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ABC gene-ranking for prediction of drug-induced cholestasis in rats

Yauheniya Cherkas^a, Michael K. McMillian^{b,1}, Dhammika Amaratunga^{b,2},
Nandini Raghavan^c, Jennifer C. Sasaki^{b,*}^a Janssen Research and Development, LLC, Spring House, PA, USA^b Janssen Research and Development, LLC, Raritan, NJ 08869, USA^c Janssen Research and Development, LLC, Titusville, NJ 08540, USA

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ABSTRACT

As legacy toxicogenomics databases have become available, improved data mining approaches are now key to extracting and visualizing subtle relationships between toxicants and gene expression. In the present study, a novel “aggregating bundles of clusters” (ABC) procedure was applied to separate cholestatic from non-cholestatic drugs and model toxicants in the Johnson & Johnson (Janssen) rat liver toxicogenomics database [3]. Drug-induced cholestasis is an important issue, particularly when a new compound enters the market with this liability, with standard preclinical models often mispredicting this toxicity. Three well-characterized cholestasis-responsive genes (Cyp7a1, Mrp3 and Bsep) were chosen from a previous in-house Janssen gene expression signature; these three genes show differing, non-redundant responses across the 90+ paradigm compounds in our database. Using the ABC procedure, extraneous contributions were minimized in comparisons of compound gene responses. All genes were assigned weights proportional to their correlations with Cyp7a1, Mrp3 and Bsep, and a resampling technique was used to derive a stable measure of compound similarity. The compounds that were known to be associated with rat cholestasis generally had small values of this measure relative to each other but also had large values of this measure relative to non-cholestatic compounds. Visualization of the data with the ABC-derived signature showed a very tight, essentially identically behaving cluster of robust human cholestatic drugs and experimental cholestatic toxicants (ethinyl estradiol, LPS, ANIT and methylene dianiline, disulfiram, naltrexone, methapyrilene, phenacetin, alpha-methyl dopa, flutamide, the NSAIDs—indomethacin, flurbiprofen, diclofenac, flufenamic acid, sulindac, and nimesulide, butylated hydroxytoluene, piperonyl butoxide, and bromobenzene), some slightly less active compounds (3'-acetamidofluorene, amsacrine, hydralazine, tannic acid), some drugs that behaved very differently, and were distinct from both non-cholestatic and cholestatic drugs (ketoconazole, dipyrindamole, cyproheptadine and aniline), and many postulated human cholestatic drugs that in rat showed no evidence of cholestasis (chlorpromazine, erythromycin, niacin, captopril, dapson, rifampicin, glibenclamide, simvastatin, furosemide, tamoxifen, and sulfamethoxazole). Most of these latter drugs were noted previously by other groups as showing cholestasis only in humans. The results of this work suggest that the ABC procedure and similar statistical approaches can be instrumental in combining data to compare toxicants across toxicogenomics databases, extract similarities among responses and reduce unexplained data variation.

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* Corresponding author. Current address: Neuroscience, Innovative Medicines and Early Development Biotech Unit, AstraZeneca, 140 Portland Street, Cambridge, MA 02139, USA.

E-mail addresses: ycherka@its.jnj.com (Y. Cherkas), m.mcmillian@invitrocue.com (M.K. McMillian), damaratu@its.jnj.com (D. Amaratunga), nraghava@its.jnj.com (N. Raghavan), jennifer.sasaki@azneuro.com (J.C. Sasaki).

¹ Current address: InvitroCue, 11 Biopolis Way, Helios #12-07/08, Singapore 138667, Singapore and Department of Physiology, NUS Yong Loo Lin School of Medicine, Block MD9, 2 Medical Drive #03, Singapore 117597, Singapore.

² Current address: Independent statistical researcher and consultant, Bridgewater, NJ 08807, USA.

1. Introduction

Cholestasis, or the reduction of bile flow, can progress to serious hepatotoxicity in patients and is a particular concern in evaluation of novel drug candidates. Extrahepatic cholestasis occurs within the bile duct, forming blockages via concentrated, precipitated drugs, or by damage of the biliary epithelial cells, and loss of bile transport and flow. Although a variety of drugs are concentrated in the bile, drug-induced obstructive cholestasis is often readily observed pre-clinically, and can be avoided. Intrahepatic cholestasis associated with hepatotoxicity generally results from bile salt export pump (BSEP) inhibition, which if not adequately handled by accessory pathways such as MRP3, results in accumulation of detergent-like bile salts, that are toxic to hepatocytes. At high dose levels, novel drug candidates can bind to multiple hepatic transporters and trigger cholestatic signals. Therefore, screening for transporter inhibition in membrane vesicles is a typical step in the drug discovery and development process [21,22,25,33]. Identification and translational understanding of cholestatic responses in preclinical species is key step in the nonclinical safety assessment before initiating pharmaceutical clinical development.

The rat is a well-characterized model in which to study cholestasis, and is a principal species for preclinical toxicology studies. There are some differences from human: bile salts are conjugated by different enzymes (cholic acid and chenodeoxycholic acid are predominantly conjugated with glycine in man versus with taurine in the rat), the molecular weight “filter” for drug biliary secretion is generally lower than in humans (~350 molecular weight in rat, versus ~500 in humans), and the rat lacks a gall bladder [1,17]. This latter difference is an experimental advantage in that there is more consistent bile flow regulation in rats. Additionally, laboratory rats eat a well-defined, carbohydrate-rich rodent chow and feed most heavily during the dark cycle, more continually through the day than most humans. These feeding habits contribute to less variation in rat biliary transport and a continual and higher volume of bile flow [10]. Despite numerous species differences when comparing drug effects in rat to human, most transporter inhibition studies show remarkable consistency; for example, no rat versus human differences were observed in a large study of drug inhibition of BSEP transport [21].

A number of models of cholestasis in the rat are well studied and established: biliary ligation, which models bile duct obstruction; ANIT, which damages/destroys biliary epithelium; LPS, which down-regulates much of the hepatic metabolism including most transporters; and glucuronidated ethinyl estradiol-induced cholestasis by inhibition of BSEP [2,7,11,20,23,34,36,39]. All of these models have been characterized by gene expression on microarrays [7,11,34,39]. There is a misleading tendency to generalize the specifics of each of these different models to all forms of cholestasis. For characterization of novel drug candidates, there is a need to capture as much of the invariant cholestasis gene response as possible across a wide range of pharmaceutical structural classes.

At Janssen we have developed a predictive, 24 h treatment rat toxicogenomic database using approximately one hundred non-proprietary treatments and four times as many proprietary compound treatments, the latter having supporting exposure, clinical chemistry and histopathology data, often out to a month of dosing. We have published predictive gene signatures for non-genotoxic carcinogens [24,27] macrophage and PPAR α receptor activation [18] and particularly oxidative stress/reactive metabolite responses for detecting idiosyncratic hepatotoxicants [14,15]. We previously developed a cholestasis gene expression signature using in-house proprietary compounds that induced cholestasis in rat studies (unpublished data). In the present study, we have used three robust non-redundant genes (Cyp7a1, Mrp3 and Bsep) from this previous rat signature which have been implicated in cholesta-

sis [13,19,29,35]. Non-redundant genes differentially respond with large variances to compounds across our database, therefore a single one of these genes suffices for relevant transcriptomics information. Protein expression of MRP3 is up-regulated during cholestasis and genetic defects in MRP3 have been linked with pregnancy or estrogen hormone-induced cholestasis [28]. Mutations of the gene encoding the human bile salt export pump are implicated in progressive familial intrahepatic cholestasis type 2 (PFIC2), and inhibition of human and/or rat BSEP transporter constructs have been demonstrated to be correlated to cholestatic potential [4]. CYP7A1 is an important participant in transcriptional regulation, due to bile acid synthesis via nuclear hormone receptors and modulates cholestasis response via the classical pathway of bile enzyme synthesis [12,26,31].

From this starting point of three genes, we then applied the “aggregating bundles of clusters” (ABC) statistical approach [3] to develop a rat cholestasis gene expression signature that better discriminates cholestatic from non-cholestatic compounds. Both our unpublished proprietary signature and the present 100-gene rat cholestasis signature of this work yield good separation of compounds, and can discriminate many drugs known to cause cholestasis in man.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats, seven to eight weeks old, and approximately 275 g body weight (Charles River Laboratories, Inc.) were used for experiments. Animals were individually housed in wire-bottom cages, on a 12 h/light/dark cycle, and fed Certified Rodent Diet 5002 (LabDiet) ad libitum, with free access to water. On the day prior to dosing, animals were randomized by weight and allocated to groups ($n = 3$ rats/group). The route of administration for each test article is denoted in Table 1. Oral gavage was the most commonly utilized mode of administration as it is the typical route for the majority of compounds (predominantly pharmaceuticals) included in the training set. Animals were dosed in the morning hours, with health checks performed at 1 h and 4 h after dosing, and the end of the workday. Any animals deemed to be in poor health status were evaluated by a veterinarian: in these experiments no animals were prematurely terminated due to poor health.

Necropsy was performed on fasted rats, 24 h following dosing. Rats were killed by exsanguination, severing the vena cava under CO₂ analgesia, and liver sections (approximately 200 mg of the right medial lobe) were transferred to labeled cryo tubes and snap frozen in liquid nitrogen.

The selected dose level was the maximum tolerated dose, as elucidated from the literature [18,24]. Compound selection rationale, dose levels, route of exposure, group size, and experiment number are documented within Johnson and Johnson publically-accessible NIEHS-hosted database <http://cebs.niehs.nih.gov>.

In all instances, the animals were humanely handled and according to institutional guidelines, in accordance with the IACUC and NRC Guide for the Care and Use of Laboratory Animals.

2.2. Generation of microarray data

Total RNA was extracted from liver samples using Qiagen RNEasy Midi kits (Qiagen, Inc., Valencia, CA) as per kit instructions. The amount of RNA in the samples was determined spectrophotometrically by absorbance ratio at 260 and 280 nm. Quality of RNA in the samples was assessed using rRNA peaks determined by an Agilent 2100 Bioanalyzer.

Table 1
Figure abbreviations for cholestatic (red) and non-cholestatic (black) pharmaceuticals and model toxicants used in this study. Compounds were dosed via oral gavage (p.o.) unless otherwise noted (subcutaneous—s.c.; intraperitoneal—i.p.).

2-Acetamidofluorene	Aceta	Diclofenac	Diclo	Mifepristone	Mifep
3-Acetamidophenol	Aceta	Dieldrin	Dield	Mycophenolic Acid	Mycop
Adrenocorticotropin (s.c.)	Adren	Digoxin	Digox	Naltrexone	Naltr
Aminoglutethimide (s.c.)	Amino	Dipyridamole	Dipyr	Niacin	Niaci
Amiodarone	Amiod	Disulfiram	Disul	Nimesulide	Nimes
Amsacrine (i.p.)	Amsac	Doxorubicin	Doxor	Nizatidine	Nizat
Aniline	Anili	ErythroMC Estolate	Eryth	Paraquat	Paraq
α -naphthylisothiocyanate	ANIT	Ethinyl Estradiol	Ethin	Perhexiline	Perhe
Aspirin	Aspir	Etoposide	Etopo	Phenacetin	Phena
Atenolol	Ateno	Famotidine	Famot	Phenylephrine (i.p.)	Pheny
Bromobenzene	Bromo	Flufenamic Acid	Flufe	Piperonyl butoxide	Piper
Bromocryptine	Bromo	Fluoxetine	Fluox	Progesterone	Proge
Buspiron	Buspi	Flurbiprofen	Flurb	Puromycin	Purom
Busulfan (i.p.)	Busul	Flutamide	Fluta	Raloxifene	Ralox
Butylated hydroxytoluene	Butyl	Furosemide	Furos	Ranitidine	Ranit
Cadmium Chloride	Cadmi	Gentamycin	Genta	Rifampin	Rifam
Captopril	Capto	Glibenclamide	Glibe	Rotenone	Roten
Carbamazepine	Carba	Hydrazine Hydrate	Hydra	Simvastatin	Simva
Carmustine	Carmu	Ibuprofen	Ibupr	Spironolactone (s.c.)	Spiro
Chlorambucil	Chlor	Indomethacin (i.p.)	Indom	Stanozolol (s.c.)	Stano
Chlorpromazine	Chlor	Isoniazid	Isoni	Streptozocin (i.p.)	Strep
Cholecalciferol (s.c.)	Chole	Isoproterenol (i.p.)	Isopr	Sulfamethoxazole	Sulfa
Cisplatin	Cispl	Ketoconazole	Ketoc	Sulindac	Sulin
Clozapine	Cloza	LPS (i.p.)	LPS	Tacrine	Tacri
Cyclophosphamide	Cyclo	Mebendazole	Meben	Tamoxifen	Tamox
Cyproterone Acetate	Cypro	Metformin	Metfo	Tannic Acid	Tanni
Dacarbazine (i.p.)	Dacar	Methapyrilene	Metha	Testosterone (s.c.)	Testo
Dantrolene	Dantr	Methotrexate	Metho	Tetracycline	Tetra
Dapsone	Dapso	Methyldopa	Methy	Valproic Acid	Valpr
Dexamethasone	Dexam	Methylenedianiline	Methy	Verapamil	Verap
Dichloroacetate	Dichl	Metoprolol	Metop	Vitamin A	Vitam

Reverse transcription of RNA with a T7 promoter oligo (dT) primer, followed by in vitro transcription using the RiboBeast 1-Round Aminoallyl-aRNA Amplification Kit was used for one round of RNA amplification (Epicentre, Madison, WI). Superscript II (Invitrogen, Carlsbad, CA) was used as the reverse transcriptase, and the Rneasy96 block was used for purification (Qiagen, Valencia, CA) as previously described [24]. Purified biotin-labeled cRNA was fragmented at 94 °C for 20 min and added to Codelink hybridization buffer A and B (GE Healthcare, Chandler, AZ). Each cRNA sample was applied to two Codelink Rat Whole Genome microarrays (GE Healthcare) using the manufacturer's protocol, and hybridized at 37 °C overnight. Microarrays were washed, stained with an Alexa555-streptavidin conjugate (Invitrogen), and then scanned at 532 nm with an Agilent G2565BA Microarray Scanner (Agilent Technologies, Palo Alto, CA).

Fluorescence intensities for the microarray features were determined using Codelink EXPv4.1 software (GE Healthcare). Additional details regarding experimental procedures may be found in Ref. [18].

2.3. Preprocessing of microarray data

The data for this study was part of a larger set of toxicogenomic experiments which spanned several hybridization batches. There were several vehicle controls run concurrently in each hybridization batch to establish baseline gene expression levels. The normalization procedure consisted of the following steps within each hybridization batch: (i) spot winsorization on the log-intensities to remove outliers; (b) quantile normalization to the

median of the vehicle controls in that batch; (c) mean summarization of the log-intensities for each gene of the technical replicates for each RNA sample; (d) log ratios of the mean in (c) with the median of the vehicle control within the hybridization set. Details of the winsorization and quantile normalization can be found in Ref. [3]. The dataset consists of log-ratios of the expression levels to the median vehicle control of the corresponding hybridization batch as described above, for each sample, for the 35129 probesets on the microarray chip. All analyses described below were conducted on the probesets. Compounds that were known PPAR agonists and macrophage activators (many of the acutely hepatotoxic compounds) were omitted from analysis despite cholestatic properties, since their robust effects on gene expression interfered with subtle signatures, as noted previously [24,27]. LPS was added to the analysis since it is an important cholestatic model compound in the rat.

2.4. Public availability of microarray data

The data used in this study is from the non-proprietary Johnson & Johnson (Janssen) toxicogenomics Codelink database, available in normalized ratio format in collaboration with Strand Life Sciences, Pvt., Ltd., at the weblink: <http://pubdata.strandls.com/> in the folder “jnj codelink data/” [30].

Raw data related to this project are also publically available in the CEBS at the NIEHS website.

To access the CEBS website:

- Go to <http://cebs.niehs.nih.gov> and select the “Open CEBS” option. Select the “CEBS accession number” option under the “Search Study” area.
- Enter ‘004-00009-0000-000-3’ and select “Search.”
- The folder “J&J Compounds of Known Toxicity” will appear. Open folder by clicking on the arrow to view the studies in the database. Details on each study may be obtained by double clicking on that file.
- To access specific data by compound, organ, and Unigene ID, select “J&J Codelink Data” in the “Workflows” area.
- Data can be directly downloaded via the CEBS FTP site in two ways. Go to <http://cebs.niehs.nih.gov>.
 - Select “Download Data” option. All user submitted microarray data and quantile normalized files are available at: <ftp://157.98.192.110/ntp-cebs/datatype/microarray/j&j/004-00009-0000-000-3/> or can be found by selecting the options: **Download data > microarray > J&J > 004-00009-0000-000-3.**
- Alternatively, microarray data organized by study are available at the link: <ftp://157.98.192.110/ntp-cebs/FileList/004-00009-0000-000-3/>.

2.5. Biostatistical analysis of the microarray data

A novel ABC-like procedure was applied [3]. The following three steps were performed R times:

- N compounds were selected at random with replacement. Any repeats and/or replicates were discarded. Assume that this left N^* compounds ($N^* \leq N$). This is the “bag” of compounds for this run.
- An association score A_k was assigned to each gene k . A_k was taken to be the maximum of the correlations between the k th gene and each of the three focal genes across the N^* samples; any gene that was highly correlated to at least one of the three focal genes would have a high association score. Under the premise that genes with relatively large values of A_k were more likely to carry cholestasis related information than genes with relatively small values of

A_k , a weight w_k was calculated for each gene k : $w_k = 1/(R_k + \sqrt{G})$, where R_k was the rank of A_k .

- \sqrt{G} genes were selected using weighted random sampling without replacement with weights (w_k). This small set of genes included an overabundance of genes correlated with the three focal genes. For only these N^* compounds and G^* genes, a cluster analysis was run using a standard clustering algorithm such as Ward’s hierarchical clustering procedure to get a set of \sqrt{N} base clusters.

Once these steps were run R times, the results were collated. Of the number of times that the i th and j th compounds were both included in the same bag (in step (1) of the procedure), the proportion C_{ij} of times that they also fell into the same cluster (in step (3) of the procedure) was determined.

Let $D_{ij} = 1 - C_{ij}$. A value of D_{ij} close to zero indicated that compounds i and j often clustered together in the base clusters, thereby implying that they were relatively similar to each other. Alternatively, a value of D_{ij} close to 1 indicated that compounds i and j rarely clustered together in the base clusters, thereby implying that they were relatively dissimilar to one another. Thus D_{ij} could serve as a dissimilarity measure. Multidimensional scaling of (D_{ij}) was then used to generate a two-dimensional representation of the similarities and dissimilarities of the compounds.

Following the ABC-like procedure, a boosting procedure incorporating an elastic net approach [16,41] was applied and performed 1000 times, with frequencies calculated for all signature sets.

3. Results

Although an in-house proprietary cholestasis signature was available our goal was to generate a robust gene expression signature using only non-proprietary drugs and compounds. Table 1 provides a list of cholestatic (red) and non-cholestatic (black) compounds. Initially expression profiles of only three genes (Cyp7a1, Mrp3 and Bsep), well characterized as cholestasis-responsive genes, were examined. Their expression profiles, plotted in a three-dimensional scatterplot, with known cholestatic compounds highlighted in red, did not reveal a useful separation of the compounds in any readily interpretable fashion (Fig. 1).

We next examined the simultaneous expression profiles of all genes using several methods. One approach was to reduce the dimensionality of the data using principal component analysis and to then plot the first two components (Fig. 2). Another was to calculate the dissimilarity between every pair of compounds using either Euclidean distance (Fig. 3) or 1-correlation (Fig. 4) and to then use multidimensional scaling to display the compounds on a two-dimensional scatterplot [37]. This indicated fair separation between groups, typical of many gene expression signatures, however many compounds in the two groups overlapped, with no obvious basis for the way cholestatic and non-cholestatic compounds distributed in space. Clearly while it was insufficient to consider three key genes (Fig. 1), consideration of all genes was also unproductive (Figs. 2–4). Although such mild separations were considered acceptable in past studies [24], a better statistical analysis method was sought.

A novel approach based on the ABC (“aggregating bundles of clusters”) technique was implemented [3]. The premise of this method is that most of the genes do not contribute to the organization of the compounds but the analysis should tilt towards the genes that do. Because the three selected “focal” non-redundant genes Cyp7a1, Mrp3 and Bsep are implicated with cholestasis, other gene responses could be ranked in importance by how much they correlated to responses of these three genes. Application of the ABC procedure yielded a display of the compounds that was inter-

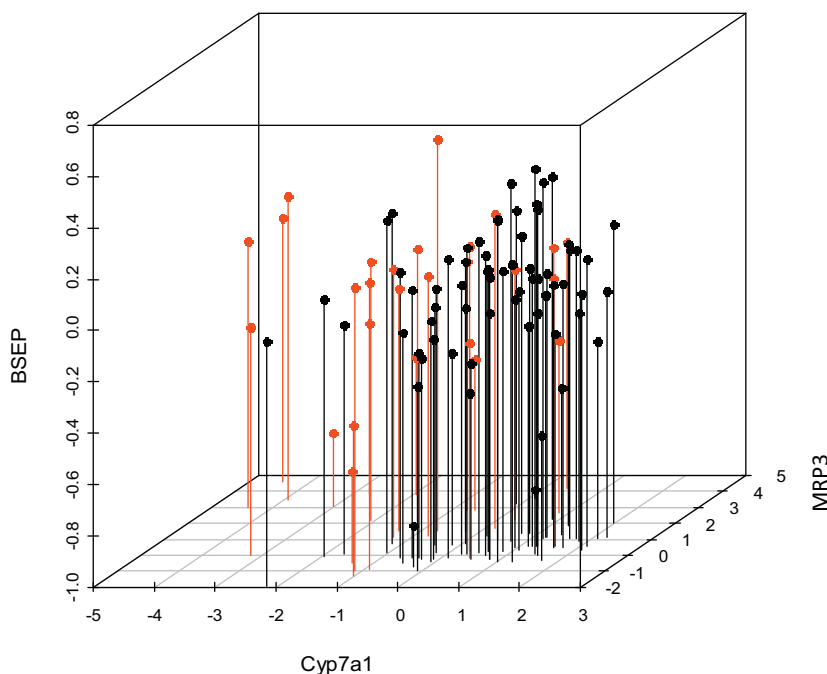


Fig. 1. mRNA expression profiles of 3 genes (Cyp7a1, MRP3 and Bsep) to cholestatic (red) and non-cholestatic (black) training set pharmaceuticals and model toxicants in male rat liver. Note that no clear separation was observed between classes.

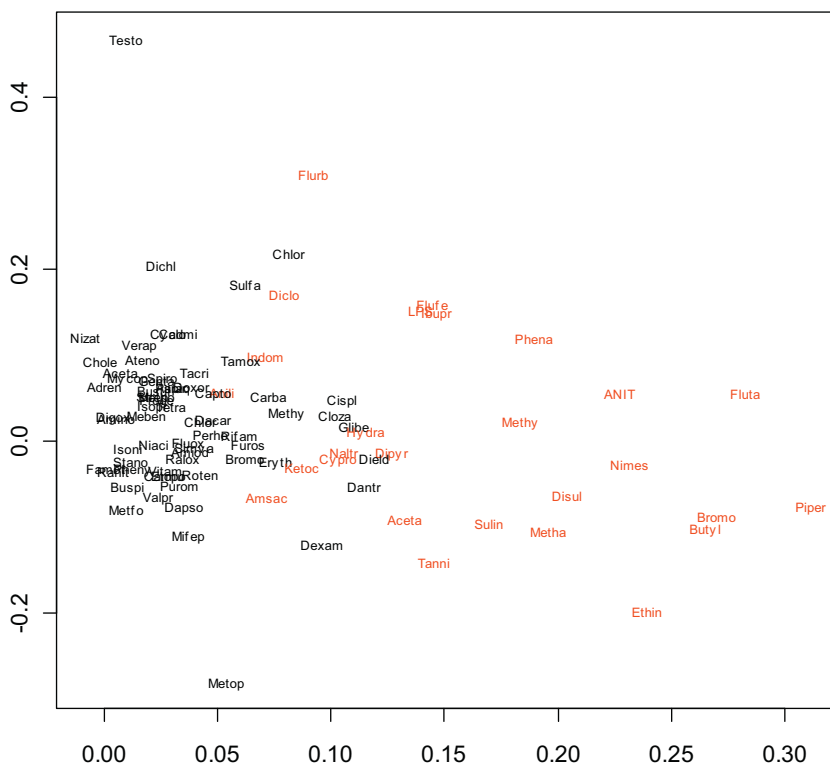


Fig. 2. Principal component analysis of training set (plot of first two components). The applied methods generate a composite representative point with arbitrary units within a two-dimensional space reflecting the similarity of one compound to another.

pretable in the context of cholestasis. Use of D_{ij} as a dissimilarity measure allowed for generation of a two-dimensional representation of similar and dissimilar compounds (Fig. 5). Compounds that are similar to each other according to (D_{ij}) are oriented close to one another while compounds that are dissimilar according to (D_{ij}) are spaced further apart. Many of the best-characterized cholestatic

compounds (depicted in red) fell into a tight cluster on the lower right-hand side of Fig. 5, and their gene responses after selection were almost identical. An expanded view of this cholestatic cluster is provided in Fig. 6. A few compounds with weaker responses, or those that act via a different mechanism, displayed as a “bridge” in this plot between the tight cluster of known cholestatic (red) com-

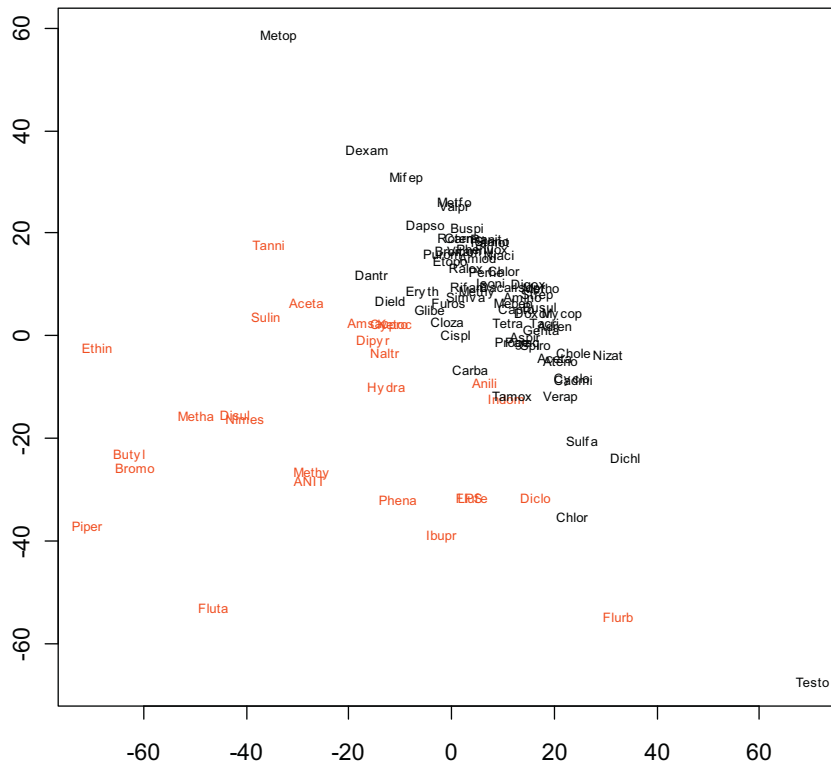


Fig. 3. Two-dimensional scatterplot of training set using multidimensional scaling to illustrate dissimilarity between every pair of pharmaceuticals and model toxicants based on Euclidean distance. The applied methods generate a composite representative point with arbitrary units within a two-dimensional space reflecting the similarity of one compound to another.

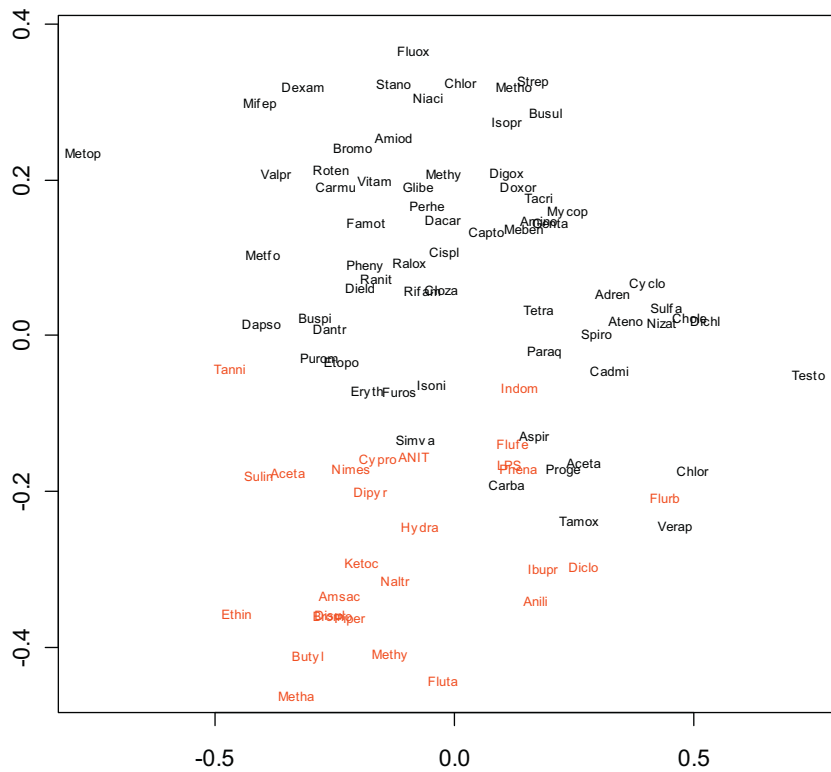


Fig. 4. Two-dimensional scatterplot of training set using multidimensional scaling to illustrate dissimilarity between every pair of pharmaceuticals and model toxicants based on 1-correlation. The applied methods generate a composite representative point with arbitrary units within a two-dimensional space reflecting the similarity of one compound to another.

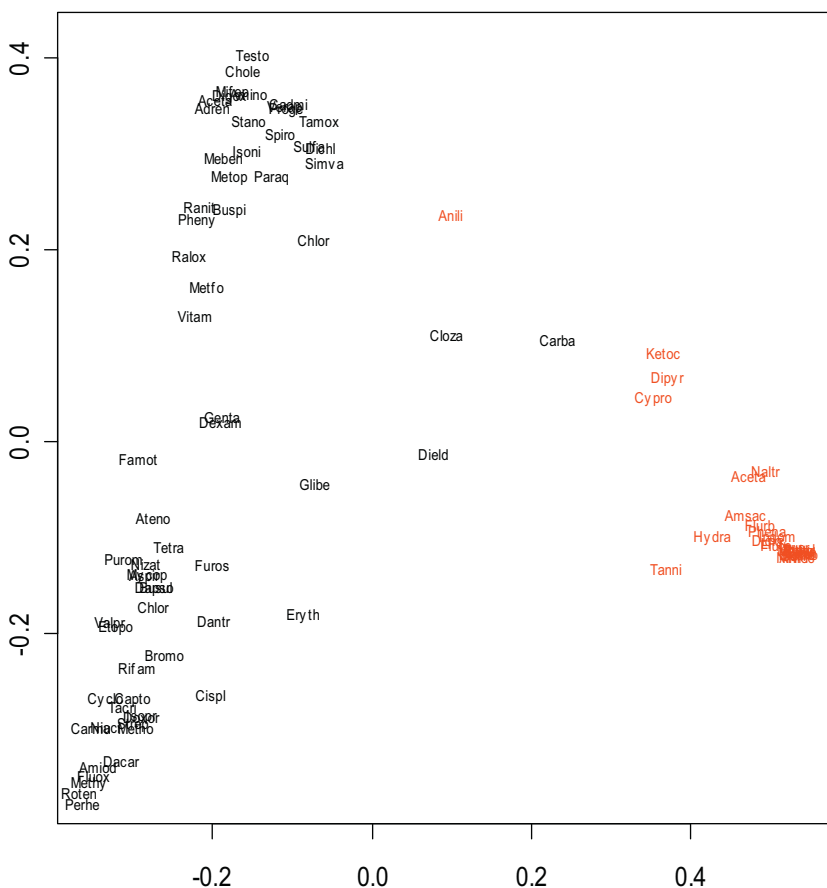


Fig. 5. Two-dimensional projection of the similarities and dissimilarities of training set pharmaceuticals and model toxicants based on multi-dimensional scaling following the ABC clustering procedure. Non-cholestatic compounds are noted in black and cholestatic compounds are noted in red. The applied statistical methods generate a composite representative point with arbitrary units within a two-dimensional space reflecting the similarity of one compound to another. A strong cholestatic (red) cluster can be noted on the lower right.

pounds on the lower right and the clearly non-cholestatic (black) compounds at bottom left in Fig. 5. Additionally there was some separation of compounds falling in the non-cholestatic left region of Fig. 5: while a number of the compounds on the top left and those closest to the cluster on the lower right are reportedly cholestatic in man, separation from non-cholestatic compounds was minimal and not investigated further in this study.

Cholestatic potential of compounds on the lower right (cholestatic) and bottom left (non-cholestatic) were confirmed via literature references. From this exercise, a ten-compound cholestatic (cholestatic in rat, and excluding LPS and gastrointestinal toxic NSAIDs) and four-compound non-cholestatic

Table 2

ABC technique-derived prediction set of rat cholestatic and non-cholestatic pharmaceuticals and model toxicants. Note the selected non-cholestatic compounds in rat are all cholestatic in human patients.

Cholestatic compounds from the right arm	Non-cholestatic compounds from the left arm
Ethinyl Estradiol	ErythroMC Estolate
Methylenedianiline	Rifampin
ANIT	Captopril
Disulfiram	Niacin
Piperonyl butoxide	
Methapyrilene	
Ketoconazole	
Bromobenzene	
Flutamide	
Nimesulide	

(non-cholestatic in rat, but cholestatic in human) compound training set (Table 2) was used to derive an improved gene expression signature for rat-specific cholestasis. We applied a boosting procedure and elastic net methodology to select a signature set of Codelink IDs [16,41]. The top set of 100 genes derived using an arbitrary cutoff with highest frequencies (i.e., those with highest statistical significance) is recorded as our final rat cholestasis signature and presented in Table 3.

Using the ABC approach, our results indicate that separation of paradigm human cholestasis compounds into cholestatic/non-cholestatic classes using rat experimental data gives as good or better separation than that observed with our proprietary Johnson & Johnson/Janssen compound-derived signature.

4. Discussion

In the present study the ABC statistical approach [3] was used with three non-redundant cholestasis indicator genes, Cyp7a1, Mrp3 and Bsep, to separate and visualize human cholestatic and non-cholestatic compounds in the rat. Although these three genes are well known to be regulated by the nuclear bile acid receptor (FXR), their different responses across the drugs tested (their non-redundant responses) suggest more complicated regulation in rats, consistent with integrated bile salt, cholesterol, sterols and other hepatic lipid metabolism pathways and the host of transcription factors involved (such as LXR, SREBP, PPAR, PXR, CAR and others, in addition to FXR and SHP; reviewed by Karagianni and Talianidis [9]). These three starting genes are recognized to

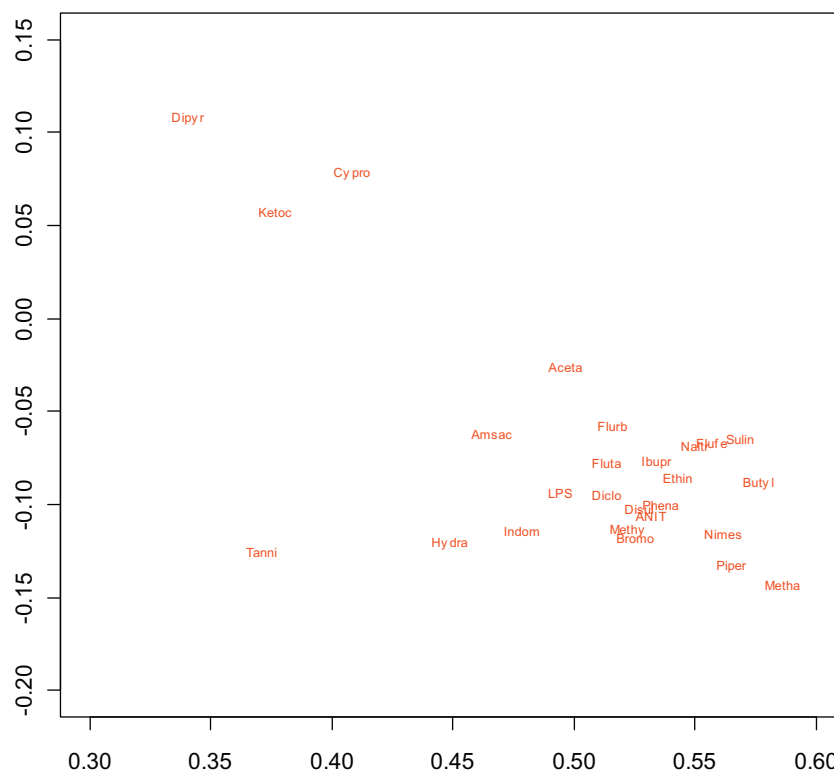


Fig. 6. Expanded view of the cholestatic (red) compounds in the right-hand “arm” of Fig. 1. Two-dimensional projection of the similarities and dissimilarities of training set pharmaceuticals and model toxicants based on multi-dimensional scaling following the ABC clustering procedure. The applied statistical methods generate a composite representative point with arbitrary units within a two-dimensional space reflecting the similarity of one compound to another.

be important in cholestasis, and were important in our previous in-house cholestasis signature based on multiple doses of proprietary compounds and histopathological evidence of cholestasis (Alex Nie, unpublished data). The separation by the ABC approach of paradigm human cholestasis compounds into cholestatic/non-cholestatic compounds in the rat was as good or better than observed with the proprietary Johnson & Johnson/Janssen compound derived signature. The ABC approach [3] was used to statistically weigh additional genes according to their similarity/dissimilarity to the three pivotal cholestasis genes and to derive predictions for potential cholestatic propensities of our paradigm compounds. The most robust cholestatic and non-cholestatic compounds in rat (all cholestatic in human) derived from this approach were used as a training set, along with a boosting procedure/elastic net approach [41], to select a broad 100-gene signature set associated with rat-specific cholestasis.

The chief advantage of the ABC approach is that it allows tight clustering of similar toxicants of interest (in this case rat cholestatic compounds) and displays the compounds along a continuum. Thus the most active rat cholestatic compounds essentially behaved identically by gene expression: cholestatic model compounds ANIT, LPS, and ethinyl estradiol, many high dose NSAIDs which can cause gut bleeding and endotoxin entry (phenacetin, flurbiprofen, sulindac, flufenamic acid, nimesulide, indomethacin, diclofenac, and ibuprofen [2,18], flutamide, disulfiram, methapyrilene, and the tool compounds piperonyl butoxide, bromobenzene, butylated hydroxytoluene, and methylene dianiline clustered together). Naltrexone, 2-acetamidofluorene, amoxicillin, hydrazine and tannic acid were slightly outside of the tight cholestatic cluster, and dipyrizidol, ketoconazole and cyproterone acetate were further removed from the cluster, showing different gene expression although quite distant from the non-cholestatic compounds.

Aniline was the only known rat cholestatic compound that fell in the “bridging” area between the cholestatic and non-cholestatic compounds. Carbamazepine, clozapine and dieldrin also fell within this bridging area, suggesting pronounced differences in gene expression but also shared features with cholestatic compounds. Erythromycin, glibenclamide, chlorpromazine, simvastatin, sulfamethoxazole, and tamoxifen (all known cholestatic drugs in human) were the closest of the non-cholestatic cluster to the cholestatic compounds, but clearly non-cholestatic in the rat, at least with acute dosing at high dose.

A number of pharmaceuticals that cause cholestasis in humans do not appear to induce cholestasis in rats: chlorpromazine and erythromycin-induced cholestasis appears to be human-specific [5,8]. Other human cholestatic compounds such as furosemide, rifampicin, niacin, captopril and dapsone showed no cholestatic gene expression response in the rat which may reflect species differences in cholestatic mechanisms such as binding and activation/inactivation of PXR, FXR, LXR and bile salt transporters [40]. Rifampin and glibenclamide reportedly block both rat BSEP and human BSEP transport in vitro and produce cholestasis in patients [25,33] but the rat appears to be much better at processing these compounds and many other human cholestasis-inducing drugs [8]. Species differences in drug effects on metabolic regulatory pathways, drug-induced changes in bile salt solubility, interference with clearance of other endogenous substrates (particularly steroid hormones and bilirubin), and precipitation of compound in the bile ducts, septicemia and localized inflammation, such as pancreatitis, and idiosyncratic/hypersensitivity and inflammation may also modulate cholestasis differently in man versus the rat [38].

The regulation of the three cholestasis indicator genes Cyp7a1, Bsep and Mrp3 used as the starting point for the ABC approach, and the responses of these three genes across Janssen toxicogenomics database compounds is different enough that several components

Table 3

Rat specific cholestasis gene signature. The top 100 genes associated with rat cholestasis, as derived by a boosting procedure with the elastic net approach. Genes were selected by comparing compounds (all known cholestatic in patients) from the two training sets in Table 2.

Codelink	Accession #	Rat gene
GE1266870	BE115850	Gstcd
GE20245	NM.013215	Akr7a3
GE1298481	BM391646	Nars
GE21882	NM.031588	Nrg1
GE14709	AI230228	Psat1
GE1147805	CB578405	Zfp57
GE12632	AI704698	Tubb2c
GE16026	AW252110	Rexo2
GE1130267	BI296275	Mmd2
GE20928	NM.031828	Kcnma1
GE16596	AW915559	Pprc1
GE20102	NM.012991	Npap60
GE1206415	BI297088	Golt1b
GE15377	BI296275	Mmd2
GE15995	AW251324	Mthfd2-201
GE18028	BI273851	Hmgn1
GE1287407	BI282107	Pbdc1
GE1262741	BE110134	Similar to ATPase inhibitory factor 1
GE1259381	NM.138515	Cyp2d4v1
GE19894	NM.012674	Spink1
GE1173529	CA507720	Pgs1
GE16306	BQ206238	Ankhd1
GE1168184	NM.031318	Tctex1
GE13574	NM.053800	Txn1
GE1185733	BF552430	NosipGE1116501 BF565830
GE17633	BE110630	Fam98a
GE19200	BF550623	Cad
GE1128152	AF507943	GTPase Ran
GE1150923	BF398625	Yars
GE17038	H33863	Mrps27
GE1106781	BU760106	Tubb6
GE20964	NM.133539	Mrpl17
GE1287274	BF405056	
GE1249888	BC380189	Map3k6
GE16626	BI302502	Fkbp11
GE1167706	NM.173102	Tubb5
GE1261576	BF416050	
GE20940	NM.024390	Hpgd
GE18776	BF406522	Cdr2
GE17115	BE096047	Ran
GE1174448	BC062045	similar to lipase, member H
GE1181134	BM389611	
GE19529	NM.134449	Prkcdpb
GE1101245	BF401159	
GE15329	AI412180	Gsr
GE1170908	BE096969	Slc35e3
GE1195574	BC373486	Lsm4
GE20274	NM.017014	Gstm1
GE14242	BI291621	Coq6
GE1305121	AI235475	EST232037
GE12903	AA946350	Gnai1
GE1206195	BI274696	similar to Fam48a protein
GE19392	BC374448	TLOAEA57YJ04
GE1166787	BM391708	
GE1291343	BQ202678	Xpo1
GE1219528	BF562712	Trmt10a
GE1135723	BM384823	Ppp1r3b
GE1237072	BQ202004	Gstm4
GE1277644	NM.031039	Gpt1
GE1236134	AW433632	
GE22230	NM.053962	Sds
GE17670	BE113943	Tigar
GE1216317	BU759545	Trmt10a
GE1210818	NM.024148	Apex1
GE1125325	X78847	GstYc2
GE1262042	BI296358	Apmmap
GE1100230	NM.138831	Slc16a10
GE12644	NM.175707	Ppil3
GE1297200	BF412090	Xkr8
GE1168155	BF288225	EST452816
GE17771	BE113165	Nek6
GE1205310	NM.133558	Cml1
GE22027	NM.053902	Kynu

Table 3 (Continued)

Codelink	Accession #	Rat gene
GE1197342	BQ194442	Krt23
GE15769	AA956532	
GE14364	BG379780	Mcee
GE20988	NM.031051	Mif
GE1158473	BF521726	Srxn1
GE16476	BM388044	
GE16012	H35647	Tnfrsf12a
GE18057	BF281848	Mthfd11
GE1284234	AI169152	Mafb
GE1130388	AI137440	
GE1167776	BF420077	
GE16853	AW918024	Nif311
GE1119096	CA505586	
GE1274350	BF388173	Ptprd
GE1201399	BF415461	
GE15155	AI409108	Nup205_predicted
GE13570	NM.053439	Ran
GE15126	AI408727	Lurap11
GE1296800	NM.184048	Taf9
GE16570	AW915407	EST346711
GE12527	BM384489	Hmgn3
GE1228263	BE098757	
GE20373	NM.017147	Cfl1
GE1236758	NM.198771	Fam3c
GE19575	NM.053962	Sds
GE1210173	BF394858	

of the rat cholestatic response are captured in our analysis. The approach allows for improved characterization of cholestasis in the rat than would be obtained using Cyp7a1 and other redundant, identically regulated genes, such as Cyp8b1.

Importantly, the 100 genes comprising our derived rat cholestasis signature would not have been obvious from most biochemical pathway analyses. There were, however, several duplicate genes with different accession numbers that were selected by the algorithm (monocyte to macrophage differentiation associated, Mmd2, tRNA methyltransferase 10a, Trmt10a, serine dehydratase, Sds, glutathione transferases, Ran, high mobility group nucleosome-binding proteins, Hmgn3, and Methylenetetrahydrofolate dehydrogenases, Mthfd), confirming the gene selections. Other transporters selected were for aromatic amino acids and nucleotide sugars, and another Cyp selected, Cyp2d4, is well known for 21-hydroxylation of steroid hormones rather than of bile salts or cholesterol. The robustness of the three starting genes and selection against strongly redundant responses means there are a limited number of tightly linked and/or well-known cholestatic signature genes.

In terms of in vivo study design, our experimental protocol was designed for tissue collection at an optimal time point for detection of robust gene expression changes. The non-proprietary paradigm compounds used in this work were generally administered as single dose, with animals necropsied 24 h later. Histopathology was not performed, as these compounds were typically well-characterized for their cholestatic potential in rat and human. Additionally, this acute treatment regimen was not expected to induce clinical manifestation of cholestasis after such a short dosing period. Exploration of dose-response and identification of additional cholestatic agents with multiple or chronic dosing regimens was not within the scope of this study: such investigations may yield additional insight and improved predictivity.

While other microarray studies of cholestasis exist for the rat, they have generally employed a single cholestatic agent. Such signatures are appropriate for the individual particular cholestatic model studied but may be more limited when applied to the variety of structural classes encountered in drug discovery and development. Comparison to ethinyl estradiol EE-treatment (24 h sampling time) showed minimal overlap with our >80 named

genes (aflatoxin aldehyde reductase, FK506 binding protein, guanine nucleotide binding protein alpha inhibiting, high mobility group nucleosomal binding domain 1, NIMA-related kinases, nucleoporins, RAN, member RAS oncogene family, Serine protease inhibitor, Kazal type, and several tubulins) [7]. Comparison with a 15 named gene for cholestasis derived by the supervised learning approach SVM (support vector machine) showed only one gene overlap—protein kinase c-binding protein [32]. Comparison with a signature for hepatobiliary injury showed pronounced differences, with dysregulated genes reflecting immune activation and changes in integrins and extracellular matrix markers [6], which were missing in our 24-h cholestasis gene signature.

The data mining approach used in the present study provides an innovative analysis of pharmaceuticals and model toxicants that induce cholestasis in the rat, and an improved, more predictive 100-gene signature beyond the original three-gene panel comprised of Cyp7a1, Mrp3 and Bsep. While applicability of this statistical approach remains to be demonstrated with other data sets, similar data mining approaches with future or existing data may yield insights into other toxicological properties, using comprehensive gene expression as surrogates for important biological responses.

Transparency document

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