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Interactions between polycyclic aromatic hydrocarbons in binary mixtures: Effects on gene expression and DNA adduct formation in precision-cut rat liver slices

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Although exposure to polycyclic aromatic hydrocarbons (PAHs) occurs mostly through mixtures, hazard and risk assessment are mostly based on the effects caused by individual compounds. The objective of the current study was to investigate whether interactions between PAHs occur, focusing on gene expression (as measured by cDNA microarrays) and DNA adduct formation. The effects of benzo[*a*]pyrene or dibenzo[*a*,*h*]anthracene (DB[*a*,*h*]A) alone and in binary mixtures with another PAH (DB[*a*,*h*]A, benzo[*b*]fluoranthene, fluoranthene or dibenzo[*a*,*l*]pyrene) were investigated using precision-cut rat liver slices. All compounds significantly modulated the expression of several genes, but overlap between genes affected by the mixture and by the individual compounds was relatively small. All mixtures showed an antagonistic response on total gene expression profiles. Moreover, at the level of individual genes, mostly antagonism was evident, with additivity and synergism observed for only a few genes. As far as DNA adduct formation is concerned, the binary mixtures generally caused antagonism. The effects in liver slices suggest a lower carcinogenic potency of PAH mixtures than estimated based on additivity of individual compounds.

Introduction

Toxicological research on hazard and risk characterization of environmental pollutants is directed towards the adverse effects of single compounds on a cell system or an organism. However, human exposure to environmental chemicals mostly involves mixtures. Therefore, it is of importance that studies are not only confined to single compounds, but also are extended to mixtures.

Through the environment, humans are daily exposed to chemical mixtures, including polycyclic aromatic hydrocarbons (PAHs). PAHs comprise a large class of structurally related compounds which are formed during the incomplete combustion of organic compounds, and many of them possess carcinogenic activity. Not only concentrations of PAHs in air vary but also the mixture composition. Although PAHs are structurally similar, they vary greatly in their carcinogenic potency, with some compounds being potent carcinogens whereas others are non-carcinogenic. Carcinogenic potency of PAHs is usually expressed relative to that of benzo[*a*]pyrene (B[*a*]P) (1,2), the prototype PAH. Not only does this require extensive animal experiments to compare carcinogenic doses but also interactive effects between compounds are not taken into account.

Similar to other chemical mixtures, it is conceivable that in PAH mixtures, the constituents interact with each other. Such interactions can lead to more adverse effects than expected based on additivity (synergism), or one PAH may repress the effects of another (antagonism). No interaction implies the addition of the effect of both PAHs (additivity) (3,4). Interactive effects may occur at all stages in PAH carcinogenicity, of which biotransformation is one of the most important.

Biotransformation is important for the metabolic activation of PAHs into their ultimate carcinogenic metabolites. PAHs are established inducers of the gene expression for several biotransformation enzymes among others, the CYP1 family of cytochromes P450, which metabolize PAHs to their reactive intermediates. These intermediates can bind to DNA and thereby lead to the formation of mutations, which may initiate carcinogenesis. PAHs can also induce epigenetic effects that may change biological processes like cellular communication (5,6) and, ultimately, may promote cancer development.

A useful applied tool in assessing overall effects caused by toxic compounds is analysis of gene expression by cDNA microarrays. By studying large numbers of genes, a transcriptomic fingerprint is obtained, which can provide information on the toxicological properties of compounds (7–9). This approach can be applied in *in vitro* cell systems for both mixtures and single compounds (10–12).

Although the liver is not the first-pass organ for PAH exposure, it plays an important role in biotransformation of xenobiotics, including PAHs. DNA adduct formation by PAHs in liver is generally higher than in lung, which makes the liver a preferred organ to study interactive effects of PAHs. Consequently, we used precision-cut liver slices, an *in vitro* model representing the liver in an *in vivo* condition. In this model, primary cells are cultured in an environment that maintains normal cell–cell and cell–matrix contacts, and cells express high levels of metabolic enzymes that are important in PAH-induced effects (13). Precision-cut liver slices are a frequently used *in vitro* model for toxicological studies (14,15), which is increasingly applied in transcriptome profiling studies (16). Compared to *in vivo* models, this *in vitro* model also offers the possibility to study effects on gene expression without inter-individual differences between control and exposure conditions.

Previously, we assessed the effects of single PAHs on gene expression and DNA adduct formation in precision-cut rat liver

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slices. We showed that each PAH induced a compound-specific gene expression response and that several PAHs induced high levels of DNA adducts. In this study, we applied the same *in vitro* model, PAHs and cDNA microarrays to assess the effects of binary PAH mixtures (10).

The aim of this study was to apply a toxicogenomics approach to assess whether interactions occur in rat precision-cut liver slices following exposure to binary PAH mixtures. We used five PAHs, selected on their carcinogenic potency and environmental abundance, namely in order of abundance benzo[*b*]fluoranthene (B[*b*]F) ≈ fluoranthene (FA) > B[*a*]P > dibenzo[*a*,*h*]anthracene (DB[*a*,*h*]A) > dibenzo[*a*,*l*]pyrene (DB[*a*,*l*]P). These PAHs also differ in their induction of *CYP1A1*, which may be important for the interactive effects in mixtures. The effects of PAHs on gene expression were examined for a total of 5700 genes by oligonucleotide arrays for individual PAHs as well as for binary mixtures of a PAH with B[*a*]P or DB[*a*,*h*]A. Effects on DNA adduct formation were determined with ^{32}P-post-labelling. PAH concentrations were selected based on DNA adduct-forming potential as determined previously (17).

Materials and methods

Chemicals

B[*a*]P (purity 97%, CAS no. 50-32-8), B[*b*]F (purity 98%, CAS no. 205-99-2), FA (purity 99%, CAS no. 206-44-0), DB[*a*,*h*]A (purity 97%, CAS no. 53-70-3), and DB[*a*,*l*]P (purity 99.6%, CAS no. 191-30-0) were obtained from Sigma-Aldich (Zwijndrecht, the Netherlands). All chemicals were dissolved in dimethyl sulfoxide (DMSO).

Preparation and exposure of precision-cut liver slices to PAHs

Rat livers (one for each experiment) were obtained from adult male Wistar albino rats (175–250 g) killed by cervical dislocation. Livers were immediately excised and slices (250 μm) were prepared using a Krumdieck tissue slicer (Alabama Research and Development Corp., Munsford, AL, USA) as previously described (14). Slices were pre-incubated for 30 min at 37°C in RPMI supplemented with 5% foetal calf serum, 0.5 mM L-methionine, 1 μM insulin, 0.1 mM hydrocothsone-21-hemisuccinate, and 50 μg/ml gentamycin in 12-well plates on a shaking gyratory in an incubator (5% CO_2 and 95% air). After pre-incubation, the slices were transferred to 12-well plates containing fresh media and a solvent control (DMSO, 0.066% v/v), B[*a*]P (3 μM), DB[*a*,*h*]A (10 μM), B[*b*]F (10 μM), FA (30 μM) or DB[*a*,*l*]P (0.3 μM), or a mixture of one of the PAHs with B[*a*]P or DB[*a*,*h*]A. Concentrations inducing a low but significant level of DNA adducts in our previous study (17) were selected for each PAH. Only FA did not induce DNA adducts and therefore the highest concentration was selected. In each of two independent experiments, three slices were used for each treatment, except for the solvent control (18 slices), B[*a*]P (8 slices), and DB[*a*,*h*]A (5 slices). After a 24-h exposure, the slices were removed from the medium and immediately frozen in liquid nitrogen. For the mixtures of B[*a*]P with B[*b*]F and B[*a*]P with FA, only one experiment was conducted.

RNA isolation, cDNA synthesis and dye labelling

After crushing the frozen liver slices under liquid nitrogen, RNA was stabilized by dissolving the crushed powder in Trizol (Gibco/BRL, Breda, The Netherlands) and isolated according to the manufacturer's manual. RNA was purified using the RNeasy mini kit (Qiagen Westburg bv., Leusden, The Netherlands) with DNase treatment, RNA quantity was determined spectrophotometrically and quality (clear 18S and 28S peaks) was assessed using a BioAnalyzer (Agilent Technologies, Breda, The Netherlands). RNA from all slices with the same treatment was pooled for each experiment.

RNA samples were reverse transcribed into cDNA in triplicate with amino-allyl-labelled deoxyuridine triphosphate (Sigma-Aldrich, St Louis, MO, USA) and subsequently labelled with one of the three dyes, namely cyanine 3 (Cy3), cyanine 5 (Cy5) or Alexa 594 (A594), as was described previously (18).

Microarray hybridization and data analysis

Dye-labelled samples were hybridized on an Operon rat oligonucleotide array containing 5700 oligonucleotides (v1.2.1, Operon; Qiagen, Venlo, The Netherlands) printed in triplicate on Corning UltraGAPS Coated Slides (Corning Life Sciences, New York, NY, USA) by the Genome Centre Maastricht (Maastricht University, Maastricht, The Netherlands). Hybridization and washing were performed according to Corning's protocol for oligonucleotide arrays as previously described (18). The labelling schedule is shown in Table I.

The microarray slides were scanned on a ScanArrayExpress (Packard Biochip Technologies, Perkin Elmer Life Sciences, Boston, MA, USA). All three channels were scanned at 100% laser power and photomultiplier tube (PMT) gain was adjusted, such that the signal of the highest fluorescent spots was just below the maximum measurable level. The images (10-micron resolution; 16-bit tiff) were processed with ImaGene 5.0 software (BioDiscovery, Inc., Los Angeles, CA, USA) to quantify spot signals. Irregular spots were manually or automatically flagged and excluded from the data analysis. If the averaged spot signal for all controls was higher than the corresponding standard deviation (SD) of that signal, the gene was used for further analysis. Of all 5700 genes studied, 2156 genes met these criteria. Among others, the *CYP1A1* gene was excluded from further analysis.

Data from ImaGene were transported into GeneSight software version 4.1.5 (BioDiscovery, Inc.) for further processing and analysis. For each spot, background was subtracted and flagged spots as well as spots with a net expression level below 20 (all three channels) were omitted. Data were log base

Table I. Labelling and hybridization design of RNA samples from liver slices exposed to the listed PAH (or PAH mixture) for each experiment and all biological replicates

Hybridization experiment	Biological replicate	Array	Cyanine 3	Cyanine 5	Alexa 594
1	1	1	Control	B[*a*]P	DB[*a*,*h*]A/DB[*a*,*l*]P
1	1	4	Control	DB[*a*,*l*]P	B[*a*]P/DB[*a*,*l*]P
1	1	5	B[*a*]P/DB[*a*,*h*]A	Control	DB[*a*,*h*]A
1	1	7	Control	DB[*a*,*h*]A/FA	FA
1	1	9	B[*a*]P/B[*b*]F	B[*b*]F	Control
2	1	2	DB[*a*,*h*]A/DB[*a*,*l*]P	Control	DB[*a*,*l*]P
2	1	3	B[*a*]P	B[*a*]P/DB[*a*,*l*]P	Control
2	1	6	DB[*a*,*h*]A	B[*a*]P/DB[*a*,*h*]A	Control
2	1	8	FA	Control	DB[*a*,*h*]A/FA
2	1	10	Control	B[*a*]P/B[*b*]F	B[*b*]F
3	2	11	Control	B[*a*]P	DB[*a*,*h*]A/DB[*a*,*l*]P
3	2	14	Control	DB[*a*,*l*]P	B[*a*]P/DB[*a*,*l*]P
3	2	15	B[*a*]P/DB[*a*,*h*]A	Control	DB[*a*,*h*]A
3	2	17	Control	DB[*a*,*h*]A/FA	FA
3	2	19	DB[*a*,*h*]A/FA	B[*a*]P/FA	Control
4	2	12	DB[*a*,*h*]A/DB[*a*,*l*]P	Control	DB[*a*,*l*]P
4	2	13	B[*a*]P	B[*a*]P/DB[*a*,*l*]P	Control
4	2	16	DB[*a*,*h*]A	B[*a*]P/DB[*a*,*h*]A	Control
4	2	18	FA	Control	B[*a*]P/FA

2-transformed and data normalization was carried out using LOWESS and centring expression differences by subtracting mean values. Expression differences between exposed and control were calculated and data of replicate spots were averaged while omitting outliers (>2 SDs). Significantly modulated genes were found using the confidence analysis tool from GeneSight (up- or down-regulation of 0.1 and 99.9% confidence limit) using the averaged data per treatment. Unsupervised clustering was performed by hierarchical clustering analysis (HCA) and principal component analysis using GeneSight tools.

Real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (RT-PCR) was performed to measure mRNA levels for a selection of genes in order to verify expression changes from the microarray experiments. Reverse transcription reaction was performed using 1 μg of total RNA using iScript cDNA Synthesis Kit (Biorad Laboratories, Hercules, CA, USA). Subsequently, RT-PCR reactions were performed using iQ SYBR Green Supermix, containing i*Taq* DNA Polymerase, deoxynucleoside triphosphates and SYBR Green I (BioRad Laboratories). All PCR reactions were performed in duplicate. β-actin mRNA was used as reference in order to normalize expression levels and to quantitate changes in gene expressions between the control and treated samples. The RT-PCR was run on the MyiQ Single-Color Real-Time PCR Detection System (BioRad Laboratories): 3 min at 95°C, 40 cycles of 95°C for 15 s and 60°C for 45 s. The following forward and reverse primers were used (operon, 5′–3′ sequences): β-actin CGTGAAAAGATGACCCAGATCA (forward) and CAGCCTG-GATGGCTACGTACA (reverse), CYP1A2 GCAGAAGGGAAGCAGTG-GAA (forward) and GGCCGTGTTGTCATTGGTAAG (reverse), CYP1A1 GGGTGGCCTTGAACTCCTTAA (forward) and CATGACTGTACCCTAG-CACTTGGT (reverse), ACOX2 CAGGAGAACCCTGCCTATAAGAAGTA (forward) and ATCCTTTGACTTTTCACATCTTGTGT (reverse), DIG1 CCGCACTGGCCCATGT (forward) and TGCGGAGTTGCTGATAGA-TAATG (reverse), ID1 TGCTACTCACGCCTCAAGGA (forward) and TCTCCACCTTGCTCACTTTGC (reverse), PFKFB1 CCCTTTTCAAGTGA-TCAGATTGTCT (forward) and AACAAGGGCAGCAGCTCCTA (reverse), SPP1 GCAGACACCACTGTAACCTAGAAGTT (forward) and AGTGGC-CATTTGCATTTCTTG (reverse) and NQO1 GCCTCATGCGTTTTTGGA-TAG (forward) and CCCCTAATCTGACCTCGTTCAT (reverse).

Dissociation curve analysis was performed and 'no-template controls' were analyzed to check for non-specific products in the reaction. For each sample, the quantity was derived from Ct = Ct(target gene) – Ct(β-actin reference). These values were log2 transformed, and the difference of each test relative to their concomitant vehicle control sample was calculated (19).

Assessing the additivity of gene expression data

To calculate the expected gene expression levels of the PAH mixture based on the individual constituents, we used the method described previously (10), which assumes additivity of the effects on gene expression. Linear regression analysis and Pearson correlation coefficients were calculated using SPSS 12.0.1 for windows (SPSS, Inc., Chicago, IL, USA). Additivity was assumed when regression analysis for the observed and expected total gene expression profile data did not show a deviation from $y = x$ (confidence interval of 2 SD). If it did deviate from $y = x$, synergism is shown by a slope > 1 and antagonism by a slope < 1.

DNA adduct analysis

After removal of the aqueous phase during RNA isolation using Trizol, the remaining phases were used for DNA isolation according to the manufacturer's protocol. DNA adduct levels were determined according to the procedure originally described by Reddy and Randerath (20) with the modifications introduced by Godschalk *et al.* (21). DNA adduct levels were quantified by comparison with standard samples with known benzo(a)pyrene diol expoxide (BPDE)–DNA adduct levels [one adduct per 10^6, 10^7 or 10^8 nucleotides (nts); detection limit one adduct per 10^8 nts]. Adduct spots on the chromatograms were located and quantified using a phosphor imager (FLA-3000; Fuji, Paris, France) and AIDA/2D densitometry software.

Expected DNA adduct levels were calculated by adding the total DNA adduct levels of both constituents, after correcting for background levels observed in solvent control samples.

Results

Gene expression analysis

Microarray analysis showed that in total 328 genes were significantly modulated after exposure to either the mixtures or the individual compounds. The highest number of affected genes was seen after exposure to the DB[*a*,*h*]A/FA mixture (80 genes). The number of modulated genes decreased as follows: B[*b*]F (60), B[*a*]P/B[*b*]F (57), B[*a*]P (52), DB[*a*,*h*]A (49), B[*a*]P/DB[*a*,*h*]A (45), DB[*a*,*h*]A/DB[*a*,*l*]P (42), FA (34), B[*a*]P/DB[*a*,*l*]P (34), B[*a*]P/FA (33) and DB[*a*,*l*]P (27). In Figure 1, it can be seen that the overlap in modulated genes for each mixture in comparison with the individual compounds was relatively small. Gene names, abbreviations, GenBank accession numbers and gene expression differences of modulated genes can be found in the supplementary data file.

HCA shows, however, that gene expression profiles between mixtures and their individual constituents are similar (Figure 2). This indicates that neither of the compounds has a dominant effect and, thus, that all compounds contribute to changes in gene expression induced by the mixtures. All treatments with DB[*a*,*h*]A, both individual as well as in mixtures, group together, indicating a distinct effect of DB[*a*,*h*]A on gene expression. Furthermore, the gene expression profile for the B[*a*]P/B[*b*]F mixture differs most extensively from the gene expression profiles brought about by the other treatments, which is most likely caused by a single analysis for this mixture compared to a duplicate analysis with dye-swaps for most other mixtures.

Assessing the additivity for gene expression profiles

The expected gene expression modulation of the mixtures was calculated by adding the responses of the individual compounds. Figure 3 shows the expected gene expression differences versus the observed differences for each mixture. All linear regression equations significantly deviated from $y = x$ and their slopes were smaller than 1, indicating an antagonistic

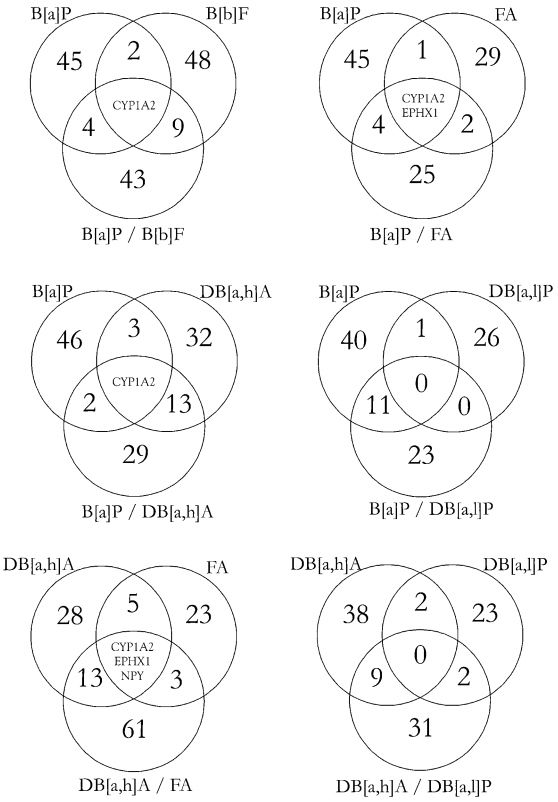

Fig. 1. Venn diagrams showing the number of differentially expressed genes in rat liver slices exposed to single PAHs and their mixtures and their overlap.

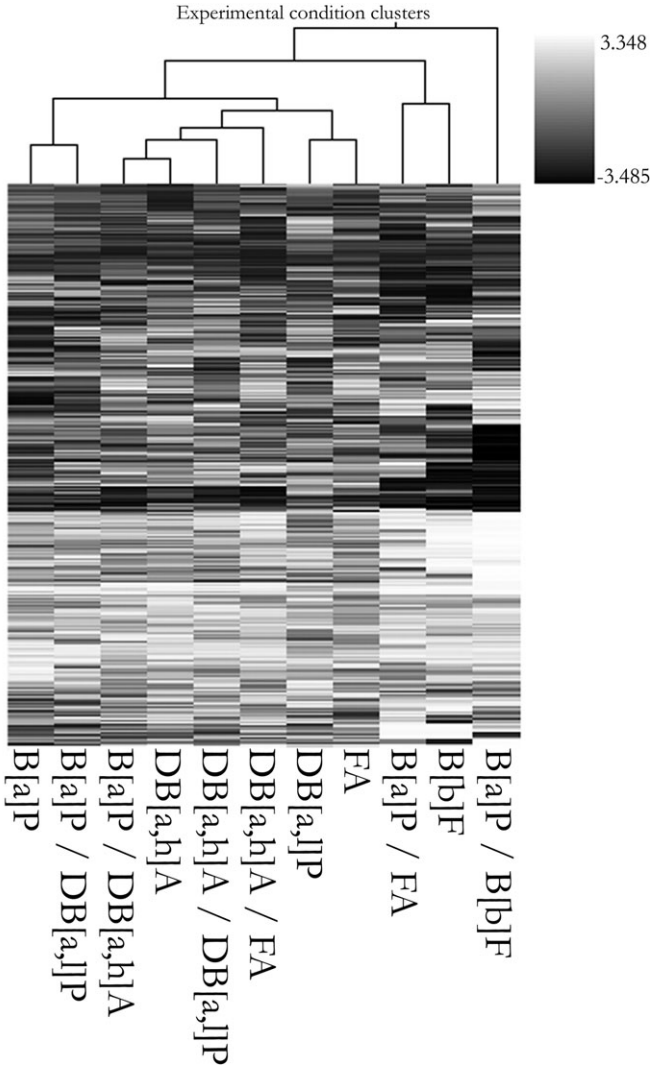

Fig. 2. HCA of gene expression differences in rat liver slices after 24-h exposure to PAHs and their mixtures with the 328 genes differentially expressed by either of the treatments. Euclidean clustering was used with average distance metric.

interaction on the total gene expression profiles of these PAHs in the mixtures.

A comparison of expected and observed gene expression differences was also performed after a more strict selection of genes, limited to those genes modulated by at least two of the three treatments for each mixture (the intersections of Figure 1). This analysis again showed that all slopes are smaller than 1, although the slopes for B[*a*]P/B[*b*]F and B[*a*]P/FA did not differ significantly from 1 (data not shown).

Assessing the additivity for single genes

In addition, at the level of individual genes, the expected expression was compared with the observed expression. Of all 328 differentially expressed genes, 21 showed lower gene expression levels than expected in most mixtures, indicating antagonism. Ten genes showed consistently no deviation between observed and expected data, suggesting additivity, and two genes showed mostly higher gene expression levels than expected, which points to synergism. These genes are shown in Table II. Thus, most genes show antagonistic effects, which is in agreement with the antagonistic effect noted for all mixtures on total gene expression profiles. The other genes show no consistent interactive effect or interactions for only one or two mixtures; they are listed in the supplemented data file.

Validation by RT-PCR

Gene expression modulation for four genes (*CYP1A2*, *ACOX2*, *DIG1*, *ID1*, *PFKFB*, *SPP* and *NQO1*) was validated by quantitative RT-PCR. The direction of modulation was similar for all genes assessed and each of the treatments (single compounds and mixtures). Only in cases with relatively low expression change, the direction was not always correct (Figure 4). The extent of modulation was slightly higher for RT-PCR compared to microarray analysis. However, the ratio between observed and expected gene expression is mostly comparable between RT-PCR and microarray analysis for each gene and mixture (data not shown). Still, the interactive effects show differences between microarray analysis and RT-PCR. This might be due to relative inaccuracy for measuring small expression changes on microarrays and the smaller SDs found in RT-PCR analysis. Generally, this indicates that the interactive results obtained by microarray analysis may be considered an accurate estimation of the interactive effect of PAH mixtures on gene expression.

Since CYP1A1 is important in the metabolism of PAHs, the expression modulation of this gene was also measured by RT-PCR (it was excluded during the gene-filtering step of the microarray analyses). The *CYP1A1* gene expression was significantly induced by all treatments, except DB[*a*,*l*]P. PAH exposure resulted in an induction of >5-fold (log2 transformed), only FA induced the *CYP1A1* expression by 2.4-fold. Interactive effects on *CYP1A1* gene expression were synergistic for all mixtures.

DNA adduct analysis

Figure 5 shows the DNA adduct levels in liver slices. Comparison of the DNA adduct level of each mixture with its expected level based on additivity of the individual compounds showed an overall significant antagonistic effect for all mixtures. This is also observed for most mixtures by individual comparison, except for the mixture B[*a*]P/FA. Bearing in mind the variation and the fact that only a single measurement for the B[*a*]P/FA mixture was carried out, no conclusion can be drawn for the interactive effects of DNA adducts by this mixture.

Discussion

This article describes a study on the effects of binary PAH mixtures on gene expression and DNA adduct formation in rat liver slices in comparison to the effects elicited by the individual components. We assessed whether the effects of binary mixtures of PAHs on gene expression and DNA adduct formation compared to the individual compounds are additive or show synergism or antagonism.

As both B[*a*]P and DB[*a*,*h*]A induced the *CYP1A1* expression in liver slices in our previous study (17), we used these compounds to assess the interactive effects of mixtures. CYP1A1 is important in PAH metabolism and its induction may increase the metabolism of non-CYP1A1 inducers and thereby cause interactive effects.

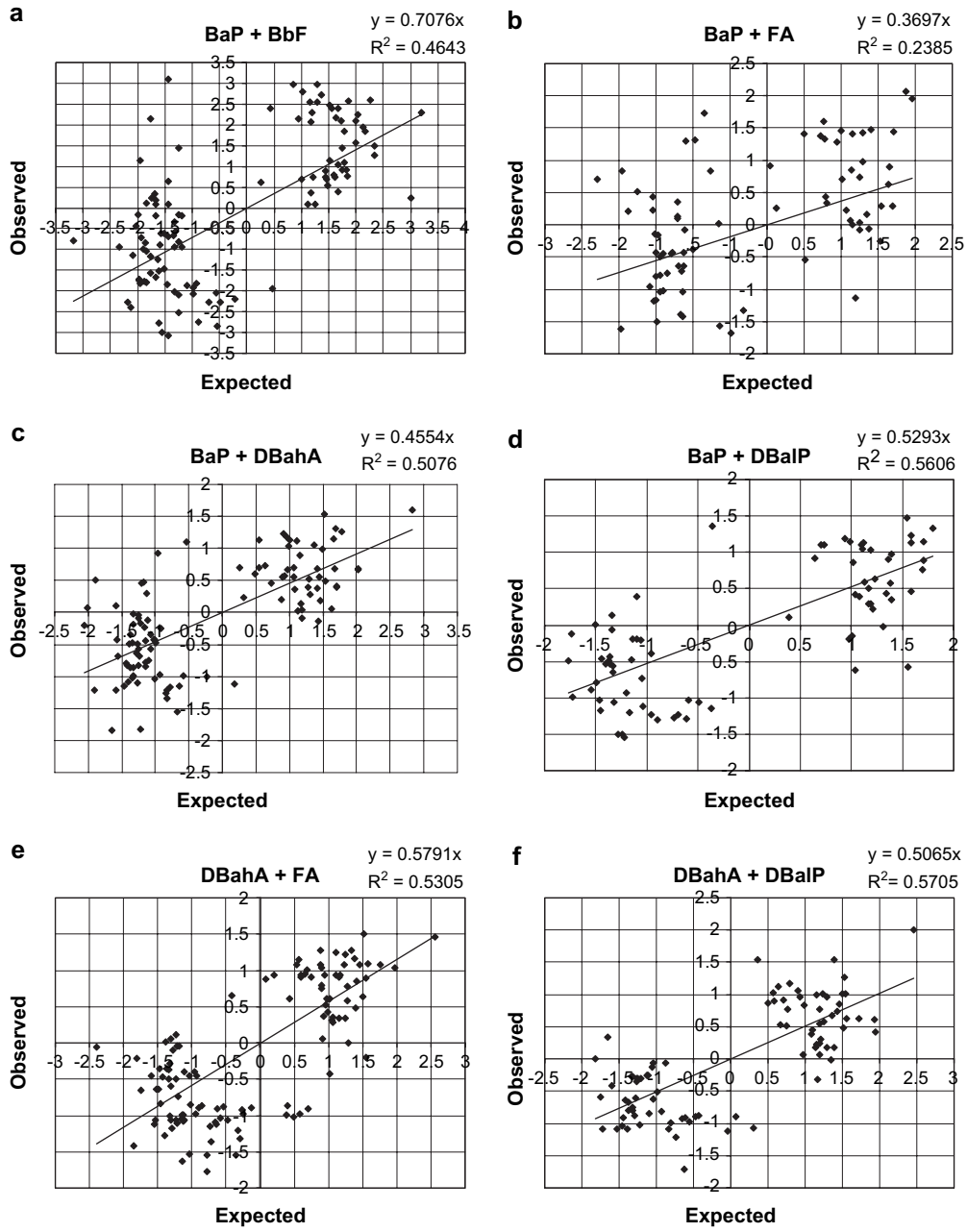

Fig. 3. Expected (*x* axis) versus observed (*y* axis) gene expression in rat liver slices exposed to PAH mixtures. Each graph is based on the genes significantly modulated by the respective individual compounds and mixture. Correlation coefficients (R^2) and the line equation are indicated in the graph.

Gene expression modulation

Each treatment caused gene expression modulation in liver slices. However, the genes significantly affected by the mixtures were frequently different from those affected by either of the constituents. This contrasts to what we observed in HepG2 cells, in which genes modulated by the individual compounds were generally also modulated by the mixtures (22). A possible explanation for this difference is that for liver slices many more genes (5700 versus 594 genes) were investigated that were not selected based on relevance to toxicology, as was the case for the HepG2 study. Thus, for the liver slice study, a more global overview on gene expression is obtained. Handriksen *et al*. (23) also noted little overlap between modulated genes by individual compounds and their mixtures. They attributed this effect to a shift from a compound-specific response to a more generic stress response.

Assessing the additivity of gene expression of PAH mixtures

The differential gene expression was lower for all mixtures than expected, indicating that all mixtures show antagonism. This is similar to what we have previously observed in HepG2 cells (10), and might be related to a competitively for receptors, including the Ah receptor. In contrast to our findings, Cherng *et al*. (24) reported a decrease in the *CYP1A1* gene expression and protein expression in B[*a*]P/1–nitropyrene mixtures compared to the individual compounds. In our study, *CYP1A1* was excluded from microarray analysis during filtering based on signal-to-noise, but we found a synergistic effect on the

Table II. Interactive effect on gene expression upon exposure to PAH mixtures

Gene symbol	Gene name	GenBank accession	B[*a*]P/B[*b*]F	B[*a*]P/DB[*a*,*h*]A	B[*a*]P/DB[*a*,*l*]P	B[*a*]P/FA	DB[*a*,*h*]A/DB[*a*,*l*]P	DB[*a*,*h*]A/FA
Additivity								
Abcd3	ATP-binding cassette, sub-family D (ALD), member 3	NM_012804			=		=	=
Aldr1	Aldehyde reductase 1 (low-K_m aldose reductase) (5.8-kb PstI fragment, probably the functional gene)	NM_012498		=			=	=
Arfgap1	ADP ribosylation factor 1 GTPase-activating protein	U35776	=		=	=		
Ccr2	Chemokine receptor CCR2 gene	NM_021866	=	=	=			
Cdh17	Cadherin 17	X78997			=		=	=
Nqo1	NAD(P)H dehydrogenase, quinone 1	NM_017000	=	=		=	=	=
Nupr1	Nuclear protein 1	AF014503	=	=			=	=
Spp1	Secreted phosphoprotein 1	NM_012881	=	=	=			
	Similar to hypothetical protein, clone 2-25	AW530379		=			=	=
	Similar to cDNA sequence AF155546	BF548312	=	=				=
Mostly antagonism								
Acox2	Acyl-coenzyme A oxidase 2, branched chain	X95189		–			–	–
Cldn7	Claudin 7	AJ011811		–		–	–	–
Cyp1a2	Cytochrome P45, 1a2	NM_012541	–	–	–	=	–	–
Dig1	Dithiolethione-inducible gene-1	U66322	–	–	=	=	–	–
Ehhadh	Enoyl coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase	K03249		–			–	–
Gilz	Glucocorticoid-induced leucine zipper	NM_031345		–			–	–
Hsd11b1	Hydroxysteroid 11-beta dehydrogenase 1	NM_017080	–	–	=		–	–
Id1	Inhibitor of DNA binding 1, helix-loop-helix protein (splice variation)	NM_012797	=	–	–	–		–
Lbp	Lipopolysaccharide-binding protein	NM_017208		–	–		–	–
Madh3	MAD homolog 3 (Drosophila)	NM_013095	=	–	–	–		
Me1	Malic enzyme 1	NM_012600	–	–	=	–		
p58/p45	Nucleoporin p58	AF000899		–			–	–
Ptpn16	Protein tyrosine phosphatase, non-receptor type 16	X84004		–			–	–
Serpind1	Leuserpin-2	NM_024382		–			–	–
Slc16a1	Solute carrier family 16, member 1	NM_012716		–			–	–
Slc16a1	Solute carrier family 16, member 1	AB047324	–			–		–
Spnb3	Beta-spectrin 3	NM_019167	–	–	–	–		
Tubb5	Tubulin, beta 5	AB011679	–	–	=	–	=	=
Ugcg	UDP-glucose:ceramide glycosyltransferase	AF047707		–			–	–
	Similar to HSPC288	BG666041		–			–	–
	Similar to complement C5 precursor (hemolytic complement)	AW917065		–	=		–	–
Mostly synergism								
Pfkfb1	6-Phosphofructo-2-kinase/ fructose-2,6-biphosphatase 1	NM_012621	+	+		+		
Rrad	Ras related associated with diabetes	U12187		+	+	+	=	=

Additivity is shown by '=', synergism by '+' and antagonism by '−'. No symbol indicates that no expected value could be calculated (see Materials and Methods). In order to judge the type of interactions on gene expression, the effects caused by the two PAHs is added (expected change) and compared to the expression of that gene in response to mixture treatment (observed change). Similar expression results in additive response (<1 SD), a higher than expected change results in a synergistic response (at least 1 SD) and a lower than expected change in an antagonistic response (at least 1 SD). A more detailed explanation is provided in our previous study (10).

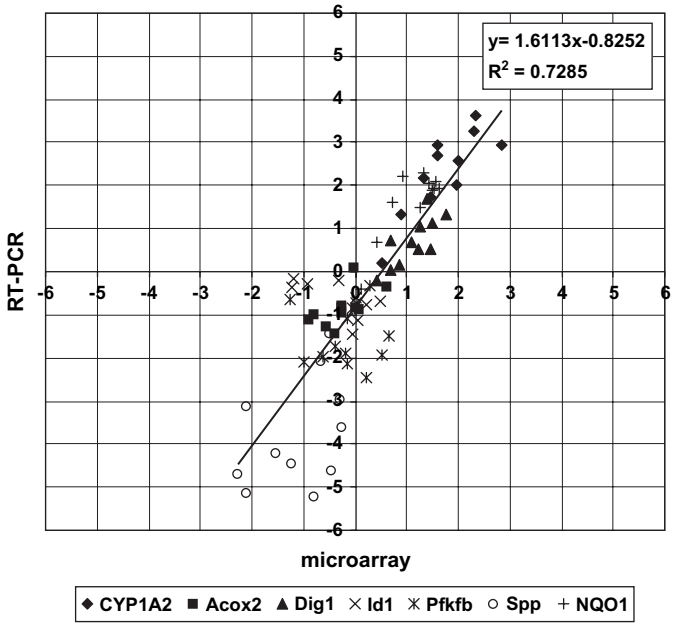

Fig. 4. Gene expression differences in rat liver slices exposed to PAH mixtures as determined by microarray analysis (*x* axis) or RT-PCR analysis (*y* axis). The correlation coefficient (R^2) and the line equation are indicated in the graph.

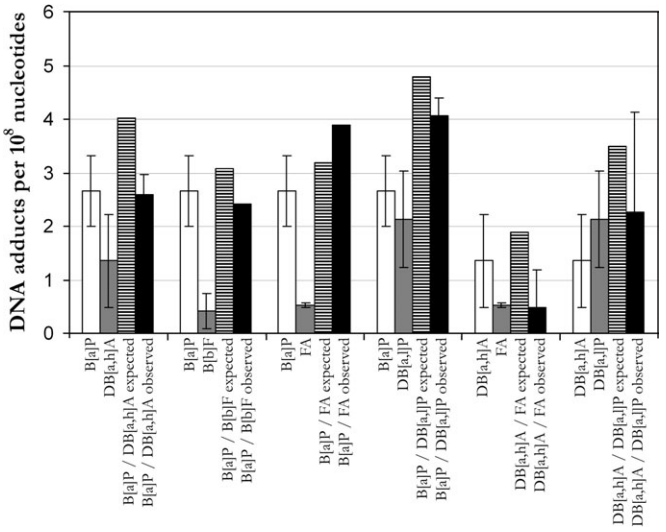

Fig. 5. DNA adduct formation in liver slices exposed to individual PAHs or their binary mixtures as measured by ^{32}P-post-labelling (detection limit one adduct per 10^8 nts). Mean adduct levels corrected for DMSO signals and their SDs are shown. ($n = 2$, except for mixtures of B[*a*]P–B[*b*]F and B[*a*]P–FA)

CYP1A1 expression by RT-PCR analysis. However, we did observe an antagonistic effect on the expression of *CYP1A2* both on the data from microarrays and RT-PCR.

Assessing the additivity of differential expression of single genes

In general, when analyzing for consistent interaction patterns for individual genes, the expression of most genes shows antagonism, which is in agreement with the observed global gene expression differences. For 21 genes, expression differences were consistently lower than expected, and many of these are involved in carcinogenesis. For example, ACOX2 and EHHADH play a role in carcinogenesis by affecting fatty acid metabolic pathways (25). This might be related to GILZ, which is involved in adipocyte differentiation (26) and ME1, which is involved in fatty acid biosynthesis. Furthermore, LPB and PTPN16 both play a role in inflammation and could be important in carcinogenesis (27,28). *CYP1A2* showed lower expression than expected and is involved in the metabolism of PAHs. Furthermore, the expression of *CLDN7* is elevated in neoplasias (29), DIG1 is involved in invasive growth (30) and, moreover, was also induced in our previous study (17). Finally, HSD11B1 contributes to the clearance of apoptotic cells (31), MADH3 plays a role in inhibition of cell cycle progression (32) and ID1 is involved in the early stages of hepatocarcinogenesis (33). So, most genes showing antagonism are involved in carcinogenesis, and this lower expression would suggest a lower carcinogenic potency of PAH mixtures than expected on the basis of the individual compounds.

Ten of the affected genes showed consistent additive response after exposure to PAH mixtures. Their corresponding proteins are involved in several functions, the most relevant being CDH17, which is up-regulated in gastric carcinomas (34), SPP1, which is de-regulated in hepatocarcinogenesis (35) and NQO1 that is controlled by the Ah receptor (36) and is involved in xenobiotic metabolism such as the generation of quinine metabolites of PAH. Increased formation of quinine metabolites may result in less epoxide metabolites and thus the antagonistic effect on DNA adduct formation.

Furthermore, for two genes, the expression differences were higher than expected, indicating synergism. These two genes are *PFKFB1*, which is involved in neoplastic transformation (37) and *RRAD*, which is up-regulated in neoplasia (38). RT-PCR analysis showed that the PAH mixtures have a synergistic effect on the expression of *CYP1A1*. This would imply increased metabolism of PAHs, but contrasts with our findings on DNA adduct formation. The relevance of *CYP1A1* for PAH metabolism is, however, questioned, as in knockout models the adduct levels even increase (39,40). By a higher expression than expected, these genes imply a higher carcinogenic potency of the PAHs mixtures than expected.

DNA adduct levels

DNA adduct levels are relatively low in liver slices exposed to PAHs, thereby approaching the limit of detection. Despite this, the data generally show an antagonistic effect on DNA adduct formation, in concordance with the effects on gene expression but in contrast to our findings in HepG2 cells (10). In those cells, however, the DNA adduct levels are 10- to 100-fold higher than in liver slices. Furthermore, the differences in cell type (liver slices versus a cell line), annual species and PAH concentrations might explain this difference in interaction on adduct levels. Different interaction on biotransformation enzymes, and thereby altered metabolism of mixtures, may explain the difference in effects in HepG2 cells and liver slices. This is shown by the antagonistic effect on the expression of *CYP1A2* in liver slices, whereas the effects on *CYP1A2* in HepG2 cells are synergistic and suggest that CYP1A2 is important for metabolic activation of PAHs. The latter agrees with other studies, showing the induction of *CYP1A2* by PAHs (41,42). Other studies have also shown an antagonistic effect of PAH mixtures on DNA adduct formation in MCF7 cells (43).

The effects of binary PAH mixtures on both gene expression and DNA adduct formation show generally antagonism. This response agrees with the antagonistic effects on gene expression modulation in HepG2 cells (10) and was also observed in previous studies with other (carcinogenic) end points (44,45).

Conclusion

This study addressed the effects of binary PAH mixtures on gene expression profiles and DNA adduct formation in rat liver slices and showed that in most cases the PAHs interact antagonistically. Similarly, at the level of individual genes, mostly antagonism was observed, with some genes showing additivity and only few genes showing synergism. These interactions are partly different to those we observed in HepG2 cells. Effects on genes involved in metabolism show primarily antagonism or additivity, and in relation to DNA adduct formation, these findings suggest diminished metabolic activation by mixtures comprising a cytochrome p450 (CYP) inducer with a non-CYP inducer than additivity would suggest. Our observations therefore suggest a lower carcinogenic potency of PAH mixtures than estimated based on the additivity of the individual constituents.

Supplementary Material

The gene expression data discussed in this publication are accessible at http://www.grat.unimaas.nl/MAdata-Staaletal-Mutagen2008.htm.

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