의학박사 학위논문

당화 GLP-1-Fc와 Dulaglutide (mutated GLP-1-Fc)의 혈당 조절 작용과 위장관계 및 심장 부작용의 비교

A glycosylated Fc-Fused GLP-1 Exhibits an Equivalent Glucose-Lowering Effect, but Lesser Gastrointestinal Side Effects than Dulaglutide

2020년 08월

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지도교수 장 학 철

이 논문을 의학박사 학위논문으로 제출함
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서울대학교대학원
임상의과학과 임상의과학전공
안 인 복

안인복의 박사 학위논문을 인준함
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논문 제목	**A glycosylated Fc-Fused GLP-1 Exhibits an Equivalent Glucose-Lowering Effect, but Lesser Gastrointestinal Side Effects than Dulaglutide**
학위 구분	**석사 □ / 박사 ■**
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1. 본인은 서울대학교가 위 저작물을 인터넷 등 정보통신망을 통해 복제·전송·배포하는 것에 동의합니다.

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제 출 일 : 2020 년 월 일
저 작 자 : 안 인 복 (인)

서울대학교총장 귀하

Abstract

A glycosylated Fc-fused GLP-1 exhibits equivalent glucose lowering effect but lesser gastrointestinal side effect than Dulaglutide

In Bok An
Department of Clinical Medical Sciences
The Graduate School
Seoul National University

Despite their attractiveness as novel antidiabetic agents, GLP-1 receptor agonists (GLP-1 RAs) have provided limited therapeutic benefits due to common drug nonadherence, due mainly to side effects such as nausea, vomiting, and abdominal pain. Considering different GLP-1 receptor density throughout the organs, binding modulation to change receptor binding mechanism could be tried for the invention of novel GLP-1 RAs with better safety profile.

I constructed a novel glycosylated Fc-fused GLP-1 RA (GLP-1-gFc) and determined binding affinity and potency using in-vitro instrumental and cell-based analyses followed by in-vivo comparison of glucose-lowering and 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.

GLP-1-gFc showed 10 times less binding affinity and 4 times less potency than dulaglutide in in-vitro. A potency-adjusted dose delayed HbA1c increase comparable to that of dulaglutide (Change % for 6 weeks: 2.4 mg/kg GLP-1-gFc, 4.34 ± 0.40 versus 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 versus 0.6 mg/kg dulaglutide, 0.04 ± 0.01; $p<0.01$) or QT interval changes (mean at 14h - 20h, mSc: 0.28 mg/kg GLP-1-gFc, 0.0 - 8.0 versus 0.07 mg/kg dulaglutide, 8.0 - 27.7; n.s.), observed as safety parameters in rats and monkeys, compared to those of dulaglutide. Glucose reductions in an oral glucose tolerance test were significant at day 3 post-dose without severe gastrointestinal adverse events and pulse rate changes in healthy subjects.

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

keywords : GLP-1-gFc, dulaglutide, Side effect, Efficacy, Binding affinity, Potency, Clinical trial

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Contents

INTRODUCTION

Diabetes is a very important metabolic syndrome with rapid growth worldwide. According to a report by WHO in 2016, about 4.22 million people, which is a prevalence of 8.5% among the adult population, are diagnosed with diabetes. Prevalence of Type 2 diabetes in the children is also steeply increasing. It is important to treat diabetes because diabetes can lead to various complications in eye, kidney, limbs, and cardiovascular (CV) system lowering the quality of life of patients and even leading to death. Diabetes is one of the top leading cause of death following after cancer and heart disease. CV diseases are known as the most important complication, which is one of the highest causes of death of Type 2 diabetes patients [1,2].

Various antidiabetic drugs have been developed to address the global diabetes epidemic. Representative drug classes include Insulins, Biguanides, Thiazolidinedions (TZDs), Sulfonylureas, Sodium/Glucose cotransporter-2 (SGLT-2) inhibitors, Dipeptidyl peptidase-4 (DPP-4) inhibitors, and Injectable incretin mimetics such as GLP-1, Gastric inhibitory peptide (GIP), Amylin analogues. Although each class of drugs have their strengths, GLP-1 receptor agonist (RA) development has been remarkable in recent decade due to the its multiple beneficial effects on beta cell function, insulin sensitivity, body weight, and the cardiovascular system [3-6], combined with their lack of life-threatening adverse effects such as hypoglycemia [7]. The seven GLP-1 receptor agonists are currently approved for the treatment of type 2 diabetes in US which consist of exenatide, liraglutide, lixisenatide as a short acting (up to once daily) analogues and exenatide extended-release, albiglutide, dulaglutide, semaglutide as a long acting (up to once weekly)

one. These GLP-1 RAs show outstanding glucose lowering efficacy in HbA1c reduction (by 0.8~1.6%) with relatively better safety profiles than other classes [8]. However, the most frequently occurred adverse effects of these GLP-1 RAs are related to gastrointestinal (GI) tract such as nausea, vomiting. These GI disorders and heart rate elevation may impede the widespread use of GLP-1 RAs [9-11].

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-1 RA-related problems [11]. In a retrospective analysis, 53.3% of included patients stopped GLP-1 RA treatment [12]. In addition, heart rate elevation has been observed in clinical trials of almost all GLP-1 RAs [1,13], likely reflecting the direct effect of peripherally administered GLP-1 RAs on cardiomyocytes [14,15], which is more pronounced and sustained for long-acting than for short-acting drugs [9]. The heart rate increase caused by long-acting GLP-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.

Side effects of GLP-1 beyond glucose control may derive from the direct/indirect effect of GLP-1 on GLP-1 receptors which are localized in various organs such as Brain, Heart, Stomach, Intestines, Kidney, etc [16,17]. Especially localization of GLP-1 receptors in Central Nervous System and Enteric Nervous System resulting in the anorexic effect [18,19] and reduction of gastric emptying [20,21] respectively, seem to be associated with the nausea and vomit of GLP-1 analog. More specifically, peripheral GLP-1 is signaling via vagal afferent neurons in the intestines to nucleus of the solitary tract

(NTS), area postrema (AP) in the brain stem, and hypothalamus promoting nauseous symptoms. Also the existence of GLP-1 receptors in heart was identified in various species such as rodent [22], monkey [23], and human [15,24] and its direct effect on the calcium homeostasis of mouse cardiac muscle cell lines was studied [14,25]. Maybe these evidences could explain the increase of heart rate in diabetes patients [9,26] by administration of GLP-1. Medicines with reduced side effects have been developed by modulating receptor binding affinity; nimotuzumab [27,28], for example, is a humanized monoclonal antibody (mAb) with 10 times the dissociation constant (Kd) for binding to epidermal growth factor receptor (EGFR) as other mAbs. The requirement for bivalent binding for stable signaling also distinguishes nimotuzumab from other mAbs. The screened Kd values of nimotuzumab, between 10^{-9} M to 10^{-8} M, shows maximal binding and internalization of mAbs in the tumor and minimal binding and internalization of mAbs in non-cancerous tissues. Due to these characteristics, nimotuzumab has not induced severe skin toxicity, hypomagnesemia, or adverse gastrointestinal (GI) effects, while showing activity similar to that of other EGFR mAbs, in preclinical and clinical studies [29].

Dulaglutide, brand name Trulicity, is a once-weekly GLP-1 RA which was approved for the treatment of T2DM in combination with diet and exercise by US UFA in 2014. According to the report by ClinCalc.com, Dulaglutide was the 232nd most commonly prescribed medication in US, with more than two million prescriptions. Dulaglutide, a IgG4 Fc fused GLP-1, has a half-life of 38.2h - 51.6h in animals [30] and approximately 5 days in human [31], making it suitable for once-weekly administration. Efficacy of glucose control measured by HbA1c reduction was 0.71 - 1.51% in their phase 3 pivotal trials without life-threatening side effects [32]. Even though its success, some concerns related to heart and gastrointestinal tract were

exist during the development phase. Regarding to the heart, dulaglutide showed concentration dependent increase in human Ether-a-go-go-Related Gene (hERG) current inhibition in hERG transfected HEK cell lines indicating potential effects on action potential of repolarization phase of cardiomyocytes. Also dulaglutide showed dose-related increase of heart rate and corrected QT interval changes in safety pharmacology, 4-wk and 13-wk chronic toxicity studies with cynomolgus monkeys [33].

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

RESEARCH DESIGN AND METHODS

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). 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[29,30]. 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).

GLP-1-Fc and GLP-1-gFc structure modeling

The binding structure of the GLP-1 receptor to each molecule was modeled using the COOT program [36]. 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 (ver. 2.0) [37] using human IgG4 Fc (PDB no. 4C54) as a template. Figures illustrating binding structure modeling were prepared using the Pymol program [38].

Preparation of GLP-1 peptide, GLP-1-Fc, GLP-1-gFc

A Fc or glycosylated Fc fused GLP-1 were produced by cell engineering technology. In brief, polynucleotide sequence encoding GLP-1-A2G-Fc and GLP-1-A2G-gFc were constructed based on the amino acids sequence of each molecules by TOP Gene Technologies Inc. (Quebec). These were subcloned into highly efficient mammalian cell expression vector, pAD15, and transfected into CHO DG44 cell line. The Final research cell bank (RCB) was selected by MTX amplification and limiting dilution cloning test for selection of single clone. Obtained RCB was underwent 3 sub-culture and 1 main culture to produce each target molecules. Media containing target molecules were purified using 3 column steps consisting of affinity chromatography, hydroxyapatite chromatography, and anion exchange chromatography followed by filtration and formulation to prepare final drug substances for studies. GLP-1 peptide was purchased from Bachem (Switzerland) and used as a control. In here, Fc is a hybrid Fc-fragment which consist of the O-glycosylated upper CH2 domain of IgD and the last CH2 and CH3 domains of IgG4. Also GLP-1 has one point amino acid substitution at n-terminal to prevent enzymatic cleavage by DPP-4.

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-specific luciferin- 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% FBS, 130 ug/mL Hygromycin B Gold, 5 ug/mL puromycin] were seeded in a T-75 flask and placed in a CO2 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 h, 20 uL/well GLP-1-gFc at various concentrations was treated and reacted in a CO_2 incubator at 37°C for 5 h. Bright-GloTM assay reagent (100 uL/well; Promega Corporation, Madison, WI, USA) was added and reacted at room temperature for 2 min, and luminescence was then measured using a luminometer (BioTek Instruments Inc., Winooski, VT, USA).

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 [39,40]. 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 analyzed 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 min for hydration. The initial step was conducted for 150 s with assay buffer. Next, 5 ug/mL recombinant human GLP-1 receptor (Abcam) was immobilized on the surface of the biosensor for 150 s and then transferred to fresh assay buffer for 150 s to establish a baseline. The association of various TA concentrations (1250-20,000 nM) was measured for 300 s, followed by dissociation measurement for 300 s 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.

Electrophoresis study (SDS-PAGE, gel-IEF) for GLP-1-gFc

Electrophoresis studies (SDS-PAGE and Gel-IEF) were conducted to identify the molecular size (kDa) and isoelectronic point (pI) of GLP-1-gFc. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of GLP-1-gFc was conducted at room temperature (RT) on the Xcell SureLock® Mini-Cell Electrophoresis system (Invitrogen, USA). One volume of NuPAGE® LDS sample buffer (Invitrogen) was mixed with three volumes of GLP-1-gFc solution to make samples for reducing or non-reducing condition. For reducing condition, the samples were heated at 70°C for 10 min. 2 ug of GLP-1-gFc was loaded to a NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm (Invitrogen). After 120 V constant current for 90 min, Gels were stained with CoomassieTM Blue

PhastGel R-350 (GE, USA) staining solution for 30 min with gentle shake. Stained gel was transferred to de-staining solution which consisted of 10% acetic acid and 30% methanol for 60 min.
Gel isoelectric focusing (IEF) was performed at 2-8°C on the Xcell SureLock® Mini-Cell Electrophoresis system (Invitrogen, USA). Appropriately diluted Novex® IEF anode buffer solution (50-fold, Invitrogen) and Novex ® pH 3-7 cathode buffer solution (10-fold, Invitrogen) were utilized as electrophoresis buffer. 10 ug of GLP-1-gFc was applied to a pH 3-7 IEF gel (1.0 mm, Invitrogen) for 1 hour at 100 V and 1 hour at 200 V, followed by 500 V for 1 hour and the focused protein was precipitated with 12% TCA for 20 min. Precipitated gels were stained with CoomassieTM Blue PhastGel R-350 (GE, USA) staining solution for 30 min with gentle shake followed by transfer to the de-staining solution for 60 min.

Size-Exclusion Chromatography (SEC) for GLP-1-gFc

The purity of GLP-1-gFc was analyzed using Waters Alliance e2695 separation module HPLC system. Blank solution, Test solution, and Standard solution of GLP-1-gFc were injected serially into the TSK-GEL G3000SWxL (7.8 * 300 mm) (TOSOH, Japan) with mobile phase of Sodium phosphate buffer containing 10% Acetonitrile. Flow rate was 0.5 mL min-1 and elution profiles were monitored at 214 nm. Column temperature was maintained at 25°C through the analysis. Test suitability was evaluated by lack of peak for blank solution and %CV (coefficient of variation) within triplicates of test or standard solution. Peak at retention time of 18 min was analyzed for present % purity of GLP-1-gFc.

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, other animals [obese (C57BL/6J-ob/ob), CD-1 mice and Sprague-Dawley (SD) rats] obtained from DBL (Eumseong, Korea), Koatech (Pyeongtaek, Korea), SLC (Shizuoka, Japan), respectively, were housed in appropriate numbers in cages with a 12/12-h 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 parameters were enrolled.

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 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 min (omitted for plasma) and centrifuging at 3000 × g for 10 min. 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-specific GLP-1 antibody (Thermo Fisher, Waltham, MA, USA) were used for antibody capture and detection, respectively. Pharmacokinetic parameters were analyzed using non-compartmental methods with Pharsight WinNonlin® software (version 12.5) (Mountain Veiw, CA, USA).

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 h post-dose. 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 h post-dose. Serum was prepared and analyzed with a GLP-1-gFc ELISA as described previously. Pharmacokinetic parameters were analyzed using non-compartmental methods with Pharsight WinNonlin® software

(version 12.5).

Evaluation of dose-dependent anti-diabetic effect of GLP-1-gFc in db/db mice

To evaluate dose-dependent anti-diabetic effect of GLP-1-gFc in the progress of type 2 diabetes, five-week-old male diabetic (C57BL/KSJ-db/db) mice were acclimated to 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 glycated hemoglobin (HbA1c) were measured at sacrifice. Glucose was analyzed using GM9 Glucose Analyser (Analox Instruments Ltd, UK). Insulin and HbA1c were measured by rat insulin radioimmunoassay (LINCO Research, MO, USA) and Vantage analyzer (Siemens, Munich, Germany), respectively.

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 to those of dulaglutide. All test materials were diluted and analyzed 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 glycated hemoglobin (HbA1c) was measured biweekly.

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 min 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.

Assessment of Anti-Diabetic/Obesity Effects in ob/ob Mice

To compare anti-diabetic and anti-obesity effect of GLP-1-gFc with dulaglutide in the obese condition, Six-week-old female obese (ob/ob) mice26 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 analyzed 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.

CTA Study

A CTA study was performed to determine the malaise effects of the nausea-inducible TAs, with modification of a previously described protocol [44,45]. Acclimated 5-week-old male DBA/2J mice were housed individually and given 10 min access to a pre-weighed 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.

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) [46]. The GLP-1-gFc doses were obtained by multiplying the dulaglutide dose by 4 (equivalent; low) and approximately 16 (high). Blood pressure, heart rate, and ECG waveforms were recorded from 2 h pre-dose to 24 h post-dose. ECG was performed for ≥30 s pre-dose (at least 30 min apart) and 2, 4, 8, 12, 16, and 24 h post-dose. ECG data were used to calculate corrected QT intervals (QTcs).

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 BMIs 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, hematological, 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) per the safety monitoring committee. The starting dose was based on the no-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, four-fold higher than the dulaglutide dose causing side effects in phase 1 clinical trials[47]. The percentages of subjects who experienced

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

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.

Pharmacokinetic Analysis

Blood samples for pharmacokinetic analysis were collected by venous puncture or indwelling venous catheter into serum separation tubes, pre-dose and between 0.25 and 648 h post-dose. Serum GLP-1-gFc concentrations were determined using a previously described GLP-1-gFc ELISA with validation. Pharmacokinetic parameters were analyzed 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 (AUClast) and maximum serum concentration (Cmax) of GLP-1-gFc were plotted with each dose to assess proportionality.

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 min. Blood samples were taken before and 0.25, 0.5, 1, 1.5, and 2 h 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 OGTT was recorded.

Statistical Analyses

SPSS 21 (IBM SPSS, Chicago, IL, USA) and SAS ver 9.4 were used to analyze the data. Outlier among the data was excluded before statistical analysis using 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 were evaluated using One-way ANOVA followed by Tukey's and Dunnett's T3 in case of parametric data. For non-parametric data, Mann-Whitney U test was performed. For the analysis of sample size and statistical power Medcalc software was used. All clinical data was evaluated only descriptively. Differences were considered statistically significant at $P < 0.05$.

RESULT

In Vitro Potency and Binding Affinity

The GLP-1-gFc and dulaglutide structures were distinguishable based on glycosylation and amino acid modification (Fig. 1A). GLP-1-gFc is glycosylated Fc-fused GLP-1 peptide with molecular size of around 70 kDa and high purity (98% by SEC-HPLC) (Fig. 2). 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 (Fig. 3 and Fig. 4). These findings were supported by three-dimensional structure prediction using Phyre web-based software (Fig. 5). 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 lesser than that of dulaglutide, with a 3.5-fold higher half maximal effective concentration (23.33 vs. 6.66 pM; Fig. 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 did dulaglutide in the dissociation phase (Fig. 1C). The Kd for GLP-1-gFc (6.43×10^{-2}) was about 10-fold higher than that for dulaglutide, whereas the association constant (4.02×10^{3}) was 1.7-fold different. This lower binding affinity of GLP-1-gFc was confirmed by biolayer interferometry (Fig. 6). Equilibrium Kds for GLP-1-gFc and dulaglutide were 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.

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 (△glucose = 241 mg/dL); all TA-treated groups showed decreases relative to this level with statistical significance only in 2.4 mg/kg GLP-1-gFc group (Fig. 7). Dulaglutide prevented an increase in the non-fasted glucose level (terminal, 348 mg/dL; △glucose = 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, △glucose = 185 mg/dL; 2.4 mg/kg: terminal glucose level = 355 mg/dL, △glucose = 80.1 mg/dL; Fig. 7A). 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; Fig. 7B). GLP-1-gFc's comparable glucose homeostatic effects to dulaglutide were confirmed and mediated by GLP-1 stimulated insulin secretion (Fig. 8). These results indicate that about four-fold more GLP-1-gFc than dulaglutide is required for equivalent anti-diabetic efficacy.

Glucose-Lowering and Weight Loss Effects in Obese Mice

GLP-1-gFc and dulaglutide delayed HbA1c increase compared to vehicle with statistical significance only in 2.4 mg/kg GLP-1-gFc group (0.9% and 1.1%, respectively, vs. 2.0%; Fig 9A). Dulaglutide decreased cumulative food intake and body weight significantly compared with vehicle (-17 g/cage and -1.9% vs. vehicle; Fig. 9B and C). GLP-1-gFc

showed weaker trends for these parameters; △body weight differed significantly between 2.5 mg/kg GLP-1-gFc and dulaglutide in weeks 1 and the gap was 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.

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 (Fig. 10A). GLP-1-gFc caused much less reduction in consumption, differing significantly from the effect of dulaglutide (Fig. 10B). 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. In contrast, overnight food intake on day 1 post-injection was dramatically reduced in the GLP-1RA-treated groups (Fig. 11). 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 (Fig. 12). Dulaglutide increased the QTc at 10-20 h, the predicted time to maximum plasma concentration (Tmax), 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 dulaglutide.

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/m². 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 h in all groups (Fig. 13A, Table 2). Serum concentrations peaked at about 36-48 h post-dose, with mean Cmax 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 AUClast and Cmax were observed (R^2 = 0.9302 and 0.9925, respectively; Fig. 13B and 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 h, respectively (Fig. 14 and Table 3).

In the OGTT, GLP−1−gFc dose−dependently decreased the area under the curve on the glucose vs. time plot (gAUC) from baseline (day −1; Fig. 15A−C). In general, this decrease was greater at 3 than at 5 days post−dose, consistent with the Tmax of 36−48 h. 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 and dose−dependency were also remarkable on day 3 (−55.7% and −53.0%, respectively, vs. baseline; Fig. 15C). In contrast to 0.24 mg/kg, reduced gAUC values by 0.08− and 0.16−mg/kg were rebounded at day 5 maybe due to insufficient drug concentration above 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 (Fig. 15D). No pulse rate change from baseline was observed on day 3 or 5 post−dose (Fig. 15E). These trends were consistent with other TEAEs related to GI symptoms (Table 4), diastolic blood pressure (Table 5), and pulse rate change (Table 6) monitored throughout the study period. These clinical results are consistent with the preclinical observations of lesser nausea/vomiting and QTc responses than glucose lowering, in contrast to dulaglutide.

DISCUSSION

GLP-1 RA side effects, especially nausea and vomiting, are not life threatening, but affect therapeutic outcomes significantly due to 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, i developed a novel long-acting GLP-1 RA with fewer side effects, and glucose-lowering ability comparable to 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 due to 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 [10]. Meier [19] 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 [48], and similar trends in other clinical trials of long-acting GLP-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 behavioral analysis of nausea and malaise [10]. Kanoski et al. reported that CTA response of

liraglutide was lesser than did exendin-4 although its comparable food intake suppression to 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 above reductions in HbA1c, fasting and non-fasting glucose levels, and increase in insulin secretion based on a supplementary study with diabetic db/db mice (Fig. 12). This difference in CTA response was more profound than that in food intake suppression in ob/ob mice. TA-induced electrophysiological signaling changes in the heart were also consistent with the CTA results. Equivalent efficacy and four-fold greater GLP-1-gFc doses did not alter the QTc, whereas dulaglutide increased it, especially at Tmax. Dulaglutide-induced QTc changes, and likely-associated heart rate increases, were observed in cynomolgus monkeys in pharmacological safety and chronic toxicology studies involving single and multiple subcutaneous injections, respectively [33] (Table 6). 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 26 weeks which have conducted as IND enabling studies that are not 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 comparable pharmacokinetics to 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 [47]. 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 (Table 6). GLP-1-gFc's glucose reduction was not related closely to 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 to other EGFR mAbs [29]. Similar to EGFRs, GLP-1Rs are expressed at different levels in various organs (e.g., heart, stomach, intestine, kidney) and the central/vagal/enteric nerves [17,22]. The vagal nerve and heart, mainly responsible to nausea/vomiting and heart rate, have low GLP-1R expression [23] (vs. the pancreas, mainly responsible to 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 [49], it would be inappropriate to simply divide vagal nerve dependent nausea/vomiting or pancreas dependent glucose lowering effect. But combining with the fact that the nausea/vomiting by GLP-1R is restrictedly related to nervous system [50] and GLP-1R expression levels are important for the maximal efficacy/potency of low-affinity molecules [51], the novel GLP-1-gFc concept could be still 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-acting GLP-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 [52], 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 [53,54]. In contrast, c-terminal fusion of Fc to produce GLP-1-Fc and GLP-1-gFc did not alter the activity of N-terminal GLP-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 [41], 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 [34,35]; the GLP-1 has one amino-acid point substitution at the N-terminal to prevent enzymatic cleavage by dipeptidyl peptidase 4 [55]. Adoption of the highly flexible IgD subclass [35,56] or an extended number of amino acids in the hinge region [30] 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 and patients. Furthermore, sex as a biological variable (SABV) [57], low animal-human translational success rates limit our findings. However, these uncertainties are alleviated by the highly conserved nature of GLP-1R across species [51,58] , the reproducibility of dulaglutide efficacy and safety profiles across healthy subjects and patient with type 2 diabetes [32], and 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, i report the development of a novel GLP-1-RA with better safety than and comparable efficacy to dulaglutide, which may provide new therapeutic options for diabetes.

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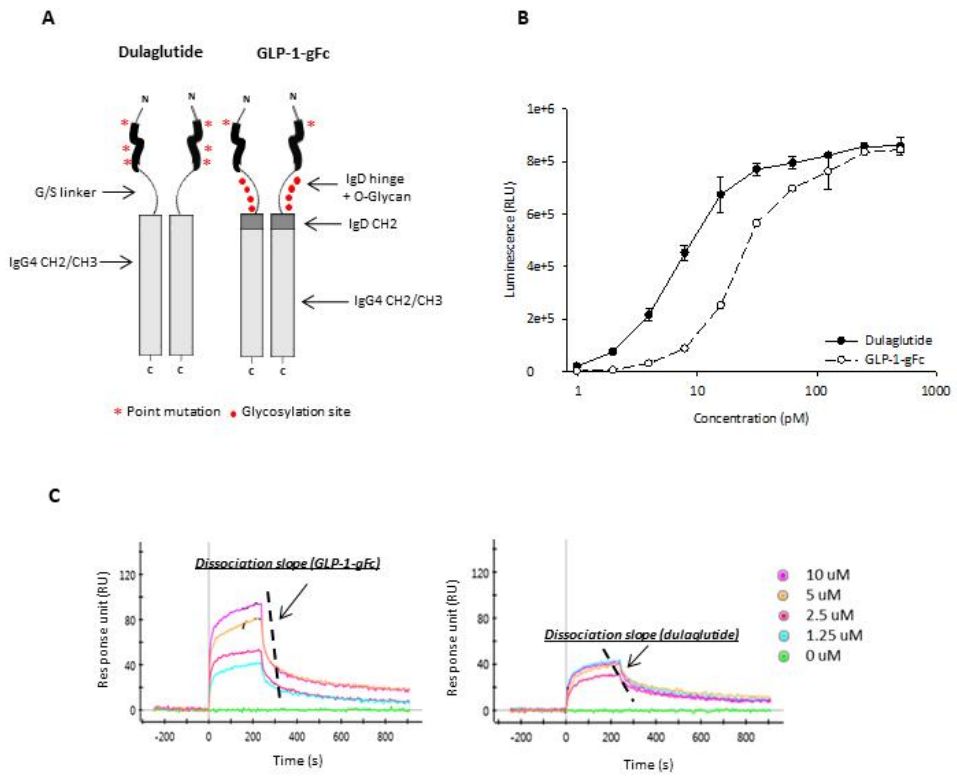
FIGURES

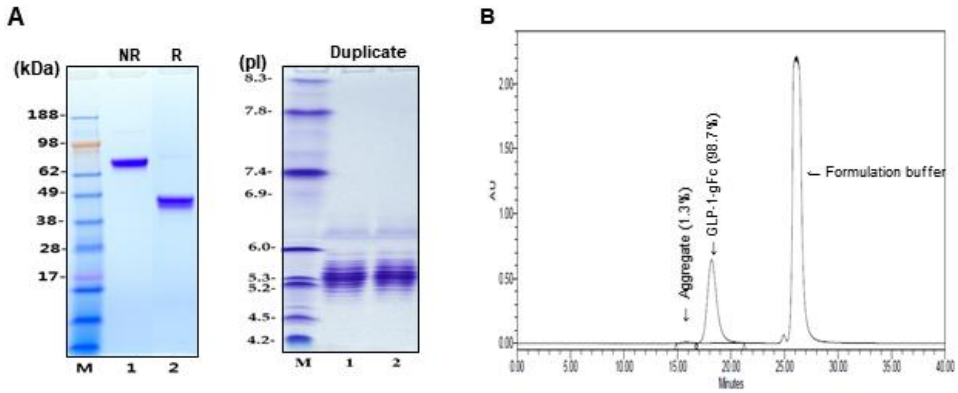
Figure 1. GLP-1-gFc had a higher dissociation constant (Kd) and lower receptor-mediated response than did dulaglutide due to structural differences.



A: Schematic diagram of the two molecules. B: cAMP dependent luminescence of various concentrations of GLP-1-gFc and dulaglutide in GLP-1R over-expressing cells. C: Sensorgrams of binding affinity, determined by surface plasmon resonance (SPR) analysis. Results (means and means ± standard errors of the mean) are representative of more

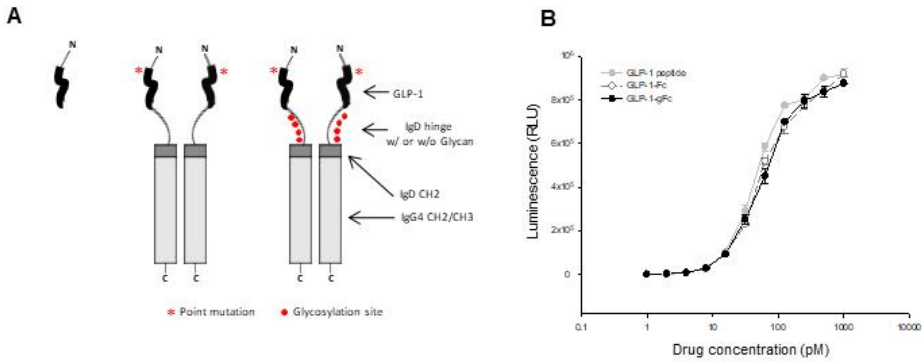
than two independent experiments. K_a, association constant; KD, dissociation constant at equilibrium; RLU, relative luminescence unit.

Figure 2. Physico-chemical characterization of the GLP-1-gFc



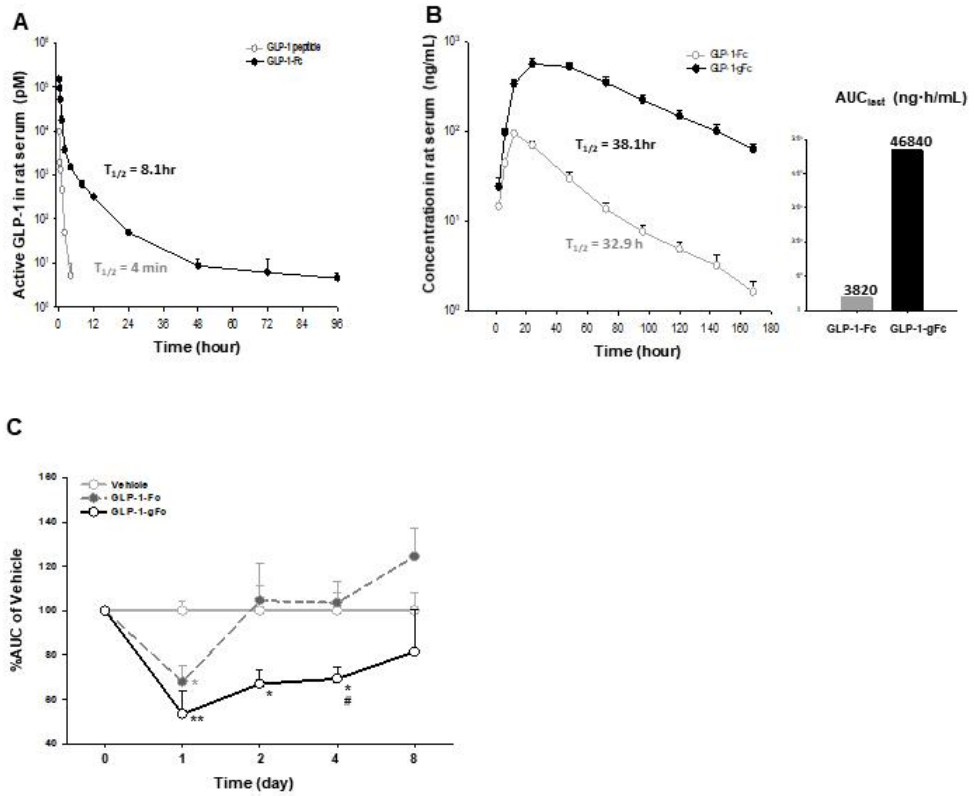
A: Representative results of SDS-PAGE (left) and gel-IEF (right). The molecular size of GLP-1-gFc was identified bewteen 62 kDa and 98 kDa. When reduced, the size was bewteen 38 kDa and 49 kDa, indicating the cleavage of S-S bond between GLP-1-gFc monomers. pI value of GLP-1-gFc was identified between 5.2 and 6.0 with main band at 5.3. Because of its glycosylated Fc, ladder shaped band was formed. B: Representative peak and purity of GLP-1-gFc measured by Size-Exclusion Chromatography. The peak of GLP-1-gFc was identified between 18 min and 19 min. The purity was 98.7% with 1.3% aggregate between 15 min and 16 min. NR, Non-reduced sample; R, Reduced sample; M, Size marker; pI, Isoelectric point;, kDa, Killodalton; AU, Absorbance units.

Figure 3. Introduction of O-glycan to the hinge region of Fc show no loss of *in-vitro* potency.



A: Schematic structure of GLP-1 peptide, GLP-1-Fc, and GLP-1-gFc. B: in-vitro activity in a transgenic cAMP-specific luciferin- and GLP-1 receptor (GLP-1R)-expressing cell line (GLP1R_cAMP/luc). Results are presented as the mean ± SEM.

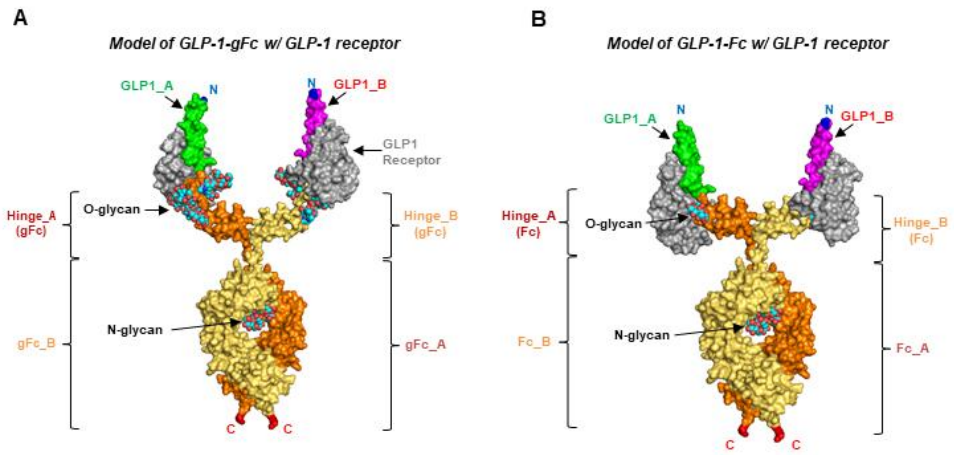
Figure 4. Comparison of pharmacokinetics and pharmacodynamics between GLP-1 peptide, GLP-1-Fc, and GLP-1-gFc to identify the effect of Fc fusion and/or O-glycosylation.



A: Pharmacokinetics of GLP-1 peptide and GLP-1-Fc after IV administration of in SD rat (n = 4/group). B: Pharmacokinetics of GLP-1-Fc and GLP-1-gFc after SC administration in SD rat (n = 4/group). PK parameters were analyzed using non-compartmental methods using Pharsight WinNonlin software (ver 12.5). C: IPGTT results of GLP-1 peptide, GLP-1-Fc, and GLP-1-gFc in CD-1 mice that were received each test molecules via SC route followed by IP

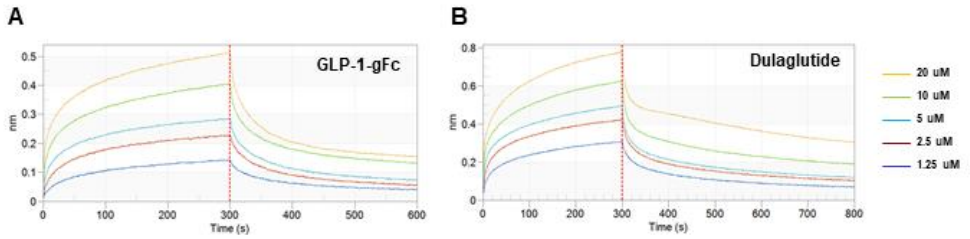
challenges of 2g/kg glucose (n = 4/group/day). The AUC of changed glucose level on each day were calculated and converted to %AUC of vehicle to plot %AUC versus time. Results are presented as the mean ± standard deviations for PK and mean ± SEM for others. * $p<0.05$, ** $p<0.01$, ***$p<0.001$ vs. vehicle group, # $p<0.05$ vs. GLP-1-Fc group. One-way ANOVA followed by Tukey's and Dunnett's T3 test as a post-hoc analysis. T1/2, half-life; AUClast, The area under the serum concentration time curve to the time of last measurable concentration.

Figure 5. Introduction of O-glycosylation to hinge region do not give any structural interference for receptor binding between GLP-1 and GLP-1Rs which is predicted by structure modeling.



A: The modeling of binding structure between GLP-1 receptor and GLP-1-gFc. B: The modeling of binding structure between GLP-1 receptor and GLP-1-Fc. Structure of the GLP1-GLP1 receptor complex (PDB 3IOL) and human IgG4 (PDB 4C54) were adopted from RCSB PDB (Protein Data Bank). The Fc and gFc, which are consist of IgD and IgG4, were obtained from Phyre v2.0 software using human IgG4 Fc (PDB 4C54) as a template. The modeling figures of binding structure were prepared by Pymol software.

Figure 6. Lower binding affinity of GLP-1-gFc than Dulaglutide was confirmed by BLI system.

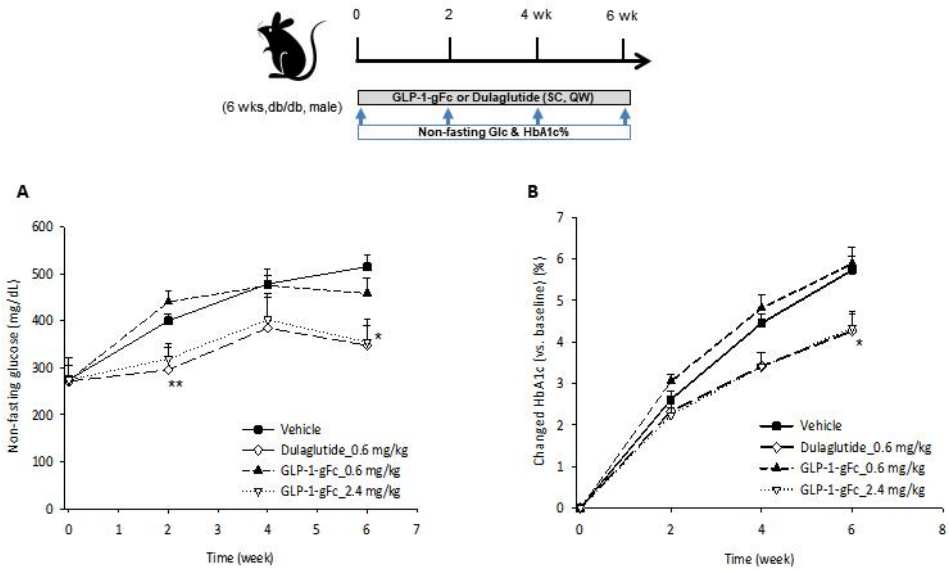


C

Test item	KD (M)	k_{on}(1/Ms)	k_{dis}(1/s)	R^2
GLP-1-gFc	4.50E-05	2.83E+03	1.32E-01	0.948
Dulaglutide	3.95E-07	3.27E+04	6.78E-03	0.903

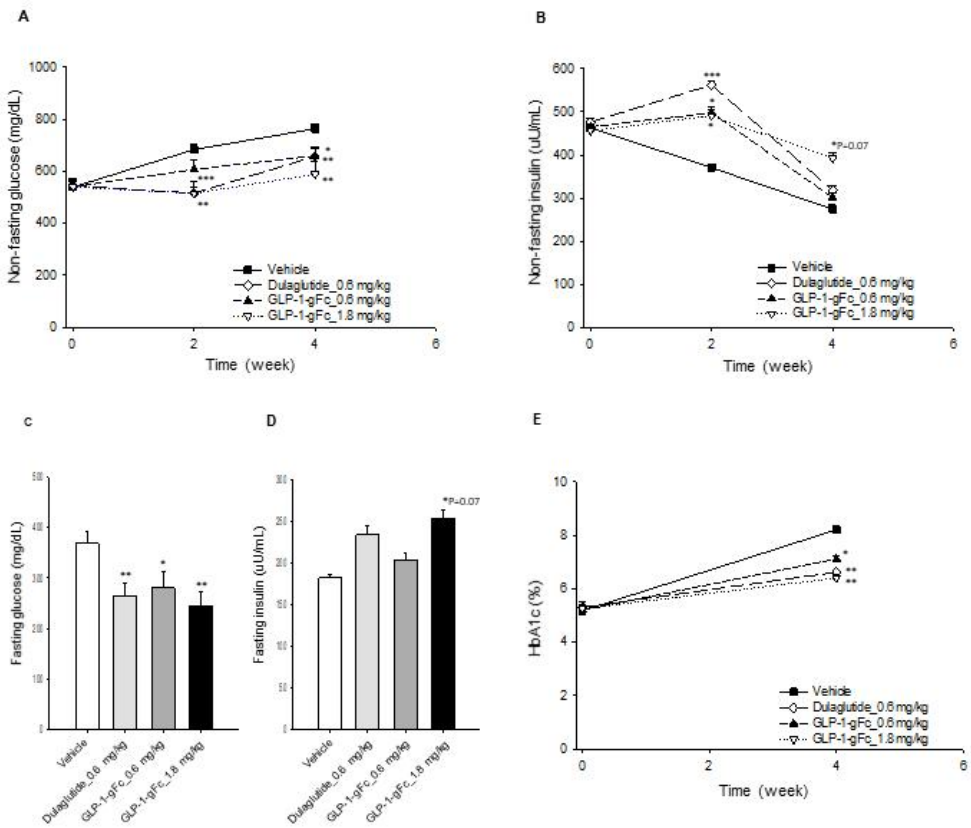
A, B: Representative sensorgrams of GLP-1-gFc and Dulaglutide's binding affinity measured by Biolayer interferometry (BLI). C: Mean of affinity parameters. The assay was repeated three times and new biosensors were used for each test article. KD, dissociation constant at equilibrium; Kon, association constant; Kdis, dissociation constant; R2, R-squared.

Figure 7. A four-times-higher dose of GLP-1-gFc had a glucose-lowering effect comparable to that of dulaglutide in diabetic (db/db) mice.



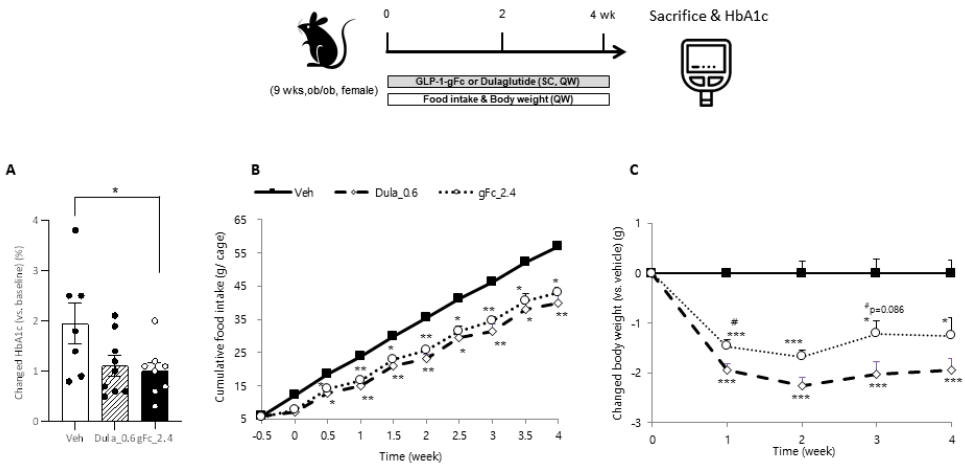
Acclimated 6-week-old male diabetic (db/db) mice were allocated to four groups based on the non-fasting blood glucose. TAs were administered subcutaneously weekly for 6 weeks. A: Non-fasting glucose level (n = 5-8/group). B: Changed HbA1c from baseline (△HbA1c) (n = 5-8/group). Results are presented as means ± standard errors of the mean. *$p < 0.05$, **$p < 0.01$ vs. vehicle, One-way ANOVA followed by Tukey's and Dunnett's T3 test as a post-hoc analysis.

Figure 8. GLP-1-gFc exhibited dose-dependent insulin secretion and glucose reduction with equivalent efficacy at 3 times higher dose than dulaglutide in male db/db mouse.



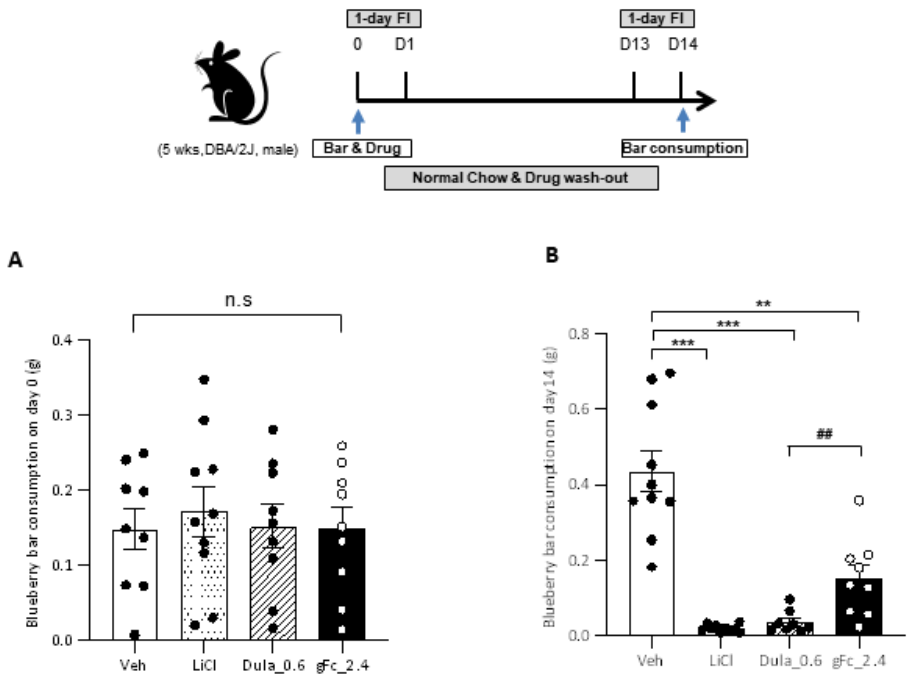
A-B: Non-fasting glucose and insulin level for 4 weeks treatment period (n = 12/group). C-D: Fasting glucose and insulin before sacrifice at 4 weeks after weekly dose of test articles (n = 11-12/group). E: Change of HbA1c for 4 weeks (n = 12/group). Results are presented as means ± standard errors of the mean. *p < 0.05, **p < 0.01, ***p < 0.01 vs. vehicle, Two-tailed student's T-test.

Figure 9. An equivalent efficacy dose of GLP-1-gFc showed similar glucose-lowering efficacy, but a weaker weight loss effect than did dulaglutide in obese (ob/ob) mice.



Acclimated 9-week-old female obese (ob/ob) mice were allocated to three groups based on the body weight. TAs were administered subcutaneously weekly for 4 weeks. A: Changed HbA1c from baseline (△HbA1c) 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 < 0.05, ** p < 0.01, *** p < 0.001 vs. vehicle, # p < 0.05 vs. 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.

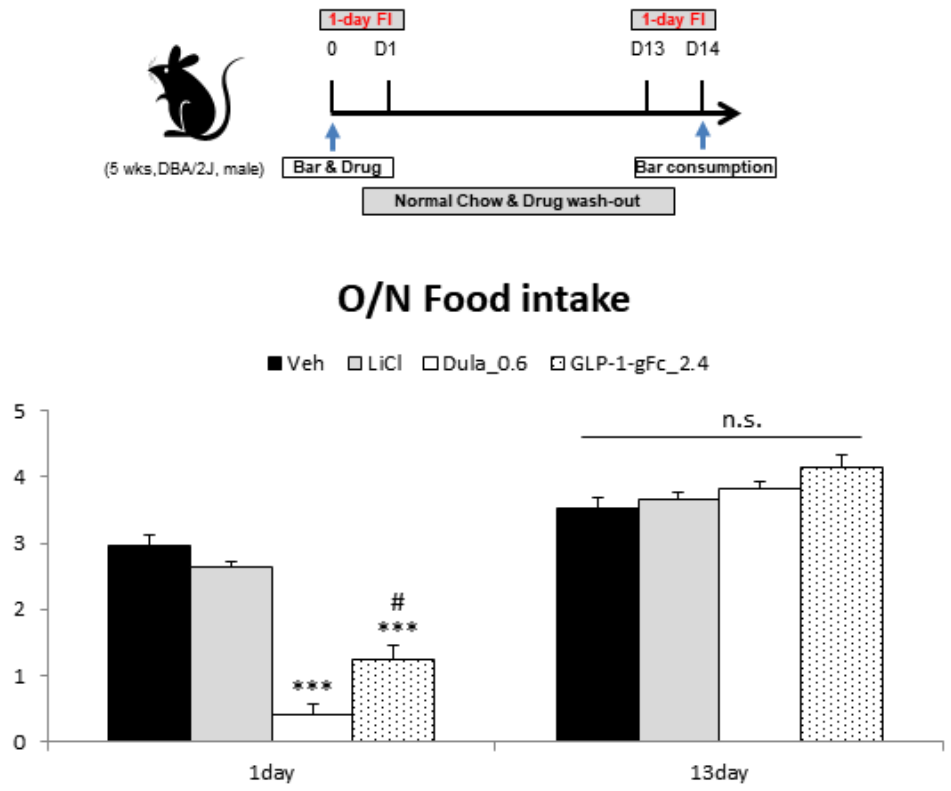
Figure 10. Compared with dulaglutide, GLP-1-gFc induced significantly lesser nausea/vomiting in mouse CTA study.



5-week-old male DBA/2J mice were given 10 min access to a blueberry bar and then consumption was weighed. By comparing the consumption of bluberry bar between before and after Test articles (TA) administration, nausea/vomit effect of TA was measured. A: Blueberry bar consumption before TA administration (n = 9-10/group). B: Blueberry bar consumption after TA administration (n = 8-10/group). TA was washed-out for 13 days to make sure the clearance of TA so that TA's anorexigenic effect could be completely excluded. Results are presented as means ± standard errors of the mean. **p < 0.01, ***p < 0.001 vs. vehicle, ##p < 0.01 vs. dulaglutide, Mann-Whitney U test was used for statistical analysis. n.s., non-significant; Dula_0.6, dulaglutide

0.6 mg/kg; gFc_2 GLP-1-gFc 2.4 mg/kg.

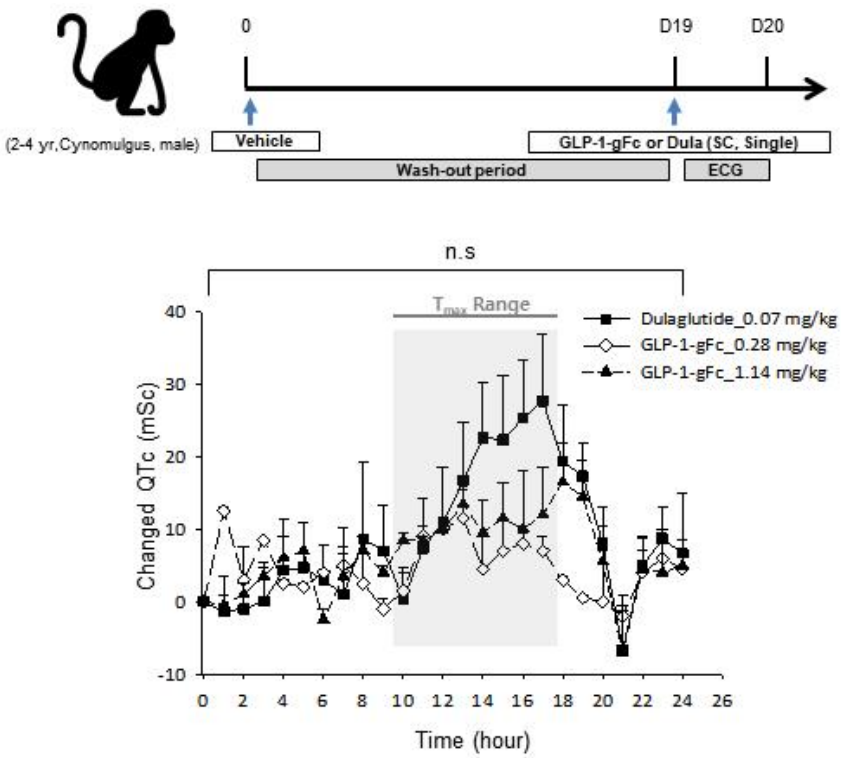
Figure 11. Confirmed drug-wash out evaluated by overnight food intake before the second exposure to blueberry bar in CTA study (n = 8-10/group).



Overnight food intake on day 1 post-injection was dramatically reduced in the GLP-1RA-treated groups. In contrast, one day before the second exposure (day 13), 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. Results are presented as means ± standard errors of the mean. ***p < 0.001 vs. vehicle, #p < 0.01 vs. dulaglutide, Mann-Whitney U test. n.s., non-significant;

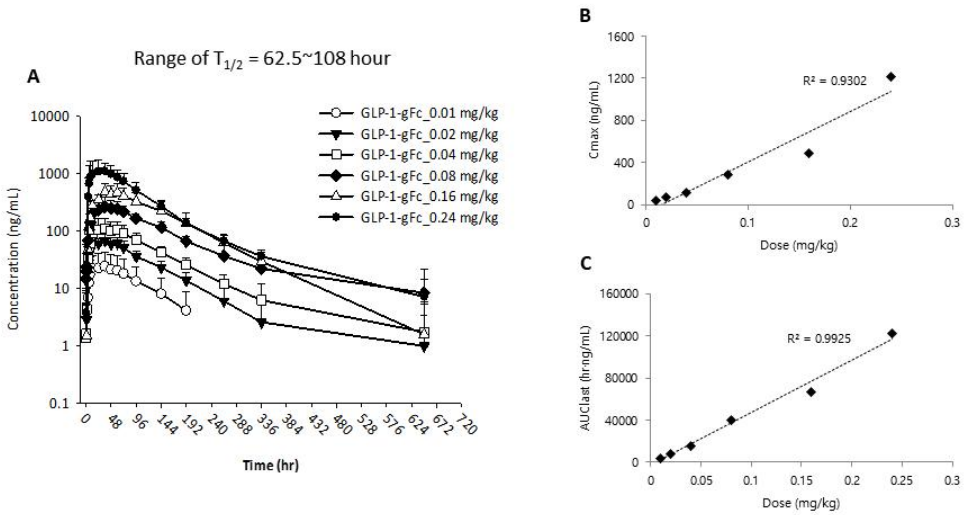
Dula_0.6, dulaglutide 0.6 mg/kg; gFc_2.4, GLP-1-gFc 2.4 mg/kg.

Figure 12. GLP-1-gFc induced lesser QT elongation responses in Telemetry implanted monkey ECG study compared with dulaglutide.



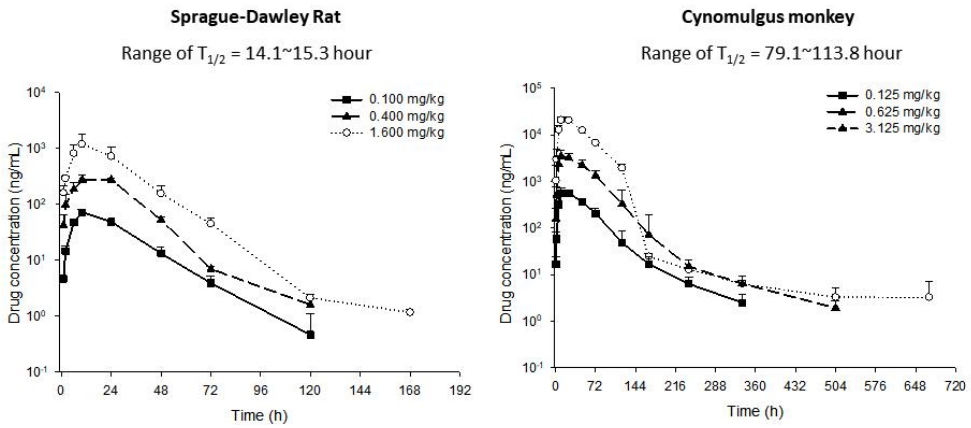
Telemetry-implanted cynomolgus monkeys (n = 2-3/group) were subcutaneously administered single doses of vehicle. After a 19-day washout period, they received single subcutaneous injections of each TAs and then Electrocardiogram (ECG) was monitored from 2 h pre-dose to 24 h post-dose. Corrected QT interval (QTc) was calculated from ECG data. Results are presented as means ± standard errors of the mean. One-way ANOVA was used for statistical analysis. n.s., non-significant; Dula_0.6, dulaglutide 0.6 mg/kg; gFc_2 GLP-1-gFc 2.4 mg/kg.

Figure 13. GLP-1-gFc exhibited dose-dependent pharmacokinetics following single subcutaneous administration in healthy men.



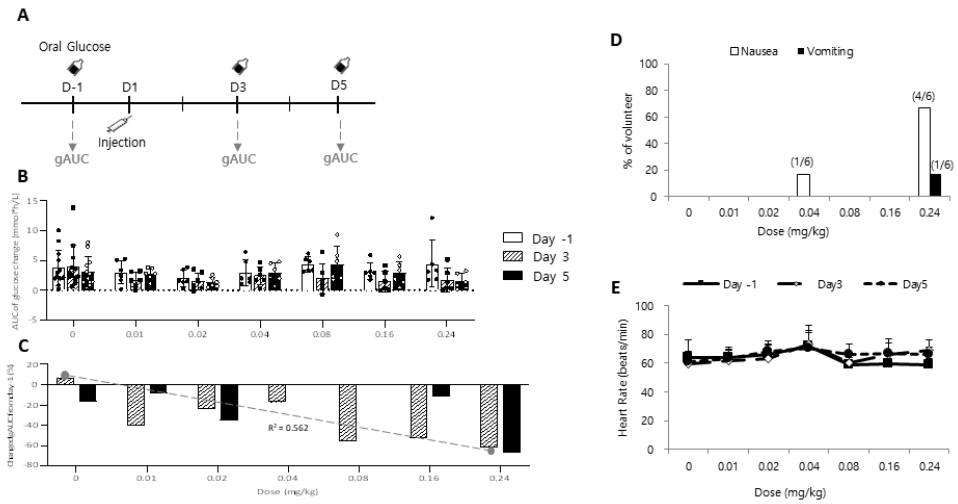
Blood samples were collected by venous puncture into serum seperation tubes, pre-dose and between 0.25 and 648 h post-dose. Serum concentrations of GLP-1-gFc were determined using a validated GLP-1-gFc ELISA. Pharmacokinetic parameters were analyzed using non-compartmental analysis (NCA) with Pharsight WinNonlin software (ver 12.5). A: Drug concentration in serum (n = 6/cohort). B: Trend line of Dose versus Cmax. C: Trend line of Dose versus AUClast. Results are presented as means ± standard deviations. Only descriptive statistics was applied.

Figure 14. GLP-1-gFc shows Dose-dependent PK profiles in SD rats and Cynomulgus monkeys (n = 3/sex/dose) after single SC administration.



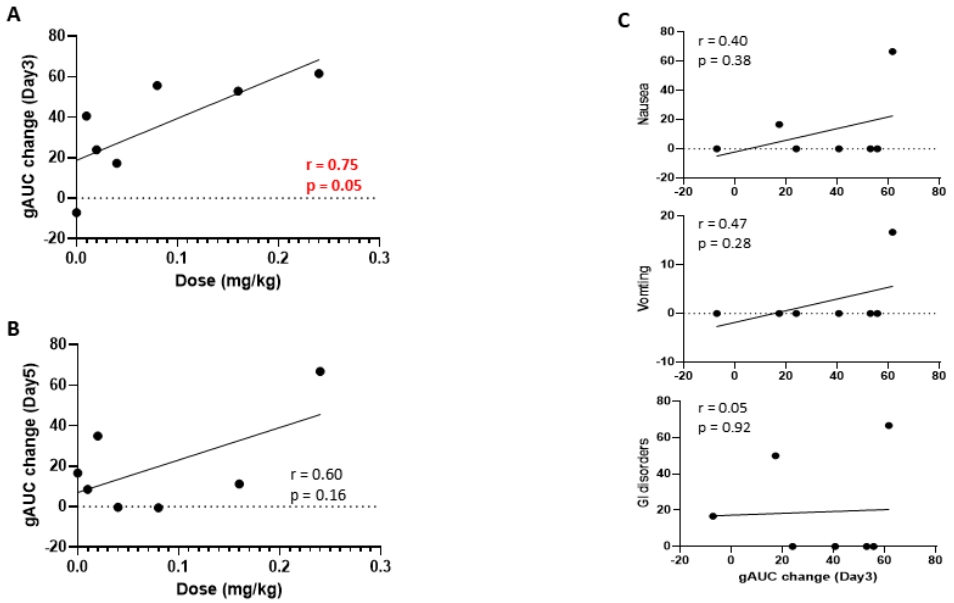
Eight-week-old male SD rats (n = 4/groups) received three different doses of GLP-1-gFc. Male and Female (n = 3/sex/dose) cynomolgus monkeys received three different doses of GLP-1-gFc. Blood samples were collected at designated time points. Collected serum samples were analyzed using GLP-1-gFc specific ELISA method where mouse Anti-human IgG4 and n-terminal specific GLP-1 antibody were used as a coating and detection antibodies. Results are presented as the mean ± standard deviations. T1/2, half-life.

Figure 15. 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. Blood samples were taken before and 0.25, 0.5, 1, 1.5, and 2 h after glucose beverage intake. B: AUC of glucose versus time plot (n = 6-12/group). C: Changed AUC of glucose versus time plot from baseline. D: % of volunteer experienced 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.

Figure 16. Pearson correlation analysis among dose, gAUC changes, and observed side effects in clinical study.



Pearson correlation analysis was conducted to show the correlations of doses, gAUC changes (at day3, day 5), and side effects (Nausea, Vomiting, GI disorders) with each others. A, B: Correlation results between gAUC change (Day 3, Day 5) and administered doses. C: Correlation results between gAUC change (Day 3), which shows strong correlation with doses, and side effects. r, pearson correlation coefficient; GI, gastro-intestinal.

TABLES

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 Min-Max	27.5±7.1 19-36	33.3±3.6 28-37	26.8±7.3 19-39	29.2±3.5 26-33	34.7±4.6 29-40	25.0±4.0 21-30	31.4±5.0 23-39	29.9±5.8 19-40
Height (cm)	Mean±SD Min-Max	177.2±5.0 173-185	180.0±4.6 171-184	176.8±7.7 164-185	183.2±8.0 173-193	175.0±6.4 165-183	179.0±6.7 170-18	180.0±6.2 170-187	178.9±6.5 164-19
Weight (kg)	Mean±SD Min-Max	73.2±6.9 64.4-84.0	74.4±9.4 60.2-87.0	77.3±11.3 60.1-89.0	87.1±14.9 69.1-110.6	78.9±6.3 71.0-86.0	75.3±12.3 61.1-91.0	83.3±8.4 70.9-96.0	79.1±10.5 60.1-110.6
BMI (kg/m^2)	Mean±SD Min-Max	23.43±3.2 19.9-28.1	23.03±3.4 18.4-27.2	24.65±2.6 20.3-27.6	25.93±3.6 19.8-29.7	25.80±2.1 23.9-28.6	23.53±4.1 19.1-29.7	25.78±2.8 20.5-29.2	24.74±3.2 18.4-29.7

Abbreviations: SD, standard of deviation; Min, minimum; Max, maximum; N, number of subjects

Table 2. Summary of pharmacokinetic parameters following single subcutaneous administration of GLP-1-gFc.

Pharmacokinetic parameters in healthy men after subcutaneous administration of 6 escalating doses of GLP-1-gFc were described.

Pharmacokinetic Parameters		Cohort 1 0.01mg/kg (N=4)*	Cohort 2 0.02mg/kg (N=6)	Cohort 3 0.04mg/kg (N=6)	Cohort 4 0.08mg/kg (N=6)	Cohort 5 0.16mg/kg (N=6)	Cohort 6 0.24mg/kg (N=6)
AUC_{0-t} (h*ng/mL)	Geom. Mean	3819.1	8185.1	13383.8	30166.2	63377.6	118174
	Geom. CV%	16	32.2	45.3	41.6	43.2	37.9
AUC_{0-inf} (h*ng/mL)	Geom. Mean	4563	9171.1	14071	31511	65946.5	120115.6
	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	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
$T_{1/2}$ (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: AUC0-inf, AUC from time 0 to infinity; Geom., Geometric; CV, coefficient of variation

† Two subjects had GLP-1-gFc serum concentrations below LLOQ at all sampling time points

Table 3. Pharmacokinetics of GLP-1-gFc after SC administration into SD rats and Cynomulgus monkeys.

PK parameters	Dose (mg/kg)	Sex	T1/2 (h)	Tmax (h)	Cmax (ng/mL)	AUClast (ng*h/mL)
SD rat	0.100	Male	14.1 ± 3.6	10.0 ± 0.0	72.4 ± 11.1	2240.0 ± 382.7
	0.400	Male	13.6 ± 2.3	14.7 ± 8.1	297.0 ± 48.8	10336.4 ± 1506.8
	1.600	Male	15.3 ± 1.9	10.0 ± 0.0	1197.2 ± 702.0	34221.2 ± 17386.9
Cynomulgus Monkey	0.125	Male	102.7 ± 86.0	19.3 ± 8.1	607.3 ± 118.6	32666.7 ± 6929.2
		Female	55.5 ± 3.3	14.7 ± 8.1	610.7 ± 198.3	39400.0 ± 7547.8
		Both	79.1 ± 60.3	17.0 ± 7.7	609.0 ± 143.7	36033.3 ± 7456.2
	0.625	Male	88.4 ± 26.0	14.7 ± 8.1	3113.3 ± 1132.4	166333.3 ± 31085.9
		Female	84.8 ± 7.3	14.7 ± 8.1	4253.3 ± 760.1	262666.7 ± 38630.7
		Both	86.6 ± 17.2	14.7 ± 7.2	3683.3 ± 1064.9	214500.0 ± 61380.0
	3.125	Male	109.3 ± 30.7	19.3 ± 8.1	20533.3 ± 1320.4	1133333.3 ± 37859.4
		Female	118.3 ± 34.6	14.7 ± 8.1	23233.3 ± 1914.0	1283333.3 ± 106926.8
		Both	113.8 ± 29.7	17.0 ± 7.7	21883.3 ± 2085.6	1208333.3 ± 109071.8

Abbreviations: h, hours; T1/2, half-life; Tmax, the time at maximum serum concentration; Cmax, maximum serum concentration; AUClast, area under the concentration-time curve from time 0 to the last measurable concentration.

Table 4. Other treatment-emergent adverse events related to Gastro-intestinal symptoms.

Treatment-emergent adverse events were observed during the 28-day study period.

	Placebo (N=12) N (%) e	Cohort 1 0.01 mg/kg (N=6) N (%) e	Cohort 2 0.02 mg/kg (N=6) N (%) e	Cohort 3 0.04 mg/kg (N=6) N (%) e	Cohort 4 0.08 mg/kg (N=6) N (%) e	Cohort 5 0.16 mg/kg (N=6) N (%) e	Cohort 6 0.24 mg/kg (N=6) N (%) e	Overall (N=48) N (%) e
Gastro-intestinal symptoms	2(16.7)2	-	-	3(50.0)5	-	-	4(66.7)17	9(18.8)24
Nausea	-	-	-	1(16.7)1	-	-	4(66.7)5	5(10.4)6
Diarrhea	-	-	-	1(16.7)1	-	-	2(33.3)3	3(6.3)4
Abdominal distension	-	-	-	-	-	-	2(33.3)4	2(4.2)3
Abdominal pain	1(8.3)1	-	-	-	-	-	1(16.7)2	2(4.2)3
Abdominal pain lower	1(8.3)1	-	-	1(16.7)2	-	-	-	1(2.1)1
Abnormal feces	-	-	-	-	-	-	1(16.7)1	1(2.1)1
Dyspepsia	-	-	-	1(16.7)1	-	-	-	1(2.1)1
Eructation	-	-	-	-	-	-	1(16.7)1	1(2.1)1
Vomiting	-	-	-	-	-	-	1(16.7)1	1(2.1)1

Abbreviations: N, number of subjects; %, portion of subjects who experienced specific symptoms; e, number of events

Table 5. Diastolic Blood pressure after single SC administration of GLP-1-gFc.

Diastolic blood pressure was monitored during the 15-day examination period.

Diastolic Blood Pressure [mmHg]		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
Predose	Mean	74.8	70.7	76.2	70.5	76.2	72.7	73.5
	SD	5.9	4.6	2.1	7.7	4.5	5.7	7.6
Day1_1h	Mean	75.3	70.3	74.0	71.0	75.0	72.5	72.8
	SD	5.6	3.8	3.2	7.5	4.4	3.9	6.2
Day1_4h	Mean	72.7	68.0	70.7	71.0	72.8	71.0	73.7
	SD	3.2	5.6	4.1	6.6	6.8	3.7	9.0
Day1_12h	Mean	73.5	68.8	74.8	75.0	74.5	73.2	73.0
	SD	3.5	6.4	4.5	5.3	6.4	5.8	4.1
Day2	Mean	73.9	70.3	73.2	70.5	74.2	73.3	71.3
	SD	2.7	5.4	6.2	5.4	7.9	2.5	7.4
Day3	Mean	74.3	67.2	76.8	72.0	71.2	73.2	70.7
	SD	4.8	4.2	5.3	4.0	3.5	4.4	7.1
Day4	Mean	73.2	72.0	74.5	70.8	74.2	72.7	72.7
	SD	3.9	5.7	4.3	7.7	4.9	3.6	1.9
Day5	Mean	72.9	69.2	74.0	71.7	76.3	74.0	72.0
	SD	2.9	5.0	5.2	3.9	3.1	4.2	3.7
Day9	Mean	74.0	71.3	72.7	72.8	75.5	75.3	71.0
	SD	6.0	3.0	6.7	4.4	6.2	3.4	5.1
Day15	Mean	73.6	71.0	76.8	68.8	73.8	74.0	72.3
	SD	3.5	5.7	10.5	3.8	5.8	4.0	3.8

Table 6. Pulse rate after single SC administration of GLP-1-gFc.

Pulse rate were monitored during the 15-day examination period.

Pulse Rate [beats/min]		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
Predose	Mean	63.9	63.7	66.3	72.0	59.0	59.3	58.7
	SD	12.0	7.2	6.1	14.7	5.3	5.9	4.5
Day1_1h	Mean	56.3	59.0	57.7	69.7	61.0	56.0	56.3
	SD	6.3	6.5	6.7	15.4	8.3	5.3	3.3
Day1_4h	Mean	62.8	64.5	68.0	62.8	62.7	63.0	61.2
	SD	10.0	4.4	8.3	13.1	4.8	8.1	5.8
Day1_12h	Mean	60.5	66.5	70.0	67.8	64.7	63.3	65.2
	SD	5.4	10.8	9.4	13.8	4.3	6.2	9.5
Day2	Mean	57.2	61.0	62.7	68.0	64.0	63.5	65.2
	SD	5.3	3.6	7.2	15.3	6.9	5.7	9.6
Day3	Mean	59.3	61.8	63.3	73.3	60.5	66.3	68.7
	SD	4.1	7.3	10.1	9.5	4.9	10.6	7.8
Day4	Mean	58.5	58.0	64.7	72.3	66.7	71.3	70.0
	SD	5.3	5.7	8.8	12.4	8.6	13.5	9.4
Day5	Mean	60.8	63.2	68.3	70.8	66.2	67.0	66.2
	SD	7.8	6.7	7.3	10.3	7.0	7.4	5.0
Day9	Mean	61.9	68.0	68.3	74.3	63.8	64.2	62.2
	SD	11.5	5.8	9.2	12.3	6.6	9.4	9.4
Day15	Mean	58.6	71.2	65.7	70.2	62.3	62.7	61.3
	SD	6.7	10.4	12.6	12.5	5.6	5.1	6.7

Table 7. Comparison of preclinical and clinical results between GLP-1-gFc and Dulaglutide.

Head to head comparison of preclinical and clinical results between GLP-1-gFc and Dulaglutide was conducted to confirm the safer profile of GLP-1-gFc than that of Dulaglutide.

Preclinical

Study	Dulaglutide	GLP-1-gFc
Safety pharmacology (CV effect in Monkey)	Dose-dependent HR increase Statistical significant QTc change	No change
4 wks repeat Tox (General Tox in Monkey)	Statistical significant QTc change	No change

Clinical

Dulaglutide

	Pbo (N=6)	0.1 mg (N=6)	0.3 mg (N=6)	1 mg (N=6)	3 mg (N=4)	6 mg (N=6)	12 mg (N=6)
AEs	1) Dyspepsia (16/18, 88.9%) 2) Nausea (12/18, 66.7%) 3) Anorexia (11/18, 61.1%) 4) Abdominal pain (9/18, 50%) 5) Dizziness (8/18, 44.4%) 6) Vomiting (8/18, 44.4%)			▪ **All subjects at the 12 mg dose experienced vomiting** ▪ **The # of TEAEs increased as the dose escalated**			
SAEs	Four SAEs in a single subject at 12 mg dose group						
Pulse rate	Statistically significant increases from baseline ($p < 0.05$), Dose-dependent						
a)DBP	-	-	Statistically significant increases from baseline				
OGTT (gAUC)		At day 5, Dose-dependent, ED50 = 2.81 mg statistically significant at doses ≥ 3 mg Maximum suppression = -29% from baseline					
PK		Mean T1/2 = 89.9 h Median Tmax = 24-48h Mean CL/f = 0.107 L/h					

GLP-1-gFc

	Pbo (N=12)	0.74 mg (N=6)	1.55 mg (N=6)	3.48 mg (N=6)	6.31 mg (N=4)	12.05 mg (N=6)	20.00 mg (N=6)
TEAEs	1) Dyspepsia (1/48, 2.1%) 2) Nausea (5/48, 10.4%) 3) Anorexia (0/48, 0%) 4) Abdominal pain (2/48, 4.2%) 5) Dizziness (2/48, 4.2%) 6) Vomiting (1/48, 2.1%)			▪ **1 subject (16.7%) at the 20 mg dose experienced vomiting** ▪ **No clear increase of # of TEAEs as the dose escalated**			
SAEs	No SAEs						
Pulse rate	No statistically significant increase from baseline						
a)DBP	No statistically significant increase from baseline						
OGTT (gAUC)	-	At day 3, Dose-dependent, ED50 = N.A No statistical significant ($p=0.2$ at doses ≥ 6.31 mg) Maximum suppression = -61.6% from baseline					
PK	-	Mean T1/2 = 73.6 h Median Tmax = 36-48h Mean CL/f = 0.150 L/h					

a) Supine diastolic blood pressure

요약 (국문초록)

당화 GLP-1-Fc와 Dulaglutide (mutated GLP-1-Fc)의 혈당 조절 작용과 위장관계 및 심장 부작용의 비교

안 인 복
서울대학교 의과대학
임상의과학과

GLP-1 수용체 작용제 (GLP-1 RA)는 매력적인 항 당뇨병 치료제임에도 불구하고 주로 메스꺼움, 구토 및 복통과 같은 부작용에 따른 낮은 복약 순응도로 인해 제한된 치료 효과를 보여왔다. 본 시험에서는 새롭게 개발된 glycosylated Fc 융합 GLP-1 RA (GLP-1-gFc)의 효능 및 부작용을 dulaglutide와 비교 평가하였다. 기기 및 세포-기반 시험관내 분석을 통해 GLP-1-gFc가 dulaglutide보다 10 배 적은 결합 친화도 및 4 배 적은 역가를 가짐을 확인하였다. 역가를 고려한 용량 (dulaglutide 용량의 4 배)에서 GLP-1-gFc는 dulaglutide와 비슷한 혈당 저하 효과를 나타냈다. 그러나, dulaglutide와 동등한 효능을 보이는 용량 및 그보다 더 높은 용량에서 dulaglutide와는 다르게 쥐의 구역질 / 구토 반응 또는 원숭이의 QT 간격

변화를 유발하지 않았다. 이러한 경향은 건강한 피험자에 대한 임상 1상 시험에서 재확인되었다; 경구 포도당 내성 시험 연구에서 포도당 조절 효과는 위장 장애 및 맥박 변화의 정도보다 훨씬 더 극 적으로 나타났다. 이러한 결과들은 GLP-1-gFc이 시판 및 개발중인 높은 활성의 GLP-1 RA보다 더 나은 안전성을 가짐으로서 당뇨병 환자에 대한 치료 혜택을 극대화할 수 있는 새로운 GLP-1 RA로 사용될 수 있음을 시사한다.

주요어 : GLP-1-gFc, dulaglutide, 부작용, 효능, 결합 친화도, 역가, 임상

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