障害の発生は予測できず，だからといって，毒性研究者が自分の思い入れのある毒性パスウェイを検討して問題なかったとしても，何の保証にもならない．何が起こるかわからない毒性の場合は，起こりうるすべての可能性を検討しておかねばならない．オミクスの中でも，トランスクリプトミクスは圧倒的に有利である．それは，遺伝子が容易に増幅できるために感度が非常に高いこと，測定対象の同定が容易である（対象を配列既知のプローブで検出するため，測定値すべて同定と定量がセットになっている）ことによる．

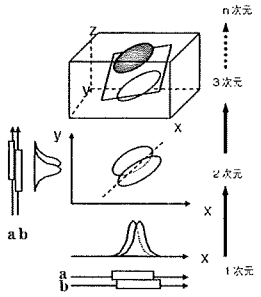
毒性学的機序が未知である新規開発薬物の有害作用を予測するためには，できるだけ多くの既存薬物のデータを蓄積しておき，これらと比較する方法がとられる．2000年以降，欧米の巨大製薬会社やベンチャー企業は，種々の薬物を投与した動物の各組織における遺伝子発現をデータベース化し始めた(2)．我が国では，製薬会社の規模が小さく，個別では対抗できないため，製薬17社が協同し，科研費の補助をうけて，官民共同のトキシコゲノミクスプロジェクトが2002年に発足した(3)．5年間で50億円を費やしたこのプロジェクトでは，150種の医薬品を投与したラット24,000匹について，肝臓（一部は腎臓も）の遺伝子発現データ約8億件を各種毒性学的データとともに取得し，統合データベースを構築した．現在，第2期のトキシコゲノミクス・インフォマティクスプロジェクトが進行中である．

蓄積されたデータを用いて，いかにして毒性予測が可能となるのであろうか．直接データを扱うと，最初に直面するのは例数に比して圧倒的に多い測定データ数の問題である．通常の科学実験では，一つの項目について5～10例をあて，統計解析で変化の有意性を検討する．しかしトランスクリプトミクスの場合，コストを考えると3～5例がせいぜいであるが，1例につき3～5万の測定値が得られてしまう．このような測定値の有意差検定は無意味に近く，第1種と第2種の過誤のどちらかが必ず極端に大きくなる．

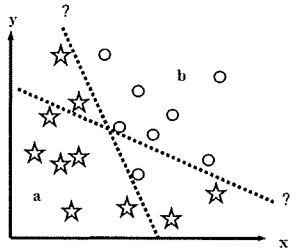
膨大なデータからどうやって意味のあるものを抽出するか，これが現在の最大の課題である．腫瘍マーカーの場合は単一の特異的な遺伝子産物から診断可能なものもあるが，薬剤性肝障害のような場合は，薬物に対する複雑な応答の結果であり，単独の変数で判定できるとは考えられない．むしろ，この複雑な過程を解析することにより，安全性を多角的に評価することが期待されている．これは多変量解析の領域である．

図Aに，多変量解析のイメージを示す．a，b2つの毒性学的フェノタイプがあるとし，遺伝子xの発現量で判別したいが，その発現には差があるものの，分布は大部分が重なっていて，少数例の測定では分別が困難である．ここで遺伝子yに注目する．これも分布が重なっているが，xとyの間に一定の関係性があれば，x-y平面上で大部分が分離される．ここでわずかに重なっている部分も，第3の遺伝子zの発現量を加味し

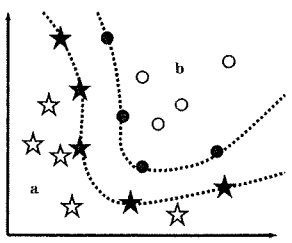
A. 多変量解析のイメージ



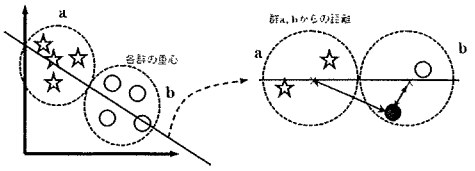
B. 2要素による線形分離



C. SVMによる分離のイメージ



D. PAMによる分離のイメージ



た3次元空間では完全に分離されている．これをn次元の空間まで拡張すれば，理想的なマーカー遺伝子が得られるが，実際はこれら遺伝子の選別が難しい．

薬物によってある毒性学的フェノタイプの有無がはっきりしている場合は，「教師付分類法」である判別分析が有効であり，マイクロアレイデータには，学習機械であるSupport Vector Machine（SVM）やPrediction Analysis of Microarrays（PAM）が適しているようである．図Bには，星印が毒性なし，丸印が毒性ありの個体で，簡単のためにx，yの2種類の遺伝子発現値をもつ場合を模式的に示す．SVMは，線形分離ができない場合（図Bの点線）でも，これを非線形写像によって高次元特徴空間に写像することによって線形分離するというもので，最適化された図Cでは，サポートベクターと呼ばれるマージン上の点（塗りつぶされている）で分類されている．SVMの場合は，サポートベクターだけが分離超平面に寄与しているが，PAMにおいては，多次元空間上の各点を，それぞれのnearest centroid（重心）からの距離でクラス確率を評価するという工夫がされている（図D）．実際，PAMを用いて，非遺伝子傷害性肝発癌のリスクを定量的に評価する試みに成功している(4)．

実際は，薬物誘起性臓器障害を単純に「ある・なし」に分けることは困難である．ある毒性が発現する用量より低い用量から遺伝子発現変化が生じることは十分考えられ，当該用量で「毒性なし」として学習させた場合には誤った結果を導く．そこで，「教師なし分類法」も重要になってくる．主成分分析Principal Component Analysis（PCA）は，なるべく少ない合成変数から多くの情報を把握するための情報の集約を達成するもので，うまく分離できたときは，毒性予測とともに，寄与率の大きい遺伝子の内容を精査することによって，毒性メカニズムにも迫ることができる．PCAによってリン脂質症の診断・予測の可能性を拓いた例を最近報告した(5)．

3. 問題点と将来展望

毒性学的・病理学的フェノタイプを個体の遺伝子型と関連づける場合には，統計学的に厳密な結論を導くことが可能である．ある疾患や薬物の有害作用に関して，患者の遺伝子型をデータベース化し，一定数以上の例数が蓄積されれば，ロジスティック回帰分析などにより，ある集団でそのフェノタイプが発生する確率を正確に評価できる．これは，特定の遺伝子型の集団における発病リスクや，治療の有効性，費用対効果が精確に評価可能となることを意味する．問題はバイオサイエンスの部分にはなく，個人情報の取り扱いや，「ヒトの健康をすべて確率的に予言してしまう社会」を容認するか否か，などが直面する課題である．

一方，トランスクリプトームとしてのトキシコゲノミクスでは状況が異なる．ある個体の遺伝子の塩基配列は確定した事実であるが，「ある個体にある薬物を投与すると遺伝子Xの発現量が1.5倍になった」という現象が確定的でないことは，研究者であれば自明であろう．データのばらつきには生物学・技術的な原因の両者があるため，施設間で再現性の保証される遺伝子リストとそれを得るための標準プロトコールの策定が重要である．理想的には，信頼性の高い少数の遺伝子をバイオマーカーとして選定したいが，それが困難な場合は，ある程度の数の遺伝子を用いて，個々の発現値には多少のぶれがあっても，最終判定にぶれの少ないアルゴリズムを採用することになる．

この再現性・信頼性の問題は，創薬における安全性の確保がレギュラトリーサイエンスと密接に関係しているため，特に重要になる．2004年に米国FDAが新薬の承認申請に当たってゲノミクスデータの任意提出を受けることを決定して以来，ゲノミクスデータを審査資料として使用するという流れとなっている(6)．わが国もそれに備えておく必要があり，現在のプロジェクトにおいても，施設間バリデーションを組み込んでいる．

オミクステクノロジーは確かに強力な手法である．将来的には，すべてのオミクステクノロジーを統合したトキシコパノミクスtoxicopanomicsも可能であるといわれ，医薬品の有害作用の検出感度はますます上昇していくであろう．しかしここで，注意すべきことがある．副作用のない薬などない．新しい技術がいたずらに薬の有害作用の部分を強調し，新薬の芽をつみ取るようなことはあってはならない．医薬品の安全性評価は，リスクとベネフィットの両者を精確に提示したうえでの客観的な判断基準を示すものであるべきだろう．

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Gene expression profiling in rat liver treated with compounds inducing elevation of bilirubin

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We have constructed a large-scale transcriptome database of rat liver treated with various drugs. In an effort to identify a biomarker for the diagnosis of elevated total bilirubin (TBIL) and direct bilirubin (DBIL), we extracted 59 probe sets of rat hepatic genes from the data for seven typical drugs, gemfibrozil, phalloidin, colchicine, bendazac, rifampicin, cyclosporine A, and chlorpromazine, which induced this phenotype from 3 to 28 days of repeated administration in the present study. Principal component analysis (PCA) using these probes clearly separated dose- and time-dependent clusters in the treated groups from their controls. Eighteen more drugs in the database, reported to elevate TBIL and DBIL, were estimated by PCA using these probe sets. Of these, 12 drugs, that is methapyrilene, thioacetamide, ticlopidine, ethinyl estradiol, alpha-naphthylisothiocyanate, indomethacin, methyltestosterone, penicillamine, allyl alcohol, aspirin, iproniazid, and isoniazid were also separated from the control clusters, as were the seven typical drugs causing elevation of TBIL and DBIL. The PC1 value showed high correlation with TBIL and DBIL. In the cases of colchicine, bendazac, chlorpromazine, gemfibrozil, and phalloidin, the possible elevation of TBIL and DBIL could be predicted by expression of these genes 24 h after single administration. We conclude that these identified 59 probe sets could be useful to diagnose the cause of elevation of TBIL and DBIL, and that toxicogenomics would be a promising approach for prediction of this type of toxicity.

Key words: bilirubin; liver; principal component analysis; rat; toxicogenomics

Introduction

The Toxicogenomics Project is a 5-year collaborative project by the National Institute of Biomedical Innovation, the National Institute of Health Science, and 15 pharmaceutical companies in Japan that started in 2002.[1] Its aim was to construct a large-scale toxicology database of transcriptomes to predict toxicity of new chemical entities in the early stage of drug development. Over 150 chemicals, mainly medicinal compounds, were selected and their gene expression in the liver was comprehensively measured 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 the Toxicogenomics Project, Japan). Recently, we identified a biomarker for the diagnosis of hepatic phospholipidosis, nongenotoxic hepatocarcinogenicity, glutathione depletion-responsive, and serum triglyceride-decreasing by using our database.[2–5]

Bilirubin is mainly a breakdown product of heme (part of the hemoglobin in the red blood cells). The heme is then turned into unconjugated bilirubin (indirect bilirubin) in the macrophages of the spleen. It is then bound to albumin and sent to the liver. In the liver it is conjugated with glucuronic acid (direct bilirubin, DBIL), making it soluble in water. Total bilirubin (TBIL) and DBIL levels can be measured in the blood, but indirect bilirubin is calculated from the

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total and direct bilirubin. Bilirubin levels reflect the balance between production and excretion.

Increased TBIL causes jaundice of the prehepatic, hepatic, and posthepatic types. The "prehepatic type" is derived from increased bilirubin production. This can be due to a number of causes, including hemolytic anemia and internal hemorrhage. If the DBIL is normal, then the problem is an excess of unconjugated bilirubin, and the location of the problem is upstream of bilirubin excretion. The "hepatic type" indicates problems in the liver, which are reflected as deficiencies in bilirubin metabolism (e.g., reduced hepatocyte uptake of bilirubin, impaired conjugation of bilirubin, and reduced hepatocyte secretion of bilirubin). Some examples would be drug-induced hepatic disorders (especially antipsychotic drugs, some sex hormones, and a wide range of other drugs), cirrhosis, and viral hepatitis. The "posthepatic type" is derived from obstruction of the bile ducts, which are reflected as deficiencies in bilirubin excretion. An obstruction such as gallstones or cancer can be located either within the liver or outside the liver.

In the present study, we selected elevation of TBIL and DBIL as a target phenotype and tried to identify candidate biomarkers that identify chemicals with the potential to cause this phenotype by using our database.

Materials and methods

Compounds

The compounds used for the data analysis are listed in Table 1, in which the chemical name, abbreviation, dosage, administration route, and vehicle used in the study are summarized.

Animal treatment

The experiments were carried out as previously described in the literature.[6] 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 five 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 h (7:00 a.m–7:00 p.m) daily, ventilated with an air-exchange rate of 15 times per hour, and maintained at 21–25 °C with a relative humidity

Table 1 List of compounds used in the present study

Compound name	*Abbreviation*	*Dose (dose level, mg/kg)*			*Administration route*	*Vehicle*	*Bilirubin*
		Low	*Middle*	*High*			
Gemfibrozil	GFZ	30	100	300	po	CO	A
Phalloidin	PHA	NA	NA	0.5	ip	SA	B
Colchicine	COL	1.5/0.5[a]	5/1.5[a]	15/5[a]	po	MC	C
Bendazac	BDZ	100/30[a]	300/100[a]	1000/300[a]	po	MC	C
Rifampicin	RIF	20	60	200	po	MC	C
Cyclosporine A	CSA	30/10[a]	100/30[a]	300/100[a]	po	CO	B
Chlorpromazine	CPZ	4.5	15	45 (150[b])	po	MC	C
Methapyrilene	MP	10	30	100	po	MC	B
Thioacetamide	TAA	4.5	15	45	po	MC	C
Ticlopidine	TCP	30	100	300	po	MC	D
Ethinyl estradiol	EE	1	3	10	po	CO	A
Alpha-naphthylisothiocyanate	ANIT	1.5	5	15	po	CO	D
Indomethacin	IM	0.5	1.6	5	po	MC	C
Methyltestosterone	MTS	30	100	300	po	MC	A
Penicillamine	PEN	100	300	1000	po	MC	A
Allyl alcohol	AA	3	10	30	po	CO	D
Aspirin	ASA	45	150	450	po	MC	D
Iproniazid	IPA	6	20	60	po	MC	B
Isoniazid	INAH	50	100	200	po	MC	C
Glibenclamide	GBC	100	300	1000	po	CO	D
Cyclophosphamide	CPA	1.5	5	15	po	MC	B
Nitrofurantoin	NFT	10	30	100	po	MC	D
Valproate	VPA	45	150	450	po	MC	D
Methotrexate	MTX	0.1	0.3	1	po	MC	C
Tetracycline	TC	100	300	1000	po	MC	C

po, peroral; CO, corn oil; A, bilirubin oxidase method; NA, not applicable; ip, intraperitoneal; SA, saline; B, vanadate oxidation method; MC, 0.5 wt/vol% methylcellulose; C, azobilirubin method; D, alkali azobilirubin method.
The animals were treated for 3, 7, 14, or 28 days, except PHA, which were treated for 3 or 7 days.
[a]As single dose/repeated dose.
[b]Extra-high dose (only single dose of CPZ).

of 40–70%. Each animal was allowed free access to water and pellet food (CRF-1, sterilized by radiation, Q4 Oriental Yeast Co., Japan). Rats in each group were orally dosed various drugs suspended or dissolved either in 0.5% methylcellulose solution or corn oil according to their dispersibility, except phalloidin, which was dissolved in saline and administered intraperitoneally. For single-dose experiments the rats were sacrificed at 3, 6, 9, and 24 h after dosing. For repeated dose experiments, the animals were treated for 3, 7, 14, or 28 days, except phalloidin, which were treated for 3 or 7 days, and they were sacrificed 24 h after the last dosing. Food was not withdrawn before sacrifice and the time of autopsy was done between 9:00 a.m–11:00 a.m. for the repeated dose group and 24 h after the single dose group. Blood samples for routine biochemical analysis were collected into heparinized tubes under ether anesthesia from the abdominal aorta after which the animals were sacrificed. As the animal experiments were performed in four different contract research organizations where different automated blood chemistry analyzers were used, TBIL and DBIL were quantified by four different methods, that is bilirubin oxidase method (TBA-120FR, Toshiba Medical Systems Corporation, Japan), vanadate oxidation method (Hitachi H7170, Hitachi High-Technologies Corporation, Japan), azobilirubin method (COBAS MIRA plus, Roche Diagnostics, Switzerland), and alkali azobilirubin method (Hitachi H7070, Hitachi High-Technologies Corporation, Japan). Because of this difference, the absolute values could not be compared, and the judgment of hyperbilirubinemia was done based on the difference from the control value. These methods are shown in Table 1 as A, B, C, and D in this order. The experimental protocols were reviewed and approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Health Sciences.

Microarray analysis

After collecting the blood 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, Texas, USA) overnight at 4 °C, and frozen at −80 °C until use. Liver samples were homoge- Q5 nized with the buffer RLT supplied in the RNeasy Mini Kit (Qiagen, Valencia, California, USA) and total RNA was isolated according to the manufacturer's instructions. Microarray analysis was conducted on three out of five 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, California, USA), containing 31,042 probe sets. The procedure was conducted basically according to the manufacturer's instructions using the Superscript Choice System (Invitrogen, Carlsbad, California, 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, New York, USA) for synthesis of biotin-labeled cRNA. Ten micrograms of fragmented cRNA were hybridized to a Rat Genome 230 2.0 Array for 18 h 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 Q6 intensities were normalized for each chip by setting the mean intensity to 500 (per chip normalization).

Microarray data analysis

To extract probe sets related to the elevation of TBIL and DBIL, we first used the gene expression data from liver from rats treated by repeated administration for 3, 7, 14, and 28 days, with gemfibrozil (GFZ), phalloidin (PHA), colchicine (COL), bendazac (BDZ), rifampicin (RIF), cyclosporine A (CSA) and chlorpromazine (CPZ), which are known to cause elevation of TBIL and DBIL, and that was confirmed in the present study.

After removing the probe sets with Affymetrix absent call in all the 48 sample sets ($N = 3$ for 4 time points and 4 dose levels for one drug), except PHA, which had 12 sample sets ($N = 3$ for 2 time points by repeated administration for 3 and 7 days, and 2 dose levels), genes differentially expressed by the treatment were extracted by Welch's ANOVA/t-test ($P < 0.05$) 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 elevation of TBIL and DBIL responsive genes. In the next step, commonly mobilized genes among these seven chemicals were selected.

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

performed using Spotfire Decision Site (Spotfire, Somerville, Massachusetts, 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.[7]

Results

Blood biochemical examination

The results of TBIL and DBIL examination of seven typical compounds (GFZ, PHA, COL, BDZ, RIF, CSA, and CPZ) known to induce their elevation in rat plasma are shown in Figure 1. In most cases, both TBIL and DBIL were elevated and this tended to progress with dose and time. In the cases of COL and BDZ, DBIL was increased with a peak at 4th day and TBIL showed the same change as COL. TBIL was increased for all sampling points at high dose of BDZ. With CPZ, the DBIL was elevated at the 15th day or later, but changes in the TBIL were obscure at any sampling point.

Microarray data analysis

Differentially expressed genes with statistically significant differences were extracted from each of the seven representative drugs elevating TBIL and DBIL as described in the Materials and methods section. The numbers of extracted probe sets were 3690 for

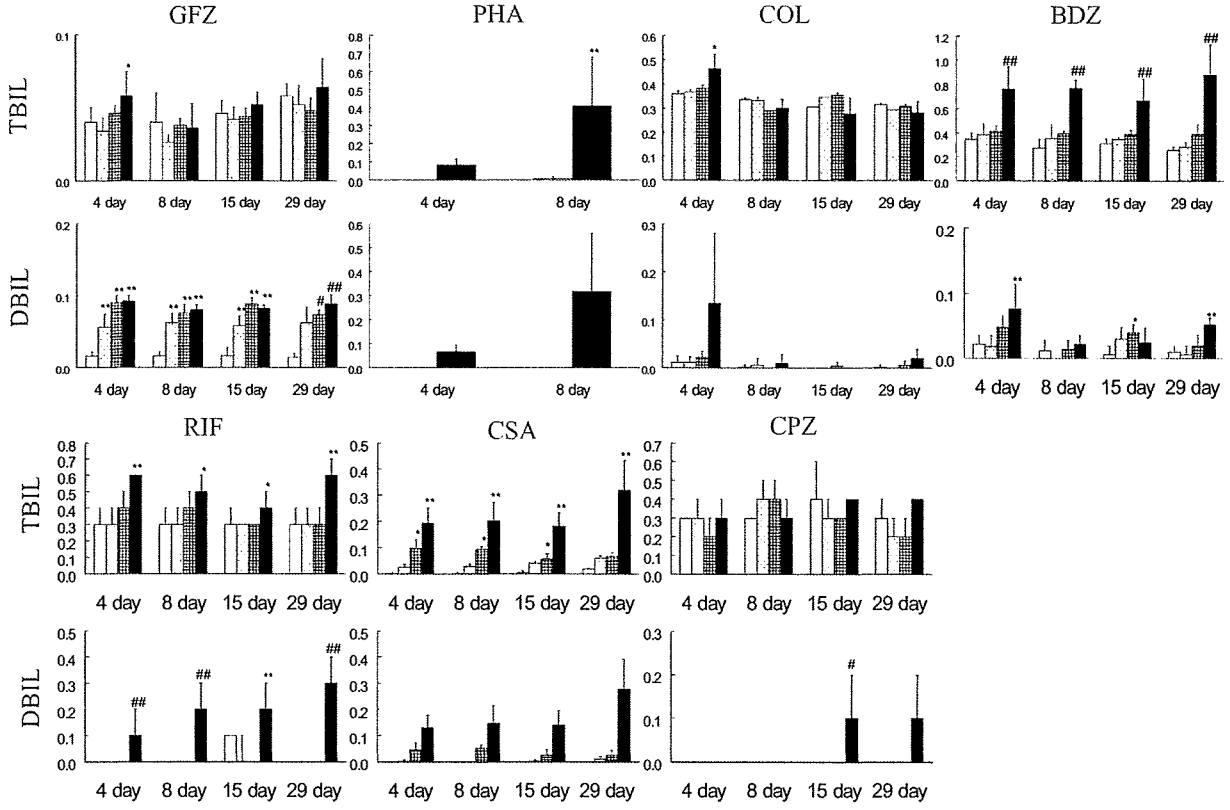

Figure 1 Plasma total bilirubin (TBIL) and direct bilirubin (DBIL) concentrations for rats treated with gemfibrozil (GFZ), phalloidin (PHA), colchicine (COL), bendazac (BDZ), rifampicin (RIF), cyclosporine A (CSA), or chlorpromazine (CPZ). Six-week-old male Sprague-Dawley rats were treated with each compound for 3, 7, 14, or 28 days, and they were sacrificed 24 h after the last dose. Blood samples were collected at sacrifice. Plasma TBIL and DBIL concentrations were estimated as described in the Materials and methods section. As the animal experiments were performed in four different contract research organizations where different automated blood chemistry analyzers were used, TBIL and DBIL were quantified by four different methods (see Table 1). Because of this difference, the absolute values could not be compared and the judgment of hyperbilirubinemia was done based on the difference from the control value. 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 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).

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, γpolypeptide 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	—

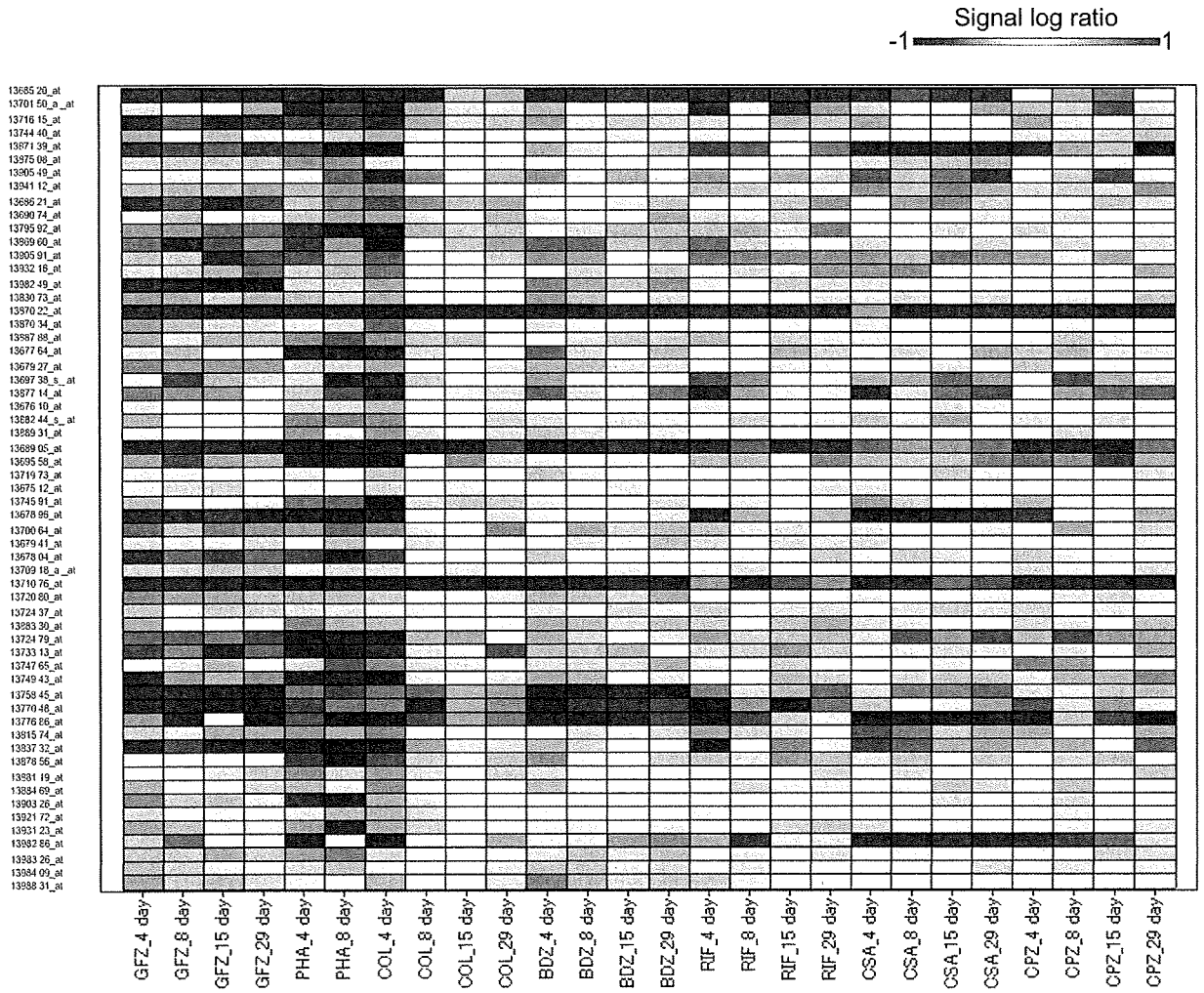

Figure 2 Heat map of the gene expression profiles of gemfibrozil (GFZ), phalloidin (PHA), colchicine (COL), bendazac (BDZ), rifampicin (RIF), cyclosporine A (CSA), and chlorpromazine (CPZ) that induced elevation of total bilirubin (TBIL) and direct bilirubin (DBIL) in the present study using the commonly 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.

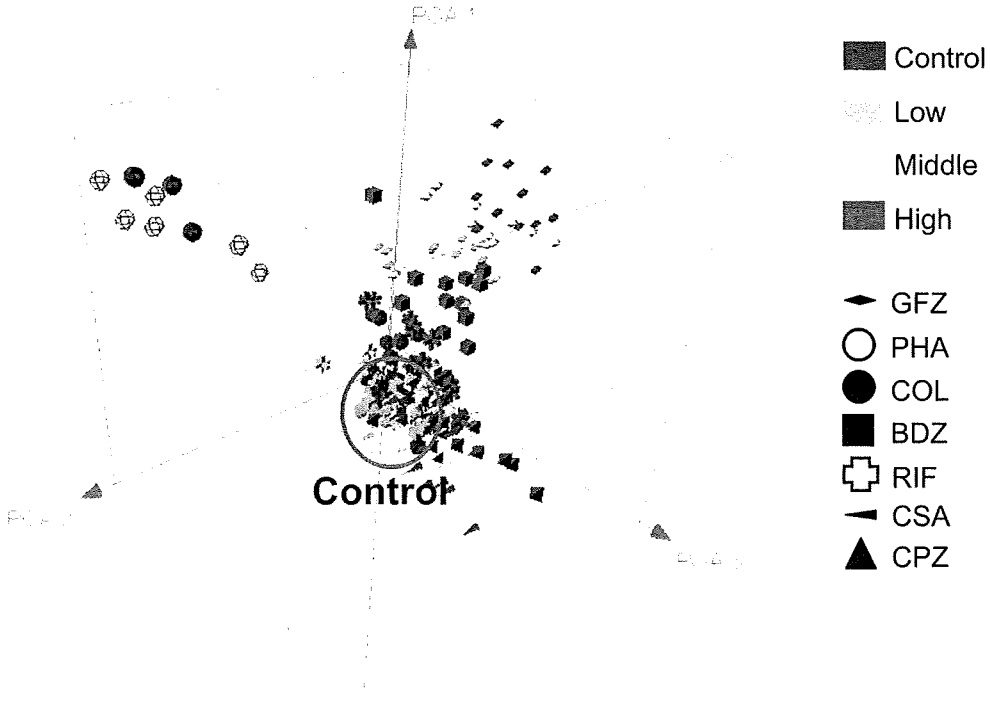

Figure 3 Principal component analysis of the gene expression profiles of gemfibrozil (GFZ), phalloidin (PHA), colchicine (COL), bendazac (BDZ), rifampicin (RIF), cyclosporine A (CSA), and chlorpromazine (CPZ), using the commonly mobilized 59 probe sets. Results are expressed as a three dimensional figure 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]

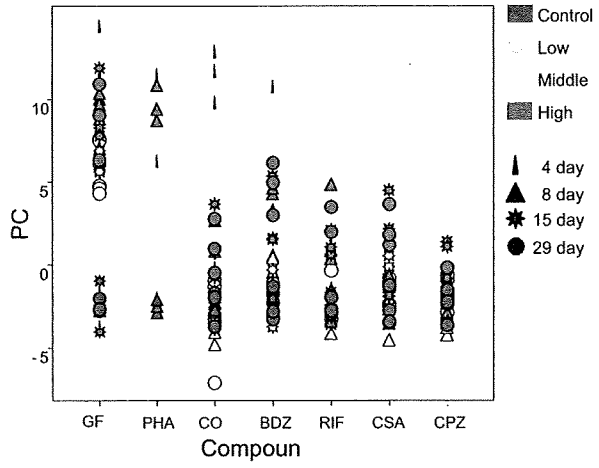

Figure 4 Principal component analysis of the same as Figure 3 but one dimensional expression using principal component 1. For each drug, each individual rat is depicted by a symbol with a different color and shape as shown on the right panel.

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.

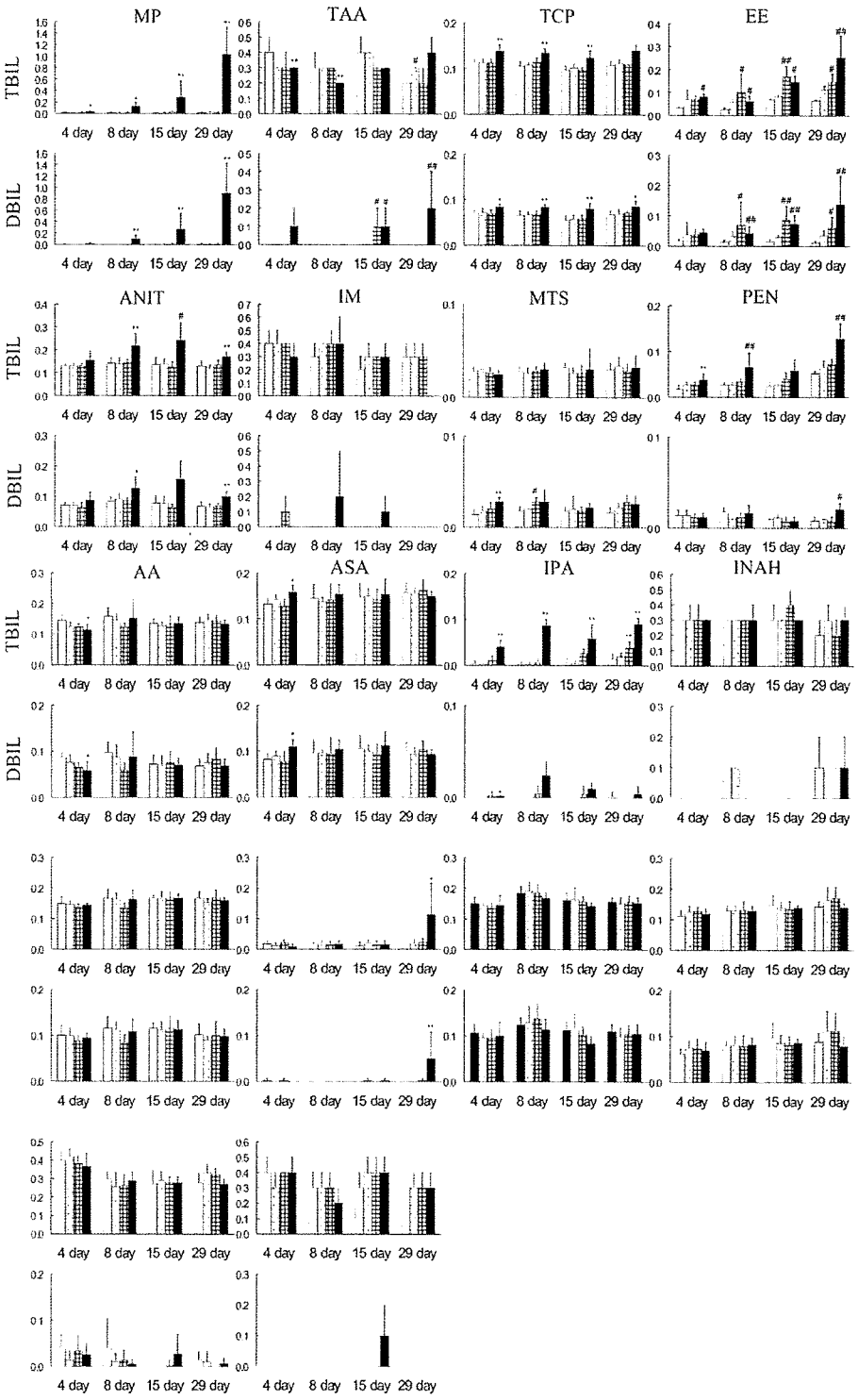

Figure 5 Plasma total bilirubin (TBIL) and direct bilirubin (DBIL) concentrations for rats treated with 18 test-drugs. Plasma TBIL and DBIL concentrations were estimated as shown in Figure 1. Open (control), dotted (low dose), checked (middle dose), and filled (high dose) columns represent plasma TBIL 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).

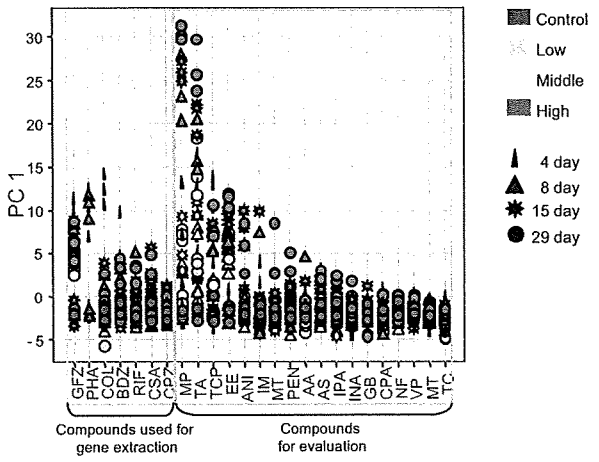

Figure 6 Principal component analysis of gene expression profiles of 18 more drugs which have been reported to elevate total and direct bilirubin, in addition to the seven typical drugs using the commonly mobilized 59 probe sets. Of the 25 compounds, the seven typical compounds used in Figures 3 and 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.

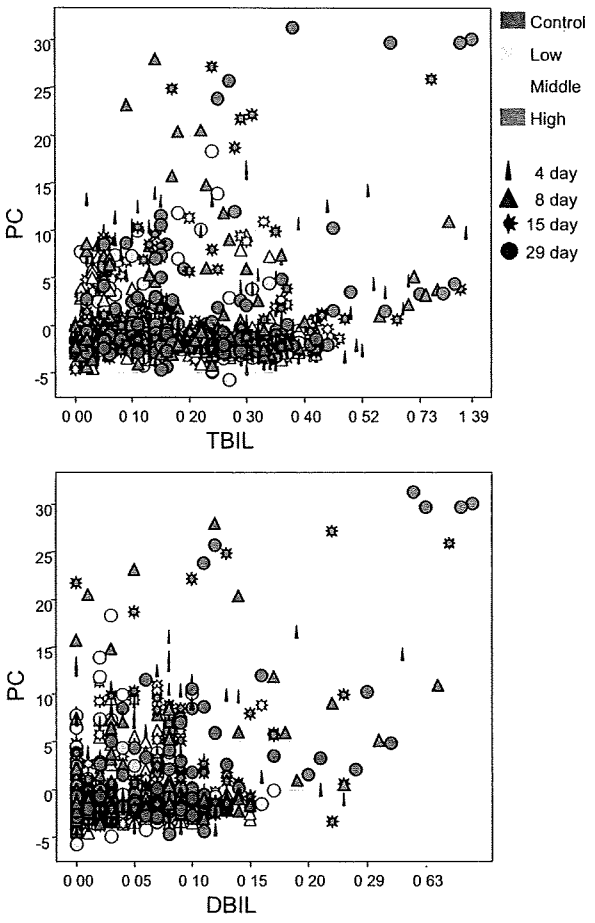

Figure 7 Correlation between the PC1 value, total bilirubin (TBIL), and direct bilirubin (DBIL). Results are expressed as a graph with TBIL or DBIL on the *y*-axis and PC1 value on the *x*. Each individual rat is depicted by a symbol with a different color and shape, as shown on the right panel. Note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

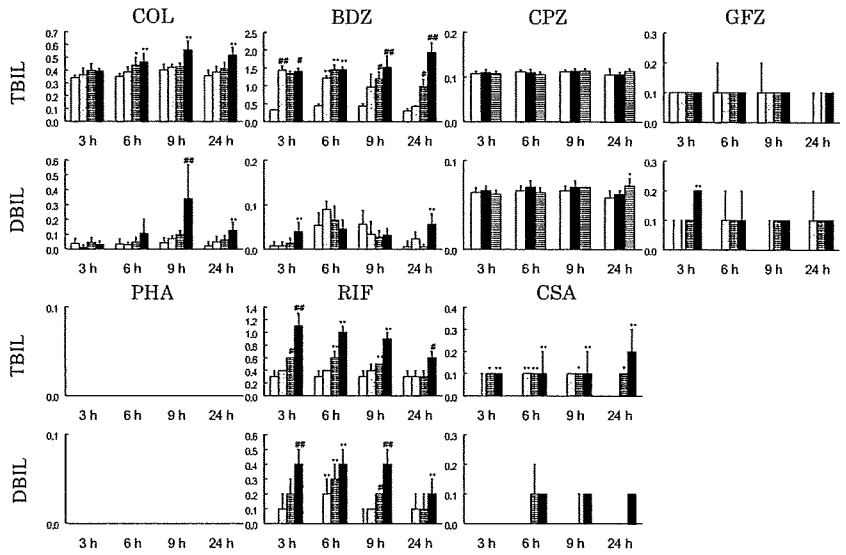

Figure 8 Plasma total bilirubin (TBIL) and direct bilirubin (DBIL) concentrations for rats after a single dose treatment with gemfibrozil (GFZ), phalloidin (PHA), colchicine (COL), bendazac (BDZ), rifampicin (RIF), cyclosporine A (CSA), and chlorpromazine (CPZ). Plasma TBIL and DBIL concentrations were 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.

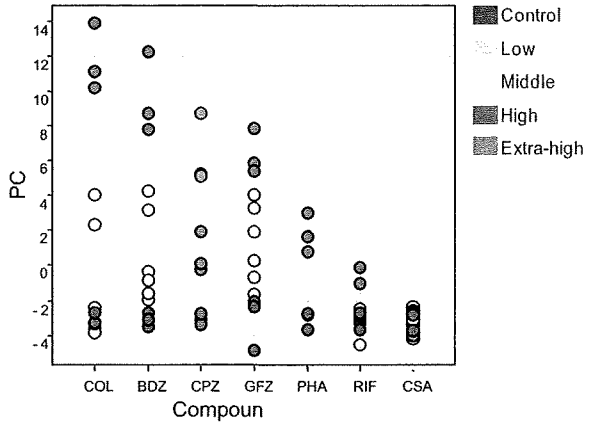

Figure 9 Principal component analysis of gene expression profiles of the samples of 24 h after single dosage using 59 probe sets. Results are expressed as a one dimensional figure with PC1 (contribution rate: 33.9 %). Each individual rat is depicted by a symbol with a different color and shape as shown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

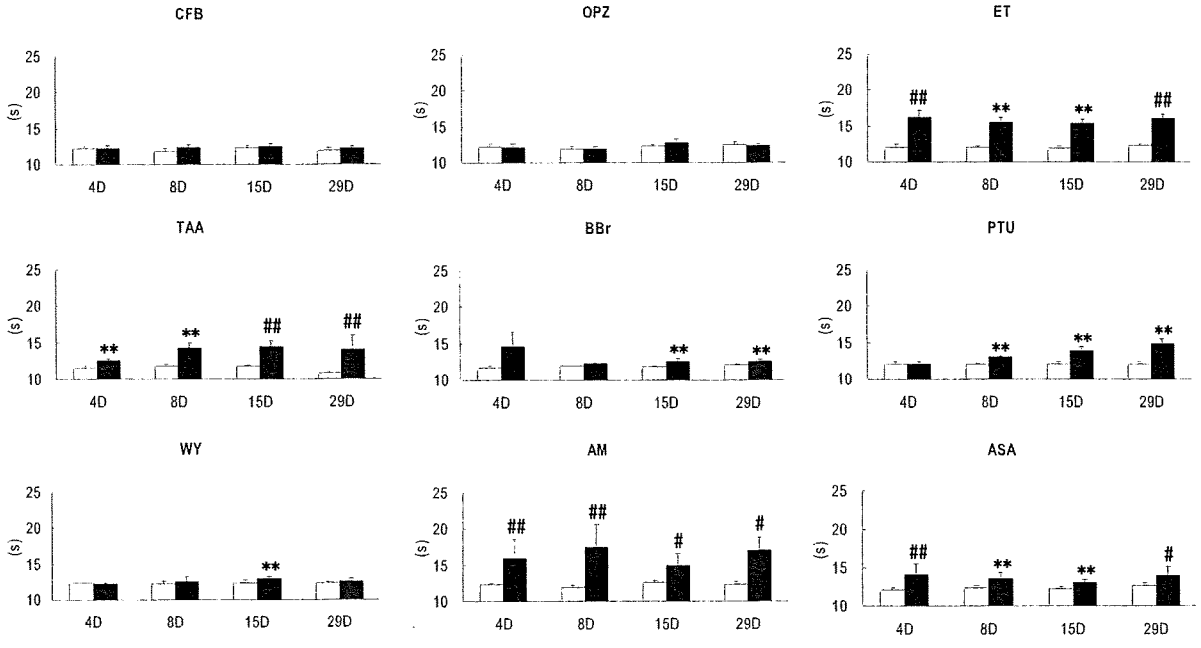

Fig. 1. Effect of hepatotoxic compounds on prothrombin time (PT).
For simplicity, the data of the highest dose are presented for each compound. Open (control) and filled (high dose) columns represent PT, which are expressed as mean ± S.D. of 5 rats each for each time and compound. Significant difference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