Cisplatin-Induced Renal Injury Is Independently Mediated by OCT2 and p53

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Abstract

**Purpose:** Tubular secretion of cisplatin is abolished in mice deficient for the organic cation transporters Oct1 and Oct2 (Oct1/2−/− mice), and these animals are protected from severe cisplatin-induced kidney damage. Since tubular necrosis is not completely absent in Oct1/2−/− mice, we hypothesized that alternate pathways are involved in the observed injury.

**Experimental Design:** Studies were done in wild-type, Oct1/2−/−, or p53-deficient animals, all on an FVB background, receiving cisplatin intraperitoneally at 15 mg/kg. Cisplatin metabolites were analyzed using mass spectrometry, and gene expression was assessed using Affymetrix microarrays and RT-PCR arrays.

**Results:** KEGG pathway analyses on kidneys from mice exposed to cisplatin revealed that the most significantly altered genes were associated with the p53 signaling network, including Cdk1 and Mdm2, in both wild-type ($P = 2.40 \times 10^{-11}$) and Oct1/2−/− mice ($P = 1.92 \times 10^{-8}$). This was confirmed by demonstrating that homozygosity for a p53-null allele partially reduced renal tubular damage, whereas loss of p53 in Oct1/2−/− mice (p53−/−/Oct1/2−/−) completely abolished nephrotoxicity. We found that pifithrin-α, an inhibitor of p53-dependent transcriptional activation, inhibits Oct2 and can mimic the lack of nephrotoxicity observed in p53−/−/Oct1/2−/− mice.

**Conclusions:** These findings indicate that (i) the p53 pathway plays a crucial role in the kidney in response to cisplatin treatment and (ii) clinical exploration of OCT2 inhibitors may not lead to complete nephroprotection unless the p53 pathway is simultaneously antagonized. *Clin Cancer Res;* 20(15); 4026–35. ©2014 AACR.

Introduction

Cisplatin is among the most widely used chemotherapeutic agents for the treatment of various cancer types. Despite its extensive clinical use, therapy with cisplatin is limited by the onset of several detrimental side effects, of which the responsible mechanisms remain poorly understood. Kidney damage to the S3 segment of the renal proximal tubules is of particular concern, as it affects more than 40% of patients, and this is considered the major dose-limiting toxicity associated with cisplatin treatment (1). Current evidence suggest that the organic cation transporter 2 (OCT2), encoded by the solute carrier gene SLC22A2, facilitates the renal secretion of cisplatin and various other, structurally diverse compounds, at the basolateral membrane of the proximal tubules (2–6). This is borne out by data indicating that the urinary excretion of cisplatin is drastically reduced in mice lacking the murine ortholog transporters Oct1 and Oct2 (Oct1/2−/−) compared with wild-type animals, and that these animals are simultaneously resistant to severe cisplatin-induced renal tubular necrosis (7, 8). Similarly, patients carrying a genetic variant of OCT2 that is associated with reduced function appear to be protected from cisplatin-induced nephrotoxicity (7, 9, 10). On the basis of these findings, strategies involving the use of OCT2 inhibitors, such as cimetidine, in combination with cisplatin-based therapy have been explored and have provided promising results both in vitro and in vivo (10–13).

Although the severity of cisplatin-induced nephrotoxicity is reduced following genetic or pharmacologic knockout of Oct1 and Oct2, renal tubular damage is not completely abolished. This suggests the existence of a secondary pathway that contributes, independently of Oct1/Oct2-mediated renal tubular drug uptake, to cisplatin-induced renal damage. A proper understanding of additional mechanisms contributing to cisplatin nephrotoxicity in Oct1/2−/− mice is of fundamental relevance to the discovery and development of translational strategies that are completely renoprotective and in turn improve treatment outcome. The aim...
of the current study was to identify common genetic factors contributing to cisplatin-induced kidney damage in both wild-type mice and Oct1/2−/− mice. We further sought to assess whether inhibition of the identified multiple molecular regulators of renal tubular damage could completely abolish cisplatin-induced nephrotoxicity.

**Materials and Methods**

**Animal experiments**

Male adult wild-type mice (8–12 weeks old), and sex- and age-matched mice with a deficiency of Oct1 and Oct2 (Oct1/2−/−), or p53 (p53−/−), all on an FVB background strain, were obtained from Taconic and bred inhouse. The p53−/− mice were cross-bred with Oct1/2−/− mice to generate mice deficient of p53, Oct1, and Oct2 (p53−/−/Oct1/2−/−). Animals were housed in a temperature-controlled environment with a 12-hour light cycle and a standard diet and water ad libitum. All animals were housed and handled in accordance with the Institutional Animal Care and Use Committee of St. Jude Children’s Research Hospital or approved by a governmental committee overseeing animal welfare at the University of Münster (Münster, Germany) and performed in accordance with national animal protection laws.

For experiments involving collection of urine, animals were single-housed in metabolic cages in a temperature-controlled environment with a 12-hour reverse light cycle. Following acclimation, a 24-hour baseline urine sample was collected, after which mice received administration of cisplatin (15 mg/kg, i.p.) and urine was collected for up to 72 hours. Platinum concentrations were determined by a validated assay based on flameless atomic absorption spectrometry (7).

**Quantitative determination of GSH1 and GSH2**

Frozen urine samples were thawed at room temperature, centrifuged at 16,000 × g for 10 minutes, and 20 μL of supernatant was injected for analysis. Concentrations of the glutathione–monoplatinum conjugate (GSH2) and glutathione–diplatinum conjugate (GSH1) were assessed with a Waters 2692 separation system and Micromass Quattro LC triple-quadrupole system. Separation was achieved on a Synergi 4 μm Polar-RP 80A column [150 × 4.6 mm using a column heater operating at 40 °C with a Waters X-Terra RP18 guard column (3.5 μm, 10 × 2.1 mm)]. The gradient mobile phase was composed of 0.1% formic acid in acetonitrile and 10 mmol/L ammonium acetate in water (pH 3.8). The flow rate was 0.9 mL/min and the separation was completed within 10 minutes. The retention times for GSH1 and GSH2 were 4.95 and 5.05 minutes, respectively. The mass spectrometer was operated in the positive mode, and controlled by Masslynx 4.1 software. The analysis was performed in MRM mode and the following mass ions (m/z) were used for detection: m/z 835 > 778.1 for GSH1 and m/z 572 > 554 for GSH2. The tandem mass spectrometry conditions were as follows: capillary voltage, 3.5 kV; cone voltage, 45 V; source temperature, 130 °C; desolvation temperature, 350 °C; cone gas flow, 10 L/h; desolvation gas flow, 900 L/h; and collision energy, 15 V.

**Gene expression analysis**

Kidneys from wild-type or Oct1/2−/− mice were harvested either before or 72 hours posttreatment with cisplatin, after which RNA was isolated for gene expression using the RNeasy mini kit (Qiagen). Gene expression was analyzed using the Mouse 430a2.0 GeneChip array (Affymetrix) and significance analysis of microarrays, using the criteria of a false discovery rate of 5%, to identify altered expression with an average fold change of ≥2.0. Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to interrogate potentially affected pathways. Confirmation of alterations in the p53 pathway was assessed using the Mouse p53 Signaling Pathway RT2 Profiles PCR Array System (SABiosciences), and evaluation of transporter gene expression was done using the Mouse Transporter RT2 Profiles PCR Array System (SABiosciences). Relative gene expression was determined using the ΔΔCt method, and normalized to a housekeeping gene (Gapdh or Ppc).

**Evaluation of nephrotoxicity**

Mice received a single dose of saline, cisplatin (15 mg/kg, i.p.), or a combination of cisplatin and pifithrin-α (2.2 mg/kg, i.p.; Enzo Life Sciences). Pifithrin-α was solubilized in 1% ethanol to circumvent the protective effects observed when cisplatin is administered in the presence of its commonly used solvent, DMSO (14). After 72 hours, kidneys were collected, fixed overnight in 10% neutral-buffered formalin, embedded in paraffin, sectioned (4 μm), and stained with hematoxylin and eosin (Lab Vision). Microscopic evaluation of nephrotoxicity was done independently by an experienced veterinary pathologist who was blinded to the treatment of the animals, as described (7). Nephrotoxicity was assessed from the percentage of observed damaged tubules and graded on a five-point scale as 0 (<10%, absent), 1 (11%–25%, minimal), 2 (26%–50%; mild), 3 (51%–75%,...
moderate), and 4 (>75%, severe). Blood urea nitrogen (BUN) and serum creatinine were measured as described (11).

Activation of p53, caspase-3, and p21 was assessed using formalin-fixed, paraffin-embedded sections of kidneys after deparaffinization in xylene, and stained as previously described (15) using the primary rabbit anti-p53 antibody (1:200 for 90 minutes; Vector Laboratories), the primary rabbit anti-mouse cleaved caspase-3 antibody (1:100 for 60 minutes; BioCare Medical), or the primary rabbit anti-p21 antibody (1:400 for 30 minutes; Abcam). Ten random high-power fields (10×) were selected per slide for immunohistochemistry scoring.

Assessment of transporter function

HEK293 cells obtained from Invitrogen (Aug 2006; no authentication was conducted by the authors upon receipt) and transfected with human OCT2, mouse Oct1, mouse Oct2, or a control vector were seeded in 6-well plates with complete DMEM containing 10% FBS and hygromycin B (100 μg/mL) at 37°C. Wells were washed once with warm PBS then preincubated for 15 minutes with DMEM containing pifithrin-α (0–100 μmol/L), followed by a 30-minute incubation with the inhibitor and [14C]TEA (0.5, 1.0, and 2 μmol/L), or cisplatin (500 μmol/L). After incubation, cells were washed twice with cold PBS, collected, and solubilized in 1 N sodium hydroxide. Radioactivity was assessed by liquid scintillation counting and platinum concentration was measured using atomic absorption spectroscopy. Uptake data were normalized to protein concentration, determined using a bicinchoninic acid protein-assay kit (Pierce Biotechnology).

Normal human kidney samples were obtained from a patient undergoing resection of a kidney tumor, as described (16). The procedure was approved by the ethics commission of the Universitätsklinikum Münster (Münster, Germany), and written consent was obtained from the patient. Proximal tubules from the human kidney sample or from wild-type mice were isolated for functional analyses (16). Organic cation transporter function and its sensitivity to inhibitory properties of pifithrin-α were evaluated according to an established protocol (16).

The contribution of OCT2 to the intracellular uptake of pifithrin-α or pifithrin-β (1 and 10 μmol/L) was assessed using HEK293 cells transfected with OCT2 or an empty vector at pH 7.4 or 6.0 in the presence or absence of 125 mmol/L NaCl. After incubation, cells were washed twice with cold PBS, collected, and lysed in 1 N sodium...
downregulated genes in wild-type mice that was largely nature comprising of 1,063 upregulated and 1,072 plex gene expression changes and a drug response sig-

Statistical analysis
Data are presented as mean values ± SE, with n referring to the number of animals, cell monolayers, or isolated tubules used in the experiments. An unpaired two-sided Student t test, and ANOVA (with Tukey post-test) were used to demonstrate statistical significance of the effects, where P < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc.).

Results
Phenotypic characterization of Oct1/2−/− mice
Since tubular necrosis is not completely absent in Oct1/2−/− mice treated with cisplatin (Fig. 1A), we hypothesized that alternate pathways are involved in the observed injury. Analysis of livers and kidneys from Oct1/2−/− mice revealed that the expression of 84 ATP-binding cassette transporter and solute carrier genes was not substantially changed compared with tissues obtained from wild-type animals, with the exception of a downregulation of Slc22a1 and Slc22a2 transcripts (Fig. 1B and C). In addition, γ-glut-

Transcriptional profiling of Oct1/2−/− mouse kidney
As a next step toward understanding the molecular mechanisms contributing to cisplatin-induced nephrotoxicity in Oct1/2−/− mice, we performed microarray analyses on kidney biopsies following cisplatin administration in vivo. Transcriptional profiling identified complex gene expression changes and a drug response sign-

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both wild-type mice (P = 2.40 × 10−11) and Oct1/2−/− mice (P = 1.92 × 10−8) involved genes associated with the p53 signaling network. This pathway has been implicated previously as a central component in DNA damage in response to cisplatin in various tissues, including the kidney (19).

Using a p53 signaling pathway array, we confirmed renal expression changes of Trp53 (p53) itself and found strong induction of multiple other well-characterized p53-dependent genes, including Ccng2 (cyclin G1), Cdk1a (p21), Mdm2, Pmaip1 (NOXA), and Sesn2, in response to cisplatin (Fig. 2B; Supplementary Table S1). None of the genes with altered expression in response to cisplatin differed among mouse genotypes in the absence of treat-

hydroxide. Levels of pifithrin-α and -β were measured using liquid chromatography-tandem mass spectrometry (17).

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As anticipated (21), the life span of p53 calcium and phosphorous levels (Supplementary Fig. S3A). Increased alkaline phosphatase, as well as slightly reduced phenotypically normal, with the exception of slightly Oct1/2 (Fig. 3B).

Loss of p53 by itself was not associated with altered urinary excretion of cisplatin (Supplementary Fig. S2C and S2D). A reduced renal expression of Oct1/2 and impaired urinary excretion of cisplatin (Supplementary Fig. S2A and S2B), presumably due to pared with FVB mice, the strain used for Oct1/2 knockout studies and to generate a line lacking p53 as well as the most commonly used strain for p53 knockout, are relatively resistant to cisplatin-induced nephrotoxicity compared with wild-type mice (Fig. 3A). C57BL/6 mice, which are relatively resistant to cisplatin-induced nephrotoxicity, as well as slightly reduced urinary excretion of cisplatin (Supplementary Fig. S2C and S2D). A similar strain dependence has been reported for bromodi chloride-induced nephrotoxicity, which is more severe in FVB mice than C57BL/6 mice (20). Therefore, p53−/− mice on histology scores observed in kidneys isolated from wild-type (WT), Oct1/2−/−, p53−/−, or p53−/−/Oct1/2−/− mice 72 hours following administration of cisplatin. Toxicity scores are based on percentage of damaged tubules: 0 (<10%, absent), 1 (11%–25%, minimal), 2 (26%–50%, mild), 3 (51%–75%, moderate), and 4 (>75%, severe). B, cumulative urinary excretion of platinum from wild-type, p53−/− or p53−/−/Oct1/2−/− mice within 72 hours after receiving cisplatin. Error bars, SE (n = number of mice), and significance was assessed by ANOVA (with Tukey post-test). *P < 0.01; **P < 0.001. 

Identification of pifithrin-α as an OCT2 inhibitor

During the course of a recently performed library screen aimed at identifying novel inhibitors of OCT2 (22), we made the serendipitous observation that the organic cation pifithrin-α, a small-molecule inhibitor of p53-dependent transcriptional activation (23), also inhibits OCT2 function as determined by its ability to reduce uptake of the OCT2 substrate, 4-[4-(dimethylamino)styryl]-N-methylpyridinium-iodide (ASP+) in HKE239 cells engineered to overexpress OCT2. We confirmed this observation by showing that pifithrin-α and its planar tricyclic degradation product pifithrin-β (24) inhibit OCT2-mediated transport of tetaethylammonium (TEA) in the same model (Fig. 4A). To identify the mechanism of this interaction, kinetic analyses were performed with and without pifithrin-α using varying concentrations of TEA as the OCT2 substrate. The resulting Dixon plot revealed that data for each substrate concentration fall on straight lines that intersect on the abscissa, suggesting that pifithrin-α is a noncompetitive inhibitor of OCT2 function with an inhibition constant (Ki) of 3.0 μmol/L (Fig. 4B). In line with this inhibitory mechanism, we found that neither pifithrin-α nor pifithrin-β were transported substrates of OCT2, irrespective of concentration, pH, or the presence of Na+ (Fig. 4C).

Pifithrin-α was also capable of completely blocking the cellular accumulation of cisplatin mediated by OCT2 in HKE293 cells (Fig. 4D). Moreover, to confirm the translational potential of these findings, we found that pifithrin-α inhibits uptake of ASP+ in freshly isolated human proximal tubular cells (Fig. 4E). Similar results were observed in murine-based models, where pifithrin-α reduced uptake of TEA in Oct1- and Oct2-overexpressing HKE293 cells (Fig. 5A) and impeded uptake of ASP+ in proximal tubular cells isolated from wild-type mice (Fig. 5B).
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Pifithrin-α ameliorates cisplatin-induced nephrotoxicity

To explore pifithrin-α further as a dual p53/OCT2-targeted agent for preventing cisplatin-induced nephrotoxicity, it was administered concomitantly with cisplatin in wild-type and Oct1/2−/− mice. Pifithrin-α was formu-
mediated apoptosis in cancer cells. Nonetheless, it should be noted that p53 is either lost or mutated in 50% of tumors, and previous reports have shown that loss of p53 function does not necessarily alter sensitivity to cisplatin chemotherapy (29). In addition, p53 can actually function as a survival factor against various chemotherapeutics (30) and therefore, intentional inhibition of p53 as a strategy to ameliorate nephrotoxicity is unlikely to alter antitumor efficacy. Furthermore, while p53−/− mice are genetically predisposed to rapid tumor development (31), temporal blockade of p53 function with pifithrin-α is not known to be associated with an increased incidence in tumorigenesis (23).

Our findings concerning the ability of pifithrin-α to offer protection against cisplatin-induced nephrotoxicity is consistent with previous reports by Wei and colleagues in C57BL/6 mice (32). A careful examination of these authors’ data reveals that the degree of renoprotection in wild-type mice receiving cisplatin with pifithrin-α is greater than that observed in p53−/− mice receiving cisplatin alone. This suggests that in this mouse model p53 inhibition can also affect cisplatin-induced nephrotoxicity by an additional, p53-independent mechanism, and it is possible that inhibition of cisplatin uptake into tubular cells by pifithrin-α contributed to the agent’s renoprotective properties. These collective data suggest that pifithrin-α could potentially be exploited further as a unique dual OCT2/p53 inhibitor for prevention of cisplatin nephrotoxicity. In this context, it is worth pointing out that pifithrin-α has also shown potential to reduce cisplatin-dependent hair cell loss in cochlear and utricular cultures (33), suggesting that pifithrin-α use may ameliorate other side effects caused by cisplatin that are associated with OCT2, such as ototoxicity (8).

The notion that combinational inactivation of the p53 pathway and OCT1 function is necessary to offer complete protection from cisplatin-induced renal damage raises new questions. First of which concerns the p53-independent mechanism responsible for renal tubular damage that appears to occur downstream of Oct1/2-mediated intracellular accumulation of cisplatin. A candidate mechanism involved is the activation of PKC-β by cisplatin in renal tubular cells, which process leads to kidney cell apoptosis and tissue damage independently of p53 (34). The second involves the identity of the regulators that modulate cisplatin accumulation in the absence of Oct1 and Oct2 that are upstream of p53. Compensatory mechanisms involving putative cisplatin transporters are unlikely since their expression levels are unchanged in Oct1/2−/− mice. However, the possibility remains that other transporters regulate accumulation of cisplatin, which in turn generates a p53-mediated response. Indeed, Ctr1 has been implicated in cisplatin-induced renal toxicity and is known to mediate a p53-response (35), although its involvement as a carrier of cisplatin has recently been brought into question (36). Interestingly, probenecid has been shown to significantly reduce the tubular secretion of cisplatin without affecting glomerular filtration, and has been shown to reduce the severity of nephrotoxicity (37–40). Although the underlying mechanism is unclear, it is conceivable that platinum-sulfhydryl complexes formed in the GGT pathway accumulate into kidney cells through an organic anion transport mechanism that is probenecid-sensitive (41). Additional studies are currently ongoing to definitively address these possibilities.

In conclusion, the results of our study indicate that p53 plays a crucial role in the kidney in response to cisplatin independently of OCT2. This finding suggests that clinical exploration of OCT2 inhibitors as an adjunct to cisplatin-based chemotherapy is unlikely to lead to complete renoprotection unless the p53 pathway is simultaneously antagonized. Future investigation will focus on the exploration of pifithrin-α, and structurally related compounds with improved pharmaceutical properties (42), as dual OCT2/p53 inhibitors for prevention of cisplatin nephrotoxicity.
Figure 6. Influence of pilifrin-α on cisplatin-induced nephrotoxicity. Levels of BUN (A) or serum creatinine (B) in wild-type (WT) or Oct1/2<sup>+/−</sup> mice 72 hours after receiving vehicle only, cisplatin alone, or a combination of cisplatin and pilifrin-α. C, degree of nephrotoxicity based on histology scores observed in kidneys isolated from wild-type or Oct1/2<sup>+/−</sup> mice after 72 hours of receiving vehicle only, cisplatin alone, or a combination of cisplatin and pilifrin-α. Numbers in parentheses represent the number of proximal tubules used per concentration. Toxicity scores are based on percentage of damaged tubules: 0 (<10%, absent), 1 (11%–25%, minimal), 2 (26%–50%, mild), 3 (51%–75%, moderate), and 4 (>75%, severe). All data are represented by mean values and SE (error bars). D, hematoxylin and eosin (H&E) staining or immunohistochemical staining of phosphorylated p53, cleaved caspase-3, or p21 in kidneys isolated from wild-type or Oct1/2<sup>+/−</sup> mice 72 hours after receiving cisplatin in the presence or absence of pilifrin-α. Activation of p53, caspase-3, and p21 was based on antibody signal intensity: Rare, low, moderate, and high.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Disclaimer
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