RESEARCH ARTICLE

Evaluation of renal protective effects of inhibiting TGF-β type I receptor in a cisplatin-induced nephrotoxicity model

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To cite this article: Bayomi HS, Elsherbiny NM, El-Gayar AM, Al-Gayyar MMH. Evaluation of renal protective effects of inhibiting TGF-β type I receptor in a cisplatin-induced nephrotoxicity model. *Eur. Cytokine Netw.* 2013; 24(4): 139-47 doi:10.1684/ecn.2014.0344

ABSTRACT. ***Purpose:*** **The use of cisplatin, the first of the platinum-containing anti-cancer drugs, is limited by the development of a myriad of adverse reactions, including nephrotoxicity. We conducted this study therefore to find out whether SB-431542, potent and specific inhibitor of type I transforming growth factor-beta receptor (TGF-βR1), could prevent or attenuate kidney damage in rats, and to elucidate its possible mechanism of action.** ***Methods:*** **Fifty rats were treated with cisplatin (10 mg/kg) in the presence (1 and 3 mg/kg) or absence of SB-431542. Morphological changes were assessed in kidney sections stained with H/E. Oxidative stress was evaluated in kidney homogenates by measuring malondialdehyde (MDA) and superoxide dismutase (SOD). Kidney samples were used for measurements of TGF-βR1, TGF-β1 and sCD93 by ELISA. Kidney tissue apoptosis was assessed by measuring caspase-3 activity.** ***Results:*** **The renal protective effect of SB-431542 was confirmed by the normal appearance of renal tissue and the relatively unaffected serum creatinine and urea levels. With SB-431542, there was significantly lower renal MDA and increased SOD compared with the cisplatin group. Furthermore, in the SB-431542 group, renal TGF-βR1, TGF-β1, sCD93 and caspase-3 levels were significantly lower.** ***Conclusions:*** **Inhibition of TGF-βR1 provides protective effects against cisplatin-induced nephrotoxicity through several mechanisms, including attenuation of oxidative stress, inhibition of pro-inflammatory cytokines, blocking of renal fibrosis markers, and anti-apoptotic effects.**

Key words: caspase-3, cisplatin, MDA, SB-431542, sCD93, SOD, TGF-β1, TGF-βR1

Chemotherapy tends to disrupt both normal and tumor cells, which triggers an endless series of serious, adverse reactions that can be life-threatening. For instance, cisplatin, the first member of a class of platinum-containing anti-cancer drugs, is a commonly used cytotoxic agent in the treatment of numerous solid tumors [1-3]. Cisplatin binds to and causes cross-linking of DNA, which ultimately triggers apoptosis. However, cisplatin's clinical usefulness is limited by the development of a myriad of adverse reactions, including serious nephrotoxicity that is reproducible in animal models [4-6]. Approximately 25–35% of patients develop evidence of nephrotoxicity following a single dose of cisplatin [7]. Hence, the search continues to find more promising antitumor agents, or combinations that would incur fewer health hazards.

A major focus of drug research is to minimize potentially serious, adverse reactions. Many previous studies have correlated the incidence of cisplatin-induced nephrotoxicity with increased levels of TGF-β [7-9]. The TGF-β signaling pathway plays an important role in controlling proliferation, differentiation and other cellular processes [10]. TGF-β1 is a most important growth factor, facilitating cell adhesion to extracellular matrix by increasing matrix production and accumulation [11]. Therefore, SB-431542, a potent and selective inhibitor of the TGF-β1 receptor (IC50 = 94 nM), was chosen for this study in order to evaluate the nephroprotective effects of blocking the TGF-β1 signaling pathway. SB-431542 specifically blocks Smad signaling, reducing gene expression relevant to fibrosis and cancer [12, 13]. In this context, we have recently reported the favorable chemoenhancing and renal protective effects, through inhibition of TNF-α signaling, with the use of docosahexaenoic acid (DHA) [14] and epigallocatechin-gallate (EGCG) [3]. Because cisplatin induces a substantial life-threatening nephrotoxicity, the goal of this study was to determine whether SB-431542 could attenuate such a severe reaction in rats. In addition, the molecular underpinnings of the possible protective effects were also investigated.

MATERIALS AND METHODS

Drugs and chemicals

Cisplatin vials (10 mg/10 mL) were obtained from (Merck Co, Whitehouse Station, NJ, USA). SB-431542 was obtained from (Selleck Chemicals, Houston, TX, USA)

doi: 10.1684/ecn.2014.0344

and was mixed with a drop of dimethylsulphoxide (DMSO) and then dissolved in normal saline and used at two concentrations (1 and 3 mg/kg).

Animal preparation and experimental design

The animal protocol was approved by the ethical committee of the Faculty of Pharmacy, University of Mansoura. Male, Sprague Dawley rats weighing 100-150 g were used. All animals in the study were maintained under standard conditions of temperature, about 25°C, with a regular 12-hour light/12-hour dark cycle, and allowed free access to food and water. Rats were fed with standard rat food. Nephrotoxicity was induced by cisplatin (10 mg/kg, ip, single dose) [15]. This evoked a significant increase in serum creatinine levels after seven days from the start of the experiment (three days after the cisplatin injection). They were classified into the following groups, with 10 rats in each:

Group (1): received 0.2 mL of intraperitoneal (ip) of phosphate-buffered saline (PBS, 10 mM, pH 7.4), on the 1st, 4th and 7th day of treatment, and served as the untreated control group.

Group (2): received SB-431542 (1 mg/kg, ip). The animals received SB-431542, on the 1st, 4th and 7th day of treatment, and served as the treated control group.

Group (3): received cisplatin (10 mg/kg, ip, on the 4th day of the experiment), 1 h prior to the injection of 0.2 mL of PBS, ip. The animals received 0.2 mL of PBS, ip, on the 1st, 4th and 7th day of treatment.

Group (4): received cisplatin (10 mg/kg, ip, on the 4th day of the experiment), 1 h prior to the injection of SB-431542 (1 mg/kg, ip). The animals received SB-431542, on the 1st, 4th and 7th day of treatment.

Group (5): received cisplatin (10 mg/kg, ip, on the 4th day of the experiment), 1 h prior to the injection of SB-431542 (3 mg/kg, ip). The animals received SB-431542 on the 1st, 4th and 7th day of treatment.

The doses and time course of experiments in this study were in the range of those used in other studies involving the same animal species [3, 14, 16, 17]. In addition, the dose was confirmed after appropriate preliminary experiments.

Collection of samples

At the end of the specified experimental period (7 or 10 days), animals were anesthetized, blood samples were collected and serum samples were separated. Finally, the animals were sacrificed and kidneys were removed. The right kidney was fixed in 10% buffered formalin for subsequent morphological analysis; the left one was used to make a 10% (w/v) homogenate in phosphate-buffered saline (PBS, pH 7.4). The homogenate was centrifuged at 3,000 rpm for 10 min at 4°C and the supernatant was removed and stored on ice for assay of malondialdehyde (MDA) concentration or superoxide anion (SOD) activity, or it was stored at –80°C for further assay.

Morphological analysis of renal tissue

The kidney was cut longitudinally; one half was fixed in 10% buffered formalin and embedded in paraffin. Five micrometer-thickness sections were cut and stained with Mayer's hematoxylin and eosin (H&E) for examination of cell structure with a light microscope [18, 19]. Renal specimens were anonymously coded and examined in a blinded manner. The morphological changes were photographed using a digital camera-aided computer system (Nikon digital camera, Japan).

Measuring renal glomerular function

Serum urea and creatinine were measured kinetically in rat serum. Kits from Dp International Co. were used.

Measuring oxidative stress in renal homogenate

Oxidative stress was evaluated by measuring the following parameters in renal homogenates:

Lipid peroxides measured as MDA. Renal homogenate levels of thiobarbituric acid reactive substances (TBARs), mainly MDA, were determined in accordance with the reported method of Satoh [20], using a kit from Biodiagnostic Company.

Superoxide dismutase (SOD) activity was determined using the phenazinemethosulfate (PMS) method [21], which depends on the ability of SOD to inhibit the PMS-mediated reaction of nitro-blue tetrazolium (NBT).

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits were used for the measurement of levels of renal TGF-βR1 (Antibodies, Atlanta, GA, USA), TGF-β and sCD93 (eBioscience Inc., San Diego, CA, USA) in accordance with the manufacturers' protocols.

Statistical analysis

The mean values ± standard error were used for quantitative variables. For comparison between two groups Student's t-test was used. Statistical computations were performed on a personal computer using Microsoft Excel 2007. Statistical significance was predefined as $P \leq 0.05$.

RESULTS

SB-431542 blocked TGF-β type I receptor (TGF-βR1) in cisplatin-treated rats

Cisplatin increased renal TGF-βR1 by 1.7- and 2.3-fold of the untreated control group levels after 7 and 10 days treatment, respectively. Treatment with SB-431542 for 7 and 10 days attenuated any increase in renal TGF-βR1 as compared to the cisplatin only-treated control group (*figure 1*), and this was in a dose-dependent manner.

Effect of blocking TGF-βR1 on rat survival in cisplatin-induced nephrotoxicity

Cisplatin reduced survival rate by up to 40% and 80% after 7 and 10 days, respectively. Treatment with 1 mg/kg SB-431542 increased survival rate to 40% after 10 days, with no remarkable change after 7 days. Treatment with 3 mg/kg SB-431542 increased survival rate to 90% after 7 days' and 70% after 10 days' treatment (*figure 2*).

Blocking TGF-βR1 attenuated cisplatin-induced impairment of renal function

Cisplatin caused a 3.6-fold increase in serum creatinine levels after 7 days of treatment and an 8-fold increase

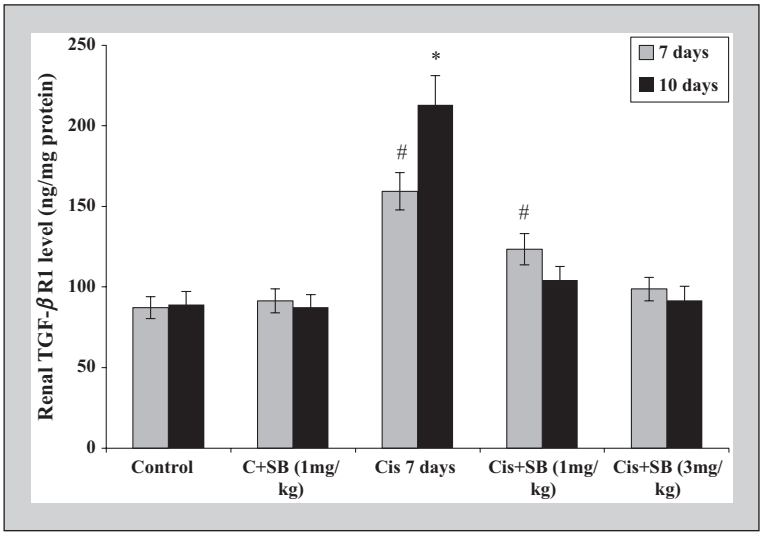

Figure 1
Effect of cisplatin (Cis, 10 mg/kg) alone, and in combination with SB-431542 (SB, 1 and 3 mg/kg), for 7 and 10 days, on renal type 1 transforming growth factor-β receptor (TGF- βR1) level. * Significant difference as compared with the rest of the groups at $p<0.05$. # Significant difference as compared with the control groups at $p<0.05$.

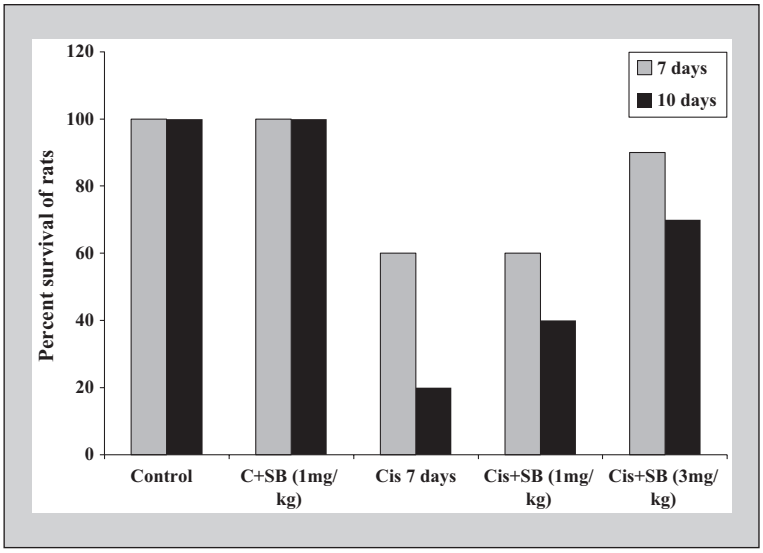

Figure 2
Effect of cisplatin (Cis, 10 mg/kg) alone, and in combination with SB-431542 (SB, 1 and 3 mg/kg), for 7 and 10 days, on rat survival.

after 10 days of treatment as compared to the untreated control group. Treatment with SB-431542 for 7 and 10 days attenuated any potential increase in serum creatinine level in a dose-dependent manner (*figure 3A*). In parallel, cisplatin increased serum urea levels by 3.8-fold after 7 days' treatment and 13.7-fold after 10 days' treatment as compared to the untreated control group. Treatment with SB-431542 attenuated the increase in serum urea level in the cisplatin group in a dose-dependent manner, but did not affect the untreated control group (*figure 3B*).

Effect of blocking TGF-βR1 on cisplatin-induced renal tissue damage

The renal protective effect of SB-431542 was examined in kidney sections stained with H&E. As shown in *figure 4*, sections of kidney from rats treated with cisplatin showed focal glomerulosclerosis, with marked shrinkage of some glomerular tufts, and tubular vacuolation with hyaline droplets. However, rats receiving both cisplatin and 1 mg/kg or 3 mg/kg SB-431542 for 7 or 10 days showed only mild glomerular effects and vacuolation of tubular epithelium.

Blocking TGF-βR1 prevented cisplatin-induced increases in renal oxidative stress

Rats receiving cisplatin for 7 days or 10 days showed 2.2- and 2.95-fold increases in renal MDA levels as compared to the untreated control group. SB-431542 treatment showed dose-dependent, lower renal MDA levels as compared to the cisplatin-only group (*figure 5A*). In parallel, cisplatin resulted in 23% and 44% reductions in renal SOD activity after 7 and 10 days, respectively. SB-431542-(1 and 3 mg/kg) treated rats showed significantly higher

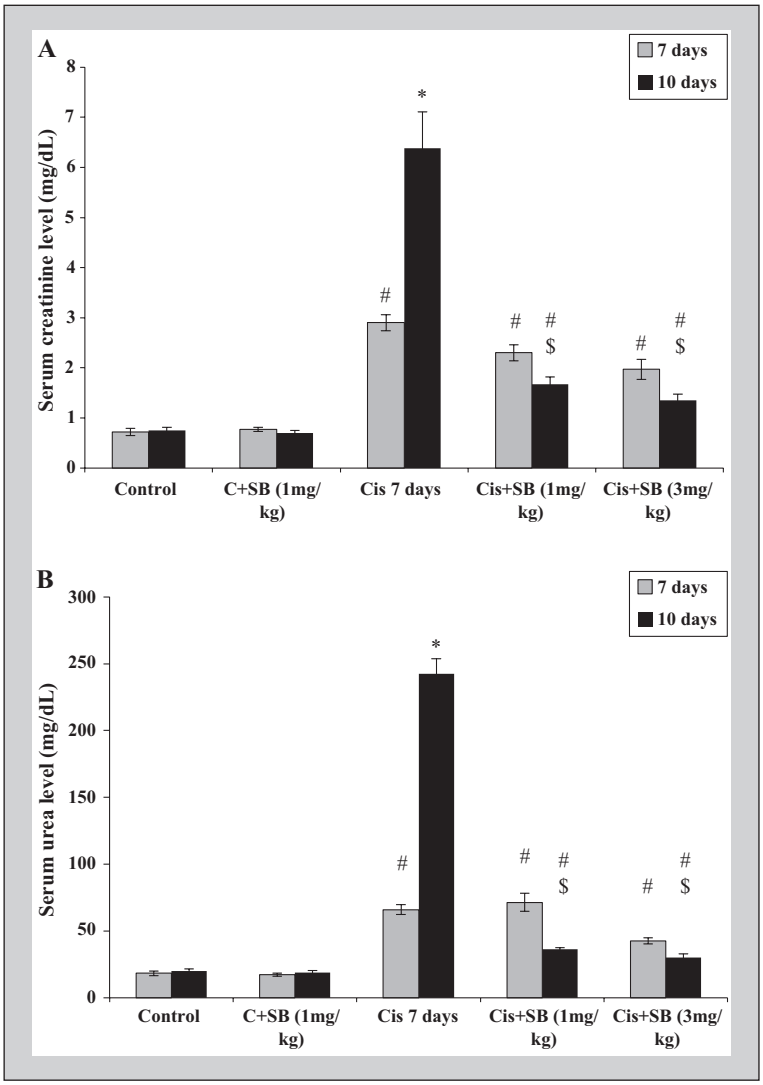

Figure 3

Effect of cisplatin (Cis, 10 mg/kg) alone, and in combination with SB-431542 (SB, 1 and 3 mg/kg), for 7 and 10 days, on serum creatinine (A) and urea (B) levels. * Significant difference as compared with the rest of the groups at $p<0.05$. # Significant difference as compared with the control groups at $p<0.05$. $ Significant difference as compared with the corresponding group after 7 days at $p<0.05$.

levels of renal SOD activity as compared to the cisplatin-only group, but did not affect the untreated control group (*figure 5B*).

Blocking TGF-βR1 attenuated cisplatin-induced increases in inflammatory markers

Cisplatin resulted in 1.7- and 2.6-fold increases in renal sCD93 levels after 7 and 10 days, respectively. Treatment with SB-431542 (1 and 3 mg/kg) attenuated possible increases in renal sCD93 as compared to the cisplatin-only group, but did not affect the untreated control group (*figure 6A*).

Blocking TGF-βR1 attenuated cisplatin-induced increases in fibrosis markers

Cisplatin increased renal TGF-β levels 1.6- and 2.1-fold, after 7 and 10 days, respectively. Treatment with SB-431542 (1 and 3 mg/kg) markedly attenuated any increases in renal TGF-β level after 7 and 10 days in the cisplatin-only group, but did not affect the untreated control group (*figure 6B*).

Blocking TGF-βR1 attenuated cisplatin-induced increases in caspase-3

Cisplatin increased renal caspase-3 levels 1.8- and 2-fold, after 7 and 10 days, respectively. Treatment with SB-431542 caused significant, dose-dependent attenuation of increases in caspase-3 levels after 7 and 10 days in the cisplatin-only group, but did not affect the untreated control group (*figure 7*).

DISCUSSION

Cisplatin is a potent and valuable chemotherapy agent used to treat a broad spectrum of malignancies. Nephrotoxicity is a major side effect that limits the use of cisplatin in many cancer patients [5]. Multiple mechanisms contribute to renal dysfunction following exposure to cisplatin, including tubular epithelial cell toxicity, vasoconstriction in the renal microvasculature, and inflammatory effects [22]. Cisplatin primarily injures the S3 segment of the proximal tubule, causing a decrease in the glomerular filtration rate [23]. Previously, cisplatin nephrotoxicity was

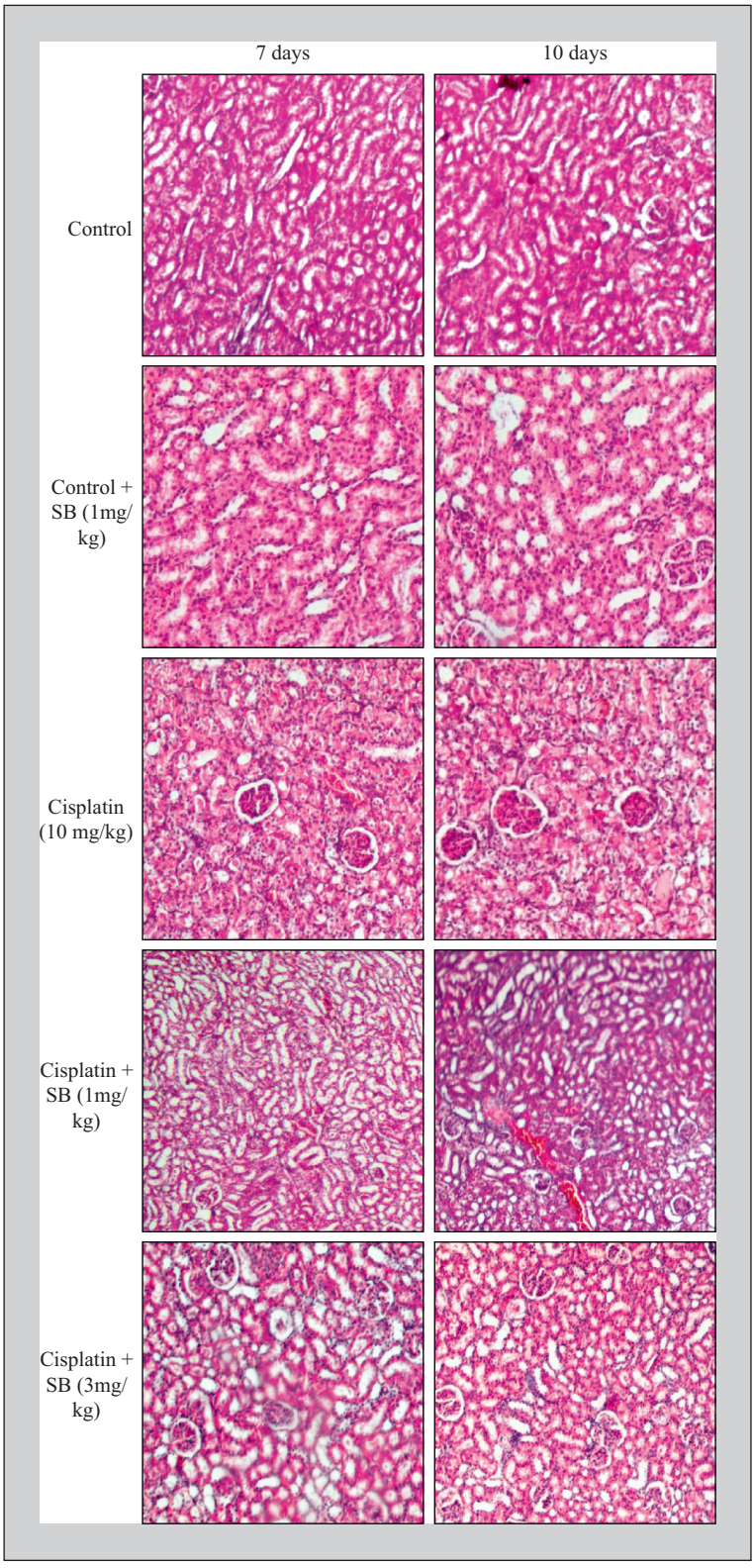

Figure 4
Effect of cisplatin (Cis, 10 mg/kg) alone and in combination with SB-431542 (SB, 1 and 3 mg/kg), for 7 and 10 days, on kidney sections stained with hematoxylin/eosin (H/E) and examined under a microscope (100x magnification).

found to increase TGF-β1 levels [24] in kidney, liver and heart. We therefore examined the effect of concomitant treatment with SB-431542. Treatment of a cisplatin group with SB-431542 completely blocked the any increase in TGF-βR1. Of note, the TGF-βR1 levels decrease caused by SB-431542 is accompanied by protection from cisplatin nephrotoxicity as indicated by the less elevated serum urea and creatinine levels and the nearly normal appearance of renal tissues stained with H&E. SB-431542 was reported to ameliorate tubulointerstitial nephropathy induced by aristolochic acid I (AAI) [25]. However, no previous studies have reported the role of SB-431542 in the treatment of cisplatin-induced nephrotoxicity.

The marked cisplatin-induced renal damage was found to be characterized by a significant increase in oxidative stress, leading ultimately to renal cell death and irreversible

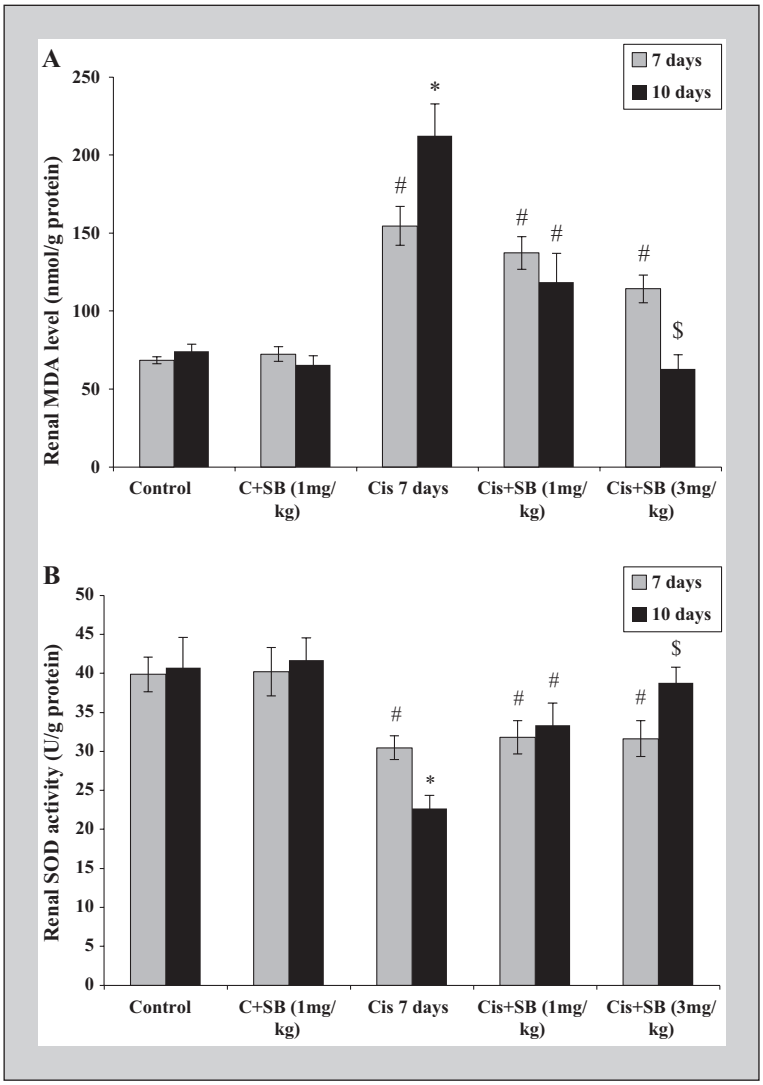

Figure 5

Effect of cisplatin (Cis, 10 mg/kg) alone, and in combination with SB-431542 (SB, 1 and 3 mg/kg), for 7 and 10 days, on renal malondialdehyde (MDA, A) and superoxide anion (SOD, B) levels. * Significant difference as compared with the rest of the groups at $p<0.05$. # Significant difference as compared with the control groups at $p<0.05$. $ Significant difference as compared with the corresponding group after 7 days at $p<0.05$.

kidney damage [26]. Excessive generation of reactive oxygen species such as malondialdehyde (MDA) is one of the mechanisms implicated in the pathogenesis of progressive renal injury. The increase in renal MDA was associated with the development of necrosis in proximal straight tubules [27]. In addition, the superoxide anion was found to attack cellular components causing damage to lipids, proteins and DNA [28]. The antioxidant effect of SB-431542 was previously reported in diabetic nephropathy as restoring the expression levels of manganese superoxide dismutase (MnSOD) and decreasing MDA levels in diabetic renal mice [29]. The present investigation revealed similar effect of SB-431542 in renal tissues of cisplatin-treated rats.

The existence of a cell-surface receptor for the Clq complement component or sCD93 was first suggested by Dickler and Kunkel [30]. Clq receptor (ClqR) activity has been reported on most leucocytes, endothelial cells, fibroblasts and platelets [31], and binding of CIq to its receptor has been reported to mediate a range of phenomena, including phagocytosis, modulation of cytokine and immuno-globulin secretion, and polymorphonuclear leucocyte-endothelium interaction [32]. However, we found that the significant increases in renal sCD93 seen after cisplatin treatment were blocked by treatment with SB-431542.

The transforming growth factor-β (TGF-β)1 superfamily is a group of multifunctional cytokines that play an important role in regulating cell growth and differentiation, cell death (apoptosis), and morphogenesis in a variety of biological systems [33, 34]. TGF-β1 activity is mediated via a heteromeric complex of TGF-β type II (TGF-βR2) and type I (TGF-βR1) serine/threonine kinase receptors [35]. We found significant increases in renal TGF-β1 levels in rats treated with cisplatin. This is in agreement with many previous studies [7-9]. However, treatment with SB-431542 significantly reduced TGF-β1 levels. SB-431542 was reported to reduce renal tubulointerstitial fibrosis induced by AAI through its epithelial to mesenchymal transition [25].

Caspases are a family of cell-death proteases that play an essential role in the execution phase of apoptosis [36]. The executioner caspase-3 has been shown to be activated by cisplatin treatment in renal proximal tubular cells [37].

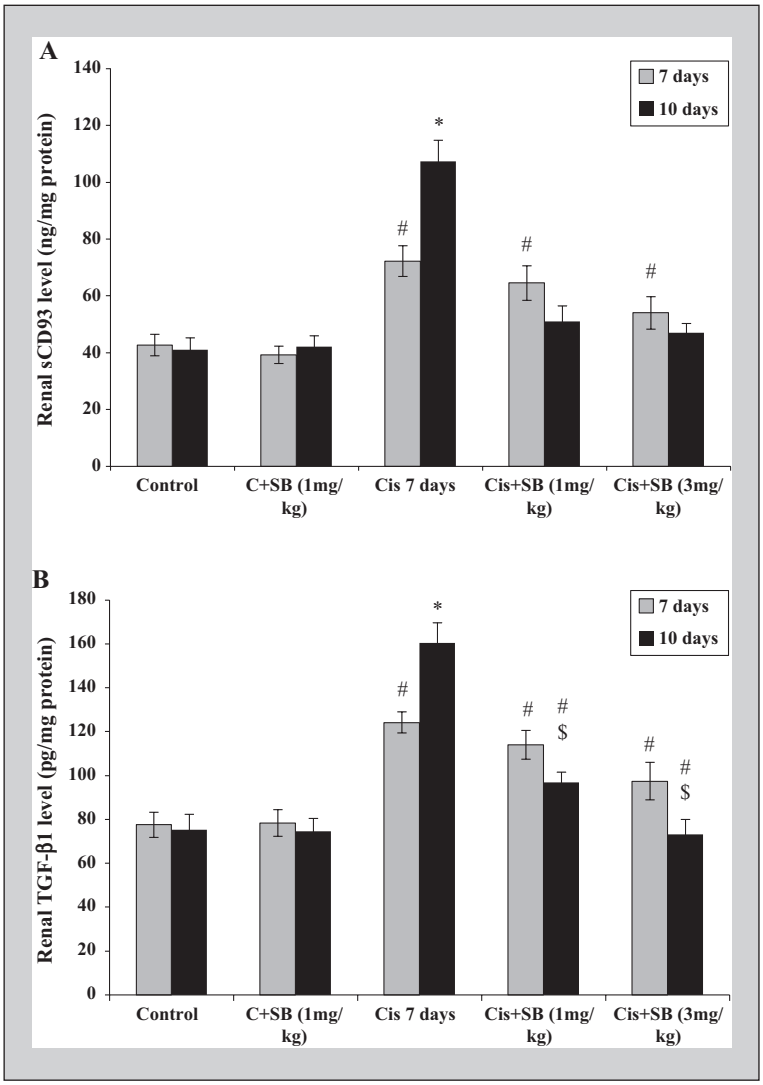

Figure 6

Effect of cisplatin (Cis, 10 mg/kg) alone, and in combination with SB-431542 (SB, 1 and 3 mg/kg), for 7 and 10 days, on renal cSD93 (A) and transforming growth factor-β1 (TGF-β1, B) levels. * Significant difference as compared with the rest of the groups at $p<0.05$. # Significant difference as compared with the control groups at $p<0.05$. $ Significant difference as compared with the corresponding group after 7 days at $p<0.05$.

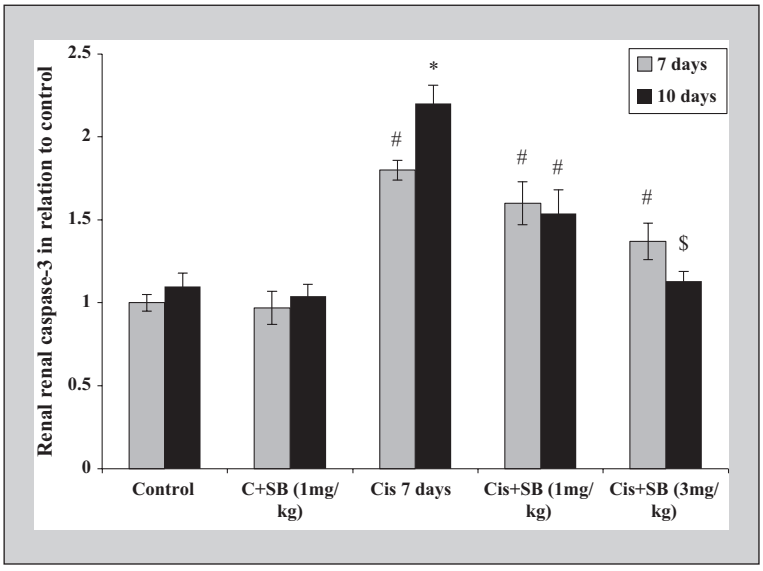

Figure 7

Effect of cisplatin (Cis, 10 mg/kg) alone, and in combination with SB-431542 (SB, 1 and 3 mg/kg), for 7 and 10 days, on renal caspase-3. * Significant difference as compared with the rest of the groups at $p<0.05$. # Significant difference as compared with the control groups at $p<0.05$. $ Significant difference as compared with the corresponding group after 7 days at $p<0.05$.

Because of the crucial role of caspases in the apoptotic pathway, abnormalities in their functions would wreak havoc in the apoptotic cascade, which can be deleterious to the cell. Of note, we found that cisplatin caused an increase in caspase-3 levels. However, SB-431542 was found to attenuate the elevated caspase-3 levels. Indeed, SB-431542 has been previously found to inhibit TGF-β-induced apoptosis in different types of cancer cells [38, 39].

CONCLUSIONS

The main findings of the current study are that blocking TGF-βR1 results in dose-dependent protection from the impairment of renal tissues function and structure in rats treated with cisplatin via multiple mechanisms including: (1) reduction of cisplatin-induced oxidative stress, as indicated by lowering of renal MDA levels, and restoration of renal SOD activity; (2) blocking of cisplatin-induced increases in renal inflammatory cytokines such as sCD93; (3) reduction of cisplatin-induced increases in renal fibrosis markers such as TGF-1β; and (4) inhibition of cisplatin-induced activation of renal caspase-3.

Disclosure. *Financial support: none. Conflict of interest: none.*

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