Tetrapeptide Melanocortin Agonist Ligands Exploring Selectivity of the mMC3R Using DPhe Substitutions in the Ac-His-Arg-DPhe-Tic-NH_2 Scaffold

A THESIS SUBMITTED TO THE FACULTY OF THE UNIVERSITY OF MINNESOTA BY

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March 2018

Abstract

There are five melanocortin receptors (MC1R-MC5R) that primarily activate the signaling pathway for adenylate cyclase. While both the MC3R and MC4R are hypothesized to be involved in regulating energy homeostasis, the MC3R functionality has been elusive to characterize due to the lack of MC3R-selective ligands. The melanocortin system has been clearly defined as a target for treating obesity or cachexia. When centrally delivered through intracerebroventricular administration, agonists decrease food intake, while antagonists increase food intake. Previous weight management therapies have focused on targeting the MC4R, though off-target cardiovascular effects may limit the clinical utility of these ligands. Therefore, obtaining a MC3R selective compound may allow for a weight regulation therapy that will not have the same cardiovascular liabilities.

MC4R-selective ligands have been heavily investigated, however MC3R-selective ligands have largely gone unexplored. A mixture-based positional scan was conducted to generate scaffolds with MC3R selectivity. The lead compound from the study was Ac-His-Arg-(pI)DPhe-Tic-NH_2, an MC3R agonist (EC_{50} 40nM) and an MC4R antagonist (pA_2 7.2). The scaffold reverses the sequence of arginine and phenylalanine residues in the His-Phe-Arg-Trp conserved sequence found in endogenous melanocortin ligands. This work presents a follow-up study investigating the position of the phenylalanine residue in efforts to create a MC3R selective agonist with decreased MC4R antagonist activity.

Acknowledgements

I would like to give a special thanks to my lab mates, because without them this would not have been possible. Thank you to my committee members, Dr. Johnson, Dr. Ambrose, Dr. Pomerantz, and Dr. Doran, for their feedback and support. Special thanks to my advisor, Dr. Carrie Haskell-Luevano for her guidance and encouragement.

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Introduction

The melanocortin receptor system contains five subtypes labeled MC1R through MC5R, which belong to the class-A family of rhodopsin-like G-protein coupled receptors[1-6]. These receptors primarily signal through the $G_{\alpha}s$ subunit, increasing production of cyclic adenosine monophosphate (cAMP)[1-6]. The MC1R is primarily involved in regulation of skin pigmentation[1, 6]. The MC2R is only activated by adrenocorticotropic hormone (ACTH) and is implicated in steroidogenesis[1]. Both the MC3R and MC4R contribute to the regulation of energy homeostasis, which have made them targets for weight regulation therapies[4, 5, 7-10]. Several single nucleotide polymorphisms (SNPs) in the MC4R[11], and double variants[12] as well as mutations in the MC3R have been associated with the onset of severe obesity[13]. The MC5R functionality in humans is still unknown, but it is known to affect the exocrine gland function in mice[3, 14].

Endogenous agonists are derived from the proopiomelanocortin (POMC) gene and include α-, β-, and γ-melanocortin stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH). The only agonist to stimulate all five receptor subtypes is ACTH; α-, β-, γ-MSH stimulate all known receptors except the MC2R[2, 15]. The His-Phe-Arg-Trp motif has been found to be the minimum sequence of endogenous agonists necessary for melanocortin activation[16-18]. The melanocortin system contains two naturally occurring antagonists, agouti-signaling protein (ASP), and agouti-related protein (AGRP). The antagonists inhibit receptor function at the MC1, MC3 and MC4 receptors[19-21]. Further studies on AGRP identified the Arg-Phe-Phe motif, as critical for antagonist pharmacology[22].

Targeting the MC3R/MC4R can lead to development of treatments for metabolic diseases such as obesity, anorexia, and cachexia. When administrated through intracerebroventricular injection (ICV), agonists that are nonselective for the MC3R and MC4R result in decreased food intake[23]. When an endogenous antagonist such as AGRP is administrated in mice it has orexigenic effects, or increased appetite, which can last upward of seven days[24]. While both receptors have a

role in energy homeostasis, the differences in phenotype of knockout mice suggest that these roles are nonredundant. The MC4R knockout models show hyperphagia, they gain fat mass, and experience both weight and length gains compared to wild type mice[25]. Comparatively, the MC3R knockout mice models exhibit increased fat mass and reduced lean mass, while maintaining similar body weight to wild type mice[25]. To further support the hypothesis of independent roles, phenotypic differences between the extremely obese double knockouts and the obese single knockout models have been observed[25].

Previous clinical trials treating obesity with MC4R drugs and follow up studies reported the side effects of hypertension and induced penile erection[26-30]. Many studies have attempted to determine how the melanocortin receptors and blood pressure are linked[27, 29, 31]. Some evidence indicates that the cardiovascular side effects seen are moderated primarily through the MC4R pathway, with very little contributions from the MC3R pathway[27]. This has been tested by central administration of α-MSH and γ-MSH, which are more selective for the MC4R and MC3R respectively[27]. When ICV injections of α-MSH are given, the elevated blood pressure seen in wild type mice is not seen in MC4R deficient mice[27]. The γ-MSH contributions to elevated blood pressure have been thought to be independent of MC3R activity[27]. However, current clinical trials of the peptide Setmelanotid (Ac-Arg-c[Cys-D-Ala-His-D-Phe-Arg-Trp-Cys]-NH_2), which targets the MC4R, have seen no indication of the adverse cardiovascular side effects[32]. Further exploring the mechanistic differences between the MC3R and MC4R, with compounds of different pharmacological profiles can help in the discovery of weight management therapies that do not have adverse cardiovascular side-effects.

While MC4R-selective agonists have been well studied as possible treatments for metabolic disease, MC3R-selective targets are. Previously reported selective compounds for the MC3R over the MC4R, have primarily been analogues of the endogenous ligands α- and γ-MSH[33-36]. In hopes of finding scaffolds with more drug-like properties, a mixture-based positional scan was previously used to rapidly scan for new scaffolds with unique pharmacology[37, 38]. Mixture-

based positional scans are a method used to efficiently and systematically explore chemical space[39, 40]. In mixture-based positional scans, multiple compounds are assayed in one well, each of the compounds in the well share the same amino acid residue in a single position. The compounds that are in active mixtures can be synthesized and tested individually. Previously, mixture-based positional scanning has successfully been used to identify tetrapeptides that rescued functionality in human MC4R containing SNPs[41], and tetrapeptides with unique pharmacology profiles[38]. Amongst the new scaffolds discovered was one of particular interest, Ac-His-Arg-(pI)DPhe-Tic-NH_2, a MC3R agonist (EC_{50} = 40nM) and a MC4R antagonist (pA_2 = 7.0)[37]. This scaffold reverses the sequence of arginine and phenylalanine residues in the His-Phe-Arg-Trp conserved sequence found in endogenous melanocortin ligands[37].

As compared to the MC3R agonist and MC4R antagonist profile seen by Ac-His-Arg-(pI)DPhe-Tic-NH_2, the tetrapeptide Ac-His-(pI)DPhe-Arg-Trp-NH_2 expresses partial agonist/antagonist pharmacology at the MC3R and is a full agonist at the MC4R[37]. Previous SAR centered around substitutions of the DPhe residues have been shown that this residue is critical in determining if melanocortin ligands exhibit MC3R or MC4R antagonism[42]. When DPhe was replaced with bulkier residues, DNal(2') and (pI)DPhe, in the synthetic agonist MTII (Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2), potent synthetic MC3R/MC4R antagonists were created[42]. Similarly, exploring substitutions on the DPhe residue in Ac-His-DPhe-Arg-Trp-NH_2 sequence showed that the DPhe residue was extremely important in switching the mMC3R functionality from an agonist to an antagonist[37]. Due to the importance of DPhe in the pharmacological profile of previous melanocortin ligands, it was hypothesized that the (pI)DPhe position in Ac-His-Arg-(pI)DPhe-Tic-NH_2 could be important to the selectivity of agonist activity in the MC3R receptor when compared with MC4R activity.

Herein, we present a SAR that investigates physicochemical properties responsible for the selectivity of the mMC3R agonism over mMC4R activity. The creation of an MC3R selective agonist with decreased agonist and antagonist activity at the MC4R would allow for a chance to

more effectively probe the activity of the MC3R *in vivo* and to develop MC3R selective therapeutics.

Experimental

Synthesis:

Peptides were synthesized using standard solid-phase fluorenyl-9-methyloxycarbonyl (Fmoc) methodology[43, 44]. Couplings were performed using a CEM Discover SPS microwave peptide synthesizer. The 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxacetyl-MBHA resin [Rink-amide-MBHA (100-200 mesh), 0.66 mequiv/g substitution], 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU), and the listed Fmoc-protected amino acids [Fmoc-His(Trt), Fmoc-Arg(Pbf), Fmoc-(pF)DPhe, and Fmoc-(pCl)DPhe] were purchased from Peptides International (Louisville, KY, USA). The Fmoc-(pBr)DPhe, Fmoc-(3,4-diCl)DPhe, Fmoc-(pCN)DPhe, Fmoc-(pMe)DPhe, Fmoc-p(NH_2)DPhe(Boc), Fmoc-(pBz)-DPhe, and Fmoc-(4-tBu)DPhe were acquired from BACHEM (San Carlos, CA, USA). Fmoc-(pI)DPhe was procured from Alfa Aesar (Tewksbury, MA,USA). Fmoc-(pCF_3)DPhe, and Fmoc-(3-Cl)DPhe was bought from Chem Cruz (Dallas, TX, USA). Fmoc-D-4,4'-Biphenylalanine and Fmoc-D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) were purchased from Synth Tech (Albony, OR, USA). Fmoc-DTyr(But) was acquired from Ad. Chem (Lousville, KY, USA). The triisopropylsilane (TIS), *N*,*N*-diisopropylethylamine (DIEA), 1,2-ethanedithiol (EDT), piperidine, pyridine, and TFA were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (MeCN), *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), and acetic anhydride were purchased from Fisher Scientific. All reagents were ACS grade or higher and used without further purification.

Peptides were synthesized from C-terminus to N-terminus on Rink-amide-MBHA resin (Peptides International, 0.66 mequiv/g) in a fritted polypropylene reaction vessel (25 mL CEM reaction vessel). Resin was swelled in DCM for at least 1 hour. Deprotection of the Fmoc protected amino acids was completed using 20% piperidine in DMF at 23°C for two minutes. A second

aliquot of 20% piperidine was added to ensure full deprotection through the assistance of microwave heating (75°C, 30W, 4 min). Amide bonds were formed by adding Fmoc-amino acids, HBTU, and DIEA. After each deprotection or coupling, resin was washed 3-5 times with DMF and confirmed with either a ninhydrin[45] or chloranil test[46]. Ninhydrin/Kaiser tests indicate the presence or lack of a free primary amine and chloranil tests indicate the presence or lack of a secondary amine. After the final peptide was added, the peptide was deprotected a final time and the N-terminus was acetylated on resin using a 3:1 mixture of acetic anhydride:pyridine.

Coupling conditions for the microwave synthesizer were as follows: 3.1:3:5 amino acid:HBTU:DiPEA. N-terminals were acetylated using a 3:1 solution of acetic acid anhydride:pyridine for 30 minutes at room temperature. Peptides were cleaved in a 91:0.3:0.3:0.3 TFA:Thioanisol:TIS:H_2O mixture. The peptide KNS2-23-9 (Ac-His-Arg-DTyr-Tic-NH_2) was cleaved in 91:0.3:0.3:0.3 TFA:EDT:TIS:H_2O mixture. After cleavage, peptides were precipitated in 0°C chilled diethyl ether. The precipitated peptide was pelleted in a centrifuge (Sorvall Legend XTR) at 4000 rpm at 4°C for 4 minutes. The peptide was washed with diethyl ether and pelleted at least three times before drying overnight in a desiccator.

The peptides were purified using RP-HPLC semipreparative chromatography in 0.1% TFA and H_2O with a C18 reverse phase column. The collected fractions were concentrated on a rotary evaporator and lyophilized three times. The pure compounds were characterized analytically using RP-HPLC with two solvent systems. Both acetonitrile and methanol 10-90% gradients were run in 0.1%TFA in water for 35 min. By integrating the area under the curve at 214 nm purity was determined for each compound. The mass was confirmed using ESI/TOF-MS (Bruker, BioTOF II, University of Minnesota Department of Chemistry Mass Spectrometry Laboratory). The peptide library was named TaMALES, for tetrapeptide melanocortin agonist ligands to explore selectivity.

Activity:

β-galactosidase assay

The purified compounds were tested for cAMP activity using a 96-well colorimetric β-galactosidase assay for agonist activity at the MC3R, MC4R, and MC5R and antagonist activity[47] at the MC4R. All β-galactosidase assays were conducted by Dr. Skye Doering. The melanocortin receptors are cloned into HEK293 cells using a pCDNA vector which was previously described[38]. Stably transfected HEK293 cells were plated with Dulbecco's Modified Eagle Medium (DMEM), with 10% bovine serum and 1% penicillin-streptomycin, into a 10 cm dish and grown until 40% confluent. When the cells reached 40% confluency (approximately 24 h later), cells were transiently transfected with 4 μg of CRE-PBKS per 10 cm plate of cells using calcium phosphate[48]. Twenty-four hours after the transfection, the cells were plated onto collagen treated Nunclon Delta Surface 96-well plates (Thermo Fischer Scientific) and incubated at 37 °C at 5% CO_2. Plates were stimulated 48 h post transfection with compounds, which were dissolved in H_2O and stored at 1000 μg/mL in -20°C. They were then incubated at 37°C with 5% CO_2 for 6 hours. Compounds were serially diluted in assay media (1.0 mL 1% bovine serum albumin [BSA] in phosphate buffered saline [PBS] and 1.0 mL of 100x isobutylmethylxanthine in 98.0 mL of DMEM) to concentrations ranging from 10^{-4} and 10^{-10} M. Positive controls included NDP-MSH (10^{-6} to 10^{-12} M) and forskolin (10 μM). Plain assay media was used as a negative control. After stimulation, media was aspirated from the plates and 50 μL of lysis buffer (250 mM Tris-HCL pH=8.0, 740 mL of DDH_2O, 10 mL of 10% Triton X-100 in water) was added to each well. Plates were stored at -80 °C for up to two weeks, until they were ready to be assayed.

Thawed plates were assessed for protein content and assayed for activity. Protein concentration was determined by adding 10 μL of cell lysate to 200 μL of BioRad dye solution (1:4 dilution with water) in a new 96-well plate. Absorbance was measured using a 96-well plate reader (Molecular Devices) at λ = 595 nm. In order to determine β-galactosidase activity, 40 μL of 0.5% BSA in PBS at 37°C, and 150 μL of the β-galactosidase substrate (60 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM KCl,, 50 mM 2-mercaptoethanol, and 660 μM 2-nitrophenyl β-D-galactosidase) were added to the remaining 40 μL of cell lysate. 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. Each compound was run in duplicate and three independent experiments were completed. All data was fitted with dose-response curves and EC_{50} values that were calculated using GraphPad Prism v4.0 software.

AlphaScreen Bioassay.

This bioassay was used to determine dose-response curves for compounds at the mMC1R. All AlphaScreen assays were conducted by Katie Freeman. The melanocortin receptors are cloned into HEK293 using a pCDNA vector. AlphaScreen (PerkinElmer) uses a direct measurement of cAMP signaling as previously described. 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.

Cells were disassociated with 1 mL of 37°C Versene solution (Gibco), resuspended in 5 mL of 37°C cell media and centrifuged (Sorvall Legend XTR centrifuge, swinging bucket rotor) at 800 rpm for 5 min at RT. The cell pellet was resuspended in Dulbecco's Phosphate Buffered Saline solution (DPBS 1x without $CaCl_2$ or $MgCl_2$, Gibco) warmed to 37°C. A hemocytometer was used to manually count the cells using 10 μL of cell mixture added to 10 μL of Trypan blue BioRad dye. The cells were centrifuged (800 rpm, 5 min, RT)., and the pellet was resuspended in stimulation buffer [Hank's Balanced Salt Solution (HBSS 10x without $NaHCO_3$ or phenol red, Gibco), 0.5 mM isobutylmehtylxanthine (IBMX), 5 mM HEPES buffer solution (1M, Gibco), and 0.1% BSA in Milli-Q water, pH = 7.4] to a final concentration of 10000 cells/μL. A solution of cells and anti-cAMP acceptor beads in stimulation buffer was than prepared (1000 cells/ μL and 0.5 μg/μL AlphaScreen anti-cAMP acceptor beads in stimulation buffer). Each 384-well microplate (OptiPlate-384, PerkinElmer) contained 10 μL of the cell/acceptor bead solution along with 5 μL of unknown compounds. Each ligand was tested from concentrations 10^{-4} to 10^{-12} M. The plate was sealed and incubated at room temperature in the dark for 2 h. Controls included NDP-MSH (10^{-6} to 10^{-12}), forskolin (10^{-4}) and the negative control, stimulation buffer.

The biotinylated cAMP/streptavidin coated donor bead mixture in lysis buffer (0.5 $\mu g/\mu L$ 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) was prepared under green light. Following the initial incubation, 10 μL of the biotinylated cAMP/streptavidin donor bead lysis buffer was added to each well. The plate was resealed and incubated for a second 2 h period in the dark. Post incubation, the plates were read by an EnSpire Alpha plate reader (PerkinElmer). Each compound was run in duplicate and three independent experiments were completed.

Physical Properties

Physical and electrostatic properties of the ligands were determined by using Maestro version 10.4.017 (Schrödinger, LLC, New York, NY, 2015). Charges were assigned using the LigPrep tool at pH 7.4. The ligand was minimized using OPLS 2005 force field[49] until there was a convergence energy difference of 0.0001. The Poisson-Boltzmann equation of electrostatics was implemented where the solvent dielectric constant $\varepsilon = 80$ and the dielectric constant of solute was $\varepsilon = 10$. Qikprop was used to determine Van der Waals energy, $\log P_{o/w,}$ PSA, hydrogen bond donors, and hydrogen bond acceptors.

Results

SAR Study

Substituents of the DPhe residue spanning a variety of electronic and physiochemical properties were tested for *in vitro* melanocortin receptor activity in the tetrapeptide template: Ac-His-Arg-DPhe-Tic-NH_2. The compounds were manually synthesized using a CEM Discover SPS microwave peptide synthesizer and standard Fmoc solid-phase peptide synthesis procedure[43]. Purification of compounds was completed using semipreparative reverse phase high pressure liquid chromatography (Table 1). Compounds were characterized through ESI-MS, confirming the mass, and RP-HPLC, verifying that all ligands are > 95% pure. Compounds activity was tested at the mouse MC3R-MC5R using a colorimetric β-galactosidase assay that measures cAMP activity (Table 2)[50]. Table 3 summarizes the activity at the mMC1R compounds which was characterized

by measuring intracellular cAMP using the Amplified Luminescent Proximity Homogenous Assay Screen (AlphaScreen, PerkinElmer). The AlphaScreen assay was conducted on mMC1R instead of the *B*-galactosidase assay because the mMC1R cell lines tend to be more impacted by the transfection conditions and duration of the β-galactosidase assay in comparison to the AlphaScreen. The increased stress levels alter the cells cAMP production. Both the β-galactosidase and AlphaScreen assays used stably transfected HEK293 cells expressing the cloned melanocortin receptor.

The tetrapeptide Ac-His-Arg-(p-I)DPhe-Tic-NH_2 was found in a mixture based positional scan and contains a unique mixed pharmacology mMC3R agonist activity (EC_{50} = 40 nM) and mMC4R antagonist activity (pA_2 = 7.0) [37, 38]. The Ac-His-Arg-(p-I)DPhe-Tic-NH_2 compound was used as a control and had mMC3R agonist activity (EC_{50} = 13 nM) and mMC4R antagonist activity (pA_2 = 7.2). The EC_{50} values of the control are slightly different from literature because the current values were determined through measuring activity using the β-galactosidase assay at the mMC3R mMC4R and mMC5R, while the literature uses the AlphaScreen assay at all tested receptors. Using the two following compounds: Ac-His-Arg-(4,4')DBip-Tic-NH_2 (**1**), Ac-His-Arg-DPhe(p-tBu)-Tic-NH_2 (**2**), were equipotent at the mMC3R (EC_{50} < 16 nM) to the Ac-His-Arg-(p-I)DPhe-Tic-NH_2 (Figure 2, Table 2). Any agonist EC_{50} values with less than three-fold difference between them are defined to be within experimental error. Compounds **1**, and **2** were all less potent antagonists at the mMC4R (6.1 < pA_2 < 6.8) compared to the control compound. All of the compounds have low nanomolar to sub-nanomolar potency at the mMC1R (Table 3) and mMC5R (Table 2). All of nanomolar potent mMC3R compounds showed partial agonist activity at the mMC4R (Figure 3), with the exception of compound **2**, compound **4,** and compound **7** which did not have any mMC4R activity at 100,000 nM (Table 2). This shows that the modified DPhe position in the His-Arg-(pI)DPhe-Tic scaffold can create mMC3R nanomolar agonists and mMC4R partial agonists, this pharmacological profile was previously seen in compounds containing the Arg-(pI)Dphe scaffold[37]. This pharmacological profile is the opposite of that seen when the DPhe position was modified in

the His-DPhe-Arg-Trp scaffold, which created mMC4R agonists that retained nanomolar potency and mMC3R partial agonists/antagonists[51]. This evidence supports the hypothesis that the switching of the DPhe and the Arg residues in His-DPhe-Arg-Trp scaffold can lead to a switch in selectivity between the mMC3R and the mMC4R.

All substituted compounds were more potent than the Ac-His-Arg-DPhe-Tic-NH_2 compound (Table 2). The two compounds that were equipotent to the control were compounds **1** and **2.** Compound **1** has agonist activity at the mMC1R (EC_{50} = 0.8 ± 0.3 nM), mMC3R (EC_{50} = 14 ± 2 nM), and mMC5R (EC_{50} = 8.4 ± 0.6 nM). At the mMC4R, compound **1** had antagonist activity (pA_2 = 6.1 ± 0.2) and partial agonist activity (EC_{50} = 600 ± 140 nM; 43% @ 100 μM). Compound **2** has nanomolar potency at the MC1R (EC_{50} = 10 ± 0.3 nM), mMC3R (EC_{50} = 16 ± 2 nM), and the mMC5R (EC_{50} = 8.4 ± 0.6 nM). Compound **2** has antagonist activity at the mMC4R (pA_2 = 6.1 ± 0.2). Compound **2** is the most selective compound for the mMC3R over the mMC4R due to the fact that it has micromolar potency as an antagonist at the mMC4R and no measurable mMC4R agonist activity. While compounds **1** and **2** have similar activity profiles and are at least 5-fold more potent at the mMC3R than other substitutions, there do not share many physical properties with the control compound, Ac-His-Arg-(p-I)DPhe-Tic-NH_2. Both (4,4')DBip (**1**) and the p-tBu (**2**) are both weak electron donating groups, but **1** contains an aromatic substituent and **2** contains an aliphatic substituent. This is dissimilar to the p-I substituent which donates electron density through π bonding, but is electronegative and known to have inductive withdrawal of electrons.

Compounds Ac-His-Arg-DPhe(p-Br)-Tic-NH_2 (**3**), Ac-His-Arg-DPhe(p-Cl)-Tic-NH_2 (**6**), Ac-His-Arg-DPhe-(3,4)diCl-Tic-NH_2 (**7**), and Ac-His-Arg-DPhe(p-F)-Tic-NH_2 (**8**) contained halogens and were chosen to explore trends in lipophilicity and electronegativity. Compound **3** has agonist activity at the MC1R (EC_{50} = 0.8 ± 0.3 nM), mMC3R (EC_{50} = 85 ± 20 nM), and the mMC5R (EC_{50} = 9.7 ± 0.5 nM), while having partial agonist activity at the mMC4R (230 ± 40 nM; 54% @ 100 μM). Compound **3** was of special interest because it has measurable partial agonist activity at the mMC4R but no measurable antagonist activity at the mMC4R. Compound **6** has agonist activity

at the MC1R (EC_{50} = 0.95 ± 0.08 nM), mMC3R (EC_{50} = 140 ± 20 nM), and the mMC5R (EC_{50} = 19 ± 6 nM). Compound **6** had partial agonist activity at the mMC4R (380 ± 50 nm; 67% @ 100 µM). Compound **7** has agonist activity at mMC1R (EC_{50} = 0.52 ± 0.03 nM), mMC3R (EC_{50} = 140 ± 70 nM), and mMC5R (EC_{50} = 70 ± 6 nM). Antagonist activity at the mMC4R (pA_2 = 6.23 ± 0.06) was reported for compound **7.** Agonist activity at the MC1R (EC_{50} = 2.2 ± 0.7 nM), mMC3R (EC_{50} = 450 ± 70 nM), and the mMC5R (EC_{50} = 5 ± 4 nM) was seen when testing compound **8**. Compound **8** had partial agonist activity at the mMC4R (560 ± 60 nm; 69% @ 100 µM). The most efficacious partial agonists were in the halogen series, specifically the compounds **6** (p-F substitution) and **8** (p-Cl substitution).

Trends in the halogen series were explored for mMC3R and mMC4R activity. The less electronegative para position substitutions of iodine and bromine (**3**) were slightly more potent at the mMC3R compared to compounds with more electronegative, smaller halogen substitutions chlorine (**6**) and fluorine (**8**). Antagonist mMC4R activity is only seen in the Ac-His-Arg-DPhe(p-I)-Tic-NH_2. The partial agonist activity is greater when compounds are substituted at the para position with chlorine and fluorine when compared to bromine and iodine (Table 2).

In order to confirm any trends in electronegativity and lipophilicity found in the halogen series, activity of the p-CF_3 substituted peptide (**4**) was compared with the p-CH_3 substituted peptide (**5**). There was no significant difference between activity at any of the melanocortin receptors tested, except for the partial agonist activity associated with compound **5**. Compound **4** has agonist activity at the MC1R (EC_{50} = 6 ± 2 nM), mMC3R (EC_{50} = 90 ± 30 nM), and the mMC5R (EC_{50} = 13 ± 4 nM), while having antagonist activity at the mMC4R (pA_2 = 6.4 ± 0.2) . Compound **5** has agonist activity at the MC1R (EC_{50} = 1.2 ± 0.3 nM), mMC3R (EC_{50} = 110 ± 20 nM), and the mMC5R (EC_{50} = 14 ± 4 nM), while having antagonist activity (pA_2 = 6.3 ± 0.2) and partial agonist activity (390 ± 90 nm; 67% @ 100 µM) at the mMC4R.

Two compounds had micromolar activity at the mMC3R, Ac-His-Arg-DPhe(p-CN)-Tic-NH_2 (**9**), and Ac-His-Arg-DTyr-Tic-NH_2 (**10**). Compound **9** showed agonist activity at the MC1R

(EC_{50} = 32 ± 3 nM), mMC3R (EC_{50} = 4000 ± 1000 nM), and the mMC5R (EC_{50} = 500 ± 200 nM). Some agonist activity at the mMC4R was detected (EC_{50} = 29% @ 100 μM) when testing compound **9**. Compound **10** showed agonist activity at the MC1R (EC_{50} = 50 ± 10 nM), mMC3R (EC_{50} = 4300 ± 800 nM), and the mMC5R (EC_{50} = 1200 ± 600 nM). The polar groups (OH) in DTyr (compound **10)** and (p-CN) in compound **9** decrease the ability of the compounds to activate the MC1R, MC3R and MC5R by greater than 100 fold compared to Ac-His-Arg-DPhe(p-I)-Tic-NH_2. Compound **9** and **10** lose antagonist activity compared to the low nanomolar mMC3R compounds (Table 2).

The main goal of this study was to develop a probe that minimizes mMC4R activity in order to study mMC3R activity thoroughly. Before the positional scan for MC3R selective compounds that developed possible mMC3R selective compounds[38], analogs of endogenous ligands γ_2-MSH and α-MSH were studied. By increasing the selectivity of the Ac-His-Arg-DPhe(p-I)-Tic-NH_2 scaffold for the mMC3R over the mMC4R, we are developing compounds that have more drug-like properties than previous endogenous analogs. These compounds could be tested *in vivo* as therapies for obesity possibly without creating undesirable side effects of previous melanocortin therapeutics primarily targeting the MC4R.

Discussion

A structure-activity relationship study was conducted by substituting (p-I)DPhe residues with a series of aromatic amino acids in the tetrapeptide scaffold Ac-His-Arg-(p-I)DPhe-Tic-NH_2. This scaffold was originally investigated for its unique pharmacological profile and the fact that it contained a 'Arg-(p-I)DPhe' motif which reversed the positions of the traditional agonist 'Phe-Arg' motif seen in the endogenous agonist sequence His-Phe-Arg-Trp[18]. It was hypothesized that the (pI)DPhe position in Ac-His-Arg-(pI)DPhe-Tic-NH_2 could be important to increasing agonist activity selective for the mMC3R receptor when compared with mMC4R. A series of mono- and di- substituted DPhe substituents were chosen to explore the physicochemical properties that contribute to selectivity of the mMC3R over the mMC4R. The tetrapeptides were characterized *in*

vitro using the stably transfected mouse MC1R, MC3R, MC4R, and MC5R in HEK293 cells (Table 2, 3).

Polar Group Substitutions

Two polar groups were incorporated into the SAR, tetrapeptide **9** and **10** which substituted (p-CN)DPhe and DTy into the tetrapeptide scaffold, respectively. Both peptides have similar pharmacological profiles at all receptors that were studied. Tetrapeptides **9** and **10** both saw 100-fold decreased potency at the mMC3R, mMC4R, and mMC5R when compared to the Ac-His-Arg-DPhe(p-I)-Tic-NH_2 control (Figure 2C). Potency at the MC1R also decreased when compared to the controls, by 40 to 63-fold, however both compound **9** and **10** were about 15-fold more potent at the mMC1R than at any other receptor. This could suggest that the polar groups are not well tolerated by the MC3R, MC4R or MC5R and that the DPhe structures are interacting with a hydrophobic pocket. Through homology modeling and mutation studies the DPhe substituent in the DPhe-Arg-Trp melanocortin agonist sequence and the Arg-Phe-Phe antagonist sequence are both predicted to interact with aromatic residues in the mMC4R binding domain[52-54]. It was also hypothesized previously that Ac-His-Arg-DPhe-Tic-NH_2 scaffold's mixed pharmacology is caused by the scaffold being a hybrid between the tripeptide AgRP sequence, "Arg-Phe-Phe" and the melanocortin agonist signal sequence "His-Phe-Arg-Trp"[37]. It could be possible that this group of tetrapeptides also binds in the same pocket, and hence more polar hydrophilic groups would have less activity.

Hydrophobic Substitutions

Hydrophobic groups such as the tetrapeptides with DBip (**1**), p-tBu (**2**) and p-Me (**5**) substitutions, experienced a range of mMC3R EC_{50} values (13 nM < EC_{50} < 110 nM) (Figure 2B). The bulkier groups of compound **1** and **2** are 8-fold more potent at the mMC3R compared to compound **5**. The most potent mMC3R hydrophobic substitutions are sterically bulky. Compounds **1** and **2**, containing substitutions DBip, and p-tBu, respectively, are 6.8-fold more potent at the mMC3R compared to the p-Me substitution. At the mMC4R all of the compounds possessing

hydrophobic substitutions were antagonists, mMC4R antagonism potency tends to increase when agonist potency at mMC3R increases. All hydrophobic groups except compound **2** show partial agonist activity at the mMC4R (Figure 3B).

Electrostatics

The electrostatics of the tetrapeptides were studied due to trends seen in halogen data series[51]. Computational modeling for a similar tetrapeptide SAR has previously allowed for the study between biophysical properties and ligand-receptor interactions by modeling data from a halogen series [51]. In Figure 6, it can be seen that the compounds that had the lowest potency at the mMC3R (Figure 6B) quite consistently have a larger build-up of negative charge at the DPhe substituent than the higher potency agonists at the mMC3R (Figure 6A). Each of the compounds in Figure 6B, **8** (p-F), **9** (p-CN), and **10** (p-OH), have an electronegative atom which is surrounded by many more electropositive atoms, this creates a dipole giving a partial negative charge on the surface of the molecules. This dipole effect is muted in other tetrapeptides due to numerous factors such as reduced electronegativity and increased symmetry between dipoles in the substituents of the other tetrapeptides. Less electronegative substitutions, such as the p-I, p-tBu, and DBip, were greater than 28-fold more potent at the mMC3R as compared to the para-fluoro substitution. Compound **4** has a p-CF_3 substituent and is 5-fold more potent than the p-F containing compound. While fluorine is extremely electronegative, the fluorine atoms in compound **4** are organized around the carbon in a symmetrical fashion canceling out each of their individual dipoles. However, the electrostatic interactions in the low nanomolar potency mMC3R agonists varies, therefore electrostatics is not sufficient to fully explain the trends seen in mMC3R potency.

Halogen Substitutions: Electronegativity and Lipophilicity

The electrostatic results match the trends seen in the halogen series, which show that the more electronegative the halogen, the lower the potency at the mMC3R. The trend in electronegativity is as follows: F > 3,4-diCl > Cl > Br > I. Potency in the mMC3R is the reverse of

the electronegativity trend, where the p-I ligand is the most potent halogen and the p-F compound is the least potent halogen (Figure 2). The antagonist activity at the mMC4R is only seen in the control ligand and the (3,4)-diCl compound (Figure 4 and 5). Both mMC3R and mMC4R activity seem to rely on electronegativity. However, there is also no statistical difference between the mMC3R agonist or mMC4R antagonist activity of the compound with a p-CF_3 (**4**) substitution and the compound with a p-CH_3 (**5**) substitution. This shows that electronegativity is not the only factor influencing the trends seen in mMC3R or mMC4R antagonist activity.

One of the other trends that was explored was that of lipophilicity. Lipophilicity increases as follows: F < Cl < Br < CF_3 < I < 3,4-diCl. The potency at the mMC3R data weakly supports a trend in lipophilicity: a more lipophilic substitution resulted in higher potency at the mMC3R (Table 2). However, the (3,4)-diCl containing compound (**7**) is not as potent as the control tetrapeptide containing p-I. There is a non-significant trend seen that $logP_{oct/water}$ was more negative as potency at the mMC3R went down (Figure 7). $LogP_{oct/water}$ values were calculated as $QPLogP_{oct/water}$ values using Maestro QikProp tool (Table 4).

Selectivity

The SAR presented created more selective tetrapeptides over the mMC4R that eliminated much of the mMC4R agonist and antagonist activity in previous tetrapeptides while keeping low nanomolar potency at the mMC3R. Three new compounds containing: (3,4)-diCl, DBip, and p-tBu, were found to be equipotent at the mMC1R, mMC3R and mMC5R compared to Ac-His-Arg-(pI)DPhe-Tic-NH_2. Compound **2** (p-tBu) was of interest because it is the most selective for the mMC3R over the mMC4R. This compound showed no notable agonist activity at the mMC4R and the mMC4R antagonist activity is at micromolar affinity compared to the low nanomolar agonist potency at the mMC3R. In general, as a compound becomes more potent at the mMC3R it also becomes a more potent antagonist at the mMC4R (Table 2). For this reason, compound **3** (p-Br) was of interest because it still contains nanomolar potency at the mMC3R but it has no measurable antagonist mMC4R activity.

The DPhe substituent correlates with mMC4R agonist activity as seen by lipophilicity trends in the halogen series, however it is likely that there are other factors influencing mMC4R activity that have not been covered in this study. In order to study trends in mMC4R activity more thoroughly, SAR at the Arg postion of the His-Arg-(pI)DPhe-Tic compound could be completed.

While trends in mMC4R agonist activity and antagonist activity were not significant in this study, the DPhe substituent contributes to mMC3R activity as seen by trends in steric interactions, lipophilicity and electrostatics. The mMC3R nanomolar agonist and mMC4R partial agonist/antagonist pharmacological profile is unique to the scaffold His-Arg-DPhe-Tic, explored in the TaMALES library. The His-Arg-DPhe-Tic scaffold reverses the order of the DPhe and Arg residues found in the common melanocortin agonist scaffold: His-DPhe-Arg-Trp. When the DPhe position of the His-DPhe-Arg-Trp scaffold was explored through SAR, compounds with mMC4R agonist activity and mMC3R partial agonist/antagonist activity were found[51]. This evidence supports the importance of the position of the DPhe residue in the His-Arg-DPhe-Tic scaffold for mMC3R selectivity over the mMC4R.

Conclusions

The SAR presented created a more selective tetrapeptides that eliminated much of the mMC4R agonist and antagonist activity while keeping low nanomolar potency at the mMC3R. The compounds are not truly selective for mMC3R due to their activity at the mMC1R and mMC5R. We propose that a combination of electrostatic properties, lipophilicity and steric factors contribute to making more potent mMC3R agonists. These compounds can be used to further investigate the physiological role of the MC3R and determine if it is a worthwhile drug target for treating metabolic diseases.

Table 1. Tetrapeptide Characterization[a]

Compound	Sequence	Observed Mass [M+1] (g/mol)	Calculated Exact Mass (g/mol)	Purity (%)	Retention Time in Acetonitrile (min)	Retention Time in Methanol (min)
-	Ac-His-DPhe-Arg-Trp-NH_2	686.4	685.3	> 98	10.11	15.60
-	Ac-His-Arg-DPhe(p-I)-Tic-NH_2	785.3	784.2	> 98	14.89	21.77
1	Ac-His-Arg-(4,4')DBip-Tic-NH_2	735.5	734.3	> 96	16.66	25.89
2	Ac-His-Arg-DPhe(p-tBu)-Tic-NH_2	715.4	714.4	> 95	17.46	27.04
3	Ac-His-Arg-DPhe(p-Br)-Tic-NH_2	737.3, 739.3	736.3, 738.3	> 97	14.89	26.07
4	Ac-His-Arg-DPhe(p-CF3)-Tic-NH_2	727.5	726.7	> 97	15.00	23.38
5	Ac-His-Arg-DPhe(p-Me)-Tic-NH_2	673.5	672.4	> 98	14.31	22.34
6	Ac-His-Arg-DPhe(p-Cl)-Tic-NH_2	693.5	692.3	> 98	14.61	22.70
7	Ac-His-Arg-(3,4-diCl)DPhe-Tic-NH_2	727.4	726.3	> 98	15.60	23.98
8	Ac-His-Arg-DPhe(p-F)-Tic-NH_2	677.5	676.3	> 95	13.55	20.82
9	Ac-His-Arg-DPhe(p-CN)-Tic-NH_2	684.3	683.3	> 98	11.50	17.25
10	Ac-His-Arg-DTyr-Tic-NH_2	675.4	674.3	> 97	10.49	15.03

[a]The pure compounds were characterized analytically using RP-HPLC with two solvent systems. Both acetonitrile and methanol 10-90% gradients were run in 0.1%TFA in water for 35 min. By integrating the area under the curve at 214 nm purity was determined for each compound. The mass was confirmed using ESI/TOF-MS

Table 2. Agonist and Antagonist Activity of DPhe Substitutions at the mMC3R, mMC4R and mMC5R[a]

#	Sequence	mMC3R Agonist EC_{50} ± SEM (nM)	Fold	mMC4R		mMC5R Agonist EC_{50} ± SEM (nM)
				Agonist EC_{50} ± SEM (nM)	Antagonist pA_2 ± SEM	
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Lys-Pro-Val-NH_2	0.42 ± 0.02	-	0.32 ± 0.03	-	0.9 ± 0.2
-	Ac-His-DPhe-Arg-Trp-NH_2	190 ± 40	-	12 ± 3	-	4.3 ± 2
-	Ac-His-Arg-DPhe-Trp-NH_2	1300 ± 300	-	3000 ± 1000	-	150 ± 60
-	Ac-His-Arg-DPhe(p-I)-Tic-NH_2	13 ± 2	1.0	70 ± 13 (32%)	7.2 ± 0.7	4.5 ± 1
1	Ac-His-Arg-(4,4')DBip-Tic-NH_2	14 ± 2	1.1	600 ± 140 (43%)	6.1 ± 0.2	8.4 ± 0.6
2	Ac-His-Arg-DPhe(p-tBu)-Tic-NH_2	16 ± 2	1.2	> 100 µM	6.8 ± 0.3	3.3 ± 0.6
3	Ac-His-Arg-DPhe(p-Br)-Tic-NH_2	85 ± 20	6.5	230 ± 40 (54%)	N/A	9.7 ± 0.5
4	Ac-His-Arg-DPhe(p-CF3)-Tic-NH_2	90 ± 30	6.9	> 100 µM	6.4 ± 0.2	13 ± 4
5	Ac-His-Arg-DPhe(p-Me)-Tic-NH_2	110 ± 20	8.5	390 ± 90 (46%)	6.3 ± 0.2	14 ± 4
6	Ac-His-Arg-DPhe(p-Cl)-Tic-NH_2	140 ± 20	11	380 ± 50 (63%)	N/A	19 ± 6
7	Ac-His-Arg-(3,4-diCl)DPhe-Tic-NH_2	250 ± 40	19	> 100 µM	6.23 ± 0.06	70 ± 6
8	Ac-His-Arg-DPhe(p-F)-Tic-NH_2	450 ± 70	35	560 ± 60 (69%)	N/A	5 ± 4
9	Ac-His-Arg-DPhe(p-CN)-Tic-NH_2	4000 ± 1000	308	29% @ 100 µM	N/A	500 ± 200
10	Ac-His-Arg-DTyr-Tic-NH_2	4300 ± 800	331	> 100 µM	N/A	1200 ± 600

[a]Data summary from colorimetric assay that gives a signal proportional to increases in cAMP. Data was collected from three independently conducted experiments, each with duplicate replicates. NDP-MSH,Ac-His-DPhe-Arg-Trp-NH_2, and forskolin were all used as positive controls. Adenylate cyclase is activated by forskolin independently of the melanocortin system. Assay media was used as a negative control. Activity for partial agonists is recorded as an EC_{50} value followed by the percentage of NDP-MSH maximum activation of the receptor at 100 μM in parenthesis. Activity for less than full agonists is denoted as a percentage of NDP-MSH maximal activation of the receptor. Compounds with less than 20% activity compared to maximal percent activation of NDP-MSH are recorded as EC_{50} > 100000 nM. Antagonist activity was tested using NDP-MSH as an agonist and ligand concentrations of: 10000, 5000, 1000, and 500nM. Dose dependent shifts in EC_{50} enables the calculation of a pA_2 value determined by Schild analysis[55]. If no shift is detected in the antagonist experiment, the outcome is denoted by "N/A" meaning there is no activity observed up to 10uM. Fold values were calculated in comparison to the mMC3R potency of c-His-Arg-DPhe(p-I)-Tic-NH_2.

Table 3. Agonist Activity of DPhe Substitutions at the mMC1R[a]

Compound	Sequence	mMC1R Agonist EC_{50} + SEM (nM)
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Lys-Pro-Val-NH_2	0.012 ± 0.002
-	Ac-His-DPhe-Arg-Trp-NH_2	10 ± 4
-	Ac-His-Arg-DPhe(p-I)-Tic-NH_2	0.8 ± 0.3
1	Ac-His-Arg-(4,4')DBip-Tic-NH_2	0.52 ± 0.03
2	Ac-His-Arg-DPhe(p-tBu)-Tic-NH_2	10 ± 3
3	Ac-His-Arg-DPhe(p-Br)-Tic-NH_2	0.8 ± 0.3
4	Ac-His-Arg-DPhe(p-CF3)-Tic-NH_2	6 ± 2
5	Ac-His-Arg-DPhe(p-Me)-Tic-NH_2	1.2 ± 0.3
6	Ac-His-Arg-DPhe(p-Cl)-Tic-NH_2	0.95 ± 0.08
7	Ac-His-Arg-(3,4-diCl)DPhe-Tic-NH_2	6 ± 2
8	Ac-His-Arg-DPhe(p-F)-Tic-NH_2	2.2 ± 0.7
9	Ac-His-Arg-DPhe(p-CN)-Tic-NH_2	32 ± 3
10	Ac-His-Arg-DTyr-Tic-NH_2	50 ± 10

[a] Data summary from intracellular cAMP readings using AlphaScreen (PerkinElmer). Data was collected from three independently conducted experiments, each with duplicate replicates. NDP-MSH, Ac-His-DPhe-Arg-Trp-NH_2, and forskolin were all used as positive controls. Adenylate cyclase is activated by forskolin independently of the melanocortin system. Assay media was used as a negative control.

Table 4. Calculated Properties of Tetrapeptides

Entry ID	Substitution	MC3R Agonist Activity (EC_{50} nM)	Portential Energy-OPLS-2005 (KJ/mol)	Hydrogen Bond Donors	Hydrogen Bond Acceptors	Polarizability ($Å^3$)	$logP_{o/w}$	Polar Surface Area (Å)
KNS2224	pI	13	-862	7.75	14.25	64	-0.399	266
KNS2231	DBip	14	-875	7.75	14.25	69	0.505	253
KNS2233	ptBu	16	-924	7.75	14.25	72	0.665	267
KNS2223	pBr	85	-856	7.75	14.25	62	-0.223	252
KNS2236	pCF_3	90	-866	7.75	14.25	64	-0.649	266
KNS2237	pCH_3	110	-872	7.75	14.25	70	-0.355	269
KNS2221	pCl	140	-776	7.75	14.25	68	0.019	269
KNS2234	(3,4,)diCl	250	-829	7.75	14.25	67	-0.121	270
KNS2222	pF	450	-778	7.75	14.25	67	-0.205	273
KNS2238	pCN	4000	-872	7.75	15.75	65	-1.034	290
KNS2239	pOH	4300	-893	8.75	15	67	-1.235	291

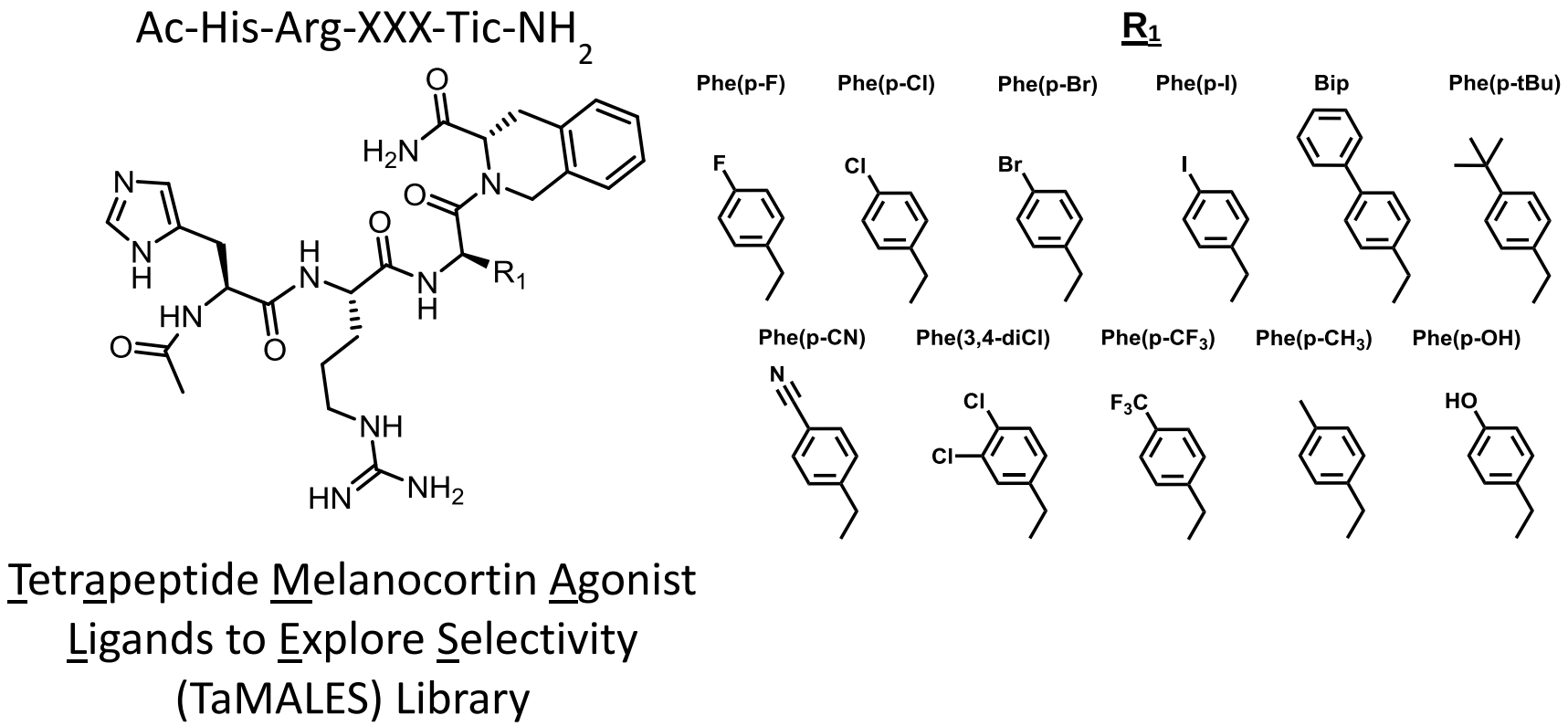

Figure 1. TaMALES library scaffold and the amino acid side chains used in the peptide library.

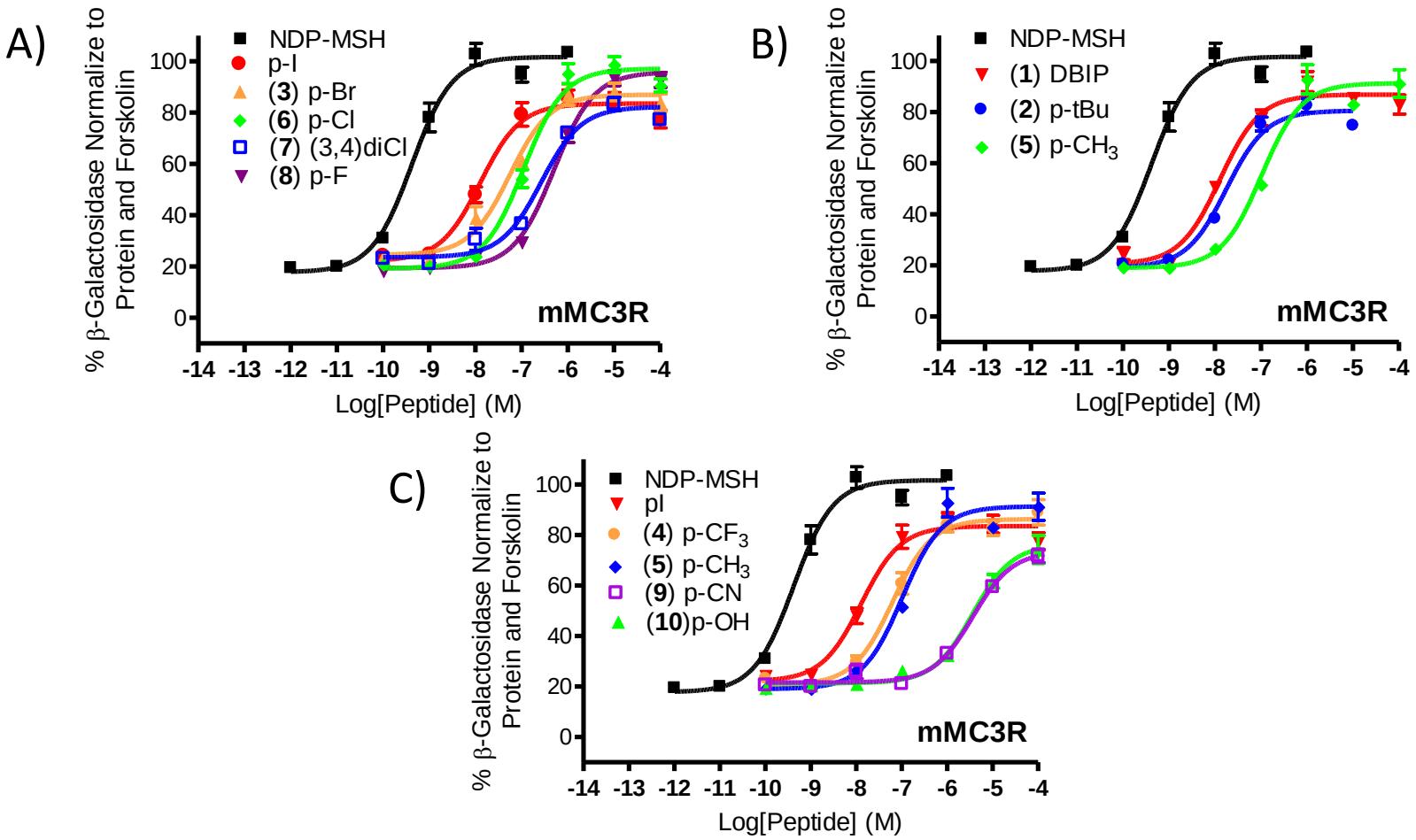

Figure 2. Illustration of agonist activity observed mouse melanocortin-3 receptor. A) Halogen series of substitutions with the Ac-His-Arg-(pI)DPhe-Tic-NH_2 control annotated as p-I. B) Hydrophobic series of substitutions. C) The Ac-His-Arg-(pI)DPhe-Tic-NH_2 control annotated as pI, the methyl substituent, p-CF_3 substituent, and the two substituents with micromolar potency (p-CN and p-OH substituent).

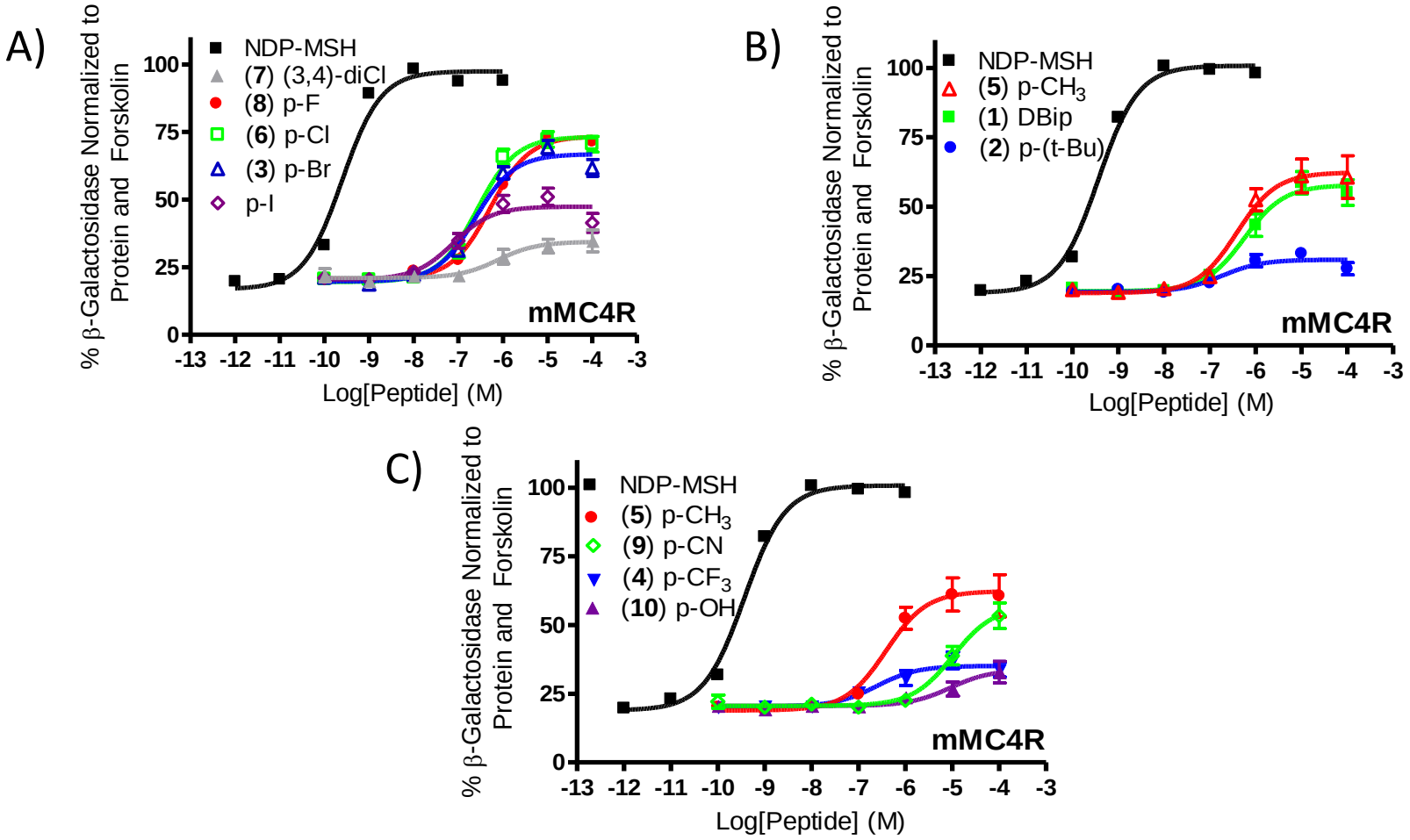

Figure 3. Illustration of agonist activity observed mouse melanocortin-4 receptor. A) Halogen series of substitutions with the Ac-His-Arg-(pI)DPhe-Tic-NH_2 control annotated as p-I. B) Hydrophobic series of substitutions. C) The methyl substituent, p-CF_3 substituent, and the two substituents with micromolar potency (p-CN and p-OH substituents).

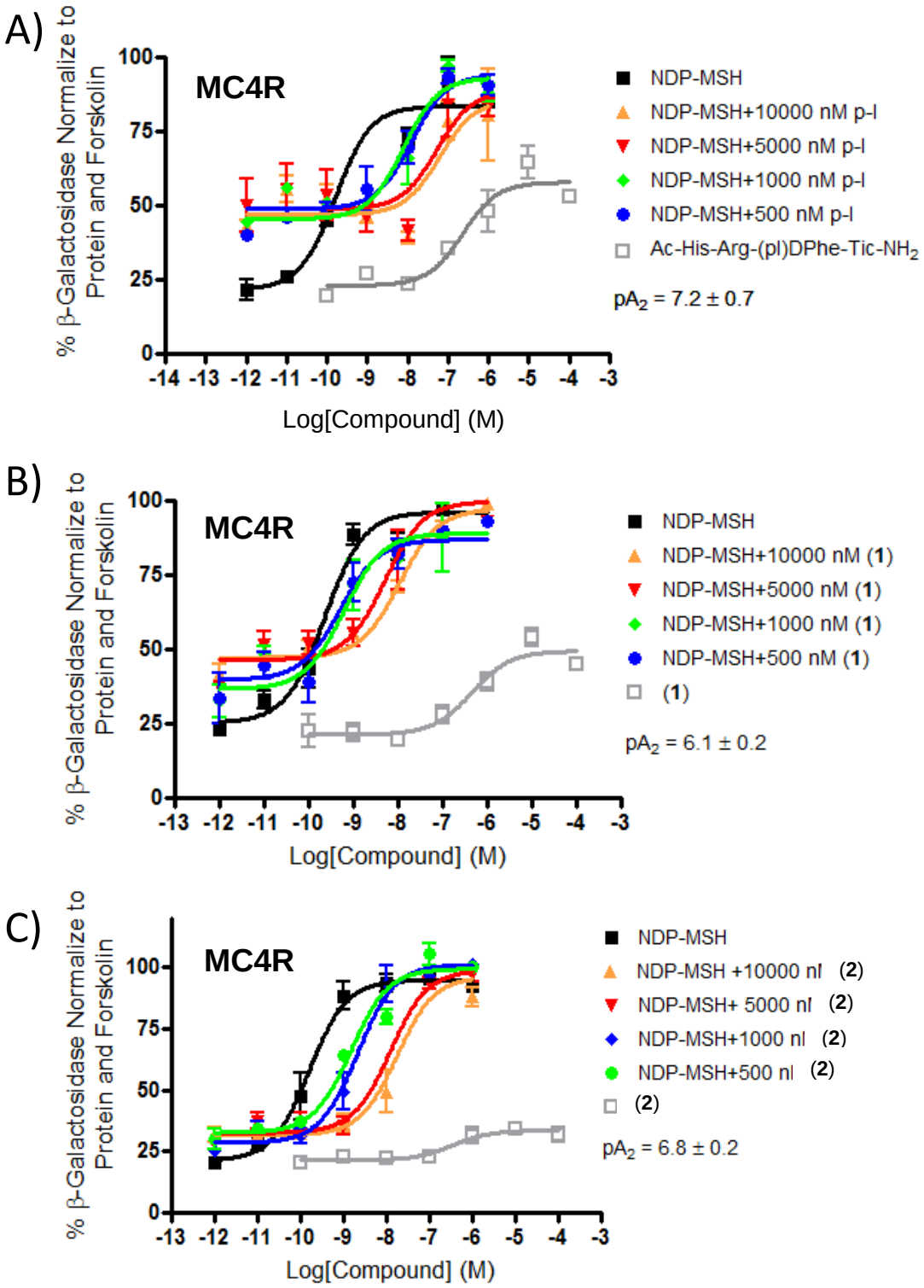

Figure 4. Illustration of antagonist activity observed at the mouse melanocortin-4 receptor from the most potent-MC3R agonists. A) Illustration of mMC4R antagonist data from the control peptide: Ac-His-Arg-(pI)DPhe-Tic-NH_2. B) Depiction of mMC4R antagonist data from Ac-His-Arg-DBip-Tic-NH_2. C) Depiction of mMC4R antagonist data from Ac-His-Arg-(p-tBu)DPhe-Tic-NH_2. The pA_2 values are calculated using Schild analysis. The $pA_2 = -Log(K_i)$.

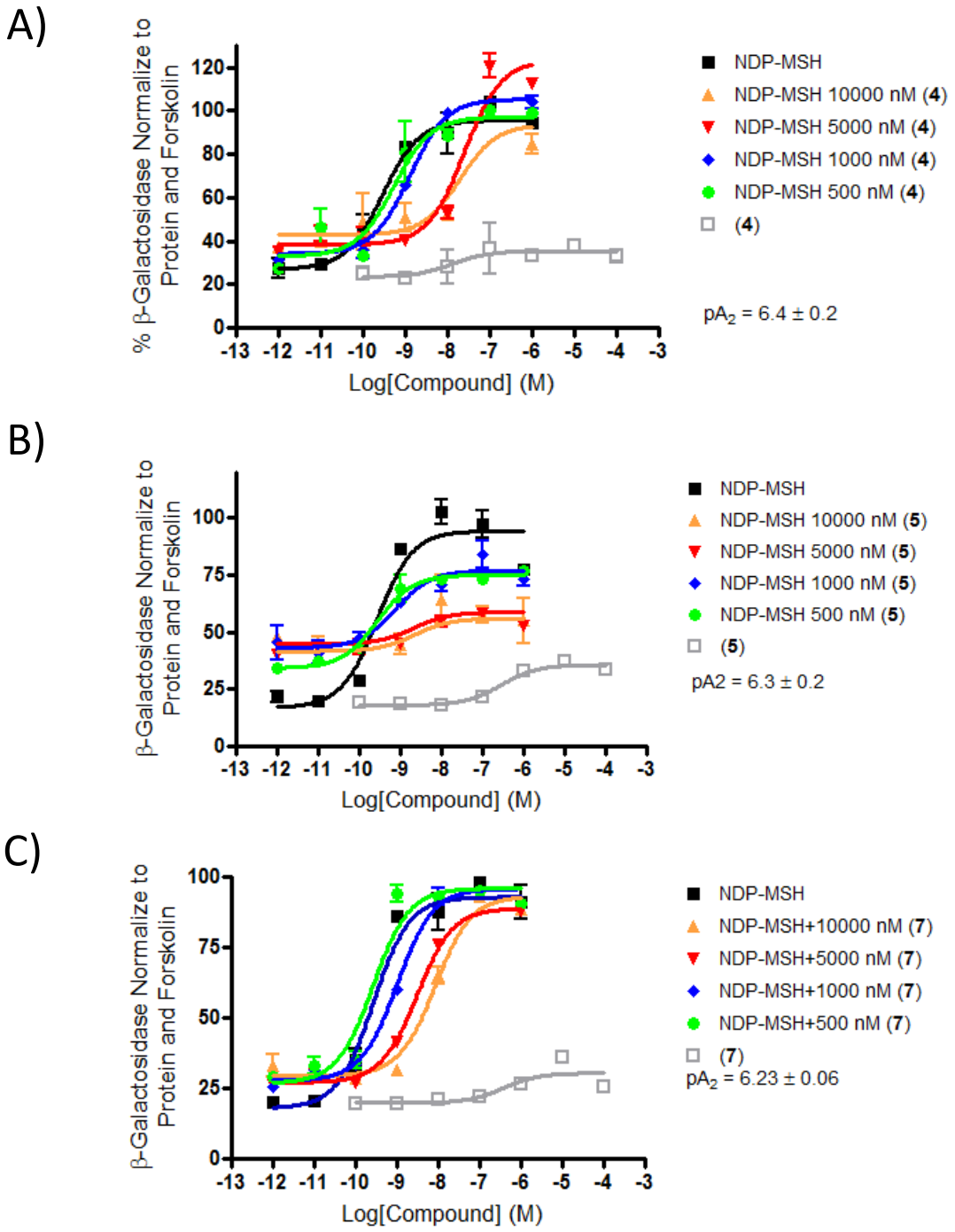

Figure 5. Illustration of antagonist activity observed at the mouse melanocortin-4 receptor.A) Illustration of mMC4R antagonist data from the control peptide: Ac-His-Arg-(pCF$_3$)DPhe-Tic-NH$_2$. . B) Depiction of mMC4R antagonist data from Ac-His-Arg-(pCH$_3$)DPhe-Tic-NH$_2$. C) Depiction of mMC4R antagonist data from Ac-His-Arg-(3,4)-dicl)DPhe-Tic-NH$_2$.. The pA$_2$ values are calculated using Schild analysis. The pA$_2$ = -Log(K$_i$).

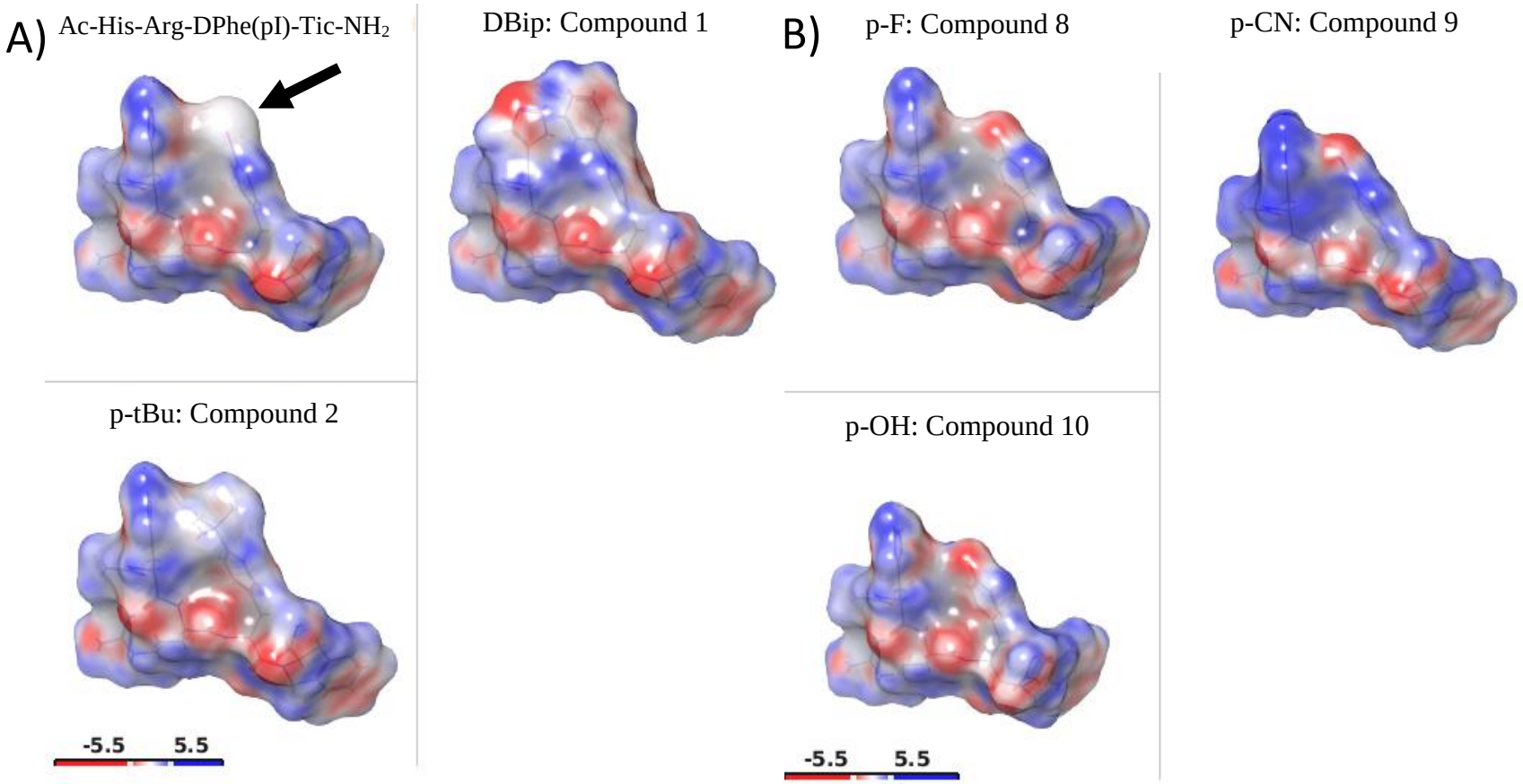

Figure 6. Electrostatic potentials calculated with Maestro. Red represents electronegative (-5.5 kT/e), blue represents electropositive (+5.5 kT/e). The molecule is oriented with the histidine residue facing upwards on the left hand side, the arginine is in the bottom left corner, the DPhenylalanine substitutions are facing upward on the right hand side and the Tic residue is in the bottom right corner. The DPhenylalanine substitutions is circled in black on the first compound. The upper **A**) Low nanomolar potency mMC3R compounds including: compound **1, 2** and the control para iodo compound. **B**) Micromolar potency mMC3R compounds including: compound **8, 9,** and **10.**

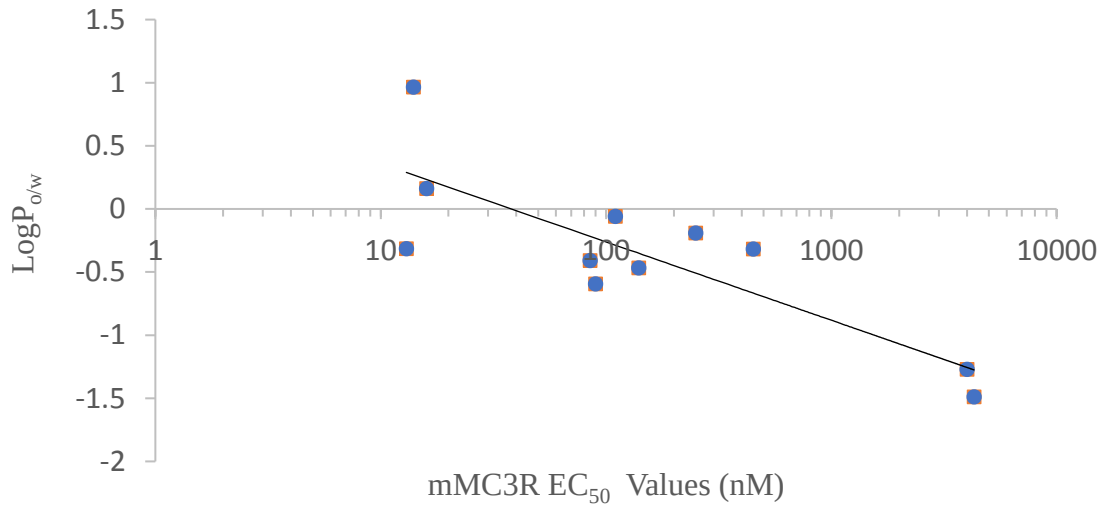

Figure 7. A scatterplot showing the potential trend between partition coefficient of octenol over water and mMC3R EC_{50} values. The x-axis is on a logarithmic scale. $LogP_{o/w}$ values were calculated in Maestro. There is a slight trend when $logP_{o/w}$ values go down the EC_{50} also decreases. The trend is not statistically significant, when it is fit with logarithmic regression line the R^2 value is 0.6866.

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