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Effect of selected food supplements on metabolism of food-born carcinogens

Vliv vybraných potravních doplňků na metabolismus karcinogenů přítomných v potravě

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Diploma Thesis

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze dne ……

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Petra Fousová

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ABSTRACT

The consumption of dietary supplements such as flavonoids may reduce risk of many civilization diseases. Flavonoids are able to modulate the activity of cytochromes P450 (CYPs), xenobiotic-metabolising phase I enzymes of biotransformation that are involved in the activation and detoxification of food-derived carcinogens. Inhibition of CYP activities by flavonoids has been extensively studied because of their potential use as agents blocking the initiation stage of carcinogenesis. On the other hand, flavonoids have been shown to enhance the activation of carcinogens and/or influence their metabolism *via* induction of specific CYPs. In the first part of this study, flavonoids dihydromyricetin and α-napthoflavone were explored for their possible effects on CYP1A1 expression and activity when administered in combination with carcinogen benzo[a]pyrene (BaP). For this purpose, liver, small intestine and colon microsomal fractions were isolated from treated rats and induction of CYP1A1 was evaluated by immunodetection and EROD activity measurements. In liver and small intestine, all combinations of BaP and flavonoids led to strong induction of CYP1A1 expression. Moreover, the CYP1A1 protein levels were almost identical to levels observed when the rats were treated with BaP alone. However, in comparison with BaP administered alone, the CYP1A1 activities significantly decreased in most of the cases when also one of the flavonoids was present. This would suggest that the selected flavonoids could be capable of reducing the potential risk of cancer development.

In addition to their involvement in activation and inhibition of CYPs, dietary flavonoids can potentially also influence phase II enzymes of biotransformation. To be able to explore whether *N*-acetyltransferases (NATs) as representatives of phase II enzymes can be modulated by flavonoids, basic methods for detection and activity measurements need to be established. Thus, the second part of this study focused on introduction and optimisation of NAT immunodetection and specific NAT activity assay. Although 3 different chicken antibodies against human/rat NATs were prepared, none of them was able to detect NATs in rat cytosol mixture. NAT activity assay based on determination of CoA amount formed during reaction of the enzyme with specific substrate was first optimised using recombinant NATs. Subsequent attempts to measure the NAT activity in rat cytosols were successful. Nevertheless, many optimisation steps still need to be performed.

Key words: flavonoids, cytochromes P450, *N*-acetyltransferase

ABSTRAKT

Užívání potravních doplňků, a to především flavonoidů, může předcházet vzniku mnoha civilizačních chorob. Flavonoidy jsou schopné modulovat aktivitu cytochromů P450 (CYPs), enzymů podílejících se na první fázi metaboslismu cizorodých látek (xenobiotik), a tak ovlivňovat aktivaci a detoxikaci potravních karcinogenů. Inhibice aktivity těchto enzymů flavonoidy je intenzivně studována především z hlediska jejich využití při potlačení iniciační fáze během vzniku rakoviny. Na druhou stranu bylo také prokázáno, že flavonoidy mohou karcinogeny aktivovat, a to právě prostřednictvím jejich indukčního efektu na cytochromy P450. V první části této diplomové práce byl studován účinek flavonoidů dihydromyricetinu a α-napthoflavonu na aktivitu a expresi CYP1A1, a to buď v kombinaci s karcinogenem benzo[a]pyrenem (BaP) nebo samostatně. Za tímto účelem byly izolovány mikrosomální frakce z premedikovaných potkaních jater, tenkého a tlustého střeva, ve kterých byla následně stanovena indukce CYP1A1 použitím metody „Western blot“ a bylo provedeno měření specifické aktivity cytochromů P450. V případech, kdy byl flavonoid podáván společně s BaP, docházelo v mikrosomech jater a tenkého střeva k silné indukci exprese CYP1A1. Množství detekovaného CYP1A1 proteinu bylo navíc téměř identické jako v případech, kdy byli potkani premedikováni pouze s BaP. Při porovnávání naměřených aktivit bylo poté zjištěno, že v téměř všech případech, kdy byl flavonoid podáván v kombinaci s BaP, byla aktivita výrazně nižší než v mikrosomech potkanů premedikovaných pouze s BaP. Tento trend naznačuje, že vybrané flavonoidy by mohly být schopné předcházet vzniku zhoubných nádorů.

Výše zmíněný inhibiční efekt flavonoidů na aktivitu CYPů naznačuje, že tyto potravní doplňky by také mohly ovlivňovat enzymy II. fáze biotransformace, jako třeba *N*-acetyltransferasy (NATy). Z tohoto důvodu byla druhá část této diplomové práce zaměřena na vývoj a optimalizaci metod určených jak k imunodetekci NATů, tak k měření jejich aktivity. Přestože byly připraveny tři specifické protilátky proti NAT, žádná nebyla následně schopná rozpoznat tyto enzymy v jaterních cytosolech potkanů. Měření aktivity NATů bylo založeno na detekci vzniklého CoA. Tato metoda byla nejdříve optimalizována za použití rekombinantních lidských NATů. Následné pokusy, kdy byly při měření specifické aktivity těchto enzymů použity jaterní cytosoly namísto rekombinantních NATů, byly úspěšné. Přesto je potřeba tuto metodu dále optimalizovat.

Klíčová slova: flavonoidy, cytochromy P450, *N*-acetyltransferasy

TABLE OF CONTENTS

1. INTRODUCTION

1.1. Biotransformation of xenobiotics

All organisms are exposed constantly to foreign compounds (xenobiotics) including both manufactured and natural chemicals. In order to be eliminated from human body, the xenobiotics usually need to undergo process known as biotransformation during which they are converted into water soluble chemicals by many different enzymes [1-2].

As first proposed in 1949 by R.T. Williams, the metabolism of drugs and environmental pollutants can be divided into phase I (functionalization) and phase II (conjugation) reactions. Phase I reactions involve hydrolysis, reduction, and oxidation. In general, the first phase of biotransformation leads to generation of more polar compounds that can either be eliminated or proceed to phase II of biotransformation. In some cases, this process can cause metabolic activation of the substrate to reactive intermediates which can react with cellular macromolecules to initiate toxic and carcinogenic events [3]. The phase I enzymes include cytochromes P450 (CYPs), sterases, alcohol dehydrogenases and other enzyme systems [4].

In phase II, the metabolite is conjugated with an endogenous polar compound (sugar, amino acid, glutathione, sulphate or phosphate) leading to a more water-soluble compound thereby making the drug more readily execrable. One of the major phase II enzyme families is uridine diphosphoglucuronosyl transferase (UGT), but also sulfotransferase (SULT), N-acetyltransferase (NAT), glutathion-S-transferase (GST), and others belong to this group of conjugating enzymes [5-6]. The reduced metabolising capacity of the phase II enzymes can lead to the manifestation of the toxic effects of clinical drugs. Although phase II reactions are generally detoxifying, the conjugates formed may also caused adverse effects (the formation of highly reactive carbenium or nitrenium cations that covalently bind to proteins and nucleic acids) [7]. Further, both external (smoking, medication, nutrition and effects of the environment) and internal (age, sex, diseases and genetics) factors are known to influence phase II enzymes [8].

In addition, enzymes catalysing biotransformation reaction often determine the intensity and duration of action of drugs and play a key role in chemical toxicity and chemical tumorigenesis [1-2]. Xenobiotic biotransformation enzymes are widely distributed throughout body and are present in several subcellular components. Within the liver (and most other organs), the enzymes catalysing xenobiotic biotransformation reaction are located primarily in the endoplasmic reticulum (microsomes) or the soluble fraction of the cytoplasm (cytosol), with lesser amounts in mitochondria, nuclei, and lysosomes.

1.1.1. Cytochrome P450

Cytochromes P450 (EC 1.14.14.1, CYPs) comprise a large superfamily of heme-containing enzymes that catalyse the monooxygenation more than 70% of medical drugs and chemical compounds. In addition, several CYPs are also involved in the biosynthesis of physiologically relevant molecules such as steroids, and fatty acids. These enzymes occur in almost every realm of life from Bacteria and Archaea to Eukaryotes [9].

Cytochromes P450 (CYPs) are so called because of the unique absorbance spectrum that is produced when CO is bound to the reduced, ferrous form of the heme. The spectrum exhibits a peak at wavelength of 450 nm; thus the name P450 for a **p**igment with an absorbance at **450** nm [10].

As already mentioned, CYPs catalyse the monooxygenase reaction, when one of the oxygen atom is inserted into a substrate, while the second oxygen is reduced to a water molecule, utilizing two electrons provided by NAD(P)H via cytochrome P450 reductase The basic stoichiometry of this process is represented by the equation:

$$\textbf{Substrate (RH)} + O_2 + \textbf{NADPH} + H^+ \rightarrow \textbf{Product (ROH)} + H_2O + \textbf{NADP}^+$$

1.1.1.1. **Cytochrome P450 structure**

CYPs are integral membrane proteins containing a single iron protoporphyrin IX (heme) prosthetic group. The heme iron of CYPs is capable of forming six bonds, four to each four pyrrole nitrogen atoms of the porphyrin ring and two to axial ligands. One ligand is a thiolate anion provided by a cysteine residue [11] and the other ligand is most probably an OH group from the water molecule or hydroxyl group of amino acid [12]. The heme iron in cytochrome P450 is usually in the ferric (Fe^{3+}) state. When reduced to the ferrous (Fe^{2+}) state, cytochrome P450 can bind ligands such as O_2 or carbon monooxide (CO).

All cytochromes P450 are triangle-shaped molecules. Half of the enzyme consists of α-helical structure and the other half is β-sheet and other structures. The mammalian proteins contain N-terminal membrane anchor sequence and internal membrane association sequences are responsible for the proteins being deeply buried in the membrane [13].

1.1.1.2. **Cytochromes P450 electron transport systems**

During catalysis, cytochrome P450 binds directly to the substrate and molecular oxygen, but it does not interact directly with NADPH or NADH. The mechanism by which cytochrome receives electrons from NAD(P)H depends on the subcellular localization of cytochrome P450. In the endoplasmic reticulum, where most of P450 enzymes involved in xenobiotic biotransformation are localized, electrons are transfered from NADPH to cytochrome P450 *via* a flavoprotein NADPH:cytochrome P450 reductase (see Figure 1, p. 4). Within this flavoprotein, electrons are transferred from NADPH to cytochrome P450 *via* FMN and FAD. Phospholipids and cytochrome b_5 play important roles in cytochrome P450 reactions. Cytochrome P450 and NADPH-cytochrome P450 reductase are embedded in the phospholipid bilayer of the endoplasmic reticulum, which facilitates their interaction. Cytochrome b_5 can donate the second of two electrons required by cytochrome P450 for the O_2 activation. Although this electron donation would be expected simply to increase the rate of catalysis of cytochrome P450, by the interaction with cytochromes P450, cytochrome b_5 can also increase the apparent affinity with which certain P450 enzymes bind their substrates [13].

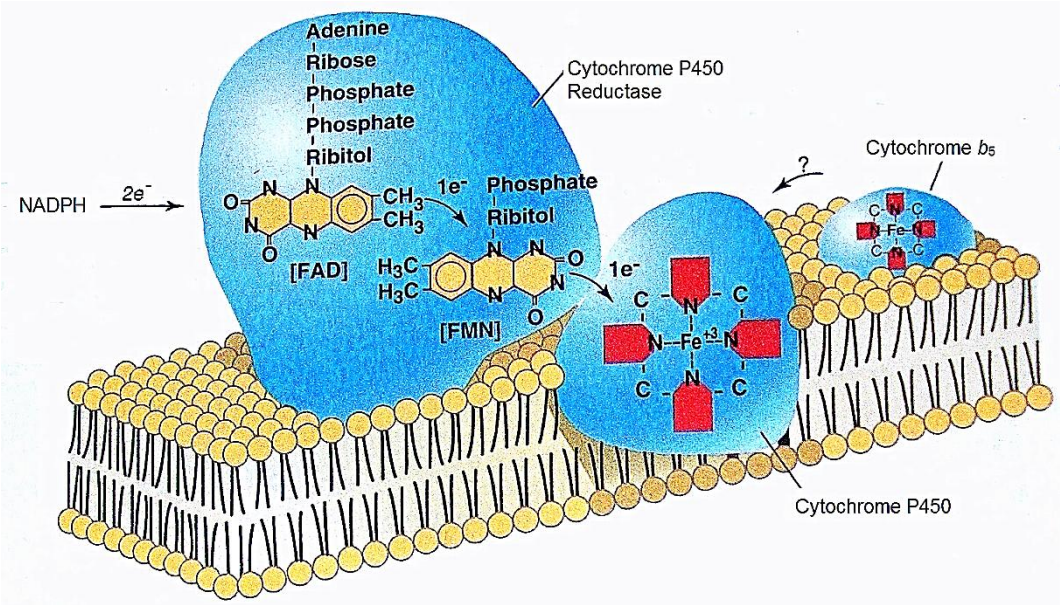

Figure 1: Components of the endoplasmic reticulum (microsomal) cytochrome P450 system. NADPH-cytochrome P450 reductase is bound by its hydrophilic tail to the membrane whereas cytochrome P450 is deeply embedded in the membrane (an integral protein). The figure was adapted from [13].

1.1.1.3. **Nomenclature of cytochrome P450**

Because large number of cytochromes P450 has been identified, a system for classification of the enzymes into functional groups and nomenclature needed to be developed. The widely used system is based on classification according to the relative identity of the amino acid sequences of the enzymes. The superfamily of CYPs is thus divided into families in which the amino acid sequence identity of the members is greater than 40%. The family is designated by the prefix „CYP“, for cytochrome P450, followed by an Arabic numeral (e.g., CYP1, CYP3, etc.). Those with sequence identity greater than 55% belong to the same subfamily and are identified by additional capital letter (e.g., CYP1A, CYP1B, etc.). The individual members of each family are then numbered in the order in which they were identified (e.g., CYP1A1, CYP3A4, etc.) [13-14].

1.1.1.4. **CYP1A**

Apparently, all mammalian species possess two inducible CYP1A enzymes with 70% homology, namely CYP1A1 and CYP1A2. CYP1A1 has been detected in the human lung, intestine, skin, lymphocytes, placenta and liver [15]. In contrast to CYP1A1, CYP1A2 is

not expressed in extrahepatic tissues, however is primarily expressed in liver at significant levels [16]. The enzymes of this subfamily are involved in the activation of procarcinogens, such as polycyclic aromatic hydrocarbons (PAH), aromatic amines and heterocyclic amines [17]. Inducers of the CYP1A enzymes include cigarette smoke, charcoal-broiled meat (sources of PAHs), cruciferous vegetables (a source of various indoles), and omeprazole (a proton-pump inhibitor used to suppress gastric acid secretion). CYP1A1 and CYP1A2 both catalyse the *O*-dealkylation of 7-ethoxyresorufin to fluorescent resorufin [3].

1.1.1.5. **Inhibition of Cytochrome P450**

To date, many inhibitors of the CYPs 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 catalytic cycle of CYP consists of at least 7 discrete steps:

1.) binding of the substrate to the ferric form of the enzyme
2.) reduction of the hem group from the ferric to the ferrous state by an electron provided by NADPH via CYP reductase
3.) binding of molecular oxygen
4.) transfer of a second electron from CYP reductase and/or cytochrome b5
5.) cleavage of the O–O bond
6.) substrate oxygenation
7.) product release

Although impairment of any of these steps can lead to inhibition of CYP enzyme activity, steps (1), (3) and (6) are particularly vulnerable to inhibition. The mechanisms of CYP inhibition can be classified into three general categories: reversible inhibition, quasi-irreversible inhibition and irreversible inhibition [18]. Among these, reversible inhibition is probably the most common mechanism responsible for the documented drug interactions. In mechanistic terms, reversible interactions arise from the competition of the CYP active site (first step of the CYP catalytic cycle). Both irreversible and quasi-irreversible inhibitions are caused by the formation of reactive metabolites.

1.1.1.6. **Induction of Cytochrome P450**

From a biological point of view, induction is an adaptive response that protects the cells from toxic xenobiotics by increasing the detoxification activity [4]. While in most cases CYP induction is the consequence of an increase in gene transcription [19], some non-transcriptional mechanisms are also known to be involved.

In drug therapy, there are two major concerns related to CYP induction. First, the induction can result in a reduction of pharmacological effects caused by increased drug metabolism. Secondly, induction may create an undesirable imbalance between 'toxification' and 'detoxification'. Like a double-edged sword, the induction of drug metabolising enzymes may lead to a decrease in toxicity through accelerated detoxification, or to an increase in toxicity caused by increased formation of reactive metabolites. Depending on the delicate balance between detoxification and activation, the enzyme induction can be a beneficial or harmful response.

In vivo, the induction of CYP1A isoforms can reduce the carcinogenicity of certain compounds. For example, intraperitoneal injection of the CYP1A inducer β-naphthoflavone inhibites tumorigenesis in the lung and mammary glands of rodents treated with 7,12-dimethylbenz[a]anthracene (DMBA), which is a highly carcinogenic compound [20]. In contrast, CYP1A isoforms can activate some compounds, such as benzo[a]pyrene, to their ultimate carcinogenic species [21]. Thus, the induction of these isoforms increases the risk of carcinogenicity.

As mentioned already, the enzymes of CYP1A subfamily are induced by aromatic hydrocarbons. The activation involves a specific receptor called the Ah receptor. After the binding of an inducing agent to the receptor, a heterodimer with the activated Ah-receptor nuclear translocator (ARNT) is formed. This heterodimer then binds to the xenobiotic response element (XRE), a transcriptional enhancer to stimulate the gene transcription of CYP1A.

In contrast to inhibitors, inducers of cytochrome P450 increase the rate of xenobiotic biotransformation. Induction of cytochrome P450 is thus expected to increase the activation of procarcinogens to DNA-reactive metabolites, leading to increased tumour formation [21].

1.1.2. Arylamine *N*-acetyltransferases

Arylamine N-acetyltransferases (NATs; EC 2.3.1.5) are defined as Phase II xenobiotic-metabolizing enzymes, adding an acetyl group from acetyl-CoA to arylamines and arylhydrazines (see Figure 2). These cytosolic enzymes were first characterized in humans as the factors responsible for the polymorphic inactivation of anti-tubercular drug isoniazid [22].

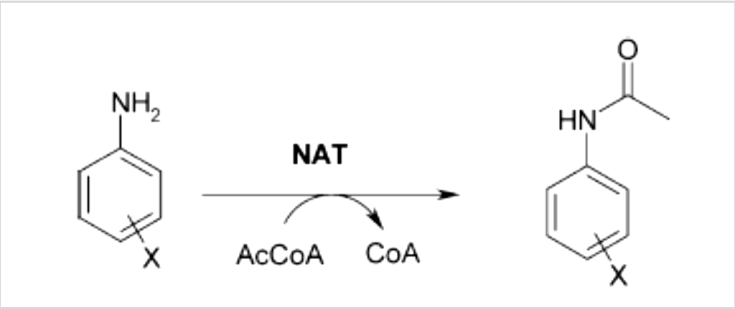

Figure 2: The basic reaction catalysed by arylamine *N*-acetyltransferase. Arylamine *N*-acetyltransferase is cytosolic conjugating enzyme which transfers an acetyl group from acetylCoA to xenobiotic acceptor substrate. NAT - Arylamine *N*-acetyltransferase, AcCoA – acetylCoA.

To date, 22 NAT-like genes have been identified in 14 different prokaryotic and eukaryotic species. It is though likely that additional genes will be discovered as more genomes become sequenced. NAT proteins are between 30 and 35 kDa in size, with variable polypeptide chain lengths (typically 290 amino acids in mammals).

The most abundant N-acetyltransferases are *N*-acetyltransferase Type I (NAT1) and *N*-acetyltransferase Type II (NAT2). The genes encoding both proteins were first isolated in 1989 by Grant and coworkers [23]. There is 82% identity at the amino acid level between mouse NAT2 and human NAT1. In rats, additionally to NAT1 and NAT2, a third rat NAT gene (*NAT3*) has been recently identified [23]. NATs have been found to have high activity in rats. Humans exhibit an intermediate activity of NATs [24].

1.1.2.1. **Structural characteristics of the NAT proteins**

Consistent with the recently published crystal structure for *Mycobacterium marinum* NAT (see Figure 3), all NATs possess a conserved cysteine, histidine and aspartate that are implicated in formation of the catalytic triad [25-27]. Specifically, a cystein-histidine-aspartate catalytic triad was identified in the N-terminus of the protein. Based on structural analysis, the protein has been divided into three domains. The first is a helical bundle, consested of amino acid 1 to approximately 90. This forms one side of a cleft in which the cysteine involved in acetyl transfer is located. All NATs are highly homologous in this region. The second domain is formed by the residues from approximately 90 to 210 and is located on the other side of the cleft. It mostly consists of β-sheet structures. The last domain at the carboxyl terminus is a combination of β-sheets and α-helices, and this region shows the greatest diversity between species (see Figure 3). All eukaryotic NATs, although having the conserved active-site triad residues, possess an additional insertion of amino acids between the second and third domains, which has been predicted to form a loop [28-29].

The structural features surrounding the triad are similar to the cysteine protease superfamily of proteins includin transglutaminases, cathepsins and caspases. To date, the crystal structure of the human NATs has not been resolved although the homology with NAT from bacteria suggests that similar features will be present [30].

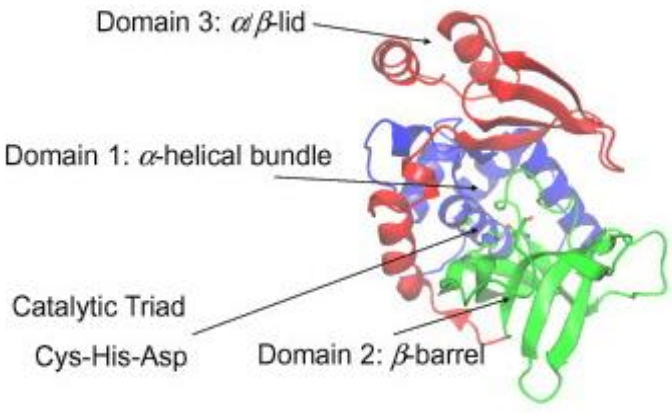

Figure 3: **The structure of NAT *Mycobacterium marinum*.** The structure of *M. marinum* has been determined [25] which led to the identification of a catalytic triad of cysteine, histidine and aspartate as leading to the activation of the cysteine in the active site. The three-domain structure has been identified in all NATs there is structural information. The figure was adapted from [25].

1.1.2.2. **Enzyme activities of NATs**

The arylamine N-acetyltransferases differ from the many other acetylCoA dependent transferases present in cells because of their ping-pong bi bi reaction mechanism [31]. The reaction takes place in two separate steps. Initially, acetyl coenzyme A binds to the enzyme and the acetyl moiety is transferred from the cofactor to a cysteine (Cys^{68} for the human isoforms) of the protein. Coenzyme A is then released. The second step involves the binding of substrate to the acetylated enzyme followed by transfer of the acetyl moiety to the substrate. Finally, the acetylated product is released from the enzyme. The first step of the reaction can proceed in the absence of arylamine substrate [32].

As well as catalysing *N*-acetylation of arylamines and hydrazines, NATs catalyse the *O*-acetylation of hyrdoxylamines and the acetylCoA-independent *N,O*-transacetylation reaction in arylhydroxamates [25]. If compounds are first *N*-hydroxylated by cytochrome P450 enzymes, the *N*-hydroxylated substrates can be then further activated by NAT-catalyzed *O*-acetylation. This bioactivation reaction leads to the formation of *N*-acetoxy metabolites that are very unstable and form spontaneously highly reactive electrophilic arylnitrenium ions. These ions can react with DNA producing DNA adducts leading to mutagenesis and initiation of cancer.

1.1.2.3. **Substrate specifity of NATs**

There is no clear structural motif that determines substrate specifity for the different isoforms of NAT. In general, p-aminobenzoic acid (PABA), p-aminobenzoyl glutamate and p-aminosalicylic acid (PAS) are considered to be specific substrates for human NAT1. These substrates can be characterised by the presence of relatively small hydrophilic substitutions in the para position of the aromatic ring. By contrast, sulfamethazine, procainamide and dapsone are acetylated primarily by human NAT2. Some compounds such as 2-aminofluorene can be substrates of both human NAT1 and NAT2 [33].

1.1.2.4. **The involvement of NATs in phatological conditions**

Humans are exposed to many toxic NAT substrates including the food-derived heterocyclic amines and arylamines such as 4-aminobiphenyl and β-naphthylamine present in tobacco smoke [30]. Because of the role of acetylation in the metabolic activation and detoxification of arylamine and heterocyclic carcinogens, acetylator status and cancer risk has been widely investigated

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1.2. Carcinogenesis

By the time of Hippocrates in the 4th century BC, many types of tumour were clinically recognized and described. Hippocrates introduced the term carcinoma from the Greek word *karkinos*, for crab: he saw cancer as crablike in its spread through the body and in its persistence. However, it was not until the end of the 18th century that cancer began to be studied systematically and intensively.

In fact, cancer comprises of more than 200 different diseases. Together, they account for about one fifth of all deaths in the industrialized countries of the Western World. In general, cancer begins with a mutational event within a single cell that can then divide abnormally leading to development of malignant tomour. The factors involved in this event include chemical carcinogen, oncogenic virus, radiation damage, endogenous genetic damage caused by oxidative insult to DNA or any host of other potential ways (e.g. chronic infections with bacteria such as *Helicobacter pylori* or with a parasite such as s *Schistosoma haemotobium, Clonorchis sinensis and Opisthorchis vivarium*, or hormonal imbalance) [34-36]. Among the main causes of cancer, 'environmental exposures' make a substantial contribution to human cancers [37]. The studies of these environmental exposures, along with the investigation of lifestyle factors and habits, led to the conclusion that the great majority (over 80%) of cancer deaths in Western industrial countries can be attributed to factors such as tobacco, alcohol, diet, infections and occupational exposures [38].

The transformation of a normal cell into a cancerous is believed to proceed through many stages over years or even decades. The stages of carcinogenesis include initiation, promotion and progression (Figure 4, p.11). The first stage of carcinogenesis has been

called initiation since 1947 [39]. It is a fast, irreversible phenomenon occurring after successive genotypical and phenotypical changes that are transmitted to daughter cells [40-41]. During promotion, the initiated cell is exposed to compounds called promotors which increased cell proliferation in susceptible tissues, contribute towards fixing mutations, enhance alterations in genetic expression and cause changes in cellular growth control [42]. Promotion is a reversible stage. Indeed, after disappearance of the promotore, a regression in cell proliferation can occur, probably by apoptosis. The last and most extended stage of carcinogenesis is called progression during which transformation into malign lesions occur [43-44]. In this process cell proliferation is independent from the presence of stimulus. Progression is characterized by irreversibility, genetic instability, faster growth, invasion, metastization, and changes in the biochemical, metabolical and morphological characteristic of cells [45-46].

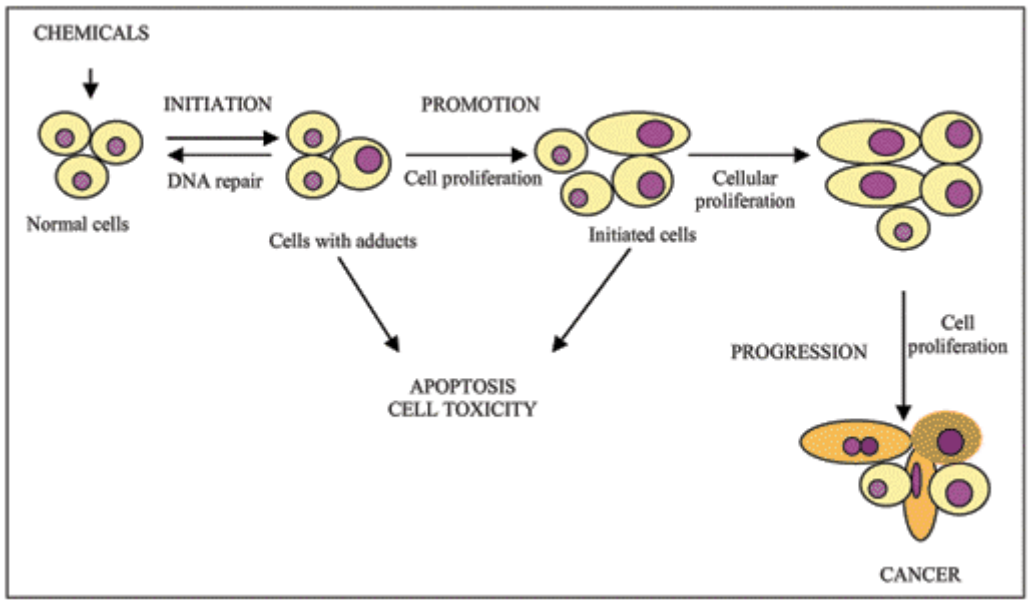


Figure 4: Scheme of chemical carcinogenesis. Chemical carcinogenesis stages and occurrences involved in each one. The figure was adapted from [47].

During metastasis, cancer cells separate from the primary tumour and migrate by the blood or lymph to different organs where they form new tumours. In fact, individual cancer cells or small groups may end up in a different tissue only to survive over long periods without net growth. Over time, they may adapt to their new environment and expand to larger metastases that threaten the life of patients. This may occur several years after the primary tumour has been removed. Generally, preferred organs for metastasis are those with extended micro capillary systems such as liver, lung, and bone [47].

1.3. Chemical carcinogens

As mention above, chemical carcinogens are part of external factors that can cause cancer. Chemical carcinogens can be classified in terms of their mechanisms of action into two major groups - genotoxic and non-genotoxic [43-44,48-49]. Genotoxic carcinogens are complete carcinogens that change the genetic information of the cell [40]. Following transmembrane diffusion, they are metabolized into electrophilic compounds that enter the nucleus and interact with nucleophilic sites (DNA, RNA and proteins) changing their structural integrity and establishing covalent bonds known as DNA-adducts [48,50]. Most genotoxic agents are microcomponents of nutrition, i.e. polycyclic aromatic hydrocarbon (PAH), heterocyclic amines (HACs), aflatoxin, and N-nitrosamine [51-52]. Non-genotoxic carcinogens act as promoters and do not need metabolic activation. Moreover, they do not react directly with DNA and do not raise adducts. Furthermore, they are negative on mutagenicity tests carried out *in vivo* and *in vitro* [43-44,49]. However, these compounds modulate growth and cell death and are tissue- and species- specific [35,43].

1.3.1. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants. Indeed, since PAHs are formed by the incomplete combustion of organic matter they are generated whenever fossil fuels or vegetation is burned. Moreover, PAHs are one of several classes of carcinogenic chemicals present in tobacco smoke [53]. PAHs themselves are chemically inert and hydrophobic. However, in mammalian cells, they undergo metabolic activation to diol-epoxides that bind covalently to cellular macromolecules, including DNA. Once DNA is modified mutations and errors in DNA replication can occur, which can initiate the carcinogenic process. Several hundred different PAHs are known including benzo[a]pyrene.

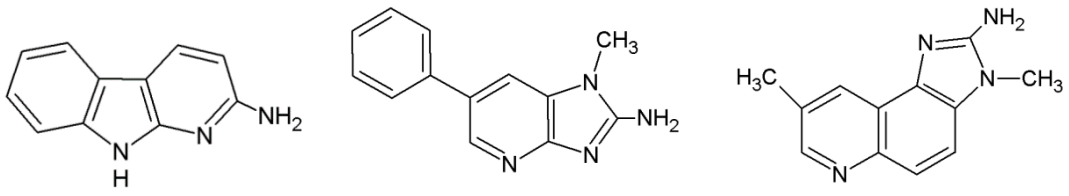
Benzo[a]pyrene BaP is the most studied PAH. Its role as an extremely potent chemical carcinogen was established. Indeed, since then, BaP has been shown to have cytotoxic, teratogenic, mutagenic and carcinogenic effects [54]. Carcinogenesis mechanism is conducted through BaP adduct formation, after being metabolically activated mainly by phase I enzymes (namely by cytochromes P450, see Chapter 1.1.1) to epoxide intermediates that are further converted to more reactive diol-epoxides by epoxide

hydrolase [47]. Many of these metabolites are further metabolized by phase II enzymes [51].

1.3.2. Heterocyclic amines

Heterocyclic amines (HCA) are the carcinogenic chemicals formed within muscle meats during most types of high temperature cooking, through a pyrolysis process from amino acids, proteins, and creatines [51]. The amount of HCAs formed in meats depends on the type of meat and the method of cooking. Many epidemiological studies have examined the interrelationships among the consumption of cooked red meat, its effect on human cancer risk of the digestive tract, prostate gland, mammary gland and the potential causal role of HCAs in the ethology of these cancers. HCA carcinogenesis mechanism begins with bioactivation of *N*-hydroxylation by cytochrome P450, especially CYP1A2. Subsequent esterification results in formation of nytrenium ions, ultimate carcinogens capable of binding guanine at position C8 causing altered DNA sequences, with leads to substitution of the base, deletion and insertion [51].

The structures of three of the most abundant HCAs identified in common cooked foods, AαC (2-amino-9Hpyrido[2,3-b]indole), MeIQ (2-amino-3,8-dimethylimidazo[45-f]quinoxaline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5b]pyridine) are shown in Figure 5.



AαC PhIP MeIQx

Figure 5: Structures of the most abundant heterocyclic amines.

1.4. Chemoprevention

Since ancient times, natural products, herbs and spices have been used for preventing several diseases, including cancer [42]. The term "chemoprevention," the inhibition or reversal of the carcinogenic process through the use of drugs or other compounds, became part of the cancer lexicon in the latter half of the 20th century [55].

In 1988, De Flora and Ramel proposed a detailed classification of inhibitors of mutagenesis and carcinogenesis. This classification takes into account the three general levels of prevention in connection with the possible mechanisms of cancer chemopreventive agents [56]. In particular, primary prevention, having the goal of preventing the occurrence of the malignant disease, includes inhibition of mutation and cancer initiation, either in the extracellular environment or inside cells, followed by inhibition of tumour promotion. Secondary prevention exploits a variety of mechanisms aimed to inhibite progression of a timely diagnosed benign tumour towards malignancy. Tertiary prevention is focused on prevention of local relapses of the disease and inhibition of invasion and metastasis [56].

It has been estimated that 35% of cancer deaths may be related to dietary factors and smoking. On the other hand, the correct lifestyle and diet are assumed to prevent 30-40% of all tumours [57]. Thus, the consumption and use of dietary supplements containing concentrated phytochemicals has increased dramatically in recent years [57]. However, the common misconception that the more of something is the better for health may reset in overdosing of individuals by these compounds [58].

1.4.1. Flavonoids

Flavonoids are part of a family of naturally occurring polyphenolic compounds and represent one of the most prevalent classes of compounds in fruits, vegetables and beverages derived from plants (tea, red wine) as well as in many dietary supplements or herbal remedies including Ginkgo Biloba, Soy Isoflavones, and Milk Thistle [59]. Moreover, extracts of many flavonoids are now available in health food stores as dietary supplements. The word „flavonoid"derivates from the Latin word „flavus"which means yellow.

These compounds can scavenge superoxide, hydroxy, and proxyradicals, breaking lipid peroxide chain reactions. Furthermore, they have also been shown to protect cells from X-ray damage, block progression of cell cycle, inhibit mutations, block prostaglandin synthesis and prevent multistage carcinogenesis in experimental animals [60].

1.4.1.1. **Structure of flavonoids**

The general structure of a flavonoid (see Figure 6a) consists of two aromatic rings (ring A and ring B) linked by three carbons that are usually in an oxygenated heterocyclic ring or C ring [59]. Based on differences in the generic structure of the heterocyclic C ring as well as the oxidation state and functional groups of the heterocyclic ring, flavonoids are classified as flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins or isoflavonoids (see Figure 6b).

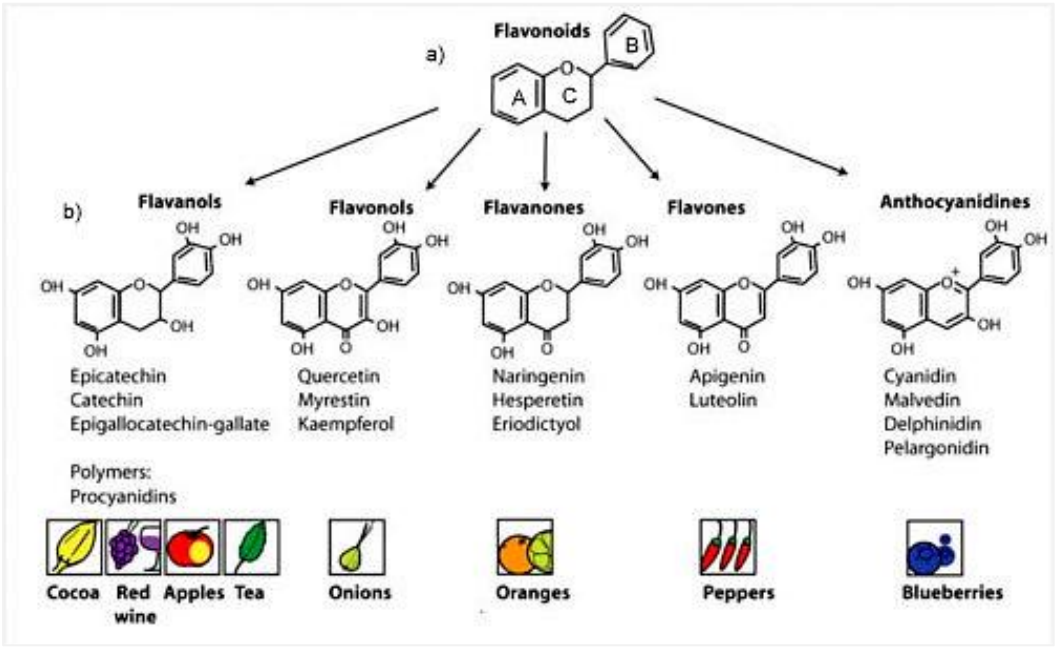


Figure 6: Structure of flavonoids. The chemical structure of flavonoids is based on oxygenous heterocyclic compound flavane **(a)**. The variol classes of flavonoids **(b)** differ in the level of oxidation and pattern of substitution of C ring, while individual compounds within a group differ in the pattern of substitution of the aromatic ring A and B. The Figure was adapted from [61].

Flavonoids are most frequently found in nature as conjugates in glycosylated or esterified forms. 80 different sugars have been identified to bind to flavonoids [59]. The polyphenolic structure of flavonoids renders them quite sensitive to oxidative enzymes and cooking conditions. Usually, natural flavonoids occur as glycosides (e.g. glucosides, rhamnoglucosides, rutinosides) and their structures can be more complex such as flavonolignans (silybin), catechin esters (epigallocatechin gallate) or prenylated chalcones (xanthohumol). The chemical structure and some activities of several flavonoids are similar to those of naturally occurring estrogens and are frequently assigned as phytoestrogens [62].

1.4.1.1. Metobolism of flavonoids

There are two major sites of flavonoid metabolism in mammalian body – colon and liver. The colon microflora converts flavonoids into phenolic acids or aglycones. Whether the whole molecule or the aglycone form of flavonoid is more effective depends on a particular flavonoid and its biological activity [63].

The absorption, metabolism and excretion of flavonoids is a complex process involving various structural modifications of the ingested flavonoid in multiple tissues and cellular compartments. Determining the bioavailability of flavonoids is critical for understanding of the effects of flavonoids as chemopreventive agents. Flavonoids are predominantly absorbed in the small intestine, with only small amounts absorbed via the gastric mucoa [64]. The glycosylation state of the flavonoid greatly affects the mechanism of flavonoid absorption. Most flavonoids in nature exist as glycosides. The first step in absorption of flavonoid-glycosides is usually hydrolysis of the sugar moiety in the gut resulting in eneration of flavonoid aglycone [65]. This hydrolysis was initially assumed to occur only in the colon by bacteria since human lack the necessary enzymes to hydrolyse the beta-glycoside linkages of flavonoid glycosides. The flavonoid aglycones generated by hydrolysis of the sugar moiety are more lipophilic, and hence are more readily absorbed in the gut by passive diffusion. [66].

The biotransformation of flavonoids continues in the enterocytes. The main metabolic transformations include conjugation of glucuronic acid (glucuronidation), methylation, acetylation and sulphation [65]. These conjugations are essentially phase II detoxification reactions resulting in increased molecular mass and improved solubility of the compound,

which enhances excretion of the compound in bile and urine. Flavonoids entering the circulation subsequently undergo phase II detoxification in the liver.

1.4.1.2. **Flavonoids and cancer**

The cancer protective effects of flavonoids have been attributed to a wide variety of mechanisms, including free radical scavenging and modifying enzymes that activate or detoxify carcinogens (see Figure 7) [67]. Although the anti-cancer effects of flavonoids predominate, one cancer type, bladder cancer, demonstrates an increased incidence with higher levels of flavonoid consumption. Three studies have examined the intake of flavonols (quercetin, luteolin, kaempferol, myricetin), green tee intake or soy isoflavones with respect to bladder cancer risk [68].

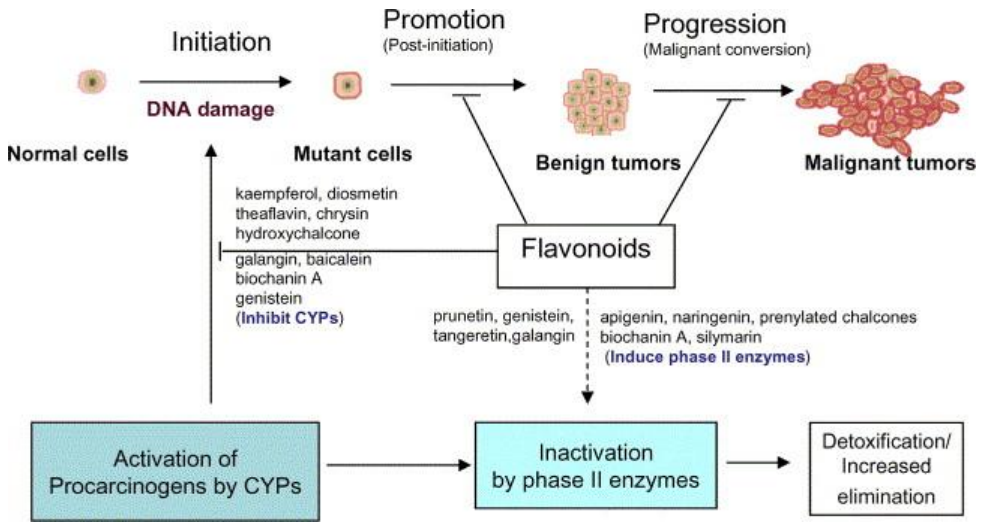


Figure 7: Effects of flavonoids in multistage carcinogenesis. Adapted from [69] .

However, numerous epidemiological studies have validated the inverse relation between the consumption of flavonoids and the risk of cancer. Indeed, flavonoids modulate various CYPs involved in carcinogen activation and scavenging reactive species formed from carcinogens by CYP-mediated reactions. Moreover, they are involved in the regulation of enzymes of phase-II responsible for xenobiotic biotransformation and colon microflora.

As mentioned above, flavonoids reduce the activation of procarcinogen substrates to carcinogens by interacting with CYPs enzymes, which makes them putative anticancer

substances [3]. *In vivo* and *in vitro* studies have shown that flavonoids can enhance or inhibit the activities of certain P450 isozymes [70].

In summary, the degree of toxicity from flavonoids depends on dose. Very high doses (≥ 1gram per kg) are likely to have toxic side effects. Since over-the-counter flavonoid supplements are recommended at doses of approximately 14mg per kg, side effects from flavonoid self-supplementation in human are unlikely [71].

1.4.1.3. **Selected flavonoid compounds**

Some individual flavonoids selected for the experimental part of this thesis are discussed bellow.

Dihydromyricetin

Didydromyricetin, also called ampelopsin or ampeloptin, is the main bioactive component extracted from *Ampelopsis grossedentata* species. It has been found that dihydromyricetin possesses many pharmacological functions such as an antioxidant, antibacterial, anticancer and disintoxicating properties. Despite the widespread use of this compound, the effect of dihydromyricetin on the CYPs remains unclear [72-73].

α - Naphthoflavone

α-naphthoflavone (ANF) or 7,8-benzoflavone is a synthetic flavonoid with the structure similar to flavones. Flavonoids can either inhibit or induce human CYPs depending on their structure. In this case, α-naphthoflavone has been shown to inhibit human CYP1A1 and 1A2 but induce CYP3A4 [74].

1.5. Chicken antibodies

Antibodies are key proteins of specific immune response. These immunoglobulins are widely used in clinical practice for determination of levels of either own antibodies (HIV test, IgE), proteins associated with various diseases (cancer markers) or low molecular weight compounds (progesterone). Moreover, they are frequently employed for detection and/or determination of various antigens (proteins) making use of techniques such as immunodiffusion, ELISA, and Western blotting [75].

Mammalian immunoglobulins are classified into five groups: IgA, IgD, IgE, IgG and IgM. On the other hand, birds possess only three classes of immunoglobulins: IgA, IgG and IgM. Because of differences between mammalian and avian IgG, Leslie and Clem [76] introduced the term IgY (standing for egg yolk) for avian IgG-like class of immunoglobulins [75]. During egg formation, blood immunoglobulins, corresponding to mammalian IgG, are concentrated in the yolk, while IgA and IgM plus other proteins are secreted into the egg white [77]. The concentration of IgG in egg yolk is 1.3-1.9 times higher than that in hen blood [78].

Recently, chicken egg yolk immunoglobulin, referred to as immunoglobulin Y (IgY) has attracted considerable attention as means to prevent and control disease. Indeed, it possesses a large number of advantages compared to treatment with mammalian IgG including cost-effectiveness, convenience and high yield [79]. Under natural conditions, the serum IgY of laying hens is deposited in large quantities in the egg yolk in order to protect the developing embryo from potential pathogens [80]. Thus, it is possible to immunize the hen against specific foreign pathogens thereby allowing the production of IgY with activity against these specific disease conditions.

1.5.1. Structure and function of chicken IgY

Although chicken IgY is the functional equivalent of mammalian IgG, there are some profound differences in their structure. The molecular mass (67–70 kDa) of the H chain in IgY is higher than that of mammals (50 kDa). The higher molecular mass of IgY is due to increased number of heavy-chain constant domains and carbohydrate chains [81]. Other differences in structure include the fact that the hinged region of IgY is much less flexible compared to mammalian IgG. IgY has an isoelectric point of pH 5.7–7.6, whereas that of

IgG is 6.1 - 8.5 [78,82]. Unlike mammalian IgG, IgY does not fix mammalian complement and does not interact with mammalian Fc and complement receptors [79]. Additionally, IgY does not bind to protein A, protein G or rheumatoid factor, so no false positives are obtained on immunoassay which is a problem with IgG-based mammalian assays [82].

1.5.2. Advantages of IgY

The most significant advantage of IgY is that the preparation of antibodies is non-invasive because IgY are isolated from chicken eggs. Due to the phylogenetic distance between birds and mammals, chicken often more succesfully produce antibodies against highly conserved mammalian proteins, which are hardly immunogenic for mammals [83]. A hen can be considered as a small "factory" for antibody production. Indeed, a hen usually lays about 300 eggs per year and the egg yolk (15 ml) contains 50–100 mg of IgY of which 2 - 10% are specific antibodies [84]. Therefore, one immunized hen produces more than 22,500 mg of IgY per year, which is equivalent to the production of 4.3 rabbits over the course of a year [85]. Another advantages of IgY lies in the possibility of developing high titre chicken antibodies even when low doses of mammalian antigen (0.001-0.01 mg/dose) are applied [79]. The maintenance costs for keeping hens are also lower than those for mammals [85]. Egg yolk provides a more hygienic, cost-efficient, convenient and rich source of antibodies compared with the traditional method of obtaining antibodies from mammalian serum. In contrast to antibiotics, the use of IgY is environmentally-friendly and elicits no undesirable side effects, disease resistance or toxic residues [75].

2. AIMS AND OBJECTIVES

The positive effect of chemoprevetive compounds (e.g. flavonoids) consumption on cancer prevention is known. On the other hand, flavonoids can enhance activation of carcinogens and/or influence their metabolism *via* induction of specific cytochromes P450, enzymes of phase I of biotransformation. The main aim of this study was to explore the effect of chemopreventive compounds in combination with carcinogen on cytochromes P450s. Moreover, flavonoids can potentially also modulate N-acetyltransferases, phase II enzymes of biotransformation. In order to investigate the effects of flavonoids on xenobiotics-metabolizing enzymes, following tasks should be accomplished:

1. To isolate liver, small intestine and colon microsomal and cytosolic fractions from rats treated with carcinogen benzo[a]pyrene (BaP), flavonoids (dihydromyricetin and α-napthoflavone) or their combinations
2. To screen various combination of chemopreventive compounds and/or carcinogen for their ability to induce the expression of cytochromes P450 (namely CYP1A1) using Western blot
3. To determine 7-ethoxyresorufin-*O*-deethylase activity of CYP1A1 in all microsomal fractions
4. To isolate and characterize specific chicken antibodies against human/rat cytosolic N-acetyltransferases for their immunodetection by Western blot
5. To establish and optimize the *N*-acetyltransferase activity assay in isolated rat cytosols

3. MATERIALS AND METHODS

3.1. Materials

BD Biosciences, USA

human arylamine N-acetyltransferase NAT1, NAT2 expressed in Supersomes™

Gentest BD Bioscience, USA

rat recombinant CYP1A1 expressed in Supersomes™

Fluka, Schwitzerland

p-nitrophenyl phosphate (pNPP), tris(hydroxymethyl)aminomethane (Tris), α-tocopherol, β-mercaptoethanol

Linde, Czech Republic

liquid nitrogen, dry ice

Millipore Corp., USA

Immobilion - NC transfer nitrocellulose membrane

Penta, Czech Republic

sodium hydroxide (NaOH), sodium azide (NaN_3), ethanol (CH_3CH_2OH)

Perkin Elmer, USA

96-well plates for fluorescence measurement

PML a. s., Czech Republic

nonfat powdered milk Laktino

Serva, Germany

acrylamide, bis-acrylamide (BIS), Coomassie Brilliant Blue R-250 (CBB), sodium dodecyl sulfate (SDS), Tris/HCl, N,N,N',N'-tetramethylethylendiamine (TEMED), Triton X-100, Tween-20

Setuza a.s., Czech Republic

Vegetol – sunflower oil

Sigma-Aldrich, USA

5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium tablets (BCIP/NBT), 7-ethoxyresorufin, alkaline phosphatase-conjugated rabbit anti-chicken IgG, dimethylsulfoxide (DMSO), guanidin/HCl, nicotinamide adenine dinucleotide phosphate-reduced (NADPH), p-nitrophenyl sulfate (pNPS), resorufin

Thermo Fisher Scientific, USA

ImjectMaleimide-Activated mcKLH, Microplate F16 MaxiSorp NUNC-IMMUNO MODULE, Microplate BCA Protein Assay Kit – Reducing Agent Compatible, PageRuler Unstained Broad Range Protein Ladder

Whatman, USA

Whatman paper, No.3

Antibody against CYP1A1 was kindly provided by prof. RNDr. Petr Hodek, CSc., Faculty of Science, Charles University in Prague, Czech Republic

All other chemicals were purchased from **Lach-Ner, Czech Republic**

3.1.1. Instruments

Analytical balance

Discovery, Ohaus, USA

Automatic micropipettes

Multi pippet Proline, Biohit, Finland

Balance

Kern EW 600-2M, Kern&Sohn, Germany

Centrifuges

Beckman Coulter – Optima TM LE-80K Ultracentrifuges, USA

Centrifuge 5418, Eppendorf, Germany

K23, Janetzki, Germany

K24, Janetzki, Germany

K70, Janetzki, Germany

Cooker

Cooker ETA, Czech Republic

Electrophoresis equipment

Mini-PROTEAN Tetra System, BioRad, UK

Incubator

IR 1500 Automatic CO_2 Incubator, Flow laboratories, UK

Luminescence Spectrofotometer

Perkin Elmer LS55, USA

Magnetic stirrer

KMO 2 basic IKA®- WERKE, IKA, Germany

Microliter syringe

Microliter syringe 702RN, Hamilton, Switzerland

Microplate reader

Microplate absorbance reader Sunrise, TECAN, Switzerland

pH meter

HI 2211, HANNA Instruments, USA

Pippetes

Nichipet EX, Nichiryo America, Inc., Japan

Power supply

EPS 301 Power supply, Amersham Biosciences, USA

Protein detection system

SNAP i.d®, Millipore corp., USA

Sonicator

Elmasonic E 30 H, P-Lab, Czech Republic

Spectrophotometr

SpectroMOM 195 D, MOM, Hungary

Vortex

MS 1 minishaker, IKA, Germany

Water purification system

Milli-Q® Simplicity 185, Millipore corp. USA

Western blot equipment

TransBlot® Turbo™ Transfer System, BioRad, UK

3.2. Methods

3.2.1. Treatment

Male Wistar rats (~ 120 g, purchased from Anlab, Germany) were allowed to acclimatize for 5 days in standard cages at 22°C with a 12h light/dark period and *ad libitum* diet and water access in the Centre for Experimental Biomodels, Prague, Czech Republic in accordance with Decree No 39/2009 Collection of Laws. The animals were then divided into eight groups (two animals per group).Control group was treated with 0.5 ml of sunflower oil only. All tested compounds were dissolved in sunflower oil and were administered by gastric gavages *p.o.* in a concentration of 120 mg/kg body weight to rats. The scheme of treatment is shown in Table 1.

- **Regimen I:** Flavonoids (α-naphthoflavone, dihydromyricetin) were administered by gastric gavage to animals. 96 hours after dosing, the animals were anaesthetized with carbon dioxide and then killed by breaking their spinal cord.
- **Regimen II:** Flavonoids **(**α-naphthoflavone and dihydromyricetin) were administered to rats and 72 hours after that, carcinogen benzo[a]pyrene (BaP) was gavaged. The treated rats were fasted overnight and 24 hours after the last treatment, animals were sacrificed.
- **Regimen III:** Benzo[a]pyrene (BaP) was administered also separately without a combination with flavonoids and 24 hours after dosing the treated animals were sacrificed.
- **Regimen IV:** Flavonoids (α-naphthoflavone, dihydromyricetin) were administered together with carcinogen BaP and the rats were sacrificed 24 hours after treatment.

Table 1: Scheme of administration

Groups	**control**	**ANF**	**dHMR**	**BaP**	**ANF→ BaP**	**dHMR→ BaP**	**BaP + ANF**	**BaP + dHMR**
Day 1	SO	ANF	dHMR	SO	ANF	dHMR	SO	SO
Day 2	-	-	-	-	-	-	-	-
Day 3	-	-	-	-	-	-	-	-
Day 4	SO	SO	SO	BaP	BaP	BaP	BaP + ANF	BaP + dHMR
Day 5	sacrifice							

SO = sunflower oil; ANF - α-naphthoflavone; dHMR - dihydromyricetin; BaP - benzo[*a*]pyrene, → indicates sequential administration; + indicates that compounds were administered together

3.2.2. Preparation of microsomal fractions

Buffer B1: 0. 15 KCl, 0.05 M Tris/HCl; pH 7.4; tocopherol in methanol (in final concentration 50 μM)

Buffer B2: 0. 1 M $Na_4P_2O_7.10H_2O$ (adjusted to pH 7.2 by 1 mM HCl)

Buffer B3: 0. 15 M KCl, 0.05 M Tris/HCl, 20% (v/v) glycerol; pH 7.4

Microsomal fractions were prepared from the liver, small intestine and colon immediately after sacrificing the rats. The small intestine was removed approximately 2 cm distal to the pylorus and divided into three parts: proximal, middle and distal (each circa 20 cm in length). Tissues from two rats were pooled for each microsomal preparation.

To isolate the microsomal fraction, standard differential centrifugation was used. The whole procedures were carried out in a cold room at 4°C, all buffers and materials were cooled down before using. The liver, all parts of small intestine and colon were thoroughly washed using buffer B1, weighted and minced with scissors. Subsequently, prepared tissues were homogenized in buffer B1 in a Potter-Elvehjem glass homogenizer and centrifuged at 600×g for 10 minutes at temperature 0-5°C (centrifuge Janetzi K-23, swing-out rotor 4x70 ml, 2000 RPM). The volume of buffer B1 depended on the tissue mass. For each 1 g of tissue, 4 ml of buffer B1 were used. Supernatants were then collected and pellets were rehomogenized in buffer B1 (for each 1 g of tissue, 250 μl of buffer B1 were used). Rehomogenates were centrifuged under the same conditions. Subsequently, both supernatants were combined and centrifuged at 15 000×g for 20 minutes at temperature 0-5°C (centrifuge Janetzki K-24, fixed-angle rotor 6x35 ml, 13 500 RPM). The supernatants were carefully separated from the pellets and were ultracentrifuged at 123 000×g for 90 minutes at 4°C (ultracentrifuge Beckman Coulter-Optima LE-80K, fixed-angle rotor Ti45, 6x64 ml, 35 000 RPM). The supernatants (cytosolic fractions) were collected and immediately frozen and stored at -80°C, the pellets were resuspended and homogenized using small Elvehjem glass homogenizer in buffer B2 (for each 1g of tissue, 2 ml of buffer B2 were used) and subsequently ultracentrifuged at 425 000×g for 90 minutes at 4°C (ultracentrifuge Beckman Coulter-Optima LE-80K, fixed-angle rotor Ti70, 8x36 ml, 60 000 RPM). After that the supernatants were removed, the final pellets were resuspended in buffer B3 (for each 1g of tissue, 200 μl of buffer B3 were used). The microsomal fractions were aliquated, immediately frozen and stored at -80°C before used.

3.2.3. Bicinchonic acid protein assay

Reagent A: 2% (w/v) $Na_2CO_3 \cdot 10\ H_2O$; 0.95% (w/v) $NaHCO_3$; 0.16% (w/v) sodium tartarate; 0.4% (w/v) NaOH, 1% (w/v) bicinchonic acid (BCA) sodium salt

Reagent B: 4% (w/v) $CuSO_4.5H_2O$

Protein concentration in microsomes was determined according to Wiechelman et co-workers [86] using Thermo Scientific™ Pierce™ BCA Protein Kit. It has been shown that cysteine, cysteine, tryptophan, tyrosine, and the peptide bonds are capable of reducing Cu^{2+} to Cu^{+}. A purple-colored reaction product is formed by the chelation of two molecules of bicinchonic acid (BCA) with the cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations.

All measurements were conducted in a 96-well transparent microplate. Intestinal and colon microsomes were diluted by water in a ratio of 1:11.5 and 1:24; liver microsomes in a ratio of 1:49. The working reagent was prepared just before use by mixing 50 parts of reagent A and 1 part of reagent B. To determine the totel protein concentration in the sample, 9 µl of the sample and 260 µl of the working reagent were added into the well. Standards for the calibration curve were prepared by diluting the bovine serum albumin (BSA) with working reagent to final concentrations of 0.125, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 mg/ml. The blank contained 9 µl of distilled water instead of the standard BSA. The standards were performed in duplicates, the samples in triplicates.

All prepared standards, samples and blanks were incubated for 30 minutes at 37˚C (IR 1500 Automativ CO_2 incubator). After incubation, the absorbance was measured at 562 nm on the microplate reader (Sunrise Absorbance Reader), protein concentration of each samples was calculated using Kim32 programme.

3.2.4. Sodium dodecyl-sulphate polyacrylamide gel electrophoresis

Buffer A: 0. 375 M Tris/HCl; pH 8.8; 0.1% (w/v) sodium dodecyl-sulphate (SDS)

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

Buffer B: 0. 125 M Tris/HCl; pH 6.8; 0.1% (w/v) SDS; 0.006% (w/v) bromphenol blue

Polymerization solution B: 30% (w/v) acrylamide; 0.8% (w/v) BIS; both in buffer B

Sample buffer: 0. 25 M Tris/HCl; 8% (w/v) SDS; 20% (v/v) 2-mercaptoethanol, 40% (v/v) glycerol; 0.004% (w/v) bromphenol blue; pH 6.8

Electrode buffer: 0. 192 M glycine; 0.025 M Tris/HCl; pH 8.3; 0.1% (w/v) SDS

For 4 gels:

Resolving gel 12%: 20.9 ml buffer A; 7.6 ml polymerization solution A; 28.5 µl tetramethylethylendiamine (TEMED); 285 µl (100 mg/ml) ammonium peroxodisulphate (APS)

Stacking gel 3%: 9 ml buffer B; 1 ml polymerization solution B; 10 µl TEMED; 200 µl (100 mg/ml) APS

Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is an analytical method used to separate components of a protein mixture based on their size using discontinues polyacrylamide gel as a support medium and an anionic detergent sodium dodecyl sulphate (SDS) to denature the proteins. The proteins being covered with SDS are negatively charged and are strongly attracted toward the anode (positively-charged electrode) when electric field is applied.

The solution for resolving gel was poured between the glasses (10x8.2; 10x7.4; 1 mm thickness), immediately overlayed with water and left to polymerize for 30 minutes at room temperature. Water was then removed and the top of the resolving gel was dried with filter paper. The stacking gel was poured onto the top of the resolving gel and the 10 wells comb was inserted between the spacers and the gel was again left to polymerize for 15 minutes at room temperature. Microsomal samples were diluted with water to the final concentration of 2 mg/ml and then mixed with sample buffer in the ratio of 3:1. The PageRuler Unstained Broad Range Protein Ladder (thermo Scientific) was diluted 3:1 with the sample buffer. All samples were subsequently boiled for 5 minutes at 100°C in a water bath. The prepared samples (20 µl) and marker (5µl) were loaded into the wells with a Hamilton syringe. The assembly was placed into the tank and filled with the electrode

buffer to the indicated level (680 ml for 4 gels). Electrophoresis was run at the constant voltage of 200 V until the dye front reached the bottom of the gel. The separated proteins were visualized either by Coomassie Brilliant Blue staining or using Western blot.

3.2.5. Coomasie Brilliant Blue staining of polyacrylamide gel

Staining bath: 0. 25% (w/v) Coomassie Brilliant Blue R-250, 46% (v/v) ethanol; 9.2 % (v/v) acetic acid
Distaining bath: 25% (v/v) ethanol, 10% (v/v) acetic acid

Polyacrylamide gel containing separated proteins was transferred into the staining bath for 1 hour at room temperature with a gentle agitation. The gel was then left to distain overnight. Stained gels were scanned on the scanner.

3.2.6. Western blotting

Transfer buffer: 0. 025 M Tris; 0.192 M glycine; pH 8.3
PBS Triton X-100: 0. 134 M NaCl; 1.8 mM $Na_2HPO_4.10H_2O$; 1mM 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

The Western blotting is used to determine the relative amounts of the protein presented in various samples. Following the electrophoresis, separated microsomal proteins are transferred to a membrane and then visualized using specific antibodies against the protein.

Following the SDS-PAGE, the stacking gel was removed and the gel was equilibrated with the transfer buffer for 30 minutes at room temperature with a gentle agitation. In the meantime, the nitrocellulose membrane and the Whatman papers were cut to correspond with the dimensions of the gel and dropped into electrode buffer, washed in water and then equilibrated in the transfer buffer for 20 minutes at room temperature. The blotting sandwich was subsequently made by placing three Whatman papers on the anode electrode plate in the centre of the cassette base followed by nitrocellulose membrane. Gel was aligned onto the membrane and covered with another three Whatman papers. The blot roller was used to remove air bubbles between the layers and finally the cassette lid was

placed on the base. The cassette was slided into the Trans-Blot® Turbo. Proteins were transferred at a constant voltage of 25 V for 7 minutes.

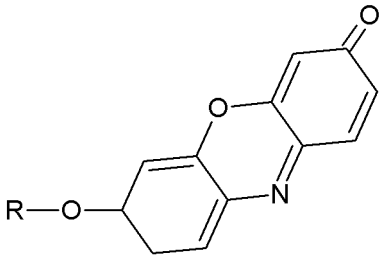
Once the transfer was completed, the membrane was blocked in 5% non-fat dried milk either for 1 hour at room temperature with a gentle agitation or overnight at 4°C. Blocking was used to prevent further non-specific binding of proteins. Then the membrane was incubated with specific primary chicken anti-rat CYP1A1/2 (anti human/rat NAT2) antibody in the concentration of 30 μg/ml in blocking solution for 2 hours at room temperature with gentle agitation, followed by 3-times extensive washing in blocking solution.

For the incubation of the membrane with the secondary antibody, Snap i.d. Protein Detection System (Millipore) was used. The membrane was placed on MilliQwet chambers with the protein side down. To eliminate the air bubbles, the roller blot was used and the blot holder was placed into the aparature. The membrane was washed three times by 15 ml of PBS Triton X-100 (the vacuum was turned on) and then incubated with secondary rabbit anti-chicken antibody conjugated with alkaline phosphatase diluted 1:1429 in PBS Triton X-100 for 10 minutes at room temperature (the vacuum was turned off).

Finally, the membrane was washed and developed using 10 ml of solution 5-bromo-4-chloro-3-indolyl phosphate/nitrobluetetrazolium (BCIP/NTB) tablet containing 10 mg substrate for alkaline phosphatase with water. The reaction was stopped by washing the membrane in water followed by drying the membrane between filter papers.

3.2.7. Ethoxyresorufin-*O*-deethylase activity assay

The substrate 7-ethoxyresorufin (see Figure 8, p.32) metabolized (dealkyled) by cytochromes P450 to a fluorescent product, resorufin (see Figure 8, p.32). The excitation wavelengths 530 nm and emission wavelengths 585 nm were used. The fluorescence was measured on Luminescence Spectrometer PerkinElmer LS-55 equipped with 96-well plate reader.



Compound	R
Resorufin	H
7-Ethoxyresorufin	C_2H_5

Figure 8: Structure of resorufin and 7-ethoxyresorufin

Dilution buffer: 0.1 M potassium phosphate; pH 7.4

Resorufin stock solution: 10 μM resorufin in methanol

Substrate: 0.2 mM 7-ethoxyresorufin in DMSO

Cofactor: 5 mM NADPH in water

The microsomal samples were diluted with potassium phosphate buffer to reach the protein concentration 0.5 mg/ml and the samples were gently vortexed. Then the specific substrate 7-ethoxyresorufin was added to stock solution to get final concentration 2.2 μM and the solution was pre-incubated for 2 minutes at room temperature. The aliquots (150 μl) of the reaction mixture were loaded into plate wells and the reaction was started by adding 17 μl of NADPH solution. The plate was put into spectrometer (Perkin Elmer LS 55 Luminescence Spectrophotometer) immediately and fluorescence was measured for 10 minutes at room temperature. Enzyme activities were quantified using resorufin standard curve prepared by diluting the resorufin stock solution by potassium phosphate buffer to achieve final concentrations of 0.0125; 0.025; 0.5; 0.1; 0.2; 0.3 and 0.4 μM. The blank contained only potassium phosphate buffer.

3.2.8. Chicken antibody preparation

To prepare antibody against NATs, peptide antigens were covalently conjugated to a carrier protein Keyhole limpet hemocyanin (KLH). Keyhole limpet hemocyanin (KLH) belongs to a group of non-heme proteins that are presented in arthropods and molluscs.

3.2.8.1. **Protein conjugation with a protein carrier**

For peptide conjugation, maleimide-activated mcKLH was used. Cysteine containing reduced sulfhydryl group react with maleimide groups from mcKLH to form stable thioester bonds (see Figure 9).

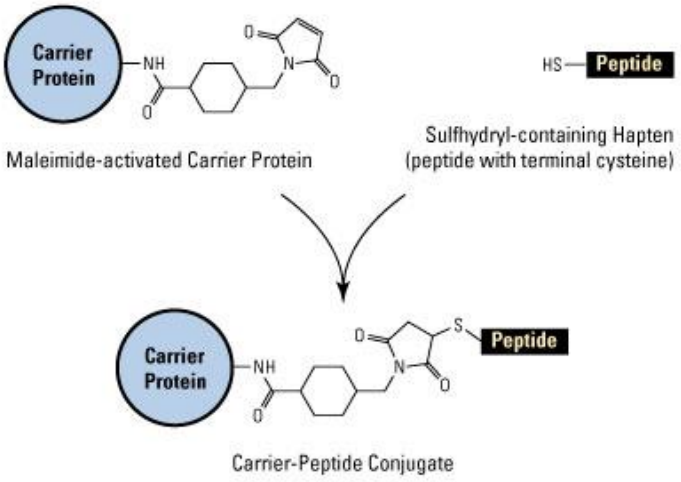


Figure 9: Reaction scheme of mcKLH conjugation.

Conjugation Buffer: 0. 1 M NaH_2PO_4; 0.15 M NaCl; pH 7.2

The Imject Maleimide Activated mcKLH solution was prepared by adding water to mcKLH (final concentration 10 mg/ml). The peptide was diluted with the conjugation buffer to make10 mg/ml solution. The peptide solution was then mixed with activated mcKLH in a ratio 1:1 (200 µl) and reaction mixture was incubated for 2 hours at room temperature under gentle agitation. Samples were aliquoted and stored at -20°C.
Prepared immunogens were used for chicken immunization. The eggs with antigen-specific IgY were collected between 5-6 weeks after immunization.

3.2.8.2. **Preparation of water-soluble fraction of yolk**

The immunoglobulin fraction was isolated from pooled egg yolks according to Hodek et al (1999). For the isolation of specific antibodies, 8 eggs collected a week after the last immunization were used. Egg yolks were separated from the whites, washed with tap water and pooled in a calibrated cylinder. The pool was diluted with tap water in a ratio 1:7 and pH of fraction was adjusted to pH 5.0 with 0.5 M HCl and the suspension of diluted yolks was frozen at -20°C. During spontaneous thawing at room temperature, the water-soluble

fraction was separated from yolk lipids. The water soluble fraction was precipitated by addition of solid NaCl 8.76 % (w/v) and pH was adjusted to 4.0 with 1.0 M HCl. Mixture was stirred for 30 minutes and then incubated 1.5 hour at room temperature without agitation. The precipitate was collected by centrifugation (Janetzki K70) at 2700×g for 20 minutes at 4°C. Supernatants were discarded and the pellets dissolved in PBS containing 0.1 % azide and stored at 4°C.

The protein concentration in the sample was determined by measuring the absorbance at 280 nm using spectrophotometer (SpectroMOM 195 D). As the blank, PBS with 0.1% sodium azide was used. For the determination of proteins concentration *c* in IgY fraction, the following equation was used:

$$c = A_{280} \cdot f \cdot n \,,$$

where A_{280} is the absorbance at 280 nm, *f* is the empiric factor = 1,094 and *n* is the dilution of the sample.

3.2.8.3. **ELISA**

Immobilization solution: 15 mM Na_2CO_3; 35 mM $NaHCO_3$; pH 9.6

Washing buffer (PBS -Tween 20): 13.4 mM NaCl; 1.8 mM Na_2HPO_4; 1 mM NaH_2PO_4; 0.1 % (v/v) Tween 20; pH 7.2

Blocking solution: 2% (w/v) egg white solution in PBS -Tween 20

PBS: 13.4 mM NaCl; 1.8 mM Na_2HPO_4; 1 mM NaH_2PO_4; pH 7.2

Substrate solution: 1 mg/ml p-nitrophenyl phosphate (pNPP); 0.02 M Na_2CO_3; 0.03 M $NaHCO_3$; 1 mM $MgCl_2$

The ELISA plates (F16 MaxiSorp NUNC-IMMUNO MODULE) were coated with 100 ng of antibody against NATs (dissolved in 100 µl of immobilization buffer) per well overnight. The plates were then washed with 200 µl washing solution (three times), then each well was loaded with 150 µl of blocking solution and incubated 1 hour at 37°C. After next washing step, 100 µl of antibody solution (2-fold serial dilutions in concentration 3.3, 10.0, 30.0 and 90.0 µg/ml) or control solution were added to plates and incubated 2 hours at 37°C. Subsequently, plates were washed and 100 µl of alkaline phosphatase-conjugated

rabbit anti-chicken IgG in PBS was added to each well (diluted in ratio 1:1) and incubated at 37°C for 1 hour. After next washing step, 100 µl of substrate solution was added and after 10 minutes. The reactions were stopped by addition of 50 µl of 3 M NaOH per well and absorbance was measured at 405 nm using ELISA reader (Microplate absorbance reader Sunrise).

3.2.9. Arylamine acetylation assay

Assay buffer: 20 mM Tris-HCl, pH 8.0

CoA stock solution: 10 mM CoA in assay

Substrate: 0.5 mM p-aminobenzoic acid in water, 0.5 mM sulfamethazine in ethanol

Quenching solution: 6.4 M guanidine-HCl, 0.1 M Tris-HCl, pH 7.3

Chromogenic detection agent: 5 mM 5.5'dithio-bis (2-nitrobenzoic acid) (DTNB) in assay buffer

The NAT catalysed acetylation of arylamines result in hydrolysis of AcCoA to give free CoA. DTNB reacts with free thiol groups in solution to produce thionitrobenzoate (TNB) which has a strong absorbance at λ=405 nm (Ridles 1983).

The reactions were set up in 96 well plates. 10 µl of recombinant NATs (0.01 µg/µl) or cytosolic samples were mixed with 25 µl of 500 µM NAT substrate p-aminobenzoic acid (PABA) or sulfamethazine and pre-incubated for 5 minutes at 37°C. AcetylCoA was then added to a final concentration of 400 µM to start the reaction in a total volume of 100 µl. The reaction was quenched with 25 µl of guanidine hydrochloride solution containing 5 mM DTNB and the absorbance was measured on a plate-reader (Microplate absorbance reader Sunrise) within 5, 10, 15, 20 and 25 minutes. The amount of CoA produced was determined from a calibration curve. Controls were carried out in which substrate, acetylCoA or NAT were replaced by the assay buffer.

4. RESULTS

4.1. Isolation of rat microsomal fractions

Microsomal fractions (MS) were isolated from liver, different sections of small intestine and colon of male Wistar rats treated with the combination of selected chemopreventive compounds (flavonoids α-naphtoflavone and dihydromyricetin) and/or carcinogen BaP as described in Chapter 3.2.1. Tissues from 2 rats were pooled for each microsomal preparation. The protein concentration of isolated microsomal fractions were determined using bicinchonic acid protein assay (see Table 2 – 4, p. 37).

Table 2: Protein concentration and tissue mass of liver microsomal fractions

Liver MS fraction	m_{tissue} [g]	$c_{protein}$ [mg/ml]
Control	18,86	44,17
α-Naphtoflavone → control	18,60	22,36
Dihydromyricetin → control	18,89	24,43
Control → BaP	20,65	27,27
α-Naphtoflavone → BaP	20,14	34,21
Dihydromyricetin → BaP	17,71	28,11
Control → (α-naphtoflavone + BaP)	13,44	20,58
Control → (dihydromyricetin + BaP)	19,57	32,46

m_{tissue} [g] - used tissue mass, $c_{protein}$ - protein concentration, BaP - benzo[a]pyrene, → indicates sequential administration; + indicates that compounds were administered together

Table 3: Protein concentration and tissue mass of small intestine microsomal fractions

Intestinal MS fraction	Proximal part		Middle part		Distal part	
	m_{tissue} [g]	$c_{protein}$ [mg/ml]	m_{tissue} [g]	$c_{protein}$ [mg/ml]	m_{tissue} [g]	$c_{protein}$ [mg/ml]
Control	2,84	1,51	4,04	7,73	3,02	8,18
α-Naphtoflavone → control	3,03	6,53	3,06	10,55	2,92	6,88
Dihydromyricetin → control	3,57	8,58	3,29	9,53	3,45	10,30
Control → BaP	3,37	3,64	3,16	9,81	3,28	10,30
α-Naphtoflavone → BaP	2,98	10,53	3,76	10,30	2,68	9,76
Dihydromyricetin → BaP	3,28	6,81	2,67	10,30	2,81	8,76
Control → (α-naphtoflavone + BaP)	2,56	10,11	2,27	6,10	1,65	4,48
Control → (dihydromyricetin + BaP)	2,05	6,11	3,24	11,81	3,63	12,40

m_{tissue} [g] - used tissue mass, $c_{protein}$ - protein concentration, BaP - benzo[a]pyrene, → indicates sequential administration; + indicates that compounds were administered together

Table 4: Protein concentration and tissue mass of colon microsomal fractions

Colon MS fraction	m_{tissue} [g]	$c_{protein}$ [mg/ml]
Control	1,88	6,20
α-Naphtoflavone → control	1,34	4,95
Dihydromyricetin → control	1,47	5,73
Control → BaP	1,15	4,38
α-Naphtoflavone → BaP	1,44	3,40
Dihydromyricetin → BaP	1,39	3,60
Control → (α-naphtoflavone + BaP)	1,20	5,23
Control → (dihydromyricetin + BaP)	1,51	7,22

m_{tissue} [g] - used tissue mass, $c_{protein}$ - protein concentration, BaP - benzo[a]pyrene, → indicates sequential administration; + indicates that compounds were administered together

4.2. CYP expression in microsomal fractions

The presence of CYP1A1 in liver, different part of small intestine and colon microsomal fractions was determined using Western blotting technique. During electrophoresis, CYP1A1 migrated in the gel to zone of relative molecular mass around 55kDa, which corresponded to the molecular weight of mammalian CYP1A1. The results of Western blot detections of CYP1A1 are shown in Figure 10, p.38. No significant induction of CYP1A1 expression was observed in rat liver microsomes when only flavonoids (α-naphtoflavone, dihydromyricetin) were administered (see Figure 10, p.38). On the other hand, treatment with either carcinogen alone or all combinations of carcinogen and chemopreventive compounds resulted in the induction of CYP1A1.

No induction of CYP1A1 expression was observed in any parts of small intestine (see Figure 10, p.38) while treating only with chemopreventive compounds. However, the combination of carcinogen and chemopreventive compounds led to induction of CYP1A1 expression. Interestingly, while strong induction of CYP1A1 expression after administration of carcinogen was seen in middle and distal part of small intestine, no induction was observed in its proximal part. This phenomenon though did not occur when repeating the experiment since CYP1A1 were detected in all parts of small intestine (data not shown).

Slightly different results were obtained in the case of colon (see Figure 10). While no induction of CYP1A1 expression was observed after administration of chemopreventive

compound α-naphtoflavone, expression of CYP1A1 after treatment with chemopreventive compound dihydromyricetin was visible. On the other hand, sequential administration of α-naphtoflavone and BaP led to much higher induction of CYP1A1 expression than in the case of dihydromyricetin and BaP. Interestingly, when administered together, induction of CYP1A1 expression by dihydromyricetin and BaP was comparable to the sequential administration but no induction could be seen in the case of α-naphtoflavone and BaP.

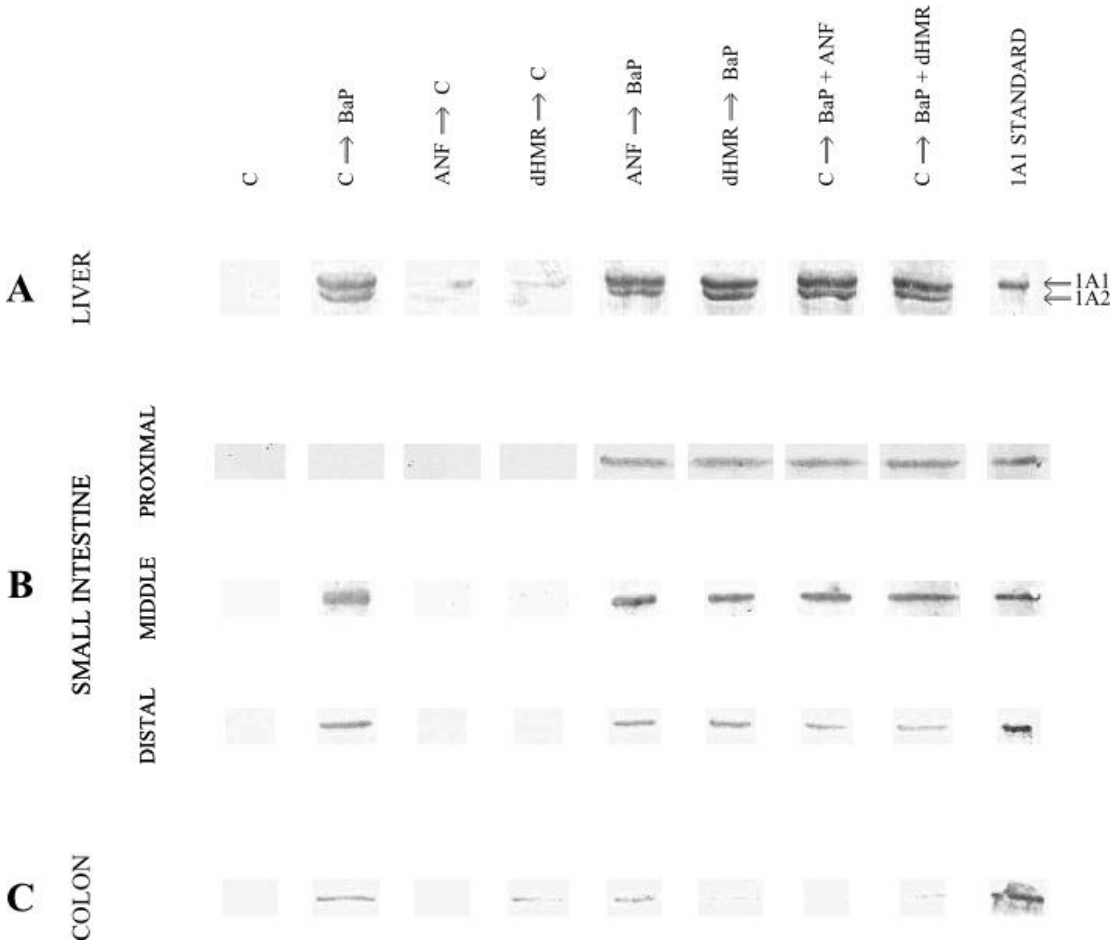


Figure 10: Summary of Western blot analysis of CYP 1A1 expression. Microsomal fraction of liver **(A)**, small intestine **(B)** and colon **(C)** after dietary exposure to flavonoids α-napthoflavone and dihydromyricetin (120 mg/kg body weight) and/or carcinogen BaP (120 mg/kg body weight) were used. The microsomal proteins (18 µg of total protein per line) separated by SDS PAGE were transferred to nitrocellulose membrane and probed with antibody against CYP1A1/2. The arrow → indicates that compounds were administered sequentially (the first one 72 hours before the second one) and the plus + indicates that compounds were administered together. C – control (sunflower oil), BaP – benzo[a]pyrene, ANF – α-napthoflavone, dHMR – dihydromyricetin, 1A1 – rat recombinant CYP1A1 expressed in Supersomes™

4.3. Effect of treatments on metabolic activity of CYP1A1

7-Ethoxyresorufin-*O*-deethylation (EROD) activity of CYP1A1, the major enzymes catalysing the deethylation of 7-ethoxyresorufin to fluorescent product resorufin was determined Formation of the resorufin was continuously measured for 10 minutes at room temperature by monitoring its fluorescence. The dealkylation rate was estimated on the basis of linear resorufin standard curve (see Figure 11).

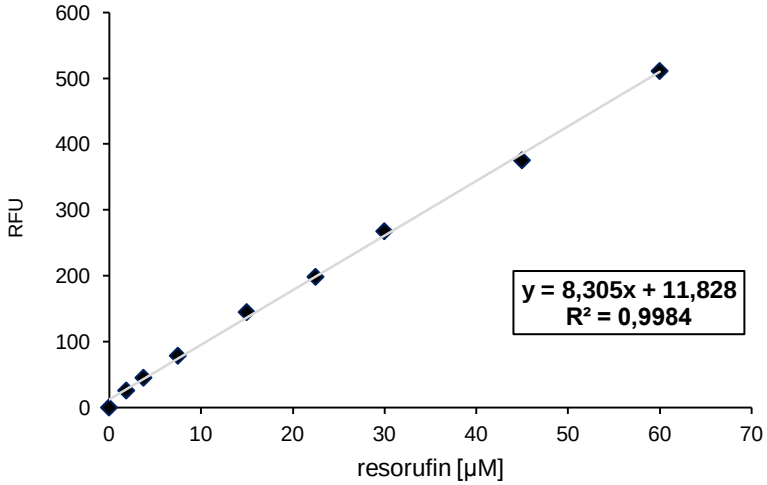


Figure 11: Calibration curve of resorufin. Calibration curve of resorufin was prepared by diluting the resorufin stock solution by potassium phosphate buffer to achieve final concentrations of 0.0125; 0.025; 0.5; 0.1; 0.2; 0.3 and 0.4 µM. RFU – relative fluorescence units

Two flavonoids (α-napthoflavon and dihydromyricetin) were tested for their effects on CYP1A1 activity in liver, small intestine and colon after *p.o* administration with/without the carcinogen benzo[a]pyrene (BaP) (for more details see Chapter 1.1.1).

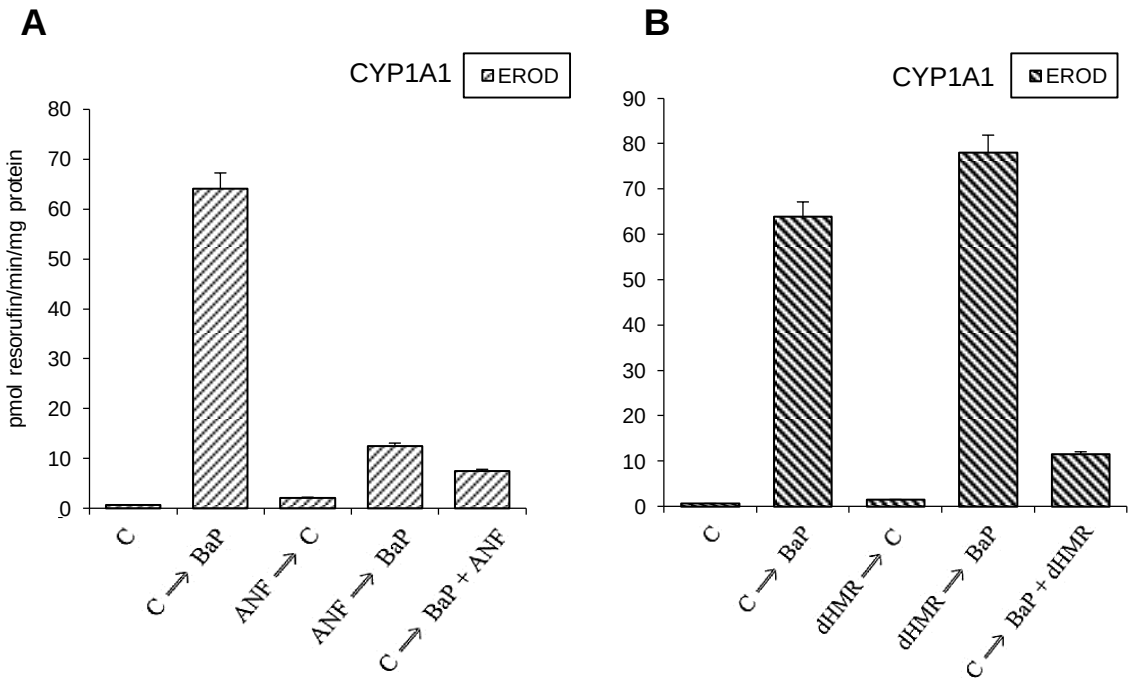


Figure 12: EROD activity of CYP1A1 in rat liver. Microsomes were isolated from rat liver after exposure to flavonoid compounds (120 mg/kg body weight) α-napthoflavone **(A)** and dihydromyricetin **(B)** and/or carcinogen benzo[a]pyrene (120 mg/kg body weight). Bars represent the means ±SD of 3 measurements. The arrow → indicates that compounds were administered sequentially (the first one 72 hours before the second one) and plus + indicates that compounds were administered together. C – control treatment (sunflower oil), BaP –benzo[a]pyrene, ANF – α-napthoflavone, dHMR – dihydromyricetin

EROD activity for CYP1A1 in respect of liver microsomes is depicted in Figure 12. Almost no EROD activity was detectable after treatment with sunflower oil (control) as well as chemopreventive compound alone. On the other hand, BaP administration led to a strong increase of EROD activity leading to even higher EROD activity when combination of BaP and dihydromyricetin was administered. α-Napthoflavon and BaP administered subsequently also enhanced specific activity (see Figure 12 A) but to a lesser extent than dihydromyricetin. In addition, a slight increase of EROD activity was observed when carcinogen and chemopreventive compound were administered together.

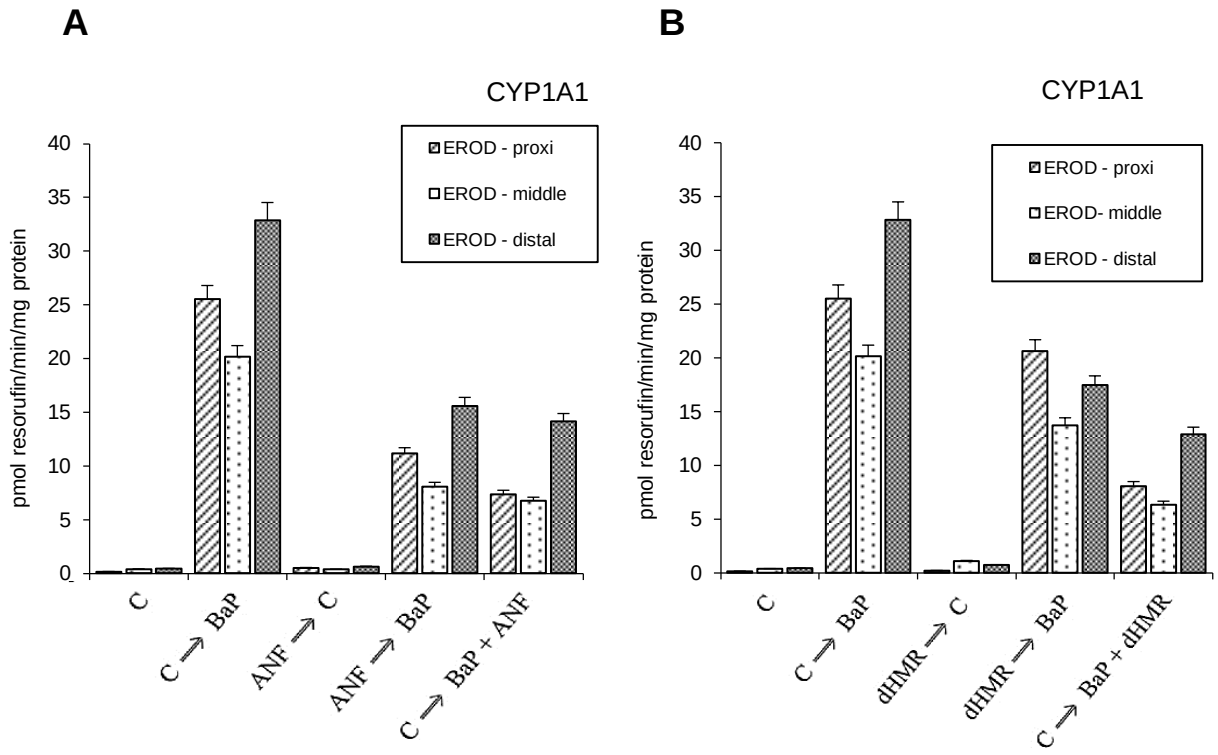


Figure 13: EROD activity of CYP1A1 in rat small intestine. Microsomes were isolated from rat small intestine after exposure to flavonoid compounds (120 mg/kg body weight) α-napthoflavone **(A)** and dihydromyricetin **(B)** and/or carcinogen benzo[a]pyrene (120 mg/kg body weight). Bars represent the means ±SD of 3 measurements. The arrow → indicates that compounds were administered sequentially (the first one 72 hours before the second one) and plus + indicates that compounds were administered together. C – control treatment (sunflower oil), BaP – benzo[a]pyrene, ANF – α-napthoflavone, dHMR – dihydromyricetin

The effects of flavonoids on CYPs in small intestine divided into three parts (proximal, middle, and distal) are shown in Figure 13. The administration of sunflower oil (control) as well as chemopreventive compound alone did not affect EROD activity. α-Napthoflavone effects on CYP1A1 activity showed similar trend as in liver (see Figure 13 A). On the other hand, EROD activity after the treatment with combined of BaP and dihydromyricetin was not higher than in case of BaP administration only. Surprisingly, the highest increase of EROD activity during that all treatments containing BaP was observed in the distal part of the small intestine.

In Figure 14, the effect of flavonoids on CYP in colon is shown. Only in BaP administration, significantly CYP activity increased compared to control rats. A slight increase of EROD activity was observed after treatment of both combinations of carcinogen and chemopreventive compound and when only dihydromyricetin was administered.

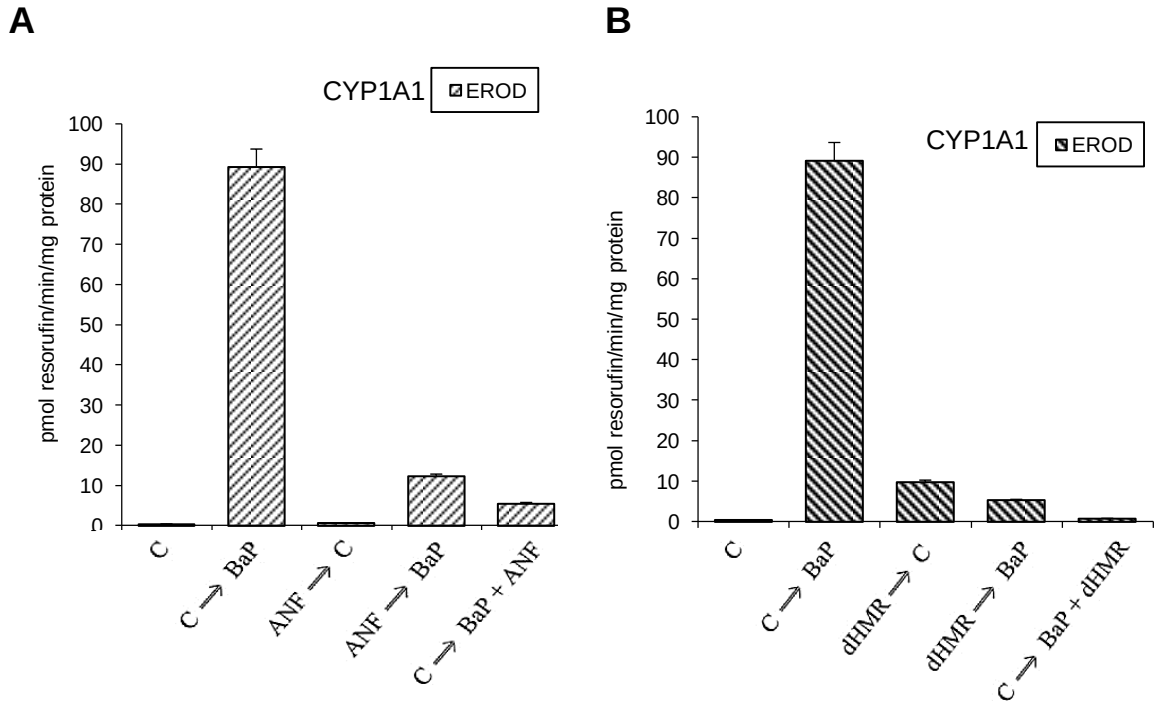


Figure 14: EROD activity of CYP1A1 in rat colon. Microsomes were isolated from rat colon after exposure to flavonoid compounds (120 mg/kg body weight) α-napthoflavone **(A)** and dihydromyricetin **(B)** and/or carcinogen benzo[a]pyrene (120 mg/kg body weight). Bars represent the means ±SD of 3 measurements. The arrow → indicates that compounds were administered sequentially (the first one 72 hours before the second one) and plus + indicates that compounds were administered together. C – control treatment (sunflower oil), BaP –benzo[a]pyrene, ANF – α-napthoflavone, dHMR – dihydromyricetin

4.4. Preparation of chicken antibody

All synthetized peptides (see Table 5), designed by prof. RNDr. Petr Hodek, CSc. were diluted with conjugation buffer and then mixed with activated mcKLH to prepare the peptide immunogen. Production of the antibodies was performed by immunization of Leghorn hens with 3 injections of NAT antigens (0.1 mg/dose/animal).

Table 5: List of the NAT peptides used for peptide immunogen preparation

Enzyme	Peptide sequence
NAT1	C-GYKNSVNK
NAT2	C-GYQSSRNK
NAT1/2	C-VPNQEFVNSD

C- means that cysteine was not originally a part of the sequence, but that it was introduced for further conjugation to the carrier protein

4.5. Isolation and characterization of specific peptides

Specific antibodies were prepared from pooled yolks of 6-8 eggs collected after immunization. As a control, pooled yolks of 6-8 eggs collected before immunization were used. After isolation of specific antibodies (for the procedure see Chapter 3.2.8), the volume of antibodies fraction were measured and the protein concentration in the samples was determined using absorbance measurement at 280 nm (see Table 6).

Table 6: The volume and protein concentration of antibodies fraction

IgY	IgY fraction (ml)	IgY concentration (mg/ml)
K	17.0	26.8
NAT1	19.9	50.1
NAT2	24.3	41.2
NAT3	24.3	41.2

The specifity of antibody fractions was tested by ELISA. For the assay antibody was diluted concentrations of 3.3, 10, 30 and 90 µg/ml. For the IgY detection, the alkaline phosphatase-conjugated rabbit anti-chicken IgG was used. With increasing concentration of IgY, the absorbance at 405 nm increased (see Figure 15) suggesting that the antibodies specifically recognize the peptide antigen.

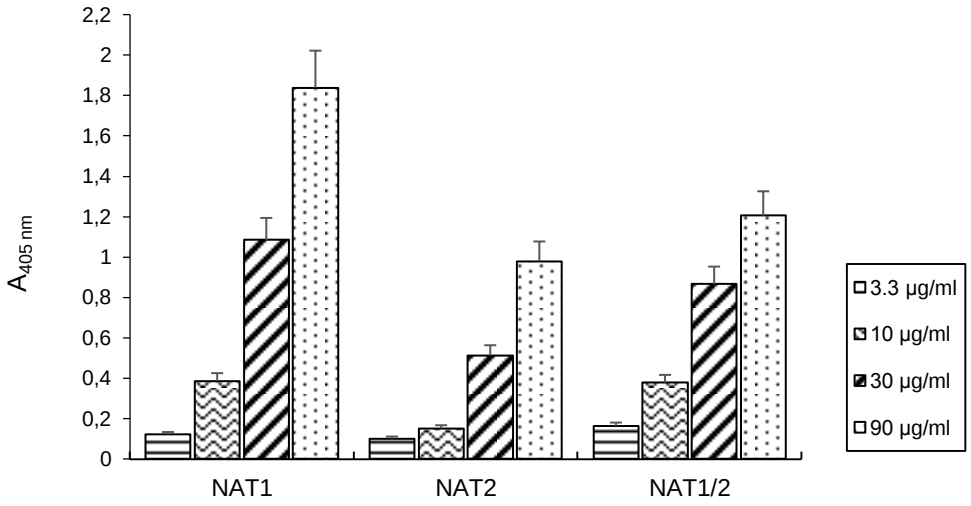


Figure 15: ELISA of NAT1, NAT2 and NAT1/2. The appropriate peptide was used as antigen. Bars represent the absorbance values at 405 nm of specific IgY fractions after control subtraction at different concentration (3.3, 10, 30 and 90 µg/ml) ± SD of 2 determinations.

4.6. Specifity of an NAT antibody

Specificity of prepared antibody raised against recombinant NATs was determined using Western blotting. Recombinant NATs were separated by SDS PAGE and transferred onto nitrocellulose membrane. The membrane was then incubated with prepared diluted IgY followed by incubation with alkaline phosphatase-conjugated rabbit anti-chicken IgG. Proteins were visualized by the substrate for alkaline phosphatase.

Prepared antibody (NAT2) was designed against human/rat NAT2. This NAT2 antibody was NAT-specific, with successful detection of recombinant human NAT2, the band of the molecular weight of 33 kDa approximately (see Figure 16, line 1). This antibody does not cross-react with recombinant human NAT1 (see Figure 16, line 2). Other anti-peptides antibodies did not specifically detect NAT enzymes on the membrane (data not shown). To be able to investigate the effect of flavonoids and carcinogen on NAT expression, rat liver cytosols were analysed by Western blot. However, none of anti-peptide antibodies detected any bands indicating the presence of NAT in rat liver cytosol (data not shown).

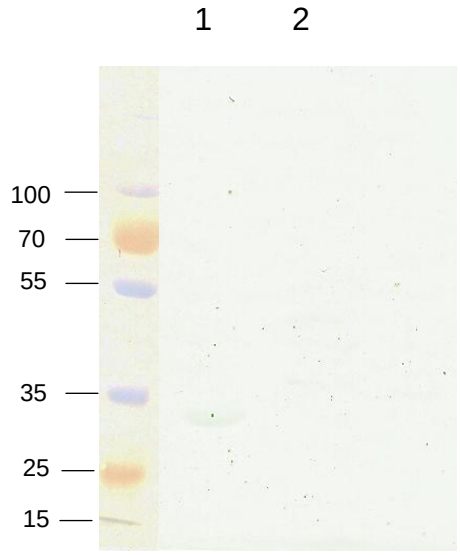


Figure 16: Immunoblots of human recombinant NATs expressed in Supersomes™. Electrophoresed human recombinant NAT2 (line 1) and NAT1 (line 2) were transferred onto nitrocellulose membrane and probed with prepared chicken anti-human/rat NAT2 antibody (10 µg/ml).

4.7. Kinetic studies of NATs

The rate of hydrolysis of acetyl-coA by NAT in the presence of a substrate (either p-aminobenzoic acid or sulfamethazine) was used to measure the NAT catalytic activity. The free CoA thiol was detected with 5,5´-dithio-bis(2-nitrobenzoic acid) (DTNB) using plate reader (Microplate absorbance reader Sunrise) and the amount of CoA produced in the reaction was determined from a calibration curve prepared from dilutions of CoA in assay buffer (see Figure 17). As controls, reaction mixture in which substrate, acetyl-coA, or NAT was replaced with assay buffer.

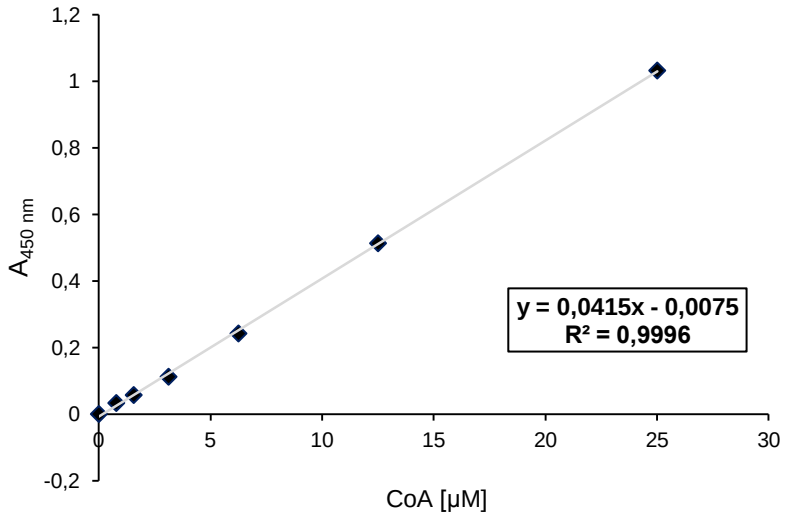


Figure 17: Calibration curve of CoA. Calibration curve of CoA was prepared by diluting the CoA stock solution by potassium phosphate buffer to achieve final concentrations of 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 µM.

Initially, the assay conditions were optimised using recombinant human NAT1 and NAT2. No spontaneous hydrolysis of Acetyl-coA in the absence of substrate or enzyme was detected. On the other hand, the formation of CoA increased over the time period when the recombinant NAT1 (see Figure 18 A, p.47) and NAT2 (see Figure 18 B, p.47) as well as substrates were present.

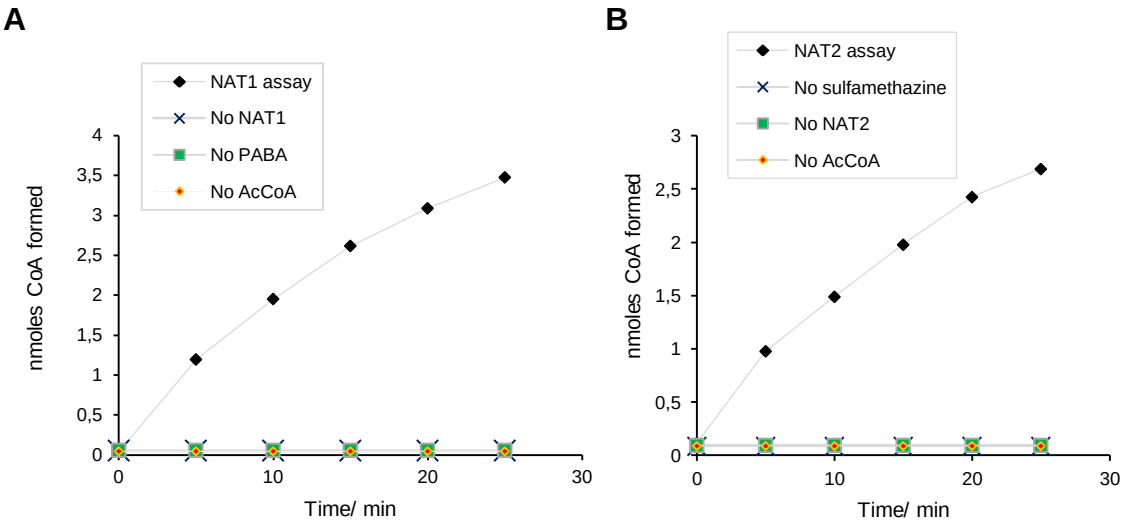


Figure 18: Measurement of recombinant human NAT1 and NAT2 activities by hydrolysis of acetyl-coA. The time courses for acetyl-coA hydrolysis with recombinant human NAT1 **(A)** and NAT2 **(B)** are shown. Recombinant human NAT (1 µg) was pre-incubated at 37°C with p-aminobenzoic acid (PABA) (500 µM) **(A)** or sulfamethazine (500 µM) (B) for 5 minutes prior to the addition of AcCoA (400 µM). The reaction was stopped after 5, 10, 15, 20 or 25 min. Reactions were performed using the same method but replacing PABA (■), NAT (×), Acetyl-coA (♦) with corresponding solution.

Following optimisation of the assay using recombinant NAT1 and NAT2, rat cytosols treated with 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) were used to screen NAT activity using the same method and substrate concentrations. While no spontaneous hydrolysis was observed in the absence of substrates (PABA as seen in Figure 19, A, p.48 or sulfamethazine as seen in Figure 19 B, p.48) or cytosols, the amount of CoA increased over the time period when the rat cytosols and substrates were present.

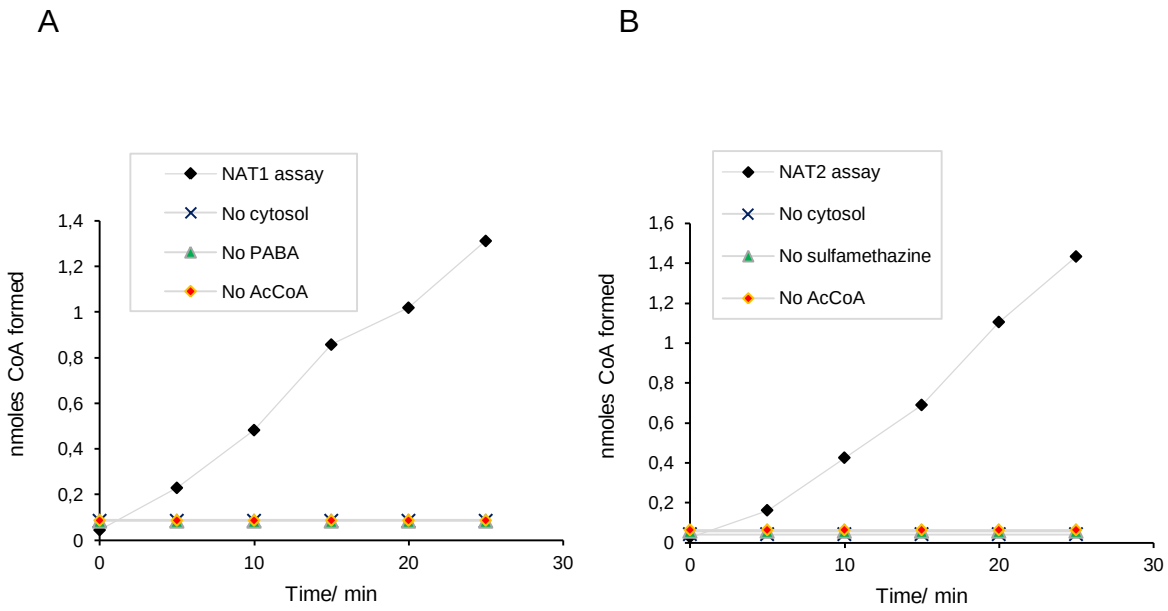


Figure 19: Measurement of hydrolysis of acetyl-coA by rat cytosols. The time courses for acetyl-CoA hydrolysis with rat cytosols treated with PhIP for NAT1 **(A)** and NAT2 **(B)** are shown. Rat cytosols (10 µl) were pre-incubated at 37°C with PABA (500 µM) **(A)** or sulfamethazine (500 µM) **(B)** for 5 minutes prior to the addition of AcCoA (400 µM). The reaction was stopped after 5, 10, 15, 20 or 25 min. Reactions were performed using the same method but replacing substrate (■), NAT (×), Acetyl-coA (♦) with corresponding solution.

5. DISCUSSION

The most prominent role in the process of carcinogenesis plays human exposure to chemical carcinogens. Carcinogenesis mechanism is conducted predominantly through DNA adduct formation, after metabolic activation of chemical carcinogens by enzymes of phase I (mainly cytochromes P450, CYPs) and phase II (among many other enzymes also N-acetyltransferases, NATs) of biotransformation. Normally during phase I, the reactive and polar groups are introduced into the exogenous compounds leading to generation of intermediates that can be further conjugated in phase II with hydrophilic endogenous molecules. This process enables to convert hydrophobic xenobiotics into more water-soluble molecules and thus helps to easily excrete them from human body. However, during biotransformation, relatively unreactive procarcinogens can be converted into electrophilic intermediates which can then react with DNA molecules and form DNA adducts mentioned above.

Many epidemilogical studies have suggested that the risk of developing cancer can be reduced by consumption of chemopreventive compounds. The most popular chemopreventive compounds are flavonoids present in fruits, vegetables and popular beverages (wine, tea, coffee). These compounds have been shown to possess antioxidant, antiviral, antitumour and anti-inflammatory properties [7]. Although flavonoids are often considered to be safe because of their „plant origin“, ingestion of flavonoids should be taken with caution. Indeed, by induction of specific CYPs, flavonoids can enhance the activation of carcinogens and/or influence their metabolism [7]. On the other hand, inhibition of CYP activities by flavonoids has been extensively studied because of their potential use as agents blocking the initiation stage of carcinogenesis [87]. More studies are though needed to investigate the potential health benefits of individual flavonoids as well as their potential harmful attributes.

Most studies on xenobiotic-metabolizing enzymes have been carried out with the liver enzymes since it is an organ with the highest capability of biotransformation [88]. However, the first barrier met by the exogenous compounds present in food or orally delivered drugs is gastroinstestinal tract. Indeed, in order to be absorbed and transferred to the whole body, xenobiotics need to first pass through the intestinal epithelium. It is thus

not surprising that the biotransformation of these compounds occurs to some extent also in small intestine and colon [57].

The first part of this study aimed to explore the possible effects of selected flavonoids (namely α-naphtoflavone and dihydromyricetin) on cytochromes P450 (CYPs) in the three organs highly exposed to xenobiotics – liver, small intestine and colon – of rat model organism.

To simulate real human intake, rats were treated *p.o.* by gastric gavages with the flavonoids (α-naphtoflavone and dihydromyricetin) and/or carcinogen benzo[a]pyrene (BaP). BaP was selected as a model carcinogen since it is known as strong inductor of CYP1A subfamily [89]. Flavonoids and BaP were administered either simultaneously or sequentially (BaP 72 hours after flavonoid, for detailed description of the setup see Chapter 3.2.1). This setup was desirable. It has been previously shown that when using the same experimental conditions, simultaneous administration of carcinogen and β-naphtoflavone (a flavonoid closely related to α-naphtoflavone) can cause either inhibition or stimulation of the carcinogen activation depending on duration of treatment [90]. Indeed, while stimulation of CYP1A1 was observed after 24 and 72 hours from administration, 48 hours treatment led to decrease of CYP1A1 activity. To explain this inhibition, potential interaction of flavonoid with CYPs preventing the carcinogen activation was suggested [90]. To preclude this phenomenon as well as explore this hypothesis, both simultaneous and sequential treatments were employed. Additionally, both flavonoids were administered separately to screen their induction potency of procarcinogen activating enzymes.

To determine whether the tested compounds affected the expression of CYP1A isoforms, Western blotting was used. In addition, the induction of CYP1A1 was evaluated by the determination of its specific activity with 7-ethoxyresorufin-O-deethylase (EROD) activity assay.

In rat liver, administration of carcinogen BaP alone as well as treatment with all combinations of BaP and chemopreventive compounds led to significant induction of CYP1A1 expression (see Figure 10A, p.38). Together with CYP1A1, CYP1A2 was also detected since the antibody used is capable of binding to both CYP1A isoforms (see Figure 10, p.38). No induction was visible when only flavonoid was administered. In all cases where the significant expression of CYP1A1 was observed, the amount of the cytochromes

seemed to be similar. However, when measuring EROD activities, differences were found (see Figure 12, p.40). Although strong increase of EROD activity after BaP administration was seen, combination of BaP and dihydromyricetin when administered sequentially increased the EROD activity even more (see Figure 12B, p.40). This could be caused by synergistic effect of dihydromyricetin and BaP. Indeed, it has been reported that the administration of flavonoids in combination with clinically used drugs may lead to increase of the drug toxicity [91]. On the other hand, when BaP was administered with dihydromyricetin simultaneously or with α-napthoflavone both sequentially and simultaneously, the EROD activity was significantly decreased as compared with BaP administered alone (see Figure 12, p.40). As already mentioned above, the level of CYP1A1 expression did not differ among different treatments with proved induction. It is thus possible that both flavonoids can modify the CYP1A1 in such a way that the enzyme irreversibly loses its activity.

The same induction pattern as in liver was observed in rat small intestine dissected into three parts – proximal, middle and distal (see Figure 10B, p.38). Moreover, similarly to liver, EROD activities in small intestine differed within the treatments even though the amounts of CYP1A1 seemed to be analogous (compare Figure 10B, p.38 with Figure 13, p.41). High increase of EROD activity was again observed when BaP was administered alone. However, in this case, all combinations of BaP and flavonoids decreased the EROD activity significantly (see Figure 13, p.41). The discrepancy between the effect of dihydromyrecitin in liver and small intestine could be explained by different environments in both tissues. It cannot be also excluded that the synergetic effect observed in liver was only an artefact. Further exploration using newly prepared microsomal fractions would be thus desirable. Surprisingly, the highest increase of EROD activity during almost all treatments containing BaP (excluding dihydromyrecitin administered with BaP sequentially) was observed in the distal part of the small intestine (see Figure 13, p.41). The different activity effect along small intestine might be caused by lower biotransformation capacity of proximal and middle part as well as higher absorption of xenobiotics from blood to distal part resulting in increase of CYP1A1 activity.

In colon, the induction of CYP1A1 expression was much lower than in case of liver and small intestine (see Figure 10C, p.38). Moreover, the induction pattern slightly differed from other tissues. For instance, dihydromyricetin administered alone induced the

CYP1A1 expression to a detectable level. The presence of CYP1A1 in the microsome fractions after treatment with dihydromyricetin alone was also confirmed by a small increase of EROD activity (see Figure 14, p.42). The explanations of this phenomenon remain unclear.

The results obtained with all tissues suggest the important role of the compound structure and its metabolism in the process of bioavailability to humans. The different effects of tested flavonoids on CYP activities in tested organs could be explained by the diverse bioavailability of particular flavonoids, which involve rate of transport to lumen, conjugation or further metabolism. Moreover, different inductive effects of flavonoids confirmed the complexity of the delivery process from the administration site to the target organs.

In this study, the hypothesis that chemopreventive compounds have an induction effect on CYP1A1 when rats are treated for 24 hours with both the compound and the carcinogen (administered simultaneously) was not confirmed. Conversely, most of the results suggest that both selected flavonoids are able to decrease the CYP1A1 activity even when administered 72 hours before the carcinogen. The only exception represents dihydromyricetin when administered simultaneously with carcinogen. The synergistic effect was though observed only in liver. Despite this controversial result, harmful attributes of dihydromyricetin and α-napthoflavone in terms of carsinogenesis could be most likely excluded.

N-acetyltransferases as the phase II enzymes are responsible for acetylation of wide variety of arylamines, arylhydroxylamines and aryl hydrazines, common components of environmental toxins [92] and drugs [93]. Therefore, many recent studies have focused on the role of NATs in the activation and detoxification of environmental carcinogens as well as the possible involvement of these carcinogens in the genetic and nongenetic regulation of NAT expression. Nevertheless, not many studies have been conducted using rats, which is on the other hand a widely used model for investigation of phase I of biotransformation.

The second part of this study aimed to introduce and optimise the methods for basic investigation of rat NATs. To be able to analyse the effects of different xenobiotics on expression changes of enzymes at protein level, the immunological method for specific NAT detection needed to be developed. Since not many specific antibodies recognizing rat

N-acetyltransferase are commercially available the preparation of own NAT antibodies was desirable. For this purpose, 3 peptides representing rat/human NATs were designed by prof. RNDr. Petr Hodek, CSc and the antibodies were subsequently prepared by immunising Leghorn hens. Chicken egg yolk antibodies (IgY) were chosen as an alternative to more frequently used mammalian ones because of several advantages e.g. the collection of antibodies is non-invasive, chicken often more successfully produce antibodies against highly conserved mammalian proteins, which are hardly immunogenic for mammals [89]. To confirm the ability of isolated IgY NAT antibodies (namely NAT1, NAT2 and NAT1/2) to bind the corresponding peptide antigens, ELISA was performed. The absorbance signal detected after addition of substrate for phosphatase labelled anti-chicken secondary antibody was increasing proportionally with concentration of NAT antibodies (see Figure 15, p.44) suggesting that the preparation of antibodies against NAT peptides was successful. We thus decided to validate these antibodies using immunodetection. For this purpose, the prepared IgY NAT antibodies were tested against recombinant human NAT1 and NAT2, the alternatives to recombinant rat NATs which were not available. This was possible since human and rat NATs share high sequence identity and the peptide antigens were designed so that the resulting antibody should recognize NATs from both species. Prepared anti-peptide NAT2 antibody was NAT-specific since it successfully detected recombinant human NAT2 (the band of the molecular weight of 33 kDa approximately, see Figure 16, line 1, p.45). Other anti-peptides antibodies did not specifically detect NAT enzymes on the membrane. The reason of unsuccessful detection of NATs could be explained by failure in selecting the peptide sequence derived from NAT.

To investigate whether prepared NAT antibodies are capable of recognizing NATs in more complex biological samples, rat liver cytosols were used. None of anti-peptide antibodies detected any bands indicating the presence of NAT in rat liver cytosol (data not shown). This could be caused by low concentration of NAT enzymes in cytosols. It cannot be also excluded that the sensitivity and specificity of prepared antibodies differ when detecting human and rat NATs. Nevertheless, in order to detect NATs in rat cytosols, more attempts need to be accomplished to generate sensitive antibodies.

Despite the fact that no NAT enzyme was detected in rat liver cytosols using Western blot, the NAT activity assay was performed. Most of the NAT activity studies are based on

analysis of formed products by HPLC. However, because of the time inconvenience, we adapted an assay which has been employed for the characterisation of prokaryotic NATs [94]. This assay is based on the observation that hydrolysis of acetyl-CoA occurs only in the presence of a substrate. According to previous studies performed by others, specific substrates – p-aminobenzoic acid for NAT1 and sulfamethazine for NAT2 – were chosen [25,94].

First, the assay conditions were optimised using recombinant human NAT1 and NAT2. The concentration of CoA in the reactions increased over the time period resulting in 2,5-3,5 nmol of formed CoA after 25 minutes from the reaction start (see Figure 18, p.47). The data demonstrate that the determination of NAT activity using DTNB gives accurate and reproducible results that are comparable with results published previously by others [94].

It has been proved that carcinogenic activity of heterocyclic amines present in cigarette smoke and foods cooked at high temperature depends on metabolic activation by N-acetyltransferases. To explore whether NAT activity can be measured in liver cytosol treated with heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), the same NAT activity assay was used. To distinguish both NAT isoforms, the same specific substrates were used. While no spontaneous hydrolysis was observed in the absence of substrates, the amount of CoA increased over the time period when the rat cytosols and substrates were present (see Figure 19, p.48). However, the amount of CoA was increased less significantly compared to assay with recombinant human NATs. The possible reasons could be differences in affinity of human and rat NATs to the specific substrates as well as much lower amount of NATs present in rat cytosol fractions in comparison with the amount of recombinant human NATs added into the reactions. However, other enzymes capable of generating CoA in the presence of specific substrates should be taken into account. The tissue cytosol samples are usually a complex mixture of different isoenzymes, some of which may have some activity toward the probe substrate. To ensure that only NATs are responsible for formation of CoA in the cytosol fractions, the specificity of the substrates should be more explored as well as the specific inhibitor of NATs would need to be designed. Indeed, although many experiments have been conducted to introduce suitable methods for in deep investigation of the effects of different carcinogens and chemopreventive compounds on NATs expression and activity, further optimizations are necessary before such study can begin.

6. CONCLUSIONS

1. Rats were p.o. administered with flavonoids α-napthoflavone and dihydromyricetin and/or carcinogen benzo[a]pyrene (BaP) and microsomal and cytosolic fractions were isolated from liver, small intestine and colon.
2. Various combination of selected flavonoids and/or BaP for their ability to induce the expression of cytochromes P450 (namely CYP1A1) were investigated using Western blot. In liver and small intestine, all combinations of BaP and flavonoids led to strong induction of CYP1A1 expression. Moreover, the CYP1A1 protein levels were almost identical to levels observed when the rats were treated with BaP alone.
3. 7-ethoxyresorufin-*O*-deethylase activity of CYP1A1 in all microsomal fractions was measured. In most of the combinations of flavonoids and BaP, the CYP1A1 activities were significantly decreased when comparing with treatements where only BaP was administered.
4. The anti-peptide chicken antibodies recognising peptides derived from human/rat NAT1, NAT2 and NAT1/2 were prepared and characterized using ELISA and Western blot. Only NAT2 antibody was able to recognize the corresponding recombiant human protein, i.e. NAT2. None of the antibodies could detect NATs present in prepared rat cytosols.
5. NAT activity assay based on determination of CoA amount formed during reaction of the enzyme with specific substrate was optimised using recombinant human NAT1 and NAT2. This optimized activity assay was then utilized for detection of NAT activity in liver cytosol gaining results similar to those obtained with recombinant NATs.

7. ABBREVIATIONS

AαC	2-amino-9Hpyrido[2,3-b]indole
AhR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
BaP	benzo[*a*]pyrene
BCA	bicinchoninic acid
BCIP/NTB	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tab.
BIS	N,N`-methylen-bis-akrylamid
BNF	β-naphthoflavone
BSA	bovine serum albumin
b.w	body weight
CYP	cytochrome P450
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EROD	7-ethoxyresorufin-*O*-deethylation
ELISA	Enzyme-linked immunosorbent assay
HAA	heterocyclic aromatic amines
HCA	heterocyclic amines
IgG	immunoglobulin (antibody) of class G
IgY	chicken antibody
kDa	kilodalton
mcKLH	maleimide activated keyhole limpet hemocyanin
MeIQ	2-Amino-3,4-dimethylimidazo[4,5-f]quinoline
MFO	mixed function oxidise
MFO	microsomal fraction
mRNA	messenger RNA
NAT	N-acetyltransferase
NADPH	nicotinamide adenine dinucleotide phosphate-reduced
PAGE	polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffer saline
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

pNPP	p-nitrophenyl phosphate
p.o.	*per os*
RPM	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorfism
SULT	cytosolic sulfotransferase
TEMED	N,N,N',N'-tetramethylethylendiamine
TRIS	tris(hydroxymethyl)aminomethane
UV	ultra violet
v/v	volume/volume percent
w/v	weight/volume percent

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Svoluji k zapůjčení této práce pro studijní účely a prosím, aby byla řádně vedena evidence zapůjčovatelů.

Jméno a příjmení s adresou	Číslo OP	Datum vypůjčení	Poznámka