Data Package Submission to the EMA

Nonclinical Enablement of Drug-Induced Kidney Injury Translational Biomarkers

The Nephrotoxicity Working Group of Critical Path Institute's Predictive Safety Testing Consortium

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LIST OF ABBREVIATIONS

AKI – Acute Kidney Injury
AUC – Area under the curve
BUN – Blood urea nitrogen
CLSI – Clinical and Laboratory Standards Institute
CTL – Control animal
C-Path – Critical Path Institute
DIKI – Drug-induced kidney injury
ELISA - Enzyme-linked immunosorbent assay
EMA – European Medicines Agency
FNIH BC – Foundation for the National Institute of Health Biomarker Consortium
FDA – U.S. Food and Drug Administration
GlA – Glomerulopathy
GLP – Good laboratory practice
HESI – Health and Environmental Sciences Institute
HyD – Hyaline droplet formation
IDI – Integrated discrimination improvement
IP – Intraperitoneal
IV – Intravenous
KIM-1 – Kidney injury molecule 1
KIP – Kidney Injury Panel
LCN2 – lipocalin-2
MAP – Multi-Analyte Profile
MSD – Meso Scale Discovery
NGAL – neutrophil gelatinase-associated lipocalin
NME – New molecular entity
NO – nitric oxide
NRI – Net reclassification index
NWG – Nephrotoxicity Working Group
OPN – osteopontin
PO – per os (by mouth)
PSTC – Predictive Safety Testing Consortium
PT – Proximal tubule
q05 – 5^{th} Quartile
q95 – 95^{th} Quartile
ROC – Receiver operator characteristic
sCr – serum creatinine
SD – Sprague Dawley Rat Strain
S1 – Segment 1
S2 – Segment 2
S3 – Segment 3
SPP1 – secreted phosphoprotein I
SQ – subcutaneous
TDN – Tubular degeneration/necrosis
TRe – Tubular cellular regeneration
RBM – Myriad Rules Based Medicine
STP – Society of Toxicologic Pathology

EXECUTIVE SUMMARY

This Data Package reports the results of Critical Path Institute's Predictive Safety Testing Consortium (PSTC) Nephrotoxicity Working Group's (NWG) analysis of urinary osteopontin (OPN) and neutrophil gelatinase-associated lipocalin (NGAL) as translational biomarkers of renal tubular epithelial degeneration/necrosis.

The aim of this work was to demonstrate the diagnostic potential of selected urinary proteins OPN and NGAL for specific histopathological diagnoses. Data was compiled from toxicology studies contributed by PSTC members where rats were dosed with well-described nephrotoxicants to generate drug-induced kidney injury (DIKI) cases and corresponding urine samples were used to measure the candidate biomarkers.

The data presented here are specifically designed to enable the early clinical exploratory use of these biomarkers by providing the nonclinical pathological and toxicological underpinning of the OPN and NGAL response. Both of these urinary biomarkers are appropriate for voluntary use in regulatory toxicology studies in rats, in conjunction with current methods for assessing nephrotoxicity, to demonstrate that drug-induced kidney injuries seen with developmental compounds in animal toxicology studies are monitorable. These data can then be used to deploy biomarker monitoring strategies in early clinical trials to ensure patient safety and enable drug development to proceed. This document is intended to provide sponsors with information to enable such applications of the biomarkers.

Both urinary OPN and NGAL outperformed serum blood urea nitrogen (BUN) and serum creatinine (sCr) in identifying cases of drug induced kidney injury characterized by renal tubular epithelial degeneration/necrosis when applied to rat toxicology studies. In addition, when urinary OPN and NGAL data are considered with BUN and sCr data, there is a significant increase in confidence to accurately diagnose the presence or absence of renal tubular epithelial degeneration/necrosis.

1 BIOMARKER PERFORMANCE

The aim of this work was to determine the diagnostic potential of urinary OPN and NGAL for specific histopathological observations of kidney injury by performing toxicology studies with well-described nephrotoxicants. The data were combined such that the histopathological diagnoses could be understood across studies and used in objective statistical tests. Biomarker measurements were conducted using commercial assays according to manufacturer specifications. The dataset predominantly represents proximal tubular injury although an effort was made to include other types of renal injury.

Two statistical approaches were used to objectively determine the performance of each biomarker. The first statistical approach was:

1) Outperformance of serum BUN and sCr using Receiver Operator Characteristic (ROC) analysis.

The ROC analysis was used to evaluate the composite sensitivity and specificity of the biomarker. This test was applied to determine whether a biomarker "outperforms" serum BUN and sCr as an indicator of nephrotoxicity. Outperformance is defined as a statistically significant difference in ROC area under the curve (AUC) such that:

a) ROC AUC for biomarker significantly greater than for sCr ($p<0.05$)

b) ROC AUC for biomarker significantly greater than for serum BUN ($p<0.05$)

The second statistical approach used to objectively determine the performance of each biomarker was:

2) Improved certainty of diagnosis when added to serum BUN and sCr using net reclassification index (NRI) and integrated discrimination improvement (IDI) analyses.

For this approach, NRI and IDI were evaluated to determine if a candidate biomarker adds incremental value to identifying positive and negative cases of nephrotoxicity. This test was implemented as the method of defining biomarkers that "improve certainty" of a nephrotoxicity diagnosis. Since this approach is very sensitive to any shifts in information from new biomarker data and many of the markers can add information to serum BUN and sCr data, a biomarker was defined as "improving certainty" as follows:

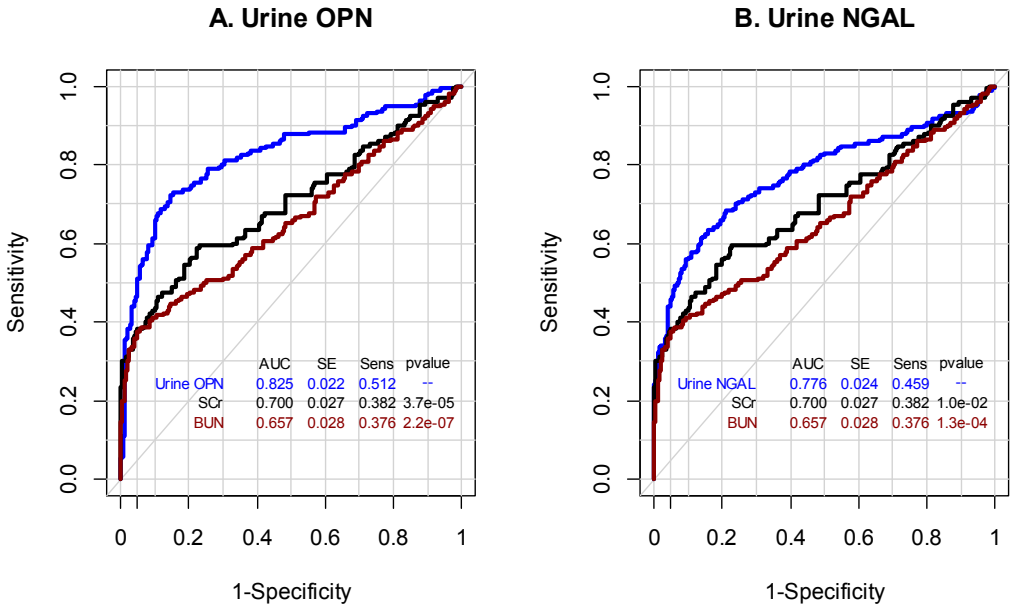
a) NRI of improved positive findings with a p-value less than $1x10^{-4}$

b) NRI of improved negative findings with a p-value less than $1x10^{-4}$

c) IDI of overall improvement with a p-value less than $1x10^{-4}$

According to the criteria defined above, the results of these studies demonstrate that urinary OPN and NGAL outperform serum BUN and sCr in identifying cases of tubular necrosis/degeneration (Figure 1). When used with serum BUN and sCr, urinary biomarkers OPN and NGAL improve diagnostic certainty of correctly identifying cases of tubular necrosis/degeneration with a performance similar to previously qualified renal biomarkers (Table 1). Total mean improvement for urinary NGAL was 0.066 or 6.6% over serum BUN and sCr alone (second to last column of Table 2). The mean improvement for OPN was 15.8%. There was also significant net improvement over serum BUN and sCr alone for identifying both positive tubular degeneration/necrosis and negative or lack of tubular degeneration/necrosis findings for both biomarkers. Each of these improvements is significant with p-values less than $1x10^{-4}$. These results indicate that OPN and NGAL add significant value to the detection, discrimination, and possibly the monitoring, of drug-induced nephrotoxicity in rats.

Figure 1 **ROC Analysis Curves to Detect Tubular Degeneration/Necrosis for OPN and NGAL compared to SCr and BUN**



Receiver operator characteristic (ROC) display of urinary biomarkers (blue), serum BUN (red), and sCr (black) sensitivity and specificity for the proposed biomarkers. A: OPN; B: NGAL, Sens=Sensitivity at 95% Specificity, p-value= p-value of marker AUC vs. SCr and BUN AUC.

Table 1 **ROC Analysis of Tubular Degeneration/Necrosis to Determine if a Biomarker Outperforms Serum BUN and sCr**

Biomarker	AUC	p-value vs. Random (AUC = 0.5)	Difference in AUC vs. sCr	p-value vs. sCr	Difference in AUC vs. BUN	p-value vs. BUN
Newly proposed biomarkers						
Urinary OPN	0.825	**<1.0E-14**	0.125	**3.70E-05**	0.167	**2.21E-07**
Urinary NGAL	0.776	**<1.0E-14**	0.076	**1.02E-02**	0.119	**1.31E-04**
Previously Qualified Biomarkers						
Urinary Kim-1	0.872	**<1.0E-14**	0.172	**4.94E-08**	0.215	**6.68E-12**
Urinary Clusterin	0.795	**<1.0E-14**	0.092	**2.61E-02**	0.116	**7.89E-04**
Urinary Albumin	0.780	**<1.0E-14**	0.079	**1.31E-02**	0.122	**4.23E-05**
Urinary Total Protein	0.769	**<1.0E-14**	0.079	**2.47E-02**	0.128	**2.01E-04**
Urinary Beta 2-µglobulin	0.755	**<1.0E-14**	0.095	**6.99E-03**	0.146	**1.53E-05**

Summary table of ROC analysis of biomarkers while evaluating sensitivity/specificity for Tubular Degeneration/Necrosis diagnoses. P-values in bold indicate statistical significance where p<0.05.

Table 2 NRI and IDI Analyses of Tubular Degeneration/Necrosis to Determine if a Biomarker Improves Certainty

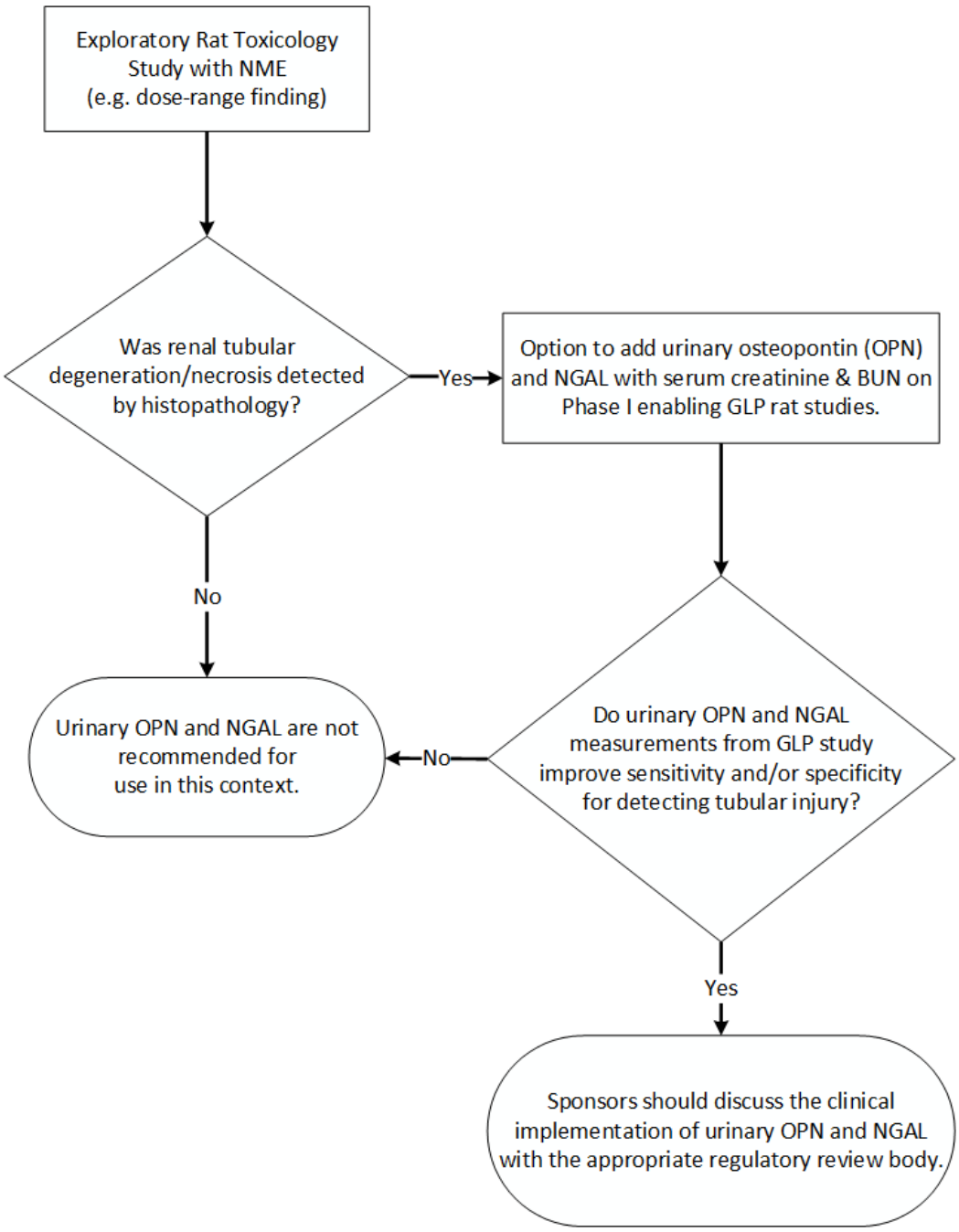
Biomarker	Percent Improved Positive Findings	p-value Positive Findings	Percent Improved Negative Findings	p-value Negative Findings	Total IDI	Total IDI p-value
Newly proposed biomarkers						
Urinary OPN	65.9	**3.45E-05**	75.6	**<1.0E-14**	0.158	**<1.0E-14**
Urinary NGAL	73.5	**8.48E-10**	64.6	**2.53E-08**	0.066	**2.37E-09**
Previously Qualified Biomarkers						
Urinary Kim-1	72.4	**5.58E-09**	87.3	**<1.0E-14**	0.253	**<1.0E-14**
Urinary Clusterin	68.1	1.15E-04	72.9	**<1.0E-14**	0.118	**6.49E-08**
Urinary Total Protein	67.6	**1.92E-05**	74.0	**<1.0E-14**	0.141	**9.79E-12**
Urinary Beta 2-µglobulin	64.3	7.23E-04	71.2	**2.11E-14**	0.134	**1.12E-11**
Urinary Albumin	61.8	2.16E-03	80.7	**<1.0E-14**	0.124	**4.17E-13**

Summary table of NRI for Positive and Negative findings and IDI analyses of all biomarkers while evaluating whether an individual biomarker improves certainty for diagnosing tubular degeneration/necrosis diagnoses. NRI and IDI are described in the methods. NRI = Percent improved − Percent Diminished. IDI = sum of mean improvement for Positive findings and mean improvement for Negative findings. For the PSTC to consider proposing a biomarker that improves certainty, each p-value for overall improvement of positive and negative findings must be less than 1.0E-04. Total IDI p-value must also be less than 1.0E-4. P-values in bold are less than 1.0E-04. NGAL and OPN meet this criterion.

2 GUIDANCE TO SPONSORS

A decision tree illustrating the recommended use of these biomarkers in a drug development program is shown in Figure 2. The sponsor is required to demonstrate that OPN and/or NGAL are responsive to the associated kidney injury specific to the developmental compound and ideally that OPN and/or NGAL can monitor injury progression and reversibility in a preclinical good laboratory practice (GLP) study. If the sponsor proposes to utilize OPN and/or NGAL to monitor patient safety in early clinical trials, it is encouraged that the strategy for doing so be developed in consultation with the appropriate regulatory review body.

Figure 2 Decision Tree



3 SUPPORTING INFORMATION

3.1 Scope of progressive qualification of nephrotoxicity biomarkers by PSTC

The nonclinical biomarker results presented here are one aspect of an undertaking by the PSTC NWG to advance the progressive understanding of translational biomarkers for drug-induced kidney injury. The PSTC NWG is currently investigating nephrotoxicity biomarkers in dog and non-human primates to more fully understand the cross-species translatability of these biomarkers. The PSTC is also carrying out complementary clinical studies, some in partnership with the Foundation for the National Institute of Health Biomarker Consortium (FNIH BC), to define clinical utility and ultimately the full translational predictivity of the analogous nonclinical biomarkers. All of these protocols, analyses plans, and results have been or are planned for submission to the FDA and EMA for regulatory qualification. Together, these data are expected to enable drug sponsors and regulatory scientists to reach agreement on appropriate safety monitoring strategies for deployment of these biomarkers in early clinical trials to overcome weaknesses associated with the use of sCr and BUN. Critical scientific and pragmatic utilization of these biomarkers will be needed to improve the safety of drug candidate development programs and overcome uncertainty in the human relevance of selected nephrotoxic changes seen in animal toxicology studies.

3.2 Review of biomarker biological mechanism and toxicological utility

The current dataset supports OPN and NGAL as translational biomarkers for renal tubular degeneration/necrosis. The following section is a critical review of the literature describing the biological mechanism and toxicological utility across nonclinical species and in humans, as well as a summary of the recommendations for the use of these biomarkers in drug development.

3.2.1 Urinary Neutrophil gelatinase-associated lipocalin (NGAL)

Neutrophil gelatinase-associated lipocalin (NGAL, also known as human neutrophil lipocalin, lipocalin-2, siderocalin, or LCN2) is a 25-kDa protein initially identified in neutrophil specific granules. NGAL is expressed in various tissues at low levels, but induced in epithelial cells with inflammation or other types of injury including malignancy (Cowland 1997). NGAL functions in iron homeostasis through binding of siderophores, leading to iron chelation and inhibition of bacterial cell growth or inhibition of apoptosis or oxidative stress in mammalian cells (Schmidt-Ott 2007). With kidney injury, NGAL is upregulated in the thick ascending limb of the loop of Henle, distal tubule and collecting duct, and is secreted into the urine as well as plasma (Paragas 2011). In mouse models, strongly increased NGAL mRNA and protein in the kidney parenchyma and urine are observed shortly after cisplatin administration or renal ischemia and precede changes in sCr (Mishra 2003, Mishra 2004). Plasma levels of NGAL are normally low, and NGAL in the glomerular filtrate is nearly completely reabsorbed by the megalin-cubilin transporter complex in the proximal tubule. With increased urinary protein

load (protein overload nephropathy), saturation of the re-absorption capacity of this complex can lead to increased urinary NGAL, and tubular back-leak can result in increases in plasma NGAL. In addition, DIKI can cause increased expression and release of NGAL as a protective mechanism as shown for other "tubular stress" proteins such as kidney injury molecule 1 (KIM-1) (Bolignano 2008). As a consequence, conditions which lead either to saturation or impairment of the re-absorption complex or to increased *de novo* expression of NGAL in kidney are expected to demonstrate the utility of NGAL as a kidney biomarker in the context of drug-development (Bolignano 2008, Cowland 1997, Devarajan 2010, Mishra 2003, Mishra 2004).

Urinary NGAL is actively being investigated and, in some cases utilized, for the prediction of acute kidney injury (AKI) in a number of clinical settings including interventional trials for AKI, the diagnosis and management of cardiorenal syndrome, and in patients undergoing cardiac surgery, in the emergency room, and in the intensive care unit (ICU). Recent reviews, from publications representing several thousand patients, summarize the promising clinical utility of NGAL for the prediction of AKI (Taub 2012, Devarajan 2014, Singer 2013, Tsigou 2013). Serum creatinine and urine output, current diagnostic measures of acute kidney injury, do not distinguish between hemodynamic changes due to reduced glomerular filtration rate (GFR) and structural kidney damage. As NGAL is rapidly upregulated following kidney tissue injury, it is a highly attractive biomarker for the sensitive monitoring of DIKI in clinical trials.

Further work to characterize several factors related to NGAL expression will assist in the confident application of this biomarker for monitoring DIKI in early clinical trials. The potential confounding influence of age, gender, diurnal and day-to-day variability, infection, chronic kidney disease and hypertension, inflammatory conditions, anemia, hypoxia and malignancies will be important to define for specific clinical trial settings.

The present work compared serum BUN and sCr to rat urinary NGAL as a biomarker useful for diagnosing and monitoring drug-induced renal tubular degeneration and/or necrosis. The importance of qualifying rat urinary NGAL is to provide a highly sensitive, readily translatable tool for detecting tubular injuries. While NGAL is an inducible marker, it has a biological role distinct from other inducible markers. For some new molecular entity (NME) development programs, NGAL may prove to be more beneficial for monitoring tubular injuries than other inducible markers, such as urinary KIM-1 or clusterin. Therefore, NGAL will provide a powerful option to monitor drug-induced renal tubular injury during drug development.

3.2.2 Urinary Osteopontin (OPN)

OPN (also known as secreted phosphoprotein I (SPP1), sialoprotein I, uropontin and others) derives its name from its role in the regulation of osteoclast function during bone formation (Tanabe 2011). In the kidney, OPN has divergent roles. OPN is a protective agent against oxidative stress and ischemia (Fuchs 2011). OPN, also has pro-inflammatory and profibrotic activity. In normal mouse, rat and human kidney, OPN is expressed at low levels in the distal nephron (thick ascending limb of the loop of Henle and distal convoluted tubules) (Hudkins 1999). With tissue injury, OPN expression has been demonstrated throughout the kidney, and OPN has proven to be a very sensitive and inducible indicator of different forms of AKI (Lyle 2012). Increased OPN mRNA and protein levels have been reported in the kidney in numerous animal models of renal injury and disease including gentamicin (Xie 2001, Irita 2011,

Lorenzen, 2008). OPN has gained recent attention as an accessible urinary protein biomarker resulting in a significant increase in activity to characterize its true value. Exploratory reagents have become commercially available to quantitatively measure OPN in the urine of rats, mice and humans.

Compared to NGAL, the characterization of OPN in clinical kidney injury and disease settings is somewhat limited. Investigations in renal transplant and critically ill patients support its utility for predicting patient outcome (Jin 2013, Lorenzen 2011). However, potential confounding variables with respect to the use of OPN in clinical trials have not yet been identified or investigated.

The present work directly compared BUN and sCr to rat urinary OPN as a biomarker useful for monitoring drug-induced renal tubular degeneration and/or necrosis. OPN differentiates itself from other inducible markers in that it acts as a protective molecule under certain stress conditions (e.g., nitric oxide (NO), oxidative stress, etc.). For some NME (NME) development programs, OPN may prove to be more beneficial for monitoring tubular injuries than other inducible markers such as urinary KIM-1 or clusterin. The specific mechanism of nephrotoxicity for a given NME would need to be evaluated, but the expectation is that OPN will provide an oxidative stress responsive marker for tubular injury. Therefore, OPN will provide a powerful option to monitor drug-induced renal tubular injury during drug development.

3.3 Model compounds and dose selection

Compounds chosen as models of DIKI in rat were selected based on literature precedent. Novel drug development compounds evaluated by PSTC NWG members were also utilized for these studies. In addition, compounds which would be expected to generate false positive data were selected.

Using our review of the published literature, the PSTC NWG primarily sought studies to model drug-induced tubular injuries. Compounds and dose ranges selected are summarized in Table 4. Several classical nephrotoxicants including cisplatin, gentamicin and doxorubicin were selected based on literature precedent where the toxicity, and in some cases the mechanism of toxicity, is well described. Model compounds that classically exhibit glomerular pathologies were selected as part of the kidney relevant compounds to broaden the variety of kidney lesions generated on-study. In addition to using the literature described doses for rat toxicology studies, dose-range finding studies were often conducted to verify the doses used in the present work. Two novel drug development compounds that exhibited nephrotoxicity in nonclinical studies were included to further diversify the chemical space tested beyond what is published in the scientific literature.

Finally, in addition to the above described toxicants, examples were intentionally sought where spurious increases or false positives might be generated to determine if non-renal tissues responding to treatment with differential mRNA expression of putative kidney injury biomarkers could contribute to false indication of renal injury. One way this could happen is by increased expression of a renal biomarker in non-renal tissue, followed by release of the protein into the circulation and then into the urine, in the absence of renal injury.

The PSTC NWG set up a query of the Entelos DrugMatrix database, a collection of histopathology and toxicogenomics data from a large, diverse collection of benchmark drugs and compounds, for a false positive nephrotoxicity biomarker gene expression response in non-renal tissues. False positive criteria included: 1) complete absence of histopathology in the kidney (elimination of true positives), 2) absence of renal biomarker expression changes in the kidney (elimination of possible false negatives after step one) and 3) presence of significant changes (>2-fold) in biomarker gene expression in a non-renal tissue (potential false positives).

While several compounds from the Entelos DrugMatrix database met the above criteria, two compounds were prioritized where multiple putative kidney biomarkers changed with treatment with a robust fold-change in marker expression. These compounds and associated gene expression changes are indicated in Table 3.

Table 3 Selected Potential False-Positive Compounds from Entelos DrugMatrix Database Query

Compound	Tissue where expression changed	Gene (biomarker)	Fold change in expression
Miconazole	Liver	Clusterin	10.8
		NGAL	156
		OPN	31
Methotrexate	Liver	KIM-1	9.6
	Spleen	OPN	8.75

3.4 Toxicology studies

Standard rat toxicology studies were designed to evaluate the performance of the proposed kidney injury biomarkers in comparison to serum BUN and sCr. Previously qualified urinary nephrotoxicity biomarkers were also measured for comparison to OPN and NGAL.

Data from eleven different rat toxicology studies, where in some cases multiple toxicants were dosed, using a total of 750 male and female rats are included in this Data Package. These studies evaluated eleven different pharmaceutical compounds including seven known and well-characterized nephrotoxicants, two known nephrotoxicants which were dosed at non-nephrotoxic levels to present the possibility of false positive biomarker results, and two non-nephrotoxic compounds. In combination, this study set represents an expansive evaluation of the possible diversity of outcomes that may be experienced in pharmaceutical development. The study designs are summarized in Table 4.

Table 4 Study Designs

Compound	Model (sub-structural)	Dose(s) mg/kg	Frequency (# doses)	Days	Route	Control Group Size	Dose Group Size (dose)	Sex	Strain
Bacitracin	Tubular injury (PT)	0, 5, 10	Daily (5)	33	SQ	15	15	M/F	SD
Cisplatin	Tubular injury (PT S3)	0, 0.1, 1.0, 2.5 0, 0.1, 1.0, 2.5 0, 1 0, 1.5 0, 1, 3	Single Single Single Daily (7) Single	22 22 29 28 42	IP IP IP IV IP	15 15 28 25 30	20 (0.01) 20 (0.1) 30 (2.5) 20 (0.01) 20 (0.1) 30 (2.5) 28 25 30	M F M F M	Wistar Wistar SD SD SD
Gentamicin	Tubular injury (PT S1/S2)	0, 50 0, 75	Daily (10) Daily (10)	39 11	SQ SQ	20 40	60 40	M M/F	SD SD
Proprietary Compound 1	Collecting duct, 2^0 tubular injury (retrograde nephropathy)	0, 1200	Daily (7)	7	PO	25	25	F	SD
Proprietary Compound 2	Glomerular injury	0, 1, 3, 10	Daily (7)	8	PO	3	3	M	SD
Doxorubicin	Glomerular Injury	0, 5, 10	Weekly (2)	15	IV	8	8	M	SD
Puromycin	Glomerular injury	0, 10, 20	Daily (14)	42	IP	30	30	M	SD
Methapyrilene	Negative control	0, 30	Daily (14)	42	PO	5	5	M	SD
α-Napthyl-isothionate	Negative control	0, 15	Daily (14)	42	PO	5	5	M	SD
Methotrexate	False positive	0, 1, 3, 10	Daily (14)	15	IP	9	9	M	SD
Miconazole	False positive	0, 50, 250, 750	Daily (7)	8	PO	20	20	M	SD

3.5 Biomarker measurement and assay validation

Measurement of selected candidate urinary biomarkers of kidney injury was performed using validated assays on Meso Scale Discovery's (MSD) chemiluminescent ELISA antibody based platform in all studies but one. One gentamicin study utilized Myriad RBM, Inc. (RBM) Luminex fluorescent microbead antibody based assay format to measure NGAL and OPN. Table 5 summarizes the assays selected for this qualification.

These assays use antibodies targeting specific isoforms and specificity was demonstrated using tissue lysates and recombinant proteins. Extensive assay validation was performed by the manufacturer and guided by CLSI standards (CLSI.org).

Assay set-up and validation at PSTC biomarker data-contributing sites focused on verification of the assay performance established by the manufacturer for the following parameters: sensitivity; dynamic range; intra- and inter-run accuracy and precision; robustness; freeze/thaw and storage stability of biomarker; and specificity, spike recovery and dilutional linearity.

Urinary Cr (uCr), serum BUN and sCr were all measured as routine clinical chemistry parameters. All urinary parameters were normalized to uCr output.

Table 5 Reagents Selected for Validation

Target of Existing Assay	Manufacturer and kit name	Assay type
Urinary NGAL	MSD Kidney Injury Panel 1 (rat) (KIP-1) RBM rat kidney MAP	Multiplex Chemiluminescent ELISA Antibody-based fluorescent microbead
Urinary OPN	MSD Kidney Injury Panel 1(rat) (KIP-1) RBM rat kidney MAP	Multiplex Chemiluminescent ELISA Antibody-based fluorescent microbead

3.6 Histopathology evaluation

Histological changes in the kidney evaluated by trained pathologists using standard industry practice as recommended by the Society of Toxicologic Pathology (STP) were the primary endpoint used as a reference standard for the biomarker performance analysis. Histopathologic diagnoses were based on a unified lexicon of morphologic terms created by the PSTC and Health and Environmental Sciences Institute (HESI) NWG in 2006 to standardize diagnoses from toxicological studies used as reference criteria for qualifying candidate nephrotoxicity biomarkers. The lexicon was organized hierarchically to stratify findings by location, primary pathologic process and secondary or downstream pathologic changes. Severity grading using a scale from 0 (no injury) to 5 (severe injury) was used to characterize dose-responsiveness of histologic changes, particularly for findings which can occur as spontaneous (background) findings in the control kidney. In this setting, identification of a test article-related change was based on an increased incidence and/or severity in treated relative to concurrent control animals. However, diagnostic performance analyses (e.g., ROC curve analysis) were dichotomized to compare scores of 0 to scores greater than 0.

Histopathology data from the eleven rat toxicology studies were curated using a series of processing steps to minimize diagnostic redundancies and optimize statistical evaluation. Study histopathology diagnoses were either made using the PSTC histopathology lexicon or mapped to the lexicon using nomenclature specified in the lexicon, and redundant diagnoses were merged using lexicon terminology. Diagnoses were evaluated within the context of each study for identification as either a primary pathologic process such as tubular degeneration, or as a secondary process such as tubular dilation (e.g. secondary to retrograde or obstructive nephropathy).

Because tubular basophilia can be either a primary manifestation of injury or a spontaneous change as part of the rat chronic progressive nephropathy, identification of tubular regeneration as a primary or drug-related finding was based on the presence of a dose-responsive change in incidence and/or severity within the study. Other common spontaneous changes (such as tubular casts, interstitial inflammation and/or fibrosis as part of the rat chronic progressive nephropathy spectrum, pelvis dilation, hyaline droplet formation in male rats or parenchymal mineralization) which occurred in the absence of tubular degeneration were similarly evaluated for dose response and were categorized as dose-responsive background findings related to test article administration or as non-dose-responsive changes consistent with background or spontaneous lesions that were unrelated to test article. Findings were considered dose-related if present within the study at a higher incidence and/or severity than in concurrent controls, and non-dose related if present at a similar incidence and/or severity in concurrent controls.

Curation of histopathology data yielded primary histopathology diagnoses of "tubular necrosis/degeneration", "tubular cell regeneration", "hyaline droplet formation, collecting duct", "glomerulopathy", and/or "other renal pathology" (representing a group of histological findings that are secondary or non-specific rather than primary test article-related renal changes). The category "other renal pathology" was not utilized in any analyses. Incidence is shown in Table 6.

Table 6 Summary and Incidence of Categorical Binning for Statistical Analysis and Biomarker Performance Evaluation

Categorical (Composite) Diagnosis	Abbreviations	NGAL	OPN
No injury	None	371	369
Tubular Degeneration/Necrosis	TDN	201	201
Tubular Cell Regeneration	Tre	129	128
Hyaline Droplet Formation	HyD	16	16
Glomerulopathy	GIA	4	4
All Liver Pathologies	Liver	56	56

Values shown for each biomarker represent the number of samples analyzed corresponding to the histomorphological diagnosis.

To provide a general sense of how biomarker values changed in treated animals where tubular degeneration/necrosis was described, median and 5th and 95th quartiles (q05 & q95) of biomarker values for OPN (Table 7) and NGAL (Table 8) are listed by study treatment. Note that the number of animals represented by some study quartiles is too small to be meaningful.

Values shown are normalized to sCr and shown as fold change from their respective untreated control cohort average. Although a diversity of toxicants was intentionally included in the dataset, diagnoses of tubular degeneration/necrosis are predominantly found in cisplatin and gentamicin treated animals. The biomarkers examined in this research may also have value beyond detection of tubular degeneration/necrosis. However, the relative lack of additional kidney pathologies, such as tubular regeneration or glomerulopathy, in the dataset, makes this determination challenging at this time (Table 6).

Table 7 OPN biomarker summary statistics for treated animals with kidney tubular degeneration/necrosis or no injury

Osteopontin										
	Total n (except CTL)	Tubular degeneration/necrosis				None				All Other
Compound		n	Median	q05	q95	n	median	q05	q95	n
Cisplatin	265	100	3.35	0.33	29.0	79	1.33	0.32	4.42	86
Gentamicin	97	80	3.81	1.09	89.5	17	2.86	0.20	8.47	0
Miconazole	67	1	1.00	1.00	1.00	5	1.00	1.00	1.00	61
Puromycin	56	10	3.97	0.55	5.90	31	1.05	0.04	2.09	15
Bacitracin	34	3	0.70	0.26	0.90	14	0.52	0.20	1.41	17
Proprietary Compound 1	24	0				6	0.82	0.60	2.53	18
Proprietary Compound 2	9	0				7	1.41	0.85	3.61	2
Methotrexate	9	0				5	1.00	0.70	3.34	4
Doxorubicin	7	7	13.4	3.10	26.7	0				0
α-Napthyl-isothionate	5	0				5	0.16	0.04	0.69	0
Methapyrilene	5	0				4	1.03	0.63	1.50	1

Untreated, control animals (CTL) are not represented in this table. Tubular degeneration/necrosis: animals with tubular degeneration/necrosis described as the primary diagnosis. None: animals without any injury noted by histopathology. All other: animals with primary renal pathologies as noted in the previous Table 6 (i.e. tubular cell regeneration; hyaline droplet formation; glomerulopathy) or liver pathology. Biomarker values shown are normalized to sCr and shown as fold-change from the average of their respective untreated control cohort.

Table 8 NGAL biomarker summary statistics for treated animals with kidney tubular degeneration/necrosis or no injury

Neutrophil gelatinase-associated lipocalin										
	Total n (except CTL)	Tubular degeneration/necrosis				None				All Other
Compound		n	Median	q05	q95	n	median	q05	q95	n
Cisplatin	267	100	1.65	0.49	6.42	80	1.00	0.48	1.71	87
Gentamicin	97	80	2.18	0.47	19.5	17	1.79	0.92	3.91	0
Miconazole	67	1	1.04	1.04	1.04	5	0.99	0.58	1.33	61
Puromycin	56	10	1.62	0.94	5.34	31	1.30	0.51	2.44	15
Bacitracin	34	3	0.74	0.00	0.98	14	0.96	0.01	1.64	17
Proprietary Compound 1	24	0				6	1.12	0.35	1.42	18
Proprietary Compound 2	9	0				7	1.30	1.12	2.45	2
Methotrexate	8	7	14.8	2.80	22.2	1	2.89	2.89	2.89	0
Doxorubicin	8	0				4	1.46	0.92	1.98	4
α-Napthyl-isothionate	5	0				5	1.59	0.87	3.72	0
Methapyrilene	5	0				4	0.81	0.60	0.96	1

Untreated, control animals (CTL) are not represented in this table. Tubular degeneration/necrosis: animals with tubular degeneration/necrosis described as the primary diagnosis. None: animals without any injury noted by histopathology. All other: animals with primary renal pathologies as noted in Table 6 (i.e. tubular cell regeneration; hyaline droplet formation; glomerulopathy) or liver pathology. Biomarker values shown are normalized to sCr and shown as fold-change from the average of their respective untreated control cohort.

4 CONCLUSIONS

Both OPN and NGAL outperformed BUN and sCr in identifying cases of drug-induced kidney injury characterized by renal tubular epithelial degeneration/necrosis when applied to standard rat toxicology studies. In addition, when OPN and NGAL data are considered with BUN and sCr data, there is a significant increase in diagnostic accuracy for discriminating the presence or absence of renal tubular epithelial degeneration/necrosis.

The data presented here are specifically intended to enable the early clinical exploratory use of OPN and NGAL by providing the nonclinical pathological and toxicological underpinning of the biomarker response. Both of these urinary biomarkers are appropriate for voluntary use in regulatory toxicology studies in rats in conjunction with current methods for assessing nephrotoxicity, to demonstrate that drug-induced kidney injuries seen with developmental compounds in animal toxicology studies are monitorable. These data can then be used to deploy biomarker monitoring strategies in early clinical trials to ensure patient safety and enable drug development to proceed.

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6 ASSAY REFERENCE DOCUMENTS

MSD Kidney Injury Panel 1 (Rat)

Product page with links to the product inserts and certificates of analysis:

http://www.mesoscale.com/CatalogSystemWeb/WebRoot/Products/ProductDetail.aspx?ItemNumber=K15162C-1

Myriad RBM Rat KidneyMAP

Product Page:

http://rbm.myriad.com/products-services/rodentmap-services/rat-kidneymap/

Myriad RBM data quality and quality control descriptions:

http://rbm.myriad.com/scientific-literature/white-papers/quality-control-white-paper/

http://rbm.myriad.com/scientific-literature/data-quality/