PROFILING OF GENE EXPRESSION IN RAT LIVER AND RAT PRIMARY CULTURED HEPATOCYTES TREATED WITH PEROXISOME PROLIFERATORS

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ABSTRACT — The Toxicogenomics project has been constructing a large-scale database of about 150 compounds exposed to rat (single dose, 3, 6, 9, 24 hrs and repeated dose for 3, 7, 14, 28 days with 3 dose levels) and rat hepatocytes (2, 8, 24 hr with 3 concentrations) and data of transcriptome in liver using GeneChip, and the related toxicological measures are being accumulated. In the present study, the data of three ligands of peroxisome proliferator activated receptor α (PPAR α), i.e., clofibrate, WY-14643 and gemfibrozil in our database were analyzed. Many of the β -oxidation-related genes were commonly induced *in vivo* and *in vitro*, whereas expression changes in genes related to cell proliferation, apoptosis, were detected *in vivo* (single and repeated dose) but not *in vitro*. Changes in those related to the immune response, coagulation and the stress response were also detectable exclusively *in vivo*. Using the genes mobilized in two or three PPAR α agonists, hierarchical clustering was performed on 32 compounds stored in our database. In the profiling of an *in vivo* single dose, benzbromarone and aspirin were located in the same cluster of the three PPAR α agonists. The clustering of *in vitro* data revealed that benzbromarone, three NSAIDs (aspirin, indomethacin and diclofenac sodium) and valproic acid belonged to the same cluster of PPAR α agonists, supporting the reports that benzbromarone, valproic acid and some NSAIDs were reported to be PPAR α agonists. Using the genes commonly up-regulated both *in vivo* and *in vitro*, principal component analysis was performed in 32 compounds, and principal component 1 was found to be the convenient parameter to extract PPAR α agonist-like compounds from the database.

KEY WORDS: Toxicogenomics, Hepatotoxicity, Peroxisome proliferator

INTRODUCTION

The Toxicogenomics Project is a 5-year collaborative project conducted by the National Institute of Health Sciences (NIHS) and 17 pharmaceutical companies in Japan which started in 2002 (Urushidani and Nagao, 2005). In April 2005, some rearrangements were made and now the project is conducted by NIHS, the National Institute of Biomedical Innovation, and 15

pharmaceutical companies. Its aim is to construct a large-scale toxicology database of transcriptome for prediction of toxicity of new chemical entities in the early stage of drug development. About 150 chemicals, mainly medicinal compounds, have been selected, and the following are examined for each. The *in vivo* test using rats consists of a single administration test (3, 6, 9 and 24 hr with 4 dose levels including vehicle control) as well as a repeated administration test (3, 7, 14

and 28 days with 4 dose levels including vehicle control) and the data of body weight, general symptoms, histopathological examination of liver and kidney, and blood biochemistry are obtained from each animal. The gene expression in liver (kidney in some cases) is comprehensively analyzed using Affymetrix GeneChip. An *in vitro* test using rat and human hepatocytes is also carried out to accomplish the bridging between the species. By the time the present study was performed, more than 100 chemicals covering wide medication categories had been finished or were ongoing, and the whole data set of 32 compounds had been stored in the database ready for analysis. We have started the analysis with three fibric acids in the database, i.e., clofibrate, WY-14643 and gemfibrozil (ligands of peroxisome proliferator-activated receptor α , PPAR α). They have been extensively studied regarding their mechanism of toxicity, as we consider them excellent model cases for evaluating the quality of our database.

MATERIALS AND METHODS

Compounds

All compounds were of the highest grade obtainable from the suppliers listed in Table 1.

In vivo studies

Male Sprague-Dawley rats were purchased from Charles River Japan Inc., (Kanagawa, Japan) at 5-weeks of age. After a 7-day quarantine and acclimatization period, the animals were divided into groups of 5 animals using a computerized stratified random grouping method based on body weight for each age. The animals were individually housed in stainless-steel cages on a 12 hr light/dark cycle. Each animal was allowed free access to water and pellet food (CRF-1, sterilized by radiation, Oriental Yeast Co., Japan). The test compounds were suspended in 0.5% methylcellulose solution or corn oil. Animals were orally administered daily at three dose levels for 1, 3, 7, 14 and 28 days. The highest dose level for each was determined in a 1-week dose-finding study (data not shown), and 1/3 and 1/10 of that were set as middle and low doses, respectively. The dose levels are given in Table 1.

Blood samples were taken at 3, 6, 9, and 24 hr after single dosing and 24 hr after repeated dosing with a needle and a heparinized syringe from the abdominal artery of animals under ether anesthesia. Plasma biochemical assessments were conducted by using COBAS MIRA plus autoanalyzer (Roche Diagnostics,

Basel, SZ). After collecting the blood, the animals were euthanized by exsanguination from the abdominal veins and arteries under ether anesthesia. Livers were collected from each animal and weighed, then a portion (about 30 mg) of each left lateral lobe was put into RNeasy® (Ambion, Austin, TX, USA) for expression profiling. The remaining liver samples were fixed in 10% buffered formalin solution for routine histological processing. Paraffin sections were stained with hematoxylin and eosin for histopathological examination. The experimental protocols were reviewed and approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Health Sciences.

In vitro studies

Hepatocytes were isolated from 6-week-old male Sprague-Dawley rats under sodium pentobarbital (120 mg/kg, ip) by a modified two-step collagenase perfusion method. The liver was perfused via the portal vein for 10 min with divalent cation-free EGTA (0.5 mM)-supplemented HEPES buffered Hank's balanced salt solution followed by a 10-min perfusion with HEPES-buffered normal Hank's balanced salt solution containing soybean trypsin inhibitor (Sigma, T-2011, 0.05 g/L) and collagenase (WAKO 034-10533, 0.5 g/L) at a flow rate of 10 - 30 ml/min. Isolated cells were washed three times by 50 g for 1 min to obtain a parenchymal cell-enriched pellet. Hepatocytes were not used when their viability assessed by trypan blue exclusion was lower than 70%. The cells were seeded into collagen-coated six-well plates (BD BioCoat® Collagen I Cellware, BD Bioscience) at a density of 1×10^6 cells/well in 2 ml HMC Bulletkit medium (CAMBREX) supplemented with 10% fetal bovine serum. Following an attachment period of 3 hr, the medium was replaced and kept overnight before drug exposure at 37°C in an atmosphere of 5% CO₂. The test compounds were added to the medium directly or as a 1,000 $\times$ stock solution in dimethylsulfoxide. The highest concentration of each compound was determined in a pilot test based on cytotoxicity (ca. 20% release of lactate dehydrogenase) and 1/5 and 1/25 of that were set as middle and lowest concentrations (data not shown). After 2, 8 and 24 hr-exposure, the cells were dissolved with RLT buffer (Qiagen) and collected for expression profiling. GeneChip® analysis was performed in a duplicated manner for each time and concentration point.

Expression profiling

The livers were homogenized using Mill Mixer

PPAR on rat liver gene expression *in vivo* and *in vitro*.

(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 Gene-Chip®RAE230A probe arrays (Affymetrix, Santa Clara, CA, USA), containing 15923 probe sets. The

Table 1.

Compound	Abbreviation	<i>in vivo</i>		<i>in vitro</i>		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	Aldrich
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	Aldrich
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 μ g 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 PPAR α 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. 1C). 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

PPAR on rat liver gene expression *in vivo* and *in vitro*.

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 PPAR α 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 PPAR α 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 PPAR α ligands. Therefore, we reasoned that this negative PC1 score could be used to estimate the PPAR α 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 PPAR α activity between the compounds can be performed. All the PPAR α 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.

PPAR on rat liver gene expression *in vivo* and *in vitro*.**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 peroxi-somal	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 al-pha 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 syn-thase 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	Acs13	2.4	5.8	3.6
* 1371886_at	Carnitine acetyltransferase	Crat	9.7	17.4	2.5
1368426_at	carnitine <i>O</i> -octanoyltransferase	Crot	2.8	3.1	2.0
1387183_at	Carnitine <i>O</i> -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
<u>Peroxisomal Protein</u>					
* 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
<u>Carbohydrate metabolism</u>					
* 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
<u>Proliferation</u>					
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	Nap111	3.3	3.9	1.2
1370826_at	Nucleosome assembly protein 1-like 1	Nap111	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	Plekhhb1	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
<u>Growth factor</u>					
1369608_at	fibroblast growth factor 16	Fgf16	1.8	1.8	1.2
<u>Apoptosis</u>					
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
<u>Cellular morphogenesis</u>					
* 1368475_at	collagen-like tail subunit of asymmetric acetylcholinesterase	Colq	7.8	7.7	2.4

PPAR on rat liver gene expression *in vivo* and *in vitro*.

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 metabolism					
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)	
<u>Lipid mobilization</u>					
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
<u>Other lipid Methabolism related</u>					
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
<u>Carbohydrate methabolism</u>					
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
<u>Proliferation</u>					
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
<u>Transcription activation and repression</u>					
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
<u>Growth factor</u>					
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
<u>Apoptosis</u>					
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

PPAR on rat liver gene expression *in vivo* and *in vitro*.**Table 3.** Continued.

Probe ID	Gene Name	Gene Symbol	Vivo		Vitro ^{e)}
			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 metabolism					
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 β -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 TNF α 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 TNF α 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 elim-

inated, 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 TNF α inhibited spontaneous TGF β 1-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_at, 1387740_at, 1387636_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.

PPAR on rat liver gene expression *in vivo* and *in vitro*.

suggest that a Kupffer cell secreting TNF α , 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 TNF α) 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 PPAR α 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 (aspirin [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 PPAR α 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.

PPAR on rat liver gene expression *in vivo* and *in vitro*.

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. PPAR α is known to function as an inhibitory factor for inflammation, and PPAR α 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- κ B from the cytosol to the nucleus. It is thus expected that inhibition of the inflammatory response by PPAR α 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 PPAR α 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 PPAR α ligand (Bichet *et al.*, 1990). As for aspirin, some NSAIDs including indomethacin, ibuprofen and fenoprofen, were reported to activate PPAR α (Lehmann *et al.*, 1997), suggesting that aspirin belongs to PPAR α 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 PPAR α 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 PPAR α so far. One possibility is that ANIT is a potential PPAR α 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 PPAR α . It should be considered that any expression changes in β -oxidation-related genes do not necessarily indicate the direct involvement of PPAR α .

The reason why the *in vitro* system was more sensitive than that of *in vivo* for detecting PPAR α 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 PPAR α activation *in vivo*, and actually, a reduction of plasma lipid was barely observed. The PPAR α 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 PPAR α 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 PPAR α activity *in vivo* from *in vitro*, and applied them to PCA. As shown in Fig. 5, PC1 appeared to have a PPAR α agonist-like attribute, and 32 chemicals were aligned by this parameter in Fig. 6. This presentation conveniently identifies potential PPAR α 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 (PPAR α 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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