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Gene Expression Analysis Reveals New Possible Mechanisms of Vancomycin-Induced Nephrotoxicity and Identifies Gene Markers Candidates

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Vancomycin, one of few effective treatments against methicillin-resistant *Staphylococcus aureus*, is nephrotoxic. The goals of this study were to (1) gain insights into molecular mechanisms of nephrotoxicity at the genomic level, (2) evaluate gene markers of vancomycin-induced kidney injury, and (3) compare gene expression responses after iv and ip administration. Groups of six female BALB/c mice were treated with seven daily iv or ip doses of vancomycin (50, 200, and 400 mg/kg) or saline, and sacrificed on day 8. Clinical chemistry and histopathology demonstrated kidney injury at 400 mg/kg only. Hierarchical clustering analysis revealed that kidney gene expression profiles of all mice treated at 400 mg/kg clustered with those of mice administered 200 mg/kg iv. Transcriptional profiling might thus be more sensitive than current clinical markers for detecting kidney damage, though the profiles can differ with the route of administration. Analysis of transcripts whose expression was changed by at least twofold compared with vehicle saline after high iv and ip doses of vancomycin suggested the possibility of oxidative stress and mitochondrial damage in vancomycin-induced toxicity. In addition, our data showed changes in expression of several transcripts from the complement and inflammatory pathways. Such expression changes were confirmed by relative real-time reverse transcription–polymerase chain reaction. Finally, our results further substantiate the use of gene markers of kidney toxicity such as KIM-1/*Havcr1*, as indicators of renal injury.

Key Words: **vancomycin; route of administration; nephrotoxicity; gene expression; inflammation; complement pathway activation.**

As the body compartment responsible for concentration and excretion of toxic metabolites and drugs, kidney is a frequent site of drug toxicity. Nephrotoxicity contributes to 8–60% of all cases of in-hospital acute kidney injury (Schetz *et al*., 2005). When assessing nephrotoxicity, the tubular secretion system, which allows transport of the drug from the blood to the urine via the tubular cells, is particularly important (Fanos *et al*., 2001). The biomarkers currently used clinically to assess nephrotoxicity are primarily changes in the level of blood urea nitrogen (BUN) and serum creatinine (CRE) (Duarte *et al*., 1993). However, such changes are only measurable after 30–50% of final tissue damage has occurred (Duarte *et al*., 1993), emphasizing the critical need for more sensitive marker(s) to detect kidney injury.

Glycopeptide antibiotics, such as vancomycin, teicoplanin, ramoplanin, and decaplanin, are glycosylated cyclic or polycyclic nonribosomal peptides. Because of their toxicity, their use is restricted to patients who are critically ill or who have a demonstrated hypersensitivity to the β-lactams. Vancomycin is one of the last available lines of defense against the Gram-positive bacterium methicillin-resistant *Staphylococcus aureus* (MRSA). After iv administration, however, nephrotoxicity has been reported in 7–17% of patients (Downs *et al*., 1989; Eng *et al*., 1989; Mellor *et al*., 1985). Vancomycin-induced renal damage requires energy-dependent transport from the blood to the tubular cells across the basolateral membrane (Fanos *et al*., 2001). In the tubular cells, vancomycin presents a pronounced lysosomal tropism (Fanos *et al*., 2001). Animal studies suggested oxidative stress might underlie the pathogenesis of vancomycin-induced toxicity (Nishino *et al*., 2003; Oktem *et al*., 2005). Despite this information, the mechanisms of vancomycin toxicity remain poorly understood, and a better appreciation of these mechanisms would be useful in designing strategies to prevent it. Our study was intended to uncover potential mechanisms of vancomycin toxicity at the genomic level.

Toxicogenomics, gene expression analysis applied to toxicology, is actively being employed in drug discovery. It is used as a screening tool to rapidly identify signs of toxicity associated with lead compounds and to understand mechanisms of chemical toxicity and action; this use is possible because compounds belonging to a single class of toxicants yield similar gene expression patterns that are distinct from other profiles generated by other classes of chemicals (Bartosiewicz

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et al., 2001). Thus, analysis of the transcriptional response in the cells of target organs can give indications of the biological and biochemical mechanisms affected by a pharmaceutical compound (Ganter *et al.*, 2005). Similarly, several research groups have attempted to identify potential biomarkers and signature profiles of toxicity for various organs (Merrick *et al.*, 2004), especially for kidneys (Amin *et al.*, 2004; Thukral *et al.*, 2005). The Thukral studies, performed in rats after ip administration of various drugs, identified several candidate genes modulated in a dose- and time-dependent manner in response to injury. They included kidney molecule injury (KIM-1/*Havcr1*), osteopontin (*Spp1*), fibrinogen alpha polypeptide (*Fga*), insulin-like growth factor binding protein 1 (*Igfbp1*), glutathione S-transferase cluster (*Gsta*), clusterin (*Clu*), lipocalin 2 (*Lcn2*), and several transporters. Some of these markers, including *Clu*, *Spp1*, and *Havcr1,* were also confirmed in a nonhuman primate model of antibiotic-induced nephrotoxicity (Davis *et al.*, 2004), suggesting that such markers are not restricted to rodent animal models and may be relevant to human applications.

In the study described herein, we have performed gene expression profiling on the kidney of mice administered various doses of vancomycin either iv or ip. The goals were to (1) gain insights into the molecular mechanisms of vancomycin-induced nephrotoxicity, (2) identify potential markers of renal toxicity, and (3) compare the gene expression responses after iv and ip delivery to identify potential differences between the two most common routes of administration in humans (iv) and in animal models (ip).

MATERIALS AND METHODS

Animals

Female BALB/c mice (Charles River Laboratories, Inc., Wilmington, MA), 6–8 weeks old, were maintained on Purina Certified Rodent Chow 5002 (Richmond, IN) and purified tap water *ad libitum* in microisolator cages under controlled lighting (12-h light/dark cycle). All animals were treated in accordance with a protocol approved by the Institutional Animal Care and Use Committee.

Experimental Designs

For the toxicity study (microarray analysis, clinical pathology, and histology), groups of six mice were administered vancomycin (Sigma, St Louis, MO) at 50, 200, or 400 mg/kg daily for 7 consecutive days; the doses were delivered iv or ip (iv-50, iv-200, iv-400, ip-50, ip-200, and ip-400). Control mice were administered a similar volume of 0.9% saline solution by both routes (iv-0 and ip-0, six mice per group). Clinical observations were recorded daily, and body weights were measured on days 1, 3, 5, and 8. Mice were sacrificed on day 8, 24 h after the last vancomycin treatment.

For the toxicokinetic (TK) analysis, groups of 12 mice were administered iv or ip vancomycin at 50, 200, and 400 mg/kg. Five control mice remained untreated.

Sample Collection

For microarray analysis, mice were sacrificed on day 8, 24 h after the last of seven daily doses of vancomycin. The left kidney of each mouse was removed and immediately placed into 1.8 ml of RNAlater (Qiagen, Valencia, CA). After overnight incubation at room temperature, each kidney was transferred into a new tube and immediately stored at −80°C until it was processed for RNA extraction.

For clinical pathology, blood samples were collected from the retro-orbital sinus on day 3 (prior to administration of the third dose) and day 8 (prior to sacrifice). Within each experimental group (six mice), the blood from three mice was collected for clinical chemistry analysis.

For histological analysis, tissues were removed from the animal immediately after sacrifice and placed directly into 10% neutral buffered formalin.

For TK analysis, blood samples were collected, with ethylenediaminetetraacetic acid as anticoagulant, from the retro-orbital sinuses of three mice per group at 0.083, 0.25, 0.5, 1, 2, 4, 6, and 24 h after the first and the seventh dose administration. Because some animals were found moribund or dead in the iv high-dose group, samples for that particular group were collected at only four time points (0.083, 0.5, 1, and 4 h). Within 15 min of collection, blood samples were centrifuged for plasma collection and subsequently stored at −80°C until analysis.

Plasma Concentration of Vancomycin and TK Analysis

Vancomycin was extracted from mouse plasma by solid phase extraction (SPE) based on the protocol described by Farin and colleagues (Farin *et al.*, 1998). Solid phase Oasis HLB C18 microelution plates (Waters, Inc., Milford, MA) were preconditioned with 1 ml methanol followed by 1 ml water. After 50 μl of plasma and 50 μl of 20 μg/ml tinidazole (internal standard) were drawn through the wells of the SPE plate, each well was washed with 0.5 ml of water, followed by 0.5 ml of 5% methanol. Vancomycin and tinidazole were eluted from the SPE plate with 0.6 ml of 1:1 (v:v) acetonitrile:50mM KH_2PO_4 (pH 4). The solvents were removed under a nitrogen flow without heat application, and the dry residues were reconstituted in 100 μl of water. The plate was centrifuged (425 × g, 15 min) prior to high-pressure liquid chromatography (HPLC) analysis. Study samples were quantitated using a set of calibration standards (0.5, 2.5, 5, 10, 25, 50, and 100 μg/ml) prepared in blank matrix and processed simultaneously.

Samples were analyzed by HPLC with detection by ultraviolet absorbance at 282 nm. Samples (50 μl) were injected onto a Luna C8 column (4.6 × 100 mm; 5 μ; Phenomenex, Torrance, CA) and eluted at a flow rate of 1.5 ml/min with the following gradient conditions: mobile phase A = 5mM KH_2PO_4, pH 2.8, and mobile phase B = acetonitrile; 0 and 1.5 min: 97% A, 3% B; 11.5 and 14 min: 80% A, 20% B; 15 and 20 min: 97% A, 3% B.

Under these conditions, vancomycin and tinidazole eluted at 5.9 and 7.5 min, respectively. A calibration standard curve was prepared by performing weighted linear regression ($1/y$) of the peak area ratio of vancomycin/tinidazole versus the concentration of vancomycin.

TK Analysis

The measured plasma levels were subjected to noncompartmental TK analysis using WinNonlin software version 5.0 (PharSight Corp., Mountain View, CA). The TK values reported are the maximal plasma concentration (C_{max}), the time to reach the C_{max} (T_{max}), and the area under the plasma concentration versus time curve ($AUC^{0\rightarrow\infty}$). Bioavailability (F) after ip administration was calculated using the formula: $(AUC_{ip}/AUC_{iv}) \times (Dose_{iv}/Dose_{ip})$.

Clinical Chemistry

After collection at SRI International, serum samples were sent to IDEXX Veterinary Services, Inc. (West Sacramento, CA) for clinical chemistry parameter evaluation.

Histopathology

Tissues retained in 10% neutral buffered formalin were subsequently embedded into paraffin, cut approximately 5 μm thick, and stained with hematoxylin and eosin (H&E). Histopathologic examination was performed by a board-certified veterinary pathologist (D. Fairchild, SRI International).

Gene Expression Analysis

Total RNA isolation. Approximately 15–20 mg of each of the 48 kidneys were transferred into 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA) along with a stainless steel bead (Qiagen), and homogenized with a TissueLyser (Qiagen). Total RNA was extracted with 200 µl of chloroform, precipitated with 500 µl of 100% ethanol, and transferred to an RNeasy mini spin column (Qiagen) for purification according to the manufacturer's instructions. Total RNA was subsequently precipitated with ethanol, resuspended in water, and stored at −70°C. RNA integrity was examined by agarose gel electrophoresis and spectrophotometric analysis.

Affymetrix GeneChip protocols. cDNA was prepared and cleaned from 10 µg total RNA using the One-Cycle cDNA Synthesis and Clean-up module kits (Affymetrix, Santa Clara, CA). The cDNA was further processed to biotin-labeled cRNA using the IVT labeling kit (Affymetrix). The cRNA was fragmented before hybridization to the GeneChip Mouse Genome 430 2.0 Array (Affymetrix) and scanning (TGT value = 500). All procedures were performed according to the manufacturer's instructions, as described in the Affymetrix Genechip Expression Analysis Technical Manual, revision 5 (http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

Data analysis. The Affymetrix CEL files were loaded into GeneSpring GX version 7.3.1 (Agilent Technologies, Santa Clara, CA) and preprocessed using GenChip Robust Multi-Array analysis (GC-RMA) (Wu *et al*., 2004). In GeneSpring GX, an experiment was created, consisting of 48 kidney samples (six mice per group and eight experimental conditions) represented by 48 arrays and a per-gene normalization to the median was applied. A total of 9502 probes (subsequently referred to as the "probe list") were selected for further analysis from the 45,101 original probes by performing two successive levels of filtering. First, probes with a raw intensity value > 60 in 5/8 conditions were selected to eliminate those with an intensity value close to background levels. Second, a one-way ANOVA was performed using a parametric test assuming equal variances and Benjamini and Hochberg as multiple testing corrections with a False Discovery Rate = 0.01. This ANOVA analysis was used to select genes that were significantly differentially expressed in at least one of the pairwise comparisons (group-to-group comparisons of the 8 experimental groups), ensuring that the differences were due to the treatment, not due to chance; it also filtered out genes without any changes across all of the experimental conditions. All subsequent analysis performed using the above probe list employed the averaged intensity value of each gene within each experimental condition (geometric mean of the six samples for each experimental condition).

The data discussed in this publication have been deposited at the National Center for Biotechnology Information Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). They are accessible through GEO Series accession number GSE7793.

Real-Time Relative Reverse Transcription–Polymerase Chain Reaction

Total RNA (5 µg) isolated as previously described was submitted to cDNA synthesis using a High Capacity cDNA reverse transcription kit (Applied Biosystems [ABI], Foster City, CA). Of the resulting cDNA, 38 ng was subjected to reverse transcription–polymerase chain reaction (RT-PCR) using the Taqman expression assays (ABI) for *Gapdh*, *Spp1*, *Havcr1*, *C3*, *C4b*, *Cxcl1*, and *Rtn4* according to the manufacturer's instructions. PCR was performed with a LightCyler 480 (Roche Applied Science, Indianapolis, IN) using the following conditions: 1 cycle of 10 min at 95°C, and 40 cycles of 10 s at 95°C and 30 s at 60°C. For each experimental group, three samples, manually selected at random were analyzed, and each sample was measured in duplicate. Fold change expression was evaluated relative to the appropriate control samples (iv or ip administration of saline) using the comparative C_T method (Livak *et al*., 2001).

Statistical Analysis

Multiple group (> 3) comparisons (clinical pathology and organ weight data) were performed separately on the iv and ip treated samples using one-way ANOVA with Dunnett's multiple comparison test. The appropriate saline treated animals (ip-0 or iv-0) were used as controls.

TABLE 1
TK Parameters after Injection of One and Seven Doses of Vancomycin

Dose (mg/kg)	Route	Day	C_{max} (µg/ml)	T_{max} (h)	$AUC^{0\rightarrow\infty}$ (µg·h/ml)	F (%)
50	iv	1	108	0.083	51	
50	ip	1	43	0.25	51	100
50	iv	7	93	0.083	46	
50	ip	7	46	0.25	46	100
200	iv	1	417	0.083	328	
200	ip	1	161	0.50	254	77
200	iv	7	489	0.083	369	
200	ip	7	145	0.50	211	57
400	iv	1	787	0.083	1927	
400	ip	1	435	0.50	4708	244
400	iv	7	940	0.083	2439	
400	ip	7	650	0.50	4033	165

Note. C_{max}: peak serum concentration; T_{max}: time when the peak serum concentration is reached; $AUC^{0\rightarrow\infty}$: area under the plasma concentration versus time curve. F: bioavailability after ip administration.

RESULTS

Toxicokinetic Analysis

Although vancomycin is administered iv in humans, most of the published vancomycin studies in animal models are done through ip injection. We compared the efficiency of the iv and ip routes in delivering vancomycin into systemic circulation after administration of one and seven doses of vancomycin at 50, 200, and 400 mg/kg. Analysis of the TK parameters (Table 1) revealed that peak vancomycin concentrations (C_{max}) after iv administration were consistently higher than those measured after ip administration. The largest difference in C_{max} value (3.4-fold) between iv and ip administration was observed after seven doses of vancomycin at 200 mg/kg (C_{max} of 489 and 145 µg/ml, respectively). In addition, for the 200 and 400 mg/kg doses, the C_{max} was reached (T_{max}) more rapidly after iv administration than after ip administration (0.083 vs. 0.5 h). At the 200 mg/kg dose level, the $AUC^{0\rightarrow\infty}$ after iv dose administration is larger than the $AUC^{0\rightarrow\infty}$ after ip administration. In contrast, at the 400 mg/kg dose level, the $AUC^{0\rightarrow\infty}$ after ip administration was higher than that after iv administration (2.4- and 1.6-fold greater after one and seven injections, respectively). These results suggested that the route of administration has a major impact on the TK parameters.

Clinical Chemistry

Blood samples were collected on day 3 and day 8, after two and seven vancomycin injections, respectively, to evaluate 17

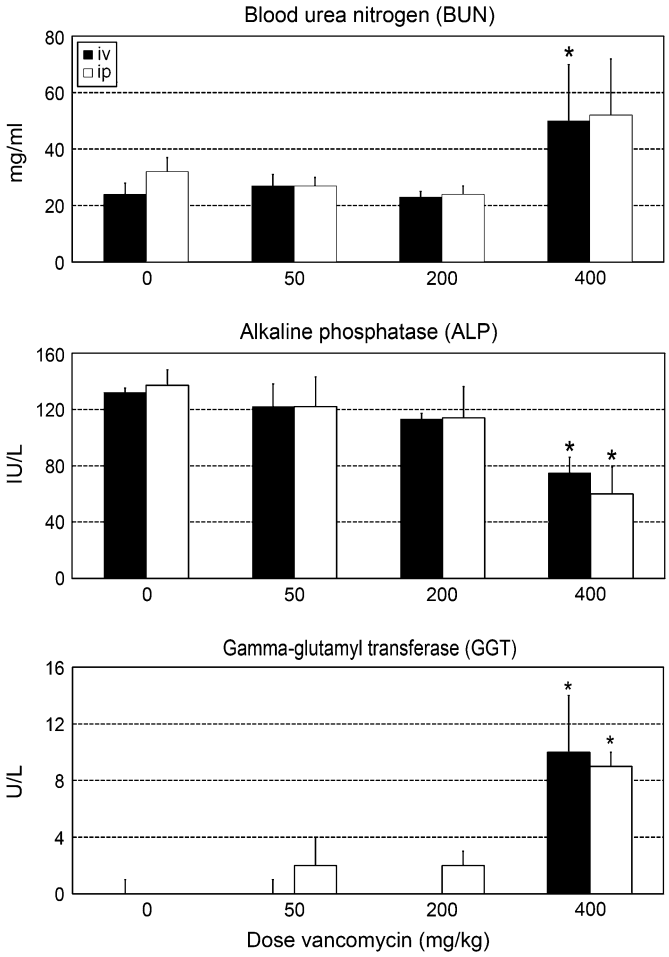

FIG. 1. Serum clinical chemistry analysis. Groups of six BALB/c mice were administered daily doses (50, 200, and 400 mg/kg) of vancomycin either iv or ip for 7 consecutive days. Control mice were given a similar volume of saline solution (0). On day 8, blood from three randomly selected mice per group was collected, and serum levels of BUN, ALP, and GGT were assessed. Results of each individual measurement are represented as well as the mean value. *$p < 0.05$, as determined by one-way ANOVA with Dunnett's multiple comparison post-test.

chemistry parameters. Most of the variations occurred only on day 8; the most important significant ($p < 0.05$) changes are presented in Figure 1. These changes occurred mainly after administration of high-dose vancomycin. There was a statistically significant increase in BUN after iv administration only, although there was a trend toward increased BUN values after ip treatment as well. Other parameters, such as alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT), were also affected by high-dose vancomycin treatment. We also observed a slight, but statistically significant ($p < 0.05$), increase in globulin (3 ± 0.2 and 3 ± 1 g/dl compared to 2 ± 0.1 and 2 ± 0.3 g/dl after iv and ip administration, respectively) and a decrease in total protein (6 ± 0.2 and 6 ± 0.6 g/dl compared to 5 ± 0.2 and 5 ± 0.4 g/dl after iv and ip administration, respectively; data not shown). CRE levels, however, were not significantly affected by vancomycin administration (data not shown), probably because such changes only take place when 30–50% of tissue damage has occurred (Duarte *et al.*, 1993).

These results suggest that high-dose vancomycin was responsible for almost all detectable signs of toxicity and that the drug's primary effects are on kidney and liver function.

Renal Histopathology

Microscopic evaluations were performed on the following tissues from BALB/c mice given saline and 400 mg/kg vancomycin: right kidney, liver caudal lobe, gross lesions (including tissue masses and abnormal regional lymph nodes), lungs with bronchi, injection site, spleen, stomach, and duodenum, small intestine (ileum, cecum, and colon), urinary bladder, mesenteric lymph nodes, heart, and adrenal glands. Kidney and liver were also evaluated in tissues from the medium (200 mg/kg) dose groups. Significant alterations were observed only in kidney tissues.

Representative sections of the proximal tubules in control and high-dose treated animals are shown in Figure 2. The primary finding in kidneys of mice given vancomycin at 400 mg/kg either iv or ip was proximal tubular necrosis (A), characterized by necrosis of proximal tubular cells, protein droplets and cellular debris within proximal tubular lumens, and stretching of tubular cells caused by loss of epithelial cells. We also observed regeneration of cuboidal cells of the proximal tubules (B). Inflammatory cell infiltration into the affected renal tissue was minimal.

Transcriptional Response in the Kidney

Overview of the host transcriptional response after vancomycin administration. The 9502 probes from the probe list (see Materials and Methods) were submitted to hierarchical clustering analysis (Pearson correlation and average linkage clustering) using the average intensity value of the six samples in each experimental group (Fig. 3). This analysis revealed two distinct nodes: the iv-400, iv-200, and ip-400 groups fell within the first node, and the remaining treatment groups (iv-0, iv-50, ip-0, ip-50, and ip-200) fell within the second major node. The finding that samples from the iv-200 group clustered with the high-dose treatment samples was surprising because, unlike the iv-400 and ip-400 mice, iv-200 animals did not exhibit signs of toxicity in clinical chemistry and histopathology assessments. This finding suggests that an iv dose of 200 mg/kg might induce toxicity identifiable with gene expression profiles even though it is not clinically detectable. It is interesting to observe that the host transcriptional response can differ depending on the route of administration.

To investigate the difference between the ip and iv routes of administration in greater detail, we performed one-way ANOVA analyses (parametric test assuming equal variances, Benjamini and Hochberg as multiple testing corrections with a False Discovery Rate = 0.01, and Tukey *post hoc* test) to

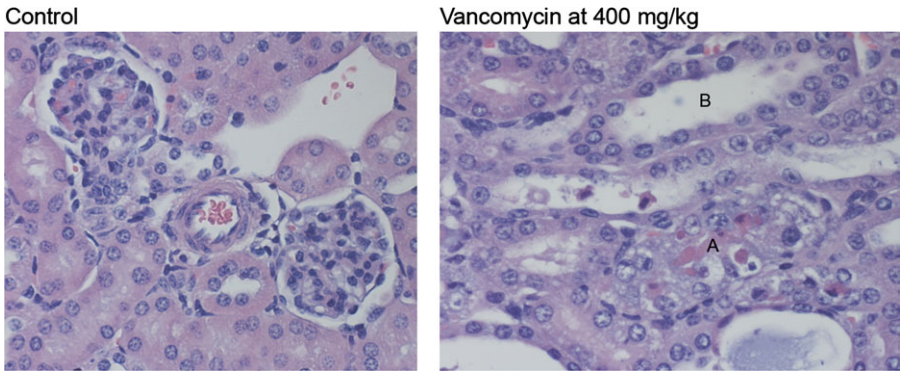

FIG. 2. Histopathological evidence of vancomycin nephrotoxicity. BALB/c mice were treated as described in Figure 1. Approximately, 24 h after the seventh vancomycin infection, the right kidney of each mouse was collected in 10% neutral buffered formalin, embedded into paraffin, and cut approximately 5 μm thick. Representative H&E-stained sections of control and high-dose treated animals (vancomycin at 400 mg/kg) are shown. The letters A and B point to a necrotic tubule and a regenerative proximal tubule, respectively.

identify how many probes were significantly differentially expressed between each dose group (50, 200, and 400 mg/kg) and the control (saline). Two independent analyses were performed, one using the samples from the iv route and one using the samples from the ip route. The results, presented in Table 2, confirmed that the two routes led to different host transcriptional profiles. After administration of low or medium doses, a larger number of probes (7 and 16 times more for 50 and 200 mg/kg, respectively) presented a significantly different intensity value compared to control animals after iv administration compared to ip (119 vs. 17 and 707 vs. 43 probes, respectively). The changes in gene expression were linked to the systemic exposure of vancomycin in mice (Table 1): on day 7, the drug levels in plasma were higher after iv administration at low and medium doses. For administration of a high dose of vancomycin, delivery via the ip route induced slightly more changes (1.5 times more; 6,763 vs. 4,347 probes) compared to the iv route. This finding is consistent with the pharmacokinetic evaluation in Table 1, which shows that the $AUC^{0\rightarrow\infty}$ was higher after ip administration of 400 mg/kg vancomycin than the $AUC^{0\rightarrow\infty}$ after iv administration. These results suggest that the differences in gene expression profile are likely to reflect a difference in drug exposure.

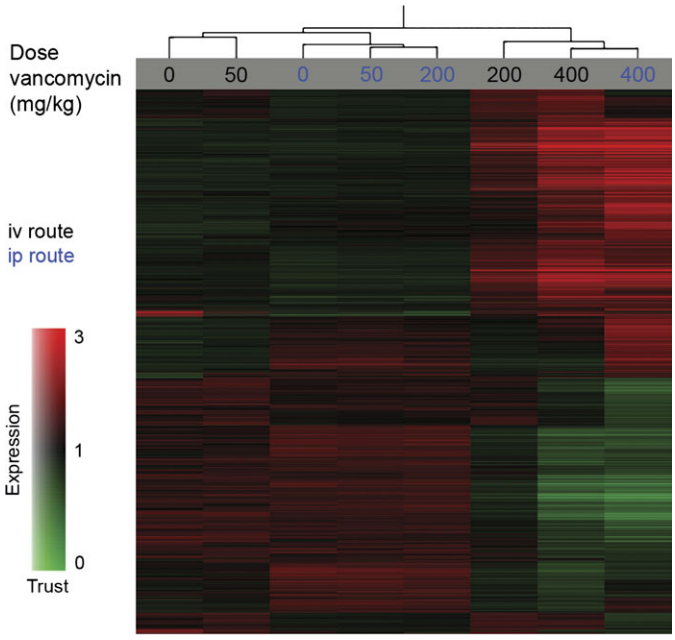

FIG. 3. Hierarchical clustering of vancomycin-treated kidney samples. The average ($n = 6$) normalized intensity value of the 9502 probes from the probe list across all the experimental conditions was represented in green (down-regulation), red (upregulation), and black (insignificant changes) on the y-axis. Experimental conditions (iv and ip administration of saline [0 mg/kg] and 50, 200, and 400 mg/kg vancomycin) are plotted on the x-axis.

Predominant changes in the kidney after high-dose vancomycin administration, independent of the route of administration. To analyze the most predominant gene expression changes associated with vancomycin treatment, we selected, from the probe list the elements presenting a greater than twofold change in intensity between iv-400 and iv-0 and between ip-400 and ip-0. This analysis identified 1537 probes composed of 53% (818/1537) downregulated and 47% (719/1537) upregulated elements. To uncover the biological themes associated with these 1537 probes, we extracted the

TABLE 2
One-Way ANOVA Analysis Indicating Number of Probes Changing after Each Experimental Condition compared with their Respective Controls (Evaluation Performed on Day 8)

	Route		
Dose vancomycin (mg/kg)	iv	ip	Common probes
50	119	17	0
200	707	43	8
400	4347	6,763	3746

TABLE 3
Most Enriched GO Term Categories among the 1537 Probes Whose Intensity Changed by at least Twofold after Administration of High-Dose Vancomycin compared with Saline, Independently of the Delivery Route (Evaluation Performed on Day 8)

Annotation cluster	Enrichment score	Number of genes	EASE score[a]
818 downregulated probes			
Cluster #1	16.93	52	≤ 1.1E−16
Amino acid and derivative metabolism, nitrogen compound metabolism, amine metabolism, amino acid metabolism			
Cluster #2	16.31	22	≤ 3.2E−16
Amino acid catabolism, amine catabolism, nitrogen compound catabolism			
Cluster #3	8.19	164	≤ 1.5E−8
Transport, establishment of localization, localization			
Cluster #4	8.09	39	≤ 5.4E−6
Organelle inner membrane, mitochondrial inner membrane, mitochondrial membrane, mitochondrial envelope, organelle envelope, envelope, organelle membrane			
Cluster #5	6.24	14	≤ 4.7E−7
Amino acid biosynthesis, amine biosynthesis, nitrogen compound biosynthesis			
Cluster #6	4.71	24	≤ 3.8E−4
Glucose metabolism, hexose metabolism, monosaccharide metabolism, cellular carbohydrate metabolism			
Cluster #7	4.03	10	≤ 5.3E−3
Pyruvate metabolism, gluconeogenesis, hexose biosynthesis, alcohol bioxynthesis, monosaccharide biosynthesis, carbohydrate biosynthesis			
719 upregulated probes			
Cluster #1	16.87	76	≤ 4.6E−17
Response to biotic stimulus, immune response, defense response			
Cluster #2	4.03	12	≤ 5.3E−4
Wound healing, blood coagulation, coagulation, hemostatis, regulation of body fluids			

[a]Modified Fisher Exact *p* value.

gene ontology (GO) terms with EASE, a program for calculating the statistical enrichment of GO annotations (Hosack *et al*., 2003). GO is a hierarchical, structured vocabulary that describes gene products in terms of their biological process, cellular component, and molecular function (Ashburner *et al*., 2000). Using the Affymetrix mouse 430_2 as a background list and high classification stringency, we identified several clusters and selected the ones presenting an enrichment score greater than 4 (Table 3).

Among the 818 downregulated probes (Supplementary Table 1), seven clusters presented an enrichment score > 4 (Table 3). The predominant changes affected genes involved in metabolism/catabolism of amine, amino acid, and nitrogen compounds. Among the downregulated genes, a large number of transport-related genes (*Slc14a2*, *Slc38a2*, *Slc39a14*, *Slc39a6*, *Slc41a2*, *Slc44a1*, *Slc44a2*, *Slc44a3*, *Slc7a1*, *Slco3a1*, *Aqp11*, and *Kcnk1*) and several mitochondrial genes (*Acad9*, *Acad10*, *Acad11*, *Acadsb*, *Car5a*, *Bcat2*, *Acaa2*, *Shmt2*, and *Sod2*) were significantly enriched. These results suggest that vancomycin treatment altered the overall metabolic activity of the kidney. More specifically, it affected the function (reabsorption) of the proximal tubule cells and of their energy-producing mitochondria.

Among the 719 upregulated probes (Supplementary Table 2), two clusters presented an enrichment score > 4 (Table 3). The most enriched cluster (cluster #1, enrichment score 16.87) consisted of genes involved in immune response/defense, mainly several histcompatibility 2 genes (H2), several components of the complement pathway (*C4b*, *C3*, *C1qa*, *Cfi*, *C1r*, *C1s*, *Cfh*, and *C1qb*) and the proteasome (*Psme1*, *Psme2*, *Psmb9*, and *Psmb8*), as well as chemokines (*Cxcl16*) and interferon-related genes (*Irf1*, *Irf8*, *Ifitm1*, *If203*, *Ifi205*, *Ifi27*, *Ifi35*, *Ifitm3*, *Mpa2l*, and *Ifit2*). These results suggest that inflammation and complement activation might be involved in vancomycin-induced pathogenesis, as they are in renal ischemia/reperfusion (I/R) (Thurman, 2007). Because adhesion molecules also participate in the kidney injury after I/R (Thurman, 2007), we evaluated the expression profile of intracellular adhesion molecule (*Icam1*), E-selectin (*Sele*), and P-selectin (*Selp*) after vancomycin treatment. Only *Icam1* was present in the probe list, and its expression was upregulated after iv and ip administration of vancomycin at 400 mg/kg (≥ 8-fold compared to iv-0 or ip-0) and after iv administration of 200 mg/kg (> 2-fold compared to iv-0; Supplementary Table 2).

Using relative real-time RT-PCR, we next verified the expression of two key components of the complement pathway (*C3* and *C4b*), as well as expression of the chemokine *Cxcl1*, a known early biomarker of ischemic acute renal failure (Molls *et al*., 2006). Verification was performed on three randomly selected samples from each experimental group. The results, presented in Table 4, confirmed the microarray data showing higher expression of these genes after both iv and ip administration of vancomycin at 400 mg/kg. None of the samples from the low dose iv or ip treated animals expressed

TABLE 4
Relative Real-time RT-PCR Measurements[a] compared with Microarray Data (Evaluation Performed on Day 8)

	Vancomycin administration route (dose in mg/kg)					
Gene symbol	iv-50	iv-200	iv-400	ip-50	ip-200	ip-400
A. Complement pathway and inflammation						
C3	1 ± 0 (0.8 ± 0.3)[b]	6 ± 4 (3.5 ± 2.2)	16 ± 7 (9.2 ± 3.4)	1 ± 0 (0.7 ± 0.2)	1 ± 0 (0.7 ± 0.2)	29 ± 11 (12 ± 3)
C4b	1 ± 0 (0.8 ± 0.1)	10 ± 9 (6 ± 6)	20 ± 4 (12 ± 4)	1 ± 0 (1 ± 0.2)	1 ± 0 (1 ± 0.2)	26 ± 4 (15 ± 5)
Cxcl1	0 ± 0 (N/A)	2 ± 2 (N/A)	10 ± 6 (N/A)	1 ± 0 (N/A)	1 ± 0 (N/A)	190 ± 94 (N/A)
B. Potential biomarkers						
Spp1	1 ± 0 (0.8 ± 0.1)	14 ± 12 (1.5 ± 0.4)	31 ± 16 (1.6 ± 0.2)	1 ± 0 (1 ± 0.1)	1 ± 0 (1 ± 0.1)	32 ± 3 (1.6 ± 0.1)
Havcr1	1 ± 0 (0.4 ± 0)	11 ± 10 (3.8 ± 0.5)	22 ± 13 (9 ± 3.2)	1 ± 1 (0.7 ± 0)	3 ± 1 (1.1 ± 0.1)	23 ± 5 (7.8 ± 1.8)
Rtn4	0.8 ± 0 (0.8 ± 0.1)	1.8 ± 0.6 (1.3 ± 0.2)	3.4 ± 1 (2.3 ± 0.4)	1.2 ± 0.2 (0.9 ± 0.1)	1.3 ± 0.1 (0.9 ± 0)	5.8 ± 2.5 (2.8 ± 0.8)

[a]Fold expression change relative to the appropriate control samples (iv-0 or ip-0). Results are the average ± SD of the measurements obtained from three animals per experimental group.
[b]Number in parentheses are the average normalized intensity value obtained by microarray ± SD ($n = 6$). N/A: not available.

C3, *C4b*, or *Cxcl1,* and only iv administration of vancomycin at 200 mg/kg upregulated expression of these genes.

Specific changes induced by vancomycin treatment. We first analyzed expression of genes involved in the oxidative stress response because it was recently suggested to be a mechanism underlying the pathogenesis of vancomycin-induced nephrotoxicity (Nishino *et al*., 2003; Oktem *et al*., 2005). In our probe list, we identified 12 genes that were involved in reactive oxygen species (ROS) metabolism or possessed antioxidant properties. The average ($n = 6$) expression of these genes differed by at least twofold in high-dose vancomycin-treated mice compared to control animals (Table 5). The abundance of most of these transcripts decreased after high-dose vancomycin treatment, especially those encoding proteins that belong to the three main cellular antioxidant groups: superoxide dismutase (*Sod2*, *Sod3*), catalase (*Cat*), and glutathione peroxidase (*Gpx6*, *Gstk1*). In addition, we observed upregulation of heme oxygenase-1 (*Hmox1*; Supplementary Table 2 and Fig. 4), which is regarded as a sensitive and reliable indicator of cellular oxidative stress (Abraham *et al*., 2007). Taken together, these results suggested that vancomycin was inducing an imbalance of the oxidative stress response globally in favor of decreased ROS elimination.

The expression levels of several genes implicated in the mechanism(s) of renal toxicity (Amin *et al*., 2004) in animals treated with high-dose vancomycin were compared with levels in animals treated with saline. For this purpose, we selected, from the probe list, the probes whose expression was upregulated by at least twofold in iv-400 versus iv-0 or ip-400 versus ip-0 (data not shown). The resulting list of 2758 probes was chosen to evaluate the vancomycin-induced genomic changes independent of the route of administration because of the observation (see Fig. 3) that the iv and ip routes do not induce similar gene expression profiles. Our analysis revealed that vancomycin treatment modulated the expression level of several of these previously reported genes (Amin *et al*., 2004), including, but not restricted to, hepatitis A virus cellular receptor 1 (*Havcr1*; human synonym: KIM-1), *Hmox1*, clusterin (*Clu*), osteopontin/secreted phosphoprotein (*Spp1*), and some kallikrein genes (*Klk5*, *Klk6*, *Klk9*, and *Klk24*). Figure 4 compares the expression of these markers in iv-400 and ip-400 samples to expression in their respective control groups. In most instances, the direction of the change (induction or repression) was similar to the one reported after cisplatin or gentamicin treatment (Amin *et al*., 2004) (data not shown). In three cases, however (*Gc*, *Idh1*, and *Ngfb*), vancomycin treatment induced changes in the opposite direction from treatment with cisplatin or gentamicin. The results suggest that these three genes might be vancomycin-specific in the transcriptional changes related to kidney damage.

We next undertook to identify new potential biomarkers of toxicity that exhibited a gradual dose response to vancomycin. These genes were selected from the probe list based on their constant increase or decrease in level of expression (at least 1.5-fold) between iv-0 and iv-50, iv-50 and iv-200, and iv-200 and iv-400. The four genes thus identified were small proline-rich protein 1A (*Sprr1a*), AI606844, reticulon 4 (*Rtn4*), and ADP-ribosylation factor-like 4D (*Arl4d*) (Supplementary Table 2 and unshown data). The relevance of these particular genes in kidney injury will require further investigation.

The expression of some of the previously described (*Spp1*, *Havcr1*) and herein identified potential (*Rtn4*) markers of kidney injury was verified by relative real-time RT-PCR on the tissue from three randomly selected animals from each experimental group (Table 4). This analysis confirmed the up- and downregulation of all the selected genes.

DISCUSSION

Vancomycin nephrotoxicity has been reported in humans in 7–17% of cases of MRSA infection treated with this antibiotic

TABLE 5
Normalized Intensity Value of the 12 Oxidative Stress Genes Modified by at Least Twofold after High-Dose Vancomycin compared with Control Treatment (Evaluation Performed at Day 8)

Gene symbol	Affymetrix ID	Genbank	iv-0[a]	iv-400[b]	ip-0[a]	ip-400[b]
Downregulated genes						
Aass1	423523_at	BF687395	1.0 ± 0.3	0.4 ± 0.2	1.3 ± 0.1	0.3 ± 0.2
Cat	1416430_at	NM_009804	1.1 ± 0.2	0.4 ± 0.2	1.2 ± 0.1	0.4 ± 0.1
	1416429_a_at	NM_009804	1.0 ± 0.1	0.4 ± 0.1	1.3 ± 0.2	0.5 ± 0.2
Ccs	1448615_at	AF173379	1.2 ± 0.3	0.6 ± 0.2	1.0 ± 0.1	0.4 ± 0.1
Fmo2	1422905_s_at	NM_018881	1.2 ± 0.3	0.4 ± 0.2	1.5 ± 0.5	0.3 ± 0.2
	1453435_a_at	AK009753	1.0 ± 0.2	0.3 ± 0.1	1.2 ± 0.2	0.3 ± 0.1
	1422904_at	NM_018881	1.2 ± 0.4	0.5 ± 0.2	1.4 ± 0.7	0.3 ± 0.2
	1435459_at	BM936480	0.9 ± 0.2	0.3 ± 0.1	1.1 ± 0.1	0.3 ± 0.1
Gpx6	1452135_at	AV001252	1.3 ± 0.4	0.2 ± 0.2	1.0 ± 0.3	0.1 ± 0.0
Gstk1	1452823_at	AK002661	1.0 ± 0.2	0.3 ± 0.1	1.1 ± 0.1	0.2 ± 0.1
Nox4	1451827_a_at	BC021378	1.0 ± 0.1	0.3 ± 0.1	1.2 ± 0.2	0.2 ± 0.1
	1419161_a_at	AB041034	1.0 ± 0.3	0.4 ± 0.2	1.3 ± 0.3	0.3 ± 0.2
Scd1	1415965_at	NM_009127	1.4 ± 0.4	0.2 ± 0.1	1.5 ± 0.4	0.3 ± 0.2
	1415964_at	NM_009127	1.2 ± 0.2	0.2 ± 0.1	1.1 ± 0.2	0.1 ± 0.1
Sod2	1444531_at	AI847438	1.0 ± 0.1	0.3 ± 0.1	1.3 ± 0.2	0.5 ± 0.1
	1454976_at	BQ174944	1.1 ± 0.1	0.4 ± 0.1	1.1 ± 0.1	0.4 ± 0.1
Sod3	1417633_at	NM_011435	1.2 ± 0.4	0.6 ± 0.3	1.1 ± 0.1	0.4 ± 0.1
Upregulated genes						
2310016C16Rik	1424099_at	BC019664	0.9 ± 0.2	1.9 ± 0.3	0.9 ± 0.1	2.0 ± 0.3
Vim	1438118_x_at	AV147875	0.7 ± 0.2	3.6 ± 0.8	0.9 ± 0.1	5.7 ± 1.3
	1450641_at	M24849	0.8 ± 0.3	4.6 ± 0.5	0.7 ± 0.1	5.4 ± 1.5
	1456292_a_at	AV147875	0.7 ± 0.3	4.6 ± 1.6	0.8 ± 0.2	7.0 ± 1.6

[a]iv-0 and ip-0 represent iv and ip treatment with saline solution.
[b]iv-400 and ip-400 represent iv and ip treatment with vancomycin at 400 mg/kg. Numbers presented in the table are the average normalized intensity value ± SD ($n = 6$).

(Downs *et al*., 1989; Eng *et al*., 1989; Mellor *et al*., 1985), although the mechanisms of toxicity are unclear. We used a genomic approach to obtain a global view of the transcriptional response in the kidneys of vancomycin-treated BALB/c mice and gain insights into the molecular mechanisms of vancomycin-induced kidney injury. Our study indicated involvement of oxidative stress and mitochondrial damage in vancomycin-induced kidney injury. Most importantly, our study revealed, for the first time, a potential contribution of complement pathway and inflammation in the vancomycin-induced renal toxicity. Expression of several previously identified gene-based markers of toxicity was dramatically affected by administration of high-dose vancomycin. Other genes presented a dose response to vancomycin and might be useful as potential biomarkers.

Two routes of administration were used: iv and ip. To our knowledge, our work is the first detailed comparison, at the genomic level, of responses after iv administration of vancomycin (clinical use) and ip administration (the most common route in animal studies). The doses selected in this study (50, 200, and 400 mg/kg) were extrapolated from the human dosage to mice; the recommended clinical dose of 30 mg/kg (~2 g/day for an average 70 kg person) in humans would correspond to a dose of 370 mg/kg in mice according to surface area conversion. After iv administration in human, C_{max} range from 20 to 50 μg/ml (Palmer-Toy, 2000) which also correspond to the therapeutic range (Rybak, 2006). Patients with signs of nephrotoxicity after vancomycin administration however had C_{max} values in the above range (Downs *et al*., 1989; Eng *et al*., 1989; Mellor *et al*., 1985), supporting the lack of evidence between pharmacodynamic parameters and overall patient outcome (Lee *et al*., 2007; Rybak, 2006).

Global gene expression profiling of the kidney of vancomycin-treated mice revealed that samples clustered according to severity of pathology. Samples from animals treated iv and ip at 400 mg/kg displayed similar gene expression profiles that clustered tightly together, separately from those of control and low dose groups. Clustering of the samples from animals treated at the high dose also correlated with the severe necrosis of the proximal tubules and significant alterations of serum chemistry parameters such as BUN. Samples from animals treated iv at 200 mg/kg clustered with samples from the high-dose treatment groups, although the animals exhibited no clinical signs of toxicity, but samples from the ip-200 dose group clustered with those from the control groups. This finding suggests that gene expression profiles represent an earlier marker for kidney injury than histopathology and clinical chemistry, consistent with knowledge that changes in expression of mRNA in the injured kidney cells are among the

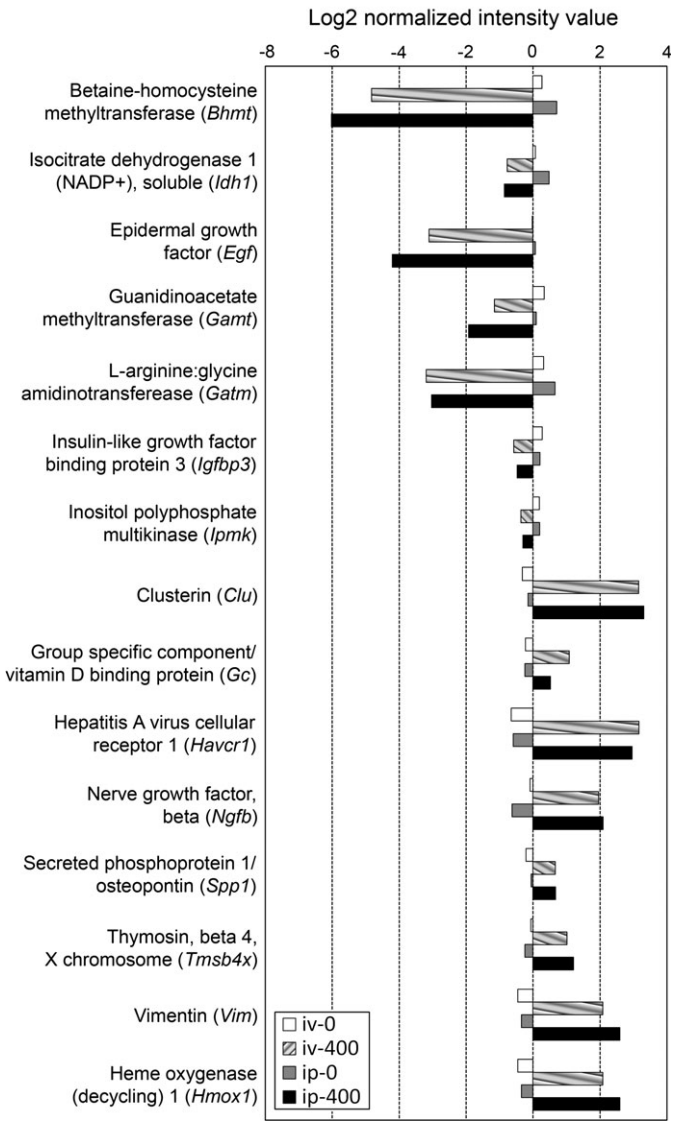

FIG. 4. Expression level of the gene-based markers of nephrotoxicity. Graph represents the expression level of 15 of the previously identified gene markers of nephrotoxicity (Amin *et al.*, 2004) after administration of high-dose vancomycin via the iv (iv-400) and ip (ip-400) routes or administration of saline (iv-0 and ip-0). Expression levels are presented as the Log_2 of the average ($n = 6$) normalized intensity value.

earliest events accompanying renal injury (Amin *et al.*, 2004). In addition, iv and ip administration of 200 mg/kg doses had different effects on the host gene transcription profiles. Because the $C_{\max}$ and the $\text{AUC}^{0\rightarrow\infty}$ were 3.4- and 1.7-fold higher after iv administration of vancomycin at 200 mg/kg than after ip administration of that dose, our hypothesis is that the $C_{\max}$ or $\text{AUC}^{0\rightarrow\infty}$ may need to reach a certain threshold before the gene expression response is affected and toxic effects are observed. This threshold would be reached after administration of iv-400, ip-400, and iv-200, but not ip-200, and this pattern indicates that the different gene expression profiles observed after iv and ip administration are likely to be associated with different levels of drug exposure. Our work thus indicates the importance of considering TK data in interpreting the results of genomics analysis.

As the major site for renal reabsorption, the proximal tubule epithelium is rich in transporters and transport mechanisms that contribute to drug clearance (Lee *et al.*, 2004). After iv and ip administration of high-dose vancomycin, the expression levels of several transport-related genes (several *Slc*, *Aqp11*, and *Kcnk1*) and genes involved in mitochondrial membrane and envelope (*Acad9*, *Acad10*, *Acad11*, *Acadsb*, *Car5a*, *Bcat2*, *Acaa2*, *Shmt2*, and *Sod2*) were significantly downregulated. These results suggested that vancomycin altered the energy-dependent renal reabsorption function of the proximal tubule cells, consistent with the observation that vancomycin might alter mitochondrial function (King *et al.*, 2004) and the fact that the basic mechanism of vancomycin nephrotoxicity depends on an energy-dependent transport process from the blood to the tubular cells across the basolateral membrane (Fanos *et al.*, 2001). Pronounced downregulation of the expression of several transporters during tubular necrosis was observed in rats after treatment with various nephrotoxic drugs (Thukral *et al.*, 2005). Like the findings of the Thukral *et al.* study, the reduction observed in our study may have resulted from loss of tubular epithelial cells during necrosis, from generalized downregulation of transport mechanism, or from a combination of both. Damage to the energy-producing mitochondria was also indirectly suggested by increased expression of genes encoding lysosomal proteins such as cathepsins (*Ctss*, *Ctsd*, *Ctsc*, and *Ctsl*; Supplementary Table 2) that degrade the worn out organelles such as mitochondria.

Mitochondria represent a major site of ROS production. Oxidative stress might be involved in the pathogenesis of vancomycin-induced nephrotoxicity (Nishino *et al.*, 2002, 2003; Oktem *et al.*, 2005), observations supported by the potential protective effects of several antioxidants (Celik *et al.*, 2005; Cetin *et al.*, 2007). Our microarray analysis revealed that high-dose vancomycin upregulated the expression level of *Hmox1*, an indicator of cellular oxidative stress (Abraham *et al.*, 2007), and downregulated the expression level of *Cat*, *Gpx6*, *Gstk1*, *Sod2*, and *Sod3,* which encode some of the main cellular antioxidants (Scandalios, 2005). Our observations correlated with the reduced antioxidant activity (super oxide dismutase, catalasse) in the kidney of vancomycin-treated rats (Cetin *et al.*, 2007; Oktem *et al.*, 2005). Depletion of genes protecting from oxidative stress was also observed in ochratoxin A–treated rats (Marin-Kuan *et al.*, 2006). All together, our results suggest that, by reducing antioxidant activity, vancomycin might increase production of ROS. Oxidative stress dysfunction might be responsible, at least partly, for the development of the observed tubular necrosis. Even though apoptosis and necrosis are often preceded by mitochondrial dysfunction (Lemasters *et al.*, 1999), we cannot rule out the possibility that the oxidative stress response might represent a secondary by-product of necrosis.

In addition to necrosis, signs of tissue repair were also detected in vancomycin-treated animals. First, we observed regeneration of cuboidal cells. Second, there was strong upregulation of nine transcripts encoding procollagen genes, including *Col4a1* and *Col4a2*; collagen IV promote repair of renal cell physiological functions after toxicant injury (Nony *et al*., 2001). Tissue repair was also suggested by upregulation of cell cycle genes (*Cdk6*, *Cdkn1a*, and *Cdkn2d*, see Supplementary Table 2), and of *Vim* (Table 5). *Vim* encodes vimentin, an intermediate filament protein that might restore transport activity in injured kidneys (Runembert *et al*., 2004). These observations are consistent with the proliferation observed following vancomycin treatment in renal proximal tubule epithelial cells (King *et al*., 2004).

Analysis of the overall kidney gene expression response for significantly enriched GO pathways revealed that several components of the complement pathway, as well as chemokines, were strongly upregulated after iv and ip administration of high-dose vancomycin. Renal I/R is a common cause of acute renal failure, recently renamed acute kidney injury (Vaidya *et al*., 2008); it triggers a robust inflammatory response and activates the complement system, both of which subsequently contribute to the resulting tissue injury (Thurman, 2007). The complement pathway is not only associated with damages after renal I/R, it is also an important mediator of local tissue injury in the kidney (Sheerin *et al*., 2008). We thus speculated that vancomycin-induced kidney injury may also be mediated by complement activation and development of an inflammatory response. Using relative real-time RT-PCR, we confirmed the increased expression of some of the complement genes (*C3* and *C4b*); we also identified increased expression of *Cxcl1*, an early biomarker of ischemic acute renal failure (Molls *et al*., 2006). *Cxcl1* is produced in a complement-dependent fashion, suggesting that complement activation mediates tissue inflammation through the generation of such a factor (Thurman *et al*., 2007). This result might contrast with the minimal inflammatory cell infiltration observed. However, because changes in expression of mRNA in the injured kidney cells are among the earliest events accompanying renal injury (Amin *et al*., 2004), microscopic tissue changes might only be detectable at later time points.

This study is the first to report that vancomycin-induced gene expression changes are enriched in the inflammation and complement pathway response, suggesting a link between vancomycin-induced nephrotoxicity and complement activation. Further work will be required to elucidate the role of the complement pathway in vancomycin-induced nephrotoxicity; it would be useful, for example, to analyze the deposition of C3 along the tubular membrane (Thurman, 2007) or the vancomycin response of complement-deficient mice using an approach similar to that of Thurman *et al.* (2003).

Several genes have been described as potential signatures for drug-induced kidney injury, especially KIM-1/*Havcr1* (Han *et al*., 2002; Ichimura *et al*., 1998). Other genes, including *Spp1* and *Clu*, are upregulated in the kidney of rats (Amin *et al*., 2004; Kharasch *et al*., 2006; Thompson *et al*., 2004) and primates (Davis *et al*., 2004), and wider use of these genes as markers has been suggested.

Administration of high-dose vancomycin upregulated the expression of *Havcr1*, *Spp1*, and *Clu*, substantiating the use of these markers as an indicator of renal injury. The induction of mRNA transcript for *Havcr1* and *Spp1* was confirmed by relative real-time RT-PCR. Several other potential biomarkers include L-arginine-glycine amidinotransferase (*Gatm*) and guanidinoacetate methyltransferase (*Gamt*) (Amin *et al*., 2004; Kharasch *et al*., 2006; Thompson *et al*., 2004), both key players in the CRE biosynthesis pathway. Although we did not observe changes in serum levels of CRE, vancomycin treatment downregulated the expression of these two genes (*Gatm* and *Gamt*), suggesting dysregulation of the CRE pathway.

Finally, our microarray analysis identified four genes whose expression profiles changed gradually as iv dose levels of vancomycin increased: *Sprr1a*, AI606844, *Rtn4*, and *Arl4d*. The relevance of these genes in kidney injury is unknown. However, markers do not always have known functional relevance; in the Fielden study for example, 37% (13/35) of the genes predicting the future onset of drug-induced renal tubular toxicity were expressed sequence tags with no similarities to other known genes (Fielden *et al*., 2005).

Future studies will be required to confirm the change in expression level of these potential gene markers and to determine whether these changes can be identified after brief exposure to vancomycin, before the onset of clinical signs of toxicity. Because vancomycin is not administered to healthy individuals, there is a need to evaluate whether these markers could be identifiable in the context of an infection such as MRSA. If so, they could make a major contribution to the application of toxicogenomics to clinical medicine. Our work represents the first step toward this goal and provides information on novel mechanisms of vancomycin-induced kidney injury that might be critical in devising ways to prevent toxicity in patients.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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