STABILIZING A TUBULYSIN ANTIBODY-DRUG CONJUGATE TO ENABLE ACTIVITY AGAINST MULTIDRUG-RESISTANT TUMORS

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SUPPLEMENTARY FIGURES

Figure S1. Affinity capture LC-MS results. In vivo stability data for anti-CD22-LC-K149C-MC-VC-PABC-MMAE (8) in Fox Chase SCID mice (1 mg/kg IV single dose).
Figure S2. Affinity capture LC-MS results. *In vivo* stability data for anti-CD22-LC-K149C-MC-VC-PABQ-tubulysin M (5) in Fox Chase SCID mice (1 mg/kg IV single dose).
SUPPLEMENTARY MATERIALS AND METHODS

Preparation of conjugates
The THIOMAB antibody (LC-K149C) was reduced in the presence of a 50-fold molar excess of DTT overnight. The reducing agent and the cysteine and GSH blocks were purified away using a HiTrap SP-HP column (GE Healthcare). The antibody was reoxidized in the presence of a 15-fold molar excess of dehydroascorbic acid (MP Biomedical) for 2.5 h. The formation of interchain disulfide bonds was monitored by LC/MS. A threefold molar excess of the linker–drug conjugates over protein was incubated with the THIOMAB for 1 h. The ADC was purified by filtration through a 0.2 μM SFCA filter (Millipore). Excess free linker–drug conjugate was removed by filtration. The conjugate was buffer-exchanged into 20 mM histidine acetate pH 5.5/240 mM sucrose by dialysis. The number of conjugated drug molecules per monoclonal antibody (mAb) was quantified by LC/MS analysis. Purity was also assessed by size-exclusion chromatography.

In vitro cytotoxicity
The non-Hodgkin lymphoma (NHL) cell lines BJAB, WSU-DLCL2 and control cell line Jurkat were obtained from the Genentech cell-line repository. All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Sigma) and 2 mM L-glutamine. On Day 0, CD22-positive cell lines, BJAB and WSU-DLCL2, were seeded at 4,000 cells per well in 40 μl RPMI-1640 culture media supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 μM cystine and 0.015 g/l L-methionine in 384-well flat clear-bottom white polystyrene tissue-culture-treated microplates (Corning). Antibody–drug conjugates, 2 mg/ml in 20 mm histidine acetate, 240 mm sucrose and 0.02% P20 pH 5.5 buffer, were transferred to cells seeded in 384-well plates using ECHO acoustic liquid handling technology (Labcyte) to create a ten-point dose–response curve in triplicate starting from 20 μg/ml with 1:3× serial dilution. Cells were cultured in a humidified incubator set at 37 °C and maintained at an atmosphere of 5% CO₂. On Day 4, cells were equilibrated to room temperature, then 40 μl per well Cell Titer-Glo II reagent (Promega) was added, the plates shaken for 10 min and then incubated for 30 min at room temperature in the dark. Luminescence was read using an EnVision 2101 Multilabel Reader (PerkinElmer). Normalized luminescence intensity data were analysed using GraphPad Prism 6, and IC₅₀ values were calculated using a four-parameter sigmoidal fit. Generation of an overexpressing Pgp cell line (BJAB.Luc/Pgp) was done and tested in cell viability studies as described previously1.

In Vivo Efficacy
All animal studies were carried out in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Genentech. The efficacy of anti-CD22 drug conjugates
was evaluated in a mouse xenograft model of BJAB.luc and BJAB.luc/pgp (human non-Hodgkin lymphoma). Cells were maintained in RPMI 1640 supplemented with 10% FBS (Sigma) and 1% L-glutamine. To establish a subcutaneous xenograft model, the tumor cells (20 million cells in 0.2mL Hank’s Balanced Salt Solution; HyClone) were inoculated subcutaneously into the flanks of female CB17 SCID mice (Charles Rivers Laboratories). When tumors reached the desired volume (180-200 mm³), the mice were divided into groups of 8 mice with similar mean tumor size and administered a single intravenous injection of antibody drug conjugate through the tail vein. Tumor growth measurements were plotted as mean tumor volume ± SEM of each group over time. To compare efficacy, percent tumor growth inhibition (% TGI) was calculated for each treatment group in relation to the vehicle control, using the following formula: % TGI = 100 x [(mean tumor volume of vehicle – mean tumor volume of treatment group) /mean tumor volume of vehicle] at the last day of the vehicle group. All treatments are well tolerated in mice with similar weight gains overtime as the vehicle group. Blood samples were collected via retro-orbital bleeds and used to derive plasma for analysis of stability by affinity capture LC-MS.

In Vivo Stability
To determine the in vivo stability of ADCs, affinity capture LC-MS was performed. Briefly, human HER2 and CD22 extracellular domain (ECD) was biotinylated and immobilized onto streptavidin-coated paramagnetic beads (Invitrogen) in a 96-well plate, and then the ECD-bead system was used to capture anti-CD22 disulfide conjugates by incubating with approximately 40 µL of mouse plasma samples for 2 h at room temperature. The captured ADCs were then washed with HBS-EP buffer (10 mM Hepes [pH 7.4], 150 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid [EDTA], 0.005% Surfactant P20) (GE Healthcare) and deglycosylated using FabRICATOR as described previously². After extensive washing of the beads with HBS-EP, water and 10% acetonitrile, the ADC analytes were eluted using 30% acetonitrile in water with 1% formic acid. A KingFisher 96 magnetic particle processor (Thermo Electron) was used to mix, wash, gather, and transfer the paramagnetic beads in the above steps. A volume of 10 µL of the eluents was analyzed by LC-MS using a TripleTOF 5600 mass spectrometer (AB Sciex). Chromatographic separation of ADCs was performed on a nanoACQUITY UPLC® system (Waters Corporation) equipped with a PS-DVB monolithic column (500 um i.d. X 5 cm) (Thermo Scientific). Raw data was deconvoluted using Analyst TF 1.6 software, and the average drug-to-antibody ratio was calculated based on the peak areas of different DAR species (DAR0–DAR2).


Optimized synthesis of tubulysin Pr (9) and its linker-drug.

To a solution of compound 2 (16.89 g, 139.32 mmol) in THF (120 mL) was added compound 1 (8.00 g, 92.9 mmol) followed by Ti(OEt)_4 (63.56 g, 278.7 mmol) at 18 °C. The mixture was heated to 80 °C for 12 h. The mixture was cooled to 20 °C, and poured into brine (100 mL). The resulting precipitate was filtered and washed with EtOAc (100 mL), and the combined organic layers were concentrated. It was purified by column chromatography on silica (solvent gradient: 0-15% EtOAc in petroleum ether) to afford the desired product compound 3 (15.0 g, 85%) as a colorless oil. LCMS (5-95, AB, 1.5 min): R_T = 0.780 min.; ^1HNMR (400 MHz, CDCl_3): δ 2.59-2.51 (m, 1H), 2.29 (s, 3H), 1.21 (s, 9H), 1.12-1.10 (m, 6H). m/z [M+H] Calculated for C_9H_20NO_S: 190.1 Found: 189.9

To a solution of compound 2 (16.89 g, 139.32 mmol) in THF (120 mL) was added compound 1 (8.00 g, 92.9 mmol) followed by Ti(OEt)_4 (63.56 g, 278.7 mmol) at 18 °C. The mixture was heated to 80 °C for 12 h. The mixture was cooled to 20 °C, and poured into brine (100 mL). The resulting precipitate was filtered and washed with EtOAc (100 mL), and the combined organic layers were concentrated. It was purified by column chromatography on silica (solvent gradient: 0-15% EtOAc in petroleum ether) to afford the desired product compound 3 (15.0 g, 85%) as a colorless oil. LCMS (5-95, AB, 1.5 min): R_T = 0.780 min.; ^1HNMR (400 MHz, CDCl_3): δ 2.59-2.51 (m, 1H), 2.29 (s, 3H), 1.21 (s, 9H), 1.12-1.10 (m, 6H). m/z [M+H] Calculated for C_9H_20NO_S: 190.1 Found: 189.9
To a solution of LDA (14.17 mL, 28.35 mmol) in diethyl ether (200 mL) was added compound 3 (4.60 g, 24.3 mmol) at -78 °C. The reaction mixture was stirred at -78 °C for 30 min under N₂. TiCl(O-i-Pr)₃ (10.15 mL, 48.6 mmol) was then added and the reaction mixture was stirred for an additional 45 min at -78 °C. Compound 4 (3.00 g, 16.2 mmol) was added in one portion. The reaction mixture was stirred under N₂ at -78 °C for 3 h. The reaction mixture was neutralized with a mixture of THF and HOAc (5.6 mL/1.4 mL), followed by addition of water (45 mL). The resulting mixture was warmed to 15 °C and filtered through celite. The filter cake was washed with EtOAc (2 x 100 mL). The organic layer was washed with brine (100 mL), dried and concentrated. The residue was purified by column chromatography on silica (33% EtOAc in petroleum ether, Rf = 0.5), to give compound 5 (3.10 g, 51%) as a light yellow oil. LCMS (5-95, AB, 1.5 min): Rₜ = 0.710 min. [M+H] m/z [M+H] Calculated for C₁₆H₂₇N₂O₄S₂: 375.1 Found: 374.9.

To a solution of NaBH₄ (0.630 g, 16.56 mmol) in THF (20 mL) was added compound 5 (3.10 g, 8.28 mmol) and Ti(O-Et)₄ (3.47 mL, 16.56 mmol) at -78 °C. The reaction mixture was stirred under N₂ at -78 °C for 3 h. The reaction mixture was neutralized using a mixture of THF/HOAc (6.4 mL/1.6 mL), followed by addition of EtOH (5.0 mL) and water (8.0 mL). The resulting mixture was warmed to 15 °C and diluted with EtOAc (50 mL). The organic layer was washed with brine (30 mL), dried and concentrated. The residue was purified by column chromatography on silica (0~50% of EtOAc in petroleum ether) to give compound 6 (2.10 g, 67%) as a light yellow solid. LCMS (5-95, AB, 1.5 min): Rₜ = 0.858 min. [M+H] m/z [M+H] Calculated for C₁₆H₂₉N₂O₄S₂: 377.2 Found: 377.0.

NaH (645.9 mg, 16.15 mmol, 60%) was suspended in DMF (60 mL) and then compound 6 (1.52 g, 4.04 mmol) was added at 0 °C. The reaction mixture was stirred at 0 °C for 30 min. PrI (27.85 g, 163.8 mmol) was then added and the reaction mixture was stirred for 2 h at 0 °C and then 6 h at 20 °C. The reaction mixture was quenched with sat. aq NH₄Cl (90.0 mL) and acidified with 1N HCl to pH = 6.0. The solution was extracted with EtOAc (2 x 150 mL). The organic layer was washed with brine (60 mL), dried and concentrated. The residue was purified by chromatography on silica (0-50% of EtOAc in petroleum ether) to afford compound 7 (1.20 g, 66%) as a light yellow solid. LCMS (5-95, AB, 1.5 min): Rₜ = 1.052 min. m/z [M+H] Calculated for C₂₀H₃₇N₂O₄S₂: 433.2 Found: 433.1.

Compound 7 (1.20 g, 2.77 mmol) was dissolved in a HCl solution in MeOH (4.0 M, 12 mL) at 20 °C. The reaction mixture was stirred at 20 °C for 30 min. The solvent was removed and the residue was purified by chromatography on silica (2% ~5% MeOH in DCM) to give compound 8 (660 mg, 72%) as a light yellow oil. LCMS (5-95, AB, 1.5 min): Rₜ = 0.804 min. m/z [M+H] Calculated for C₁₆H₂₉N₂O₃S: 329.2 Found: 329.0.

To a solution of compound 8 (300.0 mg, 0.910 mmol) and compound 9 (63.6 mg, 1.1 mmol) in MeOH (10.0 mL) was added Ti(O-i-Pr)₄ (519.2 mg, 1.83 mmol) at 20 °C. The reaction mixture was stirred at 20 °C for 5 h. NaBH₃CN (114.8 mg, 1.83 mmol) was then added and the reaction mixture was stirred for an additional 10 h at 20 °C. The reaction mixture was diluted with water (10 mL) and EtOAc (10 mL),
the precipitate was filtered and the filtrate was purified by prep-TLC (5% MeOH in DCM, Rf = 0.5) to give compound 10 (170 mg, 50%) as a light yellow oil. LCMS (5-95, AB, 1.5 min): \( R_f = 0.812 \text{ min} \). m/z [M+H] Calculated for C_{19}H_{35}N_{2}O_{3}S: 371.2 Found: 371.1.

A solution of sodium azide (12.89 g, 198.2 mmol) in water (30 mL) was mixed with DCM (50 mL) and cooled in an ice bath. \( \text{TF}_2\text{O} \) (12.8 mL, 76 mmol) was added slowly, and the resulting mixture was stirred for 3 h. The DCM phase was separated. The aqueous layer was extracted with DCM (2 x 30 mL). The organic fractions, containing the triflyl azide were cooled and washed once with saturated Na\(_2\text{CO}_3\) (30 mL) and used without further purification. Compound 11 (5.00 g, 38.12 mmol) was combined with K\(_2\text{CO}_3\) (8.02 g, 58 mmol) and cuprous sulfate pentahydrate (948.78 mg, 3.8 mmol) in distilled H\(_2\text{O}\) (50 mL) and MeOH (100 mL). The triflyl azide solution in DCM (120 mL) prepared above was added and the mixture was stirred at r.t. overnight. The organic solvents were removed under reduced pressure and the aqueous slurry was diluted with H\(_2\text{O}\) (100 mL), and acidified to pH = 6 with conc. HCl, and diluted with phosphate buffer (0.20 M pH 6.2, 150 mL). It was exacted with EtOAc (3 x 100 mL) to remove sulfonamide by-product. The aqueous phase was then acidified to pH 2.0 with conc. HCl. It was extracted with EtOAc (4 x 80 mL). The organic layers were combined, dried over Na\(_2\text{SO}_4\) and concentrated to give the desired product 12 (4.50 g, 75%) as a light yellow oil which was used without further purification.

\[ \text{HNMR (400MHz, MeOD-d4)} \delta 3.86 (d, J = 5.2 Hz, 1H), 1.92 (t, J = 3.2 Hz, 1H), 1.56-1.46 (m, 1H), 1.29-1.24 (m, 1H), 0.99 (d, J = 6.6 Hz, 3H), 0.91 (t, J = 7.6 Hz, 3H). \]

To a mixture of 12 (0.850 g, 5.41 mmol) in hexanes (60 mL) was added oxalyl chloride (3.43 g, 27.0 mmol) and DMF (0. 395 g, 5.41 mmol). The mixture was stirred for 1 h. The mixture was filtrated, and concentrated to give the crude product (0.820 g, 86%) as a yellow oil, which was used in next step without purification.

To a mixture of compound 10 (1.10 g, 2.97 mmol) in DCM (30 mL) was added DIEA (1.85 mg, 14.3 mmol), followed by 13 (0.782 g, 4.45 mmol). The mixture was stirred at 5 °C for 2 h. The mixture was concentrated to give crude product, which was purified by flash chromatography on silica (10% EtOAc in petroleum ether, Rf = 0.4) to give 14 (1.2 g, 70%) as a yellow oil. LCMS (10-80, AB, 2.0 min): \( R_f = 1.492 \text{ min} \). m/z [M+H] Calculated for C\(_{25}\)H\(_{44}\)N\(_3\)O\(_4\)S: 510.3 Found: 510.4.

To a mixture of palladium on carbon (10%, 106.4 mg) in MeOH (20 mL) was added Compound 14 (500.0 mg, 0.980 mmol) under N\(_2\). The mixture was stirred at 25 °C for 3 h under H\(_2\) (15 psi). The mixture was filtrated and the filtrate was concentrated to give the desired product (420 mg, 89%) as a yellow solid. LCMS (5-95, AB, 1.5 min): \( R_f = 0.831 \text{ min} \). m/z [M+H] Calculated for C\(_{25}\)H\(_{46}\)N\(_3\)O\(_4\)S: 484.3 Found: 484.1.
To a compound 16 (130.2 mg, 0.910 mmol) in DMF (20 mL) was added DIEA (213.8 mg, 1.65 mmol) and HATU (471.6 mg, 1.24 mmol). After the mixture stirring for 0.5 h, compound 15 (400.0 mg, 0.830 mmol) was added and stirred at 25 °C for 3 hours. The reaction mixture was diluted with water (20 mL), and the mixture was extracted with EtOAc (3x 40 mL), washed with water (3 x 30 mL), dried over anhydrous sodium sulfate, and concentrated. The residue was purified by flash column chromatography (5% MeOH in DCM, RF = 0.5) to give the desired product (380 mg, 60%) as a yellow oil. LCMS (5-95, AB, 1.5 min): RT = 0.836 min. m/z [M+H] Calculated for C_{32}H_{37}N_{4}O_{5}S: 609.4 Found: 609.2.

To a mixture of compound 17 (350.0 mg, 0.570 mmol) in THF (10 mL) and water (10 mL) was added LiOH (55.07 mg, 2.3 mmol). The mixture was stirred at 20 °C for 3 h. It was diluted with water (20 mL), cooled to 4 °C, and adjusted to pH = 3.0 with conc. HCl. The mixture was extracted with EtOAc (3 x 30 mL), and the organic layer was washed with water (2 x 40 mL), dried over anhydrous sodium sulfate, and concentrated to give crude product, which was purified by flash column chromatography (6% MeOH in DCM, RF = 0.4) to give the desired product (280 mg, 61.4%) as a brown solid. LCMS (5-95, AB, 1.5 min): RT = 1.242 min. m/z [M+H] Calculated for C_{29}H_{51}N_{4}O_{5}S: 567.4 Found: 567.5.

To a mixture of HATU (73.8 mg, 0.190 mmol) in DMF (5.0 mL) was added DIEA (45.6 mg, 0.350 mmol) and compound 18 (100.0 mg, 0.180 mmol). After the mixture was stirred for 0.5 h, compound 19 (36.6 mg, 0.180 mmol) was added at 25 °C. The mixture was stirred for another 3 h. The reaction mixture was diluted with water (10 mL), and the mixture was extracted with EtOAc (3 x 20 mL), washed with water (3 x 20 mL), dried over anhydrous sodium sulfate, and concentrated. The residue was purified by prep-TLC (10% MeOH in DCM, RF = 0.3) to give the desired product (90 mg, 59%) as a yellow oil. LCMS (5-95, AB, 1.5 min): RT = 0.811 min. HRMS (ESI): m/z [M+H] Calculated for C_{41}H_{66}N_{5}O_{6}S: 756.4732 Found: 756.4710.
To a mixture of compound 19 (50.0 mg, 0.0700 mmol) in DMF (5.0 mL) was added DIEA (17.09 mg, 0.130 mmol) and MC_VC_PAB-Cl (39.09 mg, 0.070 mmol). After the mixture was stirred for 48 h, it was filtrated and the filtrate was purified by pre-HPLC (Acetonitrile 39-69%/0.225% formic acid in water) to give the desired product (8.68 mg, 10%) as a light yellow solid. LCMS (5-95, AB, 1.5 min): R<sub>T</sub> =0.899 min. HRMS (ESI): m/z Calculated for C<sub>69</sub>H<sub>104</sub>N<sub>11</sub>O<sub>12</sub>S: 1310.7581 Found: m/z= 1310.7591.
Original route towards the synthesis of tubulysin Pr (9)

To a solution of compound 10 (120.0 mg, 0.320 mmol) in THF (4.0 mL) and was added Zn (21.1 mg, 0.320 mmol) and compound 13a (155.5 mg, 0.420 mmol) at 20 °C. After the reaction mixture was stirred at 20 °C for 24 h, it was filtered and diluted with EtOAc (15 mL) and washed with water (10 mL) and brine (10 mL). The organic layer was dried, concentrated, and purified by prep-TLC (25% EtOAc in petroleum ether, Rf = 0.6) to give compound 14a (25.0 mg, 11%) as a light yellow oil. LCMS (5-95, AB, 1.5 min): R_T =1.081 min. m/z [M+H] Calculated for C_{40}H_{56}N_{3}O_{6}: 706.4 Found: 706.1.
To a solution of compound 14a (25.0 mg, 0.040 mmol) in DMF (1.0 mL) was added piperidine (15.1 mg, 0.180 mmol) at 20 °C. After the reaction mixture was stirred under N₂ at 20 °C for 1 h, the solvent was removed and the residue was purified by prep-TLC (8% MeOH in DCM, Rf = 0.5) to give compound 15 (15.0 mg, 87.5%) as a gray oil, which was used directly in the next step. LCMS (5-95, AB, 1.5 min): Rᵣ = 0.901 min. m/z [M+H⁺] Calculated for C₂₅H₄₆N₃O₄S: 484.3 Found: 484.2.

MC-VC(R)-PABQ-tubulysin M

In a small vial, MC-Val-Cit(R)-PAB-Cl (0.025 mmol, 15 mg) and tubulysin M (2, Concorit) (1 equiv., 0.025 mmol, 21 mg) were dissolved in DMF (0.075 mL). The mixture was warmed to 40 °C for 10 minutes to solubilize contents. Added tetrabutyl ammonium iodide (0.1 equiv., 0.0025 mmol 1.5 mg) followed by N,N-diisopropylethylamine (1.5 equiv., 0.0066 mL). The clear mixture stirred at RT overnight sealed under nitrogen. The reaction was diluted and purified on HPLC under acidic conditions FA-H₂O/ACN. Isolated desired product MC-VC(R)-PABQ-tubulysin M (4.5 mg, 0.0035 mmol, 13.82%). m/z [M] Calculated for C₆₆H₉₆N₁₁O₁₃S⁺: 1282.7 Found: m/z=1282.6 [M⁺].

MC-VC-PABC-NH-tubulysin M

Combined MC-VC-PAB-PNP (1 eq., 0.014 mmol, 10.33 mg) and NH-tubulysin M (1, Concorit, 1443215-04-0) (1 eq., 10 mg, 0.014mmol, 10 mg) in DMF (0.1 mL). To this was added Hunigs base (2.5 equiv., 0.035 mmol, 6.1 uL). The reaction stirred overnight and was diluted with DMF, injected directly on HPLC for purification. 28 min method 30-70% MeCN:Water-TFA. Isolated pure product (8.34 mg, 0.006 mmol, 45% yield). HRMS (ESI): m/z [M+H⁺] Calculated for C₆₆H₉₄N₁₁O₁₅S: 1312.6652 Found=1312.6621.
MC-VC-PABC-NH-tubulysin M in d₆-DMSO