(Qiagen) and zirconium beads. Total RNA was isolated from the liver homogenate or the hepatocyte lysate using RNeasy kit. Purity of the RNA was checked by gel electrophoresis, and the OD260/280 nm ratio was between 2.0-2.2. Microarray analysis was conducted on 3 out of 5 samples for each group by using GeneChip®RAE230A probe arrays (Affymetrix, Santa Clara, CA, USA), containing 15923 probe sets. The

Table 1.

Compound	Abbreviation	*in vivo*		*in vitro*		Supplier
		Dose (mg/kg)	Vehicle	Concentration (μM)	Vehicle	
acetaminophen	APAP	300, 600, 1000	MC	300, 1000, 3000	Medium	Sigma
allopurinol	APL	15, 50, 150	MC	5.6, 28, 140	DMSO	Sigma
allyl alcohol	AA	3, 10, 30	OIL	4, 20	Medium	Tokyo kasei
alpha-naphthyl-isothiocyanate	ANIT	1.5, 5, 15	OIL	8, 40, 200	DMSO	Kanto chemical
aspirin	ASA	45, 150, 450	MC	120, 600, 3000	DMSO	Wako
benzbromarone	BBr	20, 60, 200	MC	0.6, 3, 15	DMSO	Sigma
bromobenzene	BBZ	30, 100, 300	OIL	8, 40, 200	DMSO	Tokyo kasei
carbamazepine	CBZ	30, 100, 300	MC	12, 60, 300	DMSO	Sigma
carbon tetrachloride	CCL4	10, 30, 100	OIL	300, 1000, 3000	DMSO	Wako
chlorpromazine	CPZ	4.5, 15, 45	MC	0.8, 4, 20	DMSO	Wako
clofibrate	CFB	30, 100, 300	OIL	12, 60, 300	DMSO	Wako
coumarin	CMA	15, 50, 150	OIL	12, 60, 300	DMSO	Tokyo kasei
cyclophosphamide	CPA	1.5, 5, 15	MC	8, 40, 200	Medium	Ardrich
diazepam	DZP	25, 75, 250	MC	5, 25, 125	DMSO	Wako
diclofenac sodium	DFNa	1, 3, 10	MC	16, 80, 400	DMSO	Tokyo kasei
ethionine	ET	25, 80, 250	MC	400, 2000, 10000	Medium	Tokyo kasei
gemfibrozil	GFZ	30, 100, 300	OIL	4, 20, 100	DMSO	Sigma
hexachlorobenzene	HCB	30, 100, 300	OIL	0.6, 3, 15	DMSO	Tokyo kasei
indomethacin	IM	0.5, 1.6, 5	MC	12, 60, 300	DMSO	Sigma
isoniazid	INAH	10, 50, 100	MC	400, 2000, 10000	Medium	Sigma
methapyrilene hydrochloride	MP	10, 30, 100	MC	0.6, 3, 15	Medium	Sigma
methotrexate	MTX	10, 30, 100 (Single) 0.1, 0.3, 1 (Repeated)	MC	20, 100, 500	Medium	Wako
nitrofurantoin	NFT	10, 30, 100	MC	5, 25, 125	DMSO	ICN
omeprazole	OPZ	100, 300, 1000	MC	4.8, 24, 120	DMSO	Wako
phenobarbital	PB	10, 30, 100	MC	300, 1000, 3000	Medium	Sigma
phenylbutazone	PhB	20, 60, 200	MC	16, 80, 400	DMSO	Sigma
phenytoin	PHE	60, 200, 600	MC	2.4, 12, 60	DMSO	Tokyo kasei
propylthiouracil	PTU	10, 30, 100	MC	160, 800, 4000	Medium	Tokyo kasei
rifampicin	RIF	20, 60, 200	MC	2.8, 14, 70	DMSO	Wako
thioacetamide	TAA	4.5, 15, 45	MC	400, 2000, 10000	Medium	Ardrich
valproate sodium	VPA	45, 150, 450	MC	400, 2000, 10000	Medium	Sigma
Wy-14,643	WY	10, 30, 100	OIL	8, 40, 200	DMSO	Tokyo kasei

Supplier information: Wako; Wako pure chemical industries, Osaka, Japan, Tokyo kasei; Tokyo kasei kogyo, Tokyo, Japan. ICN; ICN Biomedical Inc., Aurora, OH, USA, Sigma (St. Louis, MO, USA), Aldrich (St. Louis, MO, USA), Kanto chemical (Tokyo, Japan).

procedure was conducted basically according to the manufacturer's instructions using Superscript Choice System (Invitrogen, Carlsbad, CA, USA) and T7-(dT)24-oligonucleotide primer (Affymetrix) for cDNA synthesis, cDNA Cleanup Module (Affymetrix) for purification, and BioArray High yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA) for synthesis of biotin-labeled cRNA. Twenty μg of the fragmented cRNA was hybridized to a RAE230A probe array for 18 hr at 45°C at 60 rpm, after which the array was washed and stained by streptavidin-phycoerythrin using Fluidics Station 400 (Affymetrix) and then scanned by Gene Array Scanner (Affymetrix). The data were analyzed by using GeneSpring® version 6.1 (Silicon Genetics, Santa Clara, CA, USA). Expression data were normalized using the mean value (global normalization). Filtering of the data was performed by flags (present or marginal call) in at least half of the samples, as well as the fold change, over the concurrent control value.

Principal components analysis

To test whether profiling on *in vivo* effects of compounds can be estimated from *in vitro* transcriptome data, principal component analysis (PCA) on the data of three time points (24 hr after *in vivo* single dose, 29 day after *in vivo* repeated dose, and 24 hr after *in vitro* exposure) was independently conducted using the expression of 41 genes which changed to the same direction both *in vitro* and *in vivo* (single or repeated dose). The method of gene selection is described in the results section.

For calculation, we took the mean signals from three (*in vivo*) or two (*in vitro*) samples from each dose group and calculated the ratio for each respective control, and subsequently the values were transformed to log ratio to create a matrix. Each row corresponds to a different gene, and each column corresponds to a different condition including three different dose groups of 32 compounds. To compute the principal components, the eigenvalues and their corresponding eigenvectors were calculated from the correlation matrix of conditions. The calculations were done using the R version 2.2.0 (www.r-project.org) statistical environment.

RESULTS

Pathology

Toxicological changes observed in repeated administration of three peroxisome proliferators are summarized in Fig. 1. All three chemicals showed a significant increase in the relative liver to body weight after 4 days or later of administration (Fig. 1A). They also showed a significant decrease in triglyceride after the 4th day of administration, known to be directly related to their activity of PPARα agonist (Fig. 1B). Signs of hepatotoxicity and an increase in ALT activity were observed in the 4th day of WY-14643- and the 29th day of gemfibrozil-treated groups (Fig. 1-C). In pathological examinations, granular degeneration of hepatocytes was noted in all compounds. Furthermore, an increase in hepatocyte proliferation was observed in WY-14643 and gemfibrozil. Treatment with WY-14643 also induced necrosis of hepatocyte and hypertrophy of the bile duct (data not shown).

Analysis of mRNA expression profile

Differentially expressed genes in liver treated with clofibrate, WY-14643 and gemfibrozil were extracted as follows. For *in vivo* study, genes with present call in at least 3 out of 6 samples for each experimental set (3 each from control and treated group) were selected and used for further analysis. In the case of the *in vitro* study, genes with present call in at least 2 out of 4 samples in each experimental set (2 each from control and treated) were selected. Genes showing at least a 1.5 fold increase (or 1/1.5 fold decrease) both in middle and high dose vs. control at $p<0.05$ by Student's *t*-test were extracted for each time point. In the case of *in vitro* study, the same procedure without use of *t*-test was employed. In the next step, the common genes selected in at least 2 out of three peroxisome proliferators for single, repeated, and *in vitro* experiments were extracted. The numbers of genes extracted as above were as follows: the up-regulated genes, 115/195/89 for *in vivo*-single/*in vivo*-repeated/ *in vitro*, respectively; and the down-regulated genes, 181/221/38, for *in vivo*-single/*in vivo*-repeated/ *in vitro*, respectively. The overlapping of these genes is depicted in a Venn diagram in Fig. 2.

Comparing the extracted genes between the protocols, 71, 29, and 37 up-regulated genes were in common between the single and repeated administration, between single administration and *in vitro*, and between repeated administration and *in vitro*, respectively. For down-regulated genes, 44 genes were in common between single and repeated administration, whereas no common genes were present between *in vivo* and *in vitro*. These genes are listed in Table 2 (up-regulated) and Table 3 (down-regulated).

Most of the genes categorized in β-oxidation and

fatty acid and cholesterol synthesis, and the peroxisomal protein, were commonly changed both *in vivo* and *in vitro*. In general, genes related to lipid metabolism were commonly up-regulated *in vivo* and *in vitro* except that apolipoprotein A-IV, apolipoprotein M, and lipoprotein-binding protein were down-regulated only *in vivo* (mainly repeated dose). As for genes related to lipid metabolism other than that listed above, most of the up-regulated ones were common between *in vivo* and *in vitro*, whereas the down-regulated ones were only observed *in vivo* (single and repeated dose).

Of the genes related to carbohydrate metabolism, elevation of pyruvate dehydrogenase kinase 4 was observed both *in vivo* (single and repeated dose) and *in vitro*, whereas reduction of "pyruvate kinase liver and RBC" was observed only *in vivo* (mainly repeated dose) and others were increased *in vivo* (single and repeated dose).

The genes classified as cell proliferation were up- or down-regulated only *in vivo* (single and repeated dose). Some genes, including cyclin D1, showed more marked changes in single dose than in repeated dose. The changes of genes categorized in apoptosis were only detectable *in vivo* and their changes were also more prominent in single dose than in repeated dose.

Obvious changes were noted in the genes related to "drug and xenobiotic metabolism" and most of them occurred exclusively *in vivo* (single and repeated dose). The only observable changes in growth factor-related genes were a reduction of the *in vivo* repeated dose. Changes in the expression of genes related to "cellular morphogenesis" as well as the "stress response" were mainly noted *in vivo* (single and repeated dose). Many other genes categorized to "transcription activation and repression", "transporter", "cell adhesion", "immune response", "blood coagulation", "regulation of blood

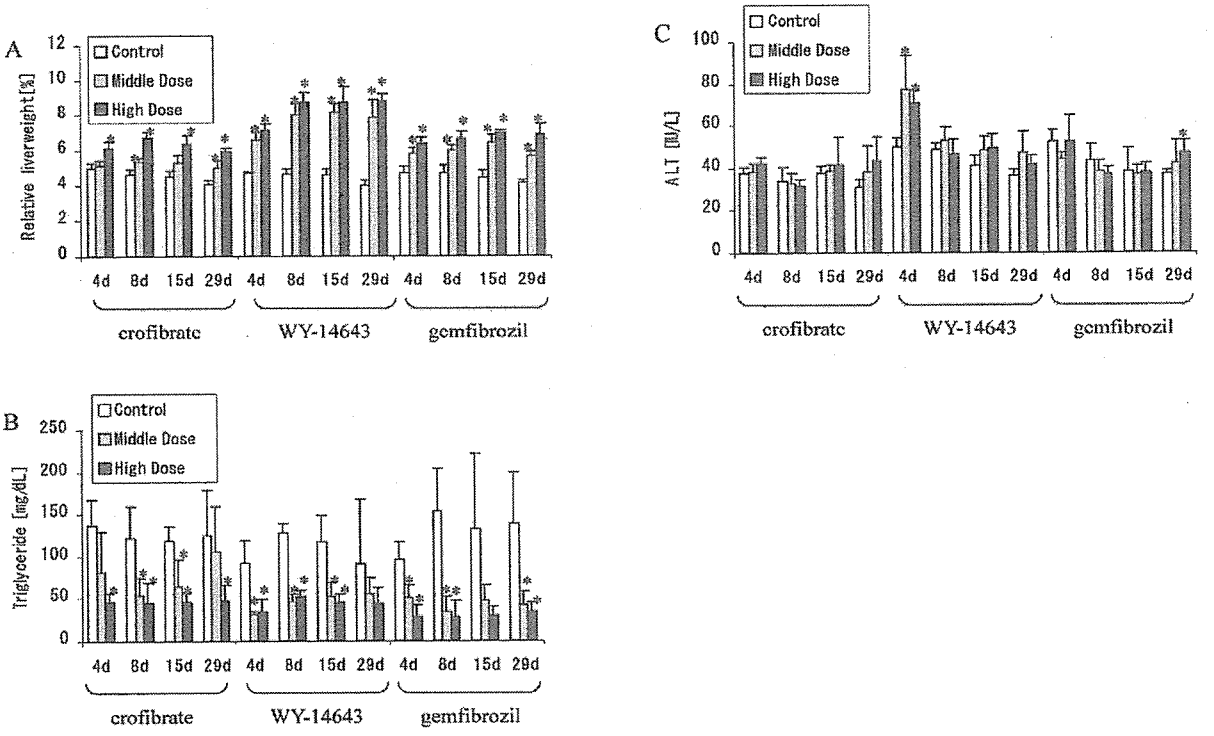

Fig. 1. Effects of clofibrate, WY-14643 and gemfibrozil on relative liver weight (A), plasma triglyceride (B) and plasma alanine amino transferase (ALT; C). Values were obtained 24 hr after repeated administration of each drug for 3, 7, 14, and 28 days. Middle and high dose was 100 and 300 mg/kg for clofibrate, 30 and 100 mg/kg for WY-14643, and 100 and 300 mg/kg for gemfibrozil, respectively. For simplicity, data of the low dose was omitted. *Significantly different from control ($p<0.05$, Student's *t*-test, N=5).

pressure" were found to be down-regulated *in vivo* without any changes *in vitro*.

Hierarchical cluster analysis

Hierarchical clustering (complete linkage method, Euclidean distance) was performed in the 32 compounds stored in our database (the number at that stage of our analysis) using the data of gene expression *in vivo* or *in vitro* (24 hr after middle and high dose). The probe sets used for analysis of *in vivo* data were 36 (up-regulated) and 35 (down-regulated) which showed a more than 1.5 fold change with $p<0.05$ by Student's *t*-test both in middle and high dose of more than 2 compounds out of clofibrate, WY-14643 and gemfibrozil. The probe sets used for analysis of *in vitro* data were 49 (up-regulated) and 6 (down-regulated) which showed a more than 1.5 fold change with $p<0.05$ by Student's *t*-test both in the middle and the high doses of more than 2 out of the three compounds. In clusters of single-dose experiments, benzbromarone and aspirin were classified into the same cluster of the three peroxisome proliferators (Fig. 3). In clusters of the *in vitro* experiments, benzbromarone, three non-steroidal anti-inflammatory drugs (aspirin, indomethacin, and diclofenac sodium), valproic acid, and ANIT were classified into the same cluster of the three peroxisome proliferators (Fig. 4).

PCA-based estimation of PPARα activity

As previously shown in Fig. 2, no common down-regulated genes existed between *in vivo* and *in vitro*, whereas 41 up-regulated genes were found to be common genes (between *in vivo* and *in vitro*) by PPARα agonist treatment. We expected that these genes could be useful for prediction of *in vivo* effects from *in vitro* effects, so PCA analysis was conducted on the 32 compounds using these 41 genes.

A projection on the first two principal components for each condition, together with the contribution rate of the first three components, is shown in Fig. 5. It was obvious from the figure that the first principal component score (PC1) with high contribution (about 60%) for all three experimental sets, was negatively correlated to the dose levels of these PPARα ligands. Therefore, we reasoned that this negative PC1 score could be used to estimate the PPARα activity in general.

Fig. 6 shows the plotting of negative PC1 values from the 32 compounds examined *in vivo* and *in vitro*. In this figure, a clearer comparison of putative PPARα activity between the compounds can be performed. All the PPARα agonists showed high scores both *in vivo* and *in vitro*. Benzbromarone, aspirin and valproic acid again showed high scores both *in vivo* and *in vitro*. It was also easy to pick up the compounds that showed high scores *in vitro* rather than *in vivo*, such as diclofenac and indomethacin.

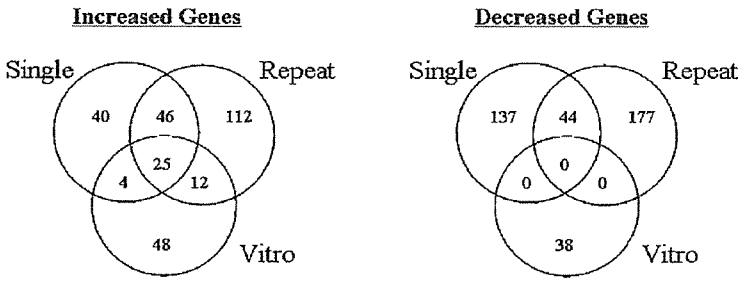

Fig. 2. Venn diagram of extracted genes. Differentially expressed genes in liver treated with clofibrate, WY-14643 and gemfibrozil were extracted as follows. For *in vivo* study (single and repeated) genes with present call in at least 3 out of 6 samples in each experimental set that show at least a 1.5-fold increase (or 1/1.5-fold decrease) for both middle and high dose vs. control (at $p<0.05$ by Student's *t*-test) were extracted for each time point. For the *in vitro* study, genes with present call at least 2 out of 4 samples in each experimental set showing the same criteria as *in vivo* (without using *t*-test) were extracted. The numbers in the diagram show the numbers of extracted genes in each category.

Table 2. Up-regulated genes that are discussed in the text.

	ProbeID	GeneName	Gene Symbol	Vivo		Vitro[c]
				Single[a]	Repeated[b]	
	β-oxidation					
*	1387783_a_at	acetyl-Coenzyme A acyltransferase 1	Acaa1	3.9	5.8	8.4
	1367735_at	acetyl-Coenzyme A dehydrogenase, long-chain	Acadl	1.6	2.0	1.8
*	1367897_at	acyl-Coenzyme A dehydrogenase, very long chain	Acadvl	2.2	2.6	2.8
	1367680_at	acyl-Coenzyme A oxidase 1, palmitoyl	Acox1	2.7	3.1	4.3
*	1367836_at	Carnitine palmitoyltransferase 1 liver	Cpt1a	5.3	7.4	6.6
*	1386946_at	carnitine palmitoyltransferase 1, liver	Cpt1a	4.3	4.6	8.5
*	1386927_at	carnitine palmitoyltransferase 2	Cpt2	5.5	6.2	3.0
*	1367659_s_at	dodecenoyl-coenzyme A delta isomerase	Dci	6.4	7.0	3.6
*	1367777_at	2,4-dienoyl CoA reductase 1, mitochondrial	Decr1	2.4	3.3	3.0
*	1370818_at	2-4-dienoyl-Coenzyme A reductase 2 peroxisomal	Decr2	3.5	3.5	2.5
*	1386885_at	enoyl coenzyme A hydratase 1, peroxisomal	Ech1	8.9	11.6	4.6
*	1368283_at	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh	7.1	8.4	83.0
	1370164_at	Hydroxyacyl-Coenzyme A dehydrogenase alpha subunit	Hadha	3.1	4.2	1.9
	1367694_at	hydroxyacyl-Coenzyme A dehydrogenase beta subunit	Hadhb	3.1	3.2	1.5
*	1388210_at	Mitochondrial acyl-CoA thioesterase 1	Mte1	24.1	32.1	3.9
*	1388211_s_at	Mitochondrial acyl-CoA thioesterase 1	Mte1	162.8	175.5	7.6
	Lipid mobilization					
	1370024_at	fatty acid binding protein 7, brain	Fabp7	2.4	3.0	1.5
*	1368150_at	solute carrier family 27 (fatty acid transporter), member 2	Slc27a2	1.7	1.9	3.3
	1398249_at	solute carrier family 25 member 20	Slc25a20	3.7	3.7	2.4
*	1367950_at	solute carrier family 22 member 5	Slc22a5	4.1	5.5	3.1
*	1367689_a_at	Cd36 antigen	Cd36	5.8	16.4	3.7
*	1386901_at	cd36 antigen	Cd36	5.2	9.6	3.8
*	1375247_at	Monoglyceride lipase	Mgll	3.2	7.4	3.3
*	1370831_at	Monoglyceride lipase	Mgll	4.2	7.4	3.5
*	1388644_at	Monoglyceride lipase	Mgll	7.2	9.4	3.9
	1368317_at	aquaporin 7	Aqp7	3.4	22.0	1.3
	Fatty acid and cholesterol synthesis					
	1367857_at	fatty acid desaturase 1	Fads1	1.5	1.8	1.2
	1368453_at	fatty acid desaturase 2	Fads2	2.4	3.8	1.3
*	1370355_at	Stearoyl-Coenzyme A desaturase 1	Scd1	1.6	2.6	2.2
	1372318_at	ELOVL family member 6 elongation of long chain fatty acids yeast	rELO2	4.6	7.2	1.5
*	1388108_at	ELOVL family member 6 elongation of long chain fatty acids yeast	rELO2	5.4	9.6	2.1
	1367767_at	3-hydroxy-3-methylglutaryl CoA lyase	Hmgcl	1.9	3.4	1.5
*	1370310_at	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	Hmgcs2	1.9	1.9	11.5
	Ohter lipid methabolism related					
*	1386880_at	acetyl-Coenzyme A acyltransferase 2	Acaa2	2.0	2.5	4.3
	1373778_at	Acetyl-Coenzyme A carboxylase beta	Acacb	2.0	3.6	1.4

Table 2. Continued.

	ProbeID	GeneName	Gene Symbol	Vivo		Vitro[c)]
				Single[a)]	Repeated[b)]	
	1367763_at	Acetyl-coenzyme A acetyltransferase 1	Acat1	2.2	3.4	2.1
	1368177_at	acyl-CoA synthetase long-chain family member 3	Acsl3	2.4	5.8	3.6
*	1371886_at	Carnitine acetyltransferase	Crat	9.7	17.4	2.5
	1368426_at	carnitine *O*-octanoyltransferase	Crot	2.8	3.1	2.0
	1387183_at	Carnitine *O*-octanoyltransferase	Crot	6.4	6.4	3.6
*	1398250_at	cytosolic acyl-CoA thioesterase 1	Cte1	537.8	971.5	1906.3
	1368206_at	peroxisomal acyl-CoA thioesterase 1	Pte1	3.6	5.8	1.5
	1369485_at	cytoplasmic acetyl-CoA hydrolase	rACH	2.3	2.9	2.7
*	1388924_at	Angiopoietin-like protein 4	Angptl4	5.3	5.6	5.5
	Peroxisomal Protein					
*	1379361_at	Peroxisomal biogenesis factor 11A	Pex11a	16.5	12.1	4.9
*	1387740_at	peroxisomal biogenesis factor 11A	Pex11a	14.8	13.3	7.4
	Carbohydrate methabolism					
*	1369150_at	pyruvate dehydrogenase kinase, isoenzyme 4	Pdk4	9.7	17.9	15.9
	1370509_at	Pyruvate dehydrogenase phosphatase isoenzyme 2	Pdp2	2.3	1.9	1.3
	1369560_at	glycerol-3-phosphate dehydrogenase 1 (soluble)	Gpd1	3.3	3.6	2.8
	1371363_at	Glycerol-3-phosphate dehydrogenase 1 soluble	Gpd1	2.4	2.3	2.2
	1387670_at	Glycerol-3-phosphate dehydrogenase 2	Gpd2	6.2	7.5	1.5
	1370870_at	Malic enzyme 1	Me1	5.0	15.0	1.2
	1370067_at	malic enzyme 1	Me1	5.1	12.0	1.1
	Proliferation					
	1371150_at	Cyclin D1	Ccnd1	2.2	1.7	1.1
	1383075_at	Cyclin D1	Ccnd1	3.7	1.6	1.1
	1368947_at	growth arrest and DNA-damage-inducible 45 alpha	Gadd45a	3.6	4.0	1.3
	1368308_at	myelocytomatosis viral oncogene homolog (avian)	Myc	2.2	5.7	1.2
	1373473_a_at	Nucleosome assembly protein 1-like 1	Nap1l1	3.3	3.9	1.2
	1370826_at	Nucleosome assembly protein 1-like 1	Nap1l1	2.0	2.2	1.1
	1387977_at	Nibrin	Nbn	3.3	2.3	1.3
	1387062_a_at	checkpoint kinase 1 homolog (S. pombe)	Chek1	2.8	2.7	1.3
	1371352_at	High mobility group protein 17	Hmgn2	1.8	1.9	1.5
	1370334_at	Evectin-1	Plekhb1	3.4	2.2	1.4
	1370413_at	Pregnancy specific beta-1-glycoprotein 4	Psg4	1.8	1.7	1.2
	1389403_at	Bone morphogenetic protein 7	Bmp7	1.7	1.9	1.2
	1368143_at	Annexin A7	Anxa7	2.3	3.5	1.0
	Growth factor					
	1369608_at	fibroblast growth factor 16	Fgf16	1.8	1.8	1.2
	Apoptosis					
	1368888_a_at	Reticulon 4	Rtn4	2.5	8.9	1.1
	1388027_a_at	Reticulon 4	Rtn4	3.5	13.3	1.1
	1387521_at	programmed cell death 4	Pdcd4	1.3	2.3	1.2
	Cellular morphogenesis					
*	1368475_at	collagen-like tail subunit of asymmetric acetylcholinesterase	Colq	7.8	7.7	2.4

Table 2. Continued.

	ProbeID	GeneName	Gene Symbol	Vivo		Vitro[c]
				Single[a]	Repeated[b]	
	1368355_at	myosin 5B	Myo5b	4.2	7.0	1.1
	1398281_at	occludin	Ocln	2.7	3.0	1.1
	1367655_at	thymosin, beta 10	Tmsb10	2.4	5.0	1.9
	Drug and xenobiotic methabolism					
	1387296_at	cytochrome P450, family 2, subfamily J, polypeptide 4	Cyp2j4	2.8	4.6	1.6
	1370706_a_at	Cytochrome P450 family 2 subfamily j polypeptide 9	Cyp2j9	1.1	2.0	1.1
*	1370397_at	Cytochrome P450 family 4 subfamily a polypeptide 14	Cyp4a14	3.4	3.3	21.9
*	1368934_at	cytochrome P450, family 4, subfamily b, polypeptide 1	Cyp4b1	6.6	6.1	9.9
	1368738_at	Cytochrome P450 subfamily 11B polypeptide 1	Cyp11b1	2.2	2.4	3.5
	1387123_at	cytochrome P450, family 17, subfamily a, polypeptide 1	Cyp17a1	5.8	8.7	4.2
	1369111_at	alcohol dehydrogenase 4 (class II), pi polypeptide	Adh4	1.4	1.2	10.7
	1370313_at	alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	Adh7	3.3	4.6	2.8
	1370613_s_at	UDP glycosyltransferase 1 family polypeptide A6	Ugt1a6	1.4	2.0	1.2
	1387759_s_at	UDP glycosyltransferase 1 family polypeptide A6	Ugt1a6	2.1	2.5	1.2
	Stress response					
	1367577_at	heat shock 27kDa protein 1	Hspb1	4.7	8.7	1.2
	1374105_at	Hypoxia induced gene 1	Hig1	2.4	1.9	1.7
	1387636_a_at	P11 protein	Cdtw1	3.3	3.6	1.3
	1368552_at	GrpE-like 1, mitochondrial	Grpel1	1.9	2.3	1.2
	1387023_at	glutathione *S*-transferase, mu type 3 (Yb3)	Gstm3	1.3	2.1	2.0
	1375357_at	Dystonia 1 torsion autosomal dominant torsin A	Dyt1	1.6	1.6	1.1
	Other probes which were used in PCA analysis					
*	1374265_at	NA	NA	4.7	5.6	2.6
*	1374556_at	Similar to spermine oxidase	NA	2.1	3.0	2.8
*	1376076_at	Transcribed locus strongly similar to NP_076005.1 RIKEN cDNA 2310016C08 gene Mus musculus	NA	5.8	5.9	4.4
*	1377037_at	Similar to peroxisomal acyl-CoA thioesterase 2B likely ortholog of mouse peroxisomal acyl-CoA thioesterase 2B	NA	17.5	10.6	7.9
*	1377867_at	Similar to Glutaminyl-peptide cyclotransferase precursor QC Glutaminyl-tRNA cyclotransferase Glutaminyl cyclase	NA	10.5	43.2	6.1
*	1383205_at	Similar to dapper2	NA	3.5	4.2	1.9
*	1388756_at	Similar to RIKEN cDNA 6330579B17 gene	NA	2.5	2.5	2.7
*	1389253_at	Vanin 1	Vnn1	14.7	29.9	5.6
*	1390383_at	Adipose differentiation-related protein	ADRP	4.7	2.4	2.0

[a]: The largest fold change among the single dose studies of CFB, WY and GFZ is shown. [b]: The largest fold change among the repeated dose studies of CFB, WY and GFZ is shown. [c]: The largest fold change among *in vitro* studies of CFB, WY and GFZ is shown. The columns are shaded when the corresponding probe sets appear in Fig. 2. *: Probes which were used in the PCA analysis.

Table 3. Down-regulated genes that are discussed in the text.

Probe ID	Gene Name	Gene Symbol	Vivo		Vitro[c]
			Single[a]	Repeated[b]	
Lipid mobilization					
1368520_at	apolipoprotein A-IV	Apoa4	−4.0	−255.0	−1.2
1386980_at	apolipoprotein M	Apom	−1.4	−5.3	−1.1
1398859_at	Lipoprotein-binding protein	Hdlbp	−1.8	−1.9	−1.1
Other lipid Methabolism related					
1387959_at	Lysophospholipase	LOC246266	−2.2	−3.4	−2.3
1370530_a_at	Phospholipase D1	Pld1	−2.0	−1.8	−1.4
1369526_at	acyl-Coenzyme A dehydrogenase, short/branched chain	Acadsb	−2.0	−2.9	−1.8
Carbohydrate methabolism					
1368651_at	Pyruvate kinase liver and RBC	Pklr	−6.1	−5.8	−1.4
1387263_at	pyruvate kinase, liver and RBC	Pklr	−11.1	−15.7	−3.1
Proliferation					
1387129_at	X-ray repair complementing defective repair in Chinese hamster cells 1	Xrcc1	−1.9	−1.4	−1.2
1372863_at	MYC binding protein 2	Mycbp2	−2.3	−3.0	−1.2
1373291_at	Deleted in liver cancer 1	Dlc1	−2.0	−2.7	−1.3
1373332_at	Casein kinase 1 delta	Csnk1d	−1.9	−1.7	−1.1
1398273_at	ephrin A1	Efna1	−2.1	−1.6	−1.3
Transcription activation and repression					
1371202_a_at	Nuclear factor I B	Nfib	−2.6	−3.2	−1.3
1388167_at	Nuclear factor I B	Nfib	−2.5	−2.8	−1.4
1370946_at	Nuclear factor I X	Nfix	−2.5	−4.1	−1.3
1368221_at	nuclear receptor subfamily 3, group C, member 1	Nr3c1	−2.3	−2.5	−1.3
1369244_at	aryl hydrocarbon receptor nuclear translocator	Arnt	−1.8	−2.2	−1.3
1372601_at	Activating transcription factor 5	Atf5	−1.9	−2.8	−1.1
1367601_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	Cited2	−3.1	−2.2	−1.3
1367602_at	Cbp p300-interacting transactivator with Glu Asp-rich carboxy-terminal domain 2	Cited2	−3.0	−2.3	−1.1
1387165_at	v-maf musculoaponeurotic fibrosarcoma (avian) oncogene homolog (c-maf)	Maf	−2.0	−3.7	−1.9
1371781_at	Signal transducer and activator of transcription 3	Stat3	−2.2	−2.6	−1.1
Growth factor					
1370830_at	Epidermal growth factor receptor	Egfr	−5.9	−3.8	−1.3
1373829_at	Fibroblast growth factor receptor 2	Fgfr2	−1.6	−2.5	−1.3
1370941_at	Platelet derived growth factor receptor alpha polypeptide	Pdgfra	−1.5	−2.7	−1.3
1367652_at	insulin-like growth factor binding protein 3	Igfbp3	−1.6	−2.6	−1.3
Apoptosis					
1369941_at	death-associated protein	Dap	−2.0	−2.8	−1.1
1369902_at	Bcl2 modifying factor	Bmf	−12.3	−22.2	−3.4
1370512_at	Androgen receptor-related apoptosis-associated protein CBL27	Cbl27	−2.9	−3.1	−1.3
1371491_at	Notch gene homolog 1 Drosophila	Notch1	−1.9	−1.9	−1.4

Table 3. Continued.

Probe ID	Gene Name	Gene Symbol	Vivo		Vitro[c]
			Single[a]	Repeated[b]	
1398362_at	Notch gene homolog 2 Drosophila	Notch2	−3.5	−5.5	−1.2
1370243_a_at	Prothymosin alpha	Ptma	−1.5	−2.5	−1.0
Cell adhesion					
1386947_at	cadherin 1	Cdh1	−2.9	−2.1	−1.2
1369224_at	cadherin 17	Cdh17	−4.5	−10.1	−1.5
1368642_at	cadherin 2	Cdh2	−2.2	−3.3	−1.2
1387259_at	Cadherin 2	Cdh2	−2.2	−2.3	−1.1
1369854_a_at	CEA-related cell adhesion molecule 1	Ceacam1	−2.0	−2.8	−1.4
1370371_a_at	CEA-related cell adhesion molecule 10	Ceacam10	−1.7	−2.2	−1.2
1370234_at	Fibronectin 1	Fn1	−2.7	−3.0	−1.1
1382027_at	Integrin beta 3 Cd61	Itgb3	−2.3	−3.5	−1.7
1372002_at	Gap junction membrane channel protein alpha 1	Gja1	−1.5	−2.2	−1.1
1367849_at	syndecan 1	Sdc1	−1.6	−2.1	−1.1
1370043_at	activated leukocyte cell adhesion molecule	Alcam	−2.4	−3.4	−1.3
1374432_at	Activated leukocyte cell adhesion molecule	Alcam	−2.1	−5.0	−1.6
1370108_a_at	Lin-7 homolog a C. elegans	Veli1	−2.5	−3.9	−3.7
1373027_at	Afadin	Af6	−1.9	−2.3	−1.2
Cellular morphogenesis					
1388459_at	Collagen type XVIII alpha 1	Col18a1	−3.0	−3.6	−1.2
1370959_at	Collagen type III alpha 1	Col3a1	−1.9	−2.6	−1.6
1376099_at	Collagen type V alpha 1	Col5a1	−2.2	−3.2	−1.4
1370895_at	Collagen type V alpha 2	Col5a2	−1.5	−2.1	−1.2
1371725_at	Myosin heavy polypeptide 9	Myh9	−2.7	−2.9	−1.2
1387402_at	myosin, heavy polypeptide 9	Myh9	−2.4	−3.3	−1.1
1369720_at	myosin Ib	Myo1b	−2.1	−2.9	−1.1
1386941_at	plectin	Plec1	−2.6	−2.7	−1.2
1370993_at	Laminin gamma 1	Lamc1	−2.2	−2.7	−1.2
1386956_at	scavenger receptor class B, member 1	Scarb1	−1.4	−3.3	−1.2
Immune response					
1371926_at	Interleukin 6 signal transducer	Il6st	−2.2	−1.9	−1.1
1368280_at	cathepsin C	Ctsc	−2.1	−3.2	−1.3
1387005_at	cathepsin S	Ctss	−1.3	−2.4	−1.1
1387893_at	Complement component 1 s subcomponent	C1s	−2.3	−13.6	−1.3
1370892_at	Complement component 4a	C4a	−2.3	−3.6	−1.1
1368558_s_at	allograft inflammatory factor 1	Aif1	−1.7	−3.3	−1.7
1370479_x_at	Alpha-2u globulin PGCL4	Obp3	−2.9	−81.0	−1.2
1387985_a_at	Alpha-2u globulin PGCL4	Obp3	−2.6	−207.5	−1.1
Coagulation					
1374320_at	Coagulation factor 5	F5	−2.5	−3.8	−1.6
1387351_at	fibrillin-1	Fbn1	−1.9	−3.2	−1.4
1371258_at	Fibrinogen alpha polypeptide	Fga	−2.1	−1.8	−1.5
1387323_at	kallikrein B, plasma 1	Klk3	−2.6	−2.7	−2.4
1369225_at	kininogen 1	Kng1	−1.8	−2.5	−1.1

Table 3. Continued.

Probe ID	Gene Name	Gene Symbol	Vivo		Vitro[c]
			Single[a]	Repeated[b]	
Drug and xenobiotic methabolism					
1387243_at	Cytochrome P450 family 1 subfamily a polypeptide 2	Cyp1a2	−2.2	−8.1	−1.2
1387913_at	Cytochrome P450 family 2 subfamily d polypeptide 22	Cyp2d22	−2.6	−4.0	−1.6
1368608_at	cytochrome P450, family 2, subfamily f, polypeptide 2	Cyp2f2	−2.0	−3.0	−1.3
1368265_at	cytochrome P450 monooxygenase CYP2T1	Cyp2t1	−2.2	−3.7	−1.3
1370387_at	Cytochrome P450 family 3 subfamily a polypeptide 13	Cyp3a13	−3.6	−50.2	−1.7
1368467_at	cytochrome P450, family 4, subfamily F, polypeptide 2	Cyp4f2	−1.7	−2.3	−1.4
1367979_s_at	cytochrome P450, subfamily 51	Cyp51	−2.1	−1.4	−1.0
1389218_at	UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	−2.9	−2.1	−1.3
1367938_at	UDP-glucose dehydrogenase	Ugdh	−2.4	−1.9	−1.2
1388410_at	UDP-glucose pyrophosphorylase 2	Ugp2	−3.0	−4.4	−1.2
Transport					
1370465_at	ATP-binding cassette sub-family B MDR TAP member 4	Abcb1	−5.5	−10.8	−1.3
1368497_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	Abcc2	−4.4	−2.5	−1.4
1369455_at	ATP-binding cassette, sub-family G (WHITE), member 5	Abcg5	−1.6	−3.1	−1.9
1369440_at	ATP-binding cassette, sub-family G (WHITE), member 8	Abcg8	−2.4	−5.6	−3.9
1398862_at	ATPase Ca transporting cardiac muscle slow twitch 2	Atp2a2	−2.6	−3.9	−1.2
1368698_at	ATPase Ca transporting plasma membrane 2	Atp2b2	−2.3	−3.4	−1.9
1387285_at	ATPase, Ca++ transporting, plasma membrane 2	Atp2b2	−2.1	−2.1	−1.7
1368621_at	aquaporin 9	Aqp9	−1.9	−8.4	−1.4
1390591_at	Na Pi cotransporter 4	Slc17a3	−1.8	−4.9	−1.5
1369746_a_at	Solute carrier family 21 member 10	Slc21a10	−1.8	−3.5	−1.6
1368461_at	solute carrier family 22 (organic anion transporter), member 8	Slc22a8	−2.0	−3.1	−1.2
1369169_at	solute carrier family 23 (nucleobase transporters), member 1	Slc23a1	−1.9	−2.6	−1.3
1368600_at	solute carrier family 26 (sulfate transporter), member 1	Slc26a1	−2.0	−2.5	−1.3
1369099_at	solute carrier family 30 (zinc transporter), member 1	Slc30a1	−1.6	−4.4	−1.3
1386960_at	solute carrier family 37 (glycerol-6-phosphate transporter), member 4	Slc37a4	−2.2	−2.3	−1.2
1368296_at	Solute carrier organic anion transporter family member 2b1	Slco2b1	−2.1	−2.4	−1.3
Stress response					
1371442_at	Hypoxia up-regulated 1	Hyou1	−2.9	−3.9	−1.6
1370665_at	Hypoxia up-regulated 1	Hyou1	−2.2	−3.3	−1.2

Table 3. Continued.

Probe ID	Gene Name	Gene Symbol	Vivo		Vitro[c]
			Single[a]	Repeated[b]	
Regulation of blood pressure					
1387811_at	angiotensinogen	Agt	−2.2	−2.1	−1.1
1369664_at	arginine vasopressin receptor 1A	Avpr1a	−3.0	−2.7	−1.4
1367801_at	endothelin converting enzyme 1	Ece1	−1.9	−2.2	−1.2
1386953_at	hydroxysteroid 11-beta dehydrogenase 1	Hsd11b1	−2.2	−4.6	−1.1
1368102_at	hydroxysteroid 11-beta dehydrogenase 2	Hsd11b2	−1.8	−3.6	−1.9
1387994_at	Hydroxysteroid 17-beta dehydrogenase 9	Hsd17b9	−1.6	−1.8	−1.3
Others					
1368490_at	CD14 antigen	Cd14	−2.1	−5.1	−1.3
1370891_at	CD48 antigen	Cd48	−1.3	−2.1	−1.2
1367709_at	CD63 antigen	Cd63	−1.4	−2.9	−1.1

[a]: The smallest ratio to control value observed in single-dose studies of CFB, WY and GFZ is shown. Negative figure means the reciprocal number of ratio; e.g., −3.0 means that one of the drugs reduced the gene expression to 1/3 of corresponding control. [b]: The smallest ratio to control value observed in repeated dose studies of CFB, WY and GFZ is shown. [c]: The smallest ratio to control value observed *in vitro* studies of CFB, WY and GFZ is shown. The columns are shaded when the corresponding probe sets appear in Fig. 2.

DISCUSSION

In the present study, analysis of gene expression in rat liver was done with three peroxisome proliferators, clofibrate, WY-14643 and gemfibrozil, stored in our database. The changes of gene expression by these compounds observed *in vivo* (single and repeated) were largely in accordance with the report by Kramer *et al.* (2003), in which the effect of clofibrate on the gene expression profile in rat liver was analyzed. Among the genes whose expression was affected, a large number of genes were overlapped between *in vivo* and *in vitro*, both in up- and down-regulated ones. Between *in vivo* and *in vitro* experiments, however, there were many common genes in up-regulated ones but none in down-regulated ones.

A large number of genes related to b-oxidation were up-regulated by a single dose, and similar changes were also noted for *in vitro* experiments. The genes that possess PPRE sequence in their promoter regions, e.g., acyl-CoA oxidase (Tugwood *et al.*,1992), carnitine palmitoyl transferase I (Brandt *et al.*, 1998), carnitine palmitoyl transferase II (Barrero *et al.*, 2003) and fatty acid desaturase 2 (Tang *et al.*, 2003) were found to be up-regulated both *in vivo* and *in vitro*. An exception was that malic enzyme (Castelein *et al.*, 1994), whose promoter region contains PPRE, was induced *in vivo* but not *in vitro*. On the other hand, there were many genes whose promoter regions had no PPRE sequence showing common induction for *in vivo* and *in vitro*. For example, CD36, a fatty acid transporter, and CYP4A14, involved in fatty acid hydroxylation, were up-regulated both *in vivo* and *in vitro*, but there has been no report that their promoter regions contain functional PPRE. Apart from their mechanism, the genes that show common changes *in vivo* and *in vitro* (as listed by the present study) are considered to be useful to assess pharmacological and toxicological effects *in vivo* from *in vitro* experiments. This will be discussed later.

There were also data suggesting the limitations of *in vitro* experiments. Among the genes modulated by administration of fibrates *in vivo*, those related to the functions of proliferation, apoptosis, immune response, transcription activation and repression, transporter, cell adhesion, blood coagulation and regulation of blood pressure, did not show any changes *in vitro*. It is well known that peroxisome proliferators are non-genotoxic carcinogens for rodents and their most convincing mechanism is presently considered to be the activation of proliferation in addition to attenuation of apoptosis (Michalik *et al.*, 2004; Boitier *et al.*, 2003). In the present study, many of the genes related to proliferation and apoptosis were mobilized by fibrates *in vivo* but not at all *in vitro*. There have been many reports describing the fact that stimulation of proliferation by peroxisome proliferators requires Kupffer cell or TNFα produced by the cell. Rose *et al.* (1997)

reported that acceleration of proliferation in rat hepatocytes by WY-14643 was attenuated by the reduction of TNFα via inactivation of Kupffer cells. It was also reported that WY-14643 failed to cause cell proliferation in hepatic parenchymal cells cultured under the condition where non-parenchymal cells had been eliminated, and that its proliferative effect was recovered when Kupffer cells were added to the culture (Parzefall *et al.*, 2001). As for the attenuation of apoptosis, it was reported that a high concentration of TNFα inhibited spontaneous TGFβ1-induced apoptosis in primary cultured hepatocytes (Rolfe *et al.*, 1997). These reports

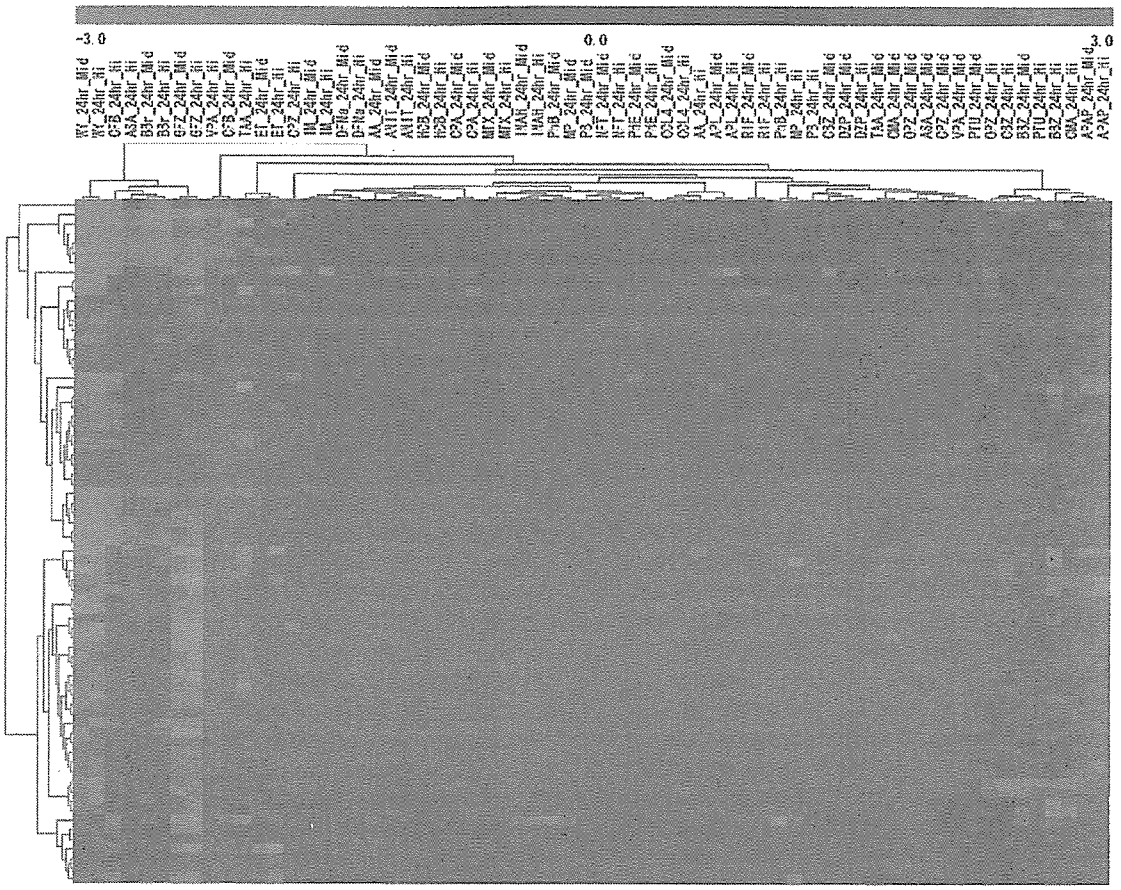

Fig. 3. A heat map view of the gene expression profile for the 32 compounds *in vivo*. Hierarchical clustering analysis of the compounds (middle and high dose, 24 hr after a single dose) was conducted using the genes that were increased (1398296_at, 1398249_at, 1390851_at, 1389551_at, 1389253_at, 1388924_at, 1388891_at, 1388756_at, 1388223_at, 1387977_at, 1387783_a_at, 1387740_at, 1387636_a_at, 1387183_at, 1387022_at, 1386927_at, 1386885_at, 1386880_at, 1379361_at, 1375845_at, 1374475_at, 1373784_at, 1373564_at, 1372134_at, 1371976_at, 1371379_at, 1370870_at, 1370818_at, 1370397_at, 1370067_at, 1368934_at, 1368283_at, 1367937_at, 1367777_at, 1367694_at, 1367659_s_at) or decreased (1398362_at, 1391485_at, 1390172_at, 1390165_at, 1390115_at, 1389218_at, 1388742_at, 1388459_at, 1387913_at, 1382944_at, 1377375_at, 1376746_at, 1376709_at, 1376593_at, 1376140_at, 1375205_at, 1374493_at, 1374320_at, 1374266_at, 1373797_at, 1372308_at, 1371368_at, 1371202_a_at, 1370043_at, 1369973_at, 1369868_at, 1368698_at, 1368642_at, 1368497_at, 1368428_at, 1368304_at, 1368036_at, 1367905_at, 1367602_at, 1367601_at) 24 hr after a single dose (complete linkage method, Euclidean distance). A cluster consisting of 3 fibrates (CFB, WY, GFZ), benzbromarone (BBr) and aspirin (ASA) was identified on the left side.

suggest that a Kupffer cell secreting TNFα, IL-1, IL-2 and IL-6 (Decker, 1990) plays an important role in hepatocyte proliferation stimulated by peroxisome proliferators. Our present results that the expression changes of genes related to proliferation or apoptosis were rarely observed for the *in vitro* system could be due to the fact that the numbers of non-parenchymal cells (including Kupffer cells) in the culture were much less than that for *in vivo* liver. Our present results also support the aforementioned concept that the Kupffer cell (or its production of TNFα) is essential for the increase of proliferation and attenuation of apoptosis caused by peroxisome proliferators.

Many genes related to cellular morphogenesis, including extracellular matrix (ECM), were down-regulated exclusively *in vivo*. Ogata *et al.* (2002, 2004) reported that the increase in mRNA of collagen type I and type III in pressure-overloaded rat heart was reduced by the administration of fenofibrate and that the proliferation of cardiac fibroblast induced by endothelin-1 was inhibited by fenofibrate. It was also reported that ETYA, a PPARα agonist, reduced the mRNA contents of elastin, tropoelastin and α-smooth muscle actin in neonatal rat lung fibroblast (McGowan

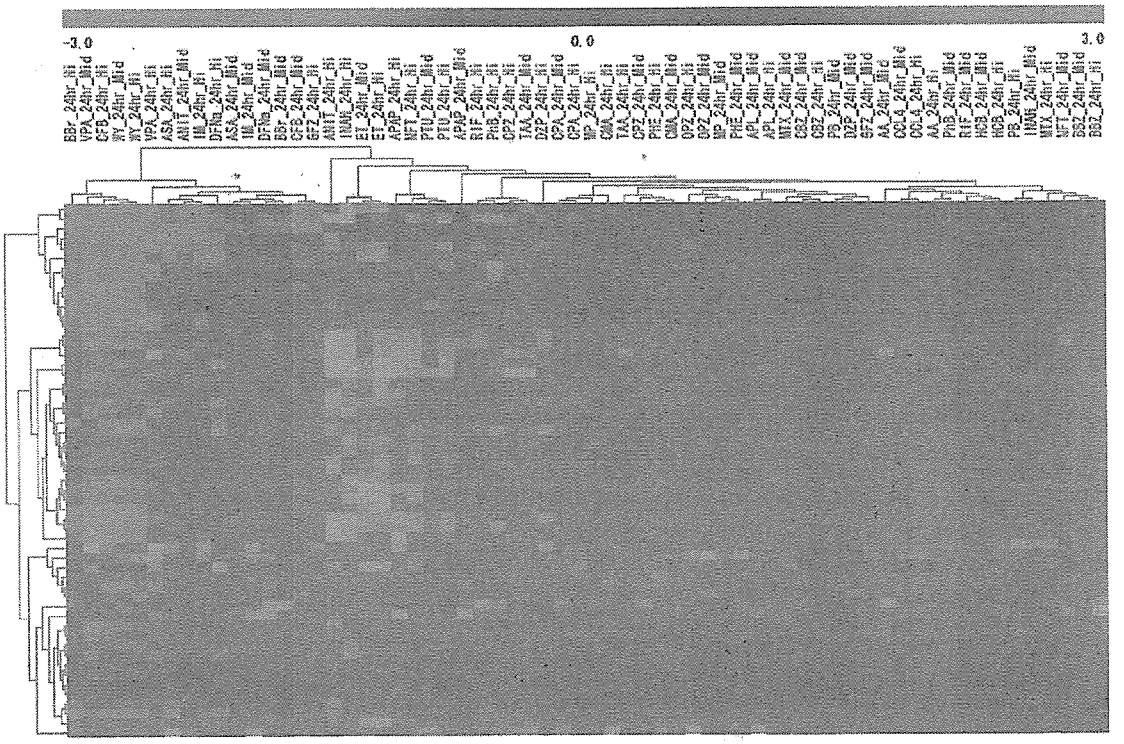

Fig. 4. A heat map view of the gene expression profile for the 32 compounds *in vitro*. Hierarchical clustering analysis of the compounds (middle & high concentrations, 24 hr of exposure) was conducted using the genes that were increased (1398310_at, 1394194_x_at, 1390383_at, 1390370_at, 1389253_at, 1388924_at, 1388756_at, 1388644_at, 1388211_s_at, 1388108_at, 1387783_a_at, 1387740_at, 1386946_at, 1386927_at, 1386901_at, 1386885_at, 1386880_at, 1383205_at, 1379361_at, 1377037_at, 1376076_at, 1374556_at, 1374478_at, 1374265_at, 1370818_at, 1370436_at, 1370397_at, 1370355_at, 1370313_at, 1370310_at, 1370237_at, 1369150_at, 1369111_at, 1368934_at, 1368797_at, 1368669_at, 1368435_at, 1368283_at, 1368150_at, 1368034_at, 1368021_at, 1367950_at, 1367897_at, 1367836_at, 1367777_at, 1367742_at, 1367689_a_at, 1367672_at, 1367659_s_at) or decreased (1387246_at, 1375791_at, 1373261_at, 1369093_at, 1368798_at, 1368342_at) in 24 hr of exposure (complete linkage method, Euclidean distance). A cluster consisting of 3 fibrates (CFB,WY, GFZ), 3 NSAIDs (asprin [ASA], indomethacin [IM] and diclofenac sodium [DFNa]), valproic acid (VPA) and benzbromarone (BBr) was identified on the left side. Although the middle dose of ANIT belonged to the same cluster, its high dose did not.

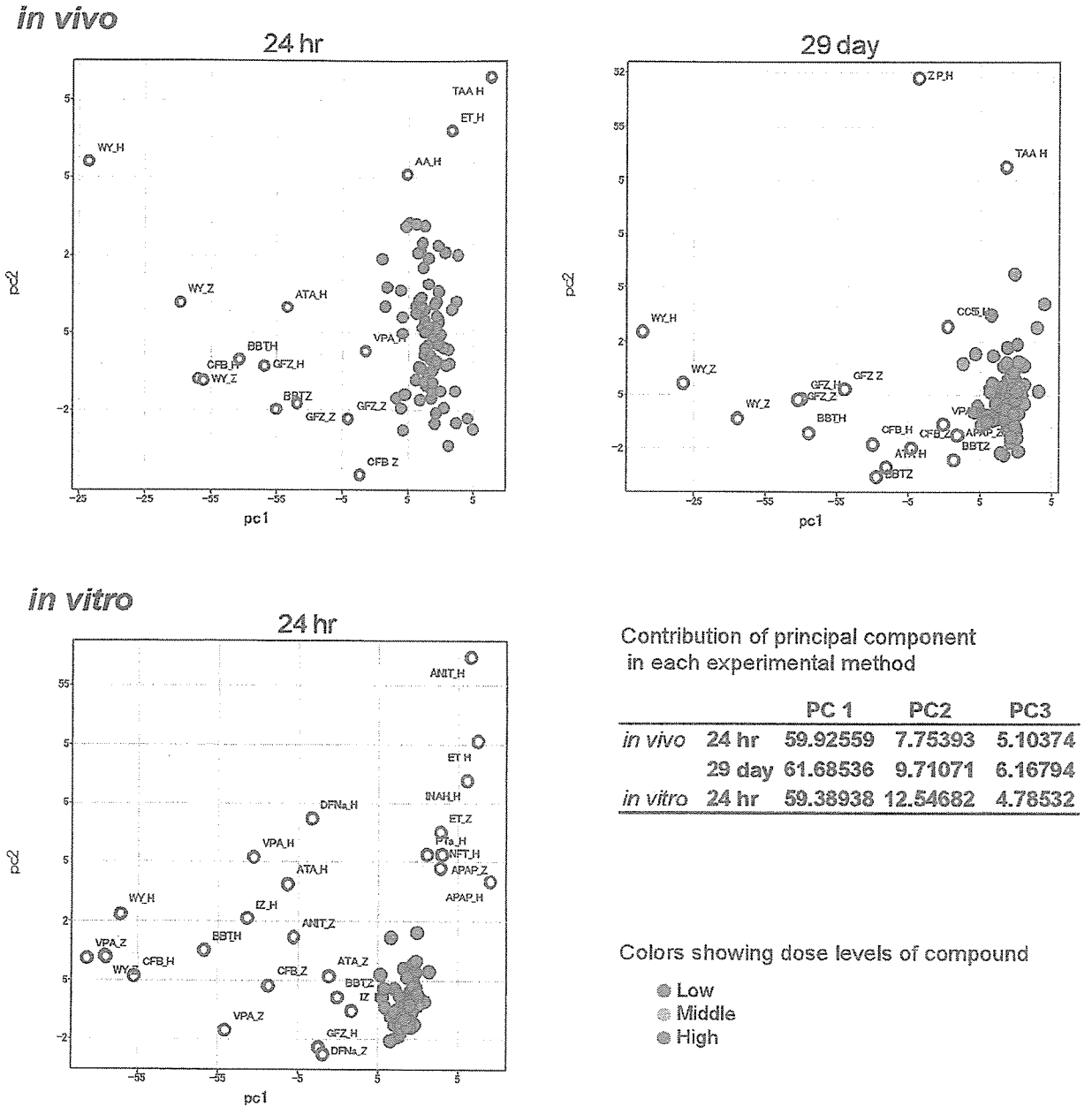

Fig. 5. Two-dimensional visualization of principal component analysis of the 32 compounds *in vivo* and *in vitro* using the commonly up-regulated 41 genes between *in vivo* and *in vitro*. The upper two panels show the *in vivo* studies and the lower left panel shows the *in vitro* study. Within each plot, the highest contributing factor to the overall variability is shown on the x-axis as the first component (PC1). The y-axis shows the second highest component (PC2). These plots show the principal separation of samples due to putative PPARα activity toward the negative direction on the x-axis, PC1. The contributions (%) of principal components for each experiment are summarized in the table on the lower right.

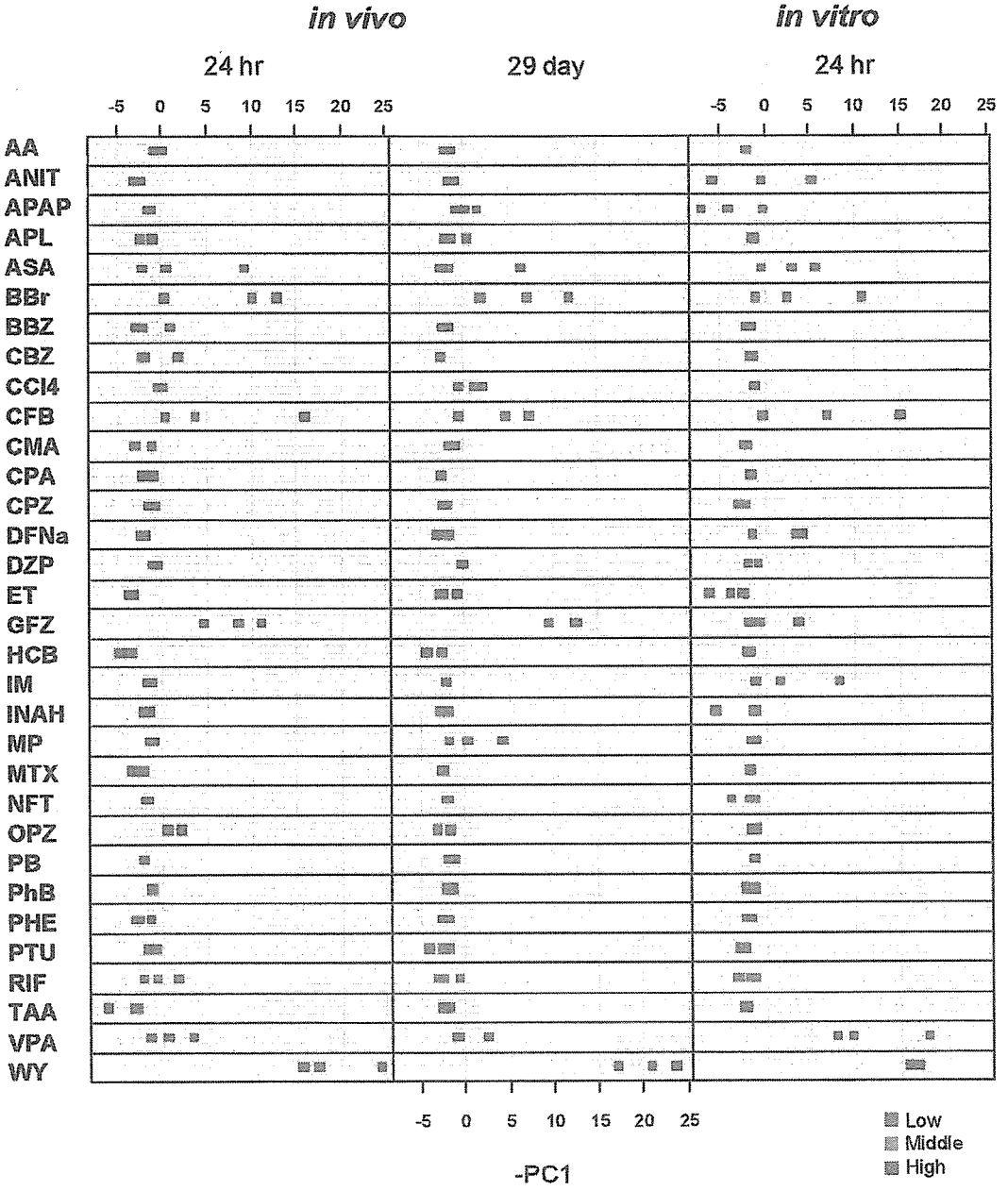

Fig. 6. Plotting of the first principal component (PC1) from the 32 compounds examined *in vivo* and *in vitro*. The left two panels show the *in vivo* studies, i.e., 24 hr after single dose and 24 hr after the last dose of the 28-day repeated dose, and the right panel shows the *in vitro* study, 24 hr exposure. The x-axis for each shows the negative value of PC1, as in Fig. 5, and the y-axis shows the compounds aligned alphabetically. The abbreviations of the compounds are found in Table 1. Note that the three fibrates (CFB, GFZ, WY), aspirin (ASA), and benzbromarone (BBr) show high values in this parameter for *in vivo* experiments and that diclofenac sodium (DFNa) and indomethacin (IM) show high values in addition to the drugs above.

et al., 1997). It appears that peroxisome proliferators also act on non-parenchymal cells other than Kupffer cells, and that they reduce the production of ECM in fibroblasts. Stellate cell, a hepatic non-parenchymal cell, possesses both of the characteristics of lipocyte and fibroblast, and its ability to produce ECM was found to be increased in liver fibrosis (Tanaka *et al.*, 1991). It is possible that production of ECM in stellate cells could be stimulated by peroxisome proliferators. Based on these ideas, it would be reasonable to conclude that the reduction of expression of genes related to ECM and cytoskeletons by peroxisome proliferators observed *in vivo* were not reproduced in the *in vitro* system, considering that these changes were a reflection of those occurring in non-parenchymal cells in liver. In the present study, relative liver weight was increased by a factor of two in peroxisome proliferators. The expression changes in genes classified to cell adhesion and cellular morphogenesis should have been associated with this obvious hypertrophy of the liver.

In the present study, down-regulation of genes classified to the immune response and coagulation was also *in vivo*-specific. PPARα is known to function as an inhibitory factor for inflammation, and PPARα agonists were reported to inhibit the expression of mRNA of fibrinogen, an acute phase protein (Kockx *et al.*, 1999; Corton *et al.*, 1998), and induction of fibrinogen gene by IL-6 (Gervois *et al.*, 2001). Moreover, it was also reported that WY-14643 inhibits induction of IL-6 and cyclooxygenase-2 by IL-1 in human aortic smooth muscle cells through inhibition of the translocation of NF-κB from the cytosol to the nucleus. It is thus expected that inhibition of the inflammatory response by PPARα agonists not only affects the hepatic parenchymal cell (producing acute phase proteins) but also affects the mechanism relating to non-parenchymal cells (including Kupffer cell) that releases inflammatory cytokines. It would be reasonable to conclude that the reason down-regulation of genes classified to the immune response and coagulation was observed *in vivo* but not *in vitro* was again due to the involvement of non-parenchymal cells. However, it might be due simply to the fact that the basal level of the mRNAs of these genes was down-regulated during our culture condition, since there was a report that PPARα agonist could inhibit the expression of fibrinogen mRNA by IL-6, using human hepatocyte culture (Gervois *et al.*, 2001).

As discussed above, the profiling of *in vivo* data represents gene expression in multiple cellular populations, whereas the profiling of *in vitro* data is focused on gene expression of hepatic parenchymal cells. The advantage of the *in vitro* system is that the direct effects of chemicals on hepatic parenchymal cells can be assessed, and in certain cases, the sensitivity and specificity of the test can be improved by eliminating noise due to gene expression of non-parenchymal cells. On the other hand, the *in vitro* system has an apparent disadvantage when indirect toxicity to parenchymal cells via non-parenchymal cells is involved or direct toxicity to non-parenchymal cells is involved.

In hierarchical clustering analysis of the *in vivo* data stored in our database, benzbromarone and aspirin were classified into the same cluster of the three peroxisome proliferators. It has been long known that benzbromarone is a PPARα ligand (Bichet *et al.*, 1990). As for aspirin, some NSAIDs including indomethacin, ibuprofen and fenoprofen, were reported to activate PPARα (Lehmann *et al.*, 1997), suggesting that aspirin belongs to PPARα agonists as well. In the hierarchical clustering of the *in vitro* data, two NSAIDs (indomethacin and diclofenac) and valproic acid were additionally located to the same cluster that included the three fibrates, and benzbromarone and aspirin. It has been reported that valproic acid induced the increase of liver weight and the activation of β-oxidation in rodents, suggesting that the drug has some PPARα agonist-like activity (Horie and Suga, 1985). Although the middle dose of ANIT belonged to the same cluster, its high dose showed a quite different profile. There is no report suggesting a relationship between ANIT and PPARα so far. One possibility is that ANIT is a potential PPARα agonist, and inconsistent results at high concentrations showed that cytotoxicity overwhelmed the inducing effects. At the middle dose of ANIT, various genes related to lipid metabolism (including β-oxidation), e.g., Acaa1, Acaa2, Cpt1a, Cpt1b, Pdk4, Ehhadh, Hmgcs2, Mte1, Cyp4a14, Cyp4b1, Cyp8b1, and Angptl4 were up-regulated more than twice of control *in vitro*. It would be interesting to examine the direct effect of ANIT on PPARα. It should be considered that any expression changes in β-oxidation-related genes do not necessarily indicate the direct involvement of PPARα.

The reason why the *in vitro* system was more sensitive than that of *in vivo* for detecting PPARα agonist-like activity is the high concentration of the drugs *in vitro*. In the standard protocol in our project, the maximal dose of the drugs *in vivo* is set to the level which the animals can tolerate for 28 days of repeated administration, while that for *in vitro* is independently determined according to the direct cytotoxicity of the cul-

tured hepatocytes. Therefore, in the case of chemicals causing severe toxicity to organs other than the liver, the practical concentration around the hepatocyte becomes much lower *in vivo* than *in vitro*. Since the main lethal cause in the case of NSAIDs is intestinal perforation, the doses employed were relatively low compared with that needed to elicit PPARα activation *in vivo*, and actually, a reduction of plasma lipid was barely observed. The PPARα activity of aspirin could possibly have been detected because its ulcerogenicity to intestine is much lower than that of the other NSAIDs.

One of the aims of the present project is the prediction of *in vivo* effects from *in vitro* experiments that have the advantages of saving chemicals, cost, and time. In the case of PPARα agonists, we could not find any common genes in down-regulated ones between *in vivo* and *in vitro*. On the other hand, 41 genes up-regulated *in vitro* were also up-regulated *in vivo*. We considered these as useful markers to predict PPARα activity *in vivo* from *in vitro*, and applied them to PCA. As shown in Fig. 5, PC1 appeared to have a PPARα agonist-like attribute, and 32 chemicals were aligned by this parameter in Fig. 6. This presentation conveniently identifies potential PPARα agonists both *in vivo* and *in vitro*. We are now incorporating this computing module into our toxicity prediction system and it will be useful in identifying other properties when appropriate marker genes are extracted.

In conclusion, our database efficiently works to classify a certain category of drugs (PPARα agonist in the present case) based on gene expression profiling. For these data, the gene expression profile *in vitro* is useful and sensitive to the direct toxicity of the chemicals in hepatic parenchymal cells, whereas indirect toxicities mediated by other cells or secondary toxicity due to pathophysiological changes such as blood pressure or inflammation in other organs might be overlooked. In order to predict *in vivo* effects from the *in vitro* system, it is important to identify genes commonly mobilized *in vivo* and *in vitro*. The scoring system (using the principal component that largely contributes the target effect) in the present study appeared to be quite useful and convenient to identify compounds with target activity among the ones stored in our database.

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GENE EXPRESSION PROFILE IN LIVER OF DIFFERING AGES OF RATS AFTER SINGLE ORAL ADMINISTRATION OF ACETAMINOPHEN

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ABSTRACT — In order to verify the influence of the rat age on hepatotoxicity, male Sprague-Dawley rats of 6 (young) and 12 (adult) weeks of age were orally administered acetaminophen (APAP), isoniazid (INH), or carbon tetrachloride (CCl4). Liver samples were obtained in a time-course manner, and changes in gene expression examined by an Affymetrix GeneChip. APAP caused more prominent hepatic injury with respect to pathology and blood biochemistry in adults than in young rats, whereas no obvious age-related differences were observed in INH- or CCl4-treated rats. Comparing gene expression in control rats, CYP3A13 was higher and GSTY2c was lower in adults, suggesting that production of the active metabolite of APAP is higher and its detoxification is lower in adults. The total amount of glutathione and total SH in rat liver was found to be higher in adult rats whereas the extent of its reduction by APAP was larger in adults. A detailed analysis of genes showing age-related differences revealed that some of them were different not in their extent but in their time course, i.e., the stress responses occurred earlier in the young than in the adult, resulting in a difference at 24 hr after dosing. These results suggest that the age-related difference in toxicity would be attributed to a higher expression of CYP3A13, producing the active metabolite of APAP as well as the lower expression of the detoxification enzyme, GSTY2c, in adult rats. Furthermore, these differences affect the time course of APAP toxicity. The present study clearly depicts the advantage of the multi-time, multi-dose protocol employed in our project for analyzing the mechanism of toxicity by gene expression profiling.

KEY WORDS: Toxicogenomics, Acetaminophen, Hepatotoxicity, Age-related difference, Rat

INTRODUCTION

The Toxicogenomics Project is a 5-year collaborative project by the National Institute of Health Sciences (NIHS) and 17 pharmaceutical companies in Japan which started in 2002 (Urushidani and Nagao, 2005). Its aim is to construct a large-scale toxicology database of transcriptome for the prediction of toxicity of new chemical entities in the early stage of drug development. About 150 chemicals, mainly medical compounds, have been selected, using both *in vitro* studies with primary hepatocyte of rat and human, and *in vivo* studies in rat. Of these, the standard protocol of *in vivo* study in TGP consists of a single administration test and a repeated administration test with multi-time and multi-dose. The gene expression of the liver is comprehensively analyzed by using Affymetrix GeneChip® with the traditional toxicity parameters. Although the ages of the rats were varied in a time-course sacrifice between 6 to 10-weeks old, we did not previously know the age-difference effect in the susceptibility of hepatotoxicity. In starting of TGP, we

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conducted an exploratory study to clarify whether the age-related difference was present in hepatotoxicity by using the first 3 chemicals in the TGP database, i.e., APAP, isoniazid (INH), and carbon tetrachloride (CCl4). Of these 3 chemicals, APAP alone showed a significant age-related difference in hepatotoxicity. In this report, we focus on the age-difference in susceptibility of APAP hepatotoxicity and elucidate the cause of the difference.

MATERIALS AND METHODS

Chemicals

APAP, INH, and CCl4 were purchased from Sigma Co. Ltd., (St. Louis, MO). All other chemicals and reagents used in the present study were of HPLC or analytical grade and are commercially available.

Animal treatment

Male Sprague-Dawley rats were purchased from Charles River Japan Inc., (Kanagawa, Japan) at 5-weeks or 11-weeks of age. After a 7-day quarantine and acclimatization period, the animals were divided into groups of 5 using a computerized stratified random grouping method based on body weights for each age. On the administration day, the animals were 6 and 12 weeks-old, respectively. The animals were individually housed in stainless-steel cages in a room that was lighted for 12 hr (7:00-19:00) daily, ventilated with an air-exchange rate of 15 times per hour, and maintained at 21 to 25°C with a relative humidity of 40 to 70%. Each animal was allowed free access to water and pellet food (CRF-1, sterilized by radiation, OrientalYeast Co., Japan).

Five rats in each group were orally administered APAP or INH by suspending in 0.5% methylcellulose solution, and with CCl4 by dissolving in corn oil. The highest dose level for each administration was determined by a 1 week dose-finding study (data not shown) and subsequently the middle and low doses were determined, i.e., 50, 300, 1000 mg/kg for APAP, 10, 50, 200 mg/kg for INH, and 30, 100, 300 mg/kg for CCl4. Blood samples were obtained at 3, 6, 9, and 24 hr post-dose with a needle and a heparinized syringe from the abdominal artery under ether anesthesia. Plasma was obtained after centrifugation and stored below −20°C until use. Aspartate aminotansferase (AST), Alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were determined by COBAS MIRA plus autoanalyzer (Roche Diagnostics, Basel, SZ). After blood collection, the animals were euthanized by exsanguination from the abdominal veins and arteries under ether anesthesia. The liver samples were collected in 10% neutral buffered formalin at necropsy and embedded in paraffin, and then sectioned and stained with hematoxylin-eosin. Histopathological evaluation of the liver specimens was conducted by using light microscopy and graded as - (no change), +/− (minimal), + (slight), ++ (moderate), and +++ (severe).

Measurement of hepatic glutathione and SH contents

The pre-weighed (ca. 0.1 g each) liver samples were homogenized with 5% 5-sulfosalicylic acid (Sigma-Aldrich), and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was used for measurement of total glutathione (GSH) content in liver using the Total Glutathione Quantification Kit (Dojindo Laboratories) according to the manufacturer's instructions. In this kit, GSH oxidized by DTNB is reduced by glutathione reductase; namely, an enzyme recycling method is employed. We modified this method in order to measure the total free SH contents in the sample. In brief, the manufacturer's instructions were followed except that the glutathione reductase solution was replaced with a buffer solution and the dilution factor for the sample and GSH standard solutions was reduced ca. 1/5 − 1/10 fold. The reaction time was 10 min at room temperature and the measurement was done by a pseudo-end point method. The results were expressed as SH equivalent to the standard GSH per wet weight (g) in both measurements.

Microarray experiment

An aliquot of the sample (about 30 mg) was obtained from the left lateral lobe of the liver of each animal immediately after sacrifice, and put into RNA later® (Ambion, Austin, TX, USA) overnight for 4°C and then frozen. Homogenization was conducted by Mill Mixer (Qiagen) and zirconium beads. The purity of the RNA was checked by gel electrophoresis, and the 260/280 nm ratio was between 2.0-2.2. Total RNA was isolated using RNeasy kit by Bio Robot 3000 (Qiagen, Valencia, CA, USA).

Microarray analysis was conducted on 3 out of 5 samples for each group by using GeneChip®RAE230A probe arrays (Affymetrix, Santa Clara, CA, USA) containing 15923 probe sets. The procedure was conducted basically according to the manufacturer's instructions. Briefly, 5 μg of total RNA was used for the synthesis of cDNA with the Superscript Choice