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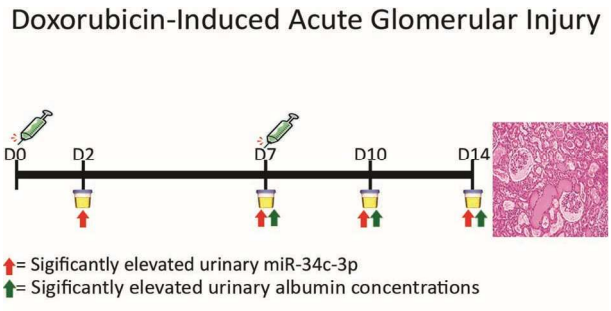
Graphical Abstract



Increased microRNA-34c-3p appeared as a novel biomarker for progressive doxorubicin nephrotoxicity in rats. MicroRNA-34c-3p alterations were more specific than and comparatively as sensitive as albuminuria for early prediction of glomerular injury.

MicroRNA-34c-3p is an early predictive biomarker for doxorubicin-induced glomerular injury progression in male Sprague-Dawley rats

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Abstract

Recently, eight urinary protein biomarkers were qualified for renal toxicity prediction in drug development; however, there are no biomarkers unique to glomerular toxicity. Albuminuria is a hallmark biomarker for primary glomerular injury but lacks specificity. MicroRNA species associated with genes that regulate kidney injury could potentially be used as biomarkers of nephrotoxicity. In this study, microRNA and protein expression changes in urine, blood, and/or kidneys, in addition to histopathology were evaluated in male Sprague-Dawley rats following weekly intravenous injections of doxorubicin (5 mg/kg/dose). Following the first administration, urinary miRNA-34c-3p was significantly increased on Day 2 and remained elevated on Day 7. Urinary osteopontin was significantly increased on day 2 only. Significant urinary albumin was detected on day 7, in the absence of histopathological findings. Following a second doxorubicin administration on day 7, significantly increased urinary kidney injury molecule 1, cystatin C, β2-microglobulin, total protein, and neutrophil gelatinase-associated lipocalin concentrations were detected on day 14. These alterations were concurrent to significant and progressive albuminuria, urinary miR-34c-3p, remarkable microscopic primary glomerular injury and secondary tubular alterations. Urinary miR-34c-3p elevations were predictive of histopathologic injury progression and outperformed the traditional renal biomarkers, serum creatinine and blood urea nitrogen, which did not increase with treatment. MiR-34c-3p was also significantly enriched in damaged glomeruli compared to adjacent nonglomerular tissue. Taken together, miR-34c-3p was identified as a highly sensitive candidate renal safety biomarker with relative specificity, particularly for early prediction of doxorubicin-induced glomerular injury progression in male Sprague-Dawley rats.

Introduction

The kidney is a major target organ for drug toxicity. The kidney receives 25% of cardiac output and it is naturally exposed to circulating drugs and chemicals as a major excretory route (1) Renal toxicity has been reported for several marketed drugs and chronic use of drugs with known renal safety liabilities may lead to acute kidney injury, renal failure, and/or mortality (2). Elevated serum creatinine (sCr) and blood urea nitrogen (BUN) concentrations are utilized as biomarkers for detecting renal injury. However, there are shortcomings for the use of these parameters in both nonclinical and clinical settings. The major concern is that significant elevations in sCr and/or BUN are not evident prior to remarkable microscopic kidney injury (3, 4) Based on the usage of these traditional renal biomarkers, early prediction of the nephrotoxic potential for drugs is often missed during preclinical drug development, contributing to increased compound attrition and substantial loss of development of potential drugs intended to deliver unique therapies to patients with unmet medical needs (5).

Recently, the US Food and Drug Administration (FDA), the European Medicines Agency (EMEA), and the Pharmaceutical and Medical Devices Agency (PMDA) approved eight novel urinary protein biomarkers for limited use in preclinical toxicology studies in rats and clinical drug development (6). Amongst these biomarkers, elevated urinary total protein (uTP), cystatin C (uCysC), and β2-Microglobulin (uB2M) concentrations may be used to predict compound related glomerular injury and impairment of renal tubular reabsorption in the rat. Because uTP, uCysC and uB2M may be remarkably elevated as a result of impairment to nonglomerular regions in functional nephrons (7), the identification of glomerular specific biomarkers represents a major gap in prediction of drug-induced progressive glomerulonephropathy. Highly sensitive and specific biomarkers for the detection and diagnosis of glomerular injury would

guide decision-making in the selection of safer drug candidates, and may also provide mechanistic insight regarding pathways involved in compound related glomerulopathies.

MicroRNAs (miRNAs) are small (~22 nucleotides in length) non-coding RNA species that selectively bind to and alter messenger RNA (mRNA) expression (8). These molecules are present in biofluids (9) and are resistant to degradation by RNases (10, 11), making them accessible for assessment. Additionally, advancements in amplification techniques enable detection of very low quantities of miRNA in biofluids. Recent interest in miRNAs as candidate renal biomarkers stems from the identification of preferentially expressed miRNAs in specific organs (12) and enrichment of a subset of miRNAs in human and rodent (13, 14) kidneys. Liver specific miR-122 is released into circulation following hepatocellular injury and it has been shown to outperform biomarkers traditionally used to monitor for liver toxicity in both animals and humans (15, 16). Recent observations in clinical cases of nephrotoxicity suggest that the application of specific urinary miRNAs as translatable biomarkers for early prediction of acute glomerular injury is feasible (17); however, this hypothesis warrants further investigation.

In order to assess the utility of miRNA measurements in urine for detection of site-specific renal toxicity the Health and Environmental Sciences Institute (HESI)[†] Biomarkers of Nephrotoxicity Committee is performing a collaborative program using toxicants specific for particular nephron segments. This study describes the protein biomarker and miRNA release profiles associated with doxorubicin nephrotoxicity over time. We hypothesized that selected miRNAs involved in doxorubicin-induced glomerular alterations represent candidate biomarkers for the early prediction of progressive glomerular injury in male Sprague-Dawley rats.

Materials and Methods

Experimental Animals

Male Sprague Dawley rats (approximately 8 weeks of age) were purchased from Charles River (Raleigh, NC) and housed in polycarbonate cages on a 12-hour (h) light-dark cycle. Rats were provided pelleted NIH-07 diet (Zeigler Brothers, Gardners, PA) *ad libitum*, with the exception of during 18 h fasting periods (overnight) during urine collections; during this period, the animals were individually housed in metabolism cages. Reverse osmosis water (HydroService and Supplies, Research Triangle Park, NC) was available to rodents, *ad libitum*, at all times. All animal use was conducted under a protocol approved by The Hamner Institutes for Health Sciences Institutional Animal Care and Use Committee.

Test Article Administration, Urine Collection, and Tissue Collection

Following arrival, rats were acclimated for approximately one week prior to the initiation of experimentation. A summary of the experimental design is given in **Table 1**. On the first morning of dosing, study day (D) 0, all rats received a bolus intravenous (*iv*) administration of either 5 mg/kg doxorubicin (DOX, Santa Cruz Biotechnology, Santa Cruz, CA) dissolved in 0.9% saline (n=6/necropsy time point; Groups 2 and 4) in a volume of 1 ml/kg or 0.9% saline, Vehicle, (n=6/necropsy time point; Groups 1 and 3). On study D7, at the same time as on D0, Groups 1 and 2 were euthanized for histological analyses, while Groups 3 and 4 rodents received an additional *iv* bolus administration of Vehicle or DOX (5 mg/kg) respectively. Groups 3 and 4 animals were euthanized on study D14. Urine samples were collected on ice, overnight, an approximately18 h collection, on nights prior to D2, D7, D10, and D14. Urine samples aliquoted for downstream miRNA applications were centrifuged at 2900 rpm at 4˚C for 10 minutes (min)

and stored at -80˚C until use. Aliquots designated for biomarker quantification were stored at -80˚C without prior centrifugation. Interim blood samples were collected by lateral saphenous venipuncture on D2 and D10. Rats were euthanized by CO_2 inhalation and exsanguination via vena cava blood collection into serum separation tubes and plasma separation tubes containing ethylenediaminetetraacetic acid (EDTA, Fisher Scientific, Pittsburgh, PA). Following humane euthanasia, representative tissues were collected and stored in formalin fixative for 48 h prior to histological processing.

Kidney, Liver, and Heart Histopathology

Formalin-fixed and paraffin-embedded kidney, liver, and heart tissues were sectioned and stained with hematoxylin and eosin (H&E). Microscopic examination of the H&E-stained slides were scored based on previously described criteria (18).

Clinical Chemistry Analysis

Measurements of BUN, sCr, and alanine aminotransferase (ALT) levels in serum; as well as urinary measurements of creatinine (uCr) and total protein concentrations were quantified with reagents from Carolina Liquid Chemistries (Winston-Salem, NC) and an Olympus AU600 (Olympus America, Center Valley, PA) clinical chemistry analyzer. Urinary concentrations of N-acetyl-beta-D-glucosaminidase (uNAG), glucose (uGlu), and gamma-glutamyl transpeptidase (uGGT) were measured utilizing Siemens reagents and the Advia 1800 instrument (Siemens Corporation, Washington DC). For each biomarker, significance was determined in Graph Pad Prism software (GraphPad Software Inc., San Diego, CA) utilizing a matched samples two way analysis of variance (ANOVA) analysis for treatment and time followed by Bonferroni multiple test correction. Significance was considered $p<0.05$.

Urinary Protein Biomarker Analysis

Isolated urine samples were utilized for the measurement of albumin (uAlb), osteopontin (uOpn), Kidney injury molecule-1 (uKIM-1), neutrophil gelatinase-associated lipocalin (uNGAL), uB2M, and uCysC using Milliplex MAP magnetic bead Rat Kidney Toxicity Panels 1 and 2 (EMD Millipore, Billerica, MA). Fluorescence was quantified using a Bio-Plex Luminex 100 (Bio-Rad, Hercules, CA) plate reader. Sample concentrations were normalized to concurrent uCr concentrations. For urinary protein concentrations values that did not fall within the standard curve, the upper and lower limit of quantification values were utilized to extrapolate protein biomarker concentrations. For each biomarker, significance was determined in Graph Pad Prism software utilizing a matched samples two way analysis of variance (ANOVA) analysis for treatment and time followed by Bonferroni multiple test correction. Significance was considered $p<0.05$.

Urinary RNA Isolation

Total RNA was extracted from 200 µl of all urine samples using the mirRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's protocol with slight alterations. Briefly, 3.5 volumes of QIAzol Lysis Reagent were added to urine samples. Post a 5-min incubation period, synthetic miRNA, *Arabidopsis thalania* miR-159a (UUUGGAUUGAAGGGAGCUCUA; Integrated DNA Technologies, Coralville, IA) was added (30 pg) to each sample. The spike-in miRNA was utilized to normalize for RNA isolation efficiency following quantitative real time PCR (qRT-PCR). Chloroform (140 µl) was added to samples which were then incubated at room temperature (RT) for three min, mixed, and centrifuged (12,000 X g at 4˚C for 15 min) in pre-spun 5 PRIME Phase Lock Gel tubes (Fisher

Scientific, Pittsburgh, PA). The upper aqueous layer of each sample was transferred to a new tube and 1.5 volumes of 100% ethanol were added. Following thorough mixing, samples were spun down in miRNeasy spin columns (8000 X g for 15 seconds at RT) discarding flow-through. Buffer RWT (700 µl) was added and spun down (8000 X g for 15 seconds at RT), discarding flow-through. Buffer RPE (500 µl) was twice added to columns and spun down (8000 X g for 15 seconds at RT), discarding flow-through. Spin columns were centrifuged at full speed for 2 min and then transferred into clean tubes. RNase-free water (12 µl) was added to the membrane of the spin-columns and centrifuged (8000 X g for 1 min at RT). Eluate was collected and stored at -80˚C until time of analysis.

MicroRNA Microarray Profiling

Three microliters of total RNA from each urine sample was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit and the Megaplex Reverse Transcription Rodent Pools Set V 3.0 (Life Technologies, Foster City, CA). The resulting cDNA was pre-amplified using TaqMan PreAmp Master Mix and Megaplex Preamp Rodent Pools Set V 3.0 (Life Technologies, Foster City, CA) following the manufacturer's instructions. The pre-amplified product was diluted 1:4 in 0.1X TE (pH 8.0). MiRNA profiling was conducted using TaqMan Rodent miRNA Set A and B v. 3.0 Arrays and Taqman Universal Master Mix II, No Uracil-N glycosylase (UNG, Life Technologies, Foster City, CA) following the manufacturer's specifications. A no template control (NTC) was also run on microarray plates. Quantitative Real-time PCR (qRT-PCR) of these arrays was performed using an Applied Biosystems 7900HT RT-PCR System (Life Technologies, Foster City, CA).

Microarray Data Analysis

Subsequent to completion of qRT-PCR, sample cycle threshold (Ct) values for each miRNA species were determined using the Applied Biosystems Expression Suite Software v1.0.1 (Life Technologies, Foster City, CA). All curves that appeared to represent background noise were removed from further analyses. The miR-159a Ct values, grouped by urine collection (D2, D7, D10, or D14) and plate set (A or B), were utilized to normalize Ct values for RNA isolation efficiency, as previously described (19). Ct values were linearized (2^{-Ct}) and then divided by sample uCr concentration. Normalized Ct values were returned to log scale and imported into Partek Genomics Suite (Partek, Inc., St. Louis, MO). One way analysis of variance (ANOVA) analysis (for treatment) was used to determine significantly altered miRNA at each urine collection time point. Significance was considered $p<0.05$. Fold changes for significantly altered miRNAs were determined by calculating $2^{-\Delta Ct}$ where ΔCt represented (individual DOX-treated normalized miRNA Ct value – average Vehicle-treated normalized miRNA Ct value).

Plasma miRNA Quantification

Plasma samples from a subset of DOX-treated experimental animals were utilized for miR-34c-3p measurement (n=4 for vehicle, Group 2 DOX-treated, and Group 4 DOX-treated samples). Total RNA was extracted from 200 µl of collected plasma using the mirRNeasy serum/plasma kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Spike-in miRNA, miR-159a, was added to each sample (30 pg) for purposes of data normalization. RNA was reverse transcribed and preamplified using a pool of miRNA specific primer/probe sets, TaqMan MicroRNA Reverse Transcription Kit, and TaqMan PreAmp Master Mix (miR-34c-3p and miR-159a; Life Technologies, Foster City, CA). Sample analysis by qRT-PCR was performed using Taqman single assay primer/probe sets and Taqman Universal Master Mix II, No UNG (Life Technologies, Foster City, CA). Samples, an NTC, and a no

reverse transcriptase control (NRTC) were run in triplicate. The resulting Ct values were normalized to the spike in control (19). Significance was determined by Student's *t* test between normalized Ct values (DOX vs Vehicle). Fold changes between DOX- and Vehicle-treated rodents were determined by calculating $2^{-\Delta Ct}$, as described for urinary miRNA.

Biomarker Performance Analysis

Receiver-operator characteristic curve (ROC) analysis was employed to compare biomarker performance, whereby the area under the ROC curve (AUROC) was used to determine the accuracy of expression levels of uAlb and miR-34c-3p at earlier time points (D2, D7, and D10), in the prediction of progressive DOX related nephrotoxicity on D14. Performance depended on how well the biomarker separated the experimental groups tested into those with and without changes in biomarker concentrations at earlier time points and the sum of individual animal renal injury severity scores on D14. AUROC represented biomarker performance according to the following scale: ≥0.90 (high), 0.80-0.90 (moderate), 0.70-0.80 (mild) and ≤0.60 (poor). The statistical significance of the AUROC values were assessed by Mann-Whitney p values, which represent the probabilities of rejecting the null hypothesis that the AUROC curve is 0.5, indicating that there is a lack of predictive power.

Paraffin-embedded Tissue miRNA Quantification

Paraffin embedded sections from a subset of experimental animals were utilized for measurement of miRNA in laser-captured renal cells. Limited studies have been published to demonstrate the most appropriate extraction method for laser-captured, paraffin-embedded renal cells in order to minimize the influence of the extraction in the variability of RNA amplification

by qRT-PCR (20). RNA extraction is the first and a critical step in downstream miRNA analysis; and pre-analytical factors such as paraffin-embedding, type of biological matrix, LCM sampling method, laser-captured sample size, and commercial extraction kit can affect the quantity and quality of RNA extracted from laser-captured, paraffin-embedded glomerular and nonglomerular tissue samples. In the study, the RNA extraction results demonstrated that 3 of 4 from samples D14 vehicle-treated animals and 4 of 4 samples from D14 DOX-treated samples) were suitable for miRNA quantification. Specifically, fresh 10-µm thick sections were cut and affixed to membrane slides (Molecular Machines & Industries, Haslett, MI). Tissue sections were de-paraffinized and stained with H&E. Laser capture microdissection (LCM) was performed utilizing an Olympus 1X81 microscope and MMI Cell Cut Plus software (Molecular Machines & Industries, Haslett, MI). Within each section, glomerular cells and adjacent nonglomerular renal cells were isolated separately and the area of tissue collected was recorded for the purpose of data normalization. RNA was extracted from isolated cells using the Qiagen FFPE miRNeasy kit (Qiagen, Valencia, CA) in accordance with the manufacturer's protocol. A spike-in control, miR-159a, was added to each sample (30 pg) to normalize for RNA extraction efficiency. Targeted reverse transcription, pre-amplification, and qRT-PCR were carried out as described for plasma samples. Ct values generated following qRT-PCR were normalized to miR-159a (19) and further normalized by dividing linearized Ct values by the area of tissue collected. Values were returned to log scale and significance was calculated between glomerular and nonglomerular normalized Ct values using a Student's *t* test. Significance was considered $p<0.05$. Fold changes between glomerular and nonglomerular miRNA concentrations were calculated for each group as well as fold change between D14 glomerular tissue and Vehicle tissue.

Results

Histopathology Findings

Animals were sacrificed for histological examination of organs on study D7 (Groups 1 and 2) and D14 (Group 3 and 4) as described in **Table 1**. No remarkable DOX-related microscopic findings were identified in the hearts of any group in this study or in kidney or liver tissues from Groups 1 or 2 (D7). Kidney and liver histopathology was observed in DOX-treated rats (Group 4; **Table 2**). Amongst the DOX-treated animals, microscopic evidence of slight-to-mild hepatocellular hypertrophy was identified, but without elevations in serum ALT (data not shown; $p>0.05$). Evidence of remarkable glomerular and tubular damage was present in the kidneys of DOX-treated animals in Group 4. Glomerular injury was characterized as mild mesangial proliferation and mild protein deposition. Tubular injury was characterized as mild-to-moderate tubular dilation and hyaline casts and droplets, slight-to-moderate tubular hypertrophy, mild vacuolization, slight-to-mild tubular basophilia, and slight tubular degeneration/necrosis. Representative images of kidney tissue obtained from Group 3 (Vehicle)-treated and Group 4 (DOX)-treated animals are shown in **Figure 1**.

Biomarker Measurements

To evaluate the utility of traditional markers for detecting renal injury in our study, BUN and sCr concentrations were measured. There were no clinically relevant increases in BUN or sCr concentration changes observed between DOX-treated groups and concurrent Vehicle-treated groups at any time point (**Supplemental Figure S1**). In addition to the traditional biomarkers analyzed, we also quantified concentrations of newer urinary protein biomarkers. A decrease in creatinine clearance was observed on D10 between Vehicle- and DOX-treated rats (uCrCl;

Supplemental Figure S2); however, the urinary biomarker trends were strongly and significantly correlated when normalized to either concurrent uCr concentrations or urine output volumes (data not shown). Timed urine collections are often impractical in the clinical setting; therefore uCr is generally utilized to normalize data parameters collected in urine specimens. For this reason, urinary biomarker data in this study was normalized to concurrent uCr concentrations. When values were outside the limit of quantification for a particular analyte, upper and lower quantification limits were utilized to extrapolate data points. Because our objective was to determine the sensitivity of urinary biomarkers for prodromal prognosis of histopathological injury, the urinary data (and subsequent statistical analyses) presented in the body of this manuscript were obtained from Group 3 and 4 animals only. As histopathological evidence of DOX-induced progressive renal injury was not apparent on D7, parallel to when the Groups 1 and 2 animals were euthanized, the data from these animals were not suitable for prodromal analyses. The mean ± SEM concentration values for all urinary protein biomarker data for Groups 1 and 2 animals are listed in **Supplemental Table 1**. A significant DOX-induced elevation of uAlb was observed on D7 ($p<0.05$); however, no other parameters showed significant elevations amongst Groups 1 and 2 on either D2 or D7.

In the present study, increased, but transient, uOpn concentrations on D2 correlated with acute DOX nephrotoxicity; the DOX related uOpn alterations were statistically significant by *t* test ($p<0.01$, **Figure 2A**) but not two way ANOVA and multiple test correction. Urinary albumin (uAlb) concentrations increased progressively from D7 through D14, reaching significance on D10 and D14 following two way ANOVA and multiple test correction (**Figure 2B**). Alterations in uAlb were highly significant on D7 by *t* test ($p<0.001$). Urinary KIM-1 (uKIM-1) concentrations were significantly elevated with DOX-treatment only on D14 (**Figure 2C**).

Similarly, increases in uCysC, uTP, uB2M, and uNGAL were significant with DOX-treatment only on D14 (**Supplemental Figure S3A-D**).

Urine samples collected on D2 and D7 from rats in Groups 3 and 4 that were humanely euthanized on D14 were utilized for analysis of additional biomarkers to determine whether they were more sensitive than uAlb as biomarkers for early identification of DOX-induced glomerular injury progression. On D2 and D7, no remarkable uNAG, uGlu, or uGGT concentration changes were observed (**Supplemental Figure 4**).

Microarray Analysis of DOX-Induced Urinary MicroRNA Alterations

In an effort to identify miRNA biomarkers for glomerular injury, alterations in Group 3 and 4 urinary miRNA species were measured by Taqman Rodent miRNA Arrays sets A and B, which contain 750 unique miRNA species (**Supplemental Table 2**). On D2, D7, and D10, there were alterations in urinary release of 29, 17, and 36 miRNA species, respectively, in DOX-treated rats. Substantial drug-related effects were observed in urinary miRNA changes on D14, with 137 miRNA species being differentially measured. Interestingly, on D2, the majority of miRNA that were differentially measured in urine represented reduced quantities in DOX-treated rats (24 miRNA). For the remainder of the study, most of DOX related urinary miRNA changes observed in this study represented increased release; only 5, 3, and 4 miRNA species were reduced in urine on D7, D10 and D14, respectively.

Various miRNA species with altered urinary release were evident on D2; however, only miR-34c-3p showed significant alteration in DOX-treated animals from D2 through D14, making it the most interesting candidate biomarker for progressive glomerular injury in this study. MiR-34c-3p demonstrated a temporal release pattern comparable to uAlb, particularly the

magnitude of change for both urinary miR-34c-3p and uAlb was progressive from D7 through D14 (**Figure 3**). The average fold changes for miR-34c-3p release into urine were 5.3-fold, 3.4-fold, 5.6-fold, and 17.5-fold on D2, D7, D10, and D14, respectively.

We additionally determined whether DOX nephrotoxicity was associated with elevated levels of miR-34c-3p in circulation. DOX-induced significant increased plasma miR-34c-3p levels were noted on both D7 and D14 ($p<0.05$ for both days). The magnitude of change was similar in urine and plasma on D7 (3.4-fold vs. 6.6-fold respectively) concurrent to clinically relevant changes in uAlb. Unlike urinary miR-34c-3p which showed progressive release between D7 and D14, no further elevation in plasma levels of miR-34c-3p was evident on D14 (4.1-fold) as compared to D7.

Biomarker Performance Analysis

In order to determine the sensitivity of urinary miR-34c-3p as compared to uAlb as a minimally invasive biomarker for prodromal prognosis of progressive DOX related kidney injury, area under the receiving-operator curves (AUROCs) for these parameters were generated utilizing data from Groups 3 and 4 animals. Neither uAlb nor miR-34c-3p showed significant predictive power on D2 (data not shown). On D7 (**Figure 4A**), D10 (**Figure 4B**), and D14 (**Figure 4C**), uAlb levels predicted renal injury amongst the DOX-treated animal (AUC=1.00, p=0.004 for each day of sample collection/analysis). The predictive value of urinary miR-34c-3p was slightly less sensitive than uAlb on D7 and D10 (AUC=0.96, p=0.024 and AUC=0.967, p=0.016 respectively) and analogous to uAlb on D14 (AUC=1.00, p=0.004).

Relative Quantity of miR-34c-3p in Glomerular and Nonglomerular Renal Tissue

Finally, we sought to determine whether or not miR-34c-3p was enriched in glomeruli as compared to adjacent nonglomerular tissue. As described in the Materials and Methods section, laser capture microdissection sampling was used to obtain glomerular and nonglomerular renal tissues from a representative subset of D14 DOX-treated as compared to Vehicle-treated animals for downstream qRT-PCR analysis.

Following, MiR-34c-3p was significantly enriched in the glomeruli of both Vehicle and DOX-treated animals. DOX administration induced a significant increase of miR-34c-3p in glomeruli (2.5-fold increase in DOX animals compared to Vehicle animals; $p<0.001$). Interestingly, however, the mean fold change of miR-34c-3p in glomerular tissue, compared to adjacent nonglomerular tissue, was reduced with treatment (**Supplemental Figure 5**). Differences of 12.4-fold and 6.3-fold were observed between glomerular and nonglomerular cells of Vehicle- and DOX-treated rats respectively.

Discussion

Since 2008-2010, novel urinary protein biomarkers have been advanced to qualification by the FDA, EMEA, and PMDA (6, 21); yet, the identification of a highly sensitive and specific biomarker for acute drug-induced minimal change glomerulonephropathy remains a challenge. The normal ranges and variability of several novel urinary renal biomarkers in male and female Sprague-Dawley rats identified by various rodent specific singleplex and multiplex protein assay/platforms have been described (22). In general, protein biomarkers present obstacles which may include characteristic posttranslational protein modification(s), instability in biological fluids, and poor conservation among species of interest (23). Based on the aforementioned characteristics, microRNAs may provide a new pathway for renal safety biomarker identification and validation. In this study, using DOX as an exemplary nephrotoxicant, we examined the induction and progression of glomerular injury in male Sprague-Dawley rats and examined urinary miRNA alterations and urinary protein biomarker concentration changes at multiple time points over a two week period.

Nephrotoxicity was initially assessed using BUN and sCr; however, there were no clinically relevant changes in these traditional biomarkers in spite of microscopic kidney histopathology findings identified on D14. These observations further demonstrate the lack of sensitivity exhibited by sCr and BUN for the detection of renal injury in our study and highlight the need for novel biomarkers for the early prediction of primary glomerular injury.

Given the absence of DOX related kidney histopathology findings on D7, we monitored for novel urinary biomarker changes indicative of glomerular and/or tubular damage (uAlb, uCysC, uTP, and uB2M) as well as tubular damage (uKIM-1, uOpn, uNGAL, uNAG, uGGT, and uGlu)

to elucidate the temporal progression of DOX nephrotoxicity. Albumin, a 69 kDa protein produced by the liver, is not typically present in the urine in appreciable concentrations (24). Based on its size, albumin is largely excluded from filtration by the glomeruli; however, low quantities of albumin are filtered by healthy kidneys and reabsorbed by proximal tubules. Therefore, the presence of albumin in the urine can be indicative of functional impairment to either glomeruli or proximal tubules. Progressive albuminuria was the most sensitive protein biomarker of kidney injury in the present study, showing significant progressive elevation from D7 through D14. In contrast, KIM-1, which is a well-established biomarker of proximal tubular injury in the rat (25), was only significantly elevated in the urine of DOX-treated animals on D14. Collectively, albuminuria was indicative of glomerular injury progression by D7, while injury to the proximal tubules appeared secondary, as evident by increased urinary KIM-1 concentrations by D14.

Of note, we observed a DOX-related elevation of uOpn levels on D2, although this change failed to reach significance by ANOVA analysis. High concentrations of uOpn were observed in 1 out of 6 Vehicle-treated rats (Group 3) on D7 (3.7 ng/ml; mean ± SEM: 0.91 ± 0.1) and D10 (1.5 ng/ml; mean ± SEM: 0.48 ± 0.2), which may serve as the basis for lack of significance in DOX-induced uOpn elevations on D2 by ANOVA and multiple test correction.. Opn is an acidic polyanion and monocyte chemoattractant with a broad constitutive tissue expression profile. Increased Opn mRNA and localized protein expression has been correlated with tubule damage (7), as is observed with progressive DOX-induced tubulointerstitial injury in rodents (26). Renal elevation of Opn has also been reported in response to glomerular injury (27, 28) and renal stone formation (29). In this study, increased uOpn concentrations “uniquely” appeared during the acute phase of DOX-induced glomerular injury progression, prior to microscopic evidence of

kidney histopathology findings as was identified on D14. To the best of our knowledge, this is the first report of increased uOpn as early as 48 hours post a single *iv* injection of DOX in male Sprague-Dawley rats. Further evaluation of acute DOX-related kidney injury would require invasive methods (i.e., electron microscopy and/or special stains such as colloidal iron stain of damaged glomeruli); invasive characterizations of DOX nephrotic syndrome in rats have been previously well-described (30). As early as 28 hours post dose, DOX-induced acute glomerular injury was associated with ultrastructural alterations (focal fusion of interdigitating foot processes and loss of glomerular polyanions as early as 3 hours post dose); a complete loss of polyanions in glomeruli at 13 days and 28 days post dose was observed. Taken together, these findings indicate that the acidic polyanion, Opn, may be released into the urine from damaged glomeruli during acute DOX glomerulonephropathy in the rat. Whereas significantly increased uOpn concentrations (by *t* test, $p<0.01$) appeared during the primary "acute" glomerular injury progression phase, insignificantly increased uOpn concentrations were detected at later time points concurrent to secondary progressive "minimal (slight)" tubular degeneration/necrosis which was identified microscopically on D14. Opn may also promote albuminuria, as has been previously reported in wild type mice but not Opn-deficient mice following LPS- or diabetes related-induced albuminuria and glomerulosclerosis (27). The acute elevation of uOpn identified in the present study, preceded clinically relevant albuminuria; thus, increased uOpn uniquely outperformed increased uAlb (the hallmark biomarker for glomerular injury) in the early prediction of DOX-induced glomerular injury. Identification of more precise kinetics and the utility of uOpn elevations as an indicator for early identification of acute DOX-induced glomerular damage warrant further investigation.

Following urinary miRNA microarray profiling, 172 unique miRNA species were identified that displayed drug related alterations at some point during our study. Because the primary objective of this study was to identify biomarker profiles with sensitivity for progressive glomerular injury as compared to albuminuria, we sought to identify which unique miRNA signatures may be associated with DOX-induced urinary albumin concentration changes prior to D7. While 29 miRNAs were altered in urine collected from DOX-treated rats on D2, only miR-34c-3p remained altered throughout the remainder of the study, making this species a potential candidate for a sensitive biomarker of progressive glomerular injury. MiR-34c-3p demonstrated a pattern of elevation in the urine that was comparable to that of albumin and was also significantly elevated in the blood of DOX-treated rats.

Besides being a potent nephrotoxicant, chronic administration of DOX can induce significant cardiomyopathy (31). Although no histopathological evidence of cardiac damage was observed in response to DOX in this study, release of miRNAs may precede evidence of injury. A recent article explored miRNA changes in the heart of Sprague-Dawley rats following weekly administration of DOX. This group found that miR-216b and miR-367 became elevated in cardiac tissue prior to histological evidence of DOX-induced cardiomyopathy (31). Neither of these species was present at quantifiable levels in the urine of vehicle or DOX-treated animals at any time throughout our study. While this group also observed cardiac elevation of miR-34c at later time points (4+ weeks following weekly DOX administrations), these changes were identified for miR-34c-5p but not *34c-3p*. No significant elevation of urinary miR-34c-5p was identified throughout the course of the present experimental DOX rat nephrotoxicity study.

Recent investigations intended to identify urinary miRNA changes associated with hepatotoxicity identified elevated urinary miR-34c-3p following treatment with acetaminophen

or carbon tetrachloride (32); however, both toxicants are known to induce renal injury, including damage to the glomeruli and basement membrane (33, 34). Although no histopathological evidence of renal injury was observed in the work conducted by Yang *et al.*[31], the data from our current study suggests that urinary miR-34c-3p is a highly sensitive biomarker for renal injury that is released prior to observable renal pathology. This may specify that Yang and colleagues feasibly detected elevated urinary miR-34c-3p due to minimal-to-mild kidney injury.

Similar to uAlb, the temporal release of miR-34c-3p into the urine of DOX-treated rats was progressive from D7 through D14, although uAlb demonstrated a greater magnitude of change. However, on D2, while uAlb levels had only increased 1.9-fold in response to DOX ($p>0.05$), urinary miR-34c-3p was more than 5-fold times greater in DOX-treated animals as compared to controls. To explore the sensitivity of albuminuria and urinary miR-34c-3p as diagnostic biomarkers for renal injury, AUROCs were determined to characterize how accurately urinary concentrations of each marker predicted the drug related renal pathology observed on D14. While neither marker could significantly predict injury on D2, both uAlb and urinary miR-34c-3p showed high prodromal value from D7 through D14. Elevated uAlb appeared to be slightly more sensitive than urinary miR-34c-3p for renal injury detection on D7 and D10; however, a compound related increase in uAlb concentrations at best, represents a sensitive yet nonspecific indicator of compound-induced glomerular injury. Regard for uAlb as a qualified novel renal safety biomarker, stemmed from its outperformance of and added value to the interpretation of remarkable BUN and sCr concentration changes (6). However, the context of use for uAlb as a renal safety biomarker includes monitoring for acute drug-induced glomerular alterations resulting in impairment of kidney tubular reabsorption and acute drug-induced tubular alterations (independent of glomerular injury) in rat toxicology studies. Further, elevated uAlb

may not reflect direct damage to the kidney. For instance, albuminuria is often observed in acute and chronic progressive disease states including, but not limited to, diabetic nephropathy and other confounding diseases (35); therefore DOX-induced increased miR-34-3p expression in damaged glomeruli further suggested that miR-34-3p may be more specific for glomerular injury, compared to uAlb.

To further explore this possibility, we performed qRT-PCR for miR-34c-3p on glomerular cells and adjacent nonglomerular cells isolated from a subset of rodents in this study. In the absence of microscopic histopathology findings, remarkable miR-34c-3p expression was detected in laser capture microdissected glomeruli as compared to surrounding nonglomerular cells from Vehicle-treated animals. DOX treatment augmented expression of miR-34c-3p in damaged glomeruli. However, the relative quantity of glomerular miR-34c-3p as compared to that expressed in the surrounding nonglomerular cells was reduced following DOX-treatment. This suggested that nonglomerular cells may show evidence of increased expression of miR-34c-3p following DOX administration; however, we observed no significant difference in the levels of nonglomerular miR-34c-3p between DOX- and Vehicle-treated animals. Taken together, these findings suggested that miR-34c-3p contributed to the maintenance of glomerular function and passively leaked into urine and blood following tissue injury, as an in situ indicator of DOX-induced alterations to glomerular filtration barrier integrity.

Currently, the only commercially available method for measurement of miR-34c-3p is qRT-PCR, a technique that is costly and time consuming, making it impractical for measurement in the clinic. However, as further understandings and confidence in the utility of miRNAs as novel biomarkers become more evident, relevant technological advances will simplify miRNA detection for monitoring renal alterations. Additionally, uncovering miRNAs which contribute to

disease progression can potentially lead to the identification of novel protein biomarkers for the treatment of a myriad of disease states including, but not limited to, nephrotic syndrome.

We utilized MirWalk, an online tool that compares multiple algorithm-based miRNA/mRNA interaction predictions to explore potential targets of miR-34c-3p (36). Many genes were predicted by at least three algorithms to be regulated by miR-34c-3p, of which several genes indeed contribute to regulation of glomerular filtration barrier integrity (37). For example, platelet derived growth factor (PDGF) signaling mechanisms are critical for mesangial cell development and contribute to mesangial cell proliferation in experimental models of glomerulonephritis (38, 39). *PDGF receptor alpha* (*PDGFRA*) is a predicted target of miR-34c-3p**.** The MiR-34c-3p predicted target *Bone morphogenetic protein 2* (*BMP2*) is expressed in mesangial cells and crosstalks with MAPK-based transcriptional events to inhibit PDGF-induced DNA synthesis via the early response gene c-*fos* (40). Other putative targets of miR-34c-3p including *Mothers against decapentaplegic homolog 2* (*SMAD2), Collagen IV*, *Fibronectin 1*, and *Integrin alpha V*, or the signal transduction pathways which they are involved in, additionally have some role in regulating glomerular function and/or are implicated in glomerular injury (41-43). Further investigation would reveal the relationship between these genes/protein products, miR-34c-3p, and the downstream consequences relative to progressive drug-induced glomerular injury.

Conclusion

This study resulted in the identification of a unique candidate renal safety biomarker (miR-34c-3p) with comparable sensitivity, as well as higher specificity, as compared to uAlb in the prediction of DOX-induced primary glomerular injury progression in male Sprague-Dawley rats. MiR-34c-3p exhibited significant enrichment in the damaged glomeruli, suggesting that this miRNA is specific for the early prediction of progressive glomerular injury. Additionally, we demonstrated that the cationic polyanion uOpn may serve as an early biomarker for progressive loss of glomerular polyanions in DOX-treated rats.

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Footnotes

†The Health and Environmental Sciences Institute (HESI) is a nonprofit institution whose mission is to engage scientists from academia, government and industry to identify and resolve global health and environmental issues.

Figure 1: On D14, kidney histopathology findings were observed in DOX-treated (B) but not Vehicle-treated (A) rats. Representative photomicrographs were taken at 200X magnification.

Figure 2: Urinary osteopontin (uOpn; A), urinary albumin (uAlb; B), urinary KIM-1 (uKIM-1; C), and urinary μ-albumin (u-μAlb; D) protein concentrations were measured in the urine obtained from Group 3 and 4 Vehicle-treated (blue) and DOX-treated (red) rats. Data represents the mean concentration ± SEM. Significance is **$p<0.01$ and ***$p<0.001$ by two way ANOVA and $^{\#\#}p<0.01$ by Student's *t* test (compared to Vehicle controls).

Figure 3: Beginning on D7, DOX related fold change release of miR-34c-3p in the urine appeared characteristically similar to the fold change albumin release into urine. Data shown represents the mean fold change +SEM.

Figure 4: Comparative AUROCs for urinary miR-34c-3p and uAlb on D7 (A), D10 (B), and D14 (C) were calculated to determine the power of candidate biomarkers to predict histopathological injury on D14. Significance was determined utilizing Mann-Whitney test (p values shown).

Table 1. Experimental Design

Study Group	Treatment	Dose (Study Day)	Necropsy (Study Day)
1	Vehicle[a]	D0	D7
2	DOX[b]	D0	D7
3	Vehicle[a]	D0, D7	D14
4	DOX[b]	D0, D7	D14

[a]0.9% Saline (n=6/time point)

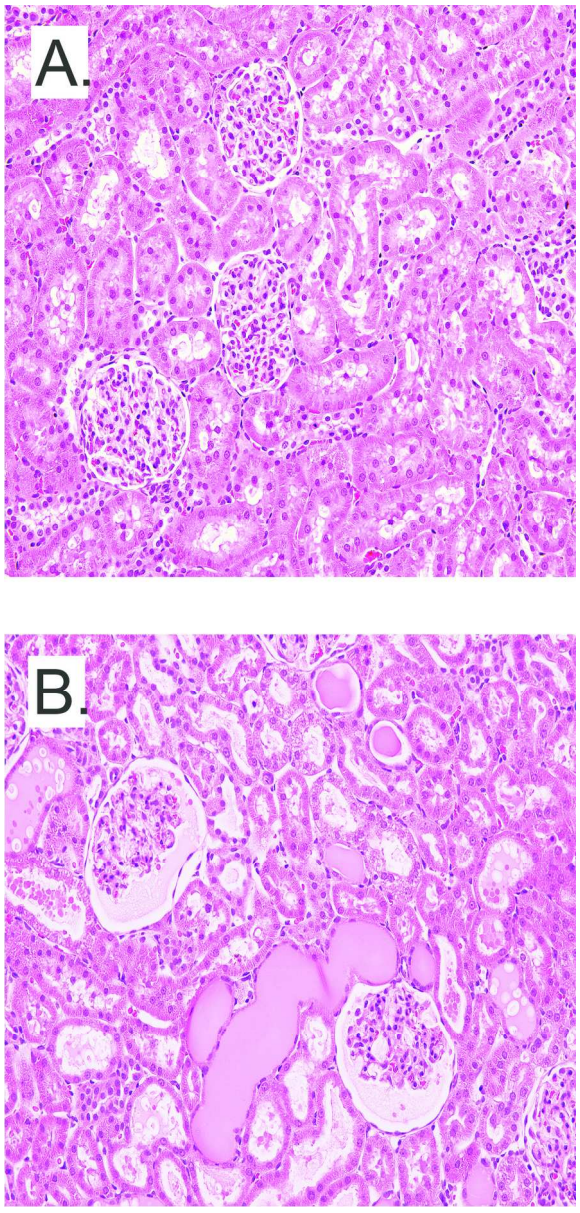
[b]Doxorubicin (5 mg/kg/wk; n=6/time point)

Table 2. DOX-Induced Histopathological Findings in Group 4 Rats[a,b]

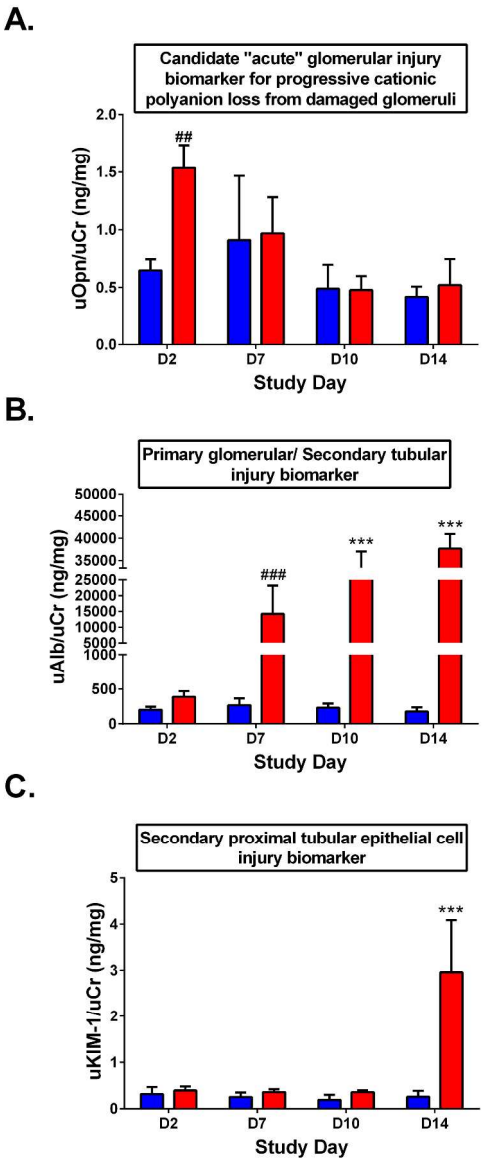
Histopathology Finding	***Injury Severity***			
Glomerular	**Normal**	**Slight**	**Mild**	**Moderate**
Mesangial Proliferation	*0/6*	*0/6*	*6/6*	*0/6*
Protein Deposition	*0/6*	*0/6*	*6/6*	*0/6*
Tubular	**Normal**	**Slight**	**Mild**	**Moderate**
Dilation	*0/6*	*0/6*	*3/6*	*3/6*
Hyaline Casts	*0/6*	*0/6*	*3/6*	*3/6*
Hypertrophy	*0/6*	*2/6*	*1/6*	*3/6*
Protein Deposition	*3/6*	*0/6*	*3/6*	*0/6*
Vacuolization	*3/6*	*0/6*	*3/6*	*0/6*
Basophilic Tubules	*0/6*	*4/6*	*2/6*	*0/6*
Hyaline Droplets	*0/6*	*6/6*	*0/6*	*0/6*
Degeneration/Necrosis	*1/6*	*5/6*	*0/6*	*0/6*
Liver	**Normal**	**Slight**	**Mild**	**Moderate**
Hypertrophy	*1/6*	*3/6*	*2/6*	*0/6*

[a]No DOX-induced renal or hepatic histological injury was observed in Groups 1-3

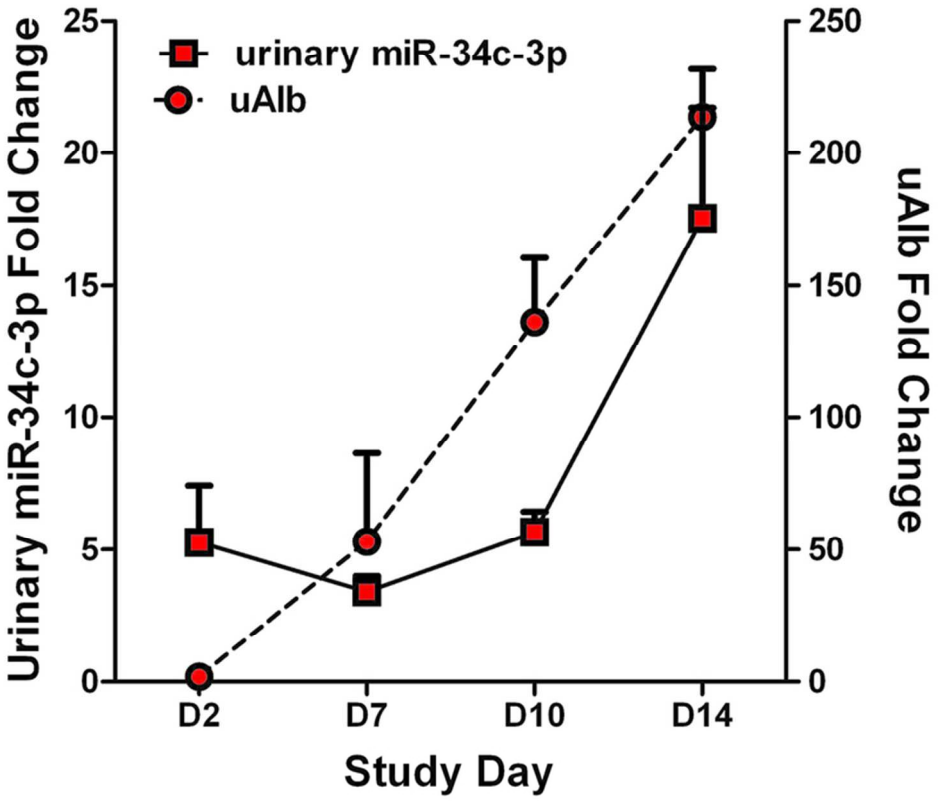
[b]No DOX-induced cardiac injury was observed in any group in this study



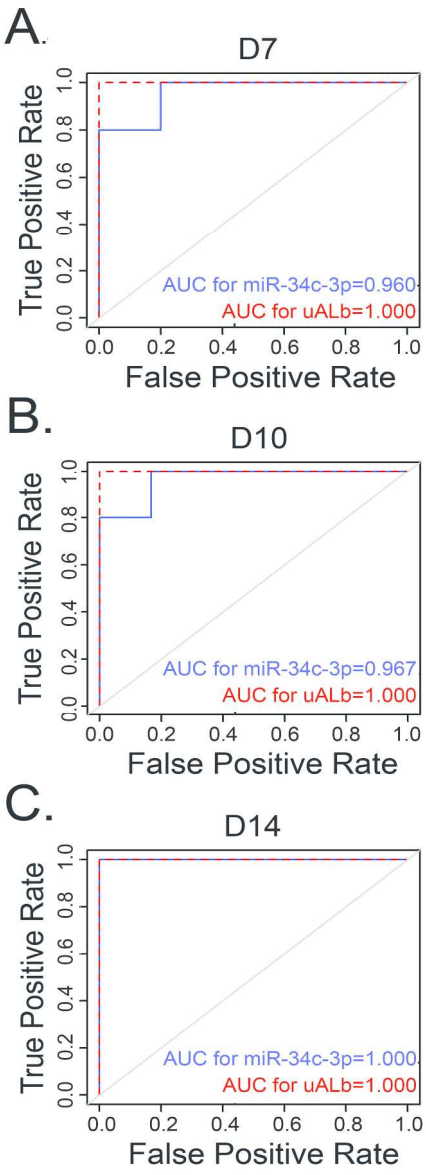
On D14, kidney histopathology findings were observed in DOX-treated (B) but not Vehicle-treated (A) rats. Representative photomicrographs were taken at 200X magnification.
133x280mm (300 x 300 DPI)



Urinary osteopontin (uOpn; A), urinary albumin (uAlb; B), urinary KIM-1 (uKIM 1; C), and urinary μ-albumin (u-μAlb; D) protein concentrations were measured in the urine obtained from Group 3 and 4 Vehicle-treated (blue) and DOX-treated (red) rats. Data represents the mean concentration ± SEM. Significance is **p<0.01 and ***p<0.001 by two way ANOVA and ##p<0.01 by Student's t test (compared to Vehicle controls).
267x580mm (300 x 300 DPI)



Beginning on D7, DOX related fold change release of miR-34c-3p in the urine appeared characteristically similar to the fold change albumin release into urine. Data shown represents the mean fold change +SEM.
72x62mm (300 x 300 DPI)



Comparative AUROCs for urinary miR-34c-3p and uAlb on D7 (A), D10 (B), and D14 (C) were calculated to determine the power of candidate biomarkers to predict histopathological injury on D14. Significance was determined utilizing Mann-Whitney test (p values shown).
233x660mm (300 x 300 DPI)