Synthesis, characterization and optimization of in vitro properties of NIR-fluorescent cyclic a-MSH peptides for melanoma imaging

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Supporting Information, Part 1

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Content

Instruments, Chemicals and Methods

All commercially available chemicals and solvents were at least of analytical grade and used, if not otherwise stated, without further purification. Fmoc-protected amino acids, Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and rink amide resin were purchased from NovaBiochem. 2-(4-((9-Fluorenylmethyloxycarbonyl)amino)piperidin-1-yl)acetic acid (Fmoc-4-APip-COOH), 8-(9-fluorenylmethyloxycarbonyl-amino)-3,6-dioxaoctanoic acid (PEG1, Fmoc-NH-PEG1-COOH), 12-(9-Fluorenylmethyloxycarbonylamino)-4,7,10-trioxa-dodecanoic acid (PEG2, Fmoc-NH-PEG2-COOH), 15-(9-Fluorenylmethyloxycarbonyl)amino-4,7,10,13-tetraoxa-pentadecanoic acid (PEG3, Fmoc-NH-PEG3-COOH), 1-(9-Fluorenylmethyloxycarbonyl)amino-3,6,9,12,15,18-hexaoxahenicosan-21-oic acid (PEG5, Fmoc-NH-PEG5-COOH) were obtained from Iris Biotech, Dichloromethane (DCM), diethylether, dimethylformamide (DMF), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), trifluoroacetic acid (TFA) and water were purchased from Carl Roth, acetonitrile (MeCN) from Häberle Labortechnik, N,N-diisopropylethylamine (DIPEA), triisopropylsilane (TIS), ascorbic acid,, 4-(Diethylamino)phenylboronic acid, 4-(Carboxymethyl)phenylboronic acid, 4-(2-carboxyethyl)benzeneboronic acid and 4-(trans-2-Carboxyvinyl)phenylboronic acid from Sigma-Aldrich, 4-carboxyphenylboronic acid, and tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) from TCI.

For HPLC chromatography, a Dionex UltiMate 3000 system was used together with Chromeleon Software (Version 6.80). For analytical chromatography, a Chromolith Performance (RP-18e, 100-4.6 mm, Merck, Germany) and for semipreparative analyses, Chromolith (RP-18e, 100-10 mm, Merck, Germany) columns were used, respectively. ESI (Electrospray Ionization) and MALDI (Matrix-Assisted Laser Desorption/Ionization) mass spectra were obtained with Finnigan MAT95Q and Bruker Daltronics Microflex spectrometers, respectively. γ-counting was performed using a 2480 Wizard gamma counter system from Perkin Elmer. For the determination of molar extinction coefficients a Cary 100 Bio system (Varian) was used to record the UV/Vis-Spectra together with 4 mL PMMA cuvettes from Sigma-Aldrich. Stock solutions of the samples were prepared from weighted samples in a mixture of H_2O/DMSO (90/10), the measurements were performed in aqueous dilution series ranging from c = 1.0 x 10^{-5} mol/L to c = 1.0 x 10^{-6} mol/L. For the determination of quantum yields a Tecan Infinite M200 Microplate reader together with a Nunc Micro-Well 96 solid plate from ThermoFisher was used to record absorbance and emission spectra. The relative fluorescence quantum yield was determined with LS-288 as reference using a|e software from Fluortools.com and the following equation :

$$\phi_x = \phi_{ref} \cdot \left(\frac{A_{ref}}{A_x}\right) \cdot \left(\frac{F_x}{F_{ref}}\right) \cdot \left(\frac{n_x}{n_{ref}}\right)^2$$

ϕ_x =Quantum yield of the probe
ϕ_{ref} =Quantum yield of the reference
A_{ref}= Absorbance of the reference at the fluorescence excitation wavelength (680 nm)
A_x = Absorbance of the probe at the fluorescence excitation wavelength (680 nm)
F = Area under the curves of the fluorescence emission spectra for the respective compound
n = Refractive index of the solvents (H_2O for probe and reference)

Confocal fluorescence microscopy was performed on a Leica TCS SP8 confocal microscope with lasers at λ = 405, 488, 552 and 638 nm. For cryosectioning a Leica CM3050 S Research Cryostat was used. Overlays of microscopies were generated directly with the operating software or afterwards using FIJI software (V1.50e).

Murine melanoma cells B16F10 (ATCC® CRL6475™) were used for cells experiments.. [^{125}I]-NDP-MSH was purchased from Perkin Elmer in a molar activity of 81.4 GBq/µmol. Dulbecco's Modified Eagle's Medium (DMEM, ATCC® 30-2002™,500 mL), PenStrep was obtained from Gibco, FCS (fetal calf serum) from BioCell and Dulbecco's phosphate buffered saline (PBS), 0.25% Trypsin, 0.02% EDTA Solution in PBS and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) from Sigma-Aldrich. Bovine serum albumin (BSA) was purchased from CarlRoth (Karlsruhe, Germany). 1,10-Phenanthrolin was obtained from Acros Organics.

All animal experiments were performed in compliance with the German animal protection laws and protocols of the local committee (Regierungspräsidium Karlsruhe, approval number: 35-9185.81/G-271/19). For the staining of solid tumor sections, male athymic nude mice (Balb/cAnNRj-Foxn1$^{nu/nu}$) were obtained from Javier (approval number G-271/19) amd injected subcutaneously (s.c.) with 1 × 10^6 B16F10 cells in the right flank. Once the tumors reached 1 cm the mice (8 to 9 weeks old) were euthanized and the tumors were excised.

Chemical Syntheses

Synthesis of fluorescent dyes D4-D9

Through a solution of dye **D2** ($C_{38}H_{47}ClN_2O_{12}S_4$, 815.39 g/mol, 200 mg, 0.2253 mmol, 1 equiv.) in water (H_2O, 17 mL) gaseous argon was vigorously bubbled from a balloon for about 30 minutes at room temperature. 4-carboxyphenylboronic acid ($HO_2CC_6H_4B(OH)_2$, 165.94 g/mol, 75 mg, 0.4520 mmol, 2.0 equiv.) and tetrakis(triphenylphosphine)palladium(0) ($Pd[(C_6H_5)_3P]_4$, 1155.56 g/mol, 18 mg, 0.0156 mmol, 0.07 equiv.) were added to the dark solution at room temperature. The reaction mixture was stirred at 100 °C for 13 h and was subsequently cooled to room temperature. The suspension was filtered through a short plug of silica and was then lyophilized. The crude product was was purified by chromatography (CH_3CN−H_2O, 20:1 to 10:1 to 5:1) to afford dye **D4** after lyophilization as green amorphous powder ($C_{45}H_{52}N_2O_{14}S_4$, 973.16 g/mol, 53 mg, 0.0545 mmol, 24%).**^{1}H NMR** (500 MHz, DMSO-d_6) δ 1.11 (s, 12H, H-10/H-11), 1.69 - 1.78 (m, 8H, H-19/H-20), 1.94 - 2.00 (m, 2H, H-17), 2.52 - 2.55 (m, 4H, H-21), 2.68 - 2.75 (m, 4H, H-16), 4.08 - 4.15 (m, 4H, H-18), 6.29 (d, *J* = 14.00 Hz, 2H, H-12), 7.02 (d, *J* = 14.00 Hz, 2H, H-13), 7.32 (d, *J* = 8.3 Hz, 2H, H-7), 7.42 (d, *J* = 8.0 Hz, 2H, H-23), 7.60 (d, *J* = 8.3 Hz, 2H, H-6), 7.62 (s, 2H, H-4), 8.20 (d, *J* = 8.0 Hz, 2H, H-24). **^{13}C NMR** (126 MHz, DMSO-d_6) δ 20.87 (CH_2, C-17), 22.26 (CH_2, C-19 or C-20), 24.16 (CH_2, C-16), 25.86 (CH_2, C-19 or C-20), 26.93 (CH_3, C-10/C-11), 43.47 (CH_2, C-18) 48.20 (C, C-3), 50.68 (CH_2, C-21), 100.78 (CH, C-12), 110.27 (CH, C-Ar, C-7), 119.71 (CH, C-Ar, C-4), 126.17 (CH, C-Ar, C-6), 129.57 (2xCH, C-Ar, C-23/C-24), 130.41 (C, C-22), 131.02 (C, C-14), 139.97 (C, C-9), 142.11 (C, C-8), 143.38 (C, C-5), 144.97 (C, C-25), 146.57 (CH, C-13), 159.87 (C, C-15), 166.92 (C, COOH), 171.33 (C, C-2). **HPLC gradient (analytical):** 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 2.897 min, purity:

95%, **MALDI-MS** (m/z) for $[M+H]^+$ calculated: 973.237, found: 973.016. **HRMS (ESI):** m/z for $[M-3H]^{3-}$ calculated: 323.0694, found: 323.0685; **Fluorescence** (PBS): λ_{max}(Ex) [nm] = 763; λ_{max} (Em) [nm] = 784.

Through a solution of dye **D2** ($C_{38}H_{47}ClN_2O_{12}S_4$, 887.50 g/mol, 200 mg, 0.2254 mmol, 1 equiv.) in water (H_2O, 20 mL) gaseous argon was vigorously bubbled from a balloon for about 30 minutes at room temperature. 4-(carboxymethyl)phenylboronic acid ($C_8H_9BO_4$, 179.97 g/mol, 73 mg, 0.4056 mmol, 1.8 equiv.) and tetrakis(triphenylphosphine)palladium(0) ($Pd[(C_6H_5)_3P]_4$, 1155.56 g/mol, 9 mg, 0.00779 mmol, 0.035 equiv.) were added to the dark solution at room temperature. The reaction mixture was stirred at 100 °C for 13 h and was subsequently cooled to room temperature. The suspension was filtered through a short plug of silica and was then lyophilized. The crude product was was purified by chromatography (CH_3CN-H_2O, 20:1 to 10:1 to 5:1) to afford **D5** after lyophilization as green amorphous powder ($C_{46}H_{54}N_2O_{14}S_4$, 987.19 g/mol, 87 mg, 0.0881 mmol, 39%).**^{1}H NMR** (600 MHz, DMSO-d_6) δ 1.11 (s, 12H, H-10/H-11), 1.68 - 1.78 (m, 8H, H-19/H-20), 1.90 - 1.99 (m, 2H, H-17), 2.54 (t, *J* = 7.2 Hz, 4H, H-21), 2.69 (br. s., 4H, H-16), 3.74 (s, 2H, H-26), 4.11 (br. s., 4H, H-18), 6.25 (d, *J* = 14.3 Hz, 2H, H-12), 7.14 (d, *J* = 14.0 Hz, 2H, H-13), 7.18 (d, *J* = 7.7 Hz, 2H, H-23), 7.29 (d, *J* = 8.8 Hz, 2H, H-7), 7.52 (d, *J* = 8.1 Hz, 2H, H-24), 7.55 - 7.61 (m, 4H, H-4/H-6); **^{13}C NMR** (151 MHz, DMSO-d_6) δ 20.94 (CH_2, C-17), 22.37 (CH_2, C-19 or C-20), 24.04 (CH_2, C-16), 25.92 (CH_2, C-19 or C-20), 27.08 (CH_3, C-10/C-11), 40.92 (CH_2, C-26), 43.46 (CH_2, C-18), 48.16 (C, C-3), 50.73 (CH_2, C-21), 100.44 (CH, C-12), 110.14 (CH, C-Ar, C-7), 119.59 (CH, C-Ar, C-4), 126.07 (CH, C-Ar, C-6), 128.87 (C, C-14), 129.85 (C, C-23), 131.15 (C, C-24), 135.42 (C, C-25), 136.88 (C, C-22), 140.12 (C, C-9), 142.18 (C, C-8), 144.82 (C, C-5), 147.39 (CH, C-13), 161.61 (C, C-15), 171.39 (C, C-2), 172.86 (COOH). **HPLC gradient (analytical):** 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 2.980 min, purity: 97%, **MALDI-MS** (m/z) for $[M+H]^+$ calculated: 987.253, found: 987.350 **HRMS (ESI):** m/z for $[M-3H]^{3-}$ calculated: 327.7413, found: 327.7404, **Fluorescence** (PBS): λ_{max}(Ex) [nm] = 759; λ_{max} (Em) [nm] = 783,:

Through a solution of **D2** ($C_{38}H_{47}ClN_2O_{12}S_4$, 887.50 g/mol, 200 mg, 0.2254 mmol, 1 equiv.) in water (H_2O, 20 mL) gaseous argon was vigorously bubbled from a balloon for about 30 minutes at room temperature. 4-(2-carboxyethyl)benzene boronic acid ($C_9H_{11}BO_4$, 193.99 g/mol, 79 mg, 0.4072 mmol, 1.8 equiv.) and tetrakis(triphenylphosphine)palladium(0) ($Pd[(C_6H_5)_3P]_4$, 1155.56 g/mol, 16 mg, 0.0138 mmol, 0.06 equiv.) were added to the dark solution at room temperature. The reaction mixture was stirred at 100 °C for 13 h and was subsequently cooled to room temperature. The suspension was filtered through a short plug of silica and was then lyophilized. The crude product was was purified by chromatography (CH_3CN-H_2O, 20:1 to 10:1 to 5:1) to afford **D6** after lyophilization as green amorphous powder ($C_{47}H_{56}N_2O_{14}S_4$, 1001.21 g/mol, 153 mg, 0.1528 mmol, 68%).**^{1}H NMR** (500 MHz, DMSO-d_6) δ 1.10 (s, 12H), 1.68 - 1.74 (m, 8H), 1.90 - 1.95 (m, 2H), 2.54 (t, *J* = 6.7 Hz, 4H), 2.66 - 2.72 (m, 6H), 2.99 (t, *J* = 6.4 Hz, 2H), 4.08 - 4.11 (m, 4H), 6.23 (d, *J* = 14.1 Hz, 2H), 7.11 - 7.17 (m, 4H), 7.28 (d, *J* = 8.3 Hz, 2H), 7.47 (d, *J* = 7.8 Hz, 2 H), 7.57 (d, *J* = 8.3 Hz, 2H), 7.61 (d, *J* = 0.98 Hz, 2H); **HPLC gradient (analytical):** 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 3.033 min, purity: 88%, **MALDI-MS** (m/z) for $[M+H]^+$ calculated: 1001.269, found: 1001.492.

HRMS (ESI): m/z for $[M-3H]^{3-}$ calculated: 332.4132, found: 332.4124. **Fluorescence** (PBS): λ_{max}(Ex) [nm] = 758; λ_{max} (Em) [nm] = 782

Through a solution of **D2** ($C_{38}H_{47}ClN_2O_{12}S_4$, 887.50 g/mol, 200 mg, 0.2254 mmol, 1 equiv.) in water (H_2O, 20 mL) gaseous argon was vigorously bubbled from a balloon for about 30 minutes at room temperature. 4-(trans-2-carboxyvinyl)phenylboronic acid ($C_9H_9BO_4$, 191.98 g/mol, 77 mg, 0.4011 mmol, 1.78 equiv.) and tetrakis(triphenylphosphine)palladium(0) ($Pd[(C_6H_5)_3P]_4$, 1155.56 g/mol, 22 mg, 0.0190 mmol, 0.08 equiv.) were added to the dark solution at room temperature. The reaction mixture was stirred at 100 °C for 13 h and was subsequently cooled to room temperature. The suspension was filtered through a short plug of silica and was then lyophilized. The crude product was was purified by chromatography (CH_3CN-H_2O, 20:1 to 10:1 to 5:1) to afford **D7** after lyophilization as green amorphous powder ($C_{47}H_{54}N_2O_{14}S_4$, 999.20 g/mol, 50 mg, 0.050 mmol, 22%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 1.12 (s, 12H, H-10/H-11), 1.68-1.75 (m, 8H, H-19/H-20), 1.91-1.99 (m, 2H, H-17), 2.50-2.55 (m, 4H, H-21), 2.65-2.75 (m, 4H, H-16), 4.05-4.15 (m, 4H, H-18), 6.26 (d, *J* = 13.9 Hz, 2H, H-12), 6.70 (d, *J* = 16.0 Hz, 1H, H-27), 7.10 (d, *J* = 13.9 Hz, 2H, H-13), 7.29 (d, *J* = 8.1 Hz, 2H, H-23 or H-7), 7.31 (d, *J* =8.1 Hz, 2H, H-7 or H-23), 7.57 (d, *J* = 8.1 Hz, 2H, H-6), 7.60 (s, 2H, H-4), 7.76 (d, *J* = 16.0 Hz, 1H, H-26), 7.95 (d, *J* = 8.1 Hz, 2H, H-24).**^{13}C NMR** (151 MHz, DMSO-d_6) δ 20.83 (CH_2, C-17), 22.42 (CH_2, C-19 or C-20), 23.71 (CH_2, C-16), 24.13 (CH_2, C-19 or C-20), 26.93 (CH_3, C-10/C-11), 43.44 (CH_2, C-18), 48.19 (C, C-3), 50.70 (CH_2, C-21), 100.55 (CH, C-12), 110.12 (CH, C-Ar, C-7), 119.63 (CH, C-4), 119.93 (CH, C-27), 126.00 (CH, C-Ar, C-6), 128.30 (CH, C-24), 129.8 (CH, C-23), 131.04 (C, C-14), 134.17 (C, C-25), 139.94 (C, C-9), 140.49 (C, C-22) ,142.01 (C, C-8), 143.34 (CH, C-26), 145.08 (C, C-5), 146.67 (CH_2 C-13), 160.55 (C, C-15), 167.55 (C, COOH), 171.25 (C, C-2), **HPLC gradient (analytical):** 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 3.047 min, purity: 84%, **MALDI-MS** (m/z) for $[M+H]^+$ calculated: 999.253, found: 998.927. **HRMS (ESI):** m/z for $[M-3H]^{3-}$ calculated: 331.7413, found: 331.7408 **Fluorescence** (PBS): λ_{max}(Ex) [nm] = 762; λ_{max} (Em) [nm] = 784

Through a solution of **D3** ($C_{40}H_{47}ClN_2O_{10}S_2$, 815.39 g/mol, 320 mg, 0.3925 mmol, 1 equiv.) in water (H_2O, 35 mL) gaseous argon was vigorously bubbled from a balloon for about 30 minutes at room temperature. Phenylboronic acid ($C_6H_5B(OH)_2$, 121.93 g/mol, 130 mg, 1.0662 mmol, 2.7 equiv.) and tetrakis(triphenylphosphine)palladium(0) ($Pd[(C_6H_5)_3P]_4$, 1155.56 g/mol, 47 mg, 0.0407 mmol, 0.1 equiv.) were added to the dark solution at room temperature. The reaction mixture was stirred at 100 °C for 13 h and was subsequently cooled to room temperature. The suspension was filtered through a short plug of silica and was then lyophilized. The crude product was was purified by chromatography (CH_3CN-H_2O, 20:1 to 10:1 to 5:1) to afford **D8** after lyophilization as green amorphous powder ($C_{46}H_{52}N_2O_{10}S_2$, 857.04 g/mol, 99 mg, 0.1155 mmol, 29%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 1.13 (s, 12H, H-10/H-11), 1.68 - 1.80 (m, 8H, H-19/H-20), 1.96 (m, 2H, H-17), 2.50-2.55 (m, 4H, H-21), 2.73 (m, 4 H, H-16), 4.15 (m., 4H, H-18), 6.34 (d, *J* = 14.1 Hz, 2H, H-12), 7.17 (d, *J* = 14.1 Hz, 2H, H-13), 7.28 (d, *J* = 7.0 Hz, 2H, H-23), 7.45 (d, *J* = 8.9 Hz, 2H, H-7), 7.57 - 7.67 (m, 3H, H-, H-24/H-25), 7.89 - 7.97 (m, 4H, H-4/H-6); **^{13}C NMR** (126 MHz, DMSO-d_6) δ 20.85 (CH_2, C-17), 22.35 (CH_2, C-19 or C-20), 24.13 (CH_2, C-16), 25.92 (CH_2, C-19 or C-20), 26.94 (CH_3, C-10/C-11), 43.60 (CH_2, C-18), 47.97 (C, C-3), 50.70 (CH_2, C-21), 101.44 (CH, C-12), 110.91 (CH, C-7), 123.24 (CH, C-

Ar, C-4 or C-6), 126.57 (C, C-5), 128.29 (CH, C-24 or C-25), 128.69 (CH, C-24 or C-25), 129.11 (CH, C-Ar, C-23), 130.59 (CH, C-4 or C-6), 132.42 (C, C-14) 138.30 (C, C-22) 140.67 (C, C-9) 145.79 (C, C-8) 147.87 (CH, C-13), 162.42 (C-15) 166.80 (COOH) 171.73 (C-2); **MALDI-MS** (m/z) for $[M+H]^+$ calculated: 857.314, found: 857.175. **HRMS (ESI):** m/z for $[M-H]^-$ calculated: 855.2991, found: 855.3003, for $[M-2H]^{2-}$ calculated: 427.1459, found: 427.1453 **Fluorescence** (PBS): λ_{max}(Ex) [nm] = 766; λ_{max} (Em) [nm] = 788.

Through a solution of **D3** ($C_{40}H_{47}ClN_2O_{10}S_2$, 815.39 g/mol, 100 mg, 0.1226 mmol, 1 equiv.) in water (H_2O, 35 mL) gaseous argon was vigorously bubbled from a balloon for about 30 minutes at room temperature. 4-(Diethylamino)phenylboronic acid ($C_{10}H_{16}BNO_2$, 193.05 g/mol, 43, 0.2227 mmol, 1.8 equiv.) and tetrakis(triphenylphosphine)palladium(0) ($Pd[(C_6H_5)_3P]_4$, 1155.56 g/mol, 10 mg, 0.0087 mmol, 0.07 equiv.) were added to the dark solution at room temperature. The reaction mixture was stirred at 100 °C for 13 h and was subsequently cooled to room temperature. The suspension was filtered through a short plug of silica and was then lyophilized. The crude product was was purified by chromatography (CH_3CN-H_2O, 20:1 to 10:1 to 5:1) to afford **D9** after lyophilization as green amorphous powder ($C_{50}H_{61}N_3O_{10}S_2$, 928.17 g/mol, 33 mg, 0.0356 mmol, 29%). **HPLC gradient (analytical):** 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 3.617 min, purity: 81%, **MALDI-MS** (m/z) for $[M+H]^+$ calculated: 928.387, found: 928.226. **HRMS (ESI):** m/z for $[M-2H]^{2-}$ calculated: 462.6826, found: 462.6826; **Fluorescence** (PBS): λ_{max}Ex [nm] = 766; λ_{max} (Em) [nm] = 792

General synthesis of peptides

Peptides were synthesized on Rink Amide AM Resin LL (initial loading: 0.34 mmol/g) by using N^{α}-Fmoc protecting groups and a standart HBTU activation strategy. The resin was swollen in DCM for 30 min, washed with DMF, the Fmoc protecting group was cleaved with piperidine (50% in DMF, 2 min washing then 5 min). The resin was washed with DMF (3×), DCM (3×) and DMF (3×), consecutively. The respective protected amino acid was coupled by using preactivated HBTU ester in DMF (4 equiv. N^{α}-Fmoc amino acid, 4 equiv. HBTU, 8 equiv. DIPEA) which was allowed to react for 3 min before being added to the resin The syringe was then shaken for 1 h. Afterwards, the reaction mixture was removed and the resin was washed with DMF (3×), DCM (3×) and DMF (3×). The same procedure was repeated for the next amino acid (Lys / Trp / Arg /D-Phe / His / Asp). For the cyclisation of the carbocylic carboxy group from asp with the amino group from Lys, the protecting groups of the latter ones were selectively cleaved. Therefore the resin was washed with DCM (3×) and a mixture of DCM/TIS/TFA (90/10/1) was added. After 20 min the reaction mixture was removed and replaced with a solution of 1% TFA in DCM to test for remaining liberated Mtt (yellow color) before being again replaced with DCM/TIS/TFA. This procedure was then repeated after every 10 min. After 55 min cleavage was finished and the resin was washed with DCM (3×) and DMF (3×). A solution of 5% DIPEA in DMF was added and the mixture was shaken for 10 min in order to neutralize remaining TFA. The resin was once again washed with DMF (3×) DCM (3×) and DMF (3×). The cyclization reaction was achieved by an overnight reaction using PyBOP (4.0 equiv.) with DIPEA (4 equiv.) in DMF. The next day the resin was washed with DMF (3×), DCM (3×) and DMF (3×). The final side chain Nle as well as the respective linker (4 equiv) were coupled as the procedure previously described. The resin was further washed

with DCM (3×) and diethyl ether (3×) before being dried under reduced pressure. The dried resin was stored at -20 °C until conjugation to the respective fluorescent dye.

General synthesis of probes P1 - P5

After deprotection of the remaining Fmoc group of the respective hemiconjugate **H1** – **H5** according to the previously described procedure, a solution of the fluorescent dye (2 equiv) DMF (0.04 mmol/mL) was premixed with PyBOP (2 equiv) in DMF (0.15 mmol/mL) and DIPEA (2 equiv). After 3 min the mixture was added to the resin (100 mg/mL of DMF) and the mixture was shaken for 3 h. Afterwards, the resin was washed with DMF (3×), DCM (3×) and DMF (3×), DCM (3×), diethyl ether (3×), consecutively and dried under reduced pressure. For cleavage of the conjugate from the resin and concomitant removal of the remaining protective groups**,** the resin was allowed to swell in DCM for 30 min and was then treated with a solution of TFA / TIS / water (950/25/25, 50 mg resin/mL) for 2 h. The solution was collected and the resin washed twice more with TFA (100 mg resin/mL). Filtrates were collected, concentrated under reduced pressure and precipitated by addition of ice cold diethyl ether. The precipitate was washed once, dissolved in a solution of H_2O/ACN (1/1), purified by semi-preparative HPLC and lyophilized to yield a green solid. Detailed HPLC conditions, yields and analytical data are listed below.

P1: HPLC gradient (semipreparative): 10-60 % MeCN + 0.1 % FA in 10 min, flow 6 mL/min, Rt = 8,00-8,50 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 5.67 min, yield 15.7 mg (9% over all, 20% coupling with dye), purity: 98%, MALDI-MS (m/z) for $[M+H]^+$ calculated: 1876.874, found: 1876.937. HRMS (ESI): m/z for $[M+2H]^{2+}$ calculated: 939.4425, found: 939.4455; **UV/Vis** (H_2O): λ_{max} (Ex) [nm] (ε $[M^{-1}cm^{-1}]$) = 740 (2.60 x 10^4); **Fluorescence** (DMSO): λ_{max}(Ex) [nm] = 817; λ_{max} (Em) [nm] = 829.

P2: HPLC gradient (semipreparative): 10-60 % MeCN + 0.1 % FA in 10 min, flow 6 mL/min, Rt = 7.00-7.50 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 5.53 min, yield 39.7 mg (21% over all, 70% coupling with dye), purity: 98%, MALDI-MS (m/z) for $[M+H]^+$ calculated: 2016.969, found: 2016.928. HRMS (ESI): m/z for $[M+2H]^{2+}$ calculated: 1009.4899, found: 1009.4926; **UV/Vis** (H_2O): λ_{max} (Ex) [nm] (ε $[M^{-1}cm^{-1}]$) = 743 (3.29 x 10^4); **Fluorescence** (DMSO): λ_{max}(Ex) [nm] = 817; λ_{max} (Em) [nm] = 829.

P3: HPLC gradient (semipreparative): 10-60-100 % MeCN + 0.1 % FA in 10 min, flow 6 mL/min, Rt = 8.00-8.75 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 5.71 min, yield 40.1 (21% over all, 30% coupling with dye), purity: 98%, MALDI-MS (m/z) for $[M+H]^+$ calculated: 2021.948, found: 2021.928. HRMS (ESI): m/z for $[M+2H]^{2+}$ calculated: 1011.9794, found: 1011.9822; **UV/Vis** (H_2O): λ_{max} (Ex) [nm] (ε $[M^{-1}cm^{-1}]$) = 733 (8.48 x 10^4); **Fluorescence** (DMSO): λ_{max}(Ex) [nm] = 815; λ_{max} (Em) [nm] = 831.

P4: HPLC gradient (semipreparative): 10-60-100 % MeCN + 0.1 % FA in 10 min, flow 6 mL/min, Rt = 8.00-8.60 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 5.69 min, yield 39.1 mg (20% over all, 33% coupling with dye) , purity: 96%, MALDI-MS (m/z) for $[M+H]^+$ calculated: 2079.990, found: 2080.469. HRMS (ESI): m/z for $[M+2H]^{2+}$ calculated: 1041.00365, found: 1041.0003; **UV/Vis** (H_2O): λ_{max} (Ex)

[nm] (ε [$M^{-1}cm^{-1}$]) = 735 (6.54 x 10^4); **Fluorescence** (DMSO): λ_{max}(Ex) [nm] = 818; λ_{max} (Em) [nm] = 831.

P5: HPLC gradient (semipreparative): 10-60-100 % MeCN + 0.1 % FA in 10 min, flow 6 mL/min, Rt = 8.00-8.75 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 5.65 min, 32,7 mg (16% over all, 82% coupling with dye), purity: 99%, MALDI-MS (m/z) for $[M+H]^+$ calculated: 2124.016, found: 2124.983. HRMS (ESI): m/z for $[M\ \ 2H]^{2-}$ calculated: 1060.9988, found: 1060.9996; **UV/Vis** (H2O): λ_{max} (Ex) [nm] (ε [$M^{-1}cm^{-1}$]) = 735 (8.35 x 10^4) **Fluorescence** (DMSO): λ_{max}(Ex) [nm] = 816; λ_{max} (Em) [nm] = 833.

General synthesis of Probes P6 – P13

Deprotection of the remaining Fmoc group of hemiconjugate **H5** or **H6** was achieved according to the previously described procedure. For cleavage of hemiconjugate **H5** or **H6** from the resin and concomitant removal of the remaining protective groups, the resin was allowed to swell in DCM for 30 min and was then treated with a solution of TFA / TIS / water (950/25/25, 50 mg resin/mL) for 2 h. The solution was collected and the resin washed twice more with TFA (50 mg resin/mL). Filtrates were collected, precipitated by addition of ice cold diethyl ether and lyophilized to obtain the respective unpurified conjugate.

A solution of the respective fluorescent dye **D4 – D9** (2.0 equiv) in DMF (0.04 mmol/mL) was premixed with a solution of PyBOP (2.4 equiv) and DIPEA (5.4 equiv)in DMF (0.04 mmol/mL). After 3 min the mixture was added to the unpurified conjugate (1 equiv) and the mixture was shaken for 1.5 h. 10 mL of ice cold ether was added to precipitate the product. The crude product was washed once more with cold ether and purified by HPLC to afford the respective probe **P6** – **P13** after lyophilization as green amorphous powder Detailed HPLC conditions, yields and analytical data are listed below.

P6: HPLC gradient (semipreparative): 10-50 % MeCN + 0.1 % TFA in 8 min, flow 4 mL/min, Rt = 6.37 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 3.66 min, yield 2.8 mg (29% over all), purity: 97%, MALDI-MS (m/z) for $[M+H]^+$ calculated: 2183.899, found: 2183.183 HRMS (ESI): m/z for $[M-2H]^{2-}$ calculated: 1090.9400, found:1090.9408; **UV/Vis** (H_2O): λ_{max} (Ex) [nm] (ε [$M^{-1}cm^{-1}$]) = 769 (18.37 x 10^4) **Fluorescence** (PBS): λ_{max}(Ex) [nm] = 770; λ_{max} (Em) [nm] = 793, ϕ_f= 0.102, ε • ϕ_f [M^{-1} cm^{-1}] =18 700.

P7: HPLC gradient (semipreparative): 10-50 % MeCN + 0.1 % TFA in 8 min, flow 4 mL/min, Rt = 6.45 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 3.707 min, yield 2.6 mg (26% over all), purity: 95%, MALDI-MS (m/z) for $[M-2H]^{2-}$ calculated: 2197.914, found: 2197.920 HRMS (ESI): m/z for $[M+2H]^{2+}$ calculated: 1097.9478, found:1097.9469; **UV/Vis** (H_2O): λ_{max} (Ex) [nm] (ε [$M^{-1}cm^{-1}$]) = 769 (17.31 x 10^4) **Fluorescence** (PBS): λ_{max}(Ex) [nm] = 766; λ_{max} (Em) [nm] = 787, ϕ_f= 0.100, ε • ϕ_f [M^{-1} cm^{-1}] = 17 300.

P8: HPLC gradient (semipreparative): 10-50 % MeCN + 0.1 % TFA in 8 min, flow 4 mL/min, Rt = 6.79 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 3.82 min, yield 3.4 mg (32% over all), purity: 97%, MALDI-MS (m/z) for $[M+H]^+$ calculated: 2211.930, found: 2211.855. HRMS (ESI): m/z for $[M-2H]^{2-}$ calculated: 1104.9557, found: 1104.9564; **UV/Vis** (H_2O): λ_{max} (Ex) [nm] (ε [$M^{-1}cm^{-1}$]) = 765 (15.98 x 10^4); **Fluorescence** (PBS): λ_{max}(Ex) [nm] = 766; λ_{max} (Em) [nm] = 787, ϕ_f= 0.100, $\varepsilon \bullet \phi_f$ [M^{-1} cm^{-1}] = 16 000

P9: HPLC gradient (semipreparative): 10-50 % MeCN + 0.1 % TFA in 8 min, flow 4 mL/min, Rt = 7.85 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 4.807 min, yield 4.9 mg (45% over all), purity: 97%,MALDI-MS (m/z) for $[M+H]^+$ calculated: 2067.975, found: 2067.925 HRMS (ESI): m/z for [M 2H]$^{2-}$ calculated: 1032.9781, found: 1032.9786, **UV/Vis** (H2O): λ_{max} (Ex) [nm] (ε [$M^{-1}cm^{-1}$]) = 769 (7.39 x 10^4); **Fluorescence** (DMSO): λ_{max}(Ex) [nm] = 778; λ_{max} (Em) [nm] = 791

P10: HPLC gradient (semipreparative): 10-50 % MeCN + 0.1 % TFA in 8 min, flow 4 mL/min, Rt = 7.26 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 4.097 min, yield 2.4 mg (21% over all), purity: 88%, MALDI-MS (m/z) for $[M+H]^+$ calculated: 2139.048, found: 2139.065 HRMS (ESI): m/z for [M 2H]$^{2-}$ calculated: . 1068.5149, found: 1068.5159, **UV/Vis** (H_2O): λ_{max} (Ex) [nm] (ε [$M^{-1}cm^{-1}$]) = 766 (6.44 x 10^4); **Fluorescence** (PBS): λ_{max}(Ex) [nm] = 782; λ_{max} (Em) [nm] = 798

P11: HPLC gradient (semipreparative): 10-50 % MeCN + 0.1 % TFA in 8 min, flow 4 mL/min, Rt = 6.52 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 3.75 min, yield 2.4 mg (28% over all), purity: 95%,MALDI-MS (m/z) for $[M+H]^+$ calculated: 2285.967, found: 2285.556 HRMS (ESI): m/z for $[M+2H]^{2+}$ calculated: 1143.9886, found: 1143.9887, **UV/Vis** (H2O): λ_{max} (Ex) [nm] (ε [$M^{-1}cm^{-1}$]) = 765 (18.13 x 10^4); **Fluorescence** (PBS): λ_{max}(Ex [nm] = 766; λ_{max} (Em) [nm] = 787, ϕ_f= 0.105, $\varepsilon \bullet \phi_f$ [M^{-1} cm^{-1}] = 19 000.

P12: HPLC gradient (semipreparative): 10-50 % MeCN + 0.1 % TFA in 8 min, flow 4 mL/min, Rt = 6.77min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 3.85 min, yield 2.7 mg (30% over all), purity: 97%, MALDI-MS (m/z) for $[M+H]^+$ calculated: 2299.982, found: 2300.096. HRMS (ESI): m/z m/z for $[M-2H]^{2-}$ calculated: 1148.9819, found: 1148.9825, **UV/Vis** (H2O): λ_{max} (Ex) [nm] (ε [$M^{-1}cm^{-1}$]) = 765 (17.67 x 10^4); **Fluorescence** (PBS): λ_{max}(Ex) [nm] = 766; λ_{max} (Em) [nm] = 787, ϕ_f= 0.108, $\varepsilon \bullet \phi_f$ [M^{-1} cm^{-1}] = 19 100

P13: HPLC gradient (semipreparative): 10-50 % MeCN + 0.1 % TFA in 8 min, flow 4 mL/min, Rt = 6.68 min. HPLC gradient (analytical): 0-100% MeCN + 0.1 % TFA in 10 min, flow 4 mL/min, Rt = 3.80 min, yield 2.0 mg (29% over all), purity: 89%, MALDI-MS (m/z) for $[M+H]^+$ calculated: 2297.967, found: 2297.489. HRMS (ESI): for $[M-2H]^{2-}$ calculated: 1147.9740, found: 1147.9747; **UV/Vis** (H2O): λ_{max} (Ex) [nm] (ε [$M^{-1}cm^{-1}$]) = 766 (14.32 x 10^4) **Fluorescence** (PBS): λ_{max}(Ex) [nm] = 766; λ_{max} (Em) [nm] = 789, ϕ_f= 0.078, $\varepsilon \bullet \phi_f$ [M^{-1} cm^{-1}] = 11 200

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In vitro competitive binding assays

The MC1R binding affinities of the respective probes were determined on B16F10 murine melanoma cells by in vitro competitive displacement experiments which were performed at least three times, each experiment performed in triplicate. The cells were cultured at 37°C in DMEM medium supplemented with 10% FCS, and 1% penicillin/streptomycin (all concentrations by volume) in a humidified atmosphere containing 5% CO_2, exchanging the medium in intervals of two or three days and splitting the cells at >75% confluence. A Millipore Multiscreen punch kit and Millipore 96 well filter plates were used. The plates were incubated with PBS/BSA (1%) solution (each well 200 µL) for one hour before use. B16F10 cells were harvested and suspended carefully in DMEM medium supplemented with HEPES (25 mM), BSA (0.2 %) and 1,10-Phenanthrolin (0.3 mM). 50 µL of a cell suspension containing 10^5 cells were seeded in each well. To this, a total volume of 50 µL was added to each well, containing 25 µL (0.018 kBq/µL) of the MC1R-specific radioligand [^{125}I]-NDP -MSH (81.4 GBq/µmol) and 25 µL of the respective probe (**P1**-**P13**) or of the respective reference (α-MSH or NDP-α-MSH). The competitor was added in 11 increasing concentrations ranging from 0.5 – 1000 nM for α-MSH, 0.025 – 50 nM for NDP-MSH, whereat the twelfth well contained no competitor to ensure 100% binding of the radioligand. After one hour of incubation at ambient temperature, the solution was filtrated and the filters were washed with cold PBS (3 times). The filters were collected and measured by γ-counting. The 50% inhibitory concentration (IC50) values of the respective probes α-MSH and NDP-α-MSH were calculated by fitting the obtained data via a nonlinear regression analysis using GraphPad Prism Software (version 5.04).

Fluorescence staining of melanoma cells and tumor lesions with P11

B16F10 cells (1× 10^5 cells per well) were seeded onto coverslips in a six-well plate at 37 °C. After two days, the cells were washed with PBS and incubated with the respective media containing probe **P11** (10 µM) with or without the peptide blockade (NDP-MSH 300 µM) for 1 h at 37 °C in 5% CO_2. The cells were washed with PBS three times and then stained for nuclei and mounted with ProLong Diamond antifade mounting reagent with DAPI. The fluorescent signal was observed and recorded at 40 × magnification under a Leica TCS SP8 confocal microscope with lasers at λ = 405 and 638 nm.

Tumors of euthanized were excised, weighed and placed into an embedding base mold. The tumors were then embedded in OCT (Tissue Tek) and quickly frozen. The tumor blocks were sectioned on a cryostat Leica (CM3050 S) to obtain 6 µm thick slices. The sections were incubated with probe **P11** (1µM) with or without NDP-MSH peptide blockade (100 µM) at room temperature for 1 hour in the dark. Tissue samples were then washed with PBS three times stained for nuclei and mounted with ProLong Diamond antifade mounting reagent with DAPI. The fluorescent signal was observed and recorded at 40 × magnification under a Leica TCS SP8 confocal microscope with lasers at λ = 405 and 638 nm.