Imaging the distribution of polymyxins in the kidney

Bo Yun¹,², Mohammad A. K. Azad¹, Jiping Wang¹, Roger L. Nation¹, Philip E. Thompson²,
Kade D. Roberts¹,², Tony Velkov¹* and Jian Li¹*

¹Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences,
Monash University, Melbourne, Victoria, Australia. ²Medicinal Chemistry, Monash Institute of
Pharmaceutical Sciences, Monash University, Melbourne, Victoria, Australia.

*Joint senior authors. Corresponding author: Jian Li, Phone: +61-3-9903-9702; Fax: +61-3-9903-9583; Email: colistin.polymyxin@gmail.com

Running Title: Distribution of polymyxins in the kidney

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Abstract

Background: Dose-limiting nephrotoxicity remains the Achilles’ heel of polymyxin B and E (also known as colistin) which are important last line antibiotics used against infections caused by multidrug-resistant (MDR) Gram-negative ‘superbugs’. An understanding of the mechanisms of nephrotoxicity, including renal tissue distribution, will be crucial for the development of safer polymyxin lipopeptide antibiotics. This is the first study to visualise the kidney distribution of polymyxin B using a mouse nephrotoxicity model and in situ immunostaining of kidney sections.

Methods: Polymyxin B nephrotoxicity in mice was induced over the course of three days (accumulated IV dose 175 mg/kg) and kidneys were harvested and frozen sectioned. The sections were fixed in cold acetone, dried and treated with 1% hydrogen peroxide. Endogenous mouse immunoglobulins were blocked and the tissue sections were treated with anti-polymyxin B mouse IgM antibody. The sections were incubated with a biotinylated anti-mouse secondary antibody conjugate followed by an AlexaFluor647-Streptavidin conjugate. Polymyxin B distribution in the kidney sections was then visualised using a fluorescence scanning microscope. Kidney sections were also subjected to hematoxylin and eosin staining to assess pathological damage from the polymyxin-induced nephrotoxicity.

Results: Immunostaining of kidney sections from a mouse with polymyxin B-induced nephrotoxicity revealed that polymyxin B distributed predominantly within the renal cortex. More specifically, polymyxin B accumulated within the proximal tubular cells.

Conclusions: The observed accumulation of polymyxin B within proximal tubular cells is consistent with the extensive renal reabsorption of polymyxins and the likely cause of the associated nephrotoxicity.
Introduction

Over the past two decades there has been a pronounced increase in the emergence of Gram-negative ‘superbugs’ which are resistant to almost all clinically available antibiotics and cause serious infections.\(^1\) This dire situation is exacerbated by a lack of novel antibiotics in the developmental pipeline of both pharmaceutical companies and research institutes, leaving the world’s population in a vulnerable state against these life-threatening infections.\(^2\) This ‘perfect-storm’ has led to the revival of the polymyxin class of antibiotics, polymyxin B and E (also known as colistin), as a last line of defence against infections caused by multidrug-resistant (MDR) Gram-negative pathogens.\(^3\) However, despite their excellent antibacterial activity, the use of polymyxins has largely been limited by the associated adverse effects. In particular nephrotoxicity poses as a major dose-limiting factor for the clinical use of polymyxins, occurring in up to 60% of all patients.\(^4\) Polymyxin-induced nephrotoxicity potentially arises from their significant reabsorption by renal tubular cells;\(^4\)-\(^6\) for both colistin and polymyxin B, only a minor portion of the drug in the body is renally eliminated.\(^5\),\(^6\) A recent synchrotron X-ray fluorescence microscopy (XFM) single-cell study from our laboratory demonstrated that polymyxins accumulate within kidney tubular cells with the intracellular concentrations approximately 2,000 - 4,000 times higher than the extracellular concentrations.\(^7\) Presently there is a lack of information regarding the kidney tissue distribution of polymyxins. Such knowledge is critical for understanding their pharmacokinetics/pharmacodynamics/toxicodynamics, and for the development of novel safer polymyxins.\(^8\),\(^9\) To the best of our knowledge, this is the first study to visualise the distribution of polymyxins in kidneys using a mouse nephrotoxicity model and \textit{in situ} immunostaining of kidney sections.
Materials and methods

Animals

The animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. Animal experiments were performed in accordance with the Australian National Health and Medical Research Council guidelines for the care and use of animals for scientific purposes. Female Swiss mice (6 to 8 weeks of age, 20 to 25 g) were used in this study. Mice had free access to food and water during the experiment.

Mouse polymyxin nephrotoxicity model and immunostaining

A mouse nephrotoxicity model was established by our group. On the day of experiment, polymyxin B or sterile saline was administered subcutaneously to mice (n = 3) over the course of three days (accumulated dose 175 mg/kg, with 35, 10, 10, 20 mg/kg administered at 2 h intervals on day one; 35, 10, 10, 15 at 2 h intervals on day two; and a final dose of 30 mg/kg on day three.) On the third day, mice were euthanized via isoflurane overdose 2 h after the last dose and kidneys were collected immediately and sectioned frozen at 12 μm. The sections were fixed in cold acetone, dried and treated with 1% hydrogen peroxide for 5 min. Blocking of endogenous mouse IgG was carried out using the Vector M.O.M kit (Vector Labs, CA, USA) as per manufacturer’s instructions. Anti-polymyxin B mouse IgM antibody (Thermo Fisher Scientific, Rockford, USA) was diluted to 1:500 and incubated with the tissue section overnight at 4°C. The sections were washed and incubated with M.O.M biotinylated anti-mouse secondary link (Vector Labs, CA, USA) for 10 min, followed by incubation with an AlexaFluor647 Streptavidin conjugate at a 1:500 dilution (Life Technologies, VIC, Australia). Kidney sections were also
subjected to hematoxylin and eosin staining.\textsuperscript{11} Sections were mounted with Dako fluorescence mounting medium (Dako, NSW, Australia) and imaged using a Metasystems VSlide Scanner.

**Results and Discussion**

Currently, dose-limiting nephrotoxicity remains a major problem for the clinical usefulness of polymyxins.\textsuperscript{5, 6} Understanding the mechanisms of polymyxin-induced nephrotoxicity is not only very important for optimising their clinical use, but also for guiding the development of novel, safer polymyxin lipopeptides. Polymyxin B was employed in this study as recent pharmacological studies demonstrated that it has significantly better pharmacokinetic/pharmacodynamic characteristics than colistin.\textsuperscript{12} A mouse polymyxin nephrotoxicity model established in our novel polymyxin discovery program was employed in the present study.\textsuperscript{10}

In this study we have examined the distribution of polymyxins in the kidney tissue of mice with polymyxin-induced nephrotoxicity (Figure 1, See hematoxylin and eosin panel showing histopathological damage). Histological abnormalities were observed in the kidneys of the polymyxin B treated mice, with marked tubular dilation and degeneration. Tubular casts were identified in the cortex and the kidneys appeared encapsulated by fibrotic tissue. The kidneys of the mice that received the saline control appeared normal. Immunostaining of the kidney sections revealed that polymyxin B predominantly accumulated in the renal cortex (Figure 1, See polymyxin B treated panel). More specifically, substantial accumulation was observed in the renal proximal tubular cells (Figure 1, See polymyxin B treated panel, magnified cortex region). Polymyxin B does not appear to accumulate in the distal tubular cells as indicated by the low
staining intensity of these cells (Figure 1, See polymyxin B treated panel, magnified cortex region). This finding is consistent with our preclinical and clinical pharmacokinetic data that polymyxins undergo significant renal reabsorption after filtration by glomeruli. The observed distribution of polymyxin B is also in line with our animal and cell culture studies which suggested substantial accumulation of colistin and polymyxin B within renal proximal tubular cells and induction of apoptosis by activation of the death receptor, mitochondrial and endoplasmic reticulum pathways. Accumulation of polymyxins within renal tubular cells is the probable cause of the associated nephrotoxicity. Further studies on the distribution of polymyxins in proximal and distal tubular cells are being conducted.

In conclusion, this study provides valuable insights into the renal tissue distribution and nephrotoxicity of polymyxins, and also serves as a discovery platform for novel polymyxin lipopeptide development programs.

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Transparency declarations

None to declare.

Disclaimer

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Figure 1. Top panels. Distribution of polymyxin B (PmB) within the mouse kidney visualised by in situ fluorescence imaging. The inset shows the cortex and medulla regions of the PmB treated kidney image magnified x10. Bottom left panel. Schematic diagram of the in situ image development procedure employed. Bottom right panel. Anatomy of the kidney.
References


