Effect of JNK inhibition on cisplatin-induced renal damage

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Abstract

Background. Cisplatin therapy is effective against many tumours, however, the nephrotoxicity of this drug is a dose-limiting factor. Apoptosis and necrosis of tubular cells and inflammatory events contribute to the cisplatin-induced nephrotoxicity. Cisplatin promotes increased production of reactive oxygen species, which can activate c-jun N-terminal kinase (JNK) that is a mediator of apoptosis and can lead to increased expression of proinflammatory mediators that could intensify the cytotoxic effects of cisplatin. In this study, we evaluated the effect that SP600125, a selective inhibitor of phosphorylated JNK (p-JNK), has on the renal damage caused by cisplatin use.

Methods. A total of 33 male Wistar rats received SP600125 (15 mg/kg/day, s.c., diluted in polyethylene glycol) for 4 days. At 24 h after the first dose, those 33 rats, plus an additional 30, were injected with cisplatin (5 mg/kg, i.p.). In addition, 18 control rats were injected with saline, and 12 rats with polyethylene glycol. At 2 and 5 days after saline or cisplatin injection, blood and urine samples were collected for measurement of creatinine, sodium and potassium, and the kidneys removed for histological, immunohistochemical and Western blot studies.

Results. Cisplatin-treated rats presented higher plasma creatinine, as well as greater immunostaining for ED1 (macrophages/monocytes), p-JNK, apoptotic cells, and tubular cell necrosis in the renal cortices and outer medullae. The increase of p-JNK expression was also confirmed by Western blot analysis. All of these alterations were attenuated by treatment with SP600125.

Conclusion. The protective effect of SP600125 on cisplatin-induced renal damage seems to be related to the reduction in the apoptotic cell death and to the restriction of renal inflammation.

Keywords: acute renal failure; apoptosis; cisplatin; c-jun-N-terminal kinase; inflammation; SP600125

Introduction

Cisplatin is an inorganic complex composed of an atom of platinum surrounded by chloride and amine groups in the cis position. Cisplatin therapy is highly effective against many tumours [1], however, the primary side effect of cisplatin use, nephrotoxicity, has been reported to be a dose-limiting factor [2]. Cisplatin is taken up by renal tubular cells, reaching its highest concentrations in the proximal tubular cells of the inner cortices and outer medullae, especially in the S3 segment [2,3]. As a result, these are the major sites of cisplatin-induced renal damage [2,4,5]. Cisplatin provokes loss of tubular epithelial cell by necrosis and apoptosis, followed by inflammatory cell infiltration and fibroproliferative changes [6–9].

Cisplatin-induced kidney damage has been correlated with the formation of free radicals and with oxidative stress, which can activate c-jun N-terminal kinase (JNK) [8,10–13]. The treatment with antioxidants and caspase inhibitors can alleviate the effects of cisplatin-related nephrotoxicity [10,11]. There is considerable evidence showing that JNK and its substrate c-jun play important roles in the regulation of apoptosis pathways [13–16] as well as in the inflammatory process [6,9]. It has been observed that the JNK-activator protein-1 (AP-1) pathway plays a crucial role in H2O2-induced apoptosis in mesangial cells [17–19]. It was also found that the inhibition of JNK decreases cardiomyocyte apoptosis and infarct size after myocardial ischemia and reperfusion in anaesthetized rats [20]. In complex with other DNA binding proteins, AP-1 regulates the transcription of numerous genes [21,22], including those of cytokines
such as interferon-γ, interleukin-2 (IL-2) and tumour necrosis factor-α (TNF-α) [23], as well as those of inflammatory enzymes such as cyclooxygenase-2 [24] and matrix metalloproteinases such as matrix metalloproteinase 13 [25].

Bennett et al. [26] recently characterized SP600125, an anthrapyrazolone [anthrax (1,9-cd) pyrazole-6 (2H)-one], as an inhibitor of JNK catalytic activity. This compound inhibits JNK-1, JNK-2 and JNK-3 with high specificity, decreasing the phosphorylation of c-jun and leading to reduced expression of proinflammatory genes. SP600125 inhibited JNK-1, -2 and -3 isoforms with similar potency; however, exhibited ≥300-fold of selectivity for these kinases, compared to mitogen-activated protein (MAP), kinases extracellular signal-regulated kinase 1 (ERK1) and p38, and the serine threonine kinase cAMP-dependent protein kinase (PKA) [26]. This selective JNK inhibitor reversibly competes with endogenous adenosine triphosphate (ATP) for the ATP-binding site in JNK [27]. Wang et al. [28] found that SP600125 inhibited lipopolysaccharide-induced transcription of IL-18 mRNA in murine peritoneal macrophages in a dose-dependent manner and that this effect can be mediated through JNK inactivation. Increased production of proinflammatory cytokines can amplify the renal inflammatory process and exacerbate cisplatin-induced kidney damage [28–31]. SP600125 activity has also been reported in models of pulmonary disease. A decrease in the lung content of interleukin-4, interleukin-13, RANTES (regulated on activation, normal T-cell expressed and secreted) and TNF-α was observed following administration of SP600125 in a model of chronic allergen exposure in sensitized Balb/c mice [29]. However, the effect of treatment of SP600125 on the evolution of the cisplatin-induced renal damage has not yet been reported.

In this study, we evaluated the effects that SP600125 has on cisplatin-induced changes in renal structure and function and in the inflammatory process observed in these animals.

Materials and methods

Animals and experimental protocols

A total of 93 male Wistar rats (180–200 g) were divided into six groups: (i) control (C; n = 18), receiving a single injection of 0.9% saline (1 ml/100 g, i.p.); (ii) SP600125 (SP; n = 12), receiving a single injection of 0.9% saline (1 ml/100 g, i.p.) and SP600125 (15 mg/kg/day, s.c.) once daily for 4 days; (iii) polyethylene glycol (PEG; n = 12), the vehicle for SP600125, receiving 40% PEG (s.c.) once daily for 4 days and a single injection of 0.9% saline (1 ml/100 g, i.p.); (iv) cisplatin (CP; n = 18), receiving a single injection of cisplatin in a volume equal to that of the saline injections (5 mg/kg, i.p.); (v) polyethylene glycol + cisplatin (PEG + CP; n = 12), receiving 40% PEG (s.c.) once daily for 4 days and a single injection of cisplatin (5 mg/kg, i.p.); and (vi) SP600125 + cisplatin (SP + CP; n = 21), receiving SP600125 (15 mg/kg, s.c.) once daily for 4 days and a single injection of cisplatin (5 mg/kg, i.p.). The PEG and SP600125 were administered at 24 h and 1 h prior to, and again at 24 and 48 h after, the injection of saline or cisplatin. On post-injection days 2 and 5, the rats were killed. The kidneys were perfused through the aorta with a phosphate-buffered solution (PBS; 0.15 M NaCl and 0.01 M sodium phosphate buffer, pH 7.4) and removed for histological and immunohistochemical studies. Blood was collected to measure plasma creatinine.

All experimental procedures were conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and the Animal Experimentation Committee of the University of São Paulo at Ribeirão Preto School of Medicine approved the study protocol.

Renal function studies

Beginning on post-injection day 1 and on post-injection day 4, the rats were placed in metabolic cages, and 24-h urine and blood samples were collected. The 24-h urine samples were used to measure creatinine, sodium and potassium. Plasma creatinine was measured by the Jaffé method and sodium and potassium were measured in plasma and urine using flame photometry (model 262; Micronal, São Paulo, Brazil). On post-injection day 5, inulin clearance studies were conducted in order to measure glomerular filtration rate (GFR) in six control rats, six cisplatin-treated rats and six cisplatin + SP600125-treated rats. The rats were anesthetized with an i.p. injection of sodium thionembutal (50 mg/kg). After tracheostomy, the femoral artery and vein were cannulated to collect blood samples and inject fluids. The ureters were cannulated to collect urine. Inulin was administered in a priming dose (12 mg/100 g), followed by a maintenance dose (30 mg/100 g/h). After an ~60-min stabilization period, urine was collected for 1 h, and blood samples were collected at 30 min and at 1 h. These samples were used to assess levels of sodium, potassium and inulin. Inulin was measured in plasma and urine using the method described by Füehr et al. [32].

Light microscopy and morphometric studies

The kidneys were removed, sectioned transversely, fixed in 4% paraformaldehyde, post-fixed in Bouin’s solution for 4–6 h and processed for paraffin embedding. Histological sections (4 μm thick) were stained with Masson’s trichrome and examined under light microscopy. Tubulointerstitial damage was defined as tubular necrosis, inflammatory cell infiltrate, tubular lumen dilation or tubular atrophy. Damage was graded [33] on a scale of 0–4 (0 = normal; 0.5 = small focal areas; 1 = involvement of <10% of the cortices and outer medullae; 2 = 10–25% involvement of the cortices and outer medullae; 3 = 25–75% involvement of the cortices and outer medullae; 4 = extensive damage involving more than 75% of the cortices and outer medullae). The morphometric studies were performed with a still camera connected to an image analyser (KS 300; Kontron, Eching, Germany). In sections obtained from each kidney, the outer medullae were evaluated in 20 grid fields measuring 0.245 mm² each. The area surrounding the tubular lumen was traced manually on a video screen and determined by computerized morphometry, thereby allowing the mean area.
per kidney to be determined [34]. We also evaluated the number of tubules with cellular necrosis from renal cortices and outer medullae by grid fields (0.245 mm$^2$).

**Antibodies**

We used the following primary antibodies: a monoclonal anti-rat ED1 antibody to a cytoplasmic antigen present in macrophages and monocytes (Serotec, Oxford, UK); a monoclonal anti-phosphorylated JNK (p-JNK, also known as activated JNK) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), a polyclonal anti-phosphorylated JNK antibody (Cell Signaling Technology, Inc., Danvers, MA, USA), a polyclonal anti-phospho-p44/42 (p-ERK) antibody (Cell Signaling Technology, Inc.) and a monoclonal anti-p-p38 antibody (Sigma-Aldrich, St Louis, MO, USA), which react specifically with the active phosphorylated form of JNK, p42 and p44 MAP kinase (ERK) and p38, respectively.

**Immunohistochemical studies**

The kidneys from control, cisplatin- and SP600125+ cisplatin-treated rats killed on post-injection days 2 and 5 were submitted to immunohistochemical studies [34,35]. The sections were incubated at 4°C overnight with 1/30 monoclonal anti-p-JNK or 1/30 polyclonal anti-p-ERK or for 1 h with 1/1000 monoclonal anti-ED1 antibodies. The reaction product was detected with an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The colour reaction was developed with 3,3′-diaminobenzidine (DAB; Sigma Chemical Company, St Louis, MO, USA). The sections were counterstained with methyl green (for ED1) or Harris haematoxylin (for p-JNK and p-ERK), dehydrated and mounted. Negative controls were created by replacing the primary antibody with equivalent concentrations of normal rabbit or mouse immunoglobulin G (IgG), for polyclonal or monoclonal antibodies, respectively, at equivalent concentrations. For evaluation of immunoperoxidase staining for p-JNK and p-ERK the renal cortices and the outer medullae were graded semiquantitatively through examination of, respectively, 20 and 15 grid fields (each field measuring 0.245 mm$^2$), and the mean score per kidney was calculated [35]. The scores mainly reflected changes in the extent, rather than the intensity, of the staining and depended on the percentage of grid fields showing positive staining: 0, no involvement or involvement of <5%; I, 5–25% involvement; II, 25–50% involvement; III, 50–75% involvement and IV, >75% involvement. It has been demonstrated that the semiquantitative scoring system is not only reproducible among different observers, but also that the data also are highly correlated with those obtained through computerized morphometry [35]. Infiltrating macrophages and monocytes were counted in the cortical and outer medullary tubulointerstitium through examination of 35 grid fields, measuring 0.245 mm$^2$ each (20 from the renal cortices and 15 from the outer medullae), and the mean counts per kidney were calculated.

**Double-labelling immunohistochemistry**

Double staining for ED1 and p-JNK was performed as reported previously [35] by first staining the sections with the p-JNK antibody using an immunoperoxidase procedure and a brown reaction product (DAB). Sections were then incubated with antibody against ED1 using an immunoperoxidase procedure and a blue reaction product. These slides were performed without counterstaining.

**Western blot studies**

The capsule was removed and the distinct regions were identified on the cut surface of a bisected kidney: a paler outer region (the cortex) and a darker inner region (the medullae). The darker inner region (inner medullae) was then trimmed off with scissors. Renal cortices and outer medullae tissue from animals killed 2 and 5 days after saline or cisplatin injections were homogenized in 2 ml Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, pH 10, 1 mM sodium pyrophosphate, 25 mM sodium fluoride, 0.001 M EDTA, pH 8) at 4°C. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After immunoblotting with anti-p-JNK polyclonal antibody (1/100), anti-p-p38 monoclonal antibody (1/1000), anti-p-ERK polyclonal antibody (1/1000) or with anti-α-tubulin (Sigma-Aldrich Chemical Company; 1/4000), blots were washed or for 1 h with 1/100 polyclonal anti-ED1 antibodies. The sections were counterstained with methyl green (for ED1) or Harris haematoxylin (for p-JNK and p-ERK), dehydrated and mounted. Negative controls were created by replacing the primary antibody with equivalent concentrations of normal rabbit or mouse immunoglobulin G (IgG), for polyclonal or monoclonal antibodies, respectively, at equivalent concentrations. For evaluation of immunoperoxidase staining for p-JNK and p-ERK the renal cortices and the outer medullae were graded semiquantitatively through examination of, respectively, 20 and 15 grid fields (each field measuring 0.245 mm$^2$), and the mean score per kidney was calculated [35]. The scores mainly reflected changes in the extent, rather than the intensity, of the staining and depended on the percentage of grid fields showing positive staining: 0, no involvement or involvement of <5%; I, 5–25% involvement; II, 25–50% involvement; III, 50–75% involvement and IV, >75% involvement. It has been demonstrated that the semiquantitative scoring system is not only reproducible among different observers, but also that the data also are highly correlated with those obtained through computerized morphometry [35]. Infiltrating macrophages and monocytes were counted in the cortical and outer medullary tubulointerstitium through examination of 35 grid fields, measuring 0.245 mm$^2$ each (20 from the renal cortices and 15 from the outer medullae), and the mean counts per kidney were calculated.

**In situ detection of apoptosis using the TUNEL assay**

The kidneys obtained from the rats killed on postinjection day 2 were stained with terminal deoxynucleotidyl transferase (TdT)-mediated deoxuryridine 5 triphosphate-biotin nick end labelling (TUNEL) using a commercial kit (Oncogene Research Products, Boston, MA, USA) [37]. Tissues treated with DNase I were used as positive controls, and sections stained without terminal nucleotidyl transferase were used as negative controls. The TUNEL-positive cells in the cortical and outer medullary tubulointerstitium were counted in 35 grid fields, measuring 0.245 mm$^2$ each (20 from the renal cortices and 15 from the outer medullae), and the mean counts per kidney were calculated.

**Lipid peroxidation in urine samples**

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) in urine samples collected from rats killed at 48 h after i.p. injections using the Lipid Peroxidation Assay Kit (Calbiochem, San Diego, CA, USA) [38]. The urinary MDA was defined as the urinary level of MDA divided by the milligrams of urinary creatinine, in order to correct the variation in urine concentration.
**Renal platinum content**

Tissues samples from rats killed 24 h after saline, cisplatin and SP600125 + cisplatin injections were analysed by graphite furnace atomic emission spectrometry (GFAAS) using a Perkin Elmer 4100 ZL model [39] in order to evaluate the renal platinum content. Before platinum analysis samples were decomposed according to McGahan and Tyczkowska [40]. Briefly, concentrated nitric acid (0.5 ml) was added to 500 mg of renal tissue and incubated overnight at room temperature. It was then boiled for 3–5 min after which 30% v/v H2O2 (0.5 ml) was added and the solution was boiled again. The resulting clear pale yellow solution was diluted to 50 ml with MilliQ water and analysed by GFAAS.

**Statistical analysis**

Data concerning plasma creatinine levels, urine volume, scores for p-JNK and p-ERK, tubulointerstitial lesions, and tubular cell necrosis were analysed statistically using the non-parametric Kruskal–Wallis test followed by the Dunn post-test because they were not normally distributed. Those data are expressed as median and interquartile range (25–75%). Data related to other parameters studied presented a normal distribution, then they were submitted to analysis of variance with the Newman–Keuls multiple comparisons test.

**Results**

**Renal function**

On post-injection days 2 and 5, cisplatin-injected rats presented elevated plasma creatinine levels and increased urine volume and greater fractional excretion of sodium and potassium. On post-injection day 2, these changes were less pronounced in the SP600125 + cisplatin-treated rats. The increases in plasma creatinine levels and fractional excretion of sodium, as well the reduction in GFR, observed on post-injection day 5 were also attenuated by the treatment with SP600125 (Tables 1 and 2, Figure 1). The PEG-, SP600125-, or PEG + cisplatin-treated rats did not present differences on any parameter of renal function studied compared with their respective controls (Tables 1 and 2).

**Light microscopy and morphometric studies**

The light microscopy study on post-injection days 2 and 5 revealed renal damage, characterized by marked tubular lumen dilation due to flattening of tubular cells with brush border loss, inflammatory cell infiltration into the interstitium, tubular cell necrosis and vacuolization. On post-injection days 2 and 5, these alterations were less intense in the kidneys from rats treated with SP600125 + cisplatin. The morphometric analyses performed on post-injection days 2 and 5 showed an increase in the tubular lumen area of the renal outer medullae of rats treated with cisplatin. On post-injection day 5, this alteration was less intense in the kidneys from rats treated with SP600125

![Fig. 1. Glomerular filtration rate (GFR) for saline-treated (control), cisplatin (CP)-treated and SP600125 + CP-treated rats at 2 days after saline or CP injections](image)

**Table 1. Parameters of renal function for saline-treated (control), SP600125 (SP), polyethylene glycol (PEG), cisplatin (CP), PEG + CP and SP600125 + CP-treated rats at 2 days after saline or CP injections**

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.49</td>
<td>9.00</td>
<td>0.66</td>
<td>49.72</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(0.47; 0.54)</td>
<td>(7.50; 10.50)</td>
<td>±0.08</td>
<td>±5.18</td>
</tr>
<tr>
<td>SP</td>
<td>0.48</td>
<td>9.00</td>
<td>0.61</td>
<td>46.96</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(0.41; 0.60)</td>
<td>(7.60; 10.20)</td>
<td>±0.10</td>
<td>±6.02</td>
</tr>
<tr>
<td>PEG</td>
<td>0.51</td>
<td>10.00</td>
<td>0.57</td>
<td>48.61</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(0.34; 0.74)</td>
<td>(8.68; 19.50)</td>
<td>±0.08</td>
<td>±5.52</td>
</tr>
<tr>
<td>CP</td>
<td>1.04</td>
<td>17.00</td>
<td>2.00</td>
<td>184.90***</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(0.88; 1.25)</td>
<td>(15.50; 21.00)</td>
<td>±0.33</td>
<td>±32.27</td>
</tr>
<tr>
<td>PEG + CP</td>
<td>1.18</td>
<td>14.00</td>
<td>1.66</td>
<td>166.38***</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(0.89; 1.21)</td>
<td>(11.61; 20.62)</td>
<td>±0.11</td>
<td>±36.66</td>
</tr>
</tbody>
</table>
| SP + CP    | 0.93| 10.00#| 1.15| 96.19#
| (n = 6)    | (0.81; 1.12) | (8.50; 12.00) | ±0.19 | ±11.47 |

P<sub>creat</sub>, plasma creatinine (mg/dl); V, urinary volume (ml/24 h); FE, fractional excretion. Data are expressed as mean ± SEM (FENa<sub>creat</sub> and FEK<sub>creat</sub>). *P < 0.05, **P < 0.01 and ***P < 0.001 vs control; #P < 0.05 vs CP.

**Table 2. Parameters of renal function for saline-treated (control), SP600125 (SP), polyethylene glycol (PEG), cisplatin (CP), PEG + CP, and SP600125 + CP-treated rats at 5 days after saline or CP injections**

<table>
<thead>
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</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.44</td>
<td>9.00</td>
<td>0.58</td>
<td>39.38</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(0.35; 0.48)</td>
<td>(7.50; 10.00)</td>
<td>±0.03</td>
<td>±4.68</td>
</tr>
<tr>
<td>SP</td>
<td>0.53</td>
<td>11.00</td>
<td>0.68</td>
<td>49.87</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(0.48; 0.59)</td>
<td>(9.00; 16.08)</td>
<td>±0.07</td>
<td>±6.31</td>
</tr>
<tr>
<td>PEG</td>
<td>0.50</td>
<td>12.00</td>
<td>0.66</td>
<td>44.39</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(0.44; 0.72)</td>
<td>(8.50; 17.50)</td>
<td>±0.04</td>
<td>±4.56</td>
</tr>
<tr>
<td>CP</td>
<td>1.11##</td>
<td>34.00</td>
<td>1.91##</td>
<td>124.40*</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(0.92; 1.30)</td>
<td>(24.50; 38.50)</td>
<td>±0.68</td>
<td>±27.55</td>
</tr>
<tr>
<td>PEG + CP</td>
<td>1.05##</td>
<td>30.00*</td>
<td>1.89##</td>
<td>101.35**</td>
</tr>
<tr>
<td>(n = 6)</td>
<td>(0.99; 1.10)</td>
<td>(25.65; 35.60)</td>
<td>±0.52</td>
<td>±19.68</td>
</tr>
<tr>
<td>SP + CP</td>
<td>0.64##</td>
<td>37.00##</td>
<td>0.88##</td>
<td>118.40##</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(0.51; 0.70)</td>
<td>(35.00; 41.00)</td>
<td>±0.15</td>
<td>±7.03</td>
</tr>
</tbody>
</table>

P<sub>creat</sub>, plasma creatinine (mg/dl); V, urinary volume (ml/24 h); FE, fractional excretion. Data are expressed as median ± interquartile range (25–75%) (P<sub>creat</sub> and V) or mean ± SEM (FENa<sub>creat</sub> and FEK<sub>creat</sub>). *P < 0.05, **P < 0.01 and ***P < 0.001 vs control; #P < 0.05 vs CP, ##P < 0.01 vs CP.
pronounced in the kidneys of rats treated with SP600125 + cisplatin (Table 3). Tubular cell necrosis was also less intense in these animals compared with cisplatin-alone-treated rats (Figure 2).

**Immunohistochemical studies**

In the immunohistochemical studies performed on post-injection days 2 and 5, higher numbers of ED1-positive cells and greater p-JNK expression were seen in the renal cortices and outer medullae of cisplatin-treated rats than in those of controls (Tables 4 and 5; Figures 3 and 4). The double-labelling immunohistochemistry for p-JNK and ED1 in the renal cortices and outer medullae of cisplatin-treated rats showed that most of the macrophages expressed p-JNK (Figure 4, insert). On post-injection day 2 and 5, SP600125 + cisplatin-treated rats presented lower numbers of ED1 positive cells in the renal cortices and outer medullae compared with cisplatin-alone-treated rats. Treatment with SP600125 also prevented the increased expression of p-JNK induced by cisplatin in the renal cortices on post-injection days 2 and 5 and in the outer medullae on post-injection day 5 (Tables 4 and 5, Figures 3 and 4). We also found greater p-ERK expression in the renal cortices and outer medullae of cisplatin-treated rats compared with controls at day 2 after cisplatin injection. SP600125 treatment did not modify the increase of p-ERK expression induced by cisplatin (Tables 4 and 5).

**Western blot**

Western blot analysis performed with anti-p-JNK, anti-p-ERK or anti-p-p38 MAPK antibodies demonstrated the presence of 46, 54-kDa (phospho-p46, phospho-p54 SAPK/JNK), 42, 44-kDa (phospho-p44/42 MAPK) and 43-kDa (p-p38 MAPK) protein lanes in the tissue samples from renal cortices and outer medullae of all groups studied. The corresponding lanes for p-JNK and p-p38 from cisplatin-treated animals killed 2 and 5 days after treatment and for p-ERK lane on day 2 were more prominent than those obtained for the control animals. The increase of p-JNK expression induced by cisplatin was attenuated by treatment with SP600125.

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**Table 3.** Score for tubulointerstitial lesions and tubular lumen area in the renal cortices and outer medullae of saline-treated (control), cisplatin (CP)-treated and SP600125 + CP-treated rats at 2 and 5 days after saline or CP injections.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 6)</th>
<th>CP-2d (n = 6)</th>
<th>SP + CP-2d (n = 6)</th>
<th>CP-5d (n = 6)</th>
<th>SP + CP-5d (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>TIL, score</td>
<td>0.00</td>
<td>3.00**</td>
<td>2.00***##</td>
<td>4.00***</td>
<td>3.00***##</td>
</tr>
<tr>
<td>(3.0; 3.5)</td>
<td>(2.0; 2.5)</td>
<td>(3.5; 4.0)</td>
<td>(3.0; 3.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLA (μm²)</td>
<td>290.50</td>
<td>682.60**</td>
<td>544.60*</td>
<td>1574.00***</td>
<td>1073.00***##</td>
</tr>
<tr>
<td>±38.11</td>
<td>±113.50</td>
<td>±67.61</td>
<td>±95.15</td>
<td>±107.60</td>
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</tbody>
</table>

TIL, tubulointerstitial lesions; TLA, tubular lumen area. Data are expressed as median and interquartile range (25–75%) (TIL) or mean±SEM (TLA).

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**Table 4.** Number of ED1-positive cells per 0.245 mm² grid field and immunostaining scores for p-JNK and p-ERK in the renal cortices of saline-treated (control), cisplatin (CP)-treated and SP600125 + CP-treated rats at 2 and 5 days after saline or CP injections.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CP</th>
<th>SP + CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED1</td>
<td>5.86</td>
<td>15.34^*</td>
<td>8.19^*</td>
</tr>
<tr>
<td>±0.90</td>
<td>±1.83</td>
<td>±1.62</td>
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<tr>
<td>p-JNK</td>
<td>0.75</td>
<td>1.27</td>
<td>1.06</td>
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<tr>
<td>(0.70; 0.93)</td>
<td>(1.06; 1.54)</td>
<td>(0.99; 1.12)</td>
<td></td>
</tr>
<tr>
<td>p-ERK</td>
<td>0.23</td>
<td>0.70^*</td>
<td>0.73^*</td>
</tr>
<tr>
<td>(0.21; 0.24)</td>
<td>(0.60; 0.83)</td>
<td>(0.63; 0.76)</td>
<td></td>
</tr>
<tr>
<td>5 days</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| ED1              | 4.72    | 26.07^* | 17.30^*#
| ±0.68            | ±5.16   | ±1.44 |
| p-JNK            | 0.74    | 1.05^* | 0.79^* |
| (0.68; 0.86)     | (1.02; 1.29) | (0.67; 0.91) |
| p-ERK            | 0.21    | 0.11 | 0.14    |
| (0.10; 0.24)     | (0.10; 0.13) | (0.09; 0.16) |

Data are expressed as mean±SEM (ED1) or as median and interquartile range (25–75%) (p-JNK and p-ERK).

---

However, the increases of p-p38 and of p-ERK in renal cortices and outer medullae from cisplatin-treated rats were not modified by SP600125 treatment. There was no difference in the intensity of the
lanes for $\alpha_1$-tubulin between the different groups, showing the equivalence of protein loading (Figures 5 and 6).

In situ detection of apoptosis

On post-injection day 2, the cisplatin-treated rats also presented higher numbers of TUNEL-positive cells in the renal cortices and outer medullae compared with the controls. This alteration was attenuated by treatment with SP600125 (Table 6, Figure 7).

Urinary MDA levels

Urinary MDA levels, expressed as MDA urine level divided by milligrams of urine creatinine, were significantly higher in cisplatin-injected rats than in those of control rats. This increase was prevented by treatment with SP600125 (Figure 8).

### Table 5. Number of ED1-positive cells per 0.245 mm² grid field and immunostaining scores for p-JNK and p-ERK in the outer medullae of saline-treated (control), cisplatin (CP)-treated and SP600125 + CP-treated rats at 2 and 5 days after saline or CP injections

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CP</th>
<th>SP + CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED1</td>
<td>3.60 ± 0.66</td>
<td>23.53**</td>
<td>10.80***</td>
</tr>
<tr>
<td>p-JNK</td>
<td>1.00</td>
<td>1.60*</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>(0.78; 1.21)</td>
<td>(1.50; 2.04)</td>
<td>(1.07; 1.81)</td>
</tr>
<tr>
<td>p-ERK</td>
<td>0.37</td>
<td>1.20***</td>
<td>1.11***</td>
</tr>
<tr>
<td></td>
<td>(0.33; 0.46)</td>
<td>(1.11; 1.35)</td>
<td>(1.04; 1.16)</td>
</tr>
<tr>
<td>5 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED1</td>
<td>4.77 ± 0.57</td>
<td>56.55***</td>
<td>46.43**</td>
</tr>
<tr>
<td>p-JNK</td>
<td>0.82</td>
<td>1.80***</td>
<td>1.20***</td>
</tr>
<tr>
<td></td>
<td>(0.70; 0.84)</td>
<td>(1.46; 2.42)</td>
<td>(1.08; 1.50)</td>
</tr>
<tr>
<td>p-ERK</td>
<td>0.37</td>
<td>0.46</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>(0.33; 0.46)</td>
<td>(0.28; 0.64)</td>
<td>(0.53; 0.57)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM (ED1) or as median and interquartile range (25–75%) (p-JNK and p-ERK).

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs control; *$P < 0.05$, and **$P < 0.01$ vs CP.

Fig. 3. Immunolocalization of ED1 in the renal outer medullae of rats killed at 2 days (A and B) or at 5 days (C and D) after CP injection (CP) (A and C) or SP600125 + CP (B and D) injections. Note that the staining for ED1 is more intense in CP-treated rats than in saline-treated or SP600125 + CP-treated rats. Original magnification: ×280.
Renal platinum content

Higher platinum renal contents, expressed as μg/g of renal tissue, were observed in the kidneys of cisplatin-injected rats at 24 h after cisplatin injection. This content was not affected by SP600125 injection, showing that this treatment did not interfere with cisplatin uptake by the renal tubular cells. Platinum values for the control group were closed or lower than the method detection limits (Figure 9).

Discussion

Our data show that cisplatin administration induces an increase in plasma creatinine levels, urinary volume and in fractional excretion of sodium and potassium as well as a reduction in GFR. Treatment with SP600125 was found to attenuate the cisplatin-induced alterations in plasma creatinine, as well as in fractional excretion of sodium and potassium. The drop in GFR, observed after cisplatin injection was also less pronounced in the SP600125 + cisplatin group. However, treatment with SP600125 did not prevent cisplatin-related alterations in urine volume. The polyuria might be related to a significant decrease in the expression of collecting duct water channels (aquaporins 2 and 3), as well as to the increase in renal sodium excretion observed in cisplatin-injected rats [41,42].

We observed that immunoreactivity to p-JNK was significantly higher in the tubular and interstitium cells of renal cortices and outer medullae obtained from cisplatin-injected rats than in those obtained from control rats. The increase of p-JNK in renal cortices and outer medullae from cisplatin-treated rats was confirmed by Western blot studies performed with an antibody for p-JNK. This antibody reacts specifically with the active phosphorylated form of the JNK also known as activated JNK. The activation of this JNK pathway might be related to the higher number of apoptotic cells present in the renal cortices and outer medullae of these rats. This hypothesis is supported by the fact that p-JNK expression and the number...
of apoptotic cells were lower in the renal cortices and outer medullae of rats treated with cisplatin + SP600125 than in those injected with cisplatin alone. Sheikh-Hamad et al. [12] found that the pro-apoptotic molecular changes present in the outer medullary tissue from the kidneys of cisplatin-treated rats were associated with a 3-fold increase in the JNK-1 activity in the outer medullae. Ferrandi et al. [20] observed that the treatment with a JNK inhibitor in rats during myocardial ischemia and reperfusion reduced myocardial apoptosis associated with JNK activation and improved post-ischaemic cardiac functional recovery. It was
observed that SP600125 can modulate cisplatin-induced apoptosis in several cancer cell lines [43–45]. SP600125 blocked the activation of caspases 8 and 3 after treatment with cisplatin alone and significantly inhibited activation of caspases 8, 9 and 3 caused by the cisplatin/17-allylamino-17-demethoxygeldanamycin combination [44]. In previous studies, we observed increased immunostaining for p-JNK in the renal cortices and outer medullae obtained from rats killed at 2 and 5 days after cisplatin injection [6].

The immunohistochemical studies also revealed an increase in ED1-positive cell counts in the renal cortices and outer medullae of cisplatin-treated rats, and this alteration was less pronounced in the rats treated with cisplatin + SP600125. It was observed that JNK can induce release of granulocyte-macrophage colony stimulating factor (GM-CSF), RANTES and...
IL-8 from airway smooth muscle cells [46]. Nath et al. [29] found a decrease in the levels of proinflammatory cytokines following pre-treatment with SP600125 in a mice model of chronic allergen exposure. Treatment with SP600125 also inhibited joint inflammation by attenuating metalloproteinase expression and joint destruction in an adjuvant arthritis model in rats. This effect was associated with the suppression of JNK activation in the synovium [47]. We observed that the treatment with SP600125 also attenuated cisplatin-induced renal histological changes (interstitial inflammatory infiltration, tubular dilation and cellular necrosis). Inhibition of JNK by SP600125 can reduce proinflammatory mediators and limit the inflammatory process, thereby attenuating the cytotoxic effects of cisplatin therapy [29,47,48]. Macrophages contribute to the loss of renal function and hinder recovery by releasing radical oxygen species, nitric oxide and various inflammatory mediators, such as platelet-derived growth factor, IL-1, TNF-α and transforming growth factor-β, as well as by stimulating cell proliferation, extracellular matrix expansion and fibrosis [31,49]. Our double-labelling studies showed that most of the macrophages expressed p-JNK.

The higher urinary MDA levels observed after cisplatin injection were also attenuated by SP600125 treatment, suggesting that the SP600125-treated rats suffered less oxidative stress [38]. This is likely due to the reduction in the inflammatory process seen in the kidneys obtained from these rats. We found the measurement of oxidative damage markers (such as MDA) in urine to be a sensitive method of evaluating oxidative stress [50].

Our data showed that the platinum concentration in kidney tissue from cisplatin plus SP600125 was not different of the rats treated with cisplatin alone, although they presented a reduction in renal damage. It has been demonstrated that cisplatin induces cellular death by necrosis and apoptosis [7]. The animals treated with SP600125 presented less inflammatory infiltration and reduced cellular apoptosis. However, the tubular cell necrosis persisted in the renal cortices and outer medullae of these rats, may be because SP600125 has some effects on apoptotic pathways and inflammation, but does not interfere with the tubular necrosis. It has been observed that the mechanism of necrosis in cisplatin nephrotoxicity is different from that of apoptosis. The necrosis is most likely attributable to glycolytic enzyme activity [4] or to rapid metabolic collapse and ATP depletion secondary to impaired mitochondrial function [51]. The reactive oxygen species play an important role in apoptosis, but does not interfere with the tubular necrosis, that is related to different mechanisms.

The results of this study show that SP600125 treatment reduces cisplatin-induced alterations in renal function and structure. This effect might be related to the reduction in the number of apoptotic cells and to the limited inflammatory response observed in the cisplatin + SP600125-treated rats. These data also suggest that the inhibition of the JNK pathway may represent a novel therapeutic approach for preventing cisplatin-induced renal damage.

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Conflict of interest statement. None declared.


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