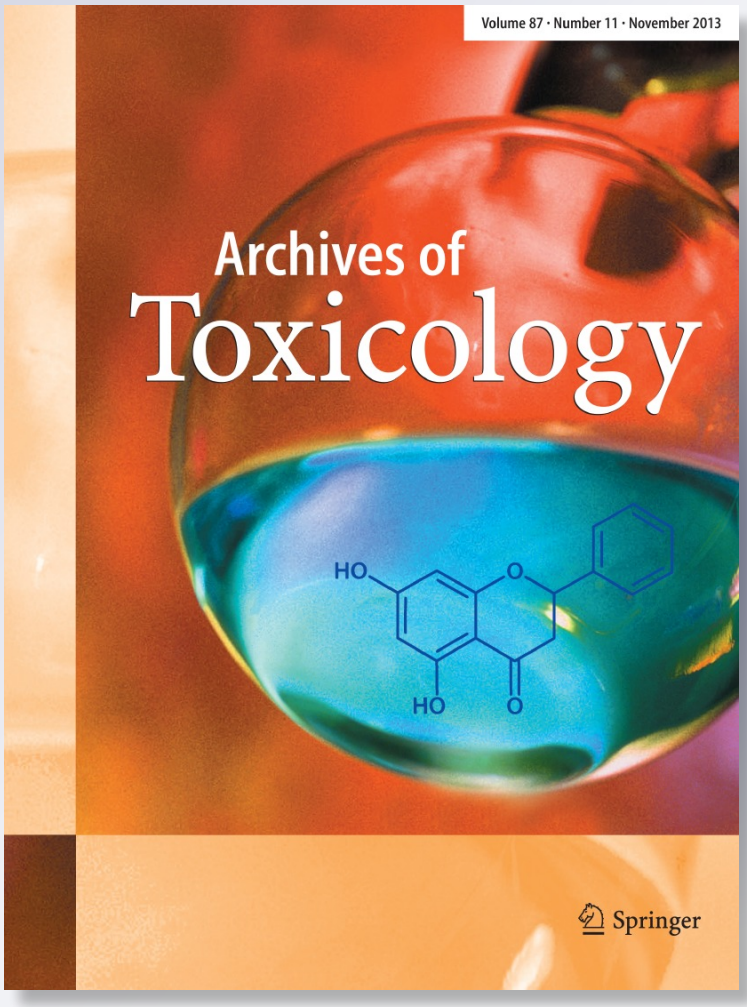
Organic anion transporter 5 (Oat5) renal expression and urinary excretion in rats treated with cisplatin: a potential biomarker of cisplatin-induced nephrotoxicity

Romina Paula Bulacio & Adriana Mónica Torres

Archives of Toxicology

ISSN 0340-5761
Volume 87
Number 11

Arch Toxicol (2013) 87:1953-1962
DOI 10.1007/s00204-013-1062-0



Springer

Arch Toxicol (2013) 87:1953–1962
DOI 10.1007/s00204-013-1062-0

ORGAN TOXICITY AND MECHANISMS

Organic anion transporter 5 (Oat5) renal expression and urinary excretion in rats treated with cisplatin: a potential biomarker of cisplatin-induced nephrotoxicity

Romina Paula Bulacio · Adriana Mónica Torres

Received: 17 December 2012 / Accepted: 23 April 2013 / Published online: 7 May 2013

Abstract Cisplatin is one of the most potent chemotherapeutic antitumor drugs used in the treatment of a wide range of solid tumors. Its primary dose-limiting side effect is nephrotoxicity. The organic anion transporter 5 (Oat5) is exclusively localized in the kidney. Oat5 urinary excretion was recently proposed as a potential early biomarker of acute kidney injury (AKI). The aim of this study was to evaluate Oat5 renal expression and its urinary excretion in rats exposed to different doses of cisplatin, in comparison with traditional markers of renal injury, like renal histology, creatinine and urea plasma levels, creatinine clearance, protein and glucose urinary levels and urinary alkaline phosphatase (AP) activity. Male Wistar rats were treated with a single injection of cisplatin at different doses of 1, 2, 5 and 10 mg/kg b.w., i.p. (Cis1, Cis2, Cis5 and Cis10, $n = 4$, respectively) and experiments were carried out 48 h after cisplatin administration. The renal expression of Oat5 was evaluated by immunohistochemistry and Western blotting. Oat5 abundance, AP activity, creatinine, glucose and proteins were assayed in urine. Creatinine clearance and creatinine and urea plasma levels were also evaluated. In this experimental model, plasma urea and creatinine levels, creatinine clearance, AP urinary activity and protein and glucose urinary levels were significantly modified only at the highest cisplatin dose of 10 mg/kg b.w., i.p., as compared to control rats. In contrast, Oat5 urinary abundance was increased in a dose-related manner after the administration of cisplatin. Oat5 urinary abundance was elevated at a dose as low as 1 mg/kg b.w., i.p., implying renal perturbation, when no modifications of traditional markers of renal injury are yet observed. Oat5 renal expression was decreased in a dose-related manner, both in homogenates and apical membranes from cisplatin-treated kidneys. The increase in urinary Oat5 excretion might explain the decrease in the amount of Oat5 molecules in the renal tubule cells. Hence, the preclinical animal results showed in this work propose that Oat5 urinary excretion might potentially serve as a non-invasive early biomarker of cisplatin-induced AKI.

Keywords Cisplatin · Oat5 · Renal damage · Urinary biomarkers

Introduction

Cisplatin (*cis*-diamminedichloroplatinum II) is one of the most potent chemotherapeutic antitumor drugs used in the treatment of a wide range of solid tumors (Miller et al. 2010). It has been used to treat a great variety of neoplasms, mainly for head and neck, testicular, ovarian, bladder and small-cell lung tumors. The principal side effects of cisplatin in man and animals include nephrotoxicity, ototoxicity, neurotoxicity and bone marrow suppression, but its primary dose-limiting side effect is nephrotoxicity (Taguchi et al. 2005; Uehara et al. 2011). The kidney accumulates cisplatin to a greater degree than other organs and is the major route for its excretion. The great accumulation of cisplatin in kidney tissue contributes to cisplatin-induced nephrotoxicity. Conversion of cisplatin to a nephrotoxic metabolite in the proximal tubule is required for cell injury. It is conjugated to glutathione and then metabolized through a γ-glutamyl transpeptidase and a cysteine-S-conjugate β-lyase-dependent pathway to a reactive thiol, which is a potent nephrotoxin (Yao et al. 2007).

R. P. Bulacio · A. M. Torres (✉)
Area Farmacología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, CONICET, Suipacha 531, 2000 Rosario, Argentina
e-mail: admotorres@yahoo.com.ar

The mechanism underlying cisplatin anticancer activity is not completely understood, but it is normally accepted that cisplatin is a DNA damaging agent which forms DNA adducts that destroy cells via various mechanisms, resulting in the induction of apoptosis (Arjumand et al. 2011). The glomerular filtration rate can decrease by 30 % after only two doses of cisplatin, and treatments must often be stopped. Actually, only 60 % of patients complete three of four cisplatin cycles. Cisplatin-induced nephrotoxicity involves enhanced oxidative stress, inflammatory reactions and tubular cell apoptosis (Yao et al. 2007; Camano et al. 2010). Its dose-limiting toxicities have encouraged the development of the non-nephrotoxic derivatives carboplatin, nedaplatin and other platinum-based drugs. Nevertheless, cisplatin is still the drug of choice in several platinum-based therapy regimens and is still one of the most commonly used antineoplastic drugs (Uehara et al. 2011; Miller et al. 2010). Hydration protocols were developed to reduce the nephrotoxicity and allowed dose escalation to therapeutic levels. In modern clinical practice, one of three types of hydration is used: saline, saline with mannitol or saline with furosemide. Though, even with vigilant hydration, a great number of patients treated with cisplatin have transitory elevation of blood urea nitrogen levels or other evidence of kidney damage in the days following cisplatin treatment (Muraki et al. 2012; Miller et al. 2010; Tiseo et al. 2007).

The transport of organic anions in the proximal tubules is achieved by basolateral and luminal transporters, moving organic anions from blood to tubular lumen into urine. A member of the organic anion transporter (Oat) family, Oat5 (Slc22a19), has been recently cloned and characterized (Youngblood and Sweet 2004; Anzai et al. 2005, 2006). This transporter is expressed exclusively in the kidneys where it is found in the apical membrane of proximal tubule cells, mainly in their late segments (S2 and S3). Oat5 has been reported to transport ochratoxin A, sulfate conjugates of steroids such as dehydroepiandrosterone sulfate and estrone-3-sulfate, dicarboxylates, for example, α-ketoglutarate and succinate, and interacts with many anionic drugs, such as bumetanide, furosemide, penicillin G, non-steroidal anti-inflammatory drugs and bromosulfophthalein (Youngblood and Sweet 2004; Anzai et al. 2005; Breljak et al. 2010; Burckhardt 2012). Our group was pioneering in detecting Oat5 in urine (Di Giusto et al. 2009). We have reported an important urinary increase in Oat5 in ischemia and mercury induced acute kidney injury (AKI), suggesting that urinary Oat5 excretion may serve as a novel and potentially valuable biomarker of AKI (Di Giusto et al. 2009; Di Giusto and Torres 2010).

The aim of this study was to evaluate Oat5 renal expression and urinary excretion in rats 48 h after exposure to different doses of cisplatin, in comparison with traditional markers of renal injury, like renal histology, creatinine and urea plasma levels and protein and glucose urinary levels, between others.

Materials and methods

Materials

Chemicals were purchased from Sigma (St. Louis, MO, USA) and were analytical grade pure. The rabbit polyclonal antibody against Oat5 was kindly provided by Prof. H. Endou and Prof. N. Anzai (Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan) (Anzai et al. 2005).

Experimental animals

Male Wistar rats (120 days, 380–430 g body weight) were used. The animals were kept in standard cages at room temperature (21–23 °C) on a 12-h light/dark cycle with free access to food and water, as outlined in the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Animals were randomly divided into five experimental groups. Rats were treated with a single injection of cisplatin at different doses of 1, 2, 5 and 10 mg/kg of body weight (b.w.), intraperitoneally (i.p.) (Cis1, Cis2, Cis5 and Cis10, $n = 4$, respectively). These doses of cisplatin were selected considering their relation to clinical doses used in chemotherapy and their nephrotoxicity, which is similar in rodents and humans at comparable doses (Bearcroft et al. 1999; dos Santos et al. 2007; Aleksunes et al. 2008; Pinches et al. 2012a, b, c; Dobyan et al. 1980; Vadiei et al. 1992; Hosohata et al. 2012). Control rats received the vehicle alone (1 mL saline/kg b.w., i.p., $n = 4$).

Immediately after the injection (saline or cisplatin), the rats were housed in their home cages with access to food and water. After 24 h, the rats were transferred to metabolic cages for urine collection for the next 24 h. During this last period, the food was withdrawn in order to improve urine sample quality by reducing contamination with waste food debris as previously described (Pinches et al. 2012b). The urinary volume (V_U) was determined gravimetrically.

The studies were performed 48 h after the injection. On the day of the experiment, the rats were anesthetized with sodium thiopental (70 mg/kg b.w., i.p.) and plasma samples were obtained by cardiac puncture and kidney tissue was also collected.

Two different sets of experimental animals were used: one for biochemical determinations and preparation of homogenates and apical membranes from kidney, another for histopathological and immunohistochemical studies.

Biochemical determinations

The urine samples were used for analyses of Oat5 abundance, alkaline phosphatase (AP) activity and creatinine (Cr_U), glucose and proteins levels. Serum samples were used to measure urea and creatinine levels (Crp).

Urine AP activity, creatinine, glucose and proteins urinary levels, as well as plasma creatinine and urea levels were determined spectrophotometrically with commercial reagent kits (Wiener Laboratory, Rosario, Argentina). Creatinine clearance (Cl_{Cr}) was calculated by the conventional formula: $Cl_{Cr} = (Cr_U \times V_U)/Crp$.

Preparation of homogenates and apical membranes from kidney

Apical membranes were isolated from kidneys by Mg/EGTA precipitation as previously described (Di Giusto et al. 2009; Di Giusto and Torres 2010). The kidneys were removed, minced and homogenized in 30 g/100 mL (v/w) of ice-cold 50 mM mannitol, 2 mM Tris HCl buffer (pH 7.10), 5 mM EGTA and 1 mM phenylmethylsulfonylfluoride (PMSF) for 5 min at top speed in a *Glas-Col* homogenizer. From this preparation, we obtained total renal homogenates, and aliquots were taken and stored at −80 °C until use. $MgCl_2$ was then added to the remaining homogenate to a final concentration of 12 mM, and the mixture was stirred in an ice bath for 15 min. The homogenate was then centrifuged (3,000×*g*, 15 min, 4 °C). The supernatant was carefully decanted and centrifuged again at 28,000×*g* for 40 min at 4 °C. The pelleted material representing apical membranes was resuspended in "experimental buffer" (50 mM mannitol, 10 mM Hepes-Tris (pH 7.50) and 1 mM PMSF) and centrifuged for 15 min at 800×*g* at 4 °C. The supernatant was finally centrifuged for 45 min at 28,000×*g*. The apical membrane pellets thus obtained were resuspended in experimental buffer. Aliquots of the membranes were stored immediately at −80 °C until used. Protein quantification of samples was performed using the method of Lowry with some modifications (Lowry et al. 1951).

Electrophoresis and immunoblotting

Homogenates (20 μg of protein), apical membranes (16 μg of protein) and urine samples (10 μL) were boiled for 3 min in the presence of 5 % 2-mercaptoethanol, 4 % sodium dodecyl sulfate (SDS) and separated by 8.5 % polyacrylamide gel (SDS-PAGE), followed by electroblotting to nitrocellulose membranes. To verify equal protein loading and transfer between lanes, Ponceau red was used as previously described (Brandoni et al. 2006). The nitrocellulose membranes were incubated with 5 % non-fat dry milk in phosphate-buffered saline containing 0.1 % Tween 20 (PBST) for 1 h. After being rinsed with PBST, the membranes were then incubated overnight at 4 °C with a non-commercial rabbit polyclonal antibody against rat Oat5 (at a dilution of 1:800). The specificity of Oat5 antibody has been described elsewhere (Anzai et al. 2005). The membranes were then incubated for 1 h with a peroxidase-coupled goat anti-rabbit IgG (Bio-Rad Laboratories, Hercules, CA, USA) after further washing with PBST. Blots were processed for detection using a commercial kit (ECL Enhanced Chemiluminescence System, Amersham, Buckinghamshire, UK). A densitometric quantification of the Western blot signal intensity of membranes was performed. For densitometry of immunoblots, samples from treated rats were run on each gel with corresponding control samples. The abundance of Oat5 in the samples from the experimental animals was calculated as percentage of the mean control value for that gel.

Histopathological and immunohistochemistry studies

The immunohistochemistry technique was performed as previously described (Brandoni et al. 2006; Di Giusto et al. 2009; Di Giusto and Torres 2010; Bulacio et al. 2012). Kidneys from different experimental groups were briefly perfused with saline, followed by perfusion with periodate-lysine-paraformaldehyde solution (0.01 M $NaIO_4$, 0.075 M lysine, 0.0375 M phosphate buffer, with 2 % paraformaldehyde, pH 6.20), through a cannula introduced in the abdominal aorta. The kidney slices were immersed in periodate-lysine-paraformaldehyde solution at 4 °C overnight. After that, the tissue was embedded in paraffin and paraffin sections were cut.

After deparaffining, some sections were used for routine hematoxylin–eosin staining, while others were incubated with 3 % H_2O_2 for 15 min (to eliminate endogenous peroxidase activity) to perform Oat5 renal immunohistochemistry. After that, the sections were incubated with blocking serum for 30 min and then with non-commercial rabbit polyclonal antibody against Oat5 (diluted 1:100) overnight at 4 °C. The sections were then rinsed with Tris-buffered saline containing 1 % Tween (TBST).

Next, the sections were incubated with horseradish peroxidase (HPR) conjugated secondary antibody against rabbit immunoglobulin for 1 h. In order to detect HPR labeling, a peroxidase substrate solution with diaminobenzidine (0.05 % diaminobenzidine in TBST with 0.05 % H_2O_2) was used. The sections were counterstained with hematoxylin before being examined by light microscope.

Negative controls with omission of primary antibody were processed.

Statistical analysis

Statistical differences between groups were evaluated by one way ANOVA followed by the Newman–Keuls test. p values of less than 0.05 were considered significant. The results are expressed as the mean ± standard error (SEM). For these analyses, GraphPad software was used.

Results

Rats injected with 1, 2 and 5 mg/kg b.w., i.p. of cisplatin showed no differences in plasma urea and creatinine levels as compared to control animals. In contrast, these parameters were significantly increased in Cis10 group in comparison with control rats as it is shown in Fig. 1a, b. Creatinine clearance was also significantly modified only in Cis10 animals (Fig. 1c). These results reflect renal dysfunction and kidney injury in rats treated with the highest dose of cisplatin. No modifications were observed in kidney/body weight ratio and in urine output in all the experimental groups. The treatment with cisplatin produced a significant decrease in body weight in all the doses evaluated in this experiment as previously described (Table 1) (Bearcroft et al. 1999; Ali et al. 2007). The decrease in body weight observed in control animals was due to the food deprivation during the 24 h of lodging in metabolic cages as it has been described by other authors (Chatamra et al. 1984; Vermeulen et al. 1997; Claassen 1994). In this connection, previous data from our laboratory showed that rats injected with saline solution at time 0 which were not put in metabolic cages and were no fasted did not showed a decrease in body weights, on the contrary, they showed in 48 h a slight increase (+0.21 %).

Histological evaluation of kidneys from rats treated with 10 mg/kg cisplatin showed tubular dilatation, tubular desquamation cells and disrupted tubular basement membranes as compared to control histology, as previously described (Yokoo et al. 2007) (Fig. 2). However, no histological injuries were detected in Cis1, Cis2 and Cis5 groups.

Organic anion transporter 5 abundance in urine was related to urinary creatinine concentrations in order to correct for variations in urine production as previously described for urinary transporters and enzymes (Heiene et al. 2001; Umenishi et al. 2002; Di Giusto and Torres 2010). Measurements of biomarkers alone are insufficient because normal physiological variations in water excretion can dilute or concentrate urinary proteins. Normalization on the basis of total protein amount is generally unsatisfactory because total protein excretion can vary broadly among various pathological states. Creatinine is excreted in the urine at relative constant rates allowing it to be used to normalize urinary excretion of a particular protein.

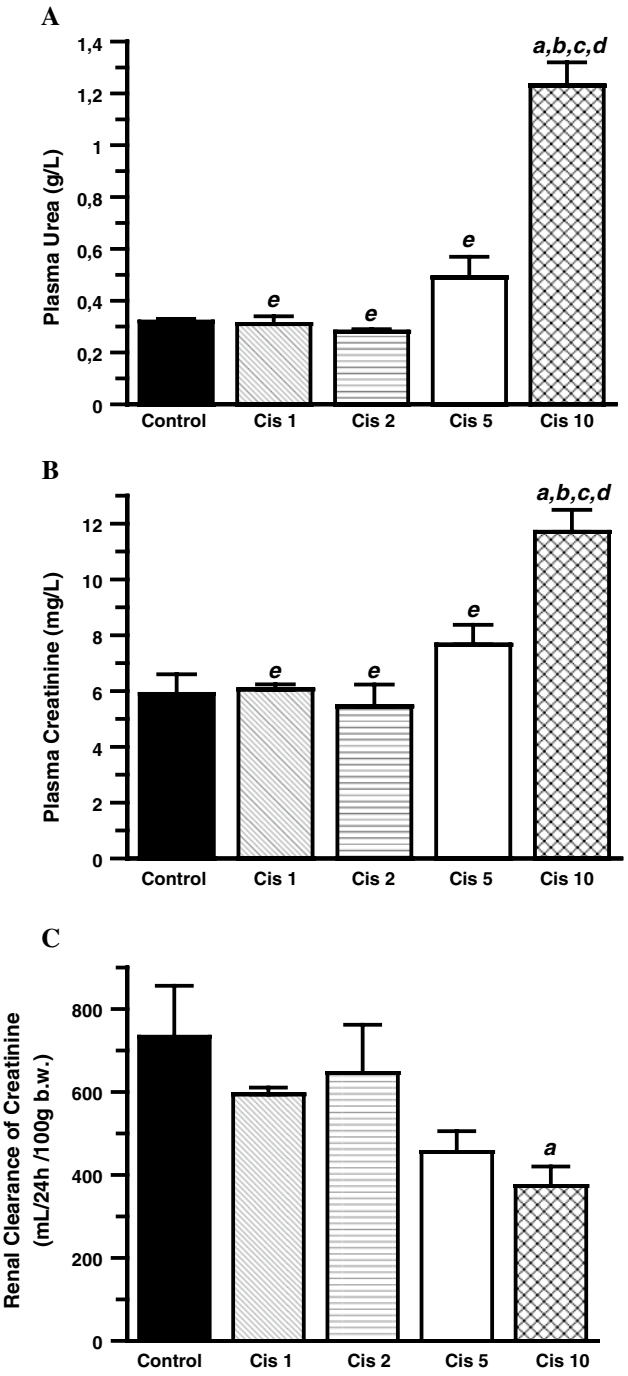


Fig. 1 **a** Urea and **b** creatinine plasma levels, and **c** renal clearance of creatinine in control and treated animals with different doses of cisplatin, Cis1 ($n = 4$), Cis2 ($n = 4$), Cis5 ($n = 4$) and Cis10 ($n = 4$), 48 h after cisplatin administration. Results are expressed as mean values ± SEM. (*a*) $p < 0.05$ versus control, (*b*) $p < 0.05$ versus Cis1, (*c*) $p < 0.05$ versus Cis2, (*d*) $p < 0.05$ versus Cis5, (*e*) $p < 0.05$ versus Cis10

Figure 3a shows that Oat5 urinary levels were significantly higher in all animals treated with cisplatin. As shown in this figure, the Oat5 urinary abundance increase

Table 1 Body weight decreased in 48 h of treatment, kidney/body weight ratio and urine volume in control and treated rats with cisplatin, Cis1, Cis2, Cis5 and Cis10, 48 h after cisplatin administration

	Control ($n = 4$)	Cis1 ($n = 4$)	Cis2 ($n = 4$)	Cis5 ($n = 4$)	Cis10 ($n = 4$)
Body weight decreased in 48 h (%)	5.81 ± 0.65	$7.98 \pm 0.69^{a,c}$	9.10 ± 0.27^{a}	8.71 ± 0.47^{a}	$10.17 \pm 0.12^{a,b}$
Kidney/body weight ratio ($\times 10^{-3}$)	6.70 ± 0.20	7.10 ± 0.10	6.80 ± 0.10	6.80 ± 0.20	6.90 ± 0.20
Urine Volume (μL/min/100 g)	2.60 ± 0.27	2.56 ± 0.28	2.93 ± 0.28	2.92 ± 0.35	3.31 ± 0.20

Body weight decreased in 48 h (%) $= -[(BW48 - BW0)/BW0] \times 100$

BW0 Initial body weight at time 0, just before the injection of saline solution (control rats) or cisplatin

BW48 Body weight after 48 h of the injection and after 24 h of putting the animals inside the metabolic cages without food

Results are expressed as means values ± SEM

[a] $P < 0.05$ versus control

[b] $P < 0.05$ versus Cis1

[c] $P < 0.05$ versus Cis10

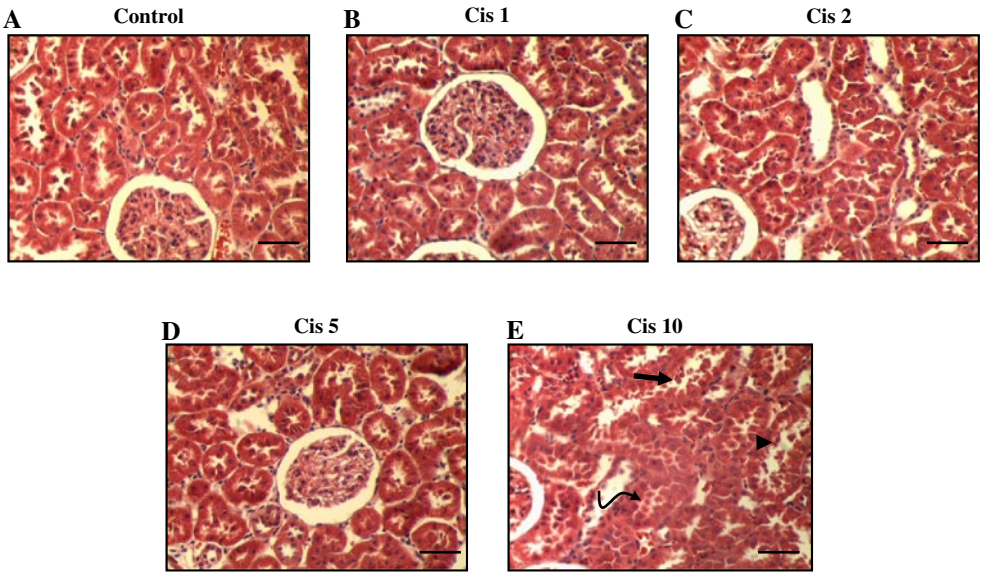


Fig. 2 Optical microscopy photos of kidney histology in control (**a**), Cis1 (**b**), Cis2 (**c**), Cis5 (**d**) and Cis10 (**e**) rats. (Hematoxylin–eosin staining). In Cis1, Cis2 and Cis5 groups, no histological damages were detected compared to control kidneys; meanwhile, in Cis10 group tubular dilatation (*arrow head*), tubular desquamation cells (*arrow*) and disrupted tubular basement membranes (*curved arrow*) were observed. These *pictures* are representatives of samples obtained from 4 animals from each experimental group. *Bars* 40 μm

is proportional to the dose of cisplatin used. To evaluate possible tubular injury, urinary activity of AP and protein and glucose urine concentration were measured and related to urinary creatinine concentration. As it is shown in Fig. 3b, c, d, all of these three parameters were only significantly increased in urine samples of Cis10-treated rats as compared to control animals, indicating that significant renal tubule damage was produced at this dose of cisplatin.

The kidney cortex homogenates and the apical membranes from control and treated animals were subjected to Western blotting for Oat5 protein. Figure 4a, b shows a decrease in Oat5 expression in homogenates as well as in apical membranes in treated animals with different doses of cisplatin as compared to control rats.

Immunohistochemistry studies showed that Oat5 labeling was associated with the apical membrane and the intracellular domains in proximal tubule cells (Fig. 5). Oat5 labeling was consistent with the density observed by Western blotting in each experimental group. Representative Oat5 labeling for Control, Cis2 and Cis10 groups is shown in Fig. 5a, b, c. Negative controls with omission of primary antibody revealed no labeling for Control, Cis2 and Cis10 groups (Fig. 5d).

Discussion

Acute kidney injury is a morbid and costly disorder, which present a very large proportion of patients progressing to chronic renal failure requiring dialysis (Star 1998). Over the last 50 years, mortality rates have remained basically unchanged for pediatric and adult patients at 50–70 and 30 %, respectively.

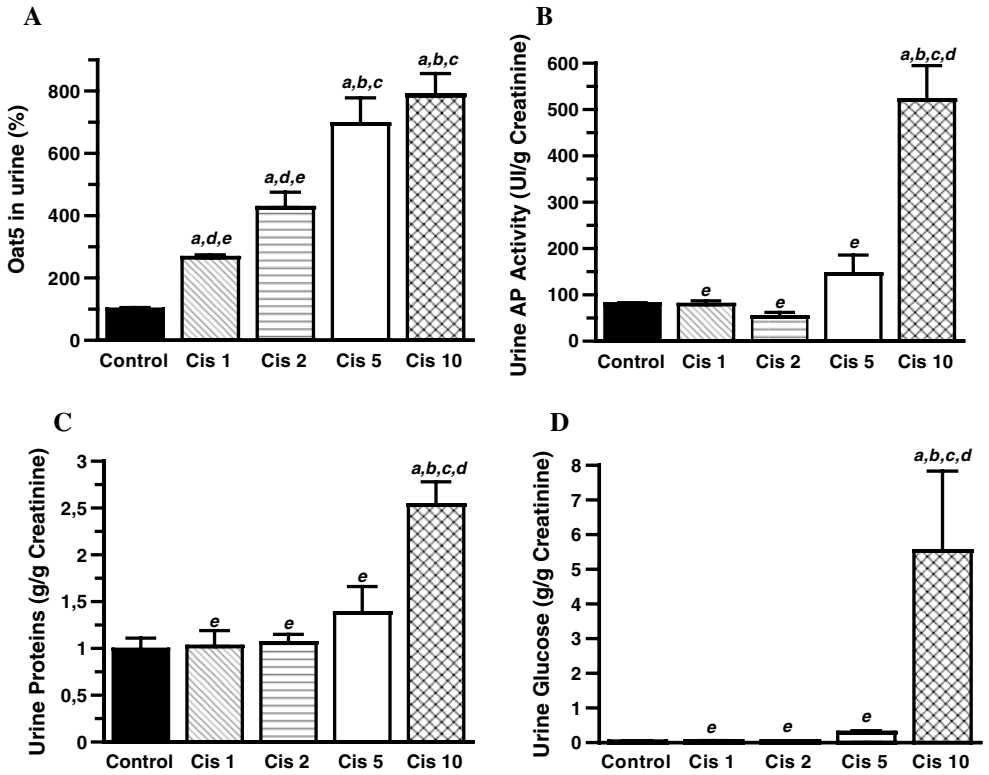


Fig. 3 Oat5 abundance in urine (**a**), AP urinary activity (**b**), protein (**c**) and glucose urinary (**d**) levels in control ($n = 4$), Cis1 ($n = 4$), Cis2 ($n = 4$), Cis5 ($n = 4$), Cis10 ($n = 4$) rats. For the assay of Oat5 abundance, urine samples were separated by SDS-PAGE and blotted onto nitrocellulose membranes. Densitometric quantification of Oat5 Western blotting from urine are expressed as arbitrary units related to urinary creatinine concentration in order to correct for variations in urine production. The mean of the control value was set as 100 %. AP urinary activity, protein and glucose urinary levels were determined using commercial kits and were related to urinary creatinine concentration in order to correct for variations in urine production. Results are expressed as mean values ± SEM. (*a*) $p < 0.05$ versus control, (*b*) $p < 0.05$ versus Cis1, (*c*) $p < 0.05$ versus Cis2, (*d*) $p < 0.05$ versus Cis5, (*e*) $p < 0.05$ versus Cis10

Many studies have reported that drug nephrotoxicity contributed to 19 % of AKI cases and there is evidence that the incidence of nephrotoxic AKI in hospitalized patients has raised over the last two decades (Uchino et al. 2005). AKI is characterized by an alteration of renal function over a period of hours to days, resulting in retention of urea, creatinine and others waste products excreted by the kidney. AKI can be caused by: a decrease in renal perfusion without cellular injury; a reduction in filtering capacity of the glomerulus; and a toxic, ischemic or obstructive insult to the renal tubule or a tubulointerstitial process with inflammation and edema. A variety of nephrotoxic agents, such as cisplatin, gentamicin, $HgCl_2$ and glycerol, among others, have been known to produce cytotoxic effects on renal tubular cells (Ferguson et al. 2008). Serum creatinine remains as the marker of choice in preclinical animal studies of kidney injury, though histopathology is frequently used. However, creatinine is an unreliable indicator during acute changes in kidney function, and its concentration might not change until about 25–50 % of kidney function has already been lost and also is not very reflective of glomerular filtration rate owing to a lot of renal and non-renal influences (Ivanišević et al. 2013). In the setting of AKI, the time relationship between changes in serum creatinine and changes in glomerular filtration rates complicates the ability to estimate accurately timing and severity of injury. Consequently, big changes in glomerular filtration rates could be associated with moderately small changes in serum creatinine in the first 24–48 h following AKI, resulting in a delayed diagnosis and intervention and also in underestimation of the degree of injury (Ferguson et al. 2008).

Urine is an ideal non-invasive source of biomarkers to diagnose and classify kidney diseases. However, conventional urine markers (casts, fractional excretion of sodium, urine osmolality, etc.) are non-specific and insensitive (Zhou et al. 2006a). The insensitivity and non-specificity of serum creatinine in addition to other conventionally used markers of renal injury, including blood urea nitrogen and urinary markers, have been obstacles in assessing for nephrotoxicity as well as in developing strategies to ameliorate injury and do not directly reflect injury to kidney

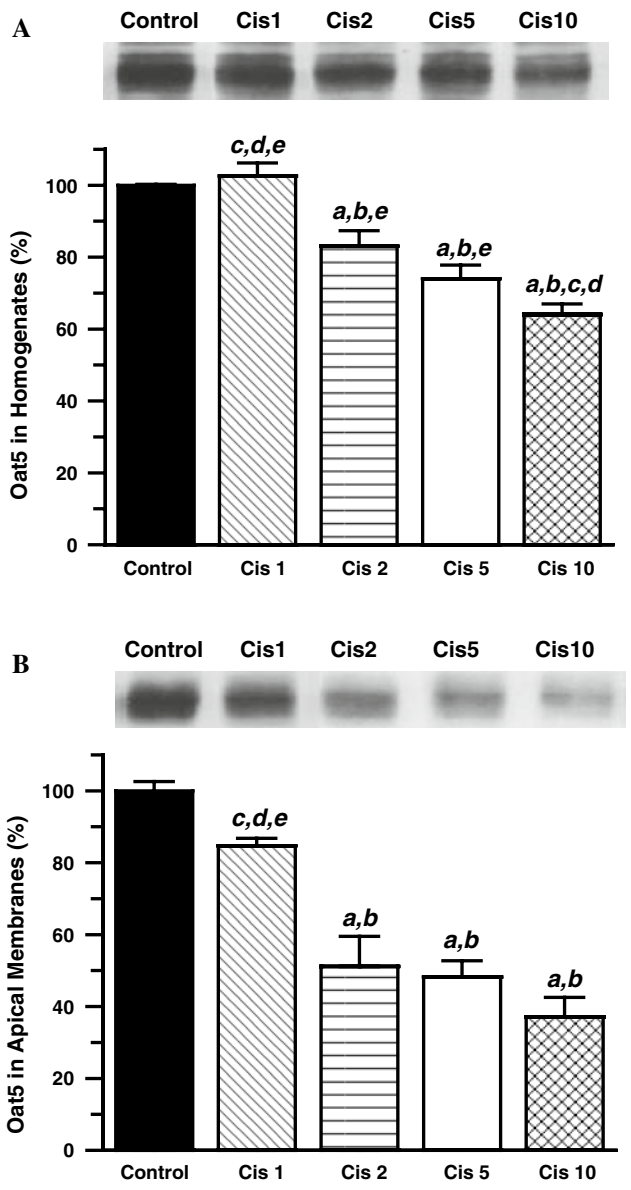


Fig. 4 Western blotting for Oat5 in homogenates (**a**, 20 μg proteins) and apical membranes (**b**, 16 μg proteins) from kidneys of control and treated rats with cisplatin, Cis1, Cis2, Cis5 and Cis10. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The mean of the control value was set as 100 %. Results are expressed as mean values ± SEM. (*a*) $p < 0.05$ versus control, (*b*) $p < 0.05$ versus Cis1, (*c*) $p < 0.05$ versus Cis2, (*d*) $p < 0.05$ versus Cis5, (*e*) $p < 0.05$ versus Cis10

cells (Vaidya et al. 2008). This delay in diagnosis and intervention predisposes to an increased morbidity and mortality besides irreversible injury resulting in chronic kidney failure (Ferguson et al. 2008).

Several previous studies have identified numerous potential biomarkers, including urinary neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1), interleukin-18 (IL-18) and *N*-acetyl-β-D-glucosaminidase (NAG) that differentiate well between individuals with and without AKI. It has been suggested that urinary NGAL levels increased 10–100-fold in rodents after cisplatin-induced nephrotoxicity and in patients with septic and ischemic AKI. Also, high levels of urinary NGAL predicted the beginning of AKI 2 h after cardiopulmonary bypass in children undergoing cardiac surgery, 2–4 days earlier than AKI was identified by changes in serum creatinine (Vaidya et al. 2008).

Our group has recently postulated urinary excretion of Oat5 as an early and novel biomarker of proximal tubule damage in ischemic and mercuric induced AKI (Di Giusto et al. 2009; Di Giusto and Torres 2010). Oat5 is an organic anion transporter that is expressed exclusively in the kidney, and it is found at the apical membranes of proximal tubules and functions as a dicarboxylate/organic anion transporter (Youngblood and Sweet 2004; Anzai et al. 2005, 2006). In ischemia–reperfusion study (Di Giusto et al. 2009), in bilateral mild subclinical ischemia group, there were no significant alterations in plasma creatinine levels and in urinary AP activity, parameters that are commonly used as biomarkers for renal injury. In contrast, there was a significantly increase in urinary Oat5 excretion. In a $HgCl_2$ dose–response study (Di Giusto and Torres 2010), plasma urea and creatinine levels and AP urinary activity were not significantly modified and no histological damage was observed in $HgCl_2$ dose of 0.2 mg/kg b.w., s.c. However, urinary excretion of Oat5 was elevated at that dose of $HgCl_2$. In both models of AKI, the abundance of Oat5 in urine was significantly higher in treated animals when none of the conventional used biomarkers of renal injury were able to predict this.

The aim of the present study was to evaluate whether Oat5 urinary excretion is a more sensitive and earlier biomarker of cisplatin-induced AKI compared to traditionally used biomarkers of renal injury. Our intention was to perform a cisplatin dose–response study, based in previous data (Yonezawa et al. 2005; Yokoo et al. 2007). In this experimental model, the kidney/body weight ratio, plasma urea and creatinine levels, creatinine clearance, AP urinary activity and protein and glucose urinary levels were significantly modified only at the highest cisplatin dose of 10 mg/kg b.w., i.p., as compared to the control rats. On the contrary, Oat5 urinary abundance was increased in a dose-related manner after cisplatin administration. The increases were around 165, 326, 593 and 686 % higher than control urinary Oat5 excretion for cisplatin doses of 1, 2, 5 and 10 mg/kg b.w., i.p., respectively. Oat5 urinary abundance was elevated at a dose as low as of 1 mg/kg b.w., i.p., allowing predicting renal perturbation, when no modifications of traditional markers of renal injury are still observed.

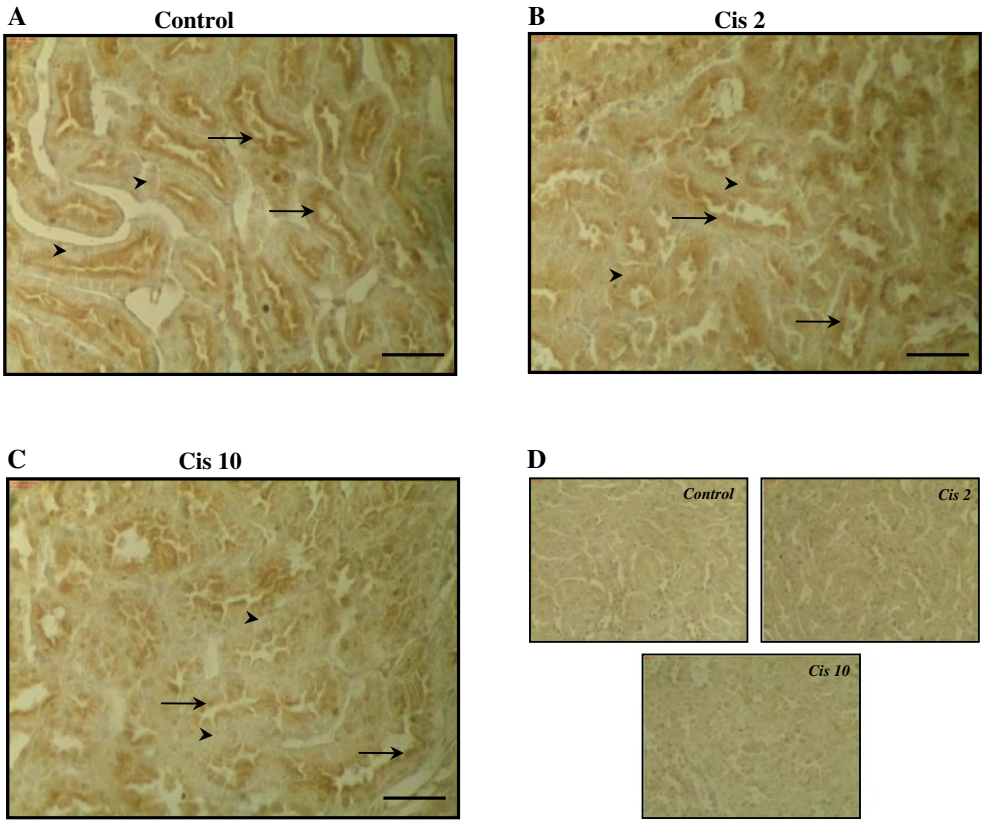


Fig. 5 Immunohistochemistry for Oat5 in renal tissue from control (**a**), Cis2 (**b**) and Cis10 (**c**) rats. Serial sections from each rat kidney were stained using a non-commercial anti-Oat5 antibody. Oat5 labeling was associated with the apical plasma membranes (*arrows*) and with intracellular domains in proximal tubule cells (*arrowheads*). A decrease in Oat5 labeling was observed both in apical plasma membranes and in the intracellular domains in treated rats with cisplatin. (**d**) Renal tissue from control, Cis2 and Cis10 rats incubated with buffer instead of anti-Oat5 antibody. These pictures are representatives of typical samples obtained from 4 animals from each experimental group. *Bars* 40 μm

In this model, Oat5 renal expression was decreased in a dose-related manner, both in homogenates and apical membranes from cisplatin-treated kidneys, suggesting a decrease in Oat5 synthesis or an increase in its degradation, probably due to the tubular damage caused by cisplatin. The ratio between the Oat5 abundance in apical membranes to the abundance in the homogenates was lower than unity (data not shown) also in a dose-related manner in rats exposed to cisplatin. The increase in urinary Oat5 excretion might explain the decrease in the amount of Oat5 molecules in the apical membranes. It has been described that urinary biomarkers as fetuin-A, Na^+/H^+ exchanger isoform 3 (NHE3) and activating transcription factor 3 (ATF3), between others, have been found in exosomes and it has been reported that they are increased in patients with AKI (du Cheyron et al. 2003; Zhou et al. 2006a, 2008). Exosomes are the internal vesicles of multivesicular bodies (MVBs) that are delivered to the extracellular fluid by fusion of the outer membrane of MVB with the plasma membrane. They are derived from all cell types that face the urinary space including glomerular podocytes, renal tubule cells and the cells lining the urinary drainage system (Zhou et al. 2006b). It is possible to postulate that Oat5 excretion into urine involves exosomal pathway. In this connection, it has been described that carriers from the same family of Oat5 (Slc22a19) such as Oat1 (Slc22a6) and Oat3 (Slc22a8) are found in urinary exosomes (Urinary exosome protein database, http://dir.nhlbi.nih.gov/papers/lkem/exosome/). The increase in Oat5 in urine following cisplatin administration could be due, at least in part, to an increase in the release of the exosomes to the urinary space caused by tubular cisplatin toxicity. Current experiments are being performed in our laboratory to evaluate this possibility. Moreover, at the higher dose of cisplatin (10 mg/kg b.w., i.p.), the tubular damage (and the consequent loss of brush border membrane) observed by the renal histopathological studies in the present work and also described by Sinha et al. (2013) might be an additional explanation for the increase in Oat5 in urine.

The kidney accumulates cisplatin to a greater degree than other organs. The S3 segment of the proximal tubule accumulates the highest concentration of cisplatin, this zone of the kidney is more susceptible to ischemic insult, and injury to this segment occurs in other toxic acute renal failure models. Uptake of cisplatin into renal cells is mainly through the organic cation transporter pathway, such as OCT2 (Yao et al. 2007; Yokoo et al. 2007). Human and rodents exhibit comparable pathological changes and time course for the toxicity induced by cisplatin (Dobyan et al. 1980), and it has been reported that the nephrotoxic effect of cisplatin is cumulative and dose dependent. The in vivo mechanisms of cisplatin nephrotoxicity are complex and

involve oxidative stress, apoptosis, inflammation and fibrogenesis. It has been reported that high concentrations of cisplatin induce necrosis in proximal tubule cells, whereas lower concentrations induce apoptosis. Hypoxia and mitochondrial injury are also involved in this nephrotoxic mechanism. Many studies have described that 48–72 h after cisplatin administration, there are impaired proximal and distal tubular reabsorption and increased vascular resistance, following a second phase that starts between 72 and 96 h after cisplatin administration, and are characterized by a decreased glomerular filtration rates (Yao et al. 2007; Miller et al. 2010).

An ideal biomarker for the detection of AKI is one that is easily obtained, easily and rapidly measured, sensitive to minor disturbances in kidney function, site specific, highly correlated with the degree of injury and indicative of injury progression and regression. A single biomarker is rarely adequate to clearly define a particular pathologic state (Rifai et al. 2006; Fliser et al. 2007). It was previously reported that 48 h after cisplatin administration, rats treated with cisplatin at a low dose of 2 mg/kg b.w., i.p., NAG activity in bladder urine was increased and KIM-1 mRNA in renal tissue was detected while plasma creatinine levels, creatinine clearance, BUN and urinary albumin levels were unchanged, as compared to control animals. At a higher dose of cisplatin (10 mg/kg b.w., i.p.), all of those parameters were significantly modified (Yonezawa et al. 2005; Yokoo et al. 2007). We have found a statistically significant elevation in urine Oat5 abundance at a cisplatin dose as low as 1 mg/kg b.w., i.p. 48 h after cisplatin administration, indicating that Oat5 urine excretion is a more sensitive biomarker than traditional and novel biomarkers of renal injury.

Hence, the preclinical animal results showed in this work in addition to the data presented before by our group (Di Giusto et al. 2009; Di Giusto and Torres 2010) propose that Oat5 urinary excretion might potentially serve as a non-invasive early biomarker of mercury, ischemic and cisplatin-induced AKI.

Kidney injury might occur under different contexts; those are the reason why a panel of carefully selected biomarkers should be most appropriate for most accurate AKI information. Development of that kind of panel will require large, well-designed studies comparing multiple biomarkers in the same set of urine samples over extended time courses, allowing this to established temporal patterns of biomarker modification. This kind of studies will also allowed to determine patterns that may be specific to the mechanism of nephrotoxicant-induced injury, population of interest and/or co-occurring disease states (diabetes, heart disease, sepsis, between others). Diverse biomarkers will probably provide different kinds of information. Some of them will reflect systemic influences; meanwhile, others will reflect the location and extent of tubular injury or reflect the functional consequences of tubular damage (Ferguson et al. 2008).

So far, nearly all studies have pointed out discovery, characterization and validation of individual biomarkers using a single model of kidney injury. This is a procedure that is necessary in the initial stages of biomarker development; nevertheless, translation to clinical applicability requires substantial additional work.

Acknowledgments This study was supported by the following grants: Fondo para la Investigación Científica y Tecnológica (FONCYT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de Rosario (UNR), Secretaría de Estado de Ciencia, and Tecnología e Innovación de Santa Fe (SECTeI). The authors thank Prof. H. Endou and Prof. N. Anzai (Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan) for kindly providing Oat5-specific antibodies and Mrs Alejandra Martínez (Area Morfología, Facultad de Ciencias Bioquímicas y Farmacéuticas, U.N.R.) for her collaboration in the present work. The authors also thank Wiener Lab Argentina for analytical kits.

References

Aleksunes LM, Augustine LM, Scheffer GL, Cherrington NJ, Manautou JE (2008) Renal xenobiotic transporters are differentially expressed in mice following cisplatin treatment. Toxicology 250:82–88

Ali BH, Al Moundhri MS, Tag Eldin M, Nemmar A, Tanira MO (2007) The ameliorative effect of cysteine prodrug L-2-oxothiazolidine-4-carboxylic acid on cisplatin-induced nephrotoxicity in rats. Fundam Clin Pharmacol 21:547–553

Anzai N, Jutabha P, Enomoto A, Yokoyama H, Nonoguchi H, Hirata T, Shiraya K, He X, Cha SH, Takeda M, Miyazaki H, Sakatada T, Tomita K, Igarashi T, Kanai Y, Endou H (2005) Functional characterization of rat organic anion transporter 5 (Slc22a19) at the apical membrane of renal proximal tubules. J Pharmacol Exp Ther 315:534–544

Anzai N, Kanai Y, Endou H (2006) Organic anion transporter family: current knowledge. J Pharmacol Sci 100:411–426

Arjumand W, Seth A, Sultana S (2011) Rutin attenuates cisplatin induced renal inflammation and apoptosis by reducing NFκB, TNF-α and caspase-3 expression in wistar rats. Food Chem Toxicol 49:2013–2021

Bearcroft CP, Domizio P, Mourad FH, André EA, Farthing MJ (1999) Cisplatin impairs fluid and electrolyte absorption in rat small intestine: a role for 5-hydroxytryptamine. Gut 44:174–179

Brandoni A, Anzai N, Kanai Y, Endou H, Torres AM (2006) Renal elimination of p aminohippurate (PAH) in response to three days of biliary obstruction in the rat. The role of OAT1 and OAT3. Biochim Biophys Acta 1762:673–682

Breljak D, Ljubojevic M, Balen D, Zlender V, Brzica H, Micek V, Kusan M, Anzai N, Sabolic I (2010) Renal expression of organic anion transporter Oat5 in rats and mice exhibits the female-dominant sex differences. Histol Histopathol 25:1385–1402

Bulacio R, Hazelhoff MH, Torres AM (2012) Renal expression and function of Oat1 and Oat3 in rats with vascular calcification. Pharmacology 90:66–77

Burckhardt G (2012) Drug transport by organic anion transporters (OATs). Pharmacol Ther 136:106–130

Camano S, Lazaro A, Moreno-Gordaliza E, Torres AM, de Lucas C, Humanes B, Lazaro JA, Milagros Gomez-Gomez M, Bosca

L, Tejedor A (2010) Cilastatin attenuates cisplatin-induced proximal tubular cell damage. J Pharmacol Exp Ther 334: 419–429

Chatamra K, Daniel PM, Lam DK (1984) The effects of fasting on core temperature, blood glucose and body and organ weights in rats. Q J Exp Physiol 69:541–545

Claassen V (1994) Neglected factors in pharmacology and neuroscience research. Biopharmaceutics, Animals Characteristics, Maintenance, Testing Conditions. Elsevier, Amsterdam, In, pp 290–320

Di Giusto G, Torres AM (2010) Organic anion transporter 5 renal expression and urinary excretion in rats exposed to mercuric chloride: a potential biomarker of mercury-induced nephropathy. Arch Toxicol 84:741–749

Di Giusto G, Anzai N, Endou H, Torres AM (2009) Oat5 and NaDC1 protein abundance in kidney and urine after renal ischemic reperfusion injury. J Histochem Cytochem 57:17–27

Dobyan DC, Levi J, Jacobs C, Kosek J, Weiner MW (1980) Mechanism of cis-platinum nephrotoxicity: II. Morphologic observations. J Pharmacol Exp Ther 213:551–556

dos Santos NA, Martins NM, Curti C, Pires Bianchi M, dos Santos AC (2007) Dimethylthiourea protects against mitochondrial oxidative damage induced by cisplatin in liver of rats. Chem Biol Interact 170:177–186

du Cheyron D, Daubin C, Poggioli J, Ramakers M, Houillier P, Charbonneau P, Paillard M (2003) Urinary measurement of Na+/H+ exchanger isoform 3 (NHE3) protein as new marker of tubule injury in critically ill patients with ARF. Am J Kidney Dis 42:497–506

Ferguson MA, Vaidya VS, Bonventre JV (2008) Biomarkers of nephrotoxic acute kidney injury. Toxicology 245:182–193

Fliser D, Novak J, Thongboonkerd V, Argilés A, Jankowski V, Girolami MA, Jankowski J, Mischak H (2007) Advances in urinary proteome analysis and biomarker discovery. J Am Soc Nephrol 18:1057–1071

Heiene R, Moe L, Mølmen G (2001) Calculation of urinary enzyme excretion, with renal structure and function in dogs with pyometra. Res Vet Sci 70:129–137

Hosohata K, Ando H, Fujimura A (2012) Urinary vanin-1 as a novel biomarker for early detection of drug-induced acute kidney injury. J Pharmacol Exp Ther 341:656–662

Ivanišević I, Peco-Antić A, Vuličević I, Hercog D, Milovanović V, Kotur-Stevuljević J, Stefanović A, Kocev N (2013) L-FABP can be an early marker of acute kidney injury in children. Pediatr Nephrol 28:963–969

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Miller RP, Tadagavadi RK, Ramesh G, Reeves WB (2010) Mechanisms of cisplatin nephrotoxicity. Toxins 2:2490–2518

Muraki K, Koyama R, Honma Y, Yagishita S, Shukuya T, Ohashi R, Takahashi F, Kido K, Iwakami S, Sasaki S, Iwase A, Takahashi K (2012) Hydration with magnesium and mannitol without furosemide prevents the nephrotoxicity induced by cisplatin and pemetrexed in patients with advanced non-small cell lung cancer. J Thorac Dis 4:562–568

Pinches M, Betts C, Bickerton S, Burdett L, Thomas H, Derbyshire N, Jones HB, Moores M (2012a) Evaluation of novel renal biomarkers with a cisplatin model of kidney injury: gender and dosage differences. Toxicol Pathol 40:522–533

Pinches MD, Betts CJ, Bickerton SJ, Beattie L, Burdett LD, Thomas HT, Derbyshire NA, Moores M (2012b) Evaluation of novel urinary renal biomarkers with a cisplatin model of kidney injury: effects of collection period. Toxicol Pathol 40:534–540

Pinches MD, Betts CJ, Bickerton SJ, Beattie L, Burdett LD, Thomas HT, Derbyshire NA, Moores M, Price SA (2012c) Evaluation of novel urinary renal biomarkers: biological variation and reference change values. Toxicol Pathol 40:541–549

Rifai N, Gillette MA, Carr SA (2006) Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat Biotechnol 24:971–983

Sinha V, Vence LM, Salahudeen AK (2013) Urinary tubular protein-based biomarkers in the rodent model of cisplatin nephrotoxicity: a comparative analysis of serum creatinine, renal histology, and urinary KIM-1, NGAL, and NAG in the initiation, maintenance, and recovery phases of acute kidney injury. J Investig Med 61:1–5

Star RA (1998) Treatment of acute renal failure. Kidney Int 54:1817–1831

Taguchi T, Nazneen A, Abid MR, Razzaque MS (2005) Cisplatin-associated nephrotoxicity and pathological events. Contrib Nephrol 148:107–121

Tiseo M, Martelli O, Mancuso A, Sormani MP, Bruzzi P, Di Salvia R, De Marinis F, Ardizzoni A (2007) Short hydration regimen and nephrotoxicity of intermediate to high-dose cisplatin-based chemotherapy for outpatient treatment in lung cancer and mesothelioma. Tumori 93:138–144

Uchino S, Kellum JA, Bellomo R, Doig GS, Morimatsu H, Morgera S, Schetz M, Tan I, Bouman C, Macedo E, Gibney N, Tolwani A, Ronco C (2005) Acute renal failure in critically ill patients: a multinational, multicenter study. JAMA 294:813–818

Uehara T, Yamate J, Torii M, Maruyama T (2011) Comparative nephrotoxicity of cisplatin and nedaplatin: mechanisms and histopathological characteristics. J Toxicol Pathol 24:87–94

Umenishi F, Summer SN, Cadnapaphornchai M, Shrier RW (2002) Comparison of three methods to quantify urinary aquaporin-2 protein. Kidney Int 62:2288–2293

Vadiei K, Siddik ZH, Khokhar AR, Al-Baker S, Sampedro F, Perez-Soler R (1992) Pharmacokinetics of liposome-entrapped cis-bis-neodecanoato-trans-R, R-1,2-diamino-cyclohexane platinum(II) and cisplatin given i.v. and i.p. in the rat. Cancer Chemother Pharmacol 30:365–369

Vaidya VS, Waikar SS, Ferguson MA, Collings FB, Sunderland K, Gioules C, Bradwin G, Matsouaka R, Betensky RA, Curhan GC, Bonventre JV (2008) Urinary biomarkers for sensitive and specific detection of acute kidney injury in humans. Clin Transl Sci 1:200–208

Vermeulen JK, de Vries A, Schlingmann F, Remie R (1997) Food deprivation: common sense or nonsense? Anim Technol 48:45–54

Yao X, Panichpisal K, Kurtzman N, Nugent K (2007) Cisplatin nephrotoxicity: a review. Am J Med Sci 334:115–124

Yokoo S, Yonezawa A, Masuda S, Fukatsu A, Katsura T, Inui K (2007) Differential contribution of organic cation transporters, OCT2 and MATE1, in platinum agent-induced nephrotoxicity. Biochem Pharmacol 74:477–487

Yonezawa A, Masuda S, Nishihara K, Yano I, Katsura T, Inui K (2005) Association between tubular toxicity of cisplatin and expression of organic cation transporter rOCT2 (Slc22a2) in the rat. Biochem Pharmacol 70:1823–1831

Youngblood GL, Sweet DH (2004) Identification and functional assessment of the novel murine organic anion transporter Oat5 (Slc22a19) expressed in kidney. Am J Physiol Renal Physiol 287:F236–F244

Zhou H, Pisitkun T, Aponte A, Yuen PS, Hoffert JD, Yasuda H, Hu X, Chawla L, Shen RF, Knepper MA, Star RA (2006a) Exosomal fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int 70:1847–1857

Zhou H, Yuen PS, Pisitkun T, Gonzales PA, Yasuda H, Dear JW, Gross P, Knepper MA, Star RA (2006b) Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int 69:1471–1476

Zhou H, Cheruvanky A, Hu X, Matsumoto T, Hiramatsu N, Cho ME, Berger A, Leelahavanichkul A, Doi K, Chawla LS, Illei GG, Kopp JB, Balow JE, Austin HA 3rd, Yuen PS, Star RA (2008) Urinary exosomal transcription factors, a new class of biomarkers for renal disease. Kidney Int 74:613–621