CHARLES UNIVERSITY IN PRAGUE

Faculty of Science

Department of Biochemistry

Effects of chemopreventive compounds on cytochrome P450s

Ph.D. Thesis

RNDr. Jitka Křížková

Supervisor: prof. RNDr. Petr Hodek, CSc.

Prague 2010

Declaration

I hereby declare that I have written this thesis on my own under the supervision of prof. RNDr. Petr Hodek, CSc. and that I have mentioned all sources used. This work has not been submitted for any other degree or award in any other university or educational establishment.

Prague ....

Co-author statement

I hereby declare that Jitka Křížková has substantially (30-90%) contributed to all 6 publications and the manuscript, which are attached to this thesis. She has carried out the majority of the experiments and has significantly participated in their planning, in the interpretation of results and writing of the publications.

Prague ....

prof. RNDr. Petr Hodek, CSc.

Acknowledgement

I would like to thank especially my supervisor prof. RNDr. Petr Hodek, CSc. for his scientific support and excellent laboratory background.

I also give my thanks to all my colleagues for their advice and friendly working environment.

Special thanks belong to my mother for her help with the stylistic revision.

This work was supported by the Czech Science Foundation (Grant No. 305/09/H008), the Ministry of Education, Youth and Sports of the Czech Republic (Grant No. MSM 0021620808) and the Grant Agency of Charles University (Grant No. 4909).

Contents

List of abbreviations

2-AAF	2-acetylaminofluorene
AαC	2-amino-9Hpyrido[2,3-b]indole
ACTH	adrenocorticotropic hormone
AhR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
BaP	benzo[a]pyrene
BCA	bicinchoninic acid
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BIS	N,N'-methylenbisacrylamide
βNF	β-naphthoflavone
BPDE	benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide
b.w.	body weight
cAMP	cyclic adenosine 3',5'-monophosphate
CYP	cytochrome P450
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EPA	Environmental Protection Agency
EROD	7-ethoxyresorufin-*O*-deethylation
HCA	heterocyclic amine
IARC	International Agency for Research on Cancer
IFP	2-amino-1,6-dimethylfuro[3,2-e]imidazo[4,5-b]pyridine
IQ	2-amino-3-methylimidazo[4,5-f]quinoline
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
3-MC	3-methylcholanthrene
2-ME	2-mercaptoethanol
MeIQ	2-amino-3,4-dimethylimidazo[4,5-f]quinoline
MeIQx	2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
MFO	mixed-function oxidase
MROD	7-methoxyresorufin-*O*-demethylation
MS	microsomal fractions
NADPH	nicotinamide adenine dinucleotide phosphate-reduced

NAT	*N*-acetyltransferase
PAGE	polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
p.o.	*per os*
PPAR	peroxisome proliferator activated receptor
PVDF	polyvinylidene fluoride
R	substrate
RAL	relative adduct labelling
ROH	hydroxylated substrate
RXR	retinoid X receptor
SDS	sodium dodecyl sulphate
SGLT-1	sodium-dependent glucose transporter
SULT	sulfotransferase
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEMED	N,N,N',N'-tetramethylethylendiamine
Tris	tris(hydroxymethyl)aminomethane
UDP	uridine diphosphate
UV	ultraviolet
v/v	volume/volume percent
w/v	weight/volume percent
WHO	World Health Organisation
XRE	xenobiotic responsive element
ε	molar absorption coefficient

1. INTRODUCTION

1.1. Cytochrome P450s

Cytochrome P450s (EC 1.14.14.1) are enzymes playing an important role in the metabolism of foreign compounds (e.g. drugs, pollutants, carcinogens) and also participating in the metabolism of hydrophobic endogenic substrates (e.g. sterols, prostaglandins, fatty acids) [Gonzalez and Gelboin, 1994; Nebert and Dieter, 2000].

These enzymes were first described by Garfinkel and Klingenberg. In 1958, they discovered a pigment in mammalian liver microsomes [Garfinkel, 1958; Klingenberg, 1958]. The term „cytochrome P450“ originates from the observation that the reduced state of this pigment forms a complex with carbon oxide exhibiting a strong absorption maximum at 450 nm [Omura and Sato, 1964].

Cytochrome P450s (CYPs) have been characterized in many organisms, including bacteria, fungi, plants, fish, and mammals [Nebert and McKinnon, 1994]. Almost all mammalian tissues contain these cytochromes present in various organelles, predominantly in the membranes of the smooth endoplasmic reticulum and mitochondria.

Cytochrome P450s are part of the mixed-function oxidase (MFO) enzyme systems, which are further composed of NADPH:cytochrome P450 reductase and membrane phospholipids. The MFO catalyzes a wide variety of reactions, including epoxidations, *N*-dealkylations, *O*-dealkylations, *S*-oxidations, and hydroxylations of aliphatic and aromatic substrates. In this system, cytochrome P450 is the terminal oxidase, the last protein in the electron transport chain.

1.1.1. Nomenclature of cytochrome P450

A nomenclature that categorizes the individual CYPs into respective families and subfamilies, based primarily on sequence similarities, has been recently adopted. Enzymes exhibiting >40% similarity in protein sequence are classified within the same family and are designated by an Arabic number (CYP1). Those with sequence identity greater than 55% belong to the same subfamily and are designated by a capital letter (CYP1A). The individual isoform is then identified using a second Arabic numeral showing the subfamily designation (CYP1A1) [Nebert and McKinnon, 1994; Nelson et al., 1996]. Genes in the same subfamily

have been found to lie on the same chromosome within the same gene cluster [Hodgson, 2004].

There are now more than 2500 cytochrome P450 sequences known. Humans have 18 families of CYP genes and 43 subfamilies. Moreover, there are 57 sequenced CYP genes and 58 pseudogenes in the human genome. The list of CYPs is continually expanding. Progress in this area can be accessed at the Internet site of the P450 Gene Superfamily Nomenclature Committee [http://drnelson.uthsc.edu/CytochromeP450.html, 25.7.2010].

Although mammals are known to have 18 CYP families, only three families (families CYP1-3) are primarily responsible for the metabolism of most of the foreign compounds (xenobiotic). The remaining families are less promiscuous in their metabolizing abilities and are often responsible for specific metabolic steps. For example, members of the CYP4 family are responsible for the end-chain hydroxylation of long-chain fatty acids. Other mammalian CYP families are involved in biosynthesis of steroid hormones. For instance, CYP19 is responsible for the aromatization of androgens to estrogen by the initial step of hydroxylation at the 19-position. [Hodgson, 2004].

1.1.2. Chemical structure of cytochrome P450

Cytochrome P450s are hemoproteins consisting of apoprotein and heme b (***Fig. 1.1***). The heme iron is located in the center of the protoporphyrin ring IX, bound to the four pyrrole nitrogen atoms. Above and below the plane of the heme, there is room for two ligands. One ligand is a thiolate anion provided by a cystein residue [Poulos et al., 1985] and the other (axial) ligand in cytochrome P450 enzymes is most probably an OH group from a water molecule or hydroxyl group of amino acids [Dawson et al., 1982].

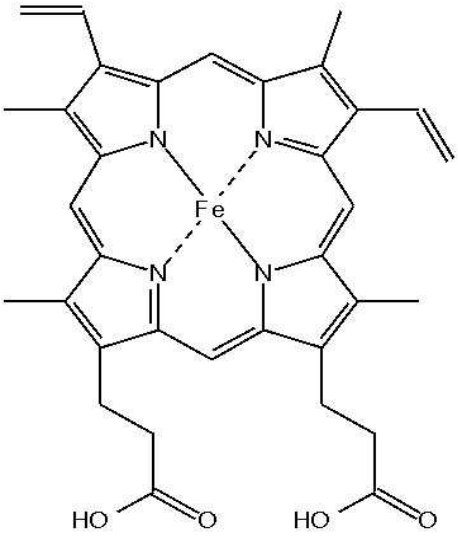


Figure 1.1 *Structure of heme b.*

The Fe^{3+} ion exists in the pentacoordinated (high-spin) and the hexacoordinated (low-spin) state. These forms are usually in equilibrium and possess different spectral properties [Jefcoate, 1978]. Changes in the spin state of hemoproteins are usually caused by ligand binding changing the state of the heme iron. Interaction with a hydrophobic substrate can lead to a shift from the low-spin to the high-spin state. In this interaction, displacement of a water

molecule by the substrate might lead to the movement of the iron ion out of the plane of the porphyrin ring, which makes the high-spin configuration favorable [Poulos et al., 1986; Lewis et al., 1989].

1.1.3. Cytochrome P450 catalytic cycle

Cytochrome P450s catalyze the incorporation of one atom of molecular oxygen into the substrate to give a product while the other oxygen atom is reduced by two electrones to give water.

The overall reactions catalyzed by CYPs can be summarized as follows:

$$\mathbf{RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+}$$

where RH is a substrate and ROH its hydroxylated metabolite.

The catalytic cycle for CYP-catalyzed reactions is expected to consist of at least eight discrete reactions (***Fig. 1.2***).

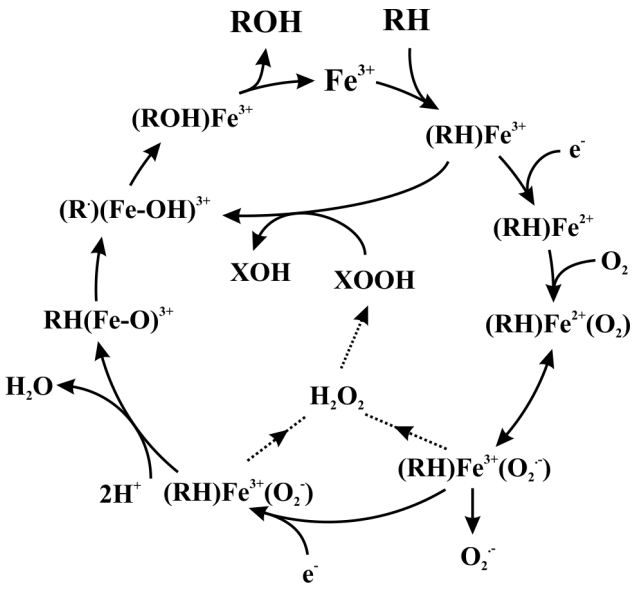


Figure 1.2 *Catalytic cycle of cytochrome P450.*

1) The first step in the reaction cycle is the binding of the substrate to the ferric (Fe^{3+}) form of the enzyme, which pertrubes the spin state equilibrium of the cytochrome P450 and facilitates the uptake of the first electron.

2) The second step involves the transfer of one electron from the NADPH:cytochrome P450 reductase to the iron of the ferric CYP enzyme to give a ferrous (Fe^{2+}) enzyme-substrate complex.
3) The reduced CYP-substrate complex then binds O_2 to form a ferrous enzyme-O_2-substrate ternary complex with the O_2 bound to the iron.
4) The addition of a second electron to this ternary complex by the reductase results in the formation of a more stable ferri-superoxide complex $Fe^{3+}O_2^-$.
5) The ferri-superoxide complex is reduced to the ferro-superoxide $Fe^{2+}O_2^-$ complex by NADPH:cytochrome P450 reductase or NADH:cytochrom b_5 reductase. This step is the last step of the activation phase of cytochrome P450 and is the rate-limiting step of the whole reaction cycle [Imai et al., 1977].
6) The next step involves the cleavage of the oxygen-oxygen bond. One of the oxygen atoms is released with the uptake of two protons at some stage, resulting in the formation of water. The retained oxygen remains associated with the heme iron as activated oxygen.
7) The activated oxygen atom associated with the iron is then inserted into the substrate, resulting in a two-electron oxidation of the substrate to the alcohol, followed by the radical recombination to form a product.
8) The product is then released, regenerating the original ferric CYP that is available to start another catalytic cycle [Hollenberg, 1992; Porter and Coon, 1991].

1.1.4. Induction of cytochrome P450s

The exposure to xenobiotics results in an increased expression of the CYP enzyme(s) usually capable of its metabolism. This adaptive response, known as induction, is a tightly regulated process that is controlled primarily at the level of transcription. Regulating the expression of CYPs allows the cell to increase the level of the necessary CYP enzymes only as needed to facilitate the excretion of xenobiotics [Ortiz de Montellano, 2005].

One type of CYP gene regulation is associated with peroxisome poliferators. Drugs act through a binding protein called PPAR or peroxisome proliferator activated receptor. When the drug is bound to this protein, it migrates to the nucleus, heterodimerizes with the retinoid X receptor (RXR) and binds to specific DNA sequences in the regulatory region of genes that are needed for peroxisome generation. The CYP4A1 gene is turned on by this mechanism. Peroxisomes oxidize fatty acids, and the CYP4A1 is a known fatty acid hydroxylase.

Another type is the tight regulation of the steroid hormones levels, which are under strict endocrine control. One example is the induction of steroid biosynthetic CYPs by the adrenocorticotropic hormone (ACTH). ACTH stimulates the production of cAMP that activates a protein kinase which phosphorylates an unidentified protein, leading to an increase in the gene transcription.

Frequently, the enzymes of the CYP1 family are induced by aromatic hydrocarbons. The activation involves a specific receptor called the Ah receptor. After the binding of an inducing agent to the receptor, a heterodimer with the activated Ah-receptor nuclear translocator (ARNT) is formed. This heterodimer then binds to a xenobiotic response element (XRE) and functions as a transcriptional enhancer to stimulate the gene transcription [http://drnelson.uthsc.edu/CytochromeP450.html, 25.7.2010].

The most extensively studied inducers of CYP1A subfamily *via* AhR are the halogenated aromatic hydrocarbons, e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), polychlorinated biphenyls, as well as polyclyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BaP) and 3-methylcholanthrene (3-MC). One of the highest affinity ligands of the AhR and the most potent inducer of CYP1A1 expression is TCDD. Due to this ligand-receptor interaction, exposure to TCDD produces a wide variety of toxic effects that are tissue and species specific. The response to TCDD is caused by the remarkably high affinity of TCDD to AhR and the resistance of this ligand to metabolism [Ortiz de Montellano, 2005].

All regulatory levels including increases in transcription, mRNA stabilization, translational efficiency increases, and post-translational protein stabilization have been shown to be involved in CYP2E1 induction [Ronis et al., 1996]. In rats, higher levels of ethanol and starvation increase transcription of CYP2E1 [Hollenberg, 2002].

Phenobarbital induces CYP2B enzymes 40-50 fold, through a phenobarbital receptor called CAR. This receptor also dimerizes with RXR as seen above with the PPAR receptor. The heterodimer binds to a phenobarbital response element in the DNA to activate the gene. [http://drnelson.uthsc.edu/CytochromeP450.html, 25.7.2010; Hollenberg, 2002; Ortiz de Montellano, 2005].

1.1.5. Inhibition of CYP

Until now, many inhibitors of the CYP enzymes have been identified. Although a lot of them are inhibitory for a number of different CYPs, some are highly selective for only one enzyme.

The nature of the catalytic cycle of the CYP enzymes (see Chapter 1.1.3., p. 11) presents a number of potential points at which inhibition of substrate metabolism can occur. The three steps that appear to be particularly susceptible to inhibition are:

- substrate binding
- the binding of molecular oxygen to the ferrous (Fe^{2+}) enzyme
- the catalytic step in which the activated oxygen is transferred from the heme iron to the substrate
- the transfer of electrons from the CYP reductases to the CYP

Based on their mechanisms, the inhibitors of the CYPs can be divided into three general categories: compounds that bind reversibly; compounds that form quasi-irreversible complexes with the iron of the heme prosthetic group; compounds that bind irreversibly to the prosthetic heme or to the protein.

Generally, reversible inhibition is probably the most common consequence of drug-drug interactions. After the elimination of the inhibitor from the body, the normal metabolic functions of the enzymes will continue as the reversible inhibition of CYPs is transient. Reversible inhibition can be further classified as competitive, noncompetitive, uncompetitive, and may involve product inhibition.

The second category of inhibitors requires catalytic activation by the enzyme to transient intermediates that then coordinate tightly to the prosthetic heme or protein in the CYP active site leading to the irreversible inhibition.

The final category of inhibitors represents those which require metabolic activation by the CYPs and are part of the class of inhibitors commonly referred to as „catalysis-dependent“, „mechanism-based“ or „suicide“ inactivators.

The insight into all three categories is very important for the development of highly selective isozyme-specific inhibitors of the CYPs. For studies probing the structure, mechanisms of action, and biological roles of specific CYPs, these inhibitors are of

substantial interest. They can also be used as therapeutic agents, because their potential is likely to modulate various CYP activities [Hollenberg, 2002].

1.1.6. CYP1A subfamily

The CYP1A subfamily consists of CYP1A1 and CYP1A2. They belong to the most important CYP enzymes involved in the activation of procarcinogens, such as polycyclic aromatic hydrocarbons, aromatic amines and heterocyclic amines [Eaton et al., 1995; Rendic and Di Carlo, 1997]. Higher CYP1A1 expression and activity seem to be associated with the risk of lung and colorectal cancer. CYP1A2 is responsible for metabolizing many frequently used drugs, such as phenacetin, caffeine, imipramine and it also activates procarcinogens. Moreover, it has been suggested that CYP1A2 plays a role in human tobacco-related cancers [Smith et al., 1996]. ***Table 1.1*** shows CYP1A substrates, inducers and inhibitors.

Table 1.1 *Substrates, inducers and inhibitors of CYP1A1 and CYP1A2.*

CYP	Endogenous substrate reactions	Xenobiotic substrates activated	Xenobiotic substrates metabolized	Inducers	Inhibitors
1A1	prostaglandin ω-2 oxidation, testosterone 6-β-hydroxylation	BaP, polycyclic aromatic hydrocarbons, PhIP	7-ethoxycoumarin, 7-ethoxyresorufin, tacrine	3-MC, βNF, TCDD, omeprazole, cigarette smoke, chrysin	9-OH-ellipticine, 7,8-benzoflavone, apigenin
1A2	prostanglandin ω-1 oxidation, testosterone 6-β-hydroxylation	2-AAF, acetaminophen, aflatoxin B, PhIP	caffeine, phenacetin, 7-ethoxyresorufin, 7-methoxyresorufin	3-MC, βNF, TCDD, omeprazole, cigarette smoke	furafylline, apigenin, isosafrole

BaP, benzo[a]pyrene; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; 3-MC, 3-methylcholanthrene; βNF, β-naphthoflavone; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin, 2-AAF, 2-acetylaminofluorene
Data source: [Guengerich and Shimada, 1991; Ortiz de Montellano, 2005; Rendic and Di Carlo, 1997; Stiborová et al., 1999].

CYP1A1 and CYP1A2 exhibit ~70% sequences identity in their coding region. Expression of CYP1A1 has been reported in a number of extrahepatic tissues including pancreas, lung, gastrointestinal tract, thymes, prostate, uterus, and mammary glands [Ortiz de Montellano, 2005]. However, the clear evidence that CYP1A1 is also expressed in human liver microsomes has been provided [Stiborová et al., 2002]. In contrast, CYP1A2 is primarily expressed in liver at significant levels [Dogra et al., 1998]. Both of these enzymes are inducible *via* Ah receptor [Gonzalez et al., 1993].

1.1.7. CYP2B subfamily

CYP2B6 is the only member of this subfamily in humans. Orthologic isoforms of CYP2B6 are CYP2B1 and CYP2B in a rat (*Rattus norvegicus*), and CYP2B4 in a rabbit (*Leporidae*), respectively. CYP2B6 is constitutive and inducible, expressed in liver [Ortiz de Montellano, 2005; Guengerich, 1992], lung [Ortiz de Montellano, 2005; Stiborová et al., 1999] and gastrointestinal tract [Stiborová et al., 1999]. This enzyme is involved in the metabolism of many clinical drugs (e.g. cyclophosphamide, barbiturates) [Hodgson, 2004] and environmental chemicals [Hodgson and Rose, 2007]. Moreover, this enzyme is responsible for the activation of organophosphates as well as some carcinogens (e.g. aflatoxin B1) [Ekins and Wrighton, 1999].

1.1.8. CYP3A subfamily

In humans, there are two major isoforms of CYP3A subfamily, CYP3A4 and CYP3A5. The level of CYP3A is estimated to be 30% of the total hepatic microsomal CYPs [Shimada et al., 1994] and up to 80% in small intestine in humans [Paine et al., 2006]. Both members are constitutive and inducible [Yan and Caldwell, 2001] and are involved in the metabolism of many important drugs (e.g. nifedipine, midazolam, erythromycin, cyclosporin). CYP3A4 is highly expressed in liver. Other tissues containing CYP3A are small intestine (3A4), lung, uterus, fetus, gastrointestinal tract and placenta. These enzymes are also responsible for the activation of some procarcinogens and mutagens including aflatoxin B1 and G1, aromatic amines, and PAHs [Ortiz de Montellano, 2005; Stiborová et al., 1999].

1.2. Biotransformation of xenobiotics

Organisms in the environment are surrounded by a large number of foreign compounds (xenobiotics) which are potentially harmful, and thus the excretion of xenobiotics from human body is desirable. Biotransformation can be defined as an enzyme-catalyzed conversion of one xenobiotic compound into another. This process usually consists of two phases. Phase I reactions include hydrolysis, reduction, and oxidation, while phase II reactions are usually conjugation reactions.

In general, the first phase of biotransformation leads to more polar compounds that may be more easily excreted from the organism. On the other hand, biotransformation can also be responsible for the metabolic activation of some substrates to reactive intermediates

(e.g. free-radicals), which then react with cellular macromolecules to initiate toxic and carcinogenic events [Guengerich and Shimada, 1991]. Major enzymes involved in phase I are for example cytochrome P450s, flavin-containing monooxygenases, peroxidases, NADPH:cytochrome P450 reductase, etc.

In phase II, the metabolite is conjugated with an endogenous polar compound (sugar, amino acid, glutathione, sulphate or phosphate) leading to a more water soluble compound. These conjugations result in the deactivation and excretion of xenobiotics from the human body.

The most important enzymes of phase II are glutathione-*S*-transferase, UDP-glucuronosyltransferase, sulphotransferase, *N*- and *O*-acetyltransferase. However, phase II enzymes involved in conjugations are not only protective but they can also activate chemical carcinogens. Examples of bioactivation roles are known for *N*-acetyltransferase [Grant et al., 1992], sulphotransferase [Yamazoe et al., 1999; Boberg et al., 1983], and UDP-glucuronosyltransferase [Kaderlik et al., 1994].

The principal organ of xenobiotic metabolism is quantitatively the liver. Other sites of xenobiotic metabolism include epithelial cells of the gastrointestinal tract, lungs, kidneys, and the skin.

Hence, most studies on xenobiotic-metabolizing enzymes have been carried out with the liver enzymes. However, it is the gastrointestinal tract which is the first barrier met by the exogenous compounds of food or orally delivered drugs. In order for them to be absorbed and transferred to the whole body, these compounds first have to pass through the intestinal epithelium. Under physiological conditions, detoxification systems have thus to minimize, within intestinal cells, the potential of damage from toxic xenobiotics [Carrière et al., 2001].

1.3. Carcinogenesis

Cancer is one of the leading diseases in the human population worldwide with one of the highest mortalities. The development of cancer is a complex process in which a large number of factors interact to disrupt normal cell growth and division. Cancer can be caused by internal factors such as heredity, immunology, and hormones as well as external factors such as chemicals (e.g. nickel, nitrosamines, aflatoxins), viruses (Human papilloma virus), bacteria (*Helicobacter pylori*), diet, and radiation (UV irradiation, ionizing radiation) [Schulz, 2005].

Among the main causes of cancer, lifestyle factors such as diet, cigarette smoke, alcohol, and sun exposure are involved. It has been reported that dietary habits are the main determinant for the genesis of cancer [Shukla and Arora, 2003].

The development of cancer is a multi-stage process involving damage to the genetic material (DNA) of cells. This damage primarily occurs in genes regulating normal cell growth and division. To develop cancer, several mutations or several stages are required. Usually there is a long latent period before cancer appears.

In the Czech Republic, neoplasms are the second most prevalent cause of death, the first being cardiovascular diseases. Most common types of cancer in the Czech Republic are malignant neoplasia of colon and rectum, bronchus and lung, breast, pancreas, female and male genital organs [http://www.czso.cz/csu/edicniplan.nsf/select_obyvatelstvo, 25.7.2010].

Nowadays, more attention is being paid to the possibility of applying chemopreventive agents to prevent cancer or reduce cancer risk of individuals. Thus, the consumption and use of dietary supplements containing concentrated chemopreventive compounds increased dramatically in recent years [Hodek et al., 2009].

For Chemoprevention, see Chapter 1.4., p 24.

1.3.1. Carcinogens

Chemical agents influencing cancer development can be classified into two major groups based on their mutagenic/non-mutagenic effects. DNA-damaging agents (genotoxic) are mutagenic in *in vitro* mutagenicity assays and are considered to produce permanent alterations in the genetic material *in vivo*, and epigenetic agents (nongenotoxic) are not mutagenic in *in vitro* assays [Hodgson, 2004].

The list of chemicals that can induce cancer is extensive. They can show high specifity for the organ in which the tumor is induced and in the molecular mechanisms through which they operate. Chemical carcinogens include organics and inorganics, fibres and particulates, and biologically active materials (e.g.hormones). The organic chemicals probably comprise the largest group [Alison, 2004].

According to the International Agency for Research on Cancer (IARC) and Environmental Protection Agency (EPA), carcinogens are divided into 5 groups (***Tab. 1.2***).

Table 1.2 *IARC and EPA classification of carcinogen.*

IARC	EPA	
1	**Group A**	**Human carcinogens**
Sufficient evidence from epidemiological studies to support a causal association between exposure to the agents and cancer		
2A	**Group B**	**Probable human carcinogens**
Group **B1** Limited epidemiological evidence that the agent causes cancer regardless of animal data Group **B2** Inadequate epidemiological evidence or no human data on the carcinogenicity of the agent and sufficient evidence in animal studies that the agent is carcinogenic		
2B	**Group C**	**Possible human carcinogens**
Absence of human data with limited evidence of carcinogenicity in animals		
3	**Group D**	**Not classifiable as to human carcinogenicity**
Agents with inadequate human and animal evidence of carcinogenicity or for which no data are available		
4	**Group E**	**Evidence of noncarcinogenicity for humans**
Agents that show no evidence for carcinogenicity in at least two adequate animal tests in different species or in both adequate epidemiologic and animal studies		

Adapted from [Hodgson, 2004].

1.3.1.1. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a class of chemicals containing two or more fused aromatic rings. They are formed during the incomplete combustion or pyrolysis of organic matter and during various industrial processes. PAHs are released into the atmosphere as a complex mixture of compounds and probably are the most widely distributed class of potent carcinogens to humans in the environment. Humans are exposed to PAHs by various means; the primary routes of exposure are the inhalation of polluted air, wood smoke, and tobacco smoke, as well as the ingestion of contaminated water and foods normally containing microgram quantities of PAHs [Alexander et al., 2008]. Based on sufficient evidence of mutagenicity/genotoxicity in experimental animals [IARC 1973, 1983, 1987], 15 PAHs are reasonably anticipated to be human carcinogens (***Tab. 1.3***).

Table 1.3 *Polycyclic aromatic hydrocarbons anticipated to be human carcinogens.*

benz[a]anthracene	7H-dibenzo[c,g]carbazole	indeno[1,2,3-cd]pyrene
benzo[b]fluoranthene	dibenzo[a,e]pyrene	5-methylchrysene
benzo[j]fluoranthene	dibenz[a,h]acridine	dibenzo[a,h]pyrene
benzo[k]fluoranthene	dibenz[a,j]acridine	dibenzo[a,i]pyrene
benzo[a]pyrene	dibenz[a,h]anthracene	dibenzo[a,l]pyrene

Benzo[a]pyrene

Although several hundred different polycyclic aromatic hydrocarbons are known, benzo[a]pyrene (BaP) is the most commonly studied and measured. According to IARC/WHO, BaP is classified as "Carcinogenic to humans" (group 1, ***Tab. 1.2***, p. 19). Moreover, BaP is also one of the most prevalent PAHs found in cigarette smoke. The main routes of BaP exposure are through the inhalation of polluted air or tobacco smoke, diet [Phillips, 1999; Phillips, 2002] and occupational exposition to e.g. coal, coke or coal-tar processing and use of coal-tar products [IARC, 1983]. It has been shown that BaP has cytotoxic, teratogenic, genotoxic, mutagenic, and carcinogenic effects in various tissues in organisms [Nebert, 1989; Ellard et al., 1991]. To covalently modify DNA, BaP requires metabolic activation, which is an essential step in the mechanism by which BaP exerts its genotoxic effects [Luch et al., 2005; Phillips, 2005]. BaP is metabolically activated mainly by CYP1A1 and CYP1B1 to epoxide intermediates that are further converted to more reactive diol-epoxides by epoxide hydrolase (***Fig. 1.3***). Many of these metabolites are further metabolized by phase II enzymes (detoxification pathway). Studies examining the carcinogenicity of BaP have identified the 7,8-oxide and 7,8-dihydrodiol as proximate carcinogens and the 7,8-diol-9,10 epoxide as a strong mutagen and ultimate carcinogen [Hodgson, 2004].

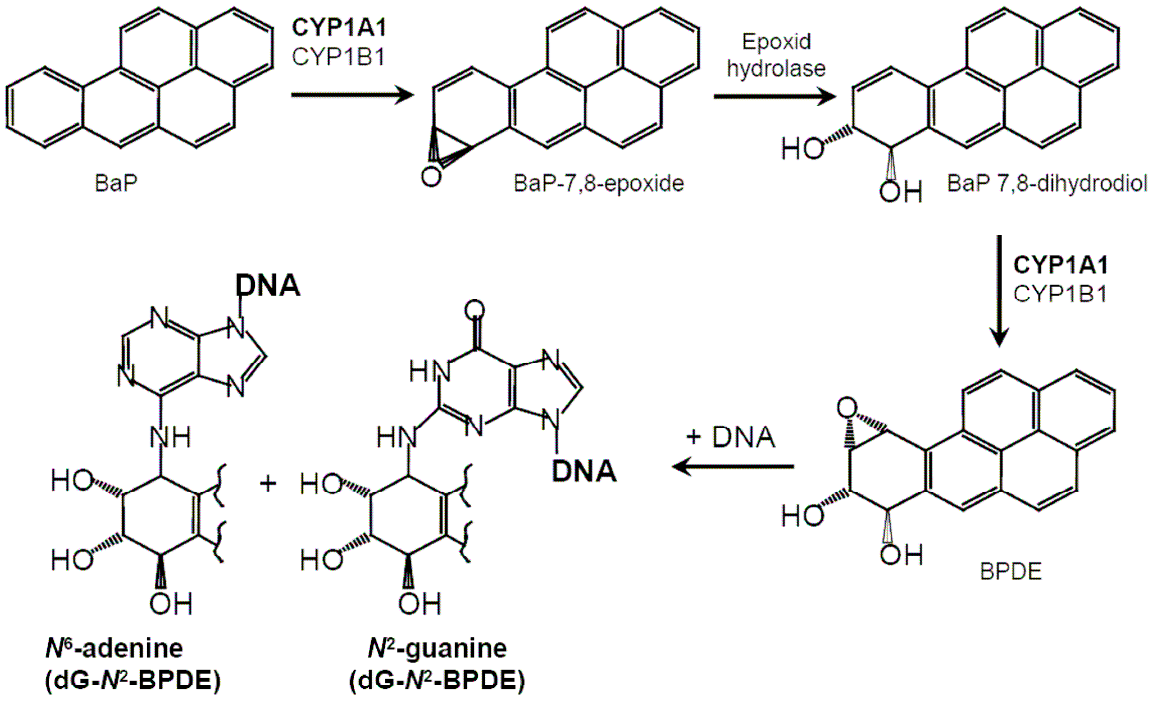


Figure 1.3 *Metabolic activation and DNA adduct formation by benzo(a)pyrene. Adapted from [Aimová et al., 2008]. BPDE, benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide*

1.3.1.2. Heterocyclic amines

Dietary exposure to heterocyclic amines (HCAs) is considered to be a potential human cancer risk. These compounds are produced during the cooking of red meat, poultry, and fish under normal household conditions [Sinha et al., 2000]. They are formed in relatively high concentrations by the condensation of creatinine with amino acids. The structures of four of the most abundant HCAs identified in common cooked foods, AαC (2-amino-9Hpyrido[2,3-b]indole), MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline), PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and IFP (2-amino-1,6-dimethylfuro[3,2-e]imidazo[4,5-b]pyridine), are shown in ***Figure 1.4***. Interestingly, IFP is the only HCA detected in cooked foods that contains an oxygen atom [Pais and Knize, 2000].

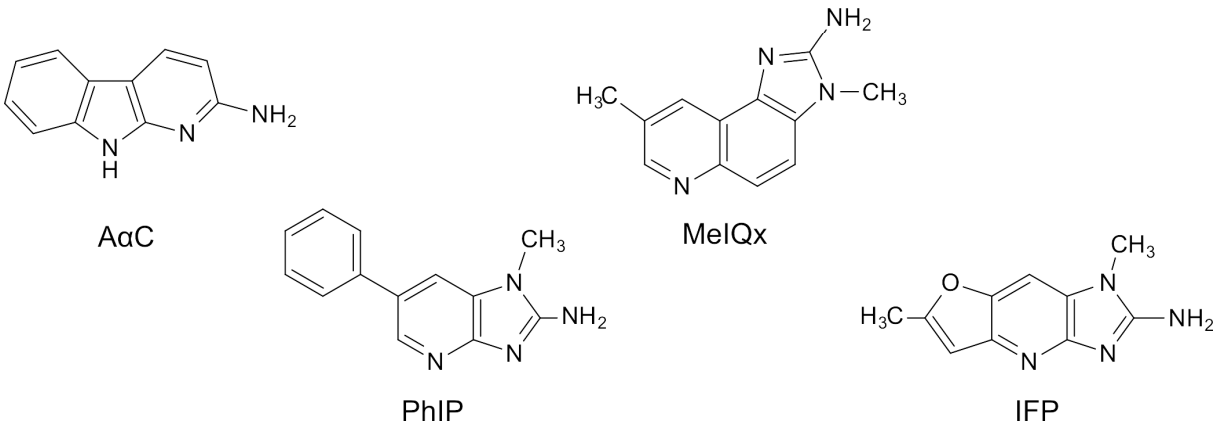


Figure 1.4 *Structures of the most abundant heterocyclic amines.*

Several of these compounds have been shown to be carcinogenic in long-term animal studies [Adamson et al., 1990; Adamson et al., 1994; Ohgaki et al., 1991; Wakabayashi et al., 1992]. Moreover, according to IARC/World Health Organisation, MeIQ, MeIQx, and PhIP are classified as „Possibly carcinogenic to humans“ (group 2B, ***Tab. 1.2***, p. 19) and IQ is classified as „Probably carcinogenic to humans“ (group 2A, ***Tab. 1.2***, p. 19) [http://monographs.iarc.fr/ENG/Classification/index.php, 25.7.2010]. The amounts of HCAs are dependent on the cooking method, time, temperature, and type of meat [Sinha et al., 2000]. Based on the results of a population-based study, the average human daily intake (randomly selected from a registered population) of HCAs is 164 ng with the highest intake being 1816 ng [Augustsson et al., 1999].

The carcinogenity of HCAs results from the metabolic activation of parent compounds to reactive species that bind to DNA forming DNA adducts, which is considered to be an initiating step in chemical carcinogenesis.

2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

PhIP administered orally can induce lymphoma tumors and tumors of small intestine, colon in rats, mammary gland tumors in female rats [Nagao et al., 1994] and prostate in males [Report on Carcinogens, Eleventh Edition]. Moreover, it has been shown that PhIP caused tumors in mice at multiple tissue sites [Report on Carcinogens, Eleventh Edition] and is mutagenic in bacterial and mammalian toxicity assays [Felton and Knize, 1991; Frandsen et al., 1991]. The role of PhIP and other HCAs in human cancer causation is of interest, in particular for cancers of colon and breast [Snyderwine, 1994; Nagao et al., 1994].

The mutagenicity/carcinogenicity of PhIP is considered to initially involve the oxidation of exocyclic amino group to its corresponding *N*-hydroxylated derivative by

cytochrome P450s (***Fig 1.5*****)** [Holme et al., 1989]. PhIP is metabolized to two major products: mutagenic 2-hydroxylamino-PhIP (N^2-OH-PhIP) and non-mutagenic 4´-hydroxylamino-PhIP (4´-OH-PhIP). Both products can be conjugated by glucuronidase and sulfotransferase to produce non-mutagenic species that are readily excreted. On the other hand, *N*-hydroxylamino-PhIP can be converted by *N*-acetyltransferases (NAT) or sulfotransferases (SULT) into an ester capable of undergoing heterolytic cleavage to produce a PhIP-nitrenium ion, which is the ultimate reactive species that binds to DNA [Turesky, 2007; Frandsen et al., 1992]. It has been shown, that the major covalent DNA adduct detected *in vivo* caused by the exposure of experimental animals and humans to PhIP is *N*-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP-C8-dG) [Frandsen et al., 1992; Lin et al., 1992]. Several previous studies have shown that hepatic CYP1A2 is responsible for the *N*-hydroxylation of PhIP, which is a major route of its metabolism and bioactivation [Ortiz de Montellano, 2005]. The study of Crofts et al. [1998] provides evidence that human CYP1A1 also produces significant levels of N^2-OH-PhIP, and thus possesses the ability to activate PhIP.

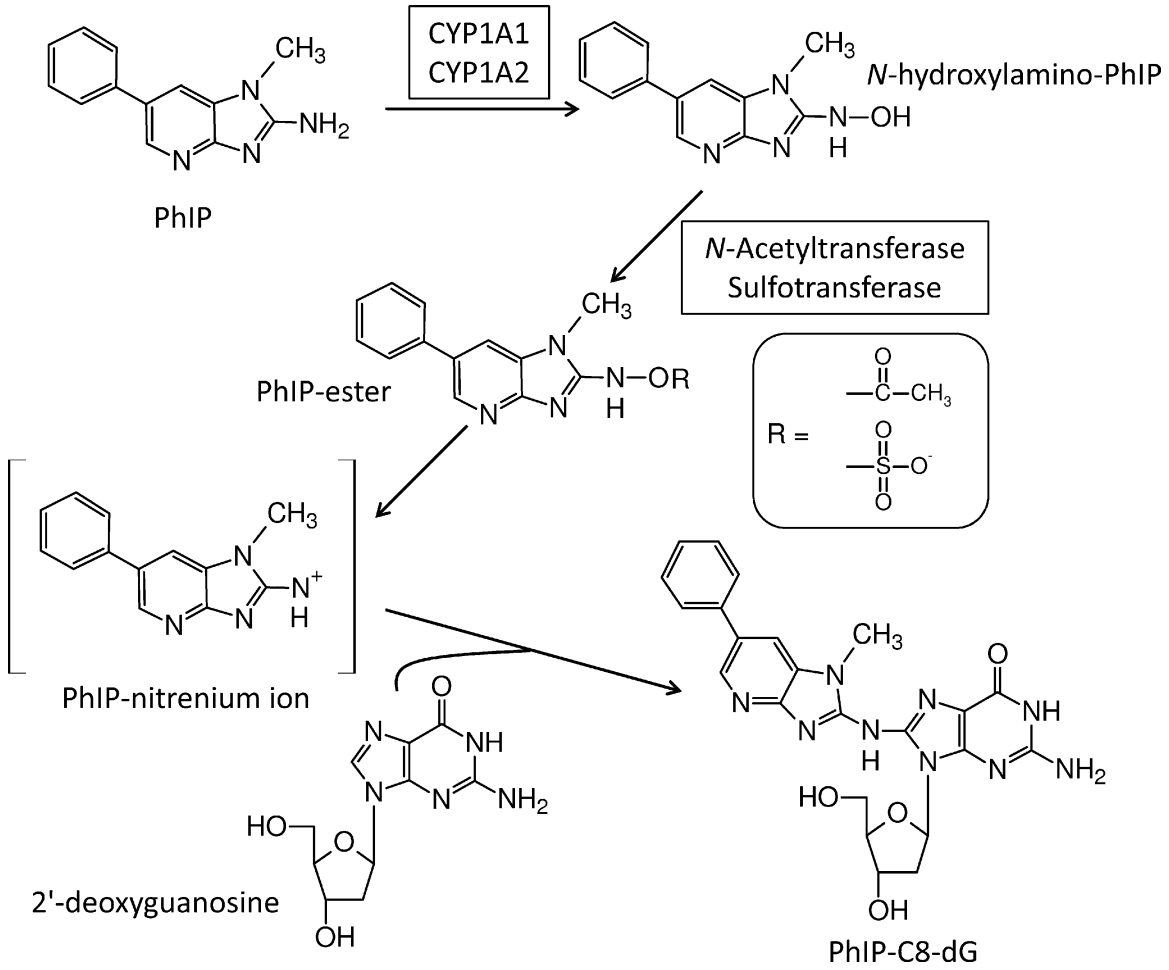


Figure 1.5 *Bioactivation of PhIP and DNA adduct formation. Adapted from [Singh et al., 2010].*

1.4. Chemoprevention

Chemoprevention is defined as the use of naturally occuring or synthetic chemical compounds to prevent cancer or reduce cancer risk. The strategy of chemoprevention is based on the use of exogenous factors (diet constituents, supplements or drugs, immunization) to enhance endogenous mechanisms that reduce the risk arising from exposure to the environmental carcinogens by affecting various stages of cancer development [Hodek et al., 2009].

Chemopreventive compounds can be classified into two groups: blocking agents and supressing agents (***Fig. 1.6***). Blocking agents prevent carcinogenic compounds from reaching or reacting with critical sites in the tissues. They can participate in the activation, detoxification or they can trap carcinogens before reaching target sites. There are three major mechanisms by which blocking agents act. Some blocking agents prevent the activation of

carcinogens or tumor promoters requiring metabolic activation. A second group of blocking agents is effective by virtue of their capacity to enhance detoxification systems. A third group of blocking agents trap reactive carcinogenic species before they reach critical target sites.

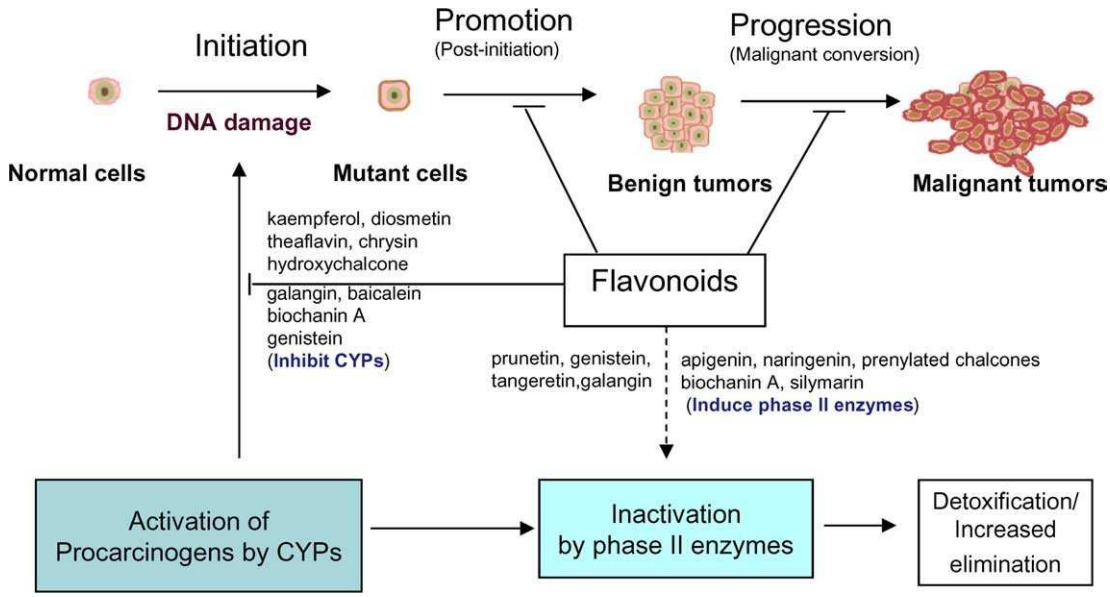


Figure 1.6 *Effects of blocking and suppressing agents in multistage carcinogenesis. Adapted from [Moon et al., 2006].*

Supressing agents prevent the evolution of the neoplastic process in tissues that otherwise would become malignant [Wattenberg, 1996]. There are several means known by which suppressing agents produce their effects. Some act by producing differentiation, others are directed specifically at counteracting the consequences of genotoxic events, in particular, oncogene activation, and a third group produces selective inhibition of proliferation of potentially malignant cells [Shukla and Pal, 2004].

Naturally occurring chemopreventive compounds might be also classified into several groups according their structure:

- vitamins (vit. A, C, E, B_2, B_6, B_{11}, B_{12})
- minerals and trace elements (Ca, P, Zn, Se, Mb)
- other compounds of plant origin:
    - sulphides from allium vegetables (onion, garlic, chives)
    - dithiolthiones and glucosinolates (converted into isothiocyanates and indoles) from cruciferous vegetable (cauliflower, cabbage, Brussel sprouts)

- terpenoids (plus limonene) from citrus fruit
- phyto-oestrogens (isoflavones and lignans) from cereals and pulses, wholegrain products, seed, fruit and berries
- flavonoids (e.g. quercetin, kaempherol, rutin, tangeritin, myricetin) from fruit and vegetable (e.g. berries, tomatoes, onion, broccoli, beans, citrus fruit)
- phenolic compounds (ellagic acid, caffeic acid, ferulic acid, resveratrol) from nuts, fruit, wine and tea.

Phytochemicals are the most popular chemopreventive compounds as their intake is widely acceptable psychologically due to their plant origin. Thus, the consumption and use of dietary supplements containing concentrated phytochemicals increased dramatically in recent years [Hodek et al., 2009]. Around 2000 natural or synthetic agents have been described in experimental systems to have chemopreventive activity [Shukla and Pal, 2004].

Besides expected beneficial effects, these food supplements may exert negative activities coming namely from: (i) their toxicity *per se*, (ii) metabolic conversion into cytotoxic, pro-oxidant or mutagenic agents, (iii) interference with endogenous metabolic pathways, (iv) interaction with other chemicals from diet, environment, or drugs, (v) induction of carcinogen activating enzymes, and (vi) effects on human intestinal microflora [Hodek et al., 2009].

1.4.1. Flavonoids

Flavonoids are a diverse group of naturally occuring phenolic compounds. They are widely distributed in most plants and are an important component of human diet [Nikolic and van Breemen, 2004]. Moreover, extracts of many flavonoids are now available in health food stores as dietary supplements. Today, more than 8000 different flavonoid structures have been identified [Pietta, 2000]. The word “flavonoid” derives from the Latin word “flavus” which means yellow. Some flavonoids are intensely colored, providing a spectrum of colors from red to blue in flowers, fruits and leaves, and other flavonoids are essentially colorless. Coloring is important for attracting insects and birds to the plant for pollination and seed dispersal.

The major sources of flavonoids are fruits (e.g. citrus fruits, rose hips, apricots, cherries, grapes, black current), vegetables (e.g. onion, broccoli, tomatoes, pepper, spinach), soy beans and herbs (e.g. *Sylibum marianum*, *Alpinia officinarum*, *Hypericum perforatum*)

[Barnes et al., 2001]. Flavonoids are also concentrated in seeds, grains, nuts and flowers, as well as in beverages, such as red wine, tea and beer [Kühnau, 1976].

In 1971, the average total intake of flavonoids in the United States was estimated to be 1 g/day [Kühnau, 1976; Scalbert and Williamson, 2000], however, recent studies have indicated that these reuslts are overestimated and that the intake varies widely. According to the study of Pietta [2000], dietary intake of flavonoids can range between 50 and 800 mg/day, depending on the consumption of vegetables, fruits, and beverages, such as red wine or tea [Pietta, 2000]. The most abundant flavonoids are the flavonols quercetin and kaempferol, which are present in many fruits and vegetables as well as in beverages. Soy beans are the main source of isoflavones containing approximately 1 mg of daidzein and genistein per g of dry beans [Reinli and Block, 1996]. The main food sources of flavanones are citrus fruits, hesperidin from oranges being the most consumed [Rousseff et al., 1987].

1.4.1.1. Structure of flavonoids

The chemical structure of flavonoids is based on an oxygenous heterocyclic compound flavane (***Fig. 1.7***), which consists of 15 carbon atoms arranged in three rings (C6-C3-C6) labeled as A, B and C. Chromane ring (A+C) can bear a second aromatic ring B in position 2 (flavonoids), 3 (isoflavonoids) or 4 (synthetic flavonoids). The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a group differ in the pattern of substitution of the ring A and B. The large number of compounds arises from various combinations of multiple hydroxyl and methoxyl groups substituting the basic flavonoid skeleton [Pietta, 2000].

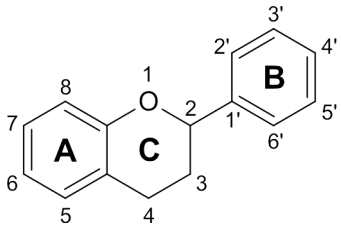


Figure 1.7 *Structure of flavane.*

Natural flavonoids usually occur as β-*O*-glycosides or β-*C*-glycosides, where the linked sugar is often galactose, glucose, rhamnose, arabinose or other sugars [Nikolic and van Breemen, 2004]. Most commonly there is one sugar, but can be two or three, and there

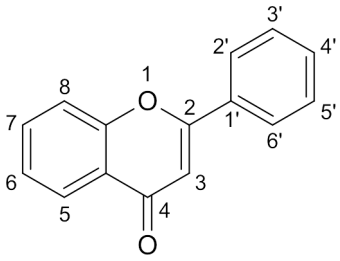
are several possible positions of substitution [Scalbert and Williamson, 2000]. Free flavonoids occur only rarely.

Flavonoids can be divided into seven main classes according to the degree of oxidation of the heterocycle, into flavones (***Tab. 1.4***), flavonols (***Tab. 1.5***), flavanones (***Tab. 1.6***), isoflavones (***Tab. 1.7***), flavanols, anthocyanins, and chalcones. Flavones differ from flavanones by the presence of a C2-C3 double bond on the C ring.

- **Flavones** mainly occur as glycosides of luteolin and apigenin. They are present in parsley and celery, in cereals (wheat and millet) and in citrus fruit skin (polymethoxylated flavones: tangeretin, nobiletin, sinesetin).
- **Flavonols** are present in food in higher amounts than other flavonoids. The most abundant are quercetin and kaempferol. Flavonols are present in onion, curly kale, leeks, broccoli, blueberries, red wine and tea (mainly in outer parts, which are exposed to sunlight), and are found mainly as glycosides with sugar moiety.
- **Flavanones** are present in tomatoes and aromatic plants (e.g. mint), and in highest concentration in citrus fruits (aglycones: e.g. naringenin, hesperetin, eriodictyol). Flavanones are usually glycosylated by disaccharides.
- **Isoflavones** are also called phytoestrogens because of their structural similarities to estrogens and their pseudohormonal properties. The most frequent isoflavones are genistein, daidzein and glycitein; they are present in leguminous plants.

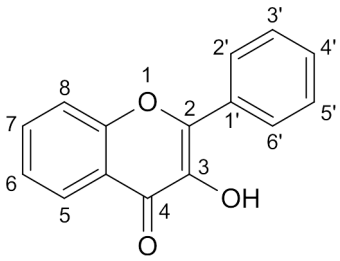
  Adapted from [Burdová, 2006].

Table 1.4 *Flavones.*



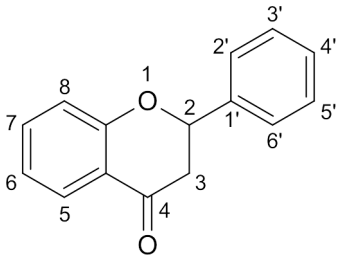
Common name	C3´	C4´	3	5	6	7	8
Flavone	H	H	H	H	H	H	H
Apigenin	OH	H	H	OH	H	OH	H
Chrysin	H	H	H	OH	H	OH	H
Diosmin	OH	OCH_3	H	OH	H	rutinose	H
Diosmetin	OH	OCH_3	H	OH	H	OH	H
Tangeretin	OH	OCH_3	H	OCH_3	OCH_3	OCH_3	OCH_3
Luteolin	OH	OH	H	OH	H	OH	H
Baicalin	H	H	H	OH	OH	glucuronide	H
Baicalein	H	H	H	OH	OH	OH	H
Acacetin	H	OCH_3	H	OH	H	OH	H

Table 1.5 *Flavonols.*



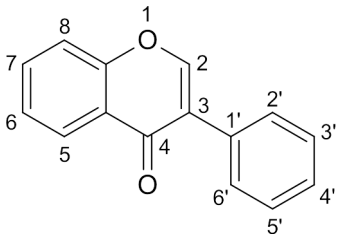
Common name	C2´	C3´	C4´	3	5	7	8
Flavonol	H	H	H	OH	H	H	H
Fisetin	H	OH	OH	OH	H	OH	H
Kaempherol	H	H	OH	OH	OH	OH	H
Quercetin	H	OH	OH	OH	OH	OH	H
Isoquercitrin	H	OH	OH	glucose	OH	OH	H
Rutin	H	OH	OH	rutinose	OH	OH	H
Galangin	H	H	H	OH	OH	OH	H
Morin	OH	H	OH	OH	OH	OH	H
Rhamnetin	H	OH	OH	OH	OH	OCH_3	H

Table 1.6 *Flavanones.*



Common name	C3´	C4´	3	5	6	7	8
Flavanone	H	H	H	H	H	H	H
Hesperetin	OH	OCH_3	H	OH	H	OH	H
Hesperidin	OH	OCH_3	H	OH	H	rutinose	H
Naringenin	H	OH	H	OH	H	OH	H
Naringin	H	OH	H	OH	H	rutinose	H

Table 1.7 *Isoflavones.*



Common name	C3´	C4´	2	5	6	7	8
Daidzein	H	OH	H	H	H	OH	H
Biochanin A	H	OCH_3	H	OH	H	OH	H
Genistein	H	OH	H	OH	H	OH	H

1.4.1.2. Metabolism of flavonoids

An understanding of the processes involved in the absorption and distribution of polyphenols is essential for determining their bioactivities *in vivo* and their significance. ***Figure 1.8, p. 32*** summarizes the flavonoid metabolism in organisms. Few studies have investigated the ability of saliva and gastric juice to alter the flavonoid structure. For instance, quercetin rutinoside, rutin, has been shown to be hydrolysed by cell-free extracts of human salivary cultures [Laires et al., 1989; Macdonald et al., 1983]. In contrast, the quercetin-3-rhamnoside, quercitrin, is not susceptible to hydrolysis, suggesting that only rutin-glycosidase-elaborating organisms occur in saliva [Macdonald et al., 1983].

There are many factors influencing the extent and rate of absorption in small intestine, such as molecular size, lipophilicity, solubility, and pKa and biological factors including gastric and intestinal transit time, lumen pH, membrane permeability, and first-pass metabolism.

Using isolated preparations of rat small intestine, it has been shown that flavonoid glycosides (luteolin-7-glucoside, kaempferol-3-glucoside, and quercetin-3-glucoside) were cleaved by rat intestinal mucosa supporting the presence of β-glucosidase. The major products transferred across the small intestinal epithelium were glucuronides of the parent aglycone or of the hydrolysed glycoside, although *O*-methylated metabolites were also observed [Spencer et al., 1999]. The study with naringenin and naringenin-7-glucoside shows that gulucuronidation and hydrolysis can occur during transfer across the small intestine [Rice-Evans and Packer, 2003].

Flavonoid glycosides are generally relatively polar in nature, thus their passive diffusion across the membranes of the small intestinal brush border is limited. Many studies, however, have suggested that flavonoid glycosides are subject to the action of β-glucosidases before their absorption in the jejunum and ileum [Spencer et al., 1999; Day and Williamson, 2001; Hollman et al., 1999; Gee et al., 2000], and it is generally believed that the removal of the glycosidic moiety is necessary before absorption of the flavonoid can take place.

Because the colon contains approximately 10^{12} microorganisms/cm^3, it has an enormous catalytic and hydrolytic potential, and the enzymatic degradation of flavonoids by the colonic microflora can result in a huge array of new metabolites. For example, bacterial enzymes may catalyze many reactions, including hydrolysis, dehydroxylation, demethylation, ring cleavage, and decarboxylation, as well as rapid deconjugation [Scheline, 1999].

Interestingly, several flavonoids (e.g. hesperetin, naringenin, diosmetin) and phenolic acids generated from flavonoids by human intestinal microflora have been observed to be effective inhibitors of the growth of *Helicobacter pylori*, a bacterium causing problems in the stomach of some patients [Bae et al., 1999].

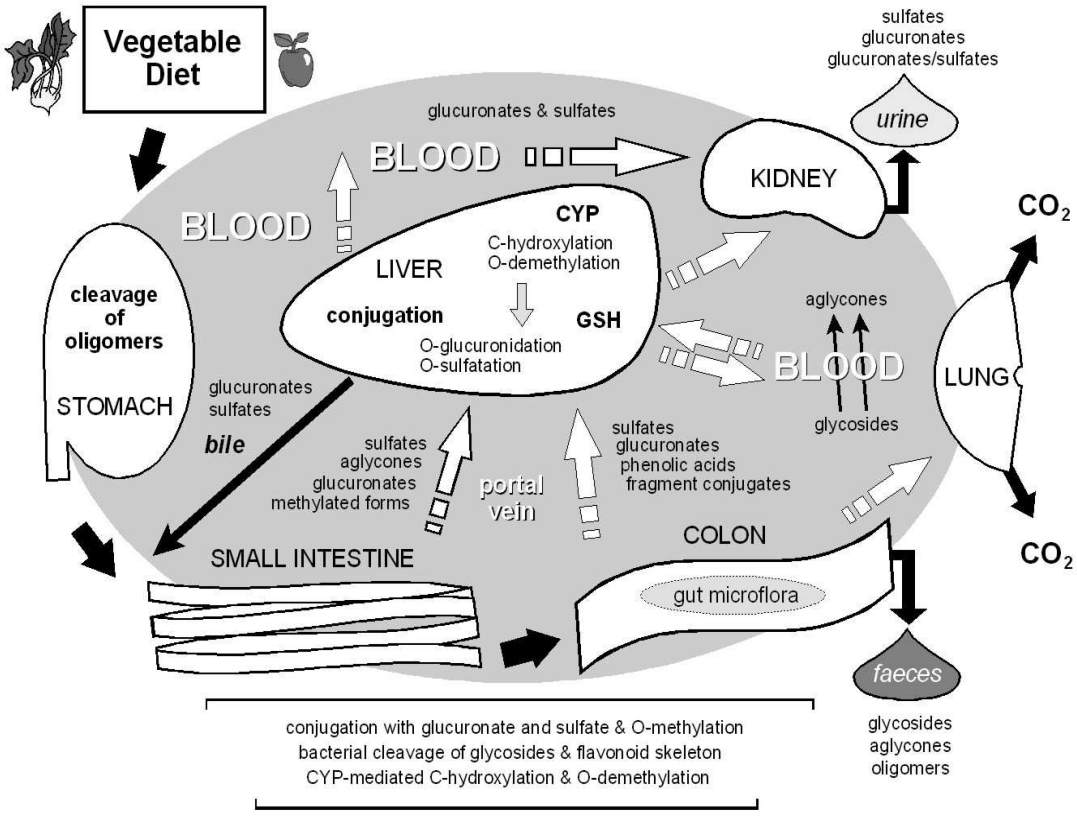


Figure 1.8 *The overall fate of flavonoids in organism. Figure by courtesy of prof. RNDr. Petr Hodek, CSc.*

1.4.1.3. Biological activities of flavonoids

In plants, flavonoids play many different roles such as attractants for pollinating insects (coloured flavonoids) and catalysts in light phase of photosynthesis. Moreover, they protect plants from UV radiation and can take part in defence mechanism against microorganisms due to their astringency (e.g. catechins, flavanols) [Pietta, 2000]. Under stress conditions, such as excessive UV light, wounding or infection, the induction of some flavonoids may occur [Bennet and Wallsgrove, 1994].

In recent years, consumption of flavonoids and phenolic compounds has been associated with lower incidence and thus prevention of various diseases.

Flavonoid compounds demonstrate a wide variety of biological activities, such as the scavenging of reactive intermediates (superoxide anion, peroxyl, alkoxyl and hydroxyl radicals) [Kandaswami and Middleton, 1994; Pietta, 2000; Rice-Evans, 2001], enzyme-modifying activity [Vernet and Siess, 1986], and antibacterial, antiinflammatory, antiallergic, antimutagenic [Birt et al., 1986] and antiviral properties [Havsteen, 1983; Xu et al., 2000].

Three structural groups enhance the stability of the flavonoid radical, and thus the flavonoid antioxidant capacity: catechol (3',4'-dihydroxyl) structure in the ring B, 2,3 double bond in conjugation with 4-oxo group and the presence of 3- and 5- hydroxyl groups [Hollman and Katan, 1997; Rice-Evans, 2001]. The glycosylation of flavonoids reduces their antioxidant activities when compared to the corresponding aglycones [Shahidi and Wanasundara, 1992].

The antioxidant properties of flavonoids are often considered to be responsible for the protective effects against coronary heart and liver diseases, photosensitivity diseases and ageing [Rietjens et al., 2005]. Many epidemiological studies and *in vivo* animal studies have suggested that flavonoids can also inhibit the development of carcinogen-induced tumors in a variety of organs [Franke et al., 1998].

Flavonoid inhibition of the immune and inflammation responses can be associated with the inhibition of various enzymes (e.g. protein tyrosine kinase, phospholipase, cyclooxygenase) that can partly regulate the formation of biological mediators responsible for the activation of endothelial cells and specialized cells involved in the inflammation (e.g. T and B lymphocytes) [Middleton and Kandaswami, 1993].

1.4.1.4. Flavonoids and cytochromes P450

Cytochrome P450 enzymes (phase I monooxygenase enzymes) are widely known for their role in the metabolism of drugs and other foreign compounds. Thus, modulation of these enzyme activities can influence the metabolism of xenobiotics. A number of naturally occurring flavonoids have been shown to modulate the CYP system. The stimulatory or inhibitory activity of flavonoids depends on the flavonoid structure, mainly on the hydroxylation pattern of both the A and the B ring [Huang et al., 1981]. On the other hand, flavonoids are metabolized by several CYP isozymes. Some flavonoids may alter the expression of CYPs (e.g. CYP1A) through their binding to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, acting as either AhR agonists or antagonists [Moon et al., 2006]. Depending on the flavonoid concentration, they can exhibit different biological activities. At lower concentrations, interaction of a flavonoid with AhR could prevent the expression of CYP1A1 and ultimately decrease the metabolic activation of some carcinogens (antagonist effect) [Turesky et al., 1991]. On the other hand, at higher concentrations, the same flavonoids might function as AhR agonists modulating gene expression [Hodek et al., 2002]. Induction of CYP1A by flavonoids proceeds by various

mechanisms, including the direct stimulation of gene expression via specific receptors and/or CYP protein, or mRNA stabilization [Lin and Lu, 1998; Shih et al., 2000].

Via modulation of CYP activity, flavonoids may also affect the plasma concentration of pharmaceutical drugs leading to an overdose or the loss of their therapeutic effects.

Many carcinogens are metabolized by CYP enzymes to either biologically inactive metabolites or to chemically reactive electrophilic metabolites that can covalently bind to DNA producing carcinogenicity. Thus, the effects of inducers of CYP enzymes on the carcinogenicity will depend on their effects on the different metabolic pathways [Conney, 2003].

A large number of potentially anticarcinogenic agents found in vegetables, may act as inhibitors in the initiation, promotion and progression of the carcinogenesis. In the study of Tan et al. [1999], PhIP (10 mg/kg) was administered in a single dose after 10 days treatment with a cabbage diet (basal diet containing 20% w/w Chinese cabbage powder). In all the tested tissues, colon, heart, lung, and liver, the amount of PhIP-DNA adducts were decreased contrary to the rats treated only with a basal diet. Since CYP1A1 is associated with detoxification of PhIP, the induction of CYP1A1 and GSTs by chemopreventive compounds from Chinese cabbage is suggested to be responsible for decrease of PhIP-DNA adduct formation. In view of this fact, one may raise a question that consumption of Chinese cabbage could enhance the carcinogenicity of PAHs, which are metabolized mainly by CYP1A1 [Tan et al., 1999].

1.4.2. Non-flavonoid chemopreventive compounds

Curcumin

Curcumin is a yellow pigment from the root of *Curcuma longa* (major component of turmeric spice), widely used as a spice and coloring agent in several foods, such as curry, mustard and potato chips as well as cosmetics and drugs [Mori et al., 2006]. It has been reported that curcumin can inhibit tumors in various organs (e.g. tongue, skin, mammary gland, forestomach, liver, duodenum and colon) of laboratory animals, during the initiation and/or promotion phases [Kelloff et al., 2000]. Recently, the beneficial effects of curcumin on human health were reviewed [Duvoix et al., 2005; Levi et al., 2001]. On the other hand, there are some studies showing possible negative activities of curcumin. For instance, curcumin may exhibit carcinogenic potential through oxidative DNA damage in the presence of CYPs and Cu(II) by its metabolite [Sakano and Kawanishi, 2002].

Diallyl sulphide

Diallyl sulphide is exclusively present in garlic, and undergoes extensive oxidations on a few positions of the molecule after ingestion in organism. Diallyl sulphide is known to be a phenobarbital type inducer, and thus induces mainly CYP2B subfamily. There are also several studies showing the ability of diallyl sulphide to induce other isoforms of CYP *in vivo* in rat and *in vitro* (namely CYP1A1 and 3A2). Davenport and Wargovich [2005] investigated the time and dose-dependent manner of CYP1A1/2 and CYP3A2 induction mediated by diallyl sulphide. Interestingly, high induction of CYP1A1 was ascertained after either single dose or prolonged administration of diallyl sulphide. Moreover, after 8 weeks treatment with 200 mg/ml dosage, hepatotoxicity in liver was observed.

Resveratrol

Resveratrol (3,4´,5-trihydroxy-trans-stilbene) has been isolated for the first time from the root of *Veratrum grandiflorum* [Šmidrkal et al., 2001]. It occurs in the vines, roots, seeds, and stalks, but its highest concentration is in the skin of red grapes. As a constituent of red wine, it may explain the “French paradox” that the incidence of coronary heart disease is relatively low in southern France despite the high dietary intake of saturated fats. Other sources of resveratrol include red beet, cabbage, broccoli, onion, and peanuts. 1 mg of resveratrol is contained in approximately 0.4-2.5 kg of fresh vegetables or 0.17-0.50 l of red wine [Šmidrkal et al., 2001]. Resveratrol is a natural phytoalexin that is abundantly expressed in plants as a defensive response against viral, microbial, fungal infections and other environmental stressors (mechanical damage, UV radiation, ozone) [Milner et al., 2001].

In association to cytochrome P450s, resveratrol was found to be AhR antagonist and is able to block AhR ligand-mediated increased expression of CYP1A1 [Ciolino and Yeh, 1999; Casper et al., 1999]. Moreover, cytochrome P450s (e.g. CYP1A1, CYP1A2 and CYP1B1) participate on resveratrol metabolism [Piver et al., 2004].

2. AIMS

The aim of our study is to expand the current knowledge of chemopreventive compounds and their role in the process of carcinogenesis. The new trend of healthy lifestyle, frequently associated with a rising consumption of dietary supplements containing chemopreventive compounds (e.g. flavonoids), evokes concerns regarding their unlimited consumption, as their side effects are not sufficiently known. These compounds have to be considered as xenobiotics exerting numerous biological activities in the body. Our study is focused on the interactions of the chemopreventive compounds with the key xenobiotic-metabolizing enzymes, cytochrome P450s (CYPs). Ingested chemopreventive compounds may induce the expression of CYPs, and thus significantly increase their potential of carcinogen activation that might, in the long run, result in the development of cancer in humans. Contrary to other studies, our research is directed at their effects from another point of view, namely at the induction persistance, repetitive and single dose *p.o.* administration, and sequential exposure of the organism to chemopreventive compounds and carcinogens. In order to address these effects of chemopreventive compounds on cytochrome P450s, several specific objectives had to be accomplished.

- To establish and optimize the immunodetection of CYP1A in microsomal samples (liver, small intestine) using a chicken anti-rat CYP1A1 and CYP1A2, as well as the assay of CYP1A1 and CYP1A2 specific activities, 7-ethoxyresorufin-*O*-deethylase and 7-methoxyresorufin-*O*-demethylase, respectively.

- To screen various groups of chemopreventive compounds for their ability to induce CYP1A1 and CYP1A2 in rat liver and small intestine, the two main organs responsible for xenobiotic metabolism and carcinogen activation, after *p.o.* administration of chemopreventive compounds.

- To examine the relevant effects on the expression of CYPs and their specific activities in different time and dose regimens of the chemopreventive compound treatment.

- To carry out a sequential study, which comprises the administration of an inducing or non-inducing carcinogen, preceded by the administration of a chemopreventive compound, and to determine the DNA-adducts formation using ^{32}P-postlabelling.

3. MATERIALS AND METHODS

3.1. Materials

Chemicals used were from:

Fluka, Switzerland

tris(hydroxymethyl)-aminomethane (Tris), 2-mercapthoethanol, methanol, α-tocopherol

Linde, Czech Republic

liquid nitrogen, dry ice

Millipore Corp., USA

Immobilon-P transfer membrane

Perkin Elmer, USA

96-well plates for fluorescence measurement

PML a.s., Czech Republic

Laktino – non-fat dried milk

Serva, Germany

acrylamide, N,N'-methylenbisacrylamide (BIS), N,N,N',N'-tetramethylethylenediamine (TEMED), Triton X-100, Coomassie brilliant blue R-250, sodium dodecyl sulphate (SDS)

Setuza a.s., Czech Republic

Vegetol – sunflower oil

Sigma–Aldrich, USA

hesperetin, hesperidin, naringin, naringenin, β-naphthoflavone, flavone, flavanone, resveratrol, baicalin, morin, rutin hydrate, biochanin A, diallyl sulphide, curcumin, 7-ethoxyresorufin, 7-methoxyresorufin, resorufin, dimethyl sulfoxide (DMSO), nicotinamide adenine dinucleotide phosphate (NADPH), SigmaMarker Wide range (6 500-205 000),

bicinchoninic acid (BCA), anti-chicken IgG-alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (BCIP/NBT)

Toronto Research Chemicals, Inc., Canada
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)

Velaz s r.o., Koleč u Kladna, Czech Republic
male Wistar rats (140-150 g)

Whatman, USA
Whatman paper, No.3

Antibodies against CYP1A1 and CYP1A2 were kindly provided by prof. RNDr. Petr Hodek, CSc., Faculty of Science, Charles University in Prague, Czech Republic

Isoquercitrin (quercetin-3-β-D-glucoside, purity 98.2%) was prepared and kindly provided by prof. Vladimír Křen, Ph.D., DrSc., FRSC, Laboratory of Biotransformation, Institute of Microbiology, Czech Academic of Sciences of the Czech Republic

All other chemicals were purchased from Lachema Brno, Czech Republic.

3.2. Instruments

Analytical balance

PESA 40SM-200, Switzerland

Automatic micropipettes

BioHit, Finland; Nichiryo, Japan

Centrifuges

Sanyo Microcentaur MSE, Great Britain; Janetzki K23, Germany; Janetzki K24, Germany; Beckman Coulter – OptimaTM LE-80K Ultracentrifuge, USA

Electronic precision balance

KERN EW600-2M, Germany

Electrophoresis

Amersham Biosciences Hoefer™ miniVE, USA

Luminescence Spectrophotometer

PerkinElmer LS55, USA

Spectrophotometers

Hewlett Packard E8453, USA; SpektroMOM 195 D, Hungary; Specord M40 Carl Zeiss Jena, Germany

Water purification system

Simplicity 185 Millipore Corp., USA

Western blotting

Whatman Biometra® Fastblot B 43, USA

Other

pH meter ATI Orion 370, USA; sonicator Ultrasonic Compact Cleaner Teson1 Tesla, Czech Republic; vortex MS 1 Minishaker, Germany; shaker IKA OS 2, Germany; water bath Julabo TW 8, Germany; heatable magnetic stirrer Variomag Monotherm, Germany; electrphoresis power supply EPS 301 Amersham Pharmacia biotech, USA;

3.3. Methods

3.3.1. Treatment

Male Wistar rats (~150 g) obtained from Velaz, Czech Republic, were housed in the Centre for Experimental Biomodels, Prague, Czech Republic in accordance with Decree No 39/2009 Collection of Laws. The rats were acclimatized for 5 days, housed in wire cages at 22°C with a 12h light/dark period and an *ad libitum* diet (ST-1 diet from Velaz, Czech Republic) and water access.

- **Regimen I:** Flavonoids (ß-naphthoflavone, flavone, flavanone, baicalin, naringenin, naringin, hesperetin, hesperidin, biochanin A, morin, rutin, quercetin, isoquercitrin), resveratrol, curcumin, diallyl sulphide dissolved in sunflower oil (1 ml) were *p.o.* administered by gastric gavage 60 mg/kg body weight, daily for 5 consecutive days. The control group was treated with 1 ml of the sunflower oil. The treated rats were fasted overnight and next day (24 hours after the last treatment) were sacrificed.

- **Regimen II:** Quercetin, isoquercitrin and ß-naphthoflavone were *p.o.* administered in a single dose 60 mg/kg b.w., dissolved in sunflower oil (1 ml). The control group was treated only with 1 ml of the sunflower oil. The treated rats were sacrificed 24, 48 and 72h after the treatment.

- **Regimen III:** β-Naphthoflavone was *p.o.* administered in a concentration of 60 mg/kg b.w. and 72h after that, one concentration of BaP (150 mg/kg b.w.) or two different concentrations of PhIP (50 and 150 mg/kg b.w.) were administered. Each compound was also administered separately. The rats were sacrificed 24h after the treatment.

3.3.2. Preparation of microsomal fractions

Buffer B1: 0.15 M KCl, 0.05 M Tris/HCl; pH 7.4; 50 μM tocopherol in methanol (just before use)

Buffer B2: 0.1 M $Na_4P_2O_7.10H_2O$; pH 7.2

Buffer B3: 0.15 M KCl, 0.05 M Tris/HCl, 20% (v/v) glycerol; pH 7.4

Microsomal fractions were prepared from rat liver and different parts of small intestine by differential centrifugation according to the method of van der Hoeven and Coon [1974]. In some experiments, the small intestine was dissected into three parts: proximal, middle and distal; each part in a length of approximately 20 cm. The proximal part started circa 2 cm under the stomach. All tissues were placed into the cold buffer B1 until used.

All buffers and materials were cooled before being used and the following procedures were carried out at 4°C.

The small intestine and liver tissues were thoroughly washed, weighed, minced with scissors and again washed in about 2 volumes of tissue mass. The prepared tissues were then homogenized with approximately 4 volumes of buffer B1 in a Potter-Elvehjem glass homogenizer. The resulting homogenates were centrifuged at 600g for 10 minutes at temperature 0-5°C (centrifuge Janetzki K-23, swing-out rotor 4x70 ml, 2 000 RPM).

Supernatants were collected and the sediments were rehomogenized in a volume equal to 1/4 of the original tissue mass. Rehomogenates were centrifuged under the same conditions. Subsequently, both supernatants were combined and centrifuged 20 minutes at 15 000g at 0-5°C (centrifuge Janetzki K-24, fixed-angle rotor 6x35 ml, 13 500 RPM). The resulting supernatants were carefully separated from the pellet and were ultracentrifuged at 123 000g for 90 minutes at 4°C (ultracentrifuge Beckman, fixed-angle rotor Ti45, 6x64 ml, 35 000 RPM).

Next, the pellets were resuspended and homogenized in 2 volumes of the original tissue mass in buffer B2 and subsequently ultracentrifuged at 425 000g for 90 minutes at 4°C (ultracentrifuge Beckman, fixed-angle rotor Ti70, 8x36 ml, 60 000 RPM).

After the supernatants were removed, the pellets were resuspended in buffer B3 in volume equal to 1/5 of the original tissue mass. The microsomal samples were then aliquated, immediately frozen and stored at –80°C until used.

3.3.3. Bicinchoninic acid protein assay

Protein concentration in microsomes was determined by the method of Smith et al. [1985]. It has been shown that cysteine, tryptophan, and tyrosine are able to reduce Cu^{2+} to Cu^{+}. BCA forms a purple-blue (absorbance maximum 562 nm) complex with Cu^{+} in alkaline environments. The color produced from this reaction is stable for at least 45 minutes and its intensity increases proportionally to the increasing protein concentrations.

Reagent A: 2% (w/v) $Na_2CO_3.H_2O$, 0.95% (w/v) $NaHCO_3$, 0.16% (w/v) sodium tartarate, 0.4% (w/v) NaOH, 1% (w/v) BCA sodium salt
Reagent B: 4% (w/v) $CuSO_4.5H_2O$

The working reagent was prepared by mixing 49 parts of reagent A and 1 part of reagent B. All the tubes needed for the assay were annealed.

Standards for the calibration curve were prepared by diluting the bovine serum albumin stock solution (1 mg/ml) with working reagent to concentrations 2, 4, 6, 10 and 20 µg/ml in a total volume of 0.5 ml. The blank contained 20 µl of distilled water instead of the standard BSA.

Intestinal and liver microsomes were diluted with water in ratio 1:49 and 10 µl of this solution were added to 490 µl of the working reagent. All prepared standards, samples and the blank were vigorously vortexed and subsequently incubated for 60 minutes at 60°C in a water bath.

The absorbance of standards and microsomal samples in cuvettes (1 cm optical path) were measured against the blank at wavelength of 562 nm (SpectroMOM 195 D) and the calibration curve was constructed based on the obtained values for serial BSA dilutions.

The protein concentrations were calculated from the regression equation of the calibration curve and were multiplied by the used dilution.

3.3.4. Cytochrome P450 concentration assay

The CYP concentrations of microsomal fractions were determined by the spectrophotometric method as decribed by Omura and Sato [1964]. The method is based on measuring the difference spectra of the complex of the reduced CYP with carbon oxide at 450 nm (Spectrometer Carl Zeiss Jena M40; slit 0, integration time 1).

Dilution buffer: 0.1 M potassium phosphate, 2% (v/v) glycerol; pH 7.5

Microsomal samples were diluted 50-times with potassium phosphate buffer in a total volume of 1 ml and solid $Na_2S_2O_4$ (approximately 3 mg) was added. After gently mixing, each sample was divided into two cuvettes, both of 1 cm optical paths. After recording the baseline within the range of 401-490 nm, the content of the sample cuvette was carefully

bubbled with CO for about 60 seconds and the difference spectrum was measured at the same range of wavelengths.

The concentrations of CYP were calculated:

$$\mathbf{c_{CYP} = (A_{450}\text{-}A_{490}/\varepsilon_{450\text{-}490}) \times dilution}$$

c_{CYP}	concentration of CYP [μmol.dm-3]
A_{450}, A_{490}	absorbance at 450 nm, resp. 490 nm
$\varepsilon_{450\text{-}490}$	molar absorption coefficient of CYP at 450 nm, resp. 490 nm; the difference is 0.091 $cm^{-1}.\mu mol^{-1}.dm^3$

3.3.5. Specific content of cytochrome P450

The specific content of CYP was calculated as a ratio CYP concentration/protein concentration:

$$\mathbf{s.c.CYP = c_{CYP}/c_{protein}}$$

$s.c._{CYP}$	specific content of CYP [nmol/mg]
c_{CYP}	concentration of CYP [μmol/l]
$c_{protein}$	protein concentration [mg/ml]

3.3.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Electrophoresis is a method for separating proteins in an electric field using a discontinuous polyacrylamide gel (PAGE) as a support medium and sodium dodecyl sulphate (SDS) as an anionic detergent. SDS denatures proteins by wrapping around the polypeptide backbone and confers a negative charge to the polypeptide. Thus, the migration of proteins is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight [Laemmli, 1970].

Buffer A: 0.375 M Tris/HCl; pH 8.8; 0.1% (w/v) SDS
Polymerization solution A: 30% (w/v) acrylamide, 0.8% (w/v) BIS, both in buffer A
Buffer B: 0.125 M Tris/HCl; pH 6.8; 0.1% (w/v) SDS, 0.0006% (w/v) bromphenol blue
Polymerization solution B: 30% (w/v) acrylamide, 0.8% (w/v) BIS, both in buffer B

Sample buffer: 0.25 M Tris/HCl, 8% (w/v) SDS, 20% (v/v) 2-mercaptoethanol, 40% (v/v) glycerol, 0.004% (w/v) bromphenol blue; pH 6.8

Electrode buffer: 0.192 M glycin, 0.025 M Tris/HCl; pH 8.3; 0.1% (w/v) SDS

Staining bath: 0.25% (w/v) Coomassie brilliant blue R-250, 46% (v/v) ethanol, 9.2% (v/v) acetic acid

Distaining bath: 25% (v/v) ethanol, 10% (v/v) acetic acid

For 2 gels:

Resolving gel 8%: 11 ml buffer A, 4 ml polymerization solution A, 15 µl TEMED, 150 µl (100 mg/ml) sodium peroxodisulphate

Stacking gel 3%: 4.5 ml buffer B, 0.5 ml polymerization solution B, 5 µl TEMED, 100 µl (100 mg/ml) sodium peroxodisulphate

The resolving gel was applied between the glasses (10 x 10.5 cm) in the sets (spacers 10.5 cm x 1 mm), immediately overlayed with water and allowed to set for 30 minutes at room temperature. Water was then removed and the stacking gel was poured onto top of the resolving gel, the comb (10 or 15 wells) was inserted and the gel was again allowed to set for 15 minutes at room temperature. Subsequently, the upper chamber of the stand with the gel plate was filled with the electrode buffer and the comb was removed.

Microsomal samples were diluted with water to the final protein concentration 1 mg/ml and then were diluted 3:1 with the sample buffer. SigmaMarker Wide range was diluted 3:1 with the sample buffer. All samples were incubated for 5 minutes at 100°C in a water bath.

Samples (25 µl) and the marker (5 µl) were loaded to the gel wells using a Hamilton syringe.

The sets of stands with glass plates were put into a tank filled with the electrode buffer and a lid was attached. Electrophoresis ran for circa 2 hours (constant voltage 150V) until the dye front reached the bottom of the gel.

The gel plates were separated and one gel without the stacking part was dropped into a staining bath for 1 hour before allowed to distain overnight. One corner of the second gel was cut away and the gel was used for the Western blotting method without staining.

3.3.7. Western blotting

The Western blotting or immunoblotting is used to determine the relative amounts of the protein present in various samples. Following the electrophoresis, separated microsomal proteins are transffered to a membrane, where they are probed using antibodies specific to the protein [Towbin et al., 1979].

Transfer buffer: 0.025 M Tris, 0.192 M glycin; pH 8.3
PBS Triton X-100: 0.134 M NaCl, 1.8 mM $Na_2HPO_4.10H_2O$, 1 mM NaH_2PO_4; pH 7.2; 0.3% (w/v) Triton X-100
Blocking solution: 5% (w/v) non-fat dried milk in PBS Triton X-100

Following the electrophoresis, the stacking gel was removed and the gel was incubated with the transfer buffer for 30 minutes with shaking. In the meantime, the PVDF membrane was cut to the dimensions of the gel and one corner was cut away to correspond to the labeled corner of the gel. The PVDF membrane was then wet in methanol for 10 seconds, then in water for 10 minutes and finally in the transfer buffer.

The first three sheets of Whatman papers were placed on an anode electrode plate, next the membrane, the gel covered with another three sheets. The gel/membrane sandwich was rolled over with a pipette to drive out any air bubbles and finally a cathode plate cover was placed on top of the assembled transfer stack. Total current was determined based on the membrane surface, 3.5 mA/cm^2 for 45 minutes.

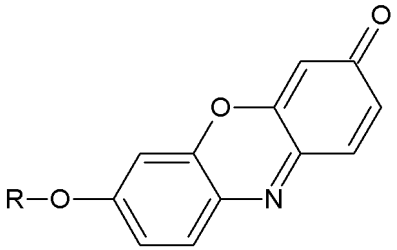
When the transfer was complete, the membrane was blocked in 5% non-fat dried milk overnight at 4°C to prevent further non-specific binding of proteins.

Next day, the membrane was incubated with the specific primary chicken anti-rat CYP1A1 or CYP1A2 antibody in the concentration 30 µg/ml in blocking solution for 2 hours with shaking, followed by 3-times extensive washing in blocking solution. These antibodies were previously treated according to the method described in Chapter 4.2, p. 56. The membrane was then incubated for 1 hour with the secondary rabbit anti-chicken antibody conjugated with alkaline phosphatase, diluted in ratio 1:1 500 in blocking solution. Finally, the membrane was washed in 5% non-fat dried milk (3x5 minutes), PBS Triton X-100 buffer (3x5 minutes) and in distilled water (3x5 minutes).

The proteins were visualized by a BCIP/NTB tablet containing 10 mg substrate for alkaline phosphatase. The reaction was stopped by washing the membrane in water. The membrane was then dried between filter papers.

3.3.8. Ethoxyresorufin-*O*-deethylase and methoxyresorufin-*O*-demethylase activity assay

CYP1A1 and CYP1A2 are the major enzymes that catalyse the *O*-deethylation (demethylation) of 7-ethoxyresorufin (7-methoxyresorufin) to resorufin, thus 7-ethoxyresorufin-*O*-deethylase (EROD) and 7-methoxyresorufin-*O*-demethylase (MROD) activity assays were used as a marker for their enzyme activity. The substrates are metabolized (dealkylated) by CYP to a fluorescent product, resorufin (***Fig 3.1***). Modified method described by Burke and Mayer [1974] was used. The excitation and emission wavelengths used were 530 nm (slit 15) and 585 nm (slit 10), respectively. The fluorescence was measured on Luminescence Spectrometer PerkinElmer LS-55 equipped with 96-well plate reader.



Compound	R
Resorufin	H
7-Ethoxyresorufin	C_2H_5
7-Methoxyresorufin	CH_3

Figure 3.1 *Structure of resorufin, 7-ethoxyresorufin and 7-methoxyresorufin.*

Dilution buffer: 0.1 M potassium phosphate; pH 7.4
Resorufin stock solution: 10 μM resorufin in methanol
Substrate: 0.2 mM 7-ethoxyresorufin (7-methoxyresorufin) in methanol or DMSO
Cofactor: 5 mM NADPH

Standards for the calibration curve were prepared by diluting the resorufin stock solution with potassium phosphate buffer to achieve final concentrations of 0.0125, 0.025, 0.05, 0.1, 0.15, 0.2, 0.3 and 0.4 μM. The blank contained only the potassium phosphate buffer. All standards and the blank were gently vortexed.

Samples were prepared by dilution of microsomal fractions with buffer to protein concentration 0.5 mg/ml. The specific substrate (7-ethoxyresorufin or 7-methoxyresorufin) was then added to this solution to the final concentration 2.2 μM. Aliquots (150 μl) of these solutions were filled into plate wells and the reaction was started by adding 17 μl NADPH solution (final concentration 0.5 mM). The fluorescence was immediately measured in ten 60 seconds cycles.

Enzyme activities were quantified by comparison with the resorufin standards.

3.3.9. Measurement of carcinogen - DNA adducts

Formation of carcinogen–DNA adducts by ^{32}P-postlabelling analysis was accomplished by prof. RNDr. Marie Stiborová, DrSc. in German Cancer Research Center in Heidelberg. Detailed description of the used method is mentioned in Stiborová et al. [2004] and Arlt et al. [2008].

4. RESULTS

4.1. Isolation and characterization of rat microsomal fractions

Microsomal fractions (MS) were isolated from liver and different parts of small intestine of male Wistar rats treated with selected chemopreventive compounds (***Fig 4.1-4.3***) and/or carcinogens (BaP, PhIP) as described in Chapter 3.3.1., p. 41. To mimic the human intake, all tested compounds were *p.o.* administred by gastric gavages to rats. The control group of rats was treated only with sunflower oil.

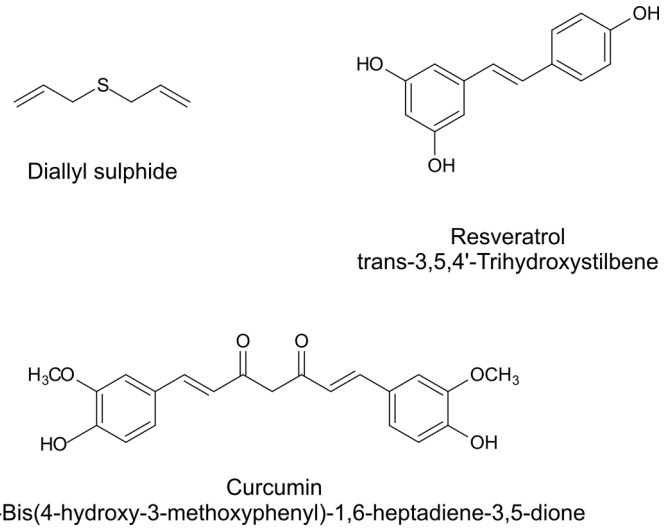


Figure 4.1 *Structures of selected non-flavonoid chemopreventive compounds.*

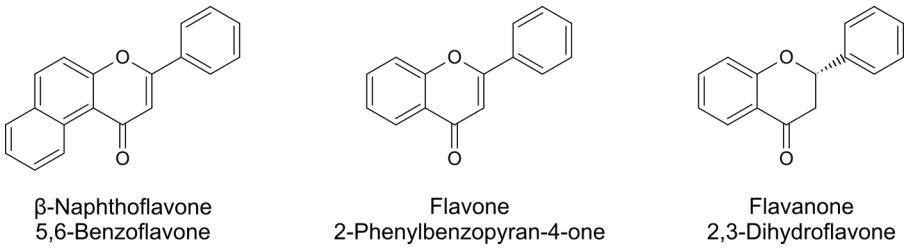


Figure 4.2 *Structures of selected non-substituted flavonoids.*

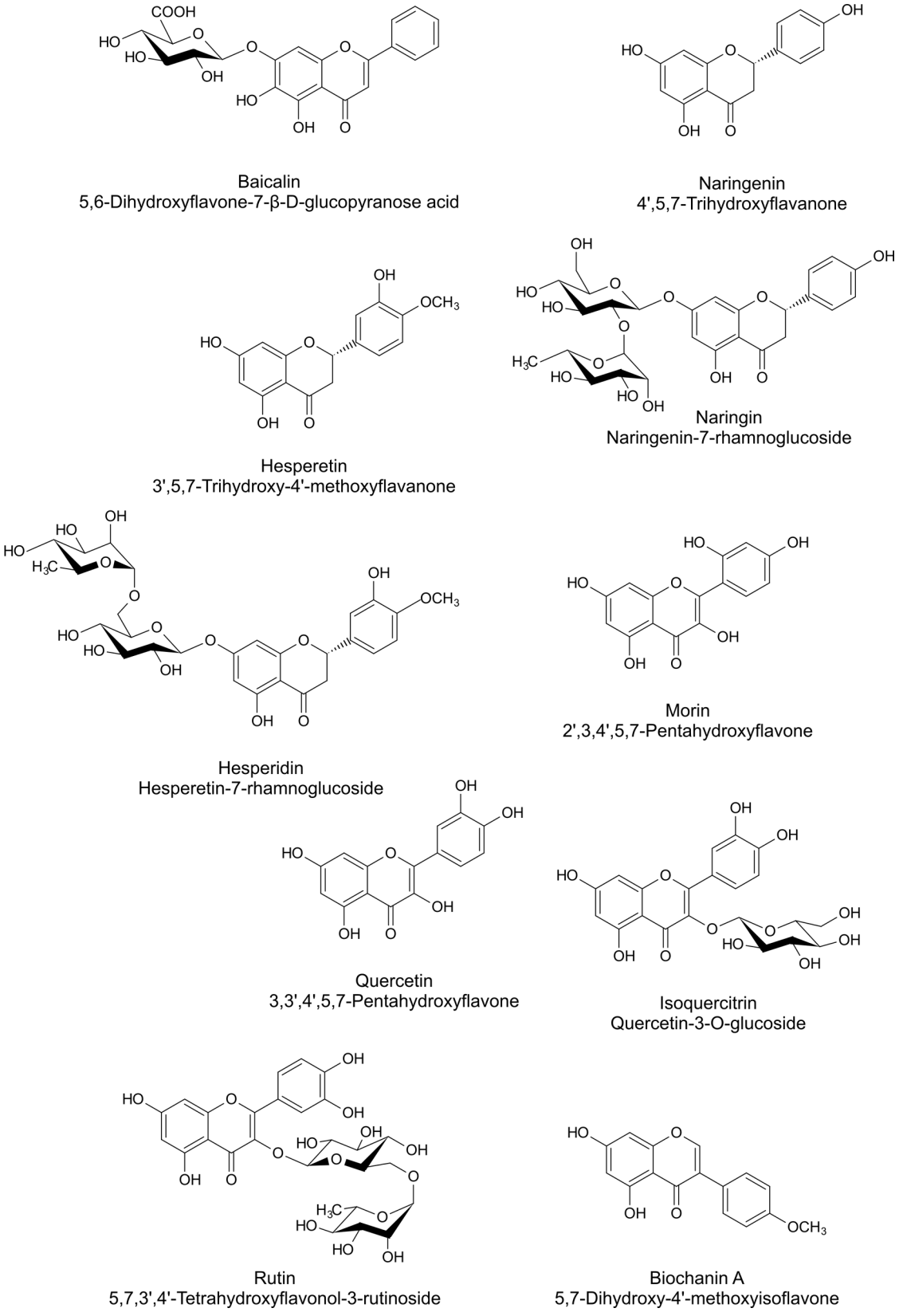


Figure 4.3 *Structures of selected flavonoids.*

Microsomal fractions were characterized for protein and CYP concentrations, using BCA protein assay and reduced CO complex determination, respectively. From the obtained values, specific CYP content was calculated. Low CYP content of intestinal microsomes did not allow us to calculate the specific CYP content, which was under the threshold of the used assay. Characteristics of intestinal and liver microsomal fractions are shown in ***Table 4.1-4.3*** and ***Table 4.4-4.6***, respectively.

Table 4.1 *Characterization of intestinal microsomal fractions from rats treated with chemopreventive compounds (Regimen I).*

Intestinal MS fraction Proximal part	**m_{tissue} [g]**	**$c_{protein}$ [mg/ml]**
Flavonoids		
ß-Naphthoflavone	7.2	21.4
Baicalin	3.4	7.1
Flavone	4.0	8.9
Flavanone	8.4	13.8
Naringenin	3.5	6.8
Naringin	3.3	21.3
Hesperidin	3.2	19.6
Hesperetin	4.2	9.3
Morin	7.9	19.0
Rutin	7.4	13.0
Quercetin	7.2	4.8
Isoquercitrin	7.5	13.5
Biochanin A	7.2	21.3
Non-flavonoids		
Curcumin	8.6	18.8
Diallyl sulphide	7.1	15.2
Resveratrol	3.0	5.3
Control	7.3	12.3

m_{tissue} – used tissue mass, $c_{protein}$ – protein concentration, c_{CYP} – cytochrome P450 concentration

Intestinal MS fraction Middle part	**m_{tissue} [g]**	**$c_{protein}$** [mg/ml]
Quercetin	8.3	21.5
Isoquercitrin	7.0	15.0
Biochanin A	7.1	25.0
Curcumin	8.8	27.8

m_{tissue} – used tissue mass, $c_{protein}$ – protein concentration, c_{CYP} – cytochrome P450 concentration

Intestinal MS fraction Distal part	**m_{tissue} [g]**	**$c_{protein}$** [mg/ml]
β-Naphthoflavone	5.4	14.2
Morin	6.1	16.3
Rutin	4.2	17.7
Quercetin	4.7	18.1
Isoquercitrin	4.8	15.2
Biochanin A	9.2	19.8
Curcumin	8.7	20.9
Diallyl sulphide	4.4	15.0

m_{tissue} – used tissue mass, $c_{protein}$ – protein concentration, c_{CYP} – cytochrome P450 concentration

Table 4.2 *Characterization of intestinal microsomal fractions from rats treated with a single dose of selected chemopreventive compounds 24, 48 and 72h after the treatment (Regimen II).*

Intestinal MS fraction	**Proximal part**		**Middle part**		**Distal part**	
	m_{tissue} [g]	**$c_{protein}$ [mg/ml]**	**m_{tissue} [g]**	**$c_{protein}$ [mg/ml]**	**m_{tissue} [g]**	**$c_{protein}$ [mg/ml]**
24h						
Quercetin	2.7	17.7	3.6	16.1	2.8	11.8
Isoquercitrin	2.7	11.7	3.4	17.5	2.5	11.5
ß-Naphthoflavone	3.0	20.5	2.7	17.9	2.1	16.0
48h						
Quercetin	2.3	11.8	3.1	9.6	3.5	8.8
Isoquercitrin	3.1	18.6	2.2	8.0	2.3	16.2
ß-Naphthoflavone	2.5	9.4	2.2	6.5	2.5	3.0
72h						
Quercetin	2.8	12.5	3.5	14.7	2.7	5.4
Isoquercitrin	3.3	12.6	3.4	21.8	3.2	10.1
ß-Naphthoflavone	3.8	26.8	3.6	25.8	2.1	6.3
control	2.8	14.5	2.9	11.7	2.1	9.0

m_{tissue} – used tissue mass, $c_{protein}$ – protein concentration, c_{CYP} – cytochrome P450 concentration

Table 4.3 *Characterization of intestinal microsomal fractions from rats treated with ß-naphthoflavone and/or carcinogens (Regimen III).*

Intestinal MS fraction	**Proximal part**		**Middle part**		**Distal part**	
	m_{tissue} [g]	**$c_{protein}$ [mg/ml]**	**m_{tissue} [g]**	**$c_{protein}$ [mg/ml]**	**m_{tissue} [g]**	**$c_{protein}$ [mg/ml]**
PhIP1	2.9	14.0	2.3	10.3	2.7	12.7
PhIP2	3.2	30.8	2.8	13.3	2.5	14.4
BaP	4.0	17.6	2.5	11.3	2.6	10.6
βNF+PhIP1	2.7	19.0	3.0	16.0	3.1	12.4
βNF+PhIP2	3.0	25.1	4.5	13.7	2.7	10.6
βNF+BaP	3.7	16.3	3.4	14.5	3.0	12.9

m_{tissue} – used tissue mass, $c_{protein}$ – protein concentration, c_{CYP} – cytochrome P450 concentration

PhIP1 = 50 mg/kg b.w. PhIP2 = 150 mg/kg b.w.

Table 4.4 *Characterization of liver microsomal fractions from rats treated with chemopreventive compounds (Regimen I).*

Liver MS fraction	m_{tissue} [g]	$c_{protein}$ [mg/ml]	c_{CYP} [µM]	s.c.$_{CYP}$ [nmol/mg]
Flavonoids				
ß-Naphthoflavone	40.6	43.4	31.4	0.72
Baicalin	19.7	26.5	17.9	0.68
Flavone	30.5	44.8	32.5	0.73
Flavanone	44.8	42.9	24.9	0.58
Naringenin	30.2	18.3	8.6	0.47
Naringin	19.4	53.4	22.2	0.42
Hesperidin	22.0	34.8	15.4	0.44
Hesperetin	21.0	41.4	18.6	0.45
Morin	36.5	45.0	23.7	0.53
Rutin	34.3	34.7	27.3	0.79
Quercetin	32.5	23.3	7.2	0.31
Isoquercitrin	33.3	36.1	27.5	0.76
Biochanin A	44.4	37.5	25.9	0.69
Non-flavonoids				
Curcumin	49.7	44.4	29.3	0.66
Diallyl sulphide	34.4	36.6	24.1	0.66
Resveratrol	24.2	19.8	17.9	0.91
Control	40.8	33.8	24.6	0.73

m_{tissue} – used tissue mass, $c_{protein}$ – protein concentration, c_{CYP} – cytochrome P450 concentration

Table 4.5 *Characterization of liver microsomal fractions from rats treated with a single dose of selected chemopreventive compounds 24, 48 and 72h after the treatment (Regimen II).*

Liver MS fraction	m_{tissue} [g]	$c_{protein}$ [mg/ml]	c_{CYP} [µM]	$s.c._{CYP}$ [nmol/mg]
24h				
Quercetin	13.6	31.2	7.6	0.24
Isoquercitrin	12.0	57.5	18.7	0.33
ß-Naphthoflavone	15.1	33.8	19.6	0.58
48h				
Quercetin	13.8	39.0	19.3	0.49
Isoquercitrin	12.9	37.7	27.6	0.73
ß-Naphthoflavone	12.3	65.1	23.8	0.37
72h				
Quercetin	13.5	34.8	36.9	1.06
Isoquercitrin	12.5	42.5	26.3	0.62
ß-Naphthoflavone	14.9	60.5	34.6	0.57
control	11.5	40.4	29.2	0.72

m_{tissue} – used tissue mass, $c_{protein}$ – protein concentration, c_{CYP} – cytochrome P450 concentration

Table 4.6 *Characterization of liver microsomal fractions from rats treated with ß-naphthoflavone and/or carcinogens (Regimen III).*

Liver MS fraction	m_{tissue} [g]	$c_{protein}$ [mg/ml]	c_{CYP} [µM]	$s.c._{CYP}$ [nmol/mg]
PhIP1	16.6	38.8	22.0	0.57
PhIP2	13.4	48.3	37.3	0.77
BaP	14.9	33.1	23.6	0.71
βNF+PhIP1	12.8	39.1	23.7	0.61
βNF+PhIP2	14.1	28.1	19.8	0.70
βNF+BaP	16.4	41.1	46.9	1.14

m_{tissue} – used tissue mass, $c_{protein}$ – protein concentration, c_{CYP} – cytochrome P450 concentration

PhIP1 = 50 mg/kg b.w. PhIP2 = 150 mg/kg b.w.

4.2. Optimization of Western blotting

For introductory experiments, Western blotting was used according to a procedure as described in Chapter 3.3., p. 46. However, after visualizing the protein transferred to the membrane, stained bands, spots, and vertical streaks occurred in the area corresponding to the relative molecular masses of 50 000 to 70 000 (***Fig. 4.4***). Since the contamination overlaps bands of CYPs, the procedure had to be optimized.

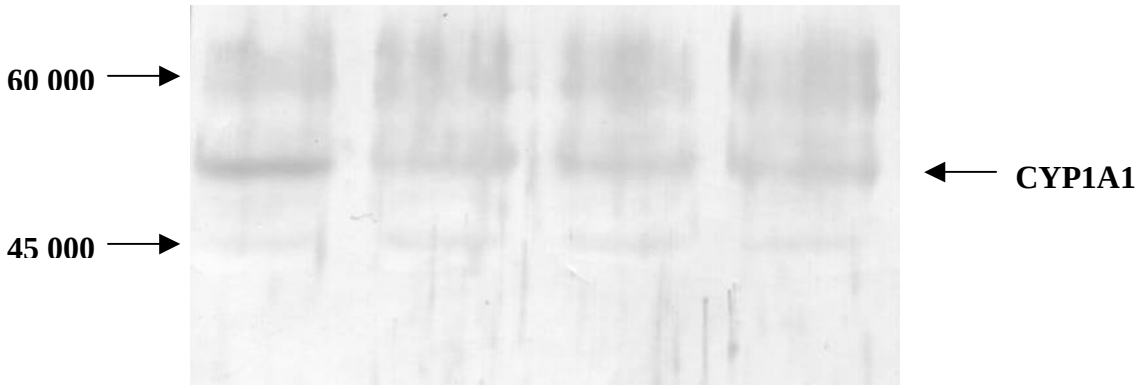


Figure 4.4 *Interference with CYP bands in CYP region. Microsomal samples were diluted with the sample buffer, transferred to the membrane and probed with primary chicken IgY antibody and secondary antibody conjugated with alkaline phosphatase. After visualizing the protein, stained bands, spots, and vertical streaks occurred.*

Because of the vertical streaks, it was suggested that the contaminants originated from the individual components of solutions for electrophoresis or impurities from the environment. The individual components of used buffers were separately loaded to the gel, transferred and probed with the same antibodies. As shown in ***Figure 4.5***, the contaminants originated from the sample buffer, especially from 2-mercaptoethanol (2-ME). The contaminant was determined to be keratin by Dr. Miroslav Šulc using MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Time-of-Flight) Mass spectrometry (data not shown).

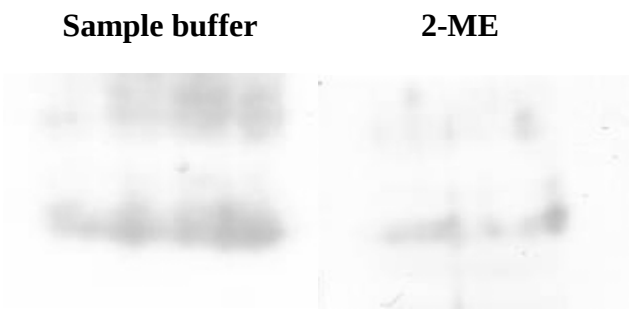


Figure 4.5 *Keratin contamination of the sample buffer, especially 2-mercaptoethanol (2-ME).*

To eliminate the keratin contamination, a purification step involving ultrafiltration of 2-mercaptoethanol on Microcon (YM-10) and Whatman (30K MWCO) centrifugal filter units by centrifugation at 13 000 RPM for 30 minutes (Sanyo Microcentaur MSE) was taken. Then the sample buffer with purified 2-mercaptoethanol was prepared. The subsequent electrophoresis and Western blot showed some amount of contamination still present.

The next step involved the incubation of the primary chicken IgY antibodies against rat CYP1A1 and CYP1A2 (30 mg/ml) with pieces of human keratinized skin (100 mg per ml of antibody) at 4°C for two days. Antibodies were then centrifuged 13 000 RPM for 5 minutes (Sanyo Microcentaur MSE) and the supernatants were put into new microtube and stored at 4°C until used. The resulting prepared antibodies were then used for detection of CYP1A1 and CYP1A2 isoforms. The successfull elimination of keratin contaminant is illustrated in ***Figure 4.6***.

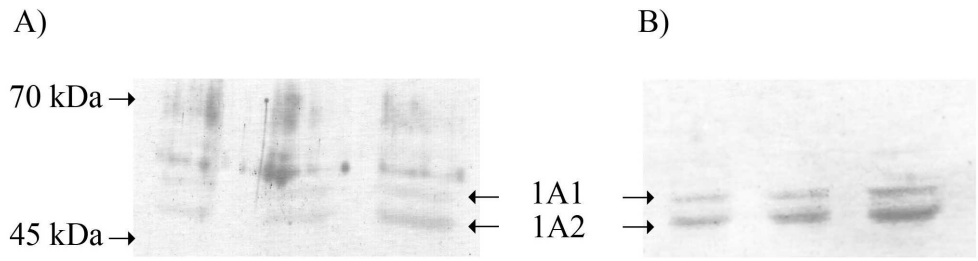


Figure 4.6 *Optimization of Western blotting. Microsomal samples were diluted with the sample buffer containing 2-mercaptoethanol. Membrane was probed without (A) or with (B) antibody incubated with pieces of keratined layer of human skin.*

4.3. Evaluation of cytochrome P450 induction

To investigate the effects on CYP1A, several approaches were taken. At first, a wide range of chemopreventive compounds were tested, namely curcumins, stilbenes, organosulphur compounds, and flavonoids. The protein level and activity of CYP1A1 and CYP1A2 was evaluated in rat small intestine and liver. Since flavonoids are frequently consumed in human diet, they were selected for sequential administration. Moreover, the different time and dose regimens of the chemopreventive compound treatment were used. All tested compounds were *p.o.* administered to male rats. The following three types of administration were applied: Regimen I: 5 day treatment (flavonoids and non-flavonoids), Regimen II: a single dose treatment (flavonoids β-naphthoflavone, quercetin, isoquercitrin), and Regimen III: sequential administration (β-naphthoflavone and/or carcinogens BaP and PhIP). In addition, the induction effects of flavonoids treated with a single dose were investigated 24, 48 and 72h after the treatment.

The induction effects of tested compounds were evaluated by the immunochemical identification of the CYP protein level and CYP specific activities with marker substrates.

Immunoblot analyses were carried out to determine CYP1A1 and CYP1A2 protein expression and subsequently to correlate the observed EROD and MROD activities, respectively, with content of the corresponding isoform. The presence of CYP1A1 and CYP1A2 isoforms in liver and intestinal microsomal fractions was determined using an optimized Western blotting method as described in Chapter 4.2., p. 56.

7-Ethoxyresorufin-*O*-deethylation (EROD) and 7-methoxyresorufin-*O*-demethylase (MROD) activity were determined to prove the activity of CYP1A1 and CYP1A2, respectively. These enzymes catalyze the deethylation and demethylation of the marker substrates to fluorescent product, resorufin. Enzyme activities were calculated from the regression equation of the calibration curve of resorufin standards.

4.3.1. Induction of CYP1A1/2 by different groups of chemopreventive compounds

Chemopreventive compounds, as a group of a wide variety of compounds are considered to have many beneficial effects on human health. On the other hand, as CYP1A1/2 are responsible for carcinogen activation, their induction by chemopreventive compounds may be associated with some possible negative effects of these compounds. Rat liver and small intestine, the two main organs responsible for the xenobiotic metabolism, were used for the evaluation of CYP induction.

Four categories of chemopreventive compounds, flavonoids, stilbenes (resveratrol), curcumins (curcumin), and organosuplhur compounds (diallyl suplhide) were chosen. Flavonoids as one of the main groups of chemopreventive compounds were studied in detail. The tested flavonoids were from the following classes: flavones (β-naphthoflavone, flavone, baicalin), flavanones (flavanone, naringin, naringenin, hesperidin, hesperetin), flavonols (quercetin, isoquercitrin, rutin, morin), and isoflavones (biochanin A). Selected chemopreventive compounds were *p.o.* administered to male rats for 5 consecutive days.

The effects of the selected compounds on CYP expression were evaluated by 7-ethoxyresorufin-*O*-deethylase (EROD) and 7-methoxyresorufin-*O*-demethylase (MROD) activities of CYP1A1 and CYP1A2, respectively, and by the immunochemical identification of the CYP protein (***Fig. 4.7-4.10***).

The induction effects of the tested compounds in small intestine are summarized in ***Figure 4.7*** and ***4.8***. In microsomal fractions from the proximal part of small intestine, a strong induction of EROD and MROD activities was observed after diallyl sulphide treatment followed by curcumin. Among flavonoids, β-naphthoflavone and morin increased EROD and MROD activities significantly. In addition, a slight increase of both activities was observed after all the treatments.

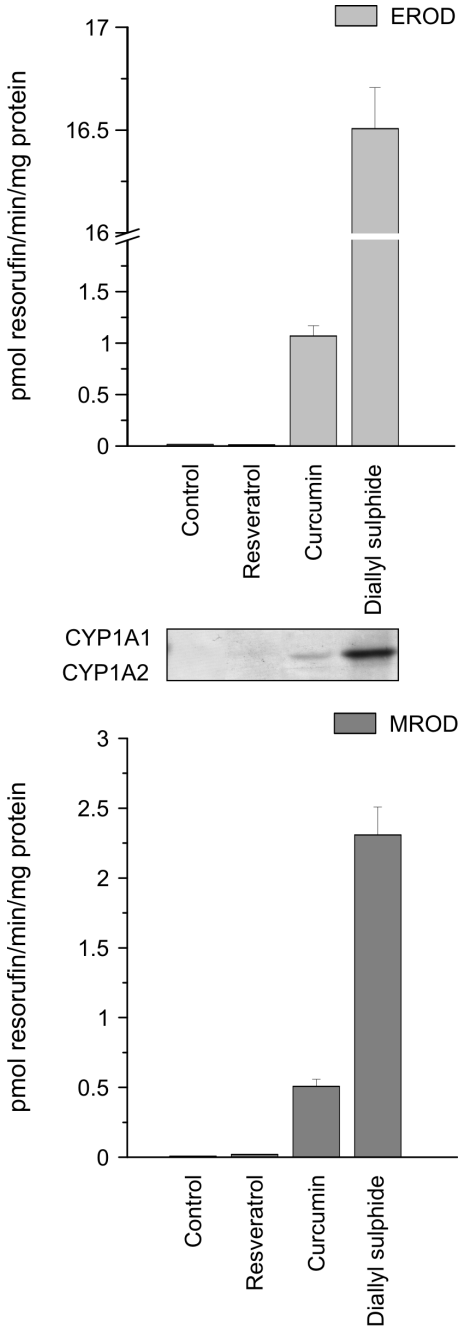


Figure 4.7 *Effects of non-flavonoid treatment in small intestine. EROD and MROD activities of CYP1A1 and CYP1A2, respectively, were determined in rat microsomes from the proximal part of small intestine after exposure to non-flavonoid chemopreventive compounds (60 mg/kg body weight) for 5 days. Bars represent the means ± SD of 3 determinations.*

Immunodetection of CYP1A1/2 in the proximal part of rat small intestine. Electrophoresed microsomal proteins (30 μg) were transferred to Immobilon-P membrane and probed with antibody against CYP1A1/2.

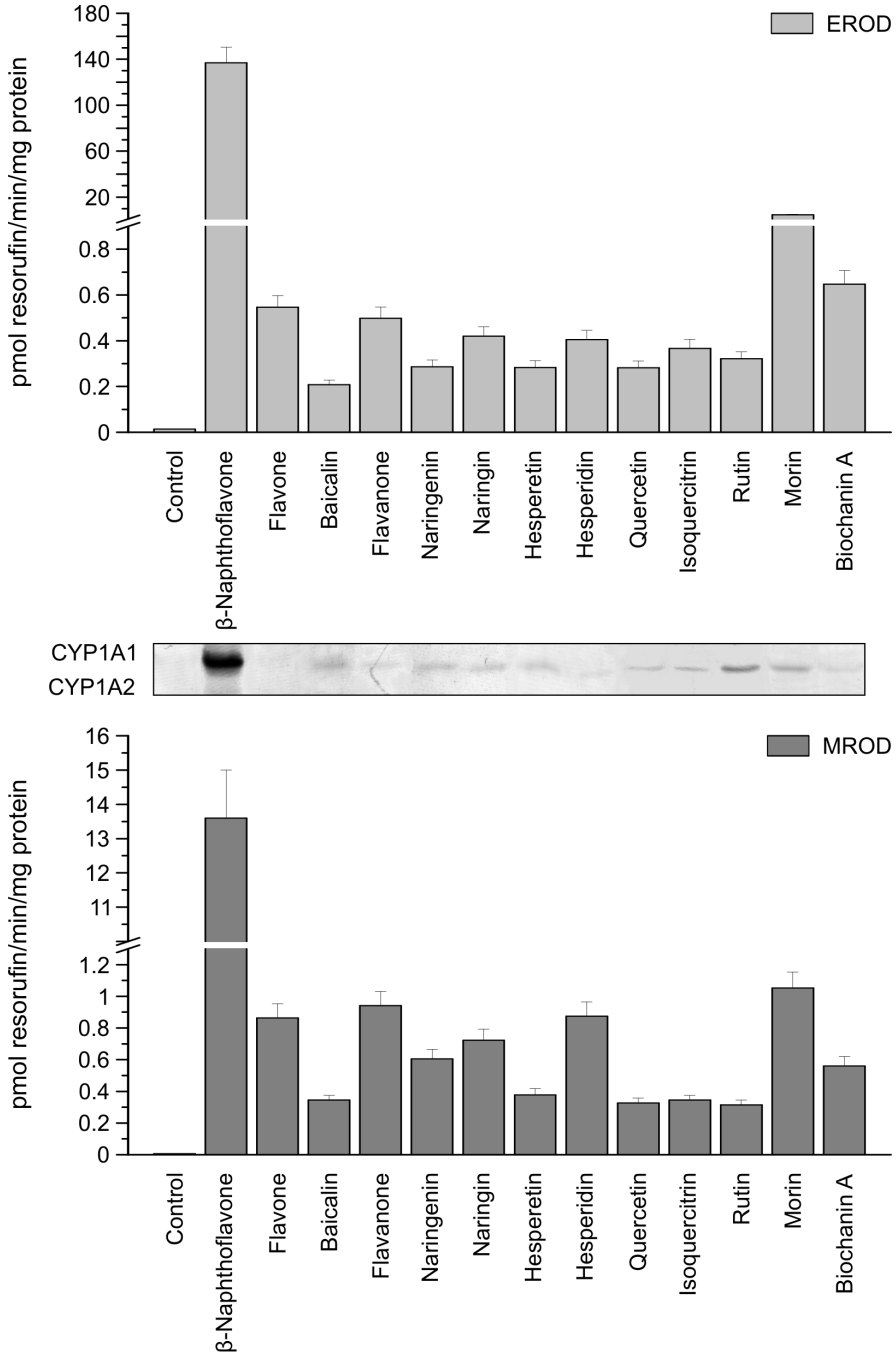


Figure 4.8 *Effects of flavonoid treatment in small intestine. EROD and MROD activities of CYP1A1 and CYP1A2, respectively, were determined in rat microsomes from the proximal part of small intestine after exposure to flavonoids (60 mg/kg body weight) for 5 days. Bars represent the means ± SD of 3 determinations.*

Immunodetection of CYP1A1/2 in the proximal part of rat small intestine. Electrophoresed microsomal proteins (30 μg) were transferred to Immobilon-P membrane and probed with antibody against CYP1A1/2.

Figures 4.9 and ***4.10*** show the induction of CYP activities in liver microsomal fractions. Contrary to intestine (***Fig 4.7, p. 60*** and ***4.8, p. 61***), the values for EROD activity in microsomes after treatment with chemopreventive compounds were lower than those for MROD. One exception was β-naphthoflavone, which was the most efficient inducer of all tested samples. Administration of diallyl sulphide, as a representeative of organusulphur compounds, caused the highest increase of both EROD and MROD activities (2-times) contrary to untreated rats. Compared to control, curcumin and resveratrol did not change the activities significantly. Of all tested flavonoids, β-naphthoflavone, as a model compound, caused the highest increase of both activities followed by the glycoside isoquercitrin. Both activities were decreased by baicalin and hesperetin.

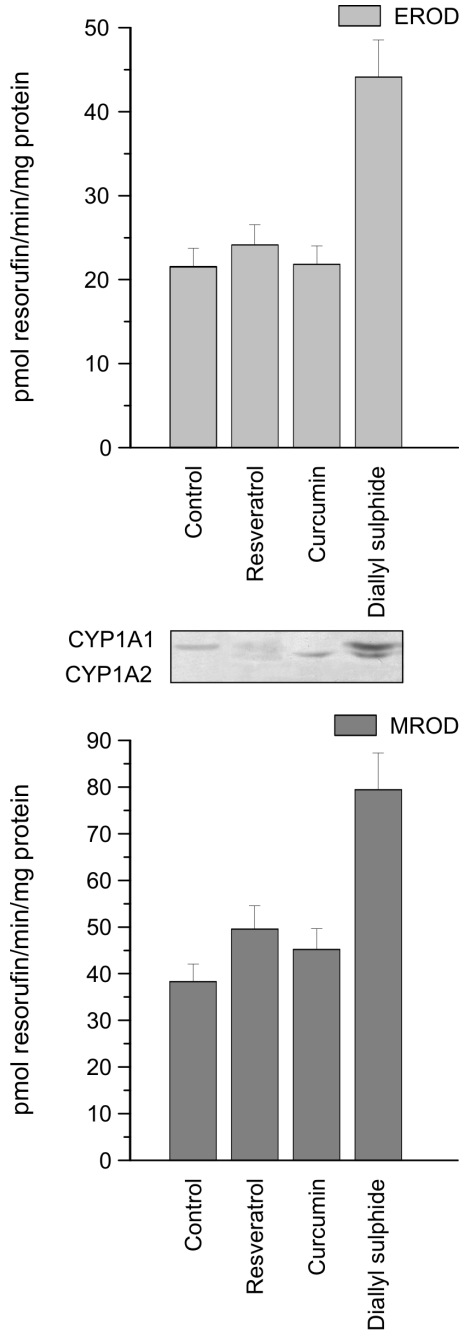


Figure 4.9 *Effects of non-flavonoid treatment in liver. EROD and MROD activities of CYP1A1 and CYP1A2, respectively, were determined in rat liver microsomes after exposure to non-flavonoid chemopreventive compounds (60 mg/kg body weight) for 5 days. Bars represent the means ± SD of 3 determinations.*

Immunodetection of CYP1A1/2 in rat liver. Electrophoresed microsomal proteins (30 μg) were transferred to Immobilon-P membrane and probed with antibody against CYP1A1/2.

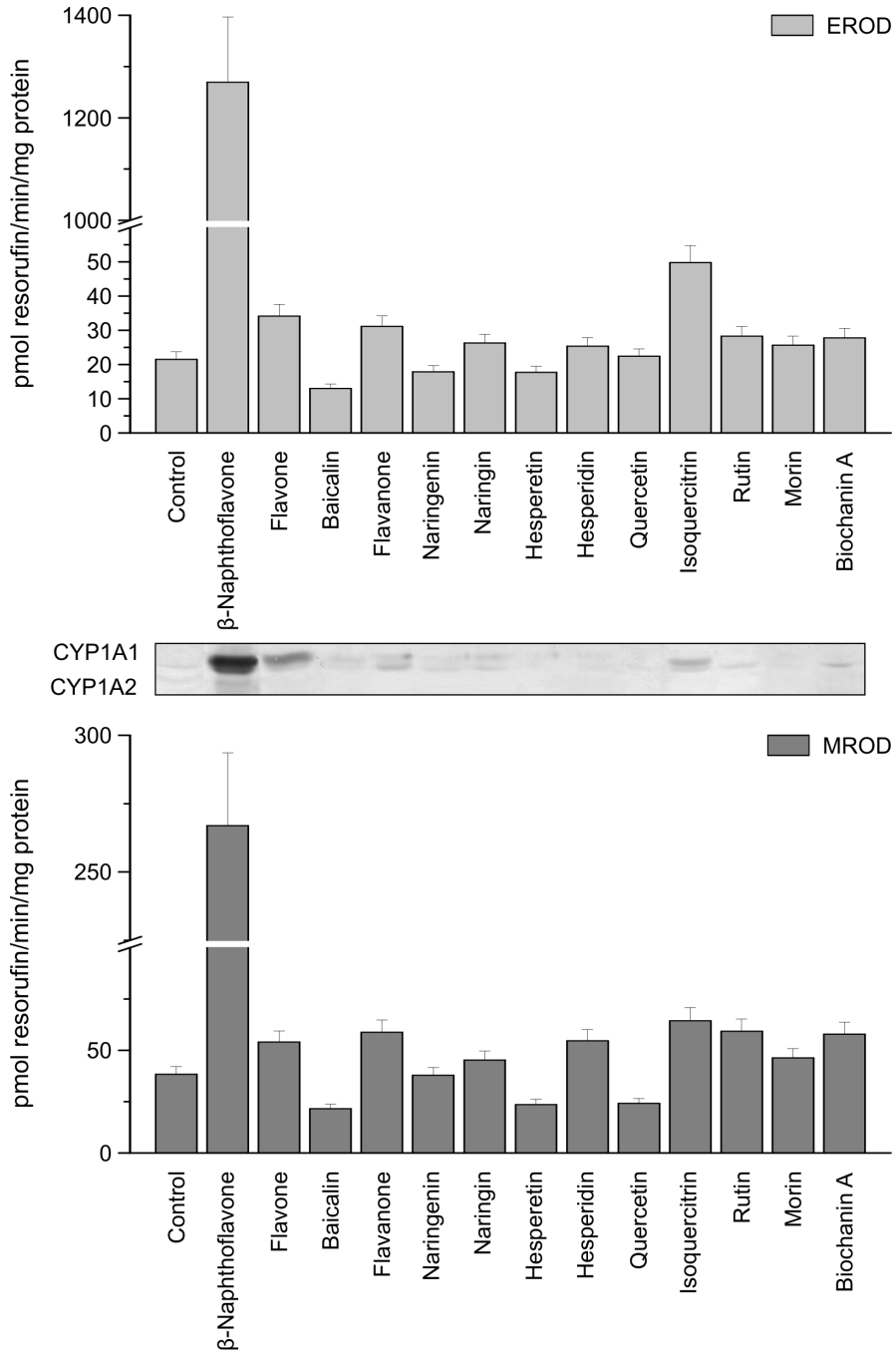


Figure 4.10 *Effects of flavonoid treatment in liver. EROD and MROD activities of CYP1A1 and CYP1A2, respectively, were determined in rat liver microsomes after exposure to flavonoids (60 mg/kg body weight) for 5 days. Bars represent the means ± SD of 3 determinations.*
Immunodetection of CYP1A/2 in rat liver. Electrophoresed microsomal proteins (30 μg) were transferred to Immobilon-P membrane and probed with antibody against CYP1A1/2.

The presence of CYP1A1 and CYP1A2 isoforms in liver (***Fig 4.7*** and ***4.8***) and intestinal microsomal fractions (***Fig 4.9*** and ***4.10***) was determined using an optimized Western blotting method.

Although CYPs in extrahepatic tissues are several times less abundant than in liver, it was possible to detect CYP1A1 in small intestine after chemopreventive compound treatment. However, in untreated rats, CYP1A1 was under the detection limit. **Figure *4.7*** and ***4.8*** illustrate that in small intestine, the expression of CYP1A1 was strongly increased by diallyl sulphide and curcumin. Flavonols (rutin, isoquercitrin), isoflavone (biochanin A) induced CYP1A1, but to a lesser extent than β-naphthoflavone. None of the flavanones (flavanone, naringin, naringenin, hesperetin, and hesperidin) caused any significant induction.

In respect of liver microsomes (***Fig 4.9*** and ***4.10***), CYP1A1 expression was highly increased after diallyl sulphide treatment, whereas CYP1A2 was induced by diallyl sulphide and curcumin. A strong induction of CYP1A1 was observed after β-naphthoflavone and isoquercitrin treatment. Moreover, both compounds also markedly induced CYP1A2. Flavone caused an increase of CYP1A1 expression, but did not induce CYP1A2. Flavanone, which differs from flavone by missing the C2-C3 double bond, caused the induction of CYP1A2. A slight induction of CYP1A2 expression was also observed after biochanin A, morin and rutin. No marked induction of CYP1A1 and CYP1A2 was observable after naringenin, naringin, hesperidin, hesperetin, baicalin and resveratrol treatment in rat liver microsomes.

4.3.2. The effects of chemopreventive compounds on CYP expression along small intestine

As the small intestine is an organ highly exposed to chemopreventive compounds, which are considered to be xenobiotics, the evaluation of CYP expression along small intestine was investigated. Thus, the tissue was firstly dissected into two parts, proximal and distal; each part in a length of approximately 20 cm. The proximal part started circa 2 cm under the stomach and the distal part was cut before large intestine. The selected chemopreventive compounds were *p.o.* administered to male rats for 5 consecutive days.

Figure 4.11 shows the same pattern of decreasing EROD activity along small intestine in all of the tested compounds including control, with the exception of rutin, which did not change any of the activities markedly. The highest decrease of both activities from the proximal part to the distal part was observed after β-naphthoflavone treatment, followed by diallyl sulphide, and morin.

Immunoblots showing the CYPs expression in the proximal and distal parts of small intestine are presented in ***Figure 4.11***. The same induction pattern, significantly lower induction in the distal part than in the proximal part, was observed for the expression of CYP1A1 in small intestine after β-naphthoflavone, diallyl sulphide and morin treatment of rats.

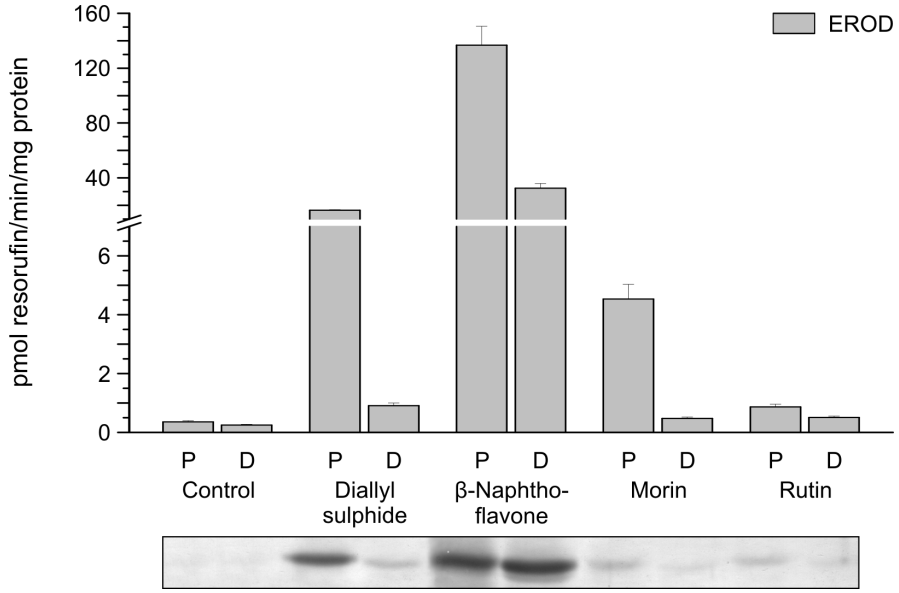


Figure 4.11 *Effects of chemopreventive compound treatment along small intestine. EROD activity of CYP1A1 was determined in the proximal (P) and distal (D) part of rat small intestine after exposure to chemopreventive compounds (60 mg/kg body weight) for 5 days. Bars represent the means ± SD of 3 determinations.*

Immunodetection of CYP1A1 in the proximal and distal part of rat small intestine. Electrophoresed microsomal proteins (30 μg) were transferred to Immobilon-P membrane and probed with antibody against CYP1A1/2.

In further study, the CYP expression was examined in three parts (proximal, middle and distal) of small intestine. One glycosylated flavonoid isoquercitrin, and its aglycone, quercetin, as representatives of flavonoids, were tested for their effects on CYP activities along small intestine after *p.o.* administration to rats.

In respect of intestinal microsomes, ***Figure 4.12*** illustrates the protein level and EROD activity of CYP1A1. The administration of isoquercitrin increased EROD activity 4-times in the middle part of small intestine compared to untreated rats, whereas no significant change was observed in other parts or after quercetin treatment. The highest increase of EROD activity is in accordance with the immunodetection of CYP1A1.

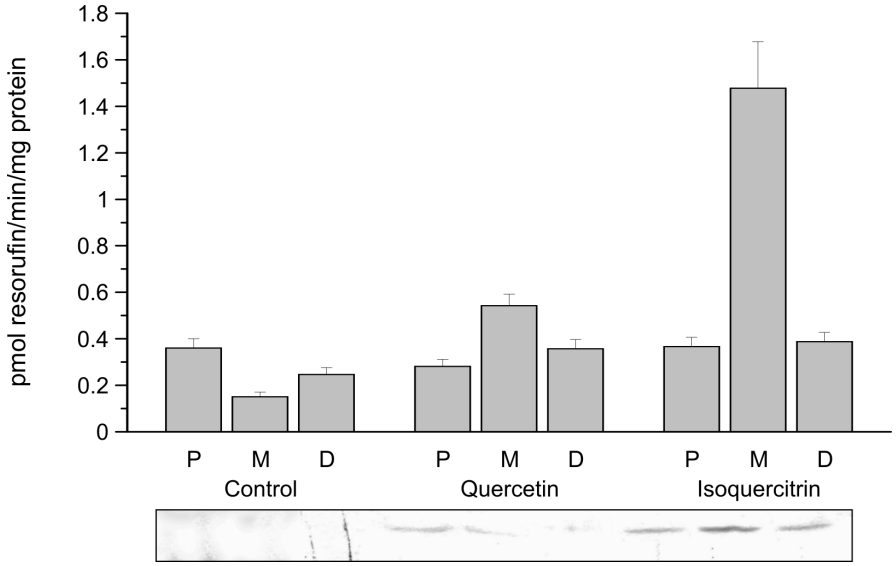


Figure 4.12 *Effects of flavonoid treatment along small intestine. EROD activity of CYP1A1 was determined in the proximal (P), middle (M) and distal (D) part of rat small intestine after exposure to flavonoids (60 mg/kg body weight) for 5 days. Bars represent the means ± SD of 3 determinations. Immunodetection of CYP1A1 in the proximal, middle and distal part of rat small intestine. Electrophoresed microsomal proteins (30 μg) were transferred to Immobilon-P membrane and probed with antibody against CYP1A1/2.*

4.3.3. The effects of a single dose treatment on CYP expression

To mimic the real human intake, it was also important to determine whether chemopreventive compounds were able to induce CY1A1/2 after a single dose (60 mg/kg) and how long the elevated levels of CYP persist. Therefore, the relevant effects on CYP expression 24, 48 and 72h after the treatment in rat small intestine (***Fig 4.13***) and liver (***Fig. 4.14***) were examined.

For these experiments, flavonoids, as the most abundant compounds in human diet or dietary supplements, especially flavonols (e.g. quercetin, isoquercitrin) were chosen.

As ***Figure 4.13*** shows, β-naphthoflavone caused a strong induction of CYP1A1 in all parts of small intestine. In addition, the highest increase of EROD activity was observed 72h after the treatment. Administration of quercetin and isoquercitrin treatment increased EROD activity mainly 72h after the treatment.

In liver microsomes (***Fig. 4.14***), the strongest induction of CYP1A1 and CYP1A2 was observed 24h after the β-naphthoflavone treatment, however, the administration of quercetin and isoquercitrin did not affect any of the specific activities.

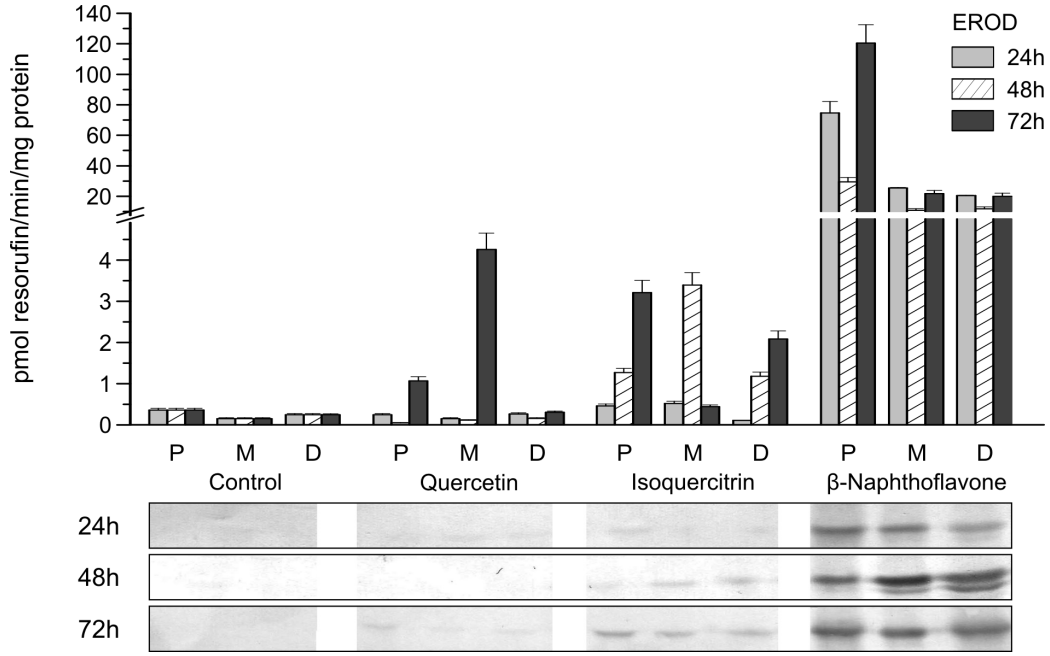


Figure 4.13 *Effects of flavonoid treatment in small intestine in different times after the treatment. EROD activity of CYP1A1 was determined in the proximal (P), middle (M) and distal (D) part of rat small intestine after a single dose treatment with flavonoids (60 mg/kg body weight) 24, 48 and 72 hours after the treatment. Bars represent the means ± SD of 3 determinations.*

Immunodetection of CYP1A1 in the proximal, middle and distal part of rat small intestine. Electrophoresed microsomal proteins (30 μg) were transferred to Immobilon-P membrane and probed with antibody against CYP1A1/2.

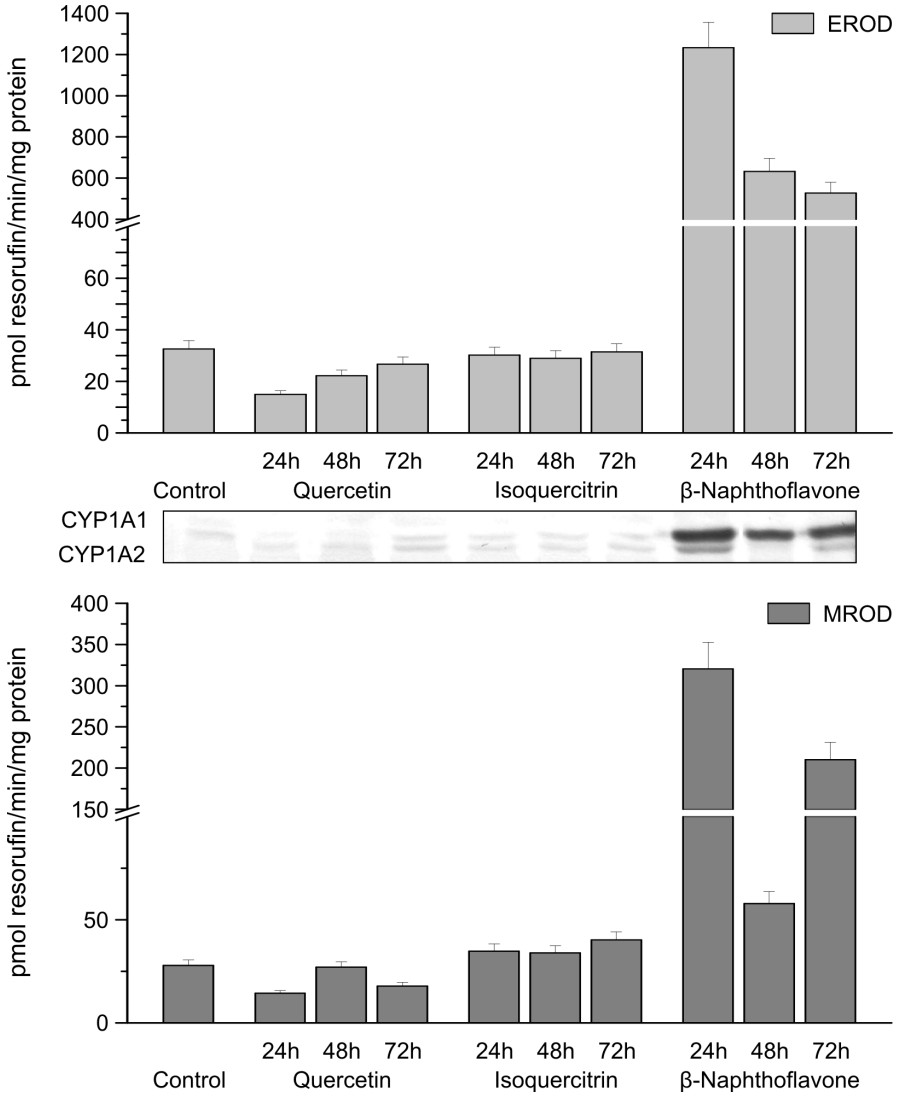


Figure 4.14 *Effects of flavonoid treatment in liver in different times after the treatment. EROD and MROD activity of CYP1A1 and CYP1A2, respectively, were determined in rat liver after a single dose treatment with flavonoids (60 mg/kg body weight) 24, 48 and 72 hours after the treatment. Bars represent the means ± SD of 3 determinations.*

Immunodetection of CYP1A1/2 in rat liver. Electrophoresed microsomal proteins (30 μg) were transferred to Immobilon-P membrane and probed with antibody against CYP1A1/2.

4.3.4. Sequential administration

Based on the results obtained in the previously described experiments, a sequential study has been launched. It comprised the administration of a carcinogen preceded by the administration of a chemopreventive compound. When the tested compounds are applied simultaneously, a chemopreventive compound can inhibit the carcinogen activation, and thus act as a health promoting agent. On the other hand, the sequential administration can reveal potential negative activities of chemopreventive compounds. Moreover, it simulates better the real human intake.

Polycyclic aromatic hydrocarbon, benzo[a]pyrene, was selected as a model carcinogen and heterocyclic amine, PhIP, as a representative of dietary carcinogens. Each compound was administered separately and/or 72h after the flavonoid inducer, β-naphthoflavone.

Figure 4.15 shows that in small intestine, the administration of β-naphthoflavone caused an increase of EROD activity and also in the expression of CYP1A1 in all intestinal parts at a low dose of PhIP (50 mg/kg). Contrary to that, a high dose of PhIP (150 mg/kg) caused a decrease of the specific EROD activity in β-naphthoflavone pretreated rats.

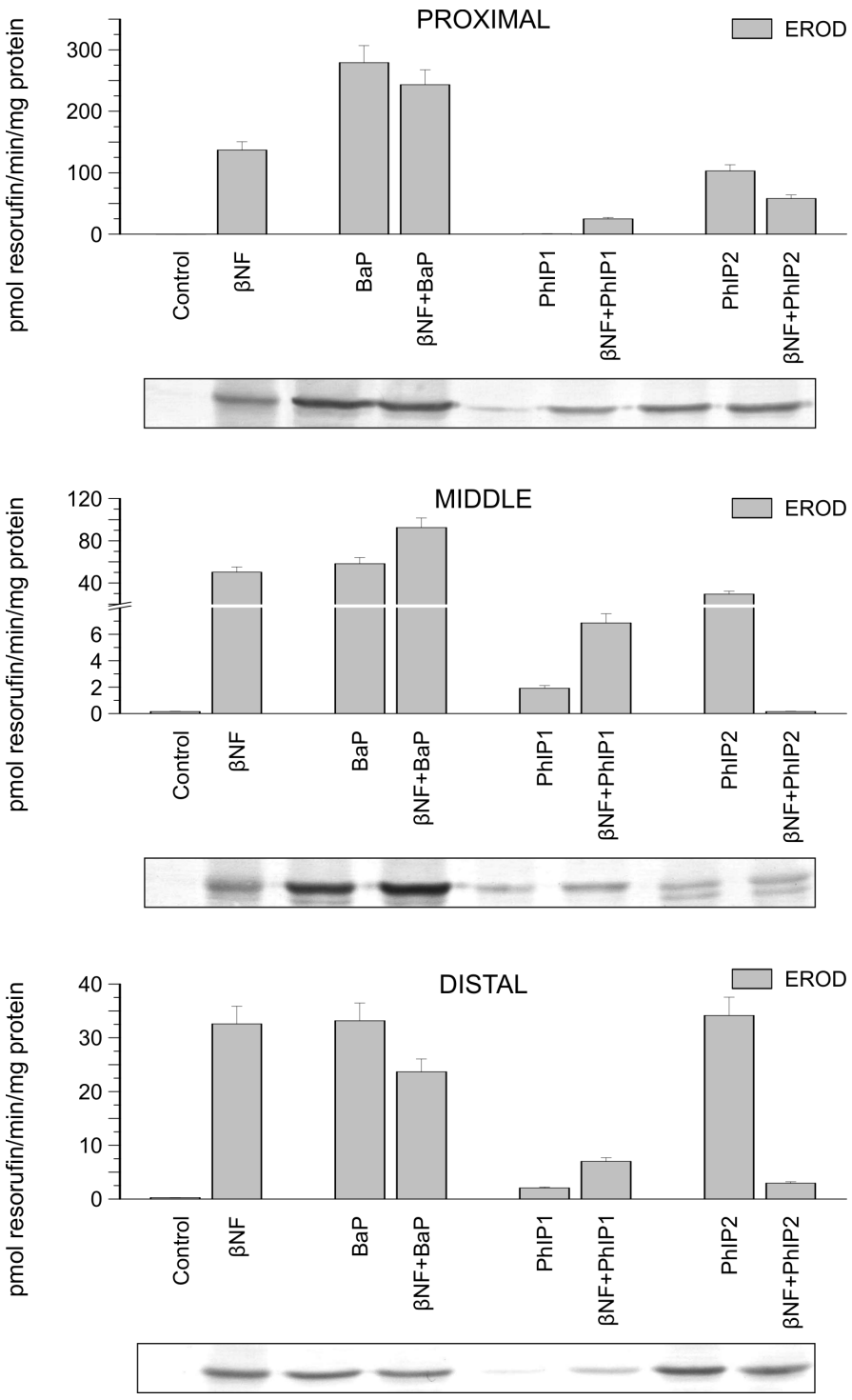


Figure 4.15 *EROD activity of CYP1A1 in small intestine. Microsomes were isolated from the proximal, middle and distal part of rat small intestine after exposure to β-naphthoflavone and/or carcinogens. Bars represent the means ± SD of 3 determinations.*

Immunodetection of CYP1A1 in the proximal, middle and distal part of rat small intestine. Electrophoresed microsomal proteins (30 μg) were transferred to Immobilon-P membrane and probed with antibody against CYP1A1/2.

Figure 4.16 illustrates that in liver, the β-naphthoflavone administration caused a significant change of EROD activity only in combination with BaP, however, it had no effects in combination with PhIP in both of the concentrations. On the other hand, immunodetection of CYP1A1 shows that BNF caused a slight increase of CYP1A1 expression in all cases.

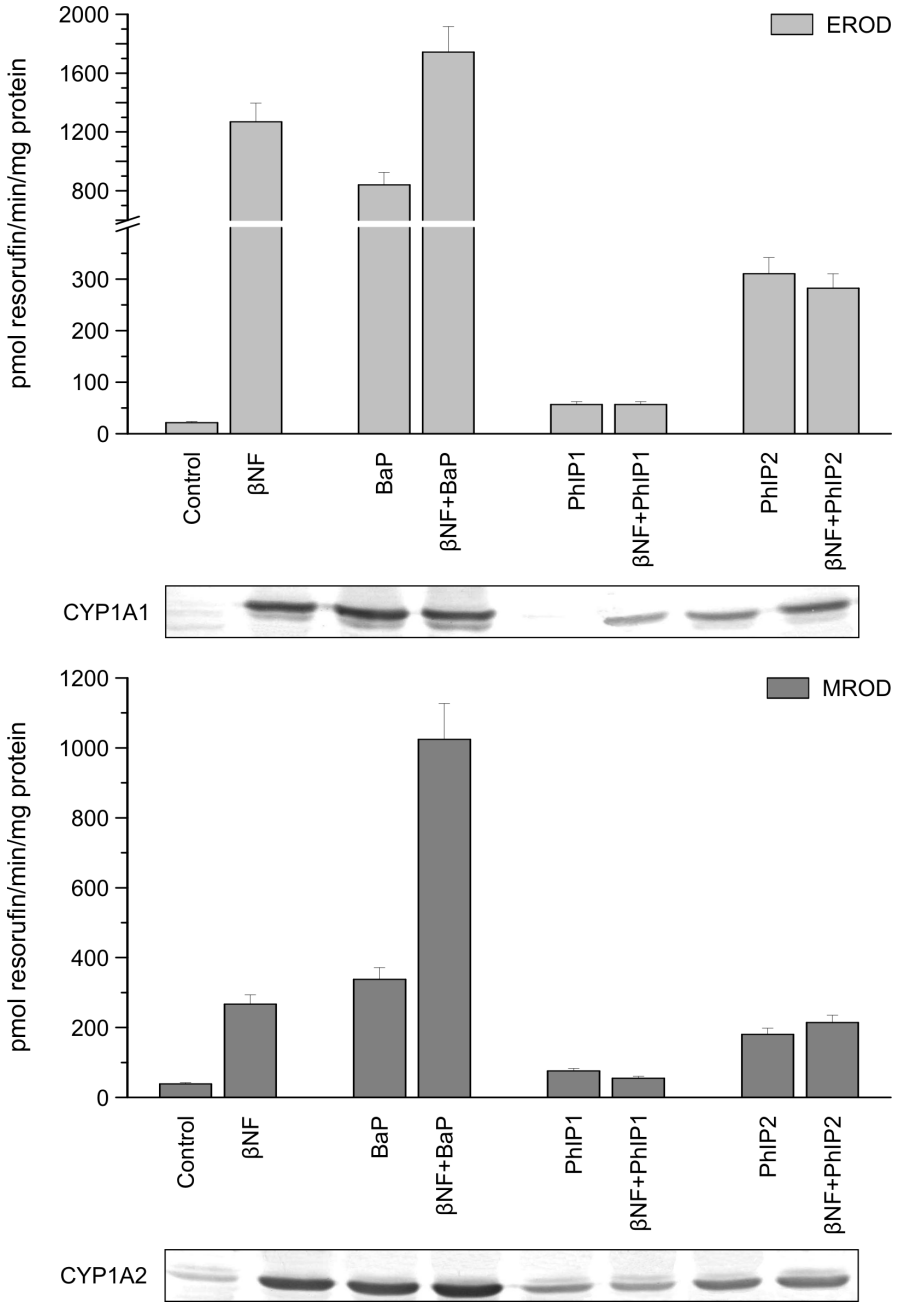


Figure 4.16 *EROD and MROD activity of CYP1A1 and CYP1A2, respectively, in liver. Microsomes were isolated from rat liver after exposure to β-naphthoflavone and/or carcinogens. Bars represent the means ± SD of 3 determinations.*

Immunodetection of CYP1A1 and CYP1A2 in liver microsomes. Electrophoresed microsomal proteins (3 μg) were transferred to Immobilon-P membrane and probed with antibodies against CYP1A1 and CYP1A2.

4.4. ^{32}P-Postlabelling

The potential of flavonoids to induce carcinogen-DNA adduct formation *in vivo* was evaluated using ^{32}P-postlabelling analysis with nuclease P1 enrichment. ^{32}P-postlabelling was accomplished by prof. RNDr. Marie Stiborová, DrSc. in German Cancer Research Center in Heidelberg.

Table 4.7 shows intestinal levels of DNA adducts with benzo[a]pyrene. Animals were either pretreated with BNF, as a inducer, followed by BaP, as a carcinogen, or BaP alone. The proximal and distal part of small intestine was examined. The total content of BaP-DNA adducts was markedly higher in the proximal part than in the distal part. In all tested samples, three BaP-DNA adducts were detected in both intestinal parts.

Table 4.7 *Benzo[a]pyrene-DNA adduct levels in small intestine.*

DNA sample Parts of small intestine	RAL /10^8 nucleotides			
	Spot 1	Spot 2	Spot 3	Total
BaP proximal	3.85	2.82	4.39	11.06
βNF+BaP proximal	5.15	2.97	3.96	12.08
BaP distal	4.40	1.56	1.64	7.60
βNF+BaP distal	4.02	1.85	2.46	8.33

β-Naphthoflavone and/or benzo[a]pyrene were administered to rats and DNA adducts were determined in the proximal and distal part of small intestine. RAL, relative adduct labelling

Spot 1 – adduct derived from 9-hydroxy-BaP

Spot 2 - the major dGp adduct (dG-N^2-BPDE)

Spot 3 – unknown adduct

5. DISCUSSION

Cancer remains one of the leading causes of death in the human population worldwide. The new trend of healthy lifestyle, frequently associated with an increasing consumption of dietary supplements containing chemopreventive compounds (*e.g.* flavonoids), evokes concerns regarding their unlimited consumption, as their side effects are not sufficiently known. Chemopreventive compounds are added to dietary supplements mainly for their potential health-beneficial activities. However, chemopreventive compounds should be considered as xenobiotics, at least interactions with drug metabolism could be expected [Hodek et al., 2009].

One of the potential health threats is that chemopreventive compounds present in dietary supplements, as well as in foods (fruits, vegetables, herbs, beverages) can modulate the activity of xenobiotic-metabolizing enzymes. Hence, chemopreventive compound-drug interactions causing an overdose or the loss of the drug therapeutic effects have been reported [Fuhr, 1998; Guo et al., 2000]. Among proteins interacting with these compounds, cytochrome P450s (CYPs), monooxygenases metabolizing xenobiotics (e.g. drugs, carcinogens) and endogenous substrates (e.g. steroids) play the most prominent role. They might inhibit or stimulate the activity of several CYPs, and/or induce an expression of certain CYPs. Furthermore, the CYP induction can lead to carcinogen activation and thus can increase the human risk of cancer development.

Chemopreventive compounds are usually tested only in artificial systems *in vitro* e.g. for mutagenicity (Ames test with *Salmonella typhimurium*) and for the desired effect confirmation in cell lines. It was shown earlier that tests on these artificial systems do not necessarily correspond with the fate of these compounds in the human body [Martignoni et al., 2004]. Moreover, most studies on xenobiotic-metabolizing enzymes have been carried out with the liver enzymes, because it is the organ with the highest capability of xenobiotic metabolism, however, the gastrointestinal tract is the first barrier met by the exogenous compounds of food or orally delivered drugs. In order for compounds to be absorbed and transferred to the whole body, they first have to pass through the intestinal epithelium. Under physiological conditions, detoxification systems have thus to minimize, within intestinal cells, the potential of damage from toxic xenobiotics [Carrière et al., 2001]. Other sites of xenobiotic metabolism include epithelial cells of the gastrointestinal tract, lungs, kidneys, and the skin. Based on the facts mentioned above, our study was focused on the effects of

chemopreventive compounds on CYPs in both crucial organs highly exposed to xenobiotics, liver and small intestine of rat as a model organism.

Flavonoids, as the most popular group of chemopreventive compunds, have the potential to modulate the activity of cytochrome P450s, mainly CYP1A subfamily [reviewed in Hodek et al., 2002; Moon et al., 2006]. The inhibition of these carcinogen-activating enzymes is considered to be one of the major health promoting effects of chemopreventive compounds. If a chemopreventive compound and carcinogen were administered simultaneously, that might lead to the inhibition of the carcinogen activation. Contrary to that, the sequential administration can cause a potential stimulation of the carcinogen activation. Since CYP1A1 and CYP1A2 are involved in the activation of food and environmental carcinogens, the intake of CYP1A inducers might increase the human risk of cancer development. The facts mentioned above led us to study the effects of chemopreventive compounds, especially flavonoids, on CYP1A subfamily.

Up to now, various studies have been carried out to obtain pharmacological data in order to elucidate the absorption, metabolism and disposition of flavonoids. However, some of the results are controversial, and the complexity of the delivery process from the administration site to the target organs, possibly via receptors, makes generalization of flavonoid metabolism hardly possible. It has been suggested, that glycoside transporters [Gee et al., 1998], β-glucosidases [Day et al., 1998], colon microflora [Parkar et al., 2008; Atkinson et al., 2005] and conjugation of aglycones to glucuronides are the key processes determining the metabolic fate of flavonoids in small intestine and liver [Zhang et al., 2007].

To determine whether the tested compounds affected the content of CYP1A isoforms, Western blotting was employed. As a severe interference hindering the CYP1A protein detection occurred, the used technique had to be optimized. In the respect of molecular mass, the source of the interference was identified to be keratin. Since the ultrafiltration separation of keratin from reagents was not successful, the primary chicken antibodies against rat CYP1A1 and CYP1A2 were incubated with pieces of human keratinized skin. Our unique arrangement managed to eliminate the antibody binding to the keratin contaminants.

In addition to Western blotting, the induction of CYP1A isoforms was further evaluated by the determination of their specific activities with marker substrates, 7-ethoxyresorufin-*O*-deethylase (EROD) and 7-methoxyresorufin-*O*-demethylase (MROD) for CYP1A1 and 1A2, respectively. EROD and MROD were used to confirm that immunodetected CYPs correspond to active CYPs.

In accordance with the previous studies showing, in general, low CYP concentrations in small intestine [Obach et al., 2001; Peters et al., 1991], the induction of CYP1A1 by flavonoids was proved to be stronger in liver than in intestinal tissues. However, CYP1A2 is mainly expressed in liver, it was not detected in any of the natural chemopreventive compounds in intestinal microsomes. Moreover, the CYP1A2 protein is known to be several times more abundant than CYP1A1 protein in control rat liver [Sesardic et al., 1990].

To investigate the effects on CYP1A properly, several approaches were applied. At first, a wide variety of chemopreventive compounds were tested, namely curcumins, stilbenes, organosulphur compounds, and flavonoids. The level and activity of CYP1A1 and CYP1A2 was evaluated to determine the ability of chemopreventive compounds to induce CYP1A in rat small intestine and liver. These two organs are the main sites of xenobiotic metabolism. Moreover, small intestine was dissected into parts and studied in detail. Since flavonoids, as a class of chemopreventive compounds, are frequently consumed in human diet, they were selected for sequential administration. In all experiments, the induction effects of chemopreventive compounds was evaluated by optimized immunodetection and fluorescent measuring of *O*-deethylase or *O*-demethylase activity with marker substrates. Another asset of this study was the use of different time and dose regimens of the chemopreventive compound treatment. To mimic better the real human intake, all tested compounds were *p.o.* administered to male rats. The following three types of administration were applied: 5 day treatment (flavonoids and non-flavonoids), a single dose treatment (flavonoids β-naphthoflavone, quercetin, isoquercitrin), and sequential administration (β-naphthoflavone and/or carcinogens BaP and PhIP). In addition, the induction effects of flavonoids applied to animals in a single dose were investigated in the time course of 24, 48 and 72h after the treatment.

In the first approach, the screening of a wide variety of chemopreventive compounds as CYP1A1 and CYP1A2 inducers was performed. Animals were treated *p.o.* by gastric gavages with the tested compounds for 5 consecutive days.

Four categories of chemopreventive compounds, flavonoids, stilbenes (resveratrol from grapes), curcumins (curcumin from the root of *Curcuma longa*), and organosuplhur compounds (diallyl suplhide from garlic) were chosen. Flavonoids as one of the main groups of chemopreventive compounds were studied further in detail. The tested flavonoids were from the following classes: flavones (β-naphthoflavone, flavone, baicalin), flavanones

(flavanone, naringin, naringenin, hesperidin, hesperetin), flavonols (quercetin, isoquercitrin, rutin, morin), and isoflavones (biochanin A).

A well known inducer of CYP1A subfamily, β-naphthoflavone, was used as a reference compound in the whole study. In both tissues, liver and small intestine, β-naphthoflavone proved its strong inducing capacity.

Although, the CYPs are several times less abundant in small intestine than in liver, it was possible to detect CYP1A1 after the chemopreventive compound treatment. In untreated rats, CYP1A1 was under the detection limit of immunoblotting. From non-flavonoid chemopreventive compounds, diallyl sulphide was the most potent CYP1A1 inducer. Flavonoids, morin, rutin and biochanin A, showed their induction effects but to a lesser extent. These results are in accordance with the determined EROD activity.

Generally, the most effective flavonoid inducers of EROD and/or MROD activities and CYP protein levels in small intestine were aglycones (β-naphthoflavone, flavone, flavanone, morin), while glycosides were inefficient. In addition, β-naphthoflavone, flavone, and flavanone as inducers of CYP1A1 are non-substituted flavonoids. Thus, this indicates the importance of the structure-function relationship for the induction effect of CYP.

In liver, EROD and MROD activities as well as CYP1A1 and CYP1A2 protein levels were markedly increased after the diallyl sulphide treatment. These findings are consistent with the study of Davenport and Wargovich [Davenport and Wargovich, 2005]. Another non-flavonoid compound, curcumin, slightly induced CYP1A2 protein level in rat liver microsomes, and this was also accompanied by an increase of MROD activity. Curcumin and its derivatives have been found as CYP inhibitors in *in vitro* tests, although *in vivo*, curcumin did not alter CYP1A activity and protein level [Mori et al., 2006; Sugiyama et al., 2006]. Among the flavonoids, flavone and flavanone administration caused an increase of both activities EROD and MROD. Moreover, immunodetection confirmed the induction of CYP1A1 by flavone and CYP1A2 by flavanone. Flavone differs from flavanone by the presence of a C2-C3 double bond on the C ring, which could be an explanation for the different effects of these two compounds on CYP protein level. These results are in accordance with another *in vivo* study in rats [Canivenc-Lavier et al., 1996].

Next part of our study was focused mainly on the CYP induction along rat small intestine in respect to the chemopreventive compound treatment. Beside liver, small intestine is an organ highly exposed to xenobiotics. Thus the evaluation of the induction effects of chemopreventive compounds, which are considered to be xenobiotics, was examined. The

tissue was dissected into either two (proximal, distal) or three (proximal, middle, distal) parts and from each part, microsomal fractions were prepared.

The above mentioned results led us to select diallyl sulphide, β-naphthoflavone, morin and rutin as efficient inducers of CYP1A1 for this part of the study. The compounds were *p.o.* administered to rats for 5 consecutive days, and microsomal fractions were tested for the CYP induction in the proximal and distal part of small intestine. After the diallyl sulphide, β-naphthoflavone, and morin administration, the same induction pattern, lower induction in the distal part than in the proximal part, was observed for the expression of CYP1A1. Moreover, obtained results correlate well with the determined EROD activity. These findings are in accordance with the study of Zhang et al. [1997], with β-naphthoflavone only. The proximal part of small intestine is either more equipped with xenobiotic response proteins or the efficient absorption or biotransformation of foreign compounds in blood significantly reduces the exposure of the distal part to xenobiotics.

On the other hand, a different pattern was observed after quercetin and isoquercitrin treatment in small intestine. In this case, three parts of small intestine were examined. Although, quercetin is aglycone and isoquercitrin glycoside, they show the same induction trend in EROD activity with the highest increase in the middle part. Moreover, the determined EROD activity after the isoquercitrin administration is in accordance with the immunodetection of CYP1A1.

The obtained data with aglycones and glycosides are ambiguous, thus the relation between their effects on CYP1A1/2 expression in small intestine cannot be simply generalized. The data obtained with the intestinal parts suggest the important role of the compound structure and its metabolism in the process of bioavailability to humans. In addition, the conjugation of xenobiotics and xenobiotic metabolites in small intestine has the potential to facilitate their excretion to the lumen of the intestine. It is thus tempting to attribute the low incidence of human small intestinal cancer to the high levels of expression of phase II enzymes in small intestinal enterocytes, relative to their expression levels in other organs of the gastrointestinal tract [Kaminsky and Zhang, 2003].

Since chemopreventive compounds are usually administered repetitively, which is the case in most studies, they might strongly induce CYP1A subfamily. This approach was used for prescreening of chemopreventive compound effects. However, to determine the induction capability of chemopreventive compounds in a low dose and the persistence of the elevated CYP levels, the following experiments included a single dose administration. The induction

effects on CYP expressions were examined in rat small intestine and liver 24, 48 and 72h after the *p.o.* treatment with the selected compounds.

For these experiments, flavonoids, as the most abundant compounds in the human diet or dietary supplements, namely flavonols (e.g. quercetin, isoquercitrin), and β-naphthoflavone, were chosen.

In small intestine, the β-naphthoflavone and quercetin administration caused the highest increase of EROD activity 72h after the treatment. Isoquercitrin increased the activity in all cases. Contrary to the observed trend in small intestine, the strongest increase of EROD activity in liver was determined 24h after the β-naphthoflavone treatment. However, the administration of quercetin and isoquercitrin did not affect any of the specific activities. The data from the specific activities EROD and MROD after the isoquercitrin treatment are in accordance with the immunodetection of CYP1A1 in both tested organs, small intestine and liver.

These results indicate that isoquercitrin affects the CYP expression relatively fast after the administration, although it is glycoside, which is considered to be cleaved before entering the intestinal cells. It has been expected that aglycone, quercetin, may have better potential to induce CYP1A1 level more rapidly, though, it was not proved. The study of Hollman et al. [1995] proposed that flavonoids glycosides, namely glucosides, can be absorbed intact via the sodium-dependent glucose transporter SGLT-1. Furthermore, the role of SGLT-1 in the transport of another quercetin glucoside (quercetin 4′-glucoside) was confirmed in the recent study using Caco-2 cell [Walgren et al., 2000; Rice-Evans and Packer, 2003].

Another concept of our study was to focus on the sequential administration of a flavonoid followed by a carcinogen. In the majority of studies, both compounds were administered simultaneously, which could under experimental conditions lead to the inhibition of the carcinogen activation by chemopreventive compounds.

Based on the results with β-naphthoflavone in the experiments mentioned above, carcinogens (BaP and PhIP) were administered 72h after the β-naphthoflavone treatment. Since benzo[a]pyrene is a known AhR agonist and strong inducer of CYP1A subfamily [Rendic and Di Carlo, 1997; Ortiz de Montellano, 2005], it was selected as one model carcinogen. The other selected carcinogen, PhIP is considered to be a weak inducer of CYP1A and is one of the most abundant heterocyclic amine in the human diet, especially in well done steaks. In addition, the role of PhIP and other heterocyclic amines in human cancer

causation is of interest, in particular for cancers of colon and breast [Snyderwine, 1994; Nagao et al., 1994].

The initial activation step of PhIP is *N*-hydroxylation catalyzed by CYP1A [Kato and Yamazoe, 1987]. In the mouse, this is principally due to CYP1A2 [Snyderwine and Battula, 1989]. Thus, the induction effect on CYP1A was examined.

In small intestine, the pretreatment of rats with β-naphthoflavone in combination with a low dose of PhIP (50 mg/kg) increased EROD activity and also CYP1A1 level in all the intestinal parts. Contrary to that, a high dose of PhIP (150 mg/kg) decreased the specific EROD activity in β-naphthoflavone pretreated rats, although the CYP1A1 level was not affected.

In liver, the combination of β-naphthoflavone and benzo[a]pyrene administration led to a strong increase of EROD and MROD activity compared to separetely administered compounds. This indicates potential synergistic effects of these two compounds. However, no marked effect of the β-naphthoflavone treatment in combination with PhIP was observed. Immunodetection illustrates that β-naphthoflavone caused a slight induction of CYP1A1 in all cases.

It has been recently suggested that the overexpression of glutathione *S*-transferases and efflux pumps in tumor tissue may reduce the reactivity of various anticancer drugs [Sau et al., 2010]. Thus, in case of treatment with both, β-naphthoflavone and benzo[a]pyrene, β-naphthoflavone might act the same way and protect the cell against entering the carcinogen.

Next part of our study was directed at carcinogen-DNA adduct formation, which is generally believed to represent an important genotoxic step in the initiation of carcinogenesis. Therefore, the level of carcinogen-DNA adducts was monitored as another marker for the risk of cancer development. Carcinogens, benzo[a]pyrene and PhIP, undergo enzymatic biotransformation *in vivo* leading to both activation and/or detoxification of the carcinogen. The balance between metabolic activation and detoxification is one of the important determinants in chemical toxicity and carcinogenicity.

BaP-DNA adducts were determined by ^{32}P-postlabelling in the proximal and distal parts of small intestine. The β-naphthoflavone pretreatment markedly increased the amount of BaP-DNA adduct 1 (adduct derived from 9-hydroxy-BaP) in the proximal part of small intestine. The total content of BaP-DNA adducts in the proximal part of small intestine was nearly twice as high as in the distal part, which is in accordance with our findings that the CYP expression is higher in the proximal part of small intestine than in the distal part.

The results obtained by ^{32}P-postlabelling correlate well with the immunodetection and determined specific activities, where the BNF pretreatment did not increase these measurements markedly in comparison to the sole BaP administration. Benzo[a]pyrene is known to be strong inducer of CYP1A subfamily, and thus it is possible that the maximal level of induction has already been exceeded.

Since the determination of PhIP-DNA adducts requires a more intricate modification of the ^{32}P-postlabelling method, samples of intestinal parts are kept for evaluation by a new method, online column-switching liquid chromatography–electrospray ionization-tandem mass spectrometry selected reaction monitoring. This recently developed method allows the rapid automation of the sample clean up and a reduction in matrix components that would otherwise interfere with the mass spectrometric analysis [Singh et al., 2010]. PhIP-DNA adducts in our samples will be determined using this new method in future cooperation with the Institute of Cancer Research in UK.

6. CONCLUSIONS

Rats, as model experimental organisms, were *p.o.* administered with selected chemopreventive compounds, and microsomal fractions were prepared from liver and different parts of small intestine. Microsomal samples were characterized for protein and CYP concentrations.

The induction effects on CYP1A1 and CYP1A2 were examined in different time and dose regimens by optimized Western blotting analysis with primary chicken antibodies, treated to eliminate their binding to keratin contamination, and activity measurements using 7-ethoxyresorufin and 7-methoxyresorufin as specific substrates for CYP1A1 and CYP1A2, respectively.

Regimen I:

The induction effects of a wide variety of chemopreventive compounds on CYP1A1 and CYP1A2 after a 5 day treatment were screened. In both tissues, diallyl sulphide was the most efficient non-flavonoid inducer of CYP1A1 and also of CYP1A2 in liver. Generally, in small intestine, the aglycones, β-naphthoflavone, flavone, flavanone, and morin, were the most effective flavonoid inducers of EROD and/or MROD activities and CYP protein levels. In rat liver microsomes, another non-flavonoid compound, curcumin, slightly increased the CYP1A2 protein level and MROD activity.

In a more detailed study, small intestine, as an organ highly exposed to xenobiotics, was dissected into two or three parts. An increased CYP1A1 level and activity in the proximal part compared to the distal part was observed after the β-naphthoflavone, morin, and rutin treatment. Quercetin and isoquercitrin behaved differently. After their administration to rats, an increase of CYP1A1 level was determined in the middle part of small intestine.

Regimen II:

The induction capability of quercetin, isoquercitrin, and β-naphthoflavone, in a single low dose and the subsequent persistence of the elevated CYP levels were investigated. In small intestine, isoquercitrin increased the CYP1A1 expression and EROD activity mainly 48 and 72h after the treatment. Contrary to that, in liver, isoquercitrin did not affect either the CYP levels or specific activities. In small intestine, the β-naphthoflavone administration increased both activities and also the CYP1A1/1A2 levels 72h after the treatment in the proximal part. However, in liver, the highest increase occurred 24h after the treatment.

The results obtained in Regimen I and Regimen II suggest the important role of the compound structure and its metabolism in the process of bioavailability to humans.

The results obtained in Regimen I and Regimen II suggest that depending on the structure, the compounds behave differently in the respect of their metabolism affecting their bioavailability.

Regimen III:

The prototypical flavonoid inducer (β-naphthoflavone) and carcinogen (benzo[a]pyrene or PhIP) were administered sequentially 72h one after the other, respectively. In small intestine, the low dose of PhIP administered to rats pretreated by β-naphthoflavone enhanced EROD activity, while the high dose had an opposite effect. In liver, the synergistic effects of β-naphthoflavone and benzo[a]pyrene administration on EROD and MROD activities was observed. Moreover, in liver, β-naphthoflavone caused a slight induction of CYP1A1 expression in all cases compared to separetely adiministered compounds.

The level of BaP-DNA adducts was evaluated by ^{32}P-postlabelling in the proximal and distal parts of small intestine. The higher CYP expression in the proximal part of small intestine than in the distal part was in accordance with our findings that the total content of BaP-DNA adducts in the proximal part is nearly twice as high as in the distal part.

The present study, focused on chemopreventive compounds administered per orally to rats, shows that the inducing ability of chemopreventive compounds is both, compound and tissue-specific. Moreover, it has been demonstrated that the absorption and metabolism of flavonoids depend on the presence and type of sugar moiety linked to aglycone. Presented data have shown that chemopreventive compounds may act as inhibitors or inducers of CYPs. Based on these demonstrated ambiguous activities on CYPs, main xenobiotic-metabolizing enzymes, the questions of safety and unlimited consumption of chemopreventive compounds arise.

References

Adamson R. H., Thorgeirsson U. P., Snyderwine E. G., Thorgeirsson S. S., Reeves J., Dalgard D. W., Takayama S., Sugimura T. (1990): Carcinogenicity of 2-amino-3-methylimidazo[4,5-f]quinoline in nonhuman primates: induction of tumors in three macaques, *Jpn J Cancer Res*, 81, 10-14.

Adamson R. H., Takayama S., Sugimura T., Thorgeirsson U. P. (1994): Induction of hepatocellular carcinoma in nonhuman primates by the food mutagen 2-amino-3-methylimidazo[4,5-f]quinoline, *Environ Health Perspect*, 102, 190-193.

Aimová D., Poljaková J., Kotrbová V., Moserová M., Frei E., Arlt V. M., Stiborová 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, *Interdisc Toxicol*, 1, 160-168.

Alexander J., Benford D., Cockburn A., Cravedi J.-P., Dogliotti E., Di Domenico A., Fernández-Cruz M. L., Fink-Gremmels J., Fürst P., Galli C., Grandjean P., Gzyl J., Heinemeyer G., Johansson N., Mutti A., Schlatter J., van Leeuwen R., Van Peteghem C., Verger P. (2008): Polycyclic Aromatic Hydrocarbons in Food. Scientific Opinion of the Panel on Contaminants in the Food Chain, *The EFSA Journal*, 724, 1-114.

Alison M. R. (ed.) (2004): The Cancer Handbook, John Wiley & Sons, Hoboken

Arlt V. M., Stiborová M., Henderson C. J., Thiemann M., Frei E., Aimová D., Singh R., Gamboa da Costa G., Schmitz O. J., Farmer P. B., Wolf C. R., Phillips D. H. (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.

Atkinson C., Frankenfeld C. L., Lampe J. W. (2005): Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health, *Exp Biol Med (Maywood)*, 230, 155-170.

Augustsson K., Skog K., Jägerstad M., Dickman P. W., Steineck G. (1999): Dietary heterocyclic amines and cancer of the colon, rectum, bladder, and kidney: a population-based study, *Lancet*, 353, 703-707.

Bae E. A., Han M. J., Kim D. H. (1999): In vitro anti-Helicobacter pylori activity of some

flavonoids and their metabolites, *Planta Med*, 65, 442-443.

Barnes J., Anderson L. A., Phillipson J. D. (2001): St John's wort (Hypericum perforatum L.): a review of its chemistry, pharmacology and clinical properties, *J Pharm Pharmacol*, 53, 583-600.

Bennet R. N., Wallsgrove R.M. (1994): Secondary metabolites in plant defence mechanisms, *New Phytol*, 127, 617-633.

Birt D. F., Walker B., Tibbels M. G., Bresnick E. (1986): Anti-mutagenesis and anti-promotion by apigenin, robinetin and indole-3-carbinol, *Carcinogenesis*, 7, 959-963.

Boberg E. W., Miller E. C., Miller J. A., Poland A., Liem A. (1983): Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1'-sulfoöxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1'-hydroxysafrole in mouse liver, *Cancer Res*, 43, 5163-5173.

Burdová K. (2006): The influence of chemopreventive compounds on food carcinogen activation, Bachelor thesis, Charles University in Prague.

Canivenc-Lavier M. C., Bentejac M., Miller M. L., Leclerc J., Siess M. H., 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.

Carrière V., Chambaz J., Rousset M. (2001): Intestinal responses to xenobiotics, *Toxicol In Vitro*, 15, 373-378.

Casper R. F., Quesne M., Rogers I. M., Shirota T., Jolivet A., Milgrom E., Savouret J. F. (1999): Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity, *Mol Pharmacol*, 56, 784-790.

Ciolino H. P., Yeh G. C. (1999): Inhibition of aryl hydrocarbon-induced cytochrome P-450 1A1 enzyme activity and CYP1A1 expression by resveratrol, *Mol Pharmacol*, 56, 760-767.

Conney A.H. (2003): Enzyme induction and dietary chemicals as approaches to cancer chemoprevention: the Seventh DeWitt S. Goodman Lecture, *Cancer Res*, 63, 7005-7031.

Crofts F. G., Sutter T. R., Strickland P. T. (1998): Metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine by human cytochrome P4501A1, P4501A2 and P4501B1, *Carcinogenesis*, 19, 1969-1973.

Davenport D. M., Wargovich M. J. (2005): Modulation of cytochrome P450 enzymes by organosulfur compounds from garlic, *Food Chem Toxicol*, 43, 1753-1762.

Dawson J. H., Andersson L. A., Sono M. (1982): Spectroscopic investigations of ferric cytochrome P-450-CAM ligand complexes. Identification of the ligand trans to cysteinate in the native enzyme, *J Biol Chem*, 257, 3606-3617.

Day A. J., DuPont M. S., Ridley S., Rhodes M., Rhodes M. J., Morgan M. R., Williamson G. (1998): Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity, *FEBS Lett*, 436, 71-75.

Day A. J., Williamson G. (2001): Biomarkers for exposure to dietary flavonoids: a review of the current evidence for identification of quercetin glycosides in plasma, *Br J Nutr*, 86, 105-110.

Dogra S. C., Whitelaw M. L., May B. K. (1998): Transcriptional activation of cytochrome P450 genes by different classes of chemical inducers, *Clin Exp Pharmacol Physiol*, 25, 1-9.

Duvoix A., Blasius R., Delhalle S., Schnekenburger M., Morceau F., Henry E., Dicato M., Diederich M. (2005): Chemopreventive and therapeutic effects of curcumin, *Cancer Lett*, 223, 181-190.

Eaton D. L., Gallagher E. P., Bammler T. K., Kunze K. L. (1995): Role of cytochrome P4501A2 in chemical carcinogenesis: implications for human variability in expression and enzyme activity, *Pharmacogenetics*, 5, 259-274.

Ekins S., Wrighton S. A. (1999): The role of CYP2B6 in human xenobiotic metabolism, *Drug Metab Rev*, 31, 719-754.

Ellard S., Mohammed Y., Dogra S., Wolfel C., Doehmer J., Parry J. M. (1991): Use of genetically engineered V79 Chinese hamster cultures expressing rat liver CYP1A1, 1A2 and 2B1 cDNAs in micronucleus assays, *Mutagenesis*, 6, 461-470.

Felton J. S., Knize M. G. (1991): Occurrence, identification, and bacterial mutagenicity of heterocyclic amines in cooked food, *Mutat Res*, 259, 205-217.

Frandsen H., Grivas S., Andersson R., Dragsted L., Larsen J. C. (1992): Reaction of the N2-acetoxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) with 2'-deoxyguanosine and DNA. Synthesis and identification of N2-(2'-deoxyguanosin-8-yl)-PhIP, *Carcinogenesis*, 13, 629-635.

Frandsen H., Rasmussen E. S., Nielsen P. A., Farmer P., Dragsted L., Larsen J. C. (1991): Metabolic formation, synthesis and genotoxicity of the N-hydroxy derivative of the food mutagen 2-amino-1-methyl-6-phenylimidazo (4,5-b) pyridine (PhIP), *Mutagenesis*, 6, 93-98.

Franke A. A., Cooney R. V., Custer L. J., Mordan L. J., Tanaka Y. (1998): Inhibition of neoplastic transformation and bioavailability of dietary flavonoid agents, *Adv Exp Med Biol*, 439, 237-248.

Garfinkel D. (1958): Studies on pig liver microsomes. I. Enzymic and pigment composition of different microsomal fractions, *Arch Biochem Biophys*, 77, 493-509.

Gee J. M., DuPont M. S., Day A. J., Plumb G. W., Williamson G., Johnson I. T. (2000): Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway, *J Nutr*, 130, 2765-2771.

Gee J. M., DuPont M. S., Rhodes M. J., Johnson I. T. (1998): Quercetin glucosides interact with the intestinal glucose transport pathway, *Free Radic Biol Med*, 25, 19-25.

Gonzalez F. J., Gelboin H. V. (1994): Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins, *Drug Metab Rev*, 26, 165-183.

Gonzalez F. J., Liu S. Y., Yano M. (1993): Regulation of cytochrome P450 genes: molecular mechanisms, *Pharmacogenetics*, 3, 51-57.

Grant D. M., Josephy P. D., Lord H. L., Morrison L. D. (1992): Salmonella typhimurium strains expressing human arylamine N-acetyltransferases: metabolism and mutagenic activation of aromatic amines, *Cancer Res*, 52, 3961-3964.

Guengerich F. P. (1992): Characterization of human cytochrome P450 enzymes, *FASEB J*, 6, 745-748.

Guengerich F. P., Shimada T. (1991): Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes, *Chem Res Toxicol*, 4, 391-407.

Havsteen B. (1983): Flavonoids, a class of natural products of high pharmacological potency, *Biochem Pharmacol*, 32, 1141-1148.

Hodek P., Křížková J., Burdová K., Šulc M., Kizek R., Hudeček J., Stiborová M. (2009): Chemopreventive compounds--view from the other side, *Chem Biol Interact*, 180, 1-9.

Hodek P., Trefil P., Stiborová M. (2002): Flavonoids-potent and versatile biologically active

compounds interacting with cytochromes P450, *Chem Biol Interact*, 139, 1-21.

Hodgson E. (ed.) (2004): A Textbook of Modern Toxicology (Third Edition), John Wiley & Sons, Hoboken

Hodgson E., Rose R. L. (2007): The importance of cytochrome P450 2B6 in the human metabolism of environmental chemicals, *Pharmacol Ther*, 113, 420-428.

Hollenberg P. F. (1992): Mechanisms of cytochrome P450 and peroxidase-catalyzed xenobiotic metabolism, *FASEB J*, 6, 686-694.

Hollenberg P. F. (2002): Characteristics and common properties of inhibitors, inducers, and activators of CYP enzymes, *Drug Metab Rev*, 34, 17-35.

Hollman P. C., Bijsman M. N., van Gameren Y., Cnossen E. P., de Vries J. H., Katan M. B. (1999): The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man, *Free Radic Res*, 31, 569-573.

Hollman P. C., de Vries J. H., van Leeuwen S. D., Mengelers M. J., Katan M. B. (1995): Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers, *Am J Clin Nutr*, 62, 1276-1282.

Hollman P. C., Katan M. B. (1997): Absorption, metabolism and health effects of dietary flavonoids in man, *Biomed Pharmacother*, 51, 305-310.

Holme J. A., Wallin H., Brunborg G., Søderlund E. J., Hongslo J. K., Alexander J. (1989): Genotoxicity of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP): formation of 2-hydroxamino-PhIP, a directly acting genotoxic metabolite, *Carcinogenesis*, 10, 1389-1396.

http://drnelson.uthsc.edu/CytochromeP450.html (July 25th 2010)

http://monographs.iarc.fr/ENG/Classification/index.php (July 25th 2010)

http://www.czso.cz/csu/edicniplan.nsf/select_obyvatelstvo (July 25th 2010)

Huang M. T., Johnson E. F., Muller-Eberhard U., Koop D. R., Coon M. J., Conney A. H. (1981): Specificity in the activation and inhibition by flavonoids of benzo[a]pyrene hydroxylation by cytochrome P-450 isozymes from rabbit liver microsomes, *J Biol Chem*, 256, 10897-10901.

IARC (1973): Some Polycyclic Aromatic Hydrocarbons and Heterocyclic Compounds, IARC

Monogr Eval Carcinog Risk Chem Hum, 3, 271

IARC (1983): Polynuclear Aromatic Compounds, Part 1. Chemical, Environmental and Experimental Data, IARC Monogr Eval Carcinog Risk Chem Hum, 32, 211

IARC (1987): Overall Evaluations of Carcinogenicity, IARC Monogr Eval Carcinog Risk Chem Hum, 7, 440

Imai Y., Sato R., Iyanagi T. (1977): Rate-limiting step in the reconstituted microsomal drug hydroxylase system, *J Biochem*, 82, 1237-1246.

Jefcoate C. R. (1978): Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical-difference spectroscopy, *Methods Enzymol*, 52, 258-279.

Kaderlik K. R., Mulder G. J., Turesky R. J., Lang N. P., Teitel C. H., Chiarelli M. P., Kadlubar F. F. (1994): Glucuronidation of N-hydroxy heterocyclic amines by human and rat liver microsomes, *Carcinogenesis*, 15, 1695-1701.

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

Kandaswami C., Middleton E. Jr. (1994): Free radical scavenging and antioxidant activity of plant flavonoids, *Adv Exp Med Biol*, 366, 351-376.

Kato R., Yamazoe, Y. (1987): Metabolic activation and covalent binding to nucleic acids of carcinogenic heterocyclic amines from cooked foods and amino acid pyrolysates, *Jpn J Cancer Res*, 78, 297-311.

Kelloff G. J., Crowell J. A., Steele V. E., Lubet R. A., Malone W. A., Boone C. W., Kopelovich L., Hawk E. T., Lieberman R., Lawrence J. A., Ali I., Viner J. L., Sigman C. C. (2000): Progress in cancer chemoprevention: development of diet-derived chemopreventive agents, J Nutr, 130, 467-471.

Klingenberg M. (1958): Pigments of rat liver microsomes, *Arch Biochem Biophys*, 75, 376-386.

Kühnau J. (1976): The flavonoids. A class of semi-essential food components: their role in human nutrition, *World Rev Nutr Diet*, 24, 117-191.

Laires A., Pacheco P., Rueff J. (1989): Mutagenicity of rutin and the glycosidic activity of cultured cell-free microbial preparations of human faeces and saliva, *Food Chem Toxicol*, 27, 437-443.

Levi M. S., Borne R. F., Williamson J. S. (2001): A review of cancer chemopreventive agents, *Curr Med Chem*, 8, 1349-1362.

Lewis D. F., Ioannides C., Parke D. V. (1989): Molecular orbital studies of oxygen activation and mechanisms of cytochromes P-450-mediated oxidative metabolism of xenobiotics, *Chem Biol Interact*, 70, 263-280.

Lin D., Kaderlik K. R., Turesky R. J., Miller D. W., Lay J. O. Jr., Kadlubar F. F. (1992): Identification of N-(Deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine as the major adduct formed by the food-borne carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, with DNA, *Chem Res Toxicol*, 5, 691-697.

Lin J.H., Lu A.Y. (1998): Inhibition and induction of cytochrome P450 and the clinical implications, *Clin Pharmacokinet*, 35, 361-390.

Luch A. (2005): Metabolic activation and detoxification of polycyclic aromatic hydrocarbons. In Luch A. (ed.): The Carcinogenic Effects of Polycyclic Aromatic Hydrocarbons, Imperial College Press, London, pp. 19-96.

Macdonald I. A., Mader J. A., Bussard R. G. (1983): The role of rutin and quercitrin in stimulating flavonol glycosidase activity by cultured cell-free microbial preparations of human feces and saliva, *Mutat Res*, 122, 95-102.

Martignoni M., de Kanter R., Grossi P., Mahnke A., Saturno G., Monshouwer M. (2004): An in vivo and in vitro comparison of CYP induction in rat liver and intestine using slices and quantitative RT-PCR, *Chem Biol Interact*, 151, 1-11.

Middleton E. Jr., Kandaswami C. (1993): Advances in Flavonoid Research, Chapman and Hall, London

Milner J. A., McDonald S. S., Anderson D. E., Greenwald P. (2001): Molecular targets for nutrients involved with cancer prevention, *Nutr Cancer*, 41, 1-16.

Moon Y. J., Wang X., Morris M. E. (2006): Dietary flavonoids: effects on xenobiotic and carcinogen metabolism, *Toxicol In Vitro*, 20, 187-210.

Mori Y., Tatematsu K., Koide A., Sugie S., Tanaka T., Mori H. (2006): Modification by

curcumin of mutagenic activation of carcinogenic N-nitrosamines by extrahepatic cytochromes P-450 2B1 and 2E1 in rats, *Cancer Sci*, 97, 896-904.

Nagao M., Ushijima T., Wakabayashi K., Ochiai M., Kushida H., Sugimura T., Hasegawa R., Shirai T., Ito N. (1994): Dietary carcinogens and mammary carcinogenesis. Induction of rat mammary carcinomas by administration of heterocyclic amines in cooked foods, *Cancer*, 74, 1063-1069.

Nebert D. W. (1989): The Ah locus: genetic differences in toxicity, cancer, mutation and birth defects, *Crit Rev Toxicol*, 20, 153-174.

Nebert D. W., Dieter M. Z. (2000): The evolution of drug metabolism, *Pharmacology*, 61, 124-135.

Nebert DW, McKinnon RA. (1994): Cytochrome P450: evolution and functional diversity, *Prog Liver Dis*, 12, 63-97.

Nelson D. R., Koymans L., Kamataki T., Stegeman J. J., Feyereisen R., Waxman D. J., Waterman M. R., Gotoh O., Coon M. J., Estabrook R. W., Gunsalus I. C., Nebert D. W. (1996): P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature, *Pharmacogenetics*, 6, 1-42.

Nikolic D., van Breemen R. B. (2004): New metabolic pathways for flavanones catalyzed by rat liver microsomes, *Drug Metab Dispos*, 32, 387-397.

Obach R. S., Zhang Q. Y., Dunbar D., Kaminsky L. S. (2001): Metabolic characterization of the major human small intestinal cytochrome p450s, *Drug Metab Dispos*, 29, 347-352.

Ohgaki H., Takayama S., Sugimura T. (1991): Carcinogenicities of heterocyclic amines in cooked food, *Mutat Res*, 259, 399-410.

Omura T., Sato R. (1964): The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties, *J Biol Chem*, 239, 2379-2385.

Ortiz de Montellano P. R. (ed.) (2005): Cytochrome P-450: Structure, Mechanism and Biochemistry. Third edition, Plenum Publishers, New York

Paine M. F., Hart H. L., Ludington S. S., Haining R. L., Rettie A. E., Zeldin D. C. (2006): The human intestinal cytochrome P450 "pie", *Drug Metab Dispos*, 34, 880-886.

Pais P., Knize M.G. (2000): Chromatographic and related techniques for the determination of

heterocyclic amines on foods, *J Chromatogr B Biomed Sci Appl*, 747, 139-169.

Parkar S. G., Stevenson D. E., Skinner M. A. (2008): The potential influence of fruit polyphenols on colonic microflora and human gut health, *Int J Food Microbiol*, 124, 295-298.

Peters W. H., Kock L., Nagengast F. M., Kremers P. G. (1991): Biotransformation enzymes in human intestine: critical low levels in the colon?, *Gut*, 32, 408-412.

Phillips D. H. (1999): Polycyclic aromatic hydrocarbons in the diet, *Mutat Res*, 443, 139-147.

Phillips D. H. (2002): Smoking-related DNA and protein adducts in human tissues, *Carcinogenesis*, 23, 1979-2004.

Phillips D.H. (2005): Macromolecular adducts as biomarkers of human exposure to polycyclic aromatic hydrocarbons. In Luch A. (ed.): The Carcinogenic Effects of Polycyclic Aromatic Hydrocarbons, Imperial College Press, London, pp. 137-169.

Pietta P. G. (2000): Flavonoids as antioxidants, *J Nat Prod*, 63, 1035-1042.

Piver B., Fer M., Vitrac X., Merillon J. M., Dreano Y., Berthou F., Lucas D. (2004): Involvement of cytochrome P450 1A2 in the biotransformation of transresveratrol in human liver microsomes, *Biochem Pharmacol*, 68, 773-782.

Porter T. D., Coon M. J. (1991): Cytochrome P-450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms, *J Biol Chem*, 266, 13469-13472.

Poulos T. L., Finzel B. C., Gunsalus I. C., Wagner G. C., Kraut J. (1985): The 2.6-A crystal structure of Pseudomonas putida cytochrome P-450, *J Biol Chem*, 260, 16122-16130.

Poulos T. L., Finzel B. C., Howard A. J. (1986): Crystal structure of substrate-free Pseudomonas putida cytochrome P-450, *Biochemistry*, 25, 5314-5322.

Reinli K., Block G. (1996): 11. Phytoestrogen content of foods - a compendium of literature values, *Nutr Cancer*, 26, 123-148.

Rendic S., Di Carlo F. J. (1997): Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors, *Drug Metab Rev*, 29, 413-580.

Report on Carcinogens, Eleventh Edition; U.S. Department of Health and Human Services, Public Health Service, National Toxicology Program

Rice-Evans C. (2001): Flavonoid antioxidants, *Curr Med Chem*, 8, 797-807.

Rice-Evans C., Packer L. (eds.) (2003): Flavonoids in Health and Disease, Second Edition, CRC Press, Boca Raton

Rietjens I. M., Boersma M. G., van der Woude H., Jeurissen S. M., Schutte M. E., Alink G. M. (2005): Flavonoids and alkenylbenzenes: mechanisms of mutagenic action and carcinogenic risk, *Mutat Res*, 574, 124-138.

Ronis M. J. J., Lindros K. O., Ingelman-Sundberg M. (1996): The CYP2E Family. In Ioannides C. (Ed.): Cytochromes P450: Metabolic and Toxicological Aspects, CRC Press: Boca Raton, pp. 211-239.

Rousseff R. L., Martin S. F., Youtsey C. O. (1987): Quantitative survey of narirutin, naringin, hesperidin, and neohesperidin in citrus, *J Agric Food Chem*, 35, 1027-1030.

Sakano K., Kawanishi S. (2002): Metal-mediated DNA damage induced by curcumin in the presence of human cytochrome P450 isozymes, *Arch Biochem Biophys*, 405, 223-230.

Sau A., Pellizzari Tregno F., Valentino F., Federici G., Caccuri A. M. (2010): Glutathione transferases and development of new principles to overcome drug resistance, *Arch Biochem Biophys*, 500, 116-122.

Scalbert A., Williamson G. (2000): Dietary intake and bioavailability of polyphenols, *J Nutr*, 130, 2073-2085.

Scheline R. R. (1999): Metabolism of oxygen heterocyclic compounds. In: CRC Handbook of mammalian metabolism of plant compounds, CRC Press, Boca Raton, pp. 243-295.

Schulz W.A. (2005): Molecular Biology of Human Cancers, Springer Science + Business Media, Dordrecht

Shahidi F., Wanasundara P. K. (1992): Phenolic antioxidants, *Crit Rev Food Sci Nutr*, 32, 67-103.

Shih H., Pickwell G.V., Quattrochi L.C. (2000): Differential effects of flavonoid compounds on tumor promoter-induced activation of the human CYP1A2 enhancer, *Arch Biochem Biophys*, 373, 287-294.

Shimada T., Yamazaki H., Mimura M., Inui Y., Guengerich F. P. (1994): Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians, *J Pharmacol Exp Ther*, 270, 414-423.

Shukla Y., Arora A. (2003): Enhancing effects of mustard oil on preneoplastic hepatic foci development in Wistar rats, *Hum Exp Toxicol*, 22, 51-55.

Shukla Y., Pal S. K. (2004): Dietary Cancer Chemoprevention: An Overview, *Int J Hum Genet*, 4, 265-276.

Singh R., Arlt V. M., Henderson C. J., Phillips D. H., Farmer P. B., Gamboa da Costa G. (2010): Detection and quantitation of N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine adducts in DNA using online column-switching liquid chromatography tandem mass spectrometry, *J Chromatogr B Analyt Technol Biomed Life Sci*, 878, 2155-2162.

Sinha R., Gustafson D. R., Kulldorff M., Wen W. Q., Cerhan J. R., Zheng W. (2000): 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, a carcinogen in high-temperature-cooked meat, and breast cancer risk, *J Natl Cancer Inst*, 92, 1352-1354.

Smith T. J., Guo Z., Guengerich F. P., Yang C. S. (1996): Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by human cytochrome P450 1A2 and its inhibition by phenethyl isothiocyanate, *Carcinogenesis*, 17, 809-813.

Smith P. K., Krohn R. I., Hermanson G. T., Mallia A. K., Gartner F. H., Provenzano M. D., Fujimoto E. K., Goeke N. M., Olson B. J., Klenk D. C. (1985): Measurement of protein using bicinchoninic acid, *Anal Biochem*, 150, 76-85.

Snyderwine E. G. (1994): Some perspectives on the nutritional aspects of breast cancer research. Food-derived heterocyclic amines as etiologic agents in human mammary cancer, *Cancer*, 74, 1070-1077.

Snyderwine E. G., Battula N. (1989): Selective mutagenic activation by cytochrome P3-450 of carcinogenic arylamines found in foods, *J Natl Cancer Inst*, 81, 223-227.

Spencer J. P. E., Chowrimootoo G., Choudhury R., Debnam E. S., Srai S. K., Rice-Evans C. (1999): The small intestine can both absorb and glucuronidate luminal flavonoids, *FEBS Lett*, 458, 224-230.

Stiborová M., Hudeček J., Hodek P., Frei E. (1999): Cytochromes P450 and human health, *Chem Listy*, 93, 229-237.

Stiborová M., Martínek V., Rýdlová H., Hodek P., Frei E. (2002): Sudan I is a potential carcinogen for humans: evidence for its metabolic activation and detoxication by human

recombinant cytochrome P450 1A1 and liver microsomes, *Cancer Res*, 62, 5678-5684.

Stiborová M., Rupertová M., Hodek P., Frei E., Schmeiser H. H. (2004): Monitoring of DNA adducts in humans and ^{32}P-postlabelling methods, *Collect Czech Chem Commun*, 69, 476-498.

Sugiyama T., Nagata J., Yamagishi A., Endoh K., Saito M., Yamada K., Yamada S., Umegaki K. (2006): Selective protection of curcumin against carbon tetrachloride-induced inactivation of hepatic cytochrome P450 isozymes in rats, *Life Sci*, 78, 2188-2193.

Šmidrkal J., Filip V., Melzoch K., Hanzlíková I., Buckiová D., Křísa B. (2001): Resveratrol, *Chem Listy*, 95, 602-609.

Tan W., Lin D. X., Xiao Y., Kadlubar F., Chen J. S. (1999): Chemoprevention of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine-induced carcinogen-DNA adducts by Chinese cabbage in rats, *World J Gastroenterol*, 5, 138-142.

Turesky R. J. (2007): Formation and biochemistry of carcinogenic heterocyclic aromatic amines in cooked meats, *Toxicol Lett*, 168, 219-227.

Turesky R. J., Lang N. P., Butler M. A., Teitel C. H., Kadlubar F. F. (1991): Metabolic activation of carcinogenic heterocyclic aromatic amines by human liver and colon, *Carcinogenesis*, 12, 1839-1845.

Vernet A., Siess M. H. (1986): Comparison of the effects of various flavonoids on ethoxycoumarin deethylase activity of rat intestinal and hepatic microsomes, *Food Chem Toxicol*, 24, 857-861.

Wakabayashi K., Nagao M., Esumi H., Sugimura T. (1992): Food-derived mutagens and carcinogens, *Cancer Res*, 52, 2092-2098.

Walgren R. A., Lin J. T., Kinne R. K., Walle T. (2000): Cellular uptake of dietary flavonoid quercetin 4′-beta-glucoside by sodiumdependent glucose transporter SGLT1, *J Pharmacol Exp Ther*, 294, 837-843.

Wattenberg L. W. (1996): Chemoprevention of cancer, *Prev Med*, 25, 44-45.

Xu H. X., Wan M., Dong H., But P. P., Foo L. Y. (2000): Inhibitory activity of flavonoids and tannins against HIV-1 protease, *Biol Pharm Bull*, 23, 1072-1076.

Yamazoe Y., Nagata K., Yoshinari K., Fujita K., Shiraga T., Iwasaki K. (1999):

Sulfotransferase catalyzing sulfation of heterocyclic amines, *Cancer Lett*, 143, 103-107.

Yan Z., Caldwell G. W. (2001): Metabolism profiling, and cytochrome P450 inhibition & induction in drug discovery, *Curr Top Med Chem*, 1, 403-425.

Zhang L., Zuo Z., Lin G. (2007): Intestinal and hepatic glucuronidation of fl avonoids, *Mol Pharm*, 4, 833-845.

Appendices

List of publications

Appendix 1

Petr Hodek, Pavel Hanuštiak, **Jitka Křížková**, Radka Mikelová, Soňa Křížková, Marie Stiborová, Libuše Trnková, Aleš Horna, Miroslava Beklová, René Kizek (2006): Toxicological aspects of flavonoid interaction with biomacromolecules, *Neuroendocrinol Lett*, 27, 14-17. **IF = 0.924**

Appendix 2

Jitka Křížková, Kamila Burdová, Petr Hodek, René Kizek, Marie Stiborová (2007): Effects of a flavonoid structure on cytochromes P450 induction, *Chem Listy*, 101, 206-208.
IF = 0.683

Appendix 3

Jitka Křížková, Kamila Burdová, Jiří Hudeček, Marie Stiborová, Petr Hodek (2008): Induction of cytochromes P450 in small intestine by chemopreventive compounds, *Neuroendocrinol Lett*, 29, 717-721. **IF = 1.359**

Appendix 4

Petr Hodek, **Jitka Křížková**, Kamila Burdová, Miroslav Šulc, René Kizek, Jiří Hudeček, Marie Stiborová (2009): Chemopreventive compounds—View from the other side, *Chem Biol Interact*, 180, 1-9. **IF = 2.457**

Appendix 5

Petr Hodek, Martina Teplá, **Jitka Křížková**, Marie Stiborová (2009): Modulation of cytochrome P450 enzyme system by selected flavonoids, *Neuroendocrinol Lett*, 30, 67-71.
IF = 1.047

Appendix 6

Jitka Křížková, Kamila Burdová, Marie Stiborová, Vladimír Křen, Petr Hodek (2009): The effects of selected flavonoids on cytochromes P450 in rat liver and small intestine, *Interdisc Toxicol*, 2, 201-204. **No impact factor yet**

Appendix 7

Jitka Křížková, Petr Hodek, Miroslav Šulc: Chicken antibodies in Western blots: How to avoid potential keratin cross-reactivity

Manuscript in preparation