

Overview of the Application of Transcription Profiling Using Selected Nephrotoxicants for Toxicology Assessment

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Microarrays allow for the simultaneous measurement of changes in the levels of thousands of messenger RNAs within a single experiment. As such, the potential for the application of transcription profiling to preclinical safety assessment and mechanism-based risk assessment is profound. However, several practical and technical challenges remain. Among these are nomenclature issues, platform-specific data formats, and the lack of uniform analysis methods and tools. Experiments were designed to address biological, technical, and methodological variability, to evaluate different approaches to data analysis, and to understand the application of the technology to other profiling methodologies and to mechanism-based risk assessment. These goals were addressed using experimental information derived from analysis of the biological response to three mechanistically distinct nephrotoxins: cisplatin, gentamicin, and puromycin aminonucleoside. In spite of the technical challenges, the transcription profiling data yielded mechanistically and topographically valuable information. The analyses detailed in the articles from the Nephrotoxicity Working Group of the International Life Sciences Institute Health and Environmental Sciences Institute suggest at least equal sensitivity of microarray technology compared to traditional end points. Additionally, microarray analysis of these prototypical nephrotoxicants provided an opportunity for the development of candidate bridging biomarkers of nephrotoxicity. The potential future extension of these applications for risk assessment is also discussed. **Key words:** cisplatin, gentamicin, nephrotoxicity, puromycin, risk assessment. *Environ Health Perspect* 112:460–464 (2004). doi:10.1289/txg.6673 available via <http://dx.doi.org/> [Online 15 January 2004]

Renal damage can be difficult to assess clinically. The kidney has excess functional capacity, and in most cases a significant proportion of the kidney must be ablated before changes in kidney function are observed (Price 1992). Currently available biomarkers, such as blood urea nitrogen or serum creatinine, are not very sensitive (Loeb 1998) because although both represent direct measures of renal function, increases in the serum concentration of these markers are generally observed only after frank renal damage. Consequently, there has been an ongoing interest in developing new, early biomarkers of renal damage (Duan et al. 1999; Taylor et al. 1997). The lysosomal enzyme *N*-acetylglucosaminidase (NAG) and the brush border enzyme γ -glutamyl-transpeptidase (GGT) have also been used to assess renal toxicity [c.f. Gibey et al. (1981) and Scherberich and Mondorf (1983)]. When tubules are damaged, NAG and GGT are excreted into the lumen of the tubules and can be detected in the urine. However, a limiting factor in the use of these enzymes, particularly NAG, is the considerable intra- and inter-individual variation in urinary

enzyme activity (Naidu and Lee 1994). Consequently, 24-hour urine collection must be obtained to allow for diurnal variation of excretion and urine volume. Recently, urinary levels of specific isoforms of glutathione *S*-transferase have been proposed as topographically specific markers of renal damage (Kilty et al. 1998).

Integration of emerging transcription profiling technologies into traditional safety assessment evaluations offers the possibility to take new steps toward understanding mechanism of target organ toxicity and elucidating putative new biomarkers of exposure/safety. In 1999 the International Life Sciences Institute's (ILSI) Health and Environmental Sciences Institute (HESI) chartered a Committee to address this concept. Members of the HESI Genomics Committee formed a working group focused on nephrotoxicity. The objective of the experimental work performed by the Nephrotoxicity Working Group was to establish the transcriptional profiles of three mechanistically and topographically distinct nephrotoxins: cisplatin, gentamicin, and puromycin. The experiments were also

designed to address biological, technical, and methodological variability; to evaluate different approaches to data analysis; and to understand the application of the technology to other profiling methodologies and to mechanism-based risk assessment. The relationship between changes in renal gene expression induced by cisplatin, gentamicin, and puromycin with conventional toxicology end points, lesion topography and potential mediators of mechanism of toxicity was also evaluated and are also discussed elsewhere in this mini-monograph (Amin et al. 2004; Thompson et al. 2004).

Experimental Overview

Three nephrotoxicants were selected for study in male Sprague-Dawley rats, cisplatin, gentamicin, and puromycin (Amin et al. 2004). Cisplatin is an antineoplastic agent used in the treatment of a variety of solid tumors, although its use is limited because of severe renal toxicity. Cisplatin is metabolized to cytotoxic intermediates by S3 proximal tubular epithelial cells and induces severe tubular and mild glomerular toxicity (Dobyan et al. 1980). Gentamicin is an antibacterial agent of the aminoglycoside class. It mediates renal toxicity via inhibition of proximal tubular epithelial cell lysosomal function, producing phospholipidosis and tubular degeneration (Vera-Roman et al. 1975). Puromycin is an aminonucleoside antibiotic with activity

This article is part of the mini-monograph "Application of Genomics to Mechanism-Based Risk Assessment."

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The authors thank members of the HESI Steering Committee for their critical review of the manuscript and the experimental designs carried out by the nephrotoxicity working group.

The authors declare they have no competing financial interests.

Received 14 August 2003; accepted 8 January 2004.

against a broad range of organisms that cause necrosis of glomerular podocytes, resulting in a severe glomerulopathy (Grond et al. 1988). Severe proteinuria results from the loss of the glomerular barrier to protein filtration, and tubular injury occurs secondary to the formation of proteinaceous casts in the proximal tubules.

A preliminary, *in vivo* study was conducted in rats exposed to a single toxic dose of cisplatin to determine the technical parameters for tissue and RNA isolation and to direct the design of the overall project. The single time point (7 days) and dose level (5 mg/kg) served as a biological replicate for a subsequent *in vivo* study with cisplatin. After an evaluation of this preliminary work, three *in vivo* studies were conducted according to the design summarized below. The dose selection was intended to provide information relating to no- or minimal-effect exposures (low), mild effects (mid), and pronounced toxicity (high). In general, five male rats were necropsied per time point and dose level. Serum and urine were collected for routine analyses to confirm nephrotoxicity. Kidneys were snap frozen in liquid nitrogen for RNA isolation. In general, RNA was isolated in a single laboratory for each study and distributed to participating laboratories for molecular analysis. Sections of each kidney were collected in 10% buffered formalin for histologic analysis (hematoxylin and eosin staining). Additional samples were frozen for potential proteomic or metabolomic analyses. Microarray analyses were performed on oligonucleotide (Affymetrix; <http://www.affymetrix.com>) and cDNA-based platforms [PHASE-I (PHASE-1 Molecular Toxicology, Inc., Santa Fe, NM), NIEHS (National Institute of Environmental Health Sciences, Research Triangle Park, NC), and Incyte Corp. (Palo Alto, CA)] at several participant's home institutions (Table 1). The complete data set is currently being submitted to ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) and will be made available on

the HESI website (<http://hesi.ilsil.org/index.cfm?pubentityid=120>). Selected transcription profiling results were confirmed using reverse transcription–polymerase chain reaction, Western blot analysis, and/or immunohistochemistry. Administration of each of the three agents produced the anticipated pathologic outcomes as confirmed by histologic or ultrastructural examination and evaluation of clinical pathology parameters (data not shown). Principal component analyses of both transcription profiling (Amin et al. 2004) and metabolomic data (Naciff et al., unpublished results) confirmed distinct expression or metabolite patterns for each nephrotoxicant, with an apparent closer similarity between cisplatin and gentamicin. In general, transcriptional analyses yielded information that provided strong topographic specificity and mechanistic information. This conclusion was not obscured by the considerable intra- and cross-platform variation. A more detailed overview of experimental design and key learnings from the nephrotoxicity working group are detailed below, and in two articles in this mini-monograph (Amin et al. 2004; Thompson et al. 2004).

Analysis of Platform Variability

In this issue, Thompson et al. (2004) discuss some of the technical and practical issues associated with interpreting data from multiple microarray platforms using data generated from the cisplatin studies. Comparison of gene expression data from four microarray platforms of individual and pooled samples, from identically dosed rats, and from two separate *in vivo* experiments provided a unique opportunity to investigate and evaluate the performance of the transcription profiling technology compared with more traditional methods of assessing toxicity.

This experimental design allowed for the investigation of numerous sources of variability, including platform technology, scanner setting, RNA isolation methods,

animal variability, and different sites for *in vivo* study completion, RNA isolation, probe generation, and array hybridization. Of these, variability due to differences in platform technology was perhaps the most difficult to manage. Comparing single-channel with dual-channel data, particularly in light of issues and errors with nomenclature and the lack of availability of full sequence information for some platforms, was problematic. In this mini-monograph, Mattes et al. (2004) addressed this issue by developing an approach to uniformly “re-annotate” microarray elements using UniGene and LocusLink IDs. A key finding in this analysis was animal variability, which demonstrated the pitfalls associated with pooling biological samples. In cisplatin-induced toxicity, a high degree of inter-animal variability was determined by assessing histologic and serum chemical parameters. For example, within the high-dose, late time-point group, one of the animals was a “low responder,” and one was essentially a “nonresponder” relative to the other three responding animals in the group. In this example, the frequency of individual animal transcript changes was reduced in low and nonresponders and increased with more severe toxicity. As blood levels of the compound were not measured, we could not definitively determine whether the variable response was due to differences in dosing or to variability in individual animal susceptibility to the toxic effects of the compound. Regardless, this analysis suggests that information may be lost by pooling samples in studies in which intra-animal variability in response is observed. This effect was accentuated at low-dose and/or early time points.

Mechanistic and Topographic Information

Amin et al. (2004) in this issue describe the application of transcription profiling for the identification of candidate molecular markers of kidney toxicity. The data set from studies with all three nephrotoxicants was

Table 1. Distribution of participants and compounds analyzed on different technical platforms in the HESI Hepatotoxicity Working Group.

Compound tested: Cisplatin		Compound tested: Gentamicin		Compound tested: Puromycin	
Organization running analysis	Gene array platform used	Organization running analysis	Gene array platform used	Organization running analysis	Gene array platform used
U.S. FDA	PHASE-1 ToxArray 700	Novartis Pharmaceuticals Corp.	Affymetrix U34A	NIEHS	NIEHS custom chip
NIEHS	NIEHS custom chip	AstraZeneca	Affymetrix U34A	U.S. FDA	PHASE-1 ToxArray 700
Pharmacia ^a	Incyte Rat GEM1	Wyeth-Ayerst Research	Affymetrix U34A		
AstraZeneca	Affymetrix U34A	Pfizer Inc	Affymetrix U34A		
Pfizer Inc	Affymetrix U34A	Amgen Inc.	PHASE-1 ToxArray 700		
		GlaxoSmithKline	Custom chip		
		NIEHS	NIEHS custom chip		
		Pharmacia ^a	Incyte Rat GEM1 and 3		
		U.S. FDA	PHASE-1 ToxArray 700		

Abbreviations: NIEHS, National Institute of Environmental Health Sciences; U.S. FDA, U.S. Food and Drug Administration.

^aNow Pfizer Inc.

generated on the NIEHS cDNA microarray platform. For this reason, and to avoid potential nomenclature and platform-specific technical issues, Amin and co-workers focused on results from the NIEHS rat array, which contains > 7,000 polymerase chain reaction-amplified and sequence-verified cDNA elements (Hamadeh et al. 2002). These analyses provided both mechanistically and topographically interesting information. Mapping individual genes whose expression was affected by the nephrotoxics into biochemical pathways provided molecular insights into the nature of the observed toxicity and of the affected cell's efforts to accommodate those effects. For example, a reduction in the levels of several mRNAs encoding proteins involved in the formation of creatinine from L-arginine (Figure 1) was observed on treatment with a dose of cisplatin that caused aberrant renal pathology. Creatinine levels in serum can be used as an indicator of glomerular filtration rate but are not particularly sensitive to the early stages of renal toxicity. In the current study, the gene encoding L-arginine-glycine amidinotransferase, a member of the creatinine biosynthesis pathway, was affected as early as the first day after treatment with cisplatin. Repression of this pathway may reflect an adaptive response to increased serum creatinine levels due to reduced glomerular filtration rate resulting from toxicity to the proximal tubule.

Work performed by the Nephrotoxicity Working Group demonstrated a number of topographically specific gene expression changes, including changes in a group of proximal tubule-expressed genes mediated by cisplatin and gentamicin, which provided a mechanistic insight into the topographically distinct nephrotoxicity induced by these chemicals. An additional example of the value and robustness of the technology was evident on evaluation of the profiling results with puromycin (Amin et al. 2004). Evidence for a mild secondary tubular lesion was detected on analysis of the transcription profiling results, which demonstrated changes in the expression of several proximal tubule associated transcripts. Although this effect had not been noticed on initial histopathological evaluation, the finding was confirmed on re-evaluation in light of the transcription profiling data.

Finally, the work performed by the Nephrotoxicity Working Group identified several potential protein biomarkers. Although molecular markers of toxicity may be useful for providing mechanistic details, protein and metabolite markers have greater use as noninvasive biomarkers of intoxication and may translate to the

clinic. As detailed by Amin et al. (2004), several particularly interesting gene expression changes could warrant further investigation as candidate protein biomarkers. In particular, kidney injury molecule-1 (KIM)-1, clusterin, and others may prove useful as kidney region-specific urinary protein markers of nephrotoxicity.

Potential Application to Risk Assessment

A primary objective of the HESI Technical Committee on the Application of Genomics to Mechanism Based Risk Assessment has been to evaluate the application of genomics for mechanism-based preclinical risk assessment. This issue engendered much discussion at the recent HESI plenary meeting (held 5–6 June 2003 in Fairfax, Virginia, USA; meeting notes are available at <http://www.ilsa.org/index.cfm?pubentityid=120>) discussed in this mini-monograph by Pennie et al. (2004). However, the relatively limited studies completed to date are insufficient to address risk assessment. Although topographical and mechanistic information was obtained from the experiments performed by the Nephrotoxicity Working Group, expression profiling in a single tissue and a single species cannot be construed as risk assessment. Additionally, in the absence of *in vivo* efficacy measurements, no therapeutic indices could be determined for any of

these studies. Finally, the likely error rates associated with microarray technology are such that it would be difficult to know how to use or interpret the data without validating each individual result. On a microarray with 10,000 elements, even a 1% error rate corresponds to 100 false results. To address this uncertainty, scientists in the nephrotoxicity and other working groups used pathway mapping to identify biochemical pathways that were affected upon treatment with a toxicant. However many biochemical pathways are underrepresented on microarrays or have only a few members that are transcriptionally regulated. In addition, a large number (> 50%) of significantly changed genes were expressed sequence tags that have not yet been functionally defined. Therefore, in spite of the useful information that may be gleaned from a microarray experiment, current methodologies in pathway mapping may not be directly applicable to an assessment of risk at this time.

For the reasons described above, there is significant concern about the misinterpretation of the significance of individual gene or pathway-specific events observed in microarray data beyond general mechanistic interpretation. Presently, there is a lack of understanding of the context of a gene expression change. For example, changes may be related to pharmacology, metabolism, adaptive, or pathology end points.

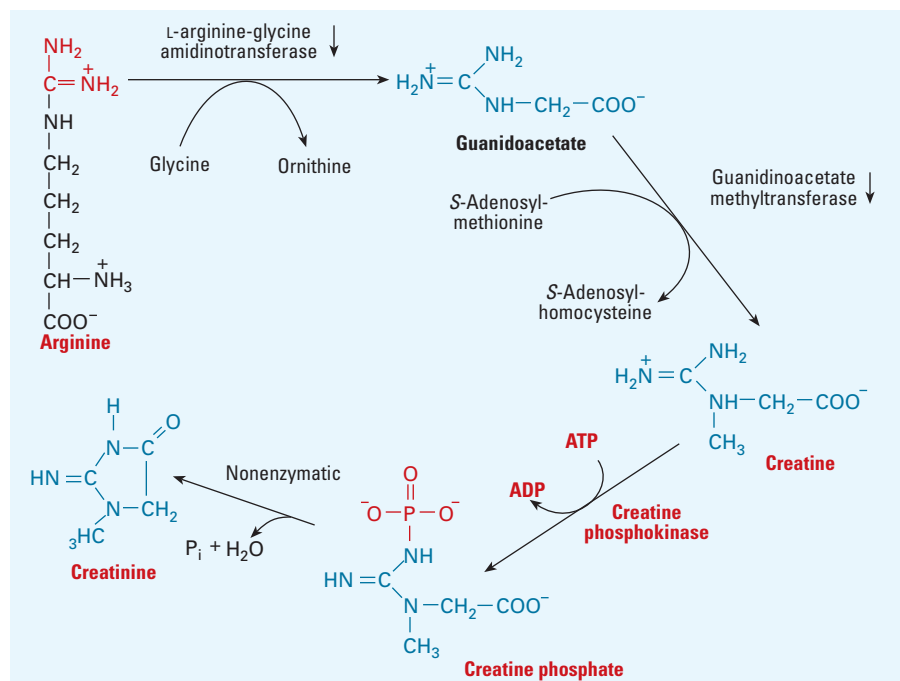


Figure 1. The creatinine biosynthesis pathway is necessary for the formation of creatinine from L-arginine. ADP, adenosine diphosphate; ATP, adenosine triphosphate. Creatinine levels in serum can be used as an indicator of glomerular filtration rate, but are not particularly sensitive to the early stages of renal toxicity. According to Amin et al. (2004), L-arginine-glycine amidinotransferase and guanidoacetate methyltransferase were repressed as early as the first day after treatment with cisplatin.

For example, in a metabolically active organ such as the liver, it may be impossible to observe a no-effect level on gene expression. In such a tissue, the smallest measurable amount of a biologically active compound would likely result in changes in the expression of genes encoding metabolism and clearance proteins, even in the complete absence of any toxicological responses. Similarly, in a drug target tissue, efficacy is just as likely as toxicity to result in gene expression changes. Until the complete function and interactivity of every gene product and every biochemical pathway is known, it would be impossible to distinguish a no-effect from a no-adverse effect level. Therefore, because of these limitations, application of profiling technologies is not currently being applied routinely in regulatory studies.

How to address some of these concerns may have been resolved at the recent HESI plenary meeting. Specifically, many contributors to the HESI consortium used principal component analysis to distinguish groups on the basis of dose, mechanism, and lesion topography. In some examples, the lowest-dose groups were indistinguishable from the controls (or, in the case of 2-channel array data, low dose vs. control was indistinguishable from control vs. control hybridizations). In such cases, the fact that evaluation of the expression level of hundreds or thousands of individual transcripts cannot distinguish between a treated and an untreated animal supports an absence of toxicity, regardless of the identity and function of the genes and pathways themselves. Figure 2 shows such an

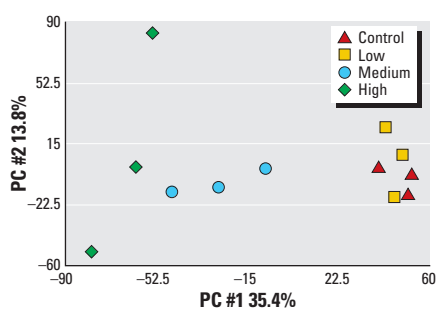


Figure 2. Principal component analysis performed on a data set of RNA profiles from rats treated with a pancreatic toxicant demonstrates separation of mid- and high-dose animals from control animals. PC, principal component. The low-dose animals, which demonstrated no observable indications of toxicity (as assessed by traditional clinical chemistry and histopathological end points), were inseparable from the control animals. In cases where the toxicity target organ is neither an efficacy target organ nor involved in metabolism or clearance of the toxicant, such evidence could be used to set a no-effect level for the compound in question.

example, wherein the transcription profile of mid- and high-dose-group animals treated with a pancreatic toxicant are clearly separated from the control vs. control hybridizations. In this example, the first principle component clearly distinguishes toxic from non- or pretoxic outcomes. The profile of the lowest-dose group, from a dose level in which no histopathological evidence for toxicity was observed, was indistinguishable from the control versus control hybridizations. In the extremely high-order theoretical space used in such analyses, a small number of nonsystematic errors in individual hybridizations would be unlikely to cause an artificial separation of these groups. Furthermore, the exact function of the gene products of the affected mRNAs is largely irrelevant. In cases where efficacy end points and toxicity were observed in the same tissue, the use of efficacious and nonefficacious structural analogs could be used to distinguish efficacy from pharmacophore-mediated toxicity. Similarly, inclusion of genetically engineered animal models in toxicogenomic experiments would also be useful in distinguishing between pharmacology (both desirable and adverse) and pharmacophore-mediated toxicity. Of course, several caveats must be placed on such a supposition. For example, this approach may be valid only in toxicity target organs that also were not targets for efficacy and also were not actively involved in the metabolism and clearance of the compound.

In an effort to distinguish toxic from nontoxic compounds, many companies have developed databases containing the expression profiles of numerous well characterized compounds. They then use pattern recognition approaches to distinguish between safe and toxic compounds to predict toxicological liabilities of novel lead compounds. However, current pattern recognition approaches are widely varied and the field is undergoing rapid development. Different databases and approaches to pattern recognition may yield different predictions of risk, raising false concerns over compounds in the absence of any evidence of toxicity. For this reason, pattern recognition is perhaps not ready for regulatory risk assessment. Development of custom medium density methodologies has gained some popularity as a potential means of querying a subset of well-understood genes in which the predictive value has been more fully characterized. Meanwhile, pattern recognition approaches applied to large-scale profiling data sets can allow for an internal assessment of the confidence in safety of an early lead compound.

Commentary

Toxicity studies in rats reproduced the topographically and mechanistically distinct renal lesions associated with administration of cisplatin, gentamicin, and puromycin. The time-course and dose-response relationships of these agents was appropriate for further study of transcriptional regulation. Our analyses suggest at least equal sensitivity of microarray technology compared to traditional end points and yielded topographically and mechanistically interpretable information. Microarray analysis of nephrotoxicants provides an avenue for development of biomarkers and for gaining additional insight into toxicologic mechanism of action. Overall, the toxicogenomic evaluation of nephrotoxicants has demonstrated potential in enhancing risk assessment through providing mechanistic information relating to the pathogenesis of toxicity.

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