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Engineering and characterization of the long-acting glucagon-like peptide-1 analogue LY2189265, an Fc fusion protein

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Abstract

Background Glucagon-like peptide-1 (GLP-1) receptor agonists are novel agents for type 2 diabetes treatment, offering glucose-dependent insulinotropic effects, reduced glucagonemia and a neutral bodyweight or weight-reducing profile. However, a short half-life (minutes), secondary to rapid inactivation by dipeptidyl peptidase-IV (DPP-IV) and excretion, limits the therapeutic potential of the native GLP-1 hormone. Recently, the GLP-1 receptor agonist exenatide injected subcutaneously twice daily established a novel therapy class. Developing long-acting and efficacious GLP-1 analogues represents a pivotal research goal. We developed a GLP-1 immunoglobulin G (IgG4) Fc fusion protein (LY2189265) with extended pharmacokinetics and activity.

Methods *In vitro* and *in vivo* activity of LY2189265 was characterized in rodent and primate cell systems and animal models.

Results LY2189265 retained full receptor activity *in vitro* and elicited insulinotropic activity in islets similar to native peptide. Half-life in rats and cynomolgus monkeys was 1.5–2 days, and serum immunoreactivity representing active compound persisted >6 days. In rats, LY2189265 enhanced insulin responses during graded glucose infusion 24 h after one dose. LY2189265 increased glucose tolerance in diabetic mice after one dose and lowered weight and delayed hyperglycaemia when administered twice weekly for 4 weeks. In monkeys, LY2189265 significantly increased glucose-dependent insulin secretion for up to a week after one dose, retained efficacy when administered subchronically (once weekly for 4 weeks) and was well tolerated.

Conclusions LY2189265 retains the effects of GLP-1 with increased half-life and efficacy, supporting further evaluation as a once-weekly treatment of type 2 diabetes.

Keywords glucagon-like peptide 1; Fc fusion protein; type 2 diabetes; incretin mimetic

Introduction

The progressive nature of β-cell dysfunction in type 2 diabetes often renders treatment inadequate over time [1], necessitating multiple oral agents and/or insulin. Treatment side effects, such as weight gain and hypoglycaemia [2],

frequently present barriers to physician administration and patient adherence, with resulting inadequate glycaemic control [3–7]. Preserving or promoting β-cell function with minimal hypoglycaemia or weight gain represents a pivotal treatment objective.

The natural incretin hormone glucagon-like peptide-1 (GLP-1) supports glucose homeostasis by enhancing glucose-dependent insulin secretion from β-cells and suppressing inappropriately elevated postprandial glucagon secretion from α-cells. In addition, GLP-1 has been demonstrated to reduce appetite and food intake and inhibit gastric emptying, which may facilitate weight management [8,9]. Rapid enzymatic inactivation by dipeptidyl peptidase-IV (DPP-IV) and excretion of GLP-1 limit its therapeutic potential [10,11]; thus analogues and long-acting formulations of analogues [12] have been developed. For example, exenatide (Byetta; Amylin Pharmaceuticals Inc., San Diego, CA, USA and Eli Lilly and Company, Indianapolis, IN, USA), approved by the US Food and Drug Administration, is a GLP-1 mimetic demonstrating many of the beneficial effects of GLP-1 in daily therapeutic use. In addition, liraglutide (Novo Nordisk, Bagsværd, Denmark, approved on January 25, 2010 by the Food and Drug Administration and will be marketed under the proprietary name Victoza®, USA) is a GLP-1 analogue administered once-daily subcutaneously [13].

Fusing GLP-1 to a larger 'carrier' moiety, hence slowing its *in vivo* clearance, might also enhance pharmacokinetics. In pre-clinical studies, linking GLP-1 to albumin substantially prolonged the half-life (to ~10–12 h) [14,15]. When GLP-1 was fused to the Fc domain of immunoglobulin, the plasma half-life of GLP-1 was substantially prolonged (~30 h) [16]. We describe the engineering and characterization of LY2189265, a DPP-IV-protected GLP-1(7–37) analogue fused to a modified immunoglobulin G (IgG4) Fc fragment; the fusion protein maintains the insulinotropic activity of the native peptide with substantially improved plasma half-life, decreased clearance and a flat profile with no burst effect, potentially allowing once-weekly dosing. The potential safety of LY2189265 was enhanced by engineering to reduce Fcγ receptor binding and immunogenic potential.

Materials and methods

Expression and purification of GLP-1-Fc

Human embryonic kidney (HEK) 293-EBNA cells were maintained in Dulbecco modified Eagle medium (DMEM)/Ham F-12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20 mM HEPES (Invitrogen), 5 µg/mL nucellin (Eli Lilly and Company), 0.4 µg/mL tropolone (Sigma–Aldrich, St Louis, MO, USA), 0.075% (w/v) F68 (Invitrogen), and 50 µg/mL geneticin (Sigma–Aldrich) (37 °C; 5–8% CO_2). DNA was added to FuGene6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) in OptiMEM (Gibco/BRL, Gaithersburg, MD, USA) and incubated (15 min, 37 °C). Concentrated expression media was loaded directly onto a Hi-Trap Protein A column (GE Healthcare, Piscataway, NJ, USA), equilibrated in phosphate-buffered saline (PBS; 3 mL/min flow rate) and washed. Pooled fractions of bound GLP-1-Fc (pH 7.4), eluted with a step gradient of 100% 50 mM Na-citrate (pH 2.2), were concentrated and loaded onto a Superdex 200 (26/60, GE Healthcare) column (PBS-equilibrated; 3 mL/min flow rate). The GLP-1-Fc fractions were characterized by SDS-PAGE and mass spectrometry, sterile-filtered (0.22 µm), assessed for concentration (absorption at 280 nm) and stored at −20 °C.

Evaluation of T-cell epitopes

Potential T-cell epitopes were identified *in silico* using EpiMatrix, a matrix-based algorithm for T-cell epitope mapping [17]. The N-terminal 64 amino acids of GLP-1-Fc were parsed into 9-mer frames overlapping by eight amino acids. Binding was then predicted to eight major histocompatibility complex class II alleles representative of human populations (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*0801, DRB1*1101, DRB1*1301 and DRB1*1501) and reported as *Z*-scores uniformly scaled for direct comparison across alleles. All scores in the top 5% (*Z*-score $\geq$1.64) were considered 'hits', and scores in the top 1% (*Z*-score $\geq$2.32) were considered highly likely for major histocompatibility complex binding. An overall EpiMatrix score for immunogenicity was calculated by determining the deviation from the number of potential T-cell epitopes predicted for random-sequence pseudoproteins per 1000 amino acid assessments. EpiMatrix *Z*-scores $\geq$1.64 (303/5664 total assessments; ~5%) were investigated further.

Antibody-dependent, cell-mediated cytotoxicity assays

Jurkat FcγIII cells (V) were created by cotransducing Jurkat T cells with the murine Maloney leukemia virus (MMLV)-based vector pLHCX (Clontech, Mountain View, CA, USA) expressing the 158V allotype of human FcγRIIIa with a hygromycin resistance cassette and the MMLV vector pLNX2, expressing human FcεRI with a neomycin resistance cassette. Dual-resistant colonies were screened by fluorescence-activated cell sorting for high FcγRIIIa expression and confirmed by anti-FcR crosslinking-induced interleukin 2 release. The reporter line Jurkat FcγRIII (V)_NFAT_Luc was created by co-electroporating the luciferase reporter under the control of the NFAT promoter (Stratagene, La Jolla, CA, USA) and pPUR (Clontech) containing the puromycin resistance cassette. Puromycin-resistant colonies were screened by anti-FcR-induced luciferase expression.

Chinese hamster ovary-K1_GLPR1 cells were plated at 2×10^4 cells/well in Costar 96-well white luminescence

plates and incubated for 1 h at 37 °C, followed by incubation with different concentrations of GLP-1-Fc for 1 h. A total of 1×10^5 Jurkat FcγRIII (V)_NFAT_Luc cells were added to each well and incubated for an additional 5 h. Luciferase activity was assayed by incubation with Pierce Steady-Glo luminescence reagent (Thermo Fisher, Rockford, IL, USA) and analysed on a Molecular Devices LMaxII luminometer (Sunnyvale, CA, USA).

Reporter gene activity with β-luciferase

HEK 293 cells (<passage 5) expressing human GLP-1 receptor and a cyclic AMP (cAMP)-responsive CRE4-luciferase system were seeded (80 000 cells/well in 80 µL) and incubated overnight in DMEM/F12 (3 : 1) medium (Gibco; no. 93-0152-DK) containing 0.25% foetal bovine serum (FBS), 50 µg/mL gentamicin and 2 mM L-glutamine. Subsequently, GLP-1-Fc or Val8-GLP-1 (in 0.5% bovine serum albumin [BSA]) was added to result in final concentrations of 0.0003 nM to 3 nM (5 h, 37 °C in 5% CO_2). Plates were read after the addition of LucLite luciferase reagent (100 µL; Packard Bioscience, Groningen, the Netherlands) and mixing in a TriLux instrument (TRILUX GmbH & Co., Arnsberg, Germany).

Reporter gene activity with β-lactamase

HEK 293 cells expressing human GLP-1 receptor and a cAMP-responsive CRE-BLAM reporter system were seeded (20 000–40 000 cells/well in 100 µL DMEM plus 10% FBS) and incubated overnight at 37 °C. Medium was subsequently replaced with plasma-free DMEM (80 µL). One day later, serum-free DMEM (20 µL) plus 0.5% BSA containing the GLP-1 agonist was added. Half-maximal effective concentration values were determined from a dose–response curve (0.00003–30 nM dilutions). After incubation (5 h), 20 µL lactamase substrate (CCF2-AM; PanVera LLC, Madison, WI, USA) was added, and fluorescence was measured 1 h later (Cytofluor Plate Reader, Applied Biosystems Inc., Foster City, CA, USA).

Insulin secretion in rat islets

After common bile duct cannulation in male Sprague–Dawley rats (250–280 g), the pancreas was distended with Hank buffer (10 mL, containing 2% BSA and 1 mg/mL Sigma type V collagenase or 0.15 mg/mL liberase [Roche]). Subsequently, tissues were digested in Hank buffer at 37 °C for 10–12 min (or 19–21 min for liberase). Purified islets (Histopaque-1077 gradient [Sigma–Aldrich], 18 min at 750 *g*) were cultured overnight in RPMI-1640 medium (Invitrogen) containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin and preconditioned by starvation in Earle balanced salt solution (EBSS) supplemented with 0.1% BSA and 2.8 mM glucose. Subsequently, islets were incubated in EBSS (Invitrogen) supplemented with 0.1% BSA, 2.8 mM to 16.8 mM glucose, and increasing levels of LY2189265 (3–6 batches of 3–4 islets/condition) with or without addition of 1 µM exendin 9–39 (Ex[9–39]; Bachem Americas Inc., Torrance, CA, USA). Insulin was measured over 90 min in supernatant using the Meso Scale Insulin Assay (Meso Scale, Gaithersburg, MD, USA).

Insulin secretion in monkey islets

Two halved cynomolgus monkey pancreata (Covance Inc., Princeton, NJ, USA) distended with Hank buffer (containing 2% BSA and 1 mg/mL collagenase [Sigma–Aldrich]) were digested (37 °C) and purified on a discontinuous Histopaque-1077 gradient (750 *g* for 18 min). After overnight culture in RPMI-1640 medium (containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin [all Invitrogen]), islets were starved in EBSS containing 2.8 mM glucose (30 min; 37 °C), and batches of three islets were then incubated for 90 min in 0.3 mL EBSS containing 16.7 mM glucose and compounds, as indicated. Supernatants were frozen (−20 °C) until electrochemiluminescent insulin assay (Meso Scale). For all *in vitro* assays, molar concentrations of GLP-1-Fc were calculated by dividing the molecular weight of the fusion protein by two because of its homodimeric nature.

Animals

For all *in vivo* studies, animals were maintained in a controlled environment (20 ± 2 °C, 50–60% humidity, 12-h light–dark cycle, lights on at 6 : 00 AM) and fed a standard chow (Mice: Purina 5008, LabDiets, St Louis, MO, USA; Rats: Purina 5001 LabDiets; cynomolgus monkeys: Certified Global Primate Diet #2055C, Harlan Laboratories, Indianapolis, IN, USA). All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval of the Eli Lilly Research Laboratories and Covance Inc. Institutional Animal Care and Use Committees.

Blood glucose and insulin level measurement

Blood glucose levels were determined by Precision-G Blood Glucose Testing System (Abbott Diagnostics, Abbott Park, IL, USA), and insulin levels were determined by radioimmunoassay (Linco Diagnostics, St Charles, MO, USA).

Pharmacokinetics in Sprague–Dawley rats and cynomolgus monkeys

Adult male rats ($n = 3$/group) received a single subcutaneous (SC) dose of 0.1 mg/kg LY2189265, and blood was collected 1, 2, 4, and 6 days later. Monkeys ($n = 3$/group)

received a single SC dose of 0.1 mg/kg LY2189265, and blood (2 mL) was collected at 0 (pre-administration), 2, 4, 8, 12, 48, 72, 96, 192, 240, 288, and 336 h after administration. Plasma samples were stabilized with 10 µL DPP-IV inhibitor/mL (Millipore, St Charles, MO, USA), and immunoreactive GLP-1-Fc concentration was determined by enzyme-linked immunosorbent assay (ELISA) using antibodies recognizing the N-terminus of GLP-1-Fc (Eli Lilly and Company) and the Fc domain (mouse anti-human IgG4; Southern Biotech, Birmingham, AL, USA). Plasma samples were diluted with equal amounts of casein/PBS and incubated for 1.5 h. Secondary antibody (1 : 2000 in blocking buffer) was added for 1 h. Optical density (450–630 nm) of 3,3′,5,5′-tetramethylbenzidine development was determined, concentrations of GLP-1-Fc were calculated using a four-parameter algorithm, and standard curves were prepared for GLP-1-Fc in rat plasma. The ELISA assay range was approximately 0.9–80 ng/mL.

Graded glucose infusion in rats

Adult male Sprague–Dawley rats (420–460 g) with femoral artery and vein cannulation were acclimated to study boxes and subsequently treated with SC vehicle (saline; $n = 18$) or LY2189265 (0.3 nmol/kg [$n = 4$], 1 nmol/kg [$n = 3$], 3 nmol/kg [$n = 7$], or 30 nmol/kg [$n = 4$]). After 24 h, fasted rats (16 h) were infused with saline (20 min), followed by low-dose glucose (50 mg/kg/min, 30 min) and finally high-dose glucose (150 mg/kg/min, 30 min). Blood samples (250 µL) were collected at −20, −10, 0, 10, 20, 30, 40, 50, and 60 min. Statistical significance was evaluated using the paired Student's *t*-test (JMP 4.04 statistical software).

Graded glucose infusion in cynomolgus monkeys

Sedated and fasted (16–18 h) cynomolgus monkeys ($n = 6$) were infused with glucose immediately after SC administration of vehicle control (PBS) or LY2189265 (1.7 nmol/kg) and 1, 5, and 7 days later. Glucose solution (20% dextrose solution, 200 mg/mL, intravenous) was infused at 10 mg/kg/min (3.0 mL/kg/h) for 20 min and then at 25 mg/kg/min (7.5 mL/kg/h) for 20 min. Blood was collected at −10, 0, 10, 20, 30, and 40 min. In a separate experiment, monkeys ($n = 6$) receiving SC vehicle or LY2189265 (1.7 nmol/kg) once weekly for 4 weeks were evaluated using this graded glucose infusion paradigm 4 days after the last LY2189265 dose.

Subchronic dosing of diabetic db/db mice for 4 weeks

Five-week-old female diabetic db/db mice (C57BL/KsOlaHsd-*Lepr*db, Harlan Laboratories) were randomly grouped ($n = 10$/group) according to body weight, and LY2189265 (10 nmol/kg) was administered subcutaneously once weekly for 4 weeks. Blood glucose was measured in conscious mice just before dosing by tail clip at each weekly injection, except for the first week, when glucose was measured 1 h after administration. Fasted insulin levels were measured on day 0 and day 26 after an overnight fast.

Statistical analysis

Unless otherwise noted, groups were compared by one-way analysis of variance followed by Dunnett test with JMP 5.1.1 statistical software (SAS Institute).

Results

The GLP-1-Fc fusion protein LY2189265 has preserved *in vitro* activity and an extended *in vivo* half-life

Direct fusion of DPP-IV-protected GLP-1 analogue (V8-GLP-1) to the human G-type immunoglobulin (IgG1) hinge region dramatically reduced *in vitro* activity (by ~95%) compared to that of free V8-GLP-1 (Figure 1A). To restore and optimize *in vitro* activity, linker sequences were added between the C-terminus of further modified GLP-1 analogues and the N-terminus of the IgG hinge, and these were tested in the *in vitro* assay. A molecule with optimal linker length and sequence was identified that demonstrated approximately 4-fold greater *in vitro* potency over that of free V8-GLP-1 (Figure 1B and C). To reduce potential complement-dependent and antibody-dependent cell-mediated cytotoxicity (ADCC), IgG1 was replaced with a modified IgG4 isotype, optimized at two selected positions (F234A and L235A) to reduce interaction with high-affinity Fc receptors, which resulted in significant reduction of dose-dependent cytotoxicity of two IgG4 versions over the IgG1 version in an ADCC assay (Figure 1D). In addition, S228 was mutated to proline to eliminate half-antibody formation, the GLP-1 R36G mutation was introduced to de-immunize the fusion protein based on the results of the EpiVax algorithm (Figure S1, Supporting information), and the C-terminal lysine of the IgG-Fc was removed. In its final version, LY2189265 had 4-fold greater GLP-1 receptor activation compared to that of V8-GLP-1 peptide (Figure 1C).

Insulin secretion from isolated rat islets was potently (2.5–3-fold) enhanced by the inclusion of 3 nM or 30 nM LY2189265 in the extracellular medium at high (16.8 mM) glucose with no significant enhancement of insulin secretion seen in the presence of low (2.8 mM) glucose (Figure 2A). Unmodified human GLP-1 (3 nM) produced a 4-fold enhancement of insulin secretion elicited by 16.8 mM glucose. Half-maximal stimulation of insulin secretion by LY2189265

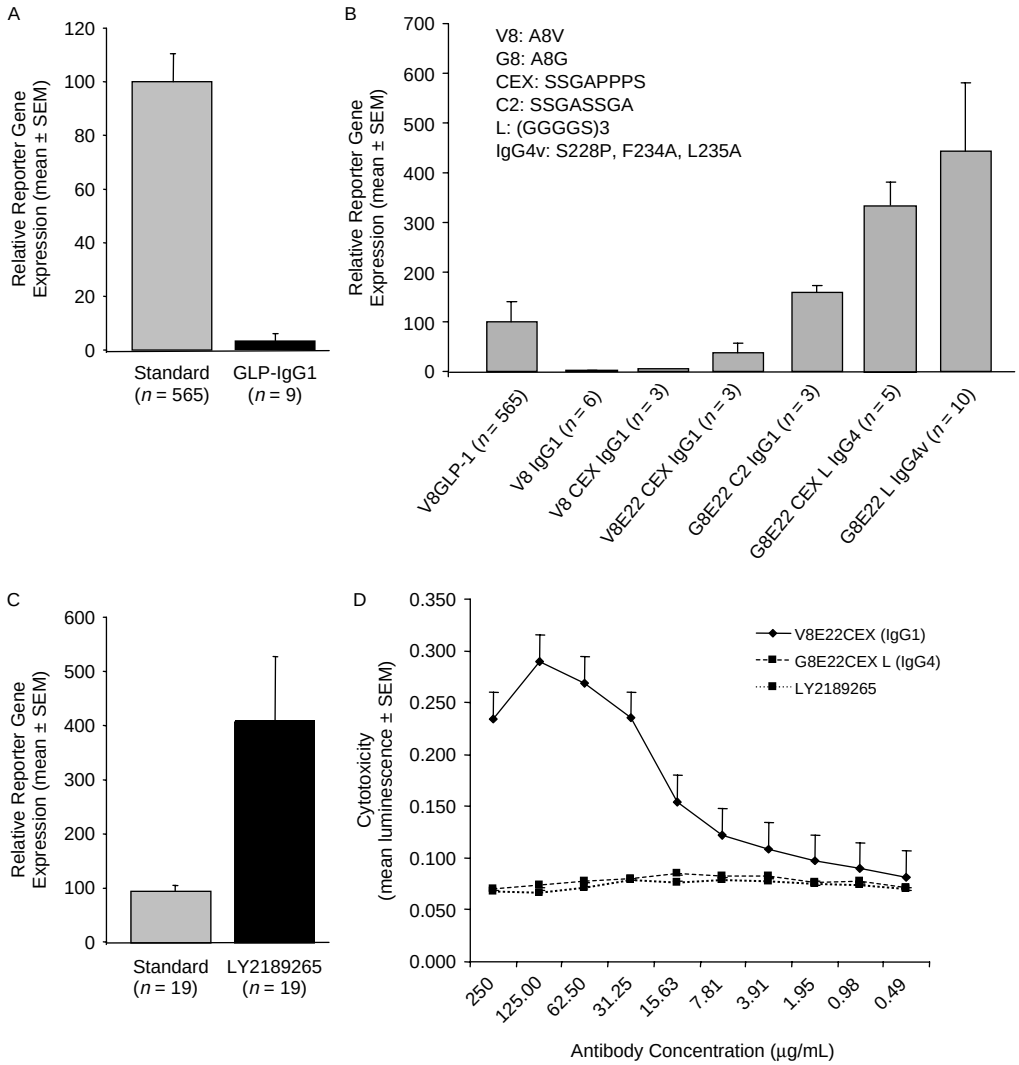

Figure 1. LY2189265 shows increased *in vitro* activity over dipeptidyl peptidase-IV (DPP-IV)-protected glucagon-like peptide-1 (GLP-1) analogue human G-type immunoglobulin (IgG1) fusion proteins without linker sequences and attenuated cytotoxicity. *In vitro* activity was assessed with a cyclic AMP (cAMP) response element transcriptional reporter (*β*-lactamase in (A) and (B), luciferase in (C)) in human embryonic kidney (HEK) 293 cells expressing the GLP-1 receptor. (A) Compared to the V8-GLP-1 peptide analogue that we used as a standard, DPP-IV-protected analogues fused to the hinge region of human IgG (GLP-IgG1) lost most of the *in vitro* activity. (B) The addition of linker sequences between GLP-1 and the IgG moiety increased activity significantly up to 4-fold that of standard peptide. (C) The fully engineered GLP-Fc molecule LY2189265 demonstrated 4-fold increased potency compared to that of the standard peptide V8-GLP-1. The amino acid sequence of GLP-1 including the linker is as follows: HGEGTFTSDVSSYLEEQAAKEFIAWLVKGGGGGGSGGGGSGGGGSA. (D) Antibody-dependent cell-mediated cytotoxicity (ADCC) associated with IgG1-Fc was attenuated by the incorporation of IgG4-Fc isotype into the GLP-1-Fc fusion protein and by mutations in the Fc*γ*R binding site to reduce its receptor-binding affinity. HEK 293 cells expressing the human GLP-1 receptor (target cells) and human peripheral blood mononuclear cells (effector cells) were exposed to several doses of LY2189265 (engineered IgG4 isotype) or GLP-1-Fc analogues V8E22CEX (IgG1 isotype) or G8E22CEXL (IgG4 isotype). Cytotoxicity was estimated by the amount of luminescence, reflecting the release of lactate dehydrogenase from lysed target cells. Results are the mean of three experiments performed in duplicate. Gene reporter activity is represented as percent activity compared to that of the standard peptide V8-GLP-1. Data points are shown as mean ± standard error of the mean (SEM). *n* = the number of independent experiments carried out in triplicate (GLP-1-Fc) or duplicate (standard)

was observed at 2.7 nM. The maximal efficacy, 4-fold stimulation, was observed at 300 nM LY2189265 (Figure 2B). The inclusion of 1 µM of the GLP-1 receptor antagonist Ex(9–39) reversed the glucose-dependent stimulation of insulin secretion observed with LY2189265, suggesting that LY2189265 acts via the islet GLP-1 receptor (Figure 2C). In islets isolated from cynomolgus monkeys, LY2189265 also increased insulin secretion in the presence of a high glucose concentration in a concentration-dependent manner (Figure 2D).

The pharmacokinetic profile of LY2189265 in rats and cynomolgus monkeys is summarized in Table 1. The half-life of LY2189265 after a single dose of 0.1 mg/kg was approximately 1.5 days in rats and >2 days in monkeys (Figure 3 and Table 1). Immunoreactivity for LY2189265 after a single dose remained detectable for several days (>6 days in rats and >14 days in monkeys) (Figure 3).

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