A Pharmacologically Active Monoclonal Antibody against the Human Melanocortin-4 Receptor: Effectiveness After Peripheral and Central Administration

Jean-Christophe Peter, Anne-Catherine Lecourt, Marjorie Weckering, Géraldine Zipfel, Michael L. Niehoff, William A. Banks, and Karl G. Hofbauer

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 human embryonic kidney 293 cells expressing the human MC4R and blocked the activity of the MC4R under basal conditions and after stimulation with α-melanocyte-stimulating hormone (α-MSH). The inverse agonist action of Agouti-related protein was significantly enhanced in the presence of mAb 1E8a. After a single intracerebroventricular injection into the third ventricle, mAb 1E8a (1 μg) increased 24-h food intake in rats. After 7 days of continuous intracerebroventricular administration, mAb 1E8a increased food intake, body weight, and fat pad weight and induced hyperglycemia. Because the complete mAb was ineffective after intravenous injection, we produced single-chain variable fragments (scFvs) derived from mAb 1E8a. In pharmacokinetic studies it was demonstrated that these scFvs crossed the blood-brain barrier and reached the hypothalamus. Consequently, the scFv 1E8a increased significantly food intake and body weight in rats after intravenous administration (300 μg/kg). The pharmacological profile of mAb 1E8a and the fact that its scFv was active after peripheral administration suggest that derivatives of anti-MC4R mAbs may be useful in the treatment of patients with anorexia or cachexia.

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 anorexia or cachexia.

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

ABBRIDATIONS: MC4R, melanocortin-4 receptor; rMC4R, rat MC4R; hMC4R, human MC4R; Ab, antibody; mAb, monoclonal Ab; α-MSH, α-melanocyte-stimulating hormone; AgRP, Agouti-related protein; scFv, single-chain variable fragment; I-scFv, iodinated scFv; RT, room temperature; HEK, human embryonic kidney; NT, N terminal; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.05% Tween 20; OD, optical density; G418, 2R,3S,4R,5R,6S)-5-amino-6-{[(1R,2S,3S,4R,6S)-4,6-diamino-3-[(2R,3R,4R,5R)-3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl]oxy-2-hydroxy cyclohexy]oxy-2-(1-hydroxyethyl)oxane-3,4-diol; BSA, bovine serum albumin; PCR, polymerase chain reaction; Ni-NTA, 20 mM imidazole, 50 mM NaHPO4, 300 mM NaCl, pH 8.0; PE, periplasmic extract; I-Alb, radioactively labeled albumin; LR-BSA, lactated Ringer's solution containing 1% BSA; %Inj/ml, percentage of the injected dose present per ml of serum; %Inj/g, percentage of the injected dose taken up per g of brain; Expt, exposure time; ANOVA, analysis of variance; SHU 9119, Asp3-Lys8-Ac-Nle-Asp-His-b-Nal(2’)-Arg-Trp-Lys-NH2.

Downloaded from jpet.aspetjournals.org at ASPET Journals on August 30, 2017
receptor (Srinivasan et al., 2004). We demonstrated 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 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 of an MC4R agonist and as noncompetitive 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 injection into the third ventricle of untreated 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 a noncompetitive antagonist. In vivo, mAb 1E8a increased food intake in rats after acute and chronic intracerebroventricular administration but was not effective after intravenous administration. To improve penetration across the blood-brain barrier, recombinant single-chain variable fragments (scFvs) were produced and evaluated in vitro and in vivo. They also acted as inverse agonists and noncompetitive antagonists and increased food intake after intravenous 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 pharmacokinetic properties, these agents could represent lead molecules for the development of therapies for patients with anorexia or cachexia.

Materials and Methods

Production of Monoclonal Antibodies. A peptide corresponding to the NT domain of the MC4R (KTSSLHLWNRSIHGLHG, residues 11–25 of the MC4R) was used as antigen. The NT peptide shares the sequence of the rat MC4R (rMC4R) and human MC4R (hMC4R) isoform from residues 2 to 15. NT3 peptide (ANSSRSGFCEQVFVKPEY, residues 26–44 of the MC3R) was synthesized as described previously (Peter et al., 2007).

C57BL/6 mice were inoculated with 25 μg of the free NT peptide emulsified in complete Freund’s adjuvant and injected subcutaneously. Four weeks later, a booster injection of 25 μg in incomplete Freund’s adjuvant was given. Four weeks later, 10 μg of peptide dissolved in NaCl 0.9% was injected intravenously 3 days before harvesting the spleen cells for fusion. Fusion was performed with polyethylene glycol 1500 (Sigma-Aldrich, St. Louis, MO) at a ratio of two spleenocytes to one SP2O myeloma cell. Hybridomas were cultivated in 96-well plates precoated with peritoneal macrophages of C57BL/6 mice at 1000 cells/well. The mixture was immunoprecipitated with 25 μg 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 plc, Cambridge, UK), and the presence of MC3R was detected by using polyclonal anti-MC3R Abs (Abcam plc) in the standard procedure described for the One-Step Complete IP-Western kit (Genscript, Piscataway, NJ).

Inhibition Experiments. Purified mAb 1E8a (25 μg) was preincubated overnight at 4°C in the presence or absence of increasing concentrations of the NT peptide (32 nM to 100 μM). MC4R membrane preparations from 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 (50 μg) were incubated with purified mAb 1E8a (25 μg) or mAb 2G2 (25 μg) overnight at 4°C. The mixture was immunoprecipitated with 25 μl 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 plc) in the standard procedure described for the One-Step Complete IP-Western kit (Genscript, Piscataway, NJ).
phase of 400 s. The kinetic parameters were calculated by using BIA evaluation software 4.1 (BIAcore AB, Uppsala, Sweden). All resonance unit values obtained with the control NT3 peptide were subtracted from those obtained with the MC4R NT peptide to compensate for nonspecific binding.

**Cell Culture.** HEK-293 cells expressing hMC4R or hMC3R were cultured in Dulbecco’s modified Eagle medium (Sigma-Aldrich) containing 10% fetal calf serum (Bioconcept, Allschwil, Switzerland), 1% penicillin/streptomycin (Invitrogen), and G418 at 600 μg/ml (Sigma-Aldrich) in a humidified atmosphere containing 5% CO₂ at 37°C.

**cAMP Assays.** Cells were transferred to 24-well culture plates 12 h before treatment, washed for 3 h with Dulbecco’s modified Eagle medium (Sigma-Aldrich), and incubated for 30 min in PBS supplemented with 0.1% bovine serum albumin (BSA) and 3-isobutyl-1-methylxanthine (Sigma-Aldrich). Cells were treated with serial dilutions of purified mAbs or scFvs for 30 min or preincubated with methylxanthine (Sigma-Aldrich). Cells were treated with serial dilutions of purificated mAbs or scFvs for 30 min and then treated with serial dilutions of α-melanocyte-stimulating hormone (α-MSH; 1.0 × 10⁻⁸ to 1.0 × 10⁻⁶ M) for 15 min or Agouti-related protein (AgRP; 1.0 × 10⁻¹² to 1.0 × 10⁻⁶ M). Subsequently, they were lysed with Biotrak cAMP lysis buffer, and cAMP was measured by using the Biotrak cAMP enzyme immunoassay system (GE Healthcare) according to the manufacturer’s instructions. Protein concentration was determined by using the BCA kit (Pierce Chemical, Rockford, IL). The concentration of cAMP was expressed in fmol cAMP/μg protein.

**Immunofluorescence.** HEK-293 cells expressing hMC4R or hMC3R were fixed for 5 min with 2% paraformaldehyde in PBS. Slides were saturated with PBS supplemented with 5% nonfat dried milk. mAbs 2G2 or 1E8a (50 μg/ml) were applied on cells for 1 h at RT. After three washes with PBS, goat anti-mouse Alexa-conjugated (1/500) (Invitrogen) was allowed to react with the fixed primary antibody for 1 h at RT. 4,6-Diamidino-2-phenylindole, dihydrochloride (1 μg/ml; Invitrogen) was used for nuclear staining. The same magnification (40×) and exposure time (500 ms) was used for each slide.

**Receptor Internalization.** HEK-293 cells expressing the hMC4R or hMC3R were fixed for 5 min with 2% paraformaldehyde in PBS. Cells were washed with ice-cold PBS and fixed at rt = 0, 10, 30, and 45 min. Concanavalin A Alexa-Fluor 488 (0.1 mg/ml; Invitrogen) and 4,6-diamidino-2-phenylindole, dihydrochloride (1 μg/ml; Invitrogen) 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 tetramethylrhodamine-5-(and 6)-isothiocyanate) confocal images were converted to grayscale. Each pixel was assigned an 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 by using Scion Image Beta 4.03.02 (downloaded from www.scioncorp.com). An increase of the OD signal indicated internalization of the MC4R.

**Cloning of cDNA Encoding the Variable Domain of the mAbs.** Total RNA was prepared from 10⁷ freshly subcloned hybridoma cells by using the RNASpert Kit (Biogentec Inc., Seabrook, TX), and first-strand cDNA was synthesized by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). The V₁H and V₁L chain domains were amplified by polymerase chain reaction (PCR) using the IgG primer set (Novagen, Gibbstown, NJ). The 50-μl PCR mixtures contained 50 ng of hybridoma cDNA, 20 pmol of each appropriate primer, 250 μM of each dNTP, 1× Taq buffer (Sigma-Aldrich), and 1 U Thermus aquaticus (Taq) polymerase. Amplification included 50 cycles of 1.5 min at 94°C, 2.5 min at 55°C, and 3 min at 72°C in a thermocycler (PTC-150; MJ Research, Watertown, MA). The amplified DNAs were ligated into the pGEMT vector (Promega, Madison, WI), and the recombinant plasmids were purified with a miniprep kit (Qiagen, Hombrechtikon, Switzerland). The DNA sequences of the cloned V₁H and V₁L inserts were determined with the ABI PRISM Cycle Sequencing kit (Applied Biosystems, Foster City, CA) 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 V₁H and V₁L genes together by PCR with overlap extensions using oligonucleotides that encoded a 15-amino-acid linker (G₄-S₄), between the C terminus of the V₁H and the N terminus of the V₁L gene. The ends of the 1E8a mAb variable gene were modified by PCR using as primers 1E8V₁HRev (5′-GTT GCA GTC GCA GCA TTC ACC TGA GC-3′), which encodes the WT wild-type sequence of the V₁H containing a NcoI site and V₁LFor (5′-ACC GGA TCC GCC TCC TGG TGA GAC ATG TG-3′), which encodes the C terminus of the VH and a part of the linker. 1E8V₁HRev (5′-GGG GGA GCA TCC GTG GTG GGC GGA TCT GGA GGT GCC GGA AGC GAC ATT GTG ATG ACC CAG-3′) and V₁LFor, containing a XhoI site that encodes six His residues (5′-GCA ATT CCT CCA GTT AGT GAT GAT GAT GAT GTT TTA TTT CCA GCT TGG TTG-3′), were used to amplify and modify the V₁H domain. The ends of the 2G2 mAb variable gene were modified by PCR using as primers 2G2V₁HRev (5′-TTG CCA TGG CCG GCG GAT TGG TAC TGC TGC TGC TAC AGC ATG CTG GA-3′), which encodes the WT wild-type sequence of the V₁H containing a NcoI site, and V₁LFor (5′-ACC GCA TCC GCC TCC TGG TGA GAC ATG TG-3′), which encodes the C terminus of the VH and part of the linker. 2G2V₁HRev (5′-GGG GGA GCA TCC GTG GTG GGC GGA TCT GGA GGT GCC GGA AGC GAC ATT GTG ATG ACC CAG-3′) and 2G2V₁LFor, containing a XhoI site that encodes six His residues (5′-GCA ATT CCT CCA GTT AGT GAT GAT GAT GAT GTT TTA TTT CCA GCT TGG TTG-3′), were used to amplify and modify the V₁L domain. The scFv gene was inserted in-frame with the Pmll sequence on the expression vector pET22b (EMD Biosciences, Darmstadt, Germany) between the NcoI and XhoI sites.

**Expression of scFvs.** Escherichia coli Rosetta bacteria transformed with pET22b (+) 1E8a or pET22b (+) 2G2 were grown in 500 ml of medium 2X YT (1.6% lactobacitron, 1% bactoyeast extract, 0.5% NaCl, pH 7.0) containing 0.15 mM ampicillin (Applichem, Darmstadt, Germany) and 0.1 mM chloramphenicol (Gebu Biotechnik, Gaiberg, Germany) until reaching an OD₆₀₀nm of 0.6 at 37°C with agitation at 200 rpm. The expression of scFv was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside (Applichem) for 4 h.

**Periplasmic Extraction.** Five hundred milliliters of bacteria cultures was centrifuged (10 min, 10,000g, 4°C), and the pellet was resuspended in 200 ml of 30 mM Tris, 1 mM EDTA, 2% sucrose, (pH 8.5). After centrifugation (10 min, 10,000g, 4°C), the pellet was resuspended in 50 ml of 5 mM MgSO₄. After a last centrifugation (10 min, 10,000g, 4°C) the supernatant corresponding to the periplasmic extract (PE) was collected. This PE was dialyzed in wash buffer Ni-NTA (20 mM imidazole, 50 mM NaHPO₄, 300 mM NaCl, pH 8.0) overnight at 4°C.

**Purification of scFvs.** The scFv proteins were purified from the PE on Ni-NTA columns according to the manufacturer’s instruction (Qiagen). After elution, purified scFvs were dialyzed in PBS (10 mM NaHPO₄, 150 mM NaCl, 27 mM KCl, pH 7.4) overnight at 4°C. The scFvs were then purified by immunoadsorption as described in Isotyping and Purification. The fractions were determined with a Micro BCA Protein Assay kit (Pierce Chemical). The quantity and purity of the purified scFv fractions were assessed by SDS-polyacrylamide gel electrophoresis analysis using 12.5% acrylamide gels followed by staining with Coomasie Brilliant Blue (Bio-Rad Laboratories) and Western blot. For Western blot analysis, the proteins were transferred from the gels onto a nitrocellulose transfer membrane using a mini-transblot system (Bio-Rad) in transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% methanol, pH 8.3).
membranes were soaked for 1 h in PBS-T supplemented with 5% nonfat milk powder and 0.1% Tween 20. This was followed by 1-h incubation with anti-His Ab conjugated to horseradish peroxidase 1/2000 (Sigma-Aldrich). The Ab was diluted in the blocking solution PBS-T milk. The proteins on the membranes were revealed by the classical procedure of the enhanced chemiluminescence reagents (ECL; GE Healthcare).

Radioactive Labeling and Purification. The scFv 1E8a was radioactively labeled with 125I by the iododehead method. In brief, an iododehead was incubated in 0.1 ml of phosphate buffer solution with 2 μCi of 125I for 5 min at RT. Five micrograms of scFv was then added in a volume of 2.2 μl. After 3 min of incubation at RT, the iodinated scFv (I-scFv) was separated from unincorporated 125I on a column of G-10 Sephadex. BSA was labeled by incubation with chloramine-T for 60 s 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 protocols approved by the local animal use committee. The blood-to-brain unidirectional influx rate (K, in units of μg/ml/min) was calculated by multiple-time regression analysis (Blasberg et al., 1983; Patlak et al., 1983). In brief, the right jugular vein and left carotid artery were exposed. At t = 0, 0.2 ml of lactated Ringer’s solution containing 1% BSA (LR-BSA) and 500,000 cpm I-scFv was injected into the jugular vein. Between 2 and 180 min after the intravenous injection, blood was collected from the carotid artery, and the mouse was immediately decapitated. Two mice were studied per time point. The arterial blood was centrifuged, and serum was collected, and results expressed as the percentage 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 were weighed, and the level of radioactivity was determined. Results were expressed as the brain/serum ratios (in units of μg/g) and plotted against exposure time (Expt), where

\[ \text{Expt} = \frac{\int [Cp(r)dr]Cp}{Cpt} \]

and Cp is the level of radioactivity in serum, and Cpt 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, K 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 ratio between brain/serum ratios and Expt measures K and the intercept of the linearity measures V (μL/g), the initial volume of distribution in brain.

In other mice, I-Alb was included in the intravenous injection. The percentage 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 intravenous injection. Whole blood was centrifuged, and 50 μl of the resulting serum was 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 were expressed as the percentage 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, and the sample was vigorously mixed and then centrifuged at 5400g for 10 min at 4°C. The supernatant and precipitate were separated and counted, and the results were expressed as the percentage 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 nonradioactive arterial whole blood or whole brain. These samples were then processed as above, and the percentage of total counts that were precipitated was determined. The mean of two 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 of saline containing 106 cpm I-scFv and 106 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 4°C in a 9-fold volume of physiological buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, and 10 mM d-glucose adjusted to pH 7.4). Dextran solution was added to the homogenate to a final concentration of 26%. An aliquot was centrifuged at 5400g for 15 min at 4°C 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.

Intracerebroventricular and Intravenous Administration. Male Sprague-Dawley rats (275–325 g) were anesthetized with isoflurane in medicinal oxygen (4% for induction and 2% for maintenance of anesthesia). A stainless-steel cannula (26 gauge, 10 mm long) was implanted into the third cerebral ventricle by using the following coordinates, relative to the Bregma: −2.3 mm anteroposterior, 0 mm lateral to the midline, and −7.5 mm ventral to the surface of the skull. The guide cannula was secured in place with three 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) (0.03 mg/kg) was given subcutaneously before and 2 days after surgery. Seven days after recovery from surgery, accuracy of the cannula placement in the third ventricle was tested by measuring the diencephalic response (immediate drinking of at least 5 ml of water in 15 min) to an intracerebroventricular injection of 20 pmol of angiotensin II in 2-μl injection volume.

Purified mAbs and scFvs were slowly (1 min) injected intracerebroventriculatly at 9:00 AM at a dose of 1 μg in a volume of 2 μl by using a Hamilton syringe. These doses were selected based on the results of comparative in vitro studies. After the injection of mAbs or scFvs, food intake was continuously recorded for 3 days by using an automatic food intake apparatus (TSE Systems, Bad Homburg, Germany) at 1-h intervals.

Osmotic minipumps (model 2002) (Charles River Laboratories, Les Oncins, France) were filled with mAbs 1E8a or 2G2 calculated to deliver 1 μg/day for 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 intracerebroventricular 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).

In the experiments with intravenous 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 300 μg/kg. After the injection of scFv or mAb, food intake was continuously recorded with an automatic food intake apparatus (TSE Systems) at 1-h intervals for 3 days.
**Data Analysis.** All data are expressed as mean ± S.D. or S.E.M. as indicated. Data were analyzed by two-way analysis of variance (ANOVA) repeated measures with Bonferroni post hoc test or Student’s t test using Prism 4 software (GraphPad Software Inc., San Diego, CA). For the cAMP concentration-response experiments, the best-fitting curves were compared for their minimum, maximum, and EC50 values using the F test. Kᵢ and EC50 were calculated by using Prism 4 software. For pharmacokinetic experiments, means are reported with standard errors. Prism 5.0 (GraphPad Software, Inc.) was used for regression analysis and comparison of 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 Vₕ 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 of the MC3R. Although the polyclonal response was high, only 10 clones were viable until subcloning and amplification (six IgG, and four IgM,κ). After purification only mAb 1E8a (IgM,κ) showed a blockade of the MC4R activity in the presence of α-MSH. mAb 2G2 (IgM,κ) was selected as an 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 Kᵢ of 1.3 × 10⁻⁸ M for the mAb 1E8a and 3.7 × 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 (Fig. 1b). When HEK-293 cells expressing hMC4R were treated with mAb 2G2 no labeling could be observed (Fig. 1c). Immunoprecipitation experiments confirmed the selectivity of mAb 1E8a for the hMC4R and rMC4R. mAb 2G2 did not immunoprecipitate the hMC4R and rMC4R (Fig. 1d). The presence of NT peptide inhibited the interaction of the mAb 1E8a with the MC4R in a concentration-dependent manner (Kᵢ of 1.3 × 10⁻⁶ M) (Fig. 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 (1 pM to 0.1 μM), no decrease in cAMP formation was measured (Fig. 2a). Conversely, when HEK-293 cells were exposed to increasing concentrations of mAb 1E8a (1 pM to 0.1 μM) an increase in cAMP formation was decreased in a concentration-dependent manner by up to 40% (EC₅₀ of 2.1 × 10⁻¹² M) (Fig. 2c).

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

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

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

---

**Fig. 1.** Immunocytochemistry of HEK-293 cells transfected with hMC4R or hMC3R. Nuclear labeling (blue) is merged with immune labeling (red). a and b, 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). c, no labeling was observed with mAb 2G2 in hMC4R expressing cells. Magnification: 400×. d, immunoprecipitation of hMC4R and rMC4R. Lanes 1 and 2 show the results obtained with hMC4R and hMC3R, respectively, with mAb 1E8a, and lanes 5 and 6 show the corresponding results with rMC4R and rMC3R, respectively. Lanes 3 and 4 show control experiments with mAb 2G2. Purified mAb 1E8a precipitated both hMC4R (lane 1) and rMC4R (lane 5) but not hMC3R or rMC3R (lanes 2 and 6), whereas 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 percentage of inhibition of the signal obtained without NT peptide in function of the [NT]. Mean ± S.D. are calculated from three independent experiments. n = 5 measurements/experiment.
Pharmacological Activity of mAbs 1E8a and 2G2 in Vivo. Rats that received an injection of 1 μg of mAb 1E8a into the third ventricle ingested 24% more food in 24 h than rats that received mAb 2G2 or BSA as controls (Fig. 4a). The body weight of rats that received mAb 1E8a was increased, whereas body weight was reduced in rats that received either mAb 2G2 or BSA (Fig. 4b).

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

Variable Domain Cloning and scFv Expression. The scFv-encoding genes derived from the variable regions [VH and VL, linked together via a short linker (G4S)3 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 Fig. 5. We confirmed that the cloned VL gene did not correspond to the aberrant transcript of the sp20 hybridomas (Carroll et al., 1988). The plasmid pET-scFv 1E8a or pET-scFv 2G2 was cloned into the Rosetta E. 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 immobilized metal ion affinity chromatography and repurified by immunoadsorption to get rid of the incorrectly folded inactive recombinant proteins (Peter et al., 2003) (Fig. 6). The affinity of these scFvs for the NT peptide was assessed by surface plasmon resonance. A Kd of 7.8 x 10^-7 M for the scFv 1E8a and 2.4 x 10^-7 M for the scFv 2G2 was calculated.

Pharmacological Effect of the scFvs 1E8a and 2G2 in Vitro. When HEK-293 cells transfected with the hMC4R were exposed to increasing concentrations of scFv 2G2 (1 pM to 0.1 μM) no decrease in cAMP formation was measured (Fig. 7a). Conversely, when HEK-293 cells were exposed to increasing concentrations of scFv 1E8a (1 pM to 0.1 μM) cAMP formation decreased in a concentration-dependent manner by up to 40% (Fig. 7c). The presence of 100 nM scFv 2G2 did not change the concentration-response curve of α-MSH (Fig. 7b), but the presence of 100 nM scFv 1E8a significantly (p < 0.001, F test) reduced the maximum effect of α-MSH (Fig. 7d).
Pharmacokinetics of scFv 1E8a. When I-scFv was injected intravenously and its clearance from blood was 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 (V_d) 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 with whole brain. For all brain regions and whole brain, the relation between brain/serum ratios and Expt was significant (r ranging from 0.869 to 0.930, p < 0.001 for all curves). No statistical difference was found among the slopes, whereas body weight was reduced in rats that received scFv 1E8a was significantly less decreased than that of rats that received scFv 2G2 (Fig. 9a). The body weight of rats that received scFv 1E8a was significantly less decreased than that of rats that received scFv 2G2 (Fig. 9b).

Pharmacological Activity of scFvs 1E8a and 2G2 in Vivo. One microgram of purified scFv 1E8a and scFv 2G2 was injected into the third ventricle of rats. Rats that received scFv 1E8a ingested 45% more food in 24 h than rats that received scFv 2G2 (Fig. 9c). The body weight of rats that received scFv 1E8a was significantly less decreased than that of rats that received scFv 2G2 (Fig. 9d).

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 overactivation 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 that acted as inverse agonists and noncompetitive antagonists (Peter et al., 2007). In the present study, we characterized the pharmacological properties of a mAb (1E8a) against this peptide sequence of the NT domain in vitro and in vivo. Another mAb (2G2) that recognized the NT peptide but not the correspond-
ing 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 HEK-293 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 that 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 that indicated that mAb 1E8a bound to hMC4R and rMC4R with high affinity, whereas the mAb 2G2 interacted only with the NT peptide.

**Fig. 5.** Nucleotidic and amino acid sequences of scFv 1E8a (top) and scFv 2G2 (bottom). The hypervariable loops are underlined.
but not with the corresponding sequence in the native MC4R.
A specific interaction of mAb 1E8a with the MC4R was sup-
ported by the observation that increasing concentrations of
the NT peptide reduced the binding of mAb 1E8a with a
\( K_i \) in the micromolar range. Compared with the
\( K_d \) of the mAb 1E8a/NT peptide binding, which is in the nM range, this
\( K_i \) indicated as better binding of mAb 1E8a to the native con-
formation 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 (1.3 \( \times \) 10\(^{-9}\) and 3.7 \( \times \) 10\(^{-8}\)
M, respectively). They are relatively high for an IgM isotype,
but other high-affinity IgM mAbs have been described pre-
viously (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 para-
tope of mAb 2G2 interacted with a conformation of the NT
peptide that the intact receptor is unable to adopt. In con-
trast, 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 as-
sessed by using adenylyl cyclase assays in HEK-293 cells transfect-
ed with hMC4R. The scFv 2G2 had no effect on basal
cAMP production. The scFv 2G2 had no effect on the concentration-
response curve of \( \alpha \)-MSH in the presence or absence of 100 nM
scFv 2G2 (△) or PBS (●). The presence of the scFv 2G2 had no effect on the
concentration-response curve of \( \alpha \)-MSH. The concentration-dependent decrease in
cAMP formation suggested an inverse ag-
gonist effect of the scFv 1E8a. The concentration-response curves obtained with
\( \alpha \)-MSH in the presence or absence of 10
nM scFv 1E8a (⊙), 100 nM scFv 1E8a
(●), or PBS (●). The reduced maximum efficacy of \( \alpha \)-MSH in the presence of
scFv 1E8a suggests that this mAb acts as a noncompetitive antagonist. Data
are presented as means ± S.D. calcu-
lated from three independent experi-
ments. **, \( p < 0.01 \); ***, \( p < 0.001 \), F
test.
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 compared with controls was recorded during the

Fig. 8. a–d, the uptake of I-scFv by cortex (a), cerebellum (b), hypothalamus (c), and hippocampus (d) in comparison with whole brain as a function of Expt. e, comparison of I-scFv and I-Alb uptake by whole brain.

Fig. 9. a, 24-h food intake in rats that received an intracerebroventricular injection of 1 μg of scFv 1E8a or 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 24 h after injection. The injection of scFv 1E8a induced a smaller loss of body weight compared with rats that received scFv 2G2. $, p < 0.05, Student's t test. c, 24-h food intake in rats that received an intravenous injection of 300 μg/kg of 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; $$$, p < 0.01. d, body weight change in rats 24 h after injection. The scFv 1E8a significantly prevented the body weight loss seen in rats that received scFv 2G2. $$, p < 0.01, Student's t test.
first 24 h. This difference is much more pronounced than that seen after intracerebroventricular injection. The stronger effect of MC4R blockade in these experiments may be because the rats had not yet recovered from the minipump implantation. The effect of mAb 1E8a under these circumstances therefore may 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 knockout mice (Fan et al., 2000), rats after central administration of antisense oligonucleotides against the MC4R (Obici et al., 2001), and 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 intravenous administration of mAb 1E8a in a dose (300 μg/kg) approximately 100-fold higher than that given intracerebroventricularly (1 μg/rat) (data not shown). This excludes the contribution of peripheral MC4R but raises the question of whether intravenously 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 intravenous 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 noncompetitive antagonist of the MC4R and showed the same efficacy as the intact mAb. The affinity of the scFv 1E8a for the NT peptide was 10 times lower than that of the intact mAb 1E8a. This difference is consistent with the higher EC50 calculated from the scFv inverse agonist activity.

The intracerebroventricular application of 1 μg of scFv 1E8a induced an increase in food intake that occurred faster and was more pronounced than that obtained with the intact mAb 1E8a. This observation could reflect a better brain penetration of scFv compared with the parent mAb. After intravenous administration in rats scFv 1E8a induced an increase in food intake after intravenous administration in rats. Such molecules therefore might represent a starting point for the development of new therapies for patients with anorexia and cachexia.

References


Banks WA, Oritus L, Ploitkin SR, and Kastin AJ (1991) Human interleukin (IL) 1α, murine IL-1α, and murine IL-1β are transported from blood to brain in the mouse by a shared saturable mechanism. J Pharmacol Exp Ther 259:988–996.


