Table 2. Plasma biochemistry and histopathological findings in rat liver treated with various prototypical hepatotoxicants.

Chemical	Dose (mg/kg)	Serum ALT activity (IU/L)		Serum AST activity (IU/L)		Histopathological findings observed in rat livers (number of animals)
		Control	Treated	Control	Treated	
Acetaminophen	1000	37.0 ± 3.1	51.0 ± 17.2	59.0 ± 6.3	76.4 ± 14.1*	Increased eosinophility of hepatocyte: central (3/5) Inflammatory infiltration: central (5/5)
Bromobenzene	300	50.8 ± 6.4	113.6 ± 49.9*	79.6 ± 7.0	481.0 ± 377.8	Hypertrophy, eosinophilic granular change (5/5) Cellular infiltration, centrilobular (5/5) Swelling, centrilobular (4/5) Necrosis, centrilobular (4/5)
Clofibrate	300	34.0 ± 5.5	38.0 ± 6.3	66.4 ± 8.0	79.0 ± 14.2	No findings
Chlorpromazine	45	32.4 ± 4.9	31.8 ± 1.8	62.2 ± 4.9	74.6 ± 8.4*	No findings
Glibenclamide	1000	32.2 ± 4.5	35.6 ± 2.9	61.4 ± 3.8	69.2 ± 4.7*	No findings
Methapyrilene	100	41.6 ± 9.5	68.4 ± 17.6*	71.8 ± 6.8	109.6 ± 25.7*	Single cell necrosis, hepatocyte (5/5) Hypertrophy, hepatocyte (5/5) Cellular infiltration, mononuclear cell, periportal (5/5) Anisoncleosis, hepatocyte (5/5)
Phenylbutazone	200	34.8 ± 4.8	49.4 ± 18.4	67.0 ± 6.3	76.6 ± 7.6	No findings
Aspirin	450	34.8 ± 5.3	44.2 ± 15.6	63.8 ± 4.5	75.6 ± 12.6	No findings
Carbon tetrachloride	300	37.2 ± 2.7	42.2 ± 4.6	66.6 ± 4.0	75.0 ± 13.3	Degeneration, hydropic: centrilobular (4/5) Cellular infiltration: centrilobular (3/5) Degeneration, fatty: centrilobular (4/5)
Coumarin	150	37.0 ± 3.9	40.2 ± 8.4	64.4 ± 8.6	85.8 ± 26.3	Hypertrophy, centrilobular (3/5)
Hexachlorobenzene	300	40.2 ± 8.7	47.0 ± 7.1	66.6 ± 3.3	69.2 ± 4.8	No findings
Perhexiline maleate	150	42.6 ± 5.9	50.0 ± 5.8	66.4 ± 5.1	71.6 ± 7.4	No findings
Thioacetamide	45	32.5 ± 3.4	137.2 ± 37.8**	63.5 ± 3.0	713.8 ± 542.6	Hypertrophy: centrilobular (5/5) Cellular infiltration, inflammatory (5/5) Change, eosinophilic hepatocyte (5/5) Necrosis, centrilobular (5/5)

Rat groups consisting of 5 animals were administered with the compounds listed in the table and euthanized 24 hr after treatment. Both blood chemistry and histopathology data are summarized using the 5 rats. Note that microarray analysis was conducted using 3 rats out of the 5. The data are presented as mean ±S.D. * and **, $p<0.05$ and $p<0.01$, respectively, determined by two-sample *t*-test.

hexachlorobenzene or perhexiline maleate did not show any histopathological changes.

Gene expression analysis for rat liver treated with various hepatotoxicants

PCA was performed using GSH probe sets for GeneChip data obtained from rat livers 24 hr after treatment with various prototypical hepatotoxicants (Fig. 4). It was obvious from the figure that a few compounds were distributed to the direction of the first principal component (PC 1) with relatively high contribution (57.6%), i.e., 300 mg/kg bromobenzene, 150 mg/kg coumarin, 1000 mg/kg acetaminophen, and 45 mg/kg thioacetamide, in that order. Rats treated with other chemicals or corresponding vehicles showed no apparent shift toward the PC 1 axis, but showed dispersed distributions along the PC 2 axis.

Glutathione content in rat livers treated with bromobenzene

From PCA using GSH probe sets, we found that bromobenzene was the most potent GSH-depletor among the compounds tested. In order to confirm this, hepatic glutathione content in the liver treated with this compound was actually quantified. It was found that the contents were significantly reduced 3, 6 and 9 hr after 300 mg/kg bromobenzene treatment (Fig. 5). It appeared that some of the treated rats showed recovery or rather rebound of GSH contents 24 hr after treatment since the mean value recovered to the control level with large variance.

Time-course of gene expression profile in rat liver treated with bromobenzene

In order to analyze the time dependent correlation between GSH contents and gene expression changes, PCA was performed by adding the data of 3, 6, and 9 hr after bromobenzene treatment to the same

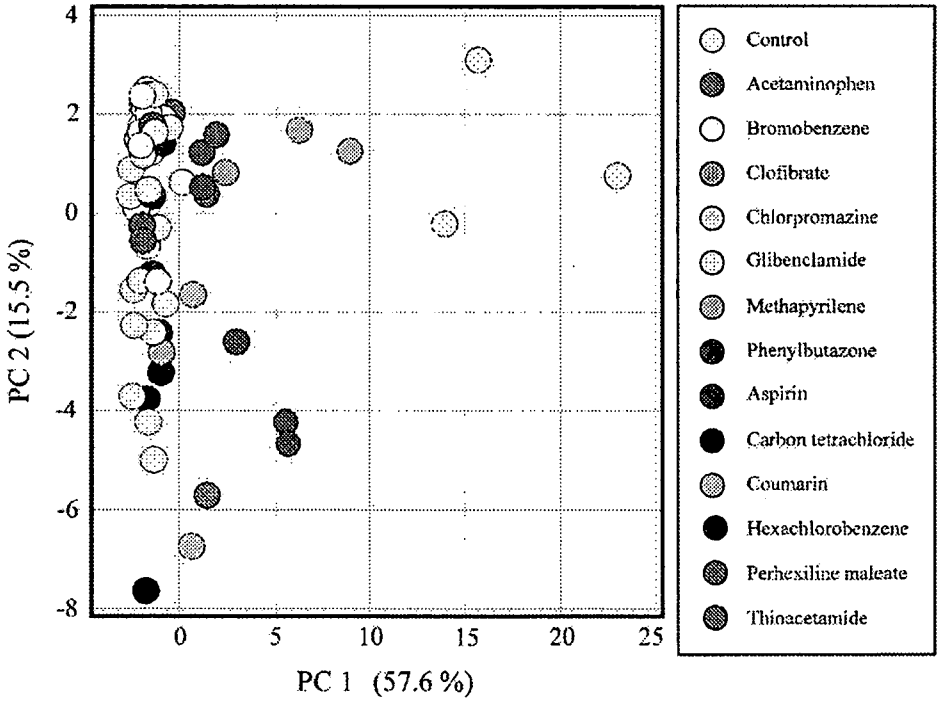

Fig. 4. PCA for GeneChip data of rat liver 24 hr after treatment with various hepatotoxicants.
PCA was performed using GSH probe sets for GeneChip data of rat livers 24 hr after treatment with various hepatotoxicants. Each spot, colored by chemical type, represents individual samples. Bromobenzene, coumarin, and acetaminophen showed apparent shift from control, suggesting a perturbation of glutathione homeostasis in the liver after treatment.

data in Fig. 4. Fig. 6 shows that signal profiles of GSH probe sets did not apparently differ from those of controls, 3 and 6 hr after 300 mg/kg bromobenzene treatment. After 9 hr, they shifted away toward both PC1 and PC2 axis, approaching the position of 24 hr on PC1 axis.

DISCUSSION

Hepatic total glutathione content was significantly decreased in all the phorone-treated groups 3 hr after treatment (Fig. 2). After acute glutathione depletion, the hepatic glutathione content gradually recovered from 6 hr in the phorone-treated group (40 and 120 mg/kg), resulting in a significantly higher glutathione content, compared to the vehicle-treated rats 24 hr after treatment. Plasma ALT activity was elevated from 9 hr after 400 mg/kg phorone treatment, suggesting slight hepatocellular injury. Since secondary undesirable effects caused by slight hepatotoxicity (other than glutathione depletion) might affect the gene expression profile, we excluded GeneChip data of the 400 mg/kg phorone-treated rats from analysis for identification of the glutathione depletion-responsive gene probe sets. Previously, candidate marker genes whose mRNA levels were inversely correlated with hepatic glutathione content were identified using L-buthionine-[S,R]-sulfoximine (BSO) as a glutathione-depleting agent (Kiyosawa *et al.*, 2004). In the present study, we used phorone as a glutathione-depleting agent instead of BSO. We identified a total of 161 probe sets, referred to as 'GSH probe sets', whose signal showed

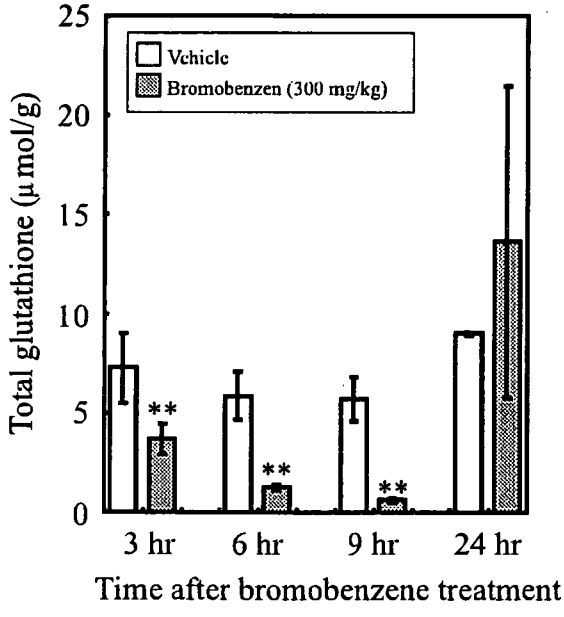

Fig. 5. Glutathione content in rat liver treated with bromobenzene.
Three rats per group were treated with 300 mg/kg bromobenzene or vehicle, and the livers were removed 3, 6, 9 and 24 hr after treatment. Hepatic glutathione content (total) was measured and the data are presented as mean ± S.D. Hepatic glutathione content was significantly decreased 3, 6 and 9 hr after bromobenzene treatment, and recovered 24 hr after treatment, although the glutathione level showed a high variability at this time point. **, $p<0.01$ determined by two-sample *t*-test.

an inverse correlation with hepatic glutathione content.

The present study had two advantages compared with the BSO study previously reported. First, the glutathione-depleting mechanism differs from phorone (a reactor to GSH thiol) and BSO (an inhibitor of gamma-glutamylcysteine synthetase). Comparing the two glutathione-depleting mechanisms, the phorone-induced one is thought to be more similar to drug-induced glutathione depletion (as in the acetaminophen overdose-induced one) where hepatic glutathione is depleted by elevated elimination, not by inhibition of glutathione synthesis. Second, the present study set multiple dose ranges and time points. The total number of rats tested in the phorone study was 36 (twelve 400 mg/kg phorone-treated rats were excluded from the gene selection procedure), whereas the previous BSO study used only 8 rats (Kiyosawa *et al.*, 2004). Thus, the GSH probe sets identified in the present study would give us more reliable information for evaluation of the potential risk of drug-induced glutathione depletion.

The GSH probe sets contained antioxidant/phase II drug-metabolizing enzymes, oxidative stress markers, transporters, metabolism-related genes, transcription factors and signal transduction-related genes, and others. GSH probe sets contain a modifier subunit of glutamate cysteine ligase gene, which encodes a key enzyme for glutathione synthesis (Moinova and Mulcahy, 1999). In addition, a prototypical oxidative stress-responsive gene, heme oxygenase 1, which is reported to be regulated by oxidative stress sensor Nrf2 (Nguyen *et al.*, 2003), was identified as GSH probe sets. Furthermore, several genes were found to be in common with previously reported gene sets identified from the BSO-induced glutathione depletion model rat, such as GTP cyclohydrolase I and HMG-CoA reductase (Kiyosawa *et al.*, 2004). On the other hand, a

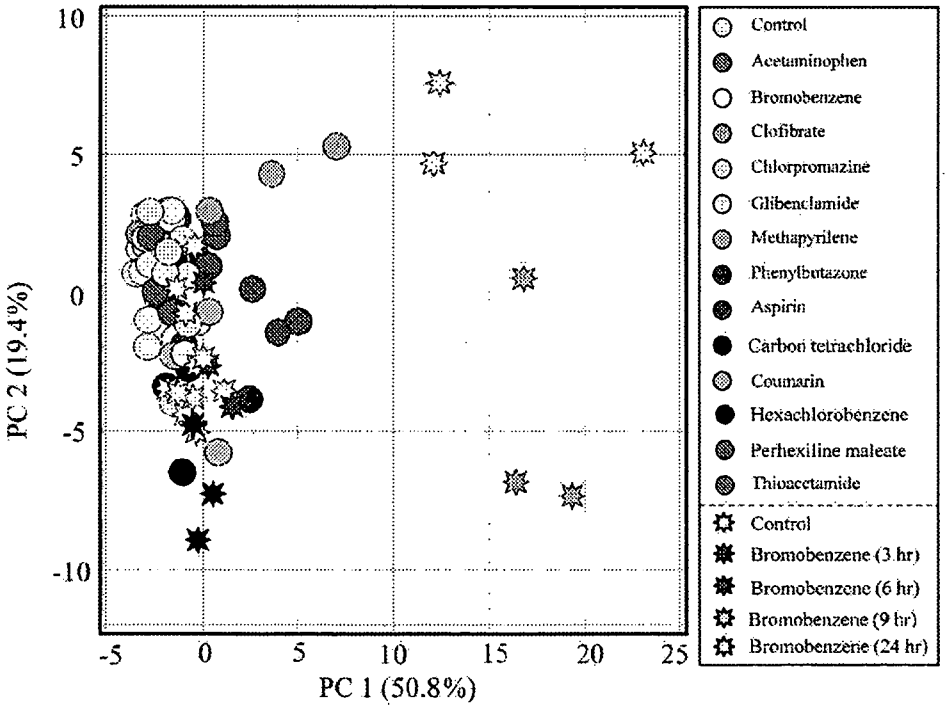

Fig. 6. Time-course of gene expression profile in rat liver treated with bromobenzene.
PCA was performed using GSH probe sets for GeneChip data of rat livers 3, 6, 9 and 24 hr after 300 mg/kg bromobenzene treatment, as well as those 24 hr after treatment with hepatotoxicants, which are the same as those shown in Fig. 4. Each spot colored by chemical types represents individual samples. Gene expression profiles of rats treated with bromobenzene did not show an apparent shift away from corresponding controls 3 and 6 hr after treatment. Those 9 and 24 hr after treatment showed an apparent shift from the controls.

difference in the content of probe sets, compared with that identified from BSO-treated rats was observed, for instance, glutathione *S*-transferase genes or metallothionein genes, which were induced by BSO but not by phorone (Kiyosawa *et al.*, 2004). Although the strain and the age of the rats were not matched between the two studies (6 week old male Crj:CD(SD)IGS rats vs. 9 week old male F344Cu/Drj rats) the difference could be mainly due to the difference of the GSH-depleting mechanism between phorone and BSO.

To examine the toxicological significance of the GSH probe sets, we conducted PCA on GeneChip data obtained from rats treated with 13 prototypical hepatotoxicants (Fig. 4). On the PCA map, rats treated with bromobenzene, coumarin, and acetaminophen showed apparent changes in hepatic gene expression profiles, and those treated with thioacetamide showed slight changes (Fig. 4). Bromobenzene-treated rats showed the most notable change in gene expression. Bromobenzene was reported to be oxidized to a reactive metabolite in liver, depleting hepatic glutathione (Chakrabarti, 1991; Heijne *et al.*, 2004).

Courmarin-treated rats showed the second most affected gene expression profile in PCA. It was reported that a single coumarin treatment reduced the hepatic content of non-protein sulfhydryl groups (Lake *et al.*, 1989), and this is thought to reflect the decrease in glutathione content. Furthermore, coumarin was shown to decrease glutathione content in rat hepatocyte as well (Lake *et al.*, 1989). Reactive metabolites generated from coumarin oxidation in liver were thought to play a role in coumarin-induced glutathione depletion and hepatotoxicity (Lake, 1984; Lake *et al.*, 1989). Since no apparent hepatotoxicity was evident in both the histopathology and plasma biochemistry data (Table 2), the PCA result would reflect the potential risk of courmarin-induced glutathione-depletion.

Acetaminophen and thioacetamide are known to deplete or reduce glutathione in liver when overdosed (Mesa *et al.*, 1996). In the present study, acetaminophen- or thioacetamide treated rat showed no dramatic change in the gene expression profile compared to bromobenzene. Considering the plasma chemistry data, rats treated with acetaminophen or thioacetamide did not show apparent hepatotoxicity within 24 hr after single dose, whereas those treated with bromobenzene apparently showed it, suggesting that glutathione depletion, expression profile of GSH probe sets, and toxicological phenotype are well correlated with each other.

We also investigated the time-course of glutathione content and gene expression profile in rat livers treated with bromobenzene, and this showed the most notable gene expression change of all of the examined hepatotoxicants (according to the PCA result). Bromobenzene rapidly depleted hepatic glutathione 3 hr after treatment, and the glutathione content was the lowest 9 hr after treatment (Fig. 5). Hepatic glutathione content recovered from initial depletion until 24 hr after the bromobenzene treatment, and such recovery has been previously reported (Chakrabarti, 1991; Heijne *et al.*, 2004). On the other hand, gene expression changes had not been apparent 3 and 6 hr after the bromobenzene treatment, but appeared 9 and 24 hr after treatment. Although hepatic glutathione content was recovered at 24 hr after the bromobenzene treatment, a changed level in gene expression was most prominent at this time point. This result depicts a characteristic of the gene expression profile in that it does not reflect the status of hepatic glutathione content itself, but the nuclear activity to maintain glutathione homeostasis in the liver against bromobenzene-induced glutathione depletion. It should be noted, that although the hepatic glutathione content was recovered 24 hr after bromobenzene treatment, the potential risk of bromobenzene-induced glutathione depletion does exist. In general, hepatic glutathione depletion caused by chemical treatments occurs immediately, followed by rapid recovery by glutathione re-synthesis (Meister and Anderson, 1983). Since the time point of sacrifice in ordinal toxicity studies is set to 24 hr after chemical treatment in many cases, measurement of glutathione content might overlook the risk of the glutathione-depleting potential of the tested chemicals, because 24 hr is enough time for the recovery of glutathione content after acute glutathione depletion. Instead, gene expression profiling is considered to be appropriate for evaluating the glutathione-depleting potential of chemicals, rather than measuring glutathione content, especially in later time points of chemical treatment. This characteristic of gene expression profiling, namely toxicogenomics analysis, would allow for safety assessment of chemicals in drug development.

In conclusion, a total of 161 probe sets of RAE 230A GeneChip, referred as GSH probe sets, were identified using phorone-treated rats for evaluation of drug-induced glutathione depletion. The significance of the identified GSH probe sets was evaluated using the TGP database, where prototypical glutathione depletors successfully showed characteristic changes in the signal levels of GSH probe sets. The time-course

of glutathione content and the gene expression profile showed that gene expression profiling could detect the glutathione-depleting potential of chemicals in later time points, e.g., 24 hr after chemical treatment, where hepatic glutathione content had recovered from acute and transient depletion at earlier time points. Therefore, toxicogenomics analysis using identified GSH probe sets would be an invaluable methodology for assessing a drug's potential risk of glutathione depletion, possibly leading to hepatotoxicity.

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GENE EXPRESSION PROFILING OF RAT LIVER TREATED WITH SERUM TRIGLYCERIDE-DECREASING COMPOUNDS

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ABSTRACT — We have constructed a large-scale transcriptome database of rat liver treated with various drugs. In an effort to identify a biomarker for interpretation of plasma triglyceride (TG) decrease, we extracted 218 probe sets of rat hepatic genes from data of 15 drugs that decreased the plasma TG level but differentially affected food consumption. Pathway and gene ontology analysis revealed that the genes belong to amino acid metabolism, lipid metabolism and xenobiotics metabolism. Principal component analysis (PCA) showed that 12 out of 15 compounds were separated in the direction of PC1, and these 12 were separated in the direction of PC2, according to their hepatic gene expression profiles. It was found that genes with either large or small eigenvector values in principal component PC 2 were those reported to be regulated by peroxisome proliferator-activated receptor (PPAR)α or constitutive androstane receptor (CAR), respectively. In fact, WY-14,643, clofibrate, gemfibrozil and benzbromarone, reported to be PPARα activators, distributed to the former, whereas propylthiouracil, omeprazole, phenobarbital, thioacetamide, methapyrilene, sulfasalazine and coumarin did to the latter. We conclude that these identified 218 probe sets could be a useful source of biomarkers for classification of plasma TG decrease, based on the mechanisms involving PPARα and CAR.

KEY WORDS: Triglyceride, Liver, CAR, PPAR, Toxicogenomics

INTRODUCTION

The toxicogenomics project was a 5-year collaborative project by the National Institute of Biomedical Innovation (NIBIO), the National Institute of Health Science (NIHS), and 15 pharmaceutical companies in Japan that started in 2002 (Urushidani and Nagao, 2005). Its aim was 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, were selected, and gene expression in liver (also kidney in some cases) was comprehensively analyzed by using Affymetrix GeneChip®. In 2007, the project was finished and the whole system, consisting of the database, the analyzing system and the prediction system, was completed and named as TG-GATEs (Genomics Assisted Toxicity Evaluation System developed by Toxicogenomics Project, Japan).

In toxicity studies, plasma triglyceride (TG) decrease is often observed. Because plasma TG level can be influenced mostly by nutritional status, decrease in food consumption is one of the factors for its change. For activator of peroxisome proliferator-activated

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receptor (PPAR)α, it is a hot field of drug development, and this drug facilitates the expression of genes related to fatty acid β–oxidation (Schoonjans *et al.*, 1996), subsequently lowering plasma TG level. Phenobarbital (PB), an antiepileptic barbiturate derivative, decreases plasma TG level but increases plasma total cholesterol without decrease in food consumption in rats (Kiyosawa *et al.*, 2004; Hall *et al.*, 1990). Kiyosawa *et al.*(2004) proposed a mechanism of serum cholesterol elevation via up-regulation of hepatic cholesterol synthesis. So far, PB is not reported to be a PPARα activator, and the mechanism of plasma TG decrease by PB is not well understood. In these cases, plasma TG decrease could be a target of the drug in one case, or a sign of toxicity in another case, each with different mechanisms. Thus, identification of the mechanisms behind plasma TG decrease during drug treatment would enable both seed discovery and interpretation of toxicity.

One of the main purposes of TG-GATEs is to identify biomarkers for toxicity evaluation. Although there have been various reports describing strategies to extract marker genes from the transcriptome data (Hibbs *et al.*, 2004; Mutlib *et al.*, 2006; Tan *et al.*, 2006), the best way has not been established. In the present study, we have started to identify candidates of potential biomarker genes for interpretation of the fundamental mechanism(s) of plasma TG decrease, since our database contains several drugs that cause plasma TG decrease.

MATERIALS AND METHODS

Animals and treatments

Male Crj:CD(SD)IGS 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 the body weight for each age. The animals were individually housed in stainless-steel cages in a room that was lighted for 12 hr (7:00-19:00) daily, ventilated with an air-exchange rate of 15 times per hour, and maintained at 21-25°C with a relative humidity of 40-70%. Each animal was allowed free access to water and pellet food (CRF-1, sterilized by radiation, Oriental Yeast Co., Japan). Rats in each group were orally administered with various drugs suspended or dissolved either in 0.5% methylcellulose solution (MC) or corn oil according to their dispersibility. At the time when the present analysis was performed, 15 compounds in our database were found to decrease the plasma triglyceride level during repeated administration (Table 1). Of these, isoniazid (INAH, 50, 100, 200 mg/kg; MC), phenobarbital (PB, 10, 30, 100 mg/kg; MC), thioacetamide (TAA, 4.5, 15, 45 mg/kg; MC), benzbromarone (BBr, 20, 60, 200 mg/kg; MC), methapyrilene (MP, 10, 30, 100 mg/kg: MC), amiodarone hydrochloride (AM, 20, 60, 200 mg/kg; MC), gemfibrozil (GFZ, 30, 100, 300 mg/kg; corn oil) and sulfasalazine (SS, 100, 300, 1000 mg/kg; MC) were purchased from Sigma Aldrich (St. Louis, MO, USA). Alpha-naphthylisothiocyanate (ANIT, 1.5, 5, 15 mg/kg; corn oil) was purchased from Kanto Chemical (Tokyo, Japan). Coumarin (CMA, 15, 50, 150 mg/kg; corn oil), propylthiouracil (PTU, 10, 30, 100 mg/kg; MC) and WY-14,643 (WY, 10, 30, 100 mg/kg; corn oil) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Carbon tetrachloride (CCL4, 30, 100, 300 mg/kg; corn oil), clofibrate (CFB, 30, 100, 300 mg/kg; corn oil) and omeprazole (OPZ, 100, 300, 1000 mg/kg; MC) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Body weights were recorded every day while food consumption was recorded every 4 days during repeated dosing and expressed as g/day. The animals were treated for 3, 7, 14 or 28 days, and they were sacrificed 24 hr after the last dosing. Blood samples were collected to heparinized tube under ether anesthesia from the abdominal aorta after which the animals were sacrificed.

Blood chemistry analysis

Heparinized blood samples were centrifuged at 1,600 × g for 20 min to obtain plasma and the concentration of TG was determined using an automated clinical analyzer (Japan Bioassay Research Center and Anpyo Center; HITACHI 7070, Hitachi Ltd., Food and Drug Safety Center: COBAS MIRA plus, Roche Diagnostics, Bozo Research Center: TBA-120FR, Toshiba Lab Medical, Tokyo, Japan).

Microarray analysis

After collecting the blood, the animals were euthanized by exsanguinations from abdominal aorta under ether anesthesia. An aliquot of the sample (about 30 mg) for RNA analysis was obtained from the left lateral lobe of the liver in each animal immediately after sacrifice, kept in RNAlater®(Ambion, Austin, TX, USA) overnight at 4°C, and frozen at −80°C until use. Liver samples were homogenized with the buffer RLT supplied in RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and total RNA was isolated according to the

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).

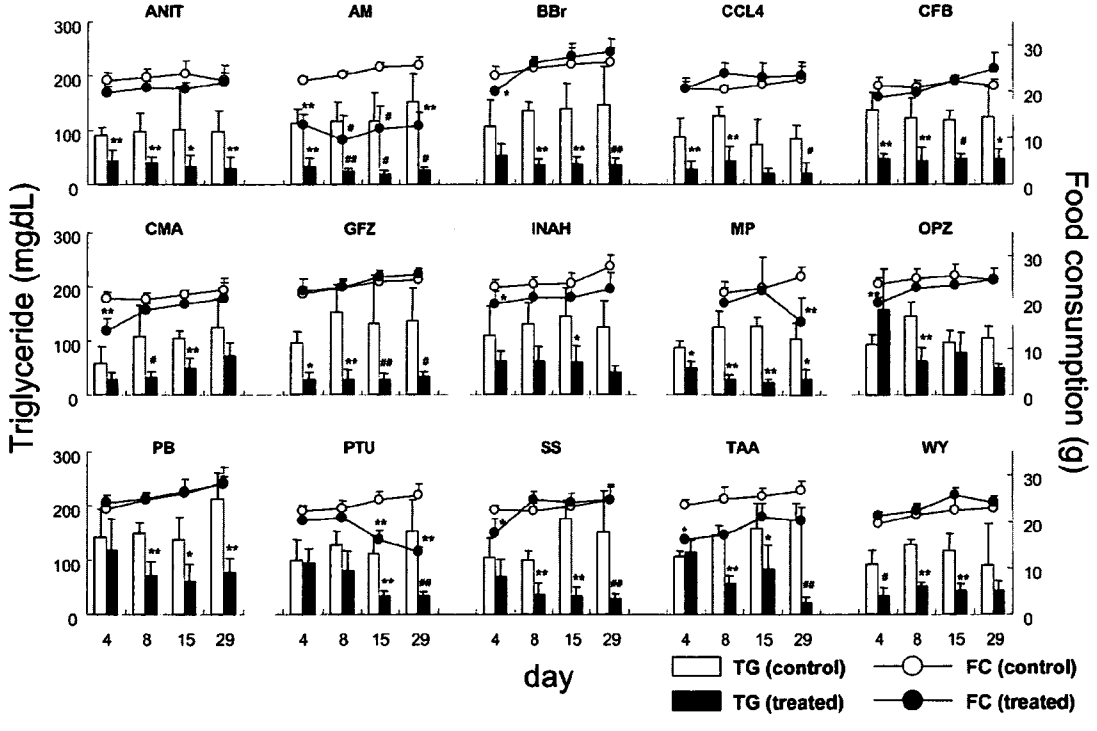

Fig. 1. Effects of TG-decreasing compounds on food consumption and plasma TG level.
Six-week-old male Sprague-Dawley rats were gavaged with each compound for 3, 7, 14 or 28 days, and they were sacrificed 24 hr after the last dosing. Food consumption was recorded every 4 days during dosing and blood samples 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

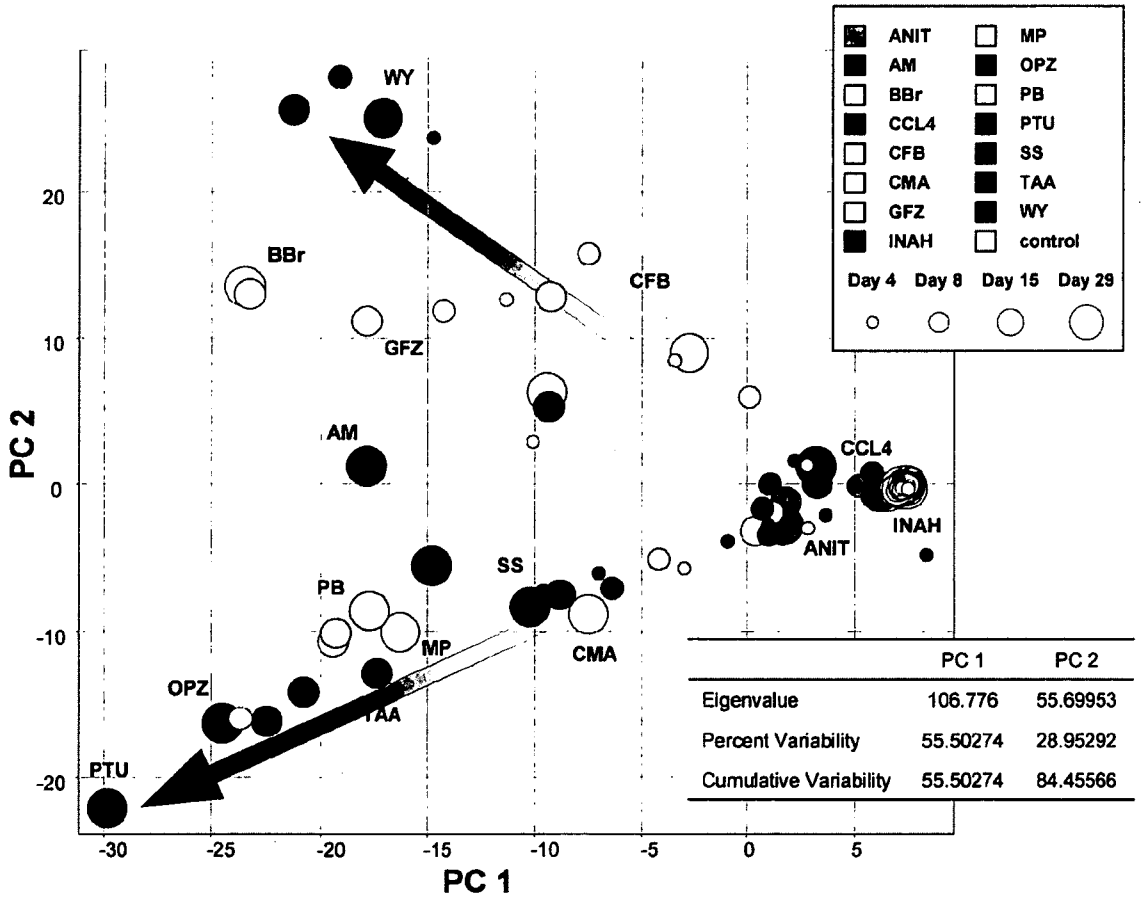

Fig. 2. Principal component analysis of gene expression profiles.
PCA of gene expression profiles was performed using identified 218 probe sets for the data of the highest dose of 15 compounds at various time points. The selection of the 218 probe sets related to plasma TG decrease is described in materials 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

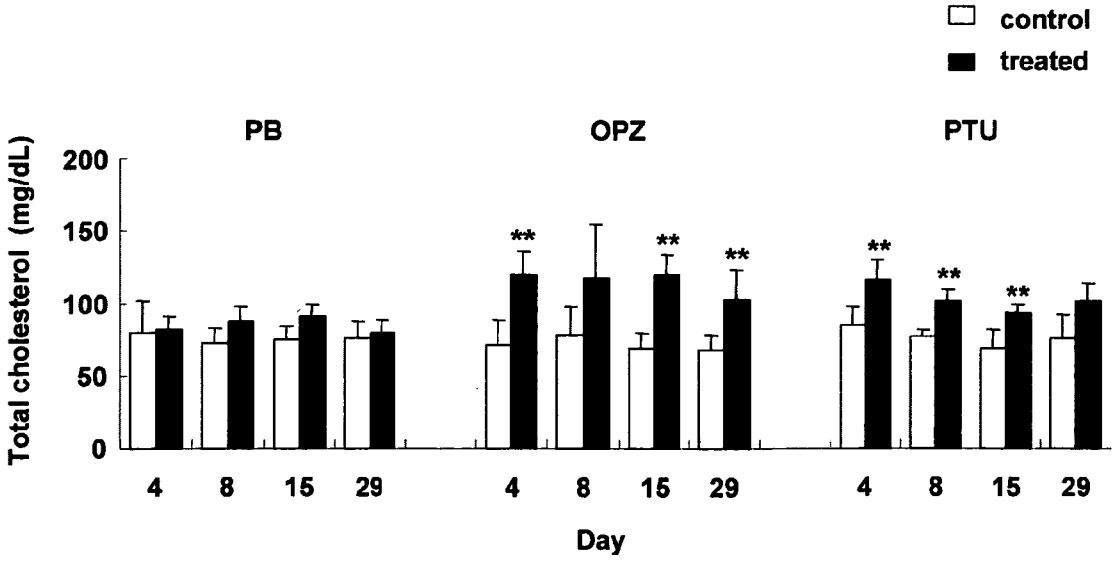

Fig. 3. Effects of PB, OPZ, PTU on plasma total cholesterol level.
Plasma total cholesterol concentrations were estimated as described in materials and methods. Open (control) and filled (treated) columns represent total plasma cholesterol concentration. Values are expressed as mean ± SD of 5 rats for 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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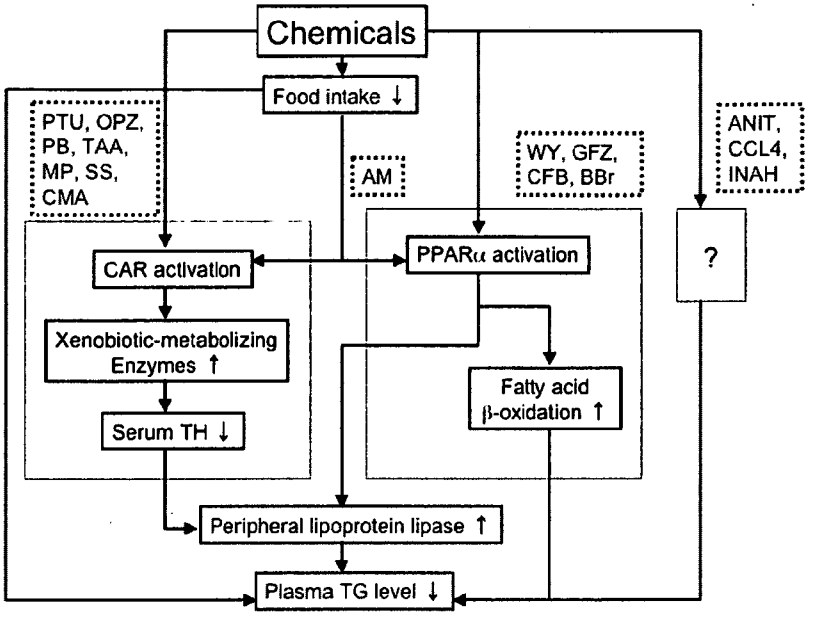

Fig. 4. Proposed TG-decreasing mechanisms of various drugs elucidated by comprehensive gene expression analysis.
Intake of chemicals induces direct or indirect CAR and/or PPARα activation. CAR activation leads to induction of hepatic xenobiotic-metabolizing enzymes, which reduces the serum thyroid hormone (TH) level. PPARα activation leads to hepatic induction of fatty acidβ-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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