Supplemental Data

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

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Journal: The Journal of Pharmacology and Experimental Therapeutics

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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'-ATTAATACGACTCAGACTATAGG-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 AGT ATCTTAGTG ATT GTG GC-3'
*R165Q Forward: 5'-G ACA GTT AAG CAG GTT GGG ATC ATC-3'
*R165W Forward: 5'-G ACA GTT AAG TGG GTT GGG ATC ATC-3'
*P299H Forward: 5'-G TGT AAT TCA ATC ATC GAT CAG TCT 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 Agel 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 ε=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 CO₂ 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 μL 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 μL of tissue homogenate or mouse plasma. After further processing, 10-20 μL 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-1μM PPPone or 10μM 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**

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<th>Mutation</th>
<th>Location</th>
<th># of probands</th>
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SUPPLEMENTAL REFERENCES


