CHARLES UNIVERSITY OF PRAGUE
FACULTY OF SCIENCE
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SELECTED CHEMOPREVENTIVE COMPOUNDS AS CYTOCHROME P450 INDUCERS

Diploma Thesis

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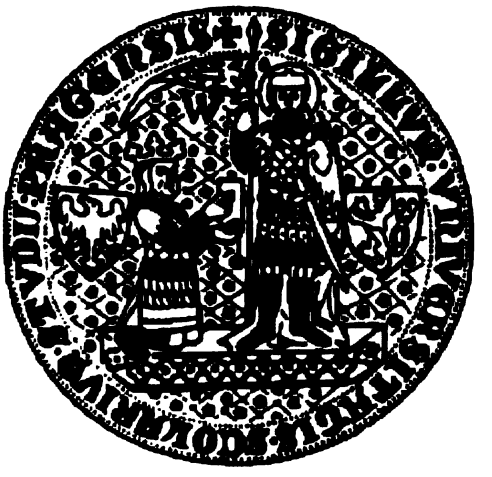

Prague 2008

Bc. Kamila Burdová

Declaration

I declare that this diploma thesis was elaborated individually under the supervising of Doc. RNDr. Petr Hodek, CSc., and that all used references were cited properly.

Prague, 5th May 2008 .....

Acknowledgement

I would like to thank to my supervisor Doc. RNDr. Petr Hodek, CSc. for his scientific support and RNDr. Jitka Křížková for her entire help in the laboratory.

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

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Abbreviations

A	absorbance
AhR	aromatic hydrocarbon receptor
ARNT	AhR nuclear translocator
BCA	bicinchoninic acid
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BIS	N,N'-methylenbisacrylamide
BSA	bovine serum albumin
CAR	constitutive androstane receptor
c_{CYP}	concentration of cytochromes P450
CO	carbon monoxide
$c_{protein}$	protein concentration
CYP	cytochrome P450
DAS	diallyl sulphide
DASO	diallyl sulphoxide
$DASO_2$	diallyl sulphone
DNA	deoxyribonucleic acid
EROD	7-ethoxyresorufin-*O*-deethylase
FAD	flavinadenine dinucleotide
FMN	flavin mononucleotide
MFO	mixed function oxygenase system
MROD	7-methoxyresorufin-*O*-demethylase
NADH	nicotinamide adenine dinucleotide - reduced
NADPH	nicotinamide adenine dinucleotide phosphate - reduced
NF1	nuclear factor 1
NR1, NR2	nuclear receptor binding site 1, 2
p.o.	*per os*
PBREM	phenobarbital-responsive enhancer module
PBS	phosphate buffered saline
PROD	7-pentoxyresorufin-*O*-dealkylase
PVDF	polyvinylidene fluoride

R	substrate
ROH	hydroxylated substrate
RPM	revolutions per minute
RXR	retinoid X receptor
$s.c._{CYP}$	specific content of cytochromes P450
SD	standard deviation
SDS	sodium dodecyl sulphate
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEMED	N,N,N',N'-tetramethylethylendiamine
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	*t*-octylphenoxypolyethoxyethanol
UV	ultraviolet
v/v	volume/volume percent
w/v	weight/volume percent
XOOH	peroxide (in general)
XRE	xenobiotic responsive element
ε	molar absorption coefficient

1 Introduction

1.1 Carcinogenesis

Cancer is worldwide spread life-threating group of diseases with one of the highest mortalities. Development of cancer is a complex process, called carcinogenesis (scheme is shown in Figure 1-1), resulting in uncontrolled cell growth and division.

As initiation is the first and critical step in the whole process, it is the most studied phase of carcinogenesis. Factors involved in this phase can be either internal, such as heredity and hormonal imbalance, or external, such as chemical carcinogens (e.g. nitrosamines, polycyclic aromatic hydrocarbons, cadmium); biological carcinogens (e.g. bacteria, viruses), and physical carcinogens (e.g. UV irradiation, X-rays).

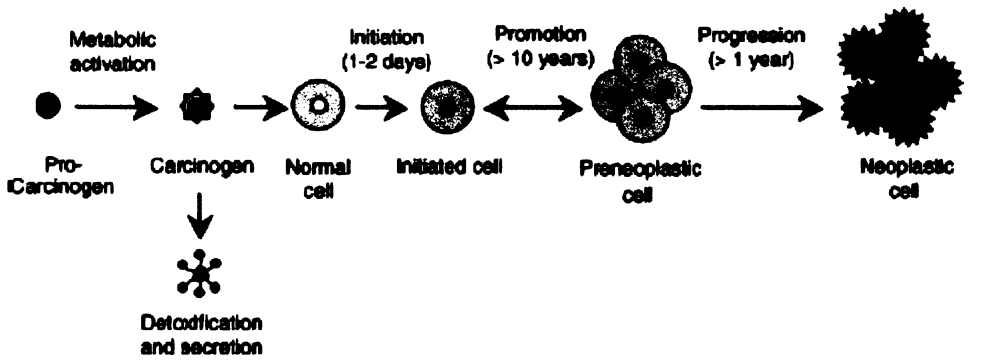


Figure 1-1 Scheme of multistep carcinogenesis (adapted and modified from [1]).

Among diseases, cancer is the second highest in incidence and mortality in the Czech Republic (in 2003 caused 27% of all deaths). Compared to European and world standards, incidence per 100 000 inhabitants is much higher as is demonstrated in Figure 1-2. In 2003 it was higher by 20% and 70%, respectively. Worldwide, higher incidence of cancer is observed in men population although in the Czech Republic, the difference is not so extensive. Concerning European and world standards, incidence of cancer in 2003 was higher in men by 34% and 25%, respectively. It was higher only by 7% in the Czech Republic [2, 3]. Moreover, mortality caused by cancer is higher in the Czech Republic than world and European standards by 84% and 22%, respectively [3].

Most common types of cancer in the Czech Republic in last years are malignant neoplasia of colon and rectum, bronchus and lung, breast, female and male genital organs, and lymphoid, haemotopoietic and related tissues. Between 1995 and 2002 deaths caused by these diagnoses decreased from 57% to 52% of all deaths caused by cancer.

Interestingly, the incidence of deaths caused by malignant neoplasia of colon and rectum increased in men population by 20% comparing data from these two years [2].

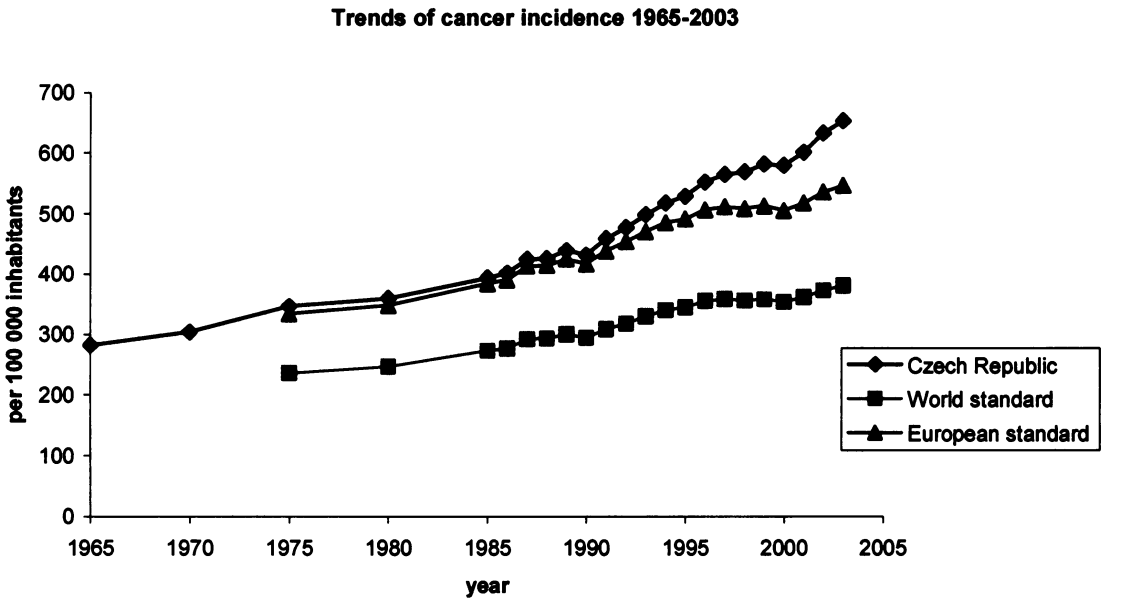


Figure 1-2 Trends in cancer incidence [3].

Due to increasing cancer incidence in Western world, there are many efforts to decrease the risk of cancer development. These involve dietary supplements that contain variety of chemopreventive compounds. Most of these compounds are of plant origin and because of this they are thought to be safe. Moreover, beneficial effects of these compounds are usually tested only in artificial systems although in human they can have undesired impact on overall balance of the organism. For example, chemopreventive compounds are able to modulate enzyme activity and change the metabolic pathways proportions. Enzymes of high importance in this case are these involved in metabolism of xenobiotics (carcinogens).

1.2 Metabolism of xenobiotics

The metabolism of xenobiotics is a multi-step process catalyzed by variety of enzymes. In general, this process can be divided into two phases.

In the first derivatization phase (**phase I**) a xenobiotic is metabolized into more polar compound to be excreted from the organism more easily and/or to be accepted by enzymes of the following second phase. Phase I reactions expose or introduce more polar group (e.g. OH, NH_2, SH, COOH) into molecule. Basically, these reactions are oxidation, reduction and hydrolysis that are catalyzed by e.g. cytochromes P450, flavin contaning monooxygenases, dehydrogenases, esterases, amidases and hydrolyses.

The metabolite, resulting from phase I, is then conjugated in the second phase (**phase II**) with endogenous polar compound (sugar, amino acid, glutathione, sulphate or phosphate) leading to more water soluble compound, making it easier to be excreted. These reactions are for example catalyzed by glutathione-*S*-transferase, sulphotransferase, methyltransferase, UDP-glucuronosyltransferase and *N*- and *O*-acyltransferase.

Conjugation phase is often followed by transport of the metabolite out of the cell, sometimes incorrectly called phase III.

Although xenobiotics are usually detoxified during this metabolizing process, some reactions produce reactive metabolites or compounds which are even more toxic than the parent compound. This process is usually called metabolic activation or bioactivation. The relationship between metabolism and toxicity of chemicals is described in Figure 1-3.

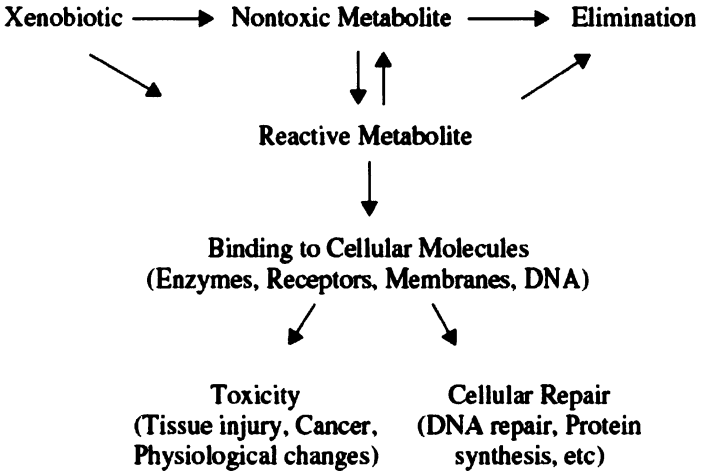


Figure 1-3 Activation and detoxication of xenobiotics. Adapted from [4].

Reactive metabolites are most frequently electrophilic compounds or radical molecules. Electrophilic compounds contain positively charged center that can easily react with nucleophilic parts of molecules, such as nucleic acids and proteins. Radicals act directly (also called free radicals) or form other radicals acting as radical generators. These radicals can interact with oxygen to produce reactive oxygen species which can damage cell membranes, DNA, etc.

Xenobiotics are usually transformed by more metabolic pathways and by variety of enzymes resulting in different metabolites. The major pathways are usually detoxifying but the minor ones forming reactive compounds are of great importance.

Almost all enzymes involved in metabolism of xenobiotics can form reactive intermediates. However, the majority of reactive metabolites are produced by oxidation reactions thus catalyzed mainly by cytochromes P450. Phase II enzymes are mainly detoxifying and therefore are not as important as phase I enzymes in the activation processes.

1.3 Cytochrome P450

Cytochromes P450 (CYP, EC 1.14.14.1) are one of the most important enzymes involved in biotransformation of xenobiotics. These enzymes together with NADPH: CYP oxidoreductase and membrane phospholipids (e.g. phosphatidylcholin) and possibly with cytochrome b_5 and NADH: cytochrome b_5 oxidoreductase (e.g. human CYP3A4, CYP3A5 and CYP2E1 [4]) compose mixed function oxygenase system (MFO).

The first experimental evidence relating to cytochromes P450 appeared in 1955 when Axelrod and Brodie identified an enzyme system in the endoplasmic reticulum of the liver which was able to oxidize xenobiotic compounds. Later, in 1958, William and Klingenberg detected carbon monoxide binding pigment in liver microsomes that was named P450 due to its properties. Reduced form of this pigment (enzyme) in complex with carbon monoxide shows strong absorption band at 450 nm.

Cytochromes P450 typically manage activation of molecular oxygen and binding of one atom of oxygen to substrate molecule while the second atom of oxygen is reduced to water. As the donor of electrons, NADPH: cytochrome P450 reductase or NADH: cytochrome b_5 reductase is involved.

Cytochromes P450 were found in a variety of living organisms including microorganisms (yeast, bacteria), animals, plants and fungi. In bacteria and yeast, soluble cytosolic forms were found. However, in eukaryotes, cytochromes P450 are transmembrane and thus insoluble, localized in inner mitochondrial membrane, smooth endoplasmic reticulum membrane, and in lower amounts also in rough endoplasmic reticulum membrane and nuclear membrane.

Until these days, more than 2000 CYPs have been found. Agreeably to new nomenclature of CYPs (proposed firstly in 1987, lastly changed in 1996), they are categorized into families and subfamilies according to their amino acid sequence similarities. Isoforms that have more than 40% of sequence identity are classified in one group (family) which is symbolized by the first number. Members of subfamily are marked with the capital letter, and have more than 55% of sequence identity in mammals and more then 46% in non-mammals. The particular isoform of cytochrome P450 is indicated by another number [4]. For example: CYP2E1, where 2 is family number, E is subfamily letter and 1 defines exact isoform of cytochrome P450. Sequences with less than 3% divergence are thought to be allelic variants of one isoform. Term "isoform" means proteins which are coded by distinct genes.

1.3.1 Structure

Cytochrome P450 contains porphyrin structure, heme-ring (non-protein part of enzyme), and apoprotein part (protein part). Heme-ring contains a central iron atom which is able to provide six valences for electron donation ligands. Four of these valences are engaged in binding of nitrogen atoms from porphyrin ring structure, protoporphyrin IX. The fifth valence is occupied by thiolate anion (negatively charged sulphur atom) from cysteine of apoprotein. The last, sixth ligand, of iron atom is usually an oxygen atom from water molecule, hydroxyl or carboxyl group from apoprotein or some exogenous compound. The structure of crystalized human CYP1A2 isoform with bound α-naphthoflavone is shown in Figure 1-4.

The apoprotein part of cytochrome usually consists of between 490 to 520 amino acids depending on the particular isoform. The protein is made of at least three functional domains. The first domain binds heme, the second binds reductase (enzyme cooperating with CYP) and the third binds substrate. The active site of protein consists of heme and substrate binding domains. Sequence of the heme binding part is highly conserved while

other parts of cytochrome sequence are rather variable except of key amino acid residues which are necessary for reductase binding. The common feature of substrate binding sequence is a high content of hydrophobic amino acids.

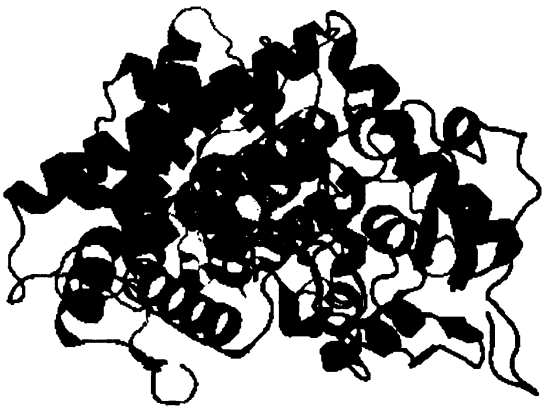


Figure 1-4 3D structure of human CYP1A2 crystalized in complex with α-naphthoflavone (black) [5].

Individual cytochrome P450 isoforms usually differ in their relative molecular weight depending on number of amino acids in sequence. Molecular weight of mammalian cytochromes P450 is spanning in the interval of 46 - 60 kDa. Proposed orientation of cytochrome P450 system with other partners in the membrane is shown in Figure 1-5.

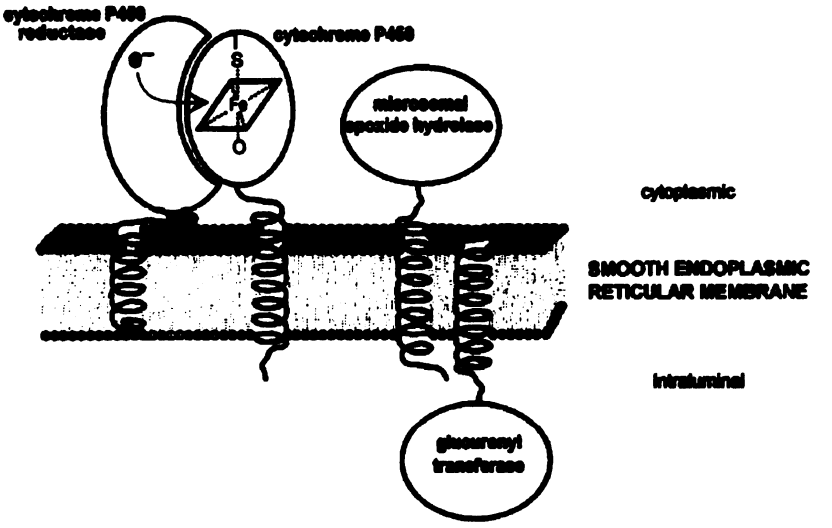


Figure 1-5 Proposed schematic representation of the orientation of cytochrome P450 and its reductase, microsomal epoxide hydrolase and glucuronyltransferase in the smooth endoplasmic reticulum membrane (adapted from [6]).

1.3.2 Function

Cytochromes P450 provide various reactions and substrate specifities depending on the particular isoform. For example, cytochromes P450 in a human are divided into two groups, the first metabolizing sterols, fatty acids, eicosanoids, vitamins and other endogenous compounds, and the second mainly involved in transformation of xenobiotics. The function of all cytochrome P450 isoforms has not been discovered yet.

The overall catalytic cycle of cytochrome P450 monoxygenase reactions is shown in Figure 1-6. This cycle proceeds through many steps in cooperation with another two enzymes associated (Figure 1-5), mainly NADPH: cytochrome P450 reductase and in minor extent NADH: cytochrome b_5 reductase. NADPH: cytochrome P450 reductase is a flavoprotein containing one mole of each flavinadenine dinucleotide (FAD) and flavin mononucleotide (FMN). Other important compounds involved in this cycle are phospholipids, mainly phosphatidylcholin, that seems to be involved in coupling of the reductase to cytochrome P450 and in binding of substrate to cytochrome P450 [4].

The first step in catalytic cycle of monooxygenase reaction of CYP is substrate binding to oxidized CYP (Fe^{3+}). Oxidized substrate free form of CYP is hexacoordinated (low spin state) and the sixth ligand of iron is usually an oxygen atom from water or from amino acid from side chains of CYP sequence. When substrate reaches the active site of enzyme then the sixth ligand is expulsed resulting in conformational changes of cytochrome structure and the iron atom is shifted to pentacoordinated form (high spin state).

The second step is one-electron reduction catalyzed by NADPH: cytochrome P450 reductase by which reduced CYP (Fe^{2+})-substrate complex is formed. This form of CYP (pentacoordinated and reduced) can bind another ligands, such as molecular oxygen or carbon monoxide.

Interaction with molecular oxygen forms ternary oxygenated complex (hexacoordinated) that can accept the second electron. Several products (complexes) are formed in this reaction. One of them is peroxide anion derivative of the substrate-bound hemoprotein. Under some conditions hydrogen peroxide (cytotoxic for cells) can be released forming oxidized CYP complex, this reaction is called uncoupling.

Regularly, dioxygen molecule is split and one oxygen atom is bound to CYP-substrate complex forming another complex where one atom of oxygen is bound to iron in heme. In another step oxygen radical is formed. This radical is highly reactive and

can pull out one electron from appropriate substrates to form hydroxyl radical and substrate radical. These radicals can recombine to form hydroxy-derivate of substrate and the oxidized (original) form of CYP (Fe^{3+}, hexacoordinated). The second atom of oxygen is reduced by two protons into a water molecule.

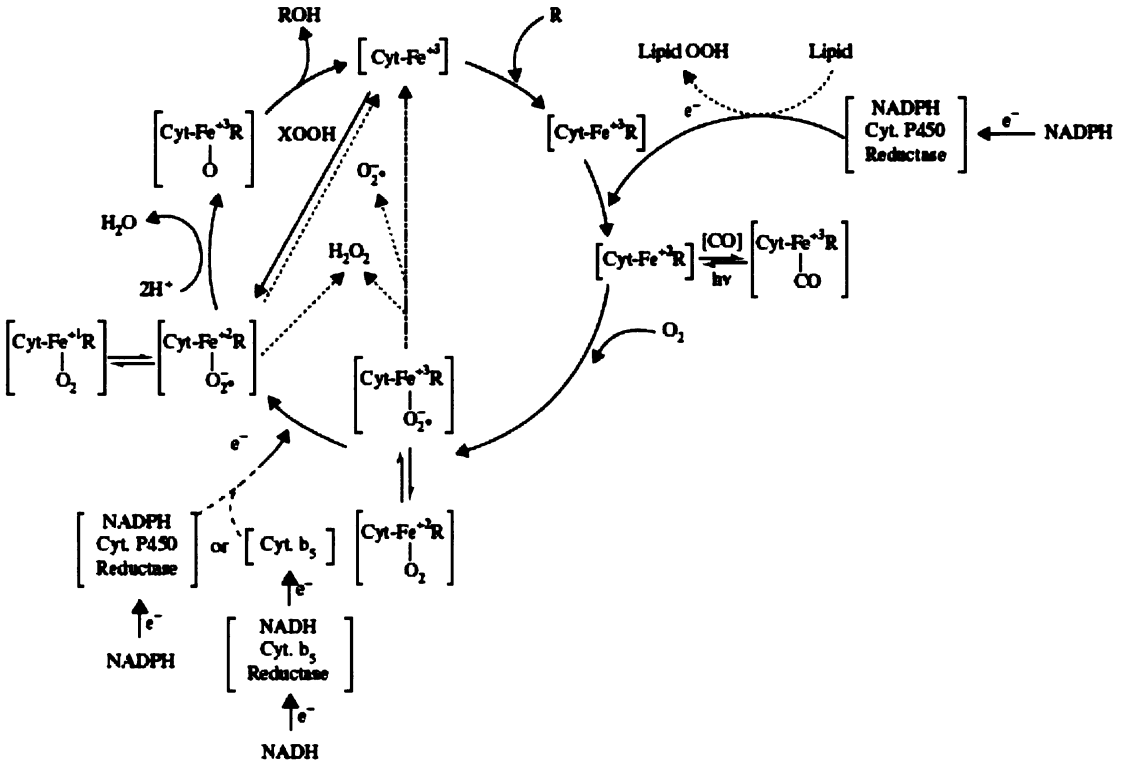


Figure 1-6 Generalized scheme of CYP monooxygenase function (adapted from [4]).

1.3.3 Modulation of cytochrome P450 activity

There are many factors influencing activity of cytochromes P450, such as nutrition, physiological, chemical and environmental effects. Since cytochromes P450 are involved in metabolism of xenobiotics and activation of carcinogens (procarcinogens), modulation of CYP activity can lead either to increased or decreased xenobiotic toxicity and carcinogen activation.

Inhibition

CYP inhibition can be either reversible or irreversible (e.g. suicidal substrate metabolized by this enzyme to reactive metabolite that damages the enzyme) involving many mechanisms.

Reversible inhibition involves no covalent binding and is usually classified into competitive, uncompetitive and non-competitive. These types are not rigidly separated and many intermediate cases exist. Two molecules competing for the same active site of the enzyme result in competitive inhibition. For CYPs are expected inhibitors, type I ligands, that binds in active site but not to heme iron. Uncompetitive inhibitors can react only with formed complex substrate-enzyme, while non-competitive inhibitors can bind either to enzyme or to complex enzyme-substrate affecting the rate of enzyme activity.

Irreversible inhibition involves in most cases formation of covalent or other stable bond, or disruption of the enzyme structure. Since this process is irreversible, enzyme cannot be regenerated.

Induction

CYP induction is based on various mechanisms including increased transcription of DNA, increased translation of mRNA to protein, and mRNA and protein (CYP) stabilization. According to this, the increase in activity is correlating with enhanced synthesis or stabilization of the enzyme, not with the activation of enzyme already synthesized.

Eukaryotic DNA transcription is also regulated on the level of chromatin structure. Generally, chromatin has two major forms, euchromatin (condensed) and heterochromatin (more relaxed). Transcription can proceed only when chromatin is not so condensed and DNA is accessible for transcriptional enzymes. Condensation of DNA is regulated by histone acetylation and cytosine methylation in DNA. [7, 8].

On the **level of transcription**, there are four major pathways involved. These inducers are classified into few groups that are differing in mechanism. These groups are: phenobarbital-type inducers, dexamethasone/rifampicin-type inducers, TCDD-type inducers and other inducers (e.g. ethanol).

Major classes of cytochrome genes are selectively regulated by ligand-activated nuclear receptors, which are responsible for transformation of signal into cellular responses. Nuclear receptors contain ligand-dependent transactivation domain (N-terminal), DNA-binding domain (containing two zinc finger motifs) and ligand binding domain (C-terminal). In most cases the ligand-dependent hetero or homo-dimerization of receptors (usually with retinoid X receptor, RXR) is required [7].

After ligand is bound to nuclear receptor, conformational change usually occurs in ligand binding site so that other molecules can bind there. These molecules are usually

co-activators or co-repressors. Co-activator activates transcription by histone acetylation (mediated by acetyltransferase), while co-repressor binding results in histone deacetylation and decreased transcription [8].

In comparison to inhibition, induction is a relatively slow process. It usually takes hours until an increase in CYP activity is apparent. This time depends on the paricular inducer and induction mechanism involved. For example induction by rifampicin, inducer of CYP3A4, 1A2 and 2C, is apparent within 24 hours, however, phenobarbital requires almost a week to reach the maximal induction [8].

The experimental part of this work is focused on CYP1A and CYP2B subfamilies, so in next part only induction mechanisms of these enzymes will be mentioned.

Aromatic hydrocarbon receptor

Aromatic hydrocarbon receptor (AhR) is cytosolic protein that can bind compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3-methylcholanthrene and polycyclic aromatic hydrocarbons forming AhR-ligand complexes. These complexes are then translocated to the nucleus and form a dimer with another protein known as AhR nuclear translocator (ARNT). Resulting complex interacts with specific DNA sequences (xenobiotic responsive elements, XREs) in the nucleus. Proposed effect of this interaction is thought to be bending of the DNA that increases level of transcription and also level of proteosynthesis [9].

This particular receptor is responsible for regulation of CYP1A and CYP1B expression [9].

Phenobarbital-type induction

There are many elements and factors cooperating in this type of induction. These include nuclear factor 1 (NF1) and nuclear receptor binding sites 1 and 2 (NR1, NR2). On DNA there is a sequence called phenobarbital-responsive enhancer module (PBREM) which can interact with heterodimer formed from the constitutive androstane receptor (CAR) and the retinoid X receptor (RXR) resulting in gene transcription. CAR and RXR are constitutively expressed receptors and their heterodimer in inactive form binds two endogenous androstane steroids. Displacement of these molecules leads to increase in activity of PBREM related genes [9].

Cytochrome subfamilies under this type of expression control are for example CYP1A, CYP2B, CYP2C and CYP3A. Of these CYP2B is most effectively induced [9].

1.3.4 Human cytochromes P450

There are extensive differences among cytochromes P450 in various species. To date, 57 cytochromes P450 divided into 18 CYP families have been identified in human genome. Of the 57, majority appears to be expressed primarily in the endoplasmic reticulum and only six are located exclusively in mitochondria [10]. In human, CYPs are mainly expressed in liver (major metabolising organ), lungs, gastrointestinal tract (e.g. small intestine) and kidney [4].

Since CYPs have many different catalytic activities, they are involved in steroidogenesis, metabolism of vitamins (A and D), eicosanoids, fatty acids and xenobiotics [9]. One particular CYP isoform usually catalyzes more reactions and thus CYP actions are overlapping. For example, xenobiotic metabolizing cytochromes are also involved in metabolism of fatty acids and steroids (oxidation).

Cytochromes involved in steroid metabolism are usually invariable among individuals in contrast to xenobiotic metabolising enzymes which vary considerably [9]. Xenobiotic metabolising enzymes are mainly of CYP families 1, 2 and 3.

In next chapters, two subfamilies (CYP1A and CYP2B), that are in particular interest of this work, will be described.

1.3.5 CYP1A subfamily

Known human members of this group are CYP1A1 and 1A2. The sequential homology in coding region of CYP1A1 and CYP1A2 proteins is ~70%. Prefered substrates for these isoforms are generally highly planar molecules. Because of its substrate specificity, CYP1A subfamily is associated with metabolic activation of many procarcinogens and mutagens, especially polycyclic aromatic hydrocarbons (CYP1A1) and heterocyclic amines (CYP1A2) [4].

Both these isoforms are inducible and, interestingly, inducers of these enzymes are typically their substrates. Beside liver, expression of CYP1A1 has been detected in many extrahepatic tissues, such as lung, kidney, gastrointestinal tract, placenta, skin [11], peripheral blood cells, thymes, prostate, uterus and mammary glands [9]. In contrary, CYP1A2 is constitutively expressed only in liver.

Orthologic isoforms in a rat (*Rattus norvegicus*) are also CYP1A1 and CYP1A2.

1.3.6 CYP2B subfamily

The only member of this family in a human is CYP2B6. This enzyme is involved in metabolism of many clinical drugs (e.g. cyclophosphamide, barbiturates) [4] and environmental chemicals [12]. Moreover, activation of organophosphates as well as some carcinogens (e.g. aflatoxin B1 [13]) is catalyzed by this enzyme.

CYP2B6 is constitutive and inducible, expressed in liver [4, 9, 11, 14], lung [9, 11] and gastrointestinal tract [11].

Orthologic isoforms of CYP2B6 are CYP2B1 and CYP2B in a rat (*Rattus norvegicus*), and CYP2B4 in a rabbit (*Leporidae*).

1.4 Flavonoids

1.4.1 Introduction

Flavonoids are naturally occurring compounds found in vegetables, nuts, fruits, medicinal plants and herbs (e.g. *Silybum marianum, Alpina officinarum, Hypericum perforatum*), and also in beverages (e.g. wine, beer, coffee). 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 system against harmful insects due to their astringency (e.g. catechins, flavanols) [15].

Structure

The chemical structure of flavonoids is based on flavane (Figure 1-7) which consists of 15 carbon atoms arranged in 3 cycles (C6-C3-C6) which are labelled A, B and C. The C ring is substituted with benzene ring (B ring) in position 2 (flavonoids), 3 (isoflavonoids) or 4 (synthetic flavonoids).

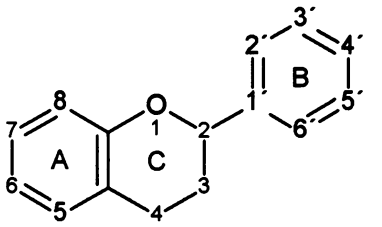
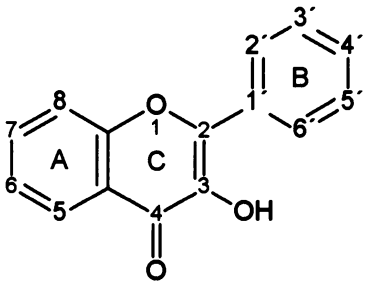


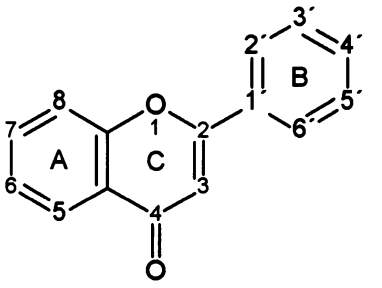
Figure 1-7 Structure of flavane.

Flavonoids can be divided into classes that differ in oxidation and substitution of C ring. These classes are: flavonols (Figure 1-8), flavones (Figure 1-9), flavanones (Figure 1-10), isoflavones (Figure 1-11), flavanols, flavanes, anthocyanidines, chalcones and flavonolignans [16, 17]. These days more than 8 000 compounds with flavonoid structure are known. The quantity of structures arises from different patterns of substitution on the skeleton (mainly by hydroxy, methoxy groups, and saccharides) [15]. In nature, flavonoids are usually linked to sugars (e.g. glucose, rhamnose, galactose) as β-glycosides (except for flavanols).



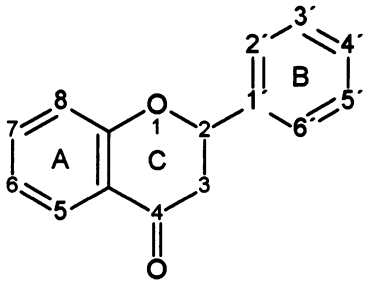
	3′	4′	3	5	6	7	8
Flavonol	H	H	OH	H	H	H	H
Quercetin	OH	OH	OH	OH	H	OH	H
Kaempferol	H	OH	OH	OH	H	OH	H
Rutin	OH	OH	rutinose	OH	H	OH	H

Figure 1-8 Flavonols.



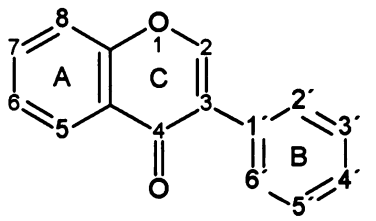
	3′	4′	3	5	6	7	8
Flavone	H	H	H	H	H	H	H
Apigenin	OH	H	H	OH	H	OH	H
Luteolin	OH	OH	H	OH	H	OH	H
Baicalein	H	H	H	OH	OH	OH	H
Baicalin	H	H	H	OH	OH	glucuronide	H
Diosmetin	OH	OCH_3	H	OH	H	OH	H
Diosmin	OH	OCH_3	H	OH	H	rutinose	H
Chrysin	H	H	H	OH	H	OH	H
Tangeretin	OH	OCH_3	H	OCH_3	OCH_3	OCH_3	OCH_3

Figure 1-9 Flavones.



	3′	4′	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

Figure 1-10 Flavanones.



	3′	4′	2	5	6	7	8
Daidzein	H	OH	H	H	H	OH	H
Genistein	H	OH	H	OH	H	OH	H
Glycitein	H	OH	H	H	OCH_3	OH	H
Biochanin A	H	OCH_3	H	OH	H	OH	H

Figure 1-11 Isoflavones.

Intake and bioavailability

Dietary intake of flavonoids has been estimated more than 25 years ago to be about 1 gram per day in USA [18]. Recent research in this area found out high variability in the intake among nationalities with different lifestyles. Nowadays, the flavonoid intake has been estimated to range from about 20 mg/day in USA to more than 70 mg/day in Holland [19]. In general, daily intake ranges from tens to hundreds of milligrams. The content of some flavonoids in food is shown in Table 1-1.

Table 1-1 Content of flavonoids in dietary sources [20].

flavonols	mg/kg fresh weight or mg/l
yellow onion	350-1200
curly kale	300-600
leek	30-225
cherry tomato	15-200
tomato	2-15
broccoli	40-100
apple	20-40
black tea infusion	30-45
green tea infusion	20-35
red wine	2-10
blueberry	30-160

flavones	mg/kg fresh weight or mg/l
parsley	240-1850
celery	20-140
flavanones	
orange juice	215-685
grapefruit juice	100-650
lemon juice	50-300
isoflavones	
soy flour	800-1800
soybeans boiled	200-900
soy milk	30-175

Beside the total intake, other important factors are composition of consumed flavonoids and their bioavailability. Composition differs with diverse fruit, vegetables and beverages eaten. Bioavailability means how many percents of orally ingested flavonoid are absorbed and can further act in organism. Normally, absorption does not exceed few percent of the ingested dose [15].

Therefore, flavonoids highly abundant in food might not be the most active in organism because they could be poorly absorbed, highly metabolized, rapidly eliminated, or have low intristic activity [20].

Metabolism of flavonoids

Most flavonoids are glycosylated in nature (except flavanols) and for a long period it was thought that glycosylated flavonoids cannot be absorbed because of their high polarity. So first step in metabolism was established to be hydrolysis by intestinal enzymes, colonic microflora and/or by enzymes in oral cavity [21].

In 90s Hollman investigated the metabolism of quercetin and its glycosides from different natural sources. In this case he indirectly discovered that some glycosides (glucosides) are also absorbed and, moreover, can be absorbed better than the aglycone (quercetin) itself [22-26]. However, in general, aglycones are better absorbed than their glycosides. The transport mechanism for glycosylated flavonoids in small intestine has been established to be sodium-dependent glucose transporter 1 [20, 25].

Interestingly, glycosylation itself does not influence further metabolic pathway and circulating metabolites. Passing through the organism majority of flavonoids resist the acidic environment in stomach and are moved unchanged to small intestine (duodenum). In small intestine only aglycones and some glucosides can be absorbed. After absorption of glucosides hydrolysis in cells can occur, for example by cytosolic *β*-glucosidases or lactase phlorizin hydrolase [20, 24].

Other glycosides (e.g. with rhamnose) pass through small intestine, are hydrolyzed by colonic microflora and then absorbed. Moreover, intestinal and colonic microflora can further degradate aglycones to aromatic acids, etc. Because of smaller exchange area and lower density of transport systems in colon, it is generally considered that flavonoids deglycosylated in colon are absorbed slowly and less efficiently. Due to this, phenolic compounds can reach the highest concentrations in colon among other dietary antioxidants (e.g. vitamins C and E that are absorbed in upper segments of small intestine) [20].

Once absorbed, partly metabolized or unchanged flavonoids are subjected to conjugation reactions (glucuronidation, sulphation, and methylation). Methylation occurs predominantly in 3' position and is catalyzed by catechol-*O*-methyltransferase which is present mainly in liver and kidney. Another two types of conjugation (catalyzed by sulphotransferases and UDP-glucuronosyltransferases) take place in liver. The proportion of these reactions varies according to the nature of compound and the dose ingested. Although, in general, the main circulating conjugates are glucuronides [20, 24].

Elimination of flavonoids and their metabolites occurs by two pathways, via the biliary or the urinary route (Figure 1-12). Large conjugates are more likely eliminated in bile, while small conjugates are excreted in urine [20]. Circulating in organism, flavonoids are usually bound to plasma proteins, mainly albumin, depending on their chemical structure [20]. For example quercetin plasma binding exceeds 99%. Excretion by urinary route correlates with plasma concentrations of parent compounds and their metabolites. Conjugated metabolites from bile can be cleaved by *β*-glucuronidases from intestinal microflora and the aglycones can be reabsorbed. This results in enterohepatic cycling and longer persistence of particular flavonoid or its metabolites in organism. Possible routes of flavonoids in organism are shown in Figure 1-12.

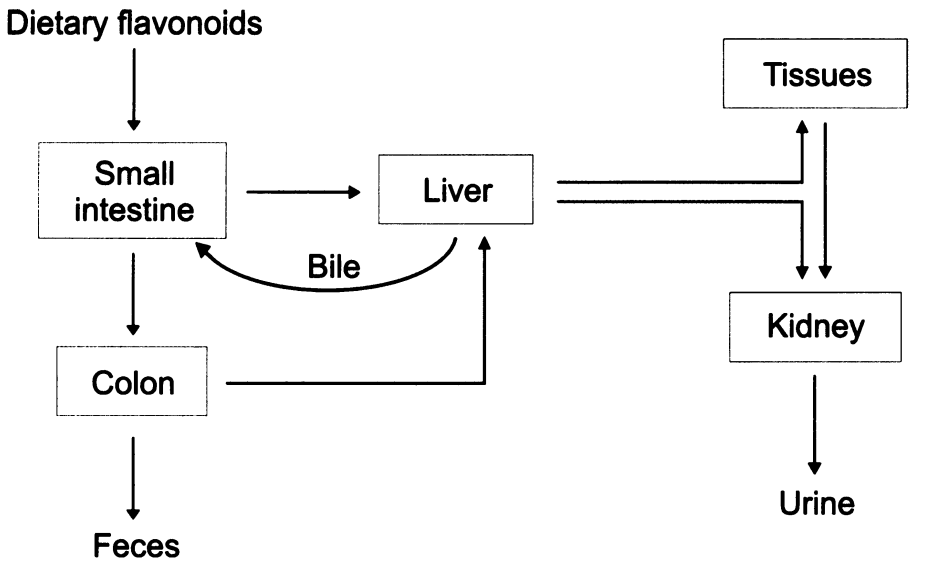


Figure 1-12 Possible routes of dietary flavonoids in humans (adapted and modified from [24]).

Biological activities

Consumption of flavonoids and phenolic compounds has been associated with lower incidence and thus prevention of diseases long time ago. Generally, flavonoids exert antibacterial, antiviral [27], antiinflammatory, antioxidant, antiangionic, analgesic, antiallergic, hepatoprotective, cytostatic, apoptotic, estrogenic and antiestrogenic properties [16]. Due to their antioxidant properties they help to protect DNA, membranes (lipids) and proteins from oxidative stress and associated diseases (e.g. cancer, cardiovascular and neurodegenerative diseases, diabetes, and osteoporosis [28]). Their antioxidative properties arise from their ability (1) to reduce free radicals formation by inhibition of enzymes or chelating of trace elements involved in free radical production, (2) to scavenge free radicals, and (3) to upregulate or protect antioxidative defences [29].

On the other hand, flavonoids exert also some negative properties, such as mutagenic (e.g. quercetin), cytotoxic [30, 31], and pro-oxidative [28, 32, 33]. There is also possible risk of iron deficiency related to flavonoids with catechol structure because of their ability to chelate iron ions in gut [28].

Majority of studies focused on flavonoids are interested in their anti-cancer activities. These arise from their antioxidant properties, abilities to modulate enzymes involved in metabolism of carcinogens [17, 28], stimulate DNA repair, inhibit cell proliferation, induce apoptosis in tumor cells, and inhibit angiogenesis [28]. However, different and sometimes contradictionary effects have been obtained by administration of

varied doses of flavonoids (or in general, phenolic compounds) under various conditions [28].

1.4.2 Flavonols

Flavonols are present in food in higher amounts than other classes of flavonoids. The most abundant are quercetin and kaempferol. Main dietary sources are onions, curly kale, leeks, broccoli, berries, apples, tea and red wine [20, 23]. The highest concentration is present in outer parts of plants because their biosynthesis is stimulated by light. Flavonols mainly occur as glycosides with sugar moiety (usually glucose or rhamnose) and usually 5-10 different glycosides are found in fruit [20].

Rutin

Rutin (quercetin-3-rutinoside, Figure 1-13) is a citrus flavonol present in buckwheat, apricots, cherries, prunes, rose hips, and in other grains (rice, wheat, beans, etc.).

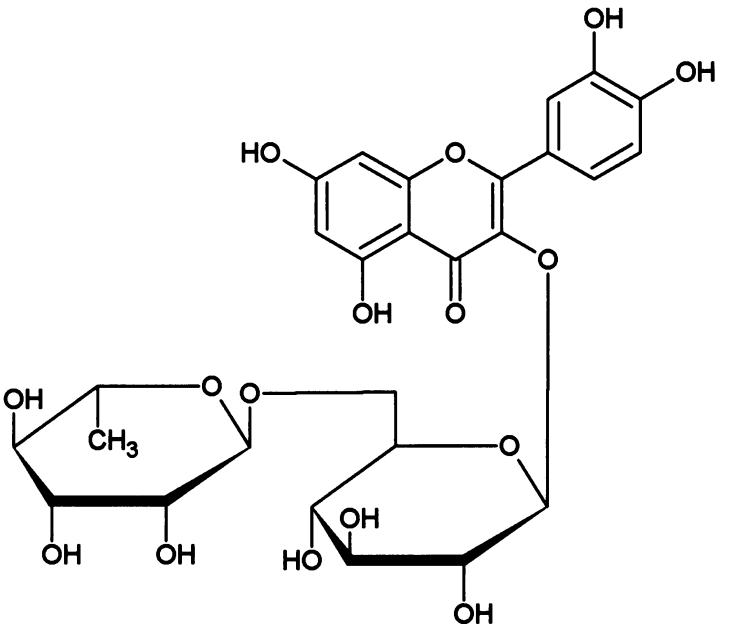


Figure 1-13 Structure of rutin.

This compound exerts antioxidant properties with potential to stabilize vitamin C and therefore is used in dietary supplements (vitamins) together with vitamin C to intensify the activity of ascorbic acid. Rutin is also capable of chelating metal ions such as iron thereby reducing oxygen radicals production. In addition, rutin exerts antiinflammatory effects and is thought to have overall preventive and healing effects including anticancer

activities. It is used in human medicine in dietary supplements for prevention of diseases due to its ability to support the circulatory system, treat chronic venous insufficiency, glaucoma, hay fever, hemorrhoids, oral herpes, cirrhosis, etc. [34].

The study of Volate et al. [35] focused on chemopreventive properties of selected compounds did not observe significant effect of rutin influencing azoxymethane induced carcinogenesis in colon.

Morin

Morin (3,5,7,2',4'-pentahydroxyflavone, Figure 1-14) is present in many fruits and vegetables, e.g. mulberries, figs, and other *Moraceae*. Morin acts as antioxidant, xanthine oxidase inhibitor, protein kinase C inhibitor, cell proliferation inhibitor and apoptosis inducer [36, 37]. Moreover, antiinflammatory (inhibition of lipooxygenase and cyclooxygenase activities in the arachidonic cascade), anticarcinogenic and antimutagenic properties were reported [38].

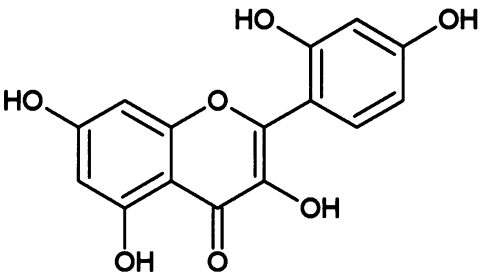


Figure 1-14 Structure of morin.

In toxicity study performed by Cho et al. [39] for 13 weeks in rats fed with diet containing 0%, 0.625%, 1.25%, 2.5% or 5.0% morin, no mortality or abnormal clinical signs were observed. However, a number of substance-related changes were observed in organ weights and parameters in hematology, serum biochemistry and histopathology.

Morin was effective to protect liver from damage caused by *N*-nitrosodiethylamine while coadministrated in long term study (16 weeks) in rats [37]. The hepatoprotective optimal dosage was found to be 500 ppm (contained in diet). Morin was also shown to lower high blood pressure in high-fructose induced hypertensis in rats [40].

On the other hand, morin as well as naringenin increased the level of lipid peroxidation and DNA strand breaks occurance in isolated rat liver nuclei [41].

1.4.3 Flavones

Flavones are less common in fruit and vegetables than flavanols and mainly occur as glycosides of luteolin and apigenin. The only edible sources are parsley, celery, cereals and skin of citrus fruits [20, 23].

Flavone

Flavone (2-phenylchromone, Figure 1-15) represents the most basic structure of flavones. Flavone acts as antioxidant and exerts antiinflammatory activities, mainly by cyclooxygenase inhibition [42].

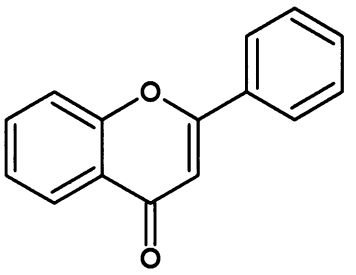


Figure 1-15 Structure of flavone.

Flavone treatment induced CYP1A1, CYP1A2 and CYP2B1/2 protein levels and activities *in vivo* in rat liver microsomes in dose-dependent manner [43-45]. The induction of CYP1A occurred as soon as 6 hours after first premedication while CYP2B was induced after a lag period [46]. Canivenc-Lavier et al. [45] observed even higher induction of CYP2B activity in rat liver microsomes after flavone premedication than after phenobarbital premedication. On the other hand, *in vitro* experiments with microsomal fractions determined flavone as CYP1A inhibitor [47].

Flavone also modulates activities and expression of phase II enzymes. Induction of glutathione-*S*-transferase and UDP-glucuronyltransferase was observed in rat liver [45, 46].

Baicalin

Baicalin (baicalein-7-*O*-glucuronide, Figure 1-16) and its aglycone baicalein are the main flavonoids of *Scutellaria baicalensis* Georgi root. This herb is used in traditional Chinese medicine as antiinflammatory agent and smooth muscle relaxant [48]. Moreover, baicalein exerts antibacterial, antiviral, antiallergic, antioxidant, and antigenotoxic effects [49]. Both baicalein and baicalin were found to impede reactive oxygen species production

and inhibit myeloperoxidase in human leukocytes [50]. The ability of baicalin and baicalein to scavenge free radicals and antioxidant properties were examined by Gao et al. [51] and Hamada et al. [52].

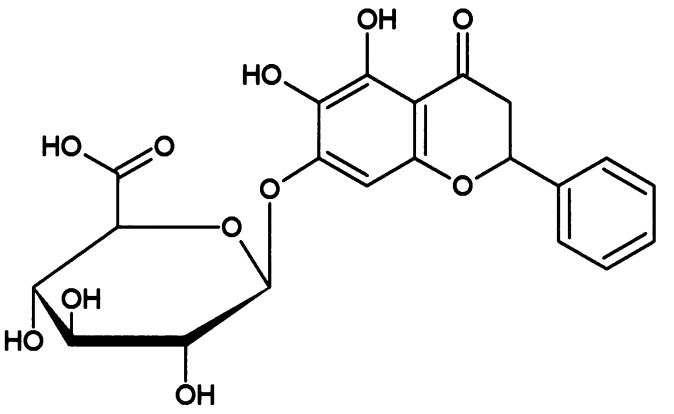


Figure 1-16 Structure of baicalin.

Baicalin induced selectively CYP1A1, CYP2B1 and CYP2C11 activities and protein levels *in vivo* in mouse liver [53]. *In vitro*, baicalien induced CYP1A1 and AhR protein levels in HepG2 and MCF-7 cells [54]. Moreover, baicalein reduced benzo(a)pyrene and aflatoxin B1 toxicities *in vitro* in *Salmonella typhimurium* and liver microsomes, and *in vivo* in mice. Stronger protective effect was observed *in vivo* [55].

Baicalin was found to be cytotoxic in leukemia-derived T cell line (Jurkat cells) increasing reactive oxygen species generation (prooxidant activity) and inducing caspase-3 activation and apoptosis via mitochondrial pathway [56].

1.4.4 Flavanones

Dietary flavanones occur mainly in citrus fruit as glycosides of naringenin (grapefruits), hesperetin (oranges) and eriodictyol (lemons). Other edible sources are tomatoes and aromatic plants (such as mint).

Flavanone

Flavanone (2,3-dihydroflavone, Figure 1-17) represents the most basic structure of flavanones. Its structure differs from flavone only by one double bond between C2 and C3.

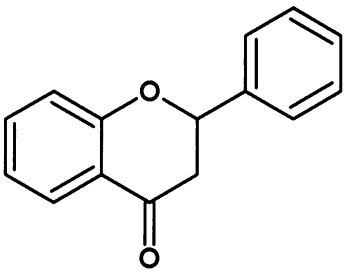


Figure 1-17 Structure of flavanone.

7-pentoxyresorufin-*O*-dealkylase activity was highly induced by flavanone premedication in rat liver microsomes [43-46]. However, in mouse liver microsomes this activity has not been altered [57]. Moreover, flavanone induced CYP1A2 protein level in rat liver microsomes although the 7-methoxyresorufin-*O*-demethylase activity was decreased [44]. On the other hand, flavanone acts *in vitro* as potent inhibitor of CYP1A subfamily [47].

Activities of phase II enzymes in rat liver, especially glutathione-*S*-transferase and UDP-glucuronyltransferase, increased after flavanone premedication [43, 45, 46].

Hesperidin, hesperetin

Hesperidin (hesperetin-7-rutinoside, hesperetin-7-rhamnoglucoside, Figure 1-18) and its aglycone hesperetin (3',5,7-trihydroxy-4-methoxyflavanone) are citrus flavanones present mainly in orange (*Citrus sinensis*).

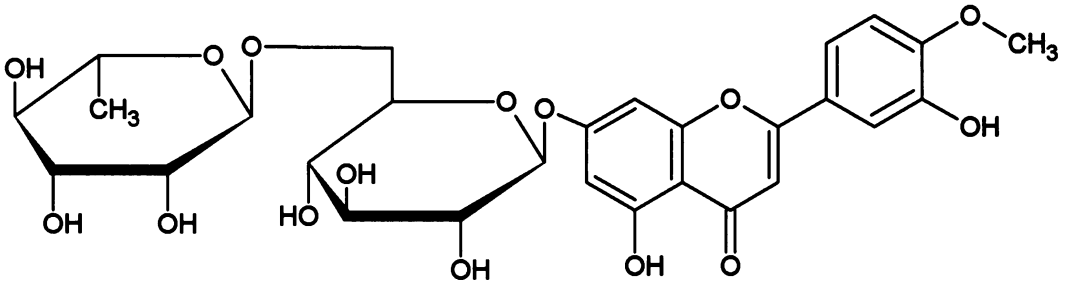


Figure 1-18 Structure of hesperidin.

Hesperidin is contained in drug product Daflon which consists of 10% hesperidin and 90% diosmin. This drug is commercially used for chronic venous insufficiency and moreover exerts antiinflammatory properties due to its constituents [58].

Doostdar et al. [59] demonstrated that hesperetin is the specific substrate and potent inhibitor of CYP1A1 and CYP1B1. These isoforms participate on metabolism of hesperetin catalyzing its demethylation.

Naringin, naringenin

Naringin (naringenin-7-rutinoside, naringenin-7-rhamnoglucoside, Figure 1-19) and its aglycone naringenin (4',5,7-trihydroxyflavanone) are citrus flavanones present mainly in grapefruit (*Citrus paradisi*). Rutinose bound to naringenin is responsible for a bitter taste of naringin. These flavanones can reach up to 10% of dry weight of grapefruits and are present in grapefruit juice >200 mg/l [60].

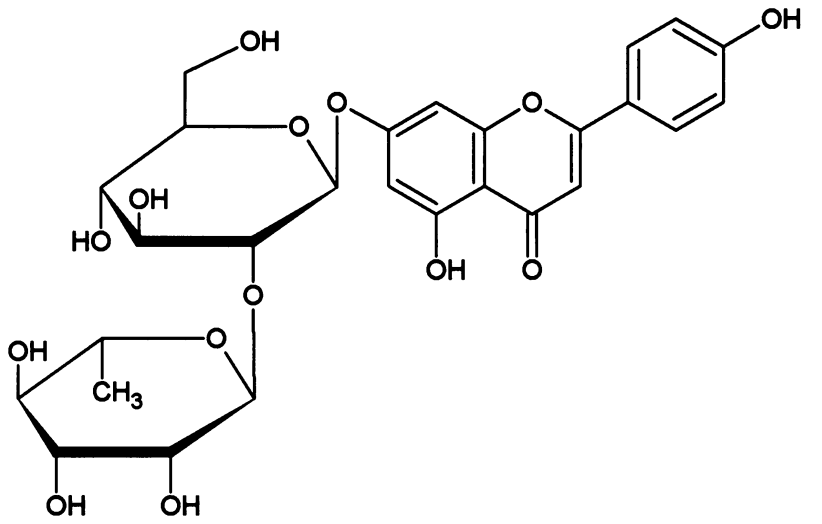


Figure 1-19 Structure of naringin.

Naringenin was found to be very poor inhibitor of human CYP1A EROD activity (IC_{50}>4 mM) [59]. As well as naringenin, naringin also decreased EROD activity by 49% and CYP1A2 protein levels in mouse liver [61]. Also other cytochrome P450 activities (MROD and PROD) were inhibited by naringenin [62].

Naringenin induced lipid peroxidation and DNA damage in isolated rat liver nuclei demonstrating its prooxidant properties [41].

1.4.5 Isoflavones

Isoflavones are almost exclusively present in leguminous plants (e.g. soy). The most abundant are genistein, daidzein and glycitein. Due to their structural similarities with estrogens and pseudohormonal properties they are sometimes called phytoestrogens. On the other hand, negative effects of isoflavones causing reproductive dysfunction in rats and male mice were reported [63]. This group also exerts antioxidant properties.

Biochanin A

Biochanin A (Figure 1-20) is found mainly in red clover (*Trifolium pratense*) and also contained in many herbal dietary supplements due to its pseudohormonal properties (in treatment of menopausal symptoms) [64]. Antiproliferative activity of biochanin A was observed in human cancer cell lines established from gastrointestinal tract in low concentrations (<20μg/ml) [65]. At higher concentrations (>40μg/ml) it was found to be cytotoxic causing DNA fragmentation indicating the apoptotic pathway.

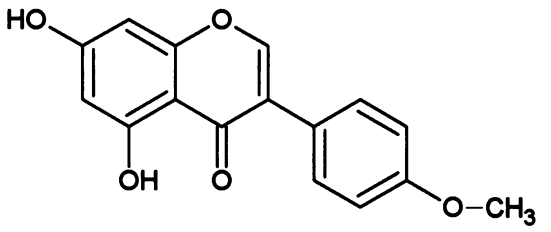


Figure 1-20 Structure of biochanin A.

Biochanin A is metabolized after absorption by *O*-demethylation to genistein. This reaction was thought to be catalyzed by gut microflora but another study [63] determined liver enzymes to be involved (e.g. CYP1A2, CYP2A6, CYP2E1, and CYP2D6*1). Hu et al. [66] found that CYP1A2 is responsible of biochanin A *O*-demethylation in human liver microsomes. Mallis et al. [67] determined the enterohepatic reuptake of soy-derived isoflavones after oral dosing in rats. The concentration of biochanin A was found to be higher than its metabolite (genistein) in plasma for more than 20 hours after per oral administration in rats [68].

Moon et al [64] investigated the effect of biochanin A treatment on gene expression of drug-metabolizing enzymes in primary human hepatocytes and human intestinal cells. In liver tissue, expression of all *CYP2* genes (CYP2A6, CYP2B6, CYP2C9 and CYP2F1) was up-regulated. Induction of CYP1A1 activity and mRNA accumulation was observed by Han et al. [69] after biochanin A pretreatment in MCF-7 cell line. Biochanin A was found to be substrate for this enzyme also competing for AhR binding.

Biochanin A was determined to inhibit benzo(a)pyrene metabolism and DNA binding in hamster embryonic cell line [70]. Thereby Lee at el. [71] investigated the influence of biochanin A on benzo(a)pyrene induced lung tumor incidence in mice. Treatment with biochanin A after benzo(a)pyrene premedication reduced lung tumor incidence by 35%.

1.4.6 Other used phenolic compounds

Phenolic compounds are based on structure containing benzene ring substituted with hydroxyl group(s) and are widely distributed in nature. Lower incidence of cancer in population with higher intake of vegetables and fruits is usually refered to phenolic compounds content. Apart from stilbenes and lignans, phenolic acids and flavonoids are two main groups of phenolic compounds. Flavonoids were discussed in chapters above.

Resveratrol

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, Figure 1-21) belongs to stilbenes and is the most abundant above others in human diet. It is present in mulberries, peanuts, grapes and red wine. The content of resveratrol in red wine spannes from 1.5 to 3 mg/l [72]. Coexistence of high-fat diet with low incidence of cardiovascular diseases in France usually called French paradox is related to moderate red wine consumption (150-300 ml/day) [72].

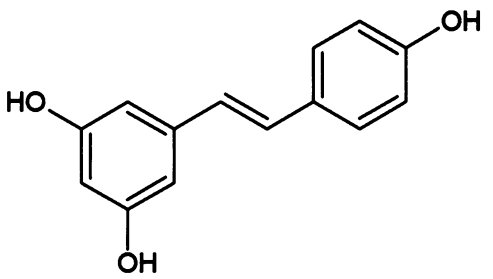


Figure 1-21 Structure of resveratrol.

Resveratrol also exerts antioxidant, cardioprotective, antiinflammatory (e.g. inhibition of COX-2), anticoagulative and anticarcinogenic properties (e.g. induction of phase II enzymes) [73].

In association to cytochrome P450 resveratrol was found to be AhR antagonist and is able to block AhR ligand-mediated increased expression of CYP1A1 [74, 75]. Resveratrol was also shown to be an irreversible inhibitor for CYP3A4 and a noncompetitive reversible inhibitor for CYP2E1 [72]. Moreover, cytochromes P450 (e.g. CYP1A1, CYP1A2 and CYP1B1) participate on resveratrol metabolism [76].

1.5 Organosulphur compounds

Organosulphur compounds are present mainly in garlic (*Allium sativum*) and onion (*Allium cepa*). These compounds can be divided into water-soluble (e.g. *S*-methylcysteine, *S*-allylcysteine) and oil-soluble (e.g. diallyl sulphide). Alliin (*S*-allylcysteine sulphoxide) and allicin (diallyl thiosulphinate) are two most important organosulphur compounds present in garlic. Alliin is formed from its precursors, γ-glutamyl-S-alk(en)yl-L-cysteines, the prime compounds present in garlic and is further metabolized by allinase (enzyme released from garlic cells by crushing or slicing), cooking or metabolism in animals to allicin and other thiosulphinates. They decompose and form many oil-soluble organosulphur compounds including diallyl sulphide, diallyl disulphide, etc. [77, 78]. The quantity of some volatile organosulphur compounds present in garlic are shown in Table 1-2 (data were obtained by water or steam distillation with solvent extraction; reviewed in [79, 80]).

Table 1-2 Quantity of some volatile organosulphur compounds present in garlic (reviewed in [79, 80]).

compound	µg/g
Diallyl sulphide	30–100
Diallyl disulphide	530–610
Diallyl trisulphide	900–1100
Allyl methyl sulphide	3.8–4.6
Allyl methyl disulphide	100
Allyl methyl trisulphide	250–270
Dimethyl disulphide	2.4–2.5
Dimethyl trisulphide	15–19
Propyl methyl disulphide	0.7–0.8

Members of both groups (water and oil soluble) are known as chemopreventive since they interfere with the process of carcinogenesis [79, 80]. Proposed mechanism explaining this chemopreventive activity include inhibition of activation enzymes (phase I), induction of detoxifying enzymes (phase II), scavenging ultimate electrophilic carcinogenic species by the sulphur atom [77], blockage of *N*-nitroso compounds formation (OSC form nitrosothiols from nitrite, one of the substrates for endogenous formation of *N*-nitroso compounds), enhancement of DNA repair system, reduction of cell proliferation and induction of apoptosis [77, 78, 81].

1.5.1 Diallyl sulphide

Diallyl sulphide (DAS, Figure 1-22) is exclusively present in garlic, and undergoes extensive oxidations on few positions of the molecule after ingestion in organism. Oxidation of sulphur atom is partly mediated by CYP2E1 producing diallyl sulphoxide (DASO) and diallyl sulphone ($DASO_2$) sequentially [82]. The last step, oxidation of terminal double bonds of $DASO_2$ leads to autocatalytic destruction of the enzyme. The first two metabolites are competitive inhibitors of CYP2E1 but not suicidal inhibitors [82]. Because of these inhibitory effects, DAS acts against cancer caused by carcinogens activated by CYP2E1, e.g. *N*-nitroso compounds [82-84].

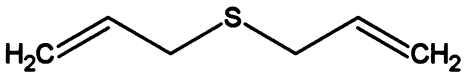


Figure 1-22 Structure of diallyl sulphide.

On the other hand, DAS is known to be phenobarbital type inducer thus inducing CYP2B subfamily. Extensive research was done *in vivo* and *in vitro* investigating the effect of DAS in dose and time-dependent manner on the level of CYP2B mRNA [85, 86], protein [82, 83] and activity [83, 86]. There are also few studies showing the ability of DAS to induce other isoforms of CYP *in vivo* in rat and *in vitro* (namely CYP1A1, and 3A2).

Davenport and Wargovich [87] investigated time and dose-dependent manner of CYP1A1/2 and CYP3A2 induction mediated by DAS. This study was carried out *in vivo* in F344 male rats and DAS was administered by gavage (per oral) dissolved in corn oil. Interestingly, high induction of CYP1A1 was ascertained after either single dose or prolonged administration of DAS. Moreover, after 8 weeks treatment with 200 mg/ml dosage, hepatotoxicity in liver was observed. The levels of CYP3A2 were not influenced by single dose of DAS but after week of administration, the induction pattern was obvious.

1.6 Curcumin

Curcumin (Figure 1-23), yellow pigment and dietary component derived from the root of *Curcuma longa L.* (major component of turmeric spice), exerts antioxidative, antiinflammatory and antiseptic properties. Moreover, curcumin interferes with many steps of carcinogenesis (inhibits tumour formation and promotion and has ability to induce apoptosis of cancer cells while healthy cells are not affected) and therefore is designated as

chemopreventive and anticarcinogenic agent. Recently, all beneficial effects of curcumin on human health were reviewed [1, 88]. On the other hand, there are some studies showing possible negative activities of curcumin. For example curcumin in the presence of CYPs (that can catalyze curcumin *O*-demethylation, e.g. 2D6, 1A1, 1A2) and copper ions cause DNA damage [89].

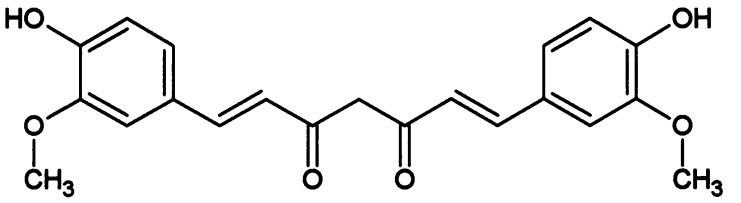


Figure 1-23 Structure of curcumin (diferuloylmethane).

High doses of curcumin (up to 12 grams per day) have been used in clinical trials. Comprise from data obtained, safe daily dose has been established up to 8 grams per day in long-term administration [1, 90]. From another studies the concentration of curcumin in intestinal lumen after ingestion of 1 gram was determined to be as high as 270 μmol/l [91].

Curcumin and its derivatives (demethoxycurcumin and bis-demethoxycurcumin) inhibited CYP1A1/2 activities *in vitro* in rat liver microsomes at lower concentrations than isothiocyanates and CYP2B1 at comparable concentrations. Due to this curcumin and its derivatives significantly inhibited activation of BaP [92, 93] and decreased the number of benzo(a)pyrene and DMBA-derived DNA adducts [93]. Moreover, curcumin was shown to inhibit aflatoxin B_1-DNA adducts formation *in vitro* by 50% [94]. This could be contributed to inhibition of CYP1A subfamily, although these data are inconsistent. Curcumin binds to AhR [95] resulting in increased CYP1A activity [96] but in another study curcumin pretreatment decreased CYP1A mRNA levels in Caco-2 cells 3 to 6-fold in time-dependent manner [91].

Generally, the influence of curcumin on cytochrome P450 activities and protein levels seems to be dose-dependent. Diet containing normal doses of curcumin (0.05-0.2%, or 0.05-0.5 g/kg) does not influence CYP1A and CYP2B protein levels [97] and activities [98] in rat liver after long-term premedication.

2 Aim

In general, this work is focused on possible negative interactions between chemopreventive compounds and food carcinogens mediated by cytochromes P450. Consequently, the modulatory effect of selected chemopreventive compounds on CYP had to be examined. To solve these tasks some specific aims had to be resolved:

- Selection of chemopreventive compounds which are possible CYP1A1, CYP1A2 and CYP2B1/2 inducers.
- Preparation of liver and intestinal microsomal fractions from rats after per oral treatment with selected chemopreventive compounds.
- Characterization of isolated microsomal fractions for CYP and protein content.
- Detection of CYP1A and CYP2B protein levels in microsomal samples by Western blotting analysis using chicken anti-rat 1A1 and chicken anti-rabbit 2B4 antibodies.
- Assay of CYP1A1, CYP1A2 and CYP2B1/2 specific activities, 7-ethoxyresorufin-*O*-deethylase, 7-methoxyresorufin-*O*-demethylase and 7-pentoxyresorufin-*O*-dealkylase, respectively.

3 Materials and Methods

3.1 Materials

Chemicals were used from:

Fluka, Switzerland

tris(hydroxymethyl)-aminomethane (Tris), 2-mercaptoethanol, methanol, sodium dodecyl sulphate, α-tocopherol

Linde, Czech Republic

liquid nitrogen, dry ice

Millipore Corp., USA

Immobilon-P transfer membrane

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

Setuza a.s., Czech Republic

Vegetol – sunflower oil

Sigma Aldrich, USA

β-naphthoflavone, flavanone, morin, rutin hydrate, biochanin A, diallyl sulphide, curcumin, 7-ethoxyresorufin, 7-methoxyresorufin, 7-pentoxyresorufin, resorufin, nicotinamide adenine dinucleotide phosphate (NADPH), SigmaMarker Wide Range (6 500 - 205 000), bicinchoninic acid (BCA), anti-chicken IgG-alkaline phosphatase conjugate, BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) Sigma*FAST* tablets

Anlab, Czech Republic

male Wistar rats (150 g)

Whatman, USA

Whatman paper, No.3

Perkin Elmer, USA

96-well plates for fluorescence measurement

Antibodies against rat CYP1A and rabbit CYP2B4 were kindly provided by Doc. RNDr. Petr Hodek, CSc., Faculty of Science, Charles University in Prague.

Other used **microsomal fractions** were isolated by Kateřina Fridrichová [99] (control and α-naphthoflavone treated rats in 2004 and 2005, respectively) and Jitka Křížková [100] (rats treated with β-naphthoflavone, baicalin, flavone, hesperidin, hesperetin, naringin, naringenin, and resveratrol in 2005). Characterizations of these microsomal fractions are shown in Tables 3-1 and 3-2.

Table 3-1 Characterization of liver microsomal fractions.

liver	$c_{protein}$ [mg/ml]	c_{CYP} [μM]	s.c. CYP [nmol/mg]	reference
α-naphthoflavone	35.5	19.9	0.56	[99]
β-naphthoflavone	19.6	8.1	0.41	[100]
Baicalin	26.5	17.9	0.68	[100]
Flavone	44.8	32.5	0.73	[100]
Hesperidin	34.8	15.4	0.44	[100]
Hesperetin	41.4	18.6	0.45	[100]
Naringenin	18.3	8.6	0.47	[100]
Naringin	53.4	22.2	0.42	[100]
Resveratrol	19.8	17.9	0.91	[100]

Table 3-2 Characterization of proximal part intestinal microsomal fractions.

proximal part	$c_{protein}$ [mg/ml]	c_{CYP} [μM]	reference
β-naphthoflavone	11.3	n.d.	[100]
Baicalin	7.1	n.d.	[100]
Flavone	8.9	n.d.	[100]
Hesperidin	19.6	n.d.	[100]
Hesperetin	9.3	n.d.	[100]
Naringenin	6.8	n.d.	[100]
Naringin	21.3	n.d.	[100]
Resveratrol	5.3	n.d.	[100]

n.d. – not detectable

Sample of **liver microsomal fraction from phenobarbital treated rats** was kindly provided by RNDr. Věra Kotrbová, Faculty of Science, Charles University in Prague

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 HoeferTM miniVE, USA

Luminescence spectrophotometer

PerkinElmer LS55, USA

Spectrophotometers

Hewlett Packard E8453, USA; SpektroMOM 195 D, Hungary; Specord Carl Zeiss Jena M40, 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 Elmasonic E30H Elma, Germany; vortex MS 1 Minishaker, Germany; shaker IKA VX 2 Janke & Kunkel, Germany; water bath Julabo TW 8, Germany; heatable magnetic stirrer Variomag Monotherm, Germany; electrophoresis power supply EPS 301 Amersham Pharmacia biotech, USA

3.3 Methods

3.3.1 Premedication

Male Wistar rats (about 150 g) in groups of 4-5 were acclimatized for 5 days. They were maintained on *ad libitum* diet. Flavonoids (flavanone, morin, rutin, β-naphthoflavone, biochanin A), diallyl sulphide and curcumin were dissolved in sunflower oil and administered by gavage *p.o.* 60 mg/kg body weight for 5 consecutive days. The control group was treated only with 1 ml of sunflower oil. The treated rats were fasted overnight and sacrificed.

3.3.2 Isolation of microsomal fractions

Buffer B1: 0.15 M KCl, 0.05 M Tris; pH 7.4; tocopherol (23.5 mg of tocopherol dissolved in 0.5 ml of methanol, added just before use)

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

Buffer B3: 0.12 M KCl, 0.04 M Tris, 20% (v/v) glycerol; pH 7.4

Rats were sacrificed and whole liver and intestinal parts (proximal part - circa 2 cm under stomach, following middle part and distal part, each in a total length about 20 cm) were removed. Liver and proximal intestinal parts were stored on ice for immediate use. Other intestinal parts (middle and distal) were deep frozen (-80°C) until use. Fractional centrifugation method was used for microsomal fraction isolation [101]. All processes were carried out at temperature 4°C.

Liver and colon tissues were washed in B1 buffer, weighed, cut into pieces by scissors and homogenized in about 4 volumes (B1) of tissue mass in Potter-Elvehjem glass homogenizer. The homogenates were than centrifuged at 600 g for 10 minutes at temperature 0-5°C (centrifuge Janetzki K-23, swing-out rotor 4x70 ml, 2 000 RPM). The supernatant was kept on ice in freezer and the pellet was rehomogenized in one fourth volume (B1) of tissue mass and centrifuged once again under the same conditions.

The supernatants were then put together and centrifuged at 15 000 g for 20 minutes at 0-5°C (centrifuge Janetzki K-24, fixed-angle rotor 6x35 ml, 13 500 RPM). The resulting supernatants were separated carefully and ultracentrifuged at 123 000 g for 90 minutes at 4°C (ultracentrifuge Beckman, fixed-angle rotor Ti45, 6x64 ml, 35 000 RPM).

The supernatants were poured off and the pellets were rehomogenized in 2 volumes (B2) of tissue mass and ultracentrifuged at 425 000 g for 90 minutes at 4°C (ultracentrifuge Beckman, fixed-angle rotor Ti70, 8x26 ml, 60 000 RPM). The supernatants were poured off and the resulting pellets were rehomogenized in one fifth volume (B3) of original tissue mass, aliquoted and stored at -80°C until use.

3.3.3 Bicinchoninic acid protein assay

Protein concentration in microsomal fractions was determined by bicinchoninic acid assay [102]. In this assay, Cu^{2+} from cupric sulphate is reduced to Cu^{+} by peptide bonds at high temperature (60°C). Then Cu^{+} ions are chelated by bicinchoninic acid in alkaline environment (pH 11.25) forming violet complex that has absorption maxima at 562 nm. Colour intensity of sample is proportional to protein concentration. Calibration was performed using dilutions of a stock solution of bovine serum albumin (BSA).

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 (added before use)
pH 11.25
Reagent B: 4% (w/v) $CuSO_4.5H_2O$

The working solution was prepared by mixing 49 parts of reagent A with 1 part of reagent B. All tubes and beakers used were reannealed.

Standards for calibration were prepared by dilution of BSA stock solution (2 mg/ml) with working reagent to concentrations from 1 µg/ml to 20 µg/ml. Blank contained 20 µl of distilled water and 980 µl of working reagent.

Microsomal fractions from rat colon and liver were diluted by 50 times and 100 times, respectively. Samples were prepared by mixing 20 µl of diluted fractions and 980 µl of working reagent.

All prepared samples, standards and blank were vortexed and incubated in water bath at 60°C for 60 minutes.

After incubation, absorbance at 562 nm was measured in cuvettes against blank (SpectroMOM 195D). Protein concentration was calculated from the calibration curve obtained.

3.3.4 Cytochrome P450 concentration assay

The concentration of cytochrome P450 was determined by differential spectroscopy described by Omura and Sato [103, 104]. The reduced form of cytochrome P450 with bound carbon monoxide has absorption peak at 450 nm.

Buffer used: 0.1 M KH_2PO_4, 20% (v/v) glycerol, pH 7.4

Microsomal samples were diluted 20 times in buffer and small amount (circa 3 mg) of $Na_2S_2O_4$ was added. This solution was gently mixed, divided into two cuvettes and one of them was bubbled with CO for about 60 seconds. Difference spectra were measured within range of 401-490 nm. The CYP concentrations were calculated from equation:

$$c_{CYP} = \frac{A_{450} - A_{490}}{\varepsilon_{450} - \varepsilon_{490}} \cdot dilution,$$

where A_{450} and A_{490} are absorbances measured at 450 nm and 490 nm, respectively, and ε_{450} and ε_{490} are molar absorption coefficients of CYP at 450 nm and 490 nm, respectively (the difference is 0.091 $cm^{-1}.\mu mol^{-1}.dm^3$).

3.3.5 Specific cytochrome P450 content

The specific content of cytochrome P450 (s.c.CYP) was calculated using CYP (c_{CYP}) and protein ($c_{protein}$) concentrations from equation:

$$s.c.CYP = \frac{c_{CYP}}{c_{protein}}.$$

3.3.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Electrophoresis is method used for separation of molecules in electric field. In this type polyacrylamide gel is used as a support medium and sodium dodecyl sulphate (SDS) as anionic detergent giving proteins uniform negative charge. Thus the migration of proteins is not determined by intrinsic electrical charge but by molecular weight [105].

Buffer A: 0.375 M Tris; pH 8.8; 0.1% (w/v) SDS

Polymerization solution A: 30% (w/v) acrylamide, 0.8% (w/v) BIS, in buffer A

Buffer B: 0.125 M Tris; 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, in buffer B

Sample buffer: 0.063 M Tris, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.003% (w/v) bromphenol blue; pH 6.8

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

Staining bath: 1.25 g Coomassie brilliant blue R-250, 270 ml ethanol, 45 ml acetic acid, distilled water to 500 ml

Destaining bath: 500 ml ethanol, 200 ml acetic acid, distilled water to 2 l

SigmaMarker Wide Range (Figure 3-1)

Resolving gel 7.5%: 12 ml buffer A, 4 ml polymerization solution A, 10 µl TEMED, 3 mg sodium peroxodisulphate

Stacking gel 4%: 9 ml buffer B, 1.4 ml polymerization solution B, 10 µl TEMED, 3 mg sodium peroxodisulphate

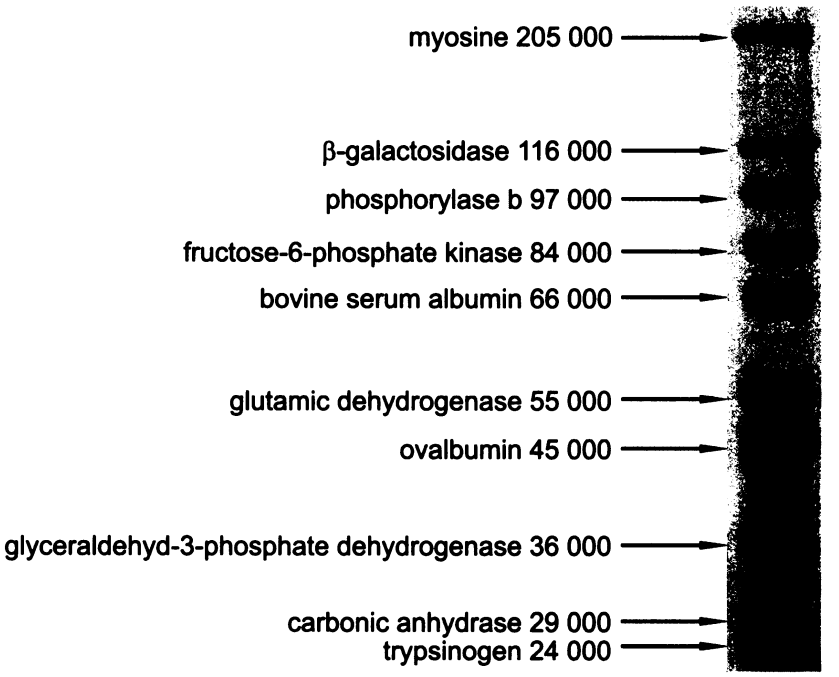


Figure 3-1 SigmaMarker Wide range separated on 7.5% gel by constant tension 110 V; numbers mean relative molecular weights of particular proteins.

Two glass plates with spacers were assembled in the stand and the resolving gel was applied between glasses, overlaid with distilled water and allowed to set at room temperature for about 30 minutes. Then water was removed, the stacking gel was applied and the well-former was inserted. The gel was allowed to set at room temperature for about 15 minutes. The stand was set into the tank, the upper chamber and tank were filled with electrode buffer and the well-former was removed.

Samples were prepared from liver and intestinal microsomal fractions by dilution to protein concentration 1 mg/ml and 2 mg/ml, respectively, and than diluted with sample buffer by 1:1 ratio. For ageing of liver microsomal fractions, samples were diluted to

cytochrome P450 concentration 0.5 μmol/l and than diluted with sample buffer 1:1. Sigma Marker was also diluted with sample buffer by 1:1 ratio. Samples and marker were then incubated at 100°C in water bath for about 5 minutes.

Prepared samples (25 μl) and marker (5-7 μl) were loaded into wells and the lid was attached. The electrophoresis was set at constant tension 110 V and ran about 2.5 hours until the dye front reached the bottom of the gel. The glass plates were separated, the stacking gel was removed and the gel was labelled by cutting of one corner of the gel.

For staining the gel was dropped into staining bath and shaken for about 1 hour (or overnight) and then destained in destaining bath as long as needed. For Western blotting see the next chapter.

3.3.7 Western blotting

In this method proteins are transferred from gel to membrane (e.g. PVDF, nitrocellulose) by electric field. Immunodetection of proteins is done by primary and secondary antibodies against particular protein and primary antibody, respectively.

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

Staining and destaining bath: see chapter about SDS-PAGE

PVDF membrane (Immobilon P transfer membrane)

After electrophoresis the gel was dropped into transfer buffer and shaken for about 30 minutes. The PVDF membrane was cut to the dimensions of the gel, wet with methanol for 10 seconds, dropped into distilled water for about 10 minutes, and finally dropped into transfer buffer. Six Whatman filter papers no.3 were cut into dimensions and wet with transfer buffer.

The electrodes were degreased with ethanol then 3 Whatman papers, membrane, gel and another 3 Whatman papers were put on the bottom electrode (Figure 3-2). Catode plate cover was placed over and current density was set according to membrane surface multiplied by 4 mA/cm^2 for 30 minutes.

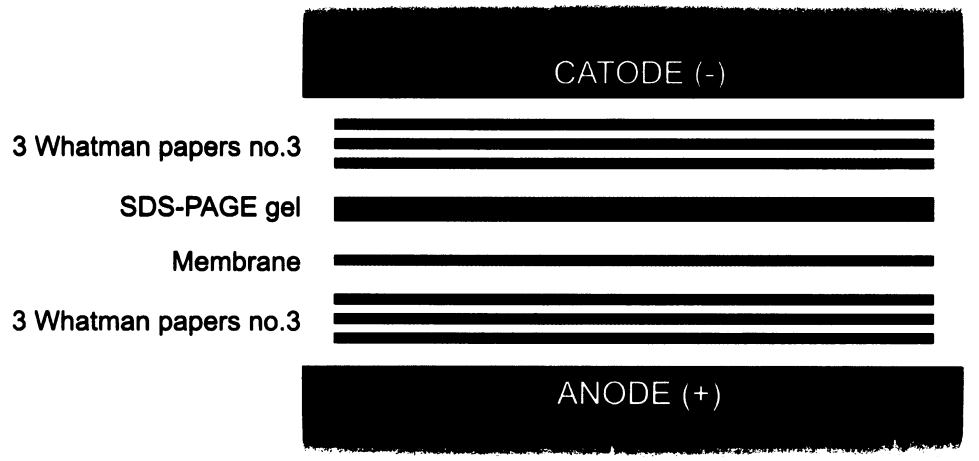


Figure 3-2 Scheme of Western blotting.

The gel was stained after transfer in staining bath and destained in destaining bath to confirm the success of transfer. The part of membrane with marker was cut off and stained/destained. The rest of membrane was dropped into blocking solution and incubated overnight at 4°C. Required volume of primary antibody was diluted in about 1 ml of PBS Triton X-100 and incubated overnight with keratinized layer of epidermis (pieces of human skin) to avoid binding of keratin from samples.

Next day the membrane was incubated with shaking in primary antibody (chicken anti-rat CYP1A or anti-rabbit CYP2B4) diluted in blocking solution to 20-30 μg/ml for 2 hours. Then the primary antibody was extensively washed out by blocking solution and the membrane was incubated in secondary rabbit antibody anti-chicken IgY conjugated with alkaline phosphatase for 1 hour (dilution 1:1 500). Then the secondary antibody was washed out by blocking solution (3x5 minutes), PBS Triton X-100 (3x5 minutes) and distilled water (3x5 minutes).

The visualisation substrate for alkaline phosphatase, BCIP/NTB Sigma*FAST* tablet, was dissolved in 10 ml of distilled water. The membrane was dropped into substrate solution, then dropped into distilled water and dried between filter papers. The scheme of protein visualization is shown in Figure 3-3.

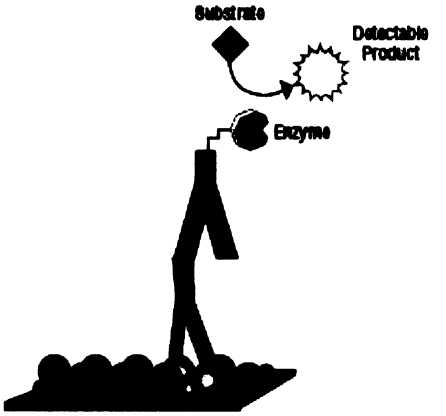
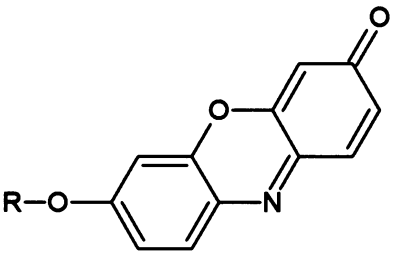


Figure 3-3 Visualization of protein bound to membrane by primary and secondary antibody, and specific substrate.

3.3.8 Cytochrome P450 activity assay

Specific substrates for particular isoforms of CYP are used in this assay (Table 3-3). These substrates are metabolized (dealkylated) by CYP to fluorescent product, resorufin (Figure 3-4). The excitation and emission wavelengths used are 530 nm (slit 15) and 585 nm (slit 10), respectively. The fluorescence was measured on Luminescence Spectrometer PerkinElmer LS-55 in 96-well plate.



compound	R
resorufin	H
7-ethoxyresorufin	C_2H_5
7-methoxyresorufin	CH_3
7-pentoxyresorufin	C_5H_{11}

Figure 3-4 Structure of resorufin, 7-ethoxyresorufin, 7-methoxyresorufin and 7-pentoxyresorufin.

Table 3-3 Specific substrates for cytochrome P450 isoforms.

method, reaction		Specific CYPs
7-ethoxyresorufin-*O*-demethylase	EROD	1A1/2
7-methoxyresorufin-*O*-deethylase	MROD	1A2
7-pentoxyresorufin-*O*-dealkylase	PROD	2B1/2

Buffer: 0.1 M KH_2PO_4; pH 7.4

Resorufin stock solution: 10 μM resorufin in methanol

0.2 mM 7-ethoxyresorufin, 7-methoxyresorufin and 7-pentoxyresorufin in methanol

5 mM NADPH

Samples were prepared by dilution of microsomal fractions with buffer to protein concentration 0.5 mg/ml (or 0.1 μM CYP for ageing study). Then the specific substrate was added to this solution to concentration 2.2 μM. 150 μl of these solutions were filled into plate wells in doublets and the reaction was started by adding 17 μl of NADPH solution (final concentration 0.5 mM). The plate was put into spectrometer immediately and fluorescence was measured in ten 60 seconds cycles for liver samples and in fifteen 120 second samples for intestinal samples.

Resorufin stock solution was diluted to concentrations: 0.0125, 0.025, 0.05, 0.1, 0.2 and 0.4 μM. Then 150 μl of these solutions were filled into wells and 17 μl of buffer was added. The fluorescence was measured and calibration curve was obtained.

4 Results

4.1 Isolation and characterization of isolated rat microsomal fractions

Microsomal fractions were isolated from liver and different small intestinal parts of male Wistar rats treated with β-naphthoflavone (positive control), selected flavonoids (biochanin A, flavanone, morin and rutin) and other chemopreventive compounds (curcumin and diallyl sulphide). Control group was treated only with sunflower oil. Microsomal fractions from control and flavanone treated rats were pooled from 5 rats, while other microsomal fractions were pooled from 4 rats. Characteristics of liver and small intestinal parts taken from rats are shown in Tables 4-1 and 4-2.

Table 4-1 Characterization of liver tissues from treated rats.

liver	m_{tissue} [g]	no.of rats	liver weight
β-naphthoflavone	40.58	4	10.15
Biochanin A	44.41	4	11.10
Curcumin	49.72	4	12.43
Diallyl sulphide	34.36	4	8.59
Flavanone	44.83	5	8.97
Morin	36.51	4	9.13
Rutin	34.25	4	8.56
Control	40.81	5	8.16

m_{tissue} = used tissue mass

Characteristics of isolated microsomal fractions from liver and intestinal parts are shown in Tables 4-3, 4-4 and 4-5. The specific content of CYP did not increase markedly in any isolated liver microsomal fraction. Small increase was observed only in liver microsomal fraction from rutin treated rats. β-naphthoflavone, biochanin A, curcumin and diallyl sulphide produced slight decrease while flavanone and morin produced stronger decrease in specific CYP content in liver microsomal fractions.

Among intestinal samples, the content of CYP was measureable only in proximal part microsomes from β-naphthoflavone treated rats. In other intestinal microsomal fractions CYP level was under the threshold of the used assay.

Table 4-2 Characterization of intestinal tissues from treated rats.

proximal part	m_{tissue} [g]	no.of rats
β-naphthoflavone	7.24	4
Biochanin A	7.15	4
Curcumin	8.64	4
Diallyl sulphide	7.07	4
Flavanone	8.35	5
Morin	7.87	4
Rutin	7.37	4
Control	7.25	5

middle part	m_{tissue} [g]	no.of rats
Biochanin A	7.14	4
Curcumin	8.83	4

distal part	m_{tissue} [g]	no.of rats
β-naphthoflavone	5.36	4
Biochanin A	9.18	4
Curcumin	8.74	4
Diallyl sulphide	4.38	4
Morin	6.07	4
Rutin	4.21	4

m_{tissue} = used tissue mass

Table 4-3 Characterization of isolated liver microsomal fractions.

liver	$c_{protein}$ [mg/ml]	c_{CYP} [µM]	**s.c. CYP** [nmol/mg]
β-naphthoflavone	43.40	31.37	0.72
Biochanin A	37.50	25.88	0.69
Curcumin	44.40	29.33	0.66
Diallyl sulphide	36.60	24.09	0.66
Flavanone	42.90	24.85	0.58
Morin	45.00	23.68	0.53
Rutin	34.70	27.33	0.79
Control	33.75	24.60	0.73

Table 4-4 Characterization of isolated proximal part intestinal microsomal fractions.

proximal part	$c_{protein}$ [mg/ml]	c_{CYP} [µM]	s.c. CYP [nmol/mg]
β-naphthoflavone	21.4	0.97	0.05
Biochanin A	21.3	n.d.	n.d.
Curcumin	18.8	n.d.	n.d.
Diallyl sulphide	15.2	n.d.	n.d.
Flavanone	13.8	n.d.	n.d.
Morin	19.0	n.d.	n.d.
Rutin	13.0	n.d.	n.d.
Control	12.25	n.d.	n.d.

n.d. – not detectable

Table 4-5 Characterization of isolated middle and distal part intestinal microsomal fractions.

middle part	$c_{protein}$ [mg/ml]	c_{CYP} [µM]
Biochanin A	25.0	n.d.
Curcumin	27.8	n.d.

distal part	$c_{protein}$ [mg/ml]	c_{CYP} [µM]
β-naphthoflavone	14.2	n.d.
Biochanin A	19.8	n.d.
Curcumin	20.9	n.d.
Diallyl sulphide	15.0	n.d.
Morin	16.3	n.d.
Rutin	17.7	n.d.

n.d. – not detectable

4.2 Ageing and degradation of liver microsomal samples

Microsomal fractions are normally stored in -80°C freezer in aliquots and used for experimental work for a long time. Since in early studies ([99], [100]) the same microsomal samples were isolated (control and β-naphthoflavone), there was a chance to compare these samples and assume the influence of ageing in freezer. Characterization of isolated liver microsomal fractions is shown in Table 4-6.

Table 4-6 Characterization of liver microsomal fractions from control rats and rats premedicated with β-naphthoflavone.

liver	$c_{protein}$ [mg/ml]	c_{CYP} [μM]	s.c. CYP [nmol/mg]	date of isolation	reference
Control	22.9	22.5	0.98	2.8.2004	[99]
Control	18.6	16.3	0.88	3.10.2005	[100]
Control	33.75	24.60	0.73	27.4.2008	this work
β-naphthoflavone	22.9	6.6	0.29	23.4.2005	[99]
β-naphthoflavone	19.6	8.1	0.41	3.10.2005	[100]
β-naphthoflavone	43.40	31.37	0.72	2.2.2008	this work

These samples were examined for their activity in alkoxyresorufin-*O*-dealkylation assay using specific CYP substrates (7-ethoxyresorufin, 7-methoxyresorufin, and 7-pentoxyresorufin). Results are shown in Figures 4-1 and 4-2. Compared to new isolation the 7-ethoxyresorufin-*O*-deethylation (EROD) activity of microsomal samples from β-naphthoflavone treated rats was significantly decreased in older samples isolated 23.4.2005 and 3.10.2005, by 86% and 59%, respectively. The 7-methoxyresorufin-*O*-demethylation (MROD) and pentoxyresorufin-*O*-dealkylation (PROD) activities were also decreased significantly but not so extensively. MROD and PROD activities of samples isolated 23.4.2005 were decreased by 63% in both assays, and activities of samples isolated 3.10.2005 were decreased by 54% and 41%, respectively.

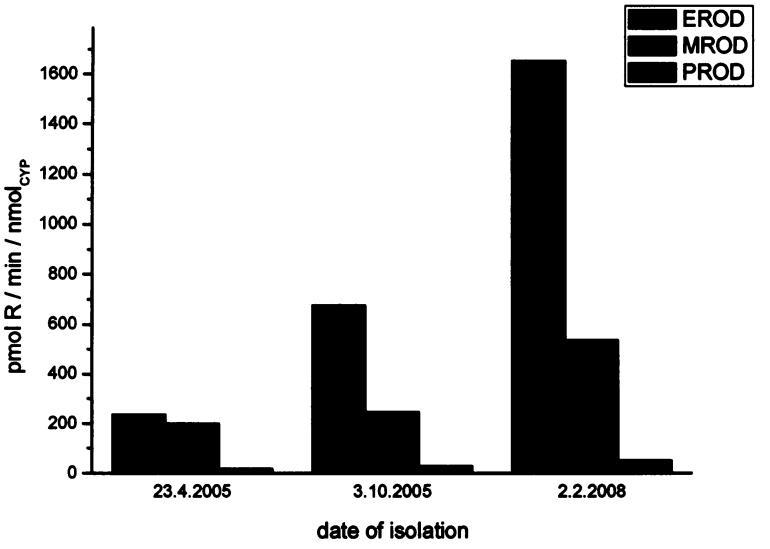


Figure 4-1 Differences among CYP activities (EROD, MROD and PROD) of isolated β-naphthoflavone liver microsomal fractions. SD±10%

EROD and PROD activities of all control liver microsomes did not decrease significantly. Interestingly, the MROD activities of older samples (isolated 23.4.2005 and 3.10.2005) were higher than these from new isolation by 42% and 13%, respectively.

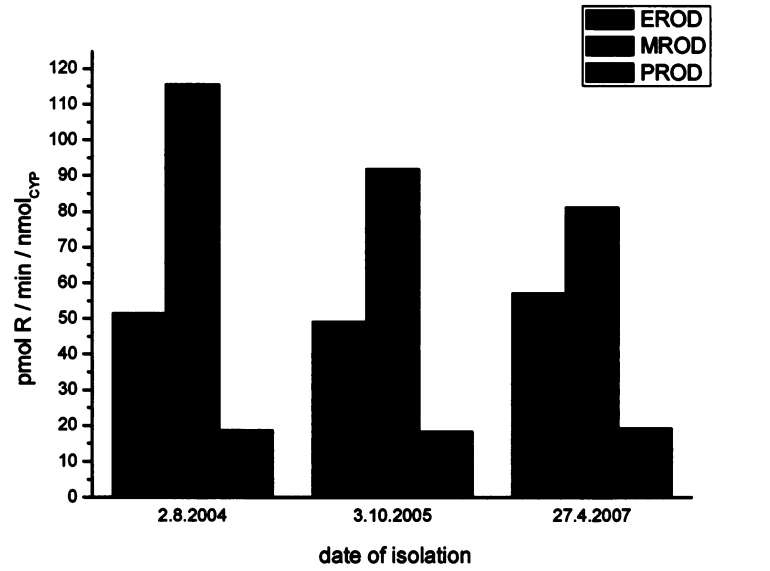


Figure 4-2 Differences among CYP activities (EROD, MROD and PROD) of isolated control liver microsomal fractions. SD±10%

All samples were also analyzed by SDS-electrophoresis (Figure 4-3) and Western blotting using immunodetection with primary antibodies anti-rat CYP1A and anti-rabbit CYP2B4 (Figures 4-4 and 4-5). Differences among EROD, MROD and PROD activities of

liver β-naphthoflavone microsomal samples correspond to results obtained from immunodetection of Western blotting. The content of CYP1A1 and CYP1A2 protein detected on PVDF membrane is decreasing with age of microsomal samples. The lower level of induction of CYP2B (measured by PROD activity) observed in newly isolated sample was confirmed by Western blotting with immunodetection (Figure 4-5).

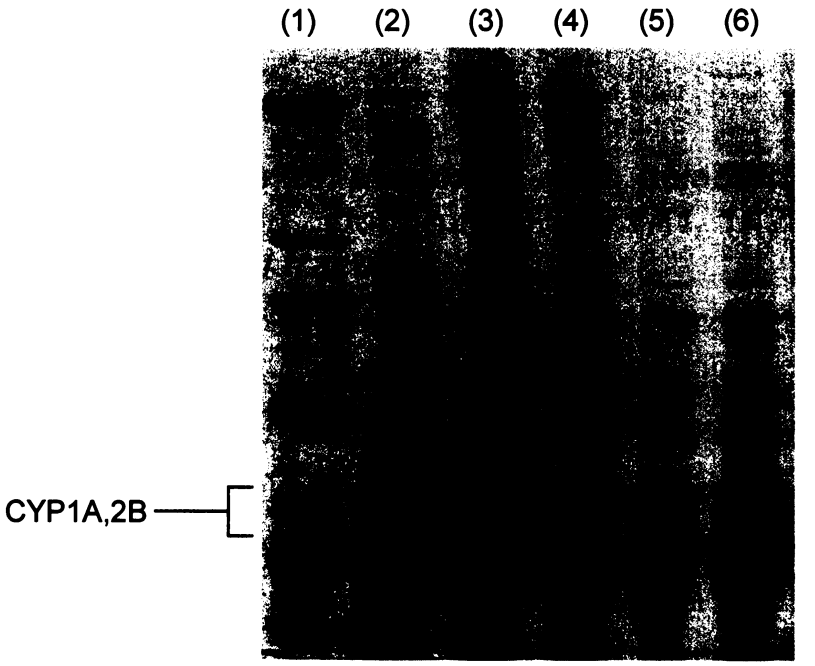


Figure 4-3 SDS-electrophoresis of liver microsomal samples. Dilution used to CYP concentration 0.5 µM (6.25 nmol CYP per well). Lane (1) SigmaMarker Wide range; (2) β-naphthoflavone microsomes isolated 2.2.2008; (3) β-naphthoflavone microsomes isolated 23.4.2005; (4) control microsomes isolated 27.4.2007; (5) control microsomes isolated 3.10.2005; (6) control microsomes isolated 2.8.2004.

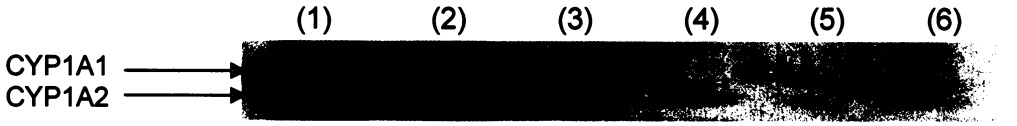


Figure 4-4 Western blotting analysis of liver microsomal samples diluted to cytochrome P450 concentration 0.5 µM (6.25 nmol CYP per well). Primary antibody used was chicken anti-rat CYP1A. Lane (1) β-naphthoflavone microsomes isolated 2.2.2008; (2) β-naphthoflavone microsomes isolated 3.10.2005; (3) β-naphthoflavone microsomes isolated 23.4.2005; (4) control microsomes isolated 27.4.2007; (5) control microsomes isolated 3.10.2005; (6) control microsomes isolated 2.8.2004.

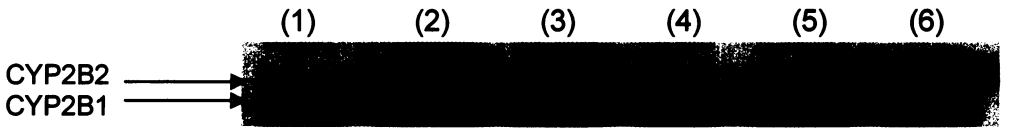


Figure 4-5 Western blotting analysis of liver microsomal samples diluted to cytochrome P450 concentration 0.5 µM (6.25 nmol CYP per well). Primary antibody used was chicken anti-rabbit CYP2B. Lane (1) β-naphthoflavone microsomes isolated 2.2.2008; (2) β-naphthoflavone microsomes isolated 23.4.2005; (3) control microsomes isolated 27.4.2007; (4) control microsomes isolated 3.10.2005; (5) control microsomes isolated 2.8.2004; (6) phenobarbital induced rat microsomes (positive control).

4.3 The influence of treatment with selected chemopreventive compounds on cytochrome P450 1A1, 1A2 and 2B1/2 activity in microsomal fractions

7-Ethoxyresorufin-*O*-deethylase (EROD), 7-methoxyresorufin-*O*-demethylase (MROD) and 7-pentoxyresorufin-*O*-dealkylase (PROD) activities were used as markers for cytochrome P450 1A and 2B subfamily induction. Dealkylation of corresponding specific CYP substrates forms a fluorescent product, resorufin. Enzymatic activities in microsomal fractions were calculated from regression equation of calibration curve obtained from resorufin standards. Resulting activities are shown in Figures 4-6 to 4-11. β-naphthoflavone microsomal fraction was used as positive control of measurement.

4.3.1 Liver microsomal fractions

The effect of unsubstituted flavonoids (α-naphthoflavone, β-naphthoflavone, flavone and flavanone) on CYP activities are shown in Figures 4-6 and 4-8. All these flavonoids are known CYP1A inducers although induction levels differ marginally. While EROD activity was induced by β-naphthoflavone 59-times, α-naphthoflavone, flavone and flavanone increased this activity only 2.5, 1.6, and 1.5-times, respectively. The induction of MROD activity by β-naphthoflavone was not as high as for EROD, only 7-times. Other unsubstituted flavonoids, α-naphthoflavone, flavone and flavanone, increased MROD activity by 2.3, 1.4 and 1.5-times, respectively.

All microsomal samples from rats treated with unsubstituted flavonoids produced an increase in PROD activity to some extent (Figure 4-8). The highest induction was found after flavone premedication, the activity increased by 650%.

Diallyl sulphide induced EROD and MROD activities 2.1-times in both assays (Figure 4-7) and PROD activity 6.3-times (Figure 4-8).

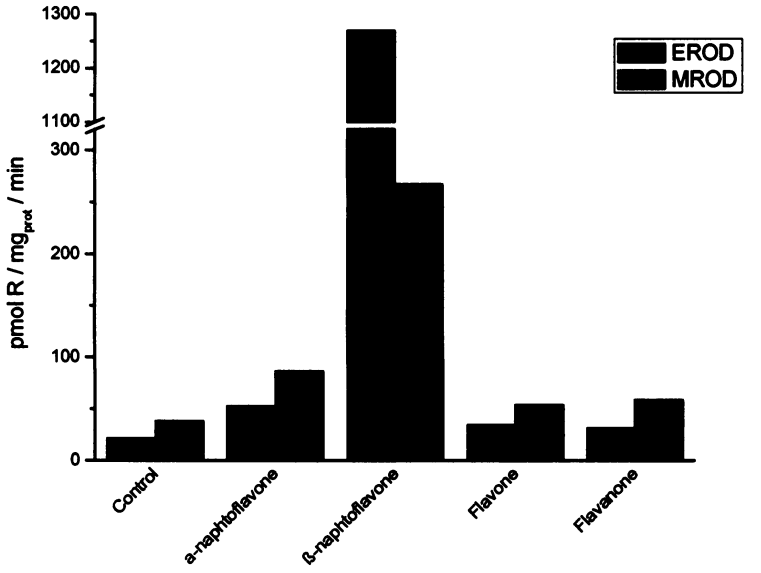


Figure 4-6 Effect of flavonoids (α-naphthoflavone, β-naphthoflavone, flavone and flavanone) premedication on EROD and MROD activities of liver microsomal fractions. SD±10%

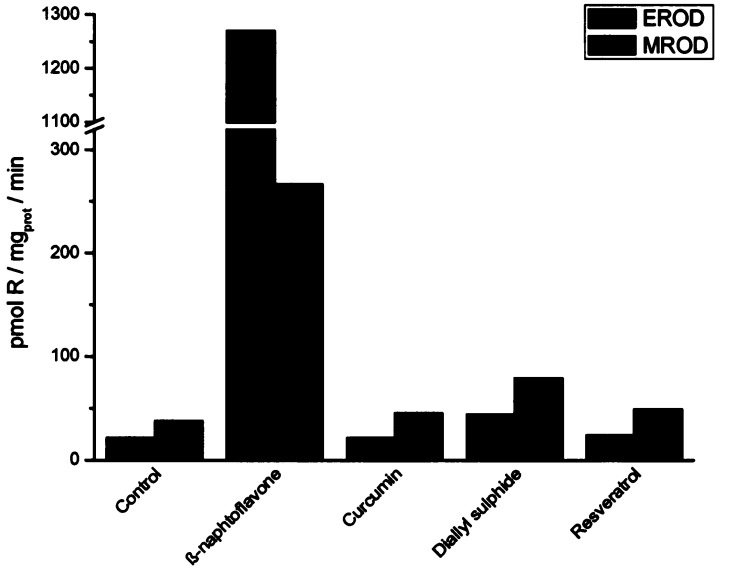


Figure 4-7 Effect of flavonoids (β-naphthoflavone) and other chemopreventive compounds (curcumin, diallyl sulphide and resveratrol) premedication on EROD and MROD activities of liver microsomal fractions. SD±10%

From other measured microsomal samples, the PROD activity was significantly induced, and EROD and MROD activities significantly decreased by baicalin. Naringin, hesperidin and resveratrol caused small increase in PROD activity (Figure 4-8).

Slight induction of EROD and MROD activities was caused by naringin, hesperidin, resveratrol and morin. Rutin and biochanin A increased the EROD and MROD activity more significantly (1.2 and 1.3-times for EROD, and 1.2 and 1.5 for MROD, Figure 4-7).

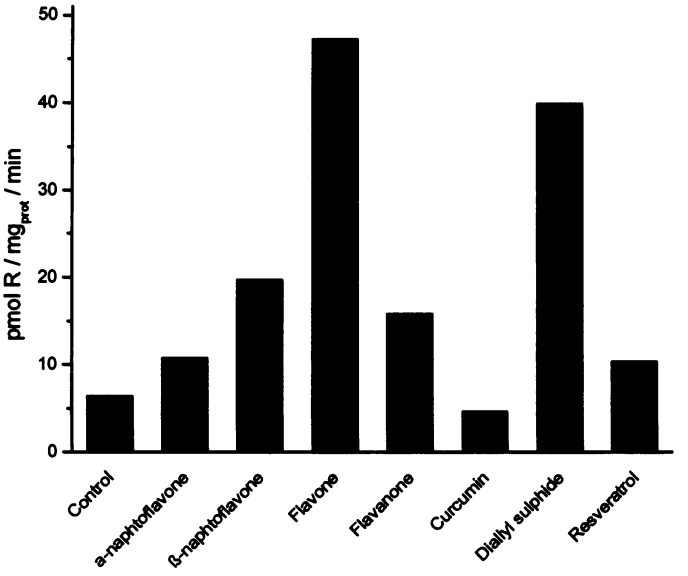


Figure 4-8 Effect of flavonoids (α-naphthoflavone, β-naphthoflavone, flavone and flavanone) and other chemopreventive compounds (curcumin, diallyl sulphide and resveratrol) premedication on PROD activities of liver microsomal fractions. SD±10%

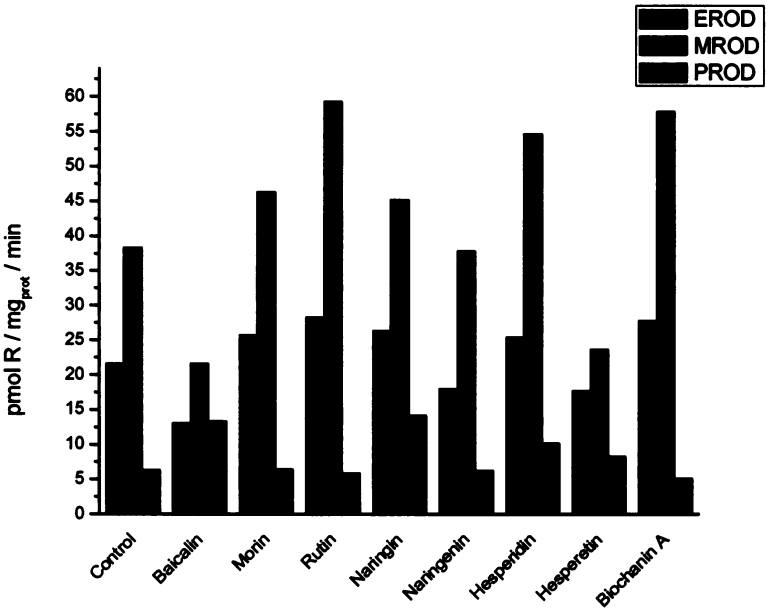


Figure 4-9 Effect of flavonoids (baicalin, morin, rutin, naringin, naringenin, hesperidin, hesperetin and biochanin A) premedication on EROD, MROD and PROD activities of liver microsomal fractions. SD±10%

4.3.2 Intestinal microsomal fractions

Measurement of EROD, MROD and PROD activities is not as simple in small intestine since the content of cytochromes P450 is really low (even not detectable). In control microsomes of rats treated only with sunflower oil, no activity was measurable. As well, all older microsomal fractions used showed no activity (except for β-naphthoflavone). However, in new isolated microsomal fractions, the activity was measurable.

The second highest induction of EROD activity after β-naphthoflavone (136.8 pmol R/mg_{prot}/min, Figure 4-10A) was found after diallyl sulphide premedication (16.5 pmol R/mg_{prot}/min). From others (curcumin, biochanin A, morin and rutin), only morin caused significant induction of EROD activity (Figure 4-10B).

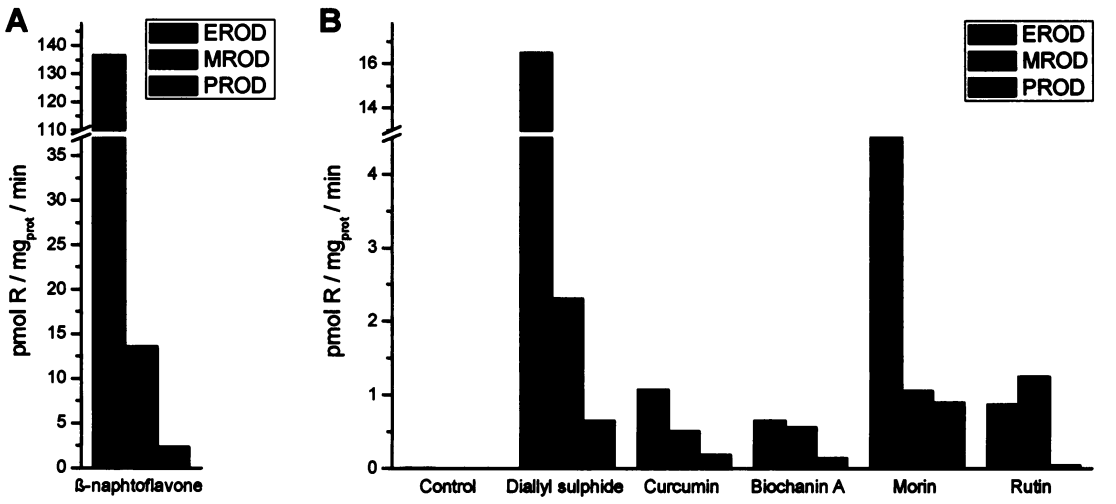


Figure 4-10 Effect of **A,** β-naphthoflavone, **B,** other flavonoids (biochanin A, morin and rutin) and chemopreventive compounds (diallyl sulphide and curcumin) premedication on EROD, MROD and PROD activities in proximal part intestinal microsomes. SD±10%

Comparing CYP activities in proximal and distal parts of small intestine the induction caused by β-naphthoflavone, diallyl sulphide and morin is rapidly decreased in distal parts but to a different extent. EROD and PROD activities of distal intestinal microsomes from β-naphthoflavone treated rats (Figure 4-11A) decreased by 76% and 42%, respectively, compared to proximal intestinal microsomes. However, after diallyl sulphide (Figure 4-11B) and morin (Figure 4-11C) treatment EROD and PROD activities decreased more rapidly by 94% and 79%, respectively, and 89% and 92%, respectively.

In other cases, activities of cytochromes P450 were not measurable in distal intestinal microsomes.

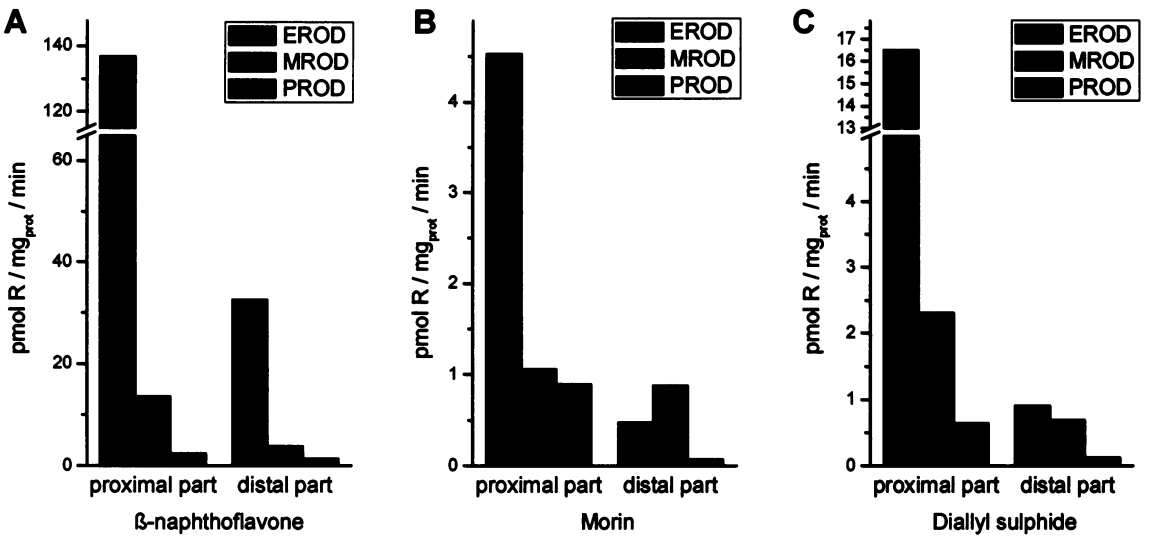


Figure 4-11 Effect of flavonoids (**A**, *β*-naphthoflavone and **B**, morin) and **C**, diallyl sulphide premedication on EROD, MROD and PROD activities in proximal and distal part intestinal microsomes. SD±10%

4.4 Effect of treatment with selected chemopreventive compounds on cytochrome 1A1/2 and 2B1/2 protein levels

To determine the effect of selected chemopreventive compounds on cytochrome P450 1A1, 1A2 and 2B protein levels, and to confirm results obtained from activity measurements, Western blotting with immunodetection was used.

Proteins of microsomal fractions were separated by SDS-electrophoresis. Cytochromes P450 1A and 2B subfamily migrated in zone with mass around 55 000 Da. The specific primary chicken antibody anti-rat CYP1A1 and anti-rabbit CYP2B4 were used. Due to high homology of CYP1A1 and 1A2 isoforms (more than 70%) this anti-rat CYP1A1 antibody recognized and detected also CYP1A2 isoform. However, the homology of rabbit CYP2B4 and rat CYP2B1/2 is not such high, the chicken anti-rabbit CYP2B4 antibody was also able to recognize rat CYP2B1/2 isoforms.

The specificity of this antibody was proved with microsomal fraction from phenobarbital treated rats (specific inducer of CYP2B1/2).

4.4.1 Liver microsomal fractions

For immunoblot analysis, microsomal fractions had to be divided into few groups: unsubstituted flavonoids (α-naphthoflavone, β-naphthoflavone, flavone and flavanone, Figures 4-13 and 4-14), non-flavonoid chemopreventive compounds (diallyl sulphide and curcumin, Figures 4-15 and 4-16), new isolated microsomal fractions (biochanin A, morin and rutin, Figures 4-17 and 4-18), citrus flavonols I (naringin and naringenin) and baicalin (Figures 4-19 and 4-20), and citrus flavonols II (hesperidin and hesperetin) and resveratrol (Figures 4-21 and 4-22). SDS-electrophoresis of new isolated liver microsomal fractions is shown in Figure 4-12.

Beside model inducer of CYP1A subfamily (β-naphthoflavone) and α-naphthoflavone, the strongest induction of CYP1A1 was observed in liver microsomes from flavone treated rats (Figure 4-13). Flavone was also the most potent inducer of cytochromes 2B1/2 (Figure 4-14). Only CYP1A2 isoform was detected in liver microsomes from control and flavanone treated rats, and flavanone induced CYP1A2 protein level.

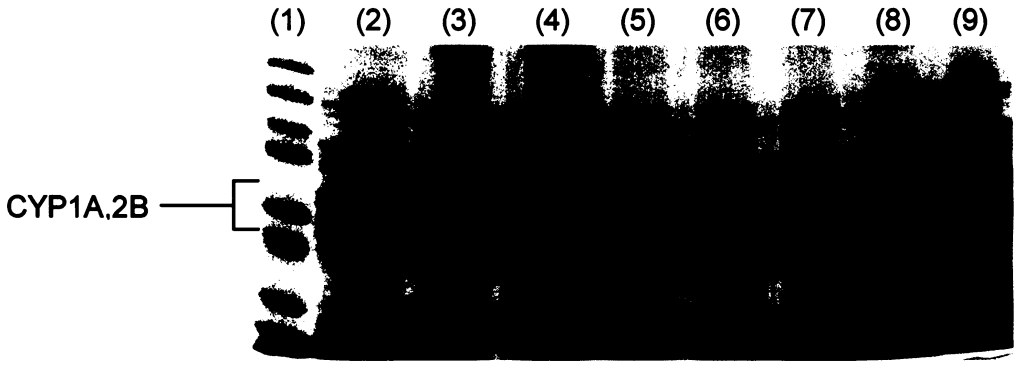


Figure 4-12 SDS-electrophoresis of 12.5 µg liver microsomal proteins from treated rats. Lane (1) SigmaMarker Wide range; (2) β-naphthoflavone; (3) control; (4) flavanone; (5) diallyl sulphide; (6) curcumin; (7) biochanin A; (8) morin; (9) rutin.

Figure 4-13 Immunodetection of CYP1A1 and CYP1A2 in liver microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rat CYP1A. Lane (1) α-naphthoflavone; (2) β-naphthoflavone; (3) control; (4) flavone; (5) flavanone.

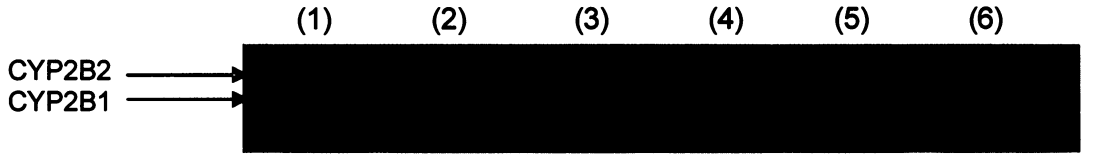


Figure 4-14 Immunodetection of CYP2B1 and CYP2B2 in liver microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rabbit CYP2B. Lane (1) phenobarbital (positive control); (2) flavanone; (3) flavone; (4) control; (5) β-naphthoflavone; (6) α-naphthoflavone.

Diallyl sulphide was determined to be potent inducer of either CYP1A1/2 or CYP2B1/2 (Figures 4-15 and 4-16). Curcumin, biochanin A, morin and rutin only slightly induced CYP1A2 isoform.

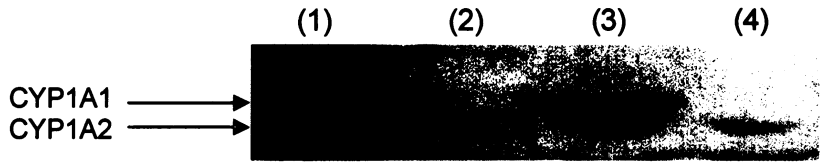


Figure 4-15 Immunodetection of CYP1A1 and CYP1A2 in liver microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rat CYP1A. Lane (1) β-naphthoflavone; (2) control; (3) diallyl sulphide; (4) curcumin.

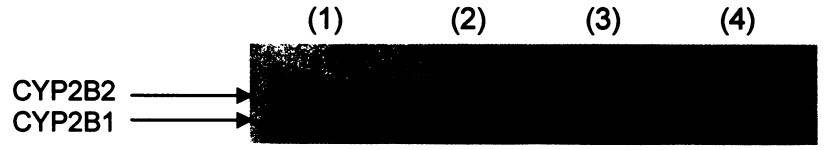


Figure 4-16 Immunodetection of CYP2B1 and CYP2B2 in liver microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rabbit CYP2B. Lane (1) β-naphthoflavone; (2) control; (3) diallyl sulphide; (4) curcumin.

Figure 4-17 Immunodetection of CYP1A1 and CYP1A2 in liver microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rat CYP1A. Lane (1) control; (2) biochanin A; (3) morin; (4) rutin.

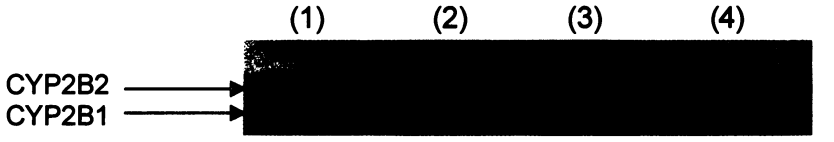


Figure 4-18 Immunodetection of CYP2B1 and CYP2B2 in liver microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rabbit CYP2B. Lane (1) control; (2) biochanin A; (3) morin; (4) rutin.

No induction of CYP1A1 and CYP1A2 was observable after naringenin, naringin, hesperidin, hesperetin, baicalin and resveratrol treatment in rat liver microsomes (Figures 4-19 and 4-21). However, premedication with baicalin, naringenin and naringin induced CYP2B1/2 isoforms (Figure 4-20). Slight induction of CYP2B1/2 was detected also in liver microsomes from hesperidin, hesperetin and resveratrol treated rats (Figure 4-22).

Figure 4-19 Immunodetection of CYP1A1 and CYP1A2 in liver microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rat CYP1A. Lane (1) control; (2) naringin; (3) naringenin; (4) baicalin.

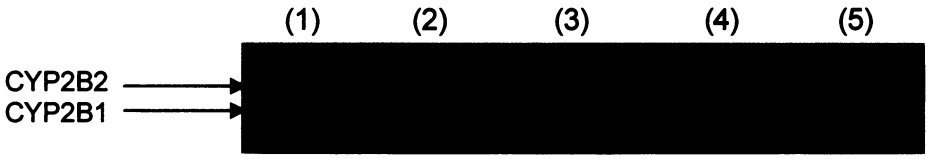


Figure 4-20 Immunodetection of CYP2B1 and CYP2B2 in liver microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rabbit CYP2B. Lane (1) control; (2) naringin; (3) naringenin; (4) baicalin; (5) phenobarbital.

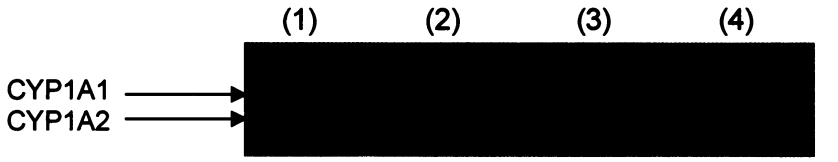


Figure 4-21 Immunodetection of CYP1A1 and CYP1A2 in liver microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rat CYP1A. Lane (1) control; (2) hesperidin; (3) hesperetin; (4) resveratrol.

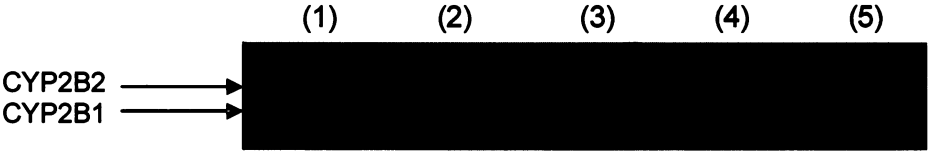


Figure 4-22 Immunodetection of CYP2B1 and CYP2B2 in liver microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rabbit CYP2B. Lane (1) phenobarbital (positive control); (2) resveratrol; (3) hesperetin; (4) hesperidin; (5) control.

4.4.2 Intestinal microsomal fractions

Since the content of cytochromes P450 is lower in small intestine, samples used for SDS-electrophoresis and following Western blotting analysis were twice as concentrated as the liver ones. SDS-electrophoreses of new intestinal microsomal samples are shown in Figures 4-23, 4-26 and 4-29.

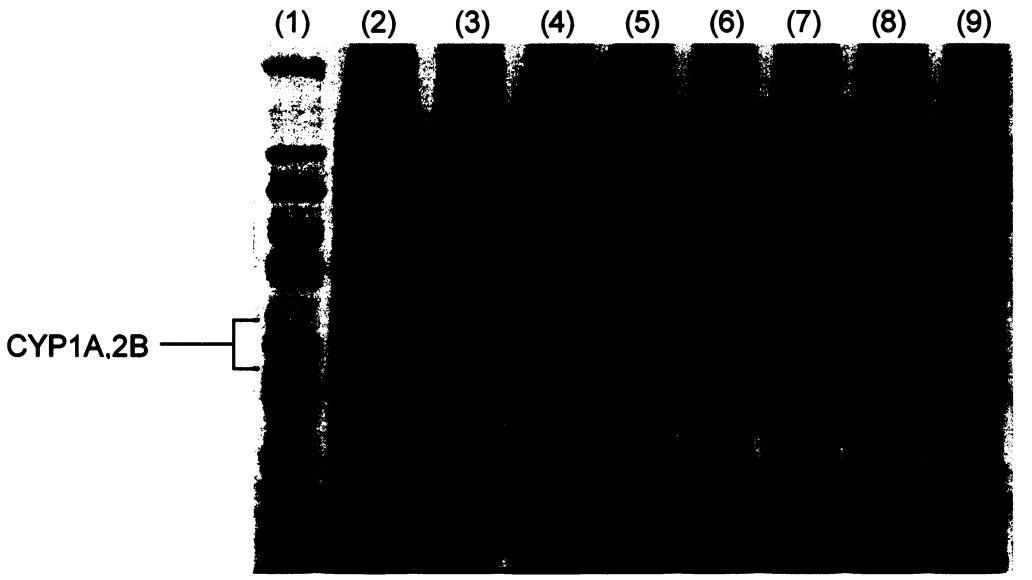


Figure 4-23 SDS-electrophoresis of 25 µg proximal part intestinal microsomal proteins from treated rats. Lane (1) SigmaMarker Wide range; (2) β-naphthoflavone; (3) control; (4) flavanone; (5) diallyl sulphide; (6) curcumin; (7) biochanin A; (8) morin; (9) rutin.

More parts of small intestine were taken in latter isolations, beside proximal part also middle and distal part, and due to this different analyses had to be done. Firstly, the effect of premedication by selected compounds on CYP1A1 and CYP2B1 in proximal parts of small intestine were determined.

In untreated rats no isoform (either CYP1A1 or CYP2B1) was detected. Interestingly, second highest induction of CYP1A1 was observed after diallyl sulphide premedication (in contrast to liver). Curcumin, morin and rutin induced CYP1A1 in proximal part of small intestine to a lesser extent (Figure 4-24). Slight CYP1A1 induction after biochanin A premedication was also observed. CYP2B1 was significantly induced by diallyl sulphide, curcumin, morin and rutin treatment (to the same extent as by β-naphthoflavone, Figure 4-25). Biochanin A caused only slight induction of CYP2B1.

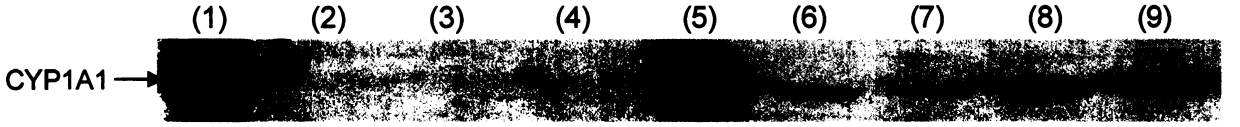


Figure 4-24 Immunodetection of CYP1A1 and CYP1A2 in proximal intestinal microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rat CYP1A. Lane (1) β-naphthoflavone; (2) control; (3) flavone; (4) flavanone; (5) diallyl sulphide; (6) curcumin; (7) biochanin A; (8) morin; (9) rutin.

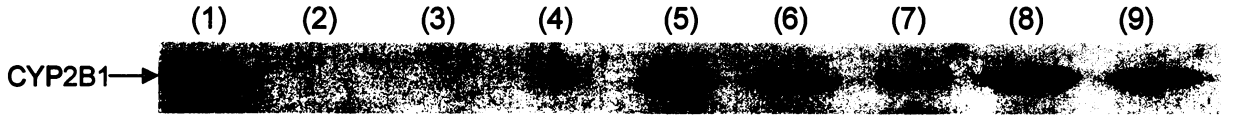


Figure 4-25 Immunodetection of CYP2B1 and CYP2B2 in proximal intestinal microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rabbit CYP2B. Lane (1) β-naphthoflavone; (2) control; (3) flavone; (4) flavanone; (5) diallyl sulphide; (6) curcumin; (7) biochanin A; (8) morin; (9) rutin.

After biochanin A and curcumin treatment, three parts of small intestine were taken (proximal, middle and distal part) for microsomal isolation. Determining the effect of these compounds in different parts of small intestine, induction of CYP1A1 was significant only in proximal parts (Figure 4-27). Induction of CYP2B1 was observed also only in proximal parts and in middle part from curcumin treated rats (Figure 4-28).

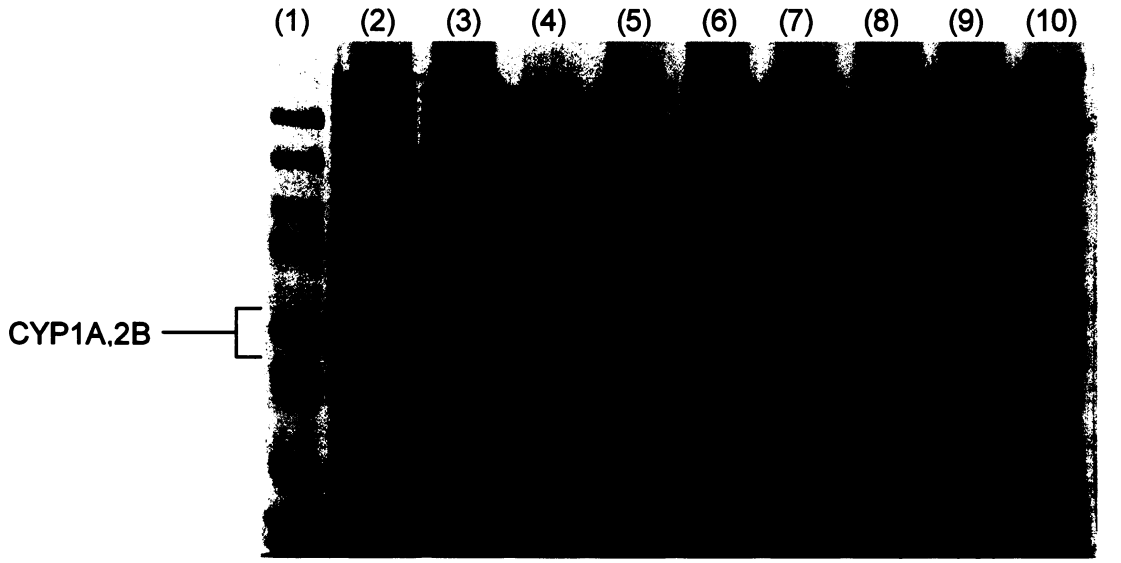


Figure 4-26 SDS-electrophoresis of 25 µg proximal, middle and distal part intestinal microsomal proteins from treated rats. Lane (1) SigmaMarker Wide range; (2) β-naphthoflavone proximal part; (3) β-naphthoflavone distal part; (4) control (proximal part); (5) biochanin A proximal part; (6) biochanin A middle part; (7) biochanin A distal part; (8) curcumin proximal part; (9) curcumin middle part; (10) curcumin distal part.

Two parts of small intestine, proximal and distal, were taken after treatment with β-naphthoflavone, diallyl sulphide, morin and rutin. Determined effect of premedication on CYP in different parts of small intestine seems to be the same. The induction of CYP1A1 and CYP2B1 detected in proximal part was much higher than in distal parts (except for

β-naphthoflavone, where the method sensitivity was overloaded, Figures 4-30 and 4-31). In the case of CYP2B1, induction in distal parts was not observed (Figure 4-31).

The highest induction of CYP2B1 and CYP1A1 (except for β-naphthoflavone) in a small intestine was observed after diallyl sulphide premedication in both parts.

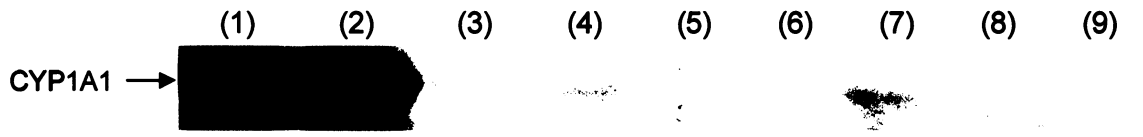


Figure 4-27 Immunodetection of CYP1A1 and CYP1A2 in proximal, middle and distal intestinal microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rat CYP1A. Lane (1) β-naphthoflavone proximal part; (2) β-naphthoflavone distal part; (3) control (proximal part); (4) biochanin A proximal part; (5) biochanin A middle part; (6) biochanin A distal part; (7) curcumin proximal part; (8) curcumin middle part; (9) curcumin distal part.

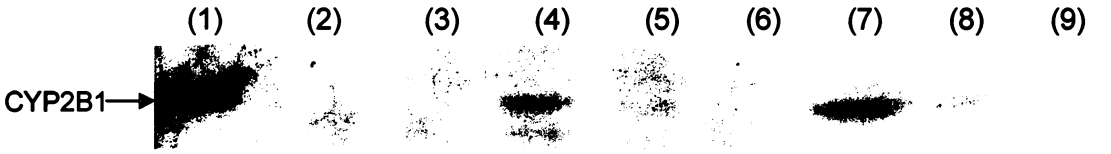


Figure 4-28 Immunodetection of CYP2B1 and CYP2B2 in proximal, middle and distal intestinal microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rabbit CYP2B. Lane (1) β-naphthoflavone proximal part; (2) β-naphthoflavone distal part; (3) control (proximal part); (4) biochanin A proximal part; (5) biochanin A middle part; (6) biochanin A distal part; (7) curcumin proximal part; (8) curcumin middle part; (9) curcumin distal part.

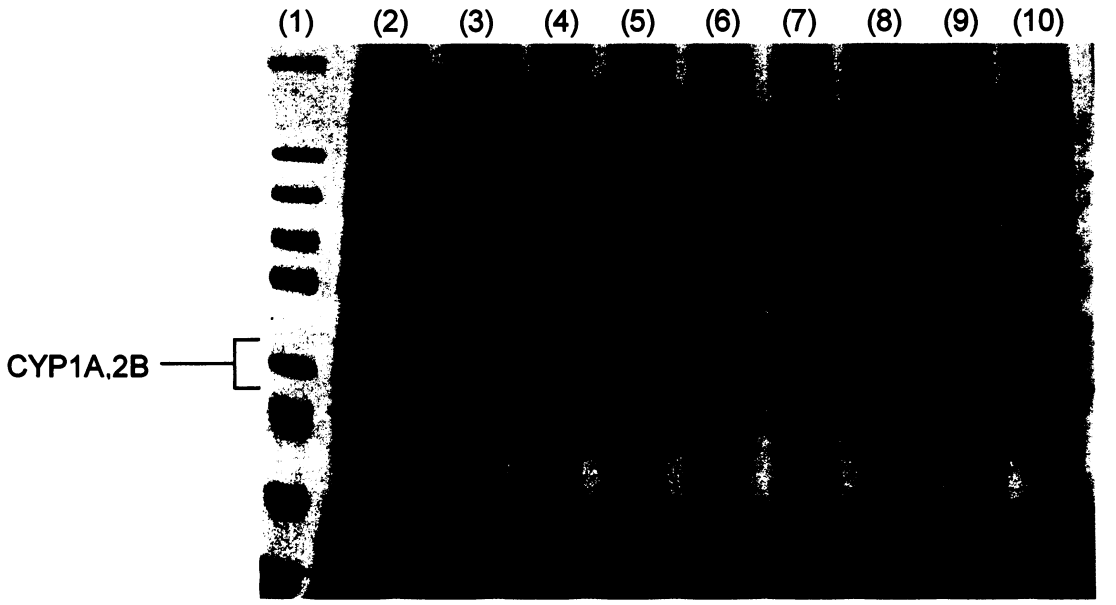


Figure 4-29 SDS-electrophoresis of 25 µg proximal and distal part intestinal microsomal proteins from treated rats. Lane (1) SigmaMarker Wide range; (2) β-naphthoflavone proximal part; (3) β-naphthoflavone distal part; (4) control (proximal part); (5) diallyl sulphide proximal part; (6) diallyl sulphide distal part; (7) curcumin distal part; (8) biochanin A distal part; (9) morin distal part; (10) rutin distal part.

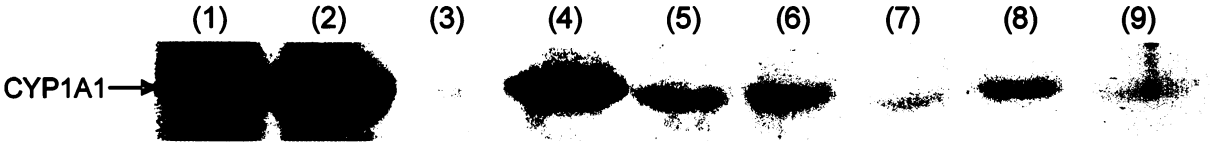


Figure 4-30 Immunodetection of CYP1A1 and CYP1A2 in proximal and distal intestinal microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rat CYP1A. Lane (1) β-naphthoflavone proximal part; (2) β-naphthoflavone distal part; (3) control (proximal part); (4) diallyl sulphide proximal part; (5) diallyl sulphide distal part; (6) morin proximal part; (7) morin distal part; (8) rutin proximal part; (9) rutin distal part.

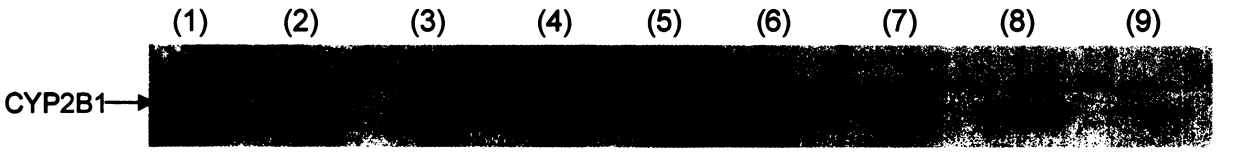


Figure 4-31 Immunodetection of CYP2B1 and CYP2B2 in proximal and distal intestinal microsomal fractions from treated rats. Electrophoresed microsomal proteins were transferred to PVDF membrane and probed with primary antibody anti-rabbit CYP2B. Lane (1) β-naphthoflavone proximal part; (2) β-naphthoflavone distal part; (3) control (proximal part); (4) diallyl sulphide proximal part; (5) diallyl sulphide distal part; (6) morin proximal part; (7) morin distal part; (8) rutin proximal part; (9) rutin distal part.

5 Discussion

Flavonoids and other natural compounds that exert some beneficial effects are more and more popular nowadays. The market with dietary supplements containing these natural compounds is growing year after year mainly in Western world. These dietary supplements are usually taken to prevent cancer, cardiac diseases (e.g. heart stroke), arthritis, etc, and, in general, to improve human health. However, interactions with drug metabolism have been reported [106-109], these supplements and their components are originally thought to be safe because of their plant origin. These dietary supplement-drug interactions can be associated with alterations of pharmacokinetic and pharmacodynamic properties of various drugs which can have clinical implications.

Chemopreventive compounds are usually tested only in artificial tests *in vitro*, e.g. for mutagenicity (*Salmonella typhimurium)* and for desired effect confirmation in cell lines. It was shown earlier that tests on these artificial systems do not correspond with human intake of such compounds [110]. In case of glycosylated flavonoids, that are usually metabolized (hydrolyzed) before absorption, the difference might be marginal.

Moreover, Jang et al. [111] has shown that dietary supplements modulate phase I and phase II enzymes. From 116 tested dietary supplements common in Korea more than 20 increased significantly protein levels of CYP1A2 in rat liver after oral administration.

Lack of tests with flavonoids and other chemopreventive compounds, done *in vivo*, lead us to test the *in vivo* effects on cytochromes P450. These enzymes are involved in metabolism of carcinogens and environmental pollutants, and are capable of their activation. Important human CYP isoforms participating in these processes are CYP1A1, CYP1A2 and CYP2B6.

Several tests with flavonoids (α-naphthoflavone, diosmin, chrysin, quercetin [99], naringin, naringenin, hesperidin, hesperetin, flavone, and baicalin [100]) have been already done in our laboratory. Western blotting analysis using primary chicken anti-rat CYP1A1 and activity assays using 7-ethoxyresorufin-*O*-deethylase and 7-methoxyresorufin-*O*-demethylase as specific substrates for CYP1A1 and CYP1A2, respectively, were done. These used methods were found to be specific enough to show relevant results.

Newly selected chemopreventive compounds were not only from the group of flavonoids (flavanone, morin, rutin and biochanin A), but also diallyl sulphide from garlic

and curcumin from the root of *Curcuma longa* L. Flavanone was chosen as the last unsubstituted flavonoid tested. Rutin is widely used for prevention of cardiovascular diseases, and is used together with vitamin C to increase its antioxidant effect. Biochanin A is from the class of isoflavones that are widely used as phytoestrogens in treatment of post-menopausal women. Curcumin has shown many anticarcinogenic properties and is used in clinical test together with anticancer drugs. Dietary supplements derived from garlic and onions are becoming more popular and one of the active compounds is diallyl sulphide.

Rats were treated *p.o.* with selected compounds (*β*-naphthoflavone, flavanone, morin, rutin, biochanin A, diallyl sulphide and curcumin) dissolved in sunflower oil in 60 mg/kg of weight dose for 5 consecutive days. Control rats were treated only with sunflower oil. After being sacrificed, microsomal fractions were prepared from liver and small intestine. In the case of morin, rutin, *β*-naphthoflavone and diallyl sulphide, two parts of small intestine were originally taken (proximal and distal part), while in case of biochanin A and curcumin, three parts were taken (proximal, middle and distal) to compare the effect of treatment along the intestine.

The obtained microsomal samples were characterized with their protein and cytochrome P450 content, and specific content of CYP. In liver, only rutin slightly increased specific CYP content. *β*-naphthoflavone did not alter this value. Biochanin A, diallyl sulphide and curcumin produced slight decrease, and flavanone and morin decreased specific CYP content markedly. In small intestine, the content of cytochromes P450 was lower and was measurable only in proximal intestinal microsomes from *β*-naphthoflavone treated rats. Moreover, presence of many proteases in this tissue might have caused degradation of CYP.

Western blotting analysis and activity assays were performed also with other isolated microsomal samples to examine the influence of flavonoid treatment on CYP2B1/2 levels and activities in rats. Since these samples are older and stored in freezer -80°C until use, the influence of storing (ageing) had to be examined. For this purpose, liver microsomal samples from *β*-naphthoflavone treated and control rats were used.

All measured activities of CYP (EROD, MROD and PROD) were significantly decreased in liver microsomal samples of *β*-naphthoflavone treated rats isolated 23.4.2005 and 3.10.2005 compared to newly isolated 2.2.2008. The highest decrease was observed in EROD activity. In contrary, the EROD and PROD activity of control liver microsomes did

not change significantly. MROD activities of control samples isolated 2.8.2004 and 3.10.2005 were higher than these from isolated 27.4.2007. The results from activity assays using 7-ethoxyresorufin, 7-methoxyresorufin and 7-pentoxyresorufin as specific substrates for CYP1A1, CYP1A2 and CYP2B1/2, respectively, mostly correlated with results of Western blotting analysis using primary chicken anti-rat CYP1A1 and chicken anti-rabbit CYP2B4 antibodies. Western blotting analysis using anti-rat CYP1A1 antibody of liver microsomes from β-naphthoflavone treated rats corresponded with decreased EROD and MROD activities. On the other hand, the stronger band of CYP2B1/2 detected in liver β-naphthoflavone microsomal sample isolated 23.4.2005 by Western blotting analysis was not accompanied by increase in PROD activity. Western blotting analysis of control samples corresponded with increased MROD activities in older samples.

The differences among isolated control liver microsomes might have occured due to the fact that Wistar rats used for previous isolations were purchased from an alternative company (Velaz, Czech Republic) than rats used for new isolations (Anlab, Czech Republic). Moreover, the conditions to which rats were exposed might have changed with diverse housing used for them. It is also possible that previously used rats varied in their genetic features and that their liver enzymes might not have been so inducible. Extensive decrease in EROD activities observed in samples with high amounts of CYP1A1 and no difference of EROD activities in control samples indicates that the rate of activity decrease may depend on CYP1A1 levels.

In liver, the 7-ethoxyresorufin-*O*-deethylase and 7-methoxyresorufin-*O*-demethylase activities were increased markedly by flavone and flavanone premedication. Western blotting analysis confirmed the induction of CYP1A1 and CYP1A2 in liver microsomes from flavone and flavanone treated rats, respectively. CYP2B1/2 activity and protein levels were rapidly induced by flavone and slightly induced by flavanone. These results are consistent with other *in vivo* studies [44, 45]. However, *in vitro* experiments have shown inhibition of many CYP isoforms by flavone and flavanone [47, 112].

Morin, rutin and biochanin A slightly increased EROD and MROD activities in rat liver microsomes. These results were confirmed by CYP1A2 protein level induction. PROD activities remained unchanged after premedication with these flavonoids although CYP2B1/2 protein levels were moderately induced.

EROD and MROD activities as well as CYP1A1 and CYP1A2 protein levels were increased in diallyl sulphide treated rat liver microsomes. These findings are consent with Davenport et al. study [87]. Diallyl sulphide was also found to be potent inducer of CYP2B subfamily that is consistent with other studies [86]. The increases of CYP2B1/2 activity and protein levels were comparable to flavone-produced induction.

Curcumin slightly induced CYP1A2 protein level in rat liver microsomes that was accompanied with slight increase in MROD activity. Curcumin and its derivatives were found as CYP inhibitors in *in vitro* tests although *in vivo* curcumin did not alter CYP1A and CYP2B activities and protein levels [97, 98]

In small intestine, the cytochrome P450 content is much lower than in liver, the major xenobiotic metabolizing organ. However, presence of phase I and phase II enzymes even in small amounts is of a great importance because small intestine is one of the first organs exposed to xenobiotics after oral ingestion. The presence and inducibility of CYP1A1 and CYP2B1 in rat small intestine has been already reported [113].

Neither CYP1A1 nor CYP2B1 were detected in control intestinal microsomes by Western blotting analysis and activity measurements. The induction of CYP1A1 protein levels observed in β-naphthoflavone treated rats was comparable in proximal and distal part of small intestine, although, 7-ethoxyresorufin-*O*-deethylase activity rapidly decreased in distal part. No difference between CYP1A1 inductions (protein levels) observed in both parts of intestine may have occured due to overloading the capacity of used method for the sake of comparing intestinal microsomal samples. 7-pentoxyresorufin-*O*-dealkylase activity also decreased along the intestine in β-naphthoflavone treated rats, while CYP2B1 protein was detected only in proximal part.

Beside model inducer, diallyl sulphide was found to be the most potent CYP1A1 inducer in small intestine. The second highest induction of CYP1A1 was observed in morin treated rats. Curcumin, biochanin A and rutin induced CYP1A1 to lesser extent. The highest induction of CYP2B1 was caused by morin premedication. Diallyl sulphide, curcumin and rutin induced CYP2B1 markedly while biochanin A produced only a small increase.

It was previously described that the amount of CYP as well as AhR protein is decreasing along the small intestine [114]. Comparing CYP activities and protein levels in

proximal and distal parts, rapid decrease occurred in all cases corresponding to these findings.

Interestingly, different rates of induction were observed in rat liver and small intestinal microsomes after premedication. Morin only slightly induced CYP1A2 in liver while the induction of CYP1A1 in small intestine was the second highest observed. On the other hand, the rate of induction between CYP1A and CYP2B by diallyl sulphide was on behalf of CYP2B in liver and on behalf of CYP1A in small intestine.

Since diallyl sulphide is suicidal substrate for CYP2E1, its anticancer properties are usually connected with prevention of nitrosamine activation catalyzed by this enzyme. Due to this fact, not much is known about its influence on other CYP isoforms. However, induction of CYP1A subfamily might increase the level of activation of polycyclic aromatic hydrocarbons and heterocyclic amines.

The level of induction depends on the route of administration. Higher induction occurs in liver after intraperitoneal administration and in small intestine after oral administration [113]. These data establish the importance of *in vivo* studies of chemopreventive compounds with oral administration imitating humans taking dietary supplements.

In further research, coadministration of chemopreventive compound and carcinogen should be performed. To imitate the model of human dietary supplement and food carcinogen intake, it should be administrated sequentially, not together. In addition to these methods used, DNA adduct formation should be examined by ^{32}P-postlabelling.

6 Conclusions

- Flavonoids (biochanin A, morin, rutin and flavanone) and other chemopreventive compounds (diallyl sulphide and curcumin) were selected.
- Experimental animals, rats, were premedicated *p.o.* with selected flavonoids and chemopreventive compounds, and the microsomal samples were prepared from liver and different parts of small intestine (proximal and distal part).
- Induction of CYP1A1/2 and CYP2B1/2 in liver and intestinal microsomal samples was determined by Western blotting analysis using chicken anti-rat CYP1A1 and chicken anti-rabbit CYP2B4 antibodies, respectively.
- Specific activity measurements were performed using 7-ethoxyresorufin, 7-methoxyresorufin and 7-pentoxyresorufin as specific substrates for CYP1A1, CYP1A2 and CYP2B1/2, respectively.
- Ageing of liver microsomal samples during storage in -80°C freezer was examined by Western blotting analysis and activity measurements.
- In liver microsomes, we demonstrated the CYP1A1 induction by flavone and diallyl sulphide, CYP1A2 induction by diallyl sulphide, biochanin A, morin, rutin and flavanone, and CYP2B1/2 induction by flavone, flavanone and diallyl sulphide.
- In small intestinal proximal microsomal samples, the highest CYP1A1 induction was observed after diallyl sulphide treatment, and second highest by morin treatment. CYP2B1 induction was observed in diallyl sulphide, curcumin, morin and rutin treated rats.
- The induction of cytochrome P450 was rapidly lower in distal part of small intestine. Thus, induction decreased along small intestine from proximal to distal part.
- Increases in specific CYP activities correlated with induction detected by Western blotting analysis.

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