The Novel GlycoPEGylated FGF21 Analog Pegozafermin Activates Human FGF Receptors and Improves Metabolic and Liver Outcomes in Diabetic Monkeys and Healthy Human Volunteers

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Nonstandard Abbreviations: ALT, alanine aminotransferase; ANOVA, analysis of variance; BCA, bicinchoninic acid; BSA, bovine serum albumin; CV, cardiovascular; DMEM, Dulbecco's Modified Eagle medium; ECG, electrocardiogram; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR, FGF receptor; HbA1c, glycated hemoglobin; HDL-c, high-density lipoprotein-cholesterol; HMW, high molecular weight; kDa, kilodalton; LDL-c, low-density lipoprotein-cholesterol; MW, molecular weight; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OGTT, oral glucose tolerance test; PBS, phosphate buffered saline; PD, pharmacodynamic; PEG, polyethylene glycol; pERK, phospho ERK; PK, pharmacokinetic; q2w, once every two weeks; qw, once per week; rhFGF21, human recombinant F21; sc, subcutaneous; SHTG, severe hypertriglyceridemia; t1/2, pharmacokinetic half-life; T2DM, type 2 diabetes mellitus; US, United States; USA, United States of America; TEAE, treatment-emergent adverse event; VLDL, very low-density lipoprotein.

Recommended Section: Drug Discovery and Translational Medicine
ABSTRACT

Pegozafermin (also known as BIO89-100) is a glycoPEGylated analog of fibroblast growth factor 21 (FGF21) under development to treat nonalcoholic steatohepatitis (NASH) and severe hypertriglyceridemia (SHTG). In cell-based assays, pegozafermin had a similar receptor engagement profile as recombinant FGF21, with approximately 8-fold higher potency at FGF receptor 1c (FGFR1c). In diabetic monkeys, once-weekly and once-every-two-week regimens of subcutaneous pegozafermin provided rapid and robust benefits on an array of metabolic biomarkers, including triglycerides, cholesterol, fasting glucose, HbA1c, adiponectin, ALT, food intake, and body weight. In a single ascending dose study in healthy volunteers, subcutaneously administered pegozafermin was associated with statistically significant improvements in triglycerides, LDL-c and HDL-c, and adiponectin, an insulin-sensitizing and anti-inflammatory adipokine. Pharmacokinetic half-lives ranged from 55 to 100 hours over the clinically relevant dose range, consistent with the expected half-life extension by glycoPEGylation. These findings provide evidence that pegozafermin is a promising candidate molecule for the treatment of patients with NASH or SHTG.
SIGNIFICANCE STATEMENT

Fibroblast growth factor 21 (FGF21) is a stress-inducible hormone that has important roles in regulating energy balance and glucose and lipid homeostasis. Studies presented here demonstrate that a novel long-acting FGF21 analog, pegozafermin, has similar pharmacologic properties as FGF21 and that repeated, subcutaneous-dosing of pegozafermin in diabetic monkeys and healthy humans improves lipid metabolism, glucose metabolism, weight and liver transaminases. These results support future development of pegozafermin for the treatment of metabolic diseases, including nonalcoholic steatohepatitis and severe hypertriglyceridemia.
INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a progressive disorder characterized by the accumulation of excess fat in the liver (steatosis) in people who drink little or no alcohol (American College of Gastroenterology). With a worldwide prevalence approaching 25% (Younossi et al., 2016), NAFLD is the most common chronic disease of the liver (El-Kader, 2015). Components of the metabolic syndrome (Eckel et al., 2005), e.g., obesity and type 2 diabetes mellitus (T2DM), are the most salient risk factors for NAFLD (El-Kader, 2015; Chaitanya Thandra et al., 2020). NAFLD has been shown in numerous studies to increase the risk of cardiovascular (CV) events, such as myocardial infarction, stroke, revascularization or death (Deprince et al., 2020).

NAFLD encompasses a wide spectrum of liver pathologies ranging from isolated hepatic steatosis to non-alcoholic steatohepatitis (NASH), in which steatosis is combined with hepatocyte ballooning and inflammation (Carr et al., 2016). NASH, in turn, can progress to cirrhosis and its complications, including hepatocellular carcinoma (Calzadilla Bertot and Adams, 2016; Lindenmeyer and McCullough, 2018). In the US, it is estimated that approximately 20% of adults with NAFLD have NASH, i.e., 3–12% of the total population (Spengler and Loomba, 2015), while the number of adults with NASH awaiting liver transplants almost tripled between 2004 and 2013 (Wong et al., 2015).

Progression of simple fatty liver to NASH and NASH to cirrhosis takes many years. Although the pathogenetic mechanisms underlying this progression remain poorly understood, numerous lines of evidence implicate alterations in hepatic and extra-hepatic lipid metabolism as central drivers (Deprince et al., 2020). Consistent with this hypothesis, severe hypertriglyceridemia (SHTG), a condition identified by triglyceride levels ≥ 500 mg/dL, is associated with an increased risk of NASH (National Cholesterol Education Program Expert Panel on Detection
and Treatment of High Blood Cholesterol in, 2002). In Western populations, SHTG has an estimated prevalence rate of 0.1–0.13 (roughly 3.4 million people in the US) (Christian et al., 2011; Retterstol et al., 2017). In addition to hepatic steatosis, SHTG is associated with other comorbidities, including pancreatitis, metabolic syndrome, hypertension, T2DM, coronary heart disease, hyperthyroidism, kidney disease and alcoholism (Retterstol et al., 2017).

Multiple new agents are under investigation for the treatment of NASH and SHTG. Among the most promising are fibroblast growth factor 21 (FGF21) analogs (Talukdar and Kharitonenkov, 2020; Tillman and Rolph, 2020). In vivo, native FGF21 is distinguished in part from other FGF family members by its ability to act in both an endocrine and paracrine fashion (Wu et al., 2010; Itoh et al., 2016). As a result, FGF21 is thought to play a key role in integrating metabolism across multiple organs, including liver, adipose tissue, skeletal muscle and the pancreas. At the molecular level, FGF21 acts through a heterodimeric receptor complex consisting of one of three FGF receptors, FGFR1c–3c, and a co-receptor, β-Klotho. Activation of these receptor complexes mediates interaction with cytosolic adaptor proteins and the RAS-MAPK, PI3K-AKT, PLCγ, and STAT intracellular signaling pathways (Ornitz and Itoh, 2015). In preclinical studies, administration of recombinant FGF21 in animal models of T2DM and NASH lowered blood glucose, increased insulin sensitivity, and reduced liver triglyceride content and steatosis (Kharitonenkov et al., 2005; Coskun et al., 2008; Xu et al., 2009; Zhang et al., 2012). FGF21 administration also improved lipid profiles in mice and monkeys, dramatically reducing triglycerides and LDL-c, increasing HDL-c, and reducing body weight via lipid catabolism in brown and white adipose tissue (Kharitonenkov et al., 2007; Schlein et al., 2016).

Native endogenous FGF21 is unsuitable for clinical use owing to poor pharmacokinetic (PK) properties, notably a short half-life (< 2 hours) (Geng et al., 2020). Several strategies to improve its PK profile have been investigated. One of these, polyethylene glycol (PEG) modification, was reported to show benefit in animal models of metabolic disease (Huang et al., 2011; Mu et al.,
2012). These observations led to the design of a novel glycoPEGylated FGF21 analog, referred to as BIO89-100 or pegozafermin, which carries two modifications that protect the molecule from rapid degradation in vivo: a 20 kDa linear PEG covalently attached via a glycosyl moiety; and an N-terminal methionine residue (Zhen et al., 2016). In this report, we describe the pharmacology, pharmacodynamics (PD), PK and safety/tolerability profile of pegozafermin in three separate systems: a cell-based assay, a nonhuman primate model (cynomolgus diabetic monkeys), and healthy human volunteers. We further describe population PK and PD models in diabetic monkeys, which support optimal dosing regimens for pegozafermin in future clinical trials.
MATERIALS AND METHODS

Cell Line Studies

Generation of β-Klotho/FGFR-overexpressing cell lines

Rat L6 myogenic cells were purchased from the American Type Culture Collection (CRL1458) and cultured in Dulbecco’s Modified Eagle medium (DMEM; 11965092, Life Technologies) supplemented with 10% fetal bovine serum (10270-106, Life Technologies) and 1% penicillin/streptomycin (15140-122, Life Technologies). Cells were cultured at 37°C under 5% CO₂, with media replenished every 48 hours.

Forty-eight hours before transduction, L6 cells were seeded into a 6-well cell-culture plates at a density of 25 × 10⁴ cells per plate. On the day of transduction, cells were incubated with 30 µL of lentivirus particles (multiplicity of infection = 2) previously thawed at 37°C. Transduction was carried out in the presence of 8 µg/mL hexadimethrine bromide (Polybrene, Sigma-Aldrich). Twenty-four hours after transduction, lentiviral particles-containing medium was replaced with fresh cell-culture medium containing 5 µg/mL puromycin. Cells were cultured for 7 days, then polyclonal populations formed were pooled together, re-seeded for cell expansion and maintained in selection with 10 µg/mL puromycin. Polyclonal cell lines were expanded and characterized, and cell banks were generated and stored under liquid nitrogen. Cells were frozen at 5 × 10⁶ cells/vial in 1 mL of 90% fetal bovine serum and 10% dimethyl sulfoxide (DMSO).

Culturing of β-Klotho/FGFR-overexpressing cell lines

Cryopreserved vials of β-Klotho/FGFR-overexpressing cells were thawed by a brief immersion in a 37°C water bath and immediately resuspended in 10 mL of L6 complete medium containing 10 µg/ml puromycin. Cells were centrifuged at 200g for 5 minutes at 4°C, and the pellet was gently resuspended in 10 mL of complete medium for L6 cells containing 10 µg/mL puromycin.
and transferred into a 60 mm cell culture dish. Cells were cultured at 37°C under 5% CO₂, with media replenished every 48 hours. Every 4 days, when cells reached approximately 90% confluency, culture medium was removed, cells were rinsed twice in ice-cold PBS and incubated for 10 minutes at 37°C under 5% CO₂ in 1 mL of Accutase solution (A6964, Sigma). Cells were then resuspended with 9 mL of L6 complete medium containing 10 µg/mL puromycin and subcultured in a new 60 mm cell culture dish with a 1:20 subcultivation ratio. Cell count and cell viability measurement were performed at every passage by Trypan Blue (T6146, Sigma) exclusion test.

**Functional phosphoERK assay**

PhosphoERK (pERK) activity was determined using the Phospho/Total ERK1/2 Whole Cell Lysate Kit from Meso Scale Diagnostics (MSD; Rockville, MD, USA). Specifically, L6 cells overexpressing β-Klotho ± FGFRs were seeded on 96-well plates at 10 × 10^3 cells/well and maintained in culture in a humidified incubator at 37°C and 5% CO₂ for two days without changes of the culture medium. Forty-eight hours later, culture medium was changed to DMEM-serum free (starvation medium) and culturing was continued for 2 hours. During the starvation period, ligand stock dilutions (100X) were prepared in 0.5 mL Eppendorf tubes in PBS, pH 7.4, containing 0.1% BSA. Two microliters (2 µL) of each stock dilution were added to 200 µL of assay medium (HBSS pH 7.4-Hepes 10 mM containing 0.1% BSA) in a well of polypropylene 96-well plate (round bottom, Corning 3365), in triplicates.

Following starvation, cells were washed two times in assay buffer, and 100 µL of ligand-containing assay buffer were transferred onto the cells using a multi-channel pipette. Treated cells were incubated for 5 minutes at 37°C. Reactions were stopped by transferring the plates on ice, and cells were washed two times with ice-cold PBS and lysed in MSD lysis buffer, according to the manufacturer’s protocol (K15107D-1, Meso Scale Discovery, Newark, DE, USA). One replicate of samples was employed for protein content determination by the
bicinchoninic acid (BCA) method. Concentration of samples were adjusted based on BCA results and samples were processed for pERK levels following the manufacturer’s protocol.

All procedures for preparation of dilution curves, cell stimulation and sample processing were performed using tubes, pipette tips and plates previously coated overnight with 1% BSA/PBS to prevent aspecific absorption of samples to plastic material. The molar concentration of pegozafermin used in the MSD assay was calculated based on a MW of 19.5 kDa.

Animal Studies

Animal Care

Spontaneously diabetic cynomolgus monkeys were housed at an environmental temperature of 23 ± 3°C and humidity of 50 ± 20%, with a 12-hour light/12-hour dark photoperiod. All animals had free access to water and were fed twice daily with a complete, nutritionally balanced diet (Beijing Keao Xieli Feed Co., Ltd., Beijing, China) enriched with seasonal fruits.

The diabetic monkey studies were conducted by Crown Bioscience (Taicang, Jiangsu Province, P.R. China). Crown Bioscience institutional animal care and use committee approved all animal procedures. All procedures related to handling, care and treatment of the animals were performed according to guidelines approved by the Association for Assessment and Accreditation of Laboratory Animal Care. After each treatment (weighing, bleeding or dosing), the animals were carefully observed until they were able to stand up and were alert. At the time of routine monitoring, the animals were checked for any effects of the compound on their behaviors, such as mobility, food and water intake, body weight changes and any other abnormal effects.

Single Dose Study

Twelve spontaneously diabetic cynomolgus monkeys 13–20 years old, with baseline blood glucose levels of 120–200 mg/dL and no prior FGF21 analog treatment, were randomly
assigned to three treatment groups: pegazafermin 2 mg/kg administered subcutaneously (n=4 males and 2 females); pegazafermin 2 mg/kg administered intravenously (n=2 males and 1 female); and vehicle administered subcutaneously (n=2 males and 1 female). PK assessments were conducted on blood samples collected immediately before dosing and 0.083, 0.5, 1, 3, 6, 9, 24, 48, 72, 96, 120, 168, 264, 336, 504, 672 and 840 hours after dosing. Each blood sample (2 mL/ per time point) was drawn from the cephalic or saphenous vein into BD Serum Separator Vacutainer® Tubes, allowed to clot for a minimum of 30 minutes at room temperature, and then centrifuged at 3500 rpm at 4°C for 10 minutes. Serum was transferred into polypropylene screw-cap vials and immediately stored at –80°C for subsequent analysis.

**Multiple Dose Study 1**

Thirty diabetic monkeys were screened, of which 24 were selected for inclusion in the study. The study population was 6–28 years old, had baseline blood glucose levels of 61–345 mg/dL, and had not received prior FGF21 analog treatment. Eligible experimental animals were stratified by endogenous FGF21 levels at baseline and by gender (5 males and 1 female per treatment group) to ensure balance and then randomized into four treatment groups. Animals in the four treatment groups received 8 qw administrations of either pegazafermin 0.1 mg/kg, pegazafermin 0.3 mg/kg, pegazafermin 1.0 mg/kg, or vehicle. Study treatments were administered by subcutaneous injections after overnight fasts. Seven days after the last study treatment (day 57), experimental animals entered a 41-day washout period for subsequent follow up.

PK assessments were conducted on blood samples collected immediately before dosing and 6, 24, 48, 72, 120 and 168 hours after dosing on day 1 and day 49; three additional PK samples were collected on day 21 (predose), 35 (predose) and 63.
**Multiple Dose Study 2**

Handling, screening, and randomization of experimental diabetic monkeys into 4 treatment groups (n=5 males and 1 female per group) were performed as described in Multiple Dose Study 1. The study population was 11–26 years old, had baseline blood glucose levels of 71–351 mg/dL, and had not received prior FGF21 analog treatment. The first and second treatment groups received five once-weekly administrations of vehicle or pegozafermin 1.0 mg/kg, respectively. The third and fourth treatment groups received three administrations of pegozafermin 1.0 mg/kg once every two weeks (q2w) or pegozafermin 2.0 mg/kg q2w, respectively. Study treatments were administered by subcutaneous injections after overnight fasts. On day 28, experimental animals entered a 2-week washout period for subsequent follow up.

PK assessments were conducted on blood samples collected immediately before dosing and 6, 24, 48, 72, 120, and 168 hours after dosing on day 1 and day 28; four additional PK samples were collected on day 14 (predose), 42 and 43. PD assessments (with the exception of oral glucose tolerance tests [OGTTs]) were conducted on blood samples collected on day –14, –1, 0, 7, 14, 21, 28, 35 and 42. OGTTs were conducted on day –1, 28 and 42.

**Assessments**

**Pegozafermin assays.** Quantitation of pegozafermin in the Single-Dose Study used a commercially available kit for the measurement of wild-type FGF21 (Biovendor, Human FGF21 ELISA, Catalog # RD191108200R). A more specific method for the quantitation of pegozafermin in monkey serum for the Multiple Dose Study 1 and 2 used a colorimetric ELISA with anti-human FGF21 as capture antibody and anti-PEG as detection antibody; the lower limit of quantitation was 1.6 ng/mL.
Blood chemistry. Serum chemistry and insulin levels were measured on an ADVIA 2400 Chemistry System (SIEMENS) and an ADVIA Centaur XP Immunoassay System (SIEMENS), respectively, using blood samples collected on day –2, –1, 0, 3, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84 and 91. HbA1c was measured on a Glycated Hemoglobin Test System (BIO-RAD, Hercules, CA, USA), using blood samples collected on day –2, 0, 14, 28, 42, 56, 70 and 84. OGTTs were performed on day –11, 31 and 52, using tail venipuncture samples collected 0, 15, 30, 60, 120 and 180 minutes after a meal of 1.0 g/kg glucose + 10 g/kg banana. Blood glucose levels in the OGTTs were assessed using an Accu-Chek Performa blood glucose meter (Roche, Bern, Switzerland).

Adiponectin quantitative sandwich enzyme immunoassay. Blood samples for adiponectin analysis were collected in 4 mL serum separator tubes and gently inverted 8–10 times. Samples were kept at room temperature and allowed to clot for approximately 30 minutes after collection. Samples were then centrifuged at 1200g for 10 minutes at approximately 4°C. The resulting serum was harvested and transferred in approximately equal amounts into two appropriately labeled 3 mL polypropylene screw-cap tubes and placed on wet ice until freezing. Samples were frozen in an upright position at approximately –70°C within 90 minutes of blood collection.

To quantify adiponectin, study samples, standards and quality controls were thawed and then pipetted into the microplate wells pre-coated with monoclonal antibody specific for HMW adiponectin. Following an incubation period to allow adiponectin to bind to the immobilized antibody, unbound substances were washed away, and an enzyme-linked monoclonal antibody specific for HMW adiponectin was added to the wells. Following another wash, a substrate solution was added to the wells, and color development was measured by absorbance at 450 nm, with a 570-nm reading subtracted for background correction. The method was developed to assess an in-well concentration of 2–250 ng HMW adiponectin per mL of prepared serum, with a minimum required dilution of 1:100. Standard curves were generated for each
plate using a 5-parameter logistic (5PL) model. Accuracy of the calibration standards ranged from 99 to 101%. Inter-assay precision (%CV) ranged from 0.6 to 1.8%; inter-assay precision of study samples ranged from 8.8 to 11.1%.

Phase I Single Ascending Dose Study in Humans

The human phase I study was supported by 89bio (San Francisco, CA, USA) and was conducted at a single study site (World Wide Clinical Trials: Early Phase Services, LLC [San Antonio, TX, USA]) in compliance with International Conference on Harmonization and Good Clinical Practice guidelines for conducting, recording and reporting clinical trials. Written consent was obtained from each participant. The informed consent form, protocol and amendments for the study were approved by the institutional review board at the study site.

The phase I study population included male and female adults 21–55 years of age, with body mass indices from 18.5–32 kg/m². Women were included only if they were sterile or postmenopausal. All subjects were in good health, as determined by medical history, ECG, physical examination findings, vital signs and clinical laboratory tests (serum chemistry, hematology, coagulation and urinalysis). Key exclusion criteria included: any significant chronic disease; any medical condition requiring chronic maintenance treatment; any malignancy diagnosis (excluding basal cell carcinoma); major trauma or surgery in the prior 2 months; acute/subacute infection or treatment with antibiotics within the prior 4 weeks; history of alcohol, drug or other substance dependence (with the exception of caffeine); and a history of smoking within the last 6 months.

The trial consisted of a Pretreatment Period from day –28 to day –1; a Treatment Period from day 1 (pegazafermin administration) to day 8; and three post-treatment clinic visits on day 15, day 22 and day 29 (End of Study Visit). Subjects remained in the investigational center from the evening on day –1 (check-in) until the morning of day 8 and then returned for the three post-
treatment visits. Following screening and check-in, eligible subjects were assigned to sequential
dosing cohorts. Subjects within each cohort were randomized to receive single, subcutaneous,
abdominal injections of pegozafermin (0.45, 1.2, 3.0, 9.1, 18.2, 39.0 and 78.0 mg [n=6 per
cohort except for the 9.1 mg dose, where n=7]) or placebo (n=2 per cohort except for the 9.1 mg
dose, where n=3).

To assess the PK of pegozafermin, blood samples (3 mL) were collected within 1 hour prior to
dosing and at 1, 2, 4, 6, 12, 18, 24, 30, 36, 48, 60, 72, 96, 120, 144, 168, 336, 504 and 672
hours postdose. Quantitation of pegozafermin in serum used an electrochemiluminescence
immunoassay (Cheng et al., 2012), with anti-human FGF21 as capture antibody and anti-PEG
as detection antibody; the lower limit of quantitation was 10 ng/mL. Noncompartmental PK
analysis was performed using Phoenix™ WinNonlin® (Version 8.1, Certara, L.P.) in conjunction
with the internet-accessible implementation of Pharsight® Knowledgebase Server™ (PKSO;
Version 4.0.4, Certara, L.P.).

PD assessments in the study included the following: triglycerides, total cholesterol, LDL-c, HDL-
c and VLDL (in blood samples collected predose on day 1 under fasted conditions and on day 8,
day 15, day 22, and day 29); blood glucose and insulin (fasting predose day 1, days 1–8, and
day 29; adiponectin (fasting predose day 1, day 8 and day 29); and endogenous FGF21
(predose day 1 and day 29). PD assessments were carried out as described above in the
animal studies.

Safety was monitored throughout the study by adverse events (including severity, seriousness,
and relatedness to study treatment) according to the current version of the National Cancer
Institute Common Terminology Criteria for Adverse Events (NCI CTCAE). In addition, safety
was monitored throughout the study by clinical laboratory tests, vital signs, physical
examination, ECG measurements, concomitant medication usage, and immunogenicity. Local
tolerability at the injection site (e.g., erythema, induration, ecchymosis, and pain) was evaluated during the first 24 hours post-dose.

**Statistical Analysis**

In the cell-based assays, data were reported as standard descriptive statistics.

In the diabetic monkey studies, mean and standard error (SE) of the experimental parameters (e.g., triglyceride levels, blood glucose, etc.) were calculated within each group. Statistical analyses were performed based on the baseline value at time zero by two-way analysis of variance (ANOVA), with repeated measures, utilizing PASW Statistics 18 (IBM SPSS Statistics, New York, NY, USA). *P*-values less than 0.05 were considered statistically significant. Monkey and human PK analyses were performed using noncompartmental analysis.

The phase I, single-ascending dose study was designed to assess safety and pharmacokinetics of pegozafermin and did not evaluate any formal hypothesis. The cohort size for each of the groups was chosen to be adequate to attain the study objectives. Dropouts and/or early withdrawals were not replaced. Results were analyzed by standard descriptive statistics using SAS software, version 9.4 or higher (SAS, Cary, NC, USA).
RESULTS

Pegozafermin Activated FGFR1c, FGFR2c and FGFR3c, but not FGFR4 in Cell-Based Assays

Isolated rat L6 cells expressing human FGFR1c/β-Klotho, FGFR2c/β-Klotho, FGFR3c/β-Klotho, FGFR4/β-Klotho or β-Klotho alone were treated in vitro with pegozafermin or human recombinant FGF21 (rhFGF21), and receptor-mediated activity was assessed by intracellular ERK phosphorylation levels (see Methods). Overall, the receptor engagement profiles of pegozafermin and rhFGF21 were highly comparable (Figure 1). Similar to rhFGF21, pegozafermin elicited pERK phosphorylation at low nanomolar concentrations in cells expressing FGFR1c/β-Klotho, FGFR2c/β-Klotho or FGFR3c/β-Klotho, but not in cells expressing FGFR4/β-Klotho or β-Klotho alone. As a positive control, FGF19 fully activated pERK in the same FGFR4/β-Klotho cell line, with an EC$_{50}$ of 1.07 ± 0.4 nM. The stabilizing modifications (i.e., PEGylation) introduced into FGF21 to create pegozafermin did not appear to adversely affect potency: in the cell-based assay, pegozafermin had approximately 8-fold greater potency than rhFGF21 at FGFR1c (EC$_{50}$, 0.6 ± 15 nM vs. 4.5 ± 1 nM) and approximately the same potency at FGFR2c and FGFR3c.

Pegozafermin Improved Metabolic Parameters in Diabetic Monkeys

Diabetic monkeys were randomly assigned to receive subcutaneous once-weekly (qw) injections of 0.1 mg/kg pegozafermin (n=6), 0.3 mg/kg pegozafermin (n=6), 1.0 mg/kg pegozafermin (n=6) or vehicle (n=6) for 8 weeks (Multiple Dose Study 1; see Methods). Treatment with pegozafermin had beneficial effects on multiple metabolic parameters (Figure 2), including triglycerides, total cholesterol (data on file), HDL-c and LDL-c. The improvements were induced rapidly and maintained throughout the treatment period, and reversed post treatment (> day 56). In general, the treatment group receiving the highest dose of pegozafermin (1.0 mg/kg) experienced the greatest benefit, although a dose response
relationship was less consistently apparent at the two lower dose groups. Pegozafermin also reversibly and robustly lowered fasting blood glucose and HbA1c levels (Figure 2). The improved glycemic profile was associated with lower fasting serum insulin levels and improved oral glucose tolerance (ie., Compared to Day -11 AUCglucose (mg/dl*min), Day 31 values were lower (P<0.05) for all treatment groups and Day 52 values were lower (P<0.05) for 0.3 and 1 mg/kg groups), suggesting the decline in blood sugar was attributable, at least in part, to increased insulin sensitivity. Pegozafermin treatment was not associated with significant alterations in mean blood pressure or heart rate in any of the pegozafermin treatment groups (Figure 2).

In addition to improving dyslipidemia and hyperglycemia, pegozafermin had beneficial effects on body weight in this nonhuman primate model of diabetes. The group receiving the highest dose (1.0 mg/kg) experienced a robust, statistically significant reduction in mean body weight, with a maximal effect of 9.3% at the end of the treatment phase, although the low pegozafermin dose groups (0.1 mg/kg and 0.3 mg/kg) exhibited relatively minor weight loss across the treatment period (Figure 2). The decreases observed in body weight were associated with reductions in food intake: within 4 days of the first subcutaneous injection of pegozafermin, animals decreased food intake by approximately 40% in the 1.0 mg/kg dose group and approximately 20% in the 0.3 mg/kg and 0.1 mg/kg dose groups. None of these effects were associated with obvious signs of gastrointestinal distress, such as vomiting or diarrhea.

Adiponectin, an insulin-sensitizing and anti-inflammatory adipokine, has been shown to decline in concentration in obesity-associated metabolic and vascular disorders (Fasshauer et al., 2004). Notably, adiponectin levels decline in patients with NAFLD, and especially NASH, which has led to the proposal that low adiponectin levels may play a role in the pathophysiological progression from NAFLD to NASH (Pagano et al., 2005; Polyzos et al., 2010; Polyzos et al., 2011). In the current study, pegozafermin substantially increased adiponectin levels throughout
the treatment period (Figure 2). In the pegozafermin 0.1 mg/kg and the pegozafermin 0.3 mg/kg treatment groups, which exhibited relatively limited reductions in body weight, adiponectin levels rose approximately 1- to 2-fold across the treatment period. In the pegozafermin 1.0 mg/kg group, adiponectin levels increased nearly 6-fold, paralleling the robust effects on insulin sensitization and glycemic control in these animals. As further evidence of potentially beneficial hepatic effects, ALT levels, a common biomarker of liver damage, declined following pegozafermin treatment.

In a second preclinical study (Multiple Dose study 2; see Methods), diabetic monkeys were randomly assigned to four treatment groups (n=6 per group): 1.0 mg/kg pegozafermin once per 2 weeks (q2w) sc for 5 weeks; 2.0 mg/kg pegozafermin q2w sc for 5 weeks; 1.0 mg/kg pegozafermin qw sc for 5 weeks; and vehicle qw sc for 5 weeks. Overall, the metabolic effects observed in this study were consistent with those observed in the first multiple dose study (see above), confirming the robust effects of pegozafermin on a wide variety of metabolic parameters (see Supplement, Figure S1). Similar to the prior study, treatment with pegozafermin led to a sharp decrease in mean serum triglyceride levels following the first dose, which remained low throughout the treatment phase. In all dose groups, percent changes from baseline in mean serum triglycerides were highly significant \((P \leq 0.01)\) at least one timepoint during the treatment phase. By contrast, there was no obvious change in triglyceride levels throughout the treatment period in the vehicle group. In general, pharmacodynamic (PD) effects tended to be more robust following treatment with the 1.0 mg/kg qw dose than the 1 mg/kg q2w dose and 2.0 mg/kg q2w dose.

**Translational Pharmacokinetic/Pharmacodynamic Modeling in Diabetic Monkeys**

To quantify the relationship between pegozafermin PK and PD markers of target engagement (e.g., triglyceride blood levels), a population PK model was developed based on data from
45 test animals treated with pegozafermin (9 from a single dose study and 36 from the two multiple-dose studies described above) [see Supplement, Table S1]. Extrapolation of this PK/PD model was then applied to support phase 1 dose selection.

Pegozafermin pharmacokinetics were best described by a three-compartment model (see Supplement, Figure S2), with parallel first-order absorption rate constants (\(k_{a1}\) and \(k_{a2}\)) and a lag time (\(t_{lag}\)) on the second absorption. PD parameters were described by in-direct response models (see Supplement, Figure S3). Both PK and PD model adequately described the data (see Supplement, Figure S4 and S5). The population estimate of clearance (CL) from the central compartment for pegozafermin was 24.3 mL/hour. Central volume of distribution (\(V_c\)) was 269 mL. In non-human primates, bioavailability of pegozafermin following subcutaneous administration was estimated at 69.4%. The estimated EC\(_{50}\) value for triglyceride lowering was 128 ng/mL, close to the average clinical concentrations from 3 to 9.1 mg for weekly dosing, based on the clinical PK results described in this report.

To enable prediction of human doses that would produce clinically meaningful PD responses, the diabetic monkey PD models were linked to a humanized PK model (i.e., based on allometric scaling), and the impact of the various pegozafermin dosing regimens on triglyceride level was assessed. In humans, near maximal response was predicted with a dose of around 27–30 mg qw. Both qw and q2w regimens were predicted to elicit clinically meaningful PD effects (simulation data not shown).

**Pegozafermin in Healthy Adults: Phase I Single Ascending Dose Study**

*Disposition and Demographic Characteristics*

In total, 58 healthy adults were randomized to study treatment (pegozafermin, \(n=43\); placebo, \(n=15\)). Among those who were dosed with pegozafermin, 6 received 0.45 mg, 6 received 1.2 mg, 6 received 3 mg, 7 received 9.1 mg, 6 received 18.2 mg, 6 received 39 mg, and
6 received 78 mg. All subjects completed the study, apart from one subject in the pegozafermin 1.2 mg cohort, who did not return for the end-of-study visit and thus withdrew on day 35. No stopping criteria were met in the study. Of the 58 enrolled subjects, 50 (86.2%) were male and 8 (13.8%) were female (4 [50%] post-menopausal and 4 [50%] surgically sterile) (see Supplement, Table S2). Ages in the study population ranged from 21 to 55 years, and ethnicity was 43.1% and 56.9% Hispanic/Latino and Not Hispanic/Latino, respectively. BMI ranged from 18.8 to 31.9 kg/m².

**Pharmacokinetics**

Pegozafermin serum concentration profiles peaked at 36 to 60 hours post dose (Figure 3). Exposures (AUC and C\(_{\text{max}}\)) were generally dose-proportional or slightly more than dose-proportional (see Supplement, Table S3). Half-life (t\(_{1/2}\)) values for the most clinically relevant dose range between 3 and 39 mg ranged from approximately 55 to 100 hours.

**Pharmacodynamic Effects**

Mean baseline levels of serum lipids, glucose and insulin were within normal ranges in the healthy volunteers (see Supplement, Table S2). Despite baseline lipid values falling within the normal limits, robust and durable effects on serum triglyceride (up to 51% reduction), HDL-c (up to 36% increase), and LDL-c (up to 37% reduction) were observed at single dose levels of 9.1 mg or higher (Figure 4). The effects were apparent by day 8 or earlier, and persisted until day 15 for most subjects, suggesting qw or q2w regimens are feasible. Dose-dependent trends were evident up to a dosage of 39 mg, without further effect in the 78 mg group. Therefore, the E\(_{\text{max}}\) dose was between 18 mg and 39 mg. A significant and long-lasting effect on serum adiponectin was observed by day 8 and persisted until day 29 at dose 9.1 mg or higher. There was no notable effect on serum glucose or insulin levels or on body weight following single administrations of pegozafermin at all studied doses.
Safety

The most frequently reported treatment-emergent adverse events (TEAEs) in the study were injection site induration (7 [16.3%] subjects on pegozafermin and 1 [6.7%] on placebo), injection site erythema (5 [11.6%] subjects on pegozafermin and 1 [6.7%] on placebo), and headache (5 [11.6%] subjects on pegozafermin and 1 [6.7%] on placebo) (Table 1). The majority of TEAEs were Grade 1. Two Grade 2 TEAEs were reported: headache in 1 subject who received pegozafermin 9.1 mg (0–2 days after dosing); and pyrexia in 1 subject who received placebo. One Grade 3 event, increased creatinine kinase, was reported in one subject who received pegozafermin 3 mg dose. The elevation in creatine kinase was observed in a subject who reported performing physical labor prior to testing, resolved without further treatment, and was assessed to be unrelated to study treatment. No Grade 4 or 5 TEAEs were reported. No liver-related stopping criteria were met in the study.

The most common treatment-related TEAEs were injection site reactions, which occurred more frequently in the high dose groups (39 mg and 78 mg) and which were all considered mild in severity (Grade 1). Apart from injection site reactions, the incidence of treatment-related TEAEs, as well as the incidence of all TEAEs, did not differ between cohorts. No clinically significant abnormalities in ECGs or out-of-range vital signs were reported. Low titer (≤ 16) anti-drug antibodies were documented in 5/43 subjects; no evidence was found that these antibodies impacted safety, PK parameters or prespecified exploratory biomarker analyses.
DISCUSSION

This report provides translational aspects of description and characterization of pegozafermin, a novel glycoPEGylated FGF21 analog. Three general lines of evidence indicate that pegozafermin may have considerable potential as a therapeutic for the treatment of NASH and SHTG. First, in cell-based assays, pegozafermin faithfully recapitulated the pharmacologic properties of rhFGF21. Specifically, pegozafermin elicited phosphorylation of ERK at low nanomolar concentrations in cells expressing FGFR1c/β-Klotho, FGFR2c/β-Klotho or FGFR3c/β-Klotho, but not in cells expressing FGFR4/β-Klotho or β-Klotho alone. This activation pattern was similar to rhFGF21 and different from FGF19. Although pegozafermin and rhFGF21 had similar potencies at FGFR2c and FGFR3c, pegozafermin had approximately 8-fold higher potency at FGFR1c, which might translate into higher clinical efficacy as well, since FGFRc1 in adipose tissue and other visceral organs appears to be the most relevant mediator of metabolic effects. Furthermore, the lack of activity of pegozafermin at FGFR4 may be promising for its safety profile, as FGFR4 is associated with bile acid metabolism, hepatocyte proliferation and NASH-related hepatocellular carcinoma (Adams et al., 2012; Yoo et al., 2017).

The second line of evidence supporting pegozafermin was its robust and beneficial effects on an array of metabolic parameters. In a nonhuman primate model of diabetes, pegozafermin improved serum triglycerides, serum cholesterol, blood glucose, insulin, insulin sensitivity, food intake, and body weight in a rapid and reversible fashion. In healthy human volunteers, pegozafermin treatment also resulted in statistically significant improvements in serum triglycerides, LDL-c and HDL-c. It will be of significant interest in the future to determine whether pegozafermin also improves blood glucose and weight in nonhealthy (e.g., NASH, SHTG, obese, diabetic, etc.) patient populations, all of which might be more sensitive to anti-glycemic and anti-obesity pharmacotherapies than the healthy study population examined in the current report.
Consistent with FGF21 and other related analogs, pegoverakin also significantly increased adiponectin levels in both the diabetic monkey system and healthy human volunteers. Such elevations have been demonstrated to impart significant metabolic benefits (Holland et al., 2013; Hui et al., 2016; Talukdar et al., 2016; Bondurant et al., 2017). Increased adiponectin is thought to benefit the liver by directly inhibiting atherogenic LDL-c uptake in endothelial cells, inhibiting pro-inflammatory signaling pathways and cytokine production, inhibiting the pro-fibrotic program, and both inhibiting de novo lipogenesis and increasing lipid oxidation (Kakino et al., 2020). It is thus noteworthy that in addition to the increased levels in adiponectin, results from the diabetic monkey studies indicated that treatment with pegoverakin generally decreased blood ALT levels, a clinically relevant signal of liver damage.

Understanding how pegoverakin and other FGF21 analogs produce such beneficial and broad-based metabolic benefits is an area of ongoing active investigation. It is known, for instance, that physiological regulation of FGF21 may function as a compensatory mechanism to raise the metabolic rate in response to the initial insult of hypercaloric diet (Fisher et al., 2010; Markan et al., 2017; Geng et al., 2020). Thus, the supraphysiological levels of FGF21 achieved following pegoverakin administration may act to persistently raise the metabolic rate, thereby leading to changes in energy utilization and the attendant improvements in metabolic parameters.

The third line of evidence supporting the therapeutic potential of pegoverakin in NASH and SHTG was the demonstration of its favorable safety and PK profiles. In healthy volunteers, single subcutaneous doses of pegoverakin appeared to be well tolerated and safe, with mild self-limiting injection site reactions comprising the bulk of relevant TEAEs. Pegoverakin showed no effects on heart rate or blood pressure in either diabetic monkeys or healthy human volunteers, in contrast to another FGF21 analog, PF-05231023 (Greenhill, 2018). Regarding its PK properties, pegoverakin exposure in the phase I study was approximately dose proportional. Over the most clinically relevant dose range (3–39 mg), $t_{1/2}$ was approximately 55–
100 hours. In general, based on a combined assessment of the PD, PK and safety profiles in this study, once-weekly or once-per-two-weeks dosing of pegozafemin may be feasible. Predictions from the translational population PK/PD model developed with data from diabetic monkeys are largely consistent with the observed \( E_{\text{max}} \) dose in the phase 1 study, with the possibility of either qw or q2w regimens eliciting clinically meaningful effects.

A potential limitation to the described animal study may be some constraints in the ability to extrapolate from non-human primates to humans. For example, in our studies with diabetic monkeys, reduced food intake associated with decrease in body weight was observed with repeated administration of pegozafemin. However, in a recent study in patients with NASH, mild increase in appetite was seen in a small proportion (16%) of subjects treated with pegozafemin, with no meaningful changes in body weight (Loomba et al., 2023). Different observations on the effect of FGF21 on food intake and body weight have been reported across species, in the literature. Reduced food intake was reported in studies with mice (Santoso et al., 2017), obese minipig model (Christoffersen et al., 2019), zebrafish (Blanco et al., 2020), and in non-human primates (Talukdar et al., 2016; Thompson et al., 2016), whereas no food effects were seen in studies with diet-induced obese mice (Xu et al., 2009) or obese rats (Coskun et al., 2008). In other studies, FGF21 resulted in increased food intake (Sarruf et al., 2010; Tillman et al., 2022). In high-fat fed obese rhesus macaque monkey, FGF21 resulted in decreased body weight but not food intake (Andersen et al., 2018) and in obese human subjects, FGF21 elicited a reduction in preferences for carbohydrates (Baruch et al., 2020). While several factors including experimental setting such as animal models, species, disease state or composition of food potentially explain the differences in findings, the ability to make any further interpretation on the correlation between overall food intake and the magnitude of changes in specific metabolic parameters are difficult to make without systematic studies. The underlying mechanisms that drive appetite and consequently food intake with pegozafemin/FGF21 need to
be studied further. Extrapolating from a limited number of healthy volunteers who received a single dose of pegozafermin to NASH/SHTG patients receiving more prolonged treatment may also present uncertainty.

A phase 1b/2a study of pegozafermin in NASH patients was recently completed (Loomba et al., 2023); the data also show that pegozafermin elicited treatment-related changes in disease or mechanism related biomarkers such as lipid and adiponectin in addition to expected pharmacokinetics based on the data as presented in this manuscript. The data presented here also provided mechanistic rationale for pegozafermin and shows generally consistent effects of pegozafermin in multiple experimental settings. Therefore, the data supports that pegozafermin has high potential as a new therapeutic for the treatment of NASH and SHTG, as well as obesity, T2DM and other metabolic diseases.
Acknowledgements

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Data Availability Statement

The individual participant data that underlie the results reported in this manuscript will not be shared due to privacy reasons.

Authorship Contributions

Participated in research design: Chen, Mansbach, Margalit, Rosenstock, Tseng

Conducted experiments: Chen, Mansbach, Margalit, Rosenstock, Tseng

Performed data analysis: Charlton, Chen, Mansbach, Margalit, Pierce, Offman, Rosenstock, Tseng

Wrote or contributed to the writing of the manuscript: Charlton, Chen, Mansbach, Margalit, Pierce, Offman, Rosenstock, Tseng
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ceramide axis controls energy expenditure and insulin action in mice. *Cell Metabolism* **17**:790-797.


activates paraventricular nucleus NUCB2/Nesfatin-1 neurons to produce satiety under fed states. Scientific reports 7:45819.


**FOOTNOTES**

a. The studies in this report were supported by 89bio, Inc., 6 Hamada Street, Herzliya, Israel, and 242 Sansome Street, San Francisco, CA, USA.


c. Lead contact

**Conflict of Interest Statement**

HM, LT, and MM are employees of and stockholders in 89bio, Inc.; AP, CYC, MR and RWC are former employees in 89bio, Inc; EO is an employee of Certara. The studies were funded by 89bio.
FIGURE LEGENDS

Figure 1. Relative effects of pegozafermin and FGF21 on FGF receptor activity. Assays were conducted in L6 rat cells expressing β-Klotho alone (top left) or β-Klotho in combination with the designated FGF receptors. Data represent mean and standard deviation of 3 replicates.

Figure 2. Pharmacodynamic effects of pegozafermin in diabetic monkeys (Multiple Dose Study 1). Shown are percent changes from baseline in triglycerides (TG), high-density lipoprotein-cholesterol (HDL-c), low-density lipoprotein-cholesterol (LDL-c), adiponectin, glucose, glycated hemoglobin (HbA1c), insulin, food intake, body weight (BW), alanine aminotransferase (ALT), mean blood pressure (MBP) and heart rate (HR). The shaded area represents the treatment period. n=5 males and 1 female for each treatment group.

Figure 3. Mean serum concentrations of pegozafermin over time following single subcutaneous injections at different dosages in healthy adults.

Figure 4. Pharmacodynamic effects of pegozafermin in healthy adults following a single subcutaneous dose. Shown are mean changes from baseline in triglycerides, LDL cholesterol, HDL cholesterol and adiponectin at the designated dosages and post-administration timepoints.
### TABLES

Table 1. Treatment-Emergent Adverse Events (Any Causality) Occurring in Two or More Subjects Who Received Pegazafermin.

<table>
<thead>
<tr>
<th>Event, n (%)</th>
<th>Placebo (N=6)</th>
<th>0.45 mg (N=6)</th>
<th>1.2 mg (N=6)</th>
<th>3 mg (N=6)</th>
<th>9.1 mg (N=7)</th>
<th>18.2 mg (N=6)</th>
<th>39 mg (N=6)</th>
<th>78 mg (N=6)</th>
<th>Pooled (N=43)</th>
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<td>1 (6.7)</td>
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Figure 1

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<th>Receptor</th>
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* Low E<sub>max</sub> (<2-fold baseline).
Figure 2

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Figure 4

Triglycerides

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LDL

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HDL

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Adiponectin

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Day 29

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* indicates statistical significance.