Netrin-1 overexpression in kidney proximal tubular epithelium ameliorates cisplatin nephrotoxicity

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Netrin-1, a multifunctional laminin-related protein is widely expressed in various tissues, including kidney. The pathophysiological roles of netrin-1 in toxic acute kidney injury are unknown. To determine the role of netrin-1 in cisplatin-induced nephrotoxicity, we used netrin-1 transgenic mice that overexpress netrin-1 in the proximal tubular epithelium using the fatty acid binding protein promoter. Administration of cisplatin caused severe renal injury in WT mice but not in netrin-1 transgenic mice. Functional improvement was associated with better preservation of morphology, reduced cytokine expression and oxidative stress in the kidney, and reduced serum and urine cytokine and chemokine levels of transgenic mice as compared with WT mice. Cisplatin induced an increase in neutrophil infiltration into the kidney of WT mice, which was not significantly reduced in netrin-1 transgenic mice. Interestingly, ischemia reperfusion induced a large increase in apoptosis in WT mice but not in netrin-1 transgenic mice (215 ± 40 vs 94 ± 20 cells/5 HPF ($\times 400$), $P < 0.0001$), which was associated with reduced caspase-3 and p53 activation in the transgenic kidney. These results suggest that netrin-1 protects renal tubular epithelial cells against cisplatin-induced kidney injury by suppressing apoptosis and inflammation.

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Cisplatin (cis-diamminedichloroplatinum(II)) is an effective chemotherapeutic drug used in the treatment of many solid-organ cancers, including those of the head, neck, lung, testis, ovary, and breast. While toxicities include ototoxicity, gastrotoxicity, myelosuppression, and allergic reactions,¹ the main dose-limiting, most common side effect of cisplatin is nephrotoxicity or acute kidney injury, which occurs in 20–30% of patients.^{2,3} Several recent studies have documented that cisplatin-induced acute kidney injury is an inflammatory disease associated with large increase in cell death by apoptosis.^{4–7} However, regulation of apoptosis and inflammation in cisplatin nephrotoxicity by endogenous molecules like netrin-1 is unknown.

Netrin-1, a diffusible laminin-related protein, is highly expressed outside the nervous system and its most abundant mRNA expression is found in the kidney.^{8,9} However, the function of netrin-1 in tissues outside the nervous system is not clear. In recent studies using both *in vitro* and *in vivo* systems, netrin-1 was shown to have a role in angiogenesis,^{10–12} cell migration,¹³ tissue morphogenesis,^{14,15} tumor progression

and growth,^{16,17} and regulation of inflammation.⁹ Our most recent studies showed that administration of recombinant netrin-1 or transgenic overexpression in kidney prevented renal dysfunction by suppressing apoptosis and inflammation.⁸ However, whether netrin-1 has any protective role in toxic acute kidney injury is unknown.

To determine the role of netrin-1 in cisplatin-induced acute kidney injury, we used mice that overexpress netrin-1 in the proximal tubular epithelial cells using the liver fatty acid binding protein promoter.¹⁸ We characterized netrin-1 transgene expression in the kidney in our earlier study.¹⁹ These transgenic mice showed normal kidney function and reproductive phenotype under basal conditions. Quantification of transgene expression by western blot showed that expression levels were more than five-fold greater as compared with wild-type mice. We subjected netrin-1 transgenic mice to cisplatin treatment and assessed kidney function. Our results show that netrin-1 transgenic mice are resistant to cisplatin-induced kidney injury. This resistance was associated with reduced cytokine production, apoptosis, and p53 activation.

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MATERIALS AND METHODS

Animals and Drug Administration

Experiments were performed using 8–10-week-old male C57BL/6 mice and netrin-1 transgenic mice that overexpress netrin-1 in kidney under control of the fatty acid binding protein promoter.^{18,19} Netrin-1 transgenic mice were in the C57BL/6 background and were backcrossed for > 30 generations. Transgenic mice were regularly bred with C57BL/6 mice from Jackson Laboratory and either littermates or C57BL/6 WT mice from Jackson Laboratory weighing ~25–30 g were used as a control. Mice were maintained on a standard diet, and water was freely available. This study was approved by IACUC at the Georgia Health Sciences University. Cisplatin was dissolved in saline at a concentration of 1 mg/ml. Mice were given a single intraperitoneal injection of either saline or cisplatin (20 mg/kg body weight). Animals were killed 72 h after cisplatin injection, and blood and kidney tissues were collected. Kidney tissues were processed for histology, TUNEL assay, neutrophil and p53 staining, and RNA isolation.

Cell Culture

Immortalized mouse proximal tubule cells, TKPTS²⁰ cells, kindly provided by Dr Bell-Reuss and Dr J Megyesi, were cultured in DMEM/F12 supplemented with glutamine, 7.5% FBS and antibiotics, and were grown to confluence and maintained at 37°C in 5% CO₂. Cells were treated with cisplatin with/without netrin-1 for 24 h and harvested for RNA isolation at the end of the incubation.

Renal Function

Renal function was assessed by measurements of blood urea nitrogen (VITROS DT60II Chemistry slides, Ortho-clinical Diagnostics) and serum creatinine (cat no: DZ072B, Diazyme Labs, USA).

Quantitation of mRNA by Real-time RT-PCR

Real-time RT-PCR was performed in an Applied Biosystems Inc. 7700 Sequence Detection System (Foster City, CA, USA). In all, 1.5 µg total RNA was reverse transcribed in a reaction volume of 20 µl using Omniscript RT kit and random primers. The product was diluted to a volume of 150 µl, and 6 µl aliquots were used as templates for amplification using the SYBR Green PCR amplification reagent (Qiagen) and gene-specific primers. The primer sets used were mouse TNFα (forward: GCATGATCCGCGACGTGGAA; reverse: AGATCCATGCCGTTG GCCAG), MCP-1 (forward: ATGCA GGTCCCTGTCATG; reverse: GCTTGAGGTGGTTGTGGA), ICAM-1 (forward: AGATCACATTCACGGTGCTG; reverse: CTTACAGAGGCAGGAAACAGG), netrin-1 (forward: AAGC CTATCACCCACCGGAAG; reverse: GCGCCACAGGAATCT TGATGC), osteopontin (forward: TCACCATTCGGATGAGT CTG-3'; reverse: ACTTGTGGCTCTGATGTTCC), hemeoxygenase-1 (HO-1) (forward: AGCATGCCCCAGGATTG; reverse: AGCTCAATGTTGAGCAGGA), and TGF-β1 (forward:

TGACGTCACCTGGAGTTGTACGG; reverse: GGTTTCATGTC ATGGATGGTGC). The amount of DNA was normalized to the β-actin signal amplified in a separate reaction (forward primer: AGAGGGAAATCGTGCGTGAC; reverse: CAATAGT GATGACCTGGCCGT).

Serum Cytokine Measurement

Serum cytokines and chemokines were measured using ELISA array kit from SA biosciences and ELISA kit from eBiosciences.

Immunohistochemical Localization of p53 and Caspase-3

Immunohistochemical localization of p53 and caspase-3 was performed as described before.¹⁹ Briefly, paraffin-embedded sections were deparaffinized and hydrated. Endogenous peroxidase activity was blocked with 90% methanol/3% H₂O₂ followed by permeabilization with 0.2% Triton X-100 in PBS. Sections were then washed and blocked with PBS containing 5% donkey serum and 1% BSA. Primary antibodies included a goat anti-caspase-3 and rabbit anti-p53 (Cell Signaling Technologies, Inc.). Primary antibodies were detected using a biotin-conjugated secondary antibody followed by ABC reagent. Color was developed using metal-enhanced DAB substrate. Peroxidase staining was visualized and photographed using an Olympus inverted microscope with a CCD camera attached to it.

TACS TdT In Situ Apoptosis Detection

To identify apoptotic cells, tissue sections were stained using TACS TdT *in situ* Apoptosis Detection kit (R&D Systems, Inc.) according to the manufacturer's instruction. Briefly, tissue sections were deparaffinized, hydrated, and washed with PBS. Sections were digested with proteinase K for 15 min at 24°C. Slides were then washed and endogenous peroxidase activity was quenched with 3% H₂O₂ in methanol. Slides were washed and incubated with TdT labeling reaction mix at 37°C for 1 h and then with streptavidin-HRP. Color was developed using TACS blue label substrate solution. Slides were washed, counterstained, and mounted with Permount. Sections were photographed, and labeled cells were counted and quantified.

Histology

Kidney tissue was fixed in buffered 10% formalin for 12 h and then embedded in paraffin wax. For assessment of injury, 5 µm sections were stained with periodic acid-Schiff (PAS). Acute tubular necrosis was assessed in the outer strip of the outer medulla and cortex using a semiquantitative scale in which the percentage of tubules showing epithelial necrosis, brush-border loss and cast formation was assigned a score: 0 = normal, 1 = <10%, 2 = 11–25%, 3 = 26–45%, 4 = 46–75%, and 5 = >76%.^{21,22} Ten fields of ×40 magnification were examined and averaged. The slides were scored in a blinded manner and de-identified. To quantify leukocyte

infiltration, sections were stained with rat anti-mouse neutrophils antibody (SEROTECH) (1:200 dilution) followed by goat anti-rat biotin conjugate. Color was developed after incubation with ABC reagent (Vector Lab). Stained sections were photographed and five $\times$ 40 fields of neutrophils were examined for quantitation of leukocytes.

Statistical Methods

All assays were performed in duplicate. The data are reported as mean $\pm$ s.e.m. Statistical significance was assessed by an unpaired, two-tailed Student's *t*-test for single comparison or ANOVA for multiple comparisons.

RESULTS

Netrin-1 Transgenic Mice Are Resistant to Cisplatin-Induced Renal Dysfunction

The role of netrin-1 in toxic acute kidney injury is unknown. To determine the role of netrin-1 in cisplatin-induced kidney injury, WT and netrin-1 transgenic mice were administered with cisplatin or saline. Renal function was determined by measuring serum creatinine. As shown in Figure 1a, WT mice developed severe renal dysfunction. Serum creatinine was increased significantly by 24 h and continued to climb up to 72 h. However, netrin-1 transgenic mice displayed lower serum creatinine levels at all time points. Saline-treated WT and netrin-1 transgenic mice did not develop any renal dysfunction. Improved renal function in the transgenic mice was associated with improved tissue morphology as determined by reduction in necrosis score as compared with WT mice (not shown). Semiquantitative scoring of tubule injury was significantly higher in WT kidneys (3.8 ± 0.1) than in kidneys from netrin-1 transgenic mice (2.4 ± 0.6) at 72 h after cisplatin administration ($P < 0.05$, $n = 4$ –6 per genotype).

Netrin-1 Overexpression Reduced Cytokine, Adhesion Molecule, and Oxidative Stress Marker Expression

Several *in vitro* and *in vivo* studies suggested that cisplatin nephrotoxicity is an inflammatory disease characterized by exacerbated inflammatory response from the kidney.^{7,23} Moreover, netrin-1 administration reduced inflammation and inhibited leukocyte migration.^{8,9} To determine whether netrin-1 overexpression reduces inflammation, expression of several cytokines, chemokines, and adhesion molecules was determined by real-time RT-PCR. Cisplatin administration induced a significant increase in MCP-1, TNF α , RANTES, ICAM-1, complement C3, osteopontin, and HO-1 (Figure 1b). Overexpression of netrin-1 significantly reduced RANTES, complement C3, ICAM-1, osteopontin, and HO-1 mRNA levels.

To determine if suppression of mRNA expression correlates with protein levels, cytokines and chemokines in serum and urine were quantified by ELISA. Administration of cisplatin increased serum levels of several cytokines (IL-6 and IL-10) and chemokines (MCP-1, MIG, eotaxin, and KC) that

Figure 1 Effect of overexpression of netrin-1 on cisplatin-induced renal dysfunction. Serum creatinine (a) was measured at different times after cisplatin or saline administration in WT and netrin-1 transgenic mice. Values are means $\pm$ s.e.m. * $P < 0.01$ vs all other groups. Values are mean $\pm$ s.e.m.; $n = 8$ –10 for each group. Cytokine and chemokine, adhesion molecule, and complement expression were quantified by real-time RT-PCR (b) and expressed as fold over saline-treated animals. * $P < 0.05$ vs WT cisplatin-treated mice.

were significantly reduced in netrin-1 transgenic mice treated with cisplatin at 24 h (Figure 2). Interestingly, IL-4 and IL-13 levels in serum are elevated in netrin-1 transgenic animals but not WT mice treated with cisplatin. A more dramatic increase of cytokine and chemokine levels was seen in urine of WT mice. As shown in Figure 3, cytokine (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IL-23, IFN γ , TNF α , and TGF β 1) and chemokine (RANTES, MCP-1, MIP-1 α , MIP-1 β ,

Figure 2 Effect of netrin-1 overexpression on serum cytokine (b) and chemokine levels (a). Cytokine and chemokine levels in serum were measured 24 h after cisplatin or saline administration in WT and netrin-1 transgenic animals. Netrin-1 overexpression suppressed cisplatin-induced cytokine and chemokine increase in serum. Values are means $\pm$ s.e.m. * P < 0.05 vs WT saline-treated mice. # P < 0.05 vs WT cisplatin-treated mice; n = 6.

MIG, eotaxin, TARC, MDC, KC, and 6Ckine) levels were significantly elevated in WT mice at 24 h after cisplatin administration. All these cytokine and chemokine levels were suppressed in netrin-1 transgenic mice except IL-4 and IL-13 which elevated in both basal and cisplatin-treated netrin-1 transgenic mice. Changes in cytokine and chemokine levels in serum are moderate as compared with urine levels which showed a dramatic increase, suggesting that renal tubular epithelial cells may be a major source of these cytokines and chemokines. These results suggest that netrin-1 is a major regulator of cytokine and chemokine expression in kidney.

To determine whether urinary cytokines were derived from tubular epithelial cells after cisplatin administration, mouse proximal tubular epithelial cells were treated with 25 μ M cisplatin and with/without netrin-1 for 24 h. Expression of TNF α was quantified by real-time RT-PCR as described in

Figure 3 Effect of netrin-1 overexpression on urine cytokine and chemokine levels. Chemokine (a) and cytokine (b) levels in urine were measured 24 h after cisplatin or saline administration in WT and netrin-1 transgenic animals. Netrin-1 overexpression suppressed cisplatin-induced cytokine and chemokine increase in urine. Values are means $\pm$ s.e.m. * P < 0.001 vs all other groups; n = 6. Netrin-1 suppresses cisplatin-induced cytokine expression in proximal tubular epithelial cells *in vitro* (c). # P < 0.05 vs cisplatin treated. n = 4.

Materials and Methods. As shown in Figure 3c, cisplatin treatment increased TNF α expression, which is significantly reduced in netrin-1-treated cells, suggesting that netrin-1 regulates cytokine and chemokine production in response to cisplatin in proximal tubular epithelial cells.

Netrin-1 Overexpression in Tubular Epithelial Cells Does Not Reduce Neutrophil Infiltration

Earlier studies suggested that intravenous administration of recombinant netrin-1 reduced neutrophil infiltration into the kidney after ischemia reperfusion⁸ and reduced leukocyte

Figure 4 Effect of netrin-1 overexpression on neutrophil infiltration. Sections were fixed and stained for neutrophils as described in Material and Methods. (a) Saline-treated WT mice. (b) Netrin-1 transgenic mice showed few neutrophils. Seventy-two hours after cisplatin administration, both WT (c) and netrin-1 transgenic mice kidney (d) had shown increased neutrophil staining. (e) Quantitative summary data of neutrophil infiltration (* $P < 0.001$ vs saline treated). $n = 4-6$.

migration during sepsis⁹ but not when netrin-1 was overexpressed in proximal tubule in an ischemia reperfusion injury model. To determine whether the observed protective effect of tubule-specific overexpression of netrin-1 is mediated, in part,

through reducing leukocyte infiltration, tissue sections were stained with an anti-neutrophil antibody (Figure 4). Saline-treated WT and transgenic mouse kidneys had very few neutrophils (Figure 4a and b). Cisplatin administration

Figure 5 Effect of netrin-1 overexpression on cisplatin-induced apoptosis. Apoptotic cells were identified using TUNEL staining (a–d). Sham-operated WT and transgenic mouse kidney did not show any apoptotic cells (a, b). Seventy-two hours after cisplatin administration, the number of apoptotic cells (blue staining) was increased dramatically in WT (c) whereas only a few cells were positive for apoptosis in transgenic animals (d). (e) Quantification of apoptotic cells. Apoptotic-positive cells were counted in five random $\times 40$ fields. * $P < 0.001$ vs saline treated. ⁺ $P < 0.001$ vs WT cisplatin treated; $n = 4-6$.

dramatically increased neutrophil infiltration in WT (Figure 4c) and in netrin-1 transgenic mice (Figure 4d). These results suggest that the observed protective effect of netrin-1 overexpression is not due to suppression of neutrophil infiltration.

Netrin-1 Overexpression Reduces Cisplatin-Induced Tubular Epithelial Cell Apoptosis

Netrin-1 was shown to be a survival factor for intestinal epithelial cells and, in the absence of netrin-1, the receptors

Figure 6 Effect of netrin-1 overexpression on cisplatin-induced activation of p53. Phospho p53 was immunolocalized in formalin-fixed sections as described in Materials and Methods (a–d). Saline-treated WT and transgenic mouse kidney did not show any p53-positive nuclei (a, b). Seventy-two hours after cisplatin administration, the number of p53-positive cells (arrows) was increased dramatically in WT (c) whereas only a few cells were positive for p53 in transgenic mice kidney (d). (e) Quantitation of p53-positive nuclei. Phospho p53-positive nuclei were counted in five random $\times 40$ fields. * $P < 0.005$ vs saline treated. + $P < 0.001$ vs WT cisplatin treated; $n = 4-6$.

DCC and UNC5B are known to induce apoptosis.^{18,24,25} Cisplatin administration is known to induce apoptosis in tubular epithelial cells. In that context, inhibition of apoptosis leads to improved renal function.^{5,26} To determine whether netrin-1 alters cisplatin-induced apoptosis, apoptotic cells were quantified using TACS TdT staining. Saline-treated WT (Figure 5a) and netrin-1 transgenic (Figure 5b) kidneys did not show any apoptotic cells. Administration of cisplatin induced a large increase in apoptosis in WT mice by 72 h (Figure 5c). However, cisplatin-induced apoptosis was drastically reduced in netrin-1 transgenic kidneys (Figure 5d). Quantification of apoptotic cells showed a significant decrease in transgenic mice as compared with WT mice treated with cisplatin (Figure 5e).

To determine the pathways through which apoptosis is suppressed, tissue sections were also stained with antibodies against phospho p53 and active caspase-3. As shown in Figure 6, cisplatin induced a large increase in phospho p53 staining in WT mice (Figure 6c), which is drastically and significantly reduced in netrin-1 transgenic mice (Figure 6d). Quantitative

data are provided in Figure 6e, which also showed a significant decrease in number of p53-positive nucleus in netrin-1 transgenic mouse kidney as compared with WT mouse kidney. Activation of p53 by cisplatin leads to the activation of caspases, and netrin-1 is known to suppress caspase activation. Therefore, we determined whether netrin-1 suppresses caspase-3 activation. As shown in Figure 7, caspase-3 staining in saline-treated WT (Figure 7a) and netrin-1 transgenic mouse kidney (Figure 7b) is not increased. However, administration of cisplatin increased active caspase-3 staining in WT mouse kidney, which is suppressed in netrin-1 transgenic mice kidney. These data suggest that suppression of p53 pathway-mediated activation of caspase may contribute to netrin-1-mediated protection of kidney against cisplatin injury.

DISCUSSION

The present study provides evidence that netrin-1, a laminin-related neuronal guidance cue, when expressed in proximal tubule cells, can prevent renal dysfunction and epithelial cell apoptosis in response to cisplatin administration.

Figure 7 Effect of netrin-1 overexpression on cisplatin-induced caspase-3 activation. Kidneys from saline-treated WT and transgenic mice did not show any staining for active caspase-3 (a, b). Seventy-two hours after cisplatin administration, tubular epithelial cells positive for active caspase-3 were increased in WT mice (c) but not in netrin-1 transgenic mice (d).

Furthermore, netrin-1 suppressed caspase-3 and p53 activation in tubular epithelial cells and reduced inflammatory cytokine expression and excretion. These results indicate that netrin-1 has a key role in toxic acute kidney injury by suppressing apoptosis and inflammatory cytokine expression.

Our earlier studies had determined that netrin-1 transgenic mice had normal kidney function and exhibit histological feature under basal conditions,¹⁹ suggesting that overexpression of netrin-1 in tubular epithelial cells is not altering normal kidney function. Our earlier studies showed that administration of cisplatin to mice rapidly induced netrin-1 in the renal tubular epithelial cells and excreted in urine.²⁷ However, the role of netrin-1 in cisplatin-induced kidney is unknown. The present study determined that netrin-1 overexpression protected against cisplatin-induced acute kidney injury. This effect is likely due to the marked suppression of apoptosis in the transgenic mice. Although several *in vitro* and *in vivo* studies in other tissues have demonstrated a role for netrin-1 in inhibiting apoptosis,^{17,18,28–30} this is the first study to show the anti-apoptotic role of netrin-1 in kidney tubules in a model of toxic acute kidney injury.

Netrin-1 receptors (DCC and UNC5) are also called dependence receptors as these receptors are known to induce apoptosis when they are not bound to netrin-1.²⁴ But the presence of netrin-1 UNC5b mediates cell survival by activating survival pathways such as like PI3K and ERK pathways. Moreover, netrin-1 is known to suppress caspase and p53 activation leading to suppression of apoptosis.^{29,31,32} Along this line, netrin-1 administration prevented p53 activation and ischemia-induced apoptosis in brain.³⁰ Both caspase and p53 are activated by cisplatin in kidney and are known to mediate kidney injury.^{5,33} Since netrin-1 is known to suppress p53 and caspase activation in other tissues,^{30,32,34} it suggests that a similar mechanism may exist in kidney as well. Our results support these findings that netrin-1-mediated protection against cisplatin-induced kidney injury is in part through suppression of caspase-3 and p53 activation (Figures 6 and 7).

Inflammatory mechanisms have a key role in cisplatin-induced kidney injury.^{6,7,23} Recent studies suggest that netrin-1 limits inflammation by inhibiting leukocyte migration via UNC5B receptors.⁹ Although intravenous administration of netrin-1 reduced ischemia reperfusion-induced neutrophil infiltration, tubule-specific overexpression in the present study did not influence neutrophil infiltration. This observation suggests that the observed protective effects in netrin-1 transgenic mice against cisplatin-induced kidney injury are not through suppression of neutrophil infiltration. It is possible that the netrin-1 expressed in proximal tubule cells may not be available to the vascular compartment to act on the leukocytes. However, netrin-1 transgenic mice did suppress the expression and excretion of several pro-inflammatory chemokines and cytokines that are known to be involved in kidney injury by acting directly on the tubular

epithelial cells. Therefore, some of the protection afforded by netrin-1 could be referable to this reduction in cytokine expression. The cell types that produce cytokines and chemokines are not clear in our study. However, earlier studies^{22,35} showed that renal epithelial cells are capable of producing several inflammatory cytokines and chemokines in response to cisplatin treatment.

Tubular epithelial cell-specific expression of netrin-1 suppressed a wide array of cytokine and chemokine expression except the Th2 cytokines IL-4 and IL-13 which were upregulated at 72 h after cisplatin treatment (data not shown). In contrast, Th2 cytokine expression is suppressed by netrin-1 in T cells.³⁶ Whether netrin-1 regulates IL-4/IL-13 expression in renal epithelial cells is unknown. IL-13 is known to suppress tubular epithelial cell apoptosis and reduce sepsis and ischemic kidney injury.^{37–39} However, whether netrin-1 mediates part of its protective effect through IL-4 and IL-13 is unknown. Th2 cytokine is also known to exert an anti-inflammatory phenotype by inducing macrophage polarization to M2 phenotype in addition to suppression of apoptosis in epithelial cells. The connection between IL-4/IL-13 and netrin-1 in renal epithelial cell functions needs to be explored further.

In summary, our findings support the view that netrin-1 is a strong pro-survival factor for proximal tubule cells and can suppress cisplatin-induced apoptosis possibly by suppressing p53 and caspase-3 activation. Netrin-1-mediated suppression of inflammatory cytokine expression and positive regulation of Th2 cytokines suggests that part of the netrin-1 effect on the kidney is mediated through IL-13. Our result suggests that netrin-1 may be a useful therapeutic molecule for treating cisplatin nephrotoxicity and other inflammatory diseases as well. The molecular mechanism by which netrin-1 and Th2 cytokine interacts warrants further investigation.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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