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A gene expression signature for oxidant stress/reactive metabolites in rat liver

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Abstract

Formation of free radicals and other reactive molecules is responsible for the adverse effects produced by a number of hepatotoxic compounds. cDNA microarray technology was used to compare transcriptional profiles elicited by training and testing sets of 15 oxidant stressors/reactive metabolite treatments to those produced by approximately 85 other paradigm compounds (mostly hepatotoxicants) to determine a shared signature profile for oxidant stress-associated hepatotoxicity. Initially, 100 genes were chosen that responded significantly different to oxidant stressors/reactive metabolites (OS/RM) compared to other samples in the database, then a 25-gene subset was selected by multivariate analysis. Many of the selected genes (e.g., aflatoxin aldehyde reductase, diaphorase, epoxide hydrolase, heme oxgenase and several glutathione transferases) are well-characterized oxidant stress/Nrf-2-responsive genes. Less than 10 other compounds co-cluster with our training and testing set compounds and these are known to generate OS/RMs as part of their mechanisms of toxicity. Using OS/RM signature gene sets, compounds previously associated with macrophage activation formed a distinct cluster separate from OS/RM and other compounds. A 69-gene set was chosen to maximally separate compounds in control, macrophage activator, peroxisome proliferator and OS/RM classes. The ease with which these 'oxidative stressor' classes can be separated indicates a role for microarray technology in early prediction and classification of hepatotoxicants. The ability to rapidly screen the oxidant stress potential of compounds may aid in avoidance of some idiosyncratic drug reactions as well as overtly toxic compounds.

Keywords: Microarray; Reactive metabolites; Oxidative stress; Nrf-2; Free radicals

1. Introduction

Oxidative stress refers to the generation of reactive oxygen species (ROS, such as superoxide, hydroxy radicals and hydrogen peroxide) and to the adaptive cellular responses to reactive oxygen species (e.g., depletion of anti-oxidant stores and activation and induction of protective and repair enzymes) [1]. Several groups have shown that most reactive electrophiles, including many hepatotoxic drug metabolites, produce effects similar, if not identical, to reactive oxygen species [2,3]. Cells appear capable of handling low doses of OS/RMs, while higher doses overwhelm protective capacity and lead to damage or death of the cells.

A common property of ROS and reactive compounds/metabolites involves inducing the expression of protective enzymes. A common site in the promoters of these oxidant stress-responsive genes has been termed the anti-oxidant response element (ARE) or electrophilic response element (EpRE) [4]. Induction of oxidant stress-responsive genes is largely dependent on binding of the transcription factor Nrf-2 to the ARE/EpRE. Under resting conditions Nrf-2 appears to be sequestered by sulfhydryl bonding to Keapl; oxidation of the sulfhydryl bonds (and activation of certain kinases) releases Nrf-2 to bind ARE/EpRE [4]. Although

Abbreviations: LPS, lipopolysaccharide; NSAIDs, nonsteroidal anti-inflammatory drugs; ROS, reactive oxygen species; OS/RM, oxidative stressor/reactive metabolite; OS/RE, oxidant stress/ reactive electrophile; ARE, anti-oxidant response element; EpRE, electrophilic response element; RT-PCR, reverse transcription polymerase chain reaction; GST, glutathione *S*-transferase; SSC, saline sodium citrate; PCA, principal components analysis

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the binding of many other transcription factors to promoter response elements is affected by cellular redox status and may contribute to gene expression, Nrf-2 binding to ARE/EpRE appears critical for induction of well-characterized oxidant stress-responsive genes, such as superoxide dismutase, catalase, glutathione synthases and transferases, and NADPH:quinone oxidoreductases [5]. Reactive metabolites formed in the liver are generally strong inducers of phase II/conjugation enzymes (glutathione transferases, UDP glucuronyltransferases, sulfotransferases), which comprise a subset of the Nrf-2-inducible genes.

The relevance of oxidative stress in drug safety evaluation of prospective pharmaceuticals is unclear. While the present study focused on high doses of hepatotoxicants, there is increasing evidence that low doses of toxicants particularly oxidant stressors, may have beneficial adaptive or hormetic effects. Mild oxidant stressors that induce protective enzymes but minimal cell damage have been touted as chemopreventive (anticancer) agents [6,7]. Effects of sulforaphane and 1,2-dithiole-3-thione, two chemopreventive compounds derived from broccoli, have been studied in Nrf-2-knockout and control mice using microarray technology [8,9]. A comprehensive list of oxidant stress- and Nrf-2-responsive genes has been compiled for mouse intestine [8] and mouse liver [9]. However, compounds that induce oxidative stress or give rise to reactive metabolites frequently are not overtly toxic, particularly in the rat.

Conversely, many innocuous or protective Nrf-2 activating compounds, such as many anti-oxidants, abruptly become hepatotoxic at high doses [10–12]. Compounds that potently induce oxidant stress-responsive genes may not be good candidates for development into therapeutic drugs, since these compounds or their metabolites are clearly reactive inside cells. Oral administration of such compounds often leads to oxidative stress and covalent binding to proteins in the gut and liver after absorption. There is evidence that some human populations may be deficient at handling oxidative stress/reactive metabolites [13–16].

In the present study, a training/testing set of OS/RMs, cDNA microarrays and commercially available clustering and gene selection algorithms were used to determine a transcriptional profile for OS/RMs. A goal of our toxicogenomics program is to be able to detect as many classes of toxicants as possible before their pathologies are manifest and prior to gene changes becoming secondary to the pathology; hence, the focus was on changes that occur 24 h after administration of compound. Using a broad coverage of compounds and a 'guilt by association' approach, the OS/RM potential of approximately 100 toxicants was characterized in rat liver.

A number of our prototypical compounds are well-characterized oxidant stressors or are documented to give rise to reactive molecules and metabolites. Reactive electrophilic compounds or metabolites that conjugate with and deplete cellular glutathione levels comprise many of the best characterized hepatotoxicants, such as bromobenzene [2,17], precocene I [18], pulegone [19] and hexachlorocyclohexane γ (lindane, [20]). Redox cycling and generation of reactive oxygen species have been postulated to explain the toxicities of NSAIDs [21,22], estrogens [23,24], troglitazone [25], aniline (in the spleen, [26]), paraquat (in the lung, [27]) and doxorubicin (in the heart, [28]). Phase II enzyme induction, in particular glutathione transferase induction, has been presented as a hallmark of OS/RMs, such as the hepatotoxins 4-methylthiazole [29] and trans-anethole [30]. Even many relatively innocuous compounds, which possess protectant anti-oxidant activity at low doses, become strong oxidant stressors/reactive electrophiles (OS/REs) and hepatotoxicants at high doses; these compounds include butylated hydroxytoluene [31], tannic acid [32], disulfiram (or rather its reactive metabolite diethyldithiocarbamate) [33] and piperonyl butoxide [34]. Additionally, OS/REs with planar structures can intercalate into DNA, and if the cells survive the resultant damage, carcinogenesis can occur. Redox cycling and reactive electrophiles appear central to the effects of carcinogens and antineoplastic drugs [35]. Macrophage and neutrophil activators, such as LPS, zymosan and concanavalin A are often viewed as profound oxidative stressors [36,37]; however, cytokines (such as TNFa) and the extra-parenchymal derived superoxide, nitric oxide and peroxynitrate result in hepatic gene expression responses distinct from those produced by intracellular generated reactive molecules [38]. Similarly, peroxisome proliferators also induce a specialized type of oxidative stress that is easily distinguished from oxidative stress caused directly by reactive compounds or their metabolites [25,38,39].

2. Materials and methods

2.1. Compounds

Compounds were obtained from Sigma–Aldrich (St. Louis, MO), except for troglitazone and WY14643, which were from Biomol (Plymouth Meeting, PA).

2.2. In vivo studies

A detailed description of in life studies was given previously [38]. Single, high doses of chosen compounds were administered to male, 7-week-old Sprague–Dawley rats (Charles River Laboratories, Inc.), and rats were killed and livers removed 24 h later. High toxic doses were selected typically as 30–50% of the published LD50s for compounds and any adverse effects noted at necropsy were recorded (Appendix A in [38]). Compounds that produced minimal gene changes in liver and no obvious toxic effects were given at a higher dose. Several compounds—including some putative oxidative stressors—

produced minimal gene changes in the liver even at the highest dose examined and were essentially indistinguishable from vehicle treatments or untreated control samples: quercetin (4 g/kg), amiodarone (1 g/kg), troglitazone (500 mg/kg), paraquat (100 mg/kg), famotidine (500 mg/kg), metformin (750 mg/kg), gabapentin (3000 mg/kg) and glycine (500 mg/kg i.p.). In all instances, the animals were humanely handled in accordance to IACUC guidelines.

2.3. RNA isolation

A strip of liver from the medial lobe (approximately 200 mg) was snap-frozen in liquid nitrogen. Liver samples were stored at −70 °C until RNA extraction. Total RNA was extracted using Qiagen RNEasy Midi kits as per kit instructions. Amount of RNA in samples was determined spectrophotometrically by absorbance ratio at 260 and 280 nm. Quality of RNA in samples was assessed using rRNA peaks determined by an Agilent 2100 Bioanalyzer.

2.4. RNA and probe preparation for microarray analysis

One round of T7 polymerase-based linear RNA amplification was performed by reverse transcription of RNA with a T7 promoter oligo(dT) primer. Cy3-dCTP labeled fluorescent cDNA probe was synthesized from the amplified RNA as described [40]. Probes were then purified with a PCR purification kit (Qiagen, Inc. Valencia, CA), vacuum-dried and resuspended in 55 μL of hybridization buffer (Version 2 hybridization buffer (Amersham Pharmacia Biotech, Piscataway, NJ) with 50% formamide) containing rat Cotl DNA (Applied Genetics Laboratories, Melbourne, FL).

2.5. Microarrays

cDNA microarrays were prepared as described previously [38]. Clones for genes of interest were obtained from Research Genetics (IMAGE consortium), sequence verified, PCR amplified, purified and spotted in duplicate on aminosilane-coated slides (Corning) using a contact pin microarrayer (Generation III Array Spotter, Molecular Dynamics). Genes were selected on the basis of clone availability (and ESTs were generally avoided) and no effort was made to enrich for toxicity-related genes. Each slide contained two identical panels of 3434-gene spots. The list of genes on our cDNA microarray (Appendix B) is available at: http://www.mimicell.com/lps/.

The sample cDNA probes were hybridized to the microarrays and washed as described previously [38]. After drying, the microarrays were scanned with a confocal laser scanner (Array Scanner, Molecular Dynamics). Intensity values for each spot of the array were obtained using Autogene software (BioDiscovery).

2.6. Microarray data normalization

Data were normalized, day-to-day hybridization differences corrected and outlier data removed as described in detail previously [38]. In short, gene responses were measured in quadruplicate, data were Spline normalized and geometric mean of controls was used to correct for day-to-day variability.

2.7. Gene selection

Fifteen compounds that have been well documented in the literature to induce oxidative stress in rat liver were chosen to be the initial OS/RM evaluation set. Clustering was used to compare gene selection methods. All data were normalized as described previously [38]. Microarray samples with selected genes were hierarchically clustered using a complete-linkage algorithm and Euclidean distance metrics. The clustering and visualization were performed with OmniViz Pro™ software version 3.7 (OmniViz Inc. Maynard, MA).

Initially, samples were clustered with all genes on the microarray; subsequently, univariate and multivariate supervised gene selection approaches were applied to identify a set of genes predictive of the OS/RM class of compounds. Selecting genes that were significantly (Student's *t*-test) changed by compounds in the OS/RM evaluation set relative to the rest of the database was a typical supervised approach. Genes with the highest significance (lowest *P*-values) were selected. Although the number of genes (100) selected was somewhat arbitrary, our intension was to elicit the best possible performance with this method as defined by unsupervised clustering. The closer the compounds in the OS/RM evaluation set clustered together (and separated from other compounds), the better the gene set performed.

A multivariate approach to gene selection was also used, which involved iterative evaluation of gene sets for their ability to separate compounds in the OS/RM evaluation sets from other compounds. Redundant genes (which change across the sample database with very similar patterns) were avoided in this approach since they did not contribute much to the total variance across the data. Linear discrimination analysis was used to evaluate the gene set at each step and a posterior error score was given to represent the performance of the gene set. Higher performance meant higher separation between OS/RM and the other classes. Genes that contributed the least to improving performance were eliminated from the gene set. Gene selection and evaluation was aided by Partek™ software (Partek Inc., St. Charles, MO). Taqman RT-PCR (ABI Prism 7900 HT Sequence Detection System and Software, Applied Biosystems, Foster City, CA) was used in several cases to confirm differential expression of genes identified by microarray analysis.

Before supervised gene selection, to evaluate the performance of a gene set objectively, the database was

divided into a training set (~70% of the samples) and a testing set (~30% of the samples). Genes were selected based on their expression pattern solely from the training set. Clustering of the training samples was conducted to initially evaluate the performance of the gene sets. The best gene set was then validated with samples from the testing set by clustering.

3. Results

A set of hepatotoxicants was selected to give broad coverage of OS/RMs: butylated hydroxytoluene (BHT), bromobenzene, disulfiram, 4-methylthiazole, piperonyl butoxide, pulegone (pennyroyal oil), 2-acetamidofluorene (2-AAF), precocene I, aniline, dieldrin, tannic acid (tannin), hexachlorocyclohexane γ (lindane), trans-anethole, ethinyl estradiol and nimesulide. The liver tissue from animals treated with the 15 chosen compounds showed transcriptional activity (Table 1) distinguishable from control liver samples. Using all the genes on the microarray (listed in Appendix B), there was no evident co-clustering of all these compounds (Fig. 1A) and eliminating unresponsive genes from the analysis (i.e., genes that did not change at least two-fold in response to any treatment) also did not cause these compounds to co-cluster (Fig. 1B).

From the chosen OS/RMs a 12-compound training set was selected with a preference for compounds that have few other acute modes of action: butylated hydroxytoluene, bromobenzene, disulfiram, 4-methylthiazole, piperonyl butoxide, pulegone, 2-acetamidofluorene, precocene I, aniline, trans-anethole, dieldrin (30 mg/kg) and hexachlorocyclohexane γ (40 mg/kg). Ethinyl estradiol was placed in a testing set for later use, as this hormone was expected to have pronounced effects on hepatic gene expression beyond its oxidant stress effects. Nimesulide, tannic acid and samples from repeated experiments with higher doses of hexachlorocyclohexane γ (65 and 80 mg/kg) and dieldrin (45 mg/kg) were also placed in the testing set.

The 100 genes that most effectively distinguished the training set compounds from all other compounds were selected using a statistical cut-off (Fig. 2). Using this gene set, all but one replicate sample from the 12 training set compounds (35 individual rat liver samples, green) tightly co-clustered and defined an OS/RM cluster (about 20% of all of our samples). All 18 testing set OS/RM samples (red) co-clustered with the training oxidant stressors indicating that the selected gene set correctly predicted oxidant stressors from outside the training set (Fig. 2). Other compounds with multiple replicates that clustered among oxidant stressors included: flutamide, phenacetin, pregnenolone 16α carbonitrile, sulindac, bile duct toxins methylenedianiline and α-naphthyl isothiocyanate (ANIT), metoprolol, fluoxetine, valproic acid and erythromycin estolate. The 100-gene set was selected for its ability to separate OS/RM compounds from compounds with other modes of toxicity. Interestingly, this same gene set was also capable of distinguishing between two subclasses of OS/RM. The first was composed of compounds, where oxidant stress is the primary mode of action, while the second class (Fig. 2 blue) has also been shown to induce macrophage activation (allyl alcohol, carbon tetrachloride, coumarin, thioacetamide, dimethylnitrosamine, galactosamine, concanavalin A, gadolinium chloride and high dose flufenamic acid, 250 mg/kg, as well as LPS and zymosan A [38]).

To further refine the OS/RM gene set, a multivariate approach (Partek) was applied to identify the most informative 25-gene subset of the 100 genes identified using the statistical cut-off (Table 2). Clustering with this smaller, ''improved'' gene set (Fig. 3) separated the training/testing compounds into two distinct clusters. One oxidant stress

Table 1
OS/RE selected for training/testing and the number of genes induced or repressed 1.5-, 2-, and 4-fold by these compounds relative to vehicle

Compound	Dose (mpk)	1.5-Fold mean ± S.E.M.	2-Fold mean ± S.E.M.	4-Fold mean ± S.E.M.
Acetamidofluorene	200	147.3 ± 4.2	44.7 ± 3.8	8 ± 1.2
Aniline	200	88.7 ± 14.1	16.3 ± 2.1	2 ± 0.5
Bromobenzene	900	506.3 ± 44	202 ± 26.3	26.3 ± 2.8
Butyl hydroxytol	1000	272.3 ± 19.7	90 ± 7.8	15.3 ± 0.7
Dieldrin	30	177 ±23.8	60.7 ± 7.5	9 ± 0.8
Dieldrin	45	153.7 ±14.3	47 ± 7.4	5.7 ± 2.4
Disulfiram	2000	214 ± 18	65 ± 8.6	8 ± 1.2
Ethinyl estradiol	500	421.3 ± 21.3	138.7 ± 10.1	21.3 ± 1.9
Hexachlorocyclohexane γ	40	180, 133	52, 26	5, 3
Hexachlorocyclohexane γ	65	123.7 ± 12.8	41.7 ± 4.8	5.7 ± 1.0
Hexachlorocyclohexane γ	80	132, 105	36, 32	1, 1
4-Methylthiazole	120	129 ± 11.1	21.3 ± 1	4.7 ± 0.7
Nimesulide	500	337.7 ± 19.3	114 ± 5	10.3 ± 1
Piperonyl butoxide	4000	290 ± 88.9	82.7 ± 41.5	9.3 ± 6.4
Precocene I	500	271, 133	69, 27	5, 3
Pulegone	400	335.3 ± 22.6	107.3 ± 19.5	11.7 ± 2.2
Tannic acid	3000	448.7 ± 21	155.7 ± 11.8	9.3 ± 0.3
Trans-anethole	600	277.3 ± 6	60 ± 6.5	4.3 ± 1.9

Numbers of changing genes represent mean of results from three rats in most cases; individual results are given where data exists for only two rats.

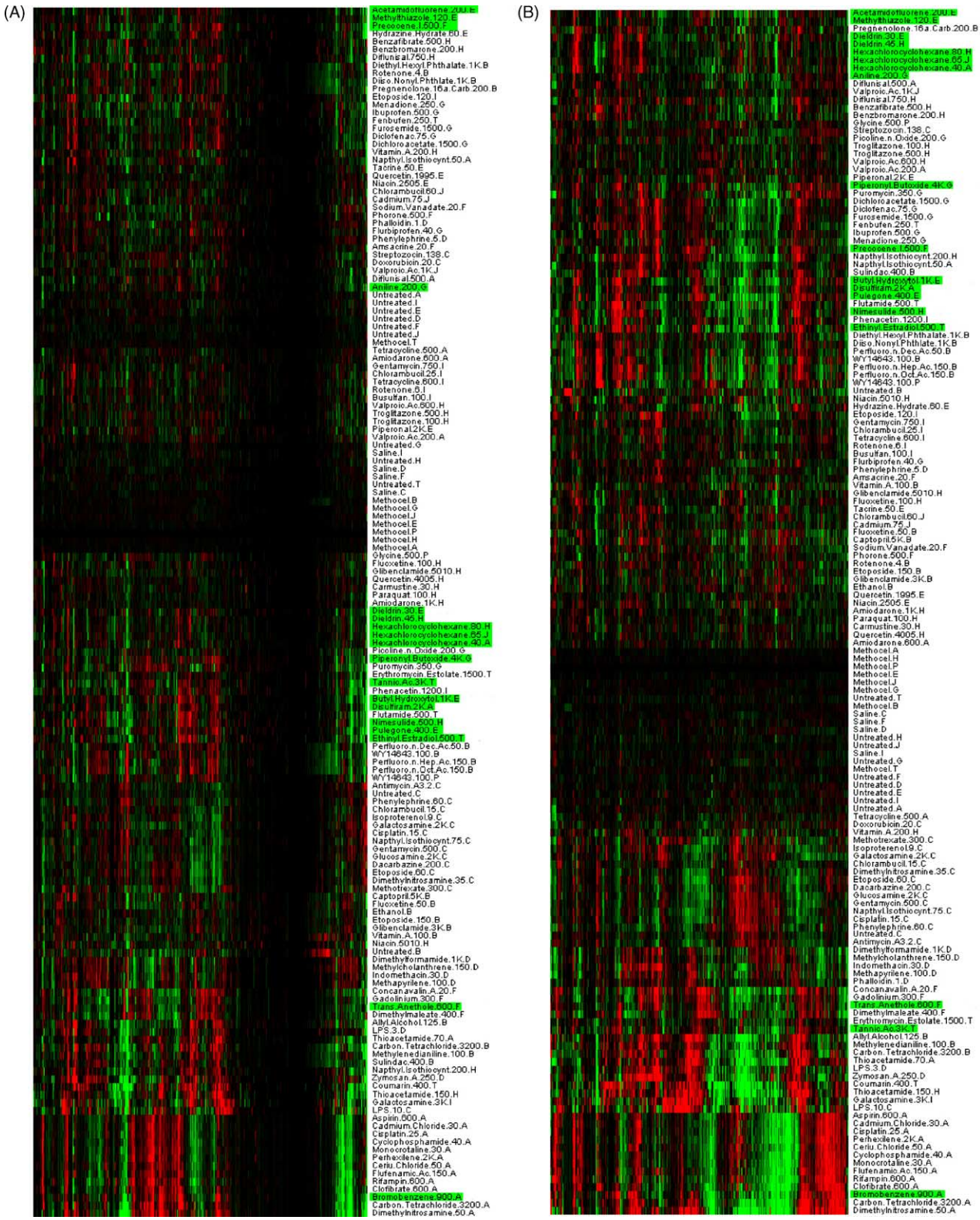

Fig. 1. (A) Clustering of Hepatotoxicants (rows) using all 3434 genes (columns) on the Rat MegaA cDNA chip. Magnitude of gene changes is given with red indicating two-fold increase and green indicating two-fold decrease in gene expression. Black indicates relatively little change. Each compound label represents the average of three replicate samples for that treatment. The number, capital letter and number lower case letter after compound name indicate dose (mg/kg), treatment group and hybridization group. Compounds highlighted in green were selected as training/testing sets of oxidant stressors/ reactive electrophiles. (B) Clustering of hepatotoxicants using only the 1050 genes that change at least two-fold in response to at least one treatment. Note that although some oxidant stressors cluster together in both A and B, there is no single cluster.

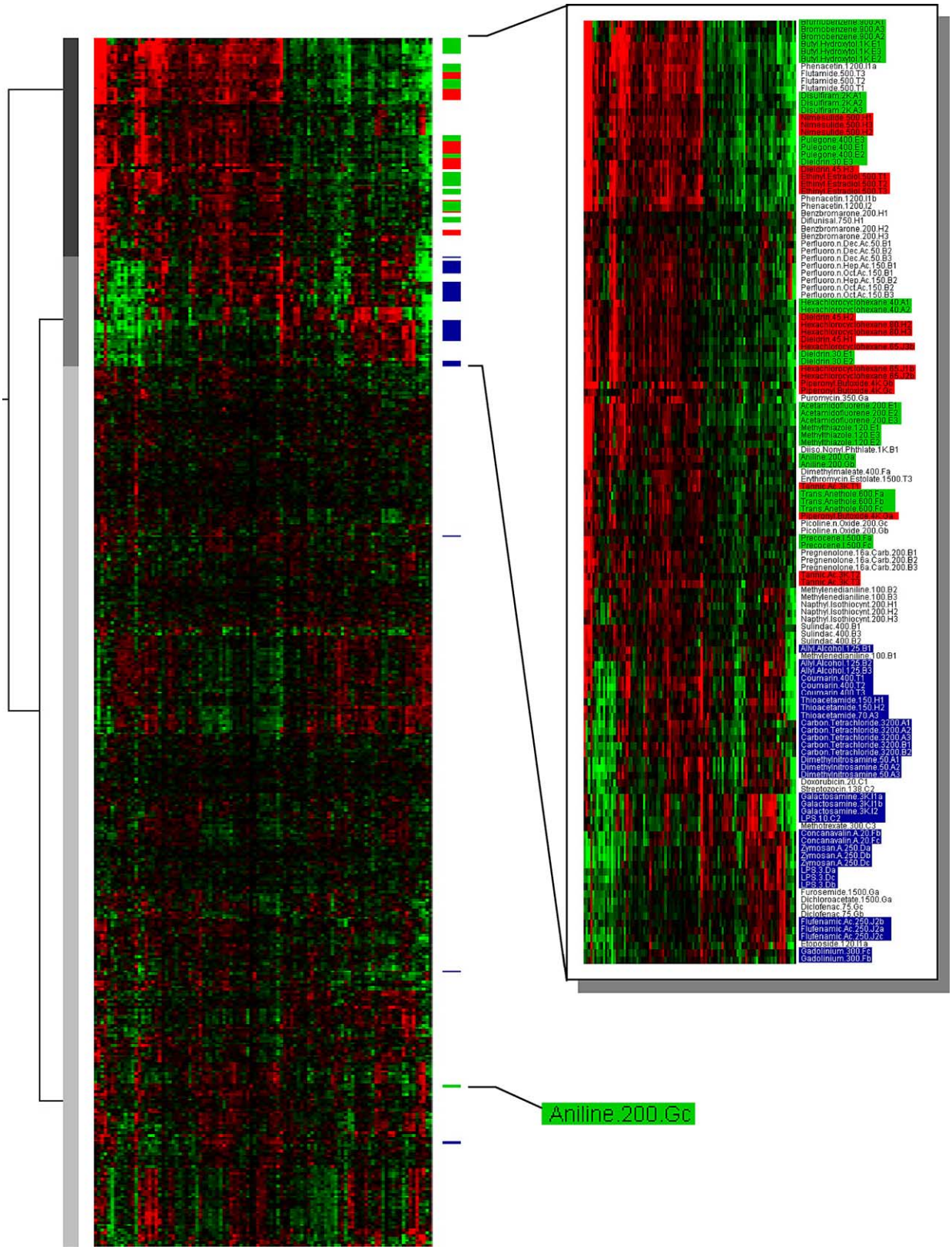

Fig. 2. Clustering of Hepatotoxicants using 100 genes expressed significantly differently between the training set of oxidant stressors/reactive electrophiles (green) and all other treatment and control groups (see Section 2 for details). Magnitude of gene changes is given with red indicating two-fold increase and green indicating two-fold decrease in gene expression. Individual replicate samples from the treatments are clustered and the blowup of the top two clusters (from the abbreviated dendrogram on the left) shows that all training set replicates except one aniline-treated sample cluster nicely together. The samples highlighted in red indicate testing set oxidant stressors; these samples also co-cluster with the oxidant stressor training set of samples. The blue highlighted samples indicate samples previously associated with macrophage activation: LPS (3 mg/kg), zymosan A (250 mg/kg), concanavalin A (20 mg/kg), gadolinium chloride

Table 2
OS/RE gene set

Accession	Gene name
AA892916	EST
BG668317	**Hsp90 alpha**
BI295979	**EST**
M17412	**LOC60380—growth and transformation-dependent protein**
NM_012580	**Hmoxi—heme oxygenase 1**
NM_012603	**Myc—v-myc avian myelocytomatosis viral oncogene homolog**
NM_012639	**Rafl—murine leukemia viral (v-raf-1) oncogene homolog 1**
NM_012731	**Ntrk2—neurotrophic tyrosine kinase, receptor, type 2**
NM_012844	**Ephx1—epoxide hydrolase 1**
NM_013215	**Afar—aflatoxin B1 aldehyde reductase**
NM_017000	**Nqo1—NAD(P)H dehydrogenase quinone 1**
NM_017013	**Gsta2—glutathione-S-transferase, alpha type 2**
NM_017014	**Gstm1—glutathione S-transferase, mu 1**
NM_017073	Gins—glutamine synthetase 1
NM_017113	Grn—granulin
NM_017305	**Gclm—glutamate cysteina ligase, modifier subunit**
NM_019195	Cd47—integrin-associated protein
NM_021766	**Pgrmd—progesterone receptor membrane component 1**
NM_022251	**Enpep—aminopeptidase A**
NM_022407	**Aldh 1 a 1—aldehyde dehydrogenase family 1, member A1**
NM_022592	**Tkt—transketolase**
NM_031325	**Ugdh—UDP-glucose dehydrogeanse**
U28504	**Slc17a1—solute carrier family 17 vesicular glutamate transporter**
X02904	**Gstp2—glutathione S-transferase, pi 2**
Y00480	Rat (diabetic BB) MHC class II alpha chain RT1.D alpha (u)

After selecting the 100 genes that most significantly separated our training set oxidative stressors from all other compounds, a multivariate algorithm (Partek) was used to select a subset of these genes that maximized variance and thus minimized redundant genes. Bolded genes represent induction and non-bolded genes repression by oxidant stressors relative to vehicle. Shaded genes are common to a 36-gene subset represented on our microarray reported to respond to Nrf-2 in mouse liver (Kwak et al, 2003).

cluster cleanly separated from all other compounds and included multiple replicates of bromobenzene, butylated hydroxytoluene, disulfiram, 2-acetamidofluorene, aniline, pulegone, 4-methylthiazole, nimesulide and ethinyl estradiol, as well as phenacetin and flutamide. The second cluster was more similar to other compounds in the database and included trans-anethole, tannic acid, precocene I, multiple doses of dieldrin and hexachlorocyclohexane γ, and piperonyl butoxide. Non-training/testing compounds in this second cluster included multiple replicates of pregnenolone 16α carbonitrile, sulindac and methylenedianiline. Again most of the macrophage activators (blue) clustered away from other compounds using these 25 genes; many of these OS/RM signature genes were repressed by macrophage activators (Fig. 3).

3.1. Nrf-2-induced genes

Lists of Nrf-2-regulated genes were recently reported for mouse intestine and liver [8,9]. Since Nrf-2 is the major transcription factor regulating the anti-oxidant/electrophile response element, the overlap between our oxidant stress 25-gene set and the reported Nrf-2-regulated genes in the mouse liver (Table 2, highlighted in grey) was investigated. Of the 25 differentially expressed genes in our set, only six genes were also regulated by Nrf-2 in the rat liver: NAD(P)H: menadione oxidoreductase (NMOR, diaphorase), microsomal epoxide hydrolase, GST mu type 2, GST alpha type, HSP90 alpha and glutamate-cysteine ligase (γ-glutamylcysteine synthetase). Even so, clustering with the 36 mouse liver-derived Nrf-2-regulated clones common to our microarray gives results similar to our oxidant stressor training set approach (compare Figs. 3 and 4). The oxidant stressors from our training/testing sets (green) separate into the same two clusters using the Nrf-2-regulated genes (Fig. 4). In addition to our testing/training oxidant stressors and flutamide, phenacetin, methylenedianiline, sulindac and pregnenolone 16α carbonitrile, multiple replicates of the NSAIDs ibuprofen and fenbufen, the bile duct toxin α-naphthyl isothiocyanate and all three replicates of allyl alcohol co-clustered using this mouse liver set of genes (Fig. 4). Similar results were obtained with the intestinal Nrf-2-induced gene set (data not shown).

3.2. Macrophage activators and peroxisome proliferators

The macrophage activators cleanly separated from other compounds even using the OS/RM signature genes (detailed above) and the peroxisome proliferators had opposite effects on many genes in macrophage activator signature gene sets [38]. Although macrophage activators and peroxisome proliferators are usually thought to be oxidative stressors in the liver, there were no genes that were common to the three signature gene sets for these classes of compounds. Even when comparing just to controls and taking the 100 most significant gene changes for each class, only three genes were found in common for these 'oxidative stressors': induced α-tubulin (tuba-1, NM_006082), repressed EST (NM_022407) and aldehyde dehydrogenase (Aldhlal, NM_022407), which was induced by OS/RM and peroxisome proliferators but repressed by macrophage activators.

On the other hand, it was relatively easy to define a subset of genes that could maximally separate macrophage activators, peroxisome proliferators and OS/RMs from each other, and from controls and other compounds (Fig. 5; Table 3).

(300 mg/kg), allyl alcohol (125 mg/kg), carbon tetrachloride (3200 mg/kg), dimethylnitrosamine (50 mg/kg), galactosamine (3000 mg/kg), thioacetamide (70 and 150 mg/kg), coumarin (400 mg/kg) and high dose flufenamic acid (250 mg/kg). These macrophage activator-like compounds cluster together; note the strong differential regulation of many genes relative to the oxidant stressor cluster.

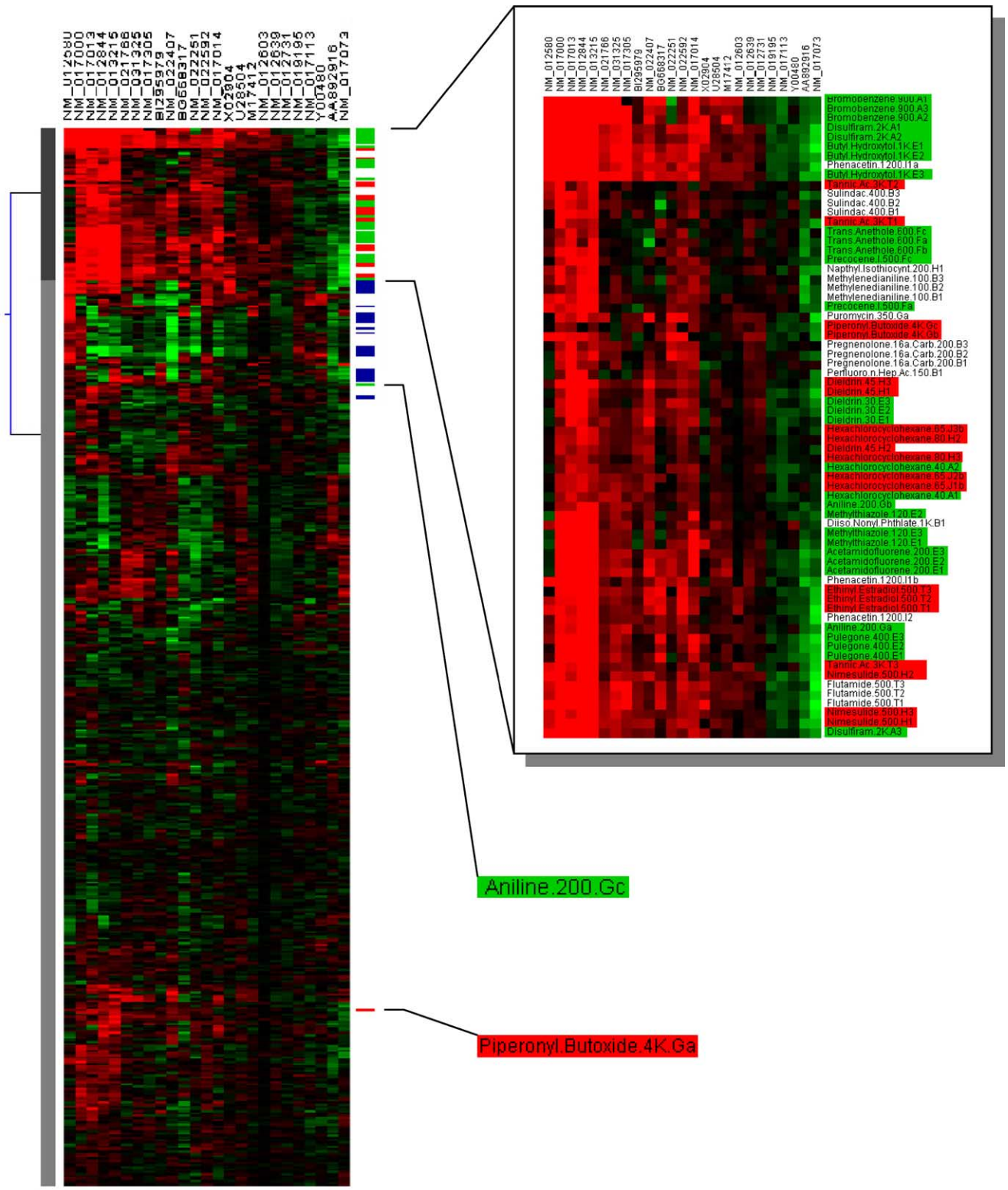

Fig. 3. Clustering of OS/RE using a subset of 25 genes (Table 2) selected from the 100 genes in Fig. 2 by a multivariate approach (Partek). The accession numbers of the 25 genes are given across the top. Individual replicate samples of the training set (green) and testing set (red) of oxidant stressors are indicated. Note the separation of oxidant stressors into two clusters. The blue highlighted samples indicate samples previously shown to be associated with macrophage activation (see Fig. 2 legend).

4. Discussion

In the present study a testing/training set approach was used to identify a gene transcriptional profile for OS/RMs in rat liver and to characterize, which of about 100 paradigm compounds are behaving as oxidative stressors at high dose 24 h after administration. It was previously determined that most genes even on a focused microarray are not useful for

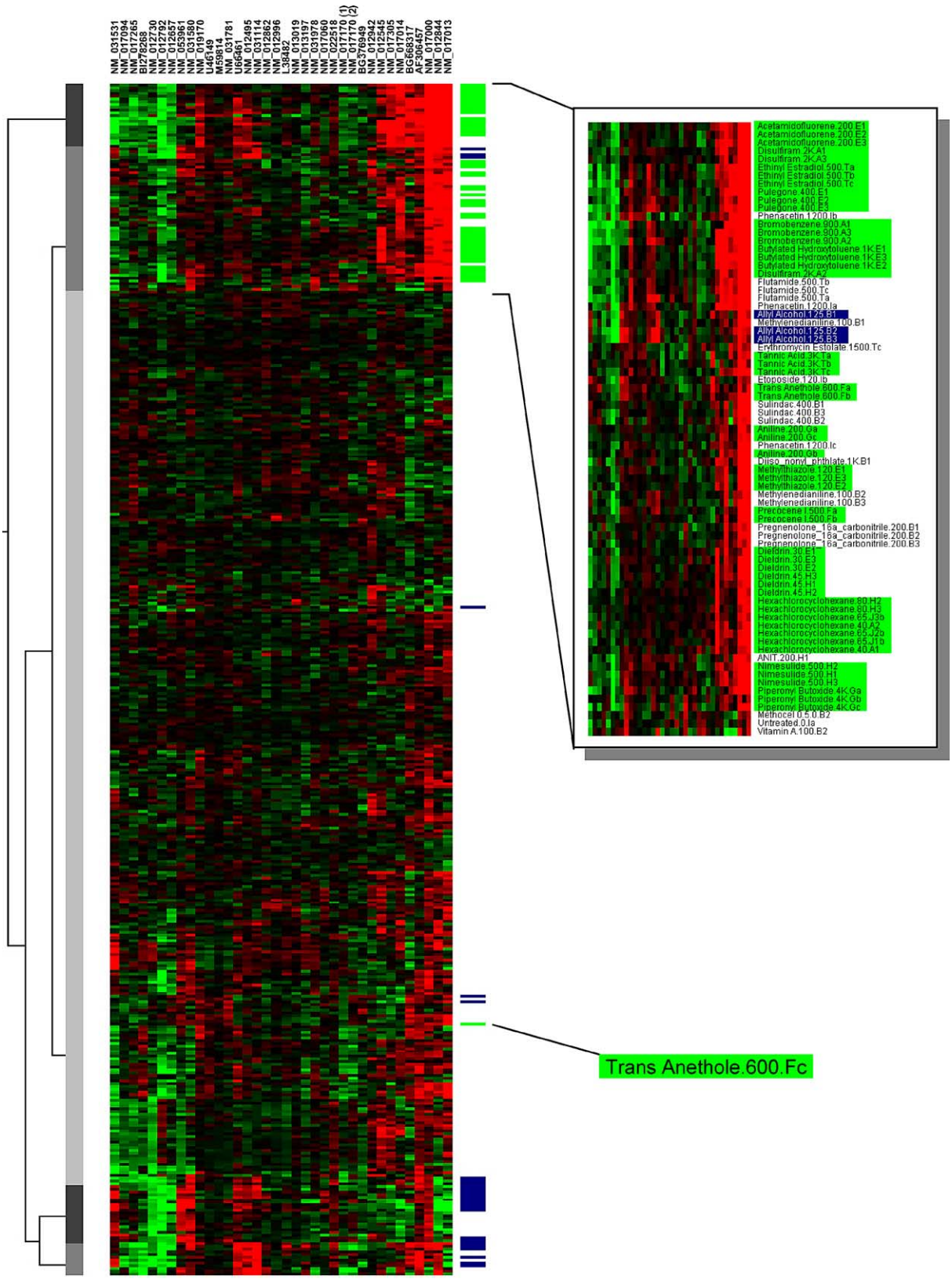

Fig. 4. Clustering of OS/REs using a list of Nrf-2-regulated genes. Our cDNA microarray contained 36 clones, which matched genes induced by Nrf-2 in mouse liver (Kwak, 2003). The accession numbers (rat/mouse) of the genes are given. Using these genes, two clusters of our training/testing sets of oxidant stressors (green) were obtained (compare to Fig. 3. The blue highlighted samples indicate samples previously associated with macrophage activation (see Fig. 2 legend).

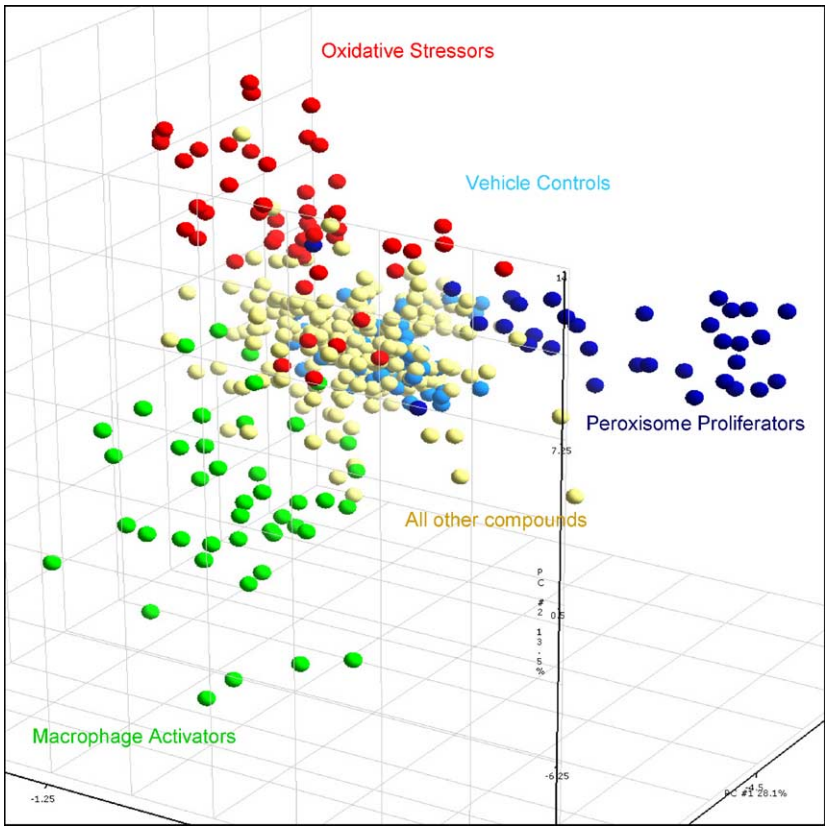

Fig. 5. Principal component analysis plot using genes from Table 3 to separate OS/RM, macrophage activators, peroxisome proliferators from each other and from controls and other compounds. Each dot represents one treated or control rat sample. Despite sharing some biochemical/metabolic endpoints, the gene expression responses for these three classes of 'oxidative stressors' are easily distinguishable.

distinguishing types of hepatotoxicities [41], and several methods to select gene sets most specific to certain types of toxicities have been attempted. Combining univariate and multivariate gene selection approaches yields a small signature set of genes for OS/RMs, and relatively few other paradigm compounds co-clustered with our training/testing set of OS/RMs using these genes. This signature gene set agrees remarkably well with oxidant stress-responsive gene sets derived in Nrf-2-knockout mice in liver and intestine [8,9]. A similar clustering of paradigm compounds was obtained using our signature gene set and using homologs on our rat microarray of the recently reported chemopreventive-induced gene set derived from a study in Nrf-2-knockout mouse liver [9].

Previously our group has characterized 24 h liver transcriptional responses to macrophage activators and peroxisome proliferators [38], two classes of compounds reported to be oxidative stressors in the liver [25,36,37,39]. Macrophage activators and peroxisome proliferators are readily detected, since their gene signature profiles are distinctly different from those obtained with other hepatotoxins; a small training set of compound-treated samples is sufficient to obtain robust gene signature profiles. In contrast, determination of gene sets sufficient for clustering of OS/RMs was dependent on a relatively large training set of known oxidant stressors. Selection of training/testing oxidant stressors was somewhat arbitrary. Literature references were used to determine compounds that gave robust transcriptional responses and avoid compounds previously found to have macrophage activation or peroxisome proliferator activities (compare [42]). A misclassified compound would probably be tolerated in such a large training/testing set and would also be apparent after clustering. OS/RM hepatotoxicants were used with as few other characteristic modes of action as possible for our training set—butylated hydroxytoluene, trans anethole, precocene I, tannic acid, piperonyl butoxide, dieldrin and hexachlorocyclohexane γ—and in addition to these relatively "pure" OS/RMs we added representative compounds for oxidant stressor classes: nimesulide, an NSAID [22], ethinyl estradiol, a steroid [23,24], 2-acetamidofluorene, a carcinogen [43], aniline, a bile duct toxin [26], bromobenzene, a solvent giving rise to reactive electrophiles [2,17] and disulfiram, a sulfhydryl-containing therapeutic drug with anti-oxidant activity at low doses but glutathione depletion and toxicity at very high dose [33]. Given that most compounds causing hepatotoxicity have been designated "oxidative stressors" by one investigator or another, it was surprising that so few additional samples co-clustered with the training set: samples treated with the NSAIDs sulindac and phenacetin [44], the steroids flutamide [45] and pregnenolone 16α carbonitrile and the bile duct toxin methylenedianiline [46].

Table 3
Gene set which maximally separates macrophage activators, peroxisome proliferators and OS/RM from each other and from controls and other compounds

Accession	Gene name
AA849738	EST192505 normalized rat muscle
AA858899	EST
AA892916	EST
AB011679	Tubb5—tubulin, beta 5
AF058791	G10—maternal G10 transcript
BE098355	EST
BE110688	EST
BF549851	EST
BG373221	EST
BG668317	Hsp90 alpha
BI285007	EST
BI285489	EST
BI295979	EST
D16478	Hadha—hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A hiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit
M11794	Mt1a—Metallothionein
M17412	LOC60380—growth and transformation-dependent protein
M30596	Me1—malic enzyme 1
M58041	Cyp2c22—cytochrome P4502c22
NM_012580	Hmoxi—heme oxygenase 1
NM_012603	Myc—v-myc avian myelocytomatosis viral oncogene homolog
NM_012639	Raf1—murine leukemia viral (v-raf-1) oncogene homolog 1 (3611-MSV)
NM_012731	Ntrk2—neurotrophic tyrosine kinase, receptor, type 2
NM_012747	Stat3—signal transducer and activator of transcription 3
NM_012844	Ephxi—epoxide hydrolase 1
NM_012930	Cpt2—carnitine palmitoyltransferase 2
NM_012998	P4hb—prolyl 4-hydroxylase, beta polypeptide
NM_013078	Otc—omithine transcarbamylase
NM_013214	Bach—brain acyl-CoA hydrolase
NM_013215	Afar—aflatoxin B1 aldehyde reductase
NM_016990	Add1—adducin 1, alpha
NM_016999	Cyp4b1—cytochrome P450, subfamily 4B, polypeptide 1
NM_017000	Nqo1—NAD(P)H dehydrogenase, quinone 1
NM_017013	Gsta2—glutathione-S-transferase, alpha type2
NM_017014	Gstml—glutathione S-transferase, mu 1
NM_017051	Sod2—superoxide dismutase 2
NM_017073	Gins—glutamine synthetase 1
NM_017075	Acati—acetyl-coenzyme A acetyltransferase 1
NM_017113	Grn—granulin
NM_017177	Chetk—choline/ethanolamine kinase
NM_017199	Ssr4—signal sequence receptor 4
NM_017305	Gclm—glutamate cysteine ligase, modifier subunit
NM_017306	Dei—dodecenoyl-coenzyme A delta isomerase
NM_017321	Ratireb—iron-responsive element-binding protein
NM_017340	RATACOA1—acyl-coA oxidase
NM_019195	Cd47—ntegrin-associated protein
NM_021766	Pgrmci—progesterone receptor membrane component 1
NM_022251	Enpep—aminopeptidase A
NM_022282	Dlg2—discs, large (Drosophila) homolog 2 (chapsyn-110)
NM_022298	Tubai—alpha-tubulin
NM_022399	Calr—calreticulin
NM_022407	Aldh1a1—aldehyde dehydrogenase family 1, member A1
NM_022592	Tkt—transketolase
NM_022594	Ech1—enoyl coenzyme A hydratase 1
NM_030656	Agxt—alanine-glyoxylate aminotransferase
NM_030850	Bhmt—betaine-homocysteine methyltransferase
NM_031315	Cte1—cytosolic acyl-CoA thioesterase 1
NM_031325	Ugdh–UDP-glucose dehydrogeanse
NM_031561	Cd36—cd36 antigen
NM_031565	Ces1—carboxylesterase 1
NM_031580	Grp58—glucose regulated protein, 58 kDa
NM_031839	Cyp2c23—arachidonic acid epoxygenase
NM_031853	Dbi—diazepam binding inhibitor
NM_032614	Txnl2—thioredoxin-like 2
U13253	Fabp5—fatty acid binding protein 5, epidermal
U19485	LOC94168—spp-24 precursor
U28504	Slc17a1—solute carrier family 17 vesicular glutamate transporter), member 1
X02904	Gstp2—glutathione S-transferase, pi 2
X05341	Acaa2—acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl- Coenzyme A thiolase)
Y00480	Rat (diabetic BB) MHC class II alpha chain RT1.D alpha (u)

Since there was minimal overlap in statistically most responsive gene sets for each of toxicity class relative to control, gene sets for each class were selected individually using the multivariate approach described in the methods and aided by Partek™ software. These gene sets were then combined to give the final gene set.

No DNA-damaging carcinogens/antineoplastic/venoocclusive agents besides 2-acetamidofluorene, which was the most transcriptionally active of this class and is a traditional hepatocarcinogen and oxidant stressor [43] clustered with the OS/RMs. This was surprising since many of these agents or their metabolites are reactive electrophiles. Most of these compounds seem selectively toxic to other tissues (endothelium, kidney, bone marrow) and the liver may not be a significant target of their toxicities even at the high doses used. Similar tissue-specific effects may explain the apparent lack of oxidant stress with the robust redox cyclers paraquat (lung) [27] and doxorubicin (heart) [28].

Additionally, other gene responses to certain classes of hepatotoxicants may overwhelm the oxidative stress component. This clearly seems to be the case with macrophage activators (such as LPS, zymosan, allyl alcohol, coumarin, thioacetamide, galactosamine and carbon tetrachloride) that cause at least a specialized type of oxidative stress (macrophage- and neutrophil-derived superoxide and nitric oxide) [36,47]. Many genes in our OS/RM signature set were either oppositely regulated or unaffected by macrophage activators (examples include aldehyde dehydrogenase, epoxide hydrolase 1 (microsomal), aminopeptidase A, glutathione- S-transferases and NAD(P)H: menadione oxidoreductase). Regulation of genes by peroxisome proliferators was frequently opposite of regulation by macrophage activators [38]. Some induced enzymes such as cytosolic epoxide hydrolase appear preferentially induced by peroxisome proliferators and overall we were

surprised that there was no shared set of induced genes for coping with oxidative stress.

Several investigators in the toxicogenomics field have emphasized traditional histopathological endpoints in concert with gene changes [48]. While this approach allows correlation between the two endpoints, there is the possible confounding effect of pathology causing gene expression changes (i.e., the response to damage rather than prediction of damage). Our approach has been to minimize gene responses to damage by focusing on an early time-point, where gene changes precede and predict damage (although there is evidence of adverse effects with some compounds at this time, Appendix A in [38]). For OS/RMs, 24 h seems an acceptable single time-point; many more gene expression changes were observed in mouse liver in response to the chemopreventive 1,2-dithiole-3-thione at 24 h than at 6 h or 6 days of treatment [9]. Moreover, while the OS/RMs in the present study are mostly hepatotoxicants at the high doses employed, we have observed robust hepatic oxidant stress gene responses to proprietary compounds that were not hepatotoxic after prolonged exposure in rodents, in agreement with published responses to chemopreventives. Most transcriptional responses appear to be adaptive, protective responses, and only when these mechanisms fail do stresses become toxic insults. Obviously the research community is interested in gene expression changes that can distinguish hepatotoxic oxidative stressors from well-tolerated oxidative stressors. Even so, a concern with compounds that have oxidant stressor or reactive electrophile properties but no overt hepatotoxicity in rats is that they may be candidates for some types of idiosyncratic drug reactions in man.

The inability of some individuals to properly deal with OS/RMs has been proposed to explain adverse reactions to many drugs, including NSAIDs (such as nimesulide, phenacetin and sulindac in the present study) [21,22], antiepileptics [16,49] and troglitazone [25] (which was not detected as an oxidant stressor at the dose used in the present study). The gradation between chemopreventive and toxic doses or reactivities of compounds may eventually be easy to gauge in rodents but less certain in the human population. All things being equal, compounds producing strong OS/RM responses should be avoided in choosing between drug candidates.

While the present study is focused on compounds that resemble OS/RMs in their effects on liver, our long-term objective is to identify gene signature profiles for as many different types of hepatotoxicity as possible using microarray gene expression data from a database that is still under development (at present it consists of the 100 or so paradigm compounds in Appendix A in [38]). Potential toxicities of uncharacterized compounds, such as therapeutic drug candidates, can be identified by a ''guilt by association'' approach using a broad coverage of paradigm toxicants. The statistical power increases with number of replicate samples (animals) and number of compounds in a toxicity class, so the database becomes more predictive and valuable as its size increases. While there are concerns (relevance of high doses necessary to see robust transcriptional responses, the ability of one time point to predict all toxicities of interest, and the reproducibility and stability of the microarray platform over time), our toxicogenomics results are consistent with expectations from the literature and show promise for evaluating a variety of potential adverse effects with therapeutic drug candidates, including the potential of a compound to produce oxidative stress.

Note in proof

The data, on which this and a prior study [38] were based, are being made publicly available in the Chemical Effects in Biological Systems (CEBS) toxicogenomics knowledgebase at the National Institute of Environmental Health Sciences (NIEHS) and in Tox/ArrayExpress at the European Bioinformatics Institute (EBI).

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