Supporting Information

Development of melanoma-targeted polymer micelles by conjugation of an MC1R specific ligand.

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Click reaction, MC1R, micelle, melanoma, melanocortin receptor, solid-phase synthesis, targeted therapies.

Supporting Information Placeholder

ABSTRACT: ABSTRACT: The incidence of malignant melanoma is rising faster than that of any other cancer in the United States. Due to its high expression on the surface of melanomas, MC1R has been investigated as a target for selective imaging and therapeutic agents against melanoma. Eight ligands were screened against cell lines engineered to over-express MC1R, MC4R or MC5R. Of these, compound 1 exhibited high (low nM) binding affinity for MC1R, and low (high nM) affinities for MC4R and MC5R. Subsequently functionalization of the ligand at the N-terminus with a alkyne for use in Cu-catalyzed click chemistry was shown not to affect the binding affinity. Finally, formation of the targeted-

polymer, as well as the targeted micelle formulation, also resulted in constructs with low nM binding affinity.

Contents:

General methods.

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in J. Biol. Chem. 1972, 247, 977-983. The following additional abbreviations are used: Aloc, allyloxycarbonyl; Boc, tert-butyloxycarbonyl; tBu, tert-butyl; CH_3CN, acetonitrile; DCM, dichloromethane; DIC, N,N'-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, N,N'-dimethylformamide; DMSO, dimethylsulfoxide; ESI-MS, Electrospray ionization - mass spectrometry; DVB, divinylbenzene; Et_2O, Diethyl ether; Fmoc, (9H-fluoren-9-ylmethoxy)carbonyl; FT-ICR, Fourier Transform - Ion Cyclotron Resonance; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate; HOBt, N-hydroxybenzotriazole; HOCt, 6-chloro-1H-hydroxybenzotriazole; MALDI, Matrix Assisted Laser Desorption Ionization - Time of Flight; 3-Mpr, 3-mercaptopropionic acid; NMI, N-methylimidazole; NMP, N-methylpyrrolidone; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl; PEGO, 19-amino-5-oxo-3,10,13,16-tetraoxa-6-azanonadecan-1-oic acid; PS, polystyrene; RP-HPLC, reverse-phase high performance liquid chromatography; SPPS, solid-phase peptide synthesis; THF, Tetrahydrofuran; TFA, trifluoroacetic acid; TPP, triphenylphoshine; Trt, triphenylmethyl (trityl).

Materials.

N-α-Fmoc-protected amino acids, HBTU, HCTU, HOCt and HOBt were purchased from Anaspec (San Jose, CA) or from Novabiochem (San Diego, CA). Rink amide Tentagel S and R resins were acquired from Rapp Polymere (Tubingen, Germany). Rink amide 1%-DVB PS resin was acquired from Novabiochem (San Diego, CA). For the N-α-Fmoc-protected amino acids, the following side chain protecting groups were used: Arg(N^g-Pbf); Asp(O-tBu); His(N^{im}-Trt); Trp(N^i-Boc); Tyr(tBu), and Lys(N^ξ-Aloc). Reagent grade solvents, reagents, and acetonitrile (ACN) for HPLC were acquired from VWR (West Chester, PA) or Aldrich-Sigma

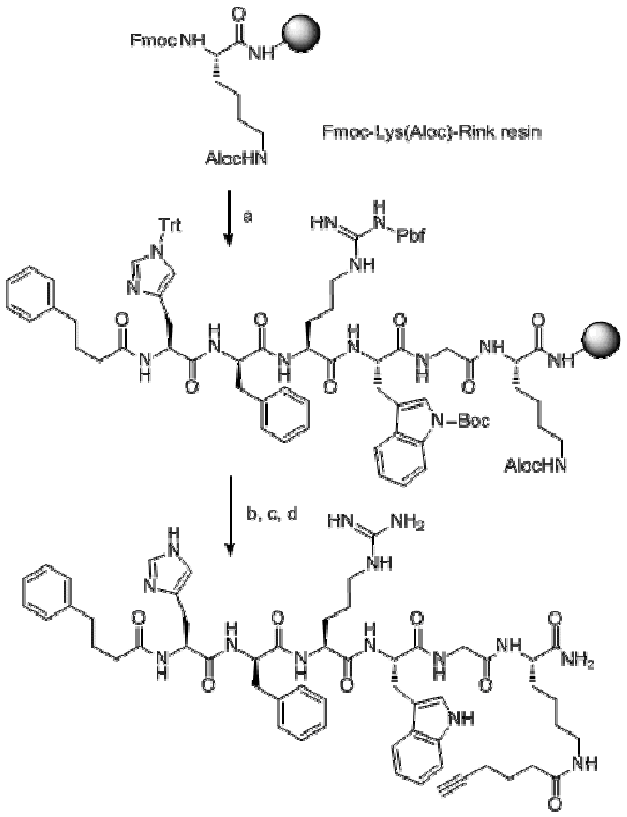
(Milwaukee, WI), and were used without further purification unless otherwise noted. N-terminal heterocyclic acids, NMI, and scavengers were obtained from Sigma-Aldrich or TCI. The solid-phase synthesis was performed in fritted syringes using a Domino manual synthesizer obtained from Torviq (Niles, MI). The C-18 Sep-Pak™ Vac RC cartdridges for solid phase extraction were purchased from Waters (Milford, MA).

General Synthesis.

All reactions were conducted under argon atmosphere using oven-dried glassware. All chemicals were obtained from commercial sources and used without further purification. 1H NMR spectra were recorded on a Bruker-DRX-300 MHz instrument with chemical shifts reported relative to TMS (0.0 ppm) and residual DMSO (2.50 ppm). Proton-decoupled 13C NMR spectra were referenced to CDCl3 (77.0 ppm) as well as DMSO (39.51 ppm). Low resolution mass spectra were obtained on AGILENT (HP) MDS 1100 using AP-ESI. High resolution mass spectra (HRMS) were recorded on a JEOL HX110A instrument. Melting points were measured using a Thomas Hoover capillary melting point apparatus and are uncorrected.

Solid-phase Synthesis.

Ligands were prepared as previously published by solid-phase synthesis as summarized in **Scheme S1** on Rink Amide Tentagel resin (0.23 mmol/g) using a Fmoc/tBu synthetic strategy and standard activations.[1,2] N-α-Fmoc amino acid was double coupled using preactivated 0.3 M HOBt or HOCt esters and HCTU/2,4,6-collidine couplings.



SCHEME S1. Synthetic route for compounds 1. a) (i) Fmoc-AA-OH (3eq), HOCt or HOBt (3eq), and DIC (3eq) in DMF/DCM (10 mL / 1g of resin) for amino acid couplings; (iii) Piperidine/DMF (1:10, 2 + 20 minutes); (iv) 4-phenylbutyric acid (6eq), and DIC (3eq) in DMF/DCM; b) (i) Pd(0)tetrakistriphenylphosphine (0.01eq), N,N'-dimethylbarbituric acid (5eq) in degassed DCM (2 x 30 minutes) (ii) 5-hexynoic acid (5eq) and DIC (3eq) in DMF/DCM for compound 1; c) (i) TFA-scavengers cocktail (91% TFA, 3% water, 3% thioanisole, 3% ethanedithiol); (ii) ether extraction; d) purification.

The Rink resin was washed with DMF, and the N-α-Fmoc protecting group was removed with 1:10 piperidine in DMF (1 X 2 min and 1 X 20 min). The resin was washed successively with DMF, DCM, DMF, a solution of 0.05 mM solution of Bromophenol Blue in 0.2 M HOBt in DMF, then DMF. The N-α-Fmoc amino acid was coupled using pre-activated 0.3 M HOBt or HOCt esters in DMF-DCM mixture (3 equiv of N-α-Fmoc amino acid, 3 equiv of HOBt or HOCt, and 3 equiv of DIC). The resin slurry was stirred for 2 hours or until the bromophenol test became negative.[3] If the test failed, the resin was washed with DMF and the amino acid was

coupled again by the HCTU/2,4,6-lutidine procedure (0.3 M solution of 3 equiv of N-α-Fmoc amino acid, 3 equiv of HCTU, and 6 equiv of 2,4,6-lutidine in DMF) for 3 hours or by a preformed symmetric anhydride (3 equiv of Nα-Fmoc amino acid and 1.5 equiv of DIC in a 1:1 DMF-DCM mixture) until Kaiser test was negative. If the couplings did not result in a negative Kaiser test, the resin was washed with DMF, and the free amino groups were capped with 50% acetic anhydride in pyridine for 10 minutes. After all coupling sequentially to the Rink amide resin, the resin was washed with DCM, dried under vacuum, and then stored in refrigerator. The final resin was washed thoroughly with DMF (3x) and DCM (7x) then cleaved. A cleavage cocktail (10 mL per 1 g of the resin) consisting of TFA (91%), H2O (3%), EDT (3%), and TA (3%) was injected into the resin and the mixture was agitated at room temperature for 4 hours. The solution was filtered, the resin was washed with TFA (2 X 3 min), the liquid phases were collected and concentrated under a stream of nitrogen, and the product was precipitated using cold Et2O. The crude product was washed three times with cold Et2O, lyophilized, purified, and characterized as described above. The pure compounds were dissolved in DI water or DMSO at approximately 1.0 mM concentrations and concentration was determined by Trp-HPLC measurement[4,5].

QC and purification.

Peptides were purified using solid-phase extraction. Briefly, C-18 Sep-Pak™ cartridges (100 mg or 500 mg) were used and pre-conditioned initially with acetonitrile, methanol, and water. After loading the compound, the column was washed with DI water, and then gradually with 5, 10, 20, 30, 50 and 70% of aqueous ACN. Fractions containing product were collected, concentrated to remove organic solvent and lyophilized. Product purity was verified by analytical RP-HPLC using a Waters Alliance 2695 Separation Model with a Waters 2487 dual wavelength detector (220 and 280 nm) on a reverse phase column (Waters XBridge C18, 3.0

X 75 mm, 3.5 mm). Peptides were eluted with a linear gradient of aqueous ACN/0.1% TFA at a flow rate of 0.3 mL/min. Purification of ligands was achieved on a Waters 600 HPLC using a reverse phase column (Waters XBridge C18, 19.0 X 250 mm, 10 mm). Peptides were eluted with a linear gradient of ACN/0.1% TFA at a flow rate of 5.0-10.0 mL/min. Separation was monitored at 230 and 280 nm. Size exclusion chromatography was performed on a borosilicate glass column (2.6 X 250 mm, Sigma, St. Louis, MO) filled with medium sized Sephadex G-25 or G-10. The compounds were eluted with an isocratic flow of 1.0 M aqueous acetic acid. Mass spectra and HPLC characterization data are provided in Supplemental Information.

The purity of products was checked by analytical PR-HPLC using a Waters Alliance 2695 Separation Model with a Waters 2487 dual wavelength detector (220 and 280 nm) on a reverse phase column (Waters XBridge C18, 3.0 X 75 mm, 3.5 μm). Peptides were eluted with a linear gradient of aqueous ACN/0.1% TFA at a flow rate of 0.3 mL/min. Purification of ligands was achieved on a Waters 600 HPLC using a reverse phase column (Waters XBridge C18, 19.0 X 250 mm, 10 μm). Peptides were eluted with a linear gradient of ACN/0.1% TFA at a flow rate of 5.0-10.0 mL/min. Separation was monitored at 230 and 280 nm. Size exclusion chromatography was performed on a borosilicate glass column (2.6 x 250 mm, Sigma, St. Louis, MO) filled with medium sized Sephadex G-25 or G-10. The compounds were eluted with an isocratic flow of 1.0 M aqueous acetic acid.

Quantitative HPLC.

The peptide concentrations were determined by monitoring absorbance of peptides against 0.5 mM solution of Tryptophan in DMSO at 280 nm. The peptides were initially dissolved in DMSO at approximately 1-5 mM concentration. Co-injections of peptide and Trp were made

on analytical HPLC with a number of different volumes and peptide concentration calculated from area under the peaks using the formula given here:

$$\text{Peptide Conc.} = \frac{[\text{Abs. of Comp.}]}{[\text{Abs. of Trp}]} \times \frac{0.5}{\frac{\sum \varepsilon_{280}(\text{Trp} + \text{Tyr} + \text{Cys} + \text{Cy5} + \ldots)}{\varepsilon_{280}(\text{Trp})}} \times \frac{\text{Vol. of Trp}}{\text{Vol. of Comp.}}$$

ξ280 of compounds were determined by summation of tryptophans (ξ280 = 5500), tyrosine (ξ280 = 1490), thiol (ξ280 = 63), and Cy5 dye (ξ280 = 5800), and normalized to extinction coefficient of one tryptophan. Other amino acids in these peptides do not absorb significantly at this wavelength. For Cy5 dye, ξ280 was determined in a similar manner by comparing the absorbances of 0.5 mM of both Trp and Cy5-malenimide ester in DMSO at 280 nm wavelength. Mass spectra and HPLC characterization data are provided in **Table S1**.

Mass Spectrometry.

Mass spectra of positive ions were recorded either with a single stage reflectron MALDI-TOF mass spectrometer (Bruker Rexlex III, Bruker Daltonics, Billerica, MA; α- cyanocinnamic acid as a matrix) in reflectron mode or with a low resolution ESI mass spectrometer (Finnigan, Thermoquest LCQ ion trap instrument, Lake Forrest, CA) and/or using high resolution Fourier transform mass spectrometer (FT-ICR MS, Bruker Apex Qh, Bremen, Germany) equipped with an ESI source. For internal calibration, an appropriate mixture of standard peptides was used with an average resolution of ca. 10,000 on the Reflex III and 60,000 on the FT-ICR instrument.

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Table S1: High resolution mass spectral data and HPLC[a]

Com-pound	Structure	R_t (k')	Calc. $[MH^+]$	Exp. $[MH^+]$
1	4-phenylbutyryl-His-DPhe-Arg-Trp-NH_2	4.14	790.4147	790.5
2	Ac-Homophenylalanine-His-DPhe-Arg-Trp-NH_2	3.89	746.4362	746.5
3	4-hydroxycinnamoyl-His-DPhe-Arg-Trp-NH_2	2.54	790.3783	790.4
4	4-phenylbutyryl-His-DPhe-Arg-Trp-Gly-Lys(hex-5-ynoyl)-NH_2	4.32	1069.5730	1069.6
5	H-Tyr-Val-Nle-Gly-His-DNal(2')-Arg-DTrp-Asp-Arg-Phe-Gly-NH_2	3.61	1601.8124	1601.8
6	H-Lys(hex-5-ynoyl)-Tyr-Val-Nle-Gly-His-DNal(2')-Arg-DTrp-Asp-Arg-Phe-Gly-NH_2	3.90	1823.9493	1824.0
7	H-Tyr-Val-Nle-Gly-His-DNal(2')-Arg-DPhe-Asp-Arg-Phe-Gly-NH_2	3.41	1562.8015	1562.5
8	H- Lys(hex-5-ynoyl)Tyr-Val-Nle-Gly-His-DNal(2')-Arg-DPhe-Asp-Arg-Phe-Gly-NH_2	3.79	1784.9384	1784.3

[a]Peptide was eluted with a linear ACN/0.1% CF3CO2H aqueous gradient (10% to 90% in 30 min) at a flow rate of 0.3 mL/min); Waters XBridge C-18 column (3.0 x 150 mm, 3.5 µm); HPLC k' = (peptide retention time - solvent retention time)/solvent retention time. All the obtained purified peptides showed purity >95% by analytical HPLC.

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