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Gene expression profiling of drug metabolism and toxicology markers using a low-density DNA microarray

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Abstract

DNA microarrays are useful tools to study changes of gene expression in response to a treatment with drugs. Here, we describe the optimization of conditions for the cDNA synthesis and hybridization protocols to be used for a low-density DNA microarray called 'Rat HepatoChips.' This DNA microarray with 59 carefully selected genes could be used to study changes in gene expression levels due to a treatment with xenobiotic. These 59 genes (including 8 housekeeping genes) have been selected among potential toxic markers involved in basic cellular processes and drug metabolism related genes. Using the optimized conditions, the results were shown to be reproducible, with 6% variation between the duplicated spots and 10% between arrays. Conditions were optimized to allow quantification with a dynamic range of four log units. In order to demonstrate the major advantage of these tool for studying gene expression, samples of control rat liver were compared with those of animals dosed with phenobarbital (PB) or pregnenolone-16α-carbonitrile (PCN), two compounds well known to induce cytochrome P450 isoforms of 2B and 3A subfamilies, respectively. This microarray has shown that other genes apart from the corresponding CYP P450 genes have been changed due to PB and PCN treatment. Apoptosis-related genes have shown to be changed due to PB and PCN treatment, which confirms results from previous work.

Keywords: Low-density microarray; Quantitative assay; Toxicogenomics; Drug metabolism; CYP2B and CYP3A inducer; Rat liver

1. Introduction

One of the main reasons for the failure of drug candidates during the development process is unforeseen toxicity or dramatic xenobiotic metabolism induction. Therefore, a molecular tool allowing to assess the potential for these possible alterations during the drug discovery process would offer the advantages of significant savings in time, money and the potential to prioritize lead compounds, based on their potential to cause toxicity earlier in the drug development process. It will also allow to introduce a drug–drug interaction risk.

The problem with the use of established *in vivo* toxicology or xenobiotic metabolism models for this purpose is that they are expensive, require a large number of animals, a large amount of compounds, and take a long time to be completed and analyzed. Even if they are simpler to be used, the same types of pitfall arise with validated *in vitro* models with the difficulty to study at the same time the different possible targets of the new tested drugs. For these reasons, only a limited number of compounds can be studied in this way. One possible mechanism or result of toxic effects is changes in gene expression. Therefore, one means of obtaining data on the potential toxicity of a drug candidate is to study its effects on gene expression.

Gene expression alterations may be either dramatic such as induction of some cytochrome P450 isoforms or more

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Abbreviations: PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; PMT, photomultiplier tube; GST Ya, gluthatione-*S*-transferase subunit Ya; GST Theta 5, gluthatione-*S*-transferase subunit theta 5; CYP, cytochrome P450; ACO, acyl-CoA oxidase; TGFβ-RII, tumor growth factor β receptor type II; JNK, Jun N-terminal kinase.

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subtle, resulting from complex interactions between cellular pathways or repair enzymes. The phenotype produced by a toxicant is the result of complex interactions from one or multiple cellular pathways, which can be detected by the analysis of the expression of multiple genes [1].

Established methods of studying gene expression, such as Northern blotting, RNase protection assays, S1 nuclease analysis and quantitative PCR have the disadvantage of being inherently serial, involving measuring a single gene at a time and being difficult to automate. Recently emerging techniques, such as differential display [2], high-density filter hybridization [3], serial analysis of gene expression (SAGE) [4] and cDNA- and oligonucleotide-based microarray hybridization give the ability to study the expression levels of multiple genes simultaneously. DNA microarrays consist of multiple DNA probes spotted and attached covalently to a modified glass slide [5–7]. These slides are then hybridized with labeled cDNA prepared from mRNA of different samples and differences in gene expression are quantified. [According to the nomenclature recommended by *Nature Genetics*, the terminology used in this manuscript describes the DNA attached (spotted) to the microarray surface as the 'probe' and the labeled cDNA which hybridizes to the array as the 'target'] (Nature Genetics Supplement, January 1999).

One of the aims of the microarray is to define the characteristic and specific pattern of gene expression elicited by a given toxicant. The potential to identify toxicant 'families' (so called molecular signature or fingerprint) based on tissue- or cell-specific gene expression in response to xenobiotic treatment could point to possible toxicity and help to elucidate the mechanism of action through identification of gene expression networks. Once signatures are determined for known compounds, gene expression profiles produced by unknown agents could be compared [1,8]. Patterns of gene expression in response to different classes of toxicants may be sufficiently unique to allow tentative identification of toxic agents. The ultimate goal is to use microarrays as a screening tool in order to determine the hepatotoxicity and side effects of new chemical entities.

In order to validate this technique, the Rat HepatoChips (AAT) was developed and used to study two prototypical cytochrome P450 isoforms inducers; PB and PCN. Molecular effects of these compounds on rat liver cells have been well characterized leading to the induction of CYP2B and CYP3A subfamilies, respectively.

PB is known to induce several enzymes involved in biotransformation including cytochrome P450 isoforms, glutathione-*S*-transferase and UDP-glucuronosyl transferase (UDPGT) isoforms [9–12]. It has also been described that administration of PB to rodents causes an increase in liver weight due to hyperplasia and hypertrophy which results in an increased incidence of liver tumors [13,14]. This pleiotropic response to PB suggests that, in addition to drug metabolizing enzymes, the expression of other genes may be affected at the transcriptional and/or post-transcriptional level [11].

In addition to the induction of CYP3A subfamily, PCN has also been shown to induce CYP2B1/2 [14,15]. Administration of PCN to rats has also been reported to cause an increase in liver weight, that was associated with increased mitosis and centrilobular hypertrophy [14].

Both PB and PCN increase the incidence of hepatic neoplasia after chronic treatment, do not damage DNA directly, and are considered as non-genotoxic hepatocarcinogens [16,17]. The mechanism(s) by which these compounds cause liver tumor formation remain to be elucidated. It has been suggested that non-genotoxic hepatocarcinogens lead to tumor growth by either an increase in cell proliferation or a decrease in cell death [18]. It has recently been reported that PB and PCN lead to a decrease in the incidence of apoptotic hepatocytes [19].

Here, we describe the optimized conditions for the cDNA synthesis and hybridization protocols to be used for a low-density microarray called 'Rat HepatoChips' from AAT and some gene expression modulations observed in this study with PB and PCN are discussed in regards to their established hepato-toxicological properties.

2. Materials and methods

2.1. Animal treatment

Female Sprague–Dawley CD rats (aged 10–12 weeks), obtained from Charles River were allowed food and water *ad libitum*. The animals were housed in groups of three or four in mesh-floored cages in accommodation maintained at $22 \pm 3^{\circ}$ with a relative humidity of 40–70% and allowed to acclimatize to these conditions for 7 days before the study. Rats were dosed orally with 100 mg/kg per day of either PB (CAS:57-30-7 and catalog number is P-5178) or PCN (CAS:1434-54-4 and catalog number is P-0543) (Sigma–Aldrich Co.) for 4 days. Control animals received corresponding quantities (5 ml/kg body weight) of the 0.56% (w/v) gum tragacanth vehicles. Following the treatment period, the animals were killed by decapitation and the livers immediately removed, snap frozen in liquid nitrogen and stored at -80° for further mRNA isolation. The animal procedures were conducted in accordance with the Animals (Scientific procedures) Act and its associated guidelines.

2.2. Isolation of mRNA

Poly $(A)^{+}$ RNA was isolated using the FastTrack 2.0 mRNA isolation kit (Invitrogen) using the manufacturer's protocol for isolating mRNA starting from 1 g snap frozen liver tissue. Denaturing agarose gel electrophoresis was used to assess the integrity and relative contamination of mRNA with ribosomal RNA.

2.3. Synthesis of labeled cDNA

Labeled cDNA were prepared using 2 μg poly $(A)^+$ RNA. A synthetic poly $(A)^+$ tailed RNA sample was spiked into the purified mRNA as an internal standard to assist in quantification and estimation of experimental variation introduced during labeling and analysis [20]. Poly $(A)^+$ RNA was added to 2 μL of oligod$T_{(12–18)}$ primer (0.5 μg/μL) (Gibco BRL, Life Technologies), Rnase-free water was used to bring the volume to 9 μL, and the mixture was denatured at 70° for 10 min and then chilled on ice for 5 min. Reverse transcription was performed by adding the following components to the annealed probe/template on ice: 4 μL of First Strand Buffer 5× (250 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) (Gibco BRL, Life Technologies), 2 μL of DTT 0.1 M (Gibco BRL, Life Technologies), 40 units of RNasin ribonuclease inhibitor (Promega), 500 μM dATP (Roche), 500 μM dTTP (Roche), 500 μM dGTP (Roche), 80 μM dCTP (Roche), 80 μM biotin-11-dCTP (NEN Life Science Product Inc.). The reaction mixture was mixed by gently flicking the tube and incubated for 5 min at room temperature, 300 units of SuperScript II^{TM} Rnase H^- Reverse Transcriptase (Gibco BRL, Life Technologies) was added to the reaction mixture and the reverse transcription allowed to proceed for 90 min at 42°. An additional 300 units of SuperScript II Rnase H^- Reverse Transcriptase was added and incubation was continued at 42° for another 90 min. The reaction was terminated by heat inactivation at 70° for 15 min. To remove RNA complementary to the cDNA, a treatment with Ribonuclease H (Gibco BRL, Life Technologies) was performed at 37° for 20 min followed by a heat denaturation at 95° for 3 min and cooled on ice before use [21]. No further cDNA purification was necessary.

2.4. Generation of the internal standard

The internal standard clone was constructed by the insertion of PCR amplified DNA fragment of the HIV-1 pol region in the pSP64 poly $(A)^+$ vector (Promega), linearizing isolated plasmid DNA with *Eco*RI and synthesizing poly $(A)^+$ tailed RNA (340 bases) complementary to the insert from the SP6 promotor by *in vitro* transcription (Promega) [22].

2.5. Rat HepatoChips design: 59 genes microarray

Genes on the Rat HepatoChips (AAT) are presented in Table 1. The selected genes are either involved in basic cellular processes, relate to drug metabolism or may have a potential to act as markers of toxicity. In order to evaluate the reliability of the experimental hybridization, several controls of positive and negative hybridization, positive and negative detection were included on the Rat HepatoChips. For normalization, an internal standard control and eight housekeeping genes were arrayed on the slides (Table 2). The Rat HepatoChips is composed of single stranded DNA probes attached to the glass support by a

Table 1
List of genes presented on the Rat HepatoChips

Gene	Function	Genbank number
Bax, Bcl-2	Apoptosis	U49729, L14680
c-jun, c-myc, Elk-1	Oncogene	X17163, Y00396, X87257
Cox 2, IL6	Inflammation	L20085, M26744
CYP1A1, CYP1B1, CYP2B1/2, CYP3A1, CYP4A1	Cytochrome P450	X00469, U09540, M34452, M10161, X07259
Enoyl CoA hydratase, PPARα	PP	K03249, M88592
ACO	PP acyl CoA oxidase	J02752
Ferritin	Iron stock	U58829
Fibronectin	Extracellular matrix	X15096
GADD153, GADD45, MGMT	DNA damage/DNA repair	U30186, L32591, M76704
Glutathione-*S*-transferase subunit Ya and theta 5, GSH reductase, HO-2, HSP70, MnSOD, ApoJ, cytochrome *c* oxidase 1, JNK-1	Oxidative stress	K01931, X67654 U73174, J05405, L16764, Y00497, M16975, M27315, L27129
Hepatocyte GF	Growth factor	D90102
Histone D-acetylase (Hdac1)	DNA transcription	NM008228
HMG CoA synthetase	Cholesterol metabolism	X52625
Telomerase, cyclin D1	Cell cycle activation	U89282, D14014
NFκB, p38, erk-1, c/EBPα, Iκβα	Transcription factor	L26267, U73142, M61177, X12752, U66479
Ornithine decarboxylase (odc)	Arginine synthesis	J04791
p53	Tumor suppressor	X13058
PCNA	Proliferation cell nuclear antigen	Y00047
Mdr-1b, transferrin, albumin	Transporters	M81855, D38380, V01222
SMP30	Senescence marker	X69021
TNF	Tumor necrosis factor	X66539
Transforming growth factor-b RII	TGF-beta receptor	L09653
UDPGT1A, UDPGT1A6	Glucuronyl transferase	J05132, D83796
Liver + control	α2-Macroglobulin	J02635

The known function of the corresponding protein and the Genbank accession number of each cDNA is described in this table.

Table 2
List of housekeeping genes included on the Rat HepatoChips

Housekeeping gene	Function	Abundance level	Accession number
α-Tubulin	Cytoskeletal protein	High	V01227
Ribosomal protein S29	Protein synthesis	Medium	X59051
Myosin heavy chain 1 (myr)	Muscle contraction	Low	X68199
Hypoxanthine guanine phosphoribosyltransferase	Nucleotide synthesis	Medium	M86443
Glyceraldehyde-3-phopshate dehydrogenase (GAPDH)	Glycolysis	High	D16554
Polyubiquitin	Cellular metabolism, development	High	D00036
Phospholipase A2	Lipid metabolism	Low	X02231
β-Actin	Cytoskeletal protein	High	V01217

The known function, the abundance and the Genbank accession number of each cDNA is described in this table.

covalent link. Each DNA probe is present in duplicate (Fig. 1). The length of the DNA probes has been optimized. The lengths are the same for all genes and are located near the 3′ end of the transcript. All probes have been designed to be gene specific and have been prepared using rat cDNAs.

2.6. Hybridization using biotinylated-labeled cDNA

The Rat HepatoChips hybridization was carried out according to the manufacturer's instructions. The hybridization was performed in a hybridization chamber (Biozym) containing the Hepatobuffer, the total biotinylated cDNA (from 2 μg poly $(A)^+$ RNA) and a positive hybridization control (biotinylated amplicons, provided in the kit at a concentration of 25 nM). Hybridization was carried out overnight at 60° in a custom slide cassette under humidity maintained by small reservoir of 3× SSC. Arrays were washed stringently four times for 2 min with buffer (10 mM maleic buffer pH 7.5, 15 mM NaCl, 0.1% Tween) at room temperature.

2.7. Indirect fluorescent detection

The presence of biotinylated hybrids on the microarray was detected using a fluorescent streptavidin conjugate.

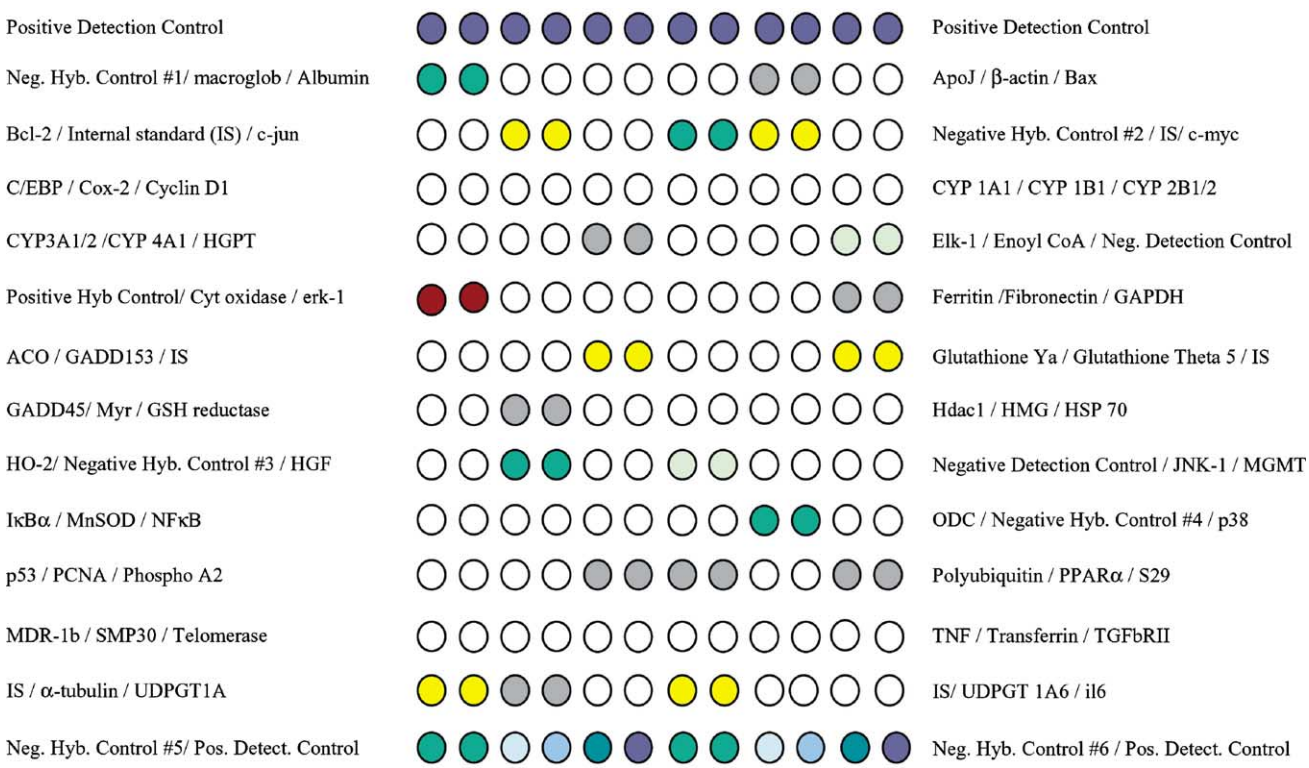

Fig. 1. Design of the Rat HepatoChips with 59 genes (including 8 housekeeping genes). Several controls were included on the microarray. Positive hybridization controls (red), negative hybridization controls (dark green), negative detection controls (light green) and positive detection controls (blue). The last row, a concentration curve of positive detection control is included (blue). The internal standard control is included on six different locations (yellow). The housekeeping genes are also located (green).

The arrays were incubated with a 1:500 dilution of cyanin 3 streptavidin conjugate (Amersham Pharmacia Biotech) in a blocking solution (100 mM maleic buffer pH 7.5, 150 mM NaCl and 0.1% (w/v) milk powder) for 45 min at room temperature. The slides were then washed stringently with buffer (10 mM maleic buffer pH 7.5, 15 mM NaCl, 0.1% Tween), five times for 2 min at room temperature then rinsed briefly with deionised water and dried at room temperature.

2.8. Imaging and data analysis

Hybridized arrays were scanned using a laser confocal scanner 'GMS 418 scanner' (Genetic Microsystem) at a resolution of 10 μm. To maximize the dynamic range of microarrays, the same arrays were scanned at different photomultiplier settings of the scanner. Using different gains will allow to quantify both the high and low copy expressed genes. After image acquisition, the scanned 16-bit images were imported into 'ImaGene 4.1' software (BioDiscovery) to quantify the signal intensities. The fluorescent signal intensity for each DNA spot (average of intensity of each pixel present within the spot) was calculated using local mean background subtraction. A signal was only accepted when the average intensity after background subtraction was at least 2.5-fold higher than the local background. The two intensity values of the duplicate DNA spots were averaged and used to calculate the intensity ratio between the reference and the test samples. Very bright element intensities (saturated signals, highly expressed genes) were deemed unsuitable for accurate quantification because they underestimated the intensity ratios and were excluded from further analysis.

There are several potential sources of experimental variation in this process. These include the synthesis and labeling of the cDNA, the hybridization and the indirect detection steps. To account for this variation the data obtained from different hybridizations were normalized in two ways. First, the values are corrected using a factor calculated from the intensity ratios of the internal standard reference and the test sample. The presence of internal standard probes at six locations of the Rat HepatoChips allows a measurement of local background and evaluation of the microarray homogeneity, which is considered in the normalization [23,24]. However, since the internal standard control does not account for the purity and the quality of the mRNA, a second step of normalization was performed based on expression levels of the housekeeping genes. This process involves calculating the average intensity for a set of housekeeping genes, the expression of which is not expected to vary. The variance of the normalized set of housekeeping genes is used to generate an estimate of expected variance, leading to a predicted confidence interval for testing the significance of the ratios obtained [25]. Ratios outside the 95% confidence interval were determined to be significantly different following the treatment. In order to make a treatment cause interpretation, data for all animals have been analyzed by ANOVA.

3. Results

3.1. Reverse transcription optimization

The first requirement for microarray data to represent the gene expression profile is that the cDNA target reflects the mRNA complexity of the original sample. This implies that the isolation poly $(A)^+$ RNA, synthesis and labeling of cDNA must be reproducible and effective [21]. Key parameters in the cDNA labeling reaction include nucleotide concentration, fraction of labeled nucleotide, type of labeled nucleotide and reaction time. We have optimized each of these variables in turn, using 2 μg of rat liver poly $(A)^+$ RNA as a standard template. The influence of each parameter was monitored by hybridization performance on microarrays. Consequently, the detection of the less abundant transcript was improved by a factor of 5.5 during these optimizations. The final protocol is given in Section 2.

A key factor was the labeling of the cDNA with biotin-dCTP. The signal intensity obtained with the incorporation of biotin dCTP was twice that obtained with biotin-dUTP (Fig. 2A). Using a final concentration of 80 μM of biotin-dCTP and an equal fraction of unlabeled and labeled biotin-dCTP, we have optimized the incubation time of the reverse transcription reaction to a total of 180 min (Fig. 2B).

3.2. Detection limit for low abundance mRNA

Detection of low copy genes in a reproducible manner is one of the challenges of microarray assays. Twenty-five percent of cellular mRNA consists of transcripts expressed at less than five copies per cell [26]. Therefore, it was important to determine the lowest number of individual RNA copies that could be detected within the mRNA cell population. To monitor the sensitivity, we carried out quantitative spiking experiments. Known amounts of the synthetic poly $(A)^+$ tailed RNA (ranging from 10,000 to 2 pg) were spiked into a background of 2 μg rat liver mRNA as internal standard. The labeled cDNA synthesis and hybridizations were processed. Hybridization of rat liver mRNA (without the added internal standard transcript) did not result in detectable signal for any probe designed to detect the internal standard (data not shown). The presence of the internal standard was reproducibly detected 2-fold above the local background for an amount of 2 pg of synthetic RNA. The detection limit for the internal standard was at a dilution of <1:1,000,000 (w/w) in rat liver mRNA (Fig. 3). This detection limit corresponds approximately to 10^7 molecules or 2 pg of specific RNA.

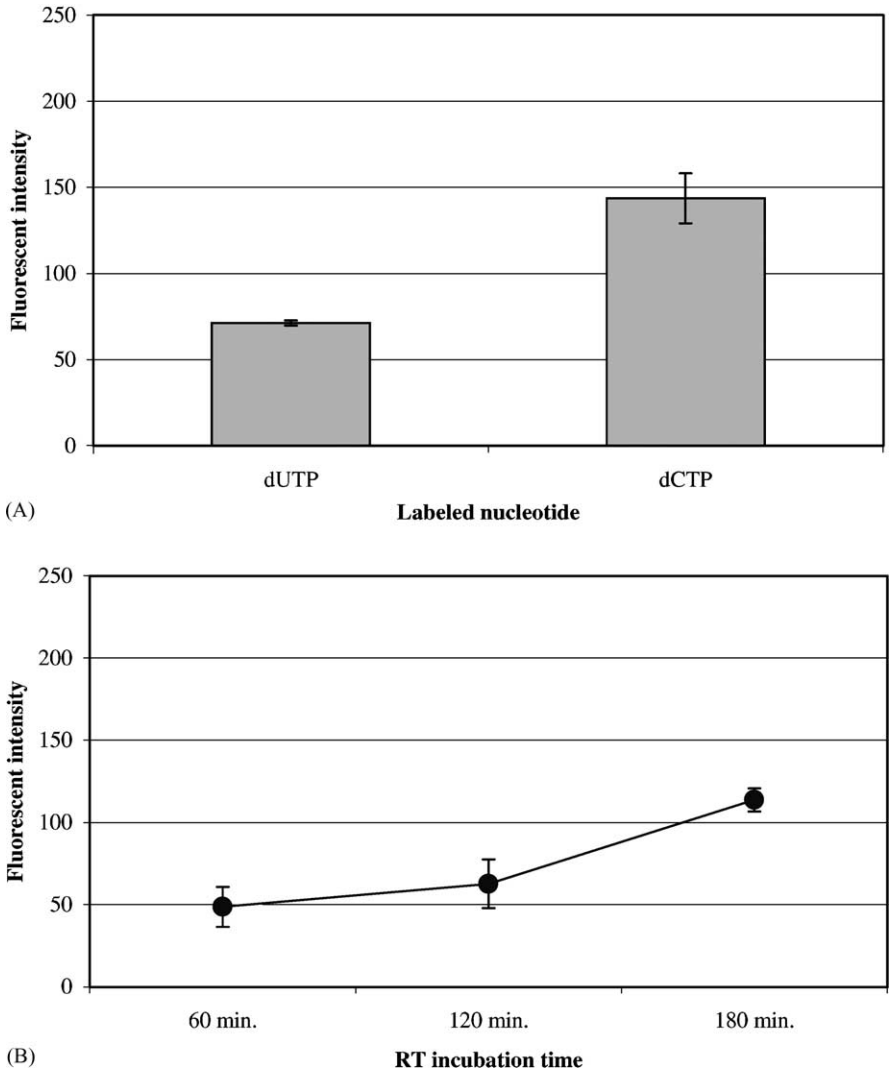

Fig. 2. Effect of reverse transcription time and labeled nucleotide incorporation on hybridization efficiency. Each condition was performed using 2 μg of mRNA as a template and was monitored by hybridization on the Rat HepatoChips. (A) Level of labeled nucleotide incorporation using biotinylated dCTP or dUTP in the reverse transcription. (B) Influence of the reaction time of reverse transcription. Three different times were analyzed: 60, 120 and 180 min.

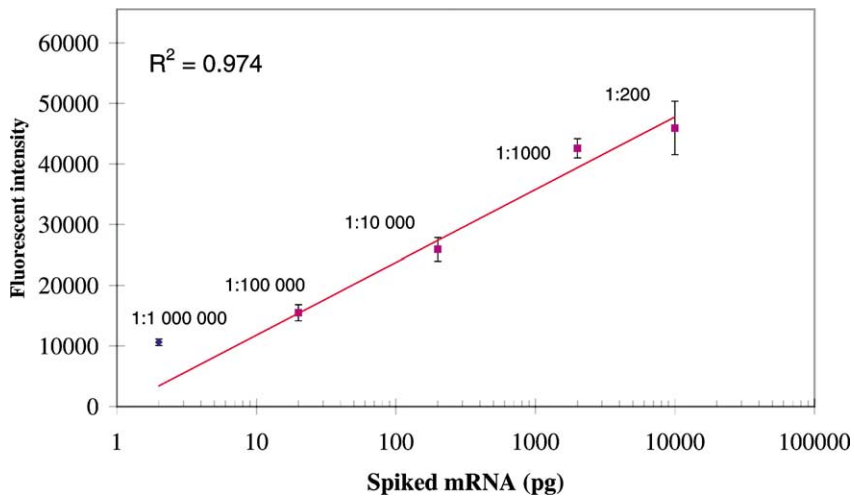

Fig. 3. Detection limit of the gene expression analysis on the Rat HepatoChips. The *in vitro* transcribed poly $(A)^+$ RNA was spiked into a background of 2 μg rat liver mRNA at dilutions from 1:1,000,000 to 1:200 (w/w). The plot represents the fluorescent intensity (signal intensity minus local background) vs. the amount of synthetic poly $(A)^+$ spiked in the reverse transcription reaction. The presence of the internal standard was reproducibly detected 2-fold above the local background (black line). The limit of detection of the internal standard was at the dilution of 1:1,000,000.

3.3. Effect of drug treatment

3.3.1. PB

In order to characterize changes in gene expression in response to PB treatment, mRNA was extracted from the liver of rats killed after 4 days of either PB or vehicle treatment. Biotinylated labeled cDNA were prepared from 2 μg mRNA by reverse transcription and hybridized on two separate arrays Rat HepatoChips spotted on the same slide. The arrays were washed at high stringency and scanned with a confocal laser scanner.

To maximize the dynamic range of microarrays and by consequence, to detect the whole gene expression spectrum, the same arrays were scanned at several different PMT settings. Fig. 4 shows the data of one replicate from the first animal at two different PMT settings (gain of 90 and 50, respectively). Acceptable hybridization signals were observed in more than 95% of probes. No hybridization signal was detected for any of the negative controls. The data were normalized first using the internal standard and then with the housekeeping genes. Scatter plots at four different PMT settings of the data for one control animal vs. one PB treated is shown in Fig. 5.

To evaluate the reliability of the microarray hybridization and the inter-animal variability, triplicates experiments were performed on the same samples of mRNA from three control and three PB-treated animals (N = 3). All slides were from the same batch. Three reverse transcription reactions for each animal were performed and pooled. Then three repeat hybridizations were performed independently with the pooled cDNA for each animal.

The signal intensity values used to calculate ratios (reference/treatment) were at least 2.5 times the background signal. This cut-off, applied to each experiment, selects preferentially genes that are moderate to strongly expressed. A disadvantage is the tendency to disregard weakly expressed genes. Several genes did not reach a high

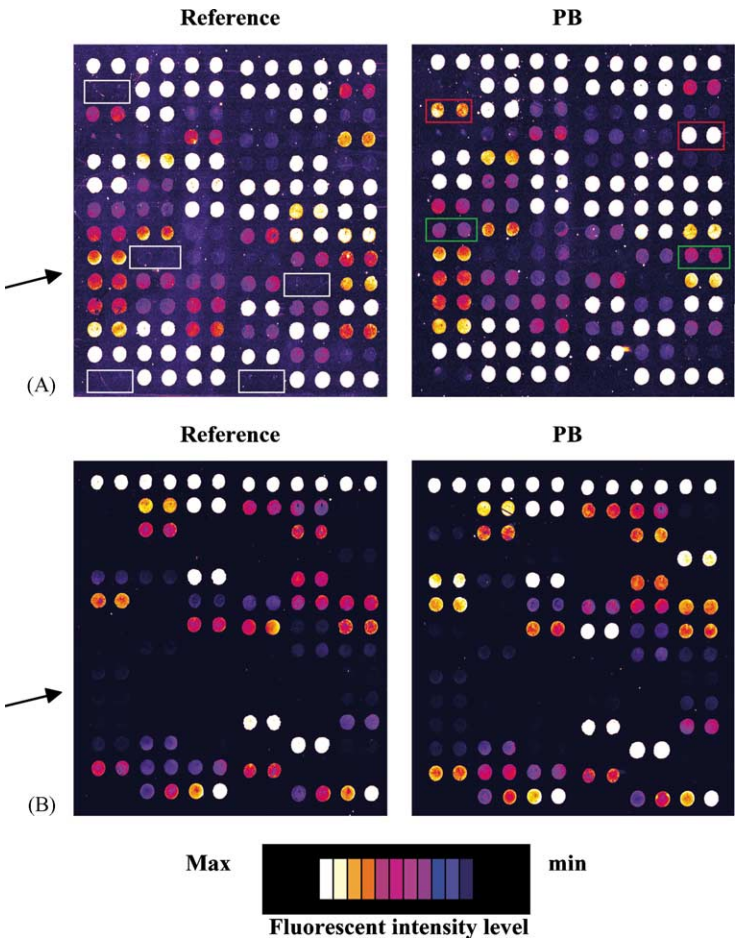

Fig. 4. Rat HepatoChips hybridized with cDNA obtained from mRNA extracted from one animal after treatment with vehicle (reference) and from one animal after treatment with PB. Fluorescence is represented in pseudocolor scale and corresponds to the expression levels of genes; (A) and (B) show the same slide with two identical arrays (one hybridized to the reference sample and one hybridized to the PB sample) but scanned with two different settings of the laser confocal scanner. (A) Laser power of 100% and PMT gain is 90. (B) Laser power of 100% and PMT gain is 50. The arrows highlight low abundance transcripts localized on the Rat HepatoChips that we could detect using the stronger setting of the confocal scanner. The white boxes contain negative hybridization control. Rat genes that display altered fluorescence intensity after PB treatment corresponding to gene activation (red boxes) or to gene repression (green boxes) are pointed out.

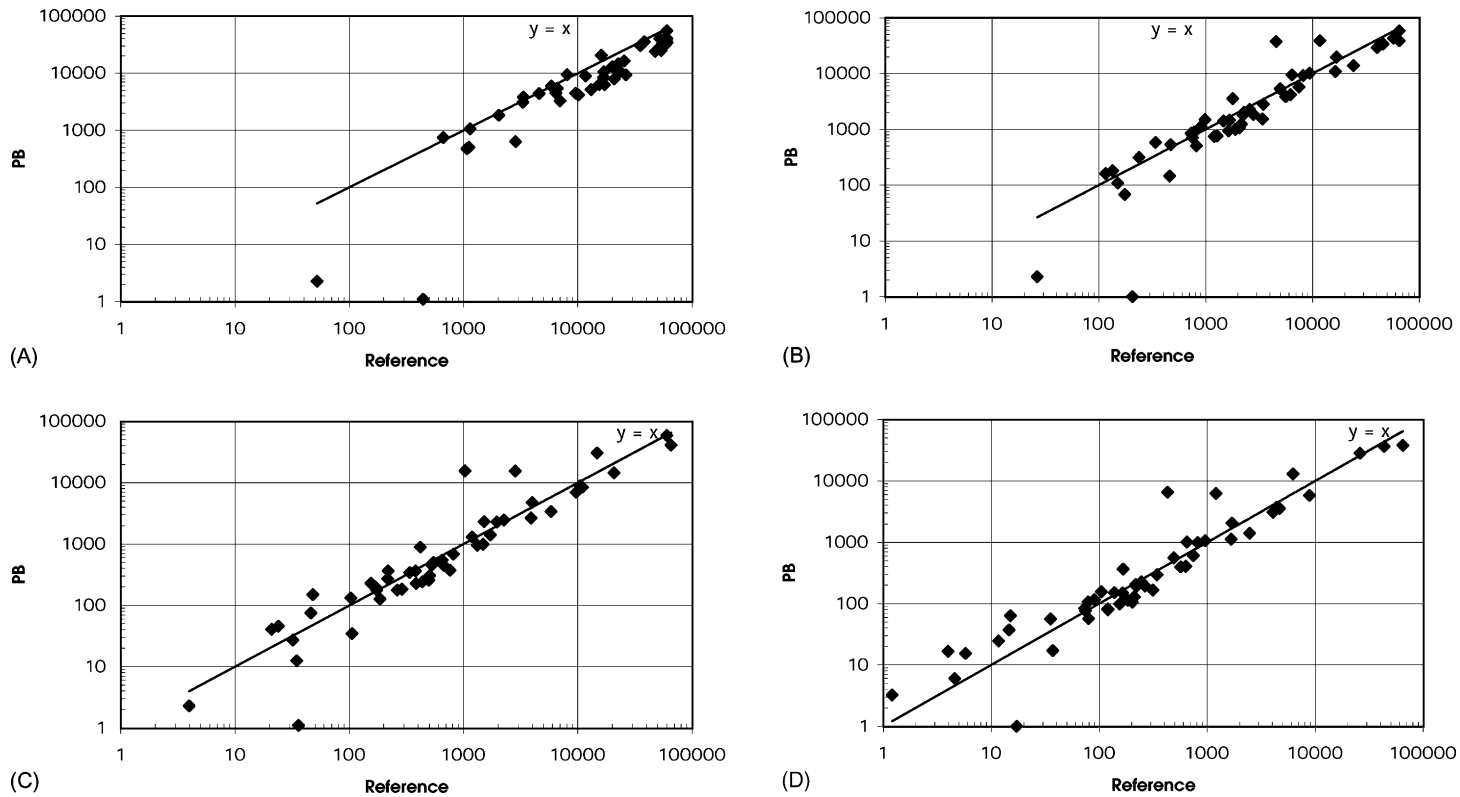

Fig. 5. Scatter plots of normalized fluorescence intensity values from Rat HepatoChips hybridized with cDNA obtained from mRNA extracted from one animal treated with vehicle (reference) and one animal treated with PB. Reference values (*x*-axis) were plotted vs. the PB values (*y*-axis). Four logarithmic scatter plots (A–D) represent the data obtained for four different settings of the laser confocal scanner. (A) Laser power of 100% and PMT gain of 90. (B) Laser power of 100% and PMT gain of 50. (C) Laser power of 100% and PMT gain of 30. (D) Laser power of 100% and PMT gain of 20.

Table 3
Gene expression pattern after *in vivo* treatment with PB from three hybridization replicate experiments from one rat

Transcript	Ratios[a]			Mean ratio[b]	Variability[c]
	1[d]	2[e]	3[f]		
Apoptose					
Bax	0.40	0.63	0.47	0.50	23.58
Bcl-2	3.30	2.03	1.87	**2.40**	32.65
Smp30	0.59	0.67	0.53	0.60	11.77
Cell cycle					
Cyclin D1	0.64	0.50	0.48	*0.54*	16.14
JNK	0.80	0.55	0.47	0.61	28.38
Telomerase	0.71	0.54	0.45	0.57	23.30
DNA damage/repair					
Mgmt	0.70	0.58	0.37	*0.55*	30.37
P450 metabolism					
CYP2B1/2	17.54	15.23	16.39	**16.39**	7.05
CYP3A1	5.29	5.35	5.13	**5.26**	2.16
CYP4A1	0.47	0.67	0.48	*0.54*	20.87
Glutathione enzymes					
GST Ya	2.78	2.09	2.80	**2.56**	15.81
GST theta 5	1.93	1.53	1.71	1.72	11.62
Peroxisome proliferator					
ACO	0.49	0.71	0.63	0.61	18.25
PPARα	0.83	0.56	0.36	0.58	40.43
Stress response					
GSH reductase	0.35	0.48	0.44	*0.42*	15.73
HO-2	0.64	0.65	0.40	*0.56*	25.13
TGFβRII	0.56	0.46	0.44	*0.49*	13.21

The data are presented as ratios (PB sample vs. reference sample) outside 95% confidence interval. Only the genes having a significant ratio in assays were taken into consideration for the statistical analyses. Mean ratios for the four most induced genes were bold and those of the most down regulated were italic.

[a] Fluorescence intensity ratio (PB sample vs. reference sample).
[b] Mean of fluorescence intensity ratio for the three hybridization replicates.
[c] Variation obtained from the three hybridization replicates (calculated as the coefficient variation).
[d] First hybridization replicate.
[e] Second hybridization replicate.
[f] Third hybridization replicate.

Table 4
Gene expression pattern (mean of three hybridization replicates) obtained after *in vivo* treatment by PB from for three rats (N = 3)

Transcript	Ratios[a]			Mean ratio[b]	Variability[c]
	1[d]	2[e]	3[f]		
Apoptose					
Bax	0.50	0.84	0.76	0.70	25.62
Bcl-2	2.40	1.50	1.60	**1.83**	26.82
Smp30	0.60	0.68	0.46	*0.58*	19.34
Cell cycle					
Cyclin D1	0.54	0.84	0.65	0.68	22.43
JNK	0.61	0.50	0.65	*0.58*	13.70
Telomerase	0.57	0.78	1.15	0.83	35.72
DNA damage/repair					
Mgmt	0.55	0.97	0.54	0.69	35.45
P450 metabolism					
CYP2B1/2	16.39	9.42	11.11	**12.31**	29.52
CYP3A1	5.26	5.41	7.29	**5.99**	18.94
CYP4A1	0.54	0.81	0.61	0.65	21.09
Glutathione enzymes					
GST Ya	2.56	5.16	3.31	**3.67**	36.46
GST theta 5	1.72	1.62	1.68	1.67	3.17
Peroxisome proliferator					
ACO	0.61	0.50	0.67	*0.59*	14.83
PPARα	0.58	0.71	0.88	0.72	20.55
Stress response					
GSH reductase	0.42	0.51	0.92	*0.62*	43.03
HO-2	0.56	0.31	0.63	*0.50*	34.17
TGFβRII	0.49	0.55	0.64	*0.56*	13.79

The data are presented as mean ratios for each rat (PB sample vs. reference sample) outside 95% confidence interval. Only the genes having a significant ratio in assays were taken into consideration for the statistical analyses. Mean ratios for the four most induced genes were bold and those of the most down regulated were italic.

[a] Mean of fluorescence intensity ratio for the three hybridization replicate experiments (PB sample vs. reference sample).
[b] Mean of fluorescence intensity ratio for the three animal.
[c] Variation obtained from the three animals (calculated as the variation coefficient).
[d] First animal.
[e] Second animal.
[f] Third animal.

and stable enough signal-to-background ratio in some of the three experiments.

The variability observed in triplicates experiments from one rat was 19% (calculated as the mean of variation coefficient for every significant gene) ranging from 2.2 to 40% (Table 3). Higher variability was obtained with genes having a low signal (e.g. Bcl-2, Mgmt, PPARα). The variability observed between animals was around 25% (calculated as the mean of variation coefficient for every significant gene). The range of observed animal variability is from 3 to 43% (Table 4). Again the most variation seen is generally associated with genes having a low expression level. Seventeen genes were found to be significantly changed when confidence interval of 95% was considered. The four most induced genes were CYP2B1/2, CYP3A1, GST Ya and Bcl-2. The most down regulated were GSH reductase, TGFβRII, Smp30, Mgmt, JNK, ACO, and HO-2. Of the 51 genes (without the housekeeping genes) present on the Rat HepatoChips, the expression of 12 genes were significantly down regulated, 5 were induced and 34 remained stable following PB treatment (Tables 3 and 4).

3.3.2. PCN

The Rat HepatoChips were also used to investigate the change of gene expression in rat liver in response to PCN treatment. Rats were dosed with vehicle or PCN at 100 mg/kg for 4 days. Rats were killed after 4 days of treatment and mRNA was extracted from the removed livers. mRNA samples from control or PCN-treated rats were labeled by reverse transcription. Biotinylated cDNA from control and PCN-treated animals was hybridized on the two separate arrays spotted on the same slide, and scanned

Table 5
Gene expression pattern (mean of three hybridization replicates) obtained after *in vivo* treatment by PCN from three rats

Transcript	Ratios[a]			Mean ratio[b]	Variability[c]
	1[d]	2[e]	3[f]		
Apoptose					
Bcl-2	2.00	2.47	1.50	**1.80**	24.47
Smp30	0.28	0.47	0.54	*0.43*	31.59
P450 metabolism					
CYP2B1/2	3.37	3.40	2.35	**3.04**	19.71
CYP3A1	11.29	12.43	15.52	**13.08**	16.74
CYP4A1	0.32	0.47	0.51	*0.43*	22.24
Glutathione enzymes					
GST Ya	2.85	6.65	3.15	**4.22**	50.06
Glucuronidation enzymes					
UDPGT1a	1.69	3.65	2.86	**2.73**	36.11
Peroxisome proliferator					
Enoyl CoA	0.60	0.79	0.87	*0.75*	18.57
Stress response					
Fibronectin	0.36	0.62	0.69	*0.56*	31.83
TGFβRII	0.25	0.54	0.79	*0.53*	51.21

The data are presented as mean ratios for each rat (PCN sample vs. reference sample) outside 95% confidence interval. Only the genes having a significant ratio in assays were taken into consideration for the statistical analyses. Ratio mean for the induced genes were bold and those of the down regulated were italic.

[a] Mean of fluorescence intensity ratio for the three hybridization replicate experiments (PCN sample vs. reference sample).

[b] Mean of fluorescence intensity ratios of the three animals.

[c] Variation obtained from three animals (calculated as the variation coefficient).

[d] First animal.

[e] Second animal.

[f] Third animal.

with a confocal laser scanner. To determine the animal-to-animal variability and to evaluate the hybridization microarray reliability on Rat HepatoChips, three hybridization replicates on three rats were performed. The ratios for all genes were normalized and ratios outside 95% confidence interval were determined to be significant as described previously. A total of 10 genes were changed in the PCN-treated rats. The five induced genes were CYP2B1/2, CYP3A1, GST Ya, UDPGT1a and Bcl-2. The five repressed genes were Smp30, CYP4A1, enoyl-CoA hydratase, fibronectin and TGFβRII (Table 5).

3.3.3. *Source of variability in microarray analysis*

The variability of the Rat HepatoChips was investigated by using the signal values of both the internal standard and the other genes present on the microarray. The spot-to-spot signal intensity variability within the array was found to be 6% for the internal standards (present as six time duplicate spots) and 6.5% for the other genes on the Rat HepatoChips. This was the case for all the microarrays (from the same batch) analyzed so far. The coefficient of variation for slides-to-slides was 10% from a same batch and 12% from different batches.

4. Discussion

In this report, we have described a fast and reliable DNA microarray with 59 genes (including 8 housekeeping genes) from a range of toxic makers and drug metabolism genes that have the potential to be used to study changes in gene expression levels due to xenobiotic treatment.

Reverse transcription conditions were optimized to obtain the best yield, hybridization efficiency and reproducibility. We determined that key factors for this optimization are the reverse transcription reaction time and the type of labeled nucleotide. Cy3 and Cy5, the most commonly used dyes incorporated into cDNA, have different quantum yield [27] and in addition, are incorporated at different levels during the reverse transcription reaction. To solve this problem, we used the same labeled nucleotide, biotin dCTP, for both the reference and the test samples. Hybridizations (control and treated) were performed on two separate identical arrays spotted on the same slide and the detection was carried out using a Cy3–streptavidin conjugate. This methodology minimizes the variation due to the use of two different fluorochromes. We also found that a longer reverse transcription reaction time (180 instead 60 min) together with biotin dCTP as the labeled nucleotide significantly enhanced the efficiency of the hybridization and consequently improved the detection of the less abundant transcripts.

The spot-to-spot variability within the Rat HepatoChips was also investigated. The variability was shown to be only 6% of the mean signal intensity. This low variability is linked to the homogenous spot intensity, their regular shape and a low homogenous background on microarray. This high reproducibility simplified image analysis and considerably enhanced the accuracy of signal detection.

The use of replicate sample hybridizations and replicate animals increase the statistical significance of findings generated by microarrays [28]. The gene expression pattern has been reproducible in three hybridizations replicates experiments with a variation coefficient of 19% in total. This variability is low and makes the array a potential semi-quantitative assay of gene expression detection. The animal-to-animal variability has shown to be 25% (calculated as the mean of variation coefficient for significant genes).

Detection of low copy genes is one of the challenges of microarray technology (25% of cellular mRNA consists of transcripts expressed in less than five copies per cell). We found that the lower detection limit of this indirect fluorescence method was obtained at a dilution of 1:1,000,000 (w/w) of the synthetic poly $(A)^+$ spiked into rat liver mRNA. This dilution correlates with 2 pg or 10^7 molecules of specific mRNA in the reaction. If we assume a homogenous population of cells and 300,000–500,000 molecules of mRNA per single mammalian cell [29,30], a detection level of 1:1,000,000 corresponds to a detection limit of less than one copy per cell. Therefore, this protocol

could detect transcripts that constitute 0.0001% of the total mRNA in a sample.

However, it should be noted that current microarray technology continues to have some difficulty in reliable detection of low copy genes. The presence of many highly abundant transcripts has a dilution effect on low abundance transcripts. Subsequent RNA amplification steps may circumvent this limitation [31,32].

In order to maximize the dynamic range of the microarray detection, different settings of the photo multiplier of the laser confocal scanner were used. The dynamic range obtained was more than four orders of magnitude.

In the past, the expression level of housekeeping genes was considered to be constant and was used to normalize microarray expression data. However, recent reports indicate that the expression levels of housekeeping genes may vary in many situations [24,33]. Using housekeeping genes alone to normalize expression data could therefore lead to erroneous conclusions. For this reason, we decided to use a well quantified internal standard to normalize differences due to efficiency variation in the reverse transcription, hybridization, microarray quality and indirect detection. However, since the internal control disregard purity and integrity of mRNA samples, a second adjustment of the data was performed based on a set of housekeeping genes whose expression is known not to be affected by experimental conditions.

To validate this microarray as a technique to study changes in gene expression after drug treatments, we selected two well known prototypical P450 inducers; PB and PCN. PB and PCN have been previously reported to induce CYP2B1/2 and CYP3A1, respectively. This microarray contains the specific DNA probes for the different P450 isoforms including CYP2B1/2 and CYP3A1 which could then be used as positive controls for the two treatments used in this study.

PB has been shown to induce other enzymes involved in biotransformation including CYP3A1, GSTs and UDPGT [11,12]. PCN is a well known CYP3A inducer in rat liver [14,15] and has also shown to induce CYP2B1/2 [14]. The data obtained using the microarray has shown that CYP2B1/2, CYP3A1 and GSTs were induced in rat livers after 4 days of PB or PCN treatment.

Results showed that genes other than CYP2B1/2, CYP3A1 and GSTs are also induced or suppressed in rat liver due to PB and PCN treatment. CYP4A1 was shown to be down regulated in rats treated for 4 days with PB or PCN. Acyl CoA oxidase was also shown to be down regulated due to PB treatment. CYP4A1 and ACO are responsible for the ω-hydroxylation of fatty acids [34] and for the peroxisomal fatty acid β-oxidation, respectively; therefore, a down regulation of these two genes could lead to cellular function alterations. However, the effect of the CYP4A1 and ACO down regulation in the liver caused by PB and PCN treatments is not known. Data have also shown that the mRNA levels of the UDPGT1a were increased after 4 days of PCN treatment. Fibronectin mRNA levels were found to be decreased after 4 days of PCN treatment. The connection between fibronectin down regulation and toxicological effect due to PCN treatment is not known.

Alterations in oxidative stress related genes in PB exposed rats are shown in Tables 3 and 4. The expression of the oxidative gene GSH-reductase is decreased after 4 days of PB treatment. It is known that glutathione (GSH) and GSH-dependant enzymes such as GST and GSH-reductase play a central role in cellular defense against toxic environmental agents [35]. This decrease in GSH-reductase mRNA levels suggest that the hepatocytes could have their cellular defense mechanisms impaired following PB treatment.

Alterations in apoptosis-related genes in PB exposed rats are shown in Tables 3 and 4. PB and PCN have previously been reported to have an influence on cell mortality by suppressing apoptosis [19]. A general increase in the level of Bcl-2 protein was previously shown in PB-treated animals [18]. Apoptosis-related genes were shown to be altered in this study due to PB and PCN treatment. The increase in Bcl-2 mRNA levels together with a decrease in bax mRNA levels are in good accordance with previous findings where PB and PCN have been shown to suppress apoptosis. Tumor growth factor-β receptor type II (TGFβ-RII) was also found to be down regulated due to PB and PCN treatment. It is known that TGFβ has diverse biological functions, including the regulation of cell proliferation, differentiation and apoptosis [36,37]. Signal transduction pathways of TGFβ through the TGFβ-RII receptor play a complex role in carcinogenesis, leading to both tumor-suppressor and oncogenic activities [38]. It is well established that many tumor cells show a decreased receptor function therefore compromising the tumor suppressor activities of TGFβ and enabling its oncogenic function [38]. This down regulation could lead to a loss of TGFβ receptor function which might be responsible for PB and PCN tumor progression effects.

Jun N-terminal kinase gene was also found to be down regulated with PB treatment. JNK was shown to be involved in a wide variety of cellular responses such as cell growth, oncogenic transformation, cell differentiation and cell death [39,40]. The observed down regulation could be correlated with the decrease in the incidence of cell death due to PB treatment.

It has been suggested that non-genotoxic hepatocarcinogens enhance the carcinogenic process by disrupting the balance between cell death and cell proliferation, leading to a selective growth advantage for some cell types [41]. These results could again support the hypothesis that the PB and PCN promote tumor development through a reduction in the incidence of cell death.

PB treatment shows a down regulation of a cell cycle related gene cyclin D1. Cyclin D1 plays a regulatory role in the progression of quiescent cells through G1 and into S-phase of the cell cycle [42]. However, PB has been shown to increase replicative DNA synthesis [14], therefore the

reason for this apparent discrepancy is not clear. However, Ramljak and co-workers showed that fumonisin B1 treatment, a hepatocarcinogen, results in cyclin D1 over expression *via* protein stabilization due to post-translational modification(s) without effect on cyclin D1 mRNA levels. To define whether cyclin D1 down regulation is a consequence of protein stabilization, further investigations to study cyclin D1 protein levels would be required.

PB and PCN treatments decreased mRNA levels of senescence marker protein-30 (SMP30). SMP30 was shown to be expressed in the cytosol of hepatocytes and renal epithelia throughout the tissue-maturing process and adulthood, but its expression is decreased thereafter [43]. SMP30 has been suggested to play a role as a Ca^{2+} binding protein [44]. The exact role of the SMP30 down regulation after PB and PCN treatment is not known. However, down regulation of SMP30 could lead to a dysregulation of Ca^{2+} homeostasis.

The present study provides new information on the extent and complexity of the overall cellular response to xenobiotics such as PB and PCN and it has also shown good correlation with previous findings in expressed genes due to PB and PCN treatment.

We have shown that a well-defined microarray could be a tool for studying changes in gene expression levels due to xenobiotic treatment. We show that this assay can detect transcripts that comprise 0.0001% of the total mRNA population. This microarray has shown that other genes apart from the corresponding CYP P450 genes have been changed due to PB and PCN treatment. However, the link between the gene expression profile and the toxic or undesired effect of drug on animals or humans is still to be made and will require the use of cluster analysis of many drugs before effective conclusions can be drawn. No doubt that the technique of DNA microarray will serve as a new screening tool in drug discovery or early pharmaceutical development.

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