

## Clofibrate-Induced Gene Expression Changes in Rat Liver: A Cross-Laboratory Analysis Using Membrane cDNA Arrays

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Microarrays have the potential to significantly impact our ability to identify toxic hazards by the identification of mechanistically relevant markers of toxicity. To be useful for risk assessment, however, microarray data must be challenged to determine reliability and interlaboratory reproducibility. As part of a series of studies conducted by the International Life Sciences Institute Health and Environmental Science Institute Technical Committee on the Application of Genomics to Mechanism-Based Risk Assessment, the biological response in rats to the hepatotoxin clofibrate was investigated. Animals were treated with high (250 mg/kg/day) or low (25 mg/kg/day) doses for 1, 3, or 7 days in two laboratories. Clinical chemistry parameters were measured, livers removed for histopathological assessment, and gene expression analysis was conducted using cDNA arrays. Expression changes in genes involved in fatty acid metabolism (e.g., acyl-CoA oxidase), cell proliferation (e.g., topoisomerase II- $\alpha$ ), and fatty acid oxidation (e.g., cytochrome P450 4A1), consistent with the mechanism of clofibrate hepatotoxicity, were detected. Observed differences in gene expression levels correlated with the level of biological response induced in the two *in vivo* studies. Generally, there was a high level of concordance between the gene expression profiles generated from pooled and individual RNA samples. Quantitative real-time polymerase chain reaction was used to confirm modulations for a number of peroxisome proliferator marker genes. Though the results indicate some variability in the quantitative nature of the microarray data, this appears due largely to differences in experimental and data analysis procedures used within each laboratory. In summary, this study demonstrates the potential for gene expression profiling to identify toxic hazards by the identification of mechanistically relevant markers of toxicity. **Key words:** cDNA array, clofibrate, cross-laboratory studies, gene expression profiling, liver, membrane array, microarray, peroxisome proliferator, rat. *Environ Health Perspect* 112:428–438 (2004). doi:10.1289/txg.6677 available via <http://dx.doi.org/> [Online 15 January 2004]

Toxicogenomics can have a significant impact on our ability to predict toxic hazards by the identification of mechanistically relevant markers of toxicity. Indeed, several recent studies have shown the potential of DNA microarray technology to reveal chemical and mechanism-specific signatures from *in vivo* and *in vitro* models treated with well-characterized toxins (Bartosiewicz et al. 2001a, 2001b; Bulera et al. 2001; Burczynski et al. 2000; Fielden and Zacharewski 2001; Hamedeh et al. 2002a, 2002b; Harries et al. 2001; Nuwaysir et al. 1999; Thomas et al. 2001; Waring et al. 2001a, 2001b). However, before the full potential of toxicogenomics can be realized, a number of important questions regarding the robustness and reproducibility of the data generated must be addressed.

To identify and address some of the issues, challenges, and opportunities afforded by the emerging field of toxicogenomics, the Health and Environmental Sciences Institute (HESI) of the International Life Sciences Institute (ILSI; <http://www.ILSI.org>) formed

a committee to develop a collaborative scientific program to address these areas (Pennie et al. 2004). Experts and advisers from academia and government laboratories participated on the committee, together with approximately 30 companies from the pharmaceutical, agrochemical, chemical, and consumer product industries. The committee was divided into several working groups conducting large-scale cross-laboratory studies in the fields of hepatotoxicity, nephrotoxicity, and genotoxicity. In addition, a fourth working group was established to address the issue of database development. The goal of the Hepatotoxicity Working Group (HWG), outlined by Ulrich et al. (2004) in this issue, was to evaluate and compare biological and gene expression responses in rats exposed to two well-studied hepatotoxins (clofibrate and methapyrilene), using a standard experimental protocol, and to address the following issues: *a*) how comparable are the biological and gene expression data from different laboratories running identical *in vivo* studies; *b*) how reproducible are the

data generated across laboratories using the same microarray platform; *c*) how do data compare using different microarray platforms; *d*) how do data compare using RNA from pooled and individual animals; *e*) do the gene expression changes demonstrate time- and dose-dependent responses that correlate with known biological markers of toxicity?

As part of the experimental program of the HWG, the biological response in rats to the model hepatotoxin clofibrate was investigated. Clofibrate, a hypolipidemic drug, was chosen as a test material for this study because of its well-characterized hepatotoxic effects in rodents. Clofibrate was developed and marketed in the 1960s for the treatment of high cholesterol and triglycerides in humans (IARC 1996; Tucker and Orton 1993) and is still used in the treatment of hypercholesterolemia. However, administration to rodents results in a characteristic increase in liver weight. This liver weight increase is due to hepatocyte hyperplasia and hypertrophy associated with distinctive morphological and biochemical effects (Tosh et al. 1989). The biochemical effects

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of clofibrate are triggered through binding of the chemical to the peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ), causing a pleiotropic response involving the induction of a number of proteins involved in fatty acid  $\beta$ -oxidation (Lapinskas and Corton 1999; Lindquist et al. 1998, for example). Clofibrate administered at a dose of 250 mg/kg/day to rats causes hepatic parenchymal cells to increase in size (Karbowski et al. 1999), induces peroxisomes, and produces liver tumors in 20–91% of chronically treated rats (Ashby et al. 1994; Doull et al. 1999; Hartig et al. 1982; Reddy and Qureshi 1979; Svoboda and Azarnoff 1979). This dose also decreases both basophilic material in the cytoplasm and vacuolation within 1 week, with maximal effects being observed at 2 weeks. For the purpose of the current study, 250 mg/kg/day was chosen as the high dose to induce peroxisome proliferation. The lower dose of 25 mg/kg/day was selected as one-tenth of the confirmed toxic dose.

To help address the goals of the HWG, the aims of this study were *a*) to investigate whether the gene expression profiles observed after exposure to clofibrate correlated with changes in known biological markers of hepatotoxicity (as identified by histopathology and clinical chemistry parameters), and *b*) to assess the reproducibility of the gene expression changes across different laboratories on a single microarray platform using RNA from identical *in vivo* studies conducted separately at different locations. In addition, comparison of gene expression data generated using RNA from pooled and individual animals was conducted, together with confirmatory analysis of a subset of genes using quantitative real-time polymerase chain reaction (PCR).

## Materials and Methods

Different components of the study were conducted in individual laboratories. Table 1 shows the involvement of each laboratory.

### Chemicals

Clofibrate [2-(*p*-chlorophenoxy)-2-methylpropionic acid ethyl ester] (CAS no. 637-07-0) was obtained from Sigma Chemical Corporation (St. Louis, MO, USA) and stored according to manufacturer instructions. The same batch was used by both laboratories performing the *in vivo* studies and had a purity of 99.7%.

### Animals and Treatment

*In vivo* studies were conducted at Abbott Laboratories (Abbott Park, IL, USA) and GlaxoSmithKline (GSK) (Ware, U.K.), as shown in Table 1. The male Sprague-Dawley rat was chosen as the test species, as

this sex, strain, and species has previously been used to demonstrate the hepatotoxic effect of clofibrate (e.g., Aberg and Appelkvist 1994; Lake et al. 1984a, 1984b; Lundgren and DePierre 1989) and is currently used by laboratories in the consortium. The animals [CrI:CD(SD)BR, also referred to as CrI:CD(SD)IGS VAF+], were obtained from Charles River Laboratories, Inc. (Margate, Kent, U.K.) for the GSK study and Charles River Laboratories, Inc. (Wilmington, MA, USA) for the Abbott study. Rats were 57 days of age (Abbott) or 60–66 days of age (GSK) and weighed 233.4–274.0 g (Abbott) or 258.0–316.0 g (GSK) at the start of treatment. Upon arrival, all rats were acclimatized for 6 days prior to beginning treatment and were randomly assigned to the treatment groups listed in Table 2. Rats were housed in groups of 4 or 2 of the same sex and treatment group in plastic solid-bottomed cages with Beekay bedding (B&K Universal Ltd, Hull, U.K.) (GSK) or suspended wire cages (Abbott). Rats were dosed once daily via gavage for a period of up to 7 days. The dose volume was 10 mL/kg. Doses for each rat were calculated based upon body weight data on the day of dosing. Food (see Table 3 for details of diet for each *in vivo* study) and water were available *ad libitum*. Rats were fasted overnight after their last treatment, euthanized the following morning under halothane/isoflurane anesthesia, and submitted for necropsy. Data collected as part of the *in vivo* studies included clinical chemistry, organ weights, and macroscopic and microscopic pathology.

### Hematology and Clinical Chemistry

Blood samples (approximately 5 mL/rat) were collected via the abdominal vein at necropsy. A range of hematology and clinical

chemistry parameters were measured (Table 4). For the study conducted at Abbott Laboratories, serum from animals was analyzed on an Abbott Aero set (Abbott Diagnostics, Abbott Park, IL, USA) according to manufacturer instructions. For the study conducted at GSK (U.K.), plasma from animals was analyzed on an Advia 1650 (Bayer Diagnostics, Tarrytown, NY, USA).

### Histology

A portion of the left lateral lobe was taken for histopathology in all studies (stored in 10% buffered saline). The right lobe (50–100 mg) was taken for measurement of acyl-CoA oxidase (ACOX) activity, and the remaining tissue was flash frozen for RNA isolation. Tissues to be examined histologically were fixed in 10% neutral-buffered formalin and subsequently sectioned and stained with hematoxylin and eosin.

### Acyl-CoA Oxidase Enzyme Assay

At necropsy, samples of liver (50–100 mg) were collected from all treatment groups for triplicate measurement of ACOX activity. Liver was collected, flash-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Analysis of ACOX activity was performed at Abbott Laboratories using spectrophotometric analysis via a modification of the method of Small et al. (1985). Briefly, pieces of frozen tissue were placed in 1 mL of 10% (w/v) sucrose, 3 mM imidazole, pH 7.4, and immediately homogenized by sonication on ice. The resulting homogenate was centrifuged at  $7,000 \times g$  for 10 min and lipid, if present, was aspirated. A portion of the supernatant was used in the Bradford assay (Bradford 1976) to assess protein concentration. Supernatant (10  $\mu\text{L}$ ) in quadruplicate, containing 10–15 mg/mL protein, was added to a 96-well plate. Two hundred

**Table 1.** Laboratories involved in the study.<sup>a</sup>

Company and location	Study
Abbott Laboratories, Chicago, Illinois, USA	<i>In vivo</i> exposures, clinical chemistry, and histopathology
GSK, Ware, Hertfordshire, U.K.	<i>In vivo</i> exposures, clinical chemistry, and histopathology
GSK, Research Triangle Park, North Carolina, USA	Gene expression analysis of pooled samples from days 1, 3, and 7
Unilever Research Sharnbrook, Bedfordshire, U.K.	Gene expression analysis of pooled samples from days 1, 3, and 7
U.S. EPA, Research Triangle Park, North Carolina, USA	Gene expression analysis of individual samples from day 7

**Table 2.** Study design.<sup>a</sup>

Group	Test material	Clofibrate dosage (mg/kg/day)	Clofibrate concentration (mg/mL)	Duration of treatment		
				1 day	3 days	7 days
T <sub>0</sub>	Vehicle <sup>b</sup>	0	0	4 <sup>c</sup>	4 <sup>c</sup>	4 <sup>c</sup>
T <sub>1</sub>	Clofibrate	25	2.5	4	4	4
T <sub>2</sub>	Clofibrate	250	25	4	4	4

<sup>a</sup>Satellite rats were also assigned to groups for toxicokinetic measurements. <sup>b</sup>0.2% hydroxypropyl methyl cellulose in sterile water. <sup>c</sup>Number of male rats.

eighty microliters of the reaction mixture (40 mM aminotriazole, pH 7.4; 11 mM potassium phosphate, monobasic; 0.2% Triton X-100; 0.08 mg horseradish peroxidase type IV-A; 0.05 mM 2,7-dichlorodihydrofluorescein diacetate) was then added and the mixture preincubated in the dark with mixing for 2 min. The reaction was started by the addition of 10  $\mu$ L of 3 mM palmitoyl-CoA, lithium salt, and the rate of dye oxidation was determined at 30°C,  $\lambda = 502$  nm for 3 min using a Spectramax plate reader (Molecular Devices, Sunnydale, CA, USA). Rates were corrected for substrate blank. The rate of oxidation was the net of substrate initiated minus nonsubstrate oxidation. Rates were normalized for protein concentration and expressed as millimolar per minute per milligram using a 2,7-dichlorodihydrofluorescein extinction coefficient of 9,100  $M^{-1}cm^{-1}$  or as fold increase relative to control values.

### RNA Isolation and Distribution

At necropsy, liver tissue was quickly chopped into small pieces and flash-frozen in liquid nitrogen. RNA isolation was performed at Abbott using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer protocol, and at GSK (U.K.) using RNeasy kits (Qiagen, Crawley, West Sussex, U.K.) according to manufacturer instructions. A portion of the RNA from the four animals

in each treatment group was pooled, using equivalent amounts. The remainder was retained as individual samples. RNA samples were aliquoted and precipitated in ethanol and ammonium acetate for shipment to the microarray analysis laboratories.

### cDNA Gene Expression Arrays

cDNA probes were prepared according to manufacturer instructions and hybridized to Atlas Rat Toxicology II arrays (Clontech, Palo Alto, CA, USA) containing 465 genes. Pooled RNA samples were analyzed by GSK (U.S.) and Unilever Research (ULR; Sharnbrook, Bedfordshire, U.K.) using triplicate arrays. The U.S. Environmental Protection Agency (U.S. EPA) analyzed RNA samples from individual animals ( $n = 4$ /group). Other differences in interlaboratory experimental protocols are noted in Table 3.

### Image Capture and Analysis

Probed arrays were developed using phosphorimagers [GSK (U.S.): Cyclone (Packard Bioscience Company, Meriden, CT, USA); U.S. EPA: FX (BioRad, Hercules, CA, USA)] or exposed to X-ray film for up to 5 days at  $-70^{\circ}C$  (ULR). After scanning, individual image files were analyzed using the AtlasImage 2.0 program (Clontech), and the intensity of each spot was determined. The complete data set is currently being submitted to ArrayExpress (EMBL-European

Bioinformatics Institute, Hinxton, U.K.; <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 web site (<http://hesi.ils.i.org/index.cfm?pubentityid=120>).

### Data Analysis

**U.S. Environmental Protection Agency.** Background was subtracted from intensity values and  $\log_2$  values were calculated for use in the analysis. For intensity values less than background, 0 was used in the analysis. Values less than twice background were flagged as not expressed. Genes that were not expressed in at least three of four arrays for at least one of three treatment groups were removed from further analysis. Normalization was carried out using a one-way analysis of variance (ANOVA) [model  $\log(\text{intensity}) = \text{array}$ ], so each array was centered around 0. Each gene was then analyzed in a one-way ANOVA [ $\log(\text{intensity}) = \text{treatment}$ ], and where the overall  $F$  test was significant, indicating some differences among treatment groups, the low- and high-dose groups were each tested for any difference from control using a  $t$  test. A significant change was deemed to have occurred where  $p < 0.05$ .

**GlaxoSmithKline.** Normalization and statistical analysis were by normalized local regression (Kepler et al. 2002). Fold change counts were accepted if the intensity of the signal was considered adequate by mean log intensity and by visual inspection of adjusted intensities and if  $p$ -values reflected consistent adjusted intensities greater than 10 pixels.

**Table 3.** Experimental procedures used in each laboratory performing the microarray analysis.

Method	U.S. EPA	GSK	ULR
Laboratory supplying RNA	Abbott (U.S.)	GSK (U.K.)	GSK (U.K.)
Source of animals	Charles River Laboratories Inc., (U.S.)	Charles River Laboratories Inc., (U.K.)	Charles River Laboratories Inc., (U.K.)
Sex/strain/species	Male Sprague-Dawley rat	Male Sprague-Dawley rat	Male Sprague-Dawley rat
Diet	LabDiet certified Rodent diet 5002 pellets (LabDiet, U.S.)	Rat and mouse no.1 expanded (SDS, U.K.)	Rat and mouse no.1 expanded (SDS, U.K.)
RNA isolation method	Trizol (Invitrogen)	RNeasy kit (Qiagen)	RNeasy kit (Qiagen)
Isotope for probe labeling	$^{32}P$	$^{33}P$	$^{33}P$
Membrane condition	New for each sample	New for each sample	Stripped and reprobbed (according to manufacturer's instructions)
Hybridization temperature	68°C	62°C	68°C
Hybridization time	15–18 hr	16 hr	16 hr
Image development time	Up to 96 hr	72 hr	Up to 120 hr
Image capture medium	Phosphorimaging plate	Phosphorimaging plate	X-ray film
Image capture device	FX (BioRad)	Cyclone (Packard Bioscience Co.)	Image Master (Amersham Biosciences, Cardiff, U.K.)
Image capture software	Quantity 1 (BioRad)	OptiQuant (Packard Bioscience Co.)	Liscap (Amersham Biosciences)
Data extraction	AtlasImage 2.0 (Clontech)	AtlasImage 2.0 (Clontech)	AtlasImage 2.0 (Clontech)
Data analysis	ANOVA	Linear regression	Manual

**Table 4.** Hematology and clinical chemistry measurements.

Hematology	Clinical chemistry
Hemoglobin	Alkaline phosphatase
Hematocrit	Alanine aminotransferase
Red blood cell count	Aspartate aminotransferase
Reticulocyte count	Triglycerides
Erythrocytic indices	Globulin
White blood cell count	Glucose
Differential white blood cell count	Urea (nitrogen)
Platelet count	Total protein
Prothrombin time	Albumin
Activated partial thromboplastin time	Creatinine
Fibrinogen	Cholesterol
	Sodium
	Potassium
	Calcium
	Total bilirubin
	Chloride
	Phosphorus
	Glutamate dehydrogenase
	Sorbitol dehydrogenase
	Gamma glutamyltransferase

**Unilever Research.** Background was subtracted from intensity values and a global normalization performed using AtlasImage 2.0 (Clontech). For intensity values less than background, zero was used in the analysis, indicating “not expressed.” All adjusted intensity values were visually inspected and genes that were not expressed in at least two of three arrays were removed from further analysis.

**Quantitative real-time polymerase chain reaction.** Total RNA was isolated from samples as previously described. Quantitative real-time PCR was conducted using TaqMan and SYBR green methods. Briefly, primers were designed for ACOX, apolipoprotein A-1 (APO-A1), catecholamine O-methyltransferase (COMT), cytochrome P450 4A1 (CYP4A1), cytosolic epoxide hydrolase (CEH), hydroxyacyl-CoA dehydrogenase (HCD), paraoxonase 1 (PON1), PPAR $\alpha$ , and phosphoenolpyruvate carboxykinase (PEPCK) with Primer Express software (PerkinElmer Applied Biosystems, Foster City, CA, USA) following the manufacturer’s advice for optimal primer design for the TaqMan reactions. For the SYBR green method, Clontech sequences were used. Primer and probe sequences are listed in Table 5. Samples were diluted to 10 ng/ $\mu$ L prior to analysis (ABI Prism 7700 Sequence Detection System; PerkinElmer). Forward and reverse primers and probes were diluted to the appropriate concentrations to make the probe/primer master mix. SYBR green primers were used at 300 nM final concentration, TaqMan primers at 900 nM, and TaqMan probes at 200 nM. The master mix was prepared according to manufacturer protocol (without probes and primers) and 30  $\mu$ L master mix, 5  $\mu$ L RNA, and 15  $\mu$ L

probe/primer mix were aliquoted per well into the gene plate. The plate was sealed and centrifuged at 3,000  $\times$  g for 10 sec. The reaction was incubated at 48°C for 30 min [reverse transcriptase (RT) step], denatured

at 95°C for 10 min (Amplitaq activation and RT denaturation), then subjected to 40 PCR cycles of 94°C for 15 sec and 60°C for 1 min. Values of fold change in expression were graphed for comparison purposes.

**Table 6.** Summary results from selected clinical chemistry and pathology parameters as determined in the two *in vivo* studies.<sup>a</sup>

Parameters	Sampling day	Clofibrate daily dose (mg/kg/day)		
		0	25	250
Total cholesterol (mmol/L)				
GSK (U.K.)	1	2.01 $\pm$ 0.40	1.58 $\pm$ 0.10* (78.6%)	0.96 $\pm$ 0.27* (47.8%)
	3	1.77 $\pm$ 0.26	1.76 $\pm$ 0.30 (99.4%)	1.31 $\pm$ 0.15* (74.0%)
	7	1.68 $\pm$ 0.15	1.91 $\pm$ 0.24 (113.7%)	1.09 $\pm$ 0.20* (64.9%)
Abbott	1	1.74 $\pm$ 0.55	1.61 $\pm$ 0.30 (92.5%)	1.22 $\pm$ 0.30 (70.1%)
	3	1.90 $\pm$ 0.19	1.61 $\pm$ 0.20* (84.7%)	1.28 $\pm$ 0.23 (67.4%)
	7	1.30 $\pm$ 0.31	1.37 $\pm$ 0.37 (105.4%)	0.88 $\pm$ 0.31 (67.7%)
Triglycerides (mmol/L)				
GSK (U.K.)	1	0.89 $\pm$ 0.11	0.73 $\pm$ 0.06 (82.0%)	0.87 $\pm$ 0.21 (97.7%)
	3	0.94 $\pm$ 0.04	1.02 $\pm$ 0.39 (108.5%)	0.70 $\pm$ 0.15* (74.5%)
	7	1.03 $\pm$ 0.46	0.95 $\pm$ 0.09 (92.2%)	0.78 $\pm$ 0.11 (75.7%)
Abbott	1	0.58 $\pm$ 0.36	0.44 $\pm$ 0.26 (75.9%)	0.89 $\pm$ 0.45 (153.4%)
	3	0.58 $\pm$ 0.20	0.53 $\pm$ 0.20 (91.4%)	0.47 $\pm$ 0.05 (81.0%)
	7	0.27 $\pm$ 0.07	0.32 $\pm$ 0.09 (118.5%)	0.39 $\pm$ 0.12 (144.4%)
Globulins (g/L)				
GSK (U.K.)	1	19.98 $\pm$ 2.63	19.60 $\pm$ 0.65 (98.1%)	18.43 $\pm$ 1.37 (92.2%)
	3	19.15 $\pm$ 1.64	21.25 $\pm$ 1.58 (110.0%)	15.98 $\pm$ 2.33* (83.3%)
	7	18.88 $\pm$ 0.71	19.13 $\pm$ 0.43 (101.3%)	15.33 $\pm$ 1.14* (81.2%)
Abbott	1	13.25 $\pm$ 1.26	12.75 $\pm$ 1.26 (96.2%)	12.75 $\pm$ 0.96 (96.2%)
	3	13.25 $\pm$ 0.50	12.50 $\pm$ 1.73 (94.3%)	11.67 $\pm$ 0.58 (88.1%)
	7	12.25 $\pm$ 0.96	12.50 $\pm$ 1.73 (102.0%)	12.00 $\pm$ 0.82 (98.0%)
Albumin (g/L)				
GSK (U.K.)	1	37.45 $\pm$ 1.01	38.55 $\pm$ 1.02 (102.9%)	39.25 $\pm$ 1.51 (104.8%)
	3	38.35 $\pm$ 1.95	39.58 $\pm$ 1.26 (103.2%)	41.88 $\pm$ 1.37* (109.2%)
	7	38.78 $\pm$ 0.42	40.78 $\pm$ 0.54* (105.2%)	43.35 $\pm$ 0.82* (111.8%)
Abbott	1	45.75 $\pm$ 1.26	43.00 $\pm$ 2.16 (94.0%)	46.25 $\pm$ 0.98 (101.1%)
	3	45.50 $\pm$ 1.73	43.50 $\pm$ 2.08 (95.6%)	44.67 $\pm$ 1.53 (98.2%)
	7	43.25 $\pm$ 1.50	43.00 $\pm$ 1.41 (99.4%)	43.50 $\pm$ 0.57 (100.6%)
Mitotic index, hepatocellular				
GSK (U.K.)	1	0/4	1/4	3/4
	3	0/4	2/4	4/4
	7	0/4	1/4	1/4
Abbott	1	0/4	3/4	2/4
	3	0/4	3/4	2/4
	7	0/4	0/4	0/4

<sup>a</sup>Data shown are the mean ( $n = 4$ )  $\pm$  SD. Numbers in parentheses are percentage of control values. \*Statistically significant at the 5% level ( $p < 0.05$ ).

**Table 5.** Primer pairs and probes used for quantitative real-time PCR.

Gene	GenBank accession no. <sup>a</sup>	Forward primer	Probe	Reverse primer
TaqMan primers and probes				
CEH	X65083	ATGAGCCCCAGTCCCAATG	FAM-ACATACACAGTGGCGAAATCCTTACCCT-TAMRA	CCGCTGTCTTGACTGCAT
ACOX	J02752	TGCTGGCATCGAAGAATGTC	FAM-GCCCGCCGACAGGCCATT-TAMRA	GGAAATCCCACTGCTGTGAGAA
CYP4A1	X07259	TCCAGGTTTGCACCAGACTCT	FAM-CCCAGACACAGCCACTCATTCT-TAMRA	TCCTCGCTCTCTGAGAA
PPAR $\alpha$	M88592	TGGAGTCCAGCATGTGAAG	FAM-CTGCAAGGGCTTCTTCGGCGA-TAMRA	CGCCAGCTTTAGCCGAATAG
COMT	M60753	CAGCAGCTCACACCACATCTC	FAM-TCTGAAGAATGAGTCAAGCTTTC-TAMRA	GGCGTGACCACCATAACC
PON1	U94856	GGTAGCAGATGGATTTGATTTCC	FAM-TGGCATTGGCATTCCCTTGATGG-TAMRA	CAGCAATTCAGCGATATAGACATACTT
PEPCK	K03248	ACAGGCAAGGTCATCATGCA	FAM-ACCCTTCGCTATGCCGCC-TAMR	TGCCGAAGTTGTAGCCAAAGA
HCD	K03249	CCTCTGAAGGAATGGCAAAGC	FAM-CAGGGCCCCACGGCAGCA-TAMR	CGCAGAAGGCTGAATCACAGT
APO-A1	X00558	GTGTGAGCTGAGCCAACACTTT	FAM-TTCTCTATAGAGTCTGTCCAGTTCGGGA-TAMRA	TAGATCTGAGGCTCAGGCTTGAT
SYBR green primer pairs				
CEH	X65083	CAGTCCCGTGCAGTCCAAATGATG		CATGTCTATAGCTAGAACAC GAAAGCC
ACOX	J02752	CTCATCTTCGAGGCTTGGAAACCAC		ATTTACCGGATAGGGACAA CAAAGGC
CYP4A1	X07259	CAAAGCCTACTTAATGTCTATGACCC		CATCAGTCGAATGGAGTC AGCCATG
PPAR $\alpha$	M88592	CAGGTTACCTTGTGAAGTACGGTG		AAATGTCACTGTCTCCAG TTCGAGG
COMT	M60753	AGCCAGTCCACAACCTGATCATGG		GTAATCCCAAGGCTGATCCATG
PON1	U94856	TGGCCCTGTGATGGTCCAATGTTG		TTATCCACAAGGCTGCAAG AGCTGAG
PEPCK	K03248	CAAGATTGGTATTGAGCTGACAGACTC		AAGCAGTGAAGTCCACC GTATCC
HCD	K03249	AATCGGATGTTGGTCCCTATTACAAC		GTGGCAATGATGGTCCAG TAAGGC
APO-A1	X00558	CAGCTAGGCCAGTACTCAGGAG		CGCAGCGCTGTGCATTACACG

<sup>a</sup>From GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/>).

## Results

### Animal Observations, Clinical Chemistry, and Histopathology

Two separate *in vivo* studies were conducted: one at Abbott Laboratories and one at GSK (U.K.). Differences in the level of biological response induced in the two *in vivo* studies were observed by variance in the percentage of liver weight increase at days 3 and 7. For example, the high-dose (250 mg/kg/day) group at day 3 demonstrated a 15% increase in liver weight relative to body weight in the GSK study, compared with a 3% liver weight increase in the Abbott study. This relationship was similarly reflected in the 7-day high-dose group, where a 31% liver weight increase was observed in the GSK study, compared

with a 15% increase in the Abbott study. Observed changes in clinical chemistry parameters also indicated differences in the biological response of the *in vivo* study concordant with the differences in liver weight increase (Table 6). For example, a significant reduction in total cholesterol levels was seen in the GSK study at the high dose for all time points. However, the Abbott study demonstrated a significant reduction at only one dose and time point. Similarly, a reduction in globulins and triglycerides was more pronounced in the GSK study, as was the increase in albumin concentrations. An increase in hepatocellular mitotic activity (as indicated by numbers of mitotic figures noted in the liver sections) was detected in both studies in the day 1, and most notably, day 3 high- and low-dose groups. However,

by day 7, the incidence of mitotic figures had decreased. An increase in clotting time after a single dose of clofibrate, associated with biosynthetic function changes in the liver after the disturbance of liver function, was also found.

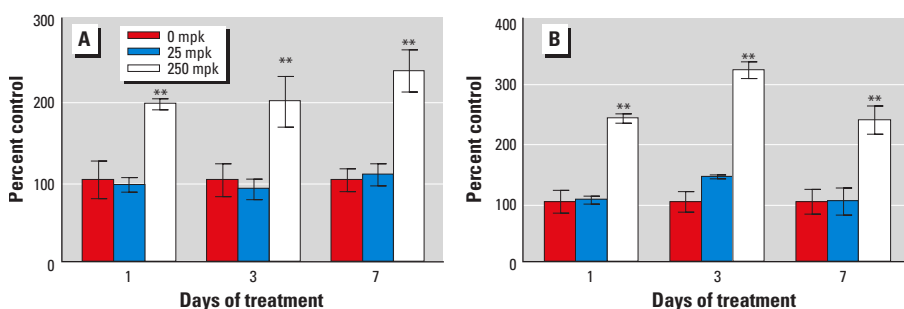
### Acyl-CoA Oxidase Activity

In both *in vivo* studies, an increase in ACOX activity 2–3 times that of the control was demonstrated at the high-dose (250 mg/kg/day) level only (Figure 1). There was no significant difference between ACOX levels in the control and low-dose (25 mg/kg/day) groups.

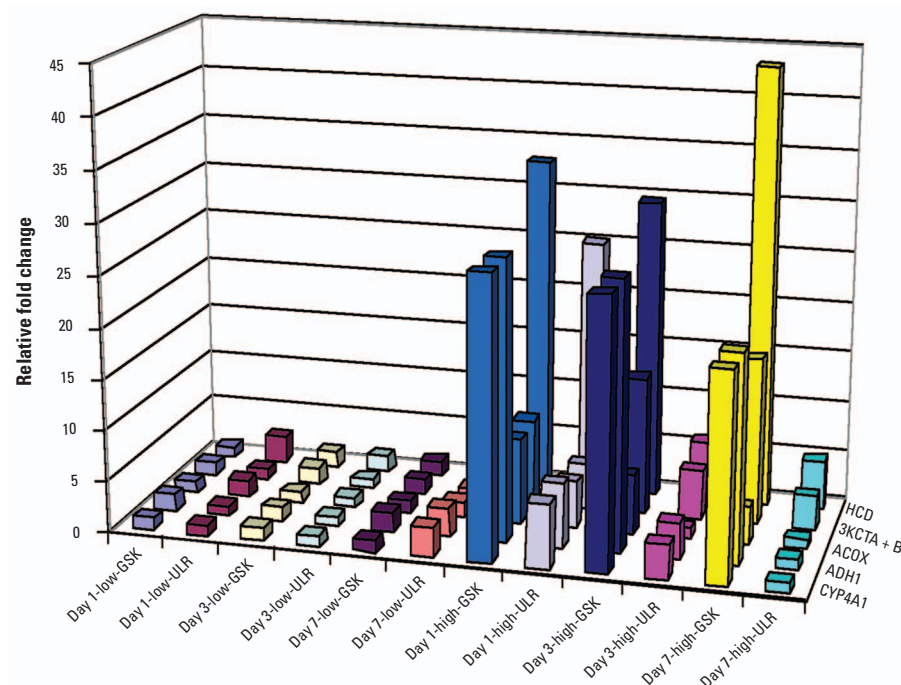
### Microarray Analysis

**Reproducibility of microarray results across different laboratories.** GSK (U.S.) and ULR analyzed pooled samples from animals treated with high (250 mg/kg/day) and low (25 mg/kg/day) doses of clofibrate for 1, 3, or 7 days at GSK (U.K.). Comparison of the gene expression data obtained from pooled RNA samples indicated that genes previously described as being regulated by clofibrate or that are associated with the histopathology findings were detected. In general, a dose-related response in gene expression was observed with the low dose, demonstrating little deviation from control levels of gene expression. The high-dose tissues demonstrated typical PPAR $\alpha$ -mediated responses associated with  $\beta$ -oxidation of fats, metabolism of bile acids and steroids, and early cell proliferation [e.g., HCD, 3-ketoacyl-CoA thiolase A + B (3KCTA+B), ACOX, aldehyde dehydrogenase 1 (ADH1), and CYP4A1]. Examples of some of these gene expression changes are shown in Figure 2. It is apparent that although the changes in gene expression observed for these five genes were demonstrated by both laboratories, there are quantitative differences in the fold change values observed between the two sites.

The upregulation of a variety of cell proliferation-associated genes (e.g., G2/M-specific cyclin B1, cyclin-dependent kinase 1, DNA topoisomerase II alpha, *c-myc* protooncogene, pololike serine-threonine protein kinase, and cell division control protein 20) began on or before day 1 and peaked at some point between days 3 and 7. By day 7, cell proliferation genes were downregulated. The chronology of this gene expression agrees with the histologic diagnoses of mitotic figures in the tissue, where an increase in mitotic figures was detected in the day 1, and most notably, day 3 high- and low-dose groups. However, by day 7, the incidence of mitotic figures had decreased. The clustering of genes associated with the G2/M transition point suggests that in the rat, the polyploid cells



**Figure 1.** ACOX activity in rat liver samples from two *in vivo* studies: (A) Abbott Laboratories and (B) GSK (U.K.). mpk, mg/kg/day. Rats received clofibrate by oral gavage at low (25 mg/kg/day) and high (250 mg/kg/day) doses for 1, 3, and 7 days. Data shown are the mean  $\pm$  SD for each group ( $n = 4$ ). Asterisks (\*\*) indicate significant ( $p < 0.01$ ) changes from control. Significance was assessed using Student *t* test.



**Figure 2.** Examples of dose- and time-related gene expression changes using cDNA microarrays after exposure to clofibrate. Rats received clofibrate by oral gavage at low (25 mg/kg/day) and high (250 mg/kg/day) doses for 1, 3, and 7 days. Data shown are the mean from pooled samples ( $n = 4$ ) run on triplicate arrays.

arrested at G2/M are those that are proceeding through the cell cycle.

Upregulation of genes representing increased  $\beta$ -oxidation of fatty acids (e.g., ACOX, 3KCTA+B) and metabolism of bile acids were also seen at 3 days as well as genes representative of peroxisome biogenesis. There was also downregulation of genes associated with movement of bile acids and xenobiotics into biliary canaliculi. At the same time that fatty acid oxidation by the liver was increasing, gluconeogenesis was decreasing, as indicated by the downregulation of PEPCK. Examples of these genes are shown in Tables 7–10 and in Figures 2 and 3.

**Reproducibility across different in vivo studies.** The U.S. EPA analyzed gene expression in individual RNA samples from day 7 high- and low-dose animals treated at Abbott. GSK (U.S.) and ULR analyzed gene expression in pooled RNA from day-7 high- and low-dose animals treated at GSK (U.K.). Gene expression data from individual animal samples indicated that 7 genes were significantly ( $p < 0.05$ ) upregulated (maximum of 7.2-fold increase), and 12 were downregulated (maximum of 4.3-fold decrease) in the high-dose group. The low-dose group generated only one statistically significant gene expression change, namely, heat shock protein 70 (HSP70). In comparison, expression changes in the 7-day pooled high-dose samples analyzed by GSK (U.S.) ranged from a 43.3-fold increase to a 3.5-fold decrease. Changes in these same samples analyzed by ULR ranged from a 4.9-fold increase to a 4.3-fold decrease. Tables 8 and 9 show statistically significant gene expression changes in the individual animal samples compared with those from the pooled samples.

### Quantitative Real-Time Polymerase Chain Reaction

Figure 3 shows the quantitative real-time PCR results for the selected genes across different doses and time points using TaqMan and SYBR green methods. These genes were selected for confirmatory studies on the basis of their known modulation (up- and downregulation) by clofibrate, both in this study and in previous studies. In addition, they were common to all microarray platforms being used within the HWG and would therefore allow cross-platform confirmatory studies to be performed. A comparison of gene expression data from day 7 high-dose samples obtained using quantitative real-time PCR versus data generated using cDNA microarrays is shown in Table 10. The genes selected for confirmatory analysis, despite showing some quantitative differences in fold changes (particularly at the

high end of the upregulated genes), demonstrated a qualitatively similar pattern of expression between the quantitative real-time PCR results and the microarray data. PPAR $\alpha$ , however, demonstrated equivocal results. Although both methods of quantitative real-time PCR on the pooled sample showed the gene to be downregulated, the GSK (U.S.) pooled sample microarray analysis indicated upregulation; the ULR pooled and U.S. EPA individual microarray analyses showed no change.

## Discussion

### Mechanism of Clofibrate Action

Clofibrate is a member of a large class of diverse chemicals known as peroxisome proliferators (PPs). Peroxisomes are subcellular organelles that perform diverse metabolic functions, including  $H_2O_2$ -derived respiration,  $\beta$ -oxidation of very long chain fatty acids, and cholesterol metabolism (Mannaerts et al. 2000; Singh 1997). PPs are thought to cause cancer by a nongenotoxic

**Table 7.** Comparison of data (top upregulated genes) obtained for pooled RNA samples from high-dose (250 mg/kg/day) 3-day clofibrate-exposed rats.<sup>a</sup>

Gene	GenBank accession no. <sup>b</sup>	GSK (U.S.) (rank/fold change)	ULR (rank/fold change)
HCD	K03249	1/465 $\uparrow$ 29.6	1/465 $\uparrow$ 5.9
CYP4A1	X07259	2/465 $\uparrow$ 26.4	8/465 $\uparrow$ 3.5
3KCTA+B	M32801, J02749	3/465 $\uparrow$ 13.6	3/465 $\uparrow$ 4.8
Estradiol 17- $\beta$ dehydrogenase 3	AF035156	4/465 $\uparrow$ 8.7	4/465 $\uparrow$ 4.7
ACOX	J02752	6/465 $\uparrow$ 5.7	7/465 $\uparrow$ 3.9
CYP17	M21208	11/465 $\uparrow$ 4.3	5/465 $\uparrow$ 4.6
ADH1	M23995	12/465 $\uparrow$ 4.2	9/465 $\uparrow$ 2.6
Pololike serine threonine protein kinase	U10188	17/465 $\uparrow$ 3.1	10/465 $\uparrow$ 2.5

Abbreviations: CYP17, cytochrome P450 subfamily 17;  $\uparrow$ , increase.

<sup>a</sup>Pooled RNA samples generated from one *in vivo* study (GSK, U.K.) were supplied to two laboratories, GSK (U.S.) and ULR, for analysis on Atlas Rat Toxicology II cDNA arrays. Rank is based on ordering genes using fold change values. Data shown are the mean from pooled samples ( $n = 4$ ) run on triplicate arrays. <sup>b</sup>From GenBank (<http://www.ncbi.nih.gov/GenBank/>).

**Table 8.** Upregulated genes from high-dose (250 mg/kg/day) 7-day clofibrate-treated rats. Comparison of data from individual and pooled samples.<sup>a</sup>

Gene	Genbank accession no. <sup>b</sup>	U.S. EPA <sup>c</sup> (fold change)	GSK (U.S.) <sup>d</sup> (fold change)	ULR <sup>d</sup> (fold change)
HCD	K03249	$\uparrow$ 7.2	$\uparrow$ 43.3	$\uparrow$ 4.9
3KCTA+B	M32801, J02749	$\uparrow$ 7.1	$\uparrow$ 16.4	$\uparrow$ 3.3
CYP4A1	X07259	$\uparrow$ 3.8	$\uparrow$ 20.3	$\uparrow$ 2.2
ACOX	J02752	$\uparrow$ 3.1	$\uparrow$ 3.6	$\uparrow$ 1.4
CYP2B1	M11251	$\uparrow$ 1.6	NC	$\downarrow$ 1.9
NAD(P)H dehydrogenase	J020608	$\uparrow$ 1.4	$\downarrow$ 1.4	ND
40S ribosomal protein S30	X62671	$\uparrow$ 1.2	$\uparrow$ 1.4	NC

Abbreviations: NC, no change; ND, not detected;  $\uparrow$ , increase;  $\downarrow$ , decrease.

<sup>a</sup>Seven genes demonstrated statistically significant ( $p < 0.05$ ) upregulation for samples obtained from individual animals ( $n = 4$ ) (U.S. EPA). These data are compared with gene expression data from pooled samples ( $n = 4$ ), run on triplicate arrays. Data shown are the mean of the replicates. <sup>b</sup>From GenBank (<http://www.ncbi.nih.gov/GenBank/>). <sup>c</sup>Individual samples. <sup>d</sup>Pooled samples.

**Table 9.** Downregulated genes from high-dose (250 mg/kg/day) 7-day clofibrate-treated rats. Comparison of data from individual and pooled samples.<sup>a</sup>

Gene	Genbank accession no. <sup>b</sup>	U.S. EPA <sup>c</sup> (fold change)	GSK (U.S.) <sup>d</sup> (fold change)	ULR <sup>d</sup> (fold change)
APO-A1	M00001	$\downarrow$ 4.3	$\downarrow$ 2.3	$\downarrow$ 4.3
PEPCK	K03243	$\downarrow$ 2.5	$\downarrow$ 2.0	$\downarrow$ 2.8
CYP1A2	K02422	$\downarrow$ 2.3	$\downarrow$ 2.7	NC
Early growth response protein 1	M18416	$\downarrow$ 2.1	$\downarrow$ 3.5	NC
Activin, beta E	AF089825	$\downarrow$ 2.0	$\downarrow$ 1.7	$\downarrow$ 1.5
G1/S-specific cyclin D1	D14014	$\downarrow$ 1.7	$\downarrow$ 1.9	ND
HSP70	Z27118	$\downarrow$ 1.7	$\downarrow$ 1.5	$\downarrow$ 1.1
CPT1	L07736	$\downarrow$ 1.7	$\uparrow$ 2.5	$\downarrow$ 4.2
Protein disulfide isomerase-related protein	M86870	$\downarrow$ 1.7	$\downarrow$ 1.6	$\downarrow$ 1.3
Tumor necrosis factor receptor 1	M63122	$\downarrow$ 1.6	$\downarrow$ 1.3	$\downarrow$ 1.2
Serine protease inhibitor	M32247	$\downarrow$ 1.5	NC	$\downarrow$ 1.1
ABC transporter	AJ003004	$\downarrow$ 1.4	$\uparrow$ 1.3	ND

Abbreviations: NC, no change; ND, not detected;  $\uparrow$ , increase;  $\downarrow$ , decrease.

<sup>a</sup>Eleven genes demonstrated statistically significant ( $p < 0.05$ ) downregulation for samples obtained from individual animals ( $n = 4$ ) (U.S. EPA). These data are compared with gene expression data from pooled samples ( $n = 4$ ) run on triplicate arrays. Data shown are the mean of the replicates. <sup>b</sup>From GenBank (<http://www.ncbi.nih.gov/GenBank/>). <sup>c</sup>Individual samples. <sup>d</sup>Pooled samples.

mechanism (Diez-Fernandez et al. 1998; Ito et al. 1992), by indirectly altering gene expression and affecting the phenotype of the target cell. The response is most notable in rodent liver, whereas humans are relatively insensitive (Hertz and Bar-Tana 1998). Many of the effects of clofibrate and other PPs are receptor mediated. Activation of PPAR $\alpha$ , a member of the nuclear superfamily highly expressed in hepatocytes, cardiomyocytes, enterocytes, and renal proximal tubule cells, has been strongly correlated with peroxisome proliferation and liver cancer (Corton et al. 2000). PPARs are ligand-activated transcription factors that control gene expression by interacting with specific DNA response elements located upstream of responsive genes. These elements, known as PP response element motifs, are found in many genes, including acyl-CoA, liver fatty acid binding protein, microsomal CYP4A, a fatty acid  $\omega$ -hydroxylase, and growth regulatory genes such as *c-myc*, *c-Ha-ras*, *fos*, *jun*, and *egr-1* (Corton et al. 2000; Vanden Heuvel 1999). The growth regulatory genes are pivotal in the progression of the cell cycle, in particular the transition from G1 to S phase. The obligatory need for PPAR $\alpha$  activation for the expression of PP-induced events is evident from observations that PPAR $\alpha$  knockout mice do not show the morphological and biochemical changes typically observed in rodents after acute or chronic administration of PPs (Lee et al. 1995).

### In Vivo Studies—Induction of Clofibrate-Induced Hepatotoxicity

To build a foundation on which to interpret gene expression data, it is important to understand how modulations in gene expression correlate with clinical chemistry and histopathological changes. The two *in vivo* studies generally produced expected outcomes with regard to changes in clinical chemistry and histopathology. For example, several dose- and time-related changes associated with a disturbance of metabolic function in the liver were observed after clofibrate treatment. These included a characteristic increase in liver weight (due

to hepatocyte hyperplasia and hypertrophy), midzonal mitoses, centrilobular hypertrophy, and changes in clinical pathology (e.g., lipid metabolism and clotting parameters), as previously reported (Kim et al. 1998; Popp et al. 1994).

However, some biological responses differed between the two *in vivo* studies. For example, at days 3 and 7, there was variance in the percentage of liver weight increase, which was also reflected in levels of ACOX activity. ACOX is an enzyme specific to peroxisomes and is involved in the oxidation of fatty acids (Kovacs et al. 2001; Lindauer et al. 1994). Under conditions of induced proliferation of the peroxisomes (e.g., administration of clofibrate), ACOX activity is extensively increased (Latruffe et al. 2001). Observed changes in clinical chemistry parameters also indicated differences in the biological response of the *in vivo* studies, concordant with the differences in liver weight increase. For example, a significant reduction in total cholesterol levels was seen in the GSK (U.K.) study at the high dose for all time points. However, the Abbott study demonstrated a significant reduction at only one dose/time point. Similarly, a reduction in globulins and triglycerides was more pronounced in the GSK (U.K.) study, as was the increase in albumin concentrations. Such differences in *in vivo* responses could be caused by a number of factors, including the source of the animals or differences in animal husbandry (e.g., diet, cage type, bedding) at the two *in vivo* exposure sites. In addition, as pharmacokinetic analysis was not performed, it is possible that difference in drug exposure occurred between the two sites, despite the fact that efforts were made to standardize the treatment protocols between the two sites. These *in vivo* differences could in turn account for some of the differences in gene expression noted between the two studies.

### Corroboration of Gene Expression Data—Comparison with Published Data

Corroboration of microarray data has become an integral and very necessary

component of most studies using the technology. One way to verify microarray data is to compare observed changes with those previously published for the same or similar models. The gene changes induced in rat liver after treatment with clofibrate have been well documented.  $\beta$ -Oxidation of fatty acids requires the presence of several enzymes located in the peroxisomes and mitochondria (Latruffe et al. 2001), and it has been shown that PPs such as clofibrate induce expression of genes encoding ACOX, enoyl-CoA hydratase, HCD (multifunctional enzyme), and ketoacyl-CoA thiolase, all of which are responsible for very long-chain fatty acid  $\beta$ -oxidation in peroxisomes (Amacher et al. 1997; Hamadeh et al. 2002a; Schoonjans et al. 1996).

Evaluation of differential gene expression data from the three laboratories performing microarray analysis in this study revealed gene expression patterns consistent with the established literature for this class of compound. For example, fatty ACOX and HCD are the first two enzymes of the peroxisomal  $\beta$ -oxidation system. Reddy et al. (1986) previously demonstrated an upregulation of these genes (9- to 15-fold) as early as 1 hr after administration of clofibrate. Although there were some quantitative (fold change) differences observed between the laboratories in this study, upregulation of these genes was seen as early as day 1 (Figure 2), with the greatest upregulation being observed with the high-dose group. In addition, HCD and 3-keto-acyl-CoA thiolase were consistently upregulated at both days 3 and 7. This is in accordance with earlier reports by Gerhold et al. (2001).

Clofibrate exposure in the current study also induced CYP4A1 activity using both microarrays and quantitative real-time PCR (Table 10; Figures 2 and 3). The transcriptional activation by clofibrate of CYP4A1 through the PPAR $\alpha$  receptor has previously been reported in liver both *in vivo* (Correia 1995; Simpson 1997) and *in vitro* (Yaacob et al. 1997).

In many studies, attention is focused on genes induced as a result of chemical

**Table 10.** Comparison of gene expression generated using quantitative real-time PCR and cDNA arrays from high-dose (250 mg/kg/day) 7-day clofibrate-treated rats.<sup>a</sup>

Gene	Genbank accession no. <sup>b</sup>	Microarray fold change			Quantitative real-time PCR	
		U.S. EPA	ULR	GSK (U.S.)	TaqMan	SYBR green
HCD	K03249	↑ 7.2	↑ 4.9	↑ 43.3	↑ 17.1	↑ 18.8
CYP4A1	X07259	↑ 3.8	↑ 2.2	↑ 20.3	↑ 81.2	↑ 15.7
ACOX	J02752	↑ 3.1	↑ 1.4	↑ 3.7	↑ 9.7	↑ 2.1
APO-A1	M00001	↓ 4.3	↓ 4.3	↓ 2.3	Did not amplify	↓ 3.2
CEH	X65083	NC	NC	↑ 8.3	↑ 11.0	↑ 21.7
PEPCK	K03243	↓ 2.5	↓ 2.8	↓ 2.0	↓ 5.9	↓ 1.9
PON1	U94856	NC	NC	↓ 1.5	↓ 2.4	↓ 1.5
PPAR $\alpha$	M88592	NC	NC	↑ 1.8	↓ 4.5	↓ 1.2

Abbreviations: NC, no change; ND, not detected; ↑, increase; ↓, decrease.

<sup>a</sup>Data from the U.S. EPA are the mean from individual animals ( $n = 4$ ). Data from GSK and ULR are from pooled samples ( $n = 4$ ) run on triplicate arrays. Data shown are the mean of replicates. Quantitative real-time PCR data are shown for TaqMan and SYBR green methods. <sup>b</sup>From GenBank (<http://www.ncbi.nih.gov/GenBank/>).

treatment. However, it is equally important to monitor those genes that have been downregulated or switched off. In this study, decreased lipid levels in serum are suggested by the downregulation of APO-A1, as demonstrated by all three laboratories. These observations were confirmed by quantitative real-time PCR (Table 10; Figure 3) and are consistent with the findings of Vu-Dac et al. (1998) and Staelens et al. (1992), who reported that the fibrates, including clofibrate, provoke a dose-dependent decrease in liver APO-A1 mRNA levels that was associated with a lower transcription rate of the APO-A1 gene.

Exposure of male rats to clofibrate for 3 days also results in a downregulation of PEPCK using microarrays (Gerhold et al. 2001). This indicates decreased lipid turnover as well as decreased gluconeogenesis mediated by PEPCK in the liver. Results

from this study are consistent with these findings. Downregulation of PEPCK was noted in the high-dose group after 1, 3, and 7 days of exposure using microarrays and quantitative real-time PCR.

### Corroboration of Gene Expression Data—Quantitative Real-Time PCR

To confirm some of key clofibrate gene changes identified using cDNA arrays, nine genes were selected for quantitative real-time PCR analysis using TaqMan and SYBR green. Using these two methods, the selected genes demonstrated dose- and time-dependent patterns of change (Figure 3). The quantitative differences observed were most likely due to the different primer sequences and PCR reaction efficiencies of the two methods.

A comparison of gene expression data obtained using quantitative real-time PCR

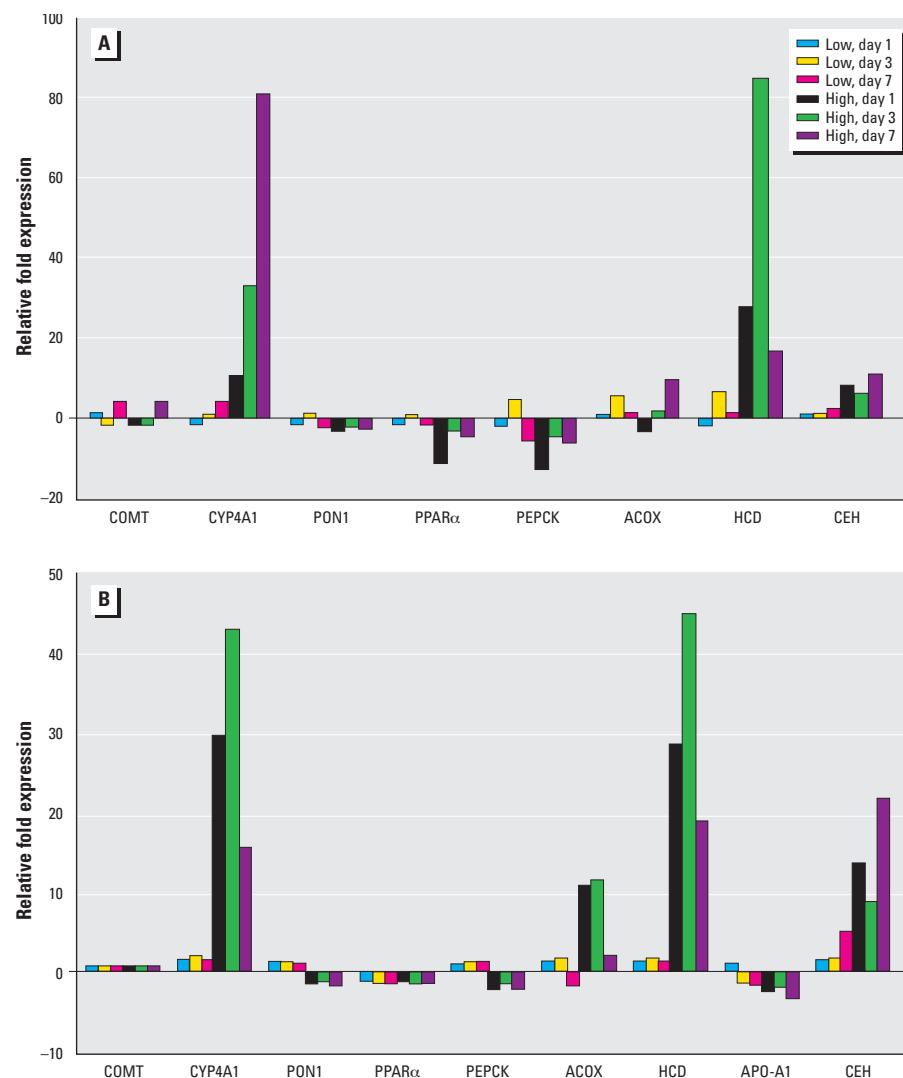
and cDNA arrays (Table 10) shows that, despite quantitative differences in fold changes (particularly at the high end of the upregulated genes), a qualitatively similar pattern of gene expression between the quantitative real-time PCR results and the microarray data was seen. Quantitative differences between cDNA array measurements and quantitative real-time PCR values are not surprising, as microarrays are, at best, semiquantitative and suffer from compression of values at high fold-changes. Gerhold et al. (2001) also reported similar quantitative differences between quantitative real-time PCR and microarray data from rats exposed to clofibrate. For example, CYP4A1 demonstrated a 9-fold upregulation using microarrays but a 47-fold upregulation using quantitative real-time PCR, a finding similar to those from this study. In addition, quantitative real-time PCR is able to detect relatively small gene expression changes deemed undetectable using microarrays (Table 10). It is therefore suggested that microarrays are a valuable way to screen many hundreds or thousands of genes that may respond to a chemical stimulus and that quantitative real-time PCR can be used to confirm observations on selected genes of particular interest.

### Comparison of Microarray Data across Laboratories

Tables 8 and 9 are lists of genes that were significantly up- and downregulated in the analysis of day 7 high- and low-dose individual animals, respectively. Although analysis of the pooled samples reported simple fold changes rather than statistically significant changes, the results from the three laboratories demonstrate a considerable degree of similarity, particularly among the genes where the expression changes were quite large. As the changes became smaller, discrepancies occur, usually in the form of one laboratory showing an opposite directional change to the other two or indicating no change. There was no clear pattern, however, with all three laboratories being the source of discordant data on at least one occasion.

### Sources of Variation—Technical

Several explanations exist for the generation of discordant data. Consequently, an important aspect of this study was the evaluation of the robustness of the gene expression changes from different laboratories performing microarray analysis. Therefore, each laboratory involved in the study performed the microarray experimental and data analysis phases using the procedures and protocols routinely used in that laboratory. Table 3 is a list of several technical differences between the protocols used in each



**Figure 3.** qRT-PCR analysis of gene expression changes using (A) TaqMan and (B) SYBR green methods. Rats received clofibrate by oral gavage at low (25 mg/kg/day) and high (250 mg/kg/day) doses for 1, 3, and 7 days. Data shown are the mean of duplicates. Note: APO-A1 failed to amplify using TaqMan.



laboratory that could have had an impact on the data generated. They include *a*) the condition of cDNA membranes (i.e., use of stripped and reprobated microarrays compared with new microarrays); *b*) the choice of radioisotope for probe labeling (i.e.,  $^{32}\text{P}$  or  $^{33}\text{P}$ ); *c*) the probe hybridization temperature (i.e.,  $62^\circ\text{C}$  vs.  $68^\circ\text{C}$ ); and *d*) the image capture system (i.e., phosphorimager compared with X-ray film).

**Reproducibility of microarray results across different laboratories.** Two of the laboratories [GSK (U.S.) and ULR] analyzed pooled RNA samples from a single *in vivo* study, thus reducing the impact of biological variability but allowing differences due to technical/experimental variations to be highlighted. A comparison of the gene expression data obtained by the two laboratories from a single source of pooled RNA samples indicated that, despite some quantitative differences [most likely due to differences in membrane use and image capture/analysis methods used in the two laboratories (Table 3)], there was generally good concordance between the gene expression data. For example, of the top 20 upregulated genes ranked by fold change values identified by each laboratory, 8 were common to both laboratories (Table 7).

However, examples of discordant data were also found. For example, microarray data from two of the three laboratories performing microarray analysis [U.S. EPA and GSK (U.S.)] indicated a downregulation of cyclin D1 (1.7-fold and 1.9-fold, respectively) and CYP1A2 (2.3-fold and 2.7-fold, respectively) for the 7-day high-dose samples, whereas no change was noted by ULR. The U.S. EPA and GSK (U.S.) used fresh membranes for each hybridization and phosphorimaging plates as opposed to the stripped and reprobated membranes and X-ray film used by ULR. Such technical variation may account for differences in the ability to detect small changes in gene expression, as seen in the ULR results. Image analysis can also be a source of variation in final data output. Where changes in gene expression are small such as the cases listed above, they can be significantly affected by local background (which can be variable across membranes) and bleedover from adjacent genes if the latter are highly expressed. Different methods of data analysis were also used by the three laboratories, and this may have also contributed to the observed differences.

### Sources of Variation—Biological

**Reproducibility across different *in vivo* studies.** Differences in gene expression data can also be biological in origin. The pooling of samples for microarray analysis has

been used in the past to defray the cost of microarray experiments, reduce the effect of biological variation, and in some cases overcome availability of limiting amounts of tissue (e.g., from embryonic organs). Unfortunately, this approach essentially produces a sample size (*n*) of one animal. Repeated microarray experiments with such pooled RNA produces technical replicates as opposed to true biological replicates and thus does not allow calculation of biologically significant changes in gene expression between different dose groups or time points.

Another possible consequence of pooling is to mask individual gene changes and leave open the possibility of introducing error due to individual outlier responses. For example, liver mitochondrial carnitine *O*-palmitoyltransferase I (CPT1) catalyses the transfer of long-chain fatty acids (LCFAs) for translocation across the mitochondrial membrane. Expression of the CPT1 gene is induced by LCFAs as well as by lipid-lowering compounds such as clofibrate (Louet et al. 2001). In this study, both laboratories examining pooled RNA samples observed an approximate 3-fold induction of CPT1 after 7-day treatment with the high dose of clofibrate. The U.S. EPA, however, when analyzing individual animals, observed a significant downregulation of this gene at the same time point. One possible biological explanation for this could be that the source of the RNA samples used by the U.S. EPA (i.e., Abbott Laboratories) was different from that providing the pooled RNA used by GSK (U.S.) and ULR [i.e., GSK (U.K.)]. As previously discussed, differences were noted in the *in vivo* response, and these could explain the observed differences. However, this type of contradictory result (also seen with CYP2B1) may also reflect the phenomenon of data skewing by individual responses. This most often occurs in pooled samples of a low *n*, where an extreme response in one outlier animal skews or nullifies changes seen in the majority. Indeed, the chances of this effect occurring are increased with the use of an outbred strain such as Sprague-Dawley. Furthermore, previous reports have suggested that some genes are hypervariable among individual animals (Gerhold et al. 2001). Such differences are most likely caused by polymorphisms in the gene regulatory regions, and therefore consideration should be given to the region of the gene selected for the probe, as probes targeted toward a polymorphic locus would demonstrate increased interindividual variability. In this study for example, one animal of the pooled samples may have responded to clofibrate with a large increase in expression

of CPT1, whereas the others showed a small downregulation of this gene.

### Individual Analysis

Running samples from multiple individual animals is more costly than using pooled samples. However, it is advantageous in that it can be used to identify outliers and also permits the calculation of statistically significant changes in gene expression. Nevertheless, a balance must still be struck between the number of individual animals that can be examined and the statistical rigor required. In this study, the *n* for each group of individual analyses was four. This is a relatively low number for computation of statistically significant changes in different exposure groups. A common consequence of using a low *n* is that variance in the level of gene expression is high, which in turn reduces the number of significant gene changes. Thus, in the analysis of individual samples in this study, only 20 genes were deemed significantly changed. Genes that might have been expected to be significantly changed but were not included PPAR $\alpha$ , enoyl-CoA hydratase, and CEH. It is this kind of discrepancy that has led many researchers to believe that observing change or lack of change in expression of single genes has little value. Instead, identification of the gene expression pathways being affected appears to be a more robust method of identifying exposure to and effects of drugs and toxicants. In this case for example, it is still clear from the significant gene changes that  $\beta$ -oxidation of fatty acids is increased, as would be expected after exposure to a PP.

In future studies careful consideration must be given to the feasibility of using RNA samples from individual animals as opposed to pooled samples. Although recent evidence suggests that selection of certain pooling schemes can provide adequate statistical power and improve efficiency and cost effectiveness (Peng et al. 2003), the weight of evidence still supports the use of individual animals where possible. Such experiments, although more time and resource intensive than the use of pooled samples, permit analysis of interindividual (biological) variation. This is an important consideration in gene expression studies, as biological variation on the whole normally exceeds experimental variation. Individual gene expression data can also be useful in trying to understand why an individual animal might have shown an outlier clinical response to the test chemical. Individual variation, which exists even in inbred strains, lies at the very heart of pharmacogenomics and is usually attributable, at least in part, to polymorphisms in key drug-metabolizing genes that alter either

the normal control of gene transcription or the final protein structure.

## Summary

The results of this study demonstrate clofibrate-induced hepatotoxicity in a rodent model identified by conventional histopathology and clinical chemistry parameters, gene expression analysis using cDNA microarrays, and confirmatory studies using quantitative real-time PCR. The results indicate that despite some variability in the quantitative nature of the data, robust gene changes relating to the mechanism of clofibrate-induced hepatotoxicity were identified across laboratories performing the microarray analysis using pooled and individual samples from two different *in vivo* studies on the same cDNA array platform. This study demonstrates the potential for gene expression profiling to identify toxic hazards by the identification of mechanistically relevant markers of toxicity. In conclusion, toxicogenomics has the potential to provide new and refined approaches to hazard identification and safety evaluation based on the identification of biologically relevant markers of toxicity.

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