

Protein Folding and Misfolding: Mechanisms and Consequences

Irish Area Section Meeting held at the Glenroyal Hotel/National University of Ireland Maynooth, Ireland, 1-2 December 2012. Organized and Edited by Gary Jones (National University of Ireland Maynooth, Ireland).

Pharmacological chaperones increase the cell-surface expression of intracellularly retained mutants of the melanocortin 4 receptor with unique rescuing efficacy profiles

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Abstract

Mutated versions of membrane proteins often fail to express at the plasma membrane, but instead are trapped in the secretory pathway, resulting in disease. The retention of these mutant proteins is thought to result from local misfolding, which prevents export from the ER (endoplasmic reticulum), targeting the receptor for degradation via the ER-associated quality control system. The rhodopsin-like G-protein-coupled MC₄R (melanocortin 4 receptor) is an example of such a membrane protein. Over 100 natural MC₄R mutations are linked with an obese phenotype and to date represent the most common monogenic cause of severe early-onset obesity. More than 80% of these mutations result in a substantial proportion of MC₄R being retained intracellularly. If these receptors were expressed at the plasma membrane, many could be functional, as mutations often occur in regions distinct from those associated with ligand or G-protein binding. Our aim is to show proof of concept that selective compounds can rescue the function of MC₄R mutants by increasing their cell-surface expression, and further to this, examine whether the rescue profile differs between mutants. Whole-cell ELISA and 96-well fluorescence-based assays with N-terminally HA (haemagglutinin)-tagged and C-terminally mCherry-tagged mutant MC₄R were used to screen a number of novel MC₄R-selective compounds. A total of four related compounds increased the cell-surface expression of wild-type and three intracellularly retained mutant MC₄R, thus acting as pharmacological chaperones. There appears to be a unique rescue efficacy profile for each compound that does not correlate with potency, suggesting distinct receptor conformations induced by the different mutations. A degree of functionality of V50M and S58C was also rescued following relocation to the cell surface.

Introduction

Several obesity-causing mutant MC₄R (melanocortin 4 receptors) have been studied in detail with respect to their

cellular localization, and subsequent ligand binding and signalling capabilities [1,2], suggesting that approximately 80% of mutations in MC₄R found in early-onset obesity result in reduced plasma membrane expression [3]. Similar to many other naturally occurring point mutations in GPCRs (G-protein-coupled receptors), the intracellular retention is thought to be a result of the cellular chaperone-mediated quality control machinery detecting an aberrant fold and clearing the misfolded proteins by the ERAD [ER (endoplasmic reticulum)-associated degradation] system [4]. Interestingly, the degree of severity of intracellular retention of these mutations can vary, with some being almost

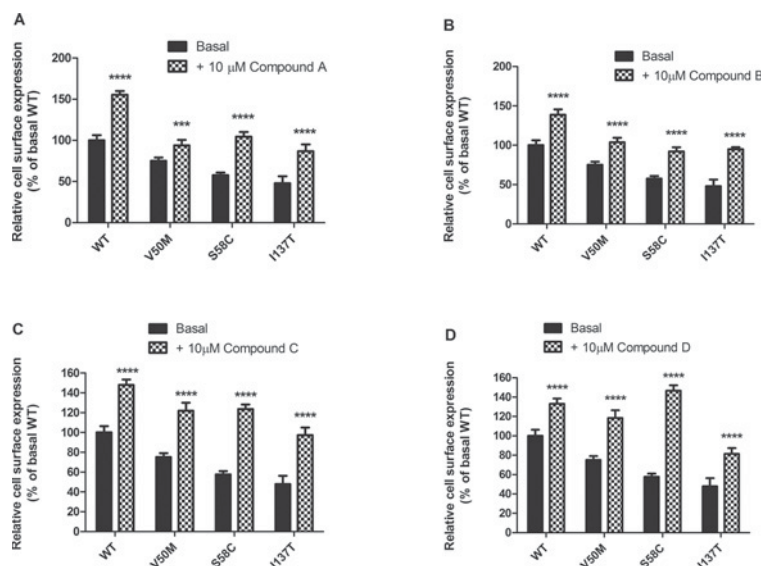
Key words: cell-surface rescue, intracellular retention, melanocortin 4 receptor (MC₄R), pharmacological chaperone, rescue efficacy profile.

Abbreviations used: ER, endoplasmic reticulum; GPCR, G-protein-coupled receptor; HA, haemagglutinin; HEK, human embryonic kidney; MC₄R, melanocortin 4 receptor; V₂R, vasopressin 2 receptor.

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Figure 1 | Novel MC₄R antagonists rescue the cell-surface expression of MC₄R mutants V50M, S58C and I137T (A) Compound A, (B) Compound B, (C) Compound C and (D) Compound D. Cell-surface expression is expressed as a percentage of basal wild-type. Results are means \pm S.E.M. ($n = 3$ performed in triplicate). *** $P < 0.0005$ and **** $P < 0.0001$ (Student's t test) compared with basal conditions of each receptor.



completely absent from the plasma membrane, whereas others retain significant cell-surface expression [5,6]. The ability to rescue the function of these mutations could be of significant therapeutic value in the treatment of severe early-onset obesity.

Evaluation of pharmacological chaperone action

Wild-type and mutant human MC₄R were tagged with an N-terminal HA (haemagglutinin) epitope and a C-terminal mCherry fluorescent protein and transiently expressed in HEK (human embryonic kidney)-293 cells. Relative plasma membrane expression was then assessed by monitoring plasma membrane HA immunoreactivity and total cellular mCherry fluorescence with the use of high-throughput confocal microscopy and whole-cell ELISA. For a quantitative measurement of cell-surface rescue, the ratio between HA immunoreactivity and the mCherry signal for each assay well was used as an index for the efficiency of plasma membrane trafficking following the addition of the rescuing compounds in order to correct for transfection efficiency and expression levels. Since MC₄R is linked to G_{αs}, functionality of receptors was determined by measuring the accumulation of the second messenger cAMP using a FRET (fluorescence resonance energy transfer)-based immunoassay (LANCER®).

Evidence to support a unique rescue profile of pharmacological chaperones

Consistent with previously published data, the point mutations in MC₄R chosen for this investigation (V50M, S58C and I137T) were found to disrupt the normal

trafficking of the receptor and resulted in reduced cell-surface expression due to retention within the cell [3,7]. Confocal microscopy (results not shown) and a semi-quantitative whole-cell ELISA confirmed that the proportion of receptor expressed at the plasma membrane differs between mutants, suggesting that the severity of trafficking defects among intracellularly retained MC₄R mutants varies. The trafficking efficiencies of the mutant receptors range from 40% to 80% compared with those measured for the wild-type receptor (Figure 1).

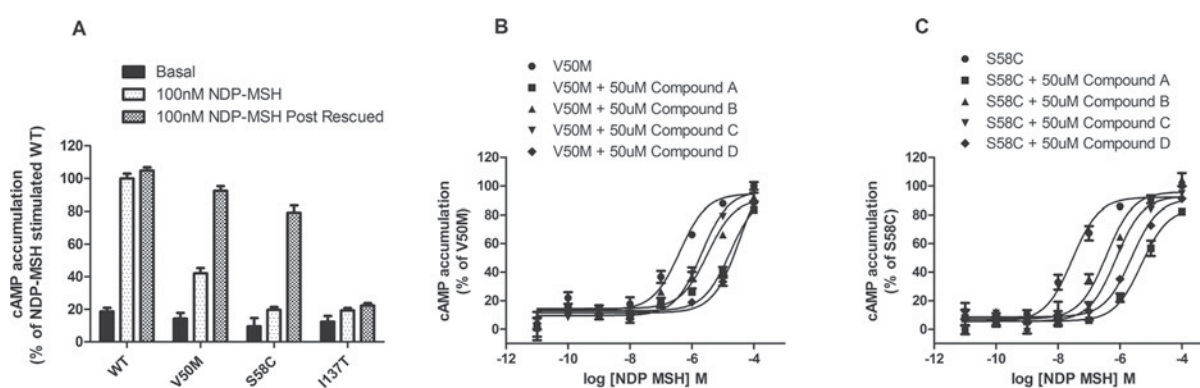
The plasma membrane expression of obesity-causing intracellularly retained mutants of MC₄R can be rescued by the addition of four cell-permeable MC₄R antagonists. Interestingly, the rescue profile of the four compounds differs between the receptors, including wild-type (Figure 1). Looking at the compounds individually, Compound A has the most marked effect on S58C and I137T, increasing their cell-surface expression by approximately 80%. Plasma membrane expression of V50M also recovers, by approximately 25%. Compound B appears to have a significantly higher rescue efficacy for I137T, increasing its appearance at the plasma membrane by almost 100% of its basal expression. A 40% recovery is seen with V50M, and a 60% recovery with S58C. Compound C rescues S58C and I137T, increasing cell-surface expression by more than 100% of their basal level; V50M shows an increase in plasma membrane expression of 60%. Lastly, Compound D displays a phenomenal preference in rescue efficacy for S58C, increasing its cell-surface expression by over 150%. Lower, but still significant, rescuing effects are seen with I137T (~70%) and V50M (~60%). The efficacy profiles of the compounds, with respect to plasma membrane expression, are summarized in Table 1.

Table 1 | Comparison of the ability of compounds A–D to rescue the cell-surface expression of MC₄R mutants V50M, S58C and I137T as measured by whole-cell ELISA

Relative cell-surface expression (% increase from basal)					
MC ₄ R	+ 0.1% DMSO	+ 10 μ M Compound A	+ 10 μ M Compound B	+ 10 μ M Compound C	+ 10 μ M Compound D
Wild-type	25 \pm 2.9	55 \pm 2.7	39 \pm 3.9	48 \pm 3.0	33 \pm 3.1
V50M	18 \pm 1.9	25 \pm 3.9	38 \pm 3.3	62 \pm 4.8	58 \pm 4.6
S58C	11 \pm 2.3	81 \pm 3.3	60 \pm 3.0	114 \pm 2.7	154 \pm 3.2
I137T	11 \pm 3.1	81 \pm 4.8	98 \pm 1.5	103 \pm 4.5	70 \pm 3.5

Figure 2 | Functionality of MC₄R mutants is restored using four novel pharmacological chaperones with unique rescue efficacy profiles that do not correlate to potency

(A) The signalling capabilities of mutant human MC₄R following rescue to the cell surface. Results are means \pm S.E.M. percentages of NDP- α -MSH ([Nle⁴, D-Phe⁷] α -melanocyte-stimulating hormone) agonist-stimulated wild-type ($n=3$ performed in triplicate). (B and C) Concentration–effect curves of NDP- α -MSH in the presence of compounds A–D on rescued V50M expressed in HEK-293 cells (B) and on rescued S58C expressed in HEK-293 cells (C).



These observations were further reinforced using a fully quantitative high-throughput 96-well fluorescence assay, which corrects for any differences in transfection efficiency and expression levels. The ratio of plasma membrane receptors to total receptor expression can be used as a direct comparison between wild-type and mutant receptors under varying conditions, giving a method for assessing the rescue efficacy of potential pharmacological chaperones. However, one ought not to overlook the possibility that such compounds, if antagonists, will stabilize an inactive conformation of receptor, thereby not only allowing release to the cell surface, but also simultaneously preventing the targeting of a properly folded receptor for degradation. The whole-cell ELISA, although only semi-quantitative, may nevertheless be a better, but more laborious, measure of receptor rescue provided that the total receptor levels do not vary markedly.

Exposure to the putative pharmacological chaperones also increases wild-type MC₄R plasma membrane expression. This is not wholly surprising, as exposure of the heavily retained wild-type δ opioid receptor to selective cell-permeable antagonists promotes almost full plasma membrane expression [8,9], and in our study the receptors are transiently expressed, meaning a greater level of protein expression. However, exposure of the wild-type V₂R (vaso-

pressin 2 receptor) to the selective cell-permeable antagonist SR121463A does not appear to promote increased cell-surface expression as measured by FACS [10], although that study was carried out using stable cell lines, where the expression levels and receptor pool reserves are likely to be lower. It is relevant, perhaps, to keep in mind that GPCRs are dynamic structures that are in a state of equilibrium between active and inactive conformations. The shifts of this equilibrium between the two conformations are likely to be different and produce different consequences for different GPCRs.

Two important controls were included in our study. In the first, the pharmacological chaperone for the V₂R failed to increase trafficking of the MC₄R mutants, indicating specificity in the effects of compounds A to D. Secondly, the addition of a high-affinity water-soluble non-permeable peptide antagonist of the MC₄R produced only a very slight increase in cell-surface expression of the mutants, indicating that inhibition of down-regulation is not the mechanism of action of the permeable small-molecule antagonists included in our study.

The results of our study also show that, following rescue to the plasma membrane, a degree of functionality could be restored to two of the three mutants examined in our study, as demonstrated by a significant increase in cAMP production upon stimulation with agonist (Figure 2), as

well as an increase in the EC_{50} and E_{max} values (results not shown). Interestingly, the rescue efficacy profiles of the compounds (Figure 1) does not appear to correlate with their potency at the two rescued receptors (Figure 2). Restored functionality of mutant receptors suggests that the cell-surface rescue of intracellularly retained MC_4R mutants using pharmacological chaperones is a promising and exciting concept that could prove to be a novel therapeutic avenue in the treatment of early-onset obesity.

Conclusions

The results of our study support the view that different MC_4R point mutations cause specific local misfolding of the receptors, leading to distinct unstable conformations that cause intracellular retention. These conformations can be rescued using MC_4R -specific antagonists, presumably by stabilizing an inactive state of the receptor. The effect varies with the mutants and the antagonists, either due to a different energetic profile of the individual mutants or through differences in the mode of interaction between receptor and antagonist, or both. The potency of the compounds at rescued receptors does not appear to correlate with rescuing efficacy.

Recently, a similar study with five cell-permeable antagonists of MC_4R were investigated for cell-surface rescue of a different cohort of intracellularly retained MC_4R mutants (including S58C) [11]. Differences in the rescuing efficacies between compounds with each mutant were again found, supporting the unique rescue profiles for individual pharmacological chaperones demonstrated in our study. A degree of functionality of some of the mutants in our study was restored upon rescue to the cell surface, indicating potential therapeutic benefits to the use of pharmacological chaperones in MC_4R -related early-onset obesity.

Since all four compounds can promote a significant proportion of the wild-type receptor to plasma membrane, pharmacological chaperones could also potentially be used in obese individuals with normal MC_4R .

Funding

This research was supported by the European Commission [grant number LSHB-CT-2007-037365 – TARGETSCREEN2].

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Received 24 April 2012
doi:10.1042/BST20110764