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TITLE PAGE

Pharmacological Characterization of Apraglutide a Novel Long-Acting Peptidic Glucagon-Like Peptide-2 Agonist for the Treatment of Short Bowel Syndrome

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Apraglutide a Novel GLP-2 Agonist

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Non-standard abbreviations:

Apraglutide, [Gly^2, Nle^{10}, D-Phe^{11}, Leu^{16}] hGLP-2 (1-33)-NH_2;

DMEM, Dulbecco's modified Eagle's medium;

DPP4, dipeptidyl peptidase-IV;

Fsc, subcutaneous bioavailability;

GLP-1, glucagon-like peptide-1;

GLP-2, glucagon-like peptide-2;

HEK, human embryonic kidney;

hGLP-1, human glucagon-like peptide-1;

hGLP-2, human glucagon-like peptide-2;

HPLC, high performance liquid chromatography;

IgG, Immunoglobulin G;

LC/MS/MS, liquid chromatography–tandem mass spectrometry;

Nle, norleucine;

PK, pharmacokinetics;

rGLP-1, rat glucagon-like peptide-1;

rGLP-2, rat glucagon-like peptide-2;

SAR, structure-activity relationship;

Vc, apparent volume of the central compartment;

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ABSTRACT

Glucagon-like peptide-2 (GLP-2) agonists have therapeutic potential in clinical indications where the integrity or absorptive function of the intestinal mucosa are compromised, such as in short bowel syndrome (SBS). Native hGLP-2, a 33-amino acid peptide secreted from the small intestine, contributes to nutritional absorption but has a very short half-life due to enzymatic cleavage and renal clearance and thus is of limited therapeutic value. The GLP-2 analogue teduglutide (Revestive®/Gattex®, Shire Inc) has been approved for use in SBS since 2012 but has a once-daily injection regimen. Pharmacokinetic (PK) and pharmacodynamic studies confirm that apraglutide, a novel GLP-2 analogue, has very low clearance, long elimination half-life and high plasma protein binding compared with GLP-2 analogues teduglutide and glepaglutide. Apraglutide and teduglutide retain potency and selectivity at the GLP-2 receptor comparable to native hGLP-2 while glepaglutide was less potent and less selective. In rat intravenous PK studies, hGLP-2, teduglutide, glepaglutide and apraglutide had clearances of 25, 9.9, 2.4 and 0.27 ml/kg/min, respectively, and elimination half-lives of 6.4, 19, 18 and 159 min, respectively. The unique PK profile of apraglutide administered via intravenous and subcutaneous routes was confirmed in monkey and minipig, and translated into significantly greater *in vivo* pharmacodynamic activity, measured as small intestinal growth in rats. Apraglutide showed greater intestinotrophic activity than the other peptides when administered at less frequent dosing intervals because of its prolonged half-life. We postulate that apraglutide offers several advantages over existing GLP-2 analogues and is an excellent candidate for the treatment of gastrointestinal diseases, such as SBS.

SIGNIFICANCE STATEMENT

Apraglutide is a potent and selective GLP-2 agonist with an extremely low clearance and prolonged elimination half-life, which differentiates it from teduglutide (the only approved GLP-2 agonist). The enhanced pharmacokinetics of apraglutide will benefit patients by enabling a reduced dosing frequency and removing the need for daily injections.

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VISUAL ABSTRACT

INTRODUCTION

Short bowel syndrome (SBS) is a severe, chronic condition characterized by malabsorption due to physical or functional loss of significant portions of the small intestine (Jeppesen 2014). Consequently, patients suffer life-threatening malnutrition, dehydration and imbalances of fluids and salts resulting from severe diarrhea.

SBS results in a process of adaptation that increases mucosal surface area (Hasosah et al. 2008; Weale et al. 2005). Non-nutritional gut stimulators, including growth factors and hormones, contribute to this. One of these, glucagon-like peptide-2 (GLP-2), is released by the intestinal L-cells into the circulation following nutrient ingestion and acts on the intestine to stimulate growth, increase absorption, promote healing and maintain intestinal epithelial integrity (Orskov et al., 1986; Xiao et al., 1999; Burrin et al., 2000, Brubaker et al. 2018).

GLP-2 stimulated growth of the small intestine and colon in mice (Drucker et al., 1996; Tsai et al., 1997; Litvak et al., 1998) but had no effect on the growth of other tissues (Drucker et al., 1996). Additional biological actions of GLP-2 on the gastrointestinal tract include enhancement of nutrient absorption, stimulation of blood flow, anti-inflammatory activity, improvement of barrier function and modulation of motility (Nagell et al., 2004; Guan et al., 2006; Meier et al., 2006; Stephens et al., 2006; Bremholm et al., 2009; Hsieh et al., 2009; Moore et al., 2010; Rowland and Brubaker, 2011).

In SBS, GLP-2 increases intestinal absorption by promoting intestinal growth, and delays accelerated gastric emptying, gastrointestinal transit and hypersecretions (Brubaker, 2018).

GLP-2 is of limited use as a therapeutic agent because of the very short circulating half-life (only ~7 min in humans) due to cleavage by dipeptidyl peptidase-IV (DPP4) as well as renal clearance (Drucker et al., 1997; Hartmann et al., 2000, Tavares et al., 2000; Hansen et al., 2007).

Consequently, pharmacologically active GLP-2 analogues with a longer half-life were developed.

The GLP-2 analogue teduglutide (Gattex®, Revestive®) has an Ala-to-Gly substitution at position 2 to eliminate the DPP4 cleavage site ([Gly^2] hGLP-2 (1-33)) and a terminal half-life of 3.0-5.5 h by the subcutaneous route in humans (Marier et al., 2008). In patients with SBS, once-daily treatment with teduglutide significantly reduced parenteral nutrition requirements and improved intestinal function (Jeppesen et al., 2011; Burness and McCormack, 2013).

Various additional analogues of GLP-2 with reduced clearance relative to hGLP-2 are under development for SBS, including apraglutide (FE 203799, VectivBio; EudraCT Numbers 2017-002486-21 and 2017-002487-41) and glepaglutide (ZP1848, Zealand Pharma; EudraCT Numbers 2015-002826-38, 2017-004394-14 and 2018-001429-26).

The GLP-2 analogue apraglutide ([Gly^2, Nle^{10}, D-Phe^{11}, Leu^{16}] hGLP-2 (1-33)-NH_2), identified through chemistry structure-activity relationship (SAR) studies of lipophilic amino acid substitutions in positions 11 and 16 of [Gly^2] hGLP-2 (1-33), showed exceptionally low clearance in i*n vivo* rat pharmacokinetic (PK) screening (Wiśniewski et al., 2011; Wiśniewski et al., 2016).

This paper presents the pharmacology and PK of apraglutide compared directly with teduglutide and glepaglutide. In particular, we show that the optimized chemical structure of apraglutide translates to a superior PK profile allowing intestinal growth to be achieved with less frequent dosing in rats.

MATERIALS AND METHODS

Peptide Synthesis. Peptides used in these studies were prepared as trifluoroacetic acid (TFA) salts by solid-phase peptide synthesis and purified by reverse-phase high performance liquid chromatography (HPLC) as previously described (Wiśniewski, et al. 2011; Wiśniewski, et al. 2016,). The peptide identities were verified by mass spectrometry. The peptide batches had purity > 90% and peptide content > 75% as measured by analytical HPLC and elemental analysis of nitrogen, respectively. Amino acid sequences of the peptides used in these studies are shown in Table 1; amino acid sequence of glepaglutide was identified from a published patent application (WO06117565A2).

Cell Culture. Human embryonic kidney 293 cells (HEK-293; ATCC, CRL-1573) were maintained in DMEM containing 5% (v/v) heat inactivated fetal bovine serum and 4 mM L-glutamine at 37°C under 5% CO_2 in a humidified atmosphere. Cells were passaged 1:3 to 1:6 by trypsinization every 3-4 days. G418 (500 μg/ml) was included in the culture medium for the cells stably transfected with the GLP-1 receptor.

Vectors. hGLP-1 receptor activity was studied in a stable cell line generated with the human GLP-1R expressing clone (pcDNA3.1-hGLP-1R encoding NM_002062) and a pCRE-Luc clone expressing a luciferase reporter gene with 2 tandem repeats of the cAMP (cyclic adenosine monophosphate) response element (CRE) in HEK293 cells.

Transient transfections in HEK293 cells were also carried out with the human GLP-2R expressing clone (pcDNA3.1-hGLP-2R encoding NM_004246) and a pCRE-Luc clone expressing a luciferase reporter gene with two tandem repeats of the CRE.

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A HEK293 stable cell line was generated expressing the rat GLP-1 receptor (pCDNA5/FRT-rGLP-1R encoding NM_012728) and transiently transfected with a pCRE-Luc clone expressing a luciferase reporter gene with two tandem repeats of the CRE.

Transient transfections in HEK293 cells were also carried out with the rat GLP-2 receptor expressing clone (pCEP4-rGLP2R encoding NM_021848) and a pCRE-Luc clone expressing a luciferase reporter gene with two tandem repeats of the CRE

Cell Transfection. For the GLP-2 receptor assays, HEK-293 cells were transiently co-transfected with human or rat GLP-2 receptor (hGLP-2 receptor, rGLP-2 receptor) and a reporter plasmid containing a luciferase gene under the control of cAMP responsive elements. Briefly, DNA plasmids containing cDNA encoding human or rat GLP-2 receptor were prepared for transfection with Lipofectamine2000™ transfection regent (Invitrogen, Carlsbad, CA). DNA-Lipofectamine complexes were added to cells at ~90% confluence and incubated for 24 h at 37°C under 5% CO_2**.**

For the cell-based hGLP-1 receptor assay, monoclonal HEK-293 cells stably co-transfected with the human GLP-1 (hGLP-1) receptor and a reporter plasmid containing a luciferase gene under the control of cAMP responsive elements were used.

Luciferase Reporter Gene Assay. Stably or transiently transfected cells grown in T-175 flasks were dispersed with 5 ml of trypsin EDTA solution and harvested in phenol red-free DMEM containing 5% heat inactivated fetal bovine serum, 4 mM L-Glutamine and plated onto 96-well white clear bottom plates at $4.5x10^4$/90 µl per well (GLP-2 receptor assay) or $2.5x10^4$/90 µl per well (GLP-1 receptor assay) and incubated overnight at 37°C under 5% CO_2.

Each compound was typically tested in serial dilutions in half log units over a final concentration range of 0.3 pM to 30 nM in the GLP-2 receptor assays and at concentrations up to

1000 nM in the GLP-1 receptor assay. The native ligands, hGLP-2 or hGLP-1, were run as positive controls at 30 nM in the respective assays and maximal efficacy of compounds was expressed relative to the positive controls run within the same assay. The assay was initiated by addition of compounds (10 µl of 10X working solutions) to each well in the 96-well plates. Blanks (compound dilution buffer) were included on each plate. After compound addition, plates were gently mixed and incubated for 5 h at 37°C under 5% CO_2. After incubation, 100 µl of reconstituted luciferase substrate (Luclite, PerkinElmer, Waltham, MA) was added to each well. The plates were sealed, gently mixed and incubated for 10 min at room temperature in the dark. Luminescence was measured on an Analyst™ (Molecular Devices, Sunnyvale, CA) plate reader using the default luminescence counting protocol. In each assay compounds were typically tested in duplicate and were tested in three or more independent assays.

Animals and Housing.

Rat: Adult male Sprague Dawley rats of approximately 225-275 g body weight were obtained from Harlan (Indianapolis, IN). Upon delivery, rats were housed in ventilated Plexiglas cages at ambient temperature 22-25 ºC and maintained on a 12-h light/12-h dark cycle. Food (rodent chow, Harlan Teklad 2018 global diet) and water were provided *ad libitum*. The rats were acclimatized to the facility for at least 2 days prior to the initiation of experiments. Rats used in the PK studies were surgically catheterized with polyethylene tubing implanted in one carotid artery for blood collection and one jugular vein for intravenous compound administration. All animal procedures were approved by the Ferring Research Institute Institutional Animal Care and Use Committee and were in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Research Council.

Monkey: Male adult cynomolgus monkeys weighing 4.64-6.39 kg were housed at Valley Biosystems (West Sacramento, CA) according to the USDA Animal Welfare Act (9CFR) and the conditions outlined in The Guide for Care and use of Laboratory Animals. An intravenous catheter was inserted in the saphenous vein for intravenous dosing. Blood samples were taken by venipuncture from a cephalic vein.

Minipig: Castrated male Yucatan minipigs weighing 62.2-67.6 kg were housed in individual cages without bedding according to the USDA Animal Welfare Act (9CFR) at Sinclair Research Center (Auxvasse, MO). The minipigs had in-dwelling ear vein catheters surgically inserted for intravenous dosing. Blood samples were taken by venipuncture.

Preparation of Dosing Solutions.

***In vitro* assays:** For the *in vitro* cell based functional assays, peptides were prepared as 1 mM stock solutions in 100% dimethyl sulfoxide and stored at -20ºC until use. Stock solutions were serially diluted to 10X working solutions in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.2% fatty acid free bovine serum albumin and 1% dimethyl sulfoxide.

PK studies: For the dosing solutions used in the *in vivo* PK studies, apraglutide and teduglutide were dissolved in 25- or 50-mM phosphate buffer without additional salts at pH 7.4. For the 5 mg/kg subcutaneous (s.c.) dose of apraglutide, to dissolve apraglutide at 10 mg FB/ml, 0.1 N sodium hydroxide was added stepwise during formulation resulting in a final pH of 7.8. hGLP-2 was dissolved in 25 mM phosphate buffer pH 7.4 isotonic with sodium chloride. Glepaglutide was dissolved in normal saline (0.9% w/v sodium chloride). For the intravenous (i.v.) PK studies, dosing solutions were prepared with single compounds or as a cassette containing a total of three to six compounds (the chemical entity being tested plus other GLP-2

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analogs from the chemistry SAR program in the same dosing solution). For the s.c. PK studies, compound dosing solutions were prepared as single compounds and animals were dosed with two to five compounds in separate dosing solutions at different s.c. sites within the same animal.

***In vivo* pharmacodynamic studies** For the *in vivo* rat intestinal growth pharmacodynamic studies, apraglutide, teduglutide and low doses (30 nmol/kg) of glepaglutide were dissolved in 25 mM phosphate buffer without additional salts at pH 7.4. Glepaglutide (high dose, 300 nmol/kg) was prepared by dissolving the peptide in sterile water. For all peptides, dosing solutions were prepared to deliver doses ≤100 nmol/kg. Dosing vials were pre-rinsed with peptide solution at the target dosing concentration to minimize peptide adsorption to the vial and then dosing solution was prepared in the pre-rinsed vial. Dosing solutions were stored (4 ºC) up to 8 days.

Pharmacokinetics. Dosing solutions were administered by i.v. bolus injection to catheterized animals or s.c. In the i.v. PK studies, blood samples were collected at multiple time points up to 6 h post injection. In the s.c. PK studies, blood samples were collected at multiple time points up to 169 h post injection. Blood was collected into tubes containing EDTA as anticoagulant and, following centrifugation, plasma was collected, frozen in dry ice and stored at -80°C until analysis.

Analysis of Plasma Peptide Concentrations in Plasma. The concentrations of GLP-2 analogues were determined using a liquid chromatography tandem mass spectrometry (LC/MS/MS) method. Standard and internal standard solutions were prepared in 50% DMSO in acetonitrile. Samples were injected into a Jupiter 5 µm 300Å C18 50 x 2.0 mm HPLC column (Phenomenex, Torrance USA) coupled to a Shimadzu LC-20AD series LC (Shimadzu, Kyoto Japan) system. Analytes were eluted using a gradient method with a mobile phase (A: 0.01% trifluoroacetic acid and 1% formic acid in water; B: 0.01% trifluoroacetic acid and 1% formic

acid in 70% acetonitrile) at a flow rate of 0.5 mL/min and detected using an API-4000 triple quadrupole mass spectrometer (Applied Biosystems, Ontario Canada) in the positive electrospray ionization mode. Analyte concentrations were calculated by linear regression analysis using the peak area ratio of analyte to the internal standard on the Applied Biosystems Analyst software version 1.4.2.

Pharmacokinetic parameters were determined by best fitting of compound concentration-time curves using a non-compartmental curve stripping method (PK Solutions 2.0™ software, Summit Research Services, Montrose, CO). The PK parameters assessed were area under the curve from time zero to infinity normalized to dose (AUC∞/unit dose), clearance (CL), maximum observed plasma concentration normalized to dose (Cmax/unit dose), subcutaneous bioavailability (Fsc), elimination half-life (T1/2), time of the maximum observed plasma concentration (Tmax) and initial apparent volume of the central compartment (Vc).

Rat Intestinal Growth. Intestinal growth, as determined by wet weight of the small intestine, was measured as a primary pharmacodynamic endpoint in normal rats.

In the once-daily dosing studies, Sprague-Dawley rats (N=6 per group) received apraglutide and teduglutide once daily for 5 days at equivalent subcutaneous doses of 3 to 1000 nmoles/kg. Rats were euthanized within 24 h after the last dose. Apraglutide, teduglutide and glepaglutide were also administered subcutaneously to Sprague-Dawley rats (N=6 per group) at equivalent doses (30 or 300 nmoles/kg) with a dosing interval of 24 h (compounds injected once-daily for 5 days), 48 h (two injections at times 0 and 48 h) or 72 h (single injection).The rats were euthanized 96 h after the first dose or 72 h after the single dose. The small intestine was flushed with saline, patted with gauze to remove excess saline and weighed. Intestinal wet weight was

normalized to body weight (small intestine weight (g)/body weight (g)). Data generated from multiple separate experiments were combined.

Data and Statistical Analysis. Concentration-response data from the *in vitro* reporter gene assays were entered into ActivityBase software (IDBS, UK) and fitted using a four-parameter concentration-response non-linear regression model to generate the concentration-response curves and determine E_{max} (maximum receptor activation expressed as % of positive control reference compound), ED_{50} (median effective dose), and Hill slope. For graphical presentation, representative curves were also generated by fitting the data to a non-linear regression equation for a sigmoidal curve with variable slope (GraphPad Prism software, La Jolla, CA).

Dose-response data from the intestinal growth assay were fitted using a three-parameter Hill equation to determine ED_{50} and E_{max} (GraphPad Prism software, La Jolla, CA):

$$Y = E_{max} * X^{nH} / (X^{nH} + ED_{50}^{nH})$$

Where Y is the effect, E_{max} is the fitted maximal effect, X is the dose (nmol/kg), n_H is the unitless Hill coefficient, and ED_{50} is the effective dose generating 50% of the E_{max}.

Data are presented as means with standard error of the mean (S.E.M.), standard deviation (S.D.) or 95% confidence interval as noted in the tables and figure legends. Analysis of variance with Newman-Keuls multiple comparison post-hoc test was used for comparisons of intestinal growth data treatment effects. P values < 0.05 were considered significant.

RESULTS

Apraglutide is Highly Potent and Selective for Activation of the GLP-2 Receptor. The *in vitro* activities of apraglutide and the other peptide analogues are summarized in Table 2 and representative concentration-response graphs are shown in Figure 1. Apraglutide was 2-fold more potent than native hGLP-2 and teduglutide for activation of the hGLP-2 receptor. Apraglutide, hGLP-2, and teduglutide had similar potency at the rGLP-2 receptor. Glepaglutide was less potent at both the hGLP-2 and rGLP-2 receptors relative to apraglutide. All peptides had ≥ 95% E_{max} at the hGLP-2 receptor and ≥ 84% E_{max} at the rGLP-2 receptor. Apraglutide and teduglutide were highly selective for the hGLP-2 receptor versus the hGLP-1 receptor with EC_{50} values > 1000 nM (the highest concentration tested) at the hGLP-1 receptor. Glepaglutide fully activated the hGLP-1 receptor and had selectivity of < 50-fold for activation of hGLP-2 receptor vs. hGLP-1 receptor (GLP-1/GLP-2 ratio = 36 versus >33,000 for apraglutide and >11,000 for teduglutide) (Table 2). Similar selectivity was observed for the rGLP-2 receptor vs the rGLP-1 receptor. This indicated that GLP-1 receptor would not play a significant role in the pharmacodynamics of apraglutide in the intestine. The peptides tested at concentrations up to 1000 nM had no activity in a recombinant human glucagon receptor assay (data not shown).

Apraglutide (1 μM) was also tested against a panel of 73 receptors, five ion channels, and three transporters (CEREP, Redmond, WA) and had no significant binding affinity (< 25% inhibition of control specific binding, results not shown) against receptors other than the GLP-2 receptor. The panels of receptors, ion channels and transporters against which apraglutide was evaluated are included as supplemental data.

Apraglutide has Unique Pharmacokinetic Properties of Very Low Clearance and Long Elimination and Terminal Half-Lives. Rat i.v. PK data for apraglutide compared with hGLP-2, teduglutide and glepaglutide are summarized in Table 3A and Figure 2A. As expected, native hGLP-2 had very rapid clearance (25 ml/kg/min) and a short elimination half-life (6.4 min). Stabilization of the DPP4 cleavage site, as exemplified by teduglutide, decreased clearance by 2.5-fold to 9.9 ml/kg/min. A further 4-fold reduction in clearance was observed with the C-terminal polylysine analog, glepaglutide (clearance 2.8 ml/kg/min). The clearance of apraglutide was 9-fold lower than that of glepaglutide. Initial i.v. PK studies, in which apraglutide was tested at 0.2 mg/kg and blood samples were taken through 2 h post-dose, demonstrated a clearance of only 0.27 ml/kg/min. The elimination half-life of apraglutide was 159 min (Table 3A). This very low clearance and long elimination half-life of apraglutide was confirmed in a follow up i.v. PK study conducted with apraglutide dosed at 0.1 mg/kg and blood samples collected through 5 h post dose (data not shown). The clearance of apraglutide in the follow up i.v. PK study was almost 50-fold lower than the clearance of teduglutide (Table 3A).

Very low clearance and long elimination half-life were also shown for apraglutide in direct PK comparisons of i.v. apraglutide and teduglutide in the monkey and minipig. Apraglutide clearance after i.v. administration was 0.046 ml/kg/min in the monkey PK study and 0.032 ml/kg/min in the minipig PK study. The clearance of apraglutide was more than 50-fold lower than that observed for teduglutide in monkey and 30-fold lower in minipig (Table 3B). Similarly, the elimination half-life of apraglutide after i.v. administration was 9-11 fold longer than that of teduglutide in both monkey (474 vs 43 min) and minipig (782 vs 88 min). Average plasma concentrations of apraglutide and teduglutide in monkey and minipig over time are shown in Figures 2B and 2C, respectively.

The PK profiles of apraglutide and teduglutide were also characterized following administration by single dose bolus s.c. injection (Table 4, Figure 3). In rats, the s.c. terminal half-life of apraglutide was appreciably longer than its elimination half-life by the i.v. route (197 min at 0.1 mg/kg i.v. *vs*. 701 min at 1 mg/kg s.c. or 1349 min at 5 mg/kg), suggesting slow release from the subcutaneous space into the circulation. The observed long value of Tmax ≥ 400 min post dose may reflect contributions of the long half-life of the peptide in the circulation and its slow absorption from the s.c. injection site. (Table 4A; Figure 3A). The subcutaneous bioavailability of apraglutide was good (74 and 54% at 1 and 5 mg/kg, respectively). In contrast, teduglutide had a terminal half-life of about 31 min, suggesting that it was absorbed fairly rapidly and also with good bioavailability.

Terminal half-life and Tmax were also notably longer for apraglutide than for teduglutide in the monkey and minipig s.c. PK studies (Table 4B). The s.c. terminal half-life of apraglutide (1941 min (32 h) in monkey and 1808 min (30 h) in minipig) was additionally longer than the i.v. elimination half-life (474 min in monkey and 782 min in minipig), suggesting slow release from the s.c. injection site also in these species. Accordingly, the observed apraglutide Tmax was over 1300 minutes (22 h) in both monkey and minipig (6-10-fold longer than those observed for teduglutide). The bioavailability of apraglutide was 32% in monkey and 43% in minipig. Average plasma concentrations of apraglutide and teduglutide over time after s.c. administration to monkey and minipig are shown in Figures 3B and 3C, respectively.

Apraglutide is Potent and Long-acting in a Pharmacodynamic Assay of Rat Intestinal Growth. To determine pharmacodynamic activity, apraglutide and the other GLP-2 analogues were tested in a rat model of intestinal growth (intact animals, gain in small intestine wet weight). In once-daily dosing studies, complete dose-response curves were generated for

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apraglutide and teduglutide (dose range tested was 3-1000 nmol/kg injected s.c. once daily for 5 days). At each dose, apraglutide had significantly greater effect on intestinal growth than teduglutide ($p < 0.05$; Figure 4). The dose-response data were fitted to a three-parameter Hill equation to estimate E_{max} and potency (ED_{50}). Apraglutide was more potent and had a greater E_{max} than teduglutide (ED_{50} 25 vs 68 nmol/kg and E_{max} 63 vs 41%, respectively, Figure 4).

We also tested the intestinotrophic activity of apraglutide, teduglutide, and glepaglutide at less frequent dosing intervals to gain insight into their duration of action. The peptides were administered once-daily (5 doses given at a dosing interval of 24 h) or every 48 h (2 doses two doses at times 0 and 48 h) at doses of 30 or 300 nmol/kg with the small intestine collected 96 h after the first dose. Additionally, peptides were tested by administration of a single dose and intestines collected 72 h post-dose. Relative to vehicle control, all peptides significantly increased small intestine weight at the 24- and 48-h dosing intervals at both doses (Figures 5A and B). In the single injection study, apraglutide was active at both doses; glepaglutide was active only at the high dose, and teduglutide was not active at either dose (Figure 5C). For all three dosing intervals, at both doses, apraglutide had significantly greater effect on small intestine weight compared with the other GLP-2 analogues (Figures 5A, B, and C).

Similarly, administration of apraglutide (3 mg/kg; 3-times per week) to male and female mice significantly increased small intestinal weight ($p<0.001$) and length ($p<0.001$) at termination at 3 weeks (Wheeler et al. 2018). Further gains were observed when animals were terminated after 10 weeks ($p<0.01$). In addition, both crypt depth and villus height and colon weight and length were markedly increased at 3 weeks of apraglutide administration ($p<0.001$) but although these effects were maintained, they did not improve further with continued apraglutide administration.

Increases in intestine weight and length were also observed in a 26-week study in rats administered apraglutide 0.5-12 mg/kg/dose. Intestine length was increased by up to 23.5% and intestine weight by up to 113.8% (data on file).

DISCUSSION

Apraglutide is a novel GLP-2 analogue with very low clearance. Its few amino acid substitutions (Table 1; Wiśniewski et al., 2011; Wiśniewski et al., 2016) confer unique PK properties, namely very low clearance and high protein binding, that enable a long *in vivo* half-life without conjugation or other major modifications to the peptide. The direct comparisons reported here for apraglutide versus native hGLP-2 and other GLP-2 analogues currently in the clinic (teduglutide) or in clinical trials (glepaglutide) for the treatment of SBS show that apraglutide has superior PK and pharmacodynamic profiles. In addition, they demonstrate that these translate to greater biological activity *in vivo.*

The attractive physiological and pharmacological activities of GLP-2 have resulted in many efforts to slow down the very rapid clearance of the native peptide so it can be developed into a therapeutic agent. GLP-2 is cleaved by the enzyme DPP4 that is widely distributed in the circulation and various tissues, including capillaries of the small intestine (Hansen et al., 1999). A variety of strategies can increase elimination and terminal half-lives of peptides to create therapeutic agents, including amino acid substitutions to prevent enzyme binding or promote protein binding, and conjugation with larger molecular entities, such as Fc fusion proteins or polyethylene glycol, to increase peptide size and reduce renal clearance (Werle and Bernkop-Schnürch, 2006; Nestor, 2009). These have been applied to the GLP-2 peptide. The GLP-2 analogue teduglutide, which is approved for the treatment of SBS, has amino acid substitutions that eliminate the DPP4 enzymatic cleavage site. Another GLP-2 analogue currently being investigated in clinical trials for the treatment of SBS, glepaglutide, has a polylysine moiety incorporated at the C-terminal to reduce renal clearance. In addition, the introduction of specific lipophilic amino acid substitutions at positions 11 and 16 unexpectedly conferred very low

systemic clearance to a third peptide analog in development for the treatment of SBS, apraglutide.

The structural modifications of apraglutide were targeted at improving the PK profile whilst preserving potency and selectivity. In cell-based assays of receptor activation, apraglutide retained potency, efficacy, and selectivity at the GLP-2 receptor comparable to native hGLP-2 and teduglutide (Table 2 and Figure 1). The cell-based reporter gene assays also showed marked selectivity of apraglutide and teduglutide for rat GLP-2 receptors over GLP-1 receptors. The GLP-1 receptor thus does not play a significant role in the pharmacodynamics of apraglutide in the intestine. Glepaglutide, was less potent than apraglutide at the GLP-2 receptor and less selective versus the GLP-1 receptor (Table 2 and Figure 1).

An extensive pre-clinical toxicology program has not revealed any safety concerns with apraglutide. After exposure of apraglutide (0.5-12 mg/kg) for up to 26 weeks in rat (n=160), all rats that received ≥3 mg/kg/dose showed minimal to moderate hyperplasia of the crypt/villus in the duodenum and jejunum compared with controls. The effects were dose-dependent, indicating that this was a treatment effect. Treatment-related minimal hyperplasia of the crypt was also observed in the colon, caecum and rectum, but fewer animals were affected. Some bile duct hyperplasia was observed in female rats, but this was not dose-related and so the relationship of this finding to apraglutide treatment is unclear (data on file). No colonic polyps were observed. However, in light of the potential risk of accelerated growth of colon polyps and neoplasms with teduglutide treatment, a comprehensive pre-clinical carcinogenicity program has been developed for apraglutide.

PK studies of apraglutide administered intravenously to rat, monkey and minipig clearly demonstrated its low clearance (Figure 2). Native hGLP-2 administered to rat was rapidly

cleared from the circulation as expected due to known mechanisms of DPP4 cleavage and renal clearance (Drucker et al., 1997; Tavares et al., 2000; Hansen et al., 2007). Stabilization of the DPP4 cleavage site of native hGLP-2, as exemplified by teduglutide, reduced clearance and further reductions in clearance were observed for glepaglutide (~10-fold reduction relative to native hGLP-2). Clearance of apraglutide was ~100-fold lower than for native hGLP-2 in rat and 30-50 fold lower than for teduglutide in rat, monkey and minipig (Table 3 and Figure 2). These results suggest that elimination of DPP4 cleavage only partly reduces clearance of native GLP-2; it must be augmented with additional modifications to obtain GLP-2 analogues with a low clearance and long half-life. The mechanisms responsible for the unique PK profile of apraglutide, including the contribution of plasma protein binding to lowering clearance, are under investigation.

Rat PK studies demonstrated that apraglutide has good s.c. bioavailability (54-74%). Apraglutide s.c terminal half-life in rat, monkey and minipig was longer than the equivalent i.v. elimination half-life, probably due to delayed absorption from the s.c. space. T_{max} and terminal half-life of apraglutide were considerably longer than those of teduglutide following s.c. administration in rat, monkey and minipig (Table 4 and Figure 3).

The present studies, in addition to confirming that the very low clearance of apraglutide is observed in several animal models, demonstrated that the prolonged terminal half-life of apraglutide translated to greater pharmacodynamic activity relative to GLP-2 analogues with shorter terminal half-lives. Intestinal growth was the primary pharmacodynamic endpoint *in vivo*. The stimulation of intestinal growth by apraglutide supports that previously reported in a mouse model of gluten sensitivity (Wheeler et al. 2018). In once-daily dosing studies in normal rats, apraglutide induced dose-related increases in small intestine growth (Figure 4) with an ED_{50} of

25 nmol/kg and a 63% increased E_{max} over vehicle control. Consistent with its prolonged terminal half-life, apraglutide showed superior stimulation of small intestine growth *in vivo* relative to comparator GLP-2 analogues. In the complete dose-response study, a direct comparison of once-daily dosing of apraglutide and teduglutide showed that apraglutide had significantly greater effect at each dose and the fitted dose-response curves showed greater E_{max} and potency for apraglutide (Figure 4). Intestine weight was normalized by body weight to decrease the rat-to-rat variation in the assessment of intestine weight. Although there was a moderate increase (≈10%) in body weight at some of the higher doses of apraglutide, this does not impact the interpretation for intestinal trophic effects.

In studies examining the efficacy of GLP-2 analogues administered either once daily or less frequently, apraglutide showed greater ability to stimulate small intestine growth in rat at each dosing interval relative to teduglutide and glepaglutide when directly compared at two doses (30 and 300 nmol/kg; Figure 5). These results show that the longer terminal half-life of apraglutide translated to greater pharmacological activity *in vivo* and suggest the potential for less-frequent dosing to achieve efficacy.

Indeed, the efficacy of apraglutide administered less frequently has been demonstrated in a neonatal piglet SBS model (Slim et al., 2018). Apraglutide was administered subcutaneously on Days 0 and 4 to pre-term piglets with 75% intestinal resection with jejunocolic anastomosis. After 7 days, significant intestinal lengthening from the post-resection measurement was observed in piglets receiving apraglutide compared with piglets fed saline (11.5 cm vs -4.5 cm; p=0.001). Similarly, piglets treated with apraglutide had significantly greater small intestinal weight on Day 7 (0.15 g/cm vs 0.12 g/cm; p=0.004), despite equivalent weight gain.

Apraglutide is expected to provide a major contribution to patient care by enabling once-weekly (instead of daily) administration, which in turn improves quality of life and treatment compliance. The reduced injection frequency provides significant clinical effects whilst increasing patient comfort and acceptability and decreasing the risk of injection site reactions. Clinical trials of once-weekly subcutaneous apraglutide in adult patients are currently underway (NCT 03415594 / NCT 03408132). Data from these clinical trials will help determine whether the benefits of the increased half-life and elevated potency of apraglutide are offset by any unwanted effects. Apraglutide had a positive safety profile in a Phase 1A trial that completed in 2017, being well tolerated in healthy human volunteers (Dimitriadou et al. 2019). Clinical evidence demonstrates that GLP-2 agonists have the potential to address unmet medical needs for patients with serious gastrointestinal disorders. Patients receiving teduglutide for SBS in clinical trials had significant and meaningful reductions in the requirement for parenteral nutritional support (Jeppesen et al. 2011; Burness and McCormack, 2013). Teduglutide was also shown to improve the symptoms of Crohn's disease, but the results were not statistically significant (Buchman et al., 2010; Blonski et al., 2013).

An additional potential therapeutic indication for GLP-2 agonists is the prevention or treatment of chemotherapy-induced intestinal damage (mucositis) that causes gastrointestinal side effects, including diarrhea and infections from intestinal bacteria. Data from animal models suggest that GLP-2 agonists can ameliorate the deleterious effects of chemotherapy and radiation on the gastrointestinal tract (Tavakkolizadeh, et al., 2000; Boushey, et al., 2001; Booth et al., 2004; Torres et al., 2007; Rasmussen et al., 2010; Kissow et al. 2012).

GLP-2 agonists may thus have several valuable therapeutic applications. An attractive feature of GLP-2 treatment is that its biological effects are largely specific for the gastrointestinal tract.

One of the first identified biological actions of GLP-2 was stimulation of intestinal growth. Although GLP-2 increased proliferation of crypt cells and decreased enterocyte apoptosis, resulting in increased mucosal surface area and villus height with normal morphology, GLP-2 did not impact the growth of the intestinal muscle layers or stimulate proliferation in other organs (Drucker et al., 1996; Tsai et al., 1997; Livak et al., 1998). In subsequent studies, GLP-2 agonists demonstrated anti-inflammatory effects in rodent models of intestinal inflammation (Boushey et al., 1999; Alavi et al., 2000; Sigalet et al., 2007; Arda-Pirincci and Bolkent, 2011; Alters, 2012).

The mechanisms involved in the intestinotrophic and anti-inflammatory effects of GLP-2 are not completely understood. GLP-2 receptor expression is most abundant in the small intestine, colon, and stomach (Munroe, 1999) with an expression pattern that is highly localized to specific cell types, including intestinal subepithelial myofibroblasts, enteroendocrine cells, and enteric and nodose ganglia of vagal neurons (Yusta, 2000; Bjerknes, 2001; Ørskov, 2005; Guan, 2006; Nelson, 2007; Koopmann, 2008). Since the GLP-2 receptor is not expressed on intestinal epithelial cells, the major site of GLP-2 proliferative and protective actions, the effects of GLP-2 are thought to be mediated indirectly through release of growth factors, including insulin-like growth factor 1, keratinocyte growth factor, ErbB ligands, and other mediators, such as vasoactive intestinal peptide, that act in a local paracrine fashion to elicit biological actions (Rowland and Brubaker, 2011).

In summary, apraglutide is a potent and selective agonist at the GLP-2 receptor with remarkably low clearance and prolonged elimination half-life endowing it with superior *in vivo* pharmacodynamic activity, as demonstrated by its ability to induce intestinal growth. Such reduction in peptide clearance by substitution of a limited number of amino acid residues is

unprecedented in the field of peptide therapeutics and may lessen challenges presented by high clearance of the native peptide. Apraglutide is in development for SBS. From a clinical perspective, the improved pharmacokinetic profile of this peptide enables less frequent drug administration, and so would provide considerable benefits in the care of patients with SBS.

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Footnotes

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FIGURE LEGENDS

Figure 1. Apraglutide is potent and selective for the GLP-2 receptor.

Apraglutide, hGLP-2, and GLP-2 analogs concentration-response curves for functional activation of hGLP-2 receptor (A) or hGLP-1 receptor (B). Data are expressed as percentage of the maximum response to the native receptor ligand (hGLP-2 or hGLP-1) for the stimulation of a cAMP (cyclic adenosine monophosphate) responsive reporter gene in HEK-293 cells co-transfected with receptor and reporter gene. Each peptide was tested in at least 3 separate experiments. Data shown are averaged duplicates from a representative experiment. Concentration-responses were fitted to the data using non-linear regression for a sigmoidal curve (GraphPad Software, La Jolla, CA).

Figure 2. Apraglutide has low clearance in rat, monkey and minipig PK studies.

Graphs show the time course of plasma peptide concentrations following i.v. bolus injection (mean±SD). Peptides were administered to (A) Sprague Dawley Rats, dose normalized to 0.2 mg/kg ($n \geq 3$) (B) Adult male cynomolgus monkeys (n=4), at a dose of 0.1 mg/kg. (C) Adult male Yucatan minipigs (n=3) apraglutide 0.025 mg/kg and teduglutide 0.1 mg/kg.

Figure 3. Apraglutide has sustained exposure levels following s.c. dosing in rat, monkey and minipig.

Graphs show the time course of apraglutide and teduglutide concentrations in plasma (mean ±SD) by the subcutaneous route. Standard error bars that are not visible are encompassed within the symbol. (A) Figure shows PK profile of apraglutide or teduglutide following s.c. bolus injection at 5mg/kg in Sprague Dawley rats ($n \geq 3$). (B) Adult male cynomolgus monkeys

received apraglutide (n=4) or teduglutide (n=4) at 0.25 mg/kg by s.c. bolus injection into the lower back. (C) Adult male Yucatan minipigs received apraglutide (n=5) and teduglutide (n=4) at a dose of 0.15 mg/kg, administered by s.c bolus injection into the neck.

Figure 4. Effect of apraglutide and teduglutide on stimulation of rat small intestine growth. Peptides were administered by s.c. injection once daily for 5 days at the doses of 3-1000 nmol/kg. On day 5 (96 h after the first dose) small intestines were collected and weighed. Intestine weight for each rat was normalized to body weight and expressed as % increase over the mean of a vehicle control group run in the same experiment. A typical experiment involved six animals per group and the results of multiple experiments were combined. Values are plotted as mean with S.E.M (n = 17-36). Standard error bars that are not visible are encompassed within the symbol. To compare the response of apraglutide to teduglutide treated groups, data for each dose were analyzed by two-way ANOVA and Newman-Keuls test for post-hoc pairwise multiple comparisons. *$p < 0.05$ for apraglutide treatment group versus the corresponding teduglutide treatment group at the same dose. Dose-response curves were fitted to sigmoidal curve with variable slope. Fitted parameters of ED_{50} (median response) and the estimated maximum response (E_{max}) with 95% confidence intervals (CI) are summarized in the accompanying table.

Figure 5. Effect of apraglutide and teduglutide on stimulation of rat small intestine growth with less frequent dosing. Apraglutide, glepaglutide or teduglutide were administered to rats by s.c. injection, at doses of 30 or 300 nmol/kg, once-daily (A) every 48 h at 0 and 48 h (B) for 5 days and small intestines were collected and weighed on day 5 (96 h after the first dose). The peptides were also tested following a single injection and small intestines were collected and weighed

72 h post injection (C). Intestine weight for each rat was normalized to body weight and expressed as % increase over the mean of a vehicle control group run in the same experiment. A typical experiment involved six animals per group and the results of multiple experiments were combined. The data are presented as mean with S.E.M (n = 6-42). Data for vehicle control groups are not plotted. The values for the mean of the vehicle groups were 0% with S.E.M value of 0.6 (n =108) for 24 h interval; 1.1% (n = 42) for the 48-h dosing interval; and 1.0% (n = 24) for the single injection with 72 h post injection collection. Data for each dosing interval was analyzed by one-way ANOVA and Newman-Keuls test for post-hoc pairwise comparisons. *$p < 0.05$ versus corresponding vehicle control; ^$p < 0.05$ versus corresponding apraglutide treatment group.

TABLE 1.

Amino acid sequence and molecular weight of hGLP-2 and GLP-2 analogues

Peptide	Mol. wt	Amino Acid Sequence																															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	
hGLP-2	3766	H	A	D	G	S	F	S	D	E	M	N	T	I	L	D	N	L	A	A	R	D	F	I	N	W	L	Q	T	K	I	T	D	OH
Glepaglutide	4316	F	**G**	**E**	G	**T**	F	S	**S**	E	**L**	**A**	T	I	L	D	**A**	L	A	A	R	D	F	I	**A**	W	L	**A**	T	K	I	T	D	**KKKKKK**NH_2
Teduglutide	3752	H	**G**	D	G	S	F	S	D	E	M	N	T	I	L	D	N	L	A	A	R	D	F	I	N	W	L	Q	T	K	I	T	D	OH
Apraglutide	3765	H	**G**	D	G	S	F	S	D	E	**Nle**	**f**	T	I	L	D	**L**	L	A	A	R	D	F	I	N	W	L	Q	T	K	I	T	D	NH_2

Amino acid substitutions relative to native hGLP-2 are shown in bold underlined letters.

hGLP-2, human glucagon-like peptide-2; Nle, norleucine; f, D-Phenylalanine

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TABLE 2.

Agonist potency and efficacy of peptides for the human and rat GLP-2 receptors and selectivity versus human GLP-1 receptor in cell-based functional assays of cAMP (cyclic adenosine monophosphate) responsive activation.

Values are mean with 95% confidence interval in parenthesis or mean ± S.E.M. of 3 or more independent assays.

Peptide	**hGLP-2 Receptor**		**hGLP-1 Receptor**		**Selectivity**[a]	**rGLP-2 Receptor**	
	EC_{50}	**E_{max}**	**EC_{50}**	**Emax**	**GLP-1/GLP-2**	**EC_{50}**	**E_{max}**
	nM	*%*	*nM*	*%*	*ratio*	*nM*	*%*
hGLP-2	0.07 (0.06-0.10)	100	517 (403-663)	71 ± 5	7300	0.09 (0.06-0.12)	100
apraglutide	0.03 (0.03-0.04)	95 ± 0	>1000	45 ± 7	>33,000	0.07 (0.05-0.10)	91 ± 3
teduglutide	0.09 (0.07-0.11)	98 ± 3	>1000	3 ± 0	>11,000	0.08 (0.07-0.09)	96 ± 1
glepaglutide	0.24 (0.17-0.34)	95 ± 1	8.7 (6.6-12)	97 ± 1	36	0.42 (0.29-0.63)	87 ± 2
hGLP-1	N.D.		0.02 (0.02-0.03)	100	N/A	N.D.	N.D.

[a]Ratio of hGLP-1 receptor EC_{50}/hGLP-2 receptor EC_{50}
EC_{50}, median response; EC_{max}, maximum response; hGLP-1, human glucagon-like peptide-1; hGLP-2, human glucagon-like peptide-2; N/A, Not applicable; N.D, Not determined; rGLP-2, rat glucagon-like peptide-2;

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TABLE 3.

PK parameters following i.v. bolus administration

(A) Rat: PK studies were conducted in male Sprague Dawley rats and PK parameters were determined by analyzing compound concentration-time curves using a non-compartmental curve stripping method.
Doses are mg free base per kg body weight. Values are mean ± S.D.

Peptide	Dose *(mg/kg)*	Elimination Half-Life *(min (h))*	Clearance *(ml/kg/min)*	Vc *(ml/kg)*	n
hGLP-2	1.0[a]	6.4 ± 0.2 (0.1)	25 ± 2.3	85 ± 15	4
teduglutide	0.2[b]	19 ± 1.9 (0.3)	9.9 ± 2.1	92 ± 31	15
glepaglutide	0.2[b]	16 ± 0.8 (0.3)	2.8 ± 0.4	44 ± 5	3
apraglutide	0.2[b]	159 ± 27 (2.7)	0.27 ± 0.04	63 ± 2	3

[a.] *Dosed as single compound*
[b.] *Dosed in cassette mode. Cassettes were prepared with 3-6 peptides per cassette*
hGLP-2, human glucagon-like peptide-2; Vc, apparent volume of the central compartment

(B) Monkey and minipig: PK studies were conducted in male cynomolgus monkeys and castrated male Yucatan minipigs, respectively. PK parameters were determined by best fitting of compound concentration-time curves using a non-compartmental curve stripping method and presented.

Doses are mg free base per kg body weight. Values are mean ± S.D.

Peptide	Dose *(mg/kg)*	Elimination Half-Life *(min (h))*	Clearance *(ml/kg/min)*	Vc *(ml/kg)*	n
apraglutide					
monkey	0.100[a]	474 ± 80 (7.9)	0.046 ± 0.008	26 ± 2	4
minipig	0.025	782 ± 365 (13.0)	0.032 ± 0.007	28 ± 4	3
teduglutide					
monkey	0.100[a]	43 ± 4 (0.7)	2.4 ± 0.3	54 ± 3	4
minipig	0.100	88 ± 11 (1.5)	0.99 ± 0.20	35 ± 4	3

a. Dosed in cassette mode
Vc, apparent volume of the central compartment.

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TABLE 4.

PK parameters following s.c. bolus administration.

(A) Rat: PK studies were conducted in male Sprague Dawley rats. PK parameters were determined by best fitting of compound concentration-time curves using a non-compartmental curve stripping.
Doses are mg free base per kg body weight. Values are mean ± S.D.

Peptide	**Dose** *(mg/kg)*	**Cmax / Dose** *((μg/ml)/(mg/kg))*	**AUC∞ /Unit Dose** *(μg/ml*h)/(mg/kg)*	**Tmax** *(h)*	**Terminal Half-Life** *(h)*	**Fsc** *(%)*	**n**
apraglutide	1	1.96 ± 0.1	45.2 ± 2.0	6.7 ± 0.6	11.7 ± 1.4	74 ± 3	3
	5	0.90 ± 0.1	32.6 ± 1.7	7.5 ± 1.0	22.5 ± 1.3	54 ± 3	4
teduglutide	1	0.54 ± 0.1	1.1 ± 0.2	0.6 ± 0.4	0.5 ± 0.1	63 ± 12	3
	5	0.54 ± 0.1	1.3 ± 03	1.0 ± 0.0	0.5 ± 0.0	74 ± 17	4

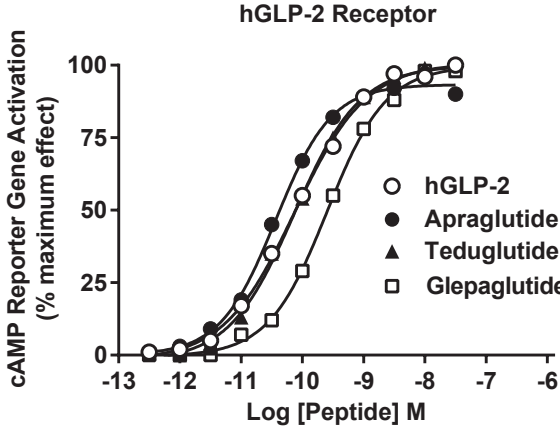
AUC∞, area under the concentration curve time zero to infinity; Cmax, maximum observed plasma concentration; Fsc, subcutaneous bioavailability; Tmax, time of the maximum observed plasma concentration

(B) Monkey and minipig: PK studies were conducted in male cynomolgus monkeys and castrated male Yucatan minipigs, respectively. PK parameters were determined by best fitting of compound concentration-time curves using a non-compartmental curve stripping.
Doses are mg free base per kg body weight. Values are mean ± S.D.

Peptide	**Dose** *(mg/kg)*	**Cmax / Dose** *((μg/ml)/(mg/kg))*	**AUC∞ /Unit Dose** *(μg/ml*h)/(mg/kg)*	**Tmax** *(h)*	**Terminal Half-Life** *(h)*	**Fsc** *(%)*	**n**
apraglutide							
monkey	0.25	2.01 ± 0.6	115.3 ± 19.0	23.3 ± 6.6	32.4 ± 6.1	32 ± 5	4
minipig	0.15	3.19 ± 1.0	227.4 ± 68.0	31.4 ± 4.1	30.1 ± 3.1	43 ± 13	5
teduglutide							
monkey	0.25	1.53 ± 0.5	5.3 ± 0.2	2.4 ± 1.4	0.9 ± 0.1	77 ± 3	4
minipig	0.15	1.26 ± 0.3	8.9 ± 1.4	5.1 ± 0.0	2.7 ± 0.8	53 ± 8	4

AUC∞, area under the concentration curve time zero to infinity; Cmax, maximum observed plasma concentration; Fsc, subcutaneous bioavailability; Tmax, time of the maximum observed plasma concentration

A


hGLP-2 Receptor
cAMP Reporter Gene Activation
(% maximum effect)
100
75
50
25
0
-13
-12
-11
-10
-9
-8
-7
-6
Log [Peptide] M
hGLP-2
Apraglutide
Teduglutide
Glepaglutide

B

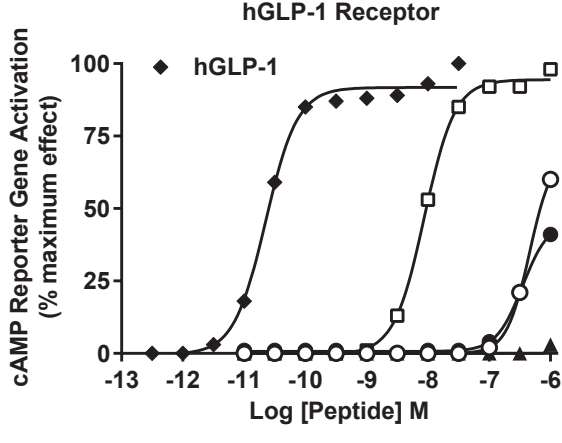

hGLP-1 Receptor
cAMP Reporter Gene Activation
(% maximum effect)
100
75
50
25
0
-13
-12
-11
-10
-9
-8
-7
-6
Log [Peptide] M
hGLP-1

Figure 1

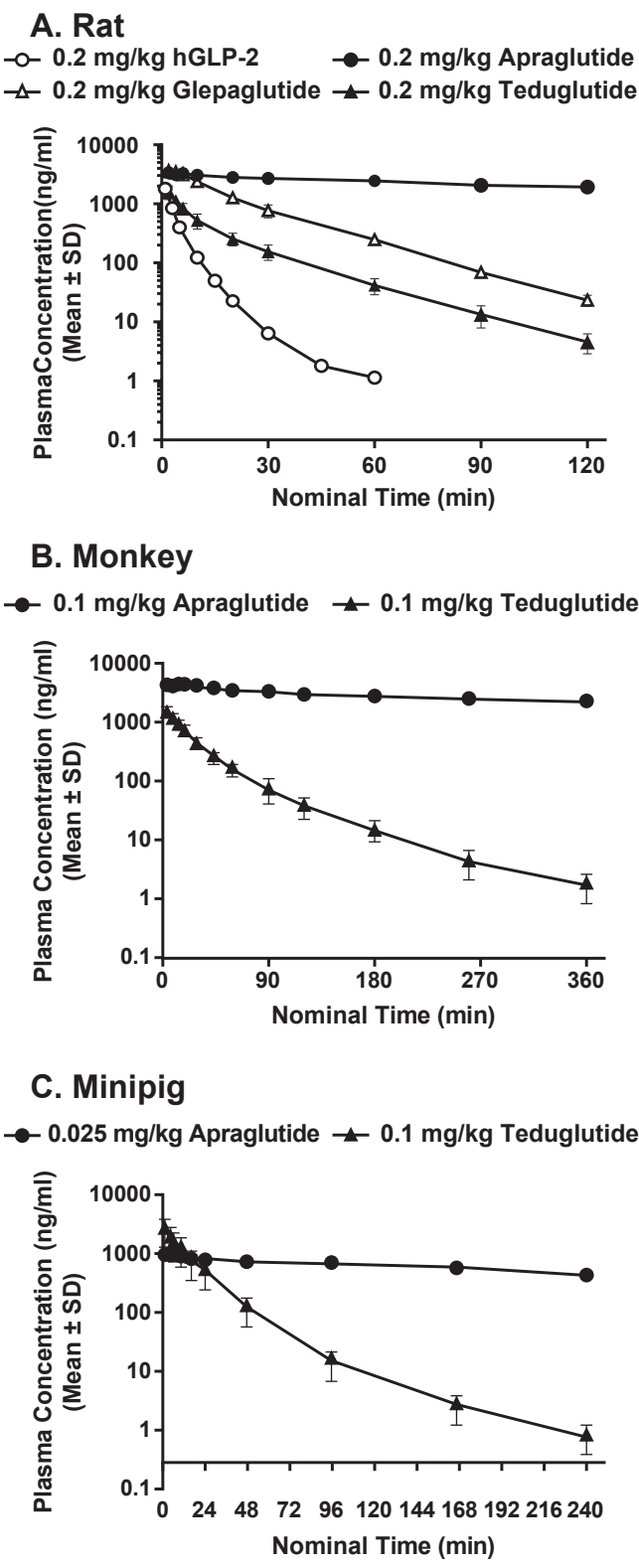

A. Rat
0.2 mg/kg hGLP-2
0.2 mg/kg Apraglutide
0.2 mg/kg Glepaglutide
0.2 mg/kg Teduglutide
PlasmaConcentration(ng/ml)
(Mean ± SD)
10000
1000
100
10
1
0.1
0
30
60
90
120
Nominal Time (min)
B. Monkey
0.1 mg/kg Apraglutide
0.1 mg/kg Teduglutide
Plasma Concentration (ng/ml)
(Mean ± SD)
10000
1000
100
10
1
0.1
0
90
180
270
360
Nominal Time (min)
C. Minipig
0.025 mg/kg Apraglutide
0.1 mg/kg Teduglutide
Plasma Concentration (ng/ml)
(Mean ± SD)
10000
1000
100
10
1
0.1
0
24
48
72
96
120
144
168
192
216
240
Nominal Time (min)

Figure 2

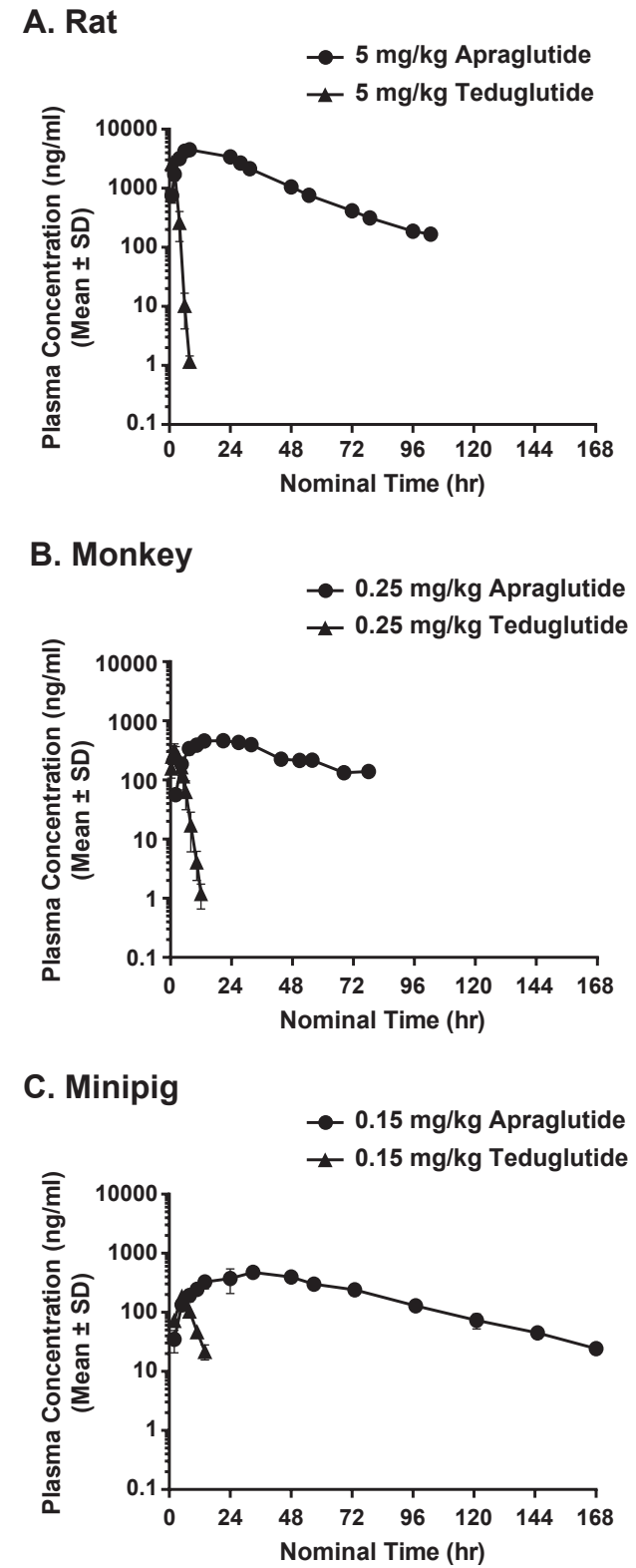
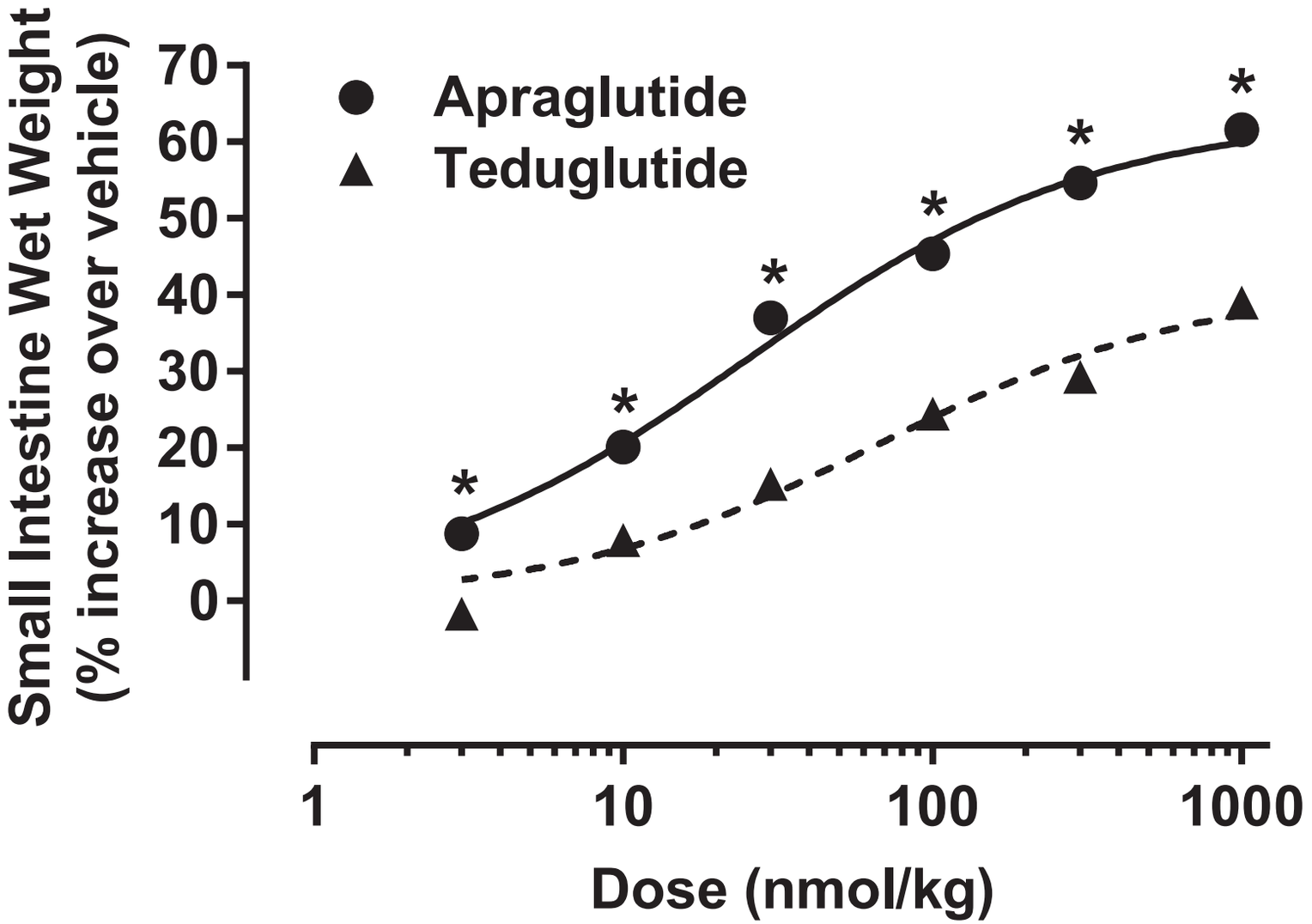

A. Rat
5 mg/kg Apraglutide
5 mg/kg Teduglutide
Plasma Concentration (ng/ml)
(Mean ± SD)
10000
1000
100
10
1
0.1
0
24
48
72
96
120
144
168
Nominal Time (hr)
B. Monkey
0.25 mg/kg Apraglutide
0.25 mg/kg Teduglutide
Plasma Concentration (ng/ml)
(Mean ± SD)
10000
1000
100
10
1
0.1
0
24
48
72
96
120
144
168
Nominal Time (hr)
C. Minipig
0.15 mg/kg Apraglutide
0.15 mg/kg Teduglutide
Plasma Concentration (ng/ml)
(Mean ± SD)
10000
1000
100
10
1
0.1
0
24
48
72
96
120
144
168
Nominal Time (hr)

Figure 3



	ED_{50}		E_{max}	
	(nmol/kg)	*(95% CI)*	*(%)*	*(95% CI)*
Apraglutide	25	(21-30)	63	(60 - 66)
Teduglutide	68	(39-97)	41	(36 - 46)

Figure 4

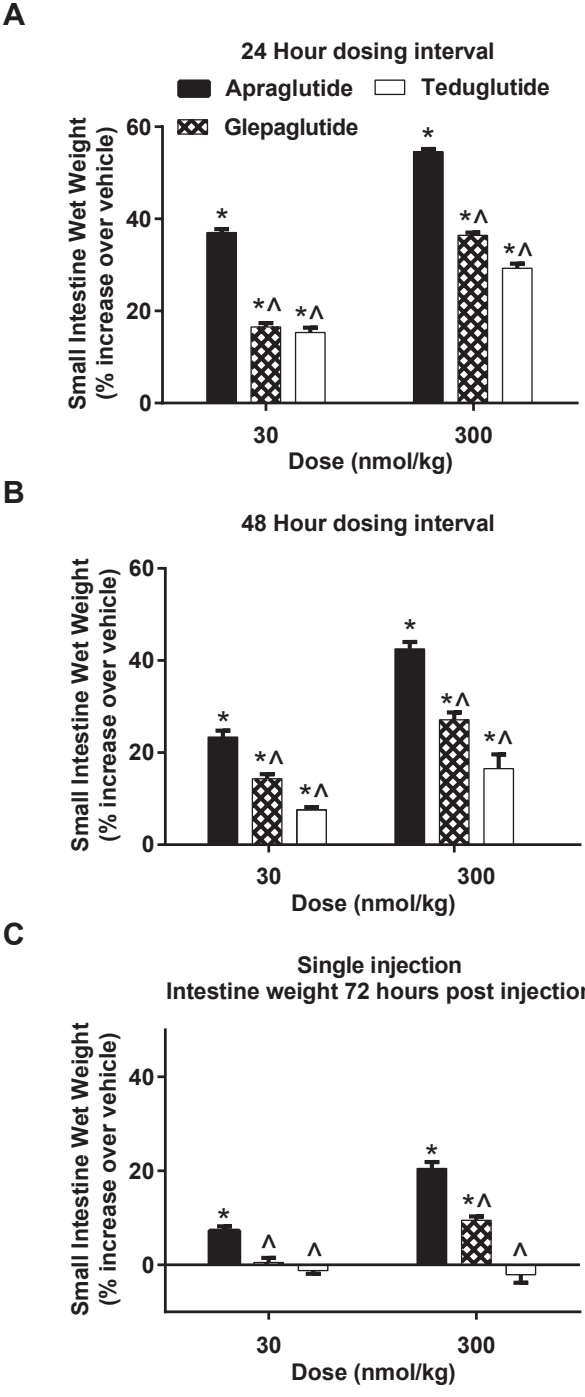

A
24 Hour dosing interval
Apraglutide
Teduglutide
Glepaglutide
Small Intestine Wet Weight
(% increase over vehicle)
60
40
20
0
*
*^
*^
*
*^
*^
30
300
Dose (nmol/kg)
B
48 Hour dosing interval
Small Intestine Wet Weight
(% increase over vehicle)
60
40
20
0
*
*^
*^
*
*^
*^
30
300
Dose (nmol/kg)
C
Single injection
Intestine weight 72 hours post injection
Small Intestine Wet Weight
(% increase over vehicle)
40
20
0
*
^
^
*
*^
^
30
300
Dose (nmol/kg)

Figure 5