SUPPORTING INFORMATION – ACS Med Chem Lett.

Multivalent Interactions: Synthesis and Evaluation of Melanotropin Multimers – Tools for Melanoma Targeting.

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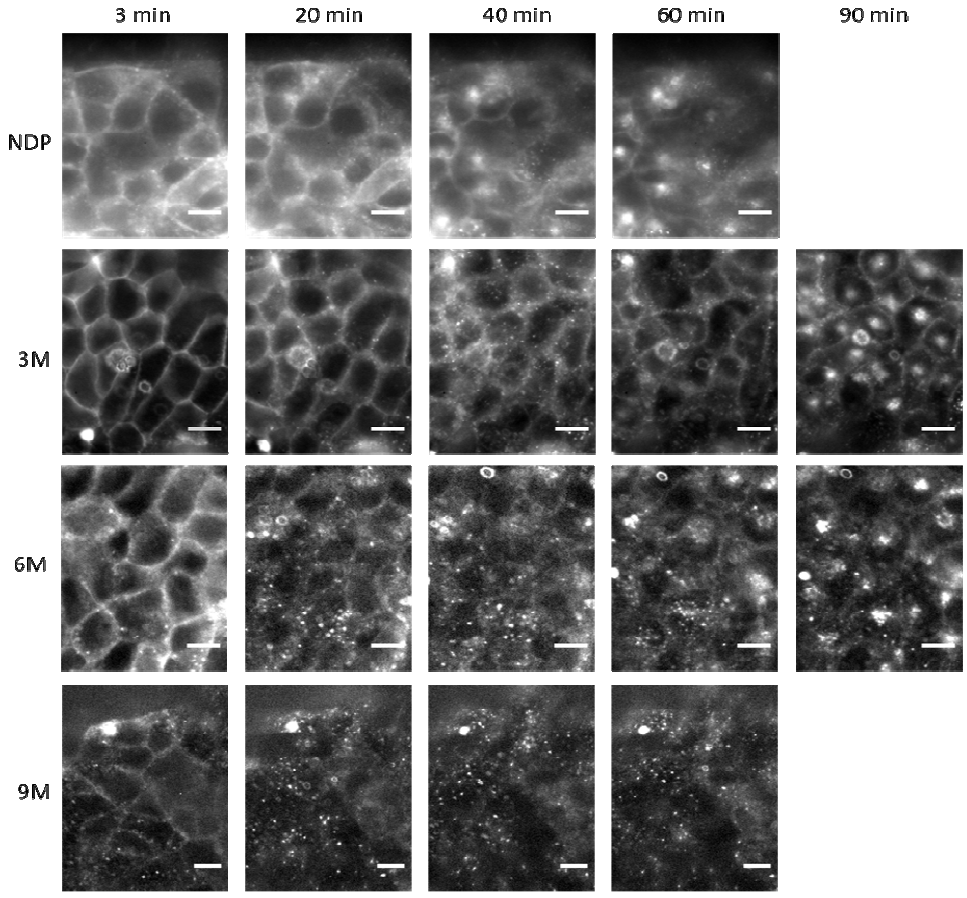
Time course.

Imaging was performed using an epifluorescence microscope. The imaging shown was performed until no more evolution was seen. At 3 minutes the ligands are at the cell surface and seem to internalize over time.



EXPERIMENTAL SECTION

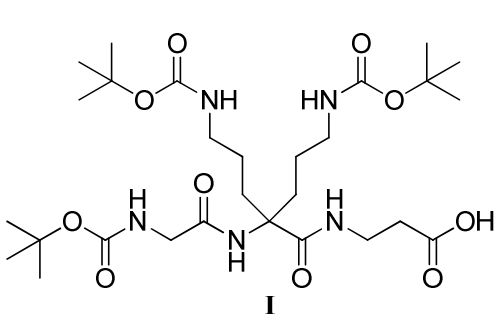
Materials and Methods.

Reagents and solvents used were reagent grade quality from commercial sources and used without further purification unless noted otherwise. Purifications by flash chromatography were performed on EMD Chemicals silica gel 60 (0.04-0.063mm). *p*-Methylbenzhydrylamine-resin (MBHA resin), (0.7 mmol/g) and Sieber amide resin (0.5 mmol/g) were purchased from ChemPep, and amino acids were purchased from ChemPep and Iris Biotech GmbH. Solid supported syntheses were performed on a Prelude synthesizer (Protein Technologies Inc.). Mass spectra were obtained with an ESI-mass spectrometer (Finnigan, thermoelectron, lcq classic), and high-resolution fast atom bombardment spectrometer (JEOL HX110 sector instrument) or by MALDI-TOF (Voyager DE-Pro). HPLC was performed on a Hewlett-Packard 1100 series liquid chromatograph (Agilent technologies) with a C8 column (SymmetryPrep, 0.78 x 30cm) or C18 column (Vydac, 1.0 x 25cm), separations were monitored at 230 and 280 nm and final purity was determined as ≥ 95% using a C18 Vydac column (0.46 x 25 cm) with a linear gradient from 0 to 100 % of MeCN, 0.1 % TFA in H_2O for the aqueous phase in 30 minutes at 1 mL/min. Reported retention times (t_r) were acquired using the same protocol.

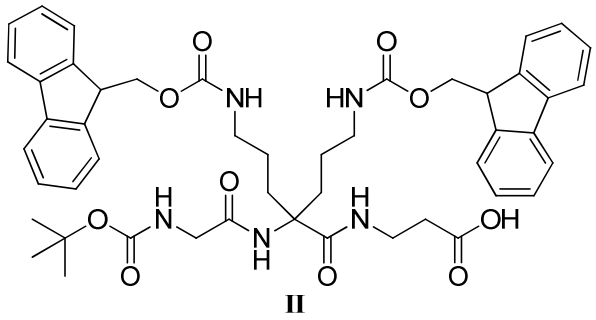
Synthesis.

The synthesis for compounds **A** (1M) and **B** (3M) was previously described[1]. The synthesis of compounds **C** (6M) and **D** (9M) was performed following the same procedure described in the previous paper thanks to scaffold I and II and the protected peptide (Figure 1), in which synthesis and characterization are also described. A scheme for the dendrimers synthesis can be found in Scheme 1.

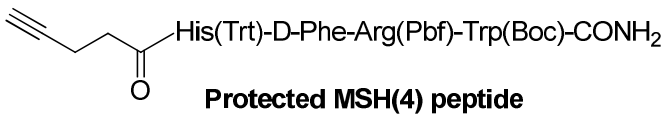
Figure 1. Scaffold I, II and protected MSH(4) peptide.



I

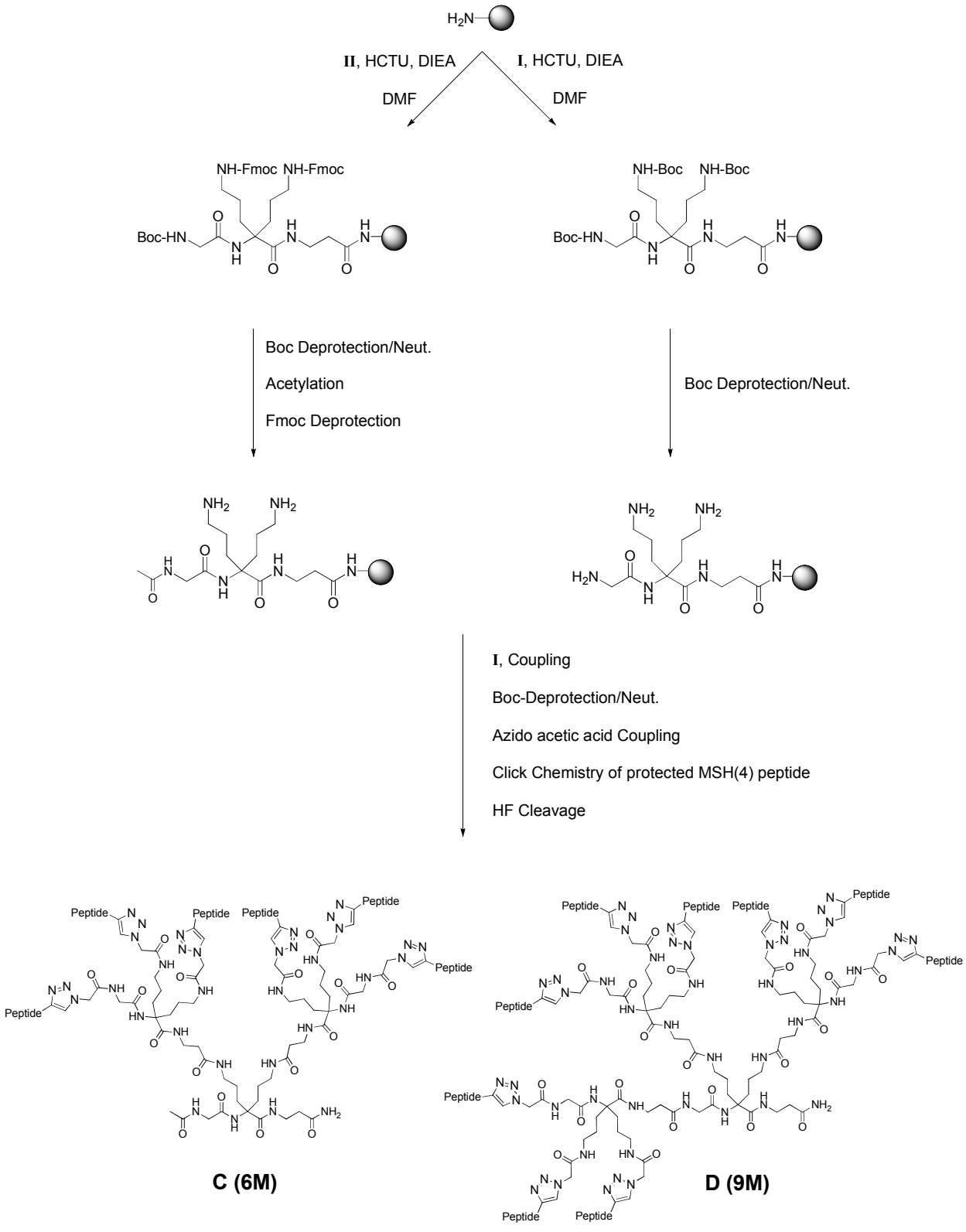


II



Protected MSH(4) peptide

Scheme 1. Synthesis of **C** and **D**.



General synthetic methods.

Coupling reactions were achieved using a 3-fold excess of amino acid, HBTU and HOBt in the presence of a 6-fold excess of DIEA in DMF (1 x 1 hr).

Boc removal was achieved using TFA (3 x 1 min) followed by DCM wash (5 x 2 min) and neutralization with 20% DIEA in DCM (2 x 2 min).

Acetylation was done with 20% acetic anhydride solution in the presence of 5% DIEA in DMF (2 x 20 min).

Fmoc removal was achieved using 20% piperidine in DMF (1 x 5 min and 1 x 20 min).

Click reaction was performed in the presence of the protected peptide (2 eq. *per* azido group), CuI (5 eq. *per* azido group), Ascorbic acid (5 eq. *per* azido group), DIEA (7 eq. *per* azido group) in a dry solution of DMF/2-6 Lutidine (8/2). The reaction is incubated until completion (monitored by IR or Kaiser Test in the presence of phenyl phosphine).

Alloc removal was performed using 0.3 eq. of $Pd(PPh_3)_4$ and 25 eq. of $PhSiH_3$ in dry DCM (2 x 30 min).

Cy5 attachment was done in DMSO after dissolution of the pure lyophilized compound at 5 mM followed by addition of 1 eq. of Cy5-NHS and 0.1 eq. of triethylamine. The reaction completion was monitored by HPLC. Once completion was reached the compound was purified by HPLC or gel filtration.

Synthesis and characterization of multimer C (6M)

The synthesis followed a Boc strategy and Fmoc strategy on MBHA resin. Coupling of compound **II** was performed followed by Boc removal and acetylation of the resulting free amine. Fmoc removal was then performed followed by coupling of **I**. Boc groups were removed and coupling of azido acetic acid was performed followed by the click reaction with the appropriate protected MSH(4) peptide. A final wash was performed using a cocktail containing 95 % TFA, 2.5 % TIS and 2.5 % H_2O (2 x 30 min) prior to proceeding to HF cleavage. Upon HF cleavage, the compound was purified by HPLC, purity >95% was confirmed by analytical HPLC and MS was performed to confirm the product molecular weight. t_r = 13.33 min; MS (MALDI): $[MH^+]$ calcd. for $C_{275}H_{357}N_{100}O_{46}$ = 5799.43 , found 5799.81.

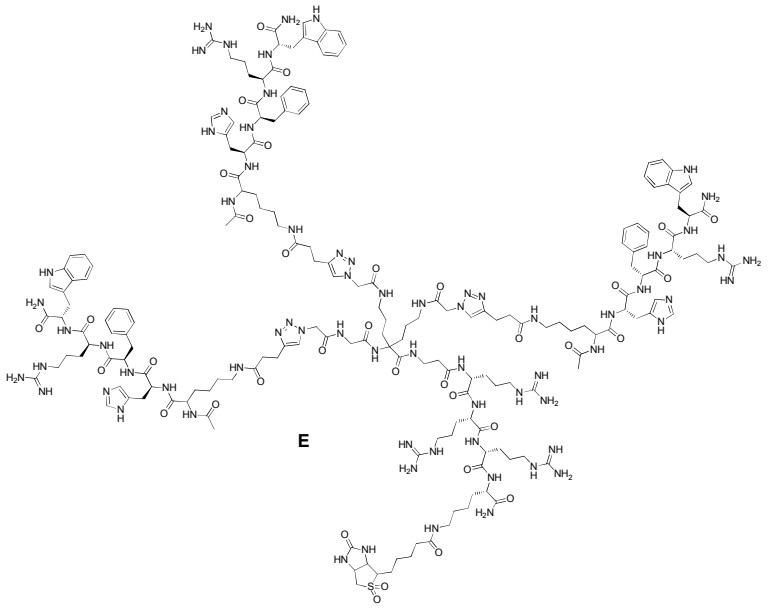
Synthesis and characterization of multimer D (9M)

The synthesis followed a Boc strategy. Coupling of compound **I** was performed followed by Boc removal and second coupling of **I**. Boc groups were removed and coupling of azido acetic acid was performed followed by the click reaction with the appropriate protected peptide. A final wash was performed using a cocktail containing 95 % TFA, 2.5 % TIS and 2.5 % (2 x 30 min) prior proceeding to HF cleavage. Upon HF cleavage, the compound is purified by HPLC, purity is confirmed by analytical HPLC and MS is performed to confirm the product molecular weight. tr = 13.66 min. MS (MALDI): [MH^+] calcd. for $C_{403}H_{518}N_{147}O_{66}$ = 8477.39 , found 8477.18.

Model molecule.

The model molecule **E** (Figure 2) is an MSH(4) trimer bearing a DArg-Arg-DArg-Lys linker and a biotin sulfone tag. The biotin sulfone was used as a substitute in place of the Cy5 dye that could potentially interfere with the readings of the TRF assay. The molecule was synthesized linearly following the established strategy by first inserting the amino acid composing the tag. t_r = 12.66 min. MS (MALDI): [MH^+] calcd. for $C_{188}H_{271}N_{70}O_{35}S$ = 4103.75 , found 4103.33.

Figure 2. Model Molecule **E**.



The model molecule **E** was assayed *via* TRF assay and an IC_{50} of 36 ± 3 nM was found, which is in the same order of magnitude as the corresponding trimer previously described[1]. Therefore, the

tag addition and its composition does not seem to affect significantly the binding, thus the sequence is selected for further use.

Synthesis and structure of molecules bearing a Cy5 tag.

Multimers bearing a Cy5 tag (Figure 3) were synthesized as followed:

The DArg-Arg-DArg-Lys sequence was first attached to the resin using regular Boc strategy. The lysine was Alloc protected to keep the synthetic scheme orthogonal. The appropriate scaffolds were then attached depending on the desired constructs and the multimer was synthesized following the established procedure (azido acetic acid attachment and click chemistry were performed). The Alloc group is then removed and the resin washed with a cocktail containing 95 % TFA, 2.5 % TIS and 2.5 % H_2O (2 x 30 min) prior to proceeding to HF cleavage. Upon HF cleavage, the compound was purified by HPLC and Cy5 attachment was performed. The Cy5 labeled multimers were purified again by HPLC and purity was confirmed by analytical HPLC. MS was performed to confirm the product molecular weight. (Cy5 labeled NDP was synthesized by addition of Cy5 onto the NDP sequence lysine side chain, no triarginine-lysine was added in this case.)

NDP-Cy5 (NDP*): tr =18.26 min. MS (ESI) $[M+3]^{3+}$ calcd. for $C_{110}H_{150}N_{23}O_{20}^{+}$ 704.3804, found 704.3801.

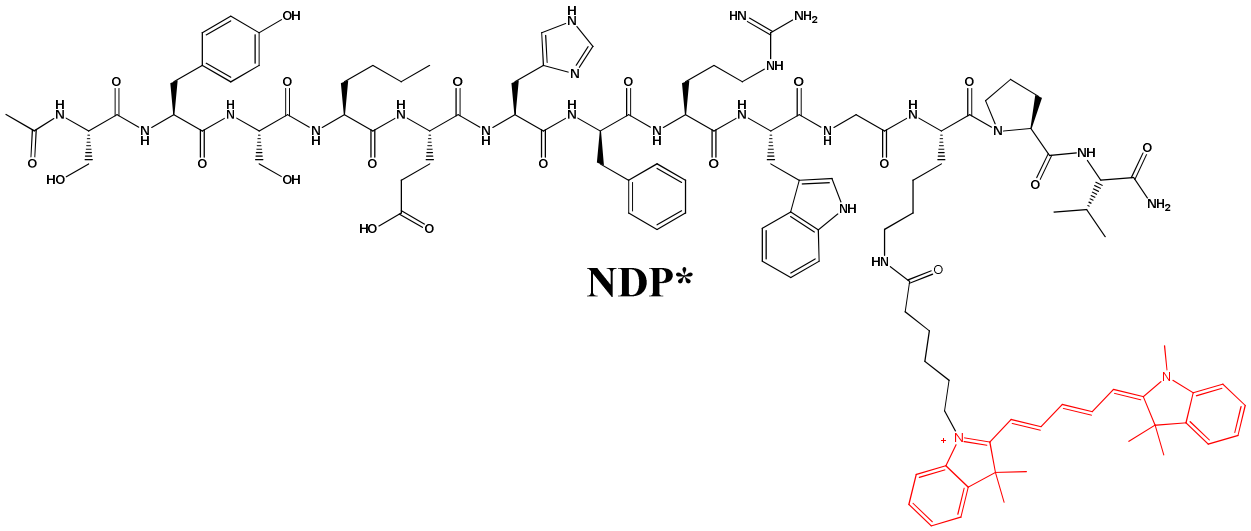
B-Cy5 (3M*): tr = 13.86 min. MS (ESI) $[M+7]^{7+}$ calcd. for $C_{186}H_{250}N_{64}O_{26}$ 543.5824, found 543.5812.

C-Cy5 (6M*): tr = 16.81 min. MS (ESI) $[M+10]^{10+}$ calcd. for $C_{331}H_{441}N_{116}O_{51}$ 686.9628, found 686.9637.

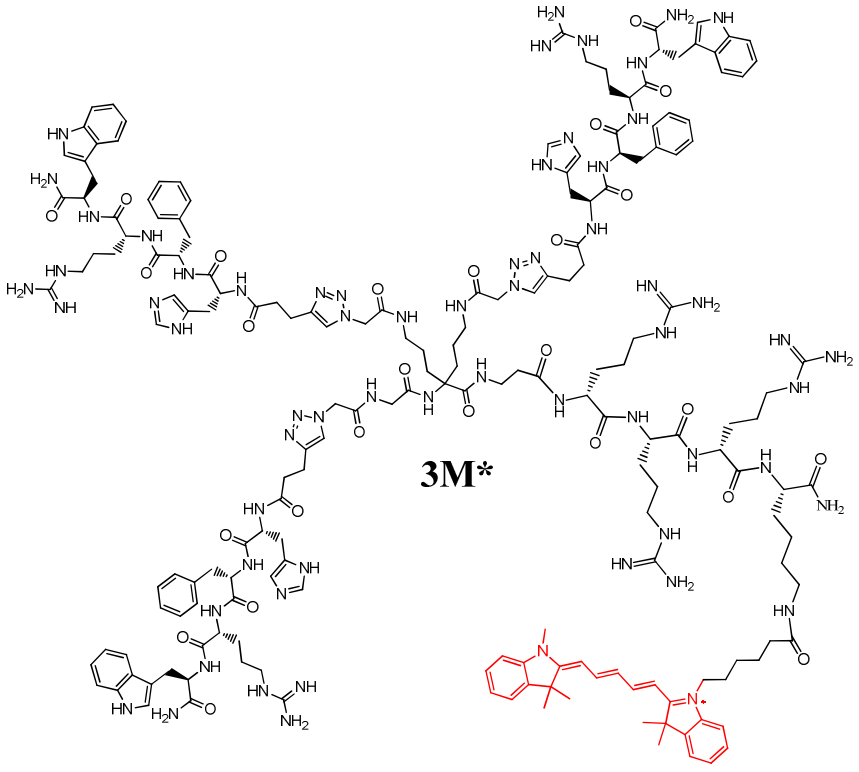
D-Cy5 (9M*): tr = 17.01 min. MS (MALDI) $[M+1]^{1+}$ calcd. for $C_{459}H_{602}N_{163}O_{71}$ 9539.8, found 9539.0.

Figure 3. Structure of investigated Cy5 labeled molecules.

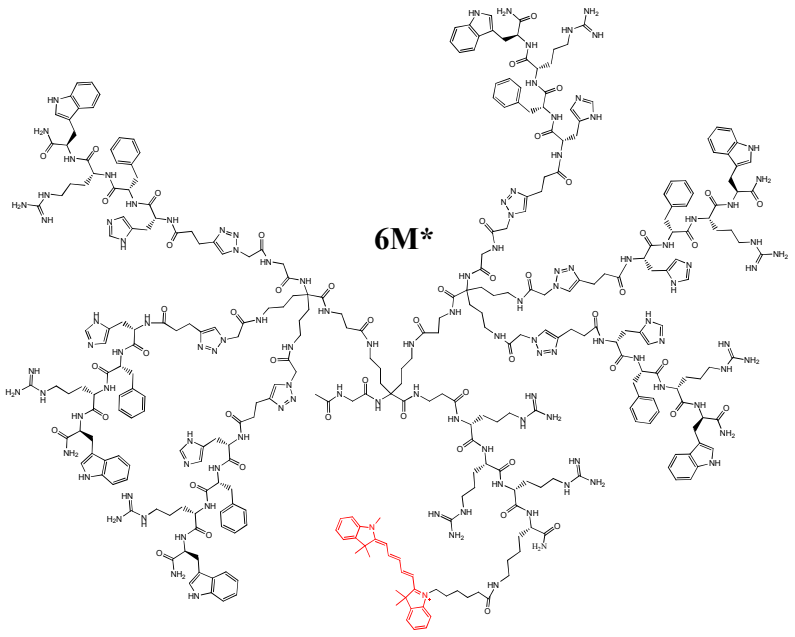
NDP-Cy5 (NDP*):



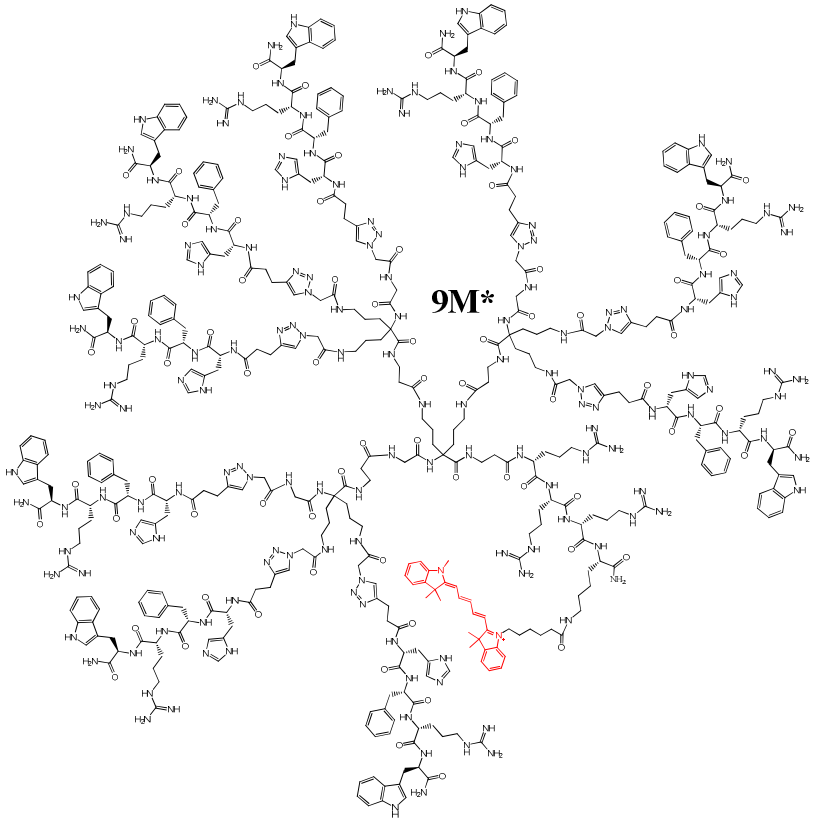
B-Cy5 (3M*):



C-Cy5 (6M*):



D-Cy5 (9M*):



Cell lines

HEK293 cells overexpressing the human MC4R were used to assess the affinity and the activity at the hMC4R, as previously described in detail[2]. A HEK293 cell clone overexpressing MC4R and the CCK2-R were used for cell viability assays and live cell imaging[2]. HEK293 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% SCS and 1% Penicillin-Streptomycin and were kept under standard conditions (37°C and 5% CO_2).

Time resolved fluorescence binding assays

HEK293 cells were seeded at 20000 cells *per* well into 96 well Costar 3603 plates 3 days before the experiment. Competition assays were performed in quadruplicate unless noted otherwise using a fixed concentration of Eu-NDP-α-MSH (10 nM, 50 μL *per* well) and 8 to 10 different concentrations of ligand. The day of the experiment, media was aspirated. Ligands were diluted in binding medium (DMEM, 1 mM 1,10-phenantroline, 200 mg/L bacitracin, 0.5 mg/L leupeptin, and 0.2 % bovine albumin serum) and added to the cells that were incubated for 2 h at 37°C. Following the incubation, cells were washed three times with wash buffer (DMEM, 20 μM EDTA, 0.2% BSA, 0.01% Tween 20), then enhancement solution (Delfia, Perkin Elmer) was added to the plates (100 uL *per* well) and incubated for 30 min at 37°C. The plates were read on a Wallac Victor instrument using the standard Eu(III) TRL measurement (340 nm excitation, 400s delay and emission collection for 400s at 615 nm), as previously described in detail[1-4]. Competitive binding data were analyzed with GraphPad Prism software using nonlinear regression analysis and fitted to a classic one site binding competition equation.

cAMP biological response immunoassays

HEK293 cells were seeded at 20000 cells *per* well into 96 well Costar 3598 plates 3 days before the experiment and incubated at 37°C in 5% CO_2. Assays were performed in triplicate in 3 different experiments. Media were aspirated and cells were pre-incubated with 1 mM 3-isobutyl-1-methylxanthine (IBMX) in DMEM at 37°C for 10 min. 30 μL of compounds and standards were added to the cells and 8 concentrations of ligand were tested (10^{-6} to 10^{-11}). Cells were incubated for 15 min at 37°C for all test agents and controls. cAMP production was measured using a chemiluminescent immunoassay kit as described in detail in the user manual (Invitrogen C10558). Briefly, cells were incubated in 60 μL of lysis buffer (provided with the cAMP assay kit) for 25 min at 37°C then transferred to precoated 96 well plates. To these plates were added

30 μL of cAMP-AP (alkaline phosphatase conjugate) and 60 μL of anti-cAMP antibody. The plates were incubated at room temperature for 1 hr and washed 5 times with wash buffer. Enhancement solution (CSPD substrate / Saphire II enhancer) was added to the plates following by incubation at room temperature in the dark for 30 min. Intensity of the signal was measured using a Wallac Victor instrument and the concentrations of cAMP were calculated after the average value of the basal condition was subtracted and based on a standard curve that was acquired during the same assay.

Live cell imaging and 3D deconvolution microscopy.

HEK293 cells were plated onto sterile #1 25 mm round cover slips, and incubated in individual cells of a 6 well plate in culture media until a confluence of 80 % was reached. The day of the experiment, a coverslip was transferred into a sterile petri dish and rinsed using a prewarmed 37^{o}C solution of Hank's buffered salt solution (HBSS). The cover slip was then transferred into a chamber containing 500 μL of HBSS (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$) and heated at 37^{o}C on the stage of an Olympus IX-71 microscope. The microscope was equipped with a 60 1.4 NA objective, and a 100 W Hg lamp as an excitation source. The focusing mechanism is motorized and axial position modulated using an infra-red feedback loop. Cell images were brought to focus using the cell autofluorescence signal at λ = 530 nm (480 nm excitation). The Cy5 autofluorescence was then recorded at λ = 640 nm (610 nM excitation) to confirm a low background. Images for the initial time course studies were acquired as single frames. Once specific time points were determined, 3D image acquisition was performed using 0.25 μM axial displacement between images; 10 image planes were acquired from the bottom to the top of the cell monolayer. The desired labeled ligand was dissolved in a pre-warmed HBSS at the desired concentration and added to the heated chamber and incubated for 2 minutes. The solution was then aspirated and the cover slip was washed twice with pre-warmed HBSS to remove unbound material. In the experiments where the FM 1-43 dye was used to identify the position of the cell limiting membrane, 2 μL of the stock solution was added 2 min prior to the desired imaging time.

To reduce the blur that comes with imaging fluorescence with high numerical aperture objectives, deconvolution was performed on 3-dimensional images, as previously detailed[5, 6].

Briefly, an empirical point spread function of the imaging light path is acquired using fluorescent microspheres for each of the filter sets used to acquire 3D distribution data. With knowledge of the PSF and 3D fluorophore distribution, an interactive approach with a non-negativity constraint is employed for deconvolving the blur within the 3D data stack[5].

Cell viability assay

HEK293 cells were plated on 6 well plates and grown to 80% confluence. The day of the assay, the media was aspirated and 2 mL of media containing ligands at varying concentrations were added as an array to the wells. At least well in which only fresh media was added was used as control. Cells were incubated for the desired times (1 hr, 24 hr, 48 hr) at 37^{o}C in 5% CO_2. The media was then aspirated and cells were harvested by incubation with 200 μL of warm 0.25 % Trypsin-EDTA for 2 minutes at room temperature followed by the addition of 2 mL of warm fresh media. The cells were transferred into a sterile centrifuge tube and centrifugation was performed for 2 minutes. The media was aspirated and the cell pellet was suspended back into 200 μL of media. 20 μL of the cell suspension and 20 μL of 0.4 % trypan blue were mixed and transferred into a hemocytometer. Live cells (yellow) and dead cells (blue) were counted to provide the % of viability. The data was then reported as a % of control.

References

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