FGF21 Analogs of Sustained Action Enabled by Orthogonal Biosynthesis Demonstrate Enhanced Antidiabetic Pharmacology in Rodents

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Fibroblast growth factor 21 (FGF21) mitigates many of the pathogenic features of type 2 diabetes, despite a short circulating half-life. PEGylation is a proven approach to prolonging the duration of action while enhancing biophysical solubility and stability. However, in the absence of a specific protein PEGylation site, chemical conjugation is inherently heterogeneous and commonly leads to dramatic loss in bioactivity. This work illustrates a novel means of specific PEGylation, producing FGF21 analogs with high specific activity and salutary biological activities. Using homology modeling and structure-based design, specific sites were chosen in human FGF21 for site-specific PEGylation to ensure that receptor binding regions were preserved. The in vitro activity of the PEGylated FGF21 analogs corresponded with the site of PEG placement within the binding model. Site-specific PEGylated analogs demonstrated dramatically increased circulating half-life and enhanced efficacy in db/db mice. Twice-weekly dosing of an optimal FGF21 analog reduced glucose levels, plasma lipids, liver triglycerides, and plasma glucagon and demonstrated important activity in insulin expression and secretion, tissue sensitivity to insulin, and the resulting carbohydrate and lipid fluxes. As a result, FGF21 holds promise as a multifaceted therapeutic that can address multiple aspects of T2D pathogenesis. As these preclinical studies have progressed, it has also become clear that the native protein has pharmaceutical limitations, a short half-life being one of the more prominent among them. Thus, despite the promise of this therapeutic, this limitation diminishes the attraction for clinical testing and has forestalled development.

In this setting, we examined the utility of applying a biosynthetic approach to site selectively install a unique amino acid that could serve as the site of attachment for a single polyethylene glycol (PEG)-based polymer. This site-directed placement of unique chemical functionality is termed ReCode (reconstituting chemically orthogonal-directed engineering) (Fig. 1). It uses a tRNA that has been modified to recognize the UAG (amber) codon and an evolved aminoacyl tRNA synthetase (O-RS), which can charge this tRNA (O-tRNA) with a novel amino acid that has chemical attributes orthogonal to those found in the naturally occurring 20 amino acids. In this way, placement of the UAG codon within the DNA encoding a protein of interest can direct site-specific insertion of that novel amino acid into the protein during translation. After purification of the protein, the novel amino acid can be conjugated with complete specificity to an array of conjugates (Fig. 2). In the instance of FGF21, the novel amino acid p-acetylphenylalanine (pAcF) was used. The ketone side chain functionality of this novel amino acid is uniquely reactive in the protein and when reacted with amino-oxy-functionalized PEG polymer, it results in a stable oxime adduct of the PEGylated protein.

Herein, we describe the identification of site-specific PEGylated FGF21 analogs that demonstrated enhanced efficacy when administrated less frequently than the native hormone. Two of these PEGylated FGF21 variants, when...
studied in db/db mice at a dose and frequency that was BW neutral, elicited a robust glucose-lowering activity that was not observed with native hormone. This antidiabetic activity was associated with the ability to increase insulin sensitivity, augment glucose catabolism, maintain pancreatic insulin content, and preserve pancreatic islet mass and function. These results extend our understanding of the physiologic activities of FGF21 while enhancing the potential for FGF21 agonism as a novel mechanism for T2D therapy.

**RESEARCH DESIGN AND METHODS**

**Selection of sites for pAcF incorporation.** A human FGF21 theoretical model was constructed by homology modeling using the crystal structures of FGF19 (Protein Data Bank code 2P33 and 2PWA) (7,8) and FGF23 (Protein Data Bank code 2P39) (7); all three belong to the FGF19 subfamily. The FGF19 subfamily members use Klotho proteins to facilitate their interactions with FGF receptors (FGFRs), rather than heparin sulfate as other FGF family members (7). Building on previous biochemical and structural actions with FGF receptors (FGFRs), rather than heparin sulfate as other FGF19 subfamily members use Klotho proteins to facilitate their interaction (7–10), amino acids with surface-exposed side chains distal from the putative receptor binding regions were selected for substitution with pAcF and subsequent PEG attachment.

**Cell line construction and production of pAcF-containing FGF21Q108samber.** Using previously described methods (6), an Escherichia coli K-12 W3110 cell line was generated to create a temperature-sensitive cell line with an additional W373R point mutation to confer lethal host phenotype at temperatures ≥37°C, which was subsequently transformed with an FGF21Q108samber plasmid into DH5α-T1 cells (Invitrogen Corp., Carlsbad, CA). FGF21Q108samberAcF protein production was confirmed through SDS-PAGE and liquid chromatography-mass spectrometry.

**GF protein purification and PEGylation.** Production and purification of PEGylated His6-FGF21 variants was accomplished as described earlier (6). The PEGylated His6-tagged FGF21 protein was >95% pure, with endotoxin levels in each variant <2 EU/mg protein by LAL analysis with EndoSafe-PTS LAL cartridges (Charles River Laboratories, Wilmington, MA).

For production of PEGylated FGF21 variants lacking the His6 tag, frozen cell paste was resuspended in 9 mL buffer per gram of paste in 50 mM Tris, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 8.0. The lysate was centrifuged at 10,000 g for 15 min at 4°C, and the pellet was washed using the same resuspension and centrifugation steps. Two additional washes were performed in 50 mM/L Tris, 100 mM/L NaCl, 1 mM/L EDTA, pH 8.0. The pellet containing purified inclusion bodies was solubilized in 9 mL buffer per gram of inclusion bodies in 20 mM/L Tris, 8 mol/L urea, 10 mM/L β-mercaptoethanol for 1 h at room temperature, and refolded in 20 mM/L Tris, pH 8.0, at a 0.91 (v/v) ratio of refold buffer:solubilized inclusion bodies at 4°C for 18–24 h. The refolded solution was processed through 0.2-μm filtration, anion-exchange capture (Q-Sepharose high-performance column; 20 mM/L Tris, pH 8.0, 0–250 mM/L NaCl gradient) and a hydrophobic interaction column (phenyl Sepharose high-performance column; binding buffer: 20 mM/L Tris, 0.55 mol/L ammonium sulfate, pH 8.0; elution buffer: 20 mM/L Tris, 2 mol/L urea, pH 8.0 gradient elution). The PEGylation and post-PEGylation purification methods were as previously described (6). All variant masses for in vitro and in vivo testing are reported as milligrams of protein without inclusion of the mass of any conjugated PEG.

**Phosphoextracellular signal-related kinase Meso Scale Discovery assay.** HEK293 β-Klotho clone #4 cells were seeded at 100,000 cells/well in a poly-n-Lys–coated 96-well plate and incubated overnight at 37°C and 5% CO2 to allow cells to adhere. The next day, fivefold serially diluted wild-type (WT) His6-tagged FGF21 and His6-tagged PEGylated FGF21 proteins were added to the cells in duplicate for 9 min at 37°C and 5% CO2. After stimulation, cold lysis buffer containing protease and phosphatase inhibitors was added to the wells and incubated on ice for 25 min to allow complete cell lysis. After centrifugation, the lysate was transferred to a 96-well Phospho/Total-ERK1/2 MultiSpot plate (Meso Scale Discovery, Gaithersburg, MD). Lysates were analyzed on a Meso Scale Discovery SE2400 reader according to protocol, and half-maximal effective concentration (EC50) values were calculated for all proteins. His6-tagged PEGylated FGF21 proteins were rank ordered by their EC50, values. The top five PEGylated FGF21 proteins, lacking the His6 tag, were also prepared and demonstrated comparable EC50 values.

**Pharmacokinetic evaluation.** Jugular vein-catheterized, male SD rats (Charles River Laboratories) were housed in individual cages in a temperature-controlled environment with a 12-h light-dark cycle with ad libitum access to water and standard rodent chow (Diet 5001; Purina). Rats were administered a single, subcutaneous injection of WT FGF21 or PEG30-FGF21 variants lacking the His6 tag (0.25 mg/kg). Blood samples were collected at various time points and analyzed by a bridging ELISA (see below) to determine compound concentrations. All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee of Ambrx, Inc. Pharmacokinetic analyses were performed using WinNonLin software (version 5.0.1; Pharsight) and noncompartmental modeling.
PEG30-FGF21 variants lacking the His6 tag.

**METHODS.**

Male db/db mice were purchased at 5 weeks of age from The Jackson Laboratory and housed eight per cage in temperature-, humidity-, and light-controlled rooms with ad libitum access to autoclaved water and food (Diet 5008; Purina). At the beginning of the experiment, animals were acclimated to a pathogen-free animal facility for 1 week, where they were maintained at 22°C on a 14-h:10-h light-dark cycle. The mice were randomized according to their glucose levels and divided into two groups (n = 8); one cohort received 0.25 mg/kg PEG30-FGF21 subcutaneously once daily for 12 days while the other was injected with vehicle. The OxyMax metabolic chamber system ran 24 hours a day, 7 days a week to collect oxygen consumption (V\textsubscript{O2}) and CO\textsubscript{2} production (V\textsubscript{CO2}) data nonstop except when the mice were dosed right after their dark cycle ended. The respiratory exchange ratio (RER) was calculated as V\textsubscript{CO2}/V\textsubscript{O2}.

**Nutrient utilization studies.** Six-week-old males were injected subcutaneously with either vehicle or PEG30-FGF21. Humulin R (3 units/kg; Eli Lilly) was administered 6 h afterward. Blood glucose from tail vein was measured by glucometer (Ultra LifeScan; OneTouch, Milpitas, CA) at the indicated time points pre- and postchallenge.

For plasma triglyceride, free fatty acid, insulin, glucagon, and liver triglyceride levels, pancreas staining/quantification, and islet functional studies were performed as previously described (11). Glucagon and insulin were measured using commercial ELISA kits (Linco Research Immunoassay, St. Charles, MO, and ALPCO Diagnostics, Windham, NH, respectively).

**Serum concentration measurements.** Standards, quality controls, and samples were spiked into 100% SD rat serum. All standards, quality controls, and study samples were then loaded onto the wells after pretreating 1:50 with assay buffer. The ARX618 in the standards, quality controls, and samples were captured by immobilized anti-PEG monoclonal antibody that had been coated onto the plate. After a wash step to remove unbound materials, biotinylated goat anti–FGF21 IgG polyclonal antibody was added to the wells. After another wash step, streptavidin sulfo tag was added for detection of the captured ARX618. After a final wash step, Meso Scale Discovery read buffer was added to all wells, and light was generated in proportion to the amount of ARX618 bound by the capture and detection reagents. The conversion of relative light units for the study samples and the quality controls to concentration was achieved through comparison with a standard curve on the same plate, which was regressed according to a four-parameter logistic regression model with a weighting factor of 1/Y\textsuperscript{2}. Results are reported in ng/mL concentration units.

**Metabolic studies in db/db mice.** Male db/db mice were purchased at 5 weeks of age from The Jackson Laboratory and housed eight per cage in temperature-, humidity-, and light-controlled rooms with ad libitum access to autoclaved water and food (Diet 5008; Purina). At the beginning of the experiment, db/db mice (8 weeks of age) were grouped based on BW and ambient glucose levels. All db/db studies were performed with WT FGF21 or PEG30-FGF21 variants lacking the His6 tag. For chronic efficacy evaluation, animals were dosed subcutaneously twice weekly (BW, WT or PEG30-FGF21) or twice daily (BID; WT FGF21) and studied for 12 days. Glucose, BW, and food consumption were measured as indicated after commencement of treatment. Plasma triglyceride, free fatty acid, insulin, glucagon, and liver triglyceride levels, pancreas staining/quantification, and islet functional studies were performed as previously described (11). Glucagon and insulin were measured using commercial ELISA kits (Linco Research Immunoassay, St. Charles, MO, and ALPCO Diagnostics, Windham, NH, respectively).

For the insulin tolerance test, db/db mice (12 weeks of age) were sorted based on blood glucose. Diet was removed at 9:00 a.m. and mice were injected subcutaneously with vehicle or PEG30-FGF21. Humulin R (3 units/kg; Eli Lilly) was administered 6 h afterward. Blood glucose from tail vein was measured by glucometer (Ultra LifeScan; OneTouch, Milpitas, CA) at the indicated time points pre- and postchallenge.

To compare insulin signaling using phospho-Akt as a marker, 12-week-old male db/db mice were grouped based on blood glucose. Diet was removed at 9:00 a.m. and mice were injected subcutaneously with vehicle or PEG30-FGF21 at 0.25 mg/kg. Saline or Humulin R (2 units/kg) was injected by tail vein the next morning at 9:00 a.m. Liver, gastrocnemius muscle, and white and brown adipose tissue were collected 5 min postinjection and snap-frozen in liquid nitrogen for phospho-Akt analysis with the whole-cell lysate phospho-Akt (Ser473) assay kit according to the manufacturer’s protocol (Meso Scale Discovery).

**Fatty acid synthesis in db/db mice.** Nine-week-old db/db mice received a subcutaneous injection of either vehicle or PEG30-FGF21 at 0.75 mg/kg. One and three hours postdose, mice received an intraperitoneal injection of \textsuperscript{3}H-H\textsubscript{2}O at 2.5 mCi/kg. Mice were killed 1 h post–\textsuperscript{3}H-H\textsubscript{2}O injection, and hepatic de novo lipogenesis (DNL) was determined as described previously (12).

**FIG. 2.** A theoretical model of FGF21 depicted as a ribbon and semitransparent surface diagram. The structure of the protein is based upon the crystal structures of FGF19 and FGF23. The FGF21 orange surface area contains its theoretical FGFR binding regions based upon the FGF:FGFR cocystal structure and the known binding relationship of FGFXX with β-Klotho. The specific FGF21 sites into which pAcF was inserted are represented as spheres. The colors of the different pAcF sites reflect the activity of their PEGylated variants in the cell-based ERK phosphorylation assay (green, sites within the most potent variants; wheat, sites within the modestly potent variants; red, sites within inactive variants). The figure was generated using the PyMOL Molecular Graphics System, version 1.3 (Schrödinger, LLC) as described in RESEARCH DESIGN AND METHODS.
RESULTS
Synthesis of PEGylated FGF21 variants. A human FGF21 theoretical model was constructed by homology modeling, using the crystal structures of FGF19 and FGF21 (7,10). Building on previous biochemical and structural work characterizing the FGF family receptor-binding interface (9), amino acids with surface-exposed side chains distal from the putative receptor binding regions were selected for substitution with pAcF and subsequent PEG attachment (see RESEARCH DESIGN AND METHODS).

On the basis of the aforementioned modeling, a series of human FGF21 variants was constructed by inserting the novel amino acid pAcF at single specific sites within the protein using the ReCode methodology. These variants were then biosynthesized, PEGylated with a 30-kDa PEG, and purified as described in RESEARCH DESIGN AND METHODS. The variants that were generated are listed in Table 1.

Cell-based functional characterization of PEGylated FGF21 variants. His₆-tagged versions of the PEGylated FGF21 variants, designated by the location of the novel amino acid insertion, were tested for in vitro activity by examining their ability to augment extracellular signal–related kinase phosphorylation (pERK) within a HEK293 cell line engineered to stably express β-Klotho. As previously described (7), FGF21 intracellular signaling requires the endogenous expression of FGFRs and the forced expression of β-Klotho. Table 1 shows the potencies with which His₆-tagged WT FGF21 and the corresponding variants induced pERK levels. There was a good correlation among the variants between pERK activity and location within the homology model. Variants Y22, K56, L99, and Y104, which are all within the modeled FGF21 binding sites, proved to be entirely inactive. In contrast, those variants most distal to the binding sites, R77, L66, Q108, and R131, proved to be most potent, whereas other variants were found to be of intermediate activity. The potency of the most active PEGylated proteins was diminished approximately fivefold compared with WT FGF21. Glucose uptake assays performed in 3T3-L1 adipocytes demonstrated the superior potency of a largely overlapping set of variants, R77, L66, E91, Q108, and R131, whereas R56, L99, and Y104 were once again found to be inactive (not shown). Therefore, we found good concordance of PEGylated variant potency using different means of assessing their functional activity in vitro.

Pharmacokinetics of PEGylated FGF21 variants. The most potent PEGylated variants, R77, L66, E91, Q108, and R131, were selected for single-dose subcutaneous testing in male rats and prepared without the His₆ tag. As shown in Supplementary Fig. 1, all of these PEGylated proteins had substantially prolonged pharmacokinetic profiles versus the WT protein. Beyond this general PEG effect on circulating FGF21 levels, important differences in pharmacokinetics were seen among the variants conjugated to the same size PEG. For example, compared with the WT FGF21–terminal half-life of 1.19 h, 30-kDa PEG differentially prolonged the half-lives of PEGylated variants across a range extending from 14.72 (R72) to 33.9 h (L66), a greater than twofold difference based solely on the site of attachment. The areas under the curve (AUCs) showed a similar range, with the WT protein displaying an AUC of 239.8 ng·h·mL⁻¹, whereas those of the PEGylated variants ranged from 3,216 ng·h·mL⁻¹ (L66) to 12,238 ng·h·mL⁻¹ (R132). These differences in circulation time were not predicted from the protein homology modeling, nor did they correlate with measured in vitro activities of the variants. Thus, the specific site of PEGylation can alter pharmacokinetics beyond the choice of PEG size itself. The combination of well-preserved in vitro activity and enhanced plasma levels warranted the further study of these five FGF21 variants in a setting where in vivo efficacy could be determined.

Table 1

<table>
<thead>
<tr>
<th>FGF21 variant</th>
<th>EC₅₀ (ng/mL)</th>
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<tbody>
<tr>
<td>WT</td>
<td>36 ± 7</td>
</tr>
<tr>
<td>R131</td>
<td>173 ± 34</td>
</tr>
<tr>
<td>E91</td>
<td>183 ± 66</td>
</tr>
<tr>
<td>Q108</td>
<td>201 ± 62</td>
</tr>
<tr>
<td>R77</td>
<td>210 ± 70</td>
</tr>
<tr>
<td>L66</td>
<td>358 ± 139</td>
</tr>
<tr>
<td>R126</td>
<td>398 ± 3</td>
</tr>
<tr>
<td>R72</td>
<td>496 ± 251</td>
</tr>
<tr>
<td>E110</td>
<td>564 ± 294</td>
</tr>
<tr>
<td>Y83</td>
<td>709 ± 0.4</td>
</tr>
<tr>
<td>H57</td>
<td>696 ± 492</td>
</tr>
<tr>
<td>P146</td>
<td>672</td>
</tr>
<tr>
<td>R135</td>
<td>754</td>
</tr>
<tr>
<td>H96</td>
<td>1403</td>
</tr>
<tr>
<td>R36</td>
<td>2054</td>
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<tr>
<td>Y104</td>
<td>&gt;10,000</td>
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<tr>
<td>L99</td>
<td>&gt;10,000</td>
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<tr>
<td>K56</td>
<td>&gt;10,000</td>
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<tr>
<td>Y22</td>
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Eighteen His₆-tagged PEGylated FGF21 proteins were rank ordered based on their potency. The five most potent PEGylated FGF21 proteins were evaluated without the His₆ tag; no significant change in EC₅₀ was observed (not shown). All variants were conjugated with 30-kDa PEG except for WT. WT FGF21 was prepared with and without the His₆ tag to serve as the appropriate comparator for the PEGylated FGF21 proteins. Data from assays performed in replicate are shown as mean ± SD. Variants with no SD values shown were only tested once in the assay.

Statistics. All data are expressed as means ± SEM. Statistical analysis was conducted using ANOVA or unpaired Student t test where appropriate. Statistical significance was defined as P < 0.05.

Acute PEG30-FGF21 administration improves insulin sensitivity in db/db mice. To determine whether PEG30-FGF21 affects insulin sensitivity in diabetic db/db mice before glucose lowering is observed, the animals were

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given a single injection of PEG30-R131 at 0.25, 0.75, or 2.5 mg/kg. This treatment resulted in reduced fasting plasma insulin and improved insulin tolerance in a dose-responsive manner 12 h postdose (Fig. 5A–C). We also profiled phospho-Akt levels in four major metabolic tissues 15 min after an intravenous injection of vehicle or insulin. The results (Fig. 5D–G) indicated that a single dose of PEGylated R131, provided 12 h in advance of insulin administration, significantly improved insulin signal transduction in liver, skeletal muscle, and white and brown adipose tissue. Together, these data demonstrated that a single dose of a potent PEG30-FGF21 variant led to a rapid improvement of insulin sensitivity in db/db mice without causing hypoglycemia, even at higher doses.

**PEG30-FGF21 alters metabolic fuel utilization.** After observing the ability of PEG30-FGF21 to dramatically reduce hyperglycemia in db/db mice without increasing BW, we hypothesized that the glucose was being taken up by various metabolic tissues and oxidatively catabolized. In order to test this proposition, we placed db/db mice in an OxyMax metabolic chamber system and injected them daily for 12 days with 0.25 mg/kg PEG30-R131 while constantly monitoring their VO2 and VCO2. These measurements were used to calculate the RER of the mice. We found that although both cohorts began the study with an RER of ~0.86, the RER of those treated with the PEG30-FGF21 variant rose to ~0.91 over the course of 4 days and maintained this elevated level throughout the treatment regimen (Fig. 6). In contrast, the control group’s RER trended down to ~0.83 during the course of the study. These results demonstrated that treatment of db/db mice with PEGylated R131 rapidly and durably increased oxidation of glucose, thereby allowing it to be removed from circulation without accumulating in insulin-responsive tissues as glycogen or lipids that would increase BW.

**DISCUSSION**

Recent studies in preclinical species have shown that FGF21, an atypical endocrine hormone–like member of the FGF superfamily, can address multiple pathophysiologic elements of T2D. These include mitigation of hyperglycemia and insulin resistance, the preservation of insulin production and pancreatic β-cell function, amelioration of dyslipidemia and altered lipid storage, and improvement of intermediary metabolism substrate fluxes (1–5). To our knowledge, no adverse effects of FGF21, including hypoglycemia at high doses, have yet been described in the literature. Thus, FGF21 has emerged as a promising candidate for the treatment of T2D.

Unfortunately, the short duration of circulation and action of native FGF21 undermines its therapeutic utility. Thus, the search for a means of prolonging the pharmacokinetics of FGF21 while maintaining its intrinsic activity led to the utilization of a novel, selective PEGylation strategy. The importance of such selectivity has been amply demonstrated, because conventional PEGylation of therapeutic proteins typically leads to the generation of heterogeneous mixtures, or less than optimal site selection (6). As a result, the specific activity is often reduced relative to the native protein, thereby necessitating higher clinical doses that lead to increased concerns about increased immunogenicity, renal vacuolation, and treatment cost.

Homology modeling allowed directed selection of pAcF site incorporation predicted to be distant from the receptor binding regions of human FGF21. Functional characterization of the PEGylated variants using cell-based assays measuring induction of ERK phosphorylation and glucose uptake validated the structural model we had produced, demonstrating that mono-PEGylation of FGF21 with a linear PEG30 polymer at putative receptor binding sites caused large losses in potency, whereas PEGylation at sites distal to those involved in FGFR interactions maintained high potency. These findings indicated that site matters in prolonging
time of action while maintaining specific activity. Recode methodology enables selection based on biological properties as opposed to conventional approaches rooted in the native placement of lysine. In addition, we showed that the same PEG30 polymer differentially improved the pharmacokinetic character of FGF21, when conjugated to different sites, perhaps due to differential absorption and proteolytic lability. These data collectively illustrated the pharmacological power in being unconstrained in site selection through biosynthesis with unique chemical functionality.

With no detectable weight change upon exposure to WT FGF21 or its PEGylated variants, db/db mice served as a good model to assess the direct metabolic actions of this therapy in a stringent rodent model of T2D. We found that BIW administration of the five most potent PEGylated FGF21 variants led to impressive glucose lowering with no sign of hypoglycemia during the 12-day study period. The rank ordering of their glucose-lowering potencies was similar to that observed in the pharmacokinetic study, with the best overall performance demonstrated by variants Q108 and R131. Such data provide strong evidence that FGF21 has direct antihyperglycemic activity and that site-specific mono-PEGylation of FGF21, as described in this work, provided molecular entities with superior pharmacological properties.

Accompanying the antihyperglycemic efficacy, treatment with PEGylated FGF21 analogs preserved pancreatic islet morphology, size, and number and improved glucose-dependent insulin secretion. These results are important because loss of islet function and mass are hallmarks of T2D (13), and the ability to mitigate endocrine pancreas deterioration promises durable antidiabetic therapy.

PEGylated FGF21 variants enhanced insulin sensitivity, as was detectable by diminished fasting insulin and improved insulin tolerance as early as 12 h after a single administration and long before significant glucose-lowering efficacy was observed. Additionally, increased insulin sensitivity was manifested by potentiation of insulin-induced Akt phosphorylation in major metabolic tissues, including liver, skeletal muscle, and adipose, which suggests that these tissues mediate the observed FGF21-induced improvements in whole-body insulin sensitivity and glucose disposal.

Acute administration of PEG30-FGF21 resulted in a rapid diminution in DNL. Though modest in scale, this change probably contributed to the reduction in hepatic steatosis seen after chronic treatment with PEGylated FGF21, which in turn would fortify its aforementioned insulin-sensitizing activity. The inhibition of hepatic DNL by PEG30-FGF21 might also mediate the observed improvement in dyslipidemia.

Our work demonstrated that FGF21 can modify nutrient utilization based on the availability of fuel substrate. In particular, when provided to hyperglycemic db/db mice, PEG30-FGF21 durably increased whole-body carbohydrate oxidative catabolism as indicated by an elevation in RER. Therefore, we proffer that PEGylated FGF21 enhances metabolic flexibility, i.e., the switch to insulin-stimulated glucose oxidation during periods of elevated circulating glucose, which has previously been shown to be impaired in T2D (14). Alleviating such a metabolic derangement could aid in the mitigation of insulin resistance.

In summary, our findings demonstrate that site-specific PEGylation of FGF21 results in the generation of potent, long-acting molecules with highly desirable antidiabetic therapeutic profiles. Such uniquely modified proteins hold significant promise in addressing the complex syndrome of T2D.
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J.M. and J.P. designed and performed research, analyzed data, and wrote the manuscript. Z.L. performed research and analyzed data. L.S. and N.L. designed and performed research and analyzed data. H.M. designed and performed research, contributed new reagents/analytic tools, and analyzed data. Q.D.-Y. performed research and analyzed data. A.-M.P. designed and performed research and analyzed data.
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