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Contribution of Organic Cation Transporter 2 (OCT2) to Cisplatin-Induced Nephrotoxicity

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Contribution of Organic Cation Transporter 2 (OCT2) to Cisplatin-Induced Nephrotoxicity

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CONTRIBUTION OF ORGANIC CATION TRANSPORTER 2 (OCT2) TO CISPLATIN-INDUCED NEPHROTOXICITY

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Kelly K. Filipski
May 2009

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I would like to first acknowledge my parents for their endless support, without them I would not be where I am today.

I would also like to show my appreciation for all the members of the Baker and Sparreboom labs, particularly Shelley Orwick for her patience and the many lessons she has taught me. I extend my gratitude to my committee members Dr. Sharyn Baker, Dr. Bernd Meibohm, Dr. Lawrence Pfeffer, and Dr. Phil Potter for their helpful suggestions.

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ABSTRACT

Cisplatin is the most widely used anticancer agent; however, the cellular pharmacokinetics are poorly understood. Cisplatin is predominantly eliminated through the urine via active secretion and is associated with nephrotoxicity. Currently, prehydration therapy is employed to prevent toxic renal side effects; however it has not been completely ameliorated. The studies described herein aim to determine the mechanism in which cisplatin enters the kidney cell from the blood and how it is subsequently secreted into the urine. Organic cation transporter 2 (OCT2) and ABCC2 are highly expressed in the kidney on the basolateral and apical membrane, respectively. We determined the contribution of OCT2 and ABCC2 to cisplatin transport and toxicity. We also evaluated the contribution of genetic variation in both transporters to cisplatin pharmacokinetics. Our results suggest a prominent role for OCT2 in the cellular accumulation of cisplatin *in vivo* and *in vitro*, whereas, ABCC2 may only play a limited role in cisplatin pharmacokinetics in conjunction with other ABC transporters. OCT2 also significantly influences cisplatin induced nephrotoxicity indicating a potential for new preventative strategies to circumvent toxicity.

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CHAPTER 1. INTRODUCTION

1.1 Cisplatin

Cis-diamminedichloroplatinum (cisplatin) was first identified in 1965 when platinum electrodes stopped cell division in *Escherichia coli* cells. Preliminary testing in mice with a dose of 8 mg/kg showed marked tumor regression.[1] Subsequent clinical trials indicated that cisplatin had promising antitumor activity and was approved by the FDA in 1978.[2,3] Cisplatin is used as the foundation of curative treatment in testicular and ovarian cancers, and is currently the most commonly used anticancer agent due to its broad spectrum of activity against other malignant solid tumors, including lung, head and neck, bladder, germ cell, endometrial, and cervical cancer.[2,4,5]

Evidence suggests that DNA is the major cytotoxic target of cisplatin. After it enters the cell, the high concentration of water and low concentration of chloride in tissue facilitates the aquation of cisplatin at one or both of the chloride groups thereby activating it.[2] Cisplatin then covalently binds to the N7 position of purine bases with the major product (approximately 50-65 % of all adducts) being intrastrand crosslinks between adjacent guanine residues. A smaller portion of product includes crosslinks between adjacent guanine and adenine bases.[3,6] These adducts cause DNA damage, such as unwinding or bending of the DNA template, potentially causing arrest of DNA synthesis and replication (**Fig 1.1**).[7] Candidate proteins induced by platinum adducts include damage recognition proteins, such as, hMSH2, hMutSα, HMG1, and HMG2. These proteins not only recognize the DNA damage, but could, in fact, promote cytotoxicity. For example, HMG1 overexpression sensitizes breast tumor cells to cisplatin by shielding the DNA adduct from repair mechanisms. Other evidence suggests that DNA adducts can sequester factors important to transcription, thus preventing the process. Together these events can lead to cell cycle arrest, p53 activation, and finally apoptosis via the activation of caspase 9 and caspase 3.[8]

1.2 Cisplatin Toxicity

Cisplatin is a potent antitumor agent; however, it is associated with a dose-limiting renal tubule dysfunction when administered in the conventional 3-weekly or 4-weekly treatment regimen.[5] Clinical markers of cisplatin induced acute renal failure include high serum creatinine, reduced serum magnesium and potassium, as well as, decreases in glomerular filtration rate. Although it has not been definitively defined, studies indicate that cisplatin nephrotoxicity can lead to long term dysfunction in glomerular filtration.[7] Chronic nephrotoxicity causes structural damage primarily to the renal tubule. In particular, tubular cells in patients treated with cisplatin have cystic dilated tubules lined by flattened epithelium with atypical nuclei.[9]

Cisplatin induced nephrotoxicity is characterized by tubular cell death with both necrosis and apoptosis contributing to the damage. The highest cisplatin concentrations

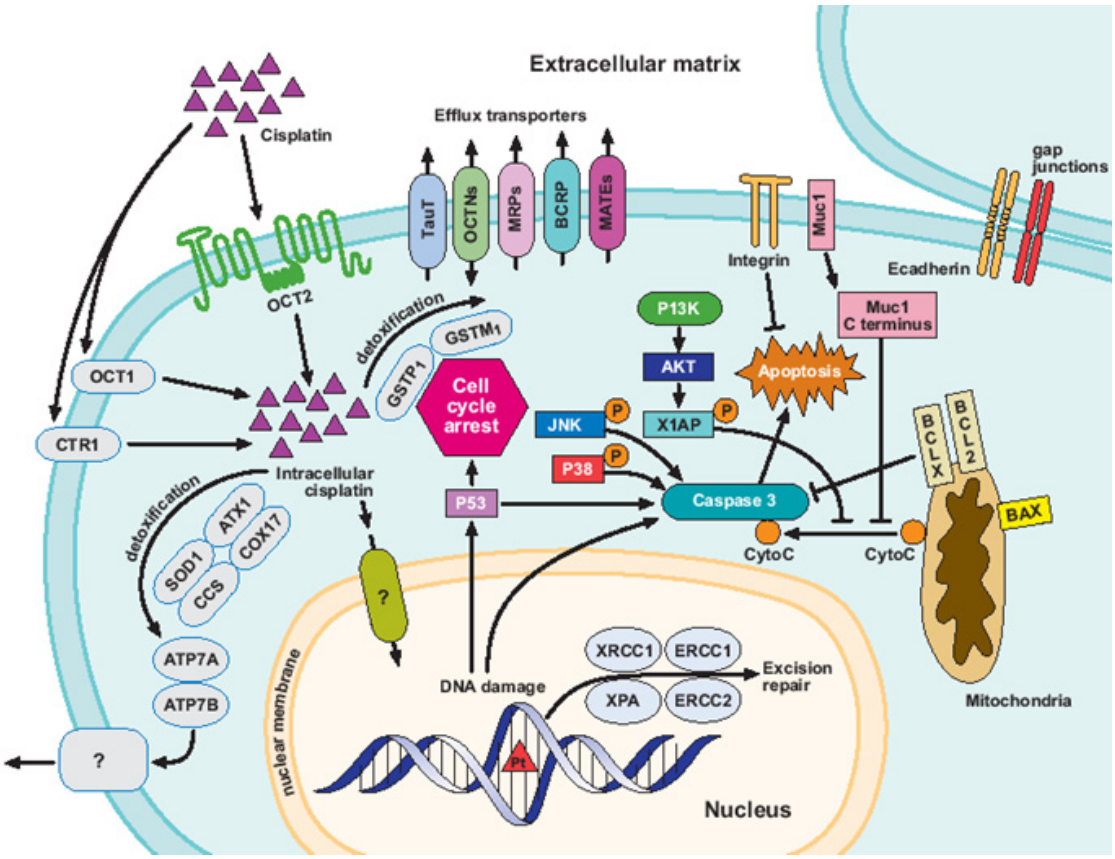


Figure 1.1 Possible Mechanisms of Cisplatin-Induced Toxicity

Possible cellular pathways involved in cisplatin-induced toxicity in cells. Cisplatin enters the cell and forms platinum-DNA adducts, initiating a cascade of events leading to cell death.

are found in the inner cortex and outer medulla proximal tubular cells.[10] Several cell death pathways have been attributed to cisplatin toxicity. Death receptor tumor necrosis factor-α (TNF-α) receptor (TNFR) 2 has been studied and shown to be involved in cisplatin toxicity by ameliorating renal failure in mice deficient in TNFR 2.[11] This result suggests an important role for TNF-α signaling. However, it is not clear whether the activation of the death receptors leads to apoptosis, or if the inflammation induced by TNF-α is the regulator of the damage.[12] Recent evidence suggests that renal tubular cells, not infiltrating inflammatory cells, contribute to the production of TNF-α after cisplatin treatment.[13] This mechanism could lead to the pathological conditions seen in cisplatin nephrotoxicity.

Other factors implicated in cisplatin induced renal damage include cell cycle regulation and the p53 pathway. The cyclin dependent kinase (CDK) inhibitor, p21, has been shown to be induced during cisplatin nephrotoxicity leading to protection of the renal tubular cells. Mice lacking p21 suffered from a more rapid onset of renal failure than wildtype animals after exposure to cisplatin indicating a renoprotective role for p21.[7,14,15] After cisplatin exposure, p53 is activated in renal tubular cells. Furthermore, mice lacking p53 are resistant to cisplatin induced renal failure while wildtype animals show an increase in p53 activity in renal tubular cells following cisplatin exposure. This resistance suggests an important role of p53 in tubular cell death. Events leading to p53 activation are not fully understood, however, genotoxic stress induced by Pt-DNA adducts may trigger its activation.[7,8,16,17]

Currently, hydration therapy is used in conjunction with cisplatin treatment to protect the kidney from cisplatin induced damage. Patients receive a prehydration, as well as, a posthydration infusion containing sodium chloride. In some cases, mannitol- or furosemide induced diuresis is used as well. This combination is thought to reduce the concentration of cisplatin in the kidney.[7,9] As mentioned previously, the reactive aquated form of cisplatin is responsible for DNA damage and subsequent cell death. The increase in the concentration of chloride in renal tubular cells may minimize the aquation of cisplatin, thereby decreasing the amount of reactive cisplatin in the kidney. This combined therapy has been shown to decrease cases of severe renal failure in patients treated with cisplatin. However, approximately one-third of patients treated with cisplatin will still experience with cisplatin induced nephrotoxicity within the first 10 days of treatment.[7,9]

1.3 Organic Cation Transporters Involved in Platinum Transport

Due to the wide use of cisplatin in the treatment of cancer, many investigators have studied the route of entry for cisplatin into the cell. In previous studies using isolated perfused rabbit proximal tubules, cisplatin accumulation in the tubular cells was temperature dependent, with the S3 segment accumulating approximately 400% more platinum than the S1 or S2 segments at 38°C. At 23°C, cisplatin concentrations were not significantly higher than zero in the tubules. These results suggested that a transport mechanism, primarily present in the S3 segment of the tubules was responsible for

cisplatin accumulation in kidney cells. In subsequent experiments, the addition of tetraethylammonium (TEA) inhibited the transport of cisplatin into tubular cells.[18] Cisplatin has also been shown to accumulate in human renal cortex slices against a concentration gradient,[19] and has the ability to competitively inhibit the active uptake of TEA by mouse kidney slices[20] and by basolateral membrane vesicles from the rat renal cortex.[21] Furthermore, cisplatin inhibits the renal clearance of organic ions from the basolateral site of the isolated-perfused rat kidney.[5,22] All of this evidence implicates a significant role for an organic cation transporter (OCT) to be the primary mechanism in which cisplatin enters the cell.

Organic cation transporters are members of the SLC22A superfamily. These transporters are polyspecific and are involved in the absorption and excretion of various endogenous and exogenous compounds. The family consists of 18 genes and includes the organic cation transporters (OCTs), organic cation/carnitine transporters (OCTNs), and organic anion transporters (OATs).[23,24] Most of the members share a common membrane topology consisting of a predicted 12 α-helical transmembrane domains and two hydrophilic loops.[23,25] Although they share common structure, the transporters in the SLC22A family show differential tissue distribution (**Fig 1.2**). The genes encoding the three organic cation transporters (*SLC22A1-3*) are all located in a cluster on chromosome 6.[23-25]

1.3.1 Organic cation transporter 1 (OCT1)

Organic cation transporter 1 was first cloned in the rat in 1994. It is primarily expressed on the sinusoidal membrane of hepatocytes. However, in rodents it is expressed on the basolateral membrane of proximal tubules and enterocytes. OCT1 typically transports organic cations, but other charged molecules and weak bases can also be transported. The main function of OCT1 is believed to be the transport of compounds through the sinusoidal membrane leading to the excretion of the compound.[26]

Transport of cisplatin via OCT1 has been previously investigated yielding controversial results. In HEK293 cells transiently transfected with human OCT1 or rat Oct1, platinum uptake was not significantly different from the empty vector control.[27,28] However, other investigators showed higher cellular accumulation of cisplatin in MDCK cells transfected with human OCT1 when compared to the empty vector control.[29] The different cell lines, as well as, different experimental conditions could explain the inconsistencies within these experiments. As mentioned previously, OCT1 is primarily found on the human hepatocytes, indicating that its role in cisplatin clearance is most likely limited.

1.3.2 Organic cation transporter 2 (OCT2)

Organic cation transporter 2 was also first identified in the rat in 1996. It is primarily located on the basolateral surface of renal proximal tubules, particularly on the

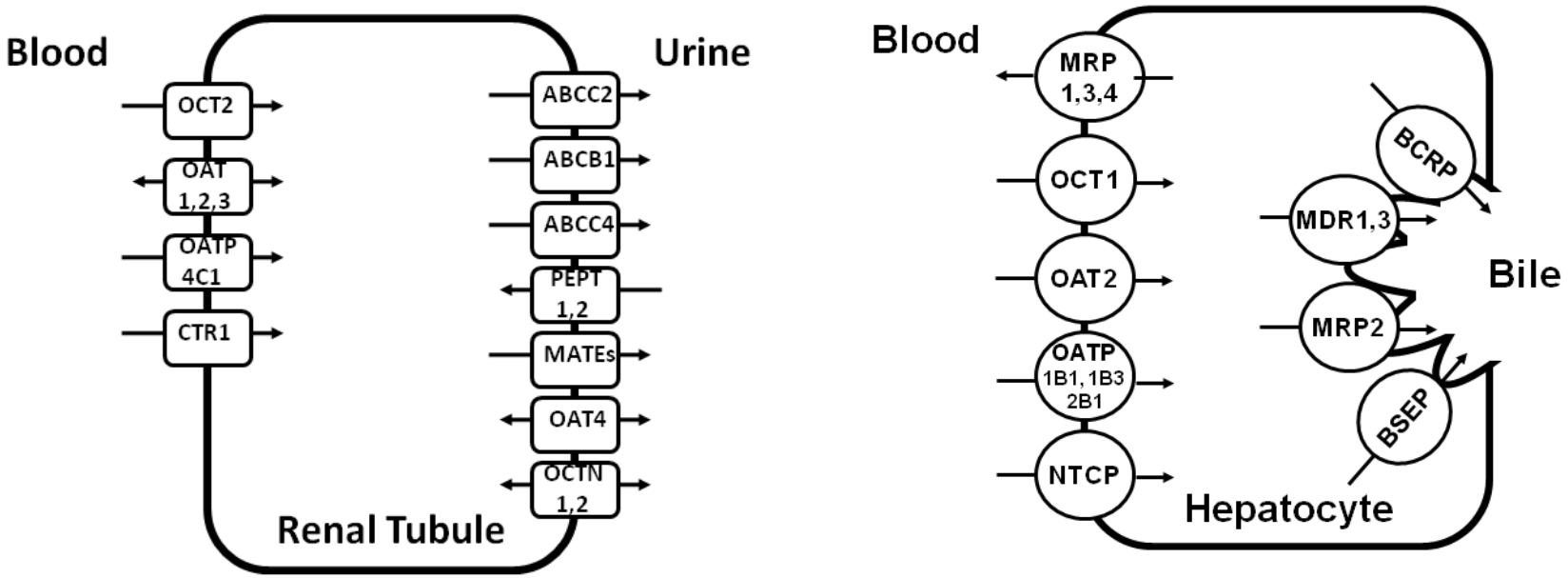


Figure 1.2 Renal and Hepatic Transporter Expression

Expression of solute carrier and ABC transporters in human kidney and liver. Human OCT2 is predominantly expressed on the basolateral surface of kidney proximal tubules, whereas, OCT1 is expressed on human hepatocytes.

S2 and S3 segments with low level expression in the brain. Previously identified OCT2 substrates include TEA, MPP, and cimetidine.[25] In rats, OCT2 expression is dependent on gender and can be regulated by testosterone.[25,30] OCT2 mediates the first step in renal elimination of compounds and may mediate the intracellular concentrations of monoamine neurotransmitters in the brain.[25]

OCT2 transport of cisplatin has been evaluated; although, inconsistent results have been published.[27-30] Currently, the role of OCT2 in cisplatin transport and toxicity is not well understood. Furthermore, several single nucleotide polymorpshisms (SNPs) have been identified in OCT2[31] (**Table 1.1**); however, the functional consequence of these SNPs to cisplatin transport has yet to be fully elucidated.

Due to the localization of OCT2 on the basolateral membrane of renal proximal tubules it is a putative candidate for cisplatin transport into the kidney.

1.4 ABCC2 (MRP2) Involvement in Cisplatin Efflux

Multidrug resistance-associated proteins were first implicated in the efflux of cisplatin after the observation that Pt-conjugated glutathione efflux is ATP dependent.[23] Subsequently, ABCC1 was isolated in cells that were cisplatin resistant with high levels of intracellular glutathione. Further studies indicated no relationship between ABCC1 expression and cisplatin cytotoxicity and accumulation in non-small cell lung cancer cell lines. ABCC1 is not expressed in the kidney, and therefore it is unlikely to be important in cisplatin clearance.[23] Subsequently, ABCC2 (MRP2) was identified as an ATP dependent conjugate export pump with sequence identity very similar to ABCC1. This transporter is localized on the apical membrane of hepatocytes and renal proximal tubules. Due to the localization, the main function of ABCC2 is in the terminal phase of detoxification. Many substrates have been identified for ABCC2 including organic anions and glutathione *S*-conjugates of lipophilic substances.[32]

In renal cells, cisplatin forms glutathione *S*-conjugates before being secreted into the urine. Therefore it is tempting to speculate that ABCC2 may be involved in the efflux of cisplatin from the renal tubules. Previous investigations have suggested ABCC2 involvement. For example, increased ABCC2 levels have been found in multiple cisplatin resistant cell lines[23] and cisplatin resistance increased with the transfection of ABCC2 into HEK293 and MDCK cells.[33] Expression of ABCC2 was associated with cisplatin resistance in patients with resected colon cancer.[23,34]

1.5 *ABCC2* Single Nucleotide Polymorphisms

Several SPNs have been identified in *ABCC2* that may contribute to functional changes in the transporter[35,36] (**Table 1.2**). However, the importance of these SNPs has not been evaluated for cisplatin clearance.

Table 1.1 Nonsynonymous single nucleotide polymorphisms identified in human OCT2

Gene Position	Region	Reference Allele	Variant Allele	Amino Acid Change
160	Exon1	C	T	Pro54Ser
481	Exon 2	T	C	Phe161Leu
493	Exon 2	A	G	Met165Val
495	Exon 2	G	A	Met165Ile
808	Exon 4	G	T	Ala270Ser
890	Exon 5	C	G	Ala297Gly
1198	Exon 7	C	T	Arg400Cys
1294	Exon 8	A	C	Lys432Gln

Table 1.2 Single nucleotide polymorphisms in *ABCC2*

Gene Position	**Region**	**Reference Allele**	**Variant Allele**	**Amino Acid Change**
-1549	5'-flanking	G	A	-
-1019	5'-flanking	A	G	-
-24	5'-UTR	C	T	-
1249	Exon 10	G	A	Val417Ile
-34	Intron 26	T	C	-
3972	Exon 28	C	T	-
4544	Exon 32	G	A	Cys1515Tyr

1.6 Scope and Objective of Dissertation

Previous literature has indicated the importance of potential transporters in the cellular accumulation and subsequent efflux of cisplatin. However, a definitive mechanism has yet to be elucidated. This study sought to identify the primary mechanism by which cisplatin is transported through the cell, as well as, the role of transporters in the renal toxicity of cisplatin. It was hypothesized that organic cation transporter 2 mediates the cellular transport, pharmacokinetics, and toxicity of cisplatin. Specifically, the objectives were as follows:

A. Elucidated the role of organic cation transporter 2 in the cellular uptake of cisplatin by creating a stable cell model expressing OCT2. Using this *in vitro* system, the transport and inhibition properties were established.

B. Determined the effects of OCT2 and ABCC2 expression and genetic variation on cisplatin pharmacokinetics and toxicity using knockout animal models and patient samples. Animal models deficient in the mouse orthologue transporters Oct1, Oct2, both Oct1/2, and Abcc2 were assessed to determine the contribution these transporters play in the urinary excretion and plasma pharmacokinetics of cisplatin. Cisplatin-induced toxicity was also evaluated in these mice. Previous investigators have reported changes in gene expression after cisplatin treatment, therefore using microarray technology, gene expression changes in the kidney of wildtype and knockout mice were assessed after cisplatin treatment. To further understand the importance of OCT2 and ABCC2 in cisplatin pharmacokinetics, genetic variation in both genes was evaluated to identify potential associations with cisplatin pharmacokinetics in patients.

C. Evaluated the role of OCT2 and ABCC2 in tumor sensitivity by studying the expression and genetic variation in the NCI 60 tumor cell lines. OCT2 expression was also determined in human tumor tissue. In order to understand the importance of OCT2 and ABCC2 to cisplatin tumor efficacy, expression of these transporters was determined in a variety of tumor samples, including the NCI 60 tumor cell lines.

CHAPTER 2. INTERACTION OF CISPLATIN WITH THE HUMAN ORGANIC CATION TRANSPORTER 2*

2.1 Introduction

Cis-diamminedichloroplatinum (cisplatin) is a commonly used anticancer drug with a broad spectrum of activity against malignant solid tumors, including lung, head and neck, bladder, germ cell, ovarian, endometrial, and cervical cancer.[4,37] Cisplatin is predominantly eliminated in urine by renal secretion in the proximal tubules.[4] Previous investigations have demonstrated that cisplatin accumulates in human renal cortex slices against a concentration gradient,[19] and that cisplatin competitively inhibits the active uptake of the cation tetraethylammonium (TEA) by mouse kidney slices[20] and by basolateral membrane vesicles from the rat renal cortex.[21] Furthermore, cisplatin inhibits the renal clearance of organic ions from a basolateral site in the isolated-perfused rat kidney.[22] These findings suggest that an organic cation transporter (OCT) likely mediates the cellular uptake of cisplatin.

The expression of the OCT2 transporter is particularly high at the basolateral membrane of renal tubular epithelial cells, and this solute carrier is considered a major transporter in the active secretion of organic cations in the kidney. The aim of the present study was to determine the contribution of OCT2 to cisplatin transport *in vitro*. Taking into consideration previously reported studies, we hypothesize that OCT2 will be the primary regulator of cisplatin transport into cells.

2.2 Materials and Methods

2.2.1 Materials

The Flp-In transfection system, Dulbecco's Modified Eagle's Medium (DMEM), phosphate-buffered saline (PBS), Lipofectamine 2000, hygromycin, zeocin, Opti-MEM reduced serum medium, TRIzol, Superscript III first strand synthesis system, and fetal bovine serum were all obtained from Invitrogen (Carlsbad, CA). The human full length cDNA clone of OCT2 was purchased from Origene (Rockville, MD). American Radiolabeled Chemicals (St. Louis, MO) provided ^{14}C-TEA, and Sigma Chemical Co. (St. Louis, MO) provided cisplatin.

2.2.2 Cell culture and transfection

The Flp-In transfection system was used to produce stably transfected cells expressing OCT2. The full length OCT2 cDNA clone was inserted into the pcDNA5/FRT (pcDNA5/FRT/OCT2) vector and transfected into HEK293 cells

* Portions of this chapter adapted with permission. Filipski, K.K., Loos, W.J., Verweij, J., & Sparreboom, A. Interaction of cisplatin with the human organic cation transporter 2. *Clin Cancer Res* **14**, 3875-3880 (2008).

containing an integrated FRT site. Cells were seeded in 10 mL of DMEM with 10% fetal bovine serum (complete DMEM). Opti-MEM I reduced serum medium was used for the transfection with 1 μg of pcDNA5/FRT/OCT2 (FLP-OCT2) or pcDNA5/FRT (empty vector), 9 μg of pOG44 and 20 μL of Lipofectamine 2000. After 24 hours, medium was replaced with complete DMEM containing hygromycin B (100 μg/mL). Medium was subsequently changed every 2-3 days. Circular colonies were visible 2 weeks after transfection. Cells were collected and re-seeded in either fresh complete DMEM containing hygromycin B, or complete DMEM containing zeocin (100 μg/mL) to test for zeocin sensitivity. Cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were split 1:10 every five days.

2.2.3 RNA extraction, cDNA synthesis and RT-PCR analysis

RNA was extracted from FLP-OCT2 and empty vector cells using the TRIzol reagent, and was reverse transcribed using the Superscript III first strand synthesis system according to the manufacturer's recommendations. Gene transcripts were quantified using SYBR green PCR mastermix. Primers were designed as previously described.[38] Reactions were carried out in 25-μL volumes using the following PCR parameters: 95ºC for 15 min, 40 cycles of 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s, followed by dissociation cycles. Each reaction was carried out in triplicate, and transcripts of each sample were normalized to the housekeeping gene, *GAPDH*.

2.2.4 Cellular uptake

All uptake experiments were performed on monolayer cultures in 6-well plates at 37ºC. Briefly, monolayers were washed once with warm PBS, then DMEM containing ^{14}C-TEA (5 μM) was added to the cells to initiate uptake. After a 5-min incubation, uptake was halted by removing the medium and washing the cells with ice-cold PBS. Cells were collected and solubilized in sodium hydroxide (1 N). Radioactivity was assessed by liquid scintillation counting, and protein concentrations were measured using a bicinchoninic acid (BCA) protein-assay kit (Pierce Biotechnology, Rockford, IL). For transport inhibition assays, cells were pre-incubated with increasing concentrations of cisplatin (up to 1000 μM) for 15 min. Cells were washed with PBS and then incubated for 10 min in medium containing cisplatin and ^{14}C-TEA (2 μM).

To determine if active uptake facilitated cisplatin transport, FLP-OCT2 and empty vector cells were treated with cisplatin (500 μM) at 4°C or 37°C. For measurements of cellular accumulation of cisplatin, FLP-OCT2 and empty vector cells were washed with warm PBS and treated with cisplatin (500 μM) in DMEM at pH 6, 7.4, or 8. A preliminary analysis revealed that the uptake of cisplatin was linear for at least 30 min (data not shown), and therefore subsequent experiments were performed using 30 min incubation periods. Concentration-dependent assays were carried out in DMEM containing cisplatin at concentrations up to 1000 μM. After incubation, cells were washed twice with ice-cold PBS and collected. Next, cells were solubilized with nitric

acid (0.2%), and total platinum was measured using flameless atomic absorption spectrometry using a validated method. Briefly, 20 μL samples were injected in duplicate into a Perkin Elmer AAnalyst 600 atomic absorption spectrometer (Perkin Elmer, Norwalk, CT) with Zeeman background correction to measure platinum content. Peak area measurements were performed at a wavelength of 265.9 nm with a slit width of 0.7 nm. Drug concentrations were determined using a linear-least squares regression analysis through linear calibration curves prepared in drug free nitric acid (0.2%). Protein measurements were done using the BCA protein assay kit.

Pt-DNA adduct experiments were done on monolayer cultures in 100 mm dishes with cells seeded in 10 mL DMEM. Cells were treated with cisplatin (500 μM) in DMEM for 30 min.[39] Cells were washed twice with ice-cold PBS and DNA was isolated as described previously. DNA concentrations were determined spectrophotometrically using the ND-1000 NanoDrop (NanoDrop Technologies) and total platinum was determined using the method described above.

2.2.5 Statistical considerations

Data are presented as mean and SEM, unless stated otherwise. Statistical analyses were performed using a two-tailed t-test, and *P* values of less than 0.05 were considered to be statistically significant. Calculations were performed using the software packages Number Cruncher Statistical Systems, version 2005 (NCSS, J. Hintze, Kaysville, UT) and GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA).

2.3 Results

2.3.1 Characterization of OCT2 overexpressed cells

Expression of *SLC22A2* was 36-fold higher in the FLP-OCT2 cells compared to the vector control cells (**Fig 2.1A**). Transport of the typical organic cation ^{14}C-TEA was 77-fold higher in the FLP-OCT2 cells when compared to vector control cells (**Fig 2.1B**). Therefore, these cells were considered to express a functional transporter and were suitable for subsequent experiments.

2.3.2 Transport studies with cisplatin

To determine if cisplatin is recognized as a substrate for OCT2, we first evaluated the effect of cisplatin on the cellular accumulation of ^{14}C-TEA. Cisplatin had a significant inhibitory effect on the transport of ^{14}C-TEA (2 μM) in FLP-OCT2 cells, and no effect on the vector control cells (**Fig 2.2**). We then examined the transport of cisplatin itself, and found that it was temperature dependent (**Fig 2.3**) and saturable in the FLP-OCT2 cells, with an apparent Michaelis-Menten constant of approximately 34 μM

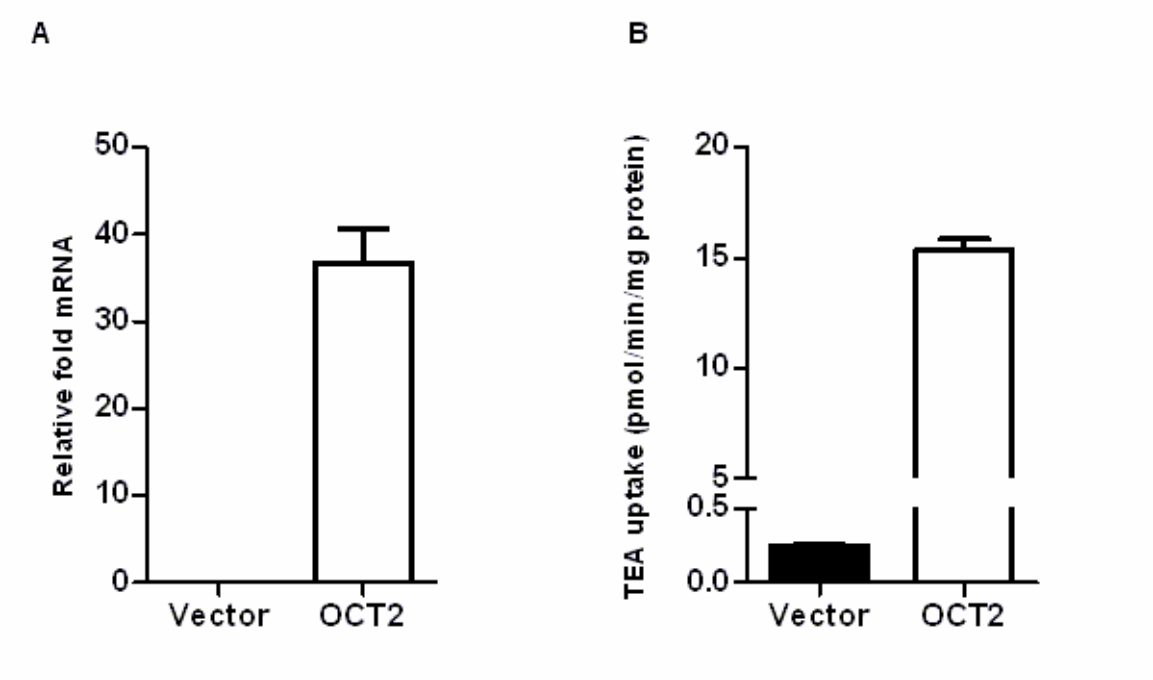


Figure 2.1 Characterization of FLP-OCT2 Cells

(A) mRNA levels of *SLC22A2* in vector control cells and FLP-OCT2 cells as determined by real time RT-PCR. Graphs show expression of *SLC22A2* normalized to *GAPDH*. **(B)** The uptake of ^{14}C-TEA in vector control cells and FLP-OCT2 cells. Cells were incubated with 5 µM ^{14}C-TEA for 5 min. Each bar represents the mean ± SEM of 3 independent experiments.

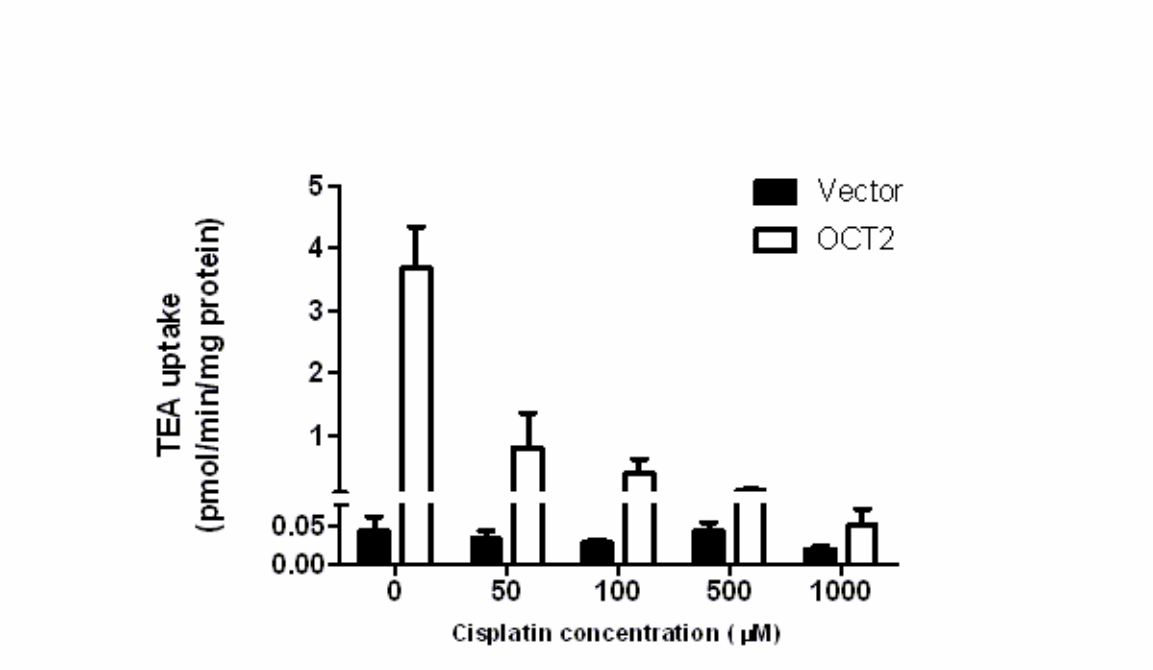


Figure 2.2 Cisplatin Inhibits the Uptake of TEA in FLP-OCT2 Cells

Inhibitory effect of cisplatin on ^{14}C-TEA transport in vector control cells and FLP-OCT2 cells. Each bar represents the mean ± SEM of 3 independent experiments.

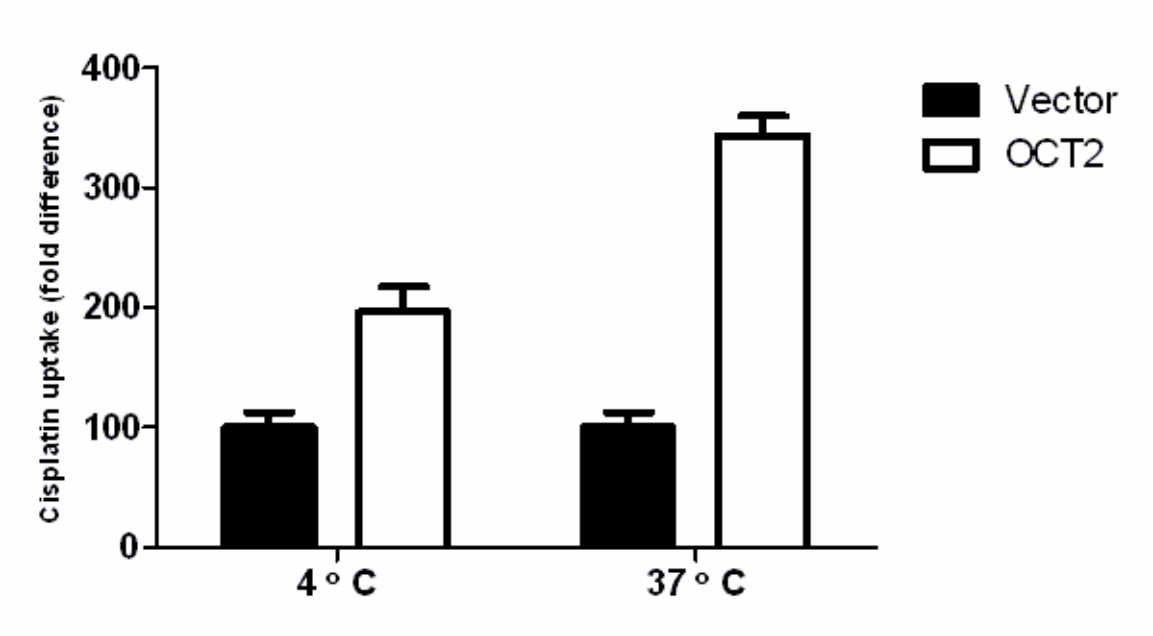


Figure 2.3 Temperature Dependent Uptake in FLP-OCT2 Cells

Comparative uptake of 500 μM cisplatin in vector control and FLP-OCT2 cells at 4°C and 37°C. Each bar represents the mean ± SEM of 3 independent experiments.

(**Fig 2.4A**). After incubation with 500 μM cisplatin for 30 min, the platinum accumulation in FLP-OCT2 cells compared to the vector control cells was about 4-fold higher ($P < 0.0001$) at neutral pH (**Fig 2.4B**) and uptake is decreased as the pH increases (**Fig 2.4C**). FLP-OCT2 cells also had a significantly higher ($P = 0.0023$) amount of Pt-DNA adducts when compared with vector control cells (**Fig 2.5**) suggesting that cisplatin is itself transported by OCT2 and not just binding to the protein.

2.4 Discussion

It has recently been suggested that human OCT2 and its rodent orthologue may play a critical role in the cellular transport of cisplatin.[28,40] However, at least one other report suggests that OCT2 is not a primary transporter handling cisplatin.[29] To address these inconsistencies, we produced a stably transfected OCT2 cell line, and performed a detailed characterization of gene overexpression and transport function. These transfected cells not only exhibited an increase in the intracellular accumulation of platinum following exposure to cisplatin (approximately 4-fold), but cisplatin almost completely inhibited the uptake of TEA, a known OCT2 substrate. These results unequivocally indicate an important role for OCT2 in the transport of cisplatin.

Although this study suggests an important contribution of OCT2 to the cellular uptake of cisplatin *in vitro*, the mechanisms by which cisplatin is eventually secreted from renal tubular cells into the lumen, and how this process is affecting the urinary excretion of cisplatin are still unclear. A number of transporters previously implicated in cisplatin transport, including ABCC2 (MRP2, cMOAT)[41] and two members of the multidrug and toxin extrusion family (MATE1 and MATE2-K),[28] are highly expressed on the luminal side of renal tubules. Our current work is thus further focused on the dynamic interplay between OCT2 and a number of suspected efflux carriers in the context of cisplatin transport using mammalian expression models.

In conclusion, this study suggests that OCT2 is a primary regulator of cisplatin transport, but additional studies are necessary to elucidate its role *in vivo*.

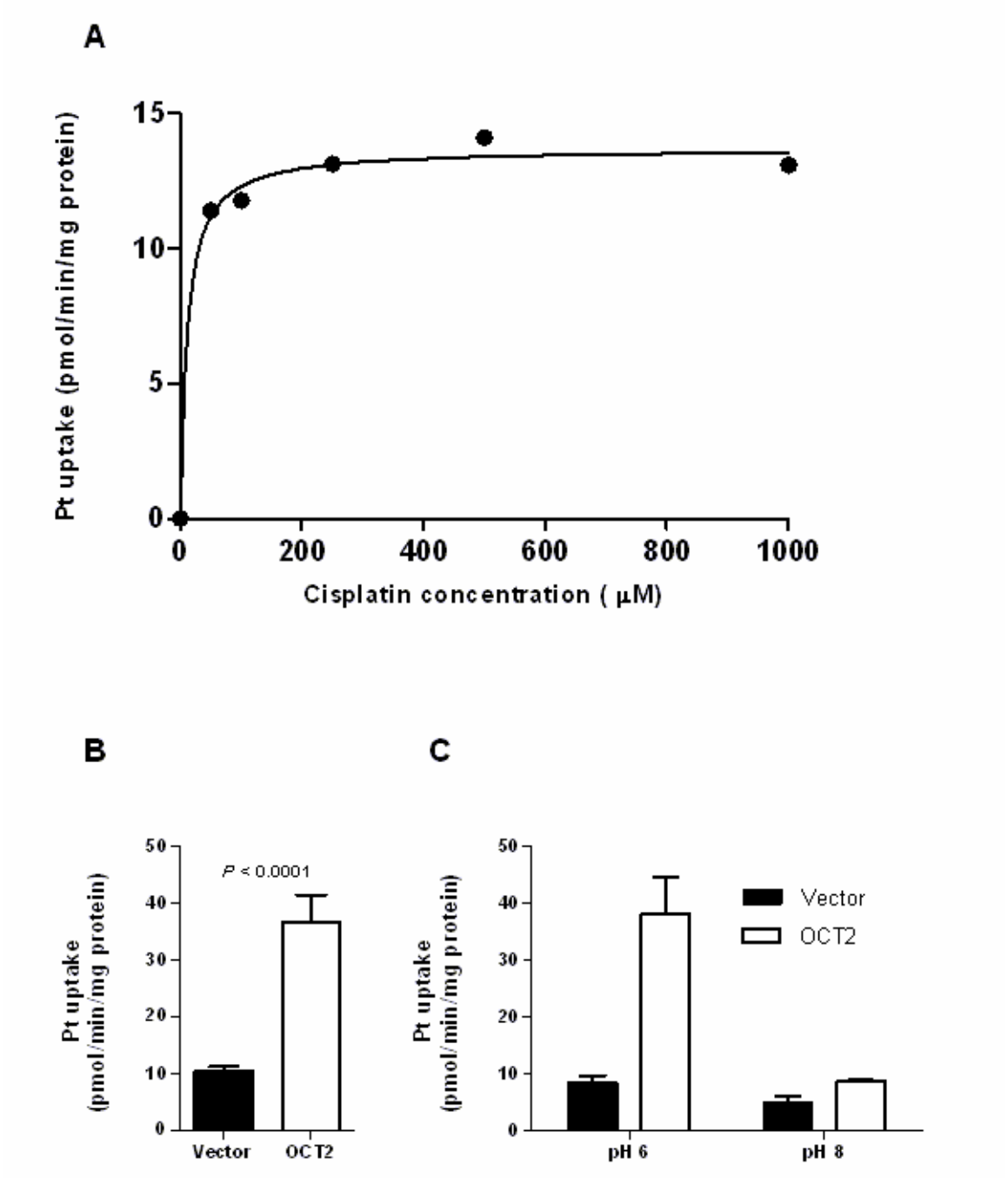


Figure 2.4 Cisplatin Uptake in FLP-OCT2 Cells

(A) Concentration dependent cisplatin uptake in FLP-OCT2 cells. **(B)** Comparative uptake of 500 μM cisplatin in vector control cells and FLP-OCT2 cells at neutral pH. **(C)** pH dependence of cisplatin uptake in FLP-OCT2 cells. Each point or bar represents the mean ± SEM of 3 independent experiments.

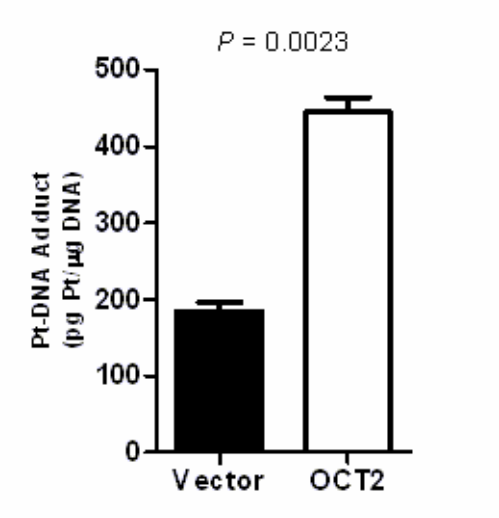


Figure 2.5 Pt-DNA Adducts in FLP-OCT2 Cells after Cisplatin Treatment

Pt-DNA adducts in vector control cells and FLP-OCT2 cells after incubation with 500 μM cisplatin. Each bar represents the mean ± SEM of 3 independent experiments

CHAPTER 3. CONTRIBUTION OF ORGANIC CATION TRANSPORTER 2 TO CISPLATIN INDUCED NEPHROTOXICITY IN MICE

3.1 Introduction

Cisplatin is among the most widely used anticancer agents and has a broad spectrum of activity. Irreversible kidney damage is an important complication of cisplatin treatment as it may limit further treatment or even threaten life. This side effect primarily affects the S3 segment of the renal proximal tubules and occurs in one-third of patients despite intensive prophylactic measures.[42] Furthermore, about 20% of all acute renal failure cases among hospitalized patients are due to cisplatin-containing chemotherapy.[43] The exact pathogenesis of cisplatin-related nephrotoxicity remains unclear. Despite having been the focus of intense investigation for many decades, the exact pathogenesis of cisplatin-related nephrotoxicity, in which quiescent proximal tubular cells are selectively damaged, remains unclear.[44] Recent studies have suggested that inflammation, oxidative stress and apoptosis probably explain part of the tissue injury, although the initiating event leading to tubular damage and mechanistic details of the drug's renal handling are still poorly understood.[45]

We and others have recently reported that the organic cation transporter 2 (OCT2), encoded by the *SLC22A2* gene, can actively transport cisplatin *in vitro*.[5,29,46,47] This transporter is predominantly expressed in human kidney at the basolateral membrane of renal proximal tubules, and is involved in the secretion of various cationic substances from the circulation into tubular cells. All experiments to date, however, have assessed a putative role of OCT2 in cisplatin-related nephrotoxicity in cultured cells. The continual identification of substrates for renal transporters using heterologous *in vitro* expression systems provides valuable information for predicting drug-drug and drug-protein interactions.[48] However, the well-recognized limitation of these preliminary determinations is that they do not indicate the true relevance of a transporter in handling a substrate in the context of whole-body disposition, renal and extrarenal transport, and glomerular filtration. Indeed, to attribute an abnormality in normal physiology to transporter perturbation, the relevance of the transporter to the disposition of a drug must first be determined *in vivo*. The aim of the current investigation was to compare the pharmacokinetics, urinary excretion, and extent of nephrotoxicity in mice lacking the ortholog transporters Oct1 [Oct1 (-/-) mice], Oct2 [Oct2 (-/-) mice], or both Oct1 and Oct2 [Oct1/2 (-/-) mice] after treatment with cisplatin. We hypothesize that Oct2 will be responsible for the renal uptake of cisplatin, and the loss of Oct2 may prevent renal tubule cells from damage after cisplatin treatment.

3.2 Materials and Methods

3.2.1 Animal experiments

Adult (8-12 week old) male Oct1(-/-), Oct2(-/-) or Oct1/2(-/-) (Taconic) mice were used along with age-matched male FVB wild-type mice. Mouse genotypes from tail biopsies were determined using real time PCR with specific probes designed for each gene (Transnetyx, Cordova, TN, USA). All animals were housed and handled in accordance with the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital. Animals were housed in a temperature controlled environment with a 12 hour light cycle and were given a standard diet and water ad libitum.

Urinary platinum excretion of cisplatin was studied in animals after they had acclimated to metabolic cages 5 days prior to drug administration. Baseline urine samples were collected sixteen hours after the onset of light, and next, mice were given a single intraperitoneal (i.p.) injection of cisplatin at a dose 10 mg/kg. Urine was collected 4, 24, 48, and 72 hours after administration, diluted with nitric acid (0.2%) and immediately analyzed for total platinum using flameless atomic absorption spectrometry. At the end of the study period, mice were euthanized and tissues harvested for gene expression and histological examination.

In separate animals, the plasma pharmacokinetics of cisplatin (10 mg/kg, i.p.) was determined using a terminal sampling procedure. Blood samples were collected by cardiac puncture after anesthesia with isoflurane in heparin-containing microtubes at 0.25, 0.75, 1, 4, and 8 hours after drug administration. Samples were immediately centrifuged at 2000 × g for 10 min and plasma was removed and stored frozen until analysis by AAS.

3.2.2 Determination of platinum concentrations

Total platinum was determined using an AAnalyst 600 atomic absorption spectrometer with Zeeman background correction (Perkin Elmer, Norwalk, CT, USA). Concentrations in unknown samples were interpolated on a linear calibration curves ranging from 0.2 to 3 μg/mL that were constructed fresh daily in the relevant matrix. The percentage deviation from nominal values and the within-run and between-run precision of quality control samples spiked with known amounts of cisplatin were always <15%. Concentrations of free, protein-unbound platinum in plasma were determined as described.[2] Pharmacokinetic parameters were calculated using PK Solutions 2.0 (Summit Research Services, Montrose, CO, USA).

3.2.3 Peripheral blood analysis

Chemistry analysis was performed on serum using a Vetscan autoanalyzer (Abaxis) to determine levels of albumin, alkaline phosphatase, alanine aminotransferase, amylase, total bilirubin, blood urea nitrogen, calcium, phosphorus, creatinine, glucose, sodium, potassium, and total protein.

3.2.4 Histological evaluation of mouse tissues

Mice were humanely euthanized and the following tissues were collected: adrenals, bone marrow, brain, heart, kidneys, large intestine, liver, lung, lymph node, pancreas, reproductive tract, salivary gland, skeletal muscle, skin, small intestine, spleen, stomach, thymus, and thyroid. After collection, tissues were fixed overnight in 10% neutral-buffered formalin. Next, the tissues were processed routinely, embedded in paraffin, sectioned (4 μm) and stained with hematoxylin and eosin. Microscopic evaluation was performed by an experienced veterinary pathologist blinded to the composition of the groups. Toxicities, including acute renal tubular necrosis, were scored as 'absent', 'rare', 'mild', 'moderate' or 'severe'.

3.2.5 Statistical calculations

Group differences in median pharmacokinetic parameters of cisplatin as a function of mouse genotype was evaluated using a Mann-Whitney U test. Variability in serum chemistry values in the studied mouse strains as a function of treatment was assessed using a Kruskal-Wallis test followed by a Kruskal-Wallis Z-test.

3.3 Results

3.3.1 Urinary platinum excretion and cisplatin pharmacokinetics

An assessment of the urinary excretion profile of cisplatin revealed that the cumulative percentage of the administered dose was very similar in Oct1(-/-) and Oct2(-/-) mice compared with wildtype mice (**Fig 3.1**). Consistent with previous findings,[49] the majority of the dose was excreted within the first 24 hours. The lack of a significant impact of the individual Oct1 and Oct2 deficiencies on the elimination of cisplatin is in line with earlier observations made for the cationic substance tetraethylammonium (TEA) in the same animal models,[50,51] and suggests that in the mouse cisplatin is recognized as a substrate by both Oct1 and Oct2. Although mice express substantial levels of both Oct1 and Oct2 in the kidney, in humans there is strong agreement that OCT2 dominates renal organic cation transport, whereas OCT1 dominates hepatic organic cation transport.[52] Therefore, we used Oct1/2(-/-) mice in all subsequent experiments as the most appropriate animal model.

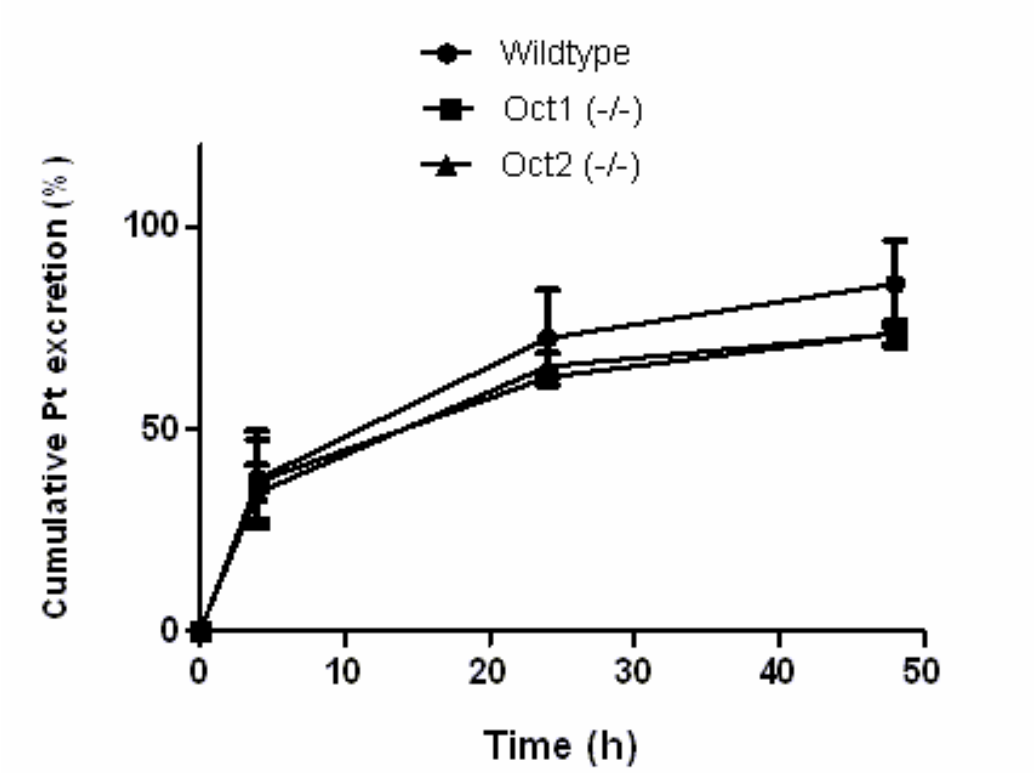


Figure 3.1 Urinary Platinum Excretion in Oct1 (-/-) and Oct2 (-/-) Mice

Effect of Oct1 and Oct2 deficiency on renal handling of cisplatin in mice. The cumulative excretion of cisplatin after drug administration (10 mg/kg; i.p.) was unchanged in Oct1 (-/-), Oct2 (-/-), and wildtype mice (n = 4-5/group). Data are shown as mean values; error bars represent ± standard error.

At 24 hours, Oct1/2 (-/-) mice had a cumulative excretion of 45% of the administered dose compared to 91% in wildtype animals ($P = 0.0008$) and only 56% of the dose was recovered after 72 hours in Oct1/2 (-/-) mice. Both the rate and extent of the cumulative urinary excretion of cisplatin was markedly reduced in the Oct1/2(-/-) mice (47%; $P = 0.0016$) (**Fig 3.2**), confirming a direct role of organic cation transporters in the renal handling of cisplatin.

The reduced renal excretion in the Oct1/2 (-/-) mice was not accompanied by measurable changes of cisplatin in plasma (**Fig 3.3A**). For example, in wildtype and Oct1/2 (-/-) mice we observed a Cmax of 26.7 ± 9.73 and 36.4 ± 8.47, respectively. Furthermore, the area under the plasma concentration curve was 50.36 µg.h/mL in wildtype and 54.9 µg.h/mL in Oct1/2 (-/-) mice. Unbound plasma platinum concentrations also were not affected (**Fig 3.3B**).

3.3.2 Histopathology and serum chemistry analysis

As predicted based on the excretion data, we found that severe, acute renal tubular necrosis was only observed in the wildtype animals (**Fig 3.4**). The lesions were characterized by dilated tubules filled with necrotic tubular epithelial cells, cellular debris and proteinaceous casts, whereas the glomeruli were histologically normal. Consistent with these histopathological observations, various physiologic hallmarks of cisplatin-related nephrotoxicity in humans, such as changes in serum alkaline phosphatase, blood urea nitrogen and serum creatinine, were specifically altered in wildtype mice undergoing cisplatin treatment (**Table 3.1**). Tissues other than kidney in the Oct1/2 (-/-) mice, including liver, brain, and pancreas, were also histologically normal (**Fig 3.5**), indicating that the impaired renal excretion in this strain does not cause exacerbated cisplatin-related side effects elsewhere.

3.4 Discussion

In our previous *in vitro* studies, we determined that human organic cation transporter 2 was involved in the uptake of cisplatin into cells. To further characterize the interaction of cisplatin with OCT2, we examined the urinary excretion, plasma pharmacokinetics, and toxicity in mice lacking the mouse orthologues Oct1, Oct2, or both Oct1 and Oct2. Although it has been speculated for several decades that net tubular secretion of cisplatin may be the initiating event in renal tubular damage,[45] our demonstration that Oct1/2 (-/-) mice exhibit impaired urinary elimination of cisplatin for the first time substantiates an important role of organic cation transporters in the *in vivo* renal handling of this compound. The most plausible explanation for the observed differences in urinary excretion of cisplatin in Oct1/2 (-/-) mice compared with wildtype mice is an impaired uptake of the drug in renal tubular cells and subsequently reduced renal tubular secretion into urine in the knockout strain.

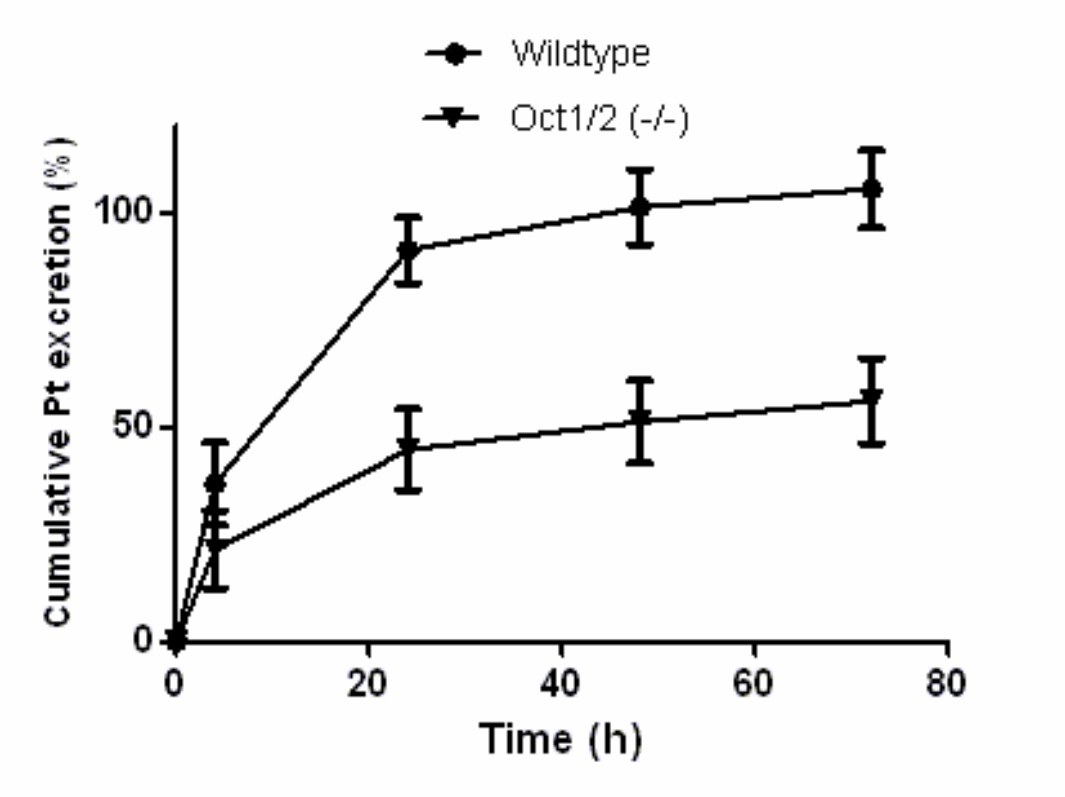


Figure 3.2 Urinary Platinum Excretion in Oct1/2 (-/-) Mice

Effect of simultaneous Oct1 and Oct2 deficiency on renal handling of cisplatin in mice. The cumulative excretion of cisplatin was reduced in Oct1/2(-/-) mice compared to wildtype mice (n = 11-12/group) after drug administration (10 mg/kg; i.p.). Data are shown as mean values; error bars represent standard error.

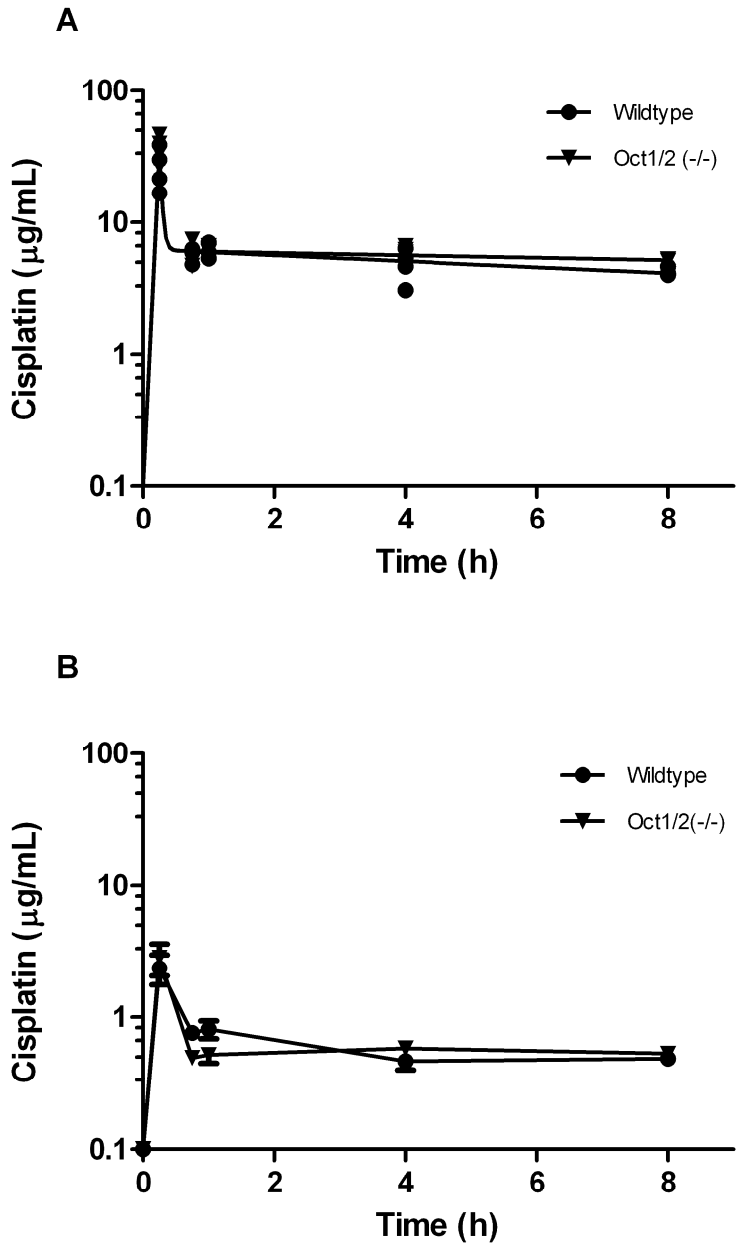


Figure 3.3 Cisplatin Plasma Concentrations

(A) Comparative concentration-time profiles of cisplatin in plasma of Oct1/2 (-/-) mice and wildtype mice (n = 4/group) after the administration of cisplatin (10 mg/kg; i.p.). Data shown as mean values with standard error along with a curve fit from a 2 compartment model. (**B**) Comparative concentration-time profiles of unbound platinum in plasma of Oct1/2(-/-) mice and wildtype mice (n = 4/group) after the administration of cisplatin (10 mg/kg, i.p.). Data shown as mean values with standard error.

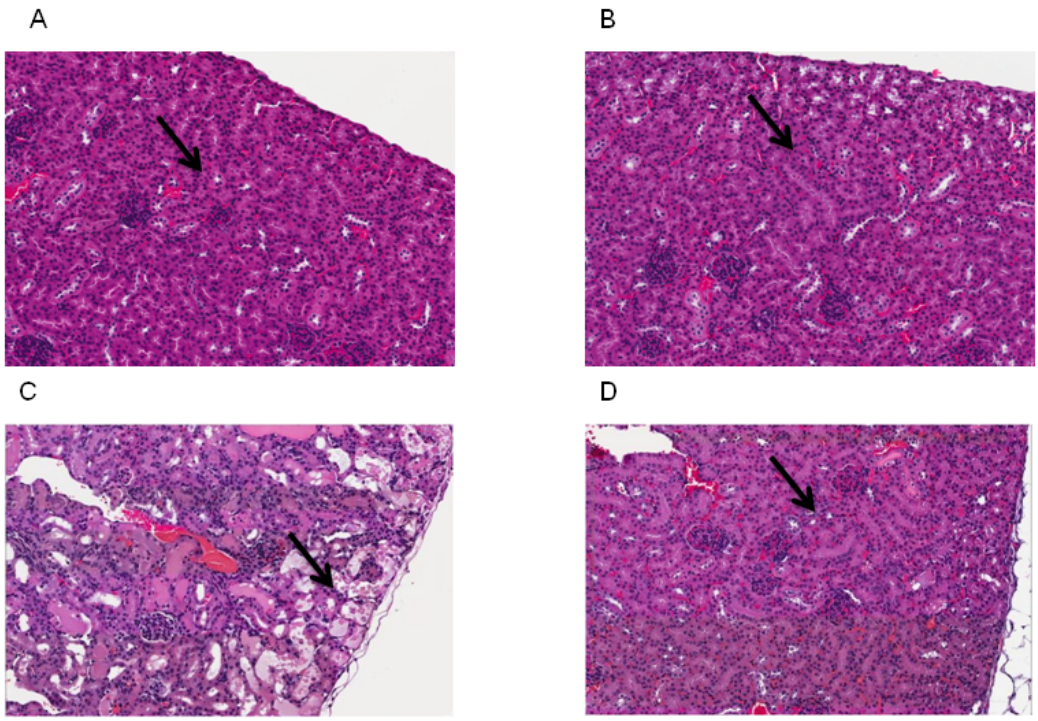


Figure 3.4 Histopathology of Wildtype and Oct1/2 (-/-) Mouse Kidney

Comparative cisplatin-related nephrotoxicity in (**A**) untreated wildtype and (**B**) untreated Oct1/2 (-/-) mice with (**C**) treated wildtype, and (**D**) treated Oct1/2 (-/-) mice 72 hours after the administration of cisplatin (10 mg/kg, i.p.) from representative animals. Severe renal tubular necrosis, characterized by dilated tubules filled with necrotic tubular epithelial cells, was observed in kidneys of all wildtype mice but in none of the Oct1/2 (-/-) mice (n = 8/group). Arrows indicate tubules.

Table 3.1 Altered serum chemistry in wildtype mice 72 hours after cisplatin administration (10 mg/kg, i.p.)

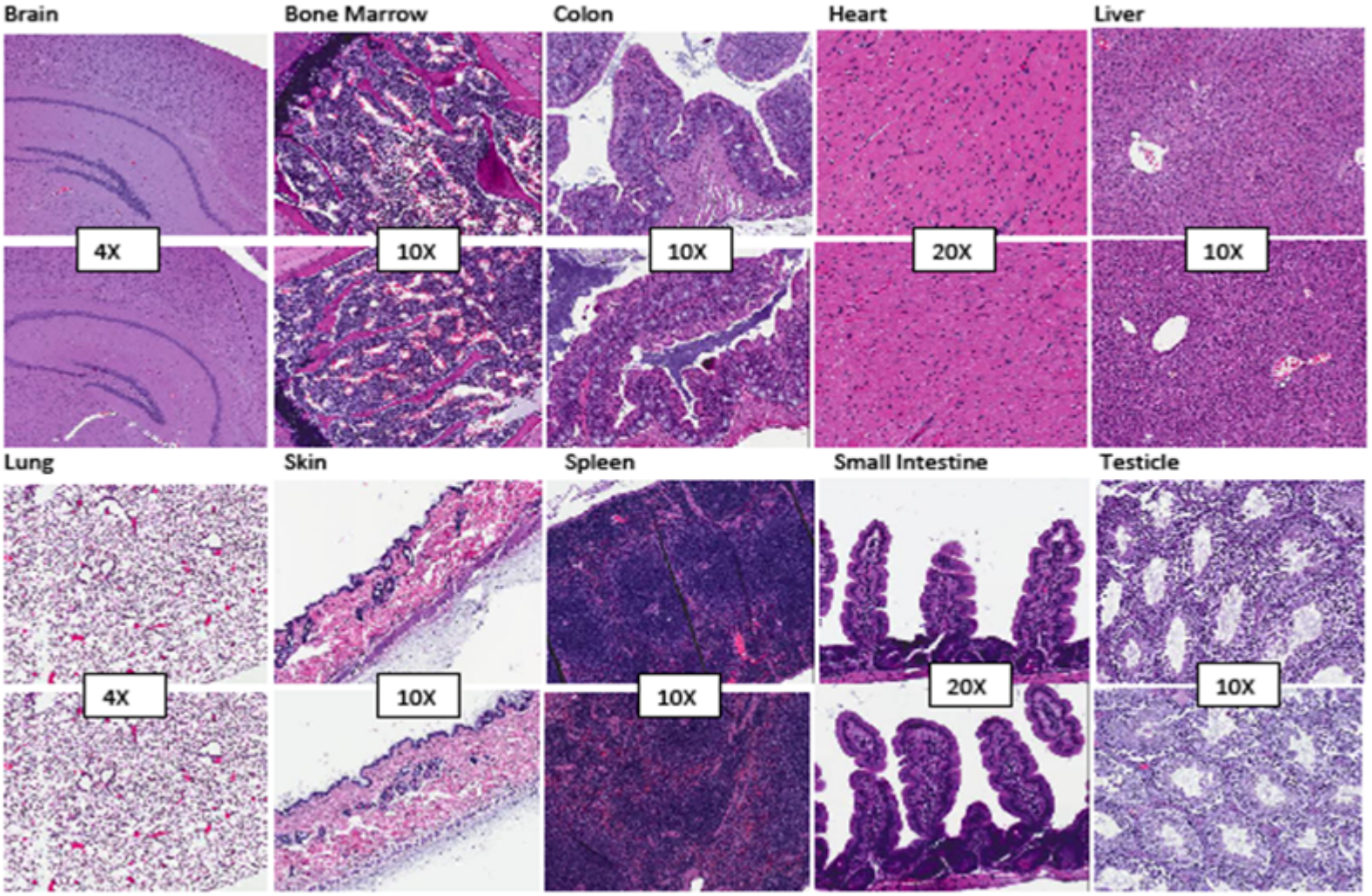
	Untreated [median (range)]		Treated [median (range)]		
Variable	**Wildtype**	**Oct1/2(-/-)**	**Wildtype**	**Oct1/2(-/-)**	**P***
Albumin (g/dL)	3.4 (3.2 – 3.4)	3.6 (3.3 – 4.1)	3.2 (1.8 – 4.5)	3.7 (3.4 – 4.3)	0.41
Alkaline phosphatase (U/L)	139 (120 – 154)	138 (108 – 167)	55 (2 – 68)**	75 (51 – 91)	0.00048
Alanine aminotransferase (U/L)	68 (50 – 98)	63 (9 – 273)	250 (108 – 493)**	139 (90 – 304)	0.018
Amylase (U/L)	1040 (983 – 1130)	874 (802 – 1090)	2570 (1130 – 3920)**	1730 (1280 – 2040)**	0.0020
Bilirubin (mg/dL)	0.30 (0.3 – 0.3)	0.35 (0.3 – 0.4)	0.20 (0.1 – 0.3)	0.30 (0.2 – 0.4)	0.070
Blood urea nitrogen (mg/dL)	24 (22 – 27)	22 (18 – 28)	157 (62 – 199)**	80 (31 – 180)	0.00096
Calcium (mg/dL)	9.4 (9.3 – 9.9)	9.8 (9.5 – 10.1)	11.4 (7.1 – 12.7)	9.8 (9.6 – 11.7)	0.13
Creatinine (mg/dL)	0.25 (0.20 – 0.30)	0.35 (0.20 – 0.50)	0.90 (0.40 – 1.60)**	0.45 (0.20 – 0.70)	0.0080
Globulin (g/dL)	1.9 (1.8 – 1.9)	2.1 (1.9 – 2.4)	2.3 (1.2 – 2.8)	2.2 (1.3 – 2.6)	0.13
Glucose (mg/dL)	215 (198 – 238)	183 (172 – 655)	149 (35 – 205)**	177 (135 – 223)	0.034
Phosphorus (mg/dL)	8.5 (8.4 – 10.1)	9.4 (8.9 – 18.1)	15.0 (5.4 – 24.2)	6.8 (5.9 – 9.0)	0.088
Potassium (mmol/L)	6.9 (6.8 – 8.0)	6.9 (3.0 – 7.8)	7.9 (6.8 – 9.7)	8.5 (6.8 – 9.7)	0.10
Sodium (mmol/L)	151 (147 – 151)	152 (151 – 152)	148 (110 – 152)	152 (148 – 154)	0.054
Total protein (g/dL)	5.3 (4.9 – 5.3)	5.6 (5.5 – 5.6)	5.6 (3.0 – 7.1)	5.6 (3.1 – 6.9)	0.19

* Kruskal-Wallis test followed by a Kruskal-Wallis Z test with Bonferroni correction.

** Different from untreated

Figure 3.5 Histopathology of Mouse Tissue

Histopathology of wildtype and Oct1/2 (-/-) mice 72 hours after the administration of cisplatin. All tissues were histologically normal. For each tissue type, images of wildtype mice are above the Oct1/2 (-/-) images.


Brain
Bone Marrow
Colon
Heart
Liver
4X
10X
10X
20X
10X
Lung
Skin
Spleen
Small Intestine
Testicle
4X
10X
10X
20X
10X

Urinary excretion of cisplatin in Oct1 (-/-) and Oct2 (-/-) mice were assessed, but did not show a significant difference when compared to wildtype littermates. In previous studies using these mice, investigators did not see a significant changes in plasma TEA concentrations in the single knockouts, when compared to wildtype mice.[51] As mentioned previously, Oct1 is more highly expressed in mouse kidney cells than in human kidney and may have some substrate overlap with Oct2. Studies identifying rat Oct1 and human OCT1 as a transporter for cisplatin have been controversial.[27-29] In this model, the lack of differences in urinary excretion in the Oct1 (-/-) and Oct2 (-/-) mice indicate at least a minor role for mouse Oct1 in cisplatin transport. Although OCT1 is primarily found only in human liver, the possible substrate overlap and high expression of mouse Oct1 in the kidney suggest a necessity for evaluating a double Oct1 and Oct2 knockout mouse. Therefore we concluded that using the Oct1/2 (-/-) mice was the best model to accurately assess cisplatin excretion and pharmacokinetics in mice.

Urinary platinum excretion was significantly altered in Oct1/2 (-/-) with only 56% of the cisplatin dose recovered in the urine after 72 hours. In spite of the currently observed changes in renal handling of cisplatin in the Oct1/2 (-/-) mice, we found that the apparent plasma clearance of cisplatin and measures of systemic exposure to total cisplatin and unbound cisplatin was unaltered in this mouse model. This paradox might be explained by the possibility that the high expression of Oct1 and Oct2 in renal tubular cells can directly control local drug levels, and thereby alter the urinary excretion of cisplatin without affecting measures of systemic exposure. The notion that the impaired urinary excretion of cisplatin in the Oct1/2 (-/-) mice was not accompanied by any evidence of other organ damage indicates a possible shunting of the primary pathway of cisplatin elimination as opposed to a dramatically altered drug distribution. A similar phenomenon has been reported previously for the anticancer and antirheumatic drug methotrexate, where deficiency of Abcc2 (Mrp2), a transporter regulating biliary secretion of this agent, is associated with increases in the extent of urinary excretion compared with wildtype mice.[53] Additional investigation is required to confirm the possibility that cisplatin is predominantly eliminated by the hepatobiliary route in the Oct1/2 (-/-) mice.

These studies using Oct1/2 (-/-) mice indicate a significant role for OCT2 in the renal handling and toxicity of cisplatin without affecting the cisplatin plasma concentrations.

CHAPTER 4. EFFECT OF CISPLATIN TREATMENT ON GENE EXPRESSION IN MICE

4.1 Introduction

Cisplatin is used for the treatment of a large spectrum of cancers, but treatment is limited by drug induced nephrotoxicity which still affects one third of patients despite prophylactic hydration procedures. Drug induced renal damage typically affects the renal proximal tubules which are responsible for active secretion and reabsorption of xenobiotics and endogenous compounds. These compounds are typically transported across the basolateral membrane by organic cation or anion transporters, and are subsequently effluxed from the apical membrane by primary active transporters. Regulation of these transporters is vitally important for the removal of toxic compounds from the blood. Previous investigators have reported changes in transporter gene expression after treatment with various compounds.[54] Furthermore, gene expression changes in important cellular pathways, particularly p53 signaling, after treatment with cytotoxic drugs has also been described.[7] In this study, we evaluated the effect of cisplatin treatment on the gene expression of transporters in kidneys of wildtype, Oct1 (-/-), Oct2 (-/-), and Oct1/2 (-/-) mice.

4.2 Materials and Methods

4.2.1 Animals

Adult (8-12 week old) male Oct1(-/-), Oct2(-/-) or Oct1/2(-/-) (Taconic) mice were used along with age-matched male FVB wild-type mice. Mouse genotypes from tail biopsies were determined using real time PCR with specific probes designed for each gene (Transnetyx, Cordova, TN, USA). All animals were housed and handled in accordance with the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital. Animals were housed in a temperature controlled environment with a 12 hour light cycle and were given a standard diet and water ad libitum. Mice were administered 10 mg/kg cisplatin via an i.p. injection while in metabolic cages. Kidneys were harvested 48 or 72 hours after drug administration.

4.2.2 Gene expression analysis of mouse kidney

RNA was extracted using the RNEasy mini kit (Qiagen, Valencia, CA, USA). RNA samples were amplified from 3 animals per group and then analyzed using the Mouse 430v2 GeneChip array (Affymetrix, Santa Clara, CA, USA). Data analysis was performed using a subset of genes including common drug-metabolizing enzymes, nuclear receptors, ATP-binding cassette transporters, and solute carrier genes. A fisher's

exact test was used to identify functionally related pathways, and probability and false discovery rates were calculated.

4.2.3 Cellular uptake

Human embryonic kidney 293 (HEK293) cells overexpressing human OCT6 were produced using the Flp-In transfection system (Invitrogen, Carlsbad, CA, USA). The full length OCT6 cDNA clone was inserted into the pcDNA5/FRT vector and transfected into HEK293 cells containing an integrated FRT site as previously described.[5] HEK293 cells overexpressing human OAT3 were provided by Dr. Yuichi Sugiyama (Tokyo, Japan). Measurements of cellular accumulation of cisplatin were performed in six well plates with monolayer cultures at 37ºC as previously reported.[5] Briefly, cell monolayers were washed with warm PBS and were treated with DMEM containing cisplatin (500 μM) for 30 min. After incubation, cells were washed twice with ice cold PBS and collected. Cells were then solubilized with nitric acid (0.2%), and total platinum was measured using flameless atomic absorption spectrometry. Protein measurements were performed using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

4.3 Results

4.3.1 Gene expression changes in Oct1 (-/-) and Oct2 (-/-) mice after cisplatin treatment

First, the gene expression changes in Oct1 (-/-), Oct2 (-/-), and wildtype animals were evaluated after treatment with cisplatin. Upon comparison of Oct1 (-/-) with wildtype gene expression, a 2-fold upregulation of *Slc22a16* in Oct1 (-/-) mice was discovered. (**Fig 4.1A**). The *Slc22a16* gene encodes the organic cation transporter 6 (OCT6), but its overexpression is unlikely to affect the renal handling of cisplatin since the drug was not found to be a substrate of OCT6 (**Fig 4.2**). In contrast, the only gene that was significantly different in Oct2 (-/-) was Oct2 (**Fig 4.1B**).

4.3.2 Gene expression changes in Oct1/2 (-/-) and wildtype mice after cisplatin treatment

Microarray analysis showed that more than 4,235 and 3,334 probe sets, respectively, exhibited a statistically significant change in cisplatin-treated wildtype mice and Oct1/2 (-/-) mice compared with untreated controls. Following correction for multiple hypotheses testing to limit the false discovery rate to 5%, 4,774 probe sets were identified, of which 4,168 had annotated biological processes, indicating that cisplatin treatment has a major effect on renal gene expression profiles. A subsequent gene ontology analysis indicated that the most significantly perturbed physiological processes in wildtype and Oct1/2 (-/-) mice were the p53 signaling pathway and the cytokine-cytokine receptor interaction pathway. This disruption is not surprising considering the

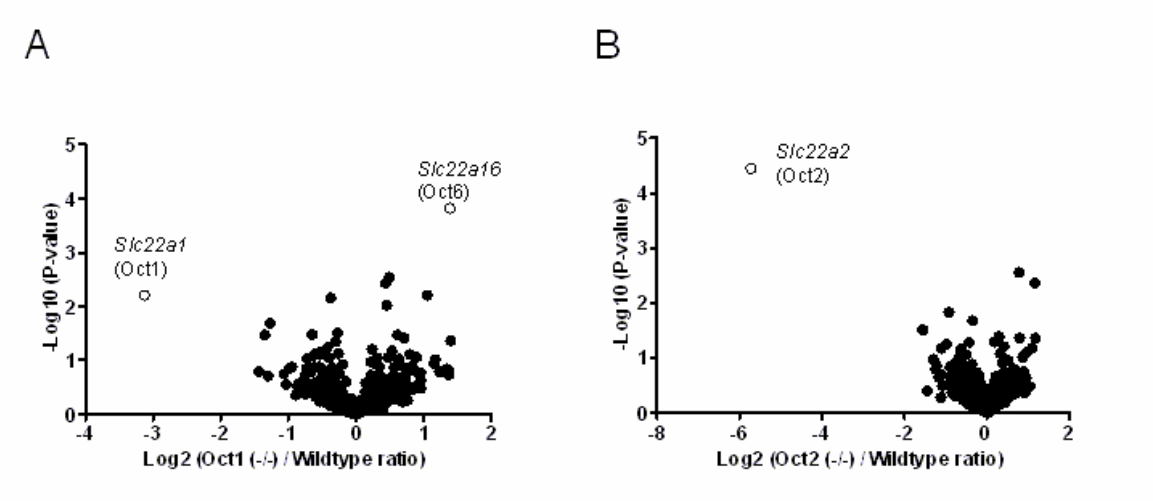


Figure 4.1 Gene Expression Changes in Oct1 (-/-) and Oct2 (-/-) Mice

Differential gene expression in the kidney of male Oct1 (-/-) mice (**A**) and Oct2 (-/-) mice (**B**) relative to wildtype FVB mice (n = 3/group) as assessed using the Affymetrix Mouse 430v2 GeneChip array. Select genes on the volcano plot include enzymes, nuclear receptors, ABC transporters, and solute carriers.

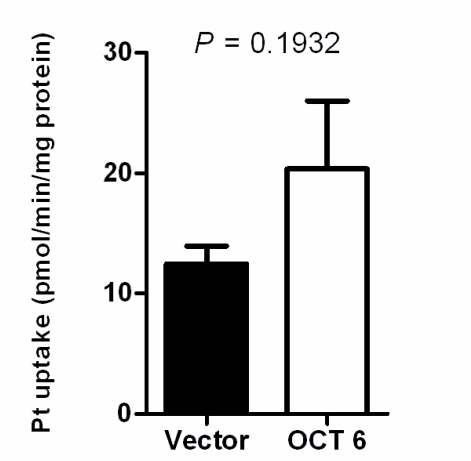


Figure 4.2 Cisplatin Accumulation in OCT6 Overexpressing Cells

Intracellular uptake of cisplatin in HEK293 cells transfected with OCT6 following incubation with 500 μM cisplatin for a period of 30 minutes. Data expressed relative to drug accumulation in cells transfected with an empty vector, which was set to 100 %. Bars represent mean values; error bars represent standard error.

important role of p53 signaling[7] and cytokines[55] in tubular apoptosis after administration of cisplatin.

Before treatment, the only gene expression changes observed to be different in wildtype and Oct1/2 (-/-) mice were the respective gene deletions (**Fig 4.3**). Cisplatin treatment was also associated with significant changes in expression of transporter genes, many of which are known to be localized in renal tubular cells. This was predominantly observed in samples originating from wildtype mice, suggesting that excessive accumulation of cisplatin in proximal tubules predisposes to these phenotypic alterations. Among the transporter genes, those known to be responsible for inward-directed transfer of molecules were typically repressed (**Fig 4.4**), whereas the expression of ATP-binding cassette transporter genes was elevated (**Fig 4.5**). For example, among the most highly reduced genes was the organic anion transporters Oat3 (between 8- and 16-fold) which does not transport cisplatin (**Fig 4.6**), whereas the P-glycoprotein-encoding *Abcb1b* gene was increased up to 28-fold. The currently observed changes in expression of renal xenobiotic transporter genes in wildtype FVB mice following treatment with cisplatin are consistent with prior findings in male C57BL/6J mice.[54]

It is interesting to note, that the copper transporter (Ctr1), which has been previously identified as a potential mechanism of cisplatin transport,[56] was unchanged in both wildtype and Oct1/2 (-/-) mice after treatment (**Fig 4.7**). This result may indicate a limited involvement for this transporter in our particular model.

4.4 Discussion

In this study we evaluated the expression changes of common drug metabolizing enzymes, ABC transporters and solute carrier genes after treatment of cisplatin in mouse kidney. Previously we reported a significant decrease in urinary platinum excretion in Oct1/2 (-/-) mice compared to wildtype animals after cisplatin treatment. The most plausible explanation for the observed differences in urinary excretion of cisplatin in Oct1/2 (-/-) mice compared with wildtype mice is an impaired uptake of the drug in renal tubular cells and subsequently reduced renal tubular secretion into urine in the knockout strain. Fifty six percent of the dose is still recovered in the urine of Oct1/2 (-/-) mice, suggesting a potential change in the gene expression of transporters at the basolateral side of the renal proximal tubule. Gene expression in the kidneys of Oct1 (-/-) and Oct2 (-/-) mice was not significantly different from wildtype animals after treatment, except for the upregulation of the organic cation transporter *Slc22a16*; however this is not a transporter for cisplatin.

Gene expression in wildtype and Oct1/2 (-/-) mice was unchanged before cisplatin treatment, but significant changes were observed in the kidney of wildtype animals after cisplatin administration. For example, an up to 11-fold downregulation of organic anion transporter *Slc22a8* and an upregulation of *Abcb1b* was observed. However, neither of these transporters has been shown to transport cisplatin *in vitro*, indicating that the gene expression changes may have been in relation to the severe toxicity observed in wildtype

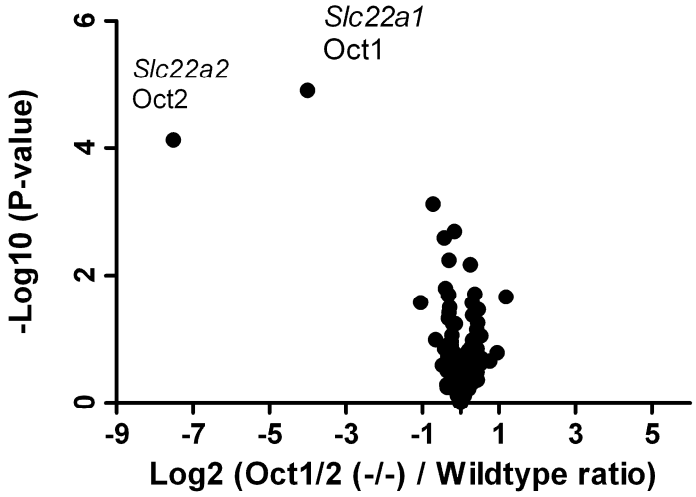


Figure 4.3 Gene Expression in Untreated Wildtype and Oct1/2 (-/-) Mice

Differential gene expression in the kidney of male Oct1/2 (-/-) mice relative to wildtype mice (n = 3/group) as assessed using the Affymetrix Mouse 430v2 GeneChip array. Select genes on the volcano plot include enzymes, nuclear receptors, ABC transporters, and solute carriers.

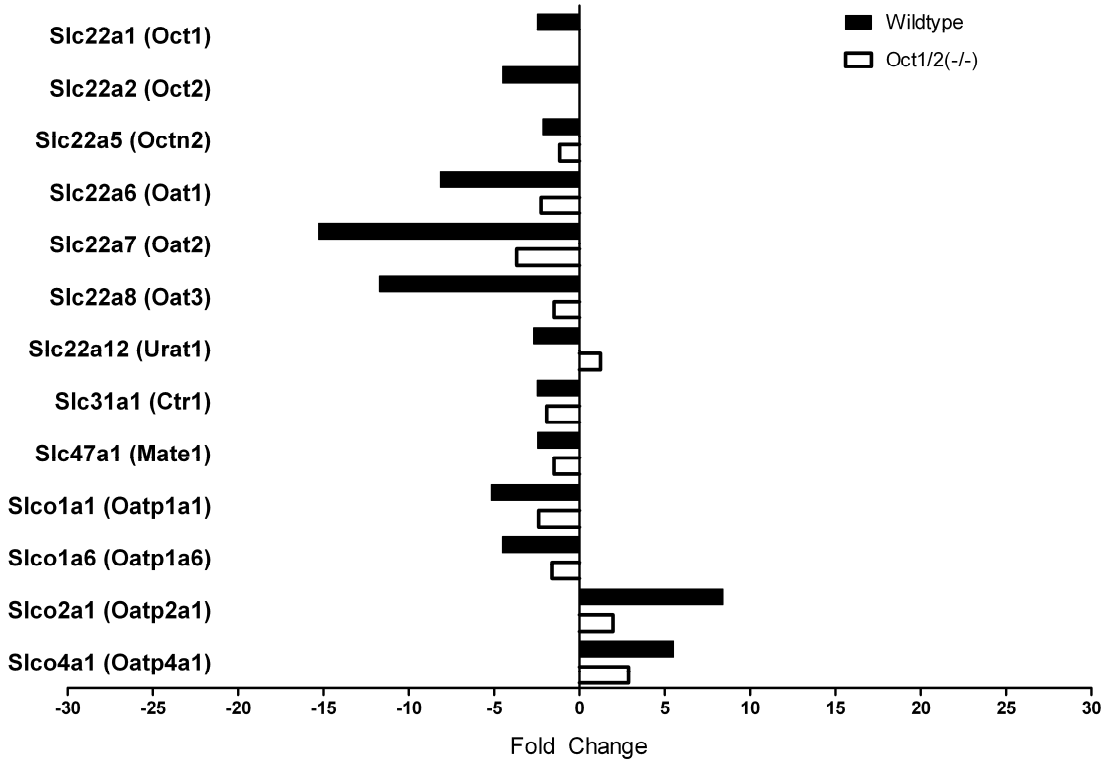


Figure 4.4 Solute Carrier Gene Expression Changes in Mice after Cisplatin Treatment

Gene expression changes in solute carriers in the kidney of male Oct1/2(-/-) and wildtype mice (n = 3/group) following treatment with cisplatin (10 mg/kg; i.p.) as determined by the Affymetrix mouse 430v2 genechip microarray. Data expressed as fold change versus untreated control in samples obtained at 72 hours after drug administration.

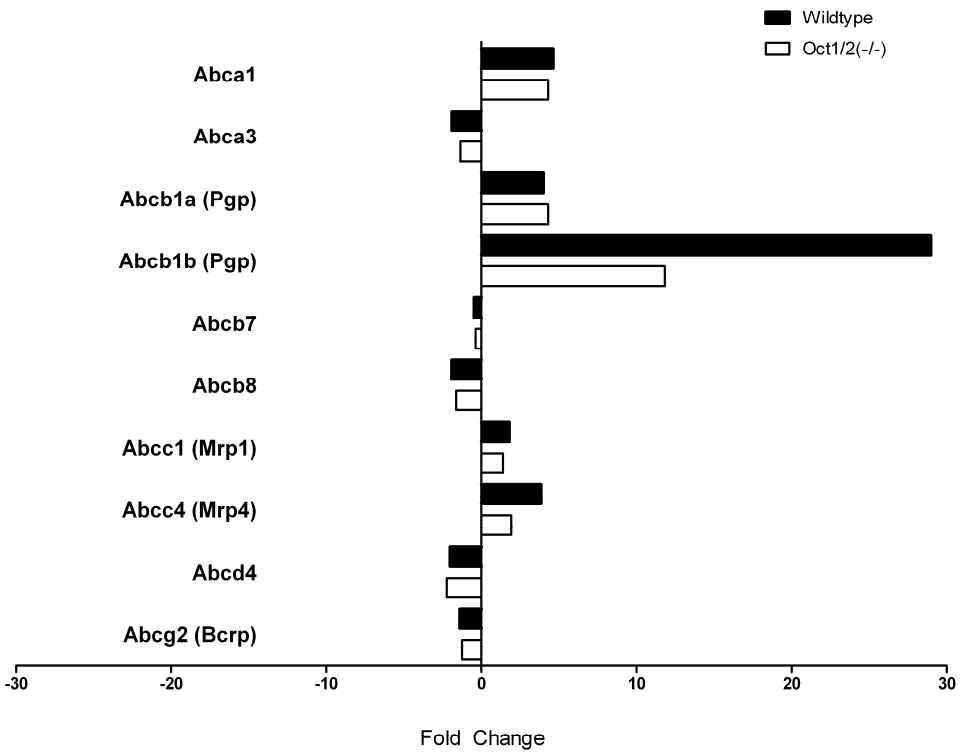


Figure 4.5 Differential Expression in ABC Transporters after Cisplatin Treatment

Gene expression changes in ABC transporters in the kidney of male Oct1/2(-/-) and wildtype mice (n = 3/group) following treatment with cisplatin (10 mg/kg; i.p.) as determined by the Affymetrix mouse 430v2 genechip microarray. Data expressed as fold change versus untreated control in samples obtained at 72 hours after drug administration.

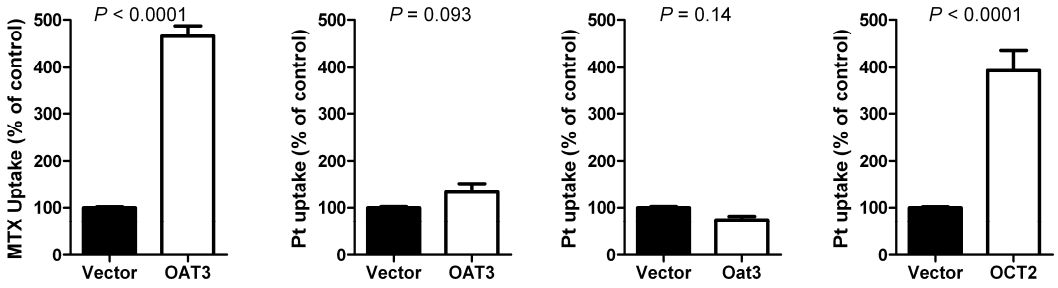


Figure 4.6 Cisplatin Uptake in OAT3 Expressing Cells

The intracellular uptake of methotrexate (MTX), a positive control, and platinum (Pt) was assessed in HEK293 cells transfected with human OAT3 and mouse Oat3 following incubation of 10 µM methotrexate or 500 µM cisplatin for a period of 30 minutes. Transport of cisplatin by OCT2 is shown for comparative purposes. Date expressed relative to drug accumulation in cells transfected with an empty vector, which was set to 100%. Data shown as mean values with standard error.

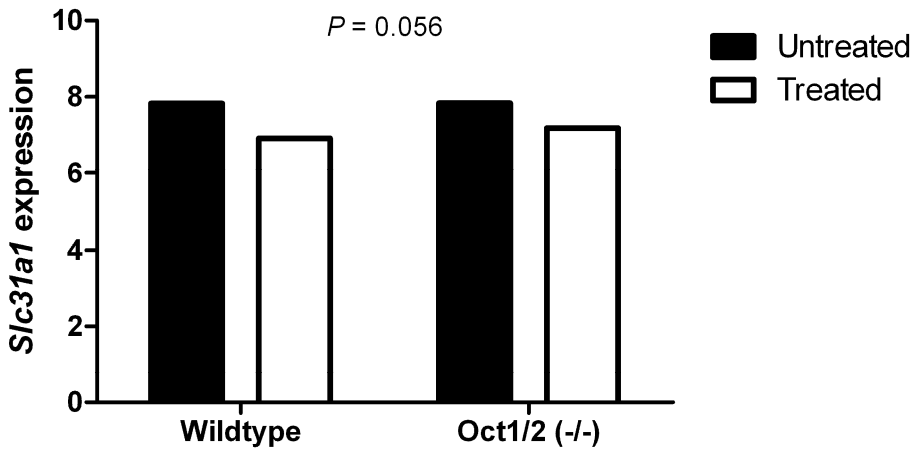


Figure 4.7 Ctr1 Expression

Gene expression changes in the copper transporter 1 (Ctr1; *Slc31a1*) in the kidney of male Oct1/2 (-/-) and wildtype mice (n = 3/group) before and after treatment with cisplatin (10 mg/kg, i.p.) as determined by the Affymetric mouse 430v2 genechip microarray. Samples were obtained at 72 hours after drug administration.

kidneys and not cisplatin transport. A recent investigation indicated that the copper transporter 1 (Ctr1; *Slc31a1*) is localized on the basolateral side of both proximal and distal tubular cells in the kidney of C57BL/6 mice, and that downregulation of Ctr1 expression by small interfering RNA was associated with decreased cisplatin uptake *in vitro*.[57] In this context, it is interesting to note that the expression of *Slc31a1* was not different in kidney samples obtained from wildtype mice and Oct1/2 (-/-) mice, and was also not affected by treatment with cisplatin. This result indicates a limited involvement for the Ctr1 transporter in the observed pharmacokinetic and pharmacodynamic changes in our particular mouse model.

Gene expression changes observed in the wildtype mice could indicate possible mechanisms to counteract the effects of cisplatin. It would be interesting to evaluate the gene expression changes in the liver Oct1/2 (-/-) mice after cisplatin treatment to identify any possible changes that could contribute to an increase in cisplatin elimination. In line with our previous toxicity and pharmacokinetic results, cisplatin did not significantly alter gene expression in the kidney of Oct1/2 (-/-) mice, but did dramatically change expression in wildtype animals.

CHAPTER 5. GENETIC VARIATION IN ORGANIC CATION TRANSPORTER 2 AND CISPLATIN PHARMACOKINETICS AND PHARMACODYNAMICS*

5.1 Introduction

Cis-diamminedichloroplatinum (cisplatin) is a commonly used anticancer drug with a broad spectrum of activity against malignant solid tumors, including lung, head and neck, bladder, germ cell, ovarian, endometrial, and cervical cancer.[2,4] Dose-limiting side effects such as renal tubular dysfunction, peripheral neuropathy, and ototoxicity, typically occur with conventional 3-weekly or 4-weekly regimens of cisplatin.[4,58] Hydration therapy has been incorporated into cisplatin treatment; however, approximately one-third of patients still show signs of acute renal toxicity.[37,59,60] Although cisplatin-induced toxicity is dose-dependent, the individual susceptibility to side effects varies considerably. Previous studies have revealed significant relationships between cisplatin pharmacokinetics and the likelihood of drug-related side effects.[4,61] Hence, identification of factors that are associated with the clearance of cisplatin could aid in predicting or adapting appropriate, individualized doses of this agent.

In our previous studies, we identified OCT2 as a primary regulator of cisplatin transport into human cells *in vitro*. *In vivo* we confirmed the role of OCT2 in mice lacking the mouse orthologue transporters Oct1 and Oct2. After cisplatin administration, mice deficient for Oct1 and Oct2 displayed a significantly lower urinary platinum excretion and did not display obvious signs of renal toxicity when compared to wildtype mice. The aims of the present study were to assess the association of variation in the gene encoding OCT2 (*SLC22A2*) with the disposition of cisplatin in adult Caucasian cancer patients. We hypothesize that functional changes in OCT2 will result in different toxicity profiles in patients carrying variant OCT2.

5.2 Materials and Methods

5.2.1 Materials

SLC22A2 primers were custom made by the Hartwell Center (St. Jude Children's Research Hospital, Memphis, TN). The ExoSAP-IT reagent was obtained from USB Corporation (Cleveland, OH).

5.2.2 Clinical studies

All patients eligible for the study had a confirmed diagnosis of a malignant solid tumor, and were between the ages of 18 and 75 years. Eligibility criteria have been

* Portions of this chapter adapted with permission. Filipski, K.K., Loos, W.J., Verweij, J., & Sparreboom, A. Interaction of cisplatin with the human organic cation transporter 2. *Clin Cancer Res* **14**, 3875-3880 (2008).

documented in detail previously.[62] The study protocols were approved by the Erasmus Medical Center review board (Rotterdam, The Netherlands), and all patients provided informed consent prior to enrollment. Cisplatin powder (Pharmachemie, Haarlem, The Netherlands) was dissolved in 250 mL of a sterile, hypertonic solution containing 3% (w/v) sodium chloride, and was administered as a 3-hour continuous intravenous infusion at doses ranging from 50 to 100 mg/m^2 with treatment cycles repeated every week or every 3 weeks. Cisplatin was administered either alone, or in combination with oral etoposide, irinotecan, oral topotecan, or docetaxel. Prior investigation has shown that the disposition of cisplatin is unaffected by concomitant administration of these chemotherapeutic agents.[63] Antiemetic prophylaxis consisted of a 5-hydroxytryptamine-3 receptor antagonist in combination with dexamethasone.

All patients had an Eastern Cooperative Oncology Group performance status 0 – 2, had no previous anticancer therapy for at least 4 weeks, and had adequate hematopoietic (absolute neutrophil count, $\geq 1.5 \times 10^9$/L and platelet count, $\geq 100 \times 10^9$/L), hepatic (total serum bilirubin, $\leq 1.25 \times$ the upper limit of institutional normal values and serum transaminase levels, $\leq 2.5 \times$ the upper limit of institutional normal values or $\leq 5 \times$ in case of liver metastases), and renal function (normal serum creatinine and/or creatinine clearance, ≥ 60 mL/min) at the time of study entry.

5.2.3 Pharmacokinetic analysis

Blood samples were drawn from the arm opposite to the infusion site and collected in 4.5-mL glass tubes containing lithium heparin as an anticoagulant. Blood samples were collected immediately before drug infusion, at 1 and 2 hours after the start of infusion, at 5 min before the end of infusion, and at 0.5, 1, 2, 3, and 18 hours after the end of infusion. In a limited number of patients, additional samples were obtained at 1.5 and 5 hour after the end of infusion. Plasma was separated by centrifugation at 3000 *g* for 10 min, and 500-µL aliquots of plasma were immediately extracted with 1000 µL of neat ice-cold (-20°C) ethanol in a 2-mL polypropylene vial in order to obtain the fraction unbound platinum.[64] The supernatant was collected by centrifugation at 23,000 *g* for 5 min.

Total platinum plasma concentrations were quantified after a 5-fold dilution of plasma in water containing 0.2% (v/v) Triton X-100 and 0.06% (w/v) cesium chloride.

Complete urine collections were obtained for a period of 24-hours after the start of drug administration. Aliquots of 750 µL drug free nitric acid (0.2%) were added to 50 µl of urine.

Aliquots of 20 µL of processed ethanolic extracts, plasma or urine were eventually injected, in duplicate, into the atomic absorption spectrometer. Samples were analyzed for platinum-containing species with a Perkin Elmer 4110 ZL spectrometer (Perkin Elmer) with Zeeman background correction using peak area signal measurements at a wavelength of 265.9 nm and a slit width of 0.7 nm. Drug concentrations were

determined by interpolation on linear calibration curves constructed from drug-free matrices derived from healthy volunteers. The lower limit of quantitations were established at 0.0300, 0.200 and 1.00 µg/mL platinum in ethanolic extracts, plasma and urine, respectively. The mean percentage deviation from nominal values (accuracy) and precision (within-run and between-run variability) of quality control samples spiked with known concentrations of cisplatin were always less than 15%.

Individual platinum plasma concentration time profiles were analyzed by a noncompartmental method using WinNonlin version 5.1 (Pharsight, Inc., Mountain View, CA). Plasma pharmacokinetic parameters assessed included peak concentration (Cmax), area under the plasma concentration-time curve extrapolated to infinity (AUC), and the systemic clearance (CL), which was calculated as dose divided by AUC. Standard noncompartmental evaluation has demonstrated that measures of systemic exposure to cisplatin are dose-proportional over the tested dose range.[63] Therefore, values for Cmax and AUC were normalized to a dose of 75 mg/m^2 without further correction. Urinary excretion was expressed as a percentage of the administered dose (in platinum equivalents) in the collection interval, whereas the apparent renal clearance (CLr) was calculated as the amount excreted in urine in the collection interval divided by the observed partial AUC within the same interval.

5.2.4 Identification of SLC22A2 *variants*

The genomic sequence of *SLC22A2* was obtained from GenBank (accession number NM 003058), and primers were designed to span exons that contain 8 known non-synonymous, single-nucleotide polymorphisms (SNPs) identified previously in a Caucasian population,[31] using Primer3 software. These SNPs include sequence variation associated with amino acid changes at the following positions: Pro54Ser (exon 1), Phe161Leu, Met165Val, Met165Ile (exon 2), Ala270Ser (exon 4), Ala297Gly (exon 5), Arg400Cys (exon 7), and Lys432Gln (exon 8). DNA was extracted from plasma, buffy coat, or whole blood using the QIAamp ultrasens virus kit and QIAamp DNA blood mini kit (Qiagen, Valencia, CA). Optimal reaction and cycle conditions were determined for each amplicon in a 25-µL reaction volume. Following PCR, samples were cleaned using ExoSAP-IT reagent and sequenced in the forward and reverse direction using the same primers used for PCR and Big Dye Terminator (version 3.1) Chemistry on Applied Biosystem 3730XL DNA Analyzers. Sequencing analysis was performed using Sequencher software version 4.7 (Gene Codes Corporation, Ann Arbor, MI).

5.2.5 Statistical considerations

Group differences in median pharmacokinetic parameters of cisplatin as a function of human OCT2-variant status was evaluated using a Mann-Whitney U test. Changes in serum creatinine before and after cisplatin treatment in patients was assessed as a function of OCT2-variant status using a Wilcoxon's signed ranks test. Two-tailed *P* values of less than 0.05 were considered as statistically significant. All statistical

calculations were performed using the software package NCSS version 2004 (Number Cruncher Statistical System, Kaysville, UT, USA).

5.3 Results

5.3.1 Cisplatin pharmacokinetics

A pharmacokinetic analysis showed that the mean clearance for total platinum (0.738 L/h) and unbound platinum (29.2 L/h) in the studied patients was in the same range as previously reported in other predominantly Caucasian populations.[2] The unbound clearance varied about 4-fold between patients (**Table 5.1**), and the observed variability was, in part, accounted for by sex (**Fig 5.1**), with men having a statistically significantly higher median unbound platinum clearance than women ($P = 0.019$).

Out of the 8 non-synonymous SNPs evaluated, only one SNP (Ala270Ser; rs316019) was identified in this patient population, with an observed minor allele frequency of 7.6%. However, none of the studied pharmacokinetic parameters were significantly associated with the presence or absence of this allele ($P > 0.05$). For example, the unbound clearances in those patients carrying the reference or the heterozygous sequence were 29.5 ± 1.00 and 31.9 ± 2.89, respectively ($P = 0.36$) (**Fig 5.2A**), whereas the values for renal clearance were 10.5 ± 0.628 and 9.50 ± 1.38, respectively ($P = 0.55$) (data not shown). The concentration time profiles were unchanged in patients carrying the reference and variant alleles (**Fig 5.2B**). This SNP was also not associated with the clearance of unbound cisplatin in males ($P = 0.62$) or females ($P = 0.25$) when analyzed separately.

5.3.2 Genotype association with cisplatin induced toxicity

Patients carrying a copy of this SNP experienced no change in serum creatinine after the first cycle of cisplatin treatment ($P = 0.12$), whereas serum creatinine significantly increased in patients carrying the reference sequence ($P = 0.00009$) (**Fig 5.3**). Three patients carrying the reference allele were considered to have changes in serum creatinine that were outliers in comparison to the rest of the cohort. One of the outliers may have been due to the use of diclofenac with cisplatin treatment. Diclofenac has been shown to interact with OCT2, ABCC2, and ABCC4 *in vitro*, although the interaction has not been completely determined.[65,66] After the patient was placed on a different pain medication, serum creatinine levels returned to the range seen in the cohort of patients carrying the reference allele. This result could implicate a drug interaction at the site of renal tubular transport of cisplatin. Nonetheless, after removal of the three patients from analysis, changes in serum creatinine were still significantly higher in the reference group.

Table 5.1 Summary of pharmacokinetic parameters for cisplatin-derived platinum*

Variable	Median	Mean ± SD	Range
Platinum dose (mg)	87.8	89.4	52-156
Infusion duration (h)	3.02	3.25	2.67-4.73
Total platinum			
Cmax (μg/mL)	2.21	2.37 ± 0.450	1.49-3.66
AUC (μg·h/mL)	31.4	32.5 ± 9.75	9.60-54.2
CL (L/h)	0.632	0.738 ± 0.461	0.163-2.74
Urinary excretion (%)	31.1	31.4 ± 10.0	9.70-62.0
CLr (L/h)	9.51	10.2 ± 4.14	3.03-24.8
Unbound platinum			
Cmax (μg/mL)	0.947	0.890 ± 0.25	0.051-1.55
AUC (μg·h/mL)	2.75	2.61 ± 0.656	0.118-4.37
CL (L/h)	27.2	29.2 ± 8.39	16.8-75.4

*Pharmacokinetic parameters were obtained with non-compartmental analysis, and normalized to 75 mg/m² cisplatin.
Abbreviations: Cmax, peak concentration; AUC, area under the plasma concentration-time curve; CL, systemic clearance; CLr, renal clearance.

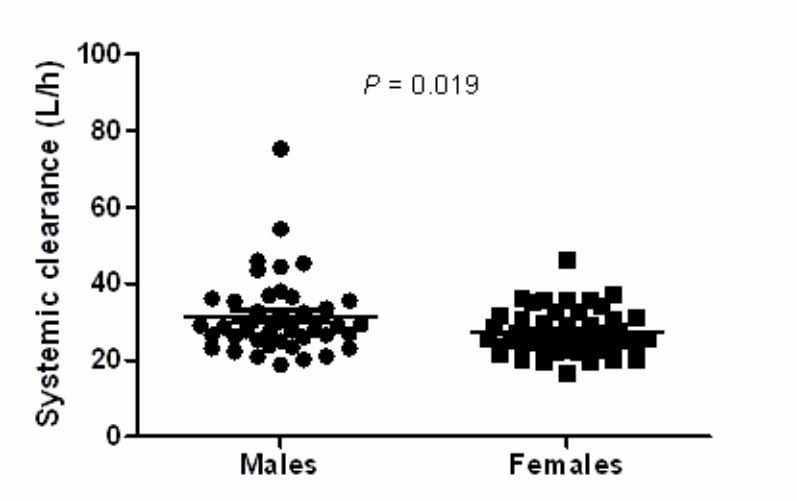


Figure 5.1 Sex Differences in Cisplatin Clearance

Influence of sex on the clearance of unbound cisplatin. Each point indicates an individual patient. The horizontal lines indicate the mean.

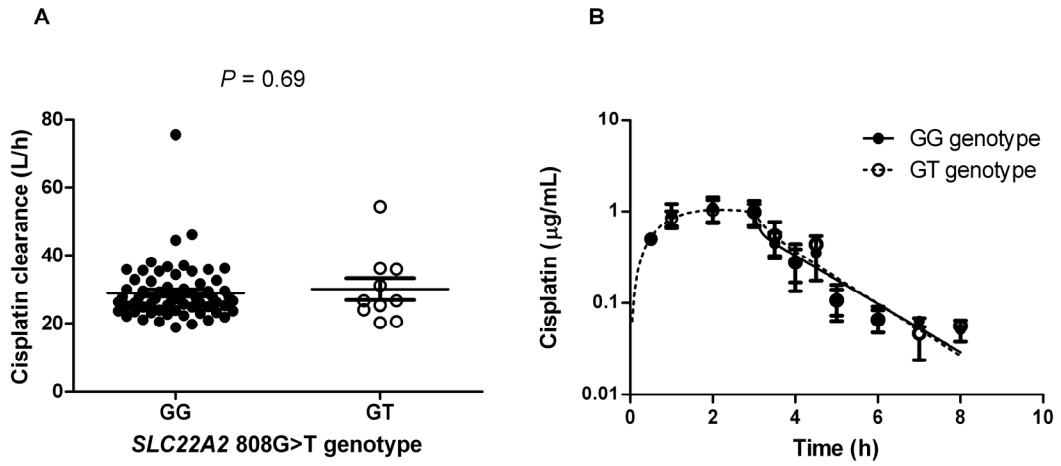


Figure 5.2 Associations between Genotype and Cisplatin Clearance

(**A**) Unchanged systemic clearance of unbound cisplatin in cancer patients as a function of *SLC22A2* 808G>T genotype status. Each point represents a patient and mean values are indicated by lines. (**B**) Concentration-time profile of unbound cisplatin as a function of SLC22A2 808G>T genotype status. Data shown as mean values ± standard error along with a curve fit from a 2-compartment model (lines).

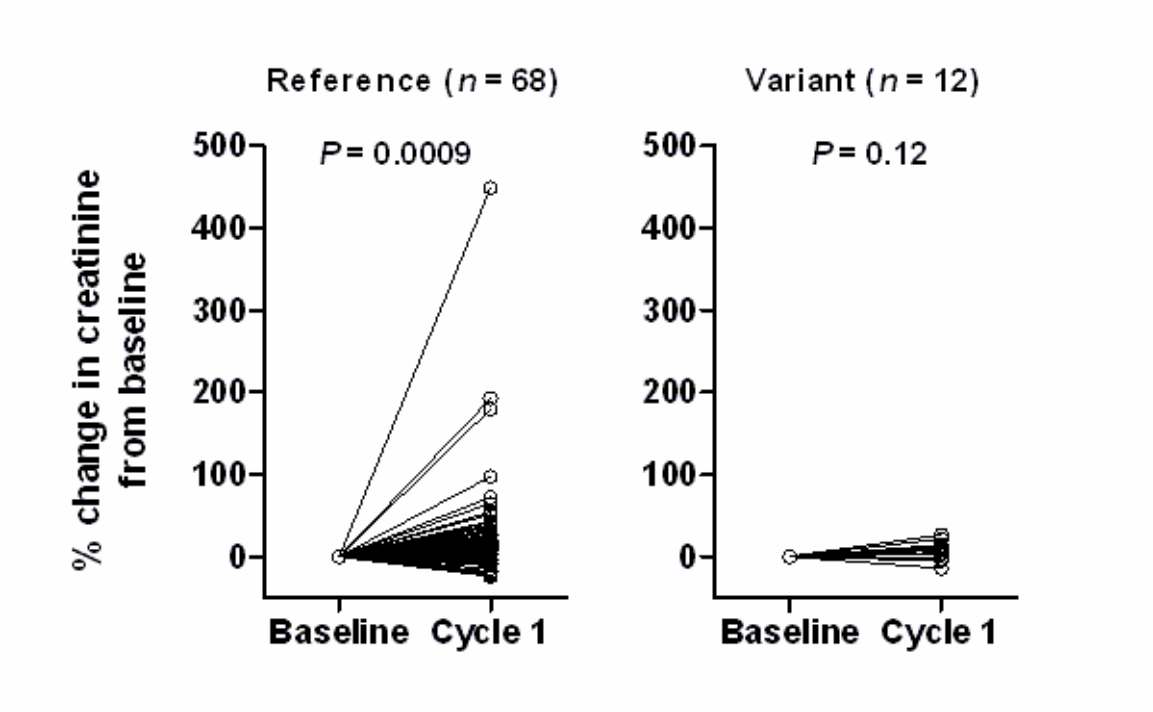


Figure 5.3 Serum Creatinine Changes

Changes in serum creatinine, a marker of nephrotoxicity, measured at baseline and after the first cycle of cisplatin treatment as a function of *SLC22A2* Ala270Ser genotype.

5.4 Discussion

In this study we found that the human organic cation transporter OCT2 is involved in the toxicity associated with cisplatin treatment. Through a pharmacokinetic analysis, we observed a high degree of interindividual variability in the clearance of cisplatin in a Caucasian population. No statistically significant associations were found between the pharmacokinetic parameters of cisplatin and a series of variants in the gene encoding OCT2 (*SLC22A2)*. However, patients carrying a variant allele in OCT2 were less likely to show increases in serum creatinine after the first cisplatin treatment cycle compared to individuals carrying the reference allele.

We and others have previously determined that human OCT2 and its rodent orthologue may play a critical role in the cellular transport of cisplatin.[28,40] Cisplatin-induced nephrotoxicity typically occurs in the S3 segment of the renal proximal tubules,[67] where OCT2 is predominantly expressed on the basolateral membrane.[68,69] This localization suggests that OCT2 may be a key regulator in the renal elimination of cisplatin. Due to the high variability in cisplatin clearance found in our patient population, as well as the known extensive interindividual variability in the incidence and severity of drug-induced toxicity, we assessed a potential contribution to this phenomenon of known SNPs in the *SLC22A2* gene. Amongst 8 variants evaluated, only one SNP (Ala270Ser; rs316019) was found in the sample population. This SNP was not associated with the plasma pharmacokinetics or the urinary excretion of cisplatin in these patients. However, this SNP was significantly associated with the serum creatinine levels in patients after the first cycle of cisplatin treatment. This observation is consistent with functional data obtained using heterologous expression models indicating that the functional activity of the Ala270Ser variant is lower than that of the OCT2 reference protein.[70] This data also correlates with our previous findings in mice, indicating a lack of function of Oct2 may protect kidney cells from cisplatin induced damage without affecting plasma platinum concentrations.

These results implicate potential new mechanisms for the prevention of cisplatin-induced nephrotoxicity by modulating the uptake of cisplatin into kidney cells. An OCT2 inhibitor may be able to block the majority of cisplatin from entering kidney tubules, perhaps shunting the drug through other elimination pathways. Future studies in patients receiving cisplatin need to be performed to understand potential toxicities that may arise with the use of an inhibitor. This study further shows the important role OCT2 plays in the renal transport and toxicity of cisplatin.

CHAPTER 6. ORGANIC CATION TRANSPORTER 2 EXPRESSION IN HUMAN TISSUE AND TUMOR

6.1 Introduction

Organic cation transporter 2 (OCT2) is predominantly expressed on the basolateral surface of the renal proximal tubules with low level expression in the brain.[25] We have previously shown that OCT2 transports cisplatin, the most widely used anticancer agent, into the cell. In mice, a deficiency in Oct2 was able to protect kidney proximal tubules from severe tubular necrosis. Furthermore, patients carrying a variant in the gene encoding OCT2 which may decrease the function of the transporter, had a lower change in serum creatinine, a marker for kidney damage, than those patients carrying the reference allele. Taken together, these results could indicate an exciting new treatment regimen to reduce cisplatin-induced toxicity by blocking OCT2. However, preventing toxicity could pose possible problems for the anti-tumor efficacy of cisplatin.

To better understand the role OCT2 plays in the anti-tumor efficacy of cisplatin, we sought to evaluate the expression of OCT2 in human tumor cell lines, as well as, a panel of human tumor tissue. Currently there is little knowledge about the importance of OCT2 in human tumors, indicating that it may play a limited role. Therefore we hypothesize that OCT2 is not highly expressed in human tumor.

6.2 Materials and Methods

6.2.1 Real time PCR

RNA and DNA from the NCI anti-cancer screening panel were provided by the National Cancer Institute tumor repository (Bethesda, MD, USA). RNA was reverse transcribed using SuperScript III first strand synthesis supermix for qRT-PCR (Invitrogen) according to manufacturer's recommendations. Tissue plates containing cDNA from 48 human tissues and 96 human cancer tissues were obtained from Origene (Rockville, MD, USA). Gene transcripts were quantified using SYBR Green PCR mastermix (Qiagen) and primers previously described.[38] Reactions were carried out in triplicate unless otherwise stated as previously reported.[5] Briefly, 25 μL volumes were used with the following PCR variables: 95ºC for 15 min then 40 cycles of 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s, followed by dissociation cycles. Transcripts of each sample were normalized to the housekeeping gene, *GAPDH*.

6.2.2 Identification of SLC22A2 *variants*

The genomic sequence of *SLC22A2* (accession no. NM 003058) was obtained by Genbank and primers were designed as previously described.[5] A single nucleotide

polymorphism (SNP) in *SLC22A2* associated with an amino acid change at position Ala270Ser (exon 4) was evaluated. Optimal reaction and cycle conditions were determined for each amplicon in a 25 μL reaction volume. As previously described,[5] PCR samples were cleaned with ExoSAP-IT reagent (USB Corporation, Cleveland, OH, USA) following PCR and sequenced in both forward and reverse directions using Big Dye Terminator (version 3.1) Chemistry on Applied Biosystems 3730XL DNA analyzers. Sequencher software version 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA) was used for sequencing analysis.

6.3 Results

6.3.1 Expression of OCT2 in normal human tissue

Using real time PCR, OCT2 expression was confirmed in a panel of normal human tissue. As expected, the tissue with the highest expression was kidney tissue when compared to the other tissues (**Fig 6.1**). Low level expression was seen in a variety of other tissues; however not to the extent of the kidney expression.

6.3.2 Expression of OCT2 in human tumor tissue

OCT2 expression was evaluated in cDNA human tumor tissue scan, as well as, the NCI 60 human tumor cell lines. OCT2 expression in the human tumor tissue again confirmed the presence of OCT2 in normal kidney and also showed low level expression in some kidney tumor types. The expression of OCT2 was absent in tumor from breast, colon, liver, lung, ovarian, prostate, and thyroid tissue, particularly when compared to the baseline expression in the kidney (**Fig 6.2A**). When looking at expression changes within tissues, colon and thyroid tumors appear to have higher expression than in the respective normal tissue (**Fig 6.2B**).

The NCI 60 human tumor panel again showed very low or absent expression levels of OCT2 (**Fig 6.3**). Two kidney cell lines, A498 and UO 31, were the only kidney lines expressing OCT2. Contrary to our tumor panel, one ovarian cancer cell line, SK OV 3, expressed OCT2 higher than any other cell line. In addition to looking at OCT2 expression, we evaluated the Ala270Ser SNP in the cell lines. Interestingly, we found a trend indicating that those cells lines carrying the variant allele had a reduced expression relative to those with the reference allele (**Fig 6.4**).

6.4 Discussion

We have previously reported that OCT2 is a primary regulator for cisplatin transport and toxicity in renal cells, implicating possible new treatment options to reduce the occurrence of cisplatin-induced nephrotoxicity. Therefore, we sought to determine the

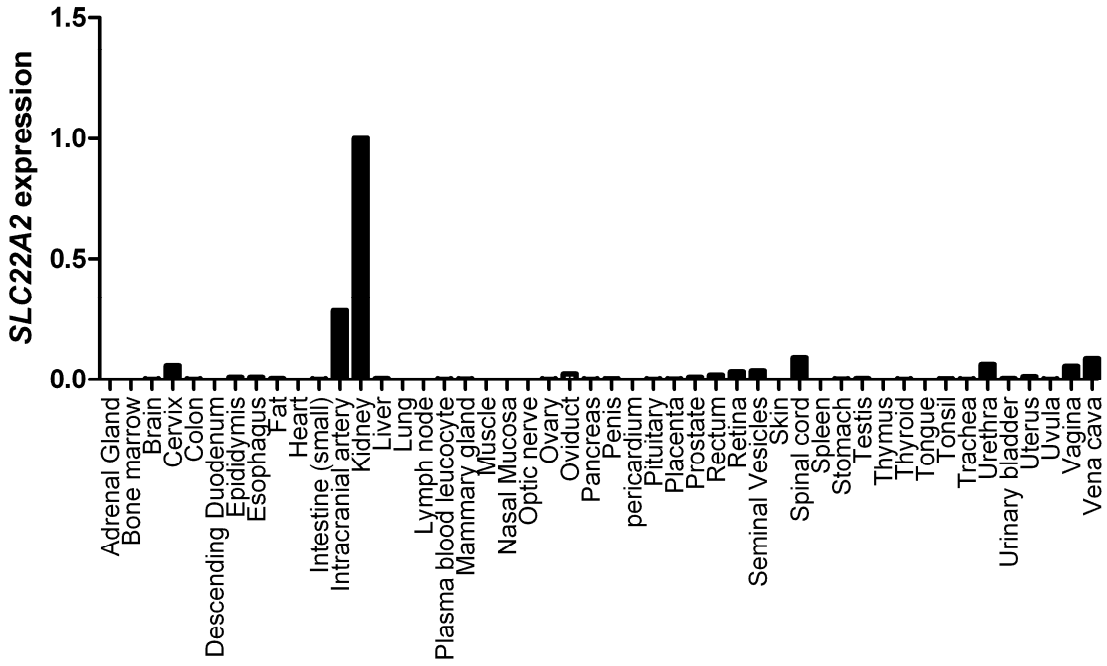
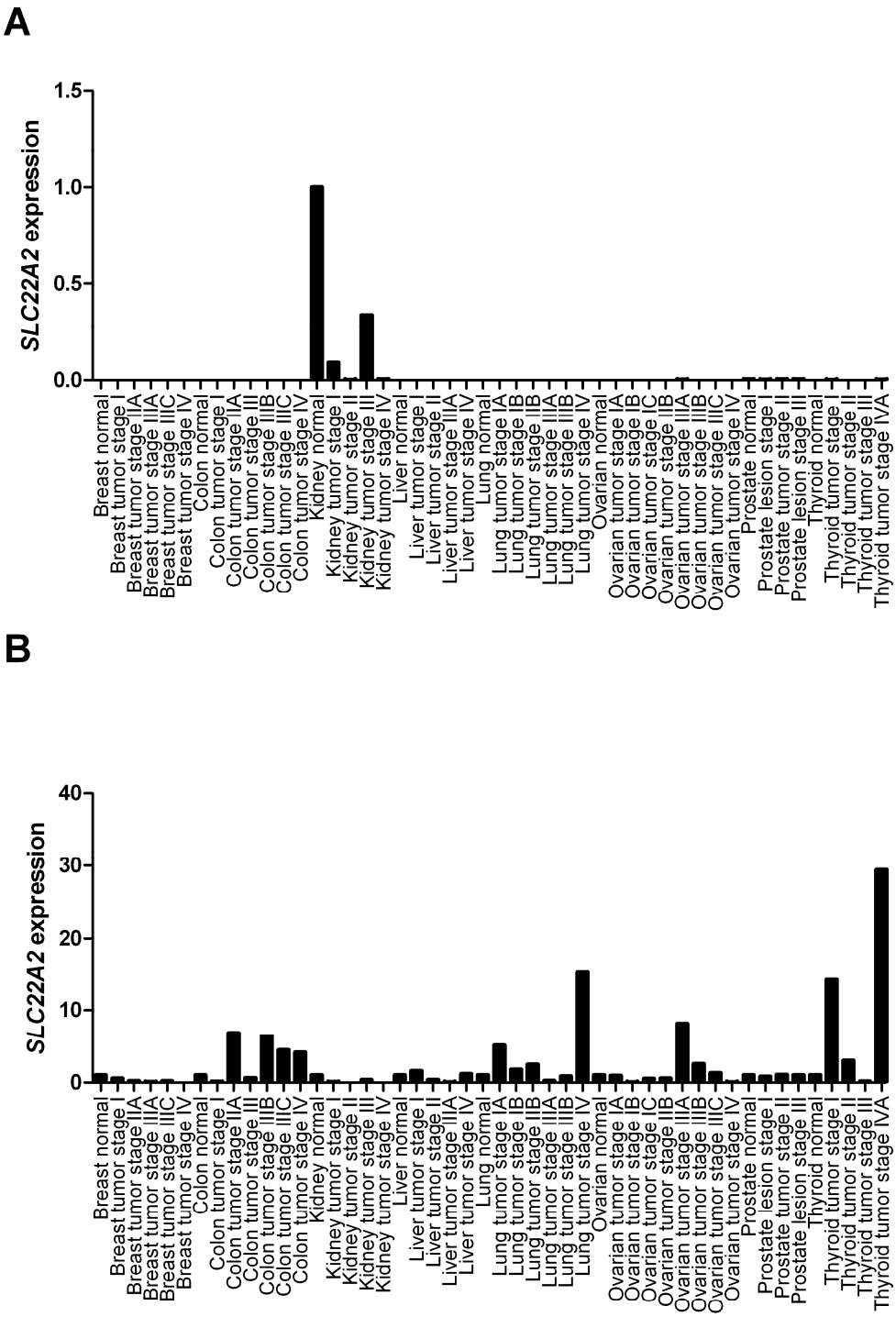


Figure 6.1 OCT2 Expression in Normal Human Tissue

Expression of *SLC22A2*, normalized to the house keeping gene *GAPDH*, in (**A**) human normal tissue. *SLC22A2* was predominantly expressed in kidney. Tissue plates containing cDNA tissues were used for real-time PCR analysis. Data are shown as mean values (bars) of duplicate plates and expressed relative to values observed in normal human kidney, which was set to a value of 1.

Figure 6.2 OCT2 Expression in Human Tumor Tissue

Expression of *SLC22A2*, normalized to the house keeping gene *GAPDH*, in human tumor samples (**A**) relative to the respective normal tissue and (**B**) relative to normal kidney. *SLC22A2* was low to absent in tumor samples. Tumor plates containing cDNA were used for real-time PCR analysis. Data are shown as mean values (bars) of duplicate plates and expressed relative to values observed in normal tissue and normal human kidney, which was set to a value of 1.


A
SLC22A2 expression
1.5
1.0
0.5
0.0
Breast normal
Breast tumor stage I
Breast tumor stage IIA
Breast tumor stage IIIA
Breast tumor stage IIIC
Breast tumor stage IV
Colon normal
Colon tumor stage I
Colon tumor stage IIA
Colon tumor stage III
Colon tumor stage IIIB
Colon tumor stage IIIC
Colon tumor stage IV
Kidney normal
Kidney tumor stage I
Kidney tumor stage II
Kidney tumor stage III
Kidney tumor stage IV
Liver normal
Liver tumor stage I
Liver tumor stage II
Liver tumor stage IIIA
Liver tumor stage IV
Lung normal
Lung tumor stage IA
Lung tumor stage IB
Lung tumor stage IIB
Lung tumor stage IIIA
Lung tumor stage IIIB
Lung tumor stage IV
Ovarian normal
Ovarian tumor stage IA
Ovarian tumor stage IB
Ovarian tumor stage IC
Ovarian tumor stage IIB
Ovarian tumor stage IIIA
Ovarian tumor stage IIIB
Ovarian tumor stage IIIC
Ovarian tumor stage IV
Prostate normal
Prostate lesion stage I
Prostate tumor stage II
Prostate lesion stage III
Thyroid normal
Thyroid tumor stage I
Thyroid tumor stage II
Thyroid tumor stage III
Thyroid tumor stage IVA
B
SLC22A2 expression
40
30
20
10
0
Breast normal
Breast tumor stage I
Breast tumor stage IIA
Breast tumor stage IIIA
Breast tumor stage IIIC
Breast tumor stage IV
Colon normal
Colon tumor stage I
Colon tumor stage IIA
Colon tumor stage III
Colon tumor stage IIIB
Colon tumor stage IIIC
Colon tumor stage IV
Kidney normal
Kidney tumor stage I
Kidney tumor stage II
Kidney tumor stage III
Kidney tumor stage IV
Liver normal
Liver tumor stage I
Liver tumor stage II
Liver tumor stage IIIA
Liver tumor stage IV
Lung normal
Lung tumor stage IA
Lung tumor stage IB
Lung tumor stage IIB
Lung tumor stage IIIA
Lung tumor stage IIIB
Lung tumor stage IV
Ovarian normal
Ovarian tumor stage IA
Ovarian tumor stage IB
Ovarian tumor stage IC
Ovarian tumor stage IIB
Ovarian tumor stage IIIA
Ovarian tumor stage IIIB
Ovarian tumor stage IIIC
Ovarian tumor stage IV
Prostate normal
Prostate lesion stage I
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Thyroid normal
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Thyroid tumor stage II
Thyroid tumor stage III
Thyroid tumor stage IVA

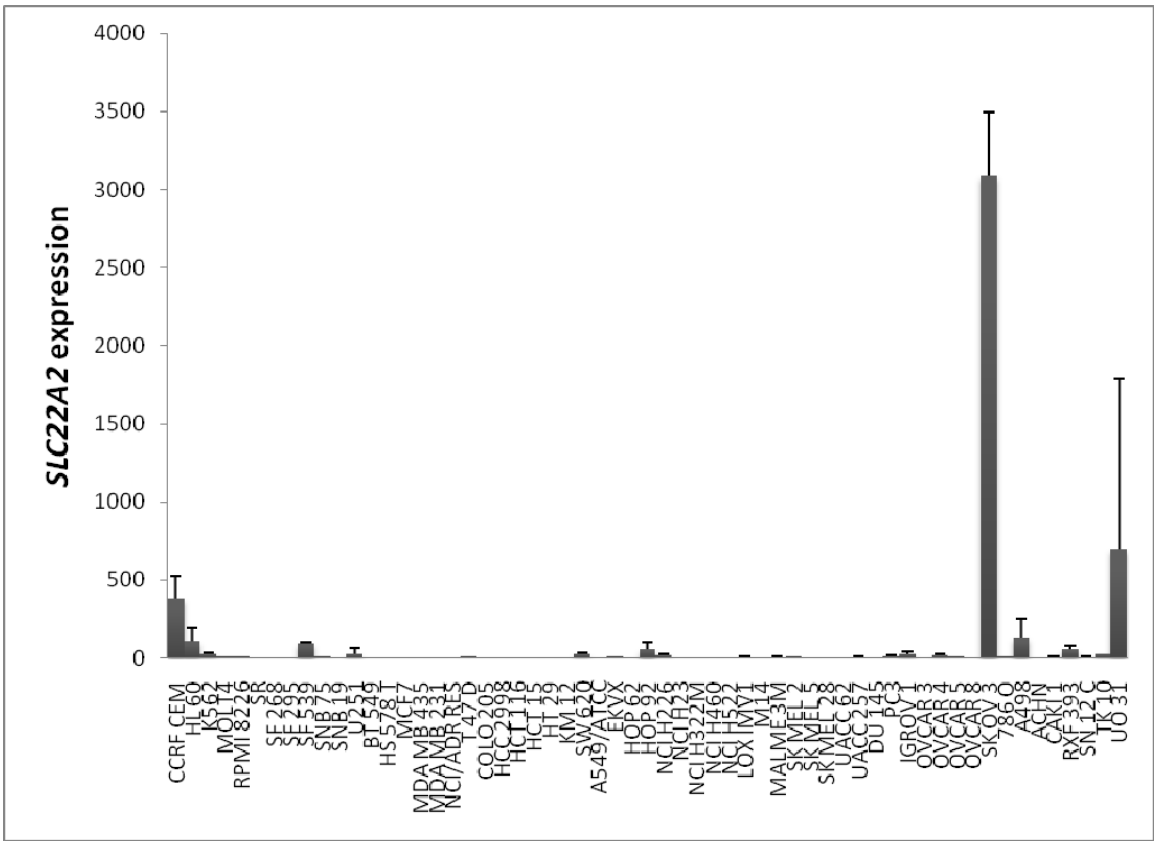


Figure 6.3 OCT2 Expression in the NCI 60 Tumor Cell Lines

Expression of *SLC22A2*, normalized to the house keeping gene *GAPDH*, in human tumor cell lines. Data are shown as mean values (bars) ± SD (n = 3), expressed relative to values observed in the HCC 2998 cell line, which was set to a value of 1.

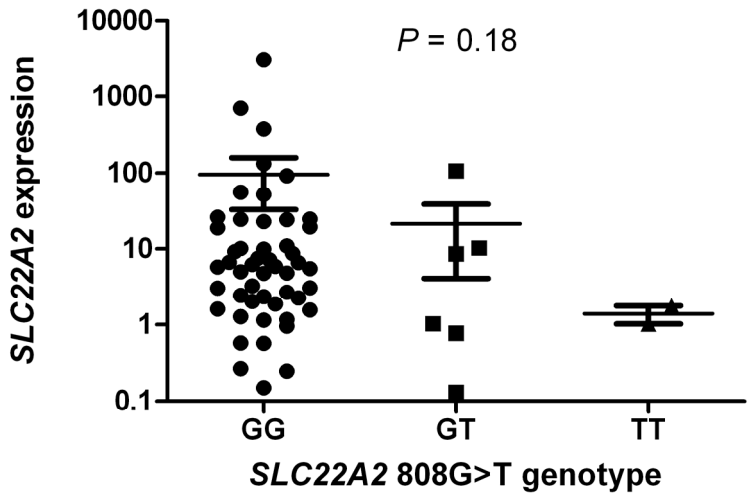


Figure 6.4 OCT2 Expression as a Function of Ala270Ser Genotype

Correlation between mRNA expression of *SLC22A2* (fold change versus the HCC 2998 cell line) and *SLC22A2* 808G>T genotype in the NCI 60 cancer cell line panel.

contribution of OCT2 to the antitumor efficacy of cisplatin by evaluating the expression of OCT2 in tumor cells and human tumor tissue. In this study, we found that OCT2 is predominantly expressed in normal human kidney; however it has very low or absent expression in almost all tumor cells and tissue, in particular in those for which treatment with cisplatin is indicated, such as lung cancer and ovarian cancer. We did find one ovarian tumor cell line with high expression of OCT2 but, this expression is contradictory when comparing it to the ovarian tumor tissue we examined. Therefore this difference may be an artifact of the cell culture process. Experiments using the cDNA from the cancer plates and the NCI 60 tumor panel may not provide a comprehensive understanding of the expression of OCT2 in tumor cells. Studying expression in tumor samples, particularly before and after cisplatin treatment could fully elucidate the role of OCT2 in human tumors. Although our findings indicate a limited involvement of OCT2 in human tumors, more elaborate investigations should be performed in the future to rule out any potential disruptions caused by an OCT2 inhibitor on cisplatin anti-tumor efficacy in patients.

CHAPTER 7. INTERACTION OF CISPLATIN WITH ABCC2

7.1 Introduction

In previous studies, we demonstrated the importance of OCT2 in the transport of cisplatin through the basolateral site in renal proximal tubules. Mice lacking the organic cation transporter were protected from severe cisplatin-induced nephrotoxicity after treatment. Urinary platinum excretion of cisplatin in these animals was reduced to approximately 56% of the dose at 72 hours, whereas wildtype animals excreted almost 100% of the dose. We also found a nonsynonymous SNP in OCT2, Ala270Ser, to be significantly associated with changes in serum creatinine after the first cycle of treatment. Patients carrying a variant allele exhibited a smaller percent change in serum creatinine compared to patients carrying the reference allele. This difference suggests that OCT2 is the primary regulator of cisplatin transport into the cell; however, the understanding of how cisplatin exits the cell via the apical membrane is poorly understood. Many transporters present on the apical membrane could be potential cisplatin transporters, for the purpose of this study we focused primarily on the multidrug resistance associated protein, ABCC2 (MRP2).

ABCC2 has been shown to be localized to the apical membrane of hepatocytes and renal proximal tubules.[32] Previous reports have shown increased ABCC2 expression in cisplatin resistant cell lines and has been associated with cisplatin resistance in patients treated with cisplatin.[23,34] In kidney cells, cisplatin can form glutathione conjugates before being eliminated. ABCC2 has also been implicated in the efflux of several glutathione conjugates,[32] suggesting an important role for this transporter in the renal excretion of cisplatin. In this study we evaluated the contribution of ABCC2 to cisplatin transport, as well as, genetic variation in the gene encoding ABCC2. We hypothesize that ABCC2 will be a key transporter in the removal of cisplatin from kidney tubule cells.

7.2 Materials and Methods

7.2.1 Materials

ABCC2 primers were custom made by the Hartwell Center (St. Jude Children's Research Hospital, Memphis, TN). The ExoSAP-IT reagent was obtained from USB Corporation (Cleveland, OH).

7.2.2 Clinical studies

All patients eligible for the study had a confirmed diagnosis of a malignant solid tumor, and were between the ages of 18 and 75 years. Eligibility criteria have been documented in detail previously.[62] The study protocols were approved by the Erasmus

Medical Center review board (Rotterdam, The Netherlands), and all patients provided informed consent prior to enrollment. Cisplatin powder (Pharmachemie, Haarlem, The Netherlands) was dissolved in 250 mL of a sterile, hypertonic solution containing 3% (w/v) sodium chloride, and was administered as a 3-hour continuous intravenous infusion at doses ranging from 50 to 100 mg/m^2 with treatment cycles repeated every week or every 3 weeks. Cisplatin was administered either alone, or in combination with oral etoposide, irinotecan, oral topotecan, or docetaxel. Prior investigation has shown that the disposition of cisplatin is unaffected by concomitant administration of these chemotherapeutic agents.[63] Antiemetic prophylaxis consisted of a 5-hydroxytryptamine-3 receptor antagonist in combination with dexamethasone.

All patients had an Eastern Cooperative Oncology Group performance status 0 – 2, had no previous anticancer therapy for at least 4 weeks, and had adequate hematopoietic (absolute neutrophil count, $\geq 1.5 \times 10^9$/L and platelet count, $\geq 100 \times 10^9$/L), hepatic (total serum bilirubin, $\leq 1.25 \times$ the upper limit of institutional normal values and serum transaminase levels, $\leq 2.5 \times$ the upper limit of institutional normal values or $\leq 5 \times$ in case of liver metastases), and renal function (normal serum creatinine and/or creatinine clearance, ≥ 60 mL/min) at the time of study entry.

7.2.3 Pharmacokinetic analysis

Blood samples were drawn from the arm opposite to the infusion site and collected in 4.5-mL glass tubes containing lithium heparin as an anticoagulant. Blood samples were collected immediately before drug infusion, at 1 and 2 hours after the start of infusion, at 5 min before the end of infusion, and at 0.5, 1, 2, 3, and 18 hours after the end of infusion. In a limited number of patients, additional samples were obtained at 1.5 and 5 hour after the end of infusion. Plasma was separated by centrifugation at 3000 *g* for 10 min, and 500-µL aliquots of plasma were immediately extracted with 1000 µL of neat ice-cold (-20°C) ethanol in a 2-ml polypropylene vial in order to obtain the fraction unbound platinum.[64] The supernatant was collected by centrifugation at 23,000 *g* for 5 min.

Total platinum plasma concentrations were quantified after a 5-fold dilution of plasma in water containing 0.2% (v/v) Triton X-100 and 0.06% (w/v) cesium chloride.

Complete urine collections were obtained for a period of 24-hours after the start of drug administration. Aliquots of 750 µL drug free nitric acid (0.2%) were added to 50 µl of urine.

Aliquots of 20 µL of processed ethanolic extracts, plasma or urine were eventually injected, in duplicate, into the atomic absorption spectrometer. Samples were analyzed for platinum-containing species with a Perkin Elmer 4110 ZL spectrometer (Perkin Elmer) with Zeeman background correction using peak area signal measurements at a wavelength of 265.9 nm and a slit width of 0.7 nm. Drug concentrations were determined by interpolation on linear calibration curves constructed from drug-free

matrices derived from healthy volunteers. The lower limit of quantitations were established at 0.0300, 0.200 and 1.00 μg/mL platinum in ethanolic extracts, plasma and urine, respectively. The mean percentage deviation from nominal values (accuracy) and precision (within-run and between-run variability) of quality control samples spiked with known concentrations of cisplatin were always less than 15%.

Individual platinum plasma concentration time profiles were analyzed by a noncompartmental method using WinNonlin version 5.1 (Pharsight, Inc., Mountain View, CA). Plasma pharmacokinetic parameters assessed included peak concentration (Cmax), area under the plasma concentration-time curve extrapolated to infinity (AUC), and the systemic clearance (CL), which was calculated as dose divided by AUC. Standard noncompartmental evaluation has demonstrated that measures of systemic exposure to cisplatin are dose-proportional over the tested dose range.[63] Therefore, values for Cmax and AUC were normalized to a dose of 75 mg/m^2 without further correction. Urinary excretion was expressed as a percentage of the administered dose (in platinum equivalents) in the collection interval, whereas the apparent renal clearance (CLr) was calculated as the amount excreted in urine in the collection interval divided by the observed partial AUC within the same interval.

7.2.4 Identification of ABCC2 *variants*

The genomic sequence of *ABCC2* was obtained from GenBank (accession no. NM 000392), and primers were designed to span exons that contain 7 known single-nucleotide polymorphisms (SNPs) using Primer3 software. These SNPs include sequence variation at the following positions: -1549 (5'-flanking), -1019 (5'-flanking, -24 (5'-UTR), 1249 (exon 10), -34 (intron 26), 3972 (exon 28), 4544 (exon 32). DNA was extracted from plasma, buffy coat, or whole blood using the QIAamp ultrasens virus kit and QIAamp DNA blood mini kit (Qiagen, Valencia, CA). Optimal reaction and cycle conditions were determined for each amplicon in a 25 μL reaction volume. Following PCR, samples were cleaned using ExoSAP-IT reagent and sequenced in the forward and reverse direction using the same primers used for PCR and Big Dye Terminator (version 3.1) Chemistry on Applied Biosystem 3730XL DNA Analyzers. Sequencing analysis was performed using Sequencher software version 4.7 (Gene Codes Corporation, Ann Arbor, MI).

7.2.5 Real time PCR

RNA and DNA from the NCI anti-cancer screening panel were provided by the National Cancer Institute tumor repository (Bethesda, MD, USA). RNA was reverse transcribed using SuperScript III first strand synthesis supermix for qRT-PCR (Invitrogen) according to manufacturer's recommendations. Gene transcripts were quantified using SYBR Green PCR mastermix (Qiagen) and primers previously described.[38] Reactions were carried out in triplicate unless otherwise stated as previously reported.[5] Briefly, 25 μL volumes were used with the following PCR variables: 95ºC for

15 min then 40 cycles of 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s, followed by dissociation cycles. Transcripts of each sample were normalized to the housekeeping gene, *GAPDH*.

7.2.6 Animal experiments

Adult (8-12 week old) male Abcc2 (-/-) (Taconic) mice were used along with age-matched male FVB wild-type mice. All animals were housed and handled in accordance with the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital. Animals were housed in a temperature controlled environment with a 12 hour light cycle and were given a standard diet and water ad libitum.

Urinary platinum excretion of cisplatin was studied in animals after they had acclimated to metabolic cages 5 days prior to drug administration. Baseline urine samples were collected sixteen hours after the onset of light, and next, mice were given a single intraperitoneal (i.p.) injection of cisplatin at a dose 10 mg/kg. Urine was collected 4, 24, 48, and 72 hours after administration, diluted with nitric acid (0.2%) and immediately analyzed for total platinum using flameless atomic absorption spectrometry. At the end of the study period, mice were euthanized and tissues harvested for histological examination.

7.2.7 Histological evaluation of mouse tissues

Mice were humanely euthanized and the following tissues were collected: adrenals, bone marrow, brain, heart, kidneys, large intestine, liver, lung, lymph node, pancreas, reproductive tract, salivary gland, skeletal muscle, skin, small intestine, spleen, stomach, thymus, and thyroid. After collection, tissues were fixed overnight in 10% neutral-buffered formalin. Next, the tissues were processed routinely, embedded in paraffin, sectioned (4 μm) and stained with hematoxylin and eosin. Microscopic evaluation was performed by an experienced veterinary pathologist blinded to the composition of the groups. Toxicities, including acute renal tubular necrosis, were scored as 'absent', 'rare', 'mild', 'moderate' or 'severe'.

7.3 Results

7.3.1 Association of cisplatin pharmacokinetics and ABCC2 *variants*

A pharmacokinetic analysis showed that the mean clearance for total platinum (0.738 L/h) and unbound platinum (29.2 L/h) in the studied patients was in the same range as previously reported in other predominantly Caucasian populations.[2]

All seven SNPs analyzed were present in this Caucasian population, however only one SNP was significantly associated with pharmacokinetic parameters. Patients carrying the variant allele for SNP -34 exhibited a higher unbound clearance of cisplatin than those patients carrying the reference allele (**Fig 7.1A**). We then analyzed haplotypes for the *ABCC2* gene and found one haplotype, AGTGTTG, was associated with an increased unbound renal clearance (**Fig 7.1B**).

7.3.2 Urinary platinum excretion and cisplatin induced toxicity

After cisplatin treatment, Abcc2 (-/-) mice had no significant difference in the cumulative platinum excretion when compared to wildtype animals (**Fig 7.2**). Futhermore, Abcc2 (-/-) mice experienced severe renal tubular necrosis like the wildtype animals (**Fig 7.3**).

7.3.3 Expression of ABCC2 in human tumor cell lines

We next evaluated the expression of ABCC2 in the NCI 60 tumor cell line panel. We found ABCC2 expressed in a variety of tumor cell lines including, colon, lung, melanoma, and renal cell lines (**Fig 7.4**). When we evaluated the effect of *ABCC2* genotype on this expression we saw only one SNP (-1019) having a moderate effect on ABCC2 expression in these cell lines (**Fig 7.5**). However, the only difference is seen in the cell lines carrying a heterozygous variant, with the homozygous variant the same as the reference. This discrepancy suggests that it is most likely a chance finding with little biological relevance.

7.4 Discussion

In this study we evaluated the role of ABCC2 in the excretion of cisplatin. In patients we found one SNP, -34 in *ABCC2* that appeared to be associated with the unbound clearance of cisplatin. However, several of the other SNPs studied had a rare allele frequency too low to be able to characterize their role in cisplatin clearance. This SNP has also not been functionally characterized or been associated with the clearance of other drugs, including irinotecan and docetaxel.[71,72] Our haplotype analysis also revealed one haplotype with potential importance to the unbound renal clearance of cisplatin. Again, this haplotype has not been reported to functionally impact the clearance by ABCC2 in patients treated with irinotecan.[71] Furthermore, the -34 SNP is not present in this haplotype. As with the single SNP analysis, several of the haplotypes exhibited very low frequencies in this population, making it difficult to fully understand the importance.

No significant difference in urinary platinum excretion was seen in Abcc2 (-/-) and wildtype animals after cisplatin administration. Previous investigators have shown that Abcc4 is upregulated in Abcc2 (-/-) mice.[73] This upregulation may indicate a compensatory mechanism between the two transporters. It would be interesting to

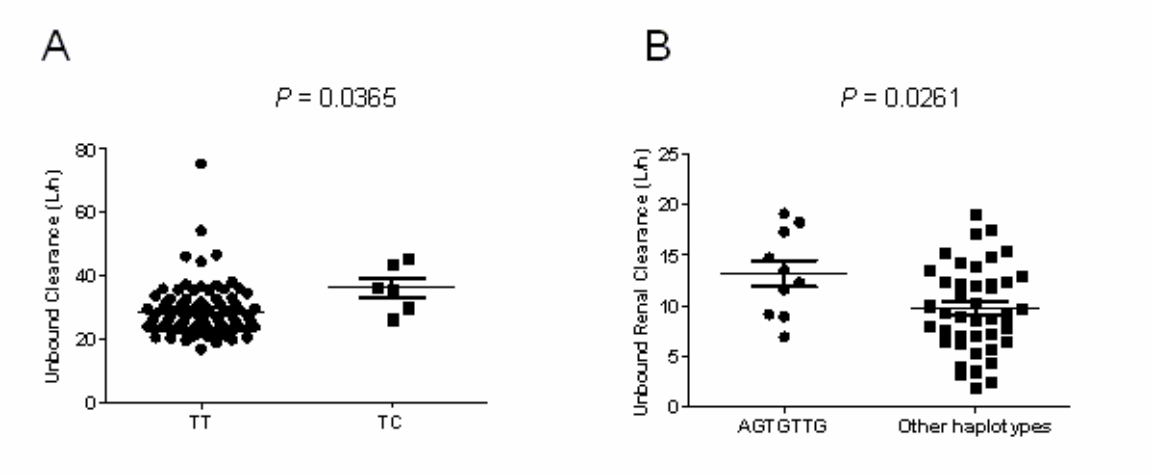


Figure 7.1 Associations between Genotype and Cisplatin Clearance

Association between **(A)** *ABCC2* -34 genotype (TT refers to the reference sequence, whereas TC is the heterozygous variant sequence) and the clearance of unbound cisplatin and **(B)** *ABCC2* haplotype and the unbound renal clearance of cisplatin. Each symbol represents an individual patient and horizontal lines indicate mean values.

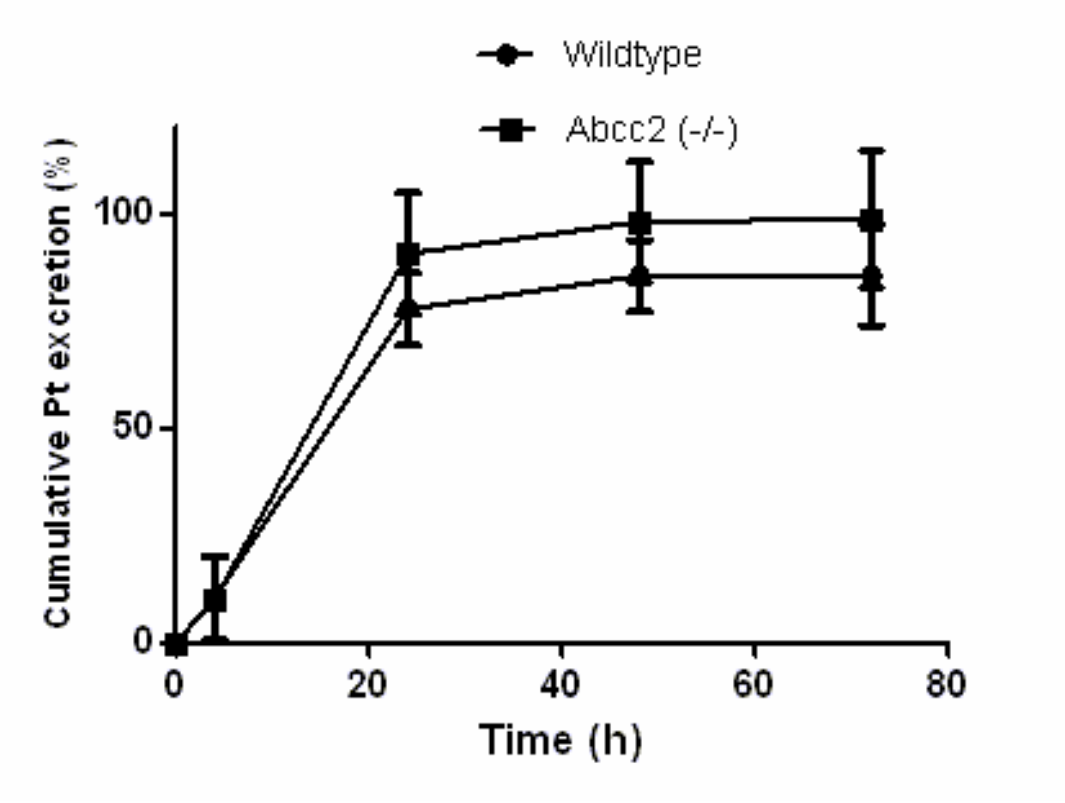


Figure 7.2 Urinary Platinum Excretion in Abcc2 (-/-) Mice

Effect of Abcc2 deficiency on renal handling of cisplatin in mice. The cumulative excretion of cisplatin after drug administration (10 mg/kg; i.p.) was unchanged in the Abcc2 (-/-) mice compared to wildtype mice (n = 4/group). Data are shown as mean values; error bars represent ± standard error.

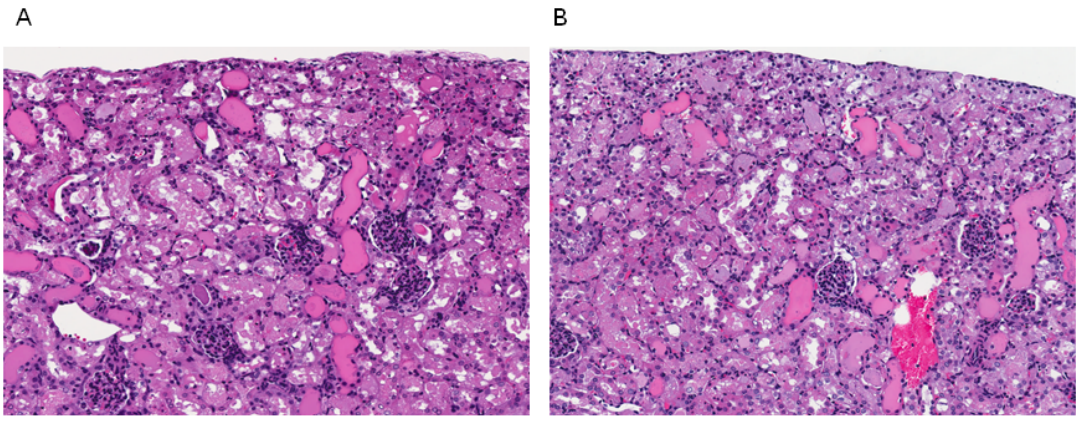


Figure 7.3 Histopathology of Abcc2 and Wildtype Kidneys

Comparative cisplatin-induced nephrotoxicity in (A) wildtype and (B) Abcc2 (-/-) mice 72 hours after administration of cisplatin (10 mg/kg; i.p.) from representative animals. Severe tubular necrosis, characterized by dilated tubules filled with necrotic tubular epithelial cells was observed in kidneys of both wildtype and Abcc2 (-/-) mice.

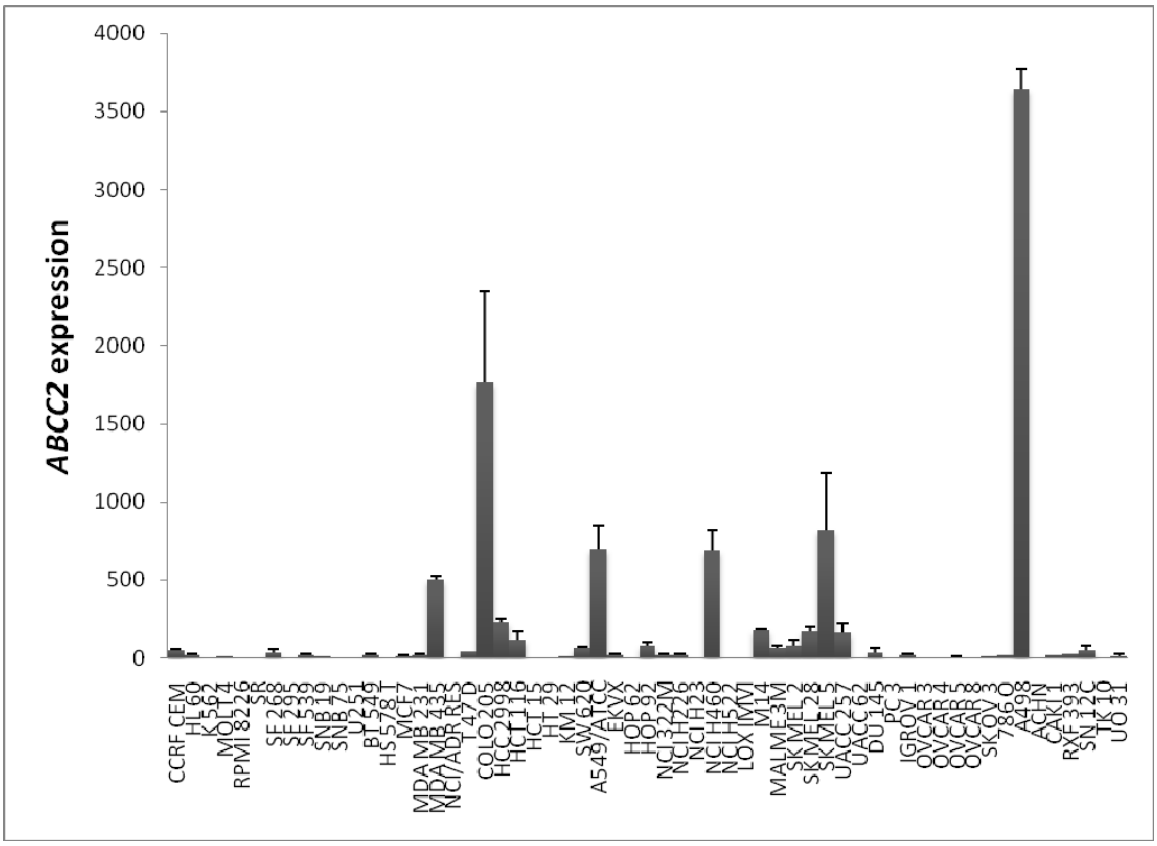


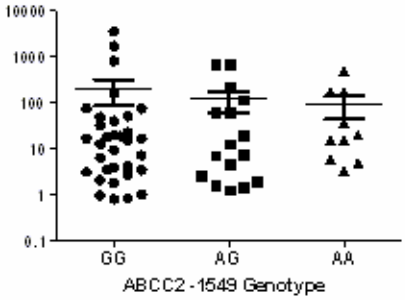
Figure 7.4 *ABCC2* Expression in the NCI 60 Tumor Cell Lines

Expression of *ABCC2*, normalized to the house keeping gene *GAPDH*, in human tumor cell lines. Data are shown as mean values (bars) ± SD (n = 3), expressed relative to values observed in the U251 cell line, which was set to a value of 1.

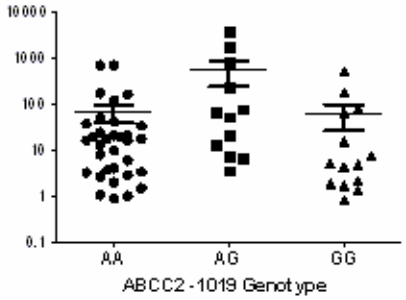
Figure 7.5 *ABCC2* Expression as a Function of *ABCC2* Genotypes

Correlation between mRNA expression of *ABCC2* (fold change versus the U251 cell line) and *ABCC2* genotype in the NCI 60 cancer cell line panel.

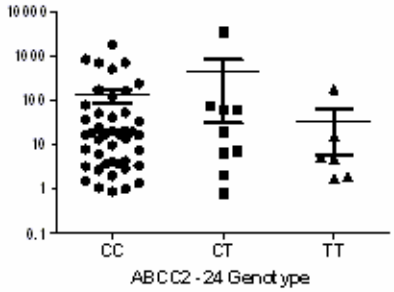
$P = 0.7831$



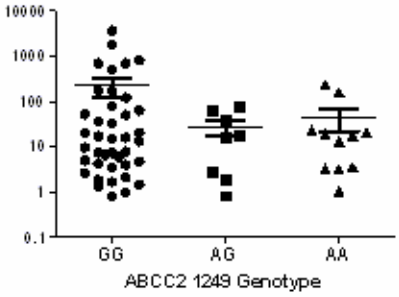
$P = 0.0158$



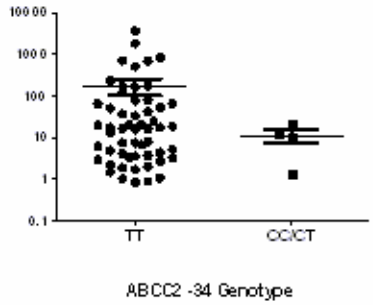
$P = 0.2560$



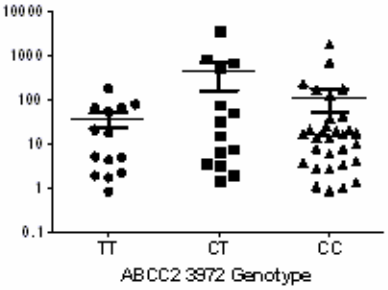
$P = 0.4595$



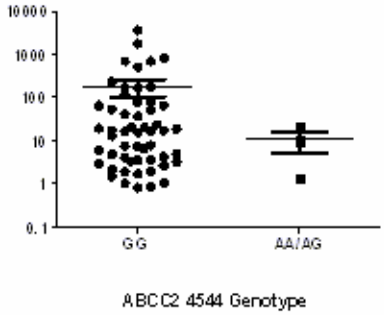
$P = 0.5560$



$P = 0.1203$



$P = 0.6061$



evaluate the urinary platinum excretion in an Abcc2/4 (-/-) mouse model. Our results may also imply a species difference in ABCC2 substrate recognition. More studies need to be done to fully understand this mechanism.

ABCC2 was found to be expressed in a variety of tumor cell lines; however, *ABCC2* genotype did not significantly affect the expression. The expression of ABCC2 in these cell lines may indicate a potential mechanism of cisplatin resistance.

In conclusion, this study suggests that ABCC2 may not play a significant role in the pharmacokinetics of cisplatin. Further investigations are necessary to understand the contribution other ABC transporters have to the renal elimination of cisplatin.

CHAPTER 8. SUMMARY AND CONCLUSIONS

The main objective of these studies were to: A) elucidate the role of organic cation transporter 2 (OCT2) in the cellular uptake of cisplatin, B) determine the effects of OCT2 and ABCC2 expression and genetic variation on cisplatin pharmacokinetics and toxicity, and C) evaluate the role of OCT2 and ABCC2 in tumor sensitivity. It was hypothesized that OCT2 would be the primary regulator of cisplatin entry into renal tubule cells, whereas, ABCC2 would subsequently efflux cisplatin into the urine.

The studies shown here provide evidence for a strong interaction of cisplatin with human OCT2. Previous investigations were inconsistent in determining the substrate affinity for OCT2,[28,29] therefore a new cell model expressing OCT2 was created. In this model cisplatin uptake was temperature dependent indicating an active transport mechanism. Also, cisplatin was able to almost completely inhibit the uptake of prototypical organic cation TEA. Furthermore, cells expressing OCT2 had increased intracellular accumulation of platinum and Pt-DNA adducts after incubation with cisplatin. These studies suggest an important role for OCT2 in the intracellular transport of cisplatin *in vitro*.

To fully understand the interaction, knockout mouse models were utilized to determine the role Oct2 plays *in vivo*. Using metabolic cages, total platinum was measured in urine to evaluate the urinary platinum excretion after cisplatin administration. In Oct1 and Oct2 single knockout mice, no difference in urinary platinum excretion was seen when compared to wildtype animals. The literature indicates an increased expression of mouse Oct1 in mouse kidney compared to human OCT1 in human kidney. Although previous studies have indicated inconsistent results determining the substrate affinity of Oct1 with cisplatin,[27-29] the unchanged platinum excretion in these animals suggests transport by Oct1. Therefore, it may serve as a compensatory mechanism in the mouse kidney. Therefore, the Oct1/2 (-/-) mice would function as a more complete model in which to understand cisplatin pharmacokinetics. The urinary platinum excretion in Oct1/2 (-/-) mice was decreased by almost half (approximately 56% of the dose after 72 hours) when compared to wildtype animals. However, there was no significant difference in plasma platinum concentrations. Serum chemistry analysis and histopathology also indicated that wildtype animals suffered severe tubular necrosis while the Oct1/2 (-/-) did not. No other tissues were affected. These results suggest that cisplatin may be eliminated through an alternate mechanism in the knockout animals, perhaps with more platinum going through the liver without adversely affecting the tissue. It would be interesting to evaluate gene expression changes in the liver, to identify any changes in transporters that may cause an increase in cisplatin transport through the liver. Furthermore, evaluating the elimination of cisplatin through the liver by assessing the platinum content in liver and feces could provide valuable information into the alternate mechanisms involved in cisplatin clearance.

The association between cisplatin pharmacokinetics and genetic variation in the gene encoding OCT2, *SLC22A2*, in patients treated with cisplatin was evaluated. In a

Caucasian population one nonsynonymous single nucleotide polymorphism in exon 4, 808G>T (Ala270Ser; rs316019), was identified. This SNP has been associated with decreased uptake *in vitro*, as well as, decreased clearance in patients receiving metformin, an OCT2 substrate.[70] As with the knockout mice, there was no difference in cisplatin plasma concentrations between patients carrying the reference allele and those with the variant allele. However, a significant difference in the change of serum creatinine, a marker for renal toxicity, after the first cycle of cisplatin was apparent. Patients carrying the reference allele had a significantly higher change in serum creatinine indicating possible renal damage in contrast to those with the variant allele. These results correspond with the mouse data indicating that less cisplatin exposure to the renal tubule cells decreases the potential for damage. To the best of our knowledge, this is the first evidence suggesting that inherited genetic variation is contributing to interindividual variability in cisplatin-induced nephrotoxicity. Even though this observation requires confirmation in a larger cohort of patients as well as further exploration in other ethnic populations, these findings are in line with those obtained in the Oct1/2 (-/-) mice. They also suggest that *a priori* genetic testing could possibly be employed in order to identify patients, eligible for treatment with cisplatin, that may or may not be at increased risk to develop acute nephrotoxicity.

Collectively, our demonstration that OCT2 plays an important role in cisplatin-related nephrotoxicity reveals a new host factor that contributes to enhanced interindividual variation in tolerability to this drug. These findings might benefit future therapeutic interventions for cisplatin-containing regimens involving the use of specific inhibitors of OCT2. Ongoing studies have indicated the possibility of inhibitors blocking the uptake of cisplatin into the cell. This could mimic the effect seen in the Oct1/2 (-/-) mice, protecting kidney tubules from accumulating damage-inducing concentrations of cisplatin. It would be interesting to establish an equitoxic dose in the Oct1/2 (-/-) mice and determine the cisplatin concentrations in the kidney and liver at several time points. Also, it is important to determine if the use of an Oct2 inhibitor could allow for similar urinary platinum excretion and toxicity profiles in wildtype and xenograft mice compared to Oct1/2 (-/-) mice treated with cisplatin. This research could develop into exciting new therapeutic regimens to prevent cisplatin-induced toxicity.

The current work on the mechanistic basis of cisplatin-induced nephrotoxicity also opens up new avenues for further evaluation of pharmacologic approaches for renoprotection through targeting of OCT2. Over the last few decades, numerous approaches have been reported to afford renoprotection during cisplatin treatment.[7] However, most of these approaches have not separately considered the possible implications on the anticancer actions of cisplatin in tumors, which is surprising considering the overlap and degree of similarity in the critical target genes for cisplatin in the kidney and tumor cell. As mentioned, cisplatin is a widely used anticancer agent. Therefore, to be able to determine new ways to prevent cisplatin induced nephrotoxicity we must understand the effect it would have on cisplatin's anticancer efficacy. In a panel of human cancer tissues and the NCI 60 cancer cell lines, it was found that OCT2 is not highly expressed in human tumors, including lung cancer and ovarian cancer, in which cisplatin is routinely used. Therefore, OCT2 may play a limited role in the uptake of

cisplatin into tumor cells, indicating it predominantly only affects renal transport. Future investigation into OCT2 expression in human tumors, particularly before and after cisplatin treatment, would provide a more comprehensive understanding into the functional importance of OCT2 in tumors. The models used in these experiments do give some insight into the expression of OCT2; however, the samples were limited and did not cover all cisplatin-treated cancer.

These studies determined the prominent transporter in the kidney transporting cisplatin from the blood; but how cisplatin is excreted into the urine needed to be investigated as well. Previous literature indicated that ABCC2 could be a potential transporter for cisplatin. In the same Caucasian patients used in the OCT2 studies, *ABCC2* variation was assessed in relation to cispatin pharmacokinetics. Only one SNP showed any significance with the unbound clearance of cisplatin. One haplotype was also associated with the unbound renal clearance of cisplatin. Interestingly, this haplotype did not contain the variant allele of the SNP identified, and also contained a variant that has been previously implicated in a reduced function for other compounds.[71] The lack of association could be due to the low number of patients expressing the particular SNPs of interest. Next the urinary platinum excretion was evaluated, but there was no difference in Abcc2 (-/-) mice compared to wildtype mice. Abcc2 (-/-) mice have been shown to overexpress Abcc4, which may act as a compensatory mechanism for Abcc2. Therefore, more transporters, perhaps using an Abcc2/4 (-/-) mouse model, need to be evaluated to understand the complex mechanism involved in the excretion of cisplatin into the urine.

In conclusion, we have identified the primary cisplatin renal transport mechanism, and shown its significance in cisplatin-induced toxicity. These studies guide future investigations into new therapeutic interventions for the prevention of nephrotoxicity associated with cisplatin containing chemotherapy.

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PUBLICATIONS

Filipski K.K., Loos W.J., Verweij J., Sparreboom A. Interaction of cisplatin with the human organic cation transporter 2. *Clin Cancer Res* 2008 Jun 15;14(12):3875-80

Hu S., Franke R.M., Filipski K.K., Hu C., Orwick S.J., de Bruijn E.A., Burger H., Baker S.D., Sparreboom A. Interaction of imatinib with human organic ion carriers. *Clin Cancer Res* 2008 May 15;14(10):3141-8

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Hu C., Filipski K.K., Zuo Z., and Sparreboom A. Inhibition of OCTN2-mediated carnitine transport by anticancer drugs. In preparation