Discovery of Mixed Pharmacology Melanocortin-3 Agonists and Melanocortin-4 Receptor Tetrapeptide Antagonist Compounds (TACOs) Based on the Sequence Ac-Xaa¹-Arg-(pI)DPhe-Xaa⁴-NH₂

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Abstract

The centrally expressed melanocortin-3 and -4 receptors (MC3R/MC4R) have been studied as possible targets for weight management therapies, with a preponderance of studies focusing on the MC4R. Herein, a novel tetrapeptide scaffold [Ac-Xaa¹-Arg-(pI)DPhe-Xaa⁴-NH₂] is reported. The scaffold was derived from results obtained from a MC3R mixture-based positional scanning campaign. From these results, a set of 48 tetrapeptides were designed and pharmacologically characterized at the mouse melanocortin-1, -3, -4, and -5 receptors. This resulted in the serendipitous discovery of nine compounds that were MC3R agonists ($EC_{50} < 1,000$ nM) and MC4R antagonists ($5.7 < pA_2 < 7.8$). The three most potent MC3R agonists, **18** [Ac-Arg-Arg-(pI)DPhe-Tic-NH₂], **1** [Ac-His-Arg-(pI)DPhe-Tic-NH₂], and **41** [Ac-Arg-Arg-(pI)DPhe-DNal(2′)-NH₂] were more potent ($EC_{50} < 73$ nM) than the melanocortin tetrapeptide Ac-His-DPhe-Arg-Trp-NH₂. This template contains a sequentially reversed "Arg-(pI)DPhe" motif with respect to the

classical "Phe-Arg" melanocortin signaling motif which results in pharmacology that is first-in-class for the central melanocortin receptors.

Graphical Abstract
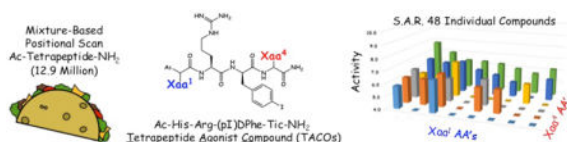
Keywords

α-MSH; NDP-MSH; Melanotropin; Obesity; Mixture-based positional scanning library; MC3R; MC4R; GPCR

Introduction

The melanocortin receptors are a family of class A, rhodopsin-like, G protein-coupled receptors (GPCRs)[1-7]. They signal primarily through the $G_{s\alpha}$ subunit which results in the accumulation of the secondary messenger cyclic adenosine monophosphate (cAMP)[1-8]. There have been five melanocortin receptor (MCRs) subtypes cloned to date, labeled MC1R through MC5R, which mediate a myriad of functions.[1-7] The MC1R is found primarily in the skin and is involved in the regulation of pigmentation.[1-2] The MC2R is involved in steroidogenesis and is activated only by the adrenocorticotropic hormone (ACTH).[9] Both the MC3R and MC4R are located in the brain and are integral in maintaining energy homoeostasis and body weight regulation.[3-4, 6, 10-12] Furthermore, the MC4R has been investigated as a weight loss drug target due to the identification of numerous single nucleotide polymorphisms in the MC4R of obese individuals, making this receptor the largest monogenic determinant of severe childhood-onset obesity.[13] The MC5R has been identified to affect the function of exocrine glands of mice,[14] however, the function of this receptor in humans remains unknown.

Stimulation of all five of the melanocortin receptors is mediated through pro-opiomelanocortin (POMC) peptide-derived products[15] with additional reports indicating they can signal through other pathways including $G_{i/o}$, MAPK, and the Kir7.1 channel.[16-18] These endogenous peptide ligands include α-, β-, and γ-melanocyte stimulating hormones (MSH) which stimulate the MC1, MC3, MC4, and MC5 receptors, and ACTH, which stimulates all five of the receptor subtypes. Extensive structure-activity relationship (SAR) studies have determined the conserved His-Phe-Arg-Trp motif found in all of the endogenous melanocortin agonists to be the minimum sequence necessary for receptor activation and has been described as the core melanocortin pharmacophore.[21-24] Receptor stimulation is inhibited at the melanocortin-1, -3, and -4 receptors by two endogenous antagonists: agouti-signaling protein (ASIP) and agouti-related protein (AGRP).[25-28] SAR studies on the 132 amino acid sequence of human AGRP have determined an Arg-Phe-Phe tripeptide sequence, AGRP(111-113), to be essential for antagonist activity.[27, 29-31] *In vivo* mouse studies have demonstrated that intracerebroventricular injection (ICV) of synthetic

melanocortin agonists can decrease food intake while MC3R/MC4R melanocortin antagonists can promote food intake.[32-33] These results support the hypothesis that the MC3R and MC4R may be viable drug targets for the treatment of metabolic diseases.

Animal knockout models indicate the two receptors have non-redundant roles in energy homeostasis. Mouse knockout models for each of the receptors results in an increase in fat mass, yet there are some phenotypic differences including the MC4R knockout mice are generally larger in size and the MC3R knockout mice are smaller in size with respect to the MC4R knockout mice.[34] However, the MC3R knockout mice have a fat to lean tissue ratio which is greater than their wild-type counterparts.[34] In addition, double knockout results in an extreme obese phenotype.[34] The phenotypic differences in the single knockout mice and the extreme obese phenotype of the double knockout suggests the receptors each have distinct roles in energy homeostasis and they may work together via a synergistic mechanism. In order to probe this hypothesis, additional melanocortin compounds that elicit diverse pharmacological profiles, such as a combination MC3R agonist/MC4R antagonist, are needed in the field.

Side effects observed when treating obesity with MC4R drugs in humans include increased blood pressure and induction of penile erection.[35-38] The hypertensive cardiovascular effects associated with central administration of the POMC derived peptides α - and γ_2-MSH were demonstrated in a rodent model may be an MC3R-independent and an MC4R-dependent processes.[35] However, these results may not correlate in humans since it was later reported that γ_2-MSH has activity at both the mouse MC3R and mouse MC5R, and this finding should be taken into consideration when interpreting *in vivo* results since the MC5R is found, among other places, in heart tissue.[39] Further confounding the issue of MC4R related side effects is the clinical candidate setmelanotide which showed no adverse effects on heart rate or blood pressure when the compound was administered to obese patients.[40] A better understanding of the underlying mechanisms by which the MC3R and MC4R affect the body metabolism could be achieved through the use of new melanocortin compounds with new selectivity and potency profiles. Reports on MC3R selective compounds have been primarily limited to analogs of α-MSH and γ-MSH.[41-46] The identification of selective MC3R compounds with more drug-like properties, may result in the development of a valuable therapeutic strategy for the current obesity epidemic, as compared to a therapeutic targeting of the activation of the MC4R which has several undesirable side effects.

Herein, we report on the discovery of a series of dual MC3R agonist/MC4R antagonist compounds that, to the best of our knowledge, are "first-in-class" chemotypes for melanocortin ligands. The resulting *in vivo* phenotype from central administration of this class of compounds is unknown; however, it is a reasonable conjecture that the observed effect on energy homeostasis and body weight regulation could be different than the sum of stimulating/blocking each receptor alone. It appears that melanocortin ligands have both an acute and longer-term mechanism of action in the regulation of food intake and energy homeostasis,[47-49] and the ligands reported herein may aid in this research as molecular probes. The use of co-therapies is not novel, and recent developments in this area illustrate the power of receptor synergy in relationship to weight-loss. This is illustrated by recent reports of a unimolecular co-agonist targeting both the glucagon-like peptide-1 (GLP-1) and

glucose-dependent insulinotropic polypeptide (GIP) receptors, in addition to, a tri-agonist targeting the GLP-1, GIP, and glucagon receptors as potential therapeutics for the treatment of "diabesity."[50-52] The discovery of the dual agonist/antagonist compounds reported herein utilized a combination of different peptide methodologies.

Classical peptide structure-activity relationship (SAR) approaches, such as truncation studies and single residue replacement scans, have yielded a variety of ligands with differing potencies and selectivity profiles at the receptor subtypes.[22, 24, 42, 53-54] These studies build upon preexisting knowledge and have been valuable in the development of potent, selective ligands. While useful, this approach has not generated a low molecular weight (M.W. < 1,000) MC3R selective ligand. An unbiased approach, such as mixture-based positional scanning libraries, may be used in order to generate new scaffolds with the desired pharmacological profile.[55] Herein, a lead compound chemotype was selected from the minimal deconvolution of a mixture-based positional scanning library campaign. This lead compound was then followed up with a double residue replacement scan to yield a series of compounds with a central MC3R agonist MC4R antagonist dual pharmacological profile.

Mixture-based positional scanning libraries have been extensively reviewed,[56-58] and have been previously validated for studying the melanocortin receptors wherein tetrapeptides were identified that rescued the function of selected human MC4R single nucleotide polymorphisms (SNPs).[55] Compounds sharing a common scaffold are assayed in mixtures where each compound within the mixture shares a common side chain at a particular position. There is a propensity for large mixtures containing only a few potent compounds to nonetheless demonstrate an overall moderate activity, since the activity of a particular mixture is the harmonic mean of the constituents.[59] These active mixtures are then identified as "hits," and combinations of "hits" can be synthesized and assayed as individual compounds. This technology allows for the rapid screening of millions of compounds/ peptides, the development of an extensive SAR, and the prioritization of individual compounds that could be studied as part of the deconvolution process. The ability to efficiently screen millions of compounds/peptides enables a larger area of chemical space to be explored in an unbiased and efficient manner. Thus, novel scaffolds can be identified that are not based upon any previously performed SAR studies, and may generate ligands with novel potency and selectivity profiles.

Herein, we describe the identification of a new synthetic tetrapeptide sequence, compound **1**, Ac-His-Arg-(pI)DPhe-Tic-NH$_2$. Analysis from the screening campaign suggested Ac-Xaa^1-Arg-(pI)DPhe-Xaa^4-NH$_2$ as a scaffold warranting further exploration due to the pharmacophore rearrangement of the Arg and Phe residues. Based on this template, it was hypothesized that substituting the first and fourth positions with several aromatic side chains previously indicated to alter the receptor selectivity and potency profiles of other melanocortin peptides, could result in the discovery of new MC3R scaffolds with novel pharmacology that could be used as molecular tools to probe the possible synergistic mechanisms between the MC3 and MC4 receptors *in vitro* and *in vivo*.[60-61]

Results

Identification of the Lead Compound 1, Ac-His-Arg-(pI)DPhe-Tic-NH$_2$

The mixture-based positional scanning library TPI924 consists of 60 individual building blocks of D-amino acids, L-amino acids, and unnatural amino acids resulting in 240 mixtures each containing 216,000 compounds with an overall library representing 12,960,000 compounds. Each member of the combinatorial library share a common Ac-tetrapeptide-NH$_2$ scaffold, and within each mixture a single residue was held constant at a specific position. For example, all peptides in the first mixture shared the structure Ac-Ala-X-X-X-NH$_2$, where X indicates a mixture of all 60 building blocks and therefore resulting in 216,000 (1×60×60×60) compounds within the mixture. The positional scanning library was constructed using the standard solid-phase synthesis N-α-tert-butyloxycarbonyl (Boc) protecting scheme, and the mixtures of compounds were synthesized using the previously reported teabag method.[62]

The library was screened using a 96-well cAMP based colorimetric β-galactosidase assay using HEK293 cells stably expressing the cloned mMC3R.[63] The primary screen assessed for mixture activity at a stimulatory concentration of 100 μg/mL. The data were normalized to both protein content and the responses of the potent synthetic agonist NDP-MSH and forskolin (a melanocortin receptor independent activator of adenylate cyclase). Inspection of the screening data resulted in the hypothesis that the putative tetrapeptide Ac-His-Arg-(pI)DPhe-Tic-NH$_2$ could serve as an unexplored MC3R chemotype that could be used to develop receptor selectivity profiles versus the MC4R. This can be considered a minimal version of the more typical deconvolution experiment wherein a set of individual compounds is produced based on a combination of the most active samples from each position of the mixture-based positional scanning library.[56-57, 64] This lead tetrapeptide sequence was prioritized for study since the sequence contained a sequence similar to that of an apparent structure reversal in the melanocortin signaling sequence residues of arginine and phenylalanine compared to the conserved His-Phe-Arg-Trp motif found in the endogenous POMC melanocortin ligands. This "Arg-(pI)DPhe" motif was reminiscent of the postulated Arg-Phe-Phe pharmacophore found in ASIP and AGRP which our laboratory has extensively studied.[29-30, 65-67]

The lead compound Ac-His-Arg-(pI)DPhe-Tic-NH$_2$, **1**, was synthesized using standard microwave assisted solid-phase N-α-fluorenylmethyloxycarbonyl (Fmoc) chemistry.[68-69] The compound was assessed for functional activity by the measurement of intracellular cAMP accumulation using the whole cell <u>A</u>mplified <u>L</u>uminescent <u>P</u>roximity <u>H</u>omogeneous <u>A</u>ssay <u>S</u>creen (AlphaScreen®, Perkin-Elmer) in the same stably transfected HEK293 cells as the initial screen in a 384-well format.[68, 70-71] Preliminary results indicated the compound was equipotent, within the 3-fold inherent error associated with this assay in our hands, to the previously reported melanocortin tetrapeptide, Ac-His-DPhe-Arg-Trp-NH$_2$ (40 vs 73 nM) at the mMC3R.[21, 60-61, 72-73] In addition, minimal agonist activity was observed at a concentration of 100 μM for the mMC4R and subsequent antagonist experiments indicated the compound demonstrated antagonist activity at the mMC4R (pA$_2$ = 7.0, K$_i$ = 100 nM).

This scaffold was named the <u>T</u>etrapeptide <u>A</u>gonist <u>C</u>ompound (TACO) scaffold, due to the propensity for this tetrapeptide to stimulate the MC3R.

Double Substitution Library Design, Synthesis, and Evaluation

The first and fourth positions were selected for further investigations as part of a double-substitution set of analogs and to perform an SAR campaign. It was hypothesized that the second and third positions should be held constant [(Ac-Xaa^1-Arg-(pI)DPhe-Xaa^4-NH$_2$] since previous studies on the linear truncated tetra- and penta-peptide analogs of α-MSH, Ac-His-DPhe-Arg-Trp-NH$_2$ and Bu-His-DPhe-Arg-Trp-Gly-NH$_2$, indicated alterations at these positions (corresponding to the DPhe and Arg positions in each of the peptides) within the His-Phe-Arg-Trp signaling motif were generally detrimental to the activity at all of the receptors.[72-76] The substitutions selected for the analogs herein contain both natural and unnatural amino acids which have been previously reported to alter either the selectivity and/or the potency at the selected melanocortin receptor subtypes in the linear Ac-His-DPhe-Arg-Trp-NH$_2$ peptide (Figure 1).[60-61, 74] For the first position of the tetrapeptide, arginine (Arg), histidine (His), biphenylalanine (Bip), β-(3-benzothienyl)-alanine (3Bal), 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), phenylalanine (Phe), D/L 2-napthylalanine [DNal(2′) and LNal(2′)] were selected (Figure 1).[60] At the fourth position, Bip, 3Bal, Tic, Phe, DNal(2′), and LNal(2′) were selected.[61] These choices resulted in a total of 48 (8×1×1×6) analogs which included the re-synthesis of the lead peptide **1** for a control; in addition, Ac-His-DPhe-Arg-Trp-NH$_2$ and NDP-MSH were included for reference and comparison purposes. All peptides were synthesized manually in a microwave using standard Fmoc solid phase peptide synthesis.[68-69]

The peptides were purified by reverse phase high pressure liquid chromatography (RP-HPLC) on a semipreparative scale and the purification was performed, whenever possible, as a mixture of two crude peptides to reduce instrument time and solvent usage by half. Pairs of RP-HPLC traces wherein the desired peptide peaks came off within 5 minutes of each other without the introduction of impurities when paired and peptides were combined for purification (Figure 2). With a modest 5 minute increase in the semi-preparative RP-HPLC method, parallel purification of two crude peptides could be achieved (Figure 2). It was estimated this effort reduced the amount of RP-HPLC time by approximately 210 hours, or 8.75 days. In addition, it reduced the amount of total solvent by 62.5 liters, of which approximately 50% was acetonitrile. Compounds were confirmed by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry at the University of Minnesota Mass Spectrometry Laboratory. They were assessed for purity by analytical RP-HPLC analysis using two different solvent systems (Supporting Information). Analytical characterization of the compounds indicated their purity >95% as indicated by UV absorbance at λ = 214 nm (Supporting Information).

The analogs synthesized herein were pharmacologically characterized for *in vitro* agonist activity using the 384-well cAMP based AlphaScreen at the mMC1R, mMC3R, mMC4R, and the mMC5R. Since the MC2R is only stimulated by ACTH, it was excluded from this study.[9] The data were normalized to NDP-MSH, and the response observed for NDP-MSH at 10^{-6} M was defined as 100% response. Additional positive (forskolin) and negative (assay

buffer) controls were included in the experiments. Compounds which failed to produce full agonist dose-response curves at the mMC3R or the mMC4R were further assessed for antagonist activity via a Schild analysis and pA_2 values were determined.[77] In a typical antagonist experiment, cells were co-treated with NDP-MSH (full dose-response, 10^{-6} to 10^{-12} M) and the compound of interest (10,000 nM, 5,000 nM, 1,000 nM, and 500 nM), and the apparent shift in NDP-MSH response was quantified. The apparent shifts in NDP-MSH's agonist activity (EC_{50} values) were recorded and a Schild analysis was performed to yield a pA_2 value [$pA_2 = -Log(K_i)$].[77]

The nine mMC3R agonists with EC_{50}'s less than 1,000 nM (**1**, **10**, **11**, **15**, **18**, **22**, **24**, **25**, and **41**) were selected for radiolabeled ^{125}I-NDP-MSH binding evaluation at both the mMC3R and mMC4R. It was hypothesized that the results from this experiment would reveal additional insight into the novel pharmacological dual mMC3R agonist/mMC4R antagonist profile that was observed. A typical experiment utilized a 12-well format. The compounds were assayed from 10^{-4} M to 10^{-10} M with a constant 100,000 cpm/well of monoiodinated ^{125}I-NDP-MSH. The data were normalized to the specific binding by a saturating concentration of unlabeled NDP-MSH that was defined as 100%. The IC_{50} value for control peptide (Ac-His-DPhe-Arg-Trp-NH_2) at the mMC3R was fitted by constraining the top and bottom, complete receptor saturation and complete radiolabel displacement, nonlinear regression parameters to those which were determined for NDP-MSH within the same experiment. This allowed for an estimation of the IC_{50} which was needed in order to numerically compare, in terms of fold difference, the observed changes in IC_{50} potencies within the receptor subtype. The calculated IC_{50} value was 50 μM at the mMC3R, and in agreement with the previously reported IC_{50} value at the human MC3R ($IC_{50} > 10$ μM) which has high receptor sequence homology to the mMC3R.[20, 78]

Overview of the SAR results at the mouse melanocortin-1, -3, -4, and -5 receptors

The double-substitution analogs produced a varied, agonist and antagonist, SAR between the four melanocortin receptor subtypes examined herein. Given the large volume of data, they are separated by receptor subtypes with the mMC1R/mMC5R together and mMC3R/mMC4R together. The combined agonist and antagonist results for the mMC3R and mMC4R are provided in Table 1 and the agonist results for the mMC1R and mMC5R are shown in Table 2. Additional figures illustrate a summary of the agonist and antagonist receptor pharmacology as a function of both substitutions at the first and fourth positions of the TACO scaffold are available in the supplementary information. The most potent agonist activity was observed at the mMC1R, followed by similar agonist activities at the mMC3R and mMC5R, while little to no agonist activity was observed at the mMC4R. As already discussed, compounds demonstrating little to no agonist activity at the mMC3R or the mMC4R were selected for antagonist activity via a Schild analysis.[77] Compounds with antagonist activity at the mMC3R were generally observed to be weak, $pA_2 < 6$, ($K_i > 1,000$ nM) whereas seventeen compounds evaluated at the mMC4R possessed $pA_2 > 6$ ($K_i < 1,000$ nM). Notably lead compound **1**, Ac-His-Arg-(pI)DPhe-Tic-NH_2, and the closely related compound **18**, Ac-Arg-Arg-(pI)DPhe-Tic-NH_2, were observed to be antagonists at the mMC4R with a pA_2 greater than 7.0 ($K_i < 100$ nM) and potent nanomolar agonists at the mMC3R ($EC_{50} = 40$ and 16 nM, respectively). Lastly, compound **41** was observed to be a

potent nanomolar agonist at the mMC3R (EC_{50} = 57 nM) which was 5-fold selective over the mMC1R and 9-fold selective over the mMC5R. In addition, this compound was a 400 nM antagonist at the mMC4R (pA_2 = 6.4).

Discussion

Mixture-Based Positional Scanning Library Deconvolution and Template Selection

A typical study using mixture-based positional scanning libraries begins with a scaffold selection where around 100 scaffolds containing mixtures ranging in number from 10s of thousands to approximately 750,000 compounds are tested. The most active mixture scaffold is then chosen for deconvolution using the method of mixture-based positional scanning. Herein, we omitted the scaffold ranking and selected a tetrapeptide scaffold since the minimally active sequence of all the POMC derived melanocortin agonists is His-Phe-Arg-Trp and previous studies about this scaffold have demonstrated a variety of SAR can be achieved.[22, 60-61, 72-73] A positional scanning tetrapeptide library is comprised of a set of systematically arranged sub-libraries representing each position in the scaffold, with fixed amino acids at that position and mixtures at the other three. Thus, there will be four sets of mixtures that enable each of the four positions to be screened to identify the most active functionalities at each of the four positions. Once this has been accomplished, the most active 2-3 different amino acid functionalities, at each of the four positions are then used to make individual tetrapeptides. Reported is what would be considered the minimalist version of the more typical deconvolution experiment.

The lead peptide sequence, Ac-His-Arg-(pI)DPhe-Tic-NH_2, was the sequence corresponding to the highest activity in each position (supplemental information). Out of the total of 60 amino acid building blocks that were incorporated in each of the four positions of the tetrapeptide library, six of those building blocks were used in the first position and four of them were used in the fourth position of the reported TACO template. A comparative analysis of the results obtained from the mixture-based positional scanning library and individual analogues reported herein yield insight into the effectiveness of the high throughput method selected. Analysis of the 24 (6×4) mixtures from the library and corresponding analogues revealed that the most potent MC3R agonists, compounds **1** and **18**, would have been identified with the traditional deconvolution of the library results (supplemental information). Furthermore, the individual peptide activity generally corresponded to the library results with the exception of a single outlier, compound **41** [Ac-Arg-Arg-(pI)DPhe-DNal(2′)-NH_2].. It is also possible an Arg substitution is favored over the His at the first position. Last, out of the 21 compounds which possessed full agonist activity at the mMC3R, potencies ranging from 16 nM to 14 μM, a total of 10 would have been identified using traditional deconvolution methods while the remaining 11 compounds would not have been part of a traditional deconvolution.

Requirements for Agonist Activity

As discussed above, compounds were most active at the mMC1R, followed by similar activities at the mMC3R and mMC5R, with minimal agonist activity at the mMC4R (Figure 3). Results from substitutions at the first position indicate the mMC1R had a preference for a

basic side chain (Arg^1 and His^1), followed by small aromatic side chains in addition to some intermediate sized aromatics [Phe^1, Tic^1, $DNal(2')^1$], and last, the remaining intermediately sized aromatics and the bulky aromatics [$Nal(2')^1$, Bip^1, $3Bal^1$] were detrimental to the SAR. This corresponded well to the original positional scanning data, which demonstrated substantially more activity for the $Ac-His-X-X-X-NH_2$ and $Ac-Arg-X-X-X-NH_2$ samples than those corresponding to the other substitutions present in the positional scanning library (supplemental information). Activity at the mMC3R and mMC5R were dependent upon having a basic side chain at the first position to produce a compound with a potency greater than 100 nM. An aromatic side chain was tolerable with the addition of either a $3Bal^4$ or Tic^4 substitution at the fourth position and resulted in a moderate (>100 nM) to weak micromolar potency. Interestingly, with the $3Bal^4$ or Tic^4 the trends that were observed with the mMC1R at the first position are consistent with the mMC3R and mMC5R. This suggests the amino acid replacements about this scaffold at the first and fourth position may be additive to the overall activity of the peptide since a step-wise increase in activity was observed when a more favorable substitution was incorporated. This would be compared to observing dramatic increases, several orders of magnitude, upon the incorporation of specific pairs of amino acid substitutions.

The amino acid substitutions at the fourth position produced similar results for agonist activities at the mMC1, mMC3, and mMC5 receptors. The rank order of the amino acid replacements as a function of their resulting potencies were similar for the three receptor subtypes ($Tic^4 > 3Bal^4 > Phe^4 = Nal(2')^4 = DNal(2')^4 \gg Bip^4$). The constrained Tic^4 substitution resulted in the most potent analogs at each of the three melanocortin receptor subtypes with potencies in the sub- to low-nanomolar ranges ($EC_{50} < 10$ nM). Again, this corresponded well to the data from the original positional scanning library screening, where $Ac-X-X-X-Tic-NH_2$ was substantially more active than other samples corresponding to the other substitutions present in the positional scanning library (supplemental information). Compounds containing the sulfur analog of tryptophan, $3Bal^4$, tended to mirror the potencies observed for the Tic^4 analogs, albeit none of these analogs were sub-nanomolar potent. Fourth position substitutions that resulted in compounds that fell within an intermediate potency range, high nanomolar to micromolar ($EC_{50} > 500$ nM), were the Phe^4, $Nal(2')^4$, and $DNal(2')^4$. With these particular substitutions, either an Arg^1 or His^1 replacement at the first position was required for the analogs to have activity at the mMC3R and mMC5R. Lastly, compounds containing the bulky Bip^4 amino acid resulted in the weakest activities with most analogs unable to produce any agonist activity at concentrations up to 100 micromolar.

Requirements for Antagonist Activity

The double substitution analogs resulted in wide-ranging antagonist activity at the mMC4R. When the same compound was assayed for antagonist activity at both the mMC3 and mMC4 receptors, antagonist activity at the mMC4R tended to be on average 8-fold more potent than the observed potency at the mMC3R, a trend observed for other melanocortin antagonists.[79-80] Similar to the SAR for the agonist activity at the mMC1, mMC3, and mMC5 receptors, the most active compounds contained a Tic^4 substitution, up to $pA_2 = 7.8$ ($K_i = 16$ nM) for **18**. The $3Bal^4$ replacement also decreased antagonist activity with respect

to the Tic[4] replacement, similar to the observed trend for agonist activity at the MC1R, MC3R and MC5R. For the Phe[4], Nal(2′)[4], and DNal(2′)[4] substitutions, either a basic Arg[1] or His[1] or, interestingly, a DNal(2′)[1] replacement was needed to achieve maximal antagonist activity (up to pA_2 = 6.9 or K_i = 126 nM for the DNal(2′)[1]/Phe[4] analog **32**). The Bip[4] substitution was generally detrimental for mMC4R antagonist activity, with only two of those analogs, **2** and **9**, able to produce detectable activity (pA_2 = 5.8 and 6.6, respectively). In addition, **8** was the only compound that resulted in full agonist activity at the mMC4R from this "focused" library and it possessed weak micromolar potency (EC_{50} = 7,900 nM).

The most potent mMC4R antagonist, **18**, had an Arg[1]/Tic[4] substitution that resulted in a pA_2 of 7.8 (K_i = 15 nM); in addition, this compound resulted in nanomolar agonist activity at the mMC3R (EC_{50} = 16 nM). The antagonist activity at the mMC4R was unexpected since studies on the linear tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 indicate antagonist activity can be conferred through substitutions such as DNal(2′) and (pI)DPhe at the second position whereas most substitutions at the first position result in agonist activity.[60, 72] A Tic[1] replacement at the first position yielded weak antagonists (pA_2 < 6, K_i > 1,000 nM). This is in contrast to the closely related peptide Tic-(pI)DPhe-Arg-Trp-NH_2 which has been reported to strongly bind to both the human MC3R and MC4R (75 and 0.30 nM, respectively), and displays potent antagonist activity at the hMC4R (pA_2 = 9.10, K_i = 0.79 nM, > 35% agonist activation with respect to maximal response).[81] In addition, a Tic substitution on a SHU9119 analog at the same relative position also resulted in potent antagonist activity.[45]

Comparison of the Activities Observed at the mMC3R and mMC4R

Further insight into the differences between the TACO scaffold and melanocortin compounds based on the endogenous melanocortin ligands can be seen from the results from the binding studies reported. Table 3 summarizes the results of two independent experiments each containing two replicates per experiment. The results are tabulated as the mean and standard error of the mean for each compound at the mMC3R and mMC4R. Also included in the table is the calculated fold-difference in IC_{50} potency based on the value observed for the control peptide Ac-His-DPhe-Arg-Trp-NH_2. The nine ligands with EC_{50} values less than 1,000 nM at the mMC3R had binding IC_{50} values less than 6,500 nM. Compared to Ac-His-DPhe-Arg-Trp-NH_2, the fold decrease in IC_{50} ranged from 9-fold for **15** to 120-fold for **24** (IC_{50} = 5,350 and 420 nM, respectively).

When compared to the mMC3R agonist function data, the binding data generally complement those observed results. That is to say, compounds that possessed nanomolar agonist function activity tended to possess similar IC_{50} values. All of the compounds possessed more potent IC_{50} values at the mMC4R than at the mMC3R. This result is consistent with the other melanocortin ligands, NDP-MSH and Ac-His-DPhe-Arg-Trp-NH_2 that were included in this study. The binding activity for the selected mMC3R TACOs at the mMC4R tended to result in a flat equipotent SAR with IC_{50} value less than a 3-fold difference, as compared to Ac-His-DPhe-Arg-Trp-NH_2 (IC_{50} = 120 nM). In addition, relative to the greater than 100-fold increases in binding affinities at the mMC3R, the most potent ligand at the mMC4R, **18**, possessed a 9-fold increase (IC_{50} = 13 nM) in binding

affinity relative to the tetrapeptide Ac-His-DPhe-Arg-Trp-NH$_2$. Perhaps the agonist selectivity and nanomolar potencies which were achieved at the mMC3R may be rationalized by the 9- to more than 100-fold increase in binding affinity IC$_{50}$ value that was observed relative to the control peptide Ac-His-DPhe-Arg-Trp-NH$_2$ (Table 3, Figure 4). All of this suggests the reported TACO scaffold is not only structurally different from compounds based on the endogenous peptides but also elicits different pharmacological profiles when corresponding residues are replaced.

Comparison of the TACO analogs to Ac-His-DPhe-Arg-Trp-NH$_2$

Seven compounds were equipotent or more potent than the tetrapeptide Ac-His-DPhe-Arg-Trp-NH$_2$ at the mMC1R, compared to four at the mMC3R, one at the mMC5R, and none at the mMC4R. In fact, only one compound, **8**, was able to produce a maximal response (EC$_{50}$ = 7,900 nM) at the mMC4R whereas all of the remaining compounds were unable to produce full agonist activity with respect to NDP-MSH and Ac-His-DPhe-Arg-Trp-NH$_2$ at concentrations up to 100 μM. In contrast, 17 members of this library were found to have antagonist activity with pA$_2$ values greater than 6.0 (K$_i$ < 1,000 nM) at the mMC4R. A prominent outcome for this library was the presence of an mMC3R agonist/mMC4R antagonist pharmacological profile as illustrated with the most potent mMC3R agonist compound **18** (Figure 3). A total of 9 compounds produced moderate to potent agonist activity (EC$_{50}$ < 1,000 nM) at the mMC3R, in addition to producing antagonist activity at the mMC4R (7.8 > pA$_2$ > 5.7). This is in contrast to previous reports on melanocortin tetrapeptides in which the SAR tends to favor MC4R potency over the MC3R.[60-61, 72-73, 76, 81-87]

Comparison to other Melanocortin Peptide Ligands

Thus far we have discussed the SAR of the new TACO scaffold, Ac-Xaa^1-Arg-(pI)DPhe-Xaa^4-NH$_2$, relative to the highly conserved endogenous "His-Phe-Arg-Trp" agonist motif.[22] It appears that peptides based on the TACO scaffold share few SAR features with melanocortin compounds based on the endogenous agonist peptides, and it is postulated some of the observed pharmacology is attributed to the unusual reversed Arg-Phe template. However, the possibility cannot be ignored that the reported TACO scaffold is a hybrid of the AgRP/AISP active tripeptide sequence "Arg-Phe-Phe" and the core melanocortin signaling sequence "His-Phe-Arg-Trp," which could help rationalize additional aspects of the observed SAR. A chimeric NDP-MSH/AGRP peptide, Ac-Ser-Tyr-Ser-Nle-Glu-His-Arg-Phe-Phe-Gly-Lys-Pro-Val-NH$_2$, is a more potent stimulator for the mMC3R over the mMC4R (EC$_{50}$ = 480 nM vs 930 nM).[88] Moreover, a series of disulfide cyclized chimeric α-MSH/ASIP analogs, template Ac-c[Cys-Arg-(X)Phe-Cys]-(X)Trp-NH$_2$ where X is D or L stereochemistry, are reported to possess hMC3R-selective non-competitive binding.[89] The unusual sequence of the TACO scaffold with respect to the endogenous "His-Phe-Arg-Trp" and "Arg-Phe-Phe" sequences gives additional insights into the requirements for receptor recognition and selectivity for the melanocortin subtypes.

Although the TACO scaffold appears to produce a pharmacology that is distinct from the profiles that have been observed for compounds based on the known endogenous ligands, both the mMC3R and mMC4R respond in a similar manner to this scaffold. In other words,

substitutions about the scaffold which are beneficial to activity, either agonist or antagonist potencies, for one receptor are beneficial to the other one. The observation is illustrated by plotting the values for each ligand studied as a function of the activities observed at the mMC4R versus the mMC3R (Figure 5). Illustrated is the absolute magnitude, either agonist EC_{50} or antagonist K_i values on a log scale, and pharmacological profile as a function of the activities observed at the mMC4R versus the mMC3R. The majority of compounds (39%) possessed pharmacological profiles wherein there was observed agonist activity at the mMC3R and antagonist activity at the mMC4R. This was followed by compounds (33%) that were neither active at the mMC3R nor mMC4R. The remaining compounds had a variety of pharmacology at the two receptor subtypes. If this scaffold tended to favor activity for the mMC4R, one would expect compounds to group in the upper-left quadrant where potencies would be high for the mMC4R and low for the mMC3R. Similar reasoning could be used for the southeast quadrant and compounds that would favor the mMC3R; however, the majority of the compounds fall within the upper-right and lower-left quadrants where either the potencies are both low or both high for the mMC3R and mMC4R.

Conclusions

Herein, a double-substitution "focused library" of analogs were pharmacologically characterized at the mouse melanocortin 1, 3, 4, and 5 receptors based upon the new and novel melanocortin chemotype Ac-Xaa[1]-Arg-(pI)DPhe-Xaa[4]-NH$_2$. The lead compound was identified from an unbiased mixture-based positional scanning library approach consisting of more than 12.9 million tetrapeptides. Notably, nine compounds reported herein possess agonist activity $EC_{50} < 1,000$ nM at the mMC3R and are competitive antagonists at the mMC4R. To our knowledge, this is the first example of a melanocortin template that yields this mixed MC3 agonist/MC4 antagonist receptor pharmacology. These compounds could serve as valuable molecular probes to help decipher the molecular mechanism(s) of how the melanocortin-3 and -4 receptors synergistically work together to maintain energy homeostasis. Furthermore, this newly discovered TACO scaffold, Ac-Xaa[1]-Arg-(pI)DPhe-Xaa[4]-NH$_2$ is distinct from the classic His-Phe-Arg-Trp melanocortin template sequence. The unique combination of switching residues and side chain replacements required to produce the reported scaffold from the classic signaling sequence illustrate the strength of mixture-based positional scanning to discover new and unbiased ligands.

Experimental Section

Mixture-Based Positional Scanning Library

TPI924 was synthesized as previously described[55] using an optimized solid-phase simultaneous multi peptide synthesis approach on p-methylbenzhydrylamine (MBHA) polystyrene resin.[62, 90-92] The library was constructed using 60 different L-, D-, and unnatural amino acids, which resulted in 240 acetylated tetrapeptide mixtures, each containing 216,000 acetylated tetrapeptides, with a total diversity of 12,960,000 acetylated tetrapeptides. The 60 different amino acids are Ala, Asp, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val, Trp, Tyr, DAla, DAsp, DGlu, DPhe, DHis, DIle, DLys, DLeu, DMet, DAsn, DPro, DGln, DArg, DSer, DThr, DVal, DTrp, DTyr, Nle, DNle,

Cha, DCha, PyrAla, DPyrAla, ThiAla, DThiAla, Tic, DTic, (pCl)Phe, (pCl)DPhe, (pI)Phe, (pI)DPhe, (pNO$_2$)Phe, (pNO$_2$)DPhe, 2-Nal, 2-DNal, β-Ala, ε-Aminocaproic acid, Met[O$_2$], dehydPro, and (3I)Tyr.[93-94] The compound mixtures were tested without further purification.

Single Tetrapeptide Set of Analogs

The single tetrapeptides described herein were synthesized manually using a combination of microwave-assisted and standard room temperature N-α-Fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis on Rink-amide MBHA resin (Peptides International, 0.35 meq/g).[68-69] The peptides were synthesized in parallel in groups of eight compounds. The resin (0.5 mmol scale) was initially swelled in dichloromethane (DCM) for 1 hr. This was followed by resin deprotection with 15 mL of 20% piperidine in N,N-dimethylformamide (DMF). The reaction was mixed via bubbling the mixture with nitrogen gas for 2 min at room temperature. The reaction vessel was drained, additional 15 mL of 20% piperidine in DMF was added, and the reaction vessel was heated to 75 °C in a microwave (Discover SPS, CEM Corporation) with 30 W power for 4 min. The reaction was allowed to cool and then washed with DMF (4 × 15 mL). Following a positive ninhydrin or chloranil test,[69] the Fmoc protected amino acid (3.1 eq), N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, Peptides International) (3 eq), and diisopropylethylamine (DIEA, Sigma-Aldrich) (5 eq) were dissolved in DMF and added to the reaction vessel. The vessel was heated to 75 °C in a microwave with 30 W power for 5 min. The amino acid building blocks used in this study were: Fmoc-Arg(Pbf)-OH (Peptides International), Fmoc-3Bal-OH (Bachem), Fmoc-Bip-OH (Synthe Tech), Fmoc-Glu(OtBu)-OH (Peptides International), Fmoc-Gly-OH (Peptides International), Fmoc-His(Trt)-OH (Peptides International), Fmoc-Lys(Boc)-OH (Peptides International), Fmoc-Nle-OH (Peptides International), Fmoc-D-4-I-Phe-OH (AnaSpec), Fmoc-Phe-OH (Peptides International), Fmoc-D-Phe-OH (Peptides International), Fmoc-Pro-OH (Peptides International), Fmoc-Nal(2′)-OH (Synthe Tech), Fmoc-D-Nal(2′)-OH (Peptides International), Fmoc-Ser(tBu)-OH (Peptides International), Fmoc-Tic-OH (Synthe Tech), Fmoc-Trp(Boc)-OH (Peptides International), Fmoc-Tyr(tBu)-OH (Peptides International), and Fmoc-Val-OH (Peptides International). For coupling Arg the equivalents of the reagents were increased Arg (5.1 eq), HBTU (5 eq), and DIEA (7 eq) as was the microwave coupling time (10 min). For coupling His, the microwave temperature was decreased to 50 °C. After coupling in the microwave, the reaction was allowed to cool and was washed in DMF (3 × 15 mL).

Following a negative ninhydrin test for primary amines, or a chloranil test for secondary amines, the entire deprotection and coupling procedure was repeated for the remaining residues.[69] After coupling the third amino acid residue, the resin was dried and then divided into eight separate reaction wells. With a semi-automatic synthesizer (LabTech 1, Advanced ChemTech), the eight resins were swelled in DCM (10 mL, 1 hr, 350 RPM) and then washed in DMF (3 × 10 mL, 1 min, 350 RPM). The resins were deprotected with 20% piperidine in DMF (10 mL for 2 min, and 10 mL for 18 min). The remaining residue was coupled using the same equivalents as above. The reactions were allowed to proceed at RT for 1 hour. Following a negative ninhydrin test for primary amines, or a chloranil test for secondary amines,[69] the resins were deprotected with 20% piperidine in DMF (10 mL 2 min, and 10

mL 18 min at RT), and subsequently acetylated with a 10 mL solution of acetic anhydride and pyridine (3:1) for 30 min at RT. The resin was then washed in DMF (3×10 mL), then in methanol (1×10 mL) and dried overnight.

The peptides were cleaved from the resin and globally deprotected in parallel. A 10 mL mixture of trifluoroacetic acid (Sigma-Aldrich), thioanisole (Fluka), triisopropylsilane (Aldrich), and water (91:3:3:3) was added to each well and allowed to shake for 1 hour at RT at 350 RPMs. The mixture was drained and collected into 50 mL Falcon tubes. Upon the addition of cold 0 °C diethyl ether a precipitant was formed. The white precipitant was pelleted using a Sorvall Legend XTR centrifuge with a swinging bucket rotor (4,000 RPM, 4 °C, 4 min). The pellet was washed with additional diethyl ether and pelleted (3 x). The pellet was allowed to dry in a desiccator overnight.

The crude peptides were purified by RP-HPLC with a photodiode array detector (Shimadzu Corp.) on a semi-preparative scale with a flow rate of 5 mL/min on a RP-HPLC C18 bonded column (Vydac 218TP1010, 1 cm $\times$ 25 cm) in pairs. Mixtures of crude peptides were combined and purified together in order to decrease instrument time and solvent usage. Typically, in order to isolate 5 to 10 mg of a pure (>95% by UV absorption at λ=214 nm) tetrapeptide for a single compound, 40 mg of crude peptide would be dissolved and injected onto a semipreparative Vydac C18 column (10 micron, 10×250 mm, Vydac Cat#218TP1010) over the course of 25 injections with a flow rate 5 mL/min in a mixture of 0.1% trifluoroacetic acid in water and acetonitrile. A typical RP-HPLC method would consist of a 10 minute run, followed by a 10 minute column flush, and then a 10 minute column equilibration for a total of 30 minutes per injection. Over the course of the purification of a single peptide, the RP-HPLC would be in use for 12.5 hours consuming 1.9 liters of acetonitrile (approximately, 50% of the total RP-HPLC solvent). The selection process in pairing crude peptides for purification consisted of first running each crude peptide on a standard 10% to 90% acetonitrile gradient in 0.1% TFA in water over 35 minutes at a rate of 1.5 mL/min using an analytical Vydac C18 column (10 micron, 4.6×250 mm, Vydac Cat#218TP104).

The collected fractions were concentrated on a rotary evaporator and subsequently lyophilized to a fine white powder. The pure compounds were analytically characterized by RP-HPLC using two different solvent systems. The analytical method was either acetonitrile or methanol in a 10% to 90% gradient in 0.1% TFA in water over 35 minutes at a rate of 1.5 mL/min using an analytical Vydac C18 column (Vydac 218TP104, 4.6 mm $\times$ 25 cm) and purity was monitored by integrating the area under the curve (AUC) at λ=214 nm; in addition, the purity by AUC was determined to be greater than 95% and is provided for each compound in SI Table 1 (supporting information). The mass was confirmed using a matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF MS) analysis using an α-cyano-4-hydroxycinnamic acid matrix (AB-Sciex 5800, University of Minnesota Department of Chemistry Mass Spectrometry Laboratory). The control peptides Ac-His-DPhe-Arg-Trp-NH$_2$ and NDP-MSH were synthesized individually using the microwave method described above. These compounds were also purified individually using the same RP-HLPC instruments.

β-Galactosidase Bioassay

This bioassay was implemented for the primary mixture-based positional scanning library TPI924. The ligands described in this study were assayed in HEK293 cells stably expressing the mouse melanocortin-1, -3, -4, and -5 receptor subtypes which were cloned into the cells using a pCDNA$_3$ vector which has been previously described by our laboratory.[79] Stably transfected HEK293 cells were plated with media (Dulbecco's Modified Eagle Medium [DMEM] supplemented with 10% Bovine Serum and 1% Penicillin Streptomycin) into a 10 cm dish such that, 24 hours later the cells reached approximately 40% confluency. Twenty-four hours post plating, the cells were transiently transfected with 4 μg CRE-PBKS per 10 cm plate of cells using the calcium phosphate method.[95] Twenty-four hours post transfection, the cells were plated onto collagen treated Nunclon Delta Surface 96-well plates (Thermo Fischer Scientific) and incubated at 37 °C with 5% CO$_2$. Forty-eight hours post transfection, the plates were stimulated with the compound mixtures. The compound mixtures were dissolved in DMF up to a concentration of 1,000 μg/mL and stored at -20 °C until use. The cell media was aspirated and to each well 40 μL of the peptide mixture from TPI924 (100 μg/mL and 50 μg/mL) in assay media (1.0 mL 1% bovine serum albumin [BSA] in phosphate buffered saline [PBS] and 1.0 mL 100x isobutylmethylxanthine in 98.0 mL DMEM). Controls included NDP-MSH (10^{-6} to 10^{-12} M), forskolin (10 μM), and plain assay media. The plates were incubated at 37 °C with 5% CO$_2$ for six hours. Post stimulation, the media was aspirated and 50 μL of lysis buffer (250 mM Tris-HCl pH=8.0, 740 mL DD H$_2$O, 10 mL 10% Triton X-100 in water) was added. The plates were stored at -80 °C for up to two weeks.

The plates were thawed, assessed for protein content, and substrate was added to develop the plates. Protein content was assessed by adding, 10 μL of cell lysate was added to 200 μL of BioRad dye solution (1:4 dilution with water) in another 96 well plate, and the absorbance was read using a 96 well plate reader (Molecular Devices) at λ = 595 nm. To the remaining 40 μL of cell lysate, 40 μL of, 37 °C, 0.5% BSA in PBS was added in addition to 150 μL of the β-galactosidase substrate (60 mM Na$_2$HPO$_4$, 1 mM MgCl$_2$, 10 mM KCl, 50 mM 2-mercaptoethanol, and 660 μM 2-nitrophenyl β-D-galactopyranoside). The plates were incubated at 37 °C and periodically read on the 96 well plate reader until the absorbance at λ = 405 nm reached approximately 1.0 relative absorbance units for the positive controls. The λ-galactosidase activity was normalized to both protein content and maximal response of the positive controls.

AlphaScreen Bioassay

This bioassay was used to produce the dose-response curves and subsequent EC$_{50}$ determination for the reported TACO library. The ligands described in this study were assayed using HEK293 cells stably expressing the mouse melanocortin-1, -3, -4, and -5 receptor subtypes which were cloned into the cells using a pCDNA$_3$ vector which has been previously described by our laboratory.[55] The cAMP signaling was directly measured using the AlphaScreen (Perkin-Elmer, Cat#6760635M) assay protocol as described by the manufacturer as previously reported.[68, 70-71] Cells were grown in an incubator at 37 °C with 5% CO$_2$ in cell media [Dulbecco's modified Eagle's medium (DMEM) containing 10%

newborn calf serum (NCS) and 1% penicillin/streptomycin] in 10 cm plates to 70-95% confluency the day of the assay.

Cells were disassociated with 1 mL 37 °C Versene solution (Gibco), re-suspended in 5 mL 37 °C cell media, and pelleted by centrifugation at 800 RPM for 5 min at RT (Sorvall Legend XTR centrifuge, swinging bucket rotor). The supernatant was subsequently aspirated and the cell pellet was re-suspended in 37 °C Dulbecco's phosphate buffered saline solution (DPBS 1X without $CaCl_2$ or $MgCl_2$, Gibco). The cells were manually counted using a hemocytometer (10 μL cell mixture added to 10 μL of Trypan blue BioRad dye). The cells were again centrifuged (800 RPM, 5 min, RT) and the supernatant was aspirated. The cell pellet was then re-suspended in a solution of freshly made stimulation buffer [Hank's Balanced Salt Solution (HBSS 10 × without $NaHCO_3$ or phenol red, Gibco), 0.5 mM isobutylmethylxanthine (IBMX), 5 mM HEPES buffer solution (1 M, Gibco), 0.1% BSA in Milli-Q water, pH=7.4] to a final concentration of 10,000 cells/μL.

A solution of cells and anti-cAMP acceptor beads in stimulation buffer was then prepared (1,000 cells/μL and 0.5 μg/μL AlphaScreen anti-cAMP acceptor beads in stimulation buffer). Ten μL of the cell/acceptor bead solution was then added to each well in a 384-well microplate (OptiPlate-384, Perkin-Elmer). To each well an additional 5 μL of the unknown ligand was added and the concentration of the ligand was adjusted such that the final concentration in the well reflected the desired concentration. The plate was then sealed and incubated at room temperature in the dark for two hours. Compounds included on each plate were NDP-MSH (10^{-6} to 10^{-12} M), forskolin (10^{-4} M) (positive controls), and stimulation buffer alone (blank control). The compounds described in this study were initially assayed from 10^{-4} to 10^{-10} M and the range was adjusted accordingly to 10^{-6}-10^{-12} M in later experiments if the compounds were more potent.

The light sensitive biotinylated cAMP/streptavidin coated donor bead mixture in lysis buffer was prepared, 30 min prior to the end of the initial two-hour plate incubation, under green light (0.5 μg/μL AlphaScreen donor beads and 0.62 μM AlphaScreen cAMP biotinylated tracer in a solution of 10% Tween-20, 5 mM HEPES, and 0.1% BSA in Milli-Q water, pH=7.4). Post initial plate incubation, 10 μL of the biotinylated cAMP/streptavidin donor bead lysis buffer was added to each well under green light. The plate was resealed, covered with aluminum foil, and incubated for a second two-hour incubation in the dark. After incubation, the plate was read via an EnSpire Alpha Plate Reader (Perkin-Elmer) using a protocol preset by the manufacturer. Duplicate replicates were measured in at least three independent experiments. The data were fitted with dose-response curves and EC_{50} values were calculated by a nonlinear regression using GraphPad Prism (v4.0) software.

^{125}I-NDP-MSH Preparation and Purification

NDP-MSH was monoradioiodinated for the use in competition binding assays. The peptide was radioiodinated with Na^{125}I using the previously described chloramine T procedure.[96] The monoradioiodinated peptide was resolved from the uniodinated and diradioiodinated peptide via RP-HPLC using a C18 reverse phase column eluted isocratically with an acetonitrile:triethylamine phosphate (pH 3.0) mobile phase. The column eluate containing the monoradioiodinated peptide was diluted in a solvent mixture (2 parts 24% acetonitrile:

76% triethylamine phosphate, pH 3.0: 1 part milliQ water) containing a 2 mg/mL bovine albumin stabilizer. The resulting yield (lot number: 150326A) was 1.9 mL containing 1126 µCi of monoiodinated NDP-MSH with a theoretical specific activity of 2175 Ci/mmol. The radioligand was stored at -80°C in a lead pig until use.

^{125}I-NDP-MSH Competitive Binding Assay

This bioassay was used to produce the dose-response curves and subsequent IC_{50} determination for selected compounds from the reported TACO library (the 9 most potent EC_{50} values at the mMC3R) at the mMC3R and mMC4R. HEK293 cells stably transfected with the selected mouse melanocortin receptors, as described above, were used in this assay. Cells were plated into 12-well treated polystyrene plates (Corning Life Sciences, Cat. # 353043) 48-hours prior to the binding experiment such that each well reached greater than 90% confluency the day of the assay. On the day of the assay, media was aspirated and a 500 µL solution of 0.1% BSA in DMEM containing the experimental compound (10^{-4} to 10^{-10} M) and a constant 100,000 cpm/well ^{125}I NDP-MSH were added to each well. The plates were incubated at 37°C with 5% CO_2 for 1 hour. Post incubation, the media was aspirated, each well was washed with 500 µL assay medium, and cells were lysed with 500 µL of 0.1 M NaOH and 500 µL of 1% Triton X-100.

Following a 10-15 minute room temperature incubation, the cell lysate mixture was transferred to 12x75 mm polystyrene tubes and the radioactivity was quantified using a WIZARD^2 Automatic Gamma Counter (Perkin-Elmer). The specific binding for each well was determined by subtracting the counts obtained from the cell lysate which was incubated with non-radioactive 10^{-6} M NDP-MSH. Each experiment determined the specific binding for NDP-MSH as a positive control. The specific binding in the presence of each compound tested was normalized to 100% relative to the specific binding determined for non-radiolabeled NDP-MSH. The data were analyzed using GraphPad Prism (v4.0; GraphPad, Inc), and dose-response curves in addition to the corresponding IC_{50} values were calculated by Prism's non-linear regression algorithm. Each reported value represents the mean and standard error of the mean (SEM) of, at least, two independent experiments each containing two experimental replicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ACTH

adrenocorticotropic hormone

AGRP

agouti-related protein

ASIP

agouti-signaling protein

GPCR

G protein-coupled receptor

cAMP

cyclic 5′-adenosine monophosphate

MC1R

melanocortin-1 receptor

MC2R

melanocortin-2 receptor

MC3R

melanocortin-3 receptor

MC4R

melanocortin-4 receptor

MC5R

melanocortin-5 receptor

MCR

melanocortin receptor

α-

β-, and γ-MSH,

α-

β-, and γ-melanocyte stimulating hormones

NDP-MSH

4-norleucine-7-D-phenylalanine, Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH2

RP-HPLC

reverse phase high-pressure liquid chromatography

Nle

norleucine, Cha, 3-Cyclohexyl-alanine

PyrAla

pyroglytamyl-alanine

ThiAla

thienylalanine

Tic
tetrahydro-isoquinoline-3-carboxylic acid

(pCl)Phe
4-chloro-phenylalanine

(pI)Phe
4-iodo-phenylalanine

(pNO$_2$)Phe
4-nitro-phenylalanine

2-Nal
3-(2-naphthyl)-L-alanine

β-Ala
3-aminopropanoic acid

Met[O$_2$]
methionine sulfone

dehydPro
3,4-dehydro-proline

(3I)Tyr
3-iodotyrosine

Bip
biphenylalanine

3Bal
3-benzothienyl-alanine

Rink-amide MBHA resin
4-(2′,4′-Dimethoxyphenyl-Fmoc-aminmethyl)-phenoxyacetamido-methylbenzhydryl amine resin

HBTU
N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate

DMEM
Dulbecco's Modified Eagle Medium

HBSS
Hank's Balanced Salt Solution

IBMX
isobutylmethylxanthine

HEPES

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

POMC

pro-opiomelanocortin
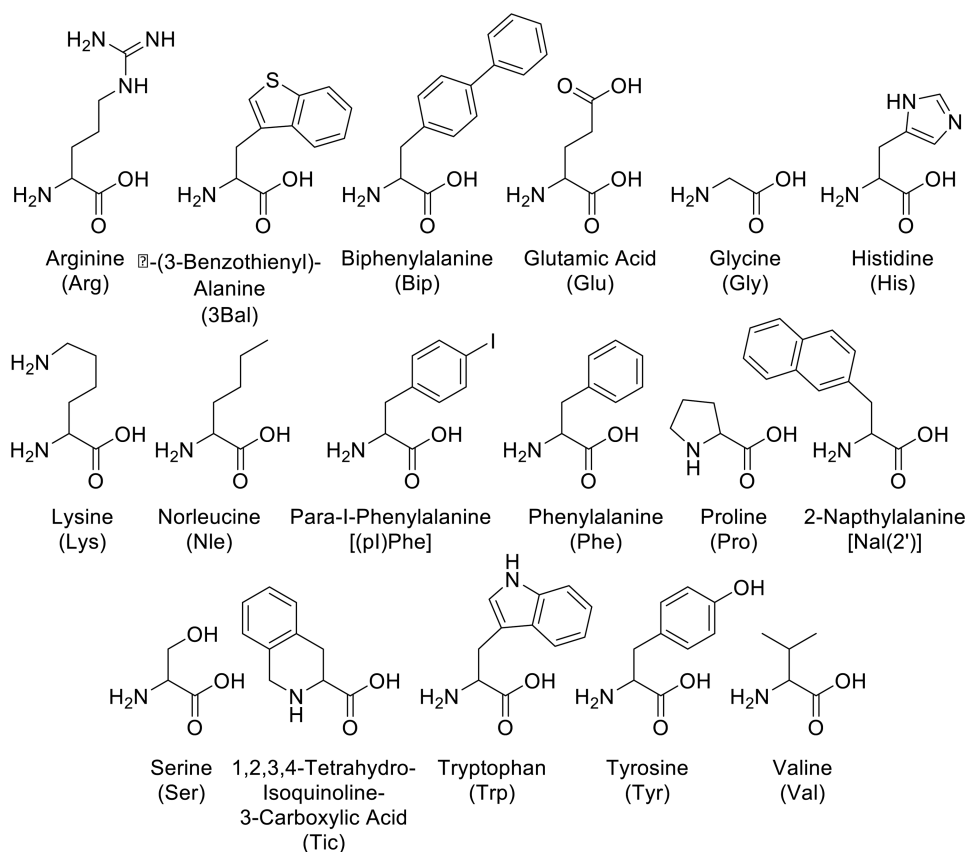
Figure 1. Amino acid building blocks for the reported focused peptide library

Structures of the building blocks used within this study. The stereochemistry is not included in this figure; however, it is included in the compound sequences.
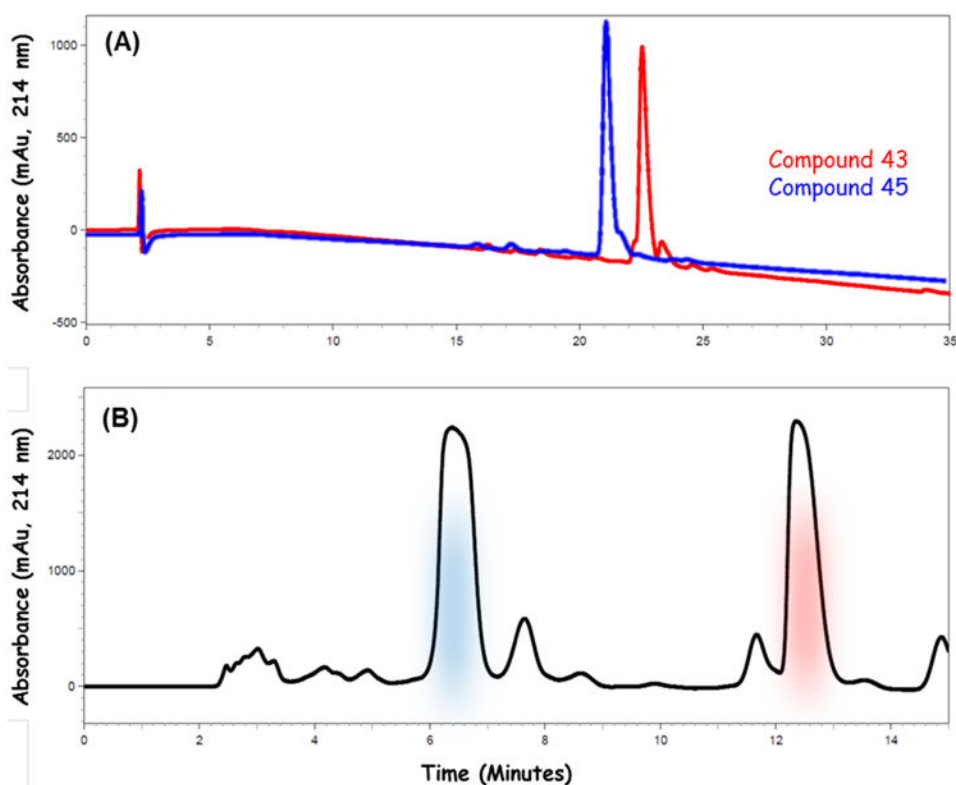
Figure 2. Parallel Purification Method Used in this Study to Purity 2 Peptides Simultaneously
Illustration of the RP-HPLC traces observed while implementing the parallel purification method used in this study. Crude peptides were selected for parallel purification when (A) overlays of the crude analytical traces (10% to 90% acetonitrile gradient in 0.1% trifluoroacetic acid in water over 35 minutes at 1.5 mL/min using an analytical 10 micron C18, 4.6 × 250 mm, Vydac Cat#218TP104) had the desired peptides within 5 minutes of each other and did not introduce impurities into the other peptide. The semipreparative parallel purification (B) could be achieved with a 15 minute separation method (typically 40% to 50% acetonitrile gradient in 0.1% trifluoroacetic acid in water over 15 minutes at 5 mL/min using a semipreparative 10 micron C18, 10 × 250 mm, Vydac Cat#218TP1010).
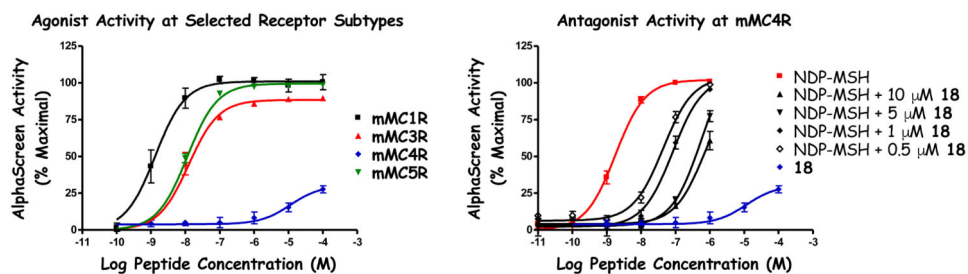
Figure 3. Illustration of Observed *In Vitro* Pharmacology for 18 at the Melanocortin Receptor Subtypes Examined in this Study

Compound **18** displayed potent nanomolar agonist activity at the mouse melanocortin-1, -3, and -5 receptor subtypes ($EC_{50} < 20$ nM) and potent 60 nanomolar antagonist activity at the mouse melanocortin-4 receptor subtype ($pA_2 = 7.8$). The observed pharmacological profile is unique to this scaffold, versus other melanocortin peptide receptor templates reported to date, and this compound produced the most potent activity within this study. A total of nine compounds in the reported library possess this mixed MCR pharmacological profile with moderate to potent agonist activity ($EC_{50} < 1,000$ nM) at the mMC3R and antagonist activity at the mMC4R ($7.8 > pA_2 > 5.7$).
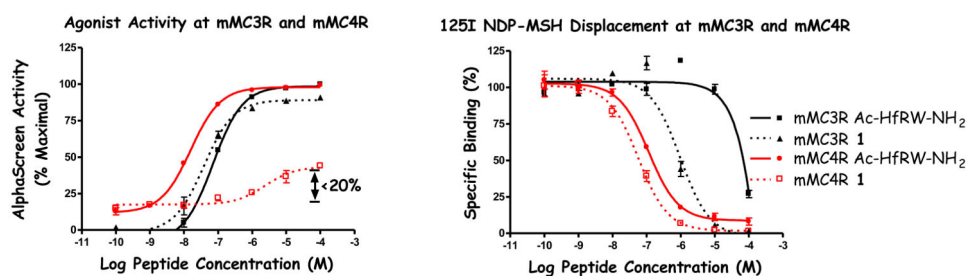
Figure 4. Illustration of *In Vitro* Agonist Activity and displacement of specific ^{125}I-NDP-MSH Binding of 1 versus Tetrapeptide HfRW at the mMC3R and mMC4R

The lead tetrapeptide **1**, sequence Ac-His-Arg-(pI)DPhe-Tic-NH$_2$, produced agonist activity comparable (within 3-fold) to the classic melanocortin tetrapeptide ligand Ac-His-DPhe-Arg-Trp-NH$_2$, labeled above as "Ac-HfRW-NH$_2$," at the mMC3R. Yet, **1** produced a binding affinity IC$_{50}$ value at the mMC3R that is 50-fold greater than the observed ^{125}I-NDP-MSH displacement value for the Ac-His-DPhe-Arg-Trp-NH$_2$ tetrapeptide. In contrast, **1** produced little, <20% of maximal, agonist activation and the Ac-HfRW-NH$_2$ ligand produced a full agonist response with a nanomolar EC$_{50}$ at the mMC4R; however, these two peptides possess similar binding displacement curves.
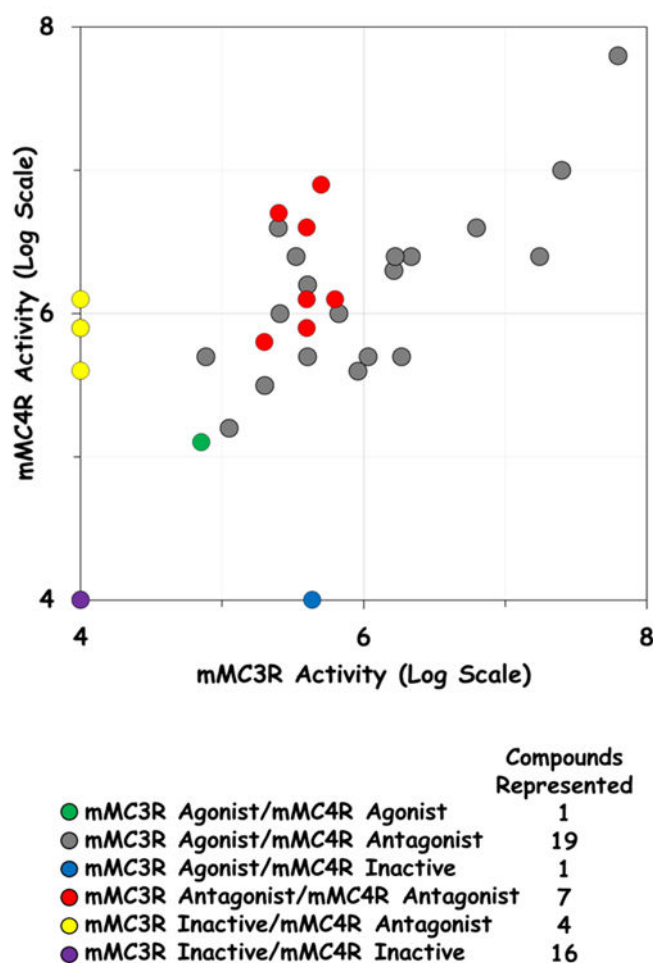
Figure 5. General overview of the potency observed for the tetrapeptide library at the mMC3R and mMC4R

Illustrated is the potency, either agonist EC_{50} or antagonist K_i values on a log scale, and pharmacological profile as a function of the activities observed at the mMC4R versus the mMC3R. 39% of the compounds possessed pharmacological profiles wherein there was observed agonist activity at the mMC3R and antagonist activity at the mMC4R. This was followed by (33%) of compounds that were essentially inactive at both the mMC3R or mMC4R, with the remaining compounds displaying a variety of pharmacological characteristics at the two receptor subtypes. The substitutions investigated in the reported scaffold, sequence Ac-Xaa^1-Arg-(pI)DPhe-Xaa^4-NH$_2$, tended to be additive for the absolute activity observed at the mMC3R and mMC4R.

Table 1

Summary of agonist and antagonist data collected at the mMC3R and mMC4R for the scaffold Ac-Xaa^1-Arg-(pI)DPhe-Xaa^4-NH$_2$

ID	Xaa^1	Xaa^4	mMC3R Agonist EC$_{50}$ ± SEM (nM)	mMC3R Antagonist pA$_2$ ± SEM	mMC4R Agonist EC$_{50}$ ± SEM (nM)	mMC4R Antagonist pA$_2$ ± SEM
NDP-MSH			0.09 ± 0.01		0.8 ± 0.1	
Ac-HfRW-NH$_2$			73 ± 10		16 ± 3	
1	His	Tic	40 ± 7		> 100,000	7.0 ± 0.2
2	Arg	Bip	45% @ 100 μM	5.3 ± 0.1	32% @ 100 μM	5.8 ± 0.1
3	His	Bip	53% @ 100 μM	N/A	43% @ 100 μM	N/A
4	Bip	Bip	> 100,000	N/A	24% @ 100 μM	N/A
5	3Bal	Bip	51% @ 100 μM	N/A	35% @ 100 μM	N/A
6	Tic	Bip	100,000	N/A	> 100,000	N/A
7	Phe	Bip	30% @ 100 μM	N/A	> 100,000	N/A
8	Nal(2′)	Bip	14,000 ± 1,100		7,900 ± 1,300	
9	DNal(2′)	Bip	30% @ 100 μM	5.6 ± 0.2	42% @ 100 μM	6.6 ± 0.1
10	Arg	3Bal	160 ± 40		24% @ 100 μM	6.6 ± 0.1
11	His	3Bal	460 ± 50		47% @ 100 μM	6.4 ± 0.1
12	Bip	3Bal	2,300 ± 920		> 100,000	N/A
13	3Bal	3Bal	2,500 ± 770		> 100,000	5.7 ± 0.3
14	Tic	3Bal	8,900 ± 2,400		> 100,000	5.2 ± 0.1
15	Phe	3Bal	930 ± 520		> 100,000	5.7 ± 0.1
16	Nal(2′)	3Bal	1,500 ± 530		> 100,000	6.0 ± 0.7
17	DNal(2′)	3Bal	4,000 ± 1,300		45% @ 100 μM	6.6 ± 0.1
18	Arg	Tic	16 ± 3		> 100,000	7.8 ± 0.3
19	Bip	Tic	13,000 ± 3,300		> 100,000	5.7 ± 0.1
20	3Bal	Tic	2,500 ± 190		37% @ 100 μM	6.2 ± 0.1
21	Tic	Tic	3,900 ± 940		36% @ 100 μM	6.0 ± 0.2
22	Phe	Tic	610 ± 200		> 100,000	6.3 ± 0.1
23	Nal(2′)	Tic	3,000 ± 940		33% @ 100 μM	6.4 ± 0.1
24	DNal(2′)	Tic	600 ± 210		32% @ 100 μM	6.4 ± 0.1

ID	Xaa[1]	Xaa[4]	mMC3R Agonist EC$_{50}$ ± SEM (nM)	mMC3R Antagonist pA$_2$ ± SEM	mMC4R Agonist EC$_{50}$ ± SEM (nM)	mMC4R Antagonist pA$_2$ ± SEM
25	Arg	Phe	540 ± 190		>100,000	5.7 ± 0.1
26	His	Phe	1,100 ± 240		>100,000	5.6 ± 0.1
27	Bip	Phe	77% @ 100 µM	N/A	>100,000	5.6 ± 0.1
28	3Bal	Phe	37% @ 100 µM	N/A	>100,000	N/A
29	Tic	Phe	>100,000	N/A	>100,000	N/A
30	Phe	Phe	50% @ 100 µM	N/A	>100,000	N/A
31	Nal(2´)	Phe	42% @ 100 µM	N/A	>100,000	N/A
32	DNal(2´)	Phe	>100,000	5.7 ± 0.1	>100,000	6.9 ± 0.1
33	Arg	Nal(2´)	51% @ 100 µM	5.8 ± 0.1	>100,000	6.1 ± 0.1
34	His	Nal(2´)	57% @ 100 µM	5.6 ± 0.1	35% @ 100 µM	6.1 ± 0.2
35	Bip	Nal(2´)	>100,000	N/A	>100,000	N/A
36	3Bal	Nal(2´)	>100,000	N/A	>100,000	N/A
37	Tic	Nal(2´)	>100,000	N/A	>100,000	5.9 ± 0.1
38	Phe	Nal(2´)	44% @ 100 µM	N/A	>100,000	5.9 ± 0.1
39	Nal(2´)	Nal(2´)	39% @ 100 µM	N/A	>100,000	N/A
40	DNal(2´)	Nal(2´)	43% @ 100 µM	5.4 ± 0.1	39% @ 100 µM	6.7 ± 0.1
41	Arg	DNal(2´)	57 ± 15		>100,000	6.4 ± 0.1
42	His	DNal(2´)	5,000 ± 2,100		50% @ 100 µM	5.5 ± 0.1
43	Bip	DNal(2´)	32% @ 100 µM	N/A	>100,000	N/A
44	3Bal	DNal(2´)	34% @ 100 µM	N/A	38% @ 100 µM	N/A
45	Tic	DNal(2´)	42% @ 100 µM	N/A	33% @ 100 µM	N/A
46	Phe	DNal(2´)	22% @ 100 µM	N/A	>100,000	6.1 ± 0.4
47	Nal(2´)	DNal(2´)	31% @ 100 µM	N/A	>100,000	N/A
48	DNal(2´)	DNal(2´)	40% @ 100 µM	5.6 ± 0.1	37% @ 100 µM	5.9 ± 0.1

Summary of the cAMP based functional data at the mMC3R and mMC4R using the AlphaScreen Technology. NDP-MSH and Ac-His-DPhe-Arg-Trp-NH2 (Ac-HlRW-NH2), in addition to assay media served as positive and negative controls, respectively. Forskolin served as an additional positive control due to the fact it activates adenylate cyclase independently of the melanocortin receptors. All of the compounds were assessed for agonist activity at up to 100 µM concentrations and values are represented as EC50 in nM. Compounds that did not produce a full agonist dose-response curve were tabulated as a percent of the NDP-MSH maximal efficacy positive control, and compounds with < 20% activity are denoted as EC50 > 100,000 nM. These experiments were performed with duplicate replicates in at least three independent experiments. Compounds were assessed for antagonist activity if they did not produce a full agonist dose-response to the maximal efficacy of NDP-MSH. Antagonist activity was assessed by using the NDP-MSH agonist and the test compound at concentrations of 10,000 nM, 5,000 nM, 1,000 nM, and 500 nM and measuring the resulting shift in EC50 values and calculating a

subsequent pA2 value [-Log(Ki)] via a Schild analysis.[77] The antagonist experiment was performed in triplicate unless no shift in EC50 activity was observed in which case it was tabulated as "N/A" indicating no activity was observed up to the 10 μM concentrations tested.

Table 2

Summary of Agonist Data Collected at the mMC1R and mMC5R for the scaffold Ac-Xaa^1-Arg-(pI)DPhe-Xaa^4-NH$_2$

ID	Xaa^1	Xaa^4	mMC1R EC$_{50}$ ± SEM (nM)	mMC5R EC$_{50}$ ± SEM (nM)
NDP-MSH			0.02 ± 0.001	0.18 ± 0.02
Ac -HfR W -NH$_2$			20 ± 1	3.0 ± 0.5
1	His	Tic	0.71 ± 0.04	17 ± 3
2	Arg	Bip	44% @ 100 µM	5,000 ± 1,600
3	His	Bip	> 100,000	69,000 ± 31,000
4	Bip	Bip	> 100,000	> 100,000
5	3Bal	Bip	> 100,000	72,000 ± 28,000
6	Tic	Bip	39% @ 100 µM	71,000 ± 29,000
7	Phe	Bip	67% @ 100 µM	68,000 ± 32,000
8	Nal(2′)	Bip	7,600 ± 1,200	8,300 ± 1,600
9	DNal(2′)	Bip	> 100,000	67,000 ± 33,000
10	Arg	3Bal	56 ± 20	210 ± 52
11	His	3Bal	70 ± 7	270 ± 67
12	Bip	3Bal	3,100 ± 490	5,500 ± 2,900
13	3Bal	3Bal	1,800 ± 590	18,000 ± 7,800
14	Tic	3Bal	38% @ 100 µM	26,000 ± 7,200
15	Phe	3Bal	330 ± 180	1,300 ± 480
16	Nal(2′)	3Bal	460 ± 210	5,400 ± 2,900
17	DNal(2′)	3Bal	210 ± 50	> 100,000
18	Arg	Tic	0.51 ± 0.08	8.8 ± 0.7
19	Bip	Tic	440 ± 70	16,000 ± 8,000
20	3Bal	Tic	72 ± 26	4,100 ± 1,300
21	Tic	Tic	93 ± 26	570 ± 110
22	Phe	Tic	8 ± 3	200 ± 70
23	Nal(2′)	Tic	54 ± 18	4,400 ± 2,700
24	DNal(2′)	Tic	7 ± 2	250 ± 70
25	Arg	Phe	55 ± 9	340 ± 90
26	His	Phe	92 ± 19	680 ± 140
27	Bip	Phe	2,600 ± 1,600	69% @ 100 µM
28	3Bal	Phe	1,200 ± 450	35% @ 100 µM
29	Tic	Phe	3,500 ± 1,200	21% @ 100 µM
30	Phe	Phe	600 ± 110	52% @ 100 µM
31	Nal(2′)	Phe	760 ± 600	49% @ 100 µM
32	DNal(2′)	Phe	540 ± 210	> 100,000
33	Arg	Nal(2′)	210 ± 100	1,000 ± 350
34	His	Nal(2′)	350 ± 90	2,900 ± 1,100
35	Bip	Nal(2′)	23% @ 100 µM	21% @ 100 µM

ID	Xaa[1]	Xaa[4]	mMC1R $EC_{50} \pm SEM$ (nM)	mMC5R $EC_{50} \pm SEM$ (nM)
36	3Bal	Nal(2′)	$12,000 \pm 4,000$	50% @ 100 μM
37	Tic	Nal(2′)	66% @ 100 μM	48% @ 100 μM
38	Phe	Nal(2′)	$1,000 \pm 750$	61% @ 100 μM
39	Nal(2′)	Nal(2′)	$3,800 \pm 2,200$	45% @ 100 μM
40	DNal(2′)	Nal(2′)	$1,200 \pm 390$	40% @ 100 μM
41	Arg	DNal(2′)	280 ± 140	500 ± 100
42	His	DNal(2′)	$3,700 \pm 2,400$	47% @ 100 μM
43	Bip	DNal(2′)	$5,300 \pm 1,100$	55% @ 100 μM
44	3Bal	DNal(2′)	36% @ 100 μM	41% @ 100 μM
45	Tic	DNal(2′)	770 ± 90	74% @ 100 μM
46	Phe	DNal(2′)	$3,800 \pm 1,500$	39% @ 100 μM
47	Nal(2′)	DNal(2′)	53% @ 100 μM	40% @ 100 μM
48	DNal(2′)	DNal(2′)	$4,000 \pm 850$	43% @ 100 μM

Summary of the cAMP based functional data at the mMC3R and mMC4R using the AlphaScreen Technology. NDP-MSH and Ac-His-DPhe-Arg-Trp-NH$_2$, in addition to assay media served as positive and negative controls, respectively. Forskolin served as an additional positive control due to the fact that it activates adenylate cyclase independently of the melanocortin receptors. All of the compounds were assessed for agonist activity at up to 100 μM concentrations. Agonist potency values are represented as EC_{50} in nM. Compounds that did not produce a full agonist dose-response curve were tabulated as a percent of the NDP-MSH maximal efficacy positive control. Compounds with < 20% activity were denoted as EC_{50} > 100,000. These data are generated from duplicate replicates in at least three independent experiments.

Table 3

Summary of ^{125}I-NDP-MSH Binding Displacement of Selected TACOs at the mMC3R and mMC4R for the scaffold Ac-Xaa^1-Arg-(pI)DPhe-Xaa^4-NH$_2$.

Compound	Xaa^1	Xaa^4	mMC3R (IC$_{50}$)		mMC4R (IC$_{50}$)	
			Mean ± SEM Fold (nM) Diff.		Mean ± SEM (nM)	Fold Diff.
NDP-MSH			5.3 ± 0.7		2.0 ± 0.2	
Ac-HfRW-NH$_2$			50,500 ± 500	1.0	121 ± 39	1.0
1	His	Tic	975 ± 225	-52	83 ± 13	1
10	Arg	3Bal	965 ± 135	-52	93 ± 6	1
11	His	3Bal	3,400 ± 300	-15	275 ± 35	2
15	Phe	3Bal	5,350 ± 250	-9	850 ± 40	7
18	Arg	Tic	550 ± 120	-92	13 ± 3	-9
22	Phe	Tic	2,500 ± 600	-20	135 ± 15	1
24	DNal(2´)	Tic	420 ± 50	-120	43 ± 7	-3
25	Arg	Phe	6,500 ± 600	-8	455 ± 85	4
41	Arg	DNal(2´)	440 ± 70	-115	220 ± 10	2

The nine most potent TACOs at the mMC3R were selected for ^{125}I NDP-MSH radiolabel competition binding experiments. The compounds were assayed at both the mMC3R and mMC4R, and the reported values are the mean and ± standard error of the mean (SEM) of two independent experiments each consisting of duplicate replicates. In addition, NDP-MSH and Ac-His-DPhe-Arg-Trp-NH$_2$ are included as experimental controls and reference purposes. The fold difference (Fold Diff.) is determined between the peptide as compared to the tetrapeptide Ac-His-DPhe-Arg-Trp-NH$_2$ (Ac-HfRW-NH$_2$) control. A negative fold difference indicates the binding affinity of the peptide is more potent than the Ac-His-DPhe-Arg-Trp-NH$_2$ (Ac-HfRW-NH$_2$) control.