Therapeutic Effects of FGF23 c-tail Fc in a Murine Preclinical Model of X-Linked Hypophosphatemia Via the Selective Modulation of Phosphate Reabsorption

Kristen Johnson, Kymberly Levine, Joseph Sergi, Jean Chamoun, Rachel Roach, Jacqueline Vekich, Mike Favis, Mark Horn, Xianjun Cao, Brian Miller, William Snyder, Dikran Aivazian, William Reagan, Edwin Berryman, Jennifer Colangelo, Victoria Markiewicz, Cedo M Bagi, Thomas P Brown, Anthony Coyle, Moosa Mohammadi, and Jeanne Magramp

1Center for Therapeutic Innovation, Pfizer, New York NY, USA
2Drug Safety Research and Development, Pfizer, Groton, CT, USA
3Comparative Medicine, Pfizer, Groton, CT, USA
4Department of Biochemistry and Molecular Pharmacology, New York University School of Medicine, New York NY, USA

ABSTRACT
Fibroblast growth factor 23 (FGF23), a genetic disorder effecting 1:20,000 that is characterized by excessive phosphate excretion, elevated FGF23 levels and a rickets/ostemalacia phenotype. FGF23 inhibits phosphate reabsorption and suppresses 1α,25-dihydroxyvitamin D (1,25D) biosynthesis, analytes that differentially contribute to bone integrity and deleterious soft-tissue mineralization. As inhibition of ligand broadly modulates downstream targets, balancing efficacy and unwanted toxicity is difficult when targeting the FGF23 pathway. We demonstrate that a FGF23 c-tail-Fc fusion molecule selectively modulates the phosphate pathway in vivo by competitive antagonism of FGF23 binding to the FGFR/αklotho receptor complex. Repeated injection of FGF23 c-tail Fc in Hyp mice, a preclinical model of XLH, increases cell surface abundance of kidney NaPi transporters, normalizes phosphate excretion, and significantly improves bone architecture in the absence of soft-tissue mineralization. Repeated injection does not modulate either 1,25D or calcium in a physiologically relevant manner in either a wild-type or disease setting. These data suggest that bone integrity can be improved in models of XLH via the exclusive modulation of phosphate. We posit that the selective modulation of the phosphate pathway will increase the window between efficacy and safety risks, allowing increased efficacy to be achieved in the treatment of this chronic disease. © 2017 American Society for Bone and Mineral Research.

KEY WORDS: FIBROBLAST GROWTH FACTOR 23; X-LINKED HYPOPHOSPHATEMIA; 1,25D; PHOSPHATE; OSTEOMALACIA

Introduction
X-linked hypophosphatemia (XLH) is the most common of the phosphate wasting diseases, affecting approximately 1:20,000 people worldwide (reviewed in Carpenter and colleagues[1]). The disease is characterized by low serum phosphate, inappropriately low levels of 1α,25-dihydroxyvitamin D (1,25D), and poor bone mineralization. XLH is typically diagnosed in children upon the appearance of a distinctive bow-legging phenotype, a consequence of the children’s “soft-bones” inability to bear weight as they begin to walk. Other disease manifestations include growth retardation, bone deformation, fractures, and bone pain, which continue into adulthood. Disease severity is variable, with some patients requiring multiple invasive surgeries during childhood. Adults suffer from persistent pain, excessive tooth abscesses, and calcification of entheses.

Currently, there is no US Food and Drug Administration (FDA)-approved standard of care for XLH patients; conventional treatments are cumbersome, not well tolerated, have variable efficacy, and harbor significant safety risks. XLH patients rely on phosphate replacement for improved bone mineralization but the persistent phosphate excretion that characterizes the disease makes it challenging to maintain the steady state of serum phosphate necessary to improve and maintain bone integrity. XLH patients receive oral phosphate at regular intervals up to five times/day in an attempt to treat their disease but the repetitive nature of phosphate administration leads to hyperparathyroidism.[1] Calcitriol, the active form of vitamin D, is used successfully to combat hyperparathyroidism; however, its...
use increases the potential for soft-tissue mineralization, an irreversible condition that can lead to tissue necrosis. Because soft-tissue mineralization can occur within multiple tissues, including the heart, this safety risk is considered more serious than the hypophosphatemic disease itself. As a consequence, physicians often underdose patients, making it extremely difficult to achieve full efficacy.\(^1\)

XHL is defined by a mutation in the phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX) but the causative factor in disease is the upregulation of the endocrine hormone, FGF23.\(^1\) FGF23 functions to decrease serum phosphate and 1,25D levels, minerals crucial for mineralization. FGF23 is secreted on osteoblasts and osteocytes in the bone, ultimately acting on the kidney and parathyroid organs (reviewed by Bergwitz and Juppner\(^2\)). Tissue specificity is achieved by the expression of α-klotho, a membrane protein that acts as a co-receptor to the FGF receptor complex through which FGF23 signals. Mechanistically, FGF23 regulates phosphate by downregulating the sodium phosphate (NaPi) transporters in the kidney\(^3,4\) thereby increasing phosphate excretion. Repression of 1,25D is achieved via modulation of enzymes responsible for the biosynthesis and degradation of vitamin D\(^3,5\). FGF23 also suppresses parathyroid hormone (PTH), though the mechanisms by which this occurs remain poorly understood.\(^2\)

FGF23 is known to be cleaved in vivo, resulting in the generation of a C-terminal (c-tail) and an N-terminal fragment.\(^9-11\) The c-tail peptide retains the ability to bind to the FGFRIc/α-klotho complex but, in contrast to the full length protein, does not induce signaling.\(^12\) Thus cleavage of FGF23 not only inactivates the protein but creates a naturally occurring competitive antagonist. The Mohammadi Laboratory (Department of Biochemistry and Molecular Pharmacology, New York University School of Medicine) has shown that exogenous delivery of the FGF23 c-tail to rats and mice increases serum phosphate levels in vivo,\(^11\) raising the possibility that it could be used as a therapeutic in phosphate wasting diseases. However, the half-life of the 72 amino acid (72aa) c-tail peptide was prohibitively short with an estimated half-life of 10 min, resulting in a return of phosphate levels to baseline 2 hours postdosing and prohibiting the assessment of a long-term impact on bone.

We generated a FGF23 c-tail Fc fusion in order to increase the half-life of the FGF23 c-tail peptide and explore the therapeutic potential of this molecule in a preclinical mouse model of XHL. We found that treatment of Hyp mice (a mouse model that harbors a mutation in the PHEX gene and mimics human disease) with the FGF23 c-tail Fc over 7 weeks is sufficient to cause dose-responsive improvement in bone quality with no evidence of soft-tissue mineralization. Interestingly, our molecule preferentially inhibits the phosphate pathway in the absence of 1,25D modulation in vivo, regardless of whether the animals are wild-type (WT) or diseased. As noted above in the current treatment paradigm, phosphate elevation is associated with bone improvement whereas elevated 1,25D can increase the risk of soft-tissue mineralization. Thus, the unique ability of FGF23 c-tail Fc to preferentially modulate the phosphate pathway in the absence of 1,25D elevation makes this molecule ideal for use as a new therapeutic in the treatment of XHL, with the potential to significantly improve bone formation in XHL patients with limited safety concerns.

### Materials and Methods

**Production of recombinant mouse and human FGF23 c-tail Fc constructs and synthetic peptide construct**

Seventy-two amino acid (72aa) human FGF23 c-tail peptide (aa 180 to 251) was synthesized by and resuspended in PBS. The mouse and human Fc-FGF23 fusion protein coding sequences were designed to contain a leader peptide, the hinge and Fc portion of human or mouse IgG1, mutations in the Fc domain that eliminate Fc binding to Fcγ receptors, a single GGGGS linker, and the C-terminal 72 amino acids of human or murine FGF23. The sequences were constructed as synthetic genes by a commercial vendor (Geneviz, South Plainfield, NJ, USA custom order) and recloned into a proprietary mammalian expression vector. The mouse fusion protein was produced by large scale transient transfections using the human embryonic kidney cell line HEK293 using the Freestyle 293 family of cells, reagents, and media (Life Technologies, Inc., Grand Island, NY, USA) as per the manufacturer’s protocols. Murine FGF23-Fc was purified by Protein A affinity (MabSelect SuRe; GE Healthcare, Piscataway, NJ, USA; 17-5438) and preparative SEC (Superdex 200pg; GE Healthcare, Piscataway, NJ, USA; 28-9893) chromatography. The final pool was formulated at approximately 5 mg/mL in 20mM Hepes, 150mM NaCl, pH 7.5. The human FGF23 fusion protein construct was transfected into a proprietary CHO cell line and a stable pool of transfectants was selected. After selection, the transfected pool was scaled to 1 L at an initial density of 1 × 10^5/mL and a production run was initiated. Use of a daily feed schedule enabled production runs of 14 to 16 days, with typical fusion protein titers of 6 to 900 mg/L. The supernatants were harvested by centrifugation and sterile filtered before purification. Human FGF23-Fc was purified by Protein A affinity (MabSelect SuRe) and ceramic hydroxyapatite (Macroprep CHT Type II, 40 μm; Bio-Rad Laboratories, Hercules, CA, USA; 157-4000) chromatography. Preliminary formulation studies were performed at 5 mg/mL and 50 to 65 mg/mL in HBS (20mM HEPES, 150mM NaCl, pH 7.5) and TMS buffer (1.2 mg/mL Tris, 40 mg/mL mannitol, 10 mg/mL sucrose, pH 7.5). The material was most stable in TMS buffer when concentrated to 50 mg/mL. All lots of human and mouse FGF23-Fc were characterized by UV absorbance, SDS-PAGE, analytical size-exclusion chromatography (analytical SEC), and endotoxin level. All preparations showed >95% purity by SDS-PAGE and analytical SEC, and endotoxin levels below 1 endotoxin unit (EU)/mg.

**Stable cell line generation**

Plasmid construction for protein expression of Klotho were made by GenScript. Protein was cloned into the pQXCVN vector (Clontech Laboratories, Palo Alto, CA, USA).

Stable cell lines were generated through retroviral transfection. HEK293T (American Type Culture Collection [ATCC], Manassas, VA, USA) cells were plated at 2 × 10^5 cells in 10-cm dishes in DMEM (CellGro, Coring Cellgro DMEM#10-013) with 10% heat-inactivated (HI) FBS and cultured overnight. The following day HEK293T cells were transfected with 5 μg of pCL10A1 retrovirus packaging vector (Novus Biologicals, Littleton, CO, USA), 5 μg plasmid DNA, and Lipofectamine 2000 (Life Technologies). The following day HEK293 and HEK293 α-Klotho cells were plated in six-well plates (Corning Inc., Corning, NY, USA) and cultured overnight. On day 4, HEK293 cells and HEK293 α-Klotho cells were infected with retrovirus containing the gene of interest with
7.5 μg/mL of Polybrene. After 48 hours cells were passaged as normal with appropriate antibiotic selection reagents.

A lentiviral GreenFire1 pGF1-EGR reporter vector (System Biosciences [SBi], Palo Alto, CA, USA) expressing destabilized copGFP reporter and firefly luciferase under early growth response (EGR) response elements, a minimal CMV promoter, and the puromycin resistance gene under the control of the EF1 α-Klotho cells. After 48 hours cells were negatively selected for and sorted on the FACSAria III (BD Biosciences, San Jose, CA, USA). Cells were allowed to recover and were expanded. The population of cells was then treated with 200nM TPA (12-O-tetradecanoyl-phorbol-13-acetate; Cell Signaling Technology, Beverly, MA, USA) for 30 min. Cells were positively selected for and sorted on the FACSAria III. Cells were then maintained as stated below.

Cells were then maintained as stated below.

Cell culture

HEK293, a human embryonic kidney cell line, was obtained from the ATCC and cultured in Eagle’s Minimum Essential Medium (Corning cellgro) containing 10% HI FBS (Life Technologies; Gibco, Grand Island, NY, USA). Cells were maintained under standard growth conditions. Cells stably expressing α-Klotho were selected and maintained in 1.2 mg/mL Geneticin (Life Technologies). Cells stably expressing α-Klotho and Egr1 reporter were selected and maintained in 1.2 mg/mL G419 (α-Klotho) and 6 μg/mL puromycin (Life Technologies). Cells were cultured and split every 72 hours at 1.3 x 10⁶ cells in 20 mL of media in a T75 flask.

Animals

Female PhexHyp-21 mice naive to any previous treatments were obtained by superovulation of C57BL/6J female mice that were fertilized with sperm from a male PhexHyp-21 at Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6J age-matched naive mice were supplied by Jackson Laboratory. Six-week-old to nine-week-old Wistar Han IGS rats were purchased from Charles River (Worcester, MA, USA). Mice were housed two to three per cage; rats were housed individually. All rodents were fed a certified rodent diet 5002 (Purina Mills, Inc., PMI Feed, Inc., St. Louis, MO, USA) and municipal drinking water ad libitum.

All procedures performed on animals in this study were in accordance with established guidelines and regulations, and were reviewed and approved by the Pfizer (or other) Institutional Animal Care and Use Committee. Pfizer animal care facilities that supported this work are fully accredited by Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Cellular reporter assay

HEK293 α-Klotho cells transfected with the Egr-1 reporter were plated in triplicate at 10,000 cells/well in 96-well, Poly-D-lysine clear well flat bottom plates (BD Biosciences). Cells were cultured overnight at 37°C in 5%CO₂. On the following day cells were pretreated with serially diluted amounts of human FGF23 c-tail Fc for 30 min. Cells were then treated with 63pM recombinant human carrier-free FGF23 (R&D Systems, Minneapolis, MN, USA) for an additional 3 hours. Luciferase expression was quantitated using Steady-Glo Luciferase reagent (Promega, San Luis Obispo, CA, USA) and measured on an Envision (PerkinElmer, Waltham, MA, USA). Data was analyzed and IC50 values were calculated using a four-parameter variable slope nonlinear regression analysis on GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Competitive binding assay

HEK93 α-klotho cells engineered and grown as stated above were grown in T75 flasks and removed from flasks using cell dissociation buffer (Gibco; Cat#13151-014) for 3 to 5 min. Cells were counted and placed in 3% BSA/1 × PBS blocking buffer (Sigma; Albumin Bovine Fraction V 7.5% solution Cat# A8412) for 1 hour on ice at 0.2 x 10⁶ cells per 200 μL for each condition. A dose-response of human FGF23 c-tail Fc peptide was added for a 15-min pretreatment on ice to each condition. Human FGF23 protein was then added for 1 hour on ice at a single dose of 0.47 μg/mL (EC80) to each Fc dose and alone. Cells were washed with 400 μL cold 0.5% BSA-PBS buffer once and then 200 μL cold 0.5% BSA-PBS buffer two additional times. Cells were centrifuged at 172 g to pellet the cells after each wash. Anti-His mouse mAb (GenScript, Piscataway, NJ, USA; Cat#A00186-100) was added alone and to each condition at 0.2 μg/200 μL in 3% BSA-PBS solution for 1 hour on ice. Cells were then washed as previously described. Anti-mouse PE secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA; Cat# 715-116-150) was then added at 1:200 in 3% BSA-PBS solution alone, with primary antibody, and at each condition and incubated for 45 min on ice. Cells were then washed as previously described. Cells were then resuspended in 200 μL of 0.5% BSA-PBS and read on the MACSQuant flow cytometer from Miltenyi Biotec (Bergisch Gladbach, Germany).

Short-term study in WT rats

Human FGF23 c-tail Fc was administered into 6-week-old to 9-week-old Wistar Han IGS rats by either subcutaneous injection (10 mg/kg, n = 5 rats; or 30 mg/kg, n = 5 rats) or i.v. administration (100 mg/kg, n = 5 rats). As a control group a solution of 12.2 mg/mL Tris, 40 mg/mL mannitol, 10 mg/mL sucrose (pH 7.5) was given (n = 5 rats). Doses of FGF23 c-tail Fc were given twice weekly by s.c. injection on days 1, 4, 8, 11, and 14. The 100 mg/kg dose was given on the same days. The control group was given an intravenous dose followed by a subcutaneous dose. Clinical chemistry parameters were evaluated in samples collected at necropsy on day 15. The animals were fasted overnight prior to blood collection.

Multidose study in Hyp mice

On day 0 of the study murine FGF23 c-tail Fc or phosphate buffered saline (vehicle control) was injected subcutaneously into 5-week-old female PhexHyp-21 mice (0 mg/kg, n = 44 mice; 3 mg/kg, n = 10 mice; 10 mg/kg, n = 15 mice). Additional C57BL/6J age-matched female mice were injected on day 0 with PBS as a second control (n = 39 mice). Mice were dosed twice a week until 12 weeks of age (day 51). Animals were euthanized on day 52. Terminal bleeds were taken by cardiac puncture. Right kidney was collected at necropsy, flash frozen in liquid nitrogen, and used to isolate total RNA using Trizol (Invitrogen, Carlsbad, CA, USA) using the manufacturer’s protocol. Mice were subject to imaging on day 1 (pretreatment) and day 50 (week 8) using a Lunar Piximus (GE Medical Systems) automated densitometer. Bone mineral density, and fat and lean body composition were assessed. Following necropsy, the right hock and the left kidney were X-rayed using a MX-20 Digital radiography system (Faxitron X-Ray LLC, Wheeling, IL, USA) and micro–computed
tomography (μCT) was conducted on the right femur to measure bone integrity.

Serum and urine chemical parameters

Phosphate, calcium, and creatinine were measured in serum and/or urine using a Siemens ADVIA 1800 Clinical Chemical Analyzer (Siemens Medical Solutions USA, Inc., Malvern, PA, USA). Quantitation of 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3] was performed using a ultra performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method similar to the method for the quantitation of the inactive form. 1α,25-Dihydroxyvitamin D2 was not assessed because feed used was only supplemented with the D3 forms of the vitamin, so D2 levels were not measurable. Urine samples were collected from group-housed non-fasted animals following an overnight collection. Fractional excretion of urine phosphate was calculated to determine the total amount of phosphate excreted. Prior to dose initiation, a group of Phex<sup>−</sup> mice were bled terminally for serum chemistry.

Renal expression of NaPi2A

The right kidney was collected at necropsy, flash frozen in liquid nitrogen, pulverized, and total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). One microgram (1 μg) of RNA was used to generate cDNA using random hexamers as per protocol of Invitrogen Superscript Vilo kit. NaPi2A expression was assessed using Invitrogen TaqMan probe Slc34a1 (Mn00441450), after normalization to the housing keeping gene B2-microglobulin.

Bone imaging and histology

A PIXImus automated densitometer was used to measure bone mineral density (BMD), bone mineral content (BMC), bone mineral area (BMA), total area (TA), total tissue mass (TTM), and percent fat tissue. μCT evaluation of bone mass, structure, and BMD was conducted on the right femur collected at scheduled necropsy. After dissection, the right femur was gently cleaned of soft tissues with a scalpel blade. All bone samples were stored in 10% buffered formalin for a minimum of 48 hours and then transferred to 70% ethanol.

Bone imaging and histology

A PIXImus automated densitometer was used to measure bone mineral density (BMD), bone mineral content (BMC), bone mineral area (BMA), total area (TA), total tissue mass (TTM), and percent fat tissue. μCT evaluation of bone mass, structure, and BMD was conducted on the right femur collected at scheduled necropsy. After dissection, the right femur was gently cleaned of soft tissues with a scalpel blade. All bone samples were stored in 10% buffered formalin for a minimum of 48 hours and then transferred to 70% ethanol. μCT analysis was performed on the hock and the cancellous bone on distal femoral metaphyses utilizing a Viva μCT-40 computed tomography system (Scanco Medical, Bassersdorf, Switzerland). The hind leg was positioned horizontally in a 21-mm holder, with the knee positioned vertically. Using a scout image for reference, a 5-mm ROI was identified to include the distal tibia and hock joint. The distal femur sample was oriented horizontally in a 21-mm holder, with the epiphyseal head facing outward. A control file, or measurement protocol, was created to define scanning parameters such as source energy, sample size, and image resolution desired. Parameters selected for this study included a source voltage of 55 kV and electric current strength (SI) of 109 μA to obtain the best contrast between bone and soft tissues. The sample area selected for three-dimensional structural analysis of cancellous bone was a 2.0 mm length of the metaphyseal secondary spongiosa, originating 0.5 mm below the epiphyseal growth plate and extending cranially.

To label the newly mineralized bone surfaces, in a separate 12 week study three mice from each group received calcein (C-0875; Sigma) and Alizarin red S (A-5533; Sigma) at 10 and 3 days, respectively, prior to euthanasia. Calcein was dissolved in 2% sodium bicarbonate/0.9% saline at 10 mg/kg and Alizarin in sterile water at 30 mg/mL. Both were dosed at 1 mL/kg as intraperitoneal injections. De-mineralized right tibia were embedded in methylmethacrylate and cut into 8-μm-thick cross-sections using a polycut sliding microtome (Leica Biosystems, Nussloch, Germany). Poor mineralization and labeling in Hyp mice prevented extensive histomorphometric analysis. Therefore, the labeling with fluorescent biomarkers was used to demonstrate difference in active mineralization between control WT mice and Hyp mice dosed with vehicle and FGF23 c-tail Fc.

Statistics

Statistical tests were conducted at the 5% and 1% significance levels. Although all in vivo studies consisted of three to 10 animals per group, several parameters (such as fractional excretion of phosphate [FEPHOS] and fractional excretion of phosphate [FECA]) required pooling of samples, thus prohibiting statistical analysis. Animal numbers and requirement for pooled samples are strictly documented within the text and figure legends. Analyses of clinical chemistry, urine, and biomarker parameters were done on measurements collected for each animal at the scheduled sampling times. A nonparametric (rank-transform) one-way analysis of variance (ANOVA) on groups were conducted, with two-sided trend tests conducted and two-sided pairwise comparisons to the Hyp control group. Average ranks are assigned to ties. The trend tests were performed sequentially using linear contrasts and the pairwise comparisons being done using Dunnett’s test.

For sequential trend tests on bone, if the initial test with all groups included in the trend analysis was significant (trend p value <0.05), then it was concluded that the “highest” dose group was different from the “lowest” dose group, and a subsequent trend test is performed on all groups except the “highest” dose group. Testing continued in this manner until a nonsignificant result was obtained or the test was performed on only the two “lowest” dose groups. All subsequent trend tests were one-sided in the direction suggested by the data in the initial test. As noted within the text, statistical significance does not necessarily represent a change that is biologically significant.

Results

Human 72aa FGF23 C-tail Fc fusion has an inhibitory potency similar to the human 72aa FGF23 C-tail peptide

The 72aa FGF23 C-tail peptide has been shown to inhibit FGF23 mediated activation of the MAPK pathway in vitro.(12) Additionally, inhibition of MAPK in Hyp mice in vivo modulates phosphate and 1,25D and improves bone mineralization, providing evidence that MAPK is a physiologically relevant pathway in FGF23-mediated hypophosphatemia.(13) In order to determine whether half-life extension engineering of the 72aa FGF23 c-tail compromised the potency of the peptide, we compared the ability of the FGF23 c-tail peptide and the FGF23 c-tail Fc to inhibit FGF23-mediated induction of Egr1, a transcription factor whose activity is upregulated downstream of the MAPK pathway following stimulation. Specifically, we pretreated HEK293 cells that were engineered to stably express both α-chlotho and an Egr1 luciferase reporter with increasing amounts of either FGF23 c-tail peptide or FGF23 c-tail Fc prior to stimulating cells with a subsaturating amount of recombinant
FGF23 and assessed luciferase reporter activity. This study took advantage of endogenous expression of FGFRs in HEK293 cells. As shown in Fig. 1A, both molecules inhibited luciferase activity in a dose-dependent manner, generating IC50s in the range of 200 nM. Therefore, c-terminal fusion of the FGF23 c-tail to a human Fc molecule devoid of effector function did not compromise functional potency of the molecule.

In order to establish that the mechanism of action for competitive inhibition was happening at the level of receptor binding, we performed cellular competitive binding assays using flow cytometry. In this assay, binding of a subsaturating dose of FGF23 to HEK293-klotho was assessed in the absence or presence of increasing amounts of the FGF23 c-tail Fc. The potency of inhibitory binding was quantitated by plotting the mean fluorescence intensity (MFI) generated at each concentration of FGF23 c-tail Fc, allowing the generation of an IC50. We noted that HEK293 cells express FGFR1, FGFR3, and FGFR4, each of which can complex with α-klotho. These data demonstrated that the FGF23 c-tail inhibited FGF23 at the level of receptor binding and quantitatively demonstrated the FGF23 c-tail Fc has comparable inhibitory binding potency to the FGF23 c-tail peptide, a molecule that has the ability to modulate phosphate in vivo (Fig. 1B).

Human FGF23 C-tail Fc modulates serum phosphate but not 1,25D in WT rats

Previous studies showed exogenous delivery of the FGF23 c-tail to rats and mice increased serum phosphate levels in vivo. We wished to extend these studies to the FGF23 c-tail Fc and include analysis of 1,25D, an additional target of FGF23. FGF23 c-tail Fc was injected subcutaneously into healthy rats twice a week over 2 weeks at 10, 30, and 100 mg/kg, assessing both phosphate and 1,25D levels in the serum on day 15, 24 hours after the final dose was administered. As seen in Fig. 2, serum phosphate was significantly modulated in a dose-dependent manner whereas

---

**Fig. 1.** Human 72aa FGF23 C-tail Fc fusion has an inhibitory potency similar to the human 72aa FGF23 C-tail peptide. (A) IC50s derived for both the FGF23 c-tail peptide and Fc fusion based on luciferase activity from a HEK293-klotho-Egr1 reporter cell line using increasing amounts of either FGF23 c-tail peptide or FGF23 c-tail Fc prior to stimulating cells with subsaturating amounts of recombinant FGF23. (B) IC50s derived from competitive binding assessment in HEK293-klotho lines using the MFI from individual samples of increasing amounts of either FGF23 c-tail peptide or Fc followed by binding of sub-saturating amounts of FGF23. Data representative of n = 3.
1,25D levels did not change at any of the administered dose levels. Hence, FGF23 c-tail Fc molecule appears to selectively modulate phosphate pathways in vivo.

Murine FGF23 c-tail Fc modulates phosphate levels in Hyp mice via regulation of NaPi2A expression

The above studies, shown in Fig. 2 demonstrated that the FGF23 c-tail modulated the phosphate pathway in a wild-type setting without affecting 1,25D levels. However, it remains unclear whether the selective modulation of the phosphate pathway would be recapitulated in the disease setting and if so whether modulation of phosphate alone would be sufficient to improve bone mineralization. In order to test this, we undertook a 7-week study in Hyp mice to assess whether treatment with the FGF23 c-tail Fc improved hypophosphatemia and bone integrity without causing soft-tissue mineralization. As in human disease, Hyp mice have elevated levels of FGF23 due to a mutation in PHEX, an endopeptidase expressed in the bone.\(^\text{[15,16]}\) For these studies mice were injected twice a week subcutaneously between the ages of 5 to 12 weeks, the active longitudinal bone growth phase of these mice. Serum/urine chemistry, bone integrity, and soft-tissue mineralization were assessed at the end of the study. Of note, though the human molecule does cross react in mice, a surrogate molecule was used for these studies in order to prevent formation of antidrug antibody. Although GFG receptors and α-klotho share a high homology between human and mouse proteins (over 86% in all cases), FGF23 homology is 72% between human and mouse, with homology of the 72aa FGF23 c-tail falling to 64%. As described in the Materials and Methods, both the human molecule and the murine surrogate were made with an effectorless IgG1 Fc. Finally, we verified that the surrogate molecule was shown to have a similar potency to the human molecule in vitro when using an all murine system versus an all human system, respectively (data not shown).

Consistent with the rat study, serum phosphate showed a trend toward elevation after treatment, 24 hours postdose at day 52 (Fig. 3A) though these changes were not statistically significant (see Discussion). Of note, serum phosphate was only modulated at the highest dose and did not reach levels seen in WT animals. Interestingly, phosphate excretion was dose-responsive and normalization occurred in the 10 mg/kg treatment group (Fig. 3B). Unfortunately, the necessity to pool urine samples resulted in 2 data points/10 animals/treatment group, thus prohibiting statistical analysis. Together, these data show that the FGF23 c-tail Fc impacted phosphate levels in diseased mice and affected phosphate excretion to a greater extent than serum phosphate (at this time point).

FGF23 modulates phosphate levels via downregulation of the sodium transporters located within the kidney; thus increasing phosphate excretion.\(^\text{[3,4]}\) In order to verify the mechanism of action by which the FGF23 c-tail Fc modulates phosphate levels, we assessed NaPi2a expression relative to B2 microglobulin from total kidney RNA in animals at the end of the study using QPCR. As shown in Fig. 3C, a dose-dependent increase in NaPi2a expression was found after treatment with the FGF23 c-tail Fc. These results verified that FGF23 c-tail Fc counteracts FGF23 function at a known target in vivo, and provided mechanistic evidence of appropriate target engagement.

Normal serum levels of 1,25D and calcium are found following treatment with the murine FGF23 c-tail Fc

1,25D levels are normally increased by hypophosphatemia via increased expression of 1αOH (CYP27B1) expression in the kidney.\(^\text{[2]}\) However, FGF23 inhibits 1,25D by decreasing 1αOH expression. Thus, in Hyp animals, which have both high levels of FGF23 and hypophosphatemia, 1,25D levels are within the normal range. Inhibition of FGF23 using an anti-FGF23 antibody cocktail over a 4-week period in Hyp mice results in a strong increase in 1,25D levels 24 hours after the final dose.\(^\text{[17]}\) Strikingly, in our study, although minor statistically significant increases were seen upon treatment in 1,25D levels using trend p value tests, no significance was found using pairwise p value analysis and overall the absolute levels were all very similar to those obtained in the C57BL/6J mice at the same time point. Therefore, as opposed to other molecules that inhibit the FGF23 pathway in the Hyp model, we found 1,25D levels did not change in a manner that would impact the biology with treatment at the 24-hour time point (Fig. 4A). In agreement with these findings, changes in CYP27B1 were not consistent across animals nor dose-dependent (data not shown). In addition, this data is consistent with the lack of 1,25D modulation seen in the WT rat study, demonstrating that that the lack of 1,25D modulation spans species.

### Fig. 2. FGF23 C-tail Fc modulates serum phosphate but not 1,25D in WT rats. Serum chemistry analysis performed 24 hours after the fifth dose in WT rats.

Data represents averages of 5 rats. *Significantly different from vehicle control \(p < 0.05\). **Significantly different from vehicle control \(p < 0.01\).
Inhibition of the FGF23 pathway in a similar model system has shown 1,25D elevation as early as 5 hours post-treatment and revealed that elevation can be sustained out to 7 days. To understand if 1,25D could be modulated by the FGF23 c-tail Fc at time points other than 24 hours, we performed a single-dose study in WT and Hyp animals (five animals/group) at 6 hours and 72 hours comparing 1,25D levels in animals treated with either vehicle or 15 mg/kg FGF23 c-tail Fc. As shown in Supporting Fig. 1, the only change we were able to detect was in the Hyp mice at 6 hours and this change, though reaching significance, was less than twofold (285 versus 509 pg/mL) and was not sustained over time. Because this response was transient and limited to the diseased state, we hypothesize that this movement may be due to the increase in phosphate, which can feedback to effect 1,25D levels.

One function of 1,25D is to increase serum calcium levels by increasing uptake within the gut. As such, the increase of 1,25D seen after inhibition of FGF23 using an anti-FGF23 antibody cocktail in Hyp mice resulted in increased serum calcium.(17) High levels of serum calcium increase the risk of soft-tissue mineralization and therefore may indicate a safety risk. We assessed serum calcium to remain at normal levels following treatment with the FGF23 c-tail Fc (Fig. 4B). Because soft-tissue mineralization is the consequence of an elevated calcium/phosphate product, normal levels of 1,25D and calcium, together with subnormal levels of serum phosphate ensures a low risk of soft-tissue mineralization. In agreement with this, no soft-tissue mineralization was observed in any animal group (data not shown).

FGF23 c-tail Fc treatment results in a significant improvement in cancellous bone and bone mineral content

Phosphate plays a major role in the mineralization of osteoid and cartilage at physes. In hypophosphatemic conditions such as XLH, this mineralization is compromised.(1) We used several radiologic techniques to assess bone mineralization in treated and nontreated Hyp mice and compared these findings to those in untreated WT controls during our 7-week study. PIXImus is an automated densitometer used to measure bone mineral density/content in live animals, thereby allowing quantitative assessment of bone parameters. Consistent with published data,(19–21) and relative to age-matched WT mice, Hyp mice displayed several skeletal abnormalities including: significantly lower bone mineral content (BMC), bone volume...
findings for these parameters strongly indicated poor bone mineralization that would eventually lead to compromised bone strength. Over the course of the study, treatment with 3 mg/kg of FGF23 c-tail Fc showed a trend of improved BMC, BV, and BMD, whereas treatment with 10 mg/kg of FGF23 c-tail Fc produced significant improvement (Table 1). Importantly, individual animals within a group behaved similarly (Supporting Fig. 2). These data were consistent with increased modulation of clinical chemistries in the 10 mg/kg treated animals and provided evidence for improved bone mineralization.

Improvement in bone mineralization and structure upon treatment of Hyp animals with 10 mg/kg FGF23 c-tail Fc

To more directly assess bone quality we imaged the cancellous bone of the distal femoral metaphysis by performing ex vivo micro-CT. Cancellous bone is normally highly vascularized and metabolically active, and is therefore suitable for assessing remodeling. As expected, the WT control mice had far more cancellous bone at the distal femoral metaphysis than did the Hyp control animals. Remarkably, a clear dose responsive increase was seen after treatment. Because bone formation typically occurs at mechanically relevant bone areas, the increase in cancellous bone observed in our study was most evident at the proximal tibial metaphysis, which transfers mechanical loads (Fig. 5A).

To confirm the μCT, we performed intravital labeling of the newly mineralized bones by fluorescent labeling in a subset of animals. Specifically, mice were injected with calcein (green) and Alizarin red (red) labels 10 and 3 days before the necropsy in order to assess active mineralization across animals of various backgrounds and treatment groups. As shown in Supporting.

Table 1. Values for Measurements of the Bone Parameters in Mice on Day 50

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C57BL/6J (Group 1)</th>
<th>C57BL/6J-Phex&lt;sup&gt;hyp/J&lt;/sup&gt; (Group 2)</th>
<th>3 mg/kg dose (Group 3)</th>
<th>10 mg/kg dose (Group 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD (g/cm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.045 ± 0.001</td>
<td>0.036 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.039 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.040 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMC (g)</td>
<td>0.42 ± 0.026</td>
<td>0.28 ± 0.019&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32 ± 0.031&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.33 ± 0.028&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMA (cm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>9.35 ± 0.660</td>
<td>7.75 ± 0.218&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.21 ± 0.576</td>
<td>8.28 ± 0.799</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significantly different from C57BL/6J control, p ≤ 0.01.
<sup>b</sup>Significantly different from C57BL/6J-Phex<sup>hyp/J</sup> control, p ≤ 0.01.
Fig. 3, defined areas of cortical bone (femur) are labeled within WT mice, whereas labeling in Hyp control mice is wide and fuzzy indicating diffuse mineralization patterns. Strikingly, both labels are much better defined in Hyp mice treated with 10 mg/kg/dose.

The poor bone quality of Hyp control animals was also evidenced by the large areas of scalloped bone surfaces, numerous pathological lacunae, and open growth plates seen throughout the entire hock joint as visualized by three-dimensional μCT. By day 50 of the study, growth plates had closed in the WT animals but remained open in the Hyp controls. Hyp mice treated with 10 mg/kg of the FGF23 c-tail also showed normalization of bone surfaces characterized by absence of these pathological lacunae during our study (Fig. 5B). In addition, closure of epiphyseal growth plates occurred after treatment with 10 mg/kg of the FGF23 c-tail Fc (Fig. 5B). Consistent with other data, Hyp mice in the 3 mg/kg group showed a moderate improvement, though the growth plates remained open in these animals.

Dose-responsive improvement in bone histology evident upon treatment with FGF23 c-tail Fc

As a final assessment of FGF23 c-tail Fc impact on the bone, we performed histologic analysis of bone architecture at the proximal tibial physis of each animal. The physis is the part of the bone responsible for bone lengthening, constituting an area that separates the metaphysis and the epiphysis, in which long bone growth occurs. As seen in Fig. 6, Hyp control animals not given FGF23 c-tail Fc had wide physes composed of cartilage. In contrast, the WT mice had thinner physes. Strikingly, there was marked improvement of the structure of physes in both treatment groups, with cartilage being replaced in a dose dependent manner by mineralized bone. Together these data demonstrate that the FGF23 c-tail treatment improved bone structure in Hyp animals during the 7 weeks of treatment.

Discussion

Current standard of care for XLH patients involves oral supplementation with phosphate and calcitriol, the active form of vitamin D. A major issue with the current treatment is achieving a balance between efficacy and unwanted toxicity. In the current study, we find that the FGF23 c-tail Fc selectively modulates the phosphate pathway without impacting 1,25D in vivo and ameliorates the bone defects present in Hyp mice, a mouse model that recapitulates XLH disease. We posit that the unique selectivity of the FGF23 c-tail Fc for the phosphate
pathway provides efficacy together with a significant safety advantage, making it an improved therapeutic option in the chronic treatment of XLH.

A defining characteristic of untreated XLH is osteomalacia, the accumulation of unmineralized bone, a trait that is recapitulated in Hyp mice. Hyp mice have somewhat larger bones with significantly lower bone volume, BMC, and BMD. Three-dimensional µCT images also showed large areas of scalloped bone surfaces, numerous lacunae throughout the hock joint, and open growth plates in untreated Hyp animals, traits indicative of poor bone quality. Multiple doses of FGF23 c-tail Fc over our 7-week study produced significant improvement in bone mass as well as bone quality across the skeleton as measured by PIXImus. There was also a dose-responsive improvement in bone surfaces, including diminished presence of pathological lacunae. At the highest dose of 10 mg/kg, growth plate closure occurred; growth plates remained open in the 3 mg/kg group. Although significant improvement was observed, we noted that bone parameters were not normalized by treatment. It remains to be determined if longer treatment or higher doses have the potential to restore the skeletal properties of the cortical and cancellous bone to a greater degree. Regardless, based on µCT, hematoxylin and eosin staining, and intravitral labeling our data indicate that multiple doses of FGF23 c-tail Fc resulted in substantial improvement in bone quality and reversed several aspects of disease similar to those which occur in XLH patients.

Interestingly, even in the presence of significant bone improvement only a moderate increase in serum phosphate was achieved at the highest treatment dose of 10 mg/kg was achieved. In contrast, we found a dose-responsive improvement in phosphate excretion that was normalized at the highest dose. The discrepancy in the magnitude of change between serum and excreted phosphate was somewhat surprising. The mechanism of action was verified because NaPi2A expression increased in a dose-dependent manner after treatment. In the pathologic states with low NaPi expression, phosphate excretion is typically high. Thus, altering NaPi receptor levels causes an immediate block to phosphate excretion, making phosphate excretion the most proximal readout of this mechanism of action. A likely explanation for why the c-tail fails to normalize the serum phosphate may be that increased phosphate ions are consumed rapidly by the phosphate-deficient bone, thus preventing detection of sustained serum phosphorus increases. This may explain why the Hyp mice dosed with 3 mg/kg showed only a partial bone improvement despite a lack of a discernible increase in serum phosphate. If this occurs as bone mineralization occurs, serum phosphate may be expected to rise over time. Indeed, patients often experience a spike in serum phosphates when the requirement of phosphate in the bone changes (because of healing or changes in growth rates). The subnormal levels of serum phosphate together with normal serum calcium levels provide a buffer for these potential spikes, limiting the chance of soft-tissue mineralization in chronic therapy.

Several preclinical studies have explored the use of small molecule inhibitors and antibodies that target the FGF23 pathway for the treatment of XLH, using the Hyp mouse model. In each case, FGF23 signaling is inhibited and
improvement of both hypophosphatemia and bone integrity is achieved. This is achieved through a variety of mechanisms including: pan inhibition of FGFRs, inhibition of the MAPK pathway, and neutralization of FGF23 itself. In each case, both phosphate and 1,25D levels are modulated. In the case of FGF23 neutralization, 1,25D modulation was sustained in a multidose 4-week study to levels approximately 10 times over wild-type levels. Consistent with the fact that 1,25D increases both calcium and phosphate absorption in the gut, serum levels of both analytes were significantly elevated in that study. Of note, at the dose that was efficacious for bone, the calcium phosphate solubility product in that study was indicative of soft-tissue mineralization. Though soft-tissue mineralization was not seen in these animals, the risk present following 4 weeks of chronic dosing suggests this toxicity would be present if the study had been extended. Additionally, the presence of excess mineralization of metaphyseal cancellous bone within the treated Hyp mice suggests that the bone was saturated for phosphate.

In contrast, our FGF23 c-tail Fc showed selective inhibition of the phosphate pathway with an absence of biologically significant 1,25D modulation. We do not yet understand the mechanism behind the preferential inhibition but speculate that it may be a consequence of antagonizing binding across several different receptor complexes, which in turn have different contributions in regulating the phosphate and 1,25D pathways. Indeed, genetic data have demonstrated unique requirements for individual FGFR/α–klotho receptor complexes in the regulation of phosphate and 1,25D. Specifically, FGFR1c/α–klotho has been shown to be the primary receptor complex responsible for mediating phosphate, whereas there is a redundant requirement for FGFR3c/α–klotho and FGFR4/α–klotho in the control of 1,25D levels. As the binding structure of FGF23 or the FGF23 c-tail to any of the FGFR/klotho complexes remains to be defined, it is difficult to predict how the c-tail may differentially interact across these receptors, and if it does act differently, whether this is at the level of binding or function. Intriguingly, preliminary data suggests that the FGF23 c-tail Fc fusion has unique competitive binding attributes across distinct receptor complexes as compared to that of the c-tail peptide. We remain actively engaged in exploring these possibilities.

Although the mechanism mediating selective modulation remains to be determined, regulation of the phosphate pathway in the absence of 1,25D modulation was a result found in both WT and diseased animals in our study. Importantly in the XLH-disease model, improved bone integrity was also achieved. Interestingly, ectopic calcifications that are found in Fgf23-null mice are no longer seen when these animals are crossed onto a 1x(OH)–deficient background. This supports the idea that 1,25D is responsible for driving this toxicity in both the standard of care and in vivo studies where FGF23 is neutralized. We posit that the FGF23 c-tail Fc offers a potential safety advantage in the chronic treatment of XLH patients because it selectively modulates the phosphate pathway in the absence of 1,25D regulation, and suggest it may be a new therapy for the treatment of both pediatric and adult XLH patients. An important next step for testing this hypothesis will be to assess the translation of our preclinical data to humans. It is interesting to think that nature has evolved to allow for the separate control of the phosphate and 1,25D pathways via the distinct action of FGF23 whereas 1,25D levels can remain to be appropriately regulated by full-length FGF23. We seek to harness the same properties in the chronic treatment of XLH.

**Disclosures**

KJ, KL, JS, JChamoun, RR, JV, MF, MH, XC, BM, WS, DA, WR, EB, JColangelo, VM, CMB, TPB, AC, and JM were all employees of Pfizer at the time this work was performed. MM has no conflicts of interest.

**Acknowledgments**

This work was supported by Pfizer Inc. Centers for Therapeutic Innovation. The Mohammadi Laboratory is primarily supported by the NIH grant DE13686. We thank Carolyn Macica, Mohammed Razzaque, and the CTI team members for helpful discussions. We thank Carol Fritz and other Biomarker team members for serum and urine chemistry analyses.


**References**


