Melatonin Attenuates Colistin-Induced Nephrotoxicity in Rats\textsuperscript{\textdagger}

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Colistin-induced nephrotoxicity is a dose-limiting adverse effect when colistin is used against Gram-negative pathogens. This study examined the nephroprotective effect of melatonin against colistin in rats. Rats (n = 7 per group) were treated intravenously twice daily with saline, colistin (at increasing doses from 0.5 to 4.0 mg/kg), melatonin (5 mg/kg), or both melatonin and colistin for 7 days. The severity of renal alteration was examined both biochemically and histologically. The effect of coadministration of melatonin on colistin pharmacokinetics was investigated. Significantly lower urinary N-acetyl-β-D-glucosaminidase excretion was observed from day 1 in the colistin-melatonin group compared to the colistin group (P < 0.0001). Plasma creatinine increased significantly (P = 0.023) only in the colistin group on day 6. Significant histological abnormalities (P < 0.0001) were detected only in the kidneys of the colistin group. Melatonin altered colistin pharmacokinetics; the total body clearance in the colistin-melatonin group (1.82 ± 0.26 ml/min/kg) was lower than in the colistin group (4.28 ± 0.93 ml/min/kg). This is the first study demonstrating the protective effect of melatonin against colistin-induced nephrotoxicity, which indicates that colistin-induced nephrotoxicity is mediated through oxidative stress. It also highlights the potential of coadministering an antioxidant to widen the therapeutic window of this very important last-line antibiotic.

The increasing prevalence of infections caused by multidrug-resistant (MDR) Gram-negative bacteria and the shortage of novel antibiotics are presenting a global medical challenge. Colistin (also known as polymyxin E), a cationic polypeptide antibiotic with activity against such pathogens, has been commercially available since the 1950s, but it was largely superseded in the 1970s due to potential toxicity, in particular nephrotoxicity. Interest in colistin has been revived over the last decade; it is now being used increasingly as a last-line therapy for infections caused by MDR Pseudomonas aeruginosa, Acinetobacter baumannii, and Klebsiella pneumoniae (7, 9, 23, 28, 29). It is important to note that colistin has never been subjected to contemporary drug development procedures; therefore, there is a significant lack of information regarding its pharmacological profile (28, 29).

Clinically, colistin is administered parenterally as sodium colistin methanesulfonate (CMS), an inactive prodrug; CMS is converted in vivo to colistin, the active antibacterial which is more toxic than CMS (4, 28, 29). Administration of CMS has been associated with nephrotoxicity in experimental animals and humans (8, 10, 11, 14, 19, 20, 22, 48). Nephrotoxicity rates in patients receiving currently recommended CMS dosage regimens are often ~45 to 55% (8, 10, 11, 14, 19, 20, 22). Although the mechanism of colistin-induced nephrotoxicity still remains unknown, it appears to be related to total dose of CMS and duration of therapy and is usually reversible upon cessation of therapy (14, 48). Notwithstanding its reversibility, nephrotoxicity is currently a major dose-limiting adverse effect impacting the clinical use of CMS. Our recent clinical pharmacokinetic and pharmacodynamic data strongly suggest that even CMS daily doses at the upper limit of the current product-recommended dosage range are suboptimal in many patients (12, 33), a situation which is destined to deteriorate with diminishing bacterial susceptibility to colistin (1, 2). Therefore, there is an urgent need to investigate approaches to ameliorate colistin-induced nephrotoxicity, thereby widening the therapeutic window to allow administration of higher doses of CMS.

Melatonin (N-acetyl-5-methoxytryptamine) has been shown to abate the renal toxicity induced by a wide range of drugs that may cause oxidative stress in kidney cells, including gentamicin, amikacin, vancomycin, and cisplatin (5, 36, 38, 43, 44). A hormone secreted by the pineal gland, melatonin exhibits substantial antioxidant activity, especially as a scavenger of hydroxyl radicals (40, 41). Oxidative stress has been suggested to have a central role in nephrotoxicity caused by many drugs, including gentamicin; mitochondrial reactive oxygen species (ROS) are able to damage many cellular macromolecules (e.g., proteins and nucleic acids) which can lead to cell death (31). Considering the safety profile of melatonin (42, 46) and its ability to reduce damage due to other nephrotoxic drugs (5, 36, 38, 41, 43, 44), our study was designed to examine whether melatonin would protect against the nephrotoxicity of colistin.

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MATERIALS AND METHODS

Chemicals and reagents. Colistin sulfate was from Zhejiang Shenghua Biology Co., Ltd. (EPS grade; Zhejiang, China). A stock solution (10 mg/ml colistin base) was prepared in sterile normal saline, stored at 4°C, and used within the stability window for colistin (25). Melatonin, trichloroacetic acid, 9-fluorenylmethyl chloroformate (FMOC-Cl), p-nitrophenol, and p-nitrophenyl N-acetyl-β-D-glucosaminide were from Sigma-Aldrich (Castle Hill, New South Wales, Australia). All other chemicals were of analytical grade.
Animals. The present study was approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee, Monash University (Parkville, Victoria, Australia). Male Sprague-Dawley rats (mean body weight ± the standard deviation [SD], 284 ± 22 g) were obtained from Monash Animal Services (Clayton, Victoria, Australia). Rats were housed individually in metabolic cages in a temperature- and humidity-controlled facility with a 12-h light-dark cycle and had free access to food and water. After 2 days of acclimation, each rat had a cannula inserted in the jugular vein and in the carotid artery.

Effect of melatonin on colistin-induced nephrotoxicity and colistin pharmacokinetics. In preliminary studies, a total of four different dosage regimens were evaluated for ability to cause colistin-induced nephrotoxicity. Two of the regimens involved twice-daily (8 h apart) intravenous administration of colistin for 4 days with cumulative doses of 5.25 and 12.0 mg/kg; these regimens did not lead to reproducible renal damage based upon biochemical evaluation (plasma creatinine and urinary N-acetyl-β-D-glucosaminidase [NAG] excretion) and histological examination. The third regimen with a cumulative dose of 39.0 mg/kg over 7 days led to severe kidney lesions in all animals; many of these lesions were considered irreversible based upon the histology. The fourth and final regimen involved administration of increasing doses of colistin twice daily (8 h apart) for 7 days with a cumulative dose of 36.5 mg/kg. Specifically, this regimen was as follows: 0.5 and 1.0 mg/kg on day 1, 1.25 and 1.25 mg/kg on day 2, 1.75 and 2.75 mg/kg on day 3, and 3.40 and 4.00 mg/kg on day 4. The nature and extent of kidney damage caused by this regimen, including the likelihood of its reversibility, were considered representative of colistin-induced nephrotoxicity in patients (14). For this reason, this regimen was used to examine the possible protective effect of melatonin.

Rats were divided into four groups (n = 7 each) and dosed via the jugular vein twice daily for 7 days with (i) saline (control group), (ii) colistin alone per the fourth regimen described above (colistin group), (iii) melatonin at 5 mg/kg (melatonin group), and (iv) melatonin at 5 mg/kg 20 min prior to each colistin dose (colistin-melatonin group); the twice-daily doses of saline, colistin, and melatonin, where relevant, were administered 8 h apart. Urine was collected into a chilling chamber at 4°C for 24-h intervals 3 days prior to commencing the treatments (baseline) and on days 1, 3, 5, and 6 thereafter. After the volume was measured, aliquots were stored at −80°C pending analysis for NAG excretion and unchanged colistin. Blood (0.3 ml) was collected via the carotid artery prior to initiating the treatments, at 30 min after the morning dose on days 1, 3, and 6, and at the time the rats were sacrificed on day 7. Plasma samples were stored at −80°C until quantification of colistin; plasma creatinine was measured at baseline and at day 6. The right kidneys were fixed in 10% neutral buffered formalin for histological quantification of colistin; plasma creatinine was measured at baseline and at day 6. The left kidneys were stored at −80°C for measurements of colistin and superoxide dismutase (SOD).

In addition, to examine the effect of melatonin on the pharmacokinetics of colistin, blood was collected prior to and at 10, 30, 60, 90, 120, 180, and 360 min after the first dose of colistin (0.5 mg/kg) from animals in the colistin and colistin-melatonin groups (n = 5 for each group). Urine was collected as described above for 6 h after colistin administration.

Biochemical evaluation and histological examination for colistin-induced nephrotoxicity. Animals were quantified as described previously (16); replicate analysis (n = 5) of a quality control containing 20.3 U/liter NAG returned a value of 20.5 ± 1.6 U/liter. Commercial kits were used to quantify the plasma creatinine (Bioassays systems, Hayward, CA) and SOD activity in the kidney homogenate (Cayman Chemical, Ann Arbor, MI). For the plasma creatinine assay, the accuracy and reproducibility were 0.26 ± 0.04 and 0.50 ± 0.04 mg/dl (n = 5) for the quality control samples containing 0.25 and 0.50 mg/dl, respectively. For the SOD assay, the accuracy and reproducibility were 0.14 ± 0.02 and 1.20 ± 0.06 U/g (n = 5) for the quality control samples containing 0.15 and 1.20 U/g, respectively.

The formalin-fixed kidneys were embedded in paraffin; sections (5 μm) were mounted on glass slides and counterstained with hematoxylin, eosin, and periodic acid-Schiff. The samples were examined by a pathologist (P. A. Hill) who was blinded to the treatment groups. Lesions were rated as follows: grade 1, mild acute tubular damage with tubular dilation, prominent nuclei, and a few pale tubular casts; grade 2, severe acute tubular damage with necrosis of tubular epithelial cells and numerous tubular casts; and grade 3, acute cortical necrosis/ infarction of tubules and glomeruli with or without papillary necrosis. The grades were given the following scores: grade 1 = 1, grade 2 = 4, and grade 3 = 10. The percentages of the kidney slices affected were scored as follows: <1% = 0, 1 to <5% = 1, 5 to <10% = 2, 10 to <20% = 3, 20 to <30% = 4, 30 to <40% = 5, and ≥40% = 6. The overall score was calculated as the product of percentage score and grade score. Finally, a semiquantitative score (SOS) for renal histological changes was assigned as follows: SOS 0 = no significant change (overall score, <1); SOS 1 = mild damage (overall score, 1 to <15); SOS 2 = mild to moderate damage (overall score, 15 to <30); SOS 3 = moderate damage (overall score, 30 to <45); SOS 4 = moderate to severe damage (overall score, 45 to <60); and SOS 5 = severe damage (overall score, 60).

HPLC analysis of colistin. The concentrations of colistin in plasma, urine, and kidneys were determined by high-pressure liquid chromatography (HPLC) (18, 24). Minor modifications for pretreatment of kidney samples involved homogenization (Kinematica polytron PT-DA 3007/2EC homogenizer; Kinematica, Luzern, Switzerland) in 5 ml of 20 mM ice-cold HEPES buffer (pH 7.2) per g of tissue, followed by ultrasonication for 10 s (Sonics VibraCell VCX500). Calibration curves in all matrices were constructed with colistin base concentrations ranging from 0.125 to 16.0 μg/ml. For quality control samples containing 0.50 and 4.00 μg of colistin/ml (n = 6), the accuracy and reproducibility were 0.49 ± 0.02 μg/ml and 4.04 ± 0.06 μg/ml for plasma, 0.51 ± 0.03 μg/ml and 3.92 ± 0.04 μg/ml for urine, and 0.51 ± 0.02 μg/ml and 3.98 ± 0.17 μg/ml for kidney homogenate. Samples with concentrations above the calibration range were reanalyzed after appropriate dilution. The limit of quantitation for colistin in plasma, urine, and kidney samples was 0.125 μg/ml.

Data analyses. All continuous variables were tested for normal distribution by using the Shapiro-Wilk test and Leven’s test for homogeneity of variance among groups. For plasma creatinine and SOD activity, the groups were compared by using one-way analysis of variance (ANOVA). For urinary NAG excretion, repeated-measures ANOVA was used. If significant differences were found (P < 0.05), the treatment groups were compared by using Tukey’s test. For the histological scores, Kruskal-Wallis one-way ANOVA by ranks was conducted. Colistin pharmacokinetics in plasma was analyzed using noncompartmental module 201 in WinNonLin (version 5.2; Pharsight Corp., Cary, NC); unpaired t test was used to compare the pharmacokinetic parameters between the colistin and colistin-melatonin groups. A P value of <0.05 was considered significant.

RESULTS

The 24-h urinary NAG excretion for the control, colistin, melatonin, and colistin-melatonin groups is illustrated in Fig. 1. No significant difference in urinary NAG excretion at baseline was observed among all four groups. There was a significant increase (P < 0.001) in urinary NAG excretion in the colistin group on days 3, 5, and 6 relative to baseline, and the excretion on days 1, 3, 5, and 6 for this group was significantly higher (P < 0.0001) than for the other groups on those respective days. Consistent with the urinary NAG excretion data, the plasma creatinine increased significantly (P = 0.023) only in the colistin group from 0.30 ± 0.11 mg/dl at baseline to 0.42 ± 0.10 mg/dl on day 6. Significant histological abnormalities (P < 0.0001) were detected in the kidneys of the colistin group that
were not present in the control, the melatonin, or colistin-melatonin group (Table 1 and Fig. 2). There was no significant difference among the groups in SOD activity in the kidneys on day 7 ($P > 0.11005$, data not shown).

Figure 3 shows the mean plasma colistin concentration-time profiles after the first intravenous dose of colistin (0.5 mg/kg) either alone or preceded by melatonin at 5 mg/kg ($n = 5$ in each group). The plasma colistin concentrations at 360 min in all rats in the colistin group were below the limit of quantification of the assay (0.125 μg/ml).

### DISCUSSION

Potential for nephrotoxicity is a major dose-limiting factor with currently recommended dosage regimens of CMS and colistin (7, 23, 29, 34). Based upon data from our recent phar-

#### TABLE 1. Renal lesions determined by histological examination

<table>
<thead>
<tr>
<th>Type of abnormality $^a$</th>
<th>% Kidney slice affected in individual rats in each group ($n = 7$)</th>
<th>Control</th>
<th>Colistin</th>
<th>Melatonin</th>
<th>Colistin-melatonin</th>
</tr>
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<tbody>
<tr>
<td>Grade 1</td>
<td>5, 0, 0, 0, 0, 0, 0</td>
<td>40, 20, 50, 0, 20, 5, 0</td>
<td>0, 0, 0, 0, 0, 0, 0</td>
<td>0, 0, 0, 0, 0, 0, 0</td>
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<tr>
<td>Grade 2</td>
<td>0, 0, 0, 0, 0, 0, 0</td>
<td>0, 10, 10, 20, 0, 0, 10</td>
<td>2, 0, 0, 0, 0, 0, 0</td>
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<tr>
<td>Grade 3</td>
<td>0, 0, 0, 0, 0, 0, 0</td>
<td>0, 10, 10, 15, 10, 0, 30</td>
<td>0, 0, 0, 0, 0, 0, 0</td>
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</tr>
<tr>
<td>SQS $^b$</td>
<td>+1, 0, 0, 0, 0, 0, 0</td>
<td>+1, +4, +2, +4, +3, +1, +5</td>
<td>+1, 0, 0, 0, 0, 0, 0</td>
<td>0, 0, 0, 0, 0, +1, 0</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Grade 1, mild acute tubular damage with tubular dilation, prominent nuclei, and a few pale tubular casts; grade 2, severe acute tubular damage with necrosis of the tubular epithelial cells and numerous tubular casts (acute tubular necrosis); grade 3, acute cortical necrosis/infarction of tubules and glomeruli with or without papillary necrosis.

$^b$ A semiquantitative score (SQS) for individual rats was used to grade lesion severity. Scores of 0, +1, +2, +3, +4, and +5 correspond to no change, mild change, mild to moderate change, moderate change, moderate to severe change, and severe change, respectively.

Figure 2. Representative photomicrographs of normal renal cortex (A), rat kidney from the colistin group with grade 1 lesions, mild acute tubular damage with tubular dilatation, prominent nuclei, and a few pale tubular casts (B), rat kidney from the colistin group showing grade 2 lesions with severe acute tubular damage with necrosis of the tubular epithelial cells and numerous tubular casts (C), rat kidney from the colistin group showing grade 3 lesions with acute cortical necrosis with necrosis and/or infarction of tubules and glomeruli (D), rat kidney from the colistin-melatonin group in which the renal cortex and medulla appeared normal (E), and rat kidney from the melatonin group without kidney damage (F). G. glomerulus (hematoxylin and eosin stain). Original magnifications: ×200 for panels A, B, C, and D and ×20 for panels E and F.

Figure 3. Mean (±SD) plasma colistin concentration versus time profiles after the first intravenous dose of colistin (0.5 mg/kg) either alone (○) or preceded by melatonin at 5 mg/kg (■) ($n = 5$ in each group). The plasma colistin concentrations at 360 min in all rats in the colistin group were below the limit of quantification of the assay (0.125 μg/ml).
Table 2. Pharmacokinetic parameters of colistin after the first intravenous dose (0.5 mg/kg) either alone or preceded by 5 mg of melatonin/kg in rats (n = 5 in each group)

<table>
<thead>
<tr>
<th>Group</th>
<th>Pharmacokinetic parameters (mean ± SD) of colistin$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL (ml/min/kg) $ V$ (ml/kg) $ t_{1/2}$ (h)</td>
</tr>
<tr>
<td>Colistin</td>
<td>4.28 ± 0.93</td>
</tr>
<tr>
<td>Colistin-melatonin</td>
<td>1.82 ± 0.26</td>
</tr>
</tbody>
</table>

$^a$ CL, clearance; $ V$, volume of distribution; $ t_{1/2}$, half-life. The $ P$ values for CL, $ V$, and $ t_{1/2}$ were 0.0005, 0.0036, and <0.0001, respectively.

Macokinetic/pharmacodynamic studies, the plasma concentrations of colistin in many patients may not be optimal with the currently recommended dosage regimens (3, 6, 33). More adequate bacterial killing may be achieved by administration of daily doses of CMS higher than those in the approved product information (33), but this may well be accompanied by increased rates and severity of nephrotoxicity. Amelioration of the potential for nephrotoxicity would serve to widen the therapeutic window and allow administration of higher daily doses for increased exposure to colistin. Given that melatonin is a highly efficient free-radical scavenger that affords protection against the nephrotoxicity induced by a number of drugs (5, 36, 38, 43, 44), the present study was designed to investigate the potential protective effects of melatonin against colistin-induced renal toxicity.

Colistin, rather than CMS, was used in the present study because it is the active antibacterial drug formed in the body after administration of CMS (4) and is substantially more toxic than CMS (15, 17, 21, 35). In addition, using colistin instead of CMS eliminated the complexity resulting from the simultaneous presence in the body of the prodrug, CMS, and the formed colistin. The renal handling of colistin and CMS are very different; whereas colistin undergoes very extensive renal tubular reabsorption, CMS undergoes renal tubular secretion (26–27). Preliminary studies for developing a rat model for colistin-induced nephrotoxicity revealed that after intravenous administration of a first dose of $ \geq 1$ mg/kg colistin, animals exhibited signs of apparent neurotoxicity, notably muscular weakness, ataxia, and labored respiration. Therefore, ascending colistin daily doses were implemented in all four regimens of the preliminary studies and did not lead to early signs of toxicity. Comparison of the four regimens showed that nephrotoxicity depended on the cumulative dose and duration of treatment, which is consistent with a recent retrospective clinical study (14).

A very important observation in the present study was the protective effect of coadministered melatonin on colistin-induced nephrotoxicity. The dose of melatonin (10 mg/kg/day, divided into two doses) used here was based upon previous studies in rats in which coadministration of melatonin was effective in protecting against renal toxicity of several other nephrotoxic drugs such as gentamicin, vancomycin, and cisplatin (5, 43, 44). It is not known why the nephroprotective effect of melatonin against these drugs has not been examined clinically. A recent study in rats (37) reported that coadministration of N-acetylcysteine seemed to ameliorate CMS-induced oxidative stress in kidney cells; however, the daily dose of the prodrug, CMS, was very low based upon animal scaling considerations (48), and no differences in histological abnormalities were observed between groups, including the CMS group. Our study is the first to demonstrate the protective effect of melatonin against colistin-induced nephrotoxicity. The increase of urinary NAG excretion in the colistin-treated rats, up to 2.4-fold of the baseline on day 6 (Fig. 1), is an indication of tubular cell injury (30). In contrast, in the colistin-melatonin group the insignificant change in urinary NAG excretion across the 6 days indicates that melatonin protected against renal tubular cell damage caused by colistin. As noted above, the daily dose of melatonin used in the present study was the same as that used in previous studies in rats (5, 43, 44). Although melatonin has been shown to have a favorable safety profile (42, 46), further investigation is warranted in order to determine whether lower doses of melatonin show a nephroprotective effect in our rat nephrotoxicity model.

Since melatonin is able to protect cell function against ROS damage (47), it is very likely that oxidative stress has a key role in colistin-induced toxicity. The urinary NAG results were in keeping with those of the histological examination, wherein the colistin group showed considerable renal damage that was essentially absent in the colistin-melatonin group (Fig. 2 and Table 1). All rats in the colistin group showed histological changes with some variation in the severity and extent of changes. Only one rat in each of the other groups (control, melatonin, and colistin-melatonin) showed mild focal tubular changes. The changes in the colistin group included acute tubular necrosis and acute cortical necrosis. The mechanisms of colistin-induced nephrotoxicity are not well understood. The focal nature of the changes and the cortical necrosis/infarction seen in the kidneys of the rats in the colistin group raise the possibility of a role for ischemia in addition to direct nephrotoxicity in the etiology of the lesions. Interestingly, an interaction of tubular, glomerular, and vascular effects has recently been proposed to be involved in aminoglycoside-induced nephrotoxicity (31). In the present study, there was no histological evidence of vascular injury or thrombosis. It would be impor-
tant to measure blood pressure in future experiments with colistin to exclude hypotension. Acute tubular necrosis is a reversible lesion with tubules able to regenerate over several days. However, acute cortical necrosis leads to irreversible renal damage with cortical scarring. The extent of the cortical necrosis will determine the severity of the chronic renal failure (39).

To our knowledge, the present study is the first to examine the effect of melatonin on the pharmacokinetics of nephrotoxic drugs, such as gentamicin, amikacin, vancomycin, and cisplatin (5, 38, 43, 44). The pharmacokinetics of colistin after administration of the first dose (0.5 mg/kg) alone in rats (Table 2) were similar to those previously reported (26). Interestingly, prior administration of melatonin (5 mg/kg) resulted in a significant alteration in the pharmacokinetics of colistin (Fig. 3 and Table 2). The significant decrease in total body clearance and the significant increase in the half-life of colistin support the proposition that melatonin may protect colistin from degradation by free radicals generated in metabolic processes. It is well known that amino acids and peptides are subject to degradation mediated by free radicals (13, 45). The impact of free radicals on the stability of colistin is under examination in our laboratory. After multiple coadministration of melatonin (10 mg/kg/day) and colistin for 7 days, the mean plasma concentration of colistin at the time of sacrificing the animals (2.99 ± 0.93 μg/ml) was ~2.5-fold higher than that in the colistin group (1.20 ± 0.90 μg/ml); this is in keeping with the pharmacokinetic differences between the groups (Table 2) discussed above. After glomerular filtration, colistin undergoes very extensive renal tubular reabsorption involving carrier-mediated pathways (26, 32), which is consistent with the fact that in the present study colistin was not quantifiable in urine on day 1. Subsequently, however, there was a very substantial increase in the urinary recovery of unchanged drug in the colistin group but not in the colistin-melatonin group; on days 3, 5, and 6, the urinary recovery in the colistin group was significantly higher than that in the colistin-melatonin group (Fig. 4). These differences across days 3 to 6 most likely occurred due to renal tubular damage caused by colistin, which decreased the ability of the kidney to undertake carrier-mediated reabsorption of the filtered colistin; the coadministration of melatonin afforded protection. The mechanism whereby melatonin modifies the pharmacokinetics, including renal handling, of colistin is under further investigation in our laboratory.

In conclusion, this is the first study to demonstrate that melatonin provides protection against colistin-induced nephrotoxicity. The unexpected effect of melatonin on the clearance of colistin may suggest an important role for free radical-mediated processes in the metabolism of colistin, a possibility that is being pursued. Further investigations are underway in our laboratory to elucidate the mechanisms of colistin-induced nephrotoxicity and protection by melatonin. The present study highlights the prospect of coadministering an antioxidant to ameliorate colistin-induced nephrotoxicity and thereby widen the therapeutic window of this important last-line antibiotic.

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