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 resurging 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; galectin-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 non-nephrotoxic 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.
Blood was collected on control mice received intraperitoneal injections of 0.1% saline BID. 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 CX5CE 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 antibiotic time course.

Urine 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 (R&D Systems, Minneapolis, MN) and Stanbio kit, respectively. Urine NGAL-to-creatinine ratio was not elevated on day 0 or 1 and was nonsignificantly elevated on day 2. Based on this, the authors anticipated significant elevation the following day and killed the mice on day 3. On day 3, urine was collected by bladder puncture with a 28-gauge needle just prior to kidney harvest. In D15 mice, urine was collected at baseline and upon death. Urine albumin was measured by ELISA (E90-134; Bethyl Laboratories, Montgomery, TX).

Tissue collection, fixation, and freezing. Kidneys were fixed or frozen for routine histologic analysis, immunofluorescence, immunoblot, and real-time PCR as previously described (15). Briefly, kidneys were harvested at death and cut in half transversely with a clean razorblade. Three half-kidneys were placed in separate Eppendorf tubes and flash-frozen in liquid nitrogen. These three half-kidneys were then stored at −80°C and used for subsequent array, RT-PCR, and immunoblot analysis. The fourth half-kidney was now cut in the sagittal plane. A quarter-kidney was fixed in formalin, and the last quarter-kidney was placed in a mold with O.C.T. compound (Tissue Tek, Torrance, CA). The mold was then immediately frozen in cold methylbutane (−80°C). The molds were removed from methylbutane and stored at −80°C, and 4 µm cryostat sections were cut for subsequent use with immunofluorescence. For formalin-fixed tissue, specimens were stored overnight in 4% phosphate-buffered formalin prior to transfer to 75% ethanol. Specimens were then brought to the core pathology lab for embedding, cutting, and periodic acid-Schiff (PAS) staining.

Pathology. PAS-stained sections for each animal were assigned a semiquantitative score for tubular injury as described by Nomura et al. (51). A blinded observer assigned a score ranging from 0 (no injury) to 3 (severe/widespread injury) for three variables: tubular dilation/ flattening, tubular casts, and tubular degeneration. Ten cortical 20× fields were randomly examined per mouse. For each variable within a field, a score of 0 was assigned when 5% or less of tubules were affected, a score of 1 when 5–33% were affected, a score of 2 when 34–66% were affected, and a score of 3 when >66% were affected. For tubular degeneration, the entire tubule was counted as affected if at least one cell was clearly apoptotic, necrotic, or undergoing mitosis. This scoring system is appropriate for observing changes in renal tubule structure; however, we employed a second more sensitive scoring system for cellular degenerative changes between control and D3 mice. Raw numbers of individual necrotic cells were counted by a blind observer in 10 cortical 40× fields (36,000 µm²/field).

TUNEL staining. Paraffin-embedded sections were stained using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) according to manufacturer’s instructions. A blinded observer counted apoptotic cells in 10 random cortical ×20 fields (n = 5 for control, D3, and D15).

Illumina gene expression array. From a frozen half-kidney described above, an ~30 µg transverse slice was cut rapidly from the kidney’s central edge on a clean glass plate over dry ice. 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 DNA 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 kidneys of mice 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 level 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, NEGs, identified by detection P value ≥ 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.
Principally 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, including DEGs with subtle changes. Although a variety of analysis techniques may be employed (31), a two-way ANOVA with imposed 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.

**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.

**Table 1. Primers for RT-PCR**

<table>
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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>RRM2</td>
<td>CCAAGCCTTAGATAGAGAAGGGA</td>
<td>TCAGGCAAACTCTAAATGTA</td>
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<td>ANLN</td>
<td>CATTAGCAAGTCTGCAAAAGTC</td>
<td>CATGGAACACATAGCAGAAG</td>
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<td>AURKA</td>
<td>TTACGTGCTTTGCTGCAAAAGG</td>
<td>ACATTGAGCTTCTGTCCTGA</td>
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<td>PRCI</td>
<td>GAGATATGTCTGAGAACAGACAGG</td>
<td>TTCTGTTGCTAGATGGCCT</td>
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<tr>
<td>LCN2</td>
<td>ACGAGTACAAACAGAAGTTCG</td>
<td>CTGCTGCTTCCATACAGGTTG</td>
</tr>
<tr>
<td>LGALS3</td>
<td>CTGTAGAATCTAGAAATCATGC</td>
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<td>CCNB1</td>
<td>TTTCTGTATTGTCAGACCT</td>
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<td>PKB5</td>
<td>GACTAGGTGTCCAGACAGC</td>
<td>CAATTAGGCAATGGGAGTCCG</td>
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<td>AGCTTGAAGGCAGGAAATCTCC</td>
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<td>PVALB</td>
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<td>AGCTTGAAGGCAGGAAATCTCC</td>
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<tr>
<td>18S</td>
<td>GTTGCTGAGCGATTGTCTC</td>
<td>GAAAGGCACTTGCCCTCTAT</td>
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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 ± 3.8 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 at baseline. 0.17 ± 0.03 mg/dL on D3, and 0.30 ± 0.06 mg/dL on D15. **D:** urinary neutrophil gelatinase-associated lipocalin (NGAL)-to-creatinine ratio increased from baseline of 60.3 to 396 ng/mg in D3 mice. **E:** urinary albumin-creatinine ratio average increased from 15.8 to 94.0 mg/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 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 dissociation 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 the highest urine albumin were considered outliers and excluded from RT-PCR analysis.

SDS-PAGE and immunoblotting of frozen mouse kidney tissue. To assess galectin-3 expression, proteins were extracted from a portion of frozen kidney and subjected to immunoblot as previously described (10, 15) with 100 μg protein per lane (n = 4 per group). Primary antibodies included polyclonal rabbit antibodies to galectin-3 (0.113 mg/ml, 1:750; Proteintech, Chicago, IL) and mouse anti-actin antibody (1:2,000; Sigma, St. Louis, MO). Secondary antibodies included 680 nm goat anti-rabbit IgG (1:10,000) and 800 nm donkey anti-mouse IgG (1:5,000; Li-Cor Biosciences, Lincoln, NE). Activity was detected using the Odyssey infrared imager (ODY-1320, Li-Cor Biosciences) and Odyssey 2.1 software. A protein molecular size ladder control was run for each membrane with Precision Plus Protein (Bio-Rad). Band density was assessed with ImageJ software (v1.44p, NIH) and normalized to actin for each lane.

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. 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.
Table 2. DEGs

<table>
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<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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<td>RRM2</td>
<td>ribonucleotide reductase M2</td>
<td>2.91</td>
<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.

evaluate nonspecific staining and autofluorescence. Sections were washed and counterstained with 4’,6-diamidino-2-phenylindole (DAPI, 1:1.000 dilution; Invitrogen, Carumillo, CA) for 5 min. Slides were mounted with Fluorogel (Biomedia, Hatfield, PA). Slides were observed and scored using an Olympus spinning disc confocal microscope at $\times 20$ and $\times 60$ magnification. Images were compiled by integration of images gathered at a z-axis increment of 0.2 µm using the accompanying software. Alexa 467 fluorescence exposure was standardized at 300 ms for $\times 60$ and 200 ms for $\times 20$, 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 levels 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...
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 nonsignificant at ×20 magnification. High-power (×40) light microscopic examination revealed an absolute increase in the number 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 comparison). 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 appreciated 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 \( \times 20 \times \) 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 and only very subtle pathologic indicators. Had we assessed gene signature at D15, we were concerned there would be a greater chance of uncovering genes differentially expressed merely as a result of injured or necrotic cells, rather than those responsible for the specific pathogenesis of or protection from injury.

Between control and D3 mice, there were 1,459 DEGs at an unadjusted \( P \leq 0.05 \) by Illumina gene expression array. Given the 16,410 genes included in the analysis, this is significantly more than expected by random chance (821 DEGs are expected by random chance at \( P \leq 0.05 \)). No fold-change threshold was imposed in the analyses because kidney tissue was harvested prior to clinically appreciable AKI and we were interested in including DEGs with subtle changes. To prioritize validation, we did impose stepwise thresholds after Benjamini and Hochberg FDR corrections. Between control and D3 mice, there were 86 DEGs associated with FDR \( P \) value < 0.2, 21 with FDR \( P \) value < 0.1, nine with FDR \( P \) value < 0.05, and one with FDR \( P \) value < 0.01. Table 2 details the 21 DEGs associated with FDR \( P \) value < 0.1 along with three other DEGs (SLC24A3, LCN2, and PVALB) of interest with expression changes at FDR \( P \) value < 0.2. A comprehensive list of DEGs is provided in Supplemental Table S1.1

Hierarchical clustering of significant DEGs clearly shows that samples from the same group cluster together (Fig. 3). The results suggest that the majority of the DEGs are upregulated upon colistin treatment (fold change ranges from 1.13 to 3.85), and a small group of genes showed decreased expression (fold change ranges from -1.11 to -2.28). Functional enrichment tests suggested that compared with all 30,854 genes in the mouse genome, DEGs with FDR \( P \) value < 0.2 (86 genes) were enriched in biological processes and networks containing genes involved in regulation of cell cycle, cellular movement, and cell death (\( P < 1 \times 10^{-6} \)). The entire GO functional enrichment analysis (BinGo) is provided in Supplemental Fig. S1. As an analogous bioinformatics method, a functional pathway analysis of the same 86 genes was performed in DAVID. After Benjamini-Hochberg FDR correction, only two pathways were enriched: cell cycle (\( P = 0.00081 \)) and the p53-signaling pathway (\( P = 0.0016 \)).

We selected 10 DEGs for array validation with RT-PCR (RRM2, ANLN, AURKA, PRC1, LGALS3, CCNB1, PBK, SLC24A3, LCN2, and PVALB). Genes were selected on the basis of their FDR, fold change, and to a lesser extent, the pathophysiologic interest to the authors. Gene expression levels were determined in D15 mice as well. Between control and D3 mice, expression of all 10 genes determined by RT-PCR followed the same pattern as that of the array (Fig. 4). Expression of the 10 DEGs by D15 either revealed partial restitution of expression toward control levels, or a more profound fold change in expression. A detailed account of RT-PCR fold changes and \( P \) values for each time point is provided in Table 3.

**Galectin-3 protein expression and distribution are 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.

**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’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

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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>
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<tr>
<td>RRM2</td>
<td>2.91</td>
<td>6.01</td>
<td>0.045</td>
<td>3.54</td>
<td>0.0016</td>
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<td>ANLN</td>
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<td>0.0011</td>
<td>9.71</td>
<td>0.00039</td>
</tr>
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<td>AURKA</td>
<td>2.00</td>
<td>2.59</td>
<td>0.0200</td>
<td>4.58</td>
<td>0.00036</td>
</tr>
<tr>
<td>PRG1</td>
<td>3.45</td>
<td>3.36</td>
<td>0.0025</td>
<td>4.96</td>
<td>0.00036</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>
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<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>
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<td>PVALB</td>
<td>0.45</td>
<td>0.47</td>
<td>0.039</td>
<td>0.68</td>
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</tbody>
</table>

1 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-

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**Fig. 5.** Expression of galectin-3 increases following colistin administration. A: immunoblot of galectin-3 and beta-actin, $n = 4$ mice per group (except 1 control mouse was excluded due to a smudge). B: densitometry of protein expression as a ratio to actin and normalized to the control group. Immunohistochemistry (IHC) of galectin-3 in control (C), D3 (D), and D15 (E) mice reveals increased staining in mice receiving colistin, ×20. Measurement bar is 100 μm. F: tubules were blindly scored, $n = 5$ for all groups. *Adjusted $P ≤ 0.05$.

**Fig. 6.** Proliferating cell nuclear antigen (PCNA) expression increases following colistin administration. Representative photos of PCNA IHC in control (A), D3 (B), and D15 (C) mice; magnification ×20, measurement bar 100 μm. Note the occasional PCNA-positive cells in control mice (arrowhead) as well as the patchy tubular distribution in D3 and D15 mice where some tubules have multiple PCNA-positive cells (arrow) and neighboring tubules are without staining. Also note many PCNA-positive nuclei appear larger than PCNA-negative nuclei. D: number of PCNA-positive cells per ×20 field in control, D3, and D15 mice. PCNA staining in control (E), D3 (F), and D15 (G) mice at ×60; measurement bar 20 μm. *Adjusted $P ≤ 0.05$; +unadjusted $P ≤ 0.05$ for nominal significance; $n = 5$ for all groups.
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). CDKN1a (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.
blocked from subsequent mitosis, as described in other models of tubular injury (54).

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 (33, 43) that 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 (50), mouse models of diabetes (29, 30, 56), and mouse models of chronic allograft nephropathy (12). Previous data suggest Galectin-3 has an anti-apoptotic role in kidney injury (29, 52), 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 (18, 60). The antibacterial mechanism of action of colistin is displacement of divalent cations Mg2+ and Ca2+ from the bacterial outer membrane (4, 16). In in vitro models, colistin has been shown to alter calcium signaling and injure the mitochondria (11). mRNA expression of calmodulin was decreased and intracellular calcium levels were increased in response to cell colistin exposure (11). 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 (39, 45). At present, the best of these assays requires 300 μl of blood for a single measurement. Given the multiple blood draws required for pharmacokinetic analysis, this would have caused excessive volume depletion in these mice. Despite this, we chose to refine a model in the mouse, as its well annotated genome is conducive for gene-based investigation.

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 (36). 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 (3, 49, 57), 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


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COLISTIN NEPHROTOXICITY


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