Supporting information for:

Antibody-PROTAC conjugates enable HER2-dependent targeted protein degradation of BRD4

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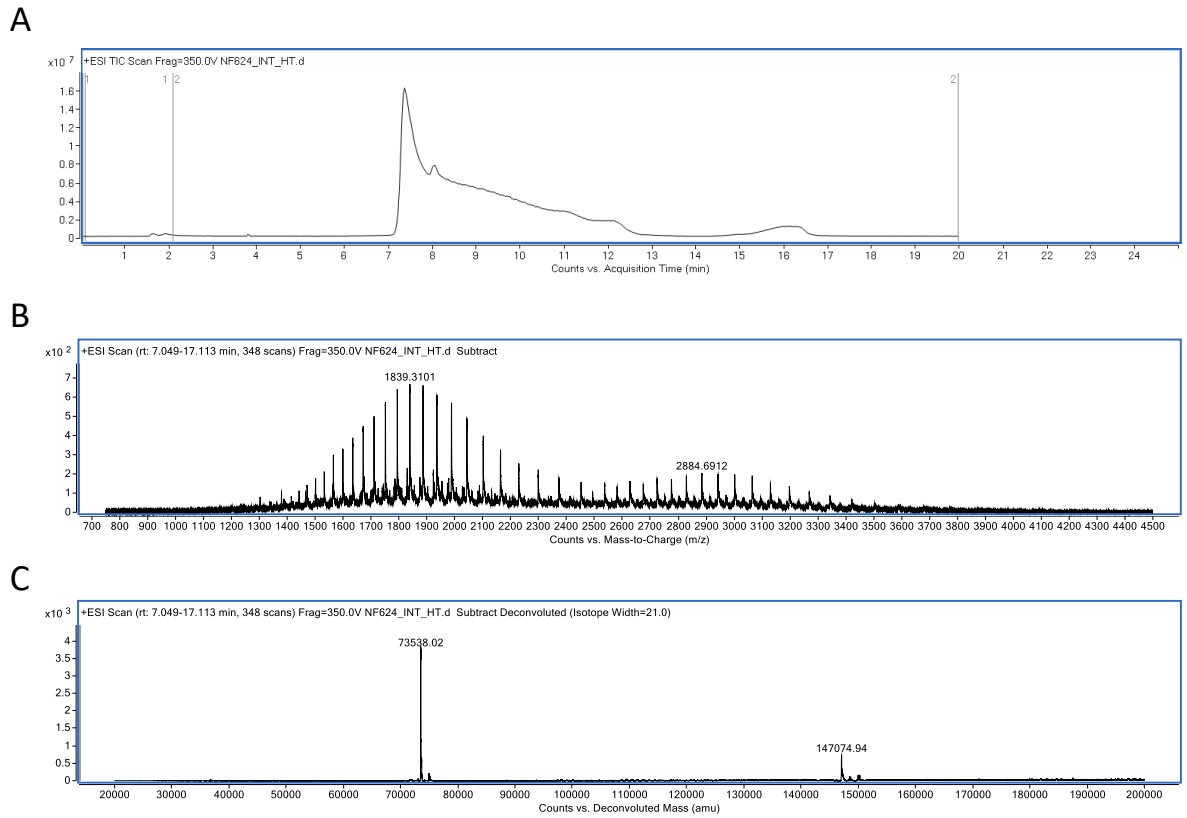


Figure S1. LC-MS analysis of trastuzumab-NGM conjugate **14**: (A) TIC, (B) non-deconvoluted ion-series, (C) deconvoluted ion series mass spectrum; observed mass of 147,075 corresponds to full antibody modified with four NGM-BCN molecules, where all NGM moieties are hydrolyzed to maleamic acids (expected 147,088), 73,538 corresponds to the isomeric form in which the hinge cysteines are bridged in an intrachain manner (expected 73,544).

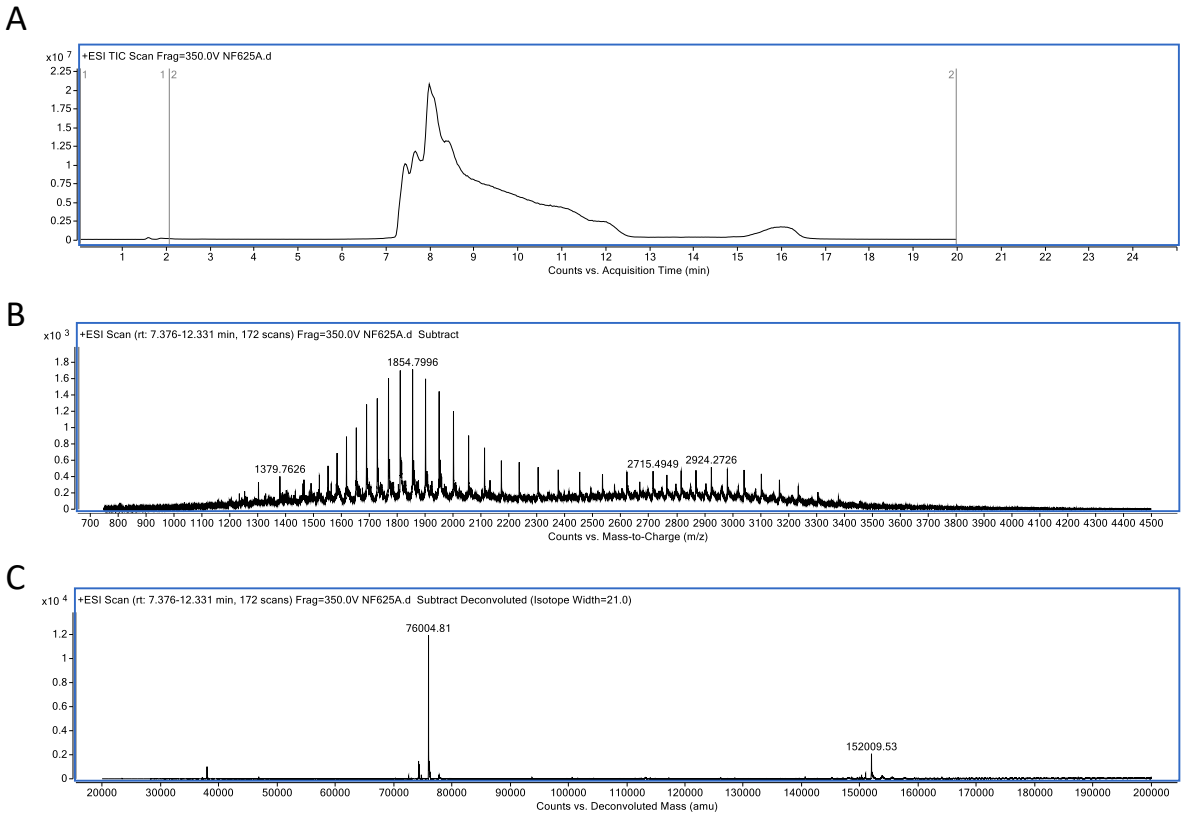


Figure S2. LC-MS analysis of trastuzumab-NGM-PROTAC conjugate **3**: (A) TIC, (B) non-deconvoluted ion-series, (C) deconvoluted ion series mass spectrum; observed mass of 152,010 corresponds to full antibody modified with four NGM-BCN-PROTAC molecules, where all NGM moieties are hydrolyzed to maleamic acids (expected 152,008), 76,005 to the isomeric form in which the hinge cysteines are bridged in an intrachain manner (expected 76,004).

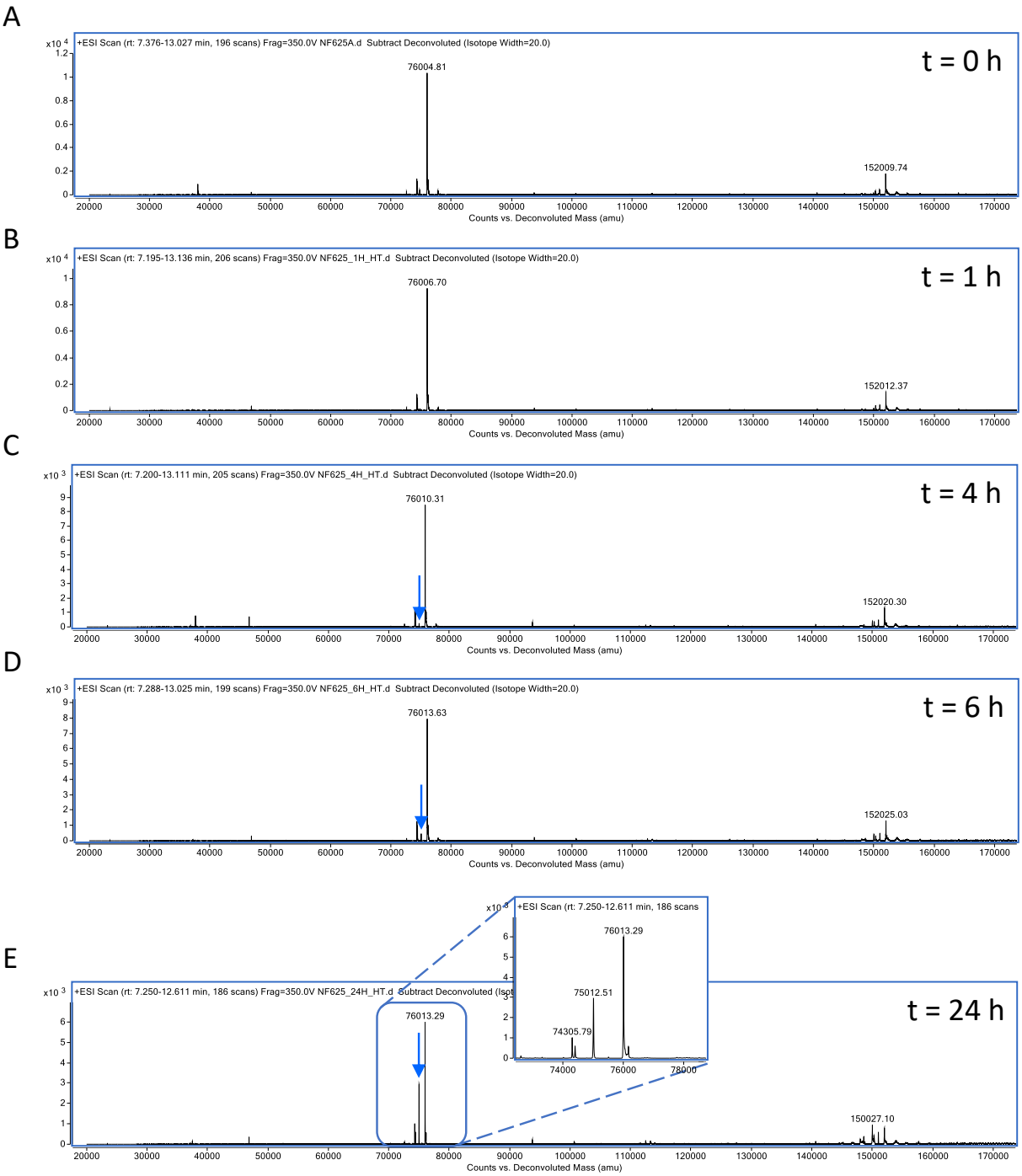


Figure S3. Deconvoluted ion series mass spectra of trastuzumab-NGM-PROTAC conjugate **3** incubated in PBS buffer (pH 7.4) at 37 °C, at different timepoints. An expanded version of the deconvoluted ion series mass spectrum is shown at 24 h, with the observed mass of 76,013 corresponding to the isomeric half antibody modified with two NGM-PROTAC molecules (expected 76,004), 75,013 corresponding to the isomeric half-antibody modified with two NGM-PROTAC molecules, where one of the two PROTAC moieties has undergone hydrolysis at the ester bond (expected 75,007); hydrolysis of both PROTAC moieties is not observed (expected 74,008).

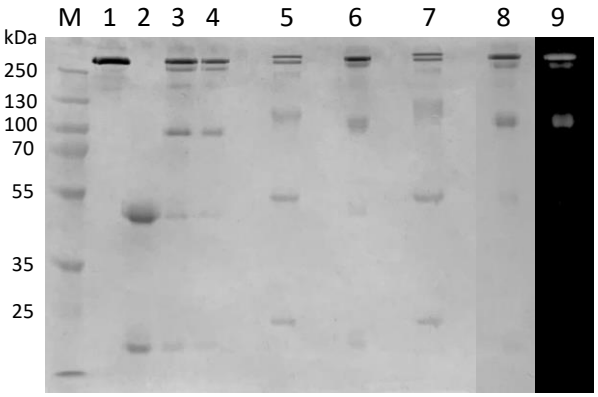


Figure S4. SDS-PAGE analysis of trastuzumab-PROTAC conjugation. M. Molecular marker, 1. Native trastuzumab, 2. Reduced trastuzumab, 3. Trastuzumab-NGM conjugate **14** after 10 min reaction, 4. Trastuzumab-NGM conjugate **14** after 18 h incubation at pH 8.5, 37 °C, 5. Trastuzumab-NGM conjugate **14** after 18 h incubation at pH 8.5, 37 °C (reducing loading buffer), 6. Trastuzumab-NGM-PROTAC conjugate **3**, 7. Trastuzumab-NGM-PROTAC conjugate **3** (reducing loading buffer), 8. Trastuzumab-NGM-PROTAC-AlexaFluor488 conjugate (AF488-**3**), 9. Trastuzumab-NGM-PROTAC-AlexaFluor488 conjugate (AF488-**3**) (picture taken on a UV transilluminator).

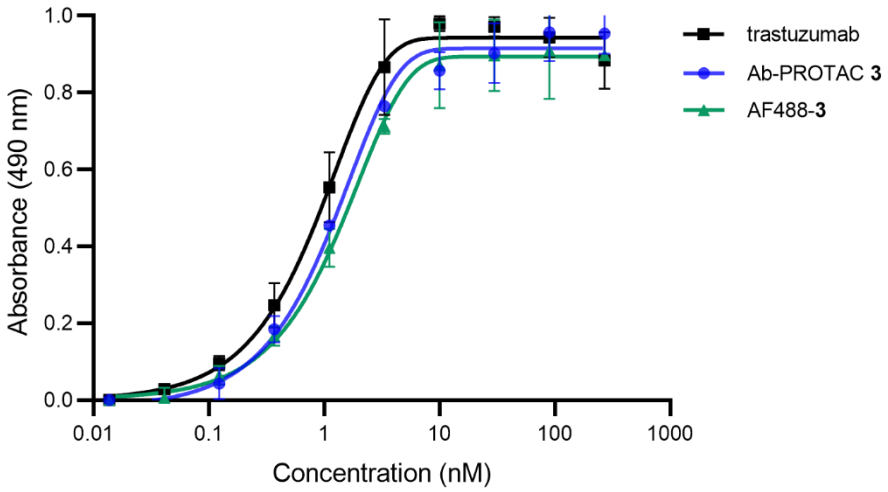


Figure S5. ELISA analysis of trastuzumab-NGM-PROTAC conjugate **3**, trastuzumab-NGM-PROTAC-AlexaFluor488 conjugate AF488-**3** and native trastuzumab against HER2.

A

$$FAR = \frac{\frac{Abs_{495}}{\varepsilon_{495}}}{\frac{Abs_{280} - (Cf \cdot Abs_{495})}{\varepsilon_{280}}} = \frac{\frac{0.893}{71{,}000}}{\frac{0.803 - (0.11 \cdot 0.893)}{215{,}380}} = 3.8$$

B

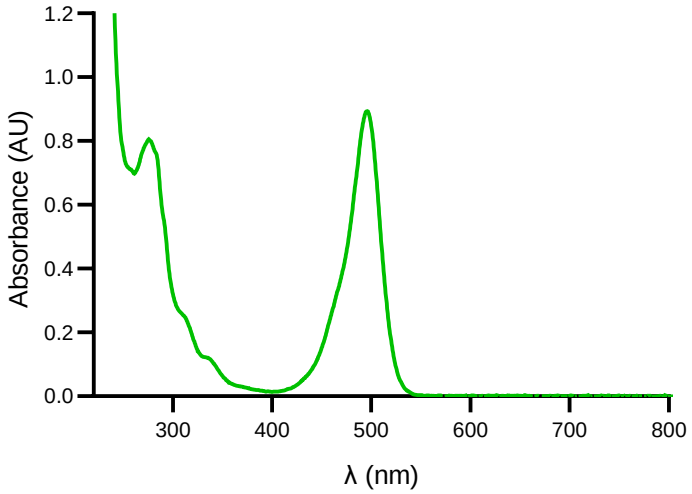


Figure S6. (A) Fluorophore-to-antibody (FAR) calculation. (B) UV/Vis absorbance of trastuzumab-PROTAC-AlexaFluor488 conjugate AF488-**3**. The fluorophore-to-antibody ratio (FAR) was determined photometrically, as described by the manufacturer. The UV/Vis absorption spectrum of the conjugate was obtained and the FAR was calculated using the formula described in (A), where *Cf* is the correction factor for the absorbance of AlexaFluor488 at 280 nm.

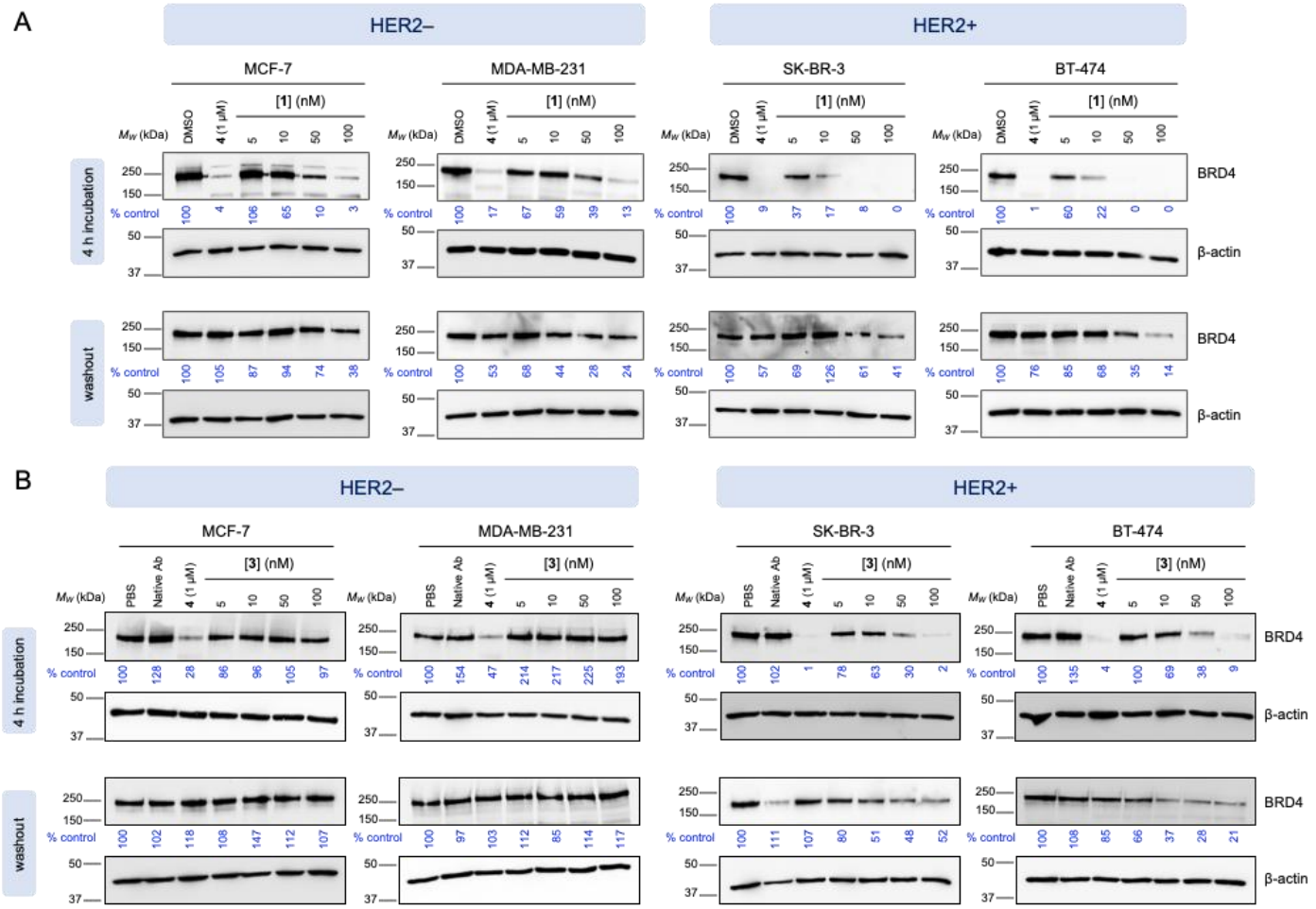


Figure S7. Monitoring of BRD4 degradation properties of PROTAC **1** (A) and Ab-PROTAC **3** (B) in MCF-7, MDA-MB-231, SK-BR-3 and BT-474 cells. Western blot analysis of BRD4 and β-actin after treatment of cells with PROTAC **1** or Ab-PROTAC **3** and control compounds. Cells were incubated with PROTAC **1** for either 4 hours in medium before harvesting cell lysates ("4 h incubation"), or for 1 hour followed by washout ("washout"), consisting of removal of medium, washing with PBS, addition of fresh medium and harvesting 23 hours later. The bands on western blots were quantitated and normalized to β-actin and negative control (DMSO or PBS).

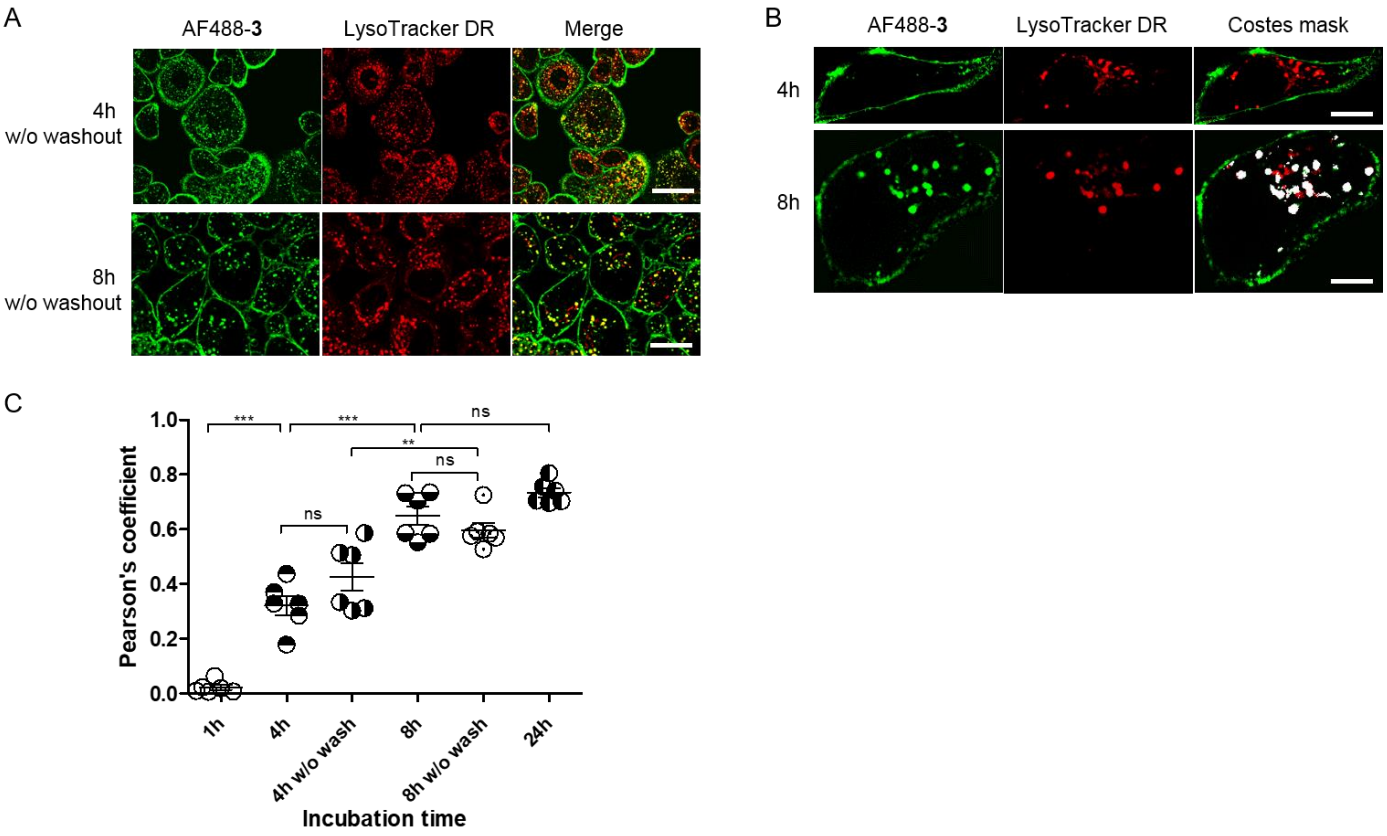
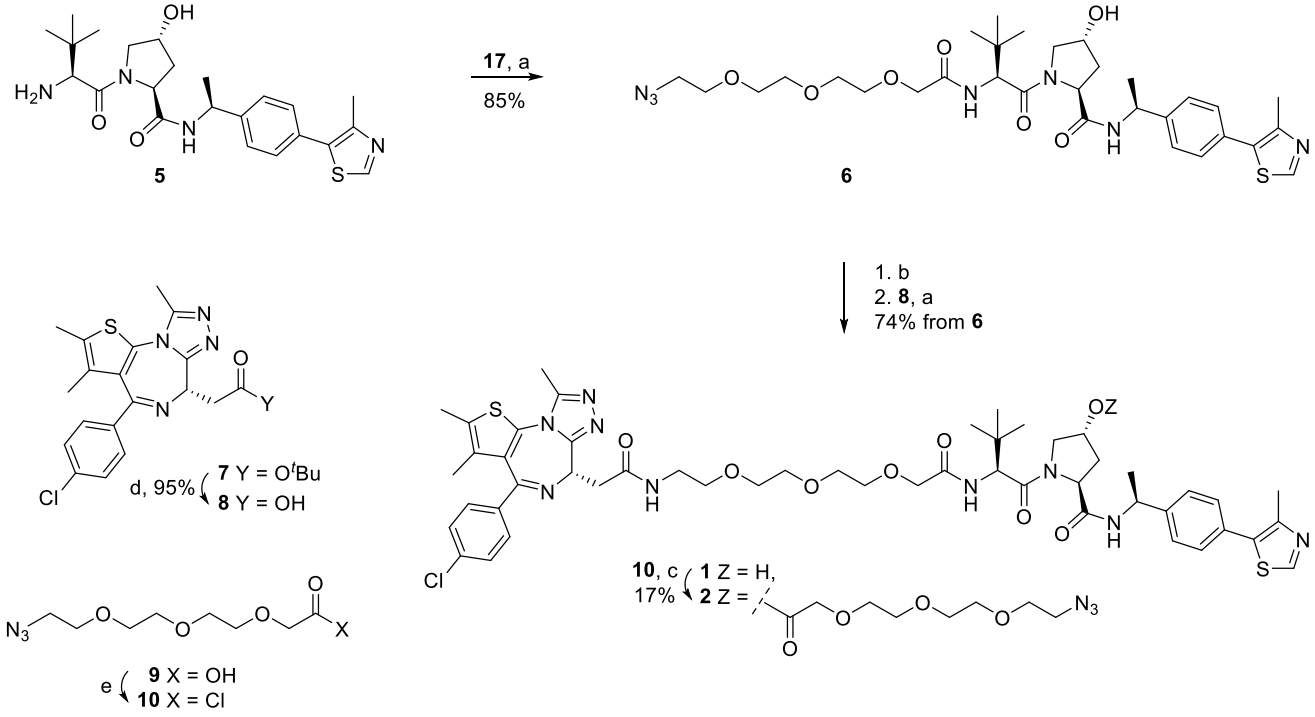


Figure S8. (A) Her2+ SK-BR-3 cells were treated with AF488-**3** (100 nM) for 4 h and 8 h without post 1 h washout. Cells were then labelled with LysoTracker DeepRed (DR) (50 nM, 30 min). Shown is a representative frame from live imaging of cells via confocal microscopy. Scale bar = 20 µm. (B) Shown is a representative single cell correlation analysis with ImageJ Costes map. White areas indicate the overlay of pixels of green colour and red colour. Scale bar = 5 µm. (C) Quantification of AF488-**3** colocalization with LysoTracker DeepRed. The Pearson's correlation coefficient is shown for various incubation time points in SK-BR-3 cells. Data are mean + SEM; n = 6 cells. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test (***P<0.0001, **P<0.001, ns is nonsignificant).

1. Chemistry and Bioconjugation

1.1. Synthesis of PROTAC 1 and azido-PROTAC 2

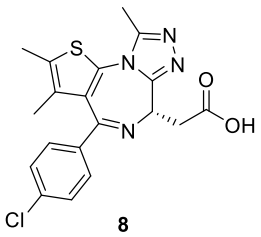


Scheme S1. Synthesis of compounds **1** and **2**. *Reagents and conditions:* (a) HATU, DIPEA, DCM, RT. (b) PPh_3, MeOH, H_2O, 50 °C. (c) Et_3N, DCM, RT. (d) TFA, DCM, RT. (e) $SOCl_2$, DCM, RT.

1.1.1 Materials and methods

All reagents were purchased from commercial sources and they were used as provided without any further purification. Pan-BET bromodomain inhibitor JQ1 (**7**) and Von Hippel-Lindau (VHL) E3 ligase ligand **5** were provided by GlaxoSmithKline Medicines Research Centre, Stevenage. Common organic solvents used in chemical reactions were purchased anhydrous from Acros Organics Ltd. over molecular sieves in AcroSeal® bottles and handled under nitrogen. Reactions were monitored by thin-layer chromatography (TLC) using aluminium sheets 20 × 20 cm with silica gel 60 F_{254} (Merck). The stains on the TLCs were visualized using UV light (254nm) and by staining with Ce-Mo or ninhydrin solutions depending on the case. Compound purification was carried out by flash chromatography employing silica gel Geduran® Si 60 (0.040-0.063 mm, Merck) as stationary phase. Nuclear Magnetic Resonance (NMR) spectra were recorded in a BRUKER AV-400 spectrometer at 400 MHz (^{1}H-NMR) and 101 MHz (^{13}C-NMR and DEPT135) in deuterated solvents. Chemical shifts (δ) are indicated in part per million (ppm) and coupling constants (J) in Hertzs (Hz). Mass spectra were recorded by the Mass Spectrometry Service (Imperial College London) in a Waters LCT Premier spectrometer employing electrospray (ESI) as ionization procedure. Data obtained are expressed in mass units (m/z). FT-IR spectra were recorded in an Agilent Cary 630 FTIR (ATR) spectrometer. Optical rotations were measured in an ADP440+ polarimeter (Bellingham+Stanley) and they are expressed in 10^{-1} deg cm^2 g^{-1}.

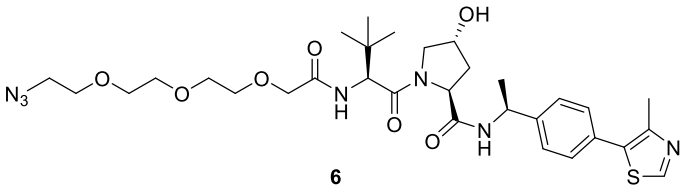
Synthesis of JQ1 carboxylic acid (8)[1]



8

A solution of JQ1 (**7**) (250 mg, 0.55 mmol) in dichloromethane (5.5 mL) was treated with trifluoroacetic acid (0.84 mL, 11 mmol) and the resulting bright yellow solution was stirred at room temperature. After 2 h, more trifluoroacetic acid was added (0.84 mL, 11 mmol) and the reaction was stirred for 2 h more. Solvents were evaporated under reduced pressure leading to acid **8**[1] (210 mg, 95%) as a bright yellow solid.

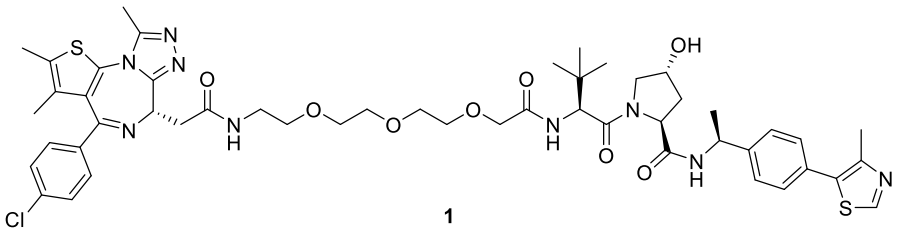
Synthesis of (2*S*,4*R*)-1-((*S*)-14-azido-2-(tert-butyl)-4-oxo-6,9,12-trioxa-3-azatetradecanoyl)-4-hydroxy-N-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (6)



6

A solution of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetic **9** (105 mg, 0.45 mmol) in dry dichloromethane (4.5 mL) under inert atmosphere was treated with HATU (222 mg, 0.59 mmol) and *N*,*N*-diisopropylethylamine (0.31 mL, 1.8 mmol) and the resulting mixture was stirred for 10 min at room temperature. VHL ligand dihydrochloride (**5**) (200 mg, 0.45 mmol) was added to the mixture and the reaction was stirred for 4 h. Dichloromethane was added, and the organic layer was washed with HCl (10%), H_2O and brine, dried (anh. Na_2SO_4), filtered an evaporated under reduced pressure. The crude mixture was purified by flash chromatography, eluting with (96:4) dichloromethane/methanol to give the azide **6** (253 mg, 85%) as a white foam. $[\alpha]_D^{20} = -52.9°$ (*c*1.0, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.82 (s, 1H), 7.55 (d, *J* = 7.7 Hz, 1H), 7.41 (d, *J* = 8.2 Hz, 2H), 7.38 (d, *J* = 8.2 Hz, 2H), 7.35 (d, *J* = 8.6 Hz, 1H), 5.08 (q, *J* = 7.0 Hz, 1H), 4.75 (t, *J* = 7.8 Hz, 1H), 4.56 (d, *J* = 8.6 Hz, 1H), 4.52 (br s, 1H), 4.07 (m, 1H), 4.03 (dd, *J* = 5.0 and 15.7 Hz, 2H), 3.72–3.61 (m, 11H), 3.39 (t, *J* = 5.0 Hz, 2H), 2.56 (s, 3H), 2.50 (m, 1H), 2.08 (m, 1H), 1.49 (d, *J* = 6.9 Hz, 3H) and 1.08 (s, 9H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ: 171.6 (C), 170.5 (C), 169.9 (C), 150.8 (CH), 147.6 (C), 143.8 (C), 132.3 (C), 130.3 (C), 129.6 (2×CH), 126.6 (2×CH), 71.3 (CH_2), 70.8 (2×CH_2), 70.6 (CH_2), 70.4 (CH_2), 70.2 (CH_2), 70.1 (CH), 58.4 (CH), 57.3 (CH), 56.7 (CH_2), 50.8 (CH_2), 49.0 (CH), 35.6 (CH_2), 35.2 ($\underline{C}(CH_3)_3$), 26.6 ($C(\underline{C}H_3)_3$), 22.4 (CH_3) and 15.8 (CH_3) ppm. FTIR (ATR) υ: 3323 (OH), 2116 (N_3) and 1668 (CO) cm^{-1}. MS (ESI) *m/z* = 682 (MNa^+). HRMS calcd for $C_{31}H_{45}N_7O_7NaS$ (MNa^+): 682.2999; found: 682.3004.

Synthesis of PROTAC 1



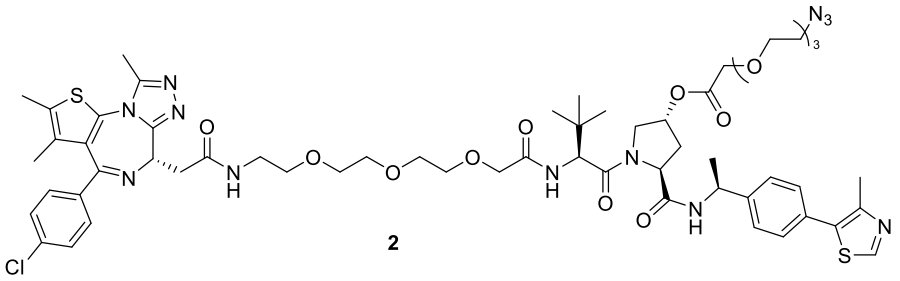
1

A solution of azide **6** (50 mg, 0.08 mmol) in methanol (0.32 mL) and H_2O (2 drops) was treated with triphenylphosphine (23 mg, 0.088 mmol) and the reaction was heated at 50 °C for 18 h. Solvents were evaporated *in vacuo*, the crude amine was dissolved in dry dichloromethane (0.8 mL) and added over a solution

[1] Kim, S. A.; Go, A.; Jo, S-H.; Park, S. J.; Jeon, y. U.; Kim, j. E.; Lee, H. K.; Park, C. H.; Lee, C-O.; Park, S. G.; Kim, P.; Park, B. C.; Cho, S, Y.; Kim, S.; Ha, J. D.; Kim, J-H.; Hwang, J. Y. A Novel Cereblon Modulator for Targeted Protein Degradation. *Eur. J. Med. Chem.* **2019**, *199*, 65–74.

of acid **8** (32 mg, 0.08 mmol), HATU (40 mg, 0.1 mmol) and *N*,*N*-diisopropyethylamine (0.56 μL, 0.32 mmol) in dry dichloromethane (0.8 mL) at room temperature and under inert atmosphere. The resulting mixture was stirred at room temperature for 1 h and then the reaction was diluted with dichloromethane. The organic layer was washed with HCl (10%), H_2O and brine, dried (anh. Na_2SO_4), filtered an evaporated under reduced pressure. The crude mixture was purified by flash chromatography, eluting with a gradient of dichloromethane/methanol [from (96:4) to (85:15)] to give PROTAC **1** (60 mg, 74%) as a colourless oil. $[\alpha]_D^{20}$ = –15.0° (*c*0.8, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.71 (s, 1H), 7.99 (m, 1H), 7.51 (d, *J* = 7.7 Hz, 1H), 7.42–7.29 (m, 8H), 7.38 (d, *J* = 8.2 Hz, 2H), 5.06 (q, *J* = 7.0 Hz, 1H), 4.79 (t, *J* = 7.8 Hz, 1H), 4.70 (m, 2H), 4.43 (br s, 1H), 4.29 (d, *J* = 15.9 Hz, 1H), 4.12 (d, *J* = 15.9 Hz, 1H), 4.06 (d, *J* = 11.0 Hz, 1H), 3.70–3.48 (m, 12H), 3.42–3.34 (m, 2H), 2.64 (s, 3H), 2.51 (s, 3H), 2.39 (s, 3H), 2.38 (m, 1H), 2.07 (m, 1H), 1.66 (s, 3H), 1.45 (d, *J* = 7.0 Hz, 3H) and 1.05 (s, 9H) ppm. ^{13}C NMR (101 MHz, $CDCl_3$) δ: 171.4 (C), 171.0 (2×C), 170.3 (C), 164.5 (C), 155.7 (C), 150.6 (CH), 150.1 (C), 148.1 (C), 143.7 (C), 137.3 (C), 136.1 (C), 132.3 (C), 132.0 (C), 131.5 (C), 131.4 (C), 130.6 (C), 130.5 (C), 130.3 (2×CH), 129.6 (2×CH), 128.9 (2×CH), 126.6 (2×CH), 71.6 (CH_2), 70.9 (CH_2), 70.4 (4×CH_2), 70.0 (CH), 58.8 (CH), 57.1 (CH_2), 56.9 (CH), 54.0 (CH), 48.9 (CH), 39.9 (CH_2), 37.6 (CH_2), 36.0 (CH_2), 35.6 (C), 26.6 (C($\underline{C}H_3$)$_3$), 22.3 (CH_3), 16.0 (CH_3), 14.5 (CH_3), 13.2 (CH_3) and 11.8 (CH_3) ppm. FTIR (ATR) υ: 3323 (OH) and 1668 (CO) cm^{-1}. MS (ESI) *m/z* = 1083 (MNa^+). HRMS calcd for $C_{50}H_{62}N_9O_8NaS_2Cl$ (MNa^+): 1038.3749; found: 1038.3760.

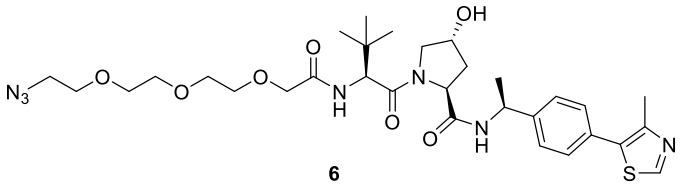
Synthesis of azido-PROTAC 2



2

A solution of 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetic acid (**9**) (21 mg, 0.09 mmol) in dry dichloromethane (0.45 mL), under inert atmosphere was treated with thionyl chloride (13 μL, 0.18 mmol) and the reaction mixture was stirred overnight at room temperature. Solvents were evaporated under reduced pressure and the resulting residue was dissolved in dry dichloromethane (0.13 mL) under inert atmosphere. Alcohol **1** (50 mg, 0.05 mmol) and triethylamine (14 μL, 0.1 mmol) were added to the solution and the reaction was stirred for 24 h at room temperature. Solvents were evaporated under reduced pressure and the resulting crude mixture was purified by flash chromatography eluting with a gradient of dichloromethane/methanol [from (96:4) to (85:15)] to give ester azido-PROTAC **2** (10.3 mg, 17%) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.66 (s, 1H), 7.63 (d, *J* = 7.8 Hz, 1H), 7.99 (m, 1H), 7.41–7.30 (m, 8H), 7.26 (d, *J* = 8.9 Hz, 1H), 7.15 (m, 1H), 5.42 (m, 1H), 5.06 (q, *J* = 7.0 Hz, 1H), 4.82 (dd, *J* = 7.0 and 8.0 Hz, 1H), 4.65 (t, *J* = 7.2 Hz, 1H), 4.52 (d, *J* = 8.9 Hz, 1H), 4.17 (d, *J* = 16.8 Hz, 1H), 4.11 (d, *J* = 16.8 Hz, 1H), 4.08 (d, *J* = 15.6 Hz, 1H), 4.03 (d, *J* = 15.6 Hz, 1H), 3.85 (dd, *J* = 4.7 and 11.7 Hz, 1H), 3.76–3.64 (m, 17H), 3.62–3.41 (m, 6H), 3.38 (t, *J* = 5.1 Hz, 2H), 3.10 (m, 1H), 2.66 (m, 1H), 2.63 (s, 3H), 2.51 (s, 3H), 2.39 (s, 3H), 2.20 (m, 1H), 1.66 (s, 3H), 1.44 (d, *J* = 7.1 Hz, 3H) and 1.05 (s, 9H) ppm. FTIR (ATR) υ: 2923 (NH), 2104 (N_3) and 1750 (CO), 1729 (CO), 1670 (CO), 1653 (CO) cm^{-1}. MS (ESI) *m/z* = 1253 (MNa^+). HRMS calcd for $C_{58}H_{75}N_{12}O_{12}NaS_2Cl$ (MNa^+): 1253.4655; found: 1253.4690.

1.1.2 NMR Spectra



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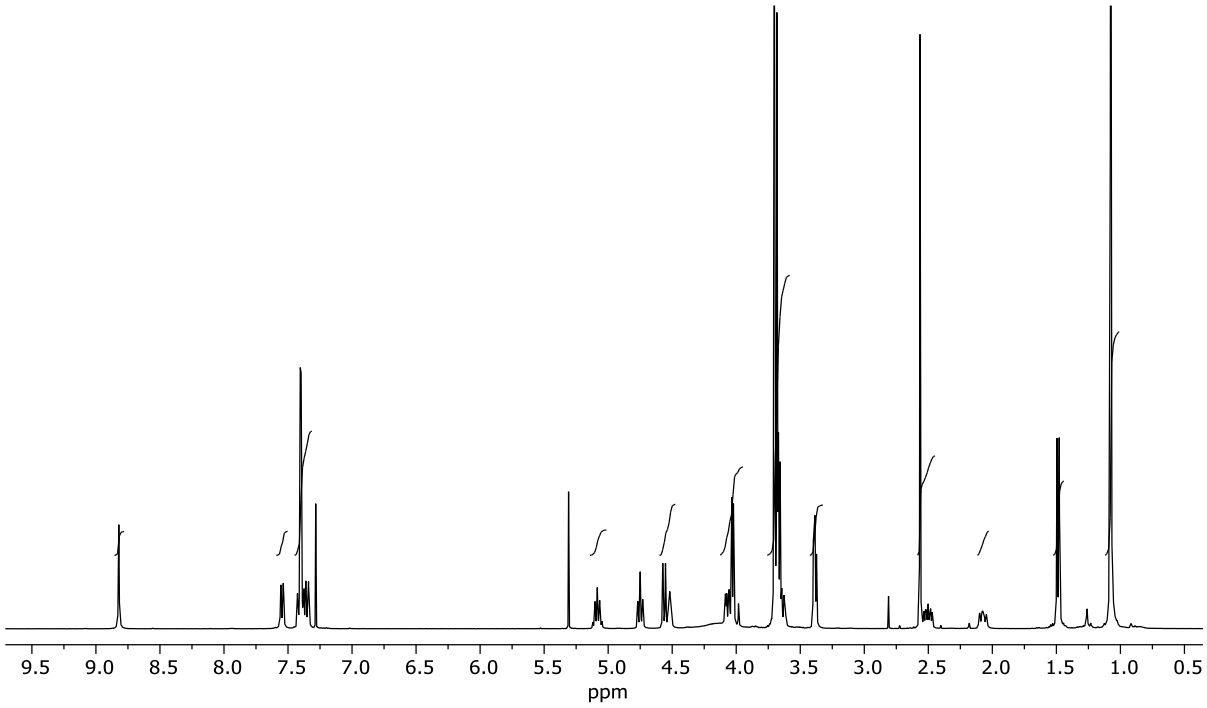

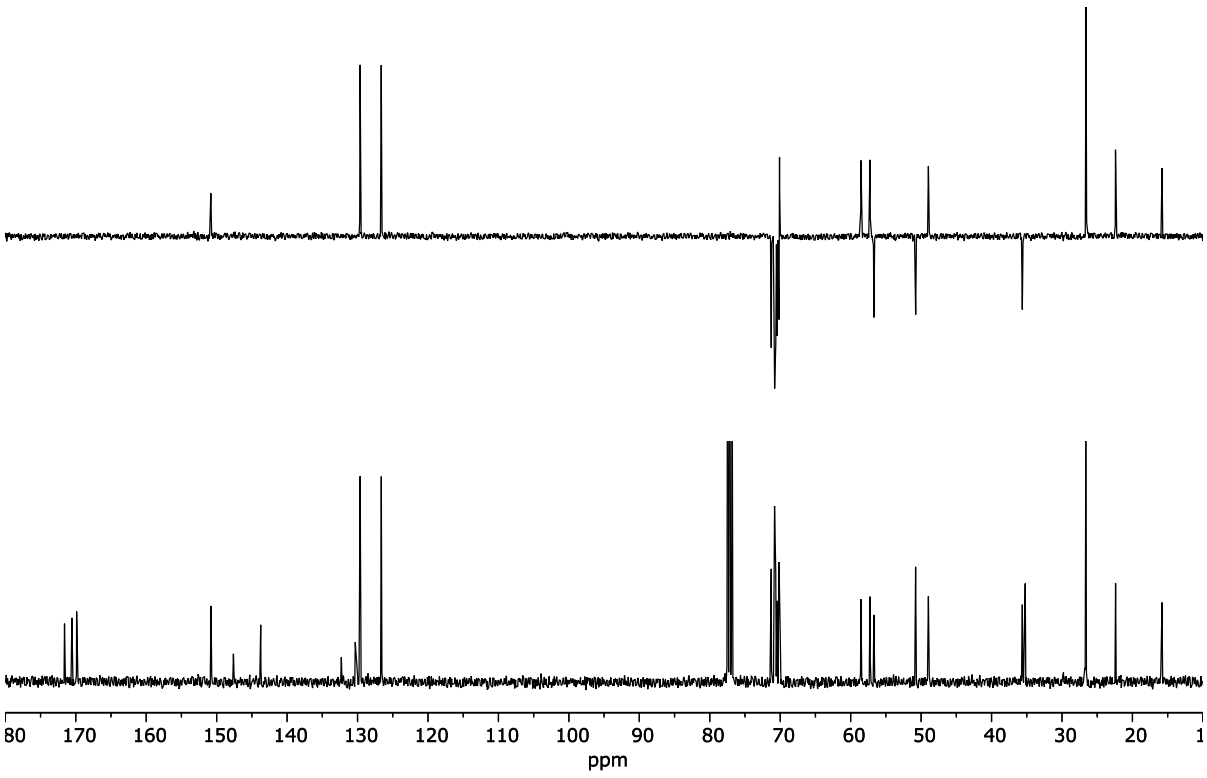

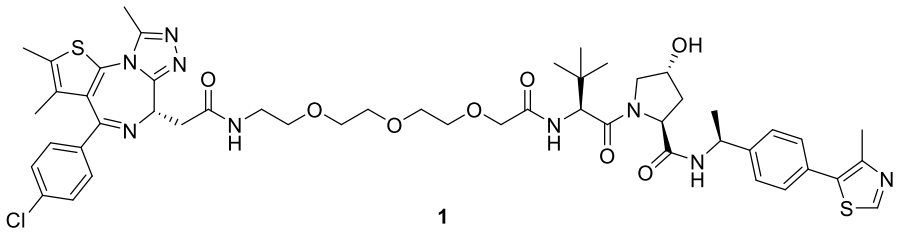

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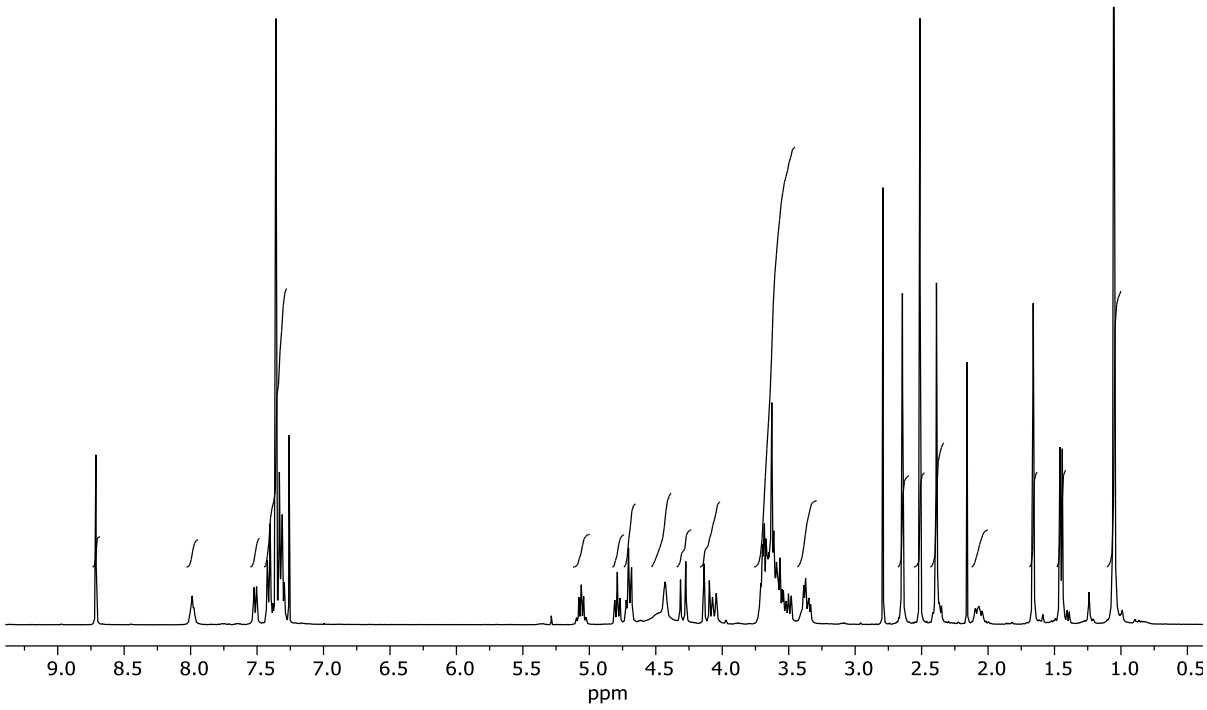
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7.5
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6.5
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3.0
2.5
2.0
1.5
1.0
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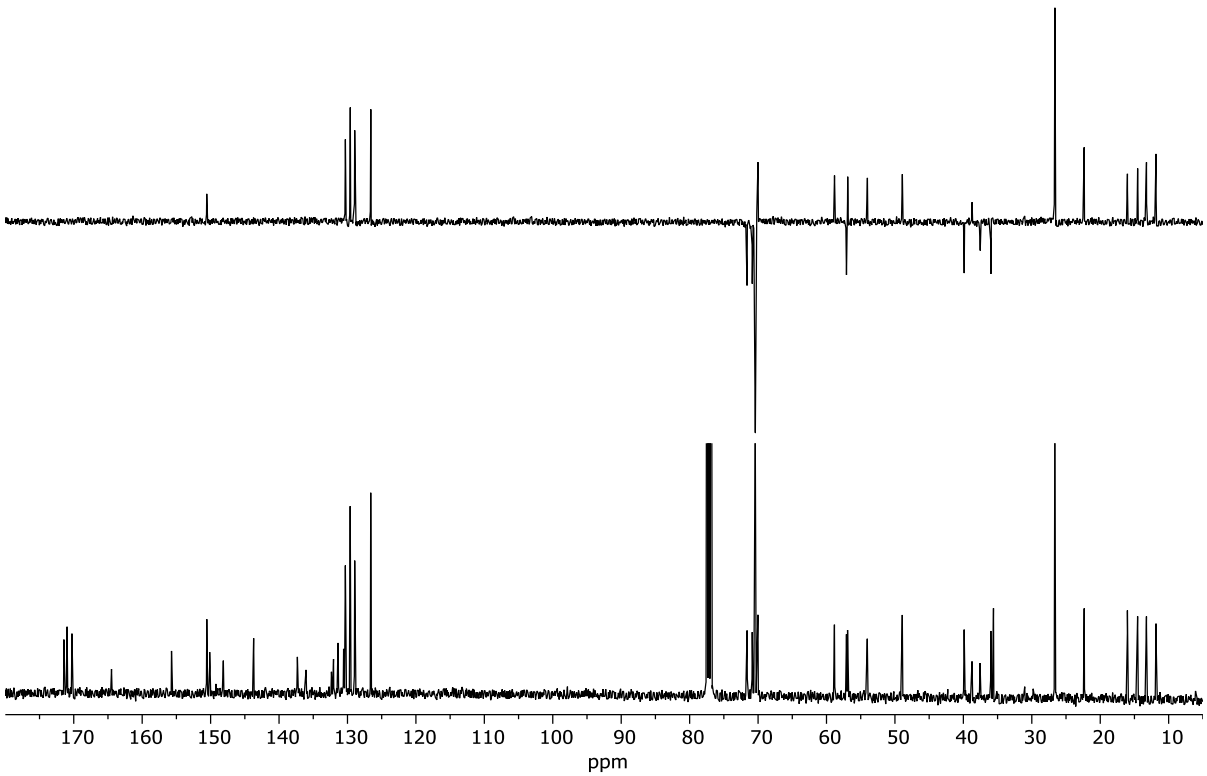
ppm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150
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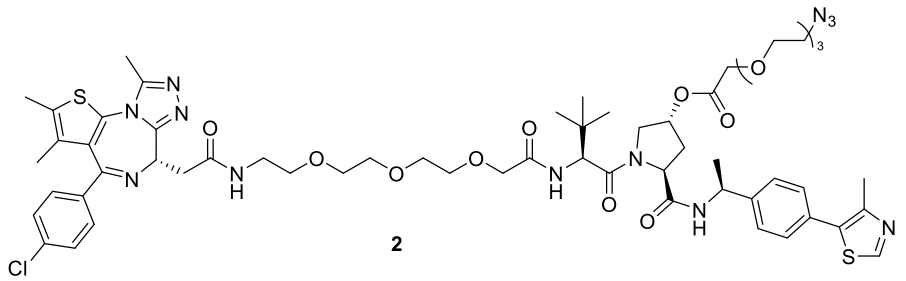

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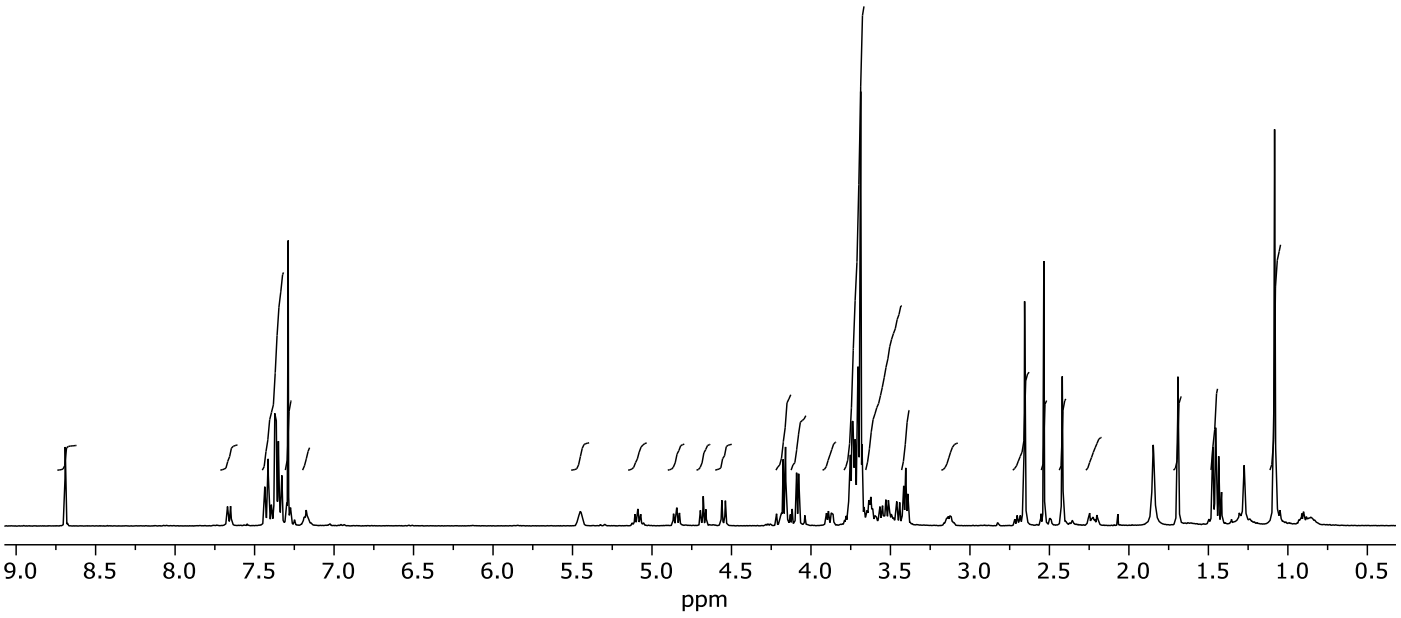
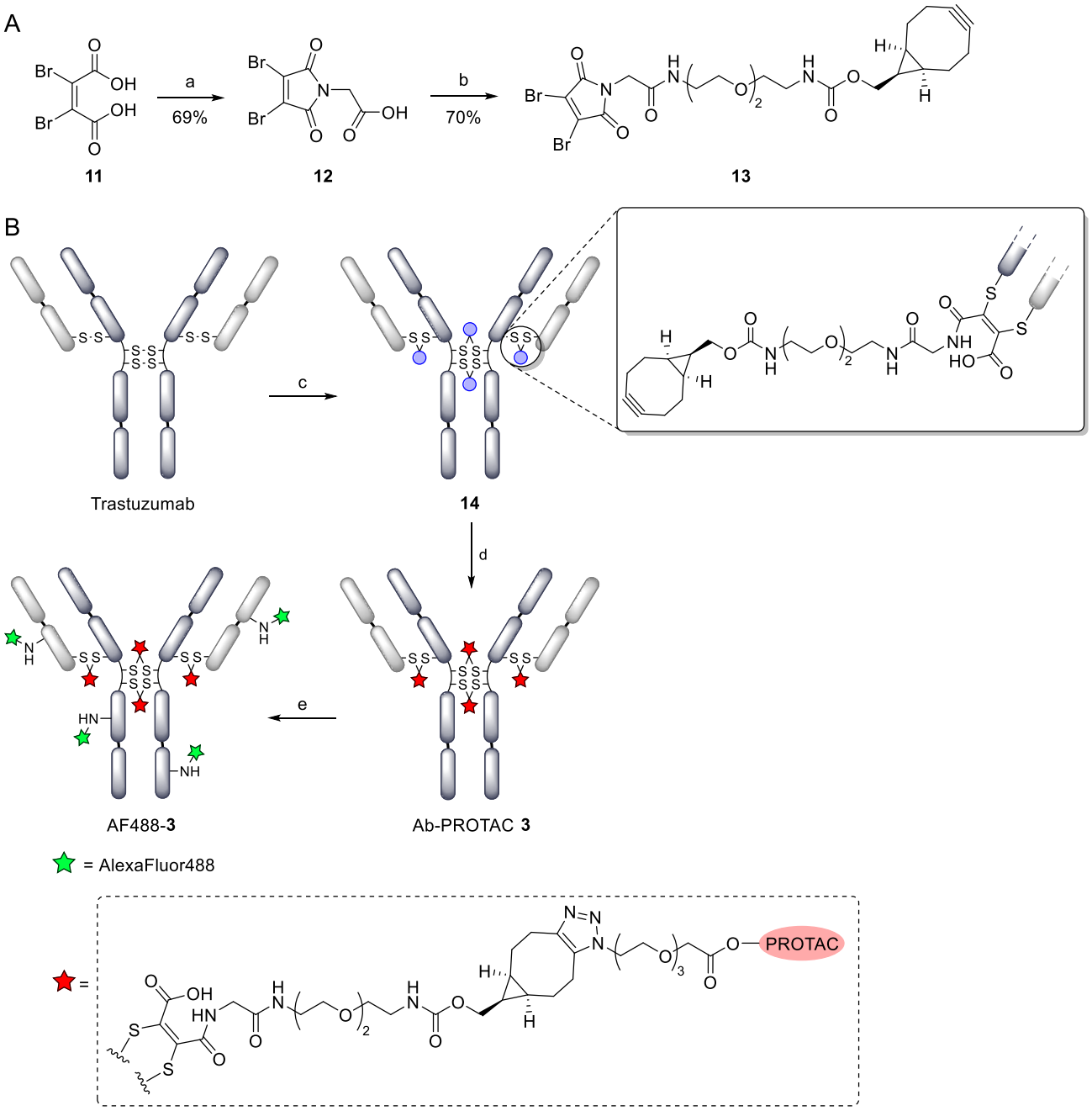


1.2. Synthesis of Ab-PROTAC 3 and AF488-3



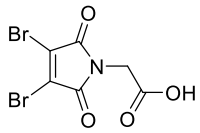
Scheme S2. Synthesis of Ab-PROTAC **3** and AF488-**3**: (A) Chemistry, (B) Bioconjugation. *Reagents and conditions:* (a) Glycine, AcOH, reflux, 3 h. (b) *N*-[(1*R*,8*S*,9*S*)-Bicyclo[6.1.0]non-4-yn-9-ylmethyloxy carbonyl]-1,8-diamino-3,6-dioxa-octane, EEDQ, MeCN, RT, 1 h. (c) 1. TCEP (6 eq.), pH 8.5, 37 °C, 2 h; 2. NGM-BCN **13** (8 eq.), 20 °C, 10 min then 37 °C, 18 h. (d) azido-PROTAC **2** (20 eq.), pH 6.9, 22 °C, 4 h. (e) AlexaFluor488 NHS ester (10 eq.), pH 7.4, 22 °C, 2 h.

1.2.1. Materials and methods – Chemistry

All chemical reagents and solvents were purchased from Sigma, AlfaAesar or VWR and used as received, without any further purification. All reactions were carried out at atmospheric pressure, under argon. Room temperature is defined as between 15-25 °C. The term *in vacuo* refers to solvent removal using Büchi rotary evaporation between 15-50 °C, at approximately 10 mm Hg. Reactions were monitored by TLC, using TLC plates pre-coated with silica gel 60 F_{254} on aluminium (Merck KGaA). Detection was by UV (254 nm and 365 nm) or chemical stain ($KMnO_4$, ninhydrin). Column chromatography was carried out using a Biotage Isolera with GraceResolvTM silica flash cartridges. ^{1}H and ^{13}C NMR spectra were recorded at ambient temperature on a Bruker Advance AMX600 instrument, operating at 600 MHz for ^{1}H and at 150 MHz for ^{13}C in the stated solvent, using $CDCl_3$

($\delta = 7.26$) or CD_3OD ($\delta = 3.31$) as the internal standard. Chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) in Hertz (Hz). The multiplicity of each signal is indicated as s-singlet, d-doublet, t-triplet, q-quartet, quin-quintet, m-multiplet (i.e. complex peak obtained due to overlap) or a combination of these. All assignments were made with the aid of DEPT, COSY, HSQC, HMBC or NOESY correlation experiments. Infra-red spectra were recorded on a Bruker ALPHA FT-IR spectrometer operating in ATR mode, with frequencies given in reciprocal centimetres (cm^{-1}). The absorptions are characterized as s (sharp), br (broad), m (medium), w (weak). Melting points were taken on a Gallenkamp apparatus and are uncorrected. High- and low-resolution mass spectra were recorded on a VG70 SE mass spectrometer, operating in mode ESI (+ or –) at the Department of Chemistry, University College London.

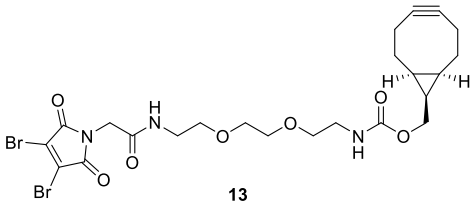
Synthesis of 2-(3,4-dibromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetic acid (12)[2]



12

To dibromomaleic acid (600 mg, 2.19 mmol) in AcOH (20 mL) was added glycine (150 mg, 1.99 mmol) and the resultant mixture was heated to reflux for 3 h. The solvent was then removed *in vacuo*, azeotroping with toluene (3 × 50 mL). Purification by column chromatography (gradient elution from CH_2Cl_2/ 1% AcOH to 10% MeOH in CH_2Cl_2/ 1% AcOH) afforded the target product as a white crystalline solid (428 mg, 1.37 mmol, 69%). mp: 207–208 °C (lit. mp 205–206 °C)[1]; ^{1}H NMR (600 MHz, CD_3OD) δ_H 4.32 (s, 2H, NCH_2); ^{13}C NMR (150 MHz, CD_3OD) δ_C 170.2 (*C*(O)OH), 164.7 (NC(O)), 130.6 (CBr), 40.6 (NCH_2); IR (solid) ν_{max}/ cm^{-1} 2989 (w), 2949 (w), 1789 (w), 1720 (s), 1586 (m); LRMS (ES+) *m/z* 316 ([^{81,81}M+H]$^+$, 48), 314 ([^{81,79}M+H]$^+$, 100), 312 ([^{79,79}M+H]$^+$, 50); HRMS (ES+) calcd for [$C_6H_3{}^{79,79}Br_2NO_4H$]$^+$ [^{79,79}M+H]$^+$ 311.8507, observed 311.8506.

Synthesis of Bicyclo[6.1.0]non-4-yn-9-ylmethyl (2-(2-(2-(2-(3,4-dibromo-2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)acetamido)ethoxy)ethoxy)ethyl)carbamate (13)



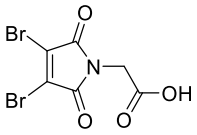
13

To compound **1** (106 mg, 0.339 mmol) in dry MeCN (18 mL) under argon was added EEDQ (102 mg, 0.412 mmol). The resultant yellow solution was stirred for 30 min at RT. After this time, *N*-[(1*R*,8*S*,9*S*)-Bicyclo[6.1.0]non-4-yn-9-ylmethyloxy carbonyl]-1,8-diamino-3,6-dioxa-octane (100 mg, 0.308 mmol) in dry MeCN (2 mL) was added and the resultant mixture was stirred for 1 h at RT. The solvent was then removed *in vacuo* and purification by column chromatography (gradient elution from 0% EtOAc in pet. ether to 100% EtOAc in pet. ether) afforded the target compound as a colourless oil (133 mg, 0.216 mmol, 70%). ^{1}H NMR (600 MHz, $CDCl_3$) δ_H 8.10 (br s, 0.5H, N*H*), 6.44 (br s, 0.5H, N*H*), 5.91 (br s, 0.5H, N*H*), 5.15 (br s, 0.5H, N*H*), 4.29 (s, 2H, NCH_2), 4.15 (d, 2H, $J = 6.6$, C(O)OCH_2), 3.70-3.56 (m, 8H, 4 × OCH_2), 3.47 (m, 2H, CH_2C(O)NHCH_2), 3.39 (m, 2H, OC(O)NHCH_2), 2.31-2.20 (m, 6H, 2 × CCH_2, 2 × CCH_2C*H*H), 1.62-1.52 (m, 2H, 2 × CCH_2CH*H*), 1.40-1.30 (m, 1H, OCH_2C*H*), 1.00-0.91 (m, 2H, 2 × OCH_2CHC*H*); ^{13}C NMR (150 MHz, $CDCl_3$) δ_C 165.4 (CH_2*C*(O)), 163.7 (*C*(O)N), 157.0 (NH*C*(O)O), 129.9 (*C*Br), 98.9 (*C*CH_2), 70.8 (OCH_2), 70.6 (OCH_2), 70.2 (OCH_2), 69.6 (OCH_2), 63.7 (C(O)OCH_2), 63.1 (C(O)OCH_2), 41.8 (NCH_2), 40.9 (OC(O)NHCH_2), 39.8

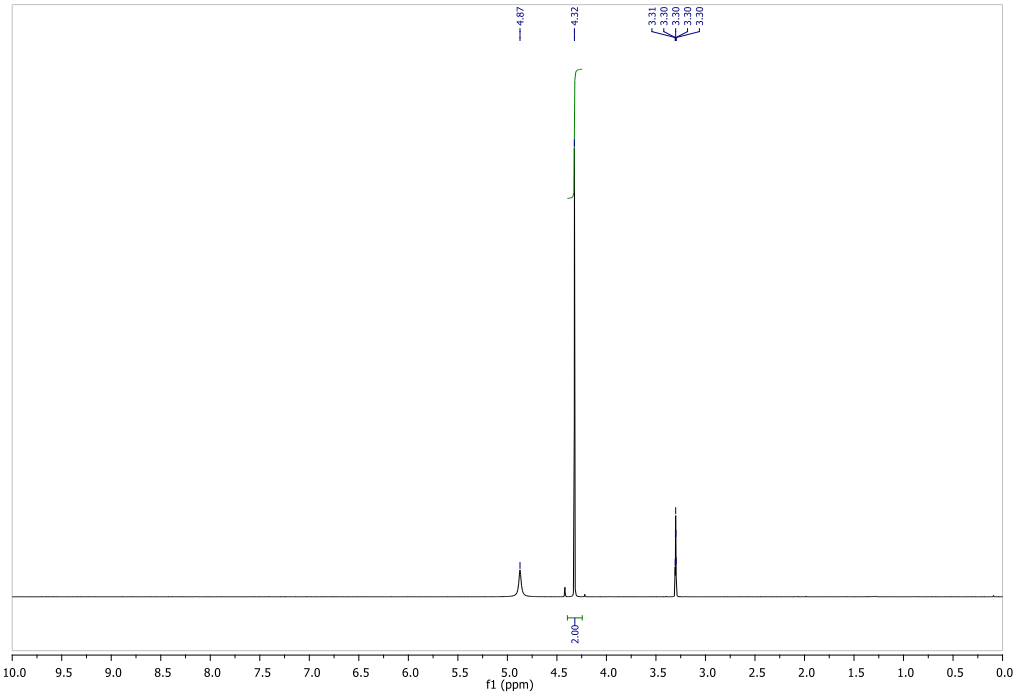
[2] Morais, M.; Nunes, J. P. M.; Karu, K.; Forte, N.; Benni, I.; Smith, M. E. B.; Caddick, S.; Chudasama, V.; Baker, J. R. Optimisation of the dibromomaleimide (DBM) platform for native antibody conjugation by accelerated post-conjugation hydrolysis, *Org. Biomol. Chem.* **2017**, *15*, 2947.

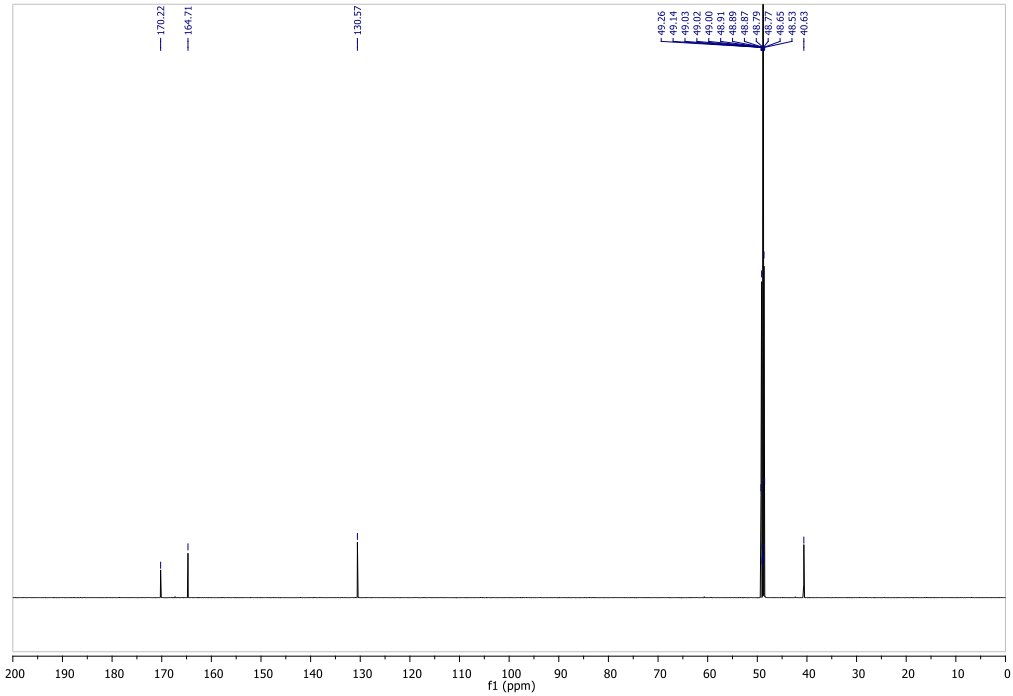
($CH_2C(O)NHCH_2$), 29.2 (CCH_2CH_2), 21.5 (CCH_2), 20.2 (OCH_2CHCH), 17.9 (OCH_2CH); IR (oil) ν_{max}/ cm^{-1} 3324 (br w), 2924 (br w), 2844 (br w), 1726 (s), 1687 (m), 1592 (w), 1539 (m); LRMS (ES+) *m/z* 1260 ($[^{81,81,81,81}2M+NH_4]^+$, 3), 1258 ($[^{81,81,81,79}2M+NH_4]^+$, 7), 1256 ($[^{81,81,79,79}2M+NH_4]^+$, 9), 1254 ($[^{81,79,79,79}2M+NH_4]^+$, 5), 1252 ($[^{79,79,79,79}2M+NH_4]^+$, 1), 644 ($[^{81,81}M+Na]^+$, 14), 642 ($[^{81,79}M+Na]^+$, 29), 640 ($[^{79,79}M+Na]^+$, 14), 622 ($[^{81,81}M+H]^+$, 77), 620 ($[^{81,79}M+H]^+$, 100), 618 ($[^{79,79}M+H]^+$, 74), 472 (18), 470 (38), 468 (18); HRMS (ES+) calcd for $[C_{23}H_{29}{}^{81,79}Br_2N_3O_7H]^+$ $[^{81,79}M+H]^+$ 620.0426, observed 620.0414.

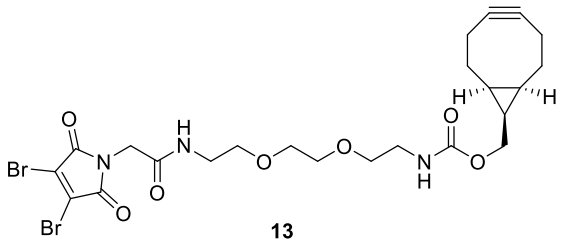
1.2.2 NMR spectra



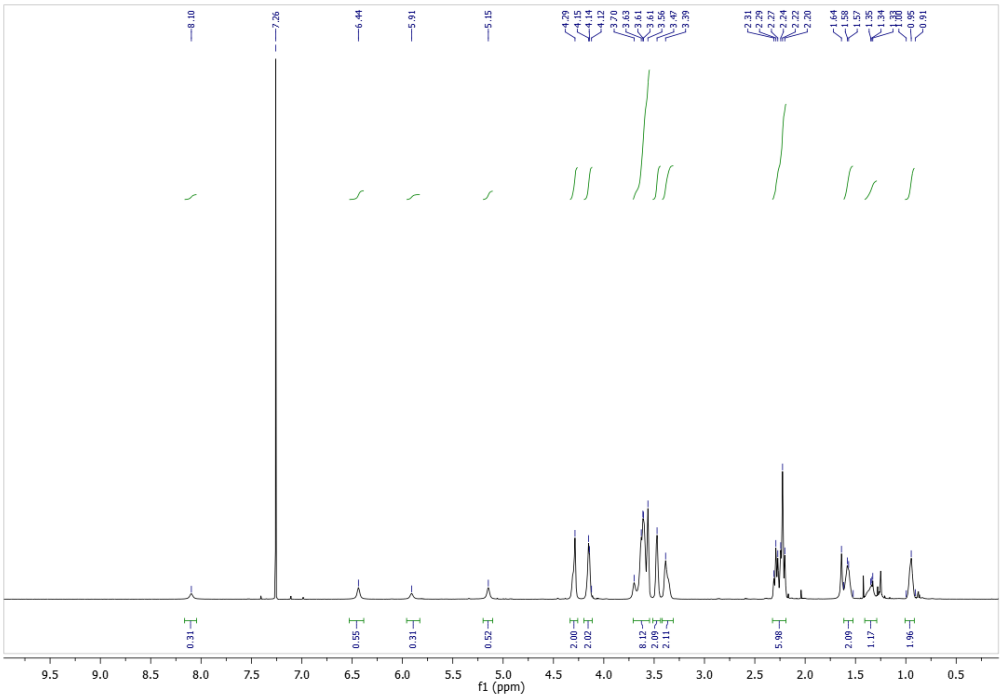
12

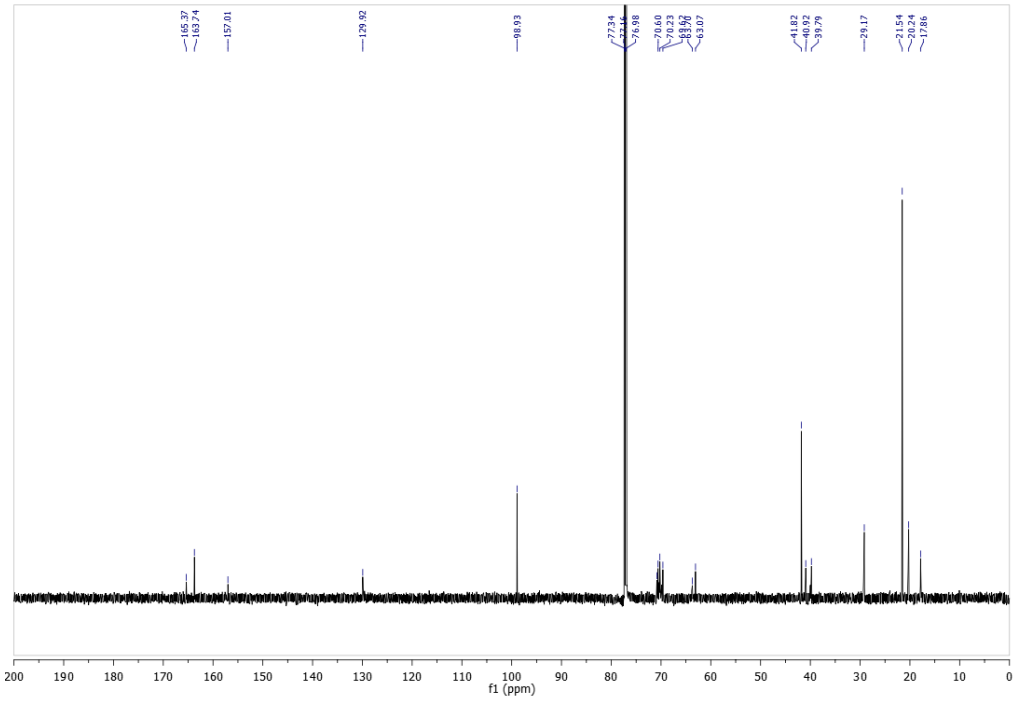



170.22
164.71
130.57
49.26
49.14
49.15
49.02
49.00
48.91
48.89
48.87
48.79
48.77
48.65
48.53
40.63
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

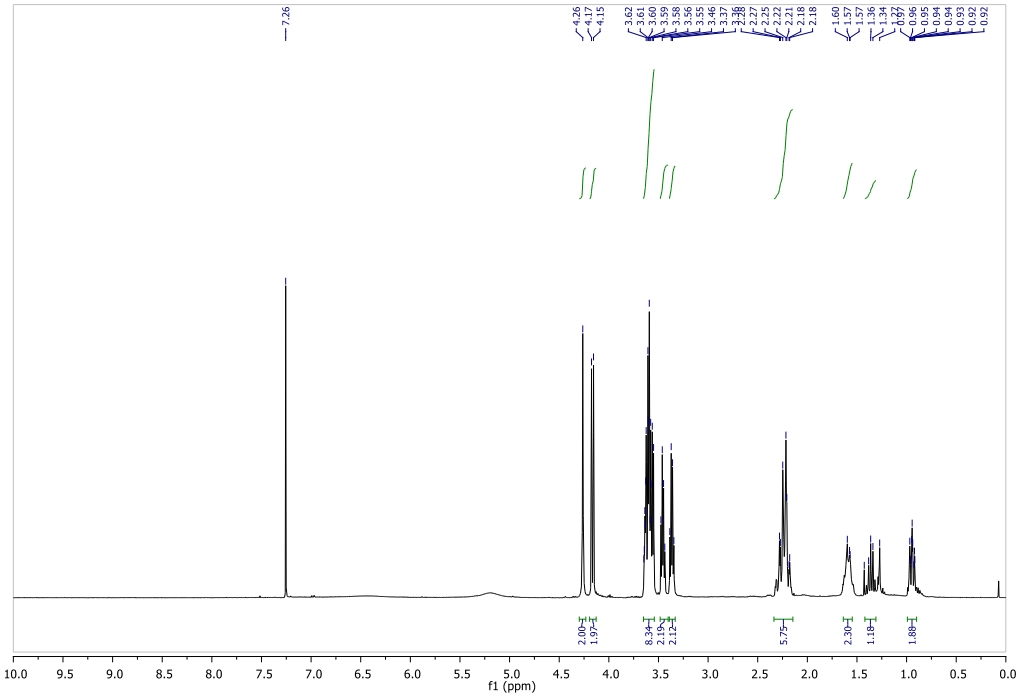

O
Br
N
O
Br
H
N
O
O
O
N
H
O
O
H
H

13


8.10
7.26
6.44
5.91
5.15
4.29
4.15
4.14
4.12
3.70
3.63
3.61
3.61
3.56
3.47
3.39
2.31
2.29
2.27
2.24
2.22
2.20
1.64
1.58
1.57
1.35
1.34
1.33
1.00
0.95
0.91
0.31
0.55
0.31
0.52
2.00
2.02
8.12
2.09
2.11
5.98
2.09
1.17
1.96
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)


165.37
163.74
157.01
129.92
98.93
77.34
77.16
76.98
70.60
70.23
69.67
69.50
63.07
41.82
40.92
39.79
29.17
21.54
20.24
17.86
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

The ^{1}H NMR spectrum of the NGM-BCN compound **13** was also recorded on a Bruker Advance AMX400 instrument operating at 400 MHz, at 60 °C to resolve extra signals obtained due to the rotameric nature of **13**; ^{1}H NMR (400 MHz, $CDCl_3$, 60 °C) δ_H 4.26 (s, 2H, NCH_2), 4.16 (d, 2H, J = 8.0, $C(O)OCH_2$), 3.65-3.55 (m, 8H, 4 × OCH_2), 3.46 (dd, 2H, J = 10.4, 5.2, $CH_2C(O)NHCH_2$), 3.37 (dd, 2H, J = 10.9, 5.5, $OC(O)NHCH_2$), 2.28-2.18 (m, 6H, 2 × CCH_2, 2 × CCH_2CHH), 1.43-1.34 (m, 2H, 2 × CCH_2CHH), 1.40-1.30 (m, 1H, OCH_2CH), 0.97-0.92 (m, 2H, 2 × OCH_2CHCH).



1.2.3 Materials and methods – Bioconjugation

Conjugation experiments were carried out in standard polypropylene Eppendorf safe-lock tubes (1.5 mL) at atmospheric pressure with mixing at the temperature stated. Reagents and solvents were purchased from commercial sources and used as supplied. All buffer solutions were prepared with doubly deionized water and filter-sterilized. All buffer solutions were degassed prior to use, where the term 'degassed' refers to the process of removing O_2 from a solution by bubbling argon through it. Borate-buffered saline (BBS) was 50 mM boric acid, 50 mM NaCl, 5 mM EDTA at pH 8.5. Ammonium acetate buffer was 0.2 M ammonium acetate at pH 6.9. Phosphate-buffered saline (PBS) was 12 mM phosphates, 140 mM NaCl at the specified pH. Ultrapure DMF was purchased from Sigma and stored under dry conditions. Ultrafiltration was carried out in Amicon® Ultra-4 Centrifugal Filter Units with a molecular weight cut-off (MWCO) of 10 kDa or in Vivaspin® 500 centrifugal concentrators (10 kDa MWCO). Centrifugation was carried out on an eppendorf 5415R fixed angle rotor centrifuge operating at 14000 rcf at 20 °C or in an eppendorf 5810 swing-bucket rotor centrifuge operating at 3220 rcf at 20 °C. Trastuzumab (Ontruzant®) was purchased from UCLH in its clinical formulation (Samsung Bioepis, lyophilised). Concentrations were determined by UV/ Vis absorbance using molecular extinction coefficient of ε_{280} = 215,380 M^{-1} cm^{-1} for trastuzumab.[3] Protein conjugation reactions were monitored by 12% glycine-SDS-PAGE with a 6% stacking gel under non-reducing conditions, unless otherwise stated. Samples were mixed

[3] Maruani, A.; Savoie, H.; Bryden, F.; Caddick, S.; Boyle, R.; Chudasama, V. Site-selective multi-porphyrin attachment enables the formation of a next-generation antibody-based photodynamic therapeutic. *Chem. Commun.* **2015**, *51*, 15304.

1:1 with SDS non-reducing loading buffer (composition for 6 x SDS: 1 g SDS, 3 mL glycerol, 6 mL 0.5 M Tris buffer pH 6.8, 2 mg bromophenol blue) or reducing loading buffer (composition for 4 x SDS: 0.8 mL β-mercaptoethanol (BME), 0.8 g SDS, 4 mL glycerol, 2.5 mL 0.5 M Tris buffer pH 6.8, 2.5 mL H_2O, 1 mg bromophenol blue) and heated at 75 °C for 5 min before applied to the gel. Samples were run at constant current (30 mA) for 40 minutes in 1 x SDS running buffer. Gels were stained with Coomassie G-250 (0.05% w/v) in 49.95% H_2O, 40% MeOH, 10% AcOH and de-stained with 10% MeOH, 10% AcOH, 80% H_2O solution. Absorbance measurements were carried out on a Carry Bio 100 UV/ Vis spectrophotometer (Varian) equipped with a temperature-controlled 12x sample holder in quartz cuvettes (Starna Scientific - 1 cm path length, volume 160 µL) at RT. Samples were baseline corrected. The UV data was analyzed using Graphpad Prism 7.03 software.

Analysis of trastuzumab conjugates by LC-MS

Trastuzumab samples (100 µL, 0.65 nmol, 6.5 µM) in ammonium acetate buffer (pH 6.9) were incubated with Rapid™ PNGase F, non-reducing format (1.0 µL, New England Biolabs) at 50 °C for 10 min. After this time, the mixture was diluted to 1.8 µM with ammonium acetate buffer (pH 6.9) and analyzed by LC-MS. Molecular masses were measured using an Agilent 6510 QTOF LC-MS system (Agilent, UK). Agilent 1200 HPLC system was equipped with an Agilent PLRP-S, 1000A, 8 µM, 150 mm x 2.1 mm column. 10 µL of trastuzumab sample (at 1.8 µM) was injected on the column using mobile phase A (water-0.1% formic acid) and B (acetonitrile-0.1% formic acid) with an eluting gradient (as shown in Table S1) at a flow rate of 300 µl/min. The oven temperature was maintained at 20 °C.

Table S1 - LC-MS mobile phase A/ B gradient elution.

Time (min)	Solvent A (%)	Solvent B (%)
0.0	85	15
1.0	85	15
1.50	68	32
2.0	68	32
7.0	50	50
9.0	5	95
10.0	5	95
12.0	85	15
15.0	85	15

Agilent 6510 QTOF mass spectrometer was operated in a positive polarity mode, coupled with an ESI ion source. The ion source parameters were set up with a VCap of 3500V, a gas temperature at 350 °C, a dry gas flow rate at 5 L/min and a nebulizer of 30 psig. MS Tof was acquired under conditions of a fragmentor at 350 V, a skimmer at 65 V and an acquisition rate at 0.5 spectra/s in a profile mode, within a scan range between 800 and 4500 *m/z*. The .data was then analysed by deconvoluting a spectrum to a zero charge mass spectra using a maximum entropy deconvolution algorithm within the MassHunter software version B.07.00.

Note: In previous work,[2,3,4] we had observed that maleamic acid conjugates undergo partial cleavage of the imide bond to release the amine and form the corresponding anhydride, under the acidic conditions and increased temperature during LC-MS

[4] Forte, N.; Livanos, M.; Miranda, E.; Morais, M.; Yang, X.; Rajkumar, V. S.; Chester, K. A.; Chudasama, V.; Baker, J. R. Tuning the Hydrolytic Stability of Next Generation Maleimide Cross-Linkers Enables Access to Albumin-Antibody Fragment Conjugates and tri-scFvs, *Bioconjugate Chem.* **2018**, *29*, 486.

analysis. In the present work, we confirmed that anhydride formation can be eliminated by analysing samples using a faster gradient to Solvent B and by maintaining the oven temperature at 20 °C (instead of 60 °C). Although this affords insufficient separation of chains in the TIC, the whole region is extracted to obtain the combined ion series.

Synthesis of trastuzumab-NGM conjugate (14)

To trastuzumab (2000 µL, 0.0458 µmol, 22.9 µM, 3.32 mg/mL) in BBS buffer (pH 8.5) was added TCEP·HCl (27.5 µL, 0.275 µmol, 10 mM solution in H_2O, 6 eq.). After 2 h at 37 °C, NGM-BCN **13** (36.6 µL, 0.366 µmol, 10 mM solution in DMF, 8 eq.) was added in the conjugation and the resultant mixture was incubated for 10 min at 20 °C. The excess reagents were then removed *via* ultrafiltration (10 kDa MWCO) into BBS buffer (pH 8.5). The concentration was determined by UV/Vis absorbance and adjusted to 20.0 µM (1950 µL, 2.90 mg/mL). The resultant solution was incubated for 18 h at 37 °C. After this time, the sample was buffer exchanged into ammonium acetate buffer (pH 6.9), *via* ultrafiltration (10 kDa MWCO).

Synthesis of trastuzumab-NGM-PROTAC conjugate (3)

To trastuzumab-NGM conjugate **14** (1200 µL, 0.036 µmol, 30.0 µM, 4.41 mg/mL) in ammonium acetate buffer (pH 6.9) was added azido-PROTAC **2** (72.0 µL, 0.720 µmol, 10 mM solution in DMF, 20 eq.) and the resultant mixture was incubated at 22 °C for 4 h. The excess reagent was then removed using a desalting column (PD Minitrap G-25, GE Healthcare), followed by ultrafiltration (10 kDa MWCO) into PBS buffer (pH 6.9).

Synthesis of trastuzumab-NGM-PROTAC-AlexaFluor488 conjugate (AF488-3)

To trastuzumab-NGM-PROTAC conjugate **3** (337 µL, 0.0135 µmol, 40 µM, 6.08 mg/ mL) in PBS buffer (pH 7.4) was added AlexaFluor488 NHS ester (13.5 µL, 0.135 µmol, 10 mM solution in DMF, 10 eq., Life Technologies) and the resultant mixture was incubated in the dark for 2 h at 22 °C. The excess fluorophore was then removed using a desalting column (PD Minitrap G-25, GE Healthcare), followed by ultrafiltration (10 kDa MWCO) into PBS buffer (pH 6.9).

Stability of trastuzumab-NGM-PROTAC conjugate 3 in PBS buffer (pH 7.4) at 37 °C

Trastuzumab-NGM-PROTAC conjugate **3** (185 µL, 0.0037 µmol, 20 µM, 3.04 mg/mL) was incubated in PBS buffer (pH 7.4) at 37 °C. The conjugate was analysed at different timepoints (t = 0, 1, 4, 6, 24 h) by LC-MS to determine the hydrolytic stability of the PROTAC moiety. The PROTAC hydrolysis (%) was then estimated from the height of the peaks obtained for the half antibody species, as determined by MassHunter software (Figure S3).

Table S2. % PROTAC hydrolysis at different timepoints, upon incubation of trastuzumab-NGM-PROTAC conjugate **3** in PBS buffer (pH 7.4) at 37 °C.

Time of incubation (h)	% PROTAC hydrolysis
0	0
1	0
4	1.2
6	2.5
24	16.5

Protocol for HER2 ELISA

A 96-well plate was coated for 1 h at RT with HER2 (Sino Biological, 100 µL/well, 0.25 µg/mL solution in PBS). After washing (3 × 0.1% Tween® 20 in PBS, followed by 3 × PBS), the wells were blocked for 1 h at RT with 5% Marvel milk powder (Premier foods) in PBS (200 µL/well). The wells were then washed and the following dilutions of native trastuzumab and conjugates Ab-PROTAC **3** and AF488-**3** were applied: 270 nM, 90 nM, 30 nM, 10 nM, 3.33 nM, 1.11 nM, 0.37 nM, 0.123 nM, 0.0412 nM, 0.0137 nM, prepared in 1% Marvel solution in 0.1% Tween® 20 in PBS (100 µL/well). The assay was then incubated at RT for 1 h, washed and the detection antibody (Anti-Human IgG, Fab specific-HRP antibody, Sigma Aldrich, 1:5000 in 1% Marvel solution in 0.1% Tween® 20 in PBS) was added (100 µL/well). After 1 h at RT, the plates were washed and *o*-phenylenediamine dihydrochloride (Sigma-Aldrich, 100 µL/well, 0.5 mg/mL in a phosphate-citrate buffer with sodium perborate) was added. Once a yellow-orange colour was observed, the reaction was stopped by addition of HCl (4M, 50 µL/well). Absorbance was immediately measured at 490 nm and was corrected by subtracting the average of negative controls (i.e. PBS had been added to some of the wells instead of HER2 or instead of the samples). Each sample was tested in triplicate and errors are shown as the standard deviation of the average. ELISA data was analyzed with Graphpad Prism 7.03 and the values have been normalized.

2. Biological Assays

2.1. General Remarks

All reagents were purchased from commercial sources and used as received without any further purification. BRD4 degrader **4** was used as positive control in the BRD4 degradation experiments and it was provided by GlaxoSmithKline Medicines Research Centre, Stevenage. The antibodies employed are summarized in Table S3.

Table S3. List of primary and secondary antibodies

Target	Species	Dilution	Catalogue no.	Supplier
BRD4	Rabbit	1:1000	13440	Cell Signaling Technology
β-actin	Rabbit	1:5000	ab8227	Abcam
HER2	Rabbit	1:1000	2165S	Cell Signaling Technology
Anti-Rabbit IgG H&L HRP	Goat	1:10000	R-05072-500	Advansta
Anti-Rabbit IgG H&L AlexaFluor®488	Goat	1:500	ab150077	Abcam

RIPA Lysis Buffer composition (20 ml final volume): NaCl (2 ml, 750 mM, final concentration 75 mM), sodium deoxycholate (1 ml, 5%, final concentration 0.25%), Triton X-100 (100 µl, final concentration 0.5%), SDS 10% (100 µl, final concentration 0.05%), 16.8 ml of H_2O Milli-Q. For cell lysis, to each 5 mL of buffer were added EDTA-free protease inhibitors solution (100 µL) and Benzonase® Nuclease (1 µL, Sigma-Aldrich, E1014).

Cell culture: All cell lines employed in this study were obtained from the Francis Crick Institute cell banking. MCF-7 and MDA-MB-231 cells were cultured in low glucose DMEM (Sigma, D6046) supplemented with 10% of FBS. SK-BR-3 cells were cultured in McCoy's 5A medium (Sigma, M8403) supplemented with 10% of FBS. BT-474 cells were cultured in high glucose DMEM (Gibco, 41966-029) supplemented with 10% of FBS. All cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

Western blot analysis: After cell treatment, the media was aspirated, and the cells were washed with PBS (2×0.5–1 mL). The cells were lysed by addition of cold RIPA-buffer (100–150 µL) containing protease inhibitor cocktail and benzonase nuclease and were scraped from the wells. After collection of the lysates on ice, these were submitted to sonication (2 min) followed by centrifugation (13000 rpm, 5 min, 4 °C) allowing the recovery of supernatant. The protein concentration was measured with a BioRad DC Protein Assay and all the concentrations were normalized to the lowest. 10-20 µg of protein extracts were fractionated by SDS-PAGE and transferred to PVDF membranes which were then blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween®-20 (TBS-T) for 1–2 h. Subsequently, the membranes were incubated overnight at 4 °C with solution of the corresponding primary antibodies (Table S3) in blocking buffer (5% skim milk in TBS-T). After washes with TBS-T (3×5 min), the membranes were incubated for 1 h at room temperature with the secondary antibody (Table S3). The bands on the membrane were detected by western fluorescent detection reagent (Merck™ Luminata™ Western HRP Chemiluminescence) and images were recorded within the ImageQuant LAS 4000 series.

2.2. Experimental procedures

2.2.1. Analysis of protein degradation/expression by western blot

Detection of HER2 and BRD4 expression: MCF-7, MDA-MB-231, SK-BR-3 and BT-474 cells were plated in 6 cm dishes at a cell density of 1.05×10^6 cell/well 24 h before harvesting in 4 mL of growth medium. After cell lysis, the lysates were processed according to the general protocols previously described and were submitted to western blot analysis.

Testing of PROTAC 1: MCF-7, MDA-MB-231, SK-BR-3 and BT-474 cells were plated in 6-well plates at a cell density of 4.8×10^5 cell/well in 2 mL of growth medium 24 h before treatment. Cells were treated separately with DMSO (2 μL), compound **4** (2 μL, 1 mM stock, final concentration 1 μM) or increasing concentrations of PROTAC **1** (2 μL of 5, 10, 50 and 100 μM stocks, final concentration of 5, 10, 50 and 100 nM). After treatment, cells were incubated for 4 h. For the washout experiment cells were incubated for 1 h after treatment and then the medium was aspirated, the cells washed with PBS, new medium was added, and the cells were incubated for further 23 h. Cells were harvested, and the lysates were processed according to the general protocols previously described. Cell lysates were submitted to western blot analysis.

Testing of Ab-PROTAC 3: MCF-7, MDA-MB-231, SK-BR-3 and BT-474 cells were plated in 12-well plates at a cell density of 1.9×10^5 cell/well in 0.99–1 mL of growth medium 24 h before treatment. Cells were treated separately with PBS (10 μL), Trastuzumab (10 μL, 10 μM stock, final concentration 100 nM), compound **4** (1 μL, 1 mM stock, final concentration 1 μM) or increasing concentrations of Ab-PROTAC **3** (10 μL of 0.5, 1, 5 and 10 μM stocks, final concentration of 5, 10, 50 and 100 nM). After treatment, cells were incubated for 4 h. For the washout experiment cells were incubated for 1 h after treatment and then the medium was aspirated, the cells washed with PBS, new medium was added, and the cells were incubated for further 23 h. Cells were harvested, and the lysates were processed according to the general protocols previously described. Cell lysates were submitted to western blot analysis.

Proteasome-dependency experiment: SK-BR-3 and BT-474 cells were plated in 12-well plates at a cell density of 1.9×10^5 cell/well in 0.99–1 mL of growth medium 24 h before treatment. Cells were treated separately with PBS (10 μL) or bortezomib (1 μL, 10 mM stock, final concentration 10 μM) and incubated for 2 hours. Cells were treated with PBS (10 μL), compound **4** (1 μL, 1 mM stock, final concentration 1 μM) or Ab-PROTAC **3** (10 μL of 10 μM stock, final concentration of 100 nM). After 1 h incubation, the medium on the wells treated with Ab-PROTAC **3** was aspirated, the cells washed with PBS and new medium was added. The cells previously treated with proteasome inhibitor were resupplemented with bortezomib (1 μL, 10 mM stock) and the cells were incubated for 23 h more. Cells were harvested and the lysates were processed according to the general protocol previously described. Cell lysates were submitted to western blot analysis.

2.2.2. Immunofluorescent Staining and Imaging

SKBR3 and MCF-7 cells grown on coverslips pre-coated with poly-D-lysine (Sigma Aldrich, A-003-E) were washed with PBS (2×1 mL/coverslip), fixed with cold 4% paraformaldehyde in PBS (1 mL/coverslip) for 15 min, washed with PBS (3×1 mL/coverslip), incubated with NH_4Cl (50 mM, 2 mL/coverslip) for 15 min and then blocked in PBS/10% BSA/0.3% Triton X-100 buffer for 2 h at RT. Coverslips were then incubated with a primary HER2 antibody (1:200 in blocking buffer) for 1 h at RT, washed by three rounds of dipping into blocking buffer (2×) and PBS (2×). Coverslips were then incubated with a

secondary AF488-conjugated goat antibody (1:500 in blocking buffer) for 1h at RT, washed by three rounds of dipping into blocking buffer (2×), PBS (2×), ultrapure water (2×), mounted on microscopy slides using Mowiol® (Sigma Aldrich, 81381, supplemented with 1 mg/mL DAPI (Invitrogen, D1306)) and dried in the dark overnight. Coverslips were then imaged using a TCS-SP5 confocal microscope (Leica) with a ×63 1.4 numerical aperture (NA) objective. Leica LAS AF image acquisition software was utilized. All subsequent Lif image files were analyzed in ImageJ (NIH).

2.2.3. Live cell confocal microscopy

Ab-PROTAC **3** trafficking in live cells was monitored by incubating SK-BR-3 and MCF-7 cells with AlexaFluor488-conjugated Ab-PROTAC **3** (AF488-**3**) in complete McCoy's 5A and low glucose DMEM, respectively. 24h before the experiment, cells were plated onto 35 mm low μ-Dishes (Ibidi, 80136) and incubated overnight. Cells were then treated with AF488-**3** (100 nM) and incubated at 37 °C for various time points (1 h, 4 h, 8 h, 24 h) w/ or w/o washout with pre-warmed complete media (2×0.5-1 mL) after 1h of incubation (see Figures 3 and S8). 30 min before imaging, cells were incubated with LysoTracker™ DeepRed (Invitrogen, L12492) (50 nM, 30 min at 37 °C). Media was then changed to pre-warmed OPTI-MEM® Reduced serum media (Gibco, 31985062) and cells were imaged using a TCS-SP5 confocal microscope (Leica) with a ×63 1.4 numerical aperture (NA) objective. Cells were maintained at 37 °C and 5% CO_2 using an environmentally controlled incubation chamber. Leica LAS AF image acquisition software was utilized. All subsequent Lif image files were analyzed in ImageJ (NIH). For measurement of colocalization, a line was drawn along the boundary of the cell to fully surround the cell. The Pearson's correlation coefficients between two channels were determined with ImageJ colocalization plugins. ImageJ Costes map was used to visualize overlayed pixels from two channels (Figure S8). All quantification analysis was performed using Prism (GraphPad Software). All error bars represent SEM. One-way ANOVA followed by a Tukey's multiple comparison test was used to evaluate the statistical significance of multiple groups of samples. **, $P < 0.001$; ***, $P < 0.0001$. $P > 0.05$ was considered ns.

3. Uncropped Blots

Figure 2A

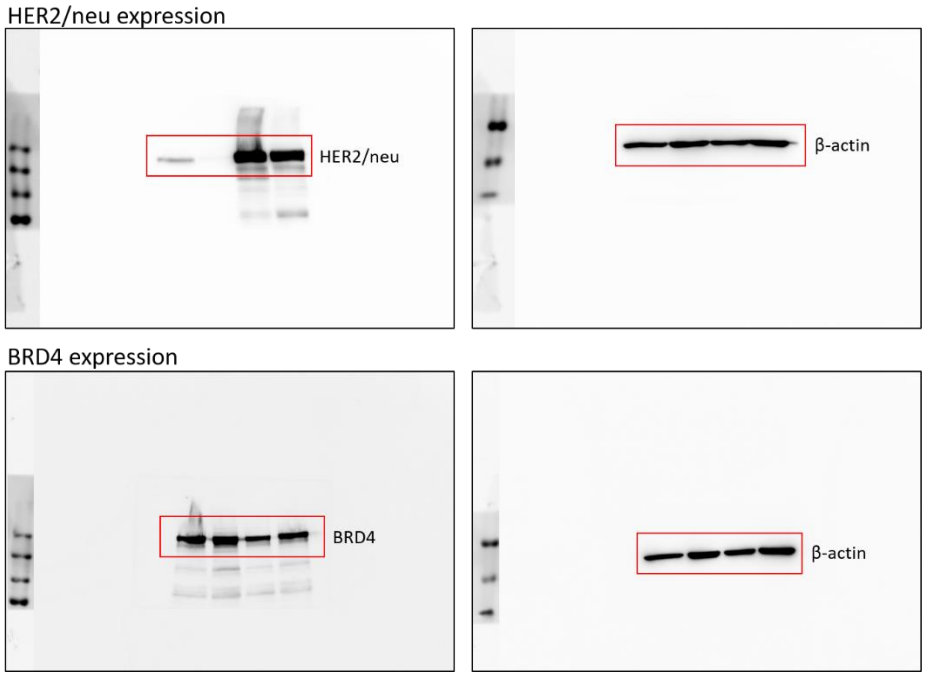


Figure 2C (4 hours)

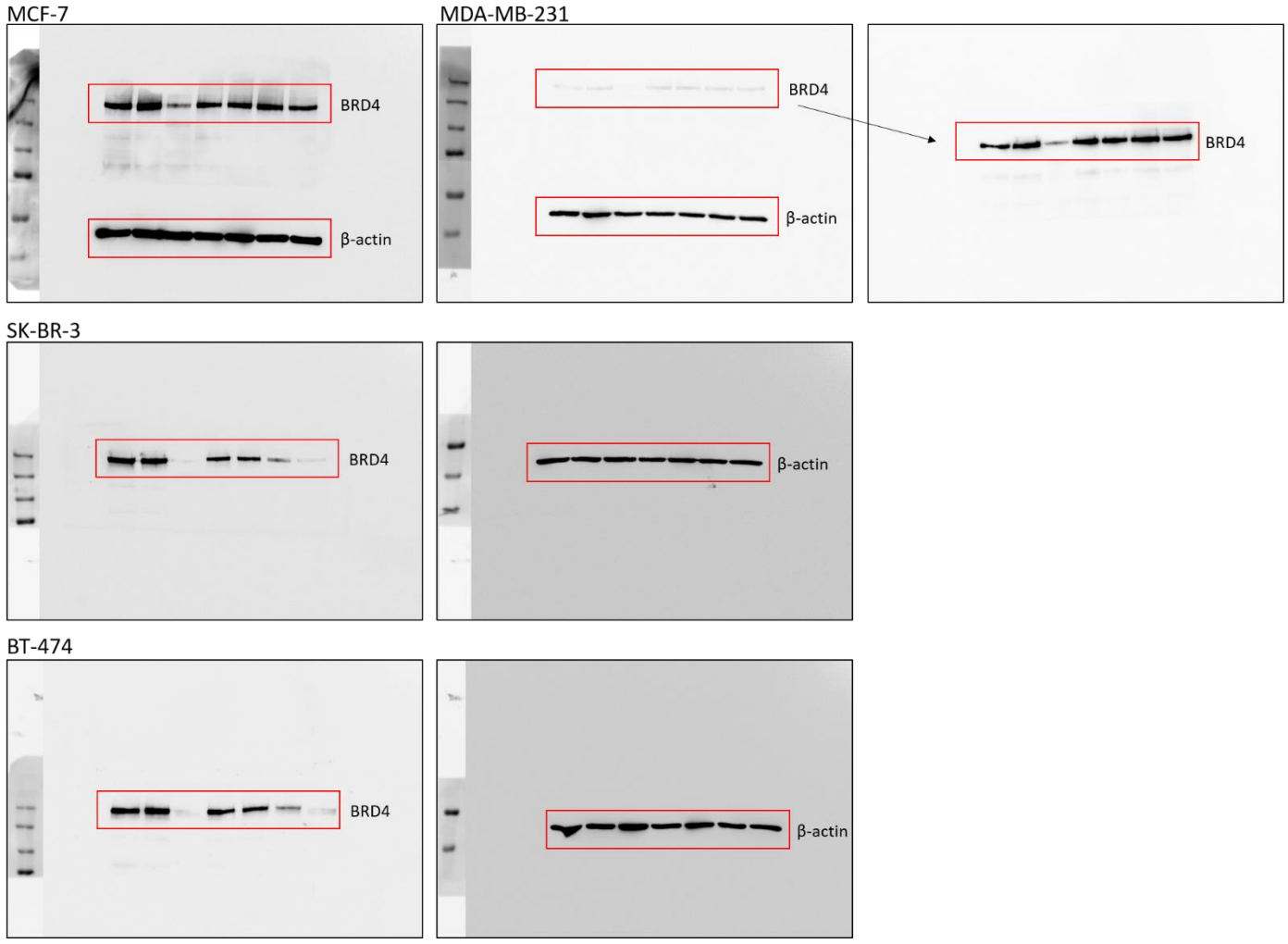


Figure 2C ("washout")

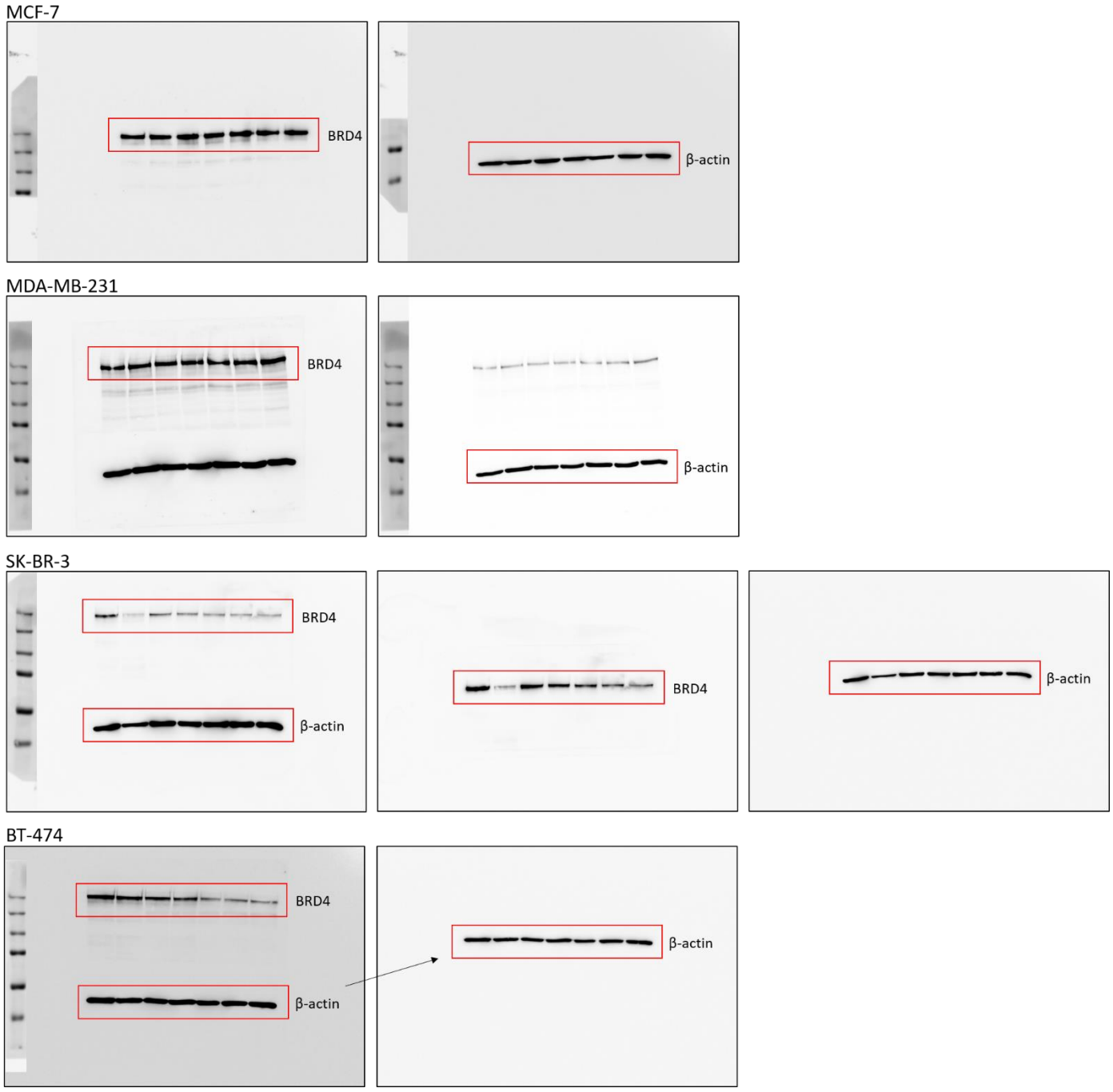


Figure 2E

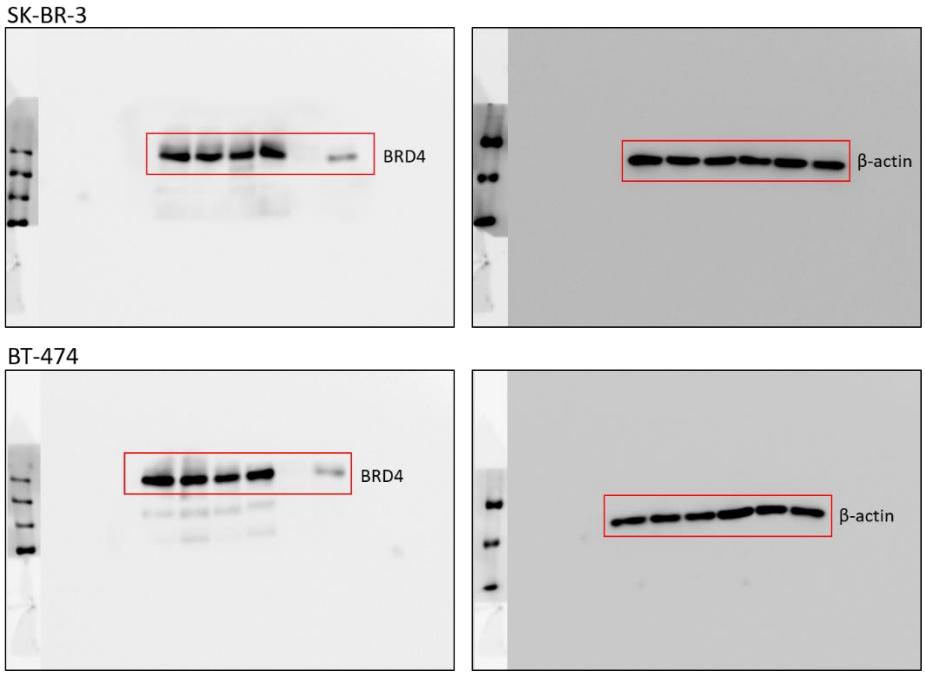


Figure S7 (4 hours)

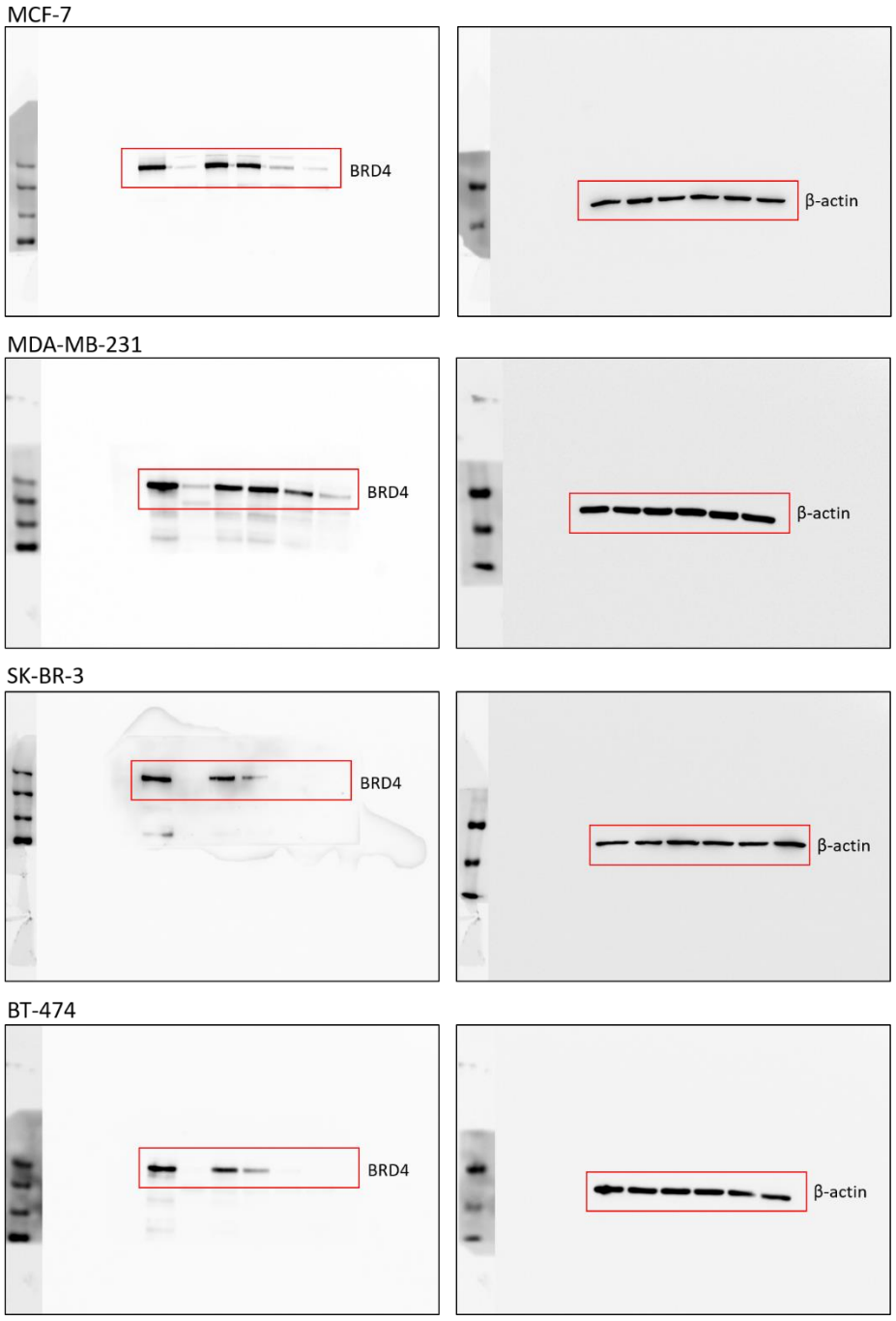


Figure S7 ("washout")