Molecular engineering of safe and efficacious oral basal insulin

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Recently, the clinical proof of concept for the first ultra-long oral insulin was reported, showing efficacy and safety similar to subcutaneously administered insulin glargine. Here, we report the molecular engineering as well as biological and pharmacological properties of these insulin analogues. Molecules were designed to have ultra-long pharmacokinetic profile to minimize variability in plasma exposure. Elimination plasma half-life of ~20 h in dogs and ~70 h in man is achieved by a strong albumin binding, and by lowering the insulin receptor affinity 500-fold to slow down receptor mediated clearance. These insulin analogues still stimulate efficient glucose disposal in rats, pigs and dogs during constant intravenous infusion and euglycemic clamp conditions. The albumin binding facilitates initial high plasma exposure with a concomitant delay in distribution to peripheral tissues. This slow appearance in the periphery mediates an early transient hepato-centric insulin action and blunts hypoglycaemia in dogs in response to overdosing.

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he availability of oral insulin would free millions of people with diabetes from the everyday burden of subcutaneous (SC) insulin injections. Furthermore, the convenience of oral insulin might facilitate earlier initiation of insulin therapy and better patient compliance leading to improved glucose control and long-term outcomes. Two key challenges need to be overcome to develop an oral insulin therapy: (1) sufficient bioavailability and (2) clinical performance including efficacy, safety, and especially low pharmacodynamic variation. Pharmacodynamic variability is particularly challenging for insulin, due to the large absorption variability associated with oral dosing in combination with a narrow therapeutic window with an overdose being associated with potentially life-threatening hypoglycaemia. The first attempt to deliver insulin orally was conducted soon after the discovery of insulin nearly 100 years ago1 and it was concluded that the variability associated with oral administration of insulin would render it of little or no therapeutic value. Nevertheless, over the years, numerous technologies have been explored, including, e.g., nanoparticles, hydrogels, liposomes, ionic liquids or ingestible devices2–6. Despite relatively high bioavailability reported in several rodent studies7, most of these attempts have not progressed past research or the preclinical stage and consequently there is currently no oral insulin therapy available5. This can at least partly be ascribed to the use of human insulin or rapid-acting insulin analogues8. In contrast, we rationalised that ultra-long acting basal insulin analogues can overcome this limitation by addressing the critical issue of variability due to accumulation to steady state combined with a protracted action profile. Thus, an elimination half-life of >24 h combined with once daily dosing will lead to a steady-state exposure level accumulated from the dose of multiple consecutive days as illustrated in Fig. 1.

Here we describe the engineering of insulin analogues for safe, efficacious, oral basal insulin therapy in man9 with no impact of food intake when the tablet is administered 30 min before a meal10 using Gastro Intestinal Permeation Enhancer Tablet I (GIPET®)11 based on sodium caprate as absorption enhancer. The combination of enhanced proteolytic stability with ultra-low receptor affinity and strong albumin binding leads to significantly improved bioavailability and ultra-long plasma half-life, while maintaining potency with regards to whole-body glucose disposal under euglycemic clamp conditions. Furthermore, we demonstrate that the inherent properties of the molecule may curtail the hypoglycaemic response to overdosing in normal dogs. Importantly, these effects are linked to the peaked absorption profile, observed both in dog and man9 which can be achieved only by oral, and not by SC administration.

**Results**

**Design of proteolytically stable ultra-long insulin.** First, we aimed at reducing the proteolytic degradation of insulin to improve the bioavailability after oral dosing. Initially, a large number of insulin analogues with substitutions at known enzymatic (primarily trypsin/chymotrypsin) cleavage sites12 were produced and screened for their rate of proteolytic degradation. Two amino acid substitutions (A14 Tyr→Glu and B25 Phe→His, named OI106) were sufficient to stabilize the insulin molecule, with an optional additional deletion of B27Thr (named OI189) conferring further stability (Fig. 2b). Concomitantly, the substitutions lowered the relative insulin receptor affinity by threefold, while considerably increasing solubility in the slightly acidic pH range (5.5–6.5) found in the small intestine13 (Supplementary Fig. 1).

Secondly, mechanisms to prolong the pharmacokinetic profile of the insulin were incorporated into the molecule. Currently available long-acting injectable insulin analogues are mostly prolonged by slowing the rate of absorption from the injection site into the circulation (e.g. insulin detemir, insulin glargine and insulin degludec)14. However, for oral insulin delivery, it is critical that the full daily dose is rapidly absorbed to avoid interaction with ingested food. Therefore, we designed insulin analogues which were protracted in the circulation with an elimination half-life of >24 h. We rationalized that this could be achieved mainly by slowing down clearance, primarily by two mechanisms: (1) introducing strong, but reversible albumin binding to reduce renal clearance and access to the insulin receptor and (2) drastically reducing the insulin receptor affinity as insulin is primarily cleared by internalization through its receptor. We hypothesised that ultra-long acting (referred to as ultra-long hereafter) insulin analogues would retain in vivo potency in spite of the low in vitro potency, albeit the in vivo effect would be executed over a prolonged time period.

**Structure of ultra-long insulin analogues.** The crystal structures of these low-affinity insulin analogues were determined to assess the effect of the substitutions and addition of albumin binders. The crystal structures of OI338 and OI320 shows a conserved insulin structure with small differences compared to the human insulin backbone, with root mean square deviations of 0.37 Å (OI338, 44C(alpha), Cβ atoms) and 0.56 Å (OI320, 41 Ca atoms) (see Supplementary Fig. 2 and Supplementary Table 1).

**Stability towards proteolytic degradation.** OI106 (A14 Tyr→Glu and B25 Phe→His) has 10-fold improvement in stability towards enzymes from rat duodenum relative to human insulin as shown in Fig. 2b. Deletion of B27 Thr (OI189) leads to additional 3–5-fold improvement.

Only limited differences in proteolytic stability are observed when comparing human insulin with OI126, OI106 with OI338 or OI189 with OI320 (Fig. 2b). Thus, the addition of the fatty acid moiety does not influence insulin stability towards degradation by these proteases.

**Insulin receptor affinity and in vitro potency.** The combination of amino acid substitutions and attachment of a strong albumin binder (C18 fatty diacid, linked via hydrophilic linker) to B29Lys reduces the insulin receptor affinity 500-fold compared to human insulin (in the presence of 1.5% HSA) (see Fig. 2a and c for structure and insulin receptor affinity). Furthermore, these insulin molecules with considerably reduced affinities for insulin receptors show proportionally low insulin-like growth factor 1 (IGF1) receptor affinities as compared to human insulin. Consistently, insulin receptor activation (tyrosine phosphorylation,
pTyr1158 hIR-A in CHO cells) and functional biological responses in primary adipocytes (lipogenesis) and hepatocytes (glycogen accumulation) from healthy rats are induced similar to human insulin, albeit with lower potencies regarding the reduced insulin receptor binding affinities (see Supplementary Figs. 3 and 4, Supplementary Tables 2 and 3).

**GIPET®I tablets for oral dosing.** Despite substantial research interest in oral administration of protein and peptide drugs, few oral formulations have been developed for large animal models with translation possibility for clinical use. In this study, we used the GIPET®I tablet system, developed by Merrion Limited[^11], based on sodium caprate as absorption enhancer. The GIPET®I tablet described in this study are identical to those used in the phase 2a clinical study[^9] with regards to composition and size. The observed absorption profiles and bioavailability in both dogs and human are comparable albeit the insulin dose was lower in the dog studies. Improvement of the GIPET®I formulation was not the aim of this study.

**Pharmacokinetics and bioavailability in Beagle dogs.** The reduced insulin receptor affinity increases the intravenous (IV) half-life in dogs from 25 min (human insulin), to 24 h (OI338) and 17 h (OI320), respectively. The strong binding of these two representative insulin analogues, OI338 and OI320, to albumin (Supplementary Fig. 5) ensures that the majority of the molecules in circulation are bound to albumin. As shown in Fig. 3a, stabilisation of the insulin backbone as well as the attachment of the fatty acid-based side chain contributed to effective plasma exposure after oral dosing. Characterization of pharmacokinetic profiles (after oral dosing in GIPET®I) shows an absolute bioavailability (F) of 3.0% for OI338 (CV 100%, single dose, n = 40) and 3.9% (CV 78%, single dose, n = 63) for OI320 (Fig. 3c), calculated relative to IV dosing. OI216 (human insulin desB30 back-bone) with an identical fatty acid side chain to OI338 and OI320 has much lower bioavailability, emphasising the importance of stabilisation of the insulin backbone (Fig. 3c). Furthermore, the oral bioavailability of insulin aspart, a marketed rapid-acting insulin analogue without stabilisation of the insulin backbone, is only 0.6% (CV 67%).
which is similar to the 17.5% for insulin glargine after SC administration (see also Table 1). c Bioavailability of different insulin analogues after oral administration in GIPET® tablets to dogs (mean +/− SD from a number of independent experiments). d Individual 12-h pharmacokinetic profiles (day 1, 7, 14) of OI338 after multiple oral daily dosing in eight dogs (each dog one colour). e Coefficient of variation of bioavailability based on area under the curves in Fig. 3d), multiple dose study. Intra/inter-individual CV based on the mean of the group was reduced from 100 to 40% after treatment to steady state.

Fig. 3 Pharmacokinetic properties of oral insulin analogues. a Pharmacokinetic profiles of OI106 (triangles), OI216 (squares) and OI338 (circles) after single oral dosing in healthy and conscious dogs (n = 8); the plotted concentrations are exposure divided by the oral dose in nmol/kg for each insulin analogue. Coefficient of variation, CV, for the AUC, was 75-100%. b Pharmacokinetic profiles of in OI338 after IV (2 nmol/kg, n = 8); peroral (105 nmol/kg, n = 4) and SC (4 nmol/kg, n = 8) administrations in dogs. CV for AUC was around 10% after IV and SC administration and ~100% after oral dosing. Note the fast absorption profile after oral administration (closed circles). After reaching C_max the oral pharmacokinetic profile almost mimics that of an IV administration (see also Table 1). c Bioavailability of different insulin analogues after oral administration in GIPET® tablets to dogs (mean +/− SD from a number of independent experiments). d Individual 12-h pharmacokinetic profiles (day 1, 7, 14) of OI338 after multiple oral daily dosing in eight dogs (each dog one colour). e Coefficient of variation of bioavailability based on area under the curves in Fig. 3d), multiple dose study. Intra/inter-individual CV based on the mean of the group was reduced from 100 to 40% after treatment to steady state.

Steady-state distribution of ultra-long insulin analogues. When administered SC, insulin is absorbed into the peripheral circulation and distributed in equal arterial concentrations to all target tissues. This contrasts with endogenously secreted insulin which reaches the liver as the first target organ in higher portal concentrations and is subject to first-pass clearance before it reaches the peripheral target tissues, such as muscle and fat, in lower concentrations. This has been demonstrated in healthy conscientious dogs with the insulin analogue. Coefficient of variation, CV, for the AUC, was 75-100%.
Table 1 Pharmacokinetic and pharmacodynamic parameters of insulin analogues in animals.

| Pharmacokinetic parameters in the dog<sup>a</sup> | Potency on whole-body glucose disposal relative to human insulin<sup>b</sup> (%) |
|-----------------------------------------------|---------------------------------------------------------------------|
|                  | T<sub>max</sub> min | T<sub>½</sub> dog oral (h) | T<sub>½</sub> dog iv (h) | Rat | Pig | Dog |
| Human insulin    |                         |                          |                         | 57 [54–60] | 45 [43–47] | 72 [58–86] |
| OI216            | 30                      | 3.8 ± 1.9                | 0.25 ± 0.1              | 45 [43–47] | 99 [89–110] | 72 [58–86] |
| OI338            | 45                      | 17 ± 2                   | 5.7 ± 0.3               | 110 [105–115] | 135 [94–203] | 106 [100–117] |
| OI 106           | 45                      | 0.7 ± 0.3                | 24 ± 0.4                | 64 [62–66] | 140 [120–170] |           |
| OI 189           | 45                      | 0.7 ± 0.3                | 0.8 ± 0.02              |       |       |     |
| OI320            | 50                      | 14 ± 2                   | 17 ± 1                  |       |       |     |

<sup>a</sup>The pharmacokinetic parameters are from non-compartmental analysis of individual profiles from either IV or oral bolus studies in dog. n = 2–4 for the IV studies and n = 8 for the oral studies. Mean ± SD is given for half-life and median for T<sub>max</sub>.

<sup>b</sup>Potency relative to human insulin at steady state during hyperinsulinaemic euglycaemic clamp (95% confidence interval).

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Fig. 4 Hyperinsulinemic, euglycemic clamp experiment. A representative dose response plot (GIR = glucose infusion rate) from a 5-h hyperinsulinemic, euglycemic clamp experiment in healthy and conscious rats performed to determine the potency of OI320 (black circles) relative to human insulin (white circles). The dots represent the average GIR during the last 60 min of the experiment in each rat (n = 6 rats per dose [group]; independent experiments). Similar dose response studies were performed in healthy and conscious pigs and dogs as indicated in (Fig. 2d) and; see Table 1 for 95% confidence interval.

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dogs by comparing intraportal versus peripheral venous infusions of human insulin, resulting in insulin concentrations in the peripheral circulation that are ≈20% higher than in the portal vein after peripheral venous infusions<sup>15</sup>. The ultra-long insulin analogues are not expected to have noticeable due to their low receptor affinity.

We modelled the steady-state distribution between the central (systemic circulation/plasma, including the liver) and peripheral (tissue/interstitial fluid, including metabolically relevant tissues for insulin such as fat and muscle) compartments to assess differences between orally administered OI338 and SC delivered human insulin (Supplementary Fig. 6). The simulations showed that a balance between elimination half-life, slow peripheral distribution and the oral administration route leads to an initial peak OI338 in the central compartment and a delayed appearance in the peripheral compartment due to slower trans-endothelial transport of the albumin bound insulin analogues. In contrast, simulations of human insulin dosed SC show a more pronounced peak in the peripheral compartment (Supplementary Fig. 6). Thus, the simulations predict that the rapid absorption of OI338 into the portal vein when dosed orally will lead to a different tissue distribution compared to human insulin dosed SC, i.e., more restricted to the central compartment in the initial phase.

In addition to the advantages that this type of distribution would have to minimize the risk of hypoglycaemia due to variations in oral absorption (see section below on hypoglycaemia assessment), it could be speculated that an insulin analogue with strong albumin binding having a rapid, peaked central (blood compartment) PK profile would mimic the pattern of insulin secreted from the pancreas directly into the portal circulation where the liver is highly exposed. This peaked PK profile upon oral delivery with greater liver effect is not expected to be the same as what was observed for insulin PEGlispro which demonstrated a constant hepatoselective effect<sup>16</sup>. Whether this more physiological profile of rapid and peaked insulin delivery to the liver (part of the blood compartment due to the fenestrated capillaries) would lead to improved metabolic control remains to be determined. Similarly, this type of insulin analogue may be expected to have an altered CNS distribution and it is not known whether this would lead to any differences in central effects compared to human insulin as has been suggested for insulin detemir<sup>17</sup>.

Pharmacokinetic and pharmacodynamic relationship. To test the accuracy of the simulations regarding the distribution of OI338 and OI320, the link between dynamic changes in the pharmacokinetic profile and the pharmacodynamic response were studied in healthy and conscious dogs during euglycemic clamp settings after a single oral administration of OI320 (Fig. 5d). OI320 shows a prolonged lag time of 6–8 h between the plasma C<sub>max</sub> and maximal pharmacodynamic effect as quantified by the GIR required to maintain euglycaemia. The lag time between C<sub>max</sub> and maximal GIR may be attributed to the delayed appearance of OI320 in the peripheral compartment where muscle and fat tissue are responsible for insulin-stimulated glucose uptake.

Assessment of hypoglycaemia after overdosing. Next, we addressed the relevant safety concerns related to the day to day variability in orally administered insulin absorption. Healthy and conscious mongrel dogs were pre-treated once daily with OI320 for three days to reach steady state and on the fourth day, OI320 was administered via the portal vein by a 40 min square wave infusion to mimic an oral absorption with up to three times the daily maintenance dose to study the hypoglycaemic response and dynamics of glucose turnover. The baseline OI320 concentration on the fourth day, 24 h after the third maintenance dose was 8009 ± 599 pM (mean ± SEM for all animals). OI320 lowered plasma glucose dose dependently; the high dose levelled around 3 mM whereas the two lower doses plateaued around 6 and 4 mM (Fig. 6a). As plasma glucose decreases, initially due to inhibition of glucose production (Ra) by the liver, glucose disappearance (Rd) also goes down slightly, indicating that there is no
stimulation of Rd by OI320 (evident by unchanged glucose clearance during the first 30–45 min, Fig. 6d). However, after 60 min Rd increases again and plateaus close to baseline. Since plasma glucose concentrations at the high OI320 doses are only half of baseline, i.e., glucose clearance has doubled, this indicates a peripheral insulin effect after one hour. In other words, Rd was affected by both glucose and insulin—the lower glucose levels bringing it down; the higher OI320 levels bringing it up.

In contrast, over-dosing with human insulin (60% of the OI320 maintenance dose) quickly affects both glucose Ra and Rd, leading to acute hypoglycaemia and requiring intervention with glucose infusion to prevent plasma glucose levels from dropping below 2.5 mM (Fig.6a). Ra actually increased which is likely a response to the decrease in plasma glucose caused by the rapid stimulation of Rd (Fig. 6b, d). The lag time between \( \text{C}_{\text{max}} \) of human insulin when infused into the portal vein for 45 min in dogs and time for maximal glucose lowering (Fig. 6a) is 0–15 min (plasma exposure shown in Supplementary Fig. 7b) thus shorter than the long lag time observed for OI320 (Fig. 5d). Thus, the delayed distribution of OI320 to the peripheral tissues after portal dosing blunts hypoglycaemia in response to overdosing in healthy and conscious dogs (additional data on arterial insulin concentration–time profiles for OI338 and OI320 presented in Supplementary Fig. 8).

The pharmacodynamic time course (GIR = glucose infusion rate required to maintain euglycemia) of orally dosed OI320 and pharmacokinetic exposure during hyperinsulinemic euglycemic clamp in healthy and conscious dogs. Note the long lag time of pharmacodynamic effect in respect to exposure. The CV for the GIR AUC was in the range 45–80% and for the pharmacokinetic AUC the CV was 45–100%.

**Methods**

**Production of insulin analogues.** Insulin precursors were expressed in Saccharomyces cerevisiae (MATa/MATalpha, leu2/leu2, Tpi1::LEU2/Tpi1::LEU2, HIS4/ his4, pep4-3/pep4-3, Cit+ ) as proinsulin-like fusion proteins consisting of the prepro-segment of the yeast mating pheromone alpha-factor, a spacer (EEAEPK) followed by the B-chain (B1-B29) linked to the A-chain (A1-A21) by a mini C-peptide AAK19. The single-chain precursors were enzymatically converted into mature two-chain desB30 insulin analogues using Achromobacter lyticus protease 1 (EC 3.4.21.50) specific for lysyl peptide bonds. The insulin analogues were chemically modified by coupling of \([2-(2-[[2-(2-[[2-((octadecanoyl-3,4-diyl)oxy)ethoxy]ethoxy)ethoxy)acetylamino] ethoxy]ethoxy]acetylamino] ethoxy]ethoxy)acetyl-O\(\text{SU}\)) to the epsilon amino group of B29 lysine.20,21 In brief, the N-hydroxysuccinimide activated side chain was dissolved in DMF and added slowly to a well stirred solution of insulin (100 mg/mL) in 0.1 M \(\text{Na}_2\text{CO}_3\) at 0–5 °C, while keeping pH at 11 by simultaneous addition of 1 M NaOH. The product was purified by Reverse-Phase High-performance liquid chromatography (RP-HPLC) and isolated by lyophilization. All insulin analogues are lacking B30 threonine except insulin aspart.

**Proteolytic degradation.** Degradation of insulin analogues was performed using duodenum lumen enzymes (prepared by filtration of duodenum lumen content) from healthy Sprague Dawley (SPD) rats. Insulin analogues –15 μM was incubated with duodenum enzymes in 100 mM Heps, pH 7.4 at 37 °C, samples were taken after 1, 15, 30, 60, 120 and 240 min and reaction quenched by addition of trifluoroacetic acid. Intact insulin analogues at each point are determined by RP-HPLC. The amount of enzyme added for the degradation was such that the half time for degradation of the reference insulin was between 5 and 15 min. The result is given as the degradation half time for the insulin analogue in rat duodenum divided by the degradation half time of the reference insulin from the same experiment (relative degradation rate).

**Tablet formulation.** Tablets were prepared according to standard pharmaceutical techniques in Good Laboratory Practice production laboratories. The tablet core composition was as follows for a final weight of 710 mg/tablet: Active ingredient

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**Fig. 5 Schematic overview of insulin levels, distribution kinetics and the kinetic/dynamic relationship.** a Schematic overview of insulin levels (arrow width) and distribution kinetics upon a oral dosing of fast acting insulin (e.g. human insulin), b oral dosing of a plasma protracted insulin (e.g. OI338 and OI320), and c SC dosing of a long acting insulin, which is protracted SC (e.g. insulin glargine or insulin degludec, or OI338/OI320 dosed SC). d The pharmacodynamic time course (GIR = glucose infusion rate required to maintain euglycemia) of orally dosed OI320 and pharmacokinetic exposure during hyperinsulinemic euglycemic clamp in healthy and conscious dogs. Note the long lag time of pharmacodynamic effect in respect to exposure. The CV for the GIR AUC was in the range 45–80% and for the pharmacokinetic AUC the CV was 45–100%.
Acid of sodium caprate is generally recognized as safe. As a food additive it is regulated [44x246]United States Pharmacopoeia as well as the European Pharmacopoeia. The free pendants rules for content uniformity and technical properties according to the to 1% in milk and it [44x238]nitrate, 0.1 M Tris pH 8.5 as precipitant (0.5 µl drops incubated over 100 µl precipitant were incubated over precipitant. OI338 (25 mg/ml in water, pH 7.5, the sitting drop vapour diffusion technique where equal volumes of insulin and [44x197]Three-dimensional structural determination

Three-dimensional structural determination. Insulin crystals were grown using the sitting drop vapour diffusion technique where equal volumes of insulin and precipitant were incubated over precipitant. OI338 (25 mg/ml in water, pH 7.5, added 0.1% (w/v) NVoy (Expedeon)) was crystallized using 0.12 M monosaccharides, 0.1 M Buffer System 1 pH 6.5, 20%(v/v) PEG500MME, precipitant at 37 °C). OI320 (8 mg/ml in water, pH 7.5) was crystallized using 0.36% (w/v) PEG20,000 (F1 from Morpheus screen, Molecular Dimensions) as precipitant (0.36 ± 0.36 µl drops incubated over 80 µl precipitant at 20 °C).

Diffraction data collection, structure determination and refinement. Diffraction data to 1.5 Å resolution were collected at 100 K on crystals first cryo-cooled in liquid nitrogen and the OI338 crystal was beforehand cryo-protected in precipitant. The OI338 data were obtained on a CCD165 detector (Mar Research) mounted on a MicroMax-007 HF rotating anode (Rigaku) using CuKα radiation (1.5418 Å). The OI320 data were collected on a Pilatus 2 M detector (Dectris) at beamline X06DA, Swiss Light Source using a wavelength of 1.00 Å. The diffraction data were auto indexed, integrated and scaled using programs from the HKL2000 [24] (OI338) and XDS [25] (OI320) suites. The OI338 crystal structure was phased by single wavelength anomalous dispersion using the sulfur atoms from insulin’s three disulfide bridges in the Autosol module of the program suite Phenix [26] where automated model-building also was applied. The OI320 crystal structure was determined with molecular replacement in the program Phaser [27] implemented in Phenix using the OI338 crystal structure as search model. Both crystal structures were refined in Phenix with rounds of manual model building in Coot [28]. The Ramachandran plots of both structures have 98% of the amino acid residues in the favoured areas and the remaining 2% in the allowed areas. The final diffraction data and refinement statistics are summarized in Supplementary Table 1.

Pharmacokinetic studies in dogs. All animal study protocols, except those involving mongrel dogs, were approved by the Danish Animal Experiments Inspectorate. The mongrel dog protocol was approved by the Vanderbilt University Institutional Animal Care and Use Committee, and housing and care followed American Association for Laboratory Animal Care guidelines. Adult male mongrel dogs (21–24 kg) purchased from a U.S. Department of Agriculture—licensed vendor was studied. All animal study protocols were approved by Novo Nordisk Ethical Review Committee, Denmark. All animal protocols are in compliance with all relevant ethical regulations. Healthy and conscious Beagle dogs (males ~ 17 kg) were fasted overnight with access to drinking water. In the morning the dog was placed on a test platform and a Venflon 20 G was inserted in the v. cephalica for blood sampling during the first 6 h post-dosing. Thereafter, the venflon was removed and the dogs were returned to their booths and were offered food and exercise in the outside run. At the later time points the dogs were led into a test room for blood sampling directly from the jugular vein using a needle and syringe.

Peroral (PO) administration: Dogs were dosed PO with the OI338/OI320/ OI216/insulin aspart GIPET® Peroral (PO) administration: Dogs were dosed PO with the OI338/OI320/OI216/insulin aspart GIPET® tablets. After administration of the tablet the dog was given 10 ml drinking water directly into the mouth by a syringe to make sure that the tablet had entered the oesophagus. Dogs were offered small walks when needed for urination and offered drinking water 120 min after the tablet was dosed.

programs from the HKL2000 [24] (OI338) and XDS [25] (OI320) suites. The OI338 crystal structure was phased by single wavelength anomalous dispersion using the sulfur atoms from insulin’s three disulfide bridges in the Autosol module of the program suite Phenix [26] where automated model-building also was applied. The OI320 crystal structure was determined with molecular replacement in the program Phaser [27] implemented in Phenix using the OI338 crystal structure as search model. Both crystal structures were refined in Phenix with rounds of manual model building in Coot [28]. The Ramachandran plots of both structures have 98% of the amino acid residues in the favoured areas and the remaining 2% in the allowed areas. The final diffraction data and refinement statistics are summarized in Supplementary Table 1.

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IV administration: Insulin was administered as an intravenous bolus via a Venflon 20 G inserted in the v. cephalica of the leg opposite to that of the sampling Venflon. SC administration: The day before the experiment the fur on a predefined area (~2 cm in diameter) of the shoulder was removed and the test compound was administered SC using a NovoPen Echo.

Glucose clamp studies in Beagle dogs. Healthy and conscious Beagle dogs (males - 17 kg) were fasted overnight with access to drinking water. In the morning the dog was placed on a test platform and shortly sedated while a temporary triple lumen catheter for blood sampling and infusion was inserted in the jugular vein after which the sedation was reversed. Plasma glucose concentrations were measured at various intervals as required to maintain a euglycaemia clamp using an automated glucose analyser (YSI 2300 Stat Plus, Yellow Springs Instruments Co., Inc., Ohio, USA), and the GIR was adjusted accordingly to maintain euglycemia. Insulin was either infused IV by primed, constant rates for determination of potency relative to human insulin (three dose levels) (Fig. 2d and Table 1) or administered PO in GIPET® tablets (Fig. 5d).

Studies in mongrel dogs. Dogs were housed according to Association for Assessment and Accreditation of Laboratory Animal Care International guidelines in a USDA-inspected facility on a 12 h:12 h light/dark cycle with male companions. Once daily, dogs were fed a weight-maintaining diet providing 28% protein, 49% carbohydrate, and 23% fat, consisting of Lab Diet canine 5006 and Pedigree Choice beef cuts in gravy 29. Licensed veterinary staff from the Vanderbilt University Medical Center supervised their care and evaluated health. Dogs were fasted for 18 h before each study. For oral insulin administration studies, eight healthy and conscious dogs were trained to take an oral placebo 2 tablets for 2 weeks before study. On the morning of study, an active insulin tablet (O1338 or O1320) was administered orally at a dose of either 120 or 180 nmol/kg. Venous insulin concentrations were measured at 10 h after dosing, with normal food administration resuming 6 h after each insulin dose. All dogs underwent four studies at 1-week intervals, receiving both analogues at both dosages with the order of analogue administration/dosage randomized within the group.

For dogs undergoing pancreatic clamps, surgery was performed under general anaesthesia approximately 16 days before the study to insert sampling catheters in the femoral artery, hepatic portal vein, and left common hepatic vein; blood was sampled at various intervals as required to maintain a euglycaemia clamp using an automated glucose analyser (YSI 2300 Stat Plus, Yellow Springs Instruments Co., Inc., Ohio, USA) while a glucose solution was infused at variable rates (0–24 h) to maintain plasma glucose at the clamp level 30.

Euglycaemic clamp in pigs. Healthy and conscious female domestic pigs (Landrace × Yorkshire × Dorset (LYD)) were used for the glucose clamp studies employing a cross-over design. In brief, human insulin or primed oral insulin analogues were infused IV at three different constant molar rates and blood samples were drawn frequently for measurements of plasma glucose concentrations (YSI 2300 Stat Plus, Yellow Springs Instruments Co., Inc., Ohio, USA) while a glucose solution was infused at variable rates (0–5 h) to maintain plasma glucose at the clamp level.

Calculation of in vivo potency and confidence intervals. The log transformed insulin infusion rates (pmol/kg/min) and mean of the corresponding GIR (mg/kg/min) during the last 60 min of the euglycaemic clamp in each animal were fitted to Sigmoidal dose-response curves and the potency of the oral basal insulin analogues relative to human insulin was calculated as the ratio between their ED50% values using GraphPad Prism’s (version 8.0.2 for Windows) log(Agonist) vs. response—Variable slope (four parameters) fitting model (Eq. (1))

\[ Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{((\log ED50 - X) \times \text{HillSlope})}) \]

Fitting constraints were shared values for Hillslope, maximal GIR response (Top) and the GIR response to no insulin infusion (Bottom). The 95% confidence interval of the ratio between means of the ED50% was calculated using Fieeler’s Theorem 32.

Quantification of insulin concentration in plasma samples. For pharmacokinetic assessment blood samples were drawn and insulin plasma concentrations were analysed using luminescence oxygen channelling immunoassay 33,34 using suitable monoclonal antibodies. The lower limit of quantification of, e.g., O1320 in dog plasma was 50 pmol/L. Regarding specificity, the cross-reactivity of antibodies to canine insulin was <1%.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Crystal structure coordinates and structure factors were deposited in the Protein Data Bank (PDB) under the following accession codes: 6S4I (O1338) and 6S4F (O1320). The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary information files. Data are available from the corresponding author upon request.

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Author contributions

F.H., P.K.N., S.L., P.M., P.G.B. and T.K. designed insulin analogues. F.H. and P.K.N. established and executed proteolytic stability screening. D.B.S. and S.L. designed and performed solubility screening. E.I. prepared crystals and solved 3-D structures. T.G. and T.K. established and executed receptor affinity screening. C.E.S. measured albumin binding. C.E.S., E.N., R.F.H., and M.K.J. characterized insulin analogues in cellular assays. L.H. designed, optimized and prepared insulin GIPET® tablets. T.P. and C.L.B. designed and executed rat experiments. S.G.N. and E.M.W. designed and executed beagle dog studies. J.K. and U.B. designed and executed IVD pig studies. M.C.M. carried out the canine studies at Vanderbilt University (Fig. 4 and a portion of Table 1). M.C.M. and A.D.C. participated in the design of those experiments and analysed and interpreted data. H.H.F.R., L.P. and C.H.C. designed and executed PK calculations, modelling and simulation. F.H., H.H.F.R., L.P., P.M., S.L., S.R. and T.K. led and coordinated the studies. F.H., H.H.F.R., M.M., C.L.B., E.N. and T.K. drafted the manuscripts. All authors read and contributed to editing of the paper.

Competing interests

All authors except M.C.M. and A.D.C. are present or past employees of Novo Nordisk and hold equity in the company. M.C.M. and A.D.C. received financial support from Novo Nordisk. Insulin analogues and formulations described in this article are covered by Novo Nordisk’s patents and patent applications. A.D.C. reports grants, personal fees, and other from Metavention, Zafgen, and Abvance; grants and/or personal fees from Thetis Pharmaceuticals, Boston Scientific, Novo Nordisk, vTv Therapeutics, Merck, Eli Lilly, and Galvani Bioelectronics; personal fees from California Institute for Biomedical Research (Calibr) and MedImmune; personal fees and other from Fractyl, Biocon and Sensulin Labs.

Additional information

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