Multiplex analysis of urinary protein biomarkers for the detection of Vancomycin-induced subacute nephrotoxicity

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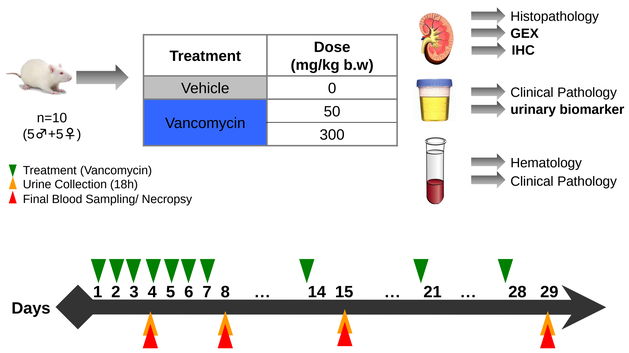
Introduction

In pharmaceutical and chemical industries, the kidney is routinely assessed during preclinical safety evaluations. The importance of the kidney as a central detoxification organ leads to a high exposure of renal tissue to drugs, reactive metabolites or environmental compounds. Traditional markers for assessing renal toxicity, such as blood urea nitrogen (BUN) and serum creatinine (SCr), are insensitive. Although both are direct measurements of renal function, increases in serum concentrations of these biomarkers occur only after substantial renal injury. For improved detection of acute nephrotoxicity, a panel of novel urinary kidney biomarkers has been approved by the FDA, EMA and PMDA. However, limited data regarding the performance of these acute markers after subacute or sub-chronic treatment are publicly available. To increase the applicability of these markers, it is important to evaluate the ability to detect these markers after 28 days or even longer.

In this study, Wistar rats were treated with three doses of Vancomycin to induce renal damage and studied for 28 days. Urine was collected under cooled conditions on an 18-hour cycle on days 4, 8, 15 and 29. Luminex® xMAP® technology-based MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panels were used to measure 14 candidate protein biomarkers simultaneously from the urine samples. Vancomycin treatment resulted in a dose-dependent increase in urinary biomarkers, specific for the observed areas within the nephron, determined histopathologically. Several biomarkers were found promising in this study, including NGAL, Cystatin C, KIM-1, Osteopontin, Clusterin and Albumin. The simultaneous measurement of these proteins with multiplex technology offered a robust and convenient method to study these biomarkers. Taken together, our data demonstrate the high accuracy and predictivity of some of these new markers for detecting subacute effects with one well described nephrotoxin, Vancomycin.

Methods



Treatment	Dose (mg/kg b.w)
Vehicle	0
Vancomycin	50
	300

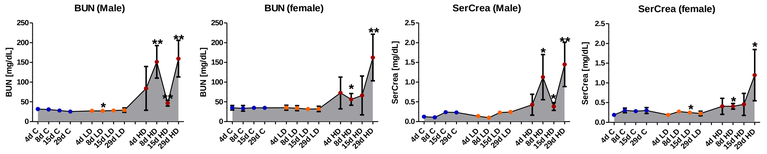
•Luminex® xMAP® technology: MILLIPLEX® MAP Rat Kidney Toxicity Magnetic Bead Panel 1 & 2 (Cat. No. RKTX1MAG-37K & RKTX2MAG-37K0)

The assays were performed as recommended in the protocol with the following proteins being measured: KIM-1, TIMP-1, VEGF, EGF, Albumin, AGP, IP-10, Osteopontin, Clusterin, Cystatin C, Calbindin 28k, β–2-Microglobulin, GSTα, and NGAL/Lipocalin-2.

• **Gene Expression Analysis (GEX)**
3 Animals/sex/group were used for gene expression analysis using Illumina Sentrix® rat Ref-12 V1 BeadChip Arrays. The Illumina® TotalPrep™-96 RNA Amplification Kit was used to synthesize biotinylated cRNA. 750 ng amplified biotinylated cRNA were hybridized onto the BeadChip under humidified conditions for 20h at 58˚ C. Fluorescence detection was carried out by confocal laser scanning with the Illumina® BeadArray Reader at 532 nm and 0.8 µm resolution.

• **Histopathology and immunohistochemical** investigations were performed according to Standard Operation Procedures (SOPs), then evaluated and interpreted.

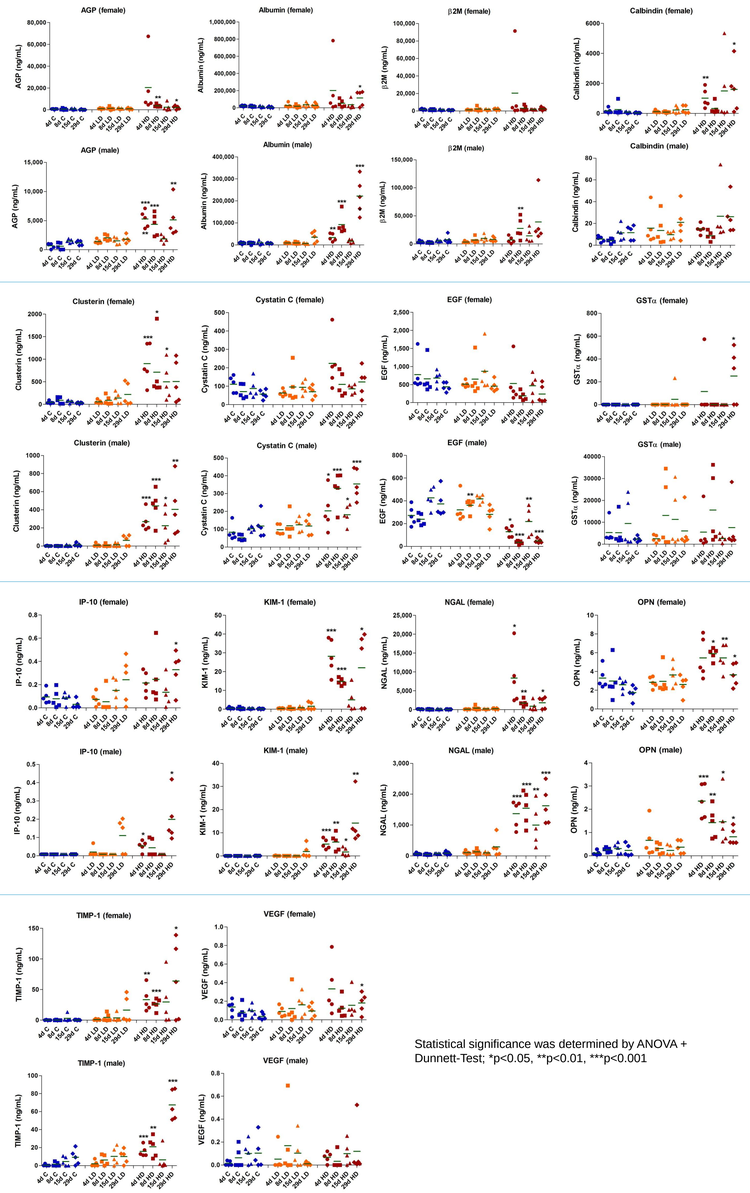
Results: Clinical Pathology -- serum creatinine (SerCrea) and blood-urea nitrogen (BUN)



Significant increases were observed at several time points in the high dose group of both genders in blood urea nitrogen (BUN) and serum creatinine (SerCrea). No changes in low dose animals could be detected.
ANOVA + Dunnett p-values:
* <0.05, ** <0.01, *** <0.001

Results: Urinary Protein Biomarker

Biomarker results were very promising and correlated strongly with histopathology. The following markers reflected the renal regeneration and strength of response best: KIM-1, Clusterin, Osteopontin (OPN), IP-10 and TIMP-1 whereas AGP, Albumin β2M, Cystatin C, EGF and NGAL are limited by gender differences. VEGF, Calbindin and GSTα demonstrated minimal changes in this approach to predicting Vancomycin-induced renal damage.

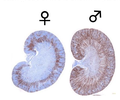


Statistical significance was determined by ANOVA + Dunnett-Test; *p<0.05, **p<0.01, ***p<0.001

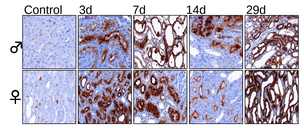
Results: Immunohistochemistry (KIM-1) and Histopathology

		Vancomycin [mg/kg]					
		male			female		
	time	0	50	300	0	50	300
Tubular Degeneration							
	day 4	/	/	++ (3/5) +++ (2/5)	/	/	++ (4/5) +++ (1/5)
	day 8	/	/	+++ (2/5) ++++ (3/5)	/	/	++ (3/5) +++ (2/5)
	day 15	/	/	++ (3/5) +++ (1/5) ++++ (1/5)	/	+ (1/5)	+ (1/5) ++ (4/5)
	day 29	/	+ (4/5)	+++ (1/5) ++++ (2/5) +++++ (2/5)	/	+ (3/5)	++ (2/5) +++ (1/5) ++++ (2/5)

+ = minimal, ++ = mild, +++ = moderate, ++++ = massiv, +++++ = servere Number of animals in each group of five is shown in parentheses



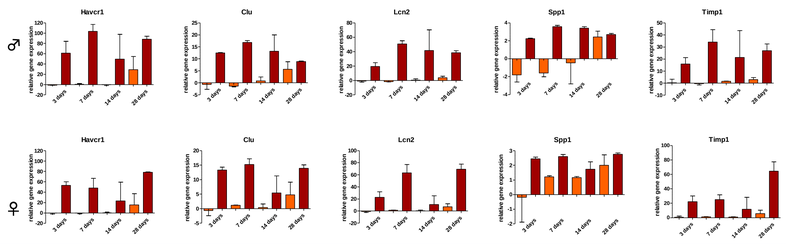
Gender specific differences in the location of KIM-1 could be identified at all time points in the high dose (here e.g. day 7).

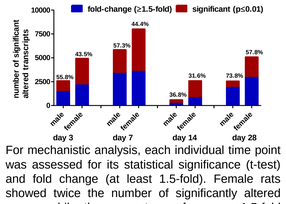


Time course of KIM-1 expression in male and female rats treated with 300 mg/kg Vancomycin. An increase in protein level could be observed up to day 7. The regeneration up to day 14 led to a reduction in KIM-1. On day 29, when massive tubular damage was again observed, KIM-1 was upregulated.

Results: Gene Expression Analysis

Transcriptional changes, reflecting urinary protein excretion, were observed for almost all biomarkers in a time- and dose-dependent manner. Low dose Vancomycin treatment group High dose Vancomycin treatment group





For mechanistic analysis, each individual time point was assessed for its statistical significance (t-test) and fold change (at least 1.5-fold). Female rats showed twice the number of significantly altered genes, while the percentage of genes >1.5-fold threshold was always lower than in males.

Three of the most prominent gene clusters identified by using the IPA™ and ToxWiz pathway analysis softwares are shown below. Time- and gender-specific alterations were observed. Cellular recovery and inflammatory responses seem to play a pivotal role.

The five genes of each time point showing the strongest fold-change (FC) for male or female rats:

Male

day 3		day7		day 14		day 28	
gene	FC	gene	FC	gene	FC	gene	FC
Havcr1	58	Havcr1	104	Lcn2	32	Havcr1	89
Lcn2	20	Lcn2	51	Havcr1	31	Fgb	44
Timp1	15	Timp1	33	Timp1	13	Lcn2	40
Fgb	14	Fgb	19	Slpi	12	Timp1	27
Clu	13	Col3a1	19	Clu	12	Il24	19
Cyp2c9	-21	Cyp2c9	-29	LOC100290142	-5	Egf	-23
Klk1	-12	Slco1a6	-19	Klk1	-4	Rgn	-23
Slco1a6	-12	Rgn	-17	Rgn	-3	Cyp2c9	-19
Ugt2B15	-9	Egf	-14	Egf	-3	Slco1a6	-19
Tnfrsf11b	-9	Klk1	-14	Cyp1a1	-3	Cml1/Nat8b	-17

Female

day 3		day 7		day 14		day 28	
gene	FC	gene	FC	gene	FC	gene	FC
Havcr1	53	Lcn2	62	Havcr1	7	Havcr1	79
Lcn2	22	Havcr1	46	Timp1	5	Lcn2	70
Timp1	22	Timp1	25	Lcn2	5	Timp1	65
Clu	13	Ubm	19	Slpi	4	Fgb	45
Adamts1	10	Cxcl11	17	Clu	4	Slc34a2	20
Cyp1a1	-11	Cyp1a1	-5	Klk1	-4	Egf	-13
LOC100290142	-7	LOC100290142	-4	Sds	-3	Slc7a12	-9
Sds	-6	Egf	-4	Egf	-2	Hnmt	-8
Slco1a6	-4	Klk1	-4	Cyp1a1	-2	Cml1/Nat8b	-7
Klk1	-4	Slco1a6	-4	Slco1a6	-2	Adh6	-7

Gene Cluster		day 3		day 7		day 14		day 28	
		No. of molecules	p-value	No. of molecules	p-value	No. of molecules	p-value	No. of molecules	p-value
Renal Tubule Injury	m	52	5.14.E-27 - 2.84E-01	65	3.93E-23 - 1.45E-01	50	5.92E-15 - 4.12E-01	58	5-94E-25 - 3-64E-01
	f	44	7.64E-11 - 4.00E-01	56	1.57E-16 - 2.72E-01	34	1.06E-09 - 2.3E-01	51	6.84E-13 - 4.62E-01
Cellular Growth and Proliferation	m	416	8.83E-25 - 2.81E-05	778	1.35E-47 - 1-58E-08	702	1-73E-50 - 8-25E-08	587	1.52E-36 - 3-03E-07
	f	671	3.77E-49 - 2-71E-07	420	4.15E-32 - 3.09E-06	776	1.30E-44 - 3-78E-07	761	2.91E-42 - 3-87E-07
Immune Cell Trafficking	m	174	1.49E-16 - 3-18E-05	340	2.14E-44 - 1.04E-08	289	1.27E-34 - 8.25E-08	245	5.11E-29 - 4.39E-07
	f	260	3.79E-29 - 3.15E-07	170	4.36E-23 - 2.65E-06	314	3-22E-33 - 3-83E-07	289	5.13E-27 - 3.95E-07

Conclusion:

In this study, acute nephrotoxicity biomarkers were investigated for their ability to detect renal damage in a 28-day toxicity study in rats.

Our data demonstrate the high accuracy and predictivity of some of these new markers, even after subacute treatment with one well described nephrotoxin, Vancomycin, which caused a very distinct kidney tubular damage.

Global transcriptomics analysis confirmed the urinary biomarker changes, in addition to identifying specific mechanistic changes, caused by Vancomycin.

Many questions remain to be addressed with regard to the applicability of these novel biomarkers. Especially for an intermittent treatment regimen, it is of major interest to discover the optimum time points for measuring biomarker excretion, especially considering the high regeneration properties of renal tissue.

The Study was funded by the German Federal Ministry of Education and Research (BMBF) together with Merck Serono Research as a part of the Biomarker Center of the BioRN Biotech Cluster.

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