Title

Mechanistic Investigation of the Pre-Clinical Pharmacokinetics and Interspecies Scaling of PF-05231023, a Fibroblast Growth Factor 21-Antibody Protein Conjugate

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RUNNING TITLE

Pharmacokinetics and Interspecies Scaling of PF-05231023

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Abbreviations used are: %F, bioavailability; AUC_{0-\infty} = area under concentration-time curve from 0 to infinity; AUC_{0-t} = area under concentration-time curve from 0 to last measured time point; CL, systemic plasma clearance; C_{max} = maximum observed plasma concentration; CT, C-terminus; ELISA, enzyme-linked immunosorbent assay; FGF21, fibroblast growth factor 21; HRP, horse radish peroxidase; i.v., intravenous; mAb, monoclonal antibody; NT, N-terminus; PBPK, physiologically based pharmacokinetic; PK, pharmacokinetics; rFGF21, recombinant fibroblast growth factor 21; s.c., subcutaneous; t_{1/2} = elimination half-life; T_{max} = time to reach C_{max}; V_c = central distribution volume; V_{ss} = volume of distribution at steady state.
ABSTRACT

PF-05231023, a long-acting fibroblast growth factor (FGF) 21 analogue, was generated by covalently conjugating two engineered [des-His1, Ala129Cys]FGF21 molecules to a non-targeting human IgG1κ scaffold. Pharmacokinetics (PK) of PF-05231023 following intravenous (i.v.) and subcutaneous (s.c.) administration were evaluated in rats and monkeys using two enzyme-linked immunosorbent assays (ELISA) with high specificity for biologically relevant intact N- (NT) and C-termini (CT) of FGF21. Intact CT of FGF21 displayed ~5-fold faster systemic plasma clearance (CL), ~2-fold lower steady-state volume of distribution (Vss) and at least 5-fold lower bioavailability compared to NT. In vitro serum stability studies in monkeys and humans suggested the principal CL mechanism for PF-05231023 was degradation by serum proteases. Direct scaling of in vitro serum degradation rates for intact CT of FGF21 underestimated in vivo CL 5-, 1.4- and 2-fold in rats, monkeys and humans, respectively. Reduced Vss and %F for intact CT relative to NT in rats and monkeys were compatible with proteolytic degradation occurring outside the plasma compartment via an unidentified mechanism. Human CL and PK profiles for intact NT and CT of FGF21 were well predicted using monkey single species allometric and Dedrick scaling. Physiologically based pharmacokinetic (PBPK) models incorporating serum stability data and an extravascular extraction term based on differential bioavailability of intact NT and CT of FGF21 in monkeys improved accuracy of human PK predictions relative to Dedrick scaling. Mechanism-based PBPK models of this nature may be highly valuable for predicting human PK of fusion proteins, synthetically conjugated proteins and other complex biologics.
INTRODUCTION

FGF21 is a 19 kDa endocrine hormone that modulates lipid and glucose homeostasis (Belouski et al., 2010; Gimeno and Moller, 2014). NT of FGF21 displays affinity for several FGF receptors, including FGFR1c, 3c and 4, whereas CT binds to the membrane associated cofactor β-klotho. Truncation of NT by six or more residues, or CT by two or more residues decreased in vitro potency more than 10-fold, suggesting potent functional activity was derived from both termini of FGF21 (Micanovic et al., 2009; Yie et al., 2009). In diabetic animal models and patients with type 2 diabetes, recombinant FGF21 (rFGF21) and its analogues demonstrated dose-dependent reductions in LDLc, apolipoproteins, fasting triglycerides, fasting insulin and body weight; and, dose-dependent elevations of HDLc and adiponectin (Adams et al., 2013; Gaich et al., 2013; Kharitonenkov et al., 2013; Smith et al., 2013). While dose-dependent reductions in blood glucose have been observed in diabetic rodent and non-human primate models, thus far only a trend towards lower fasting blood glucose has been observed in human clinical studies (Gaich et al., 2013). Although the precise mechanism by which FGF21 exerts its pharmacological actions have not been deduced, both NT and CT are believed to be important for potent regulation of these processes.

The highly desirable pharmacology profile of FGF21 has generated significant interest as a potential therapy for type 2 diabetes and/or dyslipidemia; however, the full therapeutic potential of FGF21 may be limited by its short in vivo persistence. CL of rFGF21 in mice and monkeys was comparable to the glomerular filtration rate in each respective species, resulting in half-lives on the order of 0.5 to 2 hours (Kharitonenkov et al., 2007). Furthermore, CL of NT and CT immunoreactivity was indistinguishable in mice suggesting passive renal filtration was the primary CL mechanism for rFGF21 (Hager et al., 2013). Consistent with this hypothesis,
endogenous levels of FGF21 were elevated >15-fold in patients with impaired renal function compared to controls (Stein et al., 2009).

A variety of modalities have been employed to extend the half-life of rFGF21 including pegylation, Fc-fusion, and antibody conjugation (Reitman, 2013). Site-specific conjugation of 30 kDa PEG to FGF21 demonstrated 10 to 50-fold improvements in CL/F compared to rFGF21, albeit at the expense of 5 to 10 fold reductions in \textit{in vitro} potency (Mu et al., 2012). Fusion of NT (Fc-FGF21) or CT of FGF21 (FGF21-Fc) to Fc fragment of hIgG1 right-shifted \textit{in vitro} potency 2 to 4-fold and 1000-fold, respectively (Hecht et al., 2012). The slightly reduced \textit{in vitro} potency of Fc-FGF21 was offset by a 30-fold increase in serum persistence. However, reducing passive renal filtration of FGF21 by increasing its molecular weight had the untoward consequence of uncovering latent proteolytic cleavage sites in NT and CT of FGF21, resulting in only modest enhancements in monkey CL compared to marketed Fc-fusion proteins and therapeutic monoclonal antibodies (mAb) (7.96 mL/h/kg vs. 0.2 to 0.6 mL/h/kg). Stabilization of the CT cleavage site through site directed mutagenesis (Ser171Pro) decreased CL of intact Fc-FGF21 an additional 5-fold (Hager et al., 2013). As an alternative to Fc-fusion proteins, which require NT or CT coupling of FGF21 to Fc-domain, direct chemical conjugation of FGF21 to an antibody scaffold via a bi-functional synthetic linker enabled site-specific conjugation, thereby maximizing both potency and serum persistence. Engineered site-specific linkage sites remote from the NT and CT of FGF21 preserved \textit{in vitro} potency while increasing serum persistence of FGF21 30 to 90-fold (Huang et al., 2013).

PF-05231023 (CVX-343), a long acting FGF21 analogue, consists of two molecules of [des-His1, Ala129Cys]FGF21 covalently linked to a humanized IgG1κ mAb backbone via a maleimide-azetidinone linker (Fig. 1) (Huang et al., 2013). PF-05231023 demonstrated
comparable *in vitro* potency, enhanced PK and prolonged *in vivo* pharmacodynamics relative to rFGF21. The PK of PF-05231023 in rodents and non-human primates were previously characterized using a sandwich ELISA with specificity for the mid-region of FGF21 (Huang et al., 2013). However, this assay lacked specificity for measuring the bioactive NT and CT of FGF21. In this paper, immunoassays with high specificity for intact NT and CT of FGF21 were used to characterize the PK of PF-05231023 following i.v. and s.c. administration. *In vitro* serum stability studies were used to investigate the CL mechanism for PF-05231023 and enable human PK predictions. A variety of scaling methodologies, including direct scaling of *in vitro* serum degradation rates, single species allometric scaling, Dedrick scaling and PBPK modeling that incorporated serum stability and extravascular degradation terms, were used to predict CL and PK profiles of PF-05231023 in humans.
MATERIALS AND METHODS

Reagents. PF-05231023 was produced by Pfizer Biotherapeutics Center of Excellence (St. Louis, MO). Mouse anti-IgG1κ idiotype antibody and mouse anti-FGF21 CT and NT antibodies were generated by Pfizer.

Bioanalytical Assays. Four ELISAs were used to measure NT of FGF21, CT of FGF21, total FGF21 and total IgG, respectively. Mouse anti-FGF21 mAbs recognizing NT and CT were generated using peptide immunogens, H2N-PIPDSSPLLQFGGC-OH and H2N-CGGPSQGRSPYAS-OH, respectively. Antibodies displayed high specificity for first and last two residues of the N- and C-termini of [des-His1]rFGF21, respectively. NT and CT of FGF21 were measured using an anti-idiotype capture reagent that recognized the mAb scaffold of PF-05231023, biotin labeled anti-NT or anti-CT secondary antibodies and streptavidin horse radish peroxidase (HRP). Total FGF21 was measured using an anti-FGF21 antibody that did not recognize the first 6 NT amino acids or the last 32 CT amino acids of rFGF21, designated as anti-mid region FGF21. In the total FGF21 assay, PF-05231023 was captured with the anti-mid region FGF21 mAb, and detected with an anti-hIgG-HRP labeled antibody (Huang J et al 2013). Total IgG was measured using a polyclonal anti-human IgG capture reagent and detected with an HRP-labeled polyclonal anti-human IgG reagent.

Pharmacokinetic Studies. All animal studies were conducted in accordance with animal care and use protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Pfizer, Inc. Single-dose PK of NT and CT FGF21 immunoreactivity of PF-05231023 was assessed in Wistar Hannover rats and Cynomolgus monkeys. Male rats (n = 4/route) weighing approximately 250 g received a single 10 mg/kg i.v. dose in the tail vein or s.c. dose between the shoulders. Male monkeys (n = 4/route) weighing 3-4 kg received either a single 1 mg/kg i.v.
dose in the cephalic vein or s.c. dose between the shoulders. Blood samples were collected pre-dose, 0.25, 1, 2, 6, 12 (monkeys only), 24, 48, 72, 96, 168, 240 and 336 h post-dose into EDTA tubes with 6.3 TIU/mL aprotinin. Multi-dose PK was also assessed in Wistar Hannover rats and Cynomolgus monkeys as part of the Drug Safety program. Rats (n = 3/gender/dose) weighing 250-300 g received 0.1, 1, 100, and 300 mg/kg i.v. dose in the tail vein. Monkeys (n = 3/gender/dose) weighing 3 to 4 kg received 5, 20, 100, and 300 mg/kg i.v. dose in the cephalic vein. Blood samples were collected pre-dose, 0.25, 2, 6, 24, 48, and 72 h post-dose into EDTA tubes with 6.3 TIU/mL aprotinin. Single-dose PK of total FGF21 and total IgG immunoreactivity of PF-05231023 were assessed in a separate study using Sprague Dawley rats and Cynomolgus monkeys. Male rats (n = 2) weighing 200-225 g received a single 3 mg/kg i.v. dose in the jugular vein. Male monkeys (n = 2) weighing 2.4-3.1 kg received a single 3 mg/kg i.v. dose in the cephalic vein. Blood samples were collected 0.08, 0.5, 1, 3, 5, 7, 24, 48, 72, 96, 120, 144, 192, 240, 312 and 336 h post-dose for rats, and 0.08, 0.5, 1, 4, 6, 12, 24, 36, 48, 72, 96, 120, 144, 168, 192, 240, 288, 336, 504 h post-dose for monkeys. All PK samples were stored -80 °C until analysis. Rat PK parameters for total IgG were estimated using population modeling of composite steady-state concentrations upon repeat dose administration. Blood samples were collected 0.25, 2, 6, 24, 48, 72, 672 and 1344 h post-dose following the last dose.

**FcRn Affinity.** pH 6.0 FcRn binding affinities were determined by surface plasmon resonance using previously described methods (Thorn et al., 2012). Two to four replicate experiments were performed for each molecule on a minimum of two different surfaces. Concentrations of PF-05231023 and the unconjugated IgG scaffold of PF-05231023 were varied from 0.1 to at least 5 times the steady-state $K_D$ and sequentially passed over FcRn.
Serum Stability. *In vitro* serum stability was assessed using freshly collected Wistar Hannover rat, Cynomolgus monkey or human serum. PF-05231023 was spiked into 1 mL of serum on ice, providing an initial concentration of 2 or 20 µg/mL, and incubated 37 °C for 24 h in a humidified 95%/5% O₂/CO₂ incubator. Sixty µL of sample were removed 0, 1, 2, 4, 6 and 24 h, added to 1.5 µL of 26 TIU/mL aprotinin, flash frozen on dry ice and stored -80 °C until analysis. NT and CT FGF21 immunoreactivity were measured as described above. Experiments were performed in duplicate.

**Human Pharmacokinetic Predictions.** PK data from rats and monkeys were used to predict CL and PK profiles for NT and CT FGF21 immunoreactivity in humans. CL was prospectively predicted using rat and monkey single-species allometric scaling with a fixed allometric exponent of 0.80. The validity of this exponent was assessed retrospectively by determining the three-species allometric relation in rat, monkey and human by linearizing bodyweight and CL data on a log-log scale. Mean rat, monkey and human body weights were 0.25 kg, 3.5 kg and 87 kg, respectively. Elementary Dedrick analysis was used to scale rat and monkey concentration-time profiles to human using an allometric exponent of 1.0 for volume of distribution and 0.80 for CL (Deng et al., 2011). Scaled pre-clinical data were compared to observed clinical data following a single 200 mg i.v. dose of PF-05231023 (Dong, J et al., manuscript submitted for publication).

**PBPK Modeling & Simulations.**

Standard mAb PBPK models were adapted to simulate the plasma concentration-time profiles for intact CT of FGF21 in rat, monkey and human (Baxter et al., 1995; Garg and Balthasar, 2007; Shah and Betts, 2012). Lymph flows and reflection coefficients were fitted to the mean α- and β-phase half-lives observed for human mAbs displaying linear PK in each species, and published
extravascular distribution coefficients (Shah and Betts, 2013). Vascular reflection coefficients for sinusoidal and non-sinusoidal tissues were set to 0.5 and 0.9, respectively. Lymphatic reflection coefficients were set to 0.1. Lymph flows were set to 1300 (rat), 1500 (monkey) and 2000-fold (human) slower than plasma flows in nonpulmonary tissues, and 5000 (rat) and 10000-fold (monkey and human) slower than plasma flow in pulmonary tissues (Supplemental Table 1 and Figure 1). Rate constants for returning mAbs from lymph to vasculature were set to 0.05, 0.065 and 0.014 h\(^{-1}\) for rat, monkey and human, respectively. *In vitro* serum degradation rates for CT of FGF21 were applied to vascular compartments and scaled as necessary to improve model fits. An additional CL term was applied to extravascular compartments to account for the pre-systemic metabolism of PF-05231023 in interstitial fluid spaces following s.c. administration. The pre-systemic extraction ratio in rats and monkeys was estimated by dividing s.c. %F of CT by s.c. %F of NT (representing the minimum extent absorption for intact CT of FGF21). In the rat model %F of CT was estimated to be ~1%, the maximum theoretical %F based on the LLOQ of the assay. Human extravascular extraction ratios were set equal to monkey without further scaling.
RESULTS

Monoclonal detection antibodies, displaying high specificity for the first two NT amino acids (Pro2Ile3) and the last two CT amino acids (Ala180Ser181) of FGF21, were used to determine the PK of PF-05231023. These epitopes overlapped residues required for high affinity binding and functional activity, and will henceforth be denoted as intact NT and CT of FGF21. Additional assays were designed to measure mid-region of FGF21 and IgG scaffold, and will henceforth be denoted as total FGF21 and total IgG, respectively. Single dose i.v. PK for PF-05231023 in rats and monkeys are summarized in Table 1. Plasma concentration-time profiles for intact NT and CT of FGF21 (Fig. 2 and 3) displayed biphasic kinetics with 25 to 57% and 72 to 95% of total AUC present under the $\alpha$-phase, respectively. Mean CL of intact NT and CT of FGF21 ranged from 2.6 to 3.6 mL/h/kg and 11.7 to 12.1 mL/h/kg, respectively. Mean $t_{1/2}$ for intact NT and CT of FGF21 ranged from 23 to 60 h and 3 to 6 h, respectively. $V_{ss}$ for intact NT and CT of FGF21 were 70-100 mL/kg and 38 mL/kg, respectively. As part of GLP toxicology and safety pharmacology assessments, dose-proportional increases in $C_{max}$ and AUC were observed over the entire dose range examined for intact NT and CT of FGF21 in rats from 0.1 to 300 mg/kg and monkeys from 5 to 300 mg/kg (Table 3).

Mean CL, $V_{ss}$ and $t_{1/2}$ of total IgG following i.v. administration of PF-05231023 were within four-fold of the expected range for human mAbs (Giragossian et al., 2013). Whereas, the intrinsic PK properties of the unmodified IgG scaffold, i.e., without conjugation to the synthetic linker, were within the expected range for human mAbs. Total FGF21 displayed PK parameters intermediate between intact NT of FGF21 and total IgG, compatible with proteolytic degradation occurring in NT of FGF21. The increased CL of total IgG was consistent with reduced affinity of this molecule for FcRn (Table 4). Covalently linking FGF21 to hIgG1$\kappa$ reduced the affinity of
PF-05231023 for mouse, monkey and human FcRn 5, 20 and 30-fold, respectively, compared to the unmodified scaffold.

Single dose s.c. PK for PF-05231023 are summarized in Table 2 (Fig. 2 and 3). Mean %F of intact NT and CT of FGF21 ranged from 58 to 69% and <1 to 10%, respectively. T_{max} for intact NT and CT of FGF21 was observed between 6 to 30 h and ~6 h, respectively. Half-lives for intact NT of FGF21 were 20.7 and 56.8 h in rats and monkeys, respectively. The t_{1/2} of intact CT could not be estimated due to insufficient data points with detectable exposure.

To investigate the CL mechanism for PF-05231023, in vitro serum stability of PF-05231023 was assessed in rat, monkey and human serum (Fig. 4). Percent intact NT remaining after 24 h was greater than 70%. Log-linear half-lives for intact CT in rat, monkey and human serum were estimated to be 13 h, 3.5 h and 12 h, respectively, and were concentration-independent from 2 to 20 µg/mL of PF-05231023. In vitro serum stability qualitatively reflected the differential in vivo stabilities of intact NT and CT of FGF21, and quantitatively captured in vivo stability differences for intact CT. Directly scaling in vitro serum degradation rates by multiplying the degradation rate by plasma volume accounted for 20%, 70% and 40% of in vivo CL for intact CT of FGF21 in rat, monkey and human, respectively.

Human CL of PF-05231023 was predicted by simple allometry (Table 5). Using a fixed allometric exponent of 0.80, rat and monkey single-species scaling produced human CL predictions within 1.7-fold of mean values observed in humans. Rat and monkey two-species allometric scaling resulted in human CL predictions within 2.2-fold of mean values observed in human. Observed three-species allometric exponents for intact NT and CT in rat, monkey and human were retrospectively determined to be 0.75 and 0.82, respectively (Fig. 5).
Human plasma concentration-time profiles for intact NT and CT of FGF21 were predicted using elementary Dedrick scaling of rat and monkey plasma concentrations (Fig. 6). Predicted plasma concentrations for intact NT and CT of FGF21, based on Dedrick scaling of monkey PK data, were within 3-fold of observed single dose human PK data (Dong, J et al., manuscript submitted for publication). Predicted half-lives for intact CT and NT of FGF21 were 8.2 and 103 hr, respectively, and compared favorably with observed human half-lives of 7.3±1.7 and 97±8 hr, respectively. Dedrick scaling of rat PK data resulted in less satisfactory predictions.

*In vitro* serum degradation rates, estimated *in vivo* extravascular extraction rates derived from the differential s.c. %F of the intact termini of FGF21, and intrinsic catabolic stability of total IgG were also incorporated into species-specific PBPK models to assess whether these models could improve human PK predictions for intact CT of FGF21 (Fig. 7). Similar to Dedrick scaling, rat PBPK models provided poor fits to observed PK data except when serum degradation rate was ~3-fold faster than observed rate. In contrast, the monkey PBPK model provided excellent fits to observed data after applying a modest scaling factor (1.25x). Sensitivity analyses indicated the serum degradation rate was critical for capturing the monkey plasma concentration-time profile up to 6 h post-dose, whereas the *in vivo* extravascular extraction rate was critical for capturing data beyond 6 h post-dose. For the purpose of evaluating prospective human predictions, a 1.25x scaling factor was applied to the human serum degradation rate. Since the rat extravascular extraction rate was poorly defined, monkey extravascular extraction rates were applied to the human PBPK model without further scaling.

The human PBPK model displayed improved fits for intact CT of FGF21 compared to Dedrick scaling. Predicted human plasma concentrations were on average 1.3-fold higher than mean observed concentrations between 0.5 and 73 hours post-dose. Similar to the monkey PBPK
model, sensitivity analyses indicated the serum degradation rate was critical for capturing the observed human plasma concentration-time profile up to 25 h post-dose, whereas the *in vivo* extravascular extraction rate was critical for capturing data beyond 25 h post-dose. Increasing the interstitial fluid extraction ratio from 0.825 to 0.98 adequately capture data beyond 73 hours post-dose. Extravascular extraction accounted for 6%, 2% and 4% of total CL for the intact CT of FGF21 in rats, monkeys and humans, respectively. Due to the relatively short half-life of the intact CT of FGF21, CL of total IgG contributed minimally to the overall simulation results, comprising an estimated 7%, 5% and 10% of total CL for the intact CT of FGF21 in rats, monkeys and humans, respectively. The absence of appreciable degradation of intact NT of FGF21, over the viable quantitative time-range for FGF21 related serum proteases, precluded a similar analysis for NT of FGF21.

DISCUSSION

Immunoassays with high specificity for NT and CT of FGF21 were developed to measure PK of PF-05231023 and its major metabolites. Plasma CL of total FGF21, intact NT and intact CT in monkeys was 90-, 80- and 18-fold slower than native rFGF21, and 1.6-, 1.8- and 8-fold faster than the IgG1k scaffold of PF-05231023. CL of the conjugated IgG1k scaffold was ~4-fold faster than the native unconjugated mAb (data not shown), and approached CL values expected for complete ablation of FcRn binding (Waldmann and Terry, 1990; Hakimi et al., 1991; Hutzell et al., 1991; Saravolatz et al., 1994; He et al., 1998; Garg and Balthasar, 2007). Increased CL of total IgG may be associated with reduced affinity of PF-05231023 for FcRn; however, this may not be the only factor, as CL of total IgG was predicted to be slower in rodents compared to primates based on species differences in FcRn affinity. Although it’s possible rat FcRn behaves differently than mouse, previous studies have shown only minor differences in hIgG affinity for
rat and mouse FcRn, as would be expected based on conserved FcRn binding-site homology and glycosylation patterns (Neuber et al., 2014).

Other potential sources for accelerated CL of total IgG include specific interactions with FGF receptors, anti-drug antibodies (ADA) and nonspecific interactions with endogenous molecules. PK of intact NT, intact CT and total IgG was linear in rats and monkeys across entire dose range examined, consistent with absence of appreciable target mediated disposition. Linear PK for intact NT and CT was also observed in humans across entire dose range examined from 0.5 to 200 mg (Dong, J et al., manuscript submitted). ADAs were undetectable in all species following a single dose, nor was there evidence for accelerated CL during terminal phase that would point to formation of clearing ADAs. The relatively acidic isoelectric point of PF-05231023 (pI 7.6), and absence of positively-charged clusters in the FGF19-based homology model of FGF21 suggests nonspecific interactions with negatively charged cell membranes were unlikely to contribute to accelerated CL compared to native mAb (pI = 8.5); however, nonspecific interactions with other endogenous molecules cannot be excluded.

Covalently linking rFGF21 to an antibody scaffold shifted primary CL mechanism of FGF21 from passive renal filtration to proteolytic degradation. This was supported by the fact that CL of intact NT and CT of unconjugated rFGF21 was identical in mice, and the observed correlation between endogenous FGF21 levels and renal function in humans (Hager et al., 2013; Stein et al., 2009). Differential stabilities of NT, CT and total FGF21 immunoreactivity in PF-05231023 indicated multiple proteolytic cleavage sites were present throughout FGF21 sequence. Scaled in vitro serum degradation rates for intact CT of FGF21 in monkeys and humans suggested principal CL route of PF-05231023 was degradation by serum proteases in these species; whereas in rat, serum proteases accounted for only 20% of CL.
CL of intact CT of FGF21 in PF-05231023 was comparable to CL of intact Fc-FGF21. The rate limiting cleavage site in rFGF21 in vitro (data not shown) and in vivo was identified in CT between Pro171 and Ser172 (Hager et al., 2013). Stabilization of the Pro171/Ser172 cleavage site in Fc-FGF21 by site directed mutagenesis decreased CL of Fc-FGF21 5-fold in monkeys. Consistent with results presented for PF-05231023, stabilization of the principal CT cleavage site in Fc-FGF21 shifted metabolism to other sites within FGF21 sequence, as CL of intact Fc-FGF21 was still considerably faster than CL of total Fc. In line with these observations, additional cleavage sites were putatively identified in CT of Fc-FGF21 (Hager et al., 2013).

The moderately increased molecular weight of PF-05231023 (189 kDa vs. 150 kDa) did not appear to alter apparent $V_{ss}$ or inter-compartmental CL rates compared to therapeutic mAbs. $V_{ss}$ for intact NT, total FGF21 and total IgG were comparable and 2 to 4-fold higher than plasma volume; whereas, $V_{ss}$ for intact CT was comparable to plasma volume. Reduced apparent $V_{ss}$ for intact CT, compared to other drug related products of PF-05231023, was compatible with proteolysis occurring outside the serum compartment (Richter et al., 2012). Similar findings were observed in humans, where mean $V_{ss}$ for intact NT and CT ranged from 74 to 86 mL/kg and 49 to 59 mL/kg, respectively (Dong, J et al., manuscript submitted).

%F of intact NT was similar in rats and monkeys; however, a greater than 10-fold difference was observed for %F of intact CT. Differential %F of intact NT compared to CT paralleled in vitro and in vivo stabilities of these moieties, and may be indicative of differential susceptibility to injection sites proteases and/or pre-systemic proteolysis via lymphatic system. Relatively high %F for intact NT excluded aggregation or nonspecific/specific uptake by macrophages or lymph nodes. Interestingly, species differences in %F for intact CT were not correlated with in vitro serum or in vivo stability, suggesting existence of an alternate protease.
with overlapping substrate specificity and/or differential expression of proteases between serum and extravascular tissue spaces. While the precise mechanisms responsible for reduced %F of intact CT have not been elucidated, injection site degradation and pre-systemic lymphatic extraction have been previously reported for other therapeutic proteins (Charman et al., 2000; Wang et al., 2012).

Human CL predictions based on rat and monkey single-species scaling using a fixed allometric exponent of 0.80 were within 2-fold of clinically observed values for intact NT and CT of FGF21. A fixed exponent of 0.80 was previously shown to be an appropriate single-species scaling factor for predicting human CL for a wide variety of therapeutic proteins and mAbs (Wang and Prueksaritanont, 2010). Other studies based on an overlapping dataset of therapeutic mAbs supported use of a fixed exponent ranging from 0.75 to 0.90 to predict human CL from monkey (Deng et al., 2011; Dong et al., 2011; Ling et al., 2009; Oitate et al., 2011; Oitate et al., 2012). The range of exponents reported for mAbs is not surprising, given relative insensitivity of this parameter towards single-species scaling between monkeys and humans, e.g., an exponent between 0.67 and 0.90 results in ~2-fold change in predicted human CL. Retrospective analysis of the three-species allometric relation for intact NT and CT in rats, monkeys and humans provided further support for selection of a fixed exponent of 0.80. Although both rat and monkey provided fairly good human CL predictions, in agreement with previous reports elementary Dedrick scaling of monkey plasma concentrations was qualitatively and quantitatively superior to rodent scaling (Deng et al., 2011).

In vitro serum stability generally tends to underestimate in vivo CL for biologics (Pauwels et al. 1985; Deacon et al., 1995; Hartmann et al., 2000). The poor in vitro-in vivo translation likely results from several factors, e.g., loss of intrinsic activity upon harvesting
serum, membrane associated intravascular/extravascular proteases, soluble interstitial fluid proteases and other mechanisms associated with CL of complex biologics. Species differences in serum degradation rates and extravascular extraction ratios indicated \textit{in vitro} serum stability data alone was unlikely to provide robust human CL predictions for PF-0523102 and other FGF21 analogues.

Integrating \textit{in vitro} and \textit{in vivo} measures into a PBPK modeling framework provided a flexible platform for interrogating relative contributions of various CL mechanisms, and their subsequent influence on plasma-concentration time profiles. Rate-limiting extravasation, as inferred by the $\alpha$-phase half-life of mAbs, placed significant constraints on CL contributions mediated by interstitial fluid proteases following i.v. administration. Inability of the rat PBPK model to capture the shorter than expected $\alpha$-phase half-life of intact CT, implied species differences in physiology and/or additional uncharacterized CL mechanisms. Although both rat and monkey single species scaling did an adequate job predicting human CL, the apparent disconnect in CL mechanisms between rat and monkey would prospectively point to monkey as the preferred species for human predictions, based purely on phylogeny. The observed human \textit{in vitro} serum degradation rate for intact CT was approximately two-fold slower than predicted by allometrically scaling the monkey \textit{in vitro} degradation rate. This coupled with the fact that serum degradation represents the principal CL mechanism of intact CT in monkeys and humans, explains why monkey single species allometric scaling of \textit{in vivo} CL does a fairly good job predicting human CL despite apparent differences in serum stability. In alignment with these findings, human PBPK predictions for intact CT were more accurate compared to monkey Dedrick scaling, which under-predicted human plasma concentrations by 2 to 3-fold during the terminal elimination phase.
In conclusion, immunoassays with high specificity for intact NT and CT of FGF21 demonstrated PF-05231023 was subjected to rapid proteolysis within the CT, the same region of the molecule critical for maintaining \textit{in vitro} potency through its interactions with \( \beta \)klotho. \textit{In vitro} serum stability studies suggested the principal CL route of PF-05231023 in monkeys and humans was degradation by an as yet unidentified serum protease(s). Incorporating \textit{in vitro} and \textit{in vivo} measures into PBPK models provided superior human PK predictions compared to elementary Dedrick scaling. This type of mechanism-based PBPK modeling may find broad applicability for predicting human PK of complex biologics, including fusion proteins and synthetically conjugated proteins.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Giragossian, Vage, Pelletier, Piché-Nicholas, Rajadhyaksha, Liras, Logan, Calle, Weng

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Contributed new reagents or analytic tools: Pelletier, Rajadhyaksha, Liras, Logan

Performed data analysis: Giragossian, Vage, Li, Pelletier, Piché-Nicholas, Calle, Weng.

Wrote or contributed to the writing of the manuscript: Giragossian, Piché-Nicholas, Calle, Weng
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FIGURE LEGENDS

Fig. 1. Ribbon representation of the structure of PF-05231023. Light and heavy chains of the IgG\(_{1k}\) scaffold are depicted in yellow and purple, respectively. FGF21 is depicted in grey.

Fig. 2. Mean plasma concentration-time profiles for PF-05231023 in monkeys following a single 1 mg/kg i.v. or s.c. dose. Data for total IgG and total FGF21 were dose normalized to 1 mg/kg.

Fig. 3. Mean plasma concentration-time profiles for PF-05231023 in rats following a single 10 mg/kg i.v. or s.c. dose. Data for total FGF21 and total IgG were dose normalized to 10 mg/kg. PK profile of total IgG was estimated using population modeling of composite steady-state concentrations upon repeat dose administration. ADAs (1-10% incidence) did not appear to affect the PK of total IgG in rat.

Fig. 4. *In vitro* stability of PF-05231023 in rat (open circle), monkey (closed circle) and human (triangle) serum: (A) intact NT of FGF21 and (B) intact CT of FGF21. Correlation coefficients for the intact CT of FGF21 were greater than 0.98. Symbols represent mean of two replicates.

Fig. 5. Allometric relationship for CL of PF-05231023 in rat, monkey and human. Allometric coefficients and exponents for intact NT of FGF21 (closed circles) were 0.0029 and 0.75, respectively and intact CT (open circles) of FGF21 were 0.011 and 0.82, respectively. Correlation coefficients were greater than 0.98.

Fig. 6. Predicted human PF-05231023 plasma concentration-time profiles for intact NT and intact CT of FGF21 as determined by elementary Dedrick scaling of rat (A, B) and monkey (C, D) PK data. Volume of distribution and CL were scaled with allometric exponents of 1.0 and 0.80, respectively. Observed human PK data were dose normalized to 1 mg/kg. Dotted and dashed lines denote two- and three-fold prediction limits, respectively.
Fig. 7. PBPK model predicted and observed PF-05231023 plasma concentrations for intact CT of FGF21 in (A) rat, (B) monkey and (C) human. Rat and monkey data were dose normalized to 1 mg/kg, and human data was dose normalized to 2.3 mg/kg. Serum degradation half-lives were set to 10.4 or 3.5 h for rat, 2.3 h for monkey and 9.6 h for human. The extravascular extraction ratio (EER) was set to 0.99 for rat, 0.825 for monkey and 0.825 or 0.98 for human.
TABLES

Table 1. Summary of key PK Parameters of PF-05231023 following a single i.v. dose in rats and monkeys. C_max and AUC were dosed normalized to 1 mg/kg. Rat PK parameters for total IgG were estimated using population modeling of composite steady-state concentrations upon repeat dose administration. ADAs (1-10% incidence) did not appear to affect the PK of total IgG in rat.

<table>
<thead>
<tr>
<th>Species/PK assays</th>
<th>CL (mL/h/kg)</th>
<th>V_c (mL/kg)</th>
<th>Vss (mL/kg)</th>
<th>t1/2 (h)</th>
<th>C_max/Dose (µg/mL)</th>
<th>AUC_0-t/Dose (µg*h/mL)</th>
<th>AUC_0-∞/Dose (µg*h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact CT</td>
<td>11.7 ± 0.33</td>
<td>29.6 ± 2.45</td>
<td>38.0 ± 2.90</td>
<td>6.30 ± 0.085</td>
<td>30.4 ± 2.57</td>
<td>85.5 ± 2.30</td>
<td>85.7 ± 2.30</td>
</tr>
<tr>
<td>Intact NT</td>
<td>3.59 ± 0.373</td>
<td>35.5 ± 13.6</td>
<td>70.9 ± 16.9</td>
<td>22.7 ± 2.16</td>
<td>30.3 ± 1.01</td>
<td>280 ± 26.6</td>
<td>281 ± 26.4</td>
</tr>
<tr>
<td>Total FGF21</td>
<td>2.11</td>
<td>51.9</td>
<td>111</td>
<td>97.3</td>
<td>18.5</td>
<td>466</td>
<td>474</td>
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<tr>
<td>Total IgG</td>
<td>1.29</td>
<td>30.2</td>
<td>199</td>
<td>286</td>
<td>20.5</td>
<td>699</td>
<td>775</td>
</tr>
<tr>
<td><strong>Monkeys</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact CT</td>
<td>12.1 ± 4.19</td>
<td>41.3 ± 4.18</td>
<td>37.8 ± 3.17</td>
<td>3.02 ± 1.36</td>
<td>22.2 ± 1.99</td>
<td>88.4 ± 27.3</td>
<td>89.7 ± 28.0</td>
</tr>
<tr>
<td>Intact NT</td>
<td>2.61 ± 0.421</td>
<td>41.5 ± 4.52</td>
<td>103 ± 7.39</td>
<td>59.8 ± 8.53</td>
<td>23.4 ± 1.91</td>
<td>386 ± 57.6</td>
<td>391 ± 58.0</td>
</tr>
<tr>
<td>Total FGF21</td>
<td>2.34</td>
<td>38.1</td>
<td>102</td>
<td>82.0</td>
<td>25.7</td>
<td>427</td>
<td>429</td>
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<tr>
<td>Total IgG</td>
<td>1.48</td>
<td>37.0</td>
<td>230</td>
<td>209</td>
<td>26.2</td>
<td>620</td>
<td>678</td>
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</table>
Table 2. Summary of key PK parameters for intact NT and CT in rats and monkeys following a single s.c. dose.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose (mg/kg)</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</th>
<th>AUC&lt;sub&gt;0-t&lt;/sub&gt; (µg·h/mL)</th>
<th>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (µg·h/mL)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact CT</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Intact NT</td>
<td>10</td>
<td>20.7 ± 6.07</td>
<td>30 ± 12</td>
<td>36.6 ± 14.9</td>
<td>1920 ± 794</td>
<td>1920 ± 794</td>
<td>68.3</td>
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<tr>
<td>Monkeys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact CT</td>
<td>1</td>
<td>NC</td>
<td>4.0 ± 2.3</td>
<td>1.32 ± 0.568</td>
<td>9.11 ± 4.63</td>
<td>NC</td>
<td>10.2</td>
</tr>
<tr>
<td>Intact NT</td>
<td>1</td>
<td>56.8 ± 6.88</td>
<td>6.0 ± 0.0</td>
<td>3.08 ± 1.02</td>
<td>222 ± 54.5</td>
<td>228 ± 53.7</td>
<td>58.3</td>
</tr>
</tbody>
</table>

ND: Not determined as all concentrations were below limit of detection limit. NC: not calculated due to insufficient data points.
Table 3. Mean dose-normalized $C_{\text{max}}$ and AUC$_{0-\text{last}}$ following an i.v. dose of PF-05231023 in rats and monkeys.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Total IgG</th>
<th>Intact NT</th>
<th>Intact CT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_{\text{max}}$/dose (µg/mL)</td>
<td>AUC$_{0-72\text{h}}$/dose (µg.h/mL)</td>
<td>$C_{\text{max}}$/dose (µg/mL)</td>
</tr>
<tr>
<td>Rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1$^a$</td>
<td>--</td>
<td>--</td>
<td>32.5</td>
</tr>
<tr>
<td>1$^b$</td>
<td>--</td>
<td>--</td>
<td>27.9</td>
</tr>
<tr>
<td>100</td>
<td>--</td>
<td>--</td>
<td>25.7</td>
</tr>
<tr>
<td>300</td>
<td>--</td>
<td>--</td>
<td>26.4</td>
</tr>
<tr>
<td>Monkeys</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>23.4</td>
<td>406</td>
<td>25.2</td>
</tr>
<tr>
<td>20</td>
<td>24.4</td>
<td>396</td>
<td>28.8</td>
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<tr>
<td>100</td>
<td>25.0</td>
<td>403</td>
<td>24.8</td>
</tr>
<tr>
<td>300</td>
<td>18.8</td>
<td>407</td>
<td>21.9</td>
</tr>
</tbody>
</table>

$^a$n=3; $^b$n=5; n=6 for all other groups. --, data not available.
Table 4. Mouse, monkey and human pH 6.0 FcRn binding affinities for PF-05231023 and the unconjugated IgG₁κ scaffold of PF-05231023. Data represent mean±SD from 2 to 4 experiments.

<table>
<thead>
<tr>
<th>Species</th>
<th>K_D (nM)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG₁κ</td>
<td>PF-05231023</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>2.5±0.9</td>
<td>13±3</td>
<td></td>
</tr>
<tr>
<td>Monkey</td>
<td>92±18</td>
<td>1600±200</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>75±17</td>
<td>2528±11</td>
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</tbody>
</table>
Table 5. Comparison of predicted and observed mean (SD) human CL values for PF-05231023. Monkey and rat CL values for the intact NT and CT of FGF21 were scaled to human using a fixed allometric exponent of 0.80.

<table>
<thead>
<tr>
<th>Scaling Method</th>
<th>Human CL</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>NT (L/h)</td>
</tr>
<tr>
<td>Rat Single-Species</td>
<td>0.097</td>
</tr>
<tr>
<td>Monkey Single-Species</td>
<td>0.12</td>
</tr>
<tr>
<td>Two-Species</td>
<td>0.15</td>
</tr>
<tr>
<td>Observed Human</td>
<td>0.070 (0.019)</td>
</tr>
</tbody>
</table>
Figure 1.
Figure 4

(A) % Intact NT remaining vs. Time (h)

(B) % Intact CT remaining vs. Time (h)
Figure 7

A

PF-05231023 (ng/mL)

Time (h)

100
10
1

Dotted line: 10.4 h half-life
Solid line: 3.5 h half-life

B

PF-05231023 (ng/mL)

Time (h)

100
10
1

C

PF-05231023 (ng/mL)

Time (h)

100
10
1

Dotted line: EER 0.825
Solid line: EER 0.98