A pharmacologically active monoclonal antibody against the human melanocortin-4 receptor: Effectiveness after peripheral and central administration

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Monoclonal antibody against the melanocortin-4 receptor

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Abstract

The hypothalamic melanocortin-4 receptor (MC4R) is a constituent of an important pathway regulating food intake and energy expenditure. We produced a monoclonal antibody (mAb) directed against the N-terminal domain of the MC4R and evaluated its potential as a possible therapeutic agent. This mAb (1E8a) showed specific binding to the MC4R in HEK-293 cells expressing the human MC4R and blocked the activity of the MC4R under basal conditions and after stimulation with \( \alpha \)-melanocyte stimulating hormone (\( \alpha \)-MSH). The inverse agonist action of agouti-related protein (AgRP) was significantly enhanced in the presence of mAb 1E8a. After a single intracerebroventricular (i.c.v.) injection into the third ventricle mAb 1E8a (1µg) increased 24h-food intake in rats. After 7 days of continuous i.c.v. administration mAb 1E8a increased food intake, body weight and fat pad weights and induced hyperglycemia. Because the complete mAb was ineffective after intravenous (i.v.) injection, we produced single chain variable fragments (scFv) derived from mAb 1E8a. In pharmacokinetic studies it was demonstrated that these scFv crossed the blood-brain barrier and reached the hypothalamus. Consequently, the scFv 1E8a increased significantly food intake and body weight in rats after i.v. administration (300µg/kg). The pharmacological profile of mAb 1E8a and the fact that its scFv fragment was active after peripheral administration suggest that derivatives of anti-MC4R mAbs may be useful in the treatment of patients with anorexia or cachexia.
Introduction

Antibodies (Abs) as therapeutic agents are currently receiving renewed interest in experimental and clinical medicine. Their selectivity, potency and efficacy at various targets have made them successful drugs in different indications, mainly cancer and autoimmune disorders (Kornbluth, 1998; Schuna and Megeff, 2000; Bohen et al., 2003). In previous studies in rats (Peter et al., 2007; Hofbauer et al., 2008) we used active and passive immunization to stimulate appetite by inhibiting the activity of the central melanocortin-4 receptor (MC4R).

The MC4R is part of an important central pathway of appetite regulation (Cowley, 2003; Ellacott and Cone, 2004; Adan et al., 2006). Its stimulation leads to an anorexigenic response, i.e. a decrease in appetite and food intake. Conversely, its blockade induces an orexigenic response, i.e. an increase in appetite and food intake. Blockade of the MC4R has been demonstrated to be effective in preventing anorexia and the concomitant loss of fat and lean body mass in rodent models of tumour-induced cachexia (Scarlett and Marks, 2005; DeBoer and Marks, 2006; Nicholson et al., 2006).

In all of our immunization experiments the N-terminal (NT) domain of the MC4R was used as an antigen because it is known to be essential for the constitutive activity of this receptor (Srinivasan et al., 2004). We could demonstrate that active immunization of rats against a 16-amino acid sequence of the NT domain resulted in a mild form of obesity and insulin resistance (Peter et al., 2007). Furthermore, immunization of rats against the NT peptide prevented the decrease of body weight and the loss of appetite in an acute model of anorexia induced by the intraperitoneal (i.p.) application of lipopolysaccharide (Hofbauer et
al., 2008). In a series of in vitro experiments we demonstrated that anti-MC4R Abs purified from the plasma of immunized rats acted as inverse agonists in the absence and as non-competitive antagonists in the presence of an MC4R agonist (Peter et al., 2007). Finally it could be shown that the passive transfer of purified polyclonal Abs from immunized rats by intracerebroventricular (i.c.v.) injection into the third ventricle of non-pretreated rats induced an increase in their food intake (Peter et al., 2007).

In the present study, we produced and characterized a monoclonal antibody (mAb) targeting the NT sequence of the MC4R and explored its therapeutic potential. This mAb (1E8a) acted in vitro as an inverse agonist and as a non-competitive antagonist. In vivo, mAb 1E8a increased food intake in rats after acute and chronic (i.c.v.) administration but was not effective after intravenous (i.v.) administration. In order to improve penetration across the blood-brain barrier, recombinant single chain variable fragments (scFv) were produced and evaluated in vitro and in vivo. They also acted as inverse agonists and non-competitive antagonists and increased food intake after i.v. administration. To our knowledge, this is the first report on a pharmacologically active mAb against the MC4R and its scFv. By virtue of their pharmacological profile and their pharmacokinetic properties these agents could represent lead molecules for the development of therapies for patients with anorexia or cachexia.
Methods

Production of monoclonal antibodies

A peptide corresponding to the NT domain of the MC4R (KTSLHLWNRSSHGLHG, residues 11-25 of the MC4R) was used as antigen. The NT peptide share the sequence of the rat MC4R (rMC4R) and human MC4R (hMC4R) isoform from residues 2 to 15. NT3 peptide (ASNRSGSGFCEQVFIKPEV, residues 26-44 of the MC3R) was synthesized as previously described (Peter et al., 2007).

C57BL/6 mice were immunized with 25µg of the free NT peptide emulsified in complete Freund’s adjuvant and injected subcutaneously (s.c.). Four weeks later a booster injection of 25µg in incomplete Freund’s adjuvant was given. Another four weeks later 10µg peptide dissolved in NaCl 0.9% was injected i.v. three days before harvesting the spleen cells for fusion. Fusion was performed with polyethylene glycol 1500 (Sigma, Saint Louis, MO, USA) at a ratio of 2 splenocytes for 1 SP2O myeloma cell. Hybridomas were cultivated in 96 well plates precoated with peritoneal macrophages of C57BL/6 mice 1000 cells/well. 5x10^5 cells were distributed per well in Isocoves Modified Dulbecco’s Medium (IMDM) supplemented with 10% heat inactivated fetal calf serum, 200mM glutamine, 100mM sodium pyruvate, 1% penicillin streptomycin (Omnilab, Mettmenstetten, Switzerland), 3% Hypoxanthine, Aminopterine, Thymidine (HAT, Gibco, Lucerne, Switzerland) in a humidified incubator at 37°C under an atmosphere of 5% CO₂. Secreting clones were screened by enzyme immunoassay and subcloned by limiting dilution (Oi et al., 1980).
**Enzyme immunoassay**

NT peptide (2μM) was adsorbed with carbonate buffer (Na$_2$CO$_3$ 15mM, NaHCO$_3$ 35mM, pH 9.6), on 96-well maxisorp® microtiter plates (Nunc, Roskilde, Denmark), 50μl/well, by incubating for 2h at 37°C. Plates were saturated with Phosphate Buffered Saline (PBS) (Na$_2$HPO$_4$ 10mM, NaCl 150mM, KCl 27mM, pH 7.4) supplemented with 3% dried milk (Biorad, Hercules, CA, USA) and 0.05% Tween 20 (Fluka, Buchs, Switzerland) (PBS-T milk) for 1h at 37°C. Immunized mice sera or 1:10 dilution of hybridoma culture supernatant were added to the plates and incubated for 1h at room temperature (RT). Plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and incubated with goat anti-mouse immunoglobulin H+L horseradish peroxidase conjugated (Biorad), diluted 1:5000, for 1h at 37°C. After washing the plates with PBS-T and PBS, enzymatic reactions were carried out at RT by adding 3,3′,5,5′-tetramethylbenzidine (TMB) in the presence of 0.04% H$_2$O$_2$. Reactions were stopped after 15 min by the addition of HCl (1N). Optical density (OD) was measured at 450nm by using a microplate reader Multiskan RC (Labsystem, Haverhill, MA, USA).

**Isotyping and purification**

The isotype of the selected mAbs was determined using MonoAbiD kit according to manufacturer’s instructions (Zymed Lab, Paris, France). The anti-NT mAbs were affinity-purified on activated CNBr-Sepharose 4B column (Amersham Biosciences, Uppsala, Sweden) with NT peptide coupled via the N-terminus according to manufacturer’s instructions. Culture supernatants were loaded on the column at 4°C. The mAbs were eluted with 0.2M glycine pH 2.7, collected in tubes containing 1M Tris buffer (pH 8.0), subsequently dialyzed against PBS overnight at 4°C and finally stored at -20°C.
Immunoprecipitation

HEK-293 cells stably transfected with the hMC4R or hypothalami from male Sprague-Dawley rats were prepared as described previously (Peter et al., 2005). MC4R membrane preparations (50µg) were incubated with purified mAb 1E8a (25µg) or mAb 2G2 (25µg) overnight at 4°C. The mixture was immunoprecipitated with 25µl of protein A/G agarose. The immunoprecipitated samples were loaded on a 10% polyacrylamide gel and transferred onto nitrocellulose membranes. The presence of MC4R was detected by using polyclonal anti-MC4R Abs (Abcam, Cambridge, UK) and the presence of MC3R using polyclonal anti-MC3R Abs (Abcam) in the standard procedure described in the one-Step™ Complete IP-Western kit (Genscript, Piscataway, NJ, USA).

Inhibition experiments

Purified mAb 1E8a (25µg) was preincubated overnight at 4°C in the presence or absence of increasing concentrations of the NT peptide (32nM - 100µM). MC4R membrane preparations from HEK-293 cells stably transfected with the hMC4R (50µg) were incubated with the mAb 1E8a/NT peptide mixture overnight at 4°C. The mixtures were immunoprecipitated with 25µl of protein A/G agarose. The immunoprecipitated samples were loaded on a 10% polyacrylamide gel and transferred onto nitrocellulose membranes. The presence of MC4R was detected by using polyclonal anti-MC4R Abs (Abcam) in the standard procedure described in the one-Step™ Complete IP-Western kit (Genscript). The western blot films were digitalized and resulting images were quantified using scion Image Beta 4.03.02 (downloaded from www.scioncorp.com). Inhibition percent of the interaction of the mAb 1E8a with the hMC4R was calculated as follows: 1-(OD\text{[NT]}/OD\text{ (without NT)}) \times 100 where OD\text{[NT]} is the optical density of the band corresponding to the hMC4R for a given
concentration of NT peptide and OD (without NT) is the optical density of the band corresponding to the hMC4R without NT peptide.

**Surface plasmon resonance**

Assays were performed in the BIACORE 3000 system as described previously (Peter et al., 2007). Kinetic parameters of the interaction between NT peptide and anti-MC4R mAbs were measured at 25°C in two series of experiments: first, 5 different concentrations (0.25 - 4µM) of purified mAb 1E8a were injected at a flow rate of 30µl/min for 300sec on the immobilised NT peptide or NT3 peptide as control followed by a dissociation phase of 400sec. Subsequently, 5 different concentrations (0.125 - 1µM) of purified mAb 2G2 were injected at a flow rate of 30µl/min for 300sec on the immobilised NT peptides followed by a dissociation phase of 400sec. The kinetic parameters were calculated using BIA evaluation software 4.1 (Biacore, Uppsala, Sweden). All resonance unit (RU) values obtained with the control NT3 peptide were subtracted from those obtained with the MC4R NT peptide in order to compensate for non-specific binding.

**Cell culture**

HEK-293 cells expressing hMC4R or hMC3R were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO, USA) containing 10% fetal calf serum (Bioconcept, Allschwil, Switzerland), 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) and G418 at 600 µg/ml (Sigma) in a humidified atmosphere containing 5% CO₂ at 37°C.
cAMP assays

Cells were transferred to 24-well culture plates 12h before treatment, then washed for 3h with culture medium (DMEM, Sigma) and incubated for 30min in PBS supplemented with 0.1% bovine serum albumin (BSA) and 3-isobutyl-1-methylxanthine (IBMX) (Sigma). Cells were treated with serial dilutions of purified mAbs or scFv for 30min or preincubated with fixed concentrations of mAbs or scFv for 30min and then treated with serial dilutions of α-melanocyte stimulating hormone (α-MSH, 1.0x10^{-10} M to 1.0x10^{-5} M) for 15 min or (agouti-related protein (AgRP) (1.0x10^{-12} M to 1.0x10^{-6} M). Subsequently, cells were lysed with Biotrak cAMP lysis buffer and cAMP was measured using the Biotrak cAMP enzyme immunoassay system (Amersham Bioscience, Uppsala, Sweden) according to the manufacturer's instructions. Protein concentration was determined using the BCA kit (Pierce, Rockford, IL, USA). The concentration of cAMP was expressed in fmol cAMP/μg protein.

Immunocytofluorescence

HEK-293 cells expressing hMC4R or hMC3R were fixed for 5min with 2% paraformaldehyde in PBS. Slides were saturated with PBS supplemented with 5% non-fat dried milk. mAbs 2G2 or 1E8a (50μg/ml) were applied on cells for 1h at RT. After 3 washes with PBS, goat anti-mouse alexa conjugated (1/500) (Molecular Probes, Junction City, OR, USA) was allowed to react with the fixed primary antibody for 1h at RT. 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (1μg/ml, Molecular Probes) was used for nuclear staining. The same magnification (40x) and exposure time (500msec) was used for each slide.
Receptor internalization

HEK-293 cells expressing the hMC4R were treated with 200nM of α-MSH (Bachem) conjugated with treramethylrhodamine-5-6-isothiocyanate (Molecular Probes) using manufacturer’s instructions. Cells were washed with ice-cold PBS and fixed at t=0, 10, 30 and 45min. Concanavalin A alexa-fluor 488 (0.1mg/ml, Molecular Probes) and DAPI (1µg/ml, Molecular Probes) were applied on cells for membrane labeling and nuclear staining respectively. The original green (concanavalin A alexa fluor 488) and red fluorescence (α-MSH conjugated with treramethylrhodamine-5-6-isothiocyanate) confocal images were converted to grayscale. Each pixel is assigned and intensity value ranging from 0 (black) to 255 (white). The grayscale images obtained with concanavalin A fluorescence were subtracted from grayscale images obtained with α-MSH fluorescence. The resulting images were quantified using scion Image Beta 4.03.02 (downloaded from www.scioncorp.com). An increase of the OD signal indicates internalization of the MC4R.

Cloning of cDNA encoding the variable domain of the mAbs

Total RNA was prepared from 10⁷ freshly subcloned hybridoma cells using the RNAnow kit (Biogentex Inc., Seabrook, TX, USA) and first strand cDNA was synthesized using iScript™cDNA Synthesis kit (Biorad, Hercules, CA, USA). The V_<sub>H</sub> and V_<sub>L</sub> chain domains were amplified by PCR using IgG primer set (Novagen, Gibbstown, NJ, USA). The 50µl PCR mixtures contained 50ng hybridoma cDNA, 20 pmol of each appropriate primer, 250 µM of each dNTP, 1×Taq buffer (Sigma) and 1U *Thermophilus aquaticus* (Taq) polymerase. Amplification included 50 cycles of 1.5min at 94°C, 2.5min at 55°C and 3min at 72°C in a thermocycler (PTC-150, MJ Research, Waltham, MA, USA). The amplified DNAs were ligated into the pGEMT vector (Promega, Madison, WI, USA) and the recombinant plasmids purified using miniprep kit (Qiagen, Hombrechtikon, Switzerland).
The DNA sequences of the cloned \( V_H \) and \( V_L \) inserts were determined using the ABI PRISM Cycle Sequencing kit (Applied Biosystem™, Carlsbad, CA, USA) and M13 Forward and Reverse primers. The sequences of the \( V \) genes were determined in two independent batches of RNA preparations to ensure accuracy.

Construction of the scFv genes

scFv proteins were created by joining their \( V_H \) and \( V_L \) genes together by PCR splicing with overlap extensions using oligo-nucleotides that encoded a 15 amino-acid linker (G\(_4\)-S\(_3\)) between the C-terminal of the \( V_H \) and the N-terminal of the \( V_L \) gene. The ends of the 1E8a mAb variable gene were modified by PCR using as primers, 1E8V\(_H\)Rev (5’ GTT CCA GCT GCA GCA GTC TGG ACC TGA GC3’) which encodes the N-terminal wild type sequence of the \( V_H \) containing a \( Ncol \) site and \( V_H\)For (ACC ACC GGA TCC GCC TCC GCC TGA GGA GAC TGT GAG CGT 3’) which encodes the C-terminus of the VH and a part of the linker. 1E8V\(_L\)Rev (5’ GGA GGC GGA TCC GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC GAC ATT GTG ATG ACC CAG 3’) and \( V_L\)For, containing a \( XhoI \) site which encodes 6 His residues (5’ GCA ATT CCT CGA GTT AGT GAT GGT GAT GGT GAT GTT TTA TTT CCA GCT TGG TCC 3’) were used to amplify and modify the \( V_L \) domain.

The ends of the 2G2 mAb variable gene were modified by PCR using as primers, 2G2V\(_H\)Rev (5’ TGG CCA TGG CCG CGG GAT TGG TCC AGC TGC AGC AGT CTG GA 3’) which encodes the N-terminal wild type sequence of the \( V_H \) containing a \( Ncol \) site and \( V_H\)For (ACC ACC GGA TCC GCC TCC GCC TGA GGA GAC TGT GAG CGT 3’) which encodes the C-terminus of the VH and a part of the linker. 2G2V\(_L\)Rev (5’ GGA GGC GGA TCC GGT GGT GGC GGA TCT GGA GGT GGC GGA AGC GAC ATT GTG ATG ACC CAG 3’) and 2G2V\(_L\)For, containing a \( XhoI \) site which encodes 6 His residues (5’ GCA ATT CCT CGA GTT AGT GAT GGT GAT GGT GAT GTT TTA TTT CCA GCT TGG TCC 3’)
3'), were used to amplify and modify the V_L domain. The scFv gene was inserted in frame with the PelB sequence on the expression vector pET22b (EMD Biosciences, Darmstadt, Germany) between the Ncol and Xhol sites.

**Expression of scFvs**

*Escherichia Coli* Rosetta bacterias transformed with pET22b(+)-1E8a or pET22b(+)-2G2 were grown in 500ml of medium 2xYT (bactotryptone 1.6%, bactoyeast extract 1%, NaCl 0.5%, pH 7.0) containing ampicilline 0.15mM (Applichem, Darmstadt, Germany) and chloramphenicol 0.1mM (Gerbu Biotechnik, Gaiberg, Germany) until an OD_{600nm} of 0.6 at 37°C with agitation at 200rpm. The expression of scFv was induced by adding 1mM IPTG (Applichem) at RT during 4h.

**Periplasmic extraction**

500ml of bacteria cultures were centrifuged (10min, 10,000g, 4°C) and the pellet was resuspended in 200ml of TES (Tris 30mM, EDTA 1mM, sucrose 2%, pH 8.5). After centrifugation (10min, 10,000g, 4°C), the pellet was resuspended in 50ml MgSO_4 5mM. After a last centrifugation (10min, 10,000g, 4°C) the supernatant corresponding to the periplasmic extract (PE) was collected. This PE was dialyzed in wash buffer NiNTA (imidazole 20mM, Na_2HPO_4 50mM, NaCl 300mM, pH 8.0) overnight at 4°C.

**Purification of scFv**

The scFvs were purified from the PE on NiNTA columns according to the manufacturer's instruction (Qiagen). After elution, purified scFvs were dialysed in PBS (Na_2HPO_4 10mM, NaCl 150mM, KCl 27mM, pH 7.4) overnight at 4°C. The scFvs were then purified by immunoadsorption as described previously in the *Purification of monoclonal antibody*
section. The concentrations were determined with Micro BCA Protein Assay kit (Pierce). Quality and purity of the purified scFv fractions were assessed by SDS-PAGE analysis using 12.5% acrylamide gels followed by staining with Coomassie brilliant blue (Biorad) and western blot. For western blot analysis, the proteins were transferred from the gels onto a nitrocellulose transfer membrane a mini trans-blot system (Biorad) in transfer buffer (Tris-HCl 25mM, glycine 190mM, methanol 20%, pH 8.3). The membranes were soaked 1h in PBS-T supplemented with 5% non fat milk powder and 0.1% Tween 20. This was followed by 1h incubation with anti-His\textsubscript{6} Ab conjugated to HRP 1/2000 (Sigma). The Ab was diluted in the blocking solution PBS-T milk. The proteins on the membranes were revealed by the classical procedure of the ECL reagents (Amersham Bioscience).

Radioactive labeling and purification

The scFv 1E8a was radioactively labeled with \textsuperscript{131}I by the iodobead method. In brief, an iodobead was incubated in 0.1ml of phosphate buffer solution with 2mCi of \textsuperscript{131}I for 5 min at RT. Five \textmu g of scFv were then added in a volume of 2.2\textmu l. After 3min of incubation at RT, the I-scFv was separated from unincorporated \textsuperscript{131}I on a column of G-10 Sephadex. BSA was labeled by incubation with chloramine-T for 60sec and purification of the radioactively labeled albumin (I-Alb) on a column of G-10 Sephadex.

Pharmacokinetics of brain uptake

Male CD-1 mice were anesthetized with urethane. All animal studies were done in accordance with international standards and under protocols approved by the local animal use committee. The blood-to-brain unidirectional influx rate (K\textsubscript{i}, in units of \textmu l/g-min) was calculated by multiple-time regression analysis (Blasberg et al., 1983; Patlak et al., 1983). In brief, the right jugular vein and the left carotid artery were exposed. At t = 0, 0.2ml of
lactated Ringer’s solution containing 1% BSA (LR-BSA) and 500,000cpm I-scFv was injected into the jugular vein. Between 2 and 180 min after the i.v. injection, blood was collected from the carotid artery and the mouse immediately decapitated. Two mice were studied per time point. The arterial blood was centrifuged and serum collected and results expressed as the percent of the injected dose present per ml of serum (%Inj/ml). The brain was dissected into the cortex, cerebellum, hippocampus, hypothalamus, and remainder of the brain, the regions weighed, and the level of radioactivity determined. Results were expressed as the brain/serum ratios (in units of μl/g) and plotted against exposure time (Expt), where

\[ \text{Expt} = \frac{\int_0^t C_p(\tau) d\tau}{C_p t} \]

and \( C_p \) is the level of radioactivity in serum and \( C_p t \) is the level of radioactivity in serum at time \( t \). Expt values correct for the clearance of the test substance from the blood so that the greater the clearance from blood the greater the difference between Expt and \( t \). Without this correction, Ki would be overestimated. Brain/serum ratios for whole brain were calculated by summing the brain region values for radioactivity and weight. The slope of the linear portion of the relation between brain/serum ratios and Expt measures Ki and the intercept of the linearity measures Vi (μl.g), the initial volume of distribution in brain.

In other mice, I-Alb was included in the i.v. injection. The percent of the injected dose taken up per g of brain (%Inj/g) was calculated as follows:

\[ \%\text{Inj/g} = 100(A-B)(%\text{Inj/ml})/1000 \]

where \( A \) is the brain/serum ratio for I-scFv and \( B \) is the ratio for I-Alb.
Acid precipitation

To determine whether the radioactivity in brain and serum at various times represented intact I-scFv, we performed acid precipitation on radioactivity obtained at 30 min and 4 h after i.v. injection. Whole blood was centrifuged and 50 µl of the resulting serum added to 100 µl of LR-BSA and then to 100 µl of 30% trichloroacetic acid. The sample was vigorously mixed and centrifuged at 5400g for 15 min at 4°C. The resultant supernatant and precipitate were separated and counted and the results expressed as the percent of total counts that were precipitated. Brains were homogenized in a glass homogenizer in 3 ml of LR-BSA and then centrifuged at 5400g for 10 min at 4°C. An aliquot of 0.5 ml of the supernatant was added to 0.5 ml of 30% trichloroacetic acid, the sample vigorously mixed, and then centrifuged at 5400g for 10 min at 4°C. The supernatant and precipitate were separated and counted and the results expressed as the percent of total counts that precipitated. To correct for any degradation that might have occurred during the processing for acid precipitation, we added I-scFv to non-radioactive arterial whole blood or to whole brain. These samples were then processed as above and the percent of total counts that were precipitated determined. The mean of 2 processing controls was 96% for serum and 89% for brain. The values for the biological samples were divided by the value of the processing control and multiplied by 100 to give the reported result.

Capillary depletion

Capillary depletion as modified for use in the mouse (Triguero et al., 1990; Gutierrez et al., 1993) was used to determine the degree to which I-scFv was sequestered by the vascular bed of the brain. Mice were anesthetized with urethane and given an injection into the jugular vein of 0.2 ml saline containing $10^6$ cpm of I-scFv and $10^6$ cpm I-Alb. After 2 h, arterial
blood was obtained from the carotid artery. The brain was removed and emulsified in a
glass homogenizer (8-10 strokes) at 4ºC in a 9-fold volume of physiological buffer (10mM
HEPES, 141mM NaCl, 4mM KCl, 2.8mM CaCl₂, 1mM MgSO₄, 1mM NaH₂PO₄, and 10mM
D-glucose adjusted to pH 7.4). Dextran solution was added to the homogenate to a final
conzentration of 26%. An aliquot was centrifuged at 5400g for 15min at 4ºC in a swinging
bucket rotor. The pellet containing the brain microvessels and the supernatant containing
the brain parenchyma were carefully separated. Results were expressed as capillary/serum
and parenchyma/serum ratios. Values for I-Alb were subtracted from those for I-scFv to
yield values corrected for residual vascular contamination and blood-brain barrier leakage.

**I.c.v. and i.v. administration**

Male Sprague-Dawley rats (275 to 325g) were anesthetized with isoflurane in medicinal
oxygen (4% for induction and 2% for maintenance of anesthesia). A stainless steel cannula
(26 gauge, 10mm long) was implanted into the third cerebral ventricle using the following
coordinates, relative to the Bregma: -2.3mm anteroposterior, 0mm lateral to the midline, and
-7.5mm ventral to the surface of the skull. The guide cannula was secured in place with 3
stainless steel screws and glass-ionomer cement (3M), and a stylet was inserted to seal the
cannula until use. Temgesic (Essex Chemie AG, Lucerne, Switzerland) (0.03mg/kg) was
given s.c. prior to and for 2 days postsurgery. Seven days after recovery from surgery,
accuracy of the cannula placement in the third ventricle was tested by measuring the
dipsogenic response (immediate drinking of at least 5ml water in 15min) to an i.c.v. injection
of 20pmol of angiotensin II in 2µl injection volume.

Purified mAbs and scFvs were slowly (1min) injected i.c.v. at 9:00am at a dose of 1µg in a
volume of 2µl using a Hamilton syringe. These doses were selected based on the results of
comparative *in vitro* studies. Following the injection of mAbs or scFvs, food intake was
continuously recorded during the following 3 days using an automatic food intake apparatus (TSE Systems, Bad Homburg, Germany) at one hour intervals.

Osmotic minipumps (model 2002) (Alza Corp., Charles River Laboratories Inc., Saint-Aubin-les-Elbeuf, France) were filled with mAbs 1E8a or 2G2 calculated to deliver 1µg/day during 7 days. One week after the angiotensin II test the pumps were implanted dorsally under the skin under isoflurane anesthesia and immediately connected to the i.c.v. cannula via a tube prefilled with mAbs to ensure an immediate delivery. Body weight and food intake were monitored during the treatment by using an automatic food intake apparatus (TSE Systems, Bad Homburg, Germany).

In the experiments with i.v. administration, purified mAbs 1E8a or 2G2 and scFvs 1E8a and 2G2 were injected into the tail vein of rats at 9:00 am under mild isoflurane anesthesia at a dose of 300µg/kg. Following the injection of scFv or mAb, food intake was continuously recorded using an automatic food intake apparatus (TSE Systems) at one hour intervals for 3 days.

Data analysis
All data are expressed as mean ± SD or SEM as indicated. Data were analysed by two-way ANOVA repeated measures with Bonferroni post-hoc test or by Student t-test using Graphpad Prism 4 software. For the cAMP concentration-response experiments, the best fitting curves were compared for their minimum, maximum and EC₅₀ using F-test. Ki and EC₅₀ were calculated using Graphpad Prism 4 software. For pharmacokinetic experiments, means are reported with standard errors. The Prism 5.0 (GraphPad, Inc, San Diego, CA) was used for regression analysis and to compare slopes and regression lines. Half-time
clearance from blood was calculated by multiplying by 0.301 the inverse of the slope for the relation between time and log(%Inj/ml). The Vd was computed by multiplying the antilog of the intercept by 100.
**Results**

*Selection of mAbs and selectivity of mAb 1E8a*

The mouse serum used for hybridoma production showed a pronounced response against the immunogenic peptide derived from the N-terminus of the MC4R but not against the corresponding peptide derived from the N-terminus part of the MC3R. Although the polyclonal response was high, only 10 clones were viable until subcloning and amplification (6 IgG, κ and 4 IgM, κ). After purification only mAb 1E8a (IgM, κ) showed a blockade of the MC4R activity in presence of α-MSH. mAb 2G2 (IgM, κ) was selected as isotype matched negative control for the characterization of mAb 1E8a because it did not show a blockade of the MC4R activity. When the affinity of these mAbs for the NT peptide was assessed by surface plasmon resonance, a $K_D$ of $1.3 \times 10^{-8}$M for the mAb 1E8a and $3.7 \times 10^{-8}$M for the mAb 2G2 was calculated.

Immunocytofluorescence experiments were performed to assess the binding of the mAbs to the surface of HEK-293 cells overexpressing the hMC4R. Figure 1a shows specific membrane labeling of hMC4R expressing HEK-293 cells when using the mAb 1E8a. No labeling was observed on cells expressing the hMC3R (Figure 1b). When HEK-293 cells expressing hMC4R were treated with mAb 2G2 no labeling could be observed (Figure 1c). Immunoprecipitation experiments confirmed the selectivity of mAb 1E8a for the hMC4R and rMC4R. mAb 2G2 did not immunoprecipitate the hMC4R and rMC4R (Figure 1d). The presence of NT peptide inhibited the interaction of the mAb 1E8a with the MC4R in a concentration-dependent manner ($K_i$ of $1.3 \times 10^{-6}$M) (Figure 1e).
Pharmacological activity of 1E8a and 2G2 mAbs in vitro

When HEK-293 cells transfected with the hMC4R were exposed to increasing concentrations of mAb 2G2 (1pM – 0.1µM), no decrease in cAMP formation was measured (Figure 2a). Conversely, when HEK-293 cells were exposed to increasing concentrations of mAb 1E8a (1pM – 0.1µM) cAMP formation was decreased in a concentration-dependent manner by up to 40% (EC$_{50}$ of 2.1x10$^{-12}$ M) (Figure 2c).

The presence of 100nM of mAb 2G2 did not change the concentration response curve of α-MSH (Figure 2b) but the presence of 100nM of mAb 1E8a significantly (p<0.001, F-test) reduced the maximum effect of α-MSH (Figure 2d).

When HEK-293 cells transfected with the hMC4R were exposed to increasing concentrations of AgRP (1pM – 0.1µM) a decrease in cAMP formation was measured (Figure 2e). In the presence of 100nM of mAb 1E8a, a leftward shift of the AgRP concentration-response curve was observed.

Receptor internalization

The presence of mAb 1E8a (100nM) did not influence the internalization of the MC4R expressed at the surface of HEK cells (Figure 3).

Pharmacological activity of mAbs 1E8a and 2G2 in vivo

Rats which received an injection of 1 µg of mAb 1E8a into the third ventricle ingested 24% more food in 24h than rats which received mAb 2G2 or BSA as controls (Figure 4a). The body weight of rats which received mAb 1E8a was increased whereas body weight was reduced in rats which received either mAb 2G2 or BSA (Figure 4b).

A 7 day i.c.v. infusion of 1E8a induced a significant increase in food intake (20%) compared with rats which received mAb 2G2 (Figure 4c). The body weight of rats treated with mAb
1E8a was significantly less reduced than in control rats (Figure 4d). After 7 days of continuous i.c.v. infusion, a non-significant increase of the fasting glycemia (Figure 4e) and a significant increase in fat pads weight were observed (Figure 4f).

**Variable domain cloning and scFv expression**

The scFv-encoding genes derived from the variable regions (V<sub>H</sub> and V<sub>L</sub> linked together via a short linker (G<sub>4</sub>S)<sub>3</sub> of the mAb 1E8a and mAb 2G2, with addition of a C-terminal six-His tag encoding sequence), were inserted in frame with the PelB sequence into the pET22b expression vector. The sequence of the single chain construction is represented in Figure 5. We confirmed that the cloned VL gene did not correspond to the aberrant kappa transcript of the sp20 hybridomas (Carroll et al., 1988). The plasmid pET-scFv 1E8a or pET-scFv 2G2 was cloned into the Rosetta *Escherichia Coli* strain and the recombinant protein was expressed and exported to the bacterial periplasm by its leader sequence PelB (Lei et al., 1987). The scFv 1E8a and scFv 2G2 were purified and concentrated by IMAC chromatography and repurified by immunoabsorption to get rid of the not correctly folded inactive recombinant proteins (Peter et al., 2003) (Figure 6). The affinity of these scFv for the NT peptide was assessed by surface plasmon resonance. A K<sub>D</sub> of 7.8x10<sup>-7</sup>M for the scFv 1E8a and 2.4x10<sup>-7</sup>M for the scFv 2G2 was calculated.

**Pharmacological effect of the scFv 1E8a and 2G2 in vitro**

When HEK-293 cells transfected with the hMC4R were exposed to increasing concentrations of scFv 2G2 (1pM to 0.1µM), no decrease in cAMP formation was measured (Figure 7a). Conversely, when HEK-293 were exposed to increasing concentrations of scFv 1E8a (1pM to 0.1µM) cAMP formation decreased in a concentration-dependent manner by up to 40% (Figure 7c).
The presence of 100nM of scFv 2G2 did not change the concentration-response curve of α-MSH (Figure 7b) but the presence of 100nM of scFv 1E8a significantly (p<0.001, F-test) reduced the maximum effect of α-MSH (Figure 7d).

**Pharmacokinetics of scFv 1E8a**

When iodinated scFv (I-scFv) was injected i.v. and its clearance from blood calculated, the relation between log(%Inj/ml) and time was highly significant (r=0.788, n=20, p<0.001) with a half-life of 84.6 min and a distribution volume (Vd) of 2.15 ml. This shows that I-scFv distribution was limited to the vascular space.

Figure 8 shows the uptake of I-scFv by cortex, cerebellum, hypothalamus, and hippocampus in comparison to whole brain. For all brain regions and for whole brain, the relation between brain/serum ratios and Expt was significant (r ranging from 0.869 to 0.930, n=20 mice/line; p<0.001 for all curves). No statistical difference was found among the slopes which ranged from a low of 0.046±0.005µl/g-min for cortex to a high of 0.057±0.008 µl/g-min for cerebellum. The y-intercept for cerebellum was greater than that for whole brain. The Y-intercept measures the Vi, or initial vascular space, and differences typically indicate a greater association with or binding to the luminal surface of the brain endothelial cells. Figure 8e compares I-scFv and I-albumin uptake by whole brain. For I-albumin, there was no significant correlation between brain/serum ratios and Expt so that no penetration of the blood-brain barrier was formally demonstrated. In comparison, the uptake of scFv was significant (r=0.736, n=13, p<0.005) with a slope of 0.054±0.015µl/g-min. A value of about 0.2%Inj/g was reached at 120min.

Acid precipitation corrected for processing controls gave values for serum of 102% and 96% of radioactivity representing intact I-scFv at 30min and 4h, respectively. For brain, the values were 96% and 44% at 30min and 4h.
In capillary depletion experiments, it was found that the capillary/serum ratio was $0.57 \pm 0.05 \mu l/g$ and that the parenchymal/serum ratio was $1.03 \pm 0.17 \mu l/g$. This showed that the majority of I-scFv taken up by the blood-brain barrier was not sequestered by the capillary bed, but entered the brain parenchymal space.

**Pharmacological activity of scFvs 1E8a and 2G2 in vivo**

1µg of purified scFv 1E8a and scFv 2G2 was injected into the third ventricle of rats. Rats which received scFv 1E8a ingested 45% more food in 24 h than rats which received scFv 2G2 (Figure 9a). The body weight of rats which received scFv 1E8a was significantly less decreased than that of rats that received scFv 2G2 (Figure 9a).

Purified scFv 1E8a and scFv 2G2 were injected (300µg/kg) i.v. in rats. Rats which received scFv 1E8a ingested 33% more food in 24h than rats which received scFv 2G2 (Figure 9b). The body weight of rats which received scFv 1E8a was unchanged whereas body weight was reduced in rats which received scFv 2G2 (Figure 9b).
Discussion

The MC4R is a key receptor for the regulation of food intake and energy expenditure. Its dysfunction leads to severe obesity in rodents and humans (Ste Marie et al., 2000; Farooqi et al., 2003). Conversely, its over-activation in chronic diseases such as cancer which is probably caused by high levels of circulating cytokines (Huang et al., 1999) leads to cachexia (Marks and Cone, 2001). Blockade of the MC4R in rodent models of cachexia showed a significant increase in lean body mass (Nicholson et al., 2006). Moreover, rats immunized against the NT domain of the MC4R were resistant to lipopolysaccharide-induced anorexia (Hofbauer et al., 2008).

In a previous study we demonstrated that immunization against the N-terminus of the MC4R in rats led to the generation of pharmacologically active Abs which acted as inverse agonists and non-competitive antagonists (Peter et al., 2007). In the present study, we characterised the pharmacological properties of a mAb (1E8a) against this peptide sequence of the NT domain in vitro and in vivo. Another mAb (2G2) which recognized the NT peptide but not the corresponding sequence in the native receptor served as a control throughout our experiments.

The binding properties of mAbs 1E8a and 2G2 were studied in two different in vitro experiments. By using immunocytofluorescence in HEK293 cells overexpressing the hMC3R or hMC4R, binding to the native form of the hMC4R and subtype selectivity was assessed. The mAb 1E8a bound to the hMC4R but not to the hMC3R on the cell surface, a result which was not unexpected because the N-terminal sequences of the MC3R and MC4R subtypes show a low degree of homology (Gantz et al., 1993). In general, the
sequences of extracellular domains are less well conserved than those of transmembrane domains and are therefore suitable targets for a subtype-selective pharmacological approach (Mirzadegan et al., 2003).

The mAb 2G2 showed no interaction with either hMC3R or hMC4R at the cell surface. These results are consistent with our immunoprecipitation experiments which indicated that mAb 1E8a bound to hMC4R and rMC4R with high affinity whereas the mAb 2G2 interacted only with the NT peptide but not with the corresponding sequence in the native MC4R. A specific interaction of mAb 1E8a with the MC4R was supported by the observation that increasing concentrations of the NT peptide reduced the binding of mAb 1E8a with a Ki in the µM range. As compared with the Kd of the mAb 1E8a/NT peptide binding, which is in the nM range, this Ki indicated an better binding of mAb 1E8a to the native conformation of the receptor than to the NT peptide.

The affinity of the mAb 1E8a and the mAb 2G2 for the NT peptide were in the same range (1.3x10⁻⁸M and 3.7x10⁻⁸M, respectively). They are relatively high for an IgM isotype but other high affinity IgM mAbs have been previously described (Ballard et al., 1983; Suenaga and Abdou, 1992; Cao et al., 2004). The small difference between the affinity of mAbs 1E8a and 2G2 cannot explain why the latter did not interact with the MC4R. This might indicate that the paratope of mAb 2G2 interacted with a conformation of the NT peptide that the intact receptor is unable to adopt. In contrast, mAb 1E8a is able to recognize a common structure shared by the NT peptide and the NT domain in the native MC4R.
The pharmacological properties of mAb 1E8a were assessed by using adenylyl cyclase assays in HEK-293 cells overexpressing the hMC4R. The mAb 1E8a behaved as an inverse agonist and decreased basal activity by 40% with an EC50 in the pM range. It behaved as a non-competitive antagonist in the presence of α-MSH and decreased the maximum efficacy of this agonist by 20%. A pattern of inverse agonism combined with non-competitive antagonism has been described for other Abs at G-protein coupled receptors (Peter et al., 2003; Peter et al., 2004; Peter et al., 2007). The mAb 2G2 did neither affect the constitutive activity of the MC4R nor the concentration-response curve of α-MSH.

In order to find out whether the in vitro effects of mAb 1E8a translated into efficacy in vivo purified mAb 1E8a was administered into the third ventricle of rats. The mAb 2G2 served as a control. When given at the beginning of the light phase mAb 1E8a increased 24h food intake by approximately 25%. This acute effect is not too different from that obtained with SHU 9119, a peptidic MC3/MC4R antagonist (Obici et al., 2001; Peter et al., 2007). However, the main effects on food intake were seen during the dark phase. This finding is at variance with the fact that MC4R tone is highest during the light phase when satiety prevails. The fact that AgRP expression is higher during the dark phase (Lu et al., 2002) together with our observation that mAb 1E8a strongly enhanced the effects of AgRP in vitro might offer an explanation for this result.

When mAb 1E8a was continuously infused via osmotic minipumps into the third ventricle in rats, a 70% increase of food intake as compared with controls was recorded during the first 24h. This difference is much more pronounced than that seen after i.c.v. injection. The stronger effect of MC4R blockade in these experiments may be due to the fact that rats had not yet recovered from the minipump implantation. The effect of mAb 1E8a under these
circumstances may therefore be interpreted as a treatment effect during an inflammatory response caused by a recent surgical intervention.

At the end of the 7-day treatment period with mAb 1E8a we observed a trend for an increase in glycemia and a significant increase in fat pads weight. Similar findings have been reported in MC4R knock-out mice (Fan et al., 2000), in rats after central administration of antisense oligonucleotides against the MC4R (Obici et al., 2001) and in rats immunized with the NT peptide of the MC4R (Peter et al., 2007; Hofbauer et al., 2008).

No effects on food intake were seen after single i.v. administration of mAb 1E8a in a dose (300μg/kg) approximately hundred-fold higher than that given i.c.v. (1μg/rat) (data not shown). This excludes the contribution of peripheral MC4R but raises the question whether i.v. administered mAbs can reach their supposed central sites of action. The absence of a central effect is consistent with the findings of Banks et al. (2002) who observed that after i.v. injection peak amounts of Abs in brain tissue are only 0.11% of the total administered dose. We therefore generated recombinant scFvs from mAb 1E8a and mAb 2G2. The scFv 1E8a acted as an inverse agonist and non-competitive antagonist of the MC4R and showed the same efficacy as the intact mAb. The affinity of the scFv 1E8a for the NT peptide was 10x lower than that of the intact mAb 1E8a. This difference is consistent with the higher EC\textsubscript{50} calculated from the scFv inverse agonist activity.

The i.c.v. application of 1μg of scFv 1E8a induced an increase in food intake which occurred faster and was more pronounced than that obtained with the intact mAb 1E8a. This observation could reflect a better brain penetration of scFv as compared to the parent mAb. After i.v. administration in rats scFv 1E8a induced an increase of food intake whereas
the complete mAb 1E8a was ineffective. This suggests that scFv 1E8a can cross the blood-brain barrier and penetrate brain tissue to reach its central receptors. The smaller size of the scFv may provide the explanation for these observations.

Pharmacokinetic studies were performed to further address the issue of brain penetration. Uptake of I-scFv was uniform across several brain regions and exceeded that of the vascular marker albumin. This suggests a mechanism of uptake other than entry by way of extracellular pathways (Broadwell and Sofroniew, 1993), the mechanism proposed for IgG (Banks et al., 2002; Banks et al., 2005) and IgM molecules (Banks et al., 2007). Capillary depletion showed that about 1/3 of the I-scFv retained by brain was sequestered by brain endothelial cells, while about 2/3 were located in brain parenchyma. This sequestration by capillaries also suggests that a transcytotic pathway across the blood-brain barrier is more likely than leakage into brain by the extracellular pathways.

A long half-life from brain and enzymatic resistance contributed to brain accumulation as well. We calculated that by 2h, about 0.2 percent of the i.v. injected dose had been taken up by whole brain. However, we did not correct for degradation in brain with time, so the value may be 30-50% lower than this. This would still produce brain levels that are similar to or greater than those seen with other proteins which are centrally active (Banks et al., 1991; Banks et al., 1993; Banks et al., 1996) and therefore sufficient for a pharmacological effect. However, receptor binding studies would have been needed to clearly demonstrate that the scFv fragment reached its presumed hypothalamic site of action at concentrations which were biologically active.
In this paper we report for the first time that an anti-MC4R mAb and its scFv derivative are able to modulate the MC4R activity in vitro and in vivo. The scFv 1E8a crossed the blood-brain barrier in amounts sufficient to increase food intake after i.v. administration in rats. Such molecules might therefore represent a starting point for the development of new therapies for patients with anorexia and cachexia.
References


Legends to figures

Figure 1: Immunocytochemistry of HEK-293 cells transfected with hMC4R or hMC3R. Nuclear labeling (blue) is merged with immune labeling (red). Specific membrane labeling was seen in HEK-293 cells expressing hMC4R when incubated with the mAb 1E8a (a) but not in HEK-293 cells expressing hMC3R (b). No labeling was observed with mAb 2G2 in hMC4R expressing cells (c) (400x magnification).

(d) Immunoprecipitation of hMC4R and rMC4R. Lanes 1 and 2 show the results obtained with hMC4R and hMC3R with mAb 1E8a, while lanes 5 and 6 show the corresponding results with rMC4R and rMC3R. Lanes 3 and 4 show control experiments with mAb 2G2. Purified mAb 1E8a precipitated both the hMC4R (lane 1) and the rMC4R (lane 5) but not hMC3R or rMC3R (lane 2 and 6) while mAb 2G2 was inactive (lanes 3 and 4). (e) Inhibition of hMC4R immunoprecipitation by mAb 1E8a using increasing concentration of the NT peptide. Results are presented as % of inhibition of the signal obtained without NT peptide in function of the [NT]. Mean ± SD, as calculated from 3 independent experiments, n=5 measurements/experiment.

Figure 2: Intracellular cAMP formation in HEK-293 cells transfected with hMC4R. (a) Concentration-response curves obtained with purified mAb 2G2. The mAb 2G2 had no effect on basal cAMP production. (b) Concentration-response curves obtained with α-MSH in the presence or absence of 100nM of mAb 2G2 (▲) or PBS (■). The presence of the mAb 2G2 had no effect on the concentration-response curve of α-MSH.

(c) Concentration-response curves obtained with purified mAb 1E8a. The concentration-dependent decrease in the intracellular cAMP content suggests an inverse agonist effect of mAb 1E8a. (d) Concentration-response curves obtained with α-MSH in the presence or
absence of 100nM of mAb 1E8a (♦) or PBS (■). The reduced maximum efficacy of α-MSH in the presence of mAb 1E8a suggests that this mAb acts as a non-competitive antagonist. Data are presented as means±SD calculated from 3 independent experiments. (e) Concentration-response curves obtained with AgRP in the presence of 100nM of mAb 1E8a (♦) or PBS (■). The leftward shift of the AgRP response curve in the presence of mAb 1E8a suggests that this mAb acts in synergy with AgRP. Data are presented as means±SD calculated from 3 independent experiments (***: p<0.001, F-test).

Figure 3: Time course of agonist induced internalization of MC4R. HEK-293 cells stably expressing hMC4R were exposed to 200 nM α-MSH coupled to rhodamine red for various lengths of time. (a) Confocal images show the distribution of MC4R (red) at the selected time points (t=0, 10, 30, 45min) after agonist stimulation in the (+) or absence (-) of mAb 1E8a. (b) Quantitative analysis of time course of agonist induced MC4R internalization. Digitized fluorescence intensity on the cell surface membrane and total cellular fluorescence intensity were quantified as described under Materials and Methods. The graphic represent the MC4R internalization expressed as OD as a function of time in the presence (+) or absence of (-) mAb 1E8a. The presence of mAb 1E8a did not interfere with the internalization of the hMC4R.

Figure 4: (a) 24h food intake in rats which received an i.c.v. injection of either 1µg of mAb 1E8a, 1µg of BSA or 1µg of mAb 2G2. The injection of mAb 1E8a induced a significant increase in food intake. Data are presented as mean±S.E.M. #: p<0.01 two-way ANOVA with repeated measures; *: p<0.05, **: p<0.01, ***: p<0.001 Bonferroni post-hoc test. (b) Body weight change of rats 24h after i.c.v. injections. mAb 1E8a induced an increase in body weight compared to rats which received either mAb 2G2 or BSA. $: p<0.05, $$:
p<0.01, Students’ t-test. (c) 7 day food intake in rats, which received a continuous i.c.v. infusion of either 1µg/day of mAb 1E8a or 1µg/day of mAb 2G2. The infusion of mAb 1E8a induced a significant increase in food intake. Data are presented as mean±S.E.M. ##:p<0.01 two-way ANOVA with repeated measures; *:p<0.05, **:p<0.01, Bonferroni post-hoc test. (d) Body weight change of rats during 7 days of continuous i.c.v. infusion. mAb 1E8a induced an increase in body weight compared to rats which received mAb 2G2. #: p<0.05 two-way ANOVA with repeated measures. (e) Fasting glycemia and (f) fat pads weight corrected for body weight (BW) of rats which received a continuous i.c.v. infusion of mAb 1E8 or mAb 2G2. $: p<0.05, Student’s t-test.

Figure 5: Nucleotidic and amino-acid sequences of scFv 1E8a and 2G2. The hypervariable loops are underlined.

Figure 6: Purification of the scFv 1E8a by Ni-NTA chromatography. (a) Western blot probed with an anti-His₆ Ab which revealed the presence of the His₆ tagged scFv 1E8a, and (b) SDS-PAGE electrophoresis stained with Coomassie blue, periplasmic extract (PE), flow through (FT), wash fraction (W) and the elution of the purified scFv protein (see Methods section).

Figure 7: Intracellular cAMP production in HEK-293 cells transfected with hMC4R. (a) Concentration-response curve obtained with purified scFv 2G2. The scFv 2G2 had no effect on basal cAMP production. (b) Concentration-response curves obtained with α-MSH in the presence or absence of 100nM of scFv 2G2 (▲) or PBS (■). The presence of the scFv 2G2 had no effect on the concentration-response curve of α-MSH. (c) Concentration-response curve obtained with purified scFv 1E8a. The concentration-dependent decrease
in cAMP formation suggests an inverse agonist effect of the scFv 1E8a. (d) Concentration-
response curves obtained with α-MSH in the presence or absence of 10nM of scFv 1E8a
(◊), 100nM of scFv 1E8a (♦), or PBS (■). The reduced maximum efficacy of α-MSH in the
presence of scFv 1E8a suggests that this mAb acts as a non-competitive antagonist. Data
are presented as means±SD calculated from 3 independent experiments (**, p<0.01; ***, p<0.001, F-test).

Figure 8: The uptake of I-scFv by (a) cortex, (b) cerebellum, (c) hypothalamus, and (d) hippocampus in comparison to whole brain as a function of expt. (e) Comparison of I-scFv and I-Albumin uptake by whole brain.

Figure 9: (a) 24h food intake in rats which received an i.c.v. injection of 1µg either of scFv 1E8a or 1µg of scFv 2G2. The injection of scFv 1E8a induced a significant increase in food intake. Data are presented as mean±S.E.M. #: p<0.05, two-way ANOVA with repeated measures and Bonferroni post-hoc test, **: p<0.01. (b) Body weight change in rats 24h after injection. The injection of scFv 1E8a did not induce a loss of body weight compared to rats which received scFv 2G2. $: p<0.05, Students’ t-test. (c) 24h food intake in rats which received an i.v. injection of 300µg/kg of either scFv 1E8a or scFv 2G2. The injection of scFv 1E8a induced a significant increase in food intake. Data are presented as means±S.E.M. ##: p<0.01, repeated measures two-way ANOVA with Bonferroni post-hoc test, *:p<0.05, **:p<0.01. (d) Body weight change in rats 24h after injection. The scFv 1E8a significantly prevented the body weight loss seen in rats which received scFv 2G2. $$: p<0.01, Students’ t-test.
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e.
Figure 2

(a) cAMP (% Basal) vs Log [mAb 2G2] (M)

(b) cAMP (% Maximum) vs Log [α-MSH] (M)

(c) cAMP (% Basal) vs Log [mAb 1E8a] (M)

(d) cAMP (% Maximum) vs Log [α-MSH] (M)

(e) cAMP (% Basal) vs Log [AgRP] (M)

*** indicates statistical significance.
Figure 3

- mAb 1E8a

b.

Receptor internalization (RU)

Time (min)
Figure 4

(a) Cumulative food intake (g) over time (h) for different groups:
- 1E8a (n=30)
- 2G2 (n=22)
- BSA (n=12)

(b) Changes in body weight (g) over time (day):
- BSA
- mAb 2G2
- mAb 1E8a

(c) Cumulative food intake (g) over time (h) for different groups:
- 2G2 (n=5)
- 1E8a (n=8)

(d) Changes in body weight (g) over time (day):
- 2G2
- 1E8a

(e) Glucose levels (mM) for:
- 2G2
- 1E8a

(f) Fat pads weight/EW for:
- 2G2
- 1E8a
**Figure 5**

ScFv1E8a

TTCCACGTCGACAGCAATCTGGAGCTGATGGAAGCTGGCTTTCAATGAAATATCTCTCAAGCTTTGCTGTATGTACTCATATTCTGACATAGTCT

FQLQQSQGPELVKPGASVKICSKASKGYSFTDYMN

CDR H1

ACTGGTGAAGCGAAGACAGGAAATTGGAAGCTGGCTTTCAATGAAATATCTCTCAAGCTTTGCTGTATGTACTCATATTCTGACATAGTCT

NWKQSNKSLGWIGVINPNYTSSYNQKFKGKA

CDR H2

CACATTGACTGTGACACAAATCTTTTACACACTATGCTCAGCTGACATGCTGCTGCTACTATTACTGGTACATGGTAGATGT

TLTVQSSSTAYNSLLSTEDSYNLVCARFDG

CDR H3

TACTACGTTATCTACTGTGGGGCCAAGCCACACACTCTCTACAGTCAGTTTGGAGCTGATGGGACTGACTGGTGGGTCAAGCCAGACAC

YYGYACTFFEYDYGQGTTLTVSSGVGGSGGGGGSSSD

TTTGAGATGCCCGATCCTACATCTCTCTCTCGGAGCACAGTCAACCACATGTCAGTTGCAAGGGCAAGTCAGGACATAGGAATTATATAGT

IVMTGSTSSLSASLGDRTISCRASQDISNYLWN

CDR L1

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YQKPDGGTVKLLIIYYTSLRHLHSGPSRFSGS

CDR L2

ACAGATTATTTCTCTCCCATGAGCAACTGGAGGAAGAGATATTGGGCACTTCTTTTGGGACCAGGGTAATACGCTCTCGTACACGGTGAGGGGG

TDYSLTISNLEQEDITAYFCCQQGNTLTPYTFFG

CDR L3

CCAACTGGGAAAAATAACACACCTACATCCTAC

TKLEIKHHH

ScFv 2G2

ATTCACGTCGACAGCAATCTGGAGCTGATGGAAGCTGGCTTTCAATGAAATATCTCTCAAGCTTTGCTGTATGTACTCATATTCTGACATAGT

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LRLWGQGTLTLLTVSSGGSGGGSSGDIVMTQT

CATCCCTCTGCTCTCTGTTGAGACAGATCTACATGCTCAAGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG

TSSLSASLDGRVTISCASASQGISNYLNNWYQQKPD

TGGAACTGTTAATACCTCAGTACTTTACATCAAGATTTACACTCAGGAGTCCATACAGCTGAGTGGCCATAGGACATAGTTACTGGTACTAG

GTIVKLIIIYTLSSLSHGVPFSGSSGSGTDYSOL

ATGACCACTGGGAAAAATAACACACCTACATCCTAC

ISNLPEIDTIAITYCQQYSKLPTFTGFSGT

ATCCACATCACCATCAC

HHHHHH
Figure 6

a.

Anti-His$_6$ Abs
cRFv 1E8a
26kDa

b.

scFv 1E8a
26kDa
Figure 7

(a) cAMP (% Basal) vs log [scFv 2G2] (M)

(b) cAMP (% Maximum) vs Log [α-MSH] (M)

(c) cAMP (% Basal) vs log [scFv 1E8a] (M)

(d) cAMP (% Maximum) vs Log [α-MSH] (M)
Figure 8