Calcineurin inhibitor effects on kidney electrolyte handling and blood pressure: tacrolimus versus voclosporin

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Running head: Tacrolimus vs. voclosporin: electrolytes and BP.

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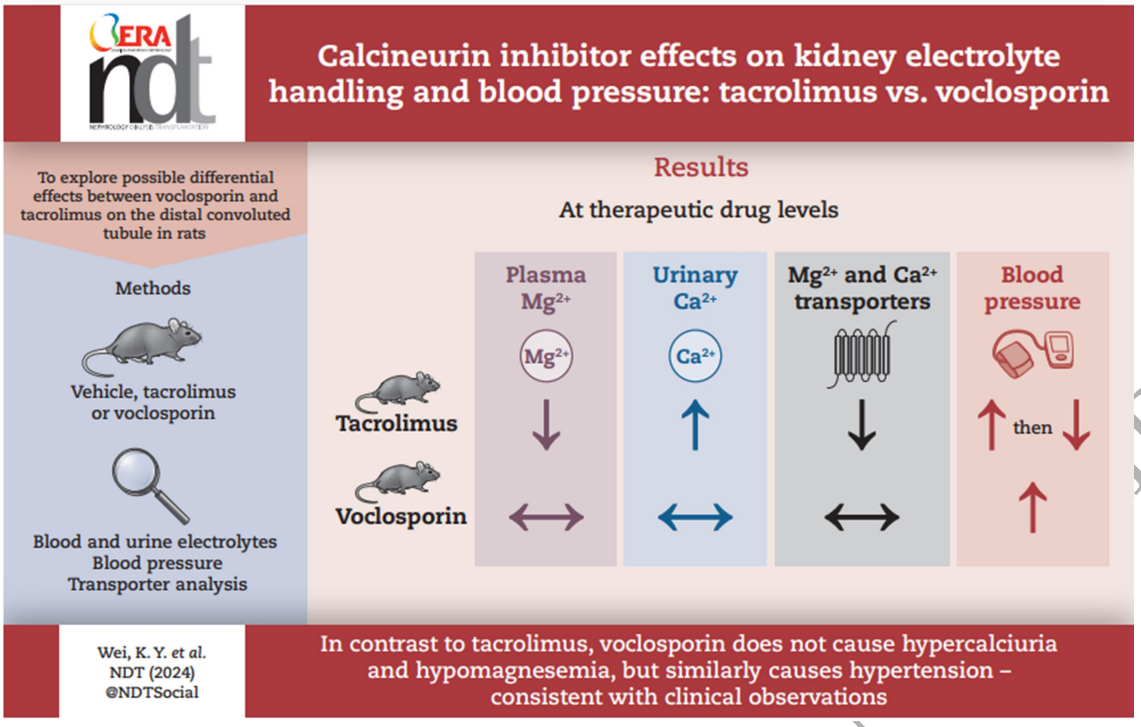
GRAPHICAL ABSTRACT



ABSTRACT

Background and hypothesis. Calcineurin inhibitors affect kidney electrolyte handling and blood pressure through an effect on the distal tubule. The second generation calcineurin inhibitor voclosporin causes hypomagnesemia and hypercalciuria less often than tacrolimus. This suggests different effects on the distal tubule, but this has not yet been investigated experimentally.

Methods. Rats were treated with voclosporin, tacrolimus or vehicle for 28 days. Dosing was based on a pilot experiment to achieve clinically therapeutic concentrations. Drug effects were assessed by electrolyte handling at day 18 and 28, thiazide testing at day 20, telemetric blood pressure recordings, and analysis of mRNA and protein levels of distal tubular transporters at day 28.

Results. Compared to vehicle, tacrolimus but not voclosporin significantly increased the fractional excretions of calcium (>4-fold), magnesium and chloride (both 1.5-fold) and

caused hypomagnesemia. Tacrolimus but not voclosporin significantly reduced distal tubular transporters at mRNA and/or protein level, including the sodium-chloride cotransporter, transient receptor melastatin 6, transient receptor potential vanilloid 5, cyclin M2, sodium-calcium exchanger and calbindin-D_{28K}. Tacrolimus but not voclosporin reduced the mRNA level and urinary excretion of epidermal growth factor. The saluretic response to hydrochlorothiazide at day 20 was similar in the voclosporin and vehicle groups, whereas it was lower in the tacrolimus group. The phosphorylated form of the sodium-chloride cotransporter was significantly higher at day 28 in rats treated with voclosporin than in those treated with tacrolimus. Tacrolimus transiently increased blood pressure, whereas voclosporin caused a gradual but persistent increase in blood pressure which was further characterized by high renin, normal aldosterone, and low endothelin-1.

Conclusions. In contrast to tacrolimus, voclosporin does not cause hypercalciuria and hypomagnesemia, but similarly causes hypertension. Our data reveal differences between the distal tubular effects of tacrolimus and voclosporin and provide a pathophysiological basis for the clinically observed differences between the two calcineurin inhibitors.

Keywords: aldosterone, calcineurin inhibitors, calcium, hypertension, hypomagnesemia, mineral metabolism

KEY LEARNING POINTS

What was known:

- Calcineurin inhibitors cause hypomagnesemia, hypercalciuria and salt-sensitive hypertension.
- The second generation calcineurin inhibitor voclosporin causes hypomagnesemia and hypercalciuria less frequently than tacrolimus, but similarly causes hypertension.

This study adds:

- At clinically therapeutic concentrations in rats, tacrolimus but not voclosporin increases the fractional excretions of calcium and magnesium leading to hypercalciuria and hypomagnesemia.
- Tacrolimus but not voclosporin significantly reduced calcium and magnesium transporters, including TRPM6, TRPV5, CNNM2, NCX1 and calbindin-D_{28K}.
- Voclosporin causes hypertension that is further characterized by high renin, normal aldosterone, and low endothelin-1.

Potential impact

- The study reveals differences between the effects of tacrolimus and voclosporin on the distal tubule.
- This study provides a pathophysiological explanation for the improved clinical safety and tolerability of voclosporin with long-term treatment.
- Future studies should further pinpoint the pathogenesis of voclosporin-induced hypertension to select the most effective anti-hypertensive therapy.

INTRODUCTION

Calcineurin inhibitors (CNIs) are commonly used drugs to prevent rejection after organ transplantation and to treat autoimmune diseases (1). Immunosuppression by CNIs comes at the cost of adverse effects including hypertension, hyperkalemia, metabolic acidosis, hypomagnesemia, and hypercalciuria. In animal studies, tacrolimus has similar adverse effects as cyclosporine (2-8). In patients, tacrolimus causes adverse effects more often than cyclosporine (9), except for hypertension which is more often seen with cyclosporine (10). These adverse effects have been attributed, at least in part, to disturbed ion reabsorption in the distal part of the nephron. Previous studies have shown that tacrolimus and cyclosporine activate the sodium-chloride cotransporter (NCC) in the distal convoluted tubule (DCT) to cause salt-sensitive hypertension (4, 6, 7). Other proposed mechanisms include vascular effects (11) and upregulation of the sodium-potassium chloride cotransporter 2 (NKCC2) (12). Hyperkalemia and metabolic acidosis may also be secondary to NCC activation (13) or altered expression of acid-base transport proteins (14). Tacrolimus and cyclosporine-induced hypercalciuria and hypomagnesemia have been attributed to downregulation of calcium and magnesium transport proteins in the DCT and connecting tubule (CNT) (2, 3, 5, 8).

Voclosporin is a second generation calcineurin inhibitor and is structurally distinct from cyclosporine with a modification of a functional group on the amino acid-1 residue. This modification enhances calcineurin binding and allows for faster elimination (15, 16). In the PROMISE trial, a phase II trial in kidney transplant recipients, voclosporin was non-inferior to tacrolimus in preventing acute rejection (17). In this study it was noted that serum magnesium levels were significantly lower in patients receiving tacrolimus than voclosporin and that this was independent of dose. Patients treated with voclosporin also had a significantly lower incidence of post-transplantation diabetes mellitus compared to

tacrolimus. Although the pathogenesis of CNI-induced post-transplantation diabetes mellitus is multifactorial (18), studies have shown that post-transplantation diabetes mellitus is also related to CNI-induced hypomagnesemia (19). In the PROMISE trial, the incidence of hypertension was similar between voclosporin and tacrolimus. Voclosporin did have a lower incidence of hypertension than cyclosporine in patients treated with these drugs for psoriasis (15, 20). In the AURA-LV phase II trial, as well as AURORA 1 and AURORA 2 phase III trials, voclosporin improved kidney outcomes in patients with active lupus nephritis when added to mycophenolate mofetil and low-dose steroids (21-23). In these trials, again, voclosporin had no significant effect on serum magnesium and potassium compared to placebo.

Together, these data suggest differential effects between voclosporin and tacrolimus on kidney electrolyte handling in the distal tubule, but this hypothesis has not yet been tested. In addition, it is unclear how voclosporin affects blood pressure. To address this, we compared the effects of tacrolimus, voclosporin, and vehicle on plasma and urinary electrolyte levels, the expression of tubular transporters and blood pressure in rats.

MATERIALS AND METHODS

Animal studies

All animal experiments were approved by the Animal Welfare Committee of the Erasmus Medical Center (protocol number 16-511-05). Male Wistar-Kyoto rats (Janvier Labs, France) were obtained at 7 weeks of age and maintained in a controlled environment with temperature at 22°C and a 12-hour light/dark cycle. Animals were housed in individual cages with access to water and standard chow (0.5% w/w NaCl; Teklad 2016; Envigo). Prior to the experiments in which we compared the effects of vehicle, tacrolimus and voclosporin, we first performed a separate experiment to investigate the single-dose pharmacokinetics of tacrolimus and voclosporin. Rats received jugular vein cannulation under inhalation anaesthesia to facilitate blood sampling. Three days after this procedure, rats were administered a single intraperitoneal injection of tacrolimus (0.5 mg/kg) and voclosporin (0.4 or 1.0 mg/kg). Blood sampling was performed 30 minutes before the injections and 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, and 24 hours after drug administration. Based on this dose finding study we selected a voclosporin dose of 0.5 mg/kg for the next experiments of which the study design is shown in Figure 1. The animals were acclimatized for at least 12 hours before being placed in metabolic cages. At baseline there were no differences between groups (Table S1). Subsequently, rats were randomly allocated and treated with vehicle (Cremophor EL: 95% ethanol: saline 5:5:90, v:v:v), tacrolimus (0.5 mg/kg; SelleckChem, The Netherlands), or voclosporin (0.5 mg/kg; provided by Aurinia Pharmaceuticals) by daily intraperitoneal injections for 28 days (n = 8–9/group). We determined these doses in a pharmacokinetic experiment to achieve a clinically therapeutic area under the concentration-time curve. After 18 days of treatment (based on (7)), blood and 24-hour urine were collected to measure trough levels of the drugs and to analyze fractional excretions of electrolytes. Thiazide testing was performed on days 19 and 20 by giving a

single intraperitoneal injection of vehicle (DMSO:saline, 10:90, v:v) and hydrochlorothiazide (25 mg/kg), respectively (24, 25). The natriuretic and chloriuretic responses were calculated from 6-hour excretions (thiazide minus vehicle) and expressed as the change in urinary sodium (ΔUNa^+) and chloride (ΔUCl^-) excretions (µmol/6h). To allow sufficient time for wash-out, rats were sacrificed on day 28, blood was collected, and kidneys were harvested and snap frozen in liquid nitrogen until further analysis.

Measurements in blood and urine

The tacrolimus concentration in whole blood was analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS), as described before (26). The voclosporin concentration in whole blood was also measured by LC-MS/MS in another laboratory (KCAS, Bioanalytical & Biomarker Services, USA) using a validated protocol for the range of 4.00–1000 ng/mL. Plasma concentrations of creatinine, urea and ions and urine concentrations of creatinine and ions were measured by the Erasmus MC clinical chemistry lab. Free calcium was measured from heparinized whole blood using a calcium-selective electrode. Estimated glomerular filtration rate (eGFR) was calculated by a recently validated plasma creatinine- and urea-based equation for rats (27). Plasma aldosterone was measured by solid-phase radioimmunoassay (lower limit of detection 12 pg/mL; Demidetec Diagnostics, Germany) and plasma renin concentration was measured by an in-house enzyme-kinetic assay (28). ELISAs were used to measure plasma endothelin-1 and urine epidermal growth factor (EGF) (R&D systems, USA).

Blood pressure measurements

After a 1-week acclimatization period, a radio-telemetry transmitter (HD-S10, Data Sciences International, USA) was implanted into the abdominal aorta under inhalation anesthesia with

isoflurane. Blood pressure and heart rate were measured through radiotelemetry by sampling for 10 seconds every 10 minutes and the daily average was used for analysis. Radiotelemetry measurements were performed during the first 18 days of treatment and 3 days before sacrifice.

Real-time PCR

Real-time PCR was performed because high-quality antibodies are not available for all distal tubular transporters. Total RNA was isolated from the kidney cortex using TRIzol Reagent (Ambion, USA) and phenol/chloroform extraction. DNase was added to eliminate genomic DNA and cDNA was converted by reverse transcription of 1 µg RNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania) according to the manufacturer's instructions. The resulting cDNA solution was diluted and 15 ng template cDNA per sample was used for subsequent qPCR. qPCR was performed using the PowerTrack™ SYBR™ Green Master Mix (Thermo Fisher Scientific). Primers are listed in Table S2. Gene expression was quantified with the Delta-Delta CT method using the housekeeping gene *Actb* for normalization (29).

Immunoblotting

Whole kidneys were homogenized in a buffer containing 0.3 M sucrose, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 1 mM sodium orthovanadate, 1% (v/v) TritonX-100, 50 mM sodium fluoride, and supplemented with phosphatase and protease inhibitor cocktails (Roche, Germany). Homogenates were centrifuged at 3,500 x g for 15 minutes at 4°C and supernatants were used for immunoblotting. Total protein concentration was measured by a colorimetric assay (DC Protein Assay, Bio-Rad, USA) and homogenates were denatured in SDS-PAGE sample

buffer (15 minutes at 65°C). An equal amount of protein was loaded in 4–20% Criterion-TGX Stain-free gels (Bio-Rad) and electrophoretically separated proteins were transferred to a 0.2 µm PVDF membrane (Bio-Rad). Next, membranes were blocked with 5% (w/v) nonfat milk or BSA (in TBS containing 0.1% (v/v) Tween-20) for 1 hour and probed overnight with the corresponding diluted primary antibodies at 4°C (Table S3). Antibody binding was detected using an HRP-conjugated secondary antibody (Table S3). After repeated washings with TBS (containing 0.1% Tween-20), signals were quantified by chemiluminescence (Clarity Western ECL substrate, Bio-Rad) using an Amersham 600 system (GE Life Sciences, USA). Optical densities were quantified using Image Studio Lite (LI-Cor Biosciences, version 5.2).

Statistics

Data are expressed as mean ± standard deviation for normally distributed data or median with interquartile ranges for non-normally distributed data. Time-dependent data (blood pressure and heart rate) are shown as mean ± standard error of the mean. For normally distributed data, comparisons were performed with two samples *t*-test (two groups) or one-way ANOVA with Scheffe or Dunnett's T3 post hoc test (three groups). For non-normally distributed data, the Mann-Whitney U or Kruskal-Wallis tests were performed. For comparisons of time-dependent data between multiple groups, a two-way ANOVA with mixed effects model was used. Statistical tests were performed using GraphPad Prism version 8 (GraphPad Software Inc., USA) and SPSS version 28.0 (IBM).

RESULTS

Dose finding study

We first examined the single dose pharmacokinetics of tacrolimus and voclosporin in rats to define dosages representing clinically relevant concentrations (Figure 2). We decided to aim for a clinically relevant area under the concentration-time curve (AUC) rather than a trough level, because the half-life of tacrolimus (0.5 mg/kg by intraperitoneal injection) in rats is much shorter than that of tacrolimus after a single oral administration (5 mg) in healthy human subjects (4.4 hours vs. 40.4 hours (30)). A minimal $AUC_{0\text{-}12h}$ threshold of 150 hr x ng/mL is recommended for the twice-daily formulation of tacrolimus in adults (31), and therefore the $AUC_{0\text{-}24h}$ threshold was estimated to be 300 ng x h/mL. When voclosporin was administrated as 23.7 mg twice daily in clinical trials, the $AUC_{0\text{-}24h}$ was 866 ng x h/mL (17). Based on pharmacokinetics data in rats, daily intraperitoneal administration of tacrolimus and voclosporin at a dose of 0.5 mg/kg is expected to achieve approximately 1.5 times the $AUC_{0\text{-}24h}$ observed in clinical studies. Furthermore, it has been shown that this dose of tacrolimus does not cause a severe decrease in eGFR in rats (32, 33). Therefore, we determined the final dosage of tacrolimus and voclosporin to be 0.5 mg/kg/day by intraperitoneal injection. After 18 days of treatment, trough levels were 2.4 ± 0.6 µg/L for tacrolimus and 25.8 ± 9.6 µg/L for voclosporin, which is in the therapeutic range for once daily dosing (Table 1 and Figure 2) (31).

Tacrolimus but not voclosporin increases urinary calcium and magnesium excretion and causes hypomagnesemia

Compared to vehicle, tacrolimus caused a >4-fold higher fractional calcium excretion, ~1.5-fold higher fractional excretions of magnesium and chloride, but without a commensurate rise in fractional sodium excretion (Figure 3A-D). Voclosporin had no effect on the fractional

excretions of magnesium, sodium and chloride. In addition to electrolytes, the urinary excretion of EGF, a distal tubule marker (34), was significantly reduced in the tacrolimus but not the voclosporin group (Figure 3E). Compared to vehicle and voclosporin, tacrolimus caused higher plasma creatinine and urea concentrations, a lower eGFR, overt hypomagnesemia, and lower plasma sodium and chloride concentrations (Table 1). Tacrolimus and voclosporin also caused a significantly higher plasma potassium and lower plasma bicarbonate concentrations compared with vehicle, but these effects were not different between the tacrolimus and voclosporin groups. Tacrolimus caused a significantly higher total plasma calcium but not free calcium. Biochemical analysis of plasma on day 28 showed similar results as on day 18, although hyperkalemia and metabolic acidosis in the CNI groups were mostly attenuated (Table S4). Food intake was significantly lower in the tacrolimus group during the first 5 days, but returned to normal thereafter, which is consistent with previous data (32) (Figure S1).

Tacrolimus but not voclosporin downregulates key transport genes in DCT and CNT

Quantitative RT-PCR analysis showed that, compared to vehicle, tacrolimus but not voclosporin caused significantly lower mRNA levels of several DCT/CNT-specific calcium and magnesium transport-related genes, including NCC (*Slc12a3*), transient receptor potential melastatin 6 (*Trpm6*), cyclin M2 (*Cnnm2*), transient receptor potential vanilloid 5 (*Trpv5*) and the sodium-calcium exchanger 1 (*Slc8a1*, Figure 4). Compared to vehicle, *Calb1*, which is the mRNA level of the cytosolic calcium-binding protein calbindin-D_{28K}, almost completely disappeared with tacrolimus and was reduced by 30% in the voclosporin group. The mRNA level of EGF, *Egf*, was significantly reduced after tacrolimus but not voclosporin treatment, which is consistent with the observed urinary EGF excretions. Compared to the vehicle group, no differences were observed in the mRNA levels of other magnesium-related

genes, including epidermal growth factor receptor (*Egfr*), the γ-subunit of the sodium-potassium ATPase (*Fxyd2*) and NEDD4 E3 ubiquitin protein ligase (*Nedd4l*), which has been implicated in NCC downregulation during hypomagnesemia (35)(Figure S2).

Tacrolimus but not voclosporin reduces the protein abundances of NCC, calbindin-D_{28K}, NCX1 and TRPV5

Compared to vehicle, tacrolimus caused a 2-fold lower abundance of total NCC, an 11-fold lower abundance of calbindin-D_{28K}, a 2.9-fold lower abundance of NCX1, and a 4.5-fold lower abundance of TRPV5 (Figure 5A-B, Figure S4). In contrast, voclosporin did not change the protein abundances of total NCC, calbindin-D_{28K}, NCX1, and TRPV5 compared to vehicle. Voclosporin did increase phosphorylated NCC (pNCC) at threonine 53, but this difference was significant only when compared to tacrolimus and not vehicle. Tacrolimus did significantly increase the ratio of pNCC to total NCC and more so than voclosporin. Eight days before the transporter analyses (at day 20), thiazide testing demonstrated significantly lower natriuretic and chloriuretic responses in tacrolimus-treated rats than in vehicle- and voclosporin-treated rats (Figure 5C). No significant differences were observed between vehicle, tacrolimus, and voclosporin in the protein abundances of other major sodium, potassium, and acid-base related transporters, including the sodium-hydrogen exchanger 3 (NHE3), sodium-potassium-chloride cotransporter 2 (NKCC2), epithelial sodium channel (ENaC), NEDD4-2, renal outer medullary potassium channel (ROMK), pendrin, and Na^+/K^+-ATPase (Figure S3).

Effects of tacrolimus and voclosporin on blood pressure and heart rate

Compared to vehicle, tacrolimus induced a rapid increase in mean arterial pressure by 10 ± 4 mmHg during the first 5 days of treatment which normalized thereafter (Figure 6A–B).

Compared to vehicle, voclosporin caused a more gradual increase in mean arterial pressure, amounting to 9 ± 2 mmHg over the 28-day treatment period. Heart rate increased significantly with tacrolimus and was higher than in both the voclosporin and vehicle groups; heart rate in the voclosporin group was also higher than in the vehicle group (Figure 6C).

Effects of tacrolimus and voclosporin on renin, aldosterone, cyclooxygenase-2 and endothelin-1

Both tacrolimus and voclosporin caused significantly higher plasma renin concentration compared to vehicle and a similar effect was observed for renin mRNA levels in the kidney cortex (Figure 7A–B). Of note, no parallel increases in plasma aldosterone levels were observed with tacrolimus or voclosporin (Figure 7C). Tacrolimus caused 60% lower COX-2 mRNA levels in kidney cortex while voclosporin caused only a trend towards reduction (Figure 7D). Compared to vehicle, voclosporin but not tacrolimus caused significantly lower plasma endothelin-1 concentrations (Figure 7E).

DISCUSSION

Prompted by observations in clinical trials (9, 10) we compared the effects of voclosporin and tacrolimus on kidney electrolyte handling and blood pressure. To do so, we induced clinically therapeutic concentrations of voclosporin and tacrolimus in rats and then studied the effects at the level of the kidney distal tubule. In contrast to tacrolimus, voclosporin did not inhibit the reabsorption of calcium and magnesium and therefore prevented hypercalciuria and hypomagnesemia. In agreement, at the tissue level, tacrolimus but not voclosporin downregulated calcium and magnesium transporters and epidermal growth factor (EGF), a distal tubule marker. We then moved on to study the effects of voclosporin and tacrolimus on blood pressure. Voclosporin caused hypertension as was also observed in clinical trials (22). In our hands tacrolimus caused hypertension only transiently followed by a normalization of blood pressure. Voclosporin-induced hypertension was further characterized by increased plasma renin concentrations, but did not lead to higher plasma aldosterone concentrations. Finally, two differences between voclosporin and tacrolimus were observed with potential relevance for blood pressure regulation, including on plasma endothelin-1 (decreased by voclosporin, increased by tacrolimus) and cyclo-oxygenase 2 (more strongly suppressed by tacrolimus). Together, our study reveals distinct differences between voclosporin and tacrolimus providing a pathophysiological basis for clinical observations (9, 10).

The downregulation of calcium and magnesium transporter-related genes and proteins in the DCT and CNT after tacrolimus treatment is in agreement with previous studies (3, 8). Mice with a DCT-specific inactivation of calcineurin (CnB1-KO mice) also exhibited hypomagnesemia and downregulation of NCC, TRPM6 and calbindin at mRNA and protein level, and NCX1, CNNM2 and EGF at mRNA level (36), which is similar to what we observed with tacrolimus. When CnB1-KO mice were treated with tacrolimus, TRPM6 and

plasma magnesium did not decrease further, whereas TRPV5 and urinary calcium reabsorption did (36). This suggests that tacrolimus specifically disrupts magnesium reabsorption in the DCT through calcineurin inhibition and affects calcium reabsorption in the late DCT and CNT, where TRPV5 and NCX1 are located (37). Mice lacking the tacrolimus-binding protein FKBP12 in the kidney were protected from tacrolimus-induced hypomagnesemia and hypercalciuria, also suggesting a role for calcineurin in mediating tacrolimus-induced DCT and CNT effects (8). The reduction of urinary EGF excretion and kidney *Egf* mRNA levels by tacrolimus could have contributed to reduced TRPM6 activity and surface expression (38). However, in a previous study treatment with EGF could not rescue TRPM6 downregulation by cyclosporine (5) suggesting that other regulators such as c-Fos may also play a role (39). A suggestion for future research is to include morphometric analysis to analyze whether the DCT and CNT effects are due to remodeling (atrophy).

Voclosporin increased pNCC more than tacrolimus suggesting higher NCC activity, whereas thiazide testing did not lead to a greater natriuretic response in voclosporin-treated animals. This discrepancy may be explained by the different time points at which the thiazide testing and transporter analysis were conducted. Based on these observations, it is difficult to assess whether voclosporin-induced hypertension is mediated by NCC, which may require longer treatment with thiazide diuretics or treatment in NCC knockout mice. An important observation was that voclosporin did increase plasma renin concentration, which likely contributed to the development of hypertension via angiotensin II. This effect may be mediated by calcineurin inhibition in renin-producing cells or through activation of the sympathetic nervous system (40-42). Of interest, we and others (43) observed that the rise in plasma renin did not translate into higher plasma aldosterone levels, which is in line with the observed lack of effect on the aldosterone-sensitive epithelial sodium channel. A possible

explanation is that CNIs inhibit the angiotensin II-induced aldosterone synthase CYP11B2 (44-46). Tacrolimus also increased plasma renin concentration, but because this occurred in the context of decreasing blood pressure, lower NCC abundance and a reduced thiazide response, this may have been secondary to salt loss. Our data suggest a biphasic response in which tacrolimus first increased NCC activity and therefore blood pressure, followed by NCC downregulation; the cause for this switch is unclear. In agreement with our observations, previous studies also showed that one week of tacrolimus increased blood pressure, whereas two weeks of tacrolimus treatment at a dose of 3 mg/kg/day by subcutaneous injection caused a secondary drop in blood pressure (7) and 28 days of tacrolimus treatment or DCT-specific deletion of calcineurin also reduced NCC abundance (33, 36). Another possible factor contributing to NCC downregulation is hypomagnesemia. In rodents treated with tacrolimus, hypomagnesemia preceded the reduction in total NCC abundance (3, 4, 47). Finally, we confirmed previous work by showing that tacrolimus significantly reduced COX-2 mRNA levels (48) and show that voclosporin did not have this effect. A difference in COX-2-derived prostanoids may also explain why tacrolimus affects tubular transport more than voclosporin (49).

The doses we used were calculated to have clinically relevant immunosuppressive effects, yet we observed a different tubular effect between tacrolimus and voclosporin. Possible explanations for the differences between voclosporin and tacrolimus include differences in intracellular binding proteins, drug potency, or pharmacokinetics. Although voclosporin and tacrolimus have different intracellular binding proteins (cyclophilin vs. FKBP12), we believe this is an unlikely explanation for the observed differences. Cyclosporine has the same binding protein as voclosporin and yet does cause similar distal tubular effects as tacrolimus, including effects on magnesium (5, 6), calcium (2, 6) and sodium transport (5, 6, 50). A

difference in potency also seems unlikely because voclosporin's potency to inhibit calcineurin and the nuclear factor of activated T cells (NFAT) is lower than that of tacrolimus but higher than that of cyclosporine (18). There are pharmacokinetic differences between voclosporin and cyclosporine that may explain the observed differences. Voclosporin preferentially engages with circulating immune cells during repeated lower dosing, whereas cyclosporine is more likely to penetrate deeper into tissues and organs (15). It is unknown if cyclosporine and tacrolimus share this pharmacokinetic behavior – measurement of calcineurin activity in kidney tissue might answer this question. Another possibility is that the lower metabolite load of voclosporin compared to tacrolimus and cyclosporine results in reduced exposure of the kidney to metabolites, thereby minimizing the potential for tubular effects (16). Together, differences in pharmacokinetic and metabolic profile may explain why the distal tubular effects of tacrolimus and cyclosporine differ from those of voclosporin.

This is the first study performing a head-to-head comparison of voclosporin and tacrolimus to delineate their effects on distal tubular function. Strengths of our study include the extensive analysis of distal tubular function, telemetric blood pressure recordings and pharmacokinetic-based dosing. However, we also acknowledge a number of limitations. First, we only studied male rats. Future studies should also include female rats to analyze whether sexual dimorphism in pharmacokinetics or tubular transport will modify the response to CNIs (51). Second, transporter profiling was performed at a later time than the peak blood pressure in the tacrolimus group and the analysis of urinary excretions. This precludes direct correlations and limits the ability to address potential distal tubular remodeling between the two time points. Future intervention studies are necessary to exactly pinpoint differences in voclosporin- and tacrolimus-induced hypertension.

In summary, our data reveal that voclosporin has a different effect on the distal tubule than tacrolimus and therefore prevents hypomagnesemia and hypercalciuria. Future studies should address whether preventing hypomagnesemia and hypercalciuria by using voclosporin instead of tacrolimus translates to long-term benefits.

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CONFLICT OF INTEREST STATEMENT

This study was supported by Aurinia Pharmaceuticals Inc.

AUTHORS' CONTRIBUTIONS

K.W., M.H.v.H., and E.J.H. designed the study; K.W., M.H.v.H., and R.v.V. contributed to data acquisition; all authors contributed to data analysis and interpretation; K.W. and E.J.H. drafted the manuscript; all authors reviewed the manuscript critically for important intellectual content and approved the final version.

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DATA AVAILABILITY STATEMENT

Data are available upon reasonable request.

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Table 1. Physiological parameters after 18 days of treatment

Parameter	Vehicle (n = 8)	Tacrolimus (n = 9)	Voclosporin (n = 9)
Body weight, g	305 ± 18	291 ± 12	303 ± 13
Food intake, g/24h	18.4 ± 1.7	20.6 ± 1.8	19.5 ± 1.6
Drug trough levels, µg/L	N/A	2.4 ± 0.6	25.8 ± 9.6
Plasma			
Creatinine, µmol/L	22 ± 2	26 ± 2*, #	23 ± 1
Urea, mmol/L	4.8 ± 0.4	6.2 ± 0.8*, #	4.8 ± 0.5
eGFR, mL/min	3.3 ± 0.2	2.6 ± 0.3*, #	3.2 ± 0.2
Na^+, mmol/L	142 (142-143)	141 (140-141)*, #	142 (142-143)
Cl^-, mmol/L	101 ± 1	99 ± 1*, #	101 ± 1
Mg^{2+}, mmol/L	0.81 ± 0.02	0.65 ± 0.04*, #	0.8 ± 0.04
Total Ca^{2+}, mmol/L	2.55 ± 0.07	2.76 ± 0.05*, #	2.61 ± 0.06
K^+, mmol/L	4.8 ± 0.2	5.4 ± 0.5*	5.2 ± 0.2*
HCO_3^-, mmol/L	25.9 ± 0.8	23.8 ± 1.2*	24.2 ± 0.7*
24-hour urine			
Urine volume, mL/day	8.9 ± 1.7	10.0 ± 3.4	7.2 ± 3.5
Na^+, µmol/day	1288 ± 94	1050 ± 373	853 ± 94*
Cl^-, µmol/day	2100 ± 178	2430 ± 527#	1811 ± 445
Mg^{2+}, µmol/day	326 ± 60	344 ± 68	288 ± 95
Ca^{2+}, µmol/day	35 ± 12	139 ± 39*, #	44 ± 15
K^+, µmol/day	1483 ± 162	1491 ± 409#	1096 ± 317

Abbreviations: Ca^{2+}, calcium; Cl^-, chloride; eGFR, estimated glomerular filtration rate; HCO_3^-, bicarbonate; FE, fractional excretion; Na^+, sodium; K^+, potassium; Mg^{2+}, magnesium.

Footnotes: *, $P < 0.05$ vs. vehicle; #, $P < 0.05$ vs. voclosporin.

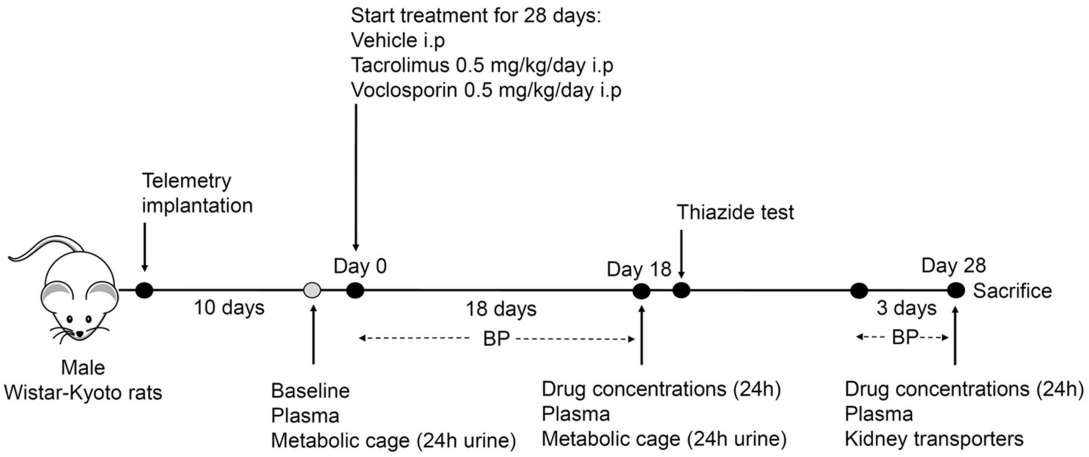

Figure 1. Study design

Overview of the study design. Following a 10-day recovery period, we collected 24-hour urine in metabolic cages, blood samples from the tail vein, and 2-day radio-telemetry measurements to assess baseline physiological parameters (Table S1). The treatment with vehicle, tacrolimus, and voclosporin started on day 0 and continued until day 28. On day 18, blood and 24-hour urine were collected to measure trough levels of the drugs and to analyze fractional excretions of electrolytes. A thiazide test was performed on days 19–20. On day 28, blood samples were collected at 8 hours and 24 hours after the last dose, and the rats were sacrificed. **Abbreviations:** BP, blood pressure; i.p., intraperitoneal

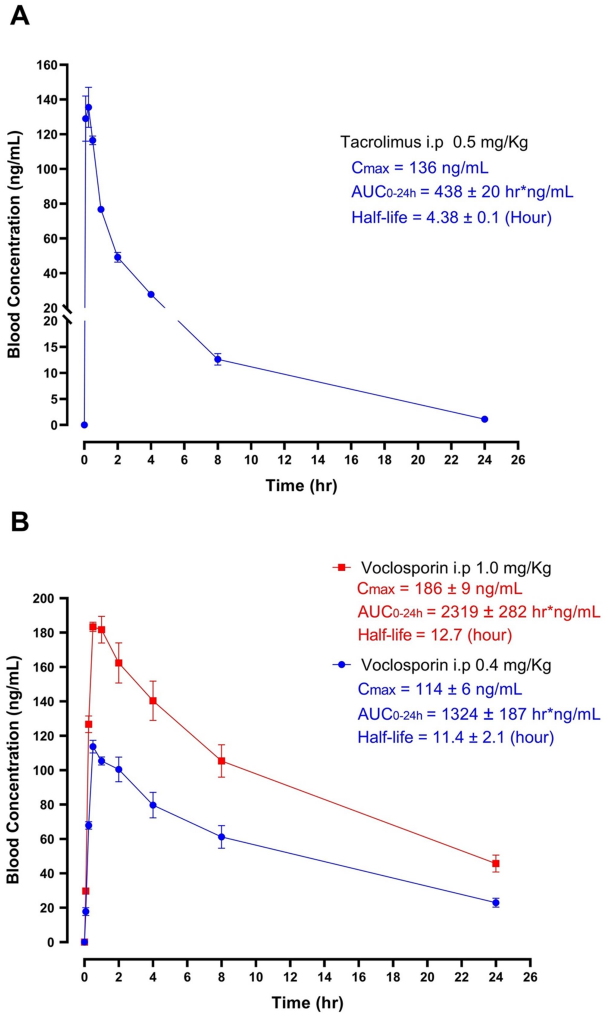

Figure 2. The single dose pharmacokinetics of tacrolimus and voclosporin in rats

Mean whole blood concentration-time profiles of (A) tacrolimus (n = 3 rats; 0.5 mg/kg by intraperitoneal injection) and (B) voclosporin (n = 3 rats; 0.4 mg/kg and 1.0 mg/kg by intraperitoneal injection) following a single dose administration in rats. Data shown are mean ± SEM. **Abbreviations:** AUC, area under the concentration-time curve; C_{max}, the maximum concentration.

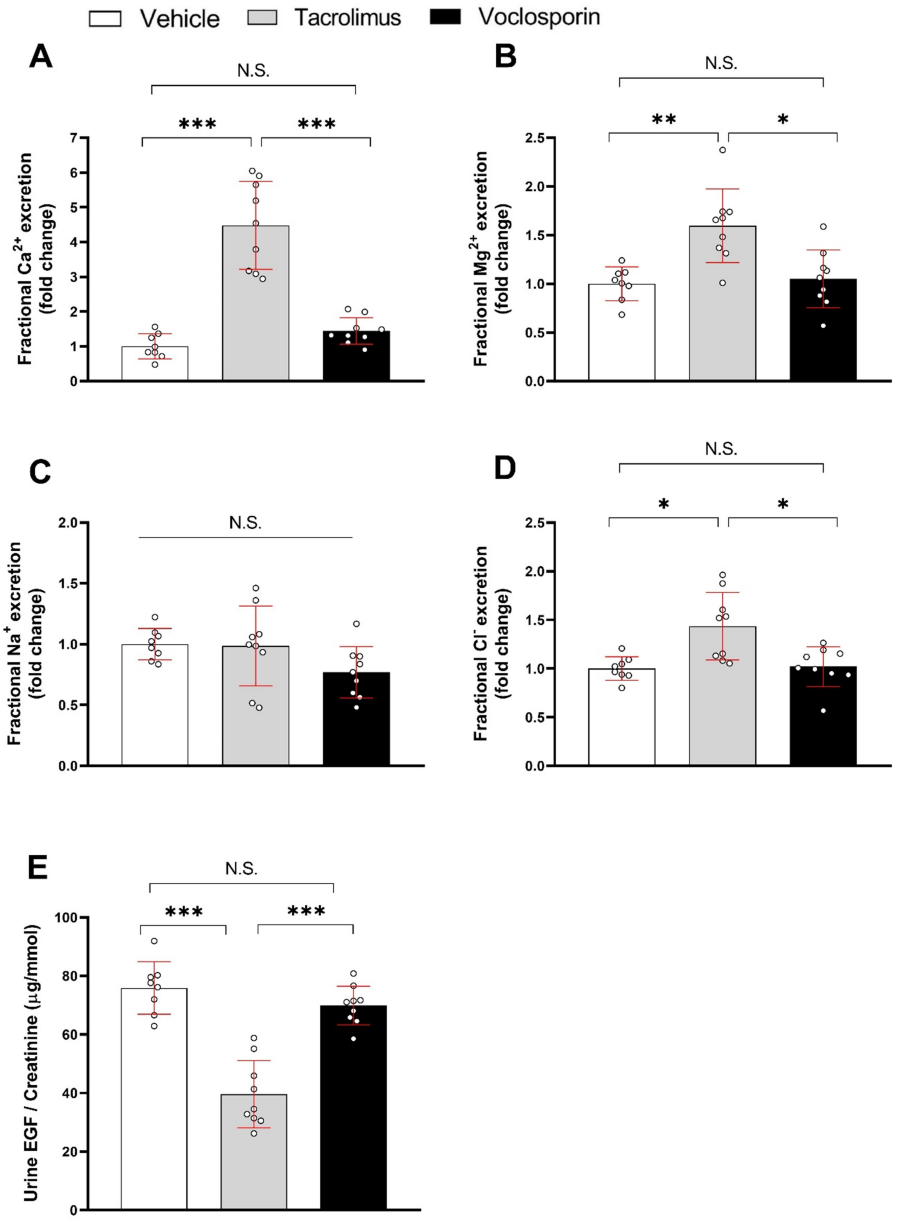

Figure 3. Effects of tacrolimus and voclosporin on fractional excretions of calcium, magnesium, sodium, chloride and urinary EGF excretion after 18 days of treatment

(A) Fractional Ca^{2+} excretion (B) Fractional Mg^{2+} excretion (C) Fractional Na^{+} excretion (D) Fractional Cl^{-} excretion (E) Urinary EGF excretion. Calculated values are normalized to the mean values of the vehicle group defined as 1.0. Shown are data points with mean ± SD (n = 8-9 rats per group. Fractional excretions of individual electrolytes were calculated by the

equation: 100 * ($U_{electrolyte}$ x $P_{Creatinine}$) / ($P_{electrolyte}$ x $U_{Creatinine}$), where $U_{electrolyte}$ is urinary excretion of electrolyte (mmol/L), $P_{Creatinine}$ is plasma creatinine (µmol/L), $P_{electrolyte}$ is plasma electrolyte concentration (mmol/L), and $U_{Creatinine}$ is urinary excretion of creatinine (mmol/L). For the fractional magnesium excretion plasma Mg^{2+} is multiplied by 0.7, because only 70% of the circulating Mg^{2+} is filterable. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; N.S., not significant; one-way ANOVA with Dunnett's T3 post hoc testing).

Abbreviations: Ca^{2+}, calcium; Cl^-, chloride; EGF, epidermal growth factor; Mg^{2+}, magnesium; Na^+, sodium;

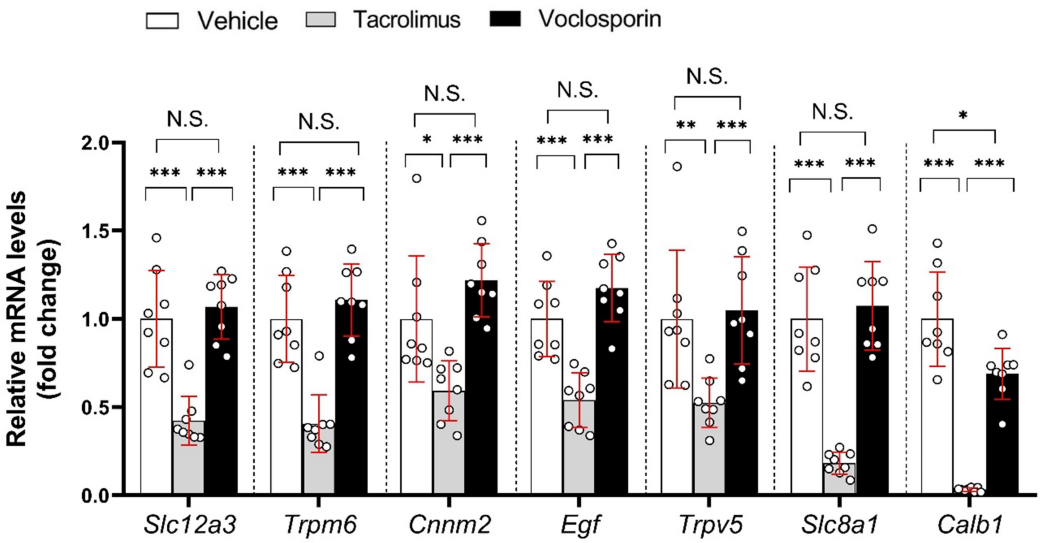

Figure 4. Effects of tacrolimus and voclosporin on mRNA levels of transport proteins in the distal convoluted tubule and connecting tubule after 18 days of treatment

Results of quantitative RT-PCR of total RNA isolated from the kidney cortex. Gene expression was normalized to *Actb* and to mean values of the vehicle group defined as 1.0. Shown are data points with mean ± SD (n = 8 rats per group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; N.S., not significant; one-way ANOVA with Dunnett's T3 post hoc testing, except for *Cnnm2* and *Trpv5* by Kruskal-Wallis test).

Abbreviations: *Calb1*, calbindin 1; *Cnnm2*, cyclin M2; *Egf*, epidermal growth factor; N.S., not significant; *Slc8a1*, solute carrier family 8 member A1; *Slc12a3,* solute carrier family 12 member A3; *Trpm6*, Transient receptor potential melastatin 6; *Trpv5*, Transient receptor potential vanilloid 5.

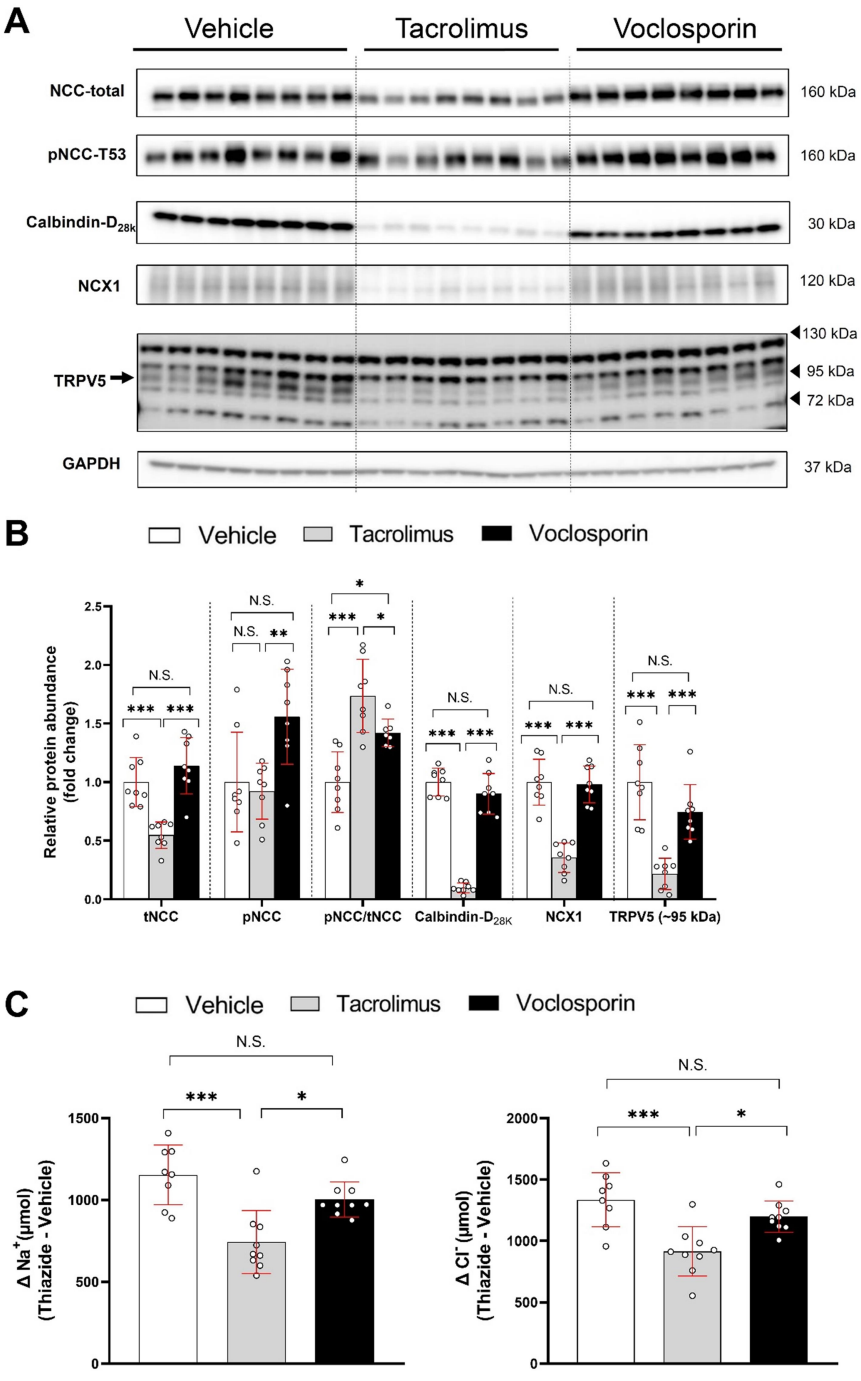

Figure 5. Effects of tacrolimus and voclosporin on distal tubular transporters and thiazide sensitivity

(A) Immunoblotting of whole kidney homogenates from vehicle-, tacrolimus- and voclosporin-treated rats

(B) Densitometry of immunoblots. Band intensities are normalized to GAPDH and to the mean intensity of the vehicle group defined as 1.0. Values displayed are mean ± SD (n = 8 rats per group, one-way ANOVA with Dunnett's T3 post hoc test)

(C) 6-hour response of a single dose of hydrochlorothiazide (25 mg/kg i.p.) on Δ urinary Na^+ and Cl^- excretion. Values displayed are mean ± SD (n = 8-9 rats per group; one-way ANOVA with Dunnett's T3 post hoc test; Δ, thiazide - vehicle; data obtained on day 19 for vehicle and on day 20 for thiazide).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; N.S., not significant.

Abbreviations: pNCC-T53, phosphorylated NCC at threonine 53; tNCC, total abundance of the sodium-chloride cotransporter; NCX1, sodium-calcium exchanger 1; TRPV5, transient receptor potential vanilloid 5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Na^+, sodium; Cl^-, chloride.

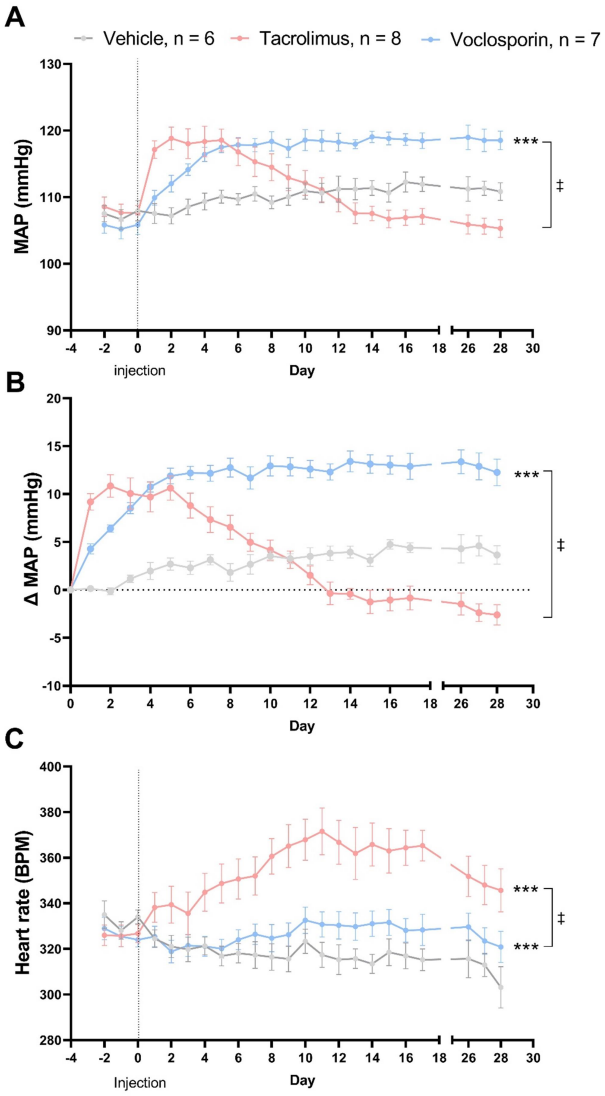

Figure 6. Effects of tacrolimus and voclosporin on mean arterial pressure and heart rate

Time course of (A) mean arterial pressure (MAP) (B) changes in MAP (C) heart rate. Data are presented as mean ± SEM (n = 6-8 rats per group; two-way ANOVA with mixed effects model analysis).

*** $P < 0.001$ vs. vehicle; ‡ $P < 0.001$ vs indicated group.

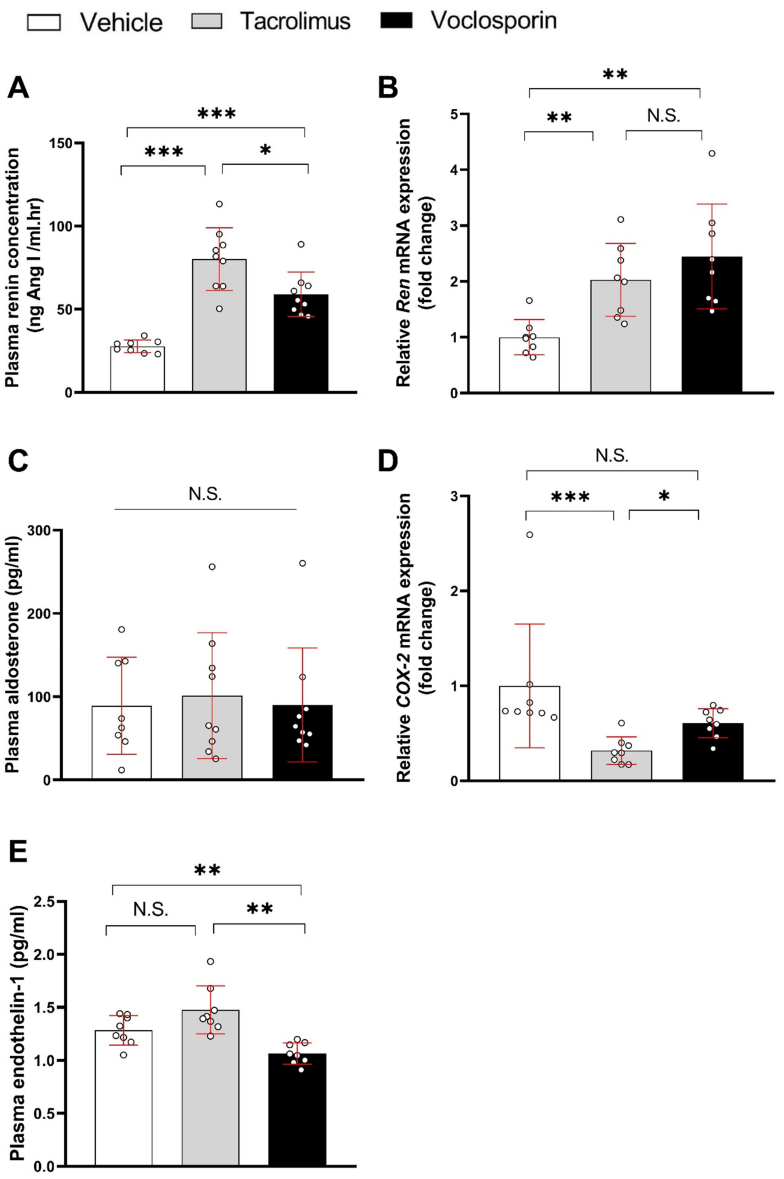

Figure 7. Effects of tacrolimus and voclosporin on renin, aldosterone, cyclooxygenase-2 and endothelin-1 after 28 days of treatment

(A) Plasma renin activity (B) Renin mRNA (C) Plasma aldosterone (D) COX-2 mRNA. Gene expression was normalized to *Actb* and to mean values of the vehicle group defined as 1.0. Shown are data points with mean ± SD (n = 8-9 rats per group, one-way ANOVA with Dunnett's T3 post hoc testing, except for plasma aldosterone and COX-2 mRNA by Kruskal-Wallis test).

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; N.S., not significant.