Published in final edited form as:

J Appl Toxicol. 2010 March ; 30(2): 172–182. doi:10.1002/jat.1484.

Age-related differences in susceptibility to cisplatin-induced renal toxicity†

P. Espandiar^{a,*}, B. Rosenzweig^a, J. Zhang^a, Y. Zhou^b, L. Schnackenberg^c, V. S. Vaidya^d, P. L. Goering^b, R. P. Brown^b, J. V. Bonventre^d, K. Mahjoob^a, R. D. Holland^c, R. D. Beger^c, K. Thompson^a, J. Hanig^a, and N. Sadrieh^a

^aCenter for Drug Evaluation and Research, FDA, Silver Spring, MD 20993, USA

^bCenter for Devices and Radiological Health, FDA, Silver Spring, MD 20993, USA

^cNational Center for Toxicological Research, FDA, Jefferson, AR 7209, USA

^dHarvard Medical School, Boston, MA 02115, USA

Abstract

Limited experimental models exist to assess drug toxicity in pediatric populations. We recently reported how a multi-age rat model could be used for pre-clinical studies of comparative drug toxicity in pediatric populations. The objective of this study was to expand the utility of this animal model, which previously demonstrated an age-dependent sensitivity to the classic nephrotoxic compound, gentamicin, to another nephrotoxicant, namely cisplatin (Cis). Sprague-Dawley rats (10, 25, 40 and 80 days old) were injected with a single dose of Cis (0, 1, 3 or 6 mg kg⁻¹ i.p.). Urine samples were collected prior and up to 72 h after treatment in animals that were ≥25 days old. Several serum, urinary and 'omic' injury biomarkers as well as renal histopathology lesions were evaluated. Statistically significant changes were noted with different injury biomarkers in different age groups. The order of age-related Cis-induced nephrotoxicity was different than our previous study with gentamicin: 80 > 40 > 10 > 25 day-old vs 10 ≥ 80 > 40 > 25-day-old rats, respectively. The increased levels of kidney injury molecule-1 (Kim-1: urinary protein/tissue mRNA) provided evidence of early Cis-induced nephrotoxicity in the most sensitive age group (80 days old). Levels of Kim-1 tissue mRNA and urinary protein were significantly correlated to each other and to the severity of renal histopathology lesions. These data indicate that the multi-age rat model can be used to demonstrate different age-related sensitivities to renal injury using mechanistically distinct nephrotoxicants, which is reflected in measurements of a variety of metabolite, gene transcript and protein biomarkers.

Keywords

cisplatin; age-related nephrotoxicity; biomarkers; Kim-1; metabonomics

INTRODUCTION

Patterns for the incidence of adverse drug reactions (ADR) in children can be dissimilar to those occurring in adults receiving the same drug. This may be due to age-related differences

†The contents of this paper do not necessarily reflect any position of the Government or the opinion of the Food and Drug Administration

*Correspondence to: P. Espandiar, Division of Applied Pharmacology Research (HFD-910), Center for Drug Evaluation and Research, Food and Drug Administration, Life Sciences Laboratory Building 64, 10903 New Hampshire Ave, Silver Spring, MD 20993, USA. parvaneh.espandiar@fda.hhs.gov.

This article is a US Government work and is in the public domain in the USA

in maturation pathways responsible for drug absorption, distribution, metabolism and excretion (ADME) (Faustman *et al.*, 2000; Olin 1998; Pirmohamed *et al.*, 1998; Lazarou *et al.*, 1998). In addition, many physiological conditions such as levels of drug metabolizing enzymes, stomach pH, gastrointestinal emptying time, levels of serum albumin and body H₂O : fat ratios are sufficiently different at a young age to cause alterations in drug ADME properties and contribute to different degrees of toxic responses (Blumer and Reed, 1992; Wershill, 1992; Weaver *et al.*, 1991; Cresteil, 1998; Heyman, 1998; Cresteil *et al.*, 1985). One of the limiting factors in understanding ADRs in pediatric populations is the lack of appropriate animal models that can be used to predict the possible consequences of exposure to drugs during the early years of human development. When developing an animal model, it is important to consider the comparability of the stages of development in animal models to that of the intended population. It has been reported that glomerular nephrogenesis in the prenatal human is comparable with that found in 8- to 14-day-old rats, the level of glomerular filtration and tubular secretion occurring in 45- to 180-day-old infants is similar with that observed in 15- to 21-day-old rats, and the completion of nephrogenesis in 35-week prenatal humans is comparable with that reported in a 4- to 6-week-old rat (Travis, 1991; Zoetis, 2003). Therefore, we designed an animal model that included different stages of development (comparable to stages of human development) and evaluated the model with various drugs to observe potential differences in age-related toxicities. Our published findings of these studies with valproic acid, a hepatotoxicant (Espandiari *et al.*, 2007b), and gentamicin, a nephrotoxicant (Espandiari *et al.*, 2007a), indicated that the pattern of age-related toxicity as measured by toxic injury biomarkers was unique to each toxicant and dependent on the animal's age. In the present study, we employed this multi-age animal model in order to evaluate whether the pattern of age-related toxicity with cisplatin (Cis; cis-dichlorodiamine-platinum II), a potent nephrotoxicant, was comparable with that seen with gentamicin, as reported in our previous study.

Cis, an antineoplastic agent, is used for the treatment of various kinds of solid tumors (Taguchi *et al.*, 2005). However, its therapeutic utility is limited due to development of side effects such as acute renal failure in approximately 20% of treated patients (Berns and Ford, 1997; Santoso *et al.*, 2003; Taguchi *et al.*, 2005; Sastry and Kellie, 2005).

The objectives of this study were to: (1) determine how the age-related toxicity of Cis compares with that of a previously tested nephrotoxic drug (gentamicin); (2) evaluate the sensitivity of several new and traditional nephrotoxicity biomarkers and compare the temporal relationship between the appearance of these biomarkers; and (3) examine the correlation between the level of urinary Kim-1 protein, Kim-1/*Havcr1* gene expression, and kidney histopathological lesions. The ultimate goal of this research is to evaluate how a multi-age-animal model could be used to predict toxicity in pediatric populations.

MATERIALS AND METHODS

Animals

Sprague-Dawley (SD) (Harlan, Indianapolis, IN, USA) 10-, 25-, 40-, or 80-day-old rats were used. The acclimation period was different for each age group: 7 days for the 33 and 73-day-old groups and 2 days for the 23-day-old group (to allow dosing at the youngest age feasible). In order to obtain 10-day-old rats, pregnant females (transferred on gestation day 15) were allowed to deliver. After birth, both female and male pups were housed with their dams and were treated beginning at 10 days of age. Male rats were used in all age groups except for the 10-day-old groups, where both female and male pups were included to increase the sample size (for serum biomarkers). All animals, except for 10-days old pups, were housed individually in plastic cages and maintained in a controlled environment (22 °C with a 12 h light-dark cycle). Rats had access to Purina rodent laboratory chow (Purina Mills, St Louis, MO, USA) and water *ad libitum*.

Chemicals

Cis was purchased from Sigma Chemical Co. (St Louis, MO, USA). The drug was dissolved in 0.9% saline at concentrations of 0, 0.2, 0.6 and 1.2 mg ml⁻¹ immediately before use. Formic acid, leucine-enkephalin and all MS standards were from Sigma Aldrich (St Louis, MO, USA). NMR solvents trimethylsilyl-2,2,-3,3-tetradeuteriopropionic acid (TMSP) and deuterium oxide (D₂O) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA).

Experimental Protocol

For collection of pre-dose urine, animals were placed in metabolism cages 12 h before the first injection. The number of animals for each age group of 25, 40 and 80 days old was 16 (four rats for each dose) and for 10-day-old pups was 32 (eight pups for each dose/sex). In this age group, for histopathology, eight kidney samples from male pups were used and, for serum biomarkers, blood from two or three female or male pups for each dose group was pooled to obtain a sufficient volume for the different assays ($n = 3/\text{group}$). Rats were given a single i.p. injection of saline (vehicle control) or 1, 3 or 6 mg kg⁻¹ Cis (injection volume for all age groups was 5 ml kg⁻¹ body weight or 0.5% of body weight). For metabolomic analysis, urine samples were collected from all age groups, except for the 10-day-old (unable to separate maternal and pup urine) at 0, 8, 24, 48 and 72 h after dosing. Seventy-four hours after treatment, all groups were anesthetized with isoflurane and terminal blood samples collected from the abdominal vena cava. The animals were then euthanized by exsanguination. At necropsy, liver, spleen, heart, intestine and kidney were removed, weighed and processed for pathology and other studies. All procedures performed during the course of the study were approved by the Center for Drug Evaluation and Research Institutional Animal Care and Use Committee and were in accord with the Guide for Care and Use of Laboratory Animals.

Pathology

A portion of each tissue collected was fixed in neutral buffered formalin, embedded in paraffin (sectioned at 5 μm) and stained with hematoxylin–eosin. Cis-induced renal lesions were evaluated by light microscopy and classified on a scale of 0–5, according to the severity of tubular cell alterations: 0 = normal histology; 1 = tubular epithelial cell degeneration only (no necrosis); 2–5 = <25, 26–50, 51–75 or >75%, respectively, of the tubular epithelial cells showing necrosis, degeneration, regeneration, tubular dilatation, protein casts, glomerular vacuolization and interstitial lymphocytic infiltration.

Sera Analysis

For clinical chemistry measurements, blood was collected at terminal necropsy (72 h post-dosing). Serum creatinine (Cr) and blood urea nitrogen (BUN) were analyzed using the VetScan analyzer (Abaxis, Inc. Union City, CA, USA).

Kidney injury molecule-1 (Kim-1)—Kim-1 protein in urine was measured by microsphere-based Luminex xMAP technology with monoclonal antibodies raised against rat Kim-1. This technique is an adaptation of a recently developed and validated sandwich enzyme-linked immunosorbent assay (ELISA) assay as described by Vaidya *et al.*, (2005, 2006). For measurements, 30 μl samples of urine from respective control and treated groups were analyzed in duplicate.

N-acetyl- β -D-glucosaminidase (NAG)—Urinary NAG protein was measured by NAG assay kit (Bio-quant, San Diego, CA, USA).

Renal papillary antigen-1 (RPA-1)—The level of urinary RPA-1 was measured by the Biotrin Rat RPA-1 EIA Assay kit (Biotrin International, Dublin, Ireland).

Renal gene expression sample processing and analysis—RNA isolation and RT-PCR analysis were carried out as previously described (Espandiari *et al.*, 2007a). Relative fold changes in renal gene expression were calculated for individual animals by dividing the amount of normalized target mRNA level by the mean in control animals in a given age and dose group. Males and females were pooled in control and dose groups of 10-day-old rats. The statistical significance of differences in relative gene expression levels between controls and dose groups was calculated using a Student's *t*-test comparison for two samples with unequal variance. The threshold for significance was set at $P < 0.05$.

NMR Analysis for Urinary Metabonomics

Urine samples (400 μ l) collected at 0, 8, 24, 48 and 72 h after dosing were combined with 200 μ l of sodium phosphate buffer (pH = 7.4) and 60 μ l of a mixture of 10 mM TMSP (sodium 3-trimethyl-silyl-[2,2,3,3,-d₄]propionate, chemical shift reference standard) and 100 mM imidazole (pH indicator). Proton (¹H) NMR spectra were acquired on a Bruker Avance spectrometer operating at 600.133 MHz for proton and equipped with a triple resonance cryoprobe. Water suppression was achieved through application of the standard Bruker 'noesyprsat' pulse sequence, which suppresses the water peak. For each sample, 32 scans were collected. NMR spectra were processed using ACD/Labs 1D NMR Manager (Toronto, Canada). The raw data zero filled to 131 072 points, multiplied by a 0.3 Hz exponential function and Fourier transformed. The transformed spectra were then phased and baseline corrected. All spectra were autoreferenced to the TMSP peak at 0.0 ppm. The spectra were overlaid in the processing window and grouped. Regions containing the resonances for water, urea and other NMR solvent peaks were removed prior to integration. The total NMR intensity without water, urea, TMSP and other solvent regions was determined for each spectrum along with the intensity of the TMSP peak for each spectrum. Spectra were integrated over 0.02–0.06 ppm widths and the table of integrals was exported as a text file for statistical analysis.

Mass Spectrometry

Urine samples were thawed at room temperature. A 100 μ l aliquot of sample was mixed with 100 μ l of a 1 : 1 mixture of acetonitrile (ACN):H₂O with constant 0.1% formic acid in a 1.5 ml polypropylene centrifuge tube and placed at 5 °C for approximately 1 h. Samples were centrifuged at 13 000 rpm for 5 min. A 40 μ l supernatant aliquot was then diluted with 180 μ l of a solution containing 0.1% formic acid and 0.5% ACN in an LC/MS vial. All samples were stored at –20 °C until analysis. Standards were prepared in the same solvents at concentrations ranging from 1.5 to 50 pg μ l^{–1}. A 2 μ l aliquot was injected into a Waters Triplequad MS. The column was a 1 $\times$ 150 Thermo Acquisil with a 0.5 μ m Phenomenex Krudcatcher filter. The 132 > 68 multiple reaction monitoring (MRM) transition was monitored using the Waters Triplequad MS and used to quantify 4-hydroxyproline. The peak quantified as 4-hydroxyproline was verified by MRM analysis of the standard.

Statistics

Various statistical analyses were performed on the collected measurements. For all comparisons, the P -value ≤ 0.05 was considered as statistically significantly different, with no consideration of the multiple testing P -value adjustment. An analysis of covariance (ANCOVA) model was used to assess the effect of Cis treatment as compared with saline on the body and kidney weight, at the end of the treatment period. For incidence and severity of kidney lesions scores, depending on the severity of kidney lesion, scores of 0–5 were assigned to each animal's kidney. Non-parametric Fisher exact tests were performed to compare observed lesion severity. The statistical significance of differences in relative gene expression levels between time-matched control and treatment groups was calculated using a Student's *t*-test comparison of fold change values between age-matched GM-treated and control groups.

All statistical analyses of NMR data were done using Statistica version 6.0 (Statsoft, Tulsa, OK, USA). Principle component analysis (PCA) based on covariance of the data was applied to the bucketed intensities. Metabolite identification within the 1D proton NMR spectra was accomplished using the Chenomx NMR Suite (Chenomx, Calgary, Canada), which has a database of >220 compounds. The concentrations obtained by Chenomx metabolite concentrations were normalized by the TMSP peak intensity divided by the total NMR intensity excluding the water, urea, TMSP and solvent regions. LC/MS raw data was processed using Waters MassLynx software. MRM intensities were evaluated and quantified in EXCEL.

RESULTS

Growth, Liver, Spleen, Heart

Cis-induced nephrotoxicity was compared in SD rats in different age groups. No deaths occurred in any of the treatment groups. The effects of Cis on body weight and kidney weight are summarized in Table 1. The rate of growth (final body weight to the initial body weight expressed as percent of control) was significantly decreased in all age groups with the highest dose of Cis (6 mg kg⁻¹). The ratio of kidney, liver, heart and spleen weights to the final body weights of the animals was determined. With the highest dose of Cis, the percentage kidney weight of only the 80-day-old rats was significantly decreased (Table 1). With the same dose of Cis, liver weights in 40- and 10-day-old rats and spleen weights in all age groups except for 80-day-old rats significantly declined (data not shown). No changes were observed in the heart in any group.

Clinical Chemistry

The highest dose of Cis (6 mg kg⁻¹) significantly increased the levels of BUN in 10-, 40- and 80-day-old rats and the levels of serum Cr in 40- and 80-day-old rats. However, in 80-day-old rats, these changes were also seen with the lower dose of Cis (3 mg kg⁻¹) (Fig. 1A and B). The significant changes were seen in BUN and serum Cr data in 10-day-old pups data from female and male pups were pooled in each dose group. At 6 mg kg⁻¹ Cis, the levels of both BUN and Cr were increased; however this increase was only statistically significant for BUN values. The lack of statistical significance for the levels of serum Cr in the 6 mg kg⁻¹ treated 10-day-old pups could be due to two male pup outliers with low levels of serum Cr. Moreover, serum Cr is not a sensitive nephrotoxic biomarker and arises only after significant nephrotoxicity has progressed. In addition, the large increase in the level of serum BUN in this group also might be due to dehydration in the 10-day-old pups at the end of the study.

Histopathology

The incidence and severity of kidney lesion scores with different doses of Cis are presented in Table 2. In general, Cis treatment caused epithelial cell injury (degeneration, regeneration, necrosis and apoptosis) in the medulla (S3 segments of proximal tubules, loops of Henle and collecting ducts). No renal lesions were observed in any control groups. The severity of Cis-induced renal injury was greater in 80- and 40-day-old compared with 25- and 10-day-old rats. Significant results were observed for 80-day-old rats for all doses, 3 and 6 mg kg⁻¹ Cis for 40-day-old rats and 6 mg kg⁻¹ for 10-day-old rats. In 80-day-old rats, the severity of the renal lesions scores were greater in rats treated with 3 mg kg⁻¹ Cis (average score = 5), as compared with those rats treated with 6 mg kg⁻¹ Cis (average score = 3.25). Since the histopathology score is an average score of four animals, it is possible that the lack of dose response in the histopathology score was due to two rats in the 6 mg kg⁻¹ Cis group responding with less sensitivity to Cis. However, it should be noted that the severity of the histopathology was quite high in both dose groups.

Gene Expression Markers of Nephrotoxicity

The effect of Cis treatment on the expression levels of four gene transcripts that are elevated in response to renal injury (Espandiari *et al.*, 2007a; Thompson *et al.*, 2004) was measured using quantitative RT-PCR assays (Fig. 2). These gene transcripts are hepatitis A virus cellular receptor 1 (*Havcr1*; also known as Kim-1), lipocalin2 (*Lcn2*), osteopontin (*Spp1*) and clusterin (*Clu*). In 80-day-old rats, Cis at doses of 3 and 6 mg kg⁻¹ induced significant elevations in the levels of all four gene transcripts. In 40-day-old rats, Kim-1/*Havcr1* mRNA levels were significantly induced by 1, 3 or 6 mg kg⁻¹ Cis, *Lcn2* and *Spp1* mRNA levels were significantly induced by 3 or 6 mg kg⁻¹ Cis, and *Clu* mRNA levels were significantly induced by 3 mg kg⁻¹ Cis. In 10-day-old rats, Cis at 6 mg kg⁻¹ increased the mRNA levels of all four transcripts, while mRNA levels of Kim-1/*Havcr1* and *Lcn2* also significantly increased at 3 mg kg⁻¹ Cis and *Clu* transcript levels increased at 1, 3 and 6 mg kg⁻¹ Cis. None of the four gene transcripts were significantly elevated in 25-day-old rats although at 6 mg kg⁻¹, Kim-1/*Havcr1*, *Lcn2* and *Spp1* mRNA levels were increased in some animals. The level of Kim-1 gene expression was very similar at 3 and 6 mg kg⁻¹ Cis in both 40- and 80-day-old rats. It is likely that, for these two age groups, maximal nephrotoxicity was reached at 3mg kg⁻¹ Cis, as evidenced by the high expression of Kim-1 and histopathology scores.

Urinary Biomarkers

Urine samples from all age groups (except 10-day-old) were collected in metabolism cages at different time points following Cis treatment and urinary nephrotoxicity biomarkers Kim-1, NAG and RPA-1 were analyzed. The results of these biomarkers were calculated as 'percentage of control' levels at the zero time point (Figs 3 and 4). No significant changes were seen with the low dose (1 mg kg⁻¹) of Cis treatment or at early time points (8 and 24 h) in any age group (data not shown). The level of Kim-1 protein in urine started to significantly increase as early as 48 h post-treatment with Cis (3 and 6 mg kg⁻¹) in 80-day-old and at 72 h in 40-day-old rats. The level of urinary NAG was significantly increased with 3 mg kg⁻¹ of Cis in 80-day-old rats at 72 h after treatment; however, with a dose of 6 mg kg⁻¹ Cis, this level significantly increased at 48 h in 80-day-olds and at 72 h in all age groups. The levels of RPA-1 were evaluated in control and 6 mg kg⁻¹ Cis-treated rats at the 0, 48 and 72 h time points only. Results showed that the level of RPA-1 protein rose significantly at 48 h post-treatment in 80-day-old rats. No significant changes in Kim-1 or RPA-1 were observed in urine from 25-day-old rats at any doses or time points.

Metabonomics Analysis (NMR Results)

The 3D PCA of NMR spectra of urine from rats treated with saline, 3 and 6 mg kg⁻¹ Cis at 0, 48 and 72 h are shown in Fig. 5. For each age group, data from the saline and the 0 time point were pooled. For visual purposes, the bin at 1.89–1.95 whose main contribution is from acetate was removed prior to PCA because several high-dose 25-day-old rats were outliers due to large amounts of acetate in the urine. Each age group is found within a circle region (Fig. 5). In these regions, 25-day-old rats cluster at one end, while 40-day-old rats cluster in the middle and then a cluster of data from 80-day-old rats. A list of select metabolites that were evaluated in each NMR spectrum (Table 3) shows that metabolites associated with energy (2-oxoglutarate, citrate and fumarate) were reduced significantly regardless of age after a toxic dose of Cis and as early as 24 h after dosing. Acetate, glucose, alanine and glutamate are all significantly increased after a toxic dose of Cis. Glucose was not significantly changed in the urine from the 25-day-old rats at any dose or time point; however, this metabolite was increased at 48 h in the 40-day-old rats in both 3 and 6 mg kg⁻¹ treatment groups and increased by a factor of almost 14 at 72 h in the 3 mg kg⁻¹ group in 80-day-old rats. Alanine was not significantly changed in the 25-day-old rats at any dose, but was significantly increased at the 48 and 72 h time points in 40- and 80-day-old rats administered 6 mg kg⁻¹ Cis and with 3 mg kg⁻¹ Cis at the 48 and 72

h in 80-day-old rats. Glutamate was also increased in urine from all three age groups in at least one time point. Acetate was decreased at 24 and 48 h in 25-day-old rats given 6 mg kg⁻¹ doses and significantly increased at 72 h for 40- and 80-day-old rats treated with 3 or 6 mg kg⁻¹ Cis doses.

LC/MS Analysis of 4-Hydroxyproline

Levels of 4-hydroxyproline (4HP) were evaluated by LC/MS since a previous study of Cis toxicity in mice showed altered levels of 4HP in urine following administration of nephrotoxic doses (unpublished). In this study, the levels of 4HP started to decrease significantly with 3 and 6 mg kg⁻¹ Cis treatments (at 24 h) in the 40-day-old animals and started to increase significantly with 3 mg kg⁻¹ (at 48 and 72 h) or 6 mg kg⁻¹ (at 48 h) Cis in the 80-day-old rats (Fig. 6). In the 25-day-old Cis-treated rats, the levels of 4HP did not change at any time point.

DISCUSSION

The physiological and biochemical differences observed at different stages of development may influence the efficacy and toxicity of a drug (Stephenson, 2005; Makri *et al.*, 2004). For example, drugs such as acetaminophen, valproic acid and lamotrigine have different toxicities in pediatric and adult patients (Insel, 1996; Dreifuss, 1987; Kapusnik-Uner *et al.*, 1996; Guberman *et al.*, 1999). The occurrence of ADRs in pediatric patients is a significant health concern (Impicciatore *et al.*, 2001; Easton *et al.*, 1998) and thus it is important to develop strategies, such as age-relevant preclinical models, to help predict such reactions in this patient population that may go undetected using standard pre-clinical models. A strategy initiated in our laboratory was to develop a multi-age rat model to detect potential age-related differences in organ injury following exposure to organ-specific toxicants. Drugs selected to be tested in this animal model were: valproic acid as a prototypic hepatotoxicant (Espandiari *et al.*, 2007b) and the prototypic nephrotoxic drugs gentamicin (Espandiari *et al.*, 2007a) and Cis (present study). In our previous studies, 10-day-old rats were found to be the most sensitive age group to both valproic acid hepatotoxicity and gentamicin nephrotoxicity (Espandiari *et al.*, 2007a, b). The observation for valproic acid seems to correlate with clinical reports indicating that infants younger than 2 years of age treated with valproic acid experience a high incidence of fatal hepatotoxicity (Anderson, 2002; Serrano *et al.*, 1999; Dreifuss *et al.*, 1989). Gentamicin and Cis were subsequently studied to assess the pattern of age-sensitivity in the kidney. These studies showed that, as in the case for valproic acid, there were age-related differences in response to treatment with nephrotoxicants. However, the order of age-related sensitivity to the toxicant was not necessarily the same with different nephrotoxicants. For gentamicin, the order of nephrotoxicity from most to least sensitive was: 10-day-old $\geq$ 80-day-old > 40-day-old > 25-day-old (Espandiari *et al.*, 2007a), whereas for Cis it was (in order of decreasing sensitivity) 80-day-old > 40-day-old > 10-day-old > 25-day-old. The findings of these studies showed that: 10-day-old pups, which were the most sensitive age group (developed the most severe lesions) following treatment with valproic acid or gentamicin, were less sensitive to Cis; 80- and 25-day-old rats, respectively, were the most and least likely age groups to develop renal lesions following treatment with Cis and gentamicin; and 40-day-old rats showed significant, but less, renal nephrotoxicity, than 80-day-old rats in responses to both nephrotoxic drugs. These studies demonstrate that age is an important factor to consider in understanding target tissue responses and predicting the safety of drug candidates in various sub-populations.

Our findings showing less pronounced Cis nephrotoxicity in young rats compared with older rats are consistent with published reports (Appenroth *et al.*, 1988; Ali *et al.*, 2008). Several potential mechanisms to account for the different age sensitivities observed between young and old rats after Cis treatment have been postulated in earlier studies. Several studies

(Appenroth and Bräunlich 1984; Appenroth *et al.*, 1990) demonstrated that Cis administration to adult rats reduced renal tubular organic ion transport, a common index for nephrotoxicity, but unexpectedly increased transport in 10- to 15-day-old rats. The investigators interpreted this increased ion excretion to the higher degree of kidney tissue regenerative capacity and activation of 'silent' nephrons in young rats, effectively countering the nephrotoxic injury. Age-related differences in Cis pharmacokinetics may be another mechanism responsible for the lower susceptibility in young rats. Decreases in both the half-life of plasma Cis and kidney tissue Cis concentrations, and increases in urinary excretion of Cis, were observed in 10-day-old rats compared with 55-day-old rats (Bräunlich and Appenroth, 1988; Appenroth *et al.*, 1988). Other studies, including our own findings (data to be published in a subsequent paper), have confirmed that young rats accumulate less Cis in kidneys compared with older rats. For example, Cis concentrations in kidneys of 21- and 49-day-old rats are 50 and 70%, respectively, of concentrations found in 168-day-old rats (Ali *et al.*, 2008). In another published study, renal cortex levels of gentamicin in 1-month-old rats were shown to be approximately 67% of levels observed in 24-month-old rats (Ali *et al.*, 1996). Similarly, using repeated daily injections up to 14 days, gentamicin concentrations in total kidney and kidney cortex of young rats were significantly lower compared with adult rats (Provoost *et al.*, 1985; Marre *et al.*, 1980). Our data regarding the correlation between kidney accumulation of cisplatin to its nephrotoxicity indicated that there was less accumulation of cisplatin in kidney tissues of the 25-day-old group compared with other age groups.

Another goal of the present study was to evaluate the utility of various biomarkers of nephrotoxicity to assess Cis-induced renal injury in the multi-age rat model. Several serum, urinary and 'omics' (genomic and metabolomics) nephrotoxicity bio-markers were evaluated in rats of various ages after treatment with Cis. Both BUN and serum Cr, which were elevated following a high dose of Cis in 40- and 80-day-old rats, correlated with the severity of renal lesions. A growing critique of these biomarkers in both preclinical and clinical environments is that increased levels of these biomarkers are detected only after a significant degree of kidney function is lost (Star, 1998). Because of the known insensitivity of serum Cr and BUN, we evaluated a battery of potentially more sensitive urinary nephrotoxic biomarkers, such as Kim-1, RPA-1 and NAG, in 25-, 40- and 80-day-old rats. In this study, urinary Kim-1 was the most sensitive urinary nephrotoxicity biomarker since a significant increase was detectable as early as 48 h after 3 mg kg⁻¹ Cis treatment in the most sensitive age group (80-day-old rats). Kim-1 (a type 1 transmembrane protein, with an immunoglobulin and mucin domain) has been reported to be increased following renal injury in both rats and humans (Ichimura *et al.*, 1998; Vaidya *et al.*, 2005; Zhou *et al.*, 2008). Several studies have reported that urinary Kim-1 can provide early evidence of drug-induced renal proximal tubular injury (Vaidya and Bonventre, 2006; Vaidya *et al.*, 2006; Zhou *et al.*, 2008) and could serve as a biomarker for monitoring kidney toxicity/disease in both pre-clinical and clinical studies. Recently, in collaborative effort by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA), seven new tests (KIM-1, Albumin, Total Protein, β 2-microglobulin, Cystatin C, Clusterin, and Trefoil Factor-3) were documented as sensitive nephrotoxic biomarkers for animal studies (preclinical new drugs development) <http://www.fda.gov/bbs/topics/NEWS/2008/NEW01850.html>. However, more studies are needed to assess whether these biomarkers are detectable in all different stages of development and demonstrate the same level of sensitivity.

The link between the appearance of Kim-1 protein in the urine, increased renal gene expression of *Kim-1/Havcr1* and the degree of kidney histopathology was investigated. The up-regulation of *Kim-1/Havcr1* gene expression and the presence of Kim-1 protein in urine has previously been reported after exposure to nephrotoxicants (Han *et al.*, 2002, 2005; Ichimura *et al.*, 2004; Kuehn *et al.*, 2002; Vaidya *et al.*, 2006; van Timmeren *et al.*, 2006; Zhou *et al.*, 2008). In our previous study using gentamicin (Espandiari *et al.*, 2007a) as well as in the present study,

increased levels of *Kim-1/Havcr1* mRNA in kidney tissue significantly correlated with the appearance of Kim-1 protein in the urine, as well as with the severity of histopathological lesions in the kidney of rats. The correlation between the levels of Kim-1 (urinary and kidney) and renal histopathological lesions is summarized in Table 4. The results of our studies show that increases in kidney *Kim-1/Havcr1* expression and urinary Kim-1 protein may be a more sensitive means to detect renal alterations induced by therapeutic drugs as compared with more traditional biomarkers such as serum level of BUN and Cr.

PCA analysis of the NMR data in this study shows that the data cluster in a clear age- and dose-related manner. In Fig. 5, all control animals are separated along PC2 by age, indicating as one would expect that the metabolic profile is dynamic and changes as the rat age increases. Following Cis treatment, the samples from Cis-treated rats cluster separately from the control samples. In addition to the age-based trajectory, the PCA scores plot also indicates that there is a dose-dependent trajectory, with the magnitude of change from the control region being greater in the animals dosed with 6 mg kg⁻¹ compared with 3 mg kg⁻¹. Since glucose was previously reported as a potential marker of nephrotoxicity in a previous metabolomics studies with valproic acid and gentamicin (Schnackenberg *et al.*, 2006; Espandiari *et al.*, 2007a), we quantified and normalized the concentrations of glucose in the NMR spectra to total NMR spectral intensity. Analysis of the metabolomic data indicates that not all of the rats responded equally to Cis treatment. Many had slightly elevated glucose levels while some levels were ten times the control value. This may indicate that some animals were more susceptible to the toxic effects of Cis. This biological variability was observed with other biomarkers in this study (such as in the levels of Kim-1 protein and *Kim-1/Havcr1* mRNA, especially in 25-day-old rats). Other metabolites were also quantified and indicated a perturbation in energy metabolism following dosing with Cis. In accordance with our previous results that showed elevated levels of 4-HP following dosing with Cis, the level of 4HP was determined in each sample. 4HP was significantly increased at 48 and 72 h post-dosing in the 80-day-old rats administered 3 and 6 mg kg⁻¹ Cis. This effect was observed as early as 48 h after treatment with 3 mg kg⁻¹ Cis, in the most sensitive age group (80-day-old rats). No significant changes were noted in the 25-day-old rats. The elevation in 4HP may be due to increased kidney epithelial cell extracellular matrix degradation and more specifically collagen degradation by Cis-mediated activation of kidney metalloproteases while glucose appears to be a general marker of renal injury and not specific to Cis-induced injury (Schnackenberg *et al.*, 2006; Espandiari *et al.*, 2007a,b).

In summary, a multi-age animal model was used to demonstrate that levels of several nephrotoxicity biomarkers were elevated in response to Cis treatment and correlated with the degree of the renal histopathology severity. The pattern of age-related toxicity for Cis was different from that previously shown for gentamicin, and these differences could be related to the relative differences in accumulation of each drug in the kidney as well as to differences in the mechanisms of toxicity of each drug. While it could be informative to evaluate each drug at all ages, it does appear with these studies that 80-day-old rats are more sensitive to nephrotoxicity induced by at least two nephrotoxicants, Cis and gentamicin. A multi-aged animal model may be useful in helping to predict potential toxicities in pediatric populations.

Acknowledgments

The authors thank Dr E Herman, A Knapton, and L Noory for assistance during this project. In addition, thanks to Dr D Portilla for supplying urine samples that led us to identify hydroxyproline as a biomarker of Cis nephrotoxicity. Work in Vaidya laboratory was supported by R00 ES016723 grant by NIEHS to VSV.

REFERENCES

- Ali BH, Bashir AK, Mugamer IT, Tanira MO. Gentamicin nephrotoxicity in the rat: influence of age and diabetes mellitus. *Hum. Exp. Toxicol* 1996;15:51–55. [PubMed: 8845210]

- Ali BH, Al-Moundhri M, Tageldin M, Al Hussein IS, Mansour MA, Nemmar A, Tanira MO. Ontogenic aspects of cisplatin-induced nephrotoxicity in rats. *Food Chem. Toxicol* 2008;46:3355–3359. [PubMed: 18790000]
- Anderson GD. Children versus adults: pharmacokinetic and adverse-effect differences. Review. *Epilepsia* 2002;43:53–59. [PubMed: 12060006]
- Appenroth D, Bräunlich H. Age differences in cisplatin nephrotoxicity. *Toxicology* 1984;28:343–353. [PubMed: 6541377]
- Appenroth D, Winnefeld K, Bräunlich H. Nephrotoxicity and pharmacokinetics of cisplatin in young and adult rats. *Biomed Biochim* 1988;47:791–797.
- Appenroth D, Gambaryan S, Gerhardt S, Kersten L, Bräunlich H. Age dependent differences in the functional and morphological impairment of kidney following cisplatin administration. *Exp. Pathol* 1990;38:231–239. [PubMed: 2387365]
- Berns JS, Ford PA. Renal toxicities of antineoplastic drugs and bone marrow transplantation. *Semin. Nephrol* 1997;17:54–66. [PubMed: 9000550]
- Blumer, JL.; Reed, MD. Principles of neonatal pharmacology. In: Yaffe, SJ.; Aranda, JV., editors. *Pediatric Pharmacology: Therapeutic Principles in Practice*. W. B. Saunders; Philadelphia: 1992. p. 164-177.
- Cresteil T. Onset of xenobiotic metabolism in children: toxicological implications. *Food Addit. Contam* 1998;15:45–51. [PubMed: 9602911]
- Cresteil T, Beaune P, Kremers P, Celier C, Guengerich FP, Leroux JP. Immunoquantification of epoxide hydrolase and cytochrome P-450 isozymes in fetal and adult human liver microsomes. *Eur. J. Biochem* 1985;151:345–350. [PubMed: 2411555]
- Dreifuss FE. Fatal liver failure in children on valproate. *Lancet* 1987;3:47–48. [PubMed: 2879132]
- Dreifuss FE, Langer DH, Moline KA, Maxwell JE. Valproic acid hepatic fatalities II. US experience since 1984. *Neurology* 1989;39:201–207. [PubMed: 2492646]
- Easton KL, Parsons BJ, Starr M, Brien JE. The incidence of drug-related problems as a cause of hospital admissions in children. *Med. J. Aust* 1998;169:356–359. [PubMed: 9803246]
- Espandiari P, Zhang J, Rosenzweig BA, Vaidya VS, Sun J, Schnackenberg L, Herman EH, Knapton A, Bonventre JV, Beger RD, Thompson KL, Hanig J. The utility of a rodent model in detecting pediatric drug-induced nephrotoxicity. *Toxicol. Sci* 2007a;99:637–648. [PubMed: 17636248]
- Espandiari P, Zhang J, Schnackenberg LK, Miller TJ, Knapton A, Herman EH, Beger RD, Hanig JP. Age-related differences in susceptibility to toxic effects of valproic acid in rats. *J. Appl. Toxicol* 2007b;28:628–637. [PubMed: 17994530]
- Faustman EM, Silbernagel SM, Fenske RA, Burbacher TM, Ponce RA. Mechanisms underlying children's susceptibility to environmental toxicants. *Environ. Health. Perspect. Rev* 2000;108:13–21.
- Guberman AH, Besag FM, Brodie MJ, Dooley JM, Duchowny MS, Pellock JM, Richens A, Stern RS, Trevathan E. Lamotrigine-associated rash: risk/benefit considerations in adults and children. *Epilepsia* 1999;40:985–991. [PubMed: 10403224]
- Han WK, Alinani A, Wu CL, Michaelson D, Loda M, McGovern FJ, Thadhani R, Bonventre JV. Human kidney injury molecule-1 is a tissue and urinary tumor marker of renal cell carcinoma. *J. Am. Soc. Nephrol* 2005;16:1126–1134. [PubMed: 15744000]
- Han WK, Bailly V, Abichandani R, Thadhani R, Bonventre JV. Kidney Injury Molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. *Kidney Int* 2002;62:237–244. [PubMed: 12081583]
- Heyman S. Gastric emptying in children. Review. *J. Nucl. Med* 1998;39:865–869. [PubMed: 9591590]
- Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL, Sanicola M. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. *Biol. Chem* 1998;13:4135–4142.
- Ichimura T, Hung CC, Yang SA, Stevens JL, Bonventre JV. Kidney injury molecule-1: a tissue and urinary biomarker for nephrotoxicant-induced renal injury. *Am. J. Physiol. Renal Physiol* 2004;286:552–563.
- Impicciatore P, Choonara I, Clarkson A, Provavi D, Pandolfini C, Bonati M. Incidence of adverse drug reactions in paediatric in/out-patients: a systematic review and meta-analysis of prospective studies. *Br. J. Clin. Pharmacol* 2001;52:77–83. [PubMed: 11453893]

- Insel, PA. Analgesic-antipyretic and antiinflammatory agents. In: Hardman, JG.; Limbird, LE.; Molinoff, PB.; Ruddon, RW.; Gilman, AG., editors. Goodman & Gilman's The Pharmacological Basis of Therapeutics. 9th edn. McGraw-Hill; New York: 1996. p. 632
- Kapusnik-Uner, JE.; Sande, MA.; Chambers, HF. Antimicrobial agents. In: Hardman, JG.; Limbird, LE.; Molinoff, PB.; Ruddon, RW.; Gilman, AG., editors. Goodman & Gilman's The Pharmacological Basis of Therapeutics. 9th edn. McGraw-Hill; New York: 1996. p. 1124-1153.
- Kuehn EW, Park KM, Somlo S, Bonventre JV. Kidney injury molecule-1 expression in murine polycystic kidney disease. *Am. J Physiol. Renal Physiol* 2002;283:1326-1336.
- Lazarou J, Pomeranz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. *JAMA* 1998;15:1200-1205. [PubMed: 9555760]
- Makri A, Goveia M, Balbus J, Parkin R. Children's susceptibility to chemicals: a review by developmental stage. *J. Toxicol. Environ. Health B Crit. Rev* 2004;7:417-435. [PubMed: 15586877]
- Marre R, Tarara N, Louton T, Sack K. Age-dependent nephrotoxicity and the pharmacokinetics of gentamicin in rats. *Eur. J. Pediatr* 1980;133:25-29. [PubMed: 7353568]
- Olin SS. Research needs: recommendations of an ILSI Working Group on age-related differences in susceptibility. *Food Addit Contam* 1998;15:53-54. [PubMed: 9602912]
- Pirmohamed M, Breckenridge AM, Kitteringham NR, Park BK. Adverse drug reactions: current status. *Br. Med. J* 1998;316:1295-1298. [PubMed: 9554902]
- Provoost AP, Adejuyigbe O, Wolff ED. Nephrotoxicity of aminoglycosides in young and adult rats. *Pediatr. Res* 1985;19:1191-1196. [PubMed: 4069829]
- Santoso JT, Lucci JA 3rd, Coleman RL, Schafer I, Hannigan EV. Saline, mannitol, and frusemide hydration in acute cisplatin nephrotoxicity: a randomized trial. *Cancer Chemother. Pharmacol* 2003;52:13-18. [PubMed: 12719883]
- Sastry J, Kellie SJ. Severe neurotoxicity, ototoxicity and nephrotoxicity following high-dose cisplatin and amifostine. *Pediatr. Hematol. Oncol* 2005;22:441-445. [PubMed: 16020136]
- Schnackenberg LK, Jones RC, Thyparambil S, Taylor JT, Han T, Tong W, Hansen DK, Fuscoe JC, Edmondson RD, Beger RD, Dragan YP. An integrated study of acute effects of valproic acid in the liver using metabolomics, proteomics, and transcriptomics platforms. *OMICS* 2006;10:1-14. [PubMed: 16584314]
- Serrano BB, Garcia Sanchez MJ, Otero MJ, Buelga DS, Serrano J, Dominguez-Gil A. Valproate population pharmacokinetics in children. *J. Clin. Pharm. Ther* 1999;24:73-80. [PubMed: 10319910]
- Star RA. Treatment of acute renal failure. *Kidney Int. Rev* 1998;54:1817-1831.
- Stephenson T. How children's responses to drugs differ from adults. *Br. J. Clin. Pharmacol* 2005;59:670-673. [PubMed: 15948930]
- Taguchi T, Nazneen A, Abid MR, Razzaque MS. Cisplatin-associated nephrotoxicity and pathological events. *Contrib. Nephrol* 2005;148:107-121. [PubMed: 15912030]
- Thompson KL, Afshari CA, Amin RP, Bertram TA, Car B, Cunningham M, Kind C, Kramer JA, Lawton M, Mirsky M, Naciff JM, Oreffo V, Pine PS, Sistare FD. Identification of platform-independent gene expression markers of cisplatin nephrotoxicity. *Environ. Health Perspect* 2004;112:488-494. [PubMed: 15033599]
- Travis, LB. The kidneys and urinary tract. In: Rudolph, AM., editor. *Pediatrics*. 19th Ed.. Appleton and Lange; Norwalk: 1991. p. 1223-1236.
- Vaidya VS, Bonventre JV. Mechanistic biomarkers for cytotoxic acute kidney injury. *Expert Opin. Drug Metab. Toxicol* 2006;2:697-713. [PubMed: 17014390]
- Vaidya VS, Bobadilla N, Bonventre JV. A microfluidics based assay to measure Kidney Injury Molecule-1 (Kim-1) in the urine as a biomarker for early diagnosis of acute kidney injury. *J. Am. Soc. Nephrol* 2005;16:192A.
- Vaidya VS, Ramirez V, Ichimura T, Bobadilla NA, Bonventre JV. Urinary kidney injury molecule-1 (Kim-1): a sensitive quantitative biomarker for early detection of kidney tubular injury. *Am. J. Physiol. Renal Physiol* 2006;290:F517-529. [PubMed: 16174863]
- van Timmeren MM, Bakker SJ, Vaidya VS, Bailly V, Schuurs TA, Damman J, Stegeman CA, Bonventre JV, van Goor H. Tubular kidney injury molecule-1 in protein-overload nephropathy. *Am. J. Physiol. Renal Physiol* 2006;291:456-464.

- Weaver LT, Austin S, Cole TJ. Small intestinal length: a factor essential for gut adaptation Review. *Gut* 1991;32:1321–1323. [PubMed: 1752463]
- Wershil, BK. Gastric function. In: Walker, WA.; Durie, PR.; Hamilton, JR.; Walker-Smith, JA.; Watkins, JB., editors. *Pediatric Gastrointestinal Disease*. Vol. Vol. 1. Mosby; St Louis, MO: 1992. p. 71-82.
- Zhou Y, Vaidya VS, Brown RP, Rosenzweig BA, Johnson AM, Thompson KL, Zhang J, Vakili Z, Miller T, Bonventre JV, Goering PL. Comparison of kidney injury molecule-1 and other nephrotoxicity biomarkers in urine and kidney after acute exposure to gentamicin, mercury, and chromium. *Toxicol. Sci* 2008;101:159–170. [PubMed: 17934191]
- Zoetis T. Species comparison of anatomical and functional renal development. *Birth Defects Res. (Pt B)* 2003;68:111–120.

Figure 1.

(A) Changes in blood urea nitrogen levels following a single treatment with 0, 1, 3 and 6 mg kg⁻¹ cisplatin in 10-, 25-, 40- or 80-day-old Sprague-Dawley rats. (B) Changes in serum creatinine levels following treatment with 0, 1, 3 and 6 mg kg⁻¹ cisplatin in 10-, 25-, 40- or 80-day-old Sprague-Dawley rats.

*Significantly higher than control group ($P < 0.05$).

Figure 2.

Changes in *Kim1/Havcr1*, *Lcn2*, *Spp1*, and *Clu* transcript levels in kidney following a single treatment with 0, 1, 3 and 6 mg kg⁻¹ cisplatin in 10-, 25-, 40- or 80-day-old Sprague-Dawley rats. The log2-fold changes are shown for each individual animal relative to the average age-matched control value. The dose groups are 0 (open circles), 1 (light gray), 3 (dark gray) and 6 (black) mg kg⁻¹ cisplatin.

*Dose groups that were statistically different from controls ($P < 0.05$).

Figure 3.

(A) Changes in level of urinary Kim-1 following treatment with 3 mg kg⁻¹ cisplatin in 25-, 40- or 80-day-old Sprague-Dawley rats. (B) Changes in urinary level of NAG following treatment with 3 mg kg⁻¹ cisplatin in 25-, 40- or 80- day old Sprague-Dawley rats.

*Significantly higher than control group ($P < 0.05$).

Figure 4.

(A) Changes in level of urinary Kim-1 following treatment with 6 mg kg^{-1} cisplatin in 25-, 40- or 80- day- old Sprague Dawley rats. (B) Changes in urinary level of NAG following treatment with 6 mg kg^{-1} cisplatin in 25-, 40- or 80-day-old Sprague–Dawley rats. (C) Changes in urinary level of RPA-1 following treatment with 6 mg kg^{-1} cisplatin in 25-, 40- or 80-day-old Sprague–Dawley rats.

*Significantly higher than control group ($P < 0.05$).

Figure 5.

Three-dimensional principle component analysis plot of NMR spectra of urine from 25-, 40-, and 80-day-old rats dosed with saline and cisplatin (3 and 6 mg kg^{-1}) at 0, 48 and 72 h. To simplify the graph, data from 0 h and saline as well as treated data at 48 and 72 h were pooled together. All symbols shown in black are for 6 mg kg^{-1} samples, in light gray for 3 mg kg^{-1} cisplatin samples and in white for control samples.

Figure 6.

Levels of 4-hydroxyproline in urine from 25-, 40-, and 80-day-old rats dosed with saline (control) and 3 or 6 mg kg⁻¹ cisplatin at 0, 24, 48 and 72 h. **P*-value < 0.05 vs control saline at 0, 24, 48, and 72 h for each age group.

Table 1

Significant differences in body and kidney weights of different age groups of SD rats following treatment with the highest dose of cisplatin

Days old	Final body wt/initial body (wt% control)	Kidney wt/final body (wt% control)
10	93 ± 4.9*	NS
25	78 ± 7.6*	NS
40	83 ± 00*	NS
80	91 ± 1.0*	83 ± 2.7*

The results are means ± SEM for four rats in each group except for 10-day-old pups ($n = 8$ for each dose group).

* Significantly different from control ($P < 0.05$). NS = not significant (significantly not different from control).

Incidence and severity of kidney lesions scores in different age group of SD rats after treatment with different doses of cisplatin

Table 2

Age	Treatment cisplatin (mg kg ⁻¹)	Nephrotoxicity lesion scores					P-value ^a	Mean lesion score	
		0	1	2	3	4			5
10 (n = 8)	0	8	0	0	0	0	0	-	0.00
	1	8	0	0	0	0	0	1.0	0.00
	3	8	0	0	0	0	0	1.0	0.00
	6	1	0	7	0	0	0	0.001	1.75
25 (n = 4)	0	4	0	0	0	0	0	-	0.00
	1	3	1	0	0	0	0	1.0	0.25
	3	4	0	0	0	0	0	1.0	0.00
	6	2	1	0	1	0	0	0.42	1.00
40 (n = 4)	0	4	0	0	0	0	0	-	0.00
	1	2	2	0	0	0	0	0.42	0.5
	3	0	2	0	0	1	1	0.02	2.75
	6	0	0	0	0	3	1	0.02	4.25
80 (n = 8)	0	4	0	0	0	0	0	-	0.00
	1	0	3	1	0	0	0	0.02	1.25
	3	0	0	0	0	0	4	0.02	5.00
	6	0	0	1	1	2	0	0.02	3.25

n = 4 rats for 25-, 40- and 80-day-old rats and n = 8 for 10-day-old pups. Equivalently, the exact tests using Wilcoxon-Mann-Whitney and Jonckheer-Terpstra procedures produced exactly the same P-values.

^aThe P-values were derived from non-parametric Fisher exact test.

Table 3

Metabolite concentrations as detected by NMR in different age group of SD rats after treatment with different doses of cisplatin

Age (days)	Metabolite	Control	3 mg kg ⁻¹ CP 24 h	3 mg kg ⁻¹ CP 48 h	3 mg kg ⁻¹ CP 72 h	6 mg kg ⁻¹ CP 24 h	6 mg kg ⁻¹ CP 48 h	6 mg kg ⁻¹ CP 72 h
25	Oxoglutarate	59 ± 21	37 ± 2.9*	34 ± 12*	43 ± 27	57 ± 7.9	30 ± 13*	19 ± 20*
	Acetate	76 ± 110	54 ± 59	80 ± 77	84 ± 59	9.3 ± 5.3*	22 ± 8.2*	93 ± 54
	Alanine	11 ± 14	6.88 ± 2.53	6.62 ± 3.78	8.9 ± 5.7	4.30 ± 0.76	6.32 ± 1.71	9.61 ± 6.08
	Citrate	170 ± 49	124 ± 45	134 ± 20	108 ± 73	123 ± 23*	74 ± 44*	67 ± 82
	Fumarate	2.10 ± 1.27	1.35 ± 0.04*	1.31 ± 0.24*	1.35 ± 0.60	1.76 ± 0.22	0.80 ± 0.07*	0.58 ± 0.23*
	Glucose	5.4 ± 1.3	9.8 ± 2.4	6.4 ± 0.8	6.8 ± 2.1	9.0 ± 2.6	11 ± 10	8.6 ± 4.0
	Glutamate	0.99 ± 0.29	1.97 ± 0.92	1.38 ± 0.68	1.6 ± 0.18*	1.55 ± 0.39	1.37 ± 0.25*	1.39 ± 0.20*
40	Oxoglutarate	118 ± 19	79 ± 34	82 ± 25	90 ± 36	110 ± 36	51 ± 35*	46 ± 36*
	Acetate	7.4 ± 12	3.6 ± 2.0	10 ± 7.7	27 ± 4.7*	18 ± 16	32 ± 23	92 ± 43*
	Alanine	1.77 ± 0.97	1.63 ± 0.55	3.06 ± 1.80	11 ± 13	3.04 ± 1.47	5.2 ± 2.1*	20 ± 5.4*
	Citrate	167 ± 37	78 ± 54*	65 ± 25*	127 ± 73	66 ± 9.8*	60 ± 53*	57 ± 61*
	Fumarate	1.68 ± 0.34	0.98 ± 0.57	1.26 ± 0.57	1.47 ± 0.67	1.09 ± 0.32*	0.64 ± 0.37*	0.50 ± 0.33*
	Glucose	7.0 ± 2.0	6.3 ± 2.7	9.4 ± 1.5*	79 ± 124	12 ± 3.0*	17 ± 4.3*	89 ± 68
	Glutamate	0.99 ± 0.29	1.88 ± 0.40*	1.33 ± 0.26	1.88 ± 1.22	2.9 ± 1.2	1.32 ± 0.36	1.89 ± 0.31*
80	Oxoglutarate	90 ± 25	57 ± 12.4*	15 ± 12*	45 ± 12*	45 ± 25*	38 ± 45	12 ± 9.0*
	Acetate	7.7 ± 12	12 ± 4.2	36 ± 15	72 ± 29*	14 ± 10	73 ± 89	50 ± 48*
	Alanine	1.97 ± 0.88	2.18 ± 0.30	4.01 ± 0.52*	20 ± 7.2*	3.1 ± 2.6	14 ± 19	11 ± 7.4
	Citrate	94 ± 24	25 ± 9.9*	14 ± 17*	17 ± 6.5*	18 ± 9.1*	16 ± 18*	6.5 ± 6.1*
	Fumarate	0.80 ± 0.30	0.40 ± 0.06*	0.26 ± 0.12*	0.27 ± 0.13*	0.49 ± 0.35	0.99 ± 1.57	0.18 ± 0.16*
	Glucose	9.7 ± 1.8	11 ± 2.2	10 ± 5.2	135 ± 20*	19 ± 14	71 ± 92	66 ± 61
	Glutamate	0.93 ± 0.44	2.02 ± 0.28*	1.52 ± 0.51	1.95 ± 0.51*	3.7 ± 3.6	13 ± 23	2.56 ± 1.64

The results are means ± SEM for four rats in each age group.

* Significantly different from control for each age group ($P < 0.05$).

Table 4

Pairwise correlations and *p*-values of Kim-1 (protein and mRNA) with renal histopathological lesion scores in different age group of SD rats treated with cisplatin

Age (days)	Urinary Kim-1/tissue <i>Kim-1/Havcr1</i>	Urinary Kim-1/lesion score	<i>Kim-1/Havcr1</i> /lesion score
10	Not done	Not done	0.82150 <i>P</i> < 0.0001
25	0.8039 <i>P</i> = 0.0003	0.99142 <i>P</i> < 0.0001	0.84118 <i>P</i> < 0.0001
40	0.88317 <i>P</i> < 0.0001	0.86287 <i>P</i> < 0.0001	0.97380 <i>P</i> < 0.0001
80	0.88087 <i>P</i> < 0.0001	0.93227 <i>P</i> < 0.0001	0.94414 <i>P</i> < 0.0001

Data were analyzed by the Fisher exact test with the software StatExact 8.