BDZ, 5398 for COL, 3473 for CPZ, 4258 for CSA, 4656 for GFZ, 4917 for PHA, and 2403 for RIF. We then selected the probe sets that were commonly changed in all the compounds and 59 probe sets were obtained. A list of these probe sets is in Table 2, where each probe is categorized by its biological function. Figure 2 shows the expression changes of these 59 probe sets at high dose of repeated administration, as a heat map of the average of log 2 ratios. It appears that the trend of the change is to decrease, and the direction of change of the probe sets is not necessarily common with these drugs. Based on gene ontology, the contents of the genes related to cellular metabolism, biosynthesis, lipid metabolism, cellular biosynthesis, cellular physiological process, response to stress, physiological process, and serine family amino acid metabolism were significantly increased (Table 3).

*Principal component analysis*

Using the 59 probe sets extracted as above, PCA was performed on the seven drugs elevating TBIL and DBIL. As shown in Figure 3, the treated samples were dose-dependently separated to form clusters from the controls, mainly toward the direction of PC1 (contribution rate: 30.4%). Of the genes contributing to PC1, those with high eigenvalue are listed in Table 4. To examine the time-dependency, all the samples were aligned on a one dimensional graph of PC1 (Figure 4). It appeared that the PC1 value generally increased with time and with the dose for these drugs. In the cases of COL and BDZ, the PC1 value increased with the peak on the 4th day. In case of CPZ, time- and dose-dependency were obscure although the group treated clearly formed a cluster separated from the control cluster.

*Verification of the probe sets using 18 test compounds*

In a survey of the literature, in addition to the seven drugs above, we identified 18 more drugs in our database that had been reported to elevate TBIL and DBIL. The data of TBIL and DBIL of the 18 compounds are shown in Figure 5. We performed PCA using the 59 probe sets on the seven typical and 18 additional drugs (25 total), and depicted them in Figure 6 as a one-dimensional graph with PC1 (contribution rate: 35.9 %). It was revealed that 12 out of 18 drugs, that is methapyrilene, thioacetamide, ticlopidine, ethinyl estradiol, alpha-naphthylisothiocyanate, indomethacin, methyltestosterone, penicillamine, allyl alcohol, aspirin, iproniazid, and isoniazid were separated from control clusters in the same way as the seven typical drugs elevating TBIL and DBIL. Of these 12 drugs, MP, TAA, EE, ANIT, PEN, and IPA showed significant elevation of TBIL and DBIL for at least one time point during repeated administration, whereas the remaining six drugs did not show any toxicologically meaningful elevation. Glibenclamide (GBC) and cyclophosphamide did not change their position very much on the PCA, whereas their extent was roughly equivalent to that of CPZ, a positive control. The remaining four drugs, that is nitrofurantoin, valproate, methotrexate, and tetracycline stayed in the same position as their controls. Reviewing the data of TBIL and DBIL of the latter six drugs with low PC1 values, it was revealed that a statistically significant elevation was absent except for CPA, which showed an increase after 28 days of repeated administration, but its absolute value was quite low (Figure 5). In general, most drugs that had high PC1 values showed high serum concentration of TBIL and DBIL. As shown in Figure 7, the PC1 value had a high correlation with TBIL and DBIL levels.

*Possibility of the distinction by samples taken at 24 h after single dose*

The above results clearly suggested that the list of extracted 59 probe sets was a useful diagnostic marker for the elevation of TBIL and DBIL in rat liver. The next question is whether the list works as a prognostic marker for drugs elevating TBIL and DBIL. To examine this possibility, we analyzed the data within 24 h of a single dose of the first seven drugs. Figure 8 shows TBIL and DBIL at 3, 6, 9, and 24 h after a single dose. Although there was some significant increase in these measures, most of them were considered to be toxicologically insignificant based on their absolute values. It was thus concluded that no severe elevation of TBIL and DBIL occurred within 24 h for a single dose. Using the gene expression data at 24 h after dosing, PCA was performed using the 59 probe sets. As shown in Figure 9, all the drugs except RIF and CSA were clearly separated from the control samples in the direction of PC1(contribution rate: 33.9%). Among these, CPZ, which showed low PC1 values in repeated dosing (Figures 4 and 6), was not distant from its control by single dosing. Interestingly, however, an excellent separation was attained when extra-high dose of CPZ was added (Figure 9).

## Discussion

Hepatotoxic adverse effects, often indicated by cholestasis, are a main concern in drug development and severe hepatotoxicity may cause a drug to be

**Table 2** List of 59 probe sets changed in seven compounds elevating of TBIL and DBIL

| *Probe ID* | *Accession No.* | *Gene title* | *Gene symbol* |
|---|---|---|---|
| Lipid metabolic process | | | |
| 1368520_at | NM_012737 | Apolipoprotein A-IV | Apoa4 |
| 1370150_a_at | NM_012703 | Thyroid hormone responsive protein | Thrsp |
| 1371615_at | BI279069 | Diacylglycerol O-acyltransferase homolog 2 (mouse) | Dgat2 |
| 1374440_at | BE098506 | Dehydrogenase/reductase (SDR family) member 8 | Dhrs8 |
| 1387139_at | NM_032082 | Hydroxyacid oxidase 2 (long chain) | Hao2 |
| 1387508_at | NM_017300 | Bile acid-Coenzyme A: amino acid N-acyltransferase | Baat |
| 1390549_at | AA859796 | Adiponectin receptor 2 | Adipor2 |
| 1394112_at | AA945123 | Hydroxyacid oxidase 1 | Hao1 |
| Transporter | | | |
| 1368621_at | NM_022960 | Aquaporin 9 | Aqp9 |
| 1369074_at | NM_130748 | Solute carrier family 38, member 4 | Slc38a4 |
| 1379592_at | AI045151 | Similar to citrin (predicted) | RGD1565889_predicted |
| 1386960_at | NM_031589 | Solute carrier family 37 (glucose-6-phosphate transporter), member 4 | Slc37a4 |
| 1390591_at | AI169163 | Solute carrier family 17 (sodium phosphate), member 3 | Slc17a3 |
| 1393216_at | BI282044 | Solute carrier family 33 (acetyl-CoA transporter), member 1 | Slc33a1 |
| 1398249_at | NM_053965 | Solute carrier family 25 (mitochondrial carnitine/ acylcarnitine translocase), member 20 | Slc25a20 |
| Ubiquitin-Proteasome | | | |
| 1383073_at | BG666028 | Ubiquitin specific protease 14 | Usp14 |
| 1398831_at | NM_031629 | Proteasome (prosome, macropain) subunit, beta type 4 | Psmb4 |
| Mitochondrial function | | | |
| 1388931_at | AA799440 | Mitochondrial ribosomal protein L13 | Mrpl13 |
| 1367941_at | NM_031326 | Transcription factor A, mitochondrial | Tfam |
| 1370918_a_at | BI275939 | ATP synthase, $H^+$ transporting, mitochondrial F1 complex, ypolypeptide 1 | Atp5c1 |
| 1372080_at | BI287936 | Inner membrane protein, mitochondrial | Immt |
| 1398326_at | BI282332 | Similar to Nur77 downstream protein 2 | MGC105647 |
| Cell proliferation/Cell cycle | | | |
| 1367764_at | NM_012923 | Cyclin G1 | Ccng1 |
| 1367927_at | BI282863 | Prohibitin | Phb |
| 1369738_s_at | NM_017334 | cAMP responsive element modulator | Crem |
| 1387714_at | AB031423 | cAMP responsive element modulator | Crem |
| 1372437_at | BM390921 | S-phase kinase-associated protein 1A | Skp1a |
| 1367512_at | AA998435 | Chromatin modifying protein 5 | Chmp5 |
| 1374591_at | AI409042 | similar to protein tyrosine phosphatase, receptor type, D (predicted) | RGD1561090_predicted |
| 1388469_at | AA945615 | Insulin-like growth factor I mRNA, 3' end of mRNA | — |
| Translation | | | |
| 1367610_at | NM_031103 | Ribosomal protein L19 | Rpl19 |
| 1388244_s_at | BG153272 | Ribosomal protein SA | Rpsa |
| 1371973_at | AI237620 | Eukaryotic translation initiation factor 3, subunit 6 | Eif3s6 |
| Metabolism | | | |
| 1371076_at | AI454613 | Cytochrome P450, family 2, subfamily b, polypeptide 15 /// Cytochrome P450, family 2, subfamily b, polypeptide 2 | Cyp2b15 /// Cyp2b2 |
| 1368905_at | NM_133586 | Carboxylesterase 2 (intestine, liver) | Ces2 |
| 1369558_at | NM_022614 | Inhibin beta C | Inhbc |
| 1387022_at | NM_022407 | Aldehyde dehydrogenase family 1, member A1 | Aldh1a1 |
| 1387034_at | NM_012619 | Phenylalanine hydroxylase | Pah |
| 1388788_at | BG664131 | Glutaryl-Coenzyme A dehydrogenase (predicted) | Gcdh_predicted |
| 1398286_at | M64755 | Cysteine sulfinic acid decarboxylase | Csad |
| Response to oxidative stress | | | |
| 1367896_at | AB030829 | Carbonic anhydrase 3 | Ca3 |
| 1370064_at | AB004454 | Presenilin 2 | Psen2 |
| Inflammatory response | | | |
| 1367804_at | NM_017170 | Serum amyloid P-component | Apcs |
| Blood coagulation | | | |
| 1388330_at | BM384958 | Vitamin K epoxide reductase complex, subunit 1 | Vkorc1 |
| 1374765_at | BI288055 | Transcribed locus, moderately similar to XP_001090810.1 fibrinogen gamma chain isoform 9 [Macaca mulatta] | — |
| Other | | | |
| 1372479_at | AI175666 | Transcribed locus | — |
| 1373313_at | BM391570 | Transcribed locus | — |
| 1374943_at | AI170809 | Transcribed locus | — |
| 1375845_at | BI290029 | Similar to Aig1 protein (predicted) | RGD1562920_predicted |
| 1377048_at | H31813 | Similar to cDNA sequence BC021917 | RGD1311026 |

*(continued)*

**Table 2** *(continued)*

| *Probe ID* | *Accession No.* | *Gene title* | *Gene symbol* |
|---|---|---|---|
| 1377686_at | AA859337 | Transcribed locus | — |
| 1381574_at | BF403907 | Similar to putative protein, with at least 6 transmembrane domains, of ancient origin (58.5 kD) (3N884) (predicted) | RGD1312038_predicted |
| 1383732_at | AA819810 | Similar to hypothetical protein MGC37914 (predicted) | RGD1307603_predicted |
| 1387856_at | BI274457 | Calponin 3, acidic | Cnn3 |
| 1388119_at | BM392140 | Similar to heterogeneous nuclear ribonucleoprotein A3 /// similar to heterogeneous nuclear ribonucleoprotein A3 (predicted) /// similar to regulator of G-protein signalling like 1 | LOC364506 /// LOC684137 /// RGD1566284_predicted |
| 1390326_at | BF564217 | Angiogenin, ribonuclease A family, member 1 | Ang1 |
| 1392172_at | AI169984 | Chemokine (C-C motif) ligand 9 | Ccl9 |
| 1393123_at | BM392153 | Complement component 8, gamma polypeptide (predicted) | C8g_predicted |
| 1398409_at | AA850428 | Transcribed locus | — |

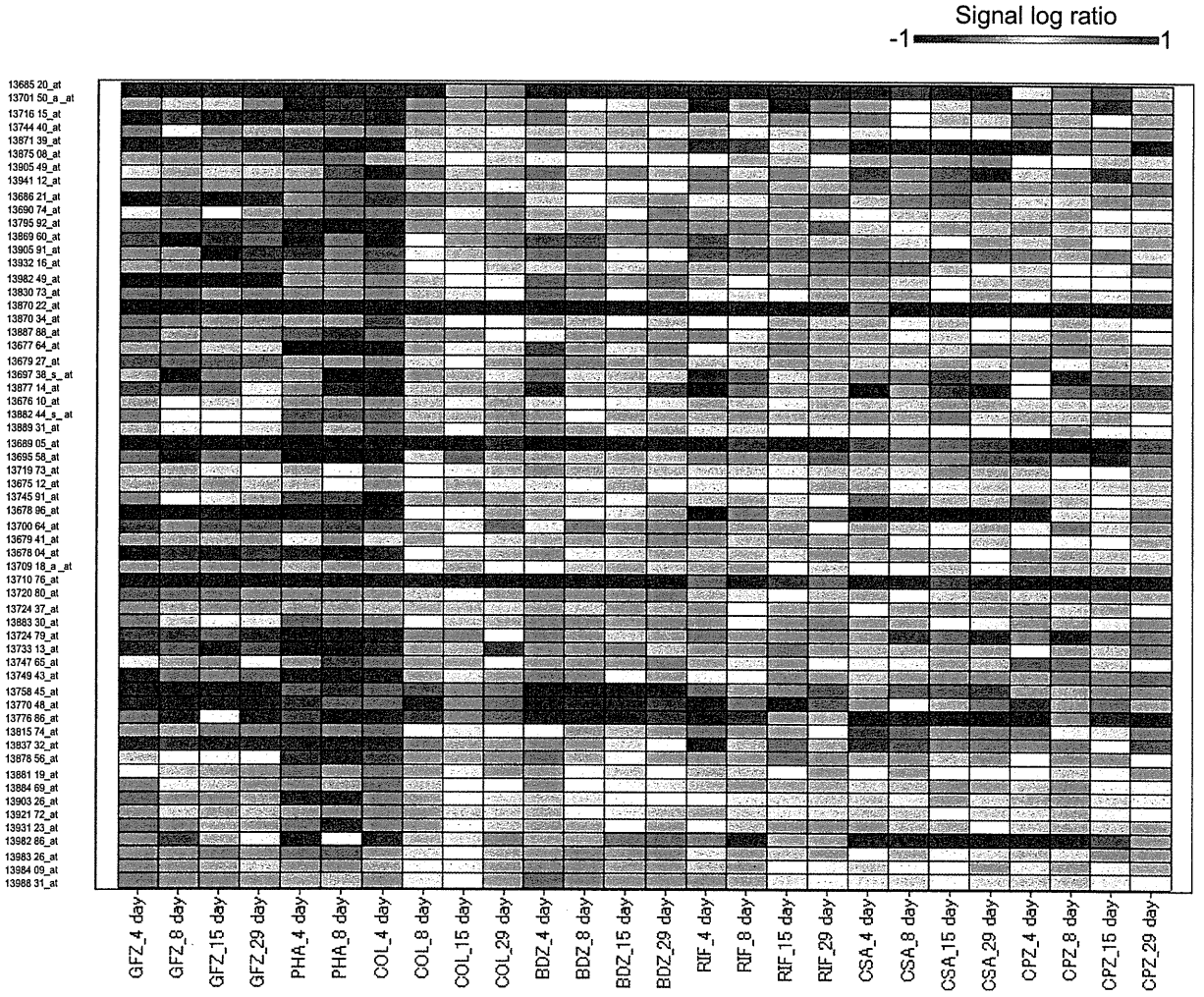


**Figure 2** Heat map of the gene expression profiles of gemfibrozil (GFZ), phalloidin (PHA), colchicine (COL), bendazac (BDZ), rifampicin (RIF), cyclosporine A (CSA), and chlorpromazine (CPZ) that induced elevation of total bilirubin (TBIL) and direct bilirubin (DBIL) in the present study using the commonly mobilized 59 probe sets. Values are expressed as average log 2 ratio, for each time point at high dosage.

**Table 3** GO analysis of identified 59 probe sets

| *Term* | *Count* | *Percent* | *P-value* |
|---|---|---|---|
| Metabolism | 32 | 51.6 | 3.5E-3 |
| Cellular metabolism | 29 | 46.8 | 1.2E-2 |
| Biosynthesis | 9 | 14.5 | 6.0E-2 |
| Lipid metabolism | 6 | 9.7 | 6.3E-2 |
| Cellular biosynthesis | 8 | 12.9 | 8.6E-2 |
| Cellular physiological process | 40 | 64.5 | 8.8E-2 |
| Response to stress | 8 | 12.9 | 8.9E-2 |
| Physiological process | 43 | 69.4 | 9.6E-2 |
| Serine family amino acid metabolism | 2 | 3.2 | 1.0E-1 |

withdrawn from the market. Cholestasis results either from a functional defect in bile formation at the level of the hepatocyte (hepatocellular cholestasis) or from an impairment in bile secretion and flow at the level of bile ductules or ducts (ductular/ductal cholestasis). Cholestatic hepatitis is the most common type of drug-induced cholestasis and is more frequent than cholestatic viral hepatitis. It is caused by a metabolic or, more frequently, immunological idiosyncrasy of a drug. Therefore, drugs causing this type of reaction are unpredictable, dose-independent hepatotoxins, and cholestasis occurs only in a small proportion of exposed individuals.

Prototypic drugs causing cholestatic hepatitis include RIF,[8] CPZ,[9–12] GFZ,[13] COL,[14–16] BDZ,[17] CSA,[18–20] and PHA.[21] Indeed, they had elevated TBIL and/or DBIL in our database. We then selected this phenotype to search for a biomarker.

The goal of our project is to extract toxicity biomarkers useful for drug development from our transcriptome database. In the course of our study, we have used a strategy to select a few genes that showed a common change in response to a certain phenotype among the database, but usually we got nothing. Even if we got a candidate, its reproducibility was poor and thus useless. We also frequently experienced such a case that the fingerprint marker genes reported from other institutes are quite different from those in our gene list. This might not be due to the problem of genomics technology, but due to the biology, that is a toxicological phenotype is a result of various biological factors, each of which contains inevitable variations. Therefore, one has to use a strategy to make a prediction based on a pattern of changes of considerable numbers of genes, in order to assure the robustness of the result.

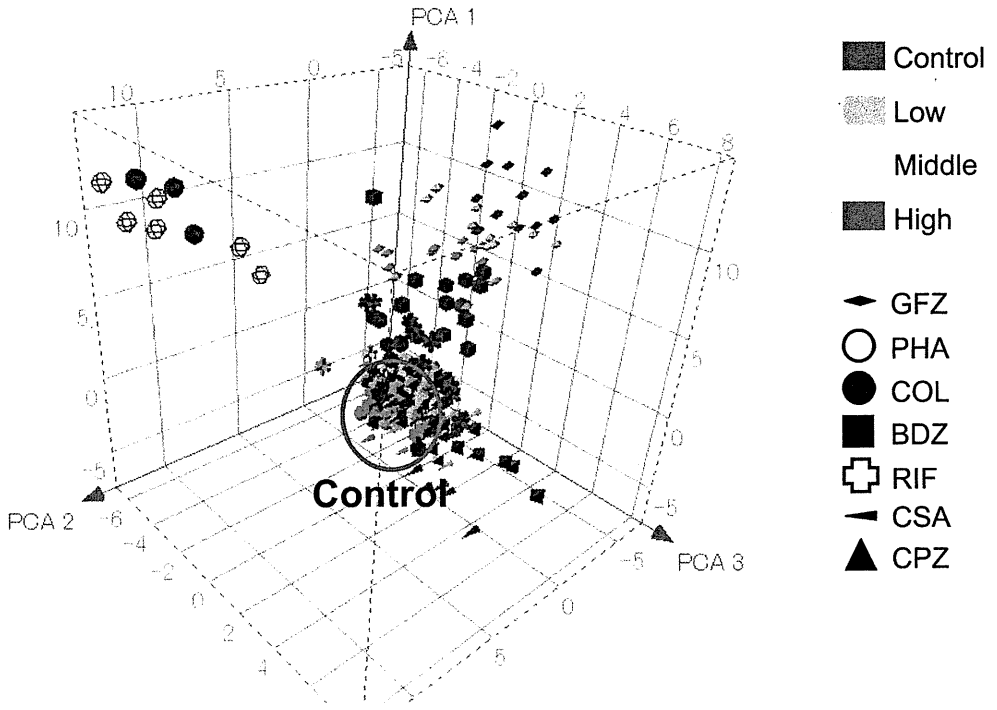


**Figure 3** Principal component analysis of the gene expression profiles of gemfibrozil (GFZ), phalloidin (PHA), colchicine (COL), bendazac (BDZ), rifampicin (RIF), cyclosporine A (CSA), and chlorpromazine (CPZ), using the commonly mobilized 59 probe sets. Results are expressed as a three dimensional figure for PC1, 2 and 3. Treated samples were dose-dependently separated from the cluster of the controls (circled by a blue line), mainly toward the direction of PC1 (contribution rate: 30.4 %). For simplicity, rats receiving the same dose with different durations (3, 7, 14, and 28 days [except PHA for 3 and 7 days], $N = 3$ for each; 12 total) are expressed by the same symbol.

**Table 4** List of 18 probe sets contributing PC1

| *Ranking* | *Probe set ID* | *Gene title* | *Eigenvalue* |
|---|---|---|---|
| 1 | 1367927_at | Prohibitin | 0.202260 |
| 2 | 1368905_at | Carboxylesterase 2 (intestine, liver) | 0.197374 |
| 3 | 1387022_at | Aldehyde dehydrogenase family 1, member A1 | 0.184622 |
| 4 | 1372080_at | Inner membrane protein, mitochondrial | 0.183434 |
| 5 | 1375845_at | Similar to Aig1 protein (predicted) | 0.180442 |
| 6 | 1398831_at | Proteasome (prosome, macropain) subunit, beta type 4 | 0.175160 |
| 7 | 1383073_at | Ubiquitin specific protease 14 | 0.175074 |
| 8 | 1398249_at | Solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20 | 0.170555 |
| 9 | 1372479_at | Transcribed locus | 0.154284 |
| 10 | 1398409_at | Transcribed locus | 0.143625 |
| 11 | 1372437_at | S-phase kinase-associated protein 1A | 0.129215 |
| 12 | 1367512_at | Chromatin modifying protein 5 | 0.121613 |
| 13 | 1367764_at | Cyclin G1 | 0.120058 |
| 14 | 1388244_s_at | Ribosomal protein SA | 0.118388 |
| 15 | 1367610_at | Ribosomal protein L19 | 0.116662 |
| 16 | 1371973_at | Eukaryotic translation initiation factor 3, subunit 6 | 0.110324 |
| 17 | 1388931_at | Mitochondrial ribosomal protein L13 | 0.109371 |
| 18 | 1367941_at | Transcription factor A, mitochondrial | 0.107880 |

There are two representative ways of classification, that is supervised and unsupervised ones. When the mechanism of action of the drug is clear, or the feature of the drug is undoubtedly designated, a supervised method, such as discriminant analysis is a powerful tool. In fact, we reported a success using this strategy.[3] However, in most of the cases of toxicological phenotypes, it is usually difficult to judge which one is positive or negative. In case of medicines, it hardly happens that a drug never causes a certain phenotype at any dose level. Otherwise, a drug developer needs to know a safety margin. In such cases, it is quite difficult to set positive/negative since the sensitivity of the detection is usually different between gene expression and toxicological phenotype. To our experience, discriminant analysis is often difficult for a phenotype induced by multiple causes or it is highly dependent on the dose level and time after dosing, since a small difference in a choice of positive/negative significantly affects the results. However, unsupervised classification such as PCA or hierarchical clustering enables one to visualize the feature of various drugs semi-quantitatively, although it is not quantitative. This could supply important information for drug development. We already reported candidate marker gene sets for diagnosis and prediction of phospholipidosis.[2]

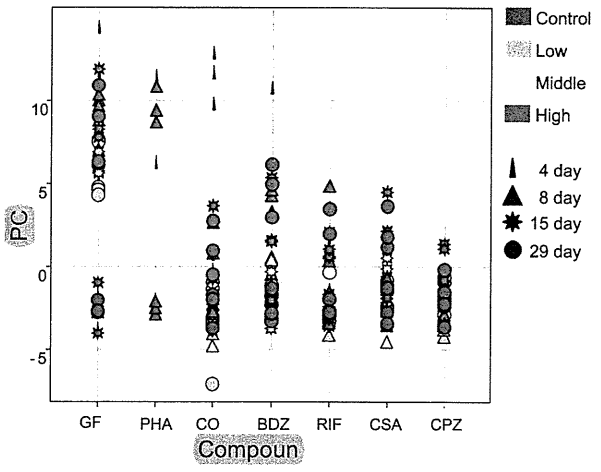


**Figure 4** Principal component analysis of the same as Figure 3 but one dimensional expression using principal component 1. For each drug, each individual rat is depicted by a symbol with a different color and shape as shown on the right panel.

In the current study, we used the latter strategy, that is we extracted probe sets for commonly mobilized genes among seven typical drugs causing cholestasis. Using these genes, we have shown that PCA clearly separated dose- and time-dependent clusters of the treated groups from their controls. To verify the usefulness of the probe sets, we explored the potential drugs causing cholestasis in the literature, and further (rarer) examples were found to be antifungal, anthelminthic, antidepressant, anticonvulsive, antihypertensive, antiarrhythmic, antidiabetic, antithyroid, antirheumatic drugs, and $H_2$-blockers. We then selected 18 drugs for verification from our database.

Of these drugs, MP,[22] TAA,[23] EE,[24–28] ANIT,[29] and PEN[30,31] showed high TBIL and DBIL as well as high PC1 by PCA using these probe sets. In the case of TCP,[32] IM,[33,34] MTS,[35–37] AA,[38] ASA,[39] IPA,[40,41] and INAH,[42] which have been reported to cause cholestasis, these were judged as negative by blood chemical examination in the current study. However, they were clearly separated from their controls by PCA using the probe sets. This suggests that the sensitivity of the diagnosis by gene expression is higher than the measurement of plasma bilirubin.

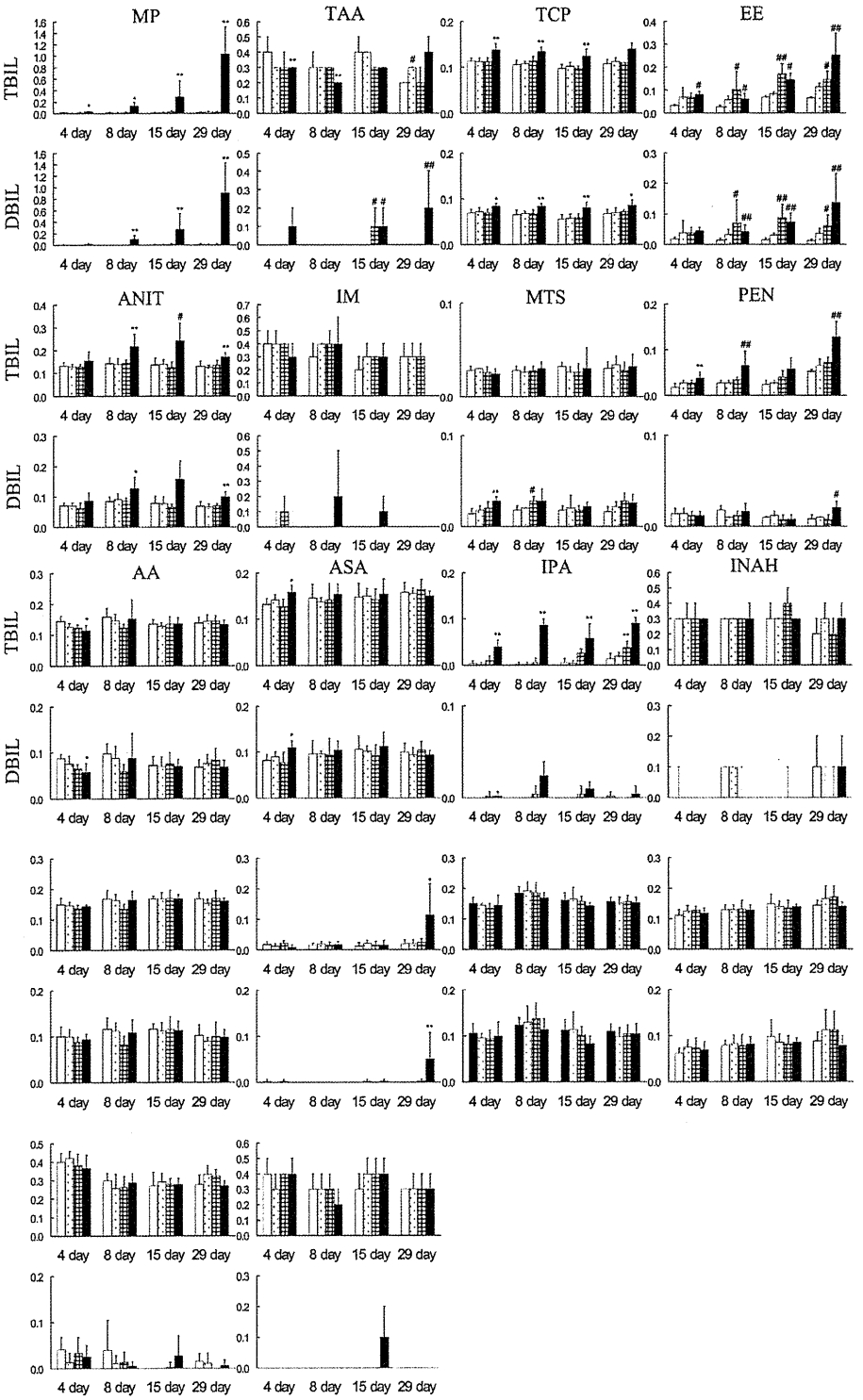


**Figure 5** Plasma total bilirubin (TBIL) and direct bilirubin (DBIL) concentrations for rats treated with 18 test-drugs. Plasma TBIL and DBIL concentrations were estimated as shown in Figure 1. Open (control), dotted (low dose), checked (middle dose), and filled (high dose) columns represent plasma TBIL and DBIL concentrations (mg/dL). Values are expressed as mean ± SD of five rats for each time and compound. Significant difference from the control rats: (*$P < 0.05$, **$P < 0.01$: Dunnett test, # $P < 0.05$, ## $P < 0.01$: Dunnett type mean rank test).

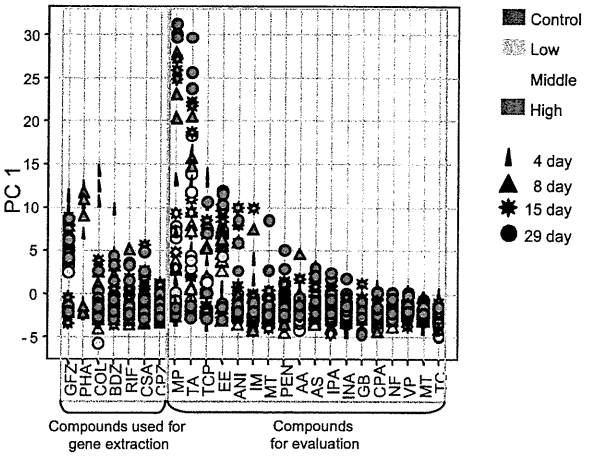


**Figure 6** Principal component analysis of gene expression profiles of 18 more drugs which have been reported to elevate total and direct bilirubin, in addition to the seven typical drugs using the commonly mobilized 59 probe sets. Of the 25 compounds, the seven typical compounds used in Figures 3 and 4, are shown on the left panel. Results are expressed as a one dimensional figure with PC1 (contribution rate: 35.9 %). For each drug, each individual rat is depicted by a symbol with a different color and shape, as shown on the right panel.

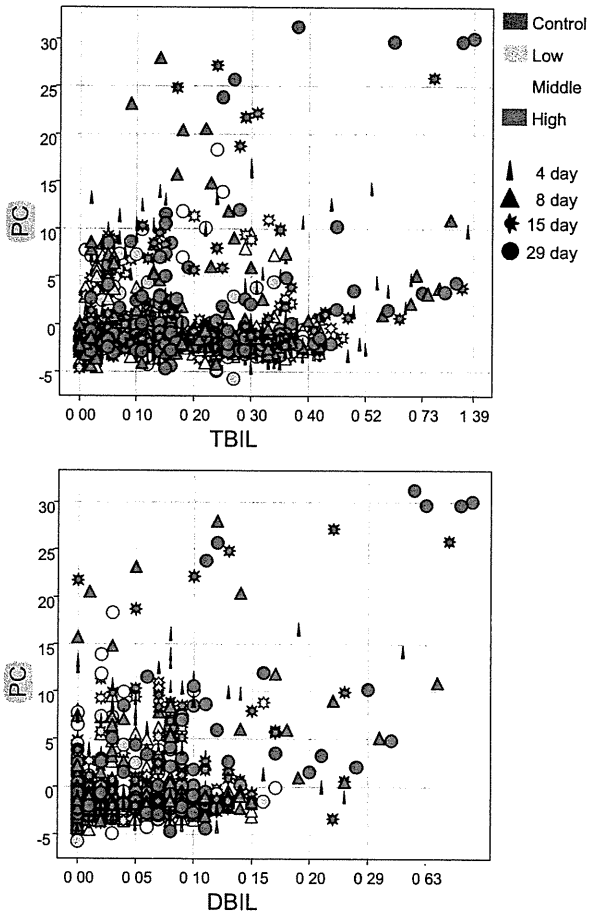


**Figure 7** Correlation between the PC1 value, total bilirubin (TBIL), and direct bilirubin (DBIL). Results are expressed as a graph with TBIL or DBIL on the *y*-axis and PC1 value on the *x*. Each individual rat is depicted by a symbol with a different color and shape, as shown on the right panel. Note that the PC1 value shows a high correlation with the TBIL and DBIL with a few exceptions.

CPA is reported to induce cholestasis,[43] but showed a low PC1 value in the current study. Stone, *et al.*[44] observed that plasma bile salt concentration was increased in rats after administration of 10 mg/kg of CPA. They suggested that CPA inhibits the uptake of bile acids from the portal blood into hepatocytes. Moreover, there was no change in liver histology in cholestasis caused by CPA,[45] suggesting that the cytotoxicity of intracellular bile acids induced by CPA is not very severe, and it might be the reason why this drug was not separated by PCA in the current study.

Based on the successful classification of the drugs by PCA using the extracted 59 probe sets, it is expected that the expression changes of these genes are directly or indirectly related to the pathogenesis of cholestasis. From experimental models of cholestasis, several mechanisms have been postulated to account for impaired bile secretion. They are 1) inhibition of $Na^+$, $K^+$-ATPase; 2) increased paracellular permeability and regurgitation into plasma of bile constituents; 3) impaired function of the cytoskeleton, mainly microfilaments; 4) alteration of intracellular calcium homeostasis; 5) alteration or mislocation of canalicular carriers; 5) ductular obstruction.[46–48] Reviewing the 59 probe sets, however, we could not identify genes related to the mechanisms listed above. It could be pointed out that it contains considerable numbers of 1) components regulating lipid metabolism, 2) transporters, 3) ubiquitin-proteasome related factors, and 4) mitochondrial components (Table 2).

The discovery that lithocholic acid (LCA) is an endogenous ligand for pregnane X receptor (PXR) suggests that bile acids may also regulate drug metabolism in the liver and intestine by the induction of CYP450 enzymes.[49,50] PXR and its human ortholog steroid and xenobiotic receptor induce the CYP3A, CYP2B, and CYP2C families of steroid- and drug-metabolizing enzymes in the liver and intestine.[51] LCA is the most efficacious bile acid that activates PXR and induces CYP3A4 to catalyze 6-hydroxylation of LCA to hyodeoxycholic acid.[49,50] Lipid metabolic process related genes and CYP2B were generally down-regulated by administration of

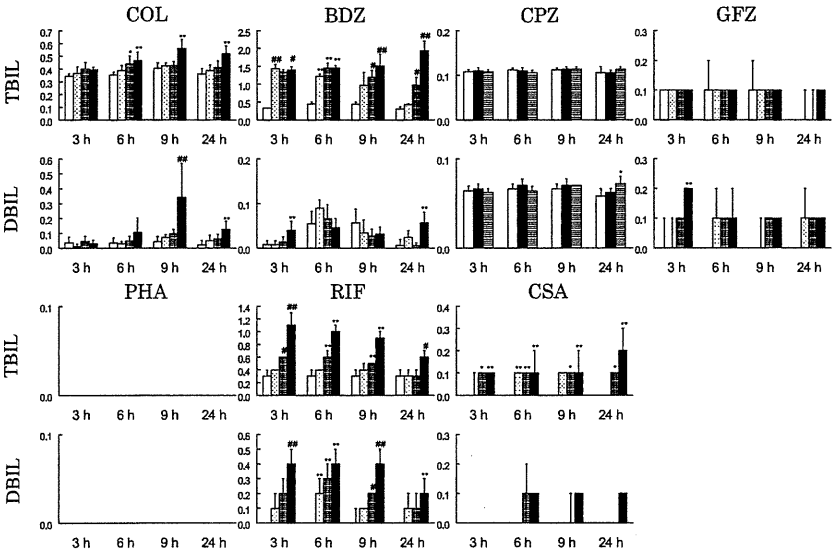


**Figure 8** Plasma total bilirubin (TBIL) and direct bilirubin (DBIL) concentrations for rats after a single dose treatment with gemfibrozil (GFZ), phalloidin (PHA), colchicine (COL), bendazac (BDZ), rifampicin (RIF), cyclosporine A (CSA), and chlorpromazine (CPZ). Plasma TBIL and DBIL concentrations were estimated as shown in Figure 1. Open (control), dotted (low dose), checked (middle dose), filled (high dose), and horizontal-striped (extra-high dose) columns represent the plasma TBIL and DBIL concentrations (mg/dL). Values are expressed as mean ± SD of five rats each for each time and compound. Significant difference from the control rats: (*$P < 0.05$, ** $P < 0.01$: Dunnett test, # $P < 0.05$, ## $P < 0.01$: Dunnett type mean rank test). Note that no pathologically meaningful elevation of TBIL and DBIL occurred yet, except for COL, BDZ, and RIF.

the positive drugs in this study. This might be a feedback response to elevated bilirubin.

Hepatic uptake and efflux processes involved in bile formation are maintained by distinct transport systems (sodium-dependent and sodium-independent transport pathways and ATP-dependent efflux pumps) expressed at the two polar surface domains of liver cells.[47] In the current study, some transporters, Aqp9, and Slc families, were mobilized by administration of the drugs causing cholestasis. This might be also a feedback response to elevated bilirubin.

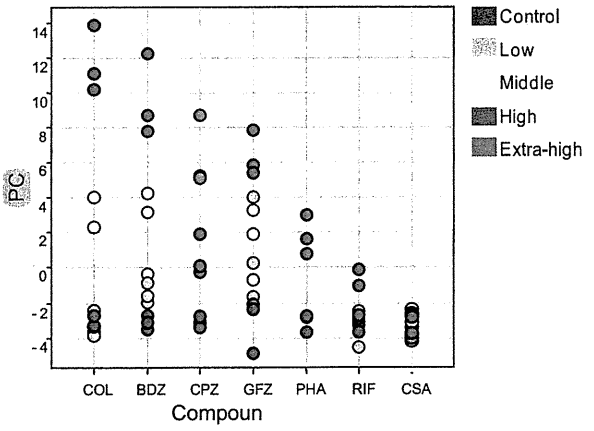


**Figure 9** Principal component analysis of gene expression profiles of the samples of 24 h after single dosage using 59 probe sets. Results are expressed as a one dimensional figure with PC1 (contribution rate: 33.9 %). Each individual rat is depicted by a symbol with a different color and shape as shown on the right panel. Note that the higher dose (magenta) showed even higher PC1 values.

At present, we cannot propose a close link between cholestasis and ubiquitin-proteasome or mitochondrial function. For the former, it was reported that ubiquitination of cytokeratin was involved in cholestasis produced by bile-duct ligation.[52] As for the mitochondrial function, it was described that cholestasis practically disturbed mitochondrial bioenergetics,[53] and bile acids were involved in the process of cell death through mitochondrial function.[54] Investigation of these factors in cholestasis would bring a new insight in the understanding of its pathogenesis. For this purpose, the gene list in the current study would supply important information.

In conclusion, we identified 59 probe sets from gene expression profiles in rat liver treated with various bilirubin-elevating compounds stored in our database. PCA using these 59 probe sets would be useful for GeneChip users to predict the risk of cholestasis in the preclinical stage of drug development. At present, it would be difficult to make an appropriate prediction by measuring these genes by another platform, such as quantitative PCR, since the procedure is

dependent on the device. However, it would be possible that these probe sets contain a biomarker(s) useful even in a clinical field. Further work is obviously necessary to improve and generalize the candidate for a marker suggested in this study.

## Acknowledgment

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*Original Article*

# Gene expression profiling in rat liver treated with various hepatotoxic-compounds inducing coagulopathy

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**ABSTRACT** — A large-scale transcriptome database of rat liver (TG-GATEs) has been established by the Toxicogenomics Project in Japan. In the present study, we focused on 8 hepatotoxic compounds within TG-GATEs, i.e., clofibrate, omeprazole, ethionine, thioacetamide, benzbromarone, propylthiouracil, Wy-14,643 and amiodarone, which induced coagulation abnormalities. Aspirin was selected as a reference compound that directly causes coagulation abnormality, but not through liver toxicity. In blood chemical examinations, for all the coagulopathic compounds there was little elevation of aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT), suggesting no severe cell death by treatment with the compounds. We extracted 344 probe sets from the data for these 8 typical drugs, which induced this phenotype at any time from 3 to 28 days of repeated administration. Principal component analysis using these probe sets clearly separated dose- and time-dependent clusters of the treated groups from their controls, except aspirin and propylthiouracil, both of which were considered to cause coagulopathy not due to their hepatotoxicity but due to their direct effects on the blood coagulation system. Reviewing the extracted genes, changes in lipid metabolism were found to be dominant. Genes related to blood coagulation were generally down-regulated by these drugs except that vitamin K epoxide reductase complex subunit 1 (Vkorc1) like 1, a paralogous gene of Vkorc1, was up-regulated. As expected, expression changes of these genes were least prominent in aspirin or propylthiouracil-treated liver. We concluded that these probe sets could be a good starting point in developing mechanism-based biomarkers for diagnosis or prognosis of hepatotoxicity-related coagulation abnormalities in the early stage of drug development.

**Key words:** Coagulopathy, Toxicogenomics, Rat, Liver

## INTRODUCTION

In toxicological examinations, abnormality in blood coagulation is often associated with hepatotoxicity, since most factors related to the coagulation system are synthesized by the liver. Although the best tests for hepatocellular damage so far are measurement of serum enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, lactate dehydrogenase, and γ-glutamyl transpeptidase (Fujii, 1997; Ozer *et al.*, 2008), liver insufficiency can be also approached through the measurement of serum fibrinogen and coagulation tests (Kerr *et al.*, 2003). If drug-induced coagulopathy is linked to hepatotoxicity by a mechanism-based interpretation, diagnosis or prognosis of liver damage during drug development would be greatly improved.

The Toxicogenomics Project is 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, 2008). Its aim was to construct a large-scale toxicology database of transcriptomes for prediction

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of toxicity of new chemical entities in the early stage of drug development. In 2007, the project 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 the Toxicogenomics Project, Japan). One mission of our project is to identify candidate biomarker genes to diagnose and/or predict certain toxicological phenotypes. Recently we identified a candidate biomarker for diagnosis of hepatic phospholipidosis (Hirode *et al.*, 2008), serum bilirubin-elevation (Hirode *et al.*, 2009), non-genotoxic hepatocarcinogenicity (Uehara *et al.*, 2008), glutathione depletion-responsive (Kiyosawa *et al.*, 2007), and serum triglyceride-decreasing (Omura *et al.*, 2007) using our database.

In order to investigate gene expression profiles reflecting the relationship between hepatotoxicity and coagulopathy, we picked nine hepatotoxic compounds from our database, i.e., aspirin (ASA), clofibrate (CFB), omeprazole (OPZ), ethionine (ET), thioacetamide (TAA), benzbromarone (BBr), propylthiouracil (PTU), Wy-14643 (WY), and amiodarone (AM), which induced coagulation abnormalities, such as increase in prothrombin time, increase in activated partial thromboplastin time, or decrease in serum fibrinogen concentration. When the commonly mobilized genes were extracted, ASA was excluded, since ASA is known to directly affect blood coagulation and platelet activation (Yip, 2004), but this is not due to an indirect effect via liver damage.

## MATERIALS AND METHODS

### Compounds

Of the compounds stored in our database, nine compounds were selected and used for the data analysis as listed in Table 1, in which the chemical name, abbreviation, dosage, administration route and vehicle used are summarized. In order to assure uniformity of the database, the highest dose was routinely determined as the maximally tolerated dose, chosen based on data derived from preliminary toxicity studies of 7 days duration. Subsequently, the middle and low doses were set as 1/3 and 1/10 of the highest, respectively.

### Animal treatment

The experiments were carried out as previously described in the literature (Takashima *et al.*, 2006). Male Crl:CD(SD) rats were purchased from Charles River Japan Inc., (Kanagawa, Japan) at 5 weeks of age. After a 7-day quarantine and acclimatization period, the animals were divided into groups of 5 animals using a computerized stratified random grouping method based on body weight for each age. The animals were individually housed in stainless-steel cages in a room that had lighting 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., Tokyo, Japan). Rats in each group were dosed orally with various drugs suspended or dissolved in either 0.5% methylcellulose solution or corn oil according to their dispersibility. The animals were treated for 3, 7, 14 or 28 days and were sacrificed 24 hr after the last dose. Under ether anesthesia, blood samples were collected upon sacrifice in tubes containing heparin lithium (for blood biochemistry), EDTA-2K (for hematology; (Advia 120, Bayer, Ramsey, MN, USA), or 1/9 vol of 3.8% citric acid (for coagulation). Prothorombin time, active partial prothrombin time, and fibrinogen were measured by an automated coagulation analyzer (Sysmex CA-5000, Sysmex, Hyogo, Japan). A routine blood biochemical analysis was performed by an

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

| Compound name | Abbreviation | Dose (Dose level, mg/kg) | | | Vehicle |
|---|---|---|---|---|---|
| | | Low | Middle | High | |
| Clofibrate | CFB | 30 | 100 | 300 | MC |
| Omeprazole | OPZ | 100 | 300 | 1,000 | MC |
| Ethionine | ET | 25 | 80 | 250 | MC |
| thioacetamide | TAA | 4.5 | 15 | 45 | MC |
| benzbromarone | BBr | 20 | 60 | 200 | MC |
| propylthiouracil | PTU | 10 | 30 | 100 | MC |
| Wy-14,643 | WY | 10 | 30 | 100 | CO |
| Amiodarone | AM | 20 | 60 | 200 | MC |
| Aspirin | ASA | 45 | 150 | 450 | MC |

MC: 0.5 w/v% methylcellulose; CO: corn oil

autoanalyzer (Hitachi 7080, Hitachi, Tokyo, Japan). The liver samples were obtained from each animal immediately after sacrifice. The experimental protocols were reviewed and approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Health Sciences.

### Microarray gene expression analysis

After collecting blood samples, the animals were euthanized by exsanguination from the 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 the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and total RNA was isolated according to the manufacturer's instructions. Microarray analysis was conducted on 3 out of 5 samples, selected based on their body weight excluding the highest and the lowest, for each group by using the GeneChip® Rat Genome 230 2.0 Arrays (Affymetrix, Santa Clara, CA, USA), containing 31042 probe sets. The procedure was conducted basically according to the manufacturer's instructions using the Superscript Choice System (Invitrogen, Carlsbad, CA, USA) using 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 were hybridized to a Rat Genome 230 2.0 Array for 18 hr at 45°C at 60 rpm, after which the array was washed and stained by streptavidin-phycoerythrin using a Fluidics Station 400 (Affymetrix) and then scanned by a Gene Array Scanner (Affymetrix). The digital image files were processed by Affymetrix Microarray Suite version 5.0 and intensities were normalized for each chip by setting the mean intensity to 500 (per chip normalization).

### Microarray data analysis

In order to extract probe sets related to hepatotoxicity-related coagulopathy, we first picked 9 compounds, i.e., ASA, CFB, OPZ, ET, TAA, BBr, PTU, WY and AM, for which coagulopathy (increased prothrombin time, activated partial thromboplastin time, or decreased serum fibrinogen concentration) was confirmed. We then excluded ASA from the analysis since ASA has been reported to directly affect blood coagulation and platelet activation (Yip, 2004), but this is not due to an indirect effect via liver damage. Although the time required for coagulopathy varied among the compounds, data in all the time points, i.e., 3, 7, 14 and 28 days of repeated administration, were used in order to not overlook any changes of gene expression preceding the occurrence of coagulation abnormality.

After removing the probe sets with Affymetrix absent call in all the 48 samples for each drug (N = 3 for 4 time points and 4 dose levels for one drug), genes differentially expressed by the treatment were extracted by one-way ANOVA ($p < 0.05$) for the dose level at one time point. This procedure was continued for all time points and the genes showing significant change at any point were combined as coagulopathy-responsive genes. In the next step, commonly mobilized genes among the above mentioned 8 chemicals were selected. Finally, probe sets without unique Entrez Gene ID were removed from the analysis and we selected only matching probes (Grade A by NetAffx). Like these compounds, gene extraction was performed on the samples treated with ASA.

The individual expression value (global mean) was converted to a ratio by the mean of the corresponding control value, and all the values with different doses and time points of the test compounds were gathered and normalized by converting them to z-scores for each gene (pergene normalization). Principal component analysis (PCA) was performed using Spotfire Decision Site (Spotfire, Somerville, MA, USA).

### Pathway and Gene Ontology (GO) analysis

The identified probe sets were subjected to GO analysis by DAVID (Database for Annotation, Visualization, and Integrated Discovery; http://david.abcc.ncifcrf.gov/) using Fisher's exact test (Dennis *et al.*, 2003).

### Statistical analysis

The results of blood chemical and hematological examinations were expressed as mean ± S.D.. They were analyzed by the Bartlett test that evaluates the homogeneity of variance. If the variances were homogeneous, 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.

## RESULTS

### Hematological and blood biochemical examinations

A summary of the hematological and blood biochem-

ical examinations is shown in Table 2. A slight increase of serum alkaline phosphatase and/or lactate dehydrogenase concentration was observed in ET, TAA, BBr, WY and AM, whereas no increase of serum AST, ALT and/or γ-glutamyl transpeptidase was observed in any of these compounds. PT was significantly increased by the ET, TAA, BBr, PTU, WY, AM and ASA treatments (Fig. 1). Activated partial thromboplastin time (APTT) was significantly increased by the CFB, OPZ, ET, BBr and AM treatments (Fig. 2). The fibrinogen concentration was significantly decreased by the CFB, OPZ, ET, TAA, PTU, WY and AM treatments (Fig. 3). In summary, coagulation abnormalities were induced by all the compounds including ASA, whereas a toxicologically significant increase of serum marker enzymes was not always associated.

## Identification of genes related to coagulopathy

Differentially expressed genes with statistical sig-

**Table 2.** Summary of clinical examinations

| Compound | PT | APTT | FBG | AST | ALT | ALP | LDH | GTP |
|---|---|---|---|---|---|---|---|---|
| CFB | – | ↑ | ↓ | – | – | – | – | – |
| OPZ | – | ↑ | ↓ | ↓ | ↓ | ↓ | – | – |
| ET | ↑ | ↑ | ↓ | – | – | ↑ | – | – |
| TAA | ↑ | – | ↓ | ↑* | ↑* | – | ↑ | – |
| BBr | ↑ | ↑ | – | – | – | – | ↑ | – |
| PTU | ↑ | – | ↓ | – | – | – | – | – |
| WY | ↑ | – | ↓ | – | – | ↑ | – | – |
| AM | ↑ | ↑ | ↓ | – | – | ↑ | – | – |
| ASA | ↑ | – | – | – | – | – | – | – |

*: only Day 4

↑: increased, ↓: decreased, –: not remarkable

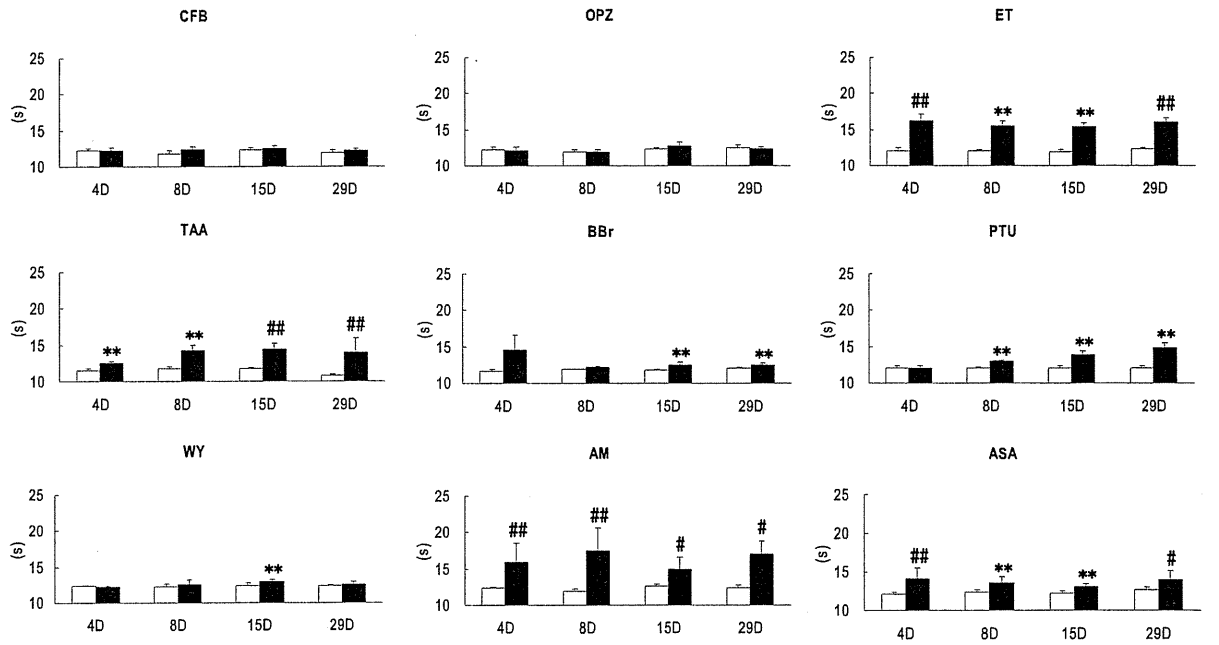


**Fig. 1.** Effect of hepatotoxic compounds on prothrombin time (PT).
For simplicity, the data of the highest dose are presented for each compound. Open (control) and filled (high dose) columns represent PT, which are expressed as mean ± S.D. of 5 rats each for each time and compound. Significant difference from control rat: (*$p < 0.05$, **$p < 0.01$: Dunnett test, #$p < 0.05$, ##$p < 0.01$: Dunnett type mean rank test).

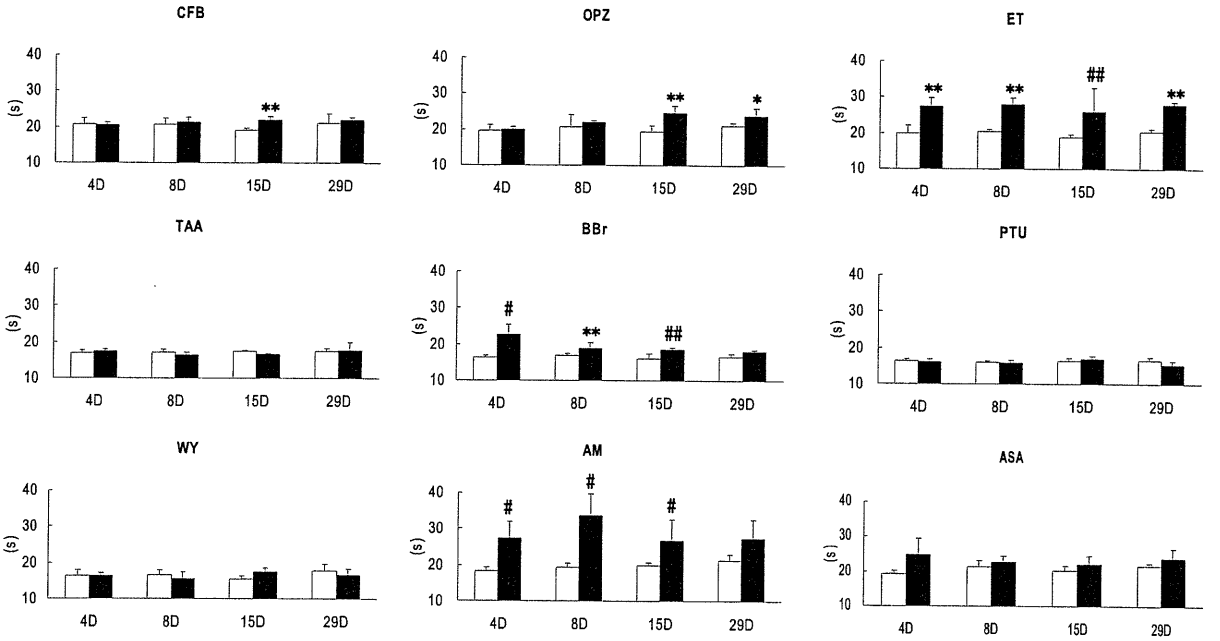


**Fig. 2.** Effect of hepatotoxic compounds on APTT.
For simplicity, the data of the highest dose are presented for each compound. Open (control) and filled (high dose) columns represent APPT, which are expressed as mean ± S.D. of 5 rats each for each time and compound. Significant difference from control rat: (*$p < 0.05$, **$p < 0.01$: Dunnett test, #$p < 0.05$, ##$p < 0.01$: Dunnett type mean rank test).

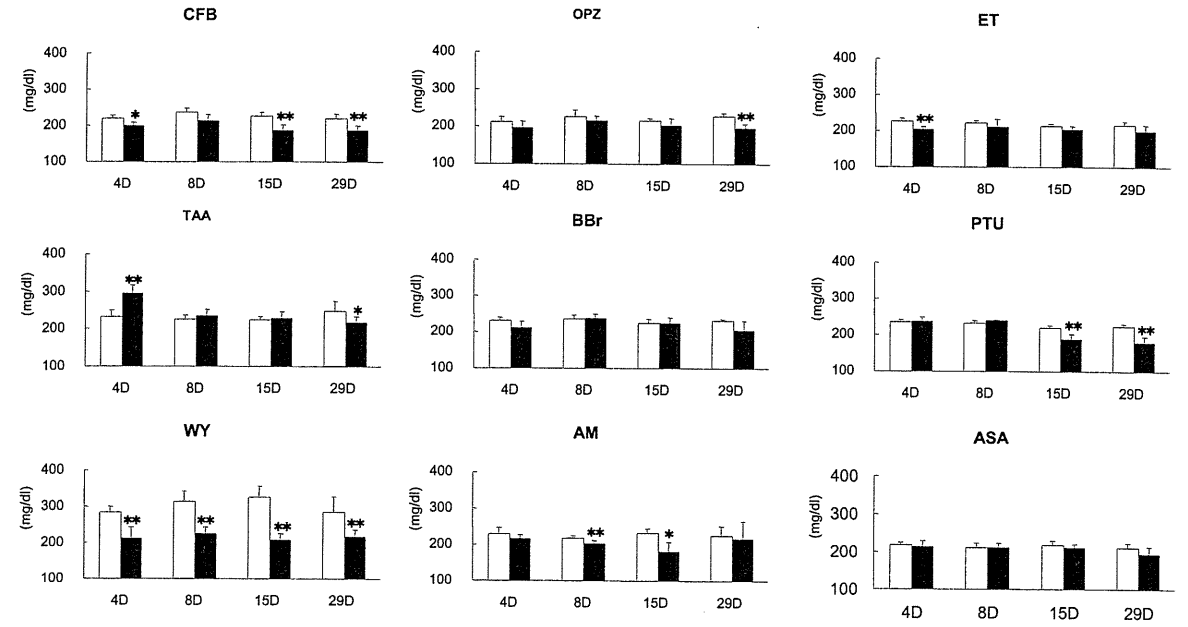


**Fig. 3.** Effect of hepatotoxic compounds in serum fibrinogen (FBG) concentration.
For simplicity, the data of the highest dose are presented for each compound. Open (control) and filled (high dose) columns represent FBG, which are expressed as mean ± S.D. of 5 rats each for each time and compound. Significant difference from control rat: (*$p < 0.05$, **$p < 0.01$: Dunnett test, #$p < 0.05$, ##$p < 0.01$: Dunnett type mean rank test).

nificance were extracted from each of the 8 compounds inducing coagulopathy as well as from ASA, as described in the Methods section. The numbers of extracted probe sets were 7,742 for CFB, 7,742 for OPZ, 10,305 for ET, 12,845 for TAA, 9,000 for BBr, 7,922 for PTU, 11,115 for WY, 7,738 for AM and 6,278 for ASA. We then selected the probe sets that were commonly changed in the 8 compounds excluding ASA, those without unique Entrez Gene ID were removed from the analysis, those with matching probes (Grade A by NetAffx) were selected, and finally 344 probe sets were obtained (see Supplemental Data). As shown in Table 3, GO analysis of these revealed that some metabolic processes were mainly changed. Genes related to blood coagulation was found to be changed by these chemicals, although their contribution was relatively low and statistically insignificant.

To check the expression profile of genes related to blood coagulation, we selected 30 such probe sets by keywords from the ones equipped in GeneChip irrespective of their statistically significant changes. Fig. 4 shows the expression changes of these probe sets at high dose of repeated administration as a heat map of the average of log 2 ratios. The mRNA levels of several genes involved in coagulation cascades, such as coagulation factors (FII, III, V, VIII, X, XII and XIIa1), thrombomodulin, plasminogen, fibrinogen (a and b), protein C, protein S, serine proteinase inhibitor (serpin), and vitamin K epoxide reductase complex subunit 1 (Vkorc1) were changed and the direction of their change tended to decrease in general. One obvious exception was vitamin K epoxide reductase complex subunit 1-like 1 (Vkorc 1l1), which is a paralogous gene of Vkorc1 and was generally up-regulated. In contrast to these drugs, animals treated with ASA or PTU did not show marked expression changes in these genes. One exception was coagulation factor III which looked largely up-regulated by ASA, but its reliability was considered to be quite low because of the large variation and lack of dose-dependency (data not shown).

## Principal component analysis (PCA)

Using the 344 probe sets extracted as above, PCA was performed on the coagulopathic 8 compounds. As shown in Fig. 5, each sample was separated from control according to the expression of these probe sets. It should be noted that liver treated with BBr, CFB and ET had a relatively large principal component PC1 (contribution rate: 38.7%), while ET showed larger PC2 (contribution rate: 12.8%). Liver treated with WY and TAA had a large value in both axes of PC. On the other hand, PTU did not change its position for PC1 and PC2, but it showed larger PC3 (contribution rate: 6.8%). Of the genes contrib-

**Table 3.** GO analysis of identified 344 probe sets

| Term | Count | Percent | p-value |
|---|---|---|---|
| Generation of precursor metabolites and energy | 39 | 10 | 4.3E-10 |
| Biosynthetic process | 66 | 18 | 4.5E-10 |
| Cellular metabolic process | 183 | 49 | 1.1E-09 |
| Catabolic process | 40 | 11 | 1.2E-09 |
| Primary metabolic process | 172 | 46 | 3.7E-06 |
| Response to external stimulus | 35 | 9.3 | 5.9E-06 |
| Response to stress | 41 | 11 | 1.6E-04 |
| Nitrogen compound metabolic process | 23 | 6.1 | 1.8E-04 |
| Translation | 26 | 6.9 | 7.7E-04 |
| Secondary metabolic process | 6 | 1.6 | 0.0045 |
| Transport | 67 | 18 | 0.0074 |
| Establishment of localization | 68 | 18 | 0.011 |
| Defense response | 19 | 5.0 | 0.012 |
| Death | 25 | 6.6 | 0.018 |
| Activation of immune response | 6 | 1.6 | 0.020 |
| Immune effector process | 7 | 1.9 | 0.043 |
| Response to endogenous stimulus | 14 | 3.7 | 0.073 |
| Regulation of immune system process | 6 | 1.6 | 0.081 |
| Response to abiotic stimulus | 9 | 2.4 | 0.084 |
| Regulation of multicellular organismal process | 13 | 3.5 | 0.086 |
| Coagulation | 5 | 1.3 | 0.098 |

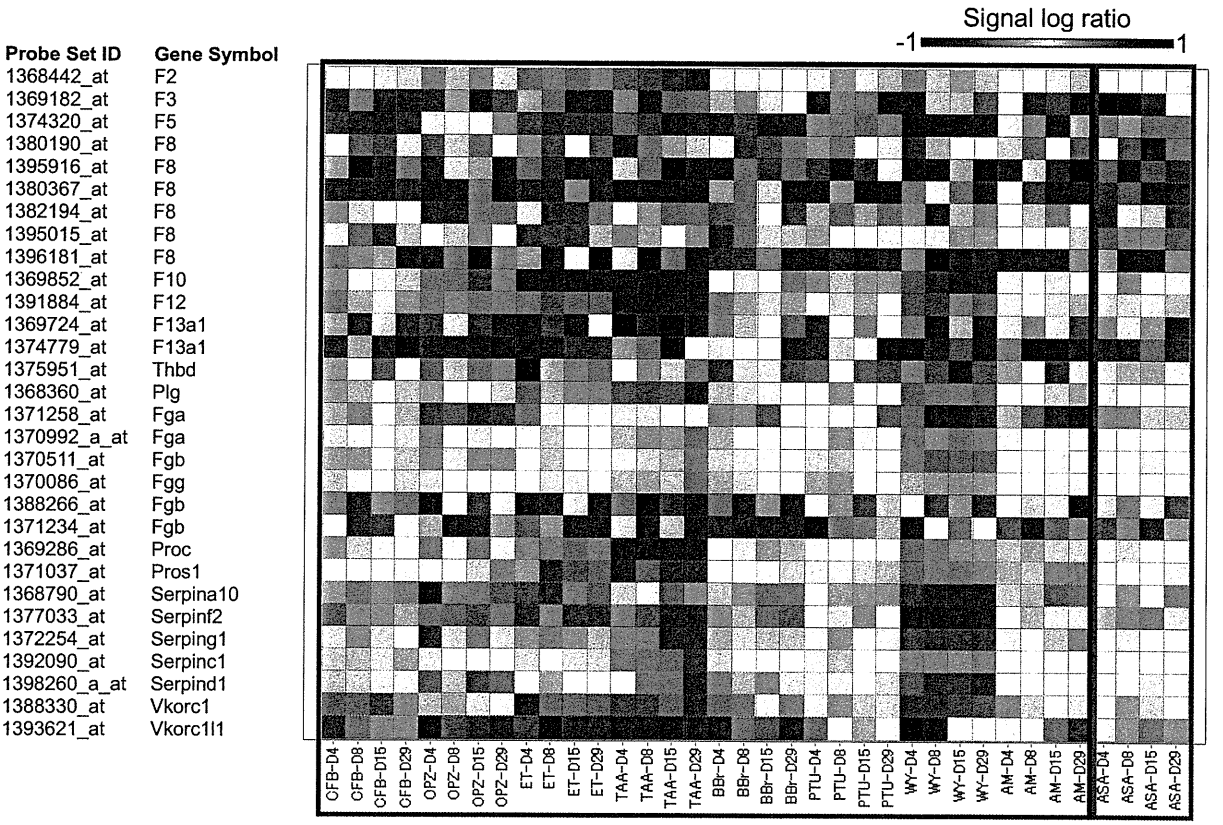


**Fig. 4.** Heat map of gene expression profiles of CFB, OPZ, ET, TAA, BBr, PTU, WY and AM, as well as ASA using coagulation-related 30 probe sets. Values are expressed as average log 2 ratio, for each time point at high dosage.

uting to PC1 and PC2, those with high eigenvalue, the top 20 for each, are listed in Table 4. It appears from the table that both components contain mainly lipid metabolism-related genes. PC1 also contains cellular stress-related genes such as prohibitin and CIDE-A, whereas PC2 are dominant in peroxisome-related genes, reflecting that PPARα agonists such as WY, CFB and BBr, are well separated from ET and TAA in the direction of PC2.

### Verification of probe sets using ASA

To examine whether these identified probe sets have specificity to coagulopathy by liver toxicity, we applied PCA using the 344 probe sets on these 8 compounds inducing coagulopathy by liver toxicity as well as ASA, which induced prolongation of prothrombin time (Fig. 1 and Table 2), as a pharmacological effect. The results were depicted as a two-dimensional graph with PC1 (contribution rate: 38.5%) and PC2 (contribution rate: 12.4%) for each axis, where ASA did not change their position very much on PCA, but shifted a little toward PC1 (Fig. 6). In order to visualize the difference between the compounds clearly, all the samples were aligned on a one-dimensional graph using PC1 (Fig. 7). It appeared that ASA as well as PTU showed the smallest shift among the compounds in spite of their obvious anti-coagulation effect.

## DISCUSSION

Abnormality of hemostasis is often associated with hepatotoxicity. However, the precise mechanism of this acquired coagulopathy remains unclear. There are a number of proposed hypotheses regarding homeostatic disturbance in liver injury, including reduced synthesis, increased consumption, and reduced clearance of coagulation factors (Peck-Radosavljevic, 2007). In the present study, drug-induced, hepatotoxicity-related coagulopathy was able to be diagnosed by PCA using 344 genes which were commonly mobilized in 8 drugs. Although the gene

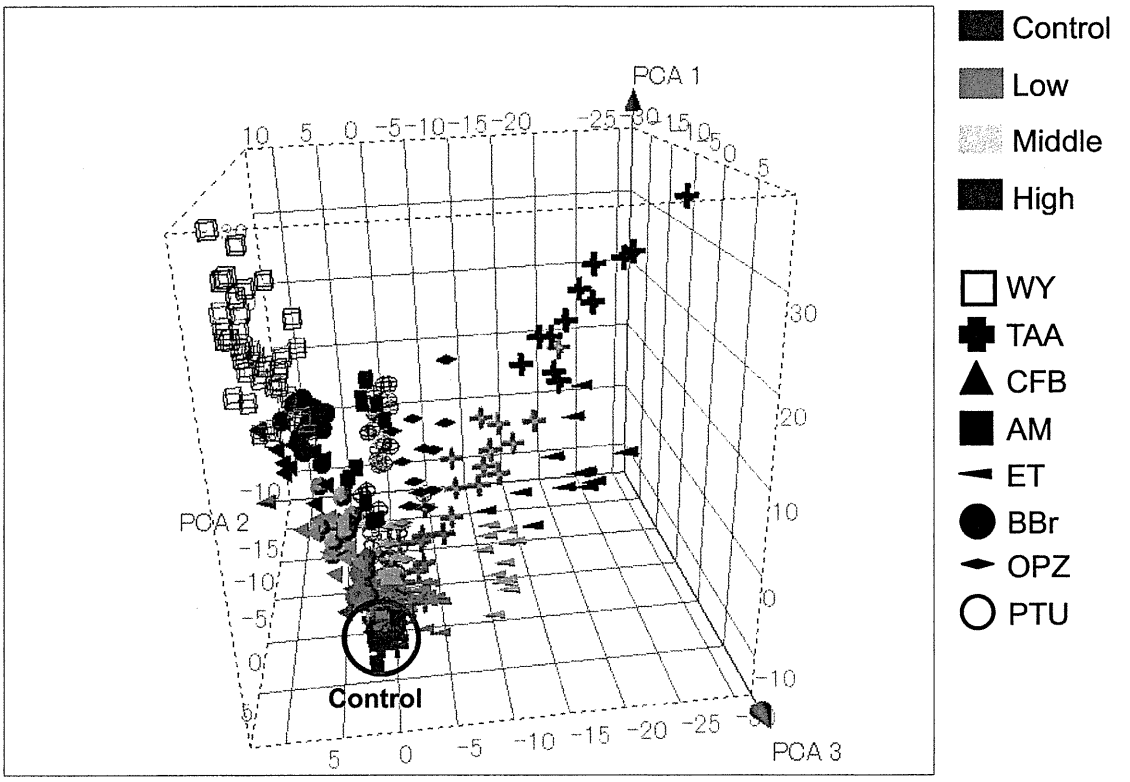


**Fig. 5.** Principal component analysis of gene expression profiles of CFB, OPZ, ET, TAA, BBr, PTU, WY and AM, using the commonly mobilized 344 probe sets. Results are expressed as a three dimensional graph for PC1, 2 and 3. Treated samples were dose-dependently separated from the cluster of the controls (circled by a blue line), mainly toward the direction of PC1 (contribution rate: 36.9%). For simplicity, rats receiving the same dose with different durations (3, 7, 14 and 28 days, N = 3 for each; 12 total) are expressed by the same symbol.

list contained several genes related to coagulation, those with higher contribution were mainly categorized as lipid metabolism and cell damage. PCA is a powerful tool as a non-supervised analysis, and it is quite useful to reveal the underlying mechanism when the principal components are suggestive. In the present case, however, the separation was considered attained by the indirect events in the hepatocytes that emerge as expression changes of these genes. Further analysis is obviously necessary to elucidate the mechanism of drug-induced coagulopathy by the present strategy.

As a part of the multifactorial role of liver in protein synthesis, most of the coagulation factors (fibrinogen, prothrombin, factor-V, -VII, -VIII, -IX, -X, -XI, -XII, -XIII, prekallikrein), natural anticoagulants (antithrombin-III, heparin cofactor-II, protein C, protein S, TFPI-1, TFPI-2), and components of the fibrinolytic system (plasminogen, α2-antiplasmin, TAFI) are produced in the liver (Watson, 1999; Berndt *et al.*, 2001; Savage *et al.*, 1992; Caldwell *et al.*, 2004). Pathophysiological changes in these can be detected by measurement of individual coagulation factors, or through the prolongation of PT or APTT, which are cumulatively responsive to most coagulation factors synthesized by the liver. Therefore, coagulation could be attributed to a vital synthetic capacity of the organ, not always, but at least to some extent. In the present study, a decrease in mRNA levels of some coagulation factors was observed, and it might be due to a malfunction of hepatocytes, not due to necrosis of hepatocytes, because no obvious leakage of hepatic enzymes was detected. This suggests it might be possible to detect a functional disturbance within hepatocytes by gene expression analysis. Another interesting point is that the pattern of changes in gene expression (Fig. 4) looks different among drugs, dose levels, and time points. A simple interpretation is that a decrease in coagulation factors or an increase in

**Table 4.** Probe sets contributing PC1 (left) and PC2 (right) in the PCA in Fig. 5. Top 20 probe sets with highest eigenvalue are listed for each component. Shaded probe sets are common in both PC1 and PC2.

| Probe sets contributing PC1 | | | | Probe sets contributing PC2 | | | |
|---|---|---|---|---|---|---|---|
| ranking | Probe set ID | Gene title | Eigenvalue | Ranking | Probe set ID | Gene title | Eigenvalue |
| 1 | 1368317_at | aquaporin 7 | 0.070009144 | 1 | 1389169_at | similar to 4631434O19Rik protein | 0.076072075 |
| 2 | 1368037_at | carbonyl reductase 1 | 0.068086758 | 2 | 1367672_at | peroxisomal multifunctional enzyme type II | 0.075988223 |
| 3 | 1367926_at | prohibitin | 0.067983584 | 3 | 1374475_at | similar to alpha/beta hydrolase-1 | 0.073630648 |
| 4 | 1371886_at | similar to carnitine acetyltransferase | 0.067583732 | 4 | 1380431_at | similar to KIAA0564 protein | 0.072382202 |
| 5 | 1367937_at | aldehyde reductase like 6 | 0.067191284 | 5 | 1388531_at | similar to 4631434O19Rik protein | 0.071730013 |
| 6 | 1390358_at | calcium channel, voltage dependent, alpha2/delta subunit 3 | 0.066297774 | 6 | 1367763_at | acetyl-coenzyme A acetyltransferase 1 | 0.06845887 |
| 7 | 1368206_at | 4,8-dimethylnonanoyl-CoA thioesterase | 0.066110077 | 7 | 1369050_at | phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide | 0.066532737 |
| 8 | 1371985_a_at | HLA-B associated transcript 5 | 0.065669124 | 8 | 1387100_at | aquaporin 3 | 0.065969965 |
| 9 | 1367767_at | 3-hydroxy-3-methylglutaryl CoA lyase | 0.065441346 | 9 | 1367897_at | acyl-Coenzyme A dehydrogenase, very long chain | 0.065740005 |
| 10 | 1398249_at | solute carrier family 25 member 20 | 0.064983662 | 10 | 1367777_at | 2,4-dienoyl CoA reductase 1, mitochondrial | 0.065545622 |
| 11 | 1388908_at | similar to Peci protein | 0.064839999 | 11 | 1370818_at | 2-4-dienoyl-Coenzyme A reductase 2, peroxisomal | 0.065292746 |
| 12 | 1370491_a_at | histidine decarboxylase | 0.064505345 | 12 | 1386885_at | enoyl coenzyme A hydratase 1 | 0.064882077 |
| 13 | 1375845_at | similar to 1500031O19Rik protein | 0.064282403 | 13 | 1375845_at | similar to 1500031O19Rik protein | 0.064743473 |
| 14 | 1387100_at | aquaporin 3 | 0.064133715 | 14 | 1368150_at | solute carrier family 27 (fatty acid transporter), member 32 | 0.063597102 |
| 15 | 1389179_at | similar to cell death activator CIDE-A | 0.06405368 | 15 | 1376296_at | acyl-coA oxidase | 0.063222767 |
| 16 | 1368977_a_at | fractured callus expressed transcript 1 | 0.063993326 | 16 | 1367680_at | fatty acid desaturase 2 | 0.062867058 |
| 17 | 1387740_at | peroxisomal membrane protein Pmp26p (Peroxin-11) | 0.063938006 | 17 | 1368453_at | carbonyl reductase | 0.062473231 |
| 18 | 1367927_at | prohibitin | 0.063680305 | 18 | 1370814_at | cytosolic acyl-CoA thioesterase 1 | 0.062293648 |
| 19 | 1374210_at | similar to RIKEN cDNA 2510027N19 | 0.063409988 | 19 | 1388211_s_at | peroxisomal membrane protein Pmp26p (Peroxin-11) | 0.061676191 |
| 20 | 1370164_at | hydroxyacyl-Coenzyme A dehydrogenase, alpha subunit | 0.063337769 | 20 | 1379361_at | enoyl-Coenzyme A, hydratase | 0.061650054 |