Abstract #1893

A Comparative Study on the Renal Toxicity of Industrial Chelates Using Novel Urinary Protein Biomarkers as Early Predictors of Nephrotoxicity

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Abstract

Toxicogenomic approaches have identified protein biomarkers of renal cell injury/repair as early predictors of renal toxicity prior to changes in renal histopathology. We used these novel biomarkers to determine if rats orally dosed with three industrial chelates exhibited altered urinary biomarker levels that preceded histopathologic changes in kidney cells. The nephrotoxicant/renal carcinogen, nitrilotriacetic acid (NTA), is known to cause rat proximal tubule cell injury/repair (3–7 weeks) followed by renal tumors (2 years) after oral dosing. A new, readily biodegradable chelate, L-glutamic acid diacetic acid tetrasodium (GLDA), which previously showed no significant microscopic renal changes (90-day, oral), and EDTA, known non-carcinogen (oral bioassay) were also included in our study. Male Wistar rats (n=10/group) were gavaged daily with sodium salts of chelates (28 days; 1000 mg/kg/day). As expected, mean urinary zinc and sodium levels were higher in all chelate-treated groups. Two animals in the NTA group were euthanized as moribund on Day 13. The surviving NTA group exhibited decreases in mean body weights, food and water consumption, and urine magnesium, and increases in the mean levels of urine calcium, total protein, lactate dehydrogenase, Kim-1, and clusterin. The latter two inducible kidney proteins are approved by FDA as predictive, early and noninvasive urinary biomarkers of kidney injury/repair. None of the above changes were seen in the GLDA group. At necropsy, bilateral kidney enlargement (mean relative kidney weights) was noted in NTA, but not GLDA group. In conclusion, this study showed that NTA, but not GLDA or EDTA, caused significant early renal cell toxicity when evaluated with urinary protein biomarkers as early predictors of nephrotoxicity. (Funding: AkzoNobel Functional Chemicals, LLC)

Introduction

Chelates are chemical agents that reversibly complex with metal ions, usually making the metals more soluble. Industrial chelates have many uses such as hard surface cleaner, water softening, autodish washing / personal care products, oil and gas field applications, etc. The nephrotoxicant / renal carcinogen, NTA (Nitrilotriacetic acid, Na3.H2O), is a common chelate known to cause rat proximal renal tubule cell injury/repair (3–7 wks) and proximal tubule cell proliferation, followed by renal tumors (2 yrs) after oral dosing (1,2). A new (readily biodegradable) chelate, Dissolvine GL (L-glutamic acid diacetic acid tetrasodium; GLDA), previously showed no significant microscopic renal changes in a 90-day oral study in rat (3). EDTA, a well known chelate used as a food additive, is a not a carcinogen but is not readily biodegradable.

Toxicogenomic approaches by the Predictive Safety Testing Consortium have identified protein biomarkers of renal cell injury/repair as early predictors of renal toxicity prior to changes in renal histopathology in rats and humans (4). In this comparative study on the above 3 chelates, we used these novel biomarkers, focusing on those approved by the FDA, to determine if rats orally dosed with industrial chelates exhibited altered urinary protein biomarker levels that preceded renal cell histopathologic changes. Since uncontrolled cell proliferation represents an important early step in the development of cancer, we measured the proximal tubule cell proliferation *in vivo* as another critical parameter of assessing potential nephrotoxicity by industrial chelates.

It was **hypothesized** that NTA exposure to rats will result in adverse effects on the levels of urinary protein biomarkers of kidney cell injury/repair and on renal cell proliferation, whereas GLDA exposure will not result in these adverse renal effects.

Study Objective

To differentiate the potential nephrotoxicity of NTA, a known nephrotoxicant and renal carcinogen, from GLDA and EDTA using selected urinary biomarker(s) and renal proximal tubular cell proliferation.

Methods

Study Design: Three chelates (from AkzoNobel BU Functional Chemicals) were tested: (1) CAS#18662-53-8, MW: 275.1, NTA-Na_3 monohydrate: 99.8%; (2) CAS#51981-21-6, MW: 351.1, L-GLDA-Na_4: 91.6%; and (3) CAS#13235-36-4, MW: 452.3, EDTA-Na_4-4H_2O: 98.7%. Male Wistar (Crl:WI(Han)) rats (n=10) were dosed daily by oral gavage for 28 days with NTA, GLDA, or EDTA (all solutions at pH 8.5; 1000 mg/kg/d) or vehicle (water, pH 8.5). Dose selection was based on a dose range finding study, literature, and OECD limit dose guidelines. Overnight urine was collected on Days 7, 14, 21, and 28. On necropsy (Day 29), kidneys were weighed. ANALYSES: **(1)** Dose formulations were analyzed (AkzoNobel Functional Chemicals, The Netherlands) by HPLC and NMR. All formulations were stable for 7 days and the concentrations were within acceptable limits (>95%). **(2)** Urine chemistry: Na^+, Ca^{2+}, Mg^{2+}, creatinine, total protein, LDH, and alkaline phosphatase were analyzed by Antech Diagnostics (NC). Urine zinc levels were measured by Inductively Coupled Plasma-Mass Spectrometry (Chemical Solutions Ltd., PA). **(3)** Urine protein biomarker levels were analyzed by Myriad RBM, Inc. (TX) using Rat KidneyMAP® v1.0 platform in a quantitative immunoassay. Biomarkers were quantitated by multiplex assays on a Luminex platform. Units were expressed as ng/mL and per mg creatinine. **(4)** Kidney cell proliferation: The number of DNA synthesizing proximal tubule cells of renal cortex was enumerated by *in vivo* Brdu labeling followed by immunostaining and counting at least 2000 cells in renal cortex bounded by the outer stripe and just below the outer margin (OSOM). **(5)** Histopathologic evaluation of H&E stained kidney sections was made by EPL, Inc., (NC). ILS, Inc., NC provided the quality assurance service. **(6)** Statistics: Provantis ™8 software system and JMP® 9.0.0 software (SAS) were used.

Results

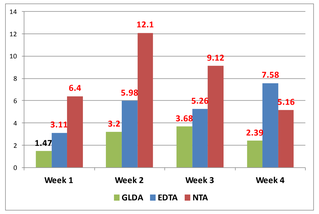
In all tables and figures, red colored numbers indicate mean values with statistically significant difference from controls

Table 1: Summary Clinical Observations / Kidney Weights / Necropsy / Histopathology

	Control	EDTA	GLDA	NTA
Body weight		↓		↓
Mortality	None	None	None	2/10
Necropsy	NR	NR	NR	NR
Kidney weight (left)/BW ratio @28 days	0.30221	0.30504	0.30782	0.35778 ↑
Kidney weight(right)/BW ratio @ 28 days	0.31434	0.31296	0.31071	0.38769 ↑
Histopathology	NR	NR	NR	NR

NR = not remarkable

Figure 1: Effect of Chelates on the Levels of Urinary Zinc (Mean Fold-Change)



Y-Axis: Urinary Zn^{2+} levels (mean ppm expressed as fold-change from concurrent control mean).

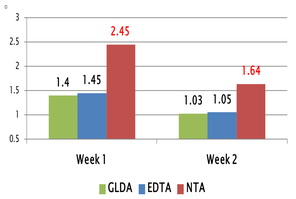
All chelates caused statistically significant increase, but NTA and GLDA caused the highest and the least increases, respectively, in Zn^{2+} excretion. The levels of Zn^{2+} eliminated by NTA were ~ **2 - 4 times** higher than by GLDA.

Table 2: Urinary Chemistry Changes – Metal Ions*

Group		Pre-study	Week 1	Week 2	Week 3	Week 4
1. Control	**Sodium**	0.821	0.522	0.740	0.668	0.607
	Calcium	0.093	0.086	0.124	0.097	0.104
	Magnesium	0.379	0.320	0.325	0.332	0.237
2. EDTA	**Sodium****	0.792	1.520 ↑	1.796 ↑	1.832 ↑	1.739 ↑
	Calcium	0.074	0.078	0.080	0.073	0.170
	Magnesium	0.271	0.196	0.216 ↓	0.166 ↓	0.117 ↓
3. GLDA	**Sodium****	0.792	1.577 ↑	1.658 ↑	1.587 ↑	1.361 ↑
	Calcium	0.101	0.087	0.092	0.107	0.103
	Magnesium	0.365	0.288	0.281	0.274	0.193
4. NTA	**Sodium****	0.839	2.037 ↑	2.210 ↑	1.947 ↑	1.846 ↑
	Calcium	0.725	0.159	0.275 ↑	0.282 ↑	0.230 ↑
	Magnesium	0.263	0.092 ↓	0.130 ↓	0.133 ↓	0.331

*Values were normalized for measured creatinine.
** All test articles were dosed as sodium salts, explaining higher sodium elimination

Figure 2: Effect of Chelates on the Urinary Levels of Kim-1



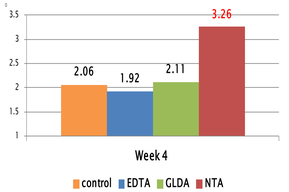
The mean levels of Kim-1 (**Y-axis)**, an FDA-approved nephrotoxicity biomarker, expressed as fold-change over concurrent controls. Only those weeks in which Kim-1 levels showed statistical significance are shown. Mean pre-study level = 0.225±0.015 ng/mg creatinine.

Table 3: Summary of Changes in the levels of FDA-Approved Urinary Biomarkers in Weeks 1 to 4

Group		Week 1 Kim-1	Week 2 Kim-1	Week 4 CLU
1. Control	Mean ± SEM	0.200 ± 0.032	0.227 ± 0.025	0.250 ± 0.015
	n	10	10	10
2. EDTA	Mean ± SEM	0.290 ± 0.041	0.239 ± 0.031	0.240 ± 0.030
	Fold change	1.45	1.05	0.96
	p value (n)	0.207 (10)	1.000 (9)	0.857 (9)
3. GLDA	Mean ± SEM	0.280 ± 0.020	0.233 ± 0.023	0.254 ± 0.027
	Fold change	1.40	1.03	1.02
	P value (n)	0.139 (10)	0.997 (10)	0.920 (10)
4. NTA	Mean ± SEM	0.489 ± 0.053	0.372 ± 0.052	0.606 ± 0.099
	Fold change	2.45 ↑	1.64 ↑	2.42 ↑
	P value (n)	0.003 (12)	0.010 (9)	0.006 (9)

Only those FDA-approved biomarkers whose levels increased significantly in at least one treatment group are presented. Kim-1 = kidney injury molecule-1, ng/mg creatinine; CLU = clusterin, µg/mg creatinine.

Figure 3: Effect of Chelates on DNA Synthesis (Cell Proliferation) in Renal Cortex



Values (**Y-axis**) represent **mean labeling index (%)** on Day 29. Kidney sections (8-10 animals per group) were evaluated microscopically after Brdu incorporation *in vivo*. Only the NTA-treated group (158% of control value) was statistically significant ($p<0.05$) from control group.

References

1. Bahnemann, R., et al (1998). *Toxicol Sci* 46:166-175.
2. NCI Bioassay report (1977). *NCI-CG-TR-6, DHEW, PHS, NIH.*
3. AkzoNobel GLP Report, GLDA, (2007).
4. Ozer, J.S., et al., (2010). *Nature Biotech.* 28(5): 486-495.

Table 4: The two FDA-approved Kidney Injury Biomarkers Whose Levels Increased in This Study

URINARY PROTEIN (EARLY BIOMARKER OF NEPHROTOXICITY)	TYPE OF PROTEIN AND RENAL CELLUAR LOCATION	DIAGNOSTIC SIGNIFICANCE IN RENAL PATHOLOGY
Kim-1	*Kidney injury molecule 1 *Type 1glycoprotein *Proximal tubular (PT) membrane *Inducible protein	*The ectodomain of Kim-1 is shed in urine after PT injury *Most sensitive and early diagnostic indicator of kidney injury *Highest sensitivity compared to conventional biomarkers *Overall indicator of tubular injury / cell repair process
CLUSTERIN	*Glycoprotein *PT and distal tubular cells *Inducible protein	*Expressed on PT & distal tubule cells after injury *Indicator of cell repair processes in general renal injury *Has been detected in renal cell carcinoma

Another biomarker, not approved by FDA, Glutathione-S-transferase-alpha (a constitutive cytoplasmic enzyme), was also tested. A statistically significant increase was noted in NTA group throughout the study. GLDA group showed a smaller increase.

Table 5: Study Conclusions

	EDTA	GLDA	NTA
1. Elevated levels of FDA approved predictive biomarkers of kidney injury/repair	No	No	Yes
2. Detection of urine chemistry changes that are indicative of impaired renal function	No	No	Yes
3. Bilateral renal enlargement (increase in relative kidney weight)	No	No	Yes
4. Increased number of DNA synthesizing renal cells (proximal tubule cell proliferation)	No	No	Yes
Overall nephrotoxicity profile of chelates based on the Weight of Evidence: **•The Profile of NTA is much different from GLDA** **• Profile of GLDA much closer to EDTA**			

Results

Clinical Observations, kidney weight and histopathology (Table 1): In the NTA group, mean body weight and food consumption were decreased during Weeks 1 & 2 and the water consumption decreased during Week 1. The GLDA group did not show these changes. During the last 2 weeks, the EDTA group had decreased body weight and food consumption. Increased (15–25%) left & right mean absolute and relative kidney weights was noted in NTA, but not GLDA or EDTA groups. No remarkable histopathologic changes were seen in kidneys.

Urine chemistry changes (Table 2; Fig. 1): Mean urine Na^+ and Zn^{2+} levels were higher (2.4 - 12.1-fold compared to control) in all chelate treated groups, with the highest and lowest changes in the NTA and GLDA group, respectively. Increased elimination of Zn^{2+} is expected because chelates in general affect urinary Zn^{2+} levels. Mean urine Ca^{2+} levels were higher in NTA, but not in GLDA group. Throughout the study, decreased mean urine Mg^{2+} level was noted in NTA and EDTA groups, but not in GLDA group. Increases in levels of urine total protein and lactate dehydrogenase were seen sometimes in the NTA, but not GLDA group (data not shown).

Summary of the urinary protein biomarkers data (Table 3; Fig. 2): Levels of two (FDA-approved) predictive protein biomarkers of nephrotoxicity were significantly elevated after treatment with NTA, but not GLDA. Levels of Kim-1 and CLU were significantly increased in NTA group in Weeks 1&2, and Week 4, respectively.

Renal cell proliferation (Fig. 3): Significantly increased mean BrdU labeling index of cortical proximal tubular cells was noted in the NTA group.

Discussion

Among the male Wistar rats dosed with 1000 mg/kg/day of chelates via oral gavage for 28 days, the NTA group exhibited clinical signs of toxicity, decreased urine Mg^{2+}, and increases in kidney weight, urine Ca^{2+}, total protein, LDH, Kim-1, and CLU. GLDA caused none of these changes. The NTA, but not the GLDA group, showed increased renal proximal tubule cell proliferation. In this study, Kim-1 and Clusterin were demonstrated to be predictive biomarkers of kidney injury / repair caused by NTA. The genes/proteins of these biomarkers are upregulated by nephrotoxic chemicals / pharmaceuticals that cause Acute Kidney Injury (4). Both proteins, expressed in rat and human, are approved by FDA and EMEA to evaluate new drugs causing kidney damage in animal studies (**Table 4**).

Conclusion

(Table 5): This urinary protein biomarker study showed that NTA caused significant early renal cell toxicity and renal cell proliferation, but GLDA did not. The order of highest to lowest severity of renal effects for the test agents studied was NTA>EDTA=GLDA.