Interaction of Cisplatin with the Human Organic Cation Transporter 2

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Abstract

Purpose: Cisplatin is predominantly eliminated in the urine through active secretion. As the solute carrier organic cation transporter 2 (OCT2) is highly expressed in the basolateral membrane of proximal tubules, we determined its contribution to cisplatin transport and assessed the relation of variation in the gene encoding OCT2 (SLC22A2) with the disposition of cisplatin.

Experimental Design: Cell lines were transfected using the Flp-In 293 system with the full-length OCT2 cDNA, and platinum concentrations were measured using flameless atomic absorption spectrometry. Pharmacokinetic data were available from 106 cancer patients, and DNA was screened for eight nonsynonymous SLC22A2 variants using direct sequencing.

Results: mRNA expression was 36-fold higher and uptake of the model substrate tetraethylammonium was significantly increased (P < 0.0001) in OCT2-transfected cells compared with empty vector-transfected controls. OCT2-mediated transport of cisplatin was saturable, and uptake was increased by >4-fold (P < 0.0001) relative to control cells. Cisplatin inhibited OCT2-mediated transport of tetraethylammonium by up to 97%. The mean ± SD systemic clearance of unbound cisplatin-derived platinum in the patient population was 29.2 ± 8.39 L/h, and renal clearance was particularly variable. Only one single nucleotide polymorphism (Ala270Se; rs316019) was identified (minor allele frequency, 7.6%), and it was not found to be associated with any of the studied pharmacokinetic variables (P > 0.05).

Conclusion: These findings support the hypothesis that OCT2 is a key renal transporter involved in cisplatin elimination. However, known variants in SLC22A2 do not substantially contribute to explaining interindividual pharmacokinetic variability, suggesting that other mechanisms, controlling OCT2 expression, might be involved.

Cis-diaminedichloroplatinum (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 cancers (1, 2). Dose-limiting side effects, such as renal tubular dysfunction, peripheral neuropathy, and otoxicity, typically occur with conventional three or four weekly regimens of cisplatin (2, 3). Hydration therapy has been incorporated into cisplatin administration to reduce cisplatin-induced toxicity (4–6). 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 (2, 7). Hence, identification of factors that are associated with the clearance of cisplatin could aid in predicting or adapting appropriate, individualized doses of this agent.

Cisplatin is predominantly eliminated in urine by renal secretion in the proximal tubules (1). Previous investigations have shown that cisplatin accumulates in human renal cortex slices against a concentration gradient (8) and that cisplatin competitively inhibits the active uptake of the cation tetraethylammonium (TEA) by mouse kidney slices (9) and by basolateral membrane vesicles from the rat renal cortex (10). Furthermore, cisplatin inhibits the renal clearance of organic cations from a basolateral site in the isolated-perfused rat kidney (11). 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 aims of the present study were to determine the contribution of OCT2 to cisplatin transport and to assess the association of variation in the gene encoding OCT2 (SLC22A2) with the disposition of cisplatin in adult Caucasian cancer patients.

Materials and Methods

Materials. 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. American Radiolabeled Chemicals provided \(^{14}C\)TEA, and Sigma provided cisplatin. SL22A2 primers were custom-made by the Hartwell Center (St. Jude Children's Research Hospital). The ExoSAP-IT reagent was obtained from USB.

**Cell culture and transfection.** The Flp-In transfection system was used to produce stably transfectant cells expressing OCT2. The full-length OCT2 cDNA clone was inserted into the pcDNA5/FRT (pcDNA5/FRT/OCT2) vector and transfected into HEK293 cells containing an integrated FRT site. Cells were seeded in 10 mL DMEM with 10% fetal bovine serum (complete DMEM). Opti-MEM I reduced serum medium was used for the transfection with 1 \(\mu\)g pcDNA5/FRT/OCT2 (FLP-OCT2) or pcDNA5/FRT (empty vector), 9 \(\mu\)g pOG44, and 20 \(\mu\)L LipofectAMINE 2000. After 24 h, medium was replaced with complete DMEM containing hygromycin B (100 \(\mu\)g/mL). Medium was subsequently changed every 2 to 3 days. Circular colonies were visible 2 weeks after transfection. Cells were collected and reseeded in either fresh complete DMEM containing hygromycin B or complete DMEM containing zeocin (100 \(\mu\)g/mL) to test for zeocin sensitivity. Cells were maintained at 37°C in a humidified atmosphere with 5% CO\(_2\) and were split 1:10 every 5 days.

**RNA extraction, cDNA synthesis, and reverse-transcription 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 described previously (12). Reactions were carried out in 25 \(\mu\)L volumes using the following PCR variables: 95°C for 15 min then 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C 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.

**Cellular uptake.** All uptake experiments were done on monolayer cultures in six-well plates at 37°C. Briefly, monolayers were washed once with warm PBS, and DMEM containing \(^{14}C\)TEA (5 \(\mu\)mol/L) was added to the cells to initiate uptake. After 5-min incubation, uptake was halted by removing the medium and washing the cells with ice-cold PBS. Cells were collected and solubilized in NaOH (1 N). Radioactivity was assessed by liquid scintillation counting, and protein concentrations were measured using a BCA protein assay kit (Pierce Biotechnology). For transport inhibition assays, cells were preincubated with increasing concentrations of cisplatin (up to 1,000 \(\mu\)mol/L) for 15 min. Cells were washed with PBS and then incubated for 10 min in medium containing cisplatin and \(^{14}C\)TEA (2 \(\mu\)mol/L).

For measurements of cellular accumulation of cisplatin, FLP-OCT2 and empty vector cells were washed with warm PBS and treated with cisplatin (500 \(\mu\)mol/L) in DMEM. A preliminary analysis revealed that the uptake of cisplatin was linear for at least 30 min (data not shown); therefore, subsequent experiments were done using 30-min incubation periods. Concentration-dependent assays were carried out in DMEM containing cisplatin at concentrations up to 1,000 \(\mu\)mol/L. The fraction unbound Pt in DMEM, determined using equilibrium dialysis, was found to range between 10% and 26% at total cisplatin concentrations of 50 and 1,000 \(\mu\)mol/L, respectively. 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 method slightly modified from that described below for analysis of human plasma and urine samples. Briefly, 20 \(\mu\)L samples were injected in duplicate into a Perkin-Elmer AAnalyst 600 atomic absorption spectrometer with Zeeman background correction to measure platinum content. Peak area measurements were done 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 \(\mu\)mol/L) in DMEM for 30 min. Cells were washed twice with ice-cold PBS and DNA was isolated as described previously (13). DNA concentrations were determined spectrophotometrically using the ND-1000 NanoDrop (NanoDrop Technologies) and total platinum was determined using the method described above.

**Clinical studies.** All patients eligible for the study had a confirmed diagnosis of a malignant solid tumor and were between ages 18 and 75 years. Eligibility criteria have been documented in detail previously (14). The study protocols were approved by the Erasmus Medical Center review board, and all patients provided informed consent before enrollment. Cisplatin powder (Pharmachemie) was dissolved in 250 mL of a sterile, hypertonic solution containing 3% (w/v) NaCl and was administered as a 3-h continuous i.v. infusion at doses ranging from 50 to 100 mg/m\(^2\)/week 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 dactinomycin. Prior investigation has shown that the disposition of cisplatin is unaffected by concomitant administration of these chemotherapeutic agents (15). Antiemetic prophylaxis consisted of a 5-hydroxytryptamine-3 receptor antagonist in combination with dexamethasone.
**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 h after the start of infusion, at 5 min before the end of infusion, and at 0.5, 1, 2, 3, and 18 h after the end of infusion. In a limited number of patients, additional samples were obtained at 1.5 and 5 h after the end of infusion. In a limited number of patients, samples were obtained at 18 h after the end of infusion. 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 h after the start of drug administration. Aliquots of 750 μL drug-free nitric acid (0.2%) were added to 50 μL urine.

Aliquots of 20 μL 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 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 mean percentage deviation from nominal values (accuracy) and precision (within-run and between-run variabilities) of quality-control samples spiked with known concentrations of cisplatin were always <15%.

**Table 1. Summary of pharmacokinetic variables for cisplatin-derived platinum**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Median</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platinum dose (mg)</td>
<td>87.8</td>
<td>89.4</td>
<td>52-156</td>
</tr>
<tr>
<td>Infusion duration (h)</td>
<td>3.02</td>
<td>3.25</td>
<td>2.67-4.73</td>
</tr>
<tr>
<td>Total platinum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (μmol/L)</td>
<td>7.67</td>
<td>7.89 ± 1.50</td>
<td>4.96-12.2</td>
</tr>
<tr>
<td>AUC (μmol/L h)</td>
<td>114</td>
<td>109 ± 35.1</td>
<td>32.0-181</td>
</tr>
<tr>
<td>CL (L/h)</td>
<td>0.632</td>
<td>0.738 ± 0.461</td>
<td>0.163-2.74</td>
</tr>
<tr>
<td>Urinary excretion (%)</td>
<td>31.1</td>
<td>31.4 ± 10.0</td>
<td>9.70-62.0</td>
</tr>
<tr>
<td>Unbound platinum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (μmol/L)</td>
<td>3.04</td>
<td>2.96 ± 0.842</td>
<td>0.171-5.18</td>
</tr>
<tr>
<td>AUC (μmol/L h)</td>
<td>8.79</td>
<td>8.67 ± 2.20</td>
<td>0.393-14.6</td>
</tr>
<tr>
<td>CL (L/h)</td>
<td>27.2</td>
<td>29.2 ± 8.39</td>
<td>16.8-75.4</td>
</tr>
<tr>
<td>CLR (L/h)</td>
<td>9.51</td>
<td>10.2 ± 4.14</td>
<td>3.03-24.8</td>
</tr>
</tbody>
</table>

NOTE: Pharmacokinetic variables were obtained with noncompartmental analysis and normalized to 75 mg/m² cisplatin. Abbreviations: $C_{\text{max}}$, peak concentration; AUC, area under the plasma concentration-time curve; CL, systemic clearance; CLR, renal clearance.

Individual platinum plasma concentration time profiles were analyzed by a noncompartmental method using WinNonlin version 5.1 (Pharsight). Plasma pharmacokinetic variables assessed included peak concentration ($C_{\text{max}}$), area under the plasma concentration-time curve extrapolated to infinity (AUC), and systemic clearance (CL), which was calculated as dose divided by AUC. Standard noncompartmental evaluation has shown that measures of systemic exposure to cisplatin are dose proportional over the tested dose range (15). Therefore, values for $C_{\text{max}}$ and AUC were normalized to a dose of 75 mg/m² 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.

**Identification of SLC22A2 variants.** The genomic sequence of SLC22A2 was obtained from Genbank (accession no. NM 003058), and primers were designed to span exons that contain eight known nonsynonymous, single nucleotide polymorphisms (SNP) identified previously in a Caucasian population (17) 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), Arg300Cys (exon 7), and Lys320Cln (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). 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 directions using the same primers used for PCR and Big Dye Terminator (version 3.1) Chemistry on Applied Biosystems 3730XL DNA Analyzers. Sequencing analysis was done using Sequencher software version 4.7 (Gene Codes).

**Statistical considerations.** Data are presented as mean and SD unless stated otherwise. Statistical analyses were done using a two-tailed t test, and P values < 0.05 were considered to be statistically significant. Calculations were done using the software packages Number Cruncher Statistical Systems version 2005 (J. Hintze) and GraphPad Prism version 5.00 (GraphPad Software).

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**Fig. 3.** A. Concentration dependence of cisplatin uptake in FLP-OCT2 cells. B. Comparative uptake of 500 μmol/L cisplatin in vector control cells and FLP-OCT2 cells. Points or columns, mean of three independent experiments; bars, SE. C. Pt-DNA adducts in vector control cells and FLP-OCT2 cells after incubation with 500 μmol/L cisplatin. Columns, mean of three observations; bars, SE.

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Results

Characterization of OCT2 overexpressed cells. Expression of SLC22A2 was 36-fold higher in the FLP-OCT2 cells compared with the vector control cells (Fig. 1A). Transport of the typical organic cation \([^{14}C]\text{TEA}\) in the FLP-OCT2 cells and vector control cells was 15 and 0.2 pmol/min/mg protein, respectively \((P < 0.0001; \text{Fig. 1B})\). Therefore, these cells were considered to express a functional transporter and were suitable for subsequent experiments.

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 \([^{14}C]\text{TEA}\). Cisplatin had a significant inhibitory effect on the transport of \([^{14}C]\text{TEA}\) (Fig. 2). We then examined the transport of cisplatin itself and found that it was saturable in the FLP-OCT2 cells, with an apparent Michaelis-Menten constant of \(2 \text{ mol/L}\) (Fig. 3A). After incubation with \(500 \text{ mol/L}\) cisplatin for 30 minutes, the platinum accumulation in FLP-OCT2 cells was 36-fold higher in the FLP-OCT2 cells compared with the vector control cells (Fig. 3B). FLP-OCT2 cells also cisplatin almost completely inhibited the uptake of TEA, a known OCT2 substrate. Furthermore, the formation of Pt-DNA adducts when compared with vector control cells (Fig. 3C), suggesting that cisplatin is itself transported by OCT2 and not just binding to the protein.

Cisplatin pharmacokinetics. A pharmacokinetic analysis showed that the mean clearance for total platinum \((0.738 \text{ L/h})\) and unbound platinum \((29.2 \text{ L/h})\) in the studied patients was in the same range as reported previously in other predominantly Caucasian populations \((1)\). The unbound clearance varied \(\sim 4\)-fold between patients (Table 1), and the observed variability was, in part, accounted for by sex (Fig. 4), with men having a statistically significantly higher median unbound platinum clearance than women \((P = 0.019)\).

SLC22A2 genotype-phenotype relations. Of the eight nonsynonymous SNPs evaluated, only one SNP (Ala\(^{270}\)Ser; rs316019) was identified in this patient population, with an observed minor allele frequency of 7.6\%. However, none of the studied pharmacokinetic variables 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 \(29.5 \pm 1.00\) and \(31.9 \pm 2.89\), respectively \((P = 0.36; \text{Fig. 5A})\), whereas the values for renal clearance were \(10.5 \pm 0.628\) and \(9.50 \pm 1.38\), respectively \((P = 0.55; \text{Fig. 5B})\). 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.

Discussion

In this study, we found that the human OCT2 is involved in the cellular uptake of the anticancer drug cisplatin. Through a pharmacokinetic analysis, we observed a high degree of interindividual variability in the clearance of cisplatin in a Caucasian population. However, no statistically significant associations were found between the pharmacokinetic variables of cisplatin and a series of variants in the gene encoding OCT2 (SLC22A2).

It has recently been suggested that human OCT2 and its rodent orthologue may play a critical role in the cellular transport of cisplatin \((18, 19)\). However, at least one other report suggests that OCT2 is not a primary transporter handling cisplatin \((20)\). To address these inconsistencies, we produced a stably transfected OCT2 cell line and did a detailed characterization of gene overexpression and transport function. Not only did these transfected cells exhibit an increase in the intracellular accumulation of platinum following exposure to cisplatin, but also cisplatin almost completely inhibited the uptake of TEA, a known OCT2 substrate. Furthermore, the formation of Pt-DNA adducts was significantly increased by the presence of OCT2. These results unequivocally indicate an important role for OCT2 in the transport of cisplatin.

Cisplatin-induced nephrotoxicity typically occurs in the S3 segment of the renal proximal tubules \((21)\), where OCT2 is
predominantly expressed on the basolateral membrane (22, 23). 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. Among eight variants evaluated, only one SNP (Ala²⁷⁰Ser; rs316019) was found in the sample population. However, this SNP was not associated with the plasma pharmacokinetics or the urinary excretion of cisplatin in these patients. This observation is consistent with functional data obtained using heterologous expression models, indicating that the level of expression and functional activity of the Ala²⁷⁰Ser variant is similar to that of the OCT2 reference protein and that only the rare variants studied here would be predicted to result in a more deleterious phenotypic change (22). However, the absence of increased frequencies of the rare variants in other common world populations and the apparent evolutionary selection acting against amino acid variants in OCT2 (17) point to the possibility that genetic variation in SLC22A2 is unlikely to play a major role in the renal elimination of cisplatin.

It is also possible that environmental factors and physiologic variables are overriding a potential effect of genetic variation in SLC22A2 in the regulation of elimination pathways for cisplatin. For example, in the current population, we noted a sexual dimorphism in the clearance of cisplatin, and further understanding of the basis for this sex dependence is warranted. Previous studies have indicated that females receiving cisplatin treatment are at a higher risk for developing treatment-related toxicities (24, 25), which is consistent with a retarded drug elimination in women. It is noteworthy in this context that OCT2 expression in rabbits, mice, and rats is known to be significantly lower in females (26–28). Furthermore, it has been reported that the renal clearance of cisplatin in rats was higher in males than in female counterparts (29). It is thus tempting to speculate that sex-dependent expression of OCT2 contributes to differential renal elimination of cisplatin in men and women and that this accounts for an increased susceptibility of cisplatin-mediated toxicity in females. To confirm a role of OCT2 in the elimination and toxicity of cisplatin, we are currently evaluating the comparative pharmacokinetics and pharmacodynamics of cisplatin in a mouse model with and without a deletion of the SLC22A2 gene.

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. Several transporters implicated previously in cisplatin transport, including ABC2 (MRP2, cMOAT; ref. 30) and two members of the multidrug and toxin extrusion family (MATE1 and MATE2-K; ref. 18), are highly expressed on the luminal side of renal tubules. Our current work is thus further focused on the dynamic interplay between OCT2 and several 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 common genetic variants in the SLC22A2 gene do not appear to substantially contribute to explaining the extensive interpatient variability in cisplatin pharmacokinetics. Further investigations are required to elucidate the contribution of other rare OCT2 variants to the elimination of cisplatin.

Disclosure of Potential Conflicts of Interest

The authors have no conflicts of interest.

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