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Kidney Injury Molecule-1 Outperforms Traditional Biomarkers of Kidney Injury in Multi-site Preclinical Biomarker Qualification Studies

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Abstract

Kidney toxicity accounts for a significant percentage of morbidity and drug candidate failure. Serum creatinine (SCr) and blood urea nitrogen (BUN) have been used to monitor kidney dysfunction for over a century but these markers are insensitive and non-specific. In multi-site preclinical rat toxicology studies the diagnostic performance of urinary kidney injury molecule-1 (Kim-1) was compared to traditional biomarkers as predictors of kidney tubular histopathologic changes, currently considered the "gold standard" of nephrotoxicity. In multiple models of kidney injury, urinary Kim-1 significantly outperformed SCr and BUN. The area under the receiver

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Disclosures

Dr. Bonventre is an inventor on KIM-1 patents, which have been licensed to Johnson & Johnson and BiogenIdec.

Details of all data (including treatment regimen, histopathology, and biomarker levels) for every animal in the study is shown in supplementary table 7.

Detailed protocol about a) Protein Extractions from Kidneys and b) Gene Expression (mRNA Extraction and RT-PCR Measurements) is available in supplementary methods.

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operating characteristic curve for Kim-1 was between 0.91 and 0.99 as compared to 0.79 to 0.9 for BUN and 0.73 to 0.85 for SCr. Thus urinary Kim-1 is the first injury biomarker of kidney toxicity qualified by the FDA and EMEA and is expected to significantly improve kidney safety monitoring.

Acute kidney injury (AKI) is a common and devastating clinical problem with an in-hospital mortality of 40 to 80 % in the intensive care setting[1]. Drug-induced nephrotoxicity plays a major role in the high incidence and prevalence of AKI in both hospitalized and non-hospitalized patients[2]. Nephrotoxicity seen in animal toxicology studies is also a major factor in the failure of drug candidates due to the lack of good kidney biomarkers for monitoring kidney injury. Traditional markers of renal injury, including serum creatinine (SCr), blood urea nitrogen (BUN), urine sediment, and urinary indices (fractional excretion of sodium, urine osmolality, etc.), have lacked the sensitivity and/or specificity to adequately detect nephrotoxicity prior to significant loss of renal function. Given "renal reserve" and each of tests sensitivity, minimal histopathologic findings are often undetectable using these traditional biomarkers. There is an urgent need for improved and non-invasive renal biomarkers to permit early detection of AKI, severity of injury, and to aid in predictive safety assessment during drug development by resolving ambiguities associated between humans and animal test species[3].

We previously isolated the gene encoding kidney injury molecule-1 (Kim-1/TIM-1/HAVCR-1) a type I cell membrane glycoprotein containing a unique six-cysteine immunoglobulin-like domain and a mucin-rich extracellular region that is conserved across species in zebrafish, rodents, dogs, primates and humans[4]. *Kim-1* mRNA levels are elevated more than any other known gene across these species after initiation of kidney injury[4, 5]. The ectodomain of Kim-1 is shed from proximal tubular kidney epithelial cells *in vitro*[6] and *in vivo* into urine in rodents[4, 5, 7-9] and humans[10-13] after injury. The primary objective of this study was to evaluate the relative sensitivity and specificity of urinary Kim-1 against BUN, SCr, and urinary NAG, using histopathology as a "gold standard" for toxicity in seventeen different multi site preclinical studies using mechanistically distinct models of renal proximal tubular damage. Specifically our aims were to (i) correlate the diagnostic performance of urinary Kim-1, BUN, SCr and urinary NAG with histopathology following a dose response and time course in 10 studies from Novartis and 6 studies from Merck using well established nephro- and hepatotoxicants and one study using 20 min bilateral ischemia/reperfusion injury as a model of kidney injury; (ii) use a composite area under the receiver operating characteristics curve (AUC-ROC) to evaluate the relative sensitivity and specificity of urinary Kim-1, BUN, SCr and NAG over subsets of histomorphologic scores using different severity grade ranges. A secondary aim was to develop, optimize and test a microbead based assay for quantitating Kim-1 to increase the analyses throughput[14].

This article is one of a series of publications describing the first submission of safety biomarkers to the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) in a Voluntary eXploratory Data Submission (VXDS) with the goal of qualifying biomarkers for improved diagnosing and monitoring of the early onset of drug-induced kidney injury in a regulatory decision-making context.

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Results

Kim-1 gene and protein expression in Novartis studies

The Kim-1 gene expression atlas in 48 organs/structures, blood and bone marrow was measured on Rat Genome 230 2.0 Array and MAS5 normalized. The average raw intensity and the standard deviation were calculated for control and high-dosed animals at various time points from numerous studies that included up to 45 active drug entities (many reference compounds and known organ toxicant such as liver, cardiac, skeletal muscle, CNS, G.I., lung, bone, testis, toxicants). Kim-1 expression was very low ("absent" according to Affymetrix standards) across all organs analyzed at the baseline (supplementary Fig. 1). Only blood cells, lymph nodes, spleen and lachrymal glands had reliably detectable baseline Kim-1 expression. In kidney, Kim-1 baseline level was very low and only after kidney toxicity was there greater than 100-fold induction of Kim-1 expression (Fig. 1A). Kim-1 expression did not change in any of the other organs demonstrating the specificity of Kim-1 for kidney injury (supplementary Fig. 1).

The Kim-1 gene expression (Fig. 1A) in the kidney was correlated with Kim-1 protein expression in the kidney (Fig. 1B) and urine (Fig. 1C) after a dose response and time course study using Gentamicin, Cisplatin, Vancomycin, Tacrolimus (proximal tubular toxicants); Puromycin, Doxorubicin (glomerular toxicants); Furosemide, Lithium (tubular and collecting duct toxicants); and α-Naphtyl isothiocyanate (ANIT) and Methapyrilene (hepatotoxicants). Kidney Kim-1 mRNA, protein and urinary Kim-1 protein levels were compared with each other for each animal (Fig. 1 A-C) and a high degree of correlation between all three entities was obtained as estimated by Pearson correlation (supplementary table 1). Kim-1 mRNA and protein in the kidney had significantly high correlation (r=0.89) followed by Kim-1 mRNA in the kidney and Kim-1 protein in the urine (0.83). The correlation between Kim-1 protein in the kidney vs. urine was also high (r=0.82).

Urinary and serum biomarker elevations in Novartis studies using 8 nephrotoxicants and 2 hepatotoxicants

Urinary Kim-1 (Fig. 1C), SCr (Fig. 1D), blood urea nitrogen BUN (Fig. 1E) and NAG (Fig. 1F) were measured from 132 nephrotoxicant-dosed animals with tubular injury (grade 1 to 3 on a 5 grade scale) and from 283 vehicle-dosed animals without proximal tubular injury. Additionally, the biomarker values for 324 dosed animals showing either no lesion or other lesions (e.g. fibrosis) or lesions in other parts of the kidney were also included thereby reaching a total of 739 animals.

A statistical analysis of the data from all of the studies shows that urinary Kim-1 outperformed SCr, BUN, and NAG in terms of early detection, sensitivity and specificity. The highest number of nephrotoxicant-treated animals scoring above the 95% specificity threshold was identified by urinary Kim-1 (79%) whereas BUN, SCr and NAG identified 52%, 44% and 52% respectively, of animals with proximal tubular injury (Fig. 1 D, E, F). Especially for the Cisplatin, Gentamicin and Vancomycin studies, urinary Kim-1 was nearly perfect in diagnosing animals with proximal tubular injury (88%) in contrast to BUN, urinary NAG and SCr, which displayed 42%, 42% and 40% of true positives. Ten of the

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very few Kim-1 false negative animals had predominantly grade 1 lesions (e.g. 1 animal in the cisplatin study, 5 animals in the Gentamicin study, 2 animals in the puromycin study, and 2 animals in the doxorubicin study). The only drug displaying an above average number of grade 1 false negative animals (14) was furosemide, which is known to induce tubular degeneration / necrosis in the S3 segment. This may reveal the specificity of Kim-1 secretion following S1 and S2 segment tubular injury as compared to injury to the S3 segment, although, Kim-1 kidney mRNA and protein levels appear elevated. The other markers except for BUN (Fig 1 E) showed a performance similar to Kim-1 for the furosemide study. The Tacrolimus-induced proximal tubular lesions could only be identified by Kim-1 (Fig 1 C). High dose Lithium resulted in kidney lesions described as collecting duct necrosis and this resulted in significant elevation of urinary NAG. There was no increase in BUN, SCr or Kim-1 consistent with no proximal tubular histopathology change. In both heptatoxicant studies, levels of Kim-1, NAG and BUN was unchanged as compared to controls. This was in agreement with histopathology assessment, which did not reveal any drug-induced kidney lesions. In contrast, SCr levels increased following high dose of the hepatotoxicant ANIT with negative kidney histopathology indicating non-specificity of SCr, especially in the context of muscle breakdown in this study.

Composite sensitivity and specificity of urinary Kim-1, urinary NAG, blood urea nitrogen and serum creatinine correlating to histopathology in kidney injury for 8 nephrotoxicants used in Novartis studies

The results of the ROC analysis (Fig. 2 and supplementary tables 2 and 3) highlight the superiority of Kim-1 for the diagnosis of proximal tubular damage. For all observed histopathology grades, Kim-1 had the highest AUC of 0.91 by exclusion analysis (inclusion analysis AUC: 0.88) compared to the current standards SCr (0.73, inclusion analysis AUC: 0.72) and BUN (0.79, inclusion analysis AUC: 0.75), but also compared to NAG (0.82, inclusion analysis AUC: 0.76) (supplementary table 2). Statistical tests confirmed that Kim-1 outperformed NAG, SCr and BUN with a p value of less than 0.001 using both exclusion and inclusion method for data analysis (supplementary table 3). For low-grade histopathology (grade 1 versus grade 0) Kim-1 maintained high diagnostic performance with an AUC of 0.88 by exclusion analysis whereas urinary NAG, BUN and SCr performed poorly with an AUC of 0.8, 0.76 and 0.67 respectively (Fig. 2C & D). The low sensitivity of BUN and SCr for detection of kidney injury has been recognized for a number of decades and these data clearly demonstrate this with a sensitivity of 0.48 for BUN, and 0.29 for SCr when there was subtle proximal tubular damage (grade 1 versus 0) following kidney toxicity. The sensitivity of urinary Kim-1 remained high at 0.71 when the proximal tubular damage was subtle (grade 1 versus 0) or 0.79 when the injury was a little more severe (grade 2 versus 0) (Fig. 2E).

Urinary and Serum Biomarker Elevations With Gentamicin-Induced Nephrotoxicity in Merck studies

Urinary Kim-1 elevations were closely correlated to gentamicin-treated kidney tubular histopathologic alterations. On days 9 and 15 of treatment, mid-dose treated animals showed mean normalized urinary Kim-1 levels that were elevated 11- and 40-fold, respectively, compared to mean concurrent control values. High-dose treated animals on days 3, 9, and 12

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demonstrated 23-, 117-, 163-fold increased levels of urinary Kim-1, respectively (Fig. 3). Tubular degeneration, necrosis and regeneration observed at days 9 and 12 in animals treated with high-dose gentamicin corresponded to an approximately 100-fold elevation of urinary Kim-1. By comparison, with lower doses there was a lower incidence and severity of tubular degeneration, necrosis and regeneration and smaller elevations in Kim-1 levels at 20mkd at day 15, or at 80mkd as early as day 3 which persisted to days 9 & 15.

With gentamicin high-dose treatment, normalized NAG activity was elevated nearly 10-fold on day 3 (Fig. 3). At days 12 and 15, NAG activity levels were elevated for the mid- and high-dose animals, with corresponding histomorphologic severity grades of 2 and 5, respectively (Fig. 3). Only high dose treated animals showed markedly elevated SCr levels exceeding 1.5 mg/dL at day 9 but were normal at day 3. Similarly, BUN elevations were seen only in high dose animals at day 9.

Urinary and Serum Biomarker Elevations with Cisplatin Induced Nephrotoxicity in Merck studies

Normalized urinary Kim-1 levels were elevated after mid-dose treatment with cisplatin both at day 3 and 8 (20- and 97-fold respectively), where severity grade 2 and 4 overall tubular damage scores were observed (Fig. 4). Similarly, normalized urinary Kim-1 was elevated in high dose cisplatin treated animals both at day 3 (11-fold) and 8 (48-fold), corresponding with severity grade 2 and 5 overall tubular damage scores respectively (Fig. 4). A mean increase of approximately 9-fold was seen in low dose animals at day 3 which trended down at day 8. With cisplatin treatment at day 3, normalized NAG values were elevated >2.0 fold for animals showing tubular grade 2 overall tubular damage at the high-dose, but not in animals with grade 2 overall tubular damage at the mid-dose. At day 8, urinary NAG activity did not change in mid and high-dose treated animals with severity grade 4 to 5 overall tubular damage (Fig. 4). There were treatment related increases in BUN and creatinine in the mid- and high-dose treatment groups at both day 3 and 8 with markedly higher elevations for both at day 8 following high dose treatment.

Urinary and Serum Biomarker Elevations with Cyclosporine A and Thioacetamide-Induced Nephrotoxicity in Merck studies

In rats treated with 0, 6, 30, or 60 mg/kg/day cyclosporin A for 3, 9, or 15 days, subtle tubular basophilia of the regenerative type at severity grades 1 and 2 was observed in most mid-dose animals at day 15, all high-dose animals on day 15, and a high-dose animal at day 9 (supplementary Fig. 2). Elevations of Kim-1 were seen in all animals with histomorphologic changes (supplementary Fig. 2) while elevated NAG activity was seen in nearly half of the animals with tubular regeneration. With cyclosporin A treatment, modest elevations in BUN were observed in all animals with histomorphologic changes on day 15 following high dose treatment whereas serum creatinine did not show increases with histomorphologic change.

Thioacetamide (TAA) has been reported as a model nephrotoxicant of proximal tubule injury [15]. A 2- and 3-day TAA study was performed using single administrations of either 50, 100, or 200 mg/kg (supplementary Fig. 3). Both liver and kidney histomorphologic

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changes including tubular degeneration and necrosis were observed at all doses and on both days. The observed tubular histopathologic changes were severity grade 1 and 2 for day 2 animals and increased in a dose-dependent manner on day 3. Urinary Kim-1 levels was the most sensitive biomarker of toxicity with 34- and 36-fold increases in concentration at days 2 and 3, respectively already seen with low dose treatment (supplementary Fig. 3). At the mid-dose Kim-1 levels were increased 12- and 6-fold at day 2 and 3, respectively and about 18-fold at the high dose on both days. Urinary NAG activity increased in a dose-dependent manner at both day 2 and 3 (supplementary Fig. 3). Significant elevations in BUN and serum creatinine were observed only on day 3 following mid- and high dose treatment.

Sensitivity and specificity of urinary Kim-1 and NAG, BUN and SCr with respect to kidney histopathology by 4 nephrotoxicants used in Merck studies

The performance of Kim-1 as measured by the area under the curve (AUC) from the (receiver operating characteristics) ROC analysis (Fig. 5, supplementary table 2) was consistently high (>0.99 by exclusion analysis and >0.96 by inclusion analysis) for all of the histomorphologic severity grade subsets (Fig. 5A and 5E) (supplementary table 2). For the analysis that uses all of the samples, the difference in AUC using between Kim-1 and NAG was 0.12 (p<0.001) by exclusion and inclusion analysis (p<0.001), between Kim-1 and serum creatinine 0.15 (p<0.001) (0.10, p<0.001 by inclusion analysis) and the difference in AUC between Kim-1 and BUN was 0.09 (p<0.001) (0.12, p<0.001 by inclusion analysis) (supplementary table 3). The difference in AUC between Kim-1 and NAG increased from 0.12 using all nephrotoxicity samples to 0.26 using a severity grade 0 and 1; between Kim-1 and BUN it increased from 0.09 using all nephrotoxicity samples to 0.22 using only the severity grade 0 and 1 nephrotoxicity sample subsets, indicating a reduced correlation of NAG and BUN to histomorphologic change when a more sensitive morphologic metric was employed (Fig. 5A, 5D and 5E). Similarly the difference in AUC between Kim-1 and serum creatinine increased from 0.15 using all nephrotoxicity samples to 0.37 using only the severity grade 0 and 1 nephrotoxicity sample subsets (Fig. 5A, 5D and 5E).

The sensitivity, or proportion of positives correctly identified at a threshold that yields 95% specificity, for Kim-1 was 0.99 for all of the nephrotoxicty histopathology severity grade subsets (Fig. 5A, 5D and 5F). The sensitivity for NAG decreased from 0.56 using all nephrotoxicity samples to 0.20 for severity grade 0 and 1 subset (supplementary table 2, Fig. 5F). Similarly the sensitivity for BUN and serum creatinine also decreased from 0.71 and 0.68 respectively using all nephrotoxicity samples to 0.45 and 0.20 using a severity grade 0 and 1 nephrotoxicity sample subset (supplementary table 2, Fig. 5F). Both inclusion and exclusion analysis show that unlike BUN, SCr, and NAG the performance of Kim-1 is uniformly high within the full range of nephrotoxicity subsets analyzed (supplementary table 2 & 3).

Merck studies for evaluating specificity of urinary Kim-1 as a biomarker for kidney injury

Kim-1 shows specificity for renal damage. Urinary Kim-1 level changes were unremarkable following toxicant induced hepatotoxicity and cardiotoxicity in rats. Bromotrichloromethane ($CBrCl_3$) induced significant hepatotoxicity in rats seen at days 2 and 4 at both low and high doses as assessed by biochemical parameters (ALT and AST) and histopathology scoring

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(necrosis and degeneration), but produced no treatment-related kidney toxicity (supplementary table 4). Urinary Kim-1 levels were similar between controls and treated $CBrCl_3$ animals with liver injury. Isoproterenol induced necrosis and degeneration of both cardiac and skeletal muscle with histomorphologic changes at 3 and 8 days following a dose of 1 mg/kg/day, yet did not show urinary Kim-1 elevations further emphasizing the specificity of Kim-1 for renal damage (supplementary table 4).

Comparison of urinary Kim-1 with other markers in the rat model of renal ischemia/reperfusion injury (I/R)

In the 20 min bilateral I/R injury model, at 3 and 6 h after reperfusion there was ~ 3 and 6-fold increase in urinary Kim-1 as compared to sham. Urinary Kim-1 levels peaked at 24 h (700-fold) and plateaued to levels persistently above baseline at 96 and 120 h (~ 70 fold) after reperfusion (Fig. 6). This time course correlated with the histological changes of the kidney with grade 1 proximal tubular damage at 3 h and 6 h, and single cell necrosis, tubular dilation and sloughing of cells in tubules of the outer stripe of the outer medulla at 9 h after I/R. At 12 and 24 h there was significant proximal tubular necrosis with associated inflammation and cast formation classified as grade 4 and 5 histopathology, respectively. Modest and transient elevations in BUN (~ 1.4 fold), SCr (~ 1.5 fold) and NAG (3.2 fold) were observed only at earlier time points (3 to 9 h) following reperfusion. Statistically significant increases in urinary NAG activity were observed at 12 h after reperfusion with ~ 5.5 fold elevation and at 18 h for BUN (~ 2.1 fold) and serum creatinine (~2.4 fold) (Fig. 6).

Threshold Determination Comparisons

The thresholds derived from different biomarker study data sets for a specific pre-defined sensitivity or specificity should be the same for practical general utility. From the Novartis and Merck studies a threshold for 95% specificity of 1.87 and 1.89 (supplementary table 2) fold increase respectively were obtained using ROC analyses. Similarly for SCr, the threshold cutoffs from the Novartis and Merck studies were 1.14-fold and 1.2-fold, for BUN 1.2 and 1.3-fold, and for NAG 1.4 and 2.4 -fold, respectively. As the threshold for 95% specificity is mainly dominated by the variance of the control animals, one can conclude that despite different rat strains, study designs and assay setups, the urinary Kim-1, SCr & BUN levels across control animals are highly reproducible, and the magnitude response necessary to signal that a significant deviation from normal equates to pathology is also consistent.

Logistic regression models were fit to assess whether Kim-1 and NAG add information to models that include SCr and BUN. The results, given in Supplementary Table 5 show that both Kim-1 and NAG provide substantial additional information. Using exclusion analysis with the binary logistic model on the Novartis data, the addition of Kim-1 was statistically significant ($p<1.0E-05$) and increased the concordance probability (C, equivalent to AUC from ROC in this case) by 0.159, the R2 by 0.37 and IDI by 0.350. The addition of NAG for the SCr+BUN model was statistically significant ($p<1.0E-05$) and increased C by 0.052, R2 by 0.105 and IDI by 0.08. For the Merck data, Kim-1 was statistically significant ($p<1.0E-05$), increased C by 0.059, R2 by 0.279, and IDI by 0.267. In the Merck data, NAG was statistically significant ($p = 0.021$) and increased C by 0.004, R2 by 0.028, and IDI by

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0.019. Results from ordinal logistic regression tended to give similar results (supplementary table 5).

Discussion

We report here the relative performance of four biomarkers to accurately assess kidney injury in thirteen structurally and mechanistically different models of renal tubular injury in rats. Irrespective of whether the kidney injury was induced by well established kidney toxicants or ischemia, urinary Kim-1 not only outperformed BUN, SCr and urinary NAG, which are conventional markers for assessing renal injury, but did so achieving an ROC-AUC of 0.91 to 0.99. The AUC for Kim-1 remained greater than 0.9 whether the entire histopathology grade of 0 to 5 were included or whether the analyzed group was restricted to histopathology grade scores of 0 and 1, demonstrating that urinary Kim-1 measurements are highly sensitive, specific and accurate in diagnosing either drug-induced kidney tubular necrosis, degeneration, and/or dilatation or regenerative basophilia when lesions are either subtle with little organ involvement, or very severe with disturbed renal function. We further show by exclusion analysis that a threshold increase of 1.87-fold of urinary Kim-1 concentration for 95 % specificity derived from one laboratory was similarly and independently defined in other laboratories using other study designs for kidney injury.

In this set of 17 studies, the increase in urinary Kim-1 was compared to histopathology, which is considered the "gold standard" for assessing preclinical renal injury. The AUC and the sensitivity of Kim-1 was nearly 1, irrespective of the mechanism of kidney injury and correlated highly with histopathologic changes even for low-grade injuries (grade 1). This is the first report demonstrating that current markers of assessing nephrotoxicity, BUN and SCr, are effective only with more severe histopathological grades (> grade 2) in preclinical studies. For example, the sensitivity of SCr was remarkably low at 0.20 for histology grades 0 to 1 and increased to only 0.56 with severity grades of 0 to 3 in the Merck studies while urinary Kim-1 was sensitive and specific for assessing subtle forms of proximal tubular damage (histology grade 0 to 1). These AUC-ROC numbers represent the exclusion data analysis approach. Exclusion analysis sets aside data values that show discordance between histopathology and early biomarker values in nephrotoxicant treated animals since further investigations would be required to unequivocally show which approach indicates truth. Exclusion analysis gives the most conservative threshold of elevation and is most appropriate for preclinical investigations since histopathology data are available. Inclusion analysis was similarly performed in parallel (Supplementary Table 2, 3 and 5) and is most similar to a clinical setting where histopathology observations are not available for biomarker correlation. An inclusion approach assumes that discordance between early biomarker values and true injuries may be attributed to false positive biomarker values. The inclusion analysis approximately doubles the Kim-1 threshold indicative of injury to 3.9-fold. Direct comparison of AUC shows a lower value for inclusion analysis compared to exclusion analysis across the curve (Supplementary Table 2).

Urinary Kim-1 has been shown in additional studies to be a sensitive and early diagnostic indicator of renal injury in a variety of acute and chronic rodent kidney injury models resulting from drugs[8, 9, 16], environmental toxicants[7, 9, 17], ischemia[8], and protein

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overload[18]. Human studies have also yielded promising results for potential utility of urinary KIM-1 as a diagnostic biomarker for AKI. Han et al. demonstrated marked expression of KIM-1 in kidney biopsy specimens from six patients with acute tubular necrosis, and found elevated urinary levels of KIM-1 after an initial ischemic renal insult, prior to the appearance of casts in the urine [10]. Liangos *et al.* reported urinary KIM-1 and NAG in 201 patients with established AKI and demonstrated that elevated levels of urinary KIM-1 and NAG were significantly associated with the clinical composite endpoint of death or dialysis requirement, even after adjustment for disease severity or comorbidity[11]. Zhang *et al.* compared the tissue expression of KIM-1 with histopathological and functional parameters of acute tubular injury (ATI) and acute cellular rejection (ACR) in renal transplant biopsies from 62 patients[19]. KIM-1 expression was present in all biopsies from patients with histological changes showing ATI and in 92 % of kidney biopsies from patients with ACR. KIM-1 staining sensitively and specifically identified proximal tubular injury and significantly correlated with declining renal function. A longitudinal prospective study conducted by van Timmeren *et al* reported that elevated urinary KIM-1 serves as an independent predictor of long-term graft loss in renal transplant recipients (n=145 patients) independent of donor age, creatinine clearance and proteinuria[13]. In our recent clinical cross sectional study comparing nine urinary biomarkers (KIM-1, NGAL, IL-18, NAG, protein, HGF, VEGF, IP-10 and Cystatin C) in 204 patients with or without acute kidney injury urinary KIM-1 had an AUC-ROC of 0.93 and was significantly higher in patients who progressed either to death or to requirement for renal replacement therapy (RRT) when compared to those who survived and did not require RRT[12] The opportunity to use the same marker for both the preclinical and clinical setting facilitates clinical monitoring of toxicity that has been demonstrated at higher doses in preclinical development or in a single test species when human relevance is suspected.

To facilitate the biomarker measurement, a sensitive, specific, reproducible and high-throughput assay is critical for its widespread use and applicability. We have previously reported development and evaluation of a sandwich ELISA assay for measurement of Kim-1 using two epitopically distinct monoclonal antibodies raised against rat Kim-1 ectodomain[8]. Nevertheless, our ELISA required adjustments of urine samples to fit to the linear range of the standard curve decreasing the throughput of the assay. Therefore, we developed the microbead based assay to measure Kim-1 in rat urine samples, and found that the dynamic range was increased to span from 4 pg/mL to 40,000 pg/mL. Other advantages of this assay include the ability to quantitate Kim-1 using only 30 μL of undiluted urine samples and reducing the assay time from 6 hours to 3.5 hours while maintaining the intra and inter assay variability to less than 10%. The easy transferability of the Kim-1 assay was demonstrated by the Kim-1 measurements of the Novartis studies conducted in a second laboratory. More recently, we have also reported the development of a rapid point of care diagnostic dipstick assay for measuring Kim-1 in rodent and human urine samples within 15 minutes[20].

In summary, we report that urinary Kim-1 levels correlate with different grades of kidney tubular histopathologies in thirteen mechanistically distinct models of acute kidney injury. Using exclusion and inclusion data analysis, Kim-1 had the highest AUC-ROC (> 0.88) as compared to BUN, SCr and NAG. Especially for low-grade toxicity (grade 1) Kim-1 was

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the only marker of the four capable of consistently detecting renal tubular injury. Urinary Kim-1 outperformed ($p<0.01$) serum creatinine, BUN and urinary NAG as biomarkers of renal tubular injury in these mechanistically distinct models of kidney injury performed at three different sites. Binary and ordinal logistic regression models for exclusion and inclusion data analysis showed that addition of Kim-1 represented a statistically significant improvement and increased the concordance probability to histopathology.

Thus, due to the striking evidence of the performance of urinary Kim-1 it was deemed qualified as a highly sensitive and specific marker of drug-induced kidney injury by both FDA and EMEA and is expected to greatly facilitate evaluation of tubular toxicity in certain preclinical and clinical settings.

Methods

Animals

Male HAN Wistar rats (275-300 g) and male Sprague Dawley (SD) rats (275-325 g) were purchased from Harlan (Indianapolis, IN) and maintained in central animal facility over wood chips free of any known chemical contaminants under conditions of 21 ± 1°C and 50-80% relative humidity at all times in an alternating 12 h light-dark cycle. These rats were fed with commercial rodent chow (Teklad rodent diet # 7012), given water *ad lib*, and were acclimated for 1-week prior to use. All animal maintenance and treatment protocols were in compliance with the Guide for Care and Use of Laboratory animals as adopted and promulgated by the National Institutes of Health and were approved by respective Institutional Animal Care and Use Committees (IACUC).

Experimental design

Novartis Studies—Ten studies using HAN Wistar rats dosed with 8 nephrotoxicants and 2 hepatotoxicants were conducted to generate dose- and time-dependent nephrotoxicity and hepatotoxicity. All studies followed a specific generic study design. Specific differences, such as differences in termination time points are displayed in supplementary table 5. Three groups of 24 Wistar Han males (11 to 12 weeks old) per study group received daily the test item, at 3 different dose levels listed in supplementary table 6. An additional group of 24 males received the vehicle under the same experimental conditions and acted as a control group. The animals were checked daily for mortality and clinical signs. Body weight and food consumption were recorded regularly during the study. Urine was collected for urinalysis and biomarker measurement as described below. For the termination time point day 1 no urine was collected and for some animals not enough urine for all investigations was possible. In total, 739 samples were available for urinary biomarker analysis. On completion of the treatment periods, six non-fasted animals per group at each time point (4 termination time points) were sampled 3 hours after dosing for laboratory investigations (blood biochemistry, urinalysis, and histopathology). The animals were sacrificed and submitted to a macroscopic post-mortem examination. Designated organs (kidneys, liver and brain) were weighed and specified tissues preserved. The kidneys and liver were processed using conventional methods for histological assessment. The right kidney of all dose groups and the liver of all animals of the control and high-dose groups and additionally of the low-

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dose and mid-dose groups for the two hepatotoxicant studies were examined microscopically following H & E staining. Histopathology of the kidneys was evaluated according to the PSTC NWG histopathology lexicon and scoring system. All studies were performed at CIT (BP 563, 27005 Evreux, France) in compliance with Animal Health regulations, in particular with the Council Directive No. 86/609/EEC of 24th November 1986.

Merck Studies—Male Sprague Dawley rats received one of four nephrotoxicants (gentamicin, cisplatin, thioacetamide, or cyclosporine), or one well-established hepatotoxicant (carbon tetrachloride), or one well established cardiotoxicant (isoproterenol) for sensitivity and specificity studies. Gentamicin sulfate was administered at 0, 20, 80, or 240 mg/kg/day (n=5 rats/dose group/time point) and the animals were necropsied on days 3, 9 or 15 for toxicity evaluation which included serum clinical chemistry (BUN, creatinine), analysis of urine Kim-1 and NAG and histomorphologic evaluation of kidneys (H&E staining) as described below. The 240 mg/kg/day gentamicin sulfate day 15 group was terminated early at treatment day 12 due to physical signs. In the cisplatin groups a single dose of cisplatin was administered intraperitoneally (i.p.) to male Sprague Dawley rats (n=5 rats/dose group/time point) at doses of 0, 0.5, 3.5 or 7 mg/kg and necropsy was performed on day 3 or 8 post-treatment. Cyclosporine A was administered subcutaneously (s.c.) at 0, 6, 30, or 60 mg/kg/day to rats (n=5/dose/time point) and necropsy was performed on day 3, 9 or 15. A single dose of thioacetamide (TAA) was administered by oral gavage at 0, 50, 100, or 200 mg/kg (n=5 rats/dose group/time point) and necropsy was performed on day 2 (24 hr post-dose) or day 3 (48 hr post-dose). Bromotrichloromethane (CBrCl3) was administered orally (p.o.) at 0, 0.03, 0.1 mL/kg to rats (n=5/dose/time point) and necropsy was performed on day 2 or 4. Isoproterenol was administered intravenously (i.v.) at 0, 0.064, 0.25, or 1 mg/kg/day to rats (n=5/dose/time point) and necropsy was performed on day 3 or 8.

Renal Ischemia-reperfusion studies—*Eighty* male Wistar (W) rats weighing approximately 270-300 g were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg) and placed on a homeothermic table to maintain core body temperature at 37°C, by means of a rectal probe attached to a temperature regulator which was in turn attached to a homeothermic blanket. A midline laparotomy was made, renal pedicles were isolated, and bilateral renal ischemia was induced by clamping the renal pedicles for 0 or 20 min as described previously [8]. Occlusion was verified visually by change in the color of the kidneys to a paler shade and reperfusion by a blush. Reperfusion commenced when the clips were removed. The rats were divided in groups of six rats each after 3, 6, 9, 12, 18, 24, 48, 72, 96 and 120 h of reperfusion. Rats (n=4) per group were immediately placed in metabolic cages at 22°C. Individual urine samples were collected at 3, 6, 9, 12, 18, 24, 48, 72, 96 and 120 h after reperfusion. Urinary N-acetyl-β-glucosaminidase (Roche diagnostics, cat # 875406) was measured spectrophotometrically[8] and urinary Kim-1 was measured using the xMAP Luminex® technology described below. Another set of rats (n=4) was sacrificed by overdose of pentobarbital (200 mg/kg, ip) at 3, 6, 9, 12, 18, 24, 48, 72, 96 and 120 h after reperfusion. Blood was collected from the dorsal aorta in heparinized tubes for measurement of blood urea nitrogen (BUN) and creatinine. One kidney was perfused via the left ventricle

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with phosphate-buffered saline and then with paraformaldehyde lysine periodate (PLP) for 10 min for histology.

Urine Collection

Novartis Studies—Urine was collected, from 2:00 pm to 8:00 pm and from 8:00 pm to 6:00 am at the days listed in table 8 from fasted animals, into tubes and kept at approximately 4°C during the collection period. The sampled urine fractions were split in 2 mL aliquots and centrifuged at 4°C for 30 minutes at 10,000 g. Urinalysis and urinary biomarker analysis (separate aliquot) were subsequently performed on the urine samples collected overnight. Urine analyses were performed with an Advia 1650 analyzer. For the termination time point day 1 no urine was collected and for some animals not enough urine for all investigations was possible. In total, 739 samples for urinary biomarker analysis were available.

Merck studies—Urine was collected prior to necropsy (18 hr +/- 2 hr collection period) and the rats were placed in standard metabolic cages and fasted prior to collection. Urine samples were collected from individual animals into containers surrounded by dry ice and were stored at -80°C until thawing for urinalysis. After the initial thawing at 22°C, samples were placed on wet ice and volume measurement was performed (precipitates were allowed to settle by gravity and were discarded). Typically, 2.5 ml urine samples were used for routine clinical chemistry urinalysis (Roche Modular Analyzer): manual specific gravity, pH, protein, glucose, creatinine, occult blood, creatinine, and ketones were measured (only creatinine shown). For the remaining urine volumes, small aliquots were made and stored at -80°C until biomarker analysis to avoid repeated freeze-thaw cycles.

Blood Collection & Clinical Chemistry

Novartis Studies—On completion of the treatment periods, six non-fasted animals per group at each time point (4 termination time points) were sampled for laboratory investigations (blood biochemistry, urinalysis, and histopathology) 3 hours after dosing. The maximum blood volume (at least 5 mL) was taken immediately before scheduled necropsy, from the retro-orbital sinus of the animals, under light isoflurane anesthesia, and collected into tubes. The tubes for determination of plasma levels of the test item were placed before and after blood sampling in wet ice. The blood sampling was split for (A) RNA extraction: 1 mL into Fastubes®; (B) Blood biochemistry: 0.7 mL into a lithium heparin tube and (C) Biomarker assays: the remaining blood was collected into sodium EDTA tubes. Clinical chemistry analyses of urine and blood were performed with an Advia 1650 device for measuring BUN (using Urease UV from Bayer) and Creatinine (using Jaffe from Bayer)

Merck Studies—Rats were fasted overnight prior to necropsy and bled from the vena cava with 2 ml collected into a serum separator tube and centrifuged 1,500 × g for 10 min at 4°C. An additional 2 ml of collected blood was placed into an EDTA collection tube and centrifuged 900 × g for 15 min at 4°C to isolate plasma. Isolated plasma and serum samples were stored at -80°C until use. Blood urea nitrogen (BUN) (mg/dL) and creatinine were measured using a standard clinical chemistry analyzer (Roche-Modular). AST (aspartate aminotransferase) (IU/L), ALT (alanine aminotransferase) (IU/L), alkaline phosphatase

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(IU/L), and creatinine kinase (IU/L) were measured using the same clinical chemistry analyzer for isoproterenol and $CBrCl_3$ toxicity studies.

Histopathology

A compendium of kidney histology images taken at low to high magnification from rat kidneys with low to severe histological damage is complied in supplementary material 1.

Novartis Studies—A microscopic examination was performed on the right upper part of the kidney of all animals and on the caudate lobes of the liver of animals of the control and high-dose groups and additionally of the low-dose and mid-dose groups for the two-hepatotoxicant studies. Histopathology of the kidneys was evaluated according to the PSTC NWG histopathology lexicon and scoring system (localized lesions with a 5 grade system. The histopathology assessment was first performed at CIT including a peer review. Subsequently about 33% of the samples were reviewed at Novartis. In case of major discrepancies, a discussion between all involved Novartis pathologists and CIT pathologists was performed for resolution. For the assessment of proximal tubular injury, the highest grade of necrosis, apoptosis, tubular degeneration and cell sloughing in the nephron segments S1 to S3 and non-localizable was assigned to the histopathology composite score “proximal tubular injury”. A severity score grading scale of 0 to 5 was employed to grade pathological lesions from 0 (no observable pathology), 1 (minimal), 2 (slight), 3 (moderate), 4 (marked), or 5 (severe).

Merck Studies—At necropsy, tissues were collected for histology soon after the last blood collection and exsanguination. The left quadriceps (3 mm section including all four muscle groups), left kidney (5 mm section including the papilla, cortex, and medulla), right lateral lobe of the liver, and heart were isolated from each animal and placed in 10% neutral buffered formalin (NBF). Tissues were fixed for a minimum of 24 hr, processed and embedded in paraffin. Embedded tissues were cut into 4-6 micron sections and stained with hematoxylin and eosin (H&E). Tissues from control, high dose animals, and organs with test article-related changes from lower dose groups, were examined microscopically by a Merck pathologist unaware of any of the biomarker data and studies were reviewed by a supervising pathologist prior as part of a final report. Histopathology of the kidneys was evaluated according to the PSTC NWG histopathology lexicon and scoring system. Diagnoses for individual animals were grouped into composite categories for statistical analysis: 1) tubular degeneration and necrosis composite, 2) tubular basophilia and regeneration composite, or 3) other composite (glomerulopathy, fibrosis and tubular dilatation). Since glomerulopathy and fibrosis was not observed in the renal studies, the composite score is considered a tubular composite. The composite score for an individual animal was derived from the highest pathology score of the diagnoses comprising a given composite.

Development and Evaluation of rat Kim-1 micro-bead assay

Coupling of beads to Kim-1 capture antibodies—The polystyrene 5.6 μm microspheres contain spectrally distinct fluorochromes. Microsphere (Bio-rad bead # 27) was coupled with monoclonal anti rat Kim-1 ectodomain antibody using a Bioplex amine

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coupling Kit from Bio-rad (cat # 171-406001). Mouse monoclonal antibody raised against rat Kim-1 ectodomain (MARKE-Trap) raised and characterized in our laboratory[8] was used as primary (capture) antibody to couple to beads.

Evaluation of the assay—The performance characteristics of the microbead based assay was evaluated similarly to the Kim-1 ELISA[8] by measuring the sensitivity, assay range, specificity, reproducibility, recovery, and interference.

Transfer of the assay for Novartis studies—The assay for measuring urinary Kim-1 in Merck study and in renal ischemia reperfusion model was performed in Vaidya/ Bonventre laboratory using the microbead technology described above. The Kim-1 assay for Novartis studies was set-up at Rules Based Medicine (Austin, Texas) using the reagents obtained from Vaidya/Bonventre laboratory as described above. The validation of the assay followed accepted procedures recommended in "The Bioanalytical Method Validation Guidance for Industry" (www.fda.gov, May 2001) with the exception that for the accuracy at the lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) a mean deviation of 30% instead of recommended 20% and in between LLOQ and ULOQ a mean deviation of 20% instead of 15% for the quality controls were accepted. The assay validation covered inter-day, inter-operator, inter-instrument reproducibility, linearity, parallelism, spike recovery, freeze-thaw stability, short-term stability, long-term stability, matrix interferences and cross-reactivity for both, urine samples and protein extracts. The LLOQ was determined as 0.058 ng/mL for urine samples and 0.1 ng/mL for protein extracts from kidney and the ULOQ was determined as 140 ng/mL for urine and 280 ng/mL for protein extracts. The Kim-1 measurements for the Merck Studies were performed in our laboratory at Brigham and Women's Hospital, Harvard Medical School (Boston, MA). The assays in both laboratories were standardized with extensive performance evaluation in terms of lowest limit of detection, dilutional linearity, recovery, precision profile and variability between the two sites. Along with the primary and secondary antibodies for Kim-1 we had sent to Rules Based Medicine recombinant protein and 30 rat urine samples each with low, medium and high values of Kim-1. The results were compared and the coefficient of variability was less than 20 % between the two sites.

Data Transformation

Measurements, which were below the lower limit of quantitation or above the upper limit of quantitation, were imputed by the respective limits. For urine analytes, measured concentrations were divided by urine creatinine concentration values from identical animals to yield a normalized value. Variables were analyzed on the log2 scale and normalized to the mean of controls measured in the same study and day for statistical analyses.

Receiver Operator Characteristic Curves

Curves were created using Receiver Operator Characteristic (ROC) analysis and summarized using reported methods[21]. Standard errors of the area under the curve (AUC) for the ROC curve were calculated using the reported formula[22]. For the purpose of ROC curve analysis, a no observable histopathology score of '0' was considered 'negative' and a histopathology score >0 was considered 'positive' for all samples. Thus, all positive grades

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of histopathology (grades 1 to 5) were treated with equal weight for the initial ROC analysis. Different severity grades of histopathologic change were grouped for a subsequent ROC analysis as indicated below. Only analyte samples taken within two days of necropsy were considered for ROC analyses. Only samples that had non-missing values for all of the candidate markers, clinical chemistry values, and histopathologic changes were used for the analyses. Our sample Exclusion model includes the union of the following sample sets: 1) Control animals with kidney Histopathology = 0; 2) Kidney toxicant treated animals with Histo>0; 3) All non-kidney toxicants (Isoproterenol and CBrCl3) treated animals with kidney Histopathology=0. Samples from animals treated with a nephrotoxicant that did not have a positive composite kidney histopathology score were excluded in this model. The reason for the exclusion is so as to not penalize markers that may be prodromal. For comparison purposes inclusion models were also fit to the data. These models included all of the data, treating samples from animals treated with a nephrotoxicant that had a negative composite kidney histopathology score as a true negative. For the most part, the effect of inclusion of these data on each marker was an increase in 95% specificity threshold and a decrease in AUC performance. The relative performance of the markers to each other was not greatly affected.

The ROC methods described were also applied to specific subsets of samples based on the severity grade of the histopathologic alteration score. The subsets used in the analyses are the following:

1. all the samples (as defined by exclusion criterion)
2. only samples with maximum composite histopathology scores of 0, 1, 2, or 3
3. only samples with maximum composite histopathology scores of 0, 1, or 2
4. only samples with maximum composite histopathology scores of 0 or 1

We state the AUC from each ROC curve or the sensitivity (at 95% specificity) range over the histomorphologic severity grade subsets. Then the difference in the AUC (AUC for biomarker – AUC for BUN or SCr) or sensitivity (SENS for biomarker – SENS for BUN or SCr) changes between the subset that includes all the nephrotoxicity samples and the subset that is restricted to samples that include histopathology severity grades 0 and 1.

Nested logistic regression models were used to assess whether Kim-1 or NAG complements or “adds value” to the standard SCr and BUN measures. Improvement gained by the addition of each marker to a model containing SCr and BUN was assessed using a p-value from a likelihood ratio test, the concordance probability, C[23], an R2[24] statistic, and integrated discrimination improvement index, IDI[25]. Models treating the histopathology score as binary and as ordered categories (ordinal logistic regression) were assessed. Note that for binary logistic regression C is equivalent to the AUC from an ROC curve. In both the binary and ordinal logistic models, the IDI was calculated as the mean of the predictions for positive samples minus the mean of predictions for the non-positive samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acknowledgments

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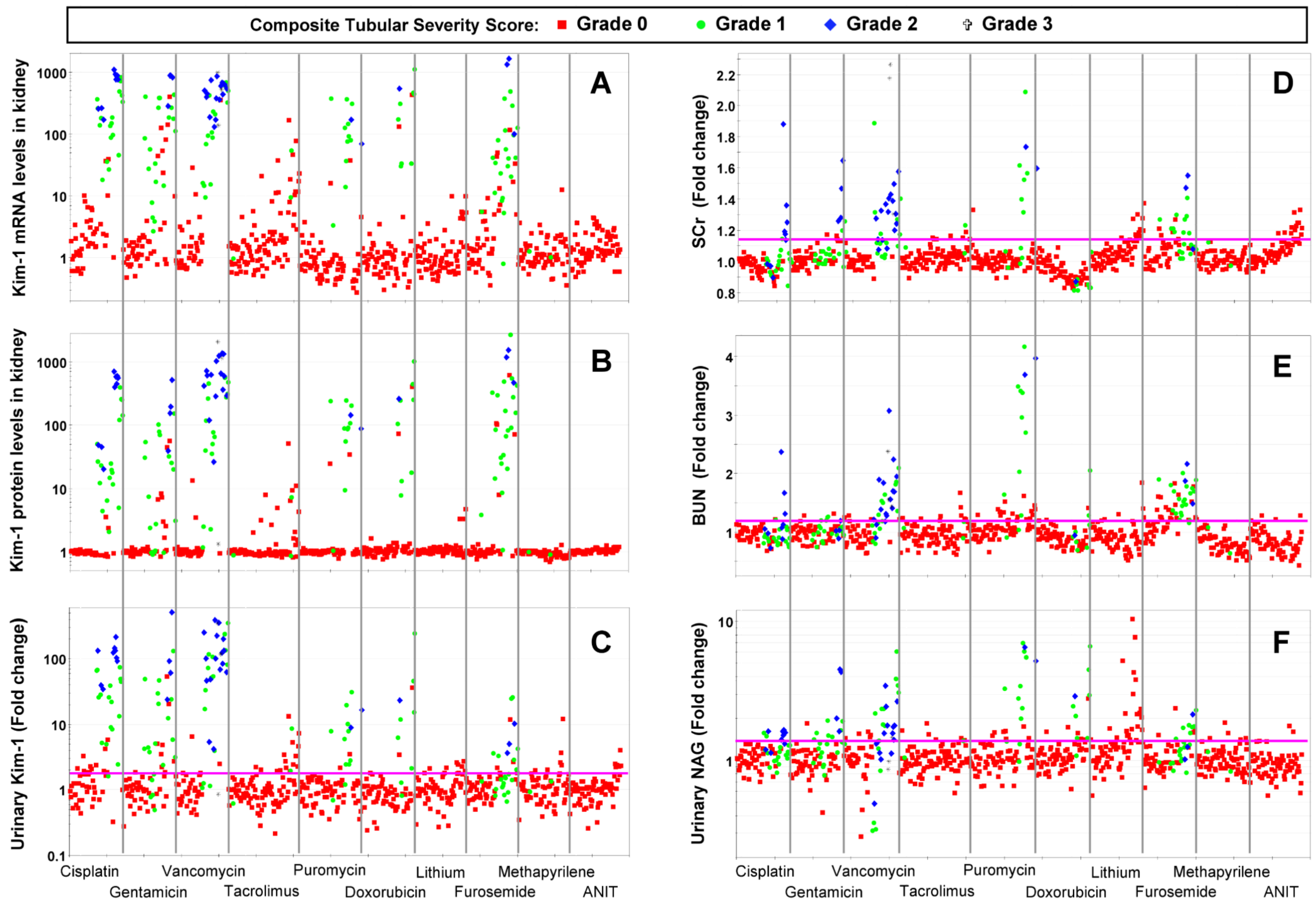

Fig. 1. Correlation of Kim-1 mRNA and protein levels in the kidney and urine respectively and comparison of urinary Kim-1 levels with serum creatinine, BUN and urinary NAG with severity grades of histopathology following a dose response and time course in 10 Novartis rat toxicology studies

Male Han Wistar rats (n=739) were dosed with a low, medium and high dose of eight mechanistically distinct nephrotoxicants and two hepatotoxicants and (A) renal Kim-1 mRNA, (B) renal Kim-1 protein and (C) urinary Kim-1 levels were measured. Conventional markers for kidney toxicity including (D) serum creatinine, (E) blood urea nitrogen and (F) urinary NAG were also measured and compared to different grades of kidney tubular histopathology. All values are represented as fold-changes versus the average values of study-matched and time-matched control animals on a logarithmic scale. The animals are ordered by study, within each study by dose-group (with increasing doses) and within each dose-group by termination time point (with increasing time). The symbols and the colors represent the histopathology readout for proximal tubular damage (red = no histopathology finding observed, green = grade 1, blue = grade 2, black = grade 3 on a 5 grade scale). The magenta lines represent the thresholds determined for 95% specificity in the ROC analysis for all histopathology grades.

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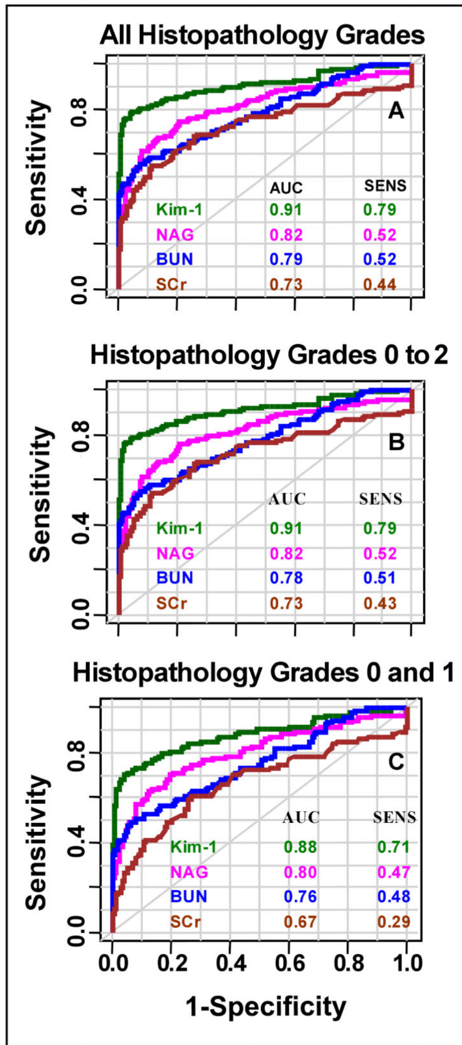

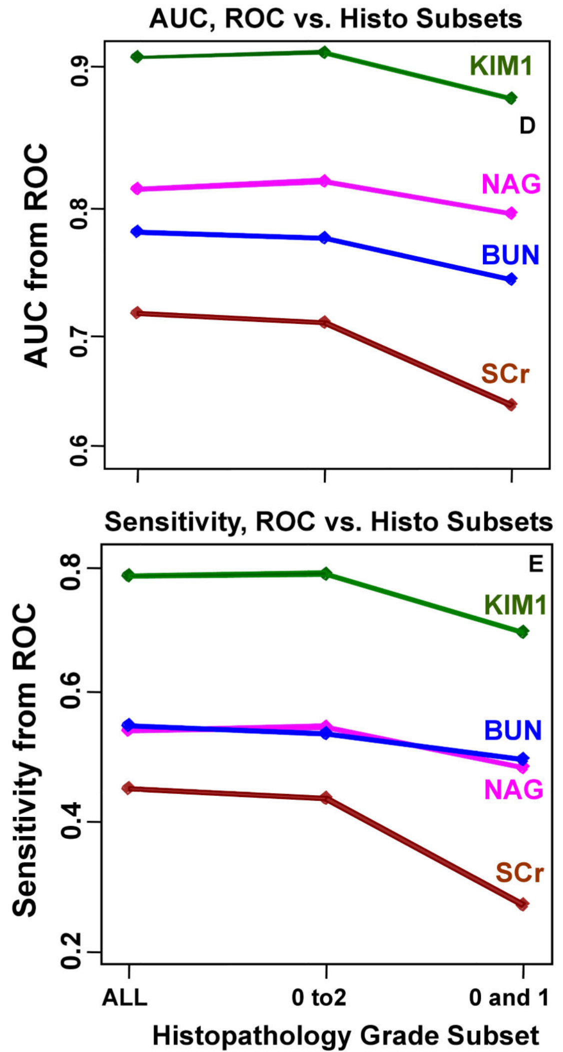

Fig. 2. Receiver operator characteristic curves for Novartis studies

Receiver operator characteristic curves from 8 different nephrotoxicant studies and 2 different hepatotoxicant studies from Novartis demonstrating sensitivity and specificity of BUN, serum creatinine, urinary Kim-1 and NAG with respect to a composite histopathology score that included (A) all histopathology grades; (B) histopathology grade 0 to 2; (C) histopathology grade 0 to 1. (D) Area under the curve and (E) Sensitivity (at 95% specificity) compared to the “gold standard”, histopathology. Animal numbers (n): nneg: 283, npos: All=132, 0 to 2=129, 0 to 1=94.

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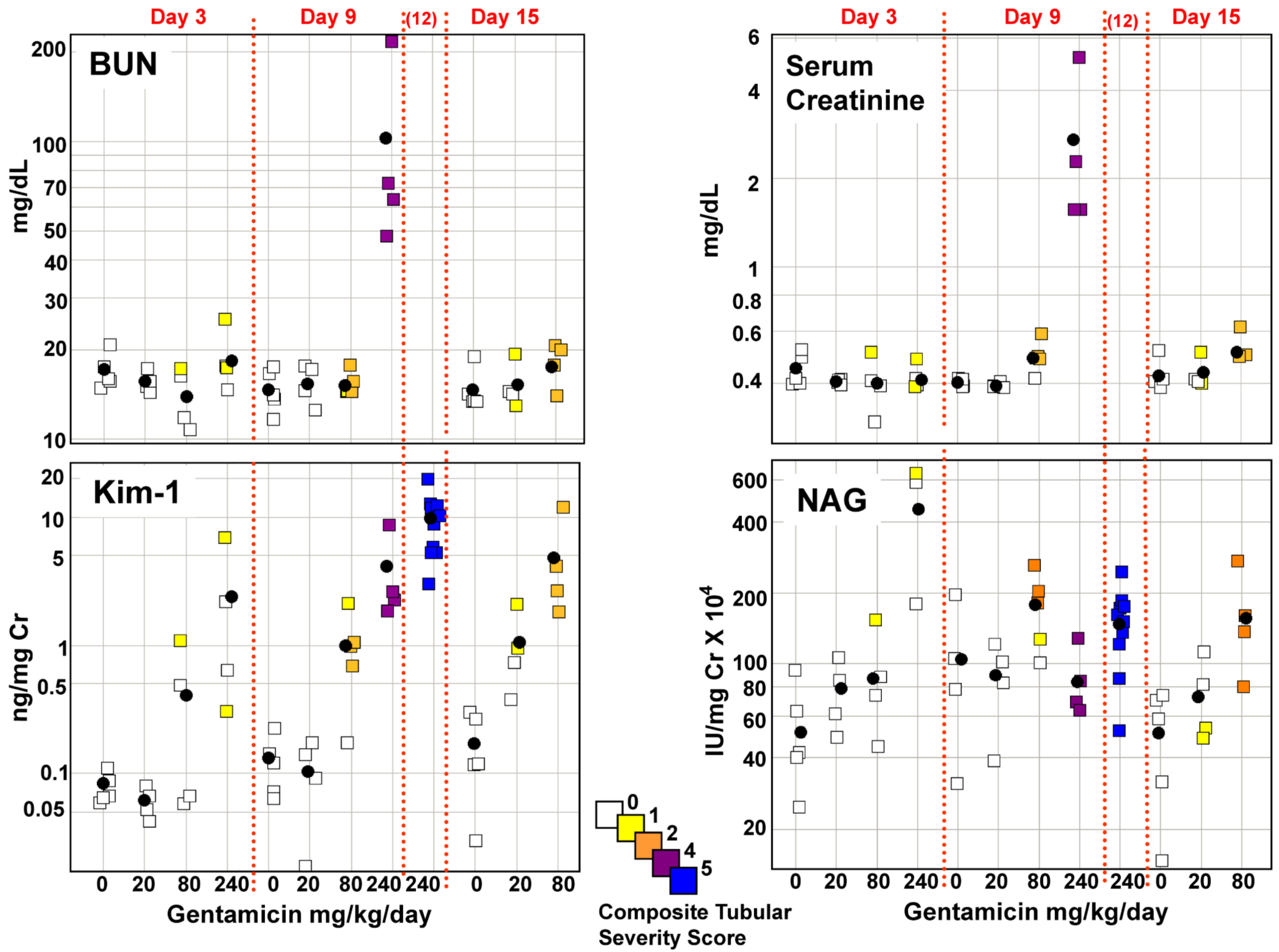

Fig. 3. Correlation of BUN, serum creatinine, urinary Kim-1 and urinary NAG with severity grades of histopathologic change following gentamicin treatment in Merck study

Male Sprague Dawley rats were administered gentamicin sulfate i.p. at 0, 20, 80, or 240 mg/kg/day (mkd) to groups of five rats/dose/time point and the animals were sacrificed on days 3, 9 or 15 for toxicity evaluation which included serum clinical chemistry (BUN, creatinine), urinary Kim-1 and NAG levels and renal histopathlogy (H&E staining). Open squares indicate grade 0 pathology and the composite tubular severity score is color coded from yellow (1), orange (2), purple (4) and blue (5). Black circles indicate average values of dose groups.

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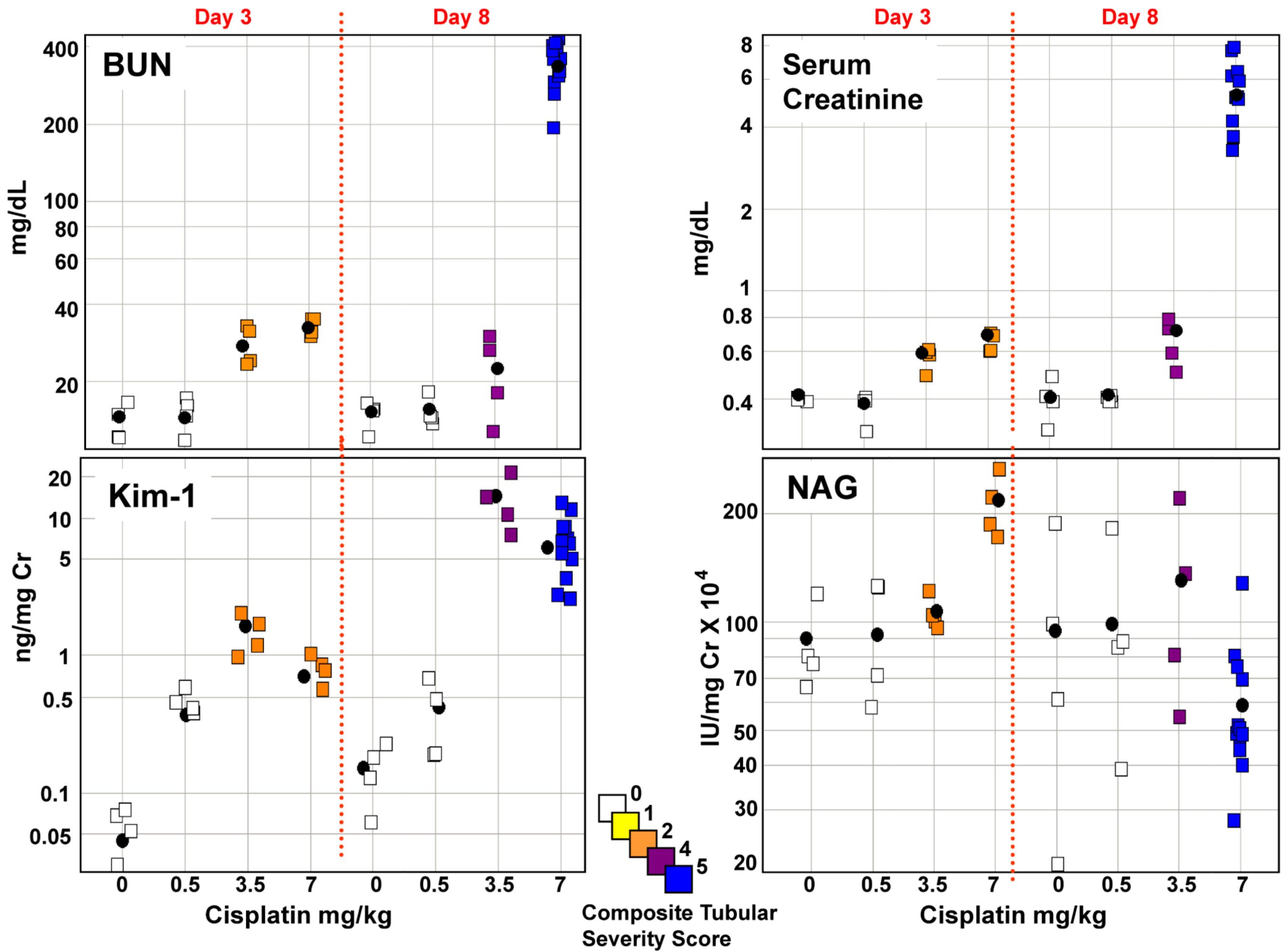
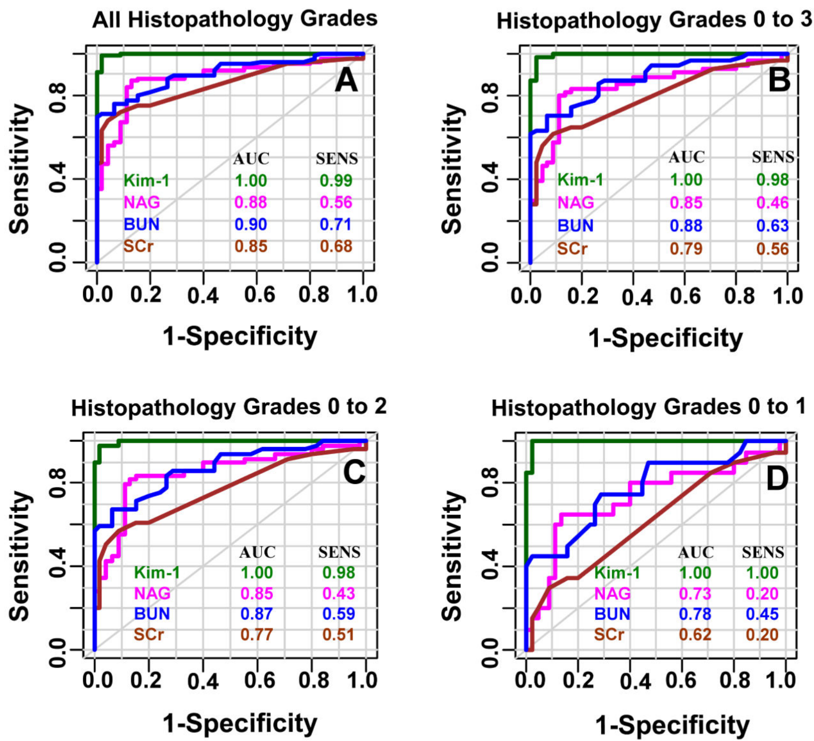


Fig. 4. Correlation of BUN, serum creatinine, urinary Kim-1 and urinary NAG with severity grades of histopathologic change following cisplatin nephrotoxicity treatment in Merck study

Male Sprague Dawley rats were administered cisplatin i.p. (n=5/dose/time point) at doses of 0, 0.5, 3.5 or 7 mg/kg and rats were sacrificed on days 3 and 8 for toxicity evaluation which included serum clinical chemistry (BUN, creatinine), urinary Kim-1 and NAG levels and renal histopathlogy (H&E staining). Open squares indicate grade 0 pathology and the composite tubular severity score is color coded from yellow (1), orange (2), purple (4) and blue (5). Black circles indicate average values of dose groups.



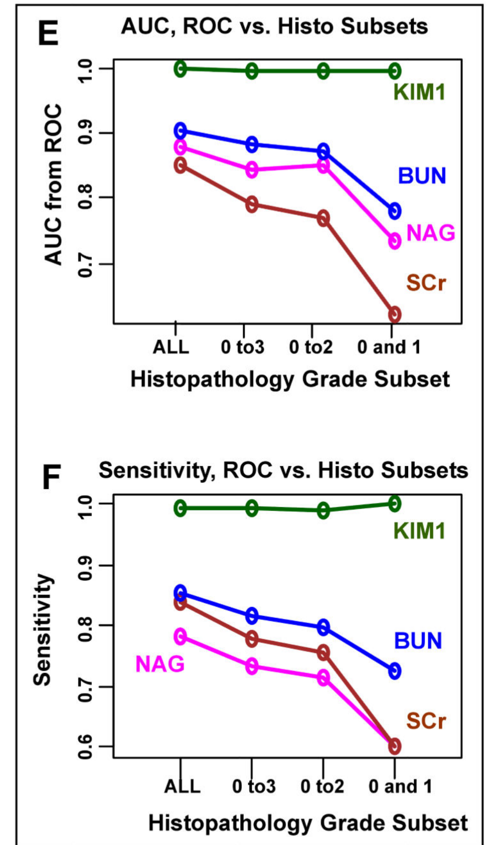


Fig. 5. Receiver operator characteristic curves for Merck studies

Receiver operator characteristic curves from four different nephrotoxicant studies demonstrating sensitivity and specificity of BUN, serum creatinine, urinary Kim-1 and NAG with respect to a composite histopathology score that included (A) all histopathology grades; (B) histopathology grade 0 to 3; (C) histopathology grade 0 to 2; (D) histopathology grade 0 and 1. (E) Area under the curve and (F) Sensitivity (at 95% specificity) of BUN, serum creatinine, urinary Kim-1 and NAG compared to the “gold standard”, histopathology. Animal number (n): nneg:45, npos: All=75, 0 to 3=54, 0 to 2=49, 0 to 1=20.

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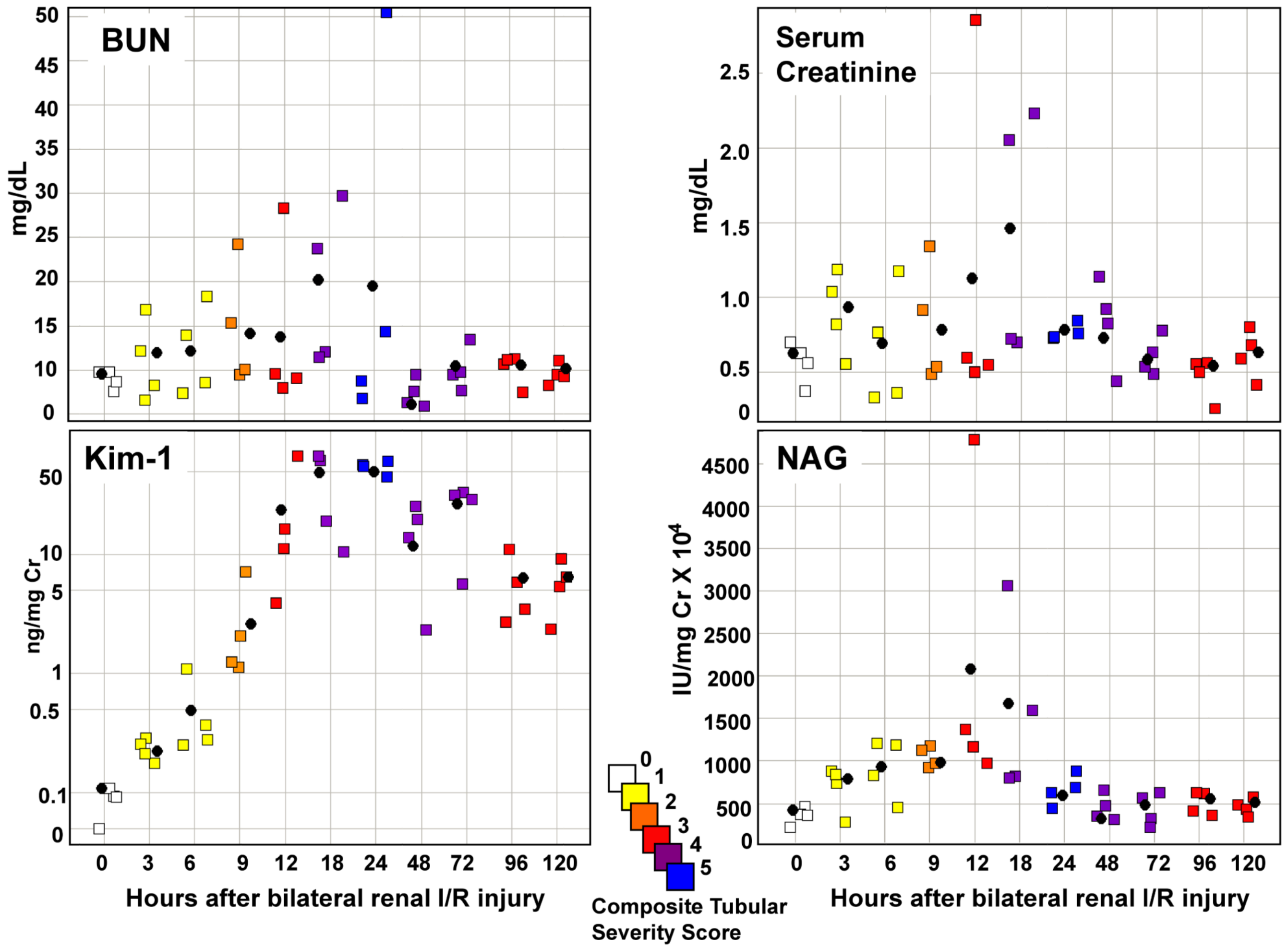

Fig. 6. Comparison of Kim-1 with routinely used biomarkers as an early diagnostic indicator of kidney injury following 20 min bilateral renal ischemia/reperfusion injury

Male Wistar rats were subjected to 0 (sham) or 20 min of bilateral ischemia by clamping the renal pedicles for 20 min and then removing the clamps and confirming reperfusion. Two hours after reperfusion the rats were placed in metabolic cages and urine, blood and tissue collected at 3, 6, 9, 12, 18, 24, 48, 72, 96 and 120 h following reperfusion. Urinary Kim-1, blood urea nitrogen, serum creatinine, and urinary N-acetyl-β-D-glucosaminidase were measured and these levels were correlated to histopathology (H&E staining) as per methods. Open squares indicate grade 0 pathology and the composite tubular severity score is color coded from yellow (1), orange (2), red (3), purple (4) and blue (5).