Site-selective multi-porphyrin attachment enables the formation of a next-generation antibody-based photodynamic therapeutic

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General Experimental

All reagents were purchased from Sigma-Aldrich, Promega, AlfaAesar, Invitrogen and were used as received. Where described below petrol refers to petroleum ether (40–60 °C). All reactions were monitored by thin-layer chromatography (TLC) on pre-coated SIL G/UV254 silica gel plates (254 μm) purchased from VWR. Flash column chromatography was carried out with Kieselgel 60M 0.04/0.063mm (200–400 mesh) silica gel. 1H and 13C NMR spectra were recorded at ambient temperature on a Jeol JNMLA400 spectrometer instrument operating at a frequency of 400 MHz for 1H and 100 MHz for 13C or on a Bruker Avance 600 instrument operating at a frequency of 600 MHz for 1H and 150 MHz for 13C in DMSO-d6, CDCl3 or CD3OD (as indicated below). The chemical shifts (δ) for 1H and 13C are quoted relative to residual signals of the solvent on the ppm scale. 1H NMR peaks are reported as singlet (s), doublet (d), triplet (t), m (multiplet), br. (broad). Coupling constants (J values) are reported in Hertz (Hz) and are H-H coupling constants unless otherwise stated. Signal multiplicities in 13C NMR were determined using the distortionless enhancement by phase transfer (DEPT) spectral editing technique. Infrared spectra were obtained on a Perkin Elmer Spectrum 100 FTIR Spectrometer operating in ATR mode with frequencies given in reciprocal centimetres (cm⁻¹). Melting points were measured with a Gallenkamp apparatus and are uncorrected. Mass spectra of organic compounds were obtained on a VG70-SE mass spectrometer.

UV-Vis spectroscopy

UV-Vis spectra were recorded on a Varian Cary 100 Bio UV/Visible spectrophotometer, operating at room temperature. Sample buffer was used as blank for baseline correction. Calculation of molecule over antibody ratio, r, follows the formula below with \( \varepsilon_{280} = 215380 \text{ M}^{-1} \text{ cm}^{-1} \) for trastuzumab, \( \varepsilon_{280} = 68590 \text{ M}^{-1} \text{ cm}^{-1} \) for trastuzumab Fab, \( \varepsilon_{345} = 9100 \text{ M}^{-1} \text{ cm}^{-1} \) for Mestra-PD, \( \varepsilon_{422} = 165175 \text{ M}^{-1} \text{ cm}^{-1} \) for the porphyrin, 0.28, as a correction factor (CF) for Mestra-PD for the absorbance at 280 nm.

\[
    r = \frac{A_\lambda/\varepsilon_\lambda}{(A_{280} - \sum CF_\lambda \times A_\lambda)/\varepsilon_{280}}
\]
With $A_\lambda$ the absorbance at the wavelength $\lambda$, and $\varepsilon_\lambda$ extinction coefficient of the relevant molecule.

**Synthesis of compounds**

**Tri-tert-butyl 2-methylhydrazine-1,1,2-tricarboxylate 2**

To a solution of methylhydrazine 1 (1.00 g, 1.14 mL 21.7 mmol), NEt$_3$ (4.34 g, 6.04 mL, 43.4 mmol) and DMAP (260 mg, 2.17 mmol) in CH$_2$Cl$_2$ (75 mL) was added Boc$_2$O (18.9 g, 86.8 mmol) and the reaction mixture stirred at 20 °C for 72 h. Then the reaction mixture was diluted with H$_2$O (80 mL), extracted with EtOAc (3 × 60 mL), the combined organic layers were dried (MgSO$_4$) and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (20% EtOAc/petrol) to afford tri-tert-butyl 2-methylhydrazine-1,1,2-tricarboxylate 2 (7.43 g, 21.5 mmol, 99%) as a yellowish oil: $^1$H NMR (600 MHz, CDCl$_3$) (major rotamer) $\delta$ 3.05 (s, 3H), 1.51–1.43 (m, 27H); $^{13}$C NMR (150 MHz, CDCl$_3$) (major rotamer) $\delta$ 154.0 (C), 150.1 (C) 83.4 (C), 81.4 (C), 35.7 (CH$_3$), 28.3 (CH$_3$), 28.1 (CH$_3$); LRMS (ES$^+$) 369 (100, [M+Na$^+$]).
Di-tert-butyl 1-methylhydrazine-1,2-dicarboxylate 3

To a solution of tri-tert-butyl 2-methylhydrazine-1,1,2-tricarboxylate 2 (2.0 g, 5.8 mmol), in dry MeCN (15 mL) was added Mg(ClO₄)₂ (0.27 g, 1.2 mmol) and the reaction mixture stirred at 20 °C for 1 h. Then the reaction mixture was diluted with 10% aq. citric acid (20 mL) and Et₂O (15 mL), extracted with Et₂O (3 × 20 mL), the combined organic layers were dried (MgSO₄) and concentrated in vacuo. The crude residue was purified by flash column chromatography (15% EtOAc/petrol) to afford di-tert-butyl 1-methylhydrazine-1,2-dicarboxylate 3 (1.3 g, 5.2 mmol, 89%) as a white solid: m.p. 54–55 °C (lit. m.p. 55–56 °C); ¹H NMR (600 MHz, CDCl₃) (major rotamer) δ 6.40 (br. s, 1H), 3.11 (s, 3H), 1.48–1.45 (m, 18H); ¹³C NMR (150 MHz, CDCl₃) (major rotamer) δ 155.9 (C), 155.3 (C) 81.3 (C), 81.1 (C), 37.6 (CH₂), 28.3 (CH₂), 28.1 (CH₃); IR (solid) 3316, 2978, 2932, 1701 cm⁻¹; LRMS (ES⁺) 269 (100, [M+Na]⁺); HRMS (ES⁺) calcd. for C₁₁H₂₂N₂O₄Na [M+Na]⁺ 269.1477, observed 269.1476.
Di-tert-butyl 1-(2-(tert-butoxy)-2-oxoethyl)-2-methylhydrazine-1,2-dicarboxylate 4

To a solution of di-tert-butyl 1-methylhydrazine-1,2-dicarboxylate 3 (0.94 g, 3.8 mmol) in DMF (20 mL) was added caesium carbonate (1.86 g, 5.7 mmol) and tert-butyl bromoacetate (1.1 g, 0.84 mL, 5.7 mmol) and the reaction mixture stirred at 20 °C for 16 h. After this time, the reaction mixture was diluted with H₂O (50 mL), extracted with Et₂O (4 × 50 mL), the combined organic layers washed with sat. aq. LiCl (2 × 30 mL), dried (MgSO₄), and concentrated in vacuo. Purification by flash column chromatography (10% Et₂O/petrol) yielded di-tert-butyl 1-(2-(tert-butoxy)-2-oxoethyl)-2-methylhydrazine-1,2-dicarboxylate 4 (1.3 g, 3.7 mmol, 98%) as a colourless oil: ¹H NMR (600 MHz, CDCl₃) δ 4.73–4.04 (m, 2H), 3.68–3.10 (m, 3H), 1.54–1.39 (m, 27H); ¹³C NMR (150 MHz, CDCl₃) (major rotamer) δ 169.2 (C), 155.2 (C), 81.9 (C), 81.6 (C), 81.1 (C), 52.7 (CH₂), 36.8 (CH₃), 28.4 (CH₃), 28.3 (CH₃), 28.2 (CH₃); IR (thin film) 2978, 1748 cm⁻¹; LRMS (ES⁺) 361 (100, [M+H]+); HRMS (ES⁺) calcd for C₁₇H₃₃O₆N₂ [M+H]+ 361.2339, observed 361.2333.
2-(4,5-Dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)acetic acid 5

To a solution of di-tert-butyl 1-(2-(tert-butoxy)-2-oxoethyl)-2-methylhydrazine-1,2-dicarboxylate 4 (1.0 g, 2.8 mmol) in CH$_2$Cl$_2$ (10 mL) was added TFA (10 mL) and the reaction mixture stirred at 20 °C for 2 h. After this time, all volatile materials were removed in vacuo. The crude residue was added to a solution of 2,3-dibromomaleic anhydride (0.75 g, 2.8 mmol) in glacial AcOH (40 mL), and the reaction mixture stirred at 20 °C for 16 h heated at 130 °C for 16 h. Then the reaction mixture was concentrated in vacuo, and purification by flash column chromatography (3% MeOH/CH$_2$Cl$_2$ with 1% AcOH) yielded 2-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)acetic acid 5 (0.65 g, 1.9 mmol, 73%) as a white solid: m.p. 210–214 °C; $^1$H NMR (600 MHz, MeOD) $\delta$ 4.96 (s, 2H), 3.62 (s, 3H); $^{13}$C NMR (150 MHz, MeOD) $\delta$ 170.2 (C), 154.8 (C), 154.0 (C), 137.4 (C), 135.7 (C), 49.5 (CH$_2$), 35.0 (CH$_3$); IR (solid) 3023, 2969, 1731, 1662 cm$^{-1}$; LRMS (ES$^-$) 338 (50, [M$^{81}$Br$^{81}$Br$^{-}$H$^{-}$]), 340 (100, [M$^{81}$Br$^{79}$Br$^{-}$H$^{-}$]), 342 (50, [M$^{79}$Br$^{79}$Br$^{-}$H$^{-}$]); HRMS (ES$^-$) calcd for C$_7$H$_5$N$_2$O$_4$ $^{79}$Br$_2$ [M$^{79}$Br$^{79}$Br$^{-}$H$^{-}$] 337.8538, observed 337.8540.
((1R,8S,9s)-Bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-(2-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)acetamido)ethoxy)ethoxy)ethylcarbamate 6

![Chemical Structure](image)

To a solution of 2-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)acetic acid 5 (86 mg, 0.25 mmol), PyBOP (0.14 g, 0.28 mmol), and DIPEA (36 mg, 0.28 mmol) in CH₂Cl₂ (5 mL). The resulting solution was stirred at 21 °C for 16 h. Then the reaction mixture was diluted with H₂O (15 mL), extracted with EtOAc (3 x 15 mL), the combined organic layers were dried (MgSO₄) and concentrated in vacuo. The crude residue was purified by flash column chromatography (neat EtOAc) to afford ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-(2-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin-1(2H)-yl)acetamido)ethoxy)ethoxy)ethylcarbamate 6 (87 mg, 0.14 mmol, 54%) as a yellowish oil: ¹H NMR (600 MHz, CDCl₃) δ 8.34 (br. s, 0.5H), 7.00 (br. s, 0.5H), 5.96 (br. s, 0.5H), 5.29 (br. s, 0.5H), 4.85–4.73 (m, 2H), 4.12 (d, J = 8.2 Hz, 2H), 3.76–3.50 (m, 11H), 3.50–3.43 (m, 2H), 3.40–3.30 (m, 2H), 2.31–2.17 (m, 6H), 1.61–1.51 (m, 2H), 1.41–1.24 (m, 1H), 1.01–0.85 (m, 2H); ¹³C NMR (150 MHz, CDCl₃) (major rotamer) δ 165.6 (C), 157.8 (C), 153.4 (C), 152.5 (C), 137.0 (C), 134.8 (C), 98.9 (C), 77.4 (CH₂), 77.2 (CH₂), 77.0 (CH₂), 70.8 (CH₂), 70.6 (CH₂), 70.5 (CH₂), 70.3 (CH₂), 70.2 (CH₂), 70.0 (CH₂), 69.6 (CH₂), 69.4 (CH₂), 63.0 (CH₂), 50.9 (CH₂), 50.3 (CH₂), 40.9 (CH₂), 39.7 (CH₂), 35.0 (CH₃), 29.1 (CH), 21.5 (CH₂), 20.2 (CH), 17.8 (CH₂); IR (thin film) 3338, 2925, 1685, 1633 cm⁻¹; LRMS (ES⁺) 651 (50, [M⁺Br⁺Br⁺H⁺]⁺), 649 (100, [M⁺Br₂⁺Br⁺H⁺]⁺), 647 (50, [M⁺Br⁺Br⁺H⁺]⁺); HRMS (ES⁺) calcd for C₂₄H₂₄N₅O₇⁺Br₂ [M⁺Br₂⁺Br⁺H⁺]⁺ 647.0716, observed 647.0713.
5-[4-[2-((2-Azidoethoxy)ethoxy)ethanaminocarbonyl]phenyl]-10,15,20-tris(N-methyl-4-pyridinium)porphyrin trichloride 9

To a stirred solution of 5-[4-[2-(2-azidoethoxy)ethoxy]ethanaminocarbonyl]phenyl]-10,15,20-tris(4-pyridyl)porphyrin$^2$ (87 mg, 0.11 mmol) in DMF (10ml) was added methyl iodide (2.0 mL, 32 mmol) via syringe. The reaction mixture was stirred at 40 °C overnight. The mixture was cooled to room temperature and cold diethyl ether (100 mL) was added. The mixture was filtered through cotton wool, and the residue redissolved in methanol. The mixture was stirred at room temperature for 3 h, and ammonium hexafluorophosphate added. The resulting solution was filtered and the precipitate redissolved in acetone. Tetrabutylammonium chloride was added, and the resulting solution filtered. The product was precipitated from diethyl ether over MeOH to yield the product as a purple solid (107 mg, 0.37 mmol, 82 %): Rf: 0.49 (silica, 1:1:8 sat. KNO$_3$ solution:water:MeCN); UV-vis (H$_2$O) $\lambda_{\text{max}}$ (nm) 422, 519, 558, 584, 643; $\varepsilon_{422} = 165,175$ M$^{-1}$ cm$^{-1}$; $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ –2.98 (s, 2H, -NH), 3.45 (m, 2H, CH$_2$-N$_3$), 3.56–3.80 (m, 10H), 4.76 (s, 9H, N-C$_H$$_3$), 8.30–8.43 (m, 4H, 5-m-Ph, 5-o-Ph), 8.96–9.25 (m, 14H, βH and 10,15,20-o-Py), 9.44–9.61 (m, 6H, 10,15,20-m-Py); $^{13}$C NMR (100 MHz, DMSO-d$_6$) $\delta$ 48.3 (CH$_2$-NH), 50.6 (N-C$_H$$_3$), 69.6 (O-C$_H$$_2$), 69.9 (O-C$_H$$_2$), 70.3 (O-C$_H$$_2$), 115.5, 116.2, 122.9, 126.2, 132.3, 132.7 (β-C), 133.0, 133.7, 134.6, 144.3 (β-C), 145.3, 148.5, 148.8, 149.0, 150.5, 158.9, 168.8 (C=O); LRMS (MALDI-TOF) m/z 287 (100[M–3Cl]$^{3+}$); HRMS(MALDI-TOF) calcd. for C$_{51}$H$_{46}$N$_{11}$O$_3$: 287.4642, observed 287.4643.
Control conjugation reactions involving trastuzumab Fab

To a solution of trastuzumab Fab (100 μL, 23 μM, 1.0 mg/mL, 1 eq) in borate buffer (25 mM sodium borate, 25 mM NaCl, 0.5 mM EDTA, pH 8.0) was added TCEP (final concentration 69 μM, 3 eq) and Mestra-PD 6 in DMSO (final concentration 115 μM, 5 eq) and the reaction mixture incubated at 4 °C for 16 h. The excess reagents were then removed by repeated diafiltration into fresh buffer using VivaSpin sample concentrators (GE Healthcare, 10000 MWCO). Following this, analysis by LCMS revealed conversion to the desired Fab-Mestra conjugate as the sole product (expected mass: 48089 Da, observed mass: 48090 Da). Then, porphyrin-N₉ ₙ ⁹ (5 eq from a 20 mM solution in DMF) was added and the reaction mixture incubated at 37 °C for 4 h. The excess reagents were then removed by repeated diafiltration into fresh PBS using VivaSpin sample concentrators (GE Healthcare, 10000 MWCO). Following this, analysis by LCMS and UV-Vis revealed conversion to the desired Fab-Mestra-Porphyrin conjugate with a porphyrin-to-antibody ratio of 1 (expected mass: 49022 Da, observed mass: 49021 Da).

(a)

(b)

(c)
Figure S1. (a) non-deconvoluted and (b) deconvoluted MS data for Fab fragment of trastuzumab reacted with Mestra-PD 6; (c) non-deconvoluted and (d) deconvoluted MS data for Fab-Mestra reacted with porphyrin 9; (e) UV-Vis trace for Fab-Mestra reacted with porphyrin 9.
Conjugation reactions involving trastuzumab mAb

Formation of conjugates 8 and 10

To a solution of trastuzumab (100 μL, 50 μM, 7.3 mg/mL, 1 eq) in borate buffer (25 mM sodium borate, 25 mM NaCl, 0.5 mM EDTA, pH 8.0) was added TCEP (final concentration 500 μM, 10 eq) and Mestra-PD 6 in DMSO (final concentration 1.0 mM, 25 eq) and the reaction mixture incubated at 4 °C for 16 h. The excess reagents were then removed by repeated diafiltration into fresh buffer using VivaSpin sample concentrators (GE Healthcare, 10000 MWCO). Following this, analysis by SDS-PAGE gel and UV-Vis revealed conversion to the desired Her-Mestra conjugate 8 with a PD-to-antibody ratio of ca. 4. Then, porphyrin-N₃ 9 (20 eq from a 20 mM solution in DMF) was added and the reaction mixture incubated at 37 °C for 4 h. The excess reagents were then removed by repeated diafiltration into fresh PBS using VivaSpin sample concentrators (GE Healthcare, 10000 MWCO). Following this, analysis by SDS-PAGE gel and UV-Vis revealed conversion to the desired Her-Mestra-Porphyrin conjugate 10 with a porphyrin-to-antibody ratio of ca. 4.1.

(a)
Figure S2. UV-Vis trace for (a) Conjugate 8, (b) Conjugate 10.

Figure S3. SDS-PAGE of conjugate 8 (lane 1) and conjugate 10 (lane 2).
Cytotoxicity Methodology

Each conjugate was diluted in the appropriate medium, but without FCS, to give a range of five concentrations varying between $2.5 \times 10^{-7}$ M and $3.0 \times 10^{-6}$ M. 400 μL of each cell line adjusted to a concentration of $10^6$ cells/mL was added to the dilutions and incubated for 1 h at 37 °C and 5% CO₂. After incubation, the cells were washed with a 4× excess of serum-free medium to eliminate any unbound conjugate. The pellets were re-suspended in 0.5 mL of the appropriate serum free medium and 100 μL aliquoted into 5 wells of a 96-well plate. Each plate was irradiated (IRR) with 20 J/cm² of light (400–700 nm) in two equal doses separated by 10 min delivered using an Oriel, 1000 W quartz tungsten halogen lamp fitted with water filter and diffuser. After irradiation, 5 μL of fetal bovine serum was added to each well and the plates were returned to the incubator overnight. After 24 h, an MTT (Thiazolyl blue; Sigma M5655) cell viability assay was performed and the results expressed as % of cell viability versus porphyrin–conjugate concentration.

Figure S4. Percentage cell viability for HER2 positive cells (BT-474) and HER2 negative cells (MDA-MB-468), in suspension, determined by MTT assay 24 h after incubation with conjugate 10 and with irradiation (IRR)( 20 J/cm² light (400–700 nm) or without irradiation (NI).
Figure S5. Percentage cell viability for HER2 positive cells (BT-474) and HER2 negative cells (MDA-MB-468), in suspension, determined by MTT assay 24 h after incubation with trastuzumab and irradiation with 20 J/cm² light (400–700 nm).
Figure S6. Percentage cell viability for HER2 positive cells (BT-474) and HER2 negative cells (MDA-MB-468), in suspension, determined by MTT assay 24 h after incubation with unconjugated porphyrin and irradiation with 20 J/cm² light (400–700 nm).

**References**