The Water-Soluble Triptolide Derivative PG490-88 Protects against Cisplatin-Induced Acute Kidney Injury

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ABSTRACT

Triptolide, a traditional Chinese medicine, has anti-inflammatory, antiproliferative, and proapoptotic properties. As interstitial inflammation and tubular apoptosis are features of cisplatin-induced acute kidney injury (AKI), we determined the effect of the water-soluble triptolide derivative 14-succinyl triptolide sodium salt (PG490-88) in a mouse model of cisplatin-induced AKI. PG490-88 resulted in a significant decrease in blood urea nitrogen (BUN), serum creatinine, and acute tubular necrosis (ATN) score, and a nonsignificant increase in tubular apoptosis score in AKI. The mitogen-activated protein kinase (MAPK) pathway is activated in AKI. On immunoblot analysis, phosphoextracellular signal-regulated kinase (p-ERK) was increased 3.6-fold in AKI and 2.0-fold inhibited by PG490-88. Phospho-c-Jun N-terminal kinase (p-JNK) was increased in AKI. PG490-88 resulted in a nonsignificant decrease in p-JNK. Phospho-p38 was not affected by cisplatin or PG490-88. MAPK phosphatase-1 (MKP-1) that negatively regulates MAPK signaling has not previously been studied in AKI. MKP-1 activity was not affected by cisplatin or PG490-88. Changes in p-ERK, p-JNK, and MKP-1 were confirmed on reverse protein phase analysis. The ERK inhibitor U0126 resulted in lower BUN and serum creatinine, suggesting a mechanistic role of ERK in AKI. The increase in interleukin-1α (IL-1α), IL-1β, IL-6, CXCL1, and IL-33 in the kidney in AKI was unaffected by PG490-88. In summary, PG490-88 protects against AKI and ATN despite no decrease in tubular apoptosis. The protection of PG490-88 against AKI was associated with a decrease in p-ERK and was independent of MKP-1 and proinflammatory cytokines. In conclusion, PG490-88 protects against cisplatin-induced AKI possibly by decreasing p-ERK.

Introduction

Cisplatin and other platinum derivatives are the most widely used chemotherapeutic agents to treat solid tumors. A known complication of cisplatin administration is acute kidney injury (AKI). The nephrotoxic effect of cisplatin is cumulative and dose-dependent and often necessitates dose reduction or withdrawal. Despite this toxicity, cisplatin remains one of the most commonly used chemotherapy drugs because of its therapeutic efficacy. Therefore, an understanding of the pathogenesis of cisplatin-induced AKI is important for the development of adjunctive therapies to prevent AKI, to lessen the need for dose decrease or drug withdrawal, and to lessen patient mortality and morbidity.

Triptolide, a dierpene triepoxide, is the major active component extracted from the medicinal plant Tripteris vilfordii Hook F (Liu, 2011). Triptolide has multiple pharmacological actions, including anti-inflammatory, immune modulatory, antiapoptotic, and antiangiogenic effects. The new water-soluble triptolide derivative 14-succinyl triptolide sodium salt (PG490-88) has the same effects as triptolide and has been approved to enter phase I clinical trials in prostate cancer in the United States (Liu, 2011). Triptolide or PG490-88 reduces disease progression in various animal models of kidney disease. For example, PG490-88 prolongs renal allograft survival in monkeys (Chen et al., 2006). Triptolide reduces cyst growth in autosomal dominant polycystic kidney disease (ADPKD) (Leuenroth et al., 2007), decreases experimental membranous nephropathy (Chen et al., 2010) and podocyte injury (Zheng et al., 2008), and attenuates diabetic nephropathy in rodents (Gao et al., 2010). Triptolide decreases interstitial fibrosis in a model of unilateral kidney

ABBREVIATIONS: ADPKD, autosomal dominant polycystic kidney disease; AKI, acute kidney injury; ATN, acute tubular necrosis; BUN, blood urea nitrogen; CXCL1, chemokine (C-X-C motif) ligand 1; DUSP, dual-specificity protein phosphatase; ERK, extracellular signal-regulated kinase; HPF, high-power field; IL, interleukin; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; MEK, mitogen-activated protein kinase kinase; MEK1, MEK kinase 1; MKP-1, MAPK phosphatase-1; PAS, periodic acid–Schiff; pBAD, Bcl-2-associated death promoter; p-ERK, phosphoextracellular signal-regulated kinase; PG490-88, 14-succinyl triptolide sodium salt; p-JNK, phospho-c-Jun N-terminal kinase; RPPA, reverse-phase protein array; SAPK, stress-activated protein kinase; SKF-86002, 6-(4-fluorophenyl)-2,3-dihydro-5-(4-pyridinyl)limidazo[2,1-b]thiazole dihydrochloride; TBST, Tris-buffered saline/Tween 20; TNF-α, tumor necrosis factor α; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminoophenyl)butadiene; XIAP, X-linked inhibitor of apoptosis protein.
obstruction (Yuan et al., 2011). The effect of triptolide on AKI is not known.

Interstitial inflammation in the kidney is a feature of cisplatin-induced AKI. Increases in proinflammatory cytokines such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), and IL-8 in the kidney have been demonstrated in rodent models of cisplatin-induced AKI (Ramesh and Reeves, 2002; Faubel et al., 2007; Akcay et al., 2011). Inhibition of TNF-α or IL-8, but not IL-1β or IL-6, is protective against cisplatin-induced AKI, suggesting that TNF-α and IL-8 are mediators of cisplatin-induced AKI (Ramesh and Reeves, 2002; Faubel et al., 2007; Akcay et al., 2011). Triptolide has anti-inflammatory effects (Liu, 2011). Specifically, triptolide inhibits IL-8 expression by bronchial epithelial cells and inhibits TNF-α and chemokines such as monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein (MIP)-1α and β in peripheral blood mononuclear cells (Liu, 2011). As a robust inflammatory response in the kidney, with increases in cytokines and chemokines, has been described with cisplatin (Faubel et al., 2007), and because triptolide can inhibit activation of proinflammatory cytokines and chemokines, we developed the hypothesis that injection of PG490-88, a water-soluble triptolide derivative, in mice would result in protection against cisplatin-induced AKI.

The rationale for studying mitogen-activated protein kinase (MAPK) signaling is that MAPK signaling is activated in cisplatin-induced AKI and is also known to be inhibited by triptolide. Cisplatin causes activation of p38 MAPK in proximal tubules both in vitro and in vivo, and the p38 MAPK inhibitor SKF-86002 [6-(4-fluorophenyl)-2,3-dihydro-5-(4-pyridinyl)imidazo[2,1-b]thiazole dihydrochloride] reduces cisplatin-induced AKI (Ramesh and Reeves, 2005). The mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor U0126 attenuates cisplatin-induced AKI by decreasing inflammation and apoptosis (Jo et al., 2005). Activation of p38, ERK, and c-Jun N-terminal kinase (JNK) occurs in mouse proximal tubules exposed to cisplatin (Arany et al., 2004), and activation of ERK and JNK occurs in cisplatin-induced AKI (Kim et al., 2012). Triptolide can activate ERK1/2, p38, and JNK1/2, resulting in antiproliferative and proapoptotic activities (Liu, 2011). However, triptolide can also decrease MAPK signaling. Triptolide inactivated ERK and p38, but not JNK, in human leukemia cells (Wan et al., 2006). Triptolide suppressed IL-1β-induced activation of ERK, p38, and JNK in an arthritic model (Kong et al., 2013). Triptolide inhibited activation of JNK and p38 in the liver (Lu et al., 2012). Thus, as activation of p38, ERK1/2, and JNK has been described in cisplatin-induced AKI, and triptolide is known to activate or inhibit p38, phospho-ERK (p-ERK) and phospho-JNK (p-JNK) signaling, depending on the cell type, the effect of triptolide on p38, ERK1/2, and JNK1/2 in cisplatin-induced AKI was determined.

Materials and Methods

Cisplatin-Induced AKI. For all mouse studies, 8–10-week-old male C57BL/6 mice weighing 20–25 g were used. All experiments were conducted with adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado at Denver. Mice were maintained on a standard diet, and water was freely available. Mice were housed five per cage under a 12-hour light and dark schedule for at least 1 week prior to cisplatin administration. Six hours before cisplatin administration, food and water were withheld. Cisplatin [cis-diaminedichloro-platinum (II)] (Sigma-Aldrich, Milwaukee, WI) was freshly prepared the day of administration in sterile normal saline at a concentration of 1 mg/ml. Mice were given 25 mg/kg i.p. body weight of cisplatin or vehicle (saline), after which the mice again had free access to food and water. We have described this model of cisplatin-induced AKI in detail elsewhere (Faubel et al., 2004; Lu et al., 2008). In brief, after 25 mg/kg cisplatin injection, blood urea nitrogen (BUN) and serum creatinine are normal on day 1 and slightly increased on day 2. On day 3 after cisplatin injection, renal dysfunction, renal tubular cell apoptosis, and acute tubular necrosis scores are severe.

Serum Creatinine and BUN Measurement. Serum creatinine and BUN were measured using a VetAce autoanalyzer (Alfa Wassermann, West Caldwell, NJ).

Histologic Examination. Paraformaldehyde (4%)-fixed and paraffin-embedded kidneys were sectioned at 4 μm and stained with periodic acid–Schiff (PAS) by standard methods. All histologic examinations were performed by the renal pathologist in a blinded fashion. Histologic changes attributed to acute tubular necrosis (ATN) score were evaluated in the outer stripe of the outer medulla on PAS-stained tissue and were quantified by counting the percentage of tubules that displayed cell necrosis, loss of brush border, cast formation, and tubule dilatation as follows: 0 = none, 1 = <10%, 2 = 10–25%, 3 = 26–45%, 4 = 46–75%, and 5 = >75%. At least 10 fields (×250) were reviewed for each slide.

Morphologic criteria were used to count apoptotic cells on PAS-stained tissue by the pathologist experienced in the evaluation of renal apoptosis. Morphologic characteristics included cellular rounding and shrinkage, nuclear chromatin compaction, and formation of apoptotic bodies (Gobe et al., 2000). Apoptotic tubular cells were quantitatively assessed per 10 HPF (×400) in the outer stripe of the outer medulla by the renal pathologist in a blinded fashion.

PG490-88. PG490-88 is a water-soluble derivative prodrug of PG490 (triptolide). PG490-88 was obtained from Myelox LLC (Vallejo, CA). PG490-88 (0.5 mg/kg) or vehicle (normal saline) was injected intraperitoneally 6 hours before injection of cisplatin.

Triptolide at a dose of 1 mg/kg has been reported to cause direct nephrotoxicity in rats (Yang et al., 2012). The LD₅₀ (95% confidence limits) for triptolide administered intravenously in mice is 0.83 mg/kg (0.72–0.97 mg/kg) (Xu et al., 2013). PG490-88 at a dose of 0.5 mg/kg causes tumor regression in mice (Fidler et al., 2003). Thus, we chose a maximum dose of PG490-88 of 0.5 mg/kg, which is lower than the LD₅₀ of triptolide in mice and is effective in causing tumor regression.

U0126. One hour before cisplatin administration, mice were injected with U0126 (G.A. Scientific, Inc., San Diego, CA) (10 mg/kg in 2% dimethysulfoxide) or the vehicle 2% dimethylsulfoxide IV in the tail vein as previously described (Jo et al., 2005). Mice were sacrificed on day 3 after cisplatin administration.

Enzyme-Linked Immunosorbent Assay. Mouse IL-1α, IL-1β, CXCL1 (also known as IL-8 or KC), IL-6, IL-33, and TNF-α immunoassay kits were obtained from R&D Systems (Minneapolis, MN). Enzyme-linked immunosorbent assay was performed according to the manufacturer’s instructions.

Immunoblotting. Whole-kidney extracts were immunoblotted as we have previously described in detail (Dursun et al., 2006). Immunoblot analyses were performed with the following antibodies: 1) a phospho-p38 MAPK (Thr180/Tyr182) polyclonal antibody that detects endogenous levels of p38 MAPK only when activated by phosphorylation at threonine 180 and tyrosine 182 (Cell Signaling, Boston, MA), and a p38 MAPK polyclonal antibody that detects endogenous levels of total p38α, β, or γ MAPK protein (Cell Signaling); 2) a phospho-stress-activated protein kinase (SAPK)JNK (Thr183/Tyr185) (SIE11) rabbit monoclonal antibody (mAb) that detects endogenous levels of p46 and p54 SAPK/JNK only when phosphorylated at Thr183 and Tyr185 (Cell Signaling), and a SAPK/JNK polyclonal antibody that detects endogenous levels of total SAPK/JNK protein (Cell Signaling); 3) a phospho-p44/42 MAPK (ERK1/2) (Th202/Tyr204) (197G2) rabbit mAb that detects endogenous levels

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of p44 and p42 MAPK (ERK1 and ERK2) when dually phosphorylated at Thr202 and Tyr204 of ERK1 (Thr185 and Tyr187 of ERK2) and singly phosphorylated at Tyr204 (Cell Signaling), and a polyclonal rabbit antibody that detects total ERK1 p44 and, to a lesser extent, ERK2 p42 (sc-93; Santa Cruz Biotechnology, Dallas, TX); 5) cleaved caspase-3 and X-linked inhibitor of apoptosis protein (XIAP) antibodies (Cell Signaling Technology); and a Bel-2-associated death promoter (pBAD) rabbit mAb that detects endogenous levels of Bad when phosphorylated at Ser136; Cell Signaling Technology); and 6) a β-actin rabbit mAb that detects endogenous levels of total β-actin protein (Cell Signaling).

Reverse-Phase Protein Array. Whole kidney was homogenized in radioimmunoprecipitation assay buffer plus protease inhibitors as described for immunoblotting. The same samples used for immunoblotting were used for reverse-phase protein array (RPPA). For each sample, whole lysates neat plus four serial dilutions (dilution factor 0.8) and one buffer control were pipetted into V-shaped ABgene 384-well plates (Thermo Fisher Scientific, Rockford, IL). Samples were printed in triplicate onto nitrocellulose-coated glass slides (Supernova slides; Grace Bio-Laboratories, Inc., Bend, OR) using an Aushon BioSystems 2470 Arrayer (Aushon BioSystems, Billerica, MA) with 185-mm pins and a single touch. Slides were stored at 4°C until use. Antibody screening was carried out as previously described (Ahmed et al., 2013). In brief, slides were incubated in blocking solution (3% bovine serum albumin; Sigma-Aldrich Co., St. Louis, MO) in Tris-buffered saline/0.1% Tween 20 (TBST) for 4 hours, followed by overnight incubation at 4°C with primary antibody. Slides were washed three times for 5 minutes in TBST, followed by incubation with Fluorescence Alexa Fluor 555 goat antirabbit (Invitrogen, Camarillo, CA) secondary antibody (1:2000) for 90 minutes at room temperature. Slides were washed three times for 5 minutes with TBST and dried for 20 minutes at 30°C. All steps during and after the incubation with secondary antibody were performed in the dark. Antibody-stained slides were scanned using a ScanArray ExpressHT Microarray Scanner (PerkinElmer Life and Analytical Sciences, Boston, MA). The intensity of each spot was quantified using the ScanArray Express software (PerkinElmer Life and Analytical Sciences). Antibody signal for each spot was normalized to the corresponding signals from staining a different slide with the general protein stain SyproRuby, following the manufacturer’s instructions (Invitrogen). Details of quantitation and review of data quality and reproducibility were as previously described (Ahmed et al., 2013).

MAPK Phosphatase-1 Assay. MAPK phosphatase-1 (MKP-1) was measured using a protein phosphatase DUSP1/MKP-1 fluorometric assay kit from CycLex Co., Ltd. (Nagano, Japan) and obtained from MBL International (Woburn, MA). The kit measures fluorescence of a substrate OMFP (3'-O-methylfluorescein phosphate). Vehicle control, phosphatase inhibitor control, and no enzyme controls were performed for each experiment per the manufacturer’s instructions. Equal amounts of protein were loaded in the assay for each experiment.

Statistical Analysis. Non-normally distributed data were analyzed by the nonparametric unpaired Mann–Whitney U test. Multiple group comparisons are performed using analysis of variance with post-test according to Newman-Keuls. A P value < 0.05 was considered statistically significant. Values are expressed as means ± S.E.

Results

PG490-88 Decreases BUN, Serum Creatinine, and ATN Score but Not Apoptosis Score. PG490-88 doses of 0.1 and 0.5 mg/kg i.p. were used. PG490-88 (0.1 mg/kg) did not functionally protect against cisplatin-induced AKI. Serum creatinine was 0.2 ± 0.02 in vehicle-treated mice, 1.76 ± 0.2 in cisplatin-induced AKI, and 1.66 ± 0.3 in cisplatin-induced AKI plus 0.1 mg/kg PG490-88 (n = 5). BUN, serum creatinine, and ATN scores were significantly decreased by PG490-88 (0.5 mg/kg) compared with vehicle (Fig. 1). The apoptosis score in cisplatin-induced AKI was increased by PG490-88, but the increase did not reach statistical significance (Fig. 1). PG490-88 had no effect on BUN, serum creatinine, ATN score, or apoptosis scores in mice treated with vehicle instead of cisplatin (Fig. 1). Representative pictures of kidney histology are shown in Fig. 2 (A–C).

To determine whether PG490-88 improves survival, mice were injected with PG490-88 (0.5 mg/kg i.p.) (n = 8) or vehicle (n = 8) 6 hours before cisplatin (25 mg/kg) injection. The number of mice that survived each day after cisplatin injection was as follows: 5/8 in the PG490-88 and vehicle groups on day 4, 5/8 in the PG490-88 group and 3/8 in the vehicle group on day 5, 2/8 in the PG490-88 and vehicle groups on day 6, and 0/8 in the PG490-88 and vehicle groups on day 7. The model of cisplatin-induced AKI is severe and irreversible, and on days 4–7, the mice become systemically ill and require sacrifice. The exact cause of death is unknown, but cisplatin is known to cause liver, gastrointestinal, myocardial, and lung injury (Sartori et al., 1991; Sheikh-Hamad et al., 1997; Wang et al., 2009; Leo et al., 2010). Thus, it is likely that the mice died of other organ toxicity in addition to AKI. Thus, although PG490-88 protected against cisplatin-induced AKI on day 3, it did not protect against the systemic toxicity of cisplatin.

PG490-88 Decreases p-ERK but Not p-JNK and p-p38 MAPK. There was an increase in p-ERK in kidney extracts from cisplatin-induced AKI that was significantly decreased by PG490-88 (Fig. 3). p-JNK was increased in cisplatin-induced AKI. PG490-88 reduced p-JNK, but the decrease was not statistically significant (Fig. 3). p-p38 MAPK was not increased in cisplatin-induced AKI and was unaffected by PG490-88 (Fig. 3).

MKP-1 Is Not Affected by Cisplatin or PG490-88. There was no change in MKP-1 activity in cisplatin-induced AKI compared with vehicle-treated controls. PG490-88 had no significant effect on MKP-1 activity (Fig. 4).

Reverse-Phase Protein Array. To determine the effect of cisplatin and PG490-88 on p-ERK and p-JNK proteins using another method, RPPA was performed on kidney extracts from days 1 and 3 after cisplatin administration. On day 1 after cisplatin administration, there were no significant changes in p-ERK and p-JNK (Fig. 5A). On day 3 after cisplatin administration, there was an increase in p-JNK that was not affected by PG490-88 and an increase in p-ERK in cisplatin-induced AKI that was decreased by PG490-88 (Fig. 5B), confirming the changes in p-ERK and p-JNK seen on immunoblot (Fig. 3). RPPA analysis confirmed that there was no change in MKP-1 (Fig. 5, A and B). In addition, RPPA analysis showed a significant increase in MEKK1 that was decreased by PG490-88.

Increase in IL-1α, IL-1β, IL-6, CXCL1, and IL-33 in the Kidney in Cisplatin-Induced AKI Is Unaffected by PG490-88. To determine the possible mechanism for the protection against cisplatin-induced AKI by PG490-88, the proinflammatory cytokines IL-1α, IL-1β, IL-6, CXCL1, IL-33, and TNF-α were studied. IL-1α, IL-1β, IL-6, CXCL1, and IL-33 were increased in cisplatin-induced AKI, but none of the cytokines were significantly decreased by PG490-88 (Table 1).

Cleaved Caspase-3, pBAD, and XIAP. Cisplatin is known to increase caspase-3, the major mediator of apoptosis, and pBAD, a proapoptotic protein (Kaushal et al., 2001). XIAP decreases caspase-3–mediated apoptosis. There was an increase in cleaved caspase-3 and pBAD but not XIAP in the kidney in cisplatin-induced AKI. PG490-88 had no effect on cleaved caspase-3, pBAD, or XIAP (Fig. 6).
U0126 Decreases BUN and Serum Creatinine. To determine whether p-ERK is a mediator of cisplatin-induced AKI, mice with cisplatin-induced AKI were treated with the ERK inhibitor U0126. U0126 functionally protected against cisplatin-induced AKI. Serum creatinine was $0.28 \pm 0.02$ in vehicle-treated mice, $0.47 \pm 0.04$ in cisplatin-induced AKI ($P < 0.05$ vs. vehicle), and $0.26 \pm 0.06$ in cisplatin-induced AKI plus U0126 10 mg/kg i.v. ($P < 0.05$ vs. cisplatin) ($n = 5–8$ per group) (Fig. 7). BUN was $28 \pm 1$ in vehicle-treated mice, $81 \pm 5$ in cisplatin-induced AKI ($P < 0.001$ vs. vehicle), and $61 \pm 5$ in cisplatin-induced AKI plus U0126 10 mg/kg i.v. ($P < 0.01$ vs. cisplatin) ($n = 5–8$ per group) (Fig. 7). In these experiments, a lower dose of cisplatin (20 mg/kg) was used compared with 25 mg/kg in the PG490-88 experiments (Fig. 1).

Discussion

The anti-inflammatory and chemotherapeutic effect of triptolide has been demonstrated in numerous cell models. Triptolide blocks lipopolysaccharide-stimulated increases in IL-1β, IL-6, and TNFα, which are known to be increased in cisplatin-induced AKI (Faubel et al., 2007). Triptolide is an efficacious therapy in animal models of autoimmune diseases such as rheumatoid arthritis (Liu, 2011). In preclinical studies in cancer, triptolide directly induces tumor cell apoptosis and enhances apoptosis induced by chemotherapeutic agents (Liu, 2011). Triptolide is effective in preclinical models of kidney disease, but has not previously been studied in AKI. PG490-88 prevents acute and chronic rejection in rat renal allografts (Pan et al., 2005) and has a synergistic effect with tacrolimus to prolong renal allograft survival in monkeys by inhibition of T-cell activation and interferon-γ (Chen et al., 2006). Triptolide reduces cyst growth in ADPKD (Leuenroth et al., 2007) by an antiproliferative effect and restoring calcium-mediated signaling in tubular cells. Triptolide has entered human studies in patients with ADPKD (see clinicaltrials.gov). Triptolide attenuates renal interstitial fibrosis in rats with unilateral ureteral obstruction (Yuan et al., 2011). Triptolide can dramatically attenuate albuminuria and renal lesion accompanied with dyslipidemia and obesity in db/db diabetic mice (Gao et al., 2010), and in experimental membranous nephropathy, triptolide decreases reactive oxygen species and p38 MAPK (Chen et al., 2010). Triptolide protects podocytes from puromycin aminonucleoside–induced injury in vivo and in vitro (Zheng et al., 2011).
et al., 2008). Triptolide protects against ischemia/reperfusion injury in the liver and lung (He et al., 2007; Wu et al., 2011).

As triptolide or PG490-88 was shown to affect cytokines and chemokines, apoptosis, proliferation, and p38 in models of kidney disease, and these molecules are known to be affected by cisplatin, the therapeutic effect of PG490-88 cisplatin in AKI was determined.

First, the effect of PG490-88 on apoptosis and necrosis of proximal tubular cells was determined. In the mouse model of cisplatin-induced AKI, there is a surge of apoptosis on day 2 and a surge of proximal tubule ATN on day 3 after cisplatin administration (Faubel et al., 2004). The relative contribution of the apoptosis versus necrosis of the proximal tubules to the functional AKI is not known. Protection against cisplatin-induced AKI by IL-33 or IL-8 inhibition was associated with a decrease in both tubular cell apoptosis and necrosis (Akcay et al., 2011). Thus, we considered the situation that the potent anti-inflammatory effect of PG490-88 may protect against cisplatin-induced AKI, but that the direct proapoptotic effect of PG490-88 may worsen the cisplatin-induced AKI. In the present study, PG490-88 resulted in a significant decrease in the ATN score and a nonsignificant increase in the tubular apoptosis score. The decrease in ATN but the lack of a decrease in apoptosis was associated with significant functional protection against cisplatin-induced AKI as measured by BUN and serum creatinine. These data suggest that the ATN that occurs in up to 75% of the proximal tubules in the present study may be more important in determining the functional derangement in cisplatin-induced AKI than the apoptosis that occurs in about 1–2 tubular cells per HPF as seen in the present study. In support of this concept, direct caspase-3 or apoptosis inhibition has not been reported to protect against....

**Fig. 3.** PG490-88 decreases p-ERK but not p-JNK or p-p38 MAPK. There was an increase in p-ERK in kidney extracts from cisplatin-induced AKI that was significantly decreased by PG490-88. p-JNK was increased in cisplatin-induced AKI. PG490-88 reduced p-JNK, but the decrease was not statistically significant. p-p38 MAPK was not increased in cisplatin-induced AKI and unaffected by PG490-88. In densitometric analysis of immunoblots, data are presented as phospho/total protein ratios plotted on the y-axis. Representative immunoblots of at least three separate experiments. *P < 0.01 versus vehicle (Veh) and PG490-88 (PG); **P < 0.05 versus cisplatin (Cis); ***P < 0.01 versus Veh, PG, and Cis + PG. NS, not significant.

**Fig. 4.** MKP-1 is not affected by cisplatin or PG490-88 (PG). There was no change in MKP-1 activity in cisplatin-induced AKI (Cis) versus vehicle-treated controls. PG490-88 had no significant effect on MKP-1 activity. N = 5–9 per group. Veh, vehicle.

**Fig. 5.** RPPA analysis. On day 1 after cisplatin administration, there were no significant changes in p-ERK or p-JNK (A). On day 3 after cisplatin administration, there was an increase in p-JNK that was not affected by PG490-88, and an increase in p-ERK in cisplatin-induced AKI that was decreased by PG490-88 (B), confirming the changes in p-ERK and p-JNK seen on immunoblot (Fig. 3). RPPA analysis confirmed that there was no change in MKP-1 (A and B). In addition, RPPA analysis showed a significant increase in MEKK1 that was decreased by PG490-88. *P < 0.05 versus vehicle (Veh) or PG490-88 alone (PG); **P < 0.05 versus cisplatin (Cis).
cisplatin-induced AKI in vivo. In fact, injection of a caspase inhibitor in mice worsens cisplatin-induced AKI (Herzog et al., 2012). In another study, decreases in renal apoptosis alone were not associated with functional protection against cisplatin-induced AKI as measured by BUN (Sridevi et al., 2013). The present study highlights the evidence that functional protection against cisplatin-induced AKI may not always be associated with a decrease in tubular apoptosis.

Next, the effect of PG490-88 on MAPK signaling was determined. Cisplatin activates many signaling pathways that are inhibited by triptolide. Cisplatin causes increased nuclear factor κB binding activity leading to prolonged JNK phosphorylation (Ramesh and Reeves, 2005; Benedetti et al., 2013). In HK cells, cisplatin increases the expression of p-ERK1/2 and p38 MAPK (Park et al., 2012a). In vivo, cisplatin increases p38 MAPK activation (Luo et al., 2008). p38 MAPK inhibition ameliorates cisplatin nephrotoxicity in mice (Ramesh and Reeves, 2005). Cellular targets of triptolide include ERK1/2, JNK-1/2, and p38 MAPK. In some cancer models, triptolide activates ERK1/2, p38 MAPK, and JNK-1/2 to exert its antiproliferative and proapoptotic properties (Liu, 2011). However, in a rat model of rheumatoid arthritis, triptolide can inhibit IL-1β-induced phosphorylation of ERK, p38, and JNK (Kong et al., 2013). Furthermore, triptolide decreased ERK phosphorylation in colon cancer cells (Liu et al., 2012). In the present study, in cisplatin-induced AKI, PG490-88 had no effect on JNK-1/2 or p38 MAPK, but resulted in a decrease in p-ERK. Increased ERK signaling has been demonstrated in cisplatin-induced AKI (Jo et al., 2005; Wang et al., 2013). The MAPK/ERK inhibitor U0126 decreased ERK1/2 phosphorylation and protected against cisplatin-induced AKI in mice (Jo et al., 2005; Wang et al., 2013). Thus, in the present study, there was an increase in p-ERK that was decreased by PG490-88 in cisplatin-induced AKI kidneys. The ERK inhibitor U0126 protects against cisplatin-induced AKI, suggesting that PG490-88—induced suppression of ERK may be a potential mechanism of protection against cisplatin-induced AKI by PG490-88.

Next, the signaling pathway upstream of MAPKs, especially as it relates to MAPK phosphatases (MKPs), was determined. MAPKs are inactivated by dephosphorylation of their conserved tyrosine and/or threonine residues in the activation loop (Lawan et al., 2013). The major group of enzymes that cause MAPK dephosphorylation are members of a protein tyrosine phosphatase family called dual-specificity protein phosphatases (DUSPs). DUSPs contain a subgroup of tyrosine phosphatases called MAPK phosphatases (MKPs) that are highly specific for MAPKs and directly dephosphorylate MAPKs. MKP-1 is a member of the MKP family that negatively regulates MAPK signaling (Wang et al., 2006). Cisplatin induces MKP-1 in several cancer cell lines, and overexpression of MKP-1 renders cancer cells resistant to cisplatin (Wang et al., 2006). Triptolide has been reported to suppress the expression of MKP-1 (Liu, 2011) or to induce MKP-1 (Park et al., 2012b). The expression of MKP-1 has not previously been reported in models of AKI. In the present study, MKP-1 activity, using both a fluorometric assay and RPPA analysis, was unaffected by AKI or PG490-88. These data suggest that both the

### Table 1

<table>
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<th>Cytokine</th>
<th>Veh (N = 9)</th>
<th>Vehicle + PG (N = 7)</th>
<th>Cisplatin (N = 18)</th>
<th>Cisplatin + PG (N = 16)</th>
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<td>820 ± 43</td>
<td>760 ± 67</td>
<td>750 ± 120</td>
<td>780 ± 103</td>
</tr>
</tbody>
</table>

PG, PG490-88. *P < 0.001 versus vehicle.

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**Fig. 6.** Cleaved caspase-3, pBAD, and XIAP. There was an increase in cleaved caspase-3 (CC-3) and pBAD but not XIAP in the kidney in cisplatin-induced AKI (Cis). PG490-88 (PG) had no effect on cleaved caspase-3, pBAD, or XIAP. *P < 0.01 versus vehicle (Veh); **P < 0.05 versus cisplatin. β-Actin was used as a loading control. Representative immunoblots of six separate experiments. (+), positive control (mouse spleen).
cause of cisplatin-induced AKI and the protective effect of PG490-88 in cisplatin-induced AKI are independent of MKP-1.

Next, mitogen-activated protein kinase kinase, which is also known as MAP2K or MEK kinase (MEKK), and is known to be upstream of MAPKs, was studied. MEKK is a serine/threonine kinase that plays a pivotal role in phosphorylating enzymes in the ERK, p38, and JNK signaling pathways (Yu and Rohan, 2000). MEKK phosphorylates MEKs, which can then phosphorylate and activate ERK (Lange-Carter et al., 1993). Cisplatin increases MEKK1 in lung cancer cells and mouse fibroblasts (Xia et al., 2013). The effect of cisplatin on MEKK1 in the kidney is not known. On RPPA analysis, we found an increase in MEKK1 in the kidney in cisplatin-induced AKI on day 1 that was decreased by PG490-88 on day 3. Thus, these data demonstrate that MEKK1, which is known to be upstream of ERK, is increased in the kidney in cisplatin-induced AKI and decreased by PG490-88. In this regard, we could not find any reports on the effect of triptolide on MEKK1.

Next, it was determined whether the protective effect of PG490-88 was related to a decrease in proinflammatory cytokines. Inflammation in the kidney with increases in proinflammatory cytokines is a feature of cisplatin-induced AKI (Faubel et al., 2007; Akcay et al., 2011). Triptolide is known to inhibit expression of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, interferon-γ, and MCP-1 by human bronchial cells and T cells (Liu, 2011). Triptolide inhibits lipopoly saccharide-induced expression of IL-6, IL-8, MCP-1, and IL-6 by fibroblasts (Lu et al., 2005). TNF-α has been shown to be a mediator of cisplatin-induced AKI (Ramesh and Reeves, 2002). We have shown increases in cytokines IL-1β, IL-6, and IL-18 (Faubel et al., 2007) and CXCL1 (also known as IL-8 or KC) (Akcay et al., 2011) in cisplatin-induced AKI. Whereas inhibition of CXCL1 is protective against cisplatin-induced AKI (Akcay et al., 2011), inhibition of IL-1β, IL-6, or IL-18 did not protect against cisplatin-induced AKI (Faubel et al., 2007). In the present study, the proinflammatory cytokines IL-1β, IL-1α, IL-6, CXCL1, and IL-33 were increased in cisplatin-induced AKI. PG490-88 had no effect on the increase in cytokines in the kidney. These data suggest that the protection against cisplatin-induced AKI by PG490-88 is independent of a decrease in IL-1β, IL-1α, IL-6, CXCL1, IL-33, or TNF-α.

In the present study, serum creatinine was measured by an alkaline picric acid (Jaffe reaction) method. Studies using the colorimetric Jaffe reaction for plasma measurements overestimate plasma creatinine due to endogenous chromogens in mice, and therefore underestimate creatinine clearance (Kepper et al., 2007). In addition, Eianner et al. (2010) found that secretion accounts for 50% of the creatinine in urine, and that creatinine clearance in mice is about twice that of inulin clearance. Thus, there are limitations to measuring serum creatinine by the Jaffe reaction in mice. The protective effect of PG490-88 was confirmed by other markers of kidney function and kidney histology, namely, BUN and ATN score.

In summary, PG490-88, a water-soluble triptolide derivative, resulted, in a significant decrease in BUN, SCr, and ATN score in cisplatin-induced AKI despite no effect on tubular apoptosis. The protection against cisplatin-induced AKI with PG490-88 was associated with a decrease in p-ERK, but was independent of a decrease in p-JNK, p-p38, and MKP-1. In addition, IL-1α, IL-1β, IL-6, IL-8, and IL-33 were significantly increased in cisplatin-induced AKI, but unaffected by PG490-88. An ERK inhibitor protected against cisplatin-induced AKI, demonstrating an injurious role of ERK in cisplatin-induced AKI and suggesting that PG490-88 may protect against cisplatin-induced AKI by decreasing p-ERK.

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