Interlaboratory Evaluation of Rat Hepatic Gene Expression Changes Induced by Methapyrilene

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Several studies using microarrays have shown that changes in gene expression provide information about the mechanism of toxicity induced by xenobiotic agents. Nevertheless, the issue of whether gene expression profiles are reproducible across different laboratories remains to be determined. To address this question, several members of the Hepatotoxicity Working Group of the International Life Sciences Institute Health and Environmental Sciences Institute evaluated the liver gene expression profiles of rats treated with methapyrilene (MP). Animals were treated at one facility, and RNA was distributed to five different sites for gene expression analysis. A preliminary evaluation of the number of modulated genes uncovered striking differences between the five different sites. However, additional data analysis demonstrated that these differences had an effect on the absolute gene expression results but not on the outcome of the study. For all users, unsupervised algorithms showed that gene expression allows the distinction of the high dose of MP from controls and low dose. In addition, the use of a supervised analysis method (support vector machines) made it possible to correctly classify samples. In conclusion, the results show that, despite some variability, robust gene expression changes were consistent between sites. In addition, key expression changes related to the mechanism of MP-induced hepatotoxicity were identified. These results provide critical information regarding the consistency of microarray results across different laboratories and shed light on the strengths and limitations of expression profiling in drug safety analysis.
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In recent years the field of toxicology has begun the process of integrating genomic technologies into drug safety evaluation to understand and possibly predict adverse drug side effects. New technologies allow for the identification and quantification of thousands of gene changes occurring in a cell in a single experiment. Currently, microarrays are state of the art technology for evaluation of global gene expression changes. Several studies using microarrays have shown that changes in gene expression provide crucial information regarding the mechanism of toxicity induced by xenobiotic agents, including methapyrilene (MP), Aroclor 1254, and acetaminophen (Hamadeh et al. 2002; Reilly et al. 2001; Waring et al. 2002; Waring and Halbert 2002). In addition several studies have shown that compounds associated with a particular mechanism of toxicity, such as DNA-damaging agents, Aryl hydoxylase (Ah)-receptor ligands, and peroxisome proliferators, yield similar gene expression profiles (Burczynski et al. 2000; Thomas et al. 2001; Waring et al. 2001). Despite the potential that microarray analysis offers to toxicology, many questions remain concerning the reliability and reproducibility of these assays. Perhaps of primary importance is the issue of whether gene expression profiles for a given compound will reproduce consistently from study to study and across different laboratories.

To begin to address this issue, the Health and Environmental Sciences Institute (HESI) of the International Life Sciences Institute (ILSI) (http://www.ILSI.org) formed a consortium. Over thirty pharmaceutical companies participate in this effort that focuses on three categories of toxicants, hepatotoxins, nephrotoxins, and genotoxins.

In the HESI Hepatotoxicity Working Group, one of the compounds used in the evaluation was MP. MP is a known hepatotoxin that causes periportal cell necrosis and carcinomas in rats (Cunningham et al. 1995; Ratra et al. 2000). MP is metabolized in liver mainly by phase I enzymes; it does not show mutagenic properties and does not induce DNA synthesis (Budroe et al. 1984). Hence, MP is believed to act through a nonmutagenic mechanism. In the current study we have investigated the effects of a low (presumably nontoxic) and a high (toxic) dose of this compound on hepatic gene expression, with the goal of evaluating changes across different time points and concentrations. Most important, we designed the study to assess the reproducibility of the gene changes across different laboratories. To minimize variation due to the performance of the *in vivo* studies that might be reflected on the gene expression results, animals were treated at one facility (Abbott Laboratories) and RNA was distributed to five different users of the Affymetrix GeneChip system: Boehringer Ingelheim Pharmaceuticals (BI), Novartis Pharma AG (Nov), Pfizer Inc (Pfi), F. Hoffmann-La Roche AG (RO), and Schering AG (Sch). The results showed that, despite some variability, robust gene expression changes were consistent between sites. In addition, key gene expression changes related to the mechanism of methapyrilene-induced hepatotoxicity were identified.

Materials and Methods

Test article and formulation. Methapyrilene hydrochloride (MP) (CAS no. 135-23-9, lot number 037F0929) was obtained from Sigma Chemical Corporation (St. Louis, MO). MP was formulated in water and prepared fresh daily.

Animals and treatments. The rationale for dose selection was based on prior knowledge. Acute gavage of 225 mg/kg MP caused hepatic necrosis, increased mitotic figures, and elevated serum enzyme levels characteristic of hepatotoxicity (Lijinsky et al. 1980). In a 3-day oral gavage study, 150 mg/kg/day MP resulted in periportal hepatic damage (Steinmetz et al. 1988). Previous results also showed that 50 mg/kg/day during 3 days resulted in

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minimal expression of single-cell necrosis with minimal mononuclear infiltrate without associated changes in clinical chemistry parameters (Waring et al. 2001). Thus, in the present study we chose 100 mg/kg/day as the high dose expected to elicit hepatotoxicity. A dose of 10 mg/kg/day was selected as the low dose with the expectation that no hepatotoxic effect would be observed.

Male Sprague-Dawley rats were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Rats were 57 days old and weighed 233.4–274.0 g at the start of the treatment. Upon arrival to Abbott Laboratories (Abbott Park, IL), all rats were acclimated for 6 days before treatment began. The two treatment groups comprising four rats each received the test compound at a concentration of 10 or 100 mg/kg, respectively. Animals in the equally sized control group received vehicle only.

Rats were dosed once daily by gavage for 7 days. The dose volume was 10 mL/kg. Doses were milligram salt per kilogram per day and were calculated for each rat on the basis of the most recent body weight data available. Rats were fasted overnight after their last treatment, euthanized under halothane anesthesia and submitted for necropsy. Each rat received its last treatment approximately 24 hr before scheduled necropsy.

In vivo *observations, pathology, and sampling.* All rats were observed twice each day during the pretreatment and treatment periods for survival and general condition. Blood samples were drawn from all rats, and clinical chemistry parameters were obtained for alanine aminotransferase (ALT), aspartate aminotransferase (AST), sorbitol dehydrogenase (SDH), alkaline phosphatase (ALKPHOS), total bilirubin (TBIL), glucose (GLU), and triglycerides (TRIG). At necropsy, liver was weighed and the percent of body weight of each organ was calculated. One part of the liver (left lateral lobe) was fixed for potential histopathology in 10% formalin and subsequently sectioned and stained with hematoxylin and eosin, while the rest of the organ was rinsed in phosphate-buffered saline, immediately flash-frozen in liquid nitrogen, and kept frozen for subsequent RNA isolation.

RNA Isolation and Distribution

Approximately 100 mg of tissue from each liver was placed into TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and homogenized. Total RNA isolation was performed exactly according to the TRIzol reagent protocol. The remaining portion of the liver was retained frozen. Following isolation, the RNA was quantitated using a BioRad SmartSpec 3000 spectrophotometer (BioRad, Hercules, CA), and the integrity of the RNA was determined using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). The RNA from the four animals in each treatment group was then pooled using equivalent amounts from each sample. The RNA was aliquoted and precipitated in ethanol and ammonium acetate for shipment to the participating DNA microarray users. In addition, RNA from individual animals was shipped to some of the DNA microarray analysis laboratories.

DNA microarray analysis. RNA samples were analyzed independently by five different Affymetrix users: Boehringer-Ingelheim Pharmaceuticals, Novartis, Pfizer Inc, F. Hoffmann-La Roche AG, and Schering AG using rat RGU34A expression probe arrays (Affymetrix, Santa Clara, CA), containing 8,799 probe sets interrogating primarily annotated genes. The rat sequences used for the design of the RGU34A expression probe array were derived from Build 34 of the UniGene database (http://www.ncbi.nih.gov/UniGene/; created from Genbank 107/dbEST 11/18/98) and supplemented with additional annotated gene sequences from Genbank 110 (http://www.ncbi.nih.gov/GenBank/). UniGene clusters are represented by an example sequence that is the most complete and most 3′ sequence in the cluster. The oligonucleotide probes are 25mers and 16 probe pairs per sequence are used. Processing of RNA and GeneChip experiments was carried out basically as recommended by Affymetrix, with some user-specific variations (Table 1) (Lockhart et al. 1996). An initial amount of 5–20 μg total RNA was used for the synthesis of double-stranded cDNA with a commercially available kit (Superscript Choice System; Invitrogen Life Technologies or Roche Molecular Biochemicals, Mannheim, Germany) in the presence of a T7-$(dT)_{24}$ DNA oligonucleotide primer. After synthesis, the cDNA was purified by phenol/chloroform/isoamylalcohol extraction and ethanol precipitation. The purified cDNA was then transcribed *in vitro* [Enzo Diagnostics, Inc. (Farmingdale, NY) or Ambion, Inc. (Austin, TX)] in the presence of biotinylated ribonucleotides to form biotin labeled cRNA. The labeled cRNA was then purified on an affinity resin (Rneasy; Qiagen, Inc., Valencia, CA), quantified and fragmented. An amount of 10–20 μg labeled cRNA was hybridized for approximately 16 hr at 45°C to an expression probe array. The array was then washed, stained with streptavidin-R-phycoerythrin (SAPE; Molecular Probes, Eugene, OR), and the signal amplified using a biotinylated goat anti-streptavidin antibody (Vector Laboratories, Burlingame, CA) followed by a final staining with SAPE. Arrays were stained using the GeneChip Fluidics Workstation 400 (Affymetrix) and then scanned twice using a confocal laser scanner [GeneArray Scanner 2500; Hewlett Packard (Palo Alto, CA) or Agilent Technologies], resulting in one average scanned image.

Data analysis. Tab-delimited files obtained from the Affymetrix Microarray Suite software, version 4.0, (*.chp files) and containing data on signal intensity [average difference (Avg Diff)] and categorical expression-level measurement (Absolute Call) were used for analysis. Data were normalized and further analyzed using Roche in-house developed software (RACE-A; F. Hoffmann-La Roche AG, Mannheim, Germany). Briefly, this software performs a normalization step on the signal intensities based on the average signal (Mean Avg Diff) of each microarray before calculating additional parameters. In the cases where biological replicates were included, RACE-A was also used to calculate the average signal (arithmetic mean), and SD for each probe set. Also, comparative analysis between control and treated was performed including fold change (Avg Diff Treated/Avg Diff Control) and a significance value (p-value), calculated using a two-tailed, unpaired t-test. Once the required statistical parameters were calculated, data were filtered and exported to MS-Excel 2002 (Microsoft, Corp., Bellevue, WA) or additional software for visualization and further analysis.

In addition, methods comprising more sophisticated algorithms and designed

Table 1. Sample preparation methods used by the contributing companies.

Gene expression analysis site	Acronym	Sample type	cDNA	IVT
Boehringer-Ingelheim Pharmaceuticals	BI	Pool	SSII, Invitrogen	Enzo–Affymetrix
Novartis Pharma AG	Nov	Pool	SSII, Invitrogen	Enzo–Affymetrix
Pfizer Inc	Pfi	Pool	SSII, Invitrogen	Enzo–Affymetrix
F. Hoffmann-La Roche	RO	Pool	AMV, Roche Molecular Biochemicals	Ambion, Inc.
F. Hoffmann-La Roche	RO	Individual	AMV, Roche Molecular Biochemicals	Ambion, Inc.
Schering AG	Sch	Pool	SSII, Invitrogen	Enzo–Affymetrix
Schering AG	Sch	Individual	SSII, Invitrogen	Enzo–Affymetrix

Abbreviations: AMV, avian myeloblastosis virus; Enzo-Affymetrix, Enzo Diagnostics, Inc. and Affymetrix, Inc.; IVT, *in vitro* transcription; SSII, Superscript II.

specifically for multivariate data analysis such as microarray data were employed. These methods share the characteristics of reducing the dimensionality of the data to a number of dimensions (components or vectors) that explain most of the variability in the data set. They are better suited to microarray analysis and generally superior in performance than gene-by-gene analysis with conventional statistical tests because they take into account the complex data structure. Such methods are known as unsupervised [hierarchical clustering and principal component analysis (PCA)] or supervised [support vector machines (SVMs)] multivariate analysis methods. Supervised methods such as SVMs are based on algorithms that learn from a selected training data set and use this previously acquired knowledge about classes to classify unknown data. The algorithm solves the classification problem while aiming to minimize the probability of false classifications for initially unknown test data. The basic idea of the SVM method and detailed explanations are described elsewhere (Cristianini and Shawe-Taylor 2000; Schölkopf et al. 1999).

Unsupervised methods such as clustering algorithms and PCA are commonly used to determine if gene expression patterns allow the discrimination of natural subpopulations that might bear a biological meaning such as treated/untreated or healthy/diseased. PCA is a mathematical technique that reduces the dimensionality of highly multivariate data. The reduced dimensions (or components) actually describe the major part of the variation in the samples and separate natural subpopulations without *a priori* knowledge (Liu et al. 2002).

Cluster analysis is a method used to organize primary data. Pairwise average-linkage cluster is a form of unsupervised hierarchical clustering commonly used for the analysis of microarray data. Relationships among objects such as experimental conditions or genes are represented by a tree whose branch lengths reflect the degree of similarity between the objects as assessed by a pairwise similarity function based on correlation coefficients (Eisen et al. 1998). The clustering tools and SVM used in this analysis are modules of RACE-A, whereas PCA was performed using SIMCA-P (Umetrics, Umea, Sweden).

The complete data set is currently being submitted to ArrayExpress (EMBL-European Bioinformatics Institute, Hinxton, UK; http://www.ebi.ac.uk/arrayexpress) and will be available for public download by the second quarter of 2004. Accession numbers referencing this data set will be available on the HESI website (http://hesi.ilsi.org/index.cfm?pubentityid=120).

Results

Clinical chemistry and histopathology. A significant change in both body weight and food consumption compared to that of control groups was seen in the high-dose, but not in the low-dose group (data not shown). Clinical chemistry values confirmed liver toxicity occurred in the high-dose rats (Table 2). There were no significant changes at the low dose. Significant increases in leakage enzyme (AST, SDH and ALKPHOS) indicate both hepatocellular and cholangiolar injury. The dose-dependent decline in serum glucose and a trend toward a decrease in triglyceride levels might indicate compromise of hepatocellular metabolic function but may also have been influenced by reductions in food consumption.

No compound-related histopathological changes were found for the low-dose group, whereas several compound-related changes were seen in livers from rats treated at the high-dose level. These included cytoplasmic vacuolation of periportal hepatocytes, minimal to mild necrosis of periportal hepatocytes, increased infiltration of portal tracts by mononuclear inflammatory cells, and hyperplasia of oval cells along portal tracts.

Comparisons across users. After microarray analysis of the RNA, it was determined that different users obtained comparable results despite possible variation in the sample processing [from total RNA up to fragmented IVT (*in vitro* transcript)] and microarray hybridization protocols. In a preliminary round of analysis using rigid cutoff values to assess which genes were modulated, the number of genes detected as regulated in the pooled samples (2-fold increase or decrease) by each user were strikingly different (Table 3). All five users analyzing the pooled RNAs detected 254 genes that were regulated simultaneously, while each user recognized an excess of 1,000 genes as up- or downregulated. The data set generated at RO appeared to be a clear outlier with nearly twice the amount of modulated genes as the other users. This may be due to the modifications introduced in the sample preparation (Table 1), but no direct evidence is available in support of this.

Further microarray results demonstrated that when individual animals were analyzed, as opposed to pooled samples, the number of genes detected as induced/repressed was generally reduced. Table 3 shows the results from microarray analysis

Table 2. Clinical chemistry values for methapyrilene-treated rats.

Rat no.	Dosage (mg/kg)	ALT (IU/L)	AST (IU/L)	SDH (IU/L)	ALKPHOS (IU/L)	TBIL (mg/dL)	GLU (mg/dL)	TRIG (mg/dL)
1001	0	42	91	10.9	79	0.1	145	53
1003	0	25	106	6.6	170	0.1	136	19
1005	0	28	103	6.7	292	0.1	132	60
1007	0	30	90	7.3	197	0.1	121	48
Average	0	31.3	97.5	7.9	21.0	0.1	133.5	45.0
SD	0	7.46	8.2	2.0	56.1	0.0	10.0	18.0
2001	10	51	168	17.4	275	0.1	102	28
2003	10	24	97	9.5	217	0.1	111	50
2005	10	23	90	9.1	235	0.1	138	29
2007	10	30	92	12.1	255	0.1	97	19
Average	10	32.0	111.8	12.0	245.5	0.1	112	31.5
SD	10	13.0	37.6	3.8	25.1	0.1	18.3	13.1
3001	100	36	162	12.3	220	0.1	123	18
3003	100	56	179	12.9	283	0.4	100	28
3005	100	193	> 410	24.2	460	0.9	105	24
3007	100	51	200	19.4	417	0.3	88	15
Average	100	84.0	180.3*	17.2*	345.0*	0.4	104.0*	21.3
SD	100	73.2	19.0	5.7	112.4	0.3	14.5	5.9

Abbreviations: ALKPHOS, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GLU, glucose; SDH, sorbitol dehydrogenase; TBIL, total bilirubin; TRIG, triglycerides.
*Significantly different from the control group using two-tailed *t* test (p-value < 0.05).

Table 3. Number of genes regulated by methapyrilene across the different companies in the pooled and individual samples at the high dose.[a]

	Pooled RNA samples							Individual RNA samples		
	BI	Nov	Pfi	RO	Sch	Common RO/Sch	Common All users	RO	Sch	Common RO/Sch
Upregulation	691	785	621	1,325	692	441	179	282	352	120
Downregulation	480	283	728	292	405	130	75	262	157	73

Abbreviations: BI, Boehringer-Ingelheim Pharmaceuticals; Nov, Novartis Pharma AG; Pfi, Pfizer Inc; RO, F. Hoffmann-La Roche AG; Sch, Schering AG.
[a]Cut-off values: 2-fold change, p-value ≤ 0.05.

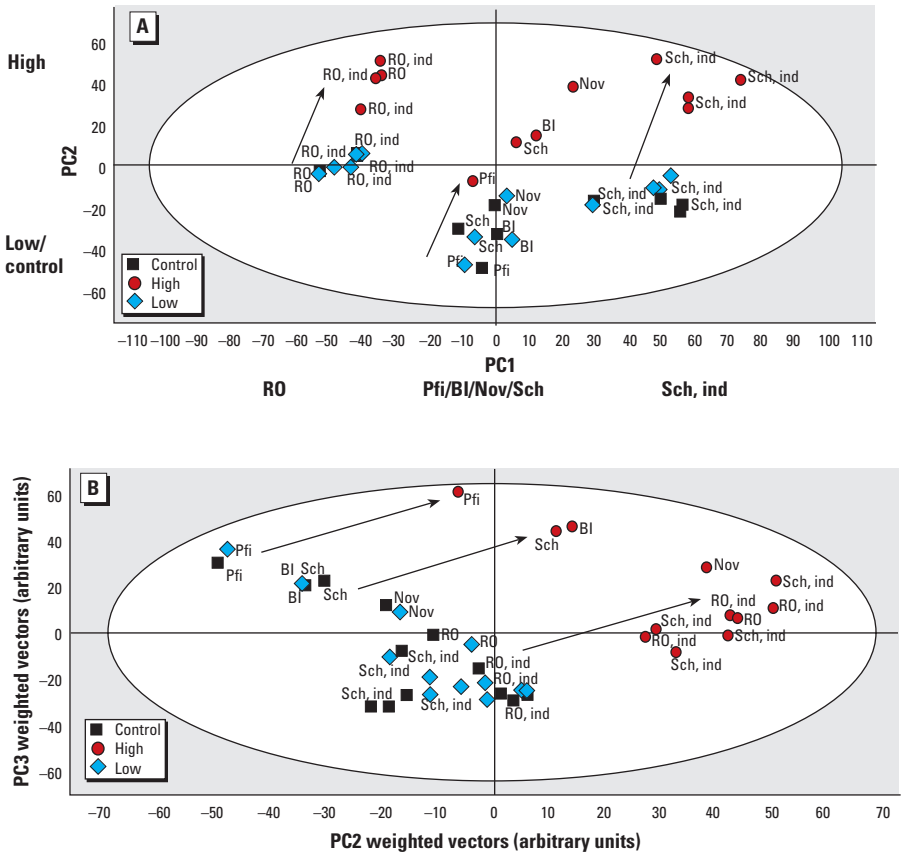

Figure 1. Principal component analysis using all expressed genes (4,846 probe sets, Avg Diff Max ≥ 200, Mean Call ≥ 0.5). Abbreviations: Bl, Boehringer-Ingelheim Pharmaceuticals; Nov, Novartis Pharma AG; Pfi, Pfizer Inc; RO, F. Hoffmann-La Roche AG; Sch, Schering AG; ind, individual; PC, principal component. All data points are from pooled samples unless otherwise indicated. (*A*) PC2 versus PC1, showing that PC1 is mainly driven by the site performing the microarray experiments. (*B*) PC3 versus PC2. Arrows show the separation of the high-dose samples from the vehicle and low-dose samples, mainly on the PC2.

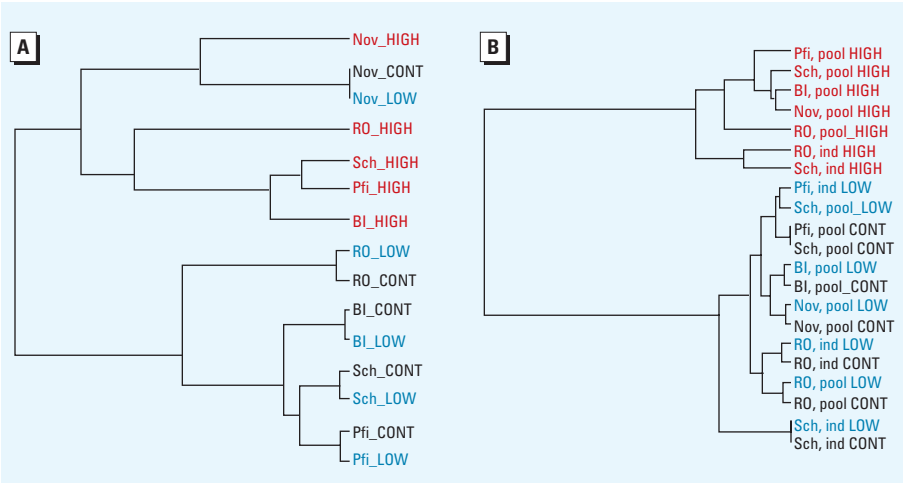

Figure 2. Hierarchical clustering using either (*A*) probe sets that were detected (4,846) or (*B*) probe sets that showed concordant regulation in the analyses performed by the two sites using RNA from individual animals (193 probe sets). Abbreviations: Bl, Boehringer-Ingelheim Pharmaceuticals; Nov, Novartis Pharma AG; Pfi, Pfizer Inc; RO, F. Hoffmann-La Roche AG; Sch, Schering AG; CONT, control; ind, individual. Each branch represents either a single chip (pooled samples) or the mean value of four replicates (individual samples). The correlation distance is represented by the distance between the samples in the dendrogram. Correlation coefficient values ranged between 0.854634 and 0.993906 in Figure 2A and between 0.490595 and 0.989861 in Figure 2B.

on pooled samples and individual animals conducted at RO and Sch. The inclusion of replicates very likely diminishes the influence of false signals. Nevertheless, there is still much disagreement among users when performing simple data analysis methods and defined cutoff values.

Whereas the gene expression analysis was not concordant between different laboratories, a critical question to be addressed is whether microarray results from all users reflected the observations from traditional toxicology markers and yielded similar mechanistic outcomes. When methapyrilene effects on the liver are examined, both histopathology and clinical chemistry analysis distinguished the high-dose animals from the low-dose and control animals. To determine if microarray analysis also distinguished between high and low dose, more sophisticated methods usually better suited to the analysis of highly multivariate microarray data were used. Among these methods, we chose to employ unsupervised as well as supervised approaches. Conversely, unsupervised methods are well suited to separate natural subpopulations in an unbiased manner. On the other hand, supervised methods allow incorporating knowledge obtained from the data (training set) to distinguish classes in the test data set. We analyzed the data using two unsupervised methods, namely PCA and hierarchical clustering. Using all expressed genes (4,846 probe sets), PCA analysis revealed a clear separation of the high-dose samples from controls and low-dose samples, despite the fact that the site differences are responsible for a large amount of variance. In this analysis, the second principal component (PC2; accounting for 15% of the variance) drives the treatment-related difference, as indicated by the arrows, whereas PC1 (accounting for 33% of the variance) showed a separation by site (Figure 1A, B). Excluding PC1 and relying exclusively on PC2 and PC3, a clear separation between high-dose–treated animals and the other two groups was achieved regardless of the site in which the sample processing was performed. Thus, the site-related differences do not mask the outcome of the classification. The low-dose samples could not be confidently distinguished from the vehicle-treated controls, a conclusion that accords with the clinical chemistry and histopathology findings.

To verify this latter conclusion, we grouped the data using another unsupervised clustering method, agglomerative hierarchical clustering. When we used the expressed genes employed for the PCA analysis, there was a tendency toward clustering by the site performing the microarray analysis (Figure 2A). An increase in statistical power

can be achieved by including the confidence information obtained from the analysis of biological replicates (Lee et al. 2000). For most users conducting these experiments, individual replicates were not available, as the RNA had been pooled. However, replicates were available from the two sites that performed microarray analysis on individual animals. Thus, we performed hierarchical agglomerative clustering using the probe sets that were regulated in common by the high dose of MP from the individual replicates obtained by Sch and RO. With this smaller subset of genes, hierarchical clustering of the treatment groups allowed the high dose to be discriminated from the controls and low-dose–treated animals (Figure 2B). Similar to the PCA analysis, the low-dose samples could not be distinguished from the control samples.

To improve the discrimination between the groups, further analysis was performed using supervised methods. Because results from biological replicates were provided by two sites (RO and Sch), it was possible to generate a training set using the profiles obtained from the individual animals. This training set consisted of two analyses (one per site) that included 4 animals in each treatment group, amounting to 24 microarrays generated from 12 animals. This training set has the limitations of being rather small and of including in each group four biological replicates (independent) and for each of these independent replicates, two processing replicates from different sites (nonindependent). The data from individual animals were analyzed using the SVM to identify probe sets that were distinct for the three different classes of treatment (vehicle, low-dose, high-dose). The training of the SVMs and the subsequent classification were performed using all probe sets on the chip (8,799). Once the SVM was thus trained, the 15 microarrays obtained from the analysis of the pooled samples (5 controls, 5 low-dose, 5 high-dose) were used as test samples and classified. In this case, samples obtained from animals treated with low or high doses of MP were correctly classified. Classification of the control animals was relatively ambiguous, as only 2 animals were correctly classified as controls, whereas the other 3 showed no similarity to either group (Figure 3). An example of some of the genes that allow the distinction between control and treated animals (low- and high-dose) is shown in Figure 4. Thus, using supervised clustering, together with biological replicates, it was possible overall to distinguish not only the high-dose–treated group, but also the low-dose–treated group from the controls. This was not unequivocally possible using clinical chemistry, histopathology, or unsupervised clustering methods.

Genes affected by MP. More important than the number of regulated genes is the determination of the identity of the regulated genes, the affected cellular pathways, and their biological significance. Some genes described previously as regulated by MP or that are associated with the histopathology findings were consistently detected by all involved users. Genes associated with cell stress, cell damage or apoptosis, and

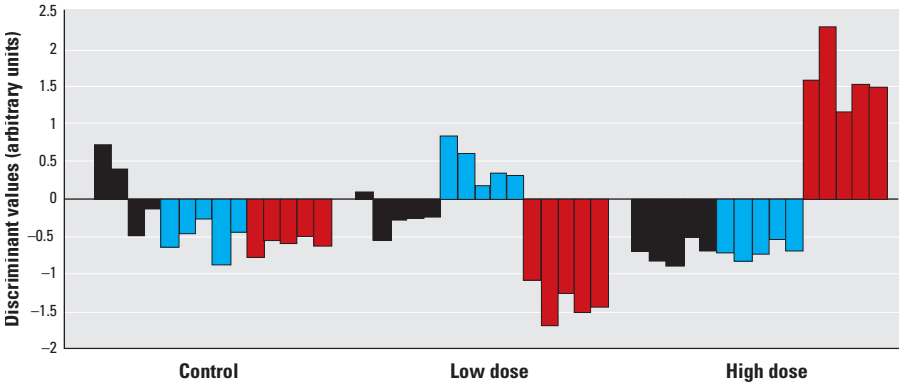

Figure 3. Classification of the pooled samples using support vector machines. The program was trained with the individual analyses provided by RO and Sch; all five pooled analyses were used as test sets. Training and classification were performed using all probe sets on the microarray. All low-dose (blue bars) and high-dose (red bars) samples were correctly classified, as well as two of the five controls (black bars). Three additional pooled controls could not be classified in any of the training groups.

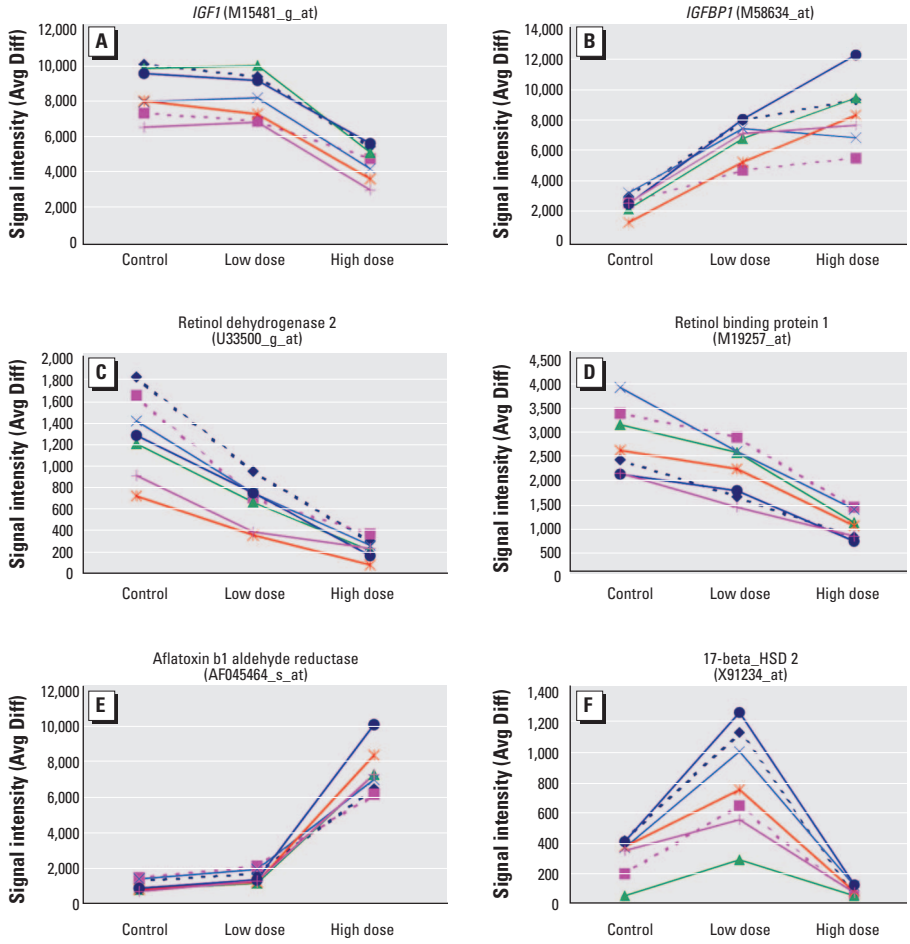

Figure 4. Examples of genes modulated at both the low and the high doses of MP. Dotted lines represent average values of the individual analyses by two users (RO and Sch) and solid lines each of the analyses of pooled samples by five users. For each panel, identification numbers in parentheses are Affymetrix probe set ID codes.

Table 4. Genes regulated by a high dose of methapyrilene.

Affymetrix probe set ID[a]	Class	Gene	Max Signal	RO (individual) Fold change	RO (individual) *t* test (*p*-value)	Sch (individual) Fold change	Sch (individual) *t* test (*p*-value)	Bl (pool)	Nov, (pool)	Pfi, (pool)	RO, (pool)	Sch, (pool)	Direction of change
X75207_s_at	Cell cycle	Cyclin D1	686	5.180	0.023	3.640	0.024	3.490	5.980	3.100	4.770	3.780	Up
D14014_g_at	Cell cycle	Cyclin D1	1,474	15.770	0.120	6.090	0.082	3.570	12.830	6.860	14.980	4.750	Up
D14014_at	Cell cycle	Cyclin D1	1,363	6.370	0.017	2.780	0.103	4.310	3.370	2.010	4.660	4.370	Up
X70871_at	Cell cycle	Cyclin D1	939	5.830	0.067	3.530	0.038	5.840	7.480	6.470	5.940	5.150	Up
E01184cds_s_at	Cyt P450	Cyt P450	4,296	0.258	0.001	0.379	0.003	0.293	0.400	0.265	0.444	0.266	Down
M21208mRNA_s_at	Cyt P450	*CYP17*	2,204	2.330	0.044	4.010	0.001	3.560	3.120	2.890	3.590	3.060	Up
K03241cds_s_at	Cyt P450	*CYP1A2*	2576	0.119	0.005	0.317	0.004	0.275	0.437	0.171	0.493	0.291	Down
J04187_at	Cyt P450	*CYP2A2*	11,191	0.383	0.007	0.450	0.001	0.307	0.405	0.392	0.319	0.319	Down
J02657_s_at	Cyt P450	*CYP2C11*	24,686	0.053	0.001	0.162	0.001	0.026	0.051	0.007	0.038	0.024	Down
M18363cds_s_at	Cyt P450	*CYP2C11*	7,563	0.013	0.012	0.048	0.000	0.017	0.019	0.013	0.015	0.020	Down
X79081mRNA_f_at	Cyt P450	*CYP2C11*	3,775	0.005	0.008	0.008	0.011	0.016	0.009	0.013	0.055	0.011	Down
J03786_s_at	Cyt P450	*CYP2C12*	7,156	8.350	0.021	2.610	0.000	4.030	2.880	6.240	6.510	5.890	Up
M33550cds_s_at	Cyt P450	*CYP2C12*	7,758	3.590	0.093	2.510	0.000	3.640	3.760	5.900	3.090	6.390	Up
rc_AA945573_f_at	Cyt P450	*CYP2C39*	16,174	0.317	0.009	0.704	0.061	0.154	0.426	0.253	0.427	0.342	Down
M31031mRNA_f_at	Cyt P450	*CYP2C39*	15,025	0.212	0.001	0.578	0.100	0.249	0.395	0.265	0.361	0.337	Down
M14775_s_at	Cyt P450	*CYP2C7*	17,548	0.043	0.000	0.448	0.108	0.210	0.275	0.129	0.121	0.152	Down
AB008424_s_at	Cyt P450	*CYP2D3*	15,558	0.376	0.003	0.763	0.036	0.276	0.439	0.204	0.260	0.350	Down
U46118_at	Cyt P450	*CYP3A9*	1,234	0.239	0.039	0.253	0.019	0.143	0.159	0.175	0.266	0.181	Down
M29853_at	Cyt P450	*CYP4B1*	2,149	13.830	0.128	9.990	0.042	13.160	14.880	18.980	28.490	8.740	Up
D00680_at	Glutathione	Glutathione peridoxidase	539	9.210	0.039	9.050	0.044	3.290	5.170	3.300	19.890	9.760	Up
L38615_g_at	Glutathione	Glutathione synthetase	1,169	2.440	0.085	26.890	0.023	7.180	2.080	3.450	6.280	2.430	Up
rc_AA945082_at	Glutathione	*GSTa2*	1,049	13.460	0.019	12.420	0.010	12.120	26.630	5.610	19.470	20.090	Up
S72506_s_at	Glutathione	*GSTyc2*	2,022	43.900	0.004	39.430	0.001	24.860	71.750	39.040	67.230	67.540	Up
S82820mRNA_s_at	Glutathione	*GSTyc2*	7,696	19.750	0.000	16.650	0.000	38.580	27.070	22.930	33.060	28.740	Up
X95189_at	Lipid metabolism	Acyl-CoA oxidase	2,963	0.249	0.093	0.476	0.152	0.269	0.323	0.120	0.407	0.452	Down
AB010428_s_at	Lipid metabolism	Acyl-CoA thioesterase 1	4,242	43.870	0.011	94.300	0.002	23.730	105.300	86.490	91.700	56.760	Up
Y09333_g_at	Lipid metabolism	Acyl-CoA thioesterase 1	5,696	14.130	0.024	12.750	0.000	18.920	25.520	10.810	190.190	16.820	Up
Y09333_at	Lipid metabolism	Acyl-CoA thioesterase 1	5,411	8.370	0.062	4.810	0.004	5.620	8.580	7.210	15.250	8.570	Up
D43623_g_at	Lipid metabolism	Carnitine palmitoyl-transferase	879	18.040	0.053	33.410	0.004	18.160	11.040	9.780	9.040	10.300	Up
M26125_at	Lipid metabolism	Epoxide hydrolase 1	18,841	4.800	0.023	1.400	0.009	3.170	2.340	3.980	5.450	3.300	Up
rc_AA893242_g_at	Lipid metabolism	Fatty acid-CoA ligase	2,968	0.306	0.012	0.412	0.017	0.431	0.667	0.307	0.424	0.422	Down
M29249cds_at	Lipid metabolism	HMG-CoA reductase	667	3.590	0.047	2.020	0.033	9.100	20.360	1.000	7.110	5.930	Up
X55286_g_at	Lipid metabolism	HMG-CoA Reductase	235	2.440	0.010	3.850	0.009	3.500	10.540	1.810	3.130	4.630	Up
J02585_at	Lipid metabolism	Stearoyl-CoA desaturase 1	3,208	0.135	0.017	0.130	0.008	0.126	0.087	0.172	0.135	0.116	Down
AB010429_s_at	Lipid metabolism	Very long chain acyl–CoA thioesterase	1,772	2.700	0.015	4.350	0.026	9.470	7.930	1.120	6.790	11.520	Up
L07114_at	Lipid transport	Apolipoprotein B binding protein	287	2.480	0.022	6.740	0.035	1.000	2.430	5.130	3.360	5.550	Up
AF072411_at	Lipid transport	*CD36*	1,077	3.240	0.005	2.660	0.030	3.400	4.650	1.910	8.820	3.160	Up
rc_AA925752_at	Lipid transport	*CD36*	1,446	3.140	0.003	2.520	0.035	3.060	4.700	2.090	5.500	3.040	Up
AB005743_g_at	Lipid transport	*CD36*	307	3.060	0.005	5.140	0.022	1.000	15.340	1.000	1.880	5.790	Up
AF072411_g_at	Lipid transport	*CD36*	1,979	2.680	0.004	3.120	0.017	3.700	4.560	2.190	7.150	4.180	Up
AB005743_at	Lipid transport	*CD36*	289	2.400	0.003	2.250	0.109	2.270	10.080	4.140	4.240	2.920	Up
K01180_at	Lipid transport	Fatty acid binding protein 2	321	6.730	0.100	4.400	0.047	13.200	15.150	5.800	5.200	4.220	Up
U02096_at	Lipid transport	Fatty acid binding protein 7	3,443	0.172	0.005	0.267	0.004	0.190	0.175	0.163	0.108	0.175	Down
L34049_g_at	Lipid transport	Megalin (LRP2)	977	0.330	0.001	0.420	0.027	0.503	0.488	0.217	0.500	0.405	Down
U89280_at	Phase 2 metabolism	17-b Hydroxysteroid dehydrogenase	6,406	0.249	0.020	0.385	0.025	0.424	0.433	0.345	0.391	0.348	Down
AF045464_s_at	Phase 2 metabolism	Aflatoxin b1 aldehyde reductase	9,840	4.810	0.016	4.000	0.000	8.260	4.840	9.310	10.710	9.340	Up
D38061exon_s_at	Phase 2 metabolism	*UGT1-6*	1,026	30.420	0.003	37.320	0.012	24.130	42.050	10.100	39.590	34.930	Up
S56936_s_at	Phase 2 metabolism	*UGT1-6*	1,166	5.560	0.022	7.810	0.005	10.630	13.400	3.950	13.050	9.200	Up
D38062exon_s_at	Phase 2 metabolism	*UGT1-7*	507	2.860	0.093	13.190	0.049	11.030	25.340	11.000	6.520	16.140	Up
J02589mRNA#2_at	Phase 2 metabolism	*UGT2B2*	1,299	0.256	0.099	0.015	0.011	0.157	0.258	0.115	0.272	0.146	Down
rc_AI169708_s_at	Phase 2 metabolism	*UGT2B2*	21,663	0.002	0.000	0.033	0.000	0.578	0.746	0.543	0.599	0.526	Down
rc_AI180442_at	Steroid metabolism	Farensyl diphosphate synthase	1,378	2.630	0.008	3.220	0.013	2.560	3.100	4.690	3.280	3.380	Up

Continued

Table 4. Continued.

Affymetrix probe set ID[a]	Class	Gene	Max signal	RO (individual) Fold change	RO (individual) *t* test (*p*-value)	Sch (individual) Fold change	Sch (individual) *t* test (*p*-value)	BI (pool)	Nov (pool)	Pfi (pool)	RO (pool)	Sch (pool)	Direction of change
M95591_g_at	Steroid metabolism	Farensyl diphosphate farnesyltransferase 1	1,040	5.280	0.040	2.770	0.033	1.520	3.660	0.613	20.130	1.280	Up
M95591_at	Steroid metabolism	Farensyl diphosphate farnesyltransferase 1	2,011	3.650	0.004	26.720	0.021	14.210	4.820	10.490	9.020	26.960	Up
M89945mRNA_g_at	Steroid metabolism	Farensyl diphosphate synthase	3,421	2.380	0.082	2.710	0.004	3.250	3.380	2.860	3.350	2.770	Up
M89945mRNA_at	Steroid metabolism	Farensyl diphosphate synthase	4,531	2.370	0.010	2.830	0.002	4.030	2.980	3.820	4.540	2.710	Up
M81225_at	Steroid metabolism	Farensyltransferase	1,063	2.360	0.013	2.380	0.019	2.340	2.240	1.930	3.430	2.100	Up
U33500_at	Steroid metabolism	Retinol dehydrogenase type II	1,256	0.315	0.008	0.510	0.005	0.437	0.420	0.324	0.402	0.495	Down
U33500_g_at	Steroid metabolism	Retinol dehydrogenase type II	1,856	0.173	0.006	0.228	0.013	0.190	0.193	0.127	0.141	0.269	Down
M19257_at	Steroid metabolism	Retinol-binding protein 1	3,941	0.327	0.001	0.420	0.000	0.352	0.353	0.394	0.331	0.382	Down
D37920_at	Steroid metabolism	Squalene epoxidase	843	1.650	0.112	31.010	0.007	20.470	7.760	13.250	42.140	3.240	Up
U30186_at	Stress/damage	*GADD153*	1,670	13.310	0.000	11.470	0.026	17.950	7.680	32.030	10.800	14.340	Up
L32591mRNA_at	Stress/damage	*GADD45a*	1,015	5.200	0.062	8.590	0.027	2.800	4.370	21.570	8.090	14.310	Up
L32591mRNA_g_at	Stress/damage	*GADD45a*	1,154	3.550	0.109	3.230	0.033	2.600	5.050	3.150	2.820	7.010	Up
rc_AI070295_g_at	Stress/damage	*GADD45a*	829	1.260	0.642	4.160	0.024	2.270	7.050	2.350	8.010	4.250	Up
rc_AI175959_at	Stress/damage	c-*jun*	1,172	3.130	0.210	2.840	0.154	3.170	2.830	2.060	4.260	6.690	Up
Y00396mRNA_at	Stress/damage	c-*myc*	643	4.640	0.061	28.430	0.011	10.070	32.140	5.320	16.280	9.300	Up
Y00396mRNA_g_at	Stress/damage	c-*myc*	988	4.300	0.094	6.090	0.029	3.600	8.890	2.610	8.420	9.070	Up
J02722cds_at	Stress/damage	Heme oxygenase 1	464	1.480	0.233	2.570	0.056	2.210	7.580	4.240	6.270	3.070	Up
M25157mRNA_i_at	Stress/damage	Superoxide dismutase	4,406	0.606	0.180	0.450	0.042	0.265	0.315	0.110	0.455	0.247	Down
S76511_s_at	Stress/damage	*BAX*	586	3.630	0.153	8.910	0.040	13.530	4.800	11.590	7.210	5.550	Up
M60921_g_at	Stress/damage	B-cell translocation gene 2	551	12.960	0.143	15.090	0.056	7.420	22.640	5.250	22.590	11.820	Up
rc_AA944156_s_at	Stress/damage	B-cell translocation gene 2	1,934	2.990	0.053	1.570	0.046	3.950	2.730	3.890	2.120	2.670	Up
U49729_at	Stress/damage	bcl12-associated X protein	285	1.010	0.144	14.080	0.010	6.840	14.270	6.630	2.700	12.100	Up
M33329_f_at	Sulfotransferase	Alcohol sulfotransferase	6,794	0.500	0.241	0.571	0.048	0.298	0.280	0.225	0.375	0.377	Down
X63410cds_f_at	Sulfotransferase	Alcohol sulfotransferase	9,853	0.364	0.006	0.610	0.034	0.254	0.324	0.253	0.233	0.431	Down
S76489_s_at	Sulfotransferase	Estrogen sulfotransferase	10,020	0.259	0.034	0.552	0.001	0.270	0.439	0.277	0.353	0.408	Down
D14989_f_at	Sulfotransferase	Hydroxysteroid sulfotransferase	5,378	0.136	0.012	0.327	0.003	0.194	0.254	0.142	0.121	0.256	Down
rc_AI169695_f_at	Sulfotransferase	Hydroxysteroid sulfotransferase	5,636	0.100	0.004	0.293	0.001	0.146	0.236	0.116	0.139	0.161	Down
D14988_f_at	Sulfotransferase	Hydroxysteroid sulfotransferase	13,597	0.206	0.000	0.405	0.005	0.189	0.244	0.139	0.178	0.187	Down
D14987_f_at	Sulfotransferase	Hydroxysteroid sulfotransferase	10,861	0.176	0.001	0.562	0.054	0.118	0.227	0.114	0.199	0.148	Down
rc_AA817987_f_at	Sulfotransferase	Hydroxysteroid sulfotransferase	8,115	0.150	0.002	0.340	0.008	0.077	0.166	0.124	0.126	0.139	Down
M31363mRNA_f_at	Sulfotransferase	Hydroxysteroid sulfotransferase	14,611	0.126	0.001	0.236	0.000	0.101	0.086	0.079	0.112	0.136	Down
rc_AA818122_f_at	Sulfotransferase	Hydroxysteroid sulfotransferase	12,159	0.089	0.052	0.380	0.007	0.065	0.129	0.083	0.165	0.127	Down
rc_AI028836_at	Sulfotransferase	Hydroxysteroid sulfotransferase	602	0.086	0.193	0.041	0.007	0.108	0.104	0.211	0.258	0.088	Down
L22339_at	Sulfotransferase	Phenol-preferring sulfotransferase	2,105	0.212	0.001	0.181	0.000	0.155	0.155	0.156	0.076	0.190	Down
L22339_g_at	Sulfotransferase	Phenol-preferring sulfotransferase	15,648	0.398	0.012	0.424	0.001	0.201	0.229	0.230	0.282	0.307	Down
AB010467_s_at	Transporter	Myosin-like protein 2	985	6.620	0.032	7.220	0.004	3.460	7.960	8.370	21.110	11.270	Up
D86086_s_at	Transporter	Myosin-like protein 2	4,083	2.860	0.053	2.520	0.014	2.740	2.770	2.570	3.870	2.990	Up
M81855_at	Transporter	P-glycoprotein/multidrug resistance 1	5,082	117.530	0.000	77.730	0.023	77.520	254.090	121.190	150.700	226.980	Up
M77479_at	Transporter	Sodium/bile acid cotransporter family	6,110	0.198	0.010	0.472	0.022	0.394	0.529	0.269	0.322	0.515	Down
rc_AI235631_at	Unknown	Expressed sequence tag	795	4.700	0.005	4.650	0.014	1.890	3.710	2.380	2.730	2.060	Up
rc_AI172452_at	Unknown	Expressed sequence tag	1,603	2.390	0.001	2.010	0.001	1.360	1.930	1.800	2.530	2.010	Up
rc_AA866240_f_at	Unknown	Expressed sequence tag	8,131	0.164	0.000	0.524	0.009	0.181	0.329	0.253	0.383	0.417	Down

oncogenesis were shown to be upregulated by all users at the high dose (Table 4). Among these genes were the *GADD* family members *GADD153* and *GADD45* (Stokes et al. 2002), the proto-oncogenes c-*myc* and c-*jun* (Hernandez et al. 1991), the antiproliferative protein PC3 (*BTG2*) (Tirone 2001), *bax* (Brunelle and Chandel 2002), and heme oxygenase-1 (Bauer et al. 2000). In addition, cyclin D1 and cyclin G1 were also induced by MP (Afshari and Barrett 1993). Genes coding for proteins involved in cholesterol biosynthesis and β-oxidation appeared consistently regulated by the treatment (i.e., mitochondrial CPT1, acyl-CoA thioester hydrolase, 3-hydroxy-3-methylglutamyl-CoA (HMG-CoA) reductase, squalene epoxidase, farnesyl diphosphate (FPP)-transferase, FPP-synthase were upregulated, while acyl-CoA desaturase, acyl-CoA synthetase, and acyl-CoA oxidase 2 were downregulated) (Ratra et al 1998b). The induction of several enzymes in the sterol metabolic pathway by the high dose was accompanied by downregulation of retinol dehydrogenase and retinol-binding protein 1 in the related retinol metabolic pathway and of the androgen/estrogen metabolic pathways (Figure 5). In addition, MP produced a marked effect in some metabolic enzymes such as the upregulation of cytochrome P-450 *(CYP)4B1*, *CYP2C12*, and aflatoxin reductase and the downregulation of *CYP1A1*, *CYP2A2*, *CYP2C11* and sulfotransferases (Ratra et al. 1998a). Additional genes involved in redox processes were affected by the treatment: glutathione *S*-transferase (*GSTyc2*), glutathione peroxidase, and glutathione synthetase were induced, whereas superoxide dismutase was repressed. A consistent induction of UDP-glucuronosyltransferase (*UDPGT*) 1–6 and a concomitant downregulation of *UDPGT2B* (3-hydroxyandrogen specific) were also observed. MP also seemed to have an effect on the expression levels of several transporters; *MDR* (P-glycoprotein), *cMOAT1* (*MRP2*) and *cMOAT2* (*MRP3*) were upregulated, whereas the expression of the sodium/taurocholate transporter was transcriptionally repressed.

As can be deduced from the cluster and PCA analyses (Figures 1 and 2), the effect of the low dose of MP is rather subtle, involving a small amount of regulated genes and moderate fold changes. This makes the distinction between low-dose treated animals and controls relatively difficult in a rather heterogeneous (different users, different protocols) set of samples comprising very few replicates to support statistical analysis (four biological replicates for individual sample analyses and five replicates for pooled samples). Nevertheless, some genes could be identified that are consistently modulated by the low dose of the compound. Among these genes, a dose-dependent decrease in acyl-CoA desaturase (EC 1.14.99.5) and in caltrin (calcium transport

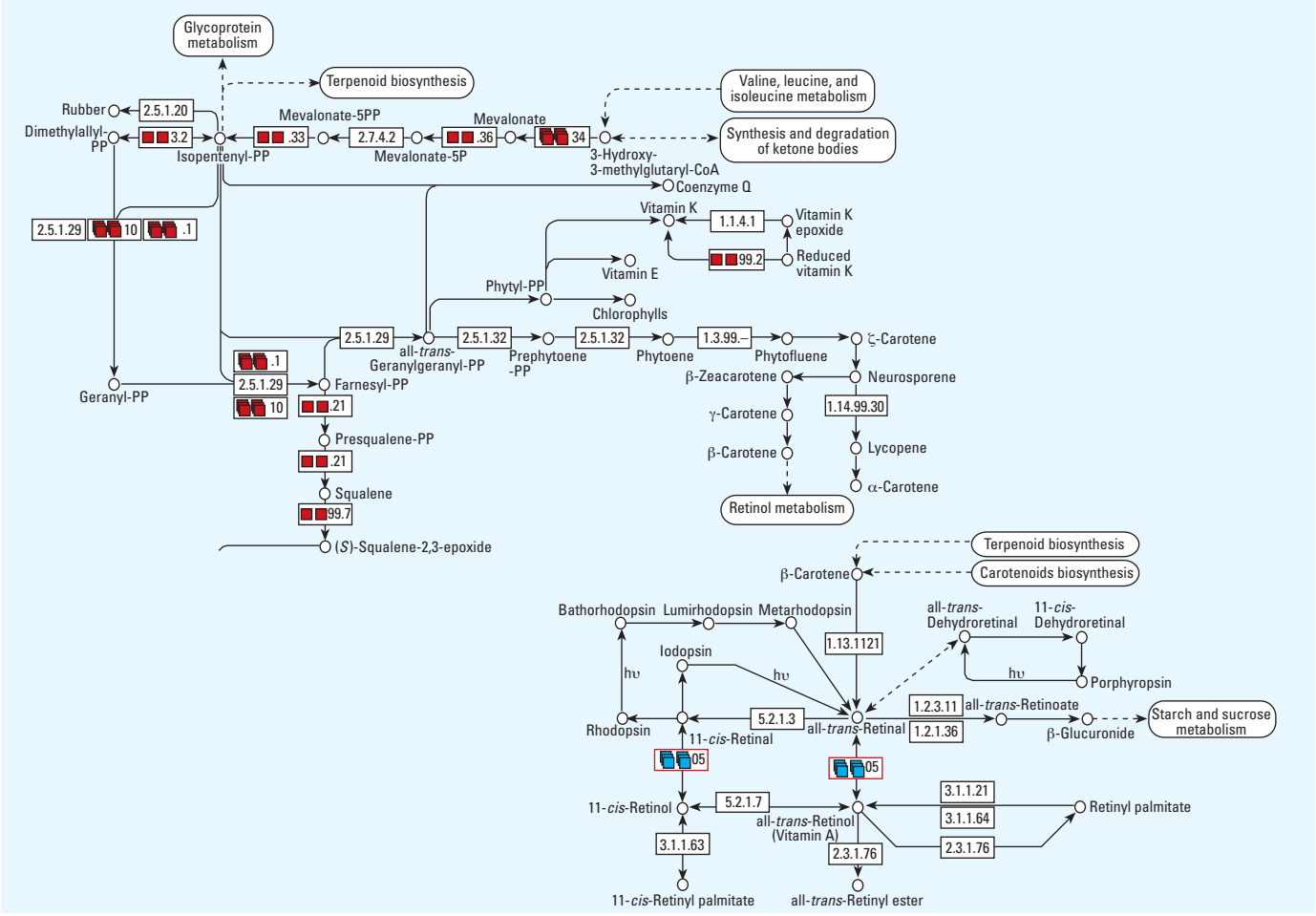

Figure 5. Projection of the gene expression results obtained using the individual animal data on the sterol/retinol metabolic pathways (KEGG). Red boxes indicate significant upregulation while blue boxes represent significant downregulation. Of the small colored boxes, boxes on the left represent the mean modulation by Roche (individual) and boxes on the right represent the mean modulation by Schering (individual). Nomenclature for the affected genes: farnesyl diphosphate farnesyltransferase (EC 2.5.1.21); farnesyl pyrophosphate synthase (EC 2.5.1.10); farnesyl pyrophosphate synthase (EC 2.5.1.1); 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34); isopentenyl-diphosphate delta-isomerase (EC 5.5.3.2); mevalonate kinase (EC 2.7.1.36); mevalonate pyrophosphate decarboxylase (EC 4.1.1.33); NAD(P)H2 dehydrogenase, quinone (EC 1.6.99.2); retinol dehydrogenase (EC 1.1.1.105); squalene monooxygenase squalene (EC 1.1499.7).

inhibitor) were observed, together with a very slight decrease in betaine–homocysteine *S*-methyltransferase (EC 2.1.1.5) and an increase in insulin-like growth factor binding protein 1 precursor (IGFBP-1) (Mohn et al. 1991).

Discussion

In this study, we were able to examine differences and similarities of results from microarray analysis obtained from a common source of RNA by several users. A preliminary evaluation of the number and identity of modulated genes uncovered striking differences between the five different Affymetrix users. This is in contrast to previous studies that have shown high reproducibility with microarray chips from the same RNA source (Waring et al. 2001). In addition, our unpublished data show that the correlation between gene expression in the liver and in the kidney from samples obtained from the same animal and prepared by the same operator following a standardized protocol is only 44%, whereas two different liver samples show a correlation of 98%. In this study some of the differences between users are likely because of different user protocols. In support of this, RO shows the most striking difference in the absolute values of regulated genes and is also the user introducing the most modifications of the sample processing and hybridization protocol (different cDNA kit, different blocking solution, etc.). Other users employing the protocols recommended by Affymetrix show less variability among them. The remaining differences are probably attributable to minor protocol deviations as well as to an expected amount of false positives. In this case the number of false positives was undoubtedly high because the samples were pooled and thus the number of replicates was low.

Further data analysis with additional tools corroborated the finding that differences between users, sample processing, and hybridization protocols affected the absolute results, but that this did not distort the major conclusion of the study. Indeed, the PCA results showed that the PC1 (accounting for 33% of the variance) was mainly site driven (Figure 1A), but this variability did not mask the effects elicited by the high dose of MP. Despite the observed differences, all users obtained similar overall results that correlated with histopathology and clinical chemistry analysis. A clear differentiation between high-dose (toxic)–treated animals and controls and low-dose–treated animals was obtained by all users, as shown using unsupervised data analysis methods (cluster analysis and PCA). Moreover, in a supervised approach it was possible to identify animals treated with high and low doses in all the pooled samples analyzed by five different users on the basis of SVM trained with data obtained from the samples processed individually. Surprisingly, three of the pooled control samples were not clearly assigned to any treatment group. There are two possible factors that could have led to this misclassification. On one hand, the training set is very small, and this type of model has an optimal performance with large data sets. Alternatively, the effects of 10 mg/kg MP are very subtle, thus making distinction of control and low-dose–treated animals rather difficult. This is even more pronounced in a heterogeneous set of data. For very slight effects a larger number of replicates might be required for optimal performance.

As stated by Hamadeh et al. (2002), the use of unsupervised analysis tools is essential to ensure that the data contain natural subpopulations and that no preconceived bias is introduced when classes of compounds are being identified. The results obtained using cluster analysis and PCA show that gene expression profiles allow the natural classification of the high dose of MP regardless of the variation introduced by the different users. Nevertheless, these unsupervised tools mainly allow the distinction of samples showing definitive histopathological findings (high-dose) from samples without findings (controls and low-dose samples). It might be argued that this is not sensitive enough for predictive toxicogenomics studies. However, using a supervised analysis method like SVM, it was possible to correctly categorize samples into vehicle, low- and high-dose classes, which was not possible with clinical chemistry or histopathology. Thus, similar to results obtained by Burczynski et al. (2000) and Thomas et al. (2001), the ability to correctly classify compounds using toxicogenomics can be greatly improved by selecting a smaller subset of the most predictive gene sets.

A number of the genes and pathways regulated by MP toxicity were similar across users. This is particularly true for the high-dose–treated animals in which the effects were more pronounced. The genes detected as transcriptionally induced or repressed are in good agreement with results from a similarly designed study by Hamadeh et al. (2002) using cDNA spotted arrays. In-depth analysis of the genes modulated by MP sheds light on the variety of cellular processes affected.

Our results provide ample proof that gene expression analysis is a suitable method to detect effects produced by a high dose (100 mg/kg) of MP. The results presented in this article are generally in good agreement with a similar study performed by Hamadeh et al. (2002) and also show signals characteristic of the compound under investigation. The decrease in cytochrome P450 after a high dose of MP was in agreement with results of previous studies that showed this compound decreased the content of CYP2C11, CYP3A and CY2A, possibly due to suicide substrate activation (Graichen et al. 1985, Ratra et al. 1998a). MP is also known to transcriptionally induce *CYP2C12* and *CYP4B1* (Hamadeh et al. 2002), as was also detected by all users analyzing the samples. In addition, several of the genes detected as modulated, including the *GADD* family, heme oxygenase and genes related to glutathione homeostasis are indicative of the oxidative stress known to be produced by MP (Ratra et al. 1998b). Also, several of the modulated genes indicate an effect of MP on lipid metabolism, which is one of the pathways affected by MP as shown in studies using *in vitro* approaches (Iype et al. 1985) and protein analysis (Man et al. 2002). Moreover, events indicative of lipid peroxidation were observed as previously published (Hamadeh et al. 2002). The induction of mitochondrial genes (i.e., *CPT1* and acyl-CoA thioester hydrolase) is also indicative of the mitochondrial proliferation that has been previously related to MP (Iype et al. 1985).

In animals treated with a low dose of MP, some genes could be identified as already being modulated after 1-week treatment with 10 mg/kg/day MP. Among these, an induction of *IGFBP-1* (Affymetrix probe set ID M58634_at) and aflatoxin B1 aldehyde reductase (Affymetrix ID AF045464_s_at), as well as the downregulation of retinol dehydrogenase type 2 (Affymetrix ID U33500_g_at) were observed. As depicted in Figure 4C, the observed downregulation of retinol dehydrogenase 2 at both the low and high doses was accompanied by the downregulation of retinol-binding protein 1 (Affymetrix ID M19257_at; Figure 4D). *IGFBP-1* (Affymetrix ID M58634_at) appeared upregulated at both doses, whereas the growth-promoting insulin-like growth factor 1 (*IGF1*, Affymetrix ID M15481_g_at) appeared downregulated only after rats were exposed to a high dose of MP (Figure 4A, B). The upregulation of *IGFBP-1* might be a protective mechanism for the known carcinogenic effect of MP because the levels of *IGFBP-1* regulate the mitogenic effects of IGFs (Kelley et al. 1996). In fact, *IGFBP-1* has been shown to inhibit hepatic preneoplasia in mice (Lu and Archer 2003).

An additional cell protection mechanism that appears stimulated after treatment with 10 mg/kg/day of MP is aflatoxin B1 aldehyde reductase (Affymetrix ID AF045464_s_at; Figure 4E). This detoxifying enzyme shows only a slight induction at the low dose and an extensive induction at the high dose, which is in agreement with its previously reported induction by a high dose (100 mg/kg/day) of MP (Hamadeh et al. 2002). 17β-Hydroxysteroid dehydrogenase type 2 (*17bHSD2*; Affymetrix ID X91234_at) shows an interesting regulation pattern, as it appears upregulated by the low dose of MP and downregulated by the high dose (Figure 4F). This enzyme is involved in the steroid conversion pathway (Akinola et al. 1996), which is one of the pathways affected by the treatment with MP (Figure 5), but the biological meaning of this finding remains unclear.

In conclusion, a high degree of user/site variability was observed with microarray analysis using the same RNA processed at different sites. Despite this, all the microarray results showed that it was nonetheless possible to distinguish toxic (i.e., histopathological findings) versus nontoxic dose levels of MP. Moreover, regardless of the user, gene expression analysis using supervised data analysis tools allowed the correct identification of the samples treated with the low dose of MP, a distinction that was not apparent from clinical chemistry or histopathology analysis.

The observed site-to-site variability did not impair the detection of molecular effects elicited by MP. In addition, crucial gene expression changes, which most likely reflect the mechanism of toxicity for MP, were observed across all user groups. These results provide critical information regarding the consistency of microarray results across different laboratories and shed light on the strengths and limitations of expression profiling in drug safety analysis.

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