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Discovery of melanocortin ligands via a double simultaneous substitution strategy based on the Ac-His-DPhe-Arg-Trp-NH₂ template

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Abstract

The melanocortin system regulates an array of diverse physiological functions including pigmentation, feeding behavior, energy homeostasis, cardiovascular regulation, sexual function, and steroidogenesis. Endogenous melanocortin agonist ligands all possess the minimal messaging tetrapeptide sequence His-Phe-Arg-Trp. Based on this endogenous sequence, the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ tetrapeptide has previously been shown to be a useful scaffold when utilizing traditional positional scanning approaches to modify activity at the various melanocortin receptors (MC1–5R). The study reported herein was undertaken to evaluate a double simultaneous substitution strategy as an approach to further diversify the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ tetrapeptide with concurrent introduction of natural and unnatural amino acids at positions 1, 2, or 4 as well as an octanoyl residue at the N-terminus. The designed library includes the following combinations: (A) double simultaneous substitution at capping group position (Ac) together with position 1, 2, or 4, (B) double simultaneous substitution at position 1 and 2, (C) double simultaneous substitution at position 1 and 4, and (D) double simultaneous substitution at position 2 and 4. Several lead ligands with unique pharmacologies were discovered in the current study including antagonists targeting the neuronal mMC3R with minimal agonist activity and ligands with selective profiles for the various melanocortin subtypes. The results suggest that the double simultaneous substitution strategy is a suitable approach in altering melanocortin receptor potency, selectivity, or converting agonists into antagonists and vice versa.

Graphical abstract

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SUPPORTING INFORMATION

Analytical data of the peptides

Conflict of Interest: The authors declare no competing financial interests.

Keywords

Melanotropin; selective ligands; alpha-MSH; MC5R; SAR

Introduction

The melanocortin system includes several endogenous agonist ligands processed from the proopiomelanocortin prohormone (POMC), melanocortin receptors (MCR), and two endogenous antagonists: agouti signaling protein (ASP) and agouti related protein (AGRP). All five known melanocortin receptor subtypes (MC1–5R) stimulate the cAMP signal transduction pathway upon agonist activation. The MC1R is mainly expressed in melanocytes and is involved in the skin pigmentation and animal coat coloration.^{1–3} The MC1R is also expressed elsewhere in the periphery including in the macrophages,^{4, 5} lymphocytes,⁶ fibroblasts, astrocytes, neutrophils, and endothelial cells.^{7–10} Small amounts of the MC1R have also been detected by *in situ* hybridization and immunohistochemistry in scattered neurons of the periaqueductal gray substance in rat and human brains.¹¹ The MC2R is activated by the adrenocorticotrophic hormone (ACTH) and is involved in the regulation of steroid release and production from the adrenal glands.¹ It has also been reported that MC2R mRNA is found in murine adipocytes regulating stress-induced lipolysis caused by ACTH.^{12, 13} The MC3R is located in the brain, placenta, gut,¹⁴ heart,¹⁵ and human monocytes.⁵ The physiological role of the MC3R involves regulating inflammation, blood pressure, cardio-protection, and energy homeostasis.^{16–19} The MC4R is mainly expressed in the brain, and interestingly it is not readily found in the periphery.^{15, 20} A role of the MC4R in obesity development, erectile function, and sexual behavior has been reported.^{21–23} The MC5R is ubiquitously expressed in the periphery and is involved in the regulation of exocrine gland function and, possibly, immune response.^{24–26}

Unlike other GPCR families, the melanocortin system is regulated by two naturally occurring antagonists in addition to endogenous agonists that are involved in regulating the plethora of physiological functions. The mouse agouti protein (ASP) has been reported to be an antagonist at the skin MC1R and centrally expressed MC4R, while AGRP is an antagonist at the MC3R and MC4R.^{27–30} All naturally occurring melanocortin agonists contain the His-Phe-Arg-Trp sequence (Table 1) that is the minimal messaging sequence to elicit pharmacological responses at MC1R, MC3R, MC4R, and MC5R. In order to study the structure-activity relationship (SAR) of both natural and synthetic peptide ligands, several approaches have been applied previously: (1) truncation studies (deletion of one amino acid from C- or N-terminus in order to generate smaller compounds), (2) positional scanning (all positions are kept constant but one that is replaced with various coding and non-coding amino acids) and (3) alanine scanning (each amino acid is replaced with Ala in order to test

contribution of that particular position in ligand-receptor interactions) (Figure 1). Our laboratory has performed extensive SAR studies applying the positional scanning approach to the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template where one structural position (including acetyl group) was subjected to a replacement with various encoded, non-encoded amino acids, and N-terminus acyl groups.^{31–36} These studies have resulted in the discovery of several compounds with novel pharmacologies and useful neurochemical probes for *in vivo* studies.^{37, 38}

Herein, we hypothesize that simultaneous substitution at two different positions of Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ may be used as a strategy to further optimize melanocortin potency and/or selectivity. We have designed and synthesized >30 compounds that included the following double simultaneous substitutions in the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template: (A) capping group position (Ac) together with position 1, 2, or 4, (B) position 1 and 2, (C) position 1 and 4, and (D) position 2 and 4 (Figure 2). We have not altered Arg³ position based on our previous study that showed the importance of the Arg residue in the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template.³⁵ The N-terminal capping position was modified to an octanoyl residue based on our previous report that this single modification resulted in improved potency at melanocortin receptors.^{34, 36} The natural and non-natural amino acids used for the double simultaneous substitution strategy at positions 1, 2, and 4 (Figure 3, Figure 4) were selected based on the degree of change previously reported in single substitution studies (either alteration of potency or selectivity).^{31–33} The aim of this SAR campaign was to study whether a synergistic pharmacological effect can be observed for the concurrent incorporation of the selected amino acids and/or capping group at specific positions of the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template that may not have been observed in a standard positional scanning approach or single substitution strategy.

One lead ligand resulting from the current double simultaneous substitution strategy, Ac-Trp-(pI)DPhe-Arg-Trp-NH₂, was originally disclosed in an academic thesis, and its pharmacology has already been further explored.^{37, 39, 40} The Ac-Trp-(pI)DPhe-Arg-Trp-NH₂ peptide, also known as SKY2–23-7 or **13** herein, was the basis of a seven-member library of tetrapeptides that led to ligands with unique pharmacological profiles at the mMC3R and mMC4R.³⁹ Furthermore, the Ac-Trp-(pI)DPhe-Arg-Trp-NH₂ peptide was utilized to probe the effect of central melanocortin ligand administration on energy homeostasis *in vivo*. It was found that Ac-Trp-(pI)DPhe-Arg-Trp-NH₂ affects energy homeostasis in male and female mice differently, therefore this tetrapeptide is a novel neurochemical probe for studying the sexual dimorphism known to exist within the melanocortin pathway.^{37, 41} Furthermore, the tetrapeptide Ac-His-(pI)DPhe-Arg-Tic-NH₂, or **31** herein, was also previously identified in a mixture based positional scanning library in which millions of compounds are screened simultaneously.⁴² The identification of the same compound by two different techniques independently helps to reciprocally verify each technique and ensure reproducibility. The current hindsight allows for a unique opportunity to present a validated method that led to useful and novel pharmacological probes.

Results and Discussion

Design and Chemical Synthesis:

A double simultaneous substitution strategy was employed utilizing the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template. This template has been extensively studied by us and others.^{31–36, 43, 44} In the double simultaneous substitution strategy, two positions are concurrently substituted in order to detect effects that may be synergetic and more effective than a single substitution. This may allow for the discovery of novel ligands that would be difficult to discover with traditional single substitution or positional scanning approaches. The current ligand-based double simultaneous substitution strategy was envisioned based on simultaneous, double and multiple substitution approaches that are frequently applied in the mutation studies of receptors.^{45–48} Double receptor mutations have identified important receptor residues that either form the binding pocket or are involved in receptor activation.^{45–48} The current double simultaneous substitution strategy allowed for the design of a highly diversified peptide library. In total, 35 unique peptide ligands were synthesized and characterized herein.

In the current report, substitutions were made at the Ac position, the His¹ position, the DPhe² position, and the Trp⁴ position. The following substitutions were simultaneously made: (A) capping group position (Ac) together with position 1, 2 or 4, (B) position 1 and 2, (C) position 1 and 4, and (D) position 2 and 4 (Figure 2, Figure 4). The residues used for the substitutions (Figure 3) were selected based on previously reports in which these substitutions resulted in large degrees of change in either potency or selectivity.^{31–34, 36}

Furthermore, the design strategy employed should allow the compounds reported herein to be good lead compounds for future ligand design due to the careful selection of the side chains utilized. Specifically, the side chains of the non-natural amino acids used in this study are readily present in many commercially available drugs suggesting that incorporation of these amino acids and side chains will aid in future ligand design and synthesis.^{49–52} For example, the Bip amino acid possesses the biphenyl group that is readily found in variety of commercially available drugs (*i.e.* a “privileged structure”), and as such is suitable for design of peptidomimetics and small molecule analogues if desired.⁵³

The peptides reported herein were synthesized using standard fluorenylmethyloxycarbonyl (Fmoc) chemistry and a parallel synthesis strategy on an automated synthesizer (Advanced ChemTech 440MOS, Louisville, KY).^{54, 55} The peptides were purified to homogeneity using semipreparative reversed-phase high-pressure liquid chromatography (RP-HPLC). The peptides possessed the correct molecular weights as determined by mass spectrometry (Supporting Material). The purity of these peptides was assessed by analytical RP-HPLC in two different solvent systems and were at least 95% pure (Supporting Material).

Biological Evaluation:

The ligands' ability to activate the melanocortin receptors was evaluated by a CRE/ β -galactosidase reporter gene assay that is primarily sensitive to G_{αs}-protein signaling and the downstream cAMP pathway as previously reported.^{32, 56, 57} Compounds were first screened for agonist qualities at the cloned mMC1R, mMC3R, mMC4R, and mMC5R (Table 2).

Ligands that were not full agonists were further assessed for antagonist qualities by Schild analysis (Table 2).⁵⁸ Reference compounds from previous single substitution positional scanning approaches are summarized in Table 3.^{31–33} Illustration of the pharmacology of peptides that possess no antagonist activity are presented in Figures 5 and Figure 6. For comparison, compounds' pharmacology will be reviewed by each substitution pattern at the melanocortin receptors below.

Double simultaneous substitution at the N-terminal capping region and position 1, 2, or 4 in the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template.

It has previously been proposed that the N-terminal acetylation or C-terminal carboxyamidation of endogenous α -MSH leads to an increase enzymatic stability towards peptidases.⁵⁹ Therefore, modification of the N-terminus has been of interest in melanocortin ligand design. Early melanocortin ligand designs included addition of fatty acid conjugates^{60, 61}, biotin⁶², and chlorotriazinylaminofluorescein⁶³ at the N-terminus of melanocortin peptides. Based upon these observations, our group has performed extensive SAR studies at the N-terminus of the His-DPhe-Arg-Trp-NH₂ tetrapeptide that included addition of various cyclic moieties, aromatic acyl modifications, polyethylene diamine diglycolic acids, and long chain fatty carboxylic acids.^{34, 36, 64} Based on these previous studies, an octanoyl residue (CH₃(CH₂)₆C=O) was chosen as the replacement of the acetyl residue in the current double simultaneous substitution strategy similar to the previously reported parent peptide **54**, octanoyl-His-DPhe-Arg-Trp-NH₂.^{34, 36} This singly substituted peptide has reported EC₅₀ values of 0.36 nM, 4.0 nM, 0.38 nM, and 0.79 nM at the mMC1R, mMC3R, mMC4R, and mMC5R, respectively (Table 3).³⁴ Peptides **2–10** were designed to probe whether an additional substitution in Octanoyl-His-DPhe-Arg-Trp-NH₂ would enhance potency or selectivity at melanocortin receptors.^{31–33}

In vitro characterization of peptides **2–10** resulted in some very interesting pharmacology when compared to the reference peptide **1**, Ac-His-DPhe-Arg-Trp-NH₂ (Table 2, Figure 5). At the mMC1R, compounds **3–7** resulted in more potent agonism with 10-, 20-, 7-, 22-, and 40-fold differences in EC₅₀ values, respectively, as compared to the reference peptide **1**. Peptides **2, 8–10** were characterized as equipotent (within 3-fold experimental error) as the reference compound **1** at the mMC1R. At the mMC3R, peptide **3** (EC₅₀ = 52 nM) was the only compound in this series that was a more potent agonist than the Ac-His-DPhe-Arg-Trp-NH₂ with an 11-fold difference. Compound **5** was characterized as equipotent agonist at the mMC3R (EC₅₀=290nM) compared to the reference peptide **1**. Compound **4** and **9** possessed low μ M stimulatory activity at the mMC3R. Ligands **2, 6, 7**, and **10** resulted in only 50%–65% maximal agonist signal compared to α -MSH at concentrations up to 100 μ M at the mMC3R. Interestingly, compounds **6–8** resulted in antagonist activity at the mMC3R with compound **6** showing the strongest antagonist characteristics (pA₂=8.3). At the mMC4R, no increases in agonist potency were observed for any compounds in this series. Compound **3** was equal potent compared to compound **1**. Compounds **4, 5, 7, 9**, and **10** were all full agonist ligands, but less potent than compound **1** at the mMC4R. Peptides **2** and **6** resulted in partial agonist activation of the mMC4R, and both acted as antagonist when measured by Schild analysis (pA₂ = 7.0 and 8.6, respectively). Compound **8** resulted in a full antagonist pharmacology with a pA₂ value of 7.3. At the mMC5R, introduction of an octanoyl residues

in peptides **3**, **6**, and **7** with substitutions 3BaI⁴, (pI)DPhe², and DBip², respectively, resulted in agonists that are equipotent to the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ peptide. Peptides **2**, **4**, **5**, **8**, **9**, and **10** (Table 2) with Bip⁴, Tic⁴, Phe⁴, DBip⁴, DHis¹, Trp¹ amino acids, respectively, resulted in agonists with decreased potencies at the mMC5R, as compared to the peptide **1**. The most significant decrease of potency compared to the Ac-His-DPhe-Arg-Trp-NH₂ was compound **9** (55-fold less potent) and compound **4** (50-fold less potent) with substitution of DHis¹ and Tic⁴, respectively.

The most interesting SAR of the current series was the conversion of agonist compounds at the mMC3R and mMC4R to antagonist compounds (Table 2). For example, the single substitution of the Ac residue to the octanoyl residue in **54** resulted in strong agonist activity at the mMC3R and mMC4R (EC₅₀ = 4.0 nM and 0.38 nM, respectively) (Table 3).³⁴ Similarly, compound **43** with a single substitution at the fourth position of Trp to DBip resulted in a weak agonist at the mMC3R and mMC4R (16 μM and 1.5 μM, respectively) (Table 3).³¹ Unexpectedly, the combination of these two substitutions in compound **8** resulted in a compound that has an antagonist pharmacology and minimal agonist activity at the mMC3R and mMC4R (pA₂ = 6.5 and 7.3, respectively) (Table 2).

Similar results can be observed at either the mMC3R or mMC4R with compounds **2**, **6**, and **7**. Specifically, compound **6** resulted in a large switch in pharmacology at the mMC4R. The singly substituted analogs **45** and **54** were both potent MC4R agonists (EC₅₀ = 25 nM and 0.38 nM, respectively), but the combination of both substitution in peptide **6** resulted in a strong nanomolar antagonist with low agonist signal (pA₂ = 8.6, corresponding to a K_i ~ 3 nM). These pharmacology changes were especially unexpected since it is hypothesized that the octanoyl analog **54** results in increased potency compared to **1** due to its ability to insert the long alkyl chain (n-octyl) into the cell membrane's lipid bilayer that results in increased peptide presentation to the binding pocket of the melanocortin receptors located on the membrane.^{36, 65} This purported mechanism would increase the local concentration of the ligand on the cell membrane and thereby increase potency, but would not modify the orthosteric binding interaction in a way that would switch pharmacology from agonist to antagonist. These surprising SAR results support the utilization of the double simultaneous substitution strategy to discover new ligands with unique pharmacologies that would have been difficult to design based on single substitution SAR (*i.e.* the combination of two single substitutions that result in agonist compounds would not be predicted to lead to antagonist compounds).

Double simultaneous substitution at positions 1 and 2 in the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template.

GPCR homology molecular modeling and MC1R mutagenesis studies have identified a putative hydrophobic receptor binding pocket built of several Phe residues proposed to interact with the amino acid corresponding to the Phe⁷ position (*i.e.* position 2 in Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂) in the natural ligand, α-MSH.^{66, 67} Several reports have been published regarding the significance of this position in the ligand binding and subsequent melanocortin stimulation.^{68–70} Because of these findings, the substitutions previously made in reference compound **45–47** (Table 3) were included in the double simultaneous

substitution strategy design.³² A previous single amino acid substitution study also reported that the His⁶ position is important in the design of ligands that are more selective for the mMC4R than the mMC3R, and therefore, the single substitutions found in compound **48–53** were also included in the double simultaneous substitution strategy design.³³

Based on these previous findings, compounds **11–22** with double simultaneous substitution at positions 1 and 2 were synthesized (Table 2). Compounds **11–17** possessed (pI)DPhe² and variety of other amino acids at position 1 in the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template which was based on previously reported single substituted analogs (Table 3). Compounds **18–22** possessed a variety of different side chains in these two positions. At the mMC1R, peptides **11** and **14** were equipotent (within 3-fold intrinsic error of the assay) as compared to the reference peptide **1**. All other compounds in this series retained full agonist profiles, but resulted in decreased potency at the mMC1R (Table 2, Figure 6). At the mMC3R, the presence of the (pI)DPhe amino acid at the 2nd position of the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template in addition to various natural and non-natural amino acids at the 1st position led to the generation of competitive antagonists (peptides **12**, **13**, **16**, and **17**) with minimal agonist activity (Figure 7), or ligands with mixed pharmacology (competitive antagonists with some stimulatory activity) as observed in the compounds **11**, **14** and **15**. Compound **18** with DBip amino acid at the 2nd position and Trp amino acid at the 1st position (similar to the compound **13**) possessed antagonist activity (pA₂ = 6.7). Interestingly, compounds **19**, **20**, and **21** possessed no activity at the mMC3R up to concentrations of 100 μM. Compound **22** resulted in 45% maximal signal when tested up concentrations of 100 μM at the mMC3R.

Similar to the pharmacological results obtained at the mMC3R, double simultaneous substitutions at positions 1 and 2 resulted in antagonist compounds **11–17** at the mMC4R when (pI)DPhe² is present in the current series (Table 2). The most potent antagonist at the mMC4R in this series was peptide **16** with a pA₂ value of 8.9. Compound **18** also possessed agonist characteristics similar to its pharmacology at the mMC3R. Compounds **19–22** that possessed Trp¹-DNal(1')², Phe¹-DBip², Phe¹-DNal(1')² or 4Pal¹-DBip² combinations were full, but less potent, agonists at the mMC4R than the reference peptide **1** (Figure 6). At the mMC5R, all compounds in this series resulted in decreased potency. Compounds **11**, **14**, **15** with concurrent combinations DHis¹-(pI)DPhe², 2Thi¹-(pI)DPhe², and Phe¹-(pI)DPhe², respectively, were the most potent analogs in this series, but still resulted in decreased potency (11- to 25- fold) as compared to the peptide **1**. The most significant decrease of potency (3300-, and 1900-fold difference) in this series was observed for the compounds **12** and **13** that possessed 3Pal¹-(pI)DPhe², and Trp¹-(pI)DPhe² simultaneous substitutions, respectively.

Two interesting SAR trends may be observed in the current series. The first trend was observed in compounds that had a DBip in the second position. Reference peptide **47** with a DBip² substitution was characterized as an agonist at all melanocortin receptors examined (Table 3). Additionally, none of the His¹ monosubstituted analogues (**48–53**) possessed an antagonist profile at the melanocortin receptors examined (Table 3).³² Interestingly, the simultaneous Trp¹-DBip² combination (**18**) resulted in a non-selective mMC3R and mMC4R antagonist with a non-selective mMC1R and mMC5R micromolar agonist profile.

Compound **20** with Phe¹, possessed no activity at the mMC3R while the peptide **22** with 4Pal¹ substitution possesses mixed pharmacology at this receptor. It seems that the agonist/antagonist profile for the DBip² double substituted tetrapeptides was dependent on the amino acid introduced in the Xaa¹ position. Based upon these three examples (peptides **18**, **20** and **22**), it can be hypothesized that competitive antagonist activity may result at the central melanocortin receptors if the first amino acid side chain corresponding to the Xaa¹-DBip² combination possesses a hetero-atom within its aromatic ring (nitrogen was present in the indole ring (Trp) or pyridine ring (4Pal)).

The second trend was observed in compounds that had a (pI)DPhe² in the second position. Reference monosubstituted analogue **45** that has (pI)DPhe in the second position was a very potent nanomolar agonist at the mMC4R and antagonist at the mMC3R (Table 3).³² However, introduction of DHis¹, 3Pal¹, Trp¹, 2Thi¹, Phe¹, 4Pal¹, or Bip¹ into the parent peptide **45** leads to the conversion of agonist activity into competitive antagonist activity at the mMC4R (Table 2). These results are very significant due to the fact that none of the His¹ monosubstituted analogues (**48–53**) possessed antagonist activity at the centrally expressed receptors, and therefore this agonist/antagonist switch would not be predicted based on additive results alone.³²

In addition to the SAR trend observed that all (pI)DPhe substituted tetrapeptides in this series possessed antagonist activity at both the mMC3R and mMC4R, many of the compound in this series were antagonist that resulted in minimal agonist activity at the mMC3R (peptides **12**, **13**, **16**, **17**, and **18**), a trait not commonly observed for mMC3R/mMC4R antagonist (Table 2, Figure 7). Peptides **16** and **17** resulted in minimal agonist activity at the mMC4R as well. Due to their uncommon pharmacological profile and their relatively “clean” antagonism, compounds from this series would be good lead compound for further development as well as good chemical probes for studying the central melanocortin receptors *in vivo*. In fact, compound **13** has already served as a lead compound in a further SAR study which resulted in the discover of two selective mMC4R antagonists over the mMC3R, and additional mMC3R antagonists that possessed minimal mMC3R agonist activity.³⁹ Furthermore, compound **13** was utilized as an *in vivo* probe and was identified to have different effects on energy homeostasis in male mice compared to female mice.^{37, 71} These already reported follow-up studies demonstrate two important lessons: 1) the importance of discovering ligands with novel *in vitro* pharmacologies and evaluating them *in vivo*, and 2) the utility of the current double simultaneous substitution strategy to discover useful lead ligands for further development and *in vivo* probes with a novel pharmacological profiles.

Double simultaneous substitution at positions 1 and 4 in the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template.

The importance of the Trp amino acid in position 4 of the Ac-His-DPhe-Arg-Trp-NH₂ has been explored previously.³¹ Examples of the effects of a single substitution on this position may be seen in compounds **37–44** in which all compounds retain full agonist pharmacology at all receptor subtypes (Table 3).³¹ Further SAR studies on the Trp positions in various melanocortin templates have shown similar trends suggesting the Trp residue does play a

role in ligand-receptor interactions.^{69, 70, 72–74} Together with the observations noted above for the significance of the His position in the Ac-His-DPhe-Arg-Trp-NH₂ template,³³ it was hypothesized that double substitution of both position 1 and position 4 would alter melanocortin potency and/or selectivity in a unique way. Therefore, compounds **23–28** that included double substitution at the positions 1 and 4 were designed, synthesized, and biologically evaluated (Table 2, Figure 6).

At the mMC1R, none of compounds **23–28** resulted in increased potency compared to compound **1**. The most potent compound in the series was peptide **23** that has a 14-fold decrease in potency compared to compound **1**. The most significant decrease in potency (>160-fold decrease as compared to **1**) has been observed for the peptide **26** with Trp at position 1 and DNal(2') at position 4 of the Ac-His¹-DPhe-Arg-Trp⁴-NH₂ template. At the mMC3R, peptides **23, 25–27** with double simultaneous substitution at the positions 1 and 4 resulted in no activity (Table 2, Figure 6). Full, but weak agonism (EC₅₀ = 12 μM) was only noticed for the peptide **28**, Ac-2Thi-DPhe-Arg-Nal(2')-NH₂, at the mMC3R. Compound **24** resulted in 50% maximal signal of α-MSH at the mMC3R. At the mMC4R, biological characterization of peptides **23–28** revealed that none of compounds were more potent than the Ac-His-DPhe-Arg-Trp-NH₂ peptide. Compound **23** was the most potent compound with an EC₅₀ of 110 nM and was 8-fold less potent than compound **1**. Compound **25** that included Trp¹ and 3Bal⁴ double substitution possessed the most significant decrease of potency in the series with a 580-fold difference. At the mMC5R, all compounds with changes at positions 1 and 4 in the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ peptide resulted in decreased potency as compared to **1**. The most potent analog in this series at the mMC5R was compound **28** that had an EC₅₀ of 76 nM and was 14-fold less potent than compound **1**. Compounds with Trp¹ and either 3Bal⁴ or DNal(2')⁴ amino acids (peptides **25–26**) are the least potent mMC5R agonists as compared to **1**, with approximately 1000-fold decreased potency.

The overall SAR trend for this series was that none of the compounds **23–28** possessed increased potency as compared to the reference peptide **1** for any receptor isoform (Table 2, Figure 6). At the mMC3R, the double simultaneous substitution approach resulted in combinations 3Pal¹-Nal(2')⁴, Trp¹-3Bal⁴, Trp¹-DNal(2')⁴, 2Thi¹-3Bal⁴ (compounds **23, 25–27**) that have minimal agonist activity up to 100 μM concentrations. Interestingly, peptide **23** is a relatively potent mMC4R agonist (EC₅₀=110 nM), and therefore selective for the mMC4R over the mMC3R (Figure 8). Comparing peptide **23** to its monosubstituted counterparts **50** and **39**, the effects of the double simultaneous substitution can be observed. The 3Pal¹ analogue **50** possessed only slight agonist activity at the mMC3R,³³ and the single substituted Nal(2')⁴ derivative **39** was characterized as a potent mMC4R agonist (EC₅₀ = 16 nM) and full mMC3R agonist (EC₅₀ = 740 nM) (Table 3).^{32, 33} The double simultaneous substitution strategy in **23** retains the potent agonism of compound **39** at the mMC4R, as well as the lack of activity of compound **50** at the mMC3R.

The double simultaneous substitution approach also led to the increased selectivity of compound **28** for the mMC5R compared to the mMC1R and mMC3R (Table 2). It is 35-fold, 163-fold, and 6-fold more potent at the mMC5R compared to the mMC1R, mMC3R,

and mMC4R, respectively. This is especially significant compared to the monosubstituted peptides **39** and **52** in which selectivity was no greater than 5-fold between the mMC1R and the mMC5R. Presumably, this change in selectivity was due to the double simultaneous substitution since it was not obtained in either singly substituted peptide.

Double simultaneous substitution at positions 2 and 4 in the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template.

The Phe position of α -MSH-based ligands has been intensively studied for over 20 years.^{18, 75–82} For example, the commonly used control antagonist SHU9119, which is a MC3R partial agonist/antagonist and MC4R antagonist, resulted from the introduction of DNal(2') at the Phe position in the MT-II agonist template (Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH₂).⁸² A similar pharmacological profile was observed when substituting the Phe position with (pI)DPhe in this template.⁸² In the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template, it has been reported that the single substitution at the second position often resulted in strong antagonists at the MC3R or MC4R while retaining an agonist activity at the MC1R and MC5R.³² Single substitutions at the fourth position have also been identified to result in changes in pharmacology as discussed above. Therefore, it was decided to simultaneously substitute both the second and fourth position of the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template herein.

Compounds **29–36** were designed with double simultaneous substitution at positions 2 and 4 in the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template (Table 2). Peptides **29–32** possessed (pI)DPhe at position 2 whereas peptides **33–36** have DBip at position 2. At the mMC1R, all compounds in this series were agonists that were either more potent (peptides **31**, **33**, **34**, and **36**) or were within 3-fold experimental error (peptides **29**, **30**, and **35**) compared to peptide **1**. The most potent analog in this series at the mMC1R was compound **34** that possessed an EC₅₀ value of 9 nM and was 16-fold more potent compared to compound **1**. At the mMC3R, peptide **31** was a full agonist with an EC₅₀ value of 2.9 μ M. All other compounds possessed antagonist characteristics with less than 60% maximal agonist activity when assayed up to 100 μ M at the mMC3R. The most potent antagonist at the mMC3R was compound **36** which had a pA₂ value of 7.6, but did possess some agonist activity (50% of maximal when assayed up to 100 μ M). Peptides **33** and **35** with combinations of different Bip isomers (L, D) were competitive antagonists with no additional stimulatory activity at the mMC3R. However, double simultaneous substitution compounds that included DBip at the 2nd position and 3Bal or Nal(2') at the 4th position (peptides **34** and **36**) led again to mixed agonist/antagonist pharmacology at the mMC3R. At the mMC4R, compounds **29**, **30**, and **33–35** were antagonists possessing some additional stimulatory activity. Peptide **30** was the most potent competitive antagonist in this series at the mMC4R (pA₂ = 8.0). Surprisingly, peptides with (pI)DPhe²-Tic⁴ combination (**31**) and (pI)DPhe²-Phe⁴ combination (**32**) were full nanomolar agonists at mMC4R, with peptide **32** being more potent than peptide **31**. In addition, peptide **36** with DBip²-Nal(2')⁴ combination was a potent mMC4R agonist with an EC₅₀ value of 52 nM. At the mMC5R, peptides **29**, **30**, **32** and **34** were equipotent agonists (within 3-fold experimental error) as compared to **1**. Compounds **31**, **33**, **35**, and **36** were all less potent compared to compound **1** at the mMC5R.

The SAR of compounds **29–36** were consistent with SAR trends of well-known studied antagonists, like SHU9119. In most cases, competitive antagonism at the centrally expressed mMC3R and mMC4R was observed with full agonism at the mMC1R and mMC5R. The combinations (pI)DPhe²-Bip⁴ (peptide **29**) and (pI)DPhe²-3Bal⁴ (peptide **30**) resulted in competitive antagonists at the mMC3R and mMC4R (with some stimulatory activity), and very potent nanomolar agonist activity at the mMC1R and mMC5R. Quite unexpectedly, peptides with the substitutions (pI)DPhe²-Tic⁴ (peptide **31**) and (pI)DPhe²-Phe⁴ (peptide **32**) were not antagonists at the centrally expressed melanocortin receptors as one would predict based on the role of the (pI)DPhe amino acid in other melanocortin antagonists. In addition, peptide **31** was more selective for the mMC1R with 120-, 26-, and 6-fold difference as compared to the mMC3R, mMC4R, and mMC5R, respectively. Interestingly, the sequence of compound **31** was more recently discovered by a mixture based positional scanning library approach.⁴² The fact that this sequence was discovered through two different techniques suggested the merit of both techniques to discover novel melanocortin ligands.

Peptide **32** that possessed Phe⁴ residue (as opposed to the cyclic residue Tic⁴ in the compound **31**) is most potent at the mMC5R (EC₅₀=18 nM) and did not elicit full agonistic response at the mMC3R. Interestingly, double substitution of (pI)DPhe²-Phe⁴ (peptide **32**) resulted in agonism at the mMC4R, with no preference over the mMC1R. The Trp⁴ residue in the Ac-His¹-DPhe²-Arg³-Trp⁴-NH₂ template appeared to play an important role in the double substituted analogues.

Conclusion

The double simultaneous substitution strategy applied herein was a useful approach for the design and synthesis of novel ligands with unique pharmacologies. Peptide **2**, that is based on the Bip⁴ amino acid and the octanoyl capping group at the N-terminus, was characterized as a competitive antagonist at the mMC4R with only some stimulatory activity observed at the neuronally expressed mMC3R. Ligands with Xaa¹-(pI)DPhe² combinations are characterized as mMC3R and mMC4R antagonists. The most selective of these compounds was peptide **16** that possesses a 66-fold preference for the mMC4R versus mMC3R. Peptides with double simultaneous substitution at positions 1 and 4 possessed no antagonist activity at the receptors examined in this study. Analogue **28** was the only representative in this series that possessed selectivity for the mMC5R versus the mMC3R, mMC1R, and mMC4R. Although the presence of the (pI)DPhe amino acid in different templates usually led to the antagonist activity, it was achievable to convert its antagonist profile into agonist activity based on the double simultaneous substitution approach (peptides **31** and **32**). The findings reported herein revealed that by introducing various concurrent substitutions into the melanocortin tetrapeptide template it was possible to alter potency, shift selectivity, and quite unexpectedly convert agonists into antagonists and vice versa.

Also, the verified results from published follow-up studies of compound **13** validates the double simultaneous substitution strategy as a useful method in the development of *in vivo* neurochemical probes to study the effects of the melanocortin system on energy homeostasis.^{37, 39} Furthermore, the independent discovery of compound **31** in a mixture based positional scanning library validates both of these methods' utility in drug and probe

discovery.⁴² The lessons learned in the current manuscript about the double simultaneous substitution strategy should be broadly applicable to various other systems where peptidic ligands have been identified, and should allow for the further discovery of novel ligands with unique pharmacologies.

Experimental

Peptide synthesis was performed using standard Fmoc methodology^{54, 55, 83} on an automated synthesizer (Advanced ChemTech 440MOS, Louisville, KY).^{52–53,86} The amino acids, Fmoc-DHis(Trt), Fmoc-His(Trt), Fmoc-DPhe, Fmoc-Trp(Boc), Fmoc-Phe, Fmoc-3-(1-naphthyl)alanine [Nal(1')], Fmoc-3-(1-naphthyl)-D-alanine [DNal(1')], Fmoc-3-(2-naphthyl)alanine [Nal(2')], Fmoc-3-(2-naphthyl)-D-alanine [DNal(2')], were purchased from Peptides International (Louisville, KY). Fmoc-3-(3-pyridinyl)alanine (3-Pal), and Fmoc-3-(4-pyridinyl)alanine (4-Pal) were purchased from Bachem (Torrance, CA). Fmoc-4-phenyl-phenylalanine (Bip), Fmoc-4-phenyl-D-phenylalanine (DBip), Fmoc-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), and Fmoc-4-iodo-D-phenylalanine (*p*-I-DPhe) were purchased from Synthetech (Albany, OR). The coupling reagents 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were purchased from Peptides International. Glacial acetic acid (HOAc), dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), and anhydrous ethyl ether were purchased from Fisher (Fair Lawn, NJ). *N,N*-dimethylformamide (DMF) was purchased from Burdick and Jackson (McGaw Park, IL). Trifluoroacetic acid (TFA), octanoic acid, 1,3-diisopropylcarbodiimide (DIC), pyridine, piperidine, and acetic anhydride were purchased from Sigma (St. Louis, MO). *N,N*-diisopropylethylamine (DIEA) and triisopropylsilane (Tis) were purchased from Aldrich (Milwaukee, WI). All reagents and chemicals were ACS grade or better and were used without further purification.

The peptides were assembled on rink-amide-MBHA resin (0.44 mequiv/g substitution), purchased from Peptides International. The synthesis was performed using a 40 well Teflon reaction block with a coarse Teflon frit. Approximately 100 mg of resin (0.044 mmol) was added to each reaction block well. The resin was allowed to swell for 2 h in DMF. An initial 5 min deprotection was performed using 25% piperidine in DMF. Then a second fresh aliquot of 25% piperidine was added, and the solution was mixed at 450 rpm for 20 mins to ensure complete deprotection. A positive Kaiser test⁸³ resulted after deprotection indicating a free amine group on the resin. The first *N*-Fmoc-protected amino acid was added to the amide-resin using the general coupling cycle as follows: 500 μ L DMF is added to each reaction well to "wet the frit", 3-fold excess *N*-Fmoc-protected amino acid starting from the C terminus is added (275 μ L of 0.5 M amino acid solution containing 0.5 M HOBt in DMF) followed by the addition of 275 μ L of 0.5 M DIC in DMF, and the reaction well volume was brought up to 3 mL using DMF. The coupling reaction was mixed for 1 hr at 450 rpm, followed by emptying of the reaction block by positive nitrogen gas pressure. A second coupling reaction was performed by the addition of 500 μ L of DMF to each reaction vessel, followed by the addition of 275 μ L of the respective amino acid (3-fold excess), 275 μ L of 0.5 M HBTU, and 225 μ L of 1 M DIEA. The reaction well volume was brought up to 3 mL with DMF and mixed at 450 rpm for 1 h.

After the second coupling cycle, the reaction block was emptied and the N-Fmoc-protected peptide-resin was washed with DMF (4.5 mL, four times). N-Fmoc deprotection was performed by the addition of 4 mL of 25% piperidine in DMF and mixing for 5 min at 450 rpms. The deprotection was driven to completion by emptying the reaction block and a second addition of 25% piperidine in DMF was mixed for 20 min at 450 rpms. The reaction well was then washed with DMF (4.5 mL, four times). The next coupling cycle was performed as described above, followed by subsequent deprotection until the desired amino acid sequence on resin was obtained. Following the final N-Fmoc deprotection, acetylation of the N-amine was performed by addition of 2 mL of acetic anhydride, 1 mL of pyridine, and 1 mL of DMF to the reaction block wells. The solution was mixed for 30 min at 450 rpms. The acetylated peptide-resin was washed with DCM (4 mL, five times) and dried thoroughly prior to cleavage from the resin.

Simultaneous deprotection of the amino acid side chains and cleavage of the crude peptide from the resin was performed by incubating the peptide-resin with 3 mL of cleavage cocktail (95% TFA, 2.5% water, and 2.5% Tis) for 3 h mixed at 450 rpms. The cleavage product was emptied from the reaction block into a cleavage block containing 7 mL collection vials under positive nitrogen gas pressure. The resin was washed with 1.5 mL of cleavage cocktail for 5 min and this solution was added to the previous cleavage solution. The peptides were transferred to pre-weighed 50 mL conical tubes and cleaved peptide was precipitated with cold (4°C) anhydrous ethyl ether (up to 50 mL). The flocculent peptide was pelleted by centrifugation (Sorval Super T21 high-speed centrifuge using the swinging bucket rotor) at 4000 rpm for 5 min, the ether was decanted off, the peptide was washed one time with cold anhydrous ethyl ether, and the crude peptide was pelleted again. The crude peptide was dried in vacuo for 48 h. A 15–30 mg sample of crude peptide was purified by RP-HPLC using a Shimadzu chromatography system with a photodiode array detector and a semipreparative RP-HPLC C18 bonded silica column (Vydac 218TP1010, 1.0 cm × 25 cm) and lyophilized. The purified peptides were at least >95% pure as determined by analytical RP-HPLC in two diverse solvent systems (*i.e.* 10% MeCN in 0.1% TFA/water and a gradient to 90% MeCN over 35 min; and 10% MeOH in 0.1% TFA/ water and a gradient to 90% MeOH over 35 min). The peptide mass was confirmed by mass spectrometry (University of Florida protein core facility) (Supplemental Materials).

Cell Culture and Transfection.

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and seeded 1 day prior to transfection at 1 to 2×10^6 cells/100 mm dish. Melanocortin receptor DNA in the pCDNA₃ expression vector (20 µg) was transfected using the calcium phosphate method.⁸⁴ Stable receptor populations were generated using G418 selection (1 mg/mL) for subsequent bioassay analysis.

CRE/β-galactosidase Reporter Gene Bioassay.

The HEK-293 cells stably expressing the melanocortin receptors were transfected with 4 µg of CRE/β-galactosidase reporter gene as previously described.⁵⁶ Briefly, 5000–15000 post-transfection cells were plated into 96-well Primera plates (Falcon) and incubated overnight. Forty-eight hours post-transfection, the cells were stimulated with 100 µL of peptide

(10^{-4} - 10^{-12} M) or forskolin (10^{-4} M) control in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 h. The assay media was aspirated, and 50 μ L of lysis buffer (250 mM Tris-HCl, pH 8.0, and 0.1% Triton X-100) was added. The plates were stored at -80°C overnight. The plates containing the cell lysates were thawed the following day. Aliquots of 10 μ L were taken from each well and transferred to another 96-well plate for relative protein determination. To the cell lysate plates, 40 μ L of phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μ L of substrate buffer (60 mM sodium phosphate, 1 mM MgCl_2 , 10 mM KCl, 5 mM β -mercaptoethanol, and 200 mg ONPG) was added to each well, and the plates were incubated at 37°C . The sample absorbance, OD_{405} , was measured using a 96 well plate reader (Molecular Devices). The relative protein was determined by adding 200 μ L of 1:5 dilution BioRad G250 protein dye:water to the 10 μ L cell lysate sample taken previously, and the OD_{595} was measured on a 96-well plate reader (Molecular Devices). Data points were normalized both to the relative protein content and nonreceptor-dependent forskolin stimulation. The antagonistic properties of these compounds were evaluated by the ability of these ligands to competitively displace the MTII agonist (Bachem) in a dose-dependent manner at concentrations up to 10 μM .⁸¹ The pA_2 ($K_i = -\log pA_2$) values were generated using the Schild analysis method.⁵⁸

Data Analysis.

The EC_{50} and pA_2 values represent the mean of duplicate data points from at least four independent experiments. The EC_{50} and pA_2 values and their associated standard error of the mean (SEM) were determined by fitting the data to a nonlinear least-squares analysis using the PRISM program (v3.0, GraphPad Inc).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Illustration of the truncation strategy approach. The concept is based on the shortening of the full-length peptide in order to obtain the minimal sequence required to elicit a pharmacological response at the target receptors. The truncation can be performed in the C-terminus to N-terminus direction (as shown) or vice versa. (B) Illustration of the positional scanning approach that is based on the altering of one amino acid position at the time while the remaining positions are kept constant. (C) Illustration of alanine scanning which is a special case of positional scanning in which the amino acid used for positional scanning is alanine.

Figure 2.

Illustration of the double simultaneous substitution technique. The strategy applied in this approach relies on the two concurrent substitutions while the other positions are kept constant. Various amino acids are simultaneously introduced at positions 1, 2, or 4 as well as the N-terminal capping position. The following combinations are performed: (A) capping group position (Ac) together with position 1, 2, or 4, (B) position 1 and 2, (C) position 1 and 4, and (D) position 2 and 4.

Figure 3.

Chemical structures of amino acids used as diversity elements in the double simultaneous substitution approach.

Figure 4.
Illustration of the double simultaneous substitution approach denoting various amino acids used at the specific positions in the Ac-His-DPhe-Arg-Trp-NH₂ tetrapeptide template.

Figure 5.

Graphical illustration of ligands that possess no antagonist activity at the melanocortin receptors. All compounds presented this figure possess full agonist profile at melanocortin receptors examined in this study.

Figure 6.

Graphical illustration of ligands that possess no antagonist activity at the melanocortin receptors. All compounds presented this figure possess full agonist profile at mMC1R, mMC4R, and mMC5R.

Figure 7. Illustration of the competitive antagonism of peptide **12** at the mMC3R as assessed by Schild analysis. Note that there is no agonist activity by compound **12** alone at concentrations up to 100 μ M.

Figure 8. Pharmacological profile of the peptide **23** that is a selective nanomolar agonist for the mMC4R, as opposed to its minimal agonist activity at the mMC3R.

Table 1.
Amino acid sequence of naturally occurring agonist ligands ACTH(1-24), α -MSH, β -MSH, γ_1 -MSH, and γ_2 -MSH.

Ligand	Amino acid sequence
ACTH(1-24)	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro
α -MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH ₂
β -MSH	Ala-Glu-Lys-Lys-Asp-Glu-Gly-Pro-Tyr-Arg-Met-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp
γ_1 -MSH	Tyr-Val-Met-His-Phe-Arg-Trp-Asp-Arg-Phe-NH ₂
γ_2 -MSH	Tyr-Val-Met-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly

Table 2.

Pharmacology data for the compound synthesized applying double simultaneous substitution approach.

Peptide	Structure	mMC1R		mMC3R		mMC4R		mMC5R	
		EC ₅₀ (nM)	Fold Diff	EC ₅₀ (nM)	Fold Diff	EC ₅₀ (nM)	Fold Diff	EC ₅₀ (nM)	Fold Diff.
	α-MSH	0.36±0.07		0.65±0.06		1.9±0.2		0.46±0.19	
	NDP-MSH	0.022±0.008		0.15±0.07		0.09±0.07		0.34±0.07	
	MTH	0.032±0.008		0.23±0.03		0.09±0.02		0.18±0.04	
1.	Ac-His-DPhe-Arg-Trp-NH ₂	140±60	1	560±220	1	13.7±1.1	1	5.3±1.6	1
2.	Octanoyl-His-DPhe-Arg- Bip -NH ₂	45±12		50% at 100 μM		40% at 100 μM pA ₂ = 7.0±0.1	Ant	62±4	12
3.	Octanoyl-His-DPhe-Arg- 3Bal -NH ₂	14.6±3.9	-10	52±7	-11	13±3		2.4±0.5	
4.	Octanoyl-His-DPhe-Arg- Tic -NH ₂	7.4±2.1	-20	6900±400	12	1700±300	120	270±20	50
5.	Octanoyl-His-DPhe-Arg- Phe -NH ₂	19.4±2.1	-7	290±60		110±10	8	26±3	5
6.	Octanoyl-His-(pI) DPhe -Arg-Trp-NH ₂	6.3±2.4	-22	50% at 100 μM pA ₂ = 8.3±0.2	Ant	30% at 100 μM pA ₂ = 8.6±0.1	Ant	5.8±1.7	
7.	Octanoyl-His- DBip -Arg-Trp-NH ₂	3.7±0.9	-40	60% at 100 μM pA ₂ = 7.2±0.2	Ant	500±200	36	15±4	
8.	Octanoyl-His-DPhe-Arg- DBip -NH ₂	180±20		pA ₂ = 6.5±0.3	Ant	pA ₂ = 7.3±0.3	Ant	31±3	6
9.	Octanoyl- DHis -DPhe-Arg-Trp-NH ₂	230±30		4200±800	7	340±30	25	290±40	55
10.	Octanoyl- Trp -DPhe-Arg-Trp-NH ₂	210±30		65 % at 100 μM		180±40	13	58±7	11
11.	Ac- DHis -(pI) DPhe -Arg-Trp-NH ₂	220±60		50% at 100 μM pA ₂ = 6.4±0.1	SA Ant	60% at 100 μM pA ₂ = 7.3±0.20	SA Ant	60±22	11
12.	Ac- 3PAL -(pI) DPhe -Arg-Trp-NH ₂	4800±1400	34	pA ₂ = 7.0±0.1	Ant	35% at 100 μM pA ₂ = 8.3±0.1	SA Ant	18000±3000	3340
13.	Ac- Trp -(pI) DPhe -Arg-Trp-NH ₂	4200±1200	30	pA ₂ = 7.2±0.04	Ant	40% at 100 μM pA ₂ = 8.3±0.3	SA Ant	10000±3000	1905
14.	Ac- 2Thi -(pI) DPhe -Arg-Trp-NH ₂	490±150		40% at 100 μM pA ₂ = 7.4±0.2	SA Ant	55% at 100 μM pA ₂ = 8.4±0.1	SA Ant	71±30	14
15.	Ac- Phe -(pI) DPhe -Arg-Trp-NH ₂	820±320	6	20% at 100 μM pA ₂ = 6.9±0.3	SA Ant	30% at 100 μM pA ₂ = 7.7±0.3	SA Ant	140±30	25
16.	Ac- 4PAL -(pI) DPhe -Arg-Trp-NH ₂	2900±1500	20	pA ₂ = 7.1±0.2	Ant	pA ₂ = 8.9±0.7	Ant	610±250	114
17.	Ac- Bip -(pI) DPhe -Arg-Trp-NH ₂	5200±1700	37	pA ₂ = 6.2±0.01	Ant	pA ₂ = 7.1±0.1	Ant	440±70	83
18.	Ac- Trp - DBip -Arg-Trp-NH ₂	1800±1000	13	pA ₂ = 6.7±0.1	Ant	45% at 100 μM pA ₂ = 7.0±0.2	SA Ant	1800±1300	340

Peptide	Structure	mMC1R			mMC3R			mMC4R			mMC5R		
		EC ₅₀ (nM)	Fold Diff.		EC ₅₀ (nM)	Fold Diff.		EC ₅₀ (nM)	Fold Diff.		EC ₅₀ (nM)	Fold Diff.	
19.	Ac-Trp-DNal(1')-Arg-Trp-NH ₂	9700±4700	70		>100 μM			12000±4000	870		3900±2200	725	
20.	Ac-Phe-DBip-Arg-Trp-NH ₂	10000±4900	71		>100 μM			5400±1600	395		500±70	94	
21.	Ac-Phe-DNal(1')-Arg-Trp-NH ₂	3900±1900	28		>100 μM			2800±1000	205		3800±2100	720	
22.	Ac-4PAL-DBip-Arg-Trp-NH ₂	1900±720	14		45% at 100 μM pA ₂ = 6.5±0.3		SA Ant	1300±200	92		1300±300	235	
23.	Ac-3PAL-DPhe-Arg-Nal(2')-NH ₂	1900±600	14		>100 μM			110±20	8		240±50	45	
24.	Ac-Trp-DPhe-Arg-Nal(1')-NH ₂	3300±1200	23		50% at 100 μM		SA	1100±300	77		3000±1200	566	
25.	Ac-Trp-DPhe-Arg-3Bal-NH ₂	8000±3000	57		>100 μM			7900±2400	576		4800±1700	905	
26.	Ac-Trp-DPhe-Arg-DNal(2')-NH ₂	23000±7000	167		>100 μM			4300±800	310		5800±600	1094	
27.	Ac-2Thi-DPhe-Arg-3Bal-NH ₂	6400±1600	45		>100 μM			1300±300	91		970±290	183	
28.	Ac-2Thi-DPhe-Arg-Nal(2')-NH ₂	2600±1300	19		12000 ±6000		22	450±190	33		76±18	14	
29.	Ac-His-(p)DDPhe-Arg-Bip-NH ₂	47±9		35% at 100 μM pA ₂ = 6.7±0.02		SA Ant		30% at 100 μM pA ₂ = 7.4±0.1	SA Ant		12.4±2.0		
30.	Ac-His-(p)DDPhe-Arg-3Bal-NH ₂	45±14		25% at 100 μM pA ₂ = 7.4±0.1		SA Ant		50% at 100 μM pA ₂ = 8.0±0.4	SA Ant		3.2±0.7		
31.	Ac-His-(p)DDPhe-Arg-Trc-NH ₂	24±4	-6		2900±400		5	640±60	46		130±30	25	
32.	Ac-His-(p)DDPhe-Arg-Phe-NH ₂	91±13			60% at 100 μM			80±7	6		18.4±5.7		
33.	Ac-His-DBip-Arg-Bip-NH ₂	15.8±6.1	-9		pA ₂ = 6.3±0.1		Ant	30% at 100 μM pA ₂ = 6.8±0.3	Ant		31.2±4.7	6	
34.	Ac-His-DBip-Arg-3Bal-NH ₂	8.9±1.2	-16		55% at 100 μM pA ₂ = 6.5±0.2		SA Ant	65% at 100 μM pA ₂ = 7.7±0.2	SA Ant		6.9±0.2		
35.	Ac-His-DBip-Arg-DBip-NH ₂	90±20			pA ₂ = 6.7±0.3		Ant	23% at 100 μM pA ₂ = 6.8±0.2	SA Ant		42±7	8	
36.	Ac-His-DBip-Arg-Nal(2')-NH ₂	17.0±5.4	-8		50% at 100 μM pA ₂ = 7.6±0.2		SA Ant	52±18	4		20.7±4.1	4	

The indicated errors represent the standard error of the mean (SEM) determined from at least three independent experiments. The antagonistic pA₂ values were determined using the Schild analysis and the agonist MTII (note that Ki = -Log pA₂). SA denotes that some stimulatory activity was observed at 100 μM concentrations, but not enough to determine an EC₅₀ value since the curve did not plateau. The percentage value associated with an SA value indicates the relative percentage of stimulation observed at a 100 μM concentration of the ligand, as compared to the maximal response generated by α-MSH. If a compound is more potent than a compound 1, the fold difference will indicate negative (-) prefix. Only differences that are more than 3 times fold (experimental error) are presented in this table.

Table 3.

Summary of the pharmacology of the reference peptides used in the double simultaneous substitution design approach from the literature.

Peptide	Structure	mMC1R EC ₅₀ (nM)	mMC3R EC ₅₀ (nM)	mMC4R EC ₅₀ (nM)	mMC5R EC ₅₀ (nM)	Ref.
37	Ac-His-DPhe-Arg-Phe-NH ₂	530±240	27000±7000	7800±2500	890±110	31
38	Ac-His-DPhe-Arg-Nal(1')-NH ₂	730±320	3500±1300	260±30	33±4	31
39	Ac-His-DPhe-Arg-Nal(2')-NH ₂	17.9±6.0	740±160	15.8±0.2	4.9±1.5	31
40	Ac-His-DPhe-Arg-DNal(2')-NH ₂	130±30	1600±600	46.2±7.9	12.1±3.3	31
41	Ac-His-DPhe-Arg-Tic-NH ₂	43±13	23000±2000	8500±900	700±100	31
42	Ac-His-DPhe-Arg-Bip-NH ₂	52±10	13000±800	2700±500	100±20	31
43	Ac-His-DPhe-Arg-DBip-NH ₂	310±70	16000±5000	1500±400	150±10	31
44	Ac-His-DPhe-Arg-3Bal-NH ₂	80±9	3700±1400	140±20	48±4	31
45	Ac-His-(p)DPhe-Arg-Trp-NH ₂	60±13	PA pA ₂ =7.3±0.2	25±10	1.6±0.4	32
46	Ac-His-DNal(1')-Arg-Trp-NH ₂	360±60	4100±100	300±70	51±4	32
47	Ac-His-DBip-Arg-Trp-NH ₂	28±11	2100±700	68±16	11.6±3.9	32
48	Ac-Phe-DPhe-Arg-Trp-NH ₂	500±100	12000±2000	71±14	140±5	33
49	Ac-Trp-DPhe-Arg-Trp-NH ₂	5400±1800	10000±2000	530±150	510±100	33
50	Ac-3Pal-DPhe-Arg-Trp-NH ₂	1200±200	SA	890±100	400±100	33
51	Ac-4Pal-DPhe-Arg-Trp-NH ₂	180±80	2300±800	130±30	93±22	33
52	Ac-2Thi-DPhe-Arg-Trp-NH ₂	200±40	3000±300	91±40	41±13	33
53	Ac-DHis-DPhe-Arg-Trp-NH ₂	290±110	6200±2500	510±60	140±20	33
54	Octanoyl-His-DPhe-Arg-Trp-NH ₂	0.36±0.24	4.0±0.7	0.38±0.06	0.79±0.25	34

SA denotes that some stimulatory activity was observed at 100 μ M concentrations, but not enough to determine an EC₅₀ value since the curve did not plateau. (PA) = Partial agonist denotes that some stimulatory activity was observed in addition to the antagonist pharmacology