ORIGINAL ARTICLE

Effect of dihydromyricetin on benzo[a]pyrene activation in rats

Petr Hodek [1], Petra Fousova [1], Eliska Brabencova [1], Michaela Moserova [1], Petr Pavek [2], Eva Anzenbacherova [3], Jaroslav Brotanek [4], Jiri Hudecek [1], Eva Frei [5], Marie Stiborova [1]

1 Department of Biochemistry, Faculty of Science, Charles University in Prague, Prague, Czech Republic
2 Department of Pharmacology and Toxicology, Faculty of Pharmacy, Charles University in Prague, Hradec Králové, Czech Republic
3 Departments of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacky University, Olomouc, Czech Republic
4 2nd Department of Internal Medicine, Thomayer University Hospital, Prague, Czech Republic
5 Division of Preventive Oncology, National Center for Tumor Diseases, German Cancer Research Center (DKFZ), Heidelberg, Germany

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

Abstract

OBJECTIVES: Flavanol dihydromyricetin (DHM) has been shown to counteract acute ethanol (EtOH) intoxication and reduce excessive EtOH consumption. Since this flavonoid is being considered for human use, the in vivo study of DHM interactions with the cytochrome P450 (CYP) multienzyme system in the respect of metabolic activation of a model food-born carcinogen, benzo[a]pyrene (BaP), is of high importance. Flavonoids of known properties, alpha-naphthoflavone (ANF) and beta-naphthoflavone (BNF) were included into the study to compare their and DHM effects on BaP-DNA adduct formation.

METHODS: The flavonoids were administered by oral gavage either 72 hrs prior or simultaneously with a single dose of BaP to experimental rats. The expression of CYP1A1/2 enzymes was examined based on the enzymatic activity with a marker substrate, 7-ethoxyresorufin, and on Western blots. The nuclease P1 version of the ^{32}P-postlabeling assay was used to detect and quantify covalent DNA adducts formed by BaP.

RESULTS: Treatment of rats with a single dose of DHM or ANF prior to or simultaneously with BaP did not produce an increase in levels of CYP1A1 and in formation of BaP-DNA adducts in liver. BNF, a known inducer of CYP1A1, showed a synergistic effect on BaP-mediated CYP1A1 induction and BaP activation in liver. Contrary to that, in small intestine the stimulatory effect of BNF on both parameters was not detected. Animal pre-treatment with DHM or ANF before BaP administration resulted in a significant elevation of BaP-DNA adducts, namely in the distal part of small intestine, while the CYP1A1 mediated 7-ethoxyresorufin-O-deethylation (EROD) was decreased markedly. It is important to note that under all regimens of animal treatment, DHM or ANF produced the higher inhibitory effect on the BaP-DNA adduct formation and BaP-induced EROD activity of

CYP1A1 when administered simultaneously than sequentially with BaP. Our data show that DHM or ANF did not enhance the BaP-activation leading to BaP-mediated genotoxicity (the formation of BaP-DNA adducts) in rat liver, however, in small intestine the pre-treatment of rats with these flavonoids may enhance BaP genotoxicity.

CONCLUSIONS: The data indicate that the intake of DHM prior to or simultaneously with the administration of BaP may increase the risk of a BaP-induced tumorigenesis in small intestine.

Abbreviations:

AHR	- aryl hydrocarbon receptor
ANF	- α-naphthoflavone
BaP	- benzo[a]pyrene
BCIP	- 5-bromo-4-chloro-3'-indolyphosphate
BPDE	- BaP-7,8-dihydrodiol-9,10-epoxide
BNF	- β-naphthoflavone
b.w.	- body weight
B5	- cytochrome b_5
CYP	- cytochrome P450
CYPOR	- NADPH:cytochrom P450 oxidoreductase
dG-N2-BPDE	- 10-(deoxyguanosin-N2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-benzo[a]pyrene
DHM	- dihydromyricetin
DMSO	- dimethyl sulfoxide
EROD	- 7-ethoxyresorufin-O-deethylation
EtOH	- ethanol
GST	- glutathione S-transferase
hrs	- hours
mEH	- microsomal epoxide hydrolase
MS	- microsomes
NADPH	- nicotinamidadeninedinucleotide phosphate (reduced)
NBT	- nitro-blue tetrazolium
p.o.	- per os
PAH	- polycyclic aromatic hydrocarbons
P-gp	- P-glycoprotein
RAL	- relative adduct labeling
SD	- standard deviation
SDS	- sodium dodecyl sulfate
UT	- untreated animals

INTRODUCTION

Dihydromyricetin, (2R,3R)-3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-2,3-dihydrochromen-4-one (DHM) depicted in Figure 1, is credited with hepatoprotective effects observed in rodents as well as with anti-tumor ability, inhibiting e.g. progression and metastasis of prostate cancer (Ni *et al.* 2012). Moreover, it is highly effective in counteracting acute EtOH intoxication, in reducing excessive EtOH consumption *via* antagonizing acute EtOH-induced potentiation of γ-aminobutyric acid receptors and in increasing activity of alcohol and acetaldehyde dehydrogenases (Shen *et al.* 2012; Chen *et al.* 2006). This compound found in Japanese Raisin Tree, *Hovenia dulcis* (Hyun *et al.* 2010), belongs to numerous groups of flavonoids, which are of a great interest since they provide a wide variety of biological activities (Hodek, 2012). In addition to their powerful antioxidant properties, they are suggested to be anti-cancer agents acting at various stages of carcinogenesis. The flavonoids are, because of their natural origin, generally accepted as health-promoting compounds and safe for the human use. Their extensive intake as food supplements may, however, cause potential threats resulting e.g. from altered metabolism of endogenic compounds and from flavonoid pro-oxidant activity (Hodek *et al.* 2009a). Much less attention is being paid to flavonoid-drug or flavonoid-carcinogen interactions. These interactions frequently arise from flavonoid abilities to modulate catalytic activity of xenobiotic metabolizing enzymes such as cytochromes P450 (CYPs). Flavonoids are inhibitors, inducers and even activators of CYPs. α-Naphthoflavone (ANF, Figure 1), may serve as a good example of versatile activities of flavonoids. ANF represents a well documented inhibitor of metabolic reactions mediated by CYPs of a 1A subfamily (Bauer *et al.* 1995), but at the same time, ANF acts as a positive modulator stimulating catalytic activity of CYP3A4 (Hodek *et al.* 2002). After the oral administration of ANF, high plasma concentrations (reached within 30 min) allow the modulation of CYP activities (Wang and Morris, 2008). However, at its lower concentrations, inefficient e.g. for CYP1A1 inhibition, ANF can initiate the expression of biotransformation enzymes (Ferguson, 2001). It has been shown that ANF increases the rate of the electron transfer from NADPH:cytochrom P450 oxidoreductase (CYPOR) to appropriate electron acceptors such as CYPs (Hodek *et al.* 2009b). Since in the endoplasmic reticulum one CYPOR molecule provides electrons for many CYPs, any raise in the CYPOR activity can stimulate the CYP1A1-mediated metabolism. Moreover, ANF was shown to induce cytochrome b_5 (B5), which similarly to CYPOR, supplies the electrons for CYP catalyzed reactions (Blaich *et al.* 1987) and most likely acts as a positive modulator of several CYPs (Borek-Dohalska *et al.* 2001; Hodek *et al.* 2002). Furthermore, ANF activates epoxide hydrolase, the enzyme which is essential for formation of an ultimate carcinogen from BaP (Glatt *et al.* 1983).

Another flavonoid, β-naphthoflavone (BNF), a structural analogue of ANF (see Figure 1), significantly elevates protein and mRNA levels of the CYP1A1 and 1A2 in rat liver. In hepatic microsomes of BNF-treated animals, the CYP1A-mediated 7-ethoxyresorufin O-deethylation (EROD) was increased by almost two orders of magnitude compared to the untreated control (Canivenc-Lavier *et al.* 1996). The CYP1A1 induction by BNF proceeds *via* an aryl hydrocarbon receptor (AHR), which binds as a heterodimer with the AHR nuclear transporter to DNA motifs, known as AHR response elements (Nebert *et al.* 2004). However, the process of the BNF-mediated CYP induction is rather slow; in rat small intestine the maximal induction of CYP1A1 was reached approx. 24 hrs after the BNF application (Zhang *et al.* 1997).

In the animal study administered flavonoids might be entirely converted to their metabolites and conju-

gates in the gastrointestinal tract (Wang and Morris, 2008). Thus the effect attributed to flavonoid is in fact caused by some of the flavonoid derivatives or degradation products. Orally administered xenobiotics are possibly absorbed to blood in their unchanged form and/or after the first-pass intestinal metabolism, further metabolized in liver by CYPs and/or conjugation enzymes, and *via* bile returned back as glucuronides and sulfates to small intestine, where they are exposed to intestinal microflora (Wang and Morris, 2008; Hodek, 2012). Hydrolytic microbial enzymes, such as sulfatase and glucuronidase, are of a particular interest since they may release parent metabolites from their conjugates. These metabolites can be reabsorbed into the enterocytes, and thus circulate between the gut lumen and the epithelial cells (Nanno *et al.* 1986). Thus, an enormous metabolic potential of intestinal microflora is involved in the overall metabolic fate of ingested compounds (Meinl *et al.* 2009; Bezirtzoglou, 2012). As CYPs are frequently involved in carcinogen activation, the effect of flavonoids on these enzymes should be always considered and carefully examined (Hodek *et al.* 2009b).

Benzo[a]pyrene (BaP) is a chemical carcinogen that is commonly used as a model carcinogenic compound in animal studies. Humans are exposed to BaP mostly in food e.g. in smoked or flame-exposed meat and/or in a cigarette smoke (Phillips, 1999). BaP has been found to cause the development of tumors in lung, skin and liver. The metabolic activation of BaP to reactive species binding to DNA proceeds similarly to other polycyclic aromatic hydrocarbons (PAH) mainly by CYP1A1 in combination with microsomal epoxide hydrolase (mEH), while its detoxification is mainly catalyzed by conjugation enzymes such as UDP-glucuronosyltransferase and glutathione S-transferase (Zhang *et al.* 2013; Fang *et al.* 2001; Aimova *et al.* 2008). The scheme in Figure 2 shows the pathways leading to formation of BaP derivatives covalently binding the DNA molecule (Stiborova *et al.* 2014). First, CYP1A1 oxidizes BaP to an epoxide that is then converted to a dihydrodiol by mEH (*i.e.* BaP-7,8-dihydrodiol); then further bio-activation 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. 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 the reaction of BPDE with DNA *in vitro* and *in vivo* (Bauer *et al.* 1995; Arlt *et al.* 2008; 2012). BaP is, however, oxidized also to other metabolites such as other dihydrodiols, BaP-diones and hydroxylated metabolites (Bauer *et al.* 1995; Indra *et al.* 2013; Stiborova *et al.* 2014). Of them, BaP-9-ol is a precursor of 9-hydroxy-BaP-4,5-epoxide that can form another adduct with deoxyguanosine in DNA (Figure 2) (Schoket *et al.* 1989; Nesnow *et al.* 1993; Fang *et al.* 2001; Phillips, 2005; Stiborova *et al.* 2014).

However, the role of CYP1A1 in BaP metabolism seems to be more complex, since this CYP has also been shown to detoxify BaP in liver and gastrointestinal tract of mice (Uno *et al.* 2004; Arlt *et al.* 2008; 2012). 3-Hydroxy-BaP formed by CYP1A1 is considered the major detoxification product of BaP oxidation (Huang *et al.* 1986). In addition to mEH, B5 plays a significant role in CYP1A1 dependent activation of BaP. This hemoprotein may, besides CYPOR, alternatively provide electron(s) required for CYP1A1 enzyme activity. Recently, the involvement of B5 in the CYP1A1-mediated activation of BaP *in vivo* has been decribed (Stiborova *et al.* 2014). Moreover, the electron transfer from reduced B5 to CYP is even faster than that from CYPOR (Schenkman and Jansson, 2003).

Treatment of animals with BaP results in an increase in the expression of CYP1A1 protein and its enzyme activity. In this respect, BaP is a prototypic PAH inducing CYP1A subfamily *via* the AHR mechanism. Although *in vitro* experiments carried out with CYP1A1-induced liver microsomes indicate a clear connection between CYP1A1 levels and DNA adduct formation, the *in vivo* studies with BaP-treated animals do not show such straightforward link (Uno *et al.* 2004; Arlt *et al.* 2008; 2012; Hodek *et al.* 2011). In animal studies, numerous additional factors, e.g. BaP transport and organ distribution, BaP-induction of conjugation enzymes participating in BaP detoxification, intestinal microflora possessing an enormous metabolic capacity, possibly contribute to the overall metabolic fate of BaP (Buesen *et al.* 2003; Gupta *et al.* 1989; Hughes and Rowland, 2000).

In the present study, the causal relation between the DHM exposure and activation of BaP, was investigated. The rat was used as an animal model. For comparison, ANF and BNF were also included for testing. To mimic the human exposure, tested compounds were admin-
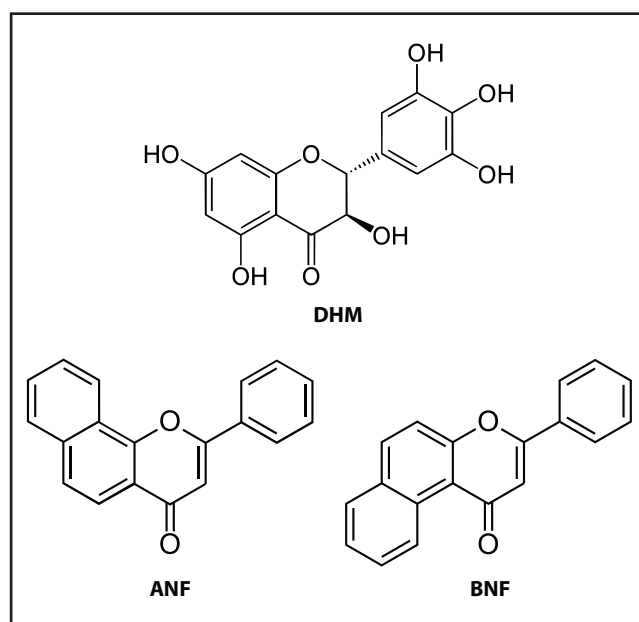
Figure 1. The structure of flavonoids: dihydromyricetin, **DHM**; α-naphthoflavone, **ANF**; β-naphthoflavone, **BNF**.

istered to rats by oral gavage. Impact of flavonoids on BaP activation was determined based on BaP-DNA adduct formation in conjunction with CYP1A1 metabolic activity.

MATERIAL AND METHODS

Chemicals

α-Naphthoflavone (7,8-benzoflavone), β-naphthoflavone (5,6-benzoflavone), NADPH, bicinchoninic acid, resorufin, 7-ethoxyresorufin, 7-methoxyresorufin, and anti-chicken IgG alkaline phosphatase conjugate, 5-bromo-4-chloro-3'-indolyphosphate/nitro-blue tetrazolium (BCIP/NBT) tablets were purchased from Sigma Chemical Co., (St. Louis, MO). Dihydromyricetin was provided from APIchem (Shanghai, PRC). Specific chicken anti-CYP1A1/2 and anti-CYP1A2 antibodies were prepared by Dr. Hodek as described (Hodek *et al.* 2013b). All chemicals used in the experiments were of analytical grade purity or better.

Animal experiments and preparation of microsomes.

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic). Male Wistar rats (150 g, AnLab, Czech Republic), were housed in groups of 3 in wire cages at 22°C with a 12 hrs light/dark period and *ad libitum* diet (ST-1 diet from

Velaz, Czech Republic) and water access. Tested flavonoids, DHM, ANF, BNF, and carcinogen, BaP, were dissolved in sunflower oil and administered *p.o.* by gastric gavages (1 ml) in two regimens: either the particular flavonoid (120 mg/kg b.w.) in a single dose 72 hrs prior the application of BaP (150 mg/kg b.w.) or the flavonoid (120 mg/kg b.w.) simultaneously with BaP (150 mg/kg b.w.). Animals of all experimental groups were sacrificed 24 hrs after the treatment with BaP. The control group was treated with 1 ml of sunflower oil only. Microsomes (MS) were prepared from 3 sections of dissected small intestine (proximal, middle, distal parts) and the whole liver, as described elsewhere (Krizkova *et al.* 2008, Hodek *et al.* 2011). Microsomal fractions were stored at −80°C before use. Protein concentrations in the microsomal fractions were determined using the bicinchoninic acid protein assay with bovine serum albumin as the standard (Weichelman *et al.* 1988). The concentration of CYP was estimated according to Omura and Sato (1964) based on the absorption of the of reduced CYP complex with carbon monoxide.

Western blot analysis

The CYP1A1 and 1A2 proteins were detected by Western blotting on the Immobilon-P membrane (Millipore, Bedford, MA) using specific chicken anti-CYP1A1/2 and anti-CYP1A2 antibodies (30 µg/ml). For sodium dodecyl sulfate (SDS)-electrophoresis (10% polyacryl-
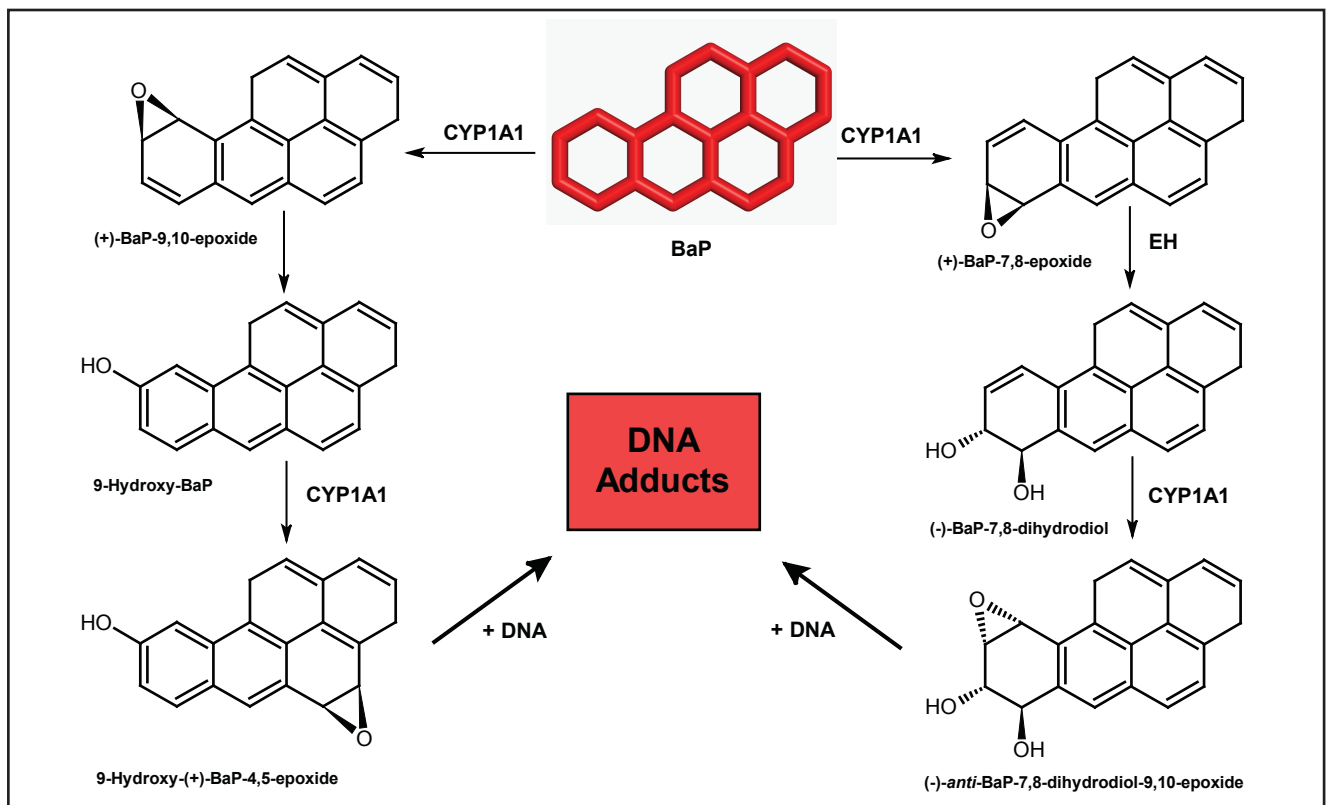
Figure 2. The metabolic activation of benzo[a]pyrene (**BaP**) *via* cytochrome P450 (**CYP**) 1A1 and epoxide hydrolase (**EH**) into an ultimate carcinogens forming adducts with DNA (Stiborova *et al.* 2014).

amide gel), 30 µg protein/well of small intestine and liver microsomes was applied. Western blotting was carried out as described earlier (Krizkova *et al.* 2009). Visualization was performed using an anti-chicken IgG alkaline phosphatase-conjugated antibody (diluted 1:2 000) and BCIP/NBT substrate tablets (10 mg) for alkaline phosphatase.

Enzyme assays

Each microsomal sample was analyzed for specific CYP1A1 activity by monitoring 7-ethoxyresorufin *O*-deethylation (EROD), according to the method described by Burke and Mayer (1974). Briefly, incubation mixtures, contained in the final volume (150 µl) 100 mM potassium phosphate buffer (pH 7.4): 0.5 mM NADPH, 0.5 mg of microsomal protein and 2.2 µM 7-ethoxyresorufin (from 1 mM stock in dimethyl sulfoxide, DMSO). The reaction was initiated by adding NADPH. The formation of resorufin was monitored by its fluorescence (excitation and emission wavelengths of 530 and 585 nm, respectively) in 1 min. intervals for 10 minutes at room temperature on luminescence spectrometer (PerkinElmer LS-55 equipped with 96-well plate reader). The dealkylation rate was estimated on the basis of a resorufin standard curve.

^{32}P-postlabeling analysis of BaP-DNA adduct

The DNA from liver and small intestine was isolated by standard phenol/chloroform extraction. DNA adducts formed from BaP were determined by ^{32}P-postlabeling analysis using the nuclease P1 enrichment version (Stiborova *et al.* 2001, 2003, 2013, 2014), and thin-layer
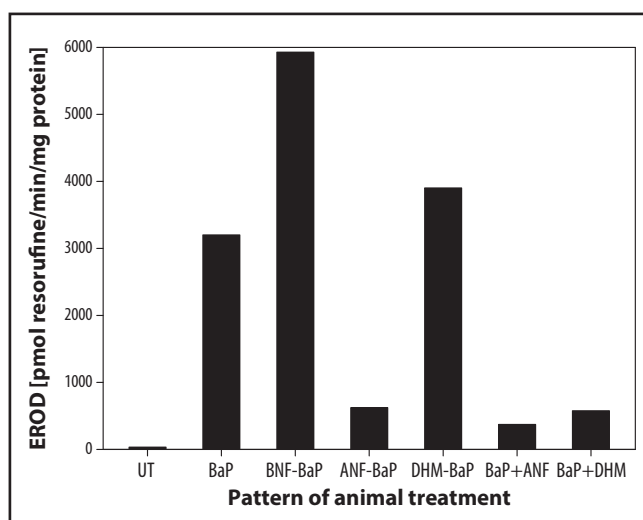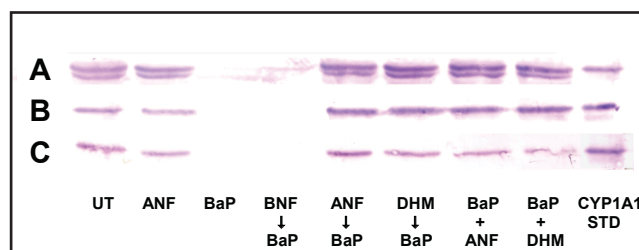
chromatography (TLC) was performed as described (Arlt *et al.* 2008). After chromatography, TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, USA) and DNA adduct levels (RAL, relative adduct labeling) were calculated as described (Stiborova *et al.* 2001, 2003; 2013, 2014). Results were expressed as DNA adducts/10^8 nucleotides.

RESULTS

To examine the effect of DHM on BaP genotoxicty, the DNA adduct formation from activated BaP was analyzed in the liver and small intestine of rats treated with these compounds. In addition to DHM or ANF and BNF, prototype CYP1A1 inhibitor and inducer, respectively, were tested, too. Flavonoids, DHM, ANF and BNF, were administered either 72 hrs before BaP animal treatment or simultaneously with BaP. In both cases animals were sacrificed 24 hrs after BaP exposure. The induction of CYP1A1/2 was evaluated in microsomal samples of liver and small intestine based on its specific metabolic activity (EROD) as well as by immunodetection of CYP1A1 protein expression.

The modulation of CYP1A1 activity and DNA adduct formation in liver

First, the induction of CYP1A1 was examined in liver microsomes. Figure 3 shows that only BNF pretreatment of BaP-administered animals significantly increased EROD activity compared to animals exposed solely to BaP. This observation suggests the synergistic effect of BNF on BaP-mediated CYP1A1 induction. In contrast to these results, when ANF or DHM were applied simultaneously with BaP a profound drop in EROD activities was detected. In that regimen both flavonoids reversed the effect of BaP. Likewise, ANF when administered 3 days before BaP decreased the EROD activity, too. The application of DHM at this sequential regimen, however, did not exert any inhibition, but EROD activity was moderately stimulated (in comparison to BaP alone). These results are in agree-


Figure 3. Determination of EROD in liver microsomes. Microsomal samples were prepared from animals exposed to BaP alone or in combination with DHM, ANF and BNF administered either 72 hrs before BaP animal treatment (BNF-BaP, ANF-BaP, DHM-BaP) or simultaneously with BaP (BaP+ANF, BaP+DHM). In all cases animals were sacrificed 24 hrs after BaP exposure. Control microsomes were prepared from animals treated with sunflower oil only (UT). Each value represents the mean of two separate analyses.

Figure 4. Immunodetection of CYP1A1/2 in liver and intestinal microsomes. Microsomal samples were prepared from liver (line **A**), proximal (line **B**) and distal (line **C**) parts of small intestine of animals exposed to BaP alone or in combination with DHM, ANF and BNF. The flavonoids were administered either 72 hrs before BaP animal treatment (BNF→BaP, ANF→BaP, DHM→BaP) or simultaneously with BaP (BaP+ANF, BaP+DHM). Control animals were treated with sunflower oil only (UT). Blots were developed with chicken antibody recognizing CYP1A1 and 1A2.

ment with immunodetection of CYP1A1 protein in liver microsomes (see Figure 4). When animals were treated solely with a single dose of BNF, DHM or ANF (no BaP), the BNF application only resulted in a large increase of EROD activity (~63% of BaP induction), while DHM showed the only slight increase in EROD activity and ANF had no effect on this CYP1A activity (data not shown).

Second, to reveal the effect of DHM on BaP activation, the DNA adduct formation by BaP, was examined. Treatment of rats with BaP resulted in formation of three BaP-derived DNA adducts (assigned as adduct
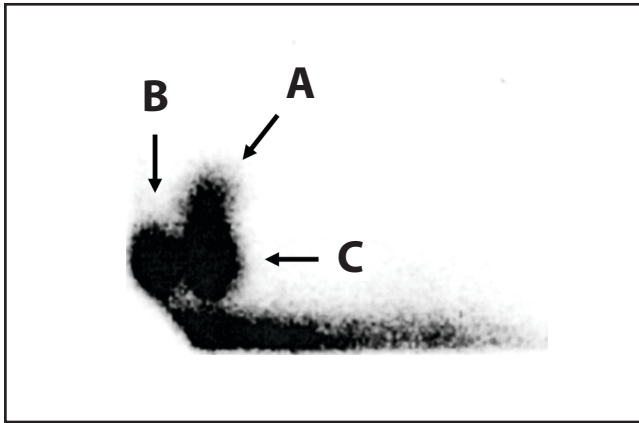
Figure 5. Representative autoradiography of BaP-derived DNA adducts formed in the liver and small intestine of rats exposed to BaP assayed by ^{32}P-postlabeling (BaP-DNA adducts formed in the liver are shown). Spots were identified as 10-(deoxyguanosin-N^2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-benzo[a]pyrene (**A**), adduct derived from a reaction of deoxyguanosine in DNA with 9-hydroxy-BaP-4,5-epoxide (**B**), and another BaP–DNA adduct of unknown structure (**C**).
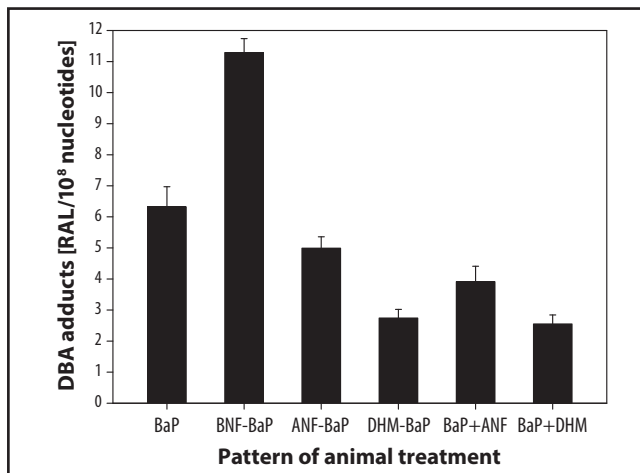
Figure 6. Formation of BaP-DNA adducts in liver. Rats were exposed to BaP alone or to BaP in combination with DHM, ANF and BNF administered either 72 hrs before BaP animal treatment (BNF-BaP, ANF-BaP, DHM-BaP) or simultaneously with BaP (BaP+ANF, BaP+DHM). In all cases animals were sacrificed 24 hrs after BaP exposure. DNA adducts were determined using ^{32}P-postlabeling assay in DNA samples from liver. Each value represents the mean of three separate analyses ± SD. Data are expressed in RAL – relative adduct labeling per 10^8 nucleosides.

spots A, B, and C in Figure 5) detectable by ^{32}P-postlabeling in the liver and the proximal and distal parts of the small intestine. Two of these adducts were tentatively identified to be dG-N^2-BPDE (adduct spot A) (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 B) (Figure 2) (Schoket *et al.* 1989; Nesnow *et al.* 1993; Fang *et al.* 2003; Stiborova *et al.* 2014). 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.* 2001). The structure of another BaP-DNA adduct (adduct spot C) has not still been characterized.

Results of analysis of liver DNA from rats exposed to flavonoids and BaP in both regimens are presented in Figure 6. Of all tested flavonoids, only BNF in combination with BaP enhanced the formation of BaP-DNA adducts above the level elicited by BaP alone. ANF reduced BaP-DNA adducts formation, when applied sequentially or in combination with BaP. Likewise, DHM in either regimen also inhibited adduct formation by BaP and this effect was even more efficient compared to ANF. This finding does not correspond to the results of CYP1A1 induction detected as EROD activity.

The modulation of CYP1A1 activity and DNA adduct formation in small intestine

The small intestine was cut into three parts, proximal, middle and distal. The CYP1A1 induction and BaP-DNA adducts were examined in proximal and distal parts to get the figure of the flavonoid – BaP interactions along the organ reflecting short and extensive exposure of xenobiotics to metabolic capacity of small intestine.

The BaP induction of CYP1A1 with/without flavonoids was assayed as EROD activity in small intestine microsomal samples (see Figure 7). Pre-treatment with BNF did not increase the BaP-mediated induction of CYP1A1 either in the proximal or distal part of small intestine, but *vice versa* a slight drop in EROD activity was detected. When other flavonoids, ANF or DHM, were also applied before BaP, they markedly reduced CYP1A1-mediated metabolism, whereas ANF was more effective than DHM in both parts of small intestine. The simultaneous administration of ANF or DHM with BaP elicited even higher reduction of EROD activity in comparison to respective samples from sequential treatment. In general, the flavonoid abilities to lower the BaP induction of CYP1A1 noticeable decline from the proximal to distal part of small intestine, with only the exception of the microsomal sample from the proximal part of animal pre-treated with DHM (prior to BaP). The immunodetection of CYP1A1 in microsomal samples of small intestine does not show any marked differences in the protein expression (Figure 4).

Tissues from the proximal and distal parts of small intestine were examined for the levels of BaP-DNA

adducts (see Figure 8). In both sections of small intestine BNF did not significantly induce the BaP-DNA adduct formation. Only a slight stimulatory effect of BNF on BaP-DNA adduct levels was found. The amount of BaP-DNA adducts was significantly higher (>2 folds) in the distal than in the proximal part of the small intestine regardless DHM or ANF administration regimen used. Moreover, in the distal part of small intestine the BaP-DNA adduct formation was stimulated, particularly with ANF, to levels substantially exceeding those obtained for BaP applied solely. On the contrary, in the proximal part of small intestine, only the pre-treatment of rats with ANF exerted a slight stimulatory effect, but in all other cases flavonoids inhibited the BaP-DNA adduct formation (in comparison to animals treated solely with BaP).

DISCUSSION

The present study is focused on the effect of DHM, the natural flavonoid, which is considered as a candidate compound for counteracting an acute EtOH intoxication and reducing excessive EtOH consumption in humans, on genotoxicity of BaP. Although DHM is a compound occurring in various plants and thus is generally believed safe for humans, its excessive consumption as a medication or food additives may raise health risk issues. Beside the acute toxicity of DHM, its possible interactions with the CYP multienzyme system, which is frequently affected by compounds of foreign origin, should be examined. As CYPs are involved in metabolism of chemical carcinogens the intake of DHM might modulate the carcinogen activation, e.g. food-derived carcinogen BaP (Hodek *et al.* 2009a).

In addition to DHM, two synthetic flavonoids, ANF and BNF, were included into the study. While BNF is a prototypical CYP1A1 inducer of CYP expression acting *via* the AHR pathway (Wiebel 1980), ANF is a powerful inhibitor of CYP1A1-mediated metabolic reactions (Bauer *et al.* 1995). However, besides the inhibition of CYP1A1, ANF might also stimulate CYP-dependent metabolism and consequently BaP genotoxicity in several ways.

The present experiments were undertaken to examine two situations, the first one, where the flavonoid was administered prior to the intake of a carcinogen BaP and the second one, where both compounds were applied simultaneously. The 72 hrs delay between administration of flavonoid and BaP was chosen to allow effective metabolism and a potential excretion of compounds used for the animal pre-treatment. Under such conditions the residual flavonoid concentration should not be inhibitory while the flavonoid elicited induction of CYPs persists, as shown in our previous work (Hodek *et al.* 2011). In the experiments of the present study the flavonoid-BaP interactions were evaluated based on the induction of CYP1A1, which plays a prominent role in BaP activation (Bauer *et al.* 1995)
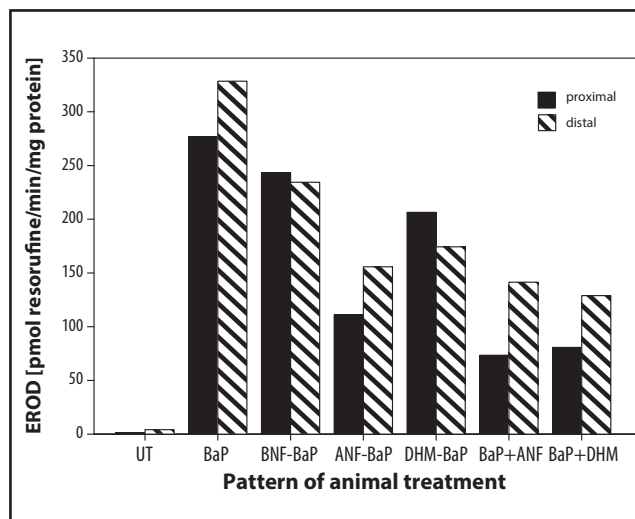
Fig. 7. Determination of EROD in intestinal microsomes. Microsomal samples were prepared from proximal (black bars) and distal (hatched bars) parts of small intestine of animals exposed to BaP alone or in combination with DHM, ANF and BNF. Flavonoids were administered either 72 hrs before BaP animal treatment (BNF-BaP, ANF-BaP, DHM-BaP) or simultaneously with BaP (BaP+ANF, BaP+DHM). In all cases animals were sacrificed 24 hrs after BaP exposure. Control microsomes were prepared from animals treated with sunflower oil only (UT). Each value represents the mean of two separate analyses.
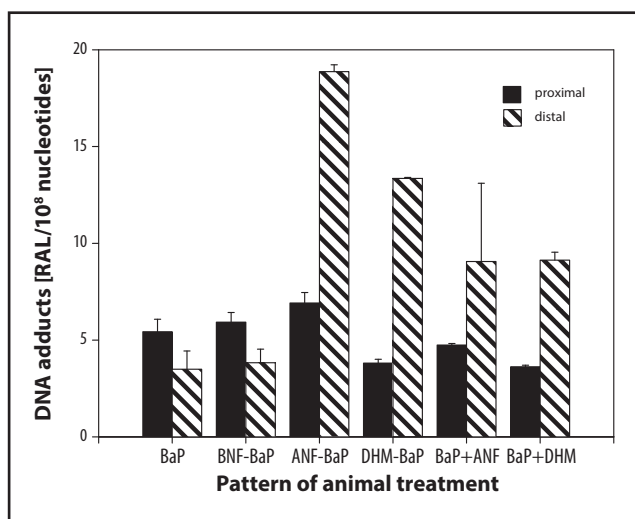
Fig. 8. Formation of BaP-DNA adducts in small intestine. Rats were exposed to BaP alone or in combination with DHM, ANF and BNF. Flavonoids were administered either 72 hrs before BaP animal treatment (BNF-BaP, ANF-BaP, DHM-BaP) or simultaneously with BaP (BaP+ANF, BaP+DHM). In all cases animals were sacrificed 24 hrs after BaP exposure. DNA adducts were determined using ^{32}P-postlabeling assay in DNA samples from proximal (black bars) and distal (hatched bars) parts of small intestine. Each value represents the mean of three separate analyses ± SD. Data are expressed in RAL – relative adduct labeling per 10^8 nucleosides.

and the BaP-DNA adduct formation that is an early sign of initiated carcinogenesis (Stiborova *et al.* 2001, 2003, 2014).

Our experiments were performed with model animals, rats, which were treated with tested compounds

in a single *p.o.* dose by gastric gavages to mimic a potential human intake. Although the liver is the major site for the metabolic activation and detoxification of PAHs in general, the intestinal epithelium plays a significant role in the first-pass metabolism of ingested xenobiotics (Kaminsky and Zhang, 2003; Thelen and Dressman, 2009). Therefore, we focused on liver and small intestine two organs, which are primarily exposed to ingested xenobiotics.

As follows from the analysis of specific EROD activity in liver microsomes, BNF is a CYP1A1 inducer enhancing the BaP induction of EROD activity. In the case of DHM or ANF at least two additional effects on BaP-induction of CYP1A1 might be regarded. These flavonoids may antagonize BaP-induction of CYP1A1 and/or inhibit CYP1A1 EROD activity. While the pre-treatment of animals with DHM or ANF (72 hrs before BaP) allows manifesting namely their agonistic or antagonistic effects on AHR, simultaneous administration of DHM or ANF with BaP may result also in the inhibition of microsomal EROD activity. Since the BaP-mediated induction of CYP1A1/2 on Western blots was not significantly impaired with any of flavonoids, the changes in CYP1A1 expression were most likely not responsible for the found reduction of EROD activity in liver microsomes. Thus, in all samples with the exception of liver microsomes from DHM-pre-treated rats, the persisted flavonoids and/or their metabolites may cause the EROD activity decline. The lack of DHM impact on the EROD activity (compared to ANF) is likely caused by the presence of multiple hydroxylation of DHM, which makes it more polar and excreted readily. A slight increase in EROD activity of this sample might be attributed to possible DHM-mediated induction of CYP1A2, which also to some extent catalyzes EROD. In agreement with this assumption, a slight elevation of the CYP1A2 level on Western blots developed with anti-CYP1A2 antibody was detected for DHM (data not shown). Present data indicate that the effect of DHM on EROD activity depended on the regimen of application: while the simultaneous administration of DHM with BaP effectively reduced BaP-mediated CYP1A1 EROD activity; the activity stimulation was detected when DHM was applied 3 days before BaP.

The CYP1A1 induction in rat livers, measured as CYP1A1/2 specific EROD activity in microsomes corresponded to an increase in the formation of BaP-DNA adducts. Hence, the increase in CYP1A1 activity after pre-medication of rats with BNF resulted in stimulation of BaP activation (see Figures 3 and 6). On the contrary, the pre-treatment of rats with DHM or ANF, regardless the application regimen used, lowered the levels of BaP-DNA adducts. That is in accordance with an observed decline in the EROD activity. In the case of DHM pre-medicated animals, however, there is an obvious discrepancy between the moderate stimulation of EROD activity (above the BaP control) and a pronounced reduction of BaP-DNA adducts formation. As

mentioned above, this phenomenon might by explained at least partially by the DHM induction of CYP1A2, which can, to some extent, contribute to EROD activity. While CYP1A2 exhibits EROD activity, this CYP is involved also in the BaP detoxification, giving rise to the formation of appreciable amounts of 3-hydroxy-BaP and of some dihydrodiols from BaP (Gautier *et al.* 1996; Bauer *et al.* 1995). Thus, our results show that DHM does not enhance the BaP activation leading to BaP-DNA adducts in rat liver.

Since the BaP activation might differ along length of small intestine (Hodek *et al.* 2011) the organ was dissected into three parts, and the proximal and distal parts were used for examination (middle part was omitted). The induction of CYP1A1 was assayed based on EROD activity and the protein expression. In contrast to liver, there is only one member of a CYP1A family, CYP1A1, in small intestine (Kaminsky and Zhang, 2003). Hence, differences in EROD activity directly reflect the CYP1A1 induction/inhibition. It should be noted that in small intestine none of BNF, DHM or ANF when applied in combination with BaP significantly induced expression of CYP1A1-mediated EROD activity more than BaP alone. The lack of the synergistic effect of BNF on BaP-mediated CYP1A1 induction is consistent with our earlier findings that either BNF or BaP already stimulated the CYP1A enzyme expression to its maximal level (Hodek *et al.* 2011). Interestingly, when comparing EROD activities of corresponding microsomal samples from the liver, proximal and distal parts of small intestine, the same pattern for all tissues may be found. The pre-treatment with DHM prior to BaP has always the lowest impact on BaP induced EROD activities, while the co-administration of ANF and BaP most efficiently reduced EROD activities. In addition, among the tissues there is a clear tendency showing the most pronounced differences among EROD activities in liver microsomes and relatively low of those in the distal part of small intestine. Apparently, the effect of flavonoids is getting weaker depending on the duration of xenobiotics exposure to intestinal tissue. In this respect it is also worth of noticing that EROD activities induced by BaP in liver microsomes are in average by 10–20 folds higher than those of small intestine.

Whereas in the proximal part of small intestine, likewise in liver, the BaP-DNA adduct formation was proportional to CYP1A1 induction, in the distal part of the small intestine the situation was more complex (see Figs. 7 and 8). In the proximal part of small intestine, in line with results of liver samples, the co-administration of ANF or DHM with BaP lowered BaP-mediated induction of CYP1A1 activity, thereby reducing the levels of BaP-DNA adducts. In the distal part of small intestine, however, ANF or DHM when applied in either combination with BaP caused a profound stimulation of BaP-DNA adduct formation. The highest effect was detected after the sequential application of ANF or DHM prior to BaP. The 5.4- and 3.8-

fold increase in BaP-DNA adduct levels found in ANF and DHM pre-treated animals, respectively, correlated reversely with the CYP1A1-mediated EROD activity. To confirm this unexpected result, the expression of CYP1A1 at a protein and at mRNA levels was determined, too. Neither Western blots (Figure 4) nor did RT-PCR of mRNA (data not shown) show any changes in CYP1A1 expression, which could explain this extensive BaP activation in the distal part of small intestine. Our findings suggest that in this particular case ANF and DHM increase the activation metabolism of BaP *via* an unknown mechanism, which does not depend on the CYP1A1 induction. Similar data resulted from experiments with *Cyp1a1(–/–)* global- and cell-specific knockout mice. In *Cyp1a1(–/–)* global-knockout mice much higher levels of BaP-DNA adduct formation in liver and small intestine than those in wild-type mice were found. Therefore, CYP1A1 was presumed to be more important in the metabolic detoxification than in activation of orally administered BaP (Uno *et al.* 2004; Shi *et al.* 2010). This hypothesis, however, cannot elucidate the increased BaP adduct formation in the distal part of small intestine detected in our experiments.

To understand the differences between the proximal and distal parts of the small intestine in flavonoid-mediated BaP-DNA adducts formation metabolic conversion of administered flavonoids should be accounted. The experiments performed by Nesnow and Bergman (1981) provide the evidence that ANF was readily metabolized to its 5,6-dihydrodiol in microsomal samples. Also other metabolites such as 7,8-dihydrodiols, and 6-, 7- and 9-hydroxy-ANF were identified (Vyas *et al.* 1983). The rapid ANF oxidation, namely by CYP1A1, results in derivatives, which are less effective inhibitors of CYP1A1-mediated metabolism of BaP than ANF (Nesnow *et al.* 1981; Bauer *et al.* 1995). When orally administered, ANF because of its lipophilicity is absorbed into the enterocytes and its fraction consequently metabolized in liver while the other underwent first-pass metabolism in small intestine (Wang and Morris, 2008). Ingested ANF is gradually along the length of small intestine converted into metabolites as well as the proximal part of small intestine is exposed to conjugated forms of ANF coming in bile. Similar concept should be regarded for BaP biotransformation. In addition, intestinal microflora is involved in a cleavage of the xenobiotic conjugates. For instance, glucuronide conjugates of BaP are hydrolyzed and mutagenic derivatives can be reabsorbed into the enterocytes, and thus circulate between the gut lumen and the epithelial cells (Nanno *et al.* 1986).

The role of ANF, DHM and their metabolites in the activation metabolism of BaP might also be explained by their interactions with P-glycoprotein (P-gp), a membrane transporter located in the apical brush border of the enterocytes (Yeh *et al.* 1992). The function of P-gp is to pump xenobiotics from the enterocytes back into the intestinal lumen and protect cells against toxic exogenous substances. As shown with Caco-2 cells, BaP and/or its metabolites are transported by intestinal cells back into the gut lumen (Buesen *et al.* 2003). The expression of this transporter and its activity might be modulated by BaP and/or ANF as well as by their metabolites (Wang *et al.* 2011). Using the intestinal Caco-2 cells exposed to BaP, the increase in P-gp expression was demonstrated (Sugihara *et al.* 2006). In addition, flavone, a flavonoid lacking hydroxyl groups like ANF, increases the P-gp expression in mice intestinal epithelial cells *in vivo* (Lohner *et al.* 2007). By analogy, ANF, flavonoid of a similar structure to flavone, might induce the P-gp expression in rat small intestine, too. In such case ANF would affect BaP genotoxicity *via* P-gp in addition to already mentioned inhibition of CYP1A1. This deduction would, however, valid for the proximal part of small intestine where the major portion of ingested ANF stays unchanged.

In the distal part of small intestine ANF is expected to be largely converted to metabolites, which are obviously not capable of inducing P-gp expression, but may modulate its transporter activity. The possibility is suggested that derivatives of ANF might affect the P-gp efflux of BaP metabolites by transporter inhibition and thus prolong the persistence of genotoxic compounds in the cell. For instance, flavonoids having a hydroxyl group on position 5 and 3, and the 2–3 double bonds have been demonstrated to inhibit efflux of carcinogens from normal cells (Bansal *et al.* 2009). Although neither the pattern of ANF metabolites formed *in vivo*, nor their impact on P-gp transport activity, has been investigated in rat small intestine, the described structure-activity relationship for the interaction of P-gp with flavonoid might give a clue. In this respect, ANF metabolites fit well with the structural requirements for the P-gp inhibition.

Thus, to understand results of the oral administration of flavonoids with BaP in our experiments a complex interplay between metabolizing enzymes and transporters, especially in small intestine, should be considered. In the proximal part of small intestine the parent flavonoids possibly inhibit CYP1A1-mediated activation of BaP and do not alter BaP-mediated induction of P-gp and its activity, while in the distal part the flavonoid metabolites lacking the CYP1A1 inhibitory ability may block P-gp transport activity. The enhanced level of BaP-DNA adducts thus results from prolonged exposure of BaP and/or its metabolites to intracellular activation enzymes. This suggestion clearly describes situation in the distal part of small intestine when ANF and BaP were applied simultaneously. Moreover, for genotoxic consequences of a sequential intake of ANF prior to BaP, the overall metabolic fate of both compounds (flavonoid and BaP) should be regarded. The delay of 72 hrs allows the second-pass metabolism of the flavonoid resulting in e.g. ANF-conjugates, which are returned back to small intestine *via* bile. The gradual microbial cleavage of ANF-conjugates, e.g. by gluc-

uronidase, along the small intestine may release ANF hydroxy-derivatives inhibiting the efflux activity of P-gp at the time when BaP genotoxic metabolites were formed. To prove this concept, additional experiments are needed, namely those with P-gp and flavonoid metabolites.

In conclusion, the results of the present study indicate a causal and organ dependent link between the BaP activation and exposure of experimental animals to flavonoids. While in liver the BNF animal pre-treatment enhanced DNA adduct formation and DHM and ANF elicited a moderate protective effect, in small intestine, namely in its distal part, flavonoids acted reversely. Particularly under a sequential administration of ANF 72 hrs prior to BaP we found an enhanced DNA adduct formation in the distal part of small intestine, which was associated with lowered BaP-induced EROD activity of CYP1A1. Depending on the organ examined, the BaP-induction of CYP1A1 might be protective against BaP-mediated DNA adduct formation as shown in the distal part of small intestine or genotoxic as detected in liver. Our results point to a potential human health risk arising from interactions of food-derived carcinogens and ingested flavonoids.

ACKNOWLEDGMENT

REFERENCES

1 Aimova D, Poljakova J, Kotrbova V, Moserova M, Frei E, Arlt VM, Stiborova M (2008). Ellipticine and benzo(a)pyrene increase their own metabolic activation *via* modulation of expression and enzymatic activity of cytochromes P450 1A1 and 1A2. Interdiscip. Toxicol. **1**: 160–168.
2 Arlt VM, Stiborova M, Henderson CJ, Thiemann M, Frei E, Aimova D, Singh R, Gamboa da Costa G, Schmitz OJ, Farmer PB, Wolf CR, Phillips DH (2008) Metabolic activation of benzo[a]pyrene *in vitro* by hepatic cytochrome P450 contrasts with detoxification in vivo: experiments with hepatic cytochrome P450 reductase null mice. Carcinogenesis **29**: 656–665.
3 Arlt VM, Poirier MC, Sykes SE, Kaarthik J, Moserova M, Stiborova M, Wolf R, Henderson CJ and Phillips DH (2012). Exposure to benzo[*a*]pyrene of hepatic cytochrome P450 reductase null (HRN) and P450 reductase conditional null (RCN) mice: detection of benzo[*a*]pyrene diol epoxide-DNA adducts by immunohistochemistry and ^{32}P-postlabelling. Toxicol Lett **213**: 160–166.
4 Bansal T, Jaggi M, Khar RK, Talegaonkar S (2009). Emerging significance of flavonoids as P-glycoprotein inhibitors in cancer chemotherapy. J Pharm Pharm Sci. **12**: 46–78.
5 Bauer E, Guo Z, Ueng YF, Bell LC, Zeldin D, Guengerich FP (1995). Oxidation of benzo[a]pyrene by recombinant human cytochrome P450 enzymes. Chem Res Toxicol. **8**: 136–142.
6 Bezirtzoglou EE. Intestinal cytochromes P450 regulating the intestinal microbiota and its probiotic profile (2012). Microb Ecol Health Dis. **23**: e18370.
7 Blaich G, Göttlicher M, Cikryt P, Metzler M (1987). Effect of 7,8-benzoflavone pretreatment on diethylstilbestrol metabolism, drug-metabolising enzymes and the aromatic hydrocarbon (Ah) receptor in male hamster liver. Eur J Drug Metab Pharmacokinet. **12**: 259–262.
8 Borek-Dohalska L, Hodek P, Sulc M, Stiborova M (2001). alpha-Naphthoflavone acts as activator and reversible or irreversible inhibitor of rabbit microsomal CYP3A6. Chem Biol Interact. **138**: 85–106.
9 Buesen R, Mock M, Nau H, Seidel A, Jacob J, Lampen A (2003). Human intestinal Caco-2 cells display active transport of benzo[a]pyrene metabolites. Chem Biol Interact. **142**: 201–221.
10 Burke MD, Mayer RT (1974). Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab. Dispos. **2**: 583–588.
11 Canivenc-Lavier MC, Bentejac M, Miller ML, Leclerc J, Siess MH, Latruffe N, Suschetet M (1996). Differential effects of nonhydroxylated flavonoids as inducers of cytochrome P450 1A and 2B isozymes in rat liver. Toxicol Appl Pharmacol. **136**: 348–353.
12 Chen SH, Zhong GS, Li AL, Li SH, Wu LK. (2006) Influence of Hovenia dulcis on alcohol concentration in blood and activity of alcohol dehydrogenase (ADH) of animals after drinking. Zhongguo Zhong Yao Za Zhi **31**: 1094–1096.
13 Fang AH, Smith WA, Vouros P, and Gupta RC (2001). Identification and characterization of a novel benzo[a]pyrene-derived DNA adduct. Biochem. Biophys. Res. Commun. **281**: 383–389.
14 Ferguson LR (2001). Role of plant polyphenols in genomic stability. Mutat. Res. **475**: 89–111.
15 Gautier JC, Lecoeur S, Cosme J, Perret A, Urban P, Beaune P, Pompon D (1996). Contribution of human cytochrome P450 to benzo[a]pyrene and benzo[a]pyrene-7,8-dihydrodiol metabolism, as predicted from heterologous expression in yeast. Pharmacogenetics. **6**: 489–499.
16 Glatt HR, Wölfel T, Oesch F. Determination of epoxide hydrolase activity in whole cells (human lymphocytes) and activation by benzoflavones (1983). Biochem Biophys Res Commun. **110**: 525–529.
17 Gupta PH, Mehta S, Mehta SK. Effects of dietary benzo(a) pyrene on intestinal phase I and phase II drug metabolizing systems in normal and vitamin A-deficient rats (1989). Biochem Int. **19**: 709–722.
18 Hodek P, Trefil P, Stiborova M (2002). Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. Chem. Biol. Interact. **139**: 1–21.
19 Hodek P, Krizkova J, Burdova K, Sulc M, Kizek R, Hudecek J, Stiborova M (2009a). Chemopreventive compounds--view from the other side. Chem. Biol. Interact. **180**:1–9.
20 Hodek P, Tepla M, Krizkova J, Sulc M, Stiborova M (2009b). Modulation of cytochrome P450 enzyme system by selected flavonoids. Neuroendocrinol. Lett. **30** (Suppl. 1): 67–71.
21 Hodek P, Krizkova J, Frei,E, Singh R, Arlt V M, Stiborova M (2011). Impact of beta-naphthoflavone on genotoxicity of food-derived carcinogens. Neuro Endocrinol. Lett. **32** (Suppl. 1): 25–34.
22 Hodek P (2012). Flavonoids in: Metabolism of Drugs and Other Xenobiotics (Anzenbacher P, Zanger UM, eds.) Wiley VCH, Weinheim, Germany 2012, pp. 543–582.
23 Hodek P, Koblihova J, Kizek R, Frei E, Arlt VM, Stiborova M. (2013a) The relationship between DNA adduct formation by benzo[*a*] pyrene and expression of its activation enzyme cytochrome P450 1A1 in rats. Environ. Toxicol. Pharmacol. **36**: 989–996.
24 Hodek P, Trefil P, Simunek J, Hudecek J, Stiborova M (2013b). Optimized protocol of chicken antibody (IgY) purification providing electrophoretically homogenous preparations. Int. J. Electrochem. Sci. **5**: 113–124.
25 Huang MT, Wood AW, Chang RL, Yagi H, Sayer JM, Jerina DM, Conney AH (1986). Inhibitory effect of 3-hydroxybenzo(a)pyrene on the mutagenicity and tumorigenicity of (+/−)-7 beta, 8 alpha-dihydroxy-9 alpha, 10 alpha-epoxy-7,8,9,10-tetrahydrobenzo(a) pyrene. Cancer Res. **46**: 558–566.
26 Hughes R, Rowland IR (2000). Metabolic activities of the gut microflora in relation to cancer. Microb Ecol Health D. **12** Suppl 2: 179–185.
27 Hyun TK, Eom SH, Yu CY, Roitsch T (2010). Hovenia dulcis--an Asian traditional herb. Planta Med. **76**: 943–949.

28 Indra R., Moserova M., Sulc M, Frei E, Stiborova M (2013). Oxidation of carcinogenic benzo[a]pyrene by human and rat cytochrome P450 1A1 and its influencing by cytochrome b₅ – a comparative study. Neuro Endocrinol Lett **34** (Suppl 2): 55–63.

29 Lohner K, Schnäbele K, Daniel H, Oesterle D, Rechkemmer G, Göttlicher M, Wenzel U (2007). Flavonoids alter P-gp expression in intestinal epithelial cells *in vitro* and *in vivo*. Mol Nutr Food Res. **51**: 293–300.

30 Kaminsky LS, Zhang Q (2003). The small intestine as a xenobiotic-metabolizing organ. Drug Metab. Disp. **31**: 1520–1525.

31 Krizkova J, Burdova K, Hudecek J, Stiborova M, Hodek P (2008). Induction of cytochromes P450 in small intestine by chemopreventive compounds. Neuroendocrinol. Lett. **29**: 717–721.

32 Krizkova J, Burdova K, Stiborova M, Kren V, Hodek P (2009). The effects of selected flavonoids on cytochromes P450 in rat liver and small intestine. Interdiscip. Toxicol. **2**: 201–204.

33 Meinl W, Sczesny S, Brigelius-Flohé R, Blaut M, Glatt H (2009). Impact of gut microbiota on intestinal and hepatic levels of phase 2 xenobiotic-metabolizing enzymes in the rat. Drug Metab Dispos. **37**: 1179–1186.

34 Nanno M, Morotomi H, Takayama H, Kuroshima T, Tanaka R, Mutai M (1986). Mutagenic activity of biliary metabolites of benzoapyrene by b-glucuronidase positive bacteria in human faeces. J Med Microbiol. **22**: 351–355.

35 Nebert DW, Dalton TP, Okey AB, Gonzalez FJ (2004). Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. J. Biol. Chem. **279**: 23847–23850.

36 Nesnow S, Bergman H (1981). Metabolism of alpha-naphthoflavone by rat liver microsomes. Cancer Res. **41**: 2621–2626.

37 Nesnow S, Easterling R, Bergman H, Roth R (1982). Inhibition of benzo(a)pyrene monooxygenase by alpha-naphthoflavone may be partially mediated by the metabolite 9-hydroxy-alpha-naphthoflavone. Toxicol Lett. **14**: 7–13.

38 Nesnow S, Ross J, Nelson G, Holden K, Erexson G, Kligerman A, Gupta RC (1993). Quantitative and temporal relationships between DNA adduct formation in target and surrogate tissues: implications for biomonitoring. Environ Health Perspect. **101** Suppl 3: 37–42.

39 Ni F, Gong Y, Li L, Abdolmaleky HM, Zhou JR. Flavonoid ampelopsin inhibits the growth and metastasis of prostate cancer *in vitro* and in mice (2012). PLoS One. **7**: e38802.

40 Omura T, Sato R (1964). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem. **239**: 2370–2378.

41 Phillips DH (1999). Polycyclic aromatic hydrocarbons in the diet. Mutat. Res. **443**: 139–147.

42 Schenkman JB and Jansson I (2003). The many roles of cytochrome b₅. Pharmacol Ther. **97**: 139–152.

43 Schoket B, Lévay K, Philips DH and Vincze I (1989). ³²P-postlabelling analysis of DNA adducts of benzo[a]pyrene formed in complex metabolic activation systems *in vitro*. Cancer Lett. **48**: 67–75.

44 Shen Y, Lindemeyer AK, Gonzalez C, Shao XM, Spigelman I, Olsen RW, Liang J (2012). Dihydromyricetin as a novel anti-alcohol intoxication medication. J Neurosci. **32**: 390–401.

45 Shi Z, Dragin N, Galvez-Peralta M, Jorge-Nebert LF, Miller ML, Wang B, Nebert DW (2010). Organ-specific roles of CYP1A1 during detoxication of dietary benzo[a]pyrene. Mol Pharmacol. **78**: 46–57.

46 Stiborova M, Bieler CA, Wiessler M, Frei E (2001). The anticancer agent ellipticine on activation by cytochrome P450 forms covalent DNA adducts. Biochem. Pharmacol. **62**: 1675–1684.

47 Stiborova M, Rupertova M, Hodek P, Frei E, Schmeiser HH (2003) Evaluation of ³²P-postlabelling as a suitable detection technique for biomonitoring of human DNA adducts. Proc. Indian Natn. Sci. Acad. **B69**: 539–556.

48 Stiborova M, Cerna V, Moserova M, Arlt VM and Frei E (2013) The effect of benzo[a]pyrene on metabolic activation of anticancer drug ellipticine in mice. Neuro Endocrinol Lett **34** (Suppl 2): 43–54.

49 Stiborova M, Moserova M, Cerna V, Indra R, Dracinsky M, Sulc M, Henderson CJ, Wolf CR, Schmeiser HH, Phillips DH, Frei E, Arlt VM (2014). Cytochrome b5 and epoxide hydrolase contribute to benzo[a]pyrene-DNA adduct formation catalyzed by cytochrome P450 1A1 under low NADPH:P450 oxidoreductase conditions. Toxicology. **318**: 1–12.

50 Sugihara N, Toyama K, Michihara A, Akasaki K, Tsuji H, Furuno K (2006). Effect of benzo[a]pyrene on P-glycoprotein-mediated transport in Caco-2 cell monolayer. Toxicology. **223**: 156–165.

51 Thelen K, Dressman JB (2009). Cytochrome P450-mediated metabolism in the human gut wall. J Pharm Pharmacol. **61**: 541–558.

52 Uno S, Dalton TP, Derkenne S, Curran CP, Miller ML, Shertzer HG, Nebert DW (2004). Oral exposure to benzo[a]pyrene in the mouse: detoxication by inducible cytochrome P450 is more important than metabolic activation. Mol Pharmacol. **65**: 1225–1237.

53 Vyas KP, Shibata T, Highet RJ, Yeh HJ, Thomas PE, Ryan DE, Levin W, Jerina DM (1983). Metabolism of alpha-naphthoflavone and beta-naphthoflavone by rat liver microsomes and highly purified reconstituted cytochrome P-450 systems. J Biol Chem. **258**: 5649–5659.

54 Wang XD, Morris ME (2008). Pharmacokinetics and bioavailability of the flavonoid 7,8-benzoflavone in rats. J. Pharm. Sci. **97**: 4546–4556.

55 Wang X, Hawkins BT, Miller DS (2011). Aryl hydrocarbon receptor-mediated up-regulation of ATP-driven xenobiotic efflux transporters at the blood-brain barrier. FASEB J. **25**: 644–652.

56 Weichelman KJ, Braun RD, Fitzpatrick JD (1988). Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. Anal. Biochem. **175**: 231–237.

57 Wiebel FJ (1980). Activation and inactivation of carcinogens by microsomal monooxygenases: Modification by benzoflavones and polycyclic aromatic hydrocarbons in Modifiers of Chemical Carcinogenesis (Slaga TJ, ed) vol 5, pp 57–84, Raven Press, New York.

58 Yeh GC, Lopaczynska J, Poore CM, Phang JM (1992). A new functional role for P-glycoprotein: efflux pump for benzo(alpha) pyrene in human breast cancer MCF-7 cells. Cancer Res. **52**: 6692–6695.

59 Zhang QY, Wikoff J, Dunbar D, Kaminsky L (1996). Characterization of rat small intestinal cytochrome P450 composition and inducibility. Drug Metab Dispos **24**: 322–328.

60 Zhang QY, Wikoff J, Dunbar D, Fasco M, Kaminsky L (1997). Regulation of cytochrome P4501A1 expression in rat small intestine. Drug. Metab. Dispos. **25**: 21–26.

61 Zhang L, Huang M, Blair IA, Penning TM. Interception of benzo[a] pyrene-7,8-dione by UDP glucuronosyltransferases (UGTs) in human lung cells (2013). Chem Res Toxicol. **26**: 1570–1578.