Activation and Involvement of p53 in Cisplatin-induced Nephrotoxicity

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Abstract
Cisplatin, a widely used chemotherapy drug, induces acute kidney injury, which limits its use and efficacy in cancer treatment. However, the molecular mechanism of cisplatin-induced nephrotoxicity is currently unclear. Using pharmacological and gene knockout models, we now demonstrate a pathological role for p53 in cisplatin nephrotoxicity. In C57BL/6 mice, cisplatin treatment induced p53 phosphorylation and protein accumulation, which was accompanied by the development of acute kidney injury. p53 was induced in both proximal and distal tubular cells and partially co-localized with apoptosis. Pifithrin-α, a pharmacological inhibitor of p53, suppressed p53 activation and ameliorated kidney injury during cisplatin treatment. Moreover, cisplatin-induced nephrotoxicity was abrogated in p53-deficient mice. Compared with wild-type animals, p53-deficient mice showed a better renal function, less tissue damage, and fewer apoptotic cells. In addition, cisplatin induced less apoptosis in proximal tubular cells isolated from p53-deficient mice than the cells from wild-type animals. Together these results suggest the involvement of p53 in cisplatin-induced renal cell apoptosis and nephrotoxicity.
Introduction

Cisplatin is one of the most widely used and most potent chemotherapeutic agents (5, 33, 39). It is being used for the treatment of cancers of the testis, ovary, head, neck, lung and many other origins. It is particularly effective in treating testicular and ovarian cancer, with an impressive cure rate (5, 33, 39). However the use of cisplatin is limited by its side effects in normal tissues, particularly injury to the kidneys (2, 7, 35). Following cisplatin treatment, over 30% of patients develop renal problems. The nephrotoxicity of cisplatin is indicated by tubular cell death, tissue damage, and the loss of renal function or acute renal failure (1, 3, 9, 16-21, 23, 24, 26, 28, 31, 32, 37, 41, 44, 45).

While tubular cell death is recognized as a major determinant of cisplatin nephrotoxicity, how these cells are terminally injured under the pathological condition is unclear. *In vivo* in animals, cisplatin induces both necrosis and apoptosis in renal tubular cells (9, 19, 21, 23, 27, 37, 41). *In vitro* in cultured tubular cells, whether apoptosis or/and necrosis is induced depends on the concentrations of cisplatin (20). Using these experimental models, recent studies have demonstrated the activation of multiple signaling pathways during cisplatin-induced tubular cell injury and nephrotoxicity (1, 3, 16, 19-21, 23, 24, 26, 28, 31, 32, 37, 41, 44, 45).

One of the cell killing pathways that are activated by cisplatin may involve p53 (5, 33, 39). p53, as a tumor suppressor protein, can induce apoptosis by transcription of pro-apoptotic genes or by direct interaction and activation of existing pro-apoptotic molecules (22, 25, 38). In cultured renal tubular cells, p53 is activated by cisplatin (6, 11-14, 30). Importantly, cisplatin-induced apoptosis is inhibited by pifithrin-α (a pharmacological inhibitor of p53) and also by a dominant negative mutant of p53 (6,
14). Mechanistically, p53 may up-regulate the pro-apoptotic genes such as PUMA-α, leading to mitochondrial outer membrane permeabilization and the release of apoptogenic factors including cytochrome c and AIF (11, 13, 30). Together, these studies have suggested the involvement of p53 and related signaling pathway in cisplatin-induced tubular cell apoptosis. Despite these findings, it is unclear whether and to what extents p53 contributes to cisplatin nephrotoxicity \textit{in vivo}. Therefore, the current study was designed to: 1) analyze p53 activation during cisplatin nephrotoxicity in C57BL/6 mice; 2) determine the effects of pifithrin-α in these animals; and 3) compare cisplatin-induced nephrotoxicity in wild-type and p53-deficient mice.
Materials and Methods

Animals. p53-deficient (C57BL/6J-Trp53^{tm1Tyj}) and wild-type C57BL/6 mice were originally from the Jackson Laboratory (Bar Harbor, ME). The animals were maintained under conditions of 12/12 hour light/dark cycle with free access to food and water in the animal facility of Veterans Affairs Medical Center at Augusta, GA. For the p53-deficient mouse colonies, genomic DNA samples were extracted from the tail for PCR-based genotyping. Male mice of 8-10 weeks were used in this study. To compare p53-deficient and p53-proficient mice, both littermates and animals from separate colonies were used and the results were similar and thus calculated together.

Cisplatin treatment of animals. In the majority of experiments, cisplatin was freshly prepared in saline at 1 mg/ml and injected intraperitoneally into mice at a dose of 30 mg/kg as previously (8, 41, 42); control animals were injected with a comparable volume of saline. In the experiment to test the effects of pifithrin-α, pifithrin-α was dissolved in DMSO and then mixed with cisplatin solution for intraperitoneal injection; control animals were injected with saline containing DMSO and the cisplatin-only group were injected saline containing DMSO and cisplatin. The dose of pifithrin-α (2.2 mg/kg) was chosen accordingly to a previous study (18).

Renal function and histology. Serum creatinine and blood urine nitrogen (BUN) were determined to monitor renal function as previously (41-43). For histology, kidneys were fixed with 4% paraformaldehyde and stained with hematoxylin-eosin (H & E). Tissues damage was indicated by tubular lysis, dilation, disruption, and cast formation. The
degree of tissue damage was scored based on the percentage of damaged tubules as previously (41-43): 0: no damage; 1: <25%; 2: 25 to 50%; 3: 50 to 75%; 4: >75%.

**TUNEL Assay.** Apoptosis in renal tissues was determined by TUNEL assay using the *in situ* Cell Death Detection kit (Roche Applied Science, Indianapolis, IN) as previously (41-43). Briefly, renal tissues were fixed with 4% paraformaldehyde and paraffin-embedded. Tissue sections of 4-µm were exposed to a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and nucleotides including FITC-labeled dUTP. The slides were examined by fluorescence microscopy.

**Immunofluorescence staining.** The immunofluorescence staining of kidney tissue was conducted as described in our recent work (41). Briefly, freshly frozen renal tissues were fixed with 4% paraformaldehyde, permeabilized in 1% Triton X-100 and then incubated with a blocking buffer. Subsequently, the samples were incubated with the primary antibodies (anti-p53 or anti-phospho-p53, Cell Signaling, Danvers, MA; anti-active caspase-3, Idun Pharmaceuticals, La Jolla, CA). Finally, the slides were exposed to Cy3 or FITC-labeled secondary antibody (Chemicon, Temecula, CA). For double staining, the tissue sections were first stained for p53 immunofluorescence and then incubated with the TUNEL reaction mixture or FITC-labeled Phaseolus Vulgaris Agglutinin (PHA) or Peanut Agglutinin (PNA) (Vector Laboratories, Burlingame, CA) as described recently (41). The staining was examined by fluorescence and confocal microscopy.
**Primary proximal tubular cell culture.** Proximal tubular cells were isolated from mice for primary culture as described recently (41). Briefly, renal cortical tissues were minced thoroughly and digested with 0.75 mg/ml collagenase 4 (Worthington, Lakewood, NJ). Proximal tubular cells were then purified by centrifugation at 2000xg for 10 minutes in DMEM/F-12 medium with 32% Percoll (Amersham, Piscataway, NJ). After washes, the cells were plated into collagen-coated dishes and cultured in DMEM/F-12 medium supplemented with 5 µg/ml transferrin, 5 µg/ml insulin, 0.05 µM hydrocortisone, 50 µM vitamin c.

**Cisplatin treatment of primary proximal tubular cells and morphological analysis.** After 5-7 days of growth, the isolated proximal tubular cells were replated at 0.3 X 10^6/35 mm dish. For experimental treatment, the cells were incubated with 30 µM cisplatin in fresh culture medium. To examine apoptosis, the cells were stained with Hoechst 33342 to monitor apoptotic morphology by phase contrast and fluorescent microscopy. The typical apoptotic morphology examined included cellular shrinkage, formation of apoptotic bodies, nuclear condensation and fragmentation. To examine long-term survival, cells after 48 hours of cisplatin treatment were returned to fresh medium without cisplatin and cultured for another 5 days.

**Statistics.** Data obtained from indicated numbers of animals or separate cell culture experiments were expressed as mean ± SD. Statistical differences between two groups were determined by student t-test with Microsoft Excel 2000. P<0.05 was considered significantly different.
Results

**Phosphorylation and nuclear accumulation of p53 during cisplatin nephrotoxicity.**

Nephrotoxicity was induced by a single injection of cisplatin in C57BL/6 mice. As shown in Fig. 1A, three days after cisplatin administration, blood urea nitrogen (BUN) increased from 60 to 192 mg/dL indicative of a severe loss of renal function. Immunoblot analysis showed an increase of total p53 protein in kidney tissue lysates after cisplatin treatment (Fig. 1B). The increase started from day 2 and intensified at day 3. In parallel to p53 accumulation, there was p53 phosphorylation (Fig. 1C). In immunofluorescence analysis, p53 accumulation and phosphorylation were shown mainly in renal cortical cell nucleus (Fig. 1C). The results suggest that p53 is phosphorylated, stabilized, and accumulated to nucleus during cisplatin nephrotoxicity.

**Localization of p53 activation in renal tubular cells during cisplatin nephrotoxicity.**

Immunofluorescence staining showed p53 induction in the cells of renal cortex and outer medulla, but not in the inner medulla (not shown). In addition, p53 was not induced in glomeruli (not shown). To further identify the cell types that induced p53, we determined whether p53 was induced in proximal tubules or distal tubules in renal cortex. To this end, we co-stained p53 with either FITC-labeled PHA or PNA, lectins that specifically bind proximal and distal tubular cells respectively (4, 34, 41). As shown in Fig. 2A, the majority of p53-positive cells were localized in PHA-stained tubules, although occasionally there were cells showing p53 staining in PNA-stained tubules. Cell counting indicated that over 80% of p53-positive cells were in PHA-stained tubules and
about 20% in PNA-stained tubules (Fig. 2B). It is suggested that, while p53 is induced in both proximal and distal tubular cells during cisplatin nephrotoxicity, proximal tubules are the main site for p53 induction.

Co-localization of p53 induction with tubular cell apoptosis.
Using in vitro models of cultured cells, previous studies have suggested the involvement of p53 in tubular cell apoptosis during cisplatin treatment (6, 11-14, 30). To determine the relationship between p53 induction and tubular cell apoptosis in vivo, we examined p53 and apoptosis in the same tissues by immunofluorescence and TUNEL assay (Fig. 3A). Three days after cisplatin injection, significant amounts of apoptotic cells were detected in renal cortical and outer medullary tissues (Fig. 3A: TUNEL). While many of the apoptotic cells showed p53 induction (arrowed), others did not. Quantitative analysis by cell counting indicated that about 50% apoptotic cells (TUNEL positive) had p53 induction (Fig. 3B).

Suppression of cisplatin nephrotoxicity by pifithrin-α.
Pifithrin-α is a pharmacological inhibitor of p53 (18). To determine the role of p53 in cisplatin nephrotoxicity in vivo, we initially examined the effects of pifithrin-α in C57BL/6 mice. As shown in Fig. 4A, cisplatin-induced p53 accumulation and phosphorylation in kidney tissues were suppressed by co-administration of pifithrin-α. Importantly, the development of acute renal failure during cisplatin treatment was ameliorated by pifithrin-α. BUN was decreased from 135 mg/dL in cisplatin-treated animals to 70 mg/dL in cisplatin+pifithrin-α treated group (Fig. 5A). Consistently, serum
Creatinine was reduced from 1.7 to 0.9 mg/dL by pifithrin-α during cisplatin treatment (Fig. 5B). Of note, compared with the results shown in Fig. 1, BUN and serum creatinine increases in cisplatin-treated animals were lower in this experiment. This was likely due to the inclusion during cisplatin injection of DMSO, the control vehicle solution for pifithrin-α in this experiment. Renal protective effects of DMSO were shown previously in a nephrotoxic model of mercuric chloride (15).

At the histological level, cisplatin induced obvious tissue damage in the kidneys, particularly in renal cortex and outer medulla (Fig. 6: Cisplatin). Co-administration of pifithrin-α afforded significant protective effects against the tissue damage. Pifithrin-α also attenuated tubular cell apoptosis during cisplatin treatment (Fig. 6A: TUNEL). The protective effects of pifithrin-α were further indicated by semi-quantitative evaluation of tissue pathology (Fig. 6B) and counting of apoptotic cells (Fig. 6C). Together these pharmacological results support a role of p53 in cisplatin nephrotoxicity in vivo.

Amelioration of cisplatin nephrotoxicity in p53-deficient mice.

To further examine the role played by p53 in cisplatin nephrotoxicity, we tested a p53 knockout mouse model. p53 deficiency in these animals was confirmed by PCR-based genotyping (not shown). In addition, cisplatin induced p53 in kidney tissues of wild-type (WT) but not the gene knockout (KO) animals (Fig. 7A). With this information, we first compared wild-type and p53-deficient mice for their renal function. Without treatment, renal function in these two genotypes of animals was similar, BUN around 50 mg/dL and serum creatinine around 0.4 mg/dL (Figs. 7B, 7C). Three days after cisplatin injection, wild-type animals developed severe renal failure, showing a BUN value of 190 mg/dL.
and serum creatinine level of 2.1 mg/dL (Figs. 7B, 7C). In the same experiments, p53-deficient mice showed an average BUN value of 135 mg/dL and serum creatinine of 1.0 mg/dL (Figs. 7B, 7C).

We further examined the histology of renal tissues collected from representative animals. Under control conditions, both wild-type and p53-deficient kidneys showed a normal healthy histology (shown in Fig. 8 only for wild-type). Cisplatin treatment induced severe tubular damage in wild-type mice (Fig. 8 top panels: p53+), which was partially suppressed in p53-deficient animals (Fig. 8 top panels: p53-). Further examination of the tissues by TUNEL assay indicated that cisplatin-induced apoptosis was markedly attenuated in p53-deficient animals (Fig. 8 middle panels: p53-). Consistently, caspase activation shown by active caspase-3 immunofluorescence was also lower in p53-deficient animals ((Fig. 8 bottom panels: p53-)

**Proximal tubular cells isolated from p53-deficient mice are resistant to cisplatin-induced apoptosis.** We hypothesized that the lower sensitivity of p53-deficient mice to cisplatin nephrotoxicity was largely due to p53 deficiency in proximal tubular cells. To test this possibility, we isolated proximal tubular cells from wild-type (p53+) and p53-deficient (p53−) mice. Primary cultures of the isolated cells were then treated with 30 µM cisplatin. Representative cell morphology was shown in Fig. 9A. Without treatment, there was a low basal level of apoptosis in both p53+ and p53− cells. After 24 hours of cisplatin treatment, significant apoptosis was detected in p53+ proximal tubular cells, which showed a typical apoptotic morphology characterized by cellular shrinkage, apoptotic body formation, and nuclear condensation and fragmentation; these cells also
had positive TNUEL staining (Fig. 9A). Apoptosis was obviously lower in p53\(^-\) cells (Fig. 9A). Under these experimental conditions, cisplatin did not induce significant necrosis as shown by the lack of propidium iodide staining (not shown). We further quantified apoptosis by cell counting. As shown in Fig. 9B, cisplatin treatment for 24 hours induced about 30% apoptosis in p53\(^+\) tubular cells, but less than 10% in p53\(^-\) cells. After 40-48 hours of cisplatin treatment, over 80% apoptosis was shown in p53\(^+\) cells, whereas about 50% was shown in p53\(^+\) cells (Fig. 9B). We further determined whether the resistance of p53\(^-\) cells to cisplatin-induced apoptosis was associated with long term survival. To this end, the cells after 48 hours of treatment were returned to cisplatin-free culture medium for recovery. In five days, some p53\(^-\) cells recovered and proliferated to reach confluence in the center area of the dish, whereas p53\(^+\) cells did not recovered or only recovered to show a few cell islands that appeared to be quite stressed and less proliferative (cell morphology not shown). For a quantitative purpose, the recovered cells were harvested to measure total protein. As shown in Fig. 9C, 43 µg protein was recovered in p53\(^-\) cells, whereas only 8 µg from the p53\(^+\) dish. Control dishes without cisplatin exposure had ~110 µg protein.
Discussion

Using pharmacological and gene knockout models, this study has provided the first in vivo evidence for a role of p53 and related signaling pathway in cisplatin nephrotoxicity. The results have characterized p53 activation during cisplatin treatment of C57BL/6 mice, demonstrated the protective effects of pifithrin-α in this in vivo model, and further shown the resistance of p53-deficient mice and isolated proximal tubular cells to cisplatin-induced acute kidney injury.

In this study, p53 activation started at day 2 of cisplatin treatment and was intensified at day 3, showing a temporal correlation with the development of renal injury and renal failure. In addition, p53 was shown to be activated in renal tubular cells, the main cell types that were injured by cisplatin. Co-staining with FITC-labeled lectins specific for proximal and distal tubules indicated that p53 was induced in both proximal and distal tubular cells under the pathological condition (Fig. 2). Although earlier work suggested that distal tubules were the primary site of apoptosis during cisplatin nephrotoxicity (23), more recent studies also showed apoptosis in proximal tubules (19, 37). Cisplatin-induced apoptosis in proximal tubules was further detected in our latest work by co-localizing apoptotic cells with PHA lectin staining. Thus the spatial analysis of p53 activation is consistent with the involvement of p53 in cisplatin-induced renal tubular cell injury. This inference is further supported by our results showing that the cells that had p53 activation underwent apoptosis as indicated by positive TUNEL staining (Fig. 3). Of note, while TUNEL staining may not be very specific for apoptosis,
it has been demonstrated to be relatively specific for apoptosis analysis in acute kidney injury induced by renal ischemia and cisplatin (17).

With clear evidence for p53 activation as indicated by its phosphorylation and protein accumulation, this study has not investigated the mechanism of p53 activation during cisplatin nephrotoxicity. For a mechanistic study, it would be preferable to be initially conducted in in vitro models. Nevertheless, it can be speculated that cisplatin may induce DNA damage by its intra- and inter-strand cross-linking property (5, 33, 39). This may then activate the signaling pathways of DNA damage response including ATM and ATR, leading to p53 phosphorylation and activation. Our recent work has shown the cleavage and inactivation of ATM, but not ATR, during cisplatin treatment of renal tubular cells (40). However, the role of ATM and ATR in p53 activation under these experimental conditions remains to be clarified. It is also noteworthy that, in addition of DNA damage, cisplatin may activate p53 via other injury or stress related signaling pathways(38). In this regard, our recent work demonstrated the inhibitory effects of free radical scavenging chemicals on p53 activation and cisplatin-induced nephrotoxicity, suggesting that oxidative stress may contributes to p53 activation (12).

Our results showed significant protective effects of pifithrin-α on cisplatin nephrotoxicity (Figs. 4-6). Moreover, p53-deficicent mice were shown to be resistant to cisplatin injury (Figs. 7-8). The effects of pifithrin-α and p53 deficiency were shown at the levels of renal function, histology, and tubular cell apoptosis. Nevertheless, it is important to recognize that the protective effects were only partial. This is not surprising as the pathogenesis of cisplatin nephrotoxicity is generally considered to be multifactorial. Indeed, our results showed that, while many apoptotic cells in cisplatin-
treated tissues had p53 induction, others did not induce p53 (Fig. 3), suggesting that apoptosis of the latter cell population is independent of p53. Consistently, in primary cultures of proximal tubular cells, p53-deficiency did not completely block cisplatin-induced apoptosis (Fig. 9). It is important to recognize that Tubular cell apoptosis during acute kidney injury is dynamic and progressive. Thus analysis of apoptosis at a fixed time-point (as in our study) only shows a snapshot image (17), which likely underestimates the amount of apoptotic cells developing during the whole study period of 3 days. As a result, partial protection of apoptosis may have significant effects on renal histology and function.

In addition, we noticed that the p53-deficient mice used in our experiments can be roughly divided into two groups, depending on their sensitivity to cisplatin injury. One group (about two-third of the tested animals) was clearly resistant to cisplatin injury and only showed small to moderate increases of BUN and serum creatinine at day 3 of cisplatin treatment. In contrast, the other group (about one-third of the tested animals) showed medium to severe renal injury and renal failure during cisplatin treatment, which was comparable to wild-type animals. Frequently both resistant and sensitive animals were found in the same litter, suggesting that the variation was not due to the abnormality of specific litters. It was reported that the developing kidneys of p53-deficient mice had structural abnormalities including hyperplastic cyst formation in the differentiated zone (29). Our study used young adult mice, which did not show obvious structural or functional abnormalities in the kidneys. Occasionally there were renal cysts, but the majority of the animals were normal. While the exact cause of the varied cisplatin sensitivity in p53-deficient mice is unclear, knockout of p53 is expected to promote
cancer development. According to the information from Jackson Laboratory, the p53-deficient mice develop tumors (principally lymphomas and osteosarcoma) at around 3-6 months of age. In our study, the animals were used at 8-10 weeks (2-2.5 months). Thus it is possible that some of the animals had already undergone homeostatic changes of early tumor development by the time of experiment, which may affect their sensitivity to cisplatin. It would be advantageous to use conditional p53 knockout models that specifically down-regulate p53 in renal tubular cells to further investigate this possibility and determine the role of p53 in cisplatin nephrotoxicity.

The involvement of p53 in cisplatin nephrotoxicity suggests that it is possible to protect kidneys during cisplatin chemotherapy by blocking p53. However, we have to recognize that inhibition of p53 may also limit apoptosis of cancer cells and thus reduce the therapeutic effects in tumors. In this regard, over 50% of cancers have p53 mutations, yet cisplatin is frequently effective in treating them, indicating that cisplatin therapy in these cancer types may not be stringently p53-dependent (10, 33). During cisplatin therapy of these cancers, targeting p53 should be a useful strategy for renoprotection. On the other hand, in cancers where the therapeutic effects of cisplatin depend on p53, restrictive renal delivery of p53 inhibitory chemicals, antisense oligonucleotides and siRNAs may be considered. Renal tissues have an enormous capacity of reabsorption and are thus particularly accessible to drugs including antisense oligonucleotides and siRNA (36). Whether these p53-targeting approaches can protect kidneys against cisplatin nephrotoxicity without limiting the therapeutic effects in cancers warrants further investigation.
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References


Figure Legends

**Fig. 1  p53 phosphorylation and accumulation during cisplatin nephrotoxicity.** Male C57BL/6 mice were injected with a single dose (i.p., 30 mg/kg) of cisplatin. A: Blood urea nitrogen. Blood samples were collected at indicated time points to determine the levels of blood urea nitrogen. Data are expressed as Mean ± S.D., n≥8 *, significantly different from day 0. B: Total p53 and phosphorylated p53. Kidney tissues were collected at indicated time points for immunoprecipitation of total p53. The resultant immunoprecipitates were analyzed for total p53 and phosphorylated p53 by immunoblots using specific antibodies. C: Immunofluorescence of p53 and phospho-p53 (Ser 15). Renal cortical tissues were collected from control and three day cisplatin-treated animals for immunofluorescence using specific antibodies. Arrows: positive staining of total and phosphorylated p53.

**Fig. 2  Localization of p53 in renal tubules during cisplatin nephrotoxicity.** Male Renal cortical tissues were collected three days after cisplatin injection. A: The tissues were fixed for immunofluorescence staining of p53, followed by co-staining with FITC-labeled PHA (proximal tubule marker) or PNA (distal tubule marker). Arrows: p53 staining in PHA-positive tubules. Arrowhead: p53 staining in PNA-positive tubules. B: Percentages of p53 positive cells in PHA-stained and PNA-stained tubules were determined by cell counting. Data are expressed as Mean ± S.D. (n=4).

**Fig. 3  Co-localization of p53 with apoptosis in cisplatin-treated renal cortical tissues.** Renal cortical tissues were collected three days after cisplatin injection and
subjected to p53 immunofluorescence and TUNEL staining. A: Representative image of p53 and TUNEL staining. Arrows: co-localization of p53 and TUNEL staining. B: The percentages of TUNEL positive cells with or without p53 accumulation were determined by cell counting. Data are expressed as Mean ± S.D. (n=4).

Fig. 4  Suppression of p53 activation during cisplatin treatment by pifithrin-α. C57BL/6 mice were treated for three days with cisplatin only or cisplatin together with 2.2 mg/kg pifithrin-α to collect renal tissues. A: Immunoblot analysis of p53 and phospho-p53 (Ser 15) in renal tissue lysates. B: Immunofluorescence of p53 and phospho-p53 (Ser 15) in renal cortex. Arrows: cells with p53 and phospho-p53 induction. The results are representatives of 4 separate experiments.

Fig. 5  Effects of pifithrin-α on cisplatin-induced acute renal failure. C57BL/6 mice were injected with saline as control, cisplatin, or cisplatin and 2.2 mg/kg pifithrin-α. Blood samples were collected three days later for measurements of blood urea nitrogen (A) and serum creatinine (B). Data are expressed as Mean ± S.D., n=7 for (A) and n≥5 for (B). *, statistically significant different from the control group; #, statistically significant different from the cisplatin only group.

Fig. 6  Effects of pifithrin-α on cisplatin-induced renal tissue damage and apoptosis. C57BL/6 mice were injected with saline (control), cisplatin, or cisplatin and 2.2 mg/kg pifithrin-α. Renal cortical tissues were collected three days later for analysis of histology by H & E staining and apoptosis by TUNEL assay. A: Representative images of H & E
staining and TUNEL assay. Asterisks: severely damaged tubules; arrows: TUNEL positive cells. **B.** Pathological score of tissue damage. **C.** Numbers of TUNEL positive cells per mm² cortical tissue. Data are expressed as Mean ± S.D., n=4. *, statistically significant different from the cisplatin-only group.

**Fig. 7 Renal function of wild-type and p53-deficient mice following cisplatin treatment.** Wild-type (p53⁺) and p53-deficient (p53⁻) mice were injected with 30 mg/kg cisplatin. Renal tissues and blood samples were collected three days later for analysis. **A:** Immunoblots of p53 and β-actin to show p53 induction by cisplatin in wild-type but not p53-deficient mice. **B:** Blood urea nitrogen. **C:** Serum creatinine. Data are expressed as Mean ± S.D., n≥29 for **B** and n≥22 for **C.** *, statistically significant different from control. #, statistically significant different from cisplatin-treated wild-type group.

**Fig. 8 Renal tissue damage, apoptosis and caspase activation in wild-type and p53-deficient mice following cisplatin treatment.** Wild-type (p53⁺) and p53-deficient (p53⁻) mice were injected with 30mg/kg cisplatin to collect renal cortical tissues three days later to analyze renal histology by H & E staining (top panels), apoptosis by TUNEL assay (middle panels), and caspase activation by immunofluorescence of active caspase-3. Asterisks: severely damaged tubules; arrows: TUNEL-positive cells; arrow heads: active caspase-3 staining.
Fig. 9  Cisplatin-induced apoptosis in primary cultures of proximal tubular cells isolated from wild-type and p53-deficient mice. Proximal tubular cells were isolated from wild-type (p53+) and p53-deficient (p53-) mice for primary culture, and then treated with 30 µM cisplatin. A: Representative morphology of cells after 24 hours of cisplatin treatment. Cells were fixed for Hoechst 33342 staining and TUNEL assay. Cell morphology, Hoechst staining and TUNEL staining of the same fields of cells were recorded by phase contrast and fluorescence microscopy. B: Percentages of apoptosis were determined by counting the cells with typical apoptotic morphology. C: Cell survival. After 48 hours of cisplatin treatment, the cells were returned to normal culture medium without cisplatin for 5 days and then whole cell lysates were collected for measurement of total protein. Data are expressed as Mean ± S.D., n=3. *, statistically significant different from cisplatin-treated wild-type group.
Fig. 1  p53 phosphorylation and accumulation during cisplatin nephrotoxicity
Fig. 2 Localization of p53 induction in renal tubules during cisplatin nephrotoxicity

A

B

% p53-positive cells

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Fig. 3 Co-localization of p53 induction with apoptosis

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Fig. 4 Suppression of p53 activation during cisplatin treatment by pifithrin-a

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Cisplatin: - - + + + +
Pifithrin: - - - - + +

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B

Control  Cisplatin  Cisplatin+pifithrin

p53

p-p53
Fig. 5 Renoprotective effects of pifithrin-α against cisplatin-induced acute renal failure

A

![Bar graph showing BUN (mg/dL) levels for Control, Cisplatin, and Cisplatin+pifithrin groups.](image)

B

![Bar graph showing Serum Creatinine (mg/dL) levels for Control, Cisplatin, and Cisplatin+pifithrin groups.](image)
Fig. 6  Effects of pifithrin-a on cisplatin-induced renal tissue damage and apoptosis

A  Histology

B Tubular damage score

C TUNEL positive cells/mm²

[Images of histology and TUNEL staining with control, cisplatin, and cisplatin+pifithrin groups]
Fig. 7 Renal function of wild-type and p53-deficient mice following cisplatin treatment.

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</table>

B

![BUN (mg/dL) controls vs. cisplatin treated](image)

C

![Serum Creatinine (mg/dL) controls vs. cisplatin treated](image)
Fig. 8 Renal tissue damage and apoptosis in wild-type and p53-deficient mice following cisplatin treatment
Fig. 9  Cisplatin-induced apoptosis in primary proximal tubular cells isolated from wild-type and p53-deficient mice

A

![Phase](image)

![Hoechst](image)

![TUNEL](image)

B

![% Apoptosis](image)

C

![Recovered cell protein (µg/dish)](image)