Pharmacological chaperones restore function to MC4R mutants responsible for severe early-onset obesity

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d) **Nonstandard abbreviations used**: DCPMP, N-((2R)-3(2,4-dichloroPhenyl)-1-(4-(2-((1-methoxypropan-2-ylamino)methyl)phenyl)piperazin-1-yl)-1-oxopropan-2-yl)propionamide ; E.R. , endoplasmic reticulum ; EXL2 , extracellular loop 2 ; GPCR, G protein-coupled receptors ; kel , elimination rate constant ; hMC4R , human melanocortin-4 receptor ; MPCI , 2-(5-bromo-2-
methoxyphenethyl)-N-(N-((1-ethylpiperidin-4-yl)methyl)carbamimido)-3-fluorobenzamide; MTHP, 2-(2-(2-methoxy-5-nitrobenzylthio)phenyl)-1,4,5,6-TetraHydroPyrimidine; NBP, 1-(1-(4-fluorophenyl)-2-(4-(4-(naphthalene-1-yl)butyl)piperazin-1-yl)ethyl)-4-methylpiperazin; NDI, nephrogenic diabetes insipidus; PCs, pharmacological chaperones; PPPone, 3-(4-(2-(4-fluorophenyl)-2-(4-methylpiperazin-1-yl)ethyl)piperazin-1-yl)-2-methyl-1-phenylpropan-1-one; SAR, structure-activity relationship; THIQ, Tetrahydroisoquinoline; vYFP, venus yellow fluorescent protein; WT, Wild-type

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Abstract

Heterozygous null mutations in the melanocortin-4 receptor (MC4R) cause early-onset obesity in humans, indicating that metabolic homeostasis is sensitive to quantitative variation in MC4R function. Most of the obesity-causing MC4R mutations, functionally characterized to date, lead to intracellular retention of receptors by the cell’s quality control system. Thus, recovering cell surface expression of mutant MC4Rs could have a beneficial therapeutic value. We tested a pharmacological chaperone approach to restore cell surface expression and function of ten different mutant forms of hMC4R found in obese patients. Five cell permeable MC4R-selective ligands were tested and displayed pharmacological chaperone activities, restoring cell surface targeting and function of the receptors with distinct efficacy profiles for the different mutations. Such mutation-specific efficacies suggested a structure-activity relationship between compounds and mutant receptor conformations that may open a path toward personalized therapy. In addition, one of the five pharmacological chaperones restored function to most of the mutant receptors tested. Combined with its ability to reach the central nervous system and its selectivity for the MC4R, this pharmacological chaperone may represent a candidate for the development of a targeted therapy suitable for a large subset of patients with MC4R-deficient obesity.
Introduction

Disease-causing mutations in G protein-coupled receptors (GPCR) often lead to decreased cell surface expression and concomitant loss of function as a result of improper folding (Schoneberg et al., 2004; Thompson et al., 2008). These mutant receptors, generally recognized by the cell’s quality control system within the endoplasmic reticulum (ER) and Golgi apparatus, are retained intracellularly and targeted for degradation. In many of these conformational diseases, the mutation occurs in receptor domains that do not directly affect ligand binding or G protein coupling, opening the possibility for interventions that could restore receptor function by rescuing folding and cell surface expression (Bernier et al., 2004; Conn et al., 2007). Such functional rescue has been achieved for several GPCRs, indicating that pharmacologically selective compounds, termed pharmacological chaperones (PCs), can stabilize the misfolded receptors to facilitate their export from the ER to the plasma membrane where they are active (Morello et al., 2000; Petaja-Repo et al., 2002; Noorwez et al., 2004; Bernier et al., 2006; Robben et al., 2007; Conn and Janovick, 2009). The clinical effectiveness of a PC approach has been tested for one such disease, nephrogenic diabetes insipidus (NDI), where a vasopressin antagonist, acting as PC, rescued the function of ER-retained V2-vasopressin receptor mutants and significantly improved the kidney function of NDI patients (Bernier et al., 2006).

PCs may present an attractive therapeutic option for severe early-onset morbid obesity that results from mutations in the melanocortin type 4 receptor (MC4R), a receptor that plays a pivotal role in energy homeostasis (Cone, 2005). In humans, MC4R mutations lead to an obese
phenotype similar to the homozygous nulls mouse model (Huszar et al., 1997; Chen et al., 2000) and represent the most common monogenic cause of severe early-onset obesity (Farooqi and O'Rahilly, 2006). To date, ~80 distinct mutations of MC4R were reported in the obese human population. One MC4R antagonist was previously shown to have pharmacological chaperone action on two MC4R mutants (Fan and Tao, 2009). However, the large diversity of obesity-related trafficking-defective mutations in MC4R questions the ability of a single compound to restore cell surface expression and function to all mutant forms. This idea is reinforced by the fact that mutations, which are broadly distributed throughout the receptor structure, might lead to different conformational changes. To address this question and to determine whether different PC candidates show different efficacies toward distinct mutants, we tested five cell-permeable MC4R antagonists that belong to three structurally distinct chemical classes. The compounds were studied for their ability to rescue cell surface expression and signaling activity of ten naturally-occurring mutant forms of MC4R causing obesity (Tao, 2005; Tan et al., 2009). Clear differences were found in the efficacies and potencies between compounds on each mutant, revealing unique rescue profiles for individual PCs. Importantly, one compound emerged as the most universal PC, rescuing nearly all mutations with the highest potency and efficacy. Furthermore, its bioavailability in brain, its clearance properties and its receptor subtype selectivity make it a good candidate for the development of a clinically useful drug to treat genetic obesity caused by distinct MC4R mutations in humans.
Methods

Generation of Mutant hMC4R Constructs

Ten mutant forms of hMC4R [S58C, E61K, N62S, I69T, I125K, T162I, R165Q, R165W, C271Y and P299H] were double tagged with a 3xHA tag at the N-terminus and a Venus tag at the C-terminus (see supplemental Materials and Methods).

Compound Synthesis

Compounds selected in the study were synthesized at Amicus Therapeutics (Cranbury, NJ, USA) following the procedures described previously:

2-(2-(2-Methoxy-5-nitrobenzylthio)phenyl)-1,4,5,6-TetraHydroPyrimidine (MTHP) (Millenium Pharmaceutical Inc. Patent WO02062766 (2002)) ; 3-(4-(2-(4-fluorophenyl)-2-(4-methylpiperazin-1-yl)ethy)l)piperazin-1-yl)-2-methyl-1-phenylpropan-1-one (PPPone) (Arasasingham et al., 2003) ; 2-(5-bromo-2-methoxyphenethyl)-N-((1-ethylpiperidin-4-yl)methyl)carbamimidoyl)-3-fluorobenzmide (MPCI) (Chaki et al., 2005) ; N-((2R)-3(2,4-dichloroPhenyl)-1-(4-2,4-dichlorophenyl-2-(4-(N-(2-(4-flurophenyl)-2-(4-(4-(naphthalene-1-yl)butyl)piperazin-1-yl)ethyl)-4-methylpiperazin (NBP) (Vos et al., 2006).

Structural Modeling

The homology modeling of the inactive conformation of human MC4R was done as previously described (Tan et al., 2009) using β2-adrenergic receptor fused with T4 lysozyme (2rh1 PDB
entrye) as a structural template. 3D structures of five small-molecule antagonists (including four stereoisomers of PPPone and two stereoisomers of both DCPMP and NBP) were generated by QUANTA (Accelrys). pKa of charged groups were calculated by Marvin software (http://www.chemaxon.com/marvin/sketch/index.html). Conformational analysis of the ligands was performed using grid scan search for torsion angles of all rotatable bonds. The lowest energy conformations of all stereoisomers of all ligands (except one stereoisomer of NBP with $\Delta E=2.2$ kcal/mol) were used for docking (see supplemental Materials and Methods).

Transfection and Cell Culture

HEK293T cells were transiently transfected with plasmids encoding chimeric WT or mutant hMC4Rs using Fugene 6 (Roche Applied Science, Laval, Qc, Canada) as the transfection agent. Cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin/streptomycin for 24 hours. Transiently expressing cells were incubated in the presence or absence of antagonist for 12 hours prior to flow cytometry or cAMP detection assays.

Quantitative Assessment of WT and Obesity-associated Mutant hMC4R Membrane Expression by Flow Cytometry

Forty-eight hours after transfection, cells were harvested, rinsed once in 1X Dulbecco-PBS and transferred into 1X Tyrode [140 mM NaCl, 2.7 mM KCl, 1 mM CaCl$_2$, 12 mM NaHCO$_3$, 5.6 mM D-Glucose, 0.49 mM MgCl$_2$, 0.37 mMNaHPO$_4$, 25 mM Hepes pH 7.4] supplemented with 1% BSA (Sigma-Aldricht, Oakville, ON, Canada) (Tyrode/BSA) containing mouse monoclonal anti-HA antiboby (HA.11, Covance, Cedarlane, Burlington, ON, Canada) (1:1000) to label cell
surface receptors. After one-hour incubation at room temperature, cells were washed once and re-suspended in Tyrode/BSA containing anti-mouse Alexa 647 secondary antibody (1:1000) (Invitrogen Canada Inc, Burlington, ON, Canada). After one-hour incubation at room temperature, cells were washed with Tyrode/BSA, re-suspended in Tyrode, and kept on ice. Right before analysis, Propidium iodide (PI) was added in each sample to exclude labelled non-viable cells. Cells were analyzed through a LSR II flow cytometer (BD Biosciences, Mississauga, ON, Canada) set to detect YFP, PI and Alexa 647nm in distinct channels.

For agonist-promoted endocytosis experiment, transiently transfected cells were incubated with 100nM NDP-α-MSH and cell surface expression was monitored after different time of agonist exposure (30 min, 1 hr, 2hr, 4hr, 6hr, 8hr and 22hr).

**Cyclic AMP Assay**

Intracellular cyclic 3’-5’ adenosine monophosphate (cAMP) accumulation was measured using a competitive immunoassay based on HTRF (Homogeneous Time-Resolved Fluorescence) technology (cAMP dynamic-2, Cis-Bio, Bedford, MA, USA).

Each double-tagged construct was transiently transfected into HEK293T cells. Thirty-six hours after transfection, cells were treated in the presence or absence of 10 μM antagonist for 12 hours. Cells were then collected and washed (1XD-PBS pH 7.4, 0.1% glucose). 40,000 cells/well were then dispensed in 96-well plates in cAMP buffer [1XD-PBS, 1% BSA, 0.1% Glucose, 0.75 mM 3-isobutyl-1methyl-xanthine (IBMX, Sigma-Aldricht, Oakville, ON, Canada)] and incubated for 15 min at 37°C in the presence of 100 nM of NDP-α-MSH (Sigma-Aldricht, Oakville, ON, Canada). 10,000 cells were transferred in 384-well plates, lysed and incubated with cAMP
labeled with the dye d2 and anti-cAMP M-Antidody labeled with Cryptate following the
manufacturer’s protocol.

Reading of HTRF signal was performed on Artemis TR-FRET plate reader (Cosmo Bio USA,
Carlsbad, CA, USA).

**Affinity for Melanocortin Receptors**

The affinities (IC₅₀ values) of DCPMP for recombinant human MC1R, MC3R, MC4R, and
MC5R were determined at MDS Pharma Services using traditional radioligand displacement
assays. The assay conditions are accessible on the MDS website.


**Plasma and Brain Quantitation of DCPMP in C57BL/6 Mice**

All *in vivo* procedures were conducted at Eurofins Product Safety Laboratories (EPSL) under
protocols approved by the EPSL Institutional Animal Care and Use Committee and followed all
relevant guidance and regulation, including that set out in the NIH Guide for the Care and Use of
Laboratory Animals. Two doses (3 or 30 mg/kg) of DCPMP, formulated in 90% cottonseed
oil/10% EtOH, were administered to 8-week old C57BL/6 mice by a single intraperitoneal
injection. Conventional liquid chromatography tandem mass spectrometry was used to achieve
separation and detection of analytes in plasma and brain tissue (see supplemental experimental
procedures for description).

The elimination rate constant (kel) was calculated from the formula (below). The half-life (t₁/₂) is
the time taken for the plasma or brain concentration of DCPMP to fall to half its highest value.
Cp is the highest concentration of DCPMP found in plasma or brain at \( t_1 \) and Cp/2 is the concentration one half-life later at \( t_2 \).

\[
\ln \left( \frac{C_p}{2} - \frac{1}{C_p} \right) = -k_{el} \times t_{1/2} \rightarrow k_{el} = \frac{0.693}{t_{1/2}} \text{ where } t_{1/2} = [t_2 - t_1]
\]

**Statistical Analyses**

All curve fitting was conducted using non-linear regression analyses using PRISM (version 4.0c, GraphPad Inc., La Jolla, CA, USA). Data are presented as mean ±SEM and statistical significance of the differences were assessed by one-way ANOVA. Pair-wise comparisons were made by post hoc Bonferroni’s multiple comparison test. Differences with p value <0.05 were considered statistically significant.
Results

Selection and Characterization of hMC4R Mutants for PC Rescue

Ten naturally-occurring MC4R point mutations [S58C, E61K, N62S, I69T, I125K, T162I, R165Q, R165W, C271Y and P299H] that result in severe early-onset obesity in humans were used to investigate the ability of five MC4R antagonists to act as PCs. The selected mutations were chosen based on previous reports suggesting their intracellular retention (Farooqi et al., 2000; Dubern et al., 2001; Farooqi et al., 2003; Nijenhuis et al., 2003; Lubrano-Berthelier et al., 2006; Tan et al., 2009), prevalence in patient populations, and distribution throughout the receptor structural domains (Fig.1, Supplemental Table 1). Wild-type (WT) and each of the ten mutant receptors were tagged with a 3xHA epitope at the N-terminus and a venus yellow fluorescent protein (vYFP) at the C-terminus. Relative cell surface expression was then assessed by differentially monitoring cell surface HA-immunoreactivity and total cellular vYFP fluorescence using dual flow cytometry. For the WT receptor, a generally linear relationship was seen between the total and the cell surface expression (Supplemental Fig. 1A, left panel). In contrast, cell surface expression of mutant receptors only increased minimally as a result of their intracellular retention until total receptor expression reached very high levels (Supplemental Fig. 1A, right panel). Then, to limit artifacts caused by high receptor expression (bypassing the quality control system), our analysis was limited to cells expressing low levels of receptor. The ratio between HA-immunoreactivity and the vYFP signal for each individual cell was used as an index for cell surface trafficking efficiency, which has been arbitrarily fixed at 100% for WT-
hMC4R and used as a reference value.

Under basal conditions, nine mutant receptors [S58C, E61K, N62S, I69T, T162I, R165Q, R165W, C271Y, P299H] showed high levels of intracellular retention, displaying trafficking efficiencies ranging from 20% to 40% of those measured for WT receptor (Fig. 2A). Contrary to what had been previously reported (Xiang et al., 2006; Yeo et al., 2003), I125K-hMC4R was expressed at the cell surface to the same extent as the WT receptor (Fig. 2A). The reason for the difference between the two studies is unclear. It should however be noted that, despite the apparent lack of cell surface expression reported by Xiang et al. using FACS analysis, some binding of the cell impermeable [125I]NDP-α-MSH was observed by these authors (Xiang et al., 2006) as well as by the group of O’Rahilly (Yeo et al., 2003).

Importantly, the reduced cell surface trafficking of the nine other mutants was not accompanied by significant changes in their total expression levels, as illustrated by their equivalent average vYFP signals (Fig. 2A inset). To investigate the signaling capacity of the mutant receptors, cAMP accumulation was determined following agonist stimulation with 100 nM NDP-α-MSH. In contrast to the WT receptor, NDP-α-MSH did not significantly stimulate cAMP production for any of the hMC4R mutants (Fig. 2B).

Selection of MC4R Compounds and Docking in the hMC4R Model

Five MC4R antagonists with distinct structural features (Table 1) were selected as potential PCs
based on published data suggesting high affinity and selectivity for MC4R, and predicted lipophilic properties allowing penetration into intracellular compartments where mutant receptors are retained. The putative binding modes of these compounds on WT-hMC4R were assessed by virtual docking and are presented in Supplemental Fig. 2 and the major contact points listed in Table 2.

Low energy conformations of all five MC4R-selective antagonists were docked into the binding pocket of the hMC4R inactive state model, based on structure-activity relationship (SAR) studies (Arasasingham et al., 2003; Pontillo et al., 2005a; Pontillo et al., 2005b; Vos et al., 2006), knowledge of functionally important receptor residues (Pogozheva et al., 2005; Yang et al., 2009), and in accordance with geometric and polarity matching of ligand and receptor (Supplemental Fig. 2, Table 2).

Three of the antagonists (PPPone, DCPMP, and NBP) could be docked similarly to the well-characterized hMC4R-selective small-molecule agonist, THIQ (Sebhat et al., 2002) (Supplemental Fig. 2A, Table 2). DCPMP is structurally-related to THIQ, harboring a central halogen-substituted 2,4-Cl-D-Phe aromatic ring (“A”) and a phenylpiperazine moiety (“B”) that mimics the cyclohexylpiperazine of THIQ (Supplemental Fig. 2E, Table 2). The basic benzylamine group of DCPMP replaces the N-terminal basic nitrogen of THIQ. The two dipiperazine-based ligands, PPPone and NBP, also have central halogen-substituted phenyl rings (“A”), and a second aromatic function (“B”) that are essential for high binding affinity (Arasasingham et al., 2003), as well as basic nitrogens in the piperazinyl rings. The central
halogen-substituted aromatic ring of these three ligands (“A”) represents the key pharmacophore, and can occupy the same position at the bottom of the binding cavity as the p-chlorophenyl ring of THIQ, forming multiple contacts with aromatic, aliphatic, and sulfur-containing residues from TM3 (I129, C130, L133), TM5 (C196, M200), TM6 (F261, L265), and TM7 (F284, L288). At physiological pH, the benzylamine group in DCPMP (pKa=8.97) and the central piperazinyl group in NBP (pKa=8.92) are positively charged and can form ionic pairs with D126 in TM3 (Supplemental Fig. 2E, F and Table 2). Furthermore, the nitrogens in the terminal piperazinyl groups of PPPone (pKa=8.21) and NBP (pKa=7.91) are also positively charged and may be involved in ionic interactions with both D126 (TM3) and E100 (TM2) (Supplemental Fig. 2C, F and Table 2). The nitrogen from the central piperazinyl group of PPPone (pKa=7.48) would be charged only at a more acidic pH. Additional hydrophobic interactions may form between the benzyl rings of DCPMP, PPPone or the naphthyl ring of NBP and residues from TM4 (F184, I185), TM5 (C196, M200), and TM6 (L265), similar to interactions of the cyclohexyl group and the triazol ring of THIQ. The benzyl ring of DCPMP extends toward the extracellular surface where it may interact with residues from TM4 (F184, I185) as well as with residues from extracellular loop 2 (EXL2) and the N-terminus.

Docking of the structurally distinct ligands MPCI and MTHP were different than that proposed for PPPone, DCPMP, and NBP. SAR studies of MPCI indicate the functional importance of the basic group in the arylguanidine substituent in combination with the central fluorobenzyl ring (“A”) and the second aromatic ring (“B”) with lower lipophilicity. Therefore, we propose that the low energy conformations of MPCI may be docked such that the N⁺ of the
piperidinyl group forms an ion pair with E100 (TM2) and D126 (TM3), while the fluoro-benzyl ring occupies a position similar to the 4-chlorophenyl ring of THIQ (Supplemental Fig. 2D, Table 2). The docking of MTHP is more challenging. A folded conformation of the ligand was chosen, since this is energetically preferred compared to an extended conformation (ΔE>3.5 kcal/mol). Due to the small size, two molecules of MTHP can be easily accommodated within the relatively large receptor binding pocket: one molecule occupying the area between TMs 3-6 and another molecule filling the space between TMs 2, 3, and 7. The positively charged nitrogen of the ligand can form an ionic pair with D126 (TM3), if the ligand is in the first docking pose, or with E100 (TM2), if the ligand occupies the second docking pose (Supplemental Fig. 2B, Table 2).

**Cell Surface Rescue of Mutant hMC4Rs**

The ability of the selected MC4R antagonists to act as PCs was first assessed by monitoring receptor cell surface expression following 12-hour incubation with the compounds at 1 μM and 10 μM. Quantitative analysis of the flow cytometry dot plots (Supplemental Fig. 1 for WT and I69T hMC4R) revealed that cell surface expression of all mutant receptors, except for P299H, was significantly increased after incubation with at least one of the compounds (Fig. 3, left panels). Interestingly, the efficiency of the individual compounds to promote cell surface expression was mutation-dependent.

At the highest concentration tested, DCPMP and NBP showed the broadest activity, significantly increasing the cell surface targeting of all nine responsive mutants (Fig. 3 left...
panels, Table 3). PPPone increased cell surface expression of the same subset of mutant receptors, except for I125K. MTHP significantly increased cell surface levels of S58C, I125K, R165Q, and R165W, while MPCI enhanced cell surface expression of T162I, R165Q, R165W, and C271Y. In some cases, PCs increased cell surface expression at higher levels than the one of untreated WT receptor [e.g. DCPMP and NBP for S58C, I125K, R165Q, R165W; MTHP for I125K, R165Q; PPPone for I69T, R165Q].

The relative apparent potency of the compounds to promote cell surface targeting also varied among the different mutants. This is evident based on the efficiency ratios of cell surface rescue promoted by incubation with the different compounds at 1 μM and 10 μM (Table 3). DCPMP and NBP showed high relative potencies for promoting cell surface targeting (ratios >0.6) of 8/9 and 6/9 mutant receptors, respectively. In contrast, MTHP and PPPone showed lower relative potencies (ratios < 0.6) for 4/4 and 8/8 of the rescued mutants, respectively. Finally, MPCI showed an intermediary relative potency profile with half of the four rescuable mutants below and half above the 0.6 ratio. All compounds had potency ratios below 0.6 to rescue N62S, as well as low potencies to rescue R165W and R165Q, except DCPMP. In contrast, T162I, and C271Y were rescued with potency ratios above 0.6 by three of the five compounds, namely MPCI, DCPMP, and NBP. It should be noted that, although the relative potency values allows to compare the potencies when the ratio is below 1, it does not allow to conclude that compounds that have ratios of 1 have identical potencies since such a ratio simply indicates that the potency of the compound is below 1μM.
Although the compounds varied significantly in their ability to rescue the cell surface expression of different mutants, some generalities can be drawn: two mutants (R165Q and R165W) are rescued by all compounds, two compounds (DCPMP and NBP) rescue all mutants and the subset of mutations that could be partially or fully rescued, is different for each compound (Table 3). Interestingly, MTHP, DCPMP, and NBP also significantly increased the relative cell surface expression of the WT receptor with high potency (relative potency ratios of 0.74, 0.91, and 0.96, respectively), indicating that trafficking efficiency of WT-hMC4R could also be influenced by PCs.

**Functional Rescue of hMC4R Mutants with PCs**

We next investigated whether PC-mediated increases in cell surface expression could also restore signaling activity of the mutant MC4Rs (Fig. 3, right panels). In the case of I125K, none of the compounds could restore NDP-α-MSH-stimulated cAMP response, despite the fact that its cell surface expression could be further increased by three compounds (MTHP, DCPMP, and NBP), indicating that this mutant form of the receptor is unable to respond to agonist. This exception apart, compounds that increased cell surface expression also restored NDP-α-MSH-stimulated cAMP production (Fig. 3, right panels) for all mutants, confirming that rescued mutant receptors are in a conformation that can bind agonist and transduce signal. However, differences in the extent of cell surface and functional rescue were observed. For instance, although NBP very efficiently rescued the cell surface expression of six mutants, it only marginally restored significant signaling for two of them. This most likely result from persistent NBP binding due to
its high affinity (2 nM), which prevents subsequent stimulation with NDP-α-MSH. This hypothesis is supported by the observation that pre-treatment of WT-hMC4R with NBP led to a ~50% inhibition of NDP-α-MSH-stimulated cAMP production (Fig. 3, right panels).

Even for compounds that have lower affinity for the receptor, discrepancies between rescued cell surface expression and signaling activity were observed. This is illustrated by the signaling/cell surface ratio, which is taken as an indication of the signaling efficacy of the rescued receptor and is set at 1 for the WT-hMC4R (Table 4). For four of the mutants (I69T, T162I, R165Q, and R165W), at least one of the compounds restored signaling efficacy ratios to values above 0.8. For other mutations (S58C, E61K, N62S, and C271Y), none of the tested compounds promoted signaling ratios above 0.8, indicating that the conformation stabilized for these mutants may be less efficient for signaling. Interestingly, when considering each mutant receptor, the compounds that favored the highest signaling efficacy ratios were not always the same. For example, DCPMP promoted the highest signaling ratios for R165Q, T162I, and E61K, whereas MTHP and PPPone did so for R165W and I69T.

In several cases, incubation with specific compounds restored cAMP responses that were equivalent or superior to those observed for untreated WT receptor (Fig. 3, right panels). Such complete rescue was seen for different compound/mutation combinations (MTHP: R165Q, R165W; PPPone: I69T, R165Q, R165W; DCPMP: S58C, E61K, N62S, I69T, T162I, R165Q, R165W). Overall, DCPMP emerged as the compound that restored function to the largest subset of mutants. As was the case for cell surface expression, incubation of WT receptor with MTHP,
PPPone, or DCPMP also increased NDP-α-MSH-stimulated cAMP production.

**Pharmacological Characterization of Rescued Receptors**

To further explore the pharmacological properties of the rescued receptor, we performed concentration-response curves for NDP-α-MSH- and α-MSH-stimulated cAMP production in cells expressing WT or three MC4R mutants selected for their different rescue profiles (R165W, N62S, and C271Y; Table 5 and Supplemental Fig. 3). Treatment of cells expressing the WT receptor with DCPMP did not affect the EC50 of either hormone to stimulate cAMP production. For DCPMP rescued R165W, the potency of NDP-α-MSH and α-MSH was almost identical or slightly reduced compared to WT (1.2- and 4-fold, respectively) (Table 5). The potency of both agonists for the rescued N62S was reduced by approximately 5-fold compared to WT receptor. These data indicate that the DCPMP-rescued R165W and N62S have signaling potencies that are not drastically different from those of the WT receptor. In contrast, the EC50 values for both NDP-α-MSH- and α-MSH-stimulated cAMP production for the DCPMP-rescued C271Y were much higher (44- and 32-fold, respectively) than for WT receptor, indicating a significant loss of potency for this receptor mutant (Table 5).

**Pharmacological Characterization of DCPMP**

Since DCPMP efficiently rescued the largest subset of MC4R mutants, it emerges as a potential lead for the development of a therapeutic PC to treat MC4R-linked obesity. Thus, its potency,
selectivity, and *in vivo* pharmacokinetic profile were assessed. The potency of DCPMP to functionally rescue the NDP-α-MSH-stimulated cAMP production by N62S, E61K and R165W is illustrated in Fig. 4. In agreement with the weaker relative potencies of DCPMP to restore cell surface expression of N62S (0.52) and E61K (0.61) vs R165W (1.04) (see table 3), the dose-response curves of DCPMP to restore signaling activity was right shifted for N62S and E61K as compared to R165W. The lack of saturation of the responses for N62S and E61K prevented the determination of an accurate EC₅₀, but an EC₅₀ of 1.5µM was calculated for R165W. This value is 10-fold higher than that obtained for the WT receptor to enhance the signaling response above untreated condition level (data not shown). Although no direct information about the affinity of DCPMP for the mutant form of the receptor is available (i.e.: its relative potency close to 1 for both WT and R165W (Table 3) indicate that its potency is better than 1µM but not necessarily equal for the two receptor forms), the difference in its potency to promote R165W vs WT signaling could indicate that the mutation affects the affinity of the receptor for DCPMP. Alternatively, the persistence of the rescued mutant receptor functionality could be reduced when compared to the WT receptor as a consequence of an accelerated loss of cell surface receptor upon activation. Thus, a higher concentration of the PC may be needed to reach an equivalent functional steady state than with the WT receptor. In order to compare the kinetics of cell surface residency of the rescued R165W with that of the WT receptor, we assessed the kinetics of agonist-promoted endocytosis of the R165W and WT receptors following a 12 hr pre-treatment with DCPMP and compared it with that of the untreated WT receptor. As shown in Fig. 5, stimulation of the receptors with the agonist NDP-α-MSH led to a loss of cell surface receptors with comparable kinetics for the rescued R165W and the WT receptor (pre-treated or not with
DCPMP), indicating that the dynamics of cell rescued R165W once at the cell surface is identical to that of the WT receptor. It is therefore unlikely that the difference in the apparent potency of DCPMP may result from distinct kinetics of cell surface residency of the rescued receptor.

The selectivity of DCPMP for MC4R, was assessed by its ability to inhibit $[^{125}\text{I}]-\text{NDP-}\alpha\text{-MSH}$ binding to human MC1R, MC3R, MC4R, and MC5R. DCPMP displayed high affinity for MC4R with an IC$_{50}$ value of 25 nM, showing a selectivity of 100-fold or more over the three other receptor subtypes (Table 6).

To determine whether DCPMP could reach its intended target in the central nervous system, two doses of DCPMP (3 mg/kg and 30 mg/kg) were administered to 8-week old C57BL/6 mice by a single intraperitoneal injection. The presence of compound was then monitored over a 24-hour period in blood and brain (Fig.6). DCPMP showed similar kinetics in these two tissues, reaching maximal concentrations at 30 and 60 minutes post-injection for the 3 mg/kg and 30 mg/kg dose groups, respectively. Although the maximal concentrations reached in the brain were 16- and 8-fold lower than those in the plasma, the level reached following 30mg/kg administration (0.5µM) is very close to the EC$_{50}$ for PC activity determined in cells (see Fig. 4). The calculated elimination rate constants were similar for brain and plasma (3 mg/kg: 0.69 ng/ml/hr for plasma and brain; 30 mg/kg: 0.35 ng/ml/hr for plasma and 0.23 ng/ml/hr for brain). Importantly, more than 80% of the drug was cleared from brain 4 hrs and 8 hrs post-injection for the 3 mg/kg and 30 mg/kg dose groups, respectively.
Discussion

Our study revealed important mutation-specific differences in the PC action of MC4R antagonists that have distinct chemical structures. Nevertheless, almost all MC4R mutants tested were rescued by at least one PC. Indeed, only two of the ten MC4R mutants were resistant to PC treatment: P299H, the cell surface expression of which could not be restored by any PC, and I125K, which had normal cell surface expression but could not be functionally rescued by any compound despite a facilitation of its cell surface targeting by some PCs. The P299H mutation affects the proline from the conserved N/DPxxY motif and is predicted to eliminate the proline-induced kink in TM7, affecting the packing of TM1, TM2 and TM7 (Tan et al., 2009). This conformational change might result in an unstable receptor that is most likely unable to bind PCs. The lack of functional response observed for I125K is consistent with the large decrease in binding affinity of NDP-α-MSH previously reported (Yeo et al., 2003; Chen et al., 2007; Haskell-Luevano et al., 2001). However this mutation did not prevent the binding of all ligands, MTHP, DCPMP, and NBP potentiated its cell surface expression. Despite this increased cell surface expression, no signaling activity was restored by these three PCs, indicating that the conformations stabilized by the PCs are unable to bind NDP-α-MSH with high affinity and/or unable to transduce the binding into signaling.

Among the five PCs tested, four were able to rescue signaling activity of at least two of the eight rescuable mutants. The only compound that could not restore significant NDP-α-MSH responsiveness was NBP, despite a very potent and efficacious rescue of the cell surface
trafficking, most likely as a consequence of prolonged antagonist action resulting from its very high affinity. The presence of the bulky naphtyl ring as well as two positively charged N⁺ from both piperazinyl groups that form multiple interactions with receptor residues (Supplemental Fig. 2 and Table 2) may explain the higher binding affinity and slow dissociation rate of this ligand.

When considering the four compounds that rescued function, distinct mutation-dependent PC efficacy was observed. Whereas DCPMP significantly restored function to all rescuable mutants, the signaling activity of only some were significantly rescued by PPPone (N62S, I69T, R165Q, R165W), MTHP (I69T, R165Q, R165W), and MPCI (R165Q, R165W). The broader PC action of DCPMP cannot be predicted simply on the basis of the affinity of the ligand for the WT-hMC4R. Indeed, PPPone, which rescues the second largest number of mutant receptor forms, has the lowest affinity. This suggests that structural effects of the mutation as well as the binding modes of the compounds must contribute to the mutant-specific profiles of the PCs. For example, DCPMP restored the highest efficacy ratios for T162I (0.76) and R165Q (0.9), while PPPone promoted efficacy ratios of only 0.05 and 0.37 for the same mutants. In contrast, PPPone restored a higher signaling efficacy than DCPMP for I69T (0.96 vs 0.66). This mutant-dependent action of the PCs may be explained by the fact that T162 and R165 are at the bottom of TM4, a TM that is predicted by our docking data (Supplemental Fig. 2C and E, Table 2) to have more contacts with DCPMP than with PPPone. Thus, the interactions of DCPMP with F184 and I185 at the top of TM4 may compensate for the destabilization of the H-bond network between TM4 and the bottom of TM3 and ICL2 by maintaining the packing of TM4 in the receptor bundle. On the other hand, unlike DCPMP, PPPone interacts with residues of TM2 (E100, I104), interactions
that may stabilize the helix packing between TM2 and TM1, which could have been disrupted by the substitution of the hydrophobic I69 with the smaller polar threonine. Interestingly, the most broadly active compounds (DCPMP, NBP, and PPPone) share a common general binding mode that involves a greater number of receptor residues (Supplemental Fig. 2 and Table 2), indicating that the multiple interactions formed by these three piperazine-containing compounds can stabilize receptors harboring conformational defects originating from mutations in different receptor regions. Despite this general rule, the cell surface expression of two mutants, E61K and C271Y, could be restored to WT levels by only one PC (DCPMP). This may be explained by the more dramatic conformational changes predicted to result from these mutations, making them more resistant than others to stabilization by most PCs. Indeed, substitution of the TM1 E61 by a lysine residue has been proposed to promote the formation of an aberrant hydrogen bond with the free carbonyl group of I296 in TM7 that may change packing interactions of TM1 and TM7 (Tan et al., 2009). The C271Y substitution prevents the formation of a disulfide bond (C271-C277) between the end of TM6 and EXL3 (Tarnow et al., 2003).

When considering signaling efficacy in light of the cell surface rescue, two general scenarios were observed: the signaling efficacy was either proportional or significantly less than predicted from the cell surface targeting. The first scenario is exemplified by I69T, T162I, R165Q, and R165W for which some PCs restored the intrinsic signaling efficacy to levels similar or above that of untreated WT, indicating that PC treatments promoted conformations that are fully competent for signaling. This is somewhat surprising for R165Q and R165W since this arginine has been proposed to interact with D146 of the DRY motif and thus to be important for ligand-
promoted MC4R activation (Xiang et al., 2006). Our data indicate that, although R165 residue may be important in promoting a specific conformation, it is not essential for receptor activation as confirmed by the recovery of the same agonist’s potency as the WT receptor in functional assay after rescue with DCPMP (Table 5). The second scenario is exemplified by S58C, E61K, N62S, and C271Y for which none of the PCs could restore signaling efficacies above 0.75 (see Table 4). Mutations S58C, E61K and N62S are predicted to alter the H-bonding interactions between polar residues from TM1 (S58, N62), TM2 (D90, S94, N97), and TM7 (N294, S295, D298), which play a central role in receptor activation (Govaerts et al., 2005; Tan et al., 2009). For C271Y, the loss of the disulfide bond in EXL3 most likely results in a major conformational change that could alter NDP-α-MSH binding or the translation of ligand binding into receptor activation. Consistent with this notion, the potencies of NDP-α-MSH and α-MSH to stimulate the cAMP production for the DCPMP-rescued C271Y-MC4R were decreased by 44- and 32-fold, respectively, as compared with WT receptor (Table 5).

The different compounds also had different relative potencies and efficacies to promote cell surface expression and enhance cell signaling of WT-hMC4R (Table 4). This observation suggests that a fraction of native receptors fails to achieve proper folding and that the presence of a PC favors such folding. Similar effects of PCs have previously been reported for the delta-opioid (Petaja-Repo et al., 2002) and GnRH receptors (Janovick et al., 2002; Conn et al., 2007). Therefore, in addition to representing a therapeutic avenue for severely obese patients carrying MC4R mutations, PC treatment could possibly be useful for the treatment of obesity in patients with normal MC4R.
It should be noted that the ability of the PCs to restore function was assessed only for the canonical Gs-adenyl cyclase signaling pathway. Given that it has been proposed that different ligand-promoted GPCR conformations may be responsible for the activation of distinct signaling pathways (Galandrin et al., 2007; Rajagopal et al. 2010), it will be interesting to monitor the action of various PCs on the ability of mutant MC4Rs to activate other signaling effectors such as the recruitment of β-arrestin and the activation of MAPK.

To be clinically useful, antagonist PCs need to: 1) selectively bind the targeted receptor; 2) have a sufficiently high affinity for the receptor but a rapid dissociation rate that allows washout and competition by the endogenous ligand; 3) be sufficiently lipophilic to penetrate cell membranes; 4) be as potent and efficacious as possible on the largest subset of receptor mutant forms.

DCPMP possesses many of these characteristics [e.g. high selectivity toward MC4R, broad efficacy toward many mutants, ability to reach the brain in sufficient concentration and relative rapid clearance], making it an interesting lead compound for the development of a therapeutically useful PC. Nevertheless, our data clearly indicate that some mutations could be better rescued by different compounds, suggesting that pharmacogenetics may play an important role in the establishment of PCs as therapeutics. Since the current strategies to control early onset obesity are either modestly effective or very invasive (bariatric surgery), the development of clinically active PC represents an attractive avenue.
Acknowledgements

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References


Footnotes:

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Legends for Figures:

Fig. 1: Naturally-occuring mutations in human melancortin 4 receptor (hMC4R) selected in this study

Schematic representation of hMC4R with the location of the ten naturally-occuring mutations selected in the study (A) and cartoon representation of the homology model of MC4R based on the β2-adrenoreceptor structural template: top view (B) and side view (C). Mutated residues are colored red, residues in the ligand binding pocket are colored blue, receptor helices are colored grey. Position of hydrophobic membrane boundaries are shown in accordance with the OPM database (http://opm.phar.umich.edu). Figures were prepared using PyMOL (http://www.pymol.org).

Fig. 2: Characterization of cell surface expression, total expression levels and signaling capacity of ten mutant forms of hMC4R

(A) Cell surface and total expression of 3HA-MC4R-vYFP mutants measured by FACS. Venus-YFP emission represents MC4R total expression and HA-Alexa647 emission represents MC4R plasma membrane expression. The relative cell surface expression (ratio: Alexa/vYFP emission) is calculated for each individual cell in the selected gate (see Supplemental Fig. 1) and is expressed as a percentage of the value obtained in the same experiment for the WT-hMC4R in the untreated condition. The total expression (see inset) is calculated as the percentage of the mean of vYFP signal emission of each individual cell in the gate of interest (see dot plot graphs in Supplemental Fig. 1) and is expressed as a percentage of the value obtained in the same experiment for the WT-hMC4R in untreated condition. (B) NDP-α-MSH induced cAMP
accumulation expressed as the percentage of agonist stimulated cAMP production of WT-hMC4R. WT-hMC4R absolute values: (basal: 40±8 fmol/104 cells; NDP-α-MSH-stimulated cAMP production: 300±20 fmol/104 cells). For both (A and B), each bar represents the mean ±SEM of at least 3 independent experiments performed in triplicate.

**Fig. 3: Effects of MC4R antagonists on cell surface expression and on stimulated cAMP production of mutant and WT hMC4Rs**

HEK293T cells transiently expressing the indicated double-tagged hMC4R were incubated for 12 hours in the absence (untreated) or presence of antagonist compounds. Left Panel: cell surface expression of each receptor was measured by flow cytometry. Cells were incubated in the absence (untreated) or presence of 1 μM (white bar) or 10 μM (black bar) antagonist compounds. The relative membrane expression (ratio: Alexa/vYFP emission) was calculated for each individual cell in the gate of interest (see dot plot graphs in supplemental Fig. 1) and expressed as a percentage of the value obtained in the same experiment for the WT-hMC4R in the absence of antagonist. Right panel: HEK293T cells transiently expressing the indicated double-tagged hMC4R were incubated for 12 hours in the absence (grey bar, untreated) or presence (black bar) of 10 μM antagonist compounds. cAMP accumulation was measured following 100 nM NDP-α-MSH stimulation. The results are expressed as the percentage of NDP-MSH stimulated cAMP production by WT-hMC4R. Each bar represents the mean ±SEM of at least 3 independent experiments. Dashed lines represent WT-hMC4R cell surface expression (left panel) or cAMP accumulation (right panel) in untreated condition. This level is considered as reference for a full
recovery for mutant receptors. The symbol (*) indicates a significant difference from untreated condition: (*) p<0.05; (**) p<0.01; (***) p<0.001

**Fig. 4: Concentration-response curve of DCPMP treatment on mutant forms of hMC4R**

Signaling capacity of E61K-, N62S- and R165W-MC4R upon stimulation with 100 nM NDP-α-MSH, after 12 hours incubation with increasing concentrations of DCPMP. The data are expressed as the percentage of maximal level of cAMP accumulation of mutant forms of hMC4R upon agonist stimulation in the presence of DCPMP pre-incubation. Each point represents the mean ±SD of 2-3 independent experiments performed in triplicate.

**Fig. 5: Kinetic of agonist-promoted endocytosis of rescued-R165W- and WT-MC4R**

HEK293T cells transiently expressing R165W and WT double-tagged hMC4R were incubated for 12 hours in the absence (only for WT-MC4R) or presence of 10 μM DCPMP. Agonist-promoted endocytosis was induced by 100nM NDP-α-MSH maintained over time. The cell surface expression level was measured by flow cytometry following incubation with agonist for 30 min, 1hr, 2hr, 4hr, 6hr, 8hr and 22hr. The cell surface expression level is expressed as the percentage of the value measured at T0 (no agonist stimulation) in each condition. Each point represents the mean ±SD of 3 independent experiments.

**Fig. 6: Pharmacokinetic profile of the MC4R antagonist DCPMP**

Two doses of DCPMP (3 mg/kg, square, and 30 mg/kg, triangle) were administered to 8-week old C57BL/6 mice by a single intra-peritoneal injection. DCPMP concentrations in blood and
brain were measured at the indicated time. The dashed and solid lines correspond to the concentration measured in brain and blood, respectively. To derive approximate molar concentrations of DCPMP in the brain, we assumed that one gram of tissue corresponds to a volume of one ml.
### Tables

**Table 1: Antagonists selected in the study**

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<th>Structure</th>
<th>Nomenclature</th>
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Values were obtained from radioligand displacement assays by competition with 0.02 nM $[^{125}I]$ NDP-α-MSH on HEK293 cells expressing hMC4R ($K_d$: 0.5 nM; $B_{max}$: 3900 fmol/mg protein).

Bold letters indicate chemical functions used for the acronyms.
Table 2: Description of binding residues for THIQ and antagonists of the study.

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NT: N-terminus;
TM: Transmembrane domain
EXL: Extracellular loop

Residues of MC4R located at 4.5 or 4.0 Å distance from PCs docked in the models of MC4R receptor in the inactive conformation. Residues that are important for THIQ binding (2iqu PDB) are in bold. Residues presented in supplemental Fig.2 are underlined.
Table 3: Overview of efficacy and relative potency of each compound on cell surface rescue

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<th>Construct</th>
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<td>132±23</td>
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<td>R165Q</td>
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<td>344±31</td>
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<td>278±29</td>
<td>277±34</td>
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<td>N/A</td>
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</table>

*a significantly different from untreated condition, p<0.05

*b significantly different from untreated condition, p<0.01

*c significantly different from untreated condition, p<0.001

* significantly different from untreated WT hMC4R, p<0.05

**significantly different from untreated WT hMC4R, p<0.01

Untreated = relative membrane expression reported as a percentage of the value obtained in the same experiment for the hMC4R (WT) in the absence of antagonist.
Efficacy = relative surface expression level attained after overnight incubation with 10μM antagonist.

Relative Potency = ratio between the mean of the relative surface expression level obtained at 1μM and 10μM.

N/A = not applicable, since no significant rescue was observed at high concentration.

Data shown are the mean ± SEM of at least three independent experiments and are derived from Figure 3.
Table 4: Comparison of cell surface expression and signaling efficacy for each compound

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>MTHP</th>
<th>PPPone</th>
<th>MPCI</th>
<th>DCPMP</th>
<th>NBP</th>
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<tr>
<td></td>
<td>Surface expression</td>
<td>Signaling</td>
<td>Ratio</td>
<td>Surface expression</td>
<td>Signaling</td>
<td>Ratio</td>
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<tr>
<td>WT</td>
<td>100</td>
<td>100</td>
<td>1</td>
<td>172±7</td>
<td>383±41^a</td>
<td>2.23</td>
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<tr>
<td>S58C</td>
<td>40±5</td>
<td>16±2**</td>
<td>0.40</td>
<td>102±4</td>
<td>34±9</td>
<td>0.33</td>
</tr>
<tr>
<td>E61K</td>
<td>26±3</td>
<td>14±2**</td>
<td>0.54</td>
<td>37±3</td>
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<td>18±8</td>
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<td>0.56</td>
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<td>0.28</td>
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<td>I69T</td>
<td>34±11</td>
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<td>0.15</td>
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<td>0.67</td>
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<tr>
<td>T125K</td>
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<td>0.07</td>
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<td>61±17</td>
<td>16±0</td>
<td>0.26</td>
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<td>R165Q</td>
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<td>86±5</td>
<td>94±21^a</td>
<td>1.09</td>
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<tr>
<td>C271Y</td>
<td>24±3</td>
<td>4±1**</td>
<td>0.17</td>
<td>26±0.5</td>
<td>4±1</td>
<td>0.15</td>
</tr>
</tbody>
</table>

^a significantly different from untreated condition, p<0.05

^b significantly different from untreated condition, p<0.01

**significantly different from untreated WT hMC4R, p<0.01

Cells expressing WT or mutant hMC4Rs were pre-incubated in the absence or presence of 10 μM of the indicated compound for 12hrs. Following washes, cells expressing WT or mutant hMC4Rs were stimulated for 15 min at 37°C with 100 nM NDP-α-MSH.
Table 5: Efficacy and Potency of melanocortin agonists to stimulate cAMP production of WT and mutant hMC4Rs rescued by DCPMP pretreatment

<table>
<thead>
<tr>
<th>Mutation</th>
<th>location</th>
<th>untreated 10 μM DCPMP</th>
<th>untreated 10 μM DCPMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>4.01±0.82 (7)</td>
<td>3.9±0.9 (9)</td>
</tr>
<tr>
<td>N62S</td>
<td>TM 1</td>
<td>18.5±0.8 (3)*</td>
<td>275.4±57.1 (3)</td>
</tr>
<tr>
<td>R165W</td>
<td>TM 4</td>
<td>4.8±1.3 (3)</td>
<td>297.0±23.4 (3)</td>
</tr>
<tr>
<td>C271Y</td>
<td>ECL3</td>
<td>175±82.1 (4)**</td>
<td>152.9±50.2 (4) b</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Mutation</th>
<th>location</th>
<th>untreated 10 μM DCPMP</th>
<th>untreated 10 μM DCPMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>0.3±0.07 (9)</td>
<td>196.7±45.3 (9)</td>
</tr>
<tr>
<td>N62S</td>
<td>TM 1</td>
<td>2.02±0.7 (3)** a</td>
<td>367.3±82.5 (3)</td>
</tr>
<tr>
<td>R165W</td>
<td>TM 4</td>
<td>1.5±0.4 (5)* a</td>
<td>213.5±64.8 (5)</td>
</tr>
<tr>
<td>C271Y</td>
<td>ECL3</td>
<td>12.3±6.5 (3)** c</td>
<td>106.7±8.4 (3) a</td>
</tr>
</tbody>
</table>

* significantly different from treated WT hMC4R, p<0.05
b significantly different from treated WT hMC4R, \( p<0.01 \)

c significantly different from treated WT hMC4R, \( p<0.01 \)

* significantly different from untreated WT hMC4R, \( p<0.05 \)

** significantly different from untreated WT hMC4R, \( p<0.01 \)

*** significantly different from untreated WT hMC4R, \( p<0.001 \)

Data shown are the mean ± SEM of the number of experiments indicated in brackets.

EC\textsubscript{50} is the concentration of ligand that results in 50% stimulation of the maximal response (Rmax).
Table 6: Melanocortin receptor selectivity of DCPMP

<table>
<thead>
<tr>
<th>Ligand DCPMP</th>
<th>% inhibition @10μM</th>
<th>IC50 (μM)</th>
<th>MC4R selectivity</th>
</tr>
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<tbody>
<tr>
<td>MC4R</td>
<td>88</td>
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<tr>
<td>MC1R</td>
<td>15</td>
<td>&gt;10</td>
<td>&gt;400</td>
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<tr>
<td>MC3R</td>
<td>70</td>
<td>3.07</td>
<td>123</td>
</tr>
<tr>
<td>MC5R</td>
<td>68</td>
<td>4.04</td>
<td>162</td>
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</table>

The affinities (IC₅₀ values) of DCPMP for recombinant human MC1R, MC3R, MC4R, and MC5R in HEK293 cells were determined using traditional radioligand displacement assays in the presence of 0.04 nM [¹²⁵I] NDP-α-MSH for MC1R [Kₐ 0.037 nM]; 0.035 nM [¹²⁵I] NDP-α-MSH for MC3R [Kₐ 0.24 nM]; 0.02 nM [¹²⁵I] NDP-α-MSH for MC4R [Kₐ 0.5 nM]; 0.035 nM [¹²⁵I] NDP-α-MSH for MC5R [Kₐ 0.53 nM]
Fig. 1
Fig. 2
Fig. 3A

Cell surface expression (% untreated WT)

WT

S58C

E61K

N62S

I69T

I125K

1μM

10μM

Untreated

MTPH

PPPone

MPCI

DCPMP

NBP

cAMP accumulation (% untreated WT)

Untreated

MTPH

PPPone

MPCI

DCPMP

NBP

* p < 0.05

** p < 0.01

*** p < 0.001
Fig. 3B

Cell surface expression (% untreated WT)

CAMP accumulation (% untreated WT)
Fig. 5

% of cell surface expression vs. Time (hour)

- WT
- WT + DCPMP
- R165W + DCPMP
Supplemental Data

Pharmacological chaperones restore function to MC4R mutants responsible for severe early-onset obesity

P. René, C. Le Gouill, I. D. Pogozheva, G. Lee, H. I. Mosberg, I. S. Farooqi, K. J. Valenzano and M. Bouvier

Journal: The Journal of Pharmacology and Experimental Therapeutics

The PDF file includes:

SUPPLEMENTAL MATERIALS AND METHODS

SUPPLEMENTAL FIGURES

Supplemental Fig. 1. Flow cytometry dot plot graphs of cell surface expression measurement for WT and I69T hMC4Rs

Supplemental Fig. 2. Docking of the selected compounds in hMC4R model

Supplemental Fig. 3. Concentration response of WT and mutant hMC4Rs expressing cells upon NDP-α-MSH and α-MSH stimulation following DCPMP rescue

Supplemental Table 1. Number of probands reported in the literature for each mutation

SUPPLEMENTAL REFERENCE
Supplemental Materials and Methods

Generation of Mutant hMC4R Construct
Five mutant forms of hMC4R [S58C, N62S, R165Q, R165W and P299H] N-terminally tagged with 3XHA were generated by site-directed mutagenesis by overlap extension (Ho et al., 1989). Generation of 3XHA N-terminally tagged hMC4R wild-type and mutants by site-directed mutagenesis by overlap extension. This procedure involves two steps: 1- introduction of the desired base substitution into the hMC4R (WT) receptor cDNA using specifically designed complementary and overlapping primers, followed by 2- amplification of the mutated cDNA using the polymerase chain reaction (PCR). Each point mutation was inserted by PCR performed with Phusion taq polymerase (Fynzymes, NEB, Ontario, Canada) using specific primers containing the mutation complementary to opposite strands of the hMC4R (WT) template (*) and either a: T7-Forward primer (5'-ATTAAATACGACTCCTAGG-3')
or pcDNA3.1-Reverse primer (5'-AGAACGTGGACTCCAACGTCAAAG-3')
*S58C Forward: 5'-CT CTG GGT GTC ATC
TGC TTG GAG AAT ATC-3'
*N62S Forward: 5'-C ATC AGC TTG TTG GAG A
GTT ATCTTAGTG ATT GTG GC-3'
*R165Q Forward: 5'-G ACA GTT AAG C
AG GTT GGG ATC ATC-3'
*R165W Forward: 5'-G ACA GTT AAG
GG GTT GGG ATC ATC-3'
*P299H Forward: 5'-G TGT AAT TCA ATC ATC GAT C
ATG ATT TAT GCA CTC CGG AG-3'
The first fragment was generated using the primers T7-Forward primer and the reverse/antisense primer complementary to forward sequence above and the second fragment was generated using the pcDNA3.1-Reverse primer and the forward/sense primer (sequence above). The 3xHA-hMC4R (WT) cDNA (Missouri S&T cDNA Resource center, USA) served as the template in these PCR reactions. Reaction conditions were 30 cycles of 94 °C (30 s), 55 °C (1 min), and 72 °C (1 min). The fragments were then purified using the QIAGEN PCR purification kit (QIAGEN Mississauga, ON, Canada) and combined in the overlap extension reaction using T7-Forward and pcDNA3.1-Reverse primers described. Full length mutant PCR products were purified with QIAGEN gel extraction kit (QIAGEN Mississauga, ON, Canada) and inserted after restriction digest in KpnI/XhoI pcDNA3.1(+) vector.
Double tagged hMC4R(WT) with 3xHA tag at the N-terminus and a Venus tag at the C-terminus was generated by PCR using a 3HA-MC4R-Forward primer containing the start codon and BamHI restriction site upstream and a MC4R-Reverse primer containing the end of the coding sequence of hMC4R without the stop codon, a flexible linker (VGGGGS) and an AgeI restriction site downstream. The PCR product was purified and inserted into a BamHI/AgeI pcDNA3.1(+-) Venus vector. The Venus tag was added to the 3xHA-hMC4R mutant constructs by subcloning the hMC4R C-terminus, flexible linker and Venus coding sequence derived from BspEI/Xbal 3xHA-hMC4R(WT)-Venus construct.
The mutant hMC4Rs [E61K, I69T, I125K, T162I, C271Y] were subcloned in the 3xHA-hMC4R-Venus core plasmid using Bsu36I and BspEI internal restriction sites to replace the WT-hMC4R coding sequence with the mutant. All PCR products were sequenced to confirm the presence of the desired mutations and the absence of unwanted mutations.

Docking procedure
The docking of each ligand was performed in three steps. First, ligands were manually positioned inside the receptor binding cavity to satisfy the following criteria: 1- similar spatial arrangement of common pharmacophore groups in five small-molecule antagonists and a small-molecule agonist, THIQ, whose docking in MC4R has been previously justified using conformational and mutagenesis analysis (Pogozheva et al., 2005); 2- interaction of most functional groups of the ligands, especially the central halogeno-substituted aromatic ring (“A”), the second aromatic ring (“B”), and positively charged N*(of piperazine group, 1,2,5,6-tetrahydropyrimidimyl group or benzylamine group) with the most functionally important receptor residues, such as aromatic residues from TM6 (F261, F262, W258) or acidic residues from TM2 (E100) and TM3 (D122, D126), respectively; 3- minimization of steric overlap and maximization of hydrogen-bonding between receptor and ligands. Second, each docking pose of each ligand was refined using Solid Docking module of QUANTA and the most common poses were selected for further analysis. Final energy minimization of complexes of the wild type receptor in the inactive conformation with all five PCs in selected docking poses was performed with CHARMM module of QUANTA using \( \varepsilon = 10 \) and the adopted basis Newton-Raphson method (100 steps).

**Plasma and Brain Quantitation of DCPMP in C57BL/6 Mice**

Whole blood of 8-week old C57BL/6 mice was drawn into lithium heparin tubes from the inferior vena cava after CO\(_2\) euthanization at various time points over a 24-hour period. Plasma was collected by spinning blood at 2700g for 10 min at 4 °C. Brains were subsequently removed, washed in cold PBS, blotted dry, and weighed before storing on dry ice. Mouse brain tissue (~100 mg) was homogenized and extracted in 300 \( \mu \)L acetonitrile:methanol (ACN:MeOH) (70:30) using a Fast Prep homogenizer (MP Biomedical) followed by centrifugation at 10600g for 5 minutes. Verapamil solution (50 ng/mL) was added as an internal standard to 25 \( \mu \)L of tissue homogenate or mouse plasma. After further processing, 10-20 \( \mu \)L was injected on to the LC/MS. Conventional liquid chromatography tandem mass spectrometry was used to achieve separation and detection of analytes. High-performance liquid chromatography analysis was conducted using the Shimadzu SIL-HT system on a dc 18 column (50 x 4.6 mm, 5 mm; Waters Atlantis; Milford, MA) at a flow rate of 1 ml/min using gradient conditions. The mobile phase was an ACN/water/formate system. MS/MS analysis was carried out on a Sciex 3000 under positive ion mode (ESI +). The following transitions were monitored: m/z 535.2→m/z 175.1 for DCPMP and m/z 455.3→m/z 165.1 for the verapamil internal standard. To derive approximate molar concentrations of DCPMP in brain, one gram of tissue was estimated as one ml of volume.
Supplemental Figures

Supplemental Fig. 1: Flow cytometry dot plot graphs of cell surface expression measurement for WT and I69T hMC4Rs

Each double-tagged construct was transiently transfected into HEK293T cells. Cells were untreated or treated for 12 hours with two concentrations of PPPone: A-untreated condition; B-1 μM PPPone or 10 μM PPPone. Detection of cell surface expression of double-tagged receptors is performed by labeling with the mouse monoclonal anti-HA (HA.11) antibody and the anti-mouse Alexa647nm secondary antibody. Venus-YFP (v-YFP) emission represents hMC4R total expression and HA-Alexa 647 emission represents hMC4R plasma membrane expression. To limit artifacts caused by high receptor expression (bypassing the quality control system), our analysis was limited to cells expressing low levels of receptor as defined by the selected gate (dash lines).
Supplemental Fig. 2: Docking of the selected compounds in hMC4R model

Docking pose of THIQ (A) in the binding pocket of the active conformation of hMC4R (1iqu PDB file, Pogozheva et al., 2005) in comparison with proposed docking poses of different PCs in the binding pocket of the inactive conformation of hMC4R: two MTHP molecules docked simultaneously (B), PPPone (C), MPCI (D), DCPMP (E), and NBP (F). Receptor helices are colored using the rainbow feature, residues are colored by atoms with N-atoms colored dark blue, O-atoms colored red, Cl-atom colored green, F-atoms colored light blue, S-atom colored yellow, and C-atoms colored either purple (for ligands), or grey (for residues from the binding pocket), or yellow (in A: for residues whose mutations were shown to impair the THIQ binding (Pogozheva et al., 2005; Yang et al., 2009)). Two major aromatic pharmacophore groups of ligands corresponding to 4Cl-DPhe and cyclohexyl rings of THIQ are marked by “A” and “B” letters, respectively.
Supplemental Figure 3: Concentration response of WT and mutant hMC4Rs expressing cells upon NDP-α-MSH and α-MSH stimulation following DCPMP rescue

Each double-tagged construct was transiently transfected into HEK293T cells. Thirty-six hours after transfection, cells were incubated in the absence or presence of 10μM DCPMP for 12 hours. Forty-eight hours after transfection, cells were collected and washed. cAMP accumulation was measured following stimulation for 15min at 37 C with increasing concentration of NDP-α-MSH or α-MSH. Results are expressed as the mean ± SEM of at least 3 independent experiments.
**Supplemental Table 1: Number of probands reported in the literature for each mutation**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Location</th>
<th># of probands</th>
<th>Genotype</th>
<th>Reference</th>
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<td>Tan et al., 2009</td>
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<td>Tan et al., 2009</td>
</tr>
<tr>
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<td>TM3</td>
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<td>heterozygous</td>
<td>Farooqi et al., 2003</td>
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SUPPLEMENTAL REFERENCES


