A Novel Glucagon Receptor Antagonist Inhibits Glucagon-Mediated Biological Effects

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Glucagon maintains glucose homeostasis during the fasting state by promoting hepatic gluconeogenesis and glycogenolysis. Hyperglucagonemia and/or an elevated glucagon-to-insulin ratio have been reported in diabetic patients and animals. Antagonizing the glucagon receptor is expected to result in reduced hepatic glucose overproduction, leading to overall glycemic control. Here we report the discovery and characterization of compound 1 (Cpd 1), a compound that inhibits binding of 125I-labeled glucagon to the human glucagon receptor with a half-maximal inhibitory concentration value of 181 ± 10 nmol/l. In CHO cells overexpressing the human glucagon receptor, Cpd 1 increased the half-maximal effect for glucagon stimulation of adenylyl cyclase with a $K_{DB}$ of 81 ± 11 nmol/l. In addition, Cpd 1 blocked glucagon-mediated glycogenolysis in primary human hepatocytes. In contrast, a structurally related analog (Cpd 2) was not effective in blocking glucagon-mediated biological effects. Real-time measurement of glycogen synthesis and breakdown in perfused mouse liver showed that Cpd 1 is capable of blocking glucagon-induced glycogenolysis in a dosage-dependent manner. Finally, when dosed in humanized mice, Cpd 1 blocked the rise of glucose levels observed after intraperitoneal administration of exogenous glucagon. Taken together, these data suggest that Cpd 1 is a potent glucagon receptor antagonist that has the capability to block the effects of glucagon in vivo. Diabetes 53:3267–3273, 2004

Glucon is a 29–amino acid polypeptide produced in the pancreatic $\alpha$-cells and secreted in response to falling glucose levels during the fasting period (1). Glucagon increases glucose production by promoting glycogenolysis and gluconeogenesis in the liver and attenuation of the ability of insulin to inhibit these processes (2). The combined action of glucagon and insulin is responsible for maintaining whole-body glucose homeostasis (3). Both increased glucagon secretion during the fasting state and the lack of insulin-mediated suppression of glucagon production in postprandial state contribute to the elevated glucagon levels associated with the hyperglycemia observed in the diabetic state (4,5). Therefore, reducing circulating glucagon levels and inhibiting glucagon-mediated biological effects in target tissues have long been considered as means of reducing hyperglycemia in diabetes. Studies using potent peptide antagonists have demonstrated significant blood glucose-lowering effects in diabetic animal models (6,7). Furthermore, it has been demonstrated that immunoneutralization of glucagon in diabetic animals effectively diminishes glucagon-stimulated hyperglycemia (8–10). Although these reagents have shown promising results in animal models, their development for use in humans has not progressed because of limitations imposed by the delivery methods necessary to achieve significant levels of exposure for the peptide agents.

The glucagon receptor (GCGR) is a member of the family B of the seven transmembrane G-protein–coupled receptor (GPCR) superfamily (11). Other closely related members of the family include the receptors for glucagon-like peptide 1 and glucose-dependent insulinotropic peptide (GIP). Glucagon signals by binding to the receptor, which leads to activation of adenylyl cyclase and an increase in intracellular cAMP levels (12). In addition, the GCGR also couples to an intracellular $Ca^{2+}$-mediated pathway (13). Activation of the GCGR results in increased glycogenolysis and gluconeogenesis, which are responsible for increased hepatic glucose output (14,15).

Given the key role of glucagon in elevating glycemia and owing to the success of finding small-molecule inhibitors for many receptors in the GPCR family (16,17), the GCGR is a clear target for the development of small-molecule antagonists. A number of antagonists with varying degree of potency and structures have been reported in recent
years (rev. in 18). Among them, one compound (Bay-27-9955) advanced to evaluation in humans in early clinical trials and was shown to block glucagon-stimulated glucose production in healthy volunteers (19). Although Bay-27-9955 has apparently not been studied in diabetic patients, current results with the compound do provide further validation for the notion that antagonism of the GCGR may be a viable approach to regulating glycemia in humans. Thus, there is now a compelling need to identify newer and better optimized GCGR antagonists that could be considered as alternative clinical development candidates for the treatment of type 2 diabetes.

In our study, we identified compounds in a new chemical class that are potent in blocking glucagon-dependent activation of the human GCGR in human hepatocytes and in a mouse model expressing the human GCGR. Here we describe the characterization of a representative compound from this novel class of glucagon antagonists.

RESEARCH DESIGN AND METHODS

Chinese hamster ovary (CHO) cells expressing the cloned human GCGR (CHO-hGCGR) have been previously described (20,21). The CHO-hGIPR cell line was produced by transfecting human glucagon insulinotropic peptide receptor (hGIPR) cDNA (kindly provided by T. Usdin, National Institutes of Health, Bethesda, MD) into CHO cells. All cell lines were maintained in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% FBS, 1 mmol/l L-glutamine, penicillin-streptomycin (100 units/ml), and G418 (500 μg/ml) (GIBCO/Invitrogen Life Sciences). Primary human hepatocytes were obtained from In Vitro Technologies (Baltimore, MD) and maintained as hepatocyte culture medium (HCM) (Clonetics, PA). Compounds (Cpd) 1 and 2 were initially procured from Olivia Scientific (Princeton, NJ) and later synthesized in house by standard chemical synthesis (J.L.D., unpublished observations). Primary human hepatocytes were obtained from In Vitro Technologies (Baltimore, MD) and maintained in hepatocyte culture medium (HCM) (Clonetics, PA). Compounds (Cpd) 1 and 2 were initially procured from Olivia Scientific (Princeton, NJ) and later synthesized in house by standard chemical synthesis (J.L.D., unpublished observations). Stock solutions of compounds were prepared in 100% DMSO and diluted in the appropriate buffer just before being used. All other materials were from commercial sources. Male mice used in this study originated from a humanized GCGR mouse line generated by replacing the mouse GCGR gene with that of the hGCGR gene, as previously described (22). Animals were housed in a standard rodent facility with free access to standard rodent diet and water following standard institutional guidelines for animal care. All animal care and procedures were performed in accordance with institutional guidelines.

Glucagon receptor–binding assay. To determine the binding affinity of selected compounds, 2 mg of cell membranes from the CHO-hGCGR cells were incubated with 50 pmol/l (125I)-labeled glucagon (Du Pont-NEN) in a buffer containing 50 mmol/l Tris HCl (pH 7.5), 5 mmol/l MgCl2, 2 mmol/l EDTA, 12% glycerol, and 200 mg wheat germ agglutinin-coated polyvinylpyrrolidone scintillation promoter (PerkinElmer Life Sciences) with or without increasing concentrations of the compounds. Nonspecific binding was determined in the same buffer in the presence of 1 μmol/l unlabeled glucagon. After a 4- to 12-h incubation at room temperature, the radioactivity bound to the cell membranes was determined in a scintillation counter (Microbeta; PerkinElmer Life Sciences, Boston, MA). The half-maximal inhibitory concentration value (IC50) for native glucagon was 2.8 ± 0.4 nmol/l (n = 5). Adenyl cyclase assay. CAMP levels in CHO-hGCGRs or CHO-hGIPRs were determined with the aid of an adenylyl cyclase assay kit (SMP-004B, PerkinElmer Life Sciences), per the manufacturer’s instructions. To assess antagonist activity, cells were incubated with Cpd 1 for 30 min and then stimulated with glucagon (0–500 nmol/l), forskolin (1 μmol/l), or GIP (250 pmol/l) for 30 min. The cell stimulation was stopped by adding an equal amount of a detection buffer containing cell lysis agent and (125I)-labeled CAMP tracer. The amount of (125I)-CAMP bound to the plate was determined using a liquid scintillation counter (Microbeta; PerkinElmer Life Sciences) and used to quantify the amount of CAMP present in each sample. The half-maximal effect (EC50) for glucagon in CHO-hGCGRs was 0.22 ± 0.02 nmol/l (n = 5). The Kp and PA2 values were determined by the method of Arumilliashka and Schild (23).

β-Lactamase reporter gene assay. A glucagon-responsive β-lactamase reporter gene cell line was established by transfecting the hGIPR into a CHO cell line containing a β-lactamase reporter gene under the control of a CAMP-responsive promoter (24); cells that expressed the β-lactamase gene in response to stimulation with 100 mmol/l glucagon were selected as previously described (24; S.A.Q. and B.B.Z., unpublished observations). A cell line expressing the hGIPR was prepared using the same protocol, except that GIP-responsive cells were selected. To study changes in CAMP-dependent transcription in these cells, 5,000 cells per well were plated onto 96-well, clear-bottomed plates and allowed to adhere to the plate for 18–24 h. Subsequently, the medium was removed and each well was washed with PBS, 0.1% FBS; then, one of the compounds or 1% DMSO (as a control) in the same medium for 30 min. Cells were stimulated with 250 pmol/l glucagon, 1 μmol/l forskolin, or 100 pmol/l GIP (for cells expressing the hGIPR) for 4 h and then loaded with CCF2/AM (a β-lactamase substrate dye) to detect β-lactamase activity, as previously described (24).

Glycogen synthesis assay. Fresh primary human hepatocytes were plated (1.7 × 107/well) onto collagen-coated, six-well plates in HCM BulletKit medium (Clonetics); a day later, they were serum starved overnight by switching to HCM with low-glucose (1 g/l) medium. Glycogen synthesis and labeling were initiated by adding 200 mmol/l insulin and n-[U-14C]glucose (2 μCi/ml; specific activity 0.36 μCi/μmol; PerkinElmer Life Sciences) in the same medium. After being incubated for 3 h at 37°C, the cells were treated with glucagon (2 nmol/l) in presence of DMSO alone or one of the compounds for an additional 60 min. Cell glycogen contents were measured as previously described (25).

Inhibition of glycogen breakdown in perfused mouse liver. Glycogen levels in perfused mouse liver were determined as previously described (26,27). Briefly, [2-14C]pyruvate was incorporated into endogenous glycogen and 14C nuclear magnetic resonance (NMR) spectroscopy was used to monitor the synthesis and breakdown of [14C]glycogen in real time in perfused livers (26,27). To assess inhibitor activity, cells were incubated with Cpd 1 or 2 (diluted from a stock in 100% DMSO) was added to the perfusate to yield the desired concentration of compound. In a similar fashion, human glucagon prepared in normal saline was added to the perfusate at the indicated time. The amount of glycogen at a given time was estimated from the area of the C1 resonance of the glucosyl unit of glycogen. Absolute areas were normalized by the area of the glycogen C1 resonance at t = 0 (glucagon administration).

Glucagon challenge assay in mice with the human glucagon receptor. Male mice with the hGCGR (age 10–11 weeks) (22) were used in these studies. These mice had free access to standard rodent diet and water before being dosed with a compound; food was withdrawn right before the dosing vehicle or compound. Animals were given either vehicle (60% polyethylene glycol, 20% ethanol, and 20% saline) or compound suspension in the vehicle via intraperitoneal injection at the indicated dosage. At 1 h after the first intraperitoneal dose, a bolus dose of 15 μg/kg glucagon (Eli Lilly) in normal saline containing 0.5% BSA or normal saline plus 0.5% BSA was administered via a second intraperitoneal injection. Blood glucose levels were monitored using a One Touch Basic Glucometer (LifeScan) before vehicle or compound was injected (t = -60) and before the saline or glucagon intraperitoneal injection (t = 0) and at 7.5, 15, 30, and 45 min after glucagon challenge.

Statistical analysis. IC50 and EC50 values were obtained using the nonlinear regression analysis (curve fit) assuming single site binding. P values were calculated using the unpaired Student’s t test. All data were analyzed with the aid of GraphPad Prism software (GraphPad Software, San Diego, CA).

RESULTS

Identification of a selective glucagon receptor antagonist. To identify novel GCGR antagonists, we screened chemical libraries for compounds that block 125I-glucagon binding to membranes prepared from CHO-hGCGR cells. From our search, we identified N-[3-cyano-6-(1,1-dimethylpropyl)-4, 5, 6, 7-tetrahydro-1-benzothien-2-yl]-2-ethylbutanamide as a potent GCGR antagonist (Cpd 1) (Fig. 1A). Cpd 1 inhibited the binding of 125I-glucagon to the membrane with an IC50 of 181 ± 10 nmol/l (n = 3). Cpd 1 specifically interacts with the hGCGR, as it did not inhibit the binding of cognate ligands to a number of other GPCRs, such as the receptors for GIP and neuropeptide Y (data not shown). In contrast, Cpd 2, a structurally related analog, was a poor inhibitor (20 ± 1.5% inhibition at 10 μmol/l) of glucagon binding (Fig. 1B).

Binding of glucagon to its receptor resulted in activation of adenylyl cyclase and subsequently cAMP production (12), which is responsible for many of glucagon’s actions, including activation of glycogen phosphorylase and inhibi-
bition of glycogen synthase. Increasing the concentration of Cpd 1 increased the EC$_{50}$ for glucagon-dependent adenylyl cyclase activation in CHO-hGCGR cells without affecting the maximal glucagon response (Fig. 2). This result suggested that Cpd 1 is a competitive antagonist, a conclusion further supported by the Schild transformation (23) of data shown in Fig. 2 that yields a straight line with a slope not significantly different from unity. Moreover, the affinity of Cpd 1 for the GCGR, as measured by the concentration of the antagonist that shifted the agonist dosage response by twofold ($K_{DB}$), was estimated as 81 $\pm$ 11 nmol/l ($n = 3$) and $pA2$ (the negative logarithm of the inhibitor concentration that reduces the agonist response to 1 unit of glucagon to the response obtained with 0.5 units of glucagon) as 7.1 $\pm$ 0.06 (23). The effect of Cpd 1 was specific to the GCGR, as it had no effect on either forskolin-dependent cAMP production in the same cells or GIP-dependent cAMP production in cells expressing hGIPRs (data not shown). In contrast, Cpd 2 showed a <10% inhibition of glucagon, forskolin, and GIP-induced cAMP production.

Activation of the GCGR leads to increased gene expression of enzymes involved in gluconeogenesis, such as glucose 6-phosphatase and PEPCK (14,15). To establish that Cpd 1 is able to suppress glucagon-induced gene expression, we examined the agent’s effect on the induction of a cAMP-responsive β-lactamase reporter gene in CHO cells. The β-lactamase reporter gene cell line was prepared by introducing the hGCGR into cells expressing a β-lactamase reporter gene under the control of a cAMP-responsive promoter element (24). Glucagon increased β-lactamase activity in these cells with an EC$_{50}$ of 250 pmol/l. When tested in this cell line, Cpd 1 inhibited the glucagon-stimulated rise in the β-lactamase activity with an IC$_{50}$ of 1,569 $\pm$ 374 nmol/l ($n = 4$), whereas Cpd 2 had no effect in this assay. In addition, Cpd 1 had no effect on the forskolin-stimulated rise in β-lactamase activity in these cells or on GIP activity in the β-lactamase cell line expressing hGIPR.

Taken together, these data demonstrate that Cpd 1 is a selective and competitive GCGR antagonist.

Inhibition of glycogenolysis in human hepatocytes. The binding of glucagon to its receptor results in increased glycogenolysis that is believed to be responsible, at least in part, for hepatic glucose production (HGP) during fasting periods. To examine if Cpd 1 would block this effect, we first induced glycogen synthesis in human primary hepatocytes with 200 nmol/l insulin in the presence of [14C]glucose to increase the pool of labeled glycogen. Incubation of human hepatocytes under these conditions resulted in a three- to fivefold increase in the amount of radiolabeled glycogen (Fig. 3; compare the basal and insulin-stimulated responses). As expected, treatment of insulin-stimulated cells with glucagon reduced the [14C]glucose to increase the pool of labeled glycogen. Incubation of human hepatocytes under these conditions resulted in a three- to fivefold increase in the amount of radiolabeled glycogen in these cells (Fig. 3; compare the basal and insulin-stimulated responses). As expected, treatment of insulin-stimulated cells with glucagon reduced the [14C]glucose contents to basal levels. However, the addition of Cpd 1 at 10 or 30 μmol/l along with glucagon significantly attenuated the glucagon-induced glycogenolysis in this setting. Once again, Cpd 2 at the same dosages was ineffective in blocking the glucagon-induced glycogenolysis in these cells. These data demonstrate the ability of Cpd 1 to block the glucagon-dependent glycogen breakdown in human hepatocytes, a target cell population for the action of glucagon in vivo.

Inhibition of glycogenolysis in perfused liver. To demonstrate that the inhibitory effect of Cpd 1 on the
glycogenolysis observed in vitro in human hepatocytes will also result in the inhibition of glucagon-dependent glycogenolysis in vivo, the activity of the Cpd 1 was examined by $^{13}$C NMR in intact perfused liver obtained from humanized mice (22) in an ex vivo setting. In this setting, the intact liver is maintained in an isotonic environment to measure various metabolites in real time at or near-physiological conditions (26). To assess the effect of Cpd 1 under these conditions, we first labeled endogenous glycogen with the aid of $[2-^{13}$C]pyruvate. Administration of $[2-^{13}$C]pyruvate (6.7 mmol/l) over the entire duration of experiment (~150 min) resulted in a steady increase in the amount of glycogen, as indicated by changes in the levels of $[^{13}$C]glycogen in these livers (Fig. 4). Furthermore, this rise in glycogen synthesis was not affected by the introduction of Cpd 1, Cpd 2, or DMSO into the perfusate 40 min after the start of labeling. Exposure of these livers to glucagon (50 pmol/l) resulted in a rapid degradation of glycogen in the DMSO-treated livers only. However, in Cpd 1 treated livers, a significant, dosage-dependent reduction in glucagon-induced glycogenolysis was observed (Fig. 4). As observed earlier, Cpd 2, the inactive analog, had no effect in this experimental setting. In addition, vehicle-treated livers continued to synthesize glycogen at a

FIG. 3. Inhibition of glucagon-induced glycogenolysis in human primary hepatocytes. Primary human hepatocytes were incubated with $[^{14}$C]glucose for 3 h without (basal) and with 200 nmol/l insulin (Ins) to facilitate labeling of glycogen. After the labeling period, the samples were treated with 2 nmol/l glucagon (GCG) with or without Cpd 1 or 2 at the indicated dosages for 60 min. Glycogen levels in each sample were then measured as described in RESEARCH DESIGN AND METHODS. Data are means ± SE of three replicates and are taken from a representative experiment. Qualitatively similar data were obtained in at least three independent experiments; however, due to significant variance in the response to insulin in various human hepatocytes preparations, pooling of the data has been avoided. $P$ values were calculated using the unpaired Student’s $t$ test.

FIG. 4. Inhibition of glucagon-induced glycogenolysis in mouse liver. An intact liver from a male hGcGR mouse was obtained and quickly perfused with Krebs bicarbonate buffer containing 2.5% BSA at 37°C. Endogenous glycogen was labeled with the constant administration of $[2-^{13}$C]pyruvate in the same buffer that started at $t = -90$ min and continued throughout the experiment. At $t = -25$ min, Cpd 1 or 2 (at the indicated dosages) or DMSO was added to the perfusate. At $t = 0$, glucagon (GCG) or saline was introduced into the perfusate. The glycogen levels during the entire experiment were determined via $^{13}$C NMR spectra and are means ± SE of three independent determinations.
indicated times after the glucagon challenge peritoneal injection at or normal saline alone (saline) was administrated via a second intraperitoneal injection of 15 g/kg glucagon re- sulted in a rapid rise in blood glucose levels in vehicle-treated animals, whereas the administration of a similar volume of saline produced minimal change in blood glucose levels in these animals (compare vehicle/saline with vehicle/GCG in Fig. 5). However, in the animals that had been treated with Cpd 1 at 50 mg/kg (but not at 15 mg/kg) before the glucagon challenge, the effect of glucagon was significantly blunted. These data show that Cpd 1 is capable of blocking the effect of glucagon in vivo, and thus should affect the regulation of glucose homeostasis in the diabetic state.

Inhibition of glucagon-induced glucose elevation in a hGCGR mouse model. Administration of glucagon in animal models as well as in humans results in a rapid rise in plasma glucose levels due to glycogen breakdown (7,19). We used this paradigm to evaluate the potential of our compound to inhibit glucagon activity in vivo using the hGCGR mouse model (22). To test the antagonistic potential of the compound, we first treated these animals with Cpd 1 or vehicle via an intraperitoneal injection and then added a glucagon challenge 1 h later. As shown in Fig. 5, a single intraperitoneal injection of 15 g/kg glucagon resulted in a rapid rise in blood glucose levels in vehicle-treated animals, whereas the administration of a similar volume of saline produced minimal change in blood glucose levels in these animals (compare vehicle/saline with vehicle/GCG in Fig. 5). However, in the animals that had been treated with Cpd 1 at 50 mg/kg (but not at 15 mg/kg) before the glucagon challenge, the effect of glucagon was significantly blunted. These data show that Cpd 1 is capable of blocking the effect of glucagon in vivo, and thus should affect the regulation of glucose homeostasis in the diabetic state.

DISCUSSION

Blood glucose levels are maintained through the balance between glucose uptake by the peripheral tissues and glucose production and secretion by the liver. The liver plays a central role in maintaining blood glucose levels by acting as a storage depot during periods of dietary carbohydrate excess and as a glucose source when carbohydrate supply is scarce. Two pathways contribute to HGP: the breakdown of glycogen (or glycogenolysis) and gluconeogenesis from precursors such as lactate, gluconeogenic amino acids, or glycerol. This homeostatic system is under the tight control of hormones, with insulin playing a major role in suppressing HGP under fed conditions and glucagon and corticosteroids promoting HGP under fasting conditions. An inappropriately high rate of HGP is the predominant cause of fasting hyperglycemia and a major contributor to the postprandial hyperglycemia characteristic of type 2 diabetes (28,29). In addition, increased circulating glucagon levels are also linked to elevated HGP in type 2 diabetes (3,30).

Glucagon and the GCGR represent potential targets for the treatment of diabetes (31,32). Over the last two decades, encouraging progress has been made in attempts to normalize hyperglycemia by antagonizing glucagon signaling using glucagon-neutralizing antibodies, peptide glucagon analogs that function as antagonists, and nonpeptide small-molecule GCGR antagonists. High-affinity glucagon-neutralizing antibodies can effectively reduce free glucagon and lead to reduced glycemia in animal models (9–10). Extensive efforts have been made to generate linear and cyclic glucagon analog peptides. Compared with native glucagon, some of these peptide analogs have shown distinct properties in terms of their ability to bind to the GCGR and affect glucagon-stimulated cAMP production. They act as pure agonists, partial agonists/antagonists, or pure antagonists of the GCGR (33–35). It was first reported that [1-Nα-trinitrophylhistidine, 12-homoarginine]-gluca- gon, a potent antagonistic glucagon analog, significantly decreased hyperglycemia in streptozotocin-induced diabetic rats in vivo (6). Other potent glucagon analogs have also demonstrated efficacy in animal models of diabetes (7).

The discovery and development of nonpeptidyl GCGR antagonists of diverse structures have been reported over recent years (rev. in 18). The published antagonists appear to act via distinct receptor mechanisms. Skyrin, one of the earlier antagonists, appeared to functionally inhibit glucagon-stimulated cAMP production and glycogenolysis without affecting glucagon binding (36). Other antagonists inhibit both the binding and the function of glucagon in competitive or noncompetitive fashion. Most recently, Bay-27-9955, an orally absorbed and potent GCGR antagonist, has been shown to block glucagon-induced elevation of blood glucose in human (19).

Given the potential importance of this new therapeutic approach and the apparent lack of progress made with existing compounds, we sought to discover and characterize a new, and hopefully improved, class of GCGR antagonists. In this study, we showed that a new compound specifically and competitively inhibits glucagon binding and blocks glucagon-stimulated glycogenolysis in human hepatocytes.

Glucagon receptor antagonists have been shown to exhibit species specificity, as some antagonists are more potent toward the human than the murine GCGR (20) and vice versa. Therefore, the demonstration of efficacy for novel structural classes may be challenging, as compounds are prioritized based on their activities using in vitro assays in human receptors, but must be evaluated in nonhuman animal models before their introduction in humans. In this regard, a mouse model expressing the hGCGR generated with a direct replacement vector is highly valuable in evaluating the in vivo efficacy of GCGR antagonists (22). In this study with humanized mice, we characterized Cpd 1 using primary hepatocytes (F. Liu and
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G.J., unpublished observations), perfused liver, and in vivo testing. This study design provided a unique paradigm for evaluating the potency of the antagonist compounds on the hGCGR in a preclinical setting and thus should afford better correlation with the potency of antagonists in future studies in humans.

Because liver is the primary target tissue for glucagon action, we evaluated the effect of our antagonist lead compound in perfused liver using NMR spectroscopy (26,27). This method enables a real-time assessment of glycogen synthesis and glucagon-stimulated glycogenolysis in the intact organ under conditions that approximate real physiology. Phosphorus spectra were taken in the beginning and at the end of the perfusion studies to ensure the presence of consistent levels of ATP, which was used as an indicator that the perfused livers were in a healthy state. In this study, we demonstrated that Cpd 1 was able to attenuate glucagon-induced glycogen breakdown in a dosage-dependent manner. These results were complementary to similar findings using human primary hepatocytes. Furthermore, we used the glucagon challenge method to determine the in vivo activity of GCGR antagonists. This paradigm is analogous to the one used in the reported human study of Bay-27-9955 and provides an efficient means to evaluate antagonist compounds in an animal model.

Hypoglycemia is a theoretical mechanism-based safety concern for GCGR antagonists. However, in ob/ob mice treated with antiguacagon antibodies for 1 week until undetectable plasma glucagon levels were reached, significant reductions in elevated glucose and triglyceride levels were observed without hypoglycemia (9). Furthermore, GCGR knockout mice are not overtly hypoglycemic (37,38). Similarly, GCGR antisense oligonucleotides reversed hypoglycemia in diabetic rodent models without causing hypoglycemia (39,40). Of note, human glucose counterregulation involves several pathways, mediated not only by glucagon but also by catecholamines, corticosteroids, and growth hormone. Blocking the glucagon axis should not interfere with these other counterregulatory responses. Thus, severe hypoglycemia is not expected with glucagon antagonists.

In summary, Cpd 1 represents a novel class of compound that functions to block glucagon binding and antagonize biological responses elicited by glucagon in human hepatocytes. It is important to note that Cpd 1 also blocked the effects of glucagon on perfused whole liver from hGCGR mice and diminished glucagon-stimulated glucose elevations in the mouse model. These results indicate that additional optimized small-molecule GCGR antagonists can be derived and suggest that efficient blockade of glucagon action is an effective means to control HGP and should lead to efficacy in lowering fasting and postprandial hyperglycemia in type 2 diabetes.

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