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CHARLES UNIVERSITY IN PRAGUE

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# Cytochrome P450 1A subfamily induction by selected flavonoids

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# Declaration

I hereby declare that I have written this thesis on my own under the supervision of

Doc. RNDr. Petr Hodek, CSc. and that I have mentioned all sources used.

Prague 4$^{th}$ May 2006 ..................................

## Acknowledgement

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

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

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# List of abbreviations

| | |
|---|---|
| 2-AAF | 2-acetylaminofluorene |
| 2-ME | 2-mercaptoethanol |
| 3-MC | 3-methyl-cholanthrene |
| A | absorbance |
| AhR | aryl hydrocarbon receptor |
| B[a]P | benzo[a]pyrene |
| BCA | 4,4'-dicarboxy-2,2'-biquinoline disodium salt (bicinchoninic acid) |
| BCIP/NBT | 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium |
| BIS | N,N'-methylenbisacrylamide |
| BSA | bovine serum albumin |
| C | control |
| $c_{CYP}$ | concentration of cytochrome P450 |
| CO | carbon oxide |
| $c_{protein}$ | concentration of protein |
| $c_R$ | concentration of resorufin |
| CYP | cytochrome P450 |
| DNA | deoxyribonucleic acid |
| EC | enzyme class index |
| EROD | 7-ethoxyresorufin-*O*-deethylase |
| $IC_{50}$ | inhibition concentration 50% |
| MFO | mixed-function oxidase |
| $M_r$ | relative molecular weight |
| MROD | 7-methoxyresorufin-*O*-demethylase |
| MS fraction | microsomal fraction |
| NADH | nicotinamide adenine dinucleotide - reduced |
| NADPH | nicotinamide adenine dinucleotide phosphate - reduced |
| NC | nitrocelluose |
| p.o. | *per os* |
| PBS | phosphate buffered saline |
| PhIP | 2-amino-l-methyl-6-phenylimidazo[4,5-b]pyridine |

| | |
|---|---|
| PVDF | polyvinylidene fluoride |
| RH | substrate |
| ROH | hydroxylated substrate |
| RPM | revolutions per minute |
| $s.c._{CYP}$ | specific content of cytochrome P450 |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SPF | specified pathogen free |
| TCDD | 2,3,7,8-tetrachlordibenzo-p-dioxin |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| Tris | tris(hydroxymethyl)aminomethane |
| Triton X-100 | t-octylphenoxypolyethoxyethanol |
| UV | ultraviolet |
| V | volume |
| v/v | volume/volume percent |
| w/v | weight/volume percent |
| βNF | β-naphthoflavone |
| ε | molar absorption coefficient |

# 1 INTRODUCTION

## 1.1 Carcinogenesis

The genetic constitution of human populations changes only slightly within centuries and differs only moderately among different parts of the world. Thus, the increased incidence of cancer over time reflects to a large extent the environmental effects.

Chemical carcinogenesis studies the mechanisms through which chemical carcinogens induce cancer. It also involves the development and/or utilization of experimental systems, which are aimed at determining whether a substance is a potential human carcinogen [Hodgson, 2004].

The development of cancer is a complex process in which a large number of factors interact to disrupt normal cell growth and division. Cancer can be caused by internal factors such as heredity, immunology, and hormones as well as external factors such as chemicals, viruses, diet, and radiation (***Tab. 1.1***) [Schulz, 2005].

***Table 1.1*** *Types and examples of human carcinogens.*

| Type of carcinogen | Examples |
|---|---|
| Chemical carcinogens | nickel, cadmium, arsenic, nitrosamines, arylamines, trichloroethylene, B[a]P, aflatoxins, reactive oxygen species |
| Physical carcinogens | UV irradiation (specifically UVB), ionizing radiation |
| Biological carcinogens | Human papilloma virus, Epstein-Barr virus, Hepatitis virus B, *Helicobacter pylori* |
| Endogenous processes | metabolic reactions generating reactive oxygen species, chronic inflammation, DNA replication |

Among the main causes of cancer, lifestyle factors such as diet, cigarette smoke, alcohol, and sun exposure are involved. It is estimated that dietary factors are associated with 35% of all human cancers, while cigarette smoke for another 30%. The development of cancer is a multi-stage process involving damage to the genetic material (DNA) of cells. This damage primarily occurs in genes regulating normal cell growth and division. To develop

cancer, several mutations or several stages are required. Usually, there is a long latent period before cancer appears.

The Czech Republic belongs to countries with the highest incidence of the most frequent malignant neoplasms (besides those of skin), that is, the colorectal carcinoma. Neoplasms are the second most prevalent cause of death in the Czech Republic, the first being cardiovascular diseases. ***Figure 1.1*** shows six categories of malignant neoplasms which are the most frequent causes of deaths by cancer. The highest increase in the number of deaths by cancer is due to prostate malignancy. In the male population, the second highest increase in deaths is due to colon cancer [http://www.czso.cz].

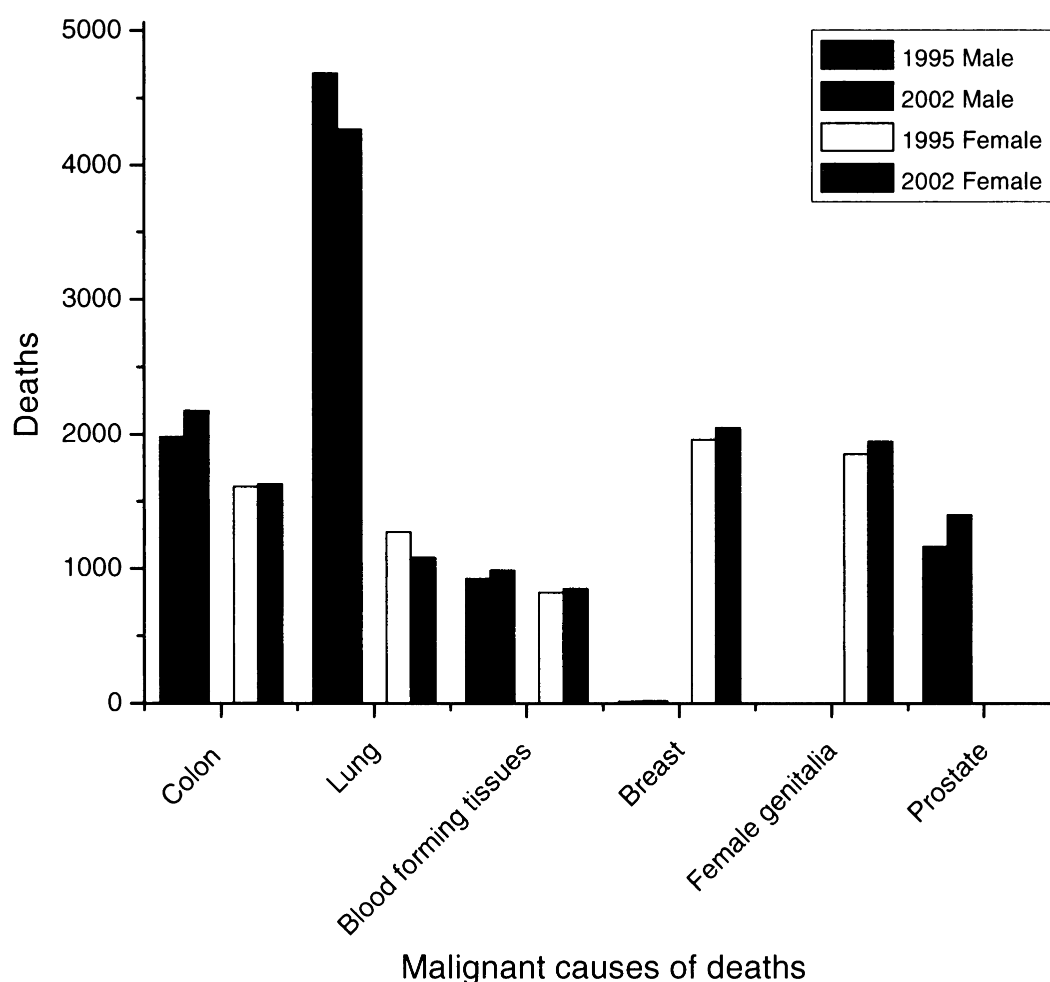



***Figure 1.1** Number of deaths by cancer. Six categories of the most frequent causes of deaths in the male and female population of the Czech Republic in 1995 and 2002. Data source: Czech Statistical Office.*

## 1.2 Biotransformation of xenobiotics

Organisms in the environment are surrounded by a large number of foreign compounds (xenobiotics) which are potentially harmful. Biotransformation can be defined as an enzyme-catalyzed conversion of one xenobiotic compound into another. This process consists of two phases. Phase I reactions include hydrolysis, reduction, and oxidation, while phase II reactions are usually conjugation reactions. The metabolism of xenobiotics is shown in ***Figure 1.2***.

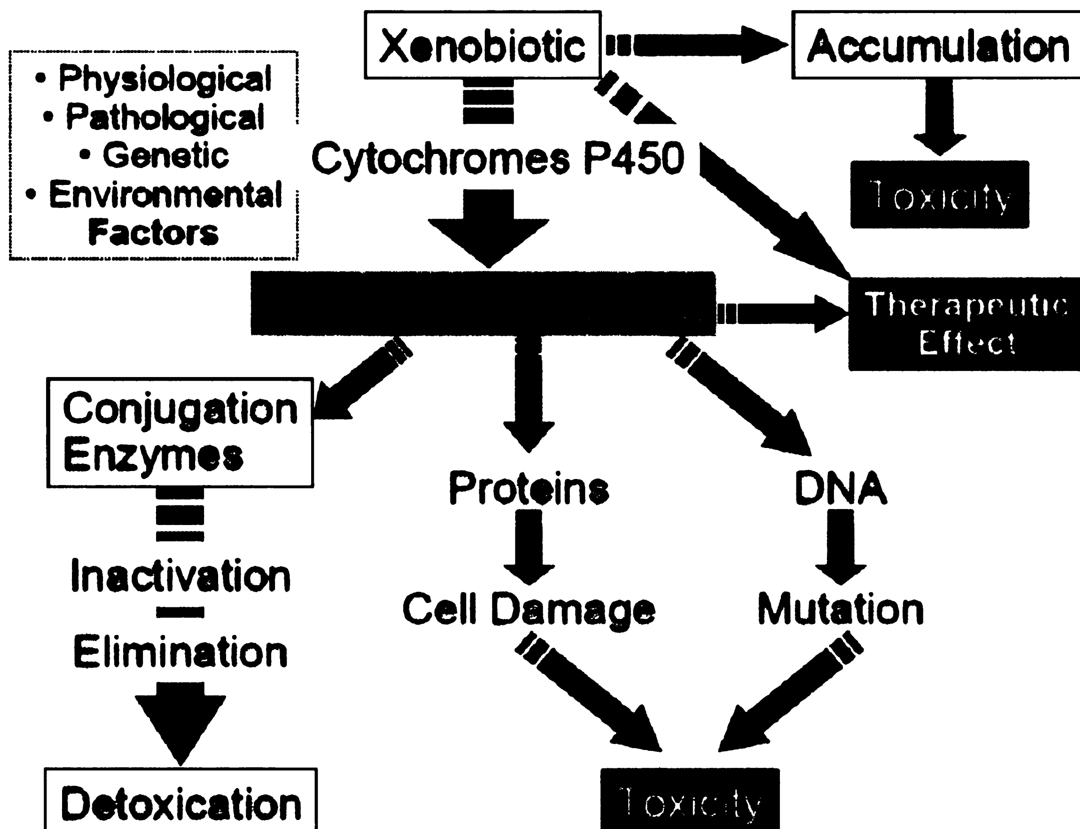



***Figure 1.2*** *The metabolism of xenobiotics. Adapted from Maurel [1996].*

In general, the first phase of biotransformation, known as detoxification, leads to more polar compounds that may be more easily excreted from the organism. On the other hand, biotransformation can be also responsible for the metabolic activation of some substrates to reactive intermediates (e.g. free-radicals), which then react with cellular macromolecules to initiate toxic and carcinogenic events [Guengerich and Shimada, 1991]. Major enzymes

involved in phase I are for example cytochromes P450, flavin-containing monooxygenases, peroxidases, NADPH:cytochrome P450 reductase, etc.

In phase II, xenobiotics with polar functional groups are conjugated with water-soluble agents by sulphation, glucuronidation, acetylation, methylation, and are conjugated with glutathione and amino acids. These conjugations result in deactivation and excretion of xenobiotics. The most important enzymes of phase II are glutathione *S*-transferase, UDP-glucuronosyltransferase, sulphotransferase, *N*- and *O*-acetyltransferase. However, phase II enzymes involved in conjugations are not only protective but also activate chemical carcinogens. Examples of bioactivation roles are known for *N*-acetyltransferase [Grant et al., 1992], sulphotransferase [Boberg et al., 1983], and UDP-glucuronosyltransferase [Kaderlik et al., 1994].

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

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

## 1.3 Cytochromes P450

Cytochromes P450 (EC 1.14.14.1) are enzymes playing an important role in the metabolism of foreign compounds (drugs, pollutants, carcinogens) and they also participate in the metabolism of hydrophobic endogenic substrates (sterols, prostaglandins, fatty acids) [Gonzalez and Gelboin, 1994; Nebert and Dieter, 2000].

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

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

Cytochromes P450 are part of the mixed-function oxidase (MFO) enzyme systems, which are further composed of NADPH:cytochrome P450 reductase and phospholipid. The MFO catalyzes a wide variety of reactions including epoxidations, *N*-dealkylations, *O*-dealkylations, *S*-oxidations, and hydroxylations of aliphatic and aromatic substrates. In this system, cytochrome P450 is the terminal oxidase.

### 1.3.1 Nomenclature of cytochrome P450

A nomenclature that categorizes the individual CYPs into respective families and subfamilies, based primarily on sequence similarities, has been adopted. Enzymes exhibiting >40% similarity in protein sequence are classified within the same family and are designated by an Arabic number (CYP1). Those with sequence identity greater than 55% belong to the same subfamily and are designated by a capital letter (CYP1A). The individual isoform is then identified using a second Arabic numeral showing the subfamily designation (CYP1A1) [Nebert and McKinnon, 1994; Nelson et al., 1996]. Genes in the same subfamily have been found to lie on the same chromosome within the same gene cluster [Hodgson, 2004].

There are now more than 2500 cytochrome P450 sequences known. Humans have 18 families of CYP genes and 43 subfamilies. Moreover, there are 57 sequenced CYP genes and 58 pseudogenes in human genome [Nelson, 2003]. The list of CYPs is continually expanding.

Progress in this area can be accessed at the Internet site of the P450 Gene Superfamily Nomenclature Committee [http://drnelson.utmem.edu/CytochromeP450.html].

Although mammals are known to have 18 CYP families, only three families (families CYP1-3) are primarily responsible for most xenobiotic metabolism. The remaining families are less promiscuous in their metabolizing abilities and are often responsible for specific metabolic steps [Hodgson, 2004].

## 1.3.2 Chemical structure of cytochrome P450

Cytochromes P450 are hemoproteins consisting of apoprotein and heme b (***Fig. 1.3***). The heme iron is located in the center of the protoporphyrin ring, bound to the four pyrrole nitrogen atoms. Above and below the plane of the heme, there is room for two ligands. One ligand is a thiolate anion provided by a cystein residue [Poulos et al., 1985] and other (axial) ligand in cytochrome P450 enzymes is most probably an OH group from a water molecule or hydroxide ion [Dawson et al., 1982].

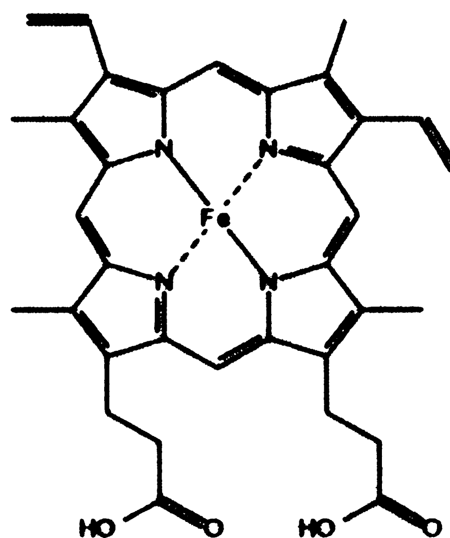


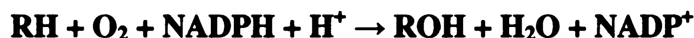
***Figure 1.3*** *Structure of heme b.*

The $Fe^{3+}$ ion exists in the pentacoordinated (high-spin) and the hexacoordinated (low-spin) state. These forms are usually in equilibrium and possess different spectral properties [Jefcoate, 1978]. Changes in the spin state of hemoproteins are usually caused by the change in ligand state of the heme iron. Interaction with a hydrophobic substrate or a ligand can lead to a shift from low-spin to high-spin state. In this interaction, displacement of a water molecule by the substrate might lead to the movement of the iron ion out of the plane of the porphyrin ring, which makes the high-spin configuration favorable [Poulos et al., 1986; Lewis et al., 1989].

## 1.3.3 Cytochrome P450 catalytic cycle

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

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



$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

where RH is a substrate and ROH its hydroxylated metabolite.

The catalytic cycle for CYP-catalyzed reactions probably consists of at least eight discrete reactions (***Fig. 1.4***).

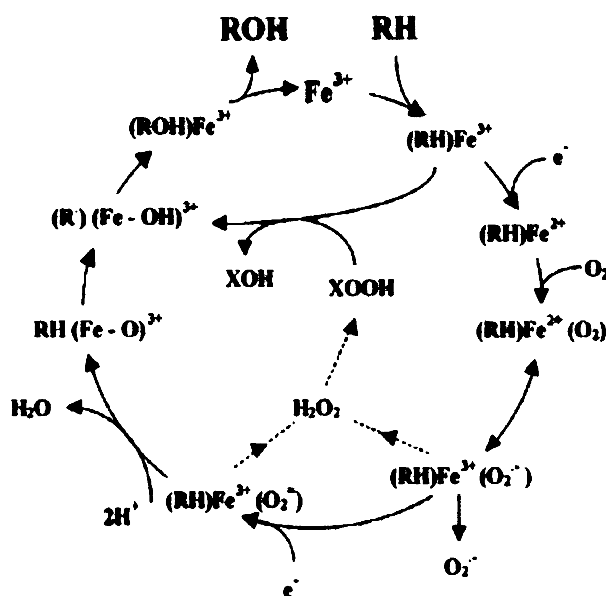



***Figure 1.4*** *Catalytic cycle of cytochrome P450.*

1) The first step in the reaction cycle is the binding of the substrate to the ferric ($Fe^{3+}$) form of the enzyme, which pertrubes the spin state equilibrium of the cytochrome and facilitates uptake of the first electron.
2) The second step involves the transfer of one electron from the NADPH:cytochrome P450 reductase to the iron of the ferric CYP enzyme to give a ferrous ($Fe^{2+}$) enzyme-substrate complex.
3) The reduced CYP-substrate complex then binds $O_2$ to form a ferrous enzyme-$O_2$-substrate ternary complex with the $O_2$ bound to the iron.
4) The addition of a second electron to this ternary complex by the reductase results in the formation of a more stable ferri-superoxide complex $Fe^{3+}O_2^-$.

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

### 1.3.4 Induction of CYP

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

One type of CYP gene regulation is associated with peroxisome poliferators. Drugs act through a binding protein or peroxisome proliferator activated receptor. When drug is bound to this protein, it migrates to the nucleus, heterodimerizes with retinoid X receptor (RXR) and binds to specific DNA sequences in the regulatory region of genes that are needed for peroxisome generation.

Another type is the tight regulation of the steroid hormones levels, which are under strict endocrine control. One example is the induction of steroid biosynthetic CYPs by adrenocorticotropic hormone, which stimulates production of cAMP.

The enzymes of the CYP1 family are induced by aromatic hydrocarbons. The activation involves a specific receptor called the Ah receptor. After binding of an inducing agent to the receptor, heterodimer with the Ah-receptor nuclear transporter (ARNT) is

formed. This heterodimer then binds to a xenobiotic response element (XRE) and functions as a transcriptional enhancer to stimulate gene transcription.

The most extensively studied inducers of CYP1A subfamily *via* AhR are the halogenated aromatic hydrocarbons (TCDD), polychlorinated biphenyls, as well as PAHs such as B[a]P and 3-MC.

Some other chemicals also induce CYPs in a different way. For instance, ethanol induces the CYP2E enzymes. Phenobarbital induces CYP2B enzymes 40-50 fold, through a phenobarbital receptor called CAR [http://drnelson.utmem.edu/P450lect.html; Hollenberg, 2002; Ortiz de Montellano, 2005].

For induction of cytochromes P450 by flavonoids see Chapter 1.4, p 19.

## 1.3.5 Inhibition of CYP

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

The nature of the catalytic cycle of the CYP enzymes (see Chapter 1.3.3, p 12) presents a number of potential points at which inhibition of substrate metabolism can occur. The three steps that appear to be particularly susceptible to inhibition are:

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

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

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

A second category of inhibitors require catalytic activation by the enzyme to transient intermediates that then coordinate tightly to the prosthetic heme in the CYP active site leading to inhibition.

A final category of inhibitors require metabolic activation by the CYPs and are part of a class of inhibitors commonly referred to as „catalysis-dependent", „suicide", or „mechanism-based" inactivators.

The insight into all three enzyme categories is very important for the development of highly selective isozyme-specific inhibitors of the CYPs. For studies probing the structure, mechanisms of action, and biological roles of specific CYPs, these inhibitors are of substantial interest. They can also be used as therapeutic agents, because of their potential as modulators of CYP activity [Hollenberg, 2002].

### 1.3.6 CYP1A

The subfamily CYP1A contains two members CYP1A1 and CYP1A2. They belong to the most important CYP enzymes involved in the activation of procarcinogens, such as polycyclic aromatic hydrocarbons, aromatic amines and heterocyclic amines [Eaton et al., 1995; Rendic and Di Carlo, 1997]. ***Table 1.2*** shows their substrates, inducers and inhibitors.

***Table 1.2*** *Substrates, inducers and inhibitors of CYP1A1 and CYP1A2.*

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

Data source: [http://cpd.ibmh.msk.su].

CYP1A1 and CYP1A2 exhibit ~70% protein sequences identity in their coding region. Expression of CYP1A1 has been reported in a number of extrahepatic tissues including pancreas, lung, gastrointestinal tract, thymes, prostate, uterus, and mammary glands [Ortiz de

Montellano, 2005]. In contrast, CYP1A2 is primarily expressed in the liver at significant levels [Dogra et al., 1998]. Both of these enzymes are inducible *via* Ah receptor [Gonzalez et al., 1993].

## 1.4 Flavonoids

### 1.4.1 Introduction

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

The major sources of flavonoids are fruits (e.g. citrus fruits, rose hips, apricots, cherries, grapes, black current), vegetables (e.g. onion, broccoli, tomatoes, pepper, spinach), soy beans and herbs (e.g. *Sylibum marianum*, *Alpinia officinarum*, *Hypericum perforatum*) [Barnes et al., 2001]. Flavonoids are also concentrated in seeds, grains, nuts and flowers, as well as in beverages, such as red wine, tea and beer [Kuhnau, 1976].

### 1.4.2 Structure

The chemical structure of flavonoids is based on an oxygenous heterocyclic compound flavane (***Fig. 1.5***), which consists of 15 carbon atoms arranged in three rings (C6-C3-C6) labeled as A, B and C. Chromane ring (A+C) can bear a second aromatic ring B in position 2 (flavonoids), 3 (isoflavonoids) or 4 (synthetic flavonoids). The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a group differ in the pattern of substitution of the ring A and B. The large number of compounds arises from various combinations of multiple hydroxyl and methoxyl groups substituting the basic flavonoid skeleton [Pietta, 2000].

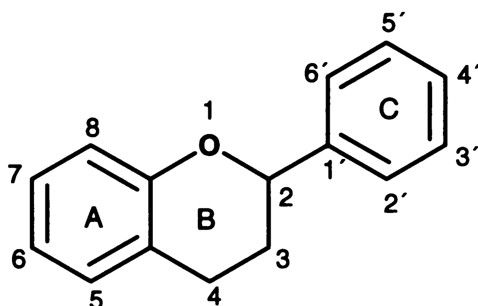


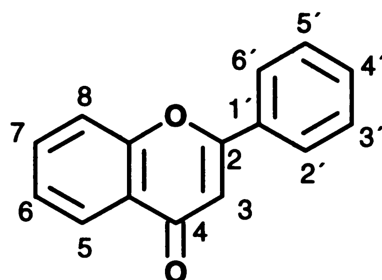

***Figure. 1.5*** *Structure of flavane.*

Natural flavonoids usually occur as β-*O*-glycosides or β-*C*-glycosides, where the linked sugar is often galactose, glucose, rhamnose, arabinose or other sugars [Nikolic et al., 2004]. Most commonly there is one sugar, but can be two or three, and there are several possible positions of substitution [Scalbert and Williamson, 2000]. Free flavonoids occur only rarely.

Flavonoids can be divided into several groups according to the degree of oxidation of the heterocycle, into flavones (***Tab. 1.3***), flavonols (***Tab. 1.4***), flavanones (***Tab. 1.5***), isoflavones (***Tab. 1.6***), isoflavanones, catechins, anthocyanidins, and chalcones. Flavones differ from flavanones by the presence of a C2-C3 double bond on the C ring.

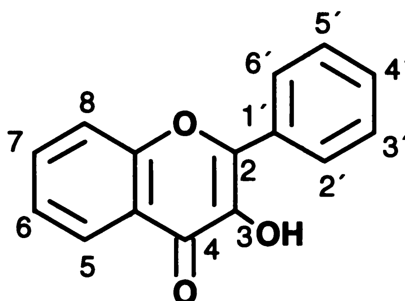
The most abundant flavonoids are the flavonols quercetin and kaempferol, which are present in many fruits and vegetables as well as in beverages. Soy beans are the main source of isoflavones containing approximately 1 mg of daidzein and genistein per g of dry beans [Reinli and Block, 1996]. The main food sources of flavanones are citrus fruits, hesperidin from oranges being the most consumed [Rousseff et al., 1987].

***Table 1.3*** *Flavones.*



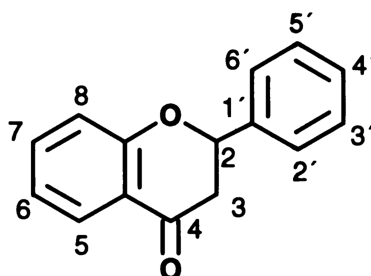

| Common name | C3´ | C4´ | 3 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| Flavone | H | H | H | H | H | H | H |
| Apigenin | OH | H | H | OH | H | OH | H |
| Chrysin | H | H | H | OH | H | OH | H |
| Diosmin | OH | OCH3 | H | OH | H | rutinose | H |
| Diosmetin | OH | OCH3 | H | OH | H | OH | H |
| Tangeretin | OH | OCH3 | H | OCH3 | OCH3 | OCH3 | OCH3 |
| Luteolin | OH | OH | H | OH | H | OH | H |
| Baicalin | H | H | H | OH | OH | glucuronide | H |
| Baicalein | H | H | H | OH | OH | OH | H |
| Acacetin | H | OCH3 | H | OH | H | OH | H |

***Table 1.4*** *Flavonols.*



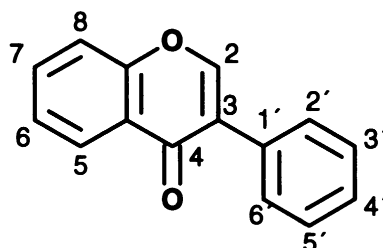

| Common name | C3´ | C4´ | 3 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| Flavonol | H | H | OH | H | H | H | H |
| Fisetin | OH | OH | OH | H | H | OH | H |
| Kaempherol | H | OH | OH | OH | H | OH | H |
| Quercetin | OH | OH | OH | OH | H | OH | H |
| Rutin | OH | OH | rutinose | OH | H | OH | H |
| Galangin | H | H | OH | OH | H | OH | H |
| Rhamnetin | OH | OH | OH | OH | H | OCH3 | H |

***Table 1.5** Flavanones.*




| Common name | C3′ | C4′ | 3 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| Flavanone | H | H | H | H | H | H | H |
| Hesperetin | OH | OCH3 | H | OH | H | OH | H |
| Hesperidin | OH | OCH3 | H | OH | H | rutinose | H |
| Naringenin | H | OH | H | OH | H | OH | H |
| Naringin | H | OH | H | OH | H | rutinose | H |

***Table 1.6** Isoflavones.*




| Common name | C3′ | C4′ | 2 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| Daidzein | H | OH | H | H | H | OH | H |
| Genistein | H | OH | H | OH | H | OH | H |

## 1.4.3 Metabolism

There are two major sites of flavonoid metabolism (***Fig. 1.6***) - liver and intestines. In the first step of metabolism, flavonoid glycosides are degraded and the linked sugar is removed by enzymes glycosidases. Resulting aglycones are expected to be able to pass through the gut wall. These enzymes are present in the food, in the cells of gastrointestinal mucosa or can be secreted by the colon microflora. The colon has an enormous catalytic and hydrolytic potential. However, some flavonoids (e.g. quercetin-3-*O*-rhamnoside) are not hydrolyzed by human enzymes but are readily hydrolyzed by the colon microflora to aglycone

(quercetin). After hydrolysis of a flavonoid derivative to the free aglycone, flavonoids are conjugated in the liver by methylation, sulphatation, glucuronidation or their combination.

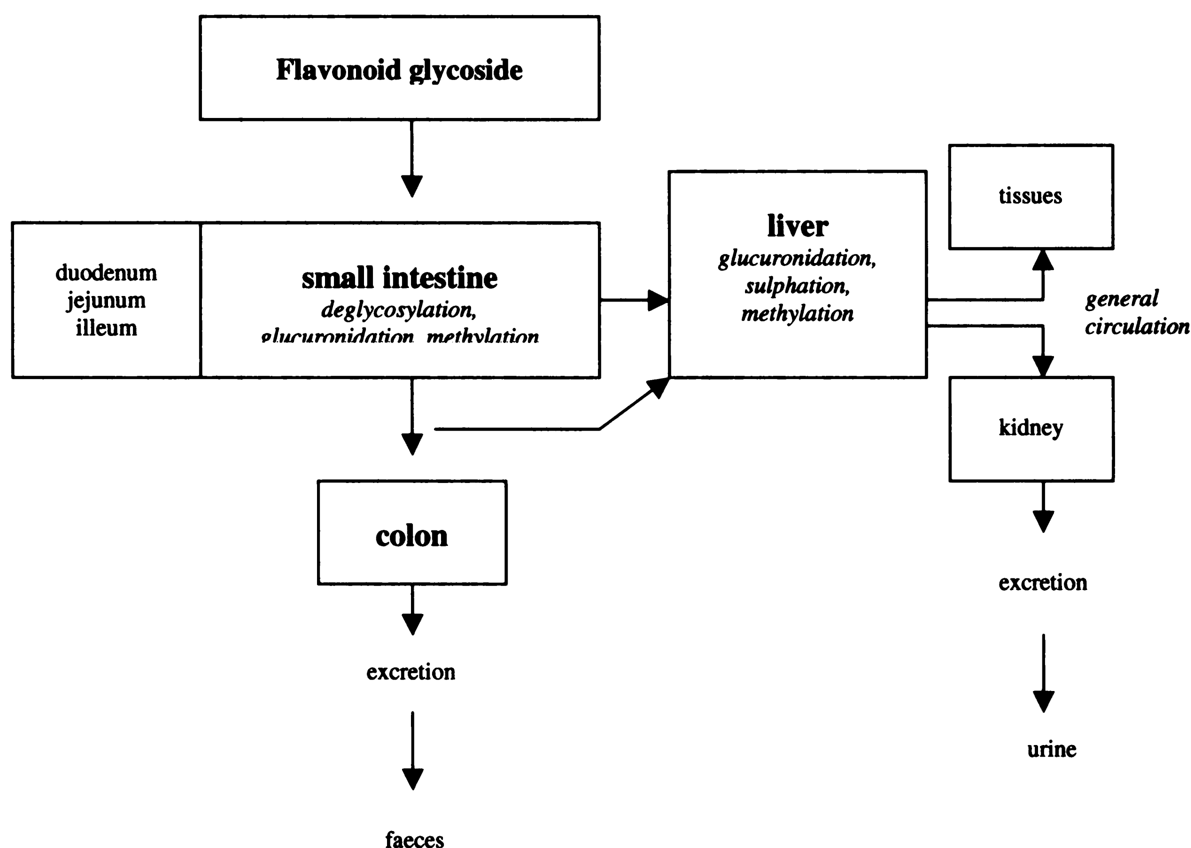



***Figure 1.6** Sites of conjugation and metabolism of flavonoids. Adapted from Rice-Evans [2001].*

The subsequent degradation of a flavonoid skeleton occurs mainly in the gut, where degradation products, phenolic acids (***Tab. 1.7***), can be absorbed and therefore can be found in urine of animals. [Kuhnau, 1976; Scalbert and Williamson, 2000; Rice-Evans, 2001].

***Table 1.7** Metabolites of flavonoid glycosides by human intestinal bacteria [Kim et al., 1998].*

| Flavonoid glycoside | Metabolite (aglycone) | Metabolite (phenolic acid) |
|---|---|---|
| Naringin | Naringenin | 4-hydroxybenzoic acid |
| | | phloroglucinol |
| | | 2,4,6-trihydroxybenzoic acid |
| | | 4-hydroxyphenylacetic acid |
| Hesperidin | Hesperetin | resorcinol |
| | | phloroglucinol |
| | | 2,4-dihydroxyphenylacetic acid |

### 1.4.4 Biological activities of flavonoids

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

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

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

Greater dietary intake may contribute to the prevention and treatment of the above mentioned diseases. Total dietary intake has been estimated to be 1 g/day, but can vary widely [Kuhnau, 1976]. Flavonoid inhibition of the immune and inflammation responses can be associated with the inhibition of various enzymes (e.g. protein tyrosine kinase, phospholipase, cyclooxygenase) that can partly regulate the formation of biological mediators responsible for the activation of endothelial cells and specialized cells involved in inflammation (e.g. T and B lymphocytes) [Middleton and Kandaswami, 1993].

Another important function of flavonoids is their action in plant defence mechanism against microorganisms. Under stress conditions, such as excessive UV light, wounding or infection, the induction of some flavonoids may occur [Bennet and Wallsgrove, 1994].

### 1.4.5 Flavonoids and cytochromes P450

Due to their ability to affect several biochemical pathways in organisms, flavonoids can also play a negative role. One of the mechanism by which these compounds may exert their effects is through the interaction with CYPs. Flavonoids can induce biosynthesis of several CYPs and/or modulate (stimulate or inhibit) their enzymatic activities [Hodek et al., 2002]. The stimulatory or inhibitory activity of flavonoids depends on the flavonoid structure, mainly on the hydroxylation pattern of both the A and the B ring [Huang et al., 1981]. On the other hand, flavonoids are metabolized by several CYP isozymes. Some flavonoids may alter the expression of CYPs (e.g. CYP1A) through binding to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, acting as either AhR agonists or antagonists [Moon et al., 2006]. Depending on the flavonoid concentration, they can exhibit deifferent biological activities. At lower concentrations, interaction of a flavonoid with AhR could prevent expression of CYP1A1 and ultimately decrease the metabolic activation of some carcinogens (antagonist effect) [Tureskey et al., 1991]. On the other hand, at higher concentrations, the same flavonoids might function as AhR agonists modulating gene expression [Hodek et al., 2002].

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

In the processes of carcinogenesis, flavonoids might increase CYP-mediated carcinogen activation by inducing CYPs or by stimulating their activities. Moreover, some flavonoid metabolites could covalently bind to DNA or proteins [Hodek et al., 2002].

### 1.4.6 Flavonoid and aromatase (CYP19)

Aromatase (CYP19) is an enzyme catalyzing aromatization reaction of the ring A of male sex steroids. It occurs in membranes of endoplasmatic reticulum of ovary, breast, testis, prostate and placenta tissues [Simpson, 2000]. Some flavonoids can stimulate or inhibit the activity of this enzyme, resulting in decreasing estrogen biosynthesis and producing antiestrogenic effects, important in breast and prostate cancers. This effect originates from the structural similarity of flavonoids and steroid hormones (estrogens). Thus, flavonoids as the steroidogenic enzyme inhibitors and the estrogen receptor modulators could help in the

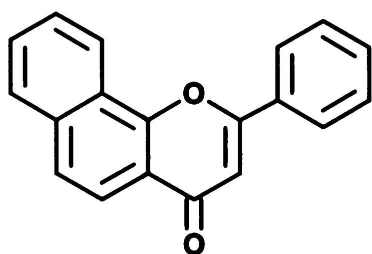
prevention and treatment of some cancers as well as menopausal symptoms [Hodek et al., 2002].

Potent aromatase inhibitors are for instance a naturally occuring flavonoid, chrysin, and some synthetic compounds like α-naphthoflavone and its 9-hydroxy derivative (***Fig. 1.7***). Their inhibitory abilities expressed as $IC_{50}$ (μM) are shown in ***Table 1.8***. Data for 4-hydroxyandrostendione, a strong steroid inhibitor of aromatase [Kellis et al., 1986], are included for comparison.

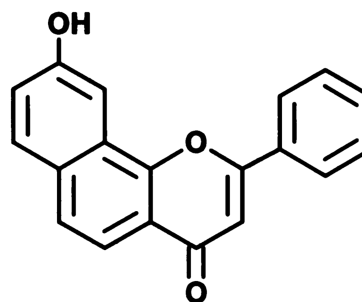
***Table 1.8*** *Characteristics of flavonoid inhibitors of human aromatase (CYP19).*

| Inhibitors | $IC_{50}$ (μM) | References |
|---|---|---|
| 9-hydroxy-α-naphthoflavone | 0,02 | Kellis et al., 1986 |
| α-naphthoflavone | 0,18 | Stresser et al., 2000 |
| chrysin | 0,70 | Stresser et al., 2000 |
| naringenin | 1,54 | Stresser et al., 2000 |
| flavone | 8,0 | Kellis and Vickery, 1984 |
| **4-hydroxyandrostendione** | **0,03** | Stresser et al., 2000 |

α-naphthoflavone




9-hydroxy-α-naphthoflavone




chrysin

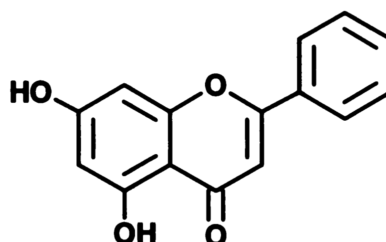
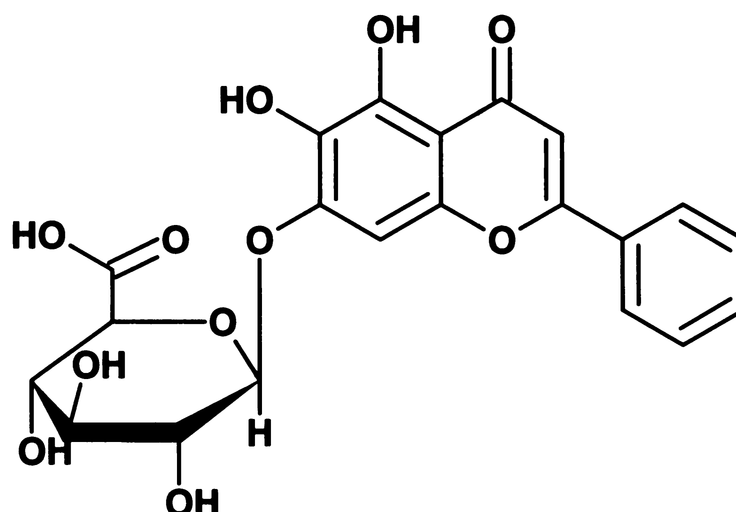



***Figure 1.7*** *Structures of aromatase inhibitors α-naphthoflavone, 9-hydroxy-α-naphthoflavone and chrysin.*

## 1.4.7 Studied flavonoids

### Baicalin

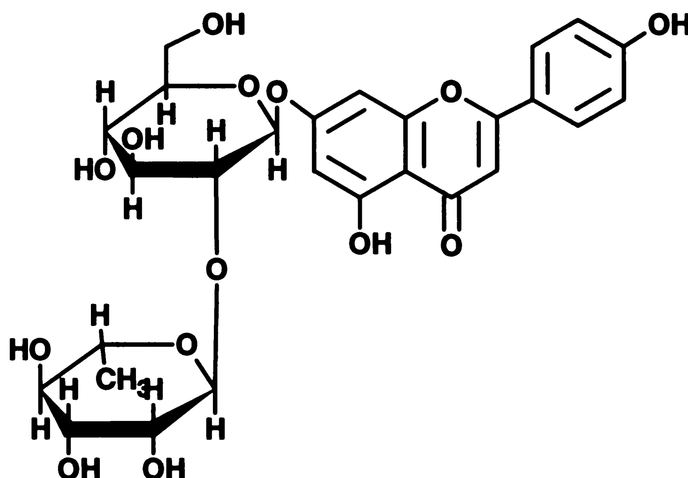



Baicalin (baicalein-7-*O*-glucuronide) and its aglycone baicalein (5,6,7-trihydroxyflavone), belonging to the flavone subfamily of flavonoids, are the main flavonoids in the root of *Scutellaria baicalensis* Georgi. This herb, being used since ancient times to treat bacterial infection, fever and allergic diseases [Shen et al., 2003], is one of the most important drugs in the traditional Chinese and Japanese medicine. Baicalin is also an active compound of drugs for the treatment of epilepsy [Hamada et al., 1993] and acute upper respiratory tract infection, acute bronchitis and light pneumonia [Feng et al., 2005].

Baicalin has been shown as an inhibitor of lipid peroxidation [Kimura et al., 1981], scavenger of several free radicals and protector of erythrocyte membrane [Shih et al., 1995]. Gao et al. (1999) suggested that baicalein and baicalin might be used to cure head injuries associated with free radical assault. In another study, the ability of baicalin to release and chelate iron ions from the liver, which could be used in drugs for the treatment of iron overload diseases, was examined [Zhang et al., 2006].

According to Hou et al. [2000] baicalin is the inducer of several CYPs including CYP1A1 in the mouse liver and enhances the 7-ethoxycoumarin-*O*-deethylase (ECOD) and aryl hydrocarbon hydroxylase (AHH) activities.

## Naringin, naringenin



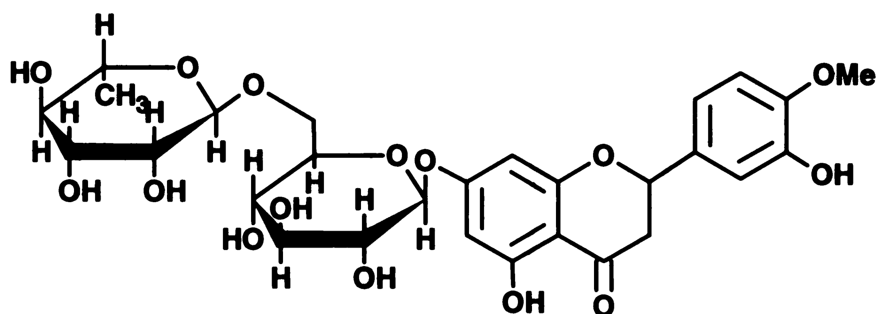

Naringin (naringenin-7-rhamnoglucoside) and its aglycone naringenin (4',5,7-trihydroxyflavanone) are one of the most abundant flavanones in citrus fruits, especially grapefruit, where the naringin is responsible for its bitter taste. Naringin can reach up to 10% of the dry weight of grapefruit and more than 200 mg/l in grapefruit juice [Mouly et al., 1994]. Erlund et al. [2002] estimated that a dietary intake can be as high as 29 mg of naringenin per day.

Both flavonoids possess antiapoptotic [Kanno et al., 2003], antioxidant [Wang and Goodman, 1999], antiproliferative [Manthey and Guthrie, 2002] and weakly estrogenic activities *in vitro* [Miksicek, 1993].

They are also able to protect hemoglobin from nitrite-induced oxidation to methemoglobin [Kumar et al., 2003]. Moreover, naringin enhances the ethanol and lipid metabolism [Seo et al., 2003] and has strong inhibitory activity against LDL oxidation thus it would be important to prevent atherosclerosis [Naderi et al., 2003].

In relation to CYPs, naringenin reduces CYP1A2 protein level and some studies show that it is an inhibitor of CYP1A2, but a very poor inhibitor of human CYP1A EROD activity ($IC_{50}$>4 μM) [Doostdar et al., 2000]. Furthermore, naringenin is effective in inhibiting the proliferation of human breast cancer cells [Fuhr, 1998] and naringin inhibits the activity of CYP isoforms (1A1, 1A2, 2B1, 2D6 and 2E1) activating NNK, a potent enviromental carcinogen to which both smokers and nonsmokers are exposed, and may afford protection against NNK-induced carcinogenesis [Bear and Teel, 2000].

## Hesperidin, hesperetin




The first description of hesperidin, 7-rhamnoglucoside of hesperetin (3',5,7-trihydroxy-4-methoxyflavanone), a major flavonoid in orange juice, was made nearly two centuries ago [Lebreton, 1828].

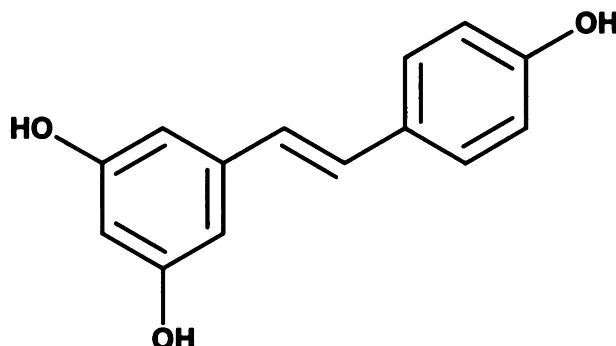
The antiinflammatory effect of hesperidin and another flavonoid, diosmin, is used in "Detralex" (90% diosmin, 10% hesperidin, Servier France), a potent cherapeutical agent for chronic venous insufficiency. Several studies have shown that the antiinflammatory properties of "Detralex" are due to its inhibition of the synthesis and biological activities of different proinflammatory mediators [Manthey et al., 2001].

According to Tanaka et al. [1997], hesperidin significantly reduces the incidence of neoplasms in the rat colon initiated with azoxymethane and also has been found to inhibit rat oral carcinogenesis. Moreover, feeding the hesperedin caused a significant reduction in the frequency of bladder carcinoma.

Levels of urinary excretion suggest that naringenin or hesperetin could be used as specific biomarkers to evaluate the consumption of coffee, wine, tea or cocoa, and citrus juices respectively [Ito et al., 2005].

Hesperetin selectively inhibits human CYP1A1 ($IC_{50}$ 0.92 μM) more than CYP1A2 and is *O*-demethylated by both human CYP1A1 and 1B1 to eriodictyol, which is then further metabolized by the same enzymes [Doostdar et al., 2000].

## Resveratrol




Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) has been isolated for the first time from the root of *Veratrum grandiflorum* [Smidrkal et al., 2001]. It occurs in the vines, roots, seeds, and stalks, but its highest concentration is in the skin of red grapes. As a constituent of red wine it may explain the "French paradox" that the incidence of coronary heart disease is relatively low in southern France despite high dietary intake of saturated fats. The amount of resveratrol in grape skins varies with the grape cultivar, its geographic origin, and exposure to fungal infection [Fremont, 2000]. Other sources of resveratrol are red beet, cabbage, broccoli, onion, and peanuts. 1 mg of resveratrol is contained in approximately 0.4-2.5 kg of fresh vegetables or 0.17-0.50 l of red wine [Smidrkal et al., 2001].

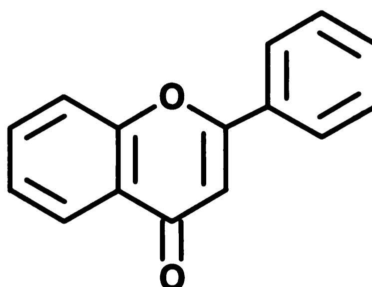
Resveratrol is a natural phytoalexin that is abundantly expressed in plants as a defensive response against viral, microbial, fungal infections and other environmental stressors (mechanical damage, UV radiation, ozone) [Milner et al., 2001].

Moreover, it exerts potent antioxidant, antimutagenic and antiinflammatory effects therefore may be responsible for the beneficial effects of wine consumption in the prevention of cardiovascular diseases [Constant, 1997]. Its ability to inhibit the growth of several cancer cell lines or tumors suggests that resveratrol can also act as an inhibitor of cancer promotion and progression.

Schneider et al. [2000] found that resveratrol exhibits an important antiproliferative effect on CaCo-2 cells, a human colonic cancer cell line. The effect is related to a decrease rate of DNA synthesis. It is also suggested that resveratrol could inhibit DNA polymerase and ribonucleotide reductase, two crucial enzymes involved in DNA biosynthesis.

Several studies suggest that resveratrol acts as an AhR antagonist and inhibits the induction of *CYP1A1* transcription by TCDD [Ciolino et al., 1998; Lee and Safe, 2001].

## Flavone




Flavone (2-phenylchromone) represents the most basic flavonoid skeleton the substitutions of which lead to other flavones, such as chrysin, diosmin, tangeretin or baicalin. In the form of glycosides, it is present in Gingko biloba, which is known for its health benefits on human organism.

Flavone is one of the most active inhibitors of cyclooxygenase, an enzyme involved in the biosynthesis of the arachidonic acid-derived mediators [Rossi et al., 1976], which are involved in stimulating the production of tissue damaging reactive oxygen species at sites of inflammation [Zallen et al., 1998].

In rat liver, the administration of flavone increases the level of the phase II enzymes, such as glutathione transferase (GST) and UDP-glucuronyltransferase (UTP). Furthermore, flavone increases ethoxyresorufin *O*-deethylase (EROD), methoxyresorufin *O*-demethylase (MROD) and pentoxyresorufin *O*-dealkylase activity (PROD). Flavone also induces the metabolism of tangeretin, a flavonoid from tangerine [Siess et al., 1989; Canivenc-Lavier et al., 1996a; Canivenc-Lavier et al., 1996b; Hollman et al., 1996; Siess et al., 1996].

# 2 AIM

Our research is focused on the flavonoid-food carcinogen interactions that might in a long run result in development of cancer in humans. In order to address this effect of flavonoids on cytochromes P450, several specific aims have to be accomplished.

1. Selection of potential CYP1A1 and CYP1A2 inducers of flavonoid structure.
2. Preparation of colon and liver microsomal samples of rats treated *p.o.* with tested flavonoids.
3. Characterization of microsomal samples in respect to protein and CYP concentrations.
4. Western blotting analysis of CYP1A isoforms induction in microsomal samples using a specific chicken anti-rat CYP1A1 antibody
5. Assay of CYP1A1 and 1A2 specific activities, 7-ethoxyresorufin-*O*-deethylase and 7-methoxyresorufin-*O*-demethylase, respectively.

# 3 MATERIALS AND METHODS

## 3.1 Materials

Chemicals used were from:

**Fluka, Switzerland**

tris(hydroxymethyl)-aminomethane (Tris), 2-mercapthoethanol, methanol, sodium dodecyl sulphate (SDS)

**Linde, Czech Republic**

liquid nitrogen, dry ice

**Millipore Corp., USA**

Immobilon-P transfer membrane, Immobilon-NC transfer membrane

**PML a.s., Czech Republic**

Laktino – non-fat dried milk

**Reanal Budapest, Hungary**

glycin

**Serva, Germany**

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

**Setuza a.s., Czech Republic**

Vegetol – sunflower oil

**Sigma–Aldrich, USA**

hesperetin, hesperidin, naringin, naringenin, β-naphthoflavone, flavone, resveratrol, baicalin, ethoxyresorufin, resorufin, methoxyresorufin, nicotinamide adenine dinucleotide phosphate (NADPH), SigmaMarker Wide range (6 500-205 000), bicinchoninic acid (BCA), anti-chicken IgG-alkaline phosphatase conjugate, BCIP/NBT tablets

**Antibodies** against CYP1A1 isoform were kindly provided by Doc. RNDr. Petr Hodek, CSc., Faculty of Science, Charles University in Prague

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

**Whatman, USA**
Whatman paper, No.3

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 32, USA

**Other**

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

## 3.3 Methods

### 3.3.1 Treatment

Male SPF Wistar rats (140-150 g) were acclimatized for 5 days. They were maintained on a *ad libitum* diet and were separated into groups per 3-4 rats. Flavonoids (baicalin, β-naphthoflavone, resveratrol, flavone, naringenin, naringin, hesperetin, hesperidin) were administred by gavage *p.o.* 60 mg/kg body weight, dissolved in sunflower oil (1 ml), daily for 5 consecutive days. The control group was treated only with 1 ml of the sunflower oil. The treated rats were fasted overnight and 24 hours after the last treatment were sacrificed.

### 3.3.2 Isolation of microsomal fractions

Buffer B1: 0.15 M KCl, 0.05 M Tris; pH 7.4; 23.5 mg tocopherol in 0.5 ml methanol/1 l buffer, just before use

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

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

Microsomal fractions were prepared, immediately after sacrificing the rats, by differential centrifugation according to the method of van der Hoeven and Coon [1974] from the colon and whole liver. The colon was removed circa 2 cm under the stomach in a total length of approximately 10 cm and all tissues were placed into the cold buffer B1 until used.

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

The liver and colon tissues were thoroughly washed, weighed, minced with scissors and again washed in about 2 volumes of tissue mass. The prepared tissues were then homogenized with approximately 4 volumes of buffer B1 in a Potter-Elvehjem glass homogenizer. The resulting homogenates were filtered through 3 layers of gauze and centrifuged at 600g for 10 minutes at temperature 0-5°C (centrifuge Janetzki K-23, swing-out rotor 4x70 ml, 2 000 RPM).

Supernatants were collected and the sediments were rehomogenized in a volume equal to 1/4 of the original tissue mass. Rehomogenates were again centrifuged under the same conditions. Subsequently, both supernatants were combined and centrifuged 20 minutes at

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

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

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

### 3.3.3 Bicinchoninic acid protein assay

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

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

The working reagent was prepared by mixing 49 parts of reagent A and 1 part of reagent B.

All the tubes needed for the assay were annealed.

Standards for the calibration curve were prepared by diluting the bovine serum albumin stock solution (1 mg/ml) with working reagent to concentrations 1, 2, 5, 10, 15 and 20 µg/ml in a total volume of 1 ml. The blank contained 20 µl of distilled water instead of the standard BSA.

Liver and colon microsomes were diluted with water in ratio 1:49 and 20 µl of this solution were added to 980 µl of the working reagent.

All prepared standards, samples and the blank were vigorously vortexed and subsequently incubated for 60 minutes in a 60°C water bath.

The absorbance of standards and microsomal samples were measured with cuvettes (1 cm optical path) against the blank at wavelength of 562 nm (SpectroMOM 195 D) and the calibration curve was made from the obtained values for standard BSA dilutions.

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

### 3.3.4 Cytochrome P450 concentration assay

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

Potassium phosphate buffer: 0.1 M $KH_2PO_4$, 2% (v/v) glycerol; pH 7.5

Microsomal samples were diluted 50-times with potassium phosphate buffer in a total volume of 1 ml and approximately 3 mg of solid $Na_2S_2O_4$ were added. Each sample was gently mixed and then divided into two cuvettes, both of 1 cm optical path. After recording the baseline within the range of 401-490 nm, the content of the sample cuvette was carefully bubbled with CO for about 60 seconds and the difference spectrum was measured at the same range of wavelengths.

The concentrations of CYP were calculated:

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

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

## 3.3.5 Specific content of cytochrome P450

The specific content of CYP was calculated from the values of CYP concentration and the values of protein concentration:

$$s.c._{CYP} = c_{CYP}/c_{protein}$$

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

## 3.3.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

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

Buffer A: 0.375 M Tris; pH 8.8; 0.1% (w/v) SDS
Polymerization solution A: 30% (w/v) acrylamide, 0.8% (w/v) BIS, both in buffer A
Buffer B: 0.125 M TRIS; pH 6.8; 0.1% (w/v) SDS, 0.0006% (w/v) bromphenol blue
Polymerization solution B: 30% (w/v) acrylamide, 0.8% (w/v) BIS, both in buffer B
Sample buffer: 0.063 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: see ***Figure 3.1***

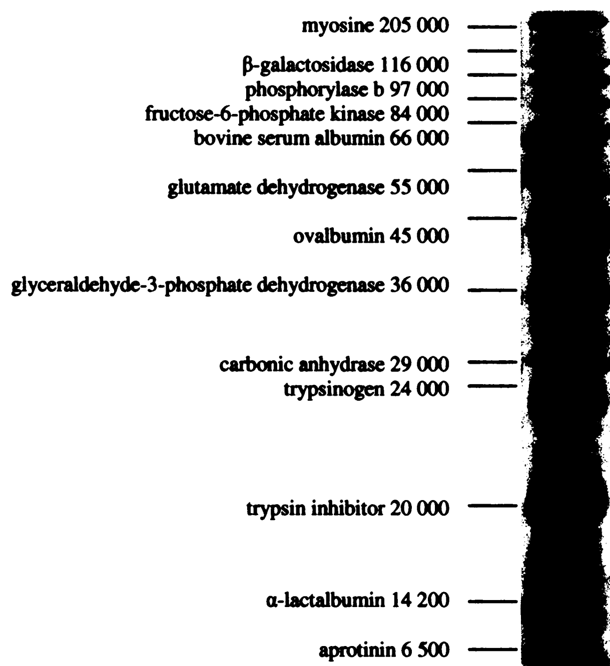



***Figure 3.1** SigmaMarker Wide range.*

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 ml polymerization solution B, 10 μl TEMED, 3 mg sodium peroxodisulphate

Two sets of two glass plates (10x10.5 cm) with side spacers (thickness 1 mm) were assembled in the stands. The resolving gel was applied between the glasses in both of the sets, immediately stacked with water and allowed to set for 30 minutes at room temperature. Overlay water was then removed and the stacking gel was poured onto top of the resolving gel, the comb (well-former) was inserted and the gel was again allowed to set for 15 minutes at room temperature. Subsequently, the upper chamber of the stand with the gel plate was filled with the electrode buffer and the comb was removed.

Microsomal samples were diluted with water to the final concentration of protein 1 mg/ml and then were diluted with the 2x concentrated sample buffer 1:1. SigmaMarker Wide range was diluted 1:1 with the 2x concentrated sample buffer. All samples and the marker were incubated for 5 minutes at 100°C in a water bath.

Samples (15 μl) and the marker (10 μl) were loaded to the gel wells using a Hamilton syringe.

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

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

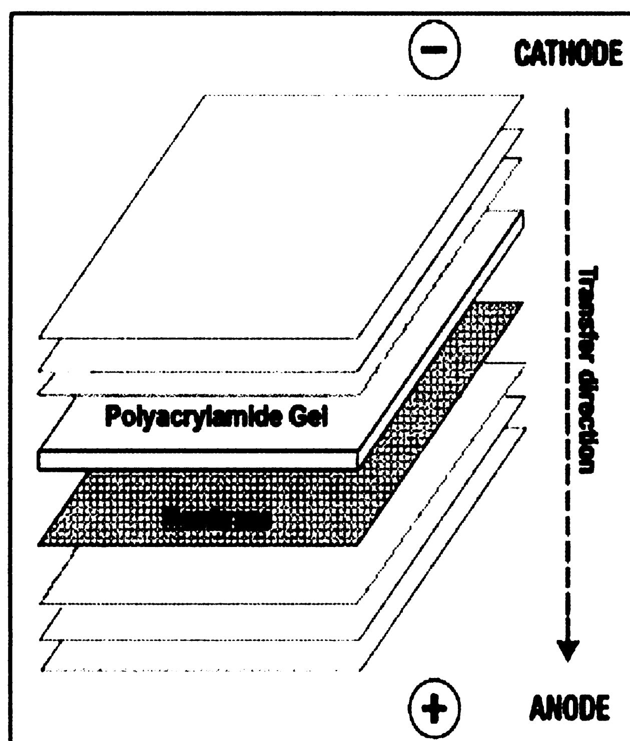
### 3.3.7 Western blotting

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

Transfer buffer: 0.025 M Tris, 0.192 M glycin; pH 8.3
PBS Triton X-100: 0.134 M NaCl, 1.8 mM $Na_2HPO_4.10H_2O$, 1 mM $NaH_2PO_4$; pH 7.2; 0.3% (w/v) Triton X-100
Blocking solution: 5% (w/v) non-fat dried milk in PBS Triton X-100
Staining and destaining bath: see page 39

Following the electrophoresis, the stacking gel was removed and the gel was incubated with the transfer buffer for 30 minutes with shaking. In the meantime, the PVDF/NC membrane was cut to the dimensions of the gel and one corner was cut away to correspond to the labeled corner of the gel. The PVDF membrane was then wet in methanol for 10 seconds, then in water for 10 minutes and finally in the transfer buffer. In the case of NC membrane used, it was laid on the Six Whatman filter papers No.3 cut to the dimensions of the gel were also soaked in the transfer buffer.

Electrodes of a transfer stack were degreased with ethanol and the first three Whatman papers were placed on an anode electrode plate, next the membrane, the gel and again three Whatman papers. The gel/membrane sandwich (***Fig. 3.2***) was rolled over with a pipette to drive out any air bubbles and finally a cathode plate cover was placed on top of the assembled transfer stack. This procedure is called semi-dry Western blotting. Current density was set




according to the membrane surface multiplied by 0.8mA for 10 minutes and multiplied by 2mA for 45 minutes.

When the transfer was complete, the gel was stained for 30 minutes using Comassie brilliant blue R-250 with consecutive destaining to assess the quality of the transfer. The membrane was stained for one minute in 0.5% Ponceau-S in 1% acetic acid. After visualizing the lanes, the marker lane was cut off, stained for 10 seconds in Comassie brilliant blue R-250 and dropped into the destaining bath.

***Figure 3.2** Semi-dry Western blotting. Gel/membrane sandwich.*

The rest of the membrane was then destained for 10 minutes in water and blocked in 5% non-fat dried milk overnight at 4°C to prevent further non-specific binding of proteins.

Next day, the membrane was incubated with the specific primary chicken anti-rat CYP1A1 antibody in the concentration 20 µg/ml for 2 hours with shaking, followed by extensive washing in 5% non-fat dried milk. This antibody diluted to the concentration 0.4 mg/ml was first incubated with pieces of human skin in the fridge overnight. The membrane was then incubated for 1 hour with the secondary rabbit anti-chicken antibody conjugated with alkaline phosphatase, diluted in ratio 1:2 000. Finally, the membrane was washed in 5% non-fat dried milk (3x5 minutes), PBS Triton X-100 buffer (3x5 minutes) and in distilled water (3x5 minutes).

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

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

CYP1A1 and CYP1A2 are the major enzymes that catalyse the deethylation (demethylation) of 7-ethoxyresorufin (7-methoxyresorufin) to resorufin, thus ethoxyresorufin-*O*-deethylase (EROD) and methoxyresorufin-*O*-demethylase (MROD) activity assay were used as a marker for their activity. Modified method described by Burke and Mayer [1974] was used.

Potassium phosphate 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) in methanol
5 mM NADPH

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

All standards and the blank were gently vortexed.

Microsomal samples were diluted with the buffer to the CYP concentration of 0.3-0.7 μM in a total volume of 0.5 ml. Then 5 μl 7-ethoxyresorufin (7-methoxyresorufin) were added and the reaction mixture was preincubated for 3 minutes at temperature 37°C. Next, the samples were gently vortexed and 50 μl NADPH were added. The reaction mixture was then again vortexed and incubated for 10 minutes at 37°C. The reaction was stopped by adding 1 ml methanol and the samples were centrifuged for 5 minutes at 4 000 RPM (Sanyo Microcentaur MSE). Aliquots (150 μl) of the resulting supernatants were placed into 96-well plates and the fluorescence was measured with excitation and emission wavelengths set at 530 nm (slit 15) and 585 nm (slit 10), respectively, using Luminescence Spectrometer PerkinElmer LS55.

Enzyme activities were quantified by comparison with the resorufin standards.

# 4 RESULTS

## 4.1 Isolation and characterization of rat microsomal fractions

Microsomal fractions were isolated from liver and colon tissues of male Wistar rats treated with naturally occurring flavonoids (baicalin, flavone, resveratrol, naringenin, naringin, hesperidin, hesperetin) and with one synthetic flavonoid, β-naphthoflavone. Control group of rats was treated only with sunflower oil. Flavone and control groups consisted of 4 rats, while other groups consisted of 3 rats. The used tissue masses and the microsomal volumes obtained by the isolation procedure are shown in ***Table 4.1*** and ***Table 4.2***.

Microsomal fractions were characterized for protein and CYP concentration, using BCA protein assay and reduced CO complex determination, respectively. From the obtained values, specific CYP contents and tissue CYP contents were calculated (***Tab. 4.1-4.4***).

***Table 4.1 Liver microsomal fractions characterized vs. original tissues.***

| Liver MS fraction | $m_{tissue}$ [g] | $V_{MS}$ [ml] | Tissue CYP content [nmol/$g_{tissue}$] | $c_{protein}$ [$mg_{protein}/g_{tissue}$] |
|---|---|---|---|---|
| β-naphthoflavone | 20.61 | 4.2 | 1.63 | 3.99 |
| Baicalin | 19.71 | 4.4 | 3.99 | 5.92 |
| Flavone | 30.52 | 7.4 | 7.88 | 10.86 |
| Resveratrol | 24.19 | 6.2 | 4.59 | 5.07 |
| Naringenin | 30.21 | 8.0 | 2.28 | 4.85 |
| Naringin | 19.36 | 4.4 | 5.05 | 12.14 |
| Hesperidin | 21.97 | 4.6 | 3.22 | 7.29 |
| Hesperetin | 20.95 | 3.8 | 3.37 | 7.51 |
| Control | 29.69 | 5.0 | 3.05 | 3.13 |

$m_{tissue}$ – used tissue mass, $V_{MS}$ – obtained microsomal volumes, $c_{protein}$ – protein concentration

***Table 4.2 Colon** microsomal fractions characterized vs. original tissues.*

| Colon MS fraction | $m_{tissue}$ [g] | $V_{MS}$ [ml] | $c_{protein}$ [$mg_{protein}/g_{tissue}$] |
|---|---|---|---|
| β-naphthoflavone | 3.60 | 1.0 | 3.14 |
| Baicalin | 3.37 | 0.4 | 0.84 |
| Flavone | 3.96 | 1.3 | 2.92 |
| Resveratrol | 2.96 | 1.3 | 2.33 |
| Naringenin | 3.46 | 1.3 | 2.55 |
| Naringin | 3.26 | 0.8 | 5.23 |
| Hesperidin | 3.24 | 1.1 | 6.65 |
| Hesperetin | 4.23 | 1.1 | 2.42 |
| Control | 4.67 | 1.0 | 0.54 |

$m_{tissue}$ – used tissue mass, $V_{MS}$ – obtained microsomal volumes, $c_{protein}$ – protein concentration

As shown in ***Table 4.1***, the tissue CYP content per $g_{tissue}$ was significantly increased by flavone and naringin in liver microsomes and to a lesser extent by other flavonoids, except β-naphthoflavone and naringenin.

***Table 4.3** Effects of flavonoids on cytochrome P450 level in the **liver** microsomal fractions.*

| Liver MS fraction | $c_{protein}$ [mg/ml] | CYP concentration [μM] | Specific CYP content [$nmol/mg_{protein}$] |
|---|---|---|---|
| β-naphthoflavone | 19.6 | 8.1 | 0.41 |
| Baicalin | 26.5 | 17.9 | 0.68 |
| Flavone | 44.8 | 32.5 | 0.73 |
| Resveratrol | 19.8 | 17.9 | 0.91 |
| Naringenin | 18.3 | 8.6 | 0.47 |
| Naringin | 53.4 | 22.2 | 0.42 |
| Hesperidin | 34.8 | 15.4 | 0.44 |
| Hesperetin | 41.4 | 18.6 | 0.45 |
| Control | 18.6 | 16.3 | 0.87 |

The specific cytochrome P450 content in liver microsomes was not significantly increased by any flavonoid (***Tab. 4.3***). Resveratrol administration caused a slight increase of the specific CYP content in liver microsomes.

***Table 4.4** Effects of flavonoids on cytochrome P450 level in the **colon** microsomal fractions.*

| Colon MS fraction | $c_{protein}$ [mg/ml] | CYP concentration [μM] |
|---|---|---|
| β-naphthoflavone | 11.3 | n.d. |
| Baicalin | 7.1 | n.d. |
| Flavone | 8.9 | n.d. |
| Resveratrol | 5.3 | n.d. |
| Naringenin | 6.8 | n.d. |
| Naringin | 21.3 | n.d. |
| Hesperidin | 19.6 | n.d. |
| Hesperetin | 9.3 | n.d. |
| Control | 2.5 | n.d. |

n.d. – not detectable; CYP content of colon microsomes was under the threshold of the used assay.

## 4.2 Effects of flavonoid treatments on metabolic activity of cytochrome P450 1A1 and 1A2 in liver microsomes

Ethoxyresorufin-*O*-deethylation (EROD) and methoxyresorufin-*O*-demethylase (MROD) activity were used as a marker for the activity of CYP 1A subfamily, the major enzymes catalyzing the deethylation and demethylation of 7-ethoxyresorufin and 7-methoxyresorufin to fluorescent product, resorufin. Enzyme activities were calculated from the regression equation of the calibration curve of resorufin standards. The effects of the selected flavonoids on CYP activities in rat liver microsomes are shown in ***Figure 4.1*** and ***4.2.***

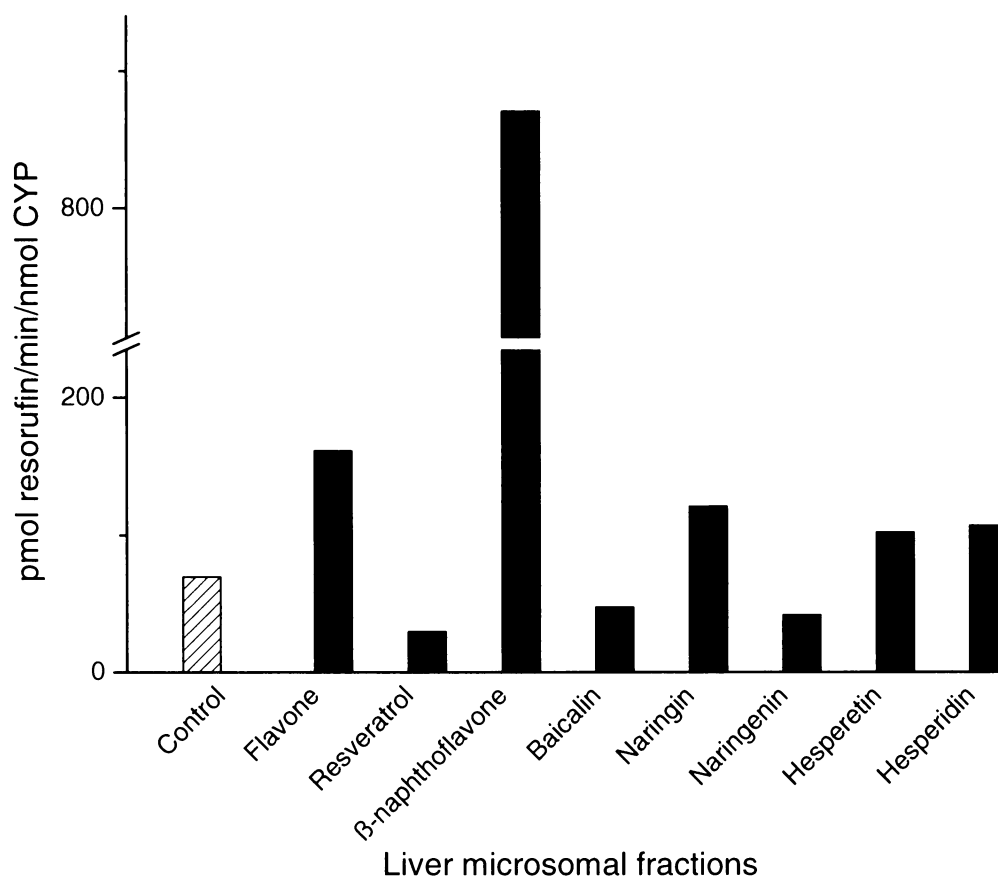



***Figure 4.1*** *Effects of flavonoids on EROD activity of CYP1A subfamily in rat liver microsomes after dietary exposure to flavonoids (60 mg/kg body weight) for 5 days. ±SD≤10%.*

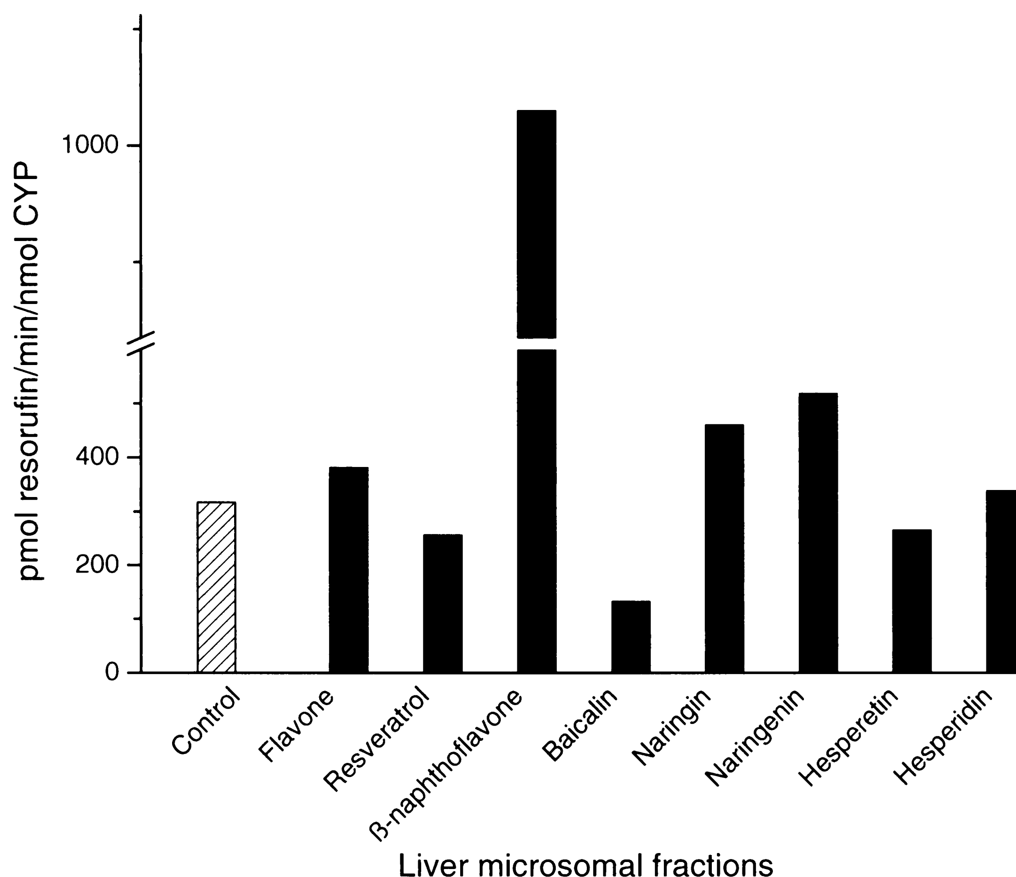



***Figure 4.2** Effects of flavonoids on MROD activity of CYP1A subfamily in rat liver microsomes after dietary exposure to flavonoids (60 mg/kg body weight) for 5 days. ±SD≤10%.*

EROD and MROD activities of CYP1A1 and CYP1A2, specifically induced by β-naphthoflavone (12.4-times, 3.3-times, resp.), was also enhanced by natural flavonoid, flavone. Naringin and hesperidin enhanced to a lesser extent both activities. By contrast, resveratrol and baicalin produced a decrease

## 4.3 Optimization of Western blotting

For introductory experiments, Western blotting was used according to a procedure previously described in the protocols of our laboratory. However, after visualizing the protein transferred to the membrane, stained bands, spots, and vertical streaks occurred in the area

corresponding to relative molecular masses of 50 to 70 000 (***Fig. 4.3***). Since contamination overlaps bands of CYP1A1 and 1A2, the procedure had to be optimized.

***Figure 4.3*** *Contamination of samples. Microsomal samples were diluted with the sample buffer, transferred to the membrane and probed with primary and secondary antibodies. After visualizing the protein, stained bands, spots, and vertical streaks occurred.*

Because of the vertical streaks, the contaminants originated from electrophoresis. Individual components of used buffers were separately loaded to the gel, transferred and probed with the same antibodies. As shown in ***Figure 4.4***, the contaminants originated from the sample buffer, especially from 2-mercaptoethanol. Molecular mass suggested the contaminant in 2-mercaptoethanol to be keratin.

**sample buffer** **2-ME**

***Figure 4.4*** *Keratin contamination of the sample buffer, especially 2-mercaptoethanol (2-ME).*

From 2-mercaptoethanol ultrafiltrated on Microcon (YM-10) and Whatman (30K MWCO) centrifugal filter units by centrifugation at 13 000 RPM for 30 minutes (Sanyo

Microcentaur MSE), the sample buffer was prepared. The subsequent electrophoresis and Western blot showed some amount of contamination still present.

The next step involved the incubation of the primary specific antibody against rat CYP1A1 with pieces of human skin at 4°C overnight. The resulting prepared antibody was then used for detection of CYP1A1 and 1A2 isoforms as described in Chapter 3.3.7, p 40. The disparity between the procedure with and without using prepared antibody is illustrated in ***Figure 4.5***.

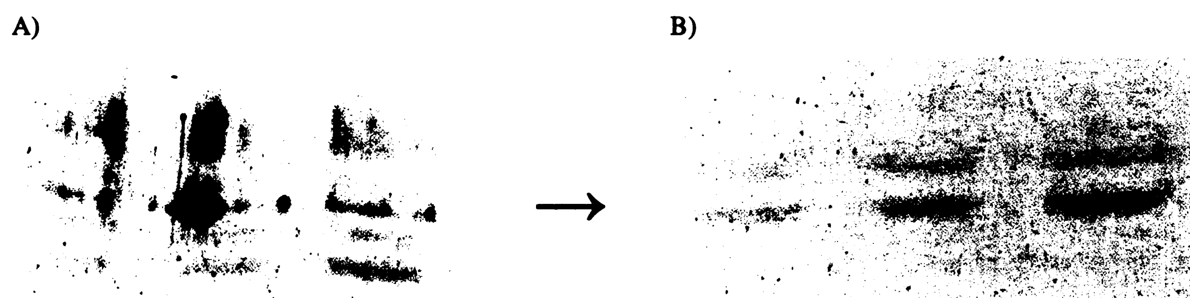



***Figure 4.5** Optimization of Western blotting. Microsomal samples were diluted with the sample buffer containing 2-mercaptoethanol. Membrane was probed without (A) or with (B) antibody incubated with pieces of keratined layer of human skin.*

## 4.4 Effects of flavonoids on CYP1A1 and CYP1A2

Immunoblot analyses were carried out to determine CYP1A protein expression and subsequently to correlate the observed EROD activities with content of the corresponding isoform.

The presence of CYP1A1 and CYP1A2 isoforms in liver and colon microsomal fractions was determined using an optimized Western blotting method. Microsomes were separated into two groups – citrus flavanones (naringenin, naringin, hesperetin, hesperidin) and others (resveratrol, baicalin, β-naphthoflavone, flavone). During electrophoresis, CYP1A1 and CYP1A2 migrated in the gel to the zone of relative molecular mass around 55 000 corresponding to the molecular weight of mammals CYP. The specific primary chicken antibody against CYP1A1 and the secondary antibody conjugated with alkaline fosfatase were used for detection of these isoforms. CYP1A1 and 1A2 can be detected by one primary specific antibody, because they exhibit more than 70% sequence similarity. Results of Western blotting are shown in ***Figure*** **4.7** and ***4.9***.

From the ***Figure 4.6*** and ***4.7*** it is clear that beside the model inducer, β-naphthoflavone, which was used as a positive control, the strongest induction effect on CYP1A1 of all natural flavonoids was determined in flavone-treated rats.

In untreated rats, both CYP isoforms were detected. Weak induction of CYP1A1 and CYP1A2 was observed in resveratrol microsomes (***Fig. 4.6, 4.7***). CYP1A2 was induced by β-naphthoflavone (***Fig. 4.6, 4.7***), naringin and hesperidin (***Fig. 4.8, 4.9***). None of the citrus flavonoids induced CYP1A1 isoform in liver.

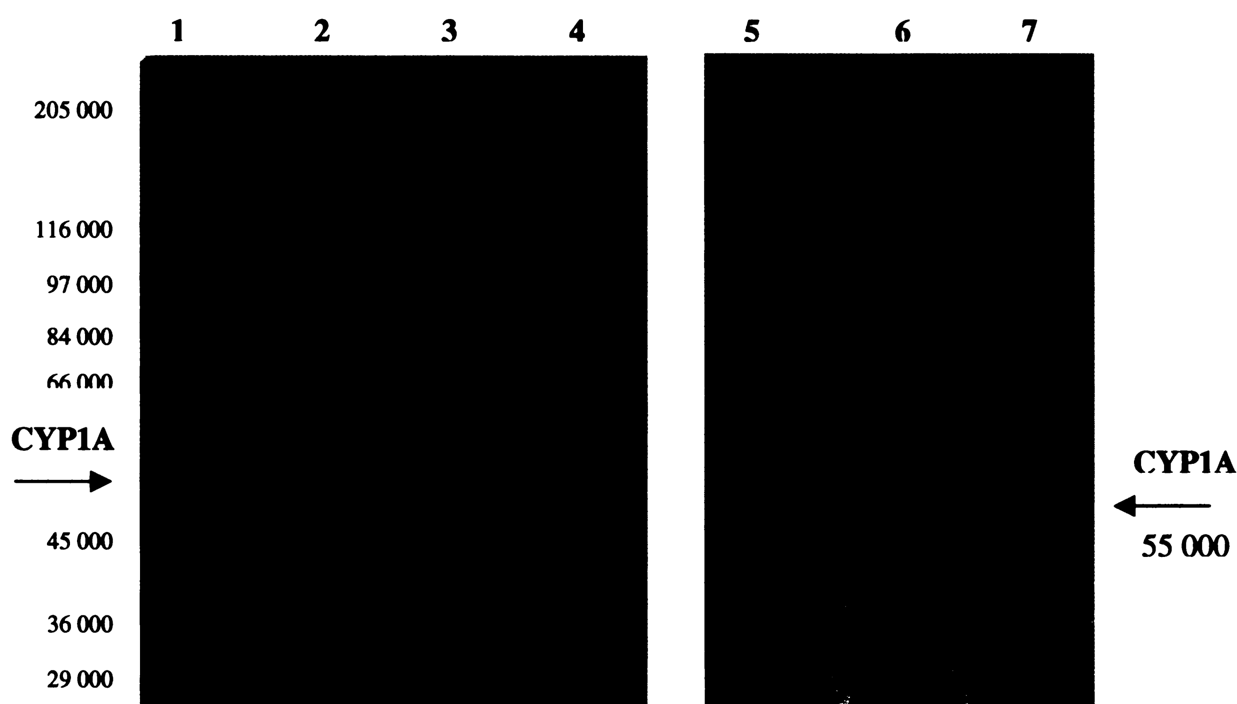



*Figure 4.6 Electrophoresis of liver microsomal fractions from flavonoids-treated rats. Microsomal proteins (7.5 μg) were electrophoresed on 7.5% SDS-polyacrylamide gel and stained. Lane 1: marker; lane 2: flavone; lane 3: control, lane 4: resveratrol; lane 5: control; lane 6: baicalin; lane 7: β-naphthoflavone.*

*Figure 4.7 Immunodetection of CYP1A1 and CYP1A2 in liver microsomes from flavonoids-treated rats. Electrophoresed microsomal proteins were transferred to Immobilon-P membrane and probed with antibody against CYP1A1 and CYP1A2. Lane 1: flavone; lane 2: control; lane 3: resveratrol; lane 4: control; lane 5: baicalin; lane 6: β-naphthoflavone.*

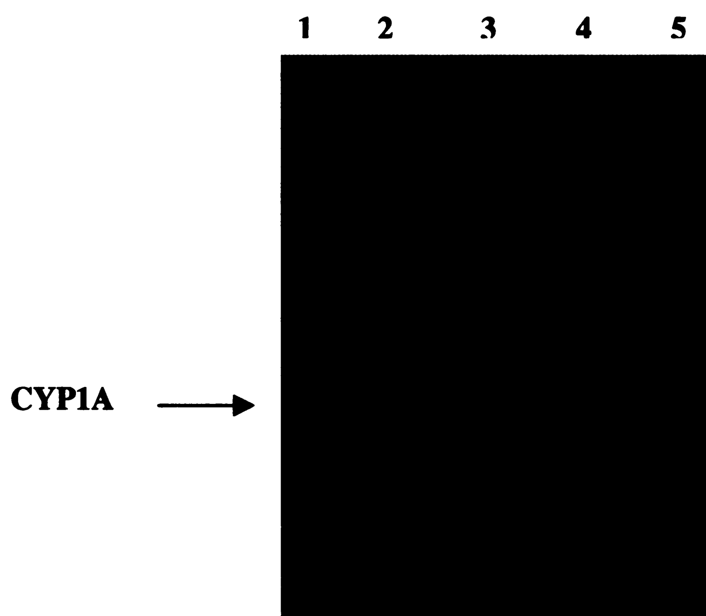



*Figure 4.8 Electrophoresis of liver microsomal fractions from flavonoids-treated rats. Microsomal proteins (7.5 μg) were electrophoresed on 7.5% SDS-polyacrylamide gel and stained. Lane 1: naringenin; lane 2: naringin; lane 3: control; lane 4: hesperidin; lane 5: hesperetin.*

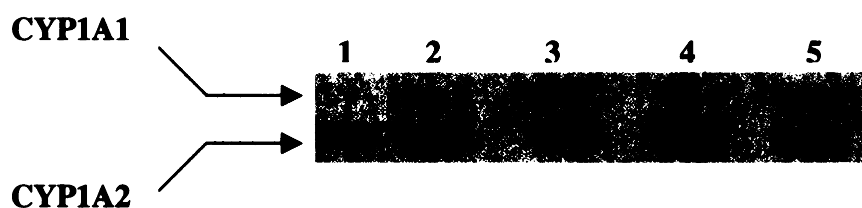



*Figure 4.9 Immunodetection of CYP1A1 and CYP1A2 in liver microsomes from flavonoids-treated rats. Electrophoresed microsomal proteins were transferred to Immobilon-P membrane and probed with antibody against CYP1A1 and CYP1A2. Lane 1: naringenin; lane 2: naringin; lane 3: control; lane 4: hesperidin; lane 5: hesperetin.*

In comparison with liver microsomes, lower induction of CYP1A1 caused by tested flavonoids was detected in colon microsomes. Western blotting performed with resveratrol (***Fig. 4.11***), hesperidin (***Fig. 4.13***) and baicalin (***Fig. 4.11***) colon microsomes showed slight induction of CYP1A1. Naringenin (***Fig. 4.12, 4.13***) and β-naphthoflavone (***Fig. 4.10, 4.11***) signficantly induced the level of CYP1A1. Except of β-naphthoflavone, none of the tested flavonoids induced CYP1A2 in colon.

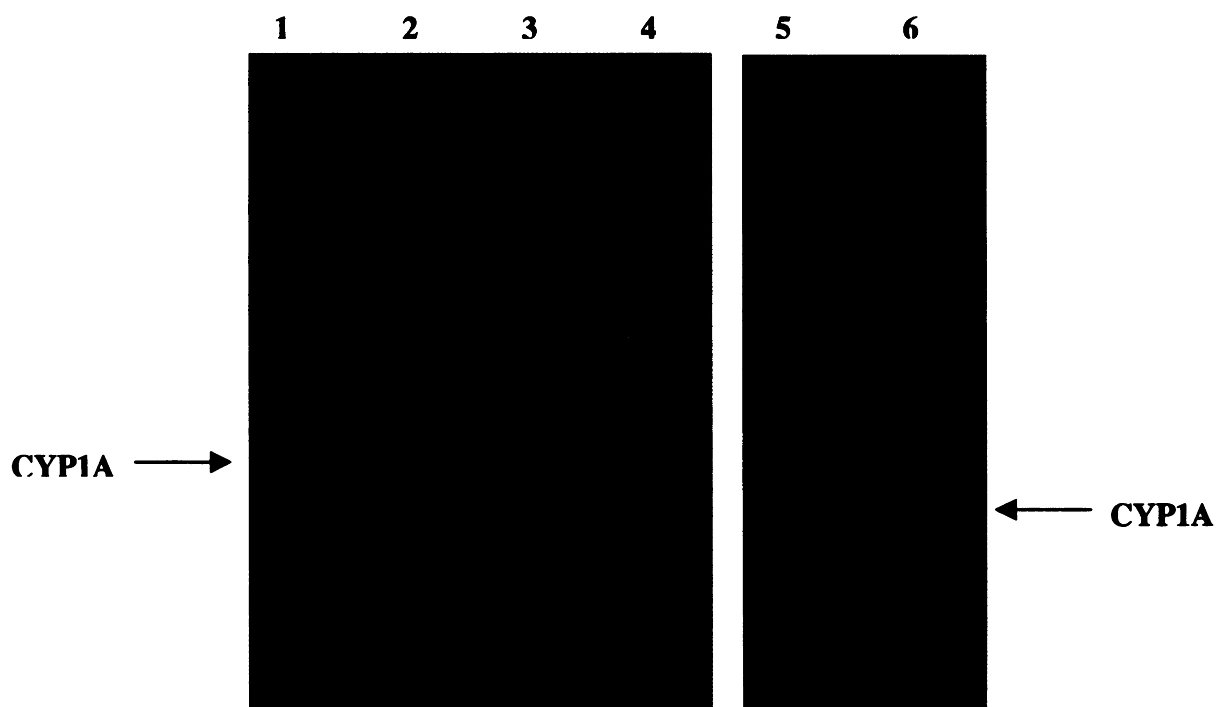



***Figure 4.10*** *Electrophoresis of* ***colon*** *microsomal fractions from flavonoids-treated rats. Microsomal proteins (7.5 μg) were electrophoresed on 7.5% SDS-polyacrylamide gel and stained. Lane 1: marker; lane 2: flavone; lane 3: resveratrol; lane 4: control; lane 5: β-naphthoflavone; lane 6: baicalin.*

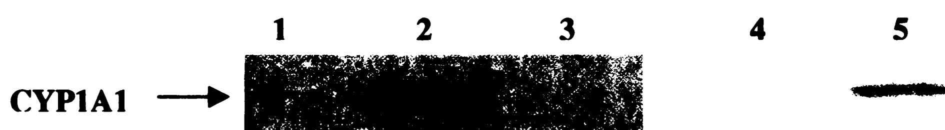



***Figure 4.11*** *Immunodetection of CYP1A1 and CYP1A2 in* ***colon*** *microsomes from flavonoids-treated rats. Electrophoresed microsomal proteins were transferred to Immobilon-P membrane and probed with antibody against CYP1A1 and CYP1A2. Lane 1: flavone; lane 2: resveratrol; lane 3: control; lane 4: baicalin; lane 5: β-naphthoflavone.*

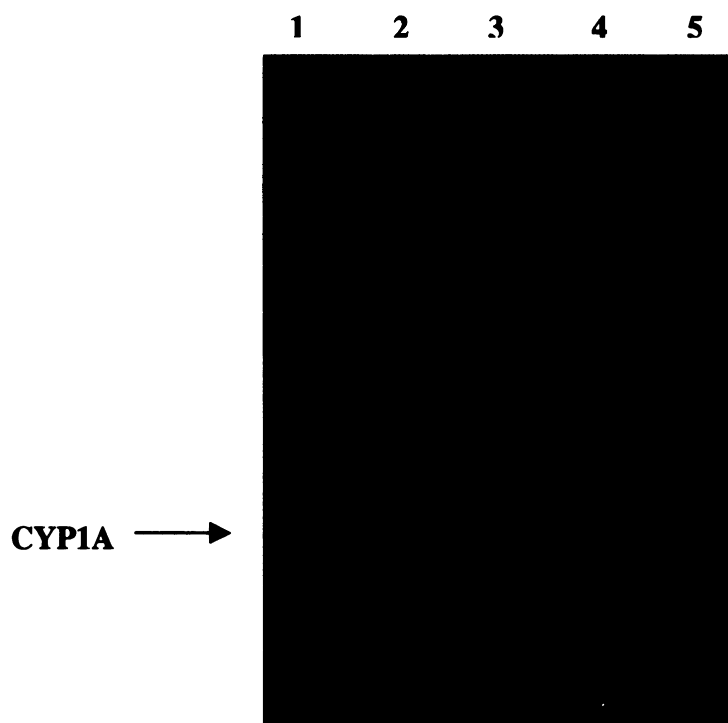



***Figure 4.12** Electrophoresis of **colon** microsomal fractions from flavonoids-treated rats. Microsomal proteins (7.5 μg) were electrophoresed on 7.5% SDS-polyacrylamide gel and stained. Lane 1: naringenin; lane 2: naringin; lane 3: control; lane 4: hesperidin; lane 5: hesperetin.*

***Figure 4.13** Immunodetection of CYP1A1 and CYP1A2 in **colon** microsomes from flavonoids-treated rats. Electrophoresed microsomal proteins were transferred to Immobilon-P membrane and probed with antibody against CYP1A1 and CYP1A2. Lane 1: control; lane 2: hesperidin; lane 3: hesperetin; lane 4: naringenin; lane 5: naringin.*

# 5 DISCUSSION

Flavonoids, naturally occurring phytochemicals, belong to popular compounds exerting a great variety of potential beneficial effects on human health. Extracts containing many flavonoids are now available in health food stores as dietary supplements designed mainly for body building programs or weight management. They are also consumed to support human immune system, and because of their antioxidant and anticancer properties. Although flavonoids are often considered to be safe due to their "plant origin", ingestion of flavonoids should be taken with caution. Flavonoids present in foods (fruits, vegetables, herbs, beverages) and supplements have the greatest potential to modulate activity of xenobiotic-metabolizing enzymes. Severe flavonoid-drug interactions causing an overdose or the loss of the drug therapeutic effects have been reported [Fuhr, 1998; Guo et al., 2000]. Among proteins interacting with flavonoids, cytochrome P450s (CYPs), monooxygenases metabolizing xenobiotics (e.g. drugs, carcinogens) and endogenous substrates (e.g. steroids) play the most prominent role. Flavonoids might inhibit or stimulate activity of several CYPs, and/or induce an expression of certain CYPs.

Although, the gastrointestinal tract is the first barrier met by exogenous compounds (drugs, pollutants, carcinogens), the effects of flavonoids on CYPs in colon have not been extensively investigated until now. The majority of flavonoid-exposure studies have been carried out with simplified systems (liver or colon cell cultures), while the whole body (compound distribution) as well as colon microflora (hydrolyzing flavonoid glycosides) were not taken into account. Moreover, it should be noted that the intestinal epithelium as a complex experimental system is responsible for the metabolic fate of the ingested compound. From animal studies, it is clear that some flavonoids are effective inhibitors of CYPs. In addition, these compounds induce expression of several CYPs (e.g. 1A1, 1A2, 2B1, 2B2) [Canivenc-Lavier et al., 1996b; Jang et al., 2004]. One can expect that flavonoids inhibit activation of a particular carcinogen, however, at the same time they may induce CYPs activating the other carcinogen.

That is why our studies were focused on research of this undesired effect that might develop namely when flavonoid and food carcinogens are not administered simultaneously. The specific aim of the Diploma Thesis was to find potential flavonoid inducers of CYP1A1 and CYP1A2, enzymes involved in the activation of ingested carcinogens. Since the epidemiological data on cancer development due to long term human exposure to dietary

flavonoids are not available, we tried to shed some light on the understanding of the potential flavonoid induction effect on CYPs by experiments conducted with laboratory rats.

Naturally occurring flavones – baicalin and flavone, flavanones – naringenin, naringin, hesperitin, hesperetin and one synthetic flavonoid, β-naphthoflavone, were selected to study the CYP1A1 and/or CYP1A2 induction. Hesperidin - hesperetin and naringin - naringenin, were selected as representatives of flavonoid glycosides and their aglycones. All four flavonoids are major components of citrus fruits, thus their dietary intake is relatively high. In addition, hesperidin is also ingested in the commercially available drug "Detralex", a potent cherapeutical agent for chronic venous insufficiency.

Structural factors of flavonoids play a decisive role in the absorption, binding to Ah-receptor and their metabolism. Hence, variation in the absorption or metabolism could cause different effects of flavonoids on CYP1A subfamily. Recent studies have shown that flavonoid glycosides are the major form in the general circulation after the intake of aglycone. Therefore, the pharmacological properties of glycosides are crucial for the overall beneficial effect of aglycones [Zhang et al., 2005].

To mimic the human flavonoid intake, the tested compounds were administred to male rats by gavage and CYP1A content was determined in isolated liver and colon microsomes. Moreover, the effects of flavonoids on the total microsomal protein and CYP content were examined. Tissue CYP content (nmol CYPs/g) in liver was significantly increased by flavone and to a lesser extent by other flavonoids, except naringenin and β-naphthoflavone. This effect could be attributed to the decrease of one or more CYP(s) along with the increase of others. Because of the much lower amount of CYPs compared to liver, the CYP concentration in colon microsomes was not detectable by the method mentioned in Chapter 3.3.4, p 38. In addition, presence of proteinases in the digestive tract could cause loss of CYP in colon samples during isolation.

To determine whether the tested flavonoids affected the content of CYP1A isoforms, Western blotting was employed. As a severe interference hindering the CYP1A protein detection has occurred, the used technique should be optimized. In the respect of molecular mass, the source of the interference was identified to be keratin contamination present mainly in 2-mercaptoethanol [Paul-Pletzer and Parness, 2001]. Since the ultrafiltration separation of keratin from reagents was not successful, the primary antibody against rat CYP1A1 was incubated with pieces of human skin. This arrangement managed to eliminate the antibody

binding to the keratin contaminants and to clear the area of relative molecular mass around 55 000, where the mammals CYP migrated during electrophoresis.

In accordance with literature data showing in general low CYP concentrations in colon [Obach et al., 2001; Peters et al., 1991], the flavonoid induction of CYP1A1 was shown to be stronger in liver than in colon tissues. CYP1A2 isoform was not detected in any of tested natural flavonoids in colon microsomes. It could be explained by the expression of CYP1A2 mainly in the liver, where CYP1A1 is expressed poorly. The CYP1A1 synthesis can be markedly induced in extrahepatic tissues [Rendic and Di Carlo, 1997]. The CYP1A2 protein is known to be several times more abundant than CYP1A1 protein in control rat liver [Sesardic et al., 1990]. In addition to Western blotting, the induction of CYP1A isoforms was further characterized by determination of their marker activities, 7-ethoxyresorufin-*O*-deethylase (EROD) and 7-methoxyresorufin-*O*-demethylase (MROD) for CYP1A1 and 1A2, respectively.

As expected, β-naphthoflavone treatments led to an increase of CYP1A1 and 1A2 protein levels in both tissues. Moreover, both activities (EROD, MROD) in liver microsomes were enhanced, thus this synthetic flavonoid was used as a positive control. The immunoblot analysis showed that only one of natural flavonoids, flavone, significantly increased the level of CYP1A1 in liver. It is also consistent with the effects of flavone on EROD and MROD activities in microsomes and also with tissue CYP content. This result correlates well with data published by others [Canivenc-Lavier et al., 1996b]. In the same manner, naringin and hesperidin slightly enhanced MROD activity and increased the CYP1A2 level in liver. In colon, the flavonoid induction effect was rather different compared to liver. Here, we show for the first time, that in colon microsomes, the most significant induction of CYP1A1 was caused by naringenin, followed by slight induction of resveratrol, hesperidin and baicalin.

Our results also show that the inducing pattern of the flavonoids varies with their structure. Three glycosides – baicalin, hesperidin, and naringin affected 7-ethoxyresorufin-*O*-deethylase (EROD) and 7-methoxyresorufin-*O*-demethylase (MROD) activity of CYP1A1 and 1A2 in liver microsomes differently. Hesperidin and naringin enhanced both activities, whereas baicalin produced a decrease. The different affection can be explained by the presence of C2-C3 single bond (ring C) in flavanones (hesperidin, naringin) and double bond in flavones (baicalin). In comparison with all results obtained for citrus flavonoids – naringin, naringenin, hesperetin and hesperidin, the direct relation of glycosides and their aglycones to enzyme induction in colon and liver were not observed. On the other hand, the most effective

inducers of EROD and MROD activities were two unsubstituted flavonoids with C2-C3 double bond in ring C, β-naphthoflavone and flavone. Accordingly to our results, the unsubstituted flavonoids are suggested that can function as inducers of several CYP (1A and 2B) enzymes [Breinholt et al., 1999, Canivenc-Lavier et al., 1996b].

Overall, the present study shows that *p.o.* administration of flavonoids to rats provided evidence for CYP induction by flavone and flavanone structures, and also that the inducing ability of flavonoid compound is both, compound and tissue-specific. In addition, our results and results obtained from the previous study, where the CYP1A1 induction by chrysin, diosmin and quercetin was observed in both tissues [Fridrichova, 2005], would be helpful in preventing the activation of ingested food carcinogens and explaining changes in drug metabolism.

# 6 CONCLUSIONS

- Natural flavonoids – baicalin, flavone, naringenin, naringin, hesperidin, hesperetin, and one synthetic flavonoid - β-naphthoflavone, were selected for studying the CYP1A induction.
- Experimental animals (rats) were treated *p.o.* with selected flavonoids and the microsomal samples were prepared from liver and colon tissues.
- Western blotting detection of the CYP1A1 and CYP1A2 proteins was optimized to eliminate the keratin causing interference.
- Induction of CYP1A1 and CYP1A2 in liver and colon microsomal samples was determined by optimized Western blotting method using specific chicken anti-rat CYP1A1 antibody.
- In liver microsomes, we demonstrated the CYP1A1 induction by flavone and the CYP1A2 induction by naringin and hesperidin.
- In colon microsomes, the highest CYP1A1 induction was observed after administration of naringenin.
- Elevation of marker activities EROD and MROD for CYP1A1 and CYP1A2, respectively, was in accordance with induction effects of unsubstituted flavonoids, β-naphthoflavone and flavone.

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