Supporting Information:

Melanocortin Antagonist Tetrapeptides with Minimal Agonist Activity at the Mouse Melanocortin-3 Receptor

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Peptide Synthesis. The peptides were synthesized using a manual microwave peptide synthesizer (Discover SPS System, CEM Corporation, Matthews, NC) applying standard solid-phase Fmoc methodology.[1, 2] Fmoc-Nω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-arginine [Fmoc-Arg(Pbf)-OH], Fmoc-$N_{(in)}$-tButyloxycarbonyl-tryptophan [Fmoc-Trp(Boc)-OH], Fmoc-Phenylalanine (Fmoc-Phe-OH), Fmoc-3-(2-naphthyl)-L-alanine [Fmoc-Nal(2')-OH], Fmoc-3-(2-naphthyl)-D-alanine [Fmoc-DNal(2')-OH], and N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were purchased from Peptides International (Louisville, KY). Fmoc-D-4-iodo-phenylalanine [Fmoc-(pI)DPhe-OH], Fmoc-L-4-4'-Biphenylalanine (Fmoc-Bip-OH), and Fmoc-L-tetrahydroisoquinoline-3-COOH (Fmoc-Tic-OH) were purchased from Synthetech (Albany, OR). Fmoc-β-(3-benzothienyl)-alanine (Fmoc-3Bal-OH) was purchased from Bachem (Torrance, CA). Anhydrous ethyl ether, methanol (MeOH), Acetonitrile (ACN), dichloromethane (DCM), and acetic anhydride were purchased from

Fisher (Fair Lawn, NJ). 1,2-Ethanedithiol (EDT) and triisopropylsilane (TIS) were purchased from Aldrich (Milwaukee, WI). The reagents N,N-dimethylformamide (DMF), piperidine, pyridine, trifluoroacetic acid (TFA), and N,N-diisopropylethylamine (DIEA) were purchased from Sigma-Aldrich (St. Louis, MO). All reagents were ACS grade or better and used without further purification.

The peptides were synthesized on rink-amide-MBHA resin (0.37 mmol/g substitution) purchased from Peptides International. Each peptide was synthesized individually using a 25 mL reaction vessel fitted with a coarse frit (CEM Corporation). Approximately 270 mg (0.1 mmol) of resin was added to the reaction vessel and allowed to swell in DCM for two hours. After swelling the resin it was then continually stirred by bubbling nitrogen gas through the mixture. The resin was washed in DMF (5 times, 15 mL, mixed 1 min) and subsequently deprotected with the addition of 15 mL 20 v/v % piperidine in DMF deprotection solution to the reaction vessel that was stirred for 2 minutes at room temperature. The reaction vessel was then drained by vacuum and an additional 15 mL of deprotection solution was added. The vessel was placed in the microwave and heated (75 °C, 30 W, 4 min). Following a positive Kaiser test,[3] the first amino acid residue was coupled. The general protocol for the growing peptide chain began with the addition of a 3.1-fold excess of the Nα-Fmoc-protected amino acid (5.1-fold for Arg) and a 3-fold excess of HBTU (5-fold for Arg) both dissolved in DMF. A 5-fold excess of DIEA (7-fold for Arg) was added, and the reaction was placed in the microwave synthesizer and heated (75 °C, 30 W) for 5 minutes (10 min for Arg). Following a negative Kaiser test,[3] the cycle was repeated beginning with the room temperature piperidine deprotection until the final amino acid was added according to the

desired peptide sequence. Following a final deprotection cycle and positive Kaiser test, the peptide was acetylated with the addition of 15 mL acetic anhydride and 5 mL pyridine, and mixed for 30 minutes at room temperature. Following a negative Kaiser test, the peptide was washed in DCM (5 times, 15 mL, mixed 1 min) and dried overnight in a desiccator. Side chain deprotection and peptide cleavage from the resin was performed by mixing the dried peptide-resin with 8 mL of cleavage cocktail (91% TFA, 3% EDT, 3% TIS, and 3% water) for a minimum of 2 hours. The peptides and cleavage solution were drained from the reaction vessel into a pre-weighed 50 mL conical tube. The cleaved resin was rinsed with an additional 2 mL of cleavage cocktail and mixed for 1 minute, the solution was drained into the conical tube, and the peptides were precipitated using cold (4 °C) anhydrous diethyl ether (up to 45 mL). The turbid mixture was placed in an ice bath for 30 minutes, followed by centrifugation t 4 °C and 4000 RPMs for 4 minutes (Sorval Super T21 high-speed centrifuge, swinging bucket rotor). The supernatant was decanted off, the crude peptide was then washed with cold (4 °C) diethylether, and again pelleted. The washing process was repeated for a total of three times. The pellet was dried in vacuo overnight. A 10-20 mg sample of the crude peptide was purified using a Shimadzu RP-HPLC gradient system with a photodiode array detector and a semipreparative RP-HPLC C18 bonded column (Vydac 218TP1010, 1 cm x 25 cm). A typical purification RP-HPLC gradient ranges from 30% to 75% acetonitrile in water with 0.1% TFA over 15 minutes. Collection times and gradients varied dependent upon impurities found in a particular crude sample. After purification the peptide was then lyophilized. Analysis of the purified peptides by analytical RP-HPLC (Vydac 218TP104, 4.6 mm x 25 cm) indicated their purity was 95% or greater and ESI-

MS (University of Minnesota Department of Chemistry Mass Spectrometry Laboratory) indicated they had the correct molecular mass.

Table analytical data for the peptides synthesized in this study.

Analog	Structure	HPLC k' (system 1)	HPLC k' (system 2)	M+1 (calcd)	Mass spectral analysis (M+1), purity %
1	Ac-Trp-(pI)DPhe-Arg-Trp-NH_2	7.3	11.1	861.27	861.29, >97%
2	Ac-Trp-(pI)DPhe-Arg-Bip-NH_2	8.5	12.7	898.29	898.30, >95%
3	Ac-Trp-(pI)DPhe-Arg-3Bal-NH_2	7.7	12.0	878.23	878.26, >97%
4	Ac-Trp-(pI)DPhe-Arg-Tic-NH_2	7.4	11.3	834.25	834.32, >96%
5	Ac-Trp-(pI)DPhe-Arg-Phe-NH_2	7.2	11.0	822.25	822.38, >95%
6	Ac-Trp-(pI)DPhe-Arg-Nal(2')-NH_2	8.0	12.2	872.27	872.26, >96%
7	Ac-Trp-(pI)DPhe-Arg-DNal(2')-NH_2	7.8	11.7	872.27	872.28, >96%

HPLC k' = (peptide retention time - solvent retention time) / solvent retention time. System 1 is a 10% to 90% gradient of acetonitrile in water containing 0.1% triflouroacetic acid over 35 minutes, and system 2 is the same gradient with methanol replacing acetonitrile. The HPLC method is run on a 250 mm x 4.6 mm C18 column (Vydac 218TP C18 10 μ) with a flow rate of 1.5 mL/min. Product purity is determined using system 2 and integrating the area under the curves of the spectra collected at 214 nm.

Functional Assay. HEK-293 cells were stably transfected with the mouse melanocortin receptors using a $pCDNA_3$ expression vector using a calcium phosphate method and G418 selection as previously reported by our laboratory.[4] Stably transfected HEK-293 cells expressing the mouse melanocortin receptors were transiently transfected with 4 μg of CRE-PBKS per 10 cm plate using the calcium phosphate crystal method.[5] Briefly, $1x10^6$ to $3x10^6$ cells were plated into 10 cm dishes 24 hours prior to the transfection such that the confluency was approximately 40% at the time of transfection. The cells were transfected with a N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffered saline solution containing 0.125 M $CaCl_2$ and 4 μg of CRE/β-galatosidase reporter gene construct and the plates were then incubated at 35°C and 3% CO_2 overnight. Twenty-four hours posttransfection the cells were plated onto collagen treated Nunclon Delta Surface 96 well plates (Thermo Fischer Scientific) and incubated at 37 °C and 5% CO_2 overnight. Forty-eight hours post-transfection the cells were stimulated with 50 μL of

peptide (10^{-4} to 10^{-10} M) or forskolin (10 μM) in assay media (1.0 mL 1% bovine serum albumin [BSA] in phosphate buffered saline [PBS] and 1.0 mL 100x isobutylmethylxanthine in 98.0 mL Dulbecco's Modified Eagle Medium [DMEM]) for 6 hours at 37 °C and 5% CO_2. The assay media was aspirated and 50 μL of lysis buffer (250 mM Tris-HCl pH=8.0, Triton X-100 in water) was added and the plates stored at -80 °C for up to 2 weeks. The plates were thawed and 10 μL sample of cell lysate was removed from each well and put into another 96 well plate for relative protein level determination. To each well 40 μL 0.5% BSA in PBS was added, followed by, the addition of 150 μL of β-galatosidase substrate solution (60 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM KCl, 50 mM 2-mercaptoethanol, and 660 μM 2-nitrophenyl β-D-galactopyranoside [ONPG]). The absorbance was read on a 96 well plate reader (Molecular Devices) at λ = 405 nm. The relative protein levels were determined through the addition of 200 μL of BioRad protein dye solution (1 dye: 4 water per manufacturer's instructions) to the 10 μL sample of cell lysate; the absorbance was recorded at λ = 595 nm on a 96 well plate reader. The β-galatosidase activity data was normalized to both protein levels and the forskolin positive controls, plotted in PRISM (v4.0, GraphPad Inc.), fitted with a sigmoidal dose-response curve, and EC_{50} values were determined. Each experiment consisted of duplicate replicates and was performed in at least three independent experiments. The values reported represent the mean with the associated SEM.

Mouse vs Human MC4R Receptor Selectivity

The question always present in scientists' minds, what is the correlation between rodent/mouse and human data? We speculate the results from this study identifying ligands for the mouse melanocortin receptors would have similar pharmacological profiles when assayed at the human melanocortin receptors, but this would need to be verified experimentally. Generally, the potency and selectivity profiles observed for peptidic ligands assayed at the mouse melanocortin receptors have been reported in the literature to possess similar SAR profiles at the human receptors. This is supported by data reported by numerous studies including both MC4R peptide ligand SAR as well as receptor mutagenesis studies. The melanocortin agonist MTII has single digit nM potencies when assayed at the hMC3R, hMC4R, and hMC5R is comparable to the subnanomolar potencies reported herein.[6] The MC3R/MC4R antagonist SHU9119 has comparable pA_2 values at the hMC3R and hMC4R (pA_2 = 8.3 and 9.3, respectively); in addition, partial agonist activity is also observed at the hMC3R when SHU9119 is assayed alone.[7] The tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 has been used in previous studies probing the function of the human MC4R, and the EC_{50} determined for the mouse MC4R in this study is comparable to the human receptor subtype[8] has a EC_{50} = 8.6 ± 0.9 nM for hMC4R,[9] has a EC_{50} = 10.2 ± 1.44 nM for mMC4R,[10] and has a EC_{50} = 0.93 ± 0.31 nM for hMC4R.

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