Structure-Activity and Mutation Studies of the Human Melanocortin System and Purification of Recombinant E. Coli TOPO 10 Plasmid cDNA

by

Morgan Robert Zingsheim
Project Advisor: Minying Cai

A Thesis Submitted in Partial Fulfillment of the Bachelors of Science degree
With Honors in
Biochemistry & Molecular Biophysics and Molecular & Cellular Biology

Department of Biochemistry & Molecular Biophysics
THE UNIVERSITY OF ARIZONA
MAY 2008

Approved by:

Dr. Victor Hruby Faculty Advisor	Undergraduate Committee Member
Date:	Date:

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ACKNOWLEDGEMENTS

This work was supported by grants from the National Institute of Health and by the US Public Health Service. I would like to thank Dr. Victor J. Hruby, Dr. Minying Cai, Mattew Dedek, and Erin Palmer. I would also like to thank Carol Bender and the Undergraduate Student Research Program. Without this program, I would not have been able to participate in the Hruby Lab. I would also like to thank Olivia Mendoza, Dr. Hazzard, and Michelle Brandon. I also appreciate the financial assistance that the University of Arizona has provided.

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ABSTRACT

The melanocortin receptors (MCRS) play a fundamental role in human behavior such as satiety, feeding, sexual and more. This causes these receptors to be targets for treating diseases such as anorexia and diabetes. The goal is to discover and synthesize peptides that are potent and selective agonists/antagonists for the MCRs. This project analyzed the BJM, JBN and QU compound series for these properties. BJM compounds do not bind to the Melanocortin Receptors. The JBN compounds show that the TNF-α antagonists derivatives bind to the MCRs. The QU compounds illustrate that small changes in the stereochemistry of the SH9119 and MT-II derivatives cause drastic changes in the binding and the agonistic/antagonist properties of the compounds. Many of the results in this study are preliminary and require repeats.

The second portion of this thesis is to isolate β-Arrestin 1-GFP recombinant E. Coli TOPO 10 cDNA. Agarose gel electrophoresis indicates a successful isolation of the cDNA, and now awaits sequence verification.

The final portion of this thesis is to determine the intracellular phosphorylation sites and amino acids of the intracellular loop/carboxyl terminus of hMC1R necessary for adenylate cyclase activation. The data was inconclusive due to the high expression levels of MCRs and requires repeats.

ABBREVIATIONS

α-MSH	α-melanotropin
β-MSH	β-melanotropin
γ-MSH	γ-melanotropin
ACTH	corticotropin
AgRP	Agouti-related protein
ASP	Agouti signaling protein
BP	Base pairs
cAMP	Adenosine 3',5'-cyclic monophosphate
CPM	Counts per minute
EDTA	Ethylene diamine tetraacetic acid
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
HEK293	Human embryonic kidney 293 (cell line)
HMCR	Human melanocortin receptor
IBMX	Isobutylmethylxanthine
MAP kinase	Mitogen-activated protein kinase
MCR	Melanocortin receptor
MSH	Melanocyte stimulating hormone
MT-II	Melanotan-2
NDP-MSH	[Nle4, DPhe7]-α-melanocyte stimulating hormone
PKA	Protein Kinase A
PKC	Protein Kinase C
POMC	Pro-opiomelanocortin
SAR	Structure activity study
TNF-α	Tumor necrosis factor-alpha

EXPERIMENTAL GOALS OF THESIS

Experimental Goal 1: Determine the MCR subtype selectivity and/or adenylate cyclase activation of BJM, JBN, and QU compounds.

Newly synthesized peptides and peptide mimetics were assessed for their ability to bind to the melanocortin receptors and activate adenylate cyclase. These peptides contain variations in their stereochemistry, and are used to determine which features are necessary for the binding and activation of the melanocortin receptors. This data can then be used to develop a better understanding of the melanocortin receptors' pharmaphores.

Experimental Goal 2: Isolate and visually confirm recombinant E. Coli TOPO 10 cDNA.

pcDNA 3.1+ DNA containing β-Arrestin-GFP was transfected into E. Coli TOPO 10. The recombinant cDNA was isolated and the molecular weight was qualitatively identified through agarose gel electrophoresis. This cDNA will be later verified by DNA sequencing and transfected into HEK293 cells for SAR studies.

Experimental Goal 3: Determine the intracellular phosphorylation sites and amino acids of intracellular loop/carboxyl terminus that are necessary for adenylate cyclase activation in MCRs.

Preliminary data from the adenylate cyclase biological activity assay on MCR mutants was inconclusive. cAMP generated in the assays was excessively high for all concentrations of the peptides tested. This is most likely due to the high expression levels of the receptors in the HEK293 cells, and requires repeat experiments at lower concentrations of peptides.

INTRODUCTION

Dr. Hruby's lab group focuses on the synthesis of peptides that are selective for the melanocortin receptors. The melanocortin receptors are members of the G-protein-coupled receptor family and are activated by the melanotropic peptides, such as melanocyte-stimulating hormones (MSHs) and corticotropin (ACTH)[1]. As of today there are 5 known receptors, known as Melanocortin-1 through Melanocortin-5 (this lab group focuses on the Melanocortin receptors 1, 3, 4 and 5). Each of these receptors has an important and specific biological role. The human Melanocortin-1 receptor (hMC1R) is found in skin cells and participates in skin pigmentation. The human Melanocortin-3 (hMC3R) receptor is present in the brain and is responsible for obesity regulation by the way of energy homeostatis and feeding behavior. The human Melanocortin-4 (hMC4R) receptor is also located in the brain and is related to hMC3R in function, but is also linked to sexual dysfunction. Finally, the human melanocortin-5 (hMC5R) receptor affects thermoregulation and is linked to regulation of endocrine function ([2,3,4,5,6,7]).

The primary melanocortin agonists consist of four peptides that are derived from a single protein, the proopiomelanocortin (POMC) gene product[8,9]. This POMC protein is processed and cleaved into seven peptide hormones. The processing consists of N-terminal acetylation, C-terminal amidation, glycosylation

and is done by prohormone convertase, as well as several others[10]. The endogenous agonists are called α-melanotropin (α-MSH), β-melanotropin (β-MSH), γ-melanotropin (γ-MSH) and ACTH, all of which contain His-Phe-Arg-Trp ([2, 11]). (see Figure 1[12])

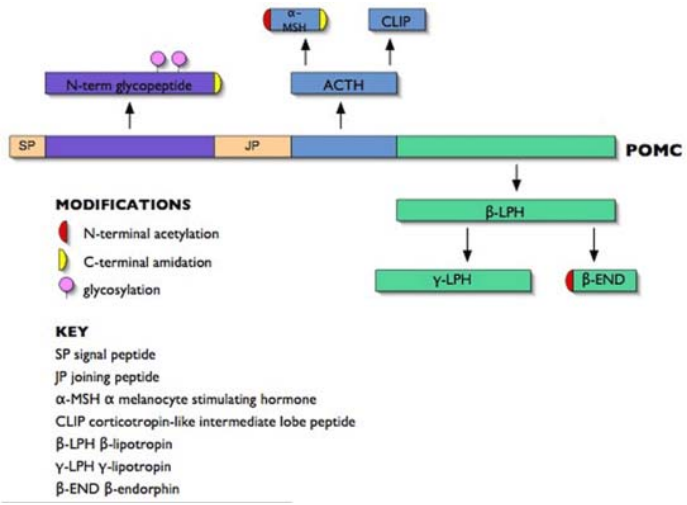


Figure 1: POMC Gene Products. The endogenous melanocortin receptor agonists are all cleaved from POMC.

The POMC protein products react with the melanocortin receptors with various levels of binding affinity and intracellular response[13]. The melanocortin receptors also respond to their endogenous antagonists which are agouti signaling protein (ASP) and agouti-related protein (AgRP).

When the melanocortin receptors are activated they primarily stimulate the cyclic adenosine monophosphate (cAMP) pathway via the G protein G_s. While this is their primary signaling route, recent research as shown that other signaling

pathways can be activated, such as the PKC pathway[14], MAP kinase[15], inositol trisphosphate[16] and others. After the melanocortin receptors have been stimulated, they are recruited by β-Arrestin mediated internalization via clathrin coated pits.

In order to determine the selectivity and potency of the peptides, and peptide mimetics, multiple bioassays are used. The whole binding assay is used to determine the binding character of the peptide analogue. This assay uses competitive binding between the ligand of interest and radioactively tagged ^{125}I-tagged α-MSH on a specific cell line. After determining if the analogue binds, a measurement of the amount of cAMP expressed in the cells to determine the potency of the ligand. The cAMP assay has the ligand interact with the receptor and then cAMP and 3H-cAMP are competitively bound to PKA to determine the cAMP accumulation as a result of the binding of the peptide to the receptor.

A final assay, the PA_2 assay will be performed. The PA_2 assay is a modified cAMP assay and essentially follows the same steps. The PA_2 assay will identify a receptor antagonist as competitive or non-competitive. A competitive antagonist occupies the same receptor binding pocket as the endogenous agonist while a non-competitive antagonist binds the receptor 'allosterically,' that is, at a location distinct from the agonist binding pocket.

MATERIALS AND METHODS

2.1 List of Peptides Tested

Peptides and peptide mimetics listed bellowed were evaluated for their ability to displace radioactively labeled [^{125}I]NDP-MSH and stimulate adenylate cyclase.

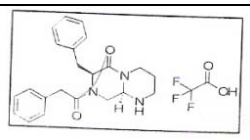
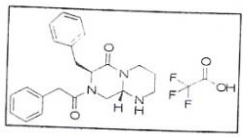
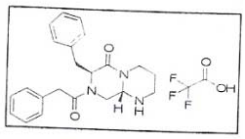
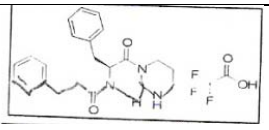
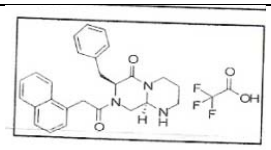
BJM Compounds		
Code	No.	Strucutre
BJM4007-1	1	
BJM4007-2	2	
BJM4008-1	3	
BJM4009-1	4	
BJM4010-1	5	

Table 1: BJM Compounds. These compounds were tested for their ability to bind to MCRs.

JBN Compounds		
Code	No.	Sequence

JBN51	6	c[{CH2C(O)}2-DNal-R-W-K]-G-K-P-V-NH_2
JBN61	7	c[{CH2C(O)}2-DNal-R-W-K]-A-K-P-V-NH_2
JBN63	8	c[{CH2C(O)}2-DNal-R-W-K]-G-A-K-P-V-NH_2
JBN69	9	c[{CH2C(O)}2-DNal-R-W-K]-β-A-K-P-V-NH_2
JBN95	10	Ac-Nle-c[D-H-DNal-R-W-K]-G-K-P-V-NH_2
JBN97	11	Ac-Nle-c[D-H-DNal-R-W-K]-A-K-P-V-NH_2
JBN99	12	Ac-Nle-c[D-H-DNal-R-W-K]-A-G-P-V-NH_2

Table 2: JBN Compounds. These TNF-α peptide derivatives were tested solely for their ability to bind to the MCRs.

QU Compounds		
Code	No.	Sequence
QU4050	13	Ac-Nle-c[Asp-His-D-Phe-Pro-Trp-Lys]-NH_2
QU3059	14	Ac-Nle-c[Asp-His-D-Phe-Trans-Xaa-Trp-Lys]-NH_2
QU4001	15	Ac-Nle-c[Asp-His-D-Phe-Cis-Xaa-Trp-Lys]-NH_2
QU3078	16	Ac-Nle-c[Asp-Trans-Xaa-D-Phe-Arg-Trp-Lys]-NH_2
QU4002	17	Ac-Nle-c[Asp-Cis-Xaa-D-Phe-Arg-Trp-Lys]-NH_2
QU4051	18	Ac-Nle-c[Asp-His-D-Nal(2')-Pro-Trp-Lys]-NH_2
QU3082	19	Ac-Nle-c[Asp-His-D-Nal(2')-Trans-Xaa-Trp-Lys]-NH_2
QU4004	20	Ac-Nle-c[Asp-His-D-Nal(2')-Cis-Xaa-Trp-Lys]-NH_2
QU3083	21	Ac-Nle-c[Asp-Trans-Xaa-D-Nal(2')-Arg-Trp-Lys]-NH_2
QU4005	22	Ac-Nle-c[Asp-Cis-Xaa-D-Nal(2')-Arg-Trp-Lys]-NH_2
QU4052	23	Ac-Tyr-Val-Nle-Gly-His-D-Phe-Pro-Trp-Asp-Arg-Phe-Gly-NH_2
QU4006	24	Ac-Tyr-Val-Nle-Gly-His-D-Phe-trans-Xaa-Trp-Asp-Arg-Phe-Gly NH_2
QU4007	25	Ac-Tyr-Val-Nle-Gly-His-D-Phe-cis-Xaa-Trp-Asp-Arg-Phe-Gly-NH_2

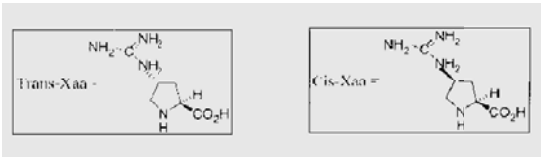


Table 3: QU Compounds. These SHU9119 and MT-II derivatives were tested for their ability to bind to MCR and stimulate the MCR cAMP response.

Cell Lines

hMC1R	In skin, involved with skin pigmentation
hMC3R	Influences obesity regulation though energy homeostasis
hMC4R	Influences feeding, regulating weight and sexual behavior.
hMC5R	Influences thermoregulation and endocrine function

2.2 Materials

The cell culture reagents were purchased from GIBCO (Carlsbad, CA) or Sigma (St. Louis, MO). The cell culture flasks and dishes were purchased from Nalgene (Rochester, NY), Corning (Lowell, MA), or BD Falcon (San Jose, CA). The HEK-293 cell line was purchased from ATCC (Manassas, VA). [^{125}I]NDP-MSH was purchased from Perkin-Elmer (Perkin-Elmer Life Science, Freemont, CA). FuGene 6 transfection agent was bought from Roche (Palo Alto, CA). Unless stated, the chemicals listed below were purchased from Sigma.

2.3 cDNA Plasmids and Mutants

B-Arrestin 1-GFP cDNA and Human Melanocortin Receptors 1, 3, 4 and 5 in the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA) were purchased from the Missouri University of Science and Technology cDNA Resource Center. The plasmid cDNA in the recombinant TOPO 10 E. coli mutants was purified according to the kit instructions of the EndoFree® Plasmid Maxi Kit (QIAGEN, Valencia, CA) or the QIAprep® Spin Miniprep Kit. For the purified β-Arrestin 1-GFP cDNA, a 5 uL sample of plasmid cDNA was visualised by agarose gel electrophoresis in a 2%

agarose gel containing ethidium bromide. The gel was ran at 120 volts for 30 minutes and photographed under UV illumination. The 1Kb Plus DNA Ladder (Biorad, Hercules, CA) was used for molecular weight comparison. The sequences of plasmid cDNA strands were verified at the University of Arizona Genomic Analysis and Technology Core by a T7 forward primer and BGH reverse primer (Invitrogen).

2.4 Cell Culture

The melanocortin receptors were transfected into Human Embryonic Kidney (HEK293) cells for biological assay use. These cells were cultured in tissue culture flasks and are grown in MEM whole medium (containing 1mM sodium pyruvate (GIBCO), 100 units/mL penicillin and streptomycin, 10% fetal bovine serum, and buffered at pH 7.4 by sodium bicarbonate). All transfected cell lines had constant selection pressure of 500µg/mL of G418 (a neomycin analogue). The cell cultures were grown in an incubator and regulated at 37 ºC, 95% RH, and 5% CO_2. After about two to three days, 100% confluent cells were transferred to 96-well plates in a sterile hood, where once confluent again, they were used for biological assays. Once 100% confluent, the cells were split to maintain their viability. The splitting procedure was done in the sterile hood. First, the remaining medium was removed from the flask. Next, the cells were rinsed in a PD buffer, and then rinsed in a PT buffer. The purpose of the PD buffer was to wash away dead and dying cells, and the PT buffer uses Trypsin to

loosen the cells contacts to the flask. After the washing phase, fresh MEM Whole medium was added to the flask, where a pipette then uses the medium to spray the cells off of the flask. The MEM whole medium with cells within it was homogenized and about 1-2 mL was added to a new flask (with G418 and new MEM Whole medium) and a new 96 well plate was also made.

2.5 Transfection of β-Arrestin-1 GFP and MCRs

Human MCR cDNA isolated from E. Coli TOPO 10 was transfected into HEK-293 cells by following the kit instructions of the Fugene 6 transfection agent. The stable cell lines were selected by growth of cells in 1mg/mL of G418 selection pressure for 30 days in the incubator. Receptor concentrations from each transfection were determined by the Bradford method (Bio-Rad, Richmond, CA), using bovine serum albumin as a standard. The cell lines were then further screened through a saturation binding assay. Cell lines containing the highest expression levels of receptors were maintained for biological assays.

2.6 Peptide and Peptide Mimetics

The peptide library presented in Table 1, Table 2, and Table 3 was mostly synthesized in the lab of Dr. Victor Hruby (University of Arizona, Tucson Arizona) by graduate and post doctoral students. The various peptides were tested for their binding ability and adenylate cyclase activation for the hMCR's 1, 3, 4, and 5 using the assays described in 2.7 and 2.9.

2.7 Competitive Binding Assay

Competition binding assays were performed on whole cells to determine the affinities (IC_{50}) of various peptide antagonist and agonists for the human melanocortin receptors. HEK293 cells that were transfected with the melanocortin receptors were seeded on 96-well plates 48 hours before the assay containing 100,000 cells/well. The cell culture medium was aspirated, washed with MEM, and then washed with binding buffer containing 100% MEM, 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10- phenanthrolone, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. Cells were then incubated for 30 minutes at 37^{e}C with different concentrations of the synthesized peptide and 20,000 cpm/well (0.1386 nm) radioactively labeled [^{125}I]NDP-MSH (Perkin-ElmerLife Science, Freemont, CA). The assay medium was aspirated and washed twice with MEM. The cells were then lysed by addition 100 µL of 0.1 M NaOH and 100 µL of 1% Triton X-100. The lysed cells were then placed into 12X75 mm glass tubes and the radioactivity was measured in a Wallac 1470 WIZARD^{TM} Gamma Counter (Wallac, Jefferson, NY).

2.8 Saturation Binding Assay

The saturation binding assays were done on whole cells to determine the receptor expression levels for transfected HEK293 cells. The cells were seeded on 96-well plates 48 hours before the assay (containing 100,000 cells/well). The

cell culture medium was aspirated, washed with MEM, and then washed with binding buffer containing 100% MEM, 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10- phenanthrolone, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. Cells were then incubated for 30 minutes at 37^{e}C and the total binding was determined by using different concentrations of radioactively labeled [^{125}I]NDP-MSH. The non-specific binding was measured by the addition of 1 μM MT-II to the radioactively labeled dilution and incubating under identical conditions. The assay medium was aspirated and washed twice with MEM. The cells were then lysed by addition 100 μL of 0.1 M NaOH and 100 μL of 1% Triton X-100. The lysed cells were then placed into 12X75 mm glass tubes and radioactivity was measured in a Wallac 1470 WIZARD™ Gamma Counter(Wallac, Jefferson, NY). Specific binding for each melanocortin receptor was determined by the difference between total binding and non-specific binding.

2.9 Adenylate Cyclase Biological Activity Assay

The adenylate cyclase biological activity assay was performed on whole cells to determine the cAMP produced by the various peptides of interest for the human melanocortin receptors. HEK293 cells that were transfected with the melanocortin receptors were seeded on 96-well plates 48 hours before the assay containing 100,000 cells/well. The cell culture medium was aspirated and then the wells were rinsed with 100 μL of MEM. 100 μL of MEM containing with 0.5 mM isobutylmethylxanthine (IBMX) was added to individual wells and incubated

for10 min at37 °C. After incubation, 25 µL aliquots of the peptides to be tested were added at various concentrations. The cells were then incubated for 10 minutes at 37 °C. 60µL of ice-cold Tris/EDTA buffer to each well to stop the reaction. The plate was wrapped in parafilm™, sealed with rubber bands and placed into boiling water for 10 minutes. The 96 well plates were then subjected to 10 minutes of centrifugation at 3,200 X g. The cAMP concentration of the supernatant was then measured by competition binding of PKA. 50 µL of the supernatant was then incubated on ice for 2 hours with 12,000 CPM[^{3}H]cAMP (Amersham Biosciences, Pittsburg, PA) in 50 µL Tris/EDTA buffer and 100 µL Tris/EDTA buffer containing 3×10^{-5}U/mL PKA. The incubated supernatant was then transferred to a Millipore MultiScreen® HTS, FB 96-well 0.65 µm glass fiber filter plate. The filter plate was then filtered using vacuum manifold, and rinsed twice with 60 µL Tris/EDTA. After filtration, 50 µL/well Optiphase Supermixscintillation cocktail(Perkin ElmerLife Science) was added to each well for six hours. Plates were then counted in a Wallac MicroBetaTriLux 1450 Luminescence counter (Perkin ElmerLife Science). The total cAMP content was determined through a comparison to a cAMP standard dilution. The cAMP stimulatory properties of the peptide of interest was evaluated by its ability to competitively dislodge the MT-II agonist at various concentrations.

2.10 PA_2 biological activity Assay

The PA_2 biological activity assay was performed on whole cells to determine if the antagonist of interest works competitively or non-competitively. This is done by measuring the cAMP produced when various concentrations of both the antagonist of interest and [^{125}I]NDP-MSH compete with each other for the binding pocket in the human melanocortin receptors. HEK293 cells that were transfected with the melanocortin receptors were seeded on 96-well plates 48 hours before the assay containing 100,000 cells/well. The cell culture medium was aspirated and then the wells were rinsed with 100 µL of MEM. 100 µL of MEM containing with 0.5 mM IBMX was added to individual wells and incubated for10 min at37 °C. After incubation, 25 µL aliquots containing both the antagonist to be tested and MT-II were added at various concentrations. The concentrations in each of the 96 well plates are listed in Table 4. The cells were then incubated for 10 minutes at 37 °C. 60µL of ice-cold Tris/EDTA buffer to each well to stop the reaction. The plate was wrapped in parafilm™, sealed with rubber bands and placed into boiling water for 10 minutes. The 96 well plates were then subjected to 10 minutes of centrifugation at 3,200 X g. The cAMP concentration of the supernatant was then measured by competition binding of PKA. 50 µL of the supernatant was then incubated on ice for 2 hours with 12,000 CPM[^{3}H]cAMP (Amersham Biosciences, Pittsburg, PA) in 50 µL Tris/EDTA buffer and 100 µL Tris/EDTA buffer containing 3×10^{-5}U/mL PKA. The incubated supernatant was then transferred to a Millipore MultiScreen® HTS, FB 96-well 0.65 µm glass fiber filter plate. The filter plate was then filtered using vacuum manifold, and rinsed

twice with 60 µL Tris/EDTA. After filtration, 50 µL/well Optiphase Supermixscintillation cocktail(Perkin ElmerLife Science) was added to each well for six hours. Plates were then counted in a Wallac MicroBetaTriLux 1450 Luminescence counter (Perkin ElmerLife Science). The total cAMP content was determined through a comparison to a cAMP standard dilution. The cAMP stimulatory properties of the peptide of interest was evaluated by its ability to competitively dislodge the MT-II agonist at various concentrations.

		Column											
		1		2		3		4		5		6	
	Row	[agonist] (nM)	[antagonist] (nM)	[agonist] (nM)	[antagonist] (nM)	[agonist] (nM)	[antagonist] (nM)	[agonist] (nM)	[antagonist] (nM)	[agonist] (nM)	[antagonist] (nM)	[agonist] (nM)	[antagonist] (nM)
Antagonist series dilution + constant [agonist]	A	0.1	10,000	0.1	1000	0.1	100	0.1	10	0.1	1	0.1	0.1
	B	1	10,000	1	1000	1	100	1	10	1	1	1	0.1
	C	10	10,000	10	1000	10	100	10	10	10	1	10	0.1
	D	100	10,000	100	1000	100	100	100	10	100	1	100	0.1
	E	1000	10,000	1000	1000	1000	100	1000	10	1000	1	1000	0.1
MT-II std	F	10,000	0	1000	0	100	0	10	0	1	0	0.1	0
No agonist	G	0	10,000	0	1,000	0	100	0	10	0	1	0	0
	H	USE	FOR	cAMP	STANDARD	USE	FOR	cAMP	STANDARD	USE	FOR	cAMP	STANDARD

Table 4: PA_2 Assay Drug Concentrations. This is a graphical layout of the various agonist and antagonist concentrations needed for the PA_2 assay.

2.11 Data Analysis

The EC_{50} and IC_{50} estimates and associated standard errors were determined using a nonlinear one-site competition least squares regression with the computer program Graphpad Prism 5.0 (GraphPad Software, San Diego, CA). These values are used to represent the intracellular cAMP activity and the binding affinity of the peptide of interest. Moreover, the IC50 is the concentration of the peptide that is half way between the maximum and minimum binding

concentration shown (in nM). The percent activity is based on the activity of MT II (a known potent agonist).

RESULTS

Peptide	no.	hMC1R			hMC3R			hMC4R			hMC5R		
		α-IC_{50},nM	β-EC_{50},nM	%max	α-IC50,nM	β-EC50,nM	%max	α-IC50,nM	β-EC50,nM	%max	α-IC50,nM	β-EC50,nM	%max
BJM4007-1	1	NB	NA	0.0	NB	NA	0.0	NB	NA	0.0	NB	NA	0.0
BJM4007-2	2	NB	NA	0.0	NB	NA	0.0	NB	NA	0.0	NB	NA	0.0
BJM4008-1	3	NB	NA	0.0	NB	NA	0.0	NB	NA	0.0	NB	NA	0.0
BJM4009-1	4	NB	NA	0.0	NB	NA	0.0	NB	NA	0.0	NB	NA	0.0
BJM4010-1	5	NB	NA	0.0	NB	NA	0.0	NB	NA	0.0	NB	NA	0.0
MT-II		2.1±2.9	81.0	ND	6.5 ±4.1	18.0	ND	3.4 ±4.6	36.0	ND	7.0 ±6.4	51.0	ND

Table 5: Binding and cAMP results for BJM Compounds. $^{\alpha}IC_{50}$= Concentration of peptide at 50% specific binding. The peptides were tested in a range of concentrations (10^{-10} to 10^{-5} M). $^{\beta}EC_{50}$ = Concentration of peptide at 50% maximal cAMP generation. The peptides were tested in a range of concentrations (10^{-10} to 10^{-5} M). NB = No binding. NA = no cAMP activity. ND = not yet determined. Experiments were done multiple times for binding assay. cAMP assays were not done, as the compound did not bind to the receptor.

Peptide	no.	hMC1R			hMC3R			hMC4R			hMC5R		
		α-IC_{50},nM	β-EC50,nM	%max	α-IC50,nM	β-EC50,nM	%max	α-IC50,nM	β-EC50,nM	%max	α-IC50,nM	β-EC50,nM	%max
JBN51	6	280±390	ND	ND	0.64±0.10	ND	ND	57±31	ND	ND	5.9±4	ND	ND
JBN61	7	17±23.3	ND	ND	2.5±0.4	ND	ND	64±0.40	ND	ND	17±0.40	ND	ND
JBN63	8	455±97.6	ND	ND	4±1.7	ND	ND	58±36	ND	ND	17±11	ND	ND
JBN69	9	280±380	ND	ND	7.5±3.5	ND	ND	51±32	ND	ND	7.6±4.5	ND	ND
JBN95	10	13±5.2	ND	ND	5±3.3	ND	ND	11±14	ND	ND	7.6±2.3	ND	ND
JBN97	11	9.5±10.1	ND	ND	7.9±2.1	ND	ND	1.6±1.8	ND	ND	5.4±2.6	ND	ND
JBN99	12	18±25	ND	ND	6±0.20	ND	ND	0.68±3.4	ND	ND	5.4±3.4	ND	ND

Table 6: Binding and cAMP results for JBN Compounds. $^{\alpha}IC_{50}$= Concentration of peptide at 50% specific binding. The peptides were tested in a range of concentrations (10^{-10} to 10^{-5} M). $^{\beta}EC_{50}$ = Concentration of peptide at 50% maximal cAMP generation. The peptides were tested in a range of concentrations (10^{-10} to 10^{-5} M). NB = No binding.

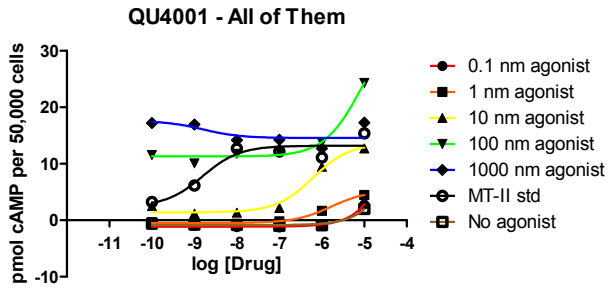
NA = no cAMP activity. ND = not yet determined. Experiments were done multiple times for binding assays. cAMP assays were not performed on this set of compounds.

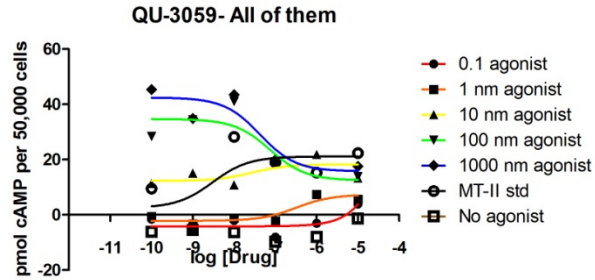
Peptide	no.	hMC1R			hMC3R			hMC4R			hMC5R		
		IC_{50}, nM	EC_{50}, nM	%max	IC_{50}, nM	EC_{50}, nM	%max	IC_{50}, nM	EC_{50}, nM	%max	IC_{50}, nM	EC_{50}, nM	%max
	MT-II	3.2			9			6			17		
QU4050	13	>5000	>1000	75	NB	NA	NA	1±0.92	200±39	13	NB	NA	NA
QU3059	14	NB	NA	NA	NB	NA	NA	53±9.47	43±7.9	3.9	588±89	2400±500	24
QU4001	15	3500±400	760±150	91	NB	NA	NA	39±0.15	11±1.9	36	NB	NA	NA
QU3078	16	2.7±0.50	820±150	77	1±0.19	14±2.5	91	39±0.15	3500±700	64	2.5±0.38	1.6±0.21	68
QU4002	17	1.1±0.18	2700±175	100	2.8±0.141	2500±487	68	5.7±0.46	41±4	89	1.1±0.42	21±3.1	100
	SHU9119		NA		0.23			1.5			16		
QU4051	18	600±115	160±28	66	NB	-	-	33±2.6	5700±1000	55	NB	NA	NA
QU3082	19	>3000	-	-	>10000	620±116	56	7.2±1.39	200±11	31	22±2.5	2401±460	59
QU4004	20	400±78	1900±3645	100	NB	NA	NA	23±1.27	250±29	7.3	NB	NA	NA
QU3083	21	2.3±0.42	600±114	25	2.3±0.11	130±24	28	1.5±0.28	200±38	17	0.7±0.17	0.3±0.058	18
QU4005	22	1.2±0.17	1700±300	89	2.2±0.19	-	-	2.5±0.54	1400±276	6.2	2.2±0.4	85±15	52
	NDP-g-MSH	0.5	NA		2			1.2			2.4		
QU4052	23	190±35	120±21	53	NB	NA	NA	270±27	210±29	46	NB	NA	NA
QU4006	24	830±152	1400±250	92	NB	NA	NA	NB	210±29	NA	NB	NA	NA
QU4007	25	1000±198	>5000	100	NB	NA	NA	NB	NA	NA	NB	NA	NA

Table 7: Binding and cAMP results for QU Compounds. $^{\alpha}IC_{50}$= Concentration of peptide at 50% specific binding. The peptides were tested in a range of concentrations (10^{-10} to 10^{-5} M). $^{\beta}EC_{50}$ = Concentration of peptide at 50% maximal cAMP generation. The peptides were tested in a range of concentrations (10^{-10} to 10^{-5} M). NB = No binding. NA = no cAMP activity. ND = not yet determined. Experiments were done multiple times for binding as well as cAMP assays.

QU3059 (Ac-Nle-c[Asp-His-D-Phe-trans-Xaa-Trp-Lys]-NH_2) on hMC4R
QU4001 (Ac-Nle-c[Asp-His-D-Phe-cis-Xaa-Trp-Lys]-NH2) on hMC4R

QU4004 (Ac-Nle-c[Asp-His-D-Nal(2')-cis-Xaa-Trp-Lys]-NH2) on hMC4R.





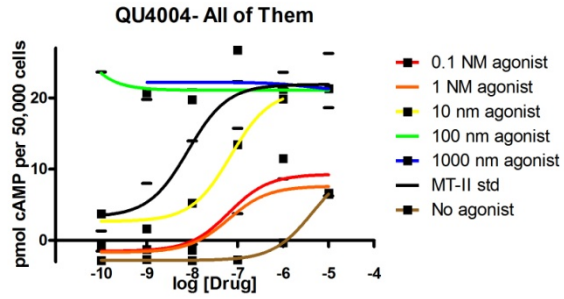


Table 8: PA_2 Assay Results. GraphPad Prism 5.0 graphs of the PA2 assays for the QU compounds 4001, 3059, and 4004.

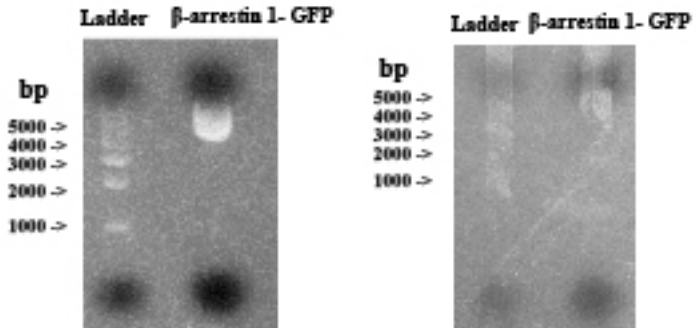


Figure 2: Agarose Gel Electrophoresis of β-Arrestin 1- GFP. To visualize the cDNA products, a 2% agarose gel containing ethidium bromide was run at 120 volts for 30 minutes. A DNA ladder was used for identifying bands based on their molecular weight. Both of the β-Arrestin-1 GFP bands correctly match the ~5000 bp band.

3.1 Binding Assay Results

The competitive binding assays were done for each peptide using [^{125}I]NDP-MSH as the competing peptide. This assay was performed according to the procedure mentioned in the materials and methods. The measured IC_{50} for each peptide is listed above in Table 5, Table 6, and Table 7. The standard deviation is shown next to each IC_{50}.

3.2 Adenylate Cyclase Assay Results

The MCRs are known to produce cAMP when activated, and therefore the adenylate cyclase assay was performed according to the procedure described in the materials and methods. The EC_{50} determined for each peptide is listed above in Table 5, Table 6, Table 7, as well as the percent MT-II cAMP accumulation. The standard deviation is shown next to each EC_{50}.

3.3 PA_2 Assay Results

The PA_2 assay was done a select set of QU compounds. This assay was performed according to the procedure described in the materials in methods.

The shift of the EC_{50} or percent max cAMP generated illustrates that the compounds most likely bind in a separate area of the binding pocket (non-competitive). As the antagonists are binding in a separate section of the binding pocket, it does not interfere with the binding of the agonist used in the assay, MT-II. This caused the result of each drug dilution to illicit the cAMP pMOL response solely based on the concentration of the MT-II.

3.4 β-Arrestin-1 GFP cDNA Agarose Gel Results

Agarose Gel Electrophoresis was performed on two cDNAs isolated from E. Coli TOPO 10 transfected with β-Arrestin 1-GFP. The molecular weight of the β-Arrestin 1-GFP band matches the 5000 bp band on the ladder. This is indicative of a successful isolation of β-Arrestin 1-GFP cDNA, as the molecular weight of the recombinant cDNA should lie around this band. The sequences of plasmid cDNA strands will be submitted to the University of Arizona Genomic Analysis and Technology Core for verification.

3.5 MCR Mutant Results

The adenylate cyclase biological activity assay for the various MCR mutants was inconclusive. cAMP generated in the assay was far too high to be used for any meaningful data acquisition. Repeat experiments will be performed.

DISCUSSION

Finding potent and selective agonists and antagonists for the human melanocortin receptors is a fundamental goal of the Hruby Lab group. Although each receptor has similarities to one another in their peptide sequence, there are definite differences. These differences allow the possibility for discovering ligands that are specific for individual receptors.

4.1 BJM, JBN and QU Compound Results

Binding assays and cAMP assays were performed and the data is listed in Table 5, 6 and 7. The results show that small changes in the stereochemistry can cause drastic changes in the binding and agonistic/antagonist ability of the compounds. The most interesting results were for compounds QU3059, QU4001, and QU4004. These compounds were SHU9119 and MT-II derivatives, which modifications caused them to be *selective* for specific MCRs (unlike SHU9119 and MT-II which bind to all of the receptors). These compounds were then tested to determine if they are competitive or non-competitive antagonists. The stereochemical differences in these peptides will be further refined so that highly selective and potent MCR peptides can be discovered.

4.2 PA_2 Assay Results

The results of the PA_2 assay for QU3059, QU4001, and QU4004 on hMC4R are shown in Table 8. The results of the PA_2 suggest that those compounds use non-competitive antagonism, possibly binding in a separate section of the binding pocket. As the antagonists are binding in a separate section of the binding pocket, it does not interfere with the binding of the agonist used in the assay, MT-II. This caused the result of each drug dilution to illicit the cAMP pMOL response solely based on the concentration of the MT-II.

4.3 β-Arrestin-1 GFP cDNA Agarose Gel Results

The agarose gel electrophoresis results are shown in Figure 2. As the isolated cDNA from the recombinant E. Coli TOPO 10 matches the 5000 bp band on the gel, it is highly likely that β-Arrestin 1-GFP cDNA was successfully isolated. This cDNA will next be verified by DNA sequencing.

4.4 MCR Mutant Results

The adenylate cyclase biological activity assay on HEK293 cell lines containing MCR mutations in the intracellular phosphorylation sites and amino acids of intracellular loop/carboxyl terminus was inconclusive. cAMP generated in the assays was excessively high for all concentrations of the well-characterized melanocortin peptides. This is most likely due to high expression levels of the receptors on the HEK293 cells. This requires repeat experiments at

lower concentrations of peptides, or on MCR mutant cell lines that have lower expression levels. These experiments are currently in progress.

4.5 Future Projects

Repeat experiments will be performed for peptides that have an unacceptable degree of standard error. The MCR mutants will have repeat adenylate cyclase biological activity assays performed. These mutants will also be tested for their ability to recruit β-Arrestin when stimulated. This will be done by constructing MCR mutants with GFP tagged β-Arrestin1 and viewing the stimulated receptors under a confocal microscope. Additional experiments will be performed on MCRs to determine their phosphorylation and desensitization differences when stimulated by various synthesized peptides.

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