Phosphorous Dysregulation Induced by MEK Small Molecule Inhibitors in the Rat Involves Blockade of FGF-23 Signaling in the Kidney

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MEK, a kinase downstream of Ras and Raf oncogenes, constitutes a high priority target in oncology research. MEK small molecule inhibitors cause soft tissue mineralization in rats secondary to serum inorganic phosphorus (iP) elevation, but the molecular mechanism for this toxicity remains undetermined. We performed investigative studies with structurally distinct MEK inhibitors GEN-A and PD325901 (PD-901) in Sprague-Dawley rats. Our data support a mechanism that involves FGF-23 signal blockade in the rat kidney, causing transcriptional upregulation of 25-hydroxyvitamin D₃ 1-alpha-hydroxylase (Cyp27b1), the rate-limiting enzyme in vitamin D activation, and downregulation of 1,25-dihydroxyvitamin D₃ 24-hydroxylase (Cyp24a1), the enzyme that initiates the degradation of the active form of vitamin D. These transcriptional changes increase serum vitamin D levels, which in turn drive the increase in serum iP, leading to soft tissue mineralization in the rat.

Key Words: MEK inhibitor; mineralization; phosphorous dysregulation; elevated vitamin D; FGF-23; PTH; NaPiIIa.

The RAF/MEK/ERK mitogen-activated protein kinase (MAPK) cascade is a molecular pathway that transduces signals from the cell surface to the nucleus, regulating diverse aspects of cellular survival, proliferation, and differentiation. Activating mutations of this pathway (EGFR, RAS, and BRAF) can lead to constitutive activation and drive tumorigenesis. As such, MEK is an attractive therapeutic target because its only known substrate is extracellular signal-regulated kinase (ERK), and several MEK small molecule inhibitors are currently in clinical trials (McCubrey et al., 2010).

Because of its central role in many cellular functions, MEK inhibition has the potential to also affect aspects of normal cellular homeostasis leading to undesired effects. In toxicology studies, a selective MEK inhibitor, PD-901, was reported to cause soft tissue and vascular mineralization in rats, which was preceded by serum inorganic phosphorus (iP) and 1,25-dihydroxyvitamin D₃ increases as early as 24 h after a single dose (Brown, 2010).

The findings observed with this MEK inhibitor, closely resemble the phenotype of Fibroblast Growth Factor Factor 23 (FGF-23) or klotho knockout mice. FGF-23 is a constitutively expressed bone-derived growth factor that inhibits phosphate reabsorption and activation by the kidney and participates in a bone-kidney axis coordinating systemic phosphate homeostasis and bone mineralization. FGF-23 null mice develop hyperphosphatemia, hypercalcemia, increased circulating 1,25-dihydroxyvitamin D₃ levels and tissue mineralization, and die after several weeks due to these effects (Shimada et al., 2004a,b). FGF-23 has also been implicated in several hypophosphatemic diseases like X-linked hypophosphatemic rickets/osteomalacia (XLH) and tumor-induced osteomalacia (Shimada et al., 2001).

Klotho, primarily expressed in the epithelial cells of the renal distal tubules, is an essential cofactor for FGF-23 signaling and is believed to be necessary for recognition of FGF-23 by target cells by increasing the affinity of the FGF receptor for its ligand (Urakawa et al., 2006). The klotho null phenotype largely overlaps with the FGF-23 null phenotype (hyperphosphatemia and increased serum levels of vitamin D). Klotho null mice have high circulating levels of FGF-23 that are possibly due to a positive feedback regulation resulting from the inability of FGF-23 to exert its biological function in the absence of klotho (Kuro-o et al., 1997; Nabeshima, 2008).

Additional evidence of involvement of the MEK pathway in the regulation of vitamin D has been reported through effects on enzyme gene expression by FGF-23. FGF-23 was shown to cause a dose-dependent decrease in Cyp27b1
(25-hydroxyvitamin D₃ 1-alpha-hydroxylase, the rate-limiting enzyme in vitamin D activation) messenger RNA (mRNA) in cultured human and mouse renal proximal tubule cells. These effects were abolished by a MEK small molecule inhibitor by blocking signal transduction through ERK1/2, thus implicating this signaling pathway (Perwed et al., 2007). Furthermore, treatment with the MEK small molecule inhibitor PD-901 resulted in an increase in the expression of Cyp27bl mRNA in the kidneys of HYP mice (hypophosphatemic mice) (Ranch et al., 2011).

GEN-A, a selective small molecule allosteric MEK inhibitor that demonstrated inhibition of tumor growth in mouse xenograft models, caused inorganic phosphate elevation followed by soft tissue mineralization in rat toxicology studies. GEN-A is structurally distinct from PD-901 and highly selective for its target, suggesting that the mechanism could potentially be an on-target effect of MEK pathway inhibition through regulation of FGF-23 activity in the kidney. Here, we provide evidence in support of this previously undetermined mechanism of MEK activity that resulted in undesired toxicity in rats.

**MATERIALS AND METHODS**

**Animal studies.** In an exploratory dose-ranging study to evaluate potential toxicologic effects, GEN-A was administered at doses of 0, 3, 10, or 30 mg/kg to male Sprague-Dawley rats (nine per group) by oral gavage once daily for 14 days, with an additional 7-day drug-free recovery period. Three males per dose group were utilized for toxicokinetic analysis, an additional three rats per group underwent terminal necropsy on day 15, and three rats per group underwent recovery necropsy on day 21. Blood was collected at necropsy via retro-orbital bleed from fasted animals for routine hematology and clinical chemistry evaluation and selected tissues (brain, eye, optic nerve, lacrimal gland, lymph nodes, heart, lung, liver, spleen, kidney, adrenal glands, pancreas, stomach, small and large intestines, and skin) were collected at necropsy and evaluated for histopathological findings.

In follow-up investigative toxicology studies, GEN-A was administered by oral gavage once daily for three consecutive days. Groups of four male rats were administered 0, 20, or 60 mg/kg of GEN-A and were necropsied at 12 h after the first dose, 24 h after the first dose, or 24 h after the third dose (96 h). PD-901 was administered as a single dose by oral gavage at 0 or 5 mg/kg to male Sprague-Dawley rats (nine per group) by oral gavage once daily for three consecutive days. Groups of four male rats were administered 0, 20, or 60 mg/kg of GEN-A and were necropsied at 12 h after dosing. The dose of 5 mg/kg was selected from the literature for causing increases in iP and tissue mineralization. Blood was collected at necropsy via retro-orbital bleed from fasted animals for routine hematology and clinical chemistry evaluation.

**Serum calcium, iP, and albumin analysis.** Serum calcium, iP, and albumin levels were measured with spectrophotometric assays on a Cobas Integra 400 Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN).

The product of Ca and iP (Ca × iP) levels was calculated and used to determine the relationship between changes in Ca × P and tissue mineralization. The relationship between Ca × iP and increased risk of tissue calcifications is well established, and it is routinely used in clinical practice.

**Vitamin D analysis.** Serum 1,25-dihydroxyvitamin D₃ levels were measured by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Calibration standards and quality control (QC) samples were prepared by spiking a known amount of 1,25-dihydroxyvitamin D₃ (Toronto Research Chemicals, Inc., ON, Canada) into water. Samples were prepared by spiking 10 µl of internal standard d₃-25-hydroxyvitamin D₃ (Sigma-Aldrich, St Louis, MO) into 1.0 ml of either sample, calibration, or QC, followed by 100 µl of 0.2% formic acid and 3 ml of methyl tert-butyl ether. The resulting samples were vortexed for 45 min and centrifuged at 1500 × g for 10 min. Supernatant (2.6 ml) was transferred to a 96-well plate, dried down, and reconstituted in 150 µl of methanol/water (1:1, vol/vol) prior to analysis by HPLC-MS/MS.

All samples were analyzed using two sets of standard curves and two sets of QCs in one batch. The HPLC-MS/MS system consisted of an Accela pump (Thermo Scientific, Waltham, MA), an H-TS-PAL autosampler (Leap Technologies, Carrboro, NC), and a AB SCIEX API 5000 (AB SCIEX, Foster City, CA) mass spectrometer with a turbo ion spray interface. A 30-µl aliquot of each sample was injected onto a reverse-phase HALO C18 column. The linear range was from 0.12 to 60 ng/ml for 1,25-dihydroxyvitamin D₃. The assay accuracy was within ± 20%.

**Serum FGF-23 and parathyroid hormone analysis.** FGF-23 and Parathyroid Hormone (PTH) levels in rat serum samples were measured using commercially available ELISA kits (Immunotopics, Inc., San Clemente, CA and Alpco, Salem, NH) following the manufacturers’ instructions.

**Phosphorylated ERK analysis.** Snap frozen rat kidney tissues were homogenized in RIPA buffer containing protease and phosphatase inhibitors (Roche Diagnostics; Sigma-Aldrich; Thermo Scientific, IL) and clarified by centrifugation. Protein concentrations of the lysates were determined using the BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL). Equal amounts of proteins were resolved on SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane, and incubated with antiphospho-ERK antibody (Cell Signaling Technology, Inc., Beverly, MA). After washing and incubating with secondary antibodies, immunoreactive proteins were visualized by the ECL detection system (GE Healthcare, Buckinghamshire, U.K.). The blot was stripped (Pierce stripping buffer) and reprobed using ERK2 antibody (BD Biosciences, Inc., Rockville, MD) to assess total ERK2 protein. Protein signal densities were quantified using an Image J System and ERK2 phosphorylation levels were expressed as a percentage of total ERK2 protein levels.

**Renal gene expression analysis by real-time PCR.** To evaluate the effects of MEK inhibitors on renal gene expression, 93 genes (including four housekeeping genes) were evaluated by real-time PCR (RT-PCR) from snap frozen kidneys obtained 12 and 24 h after dosing of vehicle and GEN-A (20 and 60 mg/kg) or PD-901 (5 mg/kg). RNA was extracted from kidney tissue using the QIAzol/TRIzol protocol (Qiagen, Valencia, CA). After the ethanol precipitation step, the RNA was further purified using the Qiagen RNeasy Mini Kit (Qiagen). The quality of the RNA was assessed by ribosomal RNA band integrity through electrophoresis on an Agilent BioAnalyzer using an RNA 6000 Nano LabChip (Agilent Technologies, Santa Clara, CA). Genomic DNA was degraded using the Genomic DNA Elimination Mixture. Complementary DNA (cDNA) was prepared using a standard cDNA synthesis reaction mixture and incubating at 42°C for 15 min, after which the reaction was stopped with a 5-min incubation at 95°C.

Real-time PCR was performed using a standard real-time PCR reaction cocktail on a custom 96-well PCR array containing custom primers for the genes of interest. The PCR reaction consisted of a two-step cycling program (1 cycle of 10 min at 95°C, followed by 40 cycles of 1 s at 95°C and 1 min at 60°C) in an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The ΔCt for each pathway-focused gene was calculated, and results were normalized to the average of four housekeeping genes for each sample. Hprt1, Ldh1, Actb, and Rplp1 (Hypoxanthine phosphoribosyltransferase 1, Lactate dehydrogenase A, beta Actin, and Ribosomal protein P1) were used as internal controls.

The fold change represents the average of four treated rat kidney samples relative to the average of four time-matched control kidney samples. Statistical p values were calculated by r-test.

**Na⁺/H⁺ immunochemistry and quantitative image analysis.** Immunochemistry (IHC) was performed on 4-µm thick formalin-fixed paraffin embedded tissue sections mounted on glass slides. All IHC steps were carried out on a Ventana Discovery XT (Ventana Medical Systems, Tucson, AZ).
autostainer. Pretreatment was performed with Cell Conditioner 1. Primary antibody, NPT2a (NaPiIIa) (Alpha Diagnostics, San Antonio, TX) was used at a concentration of 10 μg/ml and was incubated on slides for 1 h at room temperature. Ventana Rabbit OmniMap (Ventana Medical Systems) was used as the detection system. Ventana DAB and Hematoxylin II were used for chromogenic detection and counterstain.

Kidney sections were scanned at ×20 using a NanoZoomer (NDP) slide scanner (Hamamatsu Photonics). The digital images were analyzed using Definiens Developer software (Definiens AG). A rule set was custom written to analyze each image of the kidney IHC section. A low resolution (×0.2) Red-Green-Blue image was used to separate tissue from background and the cortex from the medulla, using a segmentation method (contrast split), which divides image regions depending on relative pixel intensities. The mean intensity value of the blue channel for the entire cortex was computed. Then, the kidney cortex area was subdivided into approximately 70 rectangular regions, and each region was analyzed at high resolution (×10). For each rectangular area, pixels with a blue intensity value less than 0.9 × mean intensity value of the entire cortex were classified as stained and the rest as unstained pixels. The relative stained area for the entire kidney was computed as the sum of the total stained pixels, divided by the sum of total pixels (total stained + total unstained pixels) from every subdivided region.

**Statistical analysis.** In the exploratory dose-ranging study of GEN-A, serum Ca, iP, and albumin were measured on each rat at several time points. A linear mixed effects model was fitted to the repeated measures data, with dose group, time point, and their interaction as fixed effects and rat as random effect, and the mean of each treatment group was compared with vehicle at each time point using Bonferroni’s t-test (Fitzmaurice et al., 2004). The association between Ca × iP and tissue mineralization was investigated using logistic regression (Agresti, 2002). Logistic regression is commonly used to estimate the association between a binary outcome and a set of explanatory variables. Here, for each tissue, it considers the log-odds of mineralization

$$\log\left(\frac{p}{1-p}\right) = \alpha + \beta \text{Ca} \times \text{iP},$$

where p is the probability of tissue mineralization; α and β are the intercept and slope parameters, respectively. If the slope term is significantly different from 0, it implies that an association between tissue mineralization and Ca × iP is detected.

In the follow-up investigative studies of GEN-A and PD-901, different rats were peeled off at each considered time point. Serum Ca, iP, albumin, FGF-23, PTH, and phosphorylated ERK (pERK)/total ERK reported at each time point were analyzed using one-way ANOVA, with dose group as the main effect. The mean of each treatment group was compared with the vehicle using Dunnett’s t-test (Montgomery, 2001). At each time point, RT-PCR renal gene expression for each treated group was compared with vehicle using two-sample t-test with Benjamini-Hochberg p value correction to control false discovery rate (Benjamini and Hochberg, 1995). The analysis was conducted using SAS 9.2, and all tests were conducted at 5% significance level.

**RESULTS**

**MEK Inhibitor Effects on Calcium and Phosphorus Homeostasis**

GEN-A (Fig. 1) caused a dose-proportional elevation (20–60%) in serum iP levels after three doses compared with controls (Fig. 2A); the elevation was less prominent (~25%) after 7 and 14 doses, and iP levels returned to baseline after a 7-day recovery period (at day 21). GEN-A caused only a slight

![FIG. 1. Chemical structures of GEN-A (A) and PD-901 (B).](https://academic.oup.com/toxsci/article-abstract/125/1/187/1668075)

![FIG. 2. Effect of GEN-A treatment on serum iP (A), Ca (B), and albumin (C) concentration in rats. Male Sprague-Dawley rats were treated with vehicle or GEN-A (3, 10, or 20 mg/kg) orally for 14 consecutive days, followed by a 7-day recovery period. Serum iP, Ca, and albumin were measured at 24 h after dosing on days 3, 7, and 15 of the treatment period and on day 7 of the recovery period (d21). Data represent the average of n = 6 rats (3, 7, and 15 days) or n = 3 rats (21 day) + SEM. *p < 0.05.](https://academic.oup.com/toxsci/article-abstract/125/1/187/1668075)
elevation in serum calcium levels (<10%) at 3 and 7 days of dosing compared with controls (Fig. 2B). GEN-A caused a 12% decrease in serum albumin levels at 7 days after dosing, progressing to a 27% drop at 15 days after dosing (Fig. 2C).

GEN-A caused widespread mineralization of soft tissues including the heart, stomach, lacrimal gland, cervical skeletal muscle, lymph nodes, and aorta as well as small- and medium-size arteries and arterioles in multiple tissues (Fig. 3). Tissue mineralization showed a dose-response in incidence and number of tissues and organs involved. In particular, soft tissue mineralization was observed when the calcium-phosphate product (Ca × iP) exceeded 113.2. The first tissues affected were the lacrimal gland, cervical muscle, and the stomach. All rats had mineralization in the stomach when Ca × iP was above 126.7, and Ca × iP exceeding 161 was associated with mineralization in the lymph nodes, heart, and aorta (Fig. 4).

In a follow-up 3-day time course study, both GEN-A and PD-901 were evaluated for changes in iP, calcium, and albumin. A dose of 5 mg/kg of PD-901 had been shown to increase serum iP levels and cause tissue mineralization after three daily doses in rats (Brown et al., 2005). GEN-A caused a ~30% elevation in iP at 12 and 24 h after dosing (both at 20 and 60 mg/kg), and PD-901 caused a ~25% elevation in iP at 12 h after dosing and ~45% elevation at 24 h after dosing compared with controls (Fig. 5A). PD-901 also cause a ~10% decrease in albumin at 12 h after dosing, and GEN-A at 60 mg/kg caused a ~30% decrease in albumin at 96 h after dosing (Fig. 5C).

GEN-A also caused an elevation in 1,25-dihydroxyvitamin D₃ (active form of vitamin D) levels at 12, 24 h after a single dose of 60 mg/kg compared with controls (Table 1). Furthermore, GEN-A administration caused a 50% increase in serum levels of FGF-23 at 12 h after dosing and a threefold increase at 24 h after dosing (Fig. 6A). At 96 h after dosing, FGF-23 levels were ~50% at 24 h after dosing as a result of GEN-A treatment (Fig. 6B). Effects of PD-901 on vitamin D, FGF-23 and PTH levels were not evaluated, but PD-901 has been shown to increase the levels of 1,25-dihydroxyvitamin D₃ in rats (Brown, 2010).

**MEK Inhibitor Pathway Effects**

GEN-A caused a 40% decrease in pERK at 60 mg/kg after 12 h, indicating that GEN-A interacts with its intended target in the rat kidney (Fig. 7). PD-901 effects on pERK were not determined.
Of the 93 genes evaluated, the most significant renal gene expression changes were observed for Cyp27b1, which was markedly upregulated by both MEK inhibitors at 12 h after dosing (~25-fold with GEN-A treatment and ~200-fold with PD-901 treatment), and Cyp24a1, which was markedly downregulated by both MEK inhibitors (~100-fold with GEN-A treatment and ~500-fold with PD-901) at 12 h after dosing (Fig. 8B). MAP kinase response genes, Egr, Fos, and Jun, were also downregulated at 12 h after dosing for both compounds, confirming that GEN-A and PD-901 inhibited MEK activity in rat kidney (Fig. 8A).

**FIG. 5.** Effect of GEN-A or PD-901 treatment on serum iP (A), Ca (B), and albumin (C) concentration in rats. Male Sprague-Dawley rats were treated with vehicle, GEN-A (20 or 60 mg/kg), or PD-901 (5 mg/kg) orally for 1 day (PD-901) or three consecutive days (GEN-A). Serum iP, Ca, and albumin were measured at 12 and 24 h after the first dose (GEN-A and PD-901) or at 24 h after the third dose (96 h). Time-matched vehicle control rats were used for each time point. Data represent the average of n = 4 rats ± SEM. *p < 0.05.

**TABLE 1**
1,25-Dihydroxyvitamin D\(_3\) Levels in Rat Serum at 12, 24, and 96 h After Treatment with 60 mg/kg of GEN-A. 1,25-Dihydroxyvitamin D\(_3\) Levels Were Analyzed by HPLC-MS/MS, and Data Represent Absolute Levels in n = 4 Pooled Samples

<table>
<thead>
<tr>
<th></th>
<th>12 h</th>
<th>24 h</th>
<th>96 h</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>BLQ</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
<tr>
<td>60 mg/kg</td>
<td>331 pg/ml</td>
<td>297 pg/ml</td>
<td>146 pg/ml</td>
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Note: BLQ, below limit of quantitation (<117 pg/ml).

**FIG. 6.** Effect of GEN-A treatment on serum FGF-23 (A) and PTH (B) levels in rats. Male Sprague-Dawley rats were treated with vehicle or GEN-A (20 or 60 mg/kg) orally three consecutive days. Serum FGF-23 and PTH levels were measured by ELISA at 12 and 24 h after the first dose (GEN-A) or at 24 h after the third dose (96 h). Time-matched vehicle control rats were used for each time point. Data represent the average of n = 4 rats ± SEM. *p < 0.05.

**FIG. 7.** Effect of GEN-A treatment on phospho-ERK inhibition in rat kidney. Male Sprague-Dawley rats were treated with vehicle or GEN-A at 60 mg/kg, and pERK and total ERK were measured by Western blot (A) at 12 h after treatment. Protein signal densities were quantified by densitometry (B) and phosphorylation levels were expressed as a percentage of total ERK2 protein levels. Data represent the average of n = 4 rats ± SEM. *p < 0.05.
Serum iP levels are also regulated by the type II sodium-phosphate cotransporter NaPiIIa, which is primarily localized in the brush border of proximal tubule epithelial cells and responsible for renal iP resorption; FGF-23 has a role in the expression of these transporters (Murer et al., 2003). GEN-A did not significantly alter the expression of NaPiIIa cotransporter protein in the kidney at 12 or 24 h after dosing, but it did cause a significant decrease in the expression of NaPiIIa transporter protein at 96 h after dosing (after three doses), as assessed by the area of immunolabeled kidney cortex in quantitative image analysis (Fig. 9). PD-901 effects were not evaluated.

**DISCUSSION**

MEK small molecule inhibitor GEN-A dosed orally to rats caused a dose-responsive elevation in serum iP levels and slight elevations in calcium that resulted in soft tissue mineralization after 15 days at Ca\(^{3}\)iP values exceeding \(\sim 100\) mg/dl. In addition, GEN-A also caused a decrease in albumin, which could result in an underestimation of the level of free calcium because total calcium (albumin-bound) and not free calcium was evaluated. This finding is similar to that reported for PD-901, a structurally distinct molecule with similar pharmacodynamic activity and selectivity, suggesting that this is an on-target effect and implicating the MEK pathway in phosphorus homeostasis. Furthermore, our studies demonstrated that there were differential sensitivities of tissues to mineralization.

GEN-A caused a 40% decrease in pERK in rat kidney tissue at 12 h after treatment (Fig. 7), confirming that GEN-A inhibited the target in the rat kidney. ERK phosphorylation can be driven, at least in part, by FGF-23 (injection of FGF-23 results in ERK phosphorylation in mouse kidney; Ranch et al., 2011). MEK inhibition in the rat kidney was further supported by a decrease in the expression of MEK response genes \(Egr1\), \(Jun\), and \(Fos\) 12 h after dosing (Fig. 8A). Although pERK levels in response to PD-901 were not evaluated in this study, \(Egr1\) mRNA levels were also decreased at 12 h after dosing, demonstrating target knockdown in kidney tissue with PD-901 (data not shown).

GEN-A caused an increase in 1,25-dihydroxyvitamin D\(_3\) levels in rat serum at 12, 24, and 96 h after dosing (Table 1), which is also in agreement with the increase described for PD-901 (Brown, 2010). This increase in 1,25-dihydroxyvitamin D\(_3\) caused an elevation in inorganic phosphorous levels (1,25-dihydroxyvitamin D\(_3\) increases intestinal reabsorption of phosphorus; DeLuca, 1992). To explore the mechanism responsible for the elevation in 1,25-dihydroxyvitamin D\(_3\) levels, we evaluated the gene expression of the two key enzymes involved in vitamin D regulation, \(Cyp27b1\) and \(Cyp24a1\), in rat kidney tissue. \(Cyp27b1\) was markedly upregulated at 12 h after dosing with GEN-A treatment, whereas \(Cyp24a1\) was markedly downregulated (Fig. 8). These dramatic changes in the expression of these genes and their temporal nature compared with changes in the active form of vitamin D suggest that the increase in vitamin D levels caused by MEK inhibitors in rat serum is due to transcriptional effects in the rat kidney. Our \textit{in vivo} findings are also in agreement with the blockade of FGF-23 signaling in the human and murine kidney cells by MEK inhibitors (Perwad et al., 2007; Yamashita et al., 2002). Taken together, these data further support a regulatory role for MEK in phosphorus and vitamin D regulation in the rat.

The exact molecular mechanism controlling MEK-driven transcriptional changes in the \(Cyp27b1\) and \(Cyp24a1\) genes is unknown. However, promoter analysis of the human \(Cyp27b1\) gene revealed several consensus sequences to known mammalian transcription factors, some of which are candidates for MEK pathway transcription factors: four cAMP response elements (CRE) and two activator protein-1 (AP-1) response elements (Kong et al., 1999).
The GEN-A-induced increase in FGF-23 serum levels (Fig. 6A) is likely due to a feedback mechanism in response to elevated levels because vitamin D is a potent stimulator of circulating FGF-23 levels. The administration of vitamin D has been shown to increase FGF-23 mRNA expression levels through a vitamin D response element in the FGF-23 promoter (Liu et al., 2007). PD-901 treatment has also been shown to induce the expression of FGF-23 mRNA in the mouse (Ranch et al., 2011).

PTH is a critical endocrine regulator of calcium and phosphorous concentrations. Among other things, PTH enhances the absorption of calcium and phosphorus from the intestine by increasing the production of 1,25-dihydroxyvitamin D$_3$ through transcriptional upregulation of Cyp27b1 in the kidney (Bajwa et al., 2008). GEN-A caused a 50% decrease in serum levels of PTH at 24 h after dosing (Fig. 6B), which appears to be consistent with data demonstrating that circulating FGF-23 suppresses PTH secretion and PTH gene expression through the MEK pathway (Ben-Dov et al., 2007). This decrease in PTH at 24 h is likely secondary to negative feedback from elevated FGF-23 levels combined with the release of pharmacological MEK inhibition at 24 after dosing.

Serum iP levels are also maintained by the coordinated function of the type II family of sodium-phosphate cotransporters, which include NaPiIIa, NaPiIIIb, and NaPiIIc. NaPiIIa is primarily localized in the brush border of proximal tubule epithelial cells, and it accounts for ~85% of renal iP reabsorption (Collins and Ghishan, 1994; Murer et al., 2003). The amount of cotransporter residing in the membrane, which determines the capacity of the proximal tubule to reabsorb iP, is regulated by translocation and is believed to be largely independent of changes in transcription and translation (Forster et al., 2006). Homozygous NaPiIIa knockout mice have increased urinary iP excretion, hypophosphatemia and elevated serum vitamin D levels (Beck et al., 1998). Elevated circulating FGF-23 can decrease serum iP via enhanced renal phosphate excretion that results from decreased cortical brush border membrane protein expression and activity of renal NaPiIIa and NaPiIIc and by inhibiting intestinal NaPiIIb-mediated

![Image](https://example.com/image.png)

**FIG. 9.** Effect of GEN-A treatment on NaPiIIa protein expression in rat kidney. Male Sprague-Dawley rats were treated with vehicle or GEN-A at 60 mg/kg and for three consecutive days and evaluated at 24 h after the last dose (96 h). NaPiIIa was detected in rat kidney sections by immunohistochemistry and quantified by image analysis. High magnification photomicrograph (×10) showing NaPiIIa staining in the kidney tubules for a control (A) and a treated rat (B). Quantitation of NaPiIIa expression in rat kidney (immunohistochemically stained area) after 12, 24, and 96 h of treatment with 60 mg/kg of GEN-A (C). Data represent the average of n = 4 rats + SEM. *p < 0.05.
phosphate absorption (Gattineni et al., 2009; Larsson et al., 2004).

GEN-A did not cause a detectable change in the expression of NaPiIIa mRNA in kidney tissue at 12, 24, or 96 h after dosing (data not shown) nor did GEN-A significantly alter the expression of total NaPiIIa transporter protein in the kidney at 12 or 24 h after dosing, as assessed by immunohistochemistry. However, GEN-A did cause a significant decrease in the expression of NaPiIIa protein at 96 h after dosing (after three doses), as assessed by quantitative image analysis of immunohistochemically stained kidney tissue in rats (Fig. 9). These temporal changes in NaPiIIa levels in the kidney were associated with persistent elevation of FGF-23 and may be indicative of a feedback response to elevated serum iP.

Interestingly, the pattern of phosphorous elevation in the rat caused by both GEN-A and PD-901 suggests that a compensatory mechanism is at play in this species. The elevation in phosphorous caused by GEN-A shows a gradual decrease over time (Fig. 2A), which cannot be fully explained by the drop in drug plasma exposures over time (data not shown). This compensatory mechanism may be explained by the sustained FGF-23 elevation observed over time leading to the gradual reduction in iP levels by a dual mechanism involving the release of the activating pressure on Cyp27b1 mRNA synthesis through lowered PTH levels and the drop in NaPiIIa transporter expression (Armbrecht et al., 2003; Fig. 10).

Additionally, although the collective body of research supports that phosphorous dysregulation and soft tissue mineralization are an on-target effect due to the inhibition of FGF-23 signaling through MEK, this toxicity appears to be rat specific. PD-901 did not cause these effects in mice, dogs, or monkeys even at significantly higher plasma drug levels and inhibition of MEK, and monitoring of serum Ca and iP in phase I clinical trials suggests that humans are not susceptible to this toxicity (Brown, 2010). GEN-A dosed to dogs did not cause a clear increase in serum phosphorous levels or any evidence of tissue mineralization after 7 days of dosing; however, the maximal exposures reached in dogs were only 50% of the minimum exposures that caused these effects in rats (data not shown). A time course conducted in dogs after a single dose of GEN-A revealed a slight and transient elevation in serum phosphorous levels at 6 and 9 h after dosing, which returned to baseline at 24 h after dosing (data not shown), which suggests that this mechanism could also be present in the dog, but the dog may either have not achieved exposures necessary to cause toxicity or may have a more effective compensatory mechanism that prevents the large increase in serum phosphorous levels that were observed in rats.

GEN-A was also dosed to mice, resulting in plasma exposures that surpassed the exposures that caused mineralization in rats (25% above for the area under the curve, twofold above for Cmax). GEN-A caused only a mild iP elevation in mice at 24 h after dosing (~10% above controls, compared with 30% elevation in rats). Furthermore, gene expression analysis of mouse kidney did not show an elevation in Cyp27b1 mRNA at 12 h after dosing (in contrast with the −25-fold elevation in rats at 20 mg/kg) or a downregulation of Cyp24a1 (in contrast to the −100-fold downregulation in rats at 20 mg/kg). These data may suggest the presence of a compensatory mechanism in mice (and possibly other species) that is functioning to reduce vitamin D synthesis in response to the same initial stimulus of FGF-23 pathway inhibition and may explain the species sensitivity observed.

In summary, the data provided support the hypothesis that the mechanism of phosphorous elevation and soft tissue mineralization caused by MEK small molecule inhibitors in rat involves blockade of FGF-23 signaling in the kidney. It also provides additional insight into the role of the MEK signaling pathway in the maintenance of phosphorous homeostasis in the rat.
FUNDING

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