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ORIGINAL ARTICLE

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A glycosylated Fc-fused glucagon-like peptide-1 receptor agonist exhibits equivalent glucose lowering to but fewer gastrointestinal side effects than dulaglutide

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Abstract

Aim: To evaluate the pharmacokinetic and pharmacodynamic properties of a novel glycosylated Fc-fused glucagon-like peptide-1(GLP-1-gFc) receptor agonist with distinctive receptor binding affinity, designed to improve in vivo stability and safety relative to the commercial GLP-1 analogue dulaglutide, and assess its safety profile and pharmacokinetics in healthy humans.

Materials and Methods: We constructed GLP-1-gFc and determined its binding affinity and potency using in vitro instrumental and cell-based analyses followed by in vivo comparison of the glucose-lowering and gastrointestinal side effects between GLP-1-gFc and dulaglutide. A phase 1 clinical trial was conducted to confirm the efficacy and safety profile of GLP-1-gFc.

Results: GLP-1-gFc showed 10-fold less binding affinity and 4-fold less potency than dulaglutide in in vitro. A potency-adjusted dose delayed HbA1c increase comparable with that of dulaglutide (Change for 6 weeks: 2.4 mg/kg GLP-1-gFc, 4.34 ± 0.40 vs. 0.6 mg/kg dulaglutide, 4.26 ± 0.22; n.s.). However, the equivalent efficacy dose and higher dose did not induce malaise-related responses (blueberry bar consumption, g/mouse: 2.4 mg/kg GLP-1-gFc, 0.15% ± 0.03% vs. 0.6 mg/kg dulaglutide, 0.04% ± 0.01%; $P < .01$) or QT interval changes (mean at 14-20 hours, mSc: 0.28 mg/kg GLP-1-gFc, 0.0-8.0 vs. 0.07 mg/kg dulaglutide, 8.0-27.7; n.s.), observed as safety variables in rats and monkeys, compared with those of dulaglutide. Glucose reductions in an oral glucose tolerance test were significant at day 3 postdose without severe gastrointestinal adverse events and pulse rate changes in healthy subjects.

Conclusions: These results suggest that GLP-1-gFc could be used as a novel GLP-1 receptor agonist with better safety than dulaglutide to maximize therapeutic benefits in subjects with type 2 diabetes.

KEYWORDS

drug development, dulaglutide, GLP-1 analogue, glycaemic control, type 2 diabetes

1 | INTRODUCTION

Various antidiabetic drugs have been developed to address the global diabetes epidemic. Glucagon-likepeptide-1 receptor agonist (GLP-1 RA) development has been intensive in recent decades because of their multiple beneficial effects on beta cell function, insulin sensitivity, body weight and the cardiovascular system,[1–4] combined with their lack of life-threatening adverse effects such as hypoglycaemia.[5] However, side effects such as nausea, vomiting and heart rate elevation may impede the widespread use of GLP-1 RAs.[6–8]

In a cross-sectional survey, nausea and vomiting were found to be the predominant factors leading to GLP-1 RA discontinuation reported by physicians (43.8%) and patients (45.4%-64.4%); 51.6% of patients reported these symptoms as the most bothersome GLP-1RA-related problems.[8] In a retrospective analysis, 53.3% of included patients stopped GLP-1 RA treatment.[9] In addition, heart rate elevation has been observed in clinical trials of almost all GLP-1RAs,[10,11] probably reflecting the direct effect of peripherally administered GLP-1 RAs on cardiomyocytes,[12,13] which is more pronounced and sustained for long-acting than for short-acting drugs.[6] The heart rate increase caused by long-actingGLP-1 RAs is slight, but could be a safety concern because it is a cardiovascular disease risk factor in diabetic patients with advanced heart failure. As these side effects could weaken the treatment efficacy of GLP-1 RAs, safer GLP-1 RAs are needed to enhance therapeutic outcomes.

Medicines with reduced side effects have been developed by modulating receptor binding affinity; nimotuzumab,[14,15] for example, is a humanized monoclonal antibody (mAb) with 10 times the dissociation constant (K_d) for binding to epidermal growth factor receptor (EGFR) compared with other mAbs. The requirement for bivalent binding for stable signalling also distinguishes nimotuzumab from other mAbs. Because of these characteristics, nimotuzumab has not induced severe skin toxicity, hypomagnesaemia or adverse gastrointestinal (GI) effects, while showing activity similar to that of other EGFR mAbs, in preclinical and clinical studies.[16]

In this study, we evaluated the pharmacokinetic and pharmacodynamic properties of a novel glycosylated Fc-fusedGLP-1(GLP-1-gFc) with distinctive receptor binding affinity, designed to improve in vivo stability and safety relative to the commercial GLP-1 analogue dulaglutide. We also assessed the safety profile and pharmacokinetics of GLP-1-gFc in healthy humans.

2 | RESEARCH DESIGN AND METHODS

2.1 | Materials

GLP-1-Fc and GLP-1-gFc were produced by cloning the full-length coding sequences of these molecules into plasmid vector pAD15, then transfecting the plasmid vector to Chinese hamster ovary/DHFR$^{-/-}$ cells (DG44, provided by Dr Chain of Columbia University, New York, NY, USA). Single clone selection of transfected cells and purification of secreted proteins were conducted similarly to the protocol used for other hybrid Fc-fused recombinant proteins.[17,18] An investigational product for a clinical trial was manufactured using a scaling-up process in a certified good manufacturing practice (GMP) facility under a GMP-compliant system. Dulaglutide (Trulicity) was purchased from Eli Lilly (Indianapolis, IN, USA), and LiCl for the conditioned taste avoidance (CTA) study was purchased from Sigma Aldrich (Saint Louis, MO, USA).

2.2 | GLP-1-Fc and GLP-1-gFc structure modelling

The binding structure of the GLP-1 receptor to each molecule was modelled using the COOT program.[19] The structures of the GLP1–GLP1 receptor complex and human immunoglobulin (Ig) G4 were adopted from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (PDB; nos. 3IOL and 4c54, respectively). Fc and gFc, which consist of IgD and IgG4, were obtained from Phyre (version 2.0)[20] using human IgG4 Fc (PDB no. 4C54) as a template. Figures illustrating binding structure modelling were prepared using the Pymol program.[21]

2.3 | In vitro potency analysis

To evaluate the potency of GLP-1-gFc (the degree of cyclic AMP [cAMP] induction by a GLP-1–specific response), a transgenic cAMP-specificluciferin- and GLP-1 receptor (GLP-1R)–expressing cell line (GLP1R_cAMP/luc) was constructed. After thawing and appropriate maintenance, 2×10^5 cells/mL with growth medium (90% high-glucose Dulbecco's modified Eagle medium [DMEM], 10% fetal bovine serum (FBS), 130 ug/mL Hygromycin B Gold, 5 ug/mL puromycin) were seeded in a T-75 flask and placed in a CO_2 incubator at 37°C until 70%-80% confluence was achieved. The cells were then washed with PBS, and 0.05% trypsin EDTA was added to separate them from the flask. The cells were collected and washed as needed for activity evaluation, and diluted with 0.5% FBS and high-glucose DMEM for seeding at 2×10^4 cells/80 uL/well. After CO_2 incubation at 37°C for 16 hours, 20 uL/well GLP-1-gFc at various concentrations was treated and reacted in a CO_2 incubator at 37°C for 5 hours. Bright-Glo assay reagent (100 uL/well; Promega Corporation, Madison, WI, USA) was added and reacted at room temperature for 2 minutes, and luminescence was then measured using a luminometer (BioTek Instruments Inc., Winooski, VT, USA).

2.4 | Binding affinity analysis

The binding affinity of the test articles (TAs) was evaluated by surface plasmon resonance (SPR; ProteOn XPR36; Bio-Rad Laboratories, Hercules, CA, USA) and biolayer interferometry (BLI; Octet K2; ForteBio, Fremont, CA, USA), with modification of a previously described protocol.[22,23] For the SPR analysis, a ProteOn GLC chip (Bio-Rad) was stabilized with PBS with 0.01% Tween 20 (pH 7.4), then activated with

150 uL sulfo–N-hydroxysulfosuccinimide (0.001 M) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (0.04 M; 1:1), followed by immobilization of 10 ug/mL human GLP-1R(Abcam, Cambridge, UK) diluted in acetate buffer (pH 5.0). After recording of the immobilization level and deactivation by 1 M ethanolamine-HCl (pH 8.5), different concentrations of dulaglutide and GLP-1-gFc (0, 1.25, 2.5, 5, 10 uM) were injected into each chip channel. The chip was regenerated with 25 mM NaOH, and the lack of remaining signal was checked before analysis of other analytes. Binding sensorgrams were collected, processed and analysed using ProteOn Manager software (Bio-Rad). Binding curves were fitted using the Laugmuir model.

For the BLI analysis, anti-penta His biosensors (ForteBio) were transferred to the instrument and dipped in assay buffer (1× kinetic buffer; ForteBio) for 10 minutes for hydration. The initial step was conducted for 150 seconds with assay buffer. Next, 5 ug/mL recombinant human GLP-1 receptor (Abcam) was immobilized on the surface of the biosensor for 150 seconds and then transferred to fresh assay buffer for 150 seconds to establish a baseline. The association of various TA concentrations (1250-20 000 nM) was measured for 300 seconds, followed by dissociation measurement for 300 seconds in assay buffer. The assay was repeated three times, and new biosensors were used for each TA. Based on a bivalent analyte binding curve, a classical 1:2 biomolecular interaction model was chosen to fit the data.

2.5 | Animals

All animal studies were conducted using protocols approved by the institutional animal care and use committees of Genexine (Pangyo, Korea) and Wuxi AppTec (Suzhou, China). Diabetic (C57BL/KSJ-db/db), DBA/2 mice and other animals (obese [C57BL/6J-ob/ob], CD-1 mice and Sprague–Dawley [SD] rats) obtained from DBL (Eumseong, Korea), Koatech (Pyeongtaek, Korea) and SLC (Shizuoka, Japan), respectively, were housed in appropriate numbers in cages with a 12/12-hour light/dark cycle at 20 ± 2°C. Sterilized irradiated solid animal feed (Teklad-certified irradiated global 18% protein diet, 2918C; Envigo, Huntingdon, UK) and sterilized water were provided ad libitum.

Cynomolgus monkeys obtained from Hainan Jingang Biotech (Hainan, China) were housed individually in stainless steel cages at Wuxi AppTech's animal facility. They were given monkey feed (Beijing Keao Xieli Feed Co., Ltd., Beijing, China) twice daily and reverse-osmosis-purified chlorinated water via an automated system. For the electrocardiography (ECG) study, monkeys were instrumented with transmitters (TL11M2-D70-PCT, Data Science International, Saint Paul, MN, USA) according to Wuxi's standard operating procedure, and only individuals exhibiting normal ECG variables were enrolled.

2.6 | Comparison of GLP-1 peptide, GLP-1-Fc and GLP-1-gFc pharmacokinetics in rats

Eight-week-old male SD rats were weighed and allocated to two treatment groups of each study (n = 4/group): 1 mg/kg GLP-1 peptide and GLP-1-Fc (single intravenous administration), or 0.1 mg/kg GLP-1-Fc and GLP-1-gFc (single subcutaneous administration). Blood samples were collected into dipeptidyl peptidase-4(DPP-4) inhibitor (Sigma Aldrich)-containing EDTA tube (GLP-1 peptide, GLP-1-Fc) or serum separation tubes (GLP-1-Fc, GLP-1-gFc) at designated time points, and the serum was separated by letting the blood stand at room temperature for 30 minutes (omitted for plasma) and centrifuging at $3000 \times g$ for 10 minutes. Active GLP-1 ELISA kit (Linco Research Inc., Weldon Spring Height, MO, USA) was used for plasma GLP-1 peptide and GLP-1-Fc. Serum GLP-1-Fc and GLP-1-gFc were determined using GLP-1-gFc-enzyme-linked immunosorbent assay (ELISA), in which mouse anti-human IgG4 (BD Pharmingen, San Jose, CA, USA) and biotinylated n-terminal–specificGLP-1 antibody (Thermo Fisher, Waltham, MA, USA) were used for antibody capture and detection, respectively. Pharmacokinetic variables were analysed using non-compartmental methods with Pharsight WinNonlin software (version 12.5) (Mountain View, CA, USA).

2.7 | Analysis of GLP-1-gFc pharmacokinetics in rats and monkeys

Eight-week-old male SD rats (n = 4/group) received 0.1, 0.4 and 1.6 mg/kg GLP-1-gFc, respectively, and blood was collected at 0, 2, 6, 12, 24, 48, 72, 96, 120, 144 and 168 hours postdose. To further identify gender difference, male and female (n = 3/sex/dose) cynomolgus monkeys with body weights of 2.44-4.16 kg received 0.125, 0.625 and 3.125 mg/kg GLP-1-gFc, respectively, and blood was collected at 0, 1, 2, 6, 10, 24, 48, 72, 120, 168, 240, 336, 504 and 672 hours postdose. Serum was prepared and analysed with a GLP-1-gFc ELISA as described previously. Pharmacokinetic variables were analysed using non-compartmental methods with Pharsight WinNonlin software (version 12.5).

2.8 | Evaluation of the dose-dependent antidiabetic effect of GLP-1-gFc in db/db mice

To evaluate the dose-dependent antidiabetic effect of GLP-1-gFc in the progress of type 2 diabetes, 5-week-old male diabetic (C57BL/KSJ-db/db) mice were acclimated to a feeding environment for 2 weeks. After evaluation of body weight, animals were allocated to treatment groups (n = 12/group): vehicle, 0.6 mg/kg dulaglutide, 0.6 mg/kg GLP-1-gFc and 1.8 mg/kg GLP-1-gFc. The TAs were administered subcutaneously twice a week for 4 weeks. Non-fasting blood glucose and insulin were measured biweekly, and overnight fasting blood glucose, insulin and HbA1c were measured at sacrifice. Glucose was analysed using GM9 Glucose Analyser (Analox Instruments Ltd, UK). Insulin and HbA1c were measured by rat insulin radioimmunoassay (LINCO Research, MO, USA) and Vantage analyser (Siemens, Munich, Germany), respectively.

2.9 | Determination of GLP-1-gFc dose in db/db mice

Five-week-old male diabetic (C57BL/KSJ-db/db) mice were acclimated to a feeding environment for 1 week. Non-fasting blood glucose was measured and the mice were allocated to treatment groups (n = 8/group): vehicle, 0.6 mg/kg dulaglutide (optimal dose in db/db mice), 0.6 mg/kg GLP-1-gFc and 2.4 mg/kg GLP-1-gFc.[24,25] Treatment was performed to determine the GLP-1-gFc dose with antidiabetic effects comparable with that of dulaglutide. All test materials were diluted and analysed using a GLP-1-gFc ELISA as previously described. The TAs were administered subcutaneously weekly for 6 weeks. Non-fasting blood glucose was measured weekly and HbA1c was measured biweekly.

2.10 | Comparison of intraperitoneal glucose tolerance test responses between GLP-1-Fc and GLP-1-gFc in CD-1 mice

Eight-week-old male CD-1 mice were acclimated to a feeding environment for 1 week. The mice were weighed and allocated to treatment groups (n = 4/group): vehicle, 3 mg/kg GLP-1-gFc and 3 mg/kg GLP-1-Fc. The TAs were administered subcutaneously, followed by overnight fasting and intraperitoneal challenge with 2 g/kg glucose (20% glucose solution, 10 mL/kg body mass) on days 1, 2, 4 and 8. Blood glucose was measured from the tail vein at 0, 10, 20, 30, 60, 90, 120 and 180 minutes after glucose challenge using a glucometer (Allmedicus, Anyang, Korea). Areas under the curves (AUCs) for glucose versus time were plotted with conversion to relative percentages to vehicle on each day.

2.11 | Assessment of antidiabetic/obesity effects in ob/ob mice

To compare the antidiabetic and antiobesity effects of GLP-1-gFc with dulaglutide in the obese condition, 6-week-old female obese (ob/ob) mice[26] were acclimated to a feeding environment and operating (injection and grasping) procedures for 3 weeks. Body weight was measured and the mice were allocated to treatment groups (n = 8/group): vehicle, 0.6 mg/kg dulaglutide and 2.4 mg/kg GLP-1-gFc. The TAs were diluted appropriately and analysed with a GLP-1 ELISA as described above, then administered subcutaneously weekly for 4 weeks to compare the effects of GLP-1-gFc and dulaglutide on GLP-1Rs in the pancreas and vagus nerve/brain. Food intake and body weight were measured weekly and HbA1c was measured in weeks 0 and 4.

2.12 | CTA study

A CTA study was performed to determine the malaise effects of the nausea-inducible TAs, with modification of a previously described protocol.[27,28] Acclimated 5-week-old male DBA/2 J mice were housed individually and given 10 minutes access to a preweighed blueberry bar, which was then reweighed to measure consumption. Immediately thereafter, the mice were treated (vehicle, 0.3 M LiCl [intraperitoneal], 0.6 mg/kg dulaglutide and 2.4 mg/kg GLP-1-gFc; n = 10/group) to associate the novel taste with the nauseating TA stimulus. The mice were exposed to another blueberry bar after a 14-day washout period to exclude food intake suppression by the GLP-1–derived TAs (confirmed by overnight food intake normalization). The degree of CTA response was determined by the reduction of bar consumption compared with the vehicle group.

2.13 | Evaluation of QT interval changes in monkeys

Telemetry-implanted male cynomolgus monkeys were subcutaneously administered single doses of vehicle. After a 19-day washout period, they received single subcutaneous injections of dulaglutide (0.07 mg/kg; n = 3) or GLP-1-gFc (0.28 and 1.14 mg/kg; n = 2 each) to evaluate cardiovascular effects. The dulaglutide dosage was determined by converting the clinical dose according to the body surface area (1.5 mg/65 kg × 3.08).[29] The GLP-1-gFc doses were obtained by multiplying the dulaglutide dose by 4 (equivalent; low) and 16 (high). Blood pressure, heart rate and ECG waveforms were recorded from 2 hours predose to 24 hours postdose. ECG was performed for ≥30 seconds predose (at least 30 minutes apart) and 2, 4, 8, 12, 16 and 24 hours postdose. ECG data were used to calculate corrected QT intervals (QTcs).

2.14 | Clinical study

A first-in-human, phase 1, single ascending dose, randomized, double-blind study was performed to assess the safety, tolerability and pharmacokinetics of subcutaneously administered GLP-1-gFc in healthy men, in accordance with the Declaration of Helsinki and good clinical practice. Forty-eight healthy men aged 18-40 years with a body mass index (BMI) of 18-29.9 kg/m^2 (n = 8/group [6 active drug, 2 placebo]) participated after providing written informed consent. Subjects with clinically significant pancreatic, hepatic, renal, GI, cardiovascular, respiratory, haematological, central nervous system or other disease that could influence the safety, absorption, metabolism or excretion of the active agent were excluded. GLP-1-gFc was administered in sequential doses (0.01, 0.02, 0.04, 0.08, 0.16 and 0.24 mg/kg) as per the recommendations by safety monitoring committee. The starting dose was based on the non-observed adverse effect level from a cynomolgus monkey sub-chronic toxicity study (30 mg/kg; human equivalent dose 9.75 mg/kg), with a safety factor of 1000 applied. The sub-maximum and maximum doses were adopted to check the safety profile of GLP-1-gFc administered at the equivalent efficacy dose, 4-fold higher than the dulaglutide dose causing side effects in phase 1 clinical trials.[30] The percentages of subjects who experienced

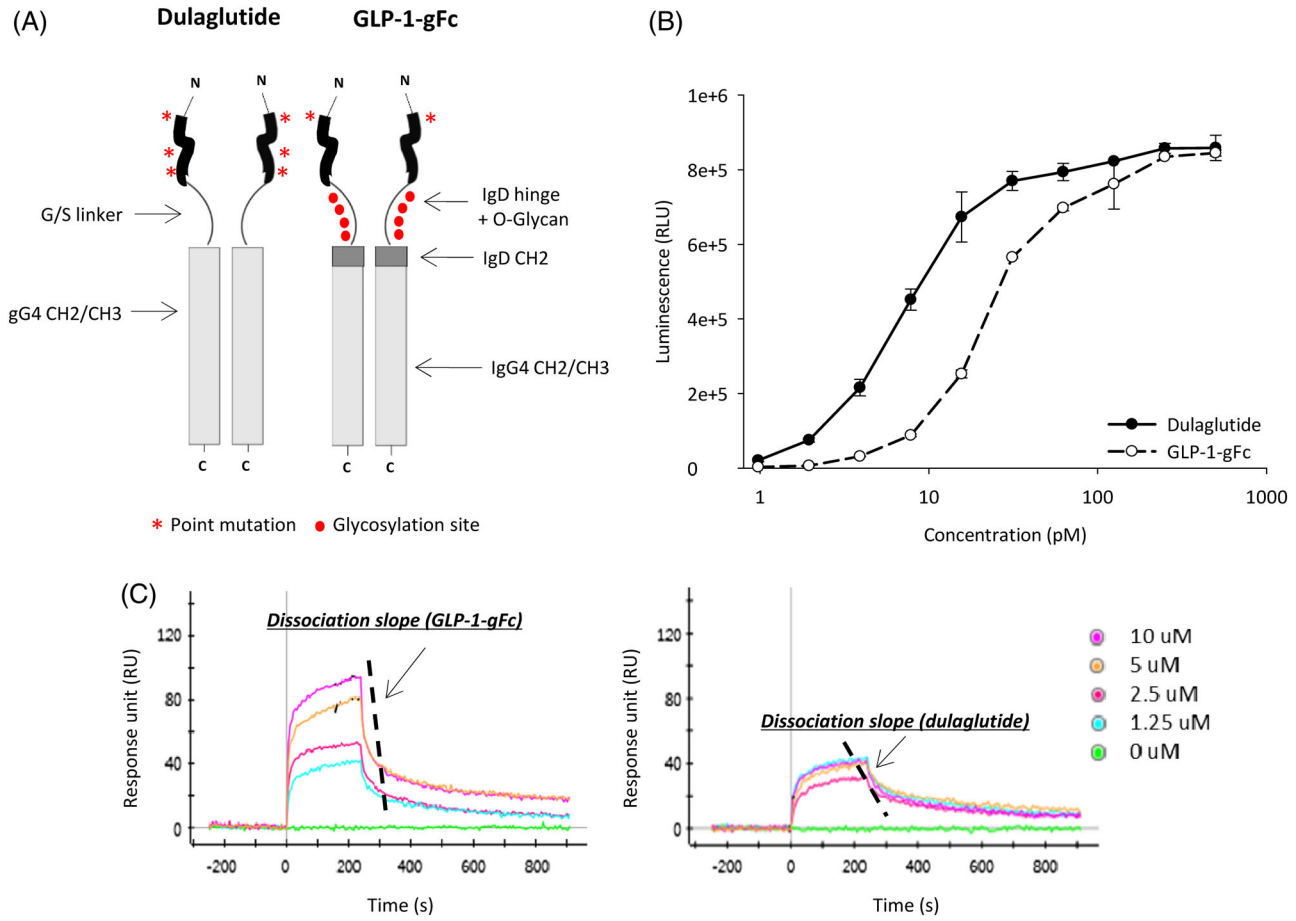

FIGURE 1 GLP-1-gFc had a higher dissociation constant (K_d) and lower receptor-mediated response than dulaglutide because of structural differences. (A) Schematic diagram of the two molecules. (B) Luminescence of various concentrations of GLP-1-gFc and dulaglutide. (C) Sensorgrams of binding affinity, determined by surface plasmon resonance analysis. Results (means and means ± standard errors of the mean) are representative of more than two independent experiments. K_a, association constant; KD, dissociation constant at equilibrium; RLU, relative luminescence unit

nausea/vomiting and pulse rate alteration during the 28-day study period were recorded.

2.15 | Safety assessment

Safety was assessed using a protocol approved by the Federal Institute for Drugs and Medical Devices (N-A-PH1-15-056). Treatment-emergent adverse effects (TEAEs), vital signs (blood pressure, pulse rate, body temperature) and 12-lead ECG data were monitored, and physical examinations and laboratory investigations, including anti-drug antibody screening, were performed. Assessments were conducted several times during the 28-day study period.

2.16 | Pharmacokinetic analysis

Blood samples for pharmacokinetic analysis were collected by venous puncture or indwelling venous catheter into serum separation tubes predose, and between 0.25 and 648 hours postdose. Serum GLP-1-gFc concentrations were determined using a previously described GLP-1-gFc ELISA with validation. Pharmacokinetic variables were analysed using non-compartmental methods with Pharsight WinNonlin software (version 12.5). The area under the serum concentration time curve to the time of last measurable concentration (AUC_{last}) and maximum serum concentration (C_{max}) of GLP-1-gFc were plotted with each dose to assess proportionality.

2.17 | Oral glucose tolerance test

After overnight fasting, subjects drank 300 mL of a commercially available oral glucose tolerance test (OGTT) beverage containing 75 g glucose within 5 minutes. Blood samples were taken before and 0.25, 0.5, 1, 1.5 and 2 hours after beverage intake with subjects seated. Glucose versus time kinetics were determined by photometric assay and electro-chemiluminescence immunoassay (Cobas c501 and e/601; Roche Diagnostics, Basel, Switzerland), respectively. The percentage of subjects with altered pulse rates on the day of the OGTT was recorded.

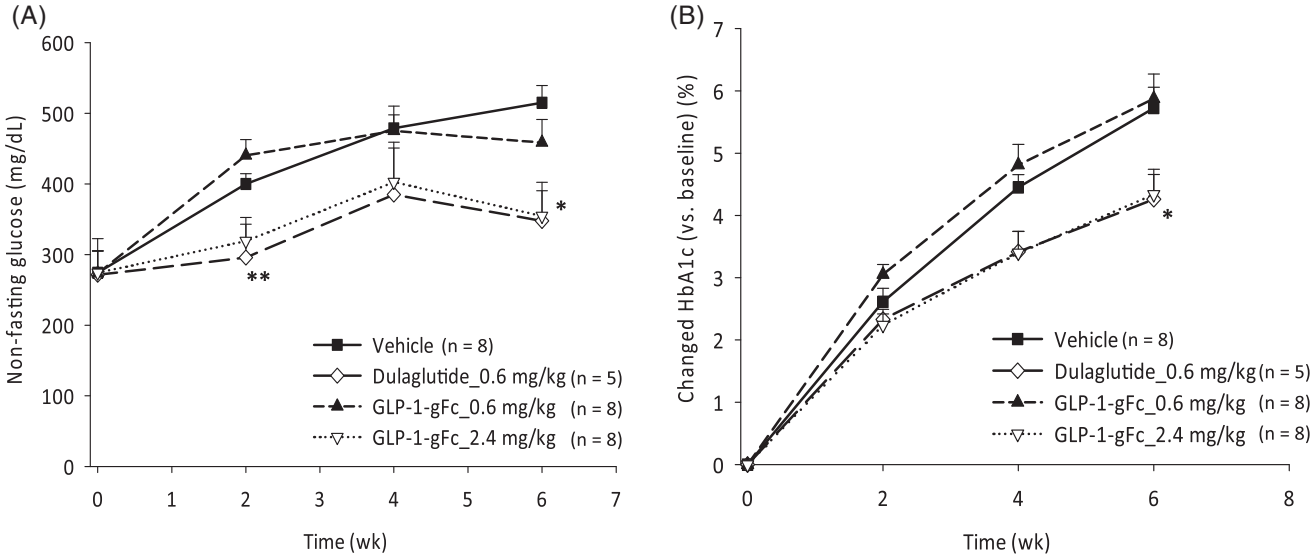

FIGURE 2 A four times higher dose of GLP-1-gFc had a glucose-lowering effect comparable with that of dulaglutide in diabetic (db/db) mice. (A) Non-fasting glucose level for 6 weeks (n = 5-8/group). (B) Changed HbA1c from baseline (ΔHbA1c) for 6 weeks (n = 5-8/group). Results are presented as means ± standard errors of the mean. *P < .05, **P < .01 versus vehicle, one-way ANOVA followed by Tukey's and Dunnett's T3 test as a post hoc analysis

2.18 | Statistical analyses

SPSS 21 (IBM SPSS, Chicago, IL, USA) and SAS version 9.4 were used to analyse the data. Outliers among the data were excluded before statistical analysis using the box-plot method. Preclinical pharmacokinetic and clinical data were expressed as mean ± standard deviations, and other data were expressed as mean ± standard errors of the mean. Normality and variances equality were evaluated by Shapiro–Wilk test and Levene's test, respectively. Statistical significance was evaluated using one-way ANOVA followed by Tukey's and Dunnett's T3 in the case of parametric data. For non-parametric data, a Mann–Whitney U test was performed. All clinical data were evaluated only descriptively. Differences were considered statistically significant at P < .05.

3 | RESULTS

3.1 | In vitro potency and binding affinity

The GLP-1-gFc and dulaglutide structures were distinguishable based on glycosylation and amino acid modification (Figure 1A). The introduction of O-glycosylation to the IgD hinge region dramatically enhanced the pharmacokinetics and pharmacodynamics of GLP-1-gFc in rodents without loss of activity (Figure S1). These findings were supported by three-dimensional structure prediction using Phyre web-based software (Figure S2). Distinct response curves were obtained for GLP-1-gFc and dulaglutide incubated with cell lines at the same molar concentration; the potency of GLP-1-gFc was less than that of dulaglutide, with a 3.5-fold higher half maximal effective concentration (23.33 vs. 6.66 pM; Figure 1B). In the SPR analysis, GLP-1-gFc and dulaglutide showed dose-dependent rapid increases in response units (RUs) in the association phase, whereas GLP-1-gFc showed a more rapid RU decrease than dulaglutide in the dissociation phase (Figure 1C). The K_d for GLP-1-gFc (6.43×10^{-2}) was 10-fold higher than for dulaglutide, whereas the association constant (4.02×10^{3}) was 1.7-fold lower. This lower binding affinity of GLP-1-gFc was confirmed by biolayer interferometry (Figure S3). Equilibrium K_ds for GLP-1-gFc and dulaglutide was 1.6×10^{-5} and 9.04×10^{-7}, respectively, indicating more rapid dissociation of GLP-1-gFc from the GLP-1R. These observations suggest that GLP-1-gFc has lower binding affinity and in vitro potency than dulaglutide because of its different structural characteristics.

3.2 | Glucose-lowering efficacy in diabetic mice

At the end of the 6-week administration period, the non-fasted glucose level in the vehicle-treated group had increased from 274 to 515 mg/dL (Δglucose = 241 mg/dL); all TA-treated groups showed decreases relative to this level with statistical significance only in the 2.4 mg/kg GLP-1-gFc group (Figure 2). Dulaglutide prevented an increase in the non-fasted glucose level (terminal, 348 mg/dL; Δglucose = 76.3 mg/dL). GLP-1-gFc had a dose-dependent effect on the delay of glucose increase (0.6 mg/kg: terminal glucose level = 459 mg/dL, Δglucose = 185 mg/dL; 2.4 mg/kg: terminal glucose level = 355 mg/dL, Δglucose = 80.1 mg/dL; Figure 2A). HbA1c changes indicated similar efficacy patterns; dulaglutide and high-dose-GLP-1-gFc meaningfully reduced terminal HbA1c levels (means, 4.26% and 4.34%, respectively; Figure 2B). GLP-1-gFc's comparable glucose homeostatic effects to dulaglutide were confirmed and mediated by GLP-1–stimulated insulin secretion (Figure S4). These results

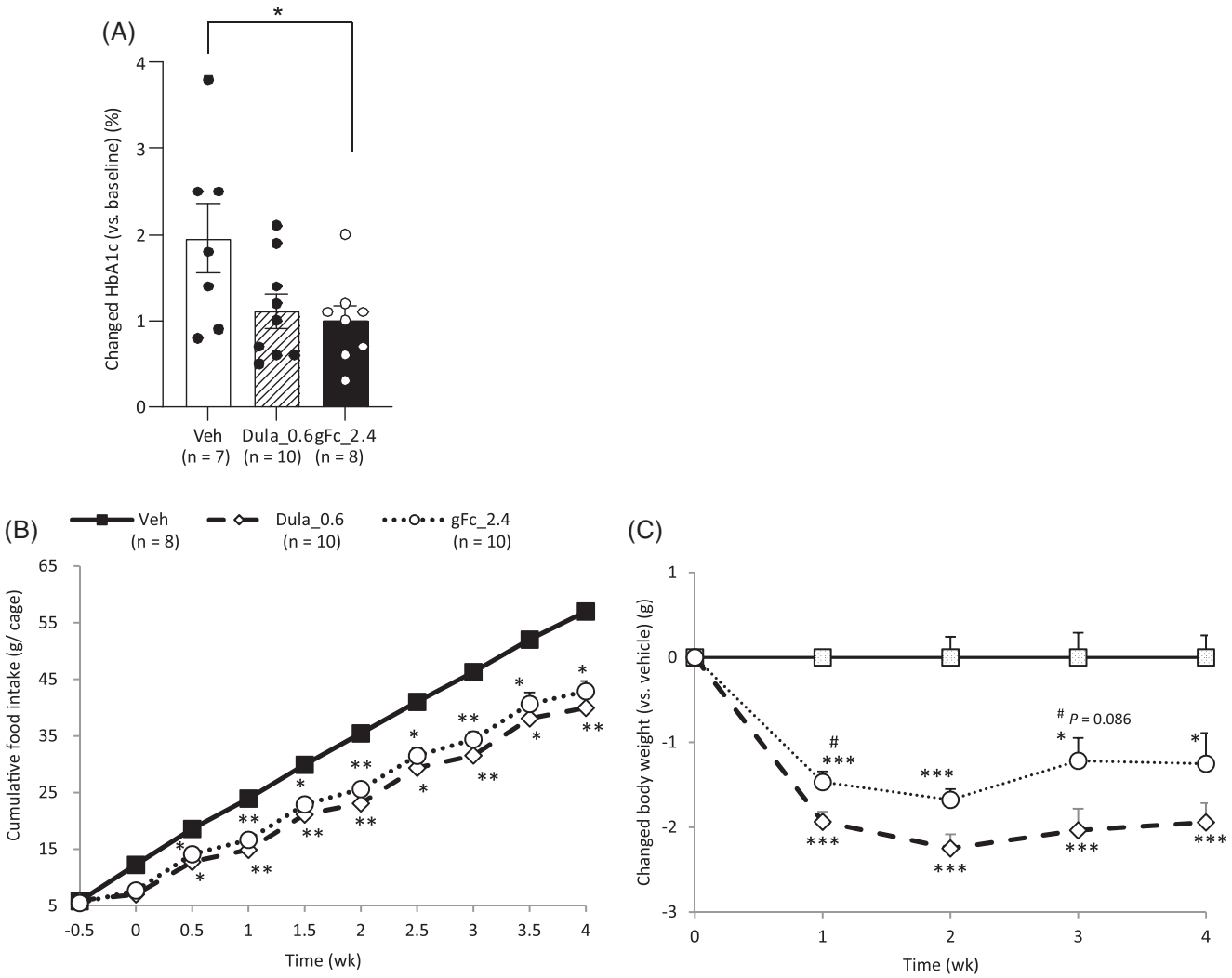

FIGURE 3 An equivalent efficacy dose of GLP-1-gFc showed similar glucose-lowering efficacy, but a weaker weight loss effect than dulaglutide did in obese (ob/ob) mice. (A) Changed HbA1c from baseline (ΔHbA1c) at week 4 (n = 7-10/group). (B) Cumulative food intake for 4 weeks (n = 4-5/cage, 2 cages). (C) Changed body weight from vehicle group for 4 weeks (n = 7-9/group). Results are presented as means ± standard errors of the mean. *P < .05, ** P < .01, *** P < .001 versus vehicle, # P < .05 versus dulaglutide, one-way ANOVA followed by Tukey's and Dunnett's T3 test as a post hoc analysis. Dula_0.6, dulaglutide 0.6 mg/kg; gFc_2.4; GLP-1-gFc 2.4 mg/kg

indicate that 4-fold more GLP-1-gFc than dulaglutide is required for equivalent antidiabetic efficacy.

3.3 | Glucose-lowering and weight-loss effects in obese mice

GLP-1-gFc and dulaglutide delayed HbA1c increase compared with vehicle with statistical significance only in the 2.4 mg/kg GLP-1-gFc group (0.9% and 1.1%, respectively, vs. 2.0%; Figure 3A). Dulaglutide decreased cumulative food intake and body weight significantly compared with vehicle (−17 g/cage and −1.9% vs. vehicle; Figure 3B,C). GLP-1-gFc showed weaker trends for these variables; Δbody weight differed significantly between 2.5 mg/kg GLP-1-gFc and dulaglutide in week 1 and the gap remained until week 4. These findings suggest that dulaglutide and GLP-1-gFc generate different receptor-mediated responses, depending on the expression of GLP-1Rs at different levels in organs.

3.4 | Malaise-related responses and risk of QT elongation

In the CTA study, first-exposure blueberry bar consumption was similar in all groups, whereas second-exposure consumption was reduced significantly in the LiCl and dulaglutide groups (Figure 4A). GLP-1-gFc caused much less reduction in consumption, differing significantly from the effect of dulaglutide (Figure 4B). One day before the second exposure, overnight food intake did not differ between the GLP-1-gFc and dulaglutide groups, confirming complete wash-out of GLP-1-RA–related food intake suppression. By contrast, overnight food

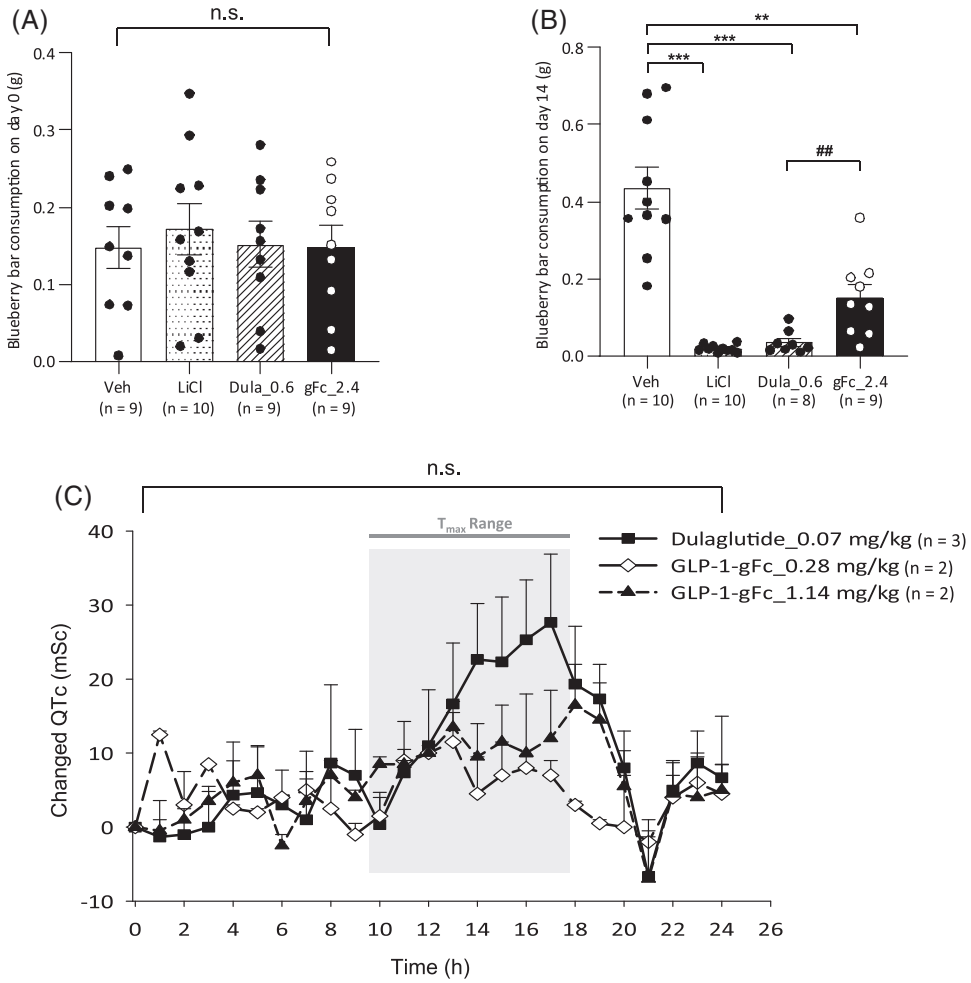

FIGURE 4 Compared with dulaglutide, GLP-1-gFc induced significantly fewer nausea/vomiting and QT elongation responses in mouse conditioned taste avoidance (CTA) (A, B) and monkey electrocardiography (ECG) (C) studies. (A) Blueberry bar consumption before TA administration (n = 9-10/group). (B) Blueberry bar consumption after TA administration (n = 8-10/group). (C) Changed corrected QT interval (QTc) after a single administration of TA (n = 2-3/group). Results are presented as means ± standard errors of the mean. $^{**}P < .01$, $^{***}P < .001$ versus vehicle, $^{\#\#}P < .01$ versus dulaglutide, Mann–Whitney$U$ test for CTA study data and one-way ANOVA for ECG data. n.s., non-significant; Dula_0.6, dulaglutide 0.6 mg/kg; gFc_2 GLP-1-gFc 2.4 mg/kg Abbreviation: TA, test articles

intake on day 1 postinjection was dramatically reduced in the GLP-1RA–treated groups (Figure S5). These results indicate that the responses to GLP-1-gFc and dulaglutide in the vagal nerve/brain differ, inconsistent with the trend observed for glucose reduction by pancreatic action.

Monkeys showed no treatment-related clinical sign after single vehicle administration, but numerically meaningful differences in QTc were observed between the GLP-1-gFc and dulaglutide groups during the ECG monitoring period (Figure 4C). Dulaglutide increased the QTc at 10-20 hours, the predicted time to maximum plasma concentration (T_{max}), whereas low and high GLP-1-gFc doses did not increase the QTc. This difference did not generate a difference in heart rate or blood pressure. Collectively, these findings suggest that GLP-1-gFc induces a milder response to GLP-1Rs in the vagal nerve/brain and heart than in the pancreas because of its attenuated receptor affinity, in contrast to the high potency of dulaglutide.

3.5 | Pharmacokinetics, OGTT efficacy, tolerability and side effects in healthy men

The mean age of the 48 healthy male subjects (46 Caucasian, 1 Asian, 1 African American) was 29.9 ± 5.8 years, the mean weight was 79.1 ± 10.5 kg, height was 178.9 ± 6.5 cm and BMI was 24.74 ± 3.15 kg/m^2. Thirty-seven subjects were non-smokers and 11 subjects were light smokers (<10 cigarettes/day). Demographic characteristics were similar among dose groups (Table 1).

Single subcutaneous doses of GLP-1-gFc were safe and well tolerated, with no severe adverse effect or antibody against GLP-1-gFc. All TEAEs were of mild to moderate intensity and had resolved by the end of the study period.

The pharmacokinetics of GLP-1-gFc followed a mono-exponential decline, with a median half-life of 62.5-108.0 hours in all groups (Figure 5A, Table 2). Serum concentrations peaked at about 36-48 hours postdose, with mean C_{max} values of 36.4, 68.2, 102.6, 242.4, 454.4 and 1087.7 ng/mL for 0.01, 0.02, 0.04, 0.08, 0.16 and 0.24 mg/kg, respectively. Dose-proportional linear increases in AUC_{last} and C_{max} were observed (R^2 = 0.9302 and 0.9925, respectively; Figure 5B,C). These findings are consistent with the pharmacokinetic profiles observed in SD rats and cynomolgus monkeys, in which GLP-1-gFc showed dose-dependent and long-lasting pharmacokinetic effects with half-lives of 14.1-15.3 and 79.1-113.8 hours, respectively (Figure S6 and Table S1).

In the OGTT, GLP-1-gFcdose-dependently decreased the AUC on the glucose versus time plot (gAUC) from baseline (day −1; Figure 6A-C). In general, this decrease was greater at 3 than at 5 days postdose,

TABLE 1 Demographics and baseline characteristics of healthy subjects

		Placebo N = 12	Cohort 1 0.01 mg/kg N = 6	Cohort 2 0.02 mg/kg N = 6	Cohort 3 0.04 mg/kg N = 6	Cohort 4 0.08 mg/kg N = 6	Cohort 5 0.16 mg/kg N = 6	Cohort 6 0.24 mg/kg N = 6	Overall N = 48
Age (years)	Mean ± SD	27.5 ± 7.1	33.3 ± 3.6	26.8 ± 7.3	29.2 ± 3.5	34.7 ± 4.6	25.0 ± 4.0	31.4 ± 5.0	29.9 ± 5.8
	Min-max	19-36	28-37	19-39	26-33	29-40	21-30	23-39	19-40
Height (cm)	Mean ± SD	177.2 ± 5.0	180.0 ± 4.6	176.8 ± 7.7	183.2 ± 8.0	175.0 ± 6.4	179.0 ± 6.7	180.0 ± 6.2	178.9 ± 6.5
	Min-max	173-185	171-184	164-185	173-193	165-183	170-18	170-187	164-19
Weight (kg)	Mean ± SD	73.2 ± 6.9	74.4 ± 9.4	77.3 ± 11.3	87.1 ± 14.9	78.9 ± 6.3	75.3 ± 12.3	83.3 ± 8.4	79.1 ± 10.5
	Min-max	64.4-84.0	60.2-87.0	60.1-89.0	69.1-110.6	71.0-86.0	61.1-91.0	70.9-96.0	60.1-110.6
BMI (kg/m^2)	Mean ± SD	23.43 ± 3.19	23.03 ± 3.38	24.65 ± 2.58	25.93 ± 3.63	25.80 ± 2.06	23.53 ± 4.12	25.78 ± 2.84	24.74 ± 3.15
	Min-max	19.9-28.1	18.4-27.2	20.3-27.6	19.8-29.7	23.9-28.6	19.1-29.7	20.5-29.2	18.4-29.7

Abbreviations: BMI, body mass index; max, maximum; min, minimum; N, number of subjects; SD, standard of deviation.

consistent with the T_{max} of 36-48 hours. Suppression of gAUC was most significant for the highest dose (−61.6% and −66.9% vs. baseline on day 3 and 5, respectively); gAUC changes induced by 0.08 and 0.16 mg/kg doses were also remarkable on day 3 (−55.7% and −53.0%, respectively, vs. baseline; Figure 6C). In contrast to 0.24 mg/kg, reduced gAUC values by 0.08 and 0.16 mg/kg were rebounded at day 5, possibly because of insufficient drug concentration above the pharmacological threshold at that time point.

GLP-1-gFc doses of 0.01-0.16 mg/kg induced almost no nausea or vomiting; one subject reported nausea after receiving the 0.04 mg/kg dose. At 0.24 mg/kg, four and one of six subjects experienced transient nausea and vomiting, respectively (Figure 6D). No pulse rate change from baseline was observed on day 3 or 5 postdose (Figure 6E). These trends were consistent with other TEAEs related to GI symptoms (Table S2) and diastolic blood pressure (Table S3) monitored throughout the study period. These clinical results are consistent with the preclinical observations of fewer nausea/vomiting and QTc responses than glucose lowering, in contrast to dulaglutide.

4 | DISCUSSION

GLP-1 RA side effects, especially nausea and vomiting, are not life-threatening, but affect therapeutic outcomes significantly because of their profound influence on drug adherence. The development of GLP-1 RAs causing fewer such effects is needed to minimize the gap in therapeutic efficacy between clinical trials and real-world practice. In this study, we developed a novel long-actingGLP-1 RA with fewer side effects, and glucose-lowering ability, comparable with that of other potent GLP-1 RAs. The introduction of O-glycosylation to the Fc hinge region enhanced the pharmacokinetic and pharmacodynamic properties without altering receptor activity, possibly because of the lack of change in steric hindrance for receptor binding. The GLP-1-gFc showed similar efficacy, but a distinctive safety profile compared with dulaglutide in preclinical and clinical studies. Our results highlight the potential of this novel antidiabetic agent candidate, which might narrow the therapeutic efficacy gap, maximizing benefits for patients.

Whether the food intake reduction caused by GLP-1 is secondary to the induction of nausea is poorly understood.[7] Meier[31] suggested that distinct mechanisms underlie nausea induction and food intake suppression. This argument is supported by the persistence of food intake suppression-related body weight reduction, in contrast to transient nausea at the beginning of treatment, in a clinical trial of liraglutide,[32] and similar trends in other clinical trials of long-actingGLP-1 RAs. Thus, the evaluation of nausea/vomiting as a side effect apart from food intake and body weight is of value. The quantitative CTA study enables the behavioural analysis of nausea and malaise: Kanoski et al[7] reported that the CTA response of liraglutide was less than exendin-4 although its food intake suppression was comparable with exendin-4. Likewise, GLP-1-gFc induced a significantly milder CTA response than dulaglutide at the equivalent efficacy dose, which is expected to show equal or higher reductions in HbA1c, fasting and non-fasting glucose levels, and increase in insulin secretion

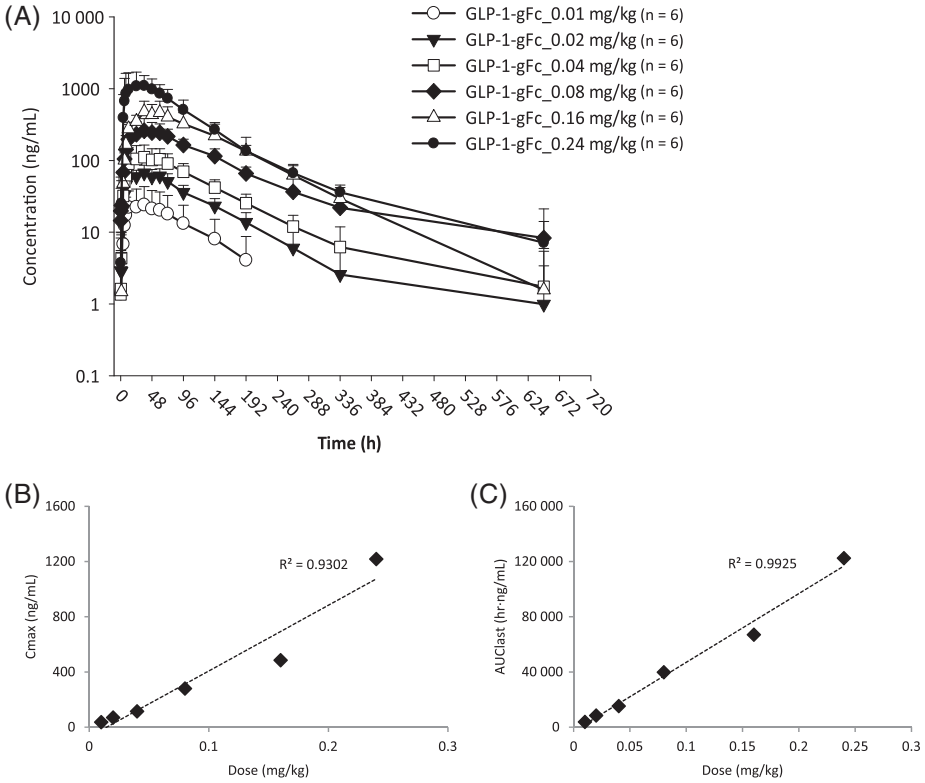

FIGURE 5 GLP-1-gFc exhibited dose-dependent pharmacokinetics following single subcutaneous administration in healthy men. (A) Drug concentration in serum (n = 6/cohort). (B) Trend line of dose versus C_{max}. (C) Trend line of dose versus AUC_{last}. Results are presented as means ± standard deviations. Only descriptive statistics were applied

TABLE 2 Summary of pharmacokinetic variables following single subcutaneous administration of GLP-1-gFc pharmacokinetic variables in healthy men after subcutaneous administration of six escalating doses of GLP-1-gFc were described

Pharmacokinetic variables		Cohort 1	Cohort 2	Cohort 3	Cohort 4	Cohort 5	Cohort 6
		0.01 mg/kg(N = 4)[a]	0.02 mg/kg(N = 6)	0.04 mg/kg(N = 6)	0.08 mg/kg(N = 6)	0.16 mg/kg(N = 6)	0.24 mg/kg(N = 6)
$AUC_{0\text{-}t}$ (h*ng/mL)	Geom. mean	3819.1	8185.1	13 383.8	30 166.2	63 377.6	118 174
	Geom. CV%	16.0	32.2	45.3	41.6	43.2	37.9
$AUC_{0\text{-}inf}$ (h*ng/mL)	Geom. mean	4563.0	9171.1	14 071.0	31 511.0	65 946.5	120 115.6
	Geom. CV%	18.1	34.4	43.7	40.2	42.6	37.6
C_{max} (ng/mL)	Geom. mean	36.4	68.2	102.6	242.4	454.4	1087.7
	Geom. CV%	13.6	27.0	54.1	50	41.6	55.6
T_{max} (h)	Median	36.0	36.0	42.0	42.0	48.0	36.0
	Min-max	24.0-36.0	36.0-60.0	12.0-60.0	24.0-60.0	36.0-60.0	8.0-36.0
Half-life (h)	Median	63.6	70.8	62.5	64.4	70.9	108.0
	Min-max	44.4-83.8	45.6-369.8	55.9-75.7	46.5-82.1	57.7-99.7	52.1-153.6

Abbreviations: $AUC_{0\text{-}inf}$, AUC from time 0 to infinity; CV, coefficient of variation; Geom., geometric; max, maximum; min, minimum.
[a]Two subjects had GLP-1-gFc serum concentrations below LLOQ at all sampling time points.

based on a supplementary study with diabetic db/db mice (Figure S4). This difference in CTA response was more profound than that in food intake suppression in ob/ob mice. TA-induced electrophysiological signalling changes in the heart were also consistent with the CTA results. Equivalent efficacy and 4-fold greater GLP-1-gFc doses did not alter the QTc, whereas dulaglutide increased it, especially at T_{max}. Dulaglutide-induced QTc changes, and probable associated heart rate increases, were observed in cynomolgus monkeys in pharmacological safety and chronic toxicology studies involving single and multiple subcutaneous injections, respectively.[33] No heart-related side effect of GLP-1-gFc was noted in a pharmacological safety study or in a chronic toxicity study involving weekly treatment of cynomolgus monkeys for 4 and 26weeks, conducted as investigational new drug enabling studies that are not

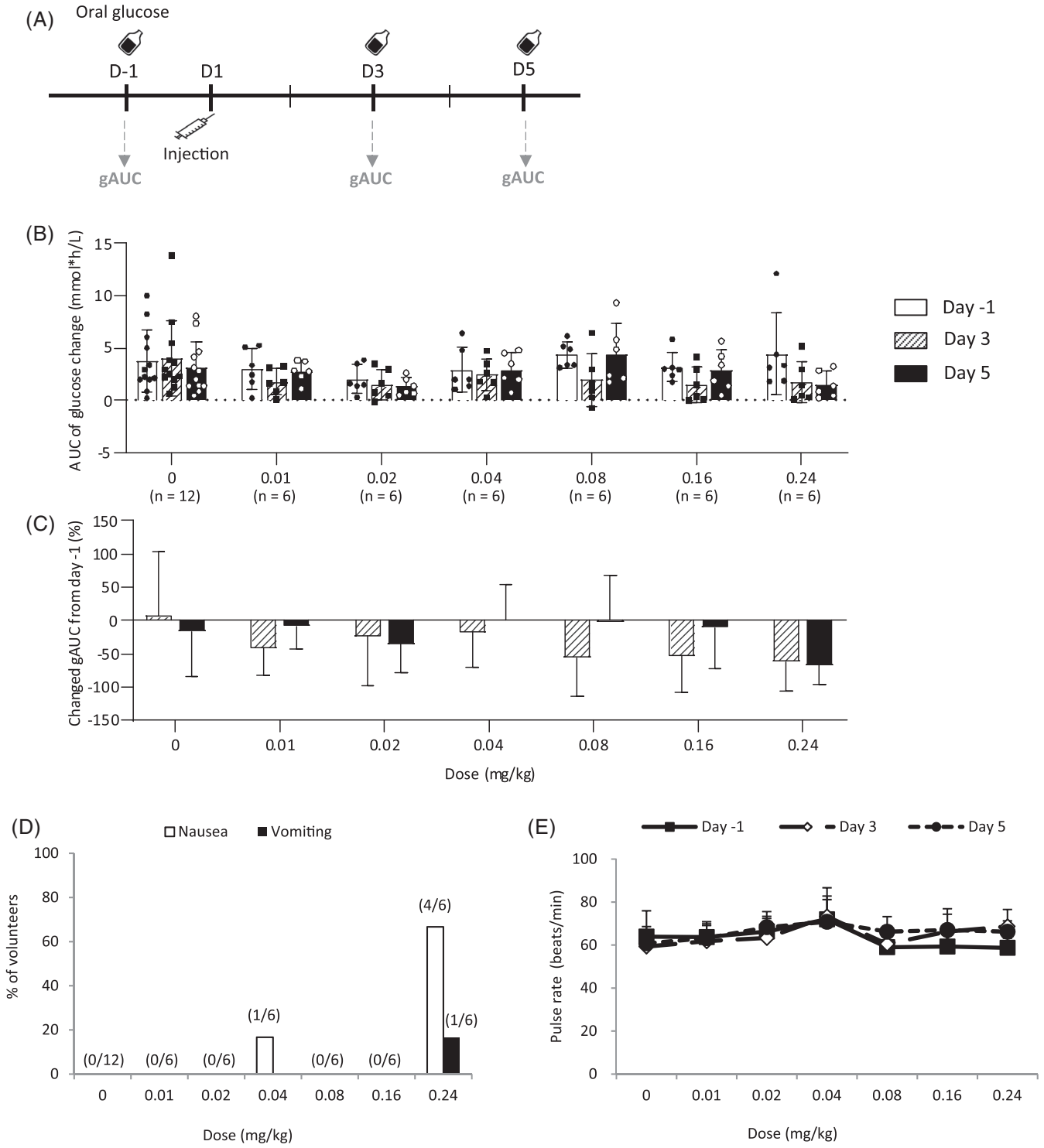

FIGURE 6 GLP-1-gFc had no remarkable effect nausea/vomiting or heart rate while showing good efficacy in an oral glucose tolerance test (OGTT). (A) Schematic diagram of OGTT. (B) AUC of glucose versus time plot (n = 6-12/group). (C) Changed AUC of glucose versus time plot from baseline. (D) Percentage of volunteers experiencing nausea or vomiting during study period. (E) Change of pulse rate in all groups on day −1, 3 and 5. Results are presented as means ± standard deviations (n = 6/cohort). Only descriptive statistics was applied. gAUC, area under the curve of glucose versus time plot

publicly disclosed. Additional investigation of the mechanisms underlying the differences in safety profile between GLP-1-gFc and dulaglutide, despite their comparable antidiabetic efficacies, is needed.

In the phase 1 clinical trial, this uncoupling of GLP-1-gFc efficacy and safety profiles was confirmed in healthy subjects. Although GLP-1-gFc's pharmacokinetics were comparable with dulaglutide, nausea/vomiting and pulse rate were not affected, except by the highest dose,

which induced nausea and vomiting in some subjects. On the other hand, the glucose-lowering effect in the OGTT was marked from the lowest dose, with 40.7% gAUC suppression observed on day 3. This finding is superior to the maximum 29% gAUC reduction caused by dulaglutide in a phase 1 clinical trial.[30] However, dulaglutide's glucose reduction was associated with side effects such as nausea/vomiting and pulse rate alterations, which increased in a dose-dependent manner from very low doses. GLP-1-gFc's glucose reduction was not related closely with side effect frequency, consistent with other GI- and heart-related trends.

Nimotuzumab, a novel EGFR mAb, has a unique safety profile, similar to GLP-1-gFc. Its low binding affinity enables it to bind stably to cancer cells with strong EGFR expression, but not normal cells with weak EGFR expression, resulting in a better safety profile with comparable efficacy with other EGFR mAbs.[16] Similar to EGFRs, GLP-1Rs are expressed at different levels in various organs (eg, heart, stomach, intestine, kidney) and the central/vagal/enteric nerves.[34,35] The vagal nerve and heart, mainly responsible for nausea/vomiting and heart rate, have low GLP-1R expression[36] (vs. the pancreas, which is mainly responsible for glucose reduction), resulting in GLP-1-gFc activity similar to that of nimotuzumab. Considering the participation of GLP-1R in vagal nerve and brain for glucose modulation,[37] it would be inappropriate to simply divide vagal nerve-dependent nausea/vomiting or pancreas-dependentglucose-lowering effects. But combined with the fact that the nausea/vomiting by GLP-1R is strictly related to the nervous system,[38] and that GLP-1R expression levels are important for the maximal efficacy/potency of low-affinitymolecules,[39] the novel GLP-1-gFc concept could still be plausible. in vitro studies using cell lines or primary cells from different organs are needed to elucidate the mechanism underlying GLP-1-gFc's activity.

Many long-actingGLP-1 RAs have been developed via Fc, human serum albumin, X-TEN and fatty acid conjugation. As such fusion partners compromise N-terminal protein activity,[40]N-terminal peptide modification is required. Peptide modification strategies have been applied to maintain the activity of dulaglutide and semaglutide,[33] whereas albiglutide and NB1001 (GLP-1-XTEN) have no modification to increase potency, resulting in dramatic losses of GLP-1 activity.[41,42] By contrast, c-terminal fusion of Fc to produce GLP-1-Fc and GLP-1-gFc did not alter the activity of N-terminalGLP-1, although no peptide modification was applied for activity enhancement. In addition, the receptor binding of GLP-1 was not influenced by the introduction of O-glycosylation to the hinge region. Whereas dulaglutide has three primary amino-acid substitutions in the GLP-1 portion to enhance durability and potency and reduce immunogenicity,[24] the hybrid Fc fragment of GLP-1-gFc consists of the CH2 domain of IgD with O-glycosylation and the last CH2 and CH3 domains of IgG4[17,18]; the GLP-1 portion has one amino-acid point substitution at the N-terminal to prevent enzymatic cleavage by DPP-4.[43] Adoption of the highly flexible IgD subclass[18,44] or an extended number of amino acids in the hinge region[24] could be considered to be reasonable contributors to these molecular characteristics, which distinguish GLP-1-gFc from dulaglutide. Additional studies, including physicochemical analyses, are needed to better understand these distinctive characteristics.

At present, it is uncertain whether these distinctive safety profiles of GLP-1-gFc would be reproduced in patients with type 2 diabetes, considering differences in physiological status and susceptibility to GLP-1 RAs between healthy subjects and patients. Furthermore, sex as a biological variable,[45] and low animal–human translational success rates limit our findings. However, these uncertainties are alleviated by the highly conserved nature of GLP-1R across species,[39,46] the reproducibility of dulaglutide efficacy and safety profiles across healthy subjects and patients with type 2 diabetes,[47] and the consistent therapeutic efficacy of GLP-1-gFc across species. Also, GLP-1-gFc's gender difference in metabolism, which was evaluated by pharmacokinetics, was not significant in rodent and non-rodent species. In conclusion, we report the development of a novel GLP-1-RA with better safety than and comparable efficacy with dulaglutide, which may provide new therapeutic options for diabetes.

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Conflict of interest

No potential conflicts of interest relevant to this article were reported.

AUTHOR CONTRIBUTIONS

IBA designed and conducted the research, statistical analysis and contributed to project progression, generated figures and wrote the manuscript. MSB managed clinical trials and processed data, generated figures, reviewed the manuscript, contributed to production and supply of GLP-1-gFc protein, and contributed to project progression. SIY commented on research design and results, contributed to project progression and reviewed the manuscript. YRC provided assistance for the in vitro instrumental and cell-based experiments, and wrote sections of the manuscript. JWW commented on clinical research design and results, contributed to project progression and reviewed the manuscript. YCS and HCJ are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity and the accuracy of the data analysis.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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