 5% to 95% B over 4.0 minutes, linear; then hold at 95% B over 1 minute. Flow rate: 2 mL/minute. Temperature: room temperature; Detection: DAD 215 nm; MS (+) range 160 -1000 daltons; injection volume 3 uL; Instrument: Waters 996 PDA.

Protocol E: Column: Ultimate XB-C18, 2.1 x 30 mm I.D., 3 µm; Mobile phase A: 0.04% TFA in water (v/v); Mobile phase B: 0.02% TFA in acetonitrile (v/v); Gradient: 10% to 80% B over 0.9 minutes then hold at 80% B over 0.6 minutes, then 10% B for 0.5 minutes. Flow rate: 1.2 mL/minute. Temperature: 50 °C. Detection: 220 nm; MS (+) range 0 -1000 daltons; injection volume 2 uL; Instrument: Shimadzu LC-MS 2010.

Protocol F: Column: Merck RP-18e, 25-2; Mobile phase A: 0.04% TFA in water (v/v); Mobile phase B: 0.02% TFA in acetonitrile (v/v); Gradient: 5% to 95% B over 0.7 minutes then hold at 95% B over 0.4 minutes, then 5% B for 0.4 minutes. Flow rate: 1.5 mL/minute. Temperature: 50 °C. Detection: 220 nm; MS (+) range 0 -1000 daltons; injection volume 2 uL; Instrument: Shimadzu LC-MS 2010.

Preparation of target compounds:

1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-(acetyloxy)-1-[4-({(2*R*,4*S*)-4-carboxy-1-[4-({*N*-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycyl}amino)phenyl]pentan-2-yl}carbamoyl)-1,3-thiazol-2-yl]-4-methyl pentan-3-yl}-*N*-methyl-*L*-isoleucinamide (LP1):

Step 1: DMF (2 mL) and DIPEA (417 µL, 2.37 mmol) was added to a vial containing *N*-Boc glycine (166 mg, 0.948 mmol) and HATU (364 mg, 0.948 mmol) . The reaction was stirred under N_2 for ~0.5 h then added by syringe to a vial containing compound **2** (203 mg, 0.237 mmol), further diluting with DMF (1.2 mL). After stirring for 2.5 h at rt the reaction was concentrated and the residue re-dissolved in DMSO (~2 mL) and H_2O (~1 mL, containing 0.02% TFA) and purified by medium pressure C18 chromatography (10% acetonitrile /90% H_2O for 5 minutes, then 10% acetonitrile to 95% acetonitrile in H_2O over 18 minutes,

each solvent containing 0.02% TFA) to yield the desired intermediate (73 mg, 35%) as a white solid. LC-MS (Protocol B): *m/z* 900.4 $[M+H]^+$; Retention time = 0.84 min.

Step 2: DCM (1 mL) and TFA (350 µL) was added to the above intermediate (35 mg, 0.039 mmol) and the reaction was stirred at rt under N_2. After 3 h the reaction was concentrated *in vacuo* and dried under high vacuum to afford the glycyl amine (45 mg, quantitative yield) as a gummy residue, which was used crude in the next step. LC-MS (Protocol B): *m/z* 800.4 $[M+H]^+$; Retention time = 0.68 min.

Step 3: 1-{6-[(2,5-dioxopyrrolidin-1-yl)oxy]-6-oxohexyl}-1H-pyrrole-2,5-dione (**6**, 31 mg, 0.101 mmol) and DMF (2.6 mL) was added to a vial containing the above intermediate (79 mg, 0.099 mmol).. DIPEA (52 µL, 0.296 mmol) was added and the reaction was stirred at rt in a capped vial for 1.5 h. An additional (10 mg, 0.048 mmol) of **6** and DIPEA (14 µL, 0.081 mmol) was added and the reaction stirred at rt for 2 h. The reaction was concentrated and purified by reverse phase chromatography (Method A) to provide the title compound **LP1** (35 mg, 26% over two steps) as a white solid. HPLC (Protocol C) *m/z* 993.4 $[M+H]^+$; Retention time = 7.02 min. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.86 (s, 1H), 8.21 – 8.03 (m, 3H), 7.81 (d, *J* = 10.0 Hz, 1H), 7.45 (d, *J* = 8.2 Hz, 2H), 7.13 (d, *J* = 8.2 Hz, 2H), 6.99 (s, 2H), 5.59 (dd, *J* = 10.7, 3.1 Hz, 1H), 4.61 (dd, *J* = 9.9, 7.4 Hz, 1H), 4.39 (s, 1H), 4.24 – 4.11 (m, 1H), 3.82 (d, *J* = 5.8 Hz, 2H), 3.38 (m, 2H), 3.02 (m, 1H), 2.95 (s, 3H), 2.88 – 2.69 (m, 2H), 2.46 (m, 1H), 2.37 (m, 1H), 2.31 – 2.17 (m, 5H), 2.13 (t, *J* = 7.5 Hz, 2H), 2.09 (s, 3H), 1.87 – 1.74 (m, 3H), 1.73 – 1.63 (m, 2H), 1.62 – 1.43 (m, 8H), 1.20 (m, 2H), 1.08 (s, 3H), 1.04 (m, 4H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.89 – 0.80 (m, 6H), 0.67 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 177.6, 175.4, 173.2, 172.9, 171.5, 171.5, 170.1, 169.8, 168.1, 160.4, 150.3, 137.4, 134.7, 134.7, 133.9, 129.7, 129.7, 124.3, 119.3, 119.3, 69.8, 67.0, 55.5,* 54.1, 52.8, 49.4, 43.1, 40.5, 39.6, 38.1, 37.5, 36.8, 36.8, 36.0, 35.4, 34.3, 29.8,* 29.6, 28.3, 26.3, 25.0, 24.3, 22.6, 21.3, 20.6, 19.7, 18.8, 16.1, 16.1, 11.4. HRMS Calcd. for $[M+H]^+$: 993.5121, Found: 993.5111.

**Value derived by HMBC correlation*

1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-[4-({(2*R*,4*S*)-4-carboxy-1-[4-({*N*-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycyl}amino)phenyl]pentan-2-yl}carbamoyl)-1,3-thiazol-2-yl]-1-hydroxy-4-methylpent an-3-yl}-*N*-methyl-*L*-isoleucinamide (LP2):

Step 1. DMF (1.5 mL) and DIPEA (54 µL, 0.31 mmol) was added to a vial containing *N*-Boc glycine (8 mg, 0.045 mmol) and HATU (17 mg, 0.045 mmol). The reaction was stirred in a capped vial for 0.5 h then added by syringe to a vial containing a solution of compound **3** (21 mg, 0.026 mmol) in DMF (1 mL). After stirring for 24 h at rt, an additional 0.003 mmol of HATU-activated *N*-Boc glycine (prepared by stirring HATU (10 mg, 0.003 mmol), *N*-Boc glycine (6 mg, 0.034 mmol) and DIPEA (36 µl, 0.207 mmol) in 0.5 mL DMF for 30 minutes) was added and the reaction mixture was stirred for 24 h. The reaction was concentrated to a crude gummy residue which was re-dissolved in DMSO (~2 mL) and purified by medium pressure C18 chromatography (10% to 95% acetonitrile in H_2O over 25 minutes, each solvent containing 0.02% TFA) to afford the desired intermediate (7 mg, 20%). LC-MS (Protocol B): *m/z* 858.5 $[M+H]^+$; Retention time = 0.71 min.

Step 2. DCM (200 µL) and TFA (80 µL) was added to a vial containing the above intermediate (7 mg, 0.007 mmol) and the reaction was stirred at rt under N_2. After 3 h the reaction was concentrated under high vacuum to afford a gummy residue.

Step 3. 1-{6-[(2,5-dioxopyrrolidin-1-yl)oxy]-6-oxohexyl}-1H-pyrrole-2,5-dione (**6**, 3.6 mg, 0.012 mmol) and DMF (0.7 mL) was added to this crude residue . DIPEA (20 µL, 0.110 mmol) was added and the reaction was stirred at rt in a capped vial for 17.5 h. The reaction was concentrated to a brown gummy residue and purified by reverse phase chromatography (Method B) to provide target compound **LP2** (3 mg, 26%) as a white solid. HPLC (Protocol C) *m/z* 951.5 $[M+H]^+$; Retention time = 6.70 min. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.95 (s, 1H), 9.79 (s, 1H), 8.64 (d, *J* = 9.2 Hz 1H), 8.01 (t, *J* = 5.8 Hz, 1H), 8.01 (s, 1H), 7.72 (d, *J* = 9.1 Hz, 1H), 7.38 (d, *J* = 8.1 Hz, 2H), 7.05 (d, *J* = 8.1 Hz, 2H), 6.92 (s, 2H), 6.08 (d, *J* = 5.7 Hz, 1H), 4.54 (t, *J* = 9.2 Hz, 1H), 4.46 (m, 1H), 4.40 (m, 1H), 4.10 (m, 1H), 3.75 (d, *J* = 5.8 Hz, 2H), 3.49 (m, 1H), 3.31 (t, *J* = 7.1 Hz, 2H), 3.09 (m, 1H), 2.96 (s, 3H), 2.77 – 2.65 (m, 2H), 2.60 (s, 3H), 2.32 (m, 1H), 2.23 (m, 1H), 2.13 – 2.02 (m, 4H), 2.01 – 1.87 (m, 2H), 1.84 – 1.69 (m, 4H), 1.52 – 1.33 (m, 9H), 1.15 (m, 2H), 1.04 (m, 1H), 0.99 (d, *J* = 7.1 Hz, 3H), 0.84 (d, *J* = 6.5 Hz, 3H), 0.82 – 0.72 (m, 6H), 0.65 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.5, 177.4, 172.9, 172.9, 172.4,* 171.5, 171.5, 170.1, 168.1, 160.6, 137.5, 134.7, 134.7, 133.8, 129.8, 129.8, 123.7, 119.3, 119.3, 72.8, 68.6, 56.0,* 54.4, 53.8, 49.2, 42.9, 40.5, 38.1, 37.9, 37.5, 36.5, 36.2, 35.5, 35.4, 35.3, 30.1, 29.9, 28.3, 26.4, 25.1, 24.5, 20.7, 20.6, 20.1, 18.6, 16.6, 15.7, 11.2. HRMS Calcd. for $[M+H]^+$: 951.5016, Found: 951.5009.

**Value derived by HMBC correlation*

(2*S*,4*R*)-4-amino-5-[4-({*N*-[(9H-fluoren-9-ylmethoxy)carbonyl]glycyl}amino)phenyl]-2-methylpentanoic acid TFA salt(8):

Step 1: A solution of (1*R*,2*S*,5*R*)-5-methyl-2-(propan-2-yl)cyclohexyl (2*S*,4*R*)-4-[(*tert*-butoxycarbonyl)amino]-2-methyl-5-(4-nitrophenyl)pentanoate[27] (800 mg, 1.63 mmol) and Pd-C (500 mg) in MeOH (30 mL) was stirred under 40 psi H_2 at rt overnight. The reaction mixture was filtered through a pad of celite and the filtrate was concentrated *in vacuo* to afford (1*R*,2*S*,5*R*)-5-methyl-2-(propan-2-yl)cyclohexyl (2*S*,4*R*)-5-(4-aminophenyl)-4-[(*tert*-butoxycarbonyl)amino]-2-methylpentanoate (750 mg, quantitative yield) as a yellow oil. LC-MS (Protocol F): *m/z* 483.2 $[M+Na]^+$; Retention time = 0.88 min.

Step 2. HATU (636 mg, 1.67 mmol) was added to a solution of the above compound (700 mg, 1.52 mmol), *N*-Fmoc glycine (497 mg, 1.67 mmol), and DIPEA (295 mg, 2.28 mmol) in DMF (20 mL) at 0 °C and the resulting solution was stirred at rt for 2 h. The reaction mixture was poured into H_2O and extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash silica gel chromatography (100% EtOAc to 10% MeOH/90% DCM) to afford (2*S*,4*R*)-4-amino-5-[4-({*N*-[(9H-fluoren-9-ylmethoxy)carbonyl]glycyl}amino)phenyl]-2-methylpentanoic acid (900 mg, 80%) as a yellow oil.

Step 3. A solution of the above compound (1.0 g, 1.351 mmol) in TFA (10 mL) was refluxed at 110 °C for 1 h. The reaction mixture was stirred at 130 °C for 15 min under microwave conditions. The reaction mixture was concentrated *in vacuo* to a crude oil which was purified by preparative HPLC (Column: Synergi 250 mm x 50mm I.D., 10 µm; Mobile phase: 25% acetonitrile in H_2O (0.1% TFA) to 55% acetonitrile in H_2O (0.1% TFA) over 30 minutes) to afford the title compound **8** (330 mg, 49%) as a white solid. LC-MS (Protocol F): *m/z* 502.3 $[M+H]^+$; Retention time = 0.73 min. ^{1}H NMR (400 MHz, DMSO-*d6*): δ 10.01 (br, 1H), 7.92-7.82 (m, 5H), 7.74-7.73 (m, 2H), 7.58-7.56 (m, 3H), 7.43-7.41 (m, 2H),7.36-7.34 (m, 2H), 7.19-7.17 (m, 2H), 4.32-4.20 (m, 3H), 3.80-3.73 (m, 2H), 2.89-2.73 (m, 1H), 1.86-1.81 (m, 1H), 1.01 (d, 3H).

1,2-dimethyl-*D*-prolyl-*N*-{(1*R*,3*R*)-1-[4-({(2*R*,4*S*)-4-carboxy-1-[4-({*N*-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoyl]glycyl}amino)phenyl]pentan-2-yl}carbamoyl)-1,3-thiazol-2-yl]-1-[(ethylcarbamoyl)oxy]-4-methylpentan-3-yl}-*N*-methyl-*L*-isoleucinamide (LP3):

Step 1: DMF (1.5 mL) and DIPEA (33 µL, 0.189 mmol) was added to a vial containing 1,2-dimethyl-*D*-prolyl-*N*-[(1*R*,3*R*)-1-[(ethylcarbamoyl)oxy]-4-methyl-1-{4-[(pentafluorophenoxy)carbonyl]-1,3-thiazol-2-yl}pentan-3-yl]-*N*-methyl-*L*-isoleucinamide (**9**, 20 mg, 0.027 mmol) and (2*S*,4*R*)-4-amino-5-[4-({*N*-[(9H-fluoren-9-ylmethoxy)carbonyl]glycyl}amino)phenyl]-2-methylpentanoic acid TFA salt (**8**, 17 mg, 0.027 mmol)and the reaction was stirred in a capped vial at rt. After stirring for 16 h at rt, the reaction was concentrated to a gummy residue which was re-dissolved in DMSO (~1.5 mL) and acetonitrile/H_2O (0.5 mL, 1/1, each containing 0.02% TFA), and purified by medium pressure C18 chromatography (10% to 95% acetonitrile in H_2O over 25 minutes, each solvent containing 0.02% TFA) to afford the target compound (19 mg, 67%) as a colorless solid. LC-MS (Protocol B): *m/z* 1051.6 $[M+H]^+$; Retention time = 0.83 min.

Step 2. DCM (1 mL) and *N,N*-diethylamine (30 µL, 0.290 mmol) was added to the above intermediate (19 mg, 0.018 mmol) and the resulting reaction was stirred at rt in a closed vial for 3 h, after which time additional *N,N*-diethylamine (130 µL) was added in 3 aliquots over the course of 4 h. After stirring for a total of 7 h at rt, the reaction was concentrated *in vacuo*, the residue re-dissolved in DMSO (~1.5 mL) and acetonitrile/H_2O (0.5 mL, 1/1, each solvent containing 0.02% TFA), and purified by medium pressure C18 chromatography (10% to 95% acetonitrile in H_2O over 25 minutes, each solvent containing 0.02% TFA) to afford the desired compound (10 mg, 62%) as a colorless solid. LC-MS (Protocol B): *m/z* 829.4 $[M+H]^+$; Retention time = 0.61 min.

Step 3. 1-{6-[(2,5-dioxopyrrolidin-1-yl)oxy]-6-oxohexyl}-1H-pyrrole-2,5-dione (**12**, 3 mg, 0.010 mmol) and DMF (1.2 mL) was added to a vial containing the above intermediate (10 mg, 0.011 mmol).. DIPEA (17 µL, 0.099 mmol) was added and the reaction was stirred at rt in a closed vial for 17 h. The reaction was concentrated and the resulting residue purified by reverse phase chromatography (Method B) to provide the target compound **LP3** (5.8 mg, 52%) as a colorless solid. HPLC (Protocol C): *m/z* 1022.8 $[M+H]^+$; Retention time = 6.92 min. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.82 (s, 1H), 8.06 (s, 1H), 8.05 (t, *J* = 5.8 Hz, 1H), 7.94 (d, *J* = 9.1 Hz, 1H), 7.74 (d, *J* = 9.8 Hz, 1H), 7.39 (d, *J* = 8.1 Hz, 2H), 7.33 (t, *J* = 5.7 Hz, 1H), 7.06 (d, *J* = 8.4 Hz, 2H), 6.92 (s, 2H), 5.40 (m, 1H), 4.53 (dd, *J* = 9.8, 7.4 Hz, 1H), 4.31 (m, 1H), 4.14 (m, 1H), 3.75 (d, *J* = 5.8 Hz, 2H), 3.31 (t, *J* = 7.1 Hz, 2H), 2.96 – 2.87 (m, 3H), 2.90 (s, 3H), 2.79 – 2.67 (m, 2H), 2.38 (m, 1H), 2.17 (m, 1H), 2.15 (s, 3H), 2.06 (t, *J* = 7.4 Hz, 4H), 1.87 (m, 1H), 1.77 – 1.54 (m, 4H), 1.54 – 1.36 (m, 8H), 1.14 (m, 2H), 1.01 (s, 3H), 0.97 – 0.90 (m, 7H), 0.85 (d, *J* = 6.5 Hz, 3H), 0.79 (d, *J* = 6.4 Hz, 3H), 0.75 (t, *J* = 7.4, Hz, 3H), 0.60 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 178.3, 175.4, 173.0, 172.9, 172.3, 171.5, 171.5, 168.2, 160.5, 155.2, 150.1, 137.5, 134.9, 134.9, 134.1, 129.8, 129.8, 124.1,

119.4, 119.4, 70.2, 67.0, 55.4,* 54.1, 53, 48.5, 43.1, 40.9, 39.6, 38.0, 37.5, 36.9, 36.9, 35.9, 35.6, 35.3, 35.3, 29.7, 29.7, 28.3, 26.4, 25.0, 24.2, 22.6, 20.5, 19.8, 17.0, 16.1, 16.0, 15.5, 11.3. HRMS Calcd. for $[M+H]^+$: 1022.5387, Found: 1022.5377.

**Value derived by HMBC correlation*

Production of Cysteine Engineered Trastuzumab Variants

Select positions were chosen in the Trastuzumab constant regions to engineer reactive cysteines to enable site-specific conjugation with a Drug: Antibody ratio equal to two (DAR2), specifically K183 on the Kappa chain (Kabat numbering) and K334, Q347, E388, K392 and L443 (EU numbering) within the Fc-region. To produce the cysteine engineered Trastuzumab variants for conjugation studies, HEK-293 cells were transiently co-transfected with heavy and light chain DNA encoding the cysteine engineered antibody variants using standard methods. A two-step purification strategy, i.e., Protein-A affinity capture followed by size exclusion chromatography (SEC), was used to isolate these cysteine variants from the concentrated HEK-293 starting material. Using these purification process, all cysteine engineered Trastuzumab variant preparations contained >97.9% peak-of-interest (POI) as determined by analytical size-exclusion chromatography (Table #KM).

Differential Scanning Calorimetry (DCS) was used to determine the thermal stability of select single cysteine engineered Trastuzumab variants. For this analysis, samples formulated in PBS-CMF pH 7.2 were dispensed into the sample tray of a MicroCal VP-Capillary DSC with Autosampler (GE Healthcare Bio-Sciences, Piscataway, NJ), equilibrated for 5 minutes at 10°C and then scanned up to 110°C at a rate of 100°C per hour. A filtering period of 16 seconds was selected. Raw data was baseline corrected and the protein concentration was normalized. Origin Software 7.0 (OriginLab Corporation, Northampton, MA) was used to fit the data to an MN2-State Model with an appropriate number of transitions. All single cysteine engineered Trastuzumab variants evaluated using DSC exhibited excellent thermal stability as determined by the first melting transition (Tm1) >65°C (Table #KM2).

Table S1

Engineered Trastuzumab Variant	%POI after ProA	%POI after Superdex 200
κK183C	94.3	99.3
K334C	ND	>99

Q347C	91.1	99.2
E388C	91.0	99.7
K392C	77.1	97.9
L443C		>99.7

Table S2

Antibody	**Tm1 (°C)**	**Tm2 (°C)**	**Tm3 (°C)**
κK183C	72.17 ± 0.029	80.78 ± 0.37	82.81 ± 0.055
Q347C	72.41 ± 0.30	82.19 ± 0.03	
E388C	72.00 ± 0.14	82.31 ± 0.02	
L443C	72.02 ± 0.06	80.98 ± 1.10	82.96 ± 0.11

Conjugation chemistry:

Size exclusion chromatography (SEC):

Analytical SEC was performed on an Agilent 1100 HPLC using a GE Superdex 200 (5/150 GL) column. An isocratic gradient was used wherein the mobile phase was phosphate buffered saline (PBS) at pH 7.4 containing with 2% acetonitrile. The flow rate was 0.25 ml/minute at ambient temperature. Typical injection size is 10 µg of ADC.

Preparative SEC was typically performed on an Akta Explorer (GE) using a Superdex 200 (10/300 GL) column. An isocratic gradient was used wherein the mobile phase was PBS at pH 7.4. The flow rate was typically 1 mL/min at ambient temperature. Typical injection size is 5 mg of ADC.

Hydrophobic interaction chromatography (HIC):

Analytical HIC was performed on an Agilent 1100 HPLC using a Butyl-NPR column (Tosoh, part# S0557-83S). Buffer A = 1.5M ammonium sulfate containing 50 mM phosphate, pH 7.0. Buffer B = 50 mM phosphate, pH 7.0, containing 20% isopropanol. A gradient from 100% A to 0% A was performed over 12 minutes. The gradient was held at 0% A for 2 minutes before re-equilibration in 100% buffer A. The flow rate 0.8 mL/min at ambient temperature. The typical injection size is 20 µg. The relative retention

index was calculated by dividing the retention time of the peak of interest by the retention time of a standard antibody (trastuzumab) injected on the same day.

LC-MS analysis: LCMS analysis was performed using an Aquity H-class UPLC connected to a Xevo G2-XS TOF mass spectrometer. Samples were reduced with TCEP immediately prior to analysis. The separation was performed using a BEH-C18 column (2.1µm x 50 mm, P/N 186002350) at 80 °C. A gradient from 10% acetonitrile to 95% acetonitrile in water (+0.1% formic acid) was performed. MS data was collected from 700-1900 m/z (positive mode). The entire protein peak was selected for deconvolution using MaxEnt software. Typical injection size is 0.1 µg.

Conjugation method A: (to hinge cysteine residues) The antibody was prepared in phosphate buffered saline (PBS, pH 7.4) containing 5 mM EDTA at a concentration of 15 mg/mL (~100 uM). TCEP (1.9-2.1 eq) was added as a 5 mM stock solution in water. The reaction was warmed to 37°C for 1h. Upon cooling to room temperature, dimethylacetamide (DMA), PBS, and the appropriate linker-payload (10 mM stock in DMA) was added such that the final protein concentration was 10 mg/mL (~66 uM), the final organic concentration was 10% (v/v), and the final linker-payload concentration was ~460 uM (~7 eq). The reaction was gently agitated or allowed to stand at rt for 1-1.5h after which time the sample was buffer exchanged into PBS using sephadex gel filtration cartridges, following the manufacturer's instructions. The crude sample was purified by preparative SEC, collecting the monomeric fractions. The resulting sample was concentrated to ~3-5 mg/mL in PBS.

Conjugation method B[1]: (to site-specific cysteines) The cysteine mutant antibody was prepared in PBS containing 20 mM EDTA at a concentration of 10 mg/mL. (~66 µM) TCEP (0.5 M stock) was added in order to give a final concentration of approximately 7 mM (100 eq). The solution was allowed to stand at ambient temperature for 12h and subsequently buffer exchanged into PBS using sephadex gel filtration cartridges, following the manufacturer's instructions. The material thus obtained (~6 mg/mL) was treated with a 50 mM stock solution of dehydroascorbic acid (DHA) in 1:1 water:EtOH to give a final

[1] Shen, B.-Q.; Xu, K.; Liu, L.; Raab, H.; Bhakta, S.; Kenrick, M.; Parsons-Reponte, K. L.; Tien, J.; Yu, S.-F.; Mai, E.; Li, D.; Tibbitts, J.; Baudys, J.; Saad, O. M.; Scales, S. J.; McDonald, P. J.; Hass, P. E.; Eigenbrot, C.; Nguyen, T.; Solis, W. A.; Fuji, R. N.; Flagella, K. M.; Patel, D.; Spencer, S. D.; Khawli, L. A.; Ebens, A.; Wong, W. L.; Vandlen, R.; Kaur, S.; Sliwkowski, M. X.; Scheller, R. H.; Polakis, P.; Junutula, J. R. Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. *Nat. Biotechnol.* **2012**, *30*, 184-189.

concentration of ~1.6 mM and allowed to stand at 4 °C overnight. The excess DHA was removed by buffer exchange (sephadex cartridges) and the resulting antibody was concentrated to ~5 mg/mL.

The antibody thus obtained was treated with DMA and the appropriate linker payload (10 mM stock in DMA) such that the final concentration was 10% (v/v), and the final linker-payload concentration was ~240 µM (~7 eq). The reaction was allowed to stand at ambient temperature for ~2h after which time the excess linker-payload was removed by sephadex filtration per the manufacturer's instructions. The resulting conjugate was purified by preparative SEC, collecting the monomeric fractions. The resulting sample was concentration to ~3-5 mg/mL in PBS.

Table S3: Characterization of ADCs

ADC	Method	LP used	mAb used	LCMS DAR	Δ mass (calc'd)	Δ mass (obs'ved.)	HIC ret.time[#] (main peak) (min)	% Agg.	Yield
ADC1	A	LP1	Tras	4.4	994	993	5.47	1.7%	78%
ADC2	A	LP1	Neg8.8	3.8	994	993	NA	0%	87%
ADC3	B	LP2	Tras-A114C	1.9	951	950	5.61	4.5%	54%
ADC4	A	LP3	Tras	4.0	1022	1022	5.91	5%	67%
ADC5	B	LP1	Tras-K392C	2.0	993	993	5.47	0%	74%
ADC6	B	LP1	Tras-K334C	2.0	995	993	5.14	0%	47%
ADC7	B	LP1	Tras-Q347C	2.0	993	993	5.96	0%	36%
ADC8	B	LP1	Tras-L443C	2.1	996	993	6.64	0%	50%
ADC9	B	LP1	Tras-E388C	2.0	994	993	5.95	0%	48%
ADC10	B	LP1	Tras-k-K183C	2.0	993	993	5.38	0%	64%

[#] The retention time of naked trastuzumab under these conditions is 4.89.

Cell lines and Cytotoxicity Assay: Cell lines were obtained from ATCC (Manassas, VA). Cells were maintained in DMEM, MEM, or RPMI media supplemented with 10% fetal bovine serum (FBS), 1% L-

glutamine, 1% sodium pyruvate. Cytotoxicity assessment was determined as reported previously.[2] Briefly, cells were treated with ADCs or compounds for 4 days, then cell viability assessed with CellTiter® 96 AQueous One MTS Solution (Promega, Madison, WI). IC_{50} values were calculated using a four-parameter logistic model with XLfit (IDBS, Bridgewater, NJ).

In vivo studies: All activities involving animals were carried out in strict accordance with federal, state, local, and institutional guidelines governing the use of laboratory animals in research and were reviewed and approved by Pfizer (or relevant) institutional animal care and use committee.

N87 Efficacy study: Female athymic nu/nu (Nude, Stock No: 002019 mice obtained from The Jackson Laboratory (Farmington, CT) were injected subcutaneously in the flank with suspensions of 1 X 10^6 of N87 cells respectively in 50% Matrigel (BD Biosciences, Franklin Lakes, NJ). Mice were randomized into study groups when tumors reached approximately 150 mg to 300 mg. Either phosphate buffered saline (PBS, Gibco, Cat#14190-144, as vehicle) or the test ADC were administered intravenously starting on day 0 for a total of four doses, 4 days apart (Q4d x4). Tumors were measured at least weekly with a calibrator (Mitutoyo, Aurora, Illinois) and the tumor mass was calculated as volume = (width X width X length)/2.

Antibody PK exposure by ligand-binding assay: The total antibody concentrations were determined by an LBA where a sheep anti-human IgG antibody (Binding Site, San Diego, CA, USA) was used for capture and a goat anti-human IgG antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA) was used for detection. Plasma concentration data for each animal was analyzed using Watson LIMS version 7.4 (Thermo).

Plasma stability method: From stock ADC solutions, 50 µg/mL of each ADC was prepared in fresh pooled male CD-1 mouse plasma with sodium heparin. In a 96-well plate, 0-min time-point was sampled immediately after the ADC plasma preparation. The plate was then placed in a - 80 ^oC freezer. The remaining ADC plasma samples in capped tubes were incubated at 37 ^oC in a 5% CO_2 controlled

[2] Loganzo, F.; Tan, X.; Sung, M.; Jin, G.; Myers, J. S.; Melamud, E.; Wang, F.; Diesl, V.; Follettie, M. T.; Musto, S.; Lam, M.-H.; Hu, W.; Charati, M. B.; Khandke, K.; Kim, K. S. K.; Cinque, M.; Lucas, J.; Graziani, E.; Maderna, A.; O'Donnell, C. J.; Arndt, K. T.; Gerber, H.-P. Tumor Cells Chronically Treated with a Trastuzumab-Maytansinoid Antibody-Drug Conjugate Develop Varied Resistance Mechanisms but Respond to Alternate Treatments. *Mol. Cancer Ther.* **2015**, *14*, 952-963.

incubator. Aliquots were removed after 24 h and 72 h and frozen at -80°C until analysis. At the time of analysis, the plasma samples were thawed and deglycosylated for 2 h at 37°C using 2 µl of IgZero (Genovis, Switzerland). The incubation time (Figure 3) is reported as the total time period for which ADCs were in contact with plasma. The immunocapture and LC-MS method are described below.

In vivo immunocapture LCMS:

For DAR evaluation and metabolite identification relied on an immunocapture high resolution LC/MS method. In short, analysis of samples from nu/nu mice (plasma) following bolus administration of ADC was performed by adding 50 µl of matrix to a 96 well lo-bind plate (Eppendorf, Hamburg, Germany) followed by deglycosylation for 1.5 h at 37 °C using 2 µl of IgZero (Genovis, Switzerland). Capture of ADC was performed using biotinylated goat anti-human FC gamma (Jackson ImmunoResearch, West Grove, PA) at a ratio of 3:1 capture:ADC for 1 h at room temperature under gentle shaking. Streptavidin T1 beads (Life Technologies, Grand Island, NY) were washed and added to the samples, mixing for 0.5 h. The samples were washed with the aid of a magnet to retain the beads, and finally the ADC samples were eluted with 50 µl of 2% formic acid. The samples were analyzed in a reduced format by treatment with 200 mM tris(2-carboxyethyl)phosphine (TCEP) for a final TCEP concentration of 20 mM. The resulting samples (2 µl) were injected onto a BEH C4 column (150µm x 50 mm, 1.7um, 300A, Waters) set at 85°C, with an autosampler set at 4°C. LC separation was achieved using a nano-acquity LC system (Waters Technology). Spectra were collected from 2-8 min using a Waters Synapt-G2S QToF equipped with an ionKey nanospray source (Waters Technology). Positive TOF-MS scan was collected over a m/z range of 800-2100 amu using MassLynx (Waters Technology) software, and was deconvoluted using the MaxEnt1 algorithm in BioPharmaLynx software (Waters Technology).

ADC Catabolism Study: Catabolism studies of ADCs were conducted using human liver S9 fraction or mouse liver crude lysosomal fraction (CLF) as the source of lysosomal enzymes. Mixed-gender human liver S9 fraction containing 20 mg/mL total protein was purchased from Celsis (now Bioreclamation IVT) and was used as received. Mouse liver CLF was prepared as per published procedures,[3] and protein pellets were reconstituted in incubation buffer to 2.5 mg/mL total protein. All experiments were carried out at 37 °C in 50 mM sodium acetate buffer adjusted to pH 5.0 with sulfuric acid, and contained

[3] Doronina, S. O.; Mendelsohn, B. A.; Bovee, T. D.; Cerveny, C. G.; Alley, S. C.; Meyer, D. L.; Oflazoglu, E.; Toki, B. E.; Sanderson, R. J.; Zabinski, R. F.; Wahl, A. F.; Senter, P. D. Enhanced activity of monomethylauristatin F through monoclonal antibody delivery: effects of linker technology on efficacy and toxicity. *Bioconj. Chem.* **2006**, *17*, 114-124.

2 mM TCEP. Incubation mixtures contained either human liver S9 fraction or mouse liver CLF in buffer (1.0 or 0.25 mg/mL, respectively) in a final volume of 200 µL. Reactions were initiated by the addition of the substrate ADC (2 µM final concentration). Samples (35 µL) were removed at 0, 0.5, 1.0, and 18 hrs and were immediately quenched in 5 volumes of cold acetonitrile containing 0.1 µM loperamide as a mass and retention time standard. These samples were centrifuged at 16,870 x *g* for 5 min, then supernatants were transferred to clean vessels for evaporation of the solvent. Residues were reconstituted in 50 µL of 5:95 acetonitrile:water (v:v) for analysis by LC/MS^n. Chromatography was achieved using a 2.7 micron Kinetix C8 2.1 x 100 mm column (Phenomenex) maintained at 45 °C, with a mobile phase composed of 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.300 mL/min. The following gradient program was used for separation: 0.00 – 1.00 min, 10% B; ramp to 70% B by 10.00 min; increase to 95% B by 10.10 min; hold at 95% B until 12.50 min; decrease to 10% B by 12.51 min, then re-equilibrate the column at 10% B until 15.00 min. An Orbitrap Elite mass spectrometer (Thermo) was equipped with a heated electrospray ion source, and was utilized in the positive-ion mode. Full-scan mass data were collected from *m/z* 200-2000 at a resolving power of 30,000, and data-dependent MS^n was collected on the most intense ions at a resolving power of 15,000 using HCD fragmentation. Data were processed using Xcalibur v. 2.2.

Figure S1. Release of cysteine-capped species **5** from ADC #1 in incubations with human liver S9 fraction (source of lysosomal enzymes). The peptide ALPAPIE is a marker for antibody degradation by these enzymes.

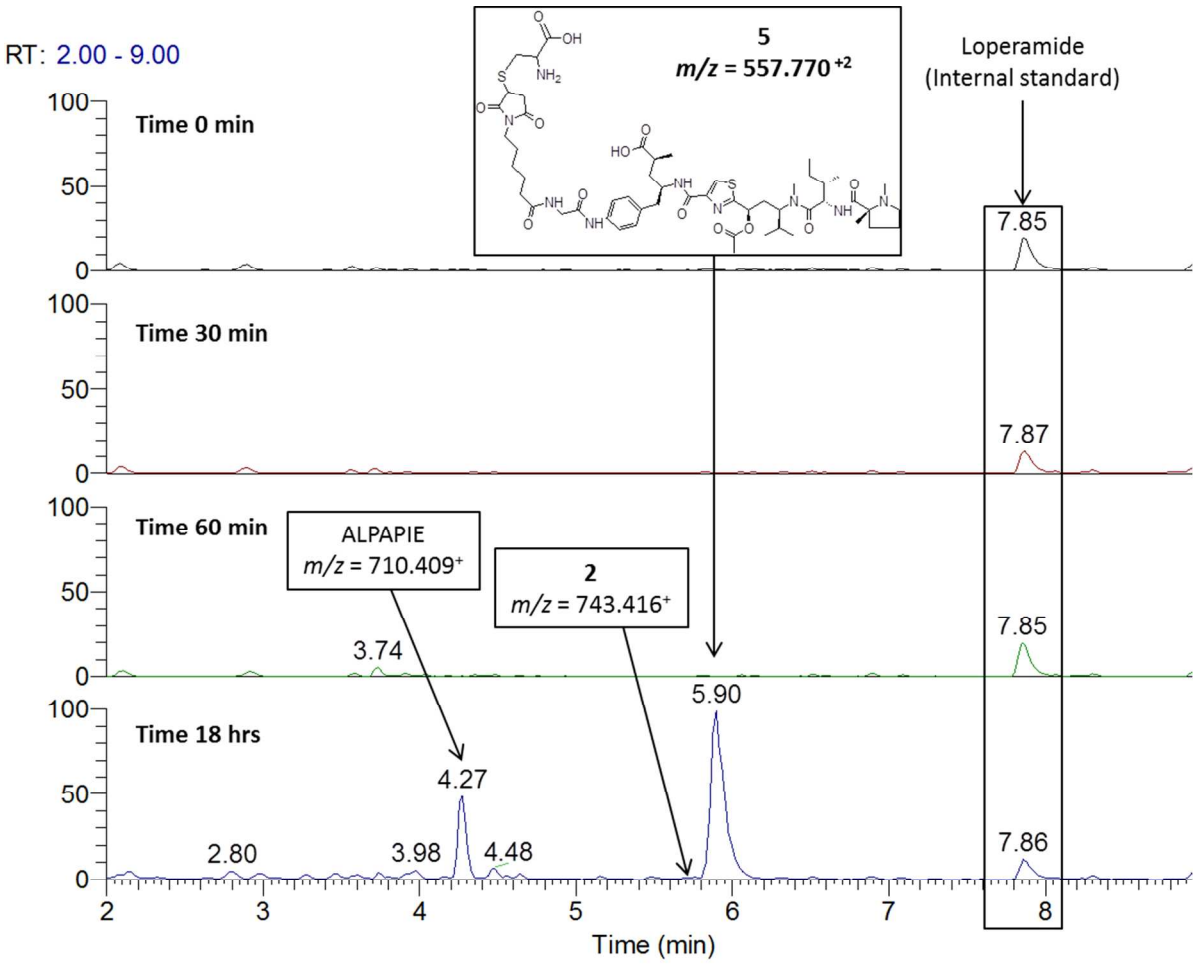


Figure S2: Immunocapture LCMS of mouse (left) and rat (ring) PK samples obtained after a 3 mpk dose of **ADC1**. LCMS indicate that the acetate at C-10 of the payload is rapidly hydrolyzed resulting in an inactive ADC. Only the LC peak is shown for clarity. Similar changes were observed on the HC. (data not shown).

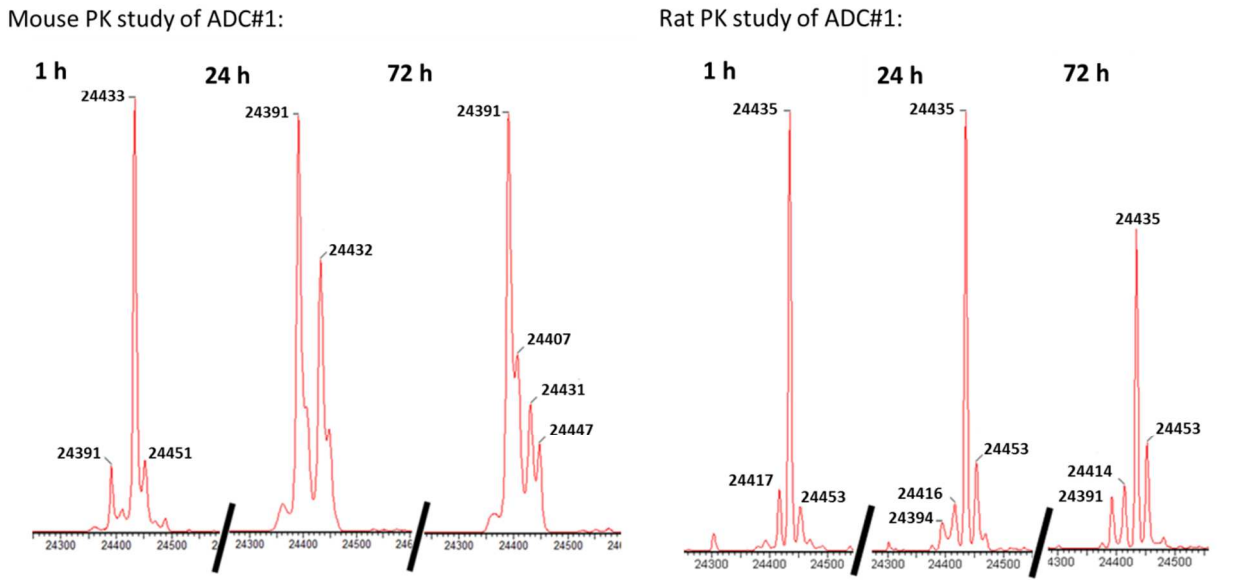
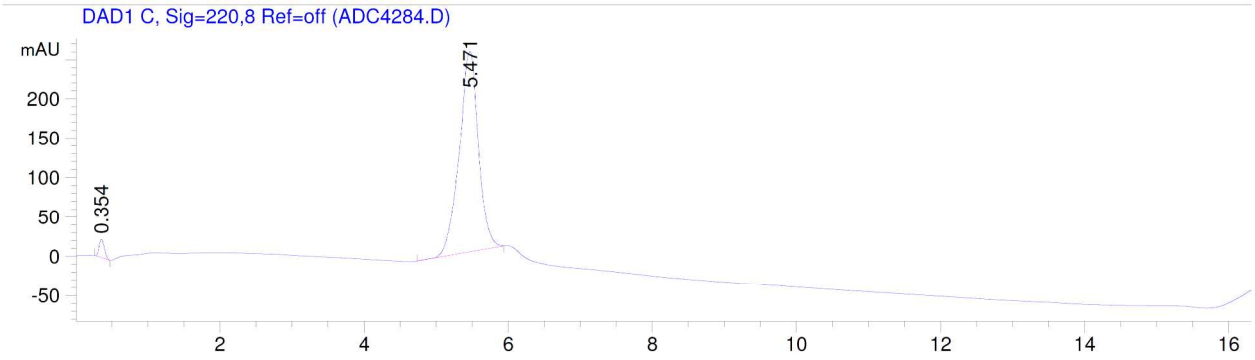
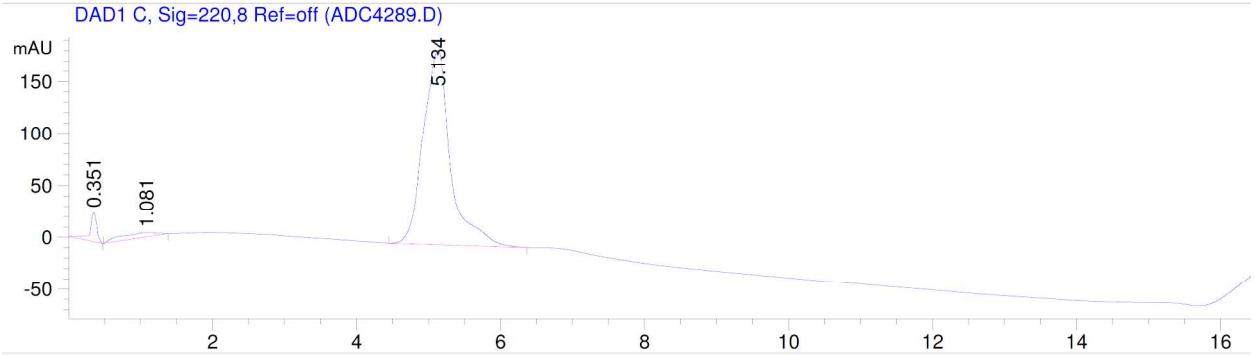


Figure S3: HIC traces for ADC#5-ADC#10

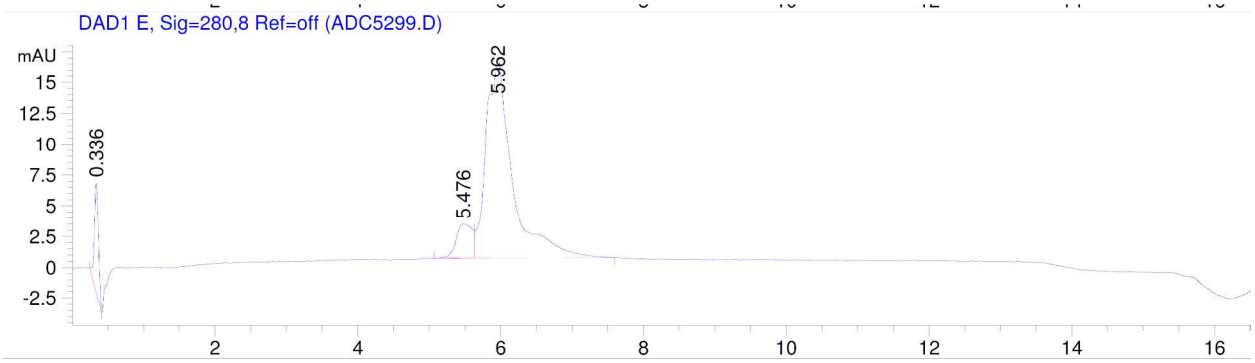
392C mutant: (ADC#5)



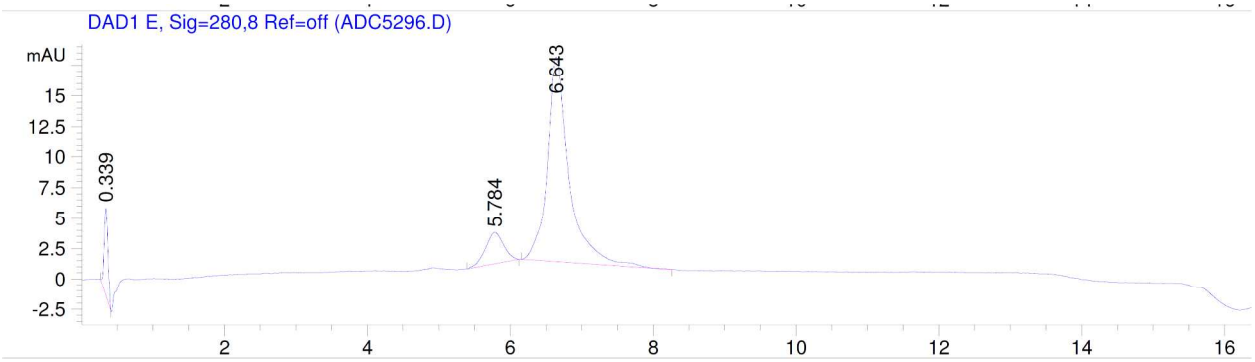
334C mutant: (ADC#6)



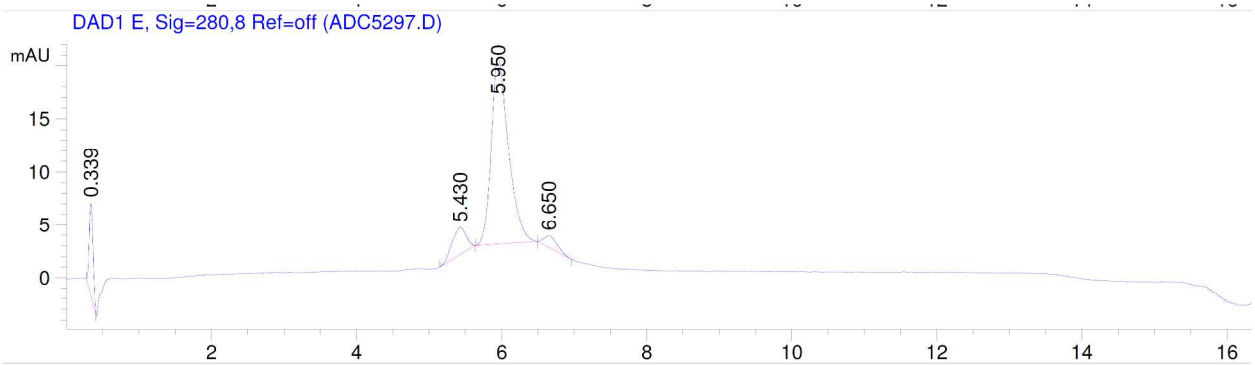
347C mutant: (ADC#7)



443C mutant: (ADC#8)



388C mutant: (ADC#9)



Kappa-183C mutant: (ADC#10)

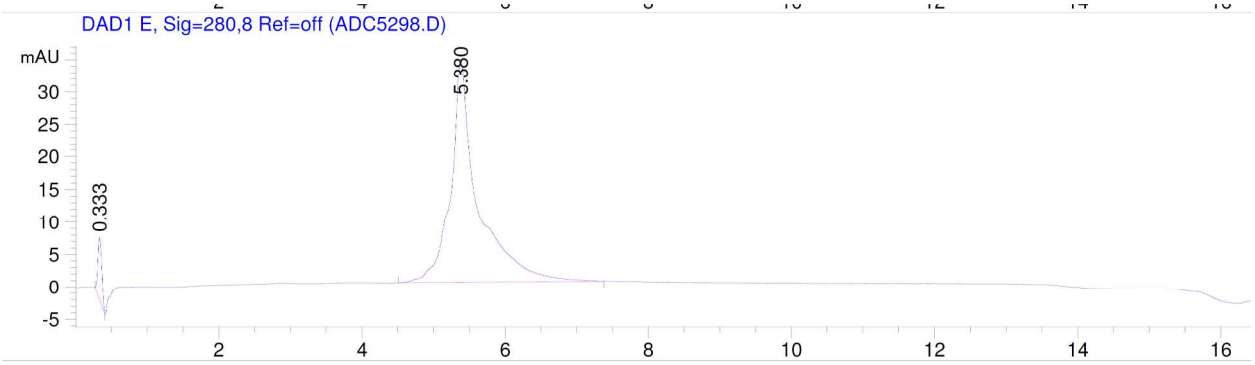


Table S4: Tubulin binding activity of payloads **2**-**4** and released species **13**. See Burke et al, Mol. Cancer Ther. **2016** for a representative analytical method.

Compound	Tubulin Competition Assay IC_{50} (nM)
2	2.5 (Ki = <0.1 nM)
3	12.3 (Ki = 3.2 nM)
4	1.9 (Ki = <0.1 nM)
5	3.3 (Ki = < 0.1 nM)