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Performance of urinary and gene expression biomarkers in detecting the nephrotoxic effects of melamine and cyanuric acid following diverse scenarios of co-exposure

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Abstract

Although standard nephrotoxicity assessments primarily detect impaired renal function, KIM-1, clusterin, NGAL, osteopontin and TIMP-1 were recently identified biomarkers proposed to indicate earlier perturbations in renal integrity. The recent adulteration of infant and pet food with melamine (MEL) and structurally-related compounds revealed that co-ingestion of MEL and cyanuric acid (CYA) could form melamine–cyanurate crystals which obstruct renal tubules and induce acute renal failure. This study concurrently evaluated the ability of multiplexed urinary biomarker immunoassays and biomarker gene expression analysis to detect nephrotoxicity in F344 rats co-administered 60 ppm each of MEL and CYA in feed or via gavage for 28 days. The biomarkers were also evaluated for the ability to differentiate the effects of the compounds when co-administered using diverse dosing schedules (*i.e.*, consecutive vs. staggered gavage) and dosing matrixes (*i.e.*, feed vs. gavage). Our results illustrate the ability of both methods to detect and differentiate the severity of adverse effects in the staggered and consecutive gavage groups at much lower doses than previously observed in animals co-exposed to the compounds in feed. We also demonstrate that these urinary biomarkers outperform traditional diagnostic methods and represent a powerful, non-invasive indicator of chemical-induced nephrotoxicity prior to the onset of renal dysfunction.

Keywords

Nephrotoxicity; Biomarkers; Melamine; Cyanuric acid, Luminex[®] bead-based; immunoassays

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Conflict of Interest

The authors declare that there are no conflicts of interest.

1. Introduction

The essential physiological role of the kidney makes it especially vulnerable to xenobiotic insults (Werner et al., 1995). Nephrotoxicity is routinely evaluated by histopathological analyses and elevated blood chemistry biomarkers such as blood urea nitrogen (BUN) and serum creatinine (SCr), which lack the sensitivity and specificity to detect early stages of renal damage (Devarajan, 2007; Duarte and Preuss, 1993; Vaidya et al., 2008). Due to the inherent shortcomings associated with these metrics, xenobiotic-induced nephrotoxicity is typically not observed until a significant portion of renal function is lost (Price, 1992). As a result, the US FDA's Critical Path Initiative sought to identify more sensitive and predictive biomarkers of nephrotoxicity that can specifically detect adverse changes in renal integrity before kidney function is compromised. Using proteomic measurements (*i.e.*, ELISA), these efforts led to the identification of seven urinary protein biomarkers (*i.e.*, kidney injury molecule-1 (KIM-1), albumin, clusterin (CLU), trefoil factor-3 (TFF3), total protein, cystatin C, and β 2-microglobulin) that were qualified by the US FDA and European Medicines Agency (EMA) as prognostic pre-pathological indicators of nephrotoxicity in preclinical rat studies (Dieterle et al., 2010; FDA, 2008). Other urinary proteins, such as osteopontin (OPN), neutrophil gelatinase-associated lipocalin (NGAL), and tissue inhibitor of metalloproteinases-1 (TIMP-1) are in exploratory phases and have been acknowledged as valuable candidate biomarkers for acute kidney injury (Kondo et al., 2009).

Luminex[®] multiplexed bead-based immunoassays and gene transcript profiling (*e.g.*, qRT-PCR) are promising methods to detect nephrotoxicity prior to the onset of renal dysfunction and before renal histopathological changes occur (Davis and Kramer, 2006; Goodsaid, 2004; Wang et al., 2008). For example, changes in rat urine protein and kidney expression levels of KIM-1, CLU, and OPN in rats have been shown to be early indicators of damage to the nephron (Dieterle et al., 2008; Endre and Westhuyzen, 2008; Ferguson et al., 2008; Hoffmann et al., 2010; Ozer et al., 2010; Parikh and Devarajan, 2008; Vaidya et al., 2008, 2010). Compared to BUN and SCr measurements, protein detection assays offer several advantages, including being potentially earlier and more sensitive endpoints (Ichimura et al., 2004; Kondo et al., 2009; Rached et al., 2008; Wang et al., 2008; Zhou et al., 2008). The benefits of gene expression profiling as compared to histopathology include being quantifiable over a wide dynamic range and adaptable to higher throughput analysis (Camacho et al., 2011; Kondo et al., 2009; Rached et al., 2008; Wang et al., 2008).

In 2007, many household animals in North America experienced acute renal failure related to the consumption of pet food that was intentionally adulterated with melamine (MEL) and structurally-related compounds, including cyanuric acid (CYA) (WHO, 2009; Brown et al., 2007; Cianciolo et al., 2008; Puschner et al., 2007; Puschner and Reimschuessel, 2011). In the following year, in China, the adulteration of baby formula and milk-related products with MEL led to approximately 300,000 cases of renal dysfunction in infants, among which more than 50,000 were hospitalized and six deaths were reported (WHO, 2009). As a result of these outbreaks concerns have increased regarding the contamination of fruits, vegetables, fresh eggs, powdered and liquid egg products, non-dairy creamers, ammonium bicarbonate and animal feed with MEL and melamine-related compounds.

Studies using animal models have demonstrated that MEL and CYA are relatively non-toxic when administered individually (Choi et al., 2010; Hammond et al., 1986; Jacob et al., 2011; Puschner et al., 2007). Subsequent animal studies, however, have shown that co-administration of MEL and CYA is extremely nephrotoxic (Dalal and Goldfarb, 2011). This combination can induce the formation of renal melamine–cyanurate crystals that can physically obstruct kidney tubules and cause acute renal failure (Dobson et al., 2008; Filigenzi et al., 2008; Puschner et al., 2007; Reimschuessel et al., 2010).

Gamboa da Costa et al. (2012) demonstrated that although male and female Fisher 344 (F344) rats fed meal containing 180 ppm each of MEL and CYA for 28 days were observed to have a small number of renal melamine–cyanurate crystals, no significant effects on animal body weight nor feed and fluid consumption were detected. However, in a related study (unpublished data), male and female F344 rats co-administered 180 ppm of each compound via oral gavage experienced significant decreases in feed and fluid consumption within 24 h of initial exposure and significant decreases in body weight within the first 2 days of dosing. Due to these severe adverse effects, animals were euthanized on day 5. Following necropsy, extensive numbers of crystals were detected in the renal tubules of all treated animals. These findings suggest that the no adverse effect level (NOAEL) for co-administration of MEL and CYA via oral gavage is much lower than that previously determined for feed (Gamboa da Costa et al., 2012). We hypothesize that the different modes of oral exposure (*e.g.*, feed vs. oral gavage) significantly influence the pharmacokinetics of MEL and CYA and thus the time-concentration profile of each compound in the kidney tubules that is available to form melamine–cyanurate crystals and induce nephrotoxic effects. This hypothesis was recently tested by (Sprando et al. (in press) to assess the onset of crystal formation and the severity of renal effects in male and female F344 rats co-administered much lower doses of MEL and CYA (*e.g.*, 60 ppm of each compound) for 28 days via feed or gavaged either consecutively (MEL and CYA administered <1 min apart) or in a staggered (CYA administered 45 min after MEL) manner to evaluate the contribution of their pharmacokinetic properties to the induction of nephrotoxic effects.

Previous studies have illustrated the ability of urinary protein biomarkers (Zhang et al., 2012) and gene expression analysis (Camacho et al., 2011) to detect the nephrotoxic effects of MEL and CYA. However, a comparison study under diverse scenarios of co-exposure is needed to further evaluate the effectiveness of these methods. Thus, this study evaluated biological samples collected from a randomly selected set of rats that were analyzed in Sprando et al. (in press) and represents the first concurrent assessment of the ability of urinary protein and kidney gene expression biomarkers to detect renal damage induced by MEL and CYA. To our knowledge, this study also represents the first evaluation of the ability of these biomarkers to differentiate the nephrotoxic effects of these two compounds when co-administered using different dosing schedules (*i.e.*, consecutive vs. staggered gavage) and different modes of oral delivery (*i.e.*, feed vs. gavage).

2. Materials and methods

2.1. Experimental design

Biological samples were obtained from 6 male [196–213 g body weight (bw)] and 6 female (138–158 g bw) F344 rats (8–10 weeks) in each treatment group that were randomly selected from the animals evaluated in Sprando et al. (in press). Briefly, one group of animals (“dosed feed”) was fed *ad libitum* with NIH-41 irradiated meal containing 60 ppm each of MEL and CYA (equivalent to 5 mg/kg bw/day). Animals in the remaining groups were gavaged daily with carboxymethylcellulose (CMC, “control gavage”) or CMC containing 60 ppm MEL (5 mg/kg bw/day) followed by 60 ppm CYA (5 mg/kg bw/day) either immediately (<1 min apart, “consecutive gavage”) or delayed (45 min after MEL, “staggered gavage”). On days 4, 14, and 21 animals were transferred to individual metabolic cages, and urine was collected for 24 h in 50-mL conical polypropylene tubes. Urine volumes were measured and stored at –80 °C until analysis. All animals were dosed for 28 days except the “staggered gavage” animals, which were removed from the study at day 9 as a consequence of treatment related effects (*e.g.*, dramatic decrease in body weight and severe dehydration). At the end of the 28-day exposure period, animals were euthanized by carbon dioxide inhalation. Blood was collected by cardiac puncture for blood chemistry

analyses and the kidneys were fixed in formalin for histopathological, wet mount, and gene expression analyses. The half kidneys (longitudinal cut) used for the gene expression studies were flash-frozen in liquid nitrogen and stored at -80°C for *ca.* 1 month, until being processed for RNA extraction. BUN and SCr levels were determined using a VetTest™ Chemistry Analyzer (IDEXX Laboratories Inc., Westbrook, ME).

2.2. Kidney toxicity biomarker analysis

Urinary levels of KIM-1, CLU, OPN, $\beta 2$ -microglobulin, and cystatin C were measured in 96-well filter plates using the MILLIPLEX MAP Rat Kidney Toxicity Assays (Millipore, Billerica, MA). Both panels rely upon Luminex® xMAP® technology, which combines multiple unique sandwich ELISA assays, carried out on antibody-conjugated microparticle beads and analyzed by flow cytometry. Urine samples obtained from 6 male and 6 female rats per group were analyzed in duplicate following the manufacturer's instructions. Briefly, the levels of KIM-1, CLU, and OPN were quantified in 12.5 μl of undiluted urine, while the levels of $\beta 2$ -microglobulin and cystatin C were quantified in 25 μl of urine sample that was diluted 1:500 in Assay Buffer. To allow binding of analytes, beads were incubated in the dark with urine samples for ~ 16 h at 4°C . Beads were washed twice with Wash Buffer and incubated with biotinylated detection antibodies at room temperature for 1 h. Following antibody incubation, beads were washed twice with 1X Wash Buffer and incubated with Streptavidin-Phycoerythrin (SA-PE) conjugates (*i.e.*, reporter) at room temperature for 30 min. Finally, beads were washed twice with Wash Buffer, re-suspended in 100 μl of Sheath Fluid, and analyzed by flow cytometry using a Bio-Plex 200 System (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's recommendations for instrument settings and calibration. The levels of bound urinary biomarker proteins were normalized to the total volume of urine collected from each animal.

2.3. Total RNA isolation, reverse-transcription, and quantitative gene expression analysis

Relative gene expression levels of the biomarkers of nephrotoxicity were assayed as described in Camacho et al. (2011). Briefly, the frozen half kidneys were macerated in liquid nitrogen using a mortar and pestle. Total RNA was isolated from 10 to 20 mg samples of frozen tissue powder using an RNeasy Mini kit, with on-column DNase I digestion, following the manufacturer's protocol (Qiagen, Valencia, CA). All RNA samples had an absorption 260/280 nm ratio greater than 2.0, as assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA), and the RNA integrity number (RIN) was 9.19 ± 0.24 (mean $\pm$ standard deviation (SD); range 8.8–9.5; $n = 12$ randomly selected RNA samples), as assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). 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 amplified in duplicate using TaqMan assays and TaqMan Fast Universal Master Mix, 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 (*Timp-1*, NM_053819; encodes TIMP-1), Rn01449972_m1 (*Spp1*, NM_012881; encodes osteopontin), Rn00562081_m1 (*Clu*, NM_053021; encodes clusterin), Rn00590612_m1 (*Lcn2*, NM_130741; encodes lipocalin-2/NGAL). 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 $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001). Data were expressed as mean $\pm$ SD.

2.4. Statistical analysis

To obtain significance values for male and female rat body weights, the slope from each treatment group was compared to the matched control group (*i.e.*, consecutive gavage vs. control gavage, staggered gavage vs. control gavage, and dosed feed vs. control feed) followed by *F*-tests on the ratio of the two Residual Sums of Squares. *T*-tests were performed to obtain significance values for urine output and urinary biomarker levels observed between treatment groups and matched control groups. In situations where the variances between the treatment and control groups were determined to be significantly different, Satterthwaite's *t*-test was used. To minimize false significances, the *p*-values were adjusted using the resampling method of Step-down Sidák to accommodate any correlation between urine output (or urinary biomarkers) and the different time points. *T*-tests were used to compare the mean kidney weights between staggered gavage and control gavage treatment groups. For the gene expression data, *t*-tests were used to compare each treatment group with the matching control (SigmaStat v3.11). In order to maintain an equal variance and normal distribution, the gene expression data were natural log-transformed before conducting the analysis. *p*-Values <0.05 were considered statistically significant for all endpoints.

3. Results and discussion

3.1. Effects of melamine and cyanuric acid co-administration in F344 rats

Table 1 summarizes the combined effects of 60 ppm each of MEL and CYA on rat body and kidney weights, renal crystal formation and blood chemistry markers (*i.e.*, BUN and SCr) in the subset of animals used in this study (the full animal data set was previously reported by Sprando et al. (in press)). Due to severe adverse effects (*i.e.*, dramatic decrease in body weight and severe dehydration), rats in the staggered gavage group were removed from the study on day 9. All animals in the consecutive gavage group survived the 28-day study and displayed only minimal effects as indicated by a 2–6% reduction in body weight (on day 9), a 7% increase in kidney weight and a moderate number of crystals in the renal tubules and collecting ducts. However, all animals in the staggered gavage group experienced more dramatic effects as shown by a 13–16% reduction in body weight (on day 9), a 50–54% increase in kidney weight, and extensive numbers of crystals in the renal tubules and collecting ducts, which were significantly larger in size compared to those observed in the consecutive gavage group. In addition, in both gavage groups, epithelial hyperplasia and inflammatory cells were detected in the collecting ducts and multiple foci of calcified material were observed throughout the renal tubules (Sprando et al., in press). Animals on the dosed feed regimen experienced no obvious adverse effects during the exposure period.

Urine output was measured on days 4, 14, and 21 as an indicator of perturbations in renal function (Fig. 1). Within 4 days, female and male rats in the staggered gavage group displayed significant increases in urine output, 2.8- and 3.6-fold, respectively. In general, urine from this group was dilute, which may represent an early sign of nephrotoxicity or indicate the onset of osmotic diuresis due to physical blockage of the nephron by melamine–cyanurate crystals (Dobson et al., 2008). Alternatively, since MEL is a known diuretic, the higher urine volume in this group may be due to a higher bioavailability of urine. In contrast to the staggered gavage group, urine from the consecutive gavage group remained concentrated throughout the study, which may indicate that the combined effects of MEL and CYA were less severe in these animals. Animals in the dosed feed group experienced no obvious effects on rat urine output during the 28-day exposure period.

Table 1 summarizes the changes in BUN and SCr levels for this set of animals. Only the staggered gavage group experienced significantly elevated levels of BUN (2.3- and 3.3-fold

in females and males, respectively) and SCr (2.5- and 3.5-fold in females and males, respectively). The changes in blood chemistry markers in each treatment group correlated with the overall severity of effects observed on body and kidney weights, urine output, and the level of melamine–cyanurate crystals detected in the renal tubules.

3.2. Urinary analysis of nephrotoxicity biomarkers

In contrast to BUN and SCr, several renal-specific biomarkers, including KIM-1 and CLU have been qualified for use in rats as earlier and more precise indicators of acute kidney injury which may also provide quantitative estimates of tissue injury (Bailey and Ulrich, 2004; Editorial, 2010; Hayes and Bradfield, 2005; Marrer and Dieterle, 2010; Thukral et al., 2005). Other urinary proteins, including OPN, NGAL, and TIMP-1 are in exploratory phases and have been acknowledged as valuable candidate biomarkers for acute kidney injury (Kondo et al., 2009).

Multiplexed bead-based immunoassays (*e.g.*, Luminex[®]) conducted on microparticle beads allow simultaneous evaluation of multiple kidney biomarkers in a single biological sample (*i.e.*, urine). We utilized the MILLIPLEX Rat Kidney Toxicity immunoassays to analyze the newly identified nephrotoxicity biomarkers in urine samples obtained from animals that were co-administered 60 ppm of each MEL and CYA either in feed or via oral gavage (*i.e.*, consecutive or staggered). Fig. 2 illustrates the urinary levels of KIM-1, CLU, and OPN in female and male rats on days 4, 14, and 21 for each group.

In general, the levels of each biomarker displayed a similar sex-specific pattern during the course of the study. Within 4 days, KIM-1 and CLU levels in males were significantly elevated in both gavage groups compared to matched controls. KIM-1 increased 19- and 8.9-fold in the staggered and consecutive gavage groups, respectively. CLU increased 20.3- and 7.6-fold in the staggered and consecutive gavage groups, respectively. In the consecutive gavage group, peak levels of both biomarkers were observed at day 14. By day 21, protein levels in this group declined but remained significantly above control levels in the case of KIM-1. In females, KIM-1 and CLU levels were also significantly elevated as early as day 4 in both gavage groups. KIM-1 increased 49.5- and 10.4-fold in the staggered and consecutive gavage groups, respectively. CLU increased 24.4- and 6.9-fold in the staggered and consecutive gavage groups, respectively. At day 4, peak levels of both biomarkers were observed in the consecutive gavage group. By day 21, protein levels in this group declined but remained significantly above control levels in the case of KIM-1 and CLU. At day 4 in male rats, the only significant increases in OPN (2.4-fold) were observed in the staggered gavage group. In contrast, at day 4, female OPN levels were significantly increased in the staggered and consecutive gavage groups, 4.8- and 2.2-fold, respectively. Similar to KIM-1 and CLU, OPN in the consecutive gavage group declined to nearly control levels by day 21. In contrast to the gavage groups, urinary levels of KIM-1, CLU, and OPN were unaltered in animals fed meal containing both MEL and CYA. Neither gavage nor feed regimens induced changes in the levels of β -2-microglobulin or cystatin C (data not shown). NGAL and TIMP-1 were not evaluated using this approach as these analytes were not available.

It has been demonstrated that MEL and CYA concentrations below 229 ppm (equivalent to 17.6 mg/kg bw/day) in feed does not induce adverse effects in male and female F344 rats, indicating that the threshold for melamine–cyanurate crystallization in renal tubules is not attained when administered below these amounts in feed for 28 days (Jacob et al., 2011). This observation is supported by our current findings of no significant changes in protein biomarker levels and no adverse effects in animals fed meal containing 60 ppm (equivalent to 5 mg/kg bw/day) of each compound.

Perturbations in biomarker levels within each treatment group correlated with observed changes in body and kidney weight measurements, levels of melamine–cyanurate crystals in renal tubules, and changes in BUN and SCr levels. These results provide additional evidence that the staggered gavage regimen induced the most severe nephrotoxic effects in animals as indicated by the dramatic and significant increase in the levels of each biomarker within 4 days. Although animals in the consecutive gavage group experienced minimal gross adverse effects in response to the compounds, it is notable that significant increases in the urinary protein levels of KIM-1, CLU, and OPN were detected in this group. BUN and SCr levels, however, were unchanged in these animals, which further demonstrate the utility of these urinary proteins as non-invasive and prognostic pre-pathological biomarkers to assess the effects of MEL and CYA co-administration. Our results also highlight the sensitivity of the urinary biomarkers compared to BUN and SCr.

In contrast to the heart and brain, the kidney has an extensive capacity to completely recover from cell death induced by toxicant exposure (Bonventre, 2003; Witzgall et al., 1994). The regenerative process of reconstituting an intact and functional proximal tubule epithelium following a toxic insult requires dedifferentiation and proliferation of the remaining viable cells that border the damaged areas (Humphreys et al., 2011; Ichimura et al., 1998; Thadhani et al., 1996). The proposed contributions of KIM-1 to the transition from normal epithelial cells to dedifferentiated cells following nephrotoxicant-induced kidney injury indicates an important role of this protein in repair processes including the proliferation and restoration of cellular architecture and function (Bonventre, 2003; Ichimura et al., 1998, 2004; Wang et al., 2008). In addition to KIM-1, CLU and OPN are also proposed to make key contributions to the repair of kidney tubules in response to cell death (Dodiya et al., 2011; Harpur et al., 2011; Persy et al., 1999; Rouse et al., 2011; Wang et al., 2008; Xie et al., 2001a,b).

The dramatic increases in urinary KIM-1, CLU, and OPN in the gavage groups may reflect both the nephrotoxic effects induced by MEL and CYA co-administration and the renoprotective responses to kidney damage. The substantial increase in urinary biomarker levels and severe adverse effects observed in the staggered gavage group on day 4 may be related to a reduction in renal repair processes. In contrast, the rise and subsequent decline in KIM-1, CLU, and OPN levels by day 21 in the consecutive gavage group may indicate that the damage induced by this dosing regimen was less severe and/or repaired. The reason for an increased repair capacity at later time points despite the continuous exposure to MEL and CYA is currently unknown.

3.3. Gene expression analysis of nephrotoxicity biomarkers

Quantitative real-time RT-PCR remains the standard for accurate evaluation of changes in gene transcript levels in response to cellular perturbations. Following co-exposure to MEL and CYA, relative changes in rat kidney gene expression for several nephrotoxicity biomarkers were measured (Fig. 3). Gene transcript analysis demonstrated that *Kim-1*, *Clu*, *Spp1* (osteopontin), *Lcn2*, and *Timp-1* were induced in the kidneys of animals on the staggered gavage regimen. Although the magnitude of gene induction varied for each biomarker, significant increases in expression were observed for all transcripts evaluated in this group. *Kim-1* was the most up-regulated gene with expression 1500- and 400-fold in females and males, respectively. *Clu*, *Spp1*, and *Lcn2* were induced at similar levels ranging between 60- to 90-fold in females and males. *Timp-1* was the least up-regulated gene in both sexes with levels elevated 12-fold above matched controls. Since animals in the remaining groups survived the duration of the exposure period, renal gene expression was evaluated at day 28. In general, low levels of gene induction were observed in the consecutive gavage group where the only significant changes were detected for *Timp-1* in females and *Clu* in males (Fig. 3). No changes in gene expression were detected in the dosed feed group (data not shown). Similar to the observed effects on urinary protein levels, expression of

biomarker genes were generally consistent with the severity of effects on body and kidney weights, blood chemistry markers, and the extent of renal crystal formation in each treatment group.

Urine biomarkers were evaluated in samples collected on days 4, 14, and 21, while histopathological assessments and gene expression analyses were conducted on samples collected on day 9 (staggered gavage group only) or 28 (remaining groups). Thus, although adverse effects were observed using both detection methods, variances in biomarker status may be due to temporal changes in the renal environment. For example, as late as day 21 in the consecutive gavage group, a time-dependent decrease in urinary biomarkers was observed whereas minimal or no changes in gene expression detected at day 28. Again, these findings may reflect both renal damage and repair processes in the tubules of animals on the consecutive gavage regimen. Similar to the proposed renoprotective attributes of KIM-1, CLU, and OPN, NGAL and TIMP-1 have also been linked to regenerative processes in response to renal damage (Miya et al., 2011; Mori et al., 2005; Sola et al., 2011; Srisawat et al., 2010; Wang et al., 2008).

A recent assessment of combined exposure to MEL and CYA established a no observable adverse effect level (NOAEL) of 69 ppm (equivalent to 8.6 mg/kg bw/day) in F344 rats fed meal containing equivalent doses of the compounds for 7 days (Jacob et al., 2011). Subsequent kidney gene expression analysis of *Kim-1*, *Clu*, *Spp1*, *Lcn2*, and *Timp-1* substantiated the proposed NOAEL in feed (Camacho et al., 2011). However, F344 rats gavaged with as low as 60 ppm (5 mg/kg bw/day) of each compound in a staggered manner experienced significant adverse effects including nephrotoxicity (Sprando et al., in press). In addition, animals gavaged consecutively with 60 ppm of each compound also experienced adverse effects, although to a significantly lesser degree and likely in a reversible fashion. These findings suggest that the threshold for renal melamine–cyanurate crystal formation is much lower when the compounds are co-administered via gavage, and illustrates the ability of both the urinary protein and gene expression biomarkers to detect these insults. The data presented in the current study substantiate the findings of Sprando et al. (in press) and illustrate the effectiveness of both urinary and genomic biomarkers to detect the differential effects of co-exposure to MEL and CYA via diverse exposure scenarios. These findings also suggest a temporal relationship exists between urinary protein and gene expression biomarker levels.

4. Conclusions

This study evaluated the utility of urinary protein and gene expression analyses to detect and differentiate the nephrotoxic effects of MEL and CYA that were orally co-administered to rats via diverse dosing schedules and dosing matrixes. Our findings demonstrate that alterations in urinary biomarker protein and renal gene expression levels following co-exposure to MEL and CYA correlated with the observed adverse effects (*e.g.*, decreases in body weight and increases kidney weight and blood chemistry biomarkers, and renal failure) reported in Sprando et al. (in press). Our findings also illustrate the ability of these alternative metrics to detect and differentiate the severity of adverse effects experienced by animals in the staggered and consecutive gavage groups at much lower doses (60 ppm of each compound) than previously observed when the compounds were co-administered in a food matrix. Future studies should include the determination of a NOAEL for gavage regimens for co-administration of MEL and CYA and a study of the mechanistic link between the increases in these biomarkers in response to the nephrotoxic effects of these compounds.

In contrast to nephrotoxics that act by altering cellular biochemical processes, melamine–cyanurate crystals physically obstruct the nephrons, which can lead to acute renal failure. Our results provide additional support to the effectiveness of these alternative biomarkers to detect nephrotoxic effects induced by diverse mechanisms. In addition, this work in rats demonstrates that analysis of urinary protein levels of nephrotoxicity biomarkers represents a powerful, non-invasive, and prognostic approach to detect adverse renal effects induced by co-exposure to MEL and CYA in rats prior to the onset of severe histopathological changes (*e.g.*, consecutive gavage group), which was not indicated by the blood chemistry measurements (*e.g.*, BUN and SCr). Our findings also further support the potential for using transcriptional analysis of nephrotoxicity biomarkers as a sensitive, fast, and quantitative method to complement histopathology analyses.

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Abbreviations

BUN	blood urea nitrogen
CYA	cyanuric acid
ELISA	enzyme-linked immunosorbent assay
KIM-1	kidney injury molecule-1
MEL	melamine
NGAL	neutrophil gelatinase-associated lipocalin
qRT-PCR	quantitative real-time reverse transcription polymerase chain reaction
SCr	serum creatinine
TIMP-1	tissue inhibitor of metalloproteinase-1

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Fig. 1.

Effects of co-administration of MEL and CYA on rat urine output. Urine output from male (black bars) and female (gray bars) F344 rats co-administered MEL and CYA was measured on day 4, 14, and 21. Feed groups were administered *ad libitum* either NIH-41 irradiated meal (control feed) or meal containing 60 ppm each of MEL and CYA (dosed feed). Gavage groups were administered carboxymethylcellulose alone (control) or carboxymethylcellulose containing MEL followed by carboxymethylcellulose containing CYA, either immediately (consecutive) or delayed 45 min after MEL (staggered). Urine samples were obtained from the same animals on each collection day. Bars represent the average relative urine output for all animals in each treatment group ($n = 6$ rats per group). Error bars represent the standard deviation of urine output in animals for each treatment group. Asterisks denote significant differences (** $p < 0.01$ or * $p < 0.05$) compared to matched control groups. Note: Due to the severity of the treatment-related effects, animals in the staggered gavage group were removed from the study after day 9 and data from this group are only depicted in day 4 graphs.

Fig. 2.

Effects of co-administration of MEL and CYA on urinary protein levels of nephrotoxicity biomarkers. Changes in levels of urinary protein biomarkers in male (black bars) and female (gray bars) F344 rats co-administered MEL and CYA were quantified using multiplexed immunoassays in urine samples collected on day 4, 14, and 21. All treatment conditions and sample collection were identical to those described in Fig. 1. Bars represent the average relative levels of urinary proteins for all animals in each treatment group ($n = 6$ rats per group). Error bars represent the standard error of the mean for each urine sample analyzed in duplicate. Asterisks denote significant differences ($***p < 0.001$, $**p < 0.01$, or $*p < 0.05$) compared to matched controls. *Note:* Due to the severity of the treatment-related effects,

animals in the staggered gavage group were removed from the study on day 9 and data from this group are only depicted in day 4 graphs.

Fig. 3.

Effects of co-administration of MEL and CYA on the expression of nephrotoxicity biomarker genes in staggered and consecutive gavage groups. Changes in kidney expression of nephrotoxicity biomarker genes in male (black bars) and female (gray bars) F344 rats co-administered MEL and CYA were quantified by qRT-PCR. All treatment conditions were identical to those described in Fig. 1. Kidney gene expression levels were normalized to the endogenous control, *Gapdh*, and the mean expression value of the control group was normalized to 1. Error bars represent the standard deviation of each kidney sample from animals in the staggered and consecutive gavage groups ($n = 6$ rats per group). Asterisks denote significant differences (** $p < 0.01$, * $p < 0.05$) compared to matched

controls. Note: Due to the severity of the treatment-related effects, animals in the staggered gavage group were removed from the study on day 9.

Table 1

Summary of body and kidney weights, melamine–cyanurate crystal formation, and blood chemistry biomarkers in rats treated with melamine and cyanuric acid for 28-days^a.

Treatment group	Body weight (g) (males/ females) ^b	Kidney weight (g) (males/ females) ^c	Kidney crystals (males/ females) ^f	BUN (mg/dL) (males/ females)	SCr (mg/dL) (males/ females)
Gavage control	219.5 ± 7.9/150.2 ± 7.8	3.09 ± 0.13/3.30 ± 0.14	–/–	20.5 ± 2.6/22.3 ± 3.6	0.4 ± 0.04/0.4 ± 0.09
Gavage consecutive	206.5 ± 6.5/147.3 ± 6.8	3.31 ± 0.29/3.52 ± 1.00	+/+	18.5 ± 2.3/22.3 ± 2.7	0.5 ± 0.12/0.4 ± 0.05
Gavage staggered ^{b,c,d}	184.3 ± 5.5/130.8 ± 9.6	6.30 ± 0.61/6.12 ± 0.28	++/++	68.5 ± 13.0/50.7 ± 12.3	1.4 ± 0.20/1.0 ± 0.26
Feed control	222.5 ± 5.1/151.7 ± 3.3	3.04 ± 0.20/3.48 ± 0.18	–/–	18.3 ± 1.2/22.2 ± 5.2	0.5 ± 0.05/0.5 ± 0.10
Feed dosed	217.5 ± 9.2/151.5 ± 6.5	3.06 ± 0.12/3.50 ± 0.18	–/–	19.8 ± 1.5/22.2 ± 4.9	0.4 ± 0.06/0.4 ± 0.08

Note: Abbreviations for average levels of melamine–cyanurate crystals observed in the kidney renal tubules: (–) no crystals observed; (+) few to moderate number of crystals detected; (++) extensive number of crystals detected.

^aData are from a pre-selected subset (60 out of 120) of animals and represent $n = 6$ rats per group.

^bValues denote measurements taken on day 9.

^cDue to the severity of the treatment-related effects, animals in the staggered gavage group were removed from the study on day 9.

^dEffects in this group were significantly different from controls ($p < 0.05$ for body weight, $p < 0.001$ for kidney weight).

^eThe weight of the left and right kidney from each animal ($n = 6$ rats per group) was averaged and normalized to overall body weight and values were multiplied by 1000.

^fRenal melamine–cyanurate crystals were detected using the wet mount method.