

Synergistic and Antagonistic Interactions of Binary Mixtures of Polycyclic Aromatic Hydrocarbons in the Upregulation of CYP1 Activity and mRNA Levels in Precision-Cut Rat Liver Slices

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ABSTRACT: The current studies investigate whether synergistic or antagonistic interactions in the upregulation of CYP1 activity occur in binary mixtures of polycyclic aromatic hydrocarbons (PAHs) involving benzo[a]pyrene and five other structurally diverse PAHs of varying carcinogenic activity. Precision-cut rat liver slices were incubated with benzo[a]pyrene alone or in combination with a range of concentrations of a second PAH, and ethoxyresorufin O-deethylase, CYP1A1 and CYP1B1 mRNA levels determined. Concurrent incubation of benzo[a]pyrene with either dibenzo[a,h]anthracene or fluoranthene in liver slices led to a synergistic interaction, at least at low concentrations, in that ethoxyresorufin O-deethylase activity was statistically higher than the added effects when the slices were incubated with the individual compounds. In contrast, benzo[b]fluoranthene and, at high doses only, dibenzo[a,l]pyrene gave rise to antagonism, whereas 1-methylphenanthrene had no effect at all concentrations studied. When CYP1A1 mRNA levels were monitored, benzo[b]fluoranthene gave rise to an antagonistic response when incubated with benzo[a]pyrene, whereas all other compounds displayed synergism, with 1-methylphenanthrene being the least effective. A similar picture emerged when CYP1B1 mRNA levels were determined, though the effects were less pronounced. In conclusion, it has been demonstrated that the benzo[a]pyrene-mediated upregulation of CYP1, at the mRNA and activity levels, is synergistically and antagonistically modulated by other PAHs. © 2016 Wiley Periodicals, Inc. *Environ Toxicol* 32: 764–775, 2017.

Keywords: polycyclic aromatic hydrocarbons; cytochrome P450; interactions; risk assessment; precision-cut tissue slices

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) comprise a major and ubiquitous class of chemical carcinogens to which humans are continuously exposed. The major sources of

human exposure for the non-smoker are food, either as a result of atmospheric deposition from air or generation by cooking procedures such as barbecuing, and to a lesser extent air (Phillips, 1999); however, exposure may be much higher in individuals exposed to these compounds occupationally, for example those working in the coke industry and in steel plants (Seidel, 2004).

PAHs are indirect carcinogens in that they require metabolic activation to genotoxic intermediates that are responsible for their carcinogenicity. Although at least three distinct metabolic pathways may generate such genotoxic intermediates,

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the principal ultimate carcinogens appear to be dihydrodiol epoxides (Luch and Baird, 2004). An initial oxidation leads to the formation of primary epoxides which are then converted to dihydrodiols by epoxide hydrolase; a second oxidation produces the dihydrodiol epoxides. The CYP1 family of cytochromes P450, in particular CYP1A1 and CYP1B1, is the dominant catalyst of both oxidation reactions (Shimada and Fujii-Kuriyama, 2004; Ioannides and Lewis, 2004), but CYP3A4 can also catalyse the second oxidation (Shimada et al., 1989).

Since the principal source of PAHs is the incomplete combustion of organic material, humans are exposed to complex mixtures of undefined composition rather than to individual compounds; carcinogenicity studies are, however, usually conducted using single pure compounds. Since PAHs display widely different carcinogenic activities, ranging from the highly carcinogenic to the non-carcinogenic, risk assessment of mixtures presents enormous difficulties as it is conceivable that a non-carcinogenic component may act as a co-carcinogen by modulating the activity of a carcinogen present in the same mixture. The default assumption used in the safety evaluation of PAH mixtures is that interactions between individual PAHs do not take place, and that carcinogenic risk can be evaluated by summation of the carcinogenic potencies of individual components or by using benzo[a]pyrene as the surrogate PAH (Relative Potency Factor, RLF) (Bostrom et al., 2002; Pufulete et al., 2004). However, the RLF approach in determining the carcinogenic risk from exposure to PAH mixtures may not reflect true carcinogenic potential, at least as far as skin cancer is concerned following topical exposure (Siddens et al., 2012). Bearing in mind that all PAHs share the same characteristics, in that they are all essentially planar compounds metabolized by the same cytochrome P450 enzymes through similar metabolic routes, it would not be unreasonable to anticipate interactions between components of a mixture. In fact, it has been documented that the cytochrome P450-catalyzed metabolism of an individual PAH may be impaired by the presence of other PAHs (Shimada and Guengerich, 2006; Shimada et al., 2007); thus the carcinogenic potential of a PAH mixture may not be reflected by the carcinogenic activity of its individual components. Indeed, studies conducted largely in the

mouse skin model have shown that one PAH can markedly influence the carcinogenic activity of another, both synergistically and antagonistically, and such interactions are not necessarily the consequence of competitive inhibition of metabolism (Slaga et al., 1978; DiGiovanni et al., 1982). For example, the tumorigenicity of benzo[a]pyrene in the mouse skin model was elevated by its isomer benzo[e]pyrene whereas it was suppressed by dibenz[a,c]anthracene (DiGiovanni et al., 1982). Similarly, a PAH-containing diesel exhaust matter that was non-tumorigenic in the mouse skin model interacted with other PAHs both synergistically and antagonistically (Courter et al., 2008). As similar interactions were noted in the extent of DNA adduct formation and bacterial mutagenicity, it is evident that PAH interactions may occur at the initiation stage of carcinogenesis encompassing both the bioactivation of a carcinogen to its reactive intermediates and their subsequent binding to DNA (Smolarek et al., 1987; Rice et al., 1988; Hughes and Phillips, 1990; Lau and Baird, 1992; Cherng et al., 2001; Marston et al., 2001; Staal et al., 2007; Tarantini et al., 2011).

An alternative site for PAH interactions is during binding to the aryl hydrocarbon (Ah) receptor, a transcription factor localized in the cytosol (Kohle and Bock, 2009). There is increasing evidence that this receptor plays a pivotal role in chemical carcinogenesis (Safe et al., 2013; Feng et al., 2013). Indeed, Ah receptor null mice were refractive to the carcinogenicity of two of the most potent polycyclic aromatic hydrocarbons, namely benzo[a]pyrene and dibenzo[a,l]pyrene (Shimizu et al., 2000; Nakatsuru et al., 2004). Moreover, experimental studies established that Arnt (aryl hydrocarbon receptor nuclear translocator), a protein that dimerizes with the Ah receptor prior to its interaction with DNA-response elements, is also required for tumour initiation by benzo[a]pyrene (Shi et al., 2009).

In the present study we evaluated the potential of five, structurally diverse, PAHs to interact with benzo[a]pyrene, the prototypic and most extensively studied PAH, using the upregulation of CYP1 as biomarker. The studies were performed using precision-cut rat liver slices, a convenient and far more preferable *in vitro* system than cancer cell lines and hepatocytes as it retains tissue architecture, cell composition and communication; the precision-cut tissue system, moreover, can be extended to human tissues (Ioannides, 2013). We have already demonstrated that this system can be used to assess the potential of PAHs, in both liver and lung, to up-regulate CYP1 (Pushparajah et al., 2007a) as well as other enzyme systems contributing to PAH metabolism (Pushparajah et al., 2007b).

MATERIALS AND METHODS

Materials

Dibenzo[a,l]pyrene (D(a,l)P) 1-methylphenanthrene (1-MP) (LGC Promochem, Middlesex, UK), rat genomic DNA

Abbreviations

1-MP	1-methylphenanthrene
Ah	aryl hydrocarbon
Arnt	aryl hydrocarbon nuclear translocator
B(a)P	benzo[a]pyrene
B(b)F	benzo[b]fluoranthene
D(a,l)P	dibenzo[a,l]pyrene
D(a,h)A	dibenzo[a,h]anthracene
F	fluoranthene
PAH	polycyclic aromatic hydrocarbon

(Novagen, Wisconsin, USA), RNase-free DNase, cell culture lysis reagent, luciferase assay reagent (Promega, Wisconsin, USA), NADPH, benzo[a]pyrene (B(a)P), fluoranthene (F), benzo[b]fluoranthene (B(b)F), dibenzo[a,h]anthracene (D(a,h)A), ethoxyresorufin and resorufin (Sigma Co. Ltd., Poole, Dorset, UK), Qiagen RNeasy Mini kits (Crawley, West Sussex, UK), AbsoluteTM QPCR Mix (Abgene, Epsom, Surrey, UK), and Earle's balanced salt solution (EBSS), foetal calf serum, gentamycin, hexamers, Superscript II and RPMI 1640 with L-glutamine culture medium (Invitrogen, Paisley, Scotland) were all purchased. Twelve-well plates were obtained from Bibby Sterilin (Helena Biosciences, Sunderland, UK).

Preparation and Incubation of Precision-Cut Tissue Slices

Male Wistar albino rats (200g) were obtained from B&K Universal Ltd (Hull, East Yorkshire, UK). The animals were housed at $22 \pm 2^\circ\text{C}$, 30–40% relative humidity in an alternating 12-hr light:dark cycle with light onset at 07.00 hr. Rats were killed by cervical dislocation, and liver was immediately excised.

Liver slices (250 μm) were immediately prepared from 8 mm cylindrical cores using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munsford, AL, USA) as previously described (Hashemi et al., 1999). The multiwell plate procedure, using 12-well culture plates, was used to culture the slices. One slice was placed in each well, in 1.5 ml of culture medium. Slices were incubated under sterile conditions on a reciprocating plate shaker housed in a humidified incubator, at a temperature of 37°C and under an atmosphere of 95% air/5% CO_2 . The slices were initially pre-incubated for 30 min to slough off any dead cells because of slicing. Three different slice pools, comprising 10 slices, were used per time point.

Enzyme Assays

Following incubation with the relevant PAHs, dissolved in DMSO (final concentration 0.5% v/v), slices were removed from the medium, homogenised, and microsomal fractions were prepared by differential centrifugation. The *O*-deethylation of ethoxyresorufin (Burke and Mayer, 1974) and protein concentration (Bradford, 1976) were determined in isolated microsomes.

Transcript Level Measurement

Two slices were used for total RNA extraction, and for each sample triplicate determinations were carried out. RNA was extracted using the Qiagen RNeasy Mini kit and was quantified using a Nanodrop spectrophotometer. Total RNA was treated with RNase-free DNase to remove genomic contamination. Reverse transcription was primed with random hexamers and carried out by Superscript II according to the

manufacturer's instructions. To ensure that DNase-treated samples were free from genomic contamination, an RT- control was carried out for every RNA sample. cDNA generated from 50 ng was amplified using AbsoluteTM QPCR Mix with 400 nM primers and 100 nM fluorogenic probe in a total reaction volume of 25 μl . Q-PCR reactions were run on the ABI7000 SDS instrument (Applied Biosystems, Warrington, UK) and quantitation was carried out using the ABI proprietary software against a standard curve generated from rat genomic DNA.

For the quantitative reverse transcription-polymerase chain reaction, the primer and TAMRA/FAM dual labelled probe were designed using the Primer Express software (Applied Biosystems, CA, USA) to amplify sequences within a single exon, so that genomic DNA could be used as a standard (Pushparajah et al., 2007b), and were purchased from MWG (Ebersberg), Germany.

Statistical Evaluation

Statistical evaluation was carried out using the Minitab package, version.14.0 (MINITAB Inc., State. College, PA). Differences among mean values of sample groups were assessed by using the 'least significant difference' (LSD) test.

The equation for estimating the LSD is:

$$\text{LSD} = t_{(\text{df})} \times \sqrt{\frac{2s^2}{n}}$$

where $t_{(\text{df})}$ is the value of the t distribution for the degrees of freedom of MS. The MS, mean squares for each source is simply the sum of squares divided by the degrees of freedom, s^2 is the estimate of the pooled error MS from the analysis of variance and n is the sample size of the means. Enzyme activities are presented as percentage (%) change over control (slices incubated with DMSO, the vehicle, alone): 'added' % change is the sum of % change caused by benzo(a)pyrene and the second PAH when slices were incubated separately with either of these PAHs, whilst 'combined' refers to the % change caused by the change in activity after incubation of the liver slices with benzo(a)pyrene in combination with an additional PAH. The possibility of an interaction caused by the binary mixture can then be obtained using the LSD value at each confidence level. If the difference in the means of 'added' and 'combined' % change is greater than the LSD value, it can be inferred that the means are statistically different, and that the difference in activity caused by a mixture of the two PAHs is significantly different from an additive response, and due to an interaction effect. LSD values were calculated for $P < 0.05$, < 0.01 and < 0.001 . Expression of mRNA levels after incubation of rat liver slices with a combination of benzo(a)pyrene and another PAH were calculated as fold-change values. These have been represented as 'alteration from additivity', i.e. the difference between the sum of 'added' and 'combined'

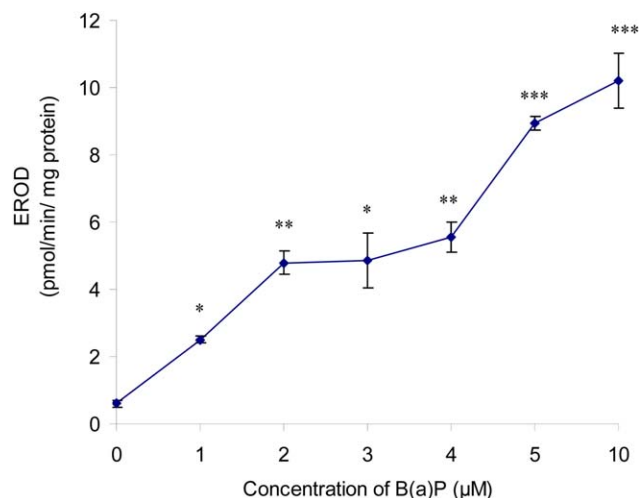


Fig. 1. Induction of ethoxyresorufin *O*-deethylase (EROD) activity by benzo[a]pyrene [B(a)P] in precision-cut rat liver slices. Rat liver slices were incubated with a range of benzo[a]pyrene concentrations (0–10 μM) for 24 hours, and EROD activity was determined in the microsomal fraction. Results are presented as mean ± SD where $n =$ three slices, from five pooled livers. *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$. [Color figure can be viewed at wileyonlinelibrary.com]

fold-change, and statistical testing was carried out once again utilizing the least significant difference test.

RESULTS

In an initial study, B(a)P caused a concentration-dependent increase in ethoxyresorufin *O*-deethylase (EROD) activity following incubation with liver slices for 24 hours (Fig. 1). All subsequent studies employed concentrations of B(a)P (0.5 and 1 μM) that were far below the concentration eliciting maximum induction.

When liver slices were incubated with a mixture of B(a)P and D(a,h)A, a synergistic interaction was noted in that the rise in EROD activity was statistically higher than the added effects observed when the slices were incubated with either of the chemicals alone, at all concentrations studied (Fig. 2). More pronounced synergism was evident when F was co-incubated with B(a)P, but in this case it was confined to the lower F concentrations (Fig. 3); no such effect was seen at the 10 μM or higher concentrations, and indeed antagonism was evident at the highest concentrations studied. In contrast, a marked antagonistic interaction was evident, at all concentrations studied, when a mixture of B(a)P and B(b)F was incubated with rat liver slices (Fig. 4). When rat liver slices were incubated with a mixture of B(a)P and D(a,l)P, at the lower concentration of the former, no statistically significant interaction was observed (Fig. 5); at the higher B(a)P concentration, however, and at the higher concentrations of D(a,l)P a significant antagonistic interaction was seen.

Finally, when the mixture of B(a)P and 1-MP was investigated, no interaction was seen at all concentrations studied (Fig. 6).

In additional studies, rat liver slices were incubated with the same PAH binary mixtures in order to investigate the effect of the modulating PAH on the B(a)P-mediated up-regulation of CYP1A1 mRNA levels (Fig. 7). When liver slices were exposed to B(a)P and D(a,h)A, at concentrations of the latter less than 1 μM, a statistically significant synergistic increase in CYP1A1 mRNA levels was observed, i.e. the rise in mRNA levels in the liver slices exposed to the combination of PAHs was higher than the sum of the levels when slices were incubated with the individual PAHs [Fig. 7(A)], but the effect was not visible at the higher concentrations of D(a,h)A. A similarly marked synergistic effect was evident when B(a)P was incubated concurrently with either D(a,l)P or F, at all concentrations studied [Figs. 7(C,D)]. A much more modest, but statistically significant, synergistic

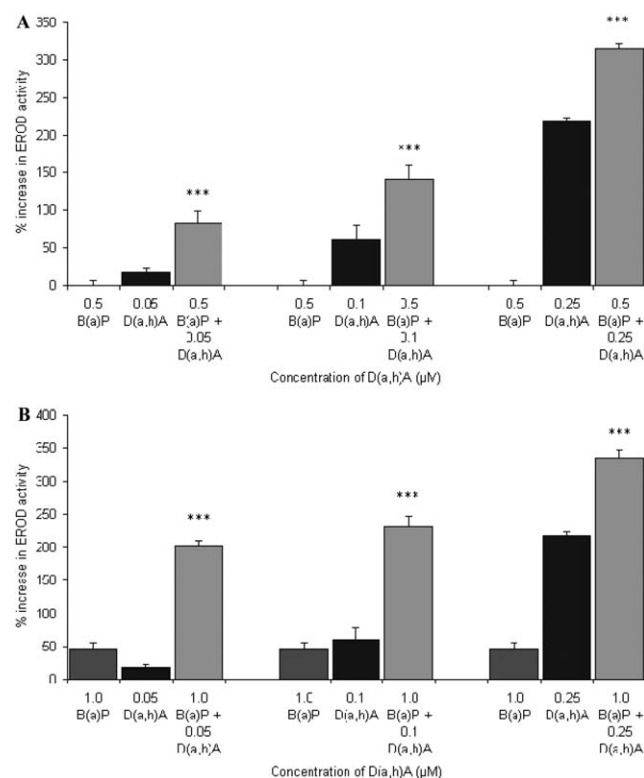


Fig. 2. Effect of dibenzo(a,h)anthracene [D(a,h)A] on B(a)P-mediated EROD activity precision-cut rat liver slices. Rat hepatic slices were incubated with either (a) 0.5 or (b) 1.0 μM B(a)P and a range of D(a,h)A concentrations (0–0.25 μM) for 24 hours, and EROD activity was determined in the microsomal fraction. The figure represents the activities as %-change where activities of control slices (incubated with the vehicle alone) correspond to 100%. Results are presented as mean ± SD where $n =$ three slices, from five pooled livers. LSD value = 11.69 at $P < 0.001$ (***), when compared with the sum of %-change in activities of slices incubated with the corresponding concentration of B(a)P and PAH alone.

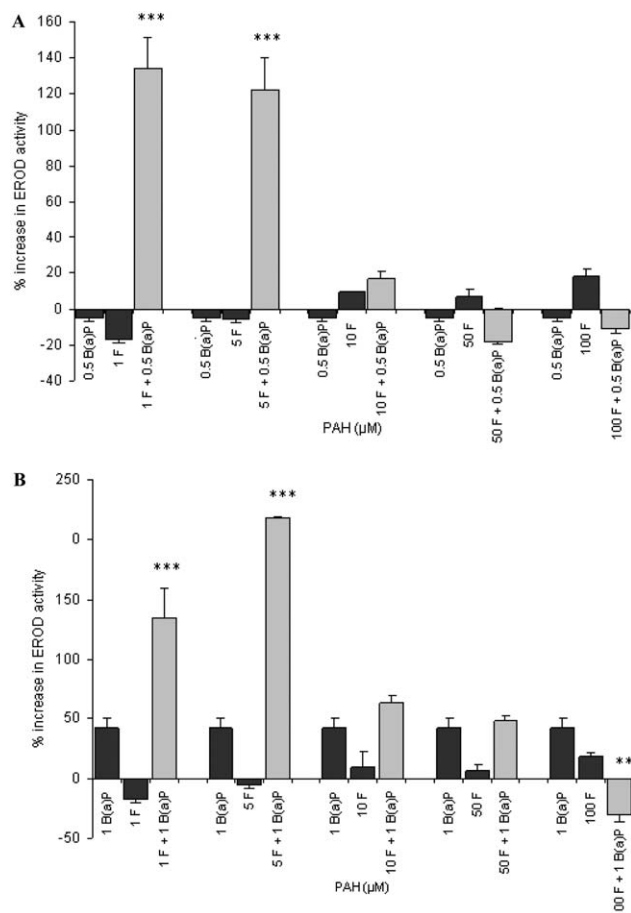


Fig. 3. Effect of fluoranthene [F] on B(a)P-mediated EROD activity in precision-cut rat liver slices. Rat hepatic slices were incubated with either (a) 0.5 or (b) 1.0 μ M B(a)P and a range of F concentrations (0–100 μ M) for 24 hours, and EROD activity was determined in the microsomal fraction. The figure represents the activities as %-change where activities of control slices (incubated with the vehicle alone) correspond to 100%. Results are presented as mean \pm SD where $n=3$ slices, from five pooled livers. LSD values = 0.79 at $P < 0.01$ (**) and 0.95 at $P < 0.001$ (***), when compared with the sum of %-change in activities of slices incubated with the corresponding concentration of B(a)P and PAH alone.

effect was noted when 1-MP served as the modulating hydrocarbon [Fig. 7(B)]. Finally, co-incubation of B(a)P with B(b)F, at all concentrations, resulted in a marked antagonistic effect, i.e. CYP1A1 mRNA levels in slices exposed to the combination of PAHs was much lower than the sum of the mRNA levels when slices were incubated with the individual PAHs [Fig. 7(E)].

A very similar picture emerged when CYP1B1 mRNA, instead of CYP1A1 mRNA, levels were monitored, although the effects were less pronounced (Fig. 8). D(a,h), at low concentrations, F, D(a,l)P and 1-MP displayed a statistically significant synergistic response when co-incubated with B(a)P,

whereas an antagonistic response was seen in the case of B(b)F (Fig. 8).

DISCUSSION

The precision-cut liver slice *in vitro* system was used to evaluate the potential of five structurally diverse PAHs to interact with B(a)P. Rat liver slices were incubated with B(a)P alone or in the presence of modulating PAHs for 24 hours, this being the incubation time required to achieve

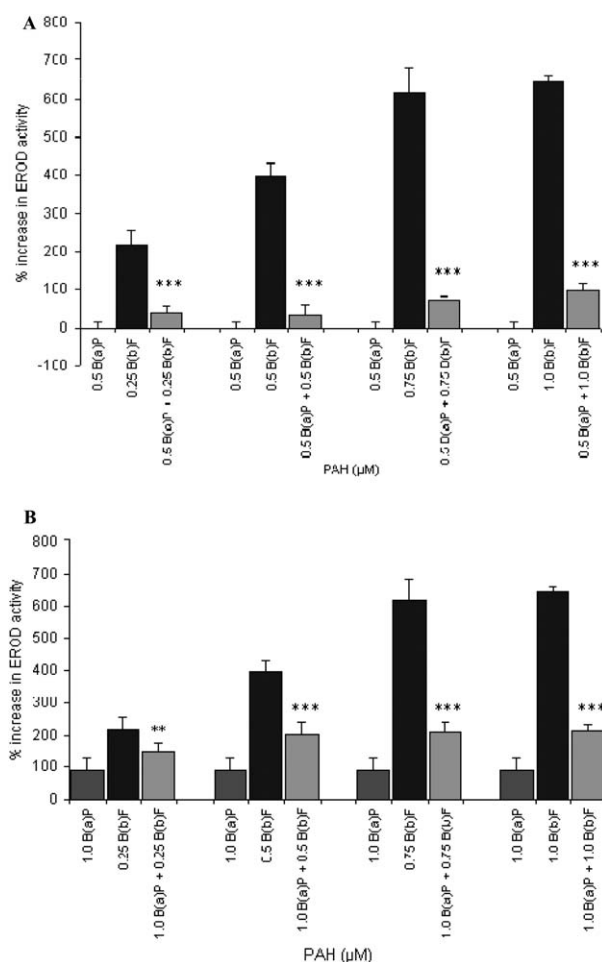


Fig. 4. Effect of benzo(b)fluoranthene [B(b)F] on B(a)P-mediated EROD activity in precision-cut rat liver slices. Rat hepatic slices were incubated with either (a) 0.5 or (b) 1.0 μ M B(a)P and a range of B(b)F concentrations (0 – 1 μ M) for 24 hours, and EROD activity was determined in the microsomal fraction. The figure represents the activities as %-change where activities of control slices (incubated with the vehicle alone) correspond to 100%. Results are presented as mean \pm SD where $n=$ three slices, from five pooled livers. LSD values = 17.68 at $P < 0.01$ (**) and 23.22 at $P < 0.001$ (***), when compared with the sum of %-change in activities of slices incubated with the corresponding concentration of B(a)P and PAH alone

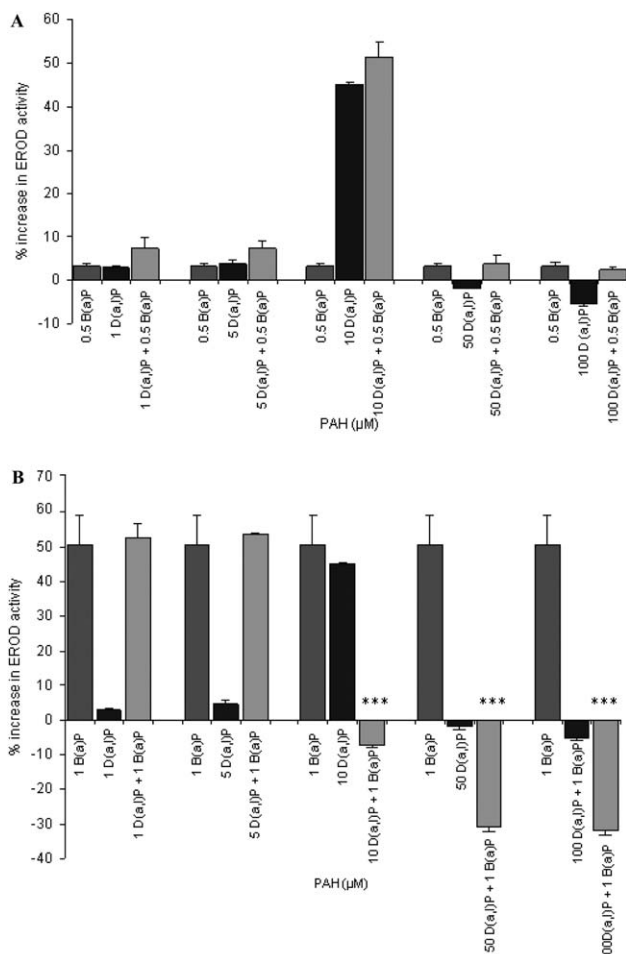


Fig. 5. Effect of dibenzo(a,l)pyrene [D(a,l)P] on B(a)P-mediated EROD activity in precision-cut rat liver slices. Rat hepatic slices were incubated with either (a) 0.5 or (b) 1.0 μM B(a)P and a range of D(a,l)P concentrations (0 – 100 μM) for 24 hours, and EROD activity was determined in the microsomal fraction. The figure represents the activities as %-change where activities of control slices (incubated with the vehicle alone) correspond to 100%. Results are presented as mean \pm SD where n = three slices, from five pooled livers. LSD value = 2.45 at $P < 0.001$ (***), when compared with the sum of %-change in activities of slices incubated with the corresponding concentration of B(a)P and PAH alone.

maximum induction of EROD, a reaction selectively catalysed by CYP1A1 (Namkung et al., 1988). Exposure of precision-cut rat liver slices to B(a)P (10 μM) led to a rise in EROD activity and CYP1A apoprotein levels that were most pronounced at 24-hours; a rise in CYP1A1 mRNA levels was evident as early as 4-hours post-incubation, but no change in EROD activity or CYP1A apoprotein levels were noted at this time point (Pushparajah et al., 2007b). Similar observations were made using non-PAH CYP1A inducers such as the glucosinolates glucoerucin and glucoraphanin

(Abdull Razis et al., 2012). In an initial study, B(a)P was shown to cause a concentration-dependent rise in EROD activity, and two low concentrations, 0.5 and 1.0 μM , were utilized in subsequent studies as they do not cause near maximal induction of this enzyme. Similarly, the choice of concentrations of the modulating PAHs was chosen based on our previous studies in which the potential of each PAH to elevate EROD activity was evaluated following similar incubations with rat liver slices for 24 hours (Pushparajah et al., 2007a). Consequently, lower concentrations were used in the case of B(b)F and D(a,h)A that are potent EROD inducers, whereas a higher range of concentrations was employed in the case of F, D(a,l)P and 1-MP which are either very weak or non-inducers. Indeed, the current studies confirm our previous findings that B(b)F and D(a,h)A are the most potent inducers of EROD activity in rat slices, whereas 1-MP is completely inactive (Pushparajah et al., 2007a).

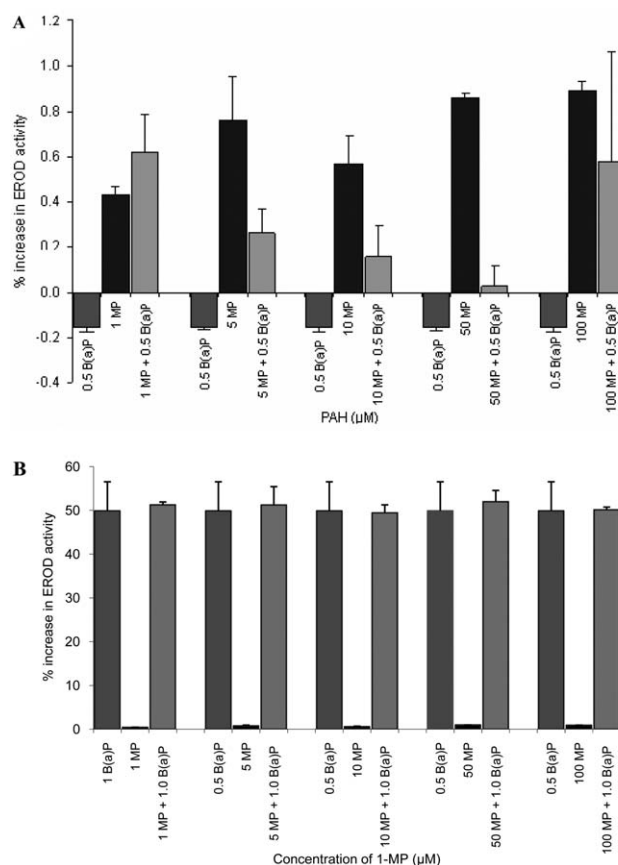


Fig. 6. Effect of 1-methylpyrene [1-MP] on B(a)P-mediated EROD activity in precision-cut rat liver slices. Rat hepatic slices were incubated with either (a) 0.5 or (b) 1.0 μM B(a)P and a range of 1-MP (0 – 100 μM) for 24 hours, and EROD activity was determined in the microsomal fraction. The figure represents the activities as %-change where activities of control slices (incubated with the vehicle alone) correspond to 100%. Results are presented as mean \pm SD where n = three slices, from five pooled livers.

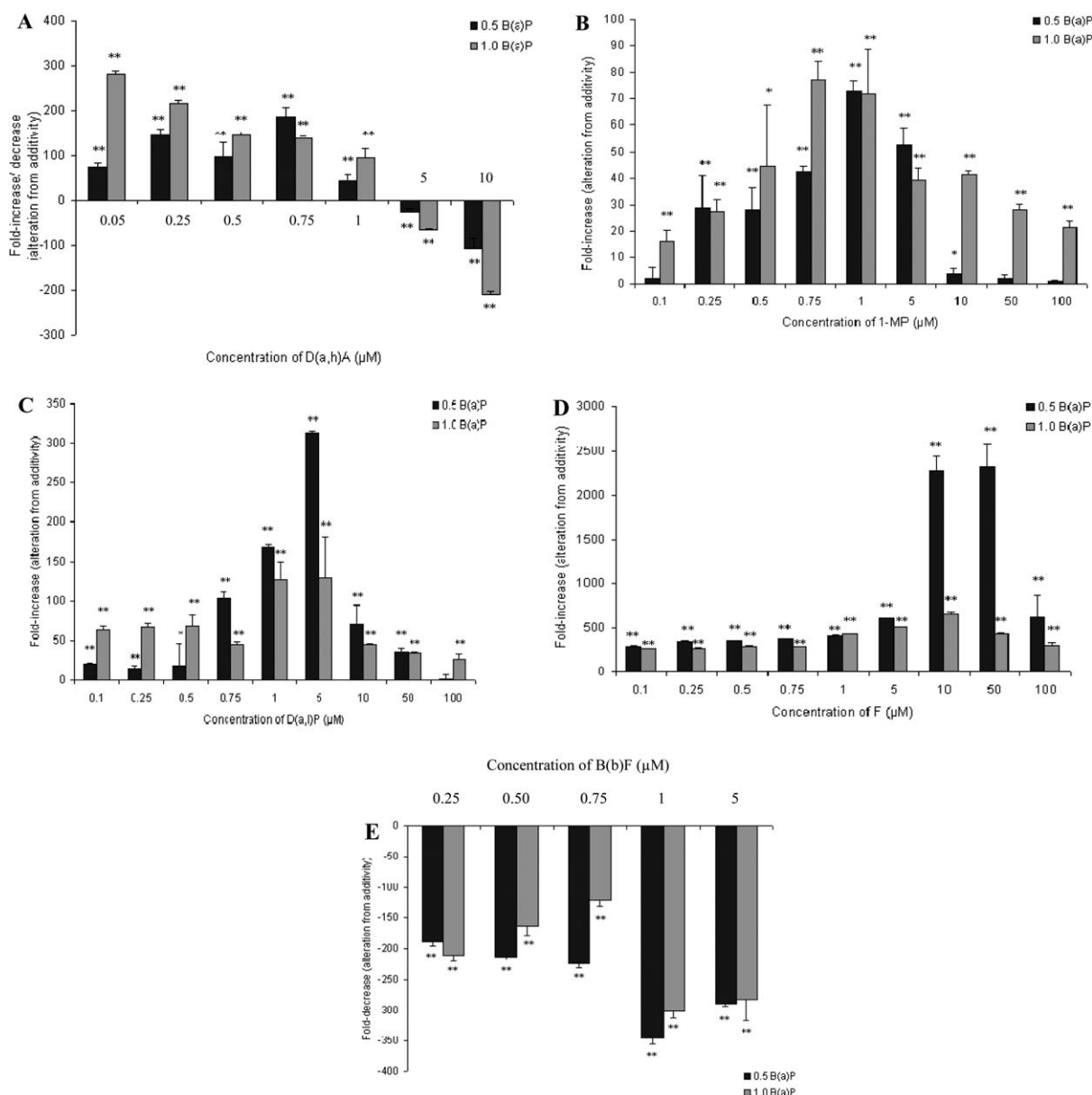


Fig. 7. CYP1A1 mRNA levels following incubation of rat liver slices with B(a)P and other PAHs alone or in combination. Rat hepatic slices were incubated in the presence of either 0.5 or 1.0 μM B(a)P and a range of concentrations of B(b)F (0–5 μM), D(a,h)A (0–10 μM) or F, D(a,l)P and 1-MP (0–100 μM) for 24 hours. Total RNA was extracted from whole liver slices, and levels of CYP1A1 mRNA were determined by quantitative RT-PCR methodology (TaqMan). Alteration from additivity of fold-change in CYP1A1 mRNA levels is shown and results are presented as mean \pm SD where n = four slices, from five pooled livers. LSD values = 57.4 at $P < 0.01$ (**) for D(a,h)A, 46.5 at $P < 0.05$ (*) and 57.6 at $P < 0.01$ (**) for D(a,l)P, 57.1 at $P < 0.01$ (**) for F, 32.7 at $P < 0.05$ (*) and 44.3 at $P < 0.01$ (**) for 1-MP and 46.9 at $P < 0.01$ (**) for B(b)F, when compared with the sum of %-change in activities of slices incubated with the corresponding concentration of B(a)P and the other PAH alone.

The B(a)P-mediated rise in EROD activity was synergistically increased by D(a,h)A and F whereas it was antagonized by B(b)F, and no effect was evident in the case of 1-MP; antagonism was also observed at high concentrations of D(a,l)P, but was confined to the higher of the two B(a)P

concentrations used. Clearly, these studies demonstrate that PAHs in binary mixtures have the potential to interact, both in a synergistic or antagonistic manner. Of relevance is the fact that interactions in the present study occurred primarily at low concentrations, circa 1 μM , of the interacting PAHs.

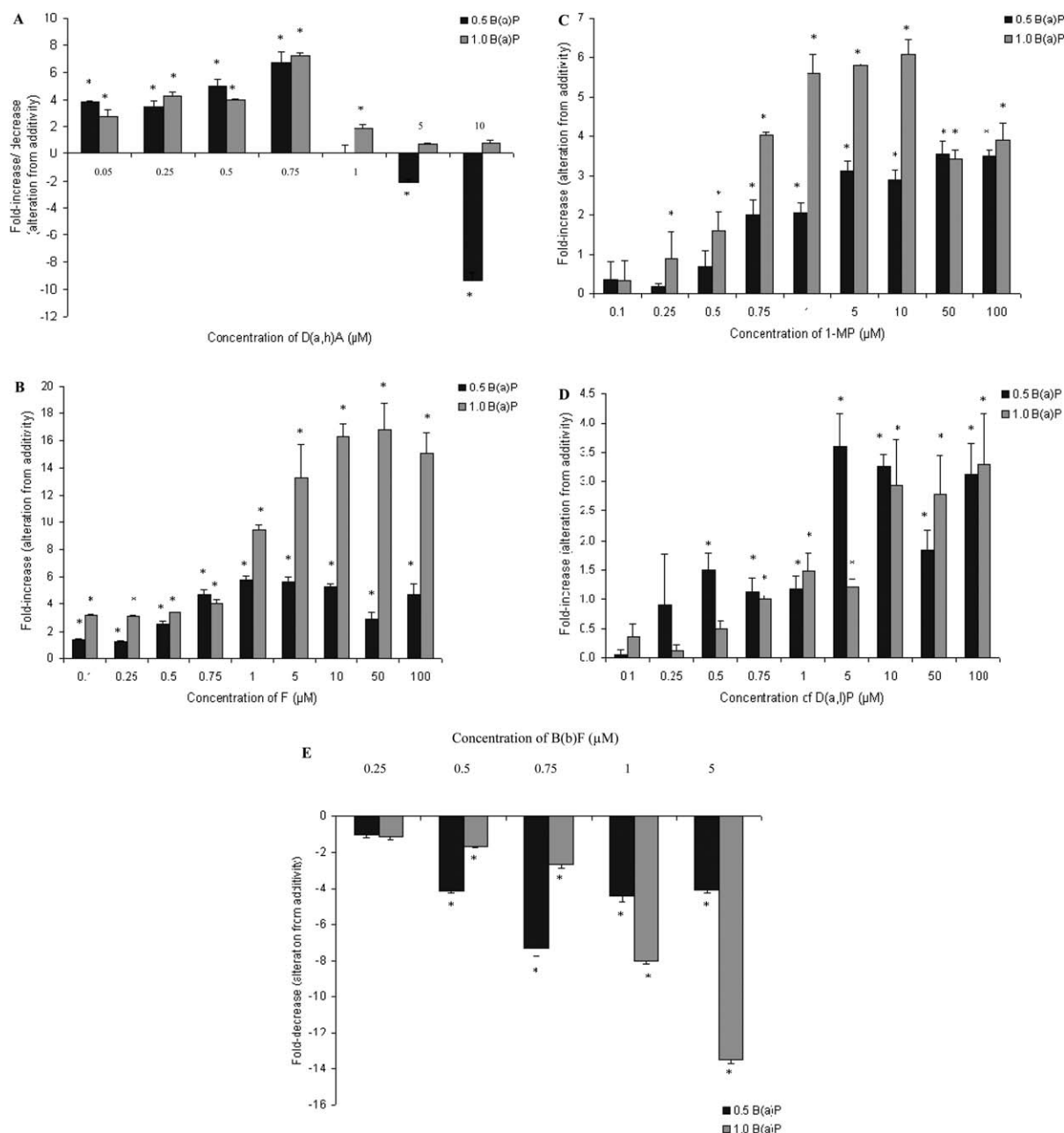


Fig. 8. CYP1B1 mRNA levels following incubation of rat liver slices with B(a)P and other PAHs alone or in combination. Rat hepatic slices were incubated in the presence of either 0.5 or 1.0 μM B(a)P and a range of concentrations of B(b)F (0–5 μM), D(a,h)A (0–10 μM) or F, D(a,l)P and 1-MP (0–100 μM) for 24 hours. Total RNA was extracted from whole liver slices, and levels of CYP1B1 mRNA were determined by quantitative RT-PCR methodology (TaqMan). Alteration from additivity of fold-change in CYP1B1 mRNA levels is shown and results are presented as mean ± SD where n = 4 slices, from five pooled livers. LSD values = 11.3 at $P < 0.05$ (*) for D(a,h)A, 7.3 at $P < 0.05$ (*) for D(a,l)P, 8.1 at $P < 0.05$ (*) for F, 3.0 at $P < 0.01$ (**) for 1-MP and 2.4 at $P < 0.05$ (*) for B(b)F, when compared with the sum of %-change in activities of slices incubated with the corresponding concentration of B(a)P and the other PAH alone.

Such interactions have also been reported in the trout following exposure to individual and mixtures of PAHs and determination of EROD activity (Basu et al., 2001). In rat

hepatoma H4IIE cells, co-incubation of benzo[a]pyrene with fluoranthene, both at 10 μM, resulted in a rise in B(a)P-DNA adducts commensurate with the present findings (Willett

et al., 1998). Similarly, fluoranthene enhanced B[a]P-DNA adduct formation in mouse skin (Rice et al., 1984). Finally, fluoranthene increased the number of tumours when applied with benzo[a]pyrene in mouse skin (Van Duuren and Goldschmidt, 1976).

Fluoranthene, at high doses only (50 and 100 μM), gave rise to antagonism; this is unlikely to be because of loss of liver slice viability as none of these PAHs, at the concentrations studied, caused any toxicity as exemplified by the leakage of lactic acid dehydrogenase (results not shown). It is more likely that at these high concentrations fluoranthene suppresses the CYP1-mediated *O*-deethylation of ethoxyresorufin directly or through mechanism-based inhibition as already established using the human orthologous enzymes (Shimada and Guengerich, 2006; Shimada et al., 2007). In these studies fluoranthene inhibited the CYP1B1-mediated EROD with an IC_{50} 0.013 μM (Shimada and Guengerich, 2006). These data are also consistent with previous reports where high concentrations (20 μM) of fluoranthene inhibited the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)- and benzo[k]fluoranthene-induced EROD activity in rat hepatoma H4IIE cells (Willett et al., 1998). As no changes at the mRNA or protein levels were evident in the same study, it may be cautiously inferred that CYP1 inhibition is the responsible mechanism. It has already been reported that the DNA-binding of PAHs in human MCF-T cells could be suppressed by a mixture of PAHs extracted from coal tar (Standard Reference Material 1597) as a result of inhibition of CYP1A1 and CYP1B1 responsible for their bioactivation (Mahadevan et al., 2007).

To establish whether similar interactions occurred at the level of transcription, liver slices were incubated for 24 hours with B(a)P alone or in combination with the other PAHs, and CYP1A1 mRNA levels monitored. All PAHs caused a synergistic increase in CYP1A1 mRNA levels when co-incubated with B(a)P, at least at the low concentrations; the only exception was B(b)F, which led to an antagonistic response at all concentrations studied. These studies imply that interactions between PAHs may occur at the level of transcription, with non-additive changes in mRNA expression being consistent with the non-additive changes in EROD expression observed. D(a,l)P also interacted synergistically at the CYP1 mRNA level, although weakly compared with the other PAHs, despite the antagonism observed at high concentrations when EROD activity was monitored. A possible explanation is that in this case the antagonistic interaction reflects inhibition of CYP1 activity, rather than a transcriptional event. We note that D(a,l)P displayed no inhibition of EROD activity catalyzed by human CYP1 enzymes at concentrations up to 1 μM (Shimada and Guengerich, 2006), consistent with our observation of no effect until D(a,l)P concentrations exceeded 10 μM . Finally, 1-MP also synergistically increased CYP1A1 mRNA levels when co-incubated with B(a)P in liver slices, but the effect was much less pronounced compared with the other PAHs stud-

ied, and was not reflected in concurrent changes in EROD activity. It is pertinent to point out that the existence of threshold levels has been proposed that must be exceeded in order for mRNA to be translated, hence activation of the gene at the mRNA level does not always lead to higher protein levels, and concurs with the data presented herein (Greenbaum et al., 2003).

As no validated chemical probe selective for CYP1B1 is currently available, no studies were undertaken at the activity level. Although CYP1B1 apoprotein can be detected in rat liver, albeit at much lower levels compared with lung (Pushparajah et al., 2007a), this was not possible in liver slices following a 24-hour incubation, presumably because of the decline in cytochrome P450 expression that occurs during the incubation of liver slices (Hashemi et al., 2000). Consequently, studies were confined to the mRNA level. A similar picture emerged as in the case of CYP1A1, in that the B(a)P-mediated elevation of CYP1B1 mRNA levels was suppressed by B(b)F but synergistically elevated with the other PAHs studied. As noted in our previous studies in rat liver, CYP1B1 mRNA levels were lower and less inducible by PAHs compared with CYP1A1 (Pushparajah et al., 2007a).

As the induction of the CYP1 family of enzymes is regulated by the Ah receptor, the possibility arises that these interactions occur during the binding and/or activation of this receptor to DNA ligand-binding form; this receptor is important in the bioactivation of PAHs although Ah-independent pathway(s) appear also to exist (Sagredo et al., 2009). The fact that these interactions were also evident at the CYP1A1/IB1 mRNA level concurs with an involvement of the Ah receptor, and synergistic interactions of PAH mixtures through Ah activation have been reported previously (Chaloupka et al., 1993; Cherng et al., 2001). It has already been reported that benzo[*g,h,i*]perylene increased the B[a]P-mediated CYP1A1 transcription in HepG2 cells as well as B[a]P DNA adducts (Cherng et al., 2001). Similarly, some aromatic amines were shown to interact synergistically with B(a)P in the transcription of Ah receptor-dependent genes (Borza et al., 2008). It is worthwhile to point out that 1-MP, which failed to interact with B(a)P, is not a ligand for this receptor, whereas B(b)F, the only compound that antagonized B(a)P in the present study, is also the only PAH that has higher affinity than B(a)P for this receptor (Pushparajah et al., 2007a). However, for firm conclusions to be drawn as to the relationship between Ah agonist activity and the nature of the interaction, additional studies are necessary employing a larger number of compounds and preferably human precision-cut liver slices. Interaction studies utilising binary mixtures of B(a)P and 7H-dibenzo[*c,g*]carbazole, a heterocyclic aromatic hydrocarbon, revealed the rat and human cells respond differently (Gábelová et al., 2013) indicating that robust risk assessments should be biased toward human-derived data. Moreover, it is logical to envisage that the carcinogenicity of PAHs in mixtures is unlikely to reflect

solely interactions between parent compounds but is likely to also involve their metabolic products. For example, metabolites such as mono- and dihydroxy-metabolites of PAHs may be potent inducers of the CYP1 family, suggesting that they are agonists of the Ah receptor (Spink et al., 2008; Gábelová et al., 2013). Similarly, they can be potent inhibitors of CYP1 activity (Shimada and Guengerich, 2006).

Finally, no apparent relationship is evident between carcinogenic potency of individual PAHs and the nature of the interaction. D(a,h)A, B(b)F and, in particular, D(a,l)P are carcinogenic chemicals; however, the first caused a synergistic interaction with B(a)P whereas the latter two displayed antagonism under the same conditions. Similarly F and 1-MP are considered to be weak or non-carcinogens; the former gave rise to a synergistic interaction whereas the latter had no such effect.

The formation of the dihydrodiol epoxide of B(a)P, the ultimate carcinogen, involves initially an oxidation catalyzed by the cytochrome P450 system to generate primary epoxides, the levels of which are modulated by epoxide hydrolase, which converts the primary epoxides to the dihydrodiol, and glutathione *S*-transferase, which detoxifies both the primary and dihydrodiol epoxides. While both of these systems are inducible by a number of PAHs including B[a]P, it is to a much lesser extent compared with CYP1 (Pushparajah et al., 2008a,b). Consequently, it is likely that the CYP-mediated epoxide formation will exceed its safe removal, thus amplifying intracellular epoxide levels. It is pertinent to point out that no statistically significant interactions were observed at the activity level when the same binary mixtures were incubated with rat liver slices under the same conditions and epoxide hydrolase was monitored with benzo[a]pyrene 4,5-oxide as the substrate, and glutathione *S*-transferase using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole as substrate (results not shown), indicating that interactions are confined to CYP1 expression. This raises the possibility that synergistic interactions at the level of CYP1 induction may be further exacerbated due to a lack of a similar synergistic response in the detoxification systems.

The current studies employing binary mixtures have shown the potential of PAHs, at low concentrations, to interact both in synergistic and antagonistic manner in the upregulation of CYP1; it would be desirable to extend these studies to ascertain whether such interactions lead to concomitant changes in the levels of DNA adducts. Thus this approach employing precision-cut liver slices may prove helpful in assessing the potential of PAH mixtures to elicit DNA damage, although more extensive studies to validate and underpin this procedure would be indispensable. A good correlation appears to exist for PAHs between the level of DNA adducts and carcinogenic potential (Phillips, 2005), and while contradictory observations have also been reported (Siddens et al., 2012), it could be cautiously inferred that interactions will impact on the carcinogenicity

of mixtures. It is imperative that studies are also conducted in precision-cut human liver slices to establish whether the same antagonistic/synergistic interactions occur as seen in the rat; it is pertinent to point out that when human and rat precision-studies were exposed to various PAHs, under identical conditions, the same profile in CYP1A1 upregulation was noted (Pushparajah et al., 2007a). Furthermore, it would be logical to envisage that interactions between PAHs are likely to also occur at post-initiation stages. Indeed in recent studies (Huang et al., 2012), F was shown to increase the B(a)P-induced expression of p53, a tumour suppressor gene.

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