Original Article

MOLECULAR METABOLISM

Discovery of ecnoglutide — A novel, long-acting, cAMP-biased glucagon-like peptide-1 (GLP-1) analog

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ABSTRACT

Objective: Glucagon-like peptide (GLP)-1 is an incretin hormone that acts after food intake to stimulate insulin production, enhance satiety, and promote weight loss. Here we describe the discovery and characterization of ecnoglutide (XW003), a novel GLP-1 analog.

Methods: We engineered a series of GLP-1 peptide analogs with an alanine to valine substitution (Ala8Val) and a γGlu-2xAEEA linked C18 diacid fatty acid at various positions. Ecnoglutide was selected and characterized in GLP-1 receptor signaling assays in vitro, as well as in db/db mice and a diet induced obese (DIO) rat model. A Phase 1, double-blind, randomized, placebo-controlled, single (SAD) and multiple ascending dose (MAD) study was conducted to evaluate the safety, tolerability, and pharmacokinetics of subcutaneous ecnoglutide injection in healthy participants. SAD doses ranged from 0.03 to 1.0 mg; MAD doses ranged from 0.2 to 0.6 mg once weekly for 6 weeks (ClinicalTrials.gov Identifier: NCT04389775).

Results: In vitro, ecnoglutide potently induced cAMP (EC_{50} = 0.018 nM) but not GLP-1 receptor internalization (EC_{50} > 10 μM), suggesting a desirable signaling bias. In rodent models, ecnoglutide significantly reduced blood glucose, promoted insulin induction, and led to more pronounced body weight reduction compared to semaglutide. In a Phase 1 trial, ecnoglutide was generally safe and well tolerated as a once-weekly injection for up to 6 weeks. Adverse events included decreased appetite, nausea, and headache. The half-life at steady state ranged from 124 to 138 h, supporting once-weekly dosing.

Conclusions: Ecnoglutide showed a favorable potency, pharmacokinetic, and tolerability profile, as well as a simplified manufacturing process. These results support the continued development of ecnoglutide for the treatment of type 2 diabetes and obesity.

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Keywords Glucagon-like peptide-1; Ecnoglutide; XW003; Peptide analog; Phase 1; Obesity; Type 2 diabetes

1. INTRODUCTION

Over the past two decades, glucagon-like peptide-1 (GLP-1) receptor agonists have emerged as an important class of medications to treat type 2 diabetes mellitus (T2DM) and obesity. GLP-1 is a peptide incretin hormone that is naturally released by gut endocrine cells in response to food intake. GLP-1 acts by signaling through the GLP-1 receptor in various cell types to promote blood sugar control, slow gastric emptying, and create a sense of fullness. In pancreatic islets, GLP-1 increases insulin production from β cells, while reducing glucagon release from α cells [1]. Native GLP-1 has a very short half-life (approximately 2 min) and is rapidly degraded by dipeptidyl peptidase-IV (DPP-4). Modified GLP-1 peptide analogs with improved stability have therefore been developed for therapeutic purposes.

Multiple generations of GLP-1 receptor agonists have been developed. The first GLP-1 peptide analog approved as an anti-diabetic agent, exenatide, was isolated from the venom of the Gila monster lizard and showed improved stability compared to the native GLP-1 peptide [2]. The half-life of exenatide after subcutaneous injection is ∼2—3 h, requiring twice daily injections. Liraglutide is a human GLP-1 analog

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Abbreviations: AEEA, 2-(2-(2-aminoethoxy)ethoxy)acetic acid; DIO, Diet induced obese; GLP-1, Glucagon-like peptide-1; MAD, Multiple ascending dose; MMRM, Mixed models for repeated measures analysis; T2DM, type 2 diabetes mellitus; SAD, Single ascending dose

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with a half-life of ~13 h, making it suitable for once-daily administration. In the last few years, dulaglutide and semaglutide have become available; these are long-acting GLP-1 analogs with >100-hour half-lives and require once weekly injection [3,4]. Compared to earlier generations of GLP-1 receptor agonists, the long-acting molecules deliver excellent efficacy while reducing the treatment burden of frequent injections. For example, a 56-week study in adults with T2DM taking metformin, thiazolidinediones, or both demonstrated that weekly treatment with 1 mg semaglutide led to a hemoglobin (Hb) A1c reduction of 1.6%, with 78% of patients lowering their HbA1c below 7%. Those patients also lost an average of 6.1 kg of body weight [5]. Semaglutide is approved as both a once daily oral tablet (Rybelsus®) and weekly injection (Ozempic®) for T2DM management, as well as a weekly injection (Wegovy®) for weight loss [6–8]. Dulaglutide is approved as a once weekly injection (Trulicity®) for T2DM [9].

Although the currently available GLP-1 receptor agonists have demonstrated impressive treatment outcomes, further improvements to this class of drugs can be envisioned. Current limitations of GLP-1 peptide analogs include the tolerability of gastrointestinal side effects, challenges with managing injections or oral dosing around meals, as well as costs and scalability of drug manufacturing. Furthermore, there are still patient populations whose glucose levels and body weight cannot be adequately controlled by current therapeutics [5].

Recent studies have suggested that "biased" GLP-1 receptor agonists may be more effective than unbiased agonists in controlling glucose and body weight [10]. Multiple intracellular signaling events occur upon GLP-1 receptor activation, including induction of cAMP production, β-arrestin recruitment, and receptor internalization via endocytosis. Biased GLP-1 receptor agonists favor cAMP generation over other downstream signaling events [11]. Recently, a dual GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) agonist, tirzepatide, was developed for the treatment of T2DM and obesity. The GLP-1 agonism activity of tirzepatide shows bias by favoring cAMP generation over β-arrestin recruitment, resulting in reduced internalization of the GLP-1 receptor and enhanced insulin secretion [10]. In a Phase 3 clinical trial, tirzepatide showed more pronounced efficacy than semaglutide at lowering HbA1c (up to mean −0.45% difference) and body weight (up to mean −5.5 kg difference) [12]. Tirzepatide weekly injection (Mounjaro®) was recently approved for the treatment of T2DM [13].

Here we describe the discovery of ecnoglutide, a novel, cAMP-biased GLP-1 peptide analog that is composed of only natural amino acid residues, with improved ease of manufacturing and greater glucose and body weight control compared to semaglutide in multiple preclinical animal models. Ecnoglutide was generally safe and well tolerated in healthy volunteers and is being further developed for the treatment of T2DM and obesity.

2. EXPERIMENTAL METHODS

2.1. Recombinant GLP-1 analog expression

GLP-1 analog expression constructs were prepared by inserting a 6-His tag, SUMO tag, and peptide gene fusion into the prokaryotic expression plasmid pET-24(+) through *BamH*I and *Xho*I restriction digestion. Plasmids were verified by gene sequencing. Peptide sequences are shown in Supplemental Table 1.

DNA was transformed into BL21 competent cells (TransGen Biotech Co., LTD, Catalog #CD601, QC, Canada) for target protein production, according to the manufacturer's instructions. Transformed cells were plated on Luria–Bertani (LB) agar containing kanamycin, following which single colonies were picked and cultured in 15 mL sterile LB medium containing kanamycin at 30 °C overnight. The bacterial suspension (50 μL) was then added to 50 mL of LB medium containing kanamycin and again incubated at 30 °C overnight. This suspension (10 mL) was then added to 1000 mL LB medium supplemented with kanamycin and incubated in a shaker at 200 rpm, 37 °C for 4 h. Isopropyl β-D-1-thiogalactopyranoside (IPTG) with a final concentration of 0.1 mol/L was added to the bacterial suspension to induce SUMO-tagged GLP-1 analog expression, and cultures were shaken at 180 rpm, 30 °C overnight. The bacterial suspension was then centrifuged at 13,000×*g* for 60 min. The bacterial yield was about 4 g bacteria per liter of fermentation broth, and the expression of GLP-1 analog as determined by SDS-PAGE was up to ~40% of total protein.

2.2. Recombinant GLP-1 analog purification

Cell slurry (100 g) was re-suspended in 500 mL of 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, then sonicated in an ultrasonic cell mill for 30 min in an ice water bath to break up the cells. The homogenate was centrifuged at 13,000×*g* for 60 min at 4 °C. The supernatant was concentrated using a Chelating Sepharose FF column equilibrated with 50 mM Tris–HCl pH 8.0, 500 mM NaCl, and 10 mM imidazole (Equilibrium Liquid 1). After rinsing with Equilibrium Liquid 1, SUMO-GLP-1 analog was eluted with 50 mM Tris–HCl pH 8.0, 50 mM NaCl, and 0.3 M imidazole (Eluent). According to SDS-PAGE analysis, the purity of the SUMO-GLP-1 analogs produced was higher than 70%. The SUMO tag was excised using Ubl-specific protease (ULP). Buffer (20 mM phosphate buffer, pH 7.4) was added to the intermediate product in a three-fold dilution, followed by addition of ULP enzyme (1:150 ratio enzyme: intermediate product) and incubation overnight at 4 °C. The digestion yield was nearly 100% according to SDS-PAGE analysis for all analogs.

The products obtained after digestion were concentrated using a Tosoh Butyl 550C column equilibrated with 20 mM Na_2HPO_4 and 0.7 M NaCl (Equilibrium Liquid 2). After rinsing with Equilibrium Liquid 2, GLP-1 analogs were eluted with 20% ethanol. Purity was about 90% according to SDS-PAGE analysis. 0.2 M Na_2HPO_4 was added to the eluted samples to a final concentration of 20 mM Na_2HPO_4. The solution was then adjusted to pH 4.8–5.0 with 1 M citric acid and precipitated overnight at 4 °C. The precipitate was centrifuged at 13,000×*g* for 30 min at 4 °C and the pellets were collected and stored at −20 °C.

2.3. Acylation of GLP-1 analogs

GLP-1 analogs were acylated with fatty acids on lysine residues, which were engineered at various positions for the different analogs. For fatty acid modification, GLP-1 analogs were dissolved in water to 4–6 mg/mL, after which 1 M sodium hydroxide was added to adjust the pH to 11.0–11.5. The final peptide concentration was quantified by HPLC. Fatty acid ester powder (Supplemental Figure 1) was weighed and dissolved in acetonitrile. Triethylamine (0.2%) was added to the peptide:fatty acid solution (1:4) and incubated at 4 °C for 1 h. The sample was then diluted 5-fold with water and adjusted to pH 4.8 with 1 M citric acid or 10% acetic acid to quench the reaction. The solution was further incubated at 4 °C for 10 min before centrifugation at 13,000×*g*, 4 °C for 30 min. The precipitate was collected and stored at −80 °C.

To deprotect the fatty acid, trifluoroacetic acid (TFA) was added to the precipitate to a final peptide concentration of about 10 mg/mL. The mixture was shaken to dissolve the precipitate and incubated for 30 min at room temperature for deprotection. 4 M NaOH was added to the reaction solution to adjust the pH to 7.5–8.5 and quench the reaction.

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For purification, a preparative HPLC (Shimadzu LC-8A) was used. The quenched reaction liquid was loaded on to a UniSil10-120 C18 column (Suzhou Nanomicro Technology Co., Ltd., Suzhou, China) equilibrated with 10 mM ammonium acetate and 20% ethanol (Equilibrium Liquid 3). After rinsing with Equilibrium Liquid 3, a gradient of 0—100% eluent containing 10 mM ammonium acetate, 80% ethanol was used for elution. Purity of the collected peak was about 90% according to RP-HPLC analysis. The elution peak was diluted 3-fold with water, then was adjusted to pH 4.8, and acid precipitation was performed at 4 °C for 30 min. After centrifugation, phosphate-buffered saline plus tween (PBST) buffer (pH 7.0) was added to reconstitute the pellet. The acylated GLP-1 analogs (M0 to M7) were stored in solution at −80 °C. The yields of each production and purification step are shown in Supplemental Table 2, with final yields between 43 and 47% for the SAD and MAD clinical batches. Chromatographic analysis (reverse phase HPLC and size exclusion chromatography [SEC]) of the integrity and purity of the SAD and MAD clinical batches of ecnoglutide are shown in Supplemental Figure 2.

2.4. In vitro activity of GLP-1 analogs in RIN-m5F cells

RIN-m5F cells (ATCC, CRL-11605, Manassas, VA, US) were grown in RPMI 1640 (Thermo Fisher Scientific, Boston, MA, US) supplemented with 10% fetal bovine serum (FBS). Cell suspension (100 μL of 1×10^5 cells/mL) was seeded into a 96-well cell culture plate and incubated overnight at 37 °C, 5% CO_2. The in vitro activity of the acylated GLP-1 analogs was measured using cAMP-Glo™ Assay kit (Promega, V1501, Madison, WI, US). GLP-1 analogs (M0 to M7 and semaglutide, Ozempic®, Novo Nordisk) were diluted to 300 ng/mL with assay medium, followed by 3-fold serial dilutions. Following removal of old medium from the cell plate, 40 μL/well of GLP-1 analog solutions were added and incubated for 15 min at 37 °C, 5% CO_2. After cells were lysed by cAMP-Glo™ Lysis Buffer, cAMP-Glo™ Detection solution (10 μL, cAMP assay kit, Promega) was added to each well and the plate was shaken at 500 rpm for 20 min at room temperature. Kinase-Glo® Reagent (KG) solution (50 μL, cAMP assay kit, Promega) was then added to each well, and then shaken at 500 rpm for 10 min at room temperature. The chemiluminescence value was recorded by SpectraMax L microplate reader (Molecular Devices). EC_{50} values were calculated by four-parameter regression using Softmax Pro software.

2.5. DiscoverX Cell Signaling Assays

For the cAMP assay, cAMP HitHunter hGLP1R-CHO-K1 cells (DiscoverX, Fremont, CA) were seeded in a total volume of 20 μL into white walled, 384-well microplates and incubated at 37 °C. Media was removed and replaced with 15 μL 2:1 HBSS/10 mM Hepes:cAMP XS+ Ab reagent (DiscoverX). Compounds (5 μL of 4×) were added to the cells and incubated at 37 °C for 30 min; vehicle concentration was 1%. Then 20 μL cAMP XS+ ED/CL lysis cocktail (DiscoverX) was added and incubated for 1 h at room temperature. Finally, 20 μL cAMP XS+ EA reagent (DiscoverX) was added and incubated for 3 h at room temperature before signal detection.

For the β-arrestin assay, PathHunter β-Arrestin hGLP1R-CHO-K1 cells (DiscoverX, Fremont, CA) were seeded in a total volume of 20 μL into white walled, 384-well microplates and incubated at 37 °C. Compounds (5 μL of 5×) were added to cells and incubated at 37 °C for 90 min; vehicle concentration was 1%. Assay signal was generated through a single addition of 12.5 or 15 μL (50% v/v) of PathHunter Detection reagent cocktail (DiscoverX), followed by one-hour incubation at room temperature.

For the G-protein coupled receptor (GPCR) internalization assay, PathHunter Activated GPCR Internalization hGLP1R-U2OS cells (DiscoverX) were seeded in a total volume of 20 μL into white walled, 384-well microplates and incubated at 37 °C. Compounds (5 μL of 5×) were added to cells and incubated at 37 °C for 90 min; vehicle concentration was 1%. Assay signal was generated through a single addition of 12.5 or 15 μL (50% v/v) of PathHunter Detection reagent cocktail (DiscoverX), followed by one hour incubation at room temperature.

For all assays, chemiluminescence was read with a PerkinElmer EnvisionTM instrument. Percent activity was calculated using the following equation: % Activity = 100% × (mean RLU of compound − mean RLU of vehicle control)/(mean RLU of MAX control − mean RLU of vehicle control). EC_{50} was calculated by the four-parameter regression using Graphpad Prism 9.3.1 software.

2.6. Binding affinity assay

Biacore™ 8K surface plasmon resonance (SPR) system (GE Healthcare Bio-Sciences Corp, Marlborough, MA) was used to monitor binding kinetics of GLP-1 analogs to their receptor. Recombinant human GLP-1 receptor protein (13944-H02H, Sino Biological, Beijing, China) was captured on a Biacore Series S sensor chip CM5 (GE Healthcare Bio-Sciences Corp) through amine coupling. A series of dilutions (100 nM—3.1 nM) of GLP-1 analogs in PBS-P pH 7.4 buffer (GE Healthcare Bio-Sciences Corp) was loaded. The kinetic data curves were fitted to a 1:1 binding model with R_{max} fitted global parameter using Biacore™ 8K Evaluation Software. Binding affinity (K_D) was calculated by dividing the dissociation constant by the association constant (k_{off}/k_{on}).

2.7. Glucose response study in db/db mice

Male db/db mice (8—9 weeks old, ~43 g, Peking University Experimental Animal Research Center, Beijing, China) were housed 5 per cage at ~20—26 °C, ~40—70% humidity, and following a 12 h:12 h light—dark cycle with free access to chow diet and water. After one to two weeks acclimation, animals were randomly regrouped ($n \geq 5$ per group) based on body weight and glucose level. A single subcutaneous injection of vehicle, ecnoglutide (M4, 0.015, 0.15 mg/kg), or semaglutide (0.15 mg/kg) at 10 mL/kg dosing volume was administered (designated time 0). Fasting (about eight hours of food restriction) and postprandial blood glucose from tail tip were measured at designated time points. Change in glucose from baseline was calculated as ΔGlucose = glucose level at tested time point minus baseline glucose before compound dosage. Data were plotted as mean value +/− standard deviation (SD). Mixed models for repeated measures (MMRM) analysis in SAS v9.4 (SAS Institute, Cary, NC) was used to compare glucose levels for treatment groups at each timepoint. Statistical analysis for insulin was performed by One-Way Analysis of Variance (ANOVA) using Graphpad Prism 9.3.1 software.

2.8. Body weight and insulin response study in db/db mice

Male db/db mice (9—11 weeks old, ~48 g, Peking University Experimental Animal Research Center, Beijing, China) were housed 5 per cage at ~20—26 °C, ~40—70% humidity, and following a 12 h:12 h light—dark cycle with free access to chow diet and water. After one to two weeks acclimation, animals were randomly regrouped (n = 10 per group) based on body weight and glucose level. Vehicle, ecnoglutide (M4, 0.005, 0.015, 0.05 mg/kg), or semaglutide (0.05 mg/kg) were subcutaneously administered at 5 mL/kg once per day for 42 days; Day 1 was designated as the first dosing day.

Body weight (BW) and food intake were recorded. Percent body weight compared to baseline was calculated as %BW = 100*(BW on Day n − BW on Day 1)/BW on Day 1. Cumulative food intake was calculated

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by summing the food intake amount in each week. Fasting serum was collected on the terminal day (Day 43) and stored at −80 °C for the measurement of serum insulin. Data were plotted as mean value +/− SD. Statistically analysis was performed by One-Way ANOVA.

2.9. Body weight response study in DIO rat

Male diet induced obese (DIO) SD rats (22-week-old, ∼650 g, Vital River Laboratories, Beijing, China) were housed at ∼20−26 °C, ∼40−70% humidity, and 12 h:12 h light−dark cycles with free access to a high fat diet (D12492, Research Diet) and water. Animals were randomly regrouped into several groups (n = 5 per group) based on body weight. Vehicle, ecnoglutide (M4, 0.025 mg/kg), and semaglutide (0.025 mg/kg) were subcutaneously administered once per day for 21 days (Day 0-Day 20). On Day 21, rats in the ecnoglutide treatment group were switched to dosing with semaglutide and rats in the semaglutide treatment group were switched to dosing with ecnoglutide. The animals were dosed for 7 days with the new treatment.

Body weight was recorded for each animal. Percent body weight compared to baseline was calculated as %BW = 100*(BW on Day n - BW on Day 0)/BW on Day 0. Data were plotted as mean value +/− SD.

2.10. Pharmacokinetics study in SD rat

Male SD rats (8−10 weeks old) were administrated ecnoglutide (M4, 1 mg/kg) or semaglutide (1 mg/kg) as a single subcutaneous (s.c.) dose (n = 3 per dose group). Plasma samples were collected at 0, 2, 4, 6, 24, 48, 72 and 96 h post-injection. Semaglutide and ecnoglutide were measured by Waters I-class Premier UPLC tandem with Sciex 6500 and quantified by ion pair (*m/z*: 1029.1/690) and (*m/z*: 1071.9/1350.4), respectively. Pharmacokinetic (PK) parameters were calculated in Excel using a non-compartment model.

2.11. Protease stability study

The reaction buffer for the pepsin stability study contained 0.005% Tween 20 and 0.001% BSA, with 20 mM citric acid-phosphate buffer of different pH values (2.6, 4.0, and 7.4). Simulated Gastric Fluid containing pepsin (SGF-pepsin) contained 5 mL 0.1 M hydrochloric acid and 0.019 g pepsin (P6887, Sigma−Aldrich). Ecnoglutide (M4) and semaglutide stock solutions were prepared at 1.33 mg/mL in phosphate buffer (PB) buffer at pH 7.4. An appropriate amount of stock solution was diluted to 0.06 mg/mL with reaction buffer at different pH values.

Samples were then mixed with SGF-pepsin and incubated at 37 °C for 0, 5, 10, 20, 35 and 50 min before the reaction was quenched with 1 M NaOH. Samples without SGF-pepsin were assigned as −0.5 min. Ecnoglutide and semaglutide content was analyzed by HPLC. The peak area at −0.5 min was normalized to 100% and the ratio of peak areas at different time points to the peak area at −0.5 min was calculated.

2.12. Phase 1 clinical study design

A first-in-human, single-center, double-blind, randomized, placebo-controlled, single and multiple ascending dose study of ecnoglutide was conducted to evaluate the safety, tolerability, PK, and pharmacodynamics (PD) of ecnoglutide in healthy participants. Up to 42 participants were to be enrolled into up to 6 SAD sequential cohorts and up to 30 participants were to be enrolled in up to 3 MAD cohorts. Participants in SAD cohorts 0.03 mg, 0.1 mg, 0.2/0.25 mg, 0.4/0.5 mg, 0.8/1.0 mg and 0.6 mg received a single s.c. dose of either ecnoglutide or matching placebo based on body weight. Participants (n = 2) in Cohort 0.03 mg received ecnoglutide open label. Once dosing of the first participant proceeded without clinically significant safety signals in the first 48 h postdose the second participant received a single s.c. dose of ecnoglutide. Participants in the remaining SAD cohorts (up to n = 8 per cohort) were randomized to receive a s.c. dose of either ecnoglutide (n = 6) or matching placebo (n = 2) on Day 1 following an overnight fast of at least 10 h. All participants were confined to the clinical research unit (CRU) from Day −1 (predose) until completion of the 120-hour postdose assessments on the morning of Day 6. The participants returned to the CRU for follow-up visits on Day 7 (±1 day), Day 8 (±1 day), Day 10 (±1 day), Day 12 (±1 day), Day 15 (±2 days), Day 22 (±2 days), Day 29 (±2 days), and for an end of study (EOS) follow-up visit on Day 36 (±2 days).

Participants in MAD cohorts 0.2 mg, 0.4 mg, and 0.6 mg received a single s.c. dose of either ecnoglutide (n = 8) or matching placebo (n = 2) once weekly for 6 weeks. Dose escalation occurred in the first two weeks for the 0.4 and 0.6 mg cohorts. All participants were confined to the CRU for Dose 1 (from Day −1 until Day 4), Dose 2 (from Day 7 until Day 11), Dose 3 (from Day 14 until Day 18), Dose 4 (from Day 21 until Day 25), Dose 5 (from Day 28 until Day 32), and for Dose 6 (from Day 35 until Day 39). Participants were to attend all other visits as outpatients. Participants return to the CRU for an EOS follow up visit on Day 71 (±2 days).

The study was conducted in accordance with the principles of the Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects); Integrated Addendum to ICH E6(R1): Guideline for Good Clinical Practice ICH E6(R2), annotated with comments by the Australian TGA (2018) and all other applicable regulatory requirements. All participants provided written informed consent.

The study was registered on the Australian New Zealand Clinical Trials Registry (ACTRN 12620000344998) and ClinicalTrials.gov (NCT04389775).

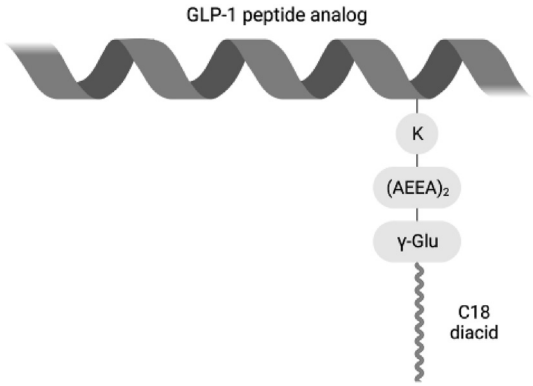

Figure 1: Schematic of GLP-1 peptide analogs. GLP-1 peptide is shown with lysine (K) at variable positions. Linker of 2-(2-(2-aminoethoxy)ethoxy)acetic acid (AEEA) and γ-glutamic acid connects the peptide lysine to C18 diacid fatty acid. Figure was created with Biorender.com.

3. RESULTS

3.1. Engineering and in vitro potency of novel GLP-1 analogs

We aimed to identify new GLP-1 peptide analogs with improved stability, tolerability, pharmacokinetic, and efficacy profiles. Bias of GLP-1 receptor agonists to induce cAMP production over β-arrestin recruitment and receptor internalization has been suggested to improve efficacy [10]. We therefore engineered GLP-1 analogs with a single amino acid substitution (Ala → Val at position 8) that has been previously reported to favor cAMP generation over GLP-1 receptor internalization [11]. Acylation is known to prolong the half-life of GLP-1

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analogs, with potential variability in this effect depending on the site of acylation. We therefore designed a series of GLP-1 analogs with lysines at different positions, allowing conjugation of a C18 diacid fatty acid via a γGlu-2xAEEA linker at various sites between amino acid 19 and 37 (Figure 1).

The panel of newly constructed GLP-1 analogs (M0 to M7) was tested for in vitro potency as GLP-1 receptor agonists using a rat islet cell line, RIN-m5F. Production of cAMP was measured following incubation with varying concentrations of the peptides. Semaglutide (Ozempic®, Novo Nordisk) was used as a comparator. Among the novel GLP-1 analogs, M2 and M4 (ecnoglutide) were the most potent at cAMP induction, with EC_{50} values of 1.996 and 2.322 ng/mL, respectively. Their potency was similar to semaglutide, which showed an EC_{50} of 2.437 ng/mL in this assay (Table 1).

3.2. Receptor binding and cAMP signaling bias of novel GLP-1 analogs

The preference of the GLP-1 analogs to induce GLP-1 receptor signaling through cAMP production compared to β-arrestin recruitment and receptor internalization in cell culture was investigated using the DiscoverX platform. Cells expressing human GLP-1 receptor were treated with varying concentrations of peptides before measuring cAMP production, β-arrestin recruitment, and receptor internalization activities using a chemiluminescent signal.

Induction of cAMP was similar for M2 and M4 compared to semaglutide. The EC_{50} values for β-arrestin recruitment were also similar between all three compounds. The maximum recruitment of β-arrestin to the GLP-1 receptor, however, was higher for semaglutide (100%) than for the novel GLP-1 analogs (54—60%). In addition, GLP-1 receptor internalization was markedly higher for semaglutide compared to M2 and M4 (Figure 2 and Table 2).

The kinetics of GLP-1 peptide analog binding to the human GLP-1 receptor were measured by surface plasmon resonance (SPR). Binding results indicated that M2 and M4 bound to the human GLP-1 receptor with ∼10—30-fold higher affinity than semaglutide (Table 2). These results indicate that M2 and M4 potently bind the human GLP-1 receptor in vitro and lead to a signaling bias towards cAMP production over β-arrestin recruitment following receptor engagement in cell culture. M4 (ecnoglutide) was chosen for further development.

3.3. Ecnoglutide shows improved efficacy compared to semaglutide in rodent models

The in vivo efficacy of ecnoglutide for controlling glucose and insulin was investigated by two studies in db/db mice.

Glucose response was measured in 8—9-week-old male db/db mice administered a single subcutaneous dose of vehicle, ecnoglutide (0.015, 0.15 mg/kg), or semaglutide (0.15 mg/kg). Fasting and post-prandial blood glucose were measured at designated timepoints. Compared to animals treated with vehicle, dosing with ecnoglutide (0.015 and 0.15 mg/kg) or semaglutide (0.15 mg/kg) decreased fasting blood glucose levels for up to 147 h post-dose. At 99 and 147 h post-dose, ecnoglutide (0.15 mg/kg) suppressed blood glucose to a significantly greater extent than semaglutide. Post-prandial blood glucose was also suppressed in mice treated with ecnoglutide (0.015 and 0.15 mg/kg) or semaglutide (0.15 mg/kg), with levels returning to baseline between 91 h (semaglutide) and 115 h (ecnoglutide) post-dose. Ecnoglutide significantly suppressed post-prandial blood glucose compared to semaglutide at 91 and 115 h post-dose for both dose levels tested (Figure 3A and B).

Insulin response was measured in 9—11-week-old db/db mice administered vehicle, ecnoglutide (0.005, 0.015, 0.05 mg/kg), or semaglutide (0.05 mg/kg) by s.c. injection once daily for 42 days. Fasting serum for insulin measurement was collected on Day 43. Compared to vehicle or semaglutide treatment, animals dosed with ecnoglutide at 0.05 mg/kg showed a significantly higher level of insulin response (Figure 3C). The lower doses of ecnoglutide did not result in a significant change in insulin levels (data not shown).

Body weight change following GLP-1 analog treatment was investigated in both db/db mice and a diet induced obese (DIO) rat model. Male db/db mice (9—11 weeks of age) were administered vehicle, ecnoglutide (0.005, 0.015, 0.05 mg/kg), or semaglutide (0.05 mg/kg) by s.c. injection once daily for 42 days. Both food intake and body weight gain were reduced for ecnoglutide (0.005, 0.015, and 0.5 mg/kg) and for semaglutide (0.05 mg/kg) compared to vehicle. On Day 41 all ecnoglutide and semaglutide dose groups had significantly reduced percent body weight compared to baseline than animals receiving vehicle alone. Percent body weight change for ecnoglutide (0.05 mg/kg) was also significantly more pronounced than for semaglutide (Figure 4A—C).

Body weight reduction and durability of the response were also measured in a DIO SD rat model. Male DIO rats (22 weeks of age) were administered vehicle, ecnoglutide, or semaglutide in a cross-over study. Rats were dosed with vehicle, ecnoglutide (0.025 mg/kg), or semaglutide (0.025 mg/kg) s.c. once daily for 21 days, after which the active treatment groups were switched to dosing with the other compound until Day 28. Both ecnoglutide and semaglutide-treated animals showed reduced body weight as percent of baseline compared to the vehicle group. When study treatments were switched,

Table 1 — In vitro potency of GLP-1 peptide analogs (cAMP induction).

Analog	Sequence modification	Fatty acid	Acylation position	Linkage	cAMP assay EC_{50} (ng/mL)[a]
Semaglutide	Aib8	C18 diacid	26	γGlu-2xAEEA	2.437
M0	Val8	C18 diacid	26	γGlu-2xAEEA	10.680
M1	Val8	C18 diacid	19	γGlu-2xAEEA	5.386
M2	Val8	C18 diacid	23	γGlu-2xAEEA	1.996
M3	Val8	C18 diacid	27	γGlu-2xAEEA	5.387
M4 (ecnoglutide)	Val8	C18 diacid	30	γGlu-2xAEEA	2.322
M5	Val8	C18 diacid	34	γGlu-2xAEEA	3.043
M6	Val8	C18 diacid	36	γGlu-2xAEEA	7.650
M7	Val8	C18 diacid	37	γGlu-2xAEEA	3.208

[a] Potency of inducing GLP-1 receptor-mediated cAMP production in RIN-m5F (rat islet cell). Abbreviations: Aib, 2-aminoisobutyric acid; Val, valine; Glu, glutamic acid; AEEA, 2-(2-(2-aminoethoxy)ethoxy)acetic acid.

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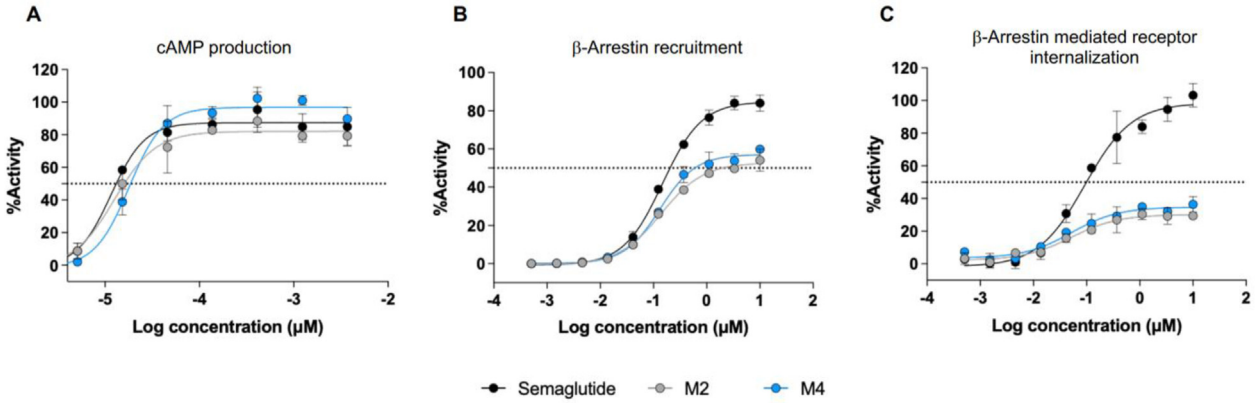

Figure 2: GLP-1 receptor signaling pathways induced by GLP-1 peptide analogs. DiscoverX Cell Signaling Assays were used to evaluate GLP-1 analogs for (**A**) cAMP induction in HitHunter hGLP1R-CHO-K1 cells (**B**) β-arrestin recruitment in PathHunter β-Arrestin hGLP1R-CHO-K1 cells and (**C**) β-arrestin-mediated GLP-1 receptor internalization in PathHunter Activated GPCR Internalization hGLP1R-U2OS cells. Percent activity was determined from the chemiluminescent readout of the assays. Mean and standard deviation are plotted.

Table 2 — Signaling bias and human GLP-1 receptor binding of GLP-1 peptide analogs.

	GLP-1 receptor signaling		
	cAMP EC_{50} (nM)	β-arrestin EC_{50} (μM) (E_{max})	Receptor internalization EC_{50} (μM)
Semaglutide	0.012	0.15 (100%)	0.093
M2	0.013	0.14 (54%)	>10
M4 (ecnoglutide)	0.018	0.13 (60%)	>10
	Binding to the human GLP-1 receptor		
	K_{on} ($M^{-1}S^{-1}$)	K_{off} (S^{-1})	K_D (M)
Semaglutide	$6.58*10^5$	$1.12*10^{-2}$	$1.70*10^{-8}$
M2	$2.20*10^6$	$1.39*10^{-3}$	$6.29*10^{-10}$
M4 (ecnoglutide)	$1.23*10^6$	$1.78*10^{-3}$	$1.45*10^{-9}$

Potency of GLP-1 analogs in DiscoverX Cell Signaling Assays is shown. Binding of GLP-1 analogs to their receptor as measured by SPR.

animals that were now receiving semaglutide began to regain body weight. In contrast, animals that were now receiving ecnoglutide maintained a stable body weight decrease from baseline (Figure 4D).

3.4. Pharmacokinetic and gastrointestinal stability profile of ecnoglutide

The pharmacokinetics (PK) of ecnoglutide were investigated in SD rat. Male rats (n = 3 per group) were administered a single s.c. dose (1 mg/kg) of ecnoglutide or semaglutide and PK parameters were measured. PK parameters, including T_{max}, C_{max}, and exposure, were similar between the two compounds (Supplemental Figure 3 and Supplemental Table 3).

GLP-1 receptor agonists have the potential to be delivered by either injection or oral administration. To evaluate the feasibility of oral dosing for ecnoglutide, in vitro gastrointestinal stability studies were conducted. The stability of ecnoglutide was tested in Simulated Gastric Fluid (SGF) containing pepsin (pH 2.6, 4.0 and 7.4) and Simulated Intestinal Fluid (SIF) with pancreatin. In SGF-pepsin, ecnoglutide showed comparable stability to semaglutide at pH 2.6 and 7.4, and improved stability compared to semaglutide at pH 4.0. Ecnoglutide also showed favorable stability in SIF with pancreatin (Figure 5 and data not shown). Taken together, the preclinical results indicate that ecnoglutide has favorable PK properties, gastrointestinal stability to support the possibility of oral dosing, and promising PD effects in vitro and in animal models. These results support the clinical development of ecnoglutide.

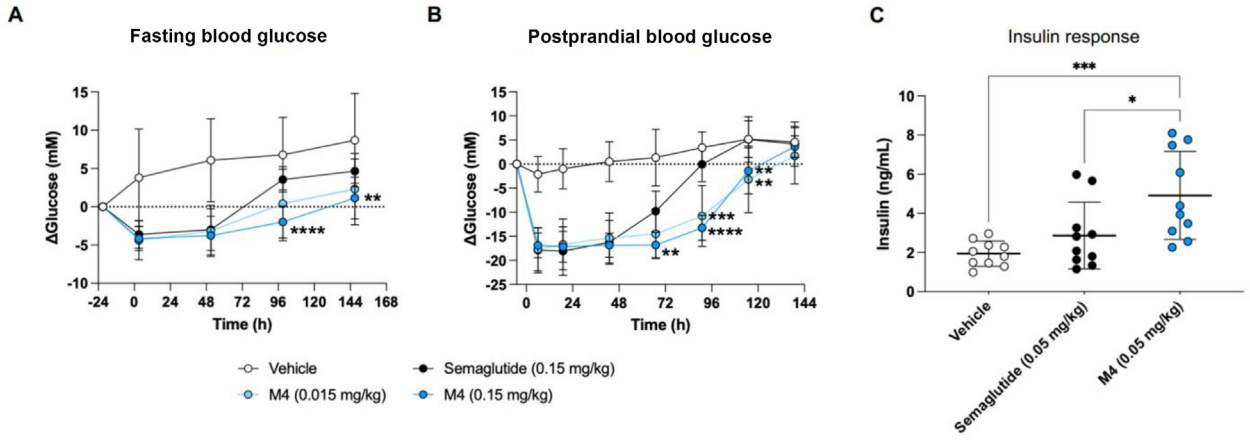

Figure 3: Glucose and insulin response in db/db mice treated with GLP-1 analogs. (A and B) Single subcutaneous dose of GLP-1 analogs in db/db mice. Dosing is at time 0 h. (C) GLP-1 analogs were dosed subcutaneously daily for 42 days. Insulin was measured on Day 43. Significance was calculated for M4 vs semaglutide using a mixed models for repeated measures (MMRM) analysis (A and B), and for M4 vs vehicle and semaglutide using one-way ANOVA (C). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. M4, ecnoglutide. Mean and standard deviation (SD) are plotted.

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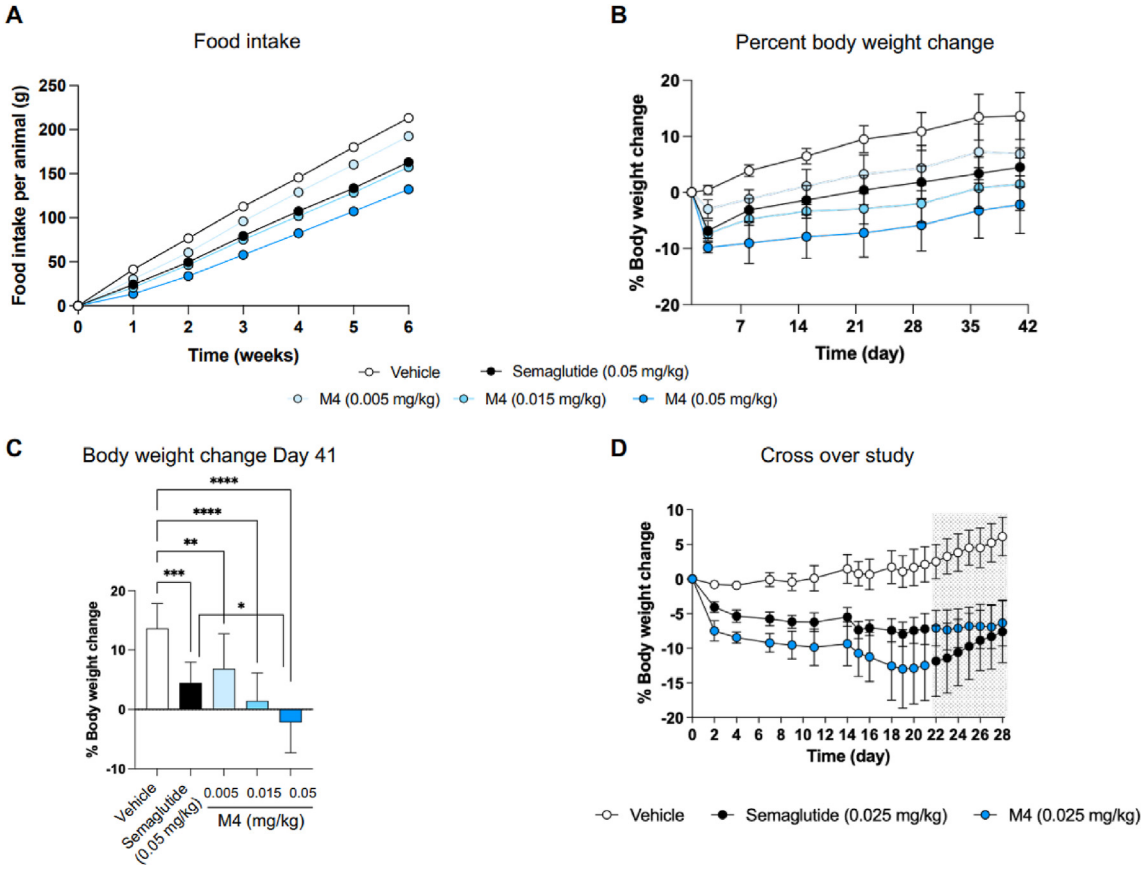

Figure 4: Body weight response in db/db mice and DIO SD rats treated with GLP-1 analogs. (A–C) Male db/db mice administered vehicle, ecnoglutide (M4), or semaglutide by s.c. injection once daily for 42 days. (D) Male DIO SD rats dosed with vehicle, ecnoglutide (M4, 0.025 mg/kg), or semaglutide (0.025 mg/kg) s.c. once daily for 21 days, after which the active treatment groups were switched to dosing with the other compound for 7 days. Grey box indicates switched treatment period. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Mean and SD are plotted for B–D.

3.5. Safety, tolerability, and PK of ecnoglutide in healthy volunteers

Ecnoglutide was evaluated in a randomized, double blind, placebo-controlled, single (SAD) (n = 36) and multiple ascending dose (MAD) (n = 28) Phase 1 study in healthy participants (Supplemental Figure 4). In the SAD portion of the study, participants received a single dose of ecnoglutide or placebo by s.c. injection. Dose levels ranged from 0.03 mg to 1.0 mg, with dosage amounts in some of the cohorts dependent on the weight of the participant. In the MAD portion of the study, participants were administered s.c. doses of ecnoglutide or placebo once weekly for 6 weeks. Doses were titrated up to target doses of 0.2, 0.4, and 0.6 mg.

Ecnoglutide was generally safe and well tolerated in healthy volunteers. The most common treatment emergent adverse events (TEAEs) in multiple dose cohorts were decreased appetite, headache, nausea, hypoglycemia, constipation, and injection site pain (Supplemental Table 4). The majority of the TEAEs were mild to moderate in severity except for one severe (Grade 3) case of decreased appetite in the highest single dose group (1.0 mg). There were no serious treatment related adverse events (TRAEs), and no TRAEs leading to study discontinuation. There were no deaths in the study.

Plasma exposure of ecnoglutide after multiple dosing at steady state (Day 36) increased dose proportionally. Systemic absorption was slow and generally consistent for all MAD cohorts, with a median T_{max} ranging from 12 to 72 h. The mean $t_{1/2}$ at steady state ranged from 124 to 138 h. The accumulation ratios on Day 36 for AUC and C_{max} were <2 for the 0.2 mg and 0.4 mg cohorts, and 2.23 and 2.11, respectively, for the 0.6 mg cohort. This suggested mild accumulation on Day 36 following 0.6 mg ecnoglutide dosing (Table 3).

Overall, these results demonstrated that ecnoglutide has a favorable clinical safety, tolerability, and PK profile. Additional clinical studies are underway to assess the efficacy of ecnoglutide in various populations.

4. DISCUSSION AND CONCLUSIONS

GLP-1 is a peptide hormone secreted from the gut in response to food intake that acts to lower blood sugar and potentiate glucose-induced insulin secretion [14]. The GLP-1 receptor is found in many organs, including pancreas, brain, heart, kidney, and gastrointestinal tract [15]. A number of GLP-1 receptor agonists (e.g., exenatide, liraglutide, semaglutide, dulaglutide) and a dual GIP/GLP-1 receptor agonist (tirzepatide) are approved to treat T2DM and/or obesity [4,13]. This class of molecules is effective at reducing mortality, improving glycemic control, reducing body weight, and improving cardiovascular risk factors. However, current GLP-1 analogs may not be efficacious for all patients, can require injection administration, can cause adverse gastrointestinal effects such as nausea and vomiting, and are expensive to manufacture.

Here we aimed to discover a novel GLP-1 peptide analog with a favorable stability, efficacy, and tolerability profile, as well as improved manufacturing feasibility. A series of analogs was developed by

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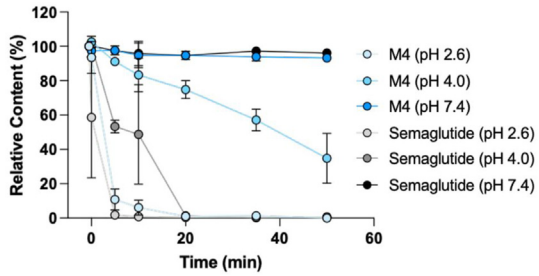

Figure 5: Gastrointestinal stability of ecnoglutide. Stability of ecnoglutide (M4) and semaglutide in pepsin at various pH levels. Mean and SD are plotted.

modification of the GLP-1 peptide, from which ecnoglutide was chosen for further development. Modifications included acylation at different positions to increase peptide half-life by promoting albumin binding and resistance to DPP-4 [16]. Acylation of lysine at position 30 was selected for ecnoglutide, while semaglutide and liraglutide are acylated at position 26. Compared to semaglutide, which incorporates an unnatural amino acid 2-aminoisobutyric acid (Aib) at position 8, ecnoglutide encodes valine at that position. This sequence has been reported to favor cAMP bias [11]. The valine substitution also facilitates manufacturing of ecnoglutide. Full-length ecnoglutide peptide is possible to synthesize from recombinant DNA without chemical conjugation. In contrast, manufacturing of semaglutide requires preparation of recombinant peptide 9—37, followed by chemical conjugation of two additional amino acids, His7 and Aib8 [17]. The manufacturing of ecnoglutide therefore takes fewer steps than semaglutide.

Analogs were tested for agonist potency, as well as for cAMP signaling bias. cAMP bias is hypothesized to enhance the efficacy of GLP-1 receptor agonists. For example, tirzepatide shows bias in its GLP-1 receptor agonist activity, with cAMP production favored over β-arrestin recruitment and receptor internalization [10]. This bias is thought to contribute the superior HbA1c reduction and weight loss results seen for tirzepatide compared to other GLP-1 receptor agonists [12,18]. Mechanistically, bias for cAMP production over receptor internalization has been associated with weaker GLP-1 receptor binding, shorter receptor residence time, and distinct conformational changes compared to the native protein [11]. These findings suggest that altered receptor engagement leads to retention of the GLP-1 receptor on the cell surface and continued signaling over a prolonged time, leading to a more robust response [11]. Downstream signaling through the cAMP pathway controls blood sugar through multiple processes, including regulation of insulin production, glucagon secretion, and glucose uptake [19]. In the present study, ecnoglutide showed similar potency to semaglutide with respect to cAMP induction, with EC_{50} values of 2.322 ng/mL and 2.437 ng/mL, respectively. Ecnoglutide and semaglutide also showed similar EC_{50} values for β-arrestin recruitment. The maximum recruitment of β-arrestin to the GLP-1 receptor, however, was higher for semaglutide than ecnoglutide and receptor internalization was much lower for ecnoglutide than for semaglutide. Ecnoglutide therefore appears to show more bias towards cAMP signaling compared to semaglutide.

Consistent with the expected increase in activity of cAMP-biased GLP-1 analogs, ecnoglutide demonstrated significantly higher efficacy compared to semaglutide in terms of blood glucose control and weight loss in preclinical models, while showing similar PK parameters. In db/

Table 3 — Summary of plasma pharmacokinetic parameters for ecnoglutide at steady state (MAD Day 36).

Parameter		Ecnoglutide dose group 0.2 mg (N = 8)	0.4 mg (N = 8)	0.6 mg (N = 7)
C_{max},ss (ng/mL)	n	7	8	6
	Mean	25.3286	46.6875	84.7333
	SD	3.4913	7.0786	10.9237
T_{max},ss (hr)	n	7	8	6
	Median	72.000	39.042	12.000
$AUC_{0\text{-inf,ss}}$ (hr*ng/mL)	n	6	8	5
	Mean	6611.5447	11658.1612	21604.9215
	SD	1017.6543	2654.8702	4555.4539
$t_{1/2,ss}$ (hr)	n	7	8	6
	Mean	124.1109	138.0929	128.6007
	SD	16.5586	23.0285	13.5973
CL/F,ss (L/hr)	n	6	8	5
	Mean	0.0575	0.0669	0.0533
	SD	0.0092	0.0108	0.0095
Vz/F,ss (L)	n	6	8	5
	Mean	10.6958	13.2773	10.1639
	SD	2.1566	2.9711	1.6094
$RA_{AUC/D}$	n	7	8	5
	Mean	1.8102	1.9709	2.2325
	SD	0.2241	0.2409	0.2413
$RA_{Cmax/D}$	n	7	8	6
	Mean	1.7192	1.9248	2.1125
	SD	0.2426	0.3312	0.2546

Abbreviations: AUC = area under plasma time—concentration curve; Cmax = maximum plasma concentration; CL/F = apparent total body clearance; RA = accumulation ratio; SD = Standard Deviation; SS = steady state; Tmax = time to reach the maximum plasma concentration; $t^1/_2$ = elimination half-life. Vz/F = apparent volume of distribution.

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db mice, ecnoglutide showed a significantly higher level of insulin response compared to semaglutide. In db/db mice, weight reduction was significantly more pronounced after 6 weeks of treatment with 0.05 mg/kg of ecnoglutide compared to the same dose of semaglutide. Furthermore, in a cross-over study, DIO SD rats receiving ecnoglutide then semaglutide began to gain weight after the treatment switch, whereas those receiving semaglutide then ecnoglutide maintained a stable reduced body weight. The weight loss effects of GLP-1 peptide analogs are beneficial to patients with T2DM, as reduced body weight can improve glycemic control and positively impact various comorbidities, including cardiovascular risk factors.

In Phase 1 SAD/MAD studies in healthy volunteers (n = 64), ecnoglutide was found to be safe and generally well tolerated when tested as once weekly s.c. dosing for up to 6 weeks. The most common TEAEs were as expected for a GLP-1 receptor agonist, including nausea, decreased appetite, and headache. In the SAD, the one sentinel participant in the highest dose group (1.0 mg) had a Grade 3 TEAE of decreased appetite, which led to emergency unblinding and a decision not to proceed with the same dose or higher dose levels. This result was consistent with the need to slowly increase the dose of GLP-1 analogs to limit gastrointestinal side effects. In the MAD cohorts, the titration period of ecnoglutide was 2 weeks before achieving the target dose for the remaining 4 weeks.

The PK of ecnoglutide was similar to semaglutide both in preclinical animal studies and in healthy volunteers. The mean $t_{1/2}$ for ecnoglutide ranged from 124 to 138 h on Day 36, compared to ~165 h hours for semaglutide [20]. In vitro testing in simulated gastrointestinal fluid indicated that ecnoglutide had significantly improved stability over semaglutide. These results suggest the potential for ecnoglutide to be developed as an oral therapeutic. An oral formulation of ecnoglutide (termed XW004) is currently being tested in a Phase 1 clinical trial. Oral dosing may be preferable for patients as it would relieve the need for injections. Indeed, semaglutide has been formulated as a tablet (Rybelsus®) for the treatment of T2DM, although its bioavailability is very low and patients must avoid taking the drug with food to allow optimal absorption [16].

In conclusion, ecnoglutide is a novel, cAMP-biased GLP-1 receptor agonist with strong binding affinity for the GLP-1 receptor and potent in vitro and in vivo efficacy. Ecnoglutide showed suitable PK for once weekly injection and gastrointestinal stability supportive of an oral formulation. Ecnoglutide was safe and generally well tolerated when administered by injection in healthy volunteers. These results support the continued development of ecnoglutide for T2DM and obesity. Phase 2 and 3 studies of ecnoglutide in participants with these conditions are recently completed or currently underway.

DECLARATION OF COMPETING INTEREST

WG, HZ, YL, JF, ZZ, QZ, RZ, BW, YL, SH, HQ, CLJ, EA, LT, MF, WZ, MKJ, SX, and HP are, or were at the time the work was conducted, employees of Sciwind Biosciences.

DATA AVAILABILITY

Data will be made available on request.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2023.101762.

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