Peptide Analogues Containing β-Amino Acids for the Melanocortin System

Viktor Flores, Dr. Anamika Singh, Huisuo Huang, and Dr. Carrie Haskell-Luevano

College of Medicine, University of Florida

The melanocortin system consists of five G-protein coupled receptors (MC1R-MC5R), four known endogenous agonists, and two known endogenous antagonists. The MC3R and MC4R play a direct role in regulating energy and weight homeostasis. When the MC3R and MC4R are stimulated by their agonist ligands, an anorexigenic response is produced. All four of the endogenous agonist ligands of the melanocortin system have the core tetrapeptide sequence His-Phe-Arg-Trp, which has been attributed to melanocortin receptor stimulation. The purpose of this study is to modify each amino acid of the enhanced tetrapeptide His-DPhe-Arg-Trp with its β-amino acid counterpart and test the selectivity and potency that each change will produce at the MC3R and MC4R. The peptides were synthesized using standard Fmoc solid phase peptide methodology. The synthesized peptides were pharmacologically characterized using β-galactosidase gene reporter assay. Compared to the conserved tetrapeptide sequence found in endogenous agonists, the β-Arg and β-Trp analogue were 400- and 1000-fold more potent at the MC4R. The βArg and βTrp analogues are potent agonist selective for the MC4R and may be useful for studying MCRs in order to design a drug that regulates weight homeostasis and food satiety.

INTRODUCTION

Melanocortin System

The melanocortin system is a complex control center that regulates various physiological pathways, which include skin pigmentation, steroidogenesis, sexual function, sebaceous lipid production, energy and weight homeostasis, and food satiety. [1,2] This system consists of five G-protein coupled receptors (MC1R-MC5R), four known endogenous agonists, and two known endogenous antagonists. [2, 3] The endogenous agonists of the melanocortin system are α-, ß-, and γ- melanocyte-stimulating hormones (α-, ß-, γ-MSH) and adrenocorticotropin hormone (ACTH). All four of these endogenous agonist ligands are derived from the posttranslational processing of the proopicomelanocortin (POMC) gene. [4,5] These hormones bind to the melanocortin receptors and activate the cyclic adenosine monophosphate (cAMP) signal transduction pathway, which generates a cascade of intracellular reactions that result in a physiological response. [6] The endogenous antagonists are Agouti and Agouti-related protein AgRP. [7, 8] These proteins inhibit the function of the agonists by blocking the agonists' ability to activate adenylyl cyclase. [6-8]

Of the five melanocortin receptors, the MC3R and MC4R have generated the greatest interest among the scientific community because of their direct role in regulating energy and weight homeostasis. [9, 10] In MC3R studies, mice null for MC3R developed higher levels of fat mass and lower levels of muscle mass despite eating a similar diet to the control mice, establishing a connection between the MC3R and energy homeostasis. [11,12] MC4R studies have revealed even more dramatic results. Knockout MC4R mice have been shown to be hyperphagic and overtly obese, proving that this receptor is critical for weight homeostasis and feeding behavior. [13-17] In addition, clinical studies have shown that people with MC4R mutations are usually morbidly obese. [16, 17] In fact, these studies suggest that mutations in the gene encoding the MC4R account for the most common form of monogenic obesity seen in humans. Up to 6% of morbidly obese individuals express a mutation in the MC4R. [17] Studies have also determined the relationship between these receptors and their endogenous agonists and antagonists. A study by Fan *et al.* concluded that food intake in mice is suppressed by agonists and activated by antagonists when either agent is administered intracerebroventricularly near the hypothalamus of the brain. [18] Following all these important findings, scientists have successfully constructed the molecular pathways that account for these observations.

Our current understanding of how the MC3R and MC4R regulate energy and weight homeostasis involves the interaction between peripheral energy stores and the arcuate nucleus of the hypothalamus. [9] The body naturally balances caloric input with caloric expenditure by storing excess energy in adipose tissue, a major peripheral storage site. An increase of adipose tissue results in an increase of leptin hormone expression. The leptin hormone is released into the bloodstream and travels to the brain, where it binds to leptin receptors expressed in POMC neurons inside the arcuate nucleus of the hypothalamus. [19, 20] The POMC neurons respond by increasing the production of α-MSH,

which mediates an anorexigenic response when it binds to the MC3R and MC4R. [5, 20] The converse patterns of events are also true; when the body is in a fasting state, POMC gene expression decreases and AgRP expression increases in AgRP/NPY neurons. [21, 22] Subsequently, AgRP binds to the MC3R and MC4R and inhibits α-MSH stimulation, which results in an orexigenic response. [6-8]

Due to the melanocortin agonists' ability to stimulate an anorexigenic response by reducing food intake, efforts to develop a therapeutic drug against obesity have aimed to design an agent that mimics the endogenous agonists' selectivity and potency for the MC3R and MC4R. The α-, β-, and γ-MSH and ACTH all share the same tetrapeptide pharmacore of His-Phe-Arg-Trp (HFRW). [23-25] This is the minimal sequence required for melanocortin receptor selectivity and stimulation. [25] Figure 1 compares the sequence of each endogenous agonist. Moreover, it was found that the stereochemical inversion of Phe into D-Phe results in a more potent and enzymatically stable tetrapeptide that targets the human MC4R. [26]

Figure 1. The endogenous agonists of the melanocortin system. This is the single letter amino acid abbreviation for the four known endogenous agonists of the melanocortin system. Encircled is the core tetrapeptide sequence of histidine-phenylalanine-arginine-tryptophan, which is necessary for melanocortin receptor stimulation.

β-Amino Acids

The purpose of this study is to modify each amino acid of the His-DPhe-Arg-Trp pharmacore with its β-amino acid counterpart and test the selectivity and potency that each change will produce on MC3R and MC4R. Unlike their naturally occurring equivalents, α-amino acids, β-amino acids differ in that they have an extra carbon between either the carbonyl group and α-carbon (known as β^3 amino acids) or the amine group and α-carbon (known as β^2 amino acids). [27] This study will utilize β^3 amino acids. Figure 2 compares the structure of α-amino acid with β^3 amino acid.

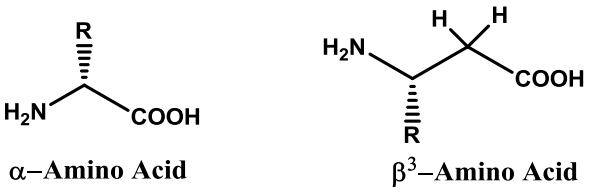

Figure 2. A α-amino acid representation is shown to the left and a β^3-amino acid representation is shown to the right. Unlike its α-amino acid counterpart, the β-amino acid has an extra carbon in the backbone of its structure.

Use of β-amino acids for drug development and peptidomimetics are promising because of three reasons: (1) Peptides made from β-amino acids, formally called β-peptides, can have multiple conformations and well defined secondary structures due to their extended backbone. [28,29] (2) β-peptides can be designed to resemble the activity-related structural features of their corresponding natural peptides. In other words, β-peptides can be designed to mimic the α-peptide's biological function. [30-33] In fact, studies have shown that β-peptides can mimic the effector response of antibodies, anticancer proteins, anti-HIV molecules, and vaccines. [30-33] (3) β-peptides are enzymatically stable against proteolytic, hydrolytic, and metabolic enzymes in mammals because these enzymes do not recognize the additional carbon in the backbone of the β-amino acid. [29,34] Thus, β-peptides would be expected to last longer *in vivo* than α-peptides, a factor that is very important when considering drug development.

METHODS

Materials

The β-amino acids (*S*)-3-Fluorenylmethoxycarbonyl-4-phenylbutyric acid-L-β-homophenylalanine (Fmoc-β-HoPhe-OH), Fluorenylmethoxycarbonyl-N_w-(2,2,5,7,8-penta-methyl-chromane-6-sulfonyl)-L-β-homoarginine (Fmoc-β-HoArg(Pmc)-OH), and (*S*)-3-Fluorenylmethoxycarbonyl-4-(3-indolyl) butyric acid-L-β-homotryptophan (Fmoc-β-HoTrp-OH) were purchased from Chem Impex International (Wood Dale, IL). The amino acids N-α-9-Fluorenylmethoxycarbonyl-Nim-Trityl-L-Histidine (Fmoc-His(Trt)-OH), 9-Fluorenylmethoxycarbonyl-L-Phenylalanine (Fmoc-Phe-OH), 9-Fluorenylmethoxycarbonyl-D-Phenylalanine

(Fmoc-D-Phe-OH), and N-α-9-Fluorenylmethoxycarbonyl-N-in-t-Butyloxycarbonyl-L-Tryptophan (Fmoc-Trp(Boc)-OH) were purchased from Peptide International (Louisville, KY). The solid support resin 4-(2',4'-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-Methylbenzhydrylamine resin (Rink Amide MBHA; 0.37 mequiv/g), and the coupling reagent 1-[bis(Dimethylamino)methylene]-1H-Benzotriazolium Hexafluorophosphate 3-Oxide (HBTU) were also purchased from Peptides International. *N*,*N*-Diisopropylethylamine (DIEA) and triisopropylsilane (TIS) were purchased from Aldrich (Milwaukee, WI). Acetonitrile (ACN), anhydrous ethyl ether, dichloromethane (DCM), glacial acetic acid (HOAc) and methanol (MeOH) were purchased from Fisher (Fair Lawn, NJ). N,N-dimethylformamide (DMF) was purchased from Burdick and Jackson (McGaw Park, IL). Trifluoroacetic acid, piperidine, and pyridine were purchased from Sigma (St. Louis, MO). 1,2-Ethanedithiol was purchased from Fluka.

Peptide Synthesis

Peptide synthesis was performed using standard Fmoc methodology on a CEM Discovery SPS microwave peptide synthesizer. [35] Approximately 270mg of Rink Amide MBHA was placed inside a reaction vessel and allowed to swell for 2 hours in dichloromethane (DCM) prior to use. The resin was washed five times with DMF and deprotected using 20% piperidine in DMF solution for 2 minutes under normal room temperature followed by another 20% piperidine in DMF treatment but this time inside the microwave synthesizer for 4 minutes at 75° C. To confirm the deprotection of the amine group, a Kaiser test was performed. [36] The growing peptide chain was synthesized on the amide resin using the following coupling steps: Three-fold excess of the appropriate Fmoc-amino acid, starting from the C-terminus of the desired peptide, was added to the reaction vessel followed by the addition of three-fold excess of HBTU and DIEA. The reaction vessel was then placed in the microwave synthesizer at a setting of 75°C for 5 minutes under N_2 bubbling. After 5 minutes, the reaction was allowed to cool down, the excess reagents were drained out, and the nascent peptide was washed with DMF four times. A Kaiser test was performed again to make sure the amino acid coupled with the resin. Following a successful coupling, the peptide was deprotected using 20% piperidine in DMF for 2 minute and then again for 4 minutes at 75°C using the microwave synthesizer. Coupling and deprotection of the peptide was repeated until the final amino acid was added. Following the deprotection of the final amino acid, the peptide was acetylated by adding 3:1 mixture of glacial acetic acid and pyridine to the vessel for 30 minutes under N_2 bubbling. This step was performed to protect the exposed N-terminus. The solution was drained out, the peptide was washed with DCM four times, and the vessel was placed inside a vacuum overnight. Cleavage of the peptide from the resin and removal of the side chain protecting groups from the amino acids was done by adding a cleavage cocktail consisting of 91% TFA, 3% EDT, 3% TIS, and 3% H_2O in the vessel for 3 hours under N_2 bubbling. The cleavage product was emptied into a preweighted 50mL conical tube and precipitated with cold anhydrous ethyl ether. The flocculent peptide was pelleted by centrifugation (Sorval Super T21 high-speed centrifuge) at 4°C and 4000rpm for 4 minutes. The anhydrous ethyl ether was decanted from the centrifuge tube and the peptide was again mixed with cold anhydrous ethyl ether and centrifuged under the same setting. This step was repeated two more times. The crude peptide inside the conical tube was placed inside a vacuum overnight.

Peptide Purification

Approximately 20mg of the dry crude peptide was purified by reverse phase high liquid performance chromatography (RP-HPLC) using a Shimadzu chromatography system with a photodiode array detector and a semipreparative RP-HPLC C_{18} bonded silica column. The major peak was collected and the solvents used to purify the crude peptide (acetonitrile, methanol, and water) were removed by rotovaporization and lyophilization. The k' for each purified peptide was calculated. In order to verify that the final product was the desired peptide, mass spectrometry was performed. The mass spectrometry results were compared to the calculated molecular mass of each peptide (University of Florida protein core facility).

β-Galactosidase Assay

The β-galactosidase assays were performed in the Haskell-Luevano laboratory. Pharmacological characterization of the synthesized peptides at the mouse melanocortin receptors mMC1R, mMC3R, mMC4R, and mMC5R was achieved using β-galactosidase gene reporter assay as described by Chen et al. [37] The mMC2R was not utilized for characterization because this receptor only selects for ACTH agonist. [38]

RESULTS

Purification of Peptides

Table 1 reports the analytical data of the synthesized peptides. The k' values indicate the HPLC retention time of the purified peptides under the solvent acetonitrile and methanol. This value is important for reproducibility purposes. The purified peptides were at least 95% pure as determined by the analytical RP-HPLC. The mass spectrometry results confirmed that the correct peptides were synthesized.

Table 1. Analytical Data for the Peptides Synthesized

Peptide	Acetonitrile k' HPLC	Methanol k' HPLC	Purity %	m/Z Calculated	m/Z Found
Ac-His-Phe-Arg-Trp-NH2	4.6	6.8	>95	685.34	686.56
Ac-His-DPhe-Arg-Trp-NH2	4.0	6.0	>95	685.34	686.62
Ac-His-βPhe-Arg-Trp-NH2	4.4	6.6	~ 95	699.36	700.57
Ac-His-DPhe-βArg-Trp-NH2	3.9	6.0	>95	699.36	700.26
Ac-His-DPhe-Arg-βTrp-NH2	4.1	6.3	>97	699.36	700.44

Note. Two solvent systems were used to determine the HPLC k' of the purified peptides: Acetonitrile and Methanol. This value was determined using Equation 1. The *calculated mass over charge* values represent the expected mass of the peptides and the *found mass over charge values* is the actual mass of the peptides determined by mass spectrometry.

Evaluation of Synthesized Peptides at Melanocortin Receptors

Table 2 summarizes the agonist pharmacology of each synthesized peptide at the mouse MC1R, MC3R, MC4R, and MC5R. NDP-MSH is a potent analogue of α-MSH, and it is used as a molecular probe for studying melanocortin receptors and comparing the potency of synthesized agonists. [39] Peptide **1** is the control tetrapeptide sequence common to all four endogenous agonists, HFRW. Stimulation from NDP-MSH resulted in 940000-, 162000-, 126000-, and 32000-fold increased potencies at MC1R, MC3R, MC4R, and MC5R respectively, relative to Peptide **1**. Peptide **2**, which has DPhe instead of Phe, resulted in 3200-, 550-, 2020-, and 1260-fold increased potencies at the MC1R, MC3R, MC4R, and MC5R relative to Peptide **1**. Peptide **3**, which replaces Phe for β-Phe, has increased potencies of 5-, 3-, and 2-fold at the MC1R, MC4R, and MC5R respectively, relative to **1**. Peptide **3** activity at the MC3R is equipotent to Peptide **1**. Peptide **4** is similar to **2**, with the only difference being that it has β-Arg instead of Arg. Potencies for **4** relative to Peptide **2** resulted in 12-, 5-, and 12-fold decrease at the MC3R, MC4R, and MC5R respectively. Peptide **4** resulted in equipotent activity at the MC1R compared to **2.** Peptide **5** is similar to **2** but contains β-Trp rather than Trp. Peptide **5** resulted in potencies of 2-, 9-, and 2-fold decrease at the MC1R, MC3R, and MC4R respectively, relative to Peptide **2**. At the MC5R, Peptide **2** and **5** had equipotent activity. Figures 3 through 6 illustrate the molar agonist EC_{50} values of each peptide at the mMC1R, mMC3R, mMC4R, and mMC5R.

Table 2. Biological Activity of Tetrapeptide Agonists at the Mouse Melanocortin Receptors

		Agonist EC50 (nM) Values			
Peptide	Peptide Structure	mMC1R	mMC3R	mMC4R	mMC5R
	NDP-MSH	0.03±0.014	0.20±0.021	0.05±0.002	0.17±0.05
1	Ac-His-Phe-Arg-Trp-NH2	28100±7480	32300±14100	6310±623	5490±788
2	Ac-His-DPhe-Arg-Trp-NH2	8.76±2.45	58.4±9.22	3.13±0.29	4.35±1.47
3	Ac-His-βPhe-Arg-Trp-NH2	5460±1300	25700±9200	2320±389	2710±550
4	Ac-His-DPhe-βArg-Trp-NH2	11.0±1.41	711±148	15.9±0.34	52.9±22.3
5	Ac-His-DPhe-Arg-βTrp-NH2	14.0±2.72	503±144	6.31±1.21	6.49±1.94

Note. The EC_{50} values represent the standard error of mean as determined from three individual experiments.

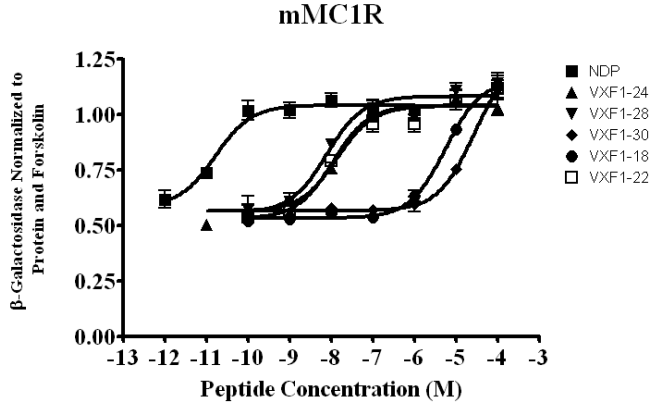

Figure 3. Tetrapeptide mMC1R stimulatory activity. Stimulatory agonist activity of each synthesized peptide at the mMC1R. The EC_{50} value for each analogue was 0.03nM (NDP-MSH), 28.1μM (Peptide 1), 8.76nM (Peptide 2), 5.46μM (Peptide 3), 11.0nM (Peptide 4), and 14nM (Peptide 5).

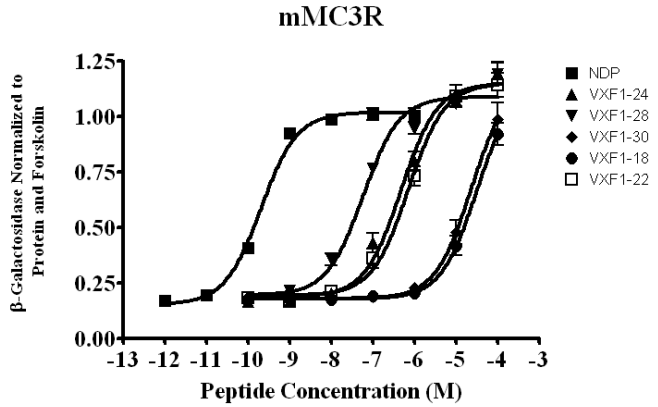

Figure 4. Tetrapeptide mMC3R Stimulatory Activity. Stimulatory agonist activity of each synthesized peptide at the mMC3R. The EC_{50} value for each analogue was 0.20nM (NDP-MSH), 32.3μM (Peptide 1), 58.4nM (Peptide 2), 25.7μM (Peptide 3), 711nM (Peptide 4), and 503nM (Peptide 5).

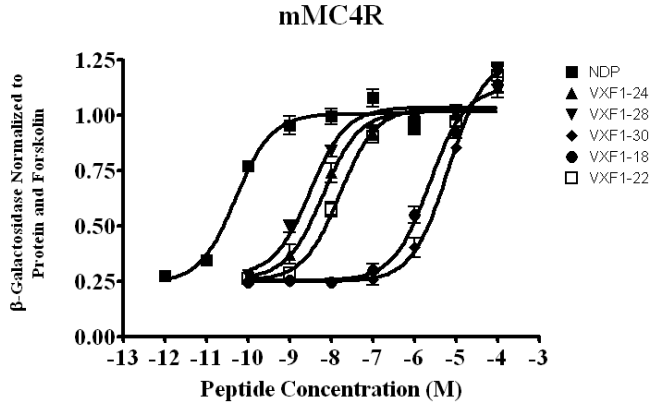

Figure 5. Tetrapeptide mMC4R Stimulatory Activity. Stimulatory agonist activity of each synthesized peptide at the mMC4R. The EC_{50} value for each analogue was 0.05nM (NDP-MSH), 6.31μM (Peptide 1), 3.13nM (Peptide 2), 2.32μM (Peptide 3), 15.9nM (Peptide 4), and 6.31nM (Peptide 5).

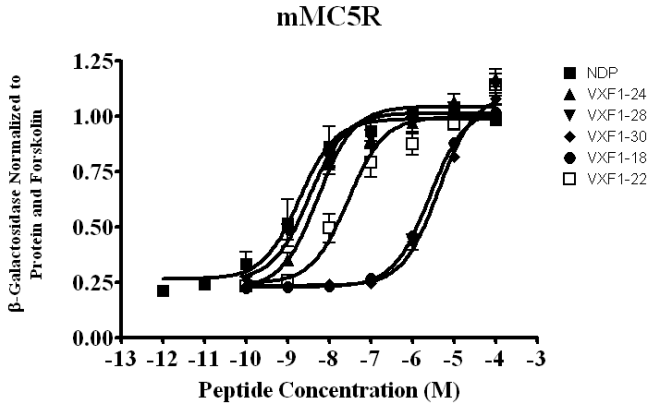

Figure 6. Tetrapeptide mMC5R Stimulatory Activity. Stimulatory agonist activity of each synthesized peptide at the mMC5R. The EC_{50} value for each analogue was 0.17nM (NDP-MSH), 5.49μM (Peptide 1), 4.35nM (Peptide 2), 2.71μM (Peptide 3), 52.9nM (Peptide 4), and 6.49nM (Peptide 5).

DISCUSSION

Melanocortin endogenous agonists' mediate their effect via action on five G-protein coupled receptor subtypes, MC1R-MC5R. This is the first study to test the pharmacological effects that β-amino acids have at the melanocortin receptors. However, this is not the first study to test β-amino acid activity in G-protein coupled receptors. Coincidentally, a study in 2003 by Nunn et al. found that β-tetrapeptide analogues of the hormone somatostatin behave as potent agonists at somatostatin sst_4 receptor—one of five G-protein coupled receptors that make up the somatostatin system, also located in the brain. [40] This study proves that peptides containing β-amino acids can also interact with G-protein coupled receptors of the melanocortin system.

Each α-amino acid of the tetrapeptide His-DPhe-Arg-Trp (with the exception of histidine) was replaced with its β-amino acid counterpart, and the potency and selectivity of each analogue was measured at the mouse melanocortin receptors. We are currently synthesizing β-His so that we can measure the potency and selectivity of Ac-**βHis**-DPhe-Arg-Trp-NH_2 and Ac- **βHis**-βPhe-βArg-βTrp-NH_2 at the mMCRs.

Characterization of β-amino Acid Containing Peptides

The pharmacological results of the β-amino acid tetrapeptides (Ac-His-**βPhe**-Arg-Trp-NH_2, Ac-His-DPhe-**βArg**-Trp-NH_2, and Ac-His-DPhe-Arg-**βTrp**-NH_2; summarized in table 2) show that each peptide was capable of stimulating the expressed melanocortin receptors. The most significant results of this study include the potency and selectivity displayed by Ac-His-DPhe-**βArg**-Trp-NH_2 and Ac-His-DPhe-Arg-**βTrp**-NH_2. The β-Arg analogue

was selective for MC1R and MC4R. Compared to the conserved tetrapeptide sequence found in endogenous agonists, Ac-His-Phe-Arg-Trp-NH_2, the β-Arg analogue was 400-fold more potent at MC4R, the receptor that pharmacologist are most interested in targeting for obesity. Moreover, the β-Arg was only 5-fold less potent at the MC4R than the enhanced tetrapeptide, Ac-His-DPhe-Arg-Trp-NH_2. The β-Trp analogue was selective for MC1R, MC4R, and MC5R; this peptide was 1000-fold more potent than Ac-His-Phe-Arg-Trp-NH_2 and only 2-fold less potent than the enhanced tetrapeptide, Ac-His-DPhe-Arg-Trp-NH_2, for MC4R. Although both β-Arg and β-Trp analogues were less potent than the already established Ac-His-DPhe-Arg-Trp-NH_2 tetrapeptide, it's important to keep in mind that these two peptides may still be useful for drug design because β-amino acid containing peptides are more enzymatically stable than pure α-amino acid containing analogues. Interestingly, none of the β-amino acid tetrapeptides showed selectivity for MC3R.

Phe Position

Another interesting result derived from this study is the importance of the Phe position for ligand efficacy. Previous studies found that stereochemically modifying His-Phe-Arg-Trp to His-DPhe-Arg-Trp, dramatically increased potency. [25,26] In our study, changing Phe to DPhe resulted in an increase potency of 2000-fold at the MC4R. Thus, this study not only confirmed the results of previous findings, but also found that changing Phe to β-Phe leaves the ligand's potency for MC4R at a 750-fold decreased potency relative to Ac-His-DPhe-Arg-Trp-NH_2.

CONCLUSION

In conclusion, the Ac-His-DPhe-**βArg**-Trp-NH_2 and Ac-His-DPhe-Arg-**βTrp**-NH_2 peptides, with ligand efficacy of 15.9nM and 6.31nM respectively, are potent agonist selective for MC4R. In addition, the potential resistance of enzymatic degradation offered by these two analogues makes them useful ligands for studying MCRs and designing a drug that can regulate weight homeostasis and food satiety.

Future Direction

We will finish synthesizing β-His so that the selectivity and potency of Ac-βHis-DPhe-Arg-Trp-NH_2 and Ac-βHis-βPhe-βArg-βTrp-NH_2 at melanocortin receptors can be characterized. All the synthesized peptides must then be tested for *in vitro* enzymatic resistivity to determine whether the inclusion of β-amino acids actually increases enzymatic stability.

ACKNOWLEDGEMENTS

I thank Dr. Carrie Haskell-Luevano for the opportunity to work in her lab. None of this would have been possible without her. I also thank Dr. Anamika Singh for her mentorship and patience and my colleagues at the Haskell-Luevano lab for their support and help.

REFERENCES

1. Haskell-Luevano, Carrie, et al. "Progress in the Development of Melanocortin Receptor Selective Ligands." *Current Pharmaceutical Design* 10 (2004): 3443–3479.

2. Gantz, Ira, and Tung M. Fung. "The Melanocortin System." *American Journal of Physiology of Endocrinology and Metabolism* 284 (2003): E468–474.

3. Holder, J. R., and C. Haskell-Luevano. "Melanocortin Ligands: 30 Years of Structure-Activity Relationship (SAR) Studies." *Medicinal Research Reviews* 24.3 (2004): 325–56.

4. Gee, C. E., et al. "Identification of Proopiomelanocortin Neurones in Rat Hypothalamus by in Situ cDNA-mRNA Hybridization." *Nature* 306.5941 (1983): 374–6.

5. Pritchard, L. E., A. V. Turnbull, and A. White. "Pro-Opiomelanocortin Processing in the Hypothalamus: Impact on Melanocortin Signalling and Obesity." *The Journal of Endocrinology* 172.3 (2002): 411–21.

6. Cone, R. D., et al. "The Melanocortin Receptors: Agonists, Antagonists, and the Hormonal Control of Pigmentation." *Recent Progress in Hormone Research* 51 (1996): 287,317; discussion 318.

7. Lu, D., et al. "Agouti Protein is an Antagonist of the Melanocyte-Stimulating-Hormone Receptor." *Nature* 371.6500 (1994): 799–802.

8. Ollmann, M. M., et al. "Antagonism of Central Melanocortin Receptors in Vitro and in Vivo by Agouti-Related Protein." *Science* 278.5335 (1997): 135–8.

9. Seeley, Randy J., Deborah L. Drazen, and Deborah J. Clegg. "The Critical Role of the Melanocortin System in the Control of Energy Balance." *Annual Review of Nutrition* 24.1 (2004): 133–49.

10. O'Rahilly, S., G. S. Yeo, and I. S. Farooqi. "Melanocortin Receptors Weigh in." *Nature Medicine* 10.4 (2004): 351–2.

11. Chen, A. S., et al. "Inactivation of the Mouse Melanocortin-3 Receptor Results in Increased Fat Mass and Reduced Lean Body Mass." *Nature Genetics* 26.1 (2000): 97–102.

12. Butler, A. A., et al. "A Unique Metabolic Syndrome Causes Obesity in the Melanocortin-3 Receptor-Deficient Mouse." *Endocrinology* 141.9 (2000): 3518–21.

13. Yeo, G. S., et al. "A Frameshift Mutation in MC4R Associated with Dominantly Inherited Human Obesity." *Nature Genetics* 20.2 (1998): 111–2.

14. Tao, Y. X., and D. L. Segaloff. "Functional Characterization of Melanocortin-4 Receptor Mutations Associated with Childhood Obesity." *Endocrinology* 144.10 (2003): 4544–51.

15. Huszar, D., et al. "Targeted Disruption of the Melanocortin-4 Receptor Results in Obesity in Mice." *Cell* 88.1 (1997): 131–41.

16. Vaisse, C., et al. "Melanocortin-4 Receptor Mutations are a Frequent and Heterogeneous Cause of Morbid Obesity." *The Journal of Clinical Investigation* 106.2 (2000): 253–62.

17. Farooqi, I. S., et al. "Clinical Spectrum of Obesity and Mutations in the Melanocortin 4 Receptor Gene." *The New England Journal of Medicine* 348.12 (2003): 1085–95.

18. Fan, W., et al. "Role of Melanocortinergic Neurons in Feeding and the Agouti Obesity Syndrome." *Nature* 385.6612 (1997): 165–8.

19. Schwartz, M. W., et al. "Identification of Targets of Leptin Action in Rat Hypothalamus." *The Journal of Clinical Investigation* 98.5 (1996): 1101–6.

20. Cowley, M. A., et al. "Leptin Activates Anorexigenic POMC Neurons through a Neural Network in the Arcuate Nucleus." *Nature* 411.6836 (2001): 480–4.

21. Mizuno, T. M., and C. V. Mobbs. "Hypothalamic Agouti-Related Protein Messenger Ribonucleic Acid is Inhibited by Leptin and Stimulated by Fasting." *Endocrinology* 140.2 (1999): 814–7.

22. Cowley, M. A., et al. "Integration of NPY, AGRP, and Melanocortin Signals in the Hypothalamic Paraventricular Nucleus: Evidence of a Cellular Basis for the Adipostat." *Neuron* 24.1 (1999): 155–63.

23. Hruby, V. J., et al. "Alpha-Melanotropin: The Minimal Active Sequence in the Frog Skin Bioassay." *Journal of Medicinal Chemistry* 30.11 (1987): 2126–30.

24. Castrucci, A. M., et al. "Alpha-Melanotropin: The Minimal Active Sequence in the Lizard Skin Bioassay." *General and Comparative Endocrinology* 73.1 (1989): 157–63.

25. Haskell-Luevano, C., et al. "Characterization of Melanocortin NDP-MSH Agonist Peptide Fragments at the Mouse Central and Peripheral Melanocortin Receptors." *Journal of Medicinal Chemistry* 44.13 (2001): 2247–52.

26. Haskell-Luevano, C., et al. "Discovery of Prototype Peptidomimetic Agonists at the Human Melanocortin Receptors MC1R and MC4R." *Journal of Medicinal Chemistry* 40.14 (1997): 2133–9.

27. Seebach, D., et al. "B-peptides: A surprise at every turn." *Chemical Communications* 21 (1997): 2015–22.

28. Beke, Tamás, et al. "Toward a Rational Design of β-peptide Structures." *Journal of Computational Chemistry* 27.1 (2006): 20–38.

29. Wu, Y. D., et al. "Theoretical Analysis of Secondary Structures of Beta-Peptides." *Accounts of Chemical Research* 41.10 (2008): 1418–27.

30. Webb, A. I., et al. "T Cell Determinants Incorporating Beta-Amino Acid Residues are Protease Resistant and Remain Immunogenic in Vivo." *Journal of Immunology (Baltimore, Md.: 1950)* 175.6 (2005): 3810–8.

31. Kritzer, J. A., et al. "Helical Beta-Peptide Inhibitors of the p53-hDM2 Interaction." *Journal of the American Chemical Society* 126.31 (2004): 9468–9.

32. Porter, E. A., et al. "Non-Haemolytic Beta-Amino-Acid Oligomers." *Nature* 404.6778 (2000): 565.

33. Stephens, O. M., et al. "Inhibiting HIV Fusion with a Beta-Peptide Foldamer." *Journal of the American Chemical Society* 127.38 (2005): 13126–7.

34. Disney, M. D., et al. "N-Linked Glycosylated Beta-Peptides are Resistant to Degradation by Glycoamidase A." *Chemistry and Biodiversity* 2.12 (2005): 1624–34.

35. Stewart, J. M.; Young, J. D. *Solid-Phase Peptide Synthesis;* 2nd ed.; Pierce Chemical Co.: Rockford, IL, 1984.

36. Kaiser, E., et al. "Color Test for Detection of Free Terminal Amino Groups in the Solid-Phase Synthesis of Peptides." *Analytical Biochemistry* 34.2 (1970): 595–8.

37. Chen, W., et al. "A Colorimetric Assay for Measuring Activation of Gs- and Gq-Coupled Signaling Pathways." *Analytical Biochemistry* 226.2 (1995): 349–54.

38. Schioth, H. B., et al. "Major Pharmacological Distinction of the ACTH Receptor from Other Melanocortin Receptors." *Life Sciences* 59.10 (1996): 797–801.

39. Sawyer, T. K., et al. "4-Norleucine, 7-D-Phenylalanine-Alpha-Melanocyte-Stimulating Hormone: A Highly Potent Alpha-Melanotropin with Ultralong Biological Activity." *Proceedings of the National Academy of Sciences of the United States of America* 77.10 (1980): 5754–8.

40. Nunn, C., et al. "Beta(2)/beta(3)-Di- and alpha/beta(3)-Tetrapeptide Derivatives as Potent Agonists at Somatostatin Sst(4) Receptors." *Naunyn-Schmiedeberg's Archives of Pharmacology* 367.2 (2003): 95–103.