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The relationship between DNA adduct formation by benzo[*a*]pyrene and expression of its activation enzyme cytochrome P450 1A1 in rat

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ABSTRACT

Benzo[*a*]pyrene (BaP) is a human carcinogen requiring metabolic activation prior to reaction with DNA. Cytochrome P450 (CYP) 1A1 is the most important hepatic and intestinal enzyme in both BaP activation and detoxification. CYP1A2 is also capable of oxidizing BaP, but to a lesser extent. The induction of CYP1A1/2 by BaP and/or β -naphthoflavone in liver and small intestine of rats was investigated. Both BaP and β -naphthoflavone induced CYP1A expression and increased enzyme activities in both organs. Moreover, the induction of CYP1A enzyme activities resulted in an increase in formation of BaP-DNA adducts detected by ^{32}P -postlabeling in rat liver and in the distal part of small intestine *in vivo*. The increases in CYP1A enzyme activity were also associated with bioactivation of BaP and elevated BaP-DNA adduct levels in *ex vivo* incubations of microsomes of both organs with DNA and BaP. These findings indicate the stimulation effects of both compounds on BaP-induced carcinogenesis.

Keywords: Benzo[*a*]pyrene; Metabolic Activation and Detoxification; Cytochrome P450 1A1/2; Induction; DNA adducts; ^{32}P -postlabeling

Abbreviations: AHR, aryl hydrocarbon receptor; BaP, benzo[*a*]pyrene; BCIP, 5-bromo-4-chloro-3'-indolyphosphate *p*-toluidine salt; BNF, β -naphthoflavone; BPDE, BaP-7,8-dihydrodiol-9,10-epoxide; CYP, cytochrome P450; dG- N^2 -BPDE, 10-(deoxyguanosin- N^2 -yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; DMSO, dimethyl sulfoxide; EROD, 7-ethoxyresorufin *O*-deethylation; NBT, nitro-blue tetrazolium chloride; mEH, microsomal epoxide hydrolase; MROD, 7-methoxyresorufin *O*-deethylation; PAH, polycyclic aromatic hydrocarbon; RAL, relative adduct labeling; TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate.

1. Introduction

Benzo[*a*]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) and has been classified as human carcinogen (Group 1) by the International Agency for Research on Cancer (IARC, 2010). BaP and other PAHs are produced mainly by incomplete combustion of organic matter and are ubiquitous in the environment, leading to measurable background levels of exposure in the general population (IARC, 2010). Beside the inhalation of polluted air, the main sources of exposure are tobacco smoke and diet (Baird et al., 2005; Hamouchene et al., 2011; Phillips and Venitt, 2012). Chronic exposure of laboratory animals to BaP has been associated with the development of cancer, primarily in the skin, stomach and lungs (IARC, 2010).

BaP requires metabolic activation catalyzed by cytochrome P450 (CYP) enzymes prior to reaction with DNA (Baird et al., 2005). Of the CYP enzymes, CYP1A1 is one of the most important enzymes in the metabolic activation of BaP to species forming DNA adducts (Baird et al., 2005; Hamouchene et al., 2011), in combination with microsomal epoxide hydrolase (mEH). First, CYP1A1 oxidizes BaP to an epoxide that is then converted to a dihydrodiol by mEH (*i.e.* BaP-7,8-dihydrodiol); then further bioactivation by CYP1A1 leads to the ultimately reactive species, BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) that can react with DNA, forming adducts preferentially at guanine residues (Fig. 1). The 10-(deoxyguanosin- N^2 -yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (dG- N^2 -BPDE) adduct is the major product of BPDE reaction with DNA *in vitro* and *in vivo* (Phillips and Venitt, 2012).

BaP is, however, oxidized also to other metabolites such as the other dihydrodiols, BaP-diones and hydroxylated metabolites (Bauer et al., 1995; Chun et al., 1996; Kim et al., 1998; Jiang et al., 2001; Zhu et al., 2008). Even though most of these metabolites are detoxification products, BaP-9-ol is a precursor of 9-hydroxy-BaP-4,5-epoxide which can form another adduct with deoxyguanosine (Fig. 1) (Schoket et al., 1989; Nesnow et al., 1993; Fang et al., 2003). Thus, the levels and activities of CYP1A1 seem to be crucial for the initiation of BaP-mediated carcinogenesis.

The expression of CYP1A1 is known to be up-regulated by the aryl hydrocarbon receptor (AHR) and BaP can bind to and activate AHR thereby enhancing its own metabolic activation (Nebert et al., 2004). Furthermore, beside BaP itself, additional AHR ligands that induce CYP1A1 can modulate BaP activation. Therefore, here we investigated the effect of the flavonoid β -naphthoflavone (BNF), an inducer of this enzyme, and another member of the CYP1A subfamily, CYP1A2, which can to some degree also participate in BaP activation (Bauer et al., 1995), on BaP-derived DNA adduct formation in rat liver and small intestine. Expression levels and activities of both CYP1A enzymes in these organs were also examined. The electrochemical method of Western blotting was used to evaluate CYP1A1 and/or 1A2 expression, whereas DNA adduct formation was investigated by the thin-layer chromatography (TLC)-³²P-postlabeling method.

2. Materials and methods

2.1. Animal experiments and isolation of microsomes

All animal experiments were conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with the Declaration of Helsinki. Male Wistar rats (150 g, AnLab, Czech Republic), were housed in groups of 3 in wire cages at 22°C with a 12 hr light/dark period and *ad libitum* diet (ST-1 diet from Velaz, Czech Republic) and water access. Tested compounds dissolved in sunflower oil (1 ml), BNF (60 mg/kg b.w.) and BaP (150 mg/kg b.w.) were administered *p.o.* by gastric gavages in a single dose. BaP was applied 72 hr after administration of a single dose of BNF. The control group was treated with 1 ml of sunflower oil only. The rats were sacrificed 24 hr after the last treatment. Microsomes were prepared from sections of small intestine and the whole liver immediately after sacrificing the rats, as described previously (Krizkova et al., 2008; Hodek et al., 2011). Small intestine was removed ~2 cm under the stomach, divided into two parts (proximal and distal), each 15-20 cm long. Tissues of 3 rats were pooled to isolate microsomes. Microsomal fractions were stored at –80°C until analysis. Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with bovine serum albumin as a standard (Weichelman et al., 1988). The concentration of CYP was estimated according to Omura and Sato (1964) based on the absorption of the complex of reduced CYP with carbon monoxide.

2.2. Determination of CYP1A protein levels by Western blotting

CYP1A1 and 1A2 were detected by the electrochemical method of Western blotting on Immobilon-P membrane (Millipore, Bedford, MA) using specific chicken anti-CYP1A1/2 and anti-CYP1A2 antibodies prepared as described previously (Stiborová et al., 2002; Hodek et al., 2013). For sodium dodecyl sulfate (SDS)-electrophoresis (8% polyacrylamide gel) 30 µg

and 15 µg protein/well of small intestine and liver microsomes, respectively, was applied. Western blotting was carried out as described earlier (Krizkova et al., 2008; Hodek et al., 2011; Stiborová et al., 2002; Hodek et al., 2013; Stiborová et al., 2002; Hodek et al., 2013; Macova et al., 2013; Vranova et al., 2013). Visualization was performed using an anti-chicken IgG alkaline phosphatase-conjugated antibody and nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) tablets containing 10 mg substrate for alkaline phosphatase.

2.3. Measurement of CYP1A1 and 1A2 enzyme activities

Each microsomal sample was analyzed for specific CYP1A1 and 1A2 activities by monitoring the following reactions: 7-ethoxyresorufin *O*-deethylation (EROD) (CYP1A1/2) and 7-methoxyresorufin *O*-deethylation (MROD) (CYP1A2), according to the method described by Burke and Mayer (1974). Briefly, incubation mixtures, containing the final volume of 150 µl, consisted of 100 mM potassium phosphate buffer (pH 7.4), 0.5 mM NADPH, 0.5 mg of microsomal protein and 2.2 µM 7-ethoxyresorufin (7-methoxyresorufin) (dissolved in 1.5 µl dimethyl sulfoxide, DMSO). The reaction was initiated by adding NADPH. The formation of resorufin was continuously measured on luminescence spectrometer (PerkinElmer LS-55 equipped with 96-well plate reader) for 10 minutes at room temperature by monitoring its fluorescence (excitation and emission wavelengths of 530 and 585 nm, respectively) (Stiborová et al., 2006; Krizkova et al., 2008; Hodek et al., 2011; Vranova et al., 2013). The dealkylation rate was estimated on the basis of a resorufin standard curve.

2.4. BaP-DNA adduct analysis by ³²P-postlabeling

The DNA was isolated from liver and small intestine by standard phenol/chloroform extraction (Stiborová et al., 2006). DNA adducts formed by BaP were determined by ³²P-

postlabeling analysis using the nuclease P1 enrichment version (Stiborová et al., 2006; Poljakova et al., 2013), and thin-layer chromatography (TLC) was performed as described (Arlt et al., 2008; 2012). After chromatography, TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, USA) and DNA adduct levels (RAL, relative adduct labeling) were calculated as described (Stiborová et al., 2006; Poljakova et al., 2013). Results were expressed as DNA adducts/ 10^8 nucleotides.

2.5 BaP-DNA adduct formation in vitro and their analysis

Incubation mixtures used to assess DNA adduct formation by BaP activated with microsomes isolated from rat liver and small intestine consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 0.5 mg of microsomal proteins and 0.5 mg of calf thymus DNA in a final volume of 750 μ l. The reaction was initiated by adding 0.1 mM BaP (dissolved in 7.5 μ l DMSO). Incubations at 37°C were carried out for 90 min; BaP-DNA adduct formation was linear up to 120 min (Arlt et al., 2008). Control incubations were carried out either without microsomes, without NADPH, without DNA, or without BaP. After the incubation, DNA was isolated from the residual water phase by the phenol/chloroform extraction method and BaP-DNA adducts analyzed with the nuclease P1 version of the 32 P-postlabeling technique as described (Stiborová et al., 2006; Arlt et al., 2008; 2012; Poljakova et al., 2013).

2.6 Statistical analyses

For statistical data analysis we used Student's *t*-test. All *P*-values are two-tailed and considered significant at the 0.05 level.

3. Results and Discussion

3.1. The effect of BaP and BNF on expression of CYP1A1/2 in rat liver and small intestine

When rats were treated with BNF or BaP, an increase in expression levels of CYP1A1 and 1A2 proteins determined with Western blotting was found in microsomes isolated from rat liver (Fig. 2), and this increase corresponded to EROD and MROD activities in these microsomes (Table 1). Pretreatment of rats with BNF prior to their treatment with BaP also resulted in elevated EROD and MROD activities in microsomes of this organ, however only the increase in CYP1A2 protein, but not CYP1A1, correlated with higher enzyme activity (see Fig. 2 and Table 1). Now, we cannot explain this phenomenon. The measurement of CYP1A1 activity seems to be more suitable to investigate the changes in CYP1A1 than determination of the CYP1A1 protein levels by Western blotting.

In microsomes of the proximal and distal parts of small intestine, BaP and BNF also induced expression of CYP1A1 and its activity, but treatment of rats with BaP had a higher induction effect on this enzyme than their pretreatment with BNF prior to treatment with BaP. The CYP1A1 expression levels and its specific activity (EROD) in small intestine were higher in its proximal part than in its distal part (Fig. 3 and Table 2).

3.2. The effect of BaP and BNF on BaP-DNA adduct formation in rat liver and small intestine

Treatment of rats with a single dose of BaP resulted in formation of three BaP-derived DNA adducts (assigned as adduct spots 1-3 in Fig. 4A and 4B) detectable by ³²P-postlabeling in the liver and the proximal and distal parts of the small intestine. Two of these adducts were tentatively identified to be dG-N²-BPDE (adduct spot 2) (Arlt et al., 2008; 2012) and the adduct derived from a reaction of deoxyguanosine in DNA with 9-hydroxy-BaP-4,5-epoxide (adduct spot 1) (Fig. 1) (Schoket et al., 1989; Nesnow et al., 1993; Fang et al., 2003). This BaP metabolite is bound to the exocyclic amino group of a guanine residue, and the site of

attachment is most likely either the 4 or the 5 position on the BaP-4,5-epoxide (Fang et al., 2003). A structure of another BaP-DNA adduct (adduct spot 3) has not been characterized as yet.

Up to 4.6-fold higher levels of BaP-DNA adducts were found in the small intestine than in the liver (Table 3). Furthermore, significantly higher levels of BaP-DNA adducts 2 and 3 were found in the proximal part of the small intestine than in its distal part, whereas levels of BaP-DNA adduct 1 in these parts of small intestine did not change. Pretreatment of rats with the CYP1A inducer BNF led to an increase in all BaP-DNA adduct levels in the liver and, to a lesser extent, in the distal part of the small intestine. However, almost no effect of this pretreatment was found on BaP-DNA adduct formation in the proximal part of small intestine (Table 3). The increase in levels of BaP-DNA adducts in the liver correlated with CYP1A1 and 1A2 enzyme activities in this organ (compare Tables 1 and 3).

Interestingly, when BaP was incubated with microsomes isolated from liver and small intestine of rats treated with BaP or with BNF prior to BaP treatment, only two BaP-DNA adducts were generated (see adduct spots 1 and 2 in Fig. 4C and 4D). Hence, formation of BaP-DNA adduct 3 found *in vivo* should be mediated not only by BaP activation with CYP enzymes present in the membrane of endoplasmic reticulum, but probably also by additional enzymes. Moreover, in contrast to results found *in vivo*, higher levels of BaP-DNA adducts were formed in *ex vivo* incubations of BaP and DNA with hepatic microsomes relative to microsomes of the small intestine (Table 4). Pretreatment of rats with BNF increased efficiencies of hepatic microsomes to generate BaP-DNA adducts (up to 2.7-fold), but either no or a low increase in efficacy of small intestine microsomes to form BaP-DNA adducts was found.

The levels of BaP-DNA adducts formed in DNA incubated with BaP and microsomes of liver corresponded to CYP1A1/2 activities (EROD and MROD) found in these microsomes (Tables 1 and 4). Likewise, higher amounts of BaP-DNA adducts formed in microsomes of

the proximal than in distal part of the small intestine correlated with expression levels of CYP1A1 and its enzyme activities in these microsomes (Fig. 3, Tables 2 and 3). However, whereas a minor, but significant, increase in BaP-DNA adduct formation was found in microsomes of the distal part of small intestine caused by pretreatment of rats with BNF prior to BaP exposure, no increase in expression of CYP1A1 and its enzyme activity was detected in these microsomes (Fig. 3, Tables 1, 2 and 4). In addition, the higher levels of BaP-DNA adducts found in the small intestine than in the liver *in vivo* contrasted with almost 70-fold higher CYP1A1 activities in liver microsomes relative to microsomes of the small intestine (Figures 1 and 2 and Table 4).

All these results suggest that not only the activity of CYP1A1 (and/or CYP1A2 in the liver) and their induction by BaP or BNF, but also additional factors influence BaP-DNA adduct formation *in vivo* in rats. This finding is consistent with suggestions of Uno et al. (2001; 2004; 2006) that the level of BaP-DNA adducts in cells depends not only on the metabolism of BaP by phase I enzymes (activation) to reactive DNA-binding species and/or on BaP-induced expression of the enzymes involved in BaP activation (*i.e.* CYP1A1). Several studies demonstrated a rather enigmatic role of CYP 1A1 in BaP activation metabolism *in vivo*. The controversial results have been found, showing a more important role of CYP1A1 *in vivo* in BaP detoxification than in its activation (Uno et al., 2001; 2004; 2006; Arlt et al., 2008; 2012). Indeed, Uno et al. (2001) showed that absence of the *Cyp1a1* enzyme protects the intact animal from BaP-mediated liver toxicity and death, by decreasing the formation of large amounts of toxic metabolites, whereas much slower metabolic clearance of BaP in *Cyp1a1(-/-)* mice leads to greater formation of BaP-DNA adducts. Using the Hepatic P450 Reductase Null (HRN) and the Reductase Conditional Null (RCN) mouse model we also showed that hepatic CYP enzymes appear to be more important for detoxification of BaP *in vivo* (Arlt et al., 2008; 2012). In HRN and RCN mice NADPH:CYP oxidoreductase, the essential electron donor to CYPs is deleted specifically in hepatocytes, resulting in a decrease

in hepatic CYP function (Henderson et al., 2003; Finn et al., 2007). We found however that the levels of dG-*N*²-BPDE adducts in livers of these mice treated with BaP were higher than in WT mice (Arlt et al., 2008; 2012). Therefore, these and other factors such as: (i) detoxification of reactive BaP metabolites by both phase I and phase II enzymes (conjugation), (ii) rate of repair of BaP-DNA adducts, and (iii) BaP-induced expression of genes of enzymes involved in BaP detoxification or in DNA damage response (Uno et al., 2004; 2006), might also influence BaP-DNA adduct levels *in vivo*. However, the impacts of those factors on BaP-DNA adduct formation in the present study *in vivo* remain to be explored.

The results of this study demonstrate that the administration of BNF, BaP or BNF prior to BaP treatment induces CYP1A enzymes in rat liver and the distal part of the small intestine which leads to increased BaP-DNA adduct formation. The results also indicate that the electrochemical method of Western blotting is a suitable tool in evaluating the expression of CYP1A enzymes which, besides other factors, contributes to the risk of BaP-induced cancer development in various organisms including humans.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1. CYP1A1/2 (EROD) and 1A2 (MROD) activities in rat liver microsomes. Reaction rates are means of three parallel experiments \pm SD. Comparison was performed by *t*-test analysis: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, different to untreated (UT).

Liver microsomes	Resorufin formation (pmol resorufin/min/mg protein)	
	CYP1A1 - EROD	CYP1A2 - MROD
UT	21.5 \pm 1.3	38.3 \pm 2.2
BNF	527.1 \pm 41.2 **	210.3 \pm 12.5 *
BaP	840.6 \pm 57.6 **	337.5 \pm 29.9 **
BaP + BNF	1742.5 \pm 113.3 ***	1024.7 \pm 37.1 **

Table 2. CYP1A1 (EROD) activity in microsomes of rat small intestine. Reaction rates are means of three parallel experiments \pm SD. Comparison was performed by *t*-test analysis: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, different to untreated (UT).

Microsomes of small intestine	CYP1A1 - EROD (pmol resorufin/min/mg protein)	
	proximal	distal
UT	1.0 \pm 0.1	0.5 \pm 0.05
BNF	120.5 \pm 9.8 ***	20.0 \pm 1.7 *
BaP	279.1 \pm 22.3 ***	33.2 \pm 2.9 **
BaP + BNF	243.2 \pm 19.5 ***	23.7 \pm 1.2 *

Table 3. BaP-DNA adduct formation in liver (**A**) and small intestine (**B**) of rats treated with BaP or with BaP and BNF. RAL - relative adduct labeling. Values are averages of three parallel experiments \pm SD (Adduct 1, 2 and 3 see Fig 4). Comparison was performed by *t*-test analysis; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, different from rats treated with BaP only.

A DNA sample	BaP-DNA adducts (RAL/10⁷ nucleotides)			
	Spot 1	Spot 2	Spot 3	Total
BaP	0.61 \pm 0.06	1.00 \pm 0.10	0.79 \pm 0.08	2.40 \pm 0.24
BaP + BNF	1.28 \pm 0.13	1.75 \pm 0.17	2.75 \pm 0.27	5.78 \pm 0.58
Folds	2.10^{***}	1.75^{***}	3.48^{***}	2.40^{***}

B DNA sample	BaP-DNA adducts (RAL/10⁷ nucleotides)				
		Spot 1	Spot 2	Spot 3	Total
BaP	proximal	3.85 \pm 0.38	2.82 \pm 0.28	4.39 \pm 0.44	11.06 \pm 1.10
	distal	4.40 \pm 0.44	1.56 \pm 0.15	1.64 \pm 0.16	7.60 \pm 0.76
BaP + BNF	proximal	5.15 \pm 0.51	2.97 \pm 0.30	3.96 \pm 0.40	12.08 \pm 1.21
	distal	4.02 \pm 0.40	1.85 \pm 0.18	2.64 \pm 0.26	8.51 \pm 0.85
Folds in proximal		1.34^{**}	1.05	0.90	1.09
Folds in distal		0.91	1.19^{**}	1.61^{***}	1.12[*]

Table 4. BaP-DNA adducts formed by liver (**A**) and small intestine microsomes (**B**) from rats treated with BaP or with BaP and BNF. RAL - relative adduct labeling. Values are averages of three parallel experiments (Adduct 1 and 2 see Fig 4). Comparison was performed by *t*-test analysis; **P* < 0.05, ***P* < 0.01, ****P* < 0.001, different from rats treated with BaP only.

A DNA sample	BaP-DNA adducts (RAL/10 ⁷ nucleotides)		
	Spot 1	Spot 2	Total
BaP	3.91 ± 0.21	1.56 ± 0.12	5.47 ± 0.51
BaP + BNF	7.06 ± 0.65	4.15 ± 0.32	11.21 ± 0.95
Folds	1.81^{***}	2.66^{***}	2.05^{***}

B DNA sample		BaP-DNA adducts (RAL/10 ⁷ nucleotides)		
		Spot 1	Spot 2	Total
BaP	proximal	0.93 ± 0.07	0.18 ± 0.02	1.11 ± 0.09
	distal	0.19 ± 0.02	0.04 ± 0.01	0.23 ± 0.02
BaP + BNF	proximal	0.90 ± 0.07	0.13 ± 0.01	1.03 ± 0.1
	distal	0.31 ± 0.03	0.07 ± 0.01	0.38 ± 0.04
Folds in proximal		0.97	0.72	0.93
Folds in distal		1.6[*]	1.75^{**}	1.65[*]

Legends to Figures

Figure 1. Proposed pathways of biotransformation and DNA adduct formation of BaP catalyzed by CYP1A1 and mEH. The two-step activation process by CYP1A1 leading to the formation of the ultimately reactive species, 9-hydroxy-BaP-4,5-epoxide, that can react with deoxyguanosine in DNA (adduct 1) is shown in the left part of the figure. The typical three-step activation process with oxidation by CYP1A1 followed by hydrolysis by mEH leads to the ultimately reactive species, BPDE, forming the dG- N^2 -BPDE adduct (adduct 2) is shown in the right part of the figure.

Figure 2. Immunodetection of induced CYP1A1 and 1A2 in liver microsomes from untreated (UT), or BNF, BaP or BaP and BNF (BaP+BNF) pretreated animals. Values are given as means of three parallel experiments. Comparison was performed by *t*-test analysis: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, different to UT.

Figure 3. Immunodetection of induced CYP1A1 in microsomes isolated from the proximal and distal parts of small intestine of rat either untreated (UT), or treated with BNF, BaP or BaP and BNF (BaP+BNF). Values are given as means of three parallel experiments. Comparison was performed by *t*-test analysis: ****P* < 0.001, different to UT.

Figure 4. BaP-DNA adduct formation in rat liver (A) and small intestine (B) *in vivo* and in incubations of BaP and DNA with rat liver (C) and small intestine (D) microsomes *ex vivo*. DNA adducts were analyzed by the ^{32}P -postlabeling method.

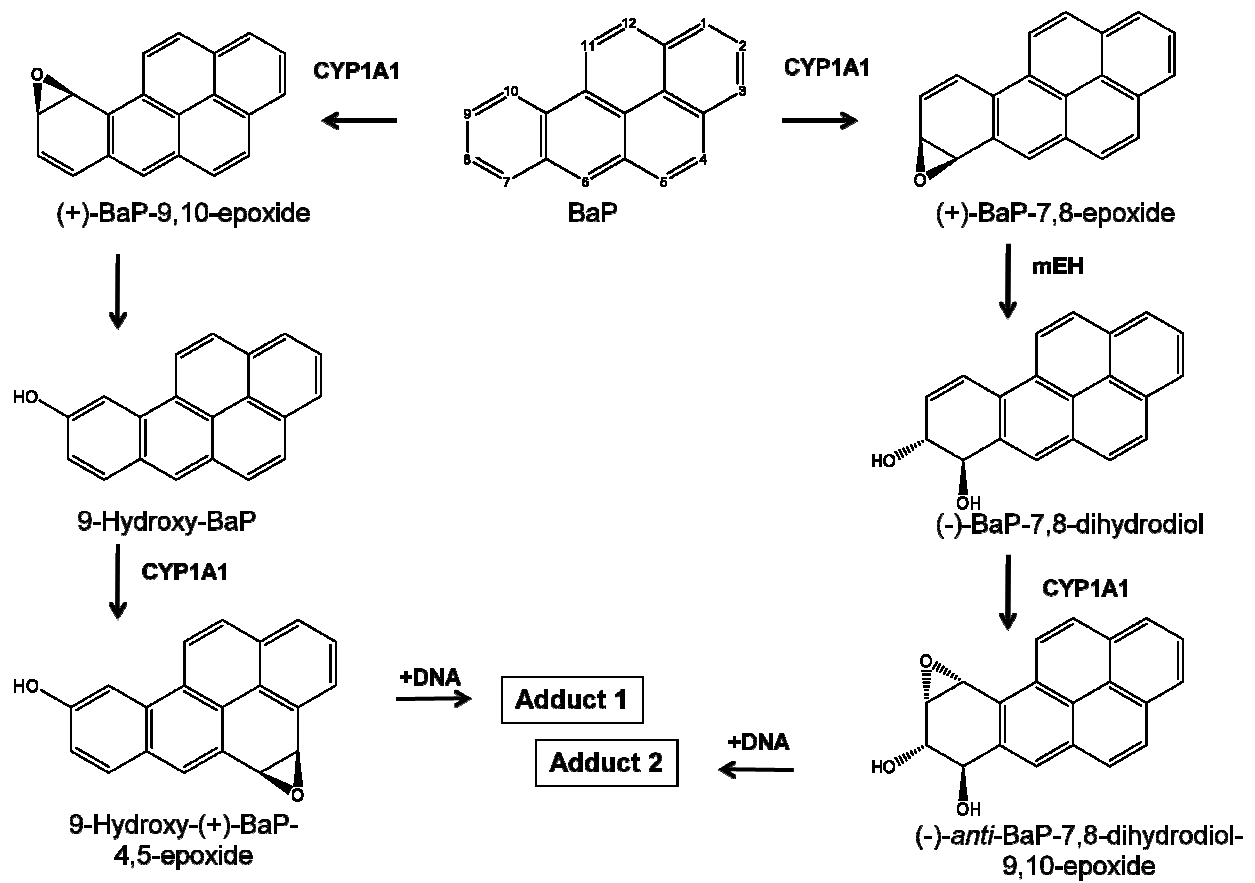
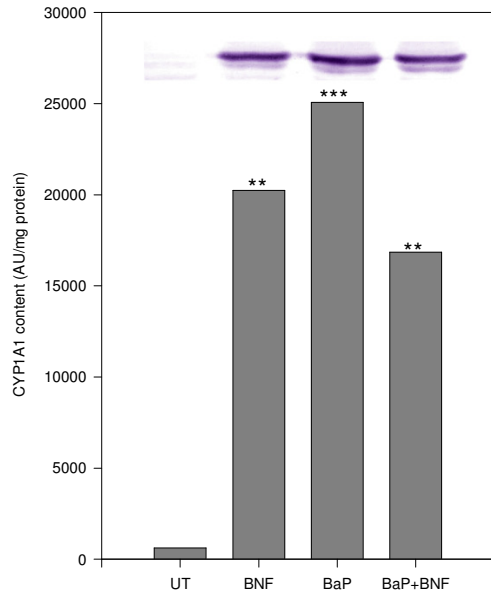


Figure 1

CYP1A1



CYP1A2

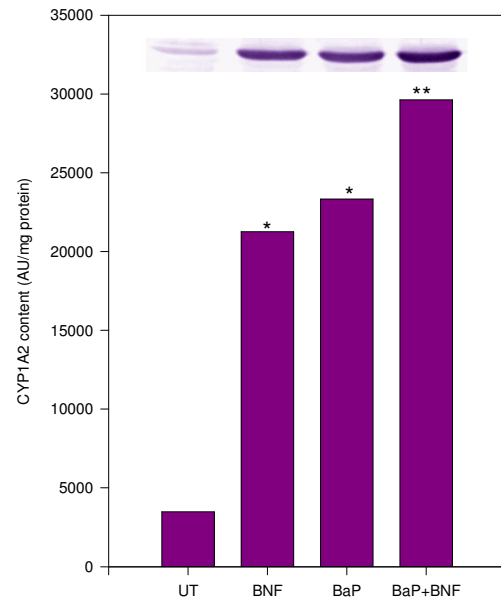


Figure 2

CYP1A1

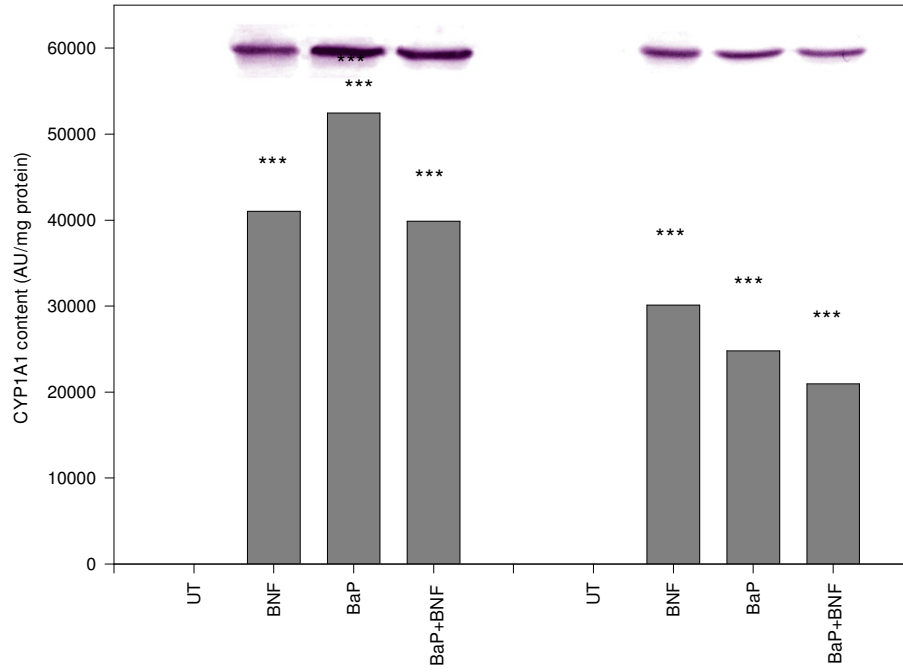


Figure 3

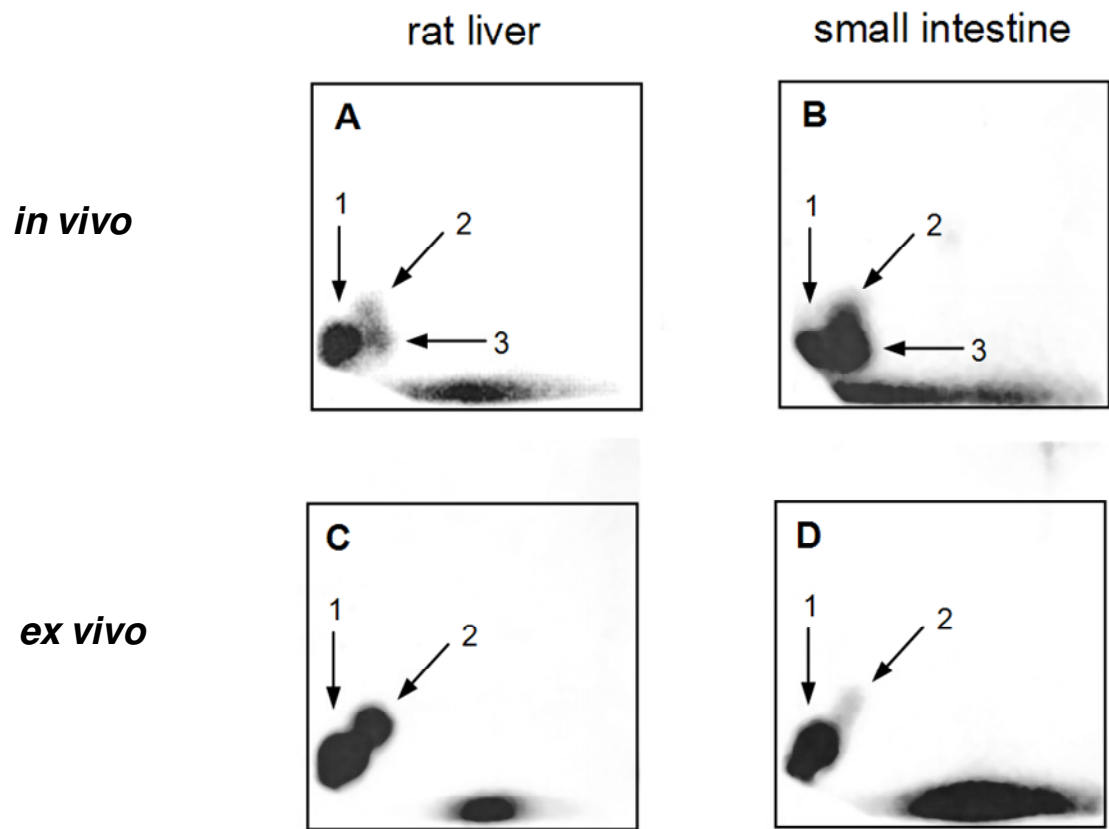


Figure 4