Workshop 5.12
Toxicogenomics – potential, validation and case studies

Development of an *In Vitro* Gene Expression Assay for Predicting Hepatotoxicity

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Summary
A significant number of compounds do not proceed in drug discovery due to toxicity issues. The purpose of this study was to evaluate whether gene expression profiles could be identified and used to classify drugs based on the mechanism of toxicity in an in vitro system. Rat hepatocytes were treated with two classes of drugs, aromatic hydrocarbon receptor (AhR) and peroxisome proliferator activated receptor (PPAR) ligands.
The results showed that a small set of genes could be identified that could be used to classify new compounds with distinct mechanisms of toxicity.

Keywords: toxicogenomics, PPAR, AhR, primary rat hepatocytes

Introduction

Toxicology studies are a major bottleneck in the development of new drug candidates (Service, 2004). One of the principle reasons for this is that the majority of toxicology studies are conducted in animals, requiring large amounts of compounds and significant resources (Kola and Landis, 2004). The development of an assay that could screen new drug candidates *in vitro* for toxicity before animal studies are conducted would result in significant savings in time and resources, and would result in fewer compounds with toxic liabilities coming through the discovery process.

Toxicogenomics is the application of gene expression analysis towards drug safety evaluation (Guerreiro et al., 2003). Currently, *in vivo* toxicogenomic studies have been used to show that drugs with similar mechanisms of toxicity produce characteristic gene profiles and to study mechanisms of toxicity (Jun et al., 2004; Waring et al., 2002). Toxicogenomics has also been applied in a predictive mode where gene expression changes can be noted before animals show changes in clinical pathology or histopathologic lesions (Ellinger-Ziegelbaur et al., 2004; Kier et al., 2004). Recent publications have applied toxicogenomics using *in vitro* systems to study toxic mechanisms and identify gene expression changes associated with certain mechanisms of toxicity (Harris et al., 2004; Sawada et al., 2005). In this study, toxicogenomics was used to determine a characteristic gene expression profile for a class of compounds, referred to as a "gene signature".

The objective of this study was to develop *in vitro* gene signatures for two relevant, drug-induced toxicities: Aryl hydrocarbon receptor (AhR) and peroxisome proliferator activated receptor (PPAR). PPARα and AhR proteins are nuclear receptors that act as transcription factors when bound to appropriate ligands (Jacobs et al., 2003). Pathology associated with PPARα binding includes an increase in peroxisomes as well as tumour development (Cattley and Roberts, 2000). AhR activation induces hepatotoxicity, immune suppression and carcinogenicity (Nebert et al., 2004; Poland and Knutson, 1982). Results from our studies show that *in vitro* gene signatures can be generated from rat hepatocytes treated with reference prototypical PPAR and AhR ligands and that these signatures can be used for *in vitro* characterisation of experimental compounds.

Animals, materials, methods

Culture conditions

Primary rat hepatocytes were obtained as previously described (Waring et al., 2002). Rat hepatocytes were plated at a density of 800,000 cells/ml with 10 ml InVitroGro™ Hepatocyte Medium (In Vitro Technologies, Baltimore, MD, USA) on 100 mm BD BioCoat™ Collagen I Cellware (Becton Dickinson, Bedford, MA, USA) at 37°C and 5% CO_2 . Hepatocytes were treated 48 hours with a TC10 dose for the three drugs of each class: PPAR (WY-14643, bezafibrate, clofibrate, troglitazone, fenoprofen, indomethacin), AhR (3-methylcholanthrene, beta-napthoflavone, aroclor, omeprazole, benzopyrene, A277249, 3-3' diindolylmethane) and negative controls (chlorpheniramine, penicillin, spectinomycin).

Drug sources

The drugs were sourced from Sigma Laboratories with the following exceptions: A-277249 was obtained from Abbott Laboratories, 3,3'diindolylmethane (DIM) was obtained from A Better Choice for Research Chemicals (ABCR, Karlsruhe, Germany), and aroclor was obtained from Monsanto (St. Louis, MO, USA).

RNA isolation

RNA was isolated from hepatocytes using TRIzol® (Invitrogen Life Technologies, Carlsbad, CA, USA) reagent according to the manufacturer's protocol. The RNA concentration was determined by absorbance at 260 nm with the Smart-Spec spectrophotometer (Bio-Rad, Hercules, CA, USA). RNA integrity was evaluated using the RNA 6000 Nano Assay with the 2100 Agilent bioanalyzer (Agilent Technologies, GmbH, Germany) according to the manufacturer's protocol.

MTT cell cytotoxicity assay

The colorimetric cell viability assay using the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl tetrazolium bromide (MTT) was used as previously described (Mossmann, 1983).

Microarrays

Microarray analysis was performed on Affymetrix RAE-230A chips according to a protocol provided by Affymetrix (Affymetrix, Santa Clara, CA, USA). The manufacturer's protocol was followed with the exception that the primer used for the reverse transcription reaction was the GeneChip® T7-Oligo(dT) Promoter Primer Kit (Affymetrix, Santa Clara, CA, USA).

Bioinformatics

The intensity data from the scanned image of the microarray were imported into Rosetta Resolver® gene expression analysis software version 4.0 (Rosetta Biosoftware, Seattle, WA, USA). Intensity based ratios were calculated in Resolver using intensity data from each treatment microarray versus its respective vehicle control. Two vehicle treated samples were pooled *in silico* for each ratio. Hierarchical clustering was done in Resolver using the agglomerative algorithm and Euclidean distance for finding significant genes with expression ratios ≥2.0 and p-values ≤0.01.

Gene signature algorithm

Each drug was tested in duplicate across three separate rat hepatocyte isolations. An experiment was defined as the data from a single microarray. A total of 18 experiments were performed for each class (3 compounds per class x 2 technical replicates x 3 rat hepatocyte isolations). Experiments were divided into two groups: training and verification. The training experiments were used to determine the gene signatures, and the verification experiments were used to verify if the signatures could be used

Tab. 1: MTT cytotoxicity data for compounds tested. Treatment dose set at 10% cytotoxicity.

Drug Classification	Drug	Treatment Dose [µM]	Gene Signature Training Set	Gene Signature Verification
Peroxisome Proliferation	Wy-14643	35	X	
	Bezafibrate	200	X	
	Clofibrate	550	X	
	Troglitazone	75		X
	Fenoprofen	300		X
	Indomethacin	235		X
AhR Ligand	3-methylcholanthrene	100	X	
	Beta-napthoflavone	75	X	
	Aroclor	100	X	
	Omeprazole	300		X
	Benzopyrene	10		X
	A-277249	108		X
	3,3' diindolylmethane (DIM)	75		X
Negative Control	Chlorpheniramine	275	X	
	Penicillin	150	X	
	Spectinomycin	90	X	

to correctly identify drugs by class. Drugs for verification were tested in duplicate with a single rat hepatocyte isolation.

The first step of the algorithm was to find significantly regulated genes for each drug of a class from among the approximately 15,000 genes represented on the microarray. Parameters were set to select genes that displayed at least a two-fold change (either up or down) with a p-value ≤0.01. Genes that were commonly regulated among the three drugs were then selected for each class. The gene list was then narrowed to include genes significantly regulated in at least 16 of the 18 total experiments within a class.

Significantly regulated genes from the negative controls were defined as those with fold-changes ≥2.0 with a p-value ≤0.01 in at least six of the negative control experiments. These genes were then removed from the PPAR or AhR gene signature lists.

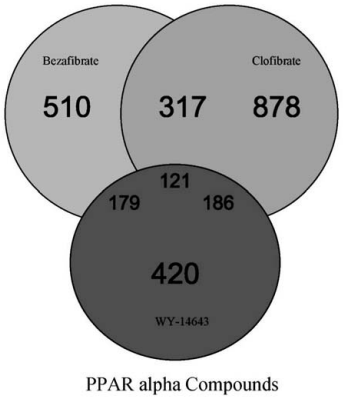

Fig. 1: Venn diagram showing that there are 121 commonly regulated genes for the PPARα training set compounds. The coloured figures can be downloaded from the ALTEX website (www.altex.ch).

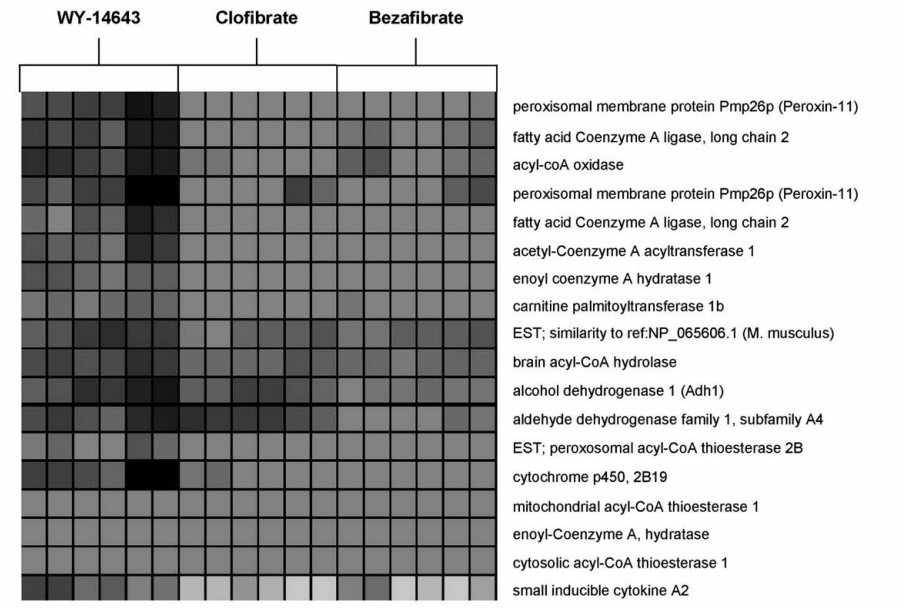

Fig. 2: Ordered cluster analysis showing gene expression for the 18 genes of the PPAR alpha gene signature. Data shown as a fold change relative to vehicle (1% DMSO), p value ≤0.01, with a range of five-fold reduced expression (light grey) to five-fold increased expression (dark grey).

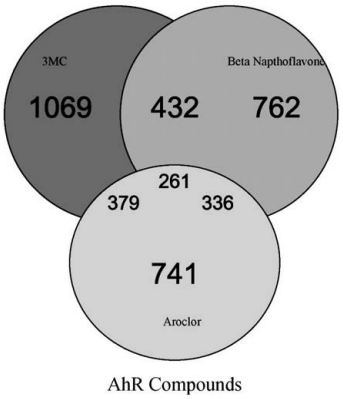

Fig. 3: Venn diagram showing that there are 261 commonly regulated genes for AhR training set compounds.

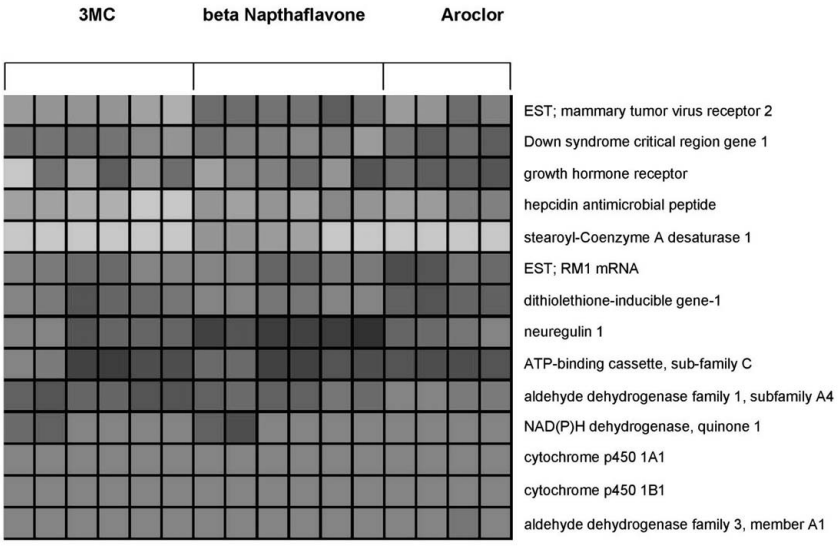

Fig. 4: Ordered cluster analysis showing gene expression for 14 genes of the AhR gene signature. Data shown as fold change relative to vehicle (1% DMSO), p ≤0.01, with a range of five-fold reduced expression (light grey) to five-fold increased expression (dark grey).

Results

Cell cytotoxicity assay

The MTT cytotoxicity results shown in table 1 are the doses calculated to result in 90% survival (10% cytotoxicity). The exceptions are benzopyrene, which was tested at 10 µM due to solubility limitations, and DIM, which was tested at 75 µM, as previously reported (Hestermann and Brown, 2003). All drugs were tested in solutions containing less than 1% DMSO vehicle.

Gene signature algorithm

Data for the PPAR training compounds are shown in figure 1 as a Venn diagram. The PPAR class contained the following significantly regulated genes across three separate isolations: 578 for bezafibrate, 878 for clofibrate, and 420 for Wy-14643. A total of 121 genes were commonly regulated by all three drugs. From the 121 common genes, 27 genes were found to be consistently regulated across the three isolations. Genes in common with negative controls were removed and the 18 remaining genes are listed in figure 2, with the experiments shown in an ordered cluster analysis. The genes for peroxisomal membrane protein and fatty acid Coenzyme A ligase are listed twice because these genes are represented on the microarray by two different probe sequences.

Data for the AhR training compounds are shown in figure 3 as a Venn diagram. The AhR class contained the following significantly regulated genes: 1069 for 3MC, 762 for beta-napthoflavone, and 741 for aroclor. A total of 261 genes were commonly regulated by all three drugs. Of the 261 common genes, 37 genes were found to be consistently regulated across the three isolations. Genes in common with negative controls were removed and the 14 remaining genes are listed in figure 4, with the experiments shown in an ordered cluster analysis. The data from two of the six aroclor experiments are not shown due to RNA degradation.

Gene signature verification

The gene signatures were verified with additional reference compounds from the same class. The hierarchical cluster analysis shown in figure 5 shows that benzopyrene, A277249, omeprazole, and DIM all cluster with the AhR training compounds and not with the negative control or the PPAR compounds. Likewise, the PPARα verification compounds indo-methacin and fenoprofen) cluster with PPARα training compounds. In contrast, troglitazone clusters with the negative control compounds and not the PPARα compounds. Finally, the AhR, PPAR, and negative control compounds all cluster separate from each other, with each class of compound producing a

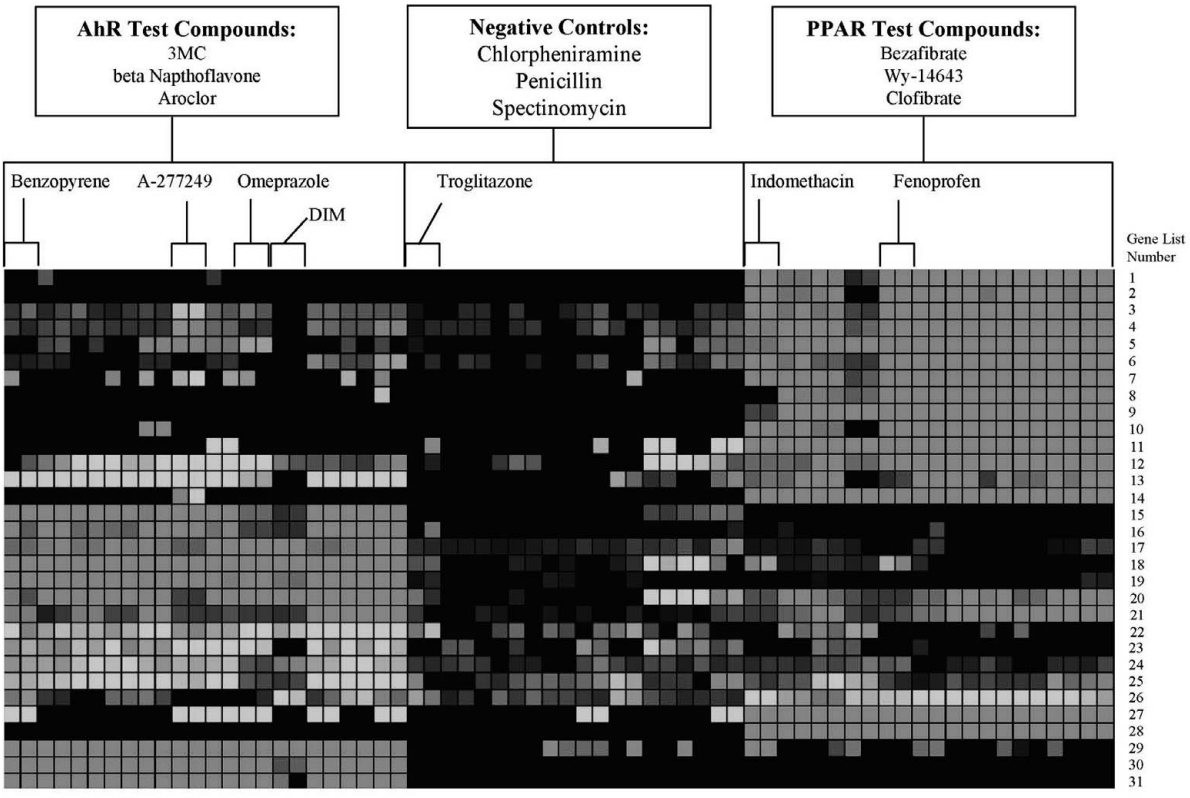

Fig. 5: Hierarchical cluster analysis showing gene expression for all experiments using the PPARα and AhR gene signatures. Data shown as fold change relative to vehicle (1% DMSO), p ≤0.01, with a range of five-fold reduced expression (light grey) to five- fold increased expression (dark grey).

unique gene expression signature. The corresponding genes are presented in order in table 2. Note that 31 genes are listed because both signatures share the gene, aldehyde dehydrogenase family 1, subfamily A4.

Discussion

The objective of this study was to develop gene signatures for two well characterised classes of compounds, PPARα and AhR agonists, and to use these signatures to correctly identify other compounds of the same class using an *in vitro* rat hepatocyte system.

Gene signatures were developed for the PPARα and AhR pathways by treating primary rat hepatocytes with class-specific compounds and measuring gene expression changes with rat DNA microarrays. The compounds were tested across three different rat hepatocyte isolations to capture biological variability and in duplicate within each isolation to capture technical variability. Three reference compounds were selected for each class and used as a training dataset to determine a gene signature. The negative control compounds were used in an attempt to identify gene expression changes that were due in part to the *in vitro* conditions. 18 genes were identified for the PPARα signature and 14 genes for the AhR signature.

Tab. 2: PPAR and AhR gene list corresponding to data in figure 5.

Gene List Number	Gene Name
1	peroxisomal membrane protein Pmp26p (Peroxin-11)
2	peroxisomal membrane protein Pmp26p (Peroxin-11)
3	fatty acid Coenzyme A ligase, long chain 2
4	acetyl-Coenzyme A acyltransferase 1
5	enoyl-Coenzyme A, hydratase
6	acyl-coA oxidase
7	fatty acid Coenzyme A ligase, long chain 2
8	brain acyl-CoA hydrolase
9	carnitine palmitoyltransferase 1b
10	cytochrome p450, 2B19
11	EST; peroxosomal acyl-CoA thioesterase 2B
12	EST; similarity to ref:NP_065606.1 (M. musculus)
13	stearoyl-Coenzyme A desaturase 1
14	mitochondrial acyl-CoA thioesterase 1
15	EST; RM1 mRNA
16	neuregulin 1
17	ATP-binding cassette, sub-family C
18	dithiolethione-inducible gene-1
19	NAD(P)H dehydrogenase, quinone 1
20	aldehyde dehydrogenase family 1, subfamily A4
21	alcohol dehydrogenase 1 (Adh1)
22	EST; mammary tumor virus receptor 2
23	growth hormone receptor
24	Down syndrome critical region gene 1
25	hepcidin antimicrobial peptide
26	small inducible cytokine A2
27	enoyl-Coenzyme A, hydratase
28	cytosolic acyl-CoA thioesterase 1
29	cytochrome p450 1A1
30	cytochrome p450 1B1
31	aldehyde dehydrogenase family 3, member A1

The gene signatures were verified using additional compounds from each class. Both omeprazole and DIM are considered to be weak non-classical AhR agonists without the structural features of AhR ligands (Hestermann and Brown, 2003; Backlund et al., 1997). The results here indicate that the gene signature is sensitive enough to identify a range of structurally diverse drugs with similar biological properties (Hestermann and Brown, 2003; Backlund et al., 1997). Troglitazone, a PPARγ agonist, clustered with the negative controls. PPARγ receptors are expressed primarily in adipose tissue and affect insulin and lipid metabolic pathways (Lebovitz and Banerji, 2001). These data suggest that the PPAR gene signature is specific for the α class of receptor and can differentiate between the PPARα and PPARγ pathways.

The genes listed in the PPAR and AhR signatures correlate with the biology of the receptors. PPAR genes associated with β-oxidation regulated in *in vivo* studies include acyl-CoA oxidase, acyl-CoA thioesterase, enoyl-CoA hydratase, fatty acid coenzyme A ligase, and aldehyde dehydrogenase (Baker et al., 2004). The genes regulated for AhR ligands include CYP1A, CYP1B, NAD(P)H reductase, and aldehyde dehydrogenase and have been previously reported to be regulated in *in vivo* studies (Waring et. al., 2001). Genes missing from the signatures include CYP4A for PPAR and glutathione-s-transferase (GST) for AhR. These genes were removed from their corresponding signature due to regulation by negative controls and inconsistent regulation across the three isolations (data not shown).

The PPAR signature was also tested against the *in vitro* conditions of drug dose and treatment time. Hepatocytes were treated with clofibrate, WY-14643, and bezafibrate for 4, 24, 48 and 72 hours. The dose for clofibrate was ten times lower than previously tested (55 μM). The low dose clofibrate treatment clustered with the negative control compounds at all time points (data not shown). The WY-14643 and bezafibrate four-hour treatment clustered with the negative controls, whereas the 24, 48, and 72-hour treatments clustered with the other PPAR compounds (data not shown). These data indicate that the signature is sensitive to dosing as well as to treatment time, with 24 hours giving similar results to 48 and 72-hour treatment times, suggesting that 24 hours is adequate for treatment.

In summary, this study demonstrates that an *in vitro* assay using primary rat hepatocytes and gene expression analysis can successfully be used to predict hepatotoxicity. An assay of this type will have significant impact on the drug discovery process by allowing drugs to be screened prior to animal studies and obtaining toxicology information earlier in the development process. By filling these needs, this *in vitro* assay will help to determine which drugs offer the best chance for success.

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