Cancer Therapy: Preclinical

Clinical Cancer Research

Influence of Oct1/Oct2-Deficiency on Cisplatin-Induced Changes in Urinary *N*-Acetyl-β-D-Glucosaminidase

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Abstract

Purpose: This study aimed to test the influence of functional renal organic cation transporters (OCT2 in humans, Oct1 and Oct2 in mice) on biomarkers of cisplatin nephrotoxicity, such as urinary activity of *N*-acetyl-β-D-glucosaminidase (NAG).

Experimental Design: Temporal cisplatin-induced nephrotoxicity was assessed by histopathology and biomarkers. Cisplatin-mediated NAG changes and survival were determined in wild-type and Oct1/2(-/-) mice. Identification of OCT2 inhibitors was done in transfected 293Flp-In cells, and the NCI_{60} cell line panel was used to assess contribution of OCT2 to cisplatin uptake in cancer cells.

Results: Classical biomarkers such as blood urea nitrogen and serum creatinine were not elevated until 72 hours after cisplatin administration and substantial kidney damage had occurred. Oct1/2(-/-) mice had 2.9-fold lower NAG by 4 hours ($P < 0.0001$) and 2.3-fold increased survival ($P = 0.0097$). Among 16 agents, cimetidine strongly inhibited uptake of tetraethylammonium bromide ($P = 0.0006$) and cisplatin ($P < 0.0001$), but did not have an influence on cisplatin uptake in SK-OV-3 cells, the cancer line with the highest OCT2 mRNA levels. In wild-type mice, cimetidine inhibited cisplatin-induced NAG changes ($P = 0.016$ versus cisplatin alone) to a degree similar to that seen in Oct1/2(-/-) mice receiving cisplatin ($P = 0.91$). Cumulative NAG activity of >0.4 absorbance units (AU) was associated with 21-fold increased odds for severe nephrotoxicity ($P = 0.0017$), which was linked with overall survival (hazard ratio, 8.1; 95% confidence interval, 2.1-31; $P = 0.0078$).

Conclusions: Cimetidine is able to inhibit OCT2-mediated uptake of cisplatin in the kidney, and subsequently ameliorate nephrotoxicity likely with minimal effect on uptake in tumor cells. *Clin Cancer Res; 16(16); 4198–206. ©2010 AACR.*

Cisplatin is a DNA-crosslinking agent that is among the most widely used anticancer drugs worldwide in both adult and pediatric populations. The dose-limiting side effects associated with cisplatin-based chemotherapy include renal tubular dysfunction (nephrotoxicity), and to a lesser extent, hearing loss (ototoxicity; ref. 1). Severe and irreversible damage to the kidney is a tremendous health problem and remains the single most 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 up to 40% of patients despite intensive prophylactic measures (2), including extensive prehydration and posthydration regimens with hypertonic saline.

Over the last three decades, various approaches have been reported to afford renoprotection during cisplatin treatment, although most of these have not been evaluated in animal models or humans (3). The clinical application of many of these strategies has been hampered by the recognition that (*a*) cisplatin has multiple intracellular targets and hence blocking a single injurious event will only have partial protective effects in the kidney, and (*b*) the renoprotective approach may diminish the antitumor effects of cisplatin given the overlap in cell death signaling pathways between normal kidney cells and tumor cells (3). Therefore, an ideal approach of renoprotection is to protect the kidneys without affecting the therapeutic effects in tumors.

Using transfected mammalian cells, we previously reported that cisplatin is a substrate for the organic cation transporter OCT2 (4), which is highly expressed at the basolateral membrane of renal tubular epithelial cells and is considered a major transporter in the active secretion of xenobiotics from the circulation into the kidney. More

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Translational Relevance

Although cisplatin is one of the most commonly prescribed chemotherapeutic agents its use is impaired by dose-limiting nephrotoxicity that occurs in 30% to 41% of all patients, despite prophylactic measures. Our study provides evidence that preventing the cellular uptake of cisplatin via renal organic cation transporters (OCT2 in humans, Oct1/Oct2 in rodents) can reduce or prevent this nephrotoxicity. This is shown with Oct1/2(-/-) mice with increased survival and decreased damage of renal proximal tubules, as determined by the acute biomarker *N*-acetyl-β-D-glucosaminidase (NAG). Screening of 16 different agents found that the OCT2 inhibitor cimetidine was a potent agent for inhibiting uptake of the prototypical substrate, tetraethylammonium bromide, and cisplatin itself. Cimetidine was capable of inhibiting cisplatin-induced increases in urinary NAG activity to levels seen with the Oct1/2(-/-) mice. This provides evidence that the inhibition of renal OCTs may be a mechanism to be exploited in a clinical environment to prevent cisplatin-induced nephrotoxicity.

recently, we found that mice lacking the ortholog transporters Oct1 and Oct2 [Oct1/2(-/-) mice] have impaired urinary excretion of cisplatin and that these animals are protected from experiencing severe cisplatin nephrotoxicity (5). This suggests that these transporters are key proteins involved in the initial step toward renal elimination of cisplatin. Based on these observations, we hypothesized that the extent to which cisplatin interacts with Oct 1 and Oct2 (or OCT2 in humans) and subsequently affects nephrotoxicity is dependent on intracellular drug concentrations in the renal tubule and subsequent activation of pathways resulting in tubular apoptosis. The objectives of the current study were to (*a*) assess the extent of renal secretion of cisplatin in wild-type mice and Oct1/2(-/-) mice, (*b*) determine the influence of cisplatin treatment on the time course of biomarkers of nephrotoxicity and overall survival, and (*c*) evaluate the effects of OCT2 inhibitors on cisplatin transport and nephrotoxicity.

Materials and Methods

Chemicals and reagents

The Flp-In transfection system, DMEM, 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. The human full length cDNA clone of OCT2 was purchased from Origene. Cisplatin and [^{14}C]-tetraethylammonium bromide (TEA) were obtained from Sigma Chemical Co. and American Radiolabeled Chemicals, respectively. All other compounds used in this study were of reagent grade or better.

Animals

Adult (8-12 weeks old) male FVB wild-type and Oct1/2(-/-) mice were purchased from Taconic. 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*.

Plasma pharmacokinetic studies

Blood samples were obtained at 0.25, 0.75, 1, 2, 4, and 8 hours after an i.p. dose of cisplatin at 10 mg/kg, centrifuged to obtain plasma, and flameless atomic absorption spectrometry (AAS) was used to determine total platinum concentrations (5). $AUC_{(0\text{-}24)}$ was calculated using noncompartmental pharmacokinetic analysis extrapolated out to 24 hours. The unbound platinum concentration was determined following ethanolic plasma-protein precipitation (6). Pharmacokinetic parameters were calculated using the software package PK Solutions 2.0 (Summit Research Services).

Urinary platinum excretion

For experiments involving collection of urine, Oct1/2(-/-) and wild-type (n = 12/group) animals were single-housed in metabolic cages in a temperature-controlled environment with a 12-hour reverse light cycle. After the animals had acclimated to metabolic cages, a 24-hour baseline urine sample was collected 16 hours after the onset of light. The next day, the mice were given a single i.p. injection of cisplatin at a dose of 10 mg/kg. Urine was collected before drug administration and at 4, 24, and 48 hours after administration. Urine samples were diluted with nitric acid (0.2%) and analyzed for total platinum using AAS (5). An estimate of the renal clearance (CL_R) of total platinum was determined as follows: $CL_R = A_{urine\ (0\text{-}24)}/AUC_{(0\text{-}24)}$, where $A_{urine\ (0\text{-}24)}$ and $AUC_{(0\text{-}24)}$ are the cumulative urinary excretion of platinum up to 24 hours and the area under the plasma concentration-time curve for protein-unbound platinum up to 24 hours, calculated using the linear trapezoidal rule, respectively. Metabolic cages were washed with a double wash cycle in a portable dishwasher using hot water and household dishwashing detergent between experiments done in different animals.

Assessment of renal biomarkers

Glomerular filtration rate (GFR) at baseline was estimated in male FVB mice and Oct1/2(-/-) mice from body weight (BW) from intraspecies allometry using the following equation: $GFR = 0.036 \times BW^{0.74 \pm 0.15}$ (7). The validity of this approach is supported by the previous finding that GFR determined from [^{14}C]inulin clearance was not significantly different between wild-type mice and Oct1/2(-/-) mice, and corresponded well to the GFR estimated from BW alone (8). Levels of blood urea nitrogen (BUN), serum

creatinine, and urinary creatinine were determined using a Vetscan autoanalyzer (Abaxis). Urinary NAG activity was chosen due to it being an effective and sensitive biomarker for nephrotoxicity (9). Urinary NAG activity was measured before and after cisplatin treatment was measured in wild-type and Oct1/2(-/-) mice using a kit from Diazyme Laboratories (Poway) with detection at 505 nm on a μQuant microplate spectrophotometer (BioTek Instruments). Subsequently cisplatin-induced (10 mg/kg, i.p.) urinary NAG activity was determined in wild-type mice administered a saline vehicle control (i.v.), cimetidine (30 mg/kg, i.v.), or nothing.

Survival analysis

Overall survival was assessed in male wild-type mice and age- and weight-matched male Oct1/2(-/-) mice after administration of cisplatin by i.p. injection at a single dose of 10 or 20 mg/kg. Overall survival was assessed by the Kaplan-Meier method, using an exact log-rank test and a Cox-Mantel proportional hazard model to determine the impact of mouse genotype and nephrotoxicity.

Histopathologic analysis

For microscopic examination, tissues were fixed in 4% phosphate-buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with H&E in accordance with standard procedures.

Cellular transport studies

The Flp-In transfection system was used as described to produce stably transfected 293Flp-In cells expressing OCT2 or an empty pcDNA5 vector (4). These cell lines were collected and reseeded in complete DMEM containing 10% fetal bovine serum and hygromycin B (100μg/mL). All uptake experiments were done on monolayer cultures in 6-well plates at 37°C, as described (4). For the transport inhibition assays, monolayers were washed once with warm PBS then preincubated with DMEM containing potential OCT2 inhibitors at initial concentrations of 1 mmol/L. This medium was removed and replaced with the same concentration of inhibitor in DMEM solution with [^{14}C]-TEA (2 μmol/L) and allowed to incubate for an additional 30 minutes at 37°C. After incubation, cells were washed twice with cold PBS, collected and solubilized in 1N sodium hydroxide. Radioactivity was assessed by liquid scintillation counting, platinum levels were measured using AAS (5), and protein concentrations were measured using a bicinchoninic acid (BCA) protein-assay kit (Pierce Biotechnology). Compounds that were found to be significant inhibitors of TEA uptake were subsequently tested at a concentration of 1 μmol/L.

The potential functional change in transport associated with a common OCT2 variant (p.270Ala>Ser) was assessed using cisplatin transport in the presence and absence of cimetidine. Briefly, HEK293 cells expressing either an empty vector (pcDNA3.1), wild-type OCT2 (containing reference sequence), or a protein variant of OCT2 containing the p.270Ala>Ser amino acid substitution were maintained and tested under similar conditions mentioned above. These cells are stably transfected and have been previously reported on (10).

Gene expression in NCI_{60} cancer cell lines

RNA from the NCI_{60} cancer cell lines were provided by the National Cancer Institute tumor repository (Bethesda). SK-OV-3 cells were from the American Type Culture Collection. RNA was reverse transcribed using SuperScript III first strand synthesis supermix for quantitative reverse transcriptase-PCR (Invitrogen) according to the manufacturer's recommendations. Gene transcripts were quantified using SYBR Green PCR mastermix (Qiagen) and primers previously described (11). Reactions were carried out in triplicate unless otherwise stated as previously reported (4). Briefly, 25 μL volumes were used with the following PCR variables: 95°C for 15 minutes then 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds followed by dissociation cycles. Transcripts of each sample were normalized to the housekeeping gene, *GAPDH*.

Primers for *SLC22A2* were designed as previously described (4). Samples were cleaned with ExoSAP-IT reagent (USB Corporation) following PCR and sequenced in both forward and reverse directions using Big Dye Terminator version 3.1 on an Applied Biosystems 3730XL DNA analyzer. Sequencher software version 4.7 (Gene Codes Corporation) was used for sequencing analysis.

Statistical analysis

All data are presented as mean and SE, unless otherwise stated. Statistical analyses were done using either ANOVA, followed by Bonferroni posthoc test, or a two-tailed *t* test where appropriate, and P values < 0.05 were considered to be statistically significant. All statistical calculations were done using the software package NCSS version 2004 (Number Cruncher Statistical System).

Results

Renal secretion of cisplatin is abolished in Oct1/2(-/-) mice

It was recently reported that in the mouse, cisplatin is recognized as a substrate by both Oct1 and Oct2 (4, 5, 12–14). To further investigate the roles of Oct1 and Oct2 in renal secretion of cisplatin, we compared the plasma and urinary pharmacokinetics of this drug in Oct1/2(-/-) mice. The renal clearance of platinum was greatly reduced in the Oct1/2(-/-) mice (15.8 ± 2.16 versus 25.9 ± 2.24 mL/h in wild-type; $P < 0.01$), although there were no differences in the estimated GFR at baseline (16.4 ± 0.21 versus 16.8 ± 0.52 mL/h). The ratio of renal clearance to GFR was about 1.54 ± 0.13 for wild-type mice, which is comparable with what has been found by others (15). In Oct1/2(-/-) mice, however, this ratio was reduced to 0.96 ± 0.13, indicating that the net tubular secretion of platinum was completely abolished in these animals ($P < 0.01$).

Renal biomarker changes in response to cisplatin

We found that in wild-type mice receiving cisplatin, the widely used biomarkers for assessing cisplatin nephrotoxicity, BUN and serum creatinine, are less than ideal because increases only occur after substantial kidney damage, and then with a time delay (Fig. 1A and B), consistent with previous findings (16). Indeed, BUN and serum creatinine did not show significant elevation in the mice until 72 hours after administration of cisplatin. This is despite the notion that histopathologic analysis indicated proximal tubular damage as early as 24 hours following drug administration (Fig. 1A, right, and B).

NAG as a biomarker of cisplatin nephrotoxicity

As an alternative to BUN and creatinine, we explored the utility of measuring urinary NAG for evaluating the role of renal transporters in cisplatin nephrotoxicity. In contrast to BUN and creatinine, we found that NAG is a sensitive quantitative biomarker for detection of cisplatin nephrotoxicity with significant changes in activity occurring as early as 4 hours ($P < 0.0001$; Fig. 2A). Cisplatin-induced changes in urinary NAG activity were much less pronounced in Oct1/2(-/-) mice compared with wild-type mice over the studied time interval (Fig. 2A), consistent with the reduced nephrotoxicity observed in the transporter-deficient animals (5). Additional analysis on mice following a single dose of cisplatin indicated that a cutoff point of >0.4 AU for the cumulative urinary NAG activity over 48 hours is highly predictive of severe nephrotoxicity, with a "severe/not-severe" odds ratio of 21.0 (95% confidence interval, 2.91-151; $P = 0.0017$).

The absence of severe cisplatin nephrotoxicity (i.e., when >75% of tubules are damaged) in Oct1/2(-/-) mice is a critical difference from the observed side effects in wild-type mice, because the severity of renal damage is linked with mortality following a single dose of cisplatin. Indeed, the decreased toxicity in the Oct1/2(-/-) mice is associated with a significantly prolonged overall survival (Fig. 2B). For example, at a cisplatin dose of 10 mg/kg, the wild-type/Oct1/2(-/-) Cox-Mantel hazard ratio was found to be 8.1 (95% confidence interval, 2.1-31; $P = 0.0078$). Interestingly, although all wild-type mice died by day 7, a subset of Oct1/2(-/-) mice seemed to be protected from cisplatin-induced death, presumably due to reduced cisplatin-induced nephrotoxicity.

Cimetidine inhibits cisplatin transport by OCT2

To explore the possible utility of chemical inhibitors of OCT2 as a strategy to prevent severe cisplatin nephrotoxicity, we screened 16 agents at varying concentrations for OCT2 inhibitory potential in transfected 293Flp-In cells using TEA as a test substrate. As shown in Supplementary

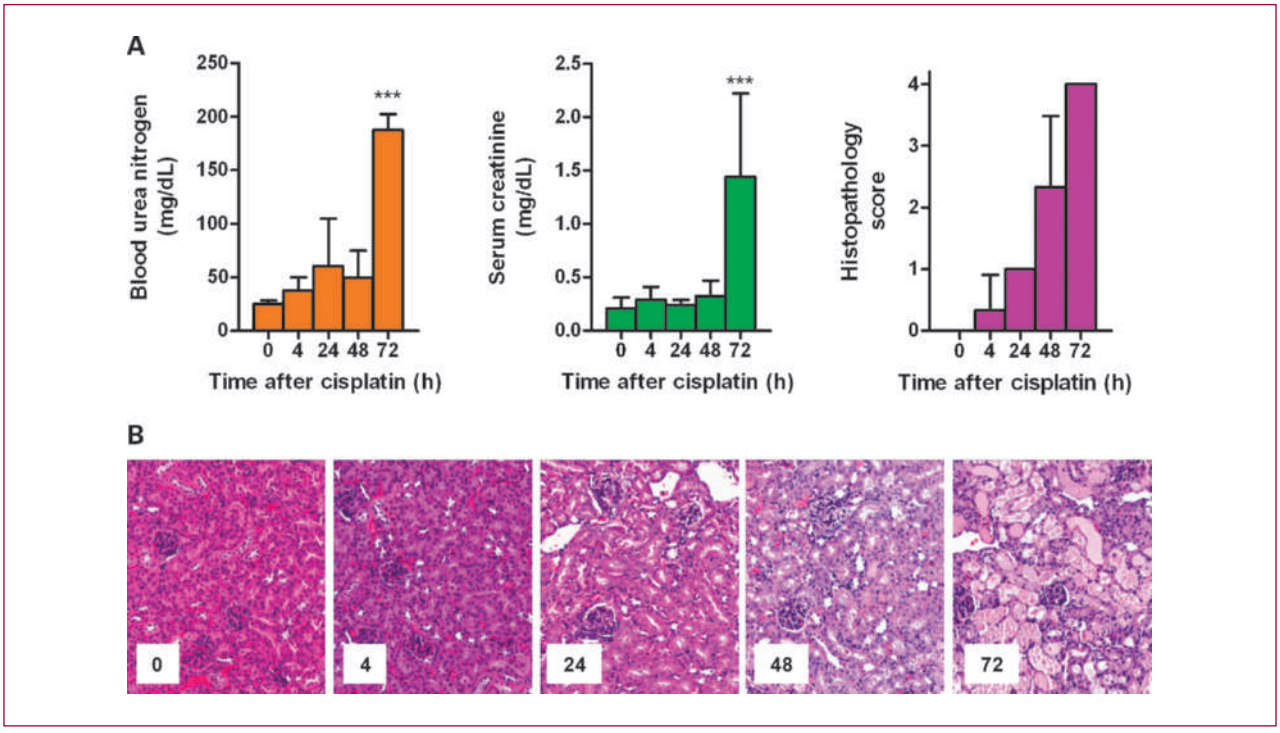

Fig. 1. Measures of nephrotoxicity in adult male FVB mice after administration of cisplatin (17.5 mg/kg, i.p.; $n = 3$ per time point). A, left, mean of blood urea nitrogen (BUN); middle, mean of serum creatinine; right, mean histopathology scores determined from the extent of acute renal tubular necrosis based on the percentage of observed damaged tubules: **0**, absent; **1**, <25% (rare); **2**, 25-50% (mild); **3**, 50-75% (moderate); **4**, >75% (severe). Bars, mean; error bars, SD. B, representative kidney histopathology before and after administration of cisplatin. ***, $P < 0.001$. Numbers indicate time after cisplatin administration in hours.

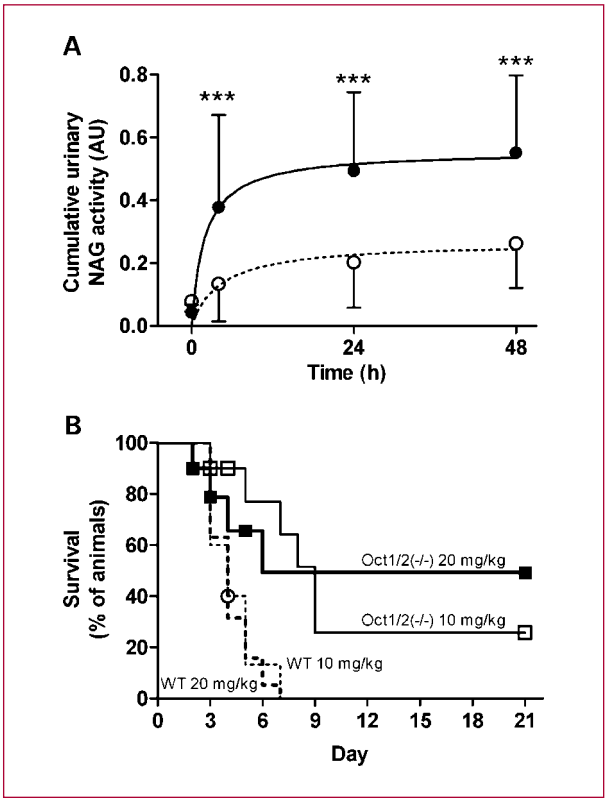

Fig. 2. Influence of Oct1/Oct2-deficiency on cisplatin-induced changes in urinary NAG and overall survival. A, cumulative urinary NAG activity in adult male FVB mice (closed symbols, solid line; $n = 12$) and Oct1/2(-/-) mice (open symbols, dotted line; $n = 12$) after administration of cisplatin (10 mg/kg, i.p.). Bars, mean; error bars, SD; AU, absorbance units. B, overall survival curves in adult male FVB wild-type (dotted lines, WT) and Oct1/2(-/-) mice (solid lines) after administration of cisplatin at a dose of 10 mg/kg (thin lines) or 20 mg/kg (thick lines); $n = 9$ to 19 per dose per genotype. Symbols are shown for censored observations. ***, $P < 0.001$.

Fig. S1, at a substrate to inhibitor concentration ratio of 1:500, all of the known OCT2 inhibitors (amantadine, cimetidine, diclofenac, imatinib, and nicotine) used in this screen showed significant inhibition of OCT2 function, as determined by intracellular accumulation of [^{14}C]-TEA (8-42% of control values). A number of supportive care drugs (aprepitant, granisetron, metoclopramide, omeprazole, and pantoprazole) and chemotherapeutic agents (docetaxel and etoposide) also showed significant inhibition of OCT2 function (by 1-29% of control). When tested at clinically relevant concentrations (1 μmol/L), however, only imatinib (75% of control, $P = 0.012$) and cimetidine (70% of control, $P = 0.0006$) were found to be inhibitors of OCT2 function (Fig. 3).

The ability of cimetidine to inhibit OCT2 function was further confirmed using cisplatin as a test substrate in three different OCT2-overexpressing cell lines (Fig. 4). These results indicated that cimetidine, at a substrate to inhibitor concentration ratio of 1:2, significantly reduced the uptake of cisplatin almost 4-fold ($P < 0.0001$). Interestingly, cisplatin transport in the absence or presence of cimetidine was not altered in HEK293 cells transfected with a common OCT2 protein variant (p.270Ala>Ser) associated with the rs316019 (808G>T) single nucleotide polymorphism (Fig. 4). This suggests that this particular OCT2 variant is not associated with altered substrate recognition for cisplatin.

Cimetidine prevents cisplatin nephrotoxicity

To obtain preliminary evidence for the usefulness of adding cimetidine to cisplatin-based therapy to ameliorate nephrotoxicity, urinary NAG activity was determined in mice receiving cisplatin (10 mg/kg i.p.) in the presence and absence of cimetidine (30 mg/kg i.v.; Fig. 5). There was a trend for slightly reduced NAG activity in wild-type mice in which cisplatin administration was preceded by an i.v. injection of saline. Although this difference was not statistically significant ($P = 0.19$), the observation is consistent with prior clinical data suggesting that i.v. hydration can reduce cisplatin nephrotoxicity to some extent (17). Most importantly, wild-type mice receiving concurrent cimetidine with cisplatin had urinary NAG values that were significantly reduced compared with mice receiving

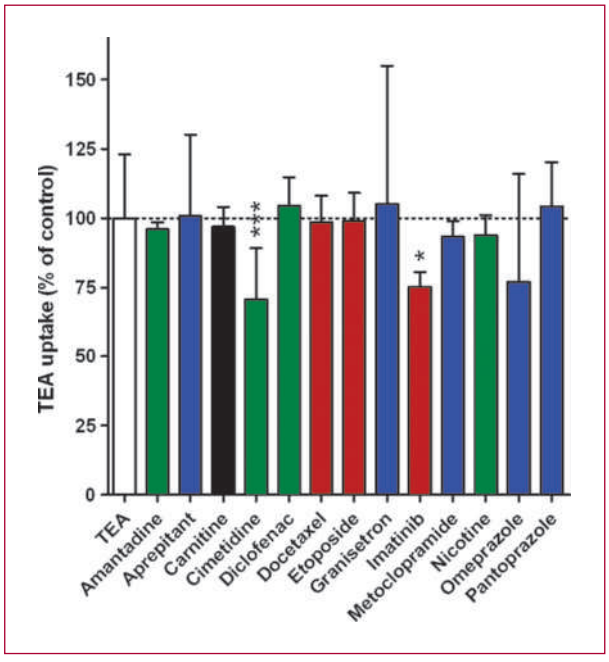

Fig. 3. Inhibition of OCT2-mediated transport of TEA by various prescription drugs at clinically relevant concentrations. Transport of [^{14}C]-TEA (2 μmol/L) during a 30-minute incubation was assessed in 293Flip-In cells transfected with an empty vector (VC) or with OCT2 in the presence and absence of the inhibitors at a substrate to inhibitor concentration ratio of 1:0.5 (1 μmol/L of inhibitor). Data represent the extent of TEA uptake in OCT2-overexpressing cells corrected for nonspecific uptake in VC cells, and are expressed as a percentage of uptake in the absence of inhibitors, which was set at 100%. Data shown as mean (bars) and SD (error bars) of at least three experiments done in triplicate. Red, cancer drugs; blue, supportive-care drugs; green, known OCT2 inhibitors; black, other. *, $P < 0.05$ versus control; ***, $P < 0.001$ versus control.

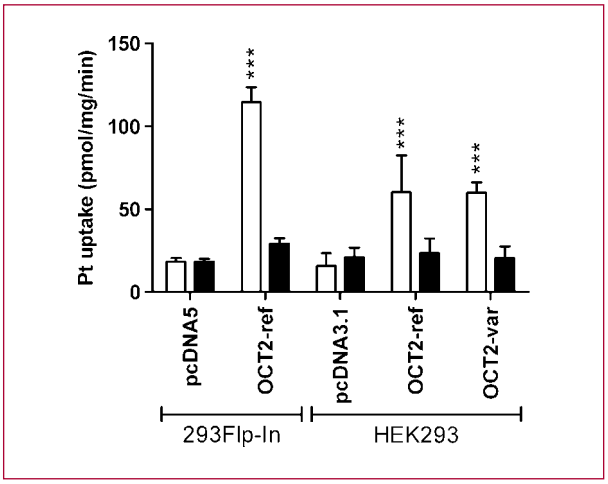

Fig. 4. Effect of cimetidine (1 mmol/L) on the accumulation of cisplatin (500 μmol/L, for 30 minutes) in 293Flp-In cells transfected with empty vector (pcDNA5) or OCT2 (OCT2-ref), or in HEK293 cells transfected with empty vector (pcDNA3.1), wild-type OCT2 (OCT2-ref), or the OCT2 p.270Ala>Ser variant (OCT2-var). Open bars, cisplatin alone; black bars, cisplatin in the presence of cimetidine. Data are shown as mean (bars) and SD (error bars) of three experiments performed in triplicate. ***, $P < 0.001$.

cisplatin only ($P = 0.016$) and were similar to levels in cisplatin treated Oct1/2(-/-) mice, as shown in Fig. 2A (0.2697 ± 0.082 and 0.2624 ± 0.141, respectively; $P = 0.91$). This suggests that cimetidine is able to completely inhibit Oct1/Oct2-mediated uptake of cisplatin in renal proximal tubular cells, and subsequently ameliorate cisplatin nephrotoxicity.

Cimetidine does not inhibit cisplatin uptake into cancer cells

The contribution of OCT2 to the uptake of cisplatin in human tumor cells is currently unknown. We previously reported that the OCT2 gene *SLC22A2* was either absent or detectable at very low levels in multiple human tumors, including those for which cisplatin is clinically used, such as lung cancer and ovarian cancer (5). In the NCI_{60} cancer cell line panel, we identified the ovarian cancer cell line SK-OV-3 as having the highest expression of *SLC22A2* (Supplementary Fig. S2). However, we found that cellular sensitivity to cisplatin in the NCI_{60} cancer cell line panel was not significantly associated with the expression of *SLC22A2* ($R^2 = 0.009$, $P = 0.47$; data not shown). We also found that expression of *SLC22A2* was approximately 175-fold lower in SK-OV-3 cells compared with our OCT2-transfected 293Flp-In cells, and that the expression of other genes of putative relevance to cisplatin transport, such as *SLC22A1* (encoding OCT1), were very low in all of the cell models tested (Fig. 6A). Although the absolute uptake of cisplatin in SK-OV-3 cells was quite substantial, the presence of an excess amount of cimetidine had no influence on the cellular uptake and retention of cisplatin in this model (Fig. 6B). This finding is consistent with the possibility that substantial overexpression of OCT2, such as that observed in our transfected 293Flp-In cells, or under normal physiologic conditions in human kidney, is required before its quantitative contribution to cisplatin transport can be discerned.

Discussion

This study directly shows *in vivo* that organic cation transporters (OCT2 in humans, Oct1 and Oct2 in mice) are essential for the active secretion of cisplatin into renal proximal tubular cells, and that these proteins play a crucial role in the development of cisplatin nephrotoxicity. Our collective *in vitro* and *in vivo* data have potentially important clinical implications for the optimization of cisplatin usage and strongly support the hypothesis that pharmacologic inhibitors of OCT2 can be used to prevent cisplatin-induced kidney damage.

A role of organic cation transporters in the renal uptake of cisplatin has been suggested previously by several studies. This was initially deduced from *in vitro* studies that showed that cisplatin could inhibit the cellular uptake of the prototypical OCT2 substrate TEA (18, 19). Furthermore, *in vivo* administration of cisplatin inhibits the renal clearance of other organic cations (20), and conversely, several organic cations can affect the renal clearance of cisplatin (21) without affecting GFR (22). Recently, *in vitro* methods using OCT2-overexpressing cells have been used to confirm that cisplatin is itself a substrate for this transporter (4). The role of Oct2 in cisplatin-induced nephrotoxicity was suggested by the increased nephrotoxicity in male rats, which have higher expression of Oct2 in the kidneys, as compared with females (23). Moreover, our previous study indicates that functional loss of the mouse ortholog transporters Oct1 and Oct2 is associated with a marked reduction in urinary excretion of platinum and

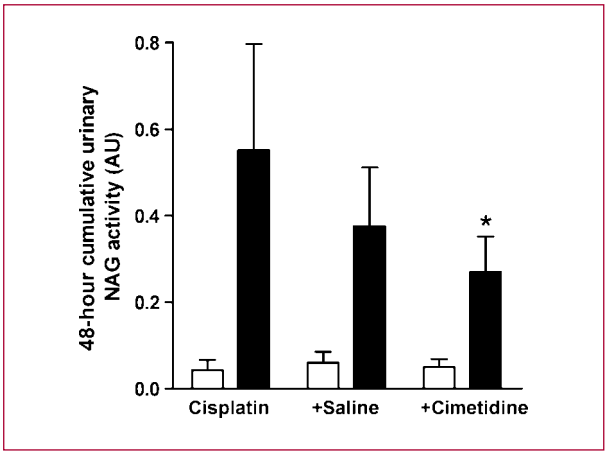

Fig. 5. Cisplatin-induced (10 mg/kg, i.p.) changes in urinary NAG activity in mice administered saline (i.v.), cimetidine (30 mg/kg, i.v.), or no injection immediately prior (n = 6-12 per treatment group). Open bars, pretreatment levels of urinary NAG activity; black bars, cumulative urinary NAG activity over 48 hours. Bars, mean; error bars, SD. *, $P < 0.05$.

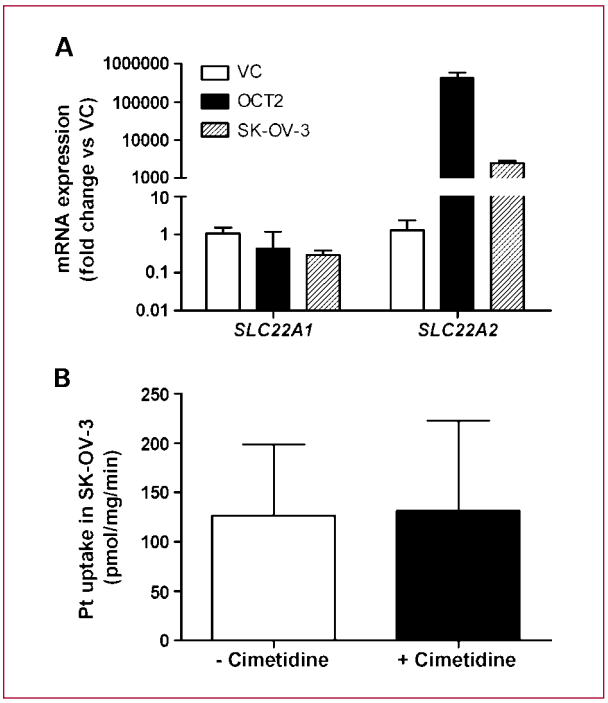

Fig. 6. The influence of OCT2 on the intracellular accumulation of cisplatin in the NCI_{60} cancer cell line with highest expression of OCT2, SK-OV-3. A, real-time PCR expression of the OCT1 gene *SLC22A1* and the OCT2 gene *SLC22A2* in 293Flip-In cells transfected with an empty vector (VC), 293Flip-In cells transfected with OCT2, and SK-OV-3 cells. B, influence of cimetidine (1 mmol/L) on the uptake of cisplatin (500 μmol/L) in SK-OV-3 cells. Data are shown as mean (bars) and SD (error bars) of three experiments done in triplicate.

nephrotoxicity, as determined by histopathologic staining (5). This renal protection with the deletion of Oct1 and Oct2 has recently been confirmed in a study evaluating cisplatin-induced toxicities (24). Our current findings that Oct1/2(-/-) mice lack renal secretion of platinum and also have an increase in the median survival time compared with wild-type mice receiving the same cisplatin dose further highlight the importance of Oct1 and Oct2 in the pharmacology and toxicology of cisplatin.

It has been long recognized that better biomarkers for cisplatin nephrotoxicity are needed both for animal studies and for use in humans where early detection of kidney injury will influence therapy and potentially morbidity and mortality. A recent report on the effects of cimetidine as an inhibitor of cisplatin-induced ototoxicity and nephrotoxicity also found inconsistencies between differing biomarkers of nephrotoxicity (24). Cimetidine pretreatment reduced levels of several biomarkers for nephrotoxicity when evaluated 4 days after administration (i.e., BUN, tubular protein casts, vacuolization), but this was not seen in all biomarkers studied (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining and proteinurea). This supports the need for a sensitive biomarker that can detect nephrotoxicity early. Here, we found that routinely used measures of kidney injury, such as BUN and serum creatinine, increase significantly only after substantial damage occurs and then with a substantial time delay, which renders BUN and creatinine useless as biomarkers in the context of our studies.

Beyond serum markers, many urinary proteins have been previously evaluated as putative noninvasive indicators of general renal injury, including α- and π-glutathione-*S*-transferases, neutrophil gelatinase-associated lipocalin, cysteine-rich protein 61, interleukin-18, clusterin, and F-actin (25). However, problems with the reliability of these proteins to identify and monitor kidney injury include instability in the urine, modification due to physicochemical properties of the urine, absence of sustained elevation, and lack of a high-throughput detection method. Based on these considerations, we evaluated cisplatin-induced changes in urinary NAG, a high-molecular-weight lysosomal enzyme that is abundant in the proximal tubular epithelia (26, 27). Due to its large size this molecule is not known to undergo filtration, but rather is released into the urine when renal proximal tubular cells are damaged (26). Urinary NAG activity has been previously used in evaluating lead-mediated damage (27) and cisplatin nephrotoxicity in rats (23). These prior studies and our current work in wild-type and Oct1/2(-/-) mice strongly support the hypothesis that urinary NAG activity is a highly sensitive, stable, and early biomarker for cisplatin nephrotoxicity, especially compared with BUN and creatinine. The ability of cimetidine to block Oct1/Oct2-mediated uptake of cisplatin in renal proximal tubular cells, and subsequently ameliorate cisplatin-induced changes in NAG activity, support further efforts to explore the use of this biomarker in rodents and patients receiving cisplatin.

Previously, cimetidine was found *in vitro* to decrease the cytotoxic effects of another platinum-based chemotherapy, oxaliplatin, in cells transfected to express OCT1, OCT2, or OCT3 (14). It should be noted, however, that although this study did find increased uptake of cisplatin in cells overexpressing OCT1 or OCT2, the effect was not as robust as in other studies (4, 13). This is most likely due to differences in transfection models and efficiency, as suggested by the authors. Although cisplatin was not as robust a substrate for these transporters as oxaliplatin, there was still increased sensitivity to cisplatin-induced toxicity in OCT1- and OCT2-overexpressing cells, further supporting the hypothesis that inhibiting OCT-mediated uptake of platinum-based chemotherapeutics can ameliorate the toxicity of these agents. Although comprehensive studies comparing mouse and human pharmacokinetics of cimetidine are not available, it should be noted that using the Food and Drug Administration (FDA) guidelines for determining human equivalent doses, a 30 mg/kg dose of cimetidine used in this study is comparable to a 200 mg dose in a human with a body surface area of ~2 m^2 (FDA. Guidance for determining human equivalent doses. http://wwwfdagov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932pdf.), which is based on interspecies conversion factors previously established (28).

Most of the approaches reported in the literature to afford renal protection during cisplatin treatment have not separately considered the possible implications on the anticancer actions of cisplatin in tumors. It is therefore imperative to conclusively show that intentional inhibition of OCT2 function does not negatively influence cisplatin effects on the tumor. The mechanism of cellular uptake of cisplatin in tumor cells is not entirely clear and may vary from one cell type to another (3). Preclinical studies have shown that a high-affinity transporter known as copper transporter 1 (CTR1, SLC31A1) plays a critical role in the uptake of cisplatin in yeast and several tumor cell lines. However, the accumulation of cisplatin in human cancer cells expressing this transporter is limited by the fact that cisplatin triggers the downregulation and proteasomal degradation of CTR1, thereby limiting its own uptake (29). Our current studies show that even the presence of an excess amount of cimetidine had no influence on the cellular uptake and retention of cisplatin in SK-OV-3 cancer cells, which carry the highest *SLC22A2* expression among the NCI_{60} cell lines. In addition to this *in vitro* observation, it is noteworthy that a quantitative real-time PCR analysis of 80 ovarian tumor specimens revealed recently that the vast majority of tumors had low (n = 12) or undetectable (n = 68) levels of *SLC22A2* (30). Of the 80 specimens, 51 were obtained from patients treated with cisplatin-containing chemotherapy, 42 of whom responded to the treatment (30). No difference, either in expression level or frequency of *SLC22A2*-positive tumors, was apparent between the nonresponders and the responders. These *in vitro* and *ex vivo* results suggest that OCT2 function is not a critical determinant of the antitumor effects of cisplatin, and thus that the use of OCT2 inhibitors should not affect the intracellular accumulation of cisplatin into tumors.

Collectively, our study suggests that the use of a pharmacologic inhibitor of OCT2 is a feasible mechanism to exploit for preventing cisplatin nephrotoxicity without influencing effects on the tumor. Furthermore, this study indicates that urinary NAG activity serves as a sensitive early biomarker for nephrotoxicity. If this dose-limiting toxicity can be alleviated in patients, the therapeutic window of cisplatin in cancer treatment can be dramatically improved. We are currently conducting an ongoing study to further elucidate the role of cimetidine in long-term survival, tumor suppression in xenograft models, and a prospective clinical trial to evaluate the effects of cimetidine as a conjunctive therapy to reduce or eliminate cisplatin nephrotoxicity. Although cimetidine will be used here as a pharmacologic tool for establishing proof of concept, our future studies will be aimed at developing and evaluating more selective agents that could provide better protection against the renal damage associated with cisplatin use.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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