Repeated Administration of Low-Dose Cisplatin in Mice Induces Fibrosis

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RUNNING HEADLINE: repeated cisplatin induces fibrosis

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Abstract

Cisplatin, a chemotherapeutic used for the treatment of solid cancers, has nephrotoxic side effects leading to acute kidney injury (AKI). Cisplatin cannot be given to patients that have comorbidities that pre-dispose them to an increased risk for AKI. Even without these comorbidities, 30% of patients administered cisplatin will develop kidney injury, requiring the oncologist to withhold or reduce the next dose, leading to a less effective therapeutic regimen. Although recovery can occur after one episode of cisplatin-induced AKI, longitudinal studies indicate that multiple episodes of AKI lead to the development of chronic kidney disease (CKD), an irreversible disease with no current treatments. The standard mouse model of cisplatin-induced AKI consists of one, high dose of cisplatin (> 20 mg/kg) that is lethal to the animal three days later. This model does not accurately reflect the dosing regimen patients receive nor does it allow for the long-term study of kidney function and biology. We have developed a repeated dosing model whereby cisplatin is given once a week for four weeks. Comparison of the repeated dosing model to the standard dosing model demonstrated that inflammatory cytokines and chemokines were induced in the repeated dosing model, but levels of cell death were lower in the repeated dosing model. The repeated dosing model had increased levels of fibrotic markers (fibronectin, TGFβ, and α-SMA) and interstitial fibrosis. These data indicate that the repeated dosing model can be used to study AKI to CKD progression, as well as the mechanisms of this progression.
Introduction

Acute kidney injury (AKI), the rapid loss of kidney function, has many medical complications, a high mortality rate, and limited therapeutic interventions beyond palliative care (45). One of the most prominent causes of AKI is pharmaceutical-induced nephrotoxicity, which accounts for 19% of all cases of AKI (44). Cisplatin is a potent chemotherapeutic utilized for the treatment of many cancers, but induces AKI in 30% of patients even in the absence of comorbidities such as advanced age or pre-existing kidney diseases (35). Clinically, cisplatin is given at low doses in multiple rounds to try to avoid nephrotoxic side effects, but even with this precaution in place, AKI still occurs (3). Blood urea nitrogen (BUN) and serum creatinine (SCr) are clinical tests utilized to monitor kidney function, but are highly insensitive (9, 10). Elevated BUN or SCr levels in patients during the course of cisplatin treatment requires that the dose of cisplatin be lowered, delayed, or alternatively that the patient be switched to a potentially less effective chemotherapeutic that lacks nephrotoxic side-effects (3). None of these options are favorable, and can result in a less efficacious cancer treatment.

Until recently, it was assumed that patients that survive AKI and don’t require dialysis are able to achieve full recovery of kidney function (23). However, recent large-scale longitudinal studies that assessed the impact of AKI on long-term renal function indicated that patients that had AKI are more likely to develop CKD and end stage renal disease (ESRD) than patients with no history of AKI. Furthermore, the occurrence of CKD/ESRD was directly proportional to the level and frequency of AKI experienced by these patients (1, 11, 12, 16, 28-30, 42). Additionally, the incidence of AKI and CKD/ESRD has significantly increased in the past decade (16, 27, 47), as the overall age of our population is also increasing (18). This is of importance because kidney function declines with normal aging even in the absence of obvious kidney disease (2). The majority of individuals receiving nephrotoxic chemotherapeutics are middle-aged or older and already have increased exposure to renal stressors, enhanced
susceptibility to injury, and decreased ability to repair following injury. With improved diagnosis and treatment of cancers, there is also increased long-term patient survival. Hence, the percentage of cancer survivors expected to develop CKD/ESRD will increase, placing a major burden on patient quality of life and our health care system.

Gaining an understanding of the cellular processes involved in the development of CKD following cisplatin-induced AKI would be useful for developing renoprotective agents. Unfortunately, the standard dosing mouse model of AKI does not allow for long-term studies. In the standard dosing model, a single, high dose of cisplatin (>20 mg/kg) is administered once to 8-10 week old mice. This dose is lethal to the mouse beyond 72 hours and as a result, examining the AKI to CKD transition is not possible. Furthermore, this model does not accurately represent the repeated dosing regimen utilized in the clinic. Thus, there is a need for a more clinically relevant mouse model that enables the study of the cellular processes involved in the progression of cisplatin-induced AKI to CKD.

We have developed a model of cisplatin-induced kidney injury that reflects the repeated low dosing of cisplatin used in the clinic, and allows for detailed analysis of repair, recovery, and long-term kidney function. Here, mice were treated with 7mg/kg of cisplatin once a week for four weeks and sacrificed 3 days after the last injection. We compared this dosing regimen to mice that were treated according to the standard dosing model of AKI (one dose of 25 mg/kg cisplatin and sacrificed three days later). We analyzed markers of kidney function and injury, inflammatory cytokines and chemokines, indicators of endoplasmic reticulum (ER) stress and cell death, and pro-fibrotic indicators. While the standard dosing model of AKI and the repeated dosing model have similarities in their effects on kidney function, kidney damage is less severe in the repeated dosing model, enabling mice to survive beyond the 24-day course of treatment. Data also indicate that interstitial fibrosis occurred in the repeated dosing model but not in the
standard dosing model. Data suggest that the increased incidence of CKD following cisplatin-induced AKI may be a result of repeated injury leading to fibrosis.

**Materials and Methods**

**Reagents and Antibodies**

The following antibodies were purchased from Cell Signaling (Beverly, MA) unless otherwise noted: cleaved caspase 3 (CC3) (#9664), C/EBBP homologous protein (CHOP) (#2895), c-Jun N-terminal kinase (JNK) (#9258), phospho-c-Jun N-terminal kinase (pJNK) (#4668), transforming growth factor-β (TGF-β) (#3712S), fibronectin (F3648, Sigma-Aldrich, St. Louis, MO), p-SMAD3 (#12747), α-tubulin (Santa Cruz, Dallas, TX, SC-23948) and β-actin (Santa Cruz, SC-47778). Cisplatin (P4394, Sigma Aldrich) was used for experiments comparing the effects of a high single dose (euthanized three days later) with the new repeated dosing model. Pharmaceutical grade cisplatin (purchased directly from the University of Louisville hospital pharmacy) was used for experiments comparing the effects of single versus repeated injury from cisplatin. Similar effects were observed for both sources of cisplatin.

**Animals**

FVB/n mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a 12-hour light/12-hour dark cycle and provided food and water *ad libitum*. Animals were maintained under standard laboratory conditions. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville and followed the guidelines of the American Veterinary Medical Association. Cisplatin at 25 mg/kg in PBS (200 μL/animal) was administered by i.p. injection. Seventy-two hours after cisplatin injection, these mice were euthanized. Another cohort of mice received either 7 or 9 mg/kg cisplatin administered by i.p. injection once a week for four weeks. For these survival studies, mice were monitored for weight loss and signs of discomfort/distress on a daily basis. Mice exhibiting a weight loss of 20% or more total body weight, or high levels of discomfort and stress
were euthanized. Mice that survived the course of treatment were sacrificed 72 h after the fourth injection of cisplatin. Serum was prepared and stored at -80°C. The kidneys were flash frozen in liquid nitrogen or fixed in 10% neutral buffered formalin.

**Blood Urea Nitrogen (BUN) and Serum Creatinine (SCr) Determination**

BUN (DIUR-500) and SCr (C7548-120) were determined using kits from Bioassay Systems (Hayward, CA) and Point Scientific Inc. (Canton, MI), respectively following the manufacturers’ instructions. For SCr, this specific assay kit employs a two-reagent enzymatic assay system to eliminate interference by endogenous creatine and ascorbic acid.

**Protein Quantification, Western Blot Analysis, and ELISAs**

Homogenates were made from kidney cortex by homogenization in Cell Extraction Buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM imidazole, 1 mM magnesium acetate, 20 mM EGTA, 10 mM β-mercaptoethanol) containing a Complete Protease Inhibitor Cocktail Tablet and Phosphatase Inhibitor Cocktail Tablets (Roche, Indianapolis, IN). Homogenates were centrifuged at 15,000 X g for 10 min at 4°C. Supernatants were removed, mixed, aliquoted, and stored at -80°C until use. Protein concentrations were determined using Bradford Reagent (Bio-Rad, Hercules CA). Kidney homogenate (40 μg) was separated on 4–12% gradient tris-glycine-SDS polyacrylamide gels and transferred to PVDF membranes that were blocked in 5% (w/v) dried milk in tris-buffered saline containing 0.05% Tween (TBST) 20 for 1 h. Membranes were incubated with 1:5000 dilutions of primary antibody overnight at 4°C. The next morning, membranes were washed 3 times for 5 min each with TBST containing 5% (w/v) dried milk. After incubation for 1 h at room temperature with secondary antibodies conjugated with horseradish peroxidase (1:50,000 in TBST containing 1.25% (w/v) dried milk), membrane proteins were detected by chemiluminiscence substrate. ELISAs for KIM-1 (DY1817, R&D systems, Minneapolis, MN) and NGAL (DY1857 R&D Systems) were performed on the urine as directed by the manufacturer.

**Gene Expression**
Total RNA was isolated using RNA-STAT 60 (TEL-TEST Inc., Friendswood, TX) combined with mini-bead-beater glass beads and Mini Bead Beater machine (Cole-Palmer, Vernon Hills, IL) following manufacturer’s protocol. cDNA was made from 1 µg of total RNA using High Capacity Reverse Transcriptase (Life Technologies, Grand Island NY) following manufacturers’ instructions. Gene specific cDNAs were quantitated using real-time PCR and predesigned TaqMan assays. Tumor necrosis factor-alpha (TNF-α) (Mm00443258_m1), interleukin 6 (IL6) (Mm00446190_m1), interleukin 1-β (IL1β) (Mm0043228_m1), C-X-C motif ligand 1 (CXCL1) (Mm04207460_m1), monocyte chemotactic protein 1 (MCP1) (Mm00441242_m1), plasminogen activator inhibitor-1 (PAI-1) (Mm00435860_m1), α- smooth muscle actin (α-SMA) (Mm1546133_m1), bone morphogenetic protein-7 (BMP-7) (Mm00432102_m1), collagen α 1(IV) chain (COL4α1) (Mm01210125_m1), cyclin-dependent kinase inhibitor 2A (CDK2NA) (Mm00491449-m1), connective tissue growth factor (CTGF) (Mm01192932_g1), α-1 type 1 collagen (COL1α1) (Mm00801666_g1), and the normalization genes (β-2 microglobulin (B2M) (Mm00437762_m1) and β-actin (Mm01205647-g1) were purchased from Life Technologies (Grand Island NY) and used in combination with 2X Gene expression Master Mix (Life Technologies).

**Histology**

Kidney sections (5µm thick) from cisplatin-treated and untreated animals were stained with H&E and PAS, and the degree of morphologic changes was determined by light microscopy in a blinded fashion by a renal pathologist. The following measures were chosen as an indication of morphologic damage to the kidney after treatment with vehicle or cisplatin: proximal tubular necrosis, loss of brush border, proximal tubule degradation, tubular casts, presence of inflammatory cells, and interstitial fibrosis. These measures were evaluated on a scale from 0 to 4, which ranged from not present (0), mild (1), moderate (2), severe (3), and very severe (4).

**Immunohistochemistry**
Kidney sections (5 μm thick) were rehydrated in Histoclear followed by an ethanol gradient. Antigen retrieval was performed in citric acid buffer pH 6.0 at 95°C in a steamer for 30 min. Endogenous peroxidases were inhibited with 3% hydrogen peroxide and dual endogenous enzyme blocker (Dako) for 10 min, followed by two 5-min PBS washes. Slides were then blocked with avidin for 10 minutes followed by a PBS wash, and then biotin for 10 minutes followed by a wash in PBS (Dako). Slides were further blocked with 5% normal goat serum in 0.1% TBST for 1 h at room temperature. α-SMA primary rabbit antibody (Abcam) was added to slides at a concentration of 0.5 μg/mL, and allowed to incubate at 4°C overnight. Biotinylated goat anti-rabbit IgG antibody (1: 25000) (Vector laboratories, BA-1000) was added to each section and incubated for 30 min at room temperature. Slides were rinsed twice with PBS (5 minutes each). Vector ABC reagent (Vector laboratories, PK-7100) was added to each section and incubated for 30 min at room temperature. Slides were rinsed 2X with PBS, followed by the addition HRP was detected with NovaRed Substrate (Vector Laboratories, SK-4800) to each section for 5-7 min. Slides were rinsed in distilled water for 5 minutes, counterstained with modified Mayer's hematoxylin (thermo scientific, 72804), then dehydrated in an ethanol gradient to Histoclear, followed by mounting with Permount (Fisher Scientific, SP15). Positive staining for α-SMA was quantified using Metamorph Image Analysis software, and percent positive pixels were calculated as follows: (threshold area/(total area-acellular area)).

**Sirius Red/ Fast Green Staining**

Kidney sections (5 μm thick) were rehydrated in Histoclear followed by an ethanol gradient. Slides were then dipped into a Coplin jar containing PBS-T (PBS+0.1% tween-20) and incubated for 5 minutes. Slides were washed with distilled water twice for 5 minutes each and then incubated in saturated picric acid containing 0.1% Sirius Red and 0.1% Fast Green. Sirius red/Direct Red 80 (cat. No-365548) and fast green FCF (cat. No. F7258) were from Sigma whereas Picric acid, saturated approximately 1.2% w/v was from Ricca Chemicals (cat.no:
Slides were washed with 5% glacial acetic water until the water ran clear. Tissue samples were then dehydrated and fixed using Permount (Fisher, F-SP15-100). Positive staining for Sirius Red was quantified using Metamorph Image Analysis software, and percent positive pixels were calculated as follows: (threshold area/(total area-acellular area)).

**Statistical Analysis Data**

Data are expressed as means ± SEM for all experiments. Multiple comparisons of normally distributed data were analyzed by one-way ANOVA, as appropriate, and group means were compared using Tukey post-tests. Single comparisons were analyzed by Student's t-test where appropriate. For statistical analysis of the survival curve, a Log-Rank (Mantel-Cox) test was used. The criterion for statistical differences was p < 0.05*, p<0.01**,p<0.001***, and p<0.0001****.

**Results**

**Effects of dosing regimens on mouse survival.** The current model used to study cisplatin-induced AKI does not allow for the analysis of long-term effects on kidney function, nor does it recapitulate the repeated nature of the dosing regimen of cisplatin in the clinic. We hypothesized that administration of a low dose of cisplatin once a week for several weeks would be more clinically relevant and allow for analysis of long-term effects on kidney function. To test this, we compared survival of mice given a single high dose of cisplatin (standard dosing model; 25 mg/kg) to mice given a dose of cisplatin (7 or 9 mg/kg) once a week for 4 weeks. All mice subjected to the standard dosing regimen were sacrificed three days after cisplatin injection due to moribund status. Mice treated with the 7 mg/kg repeated dosing regimen survived the course of treatment and were sacrificed 3 days after the fourth treatment (day 24). Ninety percent of mice treated with a 9 mg/kg repeated dosing regimen survived until day 24 (Fig1). Statistical
analyses comparing survival curves of 7 and 9 mg/kg treated mice revealed no statistical
significance, but there was statistical significance between repeated dosing and standard dosing
survival curves (Fig1). These data indicate that the repeated dosing model with 7 mg/kg
enables survival of all treated mice for long-term studies of kidney function.

**Effects of dosing regimens on kidney injury and function.** In order to assess the impact of
repeated dosing on the kidney, we measured markers of kidney function and injury in the serum
and urine of mice, respectively. BUN levels of mice treated with the standard dosing model were
significantly increased at 72 h post-treatment (Fig2A). In the repeated dosing model, BUN also
increased, but not significantly (Fig2A). SCr levels were significantly increased for both the
repeated and standard dosing models, but SCr levels were higher in the standard dosing model
(Fig2A). Urinary KIM-1 and NGAL levels were examined, as they are more sensitive biomarkers
of AKI than BUN and SCr (5, 6). Urinary KIM-1 and NGAL levels were significantly increased in
the standard dosing model, but only NGAL levels were significantly increased in the repeated
dosing model, albeit to a lesser extent than in the standard model (Fig 2B). These data indicate
that the repeated dosing model of cisplatin induced less kidney injury and less decreased
kidney function, as compared to the standard dosing model.

**Effects of dosing regimen on inflammatory cytokine and chemokine levels.** One
component of the standard dosing model of AKI is a large inflammatory response (37, 41). We
compared the levels of inflammatory cytokines and chemokines between the standard and
repeated dosing models. TNFα is a potent cytokine that mediates inflammatory tissue damage
in the kidney, and activates downstream cytokines and chemokines, particularly IL1-β, MCP-1,
and IL-6 (21, 41). CXCL1 plays a role in neutrophil recruitment to sites of tissue inflammation
(15). mRNA levels of *Tnfa, Il1β, Mcp1, and Cxcl1* were increased significantly and
approximately to the same extent in both the standard and repeated dosing models (Fig3A, C,
D, E). *Il-6* mRNA was significantly increased in the standard dosing model, but only increased 5.51±2.01-fold in the repeated dosing model (Fig3B). These data suggest similar effects of both dosing regimens on inflammatory cytokines and chemokines.

**Effect of dosing regimen on activation of ER stress and cell death proteins.** Cell death and ER stress are characteristic of cisplatin-induced AKI. It is known that inhibition or deletion of key players in pathways of apoptosis or ER stress protects the kidney from cisplatin-induced injury in the standard dosing model (22, 25, 39). Therefore, we assessed cellular markers of ER stress and cell death proteins in both models. JNK phosphorylation and activation is associated with ER stress-induced apoptosis (37, 49). We found that pJNK was elevated in both models (Fig4). CHOP is also associated with ER stress, and was also activated in both models (Fig4) (14). However, cleaved caspase 3 (CC3), a marker of apoptosis, was not increased in the repeated dosing model. (Fig4). These data suggest that while both models show similar trends in activation of ER stress proteins, there may be less cell death activation in the repeated dosing model.

**Effect of dosing regimen evident in tissue pathology.** The standard dosing model of cisplatin-induced AKI is associated with changes in kidney pathology. We compared kidney pathology of the standard and repeated dosing models by examining tubular necrosis, loss of brush borders, tubule dilation, cast formation, presence of inflammatory cells and interstitial fibrosis, all of which are indicative of kidney injury and damage. Blinded analysis by a certified pathologist indicated tubular necrosis was significantly higher in the standard dosing model, compared to the repeated dosing model (Fig5A). In contrast, there was a significant loss of brush borders (Fig5B), an increase in tubular dilation (Fig5C), and an increase in cast formation (Fig5D) in both models. Interestingly, only the repeated dosing model displayed a significant increase in the presence of inflammatory cells and interstitial fibrosis (Fig5E,F). These data
demonstrate that there are key differences in kidney pathology between the standard and repeated dosing models.

**Fibrotic markers and fibrosis in the repeated dosing model.** Since pathology of kidney sections revealed tubulointerstitial fibrosis in the repeated dosing model, but not in the standard model, we examined known markers of fibrosis in this model. Following kidney injury, TGF-β is released from immune cells (32, 37). TGF-β can then signal through its receptor leading to phosphorylation of SMAD3, thereby activating pathways that increase extracellular matrix protein deposition, particularly fibronectin (8, 40). BMP-7 is also a member of the TGF-β superfamily and works to counteract the profibrotic activity of TGF-β (33). TGF-β, p-SMAD3, and fibronectin were all increased at the protein level in the repeated dosing model (Fig6A). Conversely, Bmp-7 mRNA expression was significantly decreased (Fig6B). COL1a1, which encodes for collagen type I protein and is a transcript marker of fibrosis, also significantly increased at the message level in the repeated dosing model (4)(Fig6B). PAI-1 is produced by resident and intrarenal inflammatory cells and inhibits fibrinolysis, leading to the accumulation of scar tissue in the kidney (20). Pai-1 mRNA expression was increased in the repeated dosing model (Fig6B). CDK2NA encodes for p16, and increased expression of CDK2NA is associated with cell cycle arrest and cellular senescence (49). Cdk2na mRNA expression was significantly increased following repeated dosing (Fig6B). Furthermore, collagen deposition as the result of extracellular matrix production can be quantified with Sirius red, fast green staining (SR/FG) which stains collagen red. We performed SR/FG staining and found that collagen levels increased in the kidneys following repeated dosing of cisplatin (Fig6C). α-SMA is a marker of myofibroblasts, which are known to deposit collagen (31, 34). α-SMA IHC indicated increased myofibroblasts following repeated dosing of cisplatin (Fig6D). Taken together, these data indicate that there are alterations in key mediators of kidney fibrosis in the repeated dosing model.
Comparison of a single, low dose of cisplatin (7VVV) to repeated dosing model. To determine if fibrosis is a result of a single, low dose of cisplatin, or rather repeated injury from several low doses, mice were administered a low dose of cisplatin (7 mg/kg) followed by three weekly vehicle injections (7VVV) and compared to mice receiving 4 weekly cisplatin injections (7777). BUN and SCr both increased significantly in the repeated dosing model, but not following a single low dose (Fig7A). Likewise, levels of Tnfa and Il6 mRNA expression increased with repeated dosing (7777) of cisplatin, but not following a single dose (7VVV; Fig7C). Western blot analysis of TGF-β and fibronectin in the kidney indicated that a single dose was not sufficient to cause an increase in these fibrotic markers (Fig7B). mRNA expression of Pai-1, Cdk2na, and Col1a1 did not increase, and Bmp-7 mRNA levels did not decrease in the single, low dose regimen (Fig7C). Quantification of SR/FG staining for collagen deposition and IHC staining for α-SMA for myofibroblasts also indicated that a single low dose was insufficient to elicit changes (Fig7D,E). These data suggest that fibrosis is a result of repeated injury to the kidney by several low doses of cisplatin.

Discussion

Treatment of human cancers with cisplatin often leads to nephrotoxic side effects that are cumulative and dose dependent. AKI occurs in some individuals even after one low dose of cisplatin, and multiple episodes of AKI can cause chronic kidney disease (CKD) (13). While Kobayashi et al. performed repeated cisplatin dosing in mice to determine circadian changes related to drug administration, here we have developed a model to study the effects of multiple “hits” of cisplatin-induced AKI and the subsequent development of CKD (31). The standard dosing model of cisplatin-induced AKI has limitations that cannot be overcome for studying AKI to CKD progression. For one, a single, high dose regimen is not clinically relevant. Patients are administered multiple low doses of cisplatin over an extended period of time. Secondly, the
The standard dosing model of cisplatin induces high levels of kidney injury and cell death through apoptosis and necrosis. This in turn results in a rapid loss of kidney function. With the repeated dosing model, cleaved caspase 3 as a measure of apoptosis is low, and pathology reveals a low level of tubular necrosis. This translates to lower injury levels and a smaller decline in overall kidney function. These lower levels of injury and the maintenance of kidney function with the repeated dosing model may be key to explaining how mice treated with multiple low doses of cisplatin are able to survive for 24 days, and perhaps even beyond that.

In the standard dosing model of cisplatin-induced AKI there is a strong inflammatory response, which involves TNFα elevation and elevation of its downstream targets. In the repeated dosing model, a similar inflammatory response is observed. However, IL6 expression is not as elevated in the repeated dosing model. IL6 plays a role in mounting an effective immune response and has been indicated in AKI. Particularly, IL6 expression is correlated with the onset and severity of AKI, and has been indicated as a potential urinary and plasma biomarker of AKI (19, 36, 50). The low levels of IL6 mRNA measured in the repeated dosing model may be indicative of a less severe form of AKI, and help explain why less injury is occurring in this model.

Pathology indicates that there is a significant increase in infiltrating immune cells in the repeated dosing model, despite less injury. It has been shown that rapid increase in the
macrophage population results in the development of fibrosis (48). Macrophages are known to play a major role in mounting an effective repair response post-injury, and have also been indicated in maladaptive repair (24). While M2 macrophages play a role in normal repair, an increase in M1 macrophages has been associated with maladaptive repair (24). Future studies of this model will focus on identifying the type of infiltrating immune cells, whether M1 to M2 transition is inhibited in macrophages, as well as whether this inhibition of phenotype change is responsible for the onset of fibrosis.

The repeated administration of low dose cisplatin induces fibrosis and this is a physiologically relevant process that could be targeted therapeutically. Grgic et al. have shown that kidney function can recover after a single round of injury induced by diphtheria toxin to transgenic mice expressing the diphtheria toxin receptor in proximal tubule cells (26). However, repeated injury in this model culminated in fibrosis as determined by increased levels of TGFβ-1, fibronectin, and collagen1α1 (26). In our model of repeated cisplatin dosing, we also see increased protein levels of TGFβ, fibronectin, and increased mRNA expression of Col1α1.

G2/M arrest, cellular senescence, and fibrosis have been indicated in the ischemia-reperfusion and unilateral ureteral obstruction mouse models (49). We found increased mRNA expression of Cdk2na, which is suggestive of cellular senescence. Furthermore, pJNK has been indicated as a downstream target of G2/M cell cycle arrest, and is believed to also play a role in promoting renal fibrosis (49). While, further studies are needed to determine whether or not G2/M arrest is indeed occurring following repeated cisplatin dosing, we do observe, an increase in pJNK without evidence of apoptosis.. Thus, the increase in pJNK may be indicative of G2/M arrest occurring, and as a result, potentially maladaptive repair. Taken together, these data suggest that potential mechanisms such as senescence and G2/M arrest warrant future in-depth investigation in this model in order to identify the mechanisms by which fibrosis is induced.
Fibrosis plays a major role in our repeated dosing model and is indicative that this model can be used to perform extensive mechanistic studies of the AKI to CKD progression. For example, studies should be completed to determine the type and role for infiltrating immune cells in fibrosis in the kidney. Furthermore, it would also be worthwhile to examine kidney function, injury, fibrosis, and inflammatory markers throughout the course of cisplatin treatment, rather than just at the end of the repeated dosing regimen. This would provide further insight into when fibrosis occurs temporally. Along with determination of the temporal timeline by which fibrosis occurs, structural studies looking at remodeling of the extracellular matrix during this process would provide insight into the morphological processes that occur within kidney tissue. Fortunately, Torres et al., through application of clearing multiphoton microscopy, have gained new, detailed morphological insight into the pathophysiology involved in AKI to CKD transition (43). While Torres et al. used a dosing regimen consisting of only two, relatively high doses of cisplatin, they found that significant remodeling of the ECM was occurring, although they did not document major increases in collagen levels (43).

Fibrosis not only plays a role in CKD, but also has been indicated in cancer metastasis (17). Our repeated cisplatin dosing model could be adapted to a cancer model to also look at the fibrosis both in the kidneys and cancer. Pabla et al., have shown that repeated administration of 10 mg/kg cisplatin for four weeks does alter the tumor size in a cancer xenograft model, and also leads to an increase in BUN and SCr levels, indicating loss of kidney function (38). While they did not look at fibrosis in this model, it would be interesting to determine whether cancer-associated fibrosis occurs with our repeated dosing model. Here, we utilize mice on the FVB background rather than the C57BL/6 mouse strain as mice on the C57BL/6 background are extremely resistant to developing interstitial fibrosis (46). In addition, numerous transgenic mouse models of cancer are available on the FVB background for studying the effects of repeated low dose cisplatin in mice with cancers that develop in the
proper tumor microenvironment. Thus, the data presented in this manuscript serve as the foundation for future studies aiming to determine the impact of repeated cisplatin dosing on the tumor and the kidney. Perhaps by further elucidating the mechanism by which fibrosis occurs, we can find new, desirable targets for development of both renoprotective and cancer therapeutics.

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References:


**Figure Legends:**

Figure 1. Survival curve of animals treated with the standard dosing model and repeated dosing model. Eight-week old male FVB mice were injected (i.p.) with saline vehicle, cisplatin (7 or 9mg/kg) once a week for four weeks (repeated dosing model), or 25 mg/kg cisplatin given once (standard dosing model). Mice were monitored daily for weight loss and changes in overall well-being and were sacrificed when moribund in accordance to IACUC guidelines. At day 24, surviving mice were sacrificed and analyzed.

Figure 2. Comparison of kidney function and injury between standard dosing model and repeated dosing model. Eight-week old male FVB mice were injected (i.p.) with saline vehicle, 25 mg/kg cisplatin given once (standard dosing model), or cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model). Animals were sacrificed 72 h after last injection. Levels of (A) BUN and SCr measured in the serum. (B) KIM-1 and NGAL measured in the urine. Data expressed as mean ± SEM, n=10. Statistical significance was determined by Student’s t-test ** indicates p<0.01 and ***indicates p<0.001.

Figure 3. Comparison of inflammatory markers between standard dosing model and repeated dosing model. Eight-week old male FVB mice were injected (i.p.) with saline vehicle, 25 mg/kg cisplatin given once (standard model), or cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model). Animals were sacrificed 72 h after last injection. mRNA levels of (A) *Tnfa*, (B) *Il6*, (C) *Il1-β*, (D) *Mcp-1*, and (E) *Cxcl1* were assessed in kidney cortex via real-
time qRT-PCR and were normalized to their vehicle control. Data expressed as mean ± SEM, n=10. Statistical significance was determined by Student’s t-test *indicates p<0.05, ** indicates p<0.01, and ***indicates p<0.001.

Figure 4. Repeated dosing model shows similar increases in markers of ER stress, but not cell death markers as compared to standard dosing model. Eight-week old male FVB mice were injected (i.p.) with saline vehicle, 25 mg/kg cisplatin given once (standard model), or cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model). Animals were sacrificed 72 h after last injection. Markers of ER stress and cell death were assessed in kidney cortex homogenates via western blot analysis.

Figure 5. Qualitative analysis of kidney pathology indices. Renal histological changes were assessed on H&E and PAS stained kidney sections (5μm thick). Eight-week old male FVB mice were injected (i.p.) with saline vehicle, cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model), or 25 mg/kg cisplatin given once (standard dosing model). Animals were sacrificed 72 h after last injection. (A) Tubular necrosis, (B) loss of proximal tubule brush borders, (C) proximal tubule dilation, (D) proximal tubule cast formation, (E) presence of inflammatory cells and (F) interstitial fibrosis. For figures (A-F), scoring of the sections was performed in a blinded manner by renal pathologist Dr. Megyesi using a scale of 0-4 (0=not present, 1=mild, 2=moderate, 3=severe, and 4=very severe renal histological changes in the proximal tubules). n = 5-10; data are the mean ± SEM. Statistical significance determined by one-way ANOVA followed by Tukey’s multiple comparison test, ***indicates p<0.001.

Figure 6. Assessment of fibrosis and fibrotic markers. Eight-week old, male FVB mice were injected (i.p.) with saline vehicle or cisplatin (7 mg/kg) once a week for four weeks (repeated dosing model), Animals were sacrificed 72 h after last injection. (A) Markers of fibrosis in the kidney cortex assessed via Western blot. (B) Measurement of mRNA levels of fibrotic markers in the kidney cortex assessed via real-time qRT-PCR. (C) SR/FG staining of kidney sections
and quantitation of staining (D) α-SMA IHC staining in the kidney cortex and quantitation of staining. Data expressed as mean ± SEM, n=5-10. Statistical significance was determined by Student’s t-test, *indicates p<0.05 and ** indicates p<0.01.

**Figure 7. Comparison of single low dose and repeated dosing.** Eight-week old, male FVB mice were injected (i.p.) with saline vehicle, cisplatin (7 mg/kg) once or cisplatin (7 mg/kg) a week for four weeks (repeated dosing model), Animals were sacrificed 72 h after last injection. (A) Levels of BUN and SCr assessed in the serum (B) Markers of kidney fibrosis assessed via Western blot. (C) mRNA levels of inflammatory cytokines and fibrotic markers in the kidney cortex measured by real-time PCR. (D) SR/FG staining of kidney sections and quantitation of staining. (E) α-SMA IHC staining in kidney and quantitation of staining. Statistical significance determined by one-way ANOVA followed by Tukey’s multiple comparison test, *indicates p<0.05, ** indicates p<0.01, and ***indicates p<0.001.
Figure 1.
Figure 2

(A) 
- BUN (mg/dL)
  - Standard: 0, 25
  - Repeated: 0, 7
  - p = 0.0692

(B) 
- SCr (mg/dL)
  - Standard: 0, 25
  - Repeated: 0, 7
  - **

- KIM-1 protein (pg/mL)
  - Standard: 0, 25
  - Repeated: 0, 7
  - ***

- NGAL protein (ng/mL) x 10^5
  - Standard: 0, 25
  - Repeated: 0, 7
  - **

Cisplatin (mg/kg)
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.