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Mini-review

Chemopreventive compounds—View from the other side

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Dedicated to Prof. RNDr. Danuše Sofrová, CSc. and Prof. RNDr. Marie Tichá, CSc.

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ABSTRACT

Increasing attention is being paid to the possibility of applying chemopreventive agents for the protection of individuals from cancer risk. The beneficial potential of chemoprotective compounds is usually well documented by extensive experimental data. To assure the desired effect, these compounds are frequently concentrated to produce dietary supplements for human use. The additive and synergistic effects of other food constituents are, however, frequently ignored. Even natural chemopreventive compounds have to be considered as xenobiotics. Thus, as much attention has to be paid to their testing prior to their wide application as is usual in drug development for human treatment. Unfortunately, much of the research in this area is solely based on simplified *in vitro* systems that cannot take into account the complexity of biotransformation processes, e.g. chemopreventive compound–drug interaction, effect on metabolism of endogenic compounds. Hence, the predicted chemopreventive potential is not attained in respect of cancer prevention; moreover, the administration of high doses of chemopreventive compounds might be even detrimental for the human health.

Contents

Abbreviations: AhR, Ah receptor; B[a]P, benzo[a]pyrene; BNF, β-naphthoflavone; CYP, cytochrome P450; DEX, dexametazone; GST, glutathione S-transferase; IQ, 2-amino-3-methylimidazo(4,5-f)quinoline; NAT, N-acetyltransferase; PB, phenobarbital; PCN, pregnenolone-16α-carbonitrile; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase.

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1. Introduction

One of the most striking facts is that cancer is one of the leading causes of death in the human population worldwide. This disease is unusual since up to ten years after exposure to carcinogens usually have to pass before a detectable cancer occurs. The transformation from a normal cell into a tumor cell is a multistage process, typically a progression from a pre-cancerous lesion to malignant tumors. The cancer cell conversion is caused by both internal and external factors that may act together to initiate and/or promote the process of carcinogenesis. Besides inherited gene mutations, the age, hormonal status, and immune conditions of a given individual are the critical internal factors of cancer development. Cancer commonly results from a lifetime's exposure to various environmental factors causing gene mutations. Infectious microorganisms such as *Helicobater pylori* responsible for gastric cancer and viruses such as Rous sarcoma retrovirus containing oncogen or human DNA papillomavirus, that causes genital warts, are among well-documented biological carcinogens. A typical environmental factor is exposure to radiation (UV light, X-ray, gamma) that damages the cell DNA. However, human exposure to chemical carcinogens plays the most prominent role in the process of carcinogenesis. Such carcinogenic agents are exogenous or metabolically generated electrophiles and reactive oxygen species. These agents may arise from (normal) internal oxidative processes, or may be generated from environmental chemicals ingested via food as well as inhaled from the environment, e.g. smoking [1]. It has been estimated that 35% of cancer deaths may be related to dietary factors and smoking [2].

2. Cancer chemoprotection

While continuing the intensive search for more effective treatments of already developed tumors, today's imperative is to establish the management of cancer risk reduction in early stages of this process. The first approach to reduce cancer risk is prevention focused on the reduction in human exposure to environmental carcinogens. The second one is a protection strategy based on the use of exogenous factors (diet constituents, supplements or drugs, immunization) to enhance endogenous mechanisms that reduce the risk arising from exposure to the environmental carcinogens by affecting various stages of cancer development (both at the molecular and cellular levels). As the inherited genetic factors were shown to be responsible for only about 15% of all cancer cases [3], the number of cancers originating from the environment and lifestyle factors should be reduced significantly by the application of these two strategies. Thus, the correct lifestyle and diet are assumed to prevent 30–40% of all tumors.

Early epidemiological studies have suggested the reduction of cancer risk related to the consumption of specific types of fruits and vegetables. Moreover, diets high in fiber-containing foods are associated with a reduced incidence of cancer, especially cancer of the colon [4]. Thus, increasing evidence exists that plant-based food possesses cancer-preventive properties. The chemopreventive potential of health-promoting phytochemicals is expected to combine anti-oxidant, anti-inflammatory, immune-enhancing, and anti-hormone effects. Additionally, modifications of drug-metabolizing enzymes, influences on the cell cycle and cell differentiation, induction of apoptosis and suppression of proliferation and angiogenesis are often playing roles in the initiation and secondary modification stages of neoplastic development [5–7]. Plant chemicals thus interfere with tumor initiation, promotion and progression by acting directly on carcinogen activation, tumor cell proliferation and physiological conditions affecting the tumor growth, respectively. As multiple mechanisms are involved in the protective effects, it is difficult to identify the relative contributions of various components of a plant-based diet to overall cancer risk reduction [8]. Moreover, the synergism among these compounds may account for the final beneficial effect. By epidemiological and experimental studies, possible chemopreventive substances have been suggested: vitamin derivatives, phenolic and flavonoid agents, organic sulfur compounds, isothiocyanates, curcumins, fatty acids and terpenoids (d-limonene) [5].

3. Risk of chemopreventive compounds

Increasing attention is being paid to the possibility of applying chemopreventive agents for long-term or even life-long protection of individuals. Phytochemicals are the most popular chemopreventive compounds as their intake is widely acceptable psychologically due to their plant origin. These compounds are, in general, considered to be safe chemoprotective agents of low toxicity that are already present in the human diet. Thus, the consumption and use of dietary supplements containing concentrated phytochemicals increased dramatically in recent years. Marketing strategies advertise and often exaggerate their non-toxic therapeutic effects, most of which are not substantiated by regulated clinical trials. Moreover, the common misconception that the more of something is the better for health may result in overdosing of individuals by these compounds [9]. Chemopreventive properties of a particular compound are frequently overestimated, although the evidence is based mainly on simplified tests in artificial systems (e.g. purified enzymes, cancer cell lines) or animals exposed to high doses, exceeding by several orders of magnitude the physiologically relevant concentrations achieved after a regular human intake in normal diet. Another serious concern of chemopreventive compound testing arises from differences between experimental settings and the human exposure in respect to the administration timing. To prove experimentally, e.g. an inhibitory effect of the phytochemical on activation of a carcinogen, both compounds are administered usually simultaneously. That does not happen normally in a diet, where most often the human intake follows a sequential pattern when one compound precedes the other. Clinical data rarely, if ever, match the promising findings obtained from numerous experimental studies.

Paradoxically, the ingestion of high doses of chemopreventive compounds might be harmful for humans. Phytochemicals have to be viewed as foreign compounds (xenobiotics), and thus, their long-term administration should be considered with special care. Besides expected beneficial effects, these food supplements may exert negative activities coming namely from: (i) their toxicity per se, (ii) metabolic conversion into cytotoxic, pro-oxidant or mutagenic agents, (iii) interference with endogenous metabolic pathways, (iv) interaction with other chemicals from diet, environment, or drugs, (v) induction of carcinogen activating enzymes, and (vi) effects on human intestinal microflora. Although there are plenty of experimental and epidemiological studies trying to prove the cancer preventing effect of diet or food supplements, much less attention is being paid to trials showing no effect or even harm.

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4. Chemoprevention failure

As the studies described in the literature are focused mainly on the putative beneficial properties of chemopreventive compounds, examples of their side and adverse effects are very rare. There are only isolated reports of extensive long-term studies with humans. The following examples show some controversial effects of chemoprotective compounds.

4.1. Tamoxifen

The results of early cancer prevention trials with tamoxifen show that some epithelial cancers in breast can be prevented [10]. This chemopreventive drug reduces the risk of breast cancer by as much as 50% in high-risk women [11]. On the other hand, the use of tamoxifen increased the risk of development of endometrial cancer, stroke, pulmonary embolism and deep-vein thrombosis. As almost all chemopreventive compounds are likely to have some side effects, the appropriate risk–benefit balance should be calculated before the application of these chemicals to a population that is at risk of cancer.

4.2. β-Carotene

Several extensive lung cancer prevention trials were conducted with plant antioxidant β-carotene. This carotenoid has been shown to provide promising results in laboratory studies and to be inversely associated with cancer risk in epidemiologic studies. Although this phytochemical seems to be safe, other studies failed to show a univocal benefit, and some of them identified even harm [12,13]. Although β-carotene was proposed against lung cancer, the incidence of this cancer in high-risk individuals (smokers, asbestos-exposed workers) increased by 16% in the group supplemented with β-carotene and α-tocopherol, and by 28% in the group taking β-carotene and retinol. The increased risk of a lung cancer was limited to current smokers. In another study, the effect of β-carotene (in combination with vitamins C and/or E) on colorectal adenoma recurrence was examined [14]. In the group of non-smokers and non-drinkers, β-carotene decreased the risk of adenoma recurrence, whereas this risk was elevated in the group of smokers and even more for subjects who smoked and drank alcohol. The detailed mechanism of this effect is not clear, however, the pro-oxidant properties of β-carotene cannot be ruled out [15]. The example of β-carotene clearly shows that the preventive effect of a single nutrient may, especially at a non-physiological dose, differ from that when given as a constituent of food matrix in a regular diet.

4.3. Indole-3-carbinol

In epidemiological studies, it was shown that intake of cruciferous plants (e.g. cabbage, cauliflower, Savoy cabbage, Brussels sprouts, broccoli) is inversely correlated with the cancer risk of several organs [16]. These vegetables are a rich source of glucosinolates and their hydrolysis products, including, e.g. indoles and isothiocyanates. While they are able, by alteration in sex hormones metabolism, to inhibit the development of hormone-sensitive cancers, there is an inconsistent evidence of an inverse association between cruciferous vegetable intake and breast or prostate cancer in humans [17]. Due to the effect on steroid hormone metabolism (stimulation of estradiol 2-hydroxylation), daily consumption of these vegetables should not exceed an acceptable level to prevent hormonal imbalances. Sulforaphane and indole-3-carbinol have been implicated in a variety of anticarcinogenic mechanisms, but deleterious effects also have been reported in some experimental protocols [17]. In animal studies, chronic administration of indole-3-carbinol can promote liver tumors in initiated animals. Surprisingly, high intakes of cruciferous vegetables were associated with an increased risk of rectal cancer in a study with Dutch women [18]. Some studies suggest that the indole-3-carbinol-stimulated production of catechols from estrogens is related to an increased risk of breast cancer in women [19]. In addition, indole-3-carbinol is converted in stomach to the Ah receptor (AhR) agonist, indolo(3,2-b)carbazole. Thus, the use of indole-3-carbinol as a chemopreventive agent against namely estrogen-dependent human cancers should receive more careful evaluation before its widespread use for humans.

4.4. Flavonoids

Flavonoids are another example of chemopreventive compounds showing a double-edged activity. These phenolic compounds that are present in fruits and vegetables, as well as in popular beverages (wine, tea, coffee), have been reported to show a variety of health-promoting activities such as antioxidant, antiviral, antitumor, and anti-inflammatory compounds (for review, see [20]). Hence, flavonoids are frequently ingested in relatively large amounts as dietary factors for health maintenance. On the other hand, it has been suggested that flavonoids may act as mutagens, pro-oxidants, and inhibitors of key enzymes [9,20]. Flavonoids generate not only reactive oxygen species but also can be converted to a reactive/toxic quinone or quinone methide. Thus, these phenolic antioxidants, by their nature, can be both pro-oxidative and antioxidative [21]. The ratio of their pro-/anti-oxidative activities depends on the flavonoid structure, namely on the number and position of hydroxyl groups in the B ring [22]. Known antioxidants, quercetin, morin and naringenin can cause a single-strand DNA breakage in rat liver. In addition, quercetin has been shown to covalently bind to cellular DNA and proteins in human intestinal Caco-2 cells and hepatic Hep G2 cells [23]. Furthermore, some flavonoids exert cytotoxicity at higher concentrations as documented with promyelocytic leukemia and normal human cells [24,25]. The toxicity of flavonoids may also be caused indirectly by the inhibition of drug metabolizing enzymes resulting in potential toxic flavonoid–drug interactions [21]. Certain flavonoids, namely isoflavones, are realized to mimic natural estrogens. These “phytoestrogens” may act as endocrine-disrupting chemicals in the protection against hormone dependent cancers. However, the estrogenic activity of, e.g. genistein (at high doses) decreases fertility and causes sexual dysfunction in experimental animals [26]. With special caution, the soy-based infant formulas should be considered, since during infancy, the endocrine effects of soy phytochemicals, such as genistein and daidzein, might exert most pronounced adverse effects on, e.g. human fertility. Thus, the overall health benefit of flavonoids is uncertain, and consumption of large quantities of them in fortified foods or supplements should not be encouraged yet [27].

5. Metabolism of xenobiotics

The most pronounced and clearly detectable features are the side effects originating from interactions of the chemopreventive compounds with xenobiotic-metabolizing enzymes. Thus, from the view point of drug metabolism our understanding of the pathways involved is essential for prediction and avoidance of toxicological impacts on human health. Ingested xenobiotics are absorbed from the digestive system either directly, or after being metabolized by intestinal microflora. The metabolism of xenobiotics usually proceeds via xenobiotic-metabolizing enzymes of the phase I (functionalization) and phase II (conjugation) reactions. Although the concept of this reaction sequence is already outdated, the terminology continues to be used extensively in the literature [28]. Various xenobiotics, e.g. flavonoids, frequently undergo conjugation reactions without the need for phase I enzyme functionalization [29,30].

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Most phase I enzymes are capable of both detoxication and metabolic activation. Via the metabolic activation proximate carcinogens are often converted to electrophilic intermediates. For example, an increase in the activity of phase I enzymes, resulting from the enzyme induction or stimulation of their activities, is a balancing on the benefit/risk edge between detoxication and activation. On the contrary, the inhibition of phase I enzymes might result in an accumulation of cytotoxic compounds, impaired metabolism of endogenous compounds or fatal drug-inhibitor interactions, causing overdose or loss of the therapeutic effect of drugs.

Enzymes of phase II are usually transferases, which conjugate either unchanged xenobiotics or their metabolic products (of the phase I) with endogenous hydrophilic compounds, such as glucuronide, glutathione, acetate, sulphate, glycine, glutamine, thiocyanate, producing hydrophilic products that can be readily excreted. Although these reactions are seemingly detoxicative, spontaneous or catalyzed decomposition (rearrangement) of xenobiotic conjugates might result in the formation of highly reactive carbenium or nitrenium cations that covalently bind to proteins and nucleic acids, as discussed further.

6. Role of cytochromes P450

Cytochrome P450 (CYP) enzymes comprise 70–80% of all phase I xenobiotic-metabolizing enzymes [31]. These heme-containing monooxygenases play also a key role in the metabolism of hydrophobic endogenous substrates (e.g. sterols, prostaglandins, fatty acids). Of the 57 human CYP genes in 18 families, the members of the CYP1 to CYP4 families oxygenate thousands of xenobiotics (and some endogenous substrates), whereas other CYP families principally metabolize endogenous substrates in a highly substrate-specific manner [32]. Although CYPs generally convert ingested foreign compounds to less toxic products ready for phase II enzymes, the reactions frequently result in the formation of reactive intermediates or allow the leakage of free radicals capable of causing toxicity. CYPs are found abundantly in the liver, gastrointestinal tract, lung and kidney, organs that are highly exposed to foreign compounds from the environment. In specific tissues or organs, CYPs are present either at the basal level, and/or are inducible at elevated levels after exposure to xenobiotics. In other words, some CYPs are constitutive, others are inducible by inducers. CYP genes are regulated in a variety of ways and at multiple levels: they exhibit tissue-specific expression, they are regulated by endogenous hormones and cytokines, and respond to structurally diverse foreign chemicals, which often increase P450 protein levels by stimulating P450 gene transcription initiation [33] (for review, see [34]). For instance, the expression of *CYP1* genes can be induced by AhR, which dimerizes with the AhR nuclear translocator, in response to many polycyclic aromatic hydrocarbons. Similarly, the steroid family of orphan nuclear receptors, the constitutive androstane receptor and pregnane X receptor (both heterodimerized with the retinoid X receptor) are shown to transcriptionally activate the promoters of *CYP2B* and *CYP3A* gene expression by xenobiotics such as phenobarbital (PB)-like compounds and dexamethasone (DEX) and rifampicin-type of agents [35].

6.1. CYP induction

Based on early animal studies focused on the protection from the carcinogenic effects of carcinogens such as benzo[a]pyrene (B[a]P), 2-acetylaminofluorene, 4-dimethylaminostilbene, urethane, aflatoxin B1, diethylnitrosamine, aminoazo dyes, CYP inducers were considered to be chemopreventive compounds. Indeed, exposure of animals to CYP1A enzyme inducer, β-naphthoflavone (BNF), which stimulates the hepatic metabolism of aflatoxin B1 to aflatoxin M1, inhibits the hepatocarcinogenic activity of aflatoxin B1 [36]. In another study, pretreatment of rats with 3-methylcholanthrene (another CYP1A enzyme inducer) before administration of 2-amino-3-methylimidazo(4,5-f)quinoline (IQ) markedly decreased the formation of IQ-DNA adducts in the liver, colon, small intestine, kidneys, bladder, heart, and lung [37]. The results of this study suggested that 3-methylcholanthrene enhanced the C-hydroxylation of IQ (inactivation pathway) to a greater extent than the N-hydroxylation (activation pathway).

On the other hand, CYP inducers that are suggested to prevent chemical carcinogenesis of a particular compound may act reversely by stimulating the activation pathway of the other ones. The stimulatory effect of phenobarbital administration on the hepatocarcinogenicity of safrole is an example of an inducer of CYP2B enzymes that enhances carcinogenesis [38]. In addition, high CYP1A1 activity is also connected with a colorectal cancer [39]. Pharmacokinetic studies of several environmental toxicants have shown that CYP1A1, CYP1A2 and CYP1B1 might be beneficial or detrimental—depending on their time-specific, organ-specific, tissue-specific and cell-type-specific expression. The current state of our knowledge indicates an increasing number of CYP enzymes to be involved in the activation of environmental and diet carcinogens [40,41]. Since many chemical carcinogens are metabolized by CYP enzymes to non-carcinogenic as well as to proximate and ultimate carcinogenic metabolites, the actual status of CYPs determines the metabolic fate of the carcinogen. Thus, inducers of these enzymes play an important role in the modulation of a chemical carcinogenicity, by changing the ratio between inactive and active metabolites formed from the carcinogen by phase I enzymes. Moreover, the involvement of phase II enzyme induction (e.g., GST, UDP-glucuronosyl transferase) should be considered, too [19]. These findings suggest that animal studies and human epidemiological studies on cancer chemoprevention should be revised in view of the complexity of CYP induction as well as other impacts on the metabolism of xenobiotics.

6.2. Inhibition and stimulation of CYP activities

In addition to the effects of CYP inducers on the carcinogenicity of chemicals, chemopreventive compounds can modulate CYP activities as inhibitors by direct binding to CYP enzymes. Numerous *in vitro* studies have shown a clear connection between the CYP inhibition and the protection against a DNA-adduct formation. However, this straightforward approach to chemoprevention of carcinogen activation, usually derived from simplified experimental systems, may fail in the prediction of true chemopreventive effects in the body. For instance, a well-known procarcinogen B[a]P requires a multi-step activation towards mutagenic and carcinogenic derivatives. By CYP1A1-catalyzed metabolism, B[a]P is converted to 7,8-epoxy-7,8-dihydro-B[a]P, the precursor leading to the ultimate carcinogenic product, covalently binding DNA molecules. The inhibition of CYP1A1 catalytic activity by, e.g. natural phenolic compounds should prevent the B[a]P mutagenicity [42]. However, this CYP1A1 metabolizes B[a]P also to non-carcinogenic products such as quinones and phenols—mainly 3-hydroxy-B[a]P. Namely, 3-hydroxy-B[a]P was found to be a potent antagonist of mutagenicity and/or an inhibitor of 7,8-diol-B[a]P activation [43]. The complex effect of chemopreventive compound used in this case does not seem to be solely beneficial. Namely, in an additional study with CYP1A1 and or NADPH:CYP reductase knockout mice treated with B[a]P the unexpected high levels of B[a]P-derived DNA adducts in the liver, were found [44,45]. These data reveal an apparent paradox, whereby hepatic CYP enzymes appear to be more important for detoxication of B[a]P in vivo, despite being involved in its metabolic activation *in vitro* [45]. These results might indicate that enzymes other than CYPs

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can play a role in the B[a]P activation. Therefore the role of such, as-yet-unindetified, B[a]P-activating enzymes awaits further investigation. Hence, the potential induction of various enzymes metabolizing xenobiotics, in addition to CYPs, by tested carcinogens and chemopreventive inhibitors should be considered when these compounds are administrated simultaneously to experimental animals.

There is accumulating evidence that metabolic activity of several CYPs (e.g. family CYP1A, CYP2C, CYP3A) is stimulated by inhibitors of other CYPs. While specific activities of CYP1A1 and 1B1 were inhibited by various flavonoids, certain metabolic activities of 1A2 and CYP3A4 were also stimulated by flavonoids, α-naphthoflavone and tangeretin, respectively [46,47]. Several heterotropic co-operativity models are used to explain this stimulatory effect of flavonoids, namely in CYP3A4 [48]. Usually, the balance between the co-operativity and inhibition of these CYPs is a matter of a compound concentration. The effect of other flavonoids, quercetin and naringenin, on the mutagenicity of 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) was tested in a system expressing human CYP1A2 and NADPH:CYP reductase. Mutagenicity of MeIQ was enhanced 50% and 42% by quercetin at 0.1 and 1 μM, respectively, but suppressed 82% and 96% at 50 and 100 μM, respectively. Naringenin also increased the MeIQ-induced mutation about 37% and 22% at 0.1 and 1 μM, but suppressed it 32% and 63% at 50 and 100 μM concentrations, respectively [49]. Thus, the MeIQ-induced mutation is a concentration dependent process showing both the stimulation, at low concentrations, and the inhibition of CYP1A2 activity, at high concentrations of the flavonoid used. This example of a dose-dependent manner of the stimulation or inhibition of the carcinogen activation emphasizes the need of chemopreventive compound testing even at low concentrations, which likely occur in a human body after the compound (food) ingestion.

6.3. Xenobiotics and endogenic compound interactions

As CYPs metabolize a great variety of drugs and endogenous compounds, another concern associated with CYP induction and/or inhibition, as an approach to the cancer chemoprevention, arises from their impact on the metabolism and action of therapeutic drugs, thereby leading to drug interactions in patients. Their influence on the metabolism and action of important normal body constituents (such as vitamin D, arachidonic acid, thyroid hormone, and steroid hormones) has to be considered, too.

Since CYPs are involved in metabolism of steroids, xenobiotics in the diet may interfere with a normal hormonal status of the body. Certain classes of flavonoid compounds, as mentioned above, are assigned as phytoestrogens since their structure resembles a skeleton of estrogens. That is why such flavonoids show an estrogenic or anti-estrogenic effect in organisms. These flavonoids are able, like natural estrogens, to bind to an estrogen receptor and modulate its activity. Moreover, they also block another CYP enzyme, CYP19 (aromatase) that is the crucial enzyme of estrogen biosynthesis, and/or steroid dehydrogenases, e.g. 11β-hydroxysteroid dehydrogenase. Aromatase catalyzes a unique reaction, the aromatization of A ring of androgens, androstendione and testosterone, resulting in the formation of estrogens, estrone and estradiol, respectively. Since estrogens are known cell proliferators and their metabolites such as catechols are carcinogens, a local expression of aromatase is suggested to be closely connected with tumor initiation, promotion and progression. Thus, aromatase is a particularly attractive target for a selective inhibition of estrogen biosynthesis by chemopreventive compounds. These compounds, however, cause complex changes inducing a shift in the overall hormonal balance of an individual, resulting in various effects, e.g. infertility on one hand and retardation of cell proliferation on the other hand (for review, see [20]). In addition to inhibitory effects of flavonoids, with respect to steroidogenesis, the stimulation of CYP activities should be considered. A widely used and potent inhibitor of CYP1A1, α-naphthoflavone, stimulates CYP3A4 mediated 2-hydroxylation of 17β-estradiol and 6β-hydroxylation of progesterone and testosterone [47,50].

Much attention should also be paid to the interaction of the chemopreventive flavonoids when co-administered with drugs. The CYP3A4 enzyme, the most abundant human hepatic CYP, catalyzing the metabolism of majority of therapeutic agents is a subject of potentially severe flavonoid-drug interactions [51,52]. This issue, regarding commonly prescribed drugs and herbal medicines and diet constituents, is extensively reviewed in the literature [53,54]. The non-sedating antihistamine, terfenadine, undergoes nearly a complete presystemic elimination mediated by CYP3A4. One of the primary metabolites, terfenadine carboxylate, accounts for the drug activity. The inhibition of CYP3A4 by other compounds results in a drug overdose causing the development of a serious ventricular tachyarrhythmia. A consequent fatality has been attributed to terfenadine toxicity after consuming the drug with grapefruit juice [55]. This case clearly demonstrates the potential of various phytochemicals for serious adverse interactions, difficult to predict for all possible ingested xenobiotics.

6.4. Intestinal metabolism of xenobiotics

The oral route is the most convenient and often used means of chemopreventive compound administration. Like the liver, the small intestine is well equipped with various phase I and phase II xenobiotic-metabolizing enzymes, which contribute to the detoxification process in the body. Although the relative contributions of individual xenobiotic-metabolizing enzymes in the liver have been established, less is known about the complement of enzymes in the proximal small intestine, the major site of xenobiotic absorption. The possibly greater role of the liver than the small intestine in first-pass metabolism does not, however, detract from the capability of the small intestine to directly metabolize the orally ingested xenobiotics prior to systemic uptake, and thus to block the uptake [56]. The contribution of the gut is not routinely incorporated into *in vitro–in vivo* predictions of either clearance or drug-drug interactions, and this omission may partially explain the general underprediction trend often observed [57]. The substantial presystemic metabolism can occur as the drug passes through the small intestine containing drug biotransformation enzymes, including the CYPs as well as enzymes of intestinal microflora [58]. Bacterial azo- and nitroreductases may lead to the formation of carcinogenic aromatic amines, or the bacteria may produce glucosidases and glucuronidases releasing toxic, in their native form, aglycones from their glycoconjugates. Chemoprotective compounds such as flavonoids, which occur frequently as glycosides, may undergo microorganism-mediated cleavage, resulting in the release of aglycone. This process often leads to better absorption and/or further degradation of the aglycone chromane heterocycle. Many *in vitro* studies on the mechanisms of action of phenolic compounds concentrate on parent compounds (whether aglycones or glycosides) rather than on their metabolites, interactions of which in the body should not be ignored. Moreover, flavonoids capable of antimicrobial activities are able to shift the rather delicate balance between beneficial and potentially harmful intestinal bacteria [20].

The ingestion of chemopreventive dietary constituents makes their interaction with intestinal biotransformation enzymes obligatory, thus the better knowledge of their metabolism in the gut is essential for investigating their health effects (for review, see [59]). Although the content of intestinal CYPs is much lower than that in liver, the size of this organ makes the gut to be an important site of xenobiotic-biotransformation. In samples of human small intestines (proximal section), CYP3A4, 2C9, 2C19, and 2J2 enzymes

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were detected by Western blot, using isoform selective antibodies, as the most abundant intestinal CYPs. The expression of CYP2D6 was mostly similar to or lower than that of CYP2J2, however, present not in all individuals. The fact that the CYP1A1 apoprotein was detected only rarely in intestinal microsomes of donors suggests this CYP to be inducible. The remaining CYP enzymes examined, CYP1A2, 2A6, 2B6, and 2E1, were either not detected or detected only faintly, even after prolonged exposure [60]. These results do not take into account disease states, concomitant medications and dietary history of human donors. Thus, it is difficult to predict the enzyme status of virtually uninduced individuals. However, similarly to the hepatic counterparts, the content of CYPs in small intestine is regulated via compounds inducing expression of the particular CYP isoform and/or via mechanisms based on mRNA or protein stabilization [61]. For instance, in small intestine the AhR-dependent pathways, and numerous naturally occurring AhR agonists (e.g. flavonoids, dietary indoles) have been identified. In animal models, CYP1A1, 2B1, and 3A1 were detected in enterocytes of untreated rats and were inducible by BNF, PB, and PCN (or DEX), respectively. In addition, CYP2C was detected in untreated rats at low levels. In contrast, several CYPs that are expressed in the liver were not detected in the enterocytes of rats, including CYP2A1, 2B2, 2E1, 3A2, and 4A1. CYP1A2 mRNA was detectable only in small intestine of BNF-induced rat at levels that did not result in any detectable translation [56]. The absence of CYP2E1, an isoform involved in nitrosamine-compound induced tumorogenesis, in intestinal microsomes was also confirmed in mouse [62]. Recently, CYP2E1 was detected in esophageal mucose and large intestine of rats, but not in small intestine [63].

The conjugation of xenobiotics and xenobiotic metabolites in the small intestine has the potential to facilitate their excretion to the lumen of the intestine. It is thus tempting to attribute the low incidence of human small intestinal cancer to the high levels of expression of phase II enzymes in small intestinal enterocytes, relative to their expression levels in other organs of the gastrointestinal tract [56]. Human glutathione S-transferase (GST) levels in the gastrointestinal tract correlate inversely with the cancer risk. Several dietary compounds, such as sulforaphane-based analogues, induced GSTs in rat small intestine, and this finding possibly explains their detected anticarcinogenic effects [64]. In small intestine, UDP-glucuronosyltransferases (UGT), another II phase transferases, are expressed. As is the case with GST expression, the markedly lower expression of UGT in the human colon, relative to that in the small intestine, has been hypothesized to be a factor in the differential susceptibility to carcinogenesis of the two organs [56]. These two transferases, GST and UGT, are suggested to perform health beneficial activities by a decrease in the carcinogenic potential of some xenobiotics via the reactive intermediates scavenging and facilitating their excretion. On the contrary, other transferases, such as sulfotransferases (SULT) and N-acetyltransferases (NAT), might be involved in the formation of ultimate carcinogens. An example in point is the activation of arylamines. These compounds are not usually carcinogenic in their parent form, but require metabolic activation to reactive electrophiles. The activation process can proceed via a two-step pathway involving CYP1A2-catalyzed N-hydroxylation followed by an O-esterification step catalyzed by NATs and/or SULTs [65]. As a consequence of these reactions, the arylnitrenium ions generated from N-hydroxylamine esters, are believed to be the ultimate reactive intermediates responsible for the carcinogenic activity [66]. This pathway explains the metabolic activation of, e.g. 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), strongly mutagenic and carcinogenic heterocyclic amine produced during routine cooking of meats [67]. Moreover, taking into account the whole body, more complex fate of PhIP might occur *in vivo*. Ingested heterocyclic amines undergo initial hepatic N-oxidation and subsequent N-glucuronidation, resulting in conjugated N-hydroxy metabolites. Glucuronides can be transported to the colonic lumen, hydrolyzed by β-glucuronidases (possibly provided by the microflora), and reabsorbed. In colonic mucosa, the N-hydroxy derivatives are good substrates for O-acetylation, which spontaneously results in reactive N-acetoxyarylamines that form covalent DNA adducts [68]. Thus, in this case, the risk of a colorectal cancer development is associated with activities of both phase I and II biotransformation enzymes.

7. Testing of chemopreventive compounds

Chemopreventive compounds should target the initiation, promotion and/or progression events of the lengthy process of carcinogenesis. Besides proving their beneficial activity the safety issues of their intake should not be underestimated. Candidate compounds are tested in experimental systems mimicking interactions with crucial enzymes of the biotransformation as well as in experimental animals to better understand the underlying molecular mechanisms of their protective activities.

7.1. Experimental in vitro systems

First, it is essential to exclude the direct mutagenicity and cytotoxicity of the potential candidate compound. Their capacity to cause base-pair substitutions and frame-shift mutations in the Ames test, induce chromosomal aberrations and sister chromatid exchanges in CHO cells, and generate micronuclei in human lymphocytes (without and with activation), are tested. Although, e.g., flavonoids are regularly consumed in a human diet, one of the most abundant flavonoids, quercetin, shows mutagenicity in Ames test and ability of strand scission in DNA [69,70].

As the process of carcinogen activation is frequently attributed to CYP activities, the evaluation of an inhibitory capacity of chemopreventive compounds towards CYPs, namely of families CYP1, CYP2, and CYP3, is often the next step in chemopreventive compound testing [71]. Within, e.g. the group of flavonoids, there are several potent CYP inhibitors [20,21]. The question, however, arises, whether the CYP inhibition is the sole health-promoting activity of flavonoids. Hepatocarcinogenic activity of aflatoxin B1 may serve as an example documenting the opposite effect. CYP1A1/2 protect animals and possibly humans from the hepatocarcinogenic effects of aflatoxin B1. CYP1A1/2 are involved in the hepatic hydroxylation of aflatoxin B1 to aflatoxin M1 that is an inactivation pathway, and that inhibit aflatoxin B1-induced DNA damage [72]. In that respect, the induction of CYP1A family enzymes, another parameter examined for chemoprotective compounds, also stimulates the carcinogen inactivation. Numerous flavonoids and other dietary supplements are well-documented inducers of families CYP1, CYP2, and CYP3 [73]. Thus, on the other hand, e.g. increased CYP1A2 activity can promote the activation of food-derived mutagens (e.g. amino acid pyrolysates) [74,75].

For induction experiments focused on the expression of phase I and II enzymes of biotransformation, cell lines are frequently used. However, these studies usually provide unequivocal results depending on the cell line used [76,77]. Primary cells of biotransformation organs (liver, lung, colon) significantly differ from immortalized carcinoma cells derived from various tumor tissues (e.g. Hepa-1, HepG2, MCF-7, Caco-2) [78]. In responsive human cancer cell lines the treatment, e.g. with 2,3,7,8-tetrachlorodibenzo-p-dioxin led to the induction of CYP1A1, as determined by Western blotting, however, in primary human hepatocytes preferably CYP1A2, but not CYP1A1 were induced [78]. The sensitivity to inducers and resulting expression of individual CYPs, even between two cancer cell lines, derived from the same organ, are hardly comparable. Primary cell lines and tissue slices seem to resemble the

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conditions *in vivo* much closer than other artificial experimental systems [79].

7.2. Effect of concentration and mode of administration

Concentrations of chemopreventive compounds used *in vitro* systems often cannot realistically be attained in the body. The regular levels are much lower compared with those used in most animal studies and thus the question of biological relevance to humans must be asked, especially when the bioavailability of the compound is low [80]. It is well documented that the effect of a chemopreventive agent strictly depends on its concentration, both quantitatively and qualitatively. For instance, in the Ames test, quercetin at concentration up to 1 μM stimulates CYP1A2-mediated 2-amino-3-methylimidazo[4,5-f]quinoline (MeIQ) mutagenicity, while at concentrations much higher (50-100 μM), the activation of MeIQ is suppressed. This biphasic effect of flavonoids is due to the stimulation or the inhibition of CYP1A2 activity in a dose-dependent manner [49]. Moreover, high concentrations exert a cytotoxic effect in addition to inhibition of the metabolic activity [25].

It was pointed out that several effective cancer chemopreventive agents in one experimental setting could enhance carcinogenesis in another experimental setting [19]. This statement can be illustrated by the example of the metabolism of mutagenic heterocyclic amines. Since CYP1A1 and 1A2 compete to some extent for the initial step in the metabolism of heterocyclic amines, C-hydroxylation *versus* N-hydroxylation, respectively, the time-course and dose-dependence of indole-3-carbinol induction of CYP1A1 *versus* CYP1A2 can alter the equilibrium between the induction and the inhibition of 2-amino-3-methylimidazo[4,5-f]quinoline–DNA adduct formation in the colon [81].

The route of administration also significantly affects the chemopreventive potential. This point is illustrated by a study undertaken to elucidate the mechanism of curcumin protection. The gavage of curcumin decreased esophageal CYP2B1 and 2E1, by up to 60%, compared with the vehicle control. Similarly, intragastric treatment with 270 mg/kg curcumin decreased esophageal and gastric CYP2B1 and 2E1, but not in lung, kidney or intestine. Conversely, the level of large intestinal CYP2B1 was 2.8-fold higher in the treated rats than in control rats. Mutagenic activities of N-nitroso compound, including N-nitrosomethylbenzylamine, in the presence of esophagus and stomach S9 fractions were markedly decreased, whereas those in the presence of large intestine S9 fractions were 2.2–3.0-fold above the control. The data suggest that the dietary exposure to curcumin might suppress the esophageal or gastric carcinogenesis initiated by carcinogens activated by CYP2B1/2 and 2E1, but enhance the large-intestinal carcinogenesis [63].

Because the enzyme induction is a dose- and time-dependent phenomenon, the dose of inducing agents and the route of administration are important in determining the extent of intestinal and hepatic enzyme induction. A gavage treatment (*p.o.*) of rats for 7 days with BNF (typical *i.p.* CYP1A1 inducer) produced dose-dependent increases in intestinal CYP1A1 activity, whereas a significant increase in hepatic activity was seen only at the highest dose. These results strongly suggest that the degree of intestinal and hepatic induction may vary, depending on the oral dose of inducers [82].

7.3. Time regimen of administration

Chemoprotective compounds, such as flavonoids, may act, as enzyme inhibitors and, at the same time, as inducers. High doses of chemopreventive supplements are likely to inhibit cancer activation enzymes (CYPs) in less than an hour's delay after the administration and thus act as health promoting agents. After oral administration, e.g. of 7,8-benzoflavone, this flavonoid is rapidly absorbed and the plasma concentrations peaked within 30 min, allowing maximal inhibitory effect on carcinogen activating enzymes [83]. On the other hand, lower doses (which are much more common in the human diet) possible fail in the enzyme inhibition, however, they are sufficient to initiate the expression of biotransformation enzymes [80]. This process is much slower, showing peaks of the enzyme induction from 12 to 48 h after the agent exposure, depending on the system used. For instance, the induction of CYP1A1 in MCF-7 cells is achieved in 12 h [84], while 24 h are necessary for the maximal enzyme induction in rat small intestinal epithelial cells [85].

Another point, affecting the biotransformation capacity of a particular organ, is the persistence of the enzyme induction. Elevated levels of enzymes sustain for at least 2-3 days after the inducer administration, depending on the enzyme turn-over and speed of the inducer excretion. Some compounds are retained in a body for long periods and influence enzyme expression. In a study with *Ginkgo biloba* extract, activities of CYP enzymes were recovered almost at the control level only at 1 week after the discontinuation of the treatment with this *Ginkgo biloba* extract. At 2 weeks after the discontinuation, all parameters except GST were similar to the untreated control values [86].

Hence, an important consideration to be mentioned is the timing of administration of a model carcinogen and tested chemopreventive supplement. Some enzyme inducers, which also inhibit carcinogenesis when given together with a carcinogen, are tumor promoters when given after the carcinogen. The chemopreventive effect of a tested compound should be examined under various time regimen of the compound, preferable with carcinogen and chemopreventive compound administration to experimental animals. In experiments when both chemopreventive compounds and carcinogens are tested in mutual interactions, the precise timing of the exposure of experimental animals is essential. When the tested compounds are applied simultaneously, they can compete for the metabolizing enzymes and/or the potential reactive intermediates, formed from one compound, are quenched with the other compound. On the other hand, when the impact of induction effects is examined, the chemopreventive compound should be administered hours or days ahead the model carcinogen application.

7.4. Animal model assessment

The employment of experimental animals to study the effect of chemopreventive compounds provides numerous advantages overcoming limitations of simplified settings such as reconstituted enzyme systems, microsomes, and cell lines. Animals provide the opportunity to further examine chemopreventive compound properties in respect of their absorption (including the role of microflora), their distribution and impact on individual organs, and finally kinetics of their metabolism and excretion. Results of experiments *in vivo* might differ from those, obtained *in vitro*. For instance, genistein (soy-bean flavonoid) was tested for fetotoxic and teratogenic effects *in vitro* with rat embryo culture and after its application, all embryos were found malformed. However, when administered in a diet to rats during gestation, genistein did not show any embryo-fetal toxicity [87].

Though laboratory animals are similar to humans in only some aspects of their response to hazardous exposures to carcinogens and chemopreventive compounds, some useful scientific and public health information can be extrapolated to humans. However, the major drawbacks of animal studies consist in unrealistic doses of chemicals to test their carcinogenic or anti-carcinogenic potential, ways of compound application (not frequently attained in a human intake) and the short-term administration. Taking together all the above considerations, the biological relevance of these experiments as well as their extrapolation to human exposures is questionable.

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Consequently, clinical data possibly fail to confirm promising findings of studies with experimental animals.

Introduction of highly sensitive techniques for detection of the carcinogenesis initiation in its early stage is suggested to overcome the dose and time problems. In addition to other methods used, e.g. mass spectrometry and immunoassays, monitoring the DNA-adduct formation by ^{32}P-postlabeling allows to detect in only 5 μg of isolated DNA as little as 1 adduct/10^{10} normal nucleotides (for review, see [88]). This sensitivity makes the ^{32}P-postlabeling to be one of the superior approaches even for human biomonitoring studies. Such techniques are essential to confirm the effects observed with high concentrations in appropriate studies using physiological concentrations actually found in the body.

8. Conclusion

Epidemiological data provide a valuable source of information suggesting various compounds of plant origin to have beneficial potential against the process of carcinogenesis. For human use, these chemopreventive compounds are usually concentrated to produce dietary supplements. Although these compounds are xenobiotics, the necessity of their testing prior to wide use is often underestimated. Moreover, the entire complexity of the xenobiochemistry issue including, e.g. compound interactions, enzyme induction and synergism, presence of other enzymes, transport mechanisms, timing of intake, and dosage regimen is difficult to be simulated in experimental settings and is thus virtually ignored. Consequently, the compounds that are effective cancer chemopreventive agents in one experimental setting are inactive or can even enhance carcinogenesis in the other ones. Thus, the potentially deleterious impacts, as regards carcinogen and drug metabolism *in vivo,* might be expected. However, studies dealing with this issue in humans are sparse.

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