Peptide and Small Molecules Rescue the Functional Activity and Agonist Potency of Dysfunctional Human Melanocortin-4 Receptor Polymorphisms[†,‡]

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ABSTRACT: The melanocortin pathway, specifically the melanocortin-4 receptor and the cognate endogenous agonist and antagonist ligands, have been strongly implicated in the regulation of energy homeostasis and satiety. Genetic studies of morbidly obese human patients and normal weight control patients have resulted in the discovery of over 70 human melanocortin-4 receptor (MC4R) polymorphisms observed as both heterozygous and homozygous forms. A number of laboratories have been studying these hMC4R polymorphisms attempting to understand the molecular mechanism(s) that might explain the obese human phenotype. Herein, we have studied 13 polymorphic hMC4Rs that have been identified to possess statistically significant decreased endogenous agonist potency with synthetic peptides and small molecules attempting to identify ligands that can pharmacologically rescue the hMC4R polymorphic agonist response. The ligands examined in this study include NDP-MSH, MTII, Ac-His-DPhe-Arg-Trp-NH_2 (JRH887-9), Ac-Anc-DPhe-Arg-Trp-NH_2 (amino-2-naphtylcarboxylic acid, Anc, JRH420-12), Ac-His-(pI)DPhe-Arg-Trp-NH_2 (JRH322-18), chimeric AGRP-melanocortin based ligands (Tyr-c[Cys-His-DPhe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH_2, AMW3-130 and Ac-mini-(His-DPhe-Arg-Trp)-hAGRP-NH_2, AMW3-106), and the small molecules JB25 and THIQ. The hMC4R polymorphisms included in this study are S58C, N97D, I102S, L106P, S127L, T150I, R165Q, R165W, L250Q, G252S, C271Y, Y287Stop, and I301T. These studies resulted in the NDP-MSH, MTII, AMW3-130, THIQ, and AMW3-106 ligands possessing nanomolar to subnanomolar agonist potency at the hMC4R polymorphisms examined in this study. Thus, these ligands could generically rescue the potency and stimulatory response of the abnormally functioning hMC4Rs studied and may provide tools to further clarify the molecular mechanism(s) involving these receptor modifications.

The melanocortin system has been studied since the 1950s for the ability to induce pigmentation changes (*1*, *2*). Although the endogenous melanocortin agonists were structurally identified as the first components of the system, the five isoforms of the G-protein coupled melanocortin receptors (MC1-5Rs) and the endogenous antagonists agouti and agouti-related protein (AGRP[1]) amino acid sequences were identified in the 1990s (*3*−*11*). It was also during this decade that the MC4R was demonstrated to be involved in obesity and satiety (*12*), the agouti and AGRP proteins were shown to be competitive antagonists of the melanocortin receptors (*11*, *13*), and the central administration of melanocortin agonists and antagonists were shown to regulate rodent feeding behavior (*14*). Subsequently, the identification of human MC4R polymorphisms (*15*−*17*), genetic disruption of the POMC gene, from which the endogenous agonists are derived (*18*, *19*), and polymorphisms of the endogenous antagonist AGRP gene (*20*) were reported. These data, ranging from *in vitro* pharmacology, rodent model studies, to human patient genetic data and phenotypes support the dogma that the melanocortin system is involved in energy homeostasis.

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[1] Abbreviations: POMC, pro-opiomelanocortin; GPCR, G-Protein coupled receptor; cAMP, cyclic adenosine monophosohate; AGRP, Agouti-related protein; MSH, melanocyte stimulating hormone; ACTH, adrenocorticotropin hormone; TM, transmembrane domain; hMC4R, human melanocortin-4-receptor; NDP-MSH, 4-norleucine-7-D-phenylalanine; MTII, Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2; hAGRP, human Agouti-related protein; HEK-293, human embryonic kidney-293; CRE, cyclic adenosine monophosohate response element; DMEM, Dulbecco's modified Eagle's medium; ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

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Greater then 70 hMC4R polymorphisms have been identified in human patients, and a number of studies have been undertaken to examine these polymorphic proteins to identify possible molecular mechanisms that might result in the deregulation of human body weight. Several hypotheses have been supported by experimental data including the inability of the hMC4 polymorphic receptor to respond normally to the endogenous melanocortin agonists and AGRP antagonist, abnormal cell surface expression, modified inverse agonist AGRP function, improper protein folding, and/or truncated and non-functional hMC4R proteins. The next step, after identification of the particular molecular mechanism associated with each specific hMC4R polymorphism, is to identify and design ligands or proteins that may rescue the phenotype for the particular molecular mechanistic defect. The study presented herein attempts to identify melanocortin agonist ligands that might pharmacologically rescue hMC4R polymorphic receptors that have impaired endogenous agonist−ligand functional response. Synthetic ligands ranging from the classical NDP-MSH and MTII peptides (*21*, *22*), the tetrapeptides Ac-His-DPhe-Arg-Trp-NH_2 (JRH887-9), Ac-Anc-DPhe-Arg-Trp-NH_2 (amino-2-naphtylcarboxylic acid, Anc, JRH420-12) (*23*), Ac-His-(pI)DPhe-Arg-Trp-NH_2 (JRH322-18) (*24*), and the chimeric AGRP-melanocortin based agonist ligands Tyr-c[Cys-His-DPhe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH_2 (AMW3-130) and Ac-mini-(His-DPhe-Arg-Trp)-hAGRP-NH_2 (AMW3-106) (*25*) to the small molecule MC4R agonists JB25 (*26*) and THIQ (*27*) have been included in this study. (The rationale for ligand selection is discussed in detail for each of the ligand classes in the Results section.) On the basis of our previous results of *in vitro* pharmacological characterization of polymorphic hMC4Rs (*28*), we selected the S58C, N97D, I102S, L106P, S127L, T150I, R165Q, R165W, L250Q, G252S, C271Y, Y287Stop, and I301T hMC4Rs as targets for this study. Additionally, we performed three-dimensional homology studies of the ligands docked into the wild type hMC4R in an attempt to identify any putative ligand−receptor interactions that might complement the ligand−hMC4R polymorphic *in vitro* agonist pharmacology.

MATERIALS AND METHODS

Peptides. The peptides used in this study that were synthesized as previously reported include Ac-His-DPhe-Arg-Trp-NH_2 (JRH887-9) (*23*, *24*), Ac-Anc-DPhe-Arg-Trp-NH_2 (amino-2-naphtylcarboxylic acid, Anc, JRH420-12) (*23*), Ac-His-(pI)DPhe-Arg-Trp-NH_2(JRH322-18) (*24*), and AMW3-106 (*25*). The endogenous melanocortin peptides and synthetic agonist peptides that were purchased from commercial sources include α-MSH, NDP-MSH, MTII, ACTH-(1-24), and β-MSH, γ_2-MSH (Bachem, Torrance, CA). The small molecule JB25 was obtained through a material transfer agreement with Professor Morton Meldal (Carlsberg Laboratory, Department of Chemistry, Gamle Carlsberg Vej 10, DK-2500 Valby, Denmark) (*26*). The THIQ small molecule was obtained through a material transfer agreement with Dr. Lex Van Der Ploeg at Merck Research Laboratories (*27*).

The chimeric hAGRP-melanocortin peptide AMW3-130 was synthesized manually using standard *N*α 9-fluorenylmethoxycarbonyl (Fmoc) methodology (*29*, *30*). All amino acids and reagents were purchased from commercial sources. The Nα-Fmoc protected amino acids Cys(Trt), Tyr(tBu), Arg(Pbf), His(Trt), DPhe, Phe, Asn(Trt), Trp(Boc), and Ala were utilized. Benzotriazol-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP) and 1-hydroxybenzotriazole (HOBt) were used as coupling reagents. Dichloromethane (DCM), glacial acetic acid, methanol, acetonitrile, anhydrous ethyl ether, *N*,*N*-dimethylformamide (DMF), trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), piperidine, phenol, *N*,*N*-diisopropylethylamine (DIEA), triisopropylsilane (TIS), and 1,2-ethanedithiol (EDT) were used as reagents or solvents in the syntheses. All reagents and chemicals were of ACS grade or better and were used without further purification.

The AMW3-130 peptide was assembled on 9-fluorenylmethoxycarbonyl-Rink Amide Resin (0.40 meq/g substitution) purchased from Peptides International (Louisville, KY). The synthesis (0.26 mmol scale) was performed using a manual synthesis reaction vessel. Each synthetic cycle consisted of the following steps: (i) the removal of the Nα-Fmoc group by 20% piperidine in DMF (1 × 2min, 1 × 20 min) and (ii) a single 2 h coupling of Fmoc-amino acid (3 equiv) using BOP (3 equiv), HOBt (3 equiv) and DIEA (6 equiv) in DMF that was repeated until the peptide synthesis was complete. The presence or absence of the Nα free amino group was monitored using the Kaiser test (*31*). After synthesis was completed, the peptides were cleaved from the resin and deprotected using a cleavage cocktail consisting of 82.5% TFA, 5% H_2O, 5% EDT, 5% phenol, and 2.5% TIS for 3 h at room temperature. After cleavage and side chain deprotection, the solution was concentrated, and the peptide was precipitated and washed using cold (4 °C) anhydrous diethyl ether. The crude linear peptide was purified by reversed-phase HPLC using a Shimadzu chromatography system with a photodiode array detector and a semipreparative RP-HPLC C18 bonded silica column (Vydac 218TP1010, 1.0 × 25 cm). The purified linear peptide was oxidized to the disulfide form by reaction with 5% DMSO in H_2O. The peptide was dissolved at a concentration of 0.37 mg/mL, and the solution was allowed to react at 20 °C. The oxidation process was monitored using analytical RP-HPLC for the disappearance of the linear peptide and formation of the cyclized product. The purified peptides were at least >98% pure as determined by RP-HPLC in two diverse solvent systems and had the correct molecular mass of 1508.0 (University of Florida Protein Core Facility). HPLC $k' =$ [(peptide retention time − solvent retention time)/solvent retention time] in solvent system 1 ($k' = 5.7$ in 10% acetonitrile in 0.1% trifluoroacetic acid/water and a gradient to 90% acetonitrile over 35 min) or solvent system 2 ($k' =$ 9.9 in 10% methanol in 0.1% trifluoroacetic acid/water and a gradient to 90% methanol over 35 min). An analytical Vydac C_{18} column (Vydac 218TP104) was used with a flow rate of 1.5 mL/min. The >95% peptide purity is determined by HPLC at a wavelength of 214 λ.

hMC4R in Vitro Receptor Mutagenesis. The human wild type N-terminally Flag tagged MC4R cDNA was generously provided by Dr. Robert Mackenzie (*32*) and was subcloned into the pBluescript plasmid (Stratagene) for subsequent mutagenesis. Mutant hMC4Rs were generated using a polymerase chain reaction (PCR) based mutagenesis strategy, as previously described by our laboratory (*28*). A complementary set of PCR primers were designed containing the reported nucleotide base pair changes resulting in the

modified amino acid. After completion of the PCR reaction (95 °C 30 s, 12 cycles of 95 °C 30 s, 55 °C 1 min, 68 °C 9 min), the product was purified (Qiaquick PCR reaction, Qiagen) and eluted in water. Subsequently, the sample was cut with *Dpn*1 (Biolabs) to lineralize the wild type template DNA, leaving only nicked circularized mutant DNA. This was transformed into competent DH5α *E. coli*. Single colonies were selected and the presence of the desired mutant was checked by DNA sequencing. The DNA containing the mutant was then excised and subcloned into the *Hind*III/*Xba*I restriction sites of the $pCDNA_3$ expression vector (Invitrogen). Complete mutant hMC4R sequences were confirmed free of PCR nucleotide base errors by DNA sequencing (University of Florida Sequencing Core Facilities).

Generation of Stable Cell Lines. HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and seeded 1 day prior to transfection at $(1-2) \times 10^6$ cell/100-mm dish. Mutant and wild type DNA in $pCDNA_3$ expression vector (20 μg) were transfected using the calcium phosphate method (*33*). Stable receptor populations were generated using G418 selection (0.7−1 mg/mL) for subsequent bioassay analysis.

cAMP Based Functional Bioassay. HEK-293 cells stably expressing the polymorphic and wild type melanocortin receptors were transfected with 4 μg of CRE/β-galactosidase reporter gene as previously described (*28*, *34*, *35*). Briefly, 5,000 to 15,000 post-transfection cells were plated into 96-well Primera plates (Falcon) and incubated overnight. Forty-eight hours post-transfection, the cells were stimulated with 100 μL of peptide ($10^{-4}-10^{-12}$ M) or forskolin (10^{-4} M) control in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 h. The assay media was aspirated, and 50 μL of lysis buffer (250 mM Tris-HCl at pH 8.0 and 0.1% Triton X-100) was added. The plates were stored at −80 °C overnight. The plates containing the cell lysates were thawed the following day. Aliquots of 10 μL were taken from each well and transferred to another 96-well plate for relative protein determination. To the cell lysate plates, 40 μL of phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μL of substrate buffer (60 mM sodium phosphate, 1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, and 2 mg/mL ONPG) was added to each well, and the plates were incubated at 37 °C. The sample absorbance, OD_{405}, was measured using a 96-well plate reader (Molecular Devices). The relative protein was determined by adding 200 μL of 1:5 dilution Bio Rad G250 protein dye/water to the 10 μL cell lysate sample taken previously, and the OD_{595} was measured on a 96-well plate reader (Molecular Devices). Data points were normalized both to the relative protein content and nonreceptor dependent forskolin stimulation. For all of the ligands that possessed full agonist activity, such as NDP-MSH, MTII, and others, the maximum stimulation values at the highest concentrations were identical to the forskolin control values that indicate the maximal signal obtainable for the stable cell line. Ligands that are reported as partial agonists were determined relative to the forskolin control values, as were the ligand percent stimulation at 100 μM concentration values indicated in Table 1.

Data Analysis. Agonist EC_{50} values represent the mean of duplicate experiments performed in triplicate or more independent experiments. EC_{50} estimates and their associated standard errors of the mean were determined by fitting the data to a nonlinear least-squares analysis using the PRISM program (v4.0, GraphPad, Inc.). Statistical analysis was performed using Student's *t*-test compared to the wild type receptor values, with statistical significance defined as $p < 0.05$.

Receptor−Ligand Homology Molecular Modeling. The three-dimensional (3D) homology molecular models of human MC4R complexed with the NDP-MSH or THIQ ligands have been developed using distance geometry calculations based on the rhodopsin crystal structure (*36*) and experimentally derived distance constraints (*37*). The coordinates of these models have been deposited into the Protein Data Bank, as 2iqr and 2iqu files, respectively. Herein, we utilized a similar strategy to calculate the complexes of polymorphic hMC4Rs with the agonists studied Table 1. All these agonists were included into distance geometry calculations with DIANA (*38*) together with the receptor, hMC4R. The following distance constraints were used to generate the models presented in this study: (1) those corresponding to the receptor in the active conformation; (2) Cβ−Cβ distances between the pharmacophore tetrapeptide (His^6-$DPhe^7$-Arg^8-Trp^9) and 30 contact residues from the receptor binding pocket (at positions 44, 46, 100, 103, 122, 125, 126, 129, 130, 133, 184, 188, 189, 190, 193, 195, 197, 200, 261, 264, 265, 268, 276, 278, 280, 281, 284, 285, 288, and 292), identified as important from the previously calculated hMC4R−NDP-MSH complex; (3) internal constraints for cyclic peptides, such as the lactam bridge of MTII, the disulfide bridge in AMW3-130, and the disulfide bridges and other restraints between core residues in AMW3-106. Side chain conformers of peptide ligands were chosen to reproduce their orientation in NDP-MSH, except for the His of AGRP-derived peptides, which was unrestrained. The iterative distance geometry refinement positioned the peptides in the binding pocket in a manner similar to the position of the NDP-MSH, while removing steric overlaps and maximizing the number of H-bonds between the receptor and its ligands. Final energy minimization (50 steps) of all receptor−agonist complexes was performed with the CHARMm module of QUANTA using $\epsilon = 10$ and the adopted basis Newton−Raphson method (50 steps).

RESULTS

The 13 human MC4R polymorphisms (Figure 1) that did not respond normally, as compared to the wild type MC4R, to the endogenous POMC derived melanocortin agonists were pharmacologically tested with structurally different agonist ligands in attempts to identify a ligand structural class that might be utilized as a template for the design of polymorphic hMC4R rescue ligands. Table 1 summarizes the results of the agonist ligands pharmacologically characterized at the wild type and 13 polymorphic hMC4 receptors. On the basis of these data, we divided our results into ligands that possessed (i) essentially equipotent agonist EC_{50} values at the polymorphic and wild type hMC4Rs, which we consider to conservatively fall within the 1- to 5-fold decreased potency range, (ii) ligands that possessed 6- to 10-fold decreased potency, (iii) ligands that possessed 11- to 40-fold decreased potency, (iv) ligands that possessed 41- to 100-fold decreased potency, (v) ligands that possessed

Table 1: Summary of the Endogenous Melanocortin Agonist and Synthetic Agonist Ligand Pharmacology at the Polymorphic hMC4Rs

	WT hMC4R EC_{50} (nM)	S58C EC_{50} (nM)	N97D EC_{50} (nM)	I102S EC_{50} (nM)	L106P EC_{50} (nM)	S127L EC_{50} (nM)	T150I EC_{50} (nM)
				endogenous agonists			
α-MSH[c]	0.65 ± 0.19	15.8 ± 6.05	>100	260 ± 58	50%@100μM	5.49 ± 0.80	8.14 ± 3.2
β-MSH[c]	0.42 ± 0.13	15.3 ± 6.60	660 ± 47	150 ± 52	356 ± 53	2.22 ± 0.88	6.53 ± 2.81
γ_2-MSH[c]	73 ± 24	2360 ± 960	>100	3620 ± 1022	2660 ± 370	930 ± 170	1300 ± 760
ACTH(1-24)[c]	0.65 ± 0.15	24.4 ± 6.63	>100	480 ± 89	40%@100μM	11.4 ± 5.37	14.0 ± 4.87
				synthetic agonists			
NDP-MSH[c]	0.030 ± 0.0096	0.13 ± 0.023	1.31 ± 0.09	0.66 ± 0.21	0.42 ± 0.08	0.028 ± 0.003	0.25 ± 0.06
MTII[c]	0.027 ± 0.011	0.045 ± 0.008	2.30 ± 0.18	0.43 ± 0.07	0.92 ± 0.13	0.027 ± 0.006	0.15 ± 0.02
JRH887-9	0.93 ± 0.31	42.0 ± 3.2	5760 ± 1620	720 ± 28	91 ± 15	58.1 ± 15.3	45.6 ± 13.5
JRH420-12	2.17 ± 0.82	32.2 ± 4.10	300 ± 4.40	50 ± 12	60.6 ± 12.3	21.5 ± 5.20	41.4 ± 8.20
JRH322-18	1.25 ± 0.25	36.0 ± 17.0	80%@100μM	420 ± 114	52.6 ± 10.7	7.67 ± 1.57	13250 ± 2200
AMW3-130	0.21 ± 0.08	0.53 ± 0.22	4.02 ± 0.38	0.44 ± 0.05	0.62 ± 0.14	0.40 ± 0.12	0.90 ± 0.20
THIQ	0.23 ± 0.09	2.30 ± 0.38	43.6 ± 9.40	4.50 ± 0.95	2.90 ± 0.44	0.65 ± 0.20	2.86 ± 0.02
JB25	640 ± 170	2700 ± 860	>100	29800 ± 13750	3700 ± 420	1100 ± 670	5900 ± 2900
AMW3-106	0.45 ± 0.10	3.50 ± 0.22	820 ± 460	35.5 ± 7.54	10.0 ± 1.42	1.35 ± 0.39	4.80 ± 1.15
	R165Q EC_{50} (nM)	R165W EC_{50} (nM)	L250Q[a] EC_{50} (nM)	G252S EC_{50} (nM)	C271Y EC_{50} (nM)	Y287Stop[b] EC_{50} (nM)	I301T EC_{50} (nM)
				endogenous agonists			
α-MSH[c]	18.7 ± 10.8	21.5 ± 5.54	4.32 ± 1.10	6.11 ± 1.75	48%@100μM	partial agonist 65 ± 16	8.42 ± 1.64
β-MSH[c]	6.31 ± 2.94	7.91 ± 2.34	3.55 ± 1.67	0.53 ± 0.12	154 ± 12	partial agonist 17 ± 3.4	2.44 ± 0.44
γ_2-MSH[c]	1070 ± 330	1140 ± 350	380 ± 200	409 ± 50	58%@100μM	partial agonist 2490 ± 160	370 ± 100
ACTH(1-24)[c]	39 ± 6.2	58 ± 19	3.63 ± 0.82	10.9 ± 3.89	42%@100μM	partial agonist 150 ± 12	16 ± 2.6
				synthetic agonists			
NDP-MSH[c]	0.13 ± 0.02	0.23 ± 0.05	0.25 ± 0.07	0.081 ± 0.06	1.74 ± 0.23	partial agonist 0.80 ± 0.22	0.062 ± 0.003
MTII[c]	0.030 ± 0.003	0.15 ± 0.03	0.12 ± 0.04	0.040 ± 0.011	0.60 ± 0.11	partial agonist 0.61 ± 0.16	0.028 ± 0.004
JRH887-9	18.3 ± 3.20	26.6 ± 2.70	1.60 ± 0.31	3.90 ± 0.19	1200 ± 110	partial agonist 250 ± 70	3.40 ± 0.11
JRH420-12	14.5 ± 4.70	18.3 ± 3.00	7.20 ± 4.40	6.70 ± 0.86	790 ± 210	partial agonist 136 ± 63	11.6 ± 5.70
JRH322-18	23.0 ± 14.6	93.1 ± 32.3	8.50 ± 4.90	7.60 ± 3.90	>100	>100	6.30 ± 0.94
AMW3−130	0.094 ± 0.005	0.25 ± 0.021	0.24 ± 0.07	0.13 ± 0.02	0.80 ± 0.32	partial agonist 0.53 ± 0.08	0.13 ± 0.02
THIQ	2.60 ± 1.10	3.14 ± 0.66	1.44 ± 0.75	0.66 ± 0.19	39.7 ± 15.4	partial agonist 33.2 ± 13.0	1.10 ± 0.47
JB25	910 ± 290	5700 ± 2700	1340 ± 500	520 ± 160	75%@100μM	partial agonist 30800 ± 8580	840 ± 280
AMW3-106	3.95 ± 0.35	3.50 ± 0.42	0.97 ± 0.43	1.04 ± 0.43	45.5 ± 16.7	partial agonist 19.6 ± 1.30	2.70 ± 0.65

[a] The L250Q hMC4R is constitutively active. [b] The Y287Stop hMC4R is only able to elicit a partial agonist response. The values indicated represent the mean of at least three independent experiments with the standard error of the mean indicated; >100 indicates that an EC_{50} value was not reportable at up to 100 μM ligand concentration. A percentage value indicates that some stimulatory agonist pharmacology resulted at up to 100 μM concentration. A percentage value indicates that some stimulatory agonist pharmacology resulted at up to 100 μM concentrations, but the maximal stimulation levels were less than the control level. [c] Indicates data previously published in ref *28* but included herein for comparative purposes.

α-MSH Ac-Ser-Tyr-Ser-Met-Glu-**His-Phe-Arg-Trp**-Gly-Lys-Pro-Val-NH_2

β-MSH Ala-Glu-Lys-Lys-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-**His-Phe-Arg-Trp**-Gly-Ser-Pro-Pro-Lys-Asp

γ_2-MSH Tyr-Val-Met-Gly-**His-Phe-Arg-Trp**-Asp-Arg-Phe-Gly

ACTH(1-24) Ser-Tyr-Ser-Met-Glu-**His-Phe-Arg-Trp**-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Tyr-Pro-Asn

NDP-MSH Ac-Ser-Tyr-Ser-Nle-Glu-**His-DPhe-Arg-Trp**-Gly-Lys-Pro-Val-NH_2

MTII Ac-Nle-c[Asp-**His-DPhe-Arg-Trp**-Lys]-NH_2

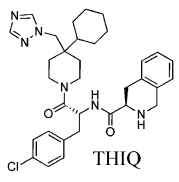
JRH887-9 Ac-**His-DPhe-Arg-Trp**-NH_2

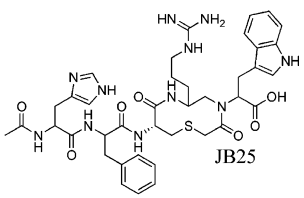
JRH420-12 Ac-**Anc-DPhe-Arg-Trp**-NH_2

JRH322-18 Ac-**His-(pI)DPhe-Arg-Trp**-NH_2

AMW3-130 Tyr-c[Cys-**His-DPhe-Arg-Trp**-Asn-Ala-Phe-Cys]-Tyr-NH_2

AMW3-106 Ac-CVRLHESCLGQQVPCCDPAATCYC-**His-DPhe- Arg-Trp**-NAFCYCR-NH_2





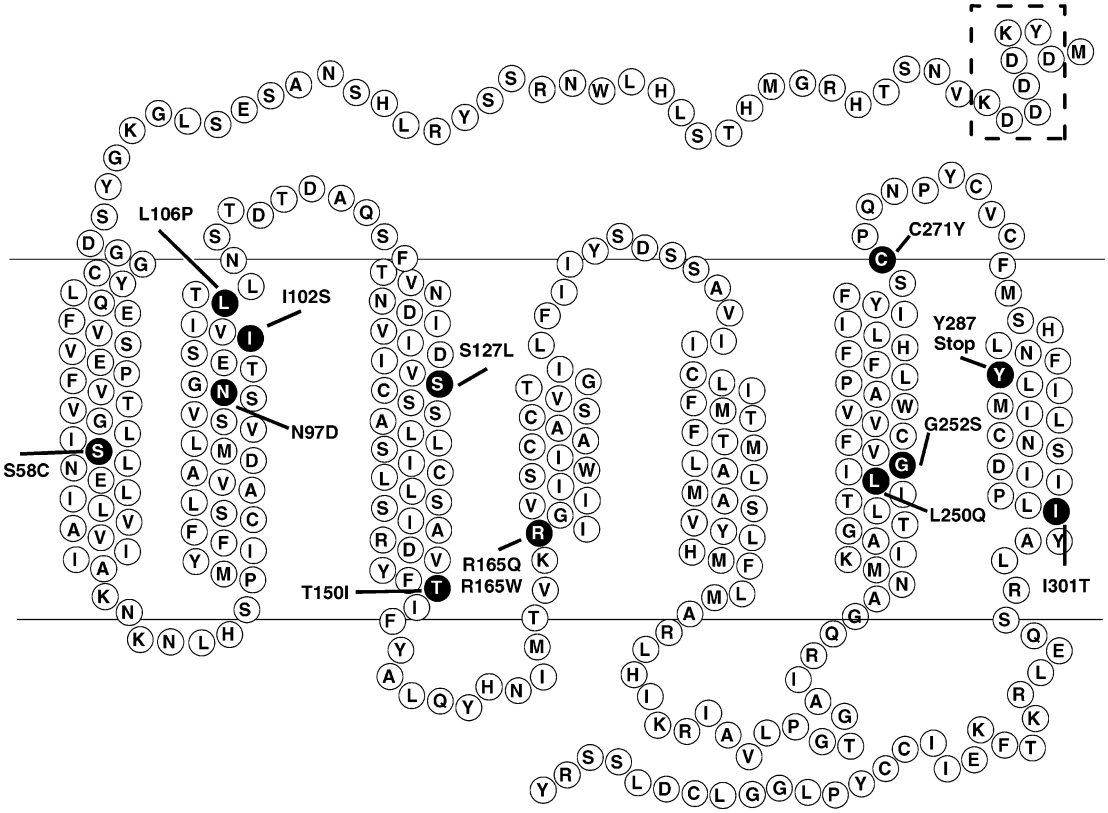


FIGURE 1: Schematic representation of the hMC4R polymorphism amino acids examined in this study. The dashed box indicates the N-terminal FLAG sequence used for antibody recognition and receptor cellular localization determination. The normal amino acid is indicated in a black circle with white text, and the hMC4R polymorphism is indicated in the text adjacent to the receptor residue.

101- to 1000-fold decreased potency, (vi) ligands that possessed >1000-fold decreased potency, (vii) ligands that were only able to slightly stimulate the polymorphic hMC4R at up to 100 μM concentrations and (viii) ligands that were unable to simulate any agonist response at up to 100 μM concentrations.

Endogenous Agonists. The POMC gene derived endogenous melanocortin agonist ligands (*39*, *40*) that were examined in this study include α-MSH, β-MSH, γ_2-MSH, and ACTH(1−24), Table 1. Human MC4R polymorphisms have been previously reported by several laboratories since the seminal publications in 1998 and 1999 (*15*−*17*). Initial characterization of these human polymorphisms were performed using typically α-MSH, the synthetic NDP-MSH agonist ligand (*21*), or both. Because it might be envisioned that one or more of the multiple endogenous melanocortin receptor agonists might respond differently to the different polymorphic hMC4Rs, our laboratory performed a side-by-side characterization of several endogenous agonists and the endogenous antagonist AGRP C-terminal fragment ligand (*28*). These data resulted in the observation that the 13 hMC4R polymorphisms in this study possessed statistically significant differences of endogenous melanocortin agonist potency of one or more of these endogenous ligands and were selected for the study herein to see if we could rescue the functional ability of the hMC4Rs (*28*). Table 1 summarizes the endogenous agonist ligand potency at the wild type and polymorphic hMC4Rs examined in this study. This data has been previously published and has been included herein for comparative purposes (*28*). The α-MSH ligand possessed 6- to 10-fold decreased agonist potency at the S127L, L250Q, and G252S hMC4R, 11- to 40-fold decreased potency at the S58C, T150I, R165Q, R165W, and I301T hMC4Rs, 100-fold decreased agonist potency at the Y287Stop hMC4R, and 400-fold decreased potency at the I102S hMC4R compared to that of the wild type hMC4R. α-MSH was only able to partially simulate the L106P and C271Y hMC4Rs but not the N97D hMC4R at up to 100 μM concentrations. The β-MSH agonist ligand possessed essentially equipotent activity at the S127L, L250Q, and G252S hMC4Rs but was 6- to 10-fold less potent at the T150I, R165Q, R165W, and I301T hMC4Rs, 11- to 40-fold less potent at the S58C and Y287Stop hMC4Rs, and 100- to 1000-fold less potent at the N97D, I102S, L106P, and C271Y hMC4Rs, as compared to the wild type hMC4R. The γ_2-MSH agonist was ca. equipotent at the L250Q and I301T hMC4Rs, as compared to the normal hMC4R sequence, 6-fold less potent at the G252S hMC4R, 10- to 40-fold less potent at the S58C, L106P, S127L, T150I, R165Q, R165W, and Y287Stop hMC4Rs, and 140-fold less potent at the I102S. At up to 100 μM concentrations, γ_2-MSH was only able to partially stimulate the C271Y hMC4R and was unable to stimulate the N97D hMC4R. The ACTH(1-24) ligand possessed 6-fold decreased potency at the L250Q hMC4R, 10- to 40-fold decreased potency at the S58C, S127L, T150I, G252S, and I301T hMC4Rs, and 41- to 100-fold decreased potency at the R165Q and R165W hMC4Rs relative to that of the wild type hMC4R. γ_2-MSH, at up to 100 μM concentrations, was only able to stimulate the L106P hMC4R ca. 40% maximal stimulation and only ca. 42% maximal simulation at the C271Y hMC4R. The N97D hMC4R was not stimulated by ACTH(1-24) at up to 100 μM concentrations.

Classical Synthetic Melanocortin Agonists NDP-MSH and MTII. The first class of synthetic ligands examined in this study, NDP-MSH (*21*) and MTII (*22*, *41*), are considered herein as the classical melanocortin agonists because they are used worldwide as compounds to characterize mutant and wild type melanocortin receptors, used for *in vivo* rodent studies, and as lead templates for ligand design strategies. NDP-MSH, although pharmacologically more potent than the endogenous α-MSH ligand and possesses prolonged biological activity (*21*, *42*), retains much of the endogenous α-MSH ligand structure in terms of consisting of linear 13 amino acids and substitutions of α-MSH Met^4 with Nle^4 and Phe^7 with DPhe. MTII is a very potent agonist peptide consisting of seven amino acids and a side chain lactam cyclization that is postulated to mimic the melanocortin agonist bioactive conformation (*22*, *41*).

NDP-MSH possessed nanomolar to subnanomolar potency at all of the hMC4R polymorphisms examined in this study. However, when comparing the fold changes of NDP-MSH at the polymorphic receptors, as compared to the wild type hMC4R, up to 58-fold decreased agonist ligand potency was observed. NDP-MSH was equipotent or possessed decreased potency up to 5-fold for the S58C, S127L, R165Q, G252S, and I301T hMC4Rs compared to that of the wild type hMC4R. A second grouping with decreased NDP-MSH potency ranging from 6- to 10-fold consisted of the T150I, R165W, and L250Q hMC4Rs. A third grouping with decreased NDP-MSH potency ranging from 11- to 40-fold contained the I102S and Y287Stop hMC4Rs. The final group of hMC4Rs that possessed 41- to 100-fold decreased NDP-MSH agonist potency consisted of the N97D and C271Y hMC4Rs.

MTII, similar to NDP-MSH, possessed subnanomolar potency at the wild type and hMC4R polymorphisms examined in this study, with the exception of the N97D hMC4R that possessed nanomolar MTII agonist potency. However, up to 85-fold decreased MTII agonist potency was observed at these polymorphic hMC4Rs and is grouped as follows: (1) equipotent (1−5-fold decreased potency range), S58C, S127L, R165Q, L250Q, G252S, and I301T, (2) 6−10-fold decreased potency, T150I, R165W, C271Y, and Y287Stop, (3) 11−40-fold decreased potency range, I102S and L106P, and finally (4) 41−100-fold decreased potency, N97D hMC4R.

Tetrapeptides. The amino acids His-Phe-Arg-Trp are conserved in all of the endogenous melanocortin agonist sequences (Table 1) and are postulated to contain key structural pharmacophore features important for melanocortin receptor molecular recognition and stimulation and, upon N-terminal acetylation and C-terminal amidation, the minimal peptide sequence that generated a physiological response in the classical frog and lizard skin bioassays (*43*, *44*). Similar to NDP-MSH, which differs from α-MSH by incorporation of a DPhe at the seven position (*21*), the $DPhe^7$-containing tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 resulted in a more potent analogue than the LPhe-containing ligand in the classical pigmentation bioassays (*45*, *46*) and interestingly, possessed nanomolar agonist potency at the human and mouse MC4R isoforms (*47*, *48*). Herein, we have included the tetrapeptides in an attempt to gain a molecular understanding of whether differences in endogenous and synthetic ligand potency center around the core tetrapeptide sequence or whether it might be attributed to ligand−receptor interactions in the N- or C-terminal ligand extensions from the His-L/DPhe-Arg-Trp tetrapeptide amino acid residues. At the human MC4R polymorphic receptors examined in this study, the control tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 (JRH887-9) possessed agonist potency ranging from nanomolar to micromolar (Table 1). The hMC4R polymorphisms that essentially retained equipotency, compared to that of the wild type, are the L250Q, G252S, and I301T receptors. Uniquely for the tetrapeptide ligands, no polymorphic receptors resulted in 6−10-fold decreased potency of JRH887-9 relative to that of the wild type hMC4R. The R165Q and R165W hMC4Rs possessed decreased tetrapeptide potency in the range of 11−40-fold. The S58C, L106P, S127L, and I301T hMC4Rs possessed 41−100-fold decreased Ac-His-DPhe-Arg-Trp-NH_2 ligand potency, and the I102S possessed 774-fold decreased potency. The most significant reduction in the control tetrapeptide potency was observed at the N97D and C271Y hMC4Rs and was >1000-fold with micromolar agonist EC_{50} values, and at the Y287Stop hMC4R with ca. 270-fold decreased potency.

The Ac-Anc-Phe-Arg-Trp-NH_2 (amino-2-naphtylcarboxylic acid, Anc, JRH420-12) tetrapeptide was selected for this study because it was observed to be greater than 4700-fold selective for the mouse MC4R versus the MC3R (*23*). Thus, identification of a potential lead molecule that already possesses MC4R selectivity for the rescue of dysfunctional hMC4R polymorphisms might be advantageous. Herein, JRH420-12 possessed nanomolar wild type hMC4R potency and ranged from low to high nanomolar agonist potency at all the polymorphic hMC4Rs examined in this study (Table 1). The L250Q, G252S, and I301T hMC4Rs were essentially equipotent (1−5-fold decreased potency range) for JRH420-12 at the wild type hMC4R. The S127L, R165Q, and R165W hMC4Rs possessed 6−10-fold decreased agonist potency for JRH420-12, whereas the S58C, I102S, L106P, and I150I hMC4Rs possessed 11−40-fold decreased potency, the Y287Stop hMC4R possessed ca. 60-fold decreased potency, and the N97D and C271Y hMC4Rs possessed ca. 140- and 360-fold decreased potency, respectively.

The Ac-His-(pI)DPhe-Arg-Trp-NH_2 (JRH322-18) tetrapeptide was selected on the basis of the unique melanocortin receptor pharmacology of being a nanomolar mMC3R antagonist with partial agonist activity and a potent nanomolar mMC4R agonist (*24*). This tetrapeptide *para*-iodo substitution of the $DPhe^7$ ring is distinct in that identical substitutions in the NDP-MSH and MTII templates results in MC3R and MC4R antagonists with partial agonist activity (*35*, *49*). Because controversy remains in regards to the role of the MC3R in energy homeostasis and the desired ligand pharmacology at the MC3R, discovery of a lead template to rescue dysfunctional hMC4 polymorphic receptors that also functions as a MC3R antagonist might be desirable. Additionally, incorporation of the iodine moiety at the DPhe side chain might provide insight into hMC4R−ligand interactions in the context of this study. Interestingly, for this JRH322-18 tetrapeptide ligand, none of the hMC4R polymorphisms examined in this study were equipotent as compared to the wild type hMC4R. The I301T, S127L, L250Q, and G252S hMC4Rs possessed between 5- to 10-fold decreased JRH322-18 agonist potency compared to that of the wild type hMC4R. The S58C and R165Q hMC4Rs

possessed 11−40-fold decreased JRH322-18 potency, whereas the L106P and R165W hMC4Rs possessed 41−100-fold decreased potency, the I102S hMC4R possessed ca. 340-fold decreased potency, and the T150I hMC4R possessed ca. 10,600-fold decreased potency compared with that of the wild type hMC4R. At the N97D hMC4R, JRH322-18, was only able to stimulate ca. 80% the maximal response at 100 μM concentrations. JRH322-18 was unable to stimulate the C271Y and Y287Stop hMC4Rs at up to 100 μM concentrations.

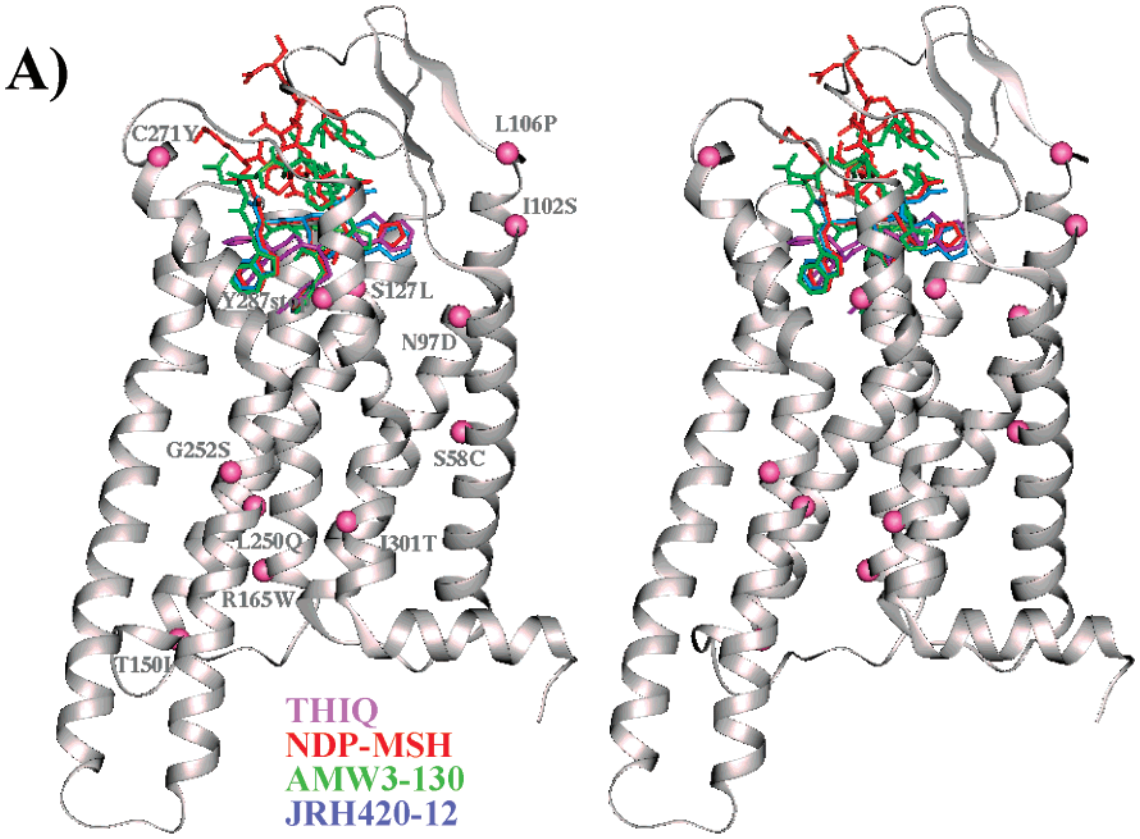
Small Molecules JB25 and THIQ. A large number of small molecule templates and scaffolds have been reported to stimulate the melanocortin receptors during the past several years. The first two small molecules reported in the literature to possess nanomolar MC4R agonist potency include the JB25 (*26*) and the Merck THIQ (*27*) ligands (Table 1). The THIQ ligand has been used in previously reported MC4R ligand−receptor related modeling studies (*37*), as a lead and reference compound in numerable ligand design strategies, and possessed subnanomolar potency at the wild type hMC4R studied herein. The JB25 molecule contains a purported reverse turn thioether peptide mimetic scaffold in the core His-Phe-Arg-Trp tetrapeptide domain and possesses 640 nM agonist potency at the wild type hMC4R (Table 1). These ligands have distinct molecular structural composition as well as notable differences in hMC4R agonist potency and were included in this study to probe the different ligand preferences that the hMC4 polymorphic receptors might favor.

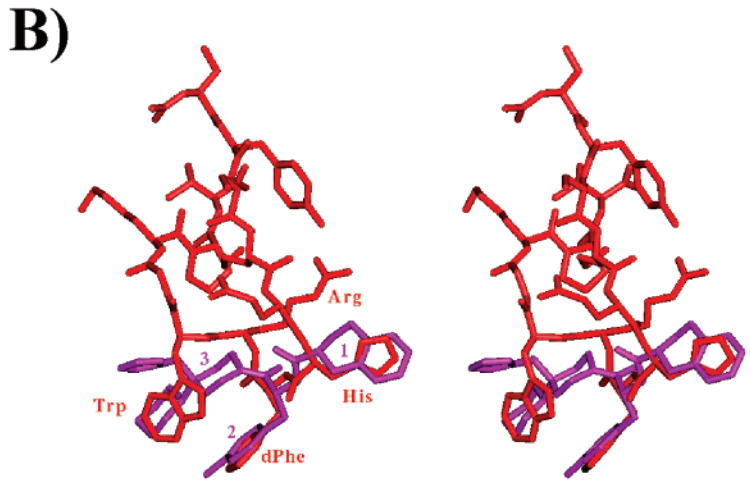
The THIQ ligand was essentially equipotent with the wild type hMC4R at the G252S, S127L, and I301T hMC4Rs. It possessed 6- to 10-fold decreased agonist potency at the S58C, and L250Q hMC4Rs and 11- to 40-fold decreased potency at the I102S, L106P, T150I, R165Q, and R165W hMC4Rs. Interestingly, a 41- to 100-fold change in potency was not observed for this ligand but 100−1000-fold decreased agonist potency was observed at the N97D, C271Y, and Y287Stop hMC4Rs. The JB25 ligand possessed equipotent agonist pharmacology compared to that of the wild type hMC4R at the S58C, S127L, R165Q, L250Q, G252S, and I301T polymorphic hMC4Rs. The L106P, T150I, and R165W hMC4Rs possessed 6−10-fold decreased JB25 agonist potency. None of the hMC4Rs examined in this study possessed 11−40-fold decreased JB25 potency, but the I102S and Y287Stop hMC4Rs possessed 41−100-fold decreased agonist potency. For this JB25 ligand, the C271Y hMC4R possessed only a 75% maximal stimulatory response at up to 100 μM concentrations and was unable to stimulate the N97D hMC4R at up to the same concentration. These data clearly demonstrate that these two melanocortin agonists possess distinct putative ligand−receptor interactions.

AGRP-Melanocortin Chimeras. Our laboratory (*25*, *50*, *51*) and another laboratory (*52*) have reported on the concept of agonist ligands that possess amino acid residues from the melanocortin agonists incorporated into various hAGRP antagonist-based templates that result in the conversion of the endogenous AGRP antagonist ligands into agonists. These chimeric ligands appear to possess unique melanocortin receptor pharmacology distinct from either the synthetic melanocortin agonists or antagonists and AGRP-based antagonists (*51*). On the basis of the chimeric ligands possessing distinct pharmacology profiles compared to those of the synthetic melanocortin agonists NDP-MSH, MTII, and the tetrapeptides examined herein, we wanted to evaluate the effect of the cyclic AMW3-130 (Tyr-c[Cys-His-DPhe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH_2) ligand comprising the AGRP(108-119) antagonist template incorporating the agonist His-DPhe-Arg-Trp residues instead of the antagonist Arg-Phe−Phe(111-113) amino acids. The AMW3-130 agonist possessed subnanomolar agonist potency at the wild type hMC4R and interestingly possessed ca. equipotent activity similar to that of the wild type hMC4R at the 12 S58C, I102S, L106P, S127L, T150I, R165Q, R165W, L250Q, G252S, C271Y, Y287Stop, and I301T hMC4Rs (Table 1). Only the N97D hMC4R possessed decreased AMW3-130 potency, relative to that of the wild type hMC4R, which was only 19-fold but still resulted in a 4 nM agonist EC_{50} value. AMW3-106 incorporates the melanocortin agonist His-DPhe-Arg-Trp residues into the mini-AGRP (*53*) antagonist template and possesses subnanomolar agonist potency at the melanocortin receptors (Table 1) (*25*). Unlike the AMW3-130 11 amino acid chimeric template, the 35 residue AMW3-106 agonist resulted in decreased polymorphic hMC4R potency up to 1800-fold at the N97D hMC4R. The S127L, L250Q, and G252S hMC4Rs possessed the equipotency of AMW3-106 compared to that of the wild type hMC4R. The S58C, R165Q, R165W, and I301T hMC4Rs possessed 6−10-fold decreased AMW3-106 potency, the L106P hMC4R possessed ca. 80-fold decreased potency, and the I102S, Y287Stop, and C271Y hMC4Rs possessed between 41- to 100-fold decreased agonist potency.

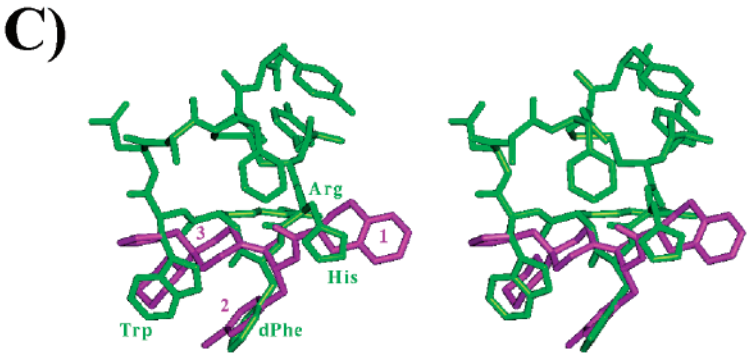
Ligand and hMC4R Computer Assisted Homology Molecular Modeling. In an effort to understand the molecular interactions between the polymorphic hMC4Rs and the different ligand structural classes, we performed 3D homology molecular modeling studies in an attempt to gain insight into putative ligand−receptor interactions that would complement the *in vitro* receptor pharmacology we observed in this study. To identify potential ligand structural similarities as well as distinctions, superimpositions of ligands docked into the binding pocket of the active hMC4R were generated. Because the THIQ ligand is the most potent small molecule examined in this study, it was overlaid with the NDP-MSH linear agonist, MTII cyclic agonist, JRH420-12 tetrapeptide, AMW3-130 chimeric ligand, and the JB25 small molecule (Figure 2 A−F). Because it is well documented that the His-L/DPhe-Arg-Trp melanocortin agonist residues are important for melanocortin receptor molecular recognition and agonist stimulation of the receptor, these residues were used as reference points for structural comparisons of the ligands examined. On the basis of the ligand functional activity at the polymorphic hMC4Rs examined herein, the NDP-MSH, AMW3-130, JRH420-12, and THIQ agonist ligands were docked into the putative active conformation of the wild type hMC4R (pdb code 1iqu) (*37*, *54*) (Figure 2A).

Several receptor mutagenesis studies have been performed on the MC4R in an attempt to identify putative ligand−receptor interactions for further drug design approaches (*35*, *37*, *48*, *54*−*60*). Noteworthy is the observation that essentially none of the human polymorphic receptors examined in this study have been previously postulated to be involved in key putative ligand−receptor interactions of the His-L/DPhe-Arg-Trp ligand residues, yet some of the polymorphic





THIQ and NDP-MSH



THIQ and AMW3-130

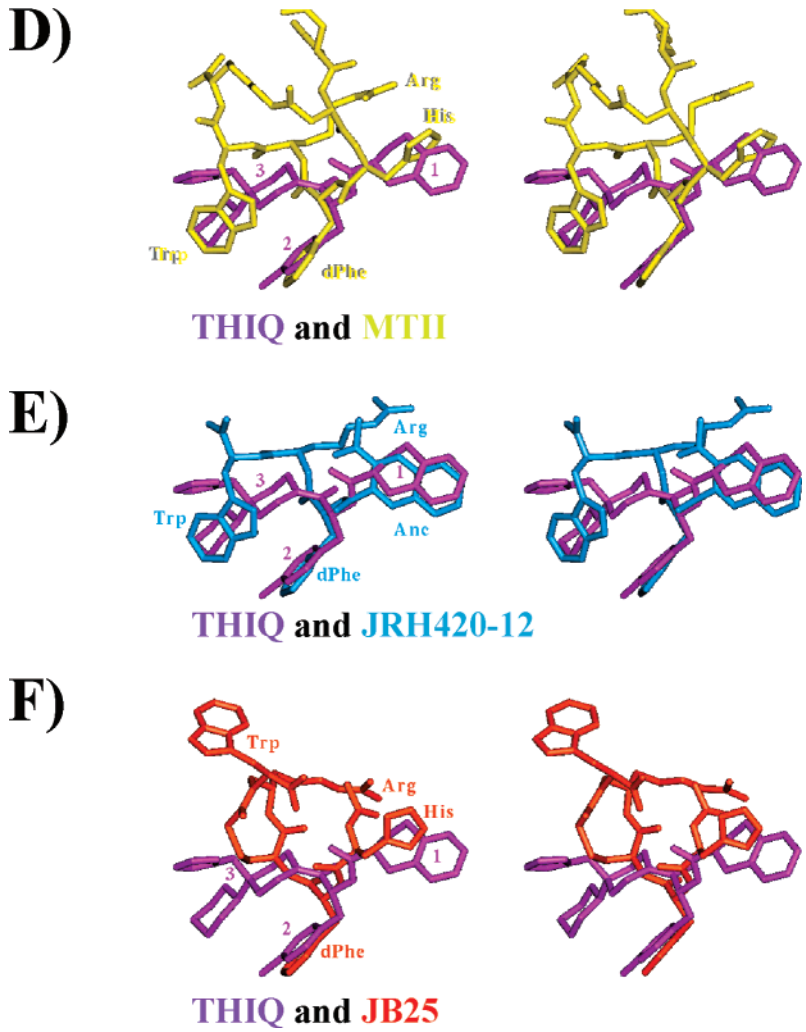


FIGURE 2: Stereoview illustrations of the ligand and hMC4R homology molecular modeling results. (A) Image of the synthetic agonist ligands THIQ (purple), NDP-MSH (red), AMW3-130 (green), and JRH420-12 (blue) docked into the active conformation of the hMC4R. The labeled hMC4R polymorphisms examined in this study are indicated as pink balls and illustrate their relative position within the hMC4R protein. (B) Superimposition of the THIQ (purple) small molecule and the NDP-MSH peptide (red). (C) Superimposition of the THIQ (purple) small molecule and the AMW-130 chimeric AGRP-melanocortin agonist peptide (green). (D) Superimposition of the THIQ (purple) small molecule and the MTII peptide (yellow). (E) Superimposition of the THIQ (purple) small molecule and the JRH420-12 tetrapeptide (blue). (F) Superimposition of the THIQ (purple) small molecule and the JB25 small molecule (red).

hMC4R amino acid modifications have dramatic effects on the endogenous agonist potency (Table 1). Thus, using homology molecular modeling techniques, insights into the role of some hMC4R polymorphic amino acid modifications are discussed below.

DISCUSSION

Identification of the possible molecular mechanism(s) by which hMC4R polymorphisms might explain the observed phenotype in human patients is a first step in trying to correct the molecular defects via a therapeutic intervention. Herein, we have selected hMC4R polymorphisms (Figure 1) that have resulted in decreased agonist potency of one or more of the multiple endogenous melanocortin receptor agonists (α-MSH, β-MSH, γ_2-MSH, ACTH(1-24)) and pharmacologically characterized them using a variety of synthetic agonist structural classes. At the onset of this study, we postulated that we might identify ligands that (a) would not be able to stimulate polymorphic hMC4Rs, (b) could correct for a single polymorphic hMC4R, (c) could correct for multiple but not all of the hMC4R polymorphisms examined, and (d) could correct for all of the hMC4R polymorphisms examined in this study.

Fortunately, all of the hMC4R polymorphisms examined in this study were stimulated by the multiple synthetic agonists examined, albeit with varying degrees of response and potency. Although it might be envisioned that single ligands may be identified and/or designed that can specifically correct for a single hMC4R polymorphism, ligands possessing such qualities were not observed in this study. Theoretically, it might be advantageous for a ligand to functionally correct a hMC4R polymorphism that does not respond normally to the endogenous melanocortin agonists yet might not have a greater affinity for the wild type hMC4R compared to that for the endogenous agonists. The majority of dysfunctional hMC4R polymorphisms are found as heterozygous genotypes in obese patients. Thus, on the basis of observations made in rodent studies (*12*, *14*, *61*), the ability to pharmacologically correct for a dysfunctional hMC4R might physiologically result in a decreased drive to eat as well as a more balanced energy homeostatic profile. The goal of a polymorphic specific ligand would be to stimulate the

heterozygous polymorphic hMC4R by mimicking the potency of the endogenous melanocortin agonists yet not effecting the normal wild type hMC4R protein. Overstimulation of the hMC4R might result in cell death, affect cellular development, and/or have an opposite unbalance in the neural energy homeostatic pathway, disturbing other neuronal circuitry resulting in an anorectic physiological state, none of which are optimal desired therapeutic endpoints (*62*). An additional undesirable phenotype for hMC4R overstimulation might also result in obesity, as observed for the morbidly obese patient possessing the L250Q constitutively active polymorphic receptor (*63*). Furthermore, because the MC4 receptor is expressed in over 100 brain nuclei (*5*, *64*, *65*), consisting of different cell types and physiological functions besides energy homeostasis, it might be envisioned that heterogeneous MC4 receptor expression levels in different brain nuclei exist. Thus, this concept raises the possibility of the need for function-specific ligands as well as hMC4R polymorphic specific ligands.

Ligands that Rescue a Group of Polymorphic hMC4Rs Postulated to be Important for Agonist-Induced Receptor Conformation(s). The synthetic ligands NDP-MSH, MTII, AMW3-130, and JB25 possessed essentially equipotent agonist activity, compared to that of the wild type hMC4R, at the S58C (TM1), S127L (TM3), R165Q (TM4), G252S (TM6), and I301T (TM7) hMC4Rs. This group of hMC4R polymorphisms, with the exception of the R165Q hMC4R, possessed less than 40-fold decreased endogenous agonist potency for the four ligands examined in this study. These hMC4R polymorphic receptor amino acids, with the exception of the S127L (TM3), are positioned outside the putative ligand binding pocket, are located closer to the cytoplasmic side of the lipid bilayer (Figures 1 and 2A), and are most likely involved in ligand-induced receptor conformational changes important for G-protein-induced signal transduction events. The S127L hMC4R residue, on the basis of our homology modeling studies, is adjacent to the Asp126 TM3 hMC4R receptor residue (Figures 1 and 2A) that has been demonstrated in melanocortin receptor mutagenesis studies to be important for ligand binding and potency (*35*, *48*). Additionally, taking into account receptor protein dynamics and movement, S127 is in the proximity of the putative agonist Arg side chain residue. Thus, it might be speculated that the Ser127 residue may be important for receptor local hydrogen bonding interactions that might shift the hMC4R from the inactive to the active receptor conformation state upon ligand binding. This concept and the importance of TM3 residues for receptor activation (*66*) are also supported by another study in which the synthetic antagonist SHU9119 (Ac-Nle-c[Asp-His-DNal(2′)-Arg-Trp-Lys]-NH_2) (*49*) is converted to an agonist by the TM3 L133M-mutated hMC4R (*60*).

Synthetic Agonist Ligands that Result in Full Agonist Pharmacology and Nanomolar Potency at the hMC4R Polymorphisms Examined Herein. Although the NDP-MSH, MTII, AMW3-130, THIQ, and AMW3-106 agonist ligands were able to elicit maximal responses for all the hMC4 polymorphic receptors examined in this study at nanomolar or subnanomolar potencies (Table 1), only the AMW3-130 ligand resulted in a maximal 19-fold decreased potency at the N97D hMC4R and was essentially equipotent at the other polymorphic hMC4Rs, as compared with the wild type hMC4R. Thus, this AMW3-130 chimeric AGRP-melanocortin agonist ligand appears to be the best generic rescue ligand that can essentially correct the hMC4 polymorphic receptors examined in this study.

Observations at Specific hMC4R Polymorphisms. The S58C polymorphism, located in the TM1 domain, was identified as a heterozygous polymorphism in an obese patient (*67*). It resulted in 14−38-fold decreased endogenous agonist potency but responded to the different synthetic agonist ligands examined in this study. The NDP-MSH, MTII, AMW3-130, and JB25 ligands essentially possessed equipotent agonist EC_{50} values the same as those at the wild type hMC4R (Table 1), whereas the AMW3-106 and THIQ ligands possessed ca. 10-fold decreased potency, and the tetrapeptides possessed 15−45-fold decreased agonist potency at this hMC4R polymorphism. Because this hMC4R Ser58 residue is too distant from the putative agonist binding pocket to be directly involved in molecular recognition and does not affect NDP-MSH binding affinity (*28*), it is most likely involved in the agonist ligand-induced conformational changes important for signal transduction.

The N97D hMC4R was identified as a homozygote polymorphism (*68*) and was unable to be stimulated by α-MSH, γ_2-MSH, and ACTH(1-24) at up to micromolar concentrations, whereas β-MSH possessed ca. 620-fold decreased agonist potency compared to the potency at the wild type hMC4R (Table 1). The AGRP-melanocortin AMW3-130 synthetic ligand possessed 19-fold decreased agonist potency at the N97D hMC4R and was the most potent stimulator of the ligands examined in this study. NDP-MSH and MTII possessed 40−85-fold decreased ligand potency, and the JRH887-9 and JRH420-12 tetrapeptides as well as the THIQ small molecule possessed 140−190-fold decreased agonist potency. The chimeric mini-AGRP-melanocortin agonist AMW3-106 possessed 1800-fold decreased agonist potency with the JRH322-18 tetrapeptide only able to generate an 80% maximal response, relative to the forskolin control, at up to 100 μM concentrations at this polymorphic hMC4R. The JB25 ligand was unable to stimulate the receptor at up to 100 μM concentrations. The N97 residue is located in TM2 and is in close proximity to the E100, D122, and D126 hMC4R acidic amino acid side chains that have been previously demonstrated by melanocortin receptor mutagenesis studies to be important for ligand molecular recognition and agonist function (*35*, *48*, *69*, *70*). Because this polymorphism possessed the most dramatic endogenous agonist dysfunctional result of not being stimulated by α-MSH, γ_2-MSH, and ACTH(1-24) at micromolar concentrations (Table 1), it is obvious that the conversion of the Asn to the Asp side chain, which is essentially the exchange of an amide moiety for a carboxylic moiety, has significant functional consequences for hMC4R ligand molecular recognition and signaling (*28*, *68*). On the basis of our homology molecular modeling studies, we can speculate that the N97D mutation may affect the arrangement of the TM2 and TM3 helical domains because of the introduction of an additional negative charge in the acid rich region. Such local receptor structural destabilization may also be attributed to the poor levels of receptor cell surface expression (*28*, *68*). Another speculative molecular disruption mechanism may be that the additional Asp carboxylic acid side chain residue in the putative ligand binding pocket, including the

hMC4R E100, D122, and D126 amino acids, the D97, may disrupt ligand molecular recognition and the ability of the ligand to transition the hMC4R from the inactive to the active receptor conformation(s) required for G-protein coupling and signal transduction. Previous binding studies using the NDP-MSH ligand used to compete radiolabeled I^{125}-NDP-MSH resulted in a only a 7-fold decreased binding affinity compared to that of the wild type hMC4R (*28*), supporting the suggestion that the N97D modification might disrupt the receptor conformational aspect of endogenous agonist ligand-induced signaling versus the endogenous agonist ligand molecule recognition event itself. However, the precise molecular mechanisms by which the Asn to Asp side chain modification of the hMC4R affects the endogenous agonist ligands remain to be verified by further experimental studies. Nonetheless, these studies have still resulted in identification that the AMW3-130 chimeric AGRP-melanocortin agonist ligand can stimulate this receptor with a 4 nM potency (Table 1).

The L106P hMC4R was reported as a heterozygous polymorphism (*68*) and was only slightly stimulated by the α-MSH and ACTH(1-24) ligands at up to 100 μM concentrations. Interestingly, however, the endogenous agonists β-MSH and γ_2-MSH were able to elicit full agonist dose−response curves, albeit with 36−330-fold decreased agonist potency compared to that of the wild type hMC4R. The AMW3-130 ligand possessed equipotency at the L106P hMC4R, as with the wild type hMC4R, and the JB25 small molecule possessed only 6-fold decreased comparative agonist potency. The synthetic ligands MTII, NDP-MSH, AMW3-106, JRH420-12, and THIQ possessed 10−40-fold decreased agonist potency, whereas the tetrapeptides JRH887-9 and JRH322-18 possessed 42−100-fold decreased agonist potency. At this human polymorphism, whereas the endogenous agonists were unable to maximally stimulate this receptor, all of the synthetic ligands examined in this study were able to elicit a full agonist response, albeit with significantly reduced potency compared to that of the wild type hMC4R in some cases. The L106P substitution located just after the TM2 helix may cause the rearrangement of the adjacent extracellular loop 1 that connects TM2 and TM3. Indeed, in agreement with our previous modeling of the hMC4R (*54*), Leu106 possesses a backbone conformation not allowed for Pro. Therefore, the L106P polymorphism may have a largely distorted structure and orientation of this loop juxtaposed to the ligand binding pocket.

The S127L hMC4R was observed in obese patients as a heterozygous polymorphism (*71*, *72*) and possessed slight but significant decreased endogenous agonist ligand potency compared to that of the wild type hMC4R. As might be anticipated on the basis of the relatively minor decreased endogenous agonist potencies, the NDP-MSH, MTII, AMW3-130, AMW3-106, JB25, and THIQ agonist ligands possessed agonist stimulation essentially equipotent to that at the wild type hMC4R. Interestingly, however, the tetrapeptides JRH420-12 and JRH322-18 possessed 6−10-fold decreased potency, and the Ac-His-DPhe-Arg-Trp-NH_2 JRH887-9 tetrapeptide possessed 62-fold decreased agonist potency. These data suggest that this residue may be important in interacting with the endogenous melanocortin conserved His-Phe-Arg-Trp residues, either in terms of molecular recognition or the agonist ligand-induced hMC4R activation conformation. Agonist ligand binding studies using NDP-MSH to competitively displace radiolabeled I^{125}-NDP-MSH demonstrated that NDP-MSH possessed affinity for the S127L equipotent to that of the wild type receptor (*28*). Unfortunately, however, these data do not directly address the affinity of the tetrapeptides and the differentiation of binding versus function, which require additional experiments to probe the molecular mechanism.

The T150I heterozygous polymorphism (*63*) is putatively located toward the intracellular domain of TM3, perhaps at the receptor−lipid bilayer interface (Figures 1 and 2A), and possessed 6−22-fold decreased endogenous agonist potency compared to that of the wild type hMC4R. The synthetic ligand AMW3-130 possessed agonist stimulation at this T150I hMC4R equipotent to that at the wild type hMC4R and possessed 6−50-fold decreased agonist potency for the NDP-MSH, MTII, JRH420-12, JRH887-9, JB25, and THIQ ligands. The Ac-His-(pI)DPhe-Arg-Trp-NH_2 JRH322-18 ligand, however, possessed 10,600-fold decreased agonist potency compared to that of the wild type hMC4R at the T150I hMC4R. This data suggest, on the basis of the location of the residue change toward the extracellular portion of TM3 and adjacent to the conserved GPCR Asp-Arg-Tyr (DRY) sequence important for GPCR signal transduction (*73*) as well as the decreased tetrapeptide agonist potency, that this residue is important for agonist ligand-induced active hMC4R conformational changes significant for signal transduction.

The heterozygous polymorphisms R165Q (*68*, *74*, *75*) and R165W (*17*, *63*, *72*, *76*) hMC4Rs were observed in obese patients. These receptors possessed ca. 6-fold decreased β-MSH potency, 15-fold decreased γ_2-MSH potency, ca. 30-fold decreased α-MSH potency, and 60−90-fold decreased ACTH(1-24) agonist potency compared to that of the wild type hMC4R. Although similar trends in decreased agonist ligand potency were observed for the endogenous ligands at these two polymorphic R165 hMC4Rs, differences in the synthetic ligand potency profiles were observed between the Gln and Trp amino acid changes. For both of these R165 hMC4Rs, the AMW3-130 ligand was essentially equipotent to that at the wild type hMC4R. The AMW3-106 and JRH420-12 ligands possessed ca. 8-fold decreased ligand potency, and the JRH887-9 (20−29-fold decreased potency) and THIQ (11−14-fold decreased potency) ligands possessed changes in agonist potency on the same order or magnitude. At the R165Q hMC4R, NDP-MSH, MTII, and JB25 ligands were essentially as equipotent as those at the wild type hMC4R, where these same ligands demonstrated 6−10-fold decreased potency at the R165W hMC4R. For the JRH322-18 ligand, 18-fold decreased potency resulted at the R165Q hMC4R, whereas this same ligand possessed 74-fold decreased agonist potency at the R165W hMC4R compared to that at the wild type hMC4R. Thus, the R165W hMC4R possessed subtle differences in some synthetic ligand agonist potency, whereas others possessed the same pharmacological profile as that at the R165Q hMC4R. On the basis of the location of the R165 amino acid toward the extracellular domain of TM4 (Figure 1), its proximity to the TM3 DRY sequence, which based upon our modeling studies putatively makes molecular contacts with the Asp residue of the DRY sequence, and the decreased potencies of the tetrapeptides examined in this study, these data support the hypothesis that this R165 hMC4R residue is important for the confor-

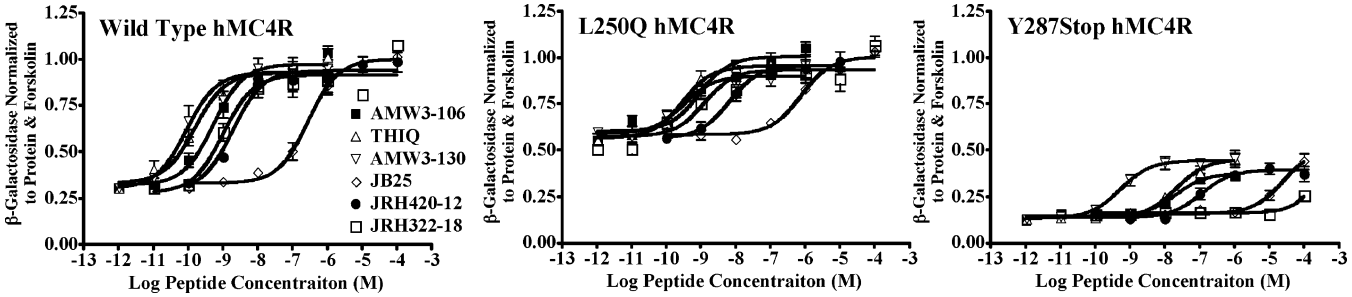


FIGURE 3: Summary of the agonist receptor pharmacology at the wild type hMC4R, the L250Q hMC4R, and the Y287Stop hMC4R. The L250Q hMC4R is constitutively active as illustrated by increased basal activity for all of the ligands. The Y287Stop hMC4R retains the ability to be partially stimulated by the agonist ligands, even though this polymorphism results in the absence of the hMC4R protein in a portion of TM7 and the entire C-terminal domain.

mation of the intracellular loop 2 appropriate for the ligand-induced active hMC4R state.

The L250Q polymorphism is the first constitutively active hMC4R identified and was observed in an extremely obese French female patient (*63*). Constitutive activation of the L250Q hMC4R resulted in elevated basal cAMP levels of the receptor in the absence of agonist stimulation (*63*, *77*). The fact that a constitutively active hMC4R mutation was found in an obese person is a paradox because chronic activation of the hMC4R and subsequently high cAMP levels should theoretically result in a normal or lean phenotype. Our laboratory has previously reported a proposed molecular mechanism involving the AGRP antagonist ligand by which this L250Q hMC4R polymorphism might be related to human obesity (*77*). It has been included in this study, however, to identify if any of the agonist ligand structural classes included herein might function as ligands to decrease the basal cAMP levels. This concept was not realized for any of these agonist ligands, however, because they all possessed equipotent or up to 8-fold decreased full agonist potency at the L250Q as compared to the same synthetic ligands at the wild type hMC4R and did not affect the basal levels of the L250Q hMC4R to any extent (Figure 3).

The heterozygous G252S hMC4R (*17*, *72*) interestingly possessed equipotency for the β-MSH endogenous agonist as the wild type hMC4R yet exhibited 6-, 9-, and 17-fold decreased agonist potency for the γ_2-MSH, α-MSH, and ACTH(1-24) ligands, respectively. These data support the hypothesis that a human MC4R polymorphism may respond differently to the endogenous melanocortin ligands. The fact that this G252S hMC4R was observed in an obese patient might support a hypothesis that the endogenous melanocortin agonist in the brain that interacts with the MC4R involved in energy homeostasis might preferentially be α-MSH and/or ACTH versus the β-MSH ligand. Further studies need to be performed to support this speculation; however, these data add to the mounting evidence to support this theory (*78*, *79*). At the G252S hMC4R, all of the synthetic agonists examined in this study were essentially as equipotent as those at the wild type hMC4R, with the largest decreased potency of 6-fold observed for the JRH322-18 ligand.

The heterozygous hMC4R C271Y amino acid polymorphism change (*68*, *75*) is putatively located at the extracellular receptor−lipid bilayer interface involving TM6 or is located in the extracellular milieu (Figures 1 and 2A). This C271 hMC4R residue has been postulated to be involved in a disulfide bridge with the Cys279 (*80*) or Cys277 (*54*) hMC4R residue or perhaps to interconvert between the two possibilities. Replacement of the homologous hMC4R Cys271 and Cys279 receptor residues with Ala amino acids resulted in a lack of α-MSH as well as Zn^{2+} metal ion-induced stimulation, leading the authors to conclude that this putative disulfide bridge is important for receptor structure and function (*80*). More recent studies suggest that a putative disulfide bridge involving the hMC4R C271 residue is essential for the correct structure of the extracellular loop 3 connecting TM6 and TM7 (*54*). The importance of this hMC4R Cys271 residue for endogenous agonist binding appears to be partially in agreement with this model, at least for the α-MSH, ACTH(1-24), and γ_2-MSH ligands that are only able to generate a slight stimulatory response at up to 100 μM concentrations. However, the endogenous β-MSH ligand is able to fully stimulate the C271Y hMC4R with an agonist potency of EC_{50} = 150 nM. On the basis of these data, we might speculate that the large N-terminal extension on the β-MSH ligand may help stabilize the appropriate conformation of this loop in the C271Y hMC4R polymorphism. Interestingly, the synthetic ligands, such as NDP-MSH, MTII, and AMW3-130, possess nanomolar to subnanomolar full agonist potency at the C271Y hMC4R, whereas the linear synthetic tetrapeptide JRH322-18 is unable to stimulate the C271Y hMC4R, and the JB25 small molecule is only able to slightly stimulate it at 100 μM concentrations. Thus, the ability of the hMC4R Cys271 amino acid side chain to participate in intramolecular disulfide interactions important for the melanocortin ligand binding and molecular recognition appears to be an important mechanism by which this polymorphism is dysfunctional. We have in this study, however, demonstrated that the synthetic cyclic ligands AMW3-130 and MTII possess subnanomolar agonist potency, supporting the hypothesis that this Cys271 residue may not be as critically important for receptor structure as previously hypothesized.

The heterozygous Y287Stop polymorphic hMC4R (*68*) truncates the hMC4R protein at the top third of the TM7 domain (Figure 1) and might be theorized to result in a loss of function. However, in the construct we have used to generate this polymorphic receptor for our studies, the hMC4R protein we characterized resulted in the ability to partially be stimulated by endogenous agonists, albeit with 30−230-fold decreased ligand potency (Figure 3 and Table 1) (*28*). This receptor was included herein to identify whether any of the synthetic ligands examined in this study might be able to elicit a full agonist response. Although this goal was not achieved because all of the ligands resulted in only partial agonists, relative to the forskolin control (Figure 3),

we did identify that the AMW3-130 agonist ligand possessed agonist stimulation at the Y287Stop essentially equipotent to that observed at the wild type hMC4R. NDP-MSH and MTII possessed ca. 20-fold decreased agonist potency, whereas, interestingly, the JRH322-18 tetrapeptide was unable to notably stimulate this receptor at up to 100 μM concentrations.

The heterozygous I301T hMC4R polymorphism (*63*) possessed a subtle but significant 5−25-fold decreased endogenous agonist potency for the endogenous ligands examined in this study and relative to their respective potency at the wild type hMC4R. Characterization of this I301T hMC4R with the synthetic ligands examined herein resulted in identification of the NDP-MSH, MTII, JRH887-9, JRH420-12, JRH322-18, AMW3-130, JB25, and THIQ as possessing pharmacology essentially equipotent to that observed for these ligands at the wild type hMC4R. Only the AMW3-106 mini-AGRP-melanocortin ligand possessed a notable 6-fold reduced potency at the I301T compared to that of the hMC4R. Thus, on the basis of the location of this I301 residue as well as the magnitude of decreased potency observed for the ligands examined, the most likely mechanism at the I301T hMC4R is to affect the ligand-induced hMC4R active conformation. This altered conformational change may be attributed to a more subtle kinetic issue versus more global effects such as being involved in TM domain conformational shifts.

In summary, we have pharmacologically characterized 13 polymorphic melanocortin-4 receptors identified in human patients that demonstrated abnormal stimulation potency by the endogenous melanocortin agonists (α-MSH, β-MSH, γ_2-MSH, and ACTH(1-24)) for the ability of synthetic peptides and small molecules to rescue the functional ability of these receptors relative to the wild type hMC4R response. This study has identified the synthetic ligands NDP-MSH, MTII, AMW3-130, THIQ, and AMW3-106 as possessing nanomolar and subnanomolar agonist potency at the hMC4Rs examined herein, but only the AMW3-130 chimeric AGRP-melanocortin agonist elicited an equipotent agonist EC_{50} value at the wild type and polymorphic hMC4Rs, with the sole exception of the N97D hMC4R, which possessed 19-fold decreased agonist potency for this ligand compared to that of the wild type hMC4R. Using the melanocortin agonist tetrapeptides, we were also able to postulate specific hMC4R polymorphisms (S58C, T150I, R165Q, R165W, C271Y, and I301T) that may be modified in the ability of the hMC4R to achieve an optimal ligand-induced active conformation important for hMC4R signal transduction. Additionally, using a variety of agonist−ligand structural templates, we were able to provide additional structure−activity relationship information that may be important for the further design of ligands to generically rescue dysfunctional hMC4Rs as well as ligands that might be able to specifically target a particular polymorphism amino acid change.

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