Table 1. List of compounds used in the present study.

Compound (abbreviation)	No. of mobilized genes (ANOVA, $p<0.05$)	Usage	Hepatotoxicity	Proposed toxicological action
alpha-naphthylisothiocyanate (ANIT)	1063	hepatotoxins	cholestasis	toxic metabolite can injure epithelial cells in biliary duct
amiodarone hydrochloride (AM)	640	antiarrhythmic, antianginal agents	phospholipidosis	oxidative stress by free radical
benzbromarone (BBr)	1720	uricosuric agents	hepatomegaly	peroxisome proliferators-like action
carbon tetrachloride (CCL4)	2262	hepatotoxins	hepatocellular carcinoma fatty liver	production of toxic metabolite that leads to peroxidative injury of membrane lipids and membrane perturbation
clofibrate (CFB)	1800	antilipemic agents	hypertrophy, hepatocellular carcinoma	peroxisome proliferator
coumarin (CMA)	1005	hepatotoxins	necrosis	formation of coumarin 3,4-epoxide
gemfibrozil (GFZ)	1943	antilipemic agents	hepatomegaly	peroxisome proliferator
isoniazid (INAH)	1482	antituberculous agents	necrosis	generation of a reactive metabolite from acetylhydrazine
methapyrilene (MP)	4910	antiallergic, hypnotic, sedative agents	carcinoma	induction of hepatic cell proliferation, lipid peroxidation of the liver
omeprazole (OPZ)	1441	antiulcer agents	elevation of serum enzyme	unknown
Phenobarbital (PB)	548	hypnotics, sedatives, anticonvulsants	hepatocellular tumor	induction of c-fos gene expression
propylthiouracil (PTU)	1544	antithyroid agents	elevation of serum enzyme	thyroxin synthesis inhibitor
sulfasalazine (SS)	860	antiinflammatory, antirheumatic, antiinfective agents	genotoxicity, carcinogenicity	antiinflammatoric, antibacterial actions, inhibition of production of cytokines
thioacetamide (TAA)	5712	hepatotoxins	carcinogenicity	hepatocarcinogen
Wy-14,643 (WY)	4307	hepatotoxins	carcinoma hepatomegaly	peroxisome proliferator

manufacturer's instructions. Microarray analysis was conducted on 3 out of 5 samples for each group by using GeneChip® RAE 230A probe arrays (Affymetrix, Santa Clara, CA, USA), containing 15,923 probe sets. The 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. Ten micrograms of 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 scanned by Gene Array Scanner (Affymetrix). The digital image files were processed by Affymetrix Microarray Suite version 5.0. Microarray image data were analyzed with GeneChip Operating Software (Affymetrix).

Statistical analysis

Plasma TG and food consumption results were expressed as means ± SD. They were analyzed by Bartlett test that evaluates the homogeneity of variance. If the variances were homogenous, ANOVA was applied. If the variances were heterogeneous, Kruskal-Wallis test was performed. When ANOVA resulted in a statistical difference between the groups, Dunnett test was applied. When Kruskal-Wallis test resulted in statistically different groups, Dunnett type mean rank test was performed. Identification of genes related to plasma TG decrease and gene expression data were analyzed by Welch ANOVA for the dose level and applied with Benjamini and Hochberg False Discovery Rate as a multiple-testing correction. In these tests, a significant level at $p<0.05$ was considered acceptable (Snedecor and Cochran, 1989).

GeneChip data were normalized by the global median normalization method using GeneSpring version 7 (Agilent Technologies Inc., Palo Alto, CA, USA). Probe sets with present or marginal call in at least 1 of 48 samples (N=3 for 4 time points and 4 dose levels) were selected. Principal component analysis (PCA) of the GeneChip data was performed using Spotfire DecisionSite ver. 8.2 (Spotfire, Somerville, MA, USA).

Pathway and Gene Ontology (GO) analysis

The identified probe sets were subjected to analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and GO analysis by DAVID (Database for Annotation, Visualization, and Integrated Discovery; http://apps1.niaid.nih.gov/david/) using Fisher's exact test (Dennis *et al.*, 2003). Level 5 analysis was adopted.

RESULTS

Plasma biochemistry and food consumption

Rats were treated with each compound (ANIT, AM, BBr, CCL4, CFB, CMA, GFZ, INAH, MP, OPZ, PB, PTU, SS, TAA and WY) by gavage for up to 4 weeks. The TG concentration and food consumption results are shown in Fig. 1. All compounds that we selected showed a TG-decreasing property during the dosing period, while their effects on food consumption differed. In BBr-, CMA-, OPZ- and SS-treated animals, food consumption transiently dropped in the first 3 days while it returned to normal thereafter. In AM-, INAH- and TAA-treated rats, food consumption was depressed throughout the dosing period. PTU-treatment decreased food consumption from day 15 whereas MP-treatment decreased at day 29. ANIT-, CFB-, CCL4, GFZ-, PB-, and WY-treatment affected the food intake only slightly.

Identification of genes related to the plasma TG-decreasing property

After filtering the probe sets (with present or marginal call in at least 1 of 48 samples), Welch ANOVA with multiple testing correction was applied to each compound to extract significantly mobilized probe sets. As shown in Table 1, the numbers of extracted probe sets varied among the compounds, from 640 (AM) to 5,712 (TAA). We then selected the probe sets that were commonly changed from more than 10 out of 15 compounds, and 218 probe sets were obtained. KEGG pathway analysis revealed that pathways related to "proteasome", "fatty acid metabolism", "amino acid metabolism", and "bile acid biosynthesis" were mainly altered in liver treated with these compounds (Table 2). GO analysis showed that some groups (other than cellular lipid metabolism) related to xenobiotics metabolism, such as carboxylic acid metabolism, and glucuronosyltransferase and aldehyde dehdrogenase activity, were also affected in liver (Table 3).

Principal component analysis (PCA)

To assess the expression profiles of identified

probe sets, PCA with 218 probe sets for data of all 15 compounds were performed. As shown in Fig. 2, each sample was separated from control according to the expression of these probe sets. Each sample tended to have a smaller value in the component PC 1 as the treatment period increased, and each was distinctly separated into either direction in component PC 2. It should be noted that liver treated with WY, BBr, GFZ and CFB had a relatively large principal component PC 2, while PTU, OPZ, PB, TAA, MP, SS and CMA showed small PC 2. Liver treated with AM for 28 days had a near zero value in PC 2. Some compounds such as ANIT, CCL4 and INAH did not change their position very much.

To elucidate which genes contributed more for each principal component, eigenvector values of each probe sets were examined. As shown in Table 4, "vanin 1", "similar to Aig1 protein", "CD36 antigen", and "cell death-inducing DNA fragmentation factor, alpha subunit-like effecter A" had large eigenvector values for PC 2. Meanwhile, "glutathione *S*-transferase A5, aldehyde dehydrogenase family 1, member A1", "liver UDP-glucuronosyltransferase, phenobarbital-inducible form", "carbonic anhydrase 2" and "cytochrome P450, family 2, subfamily b, polypeptide 15" had small values for PC 2 (Table 5). "Aldehyde dehydrogenase family 1, member A1", "glutathione *S*-transferase A5", "vanin 1", "carboxylesterase 2 (intestine, liver)" and "CD36 antigen" had smaller eigenvector values for PC 1 (Table 6).

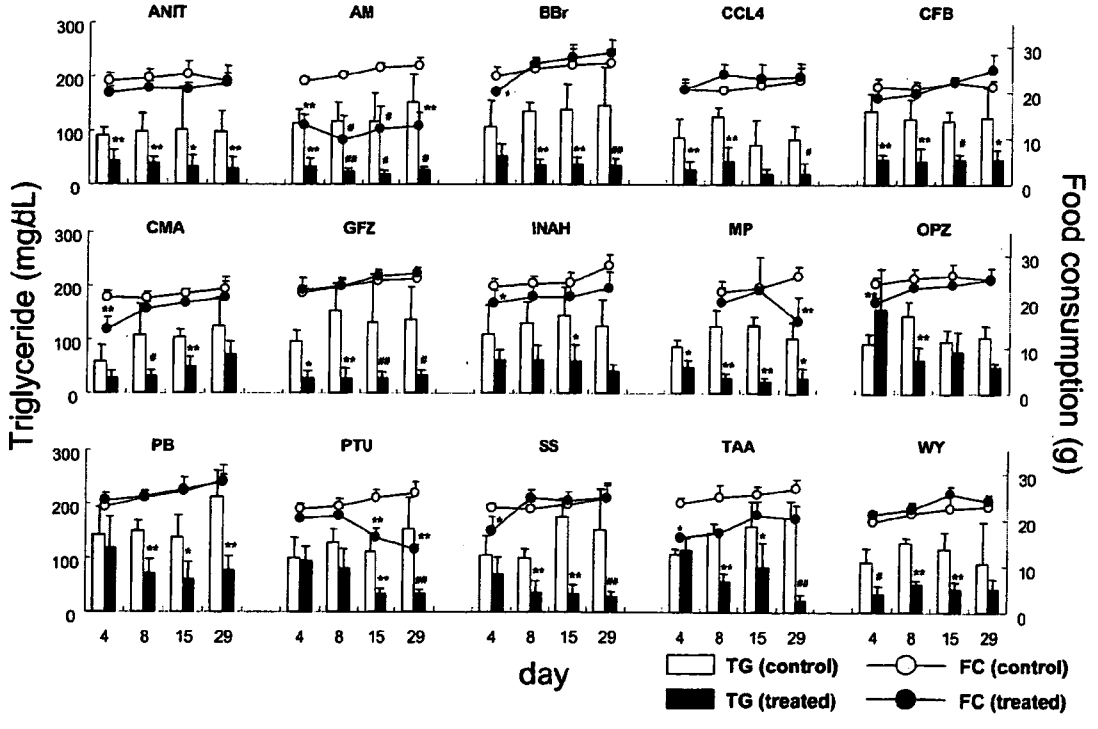

Fig. 1. Effects of TG-decreasing compounds on food consumption and plasma TG level.
Six-week-old male Sprague-Dawley rats were gavaged with each compound for 3, 7, 14 or 28 days, and they were sacrificed 24 hr after the last dosing. Food consumption was recorded every 4 days during dosing and blood samples were collected at sacrifice. Plasma TG concentrations were estimated as described in materials and methods. For simplicity, the data of the highest dose were presented for each compound. Open (control) and filled (treated) columns represent plasma TG concentration. Open (control) and filled (treated) circles represent food consumption. Values are expressed as mean ± SD of 5 rats each for each time and compound. Significant difference from control rat: (*p<0.05, **p<0.01: Dunnett test, #p<0.05, ##p<0.01: Dunnett type mean rank test). MP-food consumption on day 4 was not determined.

As PC 2 was considered to be indirectly related to cholesterol metabolism (see DISCUSSION), three compounds with the smallest values for both PC 1 and PC 2, i.e., PB, OPZ and PTU were selected, and their effects on plasma cholesterol level are shown in Fig. 3. It is obvious from the figure that OPZ and PTU, which had smaller PC 2 values than PB, significantly increased plasma cholesterol.

DISCUSSION

Lowering of the plasma TG level is often observed in rat toxicity studies. It would be useful to elucidate its mechanism not only for safety evaluation of drugs but also for finding seeds of lipid-lowering agents. In the course of our trials to identify useful toxicity biomarkers from our large-scale database, we selected plasma TG decrease as a toxicological phenotype, and picked up 15 such compounds from 40 (the number of compounds available at the time when the

Table 2. KEGG pathways of the identified 218 probe sets related to plasma TG.

Term	Count	p value
proteasome	8	1.66E-06
fatty acid metabolism	10	4.61E-06
tryptophan metabolism	9	3.44E-05
bile acid biosynthesis	4	0.00997
histidine metabolism	4	0.00997
propanoate metabolism	4	0.0224
fatty acid biosynthesis (path 2)	3	0.0236
pyruvate metabolism	4	0.0289
valine, leucine and isoleucine degradation	4	0.0289
nitrogen metabolism	3	0.0364
arginine and proline metabolism	4	0.0492

Statistically extracted 218 probe sets related to plasma TG were categorized by KEGG pathway. The terms with significantly high counts (Fisher's exact test; $p<0.05$, calculated by DAVID: http://apps1.niaid.nih.gov/david/) are presented in the table.

Table 3. Gene ontology of the identified 218 probe sets related to plasma TG.

Category	Term	Counts	p value
Biological process	carboxylic acid metabolism	15	5.25E-06
	electron transport	14	1.83E-04
	fatty acid metabolism	8	6.04E-04
	response to chemical substance	7	0.00164
	cellular lipid metabolism	10	0.0115
	amino acid metabolism	6	0.0154
	protein catabolism	11	0.0492
Molecular function	glucuronosyltransferase activity	6	5.01E-05
	aldehyde dehydrogenase activity	3	0.0249
Cellular component	endoplasmic reticulum	15	1.25E-05
	microsome	9	1.93E-04
	micorobody	5	0.00726
	peroxisome	5	0.00726
	proteasome complex (SENSU EUKARYOTA)	4	0.00829
	proteasome core complex (SENSU EUKARYOTA)	4	0.00829
	mitochondrion	15	0.0103

Statistically extracted 218 probe sets related to plasma TG were categorized by gene ontology. The terms with significantly high counts (Fisher's exact test; $p<0.05$, calculated by DAVID: http://apps1.niaid.nih.gov/david/) are presented in the table.

present analysis was performed). Since our database has a broad variety of compounds for hepatic toxicity, each compound has different properties for drug efficacy and toxicity (Table 1).

As shown in Fig. 1, the effects of these compounds on food consumption were not similar. It is well known that plasma TG levels depend largely upon nutrition, and the results suggested that there should have been multiple factors other than a simple general toxicity. To clarify the multiple mechanisms in plasma TG homeostasis, we extracted 218 probe sets as commonly changed genes in more than 10 out of 15 compounds (commonly changed in two-thirds of the compounds). The fact that there were no probe sets commonly changed in all the compounds (data not shown) also suggested that there are multiple factors involved in the mechanism of plasma TG decrease.

KEGG pathway analysis suggested that the proteasome-, fatty acid metabolism-, tryptophan metabolism-, bile acid biosynthesis-, and histidine metabolism-related pathways were involved (Table 2). Since TG is an important source of energy, it is understood

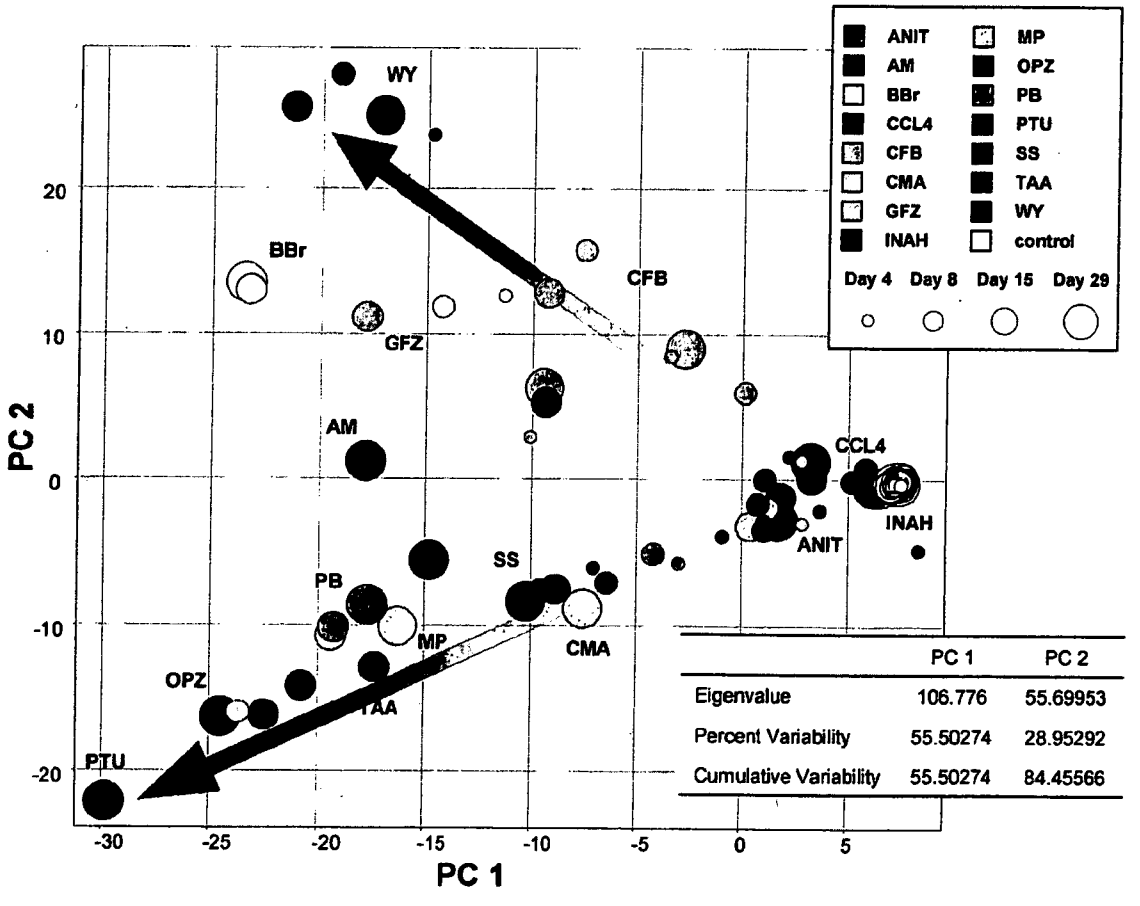

Fig. 2. Principal component analysis of gene expression profiles.
PCA of gene expression profiles was performed using identified 218 probe sets for the data of the highest dose of 15 compounds at various time points. The selection of the 218 probe sets related to plasma TG decrease is described in materials and methods. The values used in the analysis were the normalized signal values. Each spot represents the mean of the normalized gene expression value. The abbreviation for each drug is summarized in Table 1. The eigenvalue, percent variability, and cumulative variability for PC 1 and PC 2 are shown in the table on the lower right corner.

that pathways related to energy homeostasis such as fatty acid synthesis and amino acid metabolism were affected. GO analysis showed that the carboxylic acid metabolism- and glucuronosyltransferase activity-related genes were also affected by TG-decreasing compounds (Table 3). UDP-glucuronosyltransferase, one of the extracted genes, is of major importance in conjugation and subsequent elimination of potentially toxic xenobiotics (Bock *et al.*, 1990). PB and OPZ, which lowered plasma TG level in the present study, were previously reported to have the ability to induce this enzyme (Bock *et al.*, 1990; Masubuchi *et al.*, 1997). These results indicated that the xenobiotics metabolism pathway might have a role in plasma TG decrease.

To examine the basis of the gene expression profile of each sample, we performed PCA on hepatic gene expression profiles of the 15 compounds. In PCA, three clear clusters were identified (Fig. 2). All compounds except ANIT, CCL4 and INAH were uniformly dispersed into smaller PC 1 with either large or small PC 2, i.e., WY, BBr, GFZ and CFB were dispersed into small PC 1 with large PC 2, whereas PTU, OPZ, PB,MP, TAA, SS and CMA into small PC 1 with small PC 2. AM was exceptionally found in the middle position of PC 2.

In PC 2, "vanin 1", "similar to Aig1 protein", "CD36 antigen", "cell death-inducing DNA fragmentation factor, and alpha subunit-like effecter A (CIDEA)" had larger eigenvector values (Table 4), meaning that these genes made a great contribution to increasing PC 2 values in each liver sample. Vanin 1 is

Table 4. Top five probe sets with the largest eigenvector values for second principal component in the PCA shown in Fig. 2.

Ranking	Probe ID	PC 2	Description
1	1389253_at	0.610	Vanin 1 (predicted)
2	1375845_at	0.291	Similar to Aig1 protein
3	1367689_a_at	0.230	CD36 antigen
4	1389179_at	0.180	Cell death-inducing DNA fragmentation factor, alpha subunit-like effecter A (predicted)
5	1386901_at	0.153	CD36 antigen

Each eigenvector value was calculated by Spotfire DecisionSite.

Table 5. Top five probe sets with the smallest eigenvector values for second principal component in the PCA shown in Fig. 2.

Ranking	Probe ID	PC 2	Description
1	1371089_at	−0.526	Glutathione *S*-transferase A5
2	1387022_at	−0.187	Aldehyde dehydrogenase family 1, member A1
3	1370698_at	−0.113	Liver UDP-glucuronosyltransferase, phenobarbital-inducible form
4	1386922_at	−0.0742	Carbonic anhydrase 2
5	1371076_at	−0.0586	Cytochrome P450, family 2, subfamily b, polypeptide 15

Each eigenvector value was calculated by Spotfire DecisionSite.

Table 6. Top five probe sets with the smallest eigenvector values for first principal component in the PCA shown in Fig. 2.

Ranking	Probe ID	PC 1	Description
1	1387022_at	−0.630	Aldehyde dehydrogenase family 1, member A1
2	1371089_at	−0.491	Glutathione *S*-transferase A5
3	1389253_at	−0.370	Vanin 1 (predicted)
4	1368905_at	−0.163	Carboxylesterase 2 (intestine, liver)
5	1367689_a_at	−0.162	CD36 antigen

Each eigenvector value was calculated by Spotfire DecisionSite.

reported to be involved in lymphocyte migration in cell adhesion during colonization of the thymus by hematopoietic precursor cells, and also has pantetheinase activity (Pitari *et al.*, 2000). Though vanin 1 is not reported to play a role in lipid metabolism so far, it was in fact reported to be an inducible gene by PPARα (Yamazaki *et al.*, 2002).

CD36 antigen (fatty acid translocase (FAT)) is involved in regulating the uptake of fatty acid across the plasma membrane (Bonen *et al.*, 2004) and also reported to be induced by PPARα agonists in liver (Motojima *et al.*, 1998). Moreover, it has been reported that CD36 plays a role in plasma TG homeostasis via modulation of LPL activity (Goudriaan *et al.*, 2005). Thus, an increase of this gene expression would be one of the mechanisms of plasma TG level decrease in the corresponding animal.

A previous report described that CIDEA-null mice presented TG decrease in fasting condition compared with wild type (Zhou *et al.*, 2003). Because expression of the CIDEA gene was up-regulated in liver treated with TG-decreasing compounds in both principal components (data not shown), it seemed to be a compensatory reaction. In addition, compounds with large PC 2 values, i.e., WY, GFZ, CFB and BBr, are reported to be agonists of PPARα (Kunishima *et al.*, 2003; van Raalte *et al.*, 2004). Activation of PPARα induces hepatic gene expression by β-oxidation of fatty acid and hydrolysis of TG-rich lipoprotein via activation of peripheral lipoprotein lipase (LPL) (van Raalte *et al.*, 2004). In addition to activation of fatty acid β-oxidation, PPARα inhibits de novo fatty acid synthesis in liver (Schoonjans *et al.*, 1996). In peripheral tissue, it has also been reported that gene expression of APOC3 (a natural inhibitor of LPL activity) decreases by PPARα and subsequently LPL activity is increased (Staels *et al.*, 1995). Taken together, these results suggest that the trend of increasing PC 2 is linked to lowering plasma TG level via PPARα activation.

"Glutathione *S*-transferase A5", "aldehyde dehydrogenase family 1, member A1", "liver UDP-glucuronosyltransferase, phenobarbital-inducible form", "carbonic anhydrase 2", "cytochrome P450, family 2, subfamily b, polypeptide 15" had smaller eigenvector values for PC 2 (Table 5). They (except carbonic anhydrase 2) have been reported to be constitutive androstane receptor (CAR)-inducible genes (Kakizaki *et al.*, 2003). Although there is no report that PTU, OPZ, TAA, MP, SS and CMA activate CAR so far, the present results suggested that these compounds could

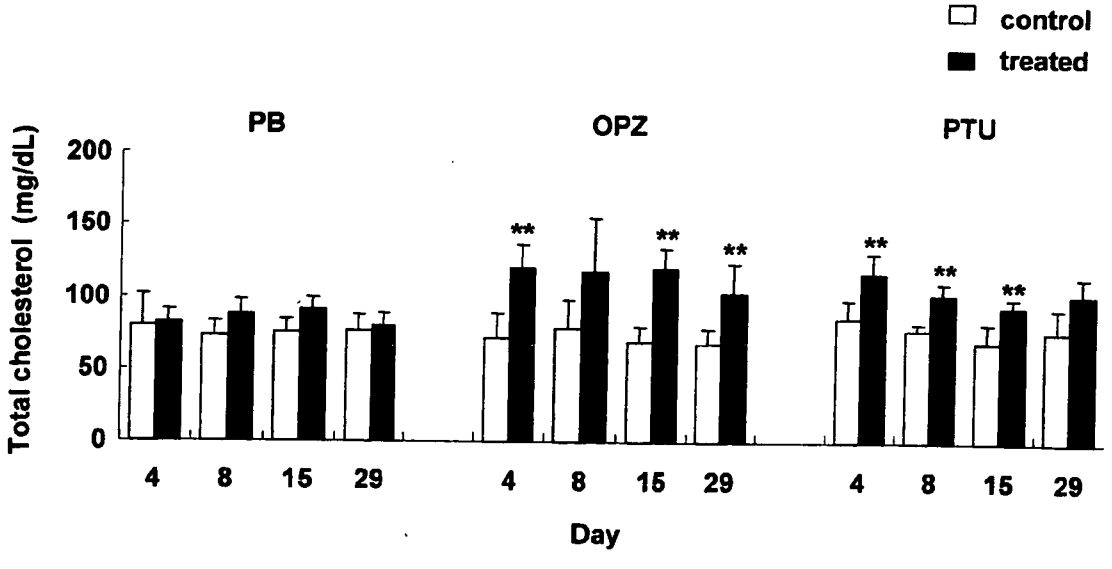

Fig. 3. Effects of PB, OPZ, PTU on plasma total cholesterol level.
Plasma total cholesterol concentrations were estimated as described in materials and methods. Open (control) and filled (treated) columns represent total plasma cholesterol concentration. Values are expressed as mean ± SD of 5 rats for each time and compound. Significant difference from control rat: (*$p<0.05$, **$p<0.01$: Dunnett test).

induce the same xenobiotics metabolizing enzymes as PB does (Kakizaki *et al.*, 2003). Meanwhile, these enzymes (such as UDP-glucuronosyltransferase) are able to metabolize not only xenobiotics but also serum thyroid hormone (TH) (Qatanani and Moore, 2005), which has a role in comprehensive regulation of energy metabolism (Weiss *et al.*, 1998). It has been reported that rats with hypothyroidism induce and activate peripheral lipoprotein lipase (LPL), the key enzyme in hydrolysis of TG-rich lipoproteins, such as chylomicron and VLDL (Kern *et al.*, 1996; Ong *et al.*, 1994). In fact, it has also been reported that PB, PTU and OPZ are able to alter blood TH level (Masubuchi *et al.*, 1997; De Sandro *et al.*, 1991). CAR is also activated by caloric restriction (Maglich *et al.*, 2004). With depletion of food, the body needs to lower its energy requirement. It might be possible that CAR was activated in rats whose food consumption was decreased (Fig. 1). Thus, identified probe sets might be indirectly related to plasma TG decrease via reduction of blood TH by CAR activation in at least two ways. Accordingly, the feature in decreased PC 2 value could be related to lowering plasma TG level via CAR activation. Moreover, it has been reported that TH is a physiological regulator of cholesterol metabolism (Weiss *et al.*, 1998; Gullberg *et al.*, 2000, 2002; Hashimoto *et al.*, 2006; Ness and Chambers, 2000) and hypercholesterolemia is found in patients with hypothyroidism (Diekman *et al.*, 2000). It was also noted in the present study that total cholesterol levels in OPZ and PTU with smaller PC 2 values were found to be increased (Fig. 3). These indirect evidences also supported the assumption that TH levels were involved in plasma TG reduction.

As shown in Table 6, genes with smaller eigenvector values for PC 1 such as "aldehyde dehydrogenase A5", "glutathione *S*-transferase A5", "vanin 1", "carboxylesterase 2" and "CD36 antigen" were important genes that contribute to shift each sample to either direction of PC 2. It appears that PC 1 shows a lowering of plasma TG level via either or both of two mechanisms (PPARα and/or CAR activation). Interestingly, AM had small PC 1 values, while near zero in PC 2. This result is supported by previous reports that AM induces the expression of PPARα target genes (McCarthy *et al.*, 2004) and lowers serum TH level (De Sandro *et al.*, 1991). Thus, these two directions might have been balanced in the case of the plasma TG reduction by AM.

It was previously reported that PPARα was also activated by fasting (Kersten *et al.*, 1999; Lee *et al.*, 2004; Leone *et al.*, 1999), as well as by CAR. An interesting question is why these 218 probe sets classify the compounds with various pharmacological and toxicological properties based on their different mechanisms. Genes with the largest or smallest eigenvector values for PC 2 such as vanin 1 and glutathione *S*-transferase A5 are related to each nuclear receptor, PPARα and CAR, respectively, rather than to plasma TG homeostasis. CD36, which was involved in regulating the uptake of fatty acid, mainly contributed to PC 1. This means that CD36 could be an important gene directly related to plasma TG level, while its background mechanisms are represented by genes like vanin 1 or glutathione *S*-transferase A5. Thus, we considered that these probe sets work to classify the compounds by PCA, based on each nuclear receptor-mediated TG-lowering mechanism.

In the present study, some compounds such as ANIT, CCL4 and INAH were not dispersed in PCA, suggesting potential involvement of other TG-decreasing mechanism(s). Because the plasma TG level is influenced by the balance of intake from diet, hepatic synthesis, secretion from liver, and metabolism at peripheral tissues, many factors remain to be investigated. Moreover, since it has been reported that the action of TH on LPL activity is in the opposite direction between human and rat (Kern *et al.*, 1996; Ong *et al.*, 1994), it is necessary to elucidate the species difference in the mechanism of plasma TG decrease.

In conclusion, we identified 218 probe sets from gene expression profiles in liver treated with various TG-decreasing compounds stored in our database. Analysis of identified probe sets suggested two mechanisms in plasma TG decrease, i.e., PPARα and CAR activation, in addition to at least one unknown mechanism. The proposed mechanisms of lowering plasma TG level elucidated by the present study are summarized and depicted in Fig. 4. Further studies, especially verifying experiments, are clearly necessary to confirm our hypothesis and to establish useful biomarker genes. The presently extracted probe sets could be a source of potential biomarkers for development of a novel hypolipidemic agent and/or interpretation of the mechanism of plasma TG reduction.

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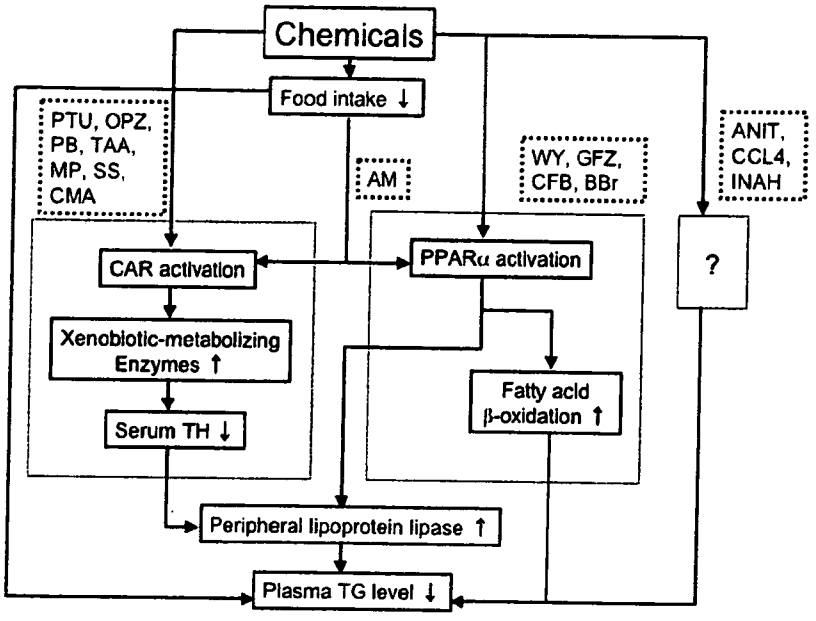

Fig. 4. Proposed TG-decreasing mechanisms of various drugs elucidated by comprehensive gene expression analysis.
Intake of chemicals induces direct or indirect CAR and/or PPARα activation. CAR activation leads to induction of hepatic xenobiotic-metabolizing enzymes, which reduces the serum thyroid hormone (TH) level. PPARα activation leads to hepatic induction of fatty acid β-oxidation. Both of the nuclear receptor activations increase peripheral lipoprotein lipase activity, which subsequently lowers the plasma TG level. PTU, OPZ, PB, TAA, MP, SS, and CMA are CAR activators, whereas WY, GFZ, CFB, and BBr are PPARα activators. AM appears to have both properties to the same extent. There must be other mechanism(s), since ANIT, CCL4, and INAH, which showed obvious TG-decreasing effects, could not be differentiated by the present analysis.

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Original Article

Gene expression profiling of methapyrilene-induced hepatotoxicity in rat

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ABSTRACT — The present study was conducted as a model case of the toxicogenomics approach for analyzing toxicological mechanisms and toxicity assessments in the early stage of drug development by comparing with classical toxicology data. Methapyrilene (MP) 100 mg/kg produced obvious histopathological changes in liver of rats by single or repeated dose up to 28 days with significant elevation of ALT and AST. In the middle dose groups (30 mg/kg MP), no apparent changes were noted in blood biochemical data by single dosing or repeated dosing up to one week, and no obvious histopathological changes were observed except a slight hypertrophy in the hepatocytes. Comprehensive gene expression changes were analyzed using Affymetrix GeneChip® and differentially expressed probe sets were statistically extracted. These contained many genes related to "glutathione metabolism", "apoptosis", "MAPK signaling pathway" and "regulation of cell cycle", which were all thought to be involved in the development of presently observed phenotypes. In the high dose groups, TGP1 scores (developed in our system in order to overview the responsiveness of drugs to multiple marker gene lists) for these categories were markedly increased from the early time point after single dose and kept their high expression throughout the repeated dose period. In the middle dose groups, the increment of the scores were noted not only at the time points when apparent pathological changes emerged, but also at the earlier stage of repeated dosing and even after single dosing. We conclude that toxicogenomics would enable a more sensitive assessment at the earlier time point than classical toxicology evaluation.

Key words: Methapyrilene; Rat; Liver; Toxicogenomics; Microarray

INTRODUCTION

The toxicogenomics approach has attracted attention in the field of drug safety assessment as a promising tool in predicting the toxicity of chemicals and analyzing the mechanism of toxicity. Classical toxicology works to characterize the emerged toxic changes, but it is not always powerful in predicting potential toxicity that has not emerged at the point of assessment but might occur in the future or to detect serious disease without apparent change in observation of the classical phenotype. On the other hand, extraction of toxicologically meaningful information from comprehensive gene expression analysis is expected to be useful since these changes precede toxicity and occur in the lower dose range.

The toxicogenomics project was a 5-year collaborative project conducted by the National Institute of Health Sciences, the National Institute of Biomedical Innovation and 15 pharmaceutical companies in Japan that started in 2002 (Urushidani and Nagao, 2005). Its aim was to construct a

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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, were selected, and gene expression in the liver (also the kidney in some cases) was comprehensively analyzed by Affymetrix GeneChip®. In 2007, the project was finished and the whole system, consisting of the database, the analyzing system and prediction system, was completed and named as TG-GATEs (Genomics Assisted Toxicity Evaluation System developed by the Toxicogenomics Project, Japan).

In the present study, we selected methapyrilene, a prototypic hepatotoxicant (Lijinsky *et al.*, 1980), as a model case of the toxicogenomics approach for analyzing the toxicological mechanism and toxicity assessment in the early stage of drug development by comparing with classical toxicology data.

MATERIALS AND METHODS

Chemical

Methapyrilene (MP) was obtained from Sigma Chemical Company (St. Louis, MO, USA) and a suspension formulation was prepared by mixing with 0.5% methylcellulose (MC) solution.

Animal and experimental design

Five-week old male Sprague-Dawley rats were obtained from Charles River Japan Inc. (Kanagawa, Japan). After a 7-day quarantine and acclimatization period, the animals (6-week old) were assigned to dosage groups (5 rats per group) using a computerized stratified random grouping method based on individual body weight. The animals were individually housed in stainless-steel cages in an animal 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 21–25°C with a relative humidity of 40–70%. Each animal was allowed free access to water and pellet diet (CRF-1, sterilized by radiation, Oriental Yeast Co., Tokyo, Japan).

According to the standard protocol in our project, rats received single or repeated doses of MP by gavage at doses of 0 (vehicle only), 10, 30 or 100 mg/kg. For the single-dose study, rats were sacrificed at 3, 6, 9 and 24 hr after dosing. For the repeated dose study, the animals were treated daily for 3, 7, 14 and 28 days, and they were sacrificed 24 hr after the last dosing. The animals were euthanized by exsanguination from the abdominal aorta under ether anesthesia after blood sampling, and liver samples were obtained from the left lateral lobe of the liver in each animal immediately after sacrifice for the following examinations.

The experimental protocols were reviewed and approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Health Sciences.

Blood chemical examination

Blood samples were collected upon sacrifice in tubes containing heparin lithium, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using an auto analyzer (Hitachi 7080).

Histopathological examination

For light microscopic examination, liver samples were fixed in 10% neutral buffered formalin, dehydrated in alcohol and embedded in paraffin. Paraffin sections were prepared and stained by a routine method using hematoxylin and eosin (H&E).

Microarray gene expression analysis

Microarray analysis was conducted on 3 out of 5 samples for each group. Total RNA was isolated from RNAlater® (Ambion, Austin, TX, USA)-preserved samples using RNeasy kit by Bio Robot 3000 (Qiagen, Valencia, CA, USA). Homogenization was conducted by Mill Mixer (Qiagen) and zirconium beads. Purity of RNA was checked by gel electrophoresis confirming that the 260/280 nm ratio was between 2.2 and 3.0. Gene expression analysis was carried out using GeneGhip® RAE230A probe arrays (Affymetrix, Santa Clara, CA, USA) containing 15,923 probe sets. The procedure was basically conducted according to the manufacture's instructions as previously reported. Microarray Analysis Suite 5.0 (MAS; Affymetrix) was used to quantify microarray signals and the intensities were normalized for each chip by setting the mean intensity to 500 (per chip normalization).

Microarray data analysis

To determine differentially expressed genes between high and time-matched control sample groups, Welch's *t* test was applied with a p cut off value of 0.01 in combination with a 1.5-fold regulation-ratio of means using GeneSpring software (Agilent Technologies, Inc., Santa Clara, CA, USA). Probe sets, which were labeled as absent by Affymetrix detection call in any of the 48 samples in single or repeated dose study, were excluded from further analysis. For the extracted probe sets, showing significant changes for at least one time point of either single or repeated administrations, pathway and GO analysis was performed using David 2.1 beta (http://david.abcc.ncifcrf.gov/) to identify overrepresented gene categories in

each gene list, and a p value of < 0.05 determined by Fisher's exact test was considered statistically significant.

Scoring of the marker gene sets (TGP1 score)

To facilitate the analytical procedures for large-scale microarray data, we developed a simple one-dimensional score, named TGP1, which is useful to overview the trend of the changes in expression of multiple biomarker gene lists all at once (Kiyosawa *et al.*, 2006). For each gene list, the signal log ratio was calculated by dividing the mean signal value of the chemical-treated group by that of the corresponding control. First, the sum of the signal log ratios for the used probe sets was calculated, and then divided by the number of probe sets used (Index 1). Next, the sum of squared signal log ratios for the used probe sets was calculated, and then divided by the number of probe sets used (Index 2). Finally, the TGP1 score was calculated by multiplying Index 1 with Index 2.

Statistical analysis

For blood chemical parameters, ANOVA followed by Dunnett's multiple comparison test or Kruskal-Wallis mean rank test followed by Mann-Whitney's U test was used as appropriate (Snedecor and Cochran, 1989).

RESULTS

Conventional toxicological parameters

Measurements of AST and ALT, representative markers of hepatotoxicity, are shown in Fig. 1 and histopathological findings are summarized in Tables 1 and 2. In the highest dose groups, both AST and ALT were elevated 3 or 6 hr after treatment, and they kept increasing with time. They also showed histopathological changes at every time point, such as hepatocellular hypertrophy and single cell necrosis in the peripheral lobular region, and obvious inflammation and anisonucleosis were noted at 24 hr after dosing (Fig. 2a). Furthermore, these lesions were aggravated and additional regenerative changes such as increased mitosis, bile duct proliferation, and hyperplasia, during repeated administrations were evident (Fig. 2b). In the middle dose groups, no apparent changes were noted in blood biochemical data by single dosing or repeated dosing up to one week, and no obvious histopathological changes were observed except a slight hypertrophy in the hepatocytes. At the time of the 15th and 29th days, single cell necrosis and infiltration of mononuclear cells were noted as in the highest group, though their severity was low. In the lowest dose groups, no obvious changes were noted either in blood biochemistry or in histopathology except for one case with hepatocyte hypertrophy in each of the 8th and 15th days.

Gene expression changes

Using the highest dose group showing obvious hepatotoxicity in terms of both blood biochemistry and histopathology, we extracted genes that showed statistically significant changes at least once in any time point in single and repeated dose experiments. For single and repeated dosing, up-regulated probe sets were 399 and 2509, respectively, and down-regulated probe sets were 235 and 876, respectively, namely, expression changes occurred in many genes in repeated dosing where obvious pathophysiological changes emerged.

Extracted genes were categorized by pathway and GO analysis and the results are summarized in Tables 3 (up-regulated) and 4 (down-regulated). Among the genes up-regulated in single dose experiments, gene ontologies or pathways related to "regulation of cell cycle", "MAPK signaling pathway" and "glutathione metabolism" were still significantly up-regulated in repeated dosing. The genes related to "apoptosis" or "ribosome" were clearly up-regulated in repeated dosing, while they were not affected by single dosing.

In single dose experiments, the number of down-regulated genes was quite a few (Table 4). Significant suppression of gene expression by repeated administration was noted in various categories, including "starch and sucrose metabolism", "steroid metabolism", "complement activation" and "complement and coagulation cascades".

We considered the pathways and GO categories of "glutathione metabolism", "apoptosis", "MAPK signaling" and "regulation of cell cycle" as most important in the toxicological mechanisms of MP. In order to overview the effects of MP on these pathways, we calculated the TGP1-score for each (Table 5). Upon calculation of the score, redundant probe sets were unified based on their reliability and dose-dependency. It is obvious from Table 5 that the marker scores of these categories markedly increased in the early stage of single dosing of the highest dose and they kept increasing during repeated dosing. In case of middle dosing, an obvious increase of scores for glutathione metabolism and apoptosis was detected not only at the 15th and 29th days with obvious pathological changes but also at the 4th and 8th days of repeated dosing and 6 hr after single dosing. In the lowest dose groups, the only detectable change was a tendency of increment in the score of apoptosis at the 8th day.

Up-regulated genes involved in glutathione metabolism, apoptosis, MAPK signaling pathway, and regulation of cell cycle

The individual gene expression change (ratio to control) in each category was depicted as heatmap (Fig. 3 to 6) for "glutathione metabolism", "apoptosis", "MAPK signaling" and "regulation of cell cycle", respectively. In general, most of the genes were dose-dependently mobilized and characteristic changes were noticed in single and repeated dosing groups. As for genes involved in glutathione biosynthesis: glutamate cysteine ligase, modifier subunit (Gclm) and glutamate-cysteine ligase, catalytic subunit (Gclc) (Fig. 3); those involved in the regulation of apoptosis: v-akt murine thymoma viral oncogene homolog 1 (Akt1) and programmed cell death 6 interacting protein (Pdcd6ip) (Fig. 4), and those belonging to heat shock proteins: heat shock 70 kD protein 1A/1B (Hspa1a/1b) and heat shock protein 8 (Hspa8) (Fig. 5), these were markedly up-regulated in the early stage of single dose, whereas little or no changes were noted in repeated dosing. Excluding these genes, the extent of up-regulation increased with repeated administration in most of the genes. Especially, those involved in glutathione metabolism: glucose-6-phosphate dehydrogenase (G6pdx), glutathione *S*-transferase M4 (Gstm4) and glutathione *S*-transferase Yc2 subunit (Yc2) (Fig. 3), those involved in regulation of apoptosis: nucleolar protein 3 (Nol3), rhoB gene (Rhob) and tribbles homolog 3 (Drosophila) (Trib3) (Fig. 4), those belonging to MAPK signaling and known as cell cycle regulators: myelocytomatosis viral oncogene homolog (avian) (Myc),

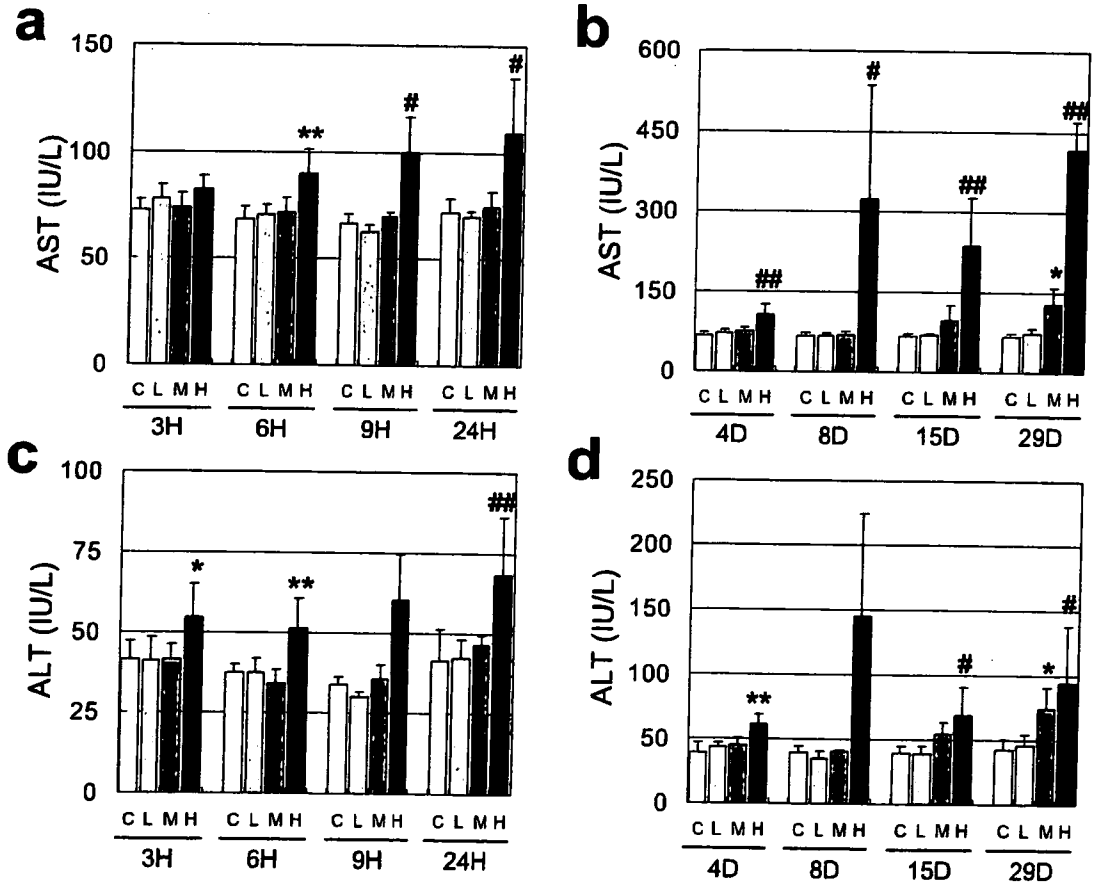

Fig. 1. Serum AST (a and b) and ALT activities (c and d) in rats treated with 10, 30 and 300 mg/kg MP in single and repeated dose studies.
Data are expressed as mean ± S.D. (n = 5). *, **Significant difference from the control group, $p < 0.05$, 0.01, by Dunnett's multiple comparison test. *, #Significant difference from the control group, $p < 0.05$, 0.01, by Mann-Whitney's U test.

Table 1. Histopathological findings in rat liver treated with MP in single dose study.

Morphology	Time Point (hrs)	3			6			9			24		
	Dose (mg/kg)	10	30	100	10	30	100	10	30	100	10	30	100
	Number of animals examined	5	5	5	5	5	5	5	5	5	5	5	5
Hepatocyte / Anisonucleosis		0	0	0	0	0	0	0	0	0	0	0	5
slight													5
Hepatocyte / Hypertrophy		0	0	1	0	0	3	0	0	4	0	1	5
slight				1			3			4		1	5
Hepatocyte / Single cell necrosis		0	0	1	0	0	3	0	0	5	0	0	5
slight				1			3			5			5
Periportal / Cellular infiltration, mononuclear cell		0	0	0	0	0	0	0	0	5	0	0	5
slight										5			5

Vehicle alone, or MP 10, 30, or 100 mg/kg was administered orally to rats, and the animals were euthanized at 3, 6, 9 and 24 hr after dosing (n = 5). The histopathological change in liver was graded into 4 categories: very slight, slight, moderate, and severe. The number of animals affected at each grade is shown.

Table 2. Histopathological findings in rat liver treated with MP in repeated dose study.

Morphology	Time Point (days)	4			8			15			29		
	Dose (mg/kg)	10	30	100	10	30	100	10	30	100	10	30	100
	Number of animals examined	5	5	5	5	5	5	5	5	5	5	5	4[a]
Hepatocyte / Alteration, cytoplasmic		0	0	0	0	0	0		0	0	0	0	1
slight													1
Hepatocyte / Anisonucleosis		0	0	3	0	0	4		0	5	0	0	4
slight				3			4			5			4
Hepatocyte / Hyperplasia		0	0	0	0	0	0		0	0	0	0	4
slight													4
Hepatocyte / Hypertrophy		0	2	5	1	2	5		3	5	1	5	4
slight			2	5	1	2	5		3	5	1	5	4
Hepatocyte / Increased mitosis		0	1	4	0	0	3		1	0	0	3	3
slight			1	4			3		1			3	3
Hepatocyte / Single cell necrosis		0	0	5	0	0	5		3	5	0	3	4
slight				5			5		3	5		3	4
Interlobular / Proliferation, bile duct		0	0	5	0	0	5		1	5	0	0	4
slight				5			5		1	4			
moderate										1			4
Periportal / Cellular infiltration, mononuclear cell		0	1	4	0	0	4		5	5	0	2	4
slight			1	4			4		5	2		2	4
moderate										3			
Periportal / Deposit, pigment		0	0	0	0	0	0		0	0	0	0	3
slight													3

Vehicle alone, or MP 10, 30, or 100 mg/kg was administered orally to rats once daily for 1, 3, 7, 14, and 28 days, and the animals were euthanized at 24 hr after dosing, namely, on 2, 4, 8, 15, and 29 days (n = 5). [a]One of the 5 rats died and was not examined histopathologically due to advanced autolysis. For more detailed information, see Table 1.

FBJ murine osteosarcoma viral oncogene homolog (Fos), v-jun sarcoma virus 17 oncogene homolog (avian) (Jun) and fibroblast growth factor 21 (Fgf21) (Fig. 5), and those related to DNA damage: growth arrest and DNA-damage-inducible 45 alpha (Gadd45a) and DNA-damage inducible transcript 3 (Ddit3) (Fig. 6), these kept up-regulated throughout the repeated dosing periods.

DISCUSSION

Methapyrilene hydrochloride is an antihistamine drug and had been used in the 1970s, but was removed from the market once it was known to be carcinogenic in rat liver (Lijinsky *et al.*, 1980; Fischer *et al.*, 1983). It is now considered to be a rat-specific carcinogen since hepatocellular carcinoma and cholangiocarcinoma were induced by administration of MP at 1000 ppm for 64 weeks, whereas no such findings were observed either in Syrian hamsters, Guinea-pigs, B6C3F1 mice, or humans (Mirsalis, 1987). As for its genotoxicity, the Ames test, DNA addition test, chromosome abnormality test (NTP, 2000) and irregular DNA synthesis test in rat and mouse (Steinmetz *et al.*, 1988) were all negative, whereas the cell transformation assay and L5178Y/TK+/– mouse lymphoma assay were positive (Turner *et al.*, 1987). Based on these observations, hepatocarcinogenicity of MP in rat has been considered to be non-genotoxic, whereas the involvement of its initiation activity cannot be completely excluded (Althaus *et al.*,

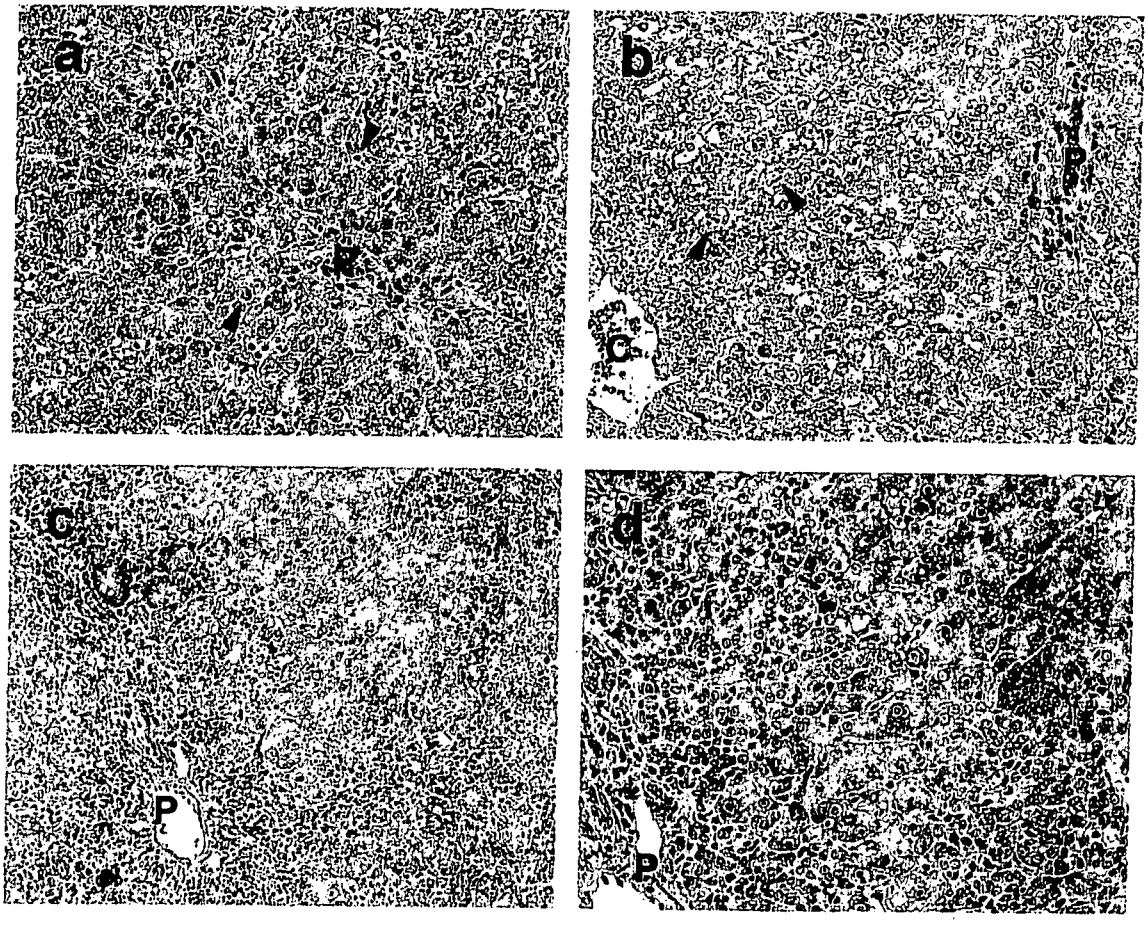

Fig. 2. Histopathological changes of liver treated with 100 mg/kg MP.
a: Hepatocellular hypertrophy and single cell necrosis (arrow head) in the periportal region (P) are observed at early time point, 24 hr after single dosing. b: Additional regenerative changes, such as increased mitosis, bile duct proliferation, and hyperplasia are evident by repeated administration.

Table 3. Gene ontology and pathway classification of extracted probe sets (up-regulation).

Exp. type	TERM[a]	Count[b]	p value[c]
Single dose study			
	GOTERM_BP_5		
	REGULATION OF NUCLEOBASE, NUCLEOSIDE, NUCLEOTIDE AND NUCLEIC ACID METABOLISM	18	6.41E-2
	TRANSCRIPTION	18	7.48E-2
	MACROMOLECULE BIOSYNTHESIS	**13**	**1.92E-2**
	PROTEIN BIOSYNTHESIS	**11**	**3.84E-2**
	REGULATION OF CELL CYCLE	**11**	**5.95E-4**
	INTRACELLULAR TRANSPORT	**9**	**8.5E-2**
	AMINO ACID METABOLISM	6	2.75E-2
	AMINE BIOSYNTHESIS	5	9.11E-3
	CELL GROWTH	5	4.86E-2
	NUCLEAR TRANSPORT	5	2.6E-3
	NUCLEOCYTOPLASMIC TRANSPORT	5	6.12E-3
	PROTEIN KINASE CASCADE	5	4.18E-2
	REGULATION OF CELL SIZE	5	4.86E-2
	RNA METABOLISM	5	4.63E-2
	POSITIVE REGULATION OF NUCLEOBASE, NUCLEOSIDE, NUCLEOTIDE AND NUCLEIC ACID METABOLISM	4	8.74E-2
	PROTEIN IMPORT	4	1.49E-2
	RNA PROCESSING	4	6E-2
	GOTERM_CC_5		
	NUCLEUS	34	7.29E-3
	KEGG_PATHWAY		
	MAPK SIGNALING PATHWAY (Rattus norvegicus)	**10**	**3.73E-2**
	GAP JUNCTION (Rattus norvegicus)	6	5.15E-2
	TGF-BETA SIGNALING PATHWAY (Rattus norvegicus)	5	8.22E-2
	ARGININE AND PROLINE METABOLISM (Rattus norvegicus)	4	3.69E-2
	GLUTATHIONE METABOLISM (Rattus norvegicus)	**4**	**2.42E-2**
Repeated dose study			
	GOTERM_BP_5		
	CELLULAR PROTEIN METABOLISM	189	2.86E-8
	MACROMOLECULE BIOSYNTHESIS	**89**	**4.92E-17**
	PROTEIN BIOSYNTHESIS	**85**	**2.13E-19**
	INTRACELLULAR TRANSPORT	**60**	**1.22E-9**
	PROTEIN TRANSPORT	47	1.48E-6
	INTRACELLULAR PROTEIN TRANSPORT	44	4.84E-7
	APOPTOSIS	38	3.07E-4
	REGULATION OF CELL CYCLE	**32**	**6.48E-4**
	REGULATION OF APOPTOSIS	31	2.99E-4
	REGULATION OF PROGRAMMED CELL DEATH	31	3.48E-4
	GOTERM_CC_5		
	VESICLE-MEDIATED TRANSPORT	30	1.48E-2
	CYTOSKELETON	64	6.02E-2
	RIBOSOME	60	1E-13
	MICROTUBULE CYTOSKELETON	41	2.59E-3
	KEGG_PATHWAY		
	MICROTUBULE ASSOCIATED COMPLEX	29	6.25E-2
	CYTOSOLIC RIBOSOME (SENSU EUKARYOTA)	26	2.79E-11
	RIBOSOME (Rattus norvegicus)	40	1.79E-24
	FOCAL ADHESION (Rattus norvegicus)	36	6.68E-2
	MAPK SIGNALING PATHWAY (Rattus norvegicus)	**33**	**7.61E-2**
	TIGHT JUNCTION (Rattus norvegicus)	27	4.25E-3
	GLUTATHIONE METABOLISM (Rattus norvegicus)	**8[d]**	**4.55E-2**

Pathway and GO analysis was performed using David 2.1 beta. Statistical significant terms are listed (Fisher's exact test, $p < 0.05$; threshold counts: greater than 10% of the number of probe sets involved in the examined gene list). Bold terms were commonly affected in both single and repeated dose studies. Shaded terms were further analyzed by scoring based on the TGP1-score.

Table 4. Gene ontology and pathway classification of extracted probe sets (up-regulation).

Exp. type TERM[a)]	Count[b)]	p value[c)]
Single dose study		
GOTERM_BP_5		
REGULATION OF NUCLEOBASE, NUCLEOSIDE, NUCLEOTIDE AND NUCLEIC ACID METABOLISM	13	9.72E-2
REGULATION OF TRANSCRIPTION	13	9.51E-2
RESPONSE TO CHEMICAL SUBSTANCE	4	8.4E-2
CHEMOTAXIS	3	5.47E-2
STEROL METABOLISM	3	6.27E-2
KEGG_PATHWAY		
STARCH AND SUCROSE METABOLISM (Rattus norvegicus)	3	4.97E-2
Repeated dose study		
GOTERM_BP_5		
CARBOXYLIC ACID METABOLISM	45	2.26E-16
ELECTRON TRANSPORT	37	2.19E-8
CELLULAR LIPID METABOLISM	34	7.06E-8
IMMUNE RESPONSE	27	5.94E-2
RESPONSE TO PEST, PATHOGEN OR PARASITE	21	4.78E-5
AMINO ACID METABOLISM	20	1.32E-7
CELLULAR CARBOHYDRATE METABOLISM	18	1.36E-3
LIPID BIOSYNTHESIS	15	2.41E-3
STEROID METABOLISM	15	1.6E-5
WOUND HEALING	14	4.27E-5
BLOOD COAGULATION	13	2.03E-6
FATTY ACID METABOLISM	13	2.59E-3
MONOSACCHARIDE METABOLISM	13	3.96E-3
AMINO ACID DERIVATIVE METABOLISM	12	2.04E-4
COENZYME METABOLISM	12	1.31E-2
COFACTOR BIOSYNTHESIS	11	1.7E-2
COMPLEMENT ACTIVATION	11	1.28E-7
HUMORAL IMMUNE RESPONSE	11	3.22E-6
AMINE CATABOLISM	10	1.37E-5
RESPONSE TO CHEMICAL SUBSTANCE	10	2.22E-2
INFLAMMATORY RESPONSE	9	3.47E-2
GOTERM_CC_5		
MITOCHONDRION	38	2.07E-5
ENDOPLASMIC RETICULUM	26	8.26E-6
MICROSOME	18	5.64E-7
KEGG_PATHWAY		
TRYPTOPHAN METABOLISM (Rattus norvegicus)	22	5.32E-12
COMPLEMENT AND COAGULATION CASCADES (Rattus norvegicus)	17	7.4E-7
FATTY ACID METABOLISM (Rattus norvegicus)	17	3.65E-7
GLYCINE, SERINE AND THREONINE METABOLISM (Rattus norvegicus)	11	5.69E-7
BUTANOATE METABOLISM (Rattus norvegicus)	9	7.11E-4
GAMMA-HEXACHLOROCYCLOHEXANE DEGRADATION (Rattus norvegicus)	9	1.29E-3
LYSINE DEGRADATION (Rattus norvegicus)	9	1.26E-5
PYRUVATE METABOLISM (Rattus norvegicus)	9	3.61E-4
STARCH AND SUCROSE METABOLISM (Rattus norvegicus)	**9**	**1.08E-4**
VALINE, LEUCINE AND ISOLEUCINE DEGRADATION (Rattus norvegicus)	9	3.61E-4