Title:

Inhibition of sodium/hydrogen exchanger 3 in the gastrointestinal tract by tenapanor reduces paracellular phosphate permeability

Authors:

Andrew J. King[1]*, Matthew Siegel[1], Ying He[1], Baoming Nie[1], Ji Wang[1], Samantha Koo-McCoy[1], Natali A. Minassian[1], Qumber Jafri[1], Deng Pan[1], Jill Kohler[1], Padmapriya Kumaraswamy[1], Kenji Kozuka[1], Jason G. Lewis[1], Dean Dragoli[1], David P. Rosenbaum[1], Debbie O'Neill[2], Allein Plain[2], Peter J. Greasley[3], Ann-Cathrine Jönsson-Rylander[4], Daniel Karlsson[4], Margareta Behrendt[4], Maria Strömstedt[4], Tina Ryden-Bergsten[5], Thomas Knöpfel[6], Eva M. Pastor Arroyo[6], Nati Hernando[6], Joanne Marks[7], Mark Donowitz[8], Carsten A. Wagner[6], R. Todd Alexander[2#], Jeremy S. Caldwell[1#]

***Corresponding author:**

Andrew J. King

Ardelyx, Inc.

34175 Ardenwood Blvd, Suite 200, Fremont, CA 94555, USA

E-mail: aking@ardelyx.com

 # Equal contributions as senior author

Affiliations:

[1]Ardelyx, Inc., Fremont, California, USA; [2]University of Alberta, Edmonton, Canada; [3]CVMD Translational Medicine Unit, Early Clinical Development, IMED Biotech Unit, AstraZeneca Gothenburg, Sweden; [4]Bioscience, Cardiovascular and Metabolic Diseases, IMED Biotech Unit, AstraZeneca Gothenburg, Sweden; [5]Cardiovascular and Metabolic Diseases, IMED Biotech Unit, AstraZeneca Gothenburg, Sweden; [6]Institute of Physiology, University of Zurich and National Center for Competence in Research Kidney Control of Homeostasis, Switzerland; [7]University College London, UK; [8]Johns Hopkins University School of Medicine, USA.

One sentence summary:

Tenapanor reduces intestinal phosphate absorption through the reduction of passive paracellular phosphate flux; this effect is mediated exclusively via on-target sodium/hydrogen exchanger isoform 3 (NHE3) inhibition.

ABSTRACT

Hyperphosphatemia is common in patients with chronic kidney disease and is increasingly recognized to associate with poor clinical outcomes. Current management using dietary restriction and oral phosphate binders often proves inadequate. Tenapanor, a minimally-absorbed, small-molecule inhibitor of the sodium/hydrogen exchanger isoform 3 (NHE3), acts locally in the gastrointestinal tract to inhibit sodium absorption. Tenapanor also reduces intestinal phosphate absorption. Here we investigated the mechanism by which tenapanor reduces gastrointestinal phosphate uptake using *in vivo* studies in rodents and translational experiments on human intestinal stem cell-derived enteroid monolayers to model ion transport physiology. Tenapanor produces its effect predominantly through reduction of paracellular phosphate absorption. This occurs by modulating tight junctions, likely a result of the intracellular proton retention accompanying NHE3 inhibition, which increases trans-epithelial electrical resistance (TEER) and reduces permeability to phosphate. NHE3 knockout monolayers replicate the phosphate phenotype of tenapanor, and tenapanor did not affect TEER or phosphate flux in the absence of NHE3. Tenapanor also prevents active transcellular phosphate absorption compensation by decreasing the expression of NaPi2b, the major active intestinal phosphate transporter. In healthy human volunteers, tenapanor 15 mg twice daily for 4 days increased stool phosphorus and decreased urinary phosphorus excretion. In conclusion, tenapanor reduces intestinal phosphate absorption, predominantly through reduction of passive paracellular phosphate flux, quantitatively the most important mechanism of intestinal phosphate absorption. Tenapanor modulates tight junctions to increase TEER, thereby reducing paracellular permeability to phosphate; this effect is mediated exclusively via on-target NHE3 inhibition.

Main text:

INTRODUCTION

Patients with chronic kidney disease (CKD) are unable to maintain fluid and mineral balance. Reduced urinary excretion of phosphate due to renal insufficiency and the resultant hyperphosphatemia are associated with multiple complications, and addressing this imbalance is increasingly recognized as an important challenge in optimizing outcomes in patients with CKD (*1*). Intestinal phosphate absorption increases linearly with increasing dietary intake and does not saturate even at extremely high luminal phosphate concentrations (*2-5*). As a result, phosphate balance is principally maintained through the regulation of urinary phosphate excretion (*6, 7*). Reabsorption of phosphate in the renal tubule is modulated such that serum phosphate concentrations are maintained within a physiologic range despite considerable variations in daily phosphate intake.

Hyperphosphatemia is a predictable consequence in patients with advanced CKD, especially in patients with end-stage renal disease (ESRD) receiving dialysis. This is, at least in part, due to sustained intestinal phosphate absorption in the face of impaired or absent urinary phosphate excretion, which cannot be fully compensated by standard dialysis regimens. This has important implications because Eelevated serum phosphate is associated with adverse outcomes in patients with CKD (*8*), including increased risk of all-cause mortality. Elevated serum phosphate levels in patients on hemodialysis (*9-12*) and in patients with CKD not on dialysis (*9-12*), associate with increased risk of all-cause mortality. In patients not on dialysis, elevated serum phosphate

has been associated with an increased risk of cardiovascular events (*13*), and CKD progression (*14*), while a recent study in a Chinese population reported that serum phosphate was anand is an independent risk factor for left ventricular hypertrophy (*15*). Furthermore, hyperphosphatemia in CKD is accompanied by increasing fibroblast growth factor 23 (FGF-23) levels concentration and secondary hyperparathyroidism, which contribute to metabolic bone disease, ectopic calcification, renal failure and progression of cardiovascular disease (*16-18*).

Oral phosphate binders, together with dietary phosphate restriction, are the primary treatment approaches for patients with ESRD receiving dialysis (*1, 19*). Restricting dietary phosphate intake can reduce the severity of hyperphosphatemia and secondary hyperparathyroidism, although adherence is typically poor and this diet can result in nutritional deficiencies (*20*). Poor compliance has also been reported with oral phosphate binders (*21*). These agents are associated with numerous side effects, such as nausea, vomiting and constipation, and the large amount of binder required to capture dietary phosphate means that treatment is associated with a high pill burden for patients, which can further compromise compliance (*22*).

Tenapanor is a first-in-class, minimally-absorbed, small-molecule inhibitor of the sodium/hydrogen exchanger isoform 3 (NHE3) that acts locally in the gastrointestinal tract. NHE3 was first identified as a drug target in CKD owing to its key role in intestinal sodium and fluid absorption (*23*). In both healthy rats and human volunteers, tenapanor increases stool sodium and reduces urinary sodium, with minimal systemic drug exposure (*23*). In nephrectomized rats fed a high-salt diet and exhibiting hypervolemia, cardiac hypertrophy and arterial stiffening, tenapanor reduced extracellular fluid volume, left ventricular hypertrophy,

albuminuria and blood pressure (*23*). In addition, tenapanor reduced intestinal absorption of radioactive phosphate, reduced urinary and serum phosphate, and both prevented ectopic calcification and lowered FGF-23 levels in a rat model of CKD (*24*).

Dietary phosphate absorption occurs predominantly in the small intestine by at least two distinct pathways: transcellular and paracellular (*25-27*). Two families of sodium-dependent phosphate solute carrier (SLC) transporters responsible for the transport of phosphate into cells have been identified: the SLC34 (type II) and SLC20 (type III) families (*25*). The type II sodium-dependent phosphate transporter 2btransporter (NaPi2b, SLC34A2)) accounts for more than 90% of sodium-dependent phosphate transport across the brush border membrane in the mouse ileum (*32*) and contributes most significantly to transcellular phosphate absorption in the intestine (*28-30*). NaPi2b has a high affinity for phosphate (K_M 10–100 μM); thus, it is saturated under most dietary conditions (*3, 31*). The type III sodium-dependent phosphate transporters (PiT) PiT1 (SLC20A1) and PiT2 (SLC20A2) are also expressed in the intestine, although their contribution to intestinal phosphate absorption is limited as shown by the minimal transcellular sodium-dependent phosphate uptake remaining in NaPi2b knockout (KO) mice (*28, 29, 32, 33*). Paracellular phosphate absorption through tight junction complexes is driven by the electrochemical phosphate gradient. An emerging body of literature demonstrates that tight junctions are dynamically regulated by signal transduction pathways, actively interact with the cytoskeleton, and can display some permeability specificity towards individual or selected ion groups (*34, 35*). In contrast to NaPi2b, paracellular phosphate absorption does not saturate, increases in a linear fashion with increasing phosphate concentration gradient and is bidirectional (*2, 36*). As luminal phosphate concentrations, even on standard diets, have been measured in the

millimolar range (*3*), far in excess of concentrations that saturate NaPi2b and higher than blood phosphate concentrationslevels, paracellular phosphate influx is probably the most quantitatively important overall mechanism of phosphate absorption under typical conditions of phosphate availability. In addition, increasing luminal phosphate concentration above levels that saturate NaPi2b saturation facilitates discrimination between these two transport pathways.

Here we delineate the mechanism by which intestinal phosphate absorption is inhibited by tenapanor, investigating its effects on both passive paracellular phosphate absorption and NaPi2b-mediated phosphate transport. Phosphate absorption has been observed along the entire length of the small intestine; however, species-specific regional variations have been described. A direct comparison of segment-specific phosphate absorption in rats and mice, using the *in vivo* loop technique, revealed it to be quantitatively highest in the duodenum of the rat and in the ileum of the mouse (*37*). Classic physiology experiments in humans, using the triple lumen catheter perfusion technique *in vivo*, showed substantial and non-saturating phosphate absorption across both the proximal and distal small intestine, although quantitatively higher proximally: more consistent with observations in rats than in mice (*2*). Therefore, to comprehensively investigate the effect of NHE3 inhibition on phosphate absorption along the length of the small intestine, we used enteroid monolayer cultures (*38*) derived from human distal duodenum and ileum to capture mechanisms of both proximal and distal absorption in humans and complemented this with experiments in the jejunum of mouse and rat. Mouse ileum, a segment where NaPi2b is highly expressed and plays a dominant role in phosphate absorption, was used to specifically assess the role of NaPi2b in the phosphate-lowering mechanism of tenapanor. We

also report the effects of tenapanor on intestinal phosphate absorption in healthy human volunteers.

RESULTS

Effects of tenapanor on passive paracellular phosphate absorption

Tenapanor reduces phosphate absorption in vivo

In a rat intestinal loop model, tenapanor reduced radioactive phosphate absorption in the jejunum, a key site of intestinal phosphate absorption in rats (*37*), versus vehicle to an amount similar level toto that observed in sodium-free conditions (**Fig. 1A**).

The effects of acute increases in luminal phosphate were examined in rats by increasing the phosphate concentration in an oral bolus, with sodium levels matched by the addition of sodium chloride. Proportionate increases in urinary phosphate excretion, an indirect marker of intestinal phosphate absorption, were seen with increasing phosphate concentration (**Fig. 1B**). This linear concentration-dependence of absorption and failure to saturate, even at high concentrations of luminal phosphate, is characteristic of passive paracellular absorption, not NaPi2b-mediated phosphate absorption (which saturates at low luminal phosphate concentrations). Tenapanor reduced phosphate absorption across all phosphate concentrationslevels, significantly decreasing urinary phosphate excretion even at high phosphate concentrations (**Fig. 1B**), suggesting that tenapanor reduces paracellular phosphate absorption. Increasing concentrations of phosphate in the bolus had minimal effect on urinary sodium excretion, and tenapanor significantly reduced urinary sodium excretion to a similar extent across all phosphate concentrations compared with vehicle (tenapanor vs vehicle, 0.15 M phosphate bolus: 8 ± 2 vs 26 ± 3 mg/4 hours, $P < 0.0001$; 0.5 M phosphate bolus: 8 ± 0.3 vs 29 ± 1 mg/4 hours, $P < 0.0001$; 1.5 M phosphate bolus: 4 ± 1 vs 39 ± 1 mg/4 hours, $P < 0.0001$).

The effects of tenapanor during chronic changes in dietary phosphate intake in rats were investigated. Increases in dietary phosphate content produced significant, linear and non-saturating increases in urinary phosphate excretion at baseline, consistent with passive paracellular absorption (**Fig. 1C**). Tenapanor significantly reduced urinary phosphate excretion after 4 days of administration, suggesting the inhibition of a constant fraction of intestinal phosphate absorption despite increasing dietary phosphate content, indicative of a reduction in paracellular, not transcellular, absorption (**Fig. 1C**). Significant reductions in urinary sodium excretion were also observed with tenapanor relative to vehicle, which were constant across varying dietary phosphate levels (tenapanor vs vehicle, 0.3% phosphate: 6 ± 1 vs 17 ± 2 mg/day, $P < 0.0001$; 0.6% phosphate: 3 ± 1 vs 19 ± 2 mg/day, $P < 0.0001$; 1.2% phosphate: 6 ± 2 vs 16 ± 2 mg/day, $P < 0.001$).

Paracellular phosphate absorption could be reduced by at least two potential mechanisms as a result of NHE3 inhibition: (i) a general decrease in paracellular water flux and diffusional driving force caused by luminal sodium and water retention (this would be accompanied by minimal change or a decrease in luminal phosphate concentration) and/or (ii) a decrease in paracellular permeability to phosphate through the tight junction (this would be accompanied by an increase in luminal phosphate concentration). To investigate this, an enteropooling study was performed in healthy rats trained to eat a high-phosphate meal (1.2%), with both ion mass and ion concentration then being measured in the cecum at defined time points. The small intestine is the primary site of phosphate absorption, therefore the concentration of phosphate and other ions in the cecum reflects the amount not absorbed in the small intestine.

In this enteropooling model, tenapanor significantly reduced urinary phosphate and sodium excretion following the high phosphate meal, reflecting a significant decrease in overall intestinal phosphate and sodium absorption (**Fig. 1D**). Tenapanor treatment resulted in significantly increased delivery of both sodium (**Fig. 1E**) and phosphate (**Fig. 1F**) to the cecum, confirming decreased absorption of these ions in the small intestine. Following vehicle treatment, very little phosphate was delivered to the cecum, even in the face of a high-phosphate load, indicating a high capacity for phosphate absorption in the small intestine (**Fig. 1F**). Luminal sodium retention observed with tenapanor treatment was accompanied by significantly increased luminal water volume (**Fig. 1G**) and luminal sodium concentration (**Fig. 1H**). In addition, the inhibition of intestinal phosphate absorption that resulted from tenapanor treatment was accompanied by an significant increase in luminal phosphate concentration (**Fig. 1I**). This suggests that tenapanor decreases paracellular permeability to phosphate rather than reducing the driving force through a dilutional effect on luminal phosphate concentration. Reduced water absorption may also contribute to decreased phosphate absorption via decreased paracellular solvent drag.

Potassium is reported to be exclusively absorbed in the small intestine via passive paracellular flux (*39*). Therefore, we also measured luminal potassium concentration in the enteropooling model to evaluate the *in vivo* ion selectivity of tenapanor. In contrast to phosphate, luminal potassium concentration was significantly decreased by tenapanor (**Fig. 1J**), consistent with greater solute dilution secondary to luminal water retention because of decreased transcellular sodium absorption, relative to any reduction in paracellular potassium flux. Combined, these

findings illustrate the *in vivo* selectivity of tenapanor for phosphate relative to potassium, another ion absorbed by the paracellular route.

Further evaluation of the ionic specificity of tenapanor included the measurement of cecal chloride, calcium and magnesium concentrations in the enteropooling model. Cecal chloride concentrations were low, indicative of high-capacity chloride absorption in the small intestine against the prevailing chemical gradient opposed by high plasma chloride concentrations (98–106 mM); consistent with a prominent role of active transcellular chloride transport (**Fig. 1K**). Tenapanor increased the luminal chloride concentration compared with vehicle control; however, the maximal increase in chloride concentration was modest (1.6-fold) compared with the maximal tenapanor induced increases in phosphate concentration (5.7-fold), demonstrating a preferential effect of tenapanor on phosphate relative to chloride. Given the low cecal concentration of chloride relative to plasma chloride concentrations, which would be insufficient to support paracellular chloride absorption, the modest increase in cecal chloride observed with tenapanor treatment is likely the result of a disruption in electroneutral transcellular sodium chloride absorption, which would be a direct result of NHE3 inhibition, rather than reduced paracellular chloride absorption.

The concentrations of divalent cations calcium (**Fig. 1L**) and magnesium (**Fig. 1M**) in the cecum were also low, comparable to their respective plasma concentrations and indicating efficient small intestinal absorption of these cations. Tenapanor did not significantly ($P > 0.05$) affect cecal concentrations of calcium or magnesium, demonstrating specificity towards inhibition of phosphate absorption relative to divalent cation absorption.

Tenapanor inhibits paracellular phosphate flux in a cellular model of the intestinal epithelial monolayer across a wide range of phosphate concentrations

We used a novel system of culturing intestinal epithelial stem cells from human or mouse gastrointestinal biopsies as monolayers, which allows the transport of ions across the intestinal epithelium to be monitored (*38*). The enteroid monolayer contains the diversity of intestinal epithelial cell lineages, models the specific gene expression patterns of each individual intestinal segment, expresses the appropriate endogenous ion transporters (for example, NHE3 and NaPi2b) in a segment-specific manner, polarizes to form tight junctions with segment-specific expression of claudins and other tight junction proteins, and generates the expected negative luminal electrical potential observed *in vivo*. The differentiated enteroid monolayer therefore enables the study of transcellular and paracellular phosphate absorption.

NHE3 is highly expressed along the length of the gastrointestinal tract (*40*) and is endogenously expressed in each segment of the intestinal monolayer model, as shown in representative images from the ileum (**Fig. S1A**). Most cells in the monolayer are absorptive cells, similar to *in vivo*, and are polarized, expressing NHE3 only at the apical surface (**Fig. S1B**). The enteroid monolayer therefore provides a model system to study the role of native NHE3 in intestinal phosphate absorption. Proton secretion coupled to sodium absorption by NHE3 results in acidification of the apical media of the monolayer. This was inhibited by tenapanor, monitored by the color change of pH-sensitive phenol red in the apical media (**Fig. S1C**). Concentration–response studies measuring apical pH using 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) dye, where a decrease in the

fluorescence emission ratio (from excitation at 490/440) reflects a decrease in pH, showed that tenapanor inhibited apical acid secretion by NHE3 with a half-maximal inhibitory concentration (IC_{50}) of 2 nM and 6 nM in human and mouse ileum monolayers, respectively (**Fig. S1D**). This is consistent with the potency of tenapanor against NHE3 in primary target assays (*23*). In human ileum and duodenum cell monolayer cultures, tenapanor caused a concentration-dependent inhibition of the NHE3-mediated recovery of intracellular pH (pHi) following acid loading (IC_{50}: ileum, 13 nM; duodenum, 9 nM) (**Fig. S1E, F**). Based on the potency of NHE3 inhibition in the intestinal monolayer culture system, subsequent experiments investigating effects on phosphate absorption used a tenapanor concentration of 1 μM to ensure complete NHE3 inhibition.

Assessment of apical-to-basolateral phosphate flux in duodenum cell monolayer cultures derived from two healthy human donors showed significant ($P < 0.0001$), linear ($R^2 = 0.989$ for donor A, $R^2 = 0.998$ for donor B) and non-saturating increases in phosphate flux with increasing apical phosphate concentrations (1–30 mM) (**Fig. 2A**). Under these conditions, NaPi2b would be saturated even at the lowest phosphate concentration. This indicates that passive paracellular absorption is the primary route of phosphate absorption in the human duodenum model, consistent with the absence of phosphate transporter expression in the duodenum epithelial cell monolayer culture, and the lack of effect of a NaPi2b inhibitor (*41*) on duodenal phosphate absorption in the monolayer (**Fig. S1G**).

The effects of apical tenapanor administration on phosphate flux were first evaluated by measuring changes in basolateral phosphate concentration following a 4-hour treatment period in duodenum cell monolayer cultures. The initial apical phosphate concentration was varied from

1 mM to 30 mM while the initial basolateral phosphate concentration was fixed at 0 mM. Phosphate absorption was strongly dependent on the apical-basolateral phosphate concentration gradient, and basolateral phosphate concentration (**Fig. 2B**) and phosphate flux (**Fig. 2C**) were significantly lower with tenapanor treatment across the range of phosphate concentrations. Tenapanor also increased trans-epithelial electrical resistance (TEER) following a 4-hour incubation in human duodenum monolayer cultures compared with vehicle (**Fig. 2D**), consistent with a decrease in paracellular permeability. Treatment with tenapanor did not affect the potential difference (−5.3 ± 0.26 mV relative to basolateral) across the monolayer compared with vehicle control (−5.5 ± 0.3 mV), importantly indicating the electrical driving force for phosphate flux is not significantly affected by tenapanor.

The effects of tenapanor on phosphate absorption in the human duodenum cell monolayers were further evaluated following overnight treatment to incorporate measurable apical-to-basolateral water flux across a range of initial apical phosphate concentrations (1–30 mM), with the initial basolateral phosphate concentration set at 1 mM. Overnight treatment with tenapanor inhibited phosphate absorption across the range of phosphate concentrations, indicated by a significantly higher retention of phosphate in the apical compartment compared with vehicle (**Fig. 2E**). Consistent with decreased phosphate permeability, the retention of phosphate in the apical chamber produced by tenapanor was accompanied by significantly higher apical phosphate concentrations compared with vehicle (**Fig. 2F**). Conversely, basolateral phosphate concentrations were significantly reduced with tenapanor exposure (**Fig. 2G**). In addition, NHE3 inhibition by tenapanor decreased apical-to-basolateral water absorption (**Fig. 2H**), as a result of decreased transcellular sodium flux. Similar results were observed in human ileum monolayers

(**Fig. S2**), showing that tenapanor also inhibits phosphate absorption in the distal small intestine of humans.

An additional and identifying characteristic of paracellular transport is bidirectional flux, based on the prevailing electrochemical gradient. Consistent with phosphate moving across human duodenum monolayer cultures through the paracellular pathway, we demonstrated basolateral-to-apical phosphate flux that was linearly dependent on the trans-epithelial phosphate concentration gradient, which is non-saturating through a 10 mM phosphate gradient (**Fig. 2I**). The magnitude of phosphate flux at a given phosphate concentration gradient was similar in both directions, although appeared to be slightly reduced in the basolateral-to-apical direction, which is likely a result of the unfavorable electrical gradient to reverse phosphate flux generated by the negative luminal potential established across the monolayer (−5.5 ± 0.3 mV relative to basolateral). Apically applied tenapanor effectively reduced basolateral-to-apical phosphate flux at all phosphate concentrations tested, comparable to the effect on apical-to-basolateral phosphate absorption (**Fig. 2I)**. Combined, these results suggest that tenapanor inhibits phosphate flux by decreasing paracellular permeability, consistent with the *in vivo* enteropooling results.

Tenapanor increases TEER when there is a favorable gradient for NHE3-mediated proton efflux

The effect of tenapanor on paracellular permeability was further evaluated by measuring TEER in the intestinal epithelial cell monolayer culture system. Proton secretion by NHE3 gradually acidifies the apical media in the monolayer cultures, which results in an unfavorable proton gradient for NHE3-mediated transport. The reduction in NHE3-mediated proton efflux in the setting of an elevated luminal proton concentration has been described in the renal tubule (*42*).

Thus, in order to initiate phosphate absorption experiments, the acidified cell culture media was replaced with media of a defined starting phosphate concentration and a neutral apical pH.

The change to neutral apical media immediately restored the proton gradient, thereby driving NHE3-mediated proton efflux. This resulted in a large and sustained reduction in TEER from baseline levels in the monolayer cultures from both the duodenum (**Fig. 3A**) and ileum (**Fig. 3B**), which was completely blocked by tenapanor (**Fig. 3A, B**). This shows that TEER is elevated when NHE3 is inhibited by tenapanor or when NHE3-mediated proton efflux is reduced by an unfavorable proton gradient, both of which result in intracellular proton accumulation.

Luminal pH modulates pHi, NHE3-mediated proton efflux, TEER and phosphate absorption

The impact of both luminal pH and tenapanor on TEER were further investigated using the cell monolayer culture system. In human duodenum monolayers, apical pH had a strong influence on TEER. Replacing the old acidified apical media (pH 6.0) with fresh media produced similar reductions in TEER from baseline under neutral (pH 7.0) and alkaline (pH 8.0) conditions, but caused an increase in TEER when acidic media (pH 5.5) was introduced (**Fig. 3C**). Fresh media, matched to the old apical media (pH 6.0), had no effect on TEER (**Fig. 3C**). Compared with the TEER of the equivalent pH control, tenapanor increased TEER at neutral and alkaline luminal pH, but not under acidic conditions, when NHE3-mediated proton efflux was unfavorable and TEER already high (**Fig. 3D**).

Further studies, using the pH-sensitive dye BCECF-AM, showed that the rate and magnitude of recovery of pHi following acid loading (a direct measure of NHE3-mediated proton efflux in the

enterocyte) were reduced with decreasing luminal pH in both duodenum and ileum monolayer cultures (**Fig. 3E, F**). Tenapanor reduced pHi recovery at each of the luminal pH values tested (pH 6, 7 and 8; **Fig. 3E, F**). Although NHE3-mediated proton efflux was reduced at pH 6.0 compared with pH 7.0, the potency of tenapanor in inhibiting NHE3 was unaffected by extracellular pH (IC_{50}: pH 6.0, 10 nM; pH 7.0, 9.5 nM) (**Fig. 3G**).

In human ileum monolayer cultures, changes in luminal pH were rapidly accompanied by changes in pHi, monitored using BCECF-AM dye, and TEER. Both occurred within 1 minute after manipulation of the apical media pH (**Fig. 3H–J**). A rapid reduction in pHi was observed at luminal pH 6.0. A higher pHi was seen when neutral (pH 7.0) or alkaline (pH 8.0) media was added, although there was little difference between pH 7.0 and 8.0 (**Fig. 3H**), similar to the effects of luminal pH on TEER. At neutral apical pH (when NHE3 is effectively effluxing protons), tenapanor significantly reduced pHi, to a similar extent observed with pH 6.0 apical media, with effects observed within 1 minute (the first time point technically possible to measure) of initiating tenapanor treatment (**Fig. 3I**). The effects of luminal pH and tenapanor on changes in TEER were similarly rapid. In monolayer cultures mounted in Ussing chambers, which permit dynamic and continuous TEER measurements, tenapanor application resulted in a near immediate increase in TEER compared with vehicle control (**Fig. 3J**). Therefore, inhibition of NHE3, by tenapanor or decreased NHE3-mediated proton efflux as a result of acidic luminal media, results in a rapid (< 1 minute) reduction in pHi and a correspondingly rapid increase in TEER.

The effects of luminal pH on phosphate absorption were also evaluated using the ileum monolayer culture system. Starting with initial apical phosphate concentrations of 10 mM or 30 mM and an initial basolateral phosphate concentration of 0 mM, phosphate flux was measured during overnight exposure to acidic (pH 6.0), neutral (pH 7.0) and alkaline (pH 8.0) apical media, with and without tenapanor. There was a strong, positive and significant relationship between apical pH and phosphate flux at initial apical phosphate concentrations of both 10 mM ($R^2 = 0.94$, $P < 0.0001$) and 30 mM ($R^2 = 0.92$, $P < 0.0001$) (**Fig. 4A**). Tenapanor reduced phosphate absorption across the pH range 6.0–8.0, with the reduction in phosphate flux being significantly higher at pH 7.0 and pH 8.0 compared with pH 6.0, but no difference between pH 7.0 and pH 8.0 (**Fig. 4B**).

To confirm that the results obtained in enteroid monolayers translate to intestinal tissue, phosphate flux measurements, by radioactive tracer, and paracellular phosphate permeability measurements, by biionic dilution potential, were made in *ex vivo* mouse jejunum mounted in Ussing chambers at pH 6.0 and 8.0. Consistent with the human duodenum monolayer results, both phosphate flux (**Fig. 4C**) and permeability (**Fig. 4D**) were increased at pH 8.0 compared with pH 6.0. Combined, these results suggest increased phosphate permeability at pH 8.0 in both human duodenum monolayer cultures and mouse jejunum; conditions where the predominant phosphate species is HPO_4^{2-}. In addition to preferential paracellular permeability to the HPO_4^{2-} species, the electrical gradient imposed by the negative lumen potential *in vivo* generates a greater driving force for absorption of the HPO_4^{2-} species, relative to the $H_2PO_4^-$ species that is more abundant at pH 6.0. Thus, the neutral-to-alkaline pH range encountered in the distal small intestine provides optimal conditions for paracellular phosphate absorption.

Biophysical assessment of paracellular permeability using dilution and biionic potential measurements

Paracellular sodium and chloride permeability and paracellular phosphate permeability were determined from sodium chloride dilution potentials and phosphate biionic potentials, respectively, in both human duodenum monolayer cultures and mouse jejunum *ex vivo* mounted in Ussing chambers at pH 8.0.

In human duodenum monolayer cultures, tenapanor significantly increased TEER (**Fig. 5A**), accompanied by a corresponding decrease in permeability to sodium (**Fig. 5B**) and chloride (**Fig. 5C**), such that sodium-to-chloride permeability (**Fig. 5D**) was unchanged, and a significant decrease in phosphate permeability (**Fig. 5E)**, confirming decreased paracellular permeability to phosphate. A consistent effect of tenapanor on paracellular permeability was observed in mouse jejunum *ex vivo* (**Fig. 5F–J**).

The preferential inhibition of intestinal phosphate (and sodium) absorption by tenapanor *in vivo*, relative to other ions including chloride, is not readily reflected in these direct biophysical measurements of paracellular permeability. It is important to recognize that measurements of paracellular ion permeability made via *in vitro* dilution potential measurements are not equivalent to *in vivo* paracellular ion flux, which is the integrated result of paracellular permeability and the prevailing electrochemical gradient. Sodium and chloride permeability was assessed in the Ussing chamber by acutely imposing an approximately 100 mM chemical gradient to enable dilution potentials to be measured; a gradient unlikely to be achieved *in vivo*

due to the high plasma concentration of both sodium (135–145 mM) and chloride (96–106 mM) opposing paracellular absorption from the lumen. To better assess the actual driving force for various ions *in vivo*, we measured ion concentrations in both the proximal and distal small intestine at defined time points in untreated, healthy rats after ingestion of a high-phosphate (1.2%) meal, which would be typical of processed food. Peak luminal intestinal sodium concentrations were observed in the distal small intestine, and approximated normal plasma sodium concentrationslevels (**Fig. 5K**). Combined with the negative luminal potential, the electrochemical gradient did not therefore provide a strong impetus for paracellular sodium absorption; in fact, there could potentially be secretion. Luminal chloride concentrations were maximal in the proximal small intestine, but still below normal plasma chloride concentrations, and decreased significantly in the distal small intestine (**Fig. 5L**). Even with a favorable electrical gradient for absorption of a negative anion, paracellular chloride absorption would not be supported in this concentration range. The absence of the requisite electrochemical gradients to drive substantial paracellular sodium and chloride flux *in vivo* suggests a prominent role for transcellular absorption of these ions, consistent with the high expression of ion exchangers for both sodium (NHE3) and chloride (SLC26A3 [DRA] and SLC26A6 [PAT1/CFEX]) in the small intestine (*43*).

In contrast, low plasma phosphate concentrationslevels, high luminal phosphate concentrations and a negative luminal potential difference across the intestinal epithelium provides a strong electrochemical gradient for luminal phosphate absorption (**Fig. 5M**). Importantly, the electrical gradient across the intestinal epithelium is lumen negative, and luminal phosphate is predominantly a divalent anion, magnifying the driving force for paracellular phosphate four-

fold relative to chloride. Combined with the absence of a low affinity, high capacity transcellular phosphate transporter, at high luminal concentrations, phosphate appears to be dependent on paracellular flux for absorption. The reliance of phosphate on paracellular absorption and the strength of the driving force for phosphate flux likely explain the preferential effect of tenapanor on phosphate absorption.

NHE3-independent changes in pHi modulate TEER

The cell monolayer culture system was used to evaluate the effects on TEER of intracellular acidification occurring independent of changes in NHE3 activity. The ionophore nigericin reduced pHi compared with control (**Fig. S3A**), and produced a similar increase in TEER relative to control as was observed with tenapanor (**Fig. S3B**). Similarly, the compounds N5,N6-bis(2-Fluorophenyl)[1,2,5]oxadiazolo[3,4-b]pyrazine-5,6-diamine (BAM15) and mesoxalonitrile 4-trifluoromethoxyphenylhydrazone (FCCP), which decrease pHi by uncoupling oxidative phosphorylation in the mitochondria (**Fig. S3C**), increased TEER compared with control (**Fig. S3D**). Moreover, neither nigericin, BAM15 nor FCCP inhibited NHE3 activity in enterocytes, as assessed by pHi recovery following acid loading. These results show that pHi is a direct regulator of TEER, independent of NHE3 activity.

NHE3 KO monolayers phenocopy tenapanor, and tenapanor does not inhibit paracellular phosphate absorption in the absence of NHE3

CRISPR/Cas9-mediated gene editing was used to produce human ileum epithelial stem cell clones with nucleotide insertions/deletions (**Fig. 6A**) resulting in a complete loss of NHE3 expression and function, confirmed by NHE3 western blotting (**Fig. 6B**), visual loss of apical

media acidification in monolayer cultures (**Fig. 6C**) and reduced recovery from intracellular acidification following acid loading (**Fig. 6D, E**). NHE3 KO monolayers were otherwise morphologically indistinguishable from control monolayers and had similar baseline TEER values.

In monolayer culture studies, the NHE3 KO clones showed the same phenotype as tenapanor-treated cells. Following overnight incubation, NHE3 KO monolayers showed reduced absorption of water (**Fig. 6F**), sodium (**Fig. 6G**) and phosphate (**Fig. 6H**), as indicated by their apical retention, compared with control monolayers (Cas9 cells with non-targeting control guide RNA). In addition, NHE3 KO cells had reduced apical proton secretion and increased apical media pH compared with the control monolayers (**Fig. 6I**). With the restoration of neutral apical media and hence NHE3-mediated proton secretion in control cells, there was a marked decrease in TEER from baseline in control cultures (**Fig. 6J**). However, no change in TEER was observed in the NHE3 KO cells.

Consistent with earlier results, tenapanor significantly inhibited apical-to-basolateral phosphate absorption in control cells **(Fig. 6K**); this was accompanied by an increased apical phosphate concentration compared with control (**Fig. 6L**). By contrast, tenapanor had little effect on phosphate absorption **(Fig. 6K**) or apical phosphate concentration (**Fig. 6L**) in NHE3 KO cells. Similarly, tenapanor administration increased TEER relative to vehicle only in control cells and had no effect on TEER in NHE3 KO cells (**Fig. 6M**). These findings indicate that the effects of tenapanor on phosphate absorption, paracellular phosphate permeability and TEER are mediated via on-target NHE3 inhibition.

CRISPR/Cas9-mediated NHE3 deletion in human duodenum monolayers (**Fig. S4A, B**) also replicated the phenotype of tenapanor in reducing apical media acidification (**Fig. S4C**), delaying recovery from intracellular acidification (**Fig. S4D**), increasing TEER (**Fig. S4E, F**) and reducing apical-to-basolateral sodium and phosphate absorption (**Fig. S4G, H**) compared with control cells. Tenapanor had no effect on TEER or apical-to-basolateral sodium and phosphate absorption in NHE3 KO duodenum monolayers (**Fig. S4F–H**). This further confirms that the effects of tenapanor on paracellular phosphate permeability, absorption and TEER are mediated via the action of tenapanor on NHE3.

Further characterization of tenapanor including effect on 24-hour urinary ion excretion and transcellular phosphate transporter NaPi2b

Effects of long-term administration of tenapanor on phosphate, sodium, chloride and potassium in vivo

To characterize the effects of tenapanor further, including effects on the renal handling of sodium and phosphate, on phosphaturic hormones and on NaPi2b expression, healthy rats were dosed for 14 days with tenapanor. Tenapanor decreased urinary sodium (**Fig. S5A**) and phosphate (**Fig. S5B**) excretion but had no effect on urinary chloride (**Fig. S5C**) or potassium (**Fig. S5D**) excretion (normalized to dietary intake of each ion to correct for any daily fluctuations in food intake). In healthy rats, tenapanor did not affect plasma sodium or phosphate concentration (**Fig. S5E**), because renal clearances of both phosphate and sodium were appropriately decreased relative to control (**Fig. S5F**). Furthermore, tenapanor had minimal

effect on phosphate-regulating hormones (FGF-23, parathyroid hormone and vitamin D) in healthy animals receiving a regular phosphate diet (**Fig. S5G**, although tenapanor does significantly reduce FGF-23 and parathyroid hormone levels in animals with CKD (*24*)) or in those on a high-phosphate diet (data not shown).

The effect of tenapanor on urinary sodium excretion was maximal on the first few days of treatment (**Fig. S5A**), and remained significantly reduced compared with vehicle for the duration of treatment. However, the reduction was attenuated in magnitude from treatment day 5, suggesting the engagement of a compensatory response to inhibition of intestinal sodium absorption. To investigate this, the expression of transcellular sodium transporters was measured by RNA sequencing (RNA-seq) along the length of the gastrointestinal tract. In tenapanor-treated rats, NHE3 expression was significantly increased in the jejunum, ileum and proximal colon (**Fig. S5H**) and epithelial sodium channel gamma subunit (ENaCγ) expression was increased in the distal colon (**Fig. S5I**), indicating an attempt to increase intestinal sodium absorption. Since NHE3 will remain inhibited in the continued presence of tenapanor, it is likely increased colonic sodium absorption via upregulated ENaC is responsible for the attenuation of the reduction of urinary sodium excretion over time. To assess whether the lack of effect of tenapanor on urinary chloride excretion (**Fig. S5C**) required a similar compensatory response as observed for sodium, we also measured the expression of the most prominent chloride exchangers and transporters along the length of the gastrointestinal tract. The expression of chloride exchangers DRA (SLC26A3 (**Fig. S5J**), and PAT1/CFEX (SLC26A6 (**Fig. S5K**) and chloride channel CFTR (**Fig. S5L**), were unchanged by tenapanor treatment. The maintenance of normal chloride balance in the absence of a transcriptional response to upregulate chloride

exchangers and transporters, in contrast to the effect on sodium transporters and channels, is consistent with minimal impact of tenapanor on intestinal chloride absorption.

Tenapanor produces a modest decrease in NaPi2b expression

The active phosphate transporter NaPi2b showed a modest (approximately 30%) but statistically significant ($P < 0.01$) decrease in mRNA expression in the distal jejunum and ileum following 14 days of tenapanor treatment (**Fig. 7A**). In addition, immunohistochemistry using an antibody specific to NaPi2b showed a modest decrease in staining intensity in the jejunum following tenapanor treatment in a separate study (**Fig. 7B**). Transcellular phosphate transport was assessed using intestinal brush border membrane vesicles (BBMV) isolated following *in vivo* tenapanor administration in rats. This transport system is predominantly driven by NaPi2b. Tenapanor had little effect on transcellular phosphate uptake, including sodium-dependent phosphate absorption, in either duodenum or jejunum BBMVs compared with control (**Fig. S6A, B**), indicating minimal functional effect of tenapanor on NaPi2b-mediated phosphate absorption. Combined, these findings indicate that NaPi2b expression and consequently transcellular phosphate absorption do not increase to compensate for the decrease in passive paracellular phosphate absorption produced by tenapanor. In addition, tenapanor did not affect sodium-dependent glucose absorption in duodenum BBMVs (**Fig. S6C**).

Tenapanor has minimal effect on NaPi2b activity in cellular and in vivo *studies*

The effects of tenapanor on NaPi2b function were also assessed in monolayer cell cultures from the mouse ileum; these had high NaPi2b expression (55 560 counts as assessed by RNA-seq), which were similar to those observed in the mouse ileum *in vivo* (30 545 counts).

In mouse ileum monolayers, evaluation of phosphate absorption at increasing initial apical phosphate concentrations (1–5 mM) with a fixed initial basolateral phosphate concentration (1 mM) showed similar decreases in apical phosphate concentration following overnight incubation, irrespective of initial starting concentration (**Fig. 7C**); phosphate was transported in the absence of a concentration gradient, consistent with transcellular flux. Final basolateral phosphate concentration was also similar despite the different starting apical phosphate concentrations (**Fig. 7C**), showing that phosphate absorption in the mouse ileum was minimally affected by the phosphate concentration gradient. This is a typical characteristic of high-affinity active transport that saturates at low phosphate concentrations of less than 1 mM, and is consistent with the known characteristics of NaPi2b.

In the absence of an apical-to-basolateral phosphate gradient, incubation of mouse ileum monolayers with NTX-9066 (*41*), a potent NaPi2b inhibitor, at 1 μM for 4 hours, 2 days or 3 days completely blocked apical-to-basolateral phosphate absorption at all time points (**Fig. 7D**). By contrast, phosphate absorption in the mouse ileum monolayer was unaffected by tenapanor (1 μM) compared with vehicle at all time points measured (**Fig. 7D**). These findings indicate that phosphate absorption in the mouse ileum monolayer cultures is active, saturates at low luminal phosphate concentrations imparting a relatively low capacity of phosphate absorption, and is mediated exclusively by NaPi2b; they also indicate that tenapanor does not affect NaPi2b-mediated ileal phosphate absorption.

The effects of tenapanor were also evaluated in the *in vivo* loop model in the ileum of wild-type and NaPi2b KO mice (*28*). Almost all (90%) phosphate absorption in the mouse ileum was mediated by NaPi2b (**Fig. 7E**), similar to the mouse ileum monolayer (**Fig. 7D**). Tenapanor produced a small, non-significant ($P > 0.05$) decrease in phosphate absorption in both wild-type and NaPi2b KO mouse ileum (**Fig. 7E**). This further suggests that the phosphate-lowering effect of tenapanor is not dependent on NaPi2b.

How tenapanor modulates paracellular phosphate permeabilityEffects of tenapanor on tight junction protein localization and trafficking

In order to clarify the mechanism by which tenapanor modulates TEER in human monolayer cultures, we investigated the potential involvement of key signaling pathways and second messengers. In Caco2 cells, NHE3 activity, via myosin II regulatory light-chain (MLC) phosphorylation, reduced TEER via the actin cytoskeleton (*48*). In our human duodenum monolayer with native NHE3 expression, however, phosphorylation of MLC was not reduced by tenapanor, and an MLC kinase inhibitor, ML-7 (10 μM), had no effect on baseline TEER or tenapanor-induced TEER changes (data not shown). A broad phospho-array panel failed to show clear activation of any classic signaling pathways. We observed that the acid-sensitive kinase Pyk2 was transiently phosphorylated by reduction in luminal pH with subsequent SRC phosphorylation, but not by tenapanor treatment. Furthermore, an extensive panel of kinase inhibitors did not convincingly affect the ability of tenapanor to modulate TEER. Tenapanor does not activate key GPCR second messengers, including intracellular calcium, cAMP or

cGMP, and established inhibitors of GPCR signaling again did not influence the effect of tenapanor on TEER (data not shown). [A1]

Finally, dDirect assessment of tight junction protein localization was performed by confocal microscopy in the presence or absence of tenapanor treatment in the human ileum monolayer model. There was no obvious change in the localization of tight junction proteins zona occludens-1, occludin, claudin 7 or claudin 3, or NHE3 (not shown), assessed following 30, 60 or 120 minutes of tenapanor treatment (**Fig. S7A–C**). As the panel of validated tight junction protein antibodies for immunocytochemistry is limited, we further evaluated the involvement of protein trafficking in the tenapanor-induced TEER effect to determine whether protein trafficking from the tight junction is responsible. Pitstop2 and dynasore (inhibitors of clathrin and dynamin, respectively) were shown to inhibit endocytosis effectively in the ileum monolayer by preventing carbachol-induced NHE3 internalization (**Fig. S7D**). However, neither pitstop2 (**Fig. S7E)** nor dynasore (**Fig. S7F**) blocked tenapanor-induced increases in TEER compared with control, indicating that endocytosis is not required for this effect of tenapanor.

The rapid increase in TEER without obvious signaling pathway or tight junction protein trafficking involvement might be consistent with the inhibition of NHE3 by tenapanor producing a pH-sensitive conformational change in the tight junction to decrease paracellular phosphate permeability. Effects of pH on TEER have been previously reported (*44*).

Tenapanor does not affect paracellular macromolecule absorption

Both ions and small macromolecules can traverse cell monolayers via paracellular pathways, but it has been proposed that ions and macromolecules are absorbed via distinct pathways (*45*).

Using radioactive tracers, the potential for tenapanor to affect paracellular macromolecule absorption was evaluated *in vivo* in rats and directly compared with its effect on phosphate ion absorption. Mannitol, a small (molecular weight 182) hydrophilic molecule thought to be exclusively absorbed via the paracellular route *in vivo*, was used as a marker of paracellular macromolecule absorption. Tenapanor significantly inhibited ^{33}P absorption at both 0.5 mg/kg and 10 mg/kg (**Fig. 8A**) but had no effect on ^{3}H-mannitol absorption (**Fig. 8B**). This suggests that tenapanor is selective for the paracellular ion absorption pathway over the macromolecule pathway. We also specifically tested the effect of tenapanor on intestinal glucose absorption, because NHE3 activity has previously been proposed to be coupled to paracellular glucose absorption (*46*). Unabsorbed glucose remaining in the luminal contents from the entire small intestine was measured at fixed time points following initiation of a standardized meal consumed over 4 hours in trained rats pretreated with tenapanor or vehicle. Dietary glucose was efficiently absorbed in the small intestine and this was unaffected by tenapanor treatment (**Fig. 8C**).

Tenapanor reduces phosphate absorption in healthy human volunteers

The effects of tenapanor on intestinal phosphate absorption were assessed in a phase 1 study conducted in healthy human volunteers. Participants received tenapanor 15 mg twice daily for 4 days, and stool and urinary phosphorus excretion over this period were compared with baseline. Tenapanor treatment significantly ($P < 0.05$) increased mean daily stool phosphorus excretion from baseline (**Fig. 8D**), which was accompanied by a comparable and significant ($P < 0.001$) decrease in mean daily urinary phosphorus excretion (**Fig. 8E**). These results confirm that the ability of tenapanor to reduce gastrointestinal phosphate absorption in rats translates successfully

into humans. Tenapanor also significantly ($P < 0.001$) reduced mean daily urinary sodium excretion (**Fig. 8F**) but had no effect on urinary potassium excretion (**Fig. 8G**), consistent with its effects in rats.

DISCUSSION

Patients with CKD receiving dialysis develop complications due to mineral imbalances, such as hyperphosphatemia, that need to be managed effectively to improve health outcomes (*9*). Current medical management of hyperphosphatemia with dietary phosphate restriction and oral phosphate binders often proves inadequate to consistently achieve serum phosphate levels within the target range (*22, 47-49*). Additional strategies to optimize serum phosphate control are required, as hyperphosphatemia is associated with poor clinical outcomes (*8-14*). Management of elevated phosphate levels using dietary restrictions alone is generally inadequate, and current pharmacotherapy with oral phosphate binders is frequently poorly tolerated, involves an unacceptable pill burden (*26, 51*) and often provides insufficient efficacy (*52, 53*).

Tenapanor is an investigational, small-molecule agent with a novel different mechanism of action from phosphate binders, and has the potential to address unmet needs in hyperphosphatemia therapy. Our investigations provide evidence for the mechanism by which tenapanor, an NHE3 inhibitor, reduces intestinal phosphate absorption. *In vivo* studies in rodents, combined with translational experiments conducted on human intestinal stem cell-derived enteroid monolayers to model intestinal ion transport physiology, indicate that the predominant

mechanism by which tenapanor produces its effect is the reduction of passive paracellular phosphate absorption, retaining activity even at high luminal phosphate concentrations.

Our findings demonstrate that tenapanor decreases the paracellular absorption of phosphate by modulating tight junctions to increase TEER, thereby reducing paracellular phosphate permeability. Experiments using monolayers of NHE3 KO cells showed that the effect of tenapanor on TEER and paracellular phosphate permeability is mediated exclusively via on-target inhibition of NHE3, and is likely the result of decreased pHi, a consequence of inhibition of proton secretion by NHE3. We have also shown that Tthe onset of action to increase TEER and reduce paracellular phosphate permeability is near immediate following NHE3 inhibition by tenapanor. Previous studies confirm that NHE3 inhibition or deletion is associated with increased TEER *(46, 50, 51)*, although an effect on phosphate absorption has not previously been described. However, rReduced urinary phosphate excretion has been reported in NHE3 KO mice (*52*), consistent with our finding of an NHE3-mediated reduction in intestinal phosphate absorption.

Our studies have also shown that tenapanor is not a direct inhibitor of the most important active phosphate transporter, NaPi2b, although repeat administration of tenapanor *in vivo* does reduce the expression of NaPi2b at the transcriptional level and does not stimulate overall transcellular phosphate uptake. The contribution of reduced NaPi2b expression to the overall phosphate-lowering effect of tenapanor is likely to be minor; however, it does indicate that tenapanor prevents increased active transcellular phosphate uptake from compensating for reduced paracellular phosphate absorption. The downregulation of NaPi2b produced by tenapanor is an

effect that further differentiates it from the commonly used phosphate binder sevelamer, which significantly increases NaPi2b expression in mice (*34*)In contrast, the commonly used phosphate binder sevelamer increases NaPi2b expression in mice (*30*), leading to enhanced transcellular phosphate absorption, which has been proposed to limit the phosphate-lowering efficacy of sevelamer (*53*).

The effects of tenapanor on overall ion balance in preclinical models and in humans appear to be specific for sodium and phosphate. We have previously reported that tenapanor does not affect the overall balance of other ions in healthy humans and rats (*23*). In the current study, we show that tenapanor significantly reduced urinary phosphate excretion in humans, accompanied by increased stool phosphate, but had no effect on urinary potassium excretion. We previously reported that tenapanor had no effect on stool potassium excretion in healthy humans (*23*), confirming the lack of effect of tenapanor on intestinal potassium absorption, an ion reliant on paracellular absorption in the small intestine. We replicate this phenotype in rats and further show that tenapanor does not affect the urinary excretion of an additional anion, chloride. Enteropooling studies measuring luminal ion concentrations in rats confirm the preferential inhibition of phosphate absorption by tenapanor relative to chloride and potassium as well as the divalent cations, calcium and magnesium, which were unaffected by tenapanor. We have previously shown that urinary calcium excretion is unaffected by tenapanor in healthy humans, consistent with this result in rats (*23*).

Although tight junction permeability can display some specificity towards individual or selected ion groups (*34*, *35*), the *in vivo* phosphate preference was not readily reflected in direct

biophysical measurements of paracellular permeability, suggesting tight junction ion specificity is not the basis of tenapanor's *in vivo* phenotypic selectivity. The reliance of phosphate on paracellular absorption likely explains the observed preferential effect of tenapanor on phosphate absorption. This is a consequence of the driving force for phosphate flux which itself is a result of a negative luminal potential applied to a divalent anion and low plasma phosphate concentrations relative to high luminal concentrations and the absence of a low-affinity, high-capacity transcellular phosphate transporter. An examination of tenapanor's effect on chloride, an anion presumably with similar inherent paracellular permeability characteristics to phosphate, highlights the *in vivo* specificity of tenapanor. Paracellular ion flux is an integrated outcome determined by paracellular permeability and the prevailing electrochemical gradient. Chloride permeability was reduced by tenapanor when measured by acutely imposing a nearly 100 mM chemical gradient to capture dilution potentials in Ussing chamber experiments *ex vivo*. However, measured luminal chloride concentrations *in vivo* were insufficient to support paracellular chloride absorption against normal plasma chloride concentrations, which are higher, indicating a prominent role of active transcellular chloride flux. Consequently, tenapanor had minimal overall impact on chloride such that 24-hour urinary chloride excretion was unchanged in the absence of a transcriptional response to upregulate chloride exchangers, transporters or channels.

Further to the assessments of ion flux, tenapanor did not affect the absorption of mannitol tracer, indicating that tenapanor does not affect the paracellular macromolecule absorption pathway under physiological conditions in the small intestine. Moreover, tenapanor had no effect on intestinal glucose absorption. This indicates a low risk of inhibiting the paracellular absorption of

dietary macromolecules and a low risk of drug–drug interactions with the few agents absorbed by this pathway.

A limitation of our study is that the method we used to follow pHi, using a pH-sensitive dye, only measured relative differences between interventions rather than an absolute quantification of pHi. This method was, however, sufficient to follow rapid dynamic pH changes that occurred following treatment with tenapanor and acidic apical media. These measurements are consistent with the ability of tenapanor to modulate tight junction phosphate permeability because of intracellular proton retention, resulting in a rapid decrease in pHi, as the effects of tenapanor on paracellular permeability were mimicked by luminal acidification and other interventions that reduce pHi. As pHi is tightly regulated within a narrow range to support normal cellular functions, it is likely that the magnitude of the effect of tenapanor on pHi is modest and probably locally confined to NHE3-expressing enterocytes. Importantly, we have previously reported in healthy volunteers that treatment with tenapanor does not affect serum bicarbonate or urinary pH, suggesting that systemic acid–base balance is not perturbed by this drug We demonstrated that the ability of tenapanor to modulate tight junction phosphate permeability is likely a result of intracellular proton retention resulting in a rapid decrease in pHi, as the effect of tenapanor on paracellular permeability as reflected by TEER could be mimicked by luminal acidification and other interventions that reduced pHi. Making direct measurements of dynamic, real-time pHi in intestinal monolayers cultured on Transwells and containing diverse cell types, some of which do not express NHE3, is technically challenging. A limitation of our study is that the method we used to follow pHi, using a pH sensitive dye, only provides a relative assessment of directional differences between interventions rather than an absolute quantification of pHi. This was

sufficient to follow the dynamic pH changes that occurred rapidly following treatment with tenapanor and acidic apical media, but does not provide an absolute value of pHi in the enterocyte. pHi is tightly regulated within a narrow range to support normal cellular functions, so it is likely that the magnitude of the effect of tenapanor on pHi is modest and probably locally confined to NHE3 expressing enterocytes. Additional mechanisms, including distinct NHE isoforms as well as bicarbonate transporters, can compensate for reduced NHE3 function at the level of the whole intestine thereby preventing systemic effects on acid–base parameters due to NHE3 inhibition. Indeed, we have previously reported in healthy volunteers that treatment with tenapanor does not affect serum bicarbonate or urinary pH, suggesting that systemic acid–base balance is not perturbed (*23*).

A further limitation to these studies is our ignorance of the identity of the paracellular phosphate pore. The rapid, almost instantaneous increase in TEER with no obvious signaling pathway or tight junction protein trafficking suggests that a direct pH-sensitive conformational change to the tight junction may decrease paracellular phosphate permeability. For example, histidine residues on the intracellular termini of tight junction claudins could be direct pH sensors for the tight junction. Consistent with this possibility, a recently developed trans-tight junction patch clamp technique, applied to measure flux across an individual claudin channel, confirms that modulation of the gating kinetics of tight junction channels is an important mechanism of barrier regulation Our extensive investigations into the link between pHi and tight junction permeability failed to identify the involvement of a second messenger or signaling pathway. The rapid, almost instantaneous increase in TEER with no obvious signaling pathway or tight junction protein trafficking involved suggests the possibility that a direct pH-sensitive conformational change in

the tight junction might decrease paracellular phosphate permeability. For example, histidine residues found on the intracellular termini of tight junction claudins could be candidates for direct pH sensing by the tight junction. (*54*). Unfortunately, the molecular identity of the paracellular phosphate pore has not yet been elucidated, preventing characterization of the specific tight junction proteins involved. Further investigation of the structural and functional response of the tight junction to NHE3 inhibition is therefore required.

To date, the molecular identity of the paracellular phosphate pore has not been elucidated, which prevents the characterization of the specific proteins involved. However, a novel trans-tight junction patch clamp technique, applied to measure flux across an individual claudin channel, has recently suggested that modulation of the gating kinetics of tight junction channels is indeed an important mechanism of barrier regulation (*58*). Further investigation of the structural and functional response of the tight junction to NHE3 inhibition is required to assess this, but beyond the scope of the current study. Connexins, a family of gap junction proteins that like claudins connect cells across the intercellular space, are pH-sensitive, providing a precedent for this possibility (*59-62*).

Our results are most consistent with pHi mediating the effect of NHE3 inhibition on altered paracellular phosphate absorption. However, our work does not exclude a reduction in intracellular sodium (or resultant potential changes in osmolality and cell volume) or chloride, which may accompany NHE3 inhibition as a result of direct inhibition of transcellular sodium absorption or indirectly through inhibition of electroneutral sodium chloride absorption, being the possible mediators. Low intracellular sodium concentrations are established and maintained by the basolateral sodium/potassium (Na^+/K^+) ATPase and the resultant electrochemical sodium

gradient provides the driving force for secondary active intestinal transport of solutes coupled to luminal sodium absorption. The Na^+/K^+ ATPase likely adapts to reduced transcellular sodium influx via NHE3, such as is encountered in periods of fasting, to buffer variations in intracellular sodium and to prevent perturbations in cell osmolality and volume. However, the specific potential contributions of intracellular sodium or chloride concentrations should be the subject of future investigation.The ability of low apical pH to mimic the effect of tenapanor and the observation that other mechanisms that decrease pHi independent of NHE3 also increase TEER are most consistent with pHi coupling the effect of NHE3 inhibition to paracellular phosphate absorption. However, the potential involvement of reductions in intracellular sodium (or resultant potential changes in osmolality and cellular volume) or chloride that may accompany NHE3 inhibition as a result of direct inhibition of transcellular sodium absorption or indirectly through inhibition of electroneutral sodium chloride absorption have not been specifically tested in this series of experiments. Low intracellular sodium concentrations are established and maintained by the basolateral sodium/potassium (Na^+/K^+) ATPase and the resultant electrochemical sodium gradient is employed as the driving force for secondary active intestinal transport of solutes coupled to luminal sodium absorption. The Na^+/K^+ ATPase likely adapts to reduced transcellular sodium influx via NHE3, such as encountered in periods of fasting, to buffer variations in intracellular sodium concentrations and prevent perturbations in osmolality and cellular volume homeostasis. The specific potential contributions of intracellular sodium or chloride concentrations should be the subject of future investigation.

We have previously shown that tenapanor reduced urinary and serum phosphate in a rat model of CKD with vascular calcification (*24*) and here show that these effects on intestinal phosphate absorption translate to healthy humans. Tenapanor, a minimally absorbed, small-molecule inhibitor of NHE3 effectively increased stool phosphorus excretion and decreased urinary phosphorus excretion in humans when one 15 mg tablet was administered twice daily. Despite currently available treatments, many patients with ESRD fail to maintain target range serum phosphate concentrations (*55, 56*), with a key contributing factor being poor adherence to phosphate binder use as a result of the high required pill burden to effectively capture dietary phosphate (*57*). Oral phosphate binders act to bind phosphate directly in the gut lumen, forming non-absorbable complexes that are excreted in the feces and reducing the availability of dietary phosphate for absorption (*65*). To capture dietary phosphate effectively, however, binders need to be administered in multi-gram quantities, which results in a high pill burden. For patients receiving hemodialysis, phosphate binders can account for almost half of the total daily pill burden (*26*). Adherence to treatment is typically low and decreases with increasing pill counts, and the high pill burden can also adversely affect health-related quality of life (*26*). Tenapanor has potential to reduce the pill burden for these patients, and thereby improve treatment compliance. In addition, tenapanor remains effective in inhibiting intestinal phosphate absorption at high luminal phosphate concentrations. Tenapanor may therefore be the first agent that can effectively inhibit intestinal phosphate absorption in the setting of a liberalized phosphate diet. This could contribute an important significant nutritional benefit to patients with ESRD.

In conclusion, tenapanor reduces intestinal phosphate absorption through the reduction of passive paracellular phosphate influx, quantitatively the most important overall mechanism of intestinal

phosphate absorption. Tenapanor modulates tight junctions to increase TEER and reduce paracellular permeability to phosphate; this effect is mediated exclusively via on-target inhibition of NHE3, and is likely the result of decreased pHi due to the inhibition of proton secretion. This novel mechanism of action means that Ttenapanor retains activity efficacy at high luminal phosphate concentrations and this translates to reduction of intestinal phosphate absorption in healthy humans. Evaluation of tenapanor in patients with ESRD and hyperphosphatemia is ongoing, to determine whether tenapanor provides offers a differentiated new approach to serum phosphate control in the clinical setting (*58*).

MATERIALS AND METHODS

Full details of materials and methods are included as Supplementary Material; an overview is provided here.

Experimental Study design

The primary objective of this work was to elucidate the mechanism by which tenapanor reduces intestinal phosphate absorption, which was explored using a combination of *in vivo* studies in healthy rodents and translational experiments on human intestinal stem cell-derived enteroid monolayers to model ion transport physiology. Intestinal phosphate absorption was assessed *in vivo* by measuring luminal and urinary phosphate concentration and excretion under various experimental conditions that manipulated the phosphate concentration gradient. Phosphate flux,

TEER and paracellular ion permeability were measured in human intestinal epithelial stem cell-derived enteroid monolayers under varying phosphate concentration gradients. The animal and cellular monolayer studies were controlled with vehicle control groups and assignment to treatment or control was randomized. Rigorous blinding of cellular and animal experiments with tenapanor was precluded due to obvious visual pharmacodynamic effects of tenapanor to prevent apical acid secretion in the monolayers and to alter fecal form in rodents; however, the analytical measurements for ion concentration were performed in a blinded manner. The translational effect of tenapanor on intestinal phosphate absorption was evaluated in a single-center, randomized, open-label study in 18 healthy human volunteers.

The primary objective of this work was to elucidate the mechanism by which tenapanor reduces intestinal phosphate absorption, which was explored using a combination of *in vivo* studies in rodents and translational experiments on human intestinal stem cell-derived enteroid monolayers to model ion transport physiology. The effects of tenapanor on phosphate uptake seen in preclinical models were then evaluated in a clinical study in healthy volunteers.

Animal studies

Animal Use

Animal experiments performed in the USA were conducted using experimental protocols approved by the Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments in the UK were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986, Amendment Regulations 2012, using protocols approved by the University College London (Royal Free Campus) Comparative Biology Unit Animal Welfare

and Ethical Review Body (AWERB) Committee. Experiments in Sweden were approved by The Regional Animal Ethics Committee for Experimental Animals, University of Gothenburg.

In vivo *loop model*

In anesthetized male Sprague Dawley (SD) rats, a 5 cm segment of jejunum was cannulated and flushed with warm 0.9% saline, then air. Uptake buffer containing $^{33}PO_4^{3-}$ plus tenapanor (10 μM) or vehicle control was instilled in the lumen and the segment tied off. Scintillation counting of plasma and initial uptake solution was used to calculate phosphate transfer from the lumen into the blood. The procedure was also performed in ileum loops of intestinal-specific NaPi2b KO mice (*28*) and wild-type littermates.

In vivo *oral phosphate bolus*

After overnight fast, male SD rats were dosed orally with tenapanor (0.5 mg/kg) or vehicle, and 1 mL of NaH_2PO_4 solution (0.15, 0.5 or 1.5 M) or water control, and immediately placed in individual metabolic cages for 4 hours. Urinary sodium and phosphate concentration, measured by ion chromatography, and urine volume were used to calculate 4-hour urinary sodium and phosphate excretion.

Changes in dietary phosphorus intake

Male SD rats, fed diets containing 0.3%, 0.6% or 1.2% phosphorus (TD.85010, TD.84122, TD.85349 respectively, Envigo, Maddison, WI), were individually housed in metabolic cages. A 24-hour urine collection was performed at baseline and following twice-daily oral treatment with tenapanor (0.5 mg/kg) or vehicle, for 4 days. Urinary sodium and phosphate concentration,

measured by ion chromatography, and urine volume were used to calculate 24-hour urinary sodium and phosphate excretion.

Enteropooling studies to measure luminal ion content

Male SD rats fed a 0.6% phosphorus diet for 6 days were dosed orally with tenapanor (0.15 mg/kg) or vehicle immediately before a final 5 g meal containing 1.2% phosphorus (TD.85349). In one cohort of animals, 4-hour urine collection was performed to determine urinary sodium and phosphate excretion. In a separate cohort, animals were euthanized at 0.5, 1 and 2 hours following dosing, and the contents of the cecum were collected for ion chromatography.

Effects of long-term administration of tenapanor on phosphate and sodium in vivo

Male SD rats fed a diet containing 0.6% phosphorus (TD.84122), were dosed orally with tenapanor (0.5 mg/kg) or vehicle twice daily for 14 days. Daily 24-hour urinary collections were made to measure urinary sodium and phosphate excretion. On the final study day, plasma was collected for measurement of sodium, phosphate, FGF-23 (C-terminal and intact), intact parathyroid hormone and vitamin D. Renal sodium and phosphate clearance and fractional excretion were calculated using standard methods. RNA was isolated from various gastrointestinal segments and NaPi2b mRNA expression was measured by qPCR.

Radioactive mannitol and phosphate absorption

Male SD rats were surgically implanted with a jugular vein catheter, recovered (1 week), fasted overnight and then dosed orally with tenapanor or vehicle along with ^{33}P- or ^{3}H-mannitol tracer

and blood samples were collected at multiple time points through 120 minutes. Plasma radioactivity was measured by liquid scintillation.

Immunohistochemistry in rat jejunum after *in vivo* treatment with tenapanor

Tissues were fixed in 4% neutral buffered formaldehyde and paraffin-embedded. The primary antibody AGGSY87 (AGG) diluted 1:1500 (0.4 µg/mL) in Da Vinci Green (Biocare medical, PD900M) was incubated for 1 hour followed by use of a Rabbit on Rodent Polymer kit (RMR622, Biocare Medical).

Intestinal epithelial stem cell monolayer culture model

Intestinal epithelial stem cell monolayers were cultured and differentiated on Transwells as described in detail by Kozuka *et al.* (*38*). Human biopsies from which stem cells were sourced were obtained according to a protocol approved by the Copernicus Group Institutional Review Board (Durham, NC, USA).

Experiments were initiated in each differentiated monolayer culture well by washing the apical and basolateral side twice with fresh supplemented basal media and phosphate-free Dulbecco's modified Eagle medium. Compounds were dosed only on the apical side of the monolayer, as detailed in the text; DMSO at an equivalent concentration was used as the control. Phosphate concentration and pH were manipulated as described in the text. Apical and basolateral ion concentrations were measured by ion chromatography, pH was measured using a pH meter, and TEER values were recorded using a Volt/Ohm meter. TEER results are reported as normalized to baseline levels. Absolute baseline TEER values are reported in **Supplementary Table S1**.

pHi recovery (NHE3 activity assay)

Stem cell-derived human intestine organoid monolayers were incubated with 10 µM pH-sensitive BCECF-AM dye for 1 hour in sodium-free buffer, then the dye was aspirated and the cells washed with sodium-free buffer. Sodium-containing buffer at different pH values levels was added to the apical compartment, in the presence and absence of tenapanor, while monitoring the intracellular BCECF signal using a fluorescent imaging plate reader (FLIPR). NHE3 activity was calculated by measuring the initial rate of change of fluorescence from the time of buffer addition to 180 seconds. It is important to note that in our experiments only relative, not absolute, changes in pH were measured.

pHi endpoint assay

Human organoid stem cell monolayers were incubated with 10 µM pH-sensitive BCECF-AM dye for 30 minutes in sodium-containing buffer, then the dye was aspirated and the cells washed with sodium-containing buffer. Tenapanor or DMSO control were added to the apical compartment, and fluorescence readings were acquired every 5 minutes for 30 minutes. The pH level was calculated from the ratio of the two fluorescent excitation wavelengths. The effects of nigericin, FCCP and BAM15 on pHi were also determined 30 minutes after application using the same method. Over the 30-minute period of intracellular BCECF intensity measurements, plates were kept at 37°C in ambient CO_2.

Biophysical assessment of paracellular permeability using dilution and biionic diffusion potential measurements

To assess the effect of tenapanor on paracellular ion permeabilities we performed paired experiments in Ussing chambers on human duodenum monolayer cultures and mouse jejunum tissue *ex vivo*. We first measured a sodium chloride (NaCl) dilution potential followed by a phosphate biionic dilution potential, and then we repeated these measurements on the same samples but in the presence of tenapanor or vehicle control. Similar methods have been described previously (*59*). NaCl dilution potentials were induced by changing the apical chamber from a Krebs Ringers solution to a low sodium chloride (55 mM) solution otherwise identical to Krebs Ringers with osmolality balanced with mannitol. Phosphate chloride biionic diffusion potentials were generated by replacing the apical solution with modified Krebs Ringers where Na_2HPO_4 replaced most of the NaCl. Osmolality was balanced with mannitol. The pH of each solution was titrated to 8.0 with 1 M sodium hydroxide, to ensure a favorable proton gradient for NHE3 mediated transport and to simplify permeability calculations by restricting the phosphate to the HPO_4^{2-} species.

Immunofluorescence and 3D confocal imaging of cultured cells

Differentiated human ileum cells in Transwell plates were treated with tenapanor (1 µM) or DMSO on the apical side. For endocytosis inhibitor treatment, cells were pre-treated with pitstop2 or dynasore for 30 minutes, then carbachol (10 µM) was added and the cells incubated for 30 minutes. Nuclei were detected by DAPI (4',6-diamidino-2-phenylindole) staining; the apical membrane and F-actin filaments were stained by adding WGA Alexa Fluor 488 conjugate and phalloidin dye, respectively, to the apical side. After staining, the membrane was cut out and mounted, and cells visualized by confocal microscopy.

CRISPR/Cas9-mediated NHE3 gene editing

Gene editing was performed using lentiviral Cas9 and single guide RNA (sgRNA) particles. For the initial transduction, organoids (broken into single cells) were mixed with Edit-R lentiviral mCMV-Blast-Cas9 nuclease particles, and transduced cells were selected with blasticidin. At 2–3 weeks post-transduction, cells with stable Cas9 overexpression were further transduced with Edit-R human lentiviral SLC9A3 sgRNAs or non-targeting control #1 sgRNA particles, and selected with puromycin. For clonal expansion of NHE3 KOs and non-targeting controls, single organoids were picked and expanded in growth medium for 2 weeks in the presence of antibiotics. Transduced cells were genotyped using PCR on isolated genomic DNA; protein expression in transduced cells was assessed using immunoblot analysis.

Brush Border Membrane Vesicle (BBMV) isolation and phosphate transport

BBMVs were prepared by the Mg^{2+}-precipitation technique (*60*-*62*) from mucosal cells scraped from the individual segments of inverted small intestine. Final membranes were resuspended at a concentration of 1–15 mg total protein, determined according to Bradford (*63*). The transport rates of ^{32}P and ^{3}H-D-glucose into BBMVs were measured as described (*64*) at 25°C in the presence of inwardly directed gradients of 100 mM NaCl or 100 mM KCl and 0.1 mM potassium phosphate or 0.1 mM D-glucose. Uptakes were measured after 90 seconds and 90 minutes to determine equilibrium values.

Tenapanor in healthy volunteers

Healthy male and female volunteers (aged 19–65 years; BMI 18–29.9 kg/m^2) were enrolled in a single-center, randomized, open-label, three-way crossover study to evaluate different tenapanor

formulations (ClinicalTrials.gov identifier: NCT02249936). The study protocol was approved by the IntegReview Institutional Review Board (Austin, TX, USA). All participants provided written informed consent and the study was conducted in accordance with the Declaration of Helsinki, and International Conference on Harmonisation and Good Clinical Practice guidelines. Volunteers received a different one of the three tenapanor formulations in each treatment period (days 1–4, 7–10 and 13–16), according to the randomization scheme. Tenapanor was administered twice daily before a standardized meal, and 24-hour urine and stool collections were made daily throughout the study period (day –2 to day 17). Data are presented for the tenapanor HCl tablet formulation.

Statistical Analysis

The data analyzed were continuous, quantitative and normally distributed, as assessed by D'Agostino-Pearson omnibus normality test. Between-group differences over time were assessed by a two-way mixed-design analysis of variance (ANOVA), and *post hoc* testing at each time point was performed using Bonferroni's procedure to correct for multiple comparisons (GraphPad Prism 6). Between-group differences (≥ 3-groups) with only one time point were assessed by one-way ANOVA and *post hoc* multiple comparisons with Dunnet's test. Pair-wise comparisons were made by Student's t-test. $P < 0.05$ was considered significant. All results are presented as mean ± standard error, except the clinical study (**Fig. 8D–G**) for which results are mean ± standard deviation.

LIST OF SUPPLEMENTARY MATERIALS

Supplementary Materials and Methods

Fig. S1. NHE3 is endogenously, highly expressed at high levels in enteroid monolayer cultures from different segments of the human and mouse intestine and is potently and completely inhibited by tenapanor.

Fig. S2. Tenapanor inhibits phosphate absorption across a range of initial apical phosphate concentrations in human ileum monolayer cultures.

Fig. S3. NHE3-independent changes in pHi modulate trans-epithelial electrical resistance in intestinal ileum monolayer cultures.

Fig. S4. Paracellular phosphate absorption in NHE3 knockout human duodenum monolayer cultures.

Fig. S5. Renal and hormonal responses to chronic *in vivo* tenapanor treatment in healthy rats.

Fig. S6. Tenapanor has little effect on phosphate uptake predominantly mediated by NaPi2b or sodium-dependent glucose uptake in rat duodenum or jejunum brush border membrane vesicles.

Fig. S7. Tenapanor does not affect the localization of tight junction specific proteins in human ileum and tenapanor-induced increases in TEER are not affected by blocking endocytosis.

Table S1. Absolute TEER (Ohm.cm^2) at baseline for all experimental conditions prior to the experimental intervention.

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Competing interests: AJK, MS, YH, BN, JW, SK-M, NAM, QJ, DP, JK, PK, KK, JL, DD, DR and JSC are current or former employees of Ardelyx and have ownership interest in Ardelyx. PJG, ACJR, DK, MB, MS and TRB are employees of AstraZeneca and have ownership interest in AstraZeneca. JM has a consultancy agreement with Ardelyx and AstraZeneca. RTA has a consultancy agreement with Ardelyx and is the Canada Research Chair in Renal Tubular Epithelial Transport Physiology. Tenapanor is covered by patent WO2010078449 (*66*).

Data and materials availability: All relevant study data are reported in the main text and Supplementary Materials.

FIGURE LEGENDS

Fig. 1. Effects of tenapanor on phosphate absorption *in vivo* in rats.

(**A**) Effect on radioactive phosphate absorption of tenapanor (10 μM) and of a sodium-free buffer (ChCl plus PFA) in the rat jejunum *in vivo* loop model with initial luminal phosphate concentration of 8 mM; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 vs vehicle (n = 5–7 per group). (**B**) Urinary phosphate excretion 4 hours after an oral bolus of varying phosphate concentrations (0.15–1.5M) with or without tenapanor (0.5 mg/kg) *in vivo* in rats; **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 vs vehicle (n = 6 per group). (**C**) Urinary phosphate excretion at different dietary phosphate levels intake at baseline and after 4 days of treatment with tenapanor (0.5 mg/kg) or vehicle *in vivo* in rats; ****P* < 0.001, *****P* < 0.0001 (n = 7 per group). (**D**) Urinary phosphate and urinary sodium excretion 4 hours after a fixed quantity (5 g) of high phosphorus (1.2%) meal in rats treated with tenapanor (0.15 mg/kg) or vehicle; *****P* < 0.0001 vs vehicle (n = 8 per group). Cecal (**E**) sodium delivery, (**F**) phosphate delivery, (**G**) water volume, (**H**) sodium concentration, (**I**) phosphate concentration, (**J**) potassium concentration, (**K**) chloride concentration, (**L**) calcium concentration, and (**M**) magnesium concentration in rats fed a fixed quantity of high phosphorus (1.2%) meal and treated with vehicle or tenapanor (0.15 mg/kg); **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 (n = 6 per time point per group).

Fig. 2. Effects of phosphate concentration gradient and tenapanor on phosphate absorption in human duodenum monolayer cultures.

(**A**) Correlation between phosphate flux and initial apical phosphate concentration in human duodenum monolayer cultures from two separate donors, A and B, and the reproducibility of phosphate absorption in donor B in experiments from two separate passages (n = 4 per group) following overnight incubation. (**B**) Basolateral phosphate concentration, (**C**) phosphate flux, and (**D**) trans-epithelial electrical resistance (TEER) at different apical phosphate concentrations (1–30 mM) in human duodenum monolayer cultures following 4 hours of treatment with tenapanor (1 μM) or vehicle with an initial starting basolateral phosphate concentration of 0 mM; **P* < 0.05, *****P* < 0.0001 vs vehicle (n = 4–16 per group). (**E**) Apical phosphate retention, (**F**) apical phosphate concentration, (**G**) basolateral phosphate concentration, and (**H**) apical fluid volume at different initial apical phosphate concentrations (1–30 mM) in human duodenum monolayer cultures following overnight treatment with tenapanor (1 μM) or vehicle with an initial starting basolateral phosphate concentration of 1 mM; data from donor B. (**I**) Effect of tenapanor (1 μM) or vehicle on bidirectional phosphate flux at varying phosphate concentration gradients (1–10 mM) in human duodenum monolayer cultures; **P* < 0.05, ****P* < 0.001, *****P* < 0.0001 vs vehicle (n = 4 per group).

Fig. 3. Trans-epithelial electrical resistance and pHi in human intestinal cell monolayer cultures.

Effects of tenapanor (1 μM) versus control on trans-epithelial electrical resistance (TEER) following the change from old acidic (pH 6.0) apical media at baseline to fresh neutral pH apical media to restore the gradient for NHE3-mediated proton efflux in (**A**) human duodenum and (**B**) human ileum cell monolayer cultures; $^{**}P < 0.01$, $^{****}P < 0.0001$ vs control (n = 3 per group). (**C**) TEER following the change from old acidic (pH 6.0) apical media at baseline to fresh apical media at different (acidic to alkaline) pH in human duodenum monolayer cultures (n = 3 per group). (**D**) Effect of tenapanor (1 μM) on TEER at varying apical media pH in human duodenum monolayer cultures normalized to equivalent pH level treated with vehicle (n = 3 per group). Recovery of pHi, initiated by the addition of a sodium-containing media and measured using pH-sensitive BCECF-AM dye following acid loading in sodium-free media in (**E**) human duodenum and (**F**) human ileum monolayer cells at different apical pH in the presence of tenapanor (1 μM) or vehicle. (**G**) Concentration–response effect of tenapanor on rate of the recovery from intracellular acidification at neutral and acidic apical pH in human ileum monolayer cultures. (**H**) Change in pHi, measured using BCECF-AM dye, over time following the change to acidic, neutral or alkaline pH apical media in human ileum monolayer cultures; $^{***}P < 0.001$, $^{****}P < 0.0001$ vs pH 6.0. Effect of tenapanor (1 μM) (vs control) on change in (**I**) pHi and (**J**) TEER over time in human ileum monolayer cultures; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control (n = 3–6 per group).

Fig. 4. Effect of apical pH on phosphate flux and effect of tenapanor on phosphate flux at different apical pH levels.

Effect of varying apical pH levels on basolateral phosphate flux in human ileum epithelial cell monolayer cultures following an overnight incubation at an initial apical phosphate concentration of 10 mM or 30 mM and an initial basolateral phosphate concentration of 0 mM, in the absence (**A**) and presence (**B**) of tenapanor (1 μM); the effect of tenapanor is represented as change from vehicle. (**C**) Phosphate flux, measured with radioactive tracer and (**D**) paracellular phosphate permeability (pPO_4^{3-}), measured by biionic dilution potential in mouse jejunum strips at pH 6.0 and pH 8.0. *P <0 .05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs control (n = 4–6 per group).

Fig. 5. Effect of tenapanor on paracellular permeability measured by direct biophysical methods, and the chemical driving forces for paracellular absorption *in vivo* in rats.

Effect of tenapanor at pH 8.0 on (**A**) TEER, (**B**) sodium permeability, pNa^+, (**C**) chloride permeability, pCl^-, (**D**) sodium-to-chloride permeability, pNa^+/pCl^- and (**E**) phosphate permeability, pPO_4^{3-} in human duodenum monolayer cultures mounted in Ussing chambers. Effect of tenapanor on (**F**) TEER, (**G**) pNa^+, (**H**) pCl^-, (**I**) pNa^+/pCl^- and (**J**) pPO_4^{3-} across mouse jejunum *ex vivo* mounted in Ussing chambers. Luminal concentrations of (**K**) sodium, (**L**) chloride and (**M**) phosphate in the proximal and distal small intestine in untreated rats trained to eat a standardized meal. $*P < 0.05$ vs control (n = 6 per group).

Fig. 6. Paracellular phosphate absorption in NHE3 knockout human ileum monolayer cultures.

(**A**) DNA sequencing showing CRISPR/Cas9-mediated gene editing of NHE3 resulting in nucleotide deletions and insertions. The sgRNA targeting human NHE3 genomic DNA exon 2 (sgNHE3-13) is shown in red and the protospacer adjacent motif (PAM) region in blue. The NHE3 knockout line (sgNHE3-13 clone 45) contains two different types of mutations (Mut 1 and Mut 2), shown in grey boxes. (**B**) Western blot showing NHE3 and beta-actin (ACTB) protein expression in control and NHE3 edited cells, (**C**) apical media showing acidification (yellow) or absence of acidification (pink) in control and NHE3 edited cells. Recovery from intracellular acidification following acid loading in the absence or presence of tenapanor in (**D**) control (non-targeting sgControl clone 4) and (**E**) NHE3 knockout (sgNHE3-13 clone 45) human ileum epithelial cell clones. (**F**) Apical volume, (**G**) apical sodium, (**H**) apical phosphate and (**I**) apical pH in control and NHE3 knockout human ileum monolayers following overnight incubation; $^{**}P < 0.01$, $^{****}P < 0.0001$ vs control (n = 6 per group). (**J**) TEER following the change from old acidic (pH 6.0) apical media at baseline to fresh neutral pH apical media to restore the gradient for NHE3 mediated proton efflux in control and NHE3 knockout monolayers; $^{****}P < 0.0001$ vs control (n = 4 per group). (**K**) Change in apical-to-basolateral phosphate absorption and (**L**) change in apical phosphate concentration with tenapanor (1 μM), normalized to the effect of vehicle in NHE3 knockout and control monolayers; $^{*}P < 0.05$, $^{***}P < 0.001$ vs vehicle (n = 4 per group). (**M**) Change in TEER with tenapanor relative to vehicle following the change to neutral pH apical media in control and NHE3 knockout monolayers; $^{****}P < 0.0001$ vs control (n = 4 per group).

Fig. 7. Effects of tenapanor on NaPi2b mRNA and protein expression *in vivo* in rats, the effects of tenapanor and a NaPi2b inhibitor on phosphate absorption in mouse ileum monolayer cultures, and the effects of tenapanor on phosphate absorption in the *in vivo* loop model in the ileum of wild-type and NaPi2b knockout mice.

(**A**) NaPi2b mRNA expression in different intestinal segments following 14 days of tenapanor (0.5 mg/kg) or vehicle treatment in rats; $^{**}P < 0.01$, $^{****}P < 0.0001$ vs vehicle (n = 8 per group). (**B**) Immunohistochemistry showing NaPi2b expression in rat jejunum following *in vivo* treatment with tenapanor or vehicle. (**C**) Apical and basolateral phosphate concentrations following overnight incubation at different initial apical phosphate concentrations (1–5 mM) in mouse ileum monolayer cultures with initial basolateral phosphate concentration of 1 mM; $^{****}P < 0.0001$ (n = 6 per group). (**D**) Apical phosphate concentrations following a 4-hour, 2-day or 3-day incubation with tenapanor (1 µM), NTX-9066 (a NaPi2b inhibitor; 1 µM) or vehicle in mouse ileum monolayer cultures; $^{****}P < 0.0001$vs initial apical phosphate concentration (1 mM). Initial basolateral phosphate concentration was set at 1 mM, so there was no phosphate concentration gradient for passive diffusion (n = 6 per group). (**E**) Phosphate absorption with tenapanor (10 µM) versus vehicle in NaPi2b knockout (KO) and control (wild-type) mouse ileum in an *in vivo* ileum loop model; $^{***}P < 0.001$ (vehicle-treated wild-type vs NaPi2b KO) (n = 4–5 per group).

Fig. 8. Effects of tenapanor on paracellular macromolecule absorption compared with phosphate absorption in rats, and on phosphate, sodium and potassium absorption in healthy human volunteers.

(**A**) Radioactive phosphate and (**B**) radioactive mannitol absorption *in vivo* in rats following treatment with different doses of tenapanor. (**C**) Small intestinal glucose content following initiation of a standardized meal in rats pretreated with vehicle or tenapanor; **P* < 0.05, ***P* < 0.01, *****P* < 0.0001 vs vehicle (n = 4–7 per group). (**D**) Stool and (**E**) urinary phosphorus, (**F**) urinary sodium and (**G**) urinary potassium excretion at baseline and after 4 days of tenapanor treatment (15 mg b.i.d.) in healthy volunteers participating in a formulation study; **P* < 0.05, ****P* < 0.001, *****P* < 0.0001 vs day −1.