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Gene Expression of Biomarkers of Nephrotoxicity in F344 Rats Co-Exposed to Melamine and Cyanuric Acid for Seven Days

Luísa Camacho[a], **Kevin P. Kelly**, **Frederick A. Beland**, and **Gonçalo Gamboa da Costa**
Division of Biochemical Toxicology, National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079, United States

Abstract

A number of studies have demonstrated that co-exposure to low levels of melamine and cyanuric acid elicits renal toxicity due to the formation of melamine cyanurate crystals in the kidney nephrons. In this work, we investigated if co-exposure of rats to these compounds leads to alterations in the expression of the genes encoding kidney injury molecule 1 (KIM-1), metallopeptidase inhibitor 1 (TIMP1), clusterin, osteopontin, and neutrophil gelatinase-associated lipocalin/lipocalin 2 (NGAL), which have been proposed as urinary biomarkers for nephrotoxicity. Six-week-old male and female F344 rats were fed *ad libitum* a diet fortified with 0 (control), 7, 23, 69, 229, or 694 ppm melamine and cyanuric acid (co-exposure groups), 1388 ppm melamine, or 1388 ppm cyanuric acid for seven days. Histopathology and clinical chemistry examination indicated marked toxicity only in the animals exposed to the two highest combined doses of melamine and cyanuric acid. Consistent with these observations, quantitative real-time polymerase chain reaction analysis of kidney tissue indicated increased expression of all genes analyzed relative to the control in both male and female rats fed daily with 229 or 694 ppm melamine and cyanuric acid. Exposure to lower levels of both compounds or to the individual compounds did not induce gene expression changes. These data indicate that quantifying the expression levels of the selected biomarker genes constitutes a useful endpoint to assess the combined toxicity of melamine and cyanuric acid in both male and female rats.

Keywords

Melamine; Cyanuric Acid; Kidney; Gene expression; Biomarkers

Introduction

Nephrotoxicity has been traditionally assessed by histopathology and clinical chemistry. More recently, an effort has been made by the scientific community to identify biomarker proteins and genes, whose levels are specifically affected by nephrotoxicants (Davis and Kramer, 2006). This effort has been mainly driven by the evidence that urinary levels of proteins, such as KIM-1, osteopontin, NGAL, and clusterin, are often more sensitive and/or occur at earlier stages of toxicity than histopathological or clinical chemistry changes

[a]Corresponding author: Division of Biochemical Toxicology, National Center for Toxicological Research, HFT-110, 3900 NCTR Road, Jefferson, AR 72079, United States. Tel: +1-870-543-7588. Fax: +1-870-543-7136. luisa.camacho@fda.hhs.gov.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

(Dieterle *et al.*, 2010; Ozer *et al.*, 2010). Given the non-invasive nature of these biomarkers, their potential use in the clinical environment has been considered by the U.S. Food and Drug Administration and the European Medicines Agency, which recently qualified several of these proteins as biomarkers for the detection of tubular or glomerular kidney injury in preclinical studies (Dieterle *et al.*, 2010).

Several studies have also demonstrated renal expression changes in the genes encoding the proteins noted above in response to insult by a variety of nephrotoxicants (Ichimura *et al.*, 2004; Kondo *et al.*, 2009; Rached *et al.*, 2008; Wang *et al.*, 2008; Zhou *et al.*, 2008). While the evaluation of the gene expression levels in kidney tissue is an invasive procedure, hampering, in principle, its translational potential, measuring changes in gene expression as a terminal endpoint presents great potential in animal studies. Compared to histopathology, gene expression assays offer several advantages, including the potential for being an earlier or more sensitive endpoint, quantitative over a wide dynamic range, suitable for high-throughput, and relatively inexpensive.

The triazines melamine and cyanuric acid, components of the industrial by-product “scrap melamine”, were found in adulterated wheat and rice gluten used in pet food that led to the illness and death of a large number of cats and dogs in 2007. While, individually, these triazines present very low toxicities, reports in the literature indicate that, when animals are exposed to mixtures of melamine and cyanuric acid, the compounds are absorbed in the GI tract, distributed systemically, precipitate in the kidney, and form nephrotoxic melamine cyanurate crystals (Dobson et al., 2008; Puschner et al., 2007); hence, the mechanism of nephrotoxicity due to co-exposure to melamine and cyanuric acid seems to be very distinct from those of other well characterized nephrotoxicants (Balakumar *et al.*, 2010; Pabla and Dong, 2008; Dirheimer and Creppy, 1991). A correlation of the renal expression levels of known candidate biomarker genes of nephrotoxicity with the kidney toxic response induced by melamine and cyanuric acid would, thus, reinforce the usefulness of genomic biomarkers of nephrotoxicity and expand their application.

In a previous study, we reported the effect of a seven-day combined exposure to melamine and cyanuric acid in F344 rats on body and kidney weights, kidney histopathology, and serum creatinine and blood urea nitrogen (BUN) levels (Jacob *et al.*, 2011). We found that, while dietary exposure to 1388 ppm melamine or cyanuric acid failed to induce any significant changes on the endpoints analyzed, co-exposure to mixtures of 229 or 694 ppm melamine and cyanuric acid led to a decrease in body weight, enlarged and pale-yellow kidneys, multiple kidney tubular histopathological lesions, deposition of melamine cyanurate crystals in the renal tubules, and elevated serum creatinine and BUN levels compared to control animals. The no-observable adverse-effect level (NOAEL) was established at 69 ppm melamine and cyanuric acid, equivalent to 8.6 mg/kg bw/day of each compound. In the present study, we expanded the list of endpoints to include the analysis of the expression levels of biomarker candidate genes of nephrotoxicity. We aimed to 1) determine if these biomarker genes could be used to assess nephrotoxicity induced by a combined exposure to melamine and cyanuric acid, and 2) determine if the changes in gene expression levels are more sensitive endpoints than those previously assessed.

Material and methods

Experimental design

The kidney tissue used in the current study was obtained from the same rats used to analyze the endpoints reported in Jacob *et al.* (2011). Briefly, six-week-old F344 rats (6 males and 6 females per dose group) were fed *ad libitum* for seven days NIH-41 irradiated meal fortified with 0 (control), 7, 23, 69, 229, or 694 ppm melamine and cyanuric acid (co-exposure

groups), 1388 ppm melamine only, or 1388 ppm cyanuric acid only. This dosing regimen resulted in the exposure of the animals to, respectively, *ca.* 0, 0.9, 2.8, 8.6, 17.6, or 29.8 mg melamine and cyanuric acid/kg bw/day, 123.7 mg melamine/kg bw/day, or 167.5 mg cyanuric acid/kg bw/day (Jacob *et al*., 2011). At necropsy, both kidneys were removed, weighed, sectioned longitudinally, and one half of each kidney was flash-frozen and stored at −80ºC until further processing. All procedures involving care and handling of animals were reviewed and approved by the National Center for Toxicological Research Institutional Animal Care and Use Committee.

Total RNA isolation and reverse-transcription

Total RNA was isolated from half of a kidney per animal. The frozen kidney tissues were macerated in liquid nitrogen using a mortar and pestle, and 10–20 mg samples of frozen tissue powder were used to isolate total RNA using an RNeasy Mini kit, with on-column DNase I digestion, following the manufacturer's protocol (Qiagen, Valencia, CA). RNA purity and concentration were assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and the RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All RNA samples had an absorption 260 nm/280 nm ratio greater than 2.0 and the RNA integrity number (RIN) was 9.19 ± 0.59 (mean ± standard deviation (SD); n = 96). One microgram of total RNA was reverse transcribed using random hexamer primers and an Advantage RT-for-PCR kit (Clontech, Palo Alto, CA), following the manufacturer's protocol. The cDNA was diluted 1:40 with nuclease-free water and stored at −20ºC.

Quantitative gene expression analysis

The gene expression levels of the biomarkers of nephrotoxiticy were quantified using quantitative real-time polymerase chain reaction (PCR). cDNAs were amplified in duplicate using TaqMan assays and a 7900HT real-time PCR detection system (Applied Biosystems, Foster City, CA), following the manufacturer's protocol. The TaqMan assays used were Rn00597703_m1 (*Kim-1/Havcr1*, NM_173149; encodes KIM-1), Rn01430875_g1 (*Timp1*, NM_053819; encodes TIMP1), Rn01449972_m1 (*Spp1*, NM_012881; encodes osteopontin), Rn00562081_m1 (*Clu*, NM_053021; encodes clusterin), Rn00590612_m1 (*Lcn2*, NM_130741; encodes NGAL). A FastStart master mix (Roche Diagnostics, Indianapolis, IN) was used, and the quantitative real-time PCR cycling conditions were set at 95ºC for 10 min for the first cycle and 15 seconds at 95ºC followed by 1 min at 60ºC for the remaining 40 cycles. The coefficient of variation between technical replicates was < 1%. Data were normalized for the endogenous control *Gapdh* (NM_017008; assay part # 4352338E) and analyzed using the ΔΔCt method (Livak and Schmittgen, 2001). To express the relative gene expression levels as a %GAPDH, the cycle threshold (Ct) values of the gene of interest were subtracted with that of *Gapdh* (ΔCt) and the $2^{-\Delta Ct} \times 100$ value was calculated. *Gapdh* levels had been determined in preliminary absolute quantitation experiments to be unchanged with treatment (data not shown). Data are expressed as mean ± SD.

Benchmark dose modeling

Benchmark doses (BMDs) and the lower 95% confidence limits (BMDLs) were calculated using Environmental Protection Agency Benchmark Dose Software (version 2.1.1; http://www.epa.gov/ncea/bmds). The calculations were conducted using Hill, linear, polynomial, and power models to fit the relative gene expression levels of *Kim-1/Havcr 1*, *Timp1*, *Clu*, *Lcn2*, and *Spp1* (mean ± SD; n = 6) and the mean doses of melamine and cyanuric acid from the entire seven-day study. The BMD was defined as the dose corresponding to a change in the mean response equal to one control standard deviation from the control mean.

Statistical analysis

Statistical significance between male and female control animals was assessed by t-tests. Statistical significance between dose groups within each sex was assessed by one-way ANOVA, followed by Dunnett's test to compare treated groups to the matching control (SigmaStat v3.11). In order to maintain an equal variance and normal data distribution, the data were natural log transformed before conducting the analyses. A p-value < 0.05 was considered statistically significant.

Results and Discussion

Table 1 summarizes the results previously reported by our group in the same set of animals used for the current study (Jacob *et al.*, 2011). All endpoints analyzed, which included body weight, kidney weight, histopathology, and blood chemistry, indicated that feeding six-week-old male and female F344 rats 229 or 694 ppm melamine and cyanuric acid for seven days resulted in alterations consistent with nephrotoxicity. Rats co-exposed to lower levels of melamine and cyanuric acid or exposed to melamine or cyanuric acid only showed no evidence of nephrotoxicity. Based on these endpoints, the NOAEL for kidney toxicity in the F344 rats was set at 69 ppm melamine and cyanuric acid in feed, which was equivalent to 8.6 mg/kg bw/day of each compound. BMD modeling was also conducted on these endpoints; kidney weights were the most sensitive endpoints modeled and afforded BMDLs of 8.4–10.9 mg/kg bw/day (Jacob *et al.*, 2011).

In the present study, we evaluated the use of quantitative expression analysis of known candidate biomarker genes as a tool to assess nephrotoxicity induced by a combined exposure to melamine and cyanuric acid. Kidneys from both male and female rats were analyzed. The use of five doses, two below the NOAEL and two above the NOAEL, allowed assessment of the shape of the dose-response curve. Furthermore, kidneys from rats treated with melamine only or cyanuric acid only were also analyzed to characterize further the effect of the individual compounds in the kidney.

The expression of all genes analyzed, which included *Kim-1/Havcr1*, *Timp1*, *Lcn2*, *Clu*, and *Spp1*, was significantly up-regulated in the kidneys of both male and female rats exposed to 229 or 694 ppm melamine and cyanuric acid (Figure 1). There were no statistically significant gene expression changes, compared to the matching control rats, in either male or female rats treated with 69 ppm or less of combined melamine and cyanuric acid, or with 1388 ppm melamine only or cyanuric acid only. From these data, a NOAEL of 69 ppm (8.6 mg/kg bw/day) was established for combined melamine and cyanuric acid, which matched the previously reported NOAEL (Jacob *et al.*, 2011). To investigate further the relative sensitivity of gene expression analysis versus the endpoints previously considered in this study, BMD modeling was also conducted, using the mean doses of melamine and cyanuric acid from the entire seven-day study and the resultant changes in relative gene expression (Table 2). The best fits were obtained with the Hill model, which led to BMDL values of 8.7–9.8 mg/kg bw/day, depending on the specific gene and sex of the animal. These values are in excellent agreement with the BMDLs for kidney weight (BMDL 8.4–10.9 mg/kg bw/day), the most sensitive endpoint previously reported. While other studies have reported gene expression changes to be a more sensitive endpoint than histopathology and/or clinical chemistry (Ichimura *et al.*, 2004; Kondo *et al.*, 2009; Rached *et al.*, 2008; Zhou *et al.*, 2008), we hypothesize that our observations may simply reflect the fact that, at exposures below 229 ppm melamine and cyanuric acid in feed, the threshold of crystallization of melamine cyanurate was not attained. Without crystal formation and consequent organ insult, alterations in gene expression levels, or any of the other endpoints, are not expected. Future studies, with a closer dose-spacing capable of affording a more continuous dose-effect, will be required to evaluate fully the relative sensitivity of these endpoints.

Kim-1/Havcr1 was the most up-regulated gene, following exposure to 694 ppm melamine and cyanuric acid, with the gene expression fold-change being >2500 in males and >75 in females, compared to the matching (same sex) control. Both *Kim-1/Havcr1* and *Lcn2* were up-regulated in a dose-dependent fashion. A similar dose-dependent pattern was reported for *Kim-1/Havcr1* gene expression in male rats exposed to different dose-levels of known nephrotoxicants (Zhou *et al.*, 2008; Chiusolo *et al.*, 2010). On the other hand, the expression levels of *Timp1*, *Clu*, and *Spp1* yielded a more dichotomous response, suggesting that an expression plateau had been reached for these genes at the 229 ppm melamine and cyanuric dose level. Rached *et al.* (2008) reported a dose-dependent up-regulation of *Kim-1/Havcr1*, *Lcn2*, *Timp1*, *Clu*, and *Spp1* in male F344 rats, in response to a 90-day treatment with the nephrotoxicant ochratoxin A; however, the *in vivo* fold-changes were smaller than the ones reported here, suggesting that even the highest ochratoxin A dose level used in that study did not maximally induce expression of the biomarker genes.

To the best of our knowledge, the current study is the first to assess gene expression changes induced by a nephrotoxicant in both male and female rats. As shown in Figure 1, the up-regulation of genes in response to co-exposure to melamine and cyanuric acid is consistently higher in males than in female rats; however, this may not necessarily indicate that males are more sensitive than females to the melamine and cyanuric acid co-exposure. Comparison of the *Gapdh*-corrected, absolute transcript levels of control animals revealed that the constitutive expression of all biomarker genes studied was higher in females than in males (Table 3). This sex difference was most prominent for gene *Kim-1/Havcr1* (>30-fold higher expression in females compared to males); thus, our data show that, unlike what was known for male rats (Ichimura *et al.* 1998; Vaidya *et al.* 2010), under our experimental conditions, seven-week-old female F344 rats constitutively express moderate levels of *Kim-1/Havcr1* (Table 3). Differences in constitutive gene expression levels between males and females were also observed for *Spp1* (*ca.* 5× more expression in females compared to males) and *Timp1*, *Clu*, and *Lcn2* (all *ca.* 2× more expression in females compared to males). Due to this sex difference, even though *Kim-1/Havcr1* was up-regulated >2500 times in males treated with 694 ppm melamine and cyanuric acid when compared with the male controls, and up-regulated >75 times in females treated with 694 ppm melamine and cyanuric acid when compared with the female controls, the maximum absolute amount of *Kim-1/Havcr1* transcripts present per unit of tissue was similar in males and females. The same holds true for the other four genes analyzed; that is, the maximum absolute amount of transcript accumulated per cell, due to co-exposure to melamine and cyanuric acid, was similar in both male and female rats. These observations further suggest that an expression plateau of genes *Timp1*, *Clu*, and *Spp1* was reached upon co-exposure to 229 ppm melamine and cyanuric acid, precluding an additional up-regulation upon co-exposure to higher levels of the mixture. The existence of a maximal level of expression of these genes had previously been speculated upon by Rached *et al.* (2008).

The very low levels of constitutive gene expression of *Kim-1/Havcr1* and *Lcn2* in male rats and *Lcn2* in female rats may be advantageous when using these candidate genes as biomarkers of nephrotoxicity, since it may result in a higher sensitivity to detect expression changes; however, the extremely low basal expression of *Lcn2* in F344 rats may be a strain-specific characteristic, since Rached *et al.* (2008) detected lipocalin-2/NGAL protein in the urine of untreated male Sprague-Dawley and Wistar rats, but failed to detect it in untreated male F344 rats.

Conclusions

In the current study, we investigated previously known candidate biomarker genes of nephrotoxicity to assess the renal injury induced by co-exposure to melamine and cyanuric

acid in male and female F344 rats. Our results show that alterations in the expression of these genes afforded NOAEL and BMDL values consistent with those previously reported. These results demonstrate that the transcriptional analyses of KIM-1, TIMP1, clusterin, osteopontin, and NGAL constitute additional sensitive endpoints to assess nephrotoxicity induced by co-exposure to melamine and cyanuric acids in rats. In contrast with other previously studied biochemical nephrotoxicants, the putative mode of action of combined melamine and cyanuric acid is based on their ability to co-precipitate and physically block the lumen of the nephrons; hence, our results expand the range of nephrotoxicant mechanisms capable of being assessed by transcriptional analysis, reinforcing the usefulness of these complementary endpoints in toxicological studies. Future studies on the combined toxicity of melamine and cyanuric acid should include the quantification of the kidney gene expression of these biomarkers of nephrotoxicity, and investigate if the products of these candidate biomarker genes are altered in the urine upon co-exposure to melamine and cyanuric acid. Such analyses should further expand our knowledge on the use of non-invasive urinary biomarkers to assess renal toxicity and on the translational potential of these biomarkers in humans.

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Abbreviations

BMD	benchmark dose
BMDL	benchmark dose (lower 95% confidence limit)
BUN	blood urea nitrogen
Ct	cycle threshold
CYA	cyanuric acid
KIM-1	kidney injury molecule 1
MEL	melamine
NGAL	neutrophil gelatinase-associated lipocalin/lipocalin 2
NOAEL	no-observable-adverse-effect level
PCR	polymerase chain reaction
TIMP1	metallopeptidase inhibitor 1

References

Balakumar P, Rohilla A, Thangathirupathi A. Gentamicin-induced nephrotoxicity: Do we have a promising therapeutic approach to blunt it? Pharmacol Res. 2010; 62:179–86. [PubMed: 20434560]

Chiusolo A, Defazio R, Zanetti E, Mongillo M, Mori N, Cristofori P, Trevisan A. Kidney injury molecule-1 expression in rat proximal tubule after treatment with segment-specific nephrotoxicants: a tool for early screening of potential kidney toxicity. Toxicol Pathol. 2010; 38:338–45. [PubMed: 20305092]

Dieterle F, Sistare F, Goodsaid F, Papaluca M, Ozer JS, Webb CP, Baer W, Senagore A, Schipper MJ, Vonderscher J, Sultana S, Gerhold DL, Phillips JA, Maurer G, Carl K, Laurie D, Harpur E, Sonee M, Ennulat D, Holder D, Andrews-Cleavenger D, Gu YZ, Thompson KL, Goering PL, Vidal JM, Abadie E, Maciulaitis R, Jacobson-Kram D, Defelice AF, Hausner EA, Blank M, Thompson A, Harlow P, Throckmorton D, Xiao S, Xu N, Taylor W, Vamvakas S, Flamion B, Lima BS, Kasper P, Pasanen M, Prasad K, Troth S, Bounous D, Robinson-Gravatt D, Betton G, Davis MA, Akunda J, McDuffie JE, Suter L, Obert L, Guffroy M, Pinches M, Jayadev S, Blomme EA, Beushausen SA, Barlow VG, Collins N, Waring J, Honor D, Snook S, Lee J, Rossi P, Walker E, Mattes W. Renal biomarker qualification submission: a dialog between the FDA-EMEA and Predictive Safety Testing Consortium. Nat Biotechnol. 2010; 28:455–62. [PubMed: 20458315]

Dirheimer G, Creppy EE. Mechanism of action of ochratoxin A. IARC Sci Publ. 1991; 115:171–86. [PubMed: 1820332]

Dobson RLM, Motlagh S, Quijano M, Cambron RT, Baker TR, Pullen AM, Regg BT, Bigalow-Kern AS, Vennard T, Fix A, Reimschuessel R, Overmann G, Shan Y, Daston GP. Identification and characterization of toxicity of contaminants in pet food leading to an outbreak of renal toxicity in cats and dogs. Toxicol Sci. 2008; 106:251–62. [PubMed: 18689873]

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:F552–63. [PubMed: 14600030]

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. J Biol Chem. 1998; 273:4135–42. [PubMed: 9461608]

Jacob CC, Reimschuessel R, Von Tungeln LS, Olson GR, Warbritton AR, Hattan DG, Beland FA, Gamboa da Costa G. Dose-response assessment of nephrotoxicity from a 7-day combined-exposure to melamine and cyanuric acid in F344 rats. Toxicol Sci. 2011; 119:391–7. [PubMed: 21030430]

Kondo C, Minowa Y, Uehara T, Okuno Y, Nakatsu N, Ono A, Maruyama T, Kato I, Yamate J, Yamada H, Ohno Y, Urushidani T. Identification of genomic biomarkers for concurrent diagnosis of drug-induced renal tubular injury using a large-scale toxicogenomics database. Toxicology. 2009; 265:15–26. [PubMed: 19761811]

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T Method. Methods. 2001; 25:402–8. [PubMed: 11846609]

Ozer JS, Dieterle F, Troth S, Perentes E, Cordier A, Verdes P, Staedtler F, Mahl A, Grenet O, Roth DR, Wahl D, Legay F, Holder D, Erdos Z, Vlasakova K, Jin H, Yu Y, Muniappa N, Forest T, Clouse HK, Reynolds S, Bailey WJ, Thudium DT, Topper MJ, Skopek TR, Sina JF, Glaab WE, Vonderscher J, Maurer G, Chibout SD, Sistare FD, Gerhold DL. A panel of urinary biomarkers to monitor reversibility of renal injury and a serum marker with improved potential to assess renal function. Nat Biotechnol. 2010; 28:486–94. [PubMed: 20458319]

Pabla N, Dong Z. Cisplatin nephrotoxicity: Mechanisms and renoprotective strategies. Kidney Int. 2008; 73:994–1007. [PubMed: 18272962]

Puschner B, Poppenga RH, Lowenstine LJ, Filigenzi MS, Pesavento PA. Assessment of melamine and cyanuric acid toxicity in cats. J Vet Diagn Invest. 2007; 19:616–24. [PubMed: 17998549]

Rached E, Hoffmann D, Blumbach K, Weber K, Dekant W, Mally A. Evaluation of putative biomarkers of nephrotoxicity after exposure to ochratoxin A *in vivo* and *in vitro*. Toxicol Sci. 2008; 103:371–81. [PubMed: 18308701]

Vaidya VS, Ozer JS, Dieterle F, Collings FB, Ramirez V, Troth S, Muniappa N, Thudium D, Gerhold D, Holder DJ, Bobadilla NA, Marrer E, Perentes E, Cordier A, Vonderscher J, Maurer G, Goering PL, Sistare FD, Bonventre JV. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. Nat Biotechnol. 2010; 28:478–85. [PubMed: 20458318]

Wang EJ, Snyder RD, Fielden MR, Smith RJ, Gu YZ. Validation of putative genomic biomarkers of nephrotoxicity in rats. Toxicology. 2008; 246:91–100. [PubMed: 18289764]

Zhou Y, Vaidya VS, Brown RP, Zhang J, Rosenzweig BA, Thompson KL, Miller TJ, Bonventre JV, Goering PL. Comparison of kidney injury molecule-1 and other nephrotoxicity biomarkers in

urine and kidney following acute exposure to gentamicin, mercury, and chromium. Toxicol Sci. 2008; 101:159–70. [PubMed: 17934191]

Highlights

- Rats fed more than 69 ppm melamine and cyanuric acid for 7 days show renal toxicity
- Candidate biomarkers of nephrotoxicity genes were up-regulated at these same doses
- Quantification of these transcripts is a sensitive endpoint to assess toxicity

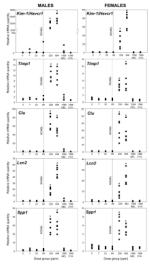

Figure 1.

Relative gene expression levels of biomarkers of nephrotoxicity (n = 6 per treatment group per sex) in kidneys of male and female F344 rats. Six-week-old animals were exposed to feed supplemented with 0 (control), 7, 23, 69, 229, or 694 ppm melamine and cyanuric acid (co-exposure groups), 1388 ppm melamine, or 1388 ppm cyanuric acid for seven days. Data were normalized for the endogenous control *Gapdh* and for the mean expression level of the matching (same sex) control by the ΔΔCt method; the control mean expression was normalized to 1. Left panel: male rats, right panel: female rats. Dashed line indicates the NOAEL as determined in Jacob *et al.* (2011). * indicates a significant group difference relative to the matching control (p-value < 0.05, one-way ANOVA followed by Dunnett's test). All genes analyzed were significantly up-regulated in both male and female rats at dose levels higher than 69 ppm combined melamine and cyanuric acid.

Table 1

Summary of the results reported in Jacob *et al.* (2011). Animals were exposed to feed supplemented with 0 (control), 7, 23, 69, 229, or 694 ppm melamine and cyanuric acid (co-exposure groups), 1388 ppm melamine, or 1388 ppm of cyanuric acid for seven days. All endpoints analyzed were affected by combined melamine and cyanuric acid, at a dose level greater than 69 ppm (NOAEL).

Endpoint	Observations per dose group
Body Weight	↔ 0, 7, 23, 69 ppm MEL + CYA ↓ 229, 694 ppm MEL + CYA ↔ 1388 ppm MEL ↔ 1388 ppm CYA
Kidney Weight	↔ 0, 7, 23, 69 ppm MEL + CYA ↑ 229, 694 ppm MEL + CYA ↔ 1388 ppm MEL ↔ 1388 ppm CYA
Kidney Crystals	− 0, 7, 23, 69 ppm MEL + CYA + 229, 694 ppm MEL + CYA + 1388 ppm MEL[1] − 1388 ppm CYA
Urinary Bladder Uroliths and Crystals	− 0, 7, 23, 69 ppm MEL + CYA + 229, 694 ppm MEL + CYA − 1388 ppm MEL − 1388 ppm CYA
Serum creatinine and BUN	↔ 0, 7, 23, 69 ppm MEL + CYA ↑229, 694 ppm MEL + CYA ↔ 1388 ppm MEL ↔ 1388 ppm CYA

Note. Abbreviations: ↔ No change; ↓ Decreased; ↑ Increased (relative to control). − Absent; + Present. MEL + CYA: co-exposure to melamine and cyanuric acid. MEL: exposure to melamine only. CYA: exposure to cyanuric acid only.

[1] A very small number of dispersed crystals was observed in 5 of the 12 rats, when the kidneys were examined by wet mount, but not by histopathology (Jacob *et al.*, 2011).

Table 2

Results of benchmark dose modeling for the relative gene expression levels of biomarkers of nephrotoxicity in kidneys of seven-week-old male and female F344 rats[a].

Gene	Sex	Model	AIC[b]	GOF[c]	BMD[d]	BMDL[d]
Kim-1/Havcr1	Male	Hill	456.8	>0.999	13.5	9.2
		Linear	472.2	0.0007	4.3	3.5
		Polynomial	467.4	0.006	7.2	4.8
		Power	465.9	0.011	7.6	5.4
	Female	Hill	201.2	0.997	16.2	9.7
		Linear	221.9	<0.0001	5.0	4.0
		Polynomial	201.0	0.601	11.6	8.3
		Power	201.4	0.527	10.8	8.4
Timp1	Male	Hill	66.9	0.967	10.2	8.7
		Linear	99.3	<0.0001	6.6	5.2
		Polynomial	99.4	<0.0001	4.5	3.1
		Power	99.3	<0.0001	6.6	5.2
	Female	Hill	50.0	0.984	15.5	9.4
		Linear	58.5	0.031	6.9	5.5
		Polynomial	60.2	0.015	8.0	4.8
		Power	59.6	0.021	9.0	5.7
Clu	Male	Hill	207.3	0.006	10.3	8.7
		Linear	249.7	<0.0001	7.9	6.2
		Polynomial	246.1	<0.0001	3.9	2.7
		Power	249.7	<0.0001	7.9	6.2
	Female	Hill	189.4	0.481	10.7	8.8
		Linear	208.5	<0.0001	9.5	7.2
		Polynomial	207.7	0.0001	5.3	3.5
		Power	208.5	<0.0001	9.5	7.2
Lcn2	Male	Hill	139.7	>0.999	15.9	9.6
		Linear	178.7	<0.0001	4.4	3.5

Gene	Sex	Model	AIC[b]	GOF[c]	BMD[d]	BMDL[d]
		Polynomial	143.0	0.354	10.5	7.9
		Power	143.8	0.248	9.5	7.7
	Female	Hill	120.8	0.970	15.2	9.8
		Linear	137.7	0.0003	5.4	4.4
		Polynomial	119.9	0.777	12.6	8.9
		Power	120.2	0.691	11.7	8.9
Spp1	Male	Hill	148.6	0.987	12.1	8.9
		Linear	175.9	<0.0001	5.6	4.5
		Polynomial	177.4	<0.0001	4.7	3.2
		Power	177.8	<0.0001	6.0	4.5
	Female	Hill	44.2	0.506	10.6	8.8
		Linear	69.3	<0.0001	8.3	6.4
		Polynomial	69.1	<0.0001	5.1	3.4
		Power	69.3	<0.0001	8.3	6.4

[a] Benchmark doses (BMDs) and the lower 95% confidence limits (BMDLs) were calculated using Environmental Protection Agency Benchmark Dose Software (version 2.1.1; http://www.epa.gov/ncea/bmds). The calculations were conducted using Hill, linear, polynomial, and power models to fit the relative gene expression levels of *Kim-1/Havcr1*, *Timp1*, *Clu*, *Lcn2*, and *Spp1* (mean ± SD; n = 6) and the mean doses of melamine and cyanuric acid from the entire seven-day study. The BMD was defined as the dose corresponding to a change in the mean response equal to one control standard deviation from the control mean.

[b] AIC, Akaike information criterion.

[c] GOF, Goodness of fit.

[d] Relative gene expression.

Table 3

Gene expression level of candidate biomarkers of nephrotoxicity in the kidney of seven-week-old control (untreated) male and female F344 rats. Data are expressed as % *Gapdh*, except *Gapdh*, which is expressed as cycle threshold (Ct) value (mean ± SD).

Gene	Females (F)	Males (M)	Fold-expression ratio (F/M)
Kim-1/Havcr1	1.21 ± 0.52	0.04 ± 0.01	34.53 *
Timp1	2.09 ± 0.51	0.94 ± 0.15	2.22 *
Clu	10.79 ± 2.23	4.73 ± 1.39	2.28 *
Lcn2	0.02 ± 0.01	0.01 ± 0.00	2.28 *
Spp1	49.28 ± 17.97	9.45 ± 2.42	5.21 *
Gapdh	22.97 ± 0.36	22.70 ± 0.56	1.01

* indicates a significant difference between males and females (p-value < 0.05, t-test).