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Changes in global gene and protein expression during early mouse liver carcinogenesis induced by non-genotoxic model carcinogens oxazepam and Wyeth-14,643

Mari Iida[1], Colleen H.Anna[1], Jennifer Hartis[2], Maribel Bruno[2], Barbara Wetmore[2], Joshua R.Dubin[3], Stella Sieber[1], Lee Bennett[1], Michael L.Cunningham[4], Richard S.Paules[1], Kenneth B.Tomer[3], Christopher D.Houle[6], Alex B.Merrick[2], Robert C.Sills[5] and Theodora R.Devereux[1,7]

[1]Laboratory of Molecular Carcinogenesis, [2]National Center for Toxicogenomics, [3]Laboratory of Structural Biology, [4]Laboratory of Pharmacology and Chemistry and [5]Laboratory of Experimental Pathology, National Institute of Environmental Health Sciences, National Institute of Health, Research Triangle Park, NC 27709 and [6]Experimental Pathology Laboratories, Inc., Research Triangle Park, NC 27709, USA

[7]To whom correspondence should be addressed
Email: devereux@niehs.nih.gov

We hypothesized that the mouse liver tumor response to non-genotoxic carcinogens would involve some common early gene and protein expression changes that could ultimately be used to predict chemical hepatocarcinogenesis. In order to identify a panel of genes to test, we analyzed global differences in gene and protein expression in livers from B6C3F1 mice following dietary treatment with two rodent carcinogens, the benzodiazepine anti-anxiety drug oxazepam (2500 p.p.m.) and the hypolipidemic agent Wyeth (Wy)-14,643 (500 p.p.m.) compared with livers from untreated mice. Male mice were exposed for 2 weeks and 1, 3 or 6 months to oxazepam or Wy-14,643 in an age-matched study design. By histopathological evaluation, no liver preneoplastic foci or tumors were detected at 6 months in treated or control groups. By cDNA microarray analysis [NIEHS Mouse Chip (8700 genes); $n = 3$ individual livers/group, four hybridizations/sample], expression of 36 genes or 220 genes were changed relative to control livers following 6 months of oxazepam or Wy-14,643 treatment, respectively. To obtain a more comprehensive picture of gene/protein expression changes, we also conducted a proteomics study by 2D-gel electrophoresis followed by matrix assisted laser desorption/ionization-mass spectrometry on cytoplasmic, nuclear, and microsomal subcellular fractions of the same liver samples utilized for the cDNA microarray analysis. Real-time PCR, western blot analysis and immunohistochemistry were utilized for validation and to expand the results to other time points. Cyp2b20, growth arrest- and DNA damage-inducible gene β (Gadd45β), tumor necrosis factor α-induced protein 2 and insulin-like growth factor binding protein 1 (Igfbp5) genes and proteins were upregulated by oxazepam, and Cyp2b20, Cyclin D1, proliferating cell nuclear antigen, Igfbp5, Gadd45β and cell death-inducing DNA fragmentation factor α subunit-like effector A exhibited higher expression after Wy-14,643 treatment. Most of these genes/proteins were also deregulated at 2 weeks. There appeared to be more distinct than common changes in the expression of carcinogenesis-related genes/proteins between the two compounds, suggesting that the major carcinogenic pathways are different for these compounds and may be distinct for different chemical classes.

Abbreviations: AFP, α-fetoprotein; Cide-A, cell death-inducing DNA fragmentation factor α subunit-like effector A; EST, expressed sequence tag; Gadd45β, growth arrest- and DNA damage-inducible gene β; HCC, hepatocellular carcinoma; Igf-I, insulin-like growth factor I; Igfbp1, insulin-like growth factor binding protein 1; Maf, v-musculoaponeurotic fibrosarcoma oncogene family, protein F; MALDI, matrix assisted laser desorption/ionization; Map3k7, mitogen activated protein kinase kinase kinase 7; MS, mass spectrometry; PP, peroxisome proliferators; PPARα, peroxisome proliferator activated receptor α; PCNA, proliferating cell nuclear antigen; Tff-1, Trefoil factor 1; Tnfaip2, tumor necrosis factor α-induced protein 2; Wy-14,643, Wyeth-14,643.

Introduction

Many chemicals cause liver tumors in 2-year bioassays with B6C3F1 mice. Current estimates predict that about two out of three of all carcinogens tested are not mutagenic and are often classified as 'non-genotoxic carcinogens' (1). The mouse liver is a common target organ of non-genotoxic carcinogens, although the mechanisms to explain the activity of most carcinogens are not known. Toxicological changes in mouse liver following administration of a variety of chemicals have been studied for a few days up to 2 weeks (2–7). However, few studies have followed the early carcinogenic process up to 6 months (8,9). While selected genes and carcinogenesis-related pathways have been examined in some models of early mouse liver carcinogenesis, little work has been done to get an overall picture of early gene and protein expression changes that cause the development of these tumors. Gene and protein expression studies provide a unique opportunity to fill this data gap.

Oxazepam, used in the treatment of anxiety in humans, has been evaluated in a National Toxicology Program 2-year bioassay (10). Oxazepam does not induce gene mutations *in vitro* in the Ames assay or sister chromatid exchanges in Chinese hamster ovary cells (10), nor does it enhance the production of micronuclei in mouse peripheral blood erythrocytes *in vivo* (11). However, male B6C3F1 mice treated with 2500 p.p.m. oxazepam in the diet had 100% incidence of hepatocellular neoplasms at 2 years with 10-fold greater tumor multiplicity than untreated mice (10). H-ras mutations were detected in only 18% of the liver neoplasms from the 2500 p.p.m. treated group compared with 63% from spontaneous tumors (12), suggesting that other genes and pathways contribute to the carcinogenic process. In a subsequent study, 58% of the hepatocellular tumors from the 2500 p.p.m. oxazepam-treated group examined had β-catenin (*Catnb* gene) mutations and increased membrane staining of the β-catenin protein. The finding of frequent *Catnb* mutations in the oxazepam-induced neoplasms was consistent with an oxidative stress mechanism of carcinogenesis (13,14). The finding of

Catnb mutations in hepatocellular adenomas suggested that this alteration was an early event and led us to investigate earlier steps in this carcinogenic process.

Another rodent liver carcinogen, Wy-14,643 (4-chloro-6-[2,3-xylidino]-2-pyrimidinylthioacetic acid), a hypolipidemic compound and potent peroxisome proliferator (PP) (15–19) also lacks significant *in vitro* genotoxicity (20–27). However, PPs cause a significant increase in the level of the H_2O_2-generating peroxisomal fatty acid oxidation in the liver (28–30). The induction of peroxisome proliferator associated receptor alpha (PPARα) in rodents treated with PPs has been linked with carcinogenesis (17,31,32). However, it is unclear whether upregulation of PPARα is required for carcinogenesis by these chemicals. Shane *et al*. (33), using Big Blue^{R} transgenic mice, reported that both oxazepam and Wy-14,643 produce G to T and G to C transversions that could clearly arise through oxidative stress. Using the Comet assay, Deutsch *et al*. (34) also reported that oxazepam and Wy-14,643 appear to produce similar types of DNA damage.

The aim of this study was to reveal both common and distinct changes associated with hepatocellular carcinogenesis by these two dissimilar compounds. We used cDNA microarray technology to compare gene expression changes derived from livers after treatment with oxazepam or Wy-14,643 for 2 weeks or 6 months. Results were expanded by real-time PCR, western blotting and immunohistochemical analyses at multiple time points up to 6 months and in oxazepam-induced neoplasms. Proteomics methods (2D-gel electrophoresis and MALDI-MS) on nuclear, cytoplasmic and microsomal subcellular fractions of livers from the 2 weeks and 6 month treatment groups were also used to identify altered expression of proteins. We propose that the use of combined global gene and protein expression technologies in conjunction with detailed histological analysis will allow for a better understanding of the mechanisms of mouse liver chemical carcinogenesis and help identify early gene/protein markers that may be indicators of carcinogenesis.

Materials and methods

Animal and experimental design

Six-week-old male B6C3F1 mice were obtained from Charles River Laboratories (Raleigh, NC). Mice were administered either 0 or 2500 p.p.m. oxazepam, or 500 p.p.m. Wy-14,643 *ad libitum* in the diet for 2 weeks, 1, 3 or 6 months in an age-matched study design. There were six mice in each group, and control mice were the same for all groups due to this design. For example, the animals in the 2 weeks treatment groups were held during the 6 months and treated only during the last 2 weeks of the study. All the age-matched controls and each treatment groups of mice were killed on the same day.

At necropsy, the mice were weighed, killed with CO_2 from a regulated source, and the livers were harvested, weighed and flash-frozen in liquid N_2 and stored at −80°C until subsequent RNA isolation or protein extraction. One section of each left lobe of liver was fixed in 10% neutral-buffered formalin, embedded in paraffin, and cut into 5 μm sections for histological and immunohistochemical staining. One specimen was stained with H&E for routine histological examinations. All mice received the highest standard of humane care in accordance with protocols approved by the NIEHS committee on animal care and the NIH Guide for the Care and Use of Laboratory Animals. In addition, frozen oxazepam-induced liver neoplasms were obtained from an earlier study (35) for real-time PCR to compare changes in gene expression with livers from earlier time points.

RNA isolation and cDNA microarray analysis

Analysis of gene expression was performed using NIEHS cDNA microarray mouse chips containing 8736 genes or expressed sequence tags (ESTs). Briefly, total RNA was isolated using TRIzol reagent, according to the protocol described by the manufacturer (Life Technologies, Gaithersburg, MD). Messenger RNA was prepared from three individual livers from each treated group and 10 from controls, and quality was tested prior to pooling of control samples and microarray hybridization. The quality of the isolated RNA was assessed by measuring the absorbance at 260 nm, analyzing the A_{260}/A_{280} ratio (1.7–1.9), and evaluating the integrity of the 28S and 18S RNA bands on 1% agarose/formaldehyde gels. Messenger RNA was isolated using Oligotex mRNA kit (Qiagen, Valencia, CA). Because of the age-matched animal study design, only one pool of mRNA from control livers was used for all microarray hybridizations.

For microarray hybridizations, each poly(A)RNA (2–4 μg) was labeled with one of the fluorescent dyes Cy3 or Cy5 (Amersham Bioscience, Piscataway, NJ), and hybridizations to the microarray chip were performed as described previously (36). Fluorescent intensities of the printed cDNA targets were measured using a GenePix 4000A microarray scanner (Axon Instruments, Foster City, CA). Images were analyzed using the Array Suite software (Scanaltics, Fairfax, VA). Gene expressions altered with each hybridization in a statistically significant manner at the 95% confidence level were termed collectively as outliers or differentially expressed genes for that hybridization. Livers from three individual animals from the 2 weeks and 6 months treatment groups, each with four replicate hybridizations were paired with pooled mRNA from 10 age-matched control mouse livers to generate the list of differential gene expressions. Messenger RNA was prepared from each control liver and quality tested prior to pooling for the hybridizations. Each RNA sample was labeled with Cy3 for two analyses with Cy5 for the other two analyses (i.e. fluorophor dye swapping). These differentially expressed genes were identified with a high degree of confidence of not being false positive by requiring a gene to be identified as differentially expressed in three or four out of the quadruplicate analyses of each sample pair. The chance of a gene being a false positive could be estimated based upon the binomial distribution, which calculates the probability of genes being included as outliers completely by random at the 95% confidence level, using MicroArray Project System (MAPS) (37,38). The probability of identifying a gene as an outlier at the 95% confidence level three or four times out of four replicate analyses would be approximately $P = 0.00048$ (four genes out of 8736) or 0.00001 (one gene out of 8736), respectively. Chance of false positives was reduced further due to removal of genes showing any fluor bias. Data were $\log_2$-transformed and used for clustering analysis. The mean $\log_2$-transformed ratio values of all the validated outlier genes from triplicate hybridizations were used for hierarchical clustering analysis with Cluster and TreeView software (39). The entire data set is available at http://dir.niehs.nih.gov/microarray/datasets.

Real-time PCR

Real-time PCR analysis was performed using a Perkin-Elmer ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR Green DNA PCR Core Reagent Kit as recommended by manufacturer. cDNAs from total liver RNA of three control mice and three treated mice were analyzed for each time point. All reactions were performed in triplicate. Each reaction was performed in a 50 μl volume with a final concentration of 4 mM $MgCl_2$, 1× SYBR Green Buffer, 300 μM dNTPs with dUTP, 200 μM primers, 0.025 U/μl of Amplitaq Gold, 10 μl of a 1:10 dilution of the cDNA and RNase free water to 21.85 μl. The thermal cycling conditions for real-time PCR were 10 min at 95°C to activate Amplitaq Gold, followed by 40 cycles of denaturation 15 s at 95°C, and annealing/extension for 30 s at 60°C. β-Actin expression was quantified to normalize the amount of cDNA in each sample. The specificity of the amplified product was monitored by its melting curve. Since the melting curve of a product is dependent upon its GC content, length and sequence composition, specific amplification can be distinguished from non-specific amplification by examining the melting curve (40,41). PCR primers were designed using PrimerExpress software (Applied Biosystems) and are shown in Table I. A non-reverse transcription control was run with every assay to assess the contamination of RNA by genomic DNA. A Student's *t*-test was used to compare the expression in liver of each gene (relative to β-actin) between the three control and treated mice.

Western blot analysis for protein expression

Cellular proteins were extracted from frozen tissues in radio-immunoprecipitation assay buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.25% Na deoxycholate, 4 μg/ml A-PMSF, 20 μg/ml aprotinin and leupeptin, 1 M NaF, 1 mM Na_3VO_4). Equal amounts of total protein (50 μg) were denatured by boiling in Laemmli sample buffer and were electrophoresed on acrylamide gels (200 V) using Mini Protean II electrophoresis tanks (Bio-Rad, Hercules, CA). Stacking gels were 4% acrylamide and resolving gels were either 4–20% (Cyclin-D1, PCNA), 7.5% [α-fetoprotein (AFP)], 10% (PPARα, β-catenin) or 15% (CIDE-A) acrylamide. After electrophoresis, proteins were transferred to PVDF membranes (Immobilon-P, 0.45 μm, Millipore, Bedford, MA, 240 mA, 1 h, 4°C). PVDF membranes were blocked for 30 min with 5% non-fat dry milk in PBS or overnight with 5% BSA in TBS/Tween 20 (TBS-T; 10 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.01% Tween 20).

Table I. Sequence of PCR primers used in real-time PCR

Gene	Primers	5′ → 3′ Sequence	Amplicon Size (bp)
Cyp2b20	Forward Reverse	TCT ACA TGA TCC ACA GTA CTT TGA ACA A TTT CTT CAG TGC CCC ATT GG	82
Sialyltransferase 1	Forward Reverse	CTA TGG GAC ATV ATT CAG GAA ATC T TGG TCA CAC AGC GTC ATC ATA A	101
Gadd45β	Forward Reverse	CGT ATC TGG ACT TGT ACT TTG CTC TT GCT CGC CAC CAG CTC TTC	95
Tff-1	Forward Reverse	GGC CCA GGA AGA AAC ATG TAT C ACT GCT GGG CGG TGA CA	77
Map3k7	Forward Reverse	GCA GCT GTG TTA AGG AGA GCA A GTA AAA AGA GCA TGT CAT TCA TAT TTC AC	84
Tnfaip2	Forward Reverse	CCC CTG TTC TCC CTT ATA AAG GTT AC GGT GTG GTG CCT GGT GTT C	85
Igf-l	Forward Reverse	CAA AAT TGA AAT GTG ACA TTG CTC TA TGG TGG GCA GGG ATA ATG A	78
Igfbp 1	Forward Reverse	CCA TCA GCA CCT ATA GCA GCA T GCA GGG CTC CTT CCA TTT CT	67
Cide-A	Forward Reverse	TCG CCC TTT TCG AGT TTC A CAG GCT GCT GGC CAT CAC	64
β-actin	Forward Reverse	GAC AGG ATG CAG AAG GAG ATT ACT G GCT GAT CCA CAT CTG CTG GAA	144

After blocking, membranes were incubated with each primary antibody overnight as follows: affinity-purified rabbit polyclonal anti-Cyclin D1 (1:250 dilution, 4°C, overnight, catalog # M-20) and goat polyclonal anti-β-catenin (1:250 dilution, 4°C, overnight, catalog # C-18), AFP (1:500 dilution, room temperature, 3 h, catalog # sc-8108) antibodies were obtained from Santa Cruz (Santa Cruz, CA). Cide-A was detected with the rabbit anti-CIDE-A polyclonal antibody (1:500 dilution, 4°C, overnight, Oncogene, Products, Boston, MA), PPARα with the affinity-purified rabbit anti-PPARα (1:500 dilution, 4°C, overnight, Affinity Bioreagents, Golden, CO) and PCNA with the PC-10 mouse monoclonal antibody (1:250 dilution, 4°C, overnight, DAKO, Carpinteria, CA). Membranes were washed and exposed to the appropriate secondary antibody (1:3000–5000 for anti-mouse, 1:10 000 for anti-rabbit or 1:20 000–40 000 for anti-goat-coupled horseradish peroxidase-conjugated, Santa Cruz). Specific proteins were visualized using ECL system (Amersham), and molecular sizing was evaluated using the Precision protein standards (Bio-Rad). The NIH Image program was used to measure the mean band densities of the western blots.

Immunohistochemistry

Immunohistochemistry staining for Cyclin D1, PCNA and β-catenin was performed on formalin-fixed paraffin sections. Briefly, sections were deparaffinized in xylene and hydrated through a graded series of ethanol to water. Antigen unmasking was accomplished by heating in 1× Antigendecloaker (Biocare Medical, Walnut Creek, CA) in a Decloaking Chamber (Biocare Medical) for 5 min and cooling for 30 min. Endogenous peroxidase activity was blocked 3% H_2O_2 for 5 min. Anti-cyclin D1 (M-20), anti-β-catenin (C-18) and PCNA (PC-10) antibody were stained by the LSAB method (Labeled Streptavidin Biotin Kit, DAKO). The signals were visualized with diaminobenzidine counterstained with hematoxylin. The primary antibody was omitted in negative control samples.

Proteomics analysis

Analysis for differential protein expression was performed using 2D-gel electrophoresis separation and mass spectrometry (MS) identification. Subcellular fractions of nuclei, cytosol and microsomes (ER) were obtained by differential centrifugation for analysis of hepatic protein expression. Liver samples ($n = 3$ mice/group) from the control group and the 2 weeks and 6 months treated groups were processed individually. The samples (1–2 g) were thawed and homogenized in 5 ml of 50 mM HEPES pH 7.6 buffer [10% glycerol, 1 mM EDTA, protease inhibitor cocktail (Calbiochem, La Jolla, CA), 1 mM DTT]. Liver homogenates were centrifuged at 10 000 *g* at 4°C for 20 min to pellet nuclei. The 10 000 *g* supernatants were ultracentrifuged at 100 000 *g* at 4°C for 1 h to pellet ER; the 100 000 g supernatant is the cytosolic fraction. Each fraction was stored at –80°C. Nuclei were isolated by the low salt lysis buffer method (42). Proteins were extracted from nuclei on ice for 20 min in Dignam buffer [420 mM NaCl, 50 mM Tris–HCl pH 7.9, 20% glycerol, 1 mM DTT, 2 mM benzamidine, 10 mM β-glycerylphosphate, 0.1 mM VaO_3, protease inhibitor cocktail (Calbiochem), 1 mM PMSF]. Nuclear and cytosolic extracts were microdialyzed (Pierce, Rockford, IL) against distilled deionized water to remove salts and proteins ≥3500 Da and lyophilized and dissolved in urea lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 1% Tergitol-10, 0.5% *n*-octyl-β-glucopyranoside, 50 mM dithiothreitol, 2 mM tributylphosphine, 0.5% ampholytes pH 3–10, trace bromophenol blue) prior to isoelectric focusing. ER proteins were dissolved directly in urea lysis buffer.

Isoelectric focusing was performed to initially separate 150 μg of protein from each subcellular fraction using immobilized pH gradient (IPG) strips 11 cm in length at pH 3–10 on an IPGphor aPPARatus (Amersham). Mass separation of reduced and alkylated (iodoacetamide) proteins was performed in 1 mm thick acrylamide Criterion® gels cast at an 8–16% gradient. Proteins were fixed and fluorescently stained with Sypro Ruby (Molecular Probes) according to the manufacturer's protocol. Stained gels were scanned (Agfa, Ridgefield, NJ; Arcus scanner) as TIFF images and analyzed for differential protein expression using PDQuest software (Bio-Rad, Richmond, CA). Gel images were aligned, background noise subtracted and a control standard gel was selected for annotation purposes. Images from each treatment group (oxazepam and Wy-14,643) at each timepoint (2 weeks and 6 months) were compared against control images for each organelle at $n = 3$/group. Mean intensity values for each protein were used to calculate fold changes. Differential proteins were selected by Student's *t*-test significance at $P < 0.05$ or by a ≥2 fold difference from control based on the PDQuest image analysis software that also considered the background and saturation levels of the protein spots.

Differentially expressed proteins selected by image analysis were excised manually from the gels with sterile pipets for MS analysis. Automated in-gel trypsin digestion was performed using a ProGest workstation (Genomic Solutions, Ann Arbor, MI) according to the manufacturer's protocol. The digests were lyophilized, reconstituted and prepared for MS analysis by the dried-droplet method using a saturated solution of recrystallized α-cyano-4-hydrocinnamic acid in 45:45:10 (v:v) ethanol:water:formic acid as the matrix. MS analyses were performed on a Voyager DE-STR MALDI/TOF spectrometer (Perseptive Biosystems, Framingham, MA) with delayed ion extraction at accelerating voltage of 20 kV and in the reflector mode with a 3.0 m flight path. A 337 nm nitrogen laser was used to desorb and ionize samples. Mass calibration was performed internally using trypsin autolysis peptides. Critical ions were searched against the Profound database (Rockefeller University, NY) for protein identification.

Results

Oxazepam at 2500 p.p.m. in the diet significantly increased the incidence of centrilobular hepatocellular hypertrophy after

Table II. Incidence of non-neoplastic histopathological changes following treatment with Oxazepam or Wy-14,643 for 2 weeks, 1 month, 3 months or 6 months

Findings	Incidence				
	Experimental time				
	2W	1M	3M	6M	Un 6M
Oxazepam					
Inflammation, mixed cell	2/6	1/6	2/5	4/10	6/18
Necrosis	0/6	0/6	1/5	0/10	1/18
Hepatocellular karyomegaly	0/6	2/6	4/5	8/12	0/18
Hepatocellular hypertrophy, centrilobular	2/6	6/6	5/5	12/12	0/18
Eosinophilic focus	0/6	0/6	0/5	1/12	0/18
Wy-14,643					
Inflammation, mixed cell	1/6	1/6	3/6	4/10	9/7
Necrosis	2/6	3/6	6/6	4/10	2/17
Hepatocellular hypertrophy	6/6	6/6	6/6	10/10	0/17
Fatty change	0/6	6/6	6/6	10/10	0/17
Hepatocellular karyomegaly	2/6	1/6	6/6	10/10	0/17
Bile stasis	0/6	0/6	6/6	6/10	0/17
Basophilic focus	0/6	0/6	1/6	0/10	0/17

Un 6M=Untreated 6 months.

2 weeks of treatment and hepatocellular karyomegaly after 1 month (Table II). By 6 months, eight of 12 oxazepam-treated mice had developed hepatocellular karyomegaly and all had centrilobular hepatocellular hypertrophy (Table II and Figure 1B). One liver from the 6 months treatment group had a small eosinophilic focus. All of the livers from mice treated with 500 p.p.m. Wy-14,643 had diffuse hepatocellular hypertrophy by 2 weeks (Figure 1C) and fatty change by 1 month (Table II). Focal areas of hepatocellular necrosis and hepatocellular karyomegaly were also apparent by 2 weeks. Bile stasis was present after 3 months. Hepatocellular karyomegaly appeared to be a treatment time-related effect for both compounds. Livers from the 6 months untreated mice had expected and commonly observed inflammatory cells small focal necrotic lesions (Table II).

To find early gene expression changes in livers of mice treated with carcinogenic doses of oxazepam and Wy-14,643, we performed cDNA microarray experiments using liver mRNA samples isolated from time-matched untreated and treated mice from each of the 2 weeks and 6 months groups. Genes that were determined to be up- or down-regulated at 95% confidence interval in at least three of four or all four replicate hybridizations from each of individual treated mouse livers ($n = 3$) analyzed or nine of 12 total hybridizations in comparison with RNA from 10 pooled age-matched control mouse liver were used to compile a list of gene changes between treatment and control mice. From the 8736 genes or ESTs present on the array platform, the expression of 36 genes and ESTs was changed relative to control in livers following 6 months of oxazepam treatment and expression of 220 genes and ESTs was changed after 6 months of Wy-14,643 treatment (the entire data set at http://dir.niehs.nih.gov/microarray/datasets).

Twenty-three known genes that appear to have a role in carcinogenesis were grouped into five major functional categories: microsomal and related enzymes; apoptosis and cell death; cell-cycle check points; growth factors and other carcinogenesis associated genes; and cell–cell interaction and cell differentiation (Table III). Of these 23 genes, all but three were outliers from at least one chemical and time point and in at least three out of four hybridizations from each individual liver. The mean value ± SE for quadruplicate reactions of three individual treated and control liver samples is shown. Growth arrest- and DNA damage-inducible gene β (Gadd45β), tumor necrosis factor α-induced protein 2 (Tnfaip2) and Cyclin D1 were outliers in nine of 12 hybridizations. Cluster analysis of these genes is shown in Figure 2. Cluster analysis of all the genes shows that individuals within each group were more similar to each other than to individuals from other treatment or time groups. (Cluster data at website http://dir.niehs.nih.gov/microarray/datasets.) The expression of many of these genes was different in the livers of mice between oxazepam and Wy-14,643 treatments, and some genes showed differential expression between 2 weeks and 6 months for each compound.

By microarray analysis Cyp4a14, Cyp4a10 and Cyp2b20 genes were highly induced in the liver by both oxazepam and Wy-14,643. All of these cytochrome P450 gene expression levels at 2 weeks were higher than at 6 months except for the Cyp2b20 gene which appeared to have a similar level of expression at 2 weeks and 6 months with Wy-14,643 treatment. Among the apoptotic and cell death genes, the v-musculoaponeurotic fibrosarcoma oncogene family member protein F (Maf) and the Cide-A gene expression levels were especially upregulated at 2 weeks in liver samples from the Wy-14,643 treatment group. Expression of Gadd45β gene and cyclin D1 gene were upregulated at 2 weeks by Wy-14,643. Expression of Tnfaip2 was upregulated by oxazepam at 6 months. Expression of trefoil factor 1 (Tff-1) and small proline-rich protein 2A gene were down-regulated at 2 weeks and 6 months by both oxazepam and Wy-14,643. Expression of insulin growth factor binding protein 1 (Igfbp1) was upregulated by Wy-14,643 at 6 months. Insulin growth factor I (Igf-I) and mitogen activated protein kinase kinase kinase 7 (Map3k7) genes were down-regulated by Wy-14,643 at 6 months. Sialyltransferase 1, which has a role in cell–cell interaction, was upregulated at 6 months by oxazepam but down-regulated at 6 months by Wy-14,643.

Real-time PCR was used to validate nine gene expression changes identified by cDNA microarray analysis (Cyp2b20, Cide-A, Gadd45β, Tnfaip2, Tff-1, Map3k7, Igf-I, Igfbp1 and Sialyltransferase 1), in addition to investigating the 1- and 3-month time points and oxazepam-induced neoplasms (Table IV). Where we found discrepancies between real-time PCR and microarray data, we considered the real-time PCR results to provide more accurate expression measurements (43). For example, we examined the real-time PCR expression of Gadd45β, Igfbp1 and Map3k7 because they were identified by microarray as outliers in the 2 weeks or 6 months Wy-14,643 treatment group. Interestingly, real-time PCR showed that Gadd45β gene was highly upregulated at 2 weeks and 6 months by both oxazepam and Wy-14,643 and in oxazepam-induced tumors. Igfbp1 expression was increased by both compounds at all time periods and very strongly in the oxazepam-induced hepatocellular tumors. Map3k7 expression, which appeared to decrease by microarray at 6 months of Wy-14,643 treatment, increased by real-time PCR at the 6-month time period and seemed to vacillate with time after both treatments. The real-time PCR results validated some of the cDNA microarray data, although the fold induction was not always the same, due at least partly to differences in the

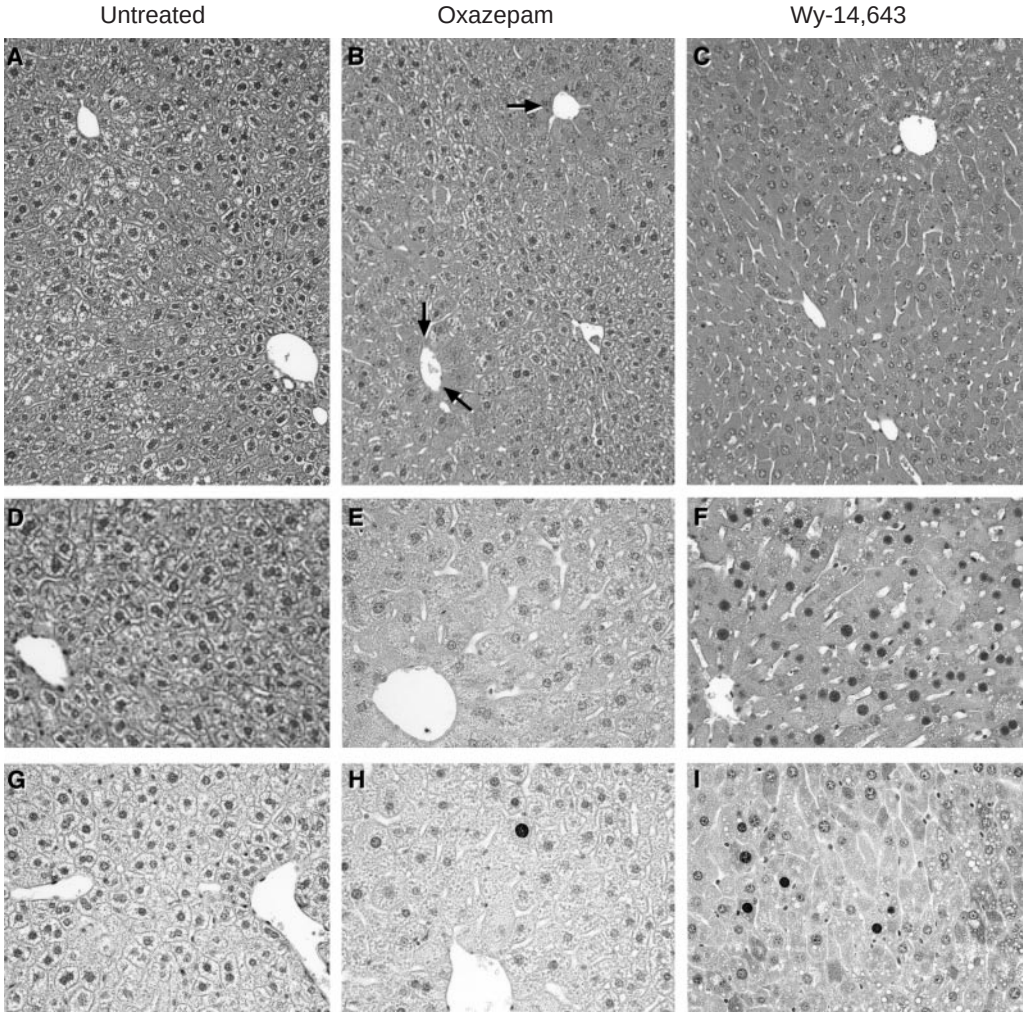

Fig. 1. H&E staining (A–C), Cyclin D1 (D–F) and PCNA (G–I) immunohistochemistry of livers from male mice treated for 6 months with oxazepam or Wy-14,643 and livers from age-matched untreated male mice. (**A**) Liver from untreated mouse showing normal tissues. H&E ×100. (**B**) Liver from male mouse treated with 2500 p.p.m. oxazepam for 6 months. H&E ×100. Centrilobular hepatocellular hypertrophy (arrows) was observed. (**C**) Liver from male mouse treated with 500 p.p.m. Wy-14,643 for 6 months. H&E ×100. Diffuse hepatocellular hypertrophy and fatty change were observed. (**D–I**) Cyclin D1 and PCNA, respectively, in liver sections 6 months after treatment with oxazepam or Wy-14,643. (E and H) In livers from oxazepam-treated mice. Immunohistochemical staining for Cyclin D1 and PCNA was negative. Magnification ×200. (F and I) Livers from Wy-14,643-treated mice showed strong positive staining in nuclei. Magnification ×200. (D and **G**) In livers from untreated mice the immunostaining was at the background level. Magnification ×200.

methodology (43). For example, the Cide-A gene was strongly upregulated by Wy-14,643, and the unusually large increases by real-time PCR were due to the very low expression in livers from untreated mice. Western blot analysis also showed increased Cide-A protein expression (Figure 3), although the intensity of the protein bands did not match the high expression measured by real-time PCR. Tff-1, having effects on both cell proliferation and apoptosis, was down-regulated only at 6 months by oxazepam (Table IV). Igf-I expression was decreased by Wy-14,643 but not oxazepam at all time points, whereas Igfbp1 was increased by both chemicals, and especially in oxazepam-induced liver neoplasms. The use of all four time points for this analysis helped to get a better picture of the time course of expression changes for these genes.

Western blot analysis was also performed to further explore and expand on the initial cDNA microarray analysis. Expression of Cyclin D1, Cide-A, PPARα, PCNA, β-catenin and AFP (upregulated by Proteomics analysis and discussed below) was evaluated in one liver sample from each treatment group and time point. PPARα and PCNA, which were not represented on the microarray chip, are known to be upregulated in liver following Wy-14,643 treatment (17,32,44,45) and were examined in this study. As shown in Figure 3, the expression of PPARα, PCNA, Cyclin D1 and CIDE-A proteins was increased in livers from Wy-14,643-treated but not oxazepam-treated mice. β-Catenin protein accumulation in oxazepam-induced mouse liver neoplasms has been reported (13,14). In the present study β-catenin expression was not upregulated during early liver carcinogenesis by either compound.

We also performed immunohistochemistry for Cyclin D1 and PCNA on tissues following treatment with oxazepam or Wy-14,643 at 2 weeks, 1, 3 and 6 months (Figure 1D–I). Strong positive staining in nuclei for Cyclin D1 was found in livers from Wy-14,643-treated mice at all times (Figure 1F, 6 months data shown), but not in any of the livers from the oxazepam treatment groups (Figure 1E). This result is consistent with the observation by cDNA microarray of a more robust induction of Cyclin D1 by Wy-14,643 treatment. PCNA may play an important role for cell-cycle control and is associated with Cyclin D1. Wy-14,643 treatment-induced

Table III. Differentially expressed and carcinogenesis-associated genes from cDNA microarray screening of livers at 2 weeks and 6 months after oxazepam and Wy-14,643 treatments

Biochemical pathway	Gene name	Gene bank acc. no.	Compound			
			Oxazepam		Wy-14,643	
			2 weeks	6 months	2 weeks	6 months
Microsomal and related enzymes	cytochrome P450, 2b20	W12874	8.92 ± 1.27*	6.61 ± 0.45*	2.80 ± 0.25*	2.73 ± 0.22*
	cytochrome P450, 4a14	AA060595	7.25 ± 0.34*	5.44 ± 0.13*	21.01 ± 0.59*	15.32 ± 0.96*
	cytochrome P450, 4a10	AA097980	5.25 ± 0.13*	4.01 ± 0.11*	15.21 ± 0.34*	13.56 ± 1.81*
	carboxylesterase 3	AA509566	1.32 ± 0.05	1.76 ± 0.11*	2.05 ± 0.12*	2.38 ± 0.26*
	glutathione *S*-transferase, π	AA108370	0.88 ± 0.03	0.66 ± 0.07	0.59 ± 0.03	0.39 ± 0.03*
	cytochrome P450, 2c40	AA146215	0.87 ± 0.07	0.55 ± 0.04*	0.35 ± 0.05*	0.26 ± 0.04*
Apoptosis/Cell death	cell death-inducing DNA fragmentation factor α-subunit like effector A (Cide-A)	W41358	0.93 ± 0.01	1.00 ± 0.01	5.78 ± 0.86*	3.90 ± 0.44*
	V-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (Maf)	AA272876	1.68 ± 0.13*	1.15 ± 0.10	16.22 ± 1.01*	5.77 ± 0.57*
	growth arrest and DNA-damage-inducible 45 beta (Gadd 45)	W64388	1.17 ± 0.01	1.47 ± 0.15	1.80 ± 0.15*	1.72 ± 0.13
	trefoil factor 1	W83072	0.42 ± 0.03*	0.41 ± 0.03*	0.41 ± 0.06*	0.43 ± 0.05*
	tumor necrosis factor, α-induced protein 2 (Tnfaip2)	AA537672	1.32 ± 0.08	1.53 ± 0.09*	0.77 ± 0.04	0.91 ± 0.03
Cell cycle check points	cyclin D1	AA111722	1.03 ± 0.07	1.12 ± 0.05	1.58 ± 0.03*	1.39 ± 0.21
	ADP-ribosylation factor 2 (Arf2)	AA266938	0.62 ± 0.06*	0.52 ± 0.03*	0.14 ± 0.01*	0.16 ± 0.03*
	a kinase (PRKA) anchor protein 8 (AKAP8)	AA290277	1.65 ± 0.29	1.31 ± 0.06	4.07 ± 0.25*	2.11 ± 0.26*
Growth factors and carcinogenesis associated genes	similar to leucine zipper, putative tumor suppressor 1	W09954	1.86 ± 0.17*	1.76 ± 0.11*	1.18 ± 0.03	1.22 ± 0.05
	insulin growth factor I (Igf-I)	W10072	1.02 ± 0.12	0.82 ± 0.03	0.61 ± 0.01	0.49 ± 0.04*
	insulin-like growth factor binding protein 1 (Igfbp1)	W83086	1.02 ± 0.03	1.25 ± 0.11	1.26 ± 0.05	3.15 ± 0.75*
	mitogen activated protein kinase kinase kinase 7 (Map3k7)	AA396734	1.08 ± 0.03	0.88 ± 0.06	0.77 ± 0.05	0.59 ± 0.06*
	glutamine synthetase	AA011759	0.90 ± 0.08	0.58 ± 0.06*	1.19 ± 0.15	0.70 ± 0.12
	small proline-rich protein 2A	AA497620	0.54 ± 0.01*	0.46 ± 0.01*	0.50 ± 0.01*	0.53 ± 0.13*
	aquaporin 8	AA272836	0.98 ± 0.04	1.45 ± 0.11	0.31 ± 0.02*	0.33 ± 0.03*
Cell–cell interaction and cell differentiation	sialyltransferase 1 (beta-galactoside alpha-2,6-sialyltransferase)	AA059687	1.17 ± 0.04	1.81 ± 0.12*	0.81 ± 0.05	0.67 ± 0.01*
	CD24a antigen (ligand for P-selectin on tumor cells)	W98974	0.98 ± 0.05	0.89 ± 0.04	2.28 ± 0.16*	3.51 ± 0.17*

Note. Numbers are mean values of fold induction/repression compared to untreated sample.
Each number is average of 12 data points (3 individual animals and 4 hybridizations per animal). 1.0 = No change.
*Significantly different from control at $P < 0.05$.

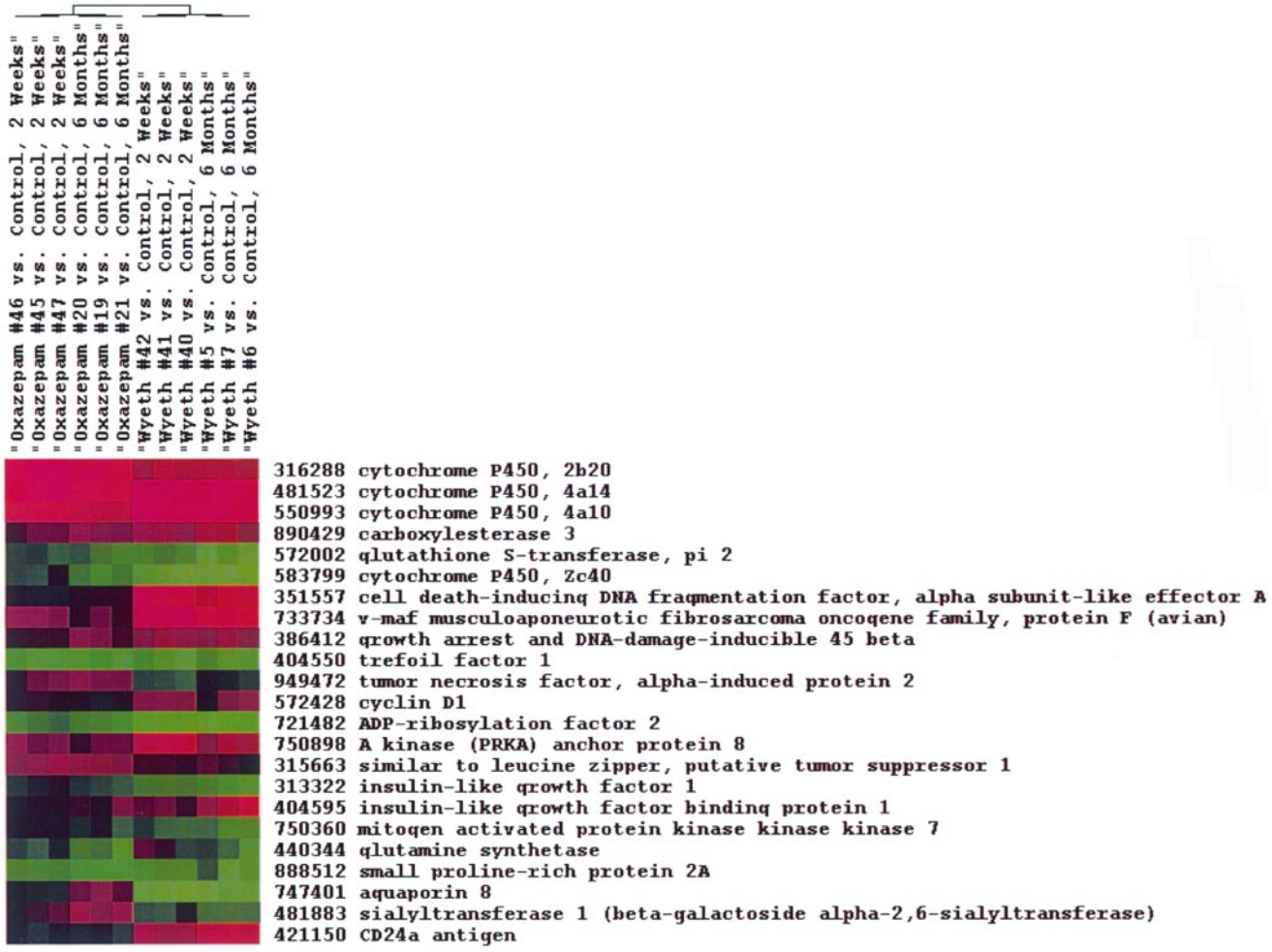

Fig. 2. Hierarchical clustering analysis of gene expression data from 23 cancer associated genes in cDNA microarray of livers from B6C3F1 mice treated with oxazepam or Wy-14,643 for 2 weeks or 6 months. Genes are listed in rows, and animals are listed in columns. The ratio of gene expression levels (treated/untreated) is color coded. Green indicates down-regulation, black means no change, and red indicates up-regulation of expression.

staining of PCNA in hepatocyte nuclei (Figure 1I) and also enhanced PCNA staining in mitotic cells (data not shown). In contrast, very low levels of PCNA staining were observed in liver cells from oxazepam-treated mice (Figure 1H).

We also performed proteomics analysis on the same liver samples using 2-D gel electrophoresis and MALDI-MS spectrometry on subcellular cytosol, microsomal and nuclei protein fractions for comparison with cDNA microarray results. Altered expression of 39 proteins was identified in liver cytosol from both treatment groups. Twenty-eight and 15 proteins were altered in the microsomal and nuclear fraction from the treated mice, respectively. A figure of all the proteins identified is shown on a Web site (http://dir/proteomics/hartis/pwrpt.htm) for reference. Table V shows a selection of identified liver protein expression changes that have been associated with carcinogenesis or were chosen for comparison with microarray results. Proteins with 2.0-fold changes or greater or changes that were significant at 95% confidence by Student's *t*-test (see Materials and methods) induced by oxazepam and Wy-14,643 treatments at 2 weeks and 6 months time points are shown. Two carcinogenesis-related proteins, AFP, a tumor marker for human hepatocellular carcinomas (HCC), and p38-2G4, which is found to be highly expressed between G_1 and mid-S phases of the cell cycle (46), were upregulated in the microsomal fraction. The upregulation of AFP by Wy-14,643 at all time points was also confirmed by western blot analysis (Figure 3). For the livers from mice treated with oxazepam, the highest expression of AFP by western blot analysis was observed at 2 weeks; and thereafter it decreased with time. Regucalcin, which helps maintain intracellular levels of Ca^{2+} by increasing calcium pump activity (47) and may play a role in regulating cell proliferation (48), was shown to be upregulated by both oxazepam and Wy-14,643 at 2 weeks and down-regulated by Wy-14,643 at 6 months in cytosol. Eukaryotic translation initiation factor 3 (TIF3) that was over-expressed in cell lines by cadmium and has an oncogenic function (49), was down-regulated in microsomes at 6 months by oxazepam and Wy-14,643. Other common gene/protein expression changes were identified by both cDNA microarray analyses and proteomics (Table V). For example, expression levels in cytosol of xenobiotic metabolism enzymes such as cytosolic epoxide hydrolase and glutathione-*S*-transferase Pi (GST π) were deregulated. Enzymes associated with oxidative stress such as glutathione peroxidase and peroxiredoxin were also detected in these fractions.

Table IV. Validation of cDNA microarray data by real-time PCR

Gene		Oxazepam					Wy-14,643			
		Experimental time					Experimental time			
		2W	1M	3M	6M	Tumor	2W	1M	3M	6M
Cyp2b20	RT-PCR	50.23**	30.30**	41.84**	49.45**	23.85**	121.42**	76.82**	78.25**	93.20**
	cDNA	8.92*	–	–	6.61*	–	2.80*	–	–	2.73*
Cide-A	RT-PCR	0.18	0.13	0.02**	0.21*	0.05**	42.13**	87.70**	105.18**	294.52**
	cDNA	0.93	–	–	1.00	–	5.78*	–	–	3.90*
Gadd45beta	RT-PCR	14.05*	–	–	17.06*	56.37*	52.75*	–	–	44.94*
	cDNA	1.17	–	–	1.47	–	1.80*	–	–	1.72
Tnfaip2	RT-PCR	1.51*	2.21*	1.95*	2.20**	0.72	0.49	0.36	1.36	0.75
	cDNA	1.32	–	–	1.53*	–	0.77	–	–	0.91
Tff-I	RT-PCR	0.50	–	–	0.19*	3.20	0.78	–	–	0.74
	cDNA	0.42*	–	–	0.41*	–	0.41*	–	–	0.43*
Igf-1	RT-PCR	0.66	0.86	0.86	0.74	0.06	0.54*	0.42**	0.35*	0.30*
	cDNA	1.02	–	–	0.82	–	0.61	–	–	0.49*
Igfbp1	RT-PCR	3.14*	2.41**	2.63*	2.21	131.90**	1.55	3.81*	9.58**	5.76**
	cDNA	1.02	–	–	1.25	–	1.26	–	–	3.15*
Map3k7	RT-PCR	1.12	2.75**	0.62	1.20	0.38*	1.72	0.62**	3.76**	1.84**
	cDNA	1.08	–	–	0.88	–	0.77	–	–	0.59*
Sialyltranferase 1	RT-PCR	2.92*	0.77	6.06*	1.24	0.05**	1.95	0.98	0.32	1.11
	cDNA	1.17	–	–	1.81*	–	0.81	–	–	0.67*

Note. Values are fold change over control. RT-PCR, real-time PCR-derived data (average of triplicate measurements from 3 individual treated or control livers); cDNA, cDNA microarray-derived data (4 hybridization from 3 individual treated livers versus control).
* Significantly different from control at $P < 0.05$.
** Significantly different from control at $P < 0.01$.

Discussion

The goal of this study is to gain a better understanding of mechanisms of mouse liver carcinogenesis by examining early time points following exposure to non-genotoxic carcinogens. In identifying important early carcinogenesis-related gene and protein expression changes, we hope to develop a panel of genes/proteins that ultimately could be screened to predict carcinogenesis for different chemical classes. We first studied the effects of oxazepam and Wy-14,643 treatments on gene and protein expression in liver because these potent rodent carcinogens have been well studied (10,12,33–35,50–55). The use of three individual livers and four hybridizations for each chemical and two time points helped minimize the number of false positives and identify subtle changes. These small changes were considered to be potentially important in our analyses of the chemical carcinogenic process (56). Indeed, the cluster analysis showed the remarkable similarity of the individual livers within each treatment and time group.

Some of the early expression changes in mouse liver carcinogenesis by oxazepam and Wy-14,643, such as induction of certain Cyp450 enzymes and increased PPARα, have been reported previously (16,31), whereas others, such as Maf and Cide-A, associated with cell death and apoptosis, Gadd45β, which controls cell-cycle checkpoints in response to stress agents, Tff-1, having effects on both cell proliferation and apoptosis (57) and Map3k7, involved in stress activated signaling, are new. Map3k7, previously known as transforming growth factor-beta-activated kinase 1, is also recognized as a mediator in TGFβ signaling pathways (58). The largest chemically induced early expression changes in liver were the induction of several cytochrome P-450 isozymes that are involved in metabolism of xenobiotics. The Cyp2b family produces oxidative stress through generation of superoxide radicals (54,59). Centrilobular hypertrophy of the liver following treatment with oxazepam, detected at 2 weeks in our study, may be associated with the strong induction of certain cytochrome P450 isoforms by this compound. The Cyp4a isoforms are associated with hepatomegaly, proliferation of parenchymal peroxisomes and hepatocarcinogenesis (16). Both Cyp4a10 and Cyp4a14 were highly upregulated in livers at all time points by both oxazepam and Wy-14,643. The increased endogenous free radicals formed by certain cytochrome P450 enzymes may result in DNA damage to cancer genes such as the β-catenin gene (60). The strong induction of these cytochrome P-450 isoforms provides evidence that increased oxidative stress plays an early role in liver carcinogenesis.

In contrast to the upregulation of the Cyp isoforms mentioned above, Cyp 2c40 and certain phase 2 metabolic enzymes including GST π were down-regulated in the livers after treatment with oxazepam and Wy-14,643. It is probable that the efficiency of the phase 2 detoxification pathways decreases early during the carcinogenic process allowing toxic metabolites to accumulate.

Cell-cycle checkpoint genes may also be deregulated early during carcinogenesis. Cyclin D1 is a key regulator of cell-cycle progression and when over-expressed can function as an oncogene. Increased levels of Cyclin D1 protein have been shown to be associated with aggressive forms of human HCC (61,62). Furthermore, Deane *et al*. have found that overexpression of cyclin D1 is sufficient to initiate transgenic mouse hepatocarcinogenesis (63). In the present study, Cyclin D1 and PCNA were significantly upregulated by Wy-14,643 treatment, but not by oxazepam at 2 weeks or 6 months (Table III, Figures 1 and 3). PCNA binds to the cdk–Cyclin D1–p21waf1 complex (64–66) to regulate the G_1/S checkpoint

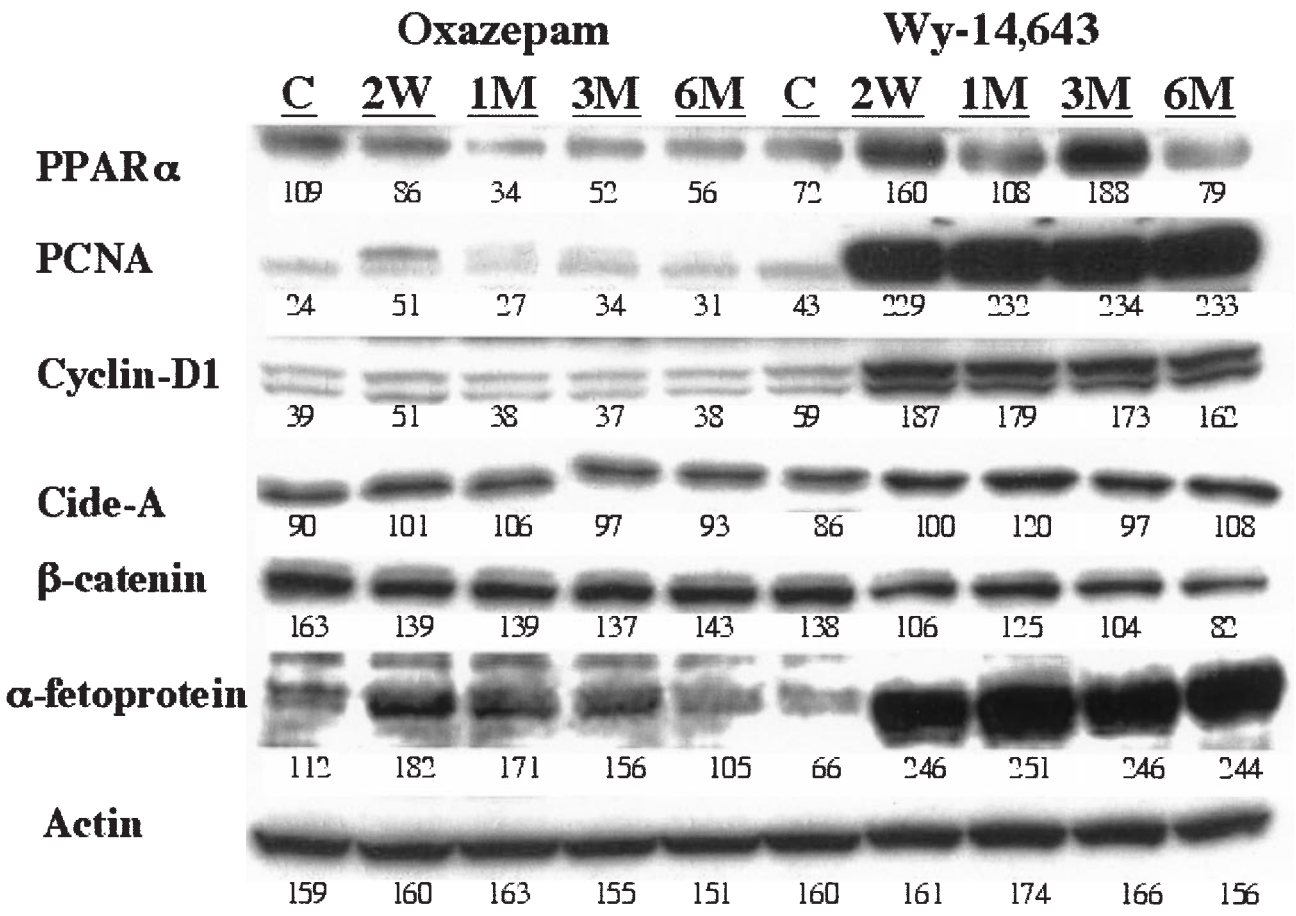

Fig. 3. Western blot analysis of liver proteins from oxazepam- and Wy-14,643-treated mice. Numbers below each blot are the mean densities of the bands. Lanes 1–5, untreated liver, and 2 weeks, 1, 3 and 6 months livers from oxazepam treatment groups; lanes 6–10, another untreated liver, and livers from 2 weeks, 1, 3 and 6 months Wy-14,643 treatment, respectively.

of the cell cycle and keep Rb in its unphosphorylated inactive form. Over-expression of Cyclin D1 and PCNA induced by Wy-14,643 probably plays a significant role in the carcinogenic response of mouse liver to Wy-14,643. Cyclin D1 over-expression in livers by 2 weeks after chronic chemical exposure is a potential gene marker, which if present, would be suggestive of a positive liver tumor response.

The formation of hepatomegaly, associated with Wy-14,643 treatment, appears to correlate with a significant increase in DNA synthesis along with an equivalent increase in hepatocellular ploidy (45). These results suggest that over-expression of Cyclin D1 and PCNA induced by Wy-14,643 may be associated with the observed increase in hepatocellular karyomegaly (Table II). Indeed, hepatocellular karyomegaly has been observed in mice and rats treated with numerous compounds (45,67–69). In addition, hepatic polyploidy has been associated with oxidative injury (70). However, while hepatocellular karyomegaly is commonly observed in rodents following chemical treatment, there is no clear linkage to carcinogenesis.

The balance between cell death and survival is likely to play an important role in early liver carcinogenesis. Previous studies have suggested that inhibition of apoptosis is an important mechanism of tumor induction by non-genotoxic hepatocarcinogens (71–73). Altered expression of Maf and Cide-A genes appear to enhance the carcinogenic process by affecting cell death. V-maf was first isolated from the provirus of the avian musculoaponeurotic fibrosarcoma virus AS42 and was recognized as a transforming gene. Maf belongs to the leucine zipper family of transcription factors, which include the activator protein 1 family, consisting of c-Fos and c-Jun partners (74,75). Over-expression of Maf was shown to cause p53-dependent cell death (74). In addition, Maf proteins have been implicated in the cell stress response by associating with electrophile response elements (76). In the present study, both Maf and Cide-A were highly upregulated at 2 weeks and 6 months by Wy-14,643. Inohara *et al.* observed that Cide-A-induced DNA fragmentation and other morphological features of apoptosis in cell lines (77). Cide-A expression was upregulated by Wy-14,643 treatment, whereas it was down-regulated by oxazepam treatment and was even lower in tumors, suggesting that the role of Cide-A in liver tumorigenesis needs further study.

TNFα also may play a role in apoptotic pathways, and the Tnfaip2 was upregulated by oxazepam at all times between 2 weeks and 6 months. Human B94 gene (TNFAIP2) has been found to be inducible by TNFα and retinoic acid, and results suggest that human TNFAIP2 is a cytokine-inducible immediate early response gene involved in tissue damage and the inflammatory response (78). However, the regulation and functions of this gene are still not fully understood.

Gadd45β was originally identified as an immediate early gene induced by interleukin (IL)-6 and IL-1 in M1 myeloid cells (79), and was subsequently shown to be upregulated by many growth factors including transforming growth factor β

Table V. Expression changes by proteomics compared to cDNA microarray

Gene (protein)	Protein (microsome)				Protein (cytosol)				Protein (nuclear)				cDNA microarray			
	OX		WY		OX		WY		OX		WY		OX		WY	
	2W	6M	2W	6M	2W	6M	2W	6M	2W	6M	2W	6M	2W	6M	2W	6M
α-fetoprotein	2.27 ± 0.28	–	1.77 ± 0.50	–	–	–	–	–	–	–	–	–	NA	NA	NA	NA
P382G4	–	2.13 ± 0.18	–	2.01 ± 0.17*	–	–	–	–	–	–	–	–	NA	NA	NA	NA
Eukaryotic translation initiation factor 3	–	0.49 ± 0.46	–	0.31 ± 0.06	–	–	–	–	–	–	–	1.35 ± 0.08	–	–	–	–
Translation initiation factor EIF-4A	–	–	–	–	–	0.48 ± 0.32	–	2.10 ± 0.23	–	–	–	–	NA	NA	NA	NA
Regucalcin	–	1.81 ± 0.44	–	2.01 ± 0.38	3.47 ± 0.34	0.94 ± 0.22	1.61 ± 0.12	0.78 ± 0.06*	–	–	–	–	NA	NA	NA	NA
Glutathione peroxidase	–	–	–	–	0.42 ± 0.36*	–	1.72 ± 0.38	–	–	–	–	–	NA	NA	NA	NA
Epoxide hydrolase	–	–	–	–	–	1.54 ± 0.55	–	5.02 ± 0.34*	–	–	–	–	1.08 ± 0.03	1.25 ± 0.05	2.28 ± 0.15	2.09 ± 0.12
Glutathinone *S*-transferase π	–	–	–	–	–	0.52 ± 0.12	–	0.37 ± 0.20*	–	–	–	–	0.88 ± 0.03	0.66 ± 0.07	0.59 ± 0.03	0.39 ± 0.03*
Peroxiredoxin	–	–	–	–	2.03 ± 0.25	–	1.58 ± 0.14	–	–	–	–	–	1.14 ± 0.09	0.97 ± 0.04	1.43 ± 0.12	1.08 ± 0.05
Cytochrome C oxidase VA	0.40 ± 0.44	–	2.01 ± 0.27	–	–	–	–	–	–	–	–	–	1.13 ± 0.06	0.96 ± 0.07	2.99 ± 0.32*	2.15 ± 0.16*
Aldehyde dehydrogenase 2	–	1.84 ± 0.68	–	0.46 ± 0.05*	0.36 ± 0.06*	–	1.99 ± 0.51	–	–	–	–	–	1.57 ± 0.02* (family 3)	1.19 ± 0.04 (family 3)	6.15 ± 0.08* (family 3)	3.56 ± 0.44* (family 3)
Malate dehydrogenase	–	–	–	–	2.17 ± 0.36	1.45 ± 0.21	1.35 ± 0.59	1.62 ± 0.12*	–	–	–	–	–	–	–	–
Enoyl CoA hydratase 1	1.54 ± 0.13	–	2.58 ± 0.11*	2.77 ± 0.07*	1.95 ± 0.45	–	1.62 ± 0.50	–	–	0.48 ± 0.55	–	0.19 ± 0.05	1.46 ± 0.17	1.44 ± 0.03	4.16 ± 0.53*	3.09 ± 0.26*
Peroxisomal acyl-CoA oxidase	–	2.73 ± 0.68	–	2.30 ± 0.16*	–	–	–	–	–	–	–	–	1.46 ± 0.09	1.45 ± 0.05	5.14 ± 0.40*	4.34 ± 0.16*
Fatty acid binding protein 1	–	2.13 ± 0.23	2.31 ± 0.41	3.49 ± 0.04*	–	1.48 ± 0.18	–	2.55 ± 0.30	–	–	–	–	1.06 ± 0.02 (protein 2)	0.93 ± 0.07 (protein 2)	1.90 ± 0.11* (protein 2)	1.58 ± 0.13 (protein 2)
Urate oxidase	–	–	–	–	–	–	–	–	–	0.18 ± 0.39	–	0.35 ± 0.08	1.21 ± 0.04	1.16 ± 0.05	0.86 ± 0.05	0.63 ± 0.05
Ketoacid dehydrogenase E1	–	0.48 ± 0.23	2.38 ± 0.20	0.70 ± 0.13*	–	–	–	–	–	–	–	–	1.15 ± 0.04	1.06 ± 0.04	1.53 ± 0.11	1.33 ± 0.10

OX: Oxazepam, WY: Wy-14,643.
*Significantly different from control at $P < 0.05$.
–: no change detected.
NA: not on array.

(TGFβ) and TNFα, and by genotoxic and oxidative stress (80–82). Furthermore, Gadd45β induction by various stimuli requires NF-κB (80,82,83). Jin *et al*. also reported that Gadd45β promoter contains three NF-κB-binding sites, and these sites are required for optimal transcriptional activation (84). In the present study, the Gadd45β gene was significantly upregulated by oxazepam and Wy-14,643 treatment at 2 weeks and 6 months and also in oxazepam-induced hepatocellular tumors by real-time PCR (Table IV). These results suggest that upregulation of Gadd45β may play a significant role in the early hepatocarcinogenic process by oxazepam and Wy-14,643.

IGF-related genes have been shown previously to be deregulated in human liver cancers (6). IGF-I and IGFBP-1 were both down-regulated in human HCC and hepatoblastomas (6,85,86). Igf-I was also down-regulated at all time points in this study after Wy-14,643 treatment. In contrast, Igfbp1 was upregulated in the livers at all time points by both compounds and strongly expressed in oxazepam-induced hepatocellular tumors. Expression of another IGF family member, Igf-II was increased in a majority of spontaneous mouse liver tumors (87), but it did not change by cDNA microarray in our study. These results suggest that some IGF-related genes play a significant role in hepatocellular carcinogenesis, and we plan to examine the expression of these genes in livers and liver tumors from other chemical treatments. The IGF axis should be investigated further to determine if early Igf-I or Igfbp1 deregulation would be potential predictors of mouse liver carcinogenesis and to understand the effects of various chemical treatments on the functions of these genes in hepatocarcinogenesis.

Because of the known increase in PPARα in liver following treatment with PPs (17,44), we examined the expression of this gene in livers in this study even though it was not present on the chip and was not detected by the proteomics analysis. We found by western blot analysis that the PPARα protein was upregulated by Wy-14,643 at all time points but down-regulated after oxazepam treatment. Other studies have shown that PPARα-knockout mice are resistant to PP-induced hepatocarcinogenesis (16). Those models suggest an essential role for PPARα in the formation of PP-induced liver tumors. However, whereas over-expression of PPARα may be sufficient for PP-induced hepatocarcinogenesis, it may not be a necessary event.

AFP is a commonly identified clinical marker of human hepatocellular cancer. Its expression in normal adult liver is very low except when a tumor such as hepatoma or teratoma is present (88). Sakairi *et al*. showed that cells in B6C3F1 mice that were treated with diethylnitrosamine and phenobarbital reacted positively for AFP with a mosaic pattern by immunohistochemistry (89). Interestingly, the mouse AFP repressor region contains potential p53 binding sites (90). In the proteomics analysis in this study, AFP was upregulated by oxazepam (2.3-fold) and Wy-14,643 (1.8-fold) treatment only at 2 weeks. By western blot analysis, AFP was upregulated by Wy-14,643 at all time points up to 6 months. After oxazepam treatment, the highest expression of AFP in liver was detected at 2 weeks, and thereafter it decreased with time. These results suggest that increased expression of AFP may be an early indicator of chemically induced mouse liver carcinogenesis, but these high levels may not always be sustained to tumor development.

Some other known interesting cancer-related genes were not identified in this study. For example, p53, Fos, Myc and Rb are known to be mutated in some HCCs (91–93), but we did not detect expression changes in these genes by the cDNA microarray screening. Moreover, we have not detected common genetic alterations or expression changes in any of these genes in mouse liver tumors (unpublished data). Additionally, we did not identify changes in β-catenin mRNA or accumulation of β-catenin protein (data not shown) in any livers examined in this study, even though *Catnb* mutations and β-catenin upregulation occur frequently in oxazepam-induced mouse liver tumors (34,94). Similarly β-catenin (CTNNB1) mutations and β-catenin protein accumulation have not been detected in human cirrhotic liver or preneoplastic lesions (95). Thus, β-catenin/Wnt signaling does not seem to be upregulated early prior to neoplastic formation by these chemicals.

The microarray screening identified 36 differentially expressed genes that were altered by oxazepam and 220 by Wy-14,643 treatments for 6 months. Much of this difference appeared to be due to the effect of Wy-14,643 as a potent PP and activator of many enzymes involved in β-oxidation of fatty acids. The proteomics methodology also discovered many proteins involved in peroxisome proliferation. PPs markedly induce the enzymes of the peroxisomal fatty acid β-oxidation cycle, especially acyl-CoA oxidase, which produces hydrogen peroxide, and the cyclic oxidation of single fatty acid molecule can result in the production of several molecules of hydrogen peroxide (96,97). As the PPs may induce many genes/proteins that function in fatty acid oxidation or maintenance of homeostasis, a majority of the genes upregulated by Wy-14,643 may not be involved in the carcinogenic process.

This study illustrates some of the advantages of using more than one method for assessing global expression changes. Cytochrome P-450 isozymes, several of which were highly induced on the cDNA microarray platform, were not detected by the proteomics technique. That methodology does not seem to work well with basic and hydrophobic membrane-bound proteins such as the cytochrome P-450s (98). In contrast, the microarray technique is limited to the genes on the chip and may underestimate expression changes (43). By combining both methods we detected expression changes in numerous genes/proteins not associated previously with liver carcinogenesis as well as many novel unidentified genes and proteins. Thus, these techniques are complimentary, and the combined data are being studied further to obtain a better understanding of the mouse hepatocarcinogenic process.

In summary, the data from our study indicate that there are more differences than common pathways to carcinogenesis by the two different non-genotoxic carcinogens oxazepam and Wy-14,643. Eventually, we would like to develop a panel of early markers for a screen that could potentially predict carcinogenesis by different chemical classes and identify those changes that are common to the carcinogenic process. Such screens using a limited panel of genes identified by clustering of cDNA microarray data have been proposed to predict toxicity of compounds (99). Further analysis of other known genes in our study set will shed more light on better understanding of early hepatocarcinogenesis. One of our future challenges is to identify the numerous unknown ESTs and proteins we detected that are associated with hepatocarcinogenesis by these chemicals.

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