NOVEL CISPLATIN KIDNEY INJURY BIOMARKERS AND

TRANSPORTER INTERACTIONS

By

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ABSTRACT OF THE DISSERTATION

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Despite the recent development of new immunotherapies and anticancer drugs, cisplatin remains an important component of chemotherapeutic regimens for the treatment of solid tumors. However, use of cisplatin is limited by nephrotoxicity, which occurs in about one-third of patients and leads to small but permanent changes in kidney function over the long-term. Current clinical markers, such as serum creatinine and estimated glomerular filtration rate, are limited in their ability to detect acute kidney injury (AKI). Additionally, a recent study reported a potential interaction *in vivo* between concurrently administered antiemetic drug, ondansetron, and cisplatin that may exacerbate cisplatin-induced nephrotoxicity. One mechanism of cisplatin kidney injury is the ability of renal transporters to mediate its urinary secretion. Presently, the degree of interaction between antiemetic drugs and cisplatin renal transporters is unknown. The purposes of this dissertation research were to assess time-dependent changes in urinary protein biomarkers in cisplatin treated oncology patients with subclinical nephrotoxicity, characterize the renal expression and regulation of the novel urinary biomarker calbindin in cisplatin-mediated acute kidney injury, and determine the extent of antiemetic drug inhibition of renal transporters involved in cisplatin secretion. In 57 patients with solid tumors receiving outpatient cisplatin therapy (≥25 mg/m^2), mean serum creatinine was unchanged following cisplatin infusion. Compared to baseline values, several novel biomarkers were significantly increased in the urine. B2M was increased 3-fold by day 3,

while KIM-1, TFF3, and calbindin were elevated 2-fold, 2-fold, and 8-fold by day 10, respectively. In a subset of 27 patients, time-dependent changes in KIM-1, calbindin, and TFF3 were assessed during early and subsequent cycles of cisplatin-containing chemotherapy. Although significant increases were seen during the early cycle, baseline biomarker levels remained elevated for KIM-1 and TFF3 and for all three biomarkers (KIM-1, TFF3, and calbindin), time-dependent changes were modest during subsequent cycles. Subsequent mechanistic studies were performed to better understand the intrarenal regulation of one biomarker, calbindin, during cisplatin AKI in mice as well as identify potential cisplatin-drug interactions due to alterations in cisplatin transporter activity *in vitro*. In mice treated with cisplatin, calbindin protein was robustly elevated in urine prior to elevations in serum creatinine and blood urea nitrogen. A time-dependent decrease in renal calbindin protein was observed on day 4 with concurrent up-regulation of calbindin mRNA. Finally, *in vitro* models revealed that 5-HT_3 antagonists used to treat cisplatin-induced emesis dose-dependently inhibited the activity of OCT2 and MATE1, two transporters responsible for cisplatin secretion. Importantly, the most potent inhibitor was ondansetron, which inhibited transport of a probe substrate ASP^+ in MATE1 overexpressing HEK293 cells at concentrations that are pharmacologically relevant (IC_{50}: 0.1 μM) and also caused accumulation and inhibition of ASP^+ transport in polarized MDCK tubule cells that overexpress OCT2 and MATE1. These data suggest that potent inhibition of MATE1-mediated efflux of cisplatin from human proximal tubules by antiemetic drugs may increase intratubular concentrations of cisplatin. This research demonstrates that novel urinary protein biomarkers are responsive to cisplatin therapy in the absence of clinically detectable AKI during early cycles, but are not reflective of progressive kidney damage during subsequent cycles of cisplatin therapy. Additionally, this research provides new mechanistic understanding of urinary calbindin release as well as the potential for antiemetic drug inhibition of renal cisplatin transporters.

DEDICATION

This dissertation is dedicated to my parents and husband.

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CHAPTER 1.2

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CHAPTER 1.3.4

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CHAPTER 2

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APPENDIX 1

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APPENDIX 2

Chang C, Hu Y, Hogan S, Mercke N, Gomez M, O'Bryant C, Bowles D, **George B**, Wen X, Aleksunes LM, Joy MS (2017) Pharmacogenomic Variants May Influence the Urinary Excretion of Novel Kidney Injury Biomarkers in Patients Receiving Cisplatin. *Int J Mol Sci*. 18(7):1333. PMID: 28640195

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CHAPTER 1: GENERAL INTRODUCTION

1.1. Human Kidney Physiology and Function

The structure and function of the kidneys are well understood and have been described in detail in *Toxicology of the Kidney* (Tarloff and Lash, 2004) and *Casarett & Doull's Toxicology: The Basic Science of Poison* (Klaassen, 2008). The following information has been adapted from these texts unless otherwise noted.

1.1.1. Kidney Function

Mammals possess two kidneys located in the retroperitoneal cavity. The kidneys are responsible for several critical functions including removal of metabolic wastes, regulation of extracellular fluid volume, electrolyte composition, and acid-base balance. In this capacity, the kidneys excrete solutes and generate urine through several processes including glomerular filtration, tubular reabsorption, and tubular secretion. Kidneys also synthesize and release hormones including renin and erythropoietin, which help to regulate blood pressure and form red blood cells, and convert vitamin D_3 to the active 1,25-dihydroxy vitamin D_3 form.

1.1.2. Kidney Structural Anatomy

The functional unit of the kidney, known as the nephron, is divided into various sections based on structure and function as depicted in Figure 1.1. Starting from the cortex region, the first segment of the nephron is known as the renal corpuscle which include the Bowman's capsule and the glomerulus, followed by the proximal convoluted tubule, proximal straight tubule, loop of Henle (descending thin limb, thin ascending limb, and thick ascending limb), distal tubule, and collecting duct.

The main blood supply to the kidneys is provided by the renal artery, which divides into interlobar, arcuate, and interlobular arteries, giving rise to arterioles that surround the glomerulus. Blood flow exits the glomerulus through the efferent arteriole. The afferent and efferent arterioles are controlled by the sympathetic nervous system and respond to nervous stimulation or hormones such as angiotensin II and vasopressin, which affect the blood flow and glomerular pressure. The glomerulus is a specialized cluster of cells comprised of endothelial cells with fenestrated cytoplasm and epithelial cells with foot processes positioned around a basement membrane. A portion (20%) of the blood entering the glomerulus is filtered into a protein-free and cell-free ultrafiltrate, which enters Bowman's space and the tubular portion of the nephron. The filtration of macromolecules is related to the molecular weight and charge; small molecules (50-65 kDa) are freely filtered whereas large molecules such as albumin are not. Neutral or cationic molecules are filtered more freely than anionic molecules.

The proximal tubules are made of three discrete segments: the S1 (Pars convoluta), S2 (transition between pars convoluta and pars recta), and S3 (the pars recta) segments. All three segments are characterized by a tall brush-border, lysosomal system and abundant mitochondria. The presence of mitochondria signals that the proximal tubule is the workhorse of the nephron with high energy demands. The proximal tubule reabsorbs approximately 60-80% of solute and water from the ultrafiltrate. Therefore, proximal tubular toxicity has a major impact on the water and solute balance of the filtrate and urine. The majority of electrolytes are reabsorbed by the proximal tubule including Na^{+}, K^{+}, HCO_3^{-}, Cl^{-}, PO_4^{3}-, Ca^{2+}, and Mg^{2+}. The proximal tubules also contain numerous transport systems, which drive the concentration-dependent transport of amino acids, glucose, as well as low-molecular-weight proteins. Proximal tubules also control and

drive secretion of various organic anions and cations through transporters from the post-glomerular blood into the tubular fluid.

The loop of Henle is comprised of the thin descending and ascending limbs, thick ascending limb which are involved in further concentrating the ultrafiltrate. Water (20%) and Na^+ and K^+ (25%) are reabsorbed in this segment. The thick ascending loop of Henle is particularly vulnerable to hypoxic injury due to high rates of Na^+/K^+-ATPase activity and oxygen demand/low supply. The distal tubular cells also contain numerous mitochondria, similar to proximal tubules, but lack the well-developed brush border and lysosomal system. Distal tubules are relatively impermeable to water and reabsorb remaining Na^+, K^+, and Cl^-. The late distal tubule, collecting tubule and ducts complete the final regulation and adjustment of urinary volume and content. The remaining Na^+ is reabsorbed along with the secretion of K^+ and H^+.

1.2. Xenobiotic Transporters and Kidney Injury

1.2.1. Introduction

An important function of the kidneys is to remove metabolic waste products and xenobiotics from the circulation. During this process, the kidneys may become vulnerable to injury. The pathways for xenobiotic elimination include glomerular filtration and tubular secretion. Drugs smaller than 50 to 65 kDa pass through the glomerulus in the unbound form (Brater 2000). Large or charged chemicals are often cleared from the circulation by transporter-mediated renal secretion. Much of the xenobiotic secretion in the kidneys occurs in proximal tubules. The uptake of organic anions and cations as well as zwitterions from the blood is the first step in renal secretion. Subsequently, chemicals can undergo active efflux by transporters on the apical brush border membrane where they are added to the ultrafiltrate or by transporters on the basolateral membrane where they re-enter the blood. Chemicals in the ultrafiltrate are either removed to the urine or reabsorbed by apical transporters and returned to the circulation. Depending on the physicochemical properties, reactivity, and propensity for binding to intracellular components, chemicals can also be retained inside tubule cells. Alterations in the rate and/or extent of uptake versus efflux can influence the net intracellular concentrations of drugs, environmental contaminants, and the formation of metabolites. Therefore, the focus of this review will be primarily on the transport mechanisms and subsequent toxicities of chemicals in proximal tubules.

1.2.2. Renal Transporters

Uptake Transporters

Proximal tubules contain several uptake proteins that are part of the solute carrier (SLC) drug transporter family, including the organic anion transporters (OATs), the organic cation transporters (OCTs), the organic cation/carnitine transporters (OCTNs), and the

organic anion transporting polypeptides (OATP). Human OAT1-3 (SLC22A6-8) proteins are largely found on the basolateral membrane of proximal tubules (Hosoyamada et al. 1999, Tojo et al. 1999, Cha et al. 2001, Enomoto et al. 2002, Kojima et al. 2002, Bahn et al. 2005, Eraly et al. 2006) although OAT2/Oat2 may also localize to the apical surface of human and rat proximal tubules (Shen et al. 2015). OAT4 (SLC22A11) and Oat5 (SLC22A10/19) proteins have been detected on the apical membrane of human and rat renal proximal tubules, respectively (Ekaratanawong et al. 2004, Anzai et al. 2005). OATs are responsible for the uptake of organic anions, such as beta-lactam antibiotics and nonsteroidal anti-inflammatory drugs (Apiwattanakul et al. 1999, Takeda et al. 1999). Endogenous dicarboxylate anions (such as alpha-ketoglutarate) are considered to be the driving force for organic anion exchange (Wolff et al. 1992, Sekine et al. 1997, Sweet et al. 1997, Bakhiya et al. 2003, Koepsell and Endou 2004). Among the OCTs, human kidneys are abundantly enriched with OCT2 (SLC22A2) protein on the basolateral membrane of proximal tubules (Karbach et al. 2000, Motohashi et al. 2002). By comparison, rodent kidneys have two isoforms (Oct1 and 2, Slc22a1 and 2, respectively) with overlapping functions on the basolateral membrane (Karbach et al. 2000). Substrates for OCT2 include the hypoglycemic drug metformin and the cancer drug cisplatin (Kimura et al. 2005, Yokoo et al. 2007, Ciarimboli et al. 2010). OCT3 (SLC22A3) is also present in human kidneys and transports cations; however, its role in nephrotoxicity remains largely unknown. OCTN1/Octn1 and OCTN2/Octn2 (SLC22A4 and 5) are both localized to the apical side of rodent and human proximal tubules (Tamai et al. 1997, Tamai et al. 2001, Tamai et al. 2004). OCTN1/Octn1 and OCTN2/Octn2 transport carnitine and cation drugs including the antiseizure drug valproic acid and the antibiotic cephaloridine (Ohashi et al. 1999, Ganapathy et al. 2000). Among OATPs, OATP4C1 (SLCO4C1) is detected at high levels on the basolateral membranes of proximal tubules (reviewed in Mikkaichi et al. 2004, Mikkaichi et al. 2004). Typical OATP

substrates include cardiac glycosides and anti-cholesterol statin drugs (Nakai et al. 2001, Mikkaichi et al. 2004).

Efflux Transporters

Renal efflux transporters include ATP-binding cassette (ABC) and SLC transporters. Multidrug resistance protein 1 (MDR1, ABCB1), multidrug resistance-associated protein 2, 4 (MRP2, 4, ABCC2, 4), and breast cancer resistance protein (BCRP, ABCG2) belong to the ABC transporter family. Multidrug and toxin extrusion 1 and 2-K (MATE1, 2-K, SLC47A1, 2) are efflux transporters in the SLC transporter family.

MDR1/P-glycoprotein (P-gp) is localized to the apical membrane of rodent and human proximal tubules (Jette et al. 1996, Ernest et al. 1997) and actively exports drugs with diverse structures including chemotherapeutic agents, steroids, cardiac glycosides, and immunosuppressant drugs (reviewed in Schinkel and Borst 1991, Tang-Wai et al. 1995). MRPs function as extrusion pumps for organic anions, including beta-lactam antibiotics, as well as glucuronide and glutathione (GSH) conjugates such as acetaminophen-glucuronide and arsenic-GSH (Schaub et al. 1997, Ghanem et al. 2005, Kato et al. 2008). Two isoforms found on the apical side of proximal tubules are MRP2 (Schaub et al. 1997, Scheffer et al. 2000) and 4 (van Aubel et al. 2002). In the kidneys, human MRP4 has 5-fold higher mRNA expression than human MRP2 as well as greater affinity for uptake of the organic anion, para-aminohippurate (PAH) in membrane vesicles (Km values: 160 μM for MRP4 and 5 mM MRP2) (Smeets et al. 2004). BCRP accepts a wide variety of organic anions as substrates including anticancer drugs, sulfated conjugates, and the Hoechst 33342 dye (Kim et al. 2002, Merino et al. 2005, Robey et al. 2005, Huang and Sadee 2006, Ando et al. 2007, Enokizono et al. 2007, Pan et al. 2007, Myllynen et al. 2008). Human BCRP protein is localized to the apical membrane of

proximal tubules but at levels lower than observed in rodent kidneys (Huls et al. 2008). MATE transporters are members of the SLC family and function as cation exchangers. Rodent Mate1 as well as human MATE1 and MATE2-K are found on brush border membranes and efflux organic cations in exchange for protons (Otsuka et al. 2005, Masuda et al. 2006, Nishihara et al. 2007, Tanihara et al. 2007). In *in vitro* models, MATE proteins typically act as uptake carriers, in particular, when non-polarized cells are acidified to pH 6.5 using ammonium chloride (Ohta et al. 2006, Terada et al. 2006). Typical substrates for MATE1 include the histamine H2 receptor antagonist, cimetidine, the antidiabetic agent, metformin, and the herbicide, paraquat (Ohta et al. 2006, Terada et al. 2006, Chen et al. 2007). MATE2-K shares overlapping substrates with MATE1 including cimetidine and metformin (Masuda et al. 2006). Many cationic drugs are eliminated using renal secretion by the OCT2/MATE pathway. Imbalances in OCT2-mediated uptake or MATE-mediated efflux can result in accumulation of cationic toxins in proximal tubules. More recently, the Na^+-dependent phosphate transporter 4 (NPT4, SLC17A3) was identified as an efflux transporter of urate and important for the pathogenesis of gout (Dehghan et al. 2008). NPT4 protein was shown to localize to the apical side of human proximal tubules and transports various organic anions including PAH and ochratoxin A (Jutabha et al. 2011).

1.2.3. Prototypical Nephrotoxicants

This section provides an overview of chemicals that injure proximal tubules using renal transport mechanisms.

1.2.3.1. Environmental Chemicals

A wide range of naturally-occurring and synthetic chemicals are introduced into the environment daily. These include nephrotoxicants such as halogenated hydrocarbons,

herbicides, mycotoxins, and heavy metals that contaminate food and water sources. Regulatory efforts have largely limited human exposure to these chemicals. Nonetheless, understanding the renal absorption and secretion of each chemical provides mechanistic insight into how transporters influence their propensity to cause proximal tubule damage.

Trichloroethylene

Solvents, such as trichloroethylene (TCE), have contaminated food, water, and air through widespread industrial use. Epidemiological studies have attributed renal cell carcinomas to the long-term exposure of industrial workers to high levels of TCE in Germany (Vamvakas et al. 1998, Charbotel et al. 2006). In rats and mice, exposure to oral and inhaled TCE caused renal tubular cytomegaly and karyomegaly as well as the formation of tumors, most notably in male rats due to their higher rate of metabolism (NTP 1988, NTP 1990). In support of this, metabolism studies identified the reactive and toxic metabolites of TCE responsible for DNA mutations and protein alkylation (Anders et al. 1988). In the liver, the majority of TCE is oxidized by cytochrome P450 enzymes (Miller and Guengerich 1982, Lash et al. 1999, Lash et al. 2006, Lash et al. 2007), while a small portion is conjugated to GSH by glutathione S-transferases to form S-1,2,-dichlorovinyl-L-cysteine (DCVC) and subsequently N-acetyl-DCVC (Lash et al. 1995, Lock et al. 1996, Birner et al. 1997, Lash et al. 1999, Lash et al. 1999, Lash et al. 2001, Lash et al. 2006). Following removal from the liver back to the circulation, DCVC and N-acetyl-DCVC accumulate in proximal tubules where N-acetyl-DCVC can be deacetylated (Uttamsingh and Anders 1999). This is important because DCVC is a substrate for renal beta lyases, which further metabolize cysteine (Cys)-containing compounds into highly reactive species that bind DNA, proteins, and lipids (Dekant et al. 1988, Hayden et al. 1991, Muller et al. 1998, Volkel and Dekant 1998). Thus, GSH-conjugated metabolites of

TCE are important mediators of renal injury (Lash et al. 1995). Rabbits administered DCVC developed dose-dependent morphological necrosis of the proximal tubules and proteinuria (Silber et al. 1986). Primary human proximal tubules exhibit apoptosis, necrosis, and proliferation following exposure to DCVC at a range of concentrations from 10 to 1000 μM (Lash et al. 2001).

Since DCVC is charged at physiological pH, it was predicted that transport proteins would be required for entry into cells (Birner et al. 1993). To determine the role of transport in the tubular damage caused by TCE metabolites, rabbit renal cortical slices were directly exposed to DCVC and N-acetyl DCVC (Wolfgang et al. 1989). Probenecid, a prototypical Oat inhibitor, prevented N-acetyl DCVC uptake by 80% and reduced cellular damage, indicating the involvement of Oats in basolateral influx. Further studies using rabbit proximal tubules demonstrated that unlabeled DCVC, PAH, and probenecid inhibited the basolateral uptake of radiolabeled DCVC by 70-85% (Figure 1.2) (Dantzler et al. 1998). Further, PAH and probenecid also attenuated the damage produced by DCVC. Similar results have revealed a role for human OAT-mediated uptake of DCVC. Chinese hamster ovary (CHO) cells stably transfected with hOAT1 exhibited uptake of DCVC that was blocked by PAH (IC_{50}: 208 μM) (Groves et al. 2003). Conversely, interference of organic cation transporters using tetraethylammonium (TEA) had no effect on DCVC uptake in rat renal proximal tubules (Lash and Anders 1989), suggesting little interaction with basolateral OCT transporters.

Multiple amino acid transporters have also been implicated in the influx of DCVC using isolated rat proximal tubule cells and LLC-PK1 cell lines (Schaeffer and Stevens 1987, Schaeffer and Stevens 1987, Lash and Anders 1989). In isolated rat proximal tubules, DCVC uptake was prevented using substrates of the system A, ASC, and L transporters,

which are proteins responsible for the uptake of amino acids such as alanine, serine, and cysteine (Lash and Anders 1989). Amino acid transporters along the apical membrane are important for DCVC reabsorption. Rabbit and rat brush-border membrane vesicles were used to assess the apical uptake of DCVC (Schaeffer and Stevens 1987, Wright et al. 1998). In rabbit brush-border membrane vesicles, [^{35}S]DCVC was transported by a saturable and Na^+-dependent process. This transport could be inhibited up to 80% by several amino acids including L-phenylalanine, L-leucine, and L-cysteine. Based on kinetic transport studies, the authors concluded that inhibition of [^{35}S]DCVC uptake by L-phenylalanine was competitive, which suggested the two chemicals shared a common transport pathway. Basolateral and apical uptake pathways allow entry of DCVC and N-acetyl DCVC into renal cells, which may contribute to the acute and chronic toxicity seen with TCE exposures.

Few studies have explored the role of efflux transporters in the disposition of TCE metabolites. Recently, membrane vesicles generated from mouse proximal tubules were used to demonstrate that Mrp2 transported N-acetyl DCVC, but not DCVC, in an ATP-dependent manner (Km 36.6 μM) (Tsirulnikov et al. 2010). Further, mouse proximal tubule-derived cells endogenously expressing Mrp2 showed marked inhibition (40-50%) of N-acetyl DCVC transport from the basolateral-to-apical direction after transfection with antisense mouse Mrp2 RNA. Transcellular transport of DCVC was not affected by transfection of anti-Mrp2 RNA, consistent with the membrane vesicle data. The authors suggest that N-acetyl DCVC may be consecutively taken up by the basolateral Oat1 transporter and excreted by Mrp2 into the ultrafiltrate. Additional studies are necessary to understand the exit pathway for other metabolites, such as DCVC, from proximal tubule cells.

Paraquat

Paraquat (PQ), a 1,1'-dimethyl-4,4'-bipyridinium cation, was discovered in 1955 and has been widely used as an herbicide since 1962. It is an effective weed killer that becomes inactivated once in contact with the soil leading to relatively low bioaccumulation in the environment. The ability of PQ to elicit toxicity extends to mammalian species as clinical cases of pulmonary and renal injury were reported as early as the 1960s. As PQ is not absorbed through intact skin, the majority of the poisoning cases are due to accidental or intentional ingestions. In 1966, Bullivant reported two of the initial human cases of accidental PQ poisoning by ingestion in New Zealand (Bullivant 1966). The peak concentration of PQ in humans was observed within 2 to 4 hours after ingestion (Houze et al. 1990). PQ rapidly distributes into tissues such as the lungs, kidneys, and liver. Pneumotoxicity is the primary cause of mortality because PQ concentrates in the lungs using the polyamine uptake system (Rose et al. 1974). Animal studies have also demonstrated that repeated, low doses of PQ can cause degeneration of the dopaminergic neurons in the substantia nigra pars compacta (McCormack et al. 2002).

The kidneys are responsible for eliminating the majority of PQ that is absorbed into the systemic circulation, and are therefore also susceptible to injury (Daniel and Gage 1966). PQ is minimally metabolized and largely excreted in the urine unchanged. Elimination of PQ occurs through a combination of glomerular filtration and active tubular secretion such that the total renal clearance exceeds the glomerular filtration rate (GFR) (Chan et al. 1998). Secretion of PQ was found to be concentration- and time-dependent, and inhibited by organic cations in rats (Chan et al. 1996). The mechanism of damage to the lungs involves redox cycling which leads to the formation of a PQ radical and superoxide (Smith 1987); a similar mechanism has been proposed for renal cells. Administration of PQ to rodents cause acute tubular degeneration, necrosis, and

hemorrhage in the kidneys within 17 to 24 h (Ecker et al. 1975, Lock 1979, Lock and Ishmael 1979). These histopathological changes eventually lead to acute renal failure, which impairs the ability of the kidneys to further excrete PQ (Beebeejaun et al. 1971, Kodagoda et al. 1973, Vale et al. 1987). Clinically, PQ toxicity presents as reduced GFR, albuminuria, and glycosuria (Vaziri et al. 1979). Ingestion of PQ at doses greater than 20 mg/kg leads to acute tubular necrosis and renal failure in humans (Vale et al. 1987).

The proximal tubules are the primary site of PQ damage, which suggests that this portion of the nephron is responsible for facilitating its secretion. In vitro studies have investigated the transport of PQ in LLC-PK1 cells. These studies pointed to basolateral OCT transporters as mediators of the uptake of PQ from the blood (Figure 1.3). In LLC-PK1 cells, the transport of PQ concentration in the basolateral-to-apical direction was saturable with time (Chan et al. 1996). Additionally, transport was inhibited by organic cations, including quinine, cimetidine, and TEA, with no change observed in the presence of organic anions. Similar results were demonstrated in primary rat proximal tubule cells (Chan et al. 1996). Mechanistic transport studies using HEK293 cells overexpressing human OCT1, 2, or 3 revealed that overexpression of OCT2, but not OCT1 or OCT3, enhanced the accumulation (12-fold) and cytotoxicity (18-fold) of PQ (Chen et al. 2007). For example, the cytotoxicity (LC_{50}) of PQ in HEK293-OCT2 cells was observed at 23 μM (compared to 417 μM in control cells). Additionally, uptake of PQ in cells transfected with a common variant of the *OCT2/SLC22A2* gene (rs316019; A270S) increased accumulation 3-fold compared to the wild-type reference allele (Chen et al. 2007). The *SLC22A2* A270S variant has an allele frequency between 10% and 20% in some populations (Kang et al. 2007). While this variant is associated with decreased metformin transport in vitro (Song et al. 2008), the interaction of PQ with

SLC22A2 A270S enhanced PQ transport. Further investigation of this variant is needed to understand the transport differences between substrates.

Apical secretion of PQ in the kidneys declines in the presence of certain cations including TEA and 1-methyl-4-phenylpyridinium (MPP^+), suggesting the involvement of the organic cation/H^+ exchangers, MATE1 and MATE2-K (Wright and Wunz 1995, Chan et al. 1996). In HEK293 cells overexpressing human MATE1 or rat Mate1, uptake of PQ was saturable and time-dependent while empty vector controls showed no activity (Chen et al. 2007). The maximal rate of PQ transport in rat Mate1 expressing cells was 2.5-fold higher than human MATE1 but the two orthologs shared similar affinity (Km values). The cytotoxicity of PQ in HEK293 cells overexpressing human MATE1 revealed an LC_{50} value of 125 μM compared to 717 μM for vector controls (Chen et al. 2007). Infusion of 50 mg/kg PQ intravenously in Mate1-deficient mice increased renal PQ concentrations by 147% at 90 min compared to wild-type mice (Li et al. 2011). Additionally, the plasma area-under-the-curve and maximal concentration of PQ in Mate1-null mice were increased 64% and 57%, respectively, compared to wild-type controls. Mate1-null mice administered PQ (20 mg/kg, intraperitoneally) had significantly higher mRNA levels of two kidney injury biomarkers, N-acetyl-Beta-D-glucosaminidase (NAG) and kidney injury molecule-1 (Kim-1) as well as enhanced necrosis and tubular degeneration compared to wild-type mice (Li et al. 2011). A direct role for MATE2-K still needs to be elucidated.

Interestingly, Mate1-null mice had reduced accumulation of PQ in their livers which correlated with an induction of liver Mdr1a mRNA expression (Li et al. 2011). However, this compensatory increase was not seen in the kidneys. Furthermore, administration of dexamethasone (a Mdr1 inducer) to rats reduced the severity of PQ-induced pulmonary damage, providing further anecdotal evidence supporting Mdr1 as an efflux transporter

for PQ (Dinis-Oliveira et al. 2006). While the authors of this study did not evaluate the effect of dexamethasone on renal accumulation of PQ, the urinary excretion of PQ was reportedly unchanged. Subsequent studies have further analyzed PQ transport and cytotoxicity *in vitro* in human proximal tubule cells and HEK293 cells overexpressing MDR1 as well as in Mdr1a/1b-null mice (Wen et al. 2014). Intracellular accumulation of PQ was reduced by 60% in MDR1-overexpressing HEK293 cells. Intracellular accumulation of PQ was increased by 50% in proximal tubule cells treated with PSC833, a specific inhibitor of MDR1 or transfected with siRNA targeted against the MDR1/ABCB1 (Wen et al. 2014). Enhanced PQ accumulation after PSC833 treatment corresponded with a 2-fold decrease in cell viability compared to non-treated human proximal tubule cells. Likewise, accumulation of PQ was 750% higher in the kidneys of Mdr1a/1b-null mice after 4 h (Wen et al. 2014). Histopathology of kidney sections from Mdr1a/1b-null mice showed increased susceptibility to PQ nephrotoxicity, as manifested by epithelial cell swelling, vacuolation, apoptosis, and necrosis compared to wild-type mice.

Ochratoxin

A number of fungal by-products, called mycotoxins, cause nephrotoxicity. These include citrinin, aflatoxin B1, ochratoxins and others. In this review, we will focus on ochratoxin due to the abundance of existing data regarding its renal transport. Ochratoxin A (OTA) is a mycotoxin produced by Aspergillus ochraceous, Aspergillus carbonarius, and Pencillium verrucosum (Harwig 1983). OTA is structurally different from ochratoxin B and C and represents the most prevalent form (O'Brien and Dietrich 2004). Human exposure to OTA results from the consumption of contaminated small grains (barley, wheat, corn), coffee beans, and grapes. OTA can cause various chronic tubule-interstitial syndromes including Balkan nephropathy (Pavlovic et al. 1979, Tapia and

Seawright 1984). Initial reports suggesting that mycotoxins cause nephrotoxicity were based on observations in pigs (Tapia and Seawright 1984, Technology 2003). Pigs fed mycotoxins consumed large amounts of water, urinated continuously, and had pain near the kidneys. Although the kidneys are an important target for OTA-induced injury, OTA can also cause neurotoxicity, teratogenicity, and immunotoxicity in rodents (Haubeck et al. 1981, Mayura et al. 1984).

OTA has a long circulating half-life (103 h in rats) due to extensive binding to albumin (99%) in the serum (Kumagai 1985). As a result, the glomerular filtration of OTA is minimal (Stojkovic et al. 1984, Hagelberg et al. 1989). OTA toxicity is observed in proximal and distal tubules due to reabsorption in these portions of the nephron following secretion and to a much lesser extent, filtration (Cooper 1979, Stein et al. 1985, Ringot et al. 2006). Male Wistar rats treated with 0.5 mg/kg of OTA had impaired proximal tubule functioning, glycosuria, and enzymuria (Kumagai 1985). OTA is carcinogenic in mice, with renal tumors particularly evident in male mice (Bendele et al. 1985). The mechanism of OTA toxicity has been characterized. OTA inhibits protein synthesis in mice by competing with phenylalanine (Creppy et al. 1984). In addition, OTA can deplete ATP production (Poor et al. 2014), covalently bind to DNA (Pfohl-Leszkowicz and Manderville 2012), as well as produce free radicals (Sorrenti et al. 2013), which collectively contribute to renal cell death.

In vitro studies have shown that OTA interacts with the same transport system as other organic anions (Figure 1-4) (Jung et al. 2001). In vesicles from the renal brush border and basolateral membranes of canine kidneys, OTA preferentially inhibited OAT-mediated transport of PAH, without affecting organic cation transport (Sokol et al. 1988). OTA produced a dose-dependent depletion of ATP in nephron segments, most

significantly in the S2 and S3 segments of the proximal tubules (Jung et al. 2001). Probenecid, an organic anion inhibitor, protected against the depletion of ATP by OTA, further supporting the involvement of the organic anion transport pathway. Similar results have been reported in isolated renal proximal tubules from rabbits (Groves et al. 1998). OAT1 was identified early as a key transporter of OTA. Rat OAT1 expressed in Xenopus oocytes mediated the sodium-independent uptake of OTA with a Km value of 2 μM (Tsuda et al. 1999). S2 segment proximal tubule cells isolated from transgenic mice stably expressing human OAT1, OAT3, or OAT4 exhibited OTA uptake, which could be competitively blocked by OAT inhibitors (PAH, probenecid, piroxicam) (Jung et al. 2001, Babu et al. 2002). OAT4 is typically found on the apical membrane of human renal proximal tubules and functions as an organic anion exchanger with the dicarboxylate ion, glutarate (Ekaratanawong et al. 2004). Stable expression of the human *OAT4/SLC22A11* gene in mouse proximal tubules increased uptake of OTA (Babu et al. 2002). The estimated Km for OAT4-mediated uptake of OTA was 23 μM, suggesting high affinity transport. Organic anions, such as probenecid, penicillin G, aspartame and others, significantly inhibited the influx of OTA by OAT4 (Babu et al. 2002). One study has explored the interaction of organic cations with peritubular uptake of OTA in single rabbit renal proximal tubules (Welborn et al. 1998). Inhibition of OTA was blocked by the organic cation TEA by only 7%. Based on these studies, the basolateral and apical uptake of OTA largely involves OAT proteins.

Other organic anion transporters that participate in the renal uptake of OTA include rat Oatp1a1 (formerly known as Oatp1) and rat Oat5. Oatp1a1 is located on the apical surface of the rat proximal tubules (Bergwerk et al. 1996). Rat Oatp1a1-mediated uptake of OTA in stably transfected CHO cells yielded a Km of 29 μM (Eckhardt et al. 1999). Rat Oat5 expressed in Xenopus laevis oocytes enabled the sodium-independent uptake

of OTA (Km 0.34 μM, Vmax 0.26 pmol/oocyte/h) (Youngblood and Sweet 2004). Immunohistochemical staining showed that Oat5 was localized to the apical membrane of proximal tubules in the corticomedullary regions as well as the S2 and S3 segments. Oat5 may have a counter ion specificity that is unique when compared to other Oats since its function was unaffected by addition of dicarboxylate-type ions and urate to the transport media for Xenopus oocytes expressing mouse Oat5 (Youngblood and Sweet 2004). Oat-K1 is a kidney-specific, organic anion carrier found on the apical side of the proximal straight tubules of the rat kidney, which enables the pH-independent reabsorption of OTA (Takeuchi et al. 2001). OTA was found to be reabsorbed in all nephron segments to varying degrees and was independent of pH in male Wistar rats (Dahlmann et al. 1998). Moreover, the reabsorption of OTA was inhibited by sulfobromopthalein, a substrate of Oat-K1 in microinfused rat proximal tubules.

Multiple efflux transporters have been investigated for their ability to remove OTA from cells. Membrane vesicles isolated from HEK cells expressing recombinant human MRP efflux transporters were used for indirect and direct measurement of OTA transport. OTA inhibited MRP1- and MRP2-mediated transport of PAH with IC_{50} values of 53 μM and 58 μM, respectively (Leier et al. 2000). Additionally, OTA was found to be a direct substrate of MRP2 in HEK-MRP2 cells with a net ATP-dependent transport rate of 1.2 nmol/mg protein/min at 200 μM. In another study, MDR1, MRP2, and BCRP were studied as potential efflux transporters of OTA using intestinal Caco-2 cells (Schrickx et al. 2006). Caco-2 cells secreted OTA to the apical compartment in a concentration-dependent manner while no effect on the absorptive ability was seen. Treatment with MRP2 and BCRP inhibitors, MK571 (35% inhibition) and Ko143 (50% inhibition), respectively, reduced OTA secretion. The MDR1 inhibitor, PSC833, did not affect OTA transport. These studies suggest the involvement of MRP2 and BCRP in OTA efflux. OTA has also

been shown to be a substrate for other ABC-transporters such as ABCA8, an anion pump and transporter of lipids (Tsuruoka et al. 2002). However, the importance of this transporter is uncertain as only faint expression has been detected in the kidneys of mice and its localization in polarized epithelial cells is not currently known (Wakaumi et al. 2005). Nonetheless, further studies in kidney cells are needed to confirm the ability of MRP2, BCRP, and ABCA8 to export OTA and reduce the extent of toxicity.

NPT4 is a Na^+-dependent phosphate transporter of urate that is also found on the apical side of human renal proximal tubules. Xenopus oocytes expressing human NPT4 demonstrated time- and concentration-dependent transport of OTA (Km 802.8 ± 137.3 μM, Vmax 518.7± 76.4 fmol/h per oocyte) (Jutabha et al. 2010). The effect of different ionic conditions on NPT4-mediated transport was also studied. While a lack of extracellular Na+ did not affect OTA transport, NPT4-mediated OTA transport was sensitive to changes in plasma membrane potential and elevation of extracellular K^+ concentrations. The presence of an outward Cl^- gradient also did not influence OTA transport.

1.2.3.2. Heavy Metals.

Many heavy metals including lead, mercury, cadmium, copper, and uranium can be toxic to the kidneys. Studies evaluating the transport of heavy metals and their metabolites have shed some light on the mechanism of toxicity. Many of the heavy metals specifically target and damage the proximal tubules, which suggests a role for active transport processes unique to this region of the nephron. In this section, we highlight two of the more commonly studied heavy metal nephrotoxicants, cadmium and mercury.

Cadmium

Cadmium (Cd^{2+}) is a divalent heavy metal pollutant to which people are exposed from various sources including cigarettes, occupational dust and fumes, food, and water (reviewed in Jarup et al. 1998). Much of the Cd^{2+} absorbed into the body is through inhalation (20-50%), whereas only 5% is absorbed by ingestion (Berglund et al. 1994, Satarug and Moore 2004). Cd^{2+} accumulates in the body due to the absence of an efficient excretory mechanism from storage sites and a long half-life of 10 to 30 years (Satarug and Moore 2004). Cd^{2+} exposure can lead to numerous conditions including neurological diseases, renal dysfunction, pulmonary complications, diabetes, cancer, immunosuppression, bone disorders, and cardiovascular diseases (Smith et al. 1976, Kazantzis 1979, Kjellstrom et al. 1979, Descotes 1992, Kjellstrom 1992, Viaene et al. 2000, Schwartz et al. 2003, Messner et al. 2009). The mechanism(s) of Cd^{2+} toxicity are still unclear but are thought to involve the indirect generation of superoxide and hydroxyl radicals inside cells. Such oxidative stress subsequently stimulates cellular proliferation and alters apoptotic and DNA repair mechanisms (Galan et al. 2001).

The kidney is one of the main targets for Cd^{2+} toxicity. Injury to proximal tubular cells leads to tubular necrosis, interstitial fibrosis, and glomerular epithelial cell hypertrophy in rats (Aughey et al. 1984). With chronic exposure, tubular damage can progress to overt renal Fanconi syndrome, denoted by glycosuria, aminoaciduria, and phosphaturia, that may culminate in renal failure (Piscator 1984, Johri et al. 2010). An interplay between the liver and kidneys has been implicated in the toxicity of Cd^{2+}. In studies using rats and chickens, the majority of circulating Cd^{2+} was bound to albumin and transported to the liver where low molecular weight (less than 7 kDa), high-affinity metal-binding proteins called metallothioneins (MTs) were induced (Weser et al. 1973, Shaikh and Smith 1976). In the liver, Cd^{2+} can dissociate from albumin and bind to MT to form Cd^{2+}-MT

complexes that are returned to the systemic circulation. Circulating Cd^{2+}-MT complexes are freely filtered through glomeruli and reabsorbed by tubular cells. Alternatively, Cd^{2+} can bind to other thiol-containing molecules such as Cys or GSH and be excreted into the bile (Cherian and Vostal 1977, Zalups 2000, Wang et al. 2010).

There is some evidence that basolateral transporters participate in the proximal tubule uptake of Cd^{2+}. Studies in Chinese hamster ovary (CHO-K1) cells transfected with rabbit Slc22a1(Oct1) or Slc22a2 (Oct2) genes have shown that Cd^{2+} dose-dependently inhibits the uptake of the OCT substrate [^{3}H]-TEA (IC50 values: Oct1 96 μM and Oct2 207 μM) (Figure 1-5) (Soodvilai et al. 2011). In this study, TEA (10 mM) also reduced Cd^{2+} (3 μM) cytotoxicity in Oct1- and Oct2-expressing CHO-K1 cells. Furthermore, administration of TEA to rats was found to decrease Cd^{2}+ accumulation by 7-fold in the kidneys. These data indirectly suggest that the OCT substrate TEA competes with Cd^{2+} for transport by Oct1 and 2. Similarly, human OCT2 has been shown to transport Cd^{2+} (Thevenod et al. 2013). The possibility of other basolateral transporters to participate in Cd^{2+} uptake has yet to be explored. Although there are no direct studies exploring OAT uptake of Cd^{2+}, studies have shown that increased basolateral uptake of cadmium (60-70%) in rat kidneys occurred after co-administration of cadmium with Cys or GSH (Zalups 2000). It is conceivable that similar to other metals such as mercury, Cys- and GSH-conjugated Cd2+ can be transported across the basolateral plasma membrane by OAT/Oats.

There is a significant evidence suggesting that Cd^{2+} enters proximal tubules across the apical surface of the plasma membrane. Following glomerular filtration, the Cd^{2+}-MT complex is taken up by megalin and cubilin, which are multiligand, endocytic receptors (Klassen et al. 2004). Megalin and cubilin are highly enriched on the brush border of proximal tubules and mediate various physiological functions such as the reabsorption of

proteins and vitamins and regulation of calcium homeostasis (reviewed in Christensen and Birn 2001, reviewed in Thevenod 2010). Upon binding, these proteins are transferred to lysosomes where they are degraded to release ionic Cd^{2+}, thereby initiating cellular damage processes (Klassen et al. 2004, Wolff et al. 2006). The ability of amino acid transporters to influx Cys-conjugates of Cd^{2+} was also recently studied (Wang et al. 2010). Amino acids that are substrates of system $B^{0,+}$, including L-cystine, and L-arginine, as well as L-methionine reduced the uptake of Cys-conjugated Cd^{2+} (Cys-S-Cd-S-Cys) by 50%. Amino acids that are not substrates of system $B^{0,+}$, such as L-aspartic acid and L-glutamate, did not modulate the uptake of Cys-conjugated Cd^{2+}. Given these data, the authors suggested that Cys-conjugates of Cd^{2+} may mimic entry of amino acids through transporters systems such as system $B^{0,+}$, in order to gain access across the apical membrane following filtration.

The apical transport of Cd^{2+} may also occur through a process of "ionic and molecular mimicry" due to size and charge similarity with essential divalent elements including iron (Fe^{2+}), zinc (Zn^{2+}) and calcium (Ca^{2+}) (reviewed in Clarkson 1993, Bridges and Zalups 2005). Transporters for these metals also recognize Cd^{2+} and consequently, the systemic levels of some essential metals affect the extent of Cd^{2+} accumulation and distribution. Cd^{2+} can be reabsorbed from the tubule lumen through zinc/iron-regulated transporters (ZRT/IRT) also called ZIP proteins. ZIP8, ZIP14, and divalent metal transporter 1 (DMT1) proteins mediate the absorption of Cd^{2+} across the apical side of mouse proximal tubule cells (He et al. 2009, Fujishiro et al. 2012). Studies have shown that silencing ZIP8, ZIP14, and DMT1 genes significantly reduced the uptake of Cd^{2+} (Fujishiro et al. 2012). Deficiencies in Fe^{2+}, Zn^{2+}, or Ca^{2+} can increase Cd^{2+} accumulation as well as susceptibility to kidney toxicity (Waalkes et al. 1991). One study revealed that low stores of Fe^{2+} enhanced the intestinal absorption of dietary Cd^{2+} in both mice and

humans (Flanagan et al. 1978). In mice fed low calcium diets, more Cd^{2+} accumulated in the kidneys following oral $CdCl_2$ compared to normal diet fed controls. Additionally, enhanced levels of intestinal MT-1 mRNA and calcium transporter 1 mRNA were observed (Min et al. 2008). This suggests that more Cd^{2+} is reabsorbed intestinally through the deficient essential metal transporter and bound to MT, which then preferentially accumulates in the kidneys. Wang et al. (2010) studied the acute effects of Fe^{2+}, Zn^{2+}, or Ca^{2+} on apical Cd^{2+} transport in isolated perfused rabbit proximal tubules (Wang et al. 2010). The presence of Fe^{2+}, Zn^{2+}, and Ca^{2+} in the lumen inhibited uptake of Cd^{2+} by 42%, 48%, and 27-69%, respectively. These studies showed that essential metals play several roles in modulating Cd^{2+}-induced cellular injury. They may competitively inhibit renal Cd^{2+} uptake and during periods of deficiency, there is enhancement of Cd^{2+} absorption from the intestine, which leads to accumulation in the kidney.

Due to the high protein binding of Cd^{2+} in the liver and kidneys (approximately 75% in humans) (Post et al. 1984), the rate of excretion into urine is low. Despite the limited renal excretion of Cd^{2+}, research has demonstrated a potential role for active transporters in its removal. Initially, it was shown that Cd^{2+} increased levels of Mdr1 protein and function in the brush border membrane of the S1 and S2 segments of rat proximal tubules (Thevenod et al. 2000). Incubation of proximal tubule cells cultured from rats with 10 μM cadmium chloride ($CdCl_2$) led to the time-dependent increase in the levels of Mdr1 protein over 72 h that protected against Cd^{2+}-induced apoptosis (Thevenod et al. 2000). The Mdr1 inhibitors, verapamil, cyclosporine A, and nifedipine, enhanced susceptibility to Cd^{2+} toxicity in rat, LLC-PK1 (Porcine Kidney) and OK (Opossum Kidney) proximal tubule cell lines (Thevenod et al. 2000, Endo et al. 2002, Kimura et al. 2005). Additionally, MDR1 inhibitors decreased the basolateral-to-apical

transport and increased the apical-to-basolateral transport of Cd^{2+} in LLC-PK1 cells overexpressing human MDR1 (Kimura et al. 2005). These results supported the participation of rat and human Mdr1/MDR1 in the apical efflux of Cd^{2+} from the kidney. More recent data support an alternate mechanism for MDR1's ability to protect against Cd^{2+} toxicity. Using MDR1-MDCK cells, it was demonstrated that MDR1 could efflux ceramide, a lipid by-product of cellular damage that contributes to renal cell apoptosis (Lee et al. 2011).

The ability of a Cd^{2+}/H^+ antiporter to efflux Cd2+ has been revealed in LLC-PK1 cells and brush border membrane vesicles from rat kidneys (Endo et al. 1996, Endo et al. 1998). A reduction in the pH of the culture media (from 7.4 to 5.5) decreased Cd^{2+} accumulation by 60% and increased the efflux of Cd^{2+} by 15% in LLC-PK1 cells treated with $CdCl_2$. An organic cation/H^+ antiport system, which may be the same as the Cd^{2+}/H^+ antiporter, has been further investigated for its ability to transport Cd^{2+} in the presence of other cations (Endo et al. 1998). In LLC-PK1 cells, treatment with N'-methylnicotinamide (NMN), cisplatin, and TEA (substrates of H^+ antiport system) increased intracellular Cd^{2+} accumulation in a concentration-dependent manner and decreased transcellular Cd^{2+} transport. Enhanced accumulation of Cd^{2+} in the presence of cations was largely due to a modest inhibition of apical Cd^{2+} efflux presumably due to competition. The accumulation of Cd^{2+} was only elevated by 25% at the highest cation concentration tested (100 μM). However, the simultaneous inhibition of basolateral transport systems, such as Oct2, was not examined which could have contributed to the limited Cd^{2+} accumulation. While not demonstrated experimentally, it is likely that the above mentioned organic cation/H^+ antiport system is Mate1/2-K, which was shown to efflux the same substrates, NMN, cisplatin, and TEA in a pH-dependent manner (Masuda et al. 2006, Tanihara et al. 2007, Nakamura et al. 2010). Recently, it was shown that mouse

Mate1 as well as human MATE1 and MATE2-K could transport Cd^{2+} and influence its cytotoxicity in HEK kidney cells (Yang et al. 2017).

Mercury

Mercury is a heavy metal pollutant that enters the environment through natural or industrial processes and is found in air, soil, and water (ATSDR 2008). The different forms of mercury in the environment encountered by humans include elemental mercury, organic mercury (methylmercury; CH_3Hg^+) and inorganic mercury (Hg^{2+}). Hg^{2+} is most likely to accumulate in the kidneys (Ashe et al. 1953, Friberg et al. 1957, Friberg 1959, Clarkson and Magos 1966, Hahn et al. 1990) and is a major contributor to mercury-induced renal damage, whereas methylmercury is more likely to cross the blood-brain barrier and cause significant adverse effects to the brain (ATSDR 2008). However, both exposures are important to nephrotoxicity as methylmercury can also accumulate in the kidney and/or be converted to Hg^{2+} within the body as evidenced by the time-dependent accumulation of Hg^{2+} in subcellular fractions of the kidneys, livers, and brains of rats exposed to methylmercury (Norseth and Clarkson 1970, Norseth and Clarkson 1970, Magos and Butler 1976, Omata et al. 1980). The renal uptake of Hg^{2+} is rapid since as much as 50% of a nontoxic dose can be found in rat kidneys within a few hours after exposure (Zalups 1993). Both Hg^{2+} and methylmercury target the proximal tubules (Berlin and Gibson 1963, Berlin and Ullberg 1963, Berlin and Ullberg 1963, Taugner et al. 1966, Norseth and Clarkson 1970, Norseth and Clarkson 1970, Magos and Butler 1976). Mercury has high affinity for sulfhydryl groups (Hughes 1957); therefore in the plasma and tissues, Hg^{2+} ions are found conjugated to thiol-containing molecules such as GSH, Cys, homocysteine (Hcy), N-acetylcysteine (NAC), and albumin. This strong affinity also contributes to the mechanism of toxicity where binding of mercuric ions to critical proteins can initiate cell injury pathways. Binding of mercuric ions to thiol-rich

proteins, such as metallothionein, also increases intracellular retention (Zalups and Cherian 1992, Zalups et al. 1993, Zalups and Koropatnick 2000). The Cys-conjugated species of both inorganic and organic mercury seem to be the primary form found in proximal tubular cells. A number of conjugated mercury species are substrates for uptake and efflux transporters, playing a role in susceptibility to mercury toxicity.

The basolateral uptake of Hg^{2+} and methylmercury into proximal tubules occurs primarily by OATs (Figure 1-6). Torres et al. (2011) showed that Oat1-null mice exposed to Hg^{2+} exhibit less renal injury as assessed by histology and biochemical endpoints including blood urea nitrogen (BUN) and serum creatinine (SCr) compared to wild-type mice (Torres et al. 2011). Furthermore, differences in the enrichment of Oat1 and Oat3 proteins in male and female rats resulted in sex-dependent susceptibility to mercury-induced kidney damage (Hazelhoff et al. 2012). Female rats have 5- and 3-fold lower levels of renal Oat1 and Oat3 protein, respectively. In turn, mercury chloride ($HgCl_2$)-exposed female rats exhibited less renal impairment than male counterparts. Human OAT1 has also been implicated in Hg^{2+} uptake. Transfection of Madin-Darby canine kidney (MDCK) cells with human OAT1 decreased cell viability after exposure to mercuric conjugates of Cys, NAC, or GSH (Cys-Hg^{2+}, NAC-Hg^{2+}, GSH-Hg^{2+}) compared to control, nontransfected cells (Aslamkhan et al. 2003, Zalups and Ahmad 2004). Interestingly, in this cellular system, the most toxic conjugate was found to be NAC-Hg^{2+} (LC_{50} 40 μM), followed by Cys-Hg^{2+} (LC_{50} 80 μM) while the least toxic was GSH-Hg^{2+} that did not reach an LC_{50} at the concentrations tested (0-100 μM). For comparison, it should be noted that the LC_{50} of $HgCl_2$ was 16 μM. Additionally, oocytes from Xenopus laevis injected with cRNA for human OAT1 showed preferential uptake of Cys-Hg^{2+}, but not the GSH-Hg^{2+} (Aslamkhan et al. 2003). PAH inhibited the uptake of Cys-Hg^{2+} in a dose-dependent manner. Methylmercury has also been shown to be transported by

OATs. Treatment of male Swiss OF1 mice with the OAT inhibitor, probenecid, reduced tubular damage from methylmercury by 80-90% (Ban and de Ceaurriz 1988). Xenopus laevis oocytes expressing rat Oat1 showed increased uptake of NAC-conjugated methylmercury (Km: 31 μM) but not Cys or GSH-conjugates. However, rat Oat3-expressing oocytes did not transport NAC, GSH-, or Cys conjugates of methylmercury (Koh et al. 2002). Additional studies in MDCK cells expressing human OAT1 showed that the human isoform could mediate the uptake of Cys-, Hcy, and NAC-*S*-conjugates of methylmercury (Zalups and Ahmad 2005, Zalups and Ahmad 2005, Zalups and Ahmad 2005). The series of *in vivo* and *in vitro* studies point to the involvement of basolaterally localized OATs/Oats in the uptake and renal toxicity of Hg^{2+}, methylmercury, and their NAC, Cys, and GSH conjugates.

After GSH-Hg^{2+} is broken down by γ-glutamyltransferase on the apical brush border membrane to form the Cys-Hg^{2+}conjugate, amino acid transporters mediate reabsorption into proximal tubule cells. This was evidenced by the reduced rate of apical disappearance flux (JD) of mercuric conjugates following inhibition of γ-glutamyltransferase in perfused S2 segments of rabbit proximal tubules (Cannon et al. 2000). Additionally, when L-lysine or cycloleucine were added to isolated, perfused S2 segments of rabbit proximal tubules, the transport rate was reduced by 50%. Most likely, multiple amino acid transporters participated in the apical influx of dicysteinylmercury including the L-cysteine, system $B^{0,+}$, system B^{0}, system ASC, and other Na^{+}-dependent and independent systems (Cannon et al. 2001). System $B^{0,+}$, a transporter of various amino acids including methionine, is localized to the apical membrane of proximal tubule cells. Similarly, Cys and Hcy conjugates of methylmercury are structurally similar to the amino acid methionine. It was proposed that conjugates of methylmercury might mimic the entry of methionine into cells (Wang et al. 2012). The uptake of Cys, GSH, and NAC

conjugates of methylmercury in perfused rabbit proximal tubule segments was inhibited by the amino acids L-methionine and L-cysteine. Compared to control Xenopus laevis oocytes, transport of the Cys-methylmercury conjugate was 10-fold greater in oocytes expressing the mouse system $B^{0,+}$ (Bridges and Zalups 2006). Further studies showed that the amino acid transporter mediated uptake of Cys-methylmercury across the apical side of the plasma membrane. In rat proximal tubule-derived NRK-52E cells grown in monolayers, uptake of Cys-methylmercury was attributed to a Na^+-independent amino acid transporter inhibited by L-cysteine, L-leucine, L-isoleucine, and L-phenylalanine on the apical side. PAH, probenecid, and glutarate did not affect uptake, suggesting apical uptake was not mediated by Oats (Zalups and Ahmad 2005).

Mercury is known to be a substrate of the apical efflux transporters, Mrp2 and Bcrp. Exposure of killifish proximal tubule cells to $HgCl_2$ reduced Mrp2 transport of fluorescein methotrexate (a substrate for MRP2) (Terlouw et al. 2002). Since Hg^{2+} accumulation was not measured in this study, this effect could be secondary to cellular toxicity. However, other results suggest that reduced Mrp2 transport by $HgCl_2$ may be the direct result of inhibited transporter function. Treatment of MDCK cells with $HgCl_2$ showed a dose-dependent induction of Mrp1 and Mrp2 mRNA and protein levels compared to vehicle-treated MDCK cells (Aleo et al. 2005). $HgCl_2$ inhibited the growth of MDCK cells, which was further enhanced in the presence of the Mrp inhibitor, MK-571 (Aleo et al. 2005). Moreover, MK-571 increased the intracellular accumulation of Hg^{2+} (30% higher), pointing to a role for Mrps in the efflux of this metal (Aleo et al. 2005). Compared to control vesicles, there was 1.5- to 2-fold greater transport of Cys-*S*-conjugates of Hg^{2+} and methylmercury in membrane vesicles expressing MRP2. *In vivo*, TR- (transport deficient) rats treated with $HgCl_2$, Cys-*S*-Hg-*S*-Cys, and Cys-methylmercury, but not methylmercury, demonstrated slightly higher renal burden and lower mercury

concentrations in the urine (4- to 10-fold) relative to control rats (Bridges et al. 2011). Recent studies examined the role of Mrp2 in the site-specificity of Hg^{2+}-induced nephropathy (Zalups et al. 2014). TR- rats lacking Abcc2/Mrp2 expression and Mrp2-null mice exhibited different patterns of nephropathy compared to control Wistar rats and FVB mice, respectively. TR- rats treated with 1.5 and 2.25 μM/kg Hg^{2+} and Mrp2-null mice treated with 18.5-19.5 μM/kg Hg^{2+} displayed cellular injury and death in the S1 and S2 segments of proximal tubules, whereas Wistar rats and wild-type mice exhibited S3 segment injury. Evidence of the differential pattern of injury and accumulation of Hg^{2+} is indicative of Mrp2 heterogeneity and heavy metal handling along the nephron in rodents. It was proposed that proximal portions of tubules may secrete Hg^{2+} into the lumen via Mrp2 for subsequent reabsorption in the distal S3 segment where the toxic effects are exerted (Zalups et al. 2014).

Interestingly, polymorphisms in the *ABCC2* gene that encodes MRP2 were associated with the differential excretion of mercury in subjects from Indonesia, the Philippines, Tanzania, and Zimbabwe (Engstrom et al. 2013). In the subgroups with the highest exposures (found in Zimbabwe), subjects with the *MRP2/ABCC2* variant (rs1885301; G/A) variant (N=146) had 166% higher concentrations of mercury in the urine than those with the wild-type allele (p=0.027). The G > A polymorphism (rs1885301) results in loss of a transcription-factor binding site (Fetal Alz-50 clone 1, FAC1) and gain of another site (FAST-1 SMAD interacting protein) in the 5' UTR. Although the functional role of these changes is unknown, they may enhance MRP2 activity. By comparison, carriers of a non-synonymous polymorphism in *MRP2/ABCC2* (rs2273697; G/A) had 2.2-fold lower urinary concentrations of mercury, suggesting decreased function. However, the influence of these SNPs on MRP2 mRNA and protein levels in kidneys as well as

susceptibility to Hg^{2+}-induced nephrotoxicity remains unclear. Nonetheless, these data further support a role for MRP2 in the excretion of mercury conjugates from the kidneys.

In addition to Mrp2/MRP2, the Bcrp transporter can participate in the efflux of mercuric species from proximal tubules. Uptake of the Cys-conjugate of Hg^{2+} in inside-out membrane vesicles expressing mouse Bcrp was 5-fold higher than control vesicles (Bridges et al. 2015). Furthermore, Bcrp-null rats treated with $HgCl_2$ have 1.5-fold higher renal concentrations of Hg^{2+} than wild-type rats. Hematologic, hepatic, and fecal burden of Hg^{2+} were also greater in Bcrp-null rats compared to wild-type rats. Histopathologic analysis, SCr, and BUN indicated increased severity of renal injury in Bcrp-null rats at the doses (1.5 and 2 μM/kg $HgCl_2$) tested. At lower doses (0.5 and 1.5 μM/kg), the decrease in urinary excretion of Hg^{2+} in Bcrp-null rats correlated well with greater renal accumulation. However, Bcrp-null rats treated with a higher dose (2 μM/kg) showed an increase in urinary excretion of Hg^{2+} compared to corresponding wild-type rats. This may be due to enhanced kidney damage or compensation by other efflux proteins such as Mrp2. Differences in the pattern of mercury-induced nephropathy in transport deficient rodent models pointed to distinct roles for Bcrp and Mrp2 in Hg^{2+} efflux. Unlike Mrp2-deficient mice and rats, which exhibit mercury-induced necrosis on the outer cortex (where S1 and S2 segments are located), Bcrp-null rats exposed to nephrotoxic doses of mercury chloride exhibited necrosis on the inner cortex and the outer strip of the outer medulla (S3 segments). These disparate findings are likely due to the segmental differences in the distribution of the Bcrp and Mrp2 proteins as well as their intrinsic ability to transport conjugated forms of mercury. Collectively, these studies point to multiple mechanisms for the elimination of Hg^{2+} from proximal tubule cells.

The uptake of mercury across the brush border membrane can be inhibited leading to a lower extent of tubular injury. Application of the heavy metal chelator, DMPS, on the apical surface of isolated rabbit proximal tubules enhanced the urinary secretion of methylmercury (Wang et al. 2012). DMPS diminished the apical influx of Cys-methylmercury suggesting that DMPS conjugates of mercury formed in the tubule lumen are not reabsorbed. Others have demonstrated that DMPS also plays a role in reducing the proximal tubular uptake and toxicity mediated by Hg^{2+}. In isolated perfused rabbit proximal tubule cells, addition of DMPS to Hg^{2+} at the basolateral or apical surface resulted in very little to no uptake of Hg^{2+} or toxicity (Zalups et al. 1998). These studies are evidence that Hg^{2+} or methylmercury bound to DMPS are poor substrates for proximal tubule transporters.

In vivo, Mrp2 plays a significant role in the secretion of Hg^{2+} and methylmercury from proximal tubule cells after administration with DMPS (Bridges et al. 2008, Bridges et al. 2008, Zalups and Bridges 2009). TR- rats that lack Abcc2/Mrp2 expression exhibited less Hg2+ in urine and feces and greater renal burden of Hg^{2+} than control Wistar rats following injection with $HgCl_2$, conjugated Hg^{2+} (Cys-Hg^{2+} or Hcy-Hg^{2+}), or methylmercury. Once treated with DMPS, the renal content of Hg^{2+} was reduced and urinary excretion was increased, however to a lesser extent in TR^- rats (7-fold higher Hg^{2+} in total renal mass, 2.5-fold lower Hg^{2+} excretion) (Bridges et al. 2008, Bridges et al. 2008, Zalups and Bridges 2009). To further understand the role of other human MRP isoforms in the disposition of DMPS-*S*-conjugates of Hg^{2+}, inside-out membrane vesicles expressing human MRP4 were used (Bridges et al. 2013). Although human MRP2-containing vesicles transported DMPS-*S*-conjugates of Hg^{2+} (1.7-fold), uptake into human MRP4-containing vesicles did not differ from controls. These data indicate that at least a portion of DMPS-induced elimination of Hg^{2+} and methylmercury occurs through

Mrp2. In addition, Bcrp may also play a role in the elimination of DMPS-Hg^{2+}. Compared to control vesicles, the DMPS-conjugate of Hg^{2+} accumulated to 5-fold greater concentrations in inside-out membrane vesicles expressing mouse Bcrp (Bridges et al. 2015). Further studies are needed to determine the affinity of human BCRP for the DMPS-conjugates of methylmercury and to assess the *in vivo* ability of BCRP to facilitate urinary secretion.

1.2.3.3. Pharmaceuticals

An important dose-limiting adverse effect of a number of pharmaceuticals is nephrotoxicity, which in turn reduces their overall utility as therapeutic agents. This section will highlight several examples of therapeutic classes and/or specific drugs that are associated with direct tubular injury due to transporter-mediated accumulation.

Beta-lactam antibiotics

Beta-lactam antibiotics include penicillins, cephalosporins, and carbapenems. Structurally, these medications are cyclic dipeptides containing lactam rings that react with bacterial carboxypeptidases to prevent cell wall synthesis (Waxman and Strominger 1983). The antibacterial spectrum of naturally-occurring beta-lactams was broadened by modifying the main structure to enhance reactivity (Hoover 1983). However, increased reactivity in the early development of cephalosporins and carbapenems resulted in a greater propensity for nephrotoxicity (Tune et al. 1996). Nephrotoxic beta-lactams can produce structural damage and proximal tubule necrosis in rabbits and various other laboratory species within 24 hours (Atkinson et al. 1966, Birnbaum et al. 1985, Tune 1993). The mechanism(s) underlying tubular injury include the ability of beta-lactams to actively accumulate in renal proximal tubular cells, acylate proteins (Tune and Fravert 1980), and/or induce lipid peroxidation (for cephaloridine) (Kuo et al. 1983, Cojocel et al.

1985, Tune et al. 1996). The side group substitutions of beta-lactams greatly affect transport rates and protein reactivity and hence contribute to the varying degrees of nephrotoxicity (Indelicato et al. 1977, Inui et al. 1983). Very few cephalosporins cause tubular damage at therapeutic doses. Cephaloridine, a first generation cephalosporin which is nephrotoxic at therapeutic doses, is no longer used. However, cephaloridine remains a classic example of how transport can be a critical determinant of drug-induced renal injury.

Single, high doses of cephaloridine lead to proximal tubule necrosis in rats, dogs, and other various laboratory species (Welles et al. 1965, Atkinson et al. 1966, Tune et al. 1989). This injury was prevented by administration of probenecid along with cephaloridine (Child and Dodds 1967). Further studies revealed that a lower concentration of cephaloridine was found in cortical regions of rabbit kidneys after probenecid treatment compared to animals treated with cephaloridine alone (Tune 1972). These data indicated that cephaloridine interacted with Oat transporters on the basolateral membrane. Mouse S3 proximal straight tubule cells stably expressing rat Oat1 were used to study the uptake of radiolabeled PAH and cephaloridine. Oat1-expressing cells treated with cephaloridine exhibited dose-dependent inhibition of [^{14}C]-PAH uptake, 2-fold increase in [^{14}C]-cephaloridine uptake, decreased viability, and increased lipid peroxidation compared to vector-expressing cells (Figure 1-7). Probenecid treatment diminished the effect of cephaloridine on cell viability as well as lipid peroxidation by 1.5- to 2-fold (Takeda et al. 1999). Other studies have also shown involvement of rat Oat3 in cephaloridine-induced nephrotoxicity (Jung et al. 2002). Proximal tubule cells stably expressing rOat3 (both basolaterally and apically) exhibited higher [^{14}C]-cephaloridine uptake and cytotoxicity that was reversed by probenecid treatment. More recent studies showed that cephaloridine inhibited organic anion uptake

in proximal tubule cells stably expressing human OAT1-3 in a competitive manner (Ki OAT1: 0.74 mM, OAT2: 2.09 mM, OAT3: 2.46 mM) (Takeda et al. 1999, Khamdang et al. 2003). Cephaloridine (5 mM) decreased the viability of proximal tubule cells expressing OAT1, OAT2, or OAT3 (30-50%), which could be reversed by probenecid in OAT1- and OAT3-expressing cells. These studies indicated that both rat and human OATs were involved in the uptake of cephaloridine, and possibly other cephalosporins, across the basolateral plasma membrane.

In studies by Takeda et. al., (2002) exploring the interaction of human OATs with cephalosporins, cephaloridine competitively inhibited organic anion uptake 31-fold in isolated proximal tubule cells expressing the human OAT4 transporter (K_i OAT4: 3.63 mM) (Takeda et al. 2002). Cephaloridine also decreased the viability of OAT4-expressing proximal tubule cells by 40%, which was modestly reversed by probenecid. OAT4 and additional transporters, such as the organic cation/carnitine transporter OCTN2, present on the apical surface likely reabsorb cephaloridine from the tubular lumen. Human OCTN2-expressing HeLa cells exhibited cephaloridine uptake compared to vector-transfected cells where cephaloridine accumulation was undetectable (Ganapathy et al. 2000). While other cephalosporins were reported to interact with the human PEPT1 and rat Pept2 peptide transporters, cephaloridine does not. More recently, mouse Octn2 was also shown to play a role in the apical uptake of cephaloridine. Experiments with juvenile visceral steatosis mice, which have a functional deficiency in the Octn2 gene, showed increased renal clearance of cephaloridine (Kano et al. 2009). Furthermore, direct uptake of cephaloridine was shown in Xenopus laevis oocytes expressing mouse Octn2 (Km: 3 mM). This accumulation of cephaloridine could be inhibited by the Octn2 substrates, carnitine (IC50: 14.8 μM) and TEA (0.68 mM).

Early studies suggest that cephaloridine might also interact with an apical cation transport system. Administration of a cationic transport inhibitor, cyanine 863, before or after cephaloridine administration to rabbits resulted in 2- to 3-fold greater susceptibility to nephrotoxicity (Wold et al. 1979). PAH and TEA accumulation were measured in slices from rabbit renal cortical tissue and accumulation of each chemical was reduced 2-fold by pretreatment with cyanine 863. However, the cationic substrates quinine and NMN showed a small or no effect, respectively, on the extent of nephrotoxicity or PAH/TEA accumulation. The authors suggested that the efflux of cephaloridine was mediated by a cationic transporter, and that inhibition of this system increased susceptibility to toxicity. However, since direct measurements of intracellular concentrations of cephaloridine were not made in this study, it was hard to conclude that a definite interaction exists. Additional studies using cells expressing specific cationic transporters, such as the MATEs, are needed to understand the efflux of cephaloridine and related beta-lactams from tubular cells.

It is important to note apparent species differences that have been reported (Kasher et al. 1983, Williams et al. 1985). Cephaloridine inhibited NMN transport across basolateral membrane vesicles isolated from rabbits; however, no differences were observed in basolateral membrane vesicles isolated from rats or dogs. Conversely, cephaloridine did inhibit NMN transport in brush-border membrane vesicles from rat and dogs (56%), suggesting similar interactions with apical cation transporters in these species.

There are very few known efflux mechanisms for cephaloridine. Future studies are unlikely given the infrequent clinical use of cephaloridine and increased availability of minimally nephrotoxic beta-lactam alternatives. Cephaloridine did not affect MRP4-mediated ATP-dependent uptake of [^{3}H]-dehydroepiandrosterone in membrane vesicles

(Ci et al. 2007). However, other cephalosporins tested in this study did show inhibitory effects. Uptake studies of membrane vesicles from Sf9 cells expressing rat Mrp2, rat Bcrp, and human MRP2 revealed increased uptake of high molecular weight cephalosporins by all three transporters (Kato et al. 2008). These apical efflux transport pathways (Bcrp and MRP2/Mrp2) may be important for the toxicity of other renally-cleared cephalosporins which warrants further study in proximal tubule cells.

Antiviral Drugs

Several potent medications are being used successfully to treat viral infections caused by cytomegalovirus, herpes virus, and retroviruses. Antiviral drugs differ in their mechanism of action. Nucleotide analogs, including cidofovir, adefovir, and tenofovir, are structurally-related to endogenous nucleotides. This structure allows the drugs to be incorporated into viral DNA by reverse transcriptase (Furman et al. 1986). Nucleotides, unlike nucleosides, contain phosphonate groups, allowing them to skip the phosphorylation step before incorporation into viral DNA (reviewed in Cihlar and Ray 2010). Despite their efficacy, the clinical use of nucleotide analogs can be limited by their propensity to cause nephrotoxicity. Thirty-five and sixty percent of the intravenously administered doses of cidofovir and adefovir, respectively, are renally secreted and have been shown to accumulate in proximal tubules at higher concentrations than in other organs (Cundy et al. 1995, Cundy et al. 1995). Dose-limiting nephrotoxicity can be observed in nearly 15% of treated patients (Shimizu et al. 2015). Renal-related side effects reported in patients prescribed antiviral agents include Fanconi syndrome, progressive declines in kidney function, diabetes insipidus, and tubular dysfunction (Shimizu et al. 2015). Most often, nephrotoxicity tends to occur in patients with pre-existing renal disease and those administered antiviral drugs for prolonged periods or prescribed concomitant nephrotoxic agents (Shimizu et al. 2015). The

pathophysiological changes are multifactorial and include acute tubular necrosis possibly through direct mitochondrial damage (Tanji et al. 2001).

Cidofovir, adefovir, and tenofovir are anionic drugs efficiently transported by human OAT1 (Figure 1-8) (Cihlar et al. 1999, Ho et al. 2000, Cihlar et al. 2001). Overexpression of human OAT1 in CHO cells caused a 500-fold increase in the extent of cytotoxicity for these drugs (Cihlar et al. 1999, Ho et al. 2000, Cihlar et al. 2001). Likewise, human OAT3 also mediated the time-dependent uptake for adefovir, cidofovir, and tenofovir (Uwai et al. 2007). It should be noted that OAT1 was found to be a more efficient transporter of adefovir, cidofovir, and tenofovir compared to OAT3. Other antiviral drugs including acyclovir and ganciclovir were also revealed as substrates of OAT1, OAT2, and OCT1 (Takeda et al. 2002, Cheng et al. 2012). By comparison, OCT2 did not mediate the uptake of adefovir, cidofovir, tenofovir, acyclovir, ganciclovir, or penciclovir (Cheng et al. 2012). Further studies compared the potency of adefovir, cidofovir, and tenofovir for hOAT1-mediated transport. From most potent to least potent are: adefovir (Km: 23.8 μM), tenofovir (Km: 33.8 μM), cidofovir (Km: 58.0 μM) (Cihlar et al. 1999). Cytotoxicity was also compared in CHO cells expressing hOAT1: adefovir (IC_{50}: 1.4 μM), cidofovir (IC_{50}: 3.0 μM), and tenofovir (IC_{50}: 21.0 μM). These data may partially explain the lower incidence of nephrotoxicity associated with tenofovir, compared to adefovir (Dkhil et al. 2014). However, other factors such as reactivity to intracellular components, likely also play a role in susceptibility to injury.

The ability of tubular cells to efficiently efflux nephrotoxic antiviral drugs is a critical determinant of the extent of toxicity. To date, a variety of renal transporters have been investigated for their ability to efflux nucleotide phosphonates. Using intact renal proximal tubules from killifish, Miller et al. (2001) tested the interaction of adefovir and

cidofovir with fluorescent substrates of efflux transporters (Miller 2001). Fluorescein-methotrexate was used as the substrate of Mrp2 whereas a fluorescent cyclosporine A analog was used as the substrate of Mdr1. Both adefovir and cidofovir reduced the apical accumulation of fluorescein-methotrexate. Notably, cidofovir inhibited transport activity more potently than adefovir (adefovir IC_{50}: 50 μM; cidofovir IC_{50}: 10 μM). However, even at high concentrations (250 μM), adefovir and cidofovir failed to alter the transport of the fluorescent cyclosporine A analog, possibly indicating no interaction with Mdr1. Although these studies indicate a potential interaction of these chemicals for the same efflux transport pathway, direct measurements or transport kinetics were not assessed. Further studies pointed to a limited role for the human MRP2 transporter in adefovir and cidofovir efflux. Inverted membrane vesicles expressing human MRP2 and BCRP showed no ATP-dependent uptake of [^{3}H]-adefovir or [^{3}H]-cidofovir (Imaoka et al. 2007).

The MRP4 transporter is likely more relevant than MRP2 for the apical efflux of nucleotide phosphonate drugs in humans. Initial evidence revealed that MRP4 was overexpressed in an adefovir-resistant human T-lymphoblast CEM cell line (Schuetz et al. 1999). Indeed, the overexpression of the human MRP4 and MRP5 transporters in HEK293 cells conferred resistance to cytotoxicity for a range of acyclic nucleoside phosphonates including adefovir, but with the exception of cidofovir (Reid et al. 2003). Inverted membrane vesicles prepared from HEK293-MRP4 cells accumulated [^{3}H]adefovir and [^{3}H]tenofovir in a concentration-dependent manner (Km > 1 mM) which was inhibited by the MRP4 substrate, dehydroepiandrosterone sulfate (Imaoka et al. 2007). By contrast, no transport of [^{3}H]cidofovir was observed. Imaoka et al. (2007) also examined the transport of antiviral agents by Mrp4 in vivo (Imaoka et al. 2007). Mrp4-null mice had reduced tubular secretion and exhibited a 2-fold increase in the kidney

accumulation of adefovir and tenofovir compared to wild-type mice. Consistent with data obtained from membrane vesicles, the kidney concentration of [^{3}H]cidofovir did not differ between Mrp4-null and wild-type mice. Taken together, these data point to MRP4 as a low affinity transporter for the secretion of adefovir and tenofovir, but not cidofovir. Although nucleotide phosphonates have similar structures, it is evident from these studies that transporter affinities among this class of medications may contribute to the varying degrees of toxicity reported.

Cisplatin

Cisplatin (cis-diamminedichloroplatinum II) is an antineoplastic drug used to treat solid tumors in the lung, testis, ovary, cervix, bladder, and colon. The primary mechanism for cisplatin's efficacy is the ability to bind DNA and form inter- and intrastrand adducts, which prevent cancer cell replication. Nephrotoxicity is a major dose-limiting side-effect of cisplatin along with peripheral neuropathy, ototoxicity, vomiting, and diarrhea. Up to a third of patients treated with high doses (100 mg/m^2) of cisplatin suffer from nephrotoxicity (Shord et al. 2006). Upon entry inside proximal tubule cells, cisplatin undergoes biotransformation to aquated intermediates which leads to intercalation of DNA and adduct formation on proteins. Early work demonstrated that cisplatin was actively transported into renal tubules (Reece et al. 1985), but it was not until recently that researchers identified the transporters responsible for this process.

Early in vitro studies highlighted the existence of a transport system for cisplatin on the basolateral side of proximal tubules (Figure 1.9) (Pan et al. 1999, Endo et al. 2000, Kolb et al. 2003). Application of cisplatin to the basolateral side of MDCK cells resulted in a loss of epithelial monolayer integrity, as measured by transepithelial electrical resistance (Ludwig et al. 2004). Furthermore, the OCT2 inhibitor, cimetidine, was able to partially

reverse the drop in electrical resistance in cisplatin-treated cells. Other studies demonstrated that cisplatin inhibited the uptake of a fluorescent OCT2 substrate, 4-[4-(dimethylamino)styryl]-N-methylpyridinium (ASP^+) into HEK293 cells stably transfected with the human OCT2 gene and in freshly isolated human proximal tubules (Ciarimboli et al. 2005). Increased platinum accumulation and injury in HEK293 cells expressing OCT2 was inhibited by cimetidine in a concentration-dependent manner (Ciarimboli et al. 2005, Yonezawa et al. 2006). Assessment of additional basolateral organic ion transporters, including OCT1, OCT3, OAT1, and OAT3, showed that only OCT1 had the potential to increase cisplatin cytotoxicity; however, to a lesser degree than OCT2. Uptake transporters on the apical side of proximal tubules, such as OCTN1 and OCTN2, did not increase transport of cisplatin suggesting they are not involved in reabsorption (Yonezawa et al. 2006).

Building on prior in vitro studies, Filipski et al. (2009) utilized double Oct1/2-null mice and demonstrated a 50% reduction in the urinary excretion of cisplatin (Filipski et al. 2009). Reduced secretion of cisplatin in the Oct1/2-null mice correlated with less acute renal tubular necrosis as well as minimal changes in BUN and SCr. There are conflicting reports regarding the importance of genetic polymorphisms in OCT2/SLC22A2 on cisplatin disposition and acute kidney injury in patients. Several studies have observed an association between a loss-of-function genetic variant at position 808G>T (rs316019) and protection from kidney injury in cisplatin-treated cancer patients (n=78 Filipski et al.; n=53 Iwata et al.; n=123 Zhang et al.) (Filipski et al. 2009, Iwata et al. 2012, Zhang and Zhou 2012). The rs316019 variant was not associated with changes in the systemic clearance or plasma concentrations of cisplatin. However, a separate study of patients with esophageal cancer (n=95) treated with cisplatin and 5-fluorouracil revealed no relationship between changes in eGFR in individuals who were homozygous wild-type

(808GG) compared to individuals carrying the variant allele (808GT+TT) (Hinai et al. 2013). Further studies are needed to validate this polymorphism as a predictor of likelihood for cisplatin kidney injury.

Recent research has also suggested that the copper transporter 1 (CTR1) is an additional basolateral uptake carrier for cisplatin. Down-regulation of endogenous CTR1 protein levels in HEK293 cells using copper chloride ($CuCl_2$) (100 μM) or siRNA significantly lowered cisplatin uptake (Pabla et al. 2009). Cellular apoptosis and necrosis were decreased in both HEK293 cells and rat proximal tubule cells treated with cisplatin after CTR1/Ctr1 knockdown. Finally, CTR1 knockdown and concomitant treatment with cimetidine resulted in the additive reduction of cisplatin uptake in HEK293 cells (Pabla et al. 2009), suggesting a combined nephroprotection due to inhibition of OCT2 and CTR1 function. However, there are conflicting studies which dispute a role for CTR1 in cisplatin uptake. In a separate study, HEK cells overexpressing hCTR1 did not increase the rate of cisplatin influx (Ivy and Kaplan 2013). The cisplatin-sensitive ovarian cancer cell line A2780 has 2-fold higher levels of human CTR1 and greater cisplatin influx compared to the resistant cell line A2780CP. However, siRNA knockdown of CTR1 in A2780 cells did not affect cisplatin uptake; it should be noted that OCT2 levels following CTR1 knockdown were not quantified since induction of this alternate uptake carrier could shift the uptake pathway for cisplatin (Ivy and Kaplan 2013). While evidence suggests a potential interaction between cisplatin and CTR1, in vivo studies are need to determine its role in the drug's renal secretion.

As described above, the transepithelial transport of organic cations is often mediated by uptake through OCT2 that is coupled to secretion by the MATE1 and 2-K transporters across the brush border membrane. There are conflicting data regarding the use of

intracellular acidification to demonstrate MATE1 uptake of cisplatin. In HEK293 cells overexpressing rat Mate1, human MATE1, or human MATE2-K, there was no difference in cisplatin uptake compared to empty vector cells under acidified intracellular conditions using 30 mM ammonium chloride for 20 min (Yokoo et al. 2007). Subsequent studies showed, however, that treatment of mouse Mate1-HEK293 cells with 30 mM ammonium chloride did markedly increase transport and accumulation of platinum 4-fold compared to empty vector cells (Nakamura et al. 2010). Differences in pH conditions, which were not always reported, could have led to the discrepant findings.

The involvement of Mate1 in cisplatin secretion has also been confirmed in vivo. Mate1-null mice treated with 15 mg/kg cisplatin intraperitoneally exhibited higher cisplatin plasma and renal concentrations and greater susceptibility to nephrotoxicity compared to wild-type mice (Nakamura et al. 2010). Additionally, the Mate1 inhibitor pyrimethamine enhanced renal injury in wild-type mice. Another study was conducted to assess the association between a genetic polymorphism in *MATE1* (rs2289669; G>A) along with the systemic exposure and toxicity of cisplatin (doses of 60 to 80 mg/m2) in patients (n=53) with advanced carcinomas (Iwata et al. 2012). The results showed no significant differences in SCr, eGFR, BUN, or plasma concentration of cisplatin among patients with and without this variant. Although there is clear evidence that cisplatin is a substrate for MATE1, the extent of MATE1 involvement in cisplatin secretion in humans requires further study.

Another important transporter associated with cisplatin extrusion from proximal tubules is Mrp2. Early studies demonstrated that Mrp2 could efflux glutathione conjugates of cisplatin from mouse cancer cells (Ishikawa and Ali-Osman 1993, Cui et al. 1999, Kawabe et al. 1999). Overexpression of the human MRP2 transporter was shown to

increase cisplatin resistance 10-fold in HEK293 cells (Cui et al. 1999) and reduce cisplatin accumulation by 30% in LLC-PK1 cells (Kawabe et al. 1999). An initial in vivo report suggested that the disposition and extent of toxicity of cisplatin were similar in Mrp2-null and wild-type mice, as demonstrated by the urinary excretion of platinum and kidney histology (Sprowl et al. 2012). However, in the study by Sprowl et al. (2012), accumulation of platinum in mouse kidneys was not assessed (Sprowl et al. 2012). More recently, Wen et al. (2014) found that cisplatin inhibited transport of the MRP2/Mrp2 substrate 5(6)-carboxy-2,'7'-dichlorofluorescein by 70 to 80% in vesicles expressing mouse Mrp2 and human MRP2 (Wen et al. 2014). Furthermore, Mrp2-null mice treated with cisplatin exhibited 2-fold higher BUN levels, a greater increase in SCr concentration, and more severe proximal tubule degeneration and necrosis compared to wild-type mice. Kidney concentrations of platinum in Mrp2-null mice were 30% higher than those observed in wild-type mice. Transgenic expression of the human MRP2/ABCC2 gene in Mrp2-null mice reduced the accumulation of platinum in the kidneys to levels observed in wild-type mice without altering plasma platinum levels. Nephrotoxicity was also reduced in humanized MRP2 mice compared to Mrp2-null mice (Wen et al. 2014). These data show direct *in vivo* evidence of the involvement of MRP2 in the efflux of platinum species and nephroprotection.

1.2.4. Summary

Transporter proteins in the kidneys coordinate the secretion and/or reabsorption of structurally-diverse chemicals including heavy metals, herbicides, mycotoxins, and drugs. Because of their critical role in the overall disposition of xenobiotics, transporters regulate the renal exposure and subsequently the toxicity of xenobiotics. Beyond the chemicals described in this review, other commonly prescribed nephrotoxic drugs as well as environmental toxins share the same renal transport pathways responsible for

clearance. These include additional antibiotics, immunosuppressant drugs, and other heavy metals. The use of in vitro models of transporter-stably expressing cell lines and renal proximal tubule cells have been a routine experimental approach thus far. These experimental tools have been instrumental in studying both transporter-mediated toxicities as well as drug-drug interactions and have been helpful alternatives to using in vivo models. Scientists in the field have a growing understanding of the potential for different carriers and pumps to contribute to chemical secretion. However, more research is needed to assess the redundancy of transporters, the influence of genetic variants on the disposition and toxicity of nephrotoxicants, the intracellular unbound concentrations of nephrotoxicants as well as the molecular mechanisms that regulate transporter expression and function in the kidneys. New renal tubule models, including a growing availability of primary human proximal tubule cells, cells transfected with multiple transporters, and kidneys-on-a-chip (Wilmer et al. 2016), hold great promise to address these critical gaps in the literature and provide novel approaches to screen for drug interactions and nephrotoxicity.

1.3. Cisplatin

1.3.1. History of Cisplatin

The following section is adapted from *Cisplatin Chemistry and Biochemistry of a Leading Anticancer Drug* (Lippert 1999). Cisplatin (*cis*-dichlorodiammineplatinum(II)) was discovered more than half a century ago. Structurally, it is made up of a central platinum molecule, two chloride and two ammonia groups arranged in a planar *cis*-configuration (Figure 1.10). Although cisplatin was originally created in 1844 by Italian chemist Michele Peyrone (known as Peyrone's chloride), the anticancer properties of cisplatin were discovered more than a century later by Barnett Rosenberg, a biophysical chemist. During Rosenberg's experiments, he found that bacteria grew 300 times their normal size when using platinum electrodes to generate electric fields (Rosenberg et al. 1965). Three years later Rosenberg showed that cisplatin cured tumors in mice (Rosenberg et al. 1969). In 1978, cisplatin was approved for the treatment for cancer by the U.S. Food and Drug Administration. Since then, many more platinum analogues have been discovered and comprise of nearly 40% of all chemotherapy treatments. The discovery of cisplatin revolutionized cancer therapy. For example, testicular cancer cure rate was 10% before cisplatin discovery but now along with early detection, the cure rate is almost 100%.

1.3.2. Cisplatin Pharmacology and Pharmacokinetics

Cisplatin enters tumor cells by either passive diffusion or specific transporter (CTR1 or OCT2 in certain kidney tumors) mediated uptake (Gately and Howell 1993, Ishida et al. 2002, Filipski et al. 2009). The intracellular chloride concentration is low (4-20 mM) resulting in aquation of cisplatin and formation of reactive, electrophilic species. The monoaquated platinum species has been found to be largely responsible for binding to DNA bases, usually guanine, to form monofunctional and difunctional DNA adducts

(Lippert 1999, Davies et al. 2000). DNA repair pathways recognize and attempt to fix the error during DNA replication, resulting in further mutations such as single and double-stranded breaks. This activates the p53 pathway (tumor suppressor gene), which causes cell cycle arrest or activation of apoptosis and tumor cell death. Side effects that manifest during therapy include nausea and vomiting, nephrotoxicity, ototoxicity, neuropathy, and myelosupression. Rarely, visual impairment, seizures, arrhythmias, acute ischemic vascular events, glucose intolerance, and pancreatitis can occur (Loehrer and Einhorn 1984). The nephrotoxicity, neuropathy, and ototoxicity are cumulative and may cause permanent damage (Loehrer and Einhorn 1984, Latcha et al. 2016).

The following section is adapted from *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (Brunton 2011). Cisplatin is administered only through the intravenous route in a chloride-containing solution over 0.5 to 2 hours. Typical dosages include 20 mg/m^2/day for 5 days, 20-30 mg weekly for 3-4 weeks, or 100 mg/m^2 once every 4 weeks. Infusion of 1-2 L of normal saline is important prior to treatment to mitigate renal toxicity. Cisplatin in combination with other chemotherapeutics is used commonly for testicular and ovarian cancer, bladder, head and neck, cervix, endometrium, lung carcinomas, anal and rectal carcinomas, and childhood neoplasms. Cisplatin also sensitizes cells to radiation therapy and enhances control of locally advanced cancers in combination with irradiation. Following intravenous administration, cisplatin has an initial plasma elimination half-life of 25-50 minutes. Concentrations of total (bound and unbound) cisplatin decline subsequently with a half-life of > 24 hours. More than 90% of platinum in the circulation is bound to plasma proteins. High concentration of cisplatin is found in the kidney, liver, intestine, and testes and has poor penetration of the central nervous system. Biliary or intestinal excretion of cisplatin is

minimal and majority of cisplatin is renally excreted. During the first 24 hours, 25% of the dose is excreted and by 5 days 43% of the dose is recovered in the urine mostly bound to proteins.

1.3.3. Cisplatin Molecular Mechanisms of Nephrotoxicity

Besides the ability of cisplatin to concentrate in certain organs due to the presence of specific transporters (Section 1.2.3.3), another major factor in the nephrotoxicity of cisplatin is the bioactivation of cisplatin (Figure 1.11). Studies in mice and rats have shown that glutathione-*S*-transferase forms cisplatin-glutathione conjugates (Sadzuka et al. 1994, Townsend et al. 2009). The glutathione-conjugates are further cleaved to cysteinyl-glycine-conjugates by gamma glutamyl transpeptidase on the surface of proximal tubule cells (Townsend and Hanigan 2002, Townsend et al. 2003). The cysteinyl-glycine-conjugates are further metabolized to cysteine-conjugates by aminodipeptidases also on the surface of proximal tubules (Townsend et al. 2003). Within cells, cysteine-*S*-conjugate beta-lyases further activate cysteine-conjugates to highly reactive thiols (Townsend and Hanigan 2002, Townsend et al. 2003, Zhang and Hanigan 2003).

The cytotoxic effects in the kidney tubules are proposed to be much the same as cisplatin's pharmacological mechanism of action. In cells with high mitochondrial density, mitochondrial DNA and proteins may also be targeted. Sensitivity of cells to cisplatin appears to correlate with the density and membrane potential of mitochondria (Qian et al. 2005, Hirama et al. 2006). The increased sensitivity of proximal tubules to cisplatin agrees with this data. Further, cisplatin may disrupt mitochondrial energetics through inhibition of fatty acid oxidation and disruption of the respiratory chain, causing decrease in intracellular ATP levels (Kruidering et al. 1997, Portilla et al. 2002, Arany et al. 2008).

Cisplatin-induced renal and tumor cell death involves multiple pathways including oxidative stress, activation of intrinsic and extrinsic apoptotic pathways and endonucleases (Miller et al. 2010). *In vitro*, generally low concentrations of cisplatin result in apoptosis whereas at higher concentrations there is necrosis (Lieberthal et al. 1996, Lee et al. 2001). *In vivo*, both apoptosis and necrosis in the kidneys have been observed (Megyesi et al. 1998, Ramesh and Reeves 2004, Miller et al. 2010). Death receptor pathways (tumor necrosis factor or Fas) can be activated by cisplatin as seen in renal epithelial cells (Tsuruya et al. 2003). Activation of caspases 3, 8, and 9 can occur as early as 12 hours after cisplatin treatment *in vitro* (Kaushal et al. 2001). P53 is involved in the induction of cell cycle arrest or apoptosis as a response to damage by cisplatin (Bassett et al. 2008). Cisplatin treatment was shown to activate p53 in the kidney *in vivo* (Wei et al. 2007) and in cells *in vitro* (Seth et al. 2005). The transcription factor Nrf2 (nuclear factor E2-related factor 2) is also up-regulated due to cisplatin-mediated oxidative stress and induces expression of cytoprotective genes to mitigate nephrotoxicity (Aleksunes et al. 2010). Autophagy has been shown to participate in cisplatin-mediated injury. Rapid expression of autophagic proteins and formation of autophagosomes was detected following cisplatin treatment in renal epithelial cells and seen to play a protective role (Kaushal et al. 2008, Periyasamy-Thandavan et al. 2008). Lastly, cell cycle regulator proteins also play an important role in tubular cell damage. Many cells enter the cell cycle following kidney injury (Megyesi et al. 1996, Price et al. 2004). Inhibition of progression of the cell cycle by p21 (a cyclin dependent kinase inhibitor) allows for enhanced repair of cisplatin-induced DNA damage (Yu et al. 2005, Price et al. 2006).

1.3.4. Urinary Protein Biomarkers of Kidney Injury in Patients Receiving Cisplatin Chemotherapy

1.3.4.1. Introduction

Despite the recent development of new immunotherapies and anticancer drugs, cisplatin continues as an important component of chemotherapeutic regimens for the treatment of solid tumors. Use of cisplatin can be limited by acute kidney injury (AKI), which occurs in about one-third of patients (Shord et al. 2006). In an attempt to decrease the incidence of AKI, clinicians employ preventive measures including hydration and diuresis to enhance cisplatin excretion and reduce renal exposure. Early histopathological studies in rats revealed the first signs of nephrotoxicity as evidenced by acute proximal tubular necrosis (Kociba and Sleight 1971). In humans, cisplatin largely injures the proximal and distal convoluted tubules of the kidneys and to some extent, the collecting ducts (Gonzales-Vitale et al. 1977, Dentino et al. 1978). The incidence of nephrotoxicity increases with the cumulative dose of cisplatin in patients and typically occurs at doses above 50 mg/m^2 (Shord et al. 2006). Clinically, cisplatin nephrotoxicity is detectable through increases in serum creatinine (SCr) and blood urea nitrogen (BUN) concentrations, as well as electrolyte imbalances. Progressive and permanent damage may occur with successive treatments. The mechanisms of cisplatin-induced kidney injury have been extensively studied. Cisplatin actively accumulates in renal tubular cells due to the presence of basolateral uptake transporters such as the copper transporter 1 and organic cation transporter 2 (Filipski et al. 2009, Pabla et al. 2009, Ciarimboli et al. 2010). Once inside cells, the chloride atoms of cisplatin become labile and are replaced by water molecules to subsequently form hydrated, electrophilic species that target cellular or mitochondrial DNA, RNA and proteins (Pascoe and Roberts 1974). Studies in rats and mice have also suggested that cisplatin is further biotransformed into highly reactive thiols that injure tubule cells (Hanigan et al. 2001,

Townsend and Hanigan 2002, Zhang and Hanigan 2003). A number of biochemical and cellular processes are perturbed leading to oxidative and nitrative stress, inflammation, lipid peroxidation, and organelle damage ultimately resulting in the activation of apoptotic or necrotic pathways (reviewed in Karasawa and Steyger 2015).

Recently, it was demonstrated that patients prescribed cisplatin have small but permanent declines in renal function (Latcha et al. 2016). Thus, there is great interest in identifying nephrotoxicity early in patients treated with cisplatin. Current clinical methods, such as SCr and estimated glomerular filtration rate (eGFR), require a substantial decline in kidney function in order to detect clinical AKI (Lan et al. 2014). However, there has been a recent surge of research activity aimed at testing the utility of urinary protein biomarkers as a noninvasive and sensitive means of diagnosing drug-induced nephrotoxicity in patients. In 2008, the U.S. Food and Drug Administration (FDA) approved seven urinary protein biomarkers for use in preclinical submissions for regulatory decision-making (Dieterle et al. 2010). These included: kidney injury molecule-1 (KIM-1), clusterin, albumin, total protein, beta 2-microglobulin (B2M), cystatin C, and trefoil factor 3 (TFF3). For clinical detection of AKI, the FDA has approved a point-of-care device, which measures tissue inhibitor of metalloproteinase 2 (TIMP2) and insulin-like growth factor binding protein 7 (IGFBP7), in critically ill patients. There are several clinical studies that have evaluated the utility of urinary proteins as biomarkers of cisplatin-mediated AKI. Moreover, there is interest in using biomarkers to diagnose subclinical AKI in patients with tubular damage in the absence of significant changes in eGFR or SCr (Ronco et al. 2012). In 2016, the U.S. FDA has encouraged the exploratory use eight protein biomarkers of AKI in early clinical trials (USFDA 2016).

The ideal biomarker would be noninvasive and obtained from available sources such as urine and should exhibit high sensitivity and specificity to diagnose patients with AKI. In addition, biomarkers should be rapidly quantified, reflect the severity of the injury and be able to stratify patients according to risk and/or prognosis. Often, the diagnostic performance of a novel biomarker includes the calculation of area under the curve receiver operating characteristic (AUC-ROC) to differentiate patients with AKI from those without AKI. The specificity and sensitivity of the biomarker are compared to a current clinical diagnostic standard. An AUC-ROC value of 0.50 indicates poor diagnostic ability because there is only a 50-50 chance that the biomarker can identify patients with AKI. An ideal biomarker would exhibit an AUC-ROC of 1.00 (100% sensitivity and 100% specificity). However, since an insensitive clinical measure, often SCr, is routinely used for calculating AKI AUC-ROC values, most novel biomarkers appear to exhibit poor performance, particularly in patients with subclinical disease (Waikar et al. 2012, Waikar et al. 2013).

The purpose of this minireview is to provide an overview of the current literature regarding the most well-studied urinary proteins that may serve as potential indicators of cisplatin-induced AKI. The discussion focuses primarily on clinical studies evaluating albumin, B2M, N-acetyl-D-glucosaminidase (NAG), KIM-1, neutrophil gelatinase-associated lipocalin (NGAL), and cystatin C. Notably, these biomarker proteins, with the exception of B2M, were encouraged by the U.S. FDA for exploratory evaluation of AKI in early clinical trials of new drugs in their 2016 Letter of Support document (USFDA 2016). Brief highlights will also address additional proteins that are emerging as possible biomarkers of cisplatin AKI. Comprehensive tables with key features of each study are included and summarized in each section.

1.3.4.2. Albumin

Albumin is a high molecular weight protein (66.5 kDa) that is not normally detected in urine due to the absence of significant filtration and the presence of reabsorption by proximal tubules (Peterson et al. 1969). However, albumin can be found in the urine of patients with glomerular and/or tubular kidney injury (Peterson et al. 1969). A clinical study comparing patients with one of the two types of injury to healthy individuals suggested that higher levels of urinary albumin were observed with glomerular injury, while moderately elevated levels of albumin excretion (less than 500 mg/24 h) tended to be a sign of tubular injury (Peterson et al. 1969). Following this work, studies were conducted to assess urinary albumin as a biomarker of cisplatin-induced AKI (Table 1.1). After cisplatin infusion, urinary albumin concentrations were elevated as early as 4 hours (Lin et al. 2013), with a peak between 4 to 10 days and decline from the peak value around or before 2 weeks (Pfaller et al. 1994, Takeda et al. 1994, Takeda et al. 1994). The magnitude of increases in albumin excretion from baseline varied greatly across clinical studies and populations of patients. In one set of patients (N=14) receiving cisplatin therapy at a dose of 20 mg/m^2 daily for 5 days, urinary albumin concentrations were elevated (2.9-fold) within 5 days after infusion compared to pretreatment levels and concentrations observed in healthy control subjects (Fleming et al. 1979). In this study, renal damage was not detected by less sensitive, routine laboratory tests such as SCr. The magnitude of changes in urinary albumin concentrations across multiple cycles of cisplatin was measured in the same set of patients and found to be similar from cycle-to-cycle when high doses of cisplatin were prescribed (40 mg/m^2 for 5 days every 3 weeks) (Daugaard et al. 1988). By comparison, in patients receiving low-dose cisplatin (20 mg/m^2 for 5 days), baseline excretion of albumin in urine for 24 h was significantly increased by the third cycle of cisplatin compared to the first cycle (Daugaard et al. 1988). Some data have suggested that early time-points (within 24 h) have no predictive

value for AKI AUC-ROC value of 0.52) (Lin et al. 2013). However, by day 4, the AUC-ROC value increased to 0.7 in patients diagnosed with clinical AKI, although time-dependent elevations in urinary albumin were seen in both AKI and no-AKI groups (Lin et al. 2013). Since urinary albumin excretion is also an indicator of glomerular injury, very large and/or delayed increases in urinary albumin may reflect more extensive damage to the entire nephron (Takeda et al. 1994, Takeda et al. 1994, Johnsson et al. 1996). Nonetheless, the current data largely support maximal increases in urinary albumin concentrations within 4 to 10 days after cisplatin infusion in patients with clinical and subclinical AKI (Table 2).

1.3.4.3. Beta-2 Microglobulin

Beta-2 microglobulin (B2M) is a low molecular weight protein (~13 kDa) that is typically reabsorbed by renal proximal tubule cells after filtration (Peterson et al. 1969). Therefore, detection of B2M in urine indicates an impairment of tubular function. B2M has become one of the most commonly utilized urinary proteins for monitoring cisplatin-induced AKI (Table 1.2). Several studies across solid tumor types and cisplatin dose ranges have observed a rise in urinary B2M levels following cisplatin treatment even in the absence of clinically detectable AKI (Cohen et al. 1981, Buamah et al. 1982, Sorensen et al. 1985, Tirelli et al. 1985, Takashi et al. 1996, George et al. 2017). As a result, B2M may not necessarily have high predictive value to distinguish between patients with clinical and subclinical AKI (Shinke et al. 2015). In one study, patients that had abnormal increases in urinary B2M did not subsequently develop nephrotoxicity (de Gislain et al. 1986). One reason could be that increased systemic production of B2M may occur in the presence of underlying malignancy. Several studies have shown that baseline urinary B2M concentrations are elevated in cancer patients who have not received chemotherapy, compared to healthy volunteers (Jones et al. 1980, Tirelli et al.

1985, George et al. 2017). Nonetheless, increases in urinary B2M following cisplatin therapy occur as early as 12 hours (Cohen et al. 1981) and generally peak between 3 and 6 days (Table 1.2) (Fleming et al. 1979, Sorensen et al. 1985, Daugaard et al. 1988, Ikeda et al. 1988, George et al. 2017). Urinary B2M levels decline about 1 to 2 weeks after cisplatin administration (Fleming et al. 1979, Ikeda et al. 1988, Takeda et al. 1994, Fuke et al. 2009) and are in the normal range at least 3-months after cessation of cisplatin-containing chemotherapy (N=35) (Brillet et al. 1994). The magnitude of B2M elevation is attenuated with successive cycles of chemotherapy (Buamah et al. 1982, Tirelli et al. 1985, Daugaard et al. 1988), although no significant correlations were observed between previous exposure to cisplatin, the dose of cisplatin, and urinary B2M levels (Cohen et al. 1981). While most of these studies have focused on urinary B2M excretion, it is important to note that serum B2M does not change significantly following cisplatin therapy (de Gislain et al. 1986).

1.3.4.4. N-Acetyl-Beta-D Glucosaminidase

N-acetyl-beta-D glucosaminidase (NAG) is a lysosomal enzyme identified in the proximal and distal tubules of rat kidneys (Le Hir et al. 1979). Due to its large molecular weight (130 kDa), NAG is not normally filtered and its presence in the urine largely reflects tubular destruction (reviewed in Hong and Chia 1998). Over the past few decades, numerous studies have evaluated urinary NAG concentrations as a surrogate marker of cisplatin AKI in patients (Table 1.3). Early work revealed that urinary NAG rises in response to cisplatin, but not carboplatin, containing chemotherapy regimens (Goren et al. 1987, Takashi et al. 1996). Urinary NAG levels generally peaked within 1 week after cisplatin treatment, typically between 3 to 5 days, with variability in maximal changes from baseline (3 to 13-fold both in the absence or presence of AKI) (Goren et al. 1987, Verplanke et al. 1994, Takashi et al. 1996). In these patients, there was no clear

relationship between maximal NAG changes and a clinical diagnosis of AKI. As a result, there is limited utility in using NAG to predict clinical AKI secondary to cisplatin. For example, in one study there was no difference in urinary NAG levels between groups of patients distinguishable by a 20% decline in eGFR (Hosohata et al. 2016). Similarly, NAG was unable to distinguish between AKI-positive (N=30) and AKI-negative (N=12) samples in lung cancer patients treated with cisplatin (Shinke et al. 2015). Long-term studies have demonstrated that urinary NAG concentrations return to normal levels by at least 3 months after 2 to 7 courses of cisplatin (Brillet et al. 1994). Furthermore, another report suggested that urinary NAG concentrations may even return to baseline as early as 2 weeks after cisplatin treatment (Takeda et al. 1994). Interestingly, there was no evidence of an increase in baseline NAG levels after additional courses of cisplatin treatment (up to 6) (Buamah et al. 1982, Tirelli et al. 1985, Pfaller et al. 1994). NAG, similar to B2M, did not correlate with accumulated cisplatin dose (Ikeda et al. 1988). Taken together, NAG concentrations increase in the urine of patients with subclinical and clinical AKI within 3 to 5 days after cisplatin treatment but do not reflect the degree of injury or the cumulative dose of cisplatin received.

1.3.4.5. Kidney Injury Molecule-1

Kidney injury molecule-1 (KIM-1) is a type I cell membrane glycoprotein (104 kDa) that is highly conserved across rodents, dogs, primates, and humans (Ichimura et al. 1998). KIM-1 is also a phosphatidylserine receptor found on renal epithelial cells that recognizes apoptotic cells and enables their clearance by phagocytosis (Ichimura et al. 2008). During proximal tubular injury, KIM-1 mRNA is up-regulated and the ectodomain of KIM-1 protein (90 kDa) is shed from the brush border membrane into the urine. KIM-1 is a prominent and promising biomarker for various etiologies of AKI and there have been several studies utilizing KIM-1 for cisplatin-induced AKI (Table 1.4). Urinary KIM-1

concentrations were shown to predictably increase in the presence of clinically detectable cisplatin-induced AKI with the peak value occurring with or following a rise in SCr. Among lung cancer patients (N=11), 10 patients were diagnosed with cisplatin-induced AKI on day 3 based on an increase in BUN > 20 mg/dL and/or a rise of 50% SCr level from baseline (Shinke et al. 2015). Urinary KIM-1 levels also exhibited time-dependent increases in these patients. In a representative patient receiving cisplatin, urinary KIM-1 concentrations peaked on day 7 as reflected in a 6-fold increase from baseline. Analysis of cisplatin AKI-positive (N=30) and AKI-negative (N=12) samples revealed a high AUC-ROC value of 0.858 suggesting a strong predictive value between urinary KIM-1 concentrations and a clinical diagnosis of AKI. Similarly, in a study of patients receiving their first cycle of cisplatin-containing chemotherapy (N=22), 8 patients developed AKI by day 3, defined by >1.5-fold elevation in SCr (Tekce et al. 2015). A significant time-dependent elevation in absolute and creatinine-normalized KIM-1 levels was detected in the AKI group and peaked on day 3. The AUC-ROC value for KIM-1 was 0.94 on day 3 after cisplatin treatment. Notably, serum KIM-1 concentrations did not differ between the AKI and non-AKI groups at any of the time points. In another study of cisplatin-naïve patients treated with cisplatin for various solid tumors (N=27), 2 patients developed a 50% increase in SCr (Pianta et al. 2017). The absolute and creatinine-normalized concentrations of KIM-1 in all patients increased between days 3 and 14, with a peak on day 7.

Interestingly many studies have also shown that patients with subclinical AKI also display time-dependent increases in urinary KIM-1 levels (Tekce et al. 2015, Pavkovic et al. 2016, George et al. 2017). However, the clinical relevance of the KIM-1 changes in subclinical AKI is not entirely clear. For example, KIM-1 displayed a diminished ability to predict nephrotoxicity at 3 days (AUC-ROC value 0.55) when using a modest change in

eGFR to diagnose nephrotoxicity (20% decline in eGFR) in urothelial carcinoma patients with AKI (N=9) or no-AKI (N=15) (Hosohata et al. 2016). Nonetheless, even patients with <20% decline in eGFR exhibited time-dependent elevations in urinary KIM-1 concentrations. Another published study (N=57) in cisplatin-treated patients with subclinical AKI supports this trend (George et al. 2017).

1.3.4.6. Neutrophil Gelatinase-Associated Lipocalin

The NGAL gene encodes for a small protein (25 kDa) bound to gelatinase that is secreted from human neutrophils (Kjeldsen et al. 2000, Xu and Venge 2000, Yang et al. 2002). NGAL sequester irons and prevents bacterial growth. In a transcriptomic study to identify ischemia-related genes, NGAL was demonstrated to be significantly induced within mouse kidneys (Mishra et al. 2003). Additional work revealed that NGAL is up-regulated and enriched in proliferating mouse proximal tubule cells after ischemia (Mishra et al. 2003). Secretion of NGAL protein in the urine has recently gained interest for use as a biomarker of AKI. There is a robust time-dependent increase in urinary NGAL protein concentrations from 0.5 to 3 days after cisplatin therapy that largely precedes elevations in SCr (Table 1.5) (Gaspari et al. 2010, Lin et al. 2013, Shahbazi et al. 2015). In one pediatric study, the dose of cisplatin correlated with post-cisplatin infusion concentrations of NGAL in urine ($r = 0.73$, N=21) (Sterling et al. 2017). Moreover, urinary levels of NGAL have been suggested as a means to differentiate patients with clinical AKI versus subclinical or no AKI. In one clinical study, significant elevations in NGAL at day 1 preceded a modest increase in SCr on day 3 (20%) prior to a maximal SCr increase on day 7 (Gaspari et al. 2010). In fact, urinary NGAL levels were significantly higher in patients with AKI at days 1, 2, 3 and 15 after cisplatin compared to those not exhibiting clinical AKI. Importantly, an increase in NGAL at day 2 was found to be a significant independent predictor of AKI. A study of patients (N=33)

revealed an AUC-ROC value for NGAL of 0.87 at 12 and 24 h (Lin et al. 2013). Other studies have also revealed similar findings with an AUC-ROC value of 0.80 at 24 h signifying important predictive value for NGAL to reflect AKI (Shahbazi et al. 2015). Recent data also suggest that elevations in urinary NGAL may even be able to detect AKI as early as 2 h (Karademir et al. 2016). Interestingly, patients with a slow recovery from AKI had earlier and higher increases in urinary NGAL levels (Gaspari et al. 2010). The time points selected for NGAL quantification are important. Several studies that assessed urinary NGAL at or past day 3 have a lesser ability to detect nephrotoxicity in patients receiving cisplatin (Shinke et al. 2015, Hosohata et al. 2016, George et al. 2017). Lastly, urinary NGAL may also identify AKI following treatment with other platinum analogs. In one study, urinary NGAL concentrations increased 2.3-fold, 2.7-fold, and 1.5-fold from baseline in response to chemotherapy containing cisplatin, carboplatin, and oxaliplatin, respectively (Seker et al. 2015). Notably, concentrations of NGAL in serum have little ability to detect cisplatin-induced AKI (Gaspari et al. 2010, Kos et al. 2013). Serum NGAL levels were similar in patients with and without cisplatin-induced AKI (Gaspari et al. 2010).

1.3.4.7. Cystatin C

Cystatin C is part of a family of cysteine proteinase inhibitors (13 kDa) expressed ubiquitously in all nucleated cells (Benohr et al. 2006). Cystatin C is primarily eliminated by glomerular filtration and therefore, serum cystatin C concentrations have been used as an endogenous marker of GFR. Since cystatin C is also catabolized by renal tubular epithelia, the presence of cystatin C in the urine indicates tubular injury. Most clinical studies investigating the relationship between cystatin C and cisplatin AKI have largely focused on changes in the serum rather than the urine (Table 1.6). Serum cystatin C concentrations were elevated 41% by day 3 in patients receiving cisplatin (N=27) (Pianta

et al. 2017). Smaller changes (1.1-fold) in serum cystatin C have been reported in other studies where no or minimal clinical AKI had occurred (Benohr et al. 2006, Kos et al. 2013, Karademir et al. 2016). Caution should be taken when interpreting changes in serum cystatin C as levels can be affected by corticosteroids, hyperthyroidism, and other conditions (Bokenkamp et al. 2002, Risch and Huber 2002, Manetti et al. 2005). Because of the modest changes observed in serum and the sensitivity of this protein to influence by concomitant diseases, cystatin C may not be an ideal biomarker for cisplatin AKI.

There is little change in urinary cystatin C concentration in the first 8 h of cisplatin treatment (Lin et al. 2013), however, 2-fold increases may be evident at later time points (day 3) (George et al. 2017). Additional work is needed to characterize the utility of urinary cystatin C concentrations for identifying subclinical and clinical AKI in oncology patients receiving cisplatin.

1.3.4.8. Calbindin

Calbindin is a 28 kDa calcium-binding protein found primarily in the distal tubules and collecting ducts of the kidneys (Bredderman and Wasserman 1974). The mechanistic role for calbindin in kidney injury is unknown, however two contradictory studies in cisplatin-treated rats have observed either an increase or decrease of calbindin in the urine and an increase in serum calbindin (Togashi et al. 2012, Won et al. 2016). Two studies have quantified urinary calbindin concentrations following cisplatin administration in cancer patients (Table 1.7). In both studies, calbindin levels were enhanced in the urine in the absence of clinically detectable AKI. There was a 23-fold increase in urinary calbindin and a 7-fold increase in serum calbindin which peaked by day 10 in cisplatin-treated patients (N=14) (Takashi et al. 1996). In this study, patients receiving the analog

carboplatin did not exhibit changes in urinary calbindin concentrations. Similarly, another study revealed a 8-fold increase in urinary calbindin by day 10 after cisplatin infusion, in the absence of SCr changes (George et al. 2017). These data suggest calbindin excretion into urine is elevated in response to cisplatin treatment, however further studies are required to understand the mechanistic role of calbindin in AKI.

1.3.4.9. Insulin-like Growth Factor Binding Protein-7 and Tissue Inhibitor of Metalloproteinase-2

Insulin-like growth factor binding protein-7 (IGFBP7) (30 kDa) and tissue inhibitor of metalloproteinases-2 (TIMP2) (68 kDa) are cell-cycle arrest proteins released into urine upon kidney injury (Bigg et al. 2001, Aregger et al. 2014). IGFBP7 is expressed in proximal tubules with increased expression upon insult, whereas TIMP2 is constitutively expressed in the distal tubules of human kidneys (Emlet et al. 2017). The U.S. FDA has approved an immunoassay, the NephroCheck® test which detects TIMP2 and IGFBP7 in the urine and calculates their product to generate an AKIRisk® Score within 20 minutes. The intended use of NephroCheck® is for the clinical risk assessment for moderate to severe AKI within the next 12 hours specifically in ICU patients (at least 21 years of age) who have or have had acute cardiovascular and/or respiratory compromise within the past 24 hours. There has been interest in assessing the utility of NephroCheck® for other causes of AKI including drug-induced toxicity. In one clinical study, 4 of 32 patients receiving cisplatin developed AKI, and the AUC-ROC value for TIMP2*IGFBP7 was 0.92 within 72 h (Schanz et al. 2017). However, another study that measured the two biomarkers individually found that there was no change in IGFBP7 concentrations 24 h after cisplatin administration, whereas a 1.1-fold increase in TIMP2 levels could be observed (Toprak et al. 2017). While 13 patients developed AKI in this study, the AUC-ROC value for the product of TIMP2*IGFBP7 was only 0.46 at 24 h. A

third study of 46 patients also showed no significant changes from baseline for either TIMP2 or IGFBP7 as well as for TIMP2*IGFBP7 at days 3 and 10 after cisplatin treatment (Chang et al. 2017). However, additional studies are required to further validate this test in cisplatin-treated patients and to find the optimal time-points for clinical use in ambulatory patients at risk of AKI.

1.3.4.10. Additional Urinary Proteins

Other promising urinary protein biomarkers have been evaluated in studies of patients receiving cisplatin. Clusterin is a secreted protein (80 kDa) up-regulated during kidney injury (Correa-Rotter et al. 1998) that possesses anti-apoptotic properties (reviewed in Rosenberg and Silkensen 1995). In two studies, there was between a 2- and 2.5-fold increase in urinary clusterin concentrations between days 7 and 10 following cisplatin treatment (George et al. 2017, Pianta et al. 2017). Similarly, the urinary protein monocyte chemotactic peptide-1 (MCP-1) (13 kDa) has shown some promise as an indicator of subclinical or clinical AKI (Prodjosudjadi et al. 1996). MCP-1 is a potent proinflammatory chemokine that plays a role in monocyte recruitment to sites of injury and was found to be increased in the proximal tubules and urine following cisplatin injury in rats (Nishihara et al. 2013). MCP-1 was elevated 1.7- to 3.8-fold between days 7-10 in patients treated with cisplatin (Shinke et al. 2015, George et al. 2017). One study also reported an AUC-ROC value of 0.85 for urinary MCP-1 and AKI on day 7 (Shinke et al. 2015).

Trefoil factor 3 (TFF3) is a small peptide hormone (~9 kDa) secreted by mucus-producing cells and enriched in proximal tubules (Yu et al. 2010, Du et al. 2013). TFF3 is involved in epithelial surface repair through inhibition of apoptosis and promotion of cell survival and migration (Kinoshita et al. 2000). Interestingly, urinary TFF3 concentrations

have been found to decrease in response to toxicant-induced renal injury in rats (carbapenem, gentamicin, cisplatin) (Yu et al. 2010, Wadey et al. 2014). Only one study has quantified urinary TFF3 levels in patients receiving cisplatin. In this study, a 2-fold increase from baseline was observed on day 10 in patients that did not exhibit clinical AKI using traditional clinical diagnostic measures (George et al. 2017). Further studies are required to understand why TFF3 protein concentrations increase in humans, but decrease in rodents, following cisplatin exposure.

The regulation of various isoforms of GST, an enzyme involved in the detoxification of cisplatin, has been studied in response to cisplatin administration. GST-alpha (51 kDa), which is primarily expressed in proximal tubules following injury (Harrison et al. 1989, reviewed in McMahon et al. 2010), was increased in response to cisplatin injury in rats (Gautier et al. 2010, Harpur et al. 2011, McDuffie et al. 2013, Won et al. 2016). Excretion of GST-pi (47 kDa), a marker for distal tubular damage (Harrison et al. 1989), was evaluated in the urine of cisplatin-treated patients without clinical AKI and found to be moderately elevated by day 10 (1.6-fold) (George et al. 2017).

There are still other biomarkers that have yet to be evaluated in cisplatin-treated patients including fatty acid-binding protein 1 (FABP1), a 15-kDa protein localized in the cytoplasmic regions of proximal tubules of human kidney (Maatman et al. 1991, Yan et al. 2009). Notably, rodents do not express FABP1 in their kidneys. However, mice expressing the human FABP1 protein had increased shedding of FABP1 into urine following cisplatin treatment that correlated with the extent of injury (Negishi et al. 2007). Quantification of FABP1 in the clinical setting of cisplatin-induced AKI is still lacking. Osteopontin (~40 kDa) has shown great promise as a biomarker in *in vivo* studies of rats treated with cisplatin (Fuchs et al. 2012, Pinches et al. 2012, Vinken et al. 2012, Wadey

et al. 2014, Pianta et al. 2017). However, only one study evaluated osteopontin in cisplatin-treated patients without clinical AKI and did not detect a significant change on day 3 or 10 (George et al. 2017). Further investigation of these additional proteins is needed to better understand their utility as biomarkers of cisplatin-induced clinical or subclinical AKI.

1.3.4.11. Conclusion

It is well known that SCr has significant limitations that hinder the sensitive and timely identification of AKI, particularly when there is only a moderate degree of damage to the kidneys. Urinary protein biomarkers play an important role in our ability to predict drug-induced injuries. In the case of cisplatin, sensitive urinary biomarkers could provide clinicians with greater information in detecting kidney injury and could aid in selecting doses for subsequent rounds of chemotherapy. The ideal AKI biomarker should be noninvasive, reflective of the degree of injury, and be unaffected by inter-individual and external variation such as concomitant medications or diseases. The implementation of novel urinary biomarkers in the clinic is lagging due to a lack of sufficient validation and inconsistencies across studies. To address these issues, larger head-to-head studies are needed to identify time-dependent responses and to establish accurate cutoff values and ranges, particularly in cancer patients. Aside from protein biomarkers, the release of microRNAs and exosomes into the urine also shows great promise for recognizing kidney injury in patients. A combination of ‘omic’ strategies, such as protein and microRNA biomarkers, may represent one option for the early and sensitive detection of AKI.

1.3.5. Antiemetic Drugs

Cisplatin is a highly emetogenic medication requiring the rigorous use of medications to control nausea and vomiting. clinical guidelines recommend the use of four different classes of medications: 5-HT_3 antagonists, NK_1 antagonists, olanzapine and dexamethasone (Hesketh et al. 2017)

1.3.5.1. 5-HT_3 Antagonists

Development of selective 5-HT_3 antagonists in the 1990s dramatically improved treatment of nausea and vomiting (Cubeddu et al. 1990, Sagar 1991, Hornby 2001). 5-HT_3 antagonists prevent the binding of serotonin to 5-HT_3 receptors in the periphery as well as centrally in the area postrema (Hornby 2001). There are many drugs that currently belong to the 5-HT_3 antagonist class, varying in generic availability, potency as well as duration of action (Table 1.8).

The first generation 5-HT_3 antagonists include dolasetron, granisetron, and ondansetron. Discovery of potent antagonists through structure-activity relationship studies led to dolasetron, which was first identified and later approved by FDA in 1997 (Sorensen et al. 1989). Dolasetron is metabolized (>99%) by carbonyl reductase to an active metabolite, hydrodolasetron, 50 times more potent than dolasetron (Boeijinga et al. 1992). Ondansetron was also discovered as a potent 5-HT_3 antagonist and approved in 1991 as the first agent in the class for treatment for chemotherapy induced nausea and vomiting (CINV) (Hagan et al. 1987). Using serotonin as a starting point, related chemical structures were developed later including tropisetron and granisetron. All first generation 5-HT_3 antagonists contain a rigid aromatic system, a coplanar hydrogen bond accepting group, and a basic center (Figure 1.12) (Hibert et al. 1990, Rizzi et al. 1990). The first generation 5-HT_3 antagonists provided complete acute emesis protection in 50-

70% of patients receiving first cycle of cisplatin (Aapro and Johnson 2005). Palonosetron is a second-generation 5-HT_3 antagonist with a much greater affinity for 5-HT_3 receptors than first generation drugs and is approved for both acute and delayed chemotherapy-induced nausea and vomiting. It has a distinct chemical structure with a long plasma half-life (> 40 h) compared to the first generation 5-HT_3 antagonists.

1.3.5.2. Dexamethasone

Concomitant use of 5-HT_3 antagonists with dexamethasone, an anti-inflammatory steroid, improved efficacy against acute emesis compared to 5-HT_3 antagonist alone and improved protection by 10 to 15% (Verweij et al. 1996). The mechanism of antiemesis by dexamethasone is not clear but is thought to involve serotonin. Dexamethasone and other corticosteroids may reduce levels of tryptophan, a precursor of serotonin (Young 1981). The anti-inflammatory properties of corticosteroids may also help prevent release of serotonin and/or inhibit prostanoid synthesis in the gastrointestinal tract (Sagar 1991, Darmani and Ray 2009). However, 5-HT_3 antagonists and dexamethasone in combination still only provided minimal protection from delayed emesis.

1.3.5.3. Olanzapine

Olanzapine is an atypical antipsychotic agent with the ability to block many receptors including dopaminergic and serotonergic receptors (Navari and Loprinzi Ch 2016). Until recently, olanzapine was only recommended in the CINV guidelines for refractory and breakthrough emesis. However in combination with a 5-HT_3 antagonist, NK_1 antagonist, and dexamethasone, olanzapine demonstrated superiority over placebo (74% vs. 45% in early period; 42% vs. 25% in later period) (Navari 2016). Due to this reason, guidelines now recommend addition of olanzapine to 5-HT_3 antagonist, NK_1 receptor antagonist, and dexamethasone in patients taking highly emetogenic agents as first-line therapy.

1.3.5.4. NK_1 Antagonists

NK_1 receptors are located in the gut and regions of the central nervous system involved in the emetic reflex. NK_1 antagonists prevent binding of substance P, a neurotransmitter involved in the regulation of inflammatory responses that is released in response to chemotherapies, to the NK_1 receptor (US and Gaddum 1931, Chang and Leeman 1970). Aprepitant was discovered as a high affinity inhibitor of the NK_1 receptor with good oral bioavailability. Aprepitant was approved in 2003 showing superior control of delayed emesis compared to ondansetron (Hesketh et al. 2003, Poli-Bigelli et al. 2003). Due to the low water-solubility, a prodrug was later approved in 2008, fosaprepitant (Hale et al. 2000).

1.3.5.5. Alternative Antiemetic Drugs

Before the discovery of 5-HT_3 antagonists, high-dose metoclopramide and prochlorperazine (antidopaminergic agents) were used to manage CINV in combination with dexamethasone in the 1960s and 1970s. This treatment only provided relief to <50% of the patients (Gralla 1983). Later it was understood that high-dose metoclopramide targets 5-HT_3 receptors rather than dopamine receptors leading to the discovery of potent 5-HT_3 antagonists. Metoclopramide and prochlorperazine are currently recommended only for breakthrough or refractory emesis or for patients with allergies to the first-line therapies.

1.4. Research Objective and Hypothesis

Taking together all the current information in this area, an important research hypothesis emerged: Novel urinary protein biomarkers can be used to detect subclinical cisplatin-induced nephrotoxicity and concurrently administered antiemetic drugs can inhibit

cisplatin renal transporters. Four specific aims of this thesis were employed to evaluate this hypothesis:

1) Assess the time-dependent changes in the urinary excretion of novel protein biomarkers in oncology patients prescribed cisplatin.
2) Determine whether the excretion of urinary protein biomarkers in oncology patients differ between early and later cycles of cisplatin chemotherapy
3) Characterize the renal expression of calbindin in cisplatin-mediated acute kidney injury in mice.
4) Determine the extent to which antiemetic drugs inhibit kidney transporters involved in cisplatin secretion.

The findings of this research will provide understanding of the performance of novel urinary protein biomarkers in a clinical setting of cisplatin-mediated acute kidney injury and mechanistic data regarding the renal regulation and expression of calbindin, as well as interaction of co-administered medications that utilize the same transport in a target organ of toxicity. Significant knowledge gaps will be filled in the fields of cisplatin-mediated kidney injury biomarkers as well as cisplatin renal transporter interactions, leading to better implementation and validation of biomarkers clinically as well as improving the prescribing of antiemetic drugs that minimize interactions with cisplatin.

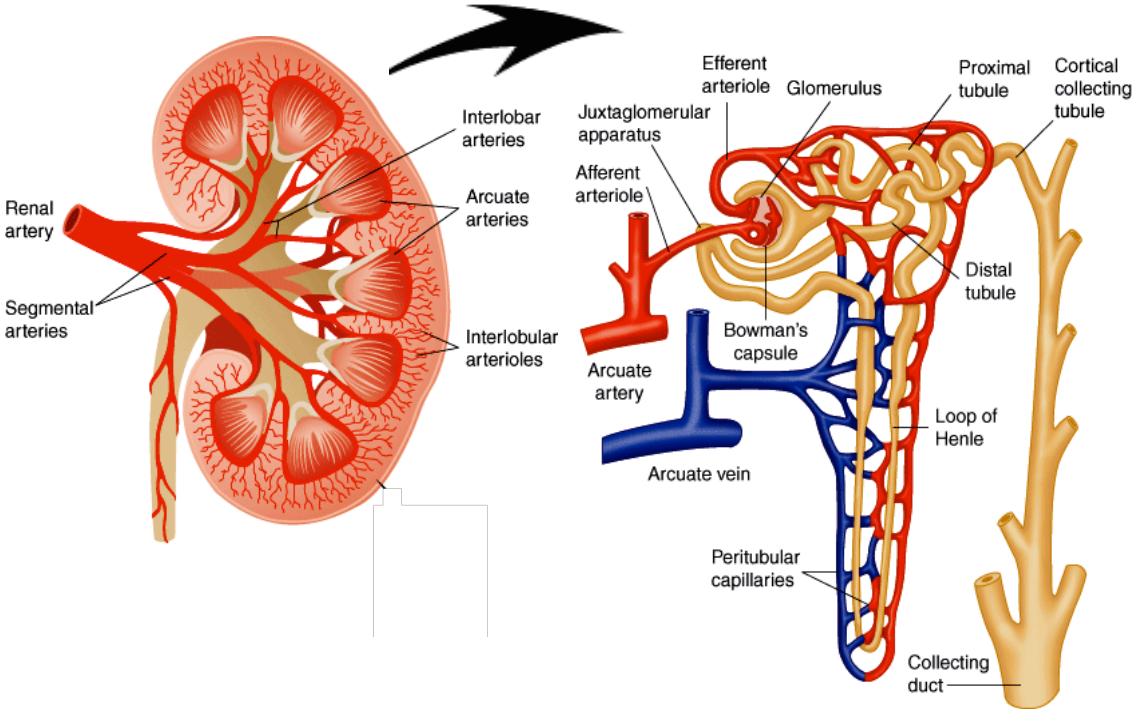

Figure 1.1. Anatomy of the Kidney and Nephron.

Schematic of the human kidney and single nephron revealing vasculature and tubular components. Image obtained from Klaassen, C. 8th Edition: Casarett & Doull's Toxicology: The Basic Science of Poisons.

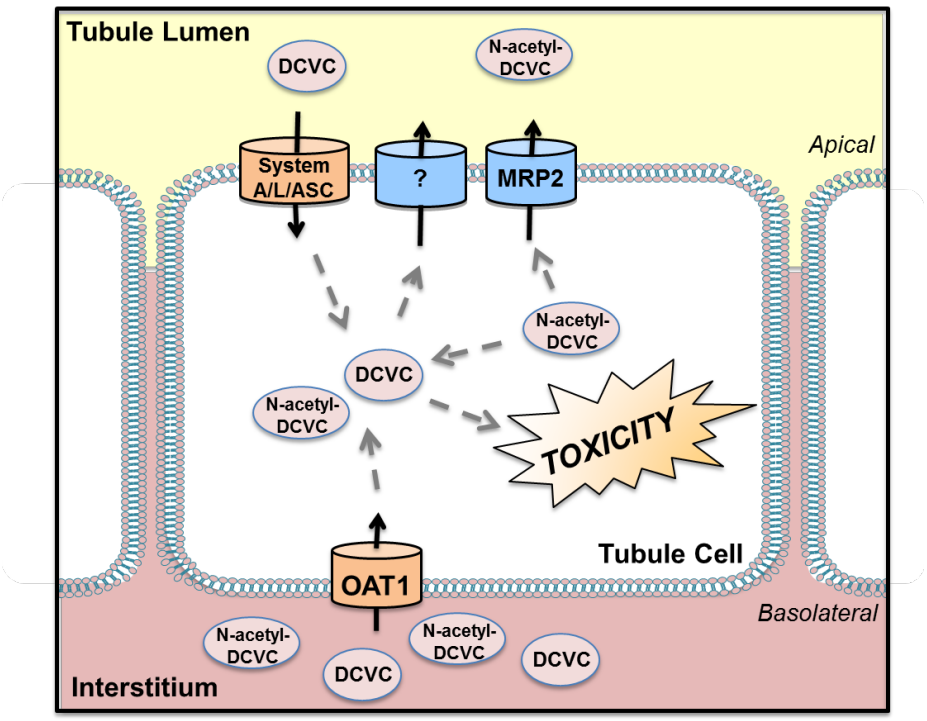

Figure 1.2. Transport of *S*-1,2,-dichlorovinyl-*L*-cysteine (DCVC) and N-acetyl-DCVC. Organic anion transporter 1 (OAT1) transports DCVC and N-acetyl-DCVC across the basolated membrane of proximal tubules while amino acid transporters, such as system ASC, A, and L, reabsorb the metabolites of trichlorethylene on the apical surface. N-acetyl-DCVC is removed from renal cells by multidrug resistance-associated protein 2 (MRP2), whereas DCVC is not.

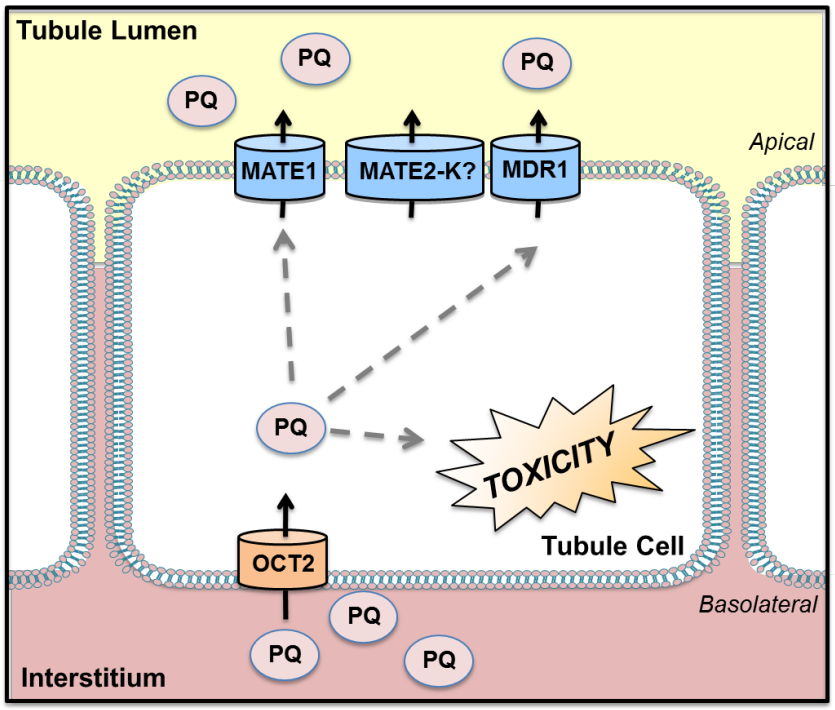

Figure 1.3. Transport of Paraquat. Organic cation transporter 2 (OCT2) is primarily responsible for the accumulation of paraquat (PQ) in proximal tubules whereby redox cycling results in toxicity. Efflux of PQ takes place through the multidrug and toxin extrusion protein 1 (MATE1) and multidrug resistance protein 1 (MDR1).

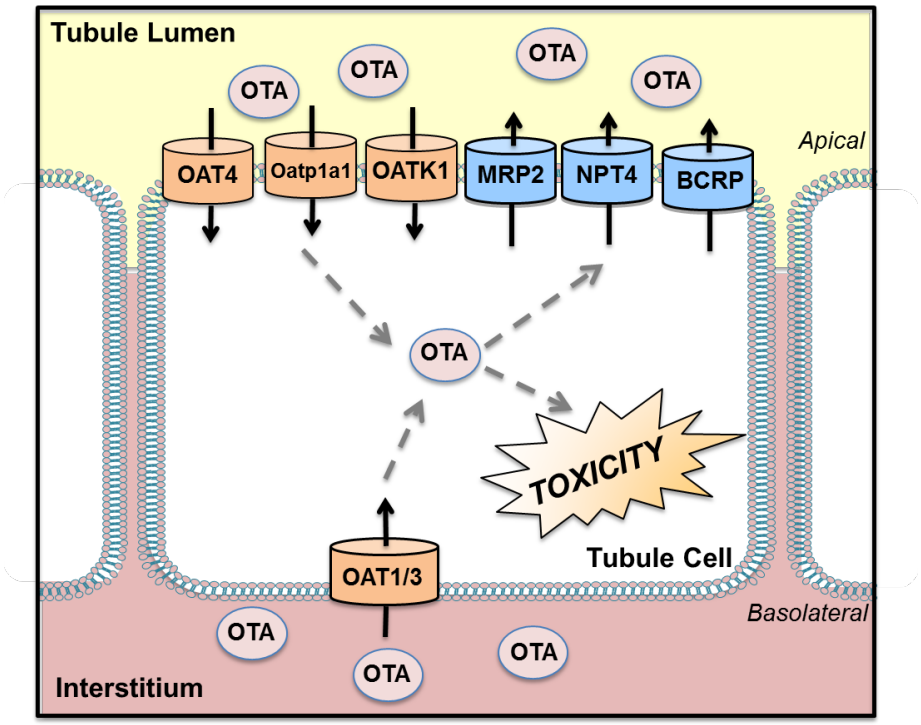

Figure 1.4. Transport of Ochratoxin. Various organic anion transporters (OATs) are involved in the uptake of ochratoxin (OTA) into proximal tubules including OAT1, 3, and 4. Other uptake transporters include the organic anion transporting polypeptide 1a1 (Oatp1a1). Efflux of OTA likely occurs through the multidrug resistance-associated protein 2 (Mrp2) and Na^+-dependent phosphate transporter (NPT4).

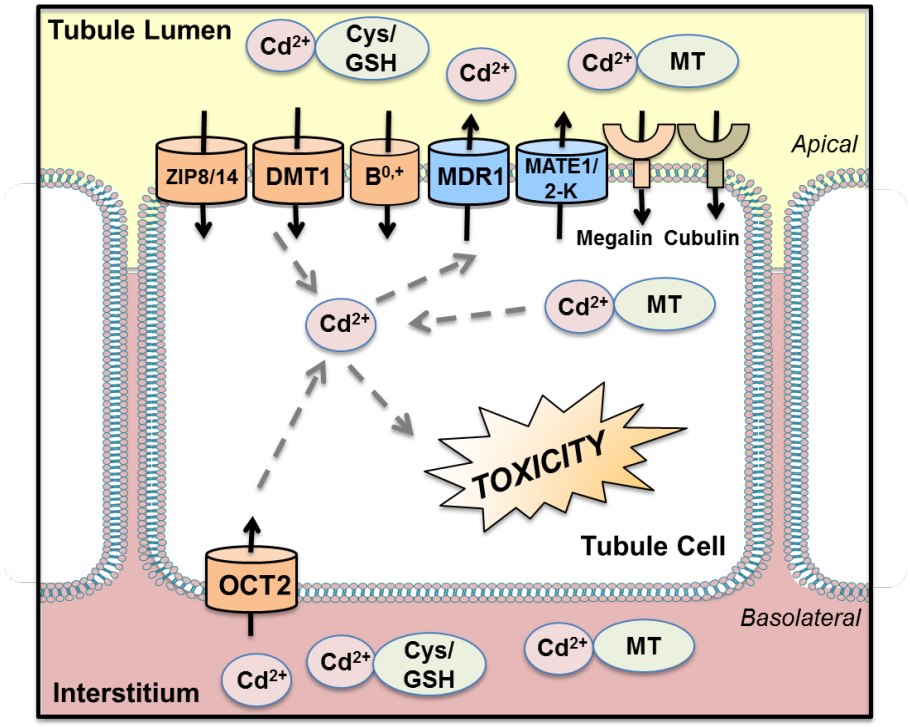

Figure 1.5. **Transport of Cadmium**. Cadmium (Cd^{2+}) is bound to albumin in the circulation after absorption. In the liver, Cd^{2+} forms a complex with metallothioneins (MT) that are released into the circulation. Cd^{2+} also binds to thiol-containing groups such as L-cysteine (Cys) and glutathione (GSH). After filtration, the Cd^{2+}-MT complexes are reabsorbed by megalin and cubulin into the proximal tubules on the apical side and undergo degradation to ionic Cd^{2+}. Cd^{2+} also displays "molecular mimicry" and uses divalent metal transporters (DMT1, divalent metal ion transporter-1) and zinc/iron proteins (ZIP8/14, zinc/iron-regulated transporter 8/14) for reabsorption. Amino acid transporters, such as system $B^{0,+}$, may also reabsorb Cys-conjugates of Cd^{2+} on the apical plasma membrane. Organic cation transporter 2 (OCT2) (and possibly Oct1 in rodents) may also influx Cd^{2+} across the basolateral side of tubule cells. Multidrug resistance protein 1 (MDR1) and multidrug and toxin extrusion protein 1 and 2-K (MATE1/2-K) contribute to the limited Cd^{2+} that is secreted.

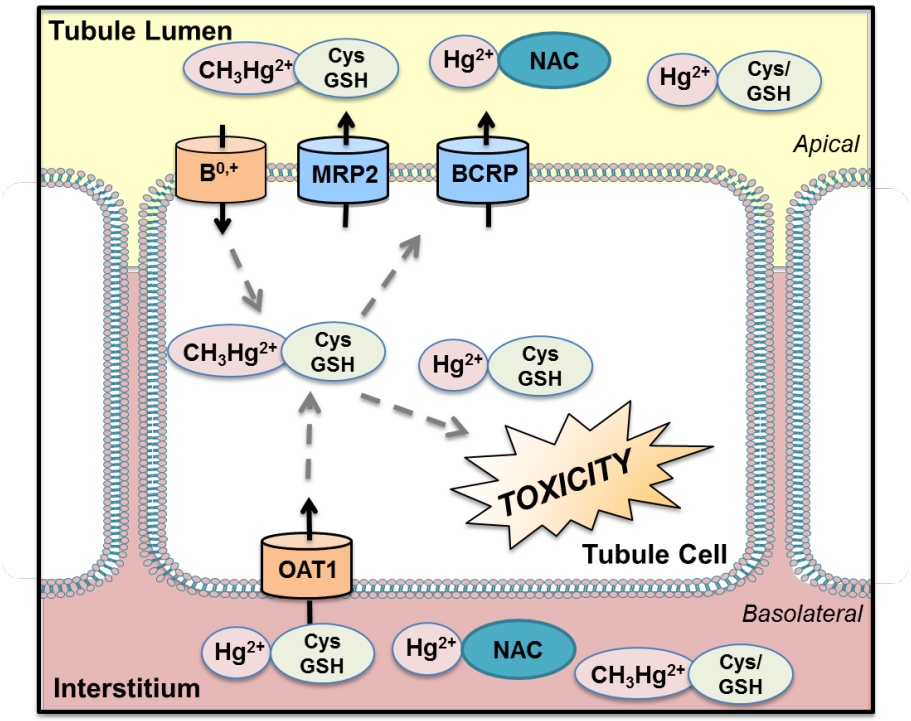

Figure 1.6. Transport of Mercury. Mercury (Hg^{2+}) and methylmercury (CH_3Hg^{2+}) bind to sulfhydryl groups including thiol-containing molecules such as glutathione (GSH), cysteine (Cys), homocysteine (Hcy), and N-acetylcysteine (NAC). These conjugated mercury species are taken up basolaterally by organic anion transporters (OAT) and apically by amino acid transporters including L -cysteine, system $B^{0,+}$, and Na^+-dependent low-affinity L -systems. Efflux transporters on the brush border membrane include multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP).

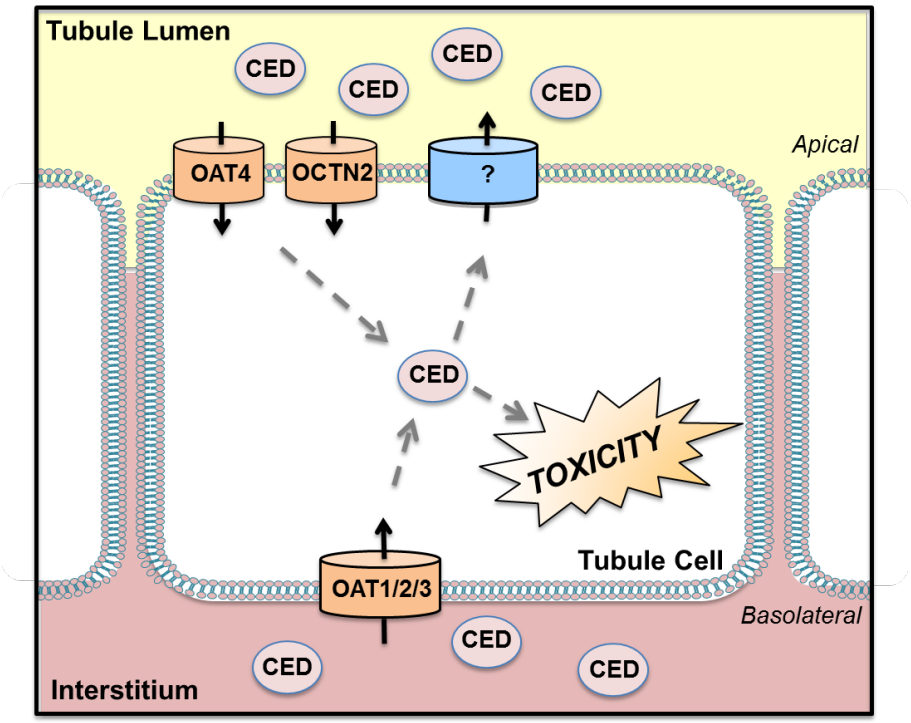

Figure 1.7. Transport of Cephaloridine. On the basolateral side of the plasma membrane, cephaloridine (CED) is taken into tubule cells by the organic anion transporters 1, 2, and 3 (OAT 1, 2, 3) whereas OAT4 and the organic cation/carnitine transporter 2 (OCTN2) mediate uptake on the apical side. Efflux transporters for cephaloridine are largely unknown and may involve multidrug and toxin extrusion protein 1 and 2-K (MATE1/2-K).

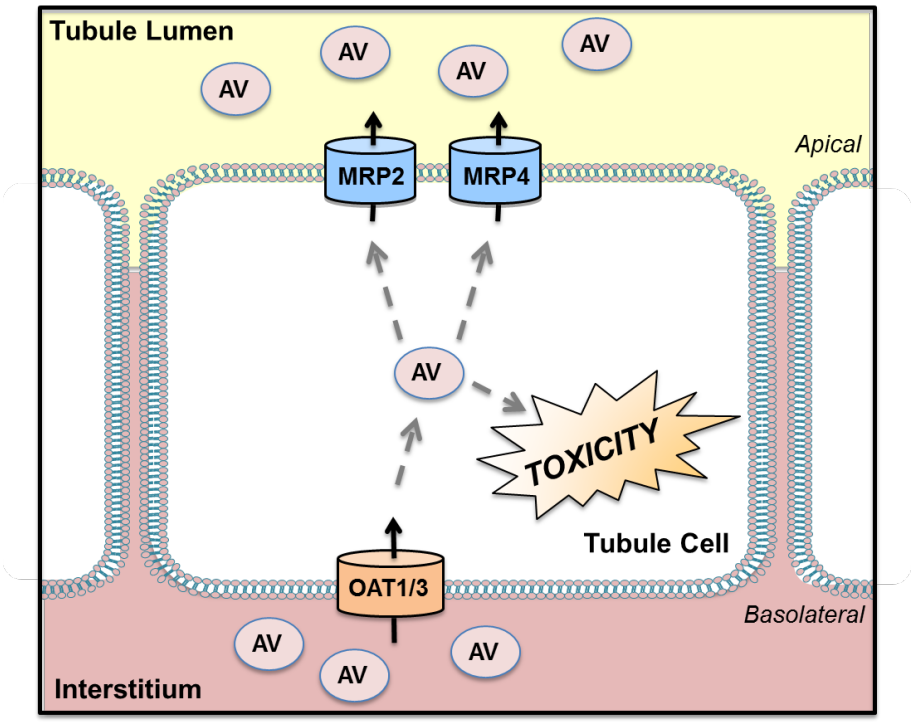

Figure 1.8. Transport of Antiviral Drugs. Anionic antiviral (AV) drugs are transported into renal cells by the organic anion transporters 1 and 3 (OAT1, 3) and removed from proximal tubules using the multidrug resistance-associated proteins 2 and 4 (MRP2, 4).

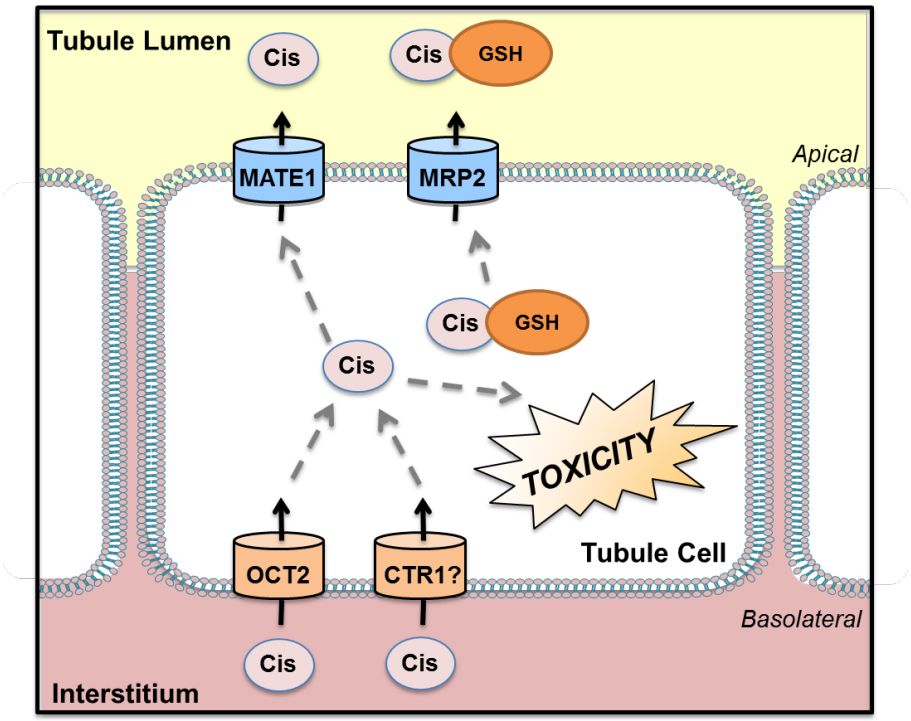

Figure 1.9. Transport of Cisplatin. Cisplatin is taken up into the proximal tubules by the organic cation transporter 2 (OCT2) and also possibly by copper transporter 1 (CTR1). Cisplatin is removed by efflux using the multidrug and toxin extrusion protein 1 (MATE1). Cisplatin also further conjugates with glutathione (GSH) in the proximal tubules and has been proposed to undergo secretion by the multidrug resistance-associated protein 2 (MRP2).

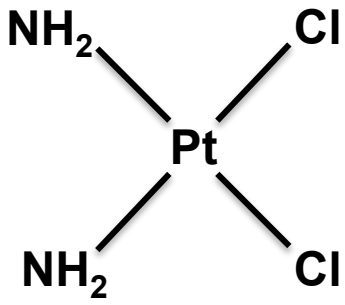

Figure 1.10. Chemical Structure of Cisplatin

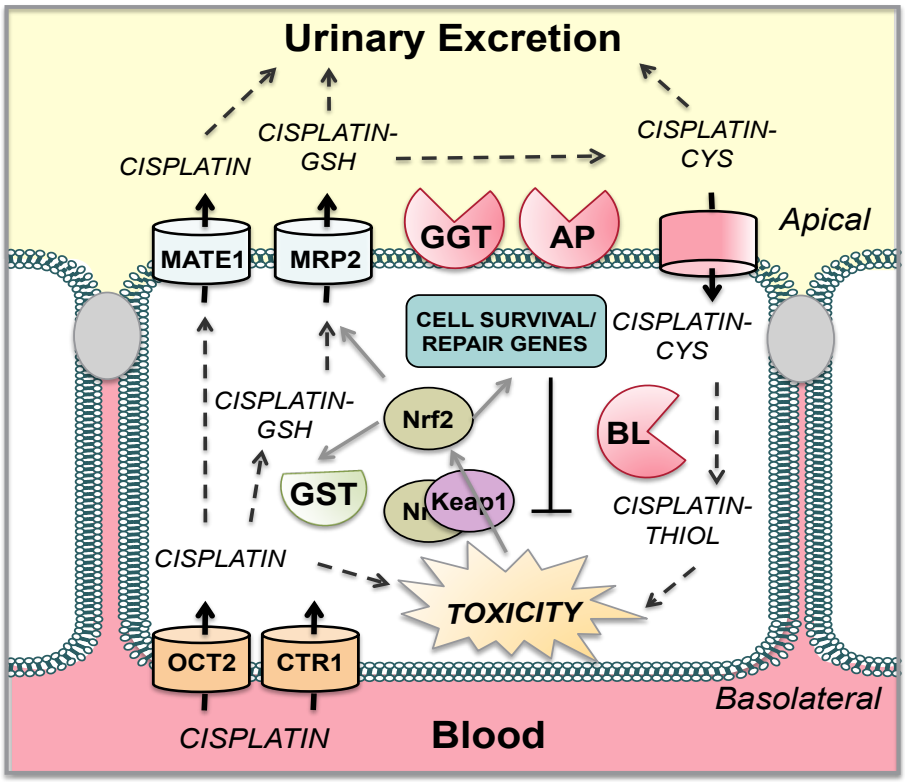

Figure 1.11. Molecular Mechanisms of Cisplatin Nephrotoxicity

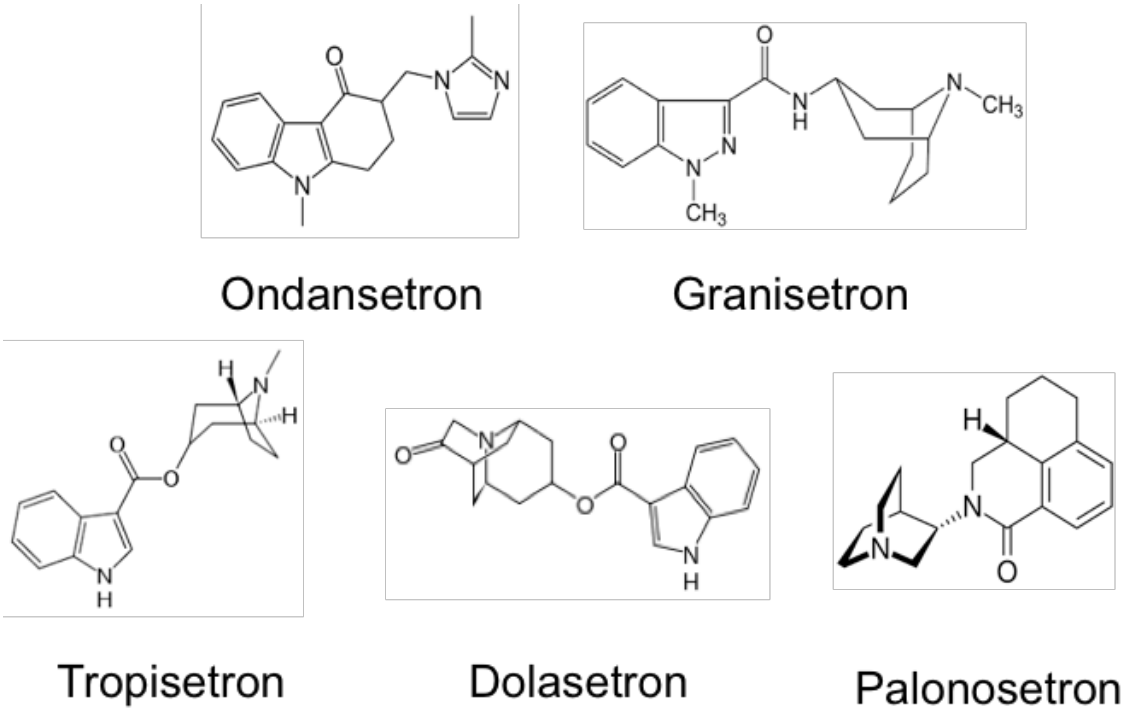

Figure 1.12. Chemical Structures of 5-HT_3 Antagonists

Table 1.1. Changes in Urinary Albumin Concentrations in Patients Prescribed Cisplatin-Containing Regimens

# Patients Analyzed	Mean Cisplatin Dose	Cisplatin Naïve (Yes/No)	# Patients with Clinical AKI	Highest Mean/Median Fold Change from Baseline During the Observation Period	Timing of Peak Change	References
3	38 mg/m^2	Unknown	N=3 23% ↓ eGFR by Day 13	U: ↑ 17-fold	Day 5-8	(Fuke et al. 2009)
8	50-75 mg/m^2 (6 cycles)	Yes	NE	U: ↑ 10-fold	Day 5	(Verplanke et al. 1994)
57	63.4 mg/m^2	Mixed	N=1	U: ↑ 2-fold	Day 10	(George et al. 2017)
33	75 mg/m^2	Yes	10/33 by Day 4 25% ↓ eGFR	U: ↑ 5.6-fold in AKI group U: ↑ 3.4-fold in no-AKI group	Day 4 AKI Day 3 no-AKI	(Lin et al. 2013)
10	100 mg/m^2 (3 cycles)	Yes	N=2	U: ↑ 2-fold	Day 1 (20 h)	(Johnsson et al. 1996)
14	100 mg/m^2 (over 5 days)	No	None	U: ↑ 2.9-fold	Day 5	(Fleming et al. 1979)
16	100 mg/m^2 (over 5 days) (2 cycles)	Unknown	None	U: Cycle 1: Up to ↑ 10-fold U: Cycle 2: Up to ↑ 5.3-fold	Cycle 1: Day 5-6 Cycle 2: Day 4-7	(Pfaller et al. 1994)
41	100 mg/ m^2 (over 5 days) (3 cycles)	Unknown	NE	U: Cycle 1: ↑ 290-fold U: Cycle 2: ↑ 28-fold U: Cycle 3: ↑ 5-fold (p>0.05)	Cycle 1: Day 9 Cycle 2: Day 9	(Daugaard et al. 1988)
23	200 mg/m^2 (over 5 days) (3 cycles)	Unknown	Mean 25% ↓ eGFR Day 9	U: Cycle 1: at least ↑ 30-fold	Cycle 1: Day 9	(Daugaard et al. 1988)
5	375 mg/m^2 (over 5 days)	Yes	None	U: ↑ 17.4-fold during U: ↑ 33-fold 1-week after	Day <5	(Takeda et al. 1994)

AKI: acute kidney injury; eGFR: estimated glomerular filtration rate; NE: Not evaluated; S: serum; U: urine. Each publication provides different definitions for the diagnosis of clinical acute kidney injury.

Table 1.2. Changes in Urinary Beta-2 Microglobulin Concentrations in Patients Prescribed Cisplatin-Containing Regimens

# Patients Analyzed	Mean Cisplatin Dose	Cisplatin Naïve (Yes/No)	# Patients with Clinical AKI	Highest Mean/Median Fold Change from Baseline During the Observation Period	Timing of Peak Change	References
35	27-145 mg	No	None	U: ↑ 8.8-fold	Day 0.5	(Cohen et al. 1981)
8	30-90 mg/m^2	Yes	N=1	U: ↑ up to 10-fold	Day 3	(Ikeda et al. 1988)
20	50 mg/m^2 (6 cycles)	Yes	Unknown	U: ↑ 2-fold	Day 0.5	(Tirelli et al. 1985)
57	63.4 mg/m^2	Mixed	N=1	U: ↑ 3.3-fold	Day 3	(George et al. 2017)
14	70 mg	Unknown	None	U: ↑ 10-fold	Day 4	(Takashi et al. 1996)
11	<80 mg/m^2	Unknown	Unknown	U: No difference between AKI and non-AKI		(Shinke et al. 2015)
19	80 mg/m^2	Yes	None	U: ↑ 6-fold	Day 1 (1-3 hr)	(de Gislain et al. 1986)
14	100 mg/m^2 (over 5 days)	No	None	U: ↑ 4.9-fold	Day <5	(Fleming et al. 1979)
41	100 mg/m^2 (over 5 days) (3 cycles)	Unknown	NE	U: Cycle 1: ↑ 474-fold U: Cycle 2: ↑ 551-fold U: Cycle 3: ↑ 63-fold	Cycle 1: Day 6 Cycle 2: Day 6 Cycle 3: Day 6	(Daugaard et al. 1988)
11	100 mg/m^2 (over 5 days) (6 cycles)	Yes	None	U: ↑ 2- to 5-fold	Day 3	(Sorensen et al. 1985)
22	100 mg/m^2 (5 cycles)	Yes	None	U: ↓ 1.7-fold	Days 21+	(Buamah et al. 1982)
12	120 mg/m^2	No	NE	U: ↑ 5.7-fold	Day <6	(Jones et al. 1980)
30	200 mg/m^2 (over 5 days) (3 cycles)	Unknown	Mean 25% ↓ eGFR Day 9	U: Cycle 1: up to 80-fold	Cycle 1: Day 9	(Daugaard et al. 1988)
5	375 mg/m^2 (over 5 days)	Yes	None	U: ↑ 13-fold	Day <5	(Takeda et al. 1994)

AKI: acute kidney injury; eGFR: estimated glomerular filtration rate; NE: Not evaluated; S: serum; UK: Unknown; U: urine. Each publication provides different definitions for the diagnosis of clinical acute kidney injury.

Table 1.3. Changes in Urinary N-acetyl-D-Glucosaminidase Concentrations in Patients Prescribed Cisplatin-Containing Regimens

# Patients Analyzed	Mean Cisplatin Dose	Cisplatin Naïve (Yes/No)	# Patients with Clinical AKI	Highest Mean/Median Fold Change from Baseline During the Observation Period	Timing of Peak Change	References
8	30-90 mg/m^2	Unknown	NE	U: Up to ↑ 2.4-fold	Day 3	(Ikeda et al. 1988)
3	38 mg/m^2	Unknown	N=3 1.3-fold ↓eGFR by Day 13	U: Up to ↑ 4.4-fold	Day 3-10	(Fuke et al. 2009)
20	50 mg/m^2 (6 cycles)	Yes	NE	U: Up to ↑ 2.6-fold	Day <4	(Tirelli et al. 1985)
8	50-75 mg/m^2 (6 cycles)	Yes	NE	U: ↑ 3-fold	Day 4	(Verplanke et al. 1994)
24	70 mg/m^2	Unknown	N=9 20% ↓ eGFR ↑ 2.1-fold by Day 6 AUC-ROC: 0.66	U: ↑ 3-fold in 20% ↓ eGFR subjects; U: ↑ 2.1-fold in others	Day 3	(Hosohata et al. 2016)
14	70 mg	Unknown	None	U: ↑ 1.9-fold	Day 4	(Takashi et al. 1996)
11	<80 mg/m^2	Unknown	Unknown	U: No difference between AKI and non-AKI		(Shinke et al. 2015)
22	100 mg/m^2 (5 cycles)	Yes	None	U: ↑ 2.2-fold	Day 1.5	(Buamah et al. 1982)
16	100 mg/m^2 (over 5 days) (2 cycles)	Unknown	None	U: Cycle 1: ↑ 3-fold U: Cycle 2: ↑ 2-fold	Day 5-6	(Pfaller et al. 1994)
12	120 mg/m^2	No	NE	U: ↑ 11.8-fold	Day 1.5-2	(Jones et al. 1980)
5	176 mg	Unknown	N=4 ↑ 1.5-fold SCr	U: ↑ 2- to 6-fold	Day 5-10	(Diener et al. 1981)
5	200 mg/m^2 (over 4 days)	Yes	None	U: Up to ↑ 5.7-fold	Day 4	(Goren et al. 1987)
30	200 mg/m^2 (over 5 days) (3 cycles)	Unknown	Mean 25% ↓ eGFR Day 9	U: Cycle 1: Up to ↑ 7-fold	Cycle 1: Day 6	(Daugaard et al. 1988)
5	375 mg/m^2 (over 5 days)	Yes	None	U: ↑ 13.6-fold	Day <5	(Takeda et al. 1994)

AKI: acute kidney injury; AUC-ROC: area under the curve receiver operating characteristic; eGFR: estimated glomerular filtration rate; NE: Not evaluated; S: serum; U: urine. Each publication provides different definitions for the diagnosis of clinical acute kidney injury.

Table 1.4. Changes in Urinary Kidney Injury Molecule-1 Concentrations in Patients Prescribed Cisplatin-Containing Regimens

# Patients Analyzed	Mean Cisplatin Dose	Cisplatin Naïve (Yes/No)	# Patients with Clinical AKI	Highest Mean/Median Fold Change from Baseline During the Observation Period	Timing of Peak Change	References
57	63.4 mg/m^2	Mixed	N=1	U: ↑ 2.8-fold	Day 10	(George et al. 2017)
24	70 mg/m^2	Unknown	N=9 20% ↓ eGFR by Day 6 AUC-ROC: 0.55	U: ↑ 2-fold	Day 3	(Hosohata et al. 2016)
22	75 mg/m^2	Yes	N=8 AUC-ROC: 0.94	U: ↑ 1.6-fold AKI U: ↑ 1.1-fold no-AKI	Day 3	(Tekce et al. 2015)
11	<80 mg/m^2	Unknown	N=10 Day 3 AUC-ROC: 0.858	U: ↑ 6-fold	Day 7	(Shinke et al. 2015)
27	80 mg/m^2	Yes	N=2	U: ↑ 4.6-fold (N=27)	Day 7	(Pianta et al. 2017)
106	Intraoperative	Unknown	N=42	U: ↑ <2-fold (N=106)	Day 2	(Pavkovic et al. 2016)

AKI: acute kidney injury; AUC-ROC: area under the curve receiver operating characteristic; eGFR: estimated glomerular filtration rate; NE: Not evaluated; S: serum; U: urine. Each publication provides different definitions for the diagnosis of clinical acute kidney injury.

Table 1.5. Changes in Urinary Neutrophil Gelatinase-Associated Lipocalin Concentrations in Patients Prescribed Cisplatin-Containing Regimens

# Patients Analyzed	Mean Cisplatin Dose	Cisplatin Naïve (Yes/No)	# Patients with Clinical AKI	Highest Mean/Median Fold Change from Baseline During the Observation Period	Timing of Peak Change	References
60	50 mg/m^2	Yes	13% ↓ eGFR Day 5	U: ↑ 1.5-fold	Day 0.08	(Karademir et al. 2016)
24	50 – 80 mg/m^2	Unknown	N=2 AUC-ROC: 0.80	U: ↑ 2.5-fold in AKI subjects U: ↑ 10-fold in no-AKI subjects	Day 2	(Shahbazi et al. 2015)
21	56 mg/m^2 Total dose=129 mg/m^2	No	N=10 >50% ↑ SCr AUC-ROC: 0.64 (Day 1) Age-adjusted	U: No Change up to Day 4		(Sterling et al. 2017)
57	63.4 mg/m^2	Mixed	N=1	U: No Change on Day 3 or 10		(George et al. 2017)
24	70 mg/m^2	Unknown	N=9 20% ↓ eGFR by Day 6 AUC-ROC: 0.64	U: ↑ 1.3-fold	Day 3	(Hosohata et al. 2016)
33	75 mg/m^2	Yes	10/33 by day 4 25% ↓ eGFR	U: ↑ 4-fold in AKI No increase in no-AKI	Day 2 AKI	(Lin et al. 2013)
11	<80 mg/m^2	Unknown	N=10 Day 3 AUC-ROC: 0.608	U: ↓ 6-fold	Day 7	(Shinke et al. 2015)
46	140 mg	Yes	N=12 25% ↑ SCr	U: ↑ >60-fold in AKI	Day 7	(Gaspari et al. 2010)
14	Unknown	Unknown	None	U: ↑ 2.3-fold cisplatin	Unknown	(Seker et al. 2015)

AKI: acute kidney injury; AUC-ROC: area-under-the curve receiver operating characteristic; eGFR: estimated glomerular filtration rate; NE: Not evaluated; S: serum; U: urine. Each publication provides different definitions for the diagnosis of clinical acute kidney injury.

Table 1.6. Changes in Serum and Urinary Cystatin C Concentrations in Patients Prescribed Cisplatin-Containing Regimens

# Patients Analyzed	Mean Cisplatin Dose	Cisplatin Naïve (Yes/No)	# Patients with Clinical AKI	Highest Mean/Median Fold Change from Baseline During the Observation Period	Timing of Peak Change	References
60	50 mg/m^2	Yes	13% ↓ eGFR Day 5	S: ↑ 1.1-fold	Day 5	(Karademir et al. 2016)
35	50 mg/m^2	Unknown	No SCr change	S: ↑ 1.1-fold	Day 5	(Benohr et al. 2006)
34	60 mg/m^2 (3 cycles)	Yes	NE	S: ↑ 1.1-fold After first cycle	Days 14-28	(Kos et al. 2013)
57	63.4 mg/m^2	Mixed	N=1	U: ↑ 1.9-fold	Day 3	(George et al. 2017)
33	75 mg/m^2	Yes	10/33 by Day 4 25% ↓ eGFR	U: No change at 8h No difference in AKI and no-AKI groups		(Lin et al. 2013)
27	80 mg/m^2	Yes	N=2	S: ↑ 41% N=9 ↑ 50%	Day 3	(Pianta et al. 2017)
123	120 mg/day	Yes	NE	S: Up to ↑ 1.1-fold	Day 21	(Zhang and Zhou 2012)

AKI: acute kidney injury; eGFR: estimated glomerular filtration rate; NE: Not evaluated; S: serum; U: urine. Each publication provides different definitions for the diagnosis of clinical acute kidney injury.

Table 1.7. Changes in Urinary Concentrations of Additional Proteins in Patients Prescribed Cisplatin-Containing Regimens

	# Patients Analyzed	Mean Cisplatin Dose	Cisplatin Naïve (Yes/No)	# Patients with Clinical AKI	Highest Mean/Median Fold Change from Baseline During the Observation Period	Timing of Peak Change	References
Calbindin	14	70 mg	Unknown	None	U: ↑ 23-fold	Day 10	(Takashi, Zhu et al. 1996)
	57	63.4 mg/m^2	Mixed	N=1	U: ↑ 8.3-fold	Day 10	(George, Wen et al. 2017)
MCP-1	11	<80 mg/m^2	Unknown	N=10 Day 3 AUC-ROC: 0.85	U: ↑ 3.8-fold	Day 7	(Shinke, Masuda et al. 2015)
	57	63.4 mg/m^2	Mixed	N=1	U: ↑ 1.7-fold	Day 10	(George, Wen et al. 2017)
Clusterin	27	80 mg/m^2	Yes	N=2	U: ↑ 2.5-fold	Day 7	(Pianta, Succar et al. 2017)
	57	63.4 mg/m^2	Mixed	N=1	U: ↑ 1.9-fold	Day 10	(George, Wen et al. 2017)
IGFBP7	45	59.9 mg/m^2	No	N=13 No difference between AKI and no AKI	U: No change Day 1		(Toprak, Cebeci et al. 2017)
IGFBP7	46	64.2 mg/m^2	No	N=1	U: No change Day 3 or 10		(Chang, Hu et al. 2017)
TIMP2 *IGFBP7	45	59.9 mg/m^2	No	N=13 AUC-ROC: 0.46	U: No change Day 1		(Toprak, Cebeci et al. 2017)
TIMP2 *IGFBP7	32	78 mg/m^2	No	N=4 <Day 3 AUC-ROC: 0.92	NE		(Schanz, Hoferer et al. 2017)
TIMP2 *IGFBP7	46	64.2 mg/m^2	No	N=1	U: No change Day 3 or 10		(Chang, Hu et al. 2017)
TIMP2	45	59.9 mg/m^2	No	N=13 No difference between AKI and no AKI	U: ↑ 1.1-fold	Day 1	(Toprak, Cebeci et al. 2017)
TIMP2	46	64.2 mg/m^2	No	N=1	U: No change Day 3 or 10		(Chang, Hu et al. 2017)
GST-pi	57	63.4 mg/m^2	Mixed	N=1	U: ↑ 1.6-fold	Day 10	(George, Wen et al. 2017)
IL-18	57	63.4 mg/m^2	Mixed	N=1	U: No Change Day 3 or 10		(George, Wen et al. 2017)
Osteopontin	57	63.4 mg/m^2	Mixed	N=1	U: No Change Day 3 or 10		(George, Wen et al. 2017)
TFF3	57	63.4 mg/m^2	Mixed	N=1	U: ↑ 2-fold	Day 10	(George, Wen et al. 2017)

AKI: acute kidney injury; AUC-ROC: area under the curve receiver operating characteristic; eGFR: estimated glomerular filtration rate; GST-pi: Glutathione-*S*-transferase-pi; IGFBP7: Insulin like growth factor binding protein-7; IL-18: Interleukin-18; MCP-1: monocyte chemotactic peptide-1; NE: Not evaluated; S: serum; TIMP2: Tissue inhibitor of metalloproteinases-2; TFF3: Trefoil factor 3; U: urine. Each publication provides different definitions for the diagnosis of clinical acute kidney injury.

Table 1.8. Properties of Antiemetic Drugs

Antiemetic Drugs	Dosing[a]	Half-Life[a]	Generic
Ondansetron	IV: 8 mg or 0.15 mg/kg Oral: 16 mg	3.9 hours	Yes
Granisetron	IV: 1 mg or 0.01 mg/kg Oral: 1-2 mg	9-11 hours	Yes
Tropisetron	IV: 5 mg Oral: 5 mg	7.3 hours	Not available in US
Dolasetron	Oral: 100 mg	7-9 hours	No
Palonosetron	IV: 0.25 mg Oral: 0.5 mg	40 hours	No
Aprepitant	**Acute Emesis**: Oral: 125 mg on day of chemotherapy IV (Fosaprepitant): 150 mg IV on day of chemotherapy **Delayed Emesis**: Oral: 125 mg on 1st day, 80 mg once daily for 2 days after chemotherapy	9-13 hours	Oral only
Netupitant	300 mg netupitant/0.5 mg palonosetron once on day of chemotherapy	80 hours	No
Rolapitant	180 mg (1-2 hours prior to chemotherapy)	7 days	No
Dexamethasone	**Acute Emesis**: 20 mg once (12 mg with NK_1 antagonist) **Delayed Emesis**: 8 mg twice daily for 3 to 4 days (8 mg once daily with NK_1 antagonist)	4 hours	Yes
Olanzapine	10 mg for 3 days	30 hours	Yes
Metoclopramide	2 mg/kg over 15 minutes, 30 minutes before chemotherapy	5-6 hours	Yes
Prochlorperazine	5 to 10 mg IM, IV, oral every 4-6 hours as needed	6-10 hours	Yes
IV: intravenous, IM: intramuscular [a]Package Insert			

CHAPTER 2: PROFILING OF KIDNEY INJURY BIOMARKERS IN PATIENTS RECEIVING CISPLATIN: TIME-DEPENDENT CHANGES IN THE ABSENCE OF CLINICAL NEPHROTOXICITY

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2.1. Abstract

The success of cisplatin-containing regimens to treat solid tumors is limited, in part, by nephrotoxicity. In rodents, several urinary proteins have emerged as more sensitive indicators of cisplatin-induced kidney injury. We sought to characterize time-dependent changes in the urinary concentrations of 12 proteins including KIM-1, calbindin, β2M, and TFF3 after cisplatin therapy. Urine was collected at baseline, 3 (range: 2-5), and 10 (range: 9-11) days from 57 patients with solid tumors receiving outpatient cisplatin therapy (≥ 25 mg/m2). Serum creatinine was largely unchanged after cisplatin infusion. However, compared to baseline values, several novel biomarkers were significantly increased in the urine including β2M, which was 3-fold higher by day 3 ($p<0.0001$). Urinary KIM-1 and TFF3 were elevated 2-fold by day 10 ($p=0.002$ and $p=0.002$, respectively) whereas calbindin levels were increased 8-fold ($p<0.0001$). We report novel time-dependent changes in the urinary excretion of noninvasive markers of subclinical kidney injury after cisplatin treatment.

2.2. Introduction

Cisplatin is a chemotherapeutic agent widely used for the treatment of solid tumors. Despite the ability of hydration and electrolyte management to reduce the incidence of acute kidney injury (AKI), up to one- third of patients receiving cisplatin therapy at doses greater than 50 mg/m^2 still experience nephrotoxicity (Shord et al. 2006). The current diagnostic criteria for AKI, as established by the Kidney Disease Improving Global Outcomes (KDIGO) and the Acute Kidney Injury Network (AKIN), are largely based on serum creatinine (SCr), which is a surrogate marker of glomerular filtration (Khwaja 2012). The estimated glomerular filtration rate GFR (eGFR), which approximates a reduction in the functional capacity of the kidney, is highly insensitive. Even with a 50% loss of functioning renal mass, eGFR values remain within the normal range. Furthermore, mounting research has revealed multiple limitations of SCr-based diagnostics including competition for tubular secretion, fluctuations during acute injury, and variation due to muscle mass and meat intake (Ciarimboli et al. 2012).

Timely detection of kidney injury may allow for earlier interventions to reduce morbidity and mortality. Several novel urinary proteins have been investigated for their ability to detect AKI in a more sensitive and specific manner. In rats, kidney injury molecule-1 (KIM-1) was elevated in the urine 24 h after a single dose of cisplatin and outperformed traditional markers of toxicity including SCr and blood urea nitrogen (BUN) (Vaidya et al. 2010, Sinha et al. 2013). Biomarkers such as urinary albumin, lipocalin 2 (also known as neutrophil- associated lipocalin (NGAL) in humans), KIM-1, and osteopontin exhibited time-dependent elevations in cisplatin-treated rats that mirrored findings from histopathological analysis (Pinches et al. 2012). In 2008, the Food and Drug Administration (FDA) approved the preclinical use of seven novel urinary proteins (KIM-1, clusterin, albumin, total protein, beta 2-microglobulin (β2M), cystatin C and trefoil

factor 3 (TFF3)) for regulatory decision-making alongside traditional markers (Dieterle et al. 2010). Other novel urinary biomarkers (tissue inhibitor of metalloproteinase 2, insulin-like growth factor binding protein 7) have been approved by the FDA for clinical detection of AKI through a point-of-care device, Nephrocheck (Vijayan et al. 2016); however, its utility, specifically for the detection of cisplatin-induced AKI, has yet to be determined. Previous studies have largely compared biomarker performance based on the clinical diagnosis of AKI or resulting from a combination of etiologies including cardiac surgery, sepsis, transplant, chronic kidney disease, renal cancer and nephrotoxic medications (Han et al. 2008, Koyner et al. 2010, Hall et al. 2011, Lin et al. 2013, Arthur et al. 2014). Few studies have prospectively examined concentrations of novel biomarkers following administration of a nephrotoxic agent in the setting of cancer or in patients that do not exhibit AKI as defined by KDIGO or AKIN (often termed subclinical nephrotoxicity). Studies in patients treated with cisplatin suggest that KIM-1 and monocyte chemoattractant protein-1 (MCP-1) can detect AKI with high sensitivity (AUROC values: 0.858 and 0.850, respectively) (Shinke et al. 2015, Pavkovic et al. 2016). Furthermore, urinary calbindin and β2M have been shown to be elevated 1468% and 903%, respectively in patients with subclinical cisplatin nephrotoxicity (n=14) (Takashi et al. 1996).

In this study, we sought to prospectively characterize time-dependent changes in twelve urinary proteins (KIM-1, calbindin, clusterin, glutathione *S*-transferase-pi (GST-pi), interleukin-18 (IL-18), MCP-1, albumin, β2M, cystatin C, NGAL, osteopontin, and TFF3) in patients receiving outpatient cisplatin therapy for various solid tumors. For this effort, two commercial ELISA-based multiplex panels were used to simultaneously quantify urinary protein concentrations prior to and at two time points after cisplatin administration. Early detection of clinical and subclinical changes in renal function would

strengthen a clinician's ability to prevent further damage and identify patients at-risk for cisplatin nephrotoxicity.

2.3. Results

Patient Characteristics.

A total of sixty patients were enrolled in the study. Three patients were excluded from analysis because they were either lacking a baseline urine collection (0 h or 0-2 h) or only had one time-point collected. Characteristics for the remaining subjects (n=57) are shown in Table 2.1. The majority of patients were Caucasian (89%) with a similar number of male and females. The mean and range of patient ages were 56.6 (26-80) years and body mass index (BMI) values were 27 (18-46) kg/m^2. The average prescribed dose and range were 63.4 (25-120) mg/m^2 and varied based on tumor type (Table 2.2).

Clinical Laboratory Parameters in Patients Receiving Cisplatin.

Using the KDIGO criteria, AKI was defined by increases in SCr more than 0.3 mg/dL over 48 h or 1.5 times baseline after one dose of cisplatin (2012). Only one patient developed AKI based on the criteria with a 2.3-fold increase in SCr from baseline. Mean SCr, BUN, and eGFR were not changed significantly 13 ± 7 days after cisplatin infusion (Table 2.3). Similarly, the ratio of urinary albumin-to-creatinine was also not changed after cisplatin. Several electrolytes, including Na^+, Ca^{2+}, and Cl^-, were significantly decreased after cisplatin therapy although they remained within normal concentration ranges (Table 2.3). The concentrations of other electrolytes, Mg^{2+}, K^+, and HCO_{3-}, did not differ in patients before and after cisplatin treatment.

Time-Dependent Changes in Urinary Protein Biomarker Concentrations following Cisplatin Infusion.

Urinary concentrations of proteins, including β2M (3.3-fold), cystatin C (1.9-fold), TFF3 (1.7-fold), KIM- 1 (1.6-fold), and albumin (1.5-fold) were significantly elevated by day 3 (range 2-5 days) compared to baseline (Figure 2.1, Table 2.4). A slight reduction in clusterin levels was also observed on day 3 ($p=0.043$). By 10 days (range 9-11 days), calbindin (8.3-fold), KIM-1 (2.8-fold), albumin (2-fold), TFF3 (2-fold), clusterin (1.9-fold), cystatin C (1.8-fold), MCP-1 (1.7-fold), and GST-pi (1.6-fold) were significantly increased (Figure 2.1, Table 2.4). β2M (1.4-fold), although still significantly increased from baseline, trended downward on day 10 compared to day 3. No change in the mean concentrations of IL-18, NGAL or osteopontin concentrations were observed at either time point. While the changes in mean concentrations of some biomarkers may not have changed, individual patients exhibited increases at Day 3 or 10. The normalization of biomarker concentrations to urinary creatinine also reflected similar time-dependent changes (Supplemental Table 2.1). β2M (5-fold), albumin (2.1-fold), KIM-1 (1.9-fold), and TFF3 (1.8-fold) were significantly increased at day 3 after normalizing to urinary creatinine. Likewise, biomarker concentrations for calbindin (8.7-fold) and KIM-1 (2.1-fold) following normalization were also significantly higher around day 10 after cisplatin.

Correlation of Clinical Laboratory Parameters to Urinary Protein Biomarker Concentrations.

The concentrations of urinary proteins at day 10 showed little correlation with changes in traditional measures of AKI, including SCr, BUN and eGFR, that were measured 13 ± 7 days after cisplatin infusion (Supplemental Table 2.2). The only relationship that was revealed was a positive correlation between urinary calbindin levels and changes in BUN concentrations ($r=0.312$, $p=0.032$) at day 10. The results were similar when the clinical

laboratory parameters were expressed as percent changes (data not shown). Strong relationships between the various protein biomarkers and urinary albumin concentrations at days 3 and 10 were observed (Table 2.5). At day 3, the strongest correlations with urinary albumin were for clusterin (r=0.671) and TFF3 (r=0.646). At day 10, the strongest correlations with urinary albumin were for KIM-1 (r=0.765) and MCP-1 (r=0.761).

Association of Patient-Specific Factors with Urinary Concentrations of Protein Biomarkers.

Several patient-specific factors were assessed for relationships with concentrations of urinary protein biomarkers including: age, sex, BMI, presence of concomitant disease, use of intravenous (IV) contrast dyes, number of cisplatin cycles, prescribed dose, and body surface area (BSA)-based dosing. There were no correlations between any of the 12 biomarkers and age or BMI (Supplemental Table 2.6 and 2.7). Urinary clusterin levels at day 3 were 5-fold lower in females than males. There were no other significant sex differences at other time-points (baseline, 3 or 10 day) or for other biomarkers (Supplemental Table 2.3). There were also no consistent correlative relationships between biomarkers and prescribed dose, BSA-based dose, or total cumulative dose received (Supplemental Table 2.8, 2.9 and 2.10).

We also assessed relationships between protein biomarker concentrations and medical record documented concomitant diseases (type 2 diabetes mellitus, hyperlipidemia, Hashimoto's thyroiditis, Human Immunodeficiency Virus, hypertension) at each time-point (Supplemental Table 2.4). Urinary albumin concentrations at baseline and at day 3 were significantly elevated in patients noted to have any of these concomitant diseases. Patients who had received IV contrast dye at least 30 days prior to cisplatin infusion had

higher urinary IL-18 protein levels at baseline as well as elevated urinary GST-pi protein levels at day 3 (Supplemental Table 2.5).

2.4. Discussion

We report novel time-dependent changes in the urinary excretion of protein biomarkers in the absence of clinical AKI in patients receiving cisplatin. Compared to baseline values, several proteins were significantly increased in urine including KIM-1, clusterin, albumin, cystatin C and TFF3, which were elevated 2-fold by day 10. Urinary β2M concentrations were elevated 3-fold by day 3 whereas calbindin levels were increased 8-fold on day 10. More moderate increases (less than 2-fold difference in means) were observed for GST-pi and MCP-1 on day 10. Overall, traditional biomarkers, eGFR, BUN, and SCr did not change significantly from before to 13 ± 7 days after administration of cisplatin.

The incidence of AKI varies between 8-40% depending on cisplatin dose, frequency of administration, and peak free platinum concentrations in plasma (Madias and Harrington 1978). Based on these estimates, we expected a higher number of AKI episodes than resulted in this study, where only one patient met the KDIGO criteria for Stage 2 AKI (Khwaja 2012). Other studies have used different AKI criteria based on arbitrary cut-offs in SCr or eGFR increase from baseline (Hosohata et al. 2016, Latcha et al. 2016). The lower incidence of AKI in this study could also be due to several factors including a limited sample size, the timing of blood collection for biomarkers, and the low to moderate cisplatin dose ranges prescribed (as low as 25 mg/mm^2). Patients were largely treatment- naïve and varied in age (26-80 years). Patients over 50 years of age tend to have a higher incidence of cisplatin nephrotoxicity (Wen et al. 2015). Additionally, African American patients are more susceptible than other races to nephrotoxicity and

were not well-represented in the current study, which was composed of largely Caucasian patients (Table 2.1) (Shord et al. 2006). AKI is normally detected within 48 h to 7 days after cisplatin administration. Due to the non-interventional nature of the study, the measurement of traditional serum biomarkers such as SCr and BUN occurred over a range wider than desired (13 ± 7 days), as clinically determined. It is possible that earlier assessment of SCr and BUN may have captured AKI in a greater number of patients.

Normal urinary protein biomarker ranges have been recently defined for healthy volunteers (Table 2.6) (Brott et al. 2014). The baseline protein concentrations for KIM-1, calbindin, clusterin, cystatin C, albumin, NGAL, osteopontin, and TFF3 in our cancer patients fell within or were close to the ranges reported. However, the values at the upper range of baseline in cancer patients tended to be higher than healthy volunteers, in particular for clusterin, β2M, cystatin C, NGAL, osteopontin and TFF3 (Table 2.6). β2M is an exception where the mean baseline concentration was elevated by 1.5-fold from the reported range in healthy volunteers (Brott et al. 2014). The mean concentrations of cystatin C, albumin, NGAL, and TFF3 post-cisplatin infusion were also within the ranges reported for healthy volunteers. Several of the top performing biomarkers had elevated levels above healthy volunteer ranges on day 3 (for β2M) and day 10 (for KIM-1 and calbindin). These findings support previous studies showing elevations in urinary β2M and calbindin in subclinical AKI and urinary KIM-1 increases in clinical AKI in patients treated with cisplatin (Takashi et al. 1996, Shinke et al. 2015).

We have reported both absolute and normalized biomarker values in this study to determine whether normalization altered the ability to detect changes in concentrations after cisplatin infusion. Overall, the top performing biomarkers were consistent whether expressed as normalized to creatinine or as absolute concentrations. Biomarker values

normalized to creatinine are often reported, due to variability in urine output between patients. The underlying assumption is that urinary creatinine excretion is constant within an individual and that biomarker excretion is linear with urinary creatinine excretion, neither of which may be the case (Mattix et al. 2002, Ciarimboli et al. 2012, Tang et al. 2015). Studies have also reported that absolute concentrations were more informative in distinguishing AKI on hospital admission; however, normalized concentrations better reflected long-term outcomes including death and dialysis (Ralib et al. 2012, Shinke et al. 2015).

Investigating the biological roles of top biomarkers could inform our understanding of the sequence of injury, functional loss, and repair processes that occur after cisplatin exposure. Biomarkers reflective of functional proximal tubule (PT) injury (β2M and cystatin C) peaked at day 3. β2M is a low-molecular weight protein filtered through glomeruli and extensively reabsorbed by PTs (Kabanda et al. 1996). β2M appears in the urine when plasma concentrations exceed the renal reabsorptive threshold (5 mg/L) or with PT damage. In this study, we observed an increase in β2M levels at day 3 that was diminished by day 10. This pattern of urinary β2M was similar in another study of cisplatin-treated patients at days 4 and 8 without overt AKI post treatment (Takashi et al. 1996). Albumin is also normally reabsorbed by the PTs. Therefore, albuminuria is often observed during direct tubular toxicity (Baines and Brunskill 2011). In this study, urinary albumin exhibited a time-dependent increase at both days 3 and 10. Furthermore, many of the biomarkers, both the top performers as well as those that did not reflect cisplatin exposure, exhibited high correlations with urinary albumin. However, as shown in this study and by others, albuminuria also occurs in the presence of concomitant diseases such as hypertension and diabetes mellitus. Thus, finding an accurate cut-off value for

predicting tubular toxicity using urinary albumin may be complicated (reviewed in (Singh 2011)).

Biomarkers with high fold changes from baseline on day 10 (KIM-1 and TFF3) have been associated with tubule repair. KIM-1 is a transmembrane protein found on the apical membrane of PTs following injury [Ichimura 1998;Vaidya 2006]. The ectodomain of KIM-1 is cleaved and shed into the tubular lumen. It can be detected in the urine of multiple species including humans. Immunohistochemistry has shown that KIM-1 is expressed in de-differentiated and regenerating PT cells (Bailly et al. 2002) and is difficult to detect in completely atrophic cells. Functionally, KIM-1 can confer phagocytic capabilities to PT cells, allowing enhanced clearance of apoptotic cell debris. Similar protective functions have been described for TFF3. TFF3 is a small peptide hormone that is secreted by mucus-producing and epithelial cells. Although its role in the kidney is largely unknown, TFF3 has been shown to play a role in mucosal and surface maintenance, inhibition of apoptosis, promotion of cell survival and migration in the lung and intestine (Taupin and Podolsky 2003). Cisplatin-treated rats showed markedly decreased levels of TFF3 in the kidneys and urine compared to control rats across two dose groups (3.5 and 7 mg/kg) at 3 and 8 days (reviewed in (Yu et al. 2010)). In patients, higher circulating and urinary TFF3 has been associated with the severity of chronic kidney disease and certain cancers (Xiao et al. 2014, Lebherz-Eichinger et al. 2015). In our study, urinary TFF3 exhibited a time-dependent increase post cisplatin, which may reflect species differences in toxicity responses.

Calbindin is a calcium-binding protein found primarily on distal tubules (DT) and collecting ducts of the kidney (Bredderman and Wasserman 1974). Although calbindin is associated with DT damage, it has also been shown to be up-regulated in vitro in an

immortalized human proximal tubule cell line, HK-2, after exposure to cisplatin (Takashi et al. 1996, Sohn et al. 2013). In a clinical study, urinary calbindin peaked in response to cisplatin (n=14, 70 mg/m^2) after 8 days but in the absence of changes in SCr, BUN, and creatinine clearance (Takashi et al. 1996). Additionally, calbindin concentrations did not change in response to administration of other chemotherapy drugs in the same patients. These findings support our data and holds promise for calbindin as a specific indicator of cisplatin induced injury even in the absence of overt functional damage.

NGAL, IL-18 and osteopontin did not show significant time-dependent changes in our study. There have been several reports suggesting these proteins are early biomarkers of AKI in cancer patients and rodents treated with cisplatin (Peres et al. 2014, Won et al. 2016). Although identified roles for these proteins in AKI are limited, they are most often associated with inflammation. Both NGAL and osteopontin are associated with the thick ascending Loop of Henle, DT and/or collecting ducts (Xie et al. 2001, Schmidt-Ott et al. 2007). Therefore, NGAL and osteopontin may not be sensitive for PT injury alone and may require a threshold of necrosis and inflammation that involves multiple nephron regions for them to be up-regulated and/or secreted. Additionally, as mentioned by Shinke et. al. (2015), it is possible that the selection of time points account for differences in the ability of these three biomarkers to detect cisplatin nephrotoxicity (Shinke et al. 2015). Previous studies in cisplatin treated patients have reported that early time points (12 h – 3 days) showed increased NGAL and IL-18 levels compared to baseline (Lin et al. 2013, Shinke et al. 2015).

Given the lack of overt nephrotoxicity, it was surprising to see statistically significant, but clinically insignificant, decreases in the serum electrolyte panel. Depletion of electrolytes including magnesium, calcium, potassium, sodium, and phosphate has been associated

with cisplatin nephrotoxicity (Arunkumar et al. 2012). The reabsorption of many electrolytes including sodium, chloride, and calcium occurs primarily in the PT and cisplatin-induced damage may explain reduced serum levels. However, total serum calcium is also affected by serum protein loss (46% protein bound) (reviewed in (Blaine et al. 2015)). The lack of significant changes in magnesium is not surprising since hypomagnesemia is more commonly associated with DT injury (Glaudemans et al. 2010). Although PT injury may contribute, it is difficult to attribute these minor electrolyte changes to kidney toxicity as they fluctuate with volumes administered and can be affected by many factors including diet, protein binding, hormones, and/or underlying disease processes such as cancer.

Elevations in urinary biomarker concentrations did not correlate with patient-specific factors including age, sex, or BMI. Although age is a factor affecting cisplatin nephrotoxicity, the age range tested in this study was wide. Furthermore, it may be possible that without overt AKI these factors may not impart as large of a role. Predictably, urinary albumin was elevated in patients with concomitant diseases at baseline and day 3. Many chronic conditions, including diabetes mellitus and hypertension, result in glomerular damage that increases urinary albumin excretion (Singh and Satchell 2011). IL-18 was significantly elevated at baseline in patients that had previously received IV contrast dye. Studies have shown that IL-18 was able to predict contrast-induced nephropathy earlier than SCr in patients undergoing percutaneous coronary intervention after 12 h (AUROC: 0.811) (He et al. 2014). The up-regulation of inflammatory cytokines, such as IL-18 and the antioxidant enzyme GST-pi, could be explained by the fact that many contrast agents induce free radicals and oxidative stress (Katholi et al. 1998). Across the different cisplatin dose parameters (prescribed dose, BSA- based dose and cumulative dose) there were no consistent

biomarker changes. Studies have shown that BSA-based dosing does not increase the accuracy of predicting cisplatin exposure (de Jongh et al. 2001). Other factors not assessed in this study, including race, tumor type, and cancer stage, may contribute to cisplatin injury. The sample size of the study limited the number of covariates that could be assessed for their influence on cisplatin injury.

In conclusion, the present study has shown that certain urinary biomarkers might be particularly sensitive for detecting cisplatin-induced subclinical AKI. Multiple urinary biomarkers (KIM-1, calbindin, β2M, clusterin, MCP-1, cystatin C, GST-pi, albumin, and TFF3) showed time-dependent elevations for detecting cisplatin exposure in the absence of clinically detectable AKI. Additionally, commonly studied biomarkers in various settings of AKI (NGAL, IL-18, osteopontin) did not show any significant changes. It should be recognized that these conclusions are based on aggregate data and thus, individual fold changes for each subject have been provided (Supplemental Table 1). Further understanding of the pathophysiological role of these proteins and whether they are involved in tubular injury or repair is necessary to utilize them as specific indicators of cisplatin-induced AKI and for selecting the correct time points for quantification. Likewise, greater investigation is needed to understand the utility of profiling multiple biomarkers, rather than one or two candidates, for detecting clinical or subclinical AKI.

2.5. Methods

Selection of Participants

A prospective study of patients receiving outpatient chemotherapy for various solid tumors at the University of Colorado Cancer Center, Aurora, CO, a National Cancer Institute-Designated Consortium Comprehensive Cancer Center was conducted. Fifty-six patients received cisplatin ≥ 25 mg/m^2 intravenously while one patient was

administered cisplatin intraperitoneally. Patients were hydrated pre- and post-treatment with saline (1-2 L). Study inclusion criteria included: 1) Age ≥ 18 years, 2) hemoglobin ≥ 10 g/dL, 3) no consumption of grapefruit juice or alcohol within 7 days, 4) no history of alcohol consumption of >14 drinks/week, 5) no history of organ transplantation or kidney dialysis, 6) willingness to comply with study, 7) not pregnant or lactating, 8) no changes in medications within previous 4 weeks, 9) normal liver function (alanine aminotransferase and aspartate aminotransferase <2x upper limit of normal) and 10) baseline eGFR > 60 mL/min/m2 (using the 4-variable Modification of Diet in Renal Disease equation) (Levey et al. 2006). Exclusion criteria included: 1) diagnosis of kidney cancer, 2) previous exposure to platinum-based chemotherapy (other than the currently prescribed regimen), 3) herbal supplement use, 4) exposure to other known nephrotoxins (including contrast agents) within the previous 30 days, and 5) concurrent use of inhibitors of transport proteins involved in cisplatin secretion into urine. The Institutional Review Boards at the University of Colorado (Protocol 12-1510) and Rutgers University (Protocol E13-716) approved the protocols for recruitment and sample collection. Twenty-eight patients were recruited before the first cycle of cisplatin therapy and twenty-nine patients were recruited before the second cycle of cisplatin therapy. The average length of time between the first and second cycles of cisplatin was 17 days (range of 6 – 34 days).

Urine Samples

Urine was collected from spontaneous voids at baseline (pre-cisplatin infusion), between 2-5 days (designated as day 3) and 9-11 days (designated as day 10) post-cisplatin infusion. For patients missing baseline urine collection, urine in the 0-2 hour timed collection after cisplatin infusion was used for analysis. Urine was centrifuged at 3000xg and supernatant was aliquoted into 2 mL collection tubes and frozen within 30-60

minutes of collection at -80℃. At the time of analysis, samples were thawed and placed on ice and centrifuged at 1500 rpm for 5 minutes. Ten µl of supernatant were pipetted for biomarker analysis.

Quantification of Urinary Protein Biomarkers

Calbindin, clusterin, KIM-1, GST-pi, IL-18, MCP-1, albumin, β2M, cystatin C, NGAL, osteopontin, and TFF3 were measured using Bio-Plex Pro RBM human kidney toxicity assay panels 1 and 2 (Bio-Rad, Life Science, Hercules, CA). Washing steps were conducted using the Bio-Plex Pro II wash station (Bio- Rad). Samples were analyzed using a Bio-Plex, MagPix Multiplex Reader (Bio-Rad), which reports the mean fluorescence intensity (MFI) proportional to the concentration of analyte bound to each bead. Concentrations were extrapolated from a known standard curve using a five-parameter logistic curve. Recommended dilutions of urine samples in dilution buffer provided in the assay kit were followed (1:10 for panel 1 and 1:50 for panel 2). Values that were above the detection limit for the Bio-Plex assays were diluted and re-analyzed. Concentrations below the limit of detection were substituted with the lower limit of quantification divided by 2 (Supplemental Table 2.12). Data are presented as absolute concentrations and normalized to urinary creatinine concentrations quantified using the DCA Vantage Analyzer (Siemens, Princeton, NJ).

Data and Statistical Analysis

Data are presented for individual patients together with the corresponding group mean ± SD (baseline, days 3 and 10). Data were tested for normality using the D'Agostino-Pearson omnibus test. Time- dependent differences among individual biomarkers were evaluated by Wilcoxon rank-sum tests for absolute concentrations and ANOVA to test normalized biomarker concentrations. Differences in patient- specific factors

(categorical) were assessed by ANOVA. Clinical laboratory values for pre- and post-cisplatin treatment were compared using paired t-tests. Differences were considered statistically significant at $p<0.05$. Pearson or Spearman correlation coefficients were used to measure the strength of association between biomarker and urinary albumin concentrations, kidney function measures or patient- specific factors (continuous). All statistical analyses and plots were done by GraphPad Prism V6 (GraphPad Software, La Jolla, CA), Partek Genomics Suite (Partek GS 6.4, St Louis, CA) or SAS 9.4 (SAS Institute Inc. Cary, North Carolina).

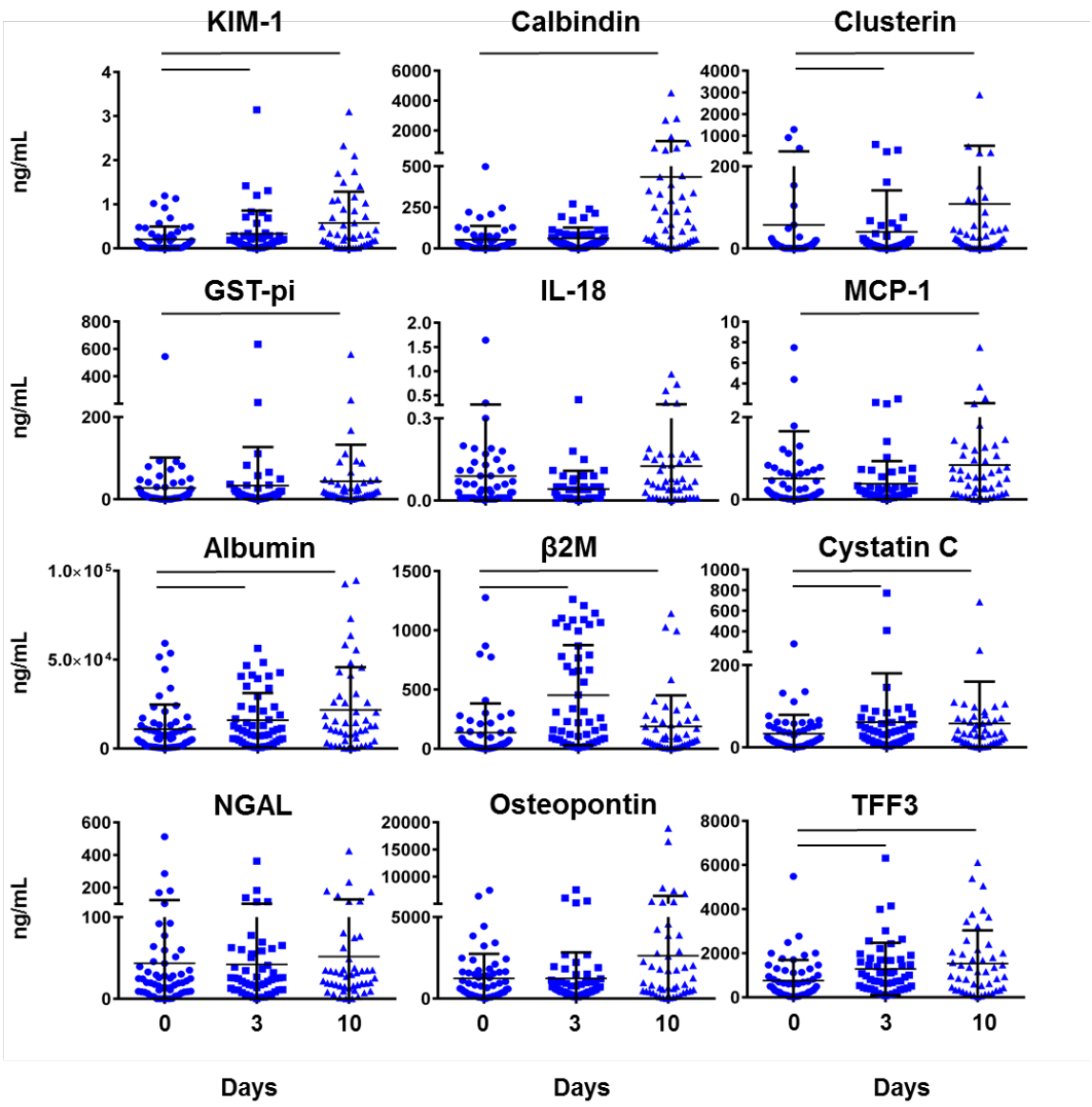

Figure 2.1. Time-Dependent Changes in Absolute Urinary Concentrations of Protein Biomarkers following Cisplatin Infusion. Urinary protein concentrations were measured using a Bio-Plex assay at baseline (n=57), 3 (range of 2-5, n=50) days and 10 (range of 9-11, n=47) days post cisplatin-infusion during the first or second cycle of chemotherapy. Concentrations were measured in urine supernatants and presented as ng/mL. Scatter plots show individual levels (n=57) and mean ± SD concentration at each time point. Solid lines represent statistically significant differences ($p<0.05$) compared to baseline protein levels.

Table 2.1. Demographic Information for Patients (n=57) Receiving Cisplatin	
Age (mean ± SD)	56.6 ± 13.2 years
Gender	Male = 29 Female = 28
Body Mass Index (mean ± SD)	27.0 ± 6.0 kg/m^2
Cisplatin Dose (mean ± SD)	61.0 ± 22.8 mg/m^2
Race	Caucasian = 51 Hispanic = 4 African American = 1 Not Stated = 1
BMI, Body mass index.	

Table 2.2. Dose of Cisplatin According to Tumor Type		
Tumor Type	Patients (n, %)	Dose (mg/m^2) mean ± SD[a]
Head/Neck	14, 25%	52.1 ± 22.5
Lung	10, 18%	66.6 ± 18.2
Genital	9, 16%	51.1 ± 22.6
Digestive	9, 16%	61.1 ± 20.1
Melanoma	6, 11%	76.0 ± 9.8
Bladder/Pelvis	4, 7%	70.0 ± 0.0
Bone/Blood	2, 4%	97.5 ± 31.8
Lymphoma/Sarcoma	2, 4%	60.0 ± 56.6
Breast	1, 2%	75.0
[a] The average length of cisplatin infusion was 1-2 h and fractioned cisplatin doses were administered to 9 out of 57 patients.		

Table 2.3. Clinical Laboratory Findings in Patients (n=57) Before and After Cisplatin Infusion

Clinical Parameter	Before Mean ± SD	After [a] Mean ± SD	P	Normal Reference Range
Mg^{2+} (mEq/L)	1.7 ± 0.2	1.6 ± 0.3	0.220	1.3 – 2.1
Na^{+} (mEq/L)	137 ± 2.4	135 ± 3.5	0.001	133 – 145
K^{+} (mEq/L)	4 ± 0.5	4 ± 0.7	0.712	3.5 – 5.1
Ca^{2+} (mEq/L)	9.6 ± 2.3	9.2 ± 2.3	0.0002	8.6 – 10.3
HCO_{3}^{-} (mEq/L)	25 ± 2.1	25 ± 2.5	0.196	21 – 31
Cl^{-} (mEq/L)	104 ± 3.3	103 ± 5.1	0.026	90 – 108
SCr (mg/dL)	0.82 ± 0.2	0.86 ± 0.2	0.330	0.70 – 1.30
BUN (mg/dL)	14.1 ± 5.4	14.5 ± 6.1	0.529	7 – 25
eGFR (mL/min/1.73m^2)	89.4 ± 24.8	90.6 ± 24.7	0.878	≥ 60
Urine Albumin/Cr (mg/mg)[b]	29.9 ± 40.6	50.7 ± 95.2	0.092	NA

[a] The time frame for assessment of clinical parameters was 13 ± 7 days post cisplatin infusion.

[b] These data were assessed on day 3 post-cisplatin infusion.

NA: not applicable

Table 2.4. Time-Dependent Changes from Baseline in Urinary Protein Biomarkers following Cisplatin Infusion[a]

Biomarker (ng/mL)	Baseline (mean ± SD)	Day 3 (mean ± SD)	P	Day 10 (mean ± SD)	P
KIM-1	0.203 ± 0.293	0.332 ± 0.524	0.024	0.575 ± 0.711	0.002
Calbindin	52.50 ± 83.59	60.63 ± 65.96	0.094	434.4 ± 856.4	<0.0001
Clusterin	57.44 ± 211.9	40.39 ± 101.0	0.043	108.2 ± 422.7	0.005
GST-pi	27.40 ± 74.07	33.43 ± 93.49	0.221	43.76 ± 88.96	0.011
IL-18	0.089 ± 0.223	0.042 ± 0.067	0.158	0.126 ± 0.189	0.061
MCP-1	0.507 ± 1.154	0.381 ± 0.549	0.330	0.838 ± 1.124	0.003
Albumin[b]	10.84 ± 13.86	15.92 ± 15.28	0.028	21.68 ± 24.08	0.008
β2M	136.9 ± 245.0	452.1 ± 422.2	<0.0001	188.1 ± 262.3	0.026
Cystatin C	32.91 ± 45.72	60.91 ± 119.1	0.015	57.71 ± 102.1	0.024
NGAL	43.45 ± 80.59	42.03 ± 59.39	0.346	51.77 ± 75.99	0.168
Osteopontin	1,235 ± 1,504	1,240 ± 1,597	0.784	2,630 ± 3,854	0.055
TFF3	755.9 ± 931.4	1,282 ± 1,187	0.001	1,531 ± 1,505	0.002

[a] Urinary biomarker concentrations were not normalized to creatinine levels for this analysis.

[b] The units for urinary albumin concentrations are μg/mL.

Table 2.5. Correlation Coefficient (r) of Biomarker and Urinary Albumin Concentrations[a]

Biomarker	Albumin 3-Day Absolute		Albumin 10-Day Absolute	
	3-Day		10-Day	
	r	P	r	P
KIM-1	0.547	<0.0001	0.765	<0.0001
Calbindin	0.459	0.00079	0.714	<0.0001
Clusterin	0.671	<0.0001	0.731	<0.0001
GST-pi	0.512	0.00015	0.557	<0.0001
IL-18	0.377	0.00762	0.731	<0.0001
MCP-1	0.553	<0.0001	0.761	<0.0001
β2M	0.424	0.00215	0.417	0.004
Cystatin C	0.539	<0.0001	0.693	<0.0001
NGAL	0.546	<0.0001	0.696	<0.0001
Osteopontin	0.435	0.00158	0.574	<0.0001
TFF3	0.646	<0.0001	0.701	0.000

[a] Urinary biomarker concentrations were not normalized to creatinine levels for this analysis.

Table 2.6. Baseline Reference Ranges for Absolute Urinary Biomarkers in Treatment Naïve Solid Tumor Patients Compared to Healthy Volunteers

	Solid Tumor (N=28)[a]	Healthy (N=39)[(Brott et. al.)]
Demographics		
Age (mean ± SD)	60.7 ± 12.2	43.9 ± 12.37
Male/Female	13/15	20/19
BMI (mean ± SD)	28.2 ± 7.2	24.4 ± 2.65
Race (% Caucasian)	93	97
Biomarkers (ng/mL)		
KIM-1	0.005 – 0.92	0.05 – 0.48
Calbindin	1.1 – 220.09	0.31 – 114.0
Clusterin	0.145 – 911.48	0 – 70
GST-pi	0.25 – 91.67	NR[c]
IL-18	0.01 – 1.64	NR
MCP-1	0.002 – 1.79	NR
Albumin[b]	0.317 – 53.6	0.4 – 62.0
β2M	0.99 – 1275.74	10 – 90
Cystatin C	0.08 – 277.68	3.2 – 75.0
NGAL	0.4 – 511.99	3 – 254
Osteopontin	1.1 – 7507.86	66 – 1230
TFF3	25.08 – 5485.37	30 – 3100

[a]These values are the baseline concentrations in cisplatin-naïve patients enrolled during cycle 1.

[b]The units for urinary albumin concentrations are µg/ml.

[c]NR: Not reported.

Supplemental Table 2.1. Time-Dependent Changes in Urinary Biomarkers Normalized to Urinary Creatinine Concentrations

Biomarker (ng/mL)	Baseline (mean ± SD)	Day 3 (mean ± SD)	P	Day 10 (mean ± SD)	P
KIM-1	0.225 ± 0.274	0.438 ± 0.461	0.004	0.479 ± 0.451	<0.001
Calbindin	60.1 ± 76.0	102.7 ± 122.3	0.032	524.3 ± 1175	0.004
Clusterin	58.96 ± 193.7	76.28 ± 171.6	0.631	83.66 ± 219.8	0.548
GST-pi	35.42 ± 70.24	66.43 ± 190.4	0.259	45.80 ± 68.91	0.456
IL-18	0.112 ± 0.222	0.061 ± 0.072	0.126	0.106 ± 0.109	0.881
MCP-1	0.518 ± 0.780	0.542 ± 0.549	0.857	0.814 ± 0.810	0.064
Albumin[a]	16.18 ± 25.17	34.51 ± 47.59	0.014	27.91 ± 36.84	0.060
β2M	202.8 ± 387.1	995.9 ± 1366	<0.001	310.9 ± 546.2	0.246
Cystatin C	49.91 ± 84.46	115.3 ± 260.1	0.079	65.81 ± 132.7	0.465
NGAL	71.60 ± 138.9	69.36 ± 70.13	0.919	57.75 ± 58.45	0.529
Osteopontin	2169 ± 5006	1794 ± 1398	0.614	2172 ± 2200	0.997
TFF3	1221 ± 1693	2222 ± 1827	0.004	1751 ± 1663	0.116

[a]The units for urinary albumin concentrations are μg/mL.

Supplemental Table 2.2. Correlation Coefficient (r) for Day 10 Biomarker Concentrations and Kidney Function Parameters[a]

	Change in BUN[b]		Change in SCr[b]		Change in eGFR[b]	
Biomarker (ng/mL)	r	P	r	P	r	P
KIM-1	0.253	0.094	-0.007	0.961	-0.020	0.897
Calbindin	0.312	0.037	0.114	0.460	-0.110	0.476
Clusterin	0.088	0.564	0.001	0.994	-0.036	0.815
GST-pi	0.032	0.835	-0.084	0.587	0.035	0.822
IL-18	0.155	0.316	-0.049	0.753	-0.049	0.755
MCP-1	0.204	0.180	0.199	0.194	-0.241	0.115
Albumin	0.097	0.527	0.109	0.482	-0.122	0.432
β2M	-0.053	0.729	0.079	0.611	-0.075	0.627
Cystatin C	0.113	0.461	-0.065	0.675	-0.020	0.897
NGAL	-0.042	0.785	0.075	0.628	-0.168	0.275
Osteopontin	0.257	0.088	0.066	0.669	-0.140	0.364
TFF3	0.168	0.270	-0.005	0.976	-0.093	0.546

[a] Urinary biomarker concentrations were not normalized to creatinine levels for this analysis.

[b] BUN: Blood Urea Nitrogen; SCr: Serum Creatinine; eGFR: Estimated Glomerular Filtration Rate

Supplemental Table 2.3. Effect of Sex on Urinary Biomarker Concentrations at Each Time Point[a]

Biomarker (ng/mL)	Baseline			3-Day			10-Day		
Gender	Male Mean ± SD	Female Mean ± SD	P	Male Mean ± SD	Female Mean ± SD	P	Male Mean ± SD	Female Mean ± SD	P
KIM-1	0.28 ± 0.38	0.13 ± 0.16	0.065	0.36 ± 0.43	0.31 ± 0.60	0.671	0.64 ± 0.74	0.51 ± 0.69	0.546
Calbindin	70.5 ± 108	35.1 ± 45.7	0.110	68.9 ± 64.9	53.6 ± 67.2	0.420	569 ± 1022	282 ± 605	0.254
Clusterin	106 ± 297	11.0 ± 21.5	0.092	71.5 ± 143	13.9 ± 16.1	0.044	174 ± 575	33.1 ± 48.8	0.257
GST-pi	31.3 ± 102	23.6 ± 29.2	0.699	21.4 ± 42.4	43.7 ± 121	0.406	45.8 ± 111	41.4 ± 56.2	0.867
IL-18	0.06 ± 0.08	0.12 ± 0.30	0.341	0.04 ± 0.08	0.04 ± 0.05	0.902	0.13 ± 0.18	0.13 ± 0.20	0.937
MCP-1	0.51 ± 0.85	0.51 ± 1.4	0.989	0.41 ± 0.57	0.36 ± 0.54	0.713	1.08 ± 1.6	0.57 ± 0.60	0.163
Albumin[b]	12.1 ± 15.7	9.6 ± 12.0	0.491	19.1 ± 17.9	13.2 ± 12.3	0.174	23.3 ± 27.7	19.8 ± 19.6	0.619
β2M	114 ± 243	159 ± 249	0.491	425 ± 389	475 ± 455	0.680	227 ± 297	144 ± 215	0.279
Cystatin C	36.1 ± 55.8	29.9 ± 34.0	0.613	82.9 ± 171	42.2 ± 33.6	0.232	72.0 ± 136	41.5 ± 34.3	0.312
NGAL	32.4 ± 55.4	54.1 ± 98.9	0.314	33.3 ± 33.8	49.5 ± 74.5	0.340	50.8 ± 90.9	52.8 ± 56.5	0.929
Osteopontin	1193 ± 1190	1275 ± 1777	0.840	1429 ± 1588	1078 ± 1616	0.444	3105 ± 4801	2090 ± 2371	0.372
TFF3	817 ± 1117	697 ± 724	0.629	1570 ± 1470	1037 ± 831	0.115	1673 ± 1457	1370 ± 1576	0.497

[a] Urinary biomarker concentrations were not normalized to creatinine levels for this analysis.

[b] The units for urinary albumin concentrations are μg/mL.

Supplemental Table 2.4. Effect of Concomitant Disease on Urinary Biomarker Concentrations[a,b]

Biomarker (ng/mL)	Baseline			3-Day			10-Day		
Presence of Concomitant Disease:	Y Mean ± SD	N Mean ± SD	P	Y Mean ± SD	N Mean ± SD	P	Y Mean ± SD	N Mean ± SD	P
KIM-1	0.23 ± 0.31	0.18 ± 0.28	0.693	0.27 ± 0.31	0.38 ± 0.64	0.516	0.67 ± 0.76	0.52 ± 0.67	0.195
Calbindin	69.4 ± 104.3	39.3 ± 58.6	0.241	57.1 ± 56.6	63.2 ± 72.1	0.808	354 ± 641.7	484 ± 966.1	0.686
Clusterin	82.2 ± 259.9	38.1 ± 160.6	0.485	62.0 ± 140.2	24.8 ± 50.0	0.232	204 ± 652.6	48.7 ± 101.3	0.232
GST-pi	43.8 ± 106.0	14.6 ± 21.7	0.170	57.4 ± 137.6	16.0 ± 21.7	0.140	52.8 ± 125.8	38.1 ± 52.4	0.545
IL-18	0.11 ± 0.32	0.07 ± 0.09	0.613	0.03 ± 0.04	0.05 ± 0.08	0.269	0.13 ± 0.21	0.12 ± 0.18	0.113
MCP-1	0.79 ± 1.6	0.12 ± 0.18	0.137	0.32 ± 0.29	0.29 ± 0.39	0.498	0.83 ± 0.85	0.42 ± 0.68	0.704
Albumin[c]	15.9 ± 15.5	6.85 ± 10.8	0.019	22.7 ± 17.4	11.0 ± 11.1	0.009	25.3 ± 25.9	19.4 ± 22.6	0.219
β2M	171 ± 223.9	110 ± 257.5	0.412	475 ± 378.4	436 ± 451.6	0.941	278 ± 303.2	133 ± 213.5	0.104
Cystatin C	35.3 ± 35.8	31.0 ± 52.3	0.832	39.1 ± 26.8	76.7 ± 153.9	0.259	49.7 ± 51.1	62.7 ± 124.1	0.589
NGAL	58.2 ± 108.7	31.9 ± 43.5	0.265	33.5 ± 30.2	48.3 ± 73.5	0.351	56.6 ± 97.8	48.8 ± 57.5	0.422
Osteopontin	1022 ± 936.4	1401 ± 1821	0.279	962 ± 1150	1441 ± 1836	0.265	3535 ± 5338	2068 ± 2288	0.296
TFF3	922 ± 752	626 ± 1035	0.303	1323 ± 963	1252 ± 1329	0.939	1772 ± 1495	1382 ± 1491	0.546

[a] Urinary biomarker concentrations were not normalized to creatinine levels for this analysis.

[b] Medical record documented concomitant diseases included Type 2 diabetes mellitus, hyperlipidemia, Hashimoto's thyroiditis, HIV, and hypertension.

[c] The units for urinary albumin concentrations are μg/mL.

Supplemental Table 2.5. Effect of Prior Intravenous Contrast Dye on Urinary Biomarker Concentrations[a]

Biomarker (ng/mL)	Baseline			3-Day			10-Day		
Contrast Used	Y Mean ± SD	N Mean ± SD	P	Y Mean ± SD	N Mean ± SD	P	Y Mean ± SD	N Mean ± SD	P
KIM-1	0.23 ± 0.32	0.21 ± 0.30	0.715	0.31 ± 0.38	0.34 ± 0.58	0.835	0.49 ± 0.53	0.61 ± 0.80	0.681
Calbindin	55.4 ± 51.4	56.1 ± 98.0	0.889	79.3 ± 74.7	55.5 ± 67.0	0.298	896 ± 1529	325 ± 561	0.060
Clusterin	10.0 ± 13.3	84.3 ± 260	0.363	44.6 ± 97.9	43.0 ± 109.8	0.878	54.8 ± 74.5	142 ± 518	0.664
GST-pi	27.2 ± 30.0	31.5 ± 90.1	0.993	85.4 ± 184.7	15.0 ± 18.5	0.038	30.2 ± 25.3	52.3 ± 107.4	0.599
IL-18	0.21 ± 0.44	0.06 ± 0.07	0.030	0.05 ± 0.07	0.04 ± 0.07	0.703	0.16 ± 0.18	0.11 ± 0.17	0.545
MCP-1	0.43 ± 0.55	0.58 ± 1.39	0.778	0.52 ± 0.67	0.36 ± 0.54	0.356	0.70 ± 0.65	0.83 ± 1.37	0.695
Albumin[b]	12.9 ± 14.3	10.1 ± 13.7	0.540	19.8 ± 18.2	14.4 ± 14.1	0.346	28.8 ± 36.1	19.1 ± 19.6	0.305
β2M	102 ± 122	169 ± 291	0.566	321 ± 264	507 ± 460	0.252	143 ± 118	208 ± 311	0.553
Cystatin C	34.4 ± 34.5	35.4 ± 52.5	0.898	48.2 ± 39.4	69.4 ± 142	0.695	50.7 ± 36.4	62.2 ± 123.7	0.814
NGAL	34.2 ± 26.3	51.2 ± 98.0	0.643	66.8 ± 102.5	37.7 ± 41.3	0.123	67.2 ± 61.8	51.0 ± 86.1	0.484
Osteopontin	1343 ± 1046	1226 ± 1702	0.771	1772 ± 2113	1115 ± 1492	0.219	4409 ± 5574	5574 1768 ± 2100	0.108
TFF3	869 ± 721	748 ± 1053	0.623	1574 ± 1015	1194 ± 1282	0.365	1938 ± 1755	1379 ± 1481	0.352

[a] Urinary biomarker concentrations were not normalized to creatinine levels for this analysis.

[b] The units for urinary albumin concentrations are μg/mL.

Supplemental Table 2.6. Correlation Coefficients (R) and P-Values of Absolute Biomarker Levels and Age[a]						
	Baseline		3-Day		10-Day	
Biomarker	R	P	R	P	R	P
KIM-1	-0.062	0.649	0.079	0.589	0.127	0.395
Calbindin	0.038	0.781	-0.146	0.313	0.013	0.929
Clusterin	-0.108	0.423	-0.070	0.630	-0.013	0.930
GSTpi	0.153	0.255	-0.030	0.839	0.141	0.346
IL-18	-0.029	0.828	-0.046	0.753	-0.049	0.745
MCP-1	0.140	0.299	0.093	0.522	0.126	0.397
Albumin	-0.100	0.457	-0.212	0.139	-0.058	0.698
B2M	-0.185	0.172	-0.066	0.655	0.006	0.969
Cystatin C	0.079	0.561	0.059	0.683	0.177	0.235
NGAL	0.148	0.271	0.157	0.276	0.060	0.690
Osteopontin	0.197	0.141	-0.034	0.817	0.216	0.144
TFF3	-0.004	0.977	-0.162	0.262	0.012	0.934

Supplemental Table 2.7. Correlation Coefficients (R) and P-Values of Absolute Biomarker Levels and Body Mass Index (BMI)						
	Baseline		3-Day		10-Day	
Biomarker	R	P	R	P	R	P
KIM-1	-0.243	0.069	-0.202	0.159	-0.189	0.203
Calbindin	0.026	0.849	0.025	0.852	-0.022	0.881
Clusterin	0.019	0.897	-0.018	0.904	0.015	0.910
GSTpi	0.015	0.920	-0.012	0.934	0.005	0.974
IL-18	-0.171	0.203	-0.146	0.279	-0.154	0.302
MCP-1	0.144	0.336	0.140	0.348	0.135	0.351
Albumin	0.139	0.351	-0.121	0.372	-0.116	0.396
B2M	0.123	0.401	-0.113	0.449	-0.100	0.459
Cystatin C	-0.098	0.470	-0.106	0.480	-0.092	0.498
NGAL	0.083	0.577	0.064	0.659	0.055	0.705
Osteopontin	0.053	0.714	-0.039	0.774	0.038	0.797
TFF3	0.034	0.813	0.033	0.828	-0.028	0.847

Supplemental Table 2.8. Correlation Coefficients (R) and P-Values of Absolute Biomarker Levels and Prescribed Dose						
	Baseline		3-Day		10-Day	
Biomarker	R	P	R	P	R	P
KIM-1	-0.053	0.693	0.171	0.239	0.021	0.889
Calbindin	-0.065	0.633	-0.047	0.748	0.122	0.415
Clusterin	-0.119	0.379	-0.085	0.56	0.048	0.747
GSTpi	-0.162	0.229	-0.227	0.113	0.015	0.92
IL-18	-0.062	0.648	-0.08	0.587	0.08	0.599
MCP-1	0.012	0.927	-0.105	0.468	-0.029	0.844
Albumin	-0.14	0.298	0.084	0.561	0.168	0.259
B2M	-0.048	0.721	**0.349**	**0.013**	-0.027	0.856
Cystatin C	-0.12	0.373	0.186	0.195	0.108	0.469
NGAL	-0.045	0.742	-0.001	0.993	0.029	0.848
Osteopontin	0.017	0.902	0.248	0.082	0.083	0.579
TFF3	-0.109	0.42	0.215	0.135	0.000	1.000

Supplemental Table 2.9. Correlation Coefficients (R) and P-Values of Absolute Biomarker Levels and Mass Based Dose

	Baseline		3-Day		10-Day	
Biomarker	R	P	R	P	R	P
KIM-1	-0.013	0.923	0.265	0.066	0.103	0.492
Calbindin	-0.048	0.722	0.008	0.958	0	0.999
Clusterin	0.051	0.706	**0.291**	**0.041**	0.178	0.232
GSTpi	-0.033	0.808	**0.292**	**0.039**	0.101	0.497
IL-18	-0.009	0.949	0.044	0.761	0.179	0.229
MCP-1	-0.094	0.485	-0.116	0.423	0.06	0.69
Albumin	-0.186	0.166	-0.165	0.253	-0.003	0.986
B2M	-0.048	0.725	-0.105	0.471	0.159	0.291
Cystatin C	0.049	0.716	-0.079	0.587	0.051	0.735
NGAL	-0.093	0.491	0.09	0.534	0.176	0.236
Osteopontin	-0.03	0.824	0.268	0.06	0.092	0.539
TFF3	-0.094	0.489	0.168	0.244	0.154	0.303

Supplemental Table 2.10. Correlation Coefficients (R) and P-Values of Absolute Biomarker Levels and Cumulative Dose Baseline

	Baseline		3-Day		10-Day	
Biomarker	R	P	R	P	R	P
KIM-1	0.011	0.938	0.21	0.147	0.011	0.943
Calbindin	-0.187	0.163	-0.084	0.563	-0.114	0.444
Clusterin	-0.054	0.688	0.114	0.43	0.031	0.836
GSTpi	-0.195	0.145	0.181	0.208	-0.068	0.649
IL-18	-0.008	0.955	-0.076	0.602	-0.034	0.822
MCP-1	-0.142	0.293	-0.179	0.212	-0.081	0.588
Albumin	-0.138	0.305	-0.16	0.267	0.013	0.929
B2M	-0.058	0.669	-0.179	0.219	0.035	0.818
Cystatin C	0.022	0.872	-0.124	0.393	-0.13	0.383
NGAL	-0.203	0.129	-0.046	0.751	-0.047	0.753
Osteopontin	-0.146	0.278	**0.311**	**0.028**	-0.054	0.72
TFF3	-0.193	0.150	0.110	0.447	0.000	0.998

Supplemental Table 2.11. Comparison of Cycle 1 and 2 of Absolute Biomarker Levels									
	Baseline			3-Day			10-Day		
Cycle	1	2		1	2		1	2	
Biomarker	Mean ± SD	Mean ± SD	P	Mean ± SD	Mean ± SD	P	Mean ± SD	Mean ± SD	P
KIM-1	0.13 ± 0.20	0.27 ± 0.35	0.072	0.36 ± 0.67	0.30 ± 0.34	0.636	0.63 ± 0.70	0.51 ± 0.73	0.554
Calbindin	41.2 ± 54.1	63.4 ± 104	0.321	66.5 ± 72.6	54.8 ± 59.5	0.536	608 ± 1018.7	238 ± 587	0.141
Clusterin	42.0 ± 171	72.3 ± 237	0.594	27.6 ± 52.5	53.2 ± 133	0.376	64.1 ± 106.4	158 ± 611	0.451
GSTpi	16.7 ± 22.1	37.7 ± 101	0.289	23.4 ± 43.6	43.5 ± 125	0.452	29.0 ± 31.0	60.6 ± 125	0.228
IL-18	0.03 ± 0.03	0.05 ± 0.09	0.546	0.16 ± 0.24	0.09 ± 0.10	0.352	0.31 ± 0.42	0.70 ± 1.55	0.224
MCP-1	0.31 ± 0.42	0.70 ± 1.55	0.205	0.39 ± 0.62	0.37 ± 0.48	0.872	1.17 ± 1.59	0.46 ± 0.45	0.050
Albumin	13.2 ± 15.1	8.57 ± 12.3	0.211	18.2 ± 15.6	13.6 ± 14.9	0.289	28.3 ± 26.4	14.1 ± 18.9	0.043
B2M	204 ± 327	72.4 ± 91.9	0.042	373 ± 416	531 ± 422	0.189	214 ± 284	158 ± 238	0.470
Cystatin C	41.1 ± 56.8	25.0 ± 30.6	0.188	65.5 ± 149	56.3 ± 81.5	0.788	73.2 ± 132	40.1 ± 49.9	0.271
NGAL	57.3 ± 100	40.1 ± 49.9	0.204	38.7 ± 34.5	30.1 ± 54.2	0.697	57.3 ± 62.7	45.3 ± 77.4	0.600
Osteopontin	1339 ± 1556	1134 ± 1473	0.612	1091 ± 1070	1389 ± 2001	0.513	3420 ± 4749	1732 ± 2274	0.135
TFF3	1000 ± 1150	521 ± 586	0.051	1320 ± 1131	1244 ± 1262	0.823	1954 ± 1788	1050 ± 922.8	0.039

Supplemental Table 2.12. Number of Values Reported Out-of-Range by the Bio-Plex Manager

Analyte	Out-of-Range Below[a]	Out-of-Range Above[b]	Range of Quantification (ng/mL)
KIM-1	38	0	0.021 - 21
Calbindin	26	0	4.4 - 1750
Clusterin	37	0	1.3 - 1250
GST-pi	13	0	0.50 - 230
IL-18	63	**0**	0.019 - 15
MCP-1	9	0	0.011 - 3.8
Albuminc	**0**	30	2.8 - 640
β2M	0	13	0.043 - 22
Cystatin C	5	0	0.16 - 40
NGAL	8	0	0.062 - 34
Osteopontin	**3**	0	3.8 - 2100
TFF3	0	6	0.075 - 98
Total	202	49	

[a]Values out-of-range below were below the limit of detection and were substituted with the lower limit of quantification divided by 2.

[b]Values out-of-range above were diluted and re-run.

[c]The units for urinary albumin concentrations are µg/mL.

CHAPTER 3: TIME-DEPENDENT CHANGES IN KIDNEY INJURY BIOMARKERS IN PATIENTS RECEIVING MULTIPLE CYCLES OF CISPLATIN

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3.1. Abstract

Current standards for diagnosing acute kidney injury (AKI) such as serum creatinine (SCr) and estimated glomerular filtration rate (eGFR) lack sensitivity and require significant changes in kidney function before changes are observed. Sensitive and novel urinary protein biomarkers reflect direct injury to tubules and are increasingly being utilized to monitor nephrotoxicity during drug development. However, there is a lack of clinical validation and assessment of the long-term performance of urinary biomarker proteins. We sought to characterize time-dependent changes in the urinary concentrations of three promising biomarkers, kidney injury molecule-1 (KIM-1), calbindin, and trefoil factor 3 (TFF3) in 27 patients with solid tumors receiving outpatient cisplatin (≥ 25 mg/m^2), during two different cycles of chemotherapy. SCr, eGFR, and blood urea nitrogen (BUN), were unchanged from baseline after both cycles of cisplatin therapy. KIM-1 (2.3-fold), calbindin (15-fold), and TFF3 (1.8-fold) significantly increased from baseline during early cycles and remained elevated prior to subsequent cisplatin administration. During subsequent cycles of cisplatin, no further changes in urinary biomarkers were observed. Modest correlations between eGFR and baseline urinary KIM-1 concentrations were observed (0.362, P=0.002) during the later cycles. Longitudinal assessment of urinary biomarker performance in the same cohort of oncology patients reveals differences in excretion between early and subsequent cycles of cisplatin-containing chemotherapy.

3.2. Introduction

Novel urinary protein biomarkers are increasingly being investigated for their ability to detect drug-induced acute kidney injury (AKI) due to limitations with current clinical measures including serum creatinine (SCr) and estimated glomerular function rate (eGFR). Several promising urinary proteins, such as kidney injury molecule-1 (KIM-1), exhibit higher sensitivity to detect kidney injury in preclinical models as well as in patients with mixed etiologies of AKI (Vaidya et al. 2008, Vaidya et al. 2010, Sinha et al. 2013). Recently, the Food and Drug Administration issued a letter of support for several novel urinary biomarkers (KIM-1, neutrophil gelatinase-associated lipocalin (NGAL), osteopontin, albumin, and total urinary protein) for use as biomarkers of drug-induced renal tubular injury in early clinical trials (USFDA 2016). However, long-term assessment of promising biomarkers in the same sets of patients is lacking, impeding clinical utilization. Our laboratory previously demonstrated significant time-dependent changes in the urinary excretion of biomarkers (KIM-1, calbindin, and trefoil factor 3, TFF3) in patients receiving cisplatin for the first or second cycle (George et al. 2017).

KIM-1 is a transmembrane protein found on the apical surface of proximal tubules. Following injury, the ectodomain of KIM-1 is shed into the tubular lumen (Ichimura et al. 1998, Vaidya et al. 2006). Calbindin is a cytosolic calcium-binding protein found primarily on the distal tubules and collecting ducts of the kidney. Calbindin concentrations have been shown to increase in the urine following cisplatin treatment in rodents, cynomolgus monkeys, as well as in oncology patients (Takashi et al. 1996, Won et al. 2016, Chen et al. 2017, George et al. 2017). TFF3 is a small peptide hormone secreted by mucus-producing epithelial cells and shown to have a role in inhibiting apoptosis and promoting cell survival and migration (Taupin and Podolsky 2003). While in rodents urinary TFF3 concentrations were shown to decrease with cisplatin-induced kidney injury (Yu et al.

2010), we have demonstrated that concentrations of TFF3 are increased in the urine of oncology patients treated with cisplatin (George et al. 2017). In the present study, we sought to compare the time-dependent changes in three biomarker proteins (KIM-1, calbindin, and TFF3) in a subset of patients during early and subsequent cycles of cisplatin chemotherapy.

3.3. Methods

Selection of participants

A prospective study of patients receiving outpatient cisplatin chemotherapy doses ≥ 25 mg/m^2 for various solid tumors at the University of Colorado Cancer Center, Aurora, CO, a National Cancer Institute-Designated Consortium Comprehensive Cancer Center was conducted. Twenty-six patients received intravenous cisplatin whereas one patient was administered cisplatin intraperitoneally. Patients were hydrated pretreatment and posttreatment with saline (1–2 L). Study inclusion criteria included: (1) age ≥ 18 years; (2) hemoglobin ≥ 10 g/dL; (3) no consumption of grapefruit juice or alcohol within 7 days; (4) no history of alcohol consumption of > 14 drinks/week; (5) no history of organ transplantation or kidney dialysis; (6) willingness to comply with study; (7) not pregnant or lactating; (8) no changes in medications within previous 4 weeks; (9) normal liver function (alanine aminotransferase and aspartate aminotransferase <2x upper limit of normal); and (10) baseline eGFR >60 mL/min/m^2 (using the four-variable Modification of Diet in Renal Disease equation) (Levey et al. 2006). Exclusion criteria included: (1) diagnosis of kidney cancer; (2) previous exposure to platinum-based chemotherapy (other than the currently prescribed regimen); (3) herbal supplement use; (4) exposure to other known nephrotoxins (including contrast agents) within the previous 30 days; and (5) concurrent use of inhibitors of transport proteins involved in cisplatin secretion into urine. The Institutional Review Boards at the University of Colorado (Protocol 12-1510)

and Rutgers University (Protocol E13-716) approved the protocols for recruitment and sample collection. For the *early cycle*, urine was collected from eighteen patients at the first cycle of cisplatin therapy and nine patients for the second cycle of cisplatin therapy. During the *subsequent cycle*, urine was collected from two patients during cycle two, five patients during cycle 3, and twenty patients at cycle 4. The average length of time between the early and subsequent cycles of cisplatin was 36 days (range, 14–70 days).

Urine samples

Urine was collected from spontaneous voids at baseline (pre-cisplatin infusion), between 2 and 5 days (designated as day 3) and 9 and 11 days (designated as day 10) post-cisplatin infusion. For patients missing baseline urine collection, urine in the 0–2 h timed collection after cisplatin infusion was used for analysis. Urine was centrifuged at 3,000x*g* and supernatant was aliquoted into 2 mL collection tubes and frozen within 30–60 min of collection at -80°C. At the time of analysis, samples were thawed and placed on ice and centrifuged at 1,500 rpm for 5 min. 12.5 µl of supernatant was pipetted for biomarker analysis.

Quantification of urinary protein biomarkers

KIM-1, calbindin, and TFF3 were measured using the Milliplex MAP Human Kidney Injury Magnetic Bead Panel 1 (MilliporeSigma, Burlington, MA). Washing steps were conducted using the Bio-Plex Pro II wash station (Bio-Rad Life Science, Hercules, CA). Samples were analyzed using a Bio-Plex, MagPix Multiplex Reader (Bio-Rad), which reports the mean fluorescence intensity proportional to the concentration of analyte bound to each bead. Concentrations were extrapolated from a known standard curve using a five-parameter logistic curve. Recommended dilutions of urine samples in dilution buffer provided in the assay kit were followed (1:2). Values that were above the

detection limit were extrapolated from the standard curve. Concentrations below the limit of detection were substituted with the lower limit of quantification divided by 2. Data are presented as absolute concentrations and normalized to urinary creatinine concentrations quantified using the DCA Vantage Analyzer (Siemens, Princeton, NJ).

Data and statistical analysis

Data are presented for individual patients together with the corresponding group mean ± SD (baseline, and days 3 and 10). Data were tested for normality using the D'Agostino-Pearson omnibus test. Time-dependent differences among individual biomarkers were evaluated by Wilcoxon rank-sum tests for absolute concentrations and analysis of variance to test normalized biomarker concentrations. Differences in patient-specific factors (categorical) were assessed by analysis of variance. Clinical laboratory values for pre-cisplatin and post-cisplatin treatment were compared using paired t-tests. Differences were considered statistically significant at $P < 0.05$. Pearson or Spearman correlation coefficients were used to measure the strength of association between biomarker and kidney function measures, or patient-specific factors. All statistical analyses and plots were done by GraphPad Prism version 6 (GraphPad Software, LaJolla, CA).

3.4. Results & Discussion

Twenty-seven patients were included in the study and patient characteristics are shown in Table 3.1. All but one patient was white (96%) with an even distribution of male and female patients. The mean and range of patient age was 59 years (range, 35 – 72 years) and body mass index (BMI) values were 26.2 kg/m^2 (range, 19.1 – 43.1 kg/m^2). The average prescribed cisplatin dose and range across initial and subsequent cycles was

59.3 mg/m^2 (range, 25 – 100 mg/m^2) and was not significantly different between early and subsequent cycles.

Using the KDIGO criteria (2012, Khwaja 2012), AKI was defined as an increase in SCr > 0.3 mg/dL over 48 h or 1.5 times baseline after one dose of cisplatin. None of the twenty-seven patients developed AKI based on this criteria during either cycle of cisplatin-containing chemotherapy. Mean SCr, BUN, eGFR, and urinary albumin-to-creatinine ratio were not significantly changed 12 ± 9 days post-cisplatin infusion compared to pre-cisplatin levels both during the initial and subsequent tracked cycles (Table 3.2). The lack of AKI in these patients could be due to several reasons. By cycle 4, patients that developed or had a tendency to develop AKI may have been identified and been prescribed a different chemotherapy regimen. Furthermore, the limited sample size, timing of blood collections, wide range of ages, and lack of racial diversity may also have contributed to a lack of AKI in this group of patients.

There were significant time-dependent changes in urinary KIM-1 concentrations during the early but not subsequent cycles of cisplatin chemotherapy (Figure 3.1 and Table 3.3). During early cycles, urinary concentrations of KIM-1 were elevated 1.7-fold and 2.3-fold at day 3 and 10 after cisplatin infusion, respectively. Although not statistically significant, baseline urinary KIM-1 concentrations were 2.6-fold higher during the subsequent cycle compared to the baseline at the early cycle. Interestingly, compared to the baseline concentration before the subsequent cisplatin cycle, there were no significant changes in absolute (Table 3.3) and normalized (Supplemental Table 3.1) mean KIM-1 urinary concentrations at day 3 and 10. However, there were 2-fold elevations at days 3 ($p=0.04$) and 10 ($p=0.03$), respectively, during the subsequent cycle compared to the earlier baseline values. Significant time-dependent changes in urinary

calbindin concentrations occurred during the early cycles but were not seen with subsequent cisplatin infusions (Figure 3.1 and Table 3.3). During the early cycles, mean calbindin concentrations in the urine were 15-fold higher on day 10 (p=0.0006) compared to baseline (Table 3.3). By comparison, there were no significant time-dependent changes during the subsequent cycle compared to the subsequent baseline and only a 3-fold elevation compared to the early baseline (p=0.0058). The baseline concentrations were not significantly different between initial and subsequent cycles. Significant changes in urinary concentrations of TFF3 also occurred during the initial cycle and not during the subsequent cycle (Figure 3.1 and Table 3.3). TFF3 levels were elevated 2-fold by day 3 from baseline (p=0.005) during the early cycle, with little to no changes during the subsequent cycle. However similar to KIM-1, there were 2.5 and 2.3-fold elevations at days 3 (p=0.0026) and 10 (p=0.0008), respectively, during the subsequent cycle compared to the earlier baseline value. Although baseline concentrations were not significantly different, there was a trend for higher baseline values during the subsequent cycle.

This is the first study to compare time-dependent changes in the novel urinary biomarkers KIM-1, calbindin, and TFF3 in the same set of patients across multiple cycles of cisplatin. Cisplatin-mediated nephrotoxicity is dose-dependent and it was recently shown that long-term consequences of cisplatin treatment result in a small but permanent decline in eGFR (Latcha et al. 2016). With all three biomarkers, KIM-1, calbindin, and TFF3, there was an initial increase without further significant increases during subsequent cycles of cisplatin. Importantly, there was a trend for increased baseline values with successive treatments indicating permanent or progressive injury. Serum creatinine was less sensitive in detecting these subtle changes. Similar trends (dampened response with successive cisplatin treatments) were seen in previously

reported urinary biomarkers (beta-2-microglobulin and N-acetyl-Beta-D-glycosminidase) (Tirelli et al. 1985). Ideally, predictors of AKI should be able to reflect the progression of injury for the long-term. From this study and others, it is evident that when utilizing novel urinary protein biomarkers for monitoring the progression of drug-induced kidney injury it is worthwhile to compare back to a treatment-naïve baseline.

Correlation between changes in kidney functional parameters and biomarkers during the subsequent cycles of cisplatin revealed significant but modest correlations between initial concentrations of urinary KIM-1 and SCr (R=0.192, P=0.037) and eGFR (0.362, P=0.002) (Table 3.4). There were also modest correlations between TFF3 and eGFR during all three time-points (Baseline: R=0.25, P=0.015; Day 3: R=0.271, P=0.011; Day 10: R=0.313, P=0.006) (Table 3.4). It is interesting to note that correlation between functional parameters (SCr and eGFR) and novel urinary biomarkers that are typically reflective of tubular injury appeared with additional cycles of cisplatin and not during the early cycles. Even better correlations may be seen in patients with clinically diagnosed AKI. There is also some evidence that initial changes in novel protein biomarkers may predict a subsequent decrease in function. This is demonstrated by a modest correlation between TFF3 concentration on day 3 during the initial cycle and subsequent change in eGFR (R=0.350, P=0.003; data not shown).

Associations of various patient-specific factors (age, gender, BMI, cisplatin dose) were tested with urinary concentrations of biomarkers during the subsequent cycles. There were no significant correlations between cisplatin dose and biomarkers during any time-point (data not shown). However, there was a significant difference in mean calbindin urinary protein concentration between male and female patients with males exhibiting 4-fold and 3.6-fold higher concentrations at baseline and day 3, respectively

(Supplemental Table 3.2). However, the disparity was diminished by day 10. This data is consistent with a previous clinical study assessing baseline biomarker values in healthy volunteers, which also identified a sex difference with higher values of both absolute and creatinine-normalized urinary calbindin concentrations in male volunteers (Brott et al. 2014). These data suggest that calbindin may be regulated by sex hormones. In fact, testosterone deficiency has been shown to increase the abundance of renal calbindin mRNA in mice which was suppressed by testosterone treatment (Hsu et al. 2010). In contrast, estrogen deficiency decreased the expression of renal calbindin in ovariectomized rats while administration of ^{17}beta-estradiol enhanced renal calbindin mRNA expression (Criddle et al. 1997). These sex differences should to be considered when setting appropriate cutoffs for clinical use.

There was a modest correlation between age and urinary KIM-1 concentrations on day 10 (R=0.147, P=0.048) (Supplemental Table 3.3). Kidney function declines with age and older patients are more sensitive to cisplatin-mediated kidney injury (Wen et al. 2015). It is reassuring that KIM-1 is possibly sensitive to subtle age-dependent kidney damage and could potentially be useful for monitoring the development of chronic kidney disease. Lastly, there was a modest correlation between baseline TFF3 and BMI (R=0.189, P=0.023) (Supplemental Table 3.4). Although very little is known about TFF3, studies suggest a possible link between TFF3 and dietary obesity (Taylor and Phillips 1997). In fact, TFF3 was found to map to Obq4, an obesity quantitative trait loci on mouse chromosome 17 (Taylor and Phillips 1997).

In summary, this study reports time-dependent changes in three urinary protein biomarkers, KIM-1, calbindin, and TFF3 during an early and subsequent cycle of cisplatin-containing chemotherapy. Unexpectedly, kidney injury biomarker

concentrations did not increase to the same magnitude during subsequent cycles of cisplatin therapy as in early cycles. KIM-1 and TFF3 concentrations never returned to the baseline values seen in earlier cycles. Larger longitudinal studies of AKI biomarkers in a more diverse population exhibiting clinical AKI are needed to understand their long-term performance and utility.

Figure 3.1.

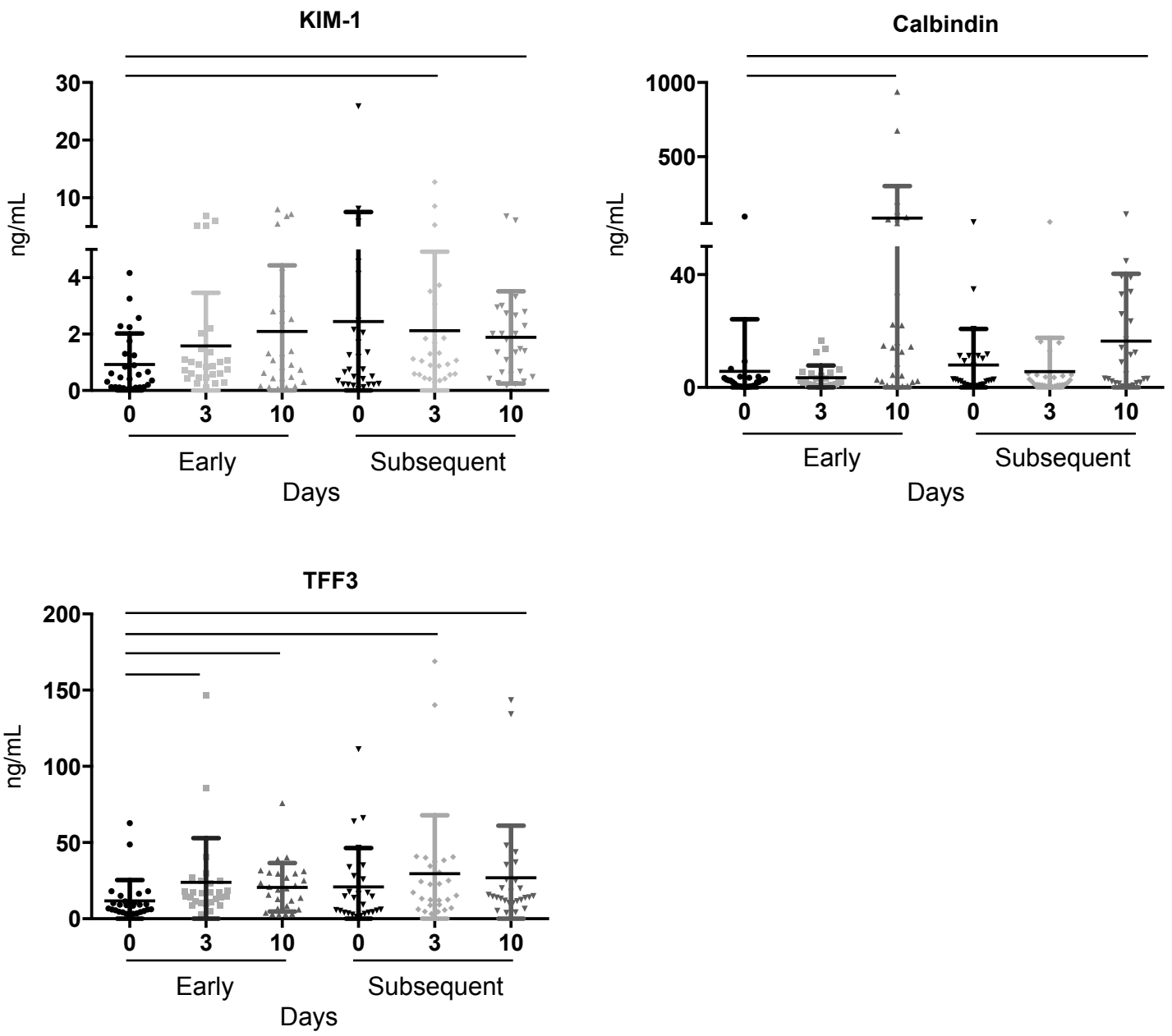
KIM-1
ng/mL
30
20
10
4
2
0
0
3
10
0
3
10
Early
Subsequent
Days
Calbindin
ng/mL
1000
500
40
0
0
3
10
0
3
10
Early
Subsequent
Days
TFF3
ng/mL
200
150
100
50
0
0
3
10
0
3
10
Early
Subsequent
Days

Figure 3.1. Time-dependent changes in absolute urinary concentrations of protein biomarkers following early and subsequent cycles of cisplatin infusion. Urinary protein concentrations for kidney injury molecule-1 (KIM-1), calbindin, trefoil factor 3 (TFF3) were quantified using a multiplex assay at baseline (n=27), 3 days (range, 2–5 days; n=27), and 10 days (range, 9–11 days; n=27) post cisplatin-infusion during early (1 or 2 cycles) or subsequent (2 or 5 cycles) of chemotherapy. Concentrations were measured in urine supernatants and presented as ng/mL. Scatter plots show individual levels (n=27) and mean ± SD concentration at each time-point. Solid lines represent statistically significant differences ($P < 0.05$) compared with baseline protein levels.

Table 3.1. Demographic Information for Patients Receiving Cisplatin During Two Cycles (N=27)	
Age (mean ± SD)	59.0 ± 9.6 years
Sex	Male = 13 Female = 14
Body Mass Index (mean ± SD)	26.2 ± 5.5 kg/m^2
Cisplatin Dose (mean ± SD)	59.3 ± 20.9 mg/m^2
Race	Caucasian = 26 Hispanic = 1

Table 3.2. Clinical Laboratory Values Pre- and Post-Cisplatin Infusion at Early and Subsequent Cycles

	Early Pre Mean ± SD	Early Post [a] Mean ± SD	P	Subsequent Pre Mean ± SD	Subsequent Post [a] Mean ± SD	P
SCr[c] (mg/dL)	0.85 ± 0.21	0.84 ± 0.23	0.774	0.83 ± 0.20	0.84 ± 0.15	0.863
BUN[c] (mg/dL)	14.5 ± 5.4	15.0 ± 5.2	0.614	12.9 ± 4.0	14.4 ± 4.3	0.191
eGFR[c] (mL/min)	90.6 ± 25.0	94.2 ± 27.2	0.445	93.3 ± 21.4	89.8 ± 18.5	0.547
Urine Albumin/Creatinine (mg/g)[b]	31.1 ± 39.7	36.8 ± 44.5	0.547	48.2 ± 72.5	22.1 ± 28.4	0.371

[a]The time frame for assessment of clinical parameters was 12 ± 9 days post cisplatin infusion.

[b]The time frame for assessment of albumin/creatinine was Day 10.

[c]SCr: Serum creatinine; BUN: Blood Urea Nitrogen; eGFR: estimated glomerular filtration rate

Table 3.3. Time-Dependent Changes in Absolute Urinary Biomarkers following Cisplatin Infusion

Biomarker (ng/mL)	Baseline (mean ± SD)	P(Ba_e)	Day 3 (mean ± SD)	P(Ba_e)	P(Ba_s)	Day 10 (mean ± SD)	P(Ba_e)	P(Ba_s)
KIM-1								
Early Cycle	0.93 ± 1.1	-	1.58 ± 1.9	0.044	-	2.09 ± 2.3	0.026	-
Subsequent Cycle	2.45 ± 5.1	0.127	2.12 ± 2.8	0.007	0.216	1.89 ± 1.6	0.003	0.157
Calbindin								
Early Cycle	5.75 ± 18.5	-	3.48 ± 4.3	0.545	-	86.9 ± 216	0.0006	-
Subsequent Cycle	7.95 ± 12.8	0.103	5.59 ± 12.0	0.447	0.364	16.4 ± 23.9	0.0058	0.113
TFF3								
Early Cycle	11.8 ± 13.6	-	24.0 ± 29.0	0.0005	-	20.6 ± 16.0	0.0124	-
Subsequent Cycle	20.8 ± 25.5	0.447	29.6 ± 38.4	0.0026	0.1331	26.9 ± 34.2	0.0008	0.1961

P Value Comparisons: Ba_e: Compared to early baseline; Ba_s: Compared to subsequent baseline

KIM-1: Kidney injury molecule-1; TFF3: Trefoil factor 3

Table 3.4. Correlation Coefficients (R) and P-Values of Absolute Biomarker Levels and Kidney Functional Parameters During Subsequent Cycle[a]

	Change in BUN[b]		Change in SCr[b]		Change in eGFR[b]	
Biomarker	R	P	R	P	R	P
KIM-1						
Baseline	0.065	0.24	0.192	0.037	0.362	0.002
3 Day	0.074	0.211	$5.8x10^{-4}$	0.913	$3.1x10^{-4}$	0.937
10 Day	0.099	0.143	0.021	0.513	0.027	0.451
Calbindin						
Baseline	0.086	0.175	0.088	0.17	0.006	0.733
3 Day	0.006	0.723	0.032	0.042	0.025	0.474
10 Day	0.009	0.66	$2.6x10^{-4}$	0.942	$4.7x10^{-4}$	0.922
TFF3						
Baseline	0.004	0.785	0.06	0.258	0.250	0.015
3 Day	0.002	0.855	0.143	0.076	0.271	0.011
10 Day	0.010	0.648	0.143	0.075	0.313	0.006

[a] Urinary biomarker concentrations were not normalized to creatinine levels for this analysis.

[b] BUN: Blood Urea Nitrogen; SCr: Serum Creatinine; eGFR: Estimated Glomerular Filtration Rate

KIM-1: Kidney injury molecule-1; TFF3: Trefoil factor 3

Supplemental Table 3.1. Time-Dependent Changes in Normalized Urinary Biomarkers following Cisplatin Infusion[a]

Biomarker (ng/mL)	Baseline (mean ± SD)	P(Ba_e)	Day 3 (mean ± SD)	P(Ba_e)	P(Ba_s)	Day 10 (mean ± SD)	P(Ba_e)	P(Ba_s)
KIM-1								
Early Cycle	1.16 ± 0.94	-	2.18 ± 1.70	0.010	-	2.16 ± 2.42	0.054	-
Subsequent Cycle	2.37 ± 3.63	0.105	2.85 ± 3.07	0.009	0.604	2.22 ± 1.65	0.007	0.844
Calbindin								
Early Cycle	3.77 ± 5.75	-	2.10 ± 2.46	0.181	-	54.2 ± 198.5	0.201	-
Subsequent Cycle	10.1 ± 32.4	0.333	3.35 ± 7.68	0.822	0.299	9.73 ± 13.75	0.047	0.959
TFF3								
Early Cycle	22.5 ± 30.6	-	43.3 ± 49.3	0.074	-	32.4 ± 32.5	0.260	-
Subsequent Cycle	25.1 ± 22.6	0.731	51.1 ± 65.9	0.050	0.062	49.0 ± 86.8	0.148	0.179

[a]Data were normalized to urinary creatinine concentrations.

Ba_e: Compared to early baseline; Ba_s: Compared to subsequent baseline

KIM-1: Kidney injury molecule-1; TFF3: Trefoil factor 3

Supplemental Table 3.2. Effect of Sex on Absolute Biomarker Levels During Subsequent Cycles

Biomarker (ng/mL)	Baseline Mean ± SD		P	3-Day Mean ± SD		P	10-Day Mean ± SD		P
Sex	Male	Female		Male	Female		Male	Female	
KIM-1	1.76 ± 1.91	3.09 ± 6.88	0.488	2.62 ± 3.71	1.66 ± 1.58	0.495	1.76 ± 2.16	2.00 ± 1.00	0.097
Calbindin	13.1 ± 16.9	3.15 ± 3.72	0.021	8.91 ± 16.4	2.51 ± 4.19	0.043	24.2 ± 30.4	9.21 ± 13.1	0.076
TFF3	21.0 ± 18.4	20.6 ± 31.4	0.280	34.0 ± 42.9	25.5 ± 34.7	0.402	27.2 ± 34.8	26.7 ± 34.9	0.650

[a] Urinary biomarker concentrations were not normalized to creatinine levels for this analysis.

KIM-1: Kidney injury molecule-1; TFF3: Trefoil factor 3

Supplemental Table 3.3. Effect of Age on Absolute Biomarker Levels During Subsequent Cycles

	Baseline		3-Day		10-Day	
Biomarker	R	P	R	P	R	P
KIM-1	0.030	0.388	0.064	0.204	0.147	0.048
Calbindin	0.002	0.829	0.049	0.266	0.087	0.135
TFF3	0.001	0.857	0.011	0.602	0.025	0.429

[a] Urinary biomarker concentrations were not normalized to creatinine levels for this analysis.

KIM-1: Kidney injury molecule-1; TFF3: Trefoil factor 3

Supplemental Table 3.4. Effect of BMI on Absolute Biomarker Levels During Subsequent Cycles

	Baseline		3-Day		10-Day	
Biomarker	R	P	R	P	R	P
KIM-1	0.087	0.135	$9.5x10^{-9}$	0.999	0.018	0.504
Calbindin	0.003	0.778	0.013	0.571	0.011	0.608
TFF3	0.189	0.023	$2.5x10^{-4}$	0.938	0.004	0.767

[a]Urinary biomarker concentrations were not normalized to creatinine levels for this analysis.

BMI: Body mass index; KIM-1: Kidney injury molecule-1; TFF3: Trefoil factor 3

CHAPTER 4: REGULATION OF RENAL CALBINDIN EXPRESSION IN MICE WITH CISPLATIN NEPHROTOXICITY

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4.1 Abstract

Calbindin is a cytosolic calcium-binding protein localized to the distal tubules and collecting ducts of the nephron. Since the discovery of calbindin protein release into the urine after kidney injury, there has been growing interest in using calbindin as an early and sensitive biomarker of nephrotoxicity. Very little is known about the intrarenal regulation of calbindin release and expression during kidney injury. We sought to characterize the time-dependent regulation of renal calbindin during cisplatin nephrotoxicity, a well-established model of kidney injury. Urine, plasma, and kidneys were collected at 24-hour time-points for 4 days from vehicle and cisplatin-treated (20 mg/kg, i.p.) male C57BL/6 mice. Preceding changes in traditional biomarkers (serum creatinine and bloor urea nitrogen), urinary calbindin and Kidney injury molecule-1 (Kim-1) concentrations were elevated by 11.6-fold and 2.5-fold, respectively by day 2. Traditional markers of renal injury, serum creatinine and blood urea nitrogen, increased in cisplatin-treated mice by day 3, confirming the presence of acute kidney injury. Concurrently, time-dependent decreases in intrarenal calbindin protein was observed on days 3 and 4. Additionally, a 200-fold upregulation of calbindin (*Calb1)* and *Kim-1* mRNA were seen on day 3. These data suggest that early loss of calbindin protein into the urine along with declines in renal calbindin levels initiate a compensatory induction of mRNA expression at later time points (days 3 and 4). Taken together, understanding the regulation of renal calbindin protein during cisplatin nephrotoxicity further enhances the utility of calbindin as a potential urinary biomarker of kidney damage.

Keywords: calbindin, cisplatin, kidney injury molecule, nephrotoxicity, acute kidney injury

Abbreviations: Acute kidney injury (AKI), plasma membrane Ca2+ ATPase 1 (PMCA1), sodium-calcium exchanger (NCX1), transient receptor potential cation channel subfamily M member 6 (TRPM6), transient receptor potential cation channel subfamily V member 5 (TRPV5), Vitamin D Receptor (VDR)

4.2 Introduction

Calbindin-D28k is a 28 kDa cytosolic calcium-binding protein localized to the distal tubules and collecting ducts in the kidneys of rodents, sheep, and humans (Palviainen et al. 2012, Iida et al. 2014). Calbindin protein is also present in high concentrations in the intestines, brain, and pancreas (Sooy et al. 2000). Initially identified as a vitamin D responsive protein in the chick intestine (Noda et al. 1978), the vitamin D-dependent regulation of calbindin was confirmed in other species including rodents. Administration of 1,25-dihydroxyvitamin D_3 (active vitamin D) to vitamin D-deficient rats caused an increase in calbindin mRNAs in the intestine and kidneys without changing vitamin D-receptor (VDR) mRNA expression in the same tissues (Huang et al. 1989). Other studies revealed that rat renal calbindin-D28k gene transcription was increased following treatment with 1,25-dihydroxyvitamin D_3 (Varghese et al. 1989). Numerous other factors have been implicated in altering the expression of renal calbindin including parathyroid hormone, sex hormones, calcium, and glucocorticoids (Gill and Christakos 1995, Criddle et al. 1997, Hoenderop et al. 2004, van Abel et al. 2005, Hsu et al. 2010).

At a functional level, calbindin is thought to regulate calcium homeostasis. Calbindin-D28k has four affinity sites for calcium binding that bear a slightly lower affinity for magnesium binding (Berggard et al. 2002, Faas and Mody 2012). Although the exact role of calbindin in the kidney is not known, it does act as a calcium buffer by binding and sequestering free calcium ions and behaves as a sensor by changing conformation upon calcium binding (Berggard et al. 2000). Conformational changes are associated with binding of various signaling proteins to calbindin *in vitro* including *myo*-inositol monophosphatase-1 (Schmidt et al. 2005) and calcium-independent binding of caspase-3 in osteoblasts, suppressing apoptosis (Bellido et al. 2000). Calbindin knockout mice fed a high calcium diet exhibit a 2- to 3-fold increase in urinary calcium without

significant changes in serum calcium, serum or urinary magnesium (Sooy et al. 2000), providing evidence supporting an *in vivo* interaction between calcium and calbindin in the kidneys.

Calbindin was uniquely identified from the urine and kidneys of ketoprofen-treated sheep (Palviainen et al. 2012), leading to further testing as a potential kidney injury biomarker. Calbindin has been increasingly examined as a urinary marker of drug-induced nephrotoxicity for ketoprofen, cyclosporine A, gentamicin, cisplatin and other xenobiotics (Betton et al. 2005, Sasaki et al. 2011, Palviainen et al. 2012, Togashi et al. 2012). Many commercially-available kidney injury multiplex panels include calbindin as a marker of distal tubule injury.

Cisplatin is a chemotherapeutic agent used for treatment of solid tumors. The utility of cisplatin is limited by dose-dependent acute kidney injury (AKI) in animals as well as in up to one-third of oncology patients (Shord et al. 2006). Two clinical studies showed that calbindin protein was elevated 8 to 23-fold from baseline in the urine of patients receiving cisplatin containing chemotherapy even in the absence of elevated serum creatinine (Takashi et al. 1996, George et al. 2017). Further, calbindin was also elevated 7-fold in the serum of these patients (Takashi et al. 1996). In primates, there was a 4.8- to 7.8-fold increase in urinary calbindin following cisplatin administration (Chen et al. 2017). Concentration-dependent increases in calbindin protein in the media and cell lysates have also been reported in HK-2 human kidney proximal tubule epithelial cells treated with cisplatin (Sohn et al. 2013). In several cisplatin-treated rats, there was a 3 to 10-fold increase in urinary calbindin (Sohn et al. 2013, Won et al. 2016). Several nephrotoxicants such as gentamicin (Hoffmann et al. 2010, Sasaki et al. 2011) and ochratoxin A (Hoffmann et al. 2010) also increased urinary calbindin protein in rats.

Taken together, these data suggest that the release of calbindin into urine is elevated in response to cisplatin treatment, however there has been little to no investigation of the intrarenal expression and regulation of calbindin in response to cisplatin-mediated kidney injury. The current study sought to assess time-dependent changes in urinary calbindin excretion and renal expression in cisplatin-treated mice by comparison with the more well-established marker of nephrotoxicity, Kim-1.

4.3. Materials and Methods

Chemicals

Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animal Treatment

Male C57BL/6 mice (9-week old) were purchased from Charles River Laboratories (Wilmington, MA). Cisplatin was dissolved in saline after heating to 50°C. Groups of mice were injected i.p. with 5 mL/kg of saline vehicle or 20 mg/kg of cisplatin after overnight fasting. The doses of cisplatin used in this study were similar to those used clinically (Shord 2006) and are routinely used to induce AKI in mice (Wen 2014). Mice were fed standard diets (Rodent Diet 20, PicoLab, St. Louis, MO) containing 0.81% calcium and Vitamin D_3 2.3 IU/g. Feed was returned 4 hours after cisplatin injection. Mice were placed into metabolism cages for collection of urine and quantification of urinary calbindin, kidney injury molecule-1 (Kim-1), creatinine, and urine output (24-hour periods between 0 and 4 days). Kidney and plasma samples were collected between 2 to 4 days after cisplatin treatment. Tissues were fixed in zinc formalin and stored at room temperature or snap frozen in liquid nitrogen and stored at -80°C.

Serum and Urine Analytes

Blood urea nitrogen (BUN) and serum and urine creatinine levels were quantified as indicators of renal injury (Thermotrace, Melbourne, Australia; Pointe Scientific, Canton, MI). Quantification of calbindin protein in mouse urine and serum was performed using an ELISA kit (Aviva Biosystems, San Diego, CA) following manufacturer recommendations for a dilution of 1:2 for urine and 1:15 for serum. Kim-1 protein concentrations in the urine were also quantified using an ELISA kit (R&D Systems) following manufacturer recommendations with diluted urine (1:15).

RNA Isolation and mRNA Quantification

Total RNA from kidneys was isolated using RNAzol (Sigma-Aldrich). The concentration of total RNA in each sample was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA was generated using the SuperScript first-strand cDNA synthesis kit (Life Technologies). The mRNA expression of mouse calbindin (*Calb1*), *Kim-1*, vitamin D receptor (*VDR*), Na^{+}/Ca^{2+} exchanger (*NCX1*), transient receptor potential cation channel subfamily M member 6 (*TRPM6*), transient receptor potential cation channel subfamily V member 5 (*TRPV5*), and plasma membrane Ca^{2+} ATPase (*PMCA1*) was quantified by quantitative PCR. Quantitative analysis of mRNA was performed with specific forward and reverse primers (Integrated DNA Technologies, Coralville, IA), 1 µg of cDNA, and SYBR Green (Applied Biosystems, Carlsbad, CA) using a ViiA7 RT-PCR system (Applied Biosystems) in 384-well plates. Supplemental Table 4.1 includes primer sequences used for each gene. C_T values were converted to $\Delta\Delta C_T$ values by comparing with a reference gene, beta-actin.

Western Blot Analysis

Kidneys were homogenized in sucrose-Tris buffer (pH 7.5) containing 2% protease inhibitors. Protein concentrations were determined by a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Western blot analysis was performed by loading 10-30 µg of protein and electrophoretically resolved using polyacrylamide gels (4-12% Bis-Tris) and transblotted overnight at 4°C onto polyvinylidine fluoride membrane (MilliporeSigma Burlington, MA). Membranes were blocked with 5% non-fat dairy milk in PBS ith 0.5% Tween-20 for 1 h. The following antibodies were diluted in 2% non-fat dairy milk in PBS with 0.5% Tween-20: calbindin (ab49899, 1:1000), Kim-1 (ab190696, 1:10000), and beta-actin (ab8227, 1:2000) (Abcam Inc, Cambridge, MA). Primary antibodies were probed using anti-rabbit HRP-conjugated secondary antibody (Sigma Aldrich, St. Louis, MO) for 1 h and SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL). Detection was performed with a FluorChem imager (ProteinSimple, Santa Clara, CA) and semiquantified using AlphaView SA version 3.4 (ProteinSimple).

Immunohistochemistry

For immunohistochemistry, tissue was embedded in paraffin and 5 µm thick sections prepared. After deparaffinization, tissue sections were quenched in 3% H_2O_2 (10 min, room temperature). Tissue sections were then blocked with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) followed by 5% serum corresponding to the source of the secondary antibody. After 2 h at room temperature, tissues sections were incubated with primary antibodies to calbindin (ab49899, 1:500; abcam), Kim-1 (AF1817, 1:500, R&D Systems). After 16 h at 4°C, tissue sections were washed and incubated with biotinylated secondary antibodies for 60 min at room temperature (Vector Laboratories). Tissue sections were then stained using a 3,3′-diaminobenzidine

peroxidase substrate kit (Vector Laboratories). After counterstaining with hematoxylin, tissue sections were dehydrated and imaged by light microscopy (VS120-S5, Olympus, Center Valley, PA).

Statistical Analysis

The software program GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA) was used for statistical analysis. Differences among groups were evaluated by Student's unpaired *t*-test (two groups) or one-way analysis of variance followed by Newman-Keuls multiple comparison tests (three or more groups). Statistical significance was set at $P<0.05$.

4.4 Results

Renal Injury

Cisplatin caused time-dependent renal injury in mice. BUN and SCr levels increased 2.8-fold and 1.4-fold, respectively on day 3 compared to saline-treated mice (day 0) (Figure 4.1A). Urinary creatinine and urine output were also significantly increased and decreased, respectively by day 3 (Figure 4.1B). Time-dependent assessment of histopathology has been previously performed and reported in a similar study (Wen 2012). The kidneys displayed progressively worsening levels of injury following cisplatin treatment. On day 2, mice kidneys had minimal (<10% of cells) levels of injury. By day 3 and 4, the kidneys progressed to mild (10-25% of cells) and moderate (25-40% of cells) degrees of injury.

Urinary and Plasma Calbindin

Absolute and creatinine normalized urinary Kim-1 concentrations were significantly increased up to 2.5-fold on days 1 and 2 (Figure 4.2). Concentrations of urinary Kim-1

protein on day 4 were similar to saline-treated mice. On day 2, absolute and creatinine-normalized urinary calbindin concentrations were significantly elevated by 11.6-fold and 7.6-fold, respectively in cisplatin-treated mice compared to saline-treated mice (day 0) (Figure 2). Plasma concentrations of calbindin were significantly decreased 1.9-fold by day 4 in cisplatin-treated mice compared to saline-treated mice (day 0) (Figure 4.2).

Intrarenal Expression of Calbindin Protein

The time-dependent expression of calbindin and Kim-1 proteins in total kidney homogenates were compared (Figure 4.3). Kim-1 protein expression increased in a time-dependent manner with a maximal elevation of 1.7-fold compared to saline-treated control mice on day 4. In contrast, calbindin protein expression decreased in cisplatin-treated mice with maximal loss (60% of control levels) on day 4.

Immunohistochemical Localization of Calbindin

Immunohistochemical analysis revealed Kim-1 staining was not detected in saline-treated mice kidney (Figure 4.4). Kim-1 staining was strongest on day 4 and overlapped with dilated and sloughing proximal tubules. In contrast, calbindin staining appeared in the cytoplasm of distal tubules and collecting ducts in both saline and cisplatin-treated mice kidney (Figure 4.4). Staining was observed both in the cortical and medullary regions of the kidney. There was large variability within each group and inconsistent staining throughout tissue sections and therefore no trend for time-dependent differences in calbindin staining was observed in cisplatin-treated mice.

Intrarenal mRNA Expression of Calbindin and Related Genes

Calb1 and *Kim-1* mRNA expression in kidneys exhibited time-dependent increases on days 3 and 4, with no notable change on day 2 (Figure 4.5). *Kim-1* mRNA expression

was increased 250-fold on day 3. *Calb1* mRNA was significantly upregulated by 200-fold on day 3 compared to saline-treated control mice. *VDR* mRNA expression was lowest in cisplatin-treated mice on day 2 compared to saline-treated mice and remained down-regulated through 4 days. The mRNA expression of calcium and magnesium channels were also quantified (Figure 4.5 and Supplemental Figure 4.2). Expression of the apical calcium import channel *TRPV5* was increased 1.5-fold compared to saline-treated mice on day 3. In contrast, mRNA expression of basolateral calcium export channels, *NCX1* and *PMCA1* were either downregulated (maximally 80% of control) or unchanged, respectively in cisplatin-treated mice on days 2 through 4. The apical magnesium channel *TRPM6* was also maximally downregulated (70% of control) on day 3 in cisplatin-treated mice compared to saline-treated mice.

4.5 Discussion

Due to the growing interest in novel biomarkers for kidney injury and the sensitive detection of calbindin protein in the urines of oncology patients receiving cisplatin, we sought to investigate the time course of expression of calbindin in mice in order to understand its regulation during nephrotoxicity. Following one dose of cisplatin (20 mg/kg), mice exhibited time-dependent elevations in injury markers including increased serum analytes (BUN and SCr), decreased urinary output, and increased urinary creatinine concentrations. Additionally, Kim-1 protein and mRNA, a well-characterized kidney injury biomarker, were up-regulated in the kidneys as well as in the urine, providing a key comparison for calbindin regulation.

Calbindin protein was elevated in mouse urine by day 2 following cisplatin administration. This is consistent with previous reports in cisplatin-treated Sprague-Dawley rats (Sohn et al. 2013, Won et al. 2016), cynomologous monkeys (Chen et al.

2017) as well as oncology patients (Takashi et al. 1996, George et al. 2017). However, two studies have reported a decrease in urinary calbindin protein following cisplatin (6-7 mg/kg) administration in Sprague-Dawley rats and WKY rats (Togashi et al. 2012, Iida et al. 2014). Though, in both studies the decrease in urinary calbindin did not display a time-dependent response, as there was a slight increase or no change in urinary calbindin concentration on days 2-3. Additionally, the time-point of decrease in urinary calbindin (day 5, 8) followed histopathological changes and therefore may not reflect the acute injury response. In contrast, the two studies showing elevated urinary calbindin secretion in Sprague-Dawley rats used higher doses of cisplatin (10-20 mg/kg) and revealed peak elevations around day 3 with no further elevation on day 5 (Sohn et al. 2013, Won et al. 2016). The decrease in serum calbindin seen in our study opposes previous reports in Sprague-Dawley rats (Togashi et al. 2012) and oncology patients (Takashi et al. 1996). Given the lack of studies assessing calbindin regulation to cisplatin-induced AKI in various species and an absence of mechanistic understanding of calbindin, it is currently difficult to ascertain whether the contrasting reports of serum calbindin are due to species differences or the design of studies.

To date, there are no studies quantifying the *in vivo* renal expression of calbindin protein following cisplatin-induced injury. In our study, we quantified calbindin expression and demonstrated for the first time a time-dependent decrease in total kidney calbindin expression compared to saline-treated mice. Immunohistochemical localization of calbindin in this study was consistent with findings from other reports. Calbindin was found to be localized to distal tubules and collecting ducts in rat and sheep (Palviainen et al. 2012, Iida et al. 2014). One previous study determined calbindin protein levels by immunohistochemical expression in the kidney following cisplatin induced kidney injury (Iida et al. 2014). In WKY rats administered 6 mg/kg of cisplatin, pathological changes

were identified mostly in the proximal tubule regions, which did not overlap with calbindin staining regions. In our study, there was large variability in calbindin staining within control and cisplatin-treated groups with no correlation between high Kim-1 and calbindin expression, making time-dependent or treatment-dependent changes difficult to identify. However, in the study by Iida et. al., the overall immunohistochemical staining showed a decreased expression of calbindin protein in the kidneys of cisplatin nephropathy rats, in alignment with our western blot results. These data suggest the potential origin of urinary calbindin to be from distal tubules in the kidney, particularly in response to cisplatin administration.

The degree and timing of mRNA induction of *Calb1* and *Kim-1* were similar following cisplatin administration. The induction of *Calb1* and *Kim-1* mRNAs on day 3 occurred alongside histopathological and serum analyte changes, indicating a response to kidney injury. While the mRNA changes in *Kim-1* coincided with an induction in protein expression, upregulation of calbindin mRNA did not result in increased protein levels at the time-points included in the study. The upregulation of *Calb1* mRNA is likely a compensatory or adaptive response to the decrease in tissue levels following continued loss into the lumen of the nephron. Although the urinary calbindin protein concentrations were attenuated on days 3 and 4 compared to day 2, they were still higher than baseline, indicating continued removal of renal calbindin into the urine.

Calbindin kidney expression is known to be regulated by vitamin D in rodents (Huang et al. 1989). Since vitamin D regulation of calbindin is relatively well-characterized (Ingersoll and Wasserman 1971, Thomasset et al. 1982, Theofan et al. 1986, Varghese et al. 1989, Gill and Christakos 1993), we sought to reveal changes in *VDR* mRNA as well as several known downstream target genes including sodium-calcium exchanger

(*NCX1*), transient receptor potential cation channel subfamily V member 5 (*TRPV5*), transient receptor potential cation channel subfamily M member 6 (*TRPM6*), and plasma membrane Ca^{2+} ATPase 1 (*PMCA1*). Localization of the proteins encoded by these genes in the distal tubule and their role in Ca^{2+} reabsorption is shown in Supplemental Figure 4.2 (adapted from (Woudenberg-Vrenken et al. 2009)). Unexpectedly, only TRPV5 was modestly up-regulated on day 3, while mRNA levels of the other genes were decreased or unchanged. Although this does not preclude vitamin D receptor involvement at the protein level, the down-regulation of various related downstream target genes suggests a more complicated mechanism of calbindin regulation. Interestingly, mice kidneys showed a tendency for TRPV5 and calbindin to colocalize (Lambers et al. 2006). Additionally, calbindin has been shown to bind TRPV5 in renal cell culture studies (Lambers et al. 2006). Cells lacking TRPV5 also impeded the translocation of calbindin to the plasma membrane fraction of cells (Lambers et al. 2006). Knockdown of *TRPV5* caused a down-regulation of calbindin mRNA in naïve mice (Renkema et al. 2005). Finally, various hormones such as parathyroid hormone (van Abel et al. 2005), vitamin D (Hoenderop et al. 2002), and testosterone (Hsu et al. 2010) elicited the same response from both TRPV5 and calbindin mRNA and protein, suggesting coordinated regulation. The concurrent mRNA up-regulation of the apical transporter *TRPV5* and the calcium-binding protein calbindin alongside the down-regulation of *NCX1* on the basolateral surface suggest cells are adapting to restore calcium intracellular concentrations. Further studies to determine urinary, intracellular, or serum calcium concentrations in this model would help to inform this hypothesis.

In summary, we demonstrate a time-dependent increase in calbindin urinary protein coinciding with a decrease in intrarenal calbindin protein expression and an up-regulation of calbindin mRNA in a mouse model of cisplatin-induced kidney injury.

Although these data support the utility of urinary calbindin as a cisplatin-induced kidney injury marker and suggest distal tubules as the source of urinary calbindin, it also raises new questions regarding the transcriptional regulation of calbindin and its role in calcium signaling in the distal tubules during cisplatin-mediated kidney injury.

Supplemental Table 4.1. Primer Sequences for Calbindin and Other Genes (5' → 3')

Gene	**Forward Primer Sequence**	**Reverse Primer Sequence**
CALB1	ACGGAAGTGGTTACCTGGAA	CACACATTTTGATTCCCTGG
VDR	CTCCTCGATGCCCACCACAAGACCTACG	GTGGGGCAGCATGGAGAGCGGAGACAG
KIM-1	ACAGACTGGAATGGCACTGT	AGTATGTACCTGGTGATAGCCAC
PMCA1b	CGCCATCTTCTGCACCATT	CAGCCATTGCTCTATTGAAAGTTC
NCX1	TGGTCTGAAAGATTCCGTGAC	AGTGACATTGCCTATAGACGC
TRPV5	ACGTGCAAGAAGACATGGGG	ACATGCAGTGCTGTCTCTCC
TRPM6	CCTTGGGGAGTCATTGAGAAC	CAGTCCCATCATCACACAGG

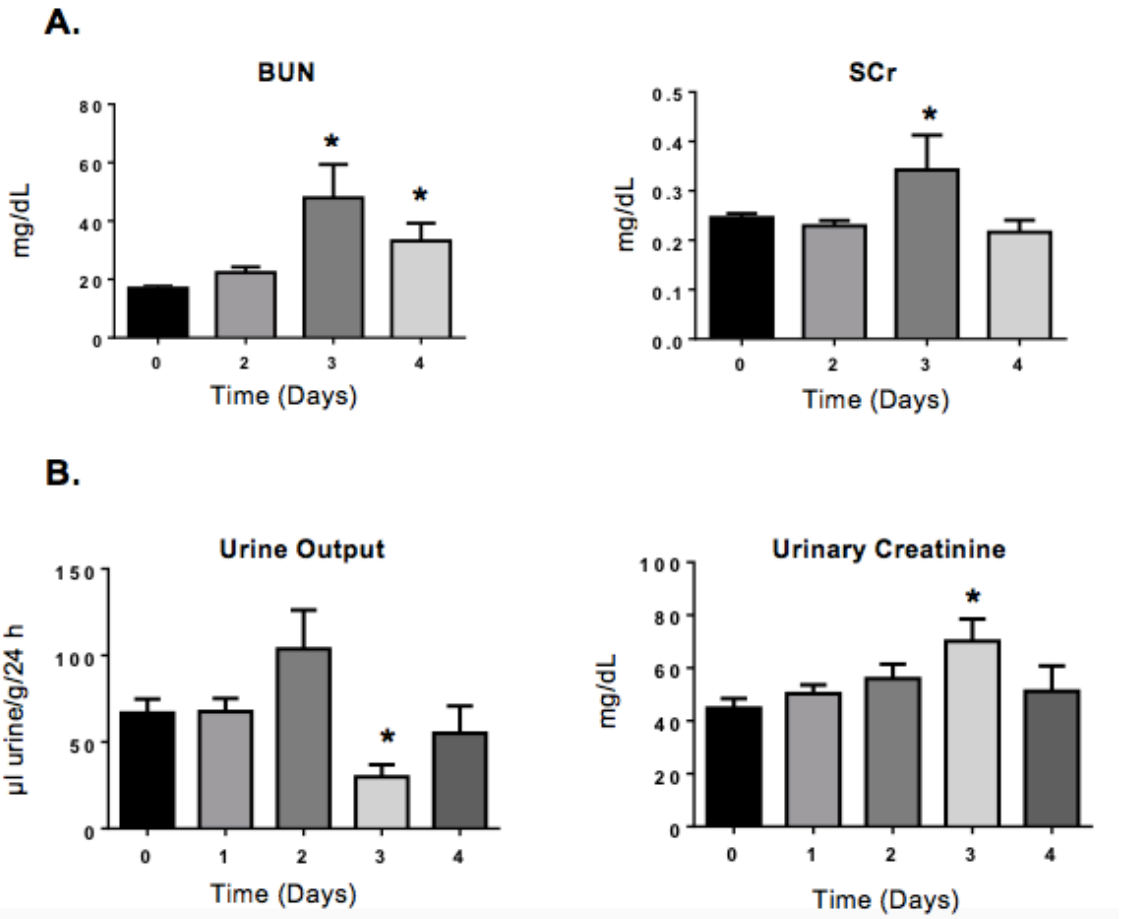


Figure 4.1. Renal Injury Markers in Mice Treated with Cisplatin. (A) Blood urea nitrogen (BUN) and serum creatinine (SCr) concentrations were quantified in C57BL/6 mice between 2 to 4 days after treatment with saline vehicle (day 0, n=15) or cisplatin 20 mg/kg i.p. (n=6 to 11). (B) Urine output and urinary creatinine concentrations were assessed in C57BL/6 mice between 1 to 4 days after treatment with saline vehicle (day 0, n=18) or cisplatin 20 mg/kg i.p. (n=5 to 6). Urine volume was quantified from mice in metabolic cages for 24 hours and was normalized to body weight. Data are presented as means ± SE. *P<0.05, compared to day 0 or saline-treated mice.

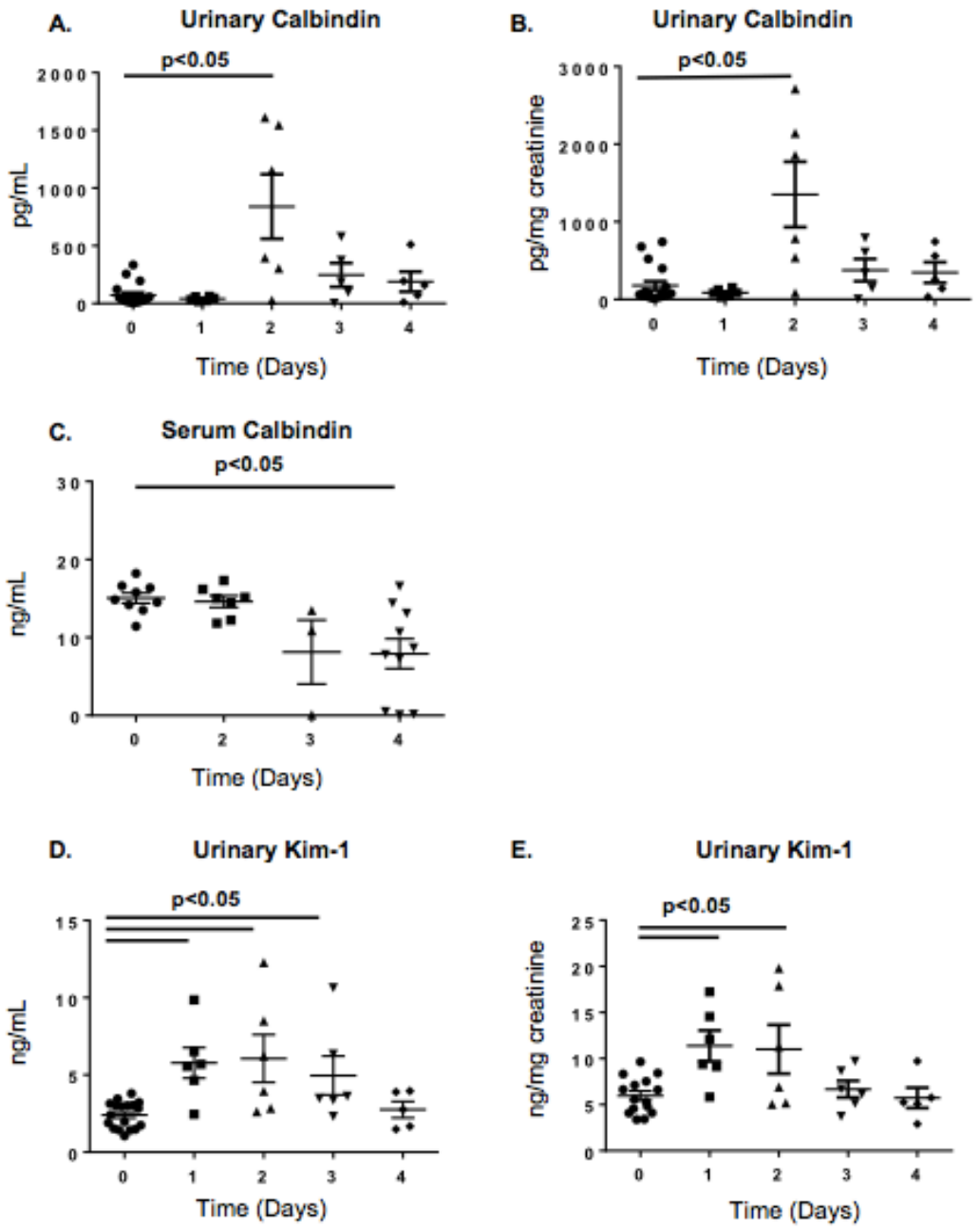


Figure 4.2. Urinary and Plasma Calbindin and Kim-1 Concentrations in Mice Treated with Cisplatin. Urine was collected in 24-hour intervals for 4 days using metabolic cages days 1 through 4 following treatment with saline vehicle (day 0, n=18) or cisplatin 20 mg/kg i.p. (n=5 to 6). A, B, C. Urinary and plasma calbindin concentrations were quantified using an ELISA kit (Aviva Biosystems, San Diego, CA). C. Plasma was collected from saline vehicle-treated (day 0, n=15) and cisplatin-treated

mice on days 2 through 4 (n=6 to 11). D and E. Kim-1 protein concentrations in urines were quantified using an ELISA kit (R&D Systems). B and E. Biomarker values were normalized to urinary creatinine quantified using a colorimetric assay (Pointe Scientific, Canton, MI). Data are presented as individual values and means ± SE. *P<0.05, compared to day 0 or saline-treated mice.

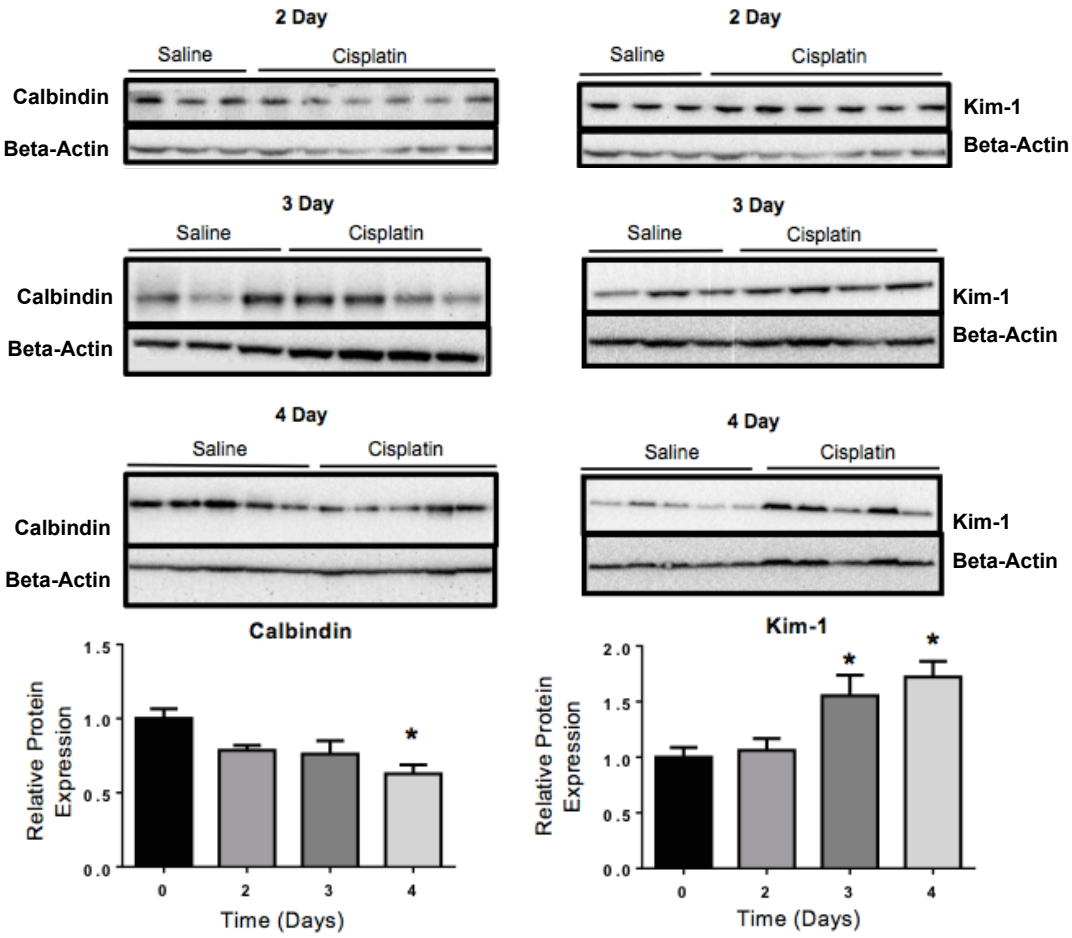


Figure 4.3. Intrarenal Expression of Calbindin and Kim-1 Proteins in Mice Treated with Cisplatin. Expression of calbindin and kidney injury molecule-1 (Kim-1) proteins in kidney from C57BL/6 mice treated with saline vehicle or cisplatin 20 mg/kg i.p. were assessed on days 2 through 4 using western blotting. Beta-actin was used as a loading control. Protein expression was semi-quantified, normalized to saline-treated mice and loading control and presented as means ± SE. *P<0.05, compared to saline-treated mice.

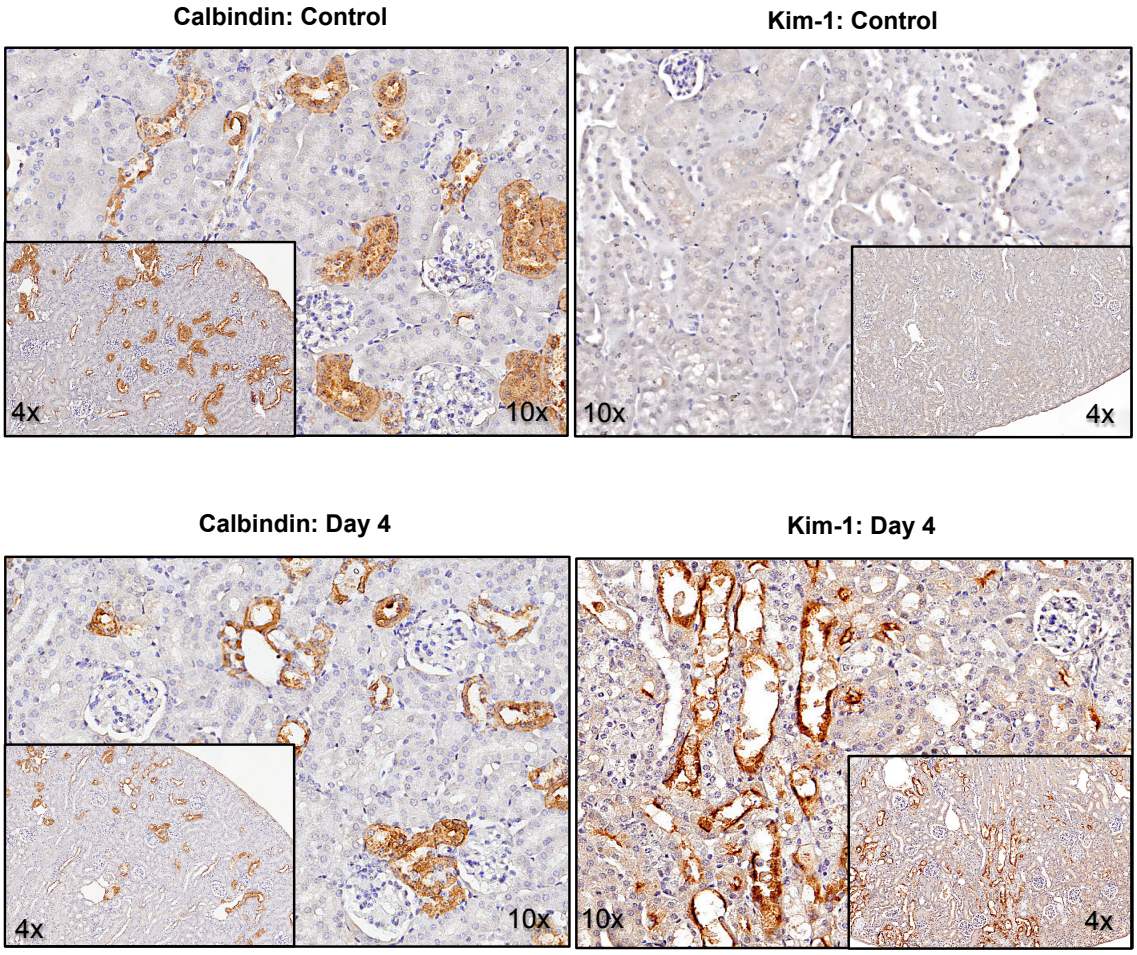


Figure 4.4. Immunohistochemical Localization of Calbindin and Kim-1 Proteins in Mice Treated with Cisplatin. Kidneys collected from saline and cisplatin (20 mg/kg) treated mice on day 4 were fixed in zinc formalin and then subjected to routine tissue processing and paraffin embedding. Sections (5 μm) were prepared and stained with antibodies against calbindin and Kim-1 as indicated in the Materials and Methods. Antibody binding was visualized using a Vectastain DAB kit (*brown staining*) and counterstained with hematoxylin (*blue staining*). Original magnification, x40.

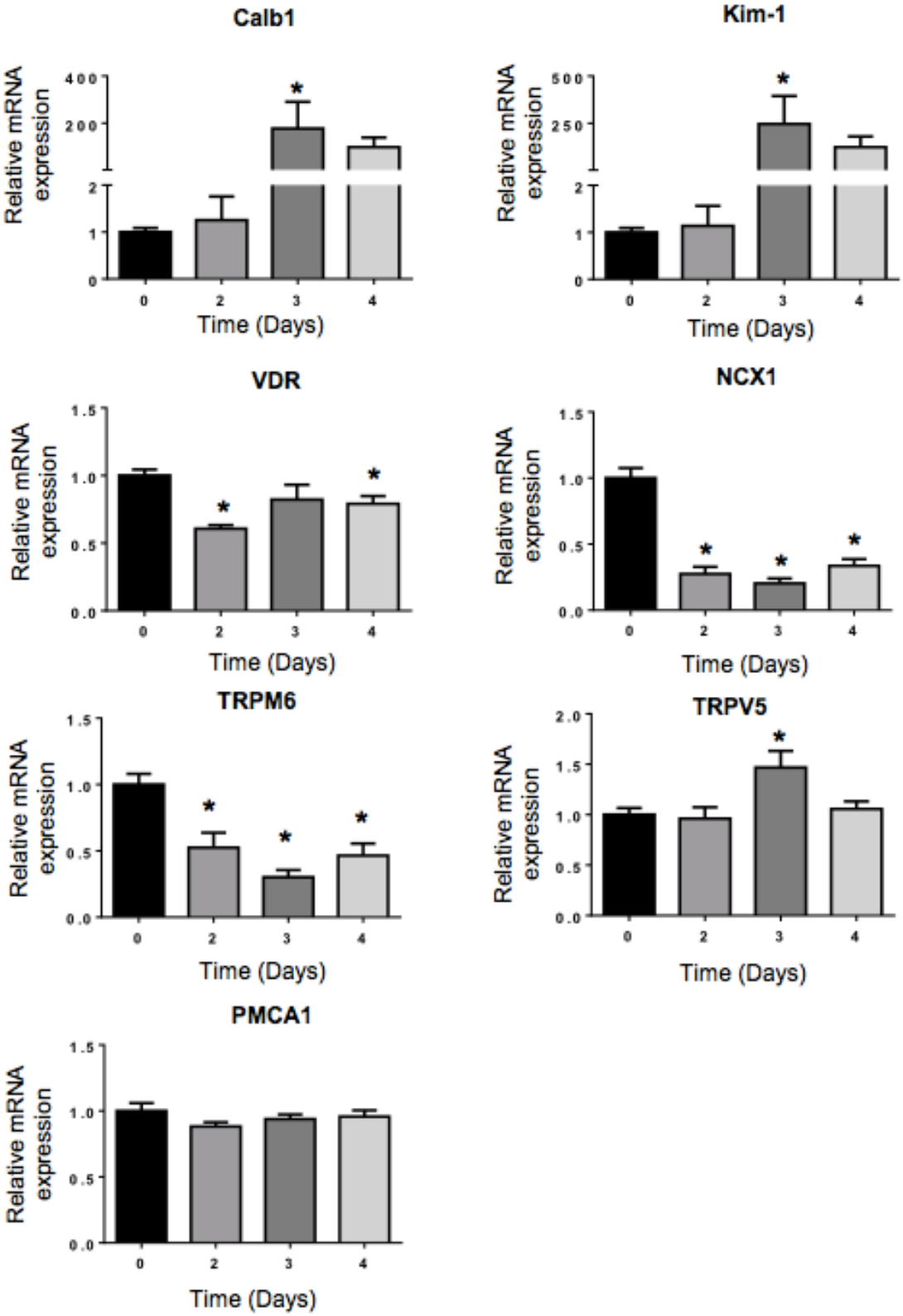
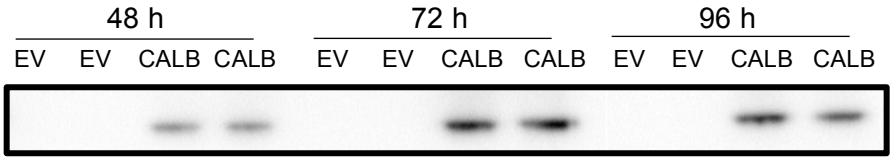
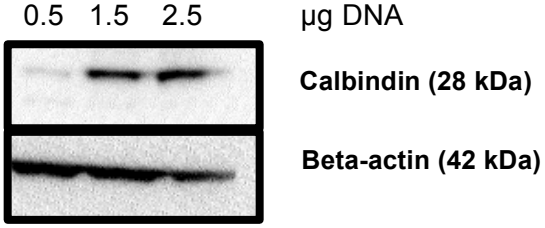

Calb1
Relative mRNA expression
400
200
2
1
0
*
0
2
3
4
Time (Days)
Kim-1
Relative mRNA expression
500
250
2
1
0
*
0
2
3
4
Time (Days)
VDR
Relative mRNA expression
1.5
1.0
0.5
0.0
*
*
0
2
3
4
Time (Days)
NCX1
Relative mRNA expression
1.5
1.0
0.5
0.0
*
*
*
0
2
3
4
Time (Days)
TRPM6
Relative mRNA expression
1.5
1.0
0.5
0.0
*
*
*
0
2
3
4
Time (Days)
TRPV5
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
*
0
2
3
4
Time (Days)
PMCA1
Relative mRNA expression
1.5
1.0
0.5
0.0
0
2
3
4
Time (Days)

Figure 4.5. mRNA Expression of Calbindin and Related Genes in Kidneys of Mice Treated with Cisplatin. mRNA expression of calbindin (*Calb1*), kidney injury molecule-1 (*Kim-1*), vitamin D receptor (*VDR*), sodium-calcium exchanger (*NCX1*), Transient receptor potential cation channel subfamily V member 5 (*TRPV5*), Transient receptor potential cation channel subfamily M member 6 (*TRPM6*), and plasma membrane Ca^{2+} ATPase 1 (*PMCA1*) was quantified using total kidney RNA from vehicle-treated control (day 0, n=14) and 20 mg/kg cisplatin-treated mice between 2 and 4 days (n=5 to 16). Data are presented as means ± SE. mRNA data were normalized to saline-treated mice and reference gene (beta-actin). *P<0.05 compared to day 0 or saline-treated mice.

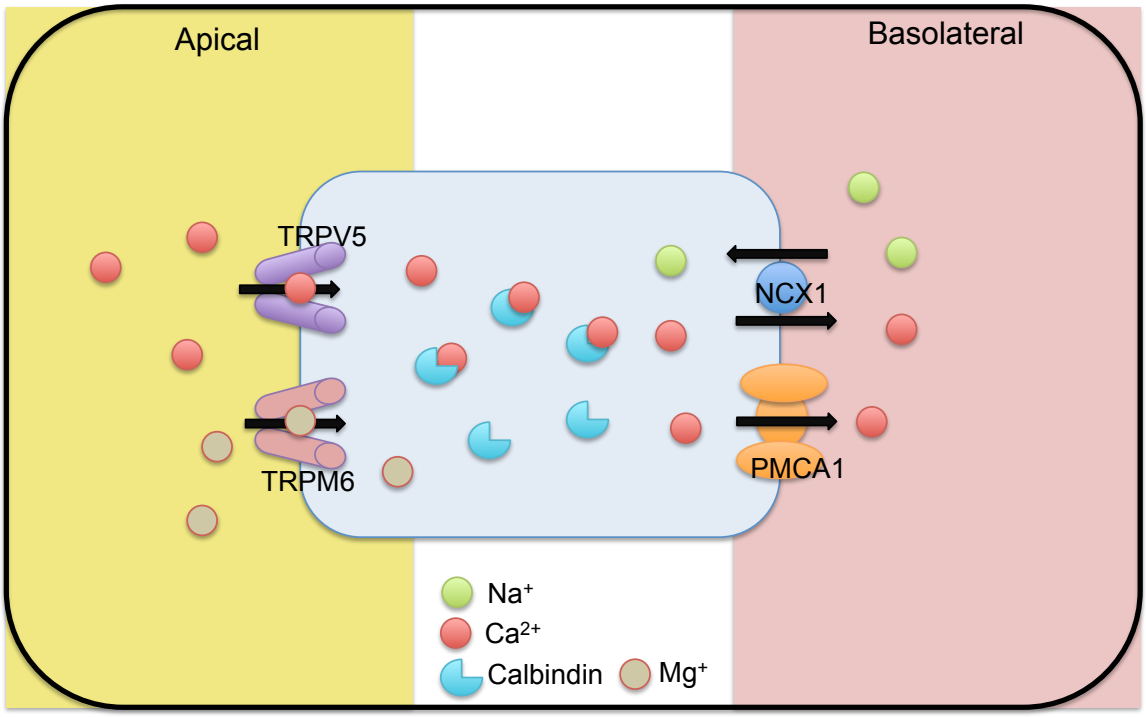
A.



B.



Supplemental Figure 4.1. Modulation of Cisplatin Cytotoxicity in Kidney Cells Expressing Calbindin. HEK293 cells were transiently transfected with empty vector (EV) or full-length human *CALB1* plasmids. (A) Time-dependent and (B) cDNA-dependent protein expression of calbindin in cell lysates was detected by Western blot analysis. Beta-actin was used as a loading control.



Supplemental Figure 4.2. Localization of Calcium Transport Proteins in the Kidney. Sodium-calcium exchanger (NCX1) and plasma membrane Ca^{2+} ATPase 1 (PMCA1) are localized on the basolateral membrane of distal tubules in the kidney and transport calcium into the blood. Transient receptor potential cation channel subfamily V member 5 (TRPV5) and transient receptor potential cation channel subfamily M member 6 (TRPM6) are localized on the apical side of distal tubules in the kidney and transport calcium and magnesium, respectively into the cytoplasm from the lumen. Calbindin protein binds calcium ions and transports them through the cytoplasm. Adapted from (Woudenberg-Vrenken et al. 2009).

CHAPTER 5: IN VITRO INHIBITION OF RENAL CISPLATIN SECRETION TRANSPORTERS BY ANTIEMETIC DRUGS

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5.1. Abstract

Cisplatin is efficacious for the treatment of solid tumors, however up to 30% of patients experience nephrotoxicity. Renal transporters, such as the uptake carrier organic cation transporter 2 (OCT2) and efflux transporter multidrug and toxin extrusion protein 1 (MATE1), regulate proximal tubule cell concentrations of cisplatin. By determining the extent of cisplatin secretion and accumulation, the activity of OCT2 and MATE1 constitute important mechanisms dictating the susceptibility of the kidneys to injury. Cisplatin is also a highly emetogenic drug that requires the concomitant use of 5-HT_3 antagonists to control nausea and vomiting. Initial published reports suggested that ondansetron could inhibit OCT2- and MATE1-mediated transport. The purpose of this study was to test structurally similar 5-HT_3 antagonist drugs for their ability to inhibit the OCT2 and MATE1 transporters. Transport of the fluorescent cation ASP^+ was assessed in HEK293 kidney cells overexpressing human OCT2 or MATE1 and double-transfected MDCK hOCT2-MATE1 cells in the presence and absence of different 5-HT_3 antagonists. The relative order of increasing potency for inhibition of ASP^+ uptake by OCT2 was palonosetron > ondansetron > granisetron > tropisetron > dolasetron and by MATE1 was ondansetron > palonosetron = tropisetron > granisetron > dolasetron. In double-transfected MDCK hOCT2-MATE1 cells, ondansetron (0.5-20 μM) inhibited the transcellular transport of ASP+ up to 64%. Concentrations of 10 and 20 μM of palonosetron, tropisetron, and dolasetron reduced the transcellular transport of ASP^+ up to 80-90%. Granisetron did not alter the transcellular transport of ASP^+ at the tested concentrations (0.5-20 μM). In double-transfected MDCK hOCT2-MATE1 cells, ondansetron (0.5, 2.5 μM) caused a significant intracellular accumulation of ASP^+. The inhibition of MATE1 transport by ondansetron and dolasetron exceeded the 0.02 cutoff for the ratio of maximum serum concentration/inhibitory concentration 50 ($Cmax/IC_{50}$ 2 and 0.06, respectively) established by the FDA guidance for *In Vitro* Metabolism and

Transporter Mediated Drug-Drug Interaction Studies, which supports further investigation of potential drug-drug interactions. Inhibition of MATE1-mediated efflux of cisplatin from human proximal tubule cells by concomitantly administered antiemetic drugs is likely an important mechanism that can influence susceptibility to nephrotoxicity.

5.2. Introduction

Cisplatin (*cis*-diamminedichloroplatinum) is one of a handful of anticancer agents approved by the FDA for the treatment of solid tumors and continues to be a mainstay of chemotherapy regimens due to its high efficacy (>90% remission rates). However, nephrotoxicity is a major dose-dependent side effect that results in the development of acute kidney injury (AKI) in up to one-third of patients treated with high doses (>50 mg/m^2), although changes in renal function have been observed at low doses as well (Shord et al. 2006). Furthermore, higher doses of cisplatin therapy are needed to overcome the increasing rate of drug resistance in cancer cells. Small but permanent changes to kidney function can occur with long-term cisplatin treatment despite preventative treatments including hydration (Latcha et al. 2016). Cisplatin-induced AKI may also force clinicians to switch to less effective anticancer agents in order to prevent chronic kidney disease.

Kidney epithelial cells take up more cisplatin from the blood compared to other cells due to the presence of high capacity membrane transporters such as the organic cation transporter 2 (OCT2) (Filipski et al. 2008). Cisplatin and its glutathione conjugates are subsequently excreted into the urine by the multidrug and toxin extrusion 1 (MATE1) and multidrug resistance-associated protein 2 (MRP2) transporters (Nakamura et al. 2010, Wen et al. 2014). Using human kidney cells transfected with the various transporter genes, we have shown that cisplatin increases cytotoxicity 3.5-fold in cells expressing OCT2 and reduces injury 3- to 4-fold in cells expressing MATE1 and MRP2 proteins, respectively compared to control cells (unpublished data). Consistent with these *in vitro* data, Oct1/2-null mice are protected from cisplatin nephrotoxicity, whereas Mate1-null mice and Mrp2-null mice exhibit enhanced kidney injury (Filipski et al. 2009, Nakamura et al. 2010, Wen et al. 2014). Clinically, a non-synonymous single-nucleotide

polymorphism (SNP, rs316019) in the *OCT2*/*SLC22A2* gene was associated with a reduced incidence of nephrotoxicity in patients receiving cisplatin (Filipski et al. 2009). It is thought that OCT2 and MATE1 work in concert to secrete the parent cisplatin (or hydrated forms), while MRP2 excretes the glutathione and toxic thiol conjugates of cisplatin. The OCT2/MATE1 cationic transport system has been previously implicated in the occurrence of other drug-drug interactions within the kidneys (Wang et al. 2008, Matsushima et al. 2009, Nies et al. 2011). However, there has been little investigation of cisplatin-drug interactions at the level of OCT2/MATE1 inhibition.

Cisplatin is also a highly emetogenic medication requiring the rigorous use of medications to control nausea and vomiting. Clinical guidelines recommend the use of three different classes of medications: 5-hydroxytryptamine (5-HT_3) antagonists, neurokinin 1 (NK_1) antagonists, and a corticosteroid. Development of selective 5-HT_3 antagonists has dramatically improved the treatment of nausea and vomiting in cancer patients (Jordan et al. 2007). Cytotoxic drugs such as cisplatin cause the release of serotonin from mucosal enterochromaffin cells in the gut, which then binds to and stimulate 5-HT_3 receptors on the vagal nerves (Smith et al. 2012). 5-HT_3 antagonists prevent the binding of serotonin to 5-HT_3 receptors. There are many drugs that currently belong to the 5-HT_3 antagonist class, varying in generic availability, potency, as well as duration of action. Initial reports suggest that the first two approved 5-HT_3 antagonists ondansetron and granisetron, can inhibit OCT2- and MATE1-mediated transport (Kido et al. 2011, Li et al. 2013, Wittwer et al. 2013). 5-HT_3 antagonists all tend to be cationic and structurally similar to each other as well as to serotonin, which is especially important for 5-HT_3 receptor antagonism. Additionally, there is a large overlap in OCT2 and MATE1 substrates and inhibitors and increased potency of inhibition for the uptake versus efflux transporter translate to different potentials for cisplatin tubular accumulation and toxicity.

Therefore, we sought to systematically compare the relative ability of existing and newer, more potent 5-HT_3 antagonists as well as other commonly co-administered antiemetic drugs to inhibit OCT2 and MATE1-mediated transport.

5.3. Materials and Methods

Chemicals

4-(4-(Dimethylamino)styryl)-N-methylpyridinium iodide (ASP^+) was purchased from Life Technologies (Grand Island, NY). Tropisetron was purchased from Abcam (Cambridge, MA) and aprepitant from Fisher Scientific (Pittsburgh, PA). All other chemicals are from Sigma-Aldrich (St. Louis, MO).

Cell Lines and Cell Culture

Empty Vector (EV) control (pcDNA5-transfected) and Flp-In human embryonic kidney (HEK)293 cell lines stably expressing human MATE1 and OCT2 transporters were generously provided by Dr. Kathy Giacomini at the University of California, San Francisco. Flp-In HEK-293 cell lines stably expressing human MRP2 or an EV plasmid were generated previously in our lab using pcDNA5/FRT vector with or without a *ABCC2* insert (Wen et al. 2017). HEK293 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM $_L$-glutamine, 100 U/mL penicillin, 100 *μ*g/mL streptomycin, and 200 *μ*g/mL hygromycin B. Vectors (pcDNA3.1+ and pcDNA3.1/Hygro(+)), and hOCT2/hMATE1 double-transfected Madin-Darby canine kidney (MDCK) cell lines were generously provided by Dr. Joanne Wang at the University of Washington, Seattle, WA. MDCK cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 500 μg/mL G418 and 200 μg/mL hygromycin B. All cell lines were cultured in a humidified incubator at 37°C with 5% CO_2.

Uptake and Efflux Inhibition Assays in HEK293 Cells

OCT2- and MATE1-overexpressing HEK293 cells were seeded in clear poly-^{D}-lysine-coated 24-well plates (Fisher Scientific, Hanover Park, IL) and grown for 24 hours until approximately 90% confluent. After washing once with pre-warmed Hank's Buffered Saline Solution (HBSS), cells were pre-incubated for 30 minutes at 37°C with various 5-HT_3 antagonists for OCT2 cells or in a 30 mM NH_4Cl solution in HBSS at pH 6.5 for MATE1 cells for intracellular acidification. Uptake into OCT2 cells was initiated by exposure to 10 μM of fluorescent substrate ASP^+ directly in the incubation media. Uptake into MATE1 cells was initiated by application of HBSS at pH 7.4 containing 5-HT_3 antagonists and 10 μM of fluorescent substrate ASP^+. After incubating for 1 min at 37°C on a shaker, substrate uptake was stopped by adding ice-cold HBSS containing 500 μM cimetidine. Media was removed and washed four times with ice-cold HBSS. Cells were lysed with 1% Triton X-100. Fluorescence was detected using Spectramax Microplate Reader (Molecular Devices, Sunnyvale, CA) at the following wavelengths (Excitation 485 nm/Emission 495 nm). Intracellular fluorescence was normalized to total protein concentration of cell lysates from each well using the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). Experiments were repeated three separate times with 3-4 replicates in each experiment.

MRP2-overexpressing and control EV cells were treated with Calcein AM (1 μM) with or without 5-HT_3 antagonists (10 and 50 μM) or positive control, MK-571 (100 μM) for 15 minutes (*uptake phase*). Cells were incubated with or without 5-HT_3 antagonists (10 and 50 μM) or positive control, MK-571 (100 μM) for 1 hour (*efflux phase*). Following incubation, intracellular fluorescence was determined using a Cellometer Vision (Nexcelom Bioscience, Lawrence, MA). Data represent 4 replicates from one experiment.

Transwell Studies in MDCK-hOCT2/hMATE1 Cells

Control and hOCT2/hMATE1-expressing MDCK cells were seeded on 0.4 μm transwell inserts (VWR, Radnor, PA) at a density of 2 x 10^5 cells/cm^2. Transport experiments were performed 3 to 5 days after seeding. The integrity of MDCK monolayers was verified by measuring transepithelial electrical resistance (TEER) >150 Ω*cm^2 using an epithelial voltohmmeter, EVOM^2 (World Precision Instruments, Sarasota, FL). Proper formation of tight junctions was also verified by measurement of passive permeability of lucifer yellow in the basolateral-to-apical (B-to-A) direction. Lucifer yellow (20 μM) was applied to the basolateral chamber for 1 hour and media were collected from the apical chamber. Lucifer yellow fluorescence was read at an excitation wavelength of 430 nm and emission wavelength of 538 nm. Average passive permeability (P_{app}) values were 7x10^{-7} cm/s, which consistent with literature values (Avdeef 2010).

After washing once with Hank's Buffered Saline Solution (HBSS) pH 7.4, transport studies were initiated after aspirating the wash buffer from both apical and basal chambers. Cells were incubated with 5-HT_3 antagonists in the apical chamber in HBSS pH 6.0 and 5-HT_3 antagonists with ASP^+ (25 μM) in the basolateral chamber in HBSS pH 7.4 and incubated at 37°C for 120 min. To measure time-dependent transcellular transport, an aliquot of the incubation medium (100 μL) from the apical chamber (receiving chamber) was collected periodically (40, 60, 90, 120 min) and replaced with equal volume of fresh buffer containing 5-HT_3 antagonist or positive control chemical at the original concentration. After 120 min, treatment media was removed and Transwells were washed three times with ice-cold HBSS. Cells were lysed with 1% Triton X-100. Fluorescence was detected using a Spectramax Microplate Reader at the following wavelengths (Excitation 485 nm/Emission 495 nm). Intracellular fluorescence was

normalized to total protein concentration of cell lysates from each transwell using the BCA assay. Experiments were performed in three individual Transwell apparatuses.

Statistical Analysis

GraphPad Prism v6 (GraphPad Software, La Jolla, CA) was used for statistical analysis. K_m and V_{max} were calculated using nonlinear regression (Michaelis-Menten enzyme kinetics equation, (Y = Vmax*X/(Km + X), fit for least squares). Groups of 3 or more were analyzed with two-way ANOVA and 2 or more were analyzed with one-way ANOVA and Dunnett's post-hoc test for multiple comparisons. IC_{50} values were calculated by a nonlinear regression to fit least squares. Differences were considered statistically significant at $p < 0.05$.

5.4. Results

Characterization of ASP^+ as a Fluorescent Substrate in HEK293 Cells Overexpressing OCT2 and MATE1

Uptake of ASP^+ in cells overexpressing OCT2 or MATE1 was characterized and optimal experimental conditions were selected including duration of uptake and concentration of ASP^+ (Figure 5.1). ASP^+ displayed time-dependent (Figure 5.1 A and B) and concentration-dependent (Figure 5.1 C) uptake in both OCT2- and MATE1-expressing cells and exhibited saturable kinetics (V_{max} for OCT2: 8.1 nmol/mg/min; V_{max} for MATE1: 3.4 nmol/mg/min). EV cells exhibited minimal uptake of ASP^+ when tested for time-dependent (Figure 5.1 A and B) and concentration-dependent (Figure 5.1 C) accumulation. One minute was selected to perform the uptake assays based upon the linear range of OCT2 and MATE1 transport with minimal accumulation in EV cells (Figure 5.1 A and B). An ASP^+ concentration of 10 μM was selected from the linear

range of uptake for OCT2 and MATE1 and provided sufficient sensitivity for fluorescence readings (Figure 5.1 C).

To ensure these conditions reflected active transport by each transporter the IC_{50} values of cimetidine, a well-established OCT2 and MATE1 inhibitor, were determined (Figure 5.2 and Table 5.1). The IC_{50} for cimetidine was 24.5 ± 4.0 μM in OCT2-expressing cells and 0.23 ± 0.2 μM in MATE1-expressing cells, in agreement with published data showing inhibition of MATE1 at lower concentrations (Kido et al. 2011, Wittwer et al. 2013).

Antiemetic Drug Inhibition of OCT2-, MATE1-, and MRP2- Mediated Transport in HEK293 Cells

Five different 5-HT_3 antagonists (ondansetron, palonosetron, granisetron, tropisetron and dolasetron) were evaluated for inhibition of OCT2 and MATE1 transport in HEK293 cells using ASP^+ as a substrate (Figure 5.2). A concentration-dependent decrease in ASP^+ uptake was observed in OCT2- and MATE1-expressing cells in the presence of all five 5-HT_3 antagonists tested (0 to 50 μM). EV cells did not show significant decreases in ASP^+ uptake with the exception of cells treated with granisetron, which exhibited a minimal decline in ASP^+ concentrations. The IC_{50} values for each 5-HT_3 antagonist and comparison to clinical plasma C_{max} is shown in Table 5.1. With the exception of granisetron, four 5-HT_3 antagonists inhibited MATE1 more potently than OCT2. OCT2-mediated transport was inhibited 72-92% while MATE1-mediated transport was inhibited 56-73% at the concentrations tested.

Concomitantly prescribed drugs for chemotherapy-induced nausea and vomiting in other antiemetic drug classes including aprepitant and dexamethasone were also tested for

inhibition of ASP^+ uptake in OCT2- and MATE1-expressing HEK293 cells (Supplemental Figure 5.1). Concentrations three times the therapeutically relevant plasma C_{max} did not inhibit OCT2- or MATE1-mediated ASP^+ uptake. Alternative antiemetic drugs used for nausea and vomiting including olanzapine, prochlorperazine, and metoclopramide were also tested for inhibition of ASP^+ uptake in OCT2- and MATE1-expressing HEK293 cells. Although none of the three drugs inhibited OCT2- and MATE1-mediated ASP^+ uptake at therapeutically relevant concentrations, all three drugs exhibited dose-dependent inhibition of OCT2 and MATE1-mediated ASP^+ uptake. Interestingly, olanzapine, prochlorperazine, and metoclopramide inhibited OCT2 more potently than MATE1 (25% maximum inhibition of OCT2 vs. 80% maximum inhibition of MATE1 for the most potent antiemetic drug, prochlorperazine).

Additionally, inhibition of MRP2 transport using calcein AM as a substrate in the presence of 5-HT_3 antagonists was also determined (Supplemental Figure 5.2). MRP2-mediated efflux assay was confirmed by testing MK-571, a positive control inhibitor at 100 μM, which caused a 90% reduction in efflux activity. There were no significant changes in the efflux of calcein AM in the presence of ondansetron, palonosetron, granisetron, tropisetron, or dolasetron in MRP2-expressing HEK293 cells.

Characterization of the Transcellular Transport and Intracellular Accumulation of ASP^+ in hOCT2/hMATE1-Expressing MDCK Cells

The protein expression of OCT2 and MATE1 was confirmed in the double transfected MDCK cells using western blotting (Figure 5.3A). The transcellular transport of the cationic probe substrate, ASP^+ (25 μM) was tested in these cells for the first time. The B-to-A transport of ASP^+ was much greater (up to 2.8-fold at 120 minutes) than apical-to-basolateral (A-to-B) transport in the hOCT2/hMATE1 double transfected cells (Figure

5.3B). The B-to-A/A-to-B efflux ratio at 120 minutes was estimated to be 2.7 for hOCT2/hMATE1 cells supporting active secretion of ASP^+. In contrast, control cells exhibited much lower ASP^+ transport in both directions compared to hOCT2/hMATE1 cells. The B-to-A transport of ASP^+ was only significantly higher compared to A-to-B in control cells at 90 (1.3-fold) and 120 minutes (1.7-fold). All further inhibition assays were performed in the B-to-A direction.

The ability of chemicals to inhibit ASP^+ flux and accumulation in hOCT2/hMATE1 double transfected cells at the specified test conditions was assessed using three positive control inhibitors (cimetidine 5 and 50 μM, pyrimethamine 1 μM and olanzapine 20 μM) (Figure 5.3C). Cimetidine is a known inhibitor of OCT2 and MATE1 with higher potency for MATE1 (Table 5.1) (Kido et al. 2011). Pyrimethamine is a MATE1 specific inhibitor (Ito et al. 2010), whereas olanzapine was found to inhibit OCT2 more potently (Supplemental Figure 5.1) (Kido et al. 2011, Wittwer et al. 2013). All three chemicals inhibited the transcellular flux of ASP^+ (18% cimetidine 5 μM, 40%-fold cimetidine 50 μM, 36% pyrimethamine 1 μM, and 28% olanzapine 20 μM at 120 minutes). At 120 minutes, cimetidine 50 μM and pyrimethamine 1 μM increased the intracellular accumulation of ASP^+ by 1.8-fold and 1.3-fold, respectively, whereas olanzapine decreased the intracellular accumulation of ASP^+ by 51%. In control cells, no significant inhibition of B-to-A transport of ASP^+ was observed at any of the time points. In control cells, there were also no significant changes in intracellular accumulation of ASP^+ compared to vehicle control cells, except for a 28% decrease in ASP^+ accumulation in the presence of olanzapine 20 μM. Interestingly, the degree of ASP^+ accumulation was similar in the absence of inhibitors in both control and hOCT2/hMATE1 double transfected cells.

Inhibition of the Transcellular Transport and Modulation of Intracellular Accumulation of ASP^+ in hOCT2/hMATE1-Expressing MDCK Cells

Five different 5-HT_3 antagonists (ondansetron, palonosetron, granisetron, tropisetron, and dolasetron) were evaluated for transcellular and intracellular inhibition of ASP^+ in hOCT2/hMATE1 double-transfected cells (Figure 5.4 and 5.5). Cimetidine (50 μM) was included with every experiment to ensure proper test conditions. Interestingly, all five 5-HT_3 antagonists exhibited varying degrees of inhibition on the transcellular B-to-A transport of ASP^+ (Figure 5.4). Ondansetron inhibited B-to-A transport of ASP^+ in a dose-dependent manner (0.5 – 20 μM) with the maximum 36% inhibition at 20 μM at 120 minutes. Palonosetron and tropisetron also displayed dose-dependent inhibition of ASP+ secretion, which was significant at 10 and 20 μM for all time-points. A maximal inhibition of 24% and 13% was seen at 120 minutes and 20 μM for palonosetron and tropisetron, respectively. Dolasetron at 10 and 20 μM inhibited ASP^+ transport 10-13% at 120 minutes only. Lastly, granisetron did not alter the B-to-A transport of ASP^+ at any concentration tested. The control cells showed no significant inhibition in the B-to-A transport of ASP^+ with any of the 5-HT_3 antagonists (data not shown).

Low concentrations (0.5 and 2.5 μM) of ondansetron resulted in 1.3-fold increase in intracellular ASP^+ accumulation, while there was no difference compared to vehicle at higher concentrations (10 and 20 μM) (Figure 5.5). However, in control cells there was a decrease (40%) in accumulation of ASP^+ at 10 and 20 μM ondansetron. In hOCT2/hMATE1 cells, tropisetron and granisetron increased ASP^+ accumulation (1.5 and 1.3-fold, respectively) at the highest concentration (20 μM). No significant changes in ASP^+ accumulation were observed with palonosetron or dolasetron.

5.5. Discussion

Cisplatin is a substrate for several renal transporters including OCT2, MATEs, and MRP2 (Filipski et al. 2009, Nakamura et al. 2010, Wen et al. 2014). Drugs that inhibit MATEs or OCTs may alter intratubular concentrations of cisplatin leading to nephrotoxic events. Inhibitors of MATE1 and MRP2 would cause drug accumulation in proximal tubule cells thereby enhancing nephrotoxicity, whereas inhibitors of OCT2 would be nephroprotective. A previous study demonstrated that ondansetron enhanced cisplatin nephrotoxicity in mice due to a greater potency for inhibiting MATE1 compared to OCT2 (Li et al. 2013). In recent years, a number of newer antiemetic drugs, especially 5-HT_3 antagonists, have been approved. They exhibit increased potency and efficacy in preventing nausea and vomiting. Currently clinical guidelines recommend the use of a three-drug regimen for highly emetogenic drugs such as cisplatin: a 5-HT_3 antagonist, NK_1 antagonist, and dexamethasone (Jordan et al. 2007, Hesketh et al. 2016). Therefore, in this study we aimed to extend the prior work investigating cisplatin-ondansetron drug interactions to determine whether other antiemetic drugs also interact with renal transporters of cisplatin.

All five 5-HT_3 antagonists demonstrated dose-dependent inhibition of MATE1 and OCT2. The 5-HT_3 antagonist class of drugs tend to be structurally similar and reflect the cationic structure of serotonin in order to effectively antagonize the 5-HT_3 receptor (Tzvetkov et al. 2012). Importantly, the increased potency of inhibition for MATE1 for the majority of 5-HT_3 antagonists suggested the potential for an *increase* in cisplatin accumulation in the tubules and nephrotoxicity. Based on the 2017 draft FDA Guidance for *In Vitro* Metabolism and Transporter-Mediated Drug-Drug Interaction Studies (FDA 2017), a drug has the potential to inhibit the transporter *in vivo* if: the C_{max} (unbound)/IC_{50} value is ≥ 0.1 for OCT2 and ≥ 0.02 for MATEs. Based on these guidelines, the MATE1 C_{max}/IC_{50}

values for ondansetron and dolasetron indicate potential *in vivo* drug interactions (Table 5.1). Furthermore, the OCT2 C_{max}/IC_{50} value for ondansetron and the MATE1 C_{max}/IC_{50} value for palonosetron also have an increased potential depending on the exact plasma C_{max} achieved.

Since the tubular secretion of cisplatin in the human kidney is mediated by the sequential action of the basolateral OCT2 and apical MATE1 transporters, double-transfected hOCT2/hMATE1 cells allow for the simultaneous inhibition of both transporters and the net intracellular accumulation of substrates. The data generated in the hOCT2/hMATE1 double transfected cells largely agree with the HEK293 data with the exception of granisetron. Ondansetron, palonosetron, and tropisetron exhibited dose-dependent inhibition of the B-to-A transport of ASP^+ with the order of potencies reflecting MATE1 inhibition seen in HEK293 cells. Surprisingly, granisetron did not exhibit any significant inhibition in hOCT2/hMATE1 MDCK cells even at concentrations 5 times above the IC_{50} generated with HEK293 cells. Because the MDCK cells resemble native tubular cells to a greater extent than HEK293 cells, there is the potential for the disposition of granisetron to be altered in the MDCK cells due to the expression of endogenous transporters. For example, MDCK cells highly express the canine P-glycoprotein (P-gp) transporter (Goh et al. 2002), which could lead to granisetron efflux. Supporting this speculation is the fact that a single nucleotide polymorphism in the human MDR1/*ABCB1* transporter improved the clinical efficacy of granisetron for treating emesis (Babaoglu et al. 2005). These data suggest that granisetron may be a substrate for canine P-gp, causing an alteration in the intracellular concentration exposed to the MATE1 transporter.

The increased intracellular accumulation of ASP^+ with ondansetron, tropisetron, and granisetron indicates MATE1 inhibition, whereas a lack of change in intracellular ASP^+ as with palonosetron indicates a similar degree of inhibition of OCT2 and MATE1 transport. This observation confirmed the higher potencies for MATE1 inhibition seen in HEK293 cells (Table 5.1). For palonosetron, the IC_{50} values were similar for OCT2 and MATE1. Dolasetron did not exhibit any significant B-to-A inhibition or intracellular accumulation of ASP^+ at the concentrations tested, which were far below the IC_{50} generated in Table 5.1.

Aprepitant and dexamethasone are the two commonly recommended antiemetic drugs concomitantly prescribed with 5-HT_3 antagonists. However, since *in vitro* inhibition was not seen for OCT2 and MATE1 even at concentrations above C_{max}, the predicted *in vivo* interaction is minimal to none. Interestingly olanzapine, prochlorperazine, and metoclopramide exhibited dose-dependent inhibition of OCT2 and MATE1 with increased potency for OCT2. Although the exact mechanism of antiemetic action is unknown, these drugs may have some ability to antagonize the 5-HT_3 receptor as well (Kast and Foley 2007, Smith et al. 2012). Recently, olanzapine was added the recommended first-line drugs for treating chemotherapy-induced nausea and vomiting (Hesketh et al. 2017). The higher potency of olanzapine for OCT2 was further confirmed with the hOCT2/hMATE1 double-transfected cells. Although there is no *in vivo* inhibition potential based on the C_{max} (Supplemental Figure 5.1), the higher potency of inhibition for OCT2 suggests that olanzapine may be nephroprotective.

A limitation of this study is the use of a single probe substrate for testing inhibitor potency. There is increasing evidence that effectiveness of cationic drugs as inhibitors of multidrug transporters can be influenced by the substrate used (Belzer et al. 2013,

Hacker et al. 2015). However, the literature shows that the effect may be more pronounced for OCT2 than MATE1. Inhibition profiles of 400 inhibitors of MATE1 with four different substrates revealed no significant influence of substrate structure on inhibitor efficacy (Martinez-Guerrero et al. 2016). However, further testing using cisplatin as the substrate is necessary to fully understand the implications of these data. Assessing cisplatin cytotoxicity or *in vivo* nephrotoxicity along with renal platinum accumulations may also be warranted.

Another limitation when comparing *in vitro* models to *in vivo* is the obvious difference in protein expression ratios of OCT2 and MATE1 between transfected cell lines and endogenous expression. The hOCT2 and hMATE1 protein levels were determined by LC-MS/MS in hOCT2 and hMATE1 double-transfected MDCK cells and were 28.6 and 6.9 fmol/μg membrane protein, respectively (Yin et al. 2015). By comparison, studies determining the protein expression in human kidney cortex and human kidney membranes reveal a more modest difference in expression between the two transporters or even greater MATE1 expression (OCT2 7.4 pmol/mg, MATE1 5.1 pmol/mg) (Prasad et al. 2016) and (OCT2 5 fmol/μg, MATE1 10 fmol/μg) (Nakamura et al. 2016). Further, cell culturing and experimental conditions may also affect the protein expression and trafficking to the apical and basolateral membranes.

Lastly, there are also many other factors that may change the concentration of tubular exposure to drugs *in vivo*, including protein binding, CYP-mediated metabolism and percent of urinary excretion. Many of the 5-HT_3 antagonists including ondansetron, tropisetron, palonosetron, and granisetron are highly metabolized (48-95%) by cytochrome P450 enzymes in the liver (Table 5.2). Recently, studies have shown that metabolites may play a larger role in drug-drug interaction than previously thought

(Isoherranen et al. 2009). Further testing with major metabolites (>25% in the circulation) is warranted and also recommended by the latest FDA guidance (Yu and Tweedie 2013, FDA 2017). Additionally, 5-HT_3 antagonists that are renally cleared at a higher proportion such as palonosetron may accumulate in the kidneys, altering exposure concentrations. *In vivo* studies are required to understand the potential implications of the effect of protein binding, metabolism, and urinary excretion.

In summary, our *in vitro* data indicate that many of the 5-HT_3 antagonists have a higher potency towards MATE1 inhibition, *raising* the potential for increased tubular concentration of nephrotoxic drugs such as cisplatin. Further, ondansetron was potent enough to increase the intracellular concentration of a probe substrate, ASP^+ at concentrations close to the clinically relevant C_{max}. This data is consistent with the previous *in vivo* study in mice where increased cisplatin-mediated nephrotoxicity was observed with concurrent administration of ondansetron. Based on the current criteria for evaluating clinical drug-drug interaction potential, other 5-HT_3 antagonists as well as antiemetic drugs in other classes pose less risk due to much lower therapeutic plasma concentrations.

Table 5.1. 5-HT_3 Antagonist 50% Inhibitory Concentration (IC_{50}) of ASP^+ for OCT2 and MATE1

$5HT_3$ Antagonist	C_{max} (μM)	OCT2 IC_{50} (μM)* Avg ± SD (N=3)	MATE1 IC_{50} (μM)* Avg ± SD (N=3)	OCT2 C_{max}/IC_{50}	MATE1 C_{max}/IC_{50}
Ondansetron	0.2 (de Wit 1996)	2.6 ± 0.9	0.1 ± 0.1	0.08	2[b]
Palonosetron	0.02[d]	2.2 ± 0.3	1.6 ± 0.6	0.009	0.01
Granisetron	0.02 (Gurpide 2007)	3.8 ± 1.6	5.0 ± 1.1	0.005	0.004
Tropisetron	0.01 (Kees 2001)	31.3 ± 6.6	1.6 ± 0.9	0.0003	0.006
Dolasetron	1.7[d]	85.4 ± 3.4	27.4 ± 2.8	0.02	0.06[b]
Cimetidine[c]	4.0(Wang 2008)	24.5 ± 4.0	0.23 ± 0.2	0.16[a]	17.4[b]

[a]Maximum serum concentration (C_{max}) /inhibitory concentration$_{50}$ (IC_{50}) > 0.1 for OCT2
[b]Maximum serum concentration (C_{max}) /inhibitory concentration$_{50}$ (IC_{50}) > 0.02 for MATE1
[c]Positive Control
[d]Package Insert
*IC_{50} for OCT2 determined in OCT2-overexpressing HEK293 cells; IC_{50} for MATE1 determined in MATE1-overexpressing HEK293 cells

Table 5.2. 5-HT_3 Antagonist Routes of Metabolism and Major Metabolites

5-HT_3 Antagonist	Route	Major Metabolites	CYPs	Urinary metabolites	Reference
Ondansetron	Hepatic oxidation (95%)	7-hydroxy/8-hydroxyondansetron	CYP1A2, CYP2D6, CYP3A4	Glucuronide or Sulfate conjugates of 7/8-hydroxyondansetron; 5% unchanged ondansetron	(Fischer et al. 1994); PI
Palonosetron	Hepatic oxidation (50%)	M4: 6-hydroxy-palonosetron M9: N-oxide-palonosetron	CYP2D6, CYP3A, CYP1A2	40% parent drug; M9 (10%), M4 (10%)	(Stoltz et al. 2004)
Granisetron	Hepatic	7-hydroxygranisetron, 9'-desmethylgranisetron	CYP1A1, CYP3A4	11% parent drug; 48% metabolites	(Nakamura et al. 2005); PI
Tropisetron	Hepatic oxidation (90%)	5, 6, and 7-hydroxytropisetron N-oxidation (trace), N-demethylation (trace)	CYP2D6	Glucuronide and sulfate conjugates of oxidation metabolites (70% of total drug), 8% unchanged tropisetron	(Fischer et al. 1994); PI
Dolasetron	99% Reduction by carbonyl reductase to hydrodolasetron	5'OH/6'OH hydrodolasetron	CYP2D6	20-31% in urine unchanged hydrodolasetron, <1% unchanged dolasetron	(Dimmitt et al. 1999, Dimmitt et al. 1999)
PI: Package Insert					

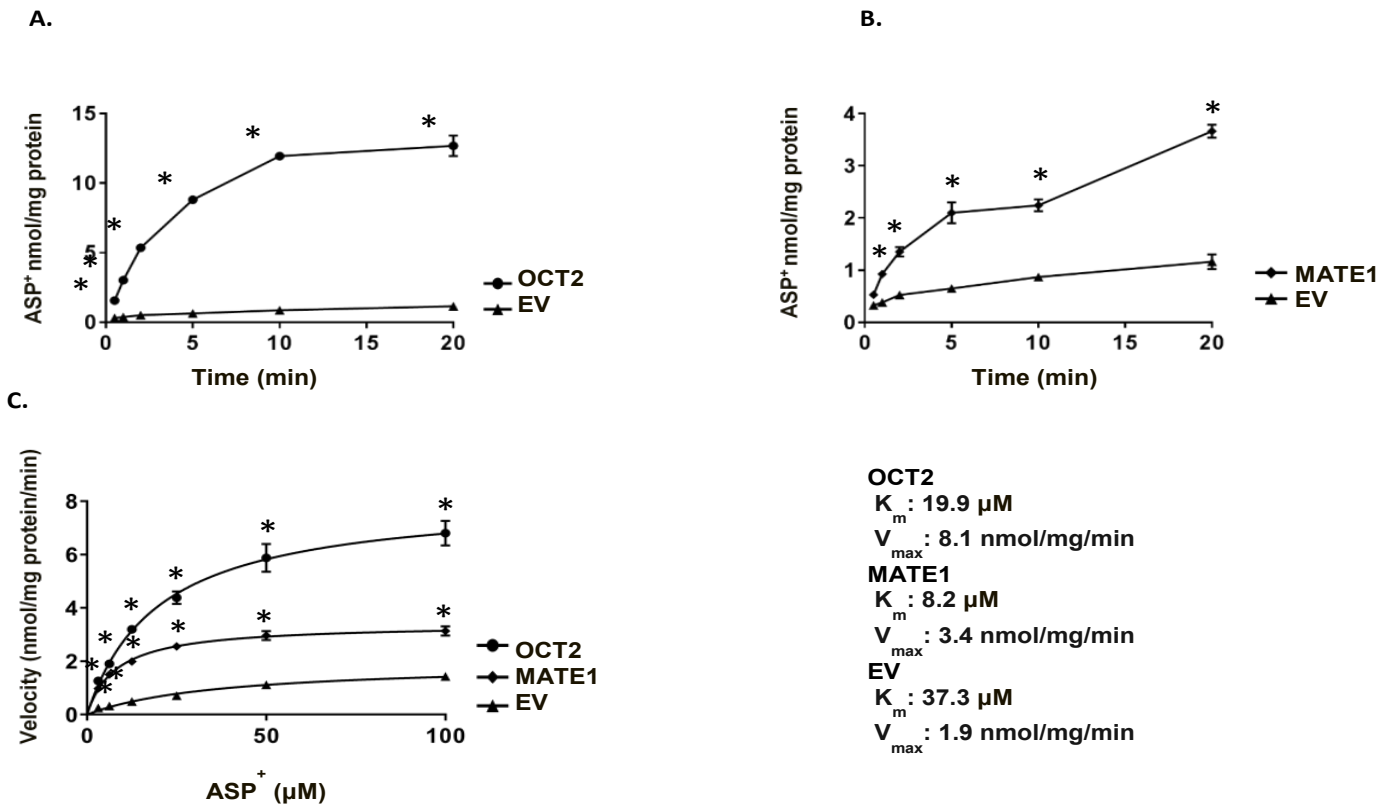


Figure 5.1. Time and Concentration-Dependent Accumulation of ASP^+ in HEK293 Cells Overexpressing OCT2 and MATE1. Cells were incubated for different time points (0.5-20 min) with 10 μM of ASP^+ for time-dependent uptake (A and B) or with serial dilutions of ASP^+ (0-100 μM) for 1 min for concentration-dependent uptake (C) on a shaker at 37°C. Fluorescence was quantified and normalized to protein concentration. Michaelis-Menten kinetic analysis was performed to calculate K_m and V_{max}. Data are presented as mean ± SE (n=3). *p<0.05 compared to Empty Vector (EV).

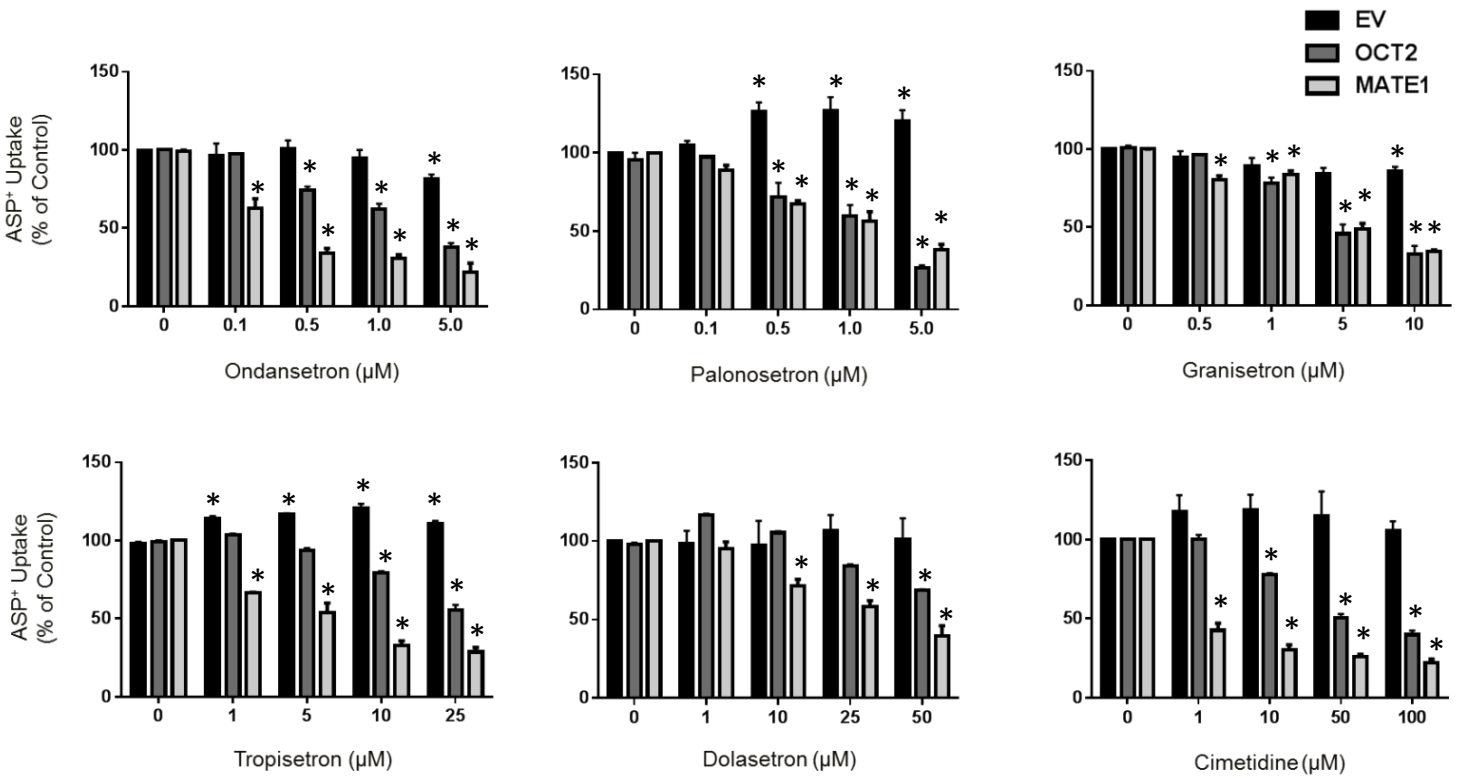


Figure 5.2. 5-HT$_3$ Antagonist Inhibition of ASP$^+$ Transport in HEK293 Cells Overexpressing OCT2 and MATE1 following 5-HT$_3$ Antagonist Treatment. Cells were incubated with ASP$^+$ (10 μM) in the presence and absence of various concentrations of 5-HT$_3$ antagonist or the positive control inhibitor, cimetidine. Fluorescence was quantified and normalized to protein concentration. Fluorescence quantified in Empty Vector (EV), OCT2, or MATE1 overexpressing cells treated with vehicle control (no inhibitor) was set to 100%. Data are expressed as mean ± SE (n=3). *p<0.05 compared to the vehicle, Hank's Balanced Salt Solution.

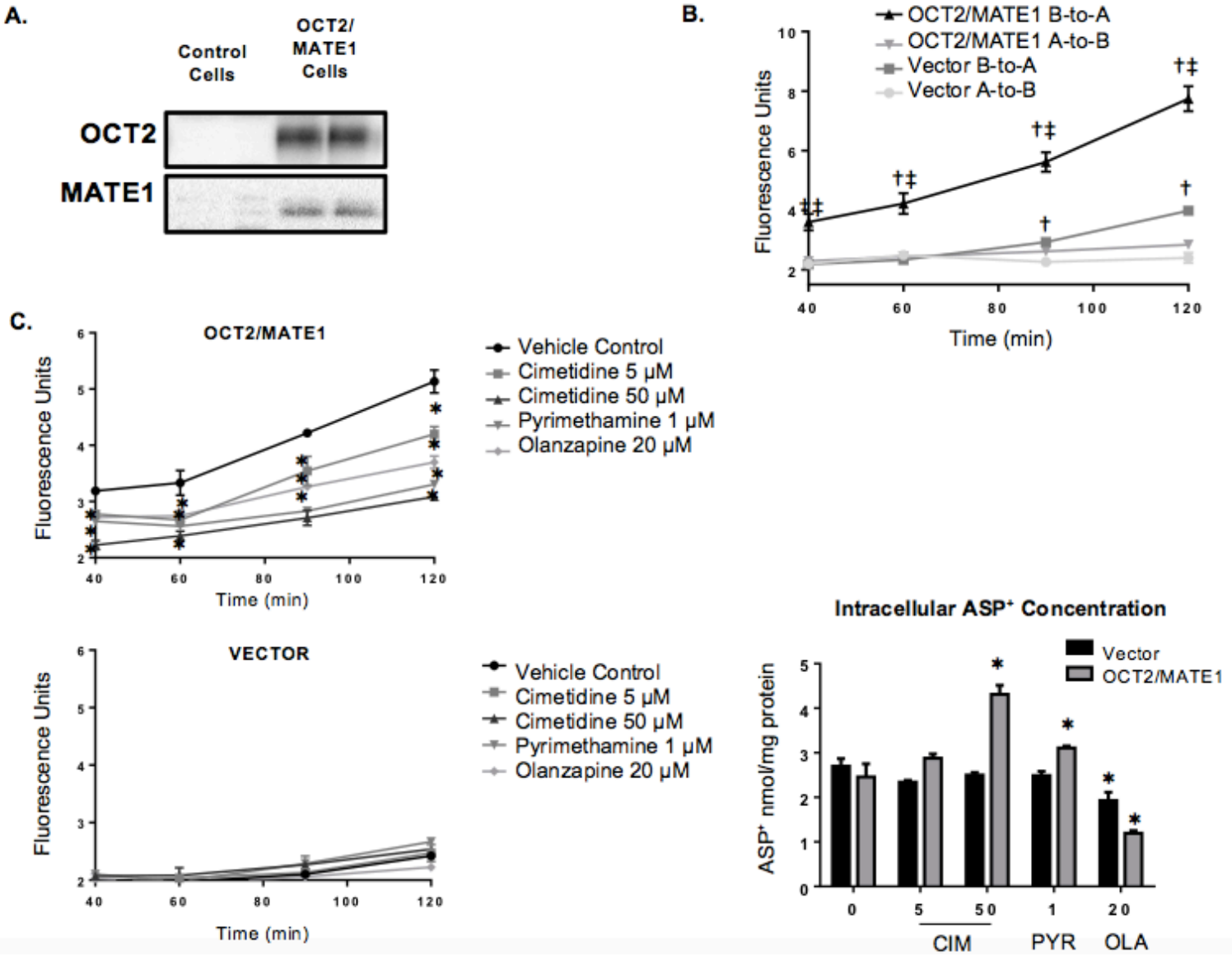

A.
Control Cells
OCT2/ MATE1 Cells
OCT2
MATE1
B.
OCT2/MATE1 B-to-A
OCT2/MATE1 A-to-B
Vector B-to-A
Vector A-to-B
Fluorescence Units
Time (min)
C.
OCT2/MATE1
Vehicle Control
Cimetidine 5 µM
Cimetidine 50 µM
Pyrimethamine 1 µM
Olanzapine 20 µM
Fluorescence Units
Time (min)
VECTOR
Vehicle Control
Cimetidine 5 µM
Cimetidine 50 µM
Pyrimethamine 1 µM
Olanzapine 20 µM
Fluorescence Units
Time (min)
Intracellular ASP+ Concentration
Vector
OCT2/MATE1
ASP+ nmol/mg protein
0
5
50
1
20
CIM
PYR
OLA

Figure 5.3. Transcellular Flux of ASP^+ in Control and OCT2/MATE1-Transfected MDCK cells. *A*. Protein expression of OCT2 and MATE1 in OCT2/MATE1 double transfected and vector control MDCK cells. *B*. Cells were incubated with ASP^+ (25 μM) in either apical or basolateral media for 120 minutes and fluorescence in apical or basolateral media was quantified. $^{\dagger}P<0.05$ compared to A-to-B. $^{\ddagger}P<0.05$ compared to vector. *C*. Cells were incubated with ASP^+ (25 μM) in basolateral media and positive control inhibitors in apical and basolateral media for 120 minutes. Apical and intracellular fluorescence were quantified and intracellular fluorescence was normalized to protein concentration. Data are expressed as mean ± SE (n=3). *p<0.05 compared to no inhibitor. CIM – Cimetidine; PYR – Pyrimethamine; OLA – Olanzapine.

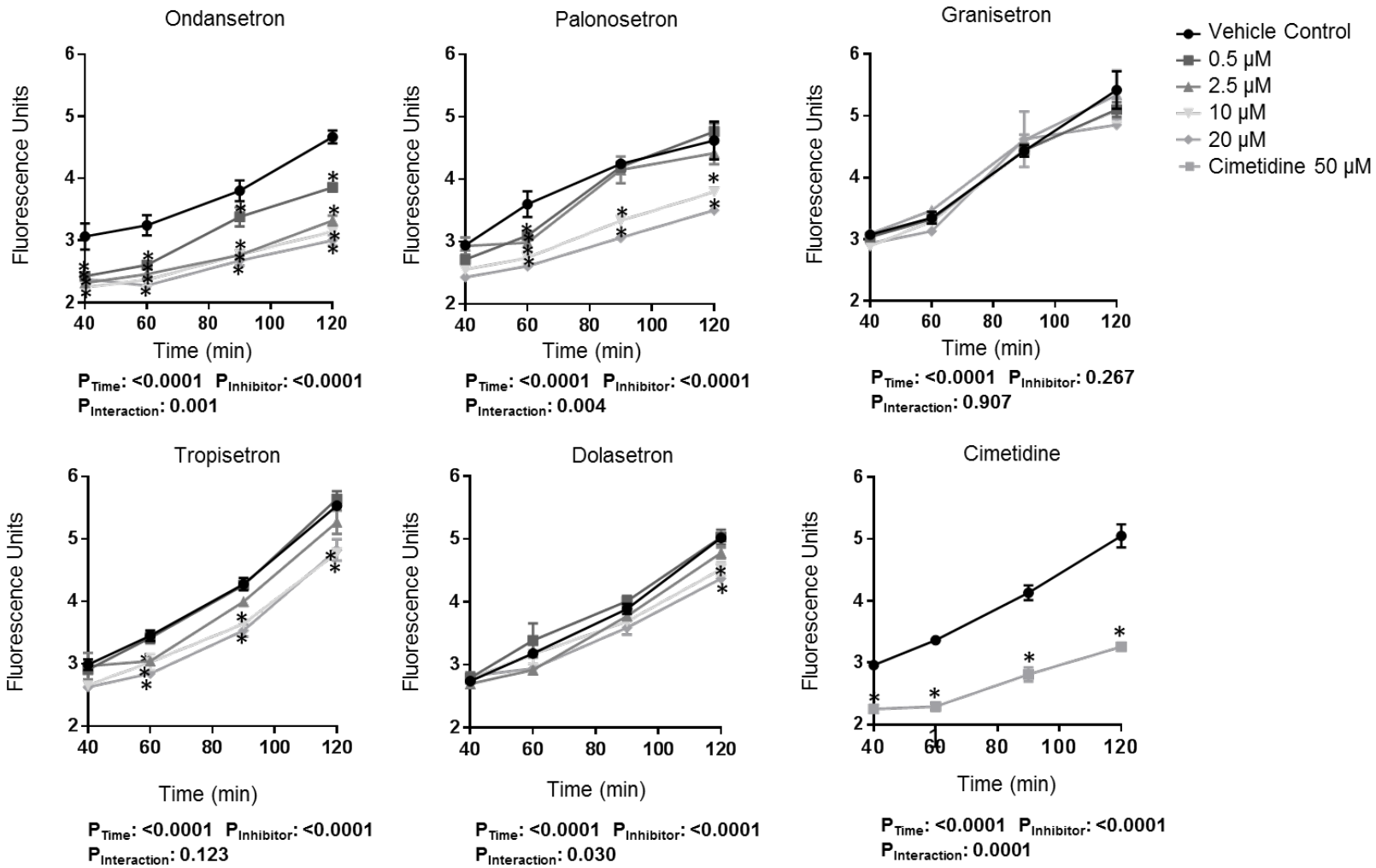

Ondansetron
Palonosetron
Granisetron
Tropisetron
Dolasetron
Cimetidine
Fluorescence Units
Time (min)
Vehicle Control
0.5 μM
2.5 μM
10 μM
20 μM
Cimetidine 50 μM
P_{Time}: <0.0001 $P_{Inhibitor}$: <0.0001
$P_{Interaction}$: 0.001
P_{Time}: <0.0001 $P_{Inhibitor}$: <0.0001
$P_{Interaction}$: 0.004
P_{Time}: <0.0001 $P_{Inhibitor}$: 0.267
$P_{Interaction}$: 0.907
P_{Time}: <0.0001 $P_{Inhibitor}$: <0.0001
$P_{Interaction}$: 0.123
P_{Time}: <0.0001 $P_{Inhibitor}$: <0.0001
$P_{Interaction}$: 0.030
P_{Time}: <0.0001 $P_{Inhibitor}$: <0.0001
$P_{Interaction}$: 0.0001

Figure 5.4. 5-HT_3 Antagonist Inhibition of Basolateral-to-Apical Transport of ASP^+ in OCT2/MATE1-Transfected MDCK Cells. Cells were incubated with ASP^+ (25 μM) in basolateral media and/or 5-HT_3 antagonists (0.5-20 μM) or 50 μM cimetidine in apical and basolateral media for 120 minutes. Apical fluorescence (40-120 min) was quantified. Data are expressed as mean ± SE (n=3). *$p<0.05$ compared to no inhibitor.

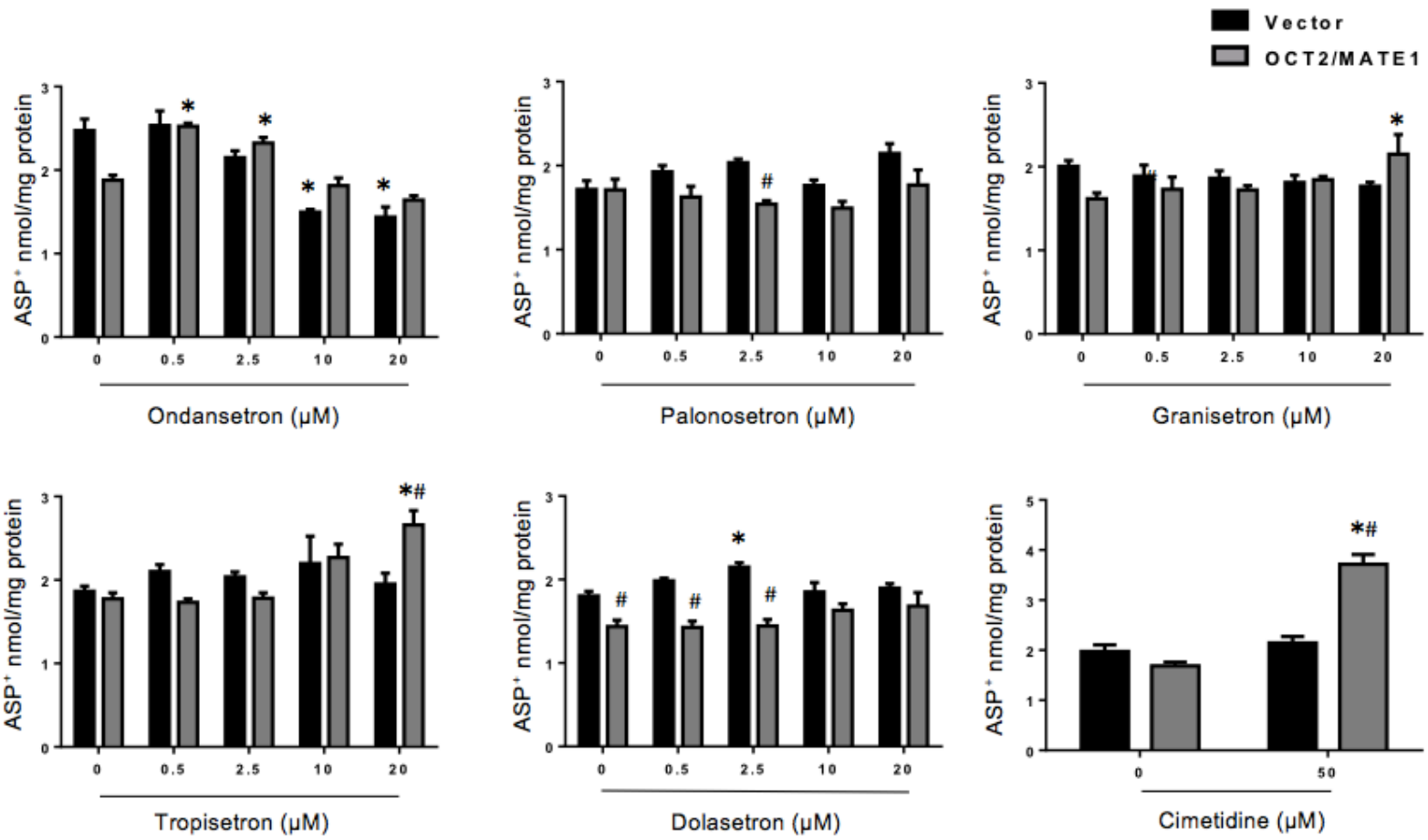
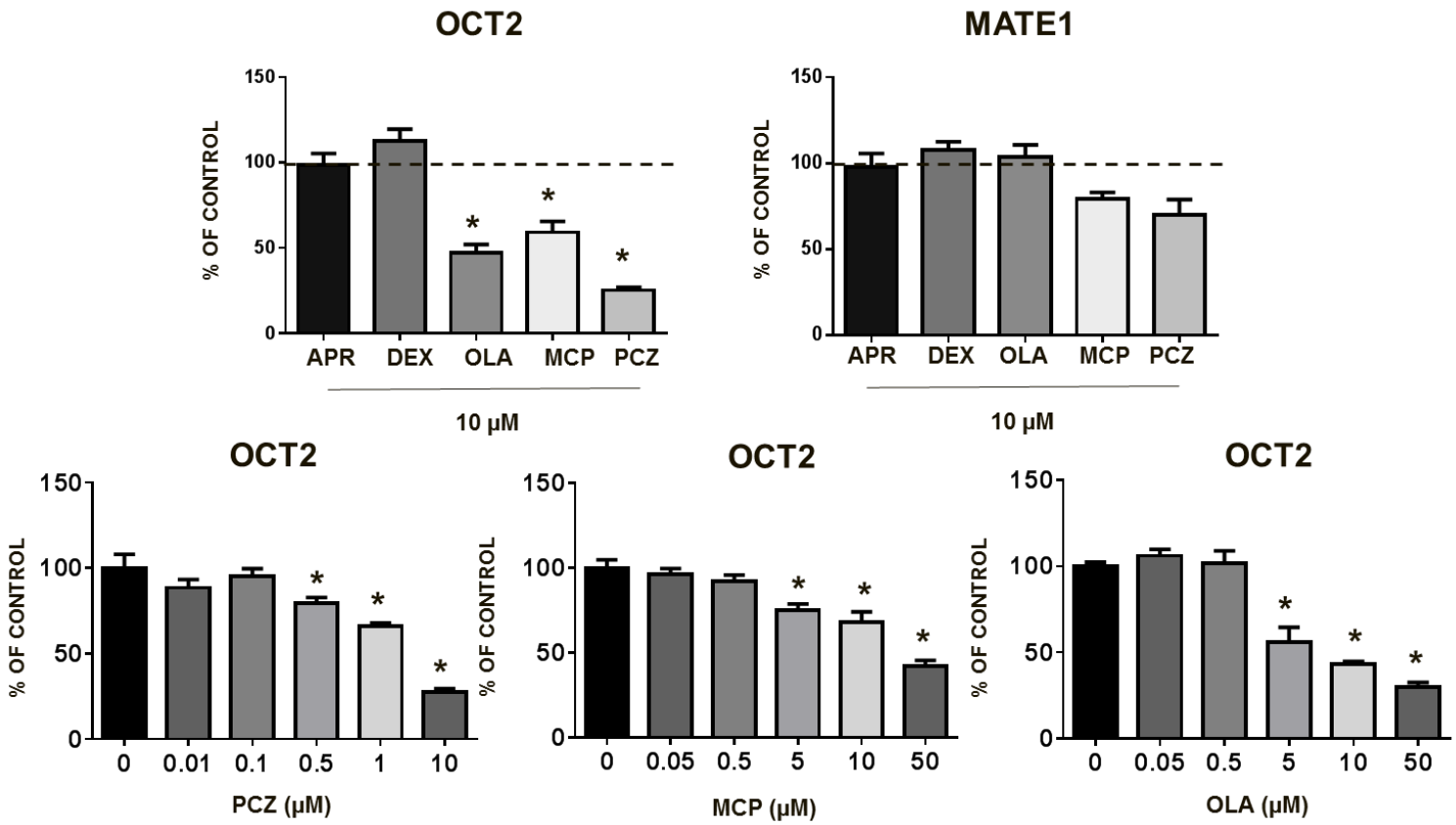
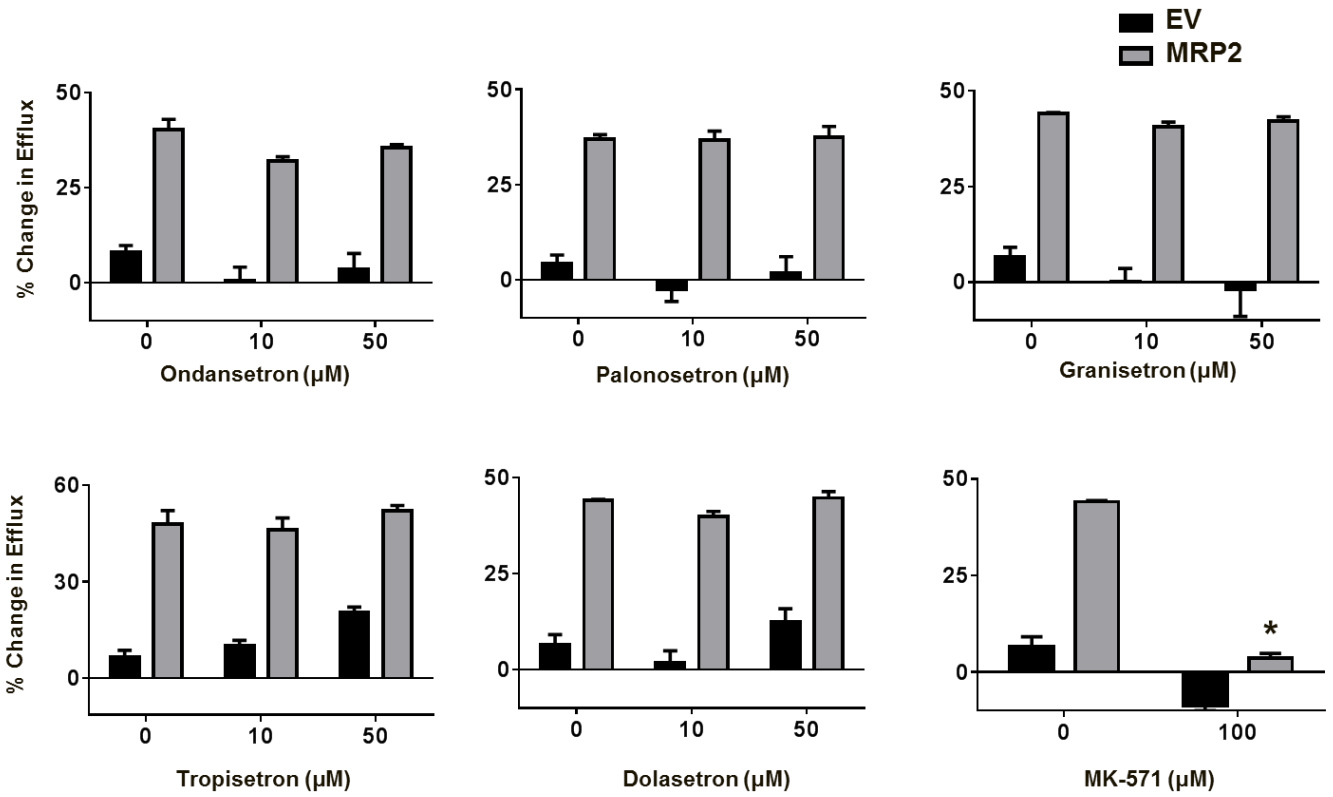

Vector
OCT2/MATE1
ASP+ nmol/mg protein
Ondansetron (μM)
Palonosetron (μM)
Granisetron (μM)
Tropisetron (μM)
Dolasetron (μM)
Cimetidine (μM)
0
0.5
2.5
10
20
50

Figure 5.5. 5-HT_3 Antagonist Accumulation of ASP^+ in OCT2/MATE1-Transfected MDCK Cells. Cells were incubated with ASP^+ (25 μM) in basolateral media and/or 5-HT_3 antagonists (0.5-20 μM) or 50 μM cimetidine in apical and basolateral media for 120 minutes. Intracellular fluorescence (120 min) was quantified and normalized to protein concentration. Data are expressed as mean ± SE (n=3). *$p<0.05$ compared to no inhibitor. $^{\#}p<0.05$ compared to vector.



Supplemental Figure 5.1. Inhibition of ASP^+ Transport in HEK293 Cells Overexpressing OCT2 and MATE1 by Other Antiemetic Drugs. Cells were incubated with ASP^+ (10 μM) in the presence and absence of other antinausea drugs (0-50 μM, APREP, aprepitant; DEXA, dexamethasone; OLA, olanzapine; MCP, Metoclopramide; PCZ, Prochlorperazine). Fluorescence was quantified and normalized to protein concentration. Data are expressed as mean ± SE (n=3). *$p<0.05$ compared to vehicle control, Hank's Balanced Salt Solution.



Supplemental Figure 5.2. 5-HT$_3$ Antagonist Inhibition of Calcein AM Transport in HEK293 Cells Overexpressing MRP2. Cells were treated with calcein AM (1 μM) for 15 min (*uptake phase*) with or without 5-HT$_3$ antagonists (10 and 50 μM) or positive control, MK-571 (100 μM) and incubated for additional 1 h with or without 5-HT$_3$ antagonist (10 and 50 μM) or MK-571 (100 μM). Intracellular fluorescence was measured using Cellometer Vision (Nexcelom). Data are represented as mean ± SE (n=4). Percent efflux of calcein was calculated as a percent change in fluorescence from uptake measurements. *$p<0.05$ compared to no inhibitor.

CHAPTER 6: OVERALL DISCUSSION AND CONCLUSIONS

Summary

The overall objectives of this dissertation research were to assess time-dependent changes in urinary protein biomarkers in cisplatin-treated oncology patients with subclinical nephrotoxicity, characterize the renal expression and regulation of the novel urinary biomarker calbindin in cisplatin-mediated acute kidney injury (AKI) and determine the extent of antiemetic drug inhibition of renal transporters involved in cisplatin secretion. Despite the recent development of new immunotherapies and gene-targeting anticancer drugs, cisplatin remains an important component of chemotherapeutic regimens for the treatment of solid tumors. However, use of cisplatin is limited by AKI, which occurs in about one-third of patients (Shord et al. 2006). Long-term impact of cisplatin treatment is a small but permanent decline in eGFR (Latcha et al. 2016). Current clinical markers, such as serum creatinine (SCr) and estimated glomerular filtration rate (eGFR), are limited in their ability to detect clinical AKI. Further, the degree of interaction of concurrently prescribed antiemetic drugs and cisplatin at renal secretory transporters is unknown.

The findings presented in the previous chapters of this dissertation support the hypotheses that novel urinary protein biomarkers can be useful in detecting subclinical cisplatin-induced nephrotoxicity and concurrently administered antiemetic drugs can inhibit cisplatin renal transporters. Four specific aims were developed to address this hypothesis: 1) Assess the time-dependent changes in the urinary excretion of novel protein biomarkers in cancer patients prescribed cisplatin (**CHAPTER 2**); 2) Determine whether the excretion of urinary protein biomarkers in cancer patients differs between early and later cycles of cisplatin chemotherapy (**CHAPTER 3**); 3) Characterize the renal expression of calbindin in cisplatin-mediated acute kidney injury in mice

(**CHAPTER 4**); 4) Determine the extent to which antiemetic drugs inhibit kidney transporters involved in cisplatin secretion (**CHAPTER 5**).

The first two aims assessed the time-dependent changes in novel urinary protein biomarkers in cancer patients prescribed cisplatin. In 57 patients with solid tumors receiving outpatient cisplatin therapy (≥25 mg/m^2), serum creatinine was unchanged following cisplatin infusion. Compared to baseline values, several novel biomarkers were significantly increased in the urine. B2M was increased 3-fold by day 3, while KIM-1, TFF3, and calbindin were elevated 2-fold, 2-fold, and 8-fold by day 10, respectively. In a subset of 27 patients, time-dependent changes in KIM-1, calbindin, and TFF3 were assessed during early and subsequent cycles of cisplatin-containing chemotherapy. Although significant increases were seen during the early cycle, biomarker levels remained elevated from baseline and further changes were modest during subsequent cycles, revealing differences in biomarker excretion during early and subsequent cycles of cisplatin therapy.

The third aim utilized a well-established model of cisplatin-induced AKI in mice to characterize the renal expression and regulation of calbindin. Calbindin was robustly elevated in the urine before there were changes in traditional renal biomarkers such as SCr and BUN. A time-dependent decrease in renal calbindin protein was observed on days 3 and 4 with a concurrent up-regulation of calbindin mRNA. These data add to the current understanding of the mechanism of calbindin urinary release following cisplatin-mediated injury.

The fourth aim utilized *in vitro* models overexpressing renal transporters OCT2 and MATE1 to determine the extent of 5-HT$_3$ antagonist drug inhibition with cisplatin renal

secretion transporters. 5-HT_3 antagonists dose-dependently inhibited the uptake of ASP^+ in cells overexpressing OCT2 or MATE1, with increased potency for MATE1 cells. In double-transfected MDCK hOCT2-MATE1 cells, ondansetron inhibited the transcellular transport of ASP^+ most potently with increased intracellular accumulation of ASP^+ demonstrated at lower concentrations. These findings suggest that potent inhibition of MATE1-mediated efflux of cisplatin from human proximal tubules by antiemetic drugs may influence susceptibility to nephrotoxicity.

Novel Urinary Biomarkers

There were several limitations in the design of clinical studies in Chapters 2 and 3, reducing the overall incidence of AKI. The sample size and cisplatin dose-range was small. Additionally, African American patients are more susceptible than other races to nephrotoxicity (Shord et al. 2006) and were not well-represented in the study, which was composed largely of Caucasian patients. AKI is normally detected within 48 h to 7 days after cisplatin administration. Due to the non-interventional nature of the study, the measurement of traditional serum biomarkers, such as SCr and BUN, occurred over a longer period than desired (13 ± 7 days). However, using the criteria of elevated SCr from baseline (>50%) within 7 days also did not change the outcome. It is possible that more consistent assessment of SCr and BUN would have captured AKI in a greater number of patients.

We primarily used absolute biomarker values for analysis in Chapters 2 and 3, although values normalized to creatinine are most often reported, due to variability in urine output between patients. However, the underlying assumption is that urinary creatinine excretion is constant within an individual and that biomarker excretion is linear with urinary creatinine excretion, neither of which may be the case. OCT2 has also been

shown to be involved in the renal secretion of creatinine, which can be inhibited by OCT2 substrates such as cisplatin (Ciarimboli et al. 2012). Absolute concentrations were more informative in distinguishing AKI on hospital admission; however, normalized concentrations better reflected long-term outcomes, including death and dialysis (Ralib et al. 2012, Shinke et al. 2015).

KIM-1. KIM-1, a well-studied kidney injury biomarker, was one of the proteins with significant changes in urinary secretion in cisplatin-treated patients on day 10 during early cycles (N=57, Chapter 2). KIM-1 is expressed on regenerating proximal tubule cells and is associated with clearance of cellular debris (Bailly et al. 2002). With additional cycles of cisplatin, KIM-1 remained elevated but did not increase proportionately after cisplatin treatment (N=27, Chapter 3). As noted previously, KIM-1 is not detected in completely atrophic cells; therefore KIM-1 may not be able to reflect progressive kidney injury (van Timmeren et al. 2007). The lack of proportional increases in KIM-1 protein may also lead to decreased clearance of damaged cells and debris, leading to the development of fibrosis or chronic kidney disease (CKD). In fact, KIM-1 protein was also present in dilated tubules in fibrotic areas of kidney biopsy samples from patients with various renal diseases (van Timmeren et al. 2007). The modest correlation between age and urinary KIM-1 concentrations on day 10 during the subsequent cycles could be indicative of an age-related increased risk for AKI (Latcha et al. 2016).

TFF3. TFF3 is a relatively understudied protein in the setting of kidney injury. In patients receiving cisplatin during early cycles, TFF3 displayed a robust time-dependent increase (N=57, chapter 2). TFF3 is a small peptide hormone secreted by epithelial cells with functions of cell surface maintenance and inhibition of apoptosis in the lung and intestine (Taupin and Podolsky 2003). TFF3 function in the kidney is unknown but in cisplatin-

treated rats, decreased levels of TFF3 were observed in the urine and kidneys (Yu et al. 2010). With subsequent cycles of cisplatin, further increases in TFF3 were not seen (N=27, chapter 3) and levels remained elevated. This may indicate a switch to an adapative environment in the kidney. However, further mechanistic understanding of TFF3 is needed to understand this phenomenon. Future studies could utilize a lower repeated dose of cisplatin *in vivo*, allowing for the development of kidney fibrosis and quantifying time-dependent changes in biomarkers.

Calbindin. The biomarker with the greatest change (8-fold) on day 10 in our studies was calbindin. Calbindin is a cytosolic calcium-binding protein located in the distal tubules and collecting ducts of the kidneys (Iida et al. 2014). The urinary changes seen our study were consistent with previous studies in cancer patients receiving cisplatin (Takashi et al. 1996). Although the function of calbindin is unknown in kidney injury, it is known to transport intracellular calcium (Berggard et al. 2000). Interestingly, we also noted a statistically significant decrease in serum calcium during early cycles of cisplatin. Further studies are needed to understand if the two events are related. However, we also observed that the robust increase in urinary calbindin was diminished during subsequent cycles of cisplatin therapy. Since very little is known about the intrarenal expression and regulation of calbindin in the setting of kidney injury, we performed an *in vivo* study using mice treated with cisplatin. As observed in cancer patients, we also noted a time-dependent increase in calbindin urinary protein in mice, validating calbindin further as a kidney-injury biomarker released in response to cisplatin. We were also able to correlate the increase in the urine with a time-dependent decline in renal expression of calbindin protein in mice, suggesting that calbindin was released from the kidneys into the urine. Interestingly, while KIM-1 immunohistochemical staining correlated well with the degree of histopathological injury, calbindin did not.

Calbindin (*Calb1*) mRNA was upregulated in response to the renal calbindin protein decrease, along with an mRNA increase in the apical calcium uptake channel, *TRPV5*. To understand whether the changes in calbindin are pathophysiological or compensating for intracellular or extracellular calcium loss, concurrent quantification of serum and intracellular calcium is necessary. Future studies to assess regulation of calbindin should examine vitamin D or parathyroid hormone pathways. Next steps should include quantification of changes in plasma 1,25-dihydroxycholecalciferol (active vitamin D) and parathyroid hormone following cisplatin administration and nuclear trafficking of vitamin D receptor and activity.

NGAL. Although many of the biomarkers assessed in our study (Chapter 2) showed time-dependent changes, others that did not. NGAL was found to have good AUC-ROC values for the detection of AKI vs. non-AKI in clinical studies assessing cisplatin-mediated AKI (Table 1.6). Notably, these studies used early time points (between 12 hours and 3 days), whereas our study utilized later time points (day 3 and 10), possibly missing any changes that occurred early on.

Studies have shown that cisplatin-induced AKI may lead to adaptive processes that occur in the body that contribute to a reduction in toxic side effects. Some mechanisms of this adapative resistance include alterations in drug uptake or efflux, increased DNA repair, and increases in intracellular thiol content such as metallothioneins. Cisplatin-induced renal injury increased the mRNA and protein levels of efflux transporters, multidrug resistance-associated proteins 2, 4 and 5 and multidrug resistant proteins 1a and 1b in mice (Aleksunes et al. 2008). Expression of organic anion and cation transporters in contrast were reduced, suggesting lower intracellular accumulation of cisplatin. Metallothioneins are low molecular weight proteins with a high density of

cysteine groups that bind and sequester heavy metals such as platinum. Renal mRNA of detoxification and cytoprotective enzymes such as NADPH:quinone oxidoreductase 1, thioredoxin reductases, epoxide hydrolase, and metallothionein 1 were upregulated following cisplatin injury in mice (Aleksunes et al. 2010). These adapative changes may also serve to protect patients from additional nephrotoxicity to an extent and explain the modest urinary biomarker changes observed following subsequent chemotherapy cycles as well as the lack of development of clinical AKI.

Future research should utilize a larger and more diverse population, capturing current or future episodes of AKI. Inclusion of multiple time-points would also be helpful in capturing the peak biomarker response. Further, since there were baseline differences in several biomarkers (Table 2.6) between cancer patients in our study and healthy volunteers (Brott et al. 2014), a head-to-head comparison of biomarker baseline concentrations and performance in these two populations would be informative. A majority of the biomarkers (KIM-1, calbindin, clusterin, B2M, cystatin C, NGAL, osteopontin, and TFF3) had higher values in oncology patients. The lack of detectable changes in inflammatory biomarkers such as IL-18 and osteopontin is also informative and can provide clues to the mechanism of injury for novel nephrotoxicants. Finally, mechanistic understanding of novel proteins such as calbindin and TFF3 will be useful in determining species differences as well as differentiating between injury and recovery phases.

Antiemetic Drugs

Our studies aimed to extend the prior work investigating cisplatin-ondansetron drug interactions to determine whether other antiemetic drugs also interact with renal transporters of cisplatin (Li et al. 2013). We demonstrated using two different cell models

that all five 5-HT_3 antagonists tested inhibit OCT2 and MATE1 activity. The higher potency for MATE1 inhibition based on ASP^+ transport indicates a potential for increased cisplatin tubular accumulation. It has been shown that MATE-mediated organic cation efflux is both the active and rate-limiting step in the secretion process (Schali et al. 1983, Pelis and Wright 2011). The recently lowered threshold for C_{max}/IC_{50} MATE1 drug-inhibitor interactions in the draft guidance (FDA 2017) indicates the growing acceptance of the critical role of MATE1 in organic cation secretion. However, testing with additional probe substrates is needed for more accurate comparison of OCT2 and MATE1 inhibitor potency. Particularly for OCT2, studies have shown that the selection of substrate impacts the potency of inhibition (Belzer et al. 2013, Hacker et al. 2015). Further studies analyzing cisplatin transport in the presence of each of the 5-HT_3 antagonists *in vitro* is needed and ongoing that may reveal differences in inhibition potencies.

Due to the *in vitro* nature of the studies, there were several limitations. Overexpressing cells may not accurately reflect endogenous transporter expression ratios and cultured cells often lose expression of other endogenous transporters that can alter the disposition of drugs. *In vitro* studies utilizing overexpressing cells can also overestimate the affinity of substrates or inhibitors for transporters due to the presence of unstirred water layers (Shibayama et al. 2015). Additionally, MDCK cells express endogenous transporters such as P-glycoprotein, which may efficiently clear 5-HT_3 antagonists that are potent substrates, affecting the degree of transporter inhibition (Goh et al. 2002). *In vitro* studies also do not account for metabolites generated by the liver, particularly important in the case of 5-HT_3 antagonists that are highly metabolized by CYP enzymes. Further urinary metabolites may play a larger role than the parent drug itself. Therefore, the intratubular concentration of 5-HT_3 antagonists that transporters are exposed to is hard to predict without pharmacokinetic or modeling studies.

Patients are administered multiple antiemetic drugs and are often switched from one 5-HT_3 antagonist to another. This raises the potential of drug interactions for medications utilizing the same pathway for clearance. There may also be alterations in drug disposition due to the patients' disease state and underlying chronic conditions such as hydration status, diabetes, hypertension or undiagnosed kidney disease. Ultimately, assessing cisplatin nephrotoxicity in patients with sensitive novel biomarkers would be informative of the interaction potential when multiple medications are administered and their metabolites are also involved.

Overall Conclusions and Implications

The findings from this research demonstrate that novel urinary protein biomarkers are responsive to cisplatin therapy in the absence of clinically detectable AKI during early cycles, but are not reflective of progressive or chronic kidney changes during subsequent cycles of cisplatin therapy. Additionally, this research provides a new mechanistic understanding of calbindin urinary release as well as the potential for antiemetic drug inhibition of renal cisplatin transporters. Due to the limitations mentioned above, direct extrapolation to human population must be exercised with caution. However, this research, along with further recommended studies, serve to steer future clinical studies in a productive direction. Understanding the performance of biomarkers in various clinical settings will enable the establishment of accurate cutoffs. Clinical use of sensitive and specific biomarkers will allow identification of AKI earlier and contribute to better therapeutic outcomes. With the growing choice of drugs, mechanistic understanding of drug-transporter and drug-drug interactions will enable prescribers to select the best combination for protection from chemotherapy-induced nausea and vomiting, while minimizing adverse events.

APPENDIX 1: PHARMACOGENOMIC VARIANTS INFLUENCE THE URINARY EXCRETION OF NOVEL KIDNEY INJURY BIOMARKERS IN PATIENTS RECEIVING CISPLATIN

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A-1.1 Abstract

Nephrotoxicity is a dose limiting side effect associated with the use of cisplatin in the treatment of solid tumors. The degree of nephrotoxicity is dictated by the selective accumulation of cisplatin in renal tubule cells due to 1) uptake by organic cation transporter 2 (OCT2) and copper transporter 1 (CTR1), 2) metabolism by glutathione *S*-transferases (GSTs) and γ-glutamyltransferase 1 (GGT1), and 3) efflux by multidrug resistance-associated protein 2 (MRP2) and multidrug and toxin extrusion protein 1 (MATE1). The purpose of this study was to determine the significance of single nucleotide polymorphisms that regulate the expression and function of transporters and metabolism genes implicated in development of acute kidney injury (AKI) in cisplatin treated patients. Changes in the kidney function were assessed using novel urinary protein biomarkers and traditional markers of kidney function. Genotyping was conducted by the QuantStudio 12K Flex Real-Time PCR System using a custom open array chip with metabolism, transport, and transcription factor polymorphisms of interest to cisplatin disposition and toxicity. Traditional and novel biomarker assays for kidney toxicity were assessed for differences according to genotype by ANOVA. Allele and genotype frequencies were determined based on Caucasian population frequencies. The polymorphisms rs596881 (*SLC22A2/*OCT2), and rs12686377 and rs7851395 (*SLC31A1/*CTR1) were associated with renoprotection and maintenance of estimated glomerular filtration rate (eGFR). Polymorphisms in *SLC22A2*/OCT2, *SLC31A1*/CTRI, *SLC47A1*/MATE1, *ABCC2/*MRP2, and *GSTP1* were significantly associated with increases in the urinary excretion of novel AKI biomarkers: KIM-1, TFF3, MCP1, NGAL, clusterin, cystatin C, and calbindin. Knowledge concerning which genotypes in drug transporters that are associated with cisplatin-induced nephrotoxicity may help to identify at-risk patients and initiate strategies to prevent AKI.

Keywords: cisplatin, nephrotoxicity, acute kidney injury, pharmacogenomics, CTR1, GGT1, GST, KEAP1, MATE1, MRP2, NRF2, OCT2

A-1.2 Introduction

While effective as a chemotherapeutic agent for various solid tumors (Coppin et al. 1996, Bolis et al. 1997, Planting et al. 1999, Rose et al. 1999, Gatzemeier et al. 2000, Hoskins et al. 2000, Pabla and Dong 2008). cisplatin has been limited by its toxicities. Cisplatin treatment can result in nephrotoxicity, ototoxicity, neurotoxicity, infections, and gastrointestinal toxicity secondary to its high emetogenic potential. Of these adverse events, acute kidney injury (AKI) is a prominent toxicity. Approximately one-third of patients treated with a single dose of cisplatin (50-100 mg/m^2) will experience kidney impairment (Lebwohl and Canetta 1998, Shiraishi et al. 2000). It is known that cisplatin selectively accumulates in the proximal tubule, leading to oxidative stress, inflammation, and vascular injury, and subsequent renal pathology (Ozkok and Edelstein 2014). The degree to which cisplatin and its metabolites accumulate and induce injury can be influenced by the coordinated activity of uptake and efflux transport proteins on the basolateral and apical membranes of the kidney proximal tubules (Bag et al. 2015). Cisplatin metabolism is also important in governing proximal tubule exposure to the parent drug and its conjugates (Townsend et al. 2003).

Single nucleotide polymorphisms in cisplatin metabolism and transport genes are hypothesized to modulate risk of nephrotoxicity. Uptake of cisplatin by kidney proximal tubule cells is governed by organic cation transporter 2 (OCT2) (Ciarimboli et al. 2010). and copper transporter 1 (CTR1) (Ishida et al. 2002) on the basolateral membrane. Studies using mice lacking the Oct1 and 2 transporters have observed reduced kidney exposure to cisplatin and attenuation of nephrotoxicity (Ciarimboli et al. 2010, Sanchez-Gonzalez et al. 2011). In the proximal tubule cell, cisplatin is metabolized by glutathione *S*-transferase pi-1 (GSTP1) and γ-glutamyltransferase 1 (GGT1) enzymes. The function of GST and GGT1 enzymes can also contribute to cisplatin induced nephrotoxicity

(Townsend et al. 2003). Metabolism of cisplatin by GST results in a cisplatin-glutathione substrate that undergoes efflux from the proximal tubule cell and is subsequently metabolized extracellularly at the apical brush border membrane by GGT1 (Townsend et al. 2003). The resultant substrate, a cisplatin-cysteinyl-glycine conjugate, is metabolized to cisplatin-cysteine by aminodipeptidase and reabsorbed into the proximal tubule cell (Townsend et al. 2003). Subsequent metabolism processes in the proximal tubule cell lead to the development of a reactive thiol that enhances nephrotoxicity (Townsend et al. 2003). This mechanism is supported by studies in mice lacking GGT, showing protection against cisplatin induced kidney injury (Hanigan et al. 2001). Cisplatin metabolites are extruded from the proximal tubule cell into urine via brush border (apical) efflux transporters including multidrug and toxin extrusion protein 1 (MATE1) and by multidrug resistance-associated protein 2 (MRP2) (Ishikawa et al. 1994). MATE1- and MRP2-null mice display increased cisplatin induced kidney toxicity and experience increased renal exposure to cisplatin (Nakamura et al. 2010, Wen et al. 2014). Although knockout studies in animals have supported genetic influences on cisplatin nephrotoxicity, there are limited studies to date that have evaluated the role of genetics of metabolism enzymes and transporters on risk of cisplatin nephrotoxicity in humans (Filipski et al. 2009).

Regulatory proteins, such as Kelch-like ECH-associated protein 1 (KEAP1) and nuclear factor (erythroid-derived 2)-like 2 (NRF2), can modulate the expression of metabolism and transport processes including GSTs and MRP2 (Friling et al. 1990, Rushmore et al. 1991, Nguyen et al. 2003). KEAP1 acts as a regulatory protein that represses the activity of NRF2 and promotes its degradation by ubiquitination (Kansanen et al. 2013). NRF2 is a transcription factor that binds to antioxidant response elements (AREs) in the regulatory regions of target genes that direct drug metabolism, transport, detoxification,

and antioxidant function (Friling et al. 1990, Rushmore et al. 1991, Nguyen et al. 2003). Mice lacking NRF2 are reported to experience enhanced cisplatin nephrotoxicity, suggesting a role for NRF2 in protecting the kidney (Aleksunes et al. 2010).

Serum creatinine has been accepted as the clinical biomarker for determination of kidney function and toxicity following cisplatin exposure. However, other novel protein biomarkers may be more sensitive and specific markers of AKI (Star 1998). Based on the current Kidney Disease Improving Global Outcomes (KDIGO) Guidelines, AKI is defined by a rapid decline in kidney function based on an increase of serum creatinine (≥ 0.3 mg/dL within 48 hours or ≥ 1.5x baseline within the previous 7 days) and a decrease in urine output (< 0.5 ml/kg/h for 6 hours) (2012, Khwaja 2012). Compared to traditional clinical markers, kidney injury molecule-1 (KIM-1), calbindin, trefoil factor 3 (TFF3), and cystatin C are urinary biomarker candidates with enhanced sensitivity for assessing kidney injury due to drugs such as cisplatin (Takashi et al. 1996, Han et al. 2002, Yu et al. 2010, Waring and Moonie 2011). Relationships between urinary biomarkers, as indicators of cisplatin kidney injury, and polymorphisms in candidate drug metabolism and transport genes have not previously been evaluated.

The current study sought to elucidate the influence of polymorphisms in drug transport, metabolism, and regulatory genes on cisplatin-induced nephrotoxicity by assessing changes in novel urinary protein biomarkers.

A-1.3 Results

Patient Characteristics

A total of 206 patients who were scheduled to receive or who had received cisplatin were recruited. This included prospective (n=57) and retrospective (n=149) patients. For

the prospective patients, a subset (n=28) were recruited prior to the first cycle of cisplatin-containing chemotherapy, while another subset (n=29) was recruited prior to the second cycle of cisplatin. The average time between first and second dose was 17 days (range of 6-34 days). Prospective patients contributed both genetics and urinary biomarker data, while retrospective patients contributed only genetics data. All patients were adequately hydrated and were not exposed to any concomitant nephrotoxins. Patient demographic and baseline laboratory characteristics are shown in Table A-1.1. The polymorphism allelic frequencies were in Hardy Weinberg Equilibrium (HWE) (Table A-1.2). The expected frequencies of minor and major alleles used in the HWE determinations were for the Caucasian (EUR) population in HapMap, which reflected the majority (92%) of patients enrolled in the study.

Associations Between SLC22A2 and SLC31A1 Variants and Estimated Glomerular Filtration Rate (eGFR) in Patients Receiving Cisplatin

Relationships between clinical kidney injury and polymorphisms in transporter, metabolism, and regulatory genes were assessed using estimated glomerular filtration rate (eGFR), a standard clinical measure of kidney function and overt nephrotoxicity. Genetic variants in the two cisplatin uptake transporters *SLC22A2* (OCT2) and *SLC31A1* (CTR1) were associated with preservation of kidney function. Patients with the CT genotype in *SLC22A2* polymorphism rs596881 exhibited positive changes in eGFR compared to individuals with the wildtype CC genotype (Figure A-1.1A, p=0.01). Similarly, increases in eGFR were observed in patients with both *SLC31A1* variants (rs12686377 and rs7851395). Patients with the GG (rs12686377) and AA (rs7851395) variant genotypes had positive changes in eGFR (p=0.01 and p=0.04) compared to patients with the wildtype genotypes AA and CC, respectively (Figures A-1.1B and C). Taken together, these data support renoprotective properties for some genetic variants

in cisplatin uptake transporters. By comparison, genetic variants for the efflux transporters MATE1 and MRP2 or metabolism genes GGT1 and GSTP1 were not associated with changes in eGFR.

Interestingly, patients with the CG genotype for a *KEAP1* variant (rs1048290) had an increase in eGFR compared to wildtype CC patients (p=0.03). In addition, patients with the GG variant (rs2886162) in the redox sensor *NFE2L2* exhibited eGFR improvement relative to wildtype AA patients (p=0.04).

Associations Between Transporter Gene Variants and Novel Urinary Biomarkers of Kidney Injury in Patients Receiving Cisplatin

Associations between transporter gene polymorphisms and novel urinary biomarkers generally suggested that variant alleles were predictive of increases in the urinary excretion of kidney injury biomarkers in patients receiving cisplatin, although there were a few notable exceptions (Table A-1.3). Significant changes in urinary biomarker excretion were associated with the uptake transporter genes *SLC22A2* and *SLC31A1*. Patients with the AC genotype for the *SLC22A2* rs316019 polymorphism exhibited higher urinary fold changes in KIM-1 at baseline (p=0.02), Day 3 (p=0.03), and Day 10 (p=0.046) compared to the wildtype CC genotype (Figure A-1.2, Table A-1.3). Patients with a CT genotype in the *SLC22A2* variant rs596881 also were associated with a significant decrease in beta 2 microglobulin (B2M) and increase in osteopontin at Day 3 after cisplatin relative to patients with the wildtype CC genotype (Table A-1.3). It should be noted that patients expressing two copies of the variant alleles for the rs316019 and rs596881 variants in *SLC22A2* did not exhibit significant changes in urinary biomarkers due to their small numbers (n=3 rs316019 and n=1 rs596881). In addition to *SLC22A2,* a positive association between *SLC31A1* and a urinary biomarker was observed.

Patients with the GG variant in rs7851395 had greater increases in urinary osteopontin levels at Day 3 after cisplatin treatment (Table A-1.3).

The AA genotype for the rs2289669 variant in the *SLC47A1* gene was associated with fold change elevations in KIM-1 (p=0.007) and monocyte chemoattractant protein-1 (MCP-1, p=0.015) at Day 3 after cisplatin administration compared to patients with the wildtype GG genotype. In addition, variants in the *ABCC2* gene were predominantly associated with higher concentrations of AKI-associated urinary biomarkers. The *ABCC2* variant rs3740066 was associated with enhanced calbindin levels in urine after cisplatin administration. Calbindin concentrations were significantly elevated by 2.2-, 1.9-, and 2.7-fold at baseline, day 3, and day 10, respectively in variant (TT vs. CC) genotype patients (Table A-1.3, Figure A-1.3). The *ABCC2* rs3740066 polymorphism was also associated with statistically significant increases in the urinary excretion of clusterin, cystatin C, and NGAL at Day 3 (Table A-1.3). In addition to the rs3740066 variant, patients with the CT genotype for the rs717620 *ABCC2* variant exhibited a 3.5-fold increase in clusterin and 2.6-fold enhancement of cystatin C concentrations at Day 3 compared to the wildtype CC genotype (Table A-1.3). Few patients were homozygous for the rs3740066 and rs717620 variants in *ABCC2* and in turn, no significant associations were observed with the excretion of urinary biomarkers following cisplatin treatment.

Association Between Cisplatin Metabolism Genes and Novel Urinary Biomarkers of Kidney Injury in Patients Receiving Cisplatin

Patients with the GG genotype for the *GSTP1* rs1695 variant had significant elevations in urinary biomarkers indicative of AKI compared to wildtype AA patients. They exhibited

2-fold or greater increases in urinary KIM-1, calbindin, and NGAL concentrations at Day 3 and urinary IL-18 at Day 10 (Table A-1.3).

Association Between Regulatory Genes and Novel Urinary Biomarkers of Kidney Injury in Patients Receiving Cisplatin

A few of the evaluated *NFE2L2* variants were associated with significant fold changes in the excretion of urinary biomarkers. Homozygous variants of *NFE2L2* rs1806649, rs1962142, rs2886162, and rs2706110 were associated with statistically significant fold-change increases in IL-18, TFF3, calbindin, and MCP-1, respectively, versus homozygous wildtypes (Table A-1.3).

The *KEAP1* polymorphism rs11085735 was associated with the excretion of several urinary biomarkers in patients who were heterozygous versus homozygous wildtype. TFF3 and cystatin C fold changes were statistically increased on Day 3 and Day 10 in patients with the AC variant genotype compared to wildtype CC patients (Table A-1.3, Figure A-1.4). Elevations in TFF3 were 2.5-, 1.8-, and 1.8-fold in patients with the AC genotype for rs11085735 compared to wildtype CC individuals at baseline, day 3, and day 10, respectively ($p=0.006$, $p=0.038$, $p=0.034$).(Table A-1.3) Cystatin C concentrations on Day 3 and 10 were enhanced by 3.3- and 2.6-fold, respectively, in patients with at least one copy of the *KEAP1* rs11085735 variant (AC) compared to patients with the reference alleles (CC) ($p=0.011$, $p=0.027$).(Table A-1.3) There were limited numbers of patients with the AA genotype for the rs11085735 polymorphism in *KEAP1*; in turn, no significant associations were observed with urinary biomarkers.

A-1.4 Discussion

The current study sought to address the role of variants in drug transport, metabolism, and regulatory genes on cisplatin-induced nephrotoxicity in cancer patients by assessing changes in the urinary concentrations of novel protein biomarkers. The results demonstrate associations that support the role of transporter and metabolizing enzyme genetics on risk or mitigation of risk of kidney injury by cisplatin. While specific variants in the uptake transporters *SLC22A2* (rs596881) and *SLC31A1* (rs12686377 and rs7851395) were associated with preserved eGFR, respectively, other variants in these genes were associated with fold-changes in novel urinary biomarkers. Variants in the efflux transporter genes *ABCC2* and *SLC47A1* were associated with elevations in novel urinary biomarkers, but not eGFR. The *SLC47A1* variant rs2289669 correlated with enhanced KIM-1 and MCP-1 concentrations. Significantly higher levels of KIM-1 at Day 3 were observed in patients with the *ABCC2* variant rs2273697. The *ABCC2* rs3740066 variant was associated with consistently enhanced fold-changes in calbindin, cystatin C, clusterin, and NGAL in patients who carried one or two alleles. Patients who carried the variants in rs1695 (*GSTP1*) has higher urinary concentrations of several biomarkers including KIM-1, calbindin, NGAL, and IL-18. Lastly, since regulatory genes such as *NFE2L2* and *KEAP1* can indirectly influence the effects of transporter and drug metabolizing enzyme genes (Atilano-Roque et al. 2016, Bai et al. 2016) associations between polymorphisms in these genes and novel urinary biomarkers were also evaluated. The *NFE2L2* polymorphism rs2886162 was consistently associated with enhanced calbindin excretion from baseline, while the *KEAP1* polymorphism rs11085735 was associated with rises in TFF3 and cystatin C concentrations. The current study is the first report to date in a cisplatin-treated patient cohort that has evaluated associations between targeted pharmacogenomics of drug transport and metabolism genes and a panel of novel urinary biomarkers of kidney injury.

The importance of the OCT2 uptake transporter in cisplatin renal clearance was previously reported in a study employing Oct1/2 mouse knockouts.(Filipski et al. 2009) These authors subsequently evaluated changes to serum creatinine after treatment with cisplatin in human cancer patients (n=78) according to the presence or absence of a copy of the nonsynonymous *SLC22A2* rs316019 variant denoting reduced OCT2 function (Filipski et al. 2009). The presence of the *SLC22A2* variant was associated with maintenance of serum creatinine. The *SLC22A2* polymorphism rs316019 (G808T; Ser270Ala) has also been associated with protection from cisplatin ototoxicity (Lanvers-Kaminsky et al. 2015) odds of hepatotoxicity and hematologic toxicity secondary to platinum chemotherapy (Qian et al. 2016) and increased metformin renal and secretory clearance (Christensen et al. 2013). In the current study, patients heterozygous vs. homozygous wildtype for the *SLC22A2* rs316019 polymorphism had associated higher concentrations and fold-changes in urinary KIM-1 at baseline, Day 3, and Day 10 as compared to wildtype homozygotes. The *SLC22A2* rs596881, rs3127573, and rs2279463 variants were associated with elevations in B2M at Day 3. Also in the current study, patients with the *SLC22A2* rs596881 variant exhibited a preservation of eGFR. A prior study has associated the *SLC22A2* rs3127573 variant with increased OCT2 function (Wanga et al. 2015). Based on our data and the supporting literature, variants in the *SLC22A2* gene were related to risk or mitigation of risk for cisplatin induced kidney injury.

A previous study has reported on a genetic variant (rs10981694) in the copper transporter protein 1 (*SLC31A1*) and cisplatin-induced ototoxicity (Xu et al. 2012). An additional study observed increased platinum resistance in lung cancer patients that was associated with two *SLC31A1* variants (rs7851395 and rs12686377) (Xu et al. 2012). The current study reported significant fold increases in eGFR in homozygous variant

versus homozygous wildtype patients (p=0.01 and p=0.04) with the previously reported variants (rs12686377 and rs7851395) in the *SLC31A1* gene. We also found higher osteopontin concentrations associated with homozygosity for the *SLC31A1* variant rs7851395 at Day 3 post cisplatin treatment. These studies support the role of copper transporter protein 1 variants on protection from changes in kidney function (eGFR), but the increase in osteopontin at Day 3 post cisplatin may reflect subacute injury prior to the later improvement in eGFR.

The transporters MATE1 and MRP2 are brush border proteins that efflux cisplatin from proximal tubule cells to urine and reduce the susceptibility to nephrotoxicity (Nakamura et al. 2010, Wen et al. 2014). A previous publication noted that the *SLC47A1* rs2289669 variant was linked to hematological toxicity secondary to platinum containing chemotherapy (Qian et al. 2016). Using oxaliplatin as a substrate, an *in vitro* study with *SLC47A1*-transfected variants in HEK-293 cells demonstrated loss of MATE1 function (Chen et al. 2009). Several publications have reported on variants in *SLC47A1* and the pharmacokinetics and pharmacodynamics of metformin (Becker et al. 2010, Stocker et al. 2013, He et al. 2015, Xiao et al. 2016). The presence of the *SLC47A1* polymorphism rs2289669 resulted in higher area under the plasma concentration time curve and lower renal clearance of metformin (He et al. 2015). In the current study, the *SLC47A1* rs2289669 variant was correlated with increases in the urinary biomarkers KIM-1 and MCP-1. Decreased function polymorphisms in *SLC47A1* are important for potentially increasing therapeutic efficacy, reducing renal clearance, and enhancing kidney toxicity of pharmaceutical substrates of MATE1.

Polymorphisms in the *ABCC2* gene have been shown to affect medication efficacy and safety. Variants in the *ABCC2* gene have been purported to influence the therapeutic

efficacy of anti-epileptic drugs (Ufer et al. 2011, Qu et al. 2012, Sha'ari et al. 2014, Ma et al. 2015). Other studies have identified an association between variants in the *ABCC2* gene and tenofovir induced kidney tubular dysfunction (Nishijima et al. 2012). The *ABCC2* rs717620 variant has previously been linked with responses to platinum chemotherapy (Qian et al. 2016). However, in cancer patients, one study failed to demonstrate a relationship between polymorphisms in *ABCC2* and cisplatin pharmacokinetics, which may be due to low statistical power or the use of traditional AKI endpoints (Sprowl et al. 2012). In the current study, several *ABCC2* variants were correlated with increased urinary biomarkers of AKI in patients receiving cisplatin; however, no relationships were observed with eGFR.

Significantly greater increases in urinary calbindin, clusterin, cystatin C, and NGAL at Day 3, and calbindin at Day 10 were observed in *ABCC2* variant rs3740066. Patients heterozygous for the *ABCC2* variant rs717620 exhibited increases in clusterin (3.5-fold) and cystatin C (2.6-fold) at Day 3. The current study in patients receiving cisplatin chemotherapy and published data from patients with epilepsy and HIV-1 support the influence of polymorphisms in *ABCC2* on biomarker changes and outcomes such as efficacy and toxicity.

Glutathione *S*-transferases metabolize platinum chemotherapeutics including cisplatin. The *GSTP1* Ile105Val polymorphism was previously purported to be associated with less neuropathy (Lecomte et al. 2006) and clinical outcomes (Stoehlmacher et al. 2004) related to oxaliplatin. The current study reported associations in the same *GSTP1* variant (rs1695) and significant increases in the urinary biomarkers KIM-1, Calbindin, and NGAL at Day 3 and IL-18 at Day 10 after cisplatin chemotherapy. While polymorphisms in *GST* metabolism genes appear to have some significance with

biomarker changes, further study is required to fully evaluate the influence of metabolism in the presence or absence of transporter haplotypes.

It is known that regulatory genes such as *NFE2L2* and *KEAP1* can influence the expression and function of transporters and drug metabolizing enzymes (Atilano-Roque et al. 2016, Bai et al. 2016). A previous publication reported a relationship between a promoter variant in *NFE2L2* and susceptibility to ototoxicity with high cumulative doses of cisplatin (Spracklen et al. 2017). The current study found links between *NFE2L2* polymorphisms and the enhanced excretion of urinary AKI biomarkers. The *NFE2L2* polymorphism rs2886162 was related to increases in calbindin at Day 10 vs. homozygous wildtypes. Additionally, homozygous variants of *NFE2L2* polymorphisms rs1806649, rs1962142, rs2886162, and rs2706110 were correlated with statistically significant fold-change increases in IL-18, TFF3, MCP-1, and cystatin C. Because *KEAP1* is related to *NRF2*, polymorphisms in *KEAP1* were also evaluated for their relationships with urinary biomarkers. TFF3 and cystatin C levels were statistically increased on Day 3 and Day 10 vs. homozygous wildtype patients for KEAP1 (rs11085735). The interaction between polymorphisms involved in cisplatin regulation, metabolism and transport has the potential to clarify mechanisms of cisplatin induced kidney injury.

The strength of the study lies in the comprehensive and targeted genomic approach to evaluating cisplatin induced kidney injury based on the hypotheses that genomics of drug transport and metabolism are a central component of this toxicity. This study incorporated polymorphisms involved in both processes as well as in regulation of these processes. Additionally, the current study evaluated kidney injury using both traditional measures and a panel of novel urinary biomarkers. However, several limitations exist

for this study. This study was primarily comprised of Caucasian patients, which reduces its generalizability to other races. Due to the limited number of non-Caucasian races, genotype frequency data (Table A-1.2) was assessed based on the published data for Caucasians. The patients in the study received low to moderate doses of cisplatin, potentially limiting our ability to detect clinical nephrotoxicity and associations between urinary biomarkers and pharmacogenetic variants. While the current study assessed relationships between genomics of selected drug metabolism and transport genes important for the disposition of cisplatin, the role of these polymorphisms in other forms of drug induced kidney injury may not be directly applicable and requires further study. This study was also not designed to study cancer specific outcomes such as progression free or overall survival based on genotype.

A-1.5 Conclusions

An improved understanding of the role of pharmacogenetic variants that regulate cisplatin transport and metabolism in renal tubule cells may help to reduce the severity of kidney injury and prevent morbidity in patients receiving treatment with cisplatin. The current study facilitates a greater understanding of the influence a patient's genotype contributes toward nephrotoxic risk in order to potentially inform about a preemptive screen to reduce the risk of kidney damage. Knowledge concerning which polymorphisms are associated with cisplatin induced nephrotoxicity will help to stratify patients at greatest risks to integrate strategies to prevent AKI and improve treatment outcomes in cancer patients.

A-1.6 Materials and Methods

General Reagents

FlexiGene® DNA Kits (ID# 51206) for DNA extraction from blood were purchased from QIAGEN Inc. (Germantown, MD). Calbindin, clusterin, KIM-1, GST-P1, IL-18, MCP-1, albumin, B2M, cystatin C, NGAL, osteopontin, and TFF3 assays (Bio-Plex Pro RBM human kidney toxicity assay panels 1 and 2) were purchased from Bio-Rad, Life Science (Hercules, CA).

Study Population

Eligible patients were greater than 18 years old and were treated with intravenous cisplatin for treatment of solid tumors in outpatient clinics at the University of Colorado Cancer Center, Aurora, CO, a National Cancer Institute – Designated Consortium Comprehensive Cancer Center. Patients received intravenous cisplatin in doses of ≥ 20 mg/m^2. Other inclusion criteria were hemoglobin ≥ 10 g/dL, no consumption of grapefruit juice or alcohol within 7 days, no history of alcohol consumption of >14 drinks/week, no history of organ transplantation or kidney dialysis, willingness to comply with study, not pregnant or lactating, no changes in medications within previous 4 weeks, and normal liver function (ALT and AST <2x ULN). As part of the standard of care protocol, patients were hydrated with 0.9% sodium chloride (1-2 L) pre- and post-cisplatin infusion. Exclusion criteria included a diagnosis of kidney cancer, previous exposure to platinum-based chemotherapy, herbal supplement use, exposure to other known nephrotoxins (including contrast agents) within previous 30 days, and concurrent use of inhibitors of cisplatin transport proteins.

Blood was collected for DNA isolation from patients who were either scheduled to receive cisplatin prospectively or who had historically received cisplatin treatment.

Patient demographics (e.g., race, age, gender, weight, body surface area), pre-chemotherapy laboratory tests (e.g., serum creatinine, estimated glomerular filtration rate (Levey et al. 2003) blood urea nitrogen, urinary albumin excretion, electrolytes, liver function tests, and complete blood count, medical and medication history, and physical examination data was collected.

The Institutional Review Boards at the University of Colorado (COMIRB Protocol 12-1510) and Rutgers University (Protocol E13-716) approved protocols for recruitment and sample collection. The investigations were carried out in accordance with the rules of the Declaration of Helsinki. (World Medical 2013)

DNA Isolation

Blood (5 mL) was collected in heparinized tubes from recruited patients who were scheduled to receive or had historically received cisplatin therapy. Whole blood underwent centrifugation at 2500x*g* for 10 minutes at room temperature to obtain buffy coats for DNA extraction. Resultant buffy coats were aliquoted into 1.5 mL tubes and frozen at -80°C until DNA isolation. DNA from buffy coats was extracted and purified per FlexiGene® DNA Handbook (QIAGEN) protocol and stored in a stock concentration of 20 ng/mL. DNA was stored at -80°C until subsequent genotyping.

Genotyping

Genotyping was performed using QuantStudio 12K Flex Real-Time PCR System at the University of Utah Genomics Core Facility. Custom Open Array Chips (Life Technologies) were designed with selected polymorphisms of interest including transporters (e.g., *SLC22A2, ABCC2, SLC47A1*), regulatory (e.g., *NFE2L2, KEAP1*) and metabolism (e.g., *GSTA1, GSTP1, GGT1*) genes. For the Chip, DNA primer sequences

were created based on polymorphism ID from NCBI polymorphism Database. Taqman Genotype Software was used to code each genotype as 0 for homozygous wildtypes (wildtype/wildtype), 1 for heterozygous (wildtype/variant), and 2 for homozygous variants (variant/variant) in order to perform statistical analyses.

Collection of Urine Samples

Urine samples for protein biomarkers were obtained from all patients who were prospectively scheduled to receive cisplatin treatment. Urine samples were collected at baseline (pre- infusion or at 0-2 h after infusion), between 2-5 days (denoted as Day 3) and 9-11 days (designated as Day 10) post-cisplatin infusion. Urine was centrifuged at 3,000*xg* and the supernatant was aliquoted for subsequent biomarker assays. All samples were frozen at -80°C within 30-60 minutes following collection. At time of analysis, samples were thawed, placed on ice and centrifuged at 1500 rpm for 5 minutes. Ten µL of supernatant was used for biomarker analyses.

Assessment of Urinary Biomarkers

Urinary samples for protein biomarkers (e.g., calbindin, clusterin, KIM-1, GST-pi, IL-18, MCP-1, albumin, B2M, cystatin C, NGAL, osteopontin, TFF3) were washed using a Bio-Plex Pro II wash station (Bio-Rad) and then analyzed using Bio-Plex, MagPix Multiplex Reader (Bio-Rad). The resultant mean fluorescence intensity (MFI) was used to calculate respective concentrations of each biomarker in the sample. Analysis was completed per protocol with dilutions of 1:10 for panel 1 and 1:50 for panel 2. When concentrations fell outside of the detection limit of the assay, they were diluted and re-analyzed or substituted with the lower limit of quantification divided by 2. The eGFR was obtained from the medical record at baseline and after the first cisplatin dose and calculated using the CKD-EPI equation. (Levey et al. 2003) Additional details on

biomarker analysis methods was previously published. (George et al. 2017) Biomarker data was not normalized to urinary creatinine as our previous study demonstrated similar findings with absolute and normalized data. (George et al. 2017) Biomarker data was assayed at baseline, and days 3 and 10 post cisplatin administration.

Statistical Analysis

Patient demographic data includes the group mean ± standard deviation. Hardy Weinberg equilibrium was used to analyze allelic frequencies by Chi Square Tests. Differences in eGFR or biomarker changes by genotype groups were evaluated by Students T-test or ANOVA with Dunn's multiple comparisons posthoc test. Differences were considered statistically significant at $p < 0.05$. Due the pilot and hypothesis generating nature of the research and the limited sample of patients who had matched genomics and urinary biomarker data, we did not apply any FDR adjustments on p values. Patients with missing values were excluded from analysis. All statistical analyses and graphs were completed by GraphPad Prism V6 (GraphPad Software, La Jolla, CA), Partek Genomics Suite (Partek GS 6.4, St. Louis, CA), and SAS 9.4 (SAS Institute Inc., Cary, NC)

Table A-1.1. Patient Characteristics (n=206) Data presented as mean ± standard deviation	
Age	53±14 years
BSA	1.9±0.3 m^2
Gender	51% male : 49% female
Weight	80±20 kg
Race	92% White: 8% Other
First Cisplatin Dose	59±25 mg/m^2
Total Dose	479±219 mg
Baseline Serum Creatinine	0.9±0.2 mg/dL
Baseline eGFR	91±21 $mL/min/1.73m^2$
Cancer Etiologies (number, %)	Genital (54, 26%) Head and Neck (41, 20%) Melanoma (31, 15%) Lung (25, 12%) Digestive (21, 10%) Urinary (18, 9%) Other (16, 8%)

Abbreviations:

Body surface area – BSA

Estimated glomerular filtration rate – eGFR (Levey et al. 2003)

Table A-1.2. Genotype and Allelic Frequencies for Transport, Metabolism, and Regulatory Genes Relevant to Cisplatin Disposition (N=206)

Gene	Variant	Homozygous Wildtype Frequency Observed		Heterozygous Frequency Observed		Homozygous Variant Frequency Observed		Undetermined	Major Allele Frequency Observed (expected)		Minor Allele Frequency Observed (expected)	
SLC22A2 (OCT2)	rs316019*	C/C	0.679	A/C	0.187	A/A	0.014	0.120	C	0.824 (0.79)	A	0.118 (0.21)
	rs3127573	A/A	0.737	A/G	0.211	G/G	0.010	0.043	A	0.858 (0.88)	G	0.10 (0.12)
	rs2279463	A/A	0.665	A/G	0.201	G/G	0.005	0.014	A	0.869 (0.88)	G	0.097 (0.12)
	rs596881	C/C	0.741	C/T	0.230	T/T	0.010	0.019	C	0.861 (0.89)	T	0.098 (0.11)
SLC31A1 (CTR1)	rs7851395	A/A	0.306	A/G	0.431	G/G	0.187	0.077	A	0.553 (0.53)	G	0.431 (0.47)
	rs12686377	C/C	0.718	A/C	0.158	A/A	0.038	0.086	C	0.847 (0.92)	A	0.194 (0.08)
SLC47A1 (MATE1)	rs2289669	G/G	0.278	A/G	0.464	A/A	0.196	0.062	G	0.527 (0.54)	A	0.443 (0.46)
ABCC2 (MRP2)	rs717620	C/C	0.603	C/T	0.258	T/T	0.043	0.096	C	0.776 (0.81)	T	0.207 (0.19)
	rs2273697*	G/G	0.531	A/G	0.325	A/A	0.053	0.091	G	0.729 (0.82)	A	0.23 (0.18)
	rs3740066	C/C	0.397	C/T	0.368	T/T	0.144	0.091	C	0.63 (0.62)	T	0.379 (0.38)
GGT1	rs4820599	A/A	0.464	A/G	0.349	G/G	0.100	0.086	A	0.681 (0.73)	G	0.316 (0.27)
GSTP1	rs1695*	A/A	0.354	A/G	0.402	G/G	0.129	0.033	A	0.63 (0.59)	G	0.372 (0.41)
KEAP1	rs11085735	C/C	0.746	A/C	0.139	A/A	0.014	0.100	C	0.864 (0.91)	A	0.118 (0.09)
	rs1048290	C/C	0.282	C/G	0.407	G/G	0.129	0.182	C	0.531 (0.68)	G	0.359 (0.32)
NFE2L2 (NRF2)	rs2886162	A/A	0.239	A/G	0.459	G/G	0.220	0.081	A	0.489 (0.58)	G	0.469 (0.42)
	rs1806649	C/C	0.512	C/T	0.311	T/T	0.053	0.124	C	0.716 (0.77)	T	0.23 (0.23)
	rs1962142	G/G	0.000	A/G	0.670	A/A	0.244	0.086	G	0 (0.92)	A	0.494 (0.08)
	rs2706110	C/C	0.560	C/T	0.344	T/T	0.086	0.010	C	0.748 (0.8)	T	0.293 (0.2)
	rs6721961	G/G	0.665	G/T	0.225	T/T	0.024	0.091	G	0.815 (0.8)	T	0.155 (0.2)

*denotes non-synonymous variant

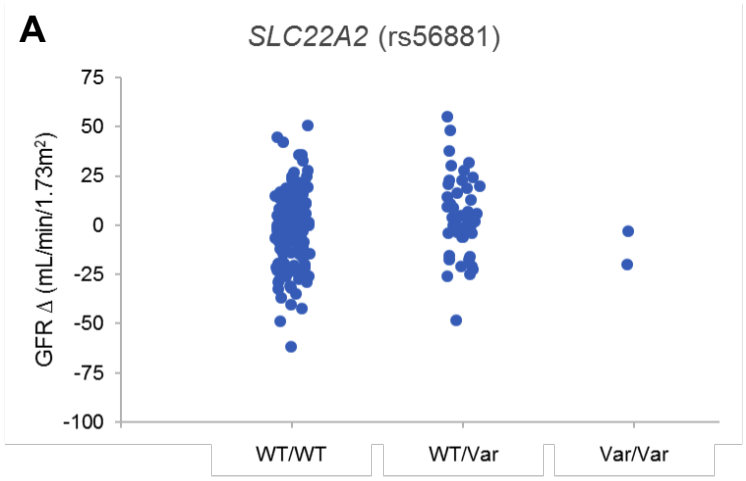
Table A-1.3. Associations between Variant Genotypes and Fold Change Increases in Urinary Biomarkers of Kidney Injury (N=57)*

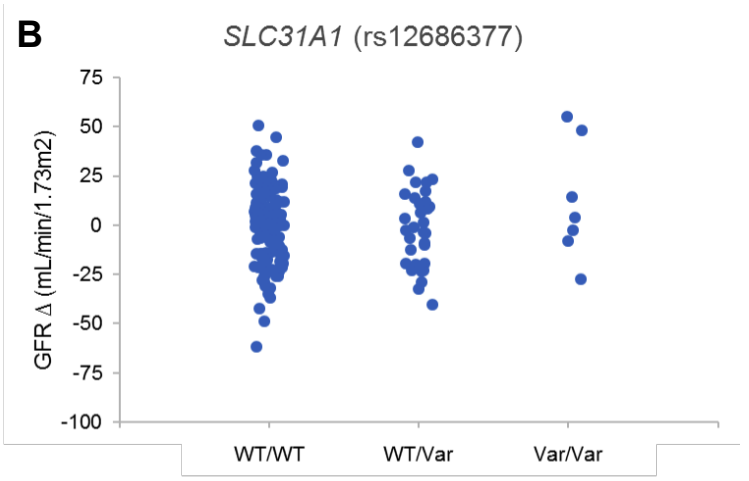
Gene	Variant	Protein Biomarker	Time	Fold Change (WT/WT vs. WT/Var)	p-value	Fold Change (WT/WT vs. Var/Var)	p-value
SLC22A2 (OCT2)	rs596881	B2M	Day 3	-2.134	0.039	2.474	0.057
		Osteopontin	Day 3	1.918	0.049	-1.052	0.972
	rs316019	KIM-1	Day 3	1.77E+171	0.038	N/A	N/A
		KIM-1	Day 10	-1.38E+84	0.046	N/A	N/A
SLC31A1 (CTR1)	rs7851395	Osteopontin	Day 3	1.341	0.488	2.509	0.015
SLC47A1 (MATE1)	rs2289669	KIM-1	Day 3	1.379	0.636	3.605	0.007
		MCP-1	Day 3	1.322	0.629	2.952	0.015
ABCC2 (MRP2)	rs717620	Clusterin	Day 3	3.534	0.024	-2.981	0.854
		Cystatin C	Day 3	2.627	0.034	1.279	0.910
	rs3740066	Calbindin	Day 3	1.900	0.017	1.068	0.883
		Calbindin	Day 10	2.732	0.023	-1.269	0.822
		Clusterin	Day 3	1.170	0.870	4.384	0.012
		Cystatin C	Day 3	1.601	0.447	3.094	0.038
		NGAL	Day 3	2.110	0.030	1.011	0.986
	rs2273697	KIM-1	Day 3	1.153	0.730	5.966	4.29E-05
		KIM-1	Day 10	1.163	0.636	2.808	0.042
		Calbindin	Day 3	1.124	0.687	2.648	0.038
		MCP-1	Day 3	-1.215	0.635	3.500	0.010
GSTP1	rs1695	KIM-1	Day 3	1.195	0.732	2.690	0.029
		Calbindin	Day 3	1.369	0.354	2.371	0.012
		IL-18	Day 10	-1.566	0.337	2.287	0.012
		NGAL	Day 3	1.294	0.572	2.569	0.027
KEAP1	rs1048290	NGAL	Day 3	-1.849	0.211	2.051	0.035
	rs11085735	Calbindin	Day 10	2.944	0.019	-17.366	0.661
		Cystatin C	Day 3	3.275	0.011	1.207	0.928
		Cystatin C	Day 10	2.600	0.027	-2.481	0.751
		TFF3	Day 3	1.747	0.038	1.466	0.619
		TFF3	Day 10	1.776	0.034	-1.629	0.685
NFE2L2	rs2886162	Calbindin	Day 10	3.486	0.029	1.53	0.710
	rs1806649	IL-18	Day 3	-1.298	0.692	3.744	0.002
		TFF3	Day 3	-1.133	0.709	2.337	0.004
	rs1962142	TFF3	Day 10	-1.638	0.028	N/A	N/A
	rs2706110	MCP-1	Day 10	1.236	0.621	3.182	0.002

*only significant figures included

Abbreviations:

Beta-2 microglobulin (B2M), Glutathione-s-transferase pi 1 (GSTP1), Interleukin 18 (IL-18), Kidney Injury Molecule 1 (KIM-1), Monocyte chemotactic protein 1 (MCP-1), Neutrophil gelatinase-associated lipocalin (NGAL), Trefoil factor 3 (TFF3), Variant (VAR), Wildtype (WT)


A
SLC22A2 (rs56881)
GFR Δ (mL/min/1.73m²)
75
50
25
0
-25
-50
-75
-100
WT/WT
WT/Var
Var/Var


B
SLC31A1 (rs12686377)
GFR Δ (mL/min/1.73m2)
75
50
25
0
-25
-50
-75
-100
WT/WT
WT/Var
Var/Var

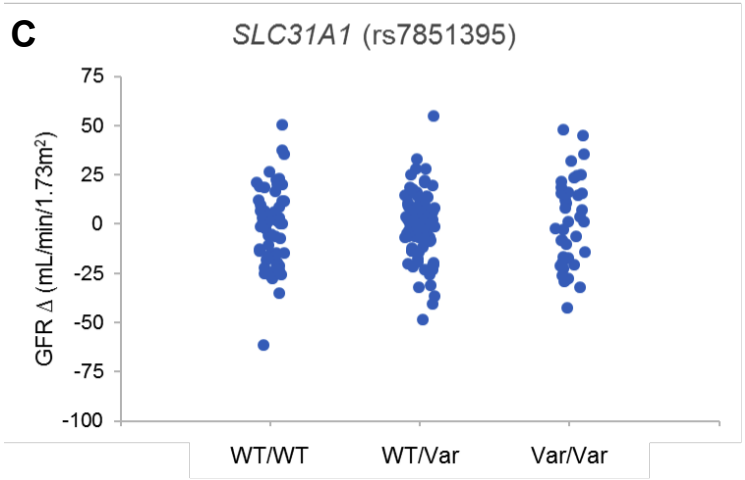
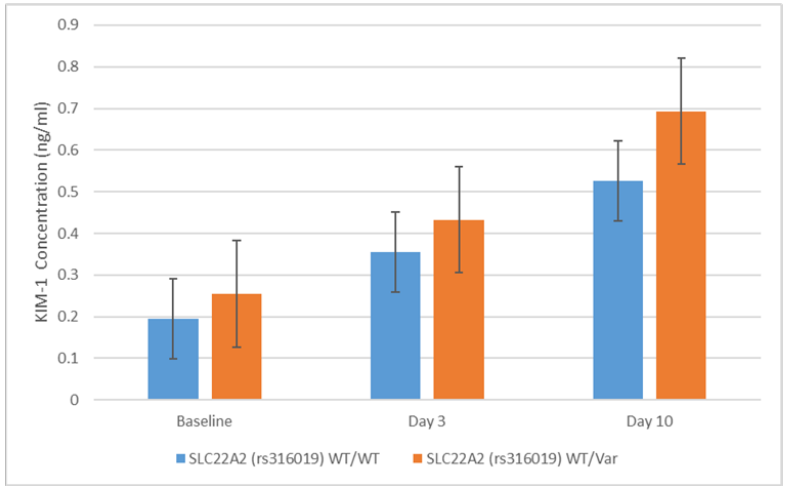

C
SLC31A1 (rs7851395)
GFR Δ (mL/min/1.73m²)
75
50
25
0
-25
-50
-75
-100
WT/WT
WT/Var
Var/Var

Figure A-1.1. A. Comparisons of *SLC22A2* (rs586881) genotypes on eGFR in ambulatory cancer patients prescribed cisplatin. Patients carrying a variant allele of rs586881 in *SLC22A2* exhibited statistically significant increases in eGFR (p=0.01). B and C. Comparisons of *SLC31A1* (rs12686377 and rs7851395) genotypes on eGFR in ambulatory cancer patients prescribed cisplatin. Homozygous variant patients for *SLC31A1* (rs12686377 and rs7851395) exhibited eGFR protection with cisplatin therapy (p=0.01 and p=0.04). Graphs indicate percent changes in eGFR from baseline.

Abbreviations:
Estimated glomerular filtration rate – eGFR, Glutathione *S*-transferase pi 1 – GSTP1, Wildtype – WT, Variant – VAR.

A


KIM-1 Concentration (ng/ml)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Baseline
Day 3
Day 10
SLC22A2 (rs316019) WT/WT
SLC22A2 (rs316019) WT/Var

B

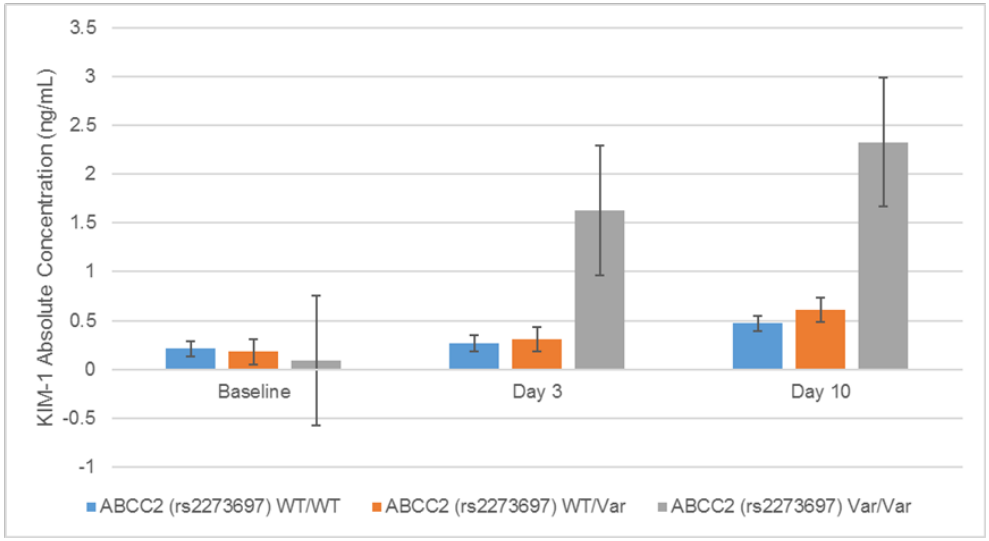

KIM-1 Absolute Concentration (ng/mL)
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
-1
Baseline
Day 3
Day 10
ABCC2 (rs2273697) WT/WT
ABCC2 (rs2273697) WT/Var
ABCC2 (rs2273697) Var/Var

Figure A-1.2. A. Comparison of absolute KIM-1 concentrations to *SLC22A2* (rs316019) genotype in ambulatory cancer patients prescribed cisplatin. Statistically significant increases of KIM-1 at baseline (p=0.02), Day 3 (p=0.03), and Day 10 (p=0.046) were demonstrated in patients expressing the *SLC22A2* rs316019 variant. B. Comparison of absolute KIM-1 concentrations to *ABCC2* (rs2273697) genotype in ambulatory cancer patients prescribed cisplatin. Statistically significant increases of KIM-1 at baseline (p=0.02), Day 3 (p=0.03) and Day 10 (p=0.046) were demonstrated in patients expressing the *ABCC2* rs2273697 variant. Error bars represent standard deviations.

Abbreviations: Kidney injury molecule 1 – KIM-1, Wildtype – WT, Variant – Var

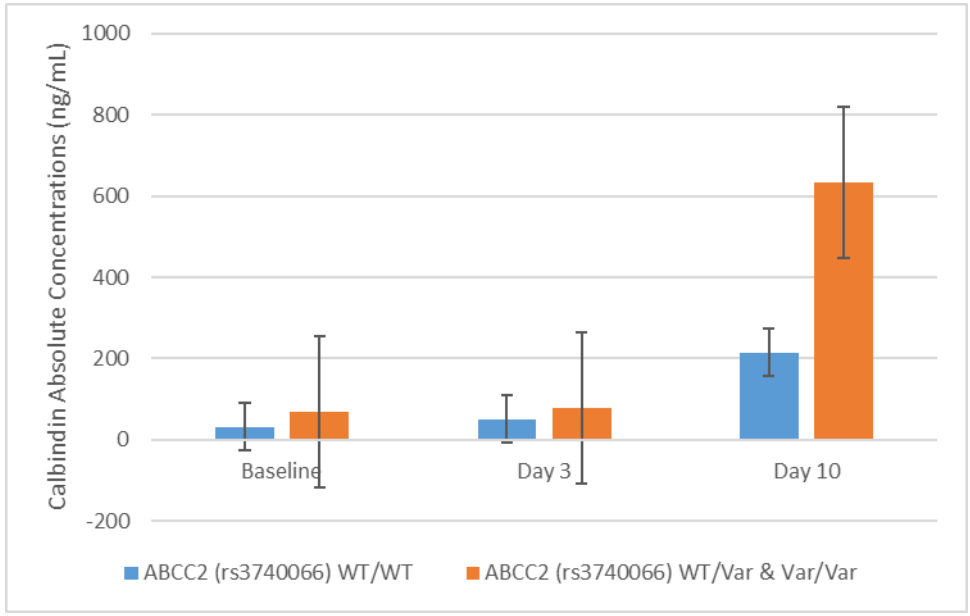
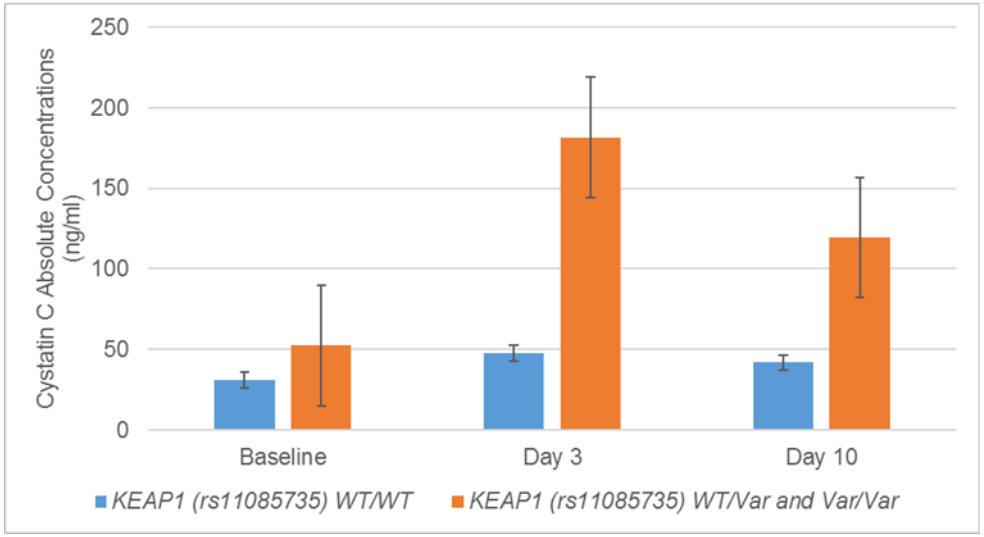


Figure A-1.3. Comparison of calbindin absolute concentrations to *ABCC2* (rs3740066) genotype in ambulatory cancer patients prescribed cisplatin. Absolute concentrations of calbindin were elevated in patients with the variant allele at all time points; statistically significant at baseline (p=0.047), Day 3 (p=0.02), and Day 10 (p=0.02) versus homozygous wildtype patients. Error bars represent standard deviations.

Abbreviations: Wild type – WT, Variant – Var

A


250
200
150
100
50
0
Cystatin C Absolute Concentrations (ng/ml)
Baseline
Day 3
Day 10
KEAP1 (rs11085735) WT/WT
KEAP1 (rs11085735) WT/Var and Var/Var

B

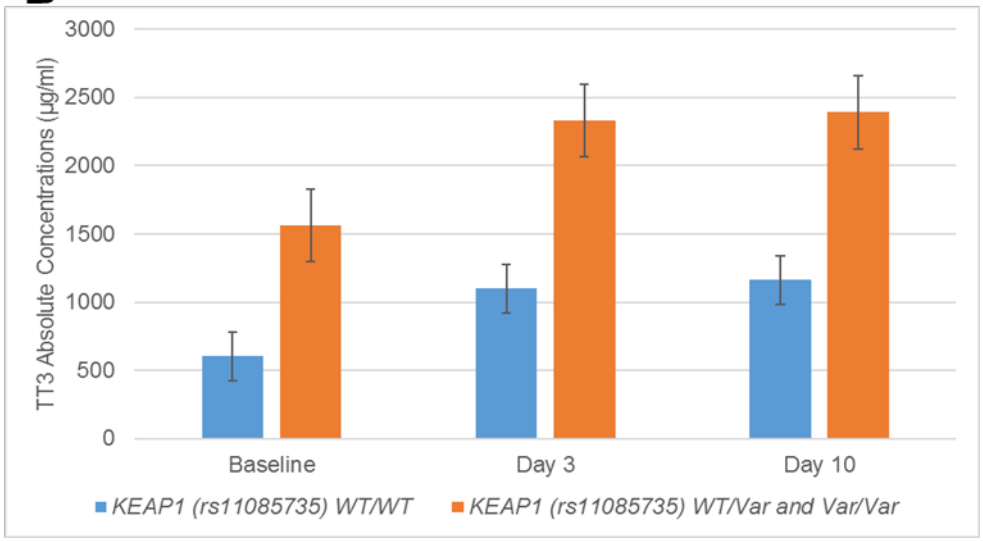

3000
2500
2000
1500
1000
500
0
TT3 Absolute Concentrations (µg/ml)
Baseline
Day 3
Day 10
KEAP1 (rs11085735) WT/WT
KEAP1 (rs11085735) WT/Var and Var/Var

Figure A-1.4. Variant alleles in *KEAP1* (rs11085735) are correlated with increased concentrations of TFF3 and cystatin C. Figure A. Absolute concentrations of cystatin C showed a statistically significant increase on Day 3 (p=0.01) and Day 10 (p=0.03) in patients expressing the variant allele of *KEAP1*. Figure B. Absolute concentrations of TFF3 were increased at baseline (p=0.01), Day 3 (p=0.03) and Day 10 (p=0.03) in patients who carried at least one variant copy of *KEAP1*. Error bars represent standard deviations.

Abbreviations: Trefoil factor 3 – TFF3, Wildtype – WT, Variant – Var

APPENDIX 2: The Product of Tissue Inhibitor of Metalloprotease 2 (TIMP2) and Insulin Growth Factor Binding Protein 7 (IGFBP7) is Elevated at Baseline in Ambulatory Cancer Patients Prescribed Cisplatin

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A-2.1 Abstract

Identifying acute kidney injury (AKI) early in patients receiving cisplatin is problematic due to a lack of sensitive indicators and inherent delay in detection imposed by traditional biomarkers such as serum creatinine. In order to prevent, limit, and manage AKI, clinicians need sensitive and specific methods to detect toxicity in a timely manner after cisplatin therapy so that interventions can be readily implemented. The purpose of this pilot study was to describe urinary concentrations of the tissue inhibitor of metalloprotease 2 (TIMP2) and insulin growth factor binding protein 7 (IGFBP7) proteins, either alone or in combination in ambulatory cancer patients at baseline and after receiving cisplatin and evaluate receiver operating characteristic (ROC) curves for predicting exposures and AKI risks. Urinary TIMP2, IGFBP7, and TIMP2*IGFBP7 product analyses failed to demonstrate any statistically significant changes from baseline to follow-up at either day 3 or day 10 post cisplatin. At baseline, 91% of ambulatory cancer patients receiving cisplatin had a TIMP2*IGFBP7 product of 43.2 $(ng/mL)^2/1000$, which was greater than in previous reports. ROC curves failed to demonstrate significant utility of TIMP2, IGFBP7, or TIMP2*IGFBP7 at day 3 and day 10 for predicting exposures. ROC curves for the combination of TIMP2*IGFBP7 (absolute and urinary creatinine normalized) in predicting decreases in kidney function by estimated glomerular filtration rate (eGFR) demonstrated good to excellent utility with values of 0.80 to 0.92. While the current study cannot support adoption of the AKI predictive value of > 0.3 for TIMP2*IGFBP7, changes in eGFR can be reasonably predicted in ambulatory cancer patients receiving cisplatin. Additional studies will be needed to determine the applicability of cut-points for cisplatin AKI predictions.

A-2.2 Introduction

Cisplatin is a platinum-based traditional chemotherapeutic agent used for the treatment of solid tumors, including lung, head and neck, ovarian, and testicular. While cisplatin is widely prescribed because of survival benefits as an anti-cancer agent, associated adverse events present multiple challenges to patients and clinicians. In particular, acute kidney injury (AKI) due to cisplatin is problematic and can occur in one-third of patients despite aggressive hydration (Akcay et al. 2010). There are four principle mechanisms described for cisplatin-induced nephrotoxicity including direct proximal tubular injury, oxidative stress, inflammation, and vascular injury (Ozkok and Edelstein 2014). A common challenge to identifying early cisplatin AKI is a lack of sensitivity and inherent delay in detection imposed by traditional biomarkers such as serum creatinine, estimated glomerular filtration rate (eGFR), blood urea nitrogen, and urine output assessments. In order to enable clinicians to prevent, limit, and manage AKI, and prevent negative effects on patient survival, sensitive and early detection methods are needed so that interventions can be implemented.

AKI is a multifactorial process that is associated with an increased risk of mortality and significant short- and long- term complications (Doyle and Forni 2016). The Kidney Disease Improving Global Outcomes (KDIGO) guidelines suggest that serum creatinine and eGFR are the best traditional biomarkers of kidney function and should be measured as often as possible after nephrotoxin exposures (Mellas 2016). However, serum creatinine and eGFR have several shortcomings inherent to creatinine homeostasis including a time-lag from injury to detection of parameter changes, as well as influences of patient covariates (age, race, muscle mass, hydration status, protein intake) on creatinine generation. Additionally, eGFR assessments assume that serum creatinine is constant within patients, which may not be true during AKI. The limitations

of traditional biomarkers for detecting changes to kidney function and damage demonstrate the need to identify other reliable biomarkers to accurately detect cisplatin-induced AKI early and with a high degree of sensitivity and specificity.

Recently, the discovery and validation of two biomarkers, tissue inhibitor of metalloprotease 2 (TIMP2) and insulin growth factor binding protein 7 (IGFBP7), have demonstrated the ability to predict which critically ill and hospitalized surgical patients are at risk for AKI (Bihorac et al. 2014). When used in conjunction and obtaining the product of the combination, TIMP2 and IGFBP7 were found to be significantly more sensitive and superior to other evaluated biomarkers and exhibited improved risk stratification of AKI (Kashani et al. 2013, Bihorac et al. 2014, Hoste et al. 2014). Both of these proteins are involved in the G1 phase of cell cycle arrest during the early phases of cell injury (Bihorac et al. 2014). Based on experimental sepsis and ischemia data, renal tubular cells enter cell cycle arrest following damage to allow time for cell repair or death (Bihorac et al. 2014).

A recent review cited drug-induced nephrotoxicity as an important area for investigation of TIMP2 and IGFBP7 utility (Vijayan et al. 2016). The purpose of this pilot study was to describe urinary concentrations of TIMP2 and IGFBP7, either alone or in combination, in ambulatory cancer patients receiving cisplatin and evaluate whether similar AKI risk assessment scores to hospitalized critically ill patients are applicable. The urinary excretion of these protein biomarkers has not been reported in cancer patients or patients in the ambulatory environment.

A-2.3 Methods

Patients scheduled to receive intravenous cisplatin ≥ 25 mg/m^2 administered over 1-2 h for solid tumors (head/neck, digestive, genital, lung, bladder, urothelial, breast, or melanoma) were recruited into a biomedical review board approved study at the University of Colorado Cancer Center. All patients received anti-emetic regimens with the chemotherapy protocol, consisting of at least oral dexamethasone and ondansetron. Other eligibility criteria included age ≥18 years, hemoglobin ≥10 g/dL, eGFR >60 mL/min^2, normal liver function (ALT and AST <2x ULN), and willingness to comply with the study. Exclusion criteria included diagnosis of kidney cancer, previous exposure to platinum-based chemotherapy, herbal supplement use, concurrent use of inhibitors of cisplatin transport proteins, consumption of grapefruit juice or alcohol within 7 days, history of alcohol consumption of >14 drinks/week, organ transplantation or kidney dialysis, pregnant or lactating, and changes to medication regimens within 4 weeks of study entry.

Traditional clinical biomarkers of kidney function (serum creatinine, eGFR, blood urea nitrogen, urinary albumin) were measured at the time of the study or abstracted from the medical record within 30 days of the study and recorded for up to 30 days after completion of the study. Additional data collection included demographics (race, age, gender) and body mass index (BMI), cancer type, and cisplatin dosing information.

Urine was collected into clean catch specimen containers and centrifuged at 3000x*g* to collect the supernatant at baseline, and on Days 3 and 10 post cisplatin dose to quantify TIMP2 and IGFBP7 concentrations. Urine was aliquoted into 2 mL collection tubes and frozen at -80˚C within 30-60 minutes of collection. At the time of analysis, samples were thawed and placed on ice and centrifuged at 1500 rpm for 5 minutes. TIMP2 (#DTM200,

R&D Systems, Minneapolis, MN) and IGFBP7 (#KA4219; Abnova, Taiwan) were quantified by commercial ELISA assays using manufacturer protocols. For TIMP2, the dynamic range of the assay was 0.156-10 ng/mL with sensitivity of 0.064 ng/mL. For IGFBP7, the dynamic range of the assay was 625-40,000 pg/mL with sensitivity of <20 pg/mL. Given the different units of TIMP2 and IGFBP7, the TIMP2*IGFBP7 product is calculated as $(ng/mL)^2/1000$. Sample concentrations were corrected for background and dilution factors. Data are presented as absolute concentrations and normalized to urinary creatinine concentrations quantified using the DCA Vantage Analyzer (Siemens, Princeton, NJ).

The TIMP2*IGFBP7 product was calculated in order to compare data to the published AKI risk score according to the NephroCheck® FDA approved product labeling. The NephroCheck® has a reportable range for AKI risk score of between 0.04 and 10.0. The studies contributing to the labeling define an AKI risk score of >0.3 in conjunction with clinical assessment as predictive for moderate or severe AKI in critically ill patients within twelve hours of assessment (Kashani et al. 2013, Bihorac et al. 2014). Studies in populations with chronic stable conditions have defined a reference range for TIMP2*IGFBP7 as 0.04-2.25 $ng/mL^2/1000$ (Chindarkar et al. 2015). Since kidney injury molecule 1 (KIM-1) has been reported as a novel biomarker for AKI secondary to cisplatin in several reports (Vaidya et al. 2006, Vaidya et al. 2010, Sinha et al. 2013, Shin et al. 2014, Wadey et al. 2014, Nan-Ya et al. 2015, Shinke et al. 2015), it was also measured and reported. The KIM-1 assay was performed using a Bio-Plex Pro RBM human kidney toxicity assay panel I (Bio-Rad, Life Science, Hercules, CA) according to manufacturer instructions. Samples are analyzed using a MagPix Multiplex Reader (Bio-Rad).

Statistical Analyses

Descriptive analyses using means ± standard deviations and percentages were reported for demographic and baseline clinical data. Differences between absolute and urinary creatinine corrected baseline and follow-up biomarkers (TIMP2, IGFBP7, TIMP2*IGFBP7) at days 3 and 10 post cisplatin were evaluated by ANOVA. Receiver Operator Curves (ROC) for AUC (AUROC) of TIMP2, IGFBP7, and TIMP2*IGFBP7 (absolute and urinary creatinine corrected) were determined from Day 0 to 3 and Day 0 to 10 post cisplatin. AUROC for prediction of reductions in eGFR using TIMP2, IGFBP7, and TIMP2*IGFBP7 (absolute and urinary creatinine corrected) were determined at baseline, day 3 and day 10 after cisplatin administration. The models for prediction of reduction in eGFR were adjusted by age, race, gender, and body mass index. Relationships between absolute TIMP2, IGFBP7, TIMP2*IGFBP7 biomarkers at day 3 and day 10 post cisplatin and eGFR and KIM-1 were assessed using correlation analyses. Data were analyzed by Instat v3.0 (GraphPad, Inc., La Jolla, CA) and SAS version 9.4 (SAS Institute, Cary, NC, USA), with p values <0.05 considered statistically significant.

A-2.4 Results

Patients (n=46) scheduled to receive outpatient chemotherapy with cisplatin were recruited to participate. The population was primarily Caucasian (89%), similar in gender (46% male; 52% female), and exhibited normal kidney function by serum creatinine, blood urea nitrogen, urinary albumin, and eGFR at baseline (**Table A-2.1**).

The results from urinary TIMP2, IGFBP7, and TIMP2*IGFBP7 product analyses failed to demonstrate any statistically significant changes from baseline to follow-up at either day 3 or 10 post cisplatin (**Table A-2.2**). However, at baseline, 91% of ambulatory cancer

patients receiving cisplatin had a TIMP2*IGFBP7 mean product of 43.2 $(ng/mL)^2/1000$, which was above the values of 0.04-2.25 $ng/mL^2/1000$ determined in healthy populations as (Chindarkar et al. 2015).

Receiver operating characteristic (AUROC) curves were applied to assess relationships between urinary concentrations of protein biomarkers pre- and post- treatment, with each ROC curve estimating the ability of the biomarker to predict exposure. **Table A-2.3** includes the absolute and urinary creatinine normalized AUC values for TIMP2, IGFBP7, and TIMP2 and IGFBP7 at day 3 and day 10. The AUROC curve estimates were higher for all three measurements at day 10 versus day 3. Receiver operating characteristic (ROC) curves were also applied to assess prediction of eGFR reductions, as a traditional marker of change in kidney function, at baseline, day 3, and day 10 after cisplatin administration (**Figure A-2.1**). The AUROC curve estimates the ability of the biomarker to predict cisplatin nephrotoxicity. **Table A-2.4** includes the absolute and urinary creatinine normalized AUROC values for TIMP2, IGFBP7, and TIMP2 and IGFBP7 at baseline, day 3 and day 10. The models using TIMP2 and IGFBP7 (absolute and normalized to urinary creatinine) demonstrated good to excellent AUROC values at day 3 and day 10 for predicting reductions in eGFR. At day 3, TIMP2 (absolute and normalized) did not predict a reduction in eGFR as well as IGFBP7 alone or combining TIMP2 and IGFBP7.

Urinary TIMP2, IGFBP7, and TIMP2*IGFBP7 product at days 3 and 10 post cisplatin exhibited a low degree of correlation with eGFR (**Table A-2.5**). The urinary TIMP2, IGFBP7 and TIMP2*IGFBP7 product at day 10 post cisplatin exhibited moderate correlation coefficients with KIM-1 (**Table A-2.5**).

A-2.5 Discussion

According to two observational cohort studies of hospitalized patients, nephrotoxicity due to drugs occurs in 14 to 26% of patients (Mehta et al. 2004, Hoste et al. 2015). Given the FDA approval of the Nephrocheck® as the first biomarker risk assessment of clinical AKI in the U.S., we evaluated TIMP2, IGFBP7, and TIMP2*IGFBP7 biomarker assessments in ambulatory cancer patients receiving cisplatin therapy. The results from the current study failed to demonstrate any statistically significant changes in these biomarkers from baseline to day 3 or day 10 after cisplatin treatment. However, the majority of the ambulatory cancer patients had a TIMP2*IGFBP7 product at baseline that exceeded the values found in patients with chronic stable conditions. Area under the receiver operating characteristic curves (AUROC) failed to demonstrate good relationships between urinary concentrations of protein biomarkers pre- and post-cisplatin treatment. However, in general, the AUROC curve estimates were higher for all three biomarker measurements at day 10 versus day 3. On the contrary, ROC curves applied to assess prediction of eGFR reduction at baseline, day 3, and day 10 after cisplatin administration demonstrated that the model that included TIMP2 and IGFBP7 product (absolute and normalized to urinary creatinine) had good to excellent AUROC values. We also reported that while the biomarker values in ambulatory cancer patients receiving cisplatin were poorly correlated with eGFR (-0.03 to 0.19) as a traditional biomarker for nephrotoxicity, there was a moderate degree of correlation with the novel biomarker KIM-1 (-0.06 to 0.31), particularly at day 10. For both ROC curve analysis, TIMP2, IGFBP7, and TIMP2*IGBP7 that were normalized to urinary creatinine exhibited higher predictive values than curves generated from absolute biomarker values at days 3 and 10 post cisplatin.

Identification of novel biomarkers that can predict and/or detect AKI has been an ongoing area for investigation given the poor specificity of traditional clinical measures including serum creatinine and eGFR. Several pre-marketing studies were conducted to support the FDA approval of TIMP2*IGFBP7 (Nephrocheck®) for the prediction of AKI (Zuo et al. 2012, Kashani et al. 2013, Bihorac et al. 2014). Kashani et al., demonstrated that TIMP2 and IGFBP7 were the best performing biomarkers out of a panel to detect AKI in hospitalized intensive care unit patients who had at least one risk factor (Kashani et al. 2013). The Sapphire study was the validation study demonstrating that TIMP2*IGFBP7 >0.3 was a major risk of AKI in intensive care unit patients (Kashani et al. 2013). Meanwhile, the Opal study was a verification study in the same population, that evaluated two different cutoff values (0.3 and 2.0) for TIMP2*IGFBP7 and reported relative risk for AKI (Hoste et al. 2014). For the Opal and Sapphire studies, the TIMP2*IGFBP7 0.3 cut-off value sensitivity was 89% and specificity was 50-53%. For the TIMP2*IGFBP7 2.0 cut-off value, sensitivity was 42-44% and specificity was 90-95%. Finally, the Topaz study validated the ability of TIMP2*IGFBP7 at the 0.3 cutoff to detect AKI in critically ill patients at high risk for developing moderate to severe AKI within 12 hours (Bihorac et al. 2014). ROC curves generated from the Opal and Topaz studies demonstrated values of 0.79 to 0.82, suggestive of a good to excellent clinical diagnostic test. While the current study did not assess AKI, per the criteria used in the described studies, ROC curves using eGFR reduction demonstrated good to excellent AUROC values for the model using TIMP2 and IGFBP7 at day 3 (0.8670 to 0.9236) and day 10 (0.7993 to 0.8435) after cisplatin treatment using absolute and normalized to urinary creatinine values, respectively.

In addition to the studies described above that contributed to FDA approval and product labeling of the Nephrocheck®, several other populations have been studied. Two

studies reported the utilizing of TIMP2*IGFBP7 for the prediction of AKI in adult surgical patients (Gocze et al. 2015, Gunnerson et al. 2016). Gunnerson, et al. reported TIMP2*IGFBP7 ROC curve of 0.84 for predicting development of moderate to severe AKI within 12 hours in surgical patients within intensive care units (Gunnerson et al. 2016). Gocze, et al., reported highest values for TIMP2*IGFBP7 in septic (1.24), transplant (0.45), and hepatic surgery (0.47) patients (Gocze et al. 2015). The ROC curve was 0.85 for predicting development of AKI. Patients with non-surgical, stable chronic medical conditions (e.g. hypertension, gout, congestive heart failure, etc.), but without AKI, were also assessed for TIMP2*IGFBP7 in reference to healthy patients (Chindarkar et al. 2015). The results demonstrated TIMP2*IGFBP7 values that were comparable to healthy patients without co-morbid conditions supporting a lack of test interference by common medical conditions (Chindarkar et al. 2015).

The concentrations of TIMP2 and IGFBP7 found in the current study, in reference to the published literature, indicate that the pathogenesis of cisplatin induced AKI may be vastly different from AKI in critically ill patients. TIMP2 and IGFBP7 proteins are both involved in G1 cell cycle arrest, which occurs in the early stages of cell injury. Other experiments have indicated that renal tubular cells enter G1 cell cycle arrest after sepsis and ischemia (Kashani et al. 2013), which are acute developments in critically ill patients. In contrast, solid tumors grow over time as a result of alterations in native DNA. To limit tumor growth, the affected cells will undergo cell cycle arrest to repair DNA as a chronic process (Hanahan and Weinberg 2000). Thus it would follow that TIMP2 and IGFBP7, originating from other distant sites and being filtered through the kidney, would be elevated at baseline in cancer patients. It is known that TIMP2 and IGFBP7 spreads from the site of injury and acts as a signal of acute stress for the rest of the body (Kashani et al. 2013).

The current study has some important limitations to note. The patient population was not stratified based on possible confounding factors such as race, gender, baseline level of kidney function, cancer diagnosis, drug metabolism/transport genotypes, and concomitant drugs that might impact TIMP2 and IGFBP7. However, because all of the biomarker results were compared against within the individual patient, it is unlikely that these would have drastically changed the results of the study. As true AKI, using criteria that includes serum creatinine and eGFR was absent in our patients, the ability to really evaluate prediction of AKI was limited. Lastly, we used separate ELISA assays to measure TIMP2 and IGFBP7, and its product, as opposed to using the NephroCheck® diagnostic device.

A-2.6 Conclusions

Despite their validation as novel biomarkers of AKI in critically ill patients, assessments of urinary TIMP2 and IGFBP7 have not been reported in cisplatin-induced nephrotoxicity. The reported data demonstrated that these biomarkers were elevated at baseline in cancer patients as compared to previous reports in other populations. While the ROC analysis that included TIMP2 and IGFBP7 did a good job at predicting reductions in eGFR, additional studies will be needed to define potential cut-points for cisplatin AKI predictions.

Table A-2.1. Patient (n=46) Demographics at Baseline

Parameter	Mean ± SD
Age (years)	56 ± 14
Race Caucasian Black Hispanic or Latino	 89% 2% 9%
Gender Male Female Not Reported	 46% 52% 2%
BSA (m^2)	1.9 ± 0.3
BMI (kg/m^2)	26.8 ± 5.9
% Cancer Types Melanoma Genital Breast Lung Head/Neck Digestive Bone/Blood Bladder/Pelvis Lymphoma/Sarcoma	 13% 13% 2% 17% 24% 18% 4% 7% 2%
Concurrent Radiation Treatment	46%
Cisplatin Dose (mg/m^2)	64.2 ± 22.4
Serum Creatinine (mg/dL)	0.8 ± 0.2
Blood Urea Nitrogen (mg/dL)	13.3 ± 4.5
Urinary Albumin (g/mL)	11.8±15.0
eGFR (mL/min/1.73 m^2)	91.0 ± 22.3
KIM-1 (ng/mL)	0.24 ± 0.32

Abbreviations:
Body mass index – BMI
Body surface area – BSA
Kidney injury molecule 1 – KIM-1

Table A-2.2. Biomarkers at Baseline, Day 3 and Day 10

	TIMP2 (ng/mL) (mean±SD)	IGFBP7 (pg/mL) (mean±SD)	TIMP2*IGFBP7 (ng/mL)^2/1000 (mean±SD)
Baseline			
Absolute	4.1 ± 4.0	8,822 ± 4,193	43.2 ± 57.0
Corrected	7.3 ± 11.7	20,040 ± 20,309	78.9 ± 164
Day 3			
Absolute	3.3 ± 3.1	7,395 ± 3,567	23.4 ± 25.6
Corrected	6.3 ± 9.9	12,919 ± 12,037	42.8 ± 74.7
Day 10			
Absolute	4.1 ± 3.4	9,468 ± 3,833	40.0 ± 43.1
Corrected	5.8 ± 9.0	11,958 ± 13,941	41.5 ± 81.3

Corrected represents normalization to urinary creatinine.

Abbreviations:

Insulin growth factor binding protein 7 – IGFBP7

Tissue inhibitor of metalloprotease 2 – TIMP2

Table A-2.3. Area Under the Receiver Operating Curve (AUROC) Values for Exposure

Biomarker	Absolute AUROC (p value)	Normalized AUROC (p value)
TIMP2		
Day 3	0.5304 (0.6314)	0.5710 (0.2484)
Day 10	0.4617 (0.5499)	0.5932 (0.1403)
IGFBP7		
Day 3	0.5905 (0.1435)	0.5868 (0.1524)
Day 10	0.5571 (0.3767)	0.6299 (0.0336)
TIMP2*IGFBP7		
Day 3	0.5641 (0.3093)	0.5943 (0.1186)
Day 10	0.4607 (0.5420)	0.6069 (0.0878)

Abbreviations:

Insulin growth factor binding protein 7 – IGFBP7

Tissue inhibitor of metalloprotease 2 – TIMP2

Table A-2.4: ROC Curve Comparisons for Predicting Decreases in eGFR

Biomarkers	Days	ROC Models*	AUROC with 95% (CI)	P values**
Absolute	baseline	TIMP2 & IGFBP7	0.7540 (0.61, 0.90)	reference
		IGFBP7	0.7659 (0.62, 0.91)	0.5137
		TIMP2	0.7480 (0.60, 0.89)	0.8857
	3 days	TIMP2 & IGFBP7	0.8670 (0.75, 0.98)	reference
		IGFBP7	0.8107 (0.67, 0.95)	0.2542
		TIMP2	0.7340 (0.57, 0.89)	0.053
	10 days	TIMP2 & IGFBP7	0.7993 (0.64, 0.95)	reference
		IGFBP7	0.8027 (0.65, 0.96)	0.4795
		TIMP2	0.7993 (0.64, 0.95)	1.000
Normalized	baseline	TIMP2 & IGFBP7	0.7605 (0.62, 0.90)	reference
		IGFBP7	0.7644 (0.62, 0.90)	0.8223
		TIMP2	0.7625 (0.62, 0.90)	0.8961
	3days	TIMP2 & IGFBP7	0.9236 (0.84, 1.00)	reference
		IGFBP7	0.8819 (0.78, 0.98)	0.2631
		TIMP2	0.7569 (0.60, 0.91)	0.0283
	10days	TIMP2 & IGFBP7	0.8435 (0.72, 0.97)	reference
		IGFBP7	0.8464 (0.72, 0.97)	0.6888
		TIMP2	0.8522 (0.73, 0.97)	0.6118

*All models were adjusted by age, race, gender, and body mass index.

** P values were used to compare receiver operator characteristic (ROC) curves using the nonparametric approach of DeLong, et al. (DeLong et al. 1988).

Abbreviations:

Insulin growth factor binding protein 7 – IGFBP7

Tissue inhibitor of metalloprotease 2 – TIMP2

Table A-2.5. Correlation Coefficients (r) Between TIMP2 and IGFBP7 Urinary Biomarkers, eGFR, and KIM-1

	eGFR	KIM-1
TIMP2 (Day 3)	0.1494	0.0178
TIMP2 (Day 10)	-0.0316	0.3069
IGFBP7 (Day 3)	-0.0463	-0.0724
IGFBP7 (Day 10)	-0.1215	0.2197
TIMP2*IGFBP7 (Day 3)	0.1907	-0.0657
TIMP2*IGFBP7 (Day 10)	-0.1349	0.3090

Abbreviations:

Estimated glomerular filtration rate – eGFR

Kidney injury molecule 1 – KIM-1

Insulin growth factor binding protein 7 – IGFBP7

Tissue inhibitor of metalloprotease 2 – TIMP2

Figure A-2.1A

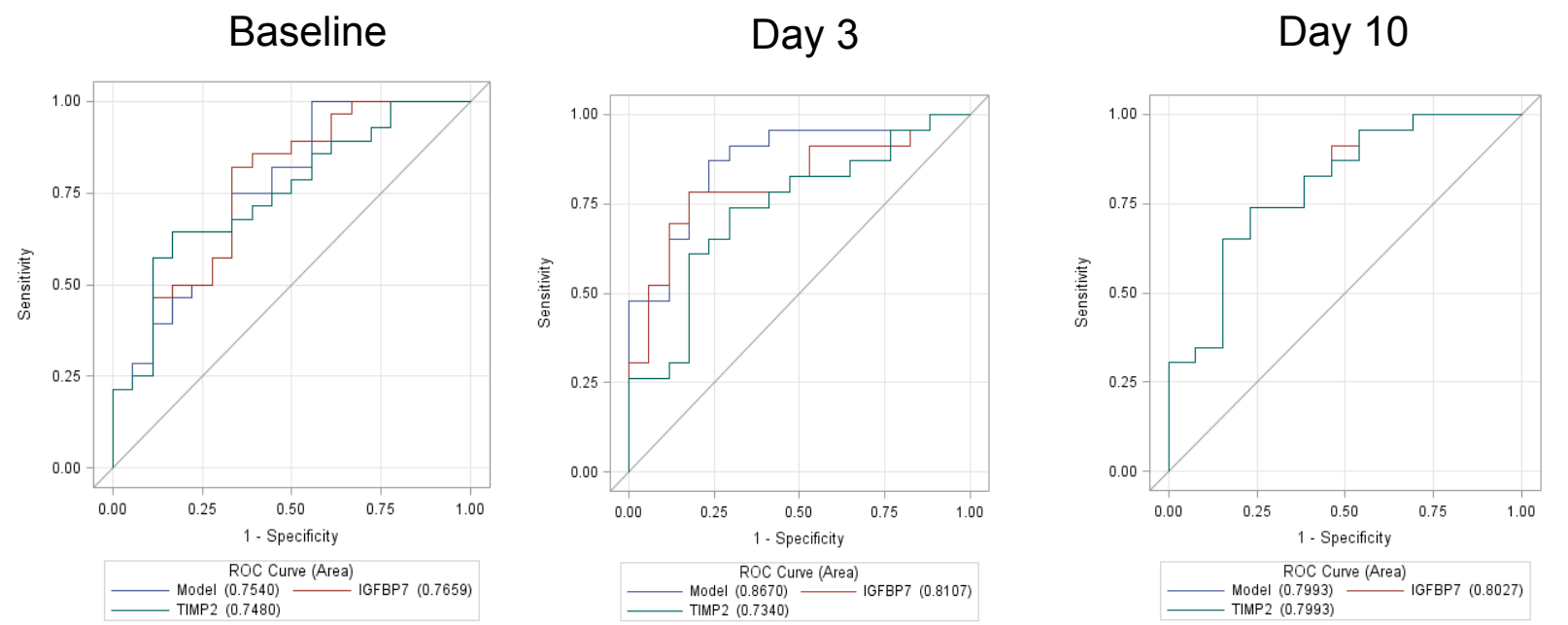
Baseline
Day 3
Day 10
Sensitivity
1 - Specificity
0.00
0.25
0.50
0.75
1.00
ROC Curve (Area)
Model (0.7540)
IGFBP7 (0.7659)
TIMP2 (0.7480)
Model (0.8670)
IGFBP7 (0.8107)
TIMP2 (0.7340)
Model (0.7993)
IGFBP7 (0.8027)
TIMP2 (0.7993)

Figure A-2.1B

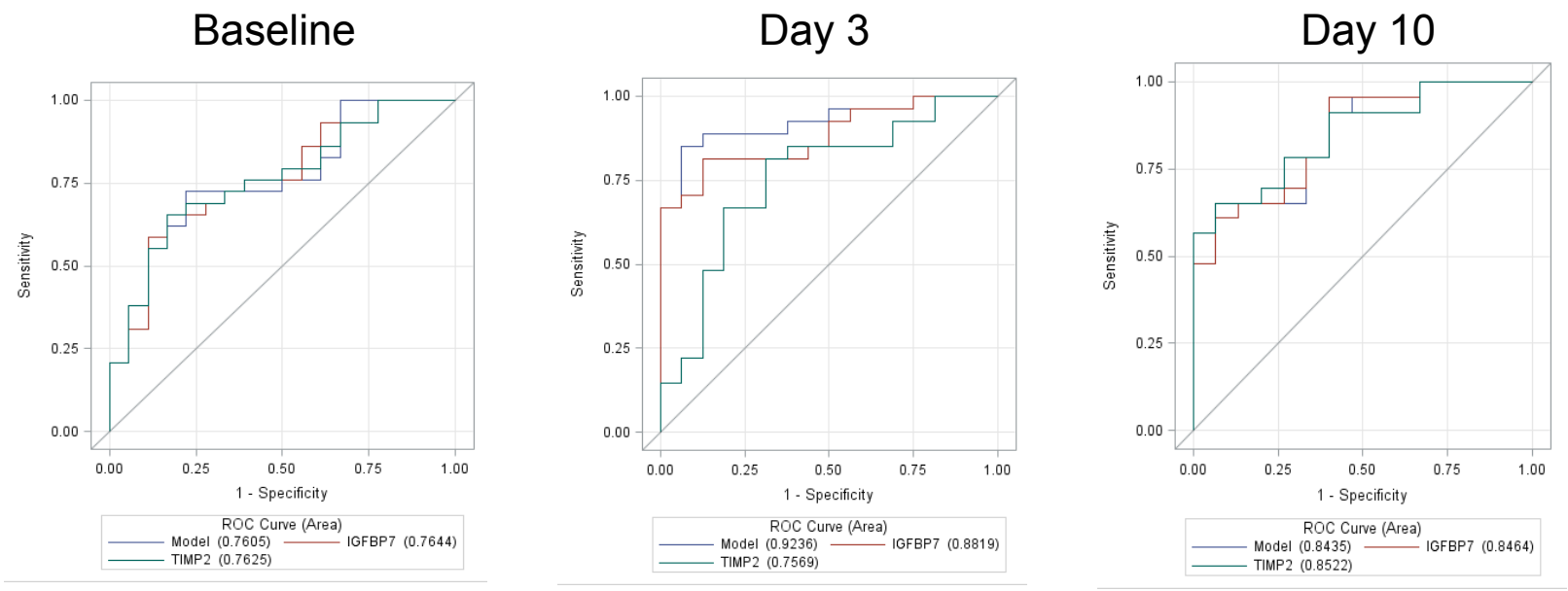

Figure A-2.1. ROC Curve Comparisons for Predicting a Decrease in eGFR using Absolute Biomarkers (TIMP2 and IGFBP7) in Ambulatory Cancer Patients Prescribed Cisplatin. Models were adjusted by age, race, gender, and body mass index. "Model" in figure indicates combined use of TIMP and IGFBP7. Area under the receiver operating characteristic curve (AUROC) values reflect predictions in detecting reductions in eGFR. Graphs show ROC curves and AUROC values for urinary biomarkers on days 3 and 10. a) ROC curves at baseline, day 3, and day using absolute biomarkers, b) ROC curves at baseline, day 3, and day 10 using absolute biomarkers.

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