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Research Article

A non-peptide radioiodinated high affinity melanocortin-4 receptor ligand

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Summary

4-Cyclohexyl-4-[(1,2,4-triazol-1-yl)methyl]piperidine was introduced into stepwise peptide synthesis procedures using Boc chemistry and derivatives of D-4-iodophenylalanine and D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid. A halogen replacement analogue (**I-Mex2**) of a known high affinity melanocortin-4 receptor selective compound resulted. It showed a subnanomolar affinity when evaluated on the melanocortin-4 receptor in competition with the α-MSH peptide analogue ^{125}I-NDP-MSH. By treatment with hexamethylditin and tetrakis(triphenylphosphine) palladium **I-Mex2** was converted to the corresponding trimethylstannyl derivative. In the next step, Na^{125}I was oxidized by an iodobead. Iododestannylation proceeded in the presence of 1 M phosphate buffer, pH 2.5, and the radioactive derivative **^{125}I-Mex2** formed was separated by HPLC at 40% radiochemical yield. Preliminary investigation showed that **^{125}I-Mex2**

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is useful as a radioligand for melanocortin-4 receptor binding studies.

Key Words: melanocortin-4 receptor radioligand; iodine-125; iododestannylation; iodobead

Introduction

The melanocortin-4 receptor (MC_4R) is expressed in the hypothalamus, penis, spinal cord, brainstem, and pelvic ganglion,[1] where it has multiple roles in the regulation of autonomic outflow, neuroendocrine functions, and behaviour.[2] Its activity is controlled by endogenous peptide and small protein ligands, which include α-melanocortin (agonist) and agouti-related protein (antagonist).[3] The melanocortin system plays major roles in the hypothalamic regulation of energy balance and sexual functions.[4] Selective agonists of the human MC_4R are of potential use for the treatment of obesity, diabetes and sexual dysfunctions.[5] Peptide/protein ligands display drawbacks, such as enzymatic instability and failure to cross the blood–brain barrier, and should preferably be replaced by non-peptide substances, which are better suited for application as drugs.[6]

Several non-peptide MC_4R selective compounds have recently been found.[7] One of them is the substance disclosed in 'example 2' of patent WO 00/74679, namely 1-(D-1,2,3,4-tetrahydroisoquinoline-3-carboxy-D-4-chlorophenylalanyl)-4-cyclohexyl-4-[(1,2,4-triazol-1-yl)methyl]piperidine.[8] In a binding assay it showed an IC_{50} of 0.92 nM on the human MC_4R, displaying at least 250-fold selective affinity for the MC_4R compared with other melanocortin receptor subtypes. Moreover, 'example 2' displayed agonistic actions. Like α-MSH it reduced appetite and stimulated sexual activity.[1,8]

To allow investigations on the mode of action of non-peptide ligands at the molecular level a radioactive derivative of a high affinity highly specific MC_4R ligand was desired and we selected 'example 2' for modification. As the label we preferred iodine-125, because of its high radioactivity and convenient introduction via iododestannylation.[9] However, the iodine atom was not included in 'example 2'; instead, in its D-4-chlorophenylalanine residue a chlorine atom is present. We here synthesized a substance where the chlorine was replaced with iodine and checked whether or not the biological properties of the obtained non-

radioactive derivative were comparable with those of the original substance. This was followed by preparation of the radioiodinated derivative.

Results and discussion

As starting material we used 4-cyclohexyl-4-[(1,2,4-triazol-1-yl)methyl]-piperidine.[8] It was first coupled with Boc-D-4-iodophenylalanine to yield protected derivative **1** (Figure 1). We used HATU (*O*-(7-azobenzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate)[10] as an activator in presence of DIEA (diisopropylethylamine). Isolation of the reaction product included extractions. The Boc protective group was then removed using trifluoroacetic acid diluted with CH_2Cl_2, which furnished **2**. Preparation of **3** was then carried out using Boc-D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, in a similar manner as for the synthesis of **1**. Removal of the Boc group of **3**, followed by HPLC

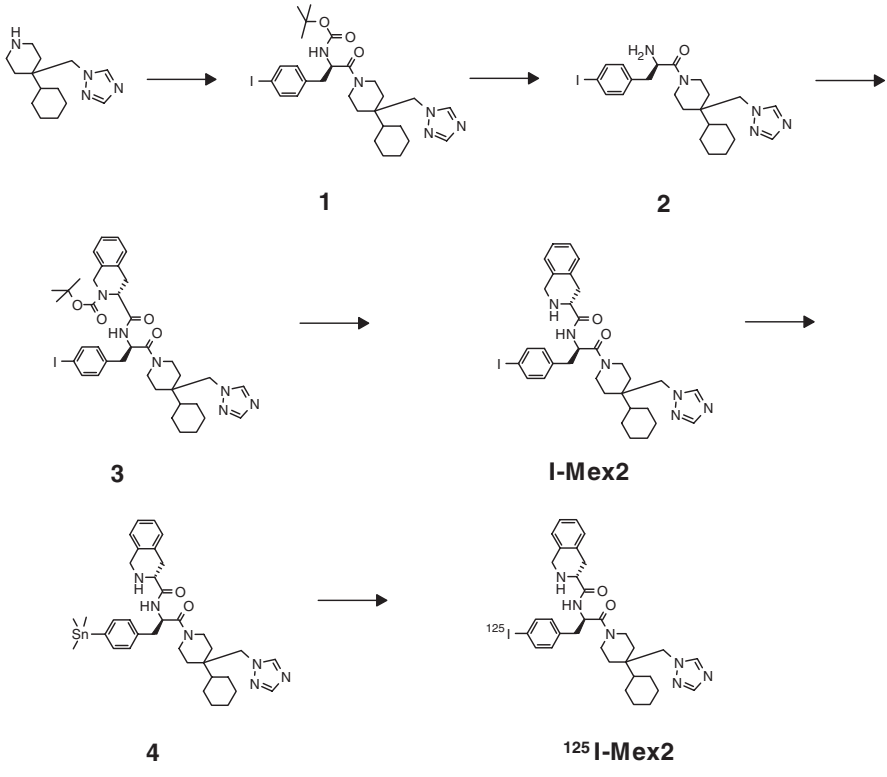

Figure 1. Synthesis of ^{125}I-Mex2

purification, resulted in the iodo analogue of WO 00/74679 'example 2', here termed **I-Mex2**.

I-Mex2 was investigated for its ability to bind to the MC_4R expressed in insect cells, by using a competition assay where the radioiodinated peptide ^{125}I-NDP-MSH ([4-^{125}I-Tyr^2, Nle^4, D-Phe^7]-α-MSH) was used as the radioligand. In these studies we found **I-Mex2** to bind with a subnanomolar affinity (i.e. K_i below 1 nM; details of this study will be published elsewhere). Labelled **I-Mex2** was thus expected to be of value for ligand–receptor interaction studies. In order to prepare it we first eliminated the trifluoroacetate ion by passing a solution of **I-Mex2** in methanol through a small Dowex 1×4 column (OH^- form). The base obtained was further dissolved in dioxane and treated with hexamethylditin and tetrakis(triphenylphosphine) palladium with heating. The trimethylstannyl derivative **4** formed showed considerable instability. It was isolated by an immediate HPLC separation, where the collected fractions were neutralized with ammonia followed by freeze-drying. The powder obtained was placed into a tightly closed vial and stored in the freezer prior to use. Mass spectrometry showed that the specific isotope pattern of the molecular peak $M + H^+$ for trimethylstannyl derivative **4** corresponded exactly to that expected (i.e. 719.3 Da for the most abundant isotope). The stability of **4** was further investigated by dissolving a small portion of it in the HPLC mobile phase used for its purification (acetonitrile–water–trifluoroacetic acid, 38:62:0.1). Infusing this solution into the mass spectrometer gave the characteristic isotope pattern around 719 Da. However, when the infusion was repeated after 17 h of storage in the mobile phase at 20°C we failed to detect any molecular ions corresponding to substance **4**. On the other hand, when stored as a powder in the freezer (−20°C) the compound appeared essentially stable for at least 1 year.

There was a large difference in the HPLC retention times between the iodine derivative **I-Mex2** and the trimethylstannyl derivative **4** on the ACE 5 Phenyl-A3152 column. The former could be eluted with 44% acetonitrile in 0.1% trifluoroacetic acid after 9.5 min, whereas **4** gave a peak after 14.6 min. (We changed the buffer to 48% acetonitrile in 0.1% trifluoracetic acid after 9.2 min of this chromatography run.) A similar chromatographic system was thus suited both for purification of trimethylstannyl derivative **4**, removing unreacted **I-Mex2**, and for isolation of radioactive compound, removing unreacted **4**. A representative example of the chromatographic separation of the labelled compound **^{125}I-Mex2** is shown in Figure 2.

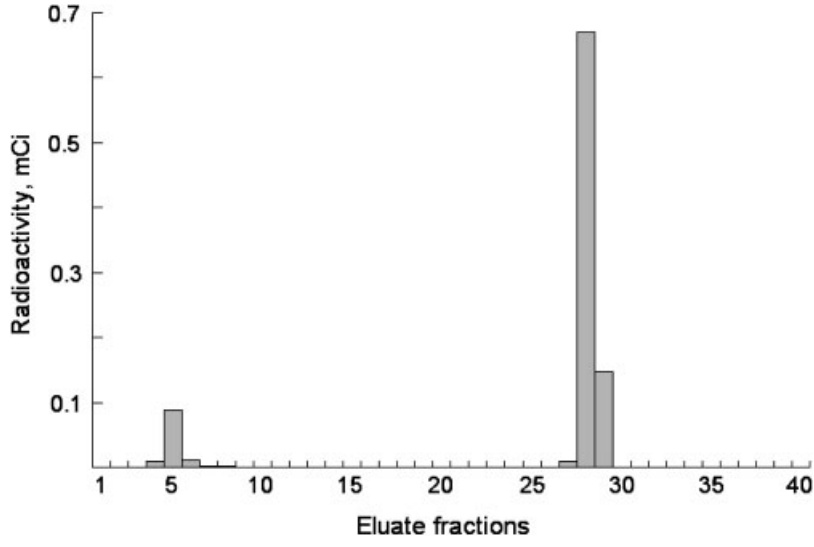

Figure 2. Separation of ^{125}I-Mex2 by HPLC (radioactivity profile). Conditions as described in the Experimental section

During our optimization of the introduction of radioactive iodine we found some factors to be important. Thus, we found iodobeads to be suited as oxidation agent; using them the oxidation of $Na^{125}I$ could be achieved in 0.05 M PBS buffer, pH 7.4, after 5 min. Replacement of the trimethylstannyl group with radioactive iodine was successful in 1 M phosphate buffer, pH 2.5. A reaction time of 30 s was found to be optimal. Even small deviations from that time led to a reduced yield. Using these conditions we obtained a reproducible radiochemical yield of about 40% of the desired product in repeated experiments.

The **^{125}I-Mex2** obtained was successfully used in preliminary radioligand binding experiments using recombinant human MC_4 receptors expressed in insect cells. Thus, adding the ligand in concentrations ranging 1 pM to 5 nM to MC_4 receptor expressing cell-membranes resulted in a binding curve suggesting binding to a saturable high affinity site, along with binding to non-saturable non-specific sites (Figure 3). The specific portion of the binding appeared to become essentially completely blocked when the binding was performed with the addition of 3 μM non-labelled **I-Mex2** (Figure 3). State of the art law of mass action computer modelling of the data suggested that **^{125}I-Mex2** bound to a single population of high affinity binding sites, with a capacity (B_{max}) of 30.6 ± 0.8 pmol/mg protein and dissociation constant (K_d) of 0.177 ± 0.012 nM ($n = 3$).

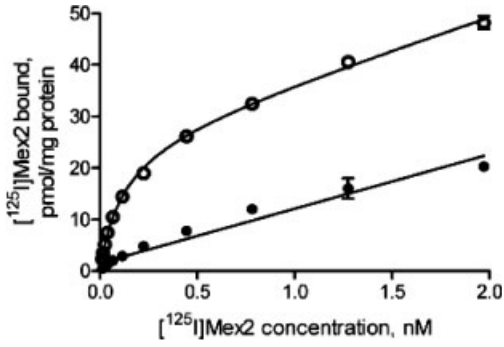

Figure 3. Saturation binding of ^{125}I-Mex2 to Sf9 cell membranes containing the MC_4 receptor. Shown is the binding of ^{125}I-Mex2 in the absence (○) and in the presence (●) of 3 μM non-labelled I-Mex2

Experimental

Chemical synthesis

Reagents were used without purification. DMF, diisopropylethylamine and HATU were from Applied Biosystems. Boc-D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid was from Neosystem, iodobeads from Pierce, and $Na^{125}I$ from Amersham Pharmacia Biotech. All other chemicals were purchased from Aldrich or Fluka. Thin layer chromatography was carried out on Merck Silica gel 60 F_{254} plates. LC/MS was performed on a Perkin Elmer instrument PE SCIEX API 150EX equipped with a Turboionspray Ion Source and a Dr Maisch ReproSil–Pur C18-AQ, 5 μ, 150 × 3 mm HPLC column, using a gradient formed from water and acetonitrile, with 5 mM ammonium acetate additive. ^{1}H NMR spectra were recorded on a Jeol JNM-EX270 spectrometer using the signal from partially deuterated DMSO present in commercial DMSO-d_6 (2.50 ppm downfield from TMS) as internal standard. Analytical HPLC was performed on a Waters system equipped with Millenium-32 Workstation, 2690 Separation Module and 996 Photodiode Array Detector. Semipreparative HPLC was carried out on a LKB system consisting of a 2150 HPLC Pump, 2152 LC Controller and a 2151 Variable Wavelength Monitor with manual collection of fractions corresponding to peaks. For radioactive work a similar system was used, which did not include a UV monitor, but which was equipped with a Gilson Microcol TDC 80 fraction collector. Aliquots (5 μl) of eluate fractions were measured using a Wallac 1470

Wizard automatic gamma counter. Freeze-drying of eluates was performed at 0.01 bar using a Lyovac GT2 Freeze-Dryer (Finn-Aqua) equipped with a Trivac D4B (Leybold Vacuum) vacuum pump and a liquid nitrogen trap.

*1-(N-Boc-*D*-4-iodophenylalanyl)-4-cyclohexyl-4-[(1,2,4-triazol-1-yl)-methyl]piperidine (***1***)*: To a solution of 4-cyclohexyl-4-[(1,2,4-triazol-1-yl)methyl]piperidine trifluoroacetate[7] (36.0 mg, 99.4 μmol) in DMF (200 μl), Boc-D-4-iodophenylalanine (38.9 mg, 99.4 μmol), HATU (41.6 mg, 109 μmol) and DIEA (51.0 μl, 298 μmol) were added. The resulting solution was stirred at room temperature for 2 h. It was then diluted with 5 ml of ethyl acetate and washed successively with 10% aqueous $KHSO_4$, saturated aqueous $NaHCO_3$ and brine (2 ml each). The organic layer was dried over Na_2SO_4 and evaporated *in vacuo* to give compound **1** as a somewhat yellow oil. ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.49, 8.52 (2 s, 1 H), 7.99, 7.95 (2 s, 1 H), 7.60 (m, 2 H), 7.13, 7.11 (2d, 1 H, $J = 7.9$, 8.6 Hz), 7.02 (m, 2 H), 4.56 (m, 1 H), 4.26, 4.23 (2 s, 2 H), 3.77–3.15 (m, 3 H), 2.85–2.6 (m, 3 H), 1.8–1.4 (m, 5 H), 1.4–0.70 (m, 10 H), 1.31, 1.28 (2 s, 9 H). MS calculated for $C_{28}H_{40}IN_5O_3$: 621; found 622 ($M + H^+$). R_f 0.74 ($CHCl_3$-MeOH, 7:1).

*1-(*D*-4-iodophenylalanyl)-4-cyclohexyl-4-[(1,2,4-triazol-1-yl)methyl]piperidine trifluoroacetate (***2***)*: Compound **1** was dissolved in CH_2Cl_2 (1 ml) and TFA (1 ml) was added. The resulting solution was stirred at room temperature for 1 h. The volatiles were removed *in vacuo* and the residue was treated with Et_2O/hexane (1:1, 3 ml) to furnish the title compound as a fine white powder (51.0 mg, 81% for 2 steps). ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.48, 8.45 (2 s, 1 H), 8.13, 8.04 (br, 3 H), 7.95, 7.92 (2 s, 1 H), 7.65 (m, 2 H), 6.98 (m, 2 H), 4.59 (m, 1 H), 4.21, 4.15 (2s, 2 H), 3.8–3.1 (m, 2 H), 3.0–2.6 (m, 4 H), 1.8–1.35 (m, 5 H), 1.35–0.92 (m, 7 H), 0.92–0.63 (m, 3 H). MS calculated for $C_{23}H_{32}IN_5O$: 521; found 522 ($M + H^+$). R_f 0.31 (*n*-BuOH-AcOH-H_2O, 4:1:1).

*1-(N-Boc-*D*-1,2,3,4-tetrahydroisoquinoline-3-carboxy-*D*-4-iodophenylalanyl)-4-cyclohexyl-4- [(1,2,4-triazol-1-yl)methyl]piperidine (***3***)*: To a solution of compound **2** (51.0 mg, 80.3 μmol) in DMF (300 μl), Boc-D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (22.3 mg, 80.3 μmol), HATU (33.6 mg, 88.0 μmol) and DIEA (41.2 μl, 241 μmol) were added. The resulting solution was stirred at room temperature for 2 h, diluted

with ethyl acetate (6 ml) and washed successively with 10% aqueous $KHSO_4$, saturated aqueous $NaHCO_3$ and brine (3 ml each). The organic layer was dried over Na_2SO_4 and evaporated in vacuo to give the title compound as a clear oil. ^{1}H NMR (270 MHz, DMSO-d_6): δ 8.51, 8.49 (2 s, 1 H), 8.08, 8.06 (2d, 1 H, $J = 8.2$, 7.9 Hz), 7.97, 7.96 (2 s, 1 H), 7.58–7.43 (m, 2 H), 7.2–7.05 (m, 4 H), 6.94–6.72 (m, 2 H), 4.9–4.2 (m, 4 H), 4.18 (s, 2 H), 3.7–2.5 (m, 8 H), 1.8–0.7 (m, 15 H), 1.27 (s, 9 H). MS calculated for $C_{38}H_{49}IN_6O_4$: 780; found 781 ($M + H^+$). R_f 0.72 ($CHCl_3$-MeOH, 7:1).

*1-(*D*-1,2,3,4-tetrahydroisoquinoline-3-carboxy-*D*-4-iodophenylalanyl)-4-cyclohexyl-4-[(1,2,4-triazol-1-yl)methyl]piperidine trifluoroacetate (***I-Mex2***)*: Compound **3** was dissolved in CH_2Cl_2 (1 ml) and TFA (1 ml) was added. The resultant solution was stirred at room temperature for 1 h. The volatiles were removed *in vacuo* and the residue was treated with Et_2O/hexane to furnish the crude product as a fine white powder (60.9 mg, 87% for two steps). For analytical and binding experiments, a 19 mg sample of raw product was dissolved in 20% acetonitrile in water + 0.1% TFA, centrifuged and the clear solution divided in three portions. Each portion was applied onto a semipreparative HPLC column (10 × 250 mm, Vydac 201HS1010), the mobile phase consisting of 26% MeCN in water + 0.1% TFA, detection at 220 nm. Eluate fractions from repeated runs containing pure putative **I-Mex2** were pooled and lyophilized. A white powder formed. Yield 8.0 mg. ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.46, 9.37 (2br, 2H), 9.01, 8.96 (2d, 1H, $J = 7.6$, 7.9 Hz), 8.52, 8.50 (2 s, 1H), 7.99, 7.96 (2 s, 1H), 7.64 (m, 2H), 7.26 (m, 4H), 7.07 (m, 2H), 5.00 (m, 1H), 4.27, 4.23 (2 s, 2H), 4.45–2.6 (m, 11H), 1.8–1.4 (m, 6H), 1.4–0.7 (m, 9H). MS calculated for $C_{33}H_{41}IN_6O_2$: 680; found 681 ($M + H^+$). R_f 0.43 (*n*-BuOH-AcOH-H_2O, 4:1:1).

*1-(*D*-1,2,3,4-tetrahydroisoquinoline-3-carboxy-*D*-4-trimethylstannylphenyl-alanyl)-4-cyclohexyl-4-[(1,2,4-triazol-1-yl)methyl]piperidine (***4***)*: Compound **I-Mex2** (3.36 mg, 4.23 μmol) was dissolved in 1 ml methanol. This solution was slowly passed five times through a column (5 × 7 mm) with Dowex 1 × 4 in OH^- form, whereafter the column was washed with 1 ml methanol, and the combined filtrate evaporated. The product obtained was dissolved in 200 μl dioxane, hexamethylditin (3 μl, 14 μmol) and tetrakis(triphenylphosphine)

palladium (0.27 mg, 0.23 μmol) were added. The mixture was stirred in argon atmosphere at 90°C for 16 h. It was then diluted with 300 μl of 0.1% aqueous trifluoroacetic acid, centrifuged, and applied immediately (in several portions) to an HPLC column (ACE 5 Phenyl-A3152, 4.6 × 250 mm); eluent—acetonitrile–water–trifluoroacetic acid (48:52:0.1), flow 1 ml/min, detection at 215 nm. Eluate fractions containing presumed **4** were collected, immediately neutralized with diluted ammonia to pH 8, pooled and lyophilized. The solid formed was dissolved in 5 ml of 30% aqueous acetonitrile and freeze dried again. This operation was repeated twice, until a fluffy powder formed. Yield 1.75 mg (58%). ^{1}H NMR (270 MHz, DMSO-d_6): δ 9.42 (br, 2H), 8.98 (m, 1H), 8.50 (s, 1H), 7.97 (m, 1H), 7.39 (m, 2H), 7.31–7.18 (m, 6H), 5.00 (m, 1H), 4.45–2.7 (m, 11H), 4.20 (s, 2H), 1.8–1.4 (m, 6H), 1.4–0.7 (m, 9H), 0.23, 0.21 (2t, $J = 27.4$ Hz, 9H). MS calculated for $C_{36}H_{50}N_6O_2Sn$: 718; found 719 ($M + H^+$).

*1-(*D*-1,2,3,4-tetrahydroisoquinoline-3-carboxy-*D*-4-^{125}iodophenylalanyl)-4-cyclohexyl-4-[(1,2,4-triazol-1-yl)methyl]piperidine trifluoroacetate (*^{125}**I-Mex2***)*: Compound **4** (65 μg, 90 nmol) was dissolved in a mixture of 40 μl methanol and 10 μl of 1 M phosphate buffer, pH 2.5. (The buffer was prepared by adding 1 M NaOH to an 1 M solution of H_3PO_4 in water to adjust pH to 2.5). One iodobead was introduced separately into a 1.5 ml volume polyethylene centrifuge vial, 0.5 ml PBS buffer (0.05 M, pH 7.4) was added, the vial shaken and the liquid pipetted away. Such washing of the iodobead was repeated twice. Then 20 μl of the above buffer and 20 μl of $Na^{125}I$ solution (2 mCi) were added to the iodobead followed by shaking on a Vortex mixer for 5 min. The solution of **4** was then added and shaken additionally for exactly 30 s. Thereafter, all liquid above the iodobead was removed in one portion using a pipette and added to 20 μl of 1 M ammonium acetate in water. The obtained solution was diluted with 30 μl methanol and 100 μl water. The resulting mixture was then applied to an HPLC column (ACE 5 Phenyl-A3152, 4.6 × 250 mm) in one portion, eluted with acetonitrile-water-trifluoroacetic acid (38:62:0.1, flow 1 ml/min), and 1 ml fractions were collected. A peak of radioactivity was detected in the 28th and 29th fractions (they contained 0.66 and 0.15 mCi of radioactivity, respectively). Samples from both fractions were diluted with buffer and used directly for receptor binding experiments.

Biological assaying

Insect cell cultures

Insect Sf9 cells were grown in 50–100 ml Sf-900 II medium (Gibco-BRL) at 27°C in small spinner bottles (250 ml) as described.[11] Recombinant baculoviruses carrying the human MC_4 receptor gene[12] were added to the cell culture ($2–3 \times 10^6$ cells/ml) and the incubation continued for additional 72 h before harvest of the cells.

Membrane preparation

After harvest cells were centrifuged at 800 *g* for 5 min and Dounce homogenized (5 times by 10 stokes with 30 s intervals) in ice-cold 20 mM Na-HEPES, 1 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 0.25 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml soybean trypsin inhibitor, pH 7.4. The homogenate was centrifuged at 700 *g* for 5 min at 4°C. The pellet was then re-homogenized and centrifuged again. The combined supernatants were collected, sedimented at 70000 *g* for 60 min at 4°C and washed once in new buffer and re-centrifuged. The final pellet was resuspended in homogenization buffer at a protein concentration of 1–3 mg of protein/ml and aliquots were stored at −80°C. Protein was determined using the Bradford method[13] with bovine serum albumin as standard.

Radioligand binding

Assays were performed by incubating insect cell membranes (5 μg protein/200 μl) in 20 mM K-Hepes (pH 7.4), 5 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mg/ml BSA with 1 pM to 5 nM ^{125}**I-Mex2** in the absence and in the presence of 3 μM non-labelled **I-Mex2**. The incubations were performed for 1 h at 25°C and were terminated by a rapid filtration through GF/B glass-fibre filters (Whatman Int. Ltd., Madistone, UK) and three washes of 5 ml of ice-cold 10 mM Na-acetate, pH 4.5, using a Brandell cell harvester. (Filters had been pre-treated with 1 mg/ml bovine serum albumin, which reduced non-specific filter binding). The radioactivity retained on the filters was then counted using a 1470 Wizard gamma counter (Wallac Oy, Turku, Finland). The results were analyzed by law of mass action computer modeling using

non-linear least-squares regression, using GraphPad PRISM^{TM} (GraphPad Software, San Diego, CA, USA).

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References

1. Van der Ploeg LHT, Martin WJ, Howard AD, Nargund RP, Austin CP, Guan X, Drisko J, Cashen D, Sebhat I, Patchett AA, Figueroa DJ, DiLella AG, Connolly BM, Weinberg DH, Tan CP, Palyha OC, Pong S-S, MacNeil T, Rosenblum C, Vongs A, Tang R, Yu H, Sailer AW, Fong TM, Huang C, Tota MR, Chang RS, Stearns R, Tamvakopoulos C, Christ G, Drazen DL, Spar BD, Nelson RJ, Maclntyre DE. *Proc Natl Acad Sci USA* 2002; **99**: 11381–11386.
2. Cone RD. In: The melanocortin receptors, Cone (ed.), Human Press, Totowa, New Jersey 2000; 405–447.
3. Nijenhuis WAJ, Oosterom J, Adan RAH. *Mol Endocrinol* 2001; **15**: 164–171.
4. Wikberg JES, Muceniece R, Mandrika I, Prusis P, Lindblom J, Post C, Skottner A. *Pharmacol Res* 2000; **42**: 393–420.
5. Ujjainwalla F, Chu L, Goulet MT, Lee B, Warner D, Wyvratt MJ. *WO patent* # 2002068387, 6 September 2002.
6. Wikberg JES. *Exp Opin Ther Patents* 2001; **11**: 61–76.
7. Andersson PM, Boman A, Seifert E, Skottner A, Lundstedt T. *Exp Opin Ther Patents* 2001; **11**: 1583–1592.
8. Bakshi RK, Barakat KJ, Nargund RP, Palucki BL, Patchett AA, Sebhat I, Ye Z, Van der Ploeg LHT. *WO patent* # 00/74679, 31 May 2000.
9. Bolton R. *J Label Compd Radiopharm* 2002; **45**: 485–528.
10. Angell YM, Garcia-Echeverria C, Rich DH. *Tetr Lett* 1994; **35**: 5981–5984.
11. Schiöth HB, Kuusinen A, Muceniece R, Szardenings M, Keinänen K, Wikberg JES. *Biochem Biophys Res Commun* 1996; **221**: 807–814.
12. Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ, DelValle J, Yamada T. *J Biol Chem* 1993; **268**: 15174–15179.
13. Bradford MM. *Anal Biochem* 1976; **72**: 248–254.

