# Applications of microarrays with toxicologically relevant genes (tox genes) for the evaluation of chemical toxicants in Sprague Dawley rats in vivo and human hepatocytes in vitro

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## Abstract

Microarrays with toxicologically relevant genes (tox genes) have been developed in our laboratory for toxicogenomics studies in rat, dog and man. The genes were chosen using published information as well as a discovery process for genes responsive to toxic treatments using transcription profiling experiments conducted with rats and dogs. In addition to published information human tox genes were derived from rat tox genes based on gene homology. Using the microarray with rat-specific tox genes, a database containing gene expression, histopathology, and clinical chemistry findings has been generated for 89 compounds. Analysis of the database indicates that treatment with toxic compounds induces specific gene expression patterns. Dose- and time-dependent response relationships in gene expression were observed for treatment with toxic compounds. Gene expression at 24 h was found to correlate well with organ toxicity observed at 72 h. Mining of the database led to the selection of specific groups of genes (predictive gene sets) whose expression patterns are predictive of organ toxicity with a high degree of accuracy (approximately 90%). The data also provide insight on toxic mechanism and gene regulation pathways. For instance, carbon tetrachloride and chloroform treatments were found to decrease the expression of the cytochrome P450 isoform 3A1 gene while enhancing the expression of the multiple drug resistance gene MDR1 in liver, clearly demonstrating that the CYP3A1 and MDR1 genes were not co-regulated as postulated by some researchers. This approach, the use of gene expression as an endpoint to define organ toxicity, is extended to the definition of human drug toxicity using primary human hepatocytes as a test system. Preliminary results demonstrate that the toxic drug, troglitazone, can be clearly distinguished from the less toxic analogues, rosiglitazone and pioglitazone based on their effects on tox gene expression in human hepatocytes. Our results with both rats in vivo and human hepatocytes in vitro suggest that microarrays with toxicologically relevant genes can be used routinely for the evaluation of chemical toxicity.

## 1. Introduction

Toxicogenomics—the use of gene expression as an endpoint to evaluate chemical and drug toxicity, is an emerging discipline in toxicology [1–12] with

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promising applications in drug discovery and development [5,13–17]. The proponents of the technology believe that gene expression provides a wealth of information, including mechanism elucidation, that can aid safety assessment. However, there are also concerns that the myriad information obtained with toxicogenomics may hinder safety assessment, as not all gene expression changes may be toxicologically relevant.

In our laboratory, we have developed an approach that we believe will allow the practical application of toxicogenomics. Instead of the use of genes from the entire genome for toxicogenomics studies, we have developed arrays with "toxicologically relevant" genes (tox genes)—genes that are known or suspected to be affected by toxicants. By focusing on the tox genes, we should alleviate the undesirable discovery of "toxicological-irrelevant" findings.

In this article, we describe the application of our microarray with tox genes in the evaluation of chemical and drug toxicity using two experimental systems: Sprague Dawley rats, a commonly used preclinical safety model, and primary human hepatocytes, a widely accepted in vitro experimental model for the evaluation of human-specific drug properties.

## 2. Materials and methods

### 2.1. Animal treatment

Tissue samples used for transcription profiling were derived from studies using male Sprague-Dawley rats treated by single intraperitoneal injection with test compounds.

The rats used in these studies were young rats (10–11 weeks of age) that were fed diets of commercial rodent chow (Purina 5001, Purina Mills, Richmond, IN or PMI Feeds, Inc. certified rodent chow, Purina Mills, Richmond, IN). Treatments were conducted at two dose levels (a projected maximum tolerated dose (MTD) level and 1/4 of the MTD level). The projected MTD level was a dose level projected to produce signs of toxicity with little or no lethality and was based on previous published or unpublished studies. In some cases experiments were repeated at lower maximum dose levels when a projected MTD dose level produced lethality. Control groups were treated with vehicle only. Three rats were usually

used for each treatment or control group at specified time points after administration of test compound (6, 24 and 72 h), rats were euthanized by $CO_2$ exposure and exsanguination. Tissues were rapidly collected (within 2 min), quick-frozen in liquid nitrogen, and stored at $-80\,^\circ$C for RNA extraction at a later time.

### 2.2. Cell culture

Human hepatocytes were isolated from livers procured but not used for transplantation as previously described [18–20]. Cells were incubated in collagen coated plates (six-well plates, $(1–1.5) \times 10^6$ cells per well) in a humidified 5% $CO_2$ atmosphere at $37\,^\circ$C in William's E medium supplemented with ITS (insulin, 5 µg/ml; transferrin, 5 µg/ml; selenous acid, 5 ng/ml), 2 mM L-glutamine, penicillin–streptomycin and 0.5 µM dexamethasone for 36–48 h followed by treatment with test materials in fresh medium for an additional 24 h. Vehicle control consisted of medium with 1% dimethylsulfoxide (the final concentration of dimethylsulfoxide in treatment medium).

After treatment, the cells were lysed with Trizol reagent (Invitrogen, Carlsbad, CA) and the lysates were stored frozen at $-80\,^\circ$C for subsequent RNA extraction.

### 2.3. Microarray preparation

Modified glass slides were printed with amine-labeled cDNA probes (300–500 base pair) using a high-throughput slide spotter (Omigrid100 GeneMachines, San Carlos, CA). The Rat CT arrays contain sequences from almost 700 rat genes with known or discovered responsiveness to toxic treatments. The Human 600 array contains sequences from almost 600 human genes with known responsiveness to toxicity or which are human homologues of discovered rat tox genes.

### 2.4. RNA isolation, probe labeling, microarray hybridization and scanning

Total RNA was isolated from rat tissue samples using Qiagen RNeasy midi kits (Qiagen, Valencia, CA) following procedures recommended by the manufacturer. Total RNA was isolated from human hepatocytes using Trizol reagent (Invitrogen, Carlsbad, CA)

and the manufacturer's recommended procedures. Targets for hybridization were prepared from the total RNA by incorporating cy3 and cy5 dye labeled nucleotides into cDNA by reverse transcription (RT). A Superscript II reverse transcriptase system (Invitrogen, Carlsbad, CA) was used for preparation of labeled cDNA from total RNA. Labeled target preparations were purified using QIAquick PCR purification kits (Qiagen, Valencia, CA) or ethanol precipitation followed by resin bead processing (Wizard DNA Binding Resin, Promega, Madison, WI). The target preparations were hybridized in pairs (control sample cy5 labeled probe and treated sample cy3 labeled probe) to Phase-1 Rat CT arrays or Phase-1 Human 600 arrays. Arrays were scanned on an Axon Instruments GenePix 4000A MicroArray Scanner and the fluorescent readings from this scanner were converted into quantitative files on a computer using GenePix software.

## 2.5. Data analysis

Expression ratio data (ratio of treated to control fluorescence signal) were normalized using the 50th percentile of the fluorescence signal distribution of all genes. Ratio data were excluded from analysis if the control channel value was <0. For analysis of correlations and predictive modeling gene expression ratios were transformed as the log of the ratio.

Data were analyzed using Phase-1 Matrix Express$^{TM}$ gene expression analysis software, and Microsoft Excel. To determine whether differential gene expression was statistically significant, a one-sample *t*-test was used to test whether ratio data was statistically significant from a ratio of one (no change). A two-sample *t*-test was used to test whether blood chemistry parameters after treatment were significantly different from control.

## 2.6. Prediction analysis and modeling

For predictive gene discovery and evaluation GeneSpring software (Version 4.1, Silicon Genetics, Redwood City, CA) was used for statistical analyses including identification of gene expressions correlating with histopathology scores using standard correlation statistical measures (e.g., Pearson and Spearman correlation measures) and predictive modeling using

the K-nearest neighbor model (KNN model used in the Predict Parameter Values tool). A complete, detailed description of the model can be obtained from Silicon Genetics (GeneSpring Advanced Analysis Techniques Manual). In brief, the model inputs are differential expression data for samples in training and test sets, classification of the samples (the classification to be predicted), a *k*-value for number of nearest neighbors, a list of genes whose expression is to be considered for the predictive model, a number of genes to be used for prediction and a *P*-value cutoff which is a *P*-value criterion that must be met for a predictive call to be made. The model first ranks the genes for discriminant ability according to a Fisher's exact test statistic computed for each gene's expression and the classification results for the training set of data. Genes are rank ordered by the discriminant score for classification. The genes used for the KNN prediction model are selected in accord with the pre-specified number of predictive genes in order of the discriminant classification score. The test samples are then classified using the KNN model the training set data and the set of genes specified. The final step in the prediction is application of a decision threshold that determines whether a KNN classification call will be made. For this decision threshold a *P*-value is calculated for the proportion of neighbors in each class against the proportions found in the training set, again using Fisher's exact test, but now a one-sided test. A *P*-value ratio (*P*-value) is set as a way of setting the level of confidence in individual sample predictions based on the ratio of *P*-values for the best class (lowest *P*-value) versus the second best class (second lowest *P*-value). If the *P*-value ratio does not exceed a specified *P*-value ratio cutoff (typically between 0.5 and 1) then no call will be made. The final result is a call (or no call) for classification of each sample in the test set using the KNN model and the parameters specified for the model. The classification prediction of the test samples is performed by the computer model in a blinded fashion, i.e. the model did not have information on the test sample classification.

Predictive genes for kidney tubular necrosis were identified using the scheme presented in Fig. 1. This scheme uses multiple training and test sets of data that can be randomly assigned from a single data set. Input genes are derived from standard statistical correlations between histopathology scores (assigned

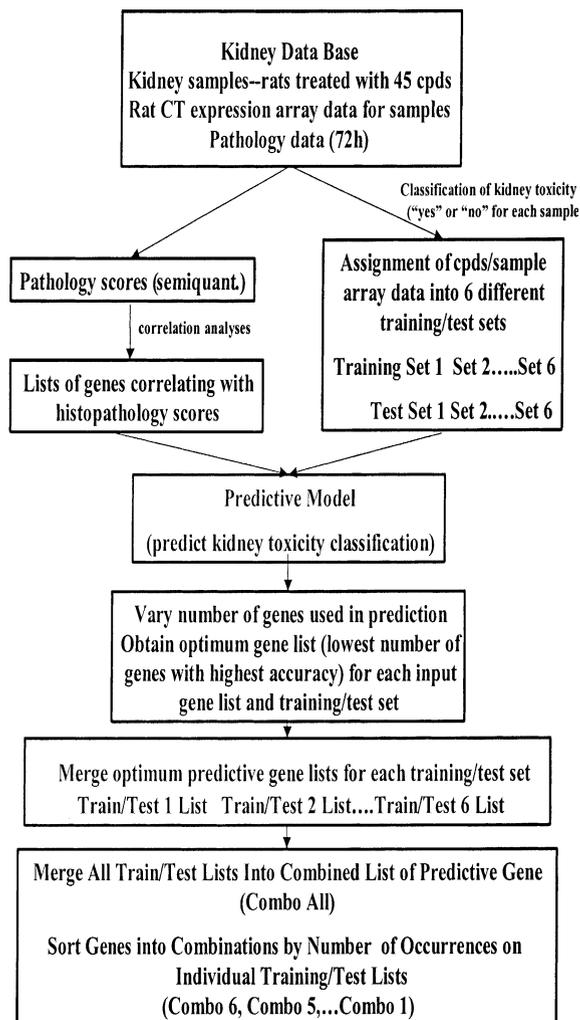## Discovery of Predictive Genes for Kidney Toxicity



Fig. 1. Schematic presentation of the approach used to discover predictive genes for organ-specific toxicity. The scheme used for the discovery of genes predictive of kidney tubular necrosis is shown.

based on occurrence and severity of tubular necrosis observed at 72 h after treatment) and gene expression (not shown). A dichotomous classification of kidney toxicity was used for predictive modeling. Each array data sample (one per animal) was assigned a "yes" classification if the compound-dose level of that sample produced kidney tubular necrosis at 72 h after treatment (as judged by histopathological analysis of kidney samples) or a "no" classification if that

compound-dose level did not produce kidney tubular necrosis at 72 h after treatment. Lists of genes, numbers of genes, $k$-values and training/test sets were run iteratively through the KNN model to produce predictions of tubular necrosis classification for the test sets. For each model iteration an optimum set of predictive genes was defined as the minimum set of genes producing the maximum classification accuracy. In the GeneSpring model genes are selected based on a discriminant score. Optimum lists of genes were merged for each training/test set using a common $k$-value (the $k$-value producing highest accuracy scores). The list of predictive genes for each training/test set were then merged and the genes were classed according to the number of occurrences in training/test set lists.

## 3. Results

The categories of tox genes selected for incorporation into the microarrays for toxicogenomics studies are shown in Table 1. We have developed such toxicogenomic arrays for rat (approximately 700 genes), dog (approximate 200 genes), and man (approximately 600 genes). Approximately half of the rat genes were discovered from analysis of data from arrays over more than 17,000 rat sequences tested

Table 1
Categories of tox genes on the rat, canine and human cDNA Phase-1 microarrays

| | |
|---|---|
| Acute phase response | Inflammation |
| Adhesion | Intracellular transport |
| Ah-response | Immunotoxicity |
| Apoptosis | Liver regeneration |
| Angiogenesis | Metastasis |
| Cell–cell communication | Mitochondrial function |
| Cell cycle | Multidrug resistance |
| Cell proliferation | Neurotoxicity |
| Cell migration | Oxidative stress |
| Chemokines | Peroxisome proliferators |
| Cholestasis | Phase I biotransformation |
| Differentiation | Phase II biotransformation |
| DNA damage/repair | Protein processing |
| DNA synthesis | Recombination |
| Fatty acid metabolism | Steatosis |
| Fibrosis | Tissue repair/regeneration |
| Growth arrest | Translation |
| Heat shock | Transcription |
| Hypoxia | Tumor suppressor |

Table 2

List of chemicals used in the generation of toxicogenomics database in Sprague Dawley rats

| | | |
|---|---|---|
| 1-Naphthylisothiocyanate[a] | Cyclosporin A[a] | Methyl methanesulfonate |
| 5-Fluorouracil[a] | Dacarbazine | Mitomycin C |
| Acetamidofluorene | Dexamethasone[a] | Naloxone[a] |
| Acetaminophen[a] | Dieldrin | Naproxen |
| Aflatoxin B1 | Diethylhexylphthalate | Nifedipine |
| Aflatoxin b1 | Diflunisal[a] | *N*-Nitroso-*N*-ethylurea |
| Allyl alcohol | Dimethylnitrosamine[a] | *N*-Nitroso-*N*-methylurea |
| Amphotericin B[a] | Dipyrone | Paraquat |
| Amsacrine | DMBA | Phalloidin |
| Atorvastatin | Doxorubicin[a] | Phenobarbital[a] |
| Azathioprine[a] | Endotoxin | Phenylhydrazine HCL[a] |
| Benzene[a] | Erythromycin estolate[a] | Polyethylene glycol[a] |
| Benzo(a)pyrene[a] | Estradiol[a] | Prednisone |
| Bromobenzene[a] | Ethanol[a] | Pregnenolone-16-alpha-carbonitrile |
| Busulfan[a] | Etoposide | Puromycin[a] |
| Cadmium chloride[a] | Flutamide | Quinidine[a] |
| Caffeine | Ganciclovir[a]T | Simvastatin |
| Carbon tetrachloride[a] | Gemfibrozil | Streptozotocin[a] |
| Carboxymethylcellulose[a] | Gentamicin[a] | Tamoxifen[a] |
| Carmustine[a] | Hydroxyurea[a]T | TCDD |
| Chlorambucil | Indomethacin | Tetracycline[a] |
| Chloroform[a]T | Isoniazid[a] | Thalidomide |
| Chlorpromazine[a] | Ketoconazole[a] | Theophylline[a] |
| Cisplatin[a]T | Lipopolysaccharide[a]T | Thioacetamide |
| Clofibrate[a] | Lovastatin | Triethylenemelamine |
| Clofibric acid | Mechlorethamine | Triethylenethiophosphoramide |
| Clozapine[a] | Melphalan | Valproic acid |
| Colchicine | Merbarone | Vincristine |
| Cycloheximide | Mercuric chloride | Wy 14,643 |
| Cyclophosphamide[a]T | Methotrexate[a] | |

[a] Indicates chemicals used for kidney toxicity prediction with T indicating kidney tubular necrosis observed at 72 h after treatment.

for transcriptional responsiveness using probes from tissue samples of rats given toxic treatments.

Chemicals used for the treatment of Sprague Dawley rats in the generation of the toxicogenomics database are shown in Table 2.

Typical results illustrating the effects of toxic compounds on gene expression in liver and kidney of the Sprague Dawley rat are illustrated with the effects of the known hepatotoxic chemical, dimethylnitrosamine (Fig. 2) and the nephrotoxic agent, lipopolysaccharide (LPS) (Fig. 3) on liver and kidney gene expression, respectively. The tox gene expression profiles 24 h after treatment are shown. The corresponding gene expression profiles for vehicle control (carboxymethylcellulose) (Figs. 2 and 3) are also shown to illustrate the increase in gene expression changes upon treatment with toxic agents.

The effects of chloroform ($CHCl_3$) and carbon tetrachloride ($CCl_4$) on the expression of cytochrome P450 isoform 3A1 (CYP3A1) and the multiple drug resistance (MDR-1A) genes in rat liver are shown in Fig. 4. MDR-1A was strongly induced and CYP3A1 was strongly suppressed in rat liver 24 h after acute exposure to either compound. These effects in gene expression were evident at 6 h for both genes after $CCl_4$ treatment, and for CYP3A1 after $CHCl_3$ treatment. At 72 h, effects in gene expression were largely diminished except for a statistically elevated increase in MDR1 after $CCl_4$ treatment (Fig. 4). The effects of $CCl_4$ and $CHCl_3$ treatments on additional tox genes in rat liver are shown in Table 3.

The database generated using toxic and nontoxic chemicals was used to identify biomarker genes with significant predictive capability for organ-specific
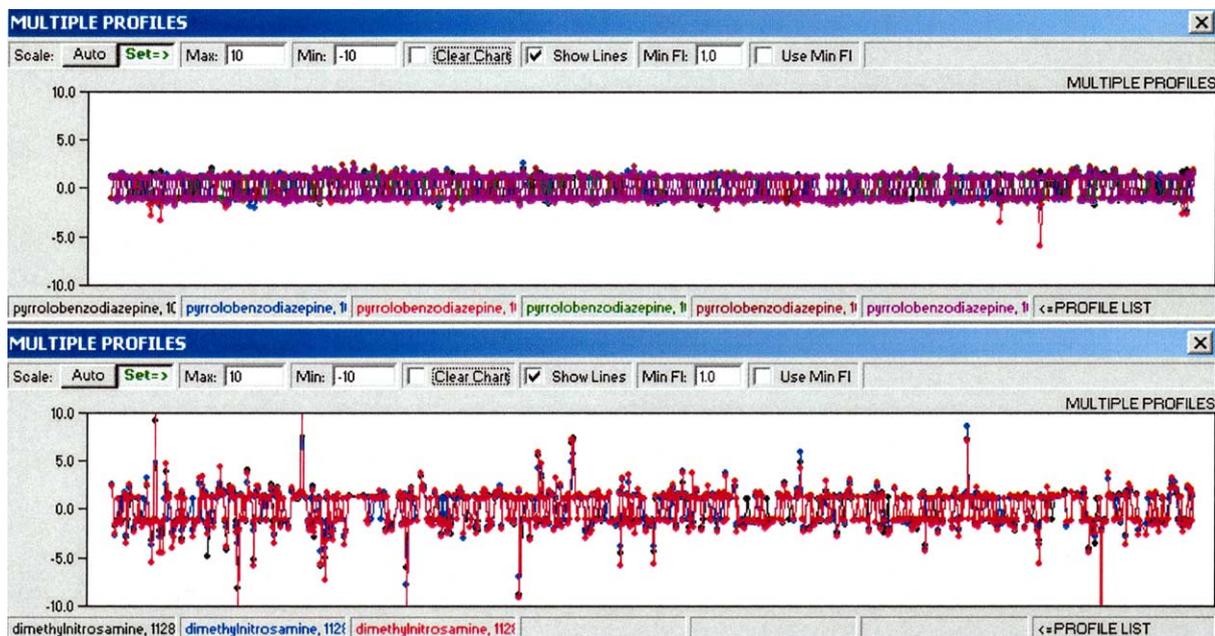
Fig. 2. Overall effects of toxic and nontoxic agents on gene expression in rat liver. The data represent differential expression ratio (no effect = ±1.0) for the 700 tox genes for individual samples from treated animals compared to samples from vehicle control or untreated animals. The toxic chemical (bottom panel: dimethylnitrosamine; 20 mg/kg) caused more gene expression changes than the vehicle control (top panel: carboxymethylcellulose). Results represent data from three animals for each treatment group.

toxicity (predictive genes). Results for the prediction of kidney tubular necrosis are presented here. This approach provided six sets of genes ranging from the most predictive set (Combo 6 occurring on all six training/test set gene lists) to the lowest predictive set (Combo 1 occurring on one training/test set gene list). Table 4 summarizes the predictive performance of the predictive gene sets for 24 h gene expression data. Accuracy increased when the unit of prediction was compound-dose (e.g. 0.950 mean accuracy and 0.894 mean geometric mean for the six sets of predictive genes (Combo 6 set)) or compound (e.g., 0.968 mean accuracy and 0.981 mean geometric mean for Combo 6 set). The predictive scores were highly significant and much higher than prediction numbers obtained using random classification. Predictive performance was also observed for 6 and 72 h expression data but the predictive performance was not as high as observed for 24 h expression data (data not shown). There was minimal overlap in predictive genes between the different time points suggesting that there are temporal sequences of gene expression changes

that represent different stages of toxic damage and repair or response processes.

Although the specific predictive genes for kidney tubular necrosis are proprietary, some perspective on these genes is provided in Table 5 which presents functional classifications of a subset of the predictive genes for kidney tubular necrosis.

Two of the compounds used in the database, cyclophosphamide and gancyclovir, produced kidney tubular necrosis at the high dose but not at the low dose employed for the database. Predictive performance for these compounds are presented in Table 6. The data show that the expression profiles of the predictive genes are concordant with the pathology dose–response.

A comparison of the effects of troglitazone, rosiglitazone, and pioglitazone on tox gene expression in cultured primary human hepatocytes is shown in Fig. 5. The results show that substantially higher numbers of genes are affected by treatment with the toxic troglitazone than the less toxic rosiglitazone and pioglitazone.
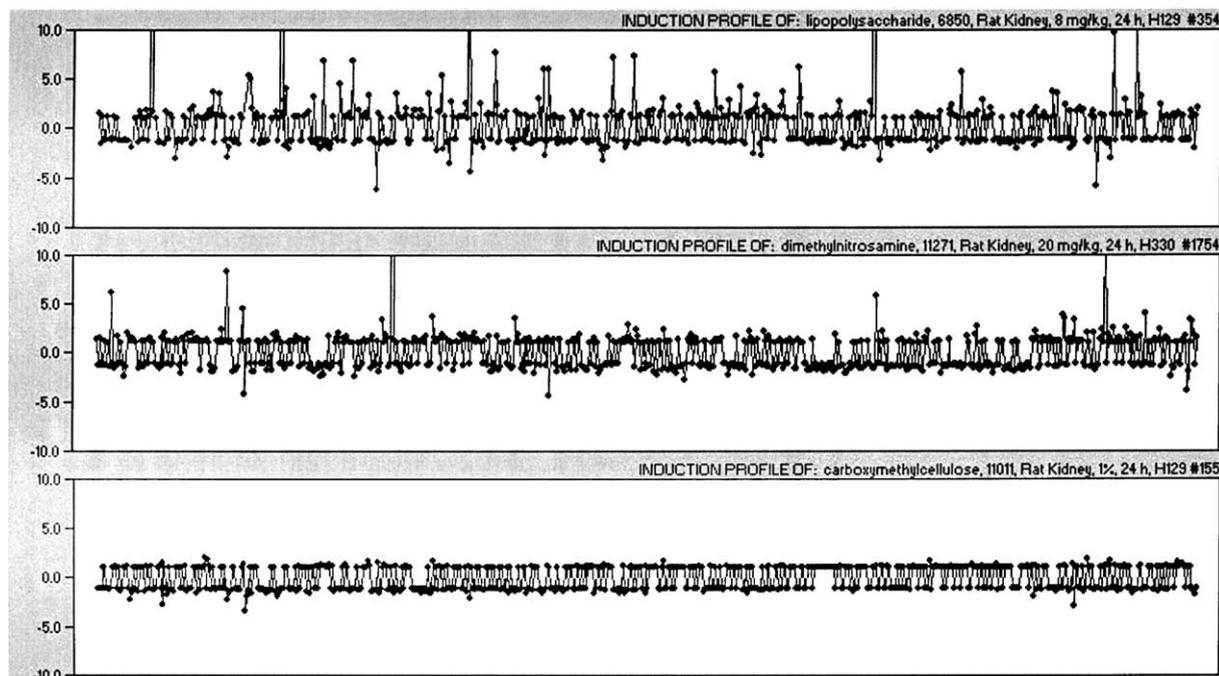
Fig. 3. Overall effects of toxic and nontoxic agents on gene expression in rat kidney. The data represent differential expression ratio (no effect = $\pm 1.0$) for the 700 tox genes. Results with the nephrotoxic lipopolysaccharide (top panel: 8 mg/kg; middle panel: 20 mg/kg) and vehicle control (bottom panel: carboxymethylcellulose) are shown.

## 4. Discussion

Gene expression changes have been used routinely to provide specific mechanistic information concerning the mode of action of a toxicant. Toxicogenomics, the use of comprehensive gene expression changes to evaluate chemical toxicity, greatly expands the scope and depth of this approach in toxicology. As gene expression represents the physiological state of a living cell, perturbations in gene expression should allow the evaluation of multiple types of toxicity, from frank toxicity to carcinogenesis [5,14,21–23]. Toxicogenomic gene expression studies are facilitated by the recent development of high density microarrays. Microarrays are now available to contain most of the known genes in the human and rat genome and the effects of a substance on gene expression of virtually all known genes can be assessed with a single microarray.

The complication of using a microarray containing genes of the entire genome is that one needs to decipher whose effects are relevant to the purpose of the experimentation. For instance, not all gene expression changes would have toxicological consequences. While fully appreciating the value of a large genomic content arrays, the use of microarrays that focus on toxicologically relevant genes can have interpretative advantages and permit practical applications of gene expression evaluation for toxicological evaluations.

A toxicogenomics database with toxicants of different structures and mode of actions is a valuable resource for the understanding of the relationship between gene expression and toxicity [7]. Using such a database, the following questions were investigated: (1) Could a toxic compound be distinguished from a nontoxic compound based on gene expression profiles of toxicologically responsive genes? (2) Could gene expression profiles provide data to guide further mechanistic evaluations? (3) Could specific genes be used as predictive biomarkers for the specific toxic endpoints?

The data show that toxicants in general produce substantial expression changes in tox genes. In
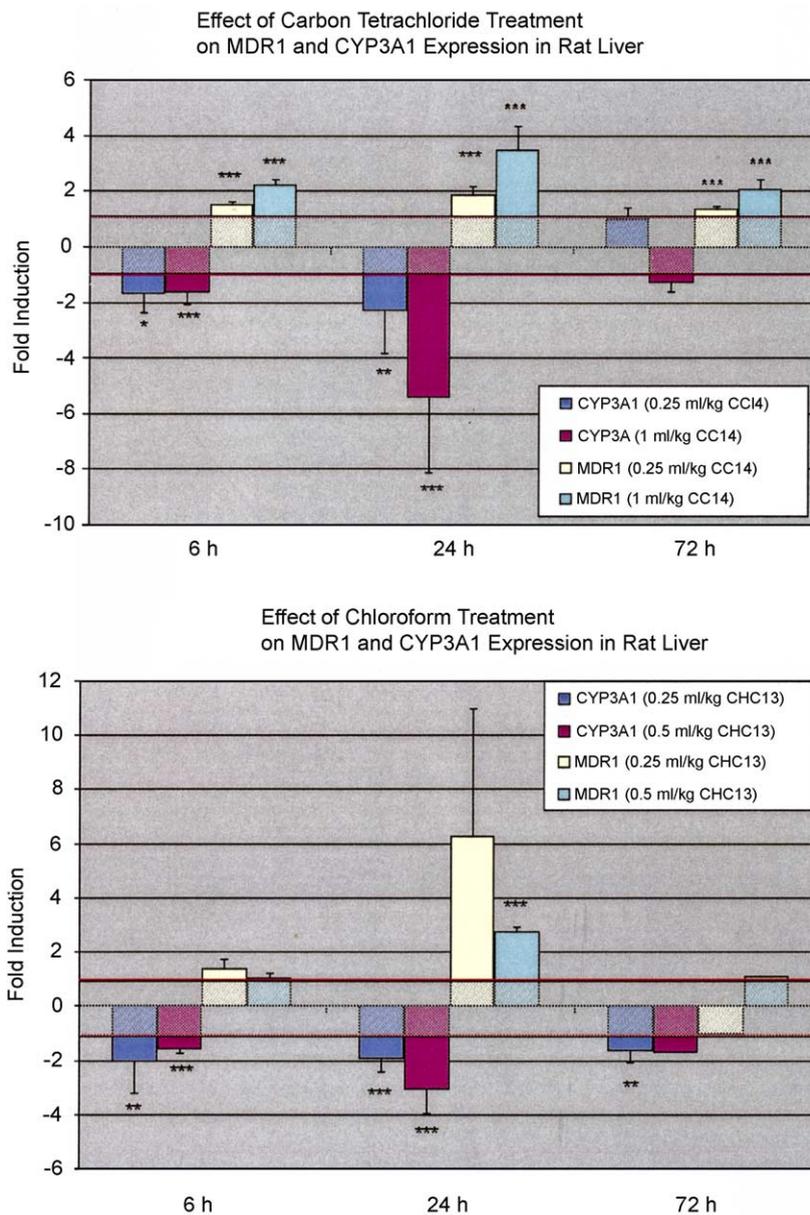
Fig. 4. Effects of carbon tetrachloride (top) and chloroform (bottom) treatments on the expression of CYP3A1 and MDR1 genes in Sprague Dawley rat livers. Differential expression (induction or suppression) compared to vehicle control samples is presented for treatments from three time points are shown. Data are mean differential expression for three separate animals with error bars for standard error of the mean (SEM). Results of a *t*-test comparison for significant difference between treated and control expression at the $P < 0.01$ (∗∗) and $P < 0.001$ (∗∗∗) significance level are also indicated.

Table 3

Effects of carbon tetrachloride (CCl$_4$) and chloroform (CHCl$_3$) on the expression of selected tox genes in livers of Sprague Dawley rats

| Genes | CCl$_4$ | | | | | | CHCL$_3$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Low | | | High | | | Low | | | High | | |
| | 6 | 24 | 72 | 6 | 24 | 72 | 6 | 24 | 72 | 6 | 24 | 72 |
| Inflammation | | | | | | | | | | | | |
| Alpha-1 acid glycoprotein | 1.30 | 4.81 | −1.12 | 1.76 | 6.14 | 1.84 | 4.04 | 3.75 | −1.25 | 1.57 | 4.17 | 1.79 |
| Alpha-2-macroglobulin | −1.30 | −2.12 | −1.05 | −1.35 | −3.17 | −2.13 | −1.19 | −1.33 | 1.09 | −1.14 | −1.28 | −1.30 |
| Interleukin-1 beta | 1.81 | 1.66 | 1.24 | 3.04 | 2.79 | 1.47 | 1.01 | 1.77 | −1.17 | 1.01 | 1.37 | 1.13 |
| Interleukin-6 | 1.23 | 1.09 | −1.07 | 1.28 | 1.25 | 1.06 | 1.11 | −1.00 | 1.07 | 1.02 | 1.11 | 1.00 |
| Macrophage inflammatory protein-1 alpha | 1.68 | 1.86 | −1.04 | 1.64 | 2.93 | 1.24 | 1.33 | 1.50 | −1.03 | 1.08 | 1.75 | −1.01 |
| Macrophage inflammatory protein-2 alpha | 1.72 | 2.04 | 1.17 | 2.27 | 3.64 | 2.00 | 1.19 | 2.77 | 1.01 | 1.14 | 2.07 | 1.08 |
| Cell prolif | | | | | | | | | | | | |
| Cyclin D1 | 1.09 | 1.82 | 1.45 | 1.09 | 2.27 | 2.28 | 1.08 | 1.21 | −1.24 | −1.14 | −1.13 | −1.42 |
| Ornithine decarboxylase | 1.06 | 135 | 1.15 | 1.42 | 2.12 | 1.23 | 1.17 | 1.37 | 1.26 | 1.09 | 1.95 | −1.29 |
| Proliferating cell nuclear antigen gene | 1.38 | 1.63 | 1.13 | 1.90 | 1.74 | 1.61 | 1.26 | 1.18 | −1.04 | 1.19 | 1.15 | −1.23 |
| Oxidative stress | | | | | | | | | | | | |
| Catalase | −1.41 | −1.52 | −1.11 | −1.89 | −3.65 | −1.52 | −1.02 | −1.18 | 1.09 | 1.06 | 2.28 | −1.57 |
| Glutathione peroxydase | −1.26 | −1.34 | 1.12 | −1.27 | −2.37 | −116 | −1.14 | −2.44 | 1.40 | −1.13 | −2.46 | 1.30 |
| Heme binding protein 23 | 151 | 1.28 | 1.11 | 1.89 | 1.55 | 1.16 | −1.14 | 1.33 | −1.24 | −1.03 | 1.26 | −1.14 |
| Superoxide dismutase Cu/Zn | −1.10 | −1.04 | −1.25 | −1.17 | −1.13 | −1.22 | −1.37 | −1.07 | −1.32 | −1.38 | −1.28 | −1.04 |
| Superoxide dismutase Mn | 1.86 | 2.34 | 1.13 | 3.18 | 3.74 | 1.23 | 1.11 | 2.07 | −1.01 | 1.05 | 1.72 | −1.09 |

Treatment with carbon tetrachloride (CCL$_4$) or chloroform (CCL$_3$) was a single dose i.p. injection at low (0.25 ml/kg body weight, CCL$_4$ and CCL$_3$) or high (1 ml/kg body weight, CCL$_4$ or 0.5 ml/kg body weight, CCL$_3$) dose levels. Liver samples were harvested at 6, 24 and 72 h after treatment. Mean differential expression, compared to control expression, for samples from three animals per treatment group with fold induction as positive values and fold suppression as negative values.

Sprague Dawley rats, a high number of genes had their expression affected by the hepatotoxic dimethyl-nitrosamine and the nephrotoxic lipopolysaccharide in liver and kidney, respectively, while very few genes were changed by treatment with the nontoxic vehicle carboxymethylcellulose. The same was observed for primary human hepatocyte cultures. The known human hepatotoxicant troglitazone caused a number of gene expression changes while substantially fewer genes were affected by the relatively nontoxic rosiglitazone and pioglitazone at equimolar dose levels. The identity of the genes that are affected by the toxicants can aid mechanistic understanding of the toxicity. While toxicology in general is an empirical discipline, it is becoming apparent that mechanistic understanding often aids safety assessment as well as the design of a nontoxic structural analog. Using two well-studied hepatotoxicants, CCl$_4$ and CCl$_3$,

we found that these two structurally similar agents induced the expression of the drug transporter gene MDR-1 and down-regulated the expression of the P450 gene CYP3A1. Our findings are novel with respect to the gene expression effects of these agents. The reciprocal effects of the two compounds on the MDR-1 and CYP3A1 gene supports the theory that these two genes are not co-regulated, as previously hypothesized by some researchers [24–27].

Treatment with these compounds also resulted in induction of the acute phase genes alpha-1 acid glycoprotein and macrophage inflammatory protein-2 alpha that coincided with the reciprocal regulation of CYP3A1 and MDR-1 (Table 3). High dose CCl$_4$ resulted in modulation of additional acute phase and cell proliferation genes at 24 h, which corresponds with the changes in histopathology observed at that time point.

Table 4
Predictive performance of gene sets for kidney tubular necrosis from 24 h expression data

| Gene list[a] | No. of genes | Accuracy, mean (range)[b] | Accuracy (rand.), mean (range)[b] | Geometric mean, mean (range)[c] |
|---|---|---|---|---|
| Combo All | 216 | 0.915 (0.861–0.945) | 0.173 (0.024–0.304) | 0.810 (0.720–0.884) |
| Combo 6 | 28 | 0.921 (0.867–0.955) | 0.203 (0.102–0.393) | 0.837 (0.660–0.953) |
| Combo 5 | 25 | 0.896 (0.829–0.929) | 0.258 (0.157–0.348) | 0.821 (0.684–0.870) |
| Combo 4 | 23 | 0.882 (0.829–0.929) | 0.235 (0.074–0.308) | 0.776 (0.700–0.925) |
| Combo 3 | 19 | 0.839 (0.778–0.911) | 0.242 (0.148–0.295) | 0.740 (0.562–0.892) |
| Combo 2 | 45 | 0.733 (0.641–0.821) | 0.240 (0.056–0.349) | 0.552 (0.343–0.663) |
| Combo 1 | 76 | 0.787 (0.667–0.884) | 0.220 (0.083–0.321) | 0.645 (0.355–0.782) |

The Combo designations represent the number of training/test set occurrences (e.g. Combo 6 gene list containing genes occurring in all six training/test set merged optimum predictive gene lists). All lists are exclusive, i.e. no genes occur in more than one set, except for the Combo All list which includes all genes from each of the Combo sets.

[a] Gene lists were obtained as described in Section 3 and Fig. 4.

[b] Mean accuracy values (proportion of calls that are correct) for all six training/test sets using the specific gene list and GeneSpring KNN model implementation with range in accuracy values as indicated. The individual animal was the unit of prediction and binary classification for kidney tubular necrosis was used (i.e., the treatment condition induced tubular necrosis or did not induce tubular necrosis). Rand. indicates values for the same training/test sets with kidney tubular necrosis classification randomly assigned to the samples. No calls were considered incorrect classifications.

[c] Mean geometric mean values for all six training/test sets using the same parameters as for accuracy. Geometric means were calculated as the square root of $TP \times TN$, where TP is the proportion of positive calls that are true positives and TN the proportion of negative calls that are true negatives.

One application of toxicogenomic profiling is identification of genes whose expression can be used as predictive biomarkers for specific types of organ toxicity. The data indicate that highly accurate predictions

Table 5
Functional categories of genes whose expressions are predictive for kidney tubular necrosis

| Functional category | Number of genes |
|---|---|
| DNA repair | 3 |
| Acute phase response | 4 |
| Transcription | 4 |
| Cytoskeleton | 5 |
| Tissue repair/regeneration | 6 |
| DNA damage | 6 |
| Cell cycle | 8 |
| Translation | 8 |
| Oxidative stress | 9 |
| Apoptosis | 10 |
| Cell proliferation | 13 |
| Inflammation | 13 |
| Unknown | 85 |

Functional categories (primarily derived from literature analysis) are presented for 174 genes identified as having predictive value for kidney tubular necrosis when their expression was measured on microarrays on samples obtained 24 h after treatment. This is a subset of the 216 genes in Table 4 that excludes expressed sequence tag-derived sequences that do not a full annotated gene sequence available.

can be obtained. It is clear from these results that the predictive gene expression patterns are related to toxicity responses rather than chemical-specific effects because they operate successfully for a number of chemically diverse structures that are primarily linked by their ability to induce kidney tubular necrosis. An interesting observation derived from these studies is that there is considerable redundancy in the predictive genes—several sets of genes have significant predictive power. This is not surprising if families of genes are involved in responding to toxic insults; however, the extent of the redundancy and patterns of expression can only be effectively assessed with toxicogenomic technologies. The data suggest that combinations of markers rather than single markers may provide superior predictive and discriminant power. An important feature of the predictive gene expression performance, with particular relevance to toxicology evaluation, is the concordance between predictive gene expression and dose–response for pathology. Results with cyclophosphamide and gancyclovir show that low doses that did not produce kidney tubular necrosis, the prediction result using the predictive genes and KNN model was nontoxic while high doses that produced toxicity were predicted as toxic. These data support a conclusion that the gene expression data can be

Table 6

Dose–response prediction of kidney tubular necrosis for cyclophosphamide and gancyclovir

| Treatment (mg/kg) | Animal | Prediction[a] | Kidney tubular necrosis[b] |
|---|---|---|---|
| CPHOS (25) | 1 | − | |
| CPHOS (25) | 2 | − | − |
| CPHOS (25) | 3 | − | |
| CPHOS (100) | 4 | + | |
| CPHOS (100) | 5 | + | + |
| CPHOS (100) | 6 | + | |
| GAN (50) | 7 | − | |
| GAN (50) | 8 | − | − |
| GAN (50) | 9 | − | |
| GAN (200) | 10 | + | |
| GAN (200) | 11 | + | + |
| GAN (200) | 12 | + | |

Sprague Dawley rats were treated with cyclophosphamide (CPHOS) and gancyclovir (GAN) with doses in mg/kg. Results with individual animals are shown. Histopathology results on tubular necrosis (pathology) at 72 h after treatment is compared to prediction results (prediction) based on gene expression (24 h after treatment) using predictive gene sets discovered from the toxicogenomics database. Results are indicated as positive for necrosis (+), negative for necrosis (−).

[a] Kidney tubular necrosis prediction is presented for biomarker expression patterns (Combo 6 predictive gene set) for kidney samples from individual animals taken at 24 h after treatment using the KNN model with independent data used for the training set (i.e. training set data not containing cyclophosphamide or gancyclovir data). A prediction of "−" (marker expression pattern predicting nontoxic) or "+" (marker expression pattern predicting kidney tubular necrosis) was made for data from each individual animal.

[b] Observed induction of kidney tubular necrosis at 72 h for the indicated compound-dose combination: −, no kidney tubular necrosis observed in 3/3 animals; +, kidney tubular necrosis observed in 3/3 animals.

interpreted for dose–response in a manner that is compatible with other, more conventional toxicology endpoints. The findings of these studies demonstrate the possibility of practical application of the predictive genes or their gene products (e.g. the corresponding proteins) for development into highly accurate, quantitative toxicity biomarkers for preclinical and clinical applications. Toxicogenomics and proteomics have been viewed as promising tools for the development of new, noninvasive biomarkers for drug toxicity [28].

One major challenge in the drug industry is that every year in the past decade, one or more marketed drugs was removed from the market or their use was severely limited due to unexpected, serious, adverse drug effects (see [29]), suggesting that the current

practice in safety assessment requires improvement. Drug-induced hepatotoxicity, resulting in fatality or a requirement for liver transplantation, is a major problem with the human toxic drugs. An example of a hepatotoxic drug that has been removed from the market due to its association with liver failures is troglitazone, a PPAR-gamma agonist developed for the treatment of type II noninsulin-dependent diabetes mellitus. Troglitazone was removed from the US market due to its association with adverse, sometimes fatal, hepatotoxicity, and is currently replaced by two apparently safe drugs, rosiglitazone and pioglitazone [3,30,31].

Following the encouraging results with rat toxicity prediction, the toxicogenomic approach is being pursued for the evaluation of human drug toxicity. Primary human hepatocytes, a well-accepted in vitro experimental system for the evaluation of human drug properties, was chosen for implementation of this approach [18–20,32–35]. As human hepatocytes have been previously applied successfully in the investigation of the metabolism and the key determinants of hepatotoxicity of troglitazone [36,37], the effects of the hepatotoxic troglitazone and the less toxic analogues rosiglitazone and pioglitazone on tox genes were compared. The tox gene expression profile obtained in human hepatocytes treated with troglitazone showed a large number of gene expression changes that were not observed when the cells when treated with the structurally similar, but apparently less hepatotoxic, rosiglitazone and pioglitazone. The fact that troglitazone, rosiglitazone and pioglitazone have similar therapeutic maximum serum levels (in the approximate range of 5–10 μM) suggest that these differences at a small multiple of the therapeutic serum level may be relevant for interpretation of human effects of these drugs. These initial results suggest that evaluation of drugs in human hepatocytes, using toxicogenomics as an endpoint, may be useful in the definition of human hepatotoxicity.

These results, and those of others, clearly demonstrate the value of toxicogenomics in toxicological evaluations. As one of the major concerns of the application of microarrays is the discovery of "toxicologically irrelevant" findings, we believe that the use of specialized microarrays such as our microarrays with tox gene is a step towards the right direction, namely, to allow practical application of toxicogenomics in routine toxicological studies. The predictive genes can
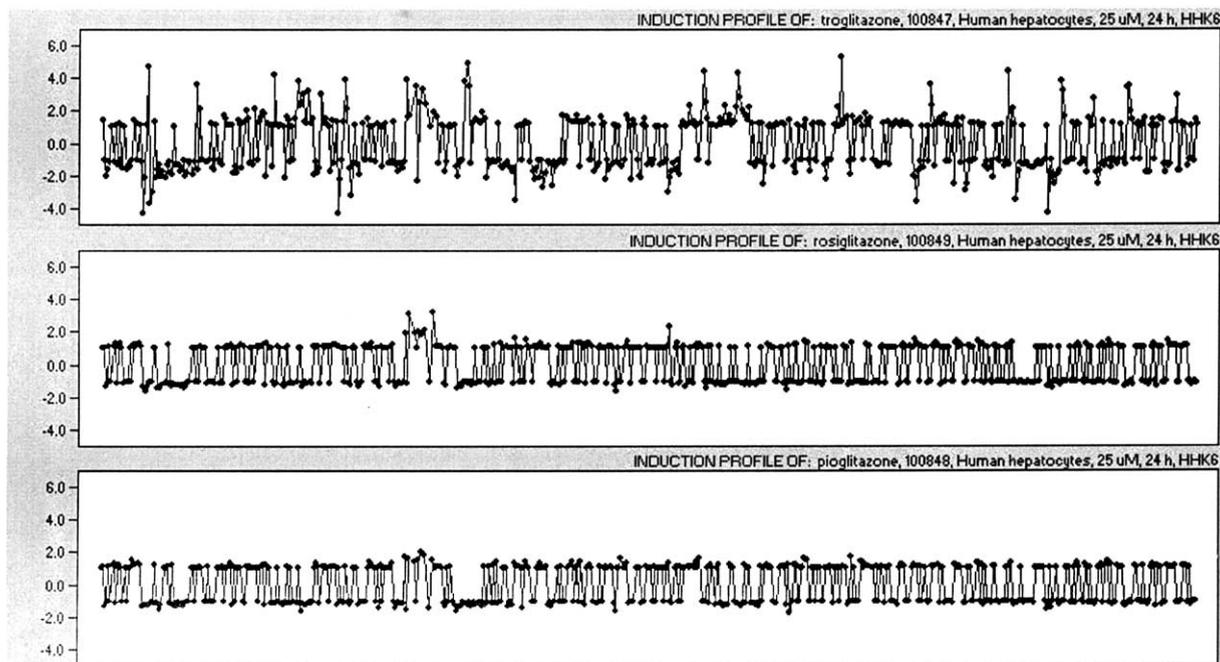
Fig. 5. Effects of toxic and nontoxic agents on tox gene expression in primary human hepatocyte cultures. The data represent differential expression ratio (no effect = ±1.0) for 600 human tox genes. Gene expression profiles of the hepatotoxic drug, troglitazone (top panel) as compared to the less toxic structural analogs, rosiglitazone (middle panel) and pioglitazone (bottom panel) are shown.

be used as specific tools to augment histopathological and clinical chemistry findings for specific types of organ toxicity. The combination of toxicogenomics and primary human cells such as human hepatocytes may be a powerful tool for the enhancement of the accuracy in the prediction of human drug toxicity which is a major unmet need in the pharmaceutical industry as evidenced by the recurring incidents of serious adverse drug reaction observed for marketed drugs.

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