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Functional selectivity of melanocortin 4 receptor peptide and non-peptide agonists: Evidence for ligand specific conformational states.

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Abstract

Agonists of the melanocortin 4 receptor (MC4R) have potential pharmaceutical benefit in the treatment of obesity and sexual dysfunction. In this study we have compared the ability of a number of peptide and non-peptide agonists to activate a FLAG-tagged human MC4 (FMC4) receptor, as measured by both cAMP accumulation and calcium mobilisation (FLIPR). In addition, we have analysed the ability of these agonists to cause receptor internalisation, as measured by FACS analysis. The endogenous agonist α-MSH increased cAMP accumulation, calcium mobilisation and receptor internalisation, in a dose-dependent manner in HEK-293 cells expressing the FMC4 receptor. The activity of the other agonists varied considerably in these assays and overall, the potency and intrinsic activity of the agonists in the cAMP accumulation assays did not correlate with their potency or intrinsic activity in either the FLIPR or receptor internalisation assays. Agonists could be clearly separated into two functional classes based on their structure. Peptide agonists β-MSH, Des-Acetyl-α-MSH, and NDP-MSH exhibited 80-112% of the maximal α-MSH response in cAMP accumulation and 62-96% in FLIPR assays, and were able to cause 75-118% of receptor internalisation induced by α-MSH. Conversely, although the non-peptide agonists exhibited 73-149% of the α-MSH response in the cAMP accumulation assays, they were significantly impaired in the FLIPR (7-40%) and receptor internalisation (-5-38%) assays. These findings demonstrate an important difference in activation and internalisation of the MC4 receptor by non-peptide versus peptide agonists and provides evidence of agonist specific conformational states.

Introduction

The melanocortin 4 receptor (MC4R) is a member of the seven transmembrane receptor family, that initiate signal transduction through activation of heterotrimeric G proteins. There are four additional members of the melanocortin receptor subfamily, distinguishable by their distribution and physiology. Melanocortin receptors are all activated by one or more pro-opiomelanocortin derived peptides, which include α- β- and γ-melanocortin stimulating hormone (MSH). In addition, the melanocortin receptors are regulated by the antagonists/inverse agonists, AGRP and agouti (Lu et al., 1994; Ollman et al., 1997).

The melanocortin receptors all signal through Gαs, which stimulates the production of cAMP by adenylate cyclase. The MC3 receptor also functions through phospholipase C (PLC) to increase intracellular calcium (Konda et al., 1994). Likewise, the MC4 receptor mediates an increase in intracellular calcium, but reportedly through a cholera toxin sensitive pathway rather than PLC (Mountjoy et al., 2001). MC4R activation of the MAP kinase pathway is, however, mediated by inositol triphosphates (Daniels et al., 2003; Vongs et al., 2004). In addition, to activating signalling pathways, α-MSH binding to the MC4 receptor activates regulatory pathways, which include receptor internalisation (Shinyama et al., 2003; Gao et al., 2003). This is of potential interest as not only is internalisation involved in receptor regulation, recent findings have suggested that it may contribute to signalling as receptors can interact with downstream effectors once internalised (Holstein et al., 2004; Daaka et al., 1998).

There has been much research in understanding the physiology of the MC4 receptor, as agonists of this receptor are of potential pharmaceutical benefit in the treatment of both obesity and sexual dysfunction. The MC4 receptor is a major regulator of energy homeostasis. Activation of the MC4 receptor by MTII reduces food intake in rodents, and conversely antagonism of the receptor by SHU9119 increases food intake (Fan et al., 1997). In addition, MC4 receptor knockout mice display hyperphagia and accelerated weight gain (Huszar et al., 1997) and over-expression of AGRP results in obesity (Butler et al., 2001). MC4 receptor agonists have also been shown to stimulate male erectile activity (Van der Ploeg et al., 2002) and female arousal (Pfaus et al., 2004)

Owing to the therapeutic potential of the MC4 receptor, considerable efforts have focused on developing peptide and non-peptide agonists. The mechanisms of how these ligands bind to the receptor have been explored in detail (Nickolls et al., 2003). However, much less is known of how they impact receptor signalling and receptor regulation. Until recently compounds acting at 7 TM receptors were divided into one of four catagories, they were either considered full or partial agonists, antagonists or inverse agonists. However, this traditional view has been challenged by recent evidence that ligands at the same GPCR can cause markedly different degrees of activation for different effector pathways. This evidence includes studies on the 5-HT_{2C} receptor (Berg et al., 1998), the β_2-adrenergic receptor (Wenzel-Seifert and Seifert, 2000) and the muscarinic acetylcholine receptor (Akam et al., 2001), also see Kenakin (2002) and Hermans (2003) for reviews of the topic. This may be of particular importance when designing non-

peptide agonists, as compounds could either mimic the effects of the endogenous agonist, or have the ability to selectively activate one pathway over another. In the work presented here we have investigated the ability of a number of peptide and non-peptide agonists to activate adenylate cyclase, mobilise intracellular calcium and internalise a FLAG-tagged MC4 receptor expressed in HEK-293 cells.

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Methods

Materials

Radiolabelled [^{125}I]NDP-MSH was obtained from Perkin Elmer Life Sciences (Boston MA). α-MSH, β-MSH, NDP-MSH, and SHU9119 were obtained from Peninsula Laboratories (San Carlos CA). AGRP(83-132), des-acetyl-α-MSH was obtained from Phoenix Pharmaceuticals (Belmont CA). The non-peptide agonist THIQ (Van der Ploeg *et al*., 2002), and those from our own compound library (Pontillo et al., 2004) were synthesized by the Chemistry Department at Neurocrine.

Construction of FLAG-tagged melanocortin 4 receptors

Human MC4 receptor cDNA in pcDNA3.1 was used as a template for creating FLAG-tagged-MC4R (FMC4R). PCR primers designed to add an EcoRI site and a FLAG tag to the 5' end of the receptor and a XhoI site to the 3' end, were added with the template DNA to PCR supermix (Invitrogen Life Technologies, Carlsbad CA) and PCR was performed as per the manufacturers instructions (95^{o}C 1min, 52^{o}C 1min, 72^{o}C 2min x25). The PCR product was ligated into the EcoRI/XhoI sites of pcDNA3.1.

Transient transfection

HEK-293 cells were maintained in DMEM with 10% FCS. Twenty-four hours prior to transfection cells were seeded at 2 X 10^6 cells/100mm dish. Eight micrograms of FMC4R DNA was transfected using Polyfect™ (Qiagen, Valencia CA) according to the manufacturers instructions. Cells were either assayed for cAMP accumulation or calcium

flux after 48 hours. The effect of pertussis toxin on signalling pathways was determined by pre-incubation of cells for 20 hours with 100ng/ml pertussis toxin.

Membrane preparation

Cells were washed once with PBS. Then 5ml of ice-cold buffer (20mM HEPES, 1mM $MgCl_2$, 1mM EDTA, pH 7.4) was used to disrupt the cell monolayer in each 100mm dish. This was transferred to a Dounce glass:glass homogeniser. The cells were homogenised at 4^{o}C by 40 strokes of the Dounce homogeniser. The homogenate was centrifuged for 10 minutes at 1,700 x g, the supernatant was collected and centrifuged at 48,000 x g (4^{o}C) for 1 hour. The resulting pellet was resuspended in buffer and aliquots were stored at –80^{o}C. Protein concentration was determined using the BioRad protein assay (BioRad, Hercules CA) using BSA as standard.

Iodinated Radioligand Saturation Binding Assays

Cell membranes (5µg protein) were incubated in duplicate with either [^{125}I]SHU9119, at concentrations ranging from 0.001nM to 5nM in a total volume of 100µl buffer (25mM HEPES, 1mM $MgCl_2$, 2.5mM $CaCl_2$, 0.5% BSA, 1% Bacitracin, (1 complete™ EDTA-free protease inhibitor tablet/50ml (Roche Molecular Biochemicals, Indianapolis, IN) pH 7.0). Non-specific binding was determined by the inclusion of 1µM SHU9119. The reaction was initiated by the addition of membranes and the plates were incubated at 25^{o}C for 2 hours. The reaction was terminated by rapid filtration using a vacuum harvester with five 100µl washes of ice cold wash buffer (25mM HEPES, 1mM $MgCl_2$, 2.5mM $CaCl_2$, 0.1% BSA, 500mM NaCl, pH 7.0). The filters were soaked in 50µl

scintillation fluid and the amount of radioactivity present was determined by liquid scintillation counting.

Ligand competition versus [^{125}I]SHU9119 and [^{125}I]NDP-MSH binding

Cell membranes (2-10µg protein) were incubated with 0.3nM iodinated radioligand and various concentrations of competitor ligand, in duplicate, in a total volume of 100µl buffer (25mM HEPES, 1mM $MgCl_2$, 2.5mM $CaCl_2$, 0.5% BSA, 1% Bacitracin, (1 complete™ EDTA-free protease inhibitor tablet/50ml) pH 7.0). Non-specific binding was determined by the inclusion of 1µM SHU9119. The reactions were initiated, incubated and terminated as described above.

cAMP accumulation assay (AlphaScreen™)

Compounds or cAMP standard (1fmol to 10nmol) were diluted in DMEM containing 1mM IBMX, but without phenol red or FBS. Agonist (5µl of 2X concentration) was added to the appropriate wells of a 384-well plate. Cells were removed from tissue culture plates using enzyme free cell dissociation buffer, washed and resuspended in DMEM containing 1mM IBMX without phenol red or FBS at a density of 600 cells/µl. Anti-cAMP acceptor beads were added to the cell suspension at a concentration of 75µg/ml and 5µl of this mixture was added to each well of the 384-well plate. The assay was incubated in the dark at 37^{o}C for 30 min and then terminated by the addition of 15µl of detection buffer containing 16.7nM biotinylated-cAMP probe and 33.3µg/ml strepavidin donor beads, 5mM HEPES, 0.3% Tween-20 and 0.1% BSA. Plates were

incubated in the dark for 1 hour before being read on the Alpha program on a Fusion microplate reader (Perkin Elmer, Boston MA).

Calcium mobilisation (FLIPR)

HEK-293 cells were transfected with FMC4R as described above. Twenty-four hours after transfection cells were plated at a density of 5 X 10^3 cells/well in black poly-D-lysine coated clear bottom 96-well plates, in DMEM containing 10% FCS. Forty-eight hours after transfection cells were loaded with Fluo-4 (Invitrogen Life Technologies, Carlsbad, CA), by replacement of the media, with fresh DMEM, containing 10% FCS, 2.5mM probenecid and Fluo-4. Cells were incubated for 1 hour at 37^{o}C before washing with 200µl/well Hanks Balanced Salt solution containing 2.5 mM probenecid. Intracellular calcium mobilisation was measured in an ImageTrak, by addition of ligands 10 seconds after the start of the assessment period. Images were acquired once every two seconds for 100 seconds.

Receptor Internalisation (FACS analysis)

Twenty four hours before experimentation cells were plated in 6-well plates at a density of 5 X 10^4 cells/well. On the day of experimentation the media was removed and replaced with media containing a given concentration of agonist (0.01nM to 1µM). Cells were incubated at 37^{o}C for 20 minutes unless otherwise stated. At the end of this incubation the plates were placed on ice and cells were washed twice with ice-cold medium without FBS. Then anti-FLAG (M2) antibody (Sigma Aldrich, St Louis MO) was added at a dilution of 1:500 in medium without FBS. Plates were placed on a slowly rotating

platform shaker (<75 rpm) at 4^{o}C for 1 hour. Cells were then washed twice with ice-cold medium without FBS. Anti-mouse AlexaFluor488 (Invitrogen Life Technologies, Carlsbad, CA) at a dilution of 1:250, in medium without FBS, was added to each well. Plates were incubated for a further hour on a slowly rotating platform shaker (<75 rpm) at 4^{o}C. Cells were then washed twice with ice-cold PBS and dissociated using 0.4ml of ice-cold PBS containing 5mM EDTA. Dissociated cells were added to 0.1ml 4% v/v formaldehyde in PBS in FACs tubes and samples were read using a FACs Caliber (BD Biosciences, San Diego CA).

Data analysis

Data were analysed using PRISM (GraphPAD Software Inc,. San Diego, CA.) and statistical significance determined using one-way ANOVA analysis of variance follwed by Tukey post-hoc analysis. $p<0.05$ determined statistical significance. Ki values were determined from IC_{50} values using the method of Cheng and Prusoff (1973).

Results

Development of FLAG-tagged MC4 receptor - The goal of this study was to determine the functional properties of a wide variety of agonists acting through the MC4 receptor. To facilitate our ability to measure one of these properties, receptor internalisation, the MC4 receptor was tagged with the FLAG epitope at the N-terminus (FMC4R). Saturation binding studies indicated that [^{125}I]SHU9119 bound specifically to FMC4R expressed in HEK-293 cells with a Bmax of 700 fmol/mg and a Kd of 0.5 nM (Figure 1a). To ensure appropriate pharmacology of the FMC4 receptor compared with the wild-type receptor, binding of agonist and antagonist peptides was assessed in competition versus [^{125}I]SHU9119 (Figure 1b). The FMC4 receptor showed no appreciable differences in ligand binding affinities compared to previously published values for the wild-type receptor (Nickolls et al., 2003). pKi±s.e.m (Ki nM) (n=3) values for ligands were as follows, α-MSH 7.42±0.16 (38.0), NDP-MSH 8.62±0.17 (2.20), AGRP(83-132) 9.41±0.16 (0.389) and SHU9119 9.72±0.11 (0.191) Function as assessed by the stimulation of cAMP accumulation was also not appreciably affected by the addition of the FLAG-tag with the EC_{50} value of 21.4nM for α-MSH which was similar to the value of 14.8nM obtained for the wild-type receptor (Nickolls et al., 2003). (Figure 1c).

α-MSH mediated MC4 receptor internalisation and calcium mobilisation – The ability of the MC4 receptor to undergo receptor internalisation in response to agonist has been documented (Shinyama et al., 2003; Gao et al., 2003), and is considered to be an important regulatory mechanism in receptor desensitisation. We assessed the ability of

the FMC4 receptor to undergo agonist dependent internalisation using FACS analysis. α-MSH (1μM) was able to induce a time-dependent decrease in the amount of receptor present at the cell surface, to a maximum of 56% receptor remaining (Figure 2). The peptide antagonists SHU9119 and AGRP(83-132) were not able to induce receptor internalisation, indeed there was a slight increase in cell surface fluorescence after cells were incubated with these compounds for 20 min at a concentration of 0.1μM, to 107±5% and 113±4% respectively. The ability of the MC4 receptor to mobilise calcium has also been reported (Mountjoy et al., 2001). We therefore assessed the ability of the FMC4 receptor to mobilise calcium. A dose-dependent increase in calcium mobilisation was observed upon addition of α-MSH, with an EC_{50} value of 130 nM (Figure 3), which was not affected by pre-treatment with PTX.

Peptide and non-peptide agonists have high binding affinity and potency and intrinsic activity in cAMP accumulation assays - A number of MC4 receptor peptide and non-peptide (Figure 4) agonists were selected to allow the functional properties of individual agonists to be assessed, these were the endogenous peptides, α-, β-, γ- and des-acetyl-α-MSH, the synthetic peptide NDP-MSH, the non-peptide agonist THIQ (Van der Ploeg et al., 2002) and five compounds from our in-house library (Pontillo et al., 2004). All compounds were able to bind to the MC4 receptor as determined in ligand/[^{125}I]NDP-MSH competition binding assays (Figure 5) and exhibited a range of affinity values (Table 1). All agonists were able to stimulate FMC4 receptor mediated accumulation of cAMP with an intrinsic activity of between 73% and 149% of the maximal α-MSH response (Figure 6a/Tables 2&3). The peptide agonists showed much stronger correlation

(r^2=0.98), between binding affinity and functional potency, than the non-peptide agonists (r^2=0.62).

Peptide agonists, but not non-peptide agonists, exhibit high intrinsic activity in calcium mobilisation assays - The ability of the MC4 receptor agonists to mobilise calcium was determined (Figure 6b). The peptide agonists were able to cause the mobilisation of calcium, with similar intrinsic activity to that of α-MSH, with the exception of γ-MSH, which was a partial agonist in both assays. The potency of α-, β-, γ- and des-acetyl-α-MSH was approximately 10-fold lower, and the potency of NDP-MSH was approximately 100-fold lower, in the calcium mobilisation assays compared to the cAMP accumulation assays, these differences were significant in all cases ($p<0.05$, Tukey post-hoc analysis). NDP-MSH was the only peptide agonist to exhibit significantly lower intrinsic activity in the calcium mobilisation assay compared to the cAMP accumulation assay ($p<0.05$, Tukey post-hoc analysis). Conversely, very little mobilisation of calcium was observed in response to the six non-peptide agonists. The intrinsic activities of all the non-peptide agonists, with the exception of 55886, were significantly lower in the FLIPR assays compared to the cAMP accumulation assays ($p<0.05$, Tukey post-hoc analysis). Conversely, there were no significant differences between the potencies of non-peptide agonists in FLIPR and cAMP accumulation assays.

Peptide agonists, but not non-peptide agonists, exhibit high intrinsic activity in receptor internalisation assays - We then determined the ability of agonists to induce FMC4 receptor internalisation using FACS analysis (Figure 6c). Again β-, des-acetyl-α- and

NDP-MSH were able to cause a similar degree of receptor internalisation to α-MSH, with potencies between 2- and 5-fold lower than observed in cAMP accumulation assays. This decrease in potency was, however, only significant in the case of NDP-MSH ($p<0.05$, Tukey post-hoc analysis). The partial agonist γ-MSH caused 75% of the α-MSH response at 1μM, which was similar to its response in the calcium mobilisation and cAMP accumulation assays. The non-peptide agonists, however, were impaired in their ability to induce receptor internalisation, with NBI-58702 exhibiting the highest intrinsic activity (38% of the α-MSH response at 1μM). All non-peptide agonists exhibited significantly lower intrinsic activity in the receptor internalisation assay compared to the cAMP accumulation assay ($p<0.05$, Tukey post-hoc analysis). There were also clear reversals in the intrinsic activities of compounds between the two assays with NBI-58704 > NBI-58702 > β-MSH = des-acetyl-α-MSH in the cAMP accumulation assay and β-MSH = des-acetyl-α-MSH > NBI-58704 = NBI-58702 in the receptor internalisation assay. Furthermore, a time course of the decrease in cell surface fluorescence induced by 1μM NBI-58702 showed no difference in kinetics to that induced by α-MSH (Figure 2). Much less variation was observed when comparing potency values between cAMP accumulation and receptor internalisation, only NBI 55886 exhibited a significant difference ($p>0.05$, Tukey post-hoc analysis) with higher potency in the receptor internalisation assay compared to the cAMP accumulation assay.

Correlation of intrinsic activity values - Correlation of intrinsic activity values revealed distinctly different profiles for peptide and non-peptide agonists (Figure 7). Overall there was no correlation between the cAMP accumulation Emax values and either the FLIPR

or internalisation Emax values. However the intrinsic activity of the peptide agonists, but not the non-peptide agonists, lay close to the line of incidence in both cases. Conversely, correlation was observed (r^2 = 0.82) between the Emax values in the FLIPR and internalisation assays, suggesting a common signalling mediator.

Discussion

Due to the therapeutic potential of MC4 receptor agonists a number of peptide and non-peptide agonists have been developed for this receptor. In this study we have investigated the ability of peptide and non-peptide agonists to activate the MC4 receptor as measured by cAMP accumulation and calcium mobilisation and also investigated their ability to regulate receptor internalisation. We have discovered that the ability of individual agonists to activate MC4 receptor signalling/regulatory pathways differs. The peptide agonists exhibited high intrinsic activity in all three assays, whereas non-peptide agonists only exhibited high intrinsic activity in the cAMP accumulation assays and were severely impaired in their ability to mobilise calcium or internalise the receptor. These data are the first to show significant differences between the ability of peptide and non-peptide agonists to signal through and regulate the MC4 receptor and are consistent with agonist specific conformational states.

Our results show that although there is strong correlation between binding affinity and functional potency in the cAMP assay for peptide agonists, this relationship is dissociated for non-peptide agonists despite a similar range of affinity values. Likewise, cAMP Emax values for the peptide agonists, but not non-peptide agonists, correlated with calcium mobilisation and receptor internalisation Emax values. Conversely, there was correlation between FLIPR and receptor internalisation Emax values for both peptide and non-peptide agonists.

Our results suggest that agonist activation of Gαs signalling and calcium mobilisation/receptor internalisation is dissociated. Receptor theory asserts that if a 7 TM receptor couples more efficiently to one pathway over another, then agonists may be expected to exhibit higher intrinsic activity and/or potency at that effector due to strength of signal (Kenakin, 1995). Therefore, the ability of a drug to activate one effector more readily than another is only indicative of functional selectivity if there are reversals in intrinsic activity and/or potency between the different effectors. Clear reversals in intrinsic activity are observed in our investigation, for example, NBI-58704 was a super-agonist (149% of the max α-MSH response) in the cAMP accumulation assay, but a very weak partial agonist in both the calcium mobilisation and receptor internalisation assays. Conversely, γ-MSH was a strong partial agonist, and des-acetyl-α-MSH and beta-MSH were full agonists in all three assay systems.

These data therefore add to the growing consensus that receptors are able to activate different pathways dependent on the agonist bound. Over the last decade a considerable number of studies have provided evidence that agonists differ in their ability to couple the same receptor to different G proteins, including the Drosophilia tyramine receptor (Robb et al., 1994), the calcitonin receptor (Watson et al., 2000), the D_2 dopamine receptor (Nickolls and Strange, 2003), and the α2-adrenergic receptor (Brink et al., 2000). More recently this phenomena has also been used to describe signalling/regulatory properties of ligands that bind to 7 TM receptors that may not be wholly G protein dependent, such as phosphorylation of the angiotensin II receptor (Thomas et al., 2000) and desensitisation of the μ opioid receptor (Blake et al., 1997).

Although peptide agonists caused marked receptor internalisation, the non-peptide agonists were severely impaired in this assay. The ability of agonists to cause receptor internalisation may be important in any long term pharmaceutical therapy, as agonist induced internalisation is ultimately linked to receptor down-regulation. Furthermore, in addition to its important role in receptor regulation, receptor internalisation may also be important for signalling. There is some evidence that at the β2 adrenergic receptor, that both GRK and β-arrestin act as scaffold proteins for the intracellular activation of the MAP kinase signalling cascade (Daaka et al., 1998). Indeed, although receptor internalisation is commonly allied to agonist activation, several studies have revealed that this is not always the case. In particular antagonist occupation of both cholecystokinin receptors (Roettger et al., 1997) and CCR5 chemokine receptors (Vila-Coro et al., 1999) can result in receptor internalisation. Likewise, consistent with the data in our study, endogenous peptide agonists of the μ-opioid receptor, but not the non-peptide agonist morphine, are able to induce receptor internalisation (Keith et al., 1996).

Even though the compounds from our in-house library are from the same series, they are structurally dissimilar from the non-peptide agonist THIQ, decreasing the likelihood that the dissociation between cAMP accumulation and calcium mobilisation/receptor internalisation is only a property of some MC4R non-peptide agonists. In addition we have previously demonstrated that THIQ occupies the α-MSH binding pocket (Nickolls et al., 2003), implying that these data are not due to a vastly different mechanism of receptor activation. Similarly the chatecholamines, norepinephrine, epinephrine and

dopamine occupy similar binding pockets in the β_2 adrenergic receptor. Biophysical studies of this receptor have demonstrated that upon catecholamine binding the receptor undergoes transitions to two kinetically distinguishable conformational states, which correlate with biological responses (Swaminath et al., 2004). Norepinephrine and epinephrine were able to induce both rapid and slow conformational changes and were also efficient at both Gs activation and receptor internalisation, whereas dopamine, which was only able to activate signalling and was inefficient at receptor internalisation, only induced a rapid conformational change.

Whether these properties of non-peptide agonists have a direct effect on their therapeutic potential has yet to be determined. Interestingly, the amount of peptide versus non-peptide agonists required to elicit feeding in rodents does not correlate with their *in vitro* profile, with non-peptides exhibiting decreased potency *in vivo* (Cepoi et al., 2004). However, further analysis of both *in vitro* and *in vivo* data are required before deductions can be drawn.

In conclusion, we have demonstrated that the melanocortin 4 receptor can exist in agonist-specific receptor conformations. Specifically we have demonstrated that non-peptide agonists of the MC4 receptor are unable to promote calcium mobilisation or receptor internalisation to the same degree as peptide agonists.

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Legends for Figures

Figure 1. a) Saturation analysis of [^{125}I]SHU9119 binding to membranes prepared from HEK-293 cells expressing the FMC4 receptor. Assays were performed as described in the experimental section, and data were best fitted by a model describing a single binding site, from which Kd and Bmax values were obtained (see text) b) Ligand/[^{125}I]SHU9119 competition binding to membranes prepared from HEK-293 cells expressing the FMC4 receptor. Ligands are represented by the following symbols; (●) NDP-MSH, (■) α-MSH, (□) AGRP(83-132), and (△) SHU9119. Competition binding was performed as described in the experimental section. The data were best fitted by a one binding site model from which Ki values were determined (see text), these were not appreciably different from those exhibited be the wild-type receptor. Graphs are representative of three independent experiments. c) α-MSH stimulation of cAMP accumulation in HEK-FMC4R cells. Alphascreen assays were performed as described in the experimental section, and data were fitted best by an equation describing a sigmoidal dose response curve from which an EC_{50} value was obtained (see text). Graphs are representative of three independent experiments.

Figure 2. Time dependent reduction in the level of FMC4 receptor detectable at the cell surface by FACS analysis upon exposure to MC4 receptor agonists. Agonists are represented by the following symbols; (■) α-MSH and (△) 58702. Data are mean±s.e.m of three independent experiments.

Figure 3. Stimulation of intracellular calcium mobilisation by α-MSH in HEK-293 cells expressing the FMC4 receptor. FLIPR experiments were performed as described in the experimental section, and data were fitted best by an equation describing a sigmoidal dose response curve. The graph is representative of six independent experiments.

Figure 4. Structures of the small molecule agonists used in this study.

Figure 5. Binding of agonists to membranes prepared from HEK-293 cells expressing the MC4 receptor. Ligand/[^{125}I]NDP-MSH] competition experiments were performed as described in the experimental section and data were best fitted by an equation describing a single binding site. Data are mean±s.e.m of 3 independent experiments. Agonists are represented by the following symbols; (●) NDP-MSH, (■) α-MSH, (▲) β-MSH, (▼) γ-MSH, (◆) des-acetyl-α-MSH, (○) THIQ, (✖) 55886, (◇) 56453, (□) 56297, (△) 58702, (▽) 58704.

Figure 6. Functional potency and intrinsic activity of MC4 receptor agonists in a) cAMP accumulation, b) calcium mobilisation and c) receptor internalisation. Assays were performed as described in the experimental section. Data were best fitted by an equation describing a sigmoidal dose response curve from with potency (Table 2) and intrinsic activity (Table 3) values were derived. Data plotted are mean±s.e.m from 3 (cAMP accumulation), 6 (Calcium mobilisation) or 4 (receptor internalisation) independent experiments. Agonists are represented by the following symbols; (●) NDP-MSH, (■)

α-MSH, (▲) β-MSH, (▼) γ-MSH, (◆) des-acetyl-α-MSH, (○) THIQ, (✖) 55886, (◇) 56453, (□) 56297, (△) 58702, (▽) 58704.

Figure 7. Correlation of intrinsic activity of MC4 receptor agonists in cAMP accumulation, calcium mobilsation and receptor internalisation assays. a) Correlation between Emax values obtained in cAMP accumulation and receptor internalisation assays. The dashed line represents the line of incidence. b) Correlation between Emax values obtained in cAMP accumulation and calcium mobilisation assays. The dashed line represents the line of incidence. c) Correlation between Emax values obtained in calcium mobilisation and receptor internalisation assays. Correlation was obtained (r^2 =0.82) (solid line). The dashed lines represent the 95% confidence intervals. Agonists are represented by the following symbols; (●) NDP-MSH, (■) α-MSH, (▲) β-MSH, (▼) γ-MSH, (◆) des-acetyl-α-MSH, (○) THIQ, (✖) 55886, (◇) 56453, (□) 56297, (△) 58702, (▽) 58704.

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	pKi±s.e.m	Ki (nM)
α-MSH	7.30±0.08	50.1
β-MSH	7.73±0.05	18.6
γ-MSH	6.23±0.02	589
Des-acetyl-α-MSH	7.52±0.06	30.2
NDP-MSH	8.40±0.14	3.98
THIQ	7.97±0.06	10.7
55886	8.02±0.10	9.55
56297	7.70±0.06	20.0
56453	7.13±0.09	74.1
58702	8.20±0.20	6.31
58704	7.87±0.03	13.5

Table 1. Binding affinities of MC4 receptor agonists to the MC4 receptor expressed in HEK-293 cells. Agonist/[^{125}I]NDP-MSH competition binding was performed as described in the experimental section. Data were fitted best by a one binding site model from which Ki values were determined. Data are mean±s.e.m of three individual experiments.

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	cAMP accumulation	Calcium mobilisation	Internalisation
α-MSH*	7.67±0.19 (21.4)	6.89±0.13 (129)	7.54±0.21 (28.8)
β-MSH*	7.95±0.20 (11.2)	6.76±0.12 [1] (174)	7.36±0.26 (43.7)
γ-MSH	6.20±0.48 (631)	>1,000	>1,000
Des-acetyl-α-MSH*	7.76±0.13 (17.4)	6.96±0.13 [1] (110)	7.52±0.28 (30.2)
NDP-MSH*	8.86±0.20 (1.38)	6.92±0.15 [1] (120)	8.14±0.13 [2,3] (7.24)
THIQ	8.88±0.12 (1.32)	6.16±0.92 (692)	9.09±0.06 (0.813)
55886	6.48±0.04 (331)	>1,000	8.46±0.64 [2] (3.47)
56297	6.89±0.07 (129)	>1,000	8.10±0.44 (7.94)
56453	6.29±0.13 (513)	7.34±0.53 (45.7)	8.28±0.66 (5.25)
58702	7.37±0.06 (43)	8.62±0.58 (2.40)	8.12±0.54 (7.59)
58704	6.69±0.11 (204)	7.75±0.55 (17.8)	6.84±0.23 (145)

Table 2. Potency of MC4 receptor agonists in cAMP accumulation, calcium mobilisation and receptor internalisation assays. Functional properties of agonists at FMC4 receptors expressed in HEK-293 cells were measured respectively by AlphaScreen™, FLIPR and

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FACS analysis, as described in the experimental section. Potency values are expressed as pEC_{50}±s.e.m (EC_{50} nM). Data are n=3 (cAMP accumulation) n=6 (calcium mobilisation) and n=4 (receptor internalisation. *ANOVA analysis showed a significant influence of the different assays on agonist potency. [1]Agonist potency was significantly different between cAMP accumulation and calcium mobilisation assay. [2]Agonist potency was significantly different between cAMP accumulation and receptor internalisation assays. [3]Agonist potency was significantly different between calcium mobilisation and receptor internalisation assays.

	cAMP accumulation	Calcium mobilisation	Internalisation
α-MSH	100	100	100
β-MSH	83±13	94±12	118±9
γ-MSH	80±7	62±7	75±5
Des-acetyl-α-MSH	84±18	96±9	107±2
NDP-MSH*	112±11	80±7 [1]	89±5
THIQ*	98±5	7±3 [1]	29±7 [2,3]
55886*	73±17	40±8	16±2 [2]
56297*	81±6	23±7 [1]	-5±18 [2]
56453*	103±8	7±2 [1]	27±20 [2]
58702*	110±14	20±5 [1]	38±16 [2]
58704*	149±15	23±7 [1]	34±11 [2]

Table 3. Intrinsic activity of MC4 receptor agonists in cAMP accumulation, calcium mobilisation and receptor internalisation assays. Functional properties of agonists at FMC4 receptors expressed in HEK-293 cells were measured respectively by AlphaScreen™, FLIPR and FACS analysis, as described in the experimental section. Intrinsic activity values are expressed as % of the maximal α-MSH response (mean±s.e.m). Data for γ-MSH in calcium and receptor internalisation assays and 55886 and 56297 in the calcium assays are the observed activity at 1μM. Data are n=3 (cAMP accumulation) n=6 (calcium mobilisation) and n=4 (receptor internalisation). *ANOVA

analysis showed a significant influence of the different assays on intrinsic activity. [1]intrinsic activity was significantly different between cAMP accumulation and calcium mobilisation assay. [2]Intrinsic activity was significantly different between cAMP accumulation and receptor internalisation assays. [3]Intrinsic activity was significantly different between calcium mobilisation and receptor internalisation assays.

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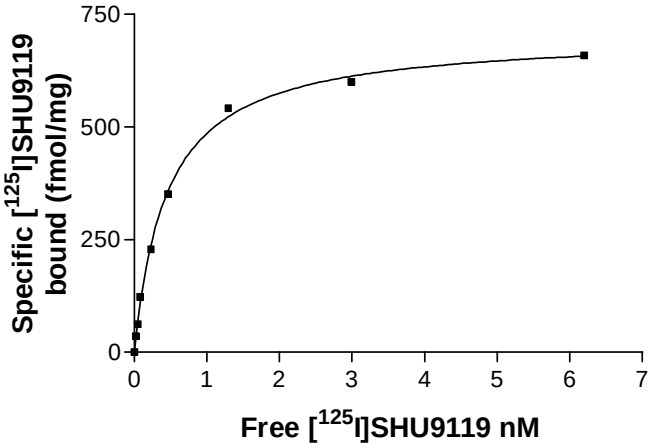
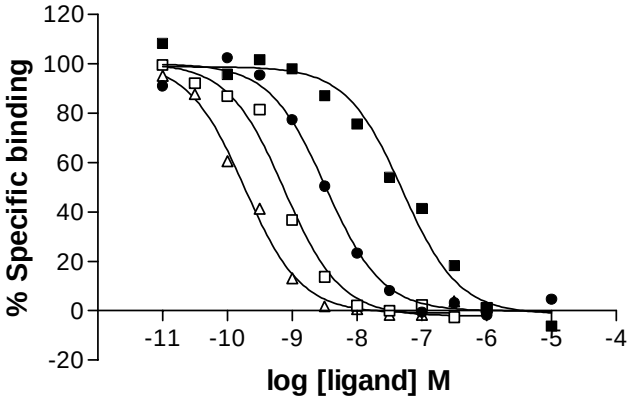
Figure 1





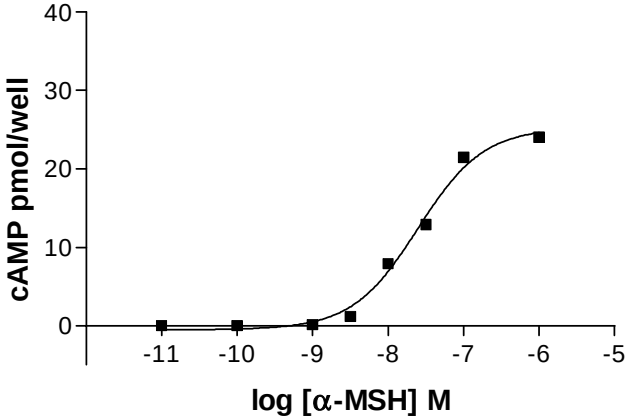


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Figure 2.

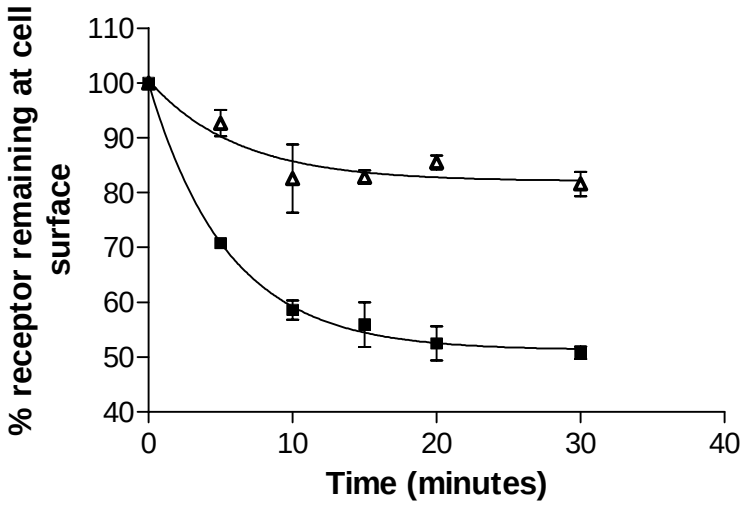

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Figure 3.

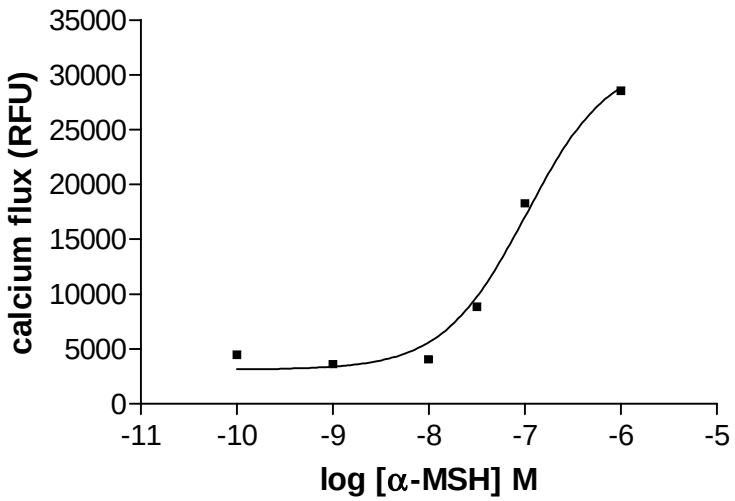

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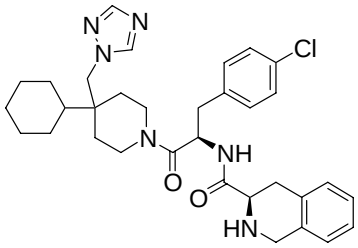
Figure 4.

THIQ



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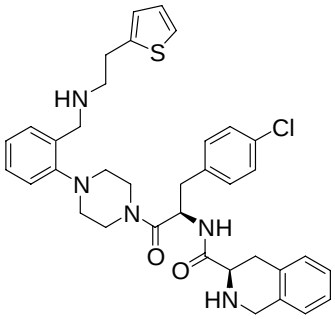

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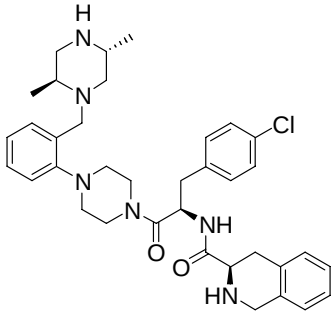

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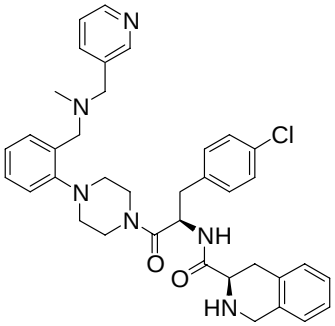

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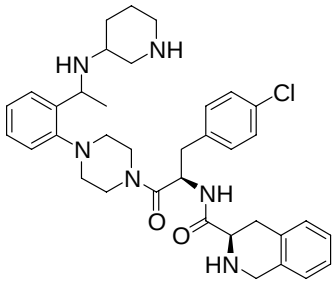

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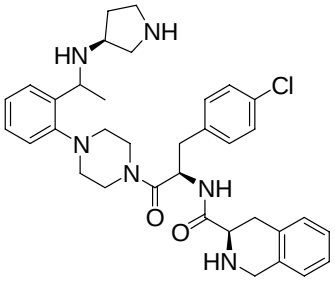

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Figure 5.

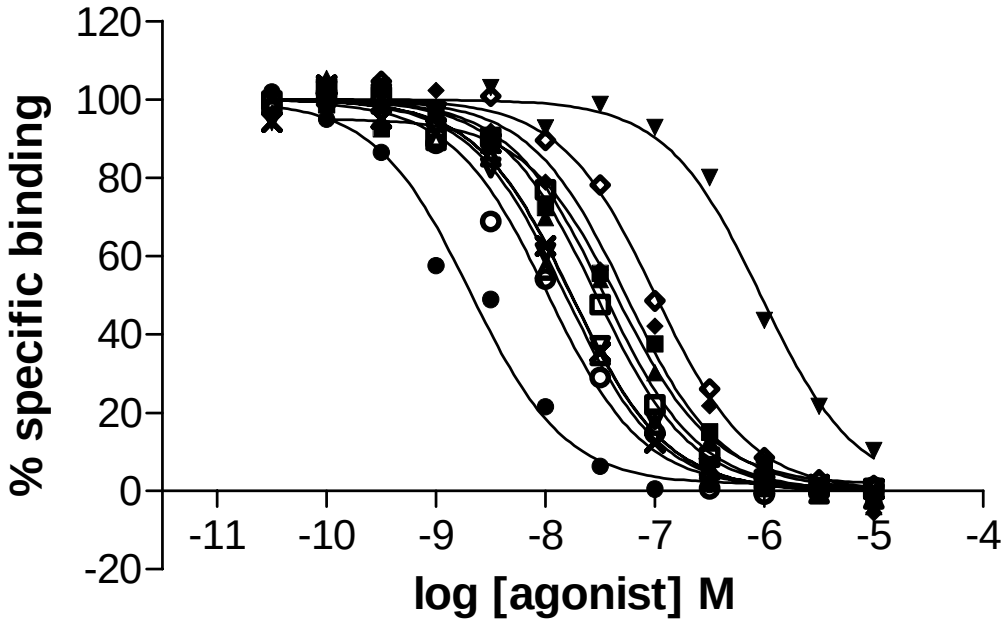

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Figure 6

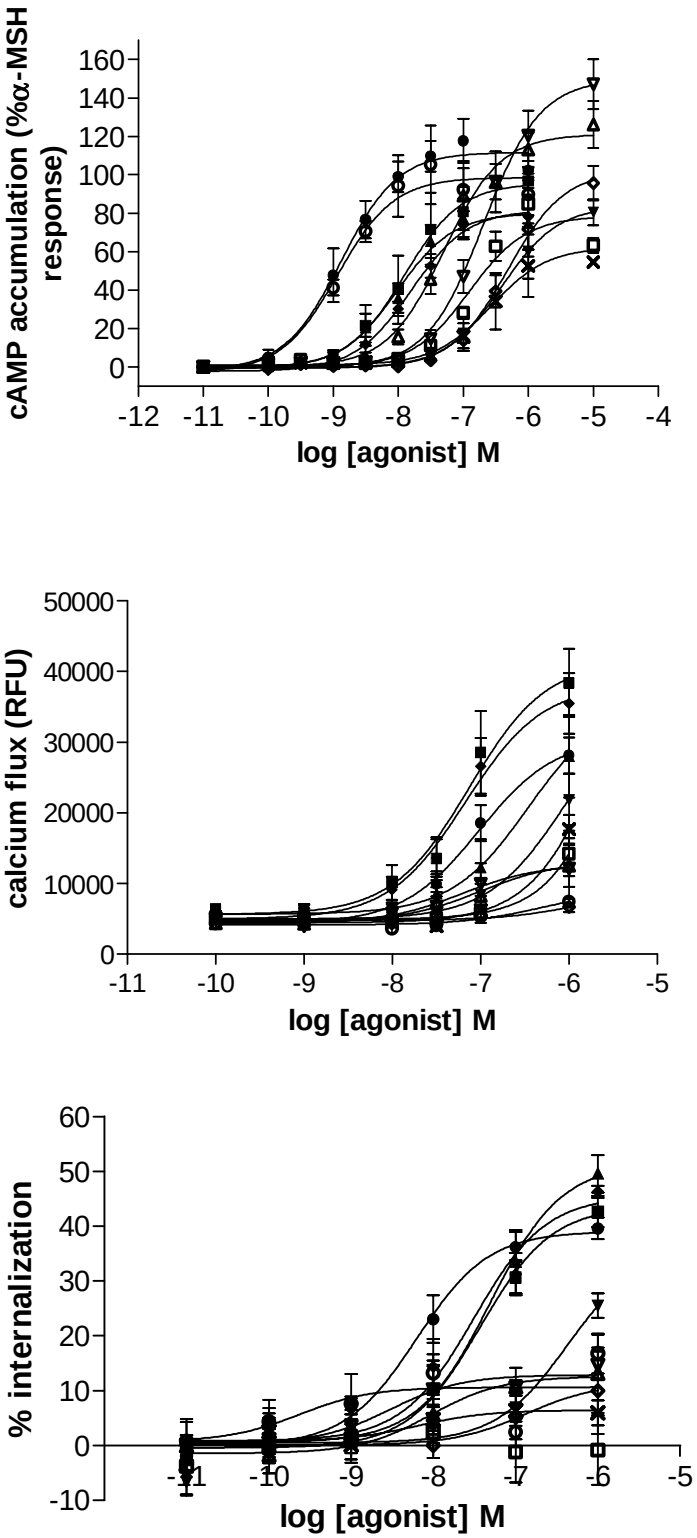

Figure 7