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The sodium-phosphate co-transporter NPT2a plays a key role in reabsorbing filtered phosphate in proximal renal tubules thereby critically contributing to phosphate homeostasis. Inadequate urinary phosphate excretion can lead to severe hyperphosphatemia as in tumoral calcinosis, and in chronic kidney disease (CKD). Pharmacological inhibition of NPT2a may therefore represent a novel approach for treating hyperphosphatemic conditions. The NPT2a-selective small molecule inhibitor, PF-06869206, was previously shown to reduce phosphate uptake in human proximal tubular cells *in vitro*. We now investigated the acute and chronic effects of the inhibitor in vivo and report that administration of PF-06869206 was well-tolerated and elicited a dose-dependent increase in fractional phosphate excretion. This phosphaturic effect lowered plasma phosphate levels in wild-type mice and in rats with CKD due to subtotal nephrectomy. PF-06869206 had no effect in Npt2a-null mice, but promoted phosphate excretion and reduced plasma phosphate in normophophatemic mice lacking Npt2c and in hyperphosphatemic mice lacking Fgf23 or Galnt3. In CKD rats, once daily administration of PF-06869206 for eight weeks induced an unabated acute phosphaturic and hypophosphatemic effect, but had no significant effect on FGF23 or PTH levels. Selective pharmacological inhibition of NPT2a thus holds promises as a novel therapeutic option for genetic and acquired hyperphosphatemic disorders.

Selective pharmacological inhibition of the sodium-dependent phosphate co-transporter NPT2a promotes phosphate excretion

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Conflict of interest:

VC, KJF and JG are employees of Pfizer, Inc. HS, MR and HJ are employees of MGH. AHN is an employee of Alcon and JK is an employee of Synchrogenix. HJ is a co-inventor on a patent describing a method to measure FGF23.

ABSTRACT

The sodium-phosphate co-transporter NPT2a plays a key role in reabsorbing filtered phosphate in proximal renal tubules thereby critically contributing to phosphate homeostasis. Inadequate urinary phosphate excretion can lead to severe hyperphosphatemia as in tumoral calcinosis, and in chronic kidney disease (CKD). Pharmacological inhibition of NPT2a may therefore represent an attractive approach for treating hyperphosphatemic conditions. The NPT2a-selective small molecule inhibitor, PF-06869206, was previously shown to reduce phosphate uptake in human proximal tubular cells *in vitro*. We now investigated the acute and chronic effects of the inhibitor in rodents and report that administration of PF-06869206 was well-tolerated and elicited a dose-dependent increase in fractional phosphate excretion. This phosphaturic effect lowered plasma phosphate levels in wild-type mice and in rats with CKD due to subtotal nephrectomy. PF-06869206 had no effect in Npt2a-null mice, but promoted phosphate excretion and reduced plasma phosphate in normophophatemic mice lacking Npt2c and in hyperphosphatemic mice lacking Fgf23 or Galnt3. In CKD rats, once daily administration of PF-06869206 for eight weeks induced an unabated acute phosphaturic and hypophosphatemic effect, but had no statistically significant effect on FGF23 or PTH levels. Selective pharmacological inhibition of NPT2a thus holds promises as a therapeutic option for genetic and acquired hyperphosphatemic disorders.

INTRODUCTION

Serum phosphate levels are maintained within normal limits through several mechanisms. These include the regulation of intestinal phosphate absorption through 1,25$(OH)_2$ vitamin D (1,25D) and the sodium-dependent phosphate transporter NPT2b (also referred to as NaPi2b, SLC34A2), as well as the reabsorption of phosphate by two renal transporters, NPT2a (NaPi2a, SLC34A1) and NPT2c (NaPi2c, SLC34A3) (1-4). Expression of the latter transporters at the apical membrane of the proximal renal tubules is controlled by parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), two hormones that consequently play important roles in regulating total phosphate balance.

PTH is produced by the parathyroid glands. It binds to the PTH/PTHrP receptor, a Gαs-coupled receptor, to stimulate cAMP formation and PKA-dependent down-stream events that include the formation of 1,25D, the biologically active vitamin D metabolite, and the down-regulation of NPT2a and NPT2c in the kidney. Consequently, lack of biologically active PTH, as in post-surgical or genetic forms of hypoparathyroidism (5, 6), and resistance to this hormone, as in the different forms of pseudohypoparathyroidism (7, 8), leads to reduced urinary phosphate excretion and thus hyperphosphatemia, as well as a reduction in 1,25D formation that contributes to the development of hypocalcemia.

FGF23, another phosphate-regulating hormone, is produced predominantly by osteocytes in bone and possibly by several other cells/tissues, at least when kidney function is impaired (9-13). FGF23 enhances the excretion of phosphate by binding with high affinity to the FGFR1 and its co-receptor KLOTHO, which reduces expression of NPT2a or NPT2c at the apical membrane of the proximal tubules (14, 15). However, unlike the rapid phosphaturic effect of PTH, administering recombinant FGF23 or increasing the endogenous levels of this phosphate-regulating hormone does not lead to increased urinary phosphate excretion until several hours after raising the circulating FGF23 levels (10, 16, 17). Furthermore, in contrast to PTH, FGF23 reduces 1,25D levels by down-regulating expression of Cyp27B1, which encodes the 1α-hydroxylase, and by up-regulating expression of Cyp24A1, which encodes the 24-hydroxylase that metabolizes 1,25D (2, 3, 10, 16). Because of these actions on vitamin D metabolism, patients with osteomalacia due to FGF23-secreting tumors show a rapid decrease of FGF23 levels after tumor removal and frequently a profound increase in 1,25D levels, besides normalization of serum phosphate (18-20).

Several different hyperphosphatemic disorders are caused by the lack of biological active FGF23 or resistance to FGF23, as in different forms of tumoral calcinosis that are caused by

homozygous or compound heterozygous, inactivating mutations in FGF23, GALNT3, or KLOTHO (21-26). In contrast, excess FGF23 production or prolonged activity due to “activating” FGF23 mutations leads to an increase in urinary phosphate excretion and thus hypophosphatemia resulting in rickets or osteomalacia, as well as diminished 1,25D levels (9, 27, 28). Urinary phosphate-wasting and thus hypophosphatemia can be caused also by homozygous or compound heterozygous mutations in NPT2c (29) and particularly in NPT2a (30). These FGF23-independent mechanisms enhancing urinary phosphate excretion are associated with mild-to-profound increases in 1,25D production leading to various degrees of hypercalcemia and hypercalciuria (29, 30). NPT2a or NPT2c thus have a critically important role in regulating serum phosphate levels and inhibitors of either transporter could therefore be useful for the treatment of different genetic disorders with hyperphosphatemia, particularly the different forms of tumoral calcinosis (21-26).

In addition to inherited disorders with lack of or resistance to PTH or FGF23, hyperphosphatemia is highly prevalent in the later stages of chronic kidney disease (CKD). During the early CKD stages in humans and mice, FGF23 levels increase only slightly, but even these minor elevations appear to be associated with a decline in serum phosphate levels, implying that FGF23 plays an important role in preventing phosphate retention when renal function has just started to decline (31-34). These findings in patients are consistent with studies in rats with mild CKD, in which administration of an anti-FGF23 antibody causes profound increases in serum phosphate levels resulting in vascular calcifications and accelerated mortality (35). As renal dysfunction progresses, FGF23 levels increase further, are associated with more rapid CKD progression, and an increased likelihood of dying prematurely (36-38).

Serum phosphate levels are typically normal or in the upper end of the normal range until glomerular filtration rate (GFR) declines below 30 mL/min/1.73m^2 (33, 34, 39). However, even these earlier stages of CKD have been associated with vascular calcifications, increased cardiovascular morbidity and mortality in humans (37) and rodents (40). Once persistently elevated, use of oral phosphate binders is approved by the FDA to reduce intestinal phosphate absorption thereby reducing serum phosphate levels. In animals with CKD, severe dietary phosphate restrictions or the use of extremely high doses of oral phosphate binders effectively reduce, but do not normalize, circulating FGF23 levels; these findings indicate nonetheless that phosphate lowering treatment strategies are effective for limiting the rise in FGF23 (41, 42). Unfortunately, an efficient reduction of serum phosphate levels in CKD patients remains challenging because of gastrointestinal side effects, pill burden and limited compliance (43, 44). Furthermore, a compensatory increase in NPT2b expression has been observed in rodent CKD models (45). Most likely because of a combination of these different reasons, oral phosphate

binders have largely failed to elicit the desired reduction in FGF23 levels, even in patients with early CKD stages (46-48). This calls into question whether a reduction in the intestinal absorption of phosphate alone is sufficient to prevent the long-term negative effects of impaired phosphate excretion. Consequently, there is a considerable need to identify additional approaches for maintaining normal serum phosphate level and preventing the rise of FGF23 in CKD. Enhancing urinary phosphate excretion may thus have potential utility in the early CKD stages and such interventions could be beneficial in patients with different forms of tumoral calcinosis and, possibly as adjunct therapy, in other disorders with increased serum phosphate levels.

We recently described the discovery of orally bioavailable selective inhibitors of NPT2a that display concentration-dependent inhibition of phosphate uptake in human proximal tubular cells *in vitro* (49). We showed that the oral PK profile of one of these inhibitors, PF-06869206, is suitable for exploring the pharmacological effects of selective NPT2a inhibition in rodents. Here we further characterize the selectivity profile and pharmacological activity of PF-06869206 *in vivo* and report that it enhanced phosphate excretion in normophosphatemic wild-type as well as in hyperphosphatemic Fgf23- and Galnt3-null mice (50, 51). Furthermore, acute and long-term treatment of CKD rats with PF-06869206 was well tolerated and it enhanced urinary phosphate excretion thereby reducing plasma phosphate level. Our data in the chronic setting and in hyperphosphatemic mice expands upon acute *in vivo* effects shown here and in a recent report (52) by demonstrating that selective NPT2a inhibition holds promises as a therapeutic approach to control serum phosphate in different genetic and acquired hyperphosphatemic disorders.

RESULTS

Inhibition of NPT2a with PF-06869206 blocks phosphate uptake in vitro

We previously reported the ability of PF-06869206 to reduce phosphate uptake in HEK293 cells stably expressing human, rat, or mouse NPT2a and in primary human proximal tubular cells, but not in cells expressing NPT2b or NPT2c (49). Here we further characterized the activity profile of PF-06869206 *in vitro* by measuring phosphate uptake in primary rat proximal renal tubule cells (PRTs) exposed to increasing concentrations of the inhibitor. PF-06869206 elicited in these cells a statistically significant, concentration-dependent reduction of ^{32}P uptake (Figure 1). The highest dose of 30μM PF-06869206 resulted in a reduction of radioactivity to 46.2±1.5% of control, compared to a reduction to 92.6±2.1% of control at the lowest dose of 30nM PF-06869206 and a reduction to 30.5±2.9% of control with 5mM of phosphonoformic acid (PFA), a non-selective inhibitor of sodium-phosphate transporters that was used as positive control (Figure 1). These results, together with the reported favorable pharmacokinetics profile of PF-06869206 in rodents (49), supported the decision to evaluate further the pharmacological activity of the NPT2a inhibitor *in vivo*.

Acute administration of PF-06869206 reduces phosphate reabsorption in wild-type mice

To characterize the *in vivo* activity of PF-06869206, we first investigated its effect in C57BL/6 mice. The single oral administration of PF-06869206 at doses ranging from 10mg/kg to 500mg/kg elicited a dose-dependent increase in the fractional excretion of phosphate index (FEI_{Pi}) (Figure 2A) that was associated with a dose-dependent decrease in serum phosphate level (Figure 2B) at 4 hours after dosing. The lowest inhibitor dose (10mg/kg) elicited a statistically significant change in FEI_{Pi} compared to vehicle-treated animals (55.8±5.5 vs. 21.3±4.2) that did not translate into a statistically significant change in serum phosphate relative to vehicle-treated animals (10.4±0.5mg/dL vs 10.5±0.6mg/dL). However, the change relative to vehicle treatment in both FEI_{Pi} and serum phosphate was statistically significant when the dose of the inhibitor was escalated to 300mg/kg and 500mg/kg, with FEI_{Pi} increasing to 198.3±20.3 and 348.0±35.9, respectively and serum phosphate decreasing to 7.2±0.7mg/dL and 4.8±0.3mg/dL, respectively (Figures 2A and 2B).

Acute administration of PF-06869206 increases phosphate excretion in wild-type and $Npt2c^{-/-}$ mice, but not in $Npt2a^{-/-}$ mice

In vivo effects of PF-06869206

To assess the specificity of PF-06869206 *in vivo*, wild-type, Npt2a$^{-/-}$, and Npt2c$^{-/-}$ mice, all in the C57BL/6 background, were treated with either vehicle or PF-06869206 at 300mg/kg. Pilot studies had shown maximal effect of the inhibitor at 2 and 4 hours after dosing, followed by a decline of the effect (data not shown); FEI_{Pi} and plasma phosphate levels were therefore measured before dosing (baseline), at 2-4 hours and at 24 hours after dosing.

Acute administration of PF-06869206 at 300mg/kg to Npt2c$^{-/-}$ mice (Figures 3A-D) led to a statistically significant 2.9-fold increase in FEI_{Pi} relative to vehicle at 2-4 hours post-dosing (168.9±12.0 vs. 58.4±9.6), which was consistent with the statistically significant increase in FEI_{Pi} relative to vehicle observed in wild-type mice treated with PF-06869206 at the same dose (116.0±12.7 vs. 26.0±4.6). Plasma phosphate was reduced by 32.6% relative to vehicle in Npt2c$^{-/-}$ mice treated with PF-06869206 at 300mg/kg (6.2±0.3 mg/dL vs. 9.2±0.2 mg/dL, which was similar to the reduction in plasma Pi relative to vehicle observed in wild-type mice treated with the 300mg/kg dose of the inhibitor (7.4±0.2 mg/dL vs. 10.1±0.2 mg/dL. At 24 hours after dosing with PF-06869206 or vehicle, FEI_{Pi} and plasma phosphate levels were back to baseline level in wild-type and in Npt2c$^{-/-}$ mice (Figures 3A-D).

In contrast to the findings in wild-type and Npt2c$^{-/-}$ mice, administration of PF-06869206 in Npt2a$^{-/-}$ mice failed to increase urinary phosphate excretion or to decrease plasma phosphate relative to vehicle or baseline levels at 2-4 hours or 24 hours after dosing (Figures 3E,F). These findings confirmed our *in vitro* data (49), namely that PF-06869206 selectively inhibits Npt2a and has no readily detectable phosphaturic activity in the absence of that transporter.

Acute administration of PF-06869206 increases calcium excretion in wild-type mice

We next determined whether the NPT2a inhibitor has an effect on the regulation of calcium homeostasis. Wild-type C57BL/6 mice were given a single dose of either vehicle or PF-06869206 (300mg/kg) by oral gavage; blood and urine samples were obtained before and 2, 4 and 24 hours after dosing. Treatment with PF-06869206 did not elicit any statistically significant changes in plasma calcium levels at any of the study timepoints when compared to treatment with vehicle (Table 1). However, a statistically significant, 5-fold increase in the fractional calcium excretion index (FEI_{Ca}) was observed at 2 hours after dosing with PF-06869206 compared to vehicle, which was associated with a 2.8 and 2.6-fold reduction in PTH levels at 2 and 4 hours after treatment, respectively. No statistically significant differences in 1,25D and FGF23 levels in vehicle- and PF-06869206-treated mice were observed at any of the study timepoints.

Acute administration of PF-06869206 promotes phosphaturia in Fgf23$^{-/-}$ mice and Galnt3$^{-/-}$ mice

To determine whether urinary phosphate excretion can be increased in the absence of FGF23, we next treated hyperphosphatemic Fgf23$^{-/-}$ mice with a single dose of PF-06869206 (200 mg/kg) or vehicle. Oral gavage with PF-06869206 elicited a 9.1-fold increase in FEI_{Pi} in Fgf23$^{-/-}$ mice relative to vehicle at 4 hours after dosing (158.1±35.0 vs. 17.4±6.8; Figure 4A). This change was associated with a 20.1% reduction in plasma phosphate levels, from 14.9±1.0 mg/dL in vehicle-treated Fgf23$^{-/-}$ mice down to 11.9±0.4mg/dL in response to treatment with PF-06869206 (Figure 4B). Differences in FEI_{Pi} or plasma phosphate between vehicle- and PF-06869206-treated mice were no longer statistically significant at 24 hours after dosing (Figures 4A,B).

GALNT3 mutations are a more frequent cause of tumoral calcinosis than *FGF23* mutations (53). We therefore tested PF-06869206 also in hyperphosphatemic Galnt3$^{-/-}$ mice, where a single dose of PF-06869206 at 300mg/kg led to a statistically significant increase in FEI_{Pi} relative to vehicle at 2-4 hours after dosing (62.5±8.8 vs. 121.1±9.2; Figure 5A). This phosphaturic effect was associated with a 21.4% reduction in plasma phosphate levels down to 9.9±0.3mg/dL in mice treated with PF-06869206 relative to vehicle (Figure 5B). Similar to the findings in wild-type mice, plasma calcium levels in vehicle- and inhibitor-treated Galnt3-null mice remained indistinguishable throughout the study (10.3±0.29 vs. 10.0±0.36 mg/dl). Baseline FEI_{Ca} was 1.7±0.19 for both groups of animals, which remained unchanged for vehicle-treated mice at 2-4 and 24 hours (1.7±0.36 and 1.9±0.27, respectively), but increased 5.4-fold to 9.2±0.43 by 2-4 hours after dosing with PF-06869206 and returned to 1.8±0.59 by 24 hours.

Acute administration of PF-06869206 promotes phosphate excretion in CKD rats

We next assessed the activity of PF-06869206 in the 5/6 nephrectomy rat model of CKD. In this model, serum creatinine was increased to 0.8±0.1 mg/dL at 8 weeks after subtotal removal of renal tissue compared to 0.3±0.01 mg/dL in sham-operated control rats. A dose-dependent increase in FE_{Pi} was observed at 4 hours after acute administration of PF-06869206 at doses ranging from 10mg/kg to 300mg/kg, with the 300mg/kg dose eliciting an 8.8-fold increase in FE_{Pi} relative to vehicle (26.7±1.7% vs. 3.1±0.7%, Figure 6A). Increased phosphaturia was associated with a dose-dependent lowering of serum phosphate levels from 6.5±0.2mg/dL in vehicle-treated CKD rats down to 4.9±0.2mg/dL in CKD rats treated with PF-06869206 at a dose of 300mg/kg (Figure 6B). Modeling of exposure-FE_{PI} relationship suggested a trend towards saturation of the effect at the highest dose of 300mg/kg PF-06869206 administered to CKD rats (data not shown).

Long-term treatment with PF-06869206 is well-tolerated and elicits sustained pharmacological response in CKD rats

To test whether repeated dosing with PF-06869206 is well-tolerated and can persistently lower plasma phosphate levels in a model of CKD, 5/6 nephrectomized rats were treated by oral gavage once daily for 8 weeks with either vehicle, PF-06869206 (300mg/kg), or losartan (50mg/kg; angiotensin receptor blocker used for the treatment of hypertension in CKD); sham-operated rats treated with vehicle served as controls (Supplemental Figure 1). Briefly, blood and urine samples were obtained 2 weeks after the second surgical intervention to measure baseline levels of intact FGF23, bioactive PTH, plasma creatinine, FE_{Pi} and plasma phosphate (Study Day 0); furthermore baseline systolic blood pressure was determined. Animals were then randomly assigned to treatment with either vehicle, PF-06869206 or losartan. Longitudinal FE_{Pi} and plasma phosphate levels were determined using urine and blood samples that were collected 4 hours after administration of the first treatment dose (Study Day 1) and at 4 hours after dosing on Study Days 7, 21, 35 and 49. Systolic blood pressure, intact FGF23, bioactive PTH, and plasma creatinine were measured on Study Days 14, 28, 42, and 56.

Baseline plasma creatinine and systolic blood pressure were increased in 5/6 nephrectomized rats compared to sham-operated animals two weeks after the second surgical intervention (0.80±0.03mg/dL vs. 0.35±0.01mg/dL, $p<0.0001$, and 171.2±4.6mmHg vs. 124.7±5.8mmHg, $p<0.0001$, respectively; see below). Furthermore, at baseline, i.e. before treatment with either vehicle, PF-06869206 or losartan, all three groups of 5/6 nephrectomized rats showed indistinguishable levels of urinary phosphate excretion (FE_{Pi}: 28.0±2.5%, 29.0±1.0%, and 29.5±2.2%, respectively); these levels were higher with statistical significance than those of sham-operated animals (FE_{Pi}: 12.1±0.9%) (Supplemental Figure 2A). However, baseline plasma phosphate levels were comparable for all four study groups, namely 7.3±0.2mg/dL for the sham-operated rats, and 7.0±0.2mg/dL, 6.8±0.2mg/dL or 6.9±0.2mg/dL for the CKD rats assigned to treatment with vehicle, PF-06869206 or losartan, respectively (Supplemental Figure 2B).

Daily dosing with PF-06869206 for 8 weeks was well-tolerated as demonstrated by weight gains that were indistinguishable throughout the entire study duration for 5/6 nephrectomized rats receiving vehicle or the NPT2a inhibitor (data not shown). No deaths occurred in the PF-06869206 treatment group. At the end of the study, organ weights (liver, kidney and heart), as well as serum liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) revealed no difference for 5/6 nephrectomized rats treated with either vehicle or PF-06869206 (data not shown).

In vivo effects of PF-06869206

On Study Day 1, 5/6 nephrectomized rats showed a statistically significant increase in FE_{Pi} associated with a decrease in plasma phosphate levels 4 hours after treatment with PF-06869206 relative to vehicle (Figure 7A,B); this is consistent with the findings in 5/6 nephrectomized rats receiving a single dose of the NPT2a inhibitor (Figure 6). The phosphaturic effect of PF-06869206 was sustained on Study Days 7, 21, 35 and 49 as indicated by the consistent increase in FE_{Pi} four hours after each dose and the corresponding reduction in plasma phosphate levels (Figures 7A,B). FE_{Pi} remained higher in vehicle- and losartan-treated 5/6 nephrectomized rats compared to sham-operated rats over the duration of treatment, resulting in a trend towards lower plasma phosphate levels that was statistically significant for selected timepoints in the 5/6 nephrectomized rats compared to sham-operated rats (Figure 7A,B).

Repeated dosing of the 5/6 nephrectomized rats with PF-06869206 did not reduce plasma creatinine levels or blood pressure relative to vehicle-treated CKD rats (Figures 8A,B). In contrast, treatment with the angiotensin receptor blocker losartan reduced blood pressure levels in 5/6 nephrectomized animals down to the level of sham-operated control rats (Figure 8B), but did not elicit any change in FE_{Pi} or plasma phosphate levels relative to vehicle-treated CKD rats throughout the 8-week study (Figures 7A,B).

FGF23 and PTH levels were increased at statistically significant levels in 5/6 nephrectomized rats relative to sham-operated animals on Study Day 0, i.e. two weeks after the second surgical intervention (503.2±47.5pg/mL vs. 281.1±22.7pg/mL, $p<0.0001$, and 144.0±14.4pg/mL vs. 53.6±12.2pg/mL, $p<0.0001$, respectively) (Figures 9A,B). Although daily dosing with PF-06869206, which continued for 8 weeks, elicited an unabated increase in FE_{Pi} along with a reduction in plasma phosphate levels in 5/6 nephrectomized rats (Figures 7A,B), no statistically significant decline over time in PTH or FGF23 levels were observed (Figures 9A,B).

DISCUSSION

Our studies with PF-06869206 showed that selective pharmacological inhibition of NPT2a promotes efficient and dose-dependent urinary excretion of phosphate, thereby lowering plasma phosphate levels. Urinary phosphate excretion increased by about 16-fold when using the highest dose of PF-06869206 (500 mg/kg), resulting in a reduction of plasma phosphate levels by approximately 6 mg/dL in wild-type C57BL/6 mice, which is consistent with recently reported findings (52). There was no obvious saturation of the phosphaturic effect suggesting that the phosphate-lowering effect could be maximized further, possibly by improving the pharmacokinetics profile or potency of the inhibitor. In addition to its phosphaturic effect, PF-06869206 increased urinary calcium excretion, but no change in plasma calcium concentration was observed, as shown also by Thomas et al. (52). The mechanism underlying the calciuric effect of the NPT2a inhibitor remains unknown. However, it was associated with a decline in PTH levels, which could be sufficient to transiently reduce distal tubular calcium reabsorption. This would imply that elevated extracellular levels of phosphate increase PTH secretion, as recently shown (54); conversely an acute reduction in blood phosphate levels can lower PTH levels. Our single-dose studies with the NPT2a inhibitor revealed no statistically significant decline in FGF23 levels, which is similar to the observations by Thomas et al. (54).

Consistent with previously reported *in vitro* data (49), we confirmed specificity of the inhibitor for NPT2a through studies with genetically altered mice. Wild-type and Npt2c-null animals revealed indistinguishable increases in urinary phosphate excretion in response to treatment with PF-06869206 (300 mg/kg) that lead in both mouse strains to similar reductions in plasma phosphate levels. Npt2a-null mice showed lower plasma phosphate levels at baseline compared to wild-type mice, as expected (55). However, Npt2a-null mice treated with PF-06869206 showed no increase in urinary phosphate excretion and thus no change in plasma phosphate level, i.e. findings that are indistinguishable from those in vehicle-treated animals. These findings confirmed *in vivo* that PF-06869206 affects only reabsorption of phosphate through NPT2a, the most prominent phosphate transporter, and that the compound does not affect the closely related transporter NPT2c.

PF-06869206 was well-tolerated in mice and rats. The lack of obvious side effects is consistent with the *in vitro* and *in vivo* selectivity of the inhibitor for NPT2a, as well as the restricted expression of this transporter to the S1-S3 segments of the renal proximal renal tubules (56). It is therefore conceivable that patients with hyperphosphatemia caused by genetic mutations in FGF23, GALNT3, or KLOTHO would benefit from long-term inhibition of NPT2a-dependent phosphate reabsorption with PF-06869206 or an analog thereof (21-26).

This conclusion is supported by our findings with Fgf23-null mice (50, 57), in which the profound elevation of plasma phosphate was effectively reduced by the NPT2a inhibitor. In comparison to vehicle-treated animals, urinary phosphate excretion increased more than 9-fold in response to a single oral dose of PF-06869206 at 200mg/kg, thereby reducing blood phosphate levels by approximately 3 mg/dL. Similar results were observed in Galnt3-null mice, a second model of tumoral calcinosis, in which urinary phosphate excretion was increased and plasma phosphate levels were reduced upon treatment with PF-06869206. Even such a moderate phosphate-lowering effect could provide meaningful clinical benefits since a recently reported patient with a compound heterozygous FGF23 mutation had shown, in response to chronic hemodialysis treatment, a similar reduction in blood phosphate levels, which resulted in a substantial improvement of his profound extraosseous calcifications (25). Long-term treatment with a NPT2a inhibitor could thus be helpful in the treatment of patients affected by different forms of tumoral calcinosis (21-26). Likewise, patients with acquired or inherited conditions that can be associated with chronically elevated serum phosphate levels may benefit from adjuvant treatment with an inhibitor of proximal tubular phosphate reabsorption similar to PF-06869206.

Furthermore, pharmacological inhibition of NPT2a is expected to increase urinary phosphate excretion in patients with early stage CKD, which could reduce the risk of vascular calcification, and possibly kidney disease progression and premature mortality. We therefore investigated rats that had undergone subtotal nephrectomy, resulting two weeks after the second surgical procedure in doubling of plasma creatinine levels, i.e. the equivalent of CKD stage 2-3 in humans. Similar to published findings in rodents with normal renal function and in 5/6 nephrectomized mice (52), our study demonstrated a dose-dependent increase in urinary phosphate excretion in 5/6 nephrectomized rats upon acute administration of PF-06869206. However, the phosphaturic effect was less pronounced than in wild-type mice resulting only in a small decline in plasma phosphate levels (<1.5 mg/dL), at the highest PF-06869206 dose tested (300mg/kg). This limited response to the NPT2a inhibitor could be related to the substantially reduced mass of functional renal tissue, possibly in combination with an increased total phosphate burden due to prolonged impairment of kidney function. Consequently PF-06869206 may need to be administered in CKD for extended periods of time.

We therefore explored the long-term efficacy of PF-06869206 using animals that had undergone 5/6 nephrectomy and thus had doubled their plasma creatinine level by the time treatment with the NPT2a inhibitor was started. At basline, vehicle-treated CKD rats showed substantially higher urinary phosphate excretion than sham-operated animals, which is likely the result of elevations in FGF23 and PTH that had occurred secondary to impaired renal function. Sustained elevations in FGF23 and PTH over the course of treatment with vehicle or losartan in

5/6 nephrectomy rats led to sustained phosphaturia at all timepoints and a trend towards lower plasma phosphate at selected timepoint compared to vehicle-treated sham-operated rats. Daily administration of PF-06869206 caused in the 5/6 nephrectomized rats a further increase in urinary phosphate excretion, as determined through the use of metabolic cages to collect urine over 4 hours after oral dosing. The phosphaturic effect of PF-06869206 was sustained over the 56 days treatment period and led to lower plasma phosphate concentrations at all investigated time points. Importantly, treatment with PF-06869206 failed to reduce plasma FGF23 and PTH concentrations, and there was no change in renal function as serum creatinine remained indistinguishable throughout the study. The NPT2a inhibitor thus failed to lower FGF23 levels despite a substantial increase in urinary phosphate excretion and a statistically significant decline in plasma phosphate levels. It is conceivable that PF-06869206 needs to be administered more frequently to maintain adequate plasma levels or that factors other than phosphate contribute to FGF23 production in CKD. Our studies confirmed those by Thomas et al. (49), namely that an acute reduction in PTH levels occurs in wild-type mice upon treatment with a single dose of PF-06869206. However, there was no PTH-lowerring effect of the NPT2a inhibitor upon repeat dosing in 5/6 nephrectomized rats. Thus, the previously observed acute effect of PF-06869206 on PTH levels in 5/6 nephrectomized mice may not be sustained (49). Additional studies are needed to further explore the role of PF-06869206 on PTH synthesis and secretion.

It is plausible that the phosphaturic response to the NPT2a inhibitor will be different in alternate rodent models of impaired renal function. For example, toxin-induced AKI or CKD, induced by a single injection of folic acid (58) or by the prolonged consumption of adenine-supplemented food (42), respectively, may show little or no phosphaturia in response to treatment with PF-06869206 because of severely impaired, toxicity-induced proximal tubular function. In contrast, mild CKD caused by homozygous Col4-ablation (32, 59) or by homozygous, podocyte-specific CTCF-ablation (60) may show a phosphaturic response to the NPT2a inhibitor that is similar to that observed in animals with normal renal function. Long-term PF-06869206 treatment may thus increase fractional phosphate excretion in other CKD models more robustly than was observed with the current 5/6 nephrectomy model, and this may lead to a decline in FGF23 levels.

In summary, PF-06869206, a selective NPT2a inhibitor, promoted urinary phosphate excretion in wild-type and Npt2c-null mice without evidence for systemic toxicity. It furthermore lowered plasma phosphate levels in hyperphosphatemic Fgf23-null mice and Galnt3-null mice, thus raising the possibility that patients with tumoral calcinosis or other hyperphosphatemic disorders could benefit from treatment with a NPT2a inhibitor. Long-term treatment with PF-

06869206 resulted in a statistically significant increase in urinary phosphate excretion in rats with CKD due to 5/6 nephrectomy that reduced plasma phosphate levels. Although the limited hypophosphatemic effect in the current CKD model failed to reduce elevated FGF23 or PTH levels, it is conceivable that other models will show a more pronounced effect. Our results provide evidence that chronic treatment with PF-06869206 or analogs thereof could be suitable for treating both genetic and acquired disorders with impaired phosphate excretion and thus hyperphosphatemia.

METHODS

Isolation of primary rat proximal renal tubular (PRT) cells

PRT cells were isolated from the kidneys of Sprague-Dawley (SD) rats using a protocol adapted from Brown et al. (61). Briefly, cortical slices were minced to approximately 1mm^3 pieces that were suspended in isolation medium (RPMI, 5% Fetal calf serum, Penicillin, Streptomycin). The suspension was passed through a 40μm nylon sieve and centrifuged. The resulting cell pellet was re-suspended in fresh isolation medium and centrifugation-resuspension steps were repeated once before the cell suspension was loaded on top of discontinuous Percoll gradients with densities of 1.04 and 1.07g/mL and centrifuged. PRT cells at the intersection of the gradients were aspirated, rinsed and re-suspended in rat renal epithelial growth medium (DMEM/Ham's F-12 basal medium 1:1, HEPES 10mM, mouse epithelial growth factor 10ng/mL, T3 5pM, ascorbic acid 3.5μg/mL, transferrin 5μg/mL, PGE 25 ng/mL, sodium selenite 8.65ng/mL, G418 100μg/mL, insulin 5μg/mL, L-glutamine 4mM, 10% fetal calf serum). The cell suspension was passed through a large bore needle three times to separate aggregated cells and the cell yield was quantified using a cell counter.

Phosphate uptake in proximal renal tubular (PRT) cells

Freshly isolated PRT cells were re-suspended in phosphate-free Krebs buffer (NaCl 140mM, KCl 5.4mM, $MgSO_4$ 1.2mM, glucose 5mM, $CaCl_2$ 2mM, HEPES 10mM, TRIS base; pH 7.4) at a density of 300,000 cells/mL. Vehicle (DMSO) or PF-06869206 (30nM to 30μM) was added to the PRT cell suspension for 30 minutes. Sodium phosphate (100 μM) labeled with ^{32}P at 1μCi/mL was then added to the cell suspension. After 5 minutes, cells were rinsed in phosphate-free Krebs buffer, transferred to scintillation vials and lysed with scintillation fluid. Cell-associated radioactivity was detected with a scintillation counter (Beckman-Coulter).

Experimental animals

Npt2a$^{-/-}$ and Npt2c$^{-/-}$ mice used in the experiments had been obtained from Jackson Laboratory, Bar Harbor, ME and Drs. Segawa and Miyamoto, Tokushima, Japan (56), respectively. Both animals had been backcrossed to the C57BL/6 background for more than ten generations; genotyping by PCR amplification of genomic DNA was performed as previously described (62). Fgf23$^{+/-}$ mice were provided by Dr. Lanske, Harvard Dental School, Boston, MA (50), and Galnt3$^{+/-}$ mice were provided by Dr. Kelly Roszko, NIH, who had obtained them from Dr. Econs, Indianapolis, IN (51); both animals are on the C57BL/6 background and were

genotyped as previously described (50, 51). To generate Fgf23$^{-/-}$ and Galnt3$^{-/-}$ mice, heterozygous males and females of either strain were mated; males and females were used for experiments. Wild-type C57BL/6 mice served as controls.

Male SD rats with 5/6 nephrectomy were obtained from Charles River Laboratories (surgery code 56NEPHREX) and used in acute dose-response studies where PF-06869206 was administered once by oral gavage. For the chronic study where the inhibitor was given once daily for 8 weeks, male SD rats were obtained from Charles River Laboratories and underwent right unilateral nephrectomy followed 7 days later by ligation of 3-5 sub-branches of the left primary renal trunk artery; sham-operated rats served as controls.

Mice and rats included in acute dosing studies were provided ad libitum with a standard rodent diet containing 0.3% phosphorus (non-phytate, 5053, LabDiet). Sham-operated and 5/6 nephrectomy rats included in the repeat dosing study were provided ad libitum with a standard rodent diet containing 0.6% phosphorus (non-phytate, 8640, Harlan Teklad). The time window of treatment administration was consistent across all studies (7-9am).

Experimental in vivo procedures

PF-06869206 was suspended in 0.5% methylcellulose and administered to mice or rats by oral gavage at a dosing volume of 10mL/kg. In acute studies where a single ascending dose of PF-06869206 was administered to C57BL/6 mice or 5/6 nephrectomy rats, animals were placed in metabolic cages immediately after compound administration; urine and blood were collected at selected end points after dosing. In single dose administration studies involving Npt2a$^{-/-}$, Npt2c$^{-/-}$, Fgf23$^{-/-}$, Galnt3$^{-/-}$ mice and wild-type control mice urinary parameters were measured in spot urine samples. In the chronic treatment study where 5/6 nephrectomy rats were dosed daily with PF-06869206 for 8 weeks, urine samples were collected in metabolic cages and urine and blood were analyzed at baseline and serially throughout the study time (see Supplemental Figure 1). In this study, blood pressure was measured via indirect tail-cuff PV plethysmography under light (0.75%) isoflurane anesthesia.

Urine and blood sample analysis

Urinary phosphate (uPi), urinary calcium (uCa), urinary creatinine (uCr), serum or plasma phosphate (Pi), plasma calcium (Ca), and serum or plasma creatinine (Cr) were measured either by using a clinical chemistry analyzer or by spectroscopical measurements using assay kits (Abcam plc, Cambridge, UK for Pi and Stanbio Laboratory, Boerne, Texas for urine creatinine). Fractional excretion of phosphate index (FEI_{Pi}) in mice was calculated as

uPi*100/(Pi * uCr) and the same formula was used to calculate fractional excretion of calcium index in these animals by replacing Pi with Ca (FEI_{Ca}). Fractional phosphate excretion (FE_{Pi}) in rats was calculated as (uPi * Cr)/(Pi * uCr) (%). Intact FGF23 and bioactive intact PTH were measured in plasma by ELISA (Immutopics, #60-6800 and #60-2700, respectively), and 1,25D was determined in plasma using a kit from MyBioSource, San Diego, CA (MBS2602146).

Statistical analysis

Mixed effects models with appropriate correlation structure, terms, and variance adjustments were performed to test for associations between treatment groups over time (Figures 3, 4, 5, 7, 8, 9). ANOVA with appropriate terms and adjustments to ensure the assumptions are met were performed to test for associations between treatment groups (Figures 1, 2, 6, Supplemental Figure 2 and Table 1). To identify a dose response relationship between treatment groups, appropriate polynomial contrasts were specified. To identify statistically significant associations between treatment groups versus a control group, Dunnett contrasts were specified. To identify any pairwise statistically significant associations between treatment groups, Tukey's contrasts were specified. All multiple comparisons used the false discovery rate (fdr) adjustment of the p-values; a p-value <0.05 was considered statistically significant. All analyses were performed using R (version 3.5).

Study approvals

Studies were conducted in accordance with the current guidelines for animal welfare and all procedures were reviewed and approved by the Institutional Animal Care and Use Committees at Pfizer, MGH and Plato BioPharma, Inc.

AUTHOR CONTRIBUTIONS

VC, HS, KJF and HJ conceived, designed, analyzed data and interpreted results of the studies. JG conducted statistical analysis of all experimental data. HS and AHN performed in vivo experiments, generated data and contributed to data analysis. JK coordinated the execution of studies at Plato BioPharma, Inc. and contributed to data analysis. MR generated and genotyped all genetically modified mice used in the studies. VC, HS, JG and HJ wrote the manuscript and all authors reviewed and/or edited the manuscript.

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The *in vitro* studies testing the effect of PF-06869206 on phosphate uptake in PRTs were conducted in the laboratory of Pr. Colin D. A. Brown, Newcastle University, Newcastle upon Tyne, UK. Newcastle University received PF-06869206 through a Service Agreement between the Pfizer CVMET/IMRU Research Unit and Solvo Biotechnology, a Contract Research Organization (CRO) that works with Newcastle University to provide its services.

The long-term *in vivo* study testing the effect of PF-06869206 in 5/6 nephrectomy rats was conducted at Plato BioPharma, Inc., Westminster, CO, USA. Plato BioPharma, Inc. is a CRO that received PF-06869206 through a Service Agreement with the Pfizer CVMET/IMRU Research Unit. Supported by NIH grants P01 DK011794 (subproject 3) and DK046718 to HJ.

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FIGURES AND FIGURE LEGENDS

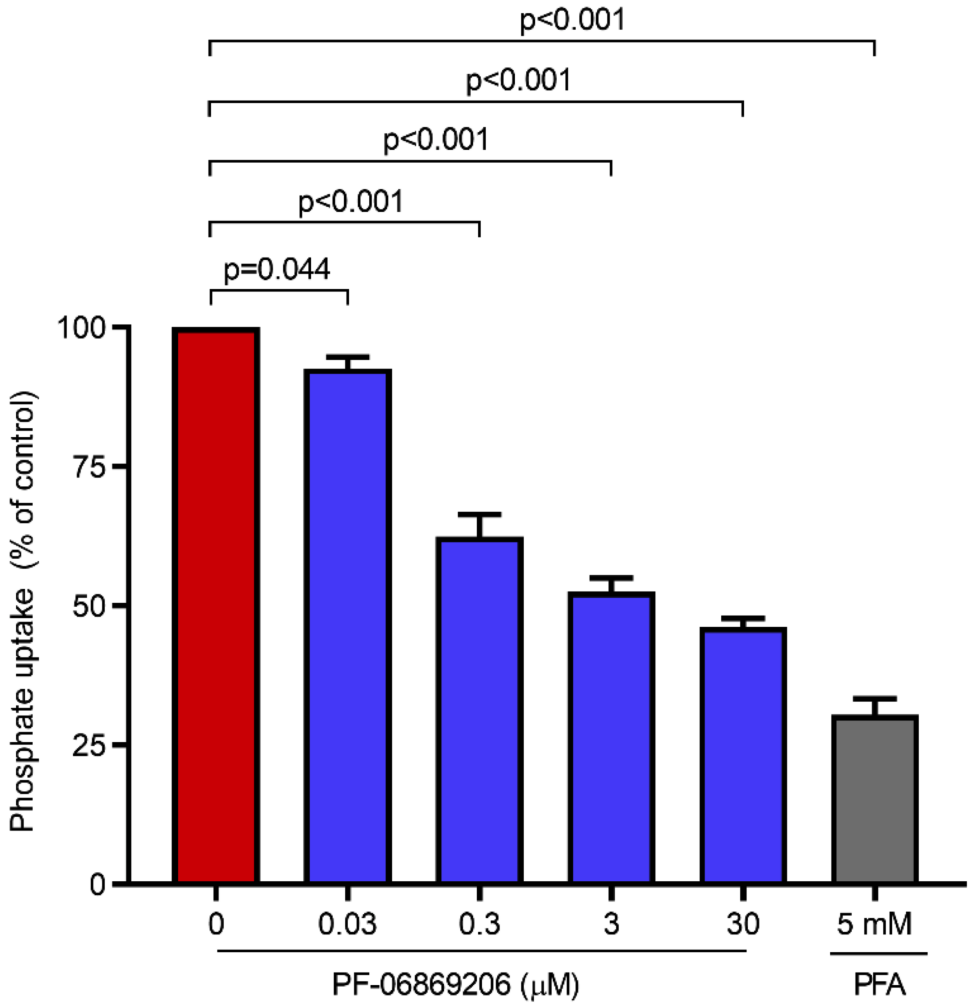

Figure 1: **PF-06869206 inhibits phosphate uptake in primary rat proximal renal tubule (PRT) cells**. PRT cells were treated with increasing doses of PF-06869206 (30nM to 30μM), vehicle (DMSO, 0 mg/kg PF-06869206) or phosphonoformic acid (PFA, 5mM, positive control) for 30 minutes. ^{32}P was then added to the cells for 5 minutes and the cells were rinsed to remove unincorporated ^{32}P. Phosphate uptake by the cells was derived from cell-associated radioactivity measured after cell lysis. Phosphate uptake data are reported as % of vehicle control and shown as Mean±SEM of cells from 3 animals; $p<0.05$ indicates a statistically significant difference (mixed effects model).

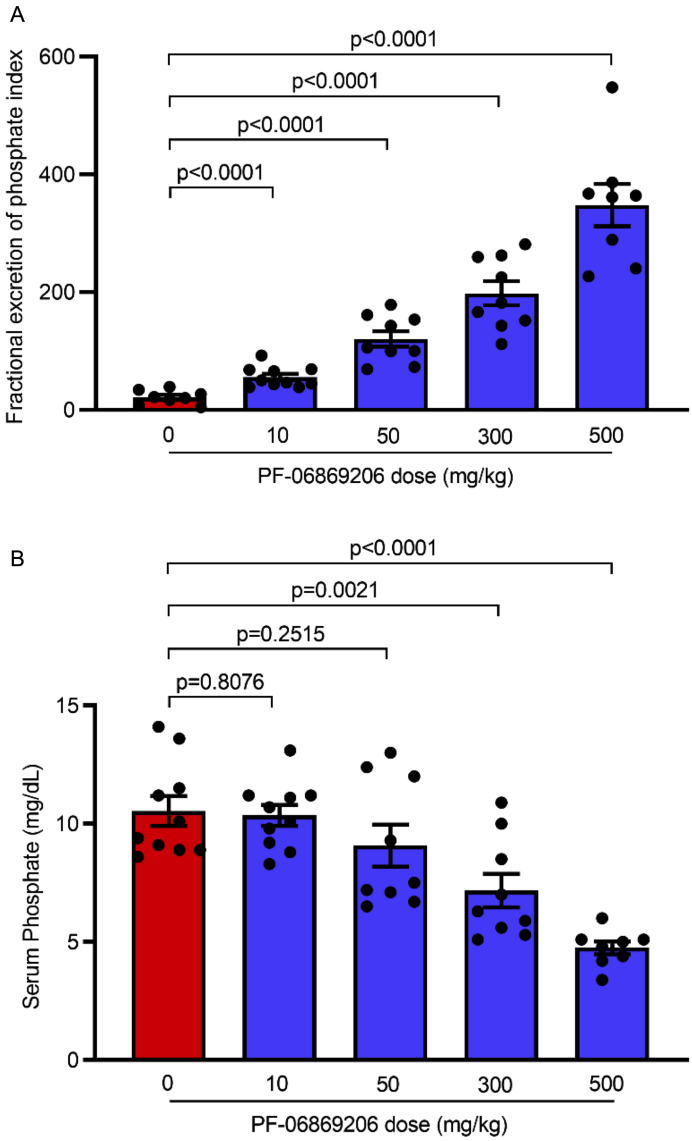

Figure 2: Acute administration of PF-06869206 elicits a dose-dependent increase in urinary phosphate excretion and reduction in serum phosphate level in C57BL/6 mice. Wild-type C57BL/6 mice were treated with a single oral dose of PF-06869206 administered at 10, 50, 300, or 500 mg/kg (n=9-10 mice per dose-group) or vehicle (0 mg/kg PF-06869206, n=8 mice) and immediately placed in metabolic cages. Fractional excretion of phosphate index (A) and serum phosphate level (B) were derived from analysis of urine and blood samples collected at 4 hours after dosing. Data are shown as individual data points and Mean±SEM for each dose group; $p<0.05$ indicates a statistically significant difference (mixed effects model).

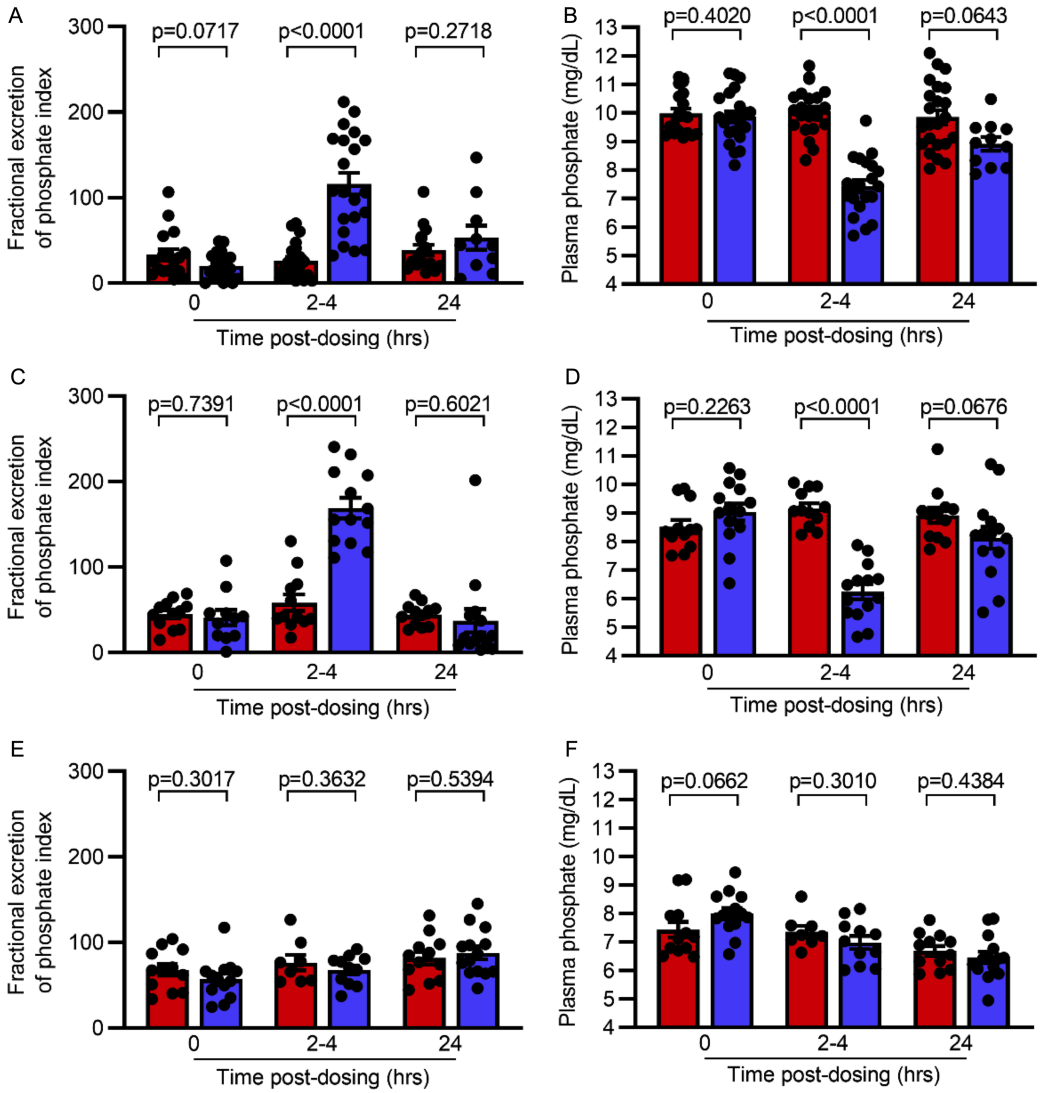

Figure 3: PF-06869206 increases urinary phosphate excretion and reduces plasma phosphate in wild-type and Npt2c-null mice, but not in Npt2a-null mice. Wild-type mice (A, B), Npt2c-null mice (C, D), and Npt2a-null mice (E, F) were treated with a single oral dose of vehicle (red bars) or PF-06869206 (300 mg/kg, blue bars). Blood and spot urine samples were obtained before treatment (n=10-14 mice per genotype), at 2-4 hours after dosing (n=8-23 mice per genotype) or 24 hours after dosing (n=4-14 mice per genotype). Fractional excretion of phosphate index (A, C, E) and plasma phosphate levels (B, D, F). Data are shown as individual data points and Mean±SEM for each experimental group; $p<0.05$ indicates a statistically significant difference (1-way ANOVA).

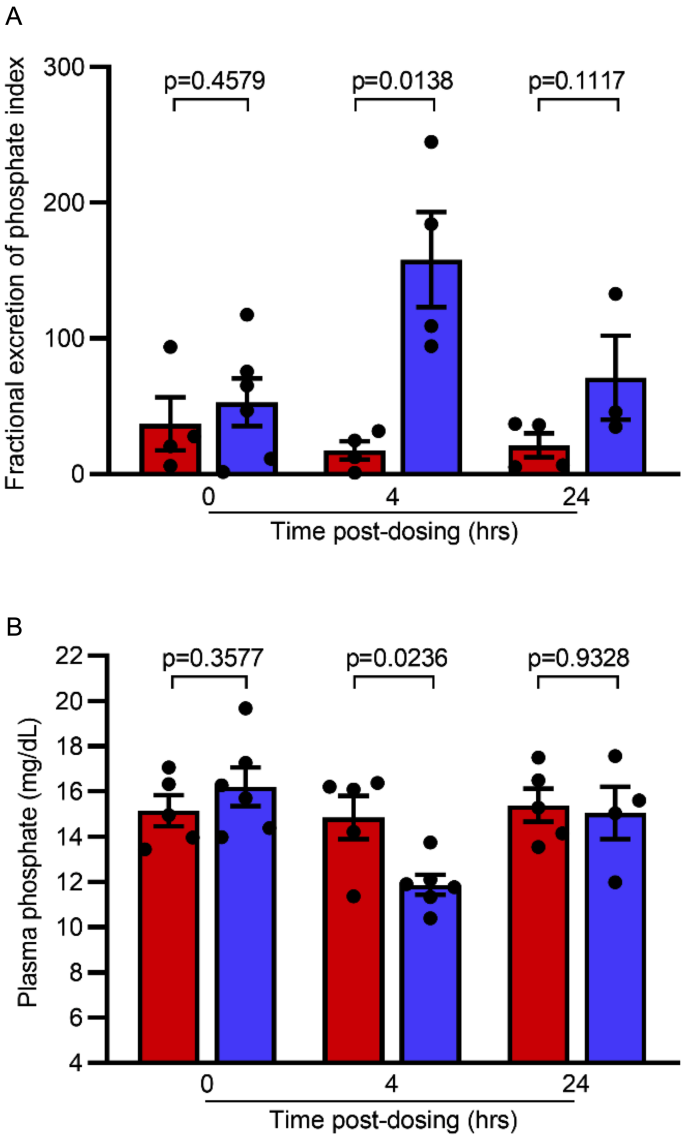

Figure 4: PF-06869206 promotes urinary phosphate excretion and reduces plasma phosphate in Fgf23-null mice. Fgf23-null mice were treated with a single oral dose of vehicle (n=5, red bars) or PF-06869206 (200mg/kg, n=6, blue bars). Blood and spot urine samples were obtained before treatment, and at 4 hours and 24 hours after dosing (n=5-6 mice per treatment group). Fractional excretion of phosphate index (A) and plasma phosphate levels (B). Data are shown as individual data points and Mean±SEM for each experimental group; $p<0.05$ indicates a statistically significant difference (1-way ANOVA).

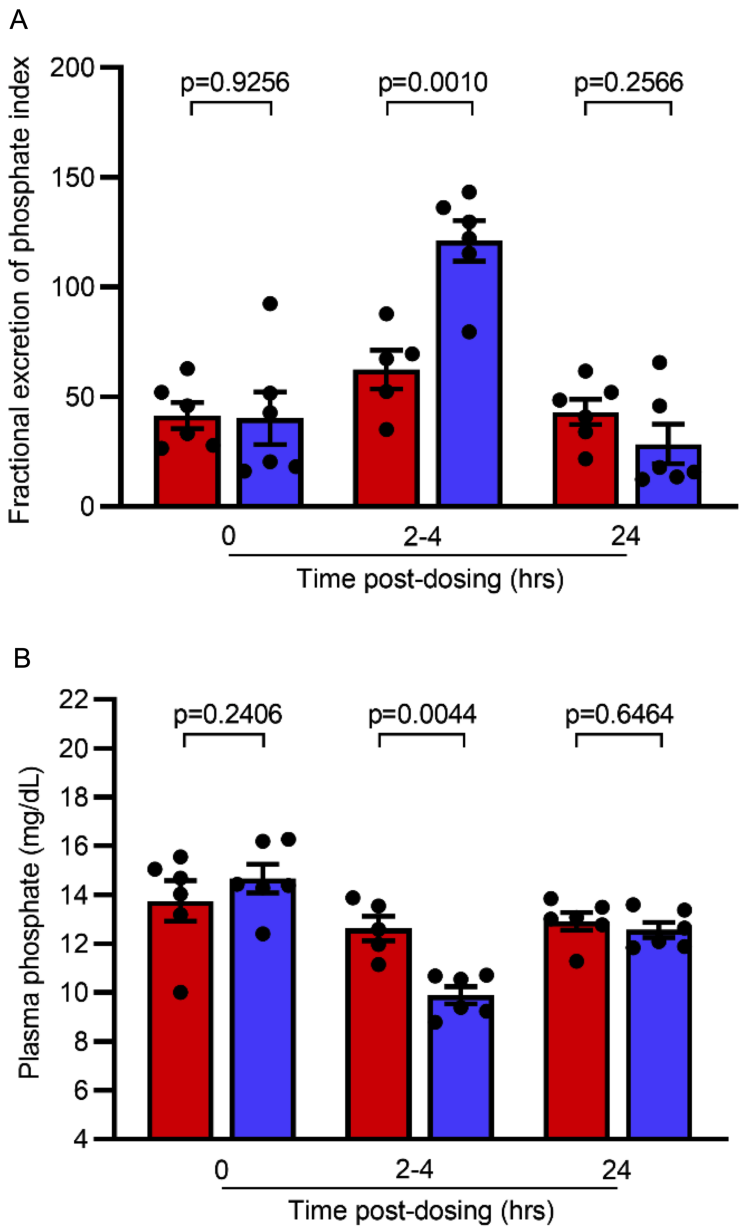

Figure 5: PF-06869206 promotes urinary phosphate excretion and reduces plasma phosphate in Galnt3-null mice. Galnt3-null mice were treated with a single oral dose of vehicle (n=6, red bars) or PF-06869206 (300mg/kg, n=6, blue bars). Blood and spot urine samples were obtained before treatment and at 2-4 or 24 hours after dosing. Fractional excretion of phosphate index (A) and plasma phosphate levels (B). Data are shown as individual data points and Mean±SEM for each experimental group; $p<0.05$ indicates a statistically significant difference (1-way ANOVA).

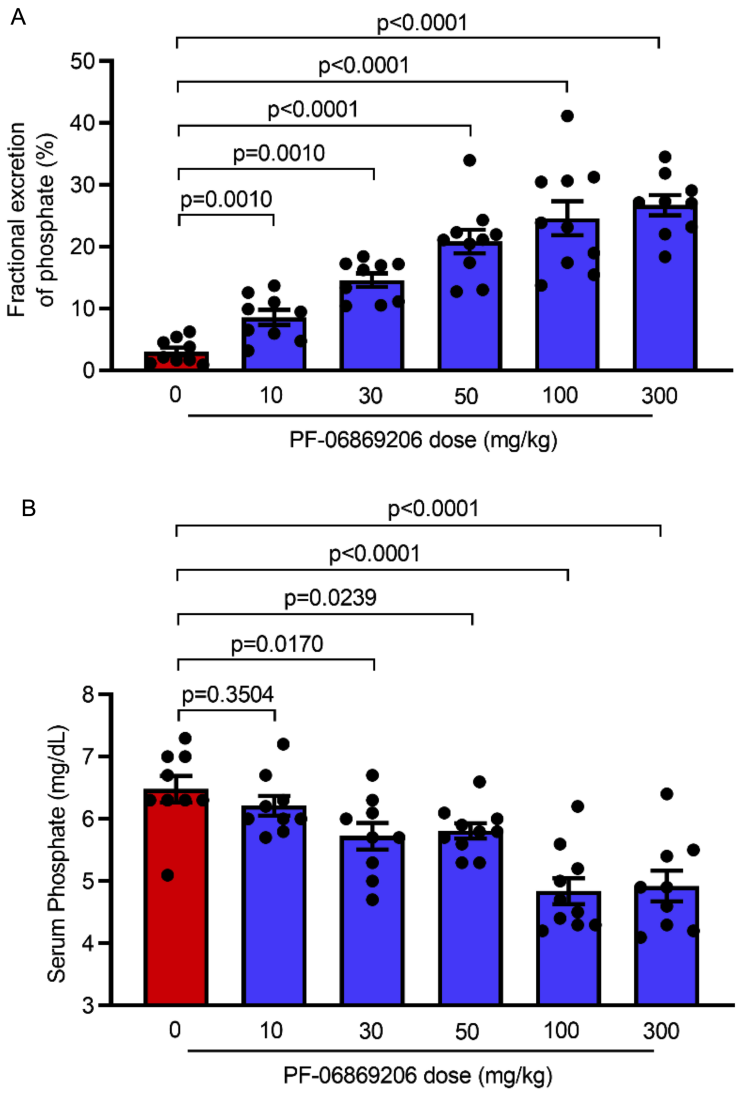

Figure 6: Acute administration of PF-06869206 dose-dependently increases fractional excretion of phosphate and reduces serum phosphate level in CKD rats. Sprague Dawley rats with CKD due to 5/6 nephrectomy were treated with a single oral dose of PF-06869206 at 10, 30, 50, 100 or 300mg/kg (n=9-10 rats per dose-group) or vehicle (0mg/kg PF-06869206, n=9 rats). Rats were placed in metabolic cages immediately after dosing and blood and urine were collected at 4 hours after dosing. Fractional excretion of phosphate (A) and serum phosphate levels (B). Data are shown as individual data points and Mean±SEM for each dose group; $p<0.05$ indicates a statistically significant difference (mixed effects model).

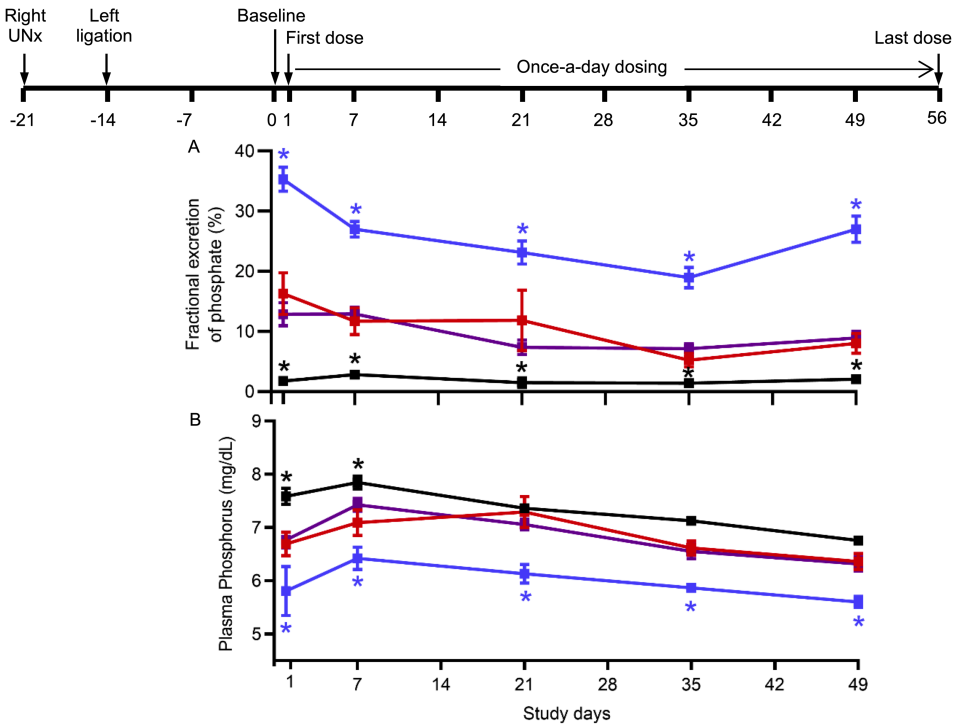

Figure 7: Long-term administration of PF-06869206 elicits sustained daily increases in fractional excretion of phosphate and reductions in plasma phosphate in CKD rats. Sprague Dawley rats with CKD due to 5/6 nephrectomy were treated once daily for 8 weeks with PF-06869206 (300mg/kg/day, n=12 rats, blue line) or vehicle (n=12 rats, red line). Fractional excretion of phosphate (A) and plasma phosphate (B) were derived from analysis of urine and blood samples collected at 4 hours after the first treatment dose on study Day 1 and on study Days 7, 21, 35 and 49. CKD rats receiving 50mg/kg/day of the clinical drug losartan (n=12 rats, pruple line) for 8 weeks were included for comparison to PF-06869206. Sham-operated rats treated with vehicle once daily for 8 weeks served as controls (n=8, black line). Data are shown as Mean±SEM, * $p<0.05$ versus Vehicle-treated 5/6 nephrectomized rats, 1-way ANOVA.

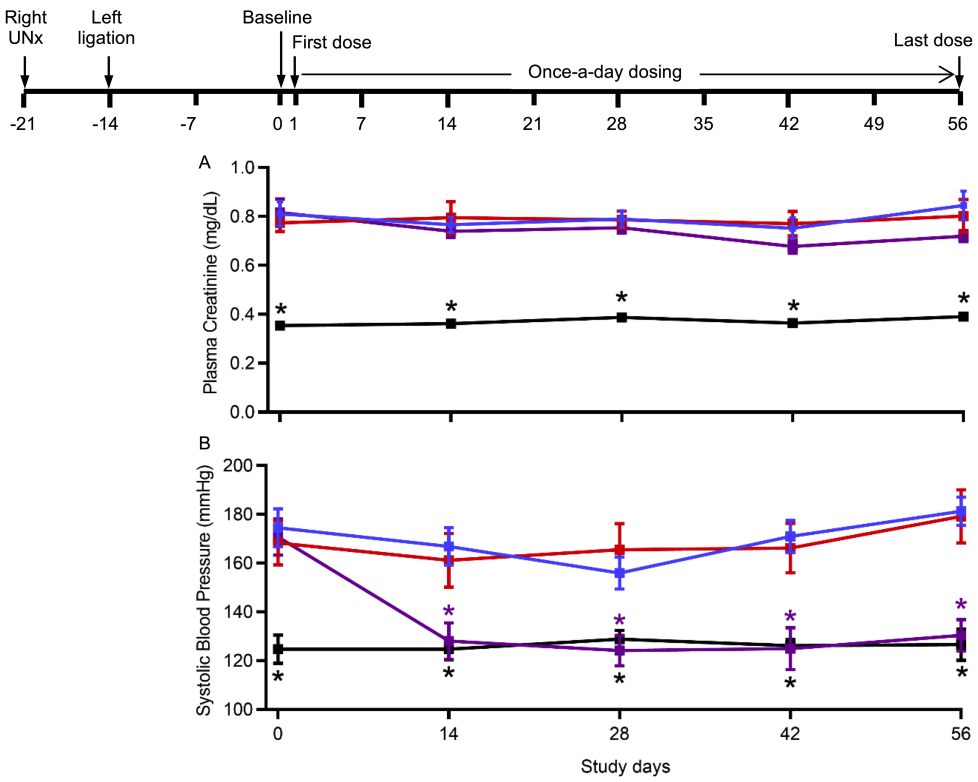

Figure 8: Long-term administration of PF-06869206 does not affect plasma creatinine level or blood pressure in CKD rats. Sprague Dawley rats with CKD due to 5/6 nephrectomy were treated once daily for 8 weeks with PF-06869206 (300mg/kg/day, n=12 rats, blue line) or vehicle (n=12 rats, red line). Plasma creatinine (A) and systolic blood pressure (B) were measured every other week from Day 0 (baseline) until Day 56 (endpoint). CKD rats receiving 50mg/kg/day of the clinical drug losartan (n=12 rats, purple line) for 8 weeks were included for comparison to PF-06869206. Sham-operated rats treated with vehicle once daily for 8 weeks served as controls (n=8, black line). Data are shown as Mean±SEM, * $p<0.05$ versus Vehicle-treated 5/6 nephrectomized rats, ANOVA.

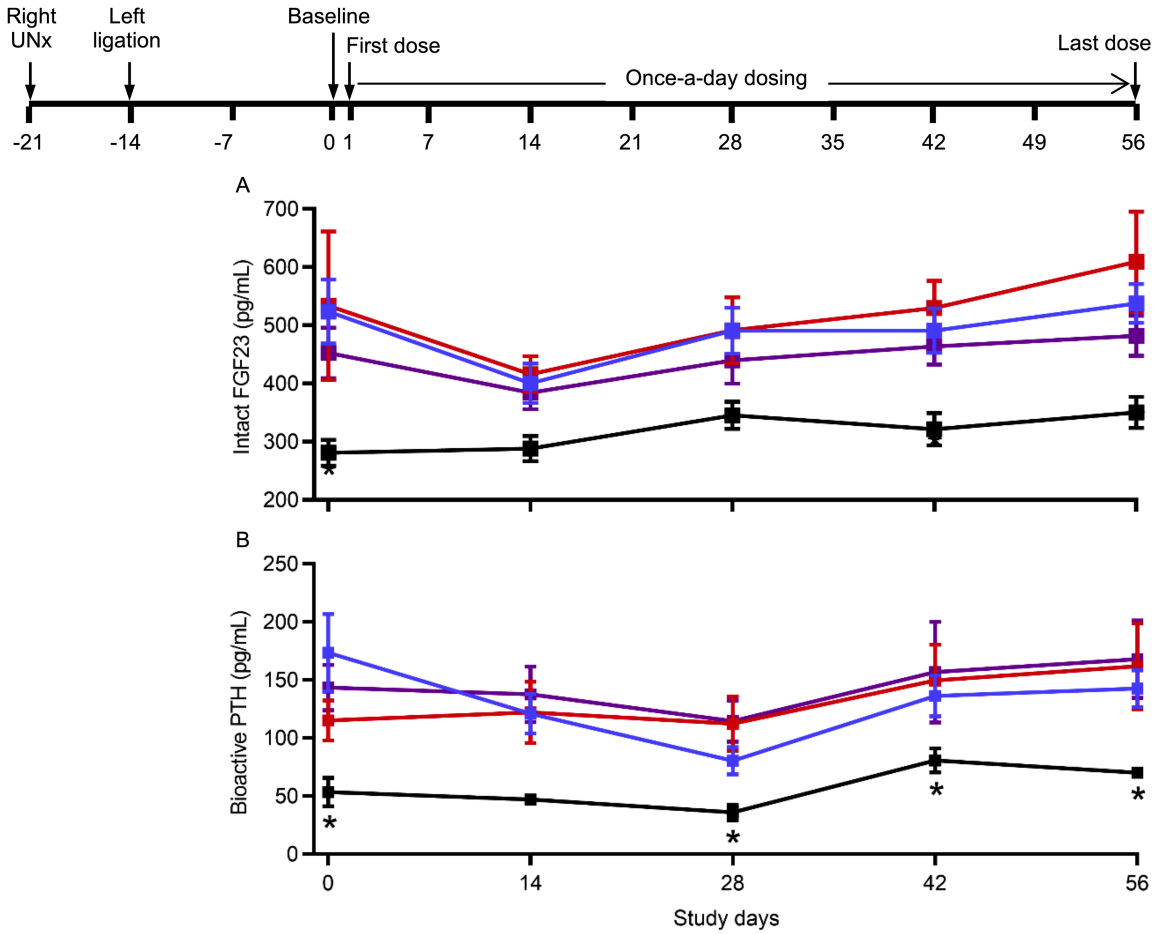

Figure 9: Long-term administration of PF-06869206 does not modulate plasma FGF23 or PTH level in CKD rats. Sprague Dawley rats with CKD due to 5/6 nephrectomy were treated once daily for 8 weeks with PF-06869206 (300mg/kg/day, n=12 rats, blue line) or vehicle (n=12 rats, red line). FGF23 (Panel A) and PTH (Panel B) were measured every other week from Day 0 (baseline) until Day 56 (endpoint). CKD rats receiving 50mg/kg/day of the clinical drug losartan (n=12 rats, purple line) for 8 weeks were included for comparison to PF-06869206. Sham-operated rats treated with vehicle once daily for 8 weeks served as controls (n=8, black line). Data are shown as Mean±SEM, * $p<0.05$ versus Vehicle-treated 5/6 nephrectomized rats, ANOVA.

Table 1: PF-06869206 acutely increases fractional excretion of calcium and decreases PTH(1-84) but does not change plasma calcium, 1,25(OH)2D or intact FGF23 in wild-type mice. Wild-type mice were treated with a single oral dose of vehicle or PF-06869206 (PF-'206, 300 mg/kg). Blood and spot urine samples were obtained in control, non-treated mice (baseline), and at 2, 4 or 24 hours after dosing. A statistically significant increase in fractional excretion of calcium index, relative to vehicle, was observed at 2 hours after treatment with PF-06869206 (*p<0.05 vs vehicle at 2 hours after dosing, 1-way ANOVA). In contrast, a statistically significant reduction in PTH(1-84) levels was observed at 2 and 4 hours (*p<0.05 vs. vehicle at 2 hours after dosing and &p<0.05 vs vehicle at 4 hours after dosing, 1-way ANOVA), while plasma calcium remained unchanged. No change was observed for $1,25(OH)_2D$ or intact FGF23.

		Plasma Ca mg/dL	**FEI_{Ca}**	**PTH(1-84)** pg/mL	**$1,25(OH)_2D$** ng/mL	**FGF23** pg/mL
Timepoint		Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Baseline		10.4 ± 0.2 *n=24*	7.9 ± 0.5 *n=20*	310.1 ± 25.4 *n=26*	3.0 ± 0.3 *n=4*	411.3 ± 31.4 *n=4*
2 hours	Vehicle	10.0 ± 0.6 *n=6*	3.6 ± 1.0 *n=6*	256.2 ± 40.1 *n=6*	2.3 ± 0.3 *n=6*	430.4 ± 44.6 *n=6*
	PF-'206	11.4 ± 0.5 *n=6*	18.3 ± 4.2 * *n=6*	91.4 ± 11.4 * *n=7*	1.8 ± 0.3 *n=6*	418.8 ± 38.4 *n=7*
4 hours	Vehicle	11.9 ± 0.3 *n=6*	3.7 ± 1.2 *n=6*	291.1 ± 28.3 *n=6*	2.6 ± 0.3 *n=6*	435.6 ± 21.0 *n=6*
	PF-'206	11.5 ± 0.3 *n=6*	5.9 ± 3.1 n=*5*	110.9 ± 29.1& *n=7*	2.7 ± 0.4 *n=6*	433.5 ± 25.1 *n=7*
24 hours	Vehicle	10.8 ± 0.6 *n=5*	4.3 ± 1.6 *n=5*	242.1 ± 41.2 *n=7*	1.7 ± 0.3 *n=7*	390.5 ± 14.6 *n=7*
	PF-'206	9.9 ± 0.5 *n=7*	5.9 ± 1.7 n=*7*	191.0 ± 43.1 *n=8*	2.4 ± 0.2 *n=8*	351.0 ± 31.9 *n=8*

*p<0.05 vs vehicle at 2 hours after dosing; &p<0.05 vs vehicle at 4 hours after dosing