

# Flucloxacillin worsens while imipenem-cilastatin protects against vancomycin induced kidney injury in a translational rat model

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## Abstract

**Background and Purpose** Vancomycin is one of the most common antibiotics administered in the hospital setting, yet acute kidney injury is a major limiting factor. Common combinations of antibiotics with vancomycin have been reported to worsen and improve vancomycin-induced kidney injury. We aimed to study the impact of flucloxacillin and imipenem-cilastatin on kidney injury when combined with vancomycin in our translational rat model. **Experimental Approach** Male Sprague-Dawley rats received allometrically scaled (1) vancomycin (2) flucloxacillin, (3) vancomycin+flucloxacillin, (4) vancomycin+imipenem-cilastatin, or (5) saline for 4 days. Vancomycin was administered intravenously and flucloxacillin or imipenem-cilastatin were administered intraperitoneally. Kidney injury was evaluated via drug accumulation and urinary biomarkers including urinary output, kidney injury molecule-1 (KIM-1), clusterin, and osteopontin. Relationships between vancomycin accumulation in the kidney and urinary kidney injury biomarkers were explored. **Key Results** Urinary output increased every study day for vancomycin+flucloxacillin; whereas in the vancomycin group it was elevated after the first dose only. In the vancomycin+flucloxacillin group, urinary KIM-1/24h increased on all days compared to vancomycin. In the vancomycin+imipenem-cilastatin group, urinary KIM-1/24h was decreased on days 1 and 2 compared to vancomycin. Similar trends were observed for clusterin. More vancomycin accumulated in the kidney with vancomycin+flucloxacillin compared to vancomycin and vancomycin+imipenem-cilastatin. The accumulation of vancomycin in the kidney tissue correlated with increasing urinary KIM-1 (4-parameter Hill Slope,  $R^2=0.7985$ ). **Conclusion and Implications** Vancomycin+flucloxacillin caused more kidney injury compared to vancomycin alone and vancomycin+imipenem-cilastatin in a translational rat model as determined by multiple kidney injury biomarkers. The combination of vancomycin+imipenem-cilastatin was nephroprotective.

**Title:** Flucloxacillin worsens while imipenem-cilastatin protects against vancomycin induced kidney injury in a translational rat model

**Running Title:** Flucloxacillin worsens while cilastatin protects against vancomycin AKI

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## CONFLICT OF INTEREST STATEMENT

MS reports ongoing research contracts with Nevakar and SuperTrans Medical as well as having filed patent US10688195B2. All other authors have no other related conflicts of interest to declare.

**Data availability statement:** Data available on request from the authors: The data that support the findings of this study are available from the corresponding author upon reasonable request. Data sharing will be subject to standard Data Use Agreements from Midwestern University.

## Abstract

### Background and Purpose

Vancomycin is one of the most common antibiotics administered in the hospital setting, yet acute kidney injury is a major limiting factor. Common combinations of antibiotics with vancomycin have been reported to worsen and improve vancomycin-induced kidney injury. We aimed to study the impact of flucloxacillin and imipenem-cilastatin on kidney injury when combined with vancomycin in our translational rat model.

### Experimental Approach

Male Sprague-Dawley rats received allometrically scaled (1) vancomycin (2) flucloxacillin, (3) vancomycin+flucloxacillin, (4) vancomycin+imipenem-cilastatin, or (5) saline for 4 days. Vancomycin was administered intravenously and flucloxacillin or imipenem-cilastatin were administered intraperitoneally.

Kidney injury was evaluated via drug accumulation and urinary biomarkers including urinary output, kidney injury molecule-1 (KIM-1), clusterin, and osteopontin. Relationships between vancomycin accumulation in the kidney and urinary kidney injury biomarkers were explored.

## Key Results

Urinary output increased every study day for vancomycin+flucloxacillin; whereas in the vancomycin group it was elevated after the first dose only. In the vancomycin+flucloxacillin group, urinary KIM-1/24h increased on all days compared to vancomycin. In the vancomycin+imipenem-cilastatin group, urinary KIM-1/24h was decreased on days 1 and 2 compared to vancomycin. Similar trends were observed for clusterin. More vancomycin accumulated in the kidney with vancomycin+flucloxacillin compared to vancomycin and vancomycin+imipenem-cilastatin. The accumulation of vancomycin in the kidney tissue correlated with increasing urinary KIM-1 (4-parameter Hill Slope,  $R^2=0.7985$ ).

## Conclusion and Implications

Vancomycin+flucloxacillin caused more kidney injury compared to vancomycin alone and vancomycin+imipenem-cilastatin in a translational rat model as determined by multiple kidney injury biomarkers. The combination of vancomycin+imipenem-cilastatin was nephroprotective.

## Keywords:

Vancomycin, flucloxacillin, imipenem-cilastatin, Acute Kidney Injury, drug induced kidney injury, pre-clinical, rodent, nephrotoxicity, biomarkers

## What is already known:

In a prospective clinical study, combination of flucloxacillin and vancomycin increased acute kidney injury as demonstrated by elevated serum creatinine levels.

Imipenem-cilastatin has decreased vancomycin-associated nephrotoxicity in pre-clinical studies.

## What this study adds:

- Increased kidney injury was confirmed when flucloxacillin was added to vancomycin as determined by multiple kidney injury biomarkers in a translational rat model.
- There is increased accumulation of vancomycin in kidney tissue that could account for the synergistic nephrotoxicity of the vancomycin+flucloxacillin combination compared to vancomycin alone.
- Imipenem-cilastatin provided some protection against vancomycin induced kidney injury.

## Clinical significance

Antibiotic combinations can worsen or protect against vancomycin induced kidney injury. Efforts should be made to advance kidney protecting agents to clinical practice and to better understand the compounded toxicity of co-nephrotoxins.

## 1 INTRODUCTION

Vancomycin is one of the most widely prescribed antibiotics in the hospital setting (Baggs, Fridkin, Pollack, Srinivasan, & Jernigan, 2016). It remains a first line therapeutic option for infections caused by methicillin-resistant *Staphylococcus aureus* (C. Liu et al., 2011; Rybak et al., 2020), enterococci and other resistant Gram positive bacteria. Kidney injury is a common adverse effect (Im et al., 2017) with rates varying from 5% to 43% depending on the population treated and concomitant medications (van Hal, Paterson, & Lodise, 2013). Less is known about how other medications increase and decrease kidney injury. Recently, a clinical trial (Combination Antibiotics for Methicillin Resistant *Staphylococcus aureus*, CAMERA2), aimed to evaluate the combination therapy of a beta-lactam (primarily flucloxacillin) with vancomycin for efficacy (Tong et al., 2020). However, the trial was stopped early as more patients in the combination therapy group developed kidney injury as defined by increased serum creatinine when compared to standard therapy (Tong et al., 2020). Further, CAMERA2 failed to identify an efficacy benefit with combination therapy, only that

toxicity was increased among the those that received vancomycin and flucloxacillin in combination. Even less is known about agents that can prevent kidney injury due to vancomycin. Imipenem-cilastatin/relebactam has been shown to decrease serum creatinine of mice that received vancomycin (He, Souza, Matvekas, Crass, & Pai, 2021) with similar findings reported in retrospective clinical studies (Hakeam, AlAnazi, Mansour, AlFudail, & AlMarzouq, 2019).

Clinical studies for evaluating acute kidney injury (AKI) with vancomycin have primarily focused on assessment of serum creatinine and various clinical classification schemes based on serum creatinine and urinary output (He et al., 2021; Nakamura, Kokuryo, Hashimoto, & Inui, 1999). While serum creatinine is a commonly used surrogate of both kidney injury and function, it is slow to change after an injury event and is neither highly sensitive nor specific for meaningful injury and functional changes. Animal studies offer the ability to study newer biomarkers that are more sensitive and specific for detecting injury and to assess drug accumulation in the kidney. For vancomycin induced kidney injury, kidney injury molecule-1 (KIM-1) has been demonstrated as the most sensitive and specific urinary biomarker for prediction of histopathologic damage (Pais et al., 2019). KIM-1 has also been shown as highly correlated with functional changes (i.e. glomerular filtration changes) due to vancomycin (Chang et al., 2022; Pais, Chang, Liu, & Scheetz, 2022).

Since the findings of nephrotoxicity as classified by serum creatinine in CAMERA2 were unexpected, the purpose of this study was to assess the biologic plausibility of increased kidney injury caused by the addition of flucloxacillin to vancomycin by employing sensitive urinary biomarkers such as KIM-1, urinary output, and vancomycin accumulation in the kidney in our translational rat model (Avedissian, Pais, O'Donnell, et al., 2019; Pais, Liu, Avedissian, et al., 2020). Simultaneously, we sought to determine if kidney injury was lessened with the addition of imipenem/cilastatin in the same rat model. To address these questions, we studied kidney injury in Sprague Dawley rats : administered saline, vancomycin alone, vancomycin plus flucloxacillin, or vancomycin plus imipenem-cilastatin.

## 2 METHODS

### 2.1 Experimental design and animals

This preclinical model replicates aspects of human vancomycin-induced kidney injury (Scheetz et al., 2021). This study was performed at Midwestern University in Downers Grove, Illinois with the approval of the Institutional Animal Care and Use Committee (IACUC protocol #s 3080 and 3151). All experiments were conducted in compliance with the Guide ("National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals. 8th edition. Washington (DC): National Academies Press (US); 2011. Available from:<https://www.ncbi.nlm.nih.gov/books/NBK54050/> doi: 10.17226/12910,")

The animal model employed for this study has been previously described (Pais et al., 2020). Male Sprague-Dawley rats (n = 45; age, approximately 9 to 10 weeks; mean ( $\pm$  SD) weight, 289 ( $\pm$  6.3) g, Envigo, Indianapolis, IN, USA) were housed in a light- and temperature-controlled room and were allowed free access to food and water for the duration of the study. Animals underwent double jugular catheterization 72 h prior to protocol initiation. Ketamine xylazine 100/10 mg kg<sup>-1</sup> was employed as a surgical anesthetic. Animals were monitored 4-6 h post-surgery for signs of pain and rescue analgesia (buprenorphine 0.02 mg kg<sup>-1</sup>) was administered subcutaneously as needed. A pre-planned protocol required that animals displaying overt signs of discomfort (severe lethargy, lack of response to stimuli, marked weight loss [i.e., greater than 15% of starting weight during the treatment period] or a significant decrease in food and water intake) were euthanized.

Following recovery from surgery, animals were transferred to metabolic cages (Nalgene, Rochester, NY) for 24-h urine collection, 24 h pre-study (day 0) and on days 1-4. Rats were assigned to one of five treatment groups vancomycin 150 mg kg<sup>-1</sup>/day (n=12), flucloxacillin 90 mg kg<sup>-1</sup>/day (n=6), vancomycin+flucloxacillin (n=10), vancomycin+ imipenem-cilastatin 90 mg kg<sup>-1</sup>/day (n=6) or saline controls (n=11). See below for rationale for numbers in each group. FDA approved fixed doses were converted to weight-based doses using a standardized patient weight of 70 kg (<https://www.fda.gov/media/72309/download>). Vancomycin 150 mg

kg-1/day was selected to allometrically scale the human dose (25 mg kg-1/day) for the rat (i.e., 25 mg kg-1 x 6.2 [rat factor] = 155 mg kg-1). Similarly, flucloxacillin 90 mg kg-1/day and imipenem-cilastatin 90 mg kg-1/day were allometrically scaled based on FDA approved human doses. Conduct of the animal protocol was not blinded as this was considered impractical; however, samples were analyzed in a blinded fashion and used objective measurements of biomarkers. Rats received the same treatment daily for 4 days. Vancomycin was administered intravenously, flucloxacillin and imipenem-cilastatin were given intraperitoneally.

A minimum sample size of n=6 per group was used based on our previous experiments where differences in total excreted urinary KIM-1 were discernable and corresponded to the “gold-standard” of histopathological change on day 3 (Pais, Liu, Avedissian, et al., 2020). Two human clinical trials CAMERA 2 and PROVIDE did not generate any signal for sex-based differences (Legg et al., 2022; Lodise et al., 2020); therefore, sex as a biological variable was not incorporated in this particular study.

## 2.2 Characteristics of animal cohorts

In keeping with the 3Rs principle to reduce the number of animals used in research, this study carried forward animal data from saline control and vancomycin only treated rats treated in the exact manner as described herein. Specifically, the saline group comprised rats with identical experimental protocols, i.e. n=8 rats from a study published in abstract form (“ACCP Abstract Booklet,” 2021) and n=3 new animals, n=11 total. Similarly, the vancomycin group included n=6 rats from the same study published in abstract form (“ACCP Abstract Booklet,” 2021) and n=6 new animals (total n=12). The vancomycin+flucloxacillin group started out with the minimum group size n=6. Due to the severe nephrotoxicity, two rats had to be euthanized on day 3 prior to dosing (i.e. they received two doses of the intervention). Hence, an additional n=4 rats were included to account for early termination due to nephrotoxicity. All animals provided data and were included in the study (total n=10). Flucloxacillin and imipenem-cilastatin groups were new and have the minimum sample size of n=6 per group.

## 2.3 Sample collection

Blood samples (0.125 mL, maximum 16 samples/animal) were drawn from a jugular vein catheter into EDTA-containing tubes, centrifuged at 3000 rpm for 10 min, (O'Donnell et al., 2017; Rhodes et al., 2016) and the resultant plasma was stored at -80°C for batch analysis. Blood was sampled daily under the #3151 protocol and each day except Day 3 under the #3080 protocol. Blood sampling was carried out for a pharmacokinetic study that will ultimately be detailed elsewhere. Urine was collected continuously, aliquoted every 24 h, centrifuged (500xg, 10 minutes at 4°C), and supernatant was stored at -80°C until batch analysis. At the end of the protocol, animals were anesthetized with ketamine/xylazine (100/10 mg kg-1, by intraperitoneal injection) and euthanized by exsanguination through the right atrium while under anesthesia. Kidneys were obtained at euthanasia, rinsed in cold saline, flash frozen and stored at -800C for vancomycin assay.

## 2.4 Urinary biomarkers

Urine samples were analyzed using MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panel 1 (EMD Millipore Corporation, Charles, MO) according to the manufacturer's protocol (Pais, Liu, Avedissian, et al., 2020). MILLIPLEX® Analyst v5.1 Flex software was used for calibration curve and analyte concentration determination.

## 2.5 Estimation of vancomycin in the kidney

Vancomycin concentrations in the kidney were analyzed by LC-MS/MS similar to our previous report (Avedissian et al., 2019) with minor noted exceptions. Samples were diluted 20x and atenolol was used as the internal standard with transitions (m/z) of 267.2 -145.0 (quantifier) and 267.2 -190.0 (qualifier). Briefly, whole kidneys were thawed on ice and homogenized using a Polytron System PT 10-35 GT (Fisher Scientific, Hampton, NH) in a 1:3 (w:v) ratio of kidney : water. The homogenate was centrifuged, and the resultant supernatant was used in the analyses. Calibration curves were prepared using control rat kidney tissue homogenates from the saline-treated group of rats. Vancomycin- containing kidney homogenates were diluted 20 x with control kidney tissue homogenate. Kidney concentrations are expressed as vancomycin

( $\mu\text{g}$ )/ kidney (g) of wet kidney tissue. Assays were linear between 1.0 and 60 mg/L. Precision was 3-6% for all measurements, including intra- and inter assay measurements. Greater than 80.27% accuracy was observed in all standards tested, with an overall mean assay accuracy of 102% and all concentrations above the lower limit of quantification were between 85 and 115%.

## 2.6 Materials

Treatments were clinical grade vancomycin (Fresenius Kabi, Lake Zurich, IL), flucloxacillin (Juno Pharmaceuticals Pty Ltd, South Yarra, VIC Australia), imipenem-cilastatin (Novation LLC, Italy Novaplus/Novation Mfd. Fresenius Kabi) and normal saline (Veterinary 0.9% Sodium Chloride Injection USP, Abbott Laboratories, North Chicago, IL).

For LC-MS/MS, analytical grade vancomycin hydrochloride (Lot#LRAC0718), and atenolol (Lot#BCBT5643) were obtained from Sigma Aldrich (St. Louis, MO), LC-MS/MS grade acetonitrile and methanol were obtained from VWR International, Radnor, PA, formic acid was obtained from Fisher Scientific, Waltham, MA and frozen, non-medicated, non-immunized, pooled Sprague-Dawley rat EDTA plasma was obtained from BioreclamationIVT, Westbury, NY, USA. Control rat kidney homogenate was prepared from saline-treated rat kidneys.

## 2.7 Data and Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Urinary output is expressed as mean  $\pm$  SD. All available urine samples were analyzed. Urinary biomarkers were standardized for urinary volume by calculating the total amount excreted in each 24-h period. Differences between groups were assessed using mixed-effects, restricted maximum likelihood estimation regression, with repeated measures occurring over days; measures were repeated at the level of the individual rat (STATA 17.0 BE, StataCorp LLC, College Station, Texas). The primary analysis reports the simple effects from joint tests of drug treatment group within each level of treatment day. Margins were calculated for a full factorial of the variables, i.e. main effects for each variable and interactions. Referent groups were pre-treatment baseline values and saline as a treatment, except where noted. Secondly, to remain agnostic to outcome variable relationship over time (and assess treatment groups over time), locally weighted scatterplot smoothing (LOWESS) trendlines with 95% confidence intervals were generated. LOWESS graphs were produced in R version 4.2.2 using ggplot2 ("H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.,"). No data were excluded as outliers. Vancomycin kidney concentrations were expressed as median (interquartile range) and compared by the non-parametric Kruskal Wallis test. Multiple Hill-type models were explored to describe the relationship between vancomycin kidney concentrations and urinary KIM-1. All tests were two-tailed, a  $p < 0.05$  was required for statistical significance. Graphs were generated using GraphPad version 9.3.1 unless specified otherwise.

## 3 RESULTS

### 3.1 Weight loss

On average (SD), rats weighed 289 g (6.3 g). Mean weight loss post catheterization between day -3 and day 1 was similar between control, flucloxacillin, vancomycin, vancomycin+flucloxacillin, vancomycin+ imipenem-cilastatin ( $10.2 \pm 6.1$  g,  $12.4 \pm 3.7$  g,  $6.7 \pm 7.0$  g,  $11.0 \pm 5.1$  g,  $7.6 \pm 4.8$  g) animals.

### 3.2 Urinary output and urinary biomarkers

Mean total urine output increased on Day 1 following the first dose in the vancomycin-treated groups. Vancomycin + flucloxacillin resulted in higher urinary volume when compared with baseline and saline-treated animals on days 3 and 4 days. Urinary output increased on every study day for vancomycin+flucloxacillin, whereas in the vancomycin group it was elevated after the first dose only. Mean urinary output is significantly increased with vancomycin+flucloxacillin compared to vancomycin and vancomycin+ imipenem-cilastatin following the 3rd dose (FIG 1a). There was a gradual increase in the mean total urine output in the flucloxacillin-treated animals compared with baseline and saline-treated animals on Days 2 and 3.

In the vancomycin group, absolute amount of urinary KIM-1 over 24 h was significantly increased from Day 1 compared to the saline and flucloxacillin control groups (Table 1, FIG 1b ). While in the vancomycin+flucloxacillin group, KIM-1 was significantly increased following the 3<sup>rd</sup> day compared to vancomycin. In the vancomycin+ imipenem-cilastatin group, KIM-1 was significantly decreased on Days 1 and 2 compared to vancomycin. Similar trends were observed in the vancomycin+flucloxacillin group for clusterin and osteopontin (FIG 1c,d). Locally weighted scatterplot smoothing (LOWESS) trendlines with 95% confidence intervals visually confirmed similar findings for all comparisons (FIG1 supplemental).

### 3.3 Vancomycin concentration in the kidney

Vancomycin concentration in rat kidney was significantly elevated after treatment with vancomycin alone or in combination (FIG 2). A relationship was identified between vancomycin concentration in the kidney tissue and KIM-1 level in the urine (FIG 3).

## 4 DISCUSSION

In this study, the combination of vancomycin+flucloxacillin caused more kidney damage compared to vancomycin alone and to vancomycin+ imipenem-cilastatin as determined by multiple kidney injury biomarkers and accumulation of vancomycin in the kidney. Imipenem-cilastatin was protective for vancomycin induced kidney injury as shown by lower KIM-1. Thus, this translational rat study reproduces the increased clinical toxicity seen in the CAMERA 2 trial (Legg et al., 2022) with combination therapy (vancomycin+flucloxacillin) which resulted in early termination of the trial. Secondly, these data accord with in pre-clinical models (Becerir, Tokgün, & Yuksel, 2021; He et al., 2021; Hori et al., 2017; Humanes et al., 2015; Im et al., 2017; Kusama et al., 1998; Nakamura et al., 1999; Toyoguchi, Takahashi, Hosoya, Nakagawa, & Watanabe, 1997) that demonstrate protection against kidney injury by cilastatin. It is also notable that previous work in our translational rat models have consistently demonstrated that piperacillin-tazobactam does not worsen vancomycin kidney injury and may in fact be protective (Avedissian, Pais, Liu, Rhodes, & Scheetz, 2019; Chang et al., 2022; Pais, Liu, Avedissian, et al., 2020; Pais, Liu, Zepcan, et al., 2020). Thus, while the exact mechanism is not yet clear, differences in toxicity exist based on the specific type of penicillin used.

The protective findings with cilastatin are notable. Cilastatin is a dehydropeptidase-1 inhibitor (DHP-1), an enzyme produced by the proximal tubules (Kahan, Kropp, Sundelof, & Birnbaum, 1983). It is commercially available in combination with imipenem, a broad spectrum carbapenem beta-lactam antibiotic, with or without relebactam, a beta-lactamase inhibitor, to prevent imipenem-induced nephrotoxicity.

In the present study, cilastatin was purchased in combination with imipenem, to translationally represent the combination that is already FDA approved and available in the hospital settings for treatment of variety of infections. Additionally, it is worthwhile to note that we specifically tested a low allometrically scaled dose of imipenem-cilastatin. A dose of 90 mg kg-1/day in the rat equates to a human dose of 14.5 mg kg-1/day of imipenem-cilastatin or 1 gram daily for a 70 kg patient ("Guidance for Industry. Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers. U.S. Department of Health and Human Services. Food and Drug Administration. Center for Drug Evaluation and Research (CDER). , " 2005), a dose that is 25% of the maximum FDA approved dose ("Primaxin Package insert. [https://www.accessdata.fda.gov/drugsatfda\\_docs/label/2016/050587s074lbl.pdf](https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/050587s074lbl.pdf). Merck 12/2016.,"). Full dose ranging experiments with urinary biomarkers are needed to provide fully quantitative results for percentage of protection available. The results here build upon those in the literature. He et al. (He et al., 2021) found lower SCr and BUN values (as AKI biomarkers) in vancomycin+imipenem-cilastatin/relebactam treated male C57BL/6J mice versus vancomycin treated animals and reported that imipenem-cilastatin/relebactam maintained a nephroprotective effect. This is consistent with absolute amount of urinary biomarker KIM-1 in our studies with imipenem-cilastatin versus vancomycin group. In their study, the investigators used 320 mg kg-1 imipenem-cilastatin/relebactam in mice (which is equivalent to 160 mg kg-1 in the rat), at approximately half the cilastatin dose (45 mg kg-1 of cilastatin component) we were able to reproduce the nephroprotective effect. Humanes et al. (Humanes et al., 2015) assessed effect of cilastatin on vancomycin nephrotoxicity in cultured renal proximal tubular epithelial cells. They reported that cilastatin protects against

proximal tubule apoptosis caused by vancomycin. Postulated mechanism of nephroprotection by cilastatin involves megalin and/or p-glycoprotein. Megalin is an endocytic receptor expressed on the apical surface of the proximal tubule cells (Christensen et al., 1995) and mediates the renal uptake of some antibiotics with consequent nephrotoxicity (Kuwahara et al., 2016; Moestrup et al., 1995; Suzuki et al., 2013). Cilastatin competitively binds to megalin and blocks uptake of vancomycin (Hori et al., 2017). A number of studies in mice, rats and in primary porcine proximal tubule cells have demonstrated that cilastatin can decrease cellular uptake of vancomycin and vancomycin concentrations in the kidney (Hori et al., 2017; Humanes et al., 2015; Kusama et al., 1998; Toyoguchi et al., 1997). Hori et al (Hori et al., 2017), assessed nephrotoxicity of vancomycin and other nephrotoxins in proximal tubule epithelial cells and in megalin knock-out mice. Both studies demonstrated that vancomycin binds to megalin and results in kidney damage; while cilastatin binds megalin and reduces damage caused by vancomycin. Others (Im et al., 2017) have suggested that the protection mediated by cilastatin is a function of upregulating p-glycoprotein. Thus, cilastatin may act both through megalin blockade as well as p-glycoprotein upregulation. A study of vancomycin and cilastatin in rabbits demonstrated that vancomycin clearance is higher in rats that received cilastatin than those that received vancomycin alone (Toyoguchi et al., 1997). Both p-glycoprotein upregulation and megalin inhibition both theoretically will increase vancomycin clearance in the urine. Finally, a Wistar albino rat study that used supratherapeutic vancomycin doses between 500-1000 mg kg<sup>-1</sup> (which allometrically translate to 80-160 mg kg<sup>-1</sup>/day in humans) demonstrated that cilastatin protects against decreasing glomerular function (measured by inulin clearance) from vancomycin (Nakamura et al., 1999). Kusama et al. demonstrated that vancomycin accumulates less in kidneys of rats treated with cilastatin (Kusama et al., 1998). Our study is the first to our knowledge to assess the nephroprotective effect of imipenem-cilastatin in a translational rat model using urinary biomarker KIM-1 that has been linked to human outcomes (Scheetz et al., 2021). These results show consistent findings across ex-vivo and in-vivo analyses of multiple species. A small retrospective study in humans (total of 227 patients) found lower rates of nephrotoxicity in patients that received imipenem-cilastatin in combination with vancomycin as opposed to patients in the meropenem (which contains no cilastatin) + vancomycin group (6.2% vs 17.1% respectively) (Hakeam et al., 2019).

Incidence of attributable acute kidney injury (AKI) caused by vancomycin exceeds 10% (Wunderink et al., 2012). Thus, it is essential for clinicians to be aware of drugs (nephroprotectants and nephrotoxins) that can potentiate or alleviate risk of vancomycin AKI. Our rat model confirms that synergistic nephrotoxicity exists, such as observed in CAMERA 2 trial (Legg et al., 2022; J. Liu et al., 2020) due to addition of flucloxacillin to vancomycin; thus we recommend against use of such combination in the hospital settings. Our animal model confirms that imipenem-cilastatin should be explored as a nephroprotectant. However, while cilastatin is available in a clinically approved preparation as imipenem-cilastatin, promotion of antimicrobial resistance will preclude regular use. Thus, the development of a -vancomycin-cilastatin preparation would be desirable, and more information will be needed on the benefits and risks of that combination.

## DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

## AUTHOR CONTRIBUTIONS

MS and GP conceived and designed the study. GP, JL, RM, SM, KV were involved in the acquisition of data. GP, MS analyzed the data. ST, JD, JL, JC, MS interpreted the data;

GP, RR, MS drafted the manuscript. ST, JD, MS revised it critically for important intellectual content.

All authors have given final approval of the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work



are appropriately investigated and resolved.

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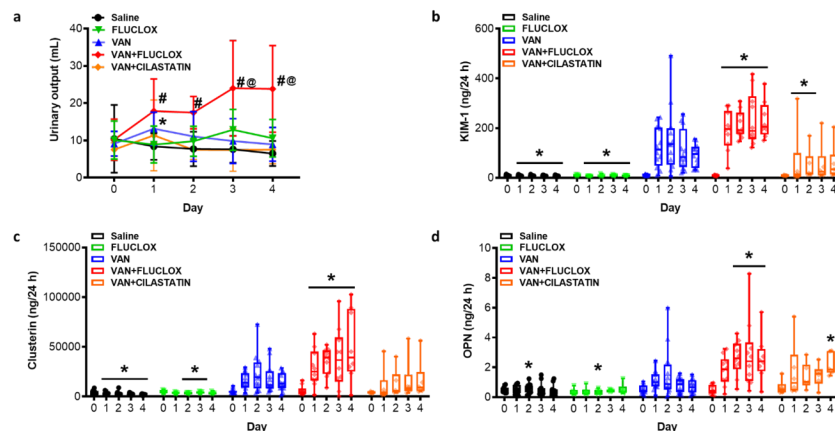
## FIGURE LEGENDS

**FIG 1** Comparison of daily urinary biomarker measurements between treatment groups and across dosing days. (a) Urinary output. Values expressed as mean  $\pm$  SD; \* vancomycin vs saline, # vancomycin+flucloxacillin vs saline, @ vancomycin+flucloxacillin vs vancomycin. Total amounts of urinary KIM-1 (b), clusterin (c), and OPN (d) in saline (n=11), flucloxacillin (n=6), vancomycin (n=12), vancomycin+flucloxacillin (n=10) and vancomycin+imipenem-cilastatin (n=6)-treated rats. Values expressed as median (min-max); \* vs vancomycin. KIM-1, kidney injury molecule-1; OPN, osteopontin.

**FIG 2** Vancomycin concentration in the kidney tissue. Values expressed as median and interquartile range.

**FIG 3** Correlation between urinary KIM-1 (ng/24 h) and vancomycin in the kidney ( $\mu$ g/g).

**FIG 1** Comparison of daily urinary biomarker measurements between treatment groups and across dosing days. (a) Urinary output. Values expressed as mean  $\pm$  SD; \* vancomycin vs saline, # vancomycin+flucloxacillin vs saline, @ vancomycin+flucloxacillin vs vancomycin. Total amounts of urinary KIM-1 (b), clusterin (c), and OPN (d) in saline (n=11), flucloxacillin (n=6), vancomycin (n=12), vancomycin+flucloxacillin (n=10) and vancomycin+imipenem-cilastatin (n=6)-treated rats. Values expressed as median (min-max); \* vs vancomycin. KIM-1, kidney injury molecule-1; OPN, osteopontin.



**FIG 2** Vancomycin concentration in the kidney tissue. Values expressed as median and interquartile range.

**FIG 3** Correlation between urinary KIM-1 (ng/24 h) and vancomycin in the kidney (µg/g).

**Table 1: Contrast means versus vancomycin as referent group**

| Dosing day                                | Treatment groups                          | Treatment groups                          |
|---|---|---|
|   | <b>Saline</b>                             | <b>FLUCLOX</b>                            |
| Urinary KIM-1 (ng/24h), mean (95% CI)     | Urinary KIM-1 (ng/24h), mean (95% CI)     | Urinary KIM-1 (ng/24h), mean (95% CI)     |
| Day 0                                     | -3.4 (-53.9 to 47.1)                      | -2.3 (-62.8 to 58.1)                      |
| Day 1                                     | -115.9 (-166.4 to -65.4)*                 | -117.5 (-178.0 to -57.0)*                 |
| Day 2                                     | -146.7 (-197.2 to -96.2)*                 | -146.6 (-207.1 to -86.1)*                 |
| Day 3                                     | -102.8 (-153.3 to -52.3)*                 | -101.1 (-161.6 to -40.6)*                 |
| Day 4                                     | -83.4 (-133.9 to -32.9)*                  | -82.5 (-143.0 to -22.0)*                  |
| Urinary clusterin (ng/24h), mean (95% CI) | Urinary clusterin (ng/24h), mean (95% CI) | Urinary clusterin (ng/24h), mean (95% CI) |
| Day 0                                     | -1547 (-12812 to 9718)                    | -825 (-14319 to 12667)                    |
| Day 1                                     | -12517 (-23782 to -1253)*                 | -12273 (-25767 to 1220)                   |
| Day 2                                     | -20360 (-31626 to -9096)*                 | -20062 (-33556 to -6570)*                 |
| Day 3                                     | -15823 (-27088 to -4558)*                 | -14261 (-27755 to -768)*                  |
| Day 4                                     | -12319 (-23585 to -1055)*                 | -11041 (-24535 to 2452)                   |
| Urinary OPN (ng/24h), mean (95% CI)       | Urinary OPN (ng/24h), mean (95% CI)       | Urinary OPN (ng/24h), mean (95% CI)       |
| Day 0                                     | -0.02 (-0.77 to 0.73)                     | -0.18 (-1.08 to 0.73)                     |
| Day 1                                     | -0.64 (-1.39 to 0.11)                     | -0.75 (-1.65 to 0.15)                     |
| Day 2                                     | -0.99 (-1.74 to -0.24)*                   | -1.25 (-2.15 to -0.35)*                   |
| Day 3                                     | -0.28 (-1.03 to 0.47)                     | -0.40 (-1.30 to 0.50)                     |
| Day 4                                     | -0.24 (-0.99 to 0.51)                     | -0.21 (-1.11 to 0.69)                     |

**Supplemental FIG 1.** Trends in urinary biomarkers visualized using locally weighted scatterplot smoothing (LOWESS) trendlines with 95% confidence intervals.

