Novel Small Molecule Glucagon-Like Peptide-1 Receptor Agonist Stimulates Insulin Secretion in Rodents and From Human Islets

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OBJECTIVE—The clinical effectiveness of parenterally-administered glucagon-like peptide-1 (GLP-1) mimetics to improve glucose control in patients suffering from type 2 diabetes strongly supports discovery pursuits aimed at identifying and developing orally active, small molecule GLP-1 receptor agonists. The purpose of these studies was to identify and characterize novel nonpeptide agonists of the GLP-1 receptor.

RESEARCH DESIGN AND METHODS—Screening using cells expressing the GLP-1 receptor and insulin secretion assays with rodent and human islets were used to identify novel molecules. The intravenous glucose tolerance test (IVGTT) and hyperglycemic clamp characterized the insulinotropic effects of compounds in vivo.

RESULTS—Novel low molecular weight pyrimidine-based compounds that activate the GLP-1 receptor and stimulate glucose-dependent insulin secretion are described. These molecules induce GLP-1 receptor-mediated cAMP signaling in HEK293 cells expressing the GLP-1 receptor and increase insulin secretion from rodent islets in a dose-dependent manner. The compounds activate GLP-1 receptor signaling, both alone or in an additive fashion when combined with the endogenous GLP-1 peptide; however, these agonists do not compete with radiolabeled GLP-1 in receptor-binding assays. In vivo studies using the IVGTT and the hyperglycemic clamp in Sprague Dawley rats demonstrate increased insulin secretion in compound-treated animals. Further, perifusion assays with human islets isolated from a donor with type 2 diabetes show near-normalization of insulin secretion upon compound treatment.

CONCLUSIONS—These studies characterize the insulinotropic effects of an early-stage, small molecule GLP-1 receptor agonist and provide compelling evidence to support pharmaceutical optimization. *Diabetes* 59:3099–3107, 2010

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Hyperglucagonemia and dysregulation of insulin secretion impair postprandial glucose homeostasis and thus are central to the pathogenesis of type 2 diabetes. Impairment of the incretin effect, including reduced secretion of glucagon-like peptide-1 (GLP-1) (1,2), is implicated in the progression of pancreatic islet dysfunction in type 2 diabetic patients. Development and use of incretin-based therapeutics may, therefore, be an effective strategy to restore normal islet function by providing insulinotropic and glucagon-suppressive capabilities. In recent years, clinical studies have shown that replacement therapy with metabolically stable GLP-1 mimetics greatly improves management of hyperglycemia, nearly correcting blood glucose regulation for some patients. For example, treatment with either exenatide or liraglutide reduces fasting hyperglycemia and results in sustained lowering of glycosylated A1C (A1C) levels (3,4). In addition, these therapies often reduce body weight and improve several cardiovascular parameters (5,6). Unfortunately, both of these GLP-1 analogues are peptides requiring administration by subcutaneous injection.

The GLP-1 receptor is a member of the class B/II family of seven transmembrane G protein-coupled receptors (GPCRs) that include receptors for peptide hormones such as secretin, GLP-1, glucose-dependent insulinotropic polypeptide (GIP), glucagon, vasoactive intestinal peptide (VIP), corticotropin-releasing factor (CRF), calcitonin, and parathyroid hormone (PTH) (7–9). Historically, the discovery of nonpeptide agonists of these receptors that could enable development of orally active pharmaceuticals has been generally unsuccessful. To a large extent, this difficulty has been attributed to the mechanisms used by class B GPCRs to recognize ligands and induce signaling. Interaction of endogenous peptide hormones with their receptors typically involves large receptor:ligand binding sites and is often initiated by receptor NH_2-terminal ectodomains (ECDs). This extracellular structure interacts with COOH-terminal residues of cognate ligands and positions the NH_2-terminus of the ligand to interact with critical determinants in receptor transmembrane regions, thereby activating heterotrimeric G-proteins and subsequently adenylyl cyclase (10–12).

The recent reports describing GLP-1 receptor activation by a series of substituted quinoxalines (13–16) and a cyclobutane derivative (17) suggest that it may be possible to develop nonpeptide GLP-1 receptor agonists. Both scaffolds activate GLP-1 receptor signaling in heterologous

GLP-1 receptor-expressing cellular systems, and the quinoxalines clearly induce glucose-stimulated insulin secretion in vitro from rodent islets and ex vivo via perfused pancreas (13,16). We now report novel, low molecular weight pyrimidines that activate the GLP-1 receptor to induce glucose-dependent insulin secretion both in vitro and in vivo. These molecules may offer therapeutic advantage because of the ability of the compounds to act alone or in combination with GLP-1. Further, these agonists nearly restore insulin secretion to normal in human diabetic islets.

RESEARCH DESIGN AND METHODS

Measurement of luciferase. HEK293 cells stably expressing human GLP-1 receptor and a 3x-CRE luciferase reporter were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium-31053 (Invitrogen, Carlsbad, CA) supplemented with 0.5% FBS, 2 mmol/l L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 20 mmol/l HEPES. For compound testing, cells were plated into 96-well poly-D-lysine, white opaque microplates. Compounds were solubilized in DMSO, diluted in medium containing 0.1% BSA fraction V substituted for 0.5% FBS, and added to cells. After a 5-h incubation, cells were harvested in Steadylite plus lysis reagent (Perkin Elmer, Waltham, MA), and luciferase activity was measured according to the manufacturer's instructions using an Envision 2104 multilabel reader. Data are expressed as a percentage of maximum stimulation induced by the GLP-1 (7–36) amide peptide (Bachem, Torrance, CA).

Measurement of cAMP. HEK293 cells stably expressing human GLP-1 receptor were used for measurement of intracellular cAMP. Two hours before compound testing, cells were resuspended in the aforementioned medium and plated in 96-well half area, solid black microplates. Compounds were then added to cells as described above. Following a 20-min incubation, cells were assayed for cAMP using the cAMP dynamic 2 kit with homogenous time-resolved fluorescence technology (Cisbio, Bedford, MA). Fluorescence was measured according to the manufacturer's instructions using an Envision 2,104 Multilabel Reader. Data are expressed as nM cAMP of the final assay solution induced by the GLP-1 receptor agonists.

Animal care, IVGTT, and hyperglycemic clamp studies. Animals were maintained in accordance with the Institutional Animal Use and Care Committee of Eli Lilly and Company and the *Guide for the Use and Care of Laboratory Animals* by the National Institutes of Health. For animal treatment, compounds were solubilized in dosing solution containing 10% ethanol/solutol, 20% polyethylene glycol-400, and 70% PBS (pH 7.4).

The IVGTT studies were performed as previously described (18). Male SD rats were purchased from Harlan (Indianapolis, IN) and group-housed three per cage in polycarbonate cages with filter tops. Rats were maintained on a 12:12 h light-dark cycle (lights on at 6:00 A.M.) at 21°C and received 2014 Teklad Global diet (Harlan, Indianapolis) and deionized water ad libitum. Rats were fasted overnight and anesthetized with 60 mg/kg pentobarbital (Lundbeck, Deerfield, IL) for the duration of the experiment. For glucose and compound administration, a catheter with a diameter of 0.84 mm (Braintree Scientific, Braintree, IL) was inserted into the jugular vein. For rapid blood collection, a larger catheter with 1.02-mm diameter (Braintree Scientific, Braintree, IL) was inserted into the carotid artery. Blood was collected for glucose and insulin levels at time 0, 2, 4, 6, 10, and 20 min after intravenous administration of the compound which was immediately followed by an intravenous glucose bolus of 0.5 g/kg. Plasma levels of glucose were determined using a Hitachi 912 clinical chemistry analyzer (Roche, Indianapolis, IN). Plasma insulin was determined using an electrochemiluminescence assay (Meso Scale, Gaithersburg, MD).

For the hyperglycemic clamp, SD rats were purchased from Taconic with surgically implanted femoral artery and vein catheters. After an overnight fast, an initial blood sample was taken at time 0 from the arterial catheter, followed by compound administration via the venous catheter. Subsequently, glucose infusion via the venous catheter was initiated, and the infusion rates were varied to maintain a blood glucose concentration of ~250 mg/dl throughout the experiment. Blood samples were collected every 5 min for glucose and every 10 min for insulin measurements over the course of 1 h. Blood samples were collected in EDTA tubes. Glucose concentrations were determined using an Accucheck Advantage Glucometer (Roche Diagnostics, Indianapolis, IN), and insulin was measured using an electrochemiluminescence assay for rat insulin (Meso Scale, Gaithersburg, MD).

Islet isolation and insulin secretion. Male SD rats weighing ~300 g were killed by CO_2 asphyxiation and subsequent cervical dislocation. The common bile duct was cannulated with a 27-gauge needle, and the pancreas was
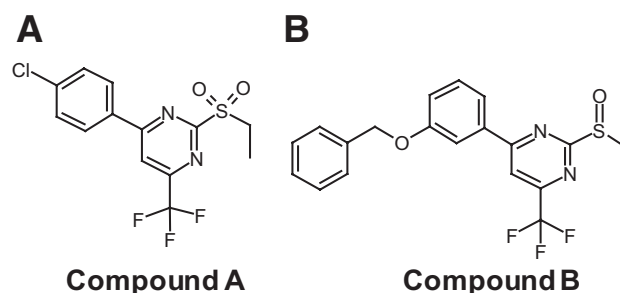

FIG. 1. The chemical structures of two pyrimidine-based GLP-1 receptor agonists, compound A (*A*) and compound B (*B*) are depicted.

distended with 10 ml of Hank's balanced salt solution buffer ([HBSS], Sigma, St. Louis, MO) containing 2% BSA (Applichem, Boca Raton, FL) and 1 mg/ml collagenase (Sigma, St. Louis, MO). Subsequently, the pancreas was removed and digested in HBSS at 37°C. Islets were purified on a Histopaque (Histopaque-1077: Histopaque-11991 mixture, Sigma, St. Louis, MO) gradient for 18 min at 750g. Islets were cultured overnight in RPMI-1640 medium containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Human islets from healthy donors were purchased from Prodo Laboratories (Irvine, CA). Human islets from a donor with type 2 diabetes (a 59-year-old male who died of a cerebrovascular stroke; islets were harvested on the day of his death, cultured overnight, and shipped the next day) were obtained from Asterand (Detroit, MI). Human islets were cultured overnight as described above. Islet perifusion experiments were carried out in a buffered, temperature controlled perifusion system as previously described (19,20). Briefly, groups of 20 islets were placed in reaction chambers and perifused with Earle's balanced salt solution (EBSS) supplemented with 10 mmol/l HEPES and 3.3 mmol/l glucose for 20–40 min. The glucose concentration in the perifusion buffer was then raised to 16.7 mmol/l to stimulate insulin secretion. Compounds were added to the perifusion buffer as indicated.

Insulin secretion in static incubation experiments was measured in EBSS (Invitrogen, Carlsbad, CA). Islets were incubated in EBSS containing 2.8 mmol/l glucose at 37°C for 30 min. Groups of three size-matched islets were then hand picked and incubated in 0.3 ml of EBSS containing the indicated amount of glucose and compounds at 37°C for 90 min. Subsequent to the perifusion and static incubation experiments, supernatants and perifusates were collected and stored at −20°C until assayed for insulin using a commercially available electrochemiluminescence assay (Meso Scale, Gaithersburg, MD).

Statistics. In vivo and islet data are represented as mean ± SEM and were compared using ANOVA followed by Dunnett test. Repeated measures analysis of variance was used to assess the statistical significance between time courses. The null hypothesis was rejected at $P < 0.05$.

RESULTS

Identification of low molecular weight GLP-1 receptor agonists. Since identification of small molecule activators of class B GPCRs remains largely elusive, we surmised that assessment of additional chemical space associated with several literature reports of GLP-1 receptor agonists (13,17,21–23) would provide a more rational group of molecules for evaluation versus high throughput screening. Accordingly, a series of three-dimensional pharmacophore models was created, leading to the identification of a small (~2000 compound) library gleaned from a variety of internal and commercial sources. This library was designed to assess key hypotheses associated with novel chemical space aiming to identify new GLP-1 receptor ligands. Compound A (Fig. 1*A*) was discovered via screening of this library using HEK293 cells stably cotransfected with the human GLP-1 receptor (NM_002062) and a 3X-cAMP response element (CRE) luciferase reporter. Compound A, which is commercially available (CAS registry number: 870083-94-6), increased luciferase in GLP-1 receptor HEK293 cells, but was inactive in parent HEK293 cells. Compound A displayed an EC_{50} value of 1.6 μmol/l
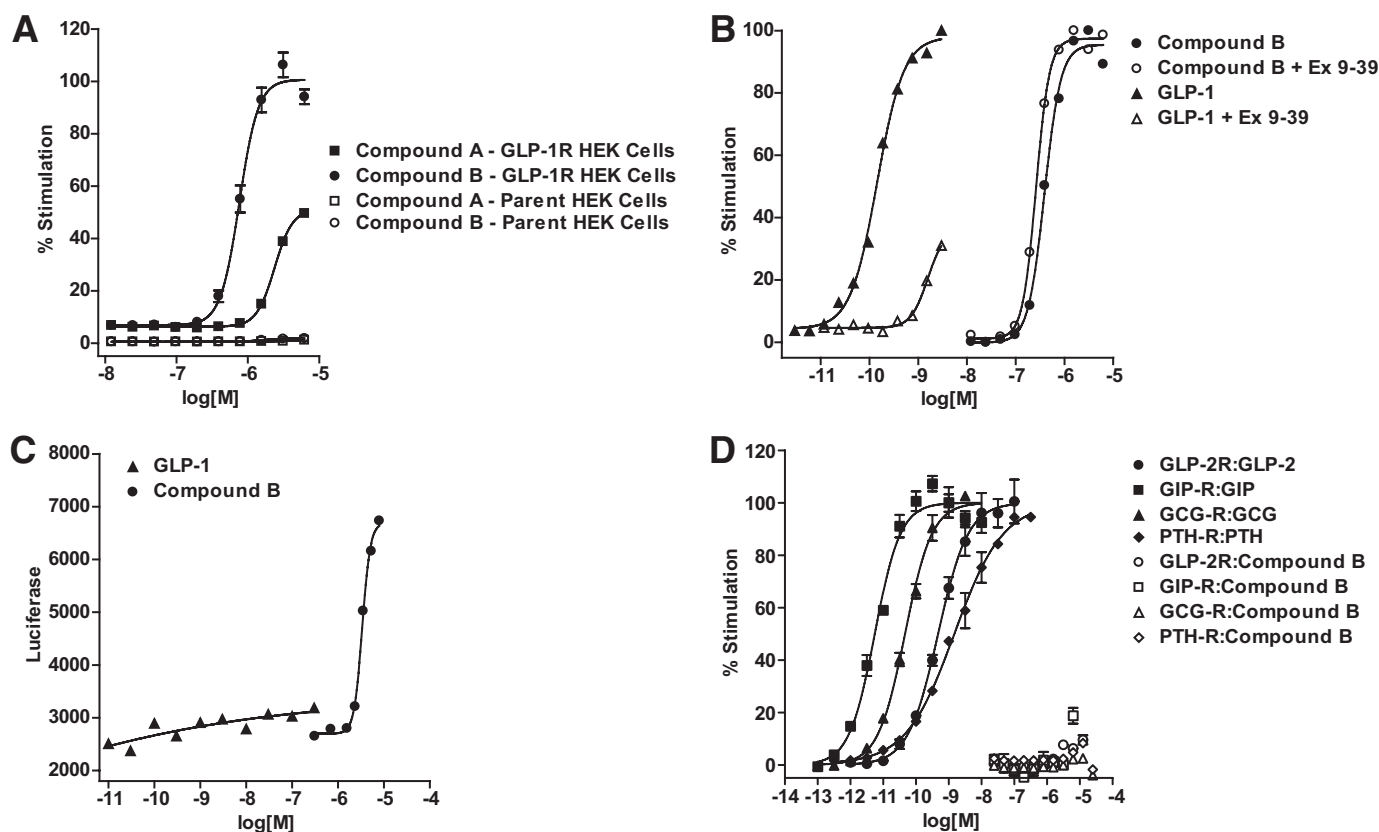
FIG. 2. Low molecular weight GLP-1 receptor agonists activate GLP-1 receptor signaling. *A*: Compound A and compound B induced GLP-1 receptor-mediated signaling in HEK293 cells coexpressing the human GLP-1 receptor and a 3x-cAMP response element-luciferase reporter, but were not active in cells lacking the GLP-1 receptor. In the human GLP-1 receptor HEK293 cells, the EC_{50} values for compounds A and B were 1.6 $\mu mol/l$ and 0.66 $\mu mol/l$, respectively; data are presented as percentages of stimulation of a maximum concentration of human GLP-1. *B*: The competitive GLP-1 receptor peptide antagonist exendin (9-39) blunted GLP-1 peptide activity but did not reduce compound B-induced signaling. *C*: Compound B was active in HEK293 cells expressing a modified form of the GLP-1 receptor lacking the NH_2-terminal ECD (Δ–ECD-GLP-1 receptor); the native GLP-1 peptide had no effect in cells expressing the Δ–ECD-GLP-1 receptor. *D*: Compound B was not active in HEK293 cells expressing the glucagon-like peptide-2 receptor (GLP-2R), glucose-dependent insulinotropic polypeptide receptor (GIP-R), glucagon receptor (GCG-R), or parathyroid hormone receptor (PTH-R).

with 46% efficacy relative to maximum stimulation by GLP-1 (Fig. 2*A*). Although this compound represented a viable starting point with moderate in vitro activity and some insulinotropic capability in ex vivo rat islet assays, structural modifications were introduced to improve potency to warrant in vivo studies. Through examination of the various regions of the molecule, Compound B (Fig. 1*B*) with a benzyloxy substituent introduced onto the phenyl ring of compound A in the meta position, along with deletion of the chlorine atom and a change of the oxidation state of the sulfur from sulfone to sulfoxide, resulted in an improved EC_{50} value of 0.66 $\mu mol/l$ and 99% efficacy (similar data were obtained using the rat GLP-1 receptor; $EC_{50} = 0.755$ $\mu mol/l$, 85% efficacy of maximum GLP-1). Neither compound A nor compound B were active in cells expressing the GLP-2, GIP, PTH, or glucagon receptors (Fig. 2*D* and data not shown).

Key structural differences between the pyrimidine series and the quinoxalines reported by Knudsen et al. (13) are evident. The pyrimidines reported here require both the trifluoromethyl and sulfonyl groups for high activity and an electron deficient heterocycle, whereas the quinoxaline-based GLP-1 receptor agonists require one or the other of these functional groups and a more electron-rich ring system. The optimized quinoxaline contains an additional electron donating t-butylamine function, providing an even more electron-rich heterocyclic ring and an addi-

tional hydrogen bond donor, not seen with these pyrimidines, whose H bond characteristics are considerably different both in nature and position. Finally, although not to the extent reported for the quinoxalines (13), it should be noted that high concentrations of compound A or compound B display lower signaling in the in vitro assays.

Compound B also showed activity in rat and human islets (Figs. 3*B* and 7*A–C*, respectively), enabling in vivo profiling of the compound. Additional details of the structure-activity relationship will be published in due course. To further evaluate compound B, assays were performed using the GLP-1 receptor peptide antagonist, exendin 9-39 (exendin-4$_{9-39}$) (24,25). This competitive antagonist binds the GLP-1 receptor ECD, but lacks the amino acids needed for interaction with extracellular loop regions to induce intracellular signaling (25,26). Treatment of GLP-1 receptor expressing HEK293 cells with exendin 9-39 blunted GLP-1 activity; however, the antagonist had no inhibitory effect on compound B-induced GLP-1 receptor signaling (Fig. 2*B*). These results suggest compound B activates the GLP-1 receptor via a different mechanism than native GLP-1. Consistent with these findings, compound B was unable to displace [^{125}I] GLP-1 binding to cell membranes expressing the human GLP-1 receptor (data not shown). Additional in vitro studies were performed to further evaluate the mechanistic differences between GLP-1 and compound B using a modified form of the GLP-1 receptor
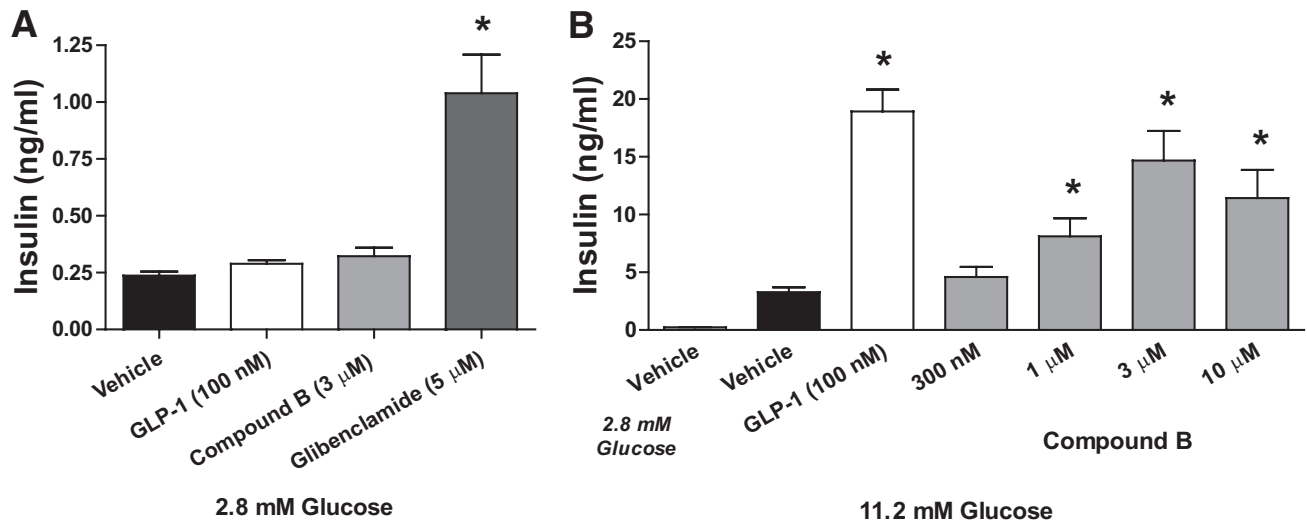
FIG. 3. Compound B increases glucose-dependent insulin secretion from SD rat islets. *A*: Insulin concentrations from static cultures of SD rat islets incubated in media containing low glucose (2.8 mmol/l) and either GLP-1 (100 nmol/l), compound B (3 μmol/l), or the sulfonylurea glibenclamide (5 μmol/l). *B*: Insulin concentrations from static cultures of SD rat islets incubated in media containing high glucose (11.2 mmol/l) and either GLP-1 (100 nmol/l) or various concentrations of compound B (0.3–10 μmol/l). All islet treatments were performed for 90 min. Results are expressed as mean ± SEM, *$P < 0.05$.

lacking the NH_2-terminal ECD (deletion of amino acids 1–138; referred to as Δ–ECD-GLP-1 receptor). In line with the established requirement of the ECD for GLP-1 binding and receptor activation, GLP-1 (tested at concentrations as high as 300 nmol/l) was not active in cells expressing the Δ–ECD-GLP-1 receptor (Fig. 2*C*); however, compound B did show activation of the truncated receptor (Fig. 2*C*), indicating the ECD is not required to induce GLP-1 receptor signaling by this small molecule. Based on these results, compound B likely interacts with an allosteric site proximal to or within the GLP-1 receptor transmembrane domains. A similar hypothesis was proposed previously for the quinoxaline series of GLP-1 receptor agonists (13,16).

Glucose-dependent insulin secretion in SD rat islets by a GLP-1 receptor low molecular weight agonist. GLP-1 is a glucose-dependent insulinotropic peptide (27,28). Accordingly, bona fide nonpeptide GLP-1 receptor agonist compounds would be predicted to act as insulin secretagogues only in conditions of elevated glucose concentrations. To evaluate these properties, static incubations of pancreatic islets isolated from SD rats were used. In low glucose (2.8 mmol/l), neither GLP-1 nor compound B increased the concentration of insulin secreted into the media after a 90-min incubation. In these conditions, however, treatment of islets with the sulfonylurea glibenclamide (glyburide) caused a fourfold increase in insulin levels (Fig. 3*A*). In islet experiments using high glucose concentrations (11.2 mmol/l), GLP-1 demonstrated insulinotropic activity, and compound B caused a robust, concentration-dependent increase in insulin secretion (Fig. 3*B*). The ability of compound B to specifically induce cAMP-mediated signaling in GLP-1 expressing cells, in combination with its glucose-dependent insulinotropic activity, supports the conclusion that compound B is a GLP-1 receptor agonist.

GLP-1 and compound B have additive mechanisms. Development of a low molecular weight GLP-1 receptor agonist that is efficacious in combination with endogenous GLP-1 or therapeutic GLP-1 mimetics may offer advantages such as improved efficacy and flexibility with com-

bination treatment regimens for type 2 diabetes. Data on compound B indicate this molecule is a GLP-1 receptor agonist that does not compete with native GLP-1 and likely interacts with an allosteric site to activate the GLP-1 receptor (see above). Therefore, experiments were undertaken to evaluate the activity of compound B in the presence of the GLP-1 peptide. For these studies, a cAMP assay using GLP-1 receptor expressing HEK293 cells and an insulin secretion assay in cultured pancreatic islets isolated from SD rats were used. Coincubation of compound B with various concentrations of GLP-1 induced both cAMP production (Fig. 4*A*) and insulin secretion (Fig. 4*B*) in an additive manner. The observation that compound B potentiates GLP-1 action supports exploring further optimization of these pyrimidine-based compounds as a novel treatment for type 2 diabetes.

Stimulation of insulin secretion in SD rats. To explore the in vivo insulinotropic effects of compound B, glucose-stimulated insulin secretion was measured in compound-treated SD rats undergoing an IVGTT and a hyperglycemic clamp. In both models, compound B induced insulin secretion over the time course of the glucose challenge (Figs. 5 and 6). In the IVGTT, fasted-SD rats implanted with dual cannulas were anesthetized and administered via a jugular vein cannula either vehicle, GLP-1 (dosed at 10 μg/kg), or compound B (dosed at 10 mg/kg) immediately followed by a bolus of glucose (administered at 0.5 g/kg). Blood samples were drawn from the carotid artery cannula for measurement of glucose (Fig. 5*A*) and insulin (Fig. 5*B*) levels at times 0, 2, 4, 6, 10, and 20 min. Both GLP-1 and compound B displayed insulin secretagogue activity in this experimental model (Fig. 5*B*). Insulin output is reported as incremental area under the curve (AUC) of plasma insulin concentrations during the 20-min time course. Compared with vehicle, animals dosed with compound B exhibited a threefold elevation in insulin AUC, whereas GLP-1 treatment increased insulin by fourfold (Fig. 5*C*). Although the IVGTT model demonstrated significant increases in circulating levels of insulin, robust changes in plasma glucose were not induced by compound B treatment. This result is likely caused by anesthesia-
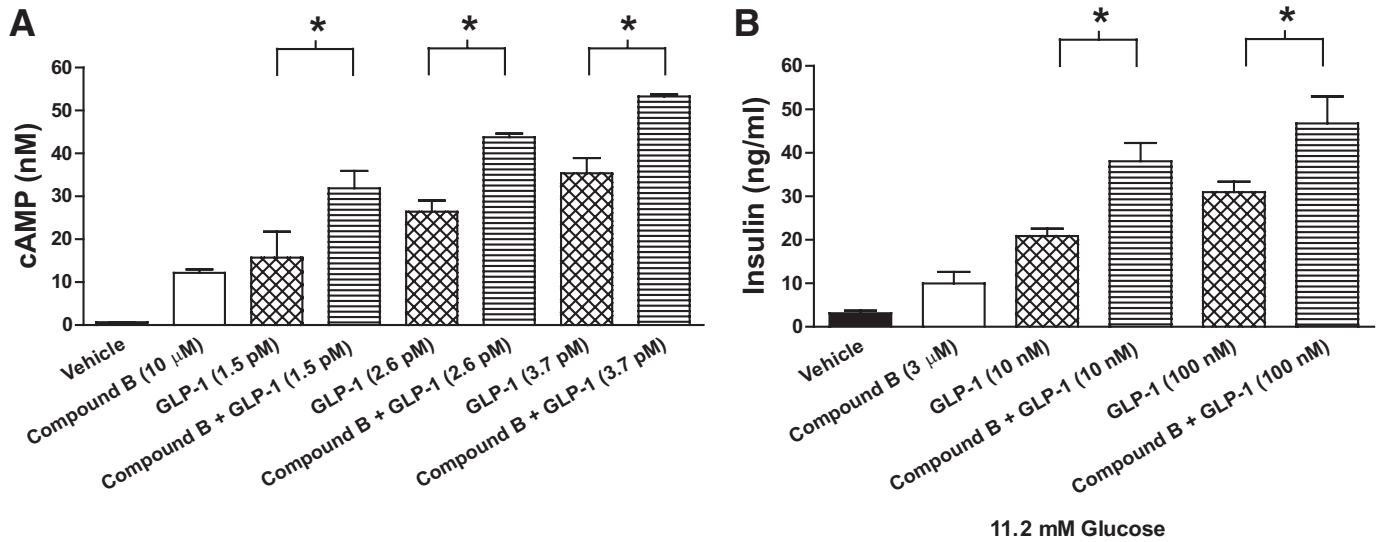
FIG. 4. GLP-1 and compound B increase GLP-1 receptor signaling in an additive manner. *A*: Combination treatment of the GLP-1 peptide and compound B increases the intracellular concentration of cAMP in HEK293 cells expressing the human GLP-1 receptor. Treatment times for the cAMP assays were 20 min. *B*: Insulin concentrations from static cultures of SD rat islets incubated in media containing high glucose (11.2 mmol/l) and compound B plus increasing concentrations of GLP-1. Islet treatments were performed for 90 min. Results are expressed as mean ± SEM, *$P < 0.05$.

induced insulin resistance in this model in which typically only very potent insulinotropic molecules, such as the GLP-1 peptide, induce a statistically significant change in glucose (unpublished data). Therefore, we further assessed the glucodynamic effects of compound B in the more sensitive (nonanesthetized SD rats) hyperglycemic clamp model that enables evaluation of changes in insulin and glucose over a longer time course. Compound B (dosed at 10 mg/kg) or vehicle was administered to animals as a single intravenous dose at the beginning of the experiment. Intravenous infusion of glucose maintained blood glucose concentrations at ~250 mg/dl (Fig. 6*A*). Compound B-treated animals required ~20% higher glucose infusion rates to maintain this glucose level (Fig. 6*B*). Measurement of serum insulin concentrations over

the next 60 min demonstrated higher insulin levels in compound B-treated animals compared with vehicle controls (Fig. 6*C*), with the $AUC_{20-60\ min}$ calculation showing an 83% increase (Fig. 6*D*). The insulin secretory response induced by compound B observed using both the IVGTT and hyperglycemic clamp methods is consistent with pharmacology demonstrated by peptide GLP-1 receptor agonists (29) and supports the results obtained from the islet assays showing that compound B is a glucose-dependent insulin secretagogue. Because oral dosing of compound B failed to show insulinotropic effects similar to those achieved by intravenous administration, additional optimization of compound B is required to improve the pharmacokinetic properties of the molecule to enable longer-term in vivo testing that will allow evaluation of
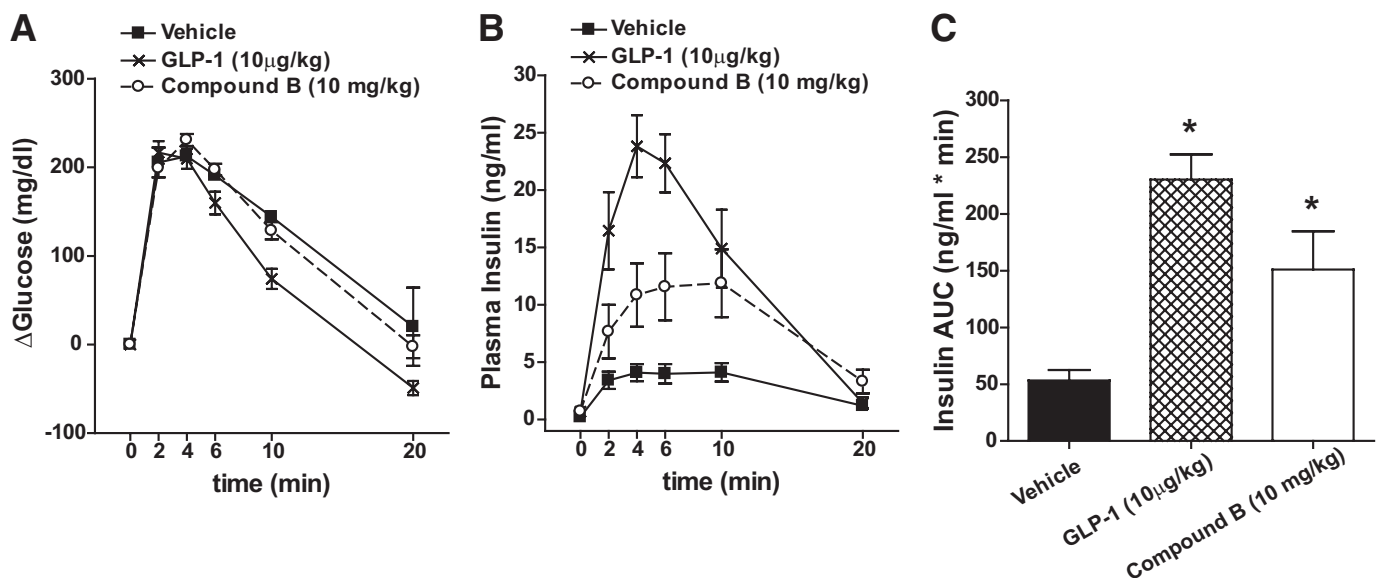

FIG. 5. Compound B increases plasma insulin levels in the SD rat IVGTT model. Time course of plasma (*A*) glucose and (*B*) insulin concentrations in fasted, anesthetized animals treated with either vehicle (■), GLP-1 (×) (10 µg/kg), or compound B (○) (dosed at 10 mg/kg) immediately before intravenous administration of a glucose bolus (0.5 g/kg). Results are expressed as mean ± SEM. *C*: AUC of the insulin excursion curves for vehicle versus the GLP-1 or compound B treatment groups, *$P < 0.05$.

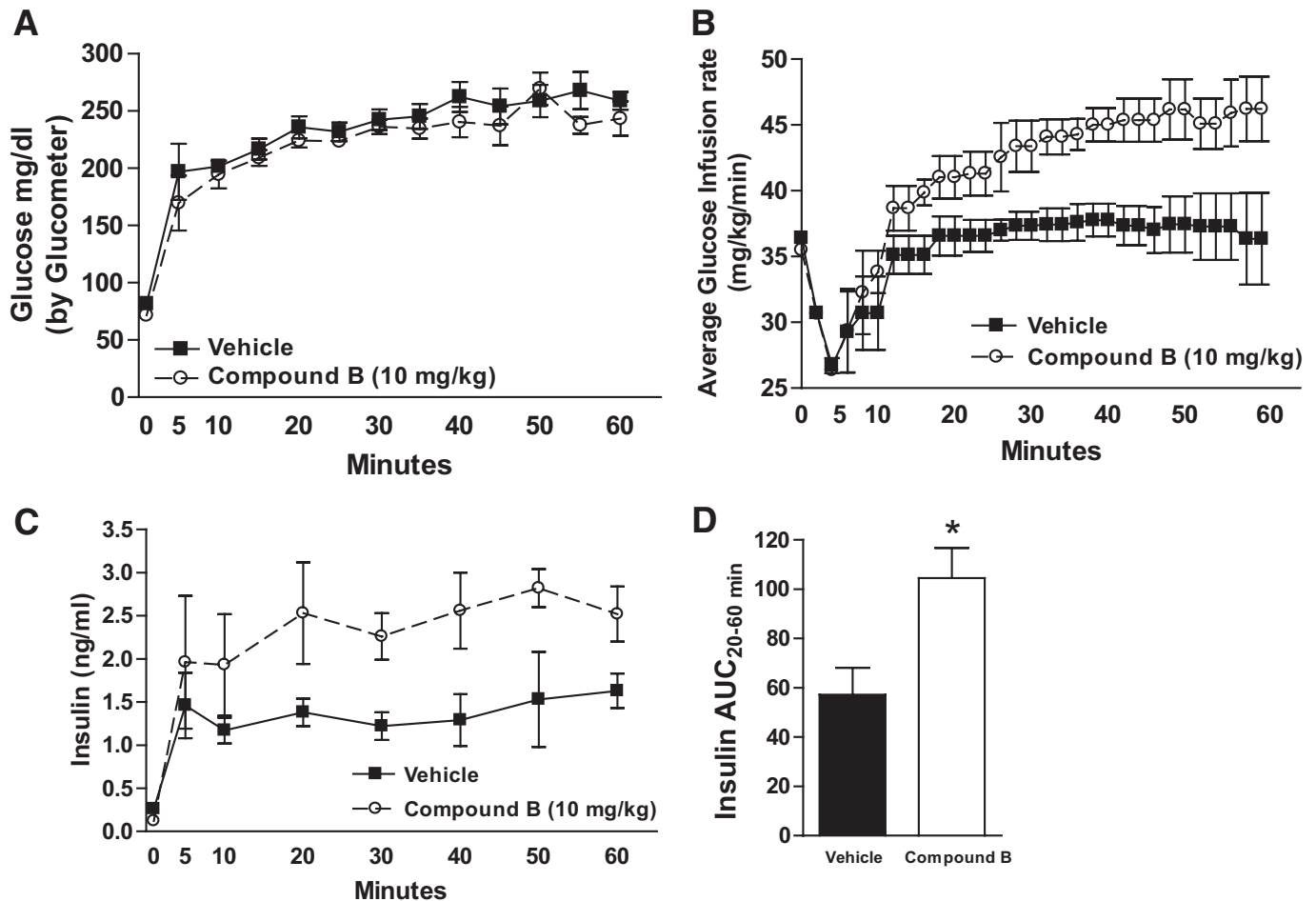FIG. 6. Compound B increases plasma insulin levels in the SD rat hyperglycemic clamp model. *A*: Intravenous dosing with vehicle (■) or 10 mg/kg compound B (○) occurred immediately before intravenous infusion of glucose. Glucose levels measured from venous blood every 5 min are shown. Results are expressed as mean ± SEM. *B*: Blood glucose concentrations of ~250 mg/dl were maintained throughout the experiment by varying the glucose infusion rates. *C*: Time course of plasma insulin concentrations in fasted animals treated with either vehicle (■) or compound B (○). Results are expressed as mean ± SEM. *D*: $AUC_{20-60\ min}$ of the insulin secretion for vehicle (*filled bar*) versus compound B-treated (*open bar*) animals, *$P < 0.05$.

efficacy, including effects on food intake and body weight, and toxicity.

Stimulation of insulin secretion from normal and diabetic human islets by compound B. To increase the probability of successfully developing novel glucose-dependent insulin secretagogues for the treatment of type 2 diabetes, ex vivo insulin secretion assays using human pancreatic islets are sometimes conducted. Similar to the insulinotropic effects in rodent islets, compound B stimulated glucose-dependent insulin release from human islets. Static cultures of islets from a healthy donor incubated with various concentrations of compound B showed increased insulin levels (Fig. 7*A*). Further, we established a perifusion system for compound testing using human islets. In this system, reaction chambers containing islets from a healthy individual were perifused first with media containing low glucose (3.3 mmol/l) followed by perifusate containing high glucose (16.7 mmol/l). For islets treated with media containing compound B, insulin levels were higher compared with controls over the time course (Fig. 7*B*) with total insulin AUC increased by twofold. Similar experiments were performed with islets harvested from a donor who had suffered from type 2 diabetes. In these assays, treatment with compound B again caused an increase in insulin release into the media (Fig. 7*C*; insulin

AUC increased by twofold). Comparison of results from the perifusion studies indicates that the increase in insulin secretion from diabetic islets achieved by compound B approached 50% of that observed with high glucose treatment of islets from the healthy donor. The observed insulin secretory properties of compound B in human islets from both normal and diabetic individuals demonstrates that application of the compound selection and preclinical testing scheme used here can result in the discovery of novel GLP-1 receptor low molecular weight agonists.

DISCUSSION

Class B GPCRs typically are activated by endocrine peptide hormones such as GLP-1, GIP, glucagon, PTH, VIP, secretin, CRF, and calcitonin (7–9). Accordingly, class B GPCRs represent some of the most attractive targets for therapeutic intervention, and this is borne out by regulatory approval of several large molecule biotherapeutics, including PTH (teriparatide, Forteo) for osteoporosis, exendin-4 (exenatide, Byetta) and liraglutide (Victoza) for type 2 diabetes, and calcitonin (calcitonin-salmon, Miacalcin) for Paget disease, hypercalcemia, and osteoporosis (12). Unfortunately, there has been much difficulty identi-
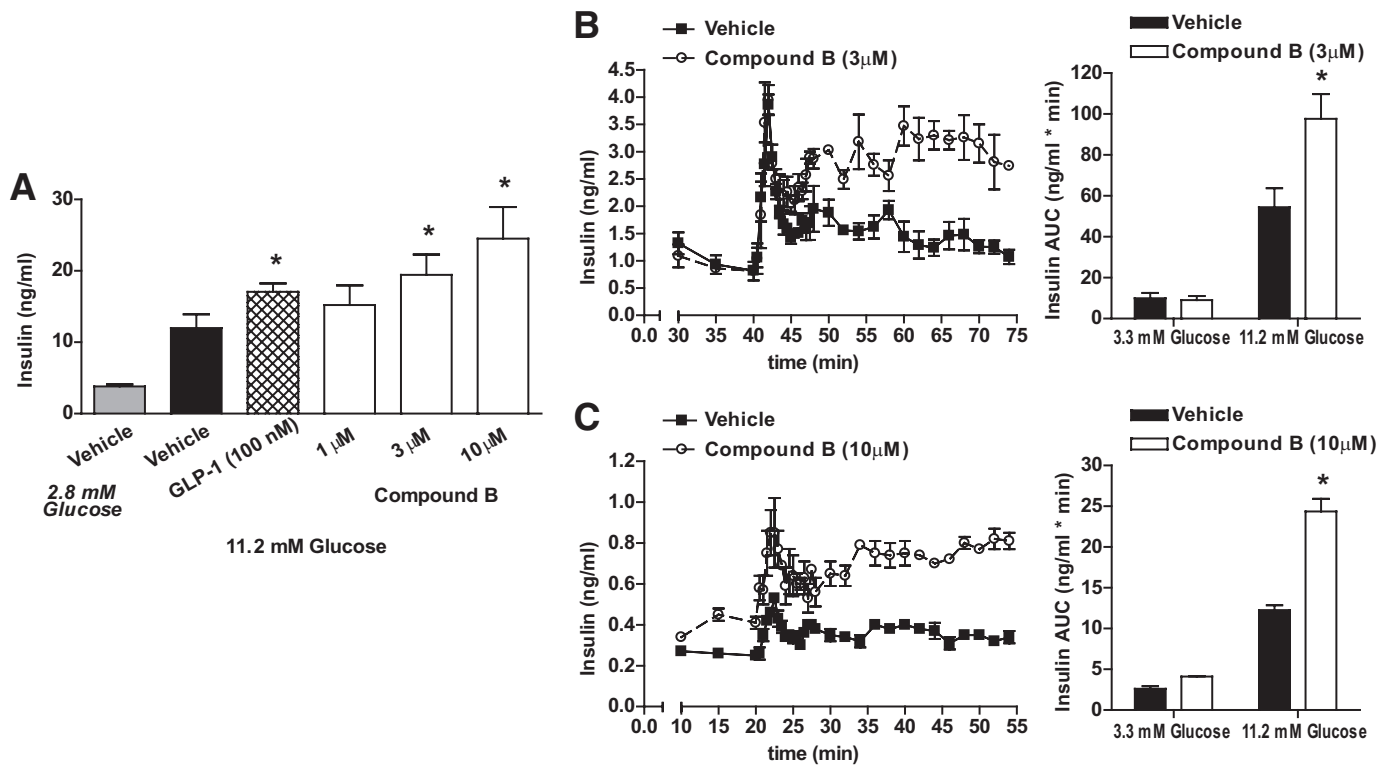
FIG. 7. Compound B enhances insulin secretion in normal and diabetic human islets. *A*: Insulin concentrations from static cultures of normal human islets incubated in media containing high glucose (11.2 mmol/l) and either GLP-1 (100 nmol/l) or various concentrations of compound B (1–10 μmol/l). The treatments were performed for 90 min, and results are expressed as mean ± SEM, *$P < 0.05$. In perifusion experiments, insulin concentrations from reaction chambers containing 20 human islets from (*B*) normal or (*C*) diabetic individuals perifused with media containing either vehicle (■) or compound B (○) (3 or 10 μmol/l) in low glucose (3.3 mmol/l) for 40 min followed by high glucose (16.7 mmol/l) for an additional 35 min. AUC of the insulin excursion for vehicle versus compound B-treated (*B*) normal and (*C*) diabetic human islets, *$P < 0.05$.

fying nonpeptide, low molecular weight organic molecules that activate class B GPCRs. The inability to modulate the activity of class B GPCRs by traditional small molecule approaches likely is caused by the unique structural architecture and activation mechanism used by these GPCRs. Ligands to these receptors typically are 30–40 amino acids in size and bind via a two-step mechanism (10–12). The initial binding event is an interaction between the COOH-terminal amino acids of ligand peptides and the ECD of class B GPCRs. The second phase of receptor activation is the interaction of the NH_2-terminal residues of the ligand with transmembrane domains of the receptor to induce an activated conformation (10–12). It appears that the main functions of the GLP-1 receptor ECD are to confer peptide selectivity and act as a high affinity binding site for the peptide ligand. This unique mechanism of activation of class B GPCRs likely has hindered conventional screening approaches for this target.

Recently, substantial progress was made in the generation of antagonists for several class B GPCRs, including the glucagon, PTH, CGRP, and CRF receptors (12,30). Small molecule antagonists of class B GPCRs have been characterized as either orthosteric antagonists or negative allosteric modulators. In contrast, minimal success has been achieved in the discovery of class B small molecule agonists; isolated examples include the discovery of small molecule agonists of the PTH receptor (31), the calcitonin receptor (32,33), and the CRF receptor (34). With the exception of low-nanomolar pyrazolopyridine calcitonin receptor partial agonists (33), class B GPCR agonist molecules have micromolar potencies. Much activity has been expended searching for GLP-1 receptor small molecule agonists, and up to this point, only two bona fide agonists have been described: low molecular weight substituted quinoxalines (13–16) and the large cyclobutane derivative, Boc-5 (17). Boc-5 is described as an orthosteric agonist, but it likely does not have suitable physiochemical properties to be developed as an orally available GLP-1 receptor agonist. The quinoxaline compounds exhibit some chemical similarity to the pyrimidines described in this report. The mechanism of action of the substituted quinoxalines appears to be analogous to compound B. Both molecules exhibit agonist and positive allosteric modulator activity at the GLP-1 receptor, and neither competes with GLP-1 for receptor binding (13). We probed the structural determinants of compound B interaction with the GLP-1 receptor using a functional GLP-1 receptor variant lacking the ECD (Δ–ECD-GLP-1 receptor). Interestingly, we observed that compound B action is independent of the GLP-1 receptor ECD, implying that compound B probably acts proximal to the transmembrane domains of the GLP-1 receptor, similar to the mechanism used by canonical small molecule agonists and allosteric modulators for class A and class C GPCRs (35). Further determinants of compound B interaction with the GLP-1 receptor have not been fully elucidated. Recent studies have defined the structure of the GLP-1 peptide-bound ECD of the GLP-1 receptor (36,37). However, there is little additional structural data to guide targeted design of low molecular weight agonists for the GLP-1 receptor, especially with regard to understanding and possibly exploiting critical transmembrane domains to modulate signaling. Computational approaches have been used to generate hypotheses

around the structural determinants of peptide and small molecule binding to the juxtamembrane regions of the GLP-1 receptor (38). The quinoxaline series of GLP-1 receptor agonists is predicted to bind in a pocket formed by the first and second extracellular loops; however, these predictions need to be confirmed by mutagenesis studies (38).

The discovery of pyrimidine-based GLP-1 receptor agonists that induce glucose-dependent insulin secretion in vitro and in vivo demonstrates that modulating class B GPCRs with low molecular weight compounds is technically feasible. However, further work is needed to improve potency and optimize pharmacokinetic properties of these molecules to enable clinical development. Future studies will also focus on characterizing the allosteric binding site and ligand-mediated conformational changes induced by compound B to potentiate receptor signaling. A better understanding of the pocket and mechanism of activation will facilitate molecular modeling strategies to develop more potent small molecule GLP-1 receptor agonists.

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