

Answer to Reviewer # 2

Question 1:

The reviewer states: *“This manuscript discusses the anticancer activity of Cilastatin. This topic is highly relevant and interest in health. It is intriguing. The manuscript is original”*.

Answer 1

Many thanks to the reviewer for their comments. Acute kidney injury associated with sepsis is a serious problem with a mortality rate ranging between 50% and 80% in the intensive care units, and there is currently no drug that can be used to protect the kidneys from damage. However, it is well known that in patients with sepsis and acute renal failure, mortality is about 75%, while in those with sepsis without AKI, mortality ranges between 27% and 32%. That is why it is very important to protect the kidneys in sepsis. The manuscript discusses the therapeutic power of cilastatin in preventing acute renal failure induced by polymicrobial sepsis.

Question 2:

The reviewer states: *“In the introduction, why sepsis can cause acute kidney injury? The author should introduce the chemical structure and molecular formula of Cilastatin. Recommendation: The author to provide additional clarification”*.

Answer 2

It is well documented that sepsis is the main cause of acute kidney injury (AKI), typically emerging within the early hours of its onset, with 30–60% of septic patients developing AKI. This fact is well reflected in some of the references cited in the article, such as numbers 4, 5 and 7. Particularly, a very recent one, number 9, reviews perfectly and extensively the fact of how sepsis is able to generate AKI, and it is perfectly summarized in figure 1 (Acute Kidney Injury in Sepsis. Int J Mol Sci 2024, 25, 5924. DOI: 10.3390/ijms25115924). In addition, sepsis is the leading cause of AKI in critically ill patients, accounting for 40-70% of cases. And the main problem is that patients with AKI already have a higher risk of death than other critically ill patients, as it is an independent risk factor associated with increased mortality (Regueira T, et al. Med Intensiva. 2011;35(7):424-32, doi: 10.1016/j.medin.2011.03.011).

Briefly, sepsis can lead to AKI due to a combination of pathophysiological factors that compromise blood flow (renal perfusion) and renal function that lead to a rapid decline in glomerular filtration rate (GFR). Recent studies have shown that inflammation and apoptosis are the primary pathological processes in septic AKI. This has been demonstrated through the progression of organ damage, including both macrovascular and microvascular dysfunction (systemic hypotension and renal vasoconstriction, not necessarily within the ischemic range), immune dysregulation and alterations in oxygen delivery, either due to decreased perfusion or impaired diffusion secondary to edema and inflammation. The

kidneys are particularly susceptible to the septic environment and this vulnerability is considered a key factor contributing to the development of AKI. The kidney plays a critical role in filtering blood and is constantly exposed to circulating inflammatory mediators, pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs), all of which activate immune responses within renal tissue. Tubular epithelial cells and resident immune cells express a wide range of pattern recognition receptors (PRRs), such as Toll-like receptors, which amplify the inflammatory cascade. This results in increased oxidative stress, mitochondrial injury, and apoptosis, contributing further to renal dysfunction. The limited regenerative capacity of renal tubular cells in the context of ongoing inflammatory insult makes the kidney especially prone to sustained injury in sepsis.

In addition, the presence of bacterial products in the systemic circulation activates inflammatory cells that infiltrate renal tissue and increases the secretion of cytokines such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and IL-6 by tubular epithelial cells, as well as leukocyte activity. Intraglomerular thrombosis and intratubular obstruction may occur, as fibrin deposits have been observed within the lumens of glomerular capillaries. Medullary tubular epithelial cells show vacuolization and increased expression of Kidney Injury Molecule-1 (KIM-1), a marker of tubular injury. Mitochondrial dysfunction and oxidative stress are also present, with increased production of reactive oxygen species (ROS) and induction of nitric oxide synthase (NOS), which can lead to damage of the endothelial barrier and glycocalyx. All these structural and functional changes contribute to the tubular dysfunction characteristic of sepsis-induced AKI.

Despite receiving approximately 20–25% of the cardiac output, the renal microcirculation is tightly regulated and highly heterogeneous, with distinct perfusion patterns between the cortex and medulla. This makes the kidney especially susceptible to even subtle alterations in systemic hemodynamics and local oxygen delivery. During sepsis, systemic inflammation, endothelial dysfunction, and microvascular dysregulation impair renal autoregulation and lead to regional ischemia, particularly in the outer medulla, which is already at the borderline of hypoxia under normal conditions.

As a result, AKI represents a serious complication of sepsis, and the consequence is that patients with AKI already have a higher risk of death than other critically ill patients, as it is an independent risk factor associated with increased mortality (Regueira T, et al. *Med Intensiva*. 2011;35(7):424-32, doi: 10.1016/j.medin.2011.03.011). A patient with sepsis complicated with AKI has a significant increase in relative mortality (70%) with respect to another without AKI (Liétor A, et al. *NefroPlus*. 2010; 3(3):9-19, doi:10.3265/NefroPlus.pre2010.Nov.10733; Poston JT, et al. *BMJ*. 2019;364:k4891. doi: 10.1136/bmj.k4891; Zhang CF, et al. *Eur Rev Med Pharmacol Sci*. 2020; 24(10): 5604-5617. doi: 10.26355/eurrev_202005_21346).

We have tried to summarize all this in a few lines in the introduction of the article, and specifically the reviewer can find it in lines: 57-63 of the new manuscript.

In response to the second question, cilastatin is the monosodium salt of [R-[R, S-(Z)]]-7-[(2-amino-2-carboxyethyl)thio]-2-[[2,2-dimethylcyclopropyl)carbonyl]amino]-2-heptenoic acid. Its empirical molecular formula is $\text{C}_{16}\text{H}_{25}\text{N}_2\text{NaO}_5\text{S}$, with a molecular weight of 280.44

Da. These data, together with the chemical structural formula of cilastatin, which is the new Figure 9, have been incorporated into the article in the Materials and methods section. The reviewer can find them between lines 782 to 796 of the new manuscript.

Question 3:

The reviewer states: *“In the methodology, What are the characteristic signs of the model of sepsis-induced kidney injury? How does the author determine the success of the model? How to judge the success of model establishment? Are Cilastatin clinically available? If it is used clinically available, what is the dose used? What is the relationship between the dose of clinically and the dose used in this study? What is base of the dose of Cilastatin used in this study?”*.

Answer 3:

Answer to the question: “In the methodology, What are the characteristic signs of the model of sepsis-induced kidney injury?”

The cecal ligation and puncture (CLP) model has been widely used in the last 30 years to study the pathophysiology of sepsis and is well described in the literature (González-Nicolás MA & Lázaro A). Compared to other models, it provides a better representation of the complexity of human sepsis where immune, biochemical, and hemodynamic responses are similar to those observed in humans (J Biomed Sci. 2017; 24(1):60. doi: 10.1186/s12929-017-0370-8). Not in vain, it has been considered the *"Gold standard"* for showing a high degree of similarity to the progression of human sepsis and tends to be the technique of choice for developing models of sepsis-induced acute kidney injury (AKI) (Trends Microbiol. 2011; 19(4):198-208. doi: 10.1016/j.tim.2011.01.001; González-Nicolas MA & Lazaro A).

Sepsis-induced AKI presents with a distinct set of histological, molecular, and functional features that differentiate it from other forms of renal injury. Unlike ischemic or nephrotoxic models, where widespread tubular necrosis is common, septic AKI is often characterized by minimal cell death and instead displays sublethal injury, including tubular epithelial cell dysfunction, loss of polarity, and cytoskeletal disorganization. One hallmark of this model is the presence of inflammatory cell infiltration, especially neutrophils and macrophages, in the interstitial space, driven by systemic and local release of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β .

Another characteristic feature is endothelial dysfunction, manifesting as loss of barrier integrity, shedding of the glycocalyx, and impaired microcirculatory perfusion. These alterations contribute to heterogeneity in renal blood flow and focal hypoxia, particularly in the outer medulla. Mitochondrial injury is also prominent, with reduced ATP production, increased generation of reactive oxygen species (ROS), and activation of cell stress pathways such as the unfolded protein response and mitophagy.

Additionally, upregulation of biomarkers such as Kidney Injury Molecule-1 (KIM-1), Neutrophil Gelatinase-Associated Lipocalin (NGAL), and increased expression of inducible nitric oxide synthase (iNOS) are molecular indicators of injury in experimental models. Functional signs include an abrupt decline in glomerular filtration rate (GFR) without

necessarily corresponding to marked tubular necrosis, supporting the notion that sepsis-associated AKI is primarily driven by inflammation, microvascular alterations, and metabolic reprogramming rather than classical ischemia.

Answer to the questions: “How does the author determine the success of the model? How to judge the success of model establishment?”

The CLP model was developed at the Getafe Hospital and was carried out by researchers from Dr. Jose Angel Lorente's group, who have a fully accredited experience in the realization of this model and others of septic damage in small and large animals. Its highly trained staff (Mario Arenillas, the author of the article is the veterinarian responsible for the animal facility), knows it and performs it to perfection, based on the greater or lesser aggressiveness required, as has been the case in the work presented here where two models have been made, the second one much more aggressive and where the animals died before 48 hours. It is a model that they have made many times and in which they have determined and studied many parameters, mainly with respect to lung damage and renal damage. And this is confirmed by some of the publications where they have made this same model precisely for studies of lung and kidney damage. Some of them are:

Seija M, et al. Role of peroxynitrite in sepsis-induced acute kidney injury in an experimental model of sepsis in rats. *Shock*. 2012; 38(4):403-10. doi: 10.1097/SHK.0b013e31826660f2.

Izquierdo-García JL, et al. A metabolomic approach for diagnosis of experimental sepsis. *Intensive Care Med*. 2011; 37(12):2023-32. doi: 10.1007/s00134-011-2359-1.

Nicolás Nin, et al. Vascular dysfunction in sepsis: effects of the peroxynitrite decomposition catalyst MnTMPyP. *Shock*. 2011; 36(2):156-61. doi: 10.1097/SHK.0b013e31821e50de.

Chacon-Cabrera A, et al. Influence of mechanical ventilation and sepsis on redox balance in diaphragm, myocardium, limb muscles, and lungs. *Transl Res*. 2014; 164(6):477-95. doi: 10.1016/j.trsl.2014.07.003.

From this accredited experience in its realization, we know that the model is well done, and its achievement was successful because the results that we saw later regarding renal damage and that we present in this article, validate the success of the model and the acute renal failure that we pursued with its realization. But, before the slaughter of the animals, clinical signs indicative of systemic deterioration were evident of a correct development of the model, including anorexia and lethargy, abnormal gait or a hunched posture, persistent curled-up position, alterations in grooming behavior, piloerection, vocalization upon handling suggestive of pain or discomfort, partially closed eyes, and changes in fecal and urinary output. These manifestations are consistent with established clinical scoring systems used to assess the severity of illness and to ensure animal welfare in experimental sepsis models.

On the other hand, in sepsis, a hypodynamic or immunosuppressive phase occurs characterized by a profound anti-inflammatory response involving reduced lymphocyte proliferation and function, as well as increased apoptosis. In an unpublished hematological

study, we observed that the CLP group exhibited a significant reduction in total leukocyte and platelet counts. Neutrophil percentages increased threefold compared to control values, while monocyte levels doubled. Conversely, lymphocyte and eosinophil percentages were significantly decreased. Notably, we observed an increase in LUCs (Large Unstained Cells), which represent a small fraction of cells (<6%) that do not take up stain during differential leukocyte counts. These cells typically include hyperactivated large leukocytes, blasts, atypical large lymphocytes, plasmocytes, and other pathological cells. An elevated LUC count (>6%), especially when accompanied by increased transaminase levels, is considered indicative of systemic infection or ongoing inflammation. All hematological parameters significantly improved in the cilastatin-treated group, showing a reduction in neutrophil and macrophage infiltration along with restoration of lymphocyte levels. Red blood cell indices remained unchanged across all experimental groups.

In addition to this, we found clear results of renal failure, with elevated creatine and urea levels, and a clear decrease in glomerular filtration, data that indicated that sepsis had indeed caused renal failure. The presence of protein in the urine also informed us of glomerular damage, and the presence of the biomarker KIM-1 that only appears when there is tubular damage were key aspects to affirm that the model was well established and successful in its development. And as we have commented, cilastatin was able to prevent inflammatory damage by decreasing each characteristic mentioned.

Answer to the question: “Are Cilastatin clinically available?”

The direct answer is no, it is not possible to use cilastatin alone for human clinical use. But it does exist in combination with an antibiotic imipenem to form the antibiotic imipenem/cilastatin (which has different trade names Primaxin, Tienam, Zienam...) which has been used in human clinical use since 1985. Therefore, cilastatin can only be used in the clinic in combination with imipenem. Its function is to inhibit the renal dehydropeptidase I enzyme so that it cannot metabolize and inactivate imipenem, preventing its absorption, increasing its urinary excretion, and reducing its concentration inside the tubular cell.

Our research group observed at the preclinical level that using cilastatin alone (without imipenem) as a therapeutic treatment, it was able to protect the kidney of rodents against toxic acute renal failure, produced for nephrotoxic drugs, which led us to think that other situations that induce renal failure, such as sepsis, could also benefit, which is the work presented here. Our results led us to patent the nephroprotective effect of cilastatin and to establish the spin off Telara Pharma, with the aim of bringing cilastatin alone to clinical use as a nephroprotective drug.

Answer to the question: “If it is used clinically available, what is the dose used?”

As mentioned above, cilastatin is not clinically available as a standalone drug, but it is used in combination with the antibiotic imipenem in the imipenem/cilastatin (I/C) formulation. A 1:1 ratio of I/C was established as the optimal dose to maintain inhibition for an interval of 8 to 10 hours (Norrby, S. R. et al. *Antimicrob. Agents Chemother.* 1983, 23, 300-307).

The dose of I/C used depends on the severity of the infection. In adults and adolescents, the usual doses are 250 mg/250 mg for mild infections every 6 hours (1 gram total per day), 500 mg/500 mg for moderate infections every 8 hours (1.5 grams total) or severe infections by very sensitive germs every 6 hours (2 grams total) and finally 1000 mg/1000 mg for severe and/or life-threatening infections every 6 or 8 hours (3- or 4-grams maximum total). The usual dose in children one year of age or older is 15/15 or 25/25 mg/kg/dose every 6 hours.

The data were obtained from the package inserts and technical data sheets of the drug imipenem/cilastatin.

Answer to the questions: “What is the relationship between the dose of clinically and the dose used in this study? What is base of the dose of Cilastatin used in this study?”.

The dose employed in this study was 150 mg/kg/day, determined based on prior experience of our research group, which has consistently yielded positive outcomes and consistent therapeutic efficacy as supported by our published studies on acute kidney injury induced by nephrotoxics such as cisplatin or gentamicin (references in the manuscript: 16, 17, 19, 23). But when we started our studies on this subject (*Kidney Int.* 2012 Sep;82(6):652-63. doi: 10.1038/ki.2012.199) we chose to administer that dose based on the literature we had available in this regard, which were old studies in which we administered imipenem/cilastatin and saw protection over nephrotoxic drugs, mainly cyclosporine and vancomycin, assessing functional values such as serum creatinine and urea. Specifically, Hammer C et al. in their article “Reduction of cyclosporin (CSA) nephrotoxicity by imipenem/cilastatin after kidney transplantation in rats” *Transplant Proc.* 1989 Feb;21(1 Pt 1):931, used 150 mg/kg/day bw showing nephroprotection; Nakamura T et al. in their articles: “Effects of fosfomycin and imipenem/cilastatin on nephrotoxicity and renal excretion of vancomycin in rats” *Pharm Res.* 1998 May;15(5):734-8. doi: 10.1023/a:1011971019868 and “Effects of fosfomycin and imipenem-cilastatin on the nephrotoxicity of vancomycin and cisplatin in rats”, *J Pharm Pharmacol.* 1999 Feb;51(2):227-32. doi: 10.1211/0022357991772187, also used a dose of 150 mg/kg/day bw and found protection against vancomycin nephrotoxicity in both studies; Sido et al. used 150 mg/kg day bw but twice a day, demonstrating its efficacy in the paper “Nephroprotective effect of imipenem/cilastatin in reducing cyclosporine toxicity” *Transplant Proc.* 1987 Feb;19(1 Pt 2):1755-8. Finally, Toyoguchi et al. in rabbits tested various doses of cilastatin for the treatment of vancomycin nephrotoxicity, 75, 150 and 300 mg/kg/day bw, showing a dose-dependent effect on protection, but no major differences in renal protection between the 150 and 300 mg/kg dose.

Based on all these studies that had shown renal protection against cyclosporine and vancomycin, mainly at a dose of 150 mg/kg, we decided to use this same dose, but only cilastatin (without imipenem) in our protection models. We were able to verify how this dose

was very effective in nephroprotection against toxins (cisplatin and gentamicin). We also performed experiments with doses of 75 mg/kg/day and 300 mg/kg/day administered to rats treated with cisplatin. The 75 mg/kg dose was shown to be less effective in nephroprotection, while the 300 mg/kg dose was shown to be slightly more effective than the 150 mg/kg dose but without significant differences, as described by Toyoguchi in rabbits for the treatment of vancomycin nephrotoxicity.

Based on all these studies, and on our own results in animal models of acute renal failure, 150mg/kg/day was the dose chosen for these studies, and we have maintained it for the study presented here, and it has also proved to be effective. We also knew that 150 mg/kg is a clinically relevant dose, as we had previously conducted an interspecies scaling using body surface area (BSA) normalization. According to FDA guidelines, the human equivalent dose (HED) of 150 mg/kg in rats corresponds to approximately 24.3 mg/kg in humans. For an average adult (70 kg), this translates to a total dose of ~1.7 grams, which is within the range of the maximum clinical dosage currently approved for cilastatin (1 gram every 8 hours, or up to 3 grams/day (or 4 maximaum) when co-administered with imipenem). Therefore, the experimental dose employed in our study is pharmacologically relevant and translationally appropriate for modeling potential clinical effects in the context of severe systemic inflammation and sepsis.

Question 4:

The reviewer states: *“In the conclusions, the clarity of the image of Western blot should be improved. What are the specific limitations of the article and future research directions? There a multiple grammar and style errors, wrongly constructed phrases and scientifically incorrect expressions”*.

Answer 4:

We have improved the images of the western blots for better and clearer visualization.

Our study, like all scientific research, has and has had some limitations, as well as methodological considerations, since the study of the pathophysiology of sepsis-associated acute kidney injury (AKI) faces several important limitations. One of the most significant is the lack of detailed information on renal histopathological changes in humans, as kidney biopsies are generally contraindicated and technically challenging in critically ill patients in the ICU setting. Beyond the clinical constraints imposed by sepsis itself, fully replicating the clinical syndrome under experimental conditions is also difficult. Available experimental models do not always accurately reproduce the complexity of septic syndrome. Nevertheless, the cecal ligation and puncture (CLP) model is widely considered the “gold standard” and is extensively described in the literature as one of the most frequently employed methods for this purpose.

Another challenge is the difficulty in obtaining reliable measurements of hemodynamic parameters in small animal models. Moreover, some rodent species have shown intrinsic resistance to endotoxins, which can limit the translational relevance of the findings. These limitations are compounded by the relatively short duration of experimental models compared to the often prolonged and clinically complex course of sepsis in human survivors.

One disadvantage of the CLP technique is the inability to precisely control the amount of fecal material extruded during cecal compression. Furthermore, intestinal microbiota composition is not uniform among individual animals or species, making inter-study comparisons challenging and requiring careful interpretation. Variations in surgical procedures and postoperative care also contribute to inconsistencies. Standardization of the model is inherently difficult and largely depends on the technique employed and the surgeon's level of experience. Additionally, the use of opioids such as buprenorphine may suppress respiration and locomotor activity, which can be misinterpreted as clinical signs of sepsis.

Another important limitation of our study was the identification of microbial species involved in the development of sepsis in the polymicrobial CLP model. These species may vary depending on the strain and species of the animal used, and this can also lead to significant differences between similar studies. In our case, we encountered logistical limitations that prevented a comprehensive analysis and characterization of the specific microbial populations contributing to the septic process.

The severity of the model has a direct impact on survival rates, as illustrated in Figure 7. Parameters such as the length of the ligated cecum, catheter gauge, and number of punctures can be adjusted to modulate the degree of severity and mortality. When performing the CLP model, all of these factors and technical parameters must be carefully considered to ensure reproducibility and consistency of results. Therefore, a thorough understanding and mastery of the procedure are essential to accurately implement the CLP model. In our experiment, we believe that this was not really a limitation, since, as we mentioned previously, the research team has extensive experience in implementing this model, although it is true that variations cannot be ruled out 100%, and the comparison in terms of damage may differ from other studies using the same model.

Additional limitations relate to space constraints associated with the use of metabolic cages, not only in terms of their physical placement but also regarding the number of units that can be managed simultaneously in a given experiment. The implementation of the 3Rs (Replacement, Reduction, and Refinement) further necessitates careful planning to minimize the number of animals used without compromising scientific validity. This was, for example, one of the reasons why we did not use a larger n in the 48-hour survival experiment (Figure 7).

Some of these limitations of the study have been incorporated into the manuscript, and the reviewer can find them in the discussion, between lines 758-767

With regard to future directions, this article highlights that cilastatin appears to be a universal nephroprotector for any type of acute renal failure of any etiology. We had already demonstrated the protective role in nephrotoxic-induced AKIs, and now we have done so in septic damage, showing that the pathways of renal protection by cilastatin are common to any type of renal damage.

This work opens the door to the study of other non-toxic acute renal failures, such as ischemia-reperfusion, or even kidney transplantation, where cilastatin may be beneficial for maintaining the donated kidney in cardiac arrest by administering it before reperfusion in the recipient. -Omic studies could also bring us closer to understanding the protective mechanism and the exact molecular pathways through which cilastatin exerts its nephroprotective action. On the other hand, this work highlights the beneficial effects of cilastatin and opens the door to future clinical use in this context, work that is being developed by the spin-off Telara Pharma (www.telarapharma.com) to bring the drug cilastatin to human clinical practice as a nephroprotective agent.

The text has been extensively revised and proofread by a style editor to correct both style and language. The changes made can be seen in change control throughout the text.

Question 5:

The reviewer states: *“The format of references is consistent, and it meets the requirements of the journal”*.

Answer 5:

Thank you very much for the review and appreciation.