# Impact of beta-naphthoflavone on genotoxicity of food-derived carcinogens

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## Abstract

**OBJECTIVES:** Benzo[a]pyrene (BaP) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) are carcinogens, which frequently occur in the human diet. Their metabolic activation to reactive species binding to DNA is mediated by cytochromes P450 (CYPs) 1A1 and 1A2. Thus, levels and activities of these CYPs are crucial for initiation of BaP- and PhPI-mediated carcinogenesis. Here, the effect of CYP1A1/2 induction due to their prototype flavonoid inducer, β-naphthoflavone (BNF), on BaP- and PhPI-derived DNA adduct formation in rats was examined.
**METHODS:** Male rats pretreated with BNF were treated with a single dose of either carcinogen by oral gavage. Nuclease P1 version of $^{32}$P-postlabeling assay and online column-switching liquid chromatography-electrospray ionization-tandem mass spectrometry were used to detect and quantify covalent DNA adducts formed by BaP and PhIP *in-vivo*, respectively. Expression of CYP1A1/2 enzymes was examined by Western blot. Enzymatic activities of CYP1A1/2 were assessed using their marker substrates (ethoxyresorufin and methoxyresorufin).
**RESULTS:** Treatment of rats with a single dose of BNF produced an increase in levels CYP1A1/2 and CYP1A1 proteins in liver and small intestine, respectively. An increase in CYP1A1 protein expression found in both organs correlated well with specific activities of these CYPs. The CYP1A1 expression levels and its specific activity in small intestine decreased along the length of the organ, being highest in its proximal part and lowest in its distal part. The BNF induction of CYP1A1/2 resulted in a significant increase in the formation of BaP- and PhIP-DNA adducts in liver and in the distal part of the small intestine, respectively. Thus, pretreatment of rats with BNF did not prevent the PhIP and BaP activation, but *vice versa*, enhanced their genotoxicity.
**CONCLUSIONS:** The results of this study demonstrate that the administration of only a single dose of CYP-inducing flavonoid prior to the intake of food carcinogens may increase the risk of a tumor formation.

ORIGINAL ARTICLE

**ABBREVIATIONS:**

| | |
|---|---|
| AHR | - aryl hydrocarbon receptor |
| BaP | - benzo[a]pyrene |
| BNF | - β-naphthoflavone |
| b.w. | - body weight |
| CYP | - cytochrome P450 |
| DMSO | - dimethyl sulfoxide |
| EROD | - 7-ethoxyresorufin-O-deethylase |
| GST | - glutathione S-transferase |
| MROD | - 7-methoxyresorufin-O-demethylase |
| MS | - microsomes |
| NADPH | - nicotinamidadeninedinucleotide phosphate (reduced) |
| NAT | - N-acetyltransferase |
| *p.o.* | - *per os* |
| PAH | - polycyclic aromatic hydrocarbons |
| PhIP | - 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine |
| UT | - untreated animals |

## INTRODUCTION

Chemoprevention is defined as the use of chemical compounds to intervene in the early precancerous stages of carcinogenesis and thereby reverse tumor formation. Many chemopreventive agents, both natural and synthetic, have been identified. Some of the most promising compounds are flavonoids, which are found in vegetables and fruits. These compounds are able to provide a wide variety of biological activities, namely as powerful antioxidants. Moreover, flavonoids are suggested to be anticancer agents acting as inhibitors of enzymes, which activate carcinogens into DNA-modifying intermediates (Hodek *et al.* 2002). However, the question arises whether flavonoids, which are generally accepted as health-promoting natural compounds and therefore used as food supplements, are really beneficial to the organism. Extensive intake of flavonoids may cause potential threats resulting from e.g. drug interactions, and may alter the metabolism of endogenic compounds. In addition, flavonoids may act as prooxidants, especially at high doses (Hodek *et al.* 2009a). Much less attention is being paid to the flavonoid ability to increase the catalytic activity of xenobiotic metabolizing enzymes, e.g. cytochromes P450 (CYPs), *via* their induction. Since flavonoids are powerful inhibitors as well as inducers of CYPs, their "double-edged sword" behavior in the process of CYP-mediated carcinogen activation should be considered (Hodek *et al.* 2009b).

Using a rat animal model, the causal relation between the flavonoid exposure and activation of food carcinogens was investigated in the present study. Two chemical carcinogens, benzo[a]pyrene (BaP) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), were selected as model compounds. While BaP is a human food-derived carcinogen present e.g. in smoked or flame-exposed meat (Phillips 1999), PhIP is formed as a product of amino acid pyrolysis during the cooking of meat and fish (Felton *et al.* 1986). These carcinogens differ in three main aspects: **i)** the enzymes required for their metabolic activation, **ii)** the target organs of their carcinogenicity and **iii)** their potential to induce CYP enzymes.

As far as the metabolic activation is concerned, BaP, similarly to other polycyclic aromatic hydrocarbons (PAH), is mainly activated to its reactive species binding to DNA by CYP1A1, while its detoxication is catalyzed by glutathione S-transferase (GST) (Fang *et al.* 2001; Aimova *et al.* 2008). Moreover, the role of CYP1A1 in BaP metabolism is 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). Heterocyclic amine PhIP is activated by its *N*-hydroxylation catalyzed predominantly by CYP1A2 (Turesky *et al.* 1998). The resulting *N*-hydroxy derivative of PhIP might undergo further metabolic activation catalyzed by *N*-acetyltransferases and sulfotransferases (Williams *et al.* 2001).

Both carcinogens differ in target organs of their carcinogenicity. Whereas BaP has been found to be a carcinogen causing development of tumors in lung, skin and liver, PhIP is a carcinogen that induces tumors of the colon, prostate and mammary gland tissues (Stuart *et al.* 2001). Both carcinogens also differ in their ability to induce CYPs. BaP is a prototypic PAH inducing CYPs of a 1A subfamily *via* the 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). In contrast to BaP, PhIP has not been shown to be an inducer either of CYP1A1 or 1A2.

In numerous *in-vitro* studies, a clear connection between the CYP inhibition and the protection against a DNA adduct formation has been shown, thus flavonoids are frequently referred to as anti-carcinogenic agents. This straightforward interpretation of flavonoid-mediated chemoprevention of a carcinogen activation arises from the simultaneous administration of both compounds to experimental animals. These simplified experiments, however, do not take into account the sequential exposure to a carcinogen and chemopreventive compound that is common in the human diet. For instance, after the oral administration of a flavonoid, 7,8-benzoflavone, its plasma concentrations peaked within 30 min, allowing the maximal inhibitory effect on carcinogen activating enzymes (Wang & Morris 2008). On the other hand, lower levels, inefficient for the enzyme inhibition, are sufficient to initiate the expression of biotransformation enzymes (Ferguson 2001). This process is much slower; for instance, it takes approx. 24 hrs after the application of 5,6-benzoflavone (BNF) to reach the maximal induction of CYP1A1 in rat small intestinal epithelial cells (Zhang *et al.* 1997).

Thus, the current study was performed to determine whether BNF, a flavonoid inducer of CYP enzymes of a 1A subfamily, could modulate BaP or PhIP genotoxicty (DNA adduct formation), when administered 72 hrs prior to the carcinogen. To mimic the human exposure, tested compounds were administered to rats by oral gavage.

## MATERIAL AND METHODS

*Chemicals*

β-Naphthoflavone (5,6-benzoflavone), NADPH, bicinchoninic acid, resorufin, 7-ethoxyresorufin, 7-methoxyresorufin, and anti-chicken IgG alkaline phosphatase conjugate, BCIP/NBT tablets were purchased from Sigma Chemical Co. (St. Louis, MO). Specific chicken anti-CYP1A1/2 antibodies were prepared by Dr. Hodek as described (Hodek *et al.* 1998). All these and other chemicals used in the experiments were of analytical 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 compounds dissolved in sunflower oil (1 ml), BNF (60 mg/kg b.w.), BaP (150 mg/kg b.w.) and PhIP (50 mg/kg b.w.) were administered *p.o.* by gastric gavages in a single dose. Each carcinogen (BaP, PhIP) was applied 72 hrs after administration of a single dose of BNF. The control group was treated with 1 ml of sunflower oil only. The rats were sacrificed 24 hrs after the last treatment. Microsomes (MS) were prepared from sections of small intestine and the whole liver immediately after sacrificing the rats, as described previously (Krizkova *et al.* 2008). Small intestine was removed ~2 cm under the stomach, divided into three parts (proximal, middle, distal), each 15–20 cm long. Tissues of 3 rats were pooled to isolate microsomes. Microsomal fractions were stored at –80 °C before use. Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with the bovine serum albumin as a standard (Weichelman *et al.* 1988). The concentration of CYP was estimated according to Omura and Sato (1964) based on the absorption of the complex of reduced CYP with carbon monoxide.

*Western blot analysis*

The CYP1A1 and 1A2 were detected by Western blotting on Immobilon-P membrane (Millipore, Bedford, MA) using specific chicken anti-CYP1A1/2 and anti-CYP1A2 antibodies. For SDS-electrophoresis (8% polyacrylamide gel) 30 µg and 15 µg protein/well of small intestine and liver microsomes, respectively, were 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 and BCIP/NBT tablets containing 10 mg substrate for alkaline phosphatase.

*Enzyme assays*

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

*PhIP-DNA adduct analysis by online column-switching liquid chromatography tandem mass spectrometry*

The DNA from liver and small intestine was isolated by standard phenol/chloroform extraction. DNA adducts formed by PhIP were determined by modified $^{32}$P-postlabeling method, using online column-switching liquid chromatography tandem mass spectrometry described by Singh *et al.* (2010). Results were expressed as adducts per $10^8$ normal (unmodified) nucleotides

*BaP-DNA adduct analysis by $^{32}$P-postlabeling*

The DNA from liver and small intestine was isolated by standard phenol/chloroform extraction. DNA adducts formed by BaP were determined by $^{32}$P-postlabeling analysis using the nuclease P1 enrichment version (Stiborova *et al.* 2001, 2003), 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). Results were expressed as DNA adducts/$10^8$ nucleotides.

*In-vitro BaP-DNA adduct formation and their analysis*

Incubation mixtures (final volume of 750 µl) used to asses DNA adduct formation by BaP activated with microsomes isolated from rat liver and small intestine consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 0.5 mg of microsomal proteins and 0.5 mg of calf thymus DNA. The reaction was initiated by adding 0.1 mM BaP (dissolved in 7.5 µl DMSO). Incubations at 37 °C were carried out for 90 min. DNA was isolated from the residual water phase by the phenol/chloroform extraction method and BaP-DNA adducts analyzed with the nuclease P1 version of the $^{32}$P-postlabeling technique as described (Stiborova *et al.* 2001; Arlt *et al.* 2008).

## RESULTS

*Time-dependence of CYP1A induction by BNF in rat liver and small intestine*

The induction of CYP1A1 and 1A2 in liver and small intestine by a single dose of orally administered flavonoid BNF was examined within the period of 72 hrs. The levels of both CYPs were determined using their specific metabolic activities as well as the expression of their proteins utilizing CYP1A1 and 1A2 specific antibodies.

In liver a peak of induced CYP1A1 specific EROD activity appeared 24 hrs after the BNF pretreatment followed by a pronounced drop at 48 hrs (Figure 1). Similarly, the induction of CYP1A2 specific MROD activity was highest at 24 hrs after the BNF administration and reduced at 72 hrs by about 35%. These data are in accordance with the immunodetection of CYP1A1 and 1A2. The marked reduction in the MROD activity at 48 hrs is reflected in blots by the reduction of the CYP1A2 band.

In small intestine, the CYP1A1 specific EROD activity and expression levels of this CYP decrease along the length of small intestine, being highest in the proximal part (duodenum) and lowest in the distal part (ileum) (Figure 2). The CYP1A1 specific activity in the proximal part of small intestine was more than 2-fold higher than in the distal part of this tissue. Middle and distal parts of the small intestine showed comparable CYP1A1 induction (data not shown). The induction of CYP1A1 specific EROD activity in all parts of small intestine persisted with some fluctuations for at least 72 hrs. The consistent decrease in all parts of small intestine detected at 48 hrs (Figure 2) corresponds to the appearance of a lower band in blots of those samples.

*Sequential administration of BNF and carcinogens*

To evaluate the effect of BNF on the BaP or PhIP genotoxicty, DNA adduct formation by both carcinogens was investigated in the liver and small intestine of BNF-pretreated rats treated with BaP and PhIP. DNA

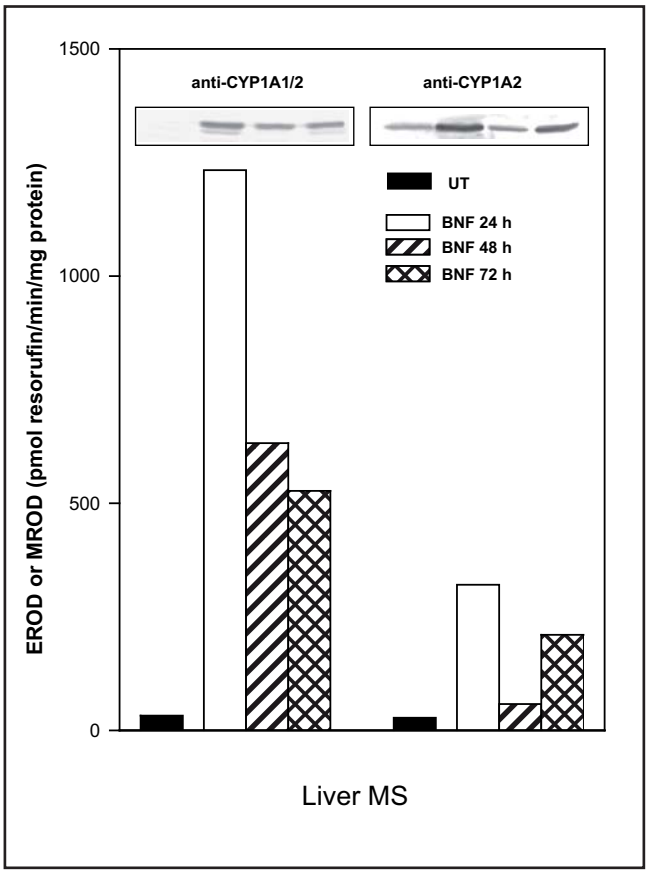


**Fig. 1.** Time-dependence of CYP1A1/2 induction in rat liver microsomes (MS). Microsomal samples were isolated from liver of rats at 24, 48 and 72 hrs after treatment with a single dose of BNF. EROD and MROD activities and CYP1A1/2 immunodetection using Western blotts were performed in rat liver microsomes by procedures described in Materials and Methods. Reaction rates of EROD and MROD are means of duplicate incubations (SD < 10%). UT – untreated rats.

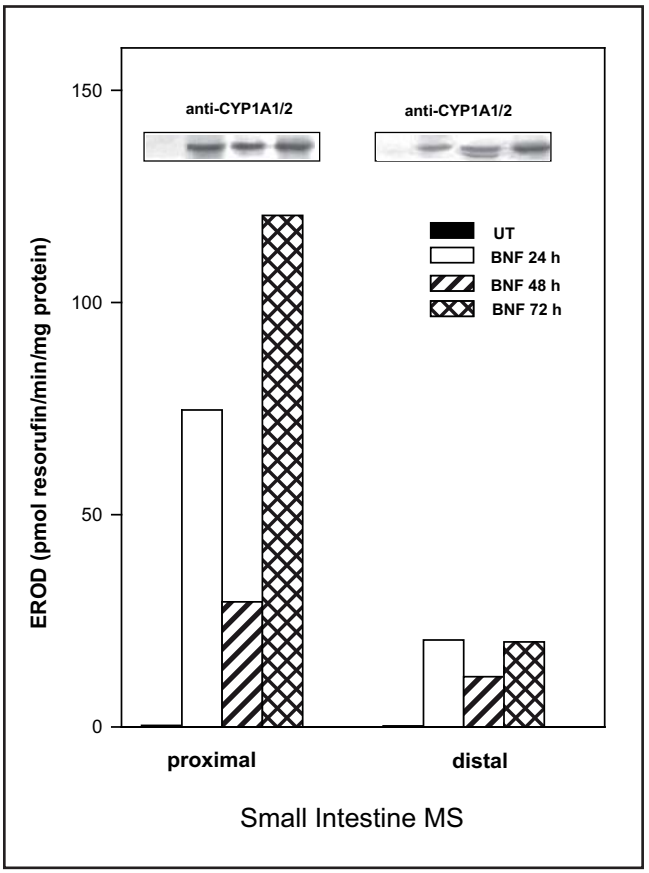


**Fig. 2.** Time-dependence of CYP1A1 induction in rat intestinal microsomes (MS.) Microsomal samples were isolated from small intestine (proximal and distal parts) of rats at 24, 48 and 72 hrs after treatment with a single dose of BNF. EROD activity and CYP1A1 immunodetection using Western blotts were performed in rat intestinal microsomes by procedures described in Materials and Methods. Reaction rates of EROD are means of duplicate incubations (SD < 10%). UT – untreated rats.

and microsomes were isolated from the tissues of liver and small intestine of exposed animals. First, the marker metabolic activities and the protein expression of CYP1A1 and 1A2 were examined in liver and small intestine microsomes.

Figure 3 illustrates that in liver, the PhIP administration had almost no effect on EROD and MROD activities. The application of PhIP to rats pretreated with BNF, however, almost wiped out the BNF induction of CYP1A1 and 1A2 specific activities to levels detected with sole PhIP administration. The immunodetection experiments confirmed the strong reduction of CYP1A1 and 1A2 expression by sequential application of BNF and PhIP.

On the contrary, the BNF induction of CYP1A in liver was significantly stimulated by subsequent BaP administration (Figure 4). An additive effect of BaP on the BNF-mediated induction was observed for both EROD and MROD activities. The immunodetection of CYP1A1 and 1A2, however, showed only a slight increase of CYP expression levels by BaP in combination with BNF, compared to BNF alone.

In small intestine, the administration of PhIP slightly elevated EROD activity and expression of CYP1A1 (Figure 5). In contrast to that, the PhIP treatment of rats pretreated with BNF resulted in a significant decrease in EROD activity and the expression of CYP1A1 in all intestinal parts. Figure 6 shows the results found in experiments utilizing combined exposure of rats to BNF and BaP. BaP exhibited a higher potency than BNF to induce the CYP1A1 activity, however, no additive effect upon sequential exposure of BNF and BaP was observed.

In additional experiments, we evaluated the effect of BNF on PhIP-DNA adduct formation. Treatment of rats with PhIP leads to formation of PhIP-DNA adducts both in the liver and in small intestine. Levels of PhIP-DNA adducts in small intestine were up to 10-fold higher than in the liver (Figure 7). A slight, but significant increase in PhIP-DNA adduct formation both

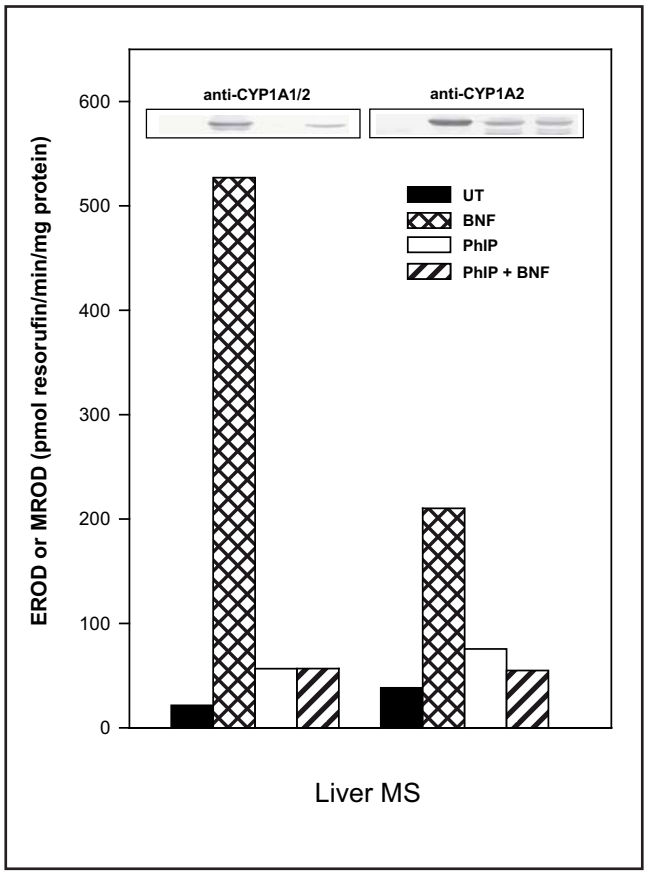


**Fig. 3.** The effect of BNF, PhIP and both these compounds on protein levels of liver CYP1A1/2 and their activities (EROD and MROD). Microsomal samples (MS) were prepared from animals either exposed to PhIP or BNF separately, or PhIP was administered 72 hrs after BNF pretreatment, as well as from untreated animals (UT). In microsones EROD and MROD assays and CYP1A1/2 immunodetection using Western blotts were performed as described in Material and Methods. Reaction rates are means of duplicate incubations (SD < 10%).

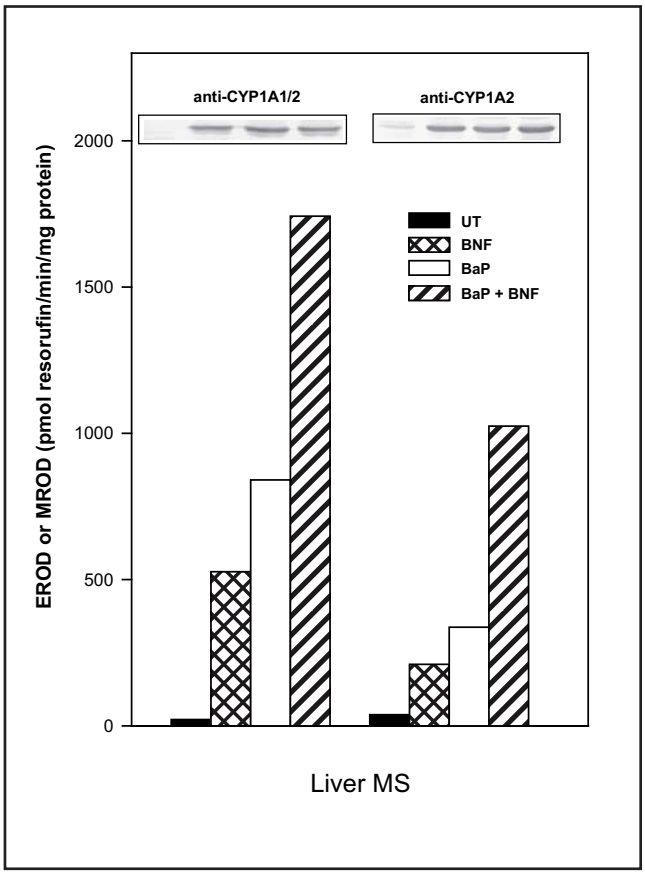


**Fig. 4.** The effect of BNF, BaP and both these compounds on protein levels of liver CYP1A1/2 and their activities (EROD and MROD). Microsomal samples (MS) were prepared from animals either exposed to BaP or BNF separately, or BaP was administered 72 hrs after BNF pretreatment, as well as from untreated animals (UT). In microsomes EROD and MROD assays and CYP1A1/2 immunodetection using Western blotts were performed as described in Material and Methods. Reaction rates are means of duplicate incubations (SD < 10%).

in the liver and mainly in small intestine was caused by pretreatment of rats with BNF. In the distal part of small intestine, BNF-pretreatment resulted in a more than 1.9-fold increase in levels of PhIP-DNA adducts relative to untreated control.

To determine the effect of BNF pretreatment of rats on the BaP activation and generation of DNA-BaP adducts, rats were exposed to BNF and BaP again in a sequential regimen (Figure 8). A slight non-significant stimulatory effect of BNF on BaP-DNA adduct levels was found in the proximal and the distal parts of small intestine. In liver, however, the BNF-pretreatment of animals resulted in a significant increase of BaP-DNA adduct formation (Figure 8).

In order to further investigate the role of BNF in the activation of BaP, the *in-vitro* experiments were carried out. Microsomes isolated from liver and small intestine of rats treated with BaP, alone or sequentially with BNF and BaP, were used in the *in-vitro* incubations containing BaP and DNA. Data shown in Figure 9 indicate that the *in-vitro* activation of BaP into DNA-binding species catalyzed by liver microsomes is considerably more efficient than BaP activation due to microsomes of small intestine. While the levels of BaP-DNA adduct formation in incubations with intestinal microsomes corresponded to those found in small intestine *in-vivo*, the levels of BaP-DNA adducts generated by BaP activation in hepatic microsomes *in-vitro* were much more higher than levels of these adducts found in liver *in-vivo* (compare Figures 8 and 9).

## DISCUSSION

The present study was designed to simulate a common situation, occurring in the human diet, when a model chemopreventive compound having a flavonoid structure (BNF) was administered to experimental animals prior to the intake of a food-derived carcinogen. Although the simultaneous presence of a chemopreventive compound and a carcinogen in the body might pre-

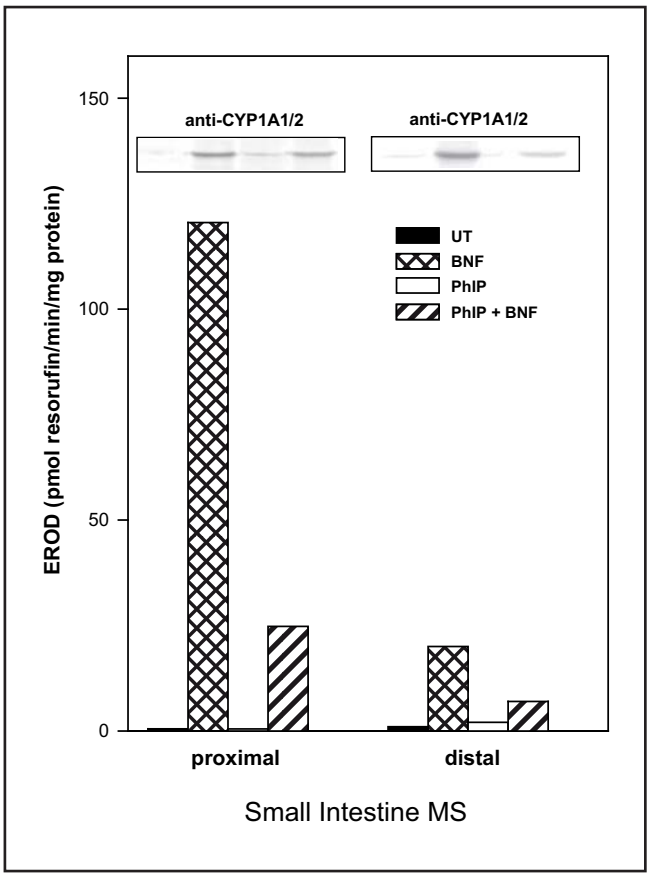


**Fig. 5.** The effect of BNF, PhIP and both these compounds on protein levels of small intestine CYP1A1 and its activity (EROD). Microsomal samples (MS) were prepared from animals either exposed to PhIP or BNF separately, or PhIP was administered 72 hrs after BNF pretreatment, as well as from untreated animals (UT). In microsomes EROD assay and CYP1A1 immunodetection using Western blotts were performed as described in Material and Methods. Reaction rates of EROD are means of duplicate incubations (SD < 10%).

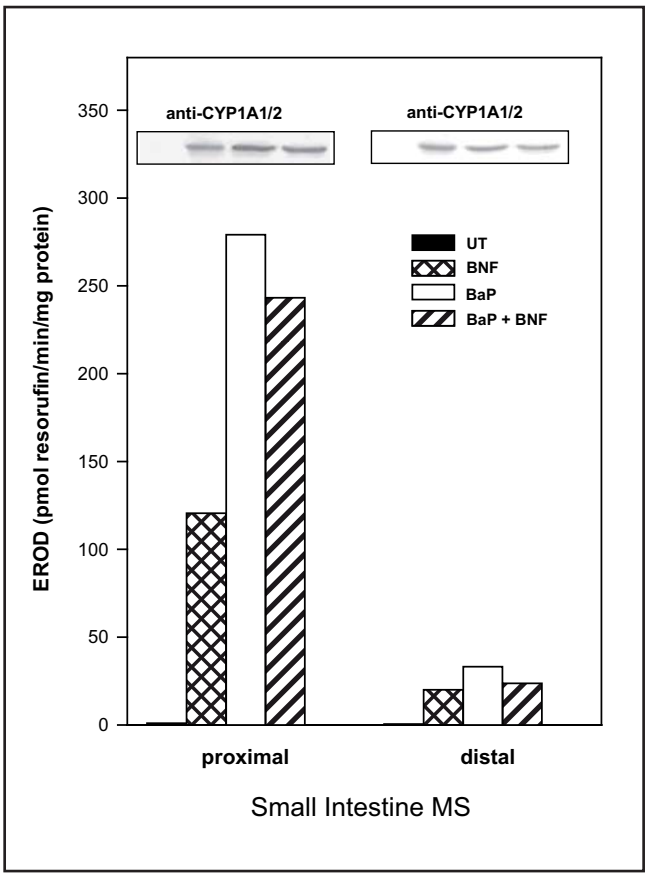


**Fig. 6.** The effect of BNF, BaP and both these compounds on protein levels of small intestine CYP1A1 and its activity (EROD). Microsomal samples (MS) were prepared from animals either exposed to BaP or BNF separately, or BaP was administered 72 hrs after BNF pretreatment, as well as from untreated animals (UT). In microsomes EROD assay and CYP1A1 immunodetection using Western blotts were performed as described in Material and Methods. Reaction rates of EROD are means of duplicate incubations (SD < 10%).

vent the carcinogen activation, and thus be beneficial to the human health, a sequential administration might result either in no chemopreventive effect or even in the stimulation of the carcinogen activation (Hodek *et al.* 2009a). However, the knowledge on the impact of the sequential administration of chemopreventive and carcinogenic compounds on the carcinogen activation *in-vivo* is rather limited. Hence, the interaction of a model flavonoid, BNF, inducing enzymes involved in metabolism of chemical carcinogens such as food-derived carcinogens BaP and PhIP, was investigated in our present study.

First, the time course of CYP1A protein induction by BNF in liver and small intestine was examined to find the optimal timing for the carcinogen administration. At the desired time point, the elevated levels of CYP1A enzymes were found, while the flavonoid inducer is mostly excreted. The flavonoid concentration should be reduced to levels, which are not inhibitory for the carcinogen activation (data not shown). Based on the results found in this work (Figures 1 and 2), the time shift of 72 hrs from the BNF-pretreatment of rats was chosen as the appropriate timing. At that time, in small intestine the CYP1A1-mediated EROD activity and its protein level did not decrease significantly. Moreover, in the proximal part of small intestine, a marked elevation of CYP1A1 occurred, which have might result from the enterohepatic flavonoid cycling *via* bile. Although induction of liver CYP1A1 and 1A2 (measured by CYP1A protein expression with Western blots and by CYP1A activities) is transient, appreciated levels of CYP1A1 and 1A2 still retained 72 hrs after the BNF administration. Hence, the 72 hrs delay from the BNF-induction was used in all sequential experiments with tested carcinogens (BaP or PhIP).

A single dose of BNF administered *p.o.* by gastric gavage to mimic the human intake by food was used in our experiments. It should be noted that even a single dose of BNF significantly induced expression of CYP1A1 and 1A2 in liver and CYP1A1 in small

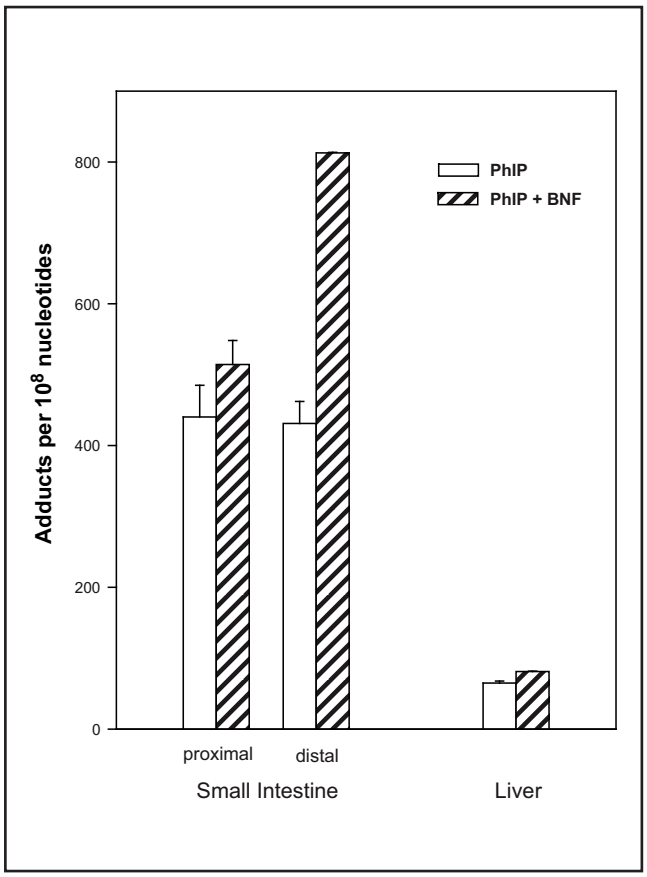


**Fig. 7.** PhIP-DNA adduct formation in liver and small intestine of rats treated with PhIP or PhIP with BNF. Rats were exposed to PhIP (50 mg/kg) 72 hrs after BNF pretreatment. In DNA samples from small intestine and livers the DNA adducts were determined using online column-switching liquid chromatography-electrospray ionization-tandem mass spectrometry (Singh *et al.* 2010). Each value represents the mean of two separate analyses. Data are expressed in adducts per $10^8$ normal (unmodified) nucleosides.

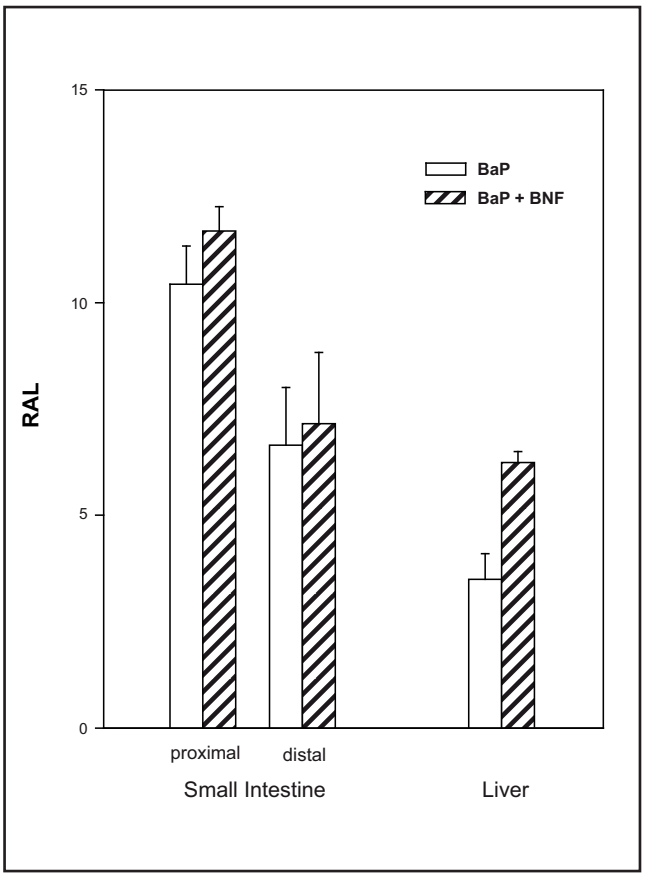


**Fig. 8.** BaP-DNA adduct formation in liver and small intestine of rats treated with BaP or BaP with BNF. Rats were exposed to BaP (60 mg/kg) 72 hrs after BNF pretreatment. In samples of DNA from small intestine and liver DNA adducts were determined using $^{32}$P-postlabeling assay. Each value represents the mean of two separate analyses. Data are expressed in RAL – relative adduct labeling per $10^8$ nucleosides.

intestine as well as corresponding enzyme activities. Whereas the induction of hepatic CYP1A1 was found to be more sensitive to *i.p.* administered BNF (Zhang *et al.* 1996), here we found that *p.o.* treatment of rats with this inducer significantly elevated CYP1A enzymes in liver. Our findings indicate that after the oral administration of BNF, the CYP1A1 specific activity (EROD) in liver was induced 10–20-folds of that determined in small intestine. In contrast to the liver, we were not able to detect any CYP1A2 expression in small intestine using CYP1A2 anti-peptide antibody (data not shown). Accordingly, *CYP1A2* mRNA has been detectable only in BNF-induced rat small intestine, at levels that did not result in any detectable translation (Kaminsky & Zhang 2003).

Of numerous food-born carcinogens, we employed PhIP as a model carcinogen. This is the compound that does not induce CYP1A enzymes (Brooks *et al.* 1994). Indeed, the results found in the present study proved that PhIP does not induce CYP1A enzymes, when administered in a low dose (50 mg/kg b.w.). Surprisingly, PhIP administered after BNF reversed the BNF induction of CYP1A enzymes to levels much lower than those induced by BNF in both liver and small intestine (measured using protein expression levels of CYP1A enzymes and CYP1A activities) (Figures 3 and 5). The mechanisms explaining this phenomenon (disappearance of CYP1A proteins) are however unclear and remain to be explored.

The second carcinogen used, BaP, in contrast to PhIP, proved to be a strong inducer of CYP1A enzymes. In liver, the organ capable of an extensive CYP expression, BNF and BaP caused the additive induction of CYP1A enzymes. No such effect, resulting from sequential administration of BNF and BaP was, however, observed in the small intestine. This finding corresponds to a rather low CYP expression capability of small intestine (compared to liver), which was possibly reached by either compound alone.

To assess the potency of BNF to modulate an acute genotoxicity of PhIP or BaP in liver and small intestine, DNA adduct formation by these compounds was analyzed. Whereas the $^{32}$P-postlabeling technique was used to detect and quantify BaP-DNA adducts, this method is not suitable to analyze PhIP-DNA adducts. Therefore, a newly developed method, online column-switching liquid chromatography-electrospray ionization-tandem mass spectrometry (Singh *et al.* 2010), was employed to detect and quantify PhIP-DNA adducts. Even though, the tested carcinogens (PhIP or BaP) were administered to animals in only a single dose allowing 24 hrs exposures, they efficiently generate DNA adducts. When comparing genotoxic potency of PhIP in small intestine and in liver, PhIP is several-fold more potent to form DNA adducts in small intestine than in liver (see Figure 7). This result confirms PhIP to be a carcinogen inducing tumors predominantly in colon of experimental animals (Kaderlik *et al.* 1994). The pretreatment of rats with BNF resulted in an increase in PhIP-DNA adduct formation in small intestine. Whereas, the levels and activities of CYP1A1 induced by BNF declined markedly along the length of small intestine (from the proximal to distal part), the levels of PhIP-DNA adducts were significantly increased in the distal part as a result of the pretreatment of rats with BNF. This contradictory observation should be viewed in the context of the whole PhIP metabolic pathway, in which PhIP is metabolized first by CYP1A enzymes into *N*-hydroxy derivative and then further activated by conjugation catalyzed with transferases (Williams *et al.* 2001). Since the presence of CYP1A2 in the small intestine was not detected in our experiments, in the intestinal activation of PhIP, CYP1A1 was involved to some extent. Moreover, the flux of activated PhIP derivates *via* bile from liver, where the principal activation of PhIP is attributed to CYP1A2, should be regarded (Turesky *et al.* 1998). Since *N*-acetyltransferase NAT2 and sulfotransferases have not been found in rat small intestine, NAT1, pres-

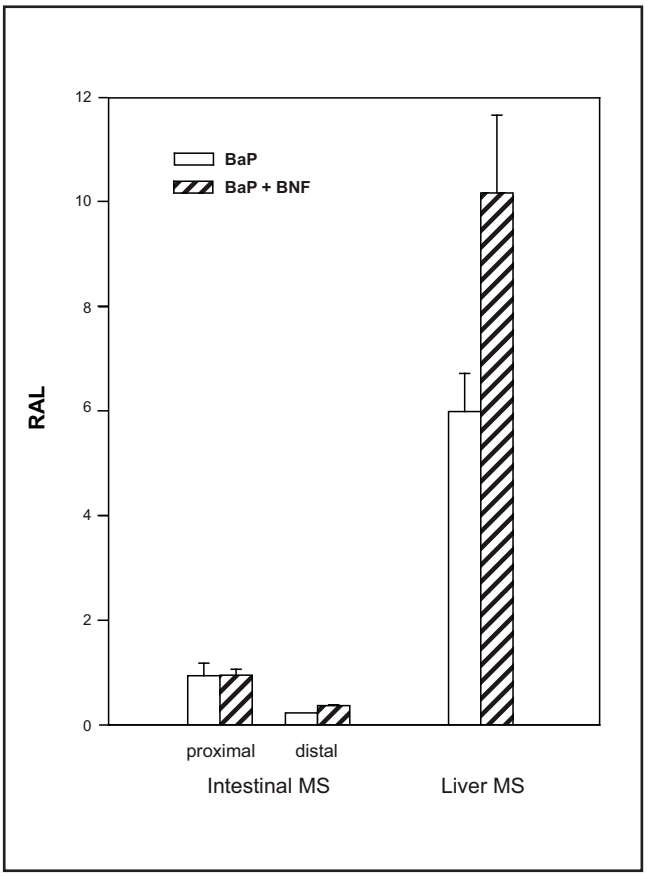


**Fig. 9.** Activation of BaP by liver and intestinal microsomes (MS) *in-vitro*. Microsomal samples (MS) were prepared from liver and small intestine of animals treated with BNF alone or sequentially with BNF and BaP. In *in-vitro* conditions BaP was activated by microsomal enzymes and DNA adducts were determined using $^{32}$P-postlabeling assay (see Material and Methods). Means of two separate analyses are expressed in RAL – relative adduct labeling per $10^7$ nucleosides.

ent in this tissue (Meinl *et al.* 2009), was possible to be involved in the acetylation of N-hydroxy derivative of PhIP. Thus, the BNF-mediated changes in PhIP mutagenicity, which is apparent mostly in the distal part of small intestine, might result from the exposure of PhIP metabolites to some intestinal transferases, possibly NAT1, that should be inducible by BNF in this part of small intestine. These suggestion needs, however, to be confirmed by further studies. Similarly to our results, the BNF-pretreatment caused an increase in levels of DNA adducts formed by another food-derived heterocyclic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline, in colon of AHR-responsive mice having rapid acetylator phenotype (Nerurkar *et al.* 1995).

Whereas small intestine was demonstrated to be a preferred organ for PhIP genotoxicity (DNA adduct formation), BaP is comparably potent to form DNA adducts in liver and in small intestine. The decrease in BaP-DNA adduct levels along the length of the small intestine corresponds to induction of the CYP1A1 enzyme (see Figure 6). The BNF-mediated induction of CYP1A enzymes correlates with the increased BaP activation in the liver and in small intestine. Likewise as BNF in the PhIP activation, this flavonoid exerted a stimulatory effect on BaP-DNA adduct formation, namely in the liver. BaP, which is a strong inducer of CYP1A subfamily, induced these CYPs in liver in addition to BNF. This cumulative induction effect, apparent also from the stimulation of EROD and MROD activities in hepatic microsomes (see Figure 4), is responsible for enhanced BaP-DNA adduct formation. In small intestine, the tissue with a low CYP induction capability, small changes in BaP-DNA adduct formation upon the BNF pretreatment reflected the saturation of the CYP1A1 induction by either compound (BNF or BaP).

Further, two experimental approaches were compared to study the activation of BaP. The first approach was based on the assessment of the BaP-DNA adduct formation *in-vivo*; the second was carried out using the *in-vitro* activation of BaP by microsomes isolated from liver and small intestine of exposed rats. It is clear from the comparison of the *in-vitro* and *in-vivo* experiments (see Figures 4, 8, 9) that the BaP-DNA adduct formation *in-vivo* is less effectively influenced by pretreatment of rats with BNF than the *in-vitro*. Namely, in the *in-vitro* experiments the stimulation of the adduct formation corresponds to the induction of CYP1A enzymes by BNF. This observation is in agreement with an assumption that the induction of enzymes involved in the metabolism of carcinogens such as BaP may reduce their genotoxicity the *in-vivo* by increasing the detoxication pathway of the carcinogen metabolism (Uno *et al.* 2004, Arlt *et al.* 2008; Shi *et al.* 2010). In small intestine, the effect of BNF and the decline in BaP-DNA adduct formation along the length of the organ are analogous both in the *in-vitro* and *in-vivo* experiments. Only a minor effect of the BNF induction on the BaP-DNA adduct formation was consistent with our finding that either BNF or BaP already stimulated the CYP1A enzyme expression to its maximal level. However, the most important conclusion follows from the comparison of these two experimental approaches. Although the results of the *in-vitro* experiments with microsomal samples may suggest the CYP enzymes of the small intestine to have low importance in the formation of BaP-DNA adducts, the data of *in-vivo* experiments show that this organ equally involved the in the BaP genotoxicity.

In conclusion, the results found in the present study support a causal relation between the carcinogen activation and exposure of experimental animals to a flavonoid inducer. Under *in-vivo* conditions, we found an unequivocal link between the BNF animal pretreatment and enhanced DNA adduct formation, namely from activated PhIP in the distal part of small intestine. Our results point to a potential human health risk arising from the sequential intake of food-derived carcinogens and chemopreventive compounds inducing CYP1A enzymes.

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