Original Article

Mechanism-based risk assessment strategy for drug-induced cholestasis using the transcriptional benchmark dose derived by toxicogenomics

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ABSTRACT — Cholestasis is one of the major causes of drug-induced liver injury (DILI), which can result in withdrawal of approved drugs from the market. Early identification of cholestatic drugs is difficult due to the complex mechanisms involved. In order to develop a strategy for mechanism-based risk assessment of cholestatic drugs, we analyzed gene expression data obtained from the livers of rats that had been orally administered with 12 known cholestatic compounds repeatedly for 28 days at three dose levels. Qualitative analyses were performed using two statistical approaches (hierarchical clustering and principle component analysis), in addition to pathway analysis. The transcriptional benchmark dose (tBMD) and tBMD 95% lower limit (tBMDL) were used for quantitative analyses, which revealed three compound sub-groups that produced different types of differential gene expression; these groups of genes were mainly involved in inflammation, cholesterol biosynthesis, and oxidative stress. Furthermore, the tBMDL values for each test compound were in good agreement with the relevant no observed adverse effect level. These results indicate that our novel strategy for drug safety evaluation using mechanism-based classification and tBMDL would facilitate the application of toxicogenomics for risk assessment of cholestatic DILI.

Key words: Toxicogenomics, Microarray, tBMD, Cholestasis, Mechanism-based

INTRODUCTION

Drug-induced liver injury (DILI) has led to the withdrawal of many drugs from the market. Wang *et al.* (2013) reported that 44% of these were withdrawn due to severe DILI, such as acute hepatitis, fatty liver, and cholestasis. Cholestasis is a condition where cytotoxic bile acids accumulate (Björnsson and Olsson, 2005) and this accounts for approximately 20% of DILI in the USA and Denmark (Padda *et al.*, 2011). In the diagnosis of DILI, cholestasis is widely evaluated clinically by measuring the levels of aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, and γ -glutamyltransferase (GGT) (Ozer *et al.*, 2008). In animal safety assessment experiments, biochemical measures of ALP, GGT, and bilirubin are utilized, in addition to histopathological findings such as bile duct injury (Fujii, 1997). However, the detection sensitivity of standard rodent evaluations of cholestatic chemicals is currently inadequate (Olson *et al.*, 2000). In particular, his-

topathological examination often underestimates the risk of DILI.

During drug development, the inhibitory effects on bile acid transporters such as the bile salt export pump (BSEP) are also evaluated. The clear correlation between inhibition of these transporters by some drugs and the incidence of cholestasis in both rats and humans (Fattering *et al.*, 2001; Funk *et al.*, 2001) has led to the development of some *in vitro* assessment methods that focus on BSEP inhibition (Ogimura *et al.*, 2011). However, not every BSEP inhibitor causes cholestasis (Morgan *et al.*, 2010) and it is therefore necessary to consider other mechanisms during these evaluations. In fact, research findings have indicated that cholestasis can be induced via several mechanisms; these include inhibition of cell-cell junctions, inhibition of microfilament activity, and even inflammation (Vinken *et al.*, 2013). For these reasons, the development of a mechanism-based and versatile method is required for the precise evaluation of the propensity of a drug to cause cholestasis.

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To address these difficulties, some research has focused on toxicogenomics using cDNA microarrays. This approach has been referred by pharmaceutical fields (Boverhof and Zacharewski, 2006) as well as some public authorities (National Research Council, 2007; Chepelev *et al.*, 2015; Organisation for Economic Co-operation and Development, 2016; Scientific Committee on Consumer Safety NfG, 2016). Toxicogenomics has the potential to detect key abnormalities and to improve understanding of biological mechanisms (Liu *et al.*, 2017). However, the current toxicogenomics methods have two crucial features that limit their practical application.

First, despite recent intense attempts to detect cholestasis using machine-learning based on toxicogenomics, the sensitivity was limited (67%) (Van den Hof *et al.*, 2014a). The use of toxicogenomics with machine-learning analysis sometimes leads to “over-fitting”, where the sensitivity during the training stage tends to decrease during validation using a new dataset. For example, Gusenleitner *et al.* (2014) conducted cross-validation analysis by machine learning using two toxicogenomics databases. However, this approach did not achieve the required level of sensitivity. Such an over-fitting problem might be caused by data heterogeneity, since several mechanisms of toxicity with different gene expression changes may underlie an adverse effect. Therefore, discriminating different gene expression patterns based on the mechanism of action would improve the sensitivity of prediction models. Indeed, Matsumoto *et al.* (2014) performed mechanism-based classification before the development of discriminators used to identify carcinogens; this approach resulted in the identification of high-performance discriminators in cross-validation using an external database. Thus, mechanism classification would be one promising approach to addressing over-fitting.

Second, the vast majority of current toxicogenomics methods involve qualitative approaches to mechanism-based classification. In contrast, quantitative methodology has been used less frequently for risk assessment. Yang *et al.* (2007) focused on dose-response effects using gene expression data and developed the transcriptional benchmark dose (tBMD) method; this was derived by applying the benchmark dose method to the estimation of transcriptional Point of Departure (tPoD) in cDNA microarray data. This tBMD approach has subsequently been used for the risk assessment of some substances (Clewell *et al.*, 2014; Dong *et al.*, 2016) and these studies found good agreement between tPoD and apical Point of Departure (aPoD), a measure that is traditionally used, such as the no observed adverse effect level (NOAEL). Therefore, the combination of mechanism-based classification

with the tBMD method could provide novel insights that would enable the pragmatic use of toxicogenomics for DILI risk assessment.

The present study aimed to develop a mechanism-based qualitative and quantitative methodology for the pragmatic safety assessment of cholestatic DILI. We employed the tBMD approach to quantitative risk assessment, followed by principal component analysis (PCA) and hierarchical clustering to analyze the mechanisms underlying cholestasis.

MATERIALS AND METHODS

Data collection

Clinical information about cholestatic chemicals was obtained from the Liver Tox database (US National Library of Medicine; <http://livertox.nlm.nih.gov/index.html>). The rat 28-day repeat dose tests in the Open TG-GATES (<http://toxico.nibiohn.go.jp>) (Igarashi *et al.*, 2015) were reviewed to determine the PoD for each cholestatic chemical. Canonical diagnostic markers (blood ALP, GTP, and bilirubin) were used as endpoints for cholestasis. If significant cholestatic marker up-regulation was observed, the maximum dose that did not cause up-regulation of these markers was defined as the cholestatic NOAEL. On the other hand, if no significant up-regulation of cholestatic markers was observed, the maximum dose was defined as NOAEL without cholestatic markers. These NOAEL were termed the aPoD. Statistical results of cholestatic marker levels were used as previously reported by National Institutes of Biomedical Innovation, Health and Nutrition (“Histopathology photograph collection”, <http://toxico.nibiohn.go.jp/english/seika.html>).

Qualitative analysis 1: mechanism classification

Data from microarrays performed using Affymetrix Rat Genome 230 2.0 chips (Santa Clara, CA, USA) were extracted from the Toxicogenomics Project-Genomics Assisted Toxicity Evaluation system (TG-GATES). The microarray data obtained from the livers of rats treated with only high doses of 12 cholestatic chemicals (Table 1) for 28 days were pre-processed by MAS5 (Gautier *et al.*, 2004) and then converted to logarithmic (base 2) fold changes ($\log_2\text{Fc}$) versus the solvent control group in order to identify differentially expressed genes. The differentially expressed genes were filtered by fold change ($\log_2\text{Fc} > 1$) and Welch's *t*-test ($p < 0.05$) and they were used in the subsequent steps if the differentially expressed genes were identified in over one-third of these 12 samples.

After extracting the differentially expressed genes, mechanism classification was performed by conduct-

Table 1. Test compounds and experimental conditions.

Name	Abbreviation	Vehicle	Route	Dose (mg/kg)		
				Low	Mid	High
Chlorpromazine	CPZ	0.5% methylcellulose	Gavage	4.5	15	45
Cyclosporine A	CSA	corn oil	Gavage	10	30	100
Diclofenac	DFNa	0.5% methylcellulose	Gavage	1	3	10
Erythromycin ethylsuccinate	EME	0.5% methylcellulose	Gavage	100	300	1000
Flutamide	FT	corn oil	Gavage	15	50	150
Imipramine	IMI	0.5% methylcellulose	Gavage	10	30	100
Ketoconazole	KC	0.5% methylcellulose	Gavage	10	30	100
Methyltestosterone	MTS	0.5% methylcellulose	Gavage	30	100	300
Naphthyl isothiocyanate	ANIT	corn oil	Gavage	1.5	5	15
Phenylbutazone	PhB	0.5% methylcellulose	Gavage	20	60	200
Sulindac	SUL	0.5% methylcellulose	Gavage	5	15	50
Tamoxifen	TMX	corn oil	Gavage	6	20	60

ing cluster analysis and PCA using R (Ver. 3.1.2, <https://cran.r-project.org/>) and its packages (cluster analysis: pvcust; PCA: prcomp, rgl). Hierarchical clustering was performed using the pvcust method (Suzuki and Shimodaira, 2006). Ward's method was used and the cluster distance was based on Euclidean distance. Statistical significance was considered when p-values were < 0.05.

Qualitative analysis 2: pathway analysis

The rank product method (Hong *et al.*, 2006) and Welch's *t*-test were used for a secondary differentially expressed gene extraction within the individual clusters identified by Qualitative analysis 1. The false discovery rate (FDR) for the rank product method and the p-value for the comparison between the control and treated rats in each classification were calculated (Welch's *t*-test). The significance thresholds applied were FDR < 0.001 (rank product), $p < 0.01$ (Welch's *t*-test), and $\log_2 F_c > 0.58$. These secondary differentially expressed genes (Supplemental Table 1) are referred to below as cholestatic differentially expressed genes.

The canonical pathway analysis and upstream regulator analysis (Krämer *et al.*, 2014) in Ingenuity Pathway Analysis (IPA; QIAGEN, North Rhine-Westphalia, Germany) were conducted using the cholestatic differentially expressed genes. The statistical significance threshold for the canonical pathway analysis was a $-\log p\text{-value} > 2$ (corresponding to $p < 0.01$). The directions of the effects on molecules predicted to be upstream of the differentially expressed genes (activation versus inhibition) in the upstream regulator analysis could be predicted by the Z-score. A Z-score > 1.96 was considered as the threshold of statistical significance.

Quantitative analysis: derivation of tPoD and comparison with aPoD

For the quantitative assessment of cholestatic effects using toxicogenomics, we analyzed microarray data obtained using three doses of these 12 cholestatic chemicals (low, middle, and high; Table 1). tBMD and the tBMD 95% lower limit (tBMDL) of each gene in the presence of each chemical were calculated by BMDexpress (Yang *et al.*, 2007). In short, the differentially expressed genes of each chemical (control, low, middle, and high doses) were imported to BMDexpress as $\log_2 F_c$ using the default settings. tBMD and tBMDL were then calculated for each cholestatic differentially expressed gene, excluding a set of 43 genes that showed differential expression for all or some treatments and were therefore regarded as non-specific. For model selection from Hill, power, linear, and polynomial, goodness-of-fit was cut off at $p > 0.1$. Cholestatic differentially expressed genes were omitted if tBMD/tBMDL was > 10 or if the lowest dose/tBMDL was > 100. Furthermore, the median values of tBMD and tBMDL in the presence of each chemical were calculated as tPoD, for comparison with the aPoD. A scatter plot was used to analyze the correlation between tPoD and aPoD. A paired *t*-test was used to analyze the statistical significance of any differences between the tPoD and aPoD values.

RESULTS

Cholestatic chemical selection

Nine known cholestatic compounds were identified using the Liver Tox database: chlorpromazine (CPZ); cyclosporine A (CSA); diclofenac (DFNa); flutamide (FT); imipramine (IMI); ketoconazole (KC); methyltestosterone (MTS); sulindac (SUL); and tamoxifen (TMX).

In addition, erythromycin ethylsuccinate (EME); naphthyl isothiocyanate (ANIT); and phenylbutazone (PhB) were also selected because these were previously reported to be cholestatic (Benjamin *et al.*, 1981; Diehl *et al.*, 1984; Ferreira *et al.*, 2003). The present study thus investigated 12 cholestatic chemicals. Table 1 summarizes these and the *in vivo* experimental conditions.

Qualitative classification of 12 cholestatic chemicals into three groups associated with inflammation, cholesterol biosynthesis, and oxidative stress

Cluster analysis initially identified two statistically significant clusters (Fig. 1A). Cluster 1 included DFNa and SUL, while cluster 2 included the other test compounds. Cluster 2 contained two minor clusters that did not differ significantly. However, PCA clarified this sub-division (Fig. 1B) by separating MTS, TMX, PhB, IMI, CPZ, KC, and EME from FT, CSA, and ANIT. These analyses classified the 12 test compounds into three groups: Group 1, DFNa and SUL; Group 2, ANIT, FT, and CSA; and Group 3, MTS, TMX, CPZ, KC, EME, PhB, and IMI.

Next, secondary cholestatic differentially expressed genes (69 probe sets in Group 1, 87 probe sets in Group 2, and 89 probe sets in Group 3) were extracted using the rank product method and Welch's *t*-test, followed by pathway analysis and upstream regulator analysis.

Fig. 2 shows the heat map for each cholestatic differentially expressed gene and Fig. 3 shows each pathway network and upstream regulators. In Group 1, inflammatory and/or immune responsive cytokines (*S100a8/9*, *Cxcl1*, *Il-7*) and signal mediators (*Jak3*, *Stat3*) were up-regulated. In Group 2, genes associated with the oxidative stress response (*Gpx2*, *Nqo1*) were identified in the upstream regulator analysis, and an apoptosis-related gene (*Hspa1a*) was also up-regulated. Both of these profiles indicated cell damage, via different mechanisms. In Group 3, phase I/II metabolic enzymes such as *Cyp2b1/2*, *Cyp4a8*, *Aldh1b1*, *Abcb1a/b*, and *Gstm3* were identified as differentially expressed genes, and nuclear receptors (the constitutive androstane receptor [CAR (NR1I3)] and the pregnane X receptor [PXR (NR1I2)]) were predicted as upstream regulators.

A summary of the canonical pathway analysis is shown in Table 2. Consistent with the upstream regulator analysis results, each chemical group was associated with specific pathways. For example, Group 1 effects were indicative of an inflammatory/damage response (role of IL-17A in psoriasis, role of JAK1 and JAK3 in γ c cytokine signaling, acute phase response signaling, IL-9 signaling, coagulation system, IL-6 signaling), Group 2 effects related to cholesterol synthesis (cholesterol biosynthesis I, cholesterol biosynthesis II [via 24,25-dihydrolanosterol], cholesterol biosynthesis III [via desmosterol]), and Group 3

Fig. 1. Cholestatic mechanism using two unsupervised methods. (A) Cluster analysis of 12 chemicals. The red boxes enclose compounds that did not differ significantly within the groups. (B) PCA of 12 chemicals. Each color represents one group: red, Group 1 (DFNa, SUL); green, Group 2 (ANIT, FT, CSA); blue, Group 3 (MTS, TMX, CPZ, KC, EME, PhB, IMI).

Mechanism-based quantitative toxicogenomic analysis of cholestasis

Fig. 2. Heat map of differentially expressed gene values for cholestatic chemicals. Each color represents the fold-change in relation to the solvent control. Some differentially expressed genes were specific for a group, while others were non-specific.

Fig. 3. Pathway analysis of three groups of cholestatic chemicals. The networks between differentially expressed genes and upstream regulators were generated using upstream regulator analysis. (A) Pathway network for Group 1. (B) Pathway network for Group 2. (C) Pathway network for Group 3. Red indicates up-regulated genes, green indicates down-regulated genes, orange indicates activated upstream-regulator molecules, and blue indicates inactivated upstream-regulator molecules. The color intensity indicates the extent of the change in level or activity.

Table 2. Summary of the canonical pathway for each classified group.

Canonical pathway		Group 1	Group 2	Group 3
Group 1-specific	Oncostatin M Signaling	3.84	0	0
	Atherosclerosis Signaling	3.28	1.02	1.03
	ErbB2-ErbB3 Signaling	3.17	0.67	1.64
	Role of IL-17A in Psoriasis	3.17	0	0
	Role of JAK1 and JAK3 in $\gamma\epsilon$ Cytokine Signaling	3.06	0	0
	Acute Phase Response Signaling	2.78	0	0.3
	Pancreatic Adenocarcinoma Signaling	2.4	0	0.45
	IL-9 Signaling	2.33	0	0
	Coagulation System	2.3	0	0
	IL-6 Signaling	2.29	0	1.08
	Alanine Biosynthesis II	2.22	0	0
	Alanine Degradation III	2.22	0	0
	iNOS Signaling	2.11	0	0
	L-serine Degradation	2.05	1.9	0
Group 2-specific	Superpathway of Cholesterol Biosynthesis	1.09	3.67	0.96
	Cholesterol Biosynthesis I	1.42	2.89	1.28
	Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	1.42	2.89	1.28
	Cholesterol Biosynthesis III (via Desmosterol)	1.42	2.89	1.28
	Leukotriene Biosynthesis	0	2.76	0
	Asparagine Biosynthesis I	0	2.38	0
Group 3-specific	Noradrenaline and Adrenaline Degradation	0.97	1.96	3.29
	Estrogen Biosynthesis	0	1.96	3.29
	PI3K/AKT Signaling	1.28	0.39	2.77
	GADD45 Signaling	1.25	0	2.56
	Glutathione-mediated Detoxification	0	0.94	2.2
	NRF2-mediated Oxidative Stress Response	0.38	1.4	2.18
	Cell Cycle Regulation by BTG Family Proteins	1	0.87	2.04
	Aryl Hydrocarbon Receptor Signaling	1.18	1.68	4.61
Groups 1/2 merged	LXR/RXR Activation	2.24	2.78	1.86
	Epoxysqualene Biosynthesis	2.22	2.08	0
Groups 2/3 merged	Xenobiotic Metabolism Signaling	0.71	7.76	11.25
	PXR/RXR Activation	1.77	5.09	11.17
	LPS/IL-1-Mediated Inhibition of RXR Function	1.54	3.5	7.62
	Histamine Degradation	1.3	2.65	4.36
	Oxidative Ethanol Degradation III	1.25	2.55	4.2
	Nicotine Degradation III	0	2.83	4.14
	Fatty Acid α -oxidation	1.23	2.51	4.14
	Putrescine Degradation III	1.21	2.47	4.07
	Melatonin Degradation I	0	2.76	4.05
	Ethanol Degradation IV	1.17	2.39	3.95
	Tryptophan Degradation X (Mammalian, via Tryptamine)	1.17	2.39	3.95
	Superpathway of Melatonin Degradation	0	2.65	3.9
	Nicotine Degradation II	0.76	2.63	3.88
	Bupropion Degradation	0	2.31	3.84
	Serotonin Degradation	0.74	2.56	3.77
	Acetone Degradation I (to Methylglyoxal)	0	2.25	3.74
	Dopamine Degradation	1.09	2.22	3.69
	Retinoate Biosynthesis I	1.02	3.46	2.09
	Ethanol Degradation II	1	2.03	3.4
	Lanosterol Biosynthesis	0	2.38	2.39

Values represent $-\log_{10}$ of the p-values calculated using IPA (Fisher's exact test). Statistically significant changes are indicated by grey shading.

effects related to oxidative stress (GADD45 signaling, glutathione-mediated detoxification, NRF2-mediated oxidative stress response). These results indicated that toxicogenomics could discriminate based on the cholestatic mechanism of action.

Quantitative analysis identified good agreement between aPoD and tPoD

The aPoD for each chemical is summarized in Table 3. Whereas six of the 12 test compounds (CSA, FT, MTS, ANIT, SUL and TMX) showed increased cholestatic markers, the other six (CPZ, DFNa, EME, IMI, KC and PhB) did not. To simplify the comparison of aPoD and tPoD, we analyzed these compounds separately.

For the six chemicals that produced increased cholestatic markers, the correlations between aPoD and tPoD are shown by scatter plots in Fig. 4 (A and B). There were no significant differences between tPoD and aPoD, and both tBMD and tBMDL highly correlated with aPoD (tBMD, $R = 0.73$; tBMDL, $R = 0.77$). For the remaining six chemicals that did not produce increased cholestatic markers, the correlations between aPoD and tPoD are shown by scatter plots in Fig. 4 (C and D). tPoD values were significantly lower than aPoD values, and both tBMD and tBMDL were very highly correlated with aPoD (tBMD, $R = 0.99$; tBMDL, $R = 0.99$). In particular, the ratio of tBMDL values to aPoD values of these 12 chemicals were within 6-fold.

DISCUSSION

The present study aimed to develop a practical tool

for cholestatic DILI risk assessment using qualitative and quantitative toxicogenomics methods, which combined unsupervised analyses and tBMD. This study resulted in two major conclusions. Firstly, known cholestatic compounds were classified into three major groups using qualitative analyses and secondly, we found that the tPoD derived from the differentially expressed gene dose-response could predict aPoD.

DILI represents a major challenge for risk assessment during the development of pharmaceuticals and other chemical substances, such as cosmetic ingredients (Vinken *et al.*, 2012). The range of potential mechanism of action involved means that DILI is not always detected by canonical methods. Thus, the present study indicated a novel qualitative and quantitative DILI assessment based on the mechanism of action.

The present mechanistic classification of 12 compounds by combined unsupervised analysis identified three mechanism of action groups. Our use of a combination of PCA and cluster analysis was shown to be useful for this classification. Although the application of PCA alone could also reveal the relationship between substance groups (Watanabe *et al.*, 2012; Furihata *et al.*, 2016), this approach requires expert judgment to achieve accurate classification. On the other hand, use of cluster analysis alone can discriminate substance groups without expert judgment; however, it often produces less separation of the sub-groups such as was observed for Groups 2 and 3. This sub-group classification was considered very important in the present study, since canonical pathway analysis identified Group 2- and 3-specific pathways. Therefore, the classification into three groups that was achieved

Table 3. Summary of aPoD for each test compound.

Name	ALP			Bilirubin			GTP			aPoD	
	Low	Middle	High	Low	Middle	High	Low	Middle	High	Cholestatic NOAEL (mg/kg/day)	NOAEL without cholestatic markers (mg/kg/day)
ANIT	-	-	+	-	-	+	-	-	+	5	N.D.
CSA	-	+	+	-	+	+	-	-	-	10	N.D.
FT	-	-	-	-	-	+	-	-	-	50	N.D.
MTS	-	-	-	-	-	+	-	-	+	100	N.D.
SUL	-	-	-	-	-	-	-	-	+	15	N.D.
TMX	-	+	+	-	-	-	-	-	-	6	N.D.
CPZ	-	-	-	-	-	-	-	-	-	N.D.	45
DFNa	-	-	-	-	-	-	-	-	-	N.D.	10
EME	-	-	-	-	-	-	-	-	-	N.D.	1,000
IMI	-	-	-	-	-	-	-	-	-	N.D.	100
KC	-	-	-	-	-	-	-	-	-	N.D.	100
PhB	-	-	-	-	-	-	-	-	-	N.D.	200

N.D., not determined; +, increased level; -, unchanged level.

Fig. 4. Correlation between tPoD and aPoD for six cholestatic chemicals. tBMD or tBMDL were plotted with aPoD as scatter plots, as indicated. (A) tBMD with cholestatic NOAEL. (B) tBMDL with cholestatic NOAEL. (C) tBMD with NOAEL without cholestatic markers (D) tBMDL with NOAEL without cholestatic markers. R = correlation coefficient, dashed line = factor 6.

by the combination of PCA and cluster analysis would be appropriate for tPoD calculation.

Pathway analyses identified three major mechanisms related to cholestasis. A previous study had reported that the inflammatory response, highlighted by Group 1 compounds in the present study, could cause cholestasis through indirect inhibition of BSEP (Hartmann *et al.*, 2002). The lipid metabolism pathway highlighted by Group 2 (Table 2) could be associated with bilirubin up-regulation (Hirode *et al.*, 2009). In addition, cholestasis

has been reported to contribute to dysfunction of cholesterol biosynthesis (Nemes *et al.*, 2016). The nuclear receptors identified by Group 3 suggested that CAR and PXR were associated with cholestasis. These receptors are key molecules for exogenous xenobiotic metabolism and endogenous lipid metabolism during adaptive and physiological responses. Chronic dysregulation of these receptors, which include the bile acid sensor, farnesoid X receptor, may be involved in the etiology of cholestasis. Some studies analyzed CSA-induced cholestatic dif-

ferentially expressed genes *in vivo* (Driessen *et al.*, 2015) and *in vitro* (Van den Hof *et al.*, 2014b, 2015) and reported similar findings relating to lipid metabolism and nuclear receptor activation. It should be mentioned that the cholestasis-related gene, *Scd1* (Flowers *et al.*, 2006), was also included in this group. These previous findings were therefore consistent with the three major mechanisms identified in the present study. Non-cholestatic chemicals were not evaluated in the present study and the identified differentially expressed genes might not therefore represent cholestasis specific changes. Further screening of genes would thus be necessary to elucidate the specific mechanisms of action of cholestatic chemicals.

The present study calculated tPoD values using each differentially expressed gene set within the different mechanism of action groups. With regards to the difference between tPoD and aPoD, tPoD values (median values of tBMD or tBMDL) were significantly lower than aPoD for chemicals that did not increase cholestatic markers (Fig. 4C, 4D), whereas there were no significant differences between tPoD and aPoD for chemicals that altered cholestatic markers (Fig. 4A, 4B). These tPoD values correlated closely with aPoD and the ratio of tBMDL values to aPoD values of these chemicals were within 6-fold. Thus, it would be better to use tBMDL to provide a conservative assessment. This result indicated that tPoD could be used to achieve quantitative assessment of DILI risk; this was consistent with a previous report by Thomas *et al.* (2013) describing the usefulness of tPoD for chemical risk assessment. However, the comparison of tPoD with aPoD in the present study was conducted using 28-days repeated toxicity test data. Thus, further studies should be performed to compare tPoD and aPoD values determined by chronic toxicity studies.

In conclusion, our findings have provided objective qualitative mechanism of action classification methods that could facilitate identification of more accurate toxicity markers. Furthermore, tPoD values can be used to assess cholestatic potential quantitatively, even if aPoD is not derived. In addition, our methodology has a major advantage over canonical binary (positive or negative) toxicogenomics methodology and could provide an alternative approach to assessing drugs during the development process. Further validation studies using an external dataset should be conducted using this approach to examine compounds associated with cholestasis and other DILI including cirrhosis, fibrosis, and lipidosis/phospholipidosis.

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