Development of A Novel Long-acting Anti-diabetic FGF21 Mimetic by Targeted Conjugation to A Scaffold Antibody

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Nonstandard abbreviations: FGF: fibroblast growth factor; FGFR: fibroblast growth factor receptor; WT: wild-type; WAT: white adipose tissue; IV: intravenously; OGTT: oral glucose tolerance test; AUC: area under the curve; RT-PCR: reverse transcription polymerase chain reaction; qPCR: quantitative RT-PCR; DIO: diet-induced obese; PPAR: peroxisome proliferator-activated receptor; PGC-1α: PPARγ coactivator-1α; SCD1: stearoyl-CoA desaturase 1; FAS: fatty acid synthase; ANOVA: analyses of variance.

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Fibroblast growth factor 21 (FGF21) improves insulin sensitivity, reduces body weight, and reverses hepatic steatosis in preclinical species. We generated long-acting FGF21 mimetics by site-specific conjugation of the protein to a scaffold antibody. Linking FGF21 through the C-terminus decreased bioactivity, while bioactivity was maintained by linkage to selected internal positions. In mice, these CovX-Bodies retain efficacy while increasing half-life up to 70-fold compared with wild-type FGF21. A preferred mid-linked CovX-Body, CVX-343, demonstrated enhanced \textit{in vivo} stability in preclinical species, and a single injection improved glucose tolerance for 6 days in \textit{ob/ob} mice. In diet-induced obese mice, weekly doses of CVX-343 reduced body weight, blood glucose and lipids levels. In \textit{db/db} mice, CVX-343 increased glucose tolerance, pancreatic \textbeta-\textit{cell} mass and proliferation. CVX-343, created by linkage of the CovX scaffold antibody to the engineered residue A129C of FGF21 protein, demonstrated superior preclinical pharmacodynamics by extending serum half-life of FGF21 while preserving full therapeutic functionality.
INTRODUCTION

Fibroblast growth factor 21 (FGF21), a member of the FGF19 subfamily, functions as an important metabolic regulator of glucose and lipid homeostasis, and the adaptive response to starvation (Kharitonenkov and Shanafelt, 2008a; Beenken and Mohammadi, 2009). FGF21 is a secreted protein abundantly expressed in liver, pancreas, adipose tissue, and skeletal muscle (Beenken and Mohammadi, 2009). Its expression is induced by fasting, ketogenic diet, peroxisome proliferator-activated receptor α (PPARα) and γ agonists (Inagaki et al., 2007; Badman et al., 2007; Muise et al., 2008), thyroid hormone (Adams et al., 2007), and thermogenic activation (Chartoumpekis et al., 2011; Hondares et al., 2011). FGF21 signals through an FGFR/βKlotho receptor complex, leading to biological effects in liver, adipose tissue, and pancreas. This tissue specific action of FGF21 is achieved by the restricted expression of βKlotho in those tissues (Kharitonenkov et al., 2008b; Kurosu et al., 2007). Although it is not clear which FGFR subtypes mediate FGF21 action in vivo, FGFR1c, 3c, and 4 have been shown to be involved in FGF21 signaling in vitro (Ogawa et al., 2007; Suzuki et al., 2008). Both the N- and C-termini of FGF21 have been shown to be important for its biological activity, with the N-terminus required for FGFR activation, and the C-terminus shown to directly bind βKlotho (Micanovic et al., 2009; Yie et al., 2009).

FGF21 stimulates glucose uptake in 3T3-L1 adipocytes and primary human adipocytes through increasing GLUT1 mRNA expression. However, in undifferentiated 3T3-L1 fibroblasts which express little or no endogenous βKlotho, FGF21 does not stimulate glucose uptake (Suzuki et al., 2008; Kharitonenkov et al., 2005). In 3T3-L1 adipocytes or fibroblasts stably expressing βKlotho, FGF21 activates FGF signaling molecules including phosphorylation of both FGFR substrate 2 (FRS2) and MAPK, and also induces GLUT1 mRNA expression in these cells.
(Ogawa et al., 2007; Suzuki et al., 2008; Kharitonenkov et al., 2005). In isolated rat islets, FGF21 increases insulin secretion and protects islet cells from gluclipotoxicity and cytokine-induced apoptosis (Wente et al., 2006). Systemic administration of FGF21 lowers blood glucose and triglycerides, reduces body weight, ameliorates hepatic steatosis, and preserves β-cell function and mass in diabetic mouse models (Kharitonenkov et al., 2005; Wente et al., 2006; Coskun et al., 2008; Xu et al., 2009a). Moreover, a significant reduction in LDL cholesterol and an elevation in HDL cholesterol were observed during FGF21 treatment in diabetic monkeys (Kharitonenkov et al., 2007). The mechanisms by which FGF21 exerts its metabolically beneficial effects are still not clear. However, increasing evidence demonstrates that FGF21 improves insulin sensitivity (Xu et al., 2009b; Berglund et al., 2009), increases energy expenditure, and enhances fat utilization (Coskun et al., 2008; Xu et al., 2009a). FGF21 has also been shown to promote browning of white adipose tissues through regulating PGC-1α (Fisher et al., 2012) and promote thermogenic activation in neonatal brown fat and primary brown adipocytes (Hondares et al., 2010). Taken together, the preclinical evidence suggests a strong therapeutic potential of FGF21 for the treatment of diabetes, obesity, and fatty liver disease.

FGF21 has a very short serum half-life, 30 mins in mouse and 2 hrs in monkey, therefore daily injection or continuous infusion of the protein is required for in vivo bioactivity. Although genetic fusion can improve the pharmacokinetic property of a protein, this involves the N- or C-terminus of the protein. In the case of FGF21, however, N- or C-terminal genetic fusion may impair the bioactivity of the protein, as the termini have been shown to be important for biological function (Micanovic et al., 2009; Yie et al., 2009). PEGylation has also been used to improve the pharmacokinetic property of proteins and peptides, although PEGylated FGF21
exhibits lower intrinsic potency compared with the wild-type (WT) protein (Mu et al., 2012). We aimed to create a long-acting FGF21 molecule with comparable in vitro potency and full physiologic efficacy of WT FGF21 protein using CovX conjugation technology. Here we describe the construction of various FGF21 CovX-Bodies and their bioactivity and stability. A preferred molecule, CVX-343, was selected for further in vivo characterization based on in vitro potency and half-life. CVX-343 exhibits in vivo efficacy comparable with WT FGF21 and prolonged pharmacodynamics in diabetic mouse models.
MATERIALS AND METHODS

Expression and purification of FGF21 proteins

Recombinant human FGF21 proteins were expressed in E. coli. The bacterial expression vector of WT human FGF21 was constructed using pET21b vector (EMD). For the lysine mutants, the WT FGF21 construct was used, and Lys-to-Arg, H1K, and S181K mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). For the cysteine mutants, an FGF21 construct lacking the first histidine residue was used, and D79C, H125C, and A129C mutations were introduced into the expression vector by using a QuikChange site-directed mutagenesis kit (Stratagene). The bacterial expression vectors were transformed into the host strain BL21- (DE3)-RIL (Stratagene). The transformed cells were grown in 1 liter of LB medium at 37°C, and expression was initiated by addition of 1 mM isopropyl β-D-thiogalactopyranoside. The cell pellets were suspended in lysis buffer (50 mM Tris, 10 mM EDTA, pH 7.5), and passed through a microfluidizer. After centrifugation at 17,000 g at 4°C for 30 min, the inclusion body pellets were solubilized with 7 M urea, 5 mM DTT, and 50 mM bis-tris propane, pH 10.5, and then diluted 10 times into 50 mM Bis-TrisTris propane, pH 8.0. The solutions were stirred for 2 days and dialyzed against 20 mM Tris-HCl, pH 7.5, followed by centrifugation at 14,000 g for 30 min. The supernatants were loaded to HiTrap Q HP (GE Healthcare) equilibrated with buffer A (20 mM Tris-HCl, pH 7.5). FGF21 proteins were eluted with a linear gradient of buffer B (20 mM Tris-HCl, 200 mM NaCl, pH 7.5). The protein fractions were then loaded onto a Ni-NTA column (GE Healthcare) pre-equilibrated with PBS. FGF21 proteins were eluted with a linear gradient from PBS to PBS buffer containing 100 mM imidazole. The fractions were concentrated and applied to a size exclusion column (Hiload 26/60, Superdex 300) equilibrated with PBS.
cysteine mutants, the free cysteine residues were confirmed using Ellman’s reagent and by RP-HPLC. Purified proteins were sterilized by 0.22 µm filter and stored at -80°C.

**Generation of FGF21 CovX-Bodies**

FGF21 CovX-Bodies were generated by covalently linking two recombinant human FGF21 proteins to the Fab of a scaffold antibody CVX-2000 through the lysine or free cysteine residue via a succinimide or maleimide linker, respectively, and purified by size exclusion chromatography. The structures of the FGF21 CovX-Bodies were described previously (Das et al., 2012). The scaffold antibody CVX-2000 was expressed in Chinese Hamster Ovary cells and purified by a standard three-column process that includes Protein A, anion exchange and cation exchange chromatographic steps. The linkers were firstly reacted with FGF21 proteins at 1:1 molar ratio in 20 mM Tris, 50 mM NaCl, pH 7.5 at room temperature for 30 mins. Then the linker attached proteins were fused to CVX-2000 at 6:1 molar ratio in 20 mM Tris, 20 mM NaCl, pH 7.0 at room temperature overnight. CovX-Bodies were purified by Sephacryl S-200 HR (GE Healthcare) in 50 mM Tris, 250 mM NaCl, pH 7.0, and diluted 1:1 with water to final solutions in 25 mM Tris, 125 mM NaCl pH 7.0 for *in vitro* and *in vivo* experiments. Purified CovX-Bodies were analyzed by 12% Bis-Tris reduced SDS-PAGE and SEC-LCMS to confirm conjugation completion.

**Cell culture and treatment**

3T3-L1 fibroblasts were obtained from ATCC and maintained in growth media containing DMEM with high glucose, 10% FBS, 2 mM L-glutamine, and 1% Pen-Strep (all from Gibco). After grown to confluence, cells were induced to differentiate in growth media supplemented with 4 µg/ml bovine insulin, 0.1 mg/ml 3-isobutyl 1-methyl xanthine, and 0.1 µg/ml dexamethasone (all from Sigma) for 3 days. Cells were then maintained in growth media to
allow full differentiation. Overnight serum starved day 10-14 differentiated 3T3-L1 adipocytes were treated with compounds in serum-free DMEM containing 0.2% BSA (Sigma) at 37°C for 6 hrs.

**Quantitative RT-PCR (qPCR)**

Total RNA was isolated from treated 3T3-L1 adipocytes and frozen WAT samples using the RNeasy mini Kit (Qiagen) according to the manufacturer’s instructions. Quantitative RT-PCR reactions were carried out using a Quantitect Probe RT-PCR kit (Qiagen) in the Applied Biosystems 7900HT Taqman machine. Primers were Mm00441480_m1 for GLUT1, Mm01244860_m1 for UCP1, Mm01208835_m1 for PGC-1α, Mm01184322_m1 for PPARγ, Mm00662319_m1 for FAS, Mm00772290_m1 for SCD1, and mM99999915_g1 for GAPDH (Applied Biosystems). RT-PCR reactions were conducted with the following conditions: reverse transcription at 50°C for 30 mins; activation at 95°C for 15 mins; and 40 cycles of denaturation at 94°C for 15 secs followed by annealing/extension at 58°C for 60 secs. The effect of the compounds was determined by a fold change in GLUT1 or UCP1 mRNA levels normalized by the GAPDH mRNA levels from each sample.

**Animals**

All animal experiments were carried out in accordance with protocols and guidelines approved by the CovX (for rodent) and Maccine Pte Ltd (for nonhuman primate) Institutional Animal Care and Use Committees and conducted in AAALAC accredited facilities. Male Swiss Webster mice and male Sprague Dawley rats were purchased from Charles River Laboratories. The cynomolgus monkeys were from Maccine Pte Ltd. Six-week-old male C57Bl/Lep/Ob and Lepr/db mice, and 12-week-old male C57Bl/6J/DIO mice, body weight 30-40 g, were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed 3-4/cage in a temperature-
controlled (22-24°C) environment with 12-h light and 12-h dark cycle. *Ob/ob* and *db/db* mice were provided with rodent chow and water *ad libitum*. DIO mice were maintained on a high fat diet (Research Diets, 60% fat, 20% carbohydrate, and 20% protein) for 2 weeks before randomization and treatment. Mice were treated with vehicle, FGF21 protein at 1 mg/kg daily, or CVX-343 at indicated doses by subcutaneous injection. Food intake and body weight were measured daily. Cumulative body weight change was calculated as the difference between the daily body weight and the baseline body weight of each mouse. At the end of the study, mice were euthanized by CO₂ asphyxiation. Terminal cardiac blood was drawn. Liver and white adipose tissue samples were snap frozen for liver triglycerides and tissue gene expression analysis, respectively.

**Pharmacokinetics in rodents and non-human primates**

Pharmacokinetics properties of compounds were assessed in male Swiss Webster mice, male Sprague Dawley rats, or cynomolgus monkeys. FGF21 CovX-Bodies were administered intravenously (IV) or subcutaneously (SC) at 3 mg/kg (n=3 for mice, and n = 2 for rats and monkeys), and blood samples were collected over a period of 5, 14 and 21 days for mice, rats, and monkeys, respectively. Serum samples were prepared and analyzed for FGF21 CovX-Body levels using an ELISA, in which the FGF21 CovX-Bodies were captured in 96-well microtiter plates coated with an anti-FGF21 mAb (Abcam), followed by detection with an anti-hIgG-HRP. Data were analyzed using WinNonlin version 4.1 to generate PK parameter estimates.

**Oral glucose tolerance test (OGTT)**

On the day of the OGTT, mice were fasted for 4-5 hrs in the morning. After baseline glucose levels were recorded, mice were gavaged with glucose (1.5 g/kg for *ob/ob* and *db/db* mice or 2 g/kg for DIO mice), and blood glucose was measured at 15, 30, 60, 90, and 120 mins after the
oral glucose challenge with One Touch glucose Meters (Life Scan, Johnson & Johnson). The glucose areas under the curves (AUC) during an OGTT from 0 - 120 mins were calculated by the linear trapezoidal method (GraphPad Prism 5, GraphPad Software, San Diego, CA).

**Serum lipids and liver triglyceride measurement**

Serum triglycerides and non-esterified fatty acid levels were analyzed using Triglyceride Reagent (Thermo Scientific) and NEFA reagents (Wako Diagnostics) respectively according to the manufacturers’ instructions. Frozen liver tissues were weighed and homogenized in chloroform/methanol (2:1) solution with a probe sonicator. After adding 0.88% KCl, the mixture was centrifuged at 4000 rpm for 5 mins. The chloroform layer was transferred to a polypropylene 96 well assay plate. After evaporation of chloroform, lipids were re-suspended in DANO solution (tert-butyl alcohol:(50% methanol/50% Triton X-100), 3:2). Triglycerides were analyzed using Triglyceride Reagent (Thermo Scientific). Liver triglyceride content was expressed as mg of triglyceride/g of liver.

**Indirect calorimetry**

Fourteen-week-old DIO mice were administered with 10 mg/kg CVX-343 or vehicle by SC injection on day 0 and 7. Immediately following the second dose, mice were placed into the Columbus instruments cage monitoring system (1 mouse per cage) (Columbus Instruments) for assessment of O₂ consumption (VO₂), CO₂ production (VCO₂), and total locomotor activity. The respiratory quotient (RQ) was calculated as VCO₂/VO₂. Heat was calculated using a formula as heat = 3.815 x VO₂ + 1.232 x VCO₂.

**Pancreatic β-cell mass and proliferation by immunohistochemistry**

Six-week-old db/db mice were administered with 10 mg/kg CVX-343 or vehicle by SC injection on day 0 and 7. On day 11, pancreas was weighed, fixed in 10% formalin, and undergone
paraffin sections. After routine deparaffinization and antigen retrieval, sections were incubated with guinea pig anti-swine insulin polyclonal antibody (Dakocytomation, 1:100) at 4°C overnight, followed by incubation with HRP conjugated goat anti-guinea pig IgG (Jackson ImmunoResearch Laboratory, 1:1000) at room temperature for 1 hr. The insulin immunostaining was detected with Nova-red (Vector Laboratory). The slides were then counterstained with hematoxylin. The whole tissue section images were acquired by Leica SCN400 scanner. The total pancreatic tissue area and insulin positive area from each section were quantified by Deffinien Tissue Studio 2.10 software. The β-cell mass was calculated as β-cell mass = insulin positive area/total pancreas area x pancreas weight. For β-cell proliferation analysis, the sections were doubly stained for PCNA and insulin. The sections were first incubated with rabbit anti-PCNA antibody (Abcam, 1:400) overnight at 4°C, followed by incubation with HRP conjugated donkey-anti-rabbit IgG (Jackson ImmunoResearch Laboratory, 1:1000) and detection by ImmPACT SG kit (Vector Laboratory). After PCNA staining, the sections were immunostained for insulin as described above. The percentage of PCNA positive β-cells were calculated as the number of PCNA and insulin positive cells/the number of insulin positive cells *100% by counting a total of 1000 β-cells for each section.

Statistical analysis

All data are represented as mean ± SEM. One-way analyses of variance (ANOVA) with Dunnett’s post-hoc analyses or two-way ANOVA (corrected for repeated measures) with Bonferroni post-tests were performed and statistical significance was set at p < 0.05.
RESULTS

Generation of bivalent FGF21 CovX-Bodies

Human FGF21 is a secreted protein of a molecular weight of ~19 kDa, consisting of 181 amino acids with four native lysine residues (K56, K59, K69, and K122) and two native cysteine residues (C75 and C93) which form an intramolecular disulfide bond. FGF21 CovX-Bodies were generated in a two-step process. First, the FGF21 protein was covalently linked at either a selected (native) lysine or (engineered) cysteine residues using various linkers, and then the FGF21 protein with attached linker was conjugated to the Fab region of a scaffold antibody CVX-2000, a humanized IgG1κ mAb, in a site-specific manner (Doppalapudi et al., 2010).

Lysine conjugates To ensure the specificity of conjugation, a number of FGF21 single lysine mutants were expressed in *E. coli*, which were made by mutating three of four native Lys to Arg to allow conjugation at the single remaining Lys residue via a succinimide linker, or mutating all four native Lys to Arg and introducing a Lys at the first (H1K) or the last residue (S181K) to allow conjugation at the N- or C-terminus of the protein.

Cysteine conjugates Three FGF21 cysteine mutants (D79C, H125C, A129C) were generated by introducing a free cysteine residue on the protein surface to allow conjugation via a maleimide linker 3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-N-(2-(2-(3-oxo-3-((4-(3-oxo-3-(2-oxoazetidin-1-yl)propyl)phenyl)amino)propoxy)ethoxy)ethyl)propanamide.

By either linking approach, the resulting protein conjugate is a bivalent FGF21 CovX-Body of a MW of ~188 kDa. The majority (> 90%) of purified material after size exclusion chromatography was composed of bivalent FGF21 CovX-Body by LCMS analysis, with the remaining being monovalent species and unconjugated scaffold antibody (<10%) (data not shown).
FGF21-A129C CovX-Body displays favorable *in vitro* activity and half-life profile

To evaluate the effect of conjugation site on the *in vitro* activity of the protein conjugates, we measured GLUT1 mRNA expression in 3T3-L1 adipocytes by qPCR following FGF21 protein or CovX-Body treatment for 6 hrs. The N-terminally linked CovX-Body had ~2-fold reduced *in vitro* activity (EC$_{50}$ = 5.9 nM), whereas the C-terminally linked CovX-Body demonstrated more than a 200-fold reduction in potency (mean EC$_{50}$ > 500 nM) compared with WT FGF21 protein (mean EC$_{50}$ = 2.9 nM, N = 26). Most of the CovX-Bodies tested, linked at Lys or Cys residues, retained *in vitro* potency similar to WT protein (Table 1).

To assess the effect of the conjugation site on the *in vivo* stability of the protein conjugates, we conducted pharmacokinetics studies by administrating 3 mg/kg FGF21 CovX-Bodies or 10 mg/kg WT FGF21 protein intravenously (IV) or subcutaneously (SC) to Swiss Webster mice. Consistent with the literature, WT FGF21 had IV and SC half-lives of 20 - 30 mins (Table 1). N-terminally linked CovX-Body showed IV and SC half-lives of 16 hrs and 11 hrs, 48- and 22-fold longer than those of WT FGF21 protein, respectively. Half-lives of the other CovX-Bodies ranged from 13 - 37 hrs depending on linkage site, with two of the Cys-linked CovX-Bodies (H125C and A129C) displaying ~70-fold increases in half-life after both IV and SC administration in comparison with WT FGF21 (Table 1).

Based in part on its favorable potency and half-life in mouse, FGF21-A129C CovX-Body, named as CVX-343, was selected for *in vivo* characterization (Figure 1A). The pharmacokinetics of this molecule was further assessed in Sprague-Dawley rats and cynomolgus monkeys. As in mice, CVX-343 exhibited prolonged residence time in both rats and monkeys, with IV half-lives of 60 and 65 hrs, SC half-lives of 39 hrs, and SC bioavailability of 52% and...
68%, respectively (Figure 1, Table 2). No adverse effects of CVX-343 were observed at the doses administered in these or subsequent studies.

**FGF21-A129C CovX-Body demonstrates prolonged pharmacodynamics in **ob/ob **mice**

Subcutaneous infusion or daily injection of WT FGF21 protein improves glucose tolerance and reduces body weight gain in diabetic mouse models (Kharitonenkov et al., 2005; Coskun T et al., 2007; Xu et al., 2009a). To determine if the FGF21-A129C CovX-Body is effective in improving glucose control with less frequent dosing, a single SC injection of CVX-343 at 3 mg/kg was given to the ob/ob mice and an OGTT was conducted 6 days after the injection. Daily SC injection of 1 mg/kg WT FGF21 protein was given as a positive control. Consistent with the literature, daily injection of WT FGF21 protein (1 mg/kg) significantly lowered the basal blood glucose levels, improved glucose tolerance, and reduced body weight gain after 6 days of treatment. Similarly, a single SC injection of CVX-343 (3 mg/kg) also significantly lowered (p < 0.001) the basal glucose levels comparable with the WT protein treated or the lean control group 6 days post dose (Figure 2A). The glucose area under the curve (AUC) of CVX-343 treated group was significantly reduced by 20% compared with the vehicle control (Figure 2B). Since the pharmacophore portion accounts for ~20% of the mass of CVX-343, 3 mg/kg of CVX-343 is equivalent to 0.6 mg/kg of FGF21 protein. To determine if the equivalent amount of the protein had any effect on glucose tolerance, a single dose of 0.6 mg/kg of FGF21 protein was administered SC to ob/ob mice, and no effect on glucose tolerance and body weight was found 72 hrs after the single injection (data not shown). Moreover, a single injection of CVX-343 at 10 mg/kg significantly reduced body weight gain compared with the vehicle control 6 days post dose, to a similar level as the daily WT FGF21 treated group (1 mg/kg) (Figure 2C). A significant reduction in liver triglycerides was also observed in the CVX-343 treated group.
compared with the vehicle control, whereas daily dosed FGF21 protein had no effect (Figure 2D). Serum triglyceride levels were unchanged by CVX-343 treatment when measured at the end of the study (data not shown). To examine the effect of the compound on gene expression in WAT, we performed qPCR and found the UCP1 expression was significantly induced by ~2.5-fold in both FGF21 and CVX-343 treated groups compared with vehicle control (Figure 2E).

**CVX-343 reduces body weight and increases energy expenditure in DIO mice**

To assess the repeat dose efficacy of the compound in DIO model, CVX-343 (10 mg/kg) was administered SC to DIO mice once a week in comparison with daily SC injection of 1 mg/kg WT FGF21 protein. WT FGF21 protein-treated group gradually lost body weight, but did not reach statistical significance compared with the vehicle treated group on day 10. However, repeat administration of CVX-343 caused a significant 7% body weight loss on day 10 compared with the vehicle control in these mice (Figure 3A). Neither WT FGF21 protein nor CVX-343 had any effect on food intake (Figure 3B). CVX-343 also significantly improved glucose tolerance 3 days after the second dose, glucose AUC reduced by 17% compared with vehicle control (Figure 3C, D). We next examined the effect of the compound on lipid metabolism, and found a significant reduction in serum triglycerides (by 48% vs. 31% for FGF21 protein, Figure 3E) and NEFA levels (by 41% vs. 34% for FGF21 protein, Figure 3F) by CVX-343 treatment on day 10 in comparison with the vehicle control. Liver triglycerides were also 34% lower, but there was no statistical significance from the vehicle control due to large variability (Figure 3G). We also examined the gene expression associated with energy expenditure. Similar to findings in the ob/ob mice, UCP1 expression was significantly induced (4-fold) in epididymal WAT by CVX-343 treatment in these mice, while WT protein treatment had minimal effect (Figure 3H). With respect to the expression of lipogenic genes in WAT, modest increases in PGC-1α (40%) and
PPARγ (40%) expression were observed, as was a 50% decrease of FAS expression, 3 days after the second injection of CVX-343 (data not shown). In the liver, CVX-343 increased PGC-1α expression (1.8-fold) and substantially decreased SCD1 expression (80%) (data not shown). These results demonstrate that weekly administration of CVX-343 achieved a similar in vivo activity profile as daily injection of the WT FGF21 protein in a diabetic mouse model.

FGF21 has been shown to reduce body weight by increasing energy expenditure (Coskun et al., 2007; Xu et al., 2009a). To determine if the FGF21-A129C CovX-Body retained this bioactivity of WT FGF21 protein to positively affect energy expenditure, DIO mice were given CVX-343 (10 mg/kg, 1x/wk) twice. Immediately following the second dose, energy expenditure parameters were monitored by indirect calorimetry. As shown in Figure 4, both O₂ consumption and CO₂ production were significantly increased by CVX-343 treatment during the dark cycle. As a result, RQ (VCO₂/VO₂) was not affected. CVX-343 treatment also significantly enhanced heat production, but had no effect on locomotor activity. These data suggest that CVX-343 is capable of positively regulating energy component which may contribute to the mechanism of body weight loss in these animals.

**CVX-343 increases pancreatic β-cell mass in db/db mice**

To determine the effect of the compound on pancreatic β-cells in vivo, we examined the β-cell mass in db/db mice treated with CVX-343 by immunohistochemistry. Repeat administration of CVX-343 (10 mg/kg, SC, 1x/wk) significantly improved glucose tolerance compared with the vehicle control in db/db mice (Figure 5A, B). However, no effect on body weight or food intake was observed by CVX-343 treatment in this model (data not shown). CVX-343 treatment resulted in a significant increase in β-cell mass by 2.4-fold compared with the vehicle control in db/db mice (Figure 5C, D), which was accompanied by an increase in β-cell proliferation.
indicated by increased PCNA positive β-cells (Figure 5E, F). These results suggest that CVX-343 has a positive effect on β-cell preservation in db/db mice, which is consistent with the β-cell protective role of FGF21 (Wente et al., 2006).
DISCUSSION

Due to renal clearance and proteolytic degradation, many biologically active proteins have short circulating half-lives, requiring frequent administration which limits their clinical utility. To overcome this shortcoming, several strategies have been utilized to improve the pharmacokinetics of native proteins and peptides, including Fc fusions, albumin fusions, PEGylation, and fusion to other scaffold proteins (Carter, 2011). Indeed, a number of Fc fusion and PEGylated proteins are approved as human therapeutics (Jevševar et al., 2010; Beck and Reichert, 2011). Although Fc fusion and PEGylation strategies effectively extend half lives of native proteins or peptides, a drawback is impaired activity of the bioactive molecules, potentially offsetting gains achieved by enhancing \textit{in vivo} stability of the construct. CovX technology offers another bioconjugation option to modulate the pharmacokinetics of proteins and peptides. A unique feature of CovX technology is flexibility in conjugation site choice by which the therapeutic molecule can be linked to the scaffold antibody in a manner to fully realize its therapeutic potential (Doppalapudi et al., 2010). In fact, we observed significant differences in pharmacological properties for FGF21 CovX-Body conjugates with varied linkage sites, demonstrating that optimal selection of these elements is critical.

This approach has an advantage over genetic fusion particularly when both termini of a protein or peptide mediate biological function. Both the N- and C-terminus of FGF21 are important for its \textit{in vitro} activity (Micanovic et al., 2009; Yie et al., 2009) and we found that certain CovX-Bodies were superior to the C-terminally linked CovX-Body in that they maintained \textit{in vitro} potency similar to the WT FGF21 protein. Some of the mid-linked FGF21 CovX-Bodies had more prolonged half-lives than the terminally linked or other mid-linked molecules. The mid-linked A129C CovX-Body, CVX-343, exhibited favorable \textit{in vitro} potency, extended half-lives
in rodents and nonhuman primates, and full \textit{in vivo} efficacy in mice. We hypothesize that CovX-Body conjugation of FGF21 at A129C leads to better protection from enzymatic digestion of proteolytic cleavage sites critical for bioactivity due to either steric hindrance afforded by the scaffold antibody and/or conformational change. An N-terminally PEGylated FGF21 has been reported to have similar \textit{in vitro} activity as native protein and \sim 4 \text{ hrs half-life in rat} (Huang et al., 2011). Similarly, an N-terminally linked CovX-Body also retained bioactivity \textit{in vitro} and demonstrated an 11-16 hrs half-life in mice. A number of mid-protein site-specific PEGylated FGF21 molecules have also been characterized, all of which had reduced \textit{in vitro} potency compared to the native protein (Mu et al., 2012). As with application of the CovX technology, these PEGylated FGF21 variants had improved half-lives ranging \sim 15-34 hrs. The enhanced pharmacokinetics of these molecules permitted less frequent administration to induce \textit{in vivo} activity comparable to wild-type protein, although the effect of the PEGylated FGF21 molecules was not assessed with less than twice weekly administration. In contrast, once weekly administration of CVX-343 was sufficient to induce persistent improvements in glucose tolerance, serum lipids, and body weight across several murine disease models, suggesting that the FGF21-A129C conjugation via the CovX scaffold technology is preferred to achieve optimal potency and pharmacokinetics profile over site-selective PEGylation, particularly at the termini of FGF21.

Consistent with improved pharmacokinetics, the pharmacodynamics of CVX-343 is sustained, evidenced by prolonged beneficial effects of the compound on glucose tolerance, body weight, and liver triglycerides in \textit{ob/ob} mice 6 days after a single SC injection. Furthermore, in DIO mice, once weekly CVX-343 reduced body weight without affecting food intake, improved glucose tolerance, and lowered serum lipids. CVX-343-induced body weight loss is probably
due to increased energy expenditure, as evidenced by the increased oxygen consumption and heat production observed in DIO mice. FGF21 promotes thermogenic activation in brown adipocytes, induces browning of WAT, and increases fatty acid oxidation in the liver (Hondares et al., 2010; Fisher et al., 2012, Coskun et al., 2007). In our work, serum NEFA levels were decreased after subchronic CVX-343 treatment, consistent with increased fatty acid oxidation. The induction of UCP1 expression in WAT by CVX-343 suggests that the compound may increase energy expenditure through browning of WAT and/or activation of brown-like adipocytes in WAT. Our findings are consistent with the actions of daily injection of FGF21 protein in diabetic mice, indicating that CVX-343 is a long acting FGF21 mimetic which promotes similar glucose and lipid control with less frequent administration.

FGF21 mimetic antibodies have recently been reported. Wu et al demonstrated that a FGFR1-specific monoclonal antibody induced FGF21 mimetic efficacy in diabetic mice (Wu et al., 2011). The antibody does not bind other isoforms of FGFR or βKlotho, but rather induces its effects by homodimerization and activation of FGFR1. The FGFR1 monoclonal antibody elicited phosphorylation of MEK and ERK in adipose and pancreas but not liver, and did not affect glucose homeostasis in a lipoatrophic model, data from which the authors concluded that the effects of FGF21 can be achieved through action on adipose tissue. However, some effects of continuous rhFGF21 infusion into the lipoatrophic mice were not mimicked by the FGFR1 monoclonal antibody (e.g. increased serum ketone bodies, decreased serum cholesterol) (Wu et al., 2011). Note that these effects were observed after once-weekly dosing of CVX-343. While the monoclonal antibody may require less frequent dosing than the CovX-Body, its lack of βKlotho-dependence may result in undesired activity on FGFR1-expressing cells that do not express βKlotho.
Another antibody which activates FGF21-regulated pathways is a monoclonal antibody which agonizes the βKlotho/FGFR1c receptor complex (mimAb1, Foltz et al., 2012). Unlike the FGFR1 antibody, mimAb1 does not bind to FGFR1c, but binds βKlotho with high affinity and activates FGFR1c-mediated signaling in a βKlotho-dependent manner. Injection of mimAb1 led to FGF21-like effects in obese cynomolgus monkeys, although differences between FGF21 and mimAb1 were reported. The binding sites of mimAb1 on the receptor complex were not identical to those of FGF21, which might result in mimAb1-induced receptor dimerization different from FGF21 (Foltz et al., 2012). Additionally, mimAb1 is highly specific to βKlotho/FGFR1c receptor complex; other FGFR isoforms through which FGF21 signals (i.e., FGFR2c and FGFR3c) are not activated (Foltz et al., 2012). Since FGFR1c is the predominant receptor expressed in adipose tissue, mimAb1-induced FGF21-like efficacy may rely on actions in adipose tissue. The study did not report serum cholesterol or liver triglycerides, two FGF21 actions which may be liver-mediated. Thus, the dependence of FGF21 on FGFR subtypes and on βKlotho might be delineated by comparison of the FGFR1-specific monoclonal antibody, the βKlotho/FGFR1c activation antibody and CVX-343, which presumably retains the receptor specificity and co-receptor dependencies of the native protein. Ultimately, therapeutic utility of these molecules will require clinical comparison.

Despite a 37 hrs SC half-life in mouse, the efficacy of CVX-343 on glucose homeostasis, body weight and liver triglycerides was sustained up to 6 days after a single injection in ob/ob mice. This apparent “pharmacokinetic-pharmacodynamic disconnect” was also reported for N-terminally PEGylated FGF21, which reduced plasma glucose and triglycerides 6 days after the last of seven daily doses, despite having a half-life of 4 hrs (Huang et al., 2011). FGF21 regulates expression of genes controlling glucose and lipid metabolism in vivo. For example,
FGF21 administration in DIO mice increased expression of insulin and leptin receptors in liver and WAT, enhanced hepatic expression of fatty acid oxidation genes, suppressed hepatic expression of de novo lipogenesis pathway genes, and increased UCP1 expression in adipose tissues (Coskun et al., 2008; Xu et al., 2009a). CVX-343 not only induced a prolonged increase in UCP1 expression, but also sustained alterations in lipid metabolism genes (e.g., FAS and PGC-1α in WAT; PGC-1α and SCD1 in liver). These results demonstrate that CVX-343, like WT FGF21 protein, regulates lipogenic pathways at the transcriptional level, and that CVX-343-induced gene expression changes persist at least 72 hrs after the last dose. Together, the data suggest that the durable effects of CVX-343 may partially rely on sustained alterations at the transcriptional level following ligand binding and receptor signaling. In mice treated with CVX-343, insulin staining and proliferative state of β cells was increased, consistent with previous reports (Mu et al., 2012; Wu et al., 2011), suggesting that the anti-diabetic effects of FGF21 mimetics in vivo may be effected not only through liver and adipose but also by enhancing β-cell secretory capacity. FGF21 has recently been reported to regulate bone homeostasis and to mediate PPARγ actions in mice (Wei et al., 2012; Dutchak et al., 2012). We did not examine these endpoints in these studies, however, given the species differences in bone remodeling between rodent and human (Brommage, 2001; Turner, 2001), the potential impact of FGF21 and FGF21 mimetics on bone mass in humans requires clinical investigation.

In summary, we report the generation and characterization of a long acting FGF21 mimic, created by utilizing proprietary CovX technology to conjugate FGF21 protein to a scaffold antibody via a proprietary linker in a site-specific manner. FGF21-A129C CovX-Body is superior to the terminally linked molecules by merit of its WT FGF21 protein-comparable potency and extended half-lives. Preclinical studies demonstrate that CVX-343 achieved
efficacy indistinguishable from daily injected FGF21 protein with once weekly dosing in diabetic mouse models.
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Wrote or contributed to the writing of the manuscript: Huang, Ishino, Talukdar, Ogilvie, and Levin.
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Figure 1. Sequence of recombinant human des-His FGF21 (A129C) and schematic of the bivalent FGF21 CovX-Body, CVX-343. The underlined C residue corresponds to the 129 position where the FGF21 protein is conjugated to the antibody scaffold (A). IV (B) and SC (C) pharmacokinetic curves in mouse (n=3), rat (n=2), and monkey (n=2) following a single administration of CVX-343 (3 mg/kg).

Figure 2. CVX-343 demonstrates sustained efficacy in ob/ob mice 6 days after a single SC administration. WT FGF21 protein (1 mg/kg) was given SC daily. CVX-343 was given once on day 0 by SC injection. OGTT was conducted on day 6. Liver and WAT samples were collected on day 6. CVX-343 (3 mg/kg) significantly improves glucose tolerance 6 days post injection (A, B). CVX-343 (10 mg/kg) significantly reduces body weight gain (C), lowers liver triglycerides (D), and increases UCP1 expression in WAT (E) 6 days post injection. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. vehicle by One-way ANOVA with Dunnett’s post-hoc analyses (B, D, E) or two-way ANOVA with Bonferroni post-tests (C).

Figure 3. CVX-343 is efficacious in DIO mice. WT FGF21 protein (1 mg/kg) was given SC daily. CVX-343 (10 mg/kg) was given on day 0 and 7 by SC injection. OGTT was conducted on day 10. Repeat administration of CVX-343 significantly reduces body weight (A) without affecting food intake (B) and improves glucose tolerance (C, D) 3 days after the second dose. On day 11, serum, liver, and WAT tissue samples were collected and analyzed for triglycerides (E), NEFA levels (F), liver triglycerides (G), and UCP1 mRNA expression in WAT (H). * p <
0.05, ** p < 0.01, *** p < 0.001 vs. vehicle by One-way ANOVA with Dunnett’s post-hoc analyses (D - H) or two-way ANOVA with Bonferroni post-tests (A).

Figure 4. CVX-343 increases energy expenditure in DIO mice. CVX-343 (10 mg/kg) was given on day 0 and 7 by SC injection. Immediately following the second dose, mice were placed into the Columbus instruments cage monitoring system. Effects of CVX-343 on O$_2$ consumption (A), CO$_2$ production (B), RQ (C), heat production (D), and locomotor activity (E). * p < 0.05 vs. vehicle by Two-way ANOVA corrected for repeated measures.

Figure 5. CVX-343 increases pancreatic β-cell mass in db/db mice. CVX-343 (10 mg/kg) was given on day 0 and 7 by SC injection. OGTT was conducted on day 10. Pancreas was fixed on day 11. Repeat administration of CVX-343 significantly improves glucose tolerance (A, B) 3 days after the second dose. (C) Immunohistochemistry images of pancreatic β-cells (brown staining). (D) Quantification of β-cell mass. (E) Immunohistochemistry images of double staining for insulin (brown) and PCNA (blue). Arrows indicate the PCNA positive β-cells. (F) Quantification of β-cell proliferation. ** p < 0.01, *** p < 0.001 vs. vehicle by One-way ANOVA with Dunnett’s post-hoc analyses.
### TABLES

Table 1. *In vitro* activity of WT FGF21 protein and FGF21 CovX-Bodies in GLUT1 qPCR assay in 3T3-L1 adipocytes and their half-lives in mouse.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean EC$_{50}$ (nM) ± SD (N)</th>
<th>IV T$_{1/2}$ (hr)</th>
<th>SC T$_{1/2}$ (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF21 protein</td>
<td>2.9 ± 4.7 (26)</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>CovX-Bodies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linking position</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-terminus</td>
<td>5.9 ± 2.3 (2)</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>K56</td>
<td>11.8 ± 14.0 (2)</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>K59</td>
<td>6.5 ± 4.2 (2)</td>
<td>13</td>
<td>ND*</td>
</tr>
<tr>
<td>D79C</td>
<td>3.3 ± 3.4 (3)</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>K122</td>
<td>4.0 ± 5.7 (3)</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>H125C</td>
<td>3.0 ± 1.6 (4)</td>
<td>37</td>
<td>32</td>
</tr>
<tr>
<td>A129C</td>
<td>3.4 ± 2.8 (11)</td>
<td>28</td>
<td>37</td>
</tr>
<tr>
<td>C-terminus</td>
<td>&gt; 500 (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

“ND” = not determined
Table 2. Mean pharmacokinetics parameter estimates of CVX-343 following a single IV and SC administration (3 mg/kg) in mouse (n=3), rat (n=2), and monkey (n=2).

<table>
<thead>
<tr>
<th>Species</th>
<th>IV</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>t_{1/2} (hr)</td>
<td>AUC (μg·hr/mL)*</td>
<td>C_{max} (μg/mL)</td>
</tr>
<tr>
<td>Mouse</td>
<td>28</td>
<td>435</td>
</tr>
<tr>
<td>Rat</td>
<td>60</td>
<td>1383</td>
</tr>
<tr>
<td>Monkey</td>
<td>65</td>
<td>1272</td>
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</tbody>
</table>

* Estimation based on IV or SC curve of a 5-day time period post dose in the mouse study, a 14-day time period post dose in the rat study, and a 21-day time period post dose in the monkey study.
Figure 3

A

Cumulative BW change (g)

Time (Days)

B

Cumulative food intake (g)

Time (Days)

C

Glucose (mg/dL)

Time (min)

D

Glucose AUC (min·mg/dL)

E

Serum TG (mg/dL)

F

Serum NEFA (mmol/L)

G

Liver TG (mg/g of liver)

H

Lipo�RNA in WAT (fold change)