Fully Human Monoclonal Antibodies Antagonizing the Glucagon Receptor Improve Glucose Homeostasis in Mice and Monkeys


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ABSTRACT

Antagonizing the glucagon signaling pathway represents an attractive therapeutic approach for reducing excess hepatic glucose production in patients with type 2 diabetes. Despite extensive efforts, there is currently no human therapeutic that directly inhibits the glucagon/glucagon receptor pathway. We undertook a novel approach by generating high-affinity human monoclonal antibodies (mAbs) to the human glucagon receptor (GCGR) that display potent antagonistic activity in vitro and in vivo. A single injection of a lead antibody, mAb B, at 3 mg/kg, normalized blood glucose levels in ob/ob mice for 8 days. In addition, a single injection of mAb B dose-dependently lowered fasting blood glucose levels without inducing hypoglycemia and improved glucose tolerance in normal C57BL/6 mice. In normal cynomolgus monkeys, a single injection improved glucose tolerance while increasing glucagon and active glucagon-like peptide-1 levels. Thus, the anti-GCGR mAb could represent an effective new therapeutic for the treatment of type 2 diabetes.

Human type 2 diabetes is characterized by multiple metabolic defects, including increased glucose production, reduced glucose disposal in insulin-sensitive tissues, and β-cell dysfunction (Consoli et al., 1989; DeFronzo et al., 1989). Recent studies suggest that gluconeogenesis is largely responsible for the overproduction of glucose in fasting type 2 diabetic patients (Magnusson et al., 1992; Basu et al., 2005). In the postprandial state, reduced suppression of gluconeogenesis and glycolysis contributes significantly to the impaired glucose tolerance in patients with type 2 diabetes (Mitrakou et al., 1992; Singhal et al., 2002).

A number of studies have demonstrated that elevated basal glucagon levels and a lack of suppression of postprandial glucagon secretion are partially responsible for the increased hepatic glucose production in patients with type 2 diabetes (Müller et al., 1970; Unger and Orci, 1975; Lins et al., 1983). Glucagon, a 29-amino acid peptide secreted by pancreatic α-cells, plays an important role in controlling endogenous glucose production. By binding and activating the glucagon receptor (GCGR), a G protein-coupled receptor (GPCR), glucagon stimulates adenylyl cyclase activity and phosphoinositol turnover. This results in the regulation of expression and activity of several key gluconeogenic and glycogenolytic enzymes, including phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase, glucose-6-phosphatase, and glycogen phosphorylase (Jiang and Zhang, 2003; Sloop et al., 2005). Therefore, reducing glucagon levels and inhibiting GCGR activity are expected to reduce excess glucose production, which would lead to improved overall glycemic control in type 2 diabetes.

Studies performed with rodent models suggest that GCGR antagonism may alleviate hyperglycemia associated with type 2 diabetes. GCGR knockout mice exhibited lower glucose levels, improved glucose tolerance without hypoglycemia, and increased insulin sensitivity (Parker et al., 2002; Gelling et al., 2003; Sørensen et al., 2006b). Furthermore,
Hepatocytes were incubated at 37°C, 5% CO2, with saturating human-modified Eagle’s medium (Sigma-Aldrich, St. Louis, MO) supplemented with Dickinson Labware; BD Biosciences, San Jose, CA) in Dulbecco’s Medium E (Invitrogen) the night before the assay. Culture medium was replaced with Williams’ Medium E (Invitrogen) for 2 to 4 h. Culture medium was replaced with Williams’. The resulting pellets were resuspended in hypotonic buffer with proteinase inhibitors and subjected to ultracentrifugation. The resulting pellets were resuspended in hypotonic buffer containing 10% sucrose. The total protein concentration of the membrane fractions was determined using the Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA).

In Vitro Pharmacology

Establishment of GCGR and Glucagon-Like Peptide-1 Receptor Recombinant Cell Lines. Recombinant human, murine, and cynomolgus monkey GCGR cDNAs were subcloned into expression vectors containing selectable markers. After transfection and appropriate drug selections, single-cell-derived stable functional cell lines were screened for glucagon-induced cAMP accumulation and specific 125I-glucagon binding. The recombinant human glucagon-like peptide-1 receptor (GLP-1R) cell line was developed as described previously (Miranda et al., 2008).

The hGCGR high-expression cell line was generated by transfecting an expression vector containing full-length hGCGR cDNA into CHO cells. A stable subclone was selected based on the level of GCGR mRNA and specific 125I-glucagon binding. Stable 293T and cynomolgus monkey GCGR cDNAs were subcloned into expression vectors. A stable subclone was selected based on the level of GCGR mRNA and specific 125I-glucagon binding. Stable 293T recombinant functional cell line. mAb A and mAb B were recombinantly expressed as human IgG2. In addition, mAb A was isotype switched from human IgG2 to mouse IgG1, generating mAb Ac.

Materials and Methods

In Vitro Pharmacology

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The hGCGR high-expression cell line was generated by transfecting an expression vector containing full-length hGCGR cDNA into CHO cells. A stable subclone was selected based on the level of GCGR mRNA and specific 125I-glucagon binding. Stable 293T hGCGR recombinant GFP cells were generated by transfecting an expression construct encoding hGCGR fused to human recombinant GFP (Stratagene, La Jolla, CA) at the C terminus. The highly expressing cells were sorted by FACS based on GFP expression.

Primary Culture of Hepatocytes. Freshly harvested primary human or cynomolgus monkey hepatocytes (InVitrogen, Carlsbad, CA) were plated onto Collagen 1 Cellware 96-well plates (Becton Dickinson Labware; BD Biosciences, San Jose, CA) in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (InVitrogen). Hepatocytes were incubated at 37°C, 5% CO2, with saturating humidity for 2 to 4 h. Culture medium was replaced with Williams’ Medium E (InVitrogen) the night before the assay.

Membrane Preparation. Cells were harvested and homogenized with hypotonic buffer with protease inhibitors and subjected to ultracentrifugation. The resulting pellets were resuspended in hypotonic buffer containing 10% sucrose. The total protein concentration of the membrane fractions was determined using the Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA).

Generation of Anti-GCGR Antibodies. Cell membrane fractions from GCGR-expressing cell lines or peptides corresponding to the extracellular regions or N-terminal extracellular domain of the hGCGR fused to an Fe fragment were used to generate antibodies to the hGCGR from Xenomouse and other sources. Hybridoma supernatants were screened for specific binding to a recombinant hGCGR cell line and concurrently counter-screened with the parental cell line using fluorometric microvolume assay technology. The ability of purified antibodies to antagonize glucagon-induced cAMP production was tested using the hGCGR recombinant functional cell line. mAb A and mAb B were recombinantly expressed as human IgG2. In addition, mAb A was isotype switched from human IgG2 to mouse IgG1, generating mAb Ac.

FACS Analysis of Recombinant GCGR-Expressing Cells with GCGR mAbs. A direct staining protocol was used for FACS analysis. mAb A and mAb B were labeled with Alexa-647 (Invitrogen) and then incubated with the cells at a final concentration of 1 μg/ml before FACS analysis.

Antibody Activity Assay. Serially diluted test antibodies were added to a functional GCGR recombinant cells and incubated at 37°C for 20 min before the addition of glucagon (Bachem, Bubendorf, Switzerland) at a final concentration of 50 pM in the presence of 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich). Cells were stimulated at 37°C for 15 min. The cAMP levels were quantified using the cAMP dynamic 2 kit (Cis Bio International, Gif-sur-Yvette, France). The homogeneous time-resolved fluorescence (HTRF) 2-step protocol was followed per manufacturer’s instructions.

Schild Analysis. Functional recombinant cells in assay buffer (F-12 medium supplemented with 5 mg/ml bovine serum albumin) were plated and incubated at 37°C overnight with saturating agonist concentration. The control or test antibody in the assay buffer was added to the cells and incubated for 30 min at 37°C before agonist stimulation. A dose range of agonist concentrations was added in the presence of IBMX. The cells were stimulated at 37°C for 15 min. The cAMP levels were measured as described above.

Human or cynomolgus monkey hepatocytes in assay buffer (Dulbecco’s modified Eagle’s medium, no glucose, 0.5% bovine serum albumin, and 250 μM IBMX) were treated with a dose range of glucagon in the presence or absence of anti-GCGR antibodies at 37°C for 10 to 15 min. After removing the assay buffer, the cells were lysed with LANCE Detection Buffer (PerkinElmer Life and Analytical Sciences, Waltham, MA). The HTRF signal was quantified by the Discovery HTRF instrument (PerkinElmer Life and Analytical Sciences).

Ligand Binding Assay. Binding assays were performed essentially as described previously (Miranda et al., 2008).

Antibody Affinity Determination. Kd and binding kinetics of hGCGR mAb B were determined by direct binding of 125I-mAb B. Four micrograms of hGCGR membrane protein per well was incubated with a concentration range of 125I-mAb B. Nonspecific binding was determined by including 1 µM unlabeled mAb B in addition to the radiolabeled antibody. After incubation for 3 h at room temperature, the binding reaction was stopped by rapid filtration, and bound radioactivity was quantified as described above.

Dissociation Rate Determination. Dissociation experiments were carried out essentially as described above, after equilibration of 0.05 nM 125I-mAb B with the GCGR membranes for 4 h. At the starting time for the dissociation reaction time point, an approximately 3000-fold excess of unlabeled mAb B was added into the well. The binding reaction was stopped at various time points by rapid filtration, and bound radioactivity was quantified as described above.

Detection of GCGR Endocytosis. Recombinant human embryonic kidney 293 cells expressing GCGR-GFP were plated at a density of approximately 1 × 104/well in dual-well chambered coverslip slides (Nalge Nunc International, Rochester, NY) 24 h before scanning and kept in an incubator at 37°C until immediately before use. Fluorescent microscopy was performed on a LSM 510 Meta Confocal inverted microscope (Carl Zeiss Inc., Thornwood, NY) using a
488-nm argon laser operating at 5 mW and collecting emissions through a 505- to 530-nm band pass filter. Scanned images were obtained using a 40× objective to image a field of living cells on a heated stage at time 0, followed by another scan of the same field 10 min after addition of ligand. For antibody studies, cells were preincubated with either mAb A or a nonantagonizing GGCR antibody at a 20-fold excess for 20 min before the time 0 scan.

In Vivo Pharmacology

**Animals.** All mouse studies were conducted at Amgen Inc. (Thousand Oaks, CA) and approved by the Institutional Animal Care and Use Committee. C57BL/6 male mice (Charles River Laboratories, Inc., Wilmington, MA), and male ob/ob male mice (The Jackson Laboratory, Bar Harbor, ME) were maintained on a 12-h light/dark cycle with free access to food and water. During the study, all blood samples taken for glucose measurement were collected from the retro-orbital sinus of nonanesthetized mice using a hand-held glucometer (LifeScan, Inc., Milpitas, CA).

The cycnomulsus monkey study was conducted at Yunnan Labora-
tory Primate Inc. (Yunnan, China) and was approved by the Insti-
tutional Animal Care and Use Committee. The monkeys were di-
vided into three groups of 10 and administered a single subcutaneous injection of vehicle or one of two different doses of mAb B. Body weight and food intake were monitored throughout the study.

**Efficacy of mAb B in ob/ob Mice.** Three days before starting the antibody injection, blood glucose levels and body weight were mea-
sured in 12-week-old male ob/ob mice to sort the animals into groups with similar distributions based on blood glucose and body weight. On day 1 (time 0), all mice were bled for blood glucose measurement and were injected intraperitoneally with vehicle or mAb B at 1 or 3 mg/kg at the dose volume of 2.0 ml/kg. Subsequent blood glu-
cose measurements were performed at 1, 3, 5, 8, and 10 days after treatment.

**Fasting Blood Glucose and Glucose Tolerance Test in C57BL/6 Mice.** The body weight of 15-week-old male C57BL/6 mice was used to sort the animals into groups having similar distribu-
tions. The mice were injected subcutaneously with either vehicle or mAb B at 1 or 10 mg/kg at a dose volume of 3.5 ml/kg. Three days after treatment, mice were fasted for 12 h, and an intraperitoneal glucose tolerance test (GTT) was performed as follows: 2 g/kg glucose at a dose volume of 3.5 ml/kg was injected after the initial fasting blood glucose was measured. To minimize stress levels in the tested animals, only 30- and 90-min blood glucose levels were measured.

**Efficacy and Safety of mAbs in Cynomolus Monkeys.** The age of animals ranged from 3 to 6 years, and body weight ranged from 3 to 6 kg. Two OGTTs were performed before the single subc-
utenaneous administration of mAb B or vehicle. Thirty male monkeys were selected based on their consistent OGTT profile from a pool of 50 (1.25 g/kg glucose at a dose volume of 10 ml/kg). Animals were ac-
climated for 42 days to all the procedures (bleeds, OGTT, and bolus gavage) before administration of the compounds. To minimize stress, the same highly experienced personnel worked with the monkeys from the beginning of the acclimation to the end of the study. Blood was collected by venipuncture from the saphenous, femoral, or cephalic vein of restrained and nonsedated monkeys. Monkeys with very low plasma glucose levels between 15 and 90 min after glucose bolus gavage were excluded. After a single dose of antibody or vehicle on day 0, three additional OGTTs were performed on days 3, 8, and 17. Plasma glucose, insulin, glucagon, and active glucagon-like pep-
tide-1 (GLP-1) levels were measured from samples collected during the OGTT at time points 60 min before glucose administration (base-
line value) and at 0, 30, 60, 90, 120, 150, and 180 min after glucose administration. For fasting plasma glucose, insulin, glucagon, and active GLP-1 levels, the baseline values for each animal were mea-
sured at 22 and 15 days before treatment. The post-treatment values were measured on days 5, 10, 14, and 19 after treatment when no OGTTs were performed.

The pharmacokinetics of mAb B were determined in normal cy-
nomolus monkeys from the efficacy study. Blood samples for pharma-
cokinetic analysis were collected from each animal in the 3 and 30 mg/kg dose groups on days 3, 5, 8, 10, 14, 17, and 19 and processed to plasma to measure the levels of mAb B.

**Plasma Analysis.** Dipeptidyl peptidase-4 (DPP-IV) inhibitor and aprotinin were added to each blood sample collected to final concen-
trations of 100 μM and 85 μg/ml, respectively. Glucose levels were analyzed using a Hitachi 7020 Automatic Analyzer (Hitachi, Tokyo, Japan). Plasma insulin, active GLP-1, and glucagon levels were measured using the Human Endocrine LINCOplex Kit, per the man-
ufacturer’s protocol (Millipore Corporation, Billerica, MA).

**Measurement of mAb B Concentration in Plasma.** The concen-
trations of mAb B in plasma samples were quantified by a sand-
wich enzyme-linked immunosorbent assay. The capture reagent was a human GGCR fragment fused to murine Fe (Amgen Inc.). Study samples were added to the coated plates after blocking nonspecific binding. Horseradish peroxidase-conjugated anti-human Fe monoclonal antibody (Amgen Inc.) was used as the secondary antibody. Colorimetric determination of the horseradish peroxidase reaction with the tetramethylbenzidine peroxide substrate solution was mea-
sured by optical density at 450 to 650 nm. The conversion of optical density units to concentrations for mAb B in the study samples was achieved through comparison with a standard curve assayed on the same plate. A five-parameter logistic-auto estimate regression model with a weighting factor of 1/Y using Watson version 7.0.0.01 (Thermo Fisher Scientific) data reduction package was used.

**Pharmacokinetic Analysis.** Pharmacokinetic parameters such as AUC were estimated from mAb B plasma concentration data via noncompartmental analysis using WinNonlin Professional software version 4.1e (Pharsight, Mountain View, CA). The AUC_{last} was esti-
mated using the linear/logarithmic trapezoidal method (for the up/
down portions of the curve, respectively) up to the last measured concentration that was above the lower detection limit.

**Statistical Analysis.** Nonlinear regression analysis was per-
formed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). IC_{50} values, p_{A2} values, and rate and equilibrium constants are reported as the mean ± S.D. of multiple independent experiments.

For the efficacy study in ob/ob mice and C57BL/6 studies, statis-
tical analyses were performed with StatView 5.0.1 (SAS Institute, Cary, NC) or GraphPad Prism 4.02 software (GraphPad Software Inc.) using an unpaired Student’s t test. For the monkey OGTT studies, glucose, insulin, glucagon, and GLP-1 levels were measured, and an analysis of variance of the log-transformed data was carried out at each day and time of measurement. In addition to the analysis at each day and time, an AUC was calculated on the differences from baseline values of each of the measurements for each of the days. All statistical analyses were done using SAS version 9.1.3 (SAS Insti-
tute) on a Windows Professional operating system. A comparison of the groups for the OGTT endpoints was done using the SAS system for mixed models. Dunnett’s test was used to compare the mAb B at 3 mg/kg and mAb B at 30 mg/kg groups with the vehicle control group. For the comparison of difference-from-baseline AUC values, proc mixed allowed for unequal variances among the groups using a random statement and a Kenward-Roger adjustment to the degrees of freedom.

**Results**

**In Vitro Pharmacological Characterization of Hu-
man Anti-Human GGCR mAbs.** In total, 89 human mono-
clonal antibodies that showed specific binding to native hGGCR were identified. mAb A, mAb Ac (derived from mAb A), and mAb B were chosen for further characterization. Most of the in vitro pharmacological data shown here are generated with mAb B. Similar results were obtained with mAb A and mAb Ac.
Fluorescence-activated cell sorting (FACS) analysis using labeled antibodies demonstrated that both mAb A and mAb B specifically bind to a recombinant cell line expressing hGCGR (Fig. 1A). No specific antibody binding was detected with the parental cell line or the cell line expressing recombinant human GLP-1R, the receptor with the highest homology to human GCGR (Jelinek et al., 1993). Additional FACS analysis showed that both mAb A and mAb B also bind recombinant GCGRs from mouse and cynomolgus monkey (data not shown).

In addition to binding specificity, the functional specificity of mAb B against GCGR and GLP-1R was evaluated. In cell-based cAMP assays, the incubation of mAb B at 20 nM, but not of control human IgG, induced a rightward shift of the glucagon-stimulated dose-response curve (EC_{50} from 0.022 to 0.51 nM) (Fig. 1B). In contrast, as shown in Fig. 1C, mAb B did not affect the EC_{50} of GLP-1-stimulated cAMP accumulation for cells expressing the human GLP-1R, demonstrating the specific antagonistic activity of mAb B toward the GCGR.

The $K_d$ of mAb B to recombinant hGCGR was determined to be 35 pM using direct saturation binding assays (Fig. 2A). In addition, kinetic analysis of mAb B dissociation demonstrated that the antibody has an off-rate of $2.9 \times 10^{-5} \, \text{s}^{-1}$, corresponding to a GCGR/antibody complex half-life of $\sim 6.7 \, \text{h}$ (Fig. 2B).

Furthermore, we evaluated the ability of mAb B to inhibit the binding of $^{125}$I-glucagon to the receptors. Figure 3, A and B, show representative competition binding curves for human and cynomolgus monkey receptors, respectively, demonstrating that mAb B has a greater potency than glucagon itself in displacing the binding of radiolabeled ligand to the receptors. Table 1 summarizes competitive binding analyses of mAb A, mAb Ac, and mAb B using recombinantly expressed human, cynomolgus monkey, and murine GCGRs. As shown, mAb A and mAb Ac are more effective in disrupting $^{125}$I-glucagon binding to the human and murine GCGRs than to cynomolgus monkey GCGR, whereas the affinities of mAb B to human, murine, and monkey GCGRs are similar.

Inhibitory properties of mAb B were further investigated by Schild analysis. Figure 3, C and D, show representative experimental results using mAb B with recombinant human and cynomolgus monkey GCGR cell lines, respectively. Increasing concentrations of mAb B induced parallel rightward shifts of the glucagon dose-response curves, which were surmountable with increasing concentrations of glucagon. This is consistent with the classical competitive interaction model of Gaddum and Schild (Gaddum, 1957; Arunlakshana and Schild, 1959). The $pA_2$ of mAb B with human and monkey receptors was 9.22 and 8.04, respectively. In addition, studies of mAb B using primary human and cynomolgus monkey hepatocytes demonstrated similar potencies and classical competitive properties ($pA_2$ of 9.19 and 8.40, respectively). Table 2 summarizes the efficacy of mAb A, mAb Ac, and mAb B in recombinant and primary cell assays. To facilitate the comparison with data generated from Schild analyses, the

![Fig. 1](https://example.com/f1.png)

**Fig. 1.** Antibody specificity study. A, FACS analysis of the parental cell line (AM1D), GLP-1R-expressing cell line, and GCGR-expressing cell line with labeled mAb A and mAb B. B, glucagon dose-response curves measured by cAMP accumulation in GCGR-expressing cells in the presence of 20 nM control IgG or mAb B. C, GLP-1 dose-response curves measured by cAMP accumulation in GLP-1R-expressing cells in the presence of 20 nM control IgG or mAb B.

![Fig. 2](https://example.com/f2.png)

**Fig. 2.** Kinetics analysis of mAb B. A, $^{125}$I-mAb B saturation binding analysis using cell membranes from human GCGR-expressing cells. B, $^{125}$I-mAb B dissociation analysis using cell membranes from human GCGR-expressing cells.
potencies of the antibodies are expressed as the pIC50, which is the negative of the logarithm of the IC50. In functional assays, mAb A was shown to be a potent antagonist of both human and murine GCGR, with pIC50s of 7.41 and 8.0 (log [M]), respectively. mAb A showed ~10-fold lower potency against the cynomolgus monkey GCGR, with a pIC50 of 6.4. Isotype switching of mAb A from human IgG2 to mouse IgG1 (mAb Ac) did not significantly alter the specificity or neutralizing activity, as illustrated in Table 2.

Moreover, we evaluated the functional capability of the antibodies to inhibit ligand-induced receptor endocytosis. Recombinant cells expressing a GFP fusion construct of hGCGR were used to monitor receptor trafficking by fluorescent confocal microscopy. Figure 4 shows the same fields scanned at time 0 (left column) and 10 min after adding 300 nM glucagon (right column), respectively. Figure 4A showed GCGR internalization induced by glucagon, clearly exhibited by loss of membrane fluorescence and accumulation of punctate staining because of formation of endosomes. Pretreatment with a nonantagonizing GCGR-binding antibody for 20 min has no major effect on punctate staining in the presence of glucagon (Fig. 4B). In contrast, the pretreatment of the antagonistic GCGR antibody, mAb A, for 20 min resulted in little to no observed endosome formation, exhibited by the maintained membrane fluorescence of the GCGR-GFP in the presence of glucagon (Fig. 4C). These results demonstrated that mAb A effectively prevented glucagon-induced GCGR endocytosis and perhaps recycling. Because mAb B was found to be a potent antagonist against GCGR across species, it was selected for in vivo pharmacological studies.

In Vivo Pharmacological Studies of Human Anti-Human GCGR mAbs in Mice and Male Cynomolgus Monkeys. We first investigated the glucose-lowering effect of an anti-GCGR mAb in ob/ob mice. As demonstrated in Fig. 5A, a single injection of mAb B at 1 or 3 mg/kg decreased blood glucose levels to a normal range in ob/ob mice fed ad libitum. Blood glucose levels returned to baseline (t = 0) at day 5 with 1 mg/kg dose, whereas the glucose-lowering effect was maintained for 8 days, returning to baseline at 10 days, with the 3 mg/kg dose. These data show that both doses achieved similar efficacy during the first 24 h, whereas the 3 mg/kg dose demonstrated a longer duration of action than the 1 mg/kg dose.

In addition, we examined the effects of an anti-GCGR mAb on glucose clearance in C57BL/6 mice. An intraperitoneal GTT was performed in normal C57BL/6 mice after a single subcutaneous injection of mAb B at a dose of 1 or 10 mg/kg. mAb B decreased fasting blood glucose levels in a dose-dependent manner (Fig. 5B) without inducing hypoglycemia. As shown in Fig. 5C, mAb B improved glucose tolerance in a dose-dependent manner (p < 0.001 for both doses at 30 and 90 min versus vehicle-treated animals).

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**TABLE 1**

<table>
<thead>
<tr>
<th>Species</th>
<th>Glucagon IC50 Mean ± S.D.</th>
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<tr>
<td></td>
<td>nM</td>
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<tr>
<td>Human GCGR</td>
<td>2.5 ± 0.47</td>
</tr>
<tr>
<td>Cynomolgus Monkey GCGR</td>
<td>21 ± 0.08</td>
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<tr>
<td>Murine GCGR</td>
<td>4.4 ± 0.23</td>
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**Fig. 3.** In vitro pharmacological characterization of mAb B. Competition analysis of mAb B binding to membrane from human (A) or cynomolgus monkey (B) GCGR-expressing cells with [125I]-glucagon. Schild analysis of mAb B inhibition of human (C) and cynomolgus monkey (D) GCGR signaling.

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106 Yan et al.
Furthermore, we studied the effect of an anti-GCGR mAb on glucose clearance in male cynomolgus monkeys. Throughout the study, the body weight and the food intake of the 30 monkeys did not change among the three groups after the administration of vehicle or mAb B dosed at 3 or 30 mg/kg (data not shown).

After the single administration of mAb B or vehicle, three OGTTs were performed on days 3, 8, and 17. Animals treated with either 3 or 30 mg/kg mAb B showed similar improvement in glucose clearance compared with vehicle-treated animals on days 3 (data not shown) and 8 (Fig. 6A). On day 17, OGTT profiles for both antibody-treated groups were similar to the group treated with vehicle (data not shown).

AUCs were determined for each OGTT performed. Day 3/11002 and Day 17 OGTTs showed stable AUCs for the three different groups (Fig. 6A, inset). At day 3, animals treated with mAb B at 3 or 30 mg/kg showed an apparent dose-dependent decrease in AUCs (Fig. 6A, inset), even though statistical significance was not achieved ($p < 0.05$).

Plasma insulin, glucagon, and active GLP-1 levels were measured from samples collected during OGTTs. Figure 6, B to D, show the data obtained from the day 8 OGTT only. No differences in insulin levels were observed between the vehicle- and mAb B-treated groups at most time points (Fig. 6B).
The glucagon levels were elevated in both mAb B-treated groups at some time points on day 8 (Fig. 6C), similar to the results obtained on day 3 (data not shown). By day 17, the glucagon levels in both mAb B-treated groups returned to pretreatment levels at most time points. On day 3, both 3 and 30 mg/kg mAb B-treated groups showed statistically significant increases in active GLP-1 levels compared with the vehicle-treated group. On day 8, only the 30 mg/kg mAb B-treated group showed significant increases in active GLP-1 (Fig. 6D). By day 17, the active GLP-1 levels returned to their pretreatment levels for both mAb B-treated groups (data not shown).

No hypoglycemia was detected in any of the monkeys treated with mAb B at either the 3 or 30 mg/kg doses (Fig. 7A), either during the fasting performed before the OGTTs or after fasting on non-OGTT days. No differences in insulin levels were observed between the vehicle- and mAb B-treated monkeys at most time points (Fig. 7B).

On days 5, 10, and 14, glucagon levels in monkeys treated with 30 mg/kg mAb B were significantly increased compared with monkeys treated with vehicle (Fig. 7C). Despite the elevated glucagon levels in the antibody-treated animals, fasting glucose remained steady during the entire treatment period, and no hyperglycemia was observed up to 19 days after treatment. As shown in Fig. 7D, day 5 active GLP-1 levels were significantly elevated in animals treated with mAb B at either dose compared with animals treated with vehicle (p < 0.001 for both doses).

Plasma concentrations of mAb B were measured in the samples obtained from the cynomolgus monkey studies and subjected for pharmacokinetic analysis. The concentration of mAb B was found to increase with dose in a nonlinear manner and a 10-fold increase in dose from 3 to 30 mg/kg resulted in 17-fold increase in exposure from 3160 ± 1040 to 53,800 ± 12,800 µg/h/ml (Fig. 8), as measured by AUC_last (area under the plasma concentration time curve from day 3 to the time of the last quantifiable concentration). In the 3 mg/kg mAb B-treated animals, the plasma concentrations of mAb B were below the assay quantitation limit for the majority of the treated animals at time points beyond day 10. In the 30 mg/kg treated animals, the plasma concentrations of mAb B were measurable for all animals up to day 14 and in 8 of 10 animals on day 17. Although blood samples were not taken at time points before day 3, the data suggest that C_max was achieved on day 3 for both the low- and high-dose groups. The pharmacokinetic profile of mAb B was consistent with the results of the OGTTs performed on days 3, 8, and 17 after administration of mAb B (Fig. 8).

**Discussion**

For human therapeutic drug development, GPCRs have traditionally been targeted using small-molecule approaches. To date, there has been limited progress in targeting this class of molecules with monoclonal antibody therapeutics. The studies reported here show the successful immunological approach to a GPCR and suggest that antagonizing hGCGR with a monoclonal antibody is a potentially effective treatment for type 2 diabetes.

Elevation of HGO contributes to the hyperglycemic state of type 2 diabetes (DeFronzo et al., 1989; Staehr et al., 2001). Moreover, both glucagon hypersecretion in the fasted state and inadequate suppression of glucagon levels in the post-
prandial state contribute to the increase of HGO (Müller et al., 1970; Unger and Orci, 1975; Reaven et al., 1987; Dunning and Gerich, 2007). In accordance, reducing glucagon levels and inhibiting GCGR activity represent attractive approaches for treating excess glucose production in diabetic patients.

Anti-glucagon antibodies have shown some efficacy in animal models (Brand et al., 1994, 1996; Sørensen et al., 2006a); however, daily injections of high doses of antibodies were required (Sørensen et al., 2006). The lack of long-term efficacy of the antibody on blood glucose lowering is probably due to a compensatory mechanism involving oversecretion of endogenous glucagon in response to the reduction of glucagon receptor signaling. Increases in circulating glucagon levels have been reported with all modalities blocking the glucagon signaling pathway, which presents technical challenges for both small-molecule GCGR inhibitors and glucagon-neutralizing mAb approaches.

Despite rising glucagon levels, treatment with neutralizing hGCGR mAbs maintained glucose-lowering efficacy. These anti-GCGR mAbs have several desirable attributes as potential therapeutic agents compared with previously pursued approaches. First, mAb B showed a higher affinity than glucagon to the GCGR ($K_d = 36$ pM versus $K_d = 100$ pM). An mAb with a higher affinity than the cognate ligand itself to the hGCGR may be necessary to sustain long-term efficacy in the context of compensatory hyperglucagonemia. This observation is supported by our studies in C57BL/6 mice and cynomolgus monkeys, in which, despite the hyperglucagonemia induced by GCGR inhibition, treatment with anti-GCGR mAb B maintained long-lasting efficacy. Second, the slow off-rate ($t_{1/2 \text{ off}} \sim 6.7 \text{ h}$) of the mAb B bound to hGCGR and the relatively long serum half-life of IgG2 probably contributes to the prolonged in vivo activity, thereby minimizing compound dosing regimens. As demonstrated in our studies, a single injection of mAb B at 3 mg/kg lowered blood glucose to the normal range in ob/ob mice for up to 8 days. Third, mAb B displayed highly specific antagonistic activity against the GCGR without inhibiting GLP-1R activity. The GLP-1R is the most closely related receptor to the GCGR, sharing 44% sequence identity (Thorens, 1992; Jelinek et al., 1993). Because the activation of the GLP-1R has several well-established beneficial effects on the control of glycemia, including stimulation of glucose-dependent insulin secretion and β-cell preservation (Drucker, 2006), the selectivity is a critical
property for ensuring the therapeutic application of anti-GCGR antibody.

Our rodent studies with an anti-GCGR mAb further support the concept that antagonism of glucagon action would be effective in reducing both fasting and postprandial glucose levels. The profound potency and prolonged half-life of mAb B against hGCGR may be advantageous over other biotherapeutic agents targeting alternative pathways, such as GLP-1 (Drucker, 2006). For example, the long-acting GLP-1 NN2211 has been shown to dose-dependently reduce blood glucose levels for 24 h in ob/ob mice. However, it failed to normalize blood glucose levels in these mice, even at a dose of 1000 µg/kg (Rolin et al., 2002). In contrast, our studies demonstrate that a single administration of mAb B (3 mg/kg) can normalize blood glucose in ob/ob mice for up to 8 days. It remains to be determined whether the efficacy of the anti-GCGR mAb in animal models can translate to clinical benefits in human studies.

Our data demonstrate that a single injection of anti-GCGR mAb B in normal monkeys improved oral glucose tolerance. Although the improvement was moderate, it is uncommon to find that a compound shows efficacy in normal monkeys after a single injection. In fact, our results were especially encouraging given that those young animal lack excess hepatic glucose production and have normal glycemic control. Even though the effect on glucose tolerance was not robust, our data from the monkey study corroborate the results from our mouse studies. Moreover, we observed that mAb B leads to elevation of both basal and postprandial active GLP-1 levels for up to 8 days in monkeys. Although the 3-fold increase in active GLP-1 levels is comparable with that achieved by a DPP-IV inhibitor (Herman et al., 2006), it is important to note that anti-GCGR mAb B treatment resulted in persistent elevation of active GLP-1, which is distinct from the DPP-IV inhibitor-mediated episodic and meal-related GLP-1 level elevation (Herman et al., 2006). It was suggested that continuous rather than intermittent activation of the GLP-1 pathway provides optimal glycemic control (Larsen et al., 2001); therefore, our anti-GCGR mAb may have advantages over DPP-IV inhibitors. In studies by Sorenson (2006), acute inhibition with a glucagon-neutralizing mAb increased hepatic glycogen formation after OGTT in ob/ob mice (Sorensen et al., 2006a). In the animals treated with anti-GCGR antibodies, the increase in GLP-1 plasma level is probably due to increased expression and processing of the proglucagon from pancreatic α-cells. In fact, the α-cell-derived active GLP-1 elevations were also observed in the GCGR knockout and GCGR ASO-treated mice. In these mice, the plasma concentrations of active GLP-1 (7-36 and 7-37 amides) were elevated 10- to 20-fold (Gelling et al., 2003; Sloop et al., 2004). In both cases, it has been hypothesized that a compensatory increase in islet α-cell PC1/3 activity that produces a shift toward GLP-1 production may contribute to the improved β-cell function (Nie et al., 2000; Gelling et al., 2003; Sloop et al., 2004). It is plausible that the elevated active GLP-1 levels and the increased rate of hepatic glycogen formation (therefore acute glucose disposal) may both contribute to greater glucose clearance during OGTT in GCGR mAb-treated animals. However, the relative contribution of GLP-1 action to the efficacy of the GCGR mAb remains to be determined.

In C57BL/6 mice, a single injection of GCGR mAb dose-dependently reduced fasting blood glucose levels after a 12-h fast without inducing hypoglycemia. However, a single dose of GCGR mAb in normal lean monkeys had no influence on overnight fasting blood glucose levels despite hyperglycemia. One explanation for the difference in the fasting blood glucose effect between the two species is that mAb B has higher inhibitory potency (~10-fold) toward mouse than monkey GCGRs, as shown by the pIC_{50} values reported in Table 2. In addition, variation in metabolism among different species may also contribute to the observed differences. First, rodents have a higher metabolic rate and need to eat considerably more than larger mammals (Yang and Bankir, 2005). Second, mice may tolerate lower fasting blood glucose levels of 2.5 mM with 10 mg/kg mAb B better than primates; thus, the compensatory mechanism may initiate at a lower threshold with rodents than primates. Third, a 12 h-fast could have a more dramatic effect on metabolic functions in mice than in monkeys, considering that mice are nocturnal and monkeys are not. Finally, glucagon may play a less important role in the overall metabolic regulation in monkeys than in mice. Further studies will be needed to better understand the differences in efficacy observed between species. Regardless, it is important to note that anti-GCGR mAb B did not induce hypoglycemia in either species.

Consistent with rodent studies, anti-GCGR mAb treatment in monkeys caused a dose-dependent increase in glucagon levels, most probably because of an adaptive mechanism compensating for reduced GCGR signaling. Even though both species showed increases in glucagon levels, it is important to point out that there are differences in islet anatomy between these species (Cabrera et al., 2006). In addition, differences in compensatory responses because of blockade of glucagon receptor signaling exist among rodents and primates. For example, rodents treated with GCGR ASOs for 4 weeks developed α-cell hyperplasia, whereas in primates, 10-week GCGR ASO treatment did not cause islet α-cell expansion (Sloop et al., 2004; Bhanot et al., 2006). Based on the unique cytoarchitecture of primate islets (Cabrera et al., 2006), the monkey may be a more relevant species than the mouse for evaluating the effects of GCGR antagonism on islet physiology.

Our anti-hGCGR mAbs are fully human antibodies (Brekke and Sandlie, 2003; Leader et al., 2008). Compared with chimeric and humanized antibodies, fully human antibodies offer a minimal risk of immunogenicity because they only contain antibody sequences of human origin. Despite the theoretical low risk of developing anti-therapeutic antibodies, the immunogenicity of fully human anti-GCGR antibody will need to be evaluated in clinical trials in humans.

With the increasing prevalence of type 2 diabetes and the limited available therapies, there is a clear demand for new and effective treatments for the disease. We successfully explored a novel approach targeting hGCGR, a well validated target for diabetic treatment. Our data demonstrate that in animal models, neutralizing GCGR mAbs are highly effective in achieving improved glycemic control without inducing hypoglycemia. Further studies are needed to evaluate the GCGR antibody as a potential new therapy for the treatment of human type 2 diabetes.

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