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Supporting Information

Evaluation of Potency and Specificity of Cryptophycin-Loaded Antibody-Drug Conjugates

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1. Material and Methods

1.1. Materials

Solvents were obtained from Acros Organics (Taufkirchen, Germany), Sigma-Aldrich (Darmstadt, Germany) and Thermo Fisher Scientific (Waltham, MA, USA) with the following quality: dimethyl sulfoxide (DMSO), dichloromethane (DCM): synthesis grade; acetonitrile (MeCN), dimethylformamide (DMF): HPLC grade. For HPLC, Millipore quality water was used. All solvents were used without further purification or drying.

Reagents were obtained from Carl Roth GmbH & Co. KG, Thermo Fisher Scientific (Waltham, MA, USA), Sigma-Aldrich (Darmstadt, Germany) and Iris Biotech GmbH (Marktredwitz, Germany). Reagents were used without further purification or drying. Mc-Val-Cit-PAB-MMAE was obtained from BroadPharm (San Diego, CA, USA). Mc-Val-Cit-PAB-PNP was obtained from Iris Biotech GmbH (Marktredwitz, Germany).

1.2. Methods

1.2.1. Hydrophobic interaction chromatography (HIC)

For hydrophobic interaction chromatography (HIC), an Agilent 1260 infinity series device (Agilent, Santa Clara, CA, USA) with variable wavelength detector (VWD) was used with a TSKgel Butyl-NRP (4.6 × 35 mm, 2.5 μM) column (Tosoh Bioscience, Griesheim, Germany). Absorbance was measured at 220 nm. Eluents consisted of eluent A (1.5 M (NH₄)₂SO₄, 25 mM tris(hydroxymethyl)aminomethane (Tris), pH = 7.5) and eluent B (25 mM Tris, pH = 7.5). Two and a half minutes of isocratic flow (0% eluent B) was followed by a 25 min gradient flow to reach 100% B. After that, the column was washed with 100% eluent B for two and a half minutes and subsequent isocratic flow 0% B for five minutes.

1.2.2. Size exclusion chromatography (SEC)

For size exclusion chromatography (SEC), an Agilent 1260 infinity series device (Agilent, Santa Clara, CA, USA) with variable wavelength detector (VWD) was used with a TSKgel SuperSW3000 (4.6 × 300 mm, 4 μM) column (Tosoh Bioscience, Griesheim, Germany). Absorbance was measured at 280 nm. Eluent consisted of 1 × PBS at pH = 7.5. Flow rate was set to 0.35 mL/min for 20 min.

1.2.3. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS)

For MALDI MS analysis, an Autoflex speed time-of-flight/time-of-flight spectrometer (Bruker Daltonics, Billerica, MA, USA) with a 2,5-dihydroxyacetophenone matrix was utilized. For sample preparation, 2 μL saturated matrix solution, 2 μL water (2 % trifluoroacetic acid) and 2 μL of protein solution were mixed and applied to the target via dried droplet method. For calibration, a protein standard was utilized. Measurement was performed in positive linear mode with an optimized method for proteins between 30 and 120 kDa.

2. Analytical Data

2.1. Characterization of ADCs

2.1.1. Hydrophobic interaction chromatograms

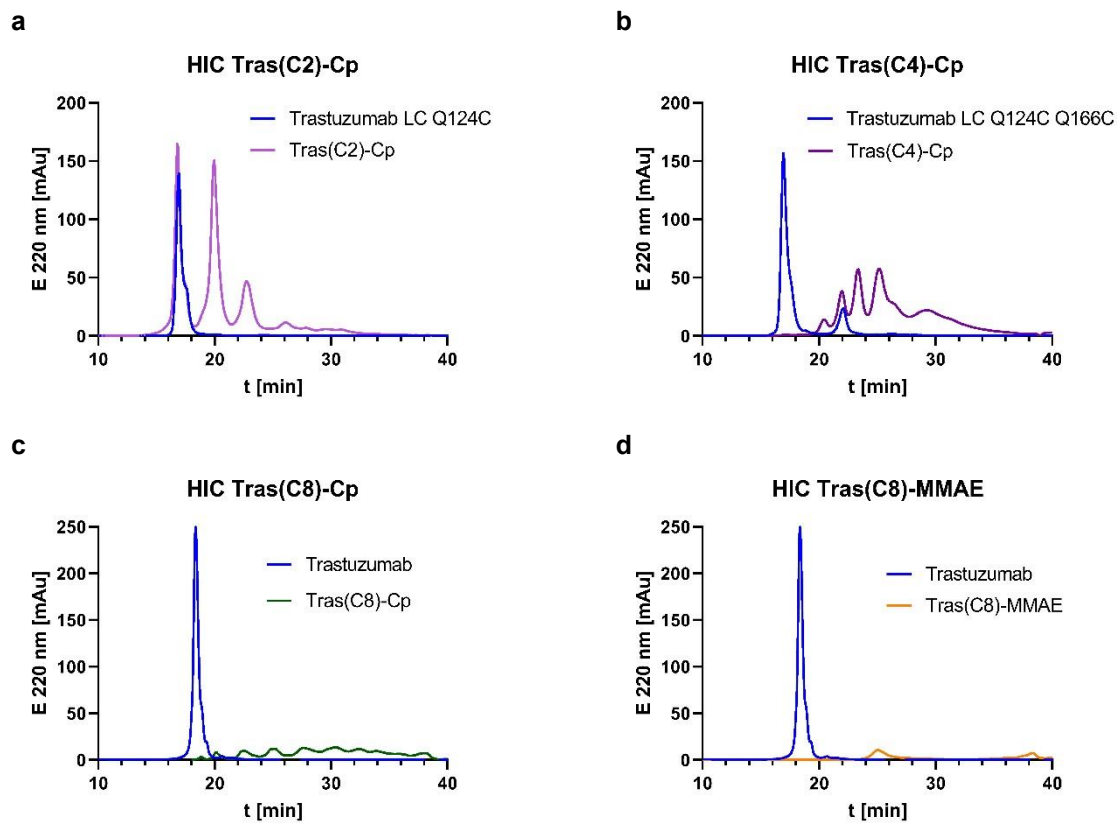


Figure S 1 Hydrophobic interaction chromatograms of generated ADCs. **a:** chromatogram of Tras(C2)-Cp. **b:** chromatogram of Tras(C2)-Cp. **c:** chromatogram of Tras(C8)-Cp. **d:** chromatogram of Tras(C8)-MMAE.

2.1.2. Size-exclusion chromatograms

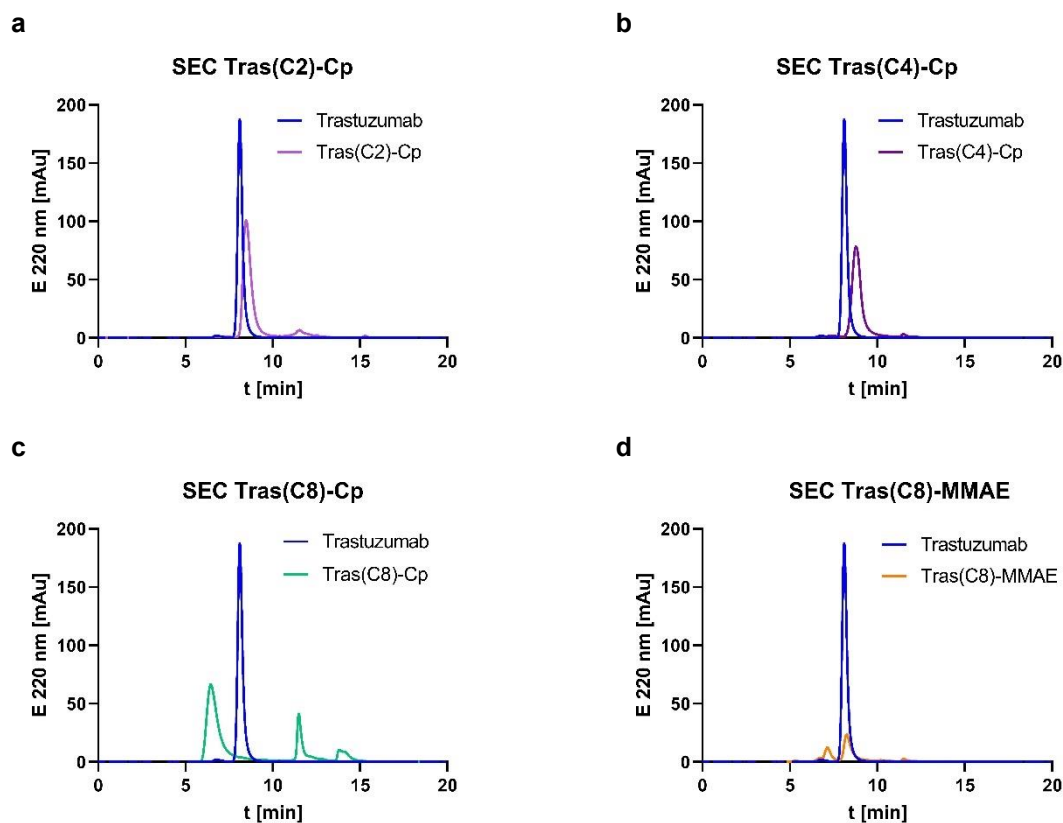


Figure S 2 Size-exclusion chromatograms of generated ADCs. a: chromatogram of Tras(C2)-Cp. b: chromatogram of Tras(C4)-Cp. c: chromatogram of Tras(C8)-Cp. d: chromatogram of Tras(C8)-MMAE.

2.1.3. MALDI MS analysis

Table S 1 Detected mass species of parental antibodies and ADCs. Division of mass difference between conjugates and parental mAb and molecular weight of corresponding toxin gave DAR values.

	Full length [M+1]		Δ [M+1]		DAR ($\Delta M/M(\text{toxin})$)				
Trastuzumab	149400								
Tras Q124C Q166C	147845								
Tras(C2)-Cp	150077	148873	2232	1028	1.8	0.8			
Tras(C4)-Cp	152311	151200	150111	4466	3355	2266	3.6	2.7	1.8
Tras(C8)-MMAE	154181	152570		4781	3170		3.6	2.4	

	HC+LC [M+1]				Δ [M+1]				DAR ($\Delta M/M(\text{toxin})$)			
Trastuzumab	74941											
Tras(C8)-MMAE	79977	78758	77563	76455	5036	3817	2622	1514	3.8	2.9	2.0	1.1
Tras(C8)-Cp	80080				5139				4.1			

	HC [M+1]			Δ [M+1]			DAR ($\Delta M/M(\text{toxin})$)		
Trastuzumab	50036								
Tras(C8)-MMAE	55594	54298	52964	5558	4262	2928	4.2	3.2	2.2
Tras(C8)-Cp	55396			5360			4.3		

	LC [M+1]	Δ [M+1]	DAR ($\Delta M/M(\text{toxin})$)
Trastuzumab	23443		
Tras(C8)-MMAE	24783	1340	1.0
Tras(C8)-Cp	24696	1253	1.0

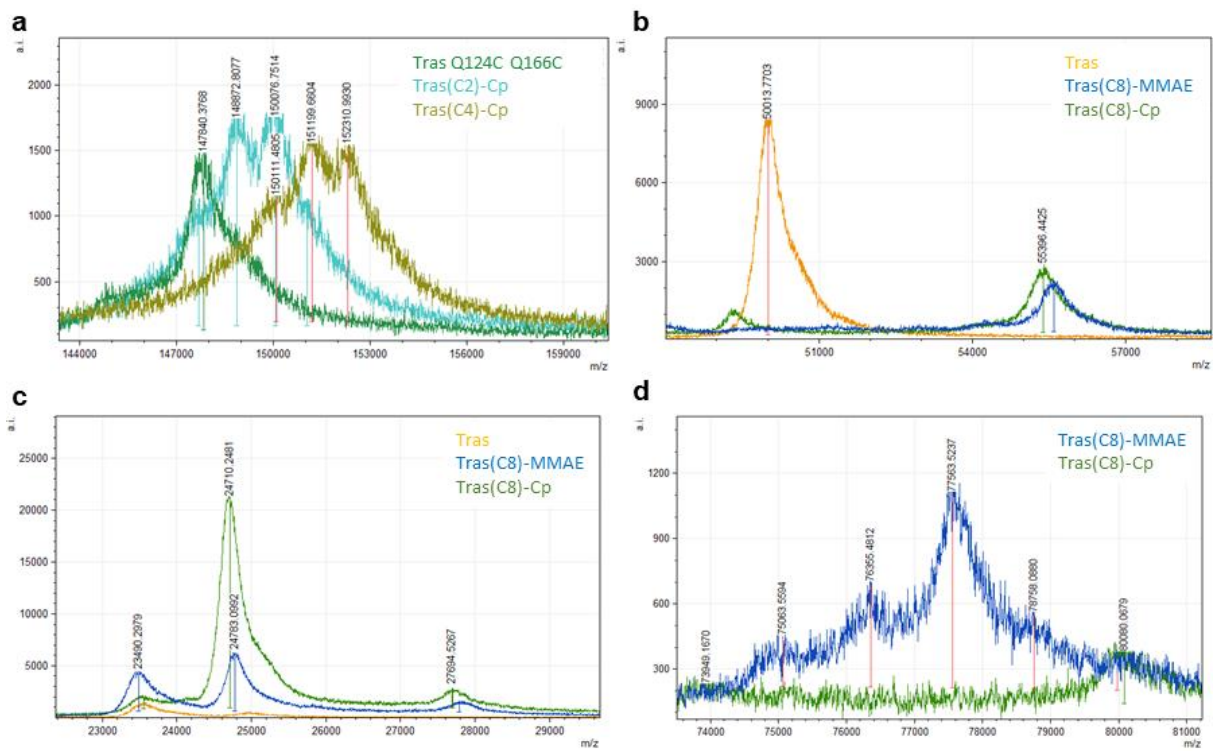


Figure S 3 MALDI mass spectra of generated ADCs. **a:** full length range, Tras Q124C Q166C, Tras(C2)-Cp, Tras(C4)-Cp. **b:** heavy chain fragments, Trastuzumab, Tras(C8)-MMAE, Tras(C8)-Cp. **c:** light chain fragment, Trastuzumab, Tras(C8)-MMAE, Tras(C8)-Cp. **d:** heavy + light chain fragment Tras(C8)-MMAE, Tras(C8)-Cp.

2.2. Cellular proliferation assays

2.2.1. Cellular proliferation assays on SK-BR-3

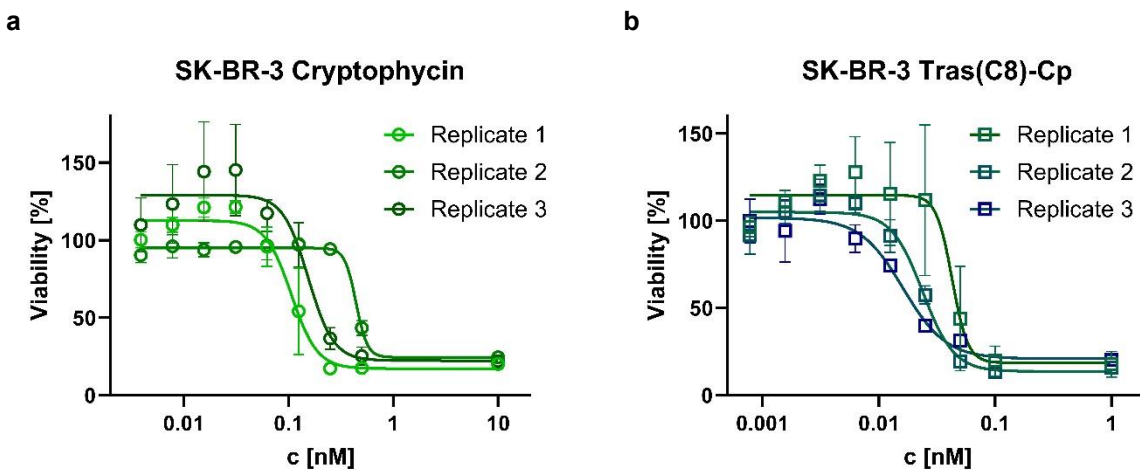


Figure S 4 Cellular proliferation assays conducted on SK-BR-3 cells. **a:** Proliferation assays conducted with cryptophycin-uD[Dap(Me)] 2. **b:** Proliferation assays conducted with Tras(C8)-Cp.

Table S 2 EC₅₀ values and maximum killing of cryptophycin-uD[Dap(Me)] 2 and Tras(C8)-Cp on SK-BR-3 cells.

SK-BR-3	EC50 [pM]			max killing [%]		
Cryptophycin	107	442	154	83	75	78
Tras(C8)-Cp	43	24	16	81	86	79

2.2.2. Cellular proliferation assays on RT112

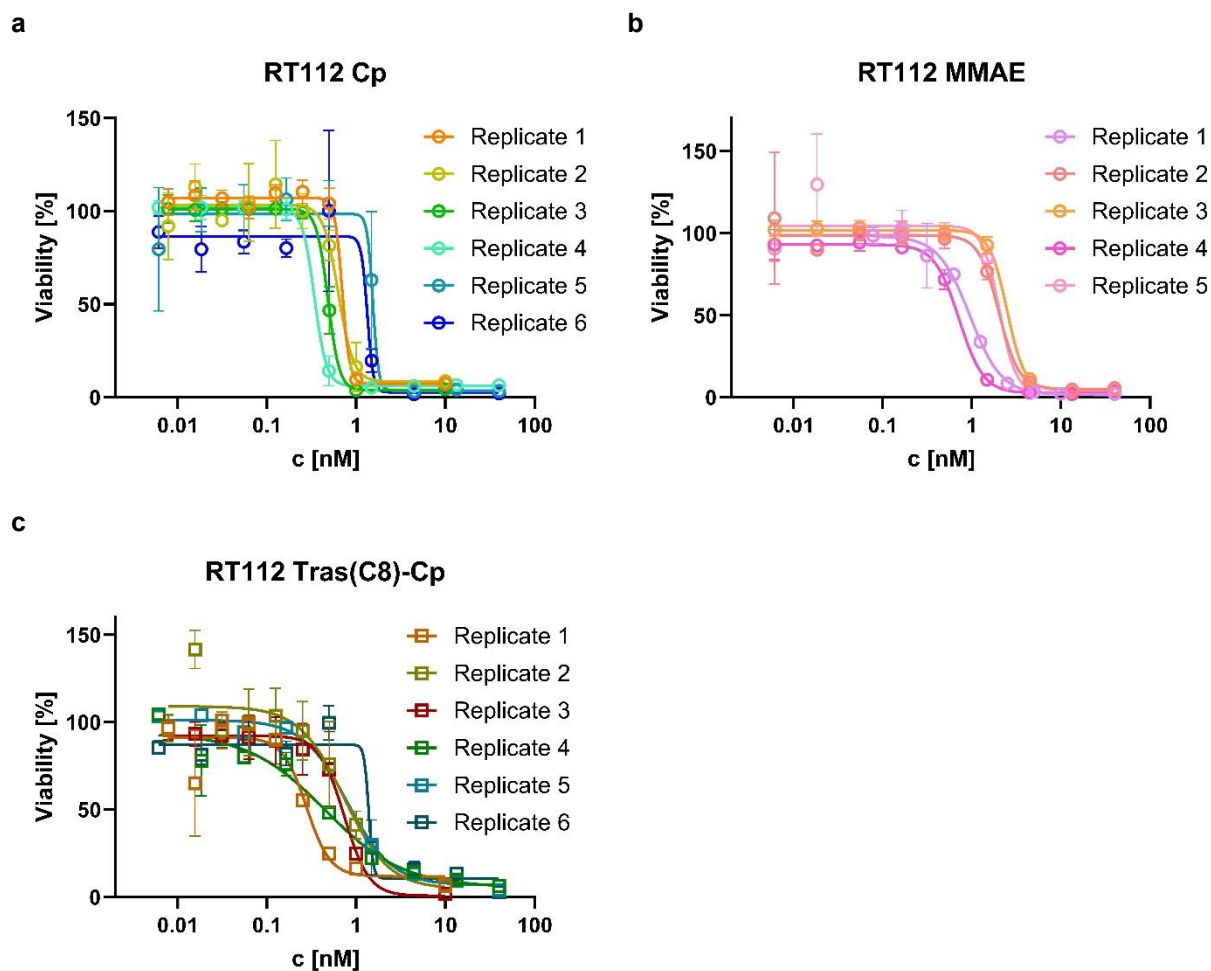


Figure S 5 Cellular proliferation assays conducted on RT112 cells. All constructs were tested in technical triplicates. **a:** Proliferation assays conducted with cryptophycin **2**. **b:** Proliferation assays conducted with MMAE **1**. **c:** Proliferation assays conducted with Tras(C8)-Cp.

Table S 3 EC₅₀ values and maximum killing of cryptophycin-uD[Dap(Me)] **2**, MMAE **1** and Tras(C8)-Cp on RT112 cells.

RT112	EC ₅₀ [pM]						max killing [%]					
Cryptophycin	697	630	483	339	1539	1332	93	91	96	94	96	97
MMAE	948	2008	2480	717	2026		98	95	96	97	98	
Tras(C8)-Cp	276	731	734	450	800	1382	88	95	99	94	93	89

3. Experimental section

3.1. Sequences of trastuzumab variants

3.1.1. Trastuzumab

HC:

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYL
QMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGLTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVS
WNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKKSCDKTHTCPPCPAPELLGG
PSVFLFPPKPKDITLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK
EYKCKVSNKALPAPIEKTKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLD
SDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG

LC:

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISLQPED
FATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVCLLNNFYPREAKVQWKVDNALQSGNSQESV
TEQDSKDYSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

3.1.2. Trastuzumab LC Q124C

LC:

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISLQPED
FATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDECLKSGTASVCLLNNFYPREAKVQWKVDNALQSGNSQESV
TEQDSKDYSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

3.1.3. Trastuzumab LC Q124C Q166C

LC:

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISLQPED
FATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDECLKSGTASVCLLNNFYPREAKVQWKVDNALQSGNSQESV
TECDKDYSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

3.1.4. Production and purification of trastuzumab variants

Expi HEK293F cells were transfected with pTT5 plasmids of respective HC and LC variants. Cells were inoculated in Expi293 expression medium 37 °C and 8 % CO₂ at 110 rpm. After 5 days, culture broth was centrifugated at 12000× g and supernatant was filtrated. Filtrate was purified utilizing an ÄKTastart (GE Healthcare Life Science, Uppsala, Sweden) equipped with a ProteinA HP column (GE Healthcare Life Science), following the instructions of the supplier. 1× PBS served as binding and washing buffer; 0.1 M citric acid at pH = 3 was used for elution. Eluate was collected in microtubes with 100 µL 1 M Tris-HCl at pH = 9.0 added per mL of eluate. After elution, proteins were transferred into a dialysis tube with a molecular weight cut off of 30000 g/mol and incubated overnight at 4 °C in 5 L of 1× PBS.

3.1.5. Generation of Tras(C8)-Cp and Tras(C8)-MMAE conjugates via Michael-Addition to hinge-region cysteines

10 molar equivalents of tris(2-carboxyethyl)phosphine (TCEP) were added to a solution of 0.5 mg of trastuzumab in 1× PBS and incubated for 2 h at 37 °C. Subsequently, 16 molar equivalents of drug-linker were added as well as DMSO to a final 10 % v/v of DMSO and incubated for 2 h at room temperature (RT). For quenching, 25 molar equivalents of *N*-acetylcysteine were added, and the solution was incubated for additional 15 min at 25 °C. Subsequently, conjugates were purified utilizing HP SpinTrap™ columns (Cytiva Europe GmbH, Freiburg, Germany) following the instructions of the supplier. Eluate was rebuffered utilizing Pur-A-Lyzer™ Mini Dialysis Kit (Merck KGaA Darmstadt, Germany) following the instructions of the supplier. Reaction progress was monitored with HIC analysis, product characterization was performed with HIC, SEC and MALDI MS.

3.1.6. Generation of thiomab-cryptophycin conjugates Tras(C2)-Cp and Tras(C4)-Cp via Michael addition to engineered cysteines in the light chain of Trastuzumab

10 molar equivalents of TCEP were added to a solution of 0.5 mg of mAb (trastuzumab LC Q124C or trastuzumab LC Q124C Q166C) in 1× PBS and incubated for 2 h at 37 °C. For regeneration of hinge region disulfide bonds, 20 molar equivalents of dehydroascorbic acid were added and incubated for 2 h at 37 °C. Subsequently, 16 molar equivalents of drug-linker were added as well as DMSO to a final 10 % v/v of DMSO and incubated for 2 h at RT. For quenching, 25 molar equivalents of *N*-acetylcysteine were added, and the solution was incubated for additional 15 min at 25 °C. Subsequently, conjugates were purified utilizing HP SpinTrap™ columns (Cytiva Europe GmbH, Freiburg, Germany) following the instructions of the supplier. Eluate was rebuffed utilizing Pur-A-Lyzer™ Mini Dialysis Kit (Merck KGaA Darmstadt, Germany) following the instructions of the supplier. Reaction progress was monitored with HIC analysis, product characterization was performed with HIC, SEC and MALDI MS.

3.1.7. Cellular binding assays on SK-BR-3 cells

Cell line utilized for binding was SK-BR-3, known to overexpress Her2,^[34] A431 cells served as negative control. 50000 cells per well were incubated with dilution rows of Trastuzumab and ADCs, respectively, in binding buffer (1× PBS with 0.1% m/v bovine serum albumin) in a U-bottom 96-well plate for 30 min at 4 °C. After washing with binding buffer, 7 µL of a 1:80 dilution of Goat anti-Human IgG Fc secondary antibody, PE, eBioscience™ (AB_465926, ThermoFisher Scientific, Darmstadt, Germany) was added to each well for staining and cells were incubated for additional 20 min at 4 °C. Subsequently, cells were washed with binding buffer and analyzed in the cytometer (CytoFLEX, Beckman Coulter GmbH, Krefeld, Germany). For data analysis, mean FI was calculated and plotted against concentration. Assay was conducted in technical triplicates.

3.1.8. Cellular binding assay for Her2 expression control

50000 cells per well were incubated 50 nM of trastuzumab in binding buffer (1× PBS with 0.1% m/v bovine serum albumin) in a F-bottom 96-well plate for 30 min at 4 °C. After washing with binding buffer, 7 µL of a 1:80 dilution of Goat anti-Human IgG Fc secondary antibody, PE, eBioscience™ (AB_465926, ThermoFisher Scientific, Darmstadt, Germany) was added to each well for staining and cells were incubated for additional 20 min at 4 °C. Subsequently, cells were washed with binding buffer and analyzed in the cytometer (CytoFLEX, Beckman Coulter GmbH, Krefeld, Germany). For data analysis, mean FI was calculated and plotted against concentration. Assay was conducted in technical triplicates.

3.1.9. Cellular viability assays

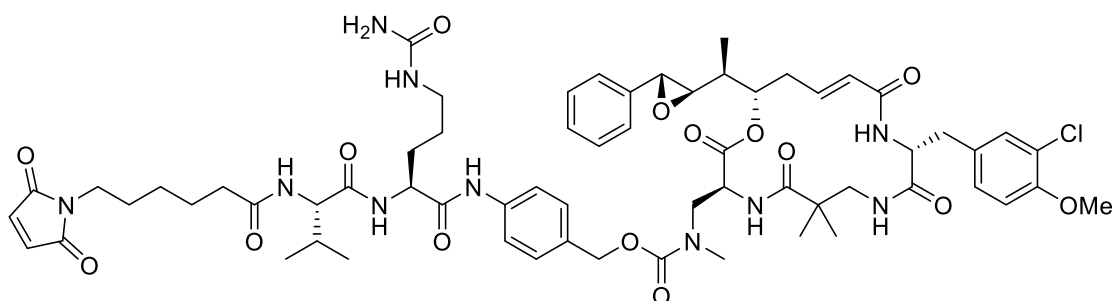
6000 cells/well were seeded in a U bottom 96 well plate in 90 µL of medium (**Table S 4**) and incubated over night at 37 °C and 5% CO₂. The next day, 10 µL of a dilution row of each sample was added and cells were incubated for 3 days at 37°C and 5% CO₂. To evaluate viability, cellular viability assay was performed utilizing CellTiter-Blue® (Promega GmbH, Walldorf, Germany) following the instructions if the supplier. Fluorescence measurement was conducted with a CLARIOstar Plus (BMG Labtech, Ortenberg, Germany) plate reader.

Table S 4 Cell lines and corresponding media utilized for cellular viability assays.

Cell line	Medium
SK-BR-3	80% McCoy's 5a + 20% FBS, DMEM, 10% FBS
JIMT-1	RPMI-1640 Medium, 10% FBS
RT112	90% RPMI 1640 + 10% heat inactivated FBS
MDA-MB468	80-90% Leibovitz' L15 + 10-20% heat inactivated FBS
MKN-45	70% medium, 20% FBS, 10% DMSO
A549	70% medium, 20% FBS, 10% DMSO

3.2. Synthesis of Mc-Val-Cit-PAB-Cryptophycin-uD[Dap(Me)] 4

Cryptophycin-uD[Dap(Me)] **2** prepared as previously described^[21] (26.0 mg, 39.6 μ mol) and Mc-Val-Cit-PAB-PNP (32.6 mg, 44.2 μ mol, 1.1 eq.) were dissolved in dichloromethane (1 mL), DMF (1 mL) and Pr_2NEt (35 μ L, 0.20 mmol, 5.0 eq.). HOBt \cdot H₂O (2.0 mg, 13 μ mol, 0.3 eq) was added to the solution. Reaction control by LC-MS showed full conversion after 22 h of stirring at 25 °C. DCM was removed under reduced pressure and the residue was diluted with acetonitrile: water (1:1, 600 μ L) and purified by RP-HPLC. The fractions were analyzed by analytical HPLC (eluent containing MeCN/H₂O with 0.1% HCO₂H), and equal fractions were pooled and lyophilized to give conjugate Mc-Val-Cit-PAB-Cryptophycin-uD[Dap(Me)] **4** (39.5 mg, 31.5 μ mol, 79%) as a colorless powder.



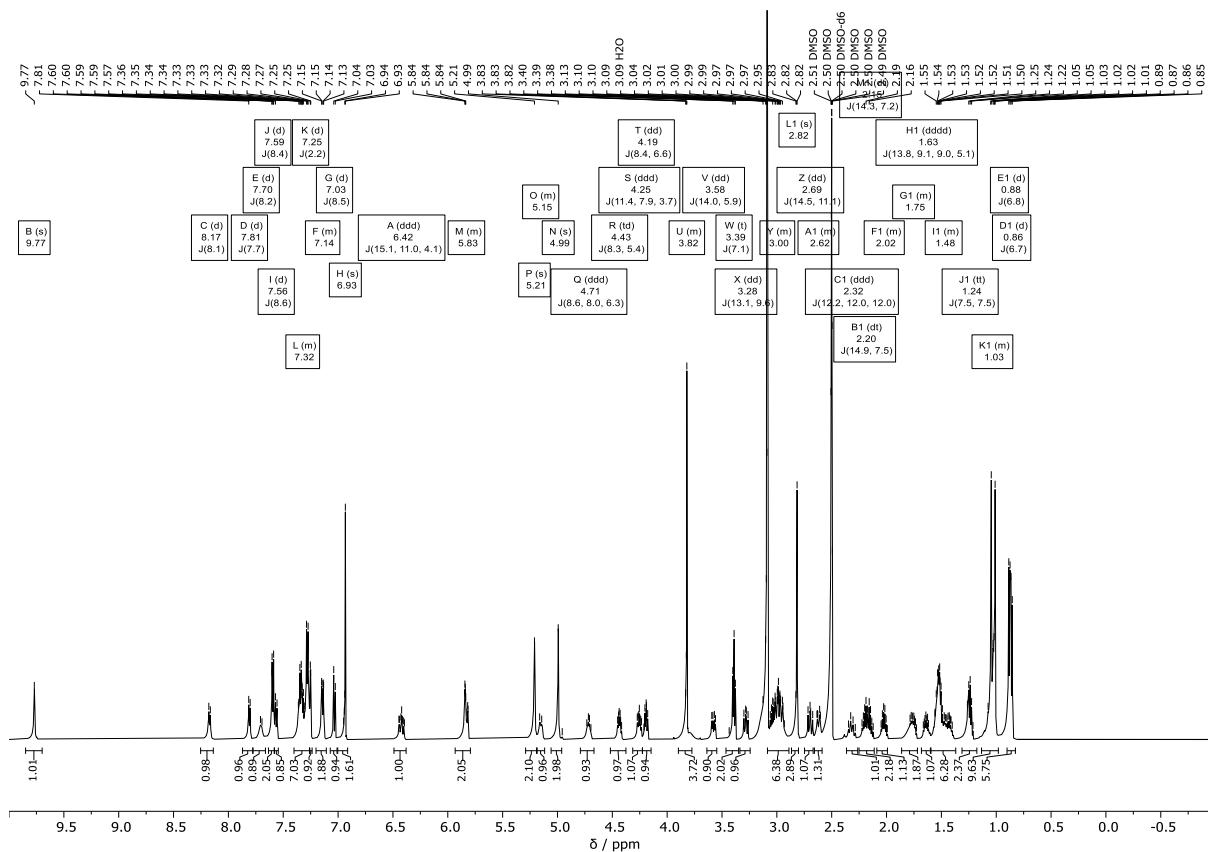
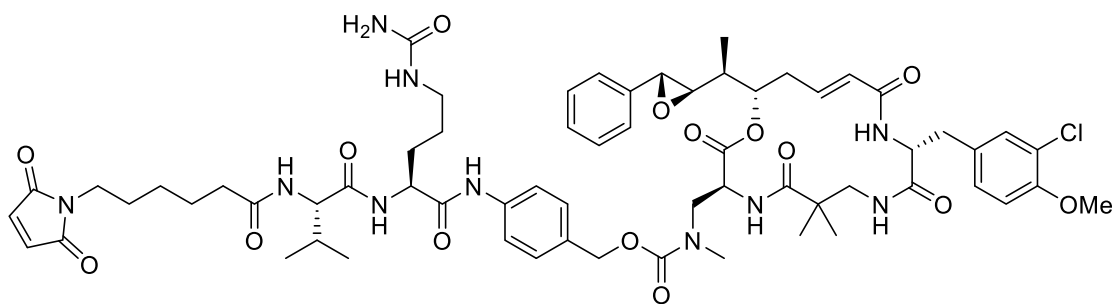
NMR spectroscopic analysis of Mc-Val-Cit-PAB-Cryptophycin-uD[Dap(Me)] **4** at 25 °C showed different signal sets due to the presence of rotamers which coalesced at 80 °C. Signal assignment for the cryptophycin core following the recently published scheme.^[21]

¹H NMR (600 MHz, 80 °C, DMSO-*d*₆): δ [ppm] = 9.77 (s, 1H, PAB-C^{ar}, *para*-NH), 8.17 (d, ³J = 8.1 Hz, 1H, uB-NH), 7.81 (d, ³J = 7.7 Hz, 1H, Cit-C^{ar}NH), 7.70 (d, ³J = 8.2 Hz, 1H, uD-NH), 7.59 (d, ³J = 8.4 Hz, 2H, PAB-C^{ar}, *meta*H), 7.56 (d, ³J = 8.6 Hz, 1H, Val-NH), 7.41 – 7.26 (m, 7H, PAB-C^{ar}, *ortho*H and uA-C^{ar}H), 7.25 (d, ⁴J = 2.2 Hz, 1H, uB-C^{ar}, ²H), 7.16 – 7.12 (m, 2H, uB-NH and uB-C^{ar}, ⁶H), 7.03 (d, ³J = 8.5 Hz, 1H, uB-C^{ar}, ⁵H), 6.93 (s, 2H, Mal-CH), 6.42 (ddd, ³J = 15.1 Hz, ³J = 11.0 Hz, ⁴J = 4.1 Hz, 1H, uA-C^βH), 5.90 – 5.79 (m, 2H, Cit-C^δNH and uA-C^αH), 5.21 (s, 2H, Cit-NH₂), 5.15 (m, 1H, uA-C^δH), 4.99 (s, 2H, PAB-C^{ar}, *ipso*-CH₂), 4.71 (ddd, ³J = 8.6 Hz, ³J = 8.0 Hz, ³J = 6.3 Hz, 1H, uD-C^αH), 4.43 (td, ³J = 8.3 Hz, ³J = 5.4 Hz, 1H, Cit-C^αH), 4.25 (ddd, ³J = 11.4 Hz, ³J = 7.9 Hz, ³J = 3.7 Hz, 1H, uB-C^αH), 4.19 (dd, ³J = 8.4 Hz, ³J = 6.6 Hz, 1H, Val-C^αH), 3.88 – 3.78 (m, 4H, uA-C^γH and uB-OCH₃), 3.58 (dd, ²J = 14.0 Hz, ³J = 5.9 Hz, 1H, uD-C^βH^AH^B), 3.39 (t, ³J = 7.1 Hz, 2H, Mal-NCH₂), 3.28 (dd, ²J = 13.1 Hz, ³J = 9.6 Hz, 1H, uC-C^βH^AH^B), 3.07 – 2.93 (m, 6H, Cit-C^γH₂, uA-C^γH, uB-C^βH^AH^B, uC-C^βH^AH^B and uD-C^βH^AH^B), 2.82 (s, 3H, uD-NCH₃), 2.69 (dd, ²J = 14.5 Hz, ³J = 11.1 Hz, 1H, uB-C^βH^AH^B), 2.62 (m, 1H, uA-C^γH^AH^B), 2.32 (ddd, ²J = 12.2 Hz, ³J = 12.1 Hz, ³J = 12.1 Hz, 1H, uA-C^γH^AH^B), 2.20 (dt, ²J = 14.9 Hz, ³J = 7.5 Hz, 1H, hexanoyl-C^αH^AH^B), 2.15 (dt, ²J = 14.3 Hz, ³J = 7.2 Hz, 1H, hexanoyl-C^αH^AH^B), 2.02 (m, 1H, Val-C^βH), 1.80 – 1.71 (m, 2H, uA-C^εH and Cit-C^βH^AH^B), 1.63 (dddd, ²J = 13.8 Hz, ³J = 9.1 Hz, ³J = 9.0 Hz, ³J = 5.1 Hz, 1H, Cit-C^βH^AH^B), 1.58 – 1.38 (m, 6H, hexanoyl-C^βH₂, hexanoyl-C^δH₂ and Cit-C^γH₂), 1.24 (tt, ³J = 7.5 Hz, ³J = 7.5 Hz, 2H, hexanoyl-C^γH₂), 1.09 – 1.00 (m, 9H, uC-C^β(CH₃)₂ and uA-C^εCH₃), 0.88 (d, ³J = 6.8 Hz, 3H, Val-C^β(CH₃)^A(CH₃)^B), 0.86 (d, ³J = 6.7 Hz, 3H, Val-C^β(CH₃)^A(CH₃)^B).

HPLC-MS (ESI+): m/z (found) 1254.2, t_R = 8.8 min.
 m/z (calc.) 1253.6 (M+H)⁺ = (C₆₃H₈₂CIN₁₀O₁₅)⁺

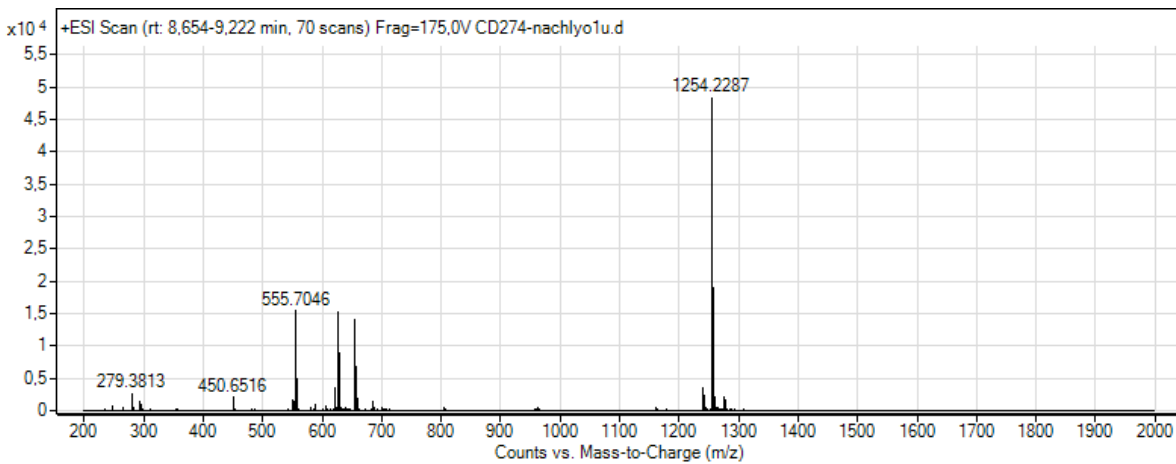
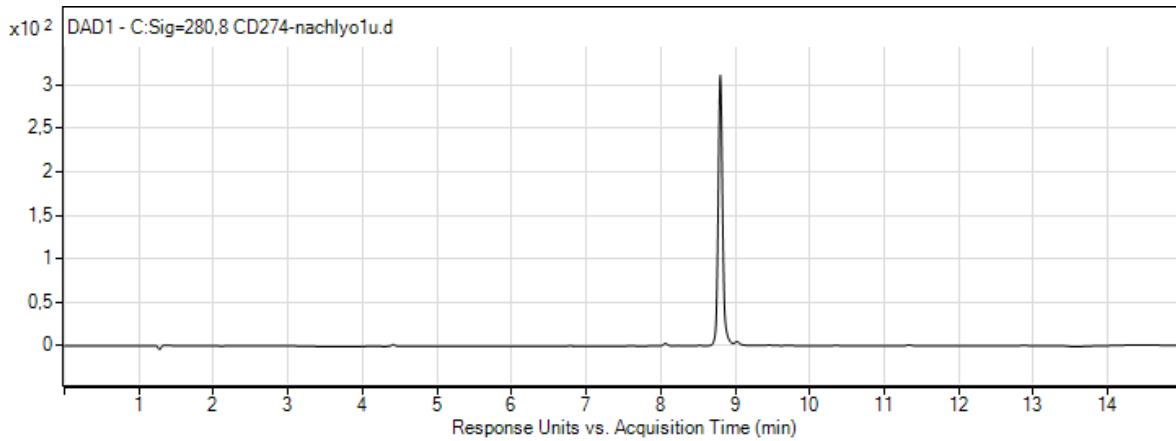
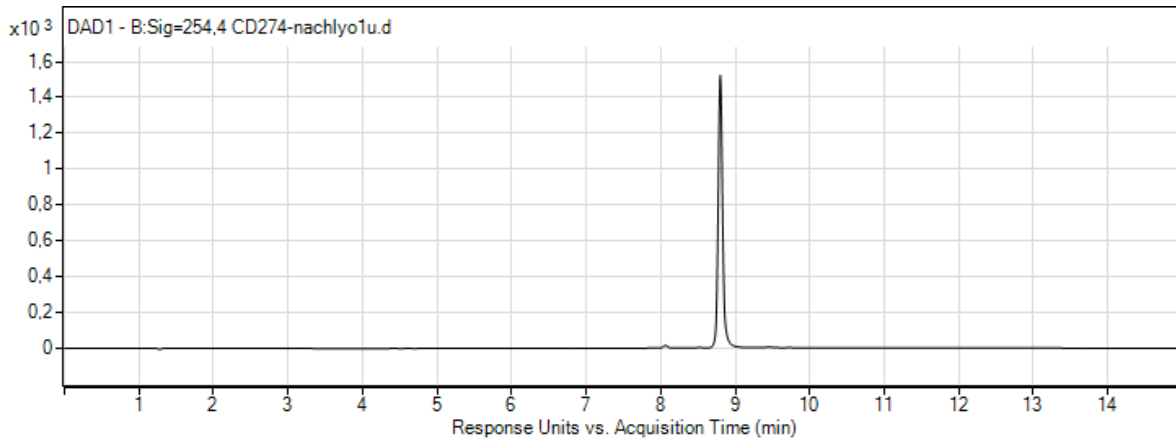
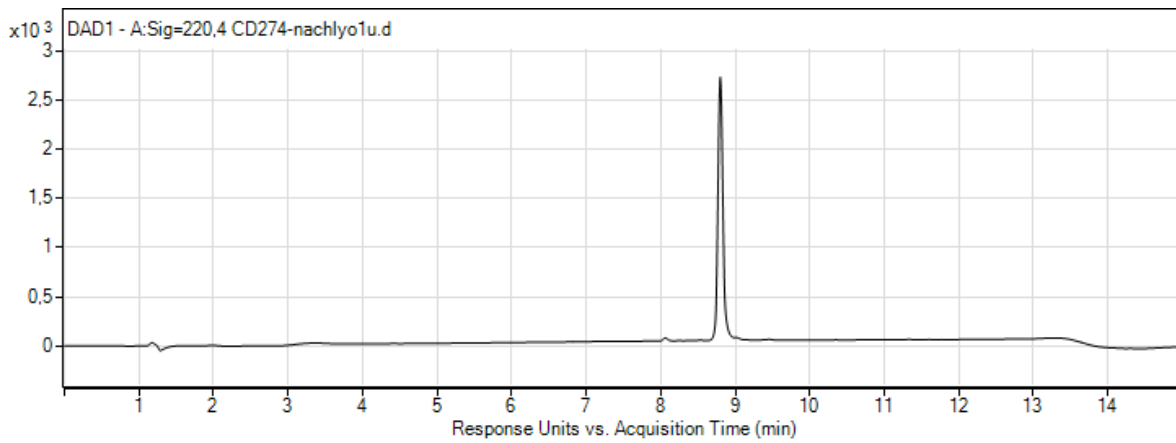
HRMS: (ESI, +) m/z (found) 1275.5490
 m/z (calc.) 1275.5464 (M+Na)⁺; (C₆₃H₈₁CIN₁₀O₁₅Na)⁺

Separation parameters RP-HPLC: 0-10 min 10% solvent B
 10-70 min 10-70% solvent B
 Eluent A: 94.9% water, 5.0% acetonitrile, 0.1% formic acid.
 Eluent B: 94.9% acetonitrile, 5.0% water, 0.1% formic acid.



NMR spectrum 1:

¹H NMR spectrum (600 MHz, 80 °C, DMSO-*d*₆) of Mc-Val-Cit-PAB-Cryptophycin-uD[Dap(Me)] 4.



HPLC-MS chromatogram 1: HPLC-MS analysis of Mc-Val-Cit-PAB-Cryptophycin-uD[Dap(Me)] 4 with detection at 220 nm, 254 nm and 280 nm (top to bottom) and associated mass spectrum (ESI, +).