Cell cycle arrest in a model of colistin nephrotoxicity

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Eadon MT, Hack BK, Alexander JJ, Xu C, Dolan ME, Cunningham PN. Cell cycle arrest in a model of colistin nephrotoxicity. Physiol Genomics 45: 877–888, 2013. First published August 6, 2013; doi:10.1152/physiolgenomics.00076.2013.—Colistin (polymyxin E) is an antibiotic prescribed with resurfacing frequency for multidrug resistant gram negative bacterial infections. It is associated with nephrotoxicity in humans in up to 55% of cases. Little is known regarding genes involved in colistin nephrotoxicity. A murine model of colistin-mediated kidney injury was developed. C57/BL6 mice were administered saline or colistin at a dose of 16 mg/kg/day in 2 divided intraperitoneal doses and killed after either 3 or 15 days of colistin. After 15 days, mice exposed to colistin had elevated blood urea nitrogen (BUN), creatinine, and pathologic evidence of acute tubular necrosis and apoptosis. After 3 days, mice had neither BUN elevation nor substantial pathologic injury; however, urinary neutrophil gelatinase-associated lipocalin was elevated (P = 0.017). An Illumina gene expression array was performed on kidney RNA harvested 72 h after first colistin dose to identify differentially expressed genes early in drug treatment. Array data revealed 21 differentially expressed genes (false discovery rate < 0.1) between control and colistin-exposed mice, including LGALS3 and CCNB1. The gene signature was significantly enriched for genes involved in cell cycle proliferation. RT-PCR, immunoblot, and immunostaining validated the relevance of key genes and proteins. This murine model offers insights into the potential mechanism of colistin-mediated nephrotoxicity. Further studies will determine whether the identified genes play a causative or protective role in colistin-induced nephrotoxicity.

Colistin; polymyxin E; nephrotoxicity; cell cycle; renal failure; galecin-3

ACUTE KIDNEY INJURY (AKI) is a common complication of hospital admissions, with modest kidney injury occurring in 13% of admissions (defined as a rise in serum creatinine > 0.5 mg/dl) and severe AKI in 1% of all admissions (creatinine rise > 2 mg/dl) (6). Even modest changes in serum creatinine are associated with increased mortality, length of stay, and healthcare costs (6). Data from the PICARD study indicate nephrotoxicity contributes to 26% of all cases of AKI (48) in the critical care setting.

Nosocomial infections with drug-resistant bacteria are an increasing problem. Colistin is an antibiotic used to treat resistant gram negative infections (16). It is administered in clinical settings as the anionic prodrug colistin methanesulfonate sodium (CMS), which undergoes hydrolysis to the cationic colistin (41). The use of colistin is increasing in the intensive care unit setting for treatment of Acinetobacter baumannii and Pseudomonas aeruginosa (40, 59). While the incidence of nephrotoxicity associated with colistin use has decreased from older estimates as high as 65% (17), rates of nephrotoxicity still range from 8 to 55% in recent clinical studies (14, 22, 35, 53, 58, 59). When corrected for confounding factors such as anemia, sepsis, and hypotension, the odds ratio for AKI with colistin is 3.31 compared with other nonnephrotoxic antibiotics (53).

Clinical predictors of colistin nephrotoxicity have been identified (34, 37), but there is presently a lack of mechanistic understanding of colistin nephrotoxicity. Gene expression signatures have been explored in rats for established models of gentamicin, cisplatin, and puromycin nephrotoxicity (1, 9). Rat models of colistin nephrotoxicity have been successfully developed with intravenous administration (62); however, other models have proved less successful after intramuscular administration with low doses (21). This may be related to the decreased conversion of CMS to colistin in rats compared with humans (8, 38). Several authors have suggested an oxidative mechanism of rat kidney injury, as both ascorbic acid (67) and melatonin (68) have ameliorated toxicity in this model. However, the gene expression signature associated with colistin nephrotoxicity has not been explored. Surprisingly, we failed to identify prior studies specifically evaluating nephrotoxicity in a murine model of colistin nephrotoxicity, although several studies assessed LD50 or renal function as part of global toxicity in mice (13, 20, 42, 63).

In this report we characterize a murine model of colistin nephrotoxicity associated with twice daily intraperitoneal injection of colistin sulfate. Because little was known about the pathogenesis of colistin nephrotoxicity, we performed a gene expression array early in the course of disease in an attempt to gain insights into the mechanism of injury. This approach revealed many differentially expressed genes (DEGs) in mice receiving colistin, which yield a cohesive and informative transcriptional signature.

METHODS

Administration of colistin to induce kidney injury in mice. We obtained 32 male C57BL/6 mice commercially and studied them at 8 wk of age (Charles River Laboratories, Boston, MA). Twenty mice were administered intraperitoneal injections of colistin sulfate (Sigma, St. Louis, MO) 16 mg/kg/day in twice daily divided doses for up to 15 consecutive days, in an effort to replicate a typical 2 wk clinical course of antibiotics. Each dose was mixed in 0.125 ml sterile water
and filtered (filter SLGG013SL; Millipore, Billerica, MA). Twelve control mice received intraperitoneal injections of 0.1% saline BID. Blood was collected on day 0, 3, 7, and 15 from mice. Nineteen mice \((n = 7\) control, \(n = 12\) colistin) were killed on day 3. Thirteen mice were killed on day 15 \((n = 5\) control, \(n = 8\) colistin). All animals were killed the morning of day 3 or 15 starting at approximately 9 AM. Blood and renal tissue were harvested at death. For clarity, groups were defined as D3 (mice killed after 6 doses or 72 h after first colistin dose), D15 (mice killed after 360 h and 30 colistin doses), or control (receiving saline). No biochemical or histologic difference could be discerned between control mice killed on day 3 and 15. Control mice killed on day 3 were analyzed for purposes of mRNA and protein expression.

Blood urea nitrogen (BUN) concentrations were determined with a Beckman CX5CME autoanalyzer. Creatinine concentrations were determined by quantitative colorimetric determination (Stanbio Creatinine procedure no. 0430, Boerne, TX). This project was approved by the Institutional Animal Care and Use Committee of the University of Chicago.

Dose selection and titration. In addition to the 32 C57BL/6 mice described above, 32 additional C57BL/6 mice were used in dose titration studies of 5 mg/kg/day \((n = 10)\), 10 mg/kg/day \((n = 10)\), and 32 mg/kg/day \((n = 12)\). These 32 mice were not used in subsequent analyses. Mice receiving 5 or 10 mg/kg/day \((n = 10\) of each) failed to achieve consistent elevation of BUN or pathologic injury by light microscopy when followed for over 1 wk. Mice receiving 32 mg/kg/day became sluggish after three doses of colistin. Three of twelve mice died before the fourth dose, we speculate, from neurotoxicity. Necropsy did not reveal an obvious cause of death, and all 12 mice were euthanized. The authors ultimately selected a 2 wk regimen of 16 mg/kg/day as it caused reproducible kidney injury. Duration of colistin therapy varies in humans depending on the type and severity of infection. The 15-day regimen was selected to approximate a 2-wk infection. The 15-day regimen was selected to approximate a 2-wk infection. The 15-day regimen was selected to approximate a 2-wk infection. The 15-day regimen was selected to approximate a 2-wk infection. The 15-day regimen was selected to approximate a 2-wk infection. The 15-day regimen was selected to approximate a 2-wk infection. The 15-day regimen was selected to approximate a 2-wk infection. The 15-day regimen was selected to approximate a 2-wk infection.

Urinary collection. Urine was collected noninvasively from control and D3 mice on day 0, 1, and 2 by placing mice on a weighing tray and patiently waiting. Urine neutrophil gelatinase-associated lipocalin (NGAL) and creatinine were measured immediately following collection by ELISA (E90-H11005, Bethyl Laboratories, Montgomery, TX). This was accomplished with proper RNA precautions and prior to the tissue thawing. Thus, tissue samples from each mouse were size-matched, centrally located, transverse slices. RNA was isolated from frozen kidney tissue \((n = 6\) for control and D3) with Trizol (GIBCO BRL, Grand Island, NY) according to the manufacturer’s instructions and further purified with the RNEasy Plus mini kit with gDNA digestion (Qiagen, Valencia, CA). RNA was run on a Bioanalyzer (Agilent 2100) by the University of Chicago Functional Genomics Facility and determined to be satisfactory quality. We randomized and independently hybridized 12 samples to two Illumina gene expression microarray chips (MouseWG-6, 48,000 probes, RefSeq plus EST). As each chip holds six samples, three control and three treated samples (D3) were hybridized to each chip. Thus, samples were not pooled. Of note, the D3 mouse with the highest NGAL was considered an outlier and excluded from the array and RT-PCR. Data were deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (GSE46410).

Array analysis. Gene expression profiles were generated from kidney samples collected after 3 days of treatment. A total of 12 samples, with six samples in the treated or control group each, were randomized and run on Illumina MouseWG-6 Expression Beadchips, which contains 48,000 probes representing 30,854 genes in the mouse genome.

The raw probe intensities were background subtracted and quartile-normalized to adjust for variation that arose from microarray technology and not from biological differences. The normalized probe signals were then summarized into gene-level expression. Signals of multiple probes that represent the same gene were averaged to obtain a gene expression profile in GenomeStudio and exported as a tab-delimited txt file. The file was imported into Partek with minimum value shifted to 1.0 and log2 transformed.

Genes that were not expressed in any sample (nonexpressed genes, NGEs, identified by detection \(P \text{ value} \geq 0.05\)) were removed from further studies to improve the sensitivity of differential gene expression detection. GenomeStudio calculates and reports a detection \(P\) value, which represents the confidence that a given transcript is expressed above the background defined by negative control probes. This detection score determines whether a transcript on the array is called detected. A value below the user-defined \(P\) value threshold of either 0.01 or 0.05 indicates a gene is detected. Therefore, genes with a detection \(P\) value < 0.05 represent those that are expressed in samples. If a gene is expressed in at least one of the 12 samples, the gene was kept for final output; if a gene is not expressed in any sample, it was excluded from subsequent analysis. After this prefiltering step, 16,410 out of 30,854 genes (53.2%) were kept for DEG detection in Partek.
Principal component analysis mapping of gene expression suggests that samples from the same chip are likely to group together, although no obvious clusters were observed. We used two-way ANOVA to detect DEGs between treated and control groups, taking into consideration the possible chip effects in evaluation of gene expression changes. In this experimental design, control and treated samples are randomized on each array. Therefore, the chip side-effects were detected by ANOVA and BeadChip was set as the random effect factor. This way, ANOVA determined whether the variations of gene expression observed between conditions (treated vs. control) result from the colistin treatment or from the chip or depleted in candidate gene lists were identified with BinGO 2.44 (http://www.psb.ugent.be/cbd/papers/BiNGO/Home.html) in the Cytoscape platform 2.8.3 (http://www.cytoscape.org/). The latest ontologies and mouse gene annotations were downloaded from the Gene Ontology (GO) official website (http://www.geneontology.org/, 10/26/2012) and used as the ontology file and the organism annotation file in BinGO. GO enrichment lists were generated for a gene list with FDR P value < 0.2 (86 genes), ranked by FDR-corrected P values. The full list of genes present on the array after removing NEGs across all samples (16,410 genes) was set as the background gene list. 

DAVID pathway enrichment analysis. Enriched or depleted pathways in the candidate gene list of FDR P value < 0.2 (86 genes) were identified with DAVID (http://david.abcc.ncifcrf.gov/) and its functional annotation tool (KEGG_PATHWAY) (27, 28). The full list of acceptable analysis techniques (5). The imposition of a fold-change threshold would have improved our positive predictive value but lowered our sensitivity.

BinGO enrichment analysis. Functional categories that are enriched or depleted in candidate gene lists were identified with BinGO 2.44 (http://www.psb.ugent.be/cbd/papers/BiNGO/Home.html) in the Cytoscape platform 2.8.3 (http://www.cytoscape.org/). The latest ontologies and mouse gene annotations were downloaded from the Gene Ontology (GO) official website (http://www.geneontology.org/, 10/26/2012) and used as the ontology file and the organism annotation file in BinGO. GO enrichment lists were generated for a gene list with FDR P value < 0.2 (86 genes), ranked by FDR-corrected P values. The full list of genes present on the array after removing NEGs across all samples (16,410 genes) was set as the background gene list.

### Table 1. Primers for RT-PCR

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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>TTATGCGCTTGGCTTACAGG</td>
<td>CATATGGTCCTCTGCTCA</td>
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<td>PRC1</td>
<td>GGATATGCTTACAGACAGATG</td>
<td>TCTCTGCTTGGGTACGCT</td>
</tr>
<tr>
<td>LCN2</td>
<td>AGGAGATCATAACGCTTACGCT</td>
<td>CTTGTCGCTCGATGAGCG</td>
</tr>
<tr>
<td>LGALS3</td>
<td>TGTATGAGACTTGTCTGAGA</td>
<td>CGGAAGATGTCAGGACAG</td>
</tr>
<tr>
<td>CCNB1</td>
<td>TTCTGTATTAGCGACACCT</td>
<td>ACTCTGTTCTGAGTCATG</td>
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<td>PBK</td>
<td>GACATAGTGCTGACACGCC</td>
<td>CAATCAGTTGTCAGGCA</td>
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<td>SLC24A3</td>
<td>TGCGATACACTGGAACCTGGA</td>
<td>GCCTAGAGGAAAGATGCTC</td>
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<td>PVALB</td>
<td>GTGGTGGAGCGGTATGTTCTG</td>
<td>AGAACGGACTTGCCCTCAT</td>
</tr>
<tr>
<td>18S</td>
<td>GACCTGGATCAGAGAGAC</td>
<td>GACCATCAGTTGTCAGGCA</td>
</tr>
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</table>

Fig. 1. Weight and biochemical changes after colistin administration. A: D3 mice lost an average of 7.41 ± 1.36% body wt over 3 days. D15 mice lost 6.40 ± 2.30% over 15 days. B: composite of BUN levels in D3 and D15 mice. Average blood urea nitrogen (BUN) was 28.7 ± 0.9 mg/dl at baseline, 28.6 ± 2.4 mg/dl on D3, 43.9 ± 5.4 mg/dl on D7; and 43.4 ± 4.1 mg/dl on D15. C: composite of serum creatinine levels in D3 and D15 mice. Average creatinine at baseline was 0.18 ± 0.02 mg/dl, 0.17 ± 0.03 mg/dl on D3, and 0.30 ± 0.06 mg/dl on D15. D: urine neutrophil gelatinase-associated lipocalin (NGAL)-to-creatinine ratio increased from baseline of 60.3 to 396 ng/mg in D3 mice. E: urine albumin-creatinine ratio average increased from 15.8 to 94.0 µg/mg in D15 mice. *P ≤ 0.05 compared with baseline.
genes present on the array after removing NEGs across all samples (16,410 genes) was set as the background gene list.

Quantitative real-time PCR. RNA was isolated and purified from frozen kidney tissue as previously described (64) (n = 6 per group). A 30 μg central transverse slice was taken from a distinct half-kidney for RNA isolation, different from that used in the array. Should two separate slices of renal tissue have the same pattern of DEGs, the authors felt this would control for sampling error and increase the strength of the data. This also controls, in part, for discrepancies between the proportion of cortex and medulla in any given sample. cDNA was generated from RNA using the iScript Advanced cDNA synthesis kit (Bio-Rad, Hercules, CA), according to the manufacturer’s instructions, and diluted fivefold before analysis. Real-time PCR was performed using the Applied Biosystems 7900 system (Foster City, CA) and SybrGreen universal mix (Bio-Rad). Each reaction was conducted in triplicate in a total volume of 20 μl with primers at 200 nM, 1 mM dNTPs, 3 mM MgCl₂, and 4 μl of sample or standard cDNA. PCR was carried out with a hot start at 95°C (10 min) followed by 40 cycles at 95°C (15 s)/57°C (60 s) and a disassociation step. Data were analyzed with standard curves and normalized to 18S expression as previously described (15). Primers were designed using Primer3 and BLAST and synthesized by Invitrogen (Camarillo, CA), with sequences provided in Table 1. Of note, the D3 mouse with the highest urine NGAL and the D15 mouse, with 10 fields per mouse) and expressed in terms of intensity on a semiquantitative scale of 0–3 (0 = none, 1 = weakly positive, 2 = positive, 3 = strongly positive) (2).

Immunohistochemistry. Paraffin-embedded sections were stained using an antibody to galectin-3 (1:200 dilution; Proteintech, Chicago, IL), proliferating cell nuclear antigen (PCNA, 1:100; Proteintech), megalin (1:100; Santa Cruz, Dallas, TX), or sodium-chloride thiazide channel (NCC, 1:1,500, a gift from the Benjamin Ko laboratory) (15) according to Proteintech’s instructions including the optional antigen retrieval step. Tubular staining intensity was scored (minimum n = 5 mice, with 10 fields per mouse) and expressed in terms of intensity on a semiquantitative scale of 0–3 (0 = none, 1 = weakly positive, 2 = positive, 3 = strongly positive) (2).

Immunofluorescence and spinning disk confocal microscopy. Frozen sections were fixed as previously described (15). Sections were incubated overnight at humidified 4°C with a primary polyclonal rabbit antibody to cyclin B1 (1:30 dilution) in sterile 0.3% BSA/PBS. Sections were washed and incubated for 2 h with secondary antibody but without primary antibody. Subsequently, sections were washed and incubated for 2 h with donkey anti-rabbit Alexa-fluor 647 (1:100). Control slides with secondary antibody but without primary antibody were prepared to

![Fig. 2. Light microscopy and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Representative photos of light microscopy of renal cortex in control (A), D3 (B), and D15 (C) mice (×20). Examples of intratubular casts are provided (white asterisk). D: significant tubular dilatation, degeneration, and cast formation was observed in D15 mice. Representative high-power photos of light microscopy of renal cortex in control (E), D3 (F) mice (×40). Examples of injured cells are given (black arrow). G: a significant increase in necrotic cells were observed in D3 mice. H: injury of both proximal and distal convoluted tubules was observed in D15 mice; however, injured distal tubules were more frequently observed. Distal tubules are labeled with sodium-chloride thiazide channel (brown), and examples are marked (white arrow). Representative photos of TUNEL staining in control (I), D3 (J), and D15 (K) mice. Examples of apoptotic cells are given (black arrow). L: a significant increase in apoptotic cells was observed in mice receiving colistin. *Adjusted P ≤ 0.05. ×20 Measurement bar = 100 μm; ×40 measurement bar is 50 μm; n = 5 for all groups, 10 images scored per mouse.](http://physiolgenomics.physiology.org/Downloaded from 10.220.32.246 on July 11, 2017)
evaluate nonspecific staining and autofluorescence. Sections were washed and counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1,000 dilution; Invitrogen, Carmaillo, CA) for 5 min. Slides were mounted with Fluorogel (Biomedia, Hatfield, PA). Slides were observed and scored using an Olympus spinning disk confocal microscope at ×20 and ×60 magnification. Images were compiled by integration of images gathered at a z-axis increment of 0.2 μm using the accompanying software. Alexa 647 fluorescence exposure was standardized at 300 ms for ×60 and 200 ms for ×20, DAPI 350 exposure was 7 ms. Immunofluorescence intensity was scored analogously to immunohistochemistry methodology given above.

Statistics. Statistical analysis was performed using the SigmaStat 10.0 software package (Systat, San Jose, CA), and graphs were prepared using Graphpad Prism 4.0 (La Jolla, CA). Unless noted otherwise, data are given as means ± SE. Groups were compared by two-tailed t-test or ANOVA with Holm-Sidak correction (for more than two groups). Adjusted P values < 0.05 were considered significant and are provided in the text. For interest, occasional unadjusted P values of nominally significant comparisons are provided and clearly denoted.

RESULTS

Characterization of a murine model of colistin nephrotoxicity. After the initial dose titration outlined in the methods, C57BL/6 mice received up to 15 days of colistin at a dose of 16 mg/kg/day in twice daily divided doses. Groups were defined as D3 (mice killed 72 h after first colistin dose), D15 (mice killed 15 days after first colistin dose), or control (receiving saline). C57BL/6 mice lost weight during their colistin regimen (Fig. 1A). Control mice had nonsignificant weight gain during the experiment (2.9 ± 1.7%, P = 0.49).

BUN did not significantly differ from baseline in D3 mice (Fig. 1B, P = 0.96). BUN was elevated on day 7 and upon death of D15 mice (P = 0.0011, P = 0.000067, respectively). There was variability of BUN measures, as a minority of mice had a higher BUN level on day 7 than day 15. Serum creatinine measures were not significantly different between baseline and day 3 but were elevated at day 15 compared with baseline (Fig. 1C, 69% increase, P = 0.019). In D3 mice, urinary NGAL-to-creatinine ratios at death were significantly elevated compared with baseline (Fig. 1D, P = 0.017). As a further index of renal injury, urinary albumin-to-creatinine ratios were collected and not significantly different from baseline in D3 mice but elevated in D15 mice (Fig. 1E, P = 0.053). Most mice were not

### Table 2. DEGs

<table>
<thead>
<tr>
<th>DEGs</th>
<th>Gene Name</th>
<th>Ratio of Treated: Control</th>
<th>Unadjusted P Value</th>
<th>Adjusted FDR*</th>
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<tr>
<td>RRM2</td>
<td>ribonucleotide reductase M2</td>
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<td>2.50E-07</td>
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<td>MNS1</td>
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*All 21 differentially expressed genes (DEGs) with false discovery rate (FDR) < 0.1 are included in order of significance. A total of 65 DEGs had an FDR between 0.1 and 0.2, of which only SLC24A3, LCN2, and PVALB are included in this table as they were validated with RT-PCR.
in the nephrotic range; this modest proteinuria may represent
deficient tubular uptake of albumin. Urine NGAL and albumin
were not significantly elevated in control mice.

Mice have significant evidence of acute tubular necrosis after
receiving colistin for 15 days and subtle pathologic injury after 3
days. PAS staining and light microscopy were performed for all
mice in the control, D3, and D15 groups (Fig. 2, A–D).
Occasional intratubular casts and degenerating cells (apoptotic
or necrotic) were seen in D3 mice; however, the difference in
histology between the control and D3 groups was nonsignifi-
cant at ×20 magnification. High-power (×40) light micro-
scopic examination revealed an absolute increase in the num-
er of necrotic cells per field in D3 mice compared with control
mice (Fig. 2, E–G; 2.3 ± 0.2 vs. 8.6 ± 1.6, P = 0.0069). In
contrast to the subtle pathologic injury observed in D3 mice,
the histology of D15 mice had markedly increased tubular
dilatation, degeneration, and cast formation compared with the
control group (Fig. 2D, adjusted P ≤ 0.05 for each compari-
son). Injury of both proximal and distal convoluted tubules was
observed in D15 mice; however, injured distal convoluted
tubules were more frequently observed (Fig. 2H). No obvious
pathologic changes in the glomeruli or vessels were appreci-
ated on light microscopy. To detect apoptotic cell death,
TUNEL staining was performed in all three groups (Fig. 2,
A nominally significant increase in apoptotic cells was discovered between control and D3 mice (0.2 vs. 1.6 cells per \( \times 20 \) field, unadjusted \( P = 0.036 \)). D15 mice had significantly more apoptotic cells per \( \times 20 \) microscopic field than control mice (6.5 cells per \( \times 20 \) field, \( P = 0.00039 \)). Notably, the distribution of apoptotic nuclei was not diffuse but seemed to cluster in particular tubules.

**Genes are differentially expressed in mice after 3 days of colistin administration.** We sought to understand the renal-specific gene signature associated with subclinical AKI due to colistin administration. D3 mice had no change in serologic indicators of AKI administration. D3 mice had no change in serologic indicators of AKI. D3 mice had a more profound fold change in expression. A detailed account of RT-PCR fold changes and values for each time point is provided in Table 3.

**Colistin nephrotoxicity engages the cell cycle.** PCNA immunohistochemistry and cyclin B1 immunofluorescence were performed for additional evidence, beyond gene signature, of colistin’s impact upon the cell cycle. PCNA is a marker of the DNA synthesis phase of the cell cycle (7). Exposure to colistin markedly increased PCNA staining of tubular cells of D3 and D15 mice (Fig. 6, A–G). Many PCNA-positive nuclei appeared to be significantly enlarged.

**CCNB1 gene expression was significantly elevated in D3 mice (3.11-fold change, \( P = 0.0092 \)) and D15 mice (4.98-fold change, \( P = 0.00028 \)). By immunofluorescence, we did not detect a total change in cyclin B1 tubular staining in D3 mice compared with control (Fig. 7, A–C), and staining was modestly but significantly increased following colistin administration. LGALS3 (galectin-3) is presently the subject of clinical trials as a marker of cardiac fibrosis (24, 61). The role of galectin-3 as a marker of kidney injury has not been fully investigated. LGALS3 mRNA expression was elevated in D3 (2.86 relative fold change to control, \( P = 0.0011 \)) and D15 mice (11.6 relative fold change to control, \( P = 0.0012 \)). Protein expression by immunoblot was increased in D15 mice (Fig. 5, A and B; 3.0 ± 0.4 D15 fold change, \( P = 0.033 \)) but did not reach significance in D3 mice compared with control (2.3 ± 0.5 fold increase, \( P = 0.069 \)). By immunohistochemistry, the proportion and intensity of tubular staining was significantly increased in D3 and D15 mice (Fig. 5, C–F; \( P = 0.000060 \) and \( P = 0.00045 \), respectively). Galectin-3 expression was increased in multiple contiguous cells of some tubules but rarely in isolated individual tubular cells. Expression seemed strongest in distal convoluted tubules based on light microscopic appearance of tubules.

**Table 3. RT-PCR time course with adjusted \( P \) values**

<table>
<thead>
<tr>
<th>Gene</th>
<th>D3 Array Fold Change</th>
<th>D3 RT-PCR Fold Change</th>
<th>D3 RT-PCR ( P ) Value</th>
<th>D15 RT-PCR Fold Change</th>
<th>D15 RT-PCR ( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRM2</td>
<td>2.91</td>
<td>6.01</td>
<td>0.045</td>
<td>3.54</td>
<td>0.0016</td>
</tr>
<tr>
<td>ANLN</td>
<td>2.83</td>
<td>4.53</td>
<td>0.0011</td>
<td>9.71</td>
<td>0.00039</td>
</tr>
<tr>
<td>AURKA</td>
<td>2.00</td>
<td>2.59</td>
<td>0.020</td>
<td>4.58</td>
<td>0.00036</td>
</tr>
<tr>
<td>PRK1</td>
<td>3.45</td>
<td>3.36</td>
<td>0.0025</td>
<td>4.96</td>
<td>0.0036</td>
</tr>
<tr>
<td>LGALS3</td>
<td>2.05</td>
<td>2.86</td>
<td>0.0033</td>
<td>11.6</td>
<td>0.0012</td>
</tr>
<tr>
<td>CCNB1</td>
<td>2.60</td>
<td>3.11</td>
<td>0.042</td>
<td>4.98</td>
<td>0.00027</td>
</tr>
<tr>
<td>PBK</td>
<td>3.52</td>
<td>3.21</td>
<td>0.0066</td>
<td>5.92</td>
<td>0.0011</td>
</tr>
<tr>
<td>SLC24A3</td>
<td>0.44</td>
<td>0.47</td>
<td>0.33</td>
<td>0.80</td>
<td>0.54</td>
</tr>
<tr>
<td>LCN2</td>
<td>3.46</td>
<td>8.20</td>
<td>0.261</td>
<td>75.38</td>
<td>0.024</td>
</tr>
<tr>
<td>PV ALB</td>
<td>0.45</td>
<td>0.47</td>
<td>0.039</td>
<td>0.68</td>
<td>0.50</td>
</tr>
</tbody>
</table>

The online version of this article contains supplemental material.
decreased in tubules and glomeruli of D15 mice (P = 0.021 and P = 0.048, respectively). While no significant difference in staining intensity was noted between control and D3 mice, there was a significant change of location of cyclin B1 from the cytoplasm to the nuclei in D3 mice (Fig. 7, D–H; 13.5 ± 3.4% vs. 55.4 ± 8.5% of nuclei in control vs. D3, P = 0.013). Seven cell cycle associated genes were upregulated in D3 mice at FDR < 0.2 (Fig. 7I), including CDKN1a (1.76-fold). Not all cell cycle genes were elevated; CDK2, CCND1, and CCNE2 were not differentially expressed. Of note, few mitotic figures were seen on light microscopy in either D3 or D15 mice.

DISCUSSION

The goal of this study was to develop a consistent model of murine nephrotoxicity to better understand the pathogenesis of colistin-induced nephrotoxicity and uncover genes contributing to or protecting from kidney injury. We determined a spe-
specific gene expression signature associated with colistin administration and subclinical kidney injury, successfully validated with RT-PCR. The enrichment of cell cycle arrest genes in this model highlights an important theme seen in other models of tubular injury.

Cell cycle progression and arrest processes are mediators of renal recovery and fibrosis following kidney injury (55, 66). The cell cycle has been implicated in models of kidney injury secondary to cisplatin (25, 55) and in models of progression from AKI to chronic kidney disease (66). The biological network modulated by CDKN1A (WAF1/CIP1/p21), which has major roles in cell cycle regulation (26, 47, 69), is upregulated upon colistin treatment. CDKN1A is a cyclin-dependent kinase inhibitor that is a direct target gene of the tumor suppressor p53 protein and plays essential roles in protection of cells against apoptosis (54). Its increase in expression suggests that injury or cellular stress caused by colistin is acting through p53 to inhibit cell cycle progression.

CCNB1 (cyclin B1) controls the progression of the cell cycle from G2 to M phase. CCNB1 gene expression normally peaks during the late G2 phase of the cell cycle with subsequent return to lower levels during M phase (19). CDKNIa (p21) inhibits the CCNB1/CDC2 complex, preventing G2 to M transition (23, 46). The upregulation of CCNB1 and CDC2 and, moreover, the lack of upregulation in CDK2, CCND, and CCNE genes point to G2/M as the particular arrest point in the cell cycle in our model (54). In addition to the increase in CCNB1 gene expression observed, we also noted a change in protein distribution, with an increase in nuclear colocalization of CCNB1. This phenomenon is also consistent with G2/M cell cycle arrest, as cyclin B1 translocates to the nucleus at the beginning of M phase (65). However, increased p21 promotes phosphorylation/dephosphorylation of certain residues on the CCNB1/CDC2 complex, preventing progression from G2 to M phase. While CCNB1 gene expression remained elevated in D15 mice, immunofluorescence staining was decreased in both tubules and glomeruli. We speculate this decrease is related to the widespread damage observed in D15 mice. Finally, we noted many enlarged PCNA-positive nuclei in both D3 and D15 mice, which is a consequence of S-phase DNA replication.

Fig. 7. Colistin engages the cell cycle. The degree of cyclin B1 tubular and glomerular immunofluorescence staining did not vary significantly between control (A) and D3 (B) mice. Measurement bar is 100 μm, ×20. C: immunofluorescence scoring, n = 5 for all groups. D: cyclin B1 tubular immunofluorescence staining in control mice is diffuse and cytoplasmic with occasional nuclei stained. E: DAPI colocalization of D, F: the proportion of nuclei staining cyclin B1 positive is increased in D3 mice. G: DAPI colocalization of F. Measurement bar is 20 μm, ×60. H: quantitation of the proportion of nuclei staining for cyclin B1 in control and D3 mice, n = 5 for both groups. I: cell cycle associated genes upregulated in the array at FDR < 0.05 (*), < 0.1 (+), and < 0.2 (^). CDK2, CCND1, and CCNE2 were not differentially expressed.
elevated in D3 mice, with subtle pathologic injury and no early time point with subclinical kidney injury. NGAL was upregulated in these mice. Despite this, we chose to refine a model in the analysis, this would have caused excessive volume depletion in the mouse model. Because colistin is a polypeptide, it could potentially be contributing to cellular injury in this model. Because colistin is a polypeptide, it could potentially interact with a vast number of proteins in the renal tubular cell and cause many downstream effects. Apoptotic, necrotic, and antioxidant pathways were not enriched among our DEGs, but these do not require transcriptional regulation to be activated. The antibacterial mechanism of action of colistin is displacement of divalent cations Mg$^{2+}$ and Ca$^{2+}$ from the bacterial outer membrane. In vitro models, colistin has been shown to alter calcium signaling and injure the mitochondria. mRNA expression of calmodulin was decreased and intracellular calcium levels were increased in response to cell colistin exposure. Analogously, genes encoding proteins that bind calcium were downregulated in the mouse model array, including MYL9, PVALB, and SLC24A3. Mitochondrial dysfunction can lead to either necrosis or apoptosis, which we observed. What our data do suggest is that tubular cells accumulate at the G2/M stage without being able to proceed to mitosis. This cell cycle arrest is potentially a protective mechanism, allowing a cell to repair damage and recover prior to advancing to the energy-expensive M phase. However, the p53 pathway causing this arrest is itself activated by a wide variety of cell stressors such as MAP kinase activation or DNA damage. In addition to causing cell cycle arrest, p53 as well as galectin-3 may also lead to apoptotic cell death.

A limitation of our model is the inability to collect pharmacokinetic data with plasma colistin levels in mice. HPLC (with or without mass spectrometry)-based colistin measurement techniques have been developed in a few highly specialized laboratories around the world. The gene and protein expression of galectin-3 were found to be upregulated in D3 and D15 mice. Galectin-3 is a lectin involved in cell growth, apoptosis resistance, and cell cycle regulation. This contributes to regulation of both the G1/S and G2/M cell cycle checkpoints via activation of p21. Galectin-3 has been found to be upregulated in rat models of AKI, mouse models of diabetes, and mouse models of chronic allograft nephropathy. Previous data suggest Galectin-3 has an anti-bacterial role in kidney injury, although the relevance of this is unclear since we did not see fibrosis in our model. Here we show that galectin-3 is upregulated during subclinical kidney injury. With the advent of commercial testing for galectin-3 in heart failure patients, galectin-3 may also prove a worthy target of investigation as an early marker of drug-induced kidney injury in patients.

The DEGs we found were quite specific to the cell cycle; however, the mechanism of injury may not be fully discernible by transcriptional signature. Several alternative processes could potentially be contributing to cellular injury in this model. Because colistin is a polypeptide, it could potentially interact with a vast number of proteins in the renal tubular cell and cause many downstream effects. Apoptotic, necrotic, and antioxidant pathways were not enriched among our DEGs, but these do not require transcriptional regulation to be activated. The antibacterial mechanism of action of colistin is displacement of divalent cations Mg$^{2+}$ and Ca$^{2+}$ from the bacterial outer membrane. In vitro models, colistin has been shown to alter calcium signaling and injure the mitochondria. mRNA expression of calmodulin was decreased and intracellular calcium levels were increased in response to cell colistin exposure. Analogously, genes encoding proteins that bind calcium were downregulated in the mouse model array, including MYL9, PVALB, and SLC24A3. Mitochondrial dysfunction can lead to either necrosis or apoptosis, which we observed. What our data do suggest is that tubular cells accumulate at the G2/M stage without being able to proceed to mitosis. This cell cycle arrest is potentially a protective mechanism, allowing a cell to repair damage and recover prior to advancing to the energy-expensive M phase. However, the p53 pathway causing this arrest is itself activated by a wide variety of cell stressors such as MAP kinase activation or DNA damage. In addition to causing cell cycle arrest, p53 as well as galectin-3 may also lead to apoptotic cell death.

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Urine NGAL was used in these experiments to identify an early time point with subclinical kidney injury. NGAL was elevated in D3 mice, with subtle pathologic injury and no elevation in BUN. However, the apoptosis, necrosis, and PCNA staining we detected in D3 mice suggest subclinical kidney injury was emerging by day 3. This is a successful illustration of how a biomarker allowed insight into the pathogenesis of kidney injury, prior to elevation of BUN and serum creatinine. The sole use of NGAL as our biomarker is the second limitation of our study. We chose NGAL as it is extremely sensitive and expressed in multiple sections of the nephron. Prior to the start of this project, it was unclear to the authors which sections of the nephron would be most affected. We found both proximal and distal tubules affected, although the distal tubules were affected to a greater extent. KIM-1 is a notable alternative with high sensitivity and success in predicting nephrotoxicity from other drugs, although it is mostly expressed in the proximal tubule. In contrast to the predominant distal convoluted tubule injury we found with colistin.

This mouse model offers an early glimpse into transcriptional events occurring in response to colistin-induced renal injury and may prove useful for the study of candidate therapeutics. The exact mechanisms by which colistin causes renal injury and activates the cell cycle remain to be elucidated and are the focus of ongoing work. Our future plan is to utilize the DEGs identified in this study as an initial filter for cell-based candidate gene association studies. It is possible that the concept of cell cycle arrest indicated in these experiments has relevance to nephrotoxic injury in patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


