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### Strain differences in susceptibility to cisplatin-induced renal fibrosis

Gabrielle B. Oropilla  
*University of Louisville*

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Strain Differences in Susceptibility  
to Cisplatin-Induced Renal Fibrosis

By  
Gabrielle Oropilla

Submitted in partial fulfillment of the requirements  
for Graduation *magna cum laude*

University of Louisville  
May 2018

## **Background**

Cisplatin is a potent chemotherapeutic used to treat a multitude of solid cancers, including lung, testicular, bladder, cervical, and ovarian (1). Despite its effective chemotherapeutic properties, cisplatin has serious side effects including bone marrow suppression, ototoxicity, anaphylaxis, and nephrotoxicity. Of these, concern for developing cisplatin-induced nephrotoxicity leading to acute kidney injury (AKI) is high. After a single dose of cisplatin, 33% of patients will develop nephrotoxicity (2). Acute kidney injury is defined as the rapid loss of renal function, and the mortality rate for all forms of AKI ranges from 20-80%, with the highest mortality in ICU patients (3). Furthermore, cisplatin-induced AKI accounts for 19% of all cases of AKI, and thus puts a significant percentage of patients at risk for increased mortality (3).

Within the human body, cisplatin causes cross-linking of DNA strands, thereby inhibiting DNA replication and gene transcription (4). As a result, cancer cells can be destroyed and the cancer spreads at a slower rate or is even halted (3). However, the effect of cisplatin is damaging within the kidney, as cisplatin substantially accumulates in the proximal tubule cells and interferes with processes that lead to severe proximal tubule cell death, ultimately resulting in kidney injury and the subsequent loss of function (5). Proximal tubular injury is just one of the four major mechanisms of the pathophysiology of cisplatin-induced AKI, alongside inflammation, oxidative stress, and vascular injury in the kidney (6).

Upon cisplatin (CDDP) administration, there is a robust pro-inflammatory response, and levels of *Tnfa*, *Il6*, *Cxcl1*, and *Mcp-1* are increased. *Tnfa* is a mediator of inflammatory tissue damage, *Il6* promotes maintenance of a chronic inflammatory state, *Cxcl1* recruits neutrophils to sites of tissue inflammation, and *Mcp-1* plays a role in macrophage recruitment and activation.

More specifically, cisplatin increases serum and urine concentrations of *Tnfa*. Recent studies on *Tnfa* -1-mice have revealed that local production of *Tnfa* by resident kidney cells rather than by bone marrow-derived infiltrating immune cells may be key in cisplatin-induced AKI. However, inflammatory cells of the immune system (T cells, macrophages, neutrophils, and mast cells) that infiltrate the kidney tissue still play a role in the development of cisplatin-induced AKI (6).

In addition to high levels of proximal tubule damaging leading to a rapid decline in kidney function and AKI, recent studies have indicated that patients with cisplatin-induced AKI are ten times more likely to develop chronic kidney disease (CKD). CKD is marked by chronic inflammation, the development of fibrosis, and a gradual decline in kidney function. Patients with severe CKD often require dialysis, which is costly and does not always improve kidney function. In addition, CKD is also associated with an increased mortality rate (3).

Ideally, physicians would want to treat cancer patients with a therapeutic agent to curtail

**Table 1: General measures for prevention and treatment of cisplatin-induced AKI.**

- (1) Determine renal function (GFR) before each session of cisplatin treatment
  - (2) Determine the risk of AKI (high risk; females, elderly patients, dehydration, patients with CKD and repeated doses of cisplatin)
  - (3) Adjust cisplatin dose according to patient's renal function
  - (4) Start hydration (with saline) before cisplatin and maintain for at least 3 days after treatment
  - (5) Watch for electrolyte wasting (e.g., Mg), replace appropriately
  - (6) Avoid concomitant nephrotoxic agents (NSAIDs, aminoglycosides, contrast agents, etc.)
  - (7) Determine renal function within 1 week of treatment
  - (8) Amifostine may be considered in patients with high risk of AKI
  - (9) Consider newer, less nephrotoxic platinumums such as carboplatin and oxaliplatin
- GFR: glomerular filtration rate, AKI: acute kidney injury, CKD: chronic kidney injury, Mg: magnesium, and NSAIDs: nonsteroid anti-inflammatory drugs.

Source: Astolfi, Laura et al (7)

nephrotoxic side effects associated with cisplatin. In doing so, this would enable oncologists to increase dose of CDDP given to improve efficacy of the treatment. Unfortunately, there are no clinically available agents, but only general measures for prevention and treatment of cisplatin-induced AKI, as

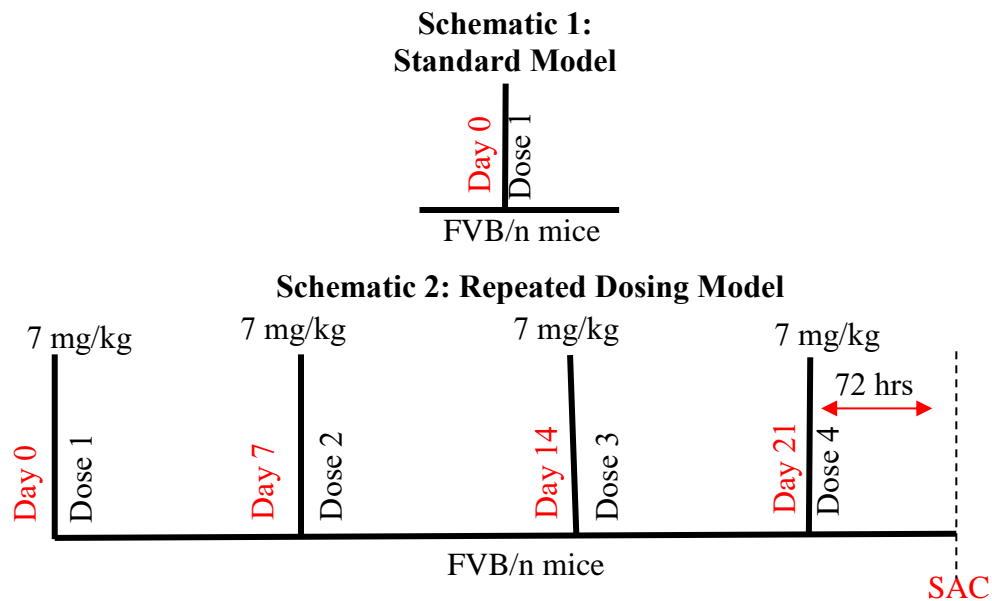
shown in Table 1 (7). Furthermore, potential therapeutics usually fail in early clinical trials (5). The reason for this is two-fold. For one, design of clinical trials for testing renoprotective/injury-ameliorating agents is poor. There are few biomarkers that can be used to determine improvement in renal injury, and often patients who are enrolled in studies have pre-existing conditions like diabetes or cardiovascular disease, that may already alter renal outcomes (5). Secondly, most experimentally designed therapeutics are tested in mouse models in such a way that may not be physiologically relevant to humans. In the Siskind lab, we believe the key to finding a renoprotective therapy is to change the mouse model of CDDP AKI.

The significance of the work proposed here is that 1.) we have developed a model of cisplatin dosing that better recapitulates the dosing regimen humans receive and may lead to more physiologically relevant outcomes, and 2.) this model can be used to determine long-term effects of cisplatin-induced kidney injury. This clinically relevant mouse model will reveal new insight to the differential effects of cisplatin, and over time, giving us the ability to identify novel therapeutic targets.

### **Significance**

Previously, the mouse model used for cisplatin-induced AKI consisted of injecting mice with one, high dose of cisplatin (20-25 mg/kg) that causes mice to die 3-4 days after treatment due to a high level of kidney injury and rapid loss of kidney function, as seen by Schematic 1. However, this model does not recapitulate the dosing regimen patients receive, which consists of administration of low doses of cisplatin over an extended period of time to curtail nephrotoxicity while maintaining therapeutic efficacy. In addition, this model does not allow for long-term studies of renal outcomes that may be associated with CKD. To address these limitations, our

laboratory has developed a repeated dosing regimen of cisplatin in which 8-week FVB/n mice are treated with 7 mg/kg cisplatin once a week for 4 weeks and are sacrificed at Day 24, as seen by Schematic 2. With this dosing regimen, FVB/n mice survive the 24-day dosing regimen and develop renal fibrosis indicative of CKD (5).



FVB/n mice are a widely used inbred strain, with white fur and blindness. While we used the FVB/n mouse strain to develop our repeated dosing model, the most commonly used strain of mouse in research, particularly kidney research, is the C57BL/6J with a phenotype of black fur. In fact, a majority of commercially available genetically manipulated mouse models are on the C57BL/6J background. The ultimate goal of developing our clinically relevant cisplatin repeated dosing regimen is to identify and test novel, therapeutic targets for the treatment of kidney injury, of which being able to use genetically manipulated mouse models would be beneficial. Thus, being able to utilize our repeated dosing regimen in C57BL/6J mice would be ideal.



FVB/n



C57BL/6J mice

Unfortunately, this strain has been shown to be resistant to renal fibrosis in some models of experimental fibrosis, including BSA-induced fibrosis and unilateral ureteral obstruction. Although it has not been reported whether or not C57BL/6J mice are resistant to developing interstitial fibrosis (the type of fibrosis that FVB/n mice develop with repeated dosing of cisplatin), this may be a potential drawback of incorporating our repeated dosing regimen into C57BL/6J mice for further experiments. Table 2 outlines the susceptibility of both C57BL/6J and FVB/n mice to different types of organ fibrosis (8).

|                   | <b>C57BL/6J mice</b> | <b>FVB/n mice</b> |
|-------------------|----------------------|-------------------|
| <b>Renal</b>      | Resistant            | Susceptible       |
| <b>Hepatic</b>    | Resistant            | Resistant         |
| <b>Cardiac</b>    | Resistant            | Resistant         |
| <b>Pulmonary</b>  | Susceptible          | Susceptible       |
| <b>Intestinal</b> | Susceptible          | Susceptible       |

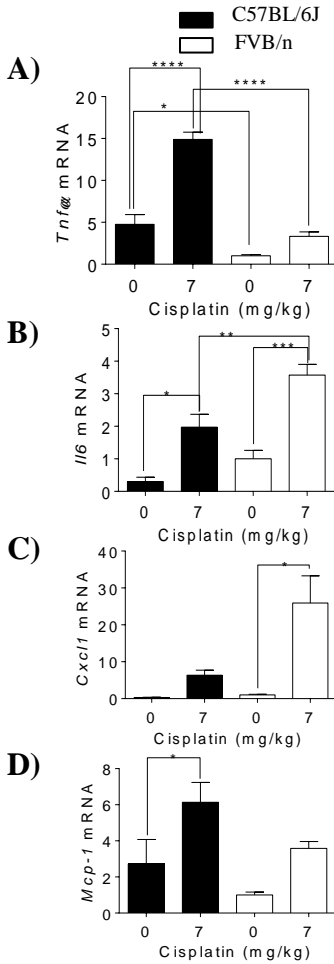
### **Preliminary Results**

Our repeated dosing model in FVB/n mice treated with 7 mg/kg of cisplatin resulted in a robust inflammatory response with elevated cytokine and chemokine levels, but low levels of cell death. We saw the development of renal fibrosis as evidenced by the increase of fibrotic markers like fibronectin, TGF $\beta$ ,  $\alpha$ -SMA IHC, and Sirius Red/Fast Green Staining. As fibrosis is a major trait of CKD, these data demonstrate that the repeated dosing model can be used to study AKI to CKD progression (9).

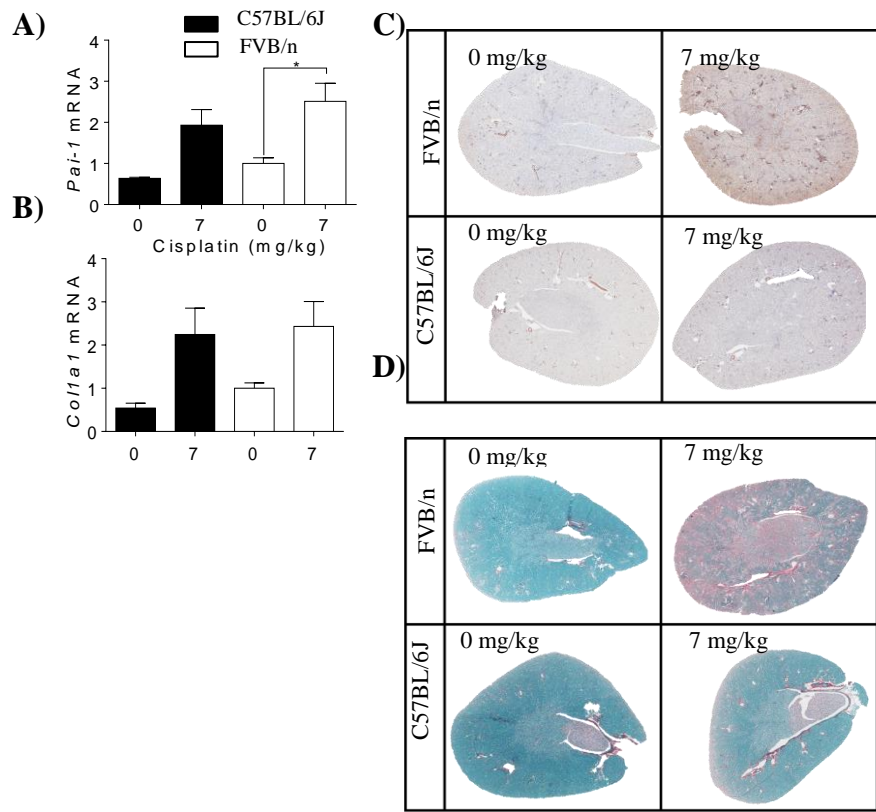
This repeated dosing model was then done in C57BL/6J mice in order to determine if the same dose of cisplatin would also lead to the development of fibrosis in this strain. However, when treated with 7 mg/kg cisplatin once a week for four weeks, preliminary results indicated that there was not a significant change in inflammatory cytokine and chemokine levels (Figure

1). Furthermore, C57BL/6J mice did not develop renal fibrosis as determined by expression *Colla1* and *Pai-1*, levels of  $\alpha$ -SMA on IHC, and Sirius Red/Fast Green Staining (Figure 2). *Colla1* (Figure 2A), encodes for collagen type 1 protein, and *Pai-1* (Figure 2B), produced by inflammatory cells and leads to the accumulation of scar tissue in the kidney, did not show a significant change in levels.  $\alpha$ -SMA IHC is a stain for myofibroblasts, which produce collagen leading to fibrosis (Figure 2C). Sirius Red/Fast Green is a stain for collagen deposition (Figure 3D). Sirius Red binds to all types of collagen, whereas fast green stains non-collagenous proteins (10). In the FVB/n mice, there was a distinct color change between the vehicle and cisplatin treated kidney section. Conversely, in the C57BL/6J mice, there is a lack of change in colors between the vehicle and cisplatin kidney sections. This raised the question of whether C57BL/6J mice are resistant to developing interstitial fibrosis overall or if this strain requires a higher dose of cisplatin in order to develop fibrosis.





**Figure 1. Inflammatory markers in C57BL/6J background mice and FVB/n background mice.** 8 wk old C57BL/6J and FVB/n mice were injected (i.p.) with saline vehicle or 7 mg/kg cisplatin once a week for four weeks. Animals were sacrificed 72 h after the last injection. Expression of (A) *Tnf $\alpha$* , (B) *Il6*, (C) *Cxcl1*, (D) *Mcp-1* was assessed in via real-time qRT-PCR and were normalized to B2M control. n=5-10; \*indicates P<0.05, \*\* P<0.01, etc.



**Figure 2. Fibrotic markers in C57BL/6J background mice and FVB/n background mice.** 8 wk old C57BL/6J and FVB/n mice were injected (i.p.) with saline vehicle and 7 mg/kg cisplatin once a week for four weeks. Animals were sacrificed 72 h after the last injection. Markers of pro-fibrotic markers in the kidney cortex were assessed by real-time qRT-PCR for (A) *Pai-1* and (B) *Col1a1*. Levels of myofibroblasts were determined by (C)  $\alpha$ -SMA IHC. Total collagen deposition was determined by (D) Sirius Red/Fast Green Staining. n=5-10; \*indicates P<0.05, \*\* P<0.01, etc.

The **Specific Aim** was to determine if C57BL/6J mice require a higher dose of cisplatin in order to develop interstitial fibrosis with repeated dosing of cisplatin. With this information, the repeated dosing regimen model could be optimized to be used to model fibrosis in this strain of mouse. Once a potential therapeutic target is found, it will be easier to purchase common knock out or genetically manipulated mice to validate the therapeutic target.

### **Materials and Methods**

\*Materials and methods were repeated in the same manner as the work performed by Sharp, et. Al (5).

*Reagents and antibodies.* The following antibodies were purchased from Cell Signaling (Beverly, MA) unless otherwise noted: cleaved caspase-3, fibronectin, and  $\beta$ -actin. Pharmaceutical grade cisplatin was purchased directly from the University of Louisville hospital pharmacy.

*Animals.* FVB/n mice and C57Bl/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a 12:12-h light-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 of the University of Louisville and followed the guidelines of the American Veterinary Medical Association. Mice received either 7 or 9 mg/kg cisplatin administered by an intraperitoneal injection once a week for 4 wk. Mice were monitored for weight loss and signs of discomfort/distress on a daily basis. All mice survived the course of treatment and were euthanized 72 h after the fourth injection of cisplatin. Serum was prepared and stored at  $-80^{\circ}\text{C}$ . The kidneys were flash frozen in liquid nitrogen or fixed in 10% neutral buffered formalin.

*Blood Urea Nitrogen (BUN) determination and Neutrophil Gelatinase Associated Lipocalin (NGAL).* BUN (DIUR-500) was determined using a kit from Bioassay Systems

(Hayward, CA) following the manufacturer's instructions. ELISA for NGAL (DY1857 R&D Systems) was performed on the urine as directed by the manufacturer.

*Protein Quantification.* Homogenates were made from kidney cortex by homogenization in Cell Extraction Buffer containing a Complete Protease Inhibitor Cocktail Tablet and Phosphatase Inhibitor Cocktail Tablet. Homogenates were centrifuged at 15,000 X *g* for 10 minutes at 4°C. Supernatants were removed, vortexed, aliquoted, and stored at -80°C until use. Protein concentrations were determined using BCA Assay (Bio-Rad, Hercules CA).

*Western Blot Analysis.* 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 0.1% Tween-20 (TBST) for 15 minutes. Membranes were incubated with 1:5000 dilutions of primary antibody overnight at 4°C. The next morning, membranes were washed 3 times for 5 minutes each with TBST containing 5% (w/v) dried milk. After incubation for 1 hour at room temperature with secondary antibodies conjugated with horseradish peroxidase (1:20,000 in TBST containing 1.25% (w/v) dried milk), membrane proteins were detected by chemiluminescence substrate.

*Gene Expression.* Total RNA was isolated using EZNA RNA Kit. 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 with predesigned TaqMan assays. Tumor necrosis factor-alpha (*Tnf-α*, Mm00443258\_m1), interleukin-6 (Il6; Mm00446190\_m1), chemokine ligand (c-x-c motif) 1 (CXCL1; Mm04207460\_m1), monocyte chemotactic protein-1 (MCP-1; Mm00441242\_m1), plasminogen activator inhibitor-1 (PAI-1; Mm00435860\_m1), collagen type 1a1 (Coll1a1; Mm00801666\_g1), and the normalization gene beta 2 microglobulin (B2M; Mm00437762\_m1)

were purchased from Life Technologies (Grand Island NY), and used in combination with iTaq (Bio-Rad).

*Immunohistochemistry.* Kidney sections (5 microns 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, S2003) for 10 minutes, followed by two 5 minute PBS washes. Slides were then blocked with avidin for 10 minutes followed by a PBS wash, and then biotin for 10 minutes followed by another wash in PBS (Dako, X0590). Slides were further blocked with 5% normal goat serum in 0.1% TBST for 1 hour at room temperature.  $\alpha$ -SMA primary rabbit antibody (Abcam, ab5694) was added to slides at a concentration of 0.5  $\mu$ g/mL, and allowed to incubate at 4°C overnight. Biotinylated goat antirabbit IgG antibody (1:25000) (Vector laboratories, BA-1000) was added to each section and incubated for 30 minutes at room temperature. Slides were rinsed twice with PBS for 5 minutes each. Vector ABC reagent (Vector laboratories, PK-7100) was added to each section and incubated for 30 minutes at room temperature. Slides were rinsed 2 times with PBS for 5 minutes each, followed by the addition of NovaRed Substrate to detect HRP (Vector Laboratories, SK-4800) to each section for 5-7 minutes. 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).

*Sirius Red/ Fast Green Staining.* Kidney sections (5 microns 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 (1.2% w/v, Ricca

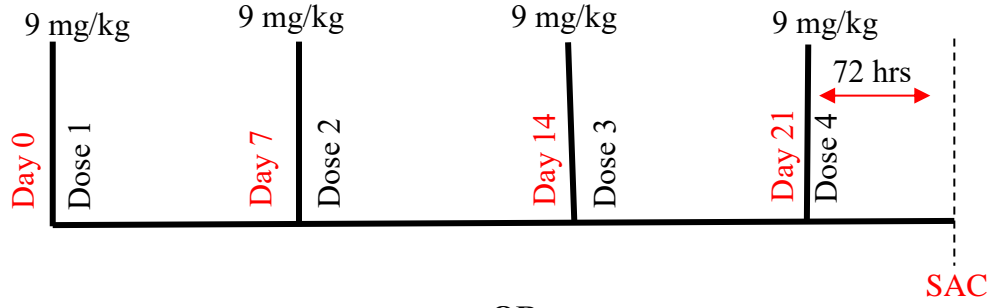
Chemicals; 5860-32) containing 0.1% Sirius Red (Sigma, 365548) and 0.1% Fast Green (Sigma, F7258). Slides were washed with 5% glacial acetic water until water ran clear. Tissue samples were then dehydrated and fixed using Permount (Fisher, F-SP15-100).

*Statistical Analysis Data.* Data are expressed as means  $\pm$  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. The criterion for statistical differences was  $p < 0.05^*$ ,  $p < 0.01^{**}$ ,  $p < 0.001^{***}$ , and  $p < 0.0001^{****}$ . For statistical analysis of the survival curve, Log-Rank (Mantel-Cox) test was used.

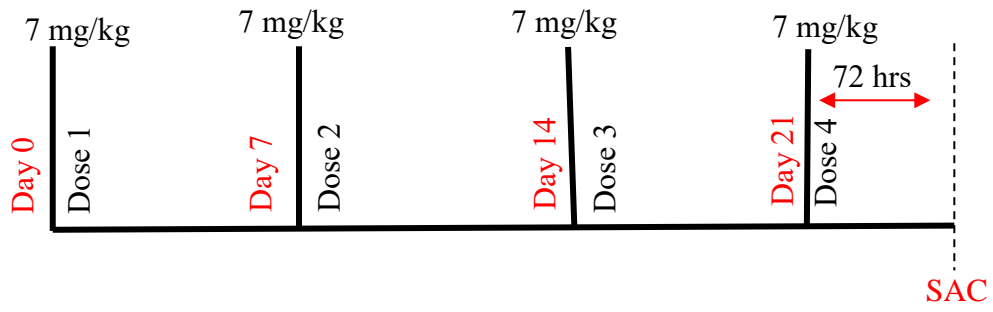
## **Results**

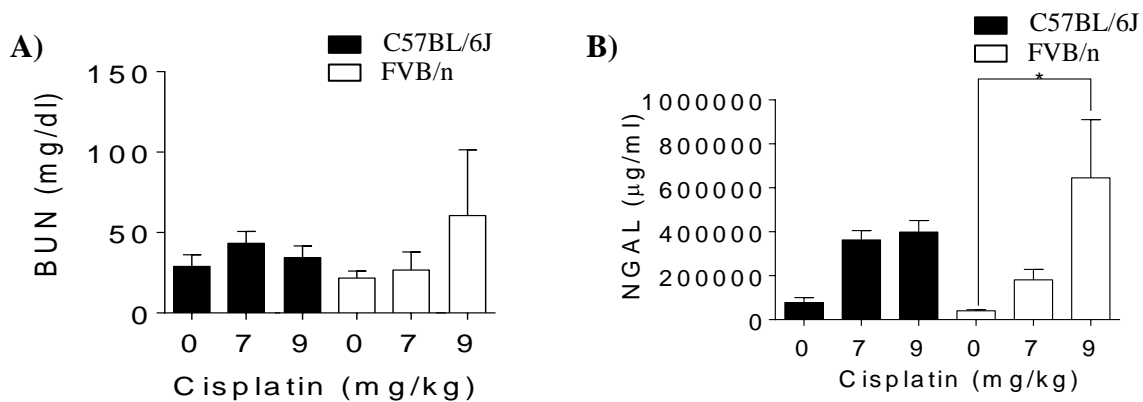
Our established repeated dosing mouse model of cisplatin-induced kidney injury reflects the dosing regimen that human patients receive. However, optimizing this model to be used in multiple strains that have variations in susceptibility to some forms of renal fibrosis remains a hurdle. Thus, we wanted to address this issue by determining optimal dose in the most common used strain of mouse- C57BL/6J. As seen in the preliminary data, 7 mg/kg cisplatin was optimal for FVB/n mice to develop fibrosis but not for C57BL/6J mice. To this end, five male C57BL/6J mice were treated with vehicle saline once a week for four weeks and ten male C57BL/6J mice (8 weeks old) were treated with 9 mg/kg of cisplatin once a week for four weeks. Both groups were sacrificed three days after the last injection at Day 24. In order to compare the data collected from the C57BL/6J mice, the same protocol was conducted with FVB/n mice with 9 mg/kg of cisplatin once a week for four weeks and sacrificed on Day 24. This was then compared to the 7 mg/kg cisplatin for C57BL/6J and FVB/n mice as seen by Schematic 3.

**Schematic 3: Repeated Dosing Model**



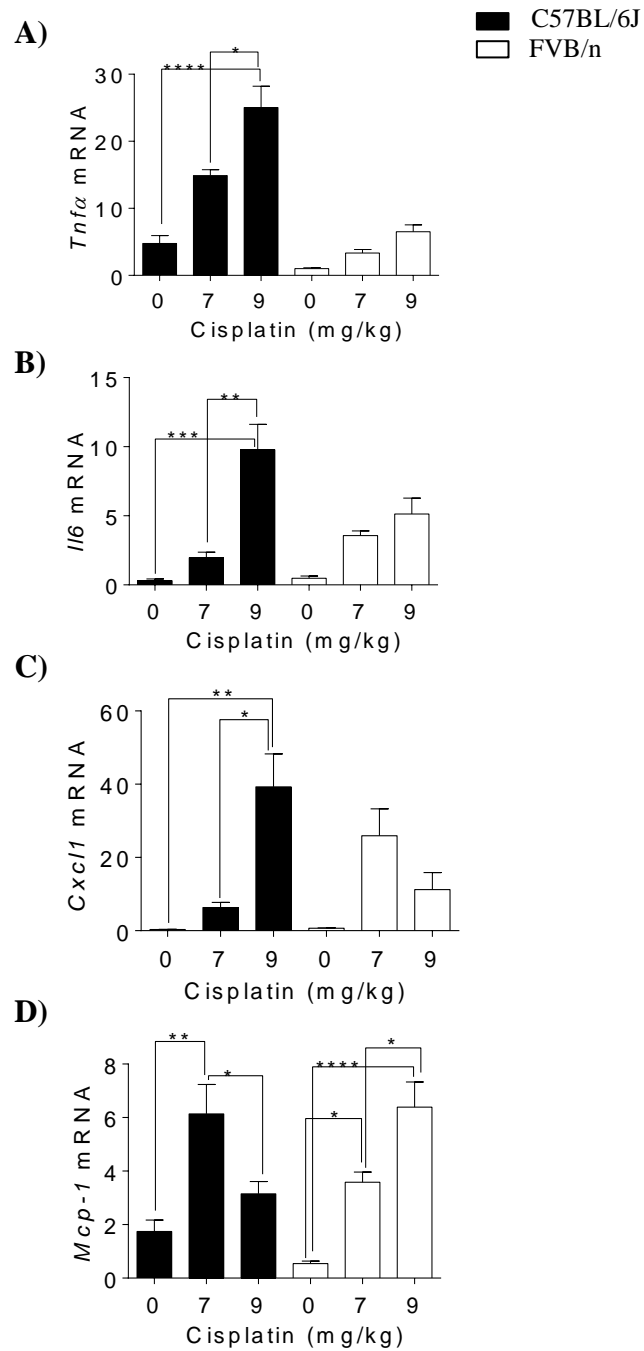
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**Figure 3. Kidney function and injury markers with repeated dosing model.** 8 wk old C57BL/6J and FVB/n mice were injected (i.p.) with saline vehicle and 7 mg/kg cisplatin once a week for four weeks. Animals were sacrificed 72 h after the last injection. Levels of (A) Blood, urea, nitrogen (BUN) as a measure of kidney function, and (B) Neutrophil gelatinase associated lipocalin (NGAL), a kidney injury marker measured in urine. n=5-10; \*indicates P<0.05, \*\* P<0.01, etc.

**Markers of kidney function and injury in the serum and urine of FVB/n and C57BL/6J mice.** Blood urea nitrogen (BUN) is a measure of kidney function, in which its levels increase in the blood when the filtering capacity of the kidney is significantly reduced. BUN levels were not significantly increased at 7 or 9 mg/kg in either strain of mouse, suggesting little decline in kidney function. However, BUN is a relatively insensitive marker of kidney injury. Thus, we measured NGAL. Neutrophil gelatinase associated lipocalin (NGAL) is a measure of kidney injury and is a sensitive biomarker of AKI. In C57BL/6J mice, NGAL levels increased with 7 mg/kg and to the same level with 9 mg/kg. However, while FVB/n mice had slightly lower NGAL at 7 mg/kg compared to C57BL/6J, at 9 mg/kg NGAL was greater.

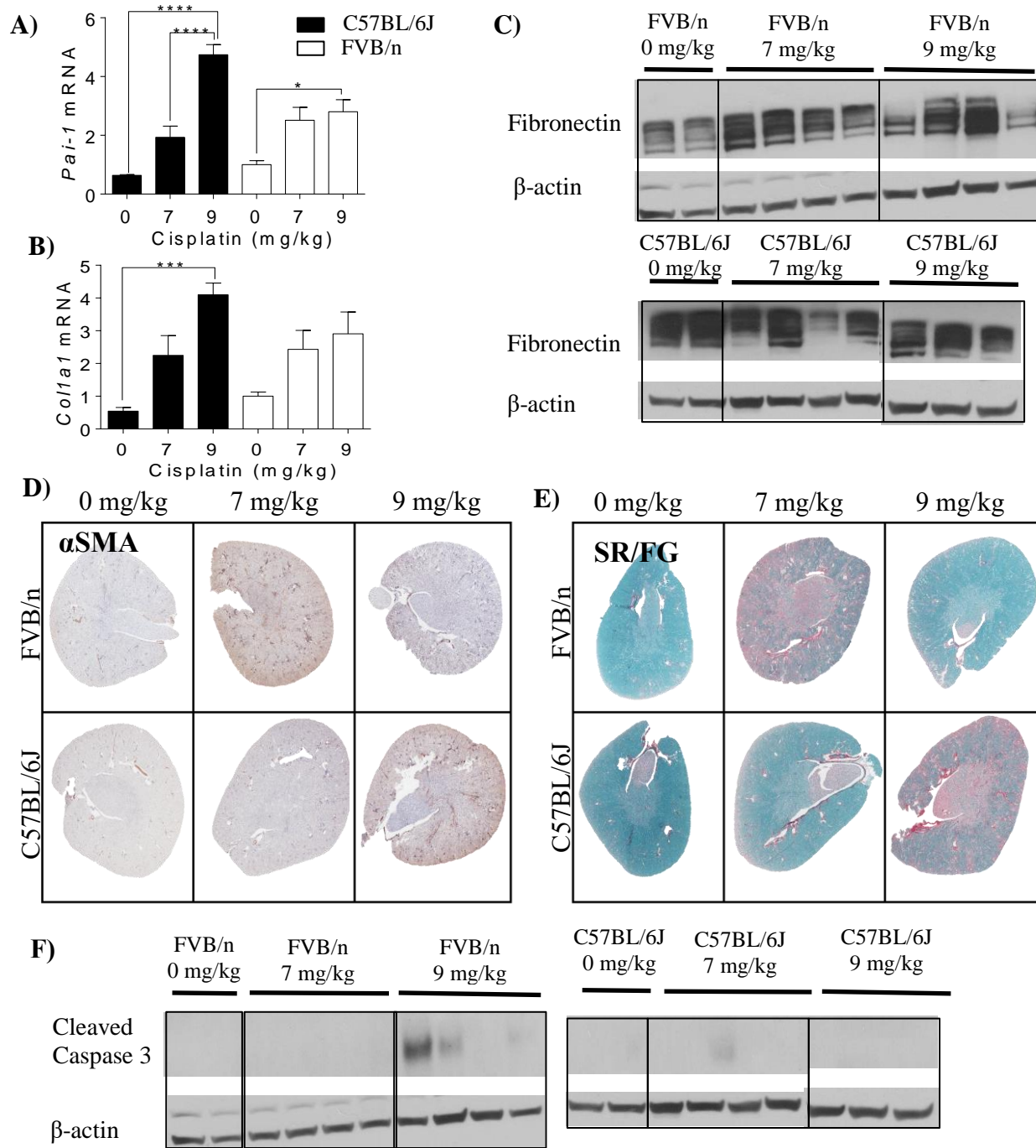


**Figure 4: Inflammatory markers in C57BL/6J and FVB/n mice.** 8 wk old C57BL/6J and FVB/n mice were injected (i.p.) with saline vehicle and 7 mg/kg cisplatin once a week for four weeks. Animals were sacrificed 72 h after the last injection. Expression of (A) *Tnfα*, (B) *IL6*, (C) *Mcp-1*, (D) *Cxcl1* was assessed in via real-time qRT-PCR and were normalized to B2M control. n=5-10; \*indicates  $P < 0.05$ , \*\*  $P < 0.01$ , etc.



**Inflammatory cytokines and chemokines in FVB/n and C57BL/6J mice.** Both strains of mice treated with repeated dosing of cisplatin had a robust inflammatory response. As previously mentioned, (A) *Tnfa* is a mediator of inflammatory tissue damage, (B) *Il6* promotes maintenance of a chronic inflammatory state, (C) *Cxcl1* recruits neutrophils to sites of tissue inflammation, and (D) *Mcp-1* plays a role in macrophage recruitment and activation.

*Tnfa* levels (A) increased approximately 15-fold after 7 mg/kg repeated dosing in C57BL/6J mice. Levels further increased with 9 mg/kg. Overall, *Tnfa* levels in FVB/n mice treated with CDDP were lower compared to those seen in C57BL/6J mice. *Tnfa* increased 5-fold at 7 mg/kg and was only 7-fold elevated with 9 mg/kg in FVB/n mice. The same trend was seen for *Il6* and *Cxcl1* levels. *Il6* levels (B) increased approximately 3-fold after 7 mg/kg repeated dosing in C57BL/6J mice and further increased with 9 mg/kg. *Il6* increased 5-fold at 7 mg/kg and was only 6-fold elevated with 9 mg/kg in FVB/n mice. Furthermore, *Cxcl1* (C) levels increased 15-fold at 7 mg/kg and was further increased at 40-fold at 9 mg/kg in C57BL/6J mice. There was a 32-fold increase at 7 mg/kg but only a 18-fold increase at 9 mg/kg for FVB/n mice. *Mcp-1* levels (D) displayed further increased levels at 7 mg/kg compared to 9 mg/kg in C57BL/6J mice. *Mcp-1* levels increased approximately 6-fold after 7 mg/kg repeated dosing in C57BL/6J mice but was only 3-fold elevated with 9 mg/kg. *Mcp-1* increased 5-fold with 7 mg/kg and was 6-fold elevated with 9 mg/kg in FVB/n mice.



**Figure 5: Fibrotic markers in C57BL/6J and FVB/n mice.** 8 wk old C57BL/6J and FVB/n mice were injected (i.p.) with saline vehicle and 7 mg/kg cisplatin once a week for four weeks. Animals were sacrificed 72 h after the last injection. Markers of pro-fibrotic markers in the kidney cortex were assessed by real-time qRT-PCR for (A) *Pai-1* and (B) *Colla1*. Markers of fibrosis were assessed by Western Blot for (A) *Fibronectin* and  $\beta$ -actin for the control. Levels of myofibroblasts were determined by (C)  $\alpha$ -SMA IHC. Total collagen deposition was determined by (D) Sirius Red/Fast Green Staining. Markers of apoptosis were assessed by Western Blot for (F) *Cleaved Caspase 3*. n=5-10; \*indicates P<0.05, \*\* P<0.01, etc.

**Fibrotic Markers in C57BL/6J and FVB/n mice.** To determine the presence of fibrosis, several markers were used. *Pai-1*, a pro-fibrotic marker, levels (A) increased approximately 2-fold after 7 mg/kg repeated dosing in both C57BL/6J and FVB/n mice. *Pai-1* levels in C57BL/6J mice were higher after 9 mg/kg repeated dosing compared to 9 mg/kg for FVB/n mice. The same trend was seen for *Colla1* (B). Levels of *fibronectin* (C) increased at both 7 mg/kg and 9 mg/kg in C57BL/6J mice. The same trend followed for FVB/n mice with increased levels of *fibronectin* at both 7 mg/kg and 9 mg/kg.

$\alpha$ -SMA IHC (D) is a stain for myofibroblasts which produce extracellular matrix and are expressed to repair and restore homeostasis after injury. In the C57BL/6J mice, there is a lack of change in colors between the vehicle and 7 mg/kg cisplatin kidney sections but a distinct change in color between the vehicle and 9 mg/kg cisplatin treated kidney section. Conversely, in the FVB/n mice, there is a distinct color change between the vehicle and 7mg/kg cisplatin treated kidney section. However, there is not a distinct change in color between the vehicle and 9 mg/kg cisplatin treated kidney section.  $\alpha$ -SMA staining occurs only at 9 mg/kg in C57BL/6J mice but at 7 mg/kg in FVB/n mice. These trends are consistent with the Sirius Red/Fast Green stain (E), which is a stain for collagen deposition that results from extracellular matrix production.

Together, these results suggest that C57BL/6J mice develop fibrosis when treated with 9 mg/kg of cisplatin. As seen in the preliminary data, 7 mg/kg of cisplatin is not a high enough dose of cisplatin in order to induce interstitial fibrosis indicative of that which a human patient develops. However, 7 mg/kg of cisplatin is the optimal dose for FVB/n mice. When treated with 9 mg/kg of cisplatin, FVB/n mice do not develop as severe of fibrosis as the mice treated with 7 mg/kg of cisplatin but instead show high levels of *Cleaved Caspase-3* (CC3), indicating cell

death and perhaps a more severe form of injury. This is further confirmed by the presence of CC3 in FVB/n mice treated with 9 mg/kg for western blot analysis (F).

## **Discussion**

Clinically, patients receive cisplatin in low doses over an extended period of time to maintain therapeutic efficacy while curtailing nephrotoxic side effects. Our repeated dosing model better recapitulates the human dosing regimen, and with this study, we have shown that it can be repeated in both FVB/n and C57BL/6J strains of mice. With repeated dosing, fibrosis indicative of CKD develops. Patients with AKI are 10x more likely to develop CKD, and in addition, other studies have shown that even patients who do not have clinical signs of AKI develop poor long-term outcomes. Studies have been conducted to determine the extent of genetic susceptibility as a risk factor for many diseases, in particular fibrosis (8). As previously displayed in Table 2, different mouse strains have varying susceptibility to developing fibrosis with the same organ. FVB/n mice have previously been shown to be susceptible to renal fibrosis, specifically interstitial fibrosis, whereas C57BL/6J are not, according to Walkin's studies (8).

However, our results show that C57BL/6J mice can develop fibrosis when administered a higher dose of cisplatin. At this higher dose of 9 mg/kg, inflammatory cytokines and chemokine levels increased (*Tnfa*, *Il6*, *Cxcl1*, and *Mcp-1*) as well as the pro-fibrotic markers *Pai-1*, *Coll1a1*, *Fibronectin*.  $\alpha$ -SMA and Sirius Red/Fast Green staining further indicated fibrosis in FVB/n and C57BL/6J mice. By studying different strains of mice, we may be better able to understand whether genetic susceptibility plays a role in fibrogenesis.

Human patients may be susceptible to developing kidney injury and eventually chronic kidney disease due to genetic factors. According to Varrier, the risk for developing AKI is

determined by patient factors and nephrotoxic exposures (11). These patient factors include hypericaemia, obesity, hypoalbuminaemia and certain genetic polymorphisms (11). AKI can also lead to end-stage renal diseases such as chronic kidney disease (CKD), with risk factors such as race, gender, age, and family history, and in particular for factors for developing CKD include being of African-American decent, older age, low birth weight, and family history of kidney disease (12). Determining whether fibrosis develops between two genetically different mice strains can lead to insight and eventually novel treatments for fibrosis and kidney injury in human patients.

With our repeated dosing model at 7 mg/kg in C57BL/6J mice, we saw that this dose was too low to develop interstitial fibrosis. However, we have now optimized this model in C57BL/6J mice with 9 mg/kg cisplatin so that they develop fibrosis. C57BL/6J mice are the most commonly used strain of mouse in kidney research and the majority of the commercially available genetically manipulated mouse models are on the C57BL/6J background. By doing so, we can now use commonly available transgenic mice to identify and test novel therapeutic targets with the more clinically relevant mouse model of cisplatin induced kidney injury.

Furthermore, this information can be translated to the differential effects of cisplatin in humans and susceptibility to drug toxicities in order to determine a patient's optimal administration and dosage.

One future direction could be to specifically study the chemokine *Cxcl1*. As seen in Figure 4, *Cxcl1* follows the same pattern of increasing levels that we see with the development of fibrosis, in which levels only increase at 7 mg/kg in FVB/n mice and only increase at 9 mg/kg in C57BL/6J mice. *Cxcl1* is a chemoattractant protein for neutrophils, indicating that neutrophils may be necessary for the development of injury and subsequent fibrosis. A study could then be

conducted using knockout mice for *Cxcl1* to then see if the mice develop fibrosis.

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