The effect of benzo[a]pyrene on metabolic activation of anticancer drug ellipticine in mice

Marie Stiborova [1], Vera Cerna [1], Michaela Moserova [1], Volker M. Arlt [2], Eva Frei [3]

1 Department of Biochemistry, Faculty of Science, Charles University, Prague, Czech Republic
2 Analytical and Environmental Sciences Division, King's College London, MRC-PHE Centre for Environmental & Health, London, United Kingdom
3 Division of Preventive Oncology, National Center for Tumor Diseases, German Cancer Research Center (DKFZ), Heidelberg, Germany

Correspondence to: Prof. RNDr. Marie Stiborova, DSc.
Department of Biochemistry, Faculty of Science, Charles University
Albertov 2030, 128 40 Prague 2, Czech Republic
TEL: +420 221951285; FAX: +420 221951283; E-MAIL: stiborov@natur.cuni.cz

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Abstract

OBJECTIVES: The aim of this study was to investigate a role of cytochrome P450 (CYP) and peroxidase in ellipticine oxidative activation in two mouse strains differing in expression of NADPH:CYP reductase (POR) [the HRN (Hepatic Cytochrome P450 Reductase Null) mice, in which POR is deleted in hepatocytes and its wild-type (WT) counterpart], and in levels of CYP1A1/2 and cytochrome b_5 that were modulated by treatment of these mouse models with a CYP1A inducer, benzo[a]pyrene (BaP).

METHODS: Ellipticine-DNA adducts were detected by ^{32}P-postlabeling. HPLC was employed for the separation and characterization of ellipticine metabolites.

RESULTS: Hepatic microsomes of HRN and WT mice activate ellipticine to form ellipticine-derived DNA adducts. A 2.2- and 10.4-fold increase in amounts of ellipticine-derived DNA adducts formed by liver microsomes was caused by exposure of HRN and WT mice to BaP, respectively. The results found and utilization of NADPH and arachidonic acid, cofactors of CYP- and cyclooxygenase (COX)-dependent enzyme systems, respectively, as well as inhibitors of CYP1A1/2 and 3A, demonstrate that the CYP1A and 3A enzymes play a major role in ellipticine activation in liver microsomes. In addition, the COX enzyme is important in ellipticine activation in liver of HRN mice.

CONCLUSION: The CYP1A and 3A enzymes activate ellipticine mainly in liver of WT mice, whereas peroxidase COX plays this role in liver of HRN mice. Treatment of mice with BaP increases an impact of CYP1A on ellipticine activation. A pattern of expression levels of these enzymes plays a crucial role in their impact on this process.

ORIGINAL ARTICLE

Abbreviations:

AHR	- aryl hydrocarbon receptor
α-NF	- α-naphthoflavone
BaP	- benzo[a]pyrene
BPDE	- BaP-7,8-dihydrodiol-9,10-epoxide
COX	- cyclooxygenase
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	- ethoxyresorufin O-deethylase
GAPDH	- glyceraldehyde phosphate dehydrogenase
HPLC	- high performance liquid chromatography
HRN	- Hepatic Cytochrome P450 Reductase Nul
i.p.	- intraperitoneally
mEH	- microsomal epoxide hydrolase
NADPH	- nicotinamidadeninedinucleotide phosphate (reduced)
POR	- NADPH:cytochrome P450 reductase
RAL	- relative adduct labeling
r. t.	- retention time
S.D.	- standard deviation
SDS-PAGE	- sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC	- thin-layer chromatography
UV	- ultraviolet
WT	- wild-type

INTRODUCTION

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Figure 1) and its derivatives are efficient anticancer compounds that function through multiple mechanisms participating in cell cycle arrest and initiation of apoptosis [for a summary see (Auclair 1987; Garbett & Graves 2004; Stiborova *et al.* 2001, 2006c, 2011; Kizek *et al.* 2012)]. Ellipticine was found (i) to arrest cell cycle progression due to modulation of levels of cyclinB1 and Cdc2, and phosphorylation of Cdc2 in human mammary adenocarcinoma MCF-7 cells, (ii) to initiate apoptosis due to formation of toxic free radicals, stimulation of the Fas/Fas ligand system and modulation of proteins of Bcl-2 family in several tumor cell lines, (iii) to induce an increase in wild-type p53 and the rescue of mutant p53 activity, and (iv) to initiate the mitochondria-dependent apoptotic processes [for a summary see (Kuo *et al.* 2005a,b; 2006; Stiborova *et al.* 2011; Kizek *et al.* 2012)]. Ellipticine also activates the p53 pathway in glioblastoma cells; its impact on these cancer cells depends on the p53 status. In a U87MG glioblastoma cell line expressing p53wt, ellipticine provoked an early G0/G1 cell cycle arrest, whereas in a U373 cell line expressing p53mt it caused arrest in S and G2/M phase (Martinkova *et al.* 2010).

The predominant molecular mechanisms of ellipticine's biological effects were suggested to be (i) intercalation into DNA and (ii) inhibition of topoisomerase II (Auclair 1987; Garbett & Graves 2004; Stiborova *et al.* 2011). We also showed that this antitumor agent forms covalent DNA adducts after its enzymatic activation with cytochromes P450 (CYP) and peroxidases (Stiborova *et al.* 2001, 2004, 2006a, 2007a,b, 2008, 2010, 2011, 2012b,c,d; Kotrbova *et al.* 2011; Kizek *et al.* 2012), suggesting an additional DNA-damaging effect of ellipticine.

Of the CYP enzymes investigated, human CYP3A4, followed by CYP1A1 and 1A2 are the most active enzymes oxidizing ellipticine to 12-hydroxy- and 13-hydroxyellipticine, the reactive metabolites that dissociate to ellipticine-12-ylium and ellipticine-13-ylium which bind to DNA (Stiborova *et al.* 2004, 2007a, 2011, 2012b,d). The CYP1A isoforms also efficiently form the other ellipticine metabolites, 7-hydroxy- and 9-hydroxyellipticine, which are the detoxification products (Figure 1). Recently we have found that cytochrome b_5 alters the ratio of ellipticine metabolites formed by CYP1A1, 1A2 and 3A4. While the amounts of the detoxification metabolites (7-hydroxy- and 9-hydroxyellipticine) were either decreased or not changed with added cytochrome b_5, 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide increased considerably. The change in amounts of metabolites resulted in an increased formation of covalent ellipticine-DNA adducts, one of the DNA-damaging mechanisms of ellipticine antitumor action (Kotrbova *et al.* 2011; Stiborova *et al.* 2012b). In addition, we found that levels of the DNA adduct formed by 13-hydroxyellipticine also increased if this ellipticine metabolite was conjugated with sulfate or acetate by human sulfotransferases 1A1, 1A2, 1A3 and 2A1, or *N,O*-acetyltransferases 1 and 2 (Moserova *et al.* 2008; Kotrbova *et al.* 2011; Stiborova *et al.* 2012b) (Figure 1). Of the mammalian peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidase and human myeloperoxidase efficiently generated ellipticine-derived DNA adducts (Poljakova *et al.* 2006, Stiborova *et al.* 2007a).

The same ellipticine-derived DNA adducts that were found in *in vitro* incubations of ellipticine with DNA and enzymes activating this drug, were generated also *in vivo*, in several tissues of mice and rats exposed to ellipticine. In both animal models, ellipticine-DNA adduct formation was mediated mainly by CYP1A and 3A enzymes, but a role of peroxidases in several organs was also proved (Stiborova *et al.* 2003a, 2003b, 2007b, 2008, 2010). The ellipticine-DNA adducts were also found in several cancer cell lines expressing CYP and/or peroxidase enzymes and in DNA of rat mammary adenocarcinoma *in vivo* (Borek-Dohalska *et al.* 2004; Poljakova *et al.* 2007, 2009, 2011, 2013; Martinkova *et al.* 2009; Stiborova *et al.* 2011).

All these results indicate that expression levels of CYP and peroxidase enzymes metabolizing ellipticine seem to be crucial for antitumor, cytostatic and genotoxic activities of this drug in individual tissues. However, the actual impacts of these enzymes *in vivo* depend on several additional factors (Stiborova *et al.* 2008, 2011). One of them might be the presence of various patterns of individual CYPs and peroxidases and/or even the presence of other proteins influencing their activities such as cytochrome b_5 in target and non-tar-

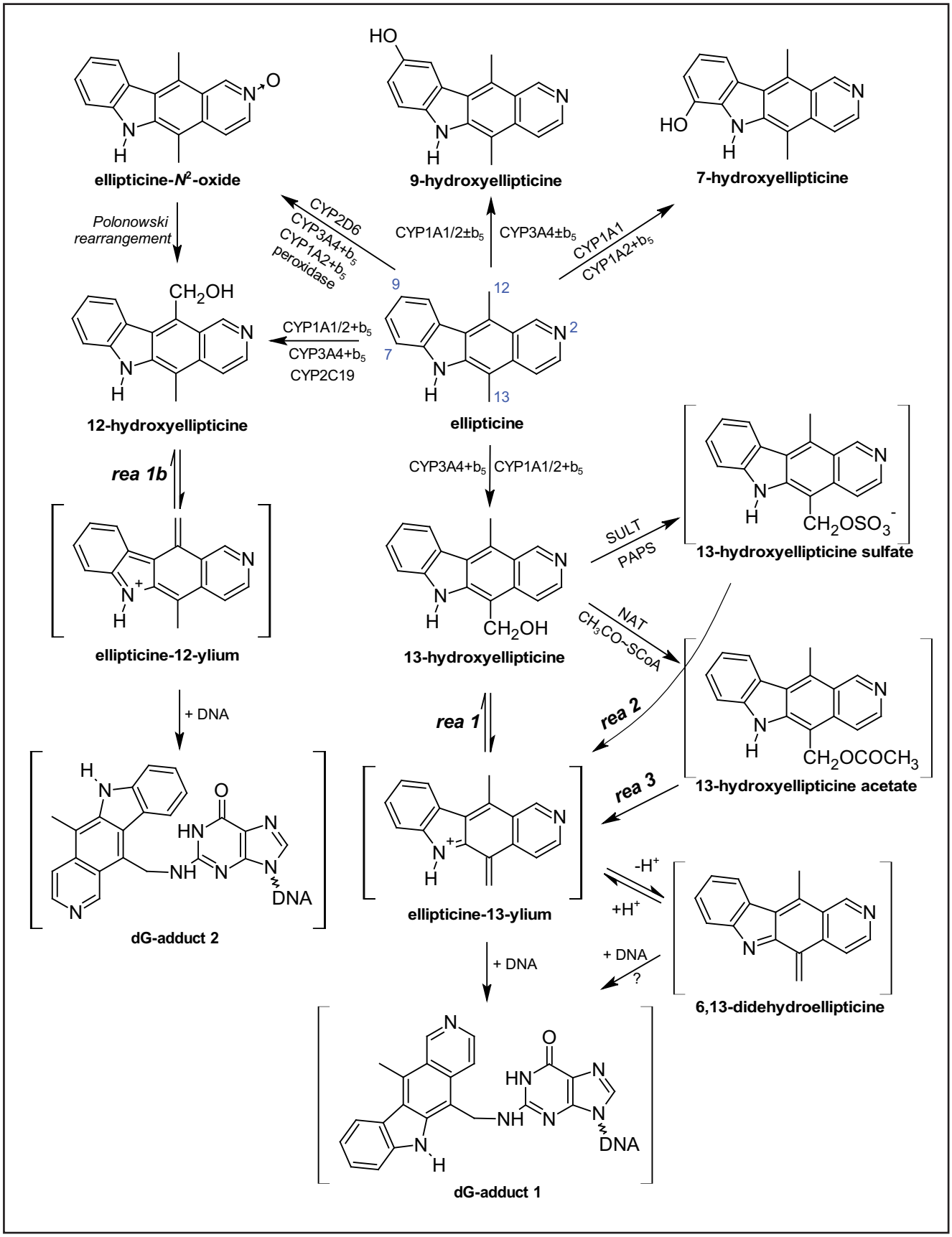

Fig. 1. Scheme of ellipticine metabolism by cytochrome P450 (CYP) enzymes showing the identified metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and/or not yet structurally characterized. The CYP enzymes predominantly oxidizing ellipticine shown in the figure were identified in our previous studies (Stiborova *et al.* 2004, 2012b; Kotrbova *et al.* 2011). Reactions (rea) 1, 2 and 3 are reactions leading to ellipticine-13-ylium from 13-hydroxyellipticine, 13-hydroxyellipticine sulfate and 13-hydroxyellipticine acetate, respectively.

get tissues (Stiborova *et al.* 2008, 2011, 2012b; Kotrbova *et al.* 2011; Kizek *et al.* 2012).

Therefore, in this study we have used two mouse strains, in which four different patterns of CYP1A1, 1A2, POR and cytochrome b_5, the enzymes that are crucial to dictate ellipticine metabolism, were expressed. Namely, beside the HRN (Hepatic Cytochrome P450 Reductase Null) mouse strain, in which NADPH:CYP reductase (POR), the unique electron donor to CYPs is deleted specifically in hepatocytes, resulting in the loss of most hepatic CYP function (Henderson *et al.* 2003; Arlt *et al.* 2008; Stiborova *et al.* 2008) and its wild-type (WT) counterpart, we have also utilized these mouse strains in which the levels of CYP1A1/2 enzymes and cytochrome b_5 protein were modulated by their treatment with a strong CYP1A1/2 inducer, benzo[a]pyrene (BaP) (Arlt *et al.* 2008, 2012; Hodek *et al.* 2011; Phillips & Venitt 2012). The use of such animal models can shed more light on the impact of these biotransformation enzymes on metabolic activation and/or detoxification of ellipticine in organisms.

The electrochemical method of Western blotting was used to estimate cytochrome b_5, CYP1A1/2 and POR protein expression in hepatic microsomes of used mouse models, whereas formation of ellipticine-derived DNA adducts or ellipticine oxidative metabolites catalyzed by these microsomes were analyzed by the ^{32}P-postlabeling or HPLC methods, respectively (Stiborova *et al.* 2001, 2006a,c, 2011).

MATERIAL AND METHODS

Animal treatment

Mouse model strains used in this study were identical to those used in our previous works (Arlt *et al.* 2008, 2012). All experiments were approved by, and conducted in accordance with, the National Institute of Health standards for the care and use of experimental animals and the University of Cincinnati Medical Center Institutional Animal Care and Use Committee. HRN (*Por*$^{lox/lox}$ + *Cre*^{ALB}) mice on a C57BL/6 background (CXR Bioscience Ltd., Dundee, UK) used in this study were derived as described previously (Henderson *et al.* 2003). Mice homozygous for loxP sites at the *Por* locus (*Por*$^{lox/lox}$) were used as wild-type (WT). BaP was dissolved in corn-oil at a concentration of 12.5 mg/ml. Groups of female HRN and WT mice (3 months old, 25–30 g) were treated intraperitoneally (*i.p.*) with 125 mg/kg body weight (n=3) of BaP daily either for 1 day (group I) or 5 days. Control mice (n=3) received corn-oil only either for 1 day or for 5 days. Animals were sacrificed 24 hours after the last dose (Arlt *et al.* 2008, 2012). Mouse livers were removed, snap frozen and stored at −80 °C until analysis.

Preparation of hepatic microsomes

Hepatic microsomes from HRN and WT mice, untreated or treated with BaP as described above, were isolated as described previously (Stiborova *et al.* 2011, 2012a; 2012b). Pooled microsomal fractions were used for further analyses.

Preparation of CYP1A1 and POR antibodies

Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA (Stiborova *et al.* 2002). Rabbit liver POR was purified as described (Arlt *et al.* 2003). Leghorn chickens were immunized subcutaneously three times (with one week interval) with rat recombinant CYP1A1 and rabbit hepatic POR antigens (0.1 mg/animal) emulsified in complete Freund's adjuvant for the first injection and in incomplete adjuvant for boosters. Immunoglobulin fraction was purified from pooled egg yolks using fractionation by polyethylene glycol 6000 (Stiborova *et al.* 2002; Hodek *et al.* 2013).

Determination of CYP1A1/2, POR and cytochrome b_5 protein levels in hepatic microsomes

Immunoquantitation of hepatic microsomal CYP1A1, CYP1A2 and POR was done essentially as described previously using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Stiborova *et al.* 2002, 2006b; Arlt *et al.* 2008). CYP and POR were probed with the chicken anti-rat CYP1A1 and chicken anti-rabbit POR polyclonal antibodies as reported (Stiborova *et al.* 2002, 2006b; Arlt *et al.* 2008). The antibody against rat recombinant CYP1A1 recognizes both CYP1A1 and CYP1A2 in mouse liver microsomes. Rat recombinant CYP1A1 and CYP1A2 (in Supersomes™, Gentest Corp., USA) and rabbit POR were used as positive controls to identify protein bands in microsomal samples. Western blot analysis was also used to determine expression of cytochrome b_5; 75 µg microsomal protein was subjected to SDS-PAGE using 15% gel. After migration, proteins were transferred onto polyvinylidene difluoride membranes. Cytochrome b_5 protein was probed with rabbit polyclonal anti-cytochrome b_5 antibody (1:750; ab69801; Abcam, MA, USA) overnight at 4°C. Glyceraldehyde phosphate dehydrogenase (GAPDH; 1:750; Millipore, MA, USA) was used as loading control. The antigen-antibody complex was visualized with an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as chromogenic substrate (Stiborova *et al.* 2002, 2006b; Arlt *et al.* 2008, 2012). The detection limit was 0.005 pmol CYP1A1 per lane (Stiborova *et al.* 2002, 2006b; Arlt *et al.* 2008) and 0.01 pmol for the other enzymes and cytochrome b_5.

Determination of CYP1A and POR enzymatic activity in hepatic microsomes

The hepatic microsomal samples were characterized for CYP1A activity using 7-ethoxyresorufin *O*-deetylation (EROD) (Stiborova *et al.* 2002, 2006b; Arlt *et al.* 2008). The activity of POR was measured as reported previously (Arlt *et al.* 2003).

Microsomal incubations

Incubation mixtures used to generate DNA adduct formation by ellipticine *in vitro* consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, pooled hepatic microsomal fraction (n=3) (0.5 mg protein) from either untreated (control) HRN or WT mice or those treated with BaP (see above), 0.1 mM ellipticine (dissolved in 7.5 µl methanol) and 0.5 mg of calf thymus DNA in a final volume of 750 µl. Incubations were also carried out in the presence of a COX cofactor, arachidonic acid (Eling & Curtis 1992; Stiborova *et al.* 2005). Mixtures then contained 0.1 mM arachidonic acid as cofactor instead of NADPH, and additionally 5 mM magnesium chloride. Incubations were carried out at 37 °C for 30 minutes; ellipticine-DNA adduct formation was found to be linear up to 30 min of incubation (Stiborova *et al.* 2001). Control incubations were carried out (*i*) without microsomes, (*ii*) without NADPH or arachidonic acid, (*iii*) without DNA and (*iv*) without ellipticine. After the incubation, DNA was isolated by a standard phenol-chloroform extraction method.

Incubation mixtures used to form the ellipticine metabolites contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase (NADPH-generation system), 0.2 mg protein of pooled hepatic microsomal fraction and 10 µM ellipticine (dissolved in 5 µl methanol) in a final volume of 500 µl. The reaction was initiated by adding the substrate. In the control incubation, ellipticine was omitted from the incubation mixture. After incubation in open glass tubes (37 °C, 20 min) the reaction was stopped by adding 100 µl of 2 M NaOH. The oxidation of ellipticine is linear up to 30 min of incubation (Stiborova *et al.* 2006a). After incubation, 5 µl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethyl acetate (2×1 ml). Analyses of ellipticine metabolites were performed by HPLC as described (Stiborova *et al.* 2004, 2006a). Recoveries of ellipticine metabolites were around 95% in the presence of microsomes without a CYP cofactor (NADPH-generation system).

DNA adduct detection by ^{32}P-postlabeling analysis

DNA adducts formed by ellipticine were determined by ^{32}P-postlabeling analysis using the nuclease P1 enrichment version and DNA adducts were resolved by thin-layer chromatography (TLC) (Stiborova *et al.* 2003a, 2004, 2007a). 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 (Stiborova *et al.* 2003a, 2004, 2007a). Results were expressed as DNA adducts/10^8 nucleotides.

Inhibition studies

The following chemicals were used to inhibit the activation of ellipticine to form DNA adducts in the presence of mouse hepatic microsomes: α-naphtoflavone (α-NF), which inhibits CYP1A1 and 1A2, ketoconazole, an inhibitor of CYP3A (Rendic & DiCarlo 1997; Arlt *et al.* 2004; Stiborova *et al.* 2005), and α-lipoic acid, which inhibits POR (Slepneva *et al.* 1995). Inhibitors were dissolved in 7.5 µl of methanol, to yield final concentrations of 0.1 mM in the incubation mixtures. Mixtures were then incubated at 37 °C for 10 min with NADPH prior to adding ellipticine, and then incubated for a further 30 min at 37 °C. After the incubation, DNA was isolated as described above.

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.

RESULTS AND DISCUSSION

Expression of hepatic CYP1A1, 1A2, POR and cytochrome b_5 in HRN and WT mice is induced by BaP

Hepatic microsomes isolated from both control (untreated) mice and mice treated with BaP were used in experiments evaluating their efficacies to activate ellipticine to species forming ellipticine-derived DNA adducts and/or to detoxify this compound to metabolites that are excreted. First, expression of CYP1A1/2, POR and cytochrome b_5, the enzymes that all dictate metabolic activation and/or detoxification of ellipticine was analyzed.

As shown in our previous study (Arlt *et al.* 2008), CYP1A1 and 1A2 enzymes are constitutively expressed in livers of untreated HRN and WT mice, with HRN mice having marginally higher levels (1.4-fold) than WT mice (Figure 2). BaP was capable to effectively induce expression of both CYPs in livers of HRN and WT mice. Whereas exposure of these mice to BaP resulted in 8.9-fold higher levels of CYP1A1/2 in livers of WT mice, its inducing effect was higher in HRN mice; a 17.8-fold increase in CYP1A1/2 protein expression was caused by BaP in this mouse strain. The increase in CYP1A1/2 levels in the WT mice was associated with a strong increase in CYP1A1/2 marker activity, ethoxyresorufin *O*-demethylation (EROD) (Figure 2C). Whereas CYP1A1/2 activity (EROD) was not detectable in untreated HRN mice, it was found in HRN mice treated with BaP. Its levels were, however, 3.4-fold lower than in WT mice treated with BaP. These findings are in concordance with previous studies, showing that expression of CYP1A1/2 is up-regulated by the aryl hydrocarbon receptor (AHR) and BaP can bind to and activate AHR, thereby enhancing metabolic activation of several genotoxic carcinogens, including BaP itself (Arlt *et al.* 2008; Hodek *et al.* 2011; Phillips & Venitt 2012).

The expression of POR was detected in liver of WT mice, while as expected, its expression in liver of HRN mice was very low, but still detectable by the

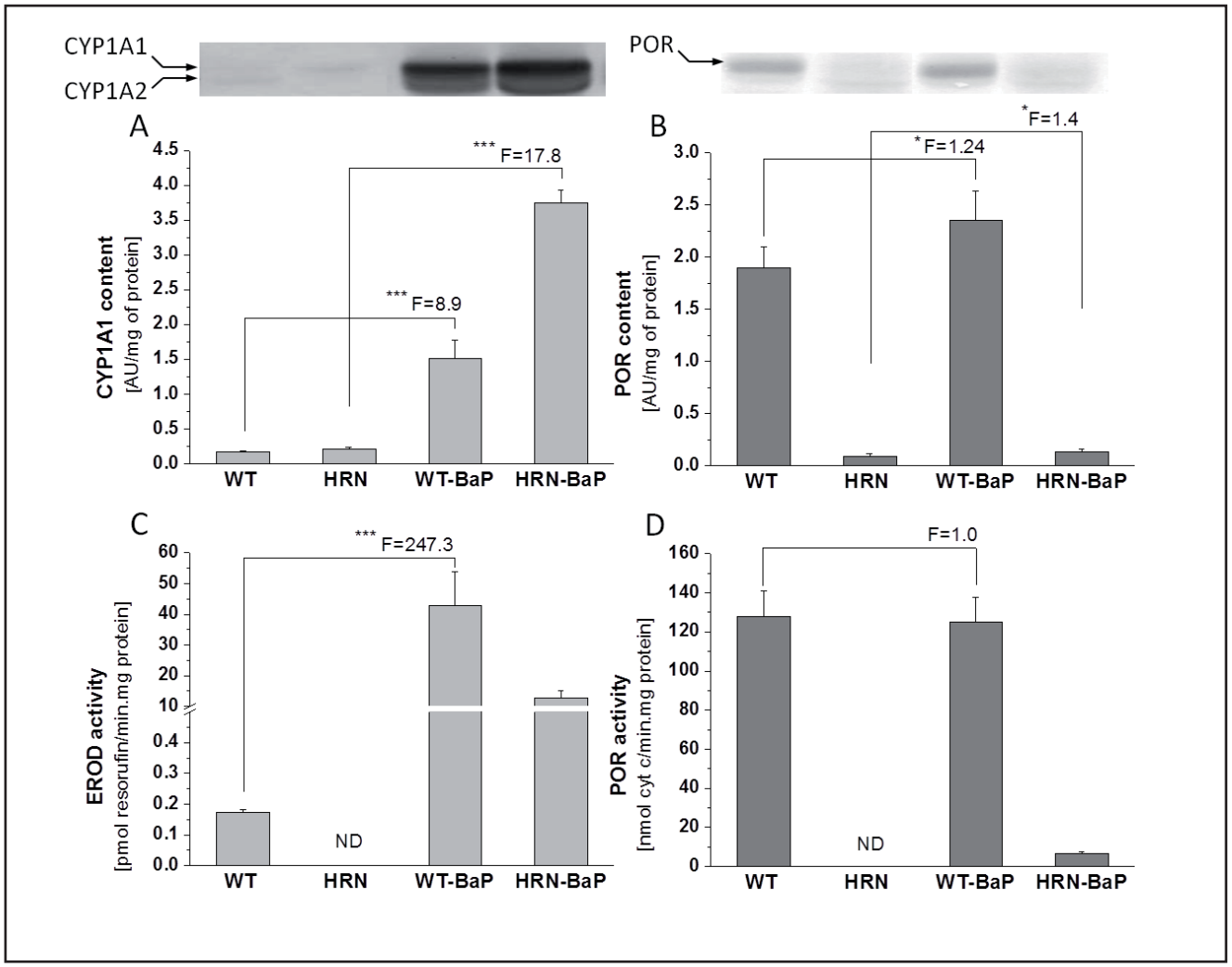

Fig. 2. Expression of cytochrome P450 (CYP) 1A1/2 (**A**) and NADPH:cytochrome P450 reductase (POR) (**B**) and their enzymatic activity [7-ethoxyresorufin *O*-deetylation (EROD) (**C**) and POR activity (**D**)] in livers of Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice, control (untreated), or treated intraperitoneally (*i.p.*) with 125 mg benzo[a]pyrene (BaP)/kg body weight daily for five days. Inset in A and B: immunoblots of microsomal CYP1A1/2 and POR from each mouse group, stained with antibody against rat CYP1A1 and rabbit POR, respectively. Pooled hepatic microsomal samples were used for analyses as described in Material and Methods. Values are given as means ± standard deviations (S.D.) (n=3). Values significantly different from untreated mice: *$p<0.05$, ***$p<0.001$. ND= not detectable. AU – arbitrary units. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as loading control (adopted from Arlt *et al.* 2008).

Western blotting method (Figure 2B). POR activity measured with cytochrome c as a substrate was, however, not detectable in hepatic microsomes of HRN mice (Figure 2D). Surprisingly, the expression level of POR was also slightly induced in liver of both WT and HRN mice treated with BaP (Figure 2B); a 1.2- and 1.4-fold increase in POR protein expression was mediated by treating the WT and HRN mice with BaP, respectively. Likewise, POR enzyme activity was detectable in hepatic microsomes from HRN mice treated with BaP (Figure 2D).

Interestingly, beside induction of expression of these enzymes by BaP, the levels of cytochrome b_5 protein has also been found to be induced by treating both mouse strains with BaP; a 1.6- and 1.7-fold increase in cytochrome b_5 protein levels was produced by BaP in livers of HRN and WT mice, respectively (Figure 3) (Arlt *et al.* 2012).

Exposure of HRN and WT mice to BaP increases activation of ellipticine to form ellipticine-derived DNA adducts

The activation of ellipticine by hepatic microsomes of HRN and WT mice was analyzed by the ^{32}P-postlabeling technique (Figure 4). In the presence of NADPH, a cofactor of POR- and CYP-dependent enzyme systems, the *ex vivo* incubations of hepatic microsomes of untreated (control) HRN and WT mice and mice treated with BaP with ellipticine and DNA led to activation of this drug to form ellipticine-derived DNA adducts (Figure 4). The DNA adduct pattern generated by ellipticine consisted of at least two adducts (spots 1

and 2 in Figure 4), which were identical to those formed *in vivo* in mice and rats treated with ellipticine (Stiborova *et al.* 2003a, 2008), each formed by 13-hydroxyellipticine (Figure 4C) or 12-hydroxyellipticine (Figure 4D) metabolites that are generated by CYP and peroxidase (see Figure 1). An additional ellipticine-derived DNA adduct, spot A, was found in DNA that had been incubated with ellipticine and mouse hepatic microsomes (Figure 4A), predominantly in microsomes isolated from HRN mice (Table 1). This ellipticine-DNA adduct was not formed by either human or rat hepatic microsomes (Stiborova *et al.* 2001, 2003b, 2004) or in mice (Figure 4E and F) or rats *in vivo* (Stiborova *et al.* 2003a, 2007b, 2008). In incubations containing hepatic microsomes of HRN and WT mice treated with BaP, an additional adduct spot, corresponding to the 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (dG-N^2-BPDE) adduct that is the major product of reaction of BaP metabolite BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) with DNA *in vitro* and *in vivo* (Arlt *et al.* 2008; Phillips & Venitt 2012) was also detected (Figure 4B). This finding indicates that residual BaP is present in microsomes isolated from livers of HRN and WT mice, and is activated by CYP1A1 in combination with microsomal epoxide hydrolase (mEH) to form this adduct. Control incubations without microsomes were free of the dG-N^2-BPDE adduct, ellipticine-DNA adducts 1 and A, but ellipticine-derived adduct 2 was always detected (data not shown). This finding is consistent with our previous results showing that this adduct is formed also non-enzymatically (Stiborova *et al.* 2001, 2003b, 2004, 2007a). In the presence of microsomes without NADPH, beside ellipticine-DNA adducts 2, a low but detectable amount of the adduct 1 (Table 1) and the dG-N^2-BPDE adduct was also found (data not shown).

Surprisingly, levels of ellipticine-derived DNA adducts formed in *ex vivo* incubations of ellipticine and DNA with hepatic microsomes from HRN mice in the presence of NADPH were only 1.4-fold lower than amounts formed by hepatic microsomes from WT mice (Figure 5 and Table 1), even though POR expression in livers of HRN mice was two orders of magnitude lower.

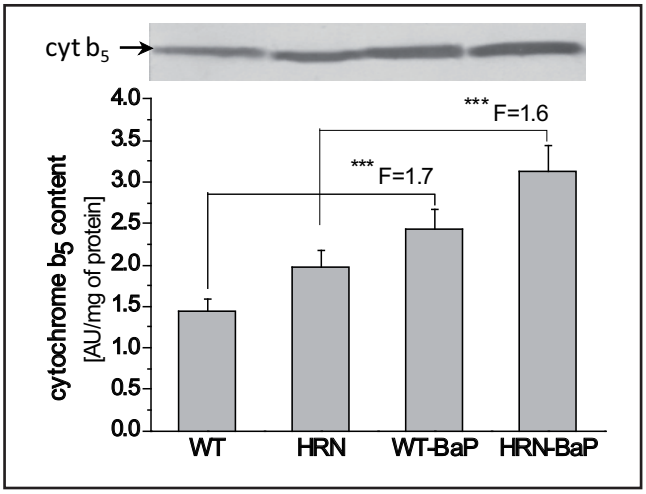

Fig. 3. Expression of cytochrome b_5 (cyt b_5) in livers of Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice, control (untreated) mice or mice treated intraperitoneally (*i.p.*) with 125 mg/kg body weight (bw) benzo[a]pyrene (BaP) for 24 hours. Pooled hepatic microsomal samples (n=3) were used for analyses as described in Material and Methods. Values significantly different from untreated mice: ***$p<0.001$. AU – arbitrary units. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as loading control (adopted from Arlt *et al.* 2012).

This finding indicates that ellipticine activation should, at least partially, be catalyzed also by enzymes, whose activities are not dependent on POR (Stiborova *et al.* 2008). Beside peroxidases that were found to activate ellipticine (see Stiborova *et al.* 2007a and the results shown below), the CYP2S1 enzyme, which is abundantly expressed in several tissues (Downie *et al.* 2005; Saarikoski *et al.* 2005; Bui *et al.* 2009a) might be such an enzyme. Namely, it was shown that it catalyzes the oxidation of compounds having polycyclic aromatic structures similar to ellipticine without participation of POR (Bui *et al.* 2009a,b). Whereas a role of a COX peroxidase is investigated in this study, the participation of CYP2S1 in ellipticine activation still awaits further examination. Therefore, the efficiency of CYP2S1 to oxidize ellipticine is planned to be investigated in our future work.

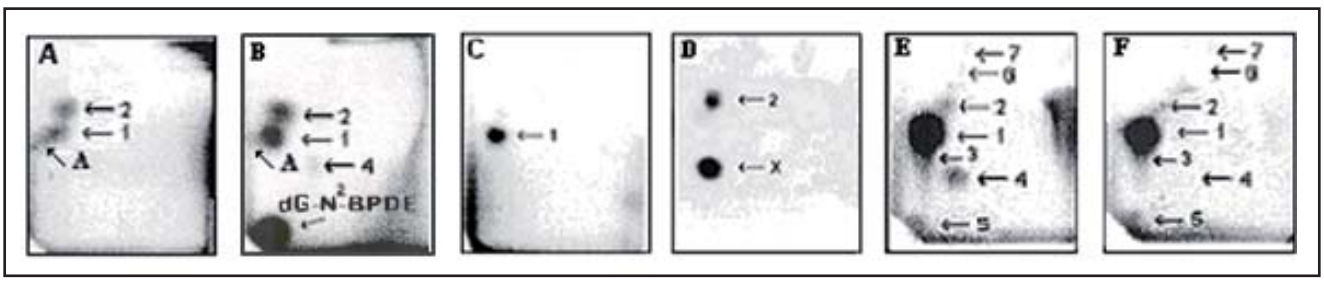

Figure 4. Autoradiographs of thin layer chromatography (TLC) maps of ^{32}P-labeled digests of calf thymus DNA reacted with ellipticine and hepatic microsomes from wild-type (WT) mice (**A**), with those of Hepatic Cytochrome P450 Reductase Null (HRN) mice treated with benzo[a]pyrene (BaP) (**B**), from calf thymus DNA reacted with 13-hydroxyellipticine (**C**) (Stiborova *et al.* 2004) and 12-hydroxyellipticine (**D**) (Stiborova *et al.* 2007a) and of DNA from livers of WT (**E**) and HRN (**F**) mice treated with 10 mg ellipticine/kg body weight (Stiborova *et al.* 2008). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay.

Tab. 1. DNA adduct formation by ellipticine activated by hepatic microsomes from either untreated (control) Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice (A) or mice treated with benzo[a]pyrene (BaP) (B).

	RAL[a] (mean/10^8 nucleotides)			
	Spot 1	**Spot 2**	**Spot A**	**Total**
HRN mice hepatic microsomes + NADPH	5.9±0.3	2.3±0.2	6.7±0.3	14.9±0.8
HRN mice hepatic microsomes + NADPH + α-lipoic acid	1.2±0.1	1.7±0.1	0.9±0.1	3.8±0.3
HRN mice hepatic microsomes + NADPH + α-NF	2.8±0.2	2.2±0.2	1.0±0.1	6.0±0.5
HRN mice hepatic microsomes + NADPH + ketoconazole	2.5±0.2	2.0±0.2	1.0±0.1	5.5±0.4
HRN mice hepatic microsomes + arachidonic acid	2.9±0.2	2.4±0.2	ND	5.3±0.4
HRN mice hepatic microsomes without cofactor	0.2±0.04	1.9±0.2	ND	2.1±0.2
WT mice hepatic microsomes + NADPH	13.0±0.9	3.2±0.2	4.8±0.4	21.0±1.5
WT mice hepatic microsomes + NADPH + α-lipoic acid	7.6±0.6	2.7±0.2	0.7±0.1	11.0±0.9
WT mice hepatic microsomes + NADPH + α-NF	6.5±0.5	2.7±0.2	1.2±0.1	10.4±0.8
WT mice hepatic microsomes + NADPH + ketoconazole	6.0±0.5	2.1±0.2	1.0±0.1	8.1±0.7
WT mice hepatic microsomes + arachidonic acid	5.7±0.4	4.6±0.3	ND	10.3±0.8
WT mice hepatic microsomes without cofactor	0.3±0.02	2.0±0.2	ND	2.3±0.2
BaP-HRN mice hepatic microsomes + NADPH	22.6±1.3	5.7±0.3	4.2±0.2	32.5±1.6
BaP-HRN mice hepatic microsomes + NADPH + α-lipoic acid	21.5±1.0	3.4±0.2	ND	24.9±1.3
BaP-HRN mice hepatic microsomes + NADPH + α-NF	30.6±1.5	6.7±0.3	ND	37.3±1.9
BaP-HRN mice hepatic microsomes + NADPH + ketoconazole	23.1±1.1	5.6±0.3	ND	28.7±1.4
BaP-HRN mice hepatic microsomes + arachidonic acid	10.1±0.5	2.3±0.1	ND	12.4±0.7
BaP-HRN mice hepatic microsomes without cofactor	0.2±0.01	1.9±0.1	ND	2.1±0.1
BaP-WT mice hepatic microsomes + NADPH	181.7±6.3	8.3±0.4	28.7±1.2	218.7±8.9
BaP-WT mice hepatic microsomes + NADPH + α-lipoic acid	82.9±4.0	5.3±0.3	4.1±0.2	92.3±4.4
BaP-WT mice hepatic microsomes + NADPH + α-NF	83.5±4.1	4.1±0.2	3.8±0.2	91.4±4.4
BaP-WT mice hepatic microsomes + NADPH + ketoconazole	164.3±8.1	7.4±0.4	26.5±1.2	198.2±9.1
BaP-WT mice hepatic microsomes + arachidonic acid	8.1±0.4	2.6±0.1	ND	10.7±0.5
BaP-WT mice hepatic microsomes without cofactor	0.5±0.03	2.5±0.1	ND	3.0±0.1

[a] Mean RAL (relative adduct labeling) ± standard deviation (S.D.) of four determinations (duplicate analyses of two independent *in vitro* incubations). ND - not detected (the detection limit of RAL was $1/10^{10}$ nucleotides). For adduct spots 1,2 and A, see Figure 4. NADPH - nicotinamidadeninedinucleotide phosphate (reduced); α-NF - α-naphthoflavone.

Treatment of both HRN and WT mice with BaP resulted in increased levels of ellipticine-derived DNA adducts formed in *ex vivo* incubations of ellipticine with hepatic microsomes of these mice relative to microsomes of untreated mice. A 2.2- and 10.4-fold increase in formation of amounts of ellipticine-derived DNA adducts was caused by exposure of HRN and WT mice to BaP, respectively, of them the levels of adduct 1 were predominantly increased. In contrast, a low (adduct 2 in both HRN and WT mice) or no increase (adduct A in HRN mice) in DNA adduct formation was produced by BaP (Table 1).

Since CYP1A, 3A and peroxidase enzymes were found to activate ellipticine (Stiborova *et al.* 2001, 2003b, 2004, 2007a), we investigated the modulation of ellipticine-derived DNA adduct formation by cofactors and selective inhibitors of these enzymes using hepatic microsomes isolated from both untreated (control) mouse strains and the animals treated with BaP.

α-Lipoic acid, a selective inhibitor of POR (Slepneva *et al.* 1995), decreased ellipticine-DNA adduct formation by 25–75% with mouse hepatic microsomes. Therefore, some activity in both HRN and WT mice is dependent on POR. α-NF, an inhibitor of CYP1A1 and 1A2 (Rendic & DiCarlo 1997), decreased the levels of ellipticine-DNA adducts generated by hepatic microsomes of untreated HRN and WT mice as well as of WT mice treated with BaP, to essentially equal extent (to 40% in microsomes of WT and BaP-treated WT mice and to 50% in those of HRN mice), but had no inhibition effect of activation of ellipticine with microsomes of HRN mice treated with BaP (Figure 5, Table 1). This finding proved a role of CYP1A in activation of WT and HRN mice, and mainly in WT mice in which CYP1A

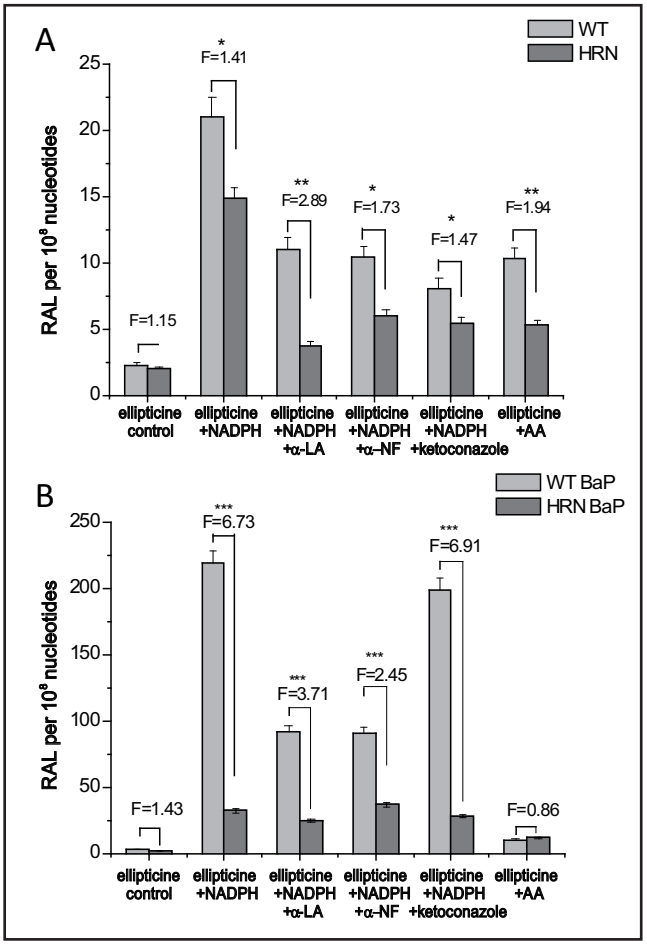

Figure 5. DNA adduct formation by ellipticine activated with microsomes isolated from livers of untreated Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice **(A)** and from those treated with BaP **(B)** as determined by thin layer chromatography (TLC) ^{32}P-postlabeling. F = fold higher DNA adducts levels in microsomes from WT mice compared to HRN mice. Columns: Mean RAL (relative adduct labeling) ± standard deviations (S.D.) shown in the figure represent total levels of DNA adducts of four determinations (duplicate analyses of two independent *in vitro* incubations). Values significantly different from HRN mice:**p*<0.05, ***p*<0.01, ****p*<0.001. Control = without cofactor; AA = arachidonic acid; α-NF = α-naphthoflavone; α-LA = α-lipoic acid. ND = not detected.

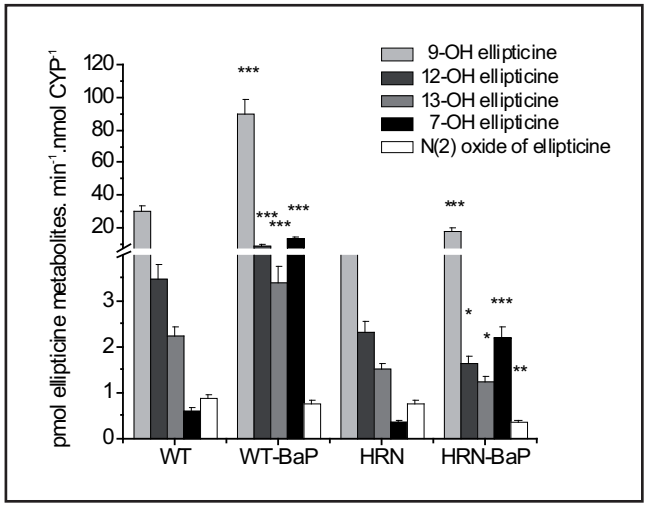

Figure 6. Levels of ellipticine metabolites formed by hepatic microsomes (0.2 mg protein) of Hepatic Cytochrome P450 Reductase Null (HRN) and wild-type (WT) mice from 10 μM ellipticine and by hepatic microsomes of HRN and WT mice treated intraperitoneally (*i.p.*) with 5×125 mg of benzo[a]pyrene (BaP) per kg of body weight. Levels of ellipticine metabolites were determined by high performance liquid chromatography (HPLC) (Stiborova *et al.* 2004, 2006a) and are averages ± standard deviations of triplicate incubations. Values significantly different from untreated mice: **p*<0.05, ***p*<0.01, ****p*<0.001.

expression was induced with BaP. Induction of CYP1A in HRN mice resulted, however, in opposite process; induced CYP1A enzymes seem to increase ellipticine detoxification (see also below). Ketoconazole, a selective inhibitor of CYP3A enzymes (Rendic & DiCarlo 1997; Ueng *et al.* 1997), inhibited formation of ellipticine-DNA adducts in hepatic microsomes of untreated (control) HRN and WT mice, by ~60%, but its effect was much lower in hepatic microsomes of BaP-treated HRN and WT mice, only by ~10% (Figure 5, Table 1). These results point additionally to CYP3A enzymes as having a role in ellipticine-DNA adduct formation in mouse livers, but their contributions to this process was decreased by induction of CYP1A1 with BaP.

Arachidonic acid, a cofactor for COX-dependent oxidation (Eling *et al.* 1990; Eling & Curtis 1992; Stiborova *et al.* 2004, 2005; Arlt *et al.* 2006), mediated formation of DNA adducts 1 and 2 by ellipticine in hepatic microsomes of all mice used, but was much less effective than NADPH (Figure 5, Table 1).

Ellipticine metabolites formed in hepatic microsomes from all mouse strains used in this work were analogous; 9-hydroxy-, 12-hydroxy-, 13-hydroxy, 7-hydroxyellipticine and N^2-oxide of ellipticine were formed (Figure 6). However, the amounts of individual metabolites in HRN and WT mice, either control (untreated) or treated with BaP, were different. In the case of untreated mice, 9-hydroxyellipticine levels were only one sixth, while the amounts of 13-hydroxy- and 12-hydroxyellipticine, were about one half in incubations with HRN microsomes compared with the levels in incubations with WT microsomes. Exposure of both HRN and WT mouse strains to BaP resulted in an increase in formation of 9-hydroxy- and 7-hydroxyellipticine, the metabolites that are considered to be detoxification products. More than 3- and 23-fold higher amounts of 9-hydroxy-and 7-hydroxyellipticine were formed by induction of CYP1A with BaP in WT mice, respectively. Likewise, treatment of HRN mice with BaP resulted in 3.8- and 5.5-fold higher levels of 9-hydroxy-and 7-hydroxyellipticine generated by hepatic microsomes of these mice, respectively (Figure 6). This result is consistent with findings of the former studies, where CYP1A1 and 1A2 were found

to be the major enzymes forming these metabolites (Stiborova *et al.* 2004; Kotrbova *et al.* 2006, 2011). However, treatment of WT mice with BaP also resulted in up to 2.5-fold increased levels of 13-hydroxy- and 12-hydroxyellipticine (Figure 6). Such an increase might result not only from induction of CYP1A enzymes by BaP, but also from induction of cytochrome b_5 by this compound (see Figure 3). Indeed, cytochrome b_5 alters the ratio of ellipticine metabolites formed by CYP1A1 and 1A2 increasing the amounts of 13-hydroxy- and 12-hydroxyellipticine (Kotrbova *et al.* 2011). The increased formation of both these ellipticine metabolites was associated with increased levels of ellipticine-DNA adducts that are generated by reaction of DNA with these two metabolites (see Figs. 5, 6 and Table 1 and the results shown in Kotrbova *et al.* 2011 and Stiborova *et al.* 2012b). In the case of HRN mice, no increase in 13-hydroxy- and 12-hydroxyellipticine formation was found by treatment of these mice with BaP; a pattern of CYP1A1/2, POR and cytochrome b_5 seems not to be appropriate to favor formation of these metabolites. Nevertheless, ellipticine-derived DNA adducts were increased when HRN mice were treated with BaP (Figs. 5, 6 and Table 1). There might be at least one reason for the above finding; activities of additional enzymes, beside CYPs, which are present in hepatic microsomes of HRN mice and are capable of activating ellipticine such as COX (Stiborova *et al.* 2007a) are induced by BaP. Indeed, an increase in formation of ellipticine-derived DNA adducts, predominantly the adduct 1, in *ex vivo* incubations of ellipticine with microsomes of HRN mice treated with BaP and arachidonic acid, a cofactor of COX, was found. Formation of adduct 1 from ellipticine13-ylium generated from COX-dependent formation of 6,13-didehydroellipticine (ellipticine mehylene-imine) (Figure 1) should therefore also occur in hepatic microsomes of HRN mice treated with BaP.

CONCLUSION

Using mouse models in which expression of enzymes metabolizing ellipticine were modulated either by deletion in liver (POR) or by induction with BaP (CYP1A1/2 and cytochrome b_5), we confirm a major role of CYP1A and 3A and participation of COX in ellipticine activation in these mice. Treatment of mice with BaP increases an impact of CYP1A on ellipticine activation. The results found also emphasize that a pattern of expression levels of these enzymes plays a crucial role in their impact on this process. Therefore, this study forms the basis to further predict the susceptibility of human cancers to ellipticine and suggests this alkaloid for treatment in combination with CYP gene transfer (Ma & Waxman 2007; Lu *et al.* 2009) increasing the anticancer potential of this pro-drug.

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