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Phytoestrogens as natural prodrugs in cancer prevention: towards a mechanistic model

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Abstract It has been widely acknowledged that regular consumption of fresh fruits and vegetables is linked with a relatively low incidence of cancers (e.g. breast, cervix, and colon). Notably, dietary polyphenolic compounds that show some structural similarity to human estrogen, e.g. isoflavones, coumestans, lignans, flavones, have been proposed to play a role in cancer prevention. However, at present there is no satisfactory explanation for the cancer preventative properties of this group of compounds. Whereas polyphenolic compounds have been shown to inhibit proliferation of tumour cells in vitro, the results of in vivo tests have mostly been disappointing in this respect. It seems that mammalian phase II detoxification mechanisms make that dietary polyphenols are rapidly and effectively removed from the body, i.e. their concentration in the blood plasma hardly ever reaches levels high enough to have a possible effect on tumour growth. The polymethoxyflavones nobiletin and tangeretin, common constituents of *Citrus* peel, are better absorbed than polyhydroxy flavonoids, and maintain their biological activity for a longer period of time. The compounds are known to be substrates for the estrogen-converting cytochrome P450 enzymes CYP1A1 and CYP1B1, which are typically overexpressed in a range of tumour tissues. The enzymes catalyse regioselective hydroxylation and dealkylation of the polymethoxyflavones, resulting in reaction products that appear to inhibit cell proliferation via interference with the MAPK/ERK cell signalling pathway.

Keywords Flavonoids · Chemoprevention · Cancer · Polymethoxy flavones · Cell signalling · Cytochrome P450

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Introduction

Carcinogenesis is a multistage process which is usually subdivided into three phases: initiation, promotion and progression. Whereas in discussions on

cancer diagnosis or cancer treatment the emphasis is on the latter two phases, cancer prevention focuses mainly on factors that affect initiation of carcinogenesis. The systematic review 'Food, Nutrition and the Prevention of Cancer: a global perspective' (WCRF/AICR 2007) is widely recognised as the most authoritative source on food, nutrition, and cancer prevention. In the report, an expert panel presents a review and assessment of all the relevant research on food, nutrition, and physical activity, designed to reduce the risk of cancer. The panel has used a broad, integrative approach, and has attempted to find patterns of food consumption, and other lifestyle choices that enable recommendations designed to prevent cancer. One of the recommendations is to eat at least five portions/servings of a variety of non-starchy vegetables and of fruits every day, and relatively unprocessed cereals (grains) or pulses (legumes) with every meal. Another recommendation is to try and meet nutritional needs through diet alone, rather than by dietary supplements. Vegetables and fruits contain vitamins, minerals, dietary fibre, and other bioactive compounds, salicylates, flavonoids, glucosinolates, terpenes, lignans, and isoflavones. Whereas all of these groups of compounds have been shown in laboratory experiments to have potentially beneficial health effects when they are included in diets, their bioavailability is variable and there is no consensus opinion on their ultimate health effects. Further epidemiological studies have shown statistically significant decreases in the incidence of cancers correlate with an increased consumption of fruit and/or vegetables that are rich in polyphenols, notably stilbenoids, flavonoids or lignans (Birt et al. 2001; Wang 2002; Adlercreutz 2007; Benetou et al. 2008). Particularly the flavonoids have received a growing interest over the past two decades as possible cancer preventive compounds (Gates et al. 2007).

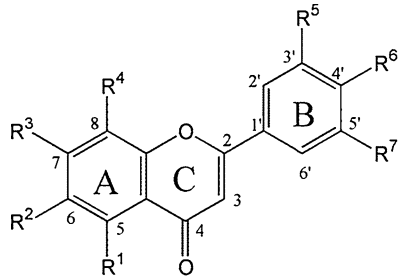
It is unrealistic to expect that these dietary compounds act on the later stages of carcinogenesis and can remove tumours, i.e. that they act as a natural chemotherapy. Accepting that theory would imply accepting that our food contains compounds that, at micromolar concentrations, are selectively and aggressively cytotoxic for tumours but not for any other rapidly dividing cells. It is far more likely that cancer preventative agents play a role in modulating cell signalling processes during the initiation phase of carcinogenesis, and thus prevent formation of tumours.

Several possible mechanisms of the cancer preventive action by flavonoids have been proposed. The most widely discussed mechanistic model works through antioxidation—polyhydroxy flavonoids can act as radical scavengers and may thus inhibit oxidation of DNA thereby preventing mutations. Quercetin and luteolin have been shown to inhibit oxidative DNA damage induced by UV light irradiation in HL-60 cells with IC_{50} <1 μM (Cai et al. 1997; Giles and Wei 1997). The in vitro antioxidant activity of flavonoids is related to the number and position of hydroxyl groups. However, giving large doses of dietary antioxidant supplements to human subjects has demonstrated little or no preventative or therapeutic effect (Halliwell 2000). In addition, the antioxidant levels in the human body are carefully regulated, and high doses of dietary antioxidants have little effect on the body's total antioxidant capacity (Halliwell 2013).

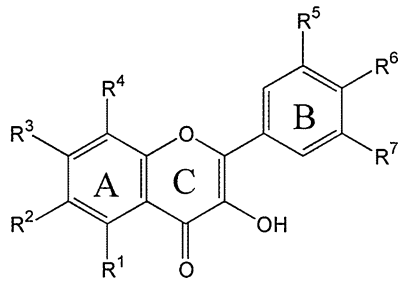
A second model emphasises estrogenic and antiestrogenic activity of a range of dietary compounds, grouped together by the pharmacological activity under the general term 'phytoestrogens'. Phytoestrogens, chemically belong to the compound classes (Table 1) isoflavones, coumestrans, lignans, or flavones, and share some structural similarities with steroid hormones (Fig. 1).

Endogenous estrogens, such as 17 β-estradiol, are implicated in the development of several types of cancer, possibly via a receptor-dependent pathway of estrogen-induced cell growth. In some cases phytoestrogens are sufficiently similar to compete with endogenous hormone for binding with estrogen receptors, and so either exert estrogenic activity in their own right or, conversely, antagonise the proliferative effect that endogenous steroid hormones have on some tumours. The synthetic 'anti-hormones' tamoxifen and raloxifene, which interrupt the hormone stimulus and thus slow the process of progression, have recently been approved as preventive drugs for women who are at moderate to high risk of developing breast cancer (NICE 2013). These compounds are cytostatic rather than cytotoxic, and are only effective until actual hormone independence occurs later in the carcinogenic pathway. However, natural phytoestrogens might be unlikely to exert significant cancer prevention directly via anti-estrogenic mechanisms since in vitro studies have suggested that their binding affinity for the estrogen receptors is comparatively weak (Birt et al. 2001).

Table 1 Structures of the flavones and flavonols discussed in this review



Flavones



Flavonols

Compound class	R^1	R^2	R^3	R^4	R^5	R^6	R^7
Flavones							
Chrysin	OH	H	OH	H	H	H	H
Baicalein	OH	OH	OH	H	H	H	H
Apigenin	OH	H	OH	H	H	OH	H
Luteolin	OH	H	OH	H	OH	OH	H
6-OH luteolin	OH	OH	OH	H	OH	OH	H
Diosmetin	OH	H	OH	H	OH	OMe	H
Eupatorin	OH	OMe	OMe	H	OH	OMe	H
Cirsiliol	OH	OMe	OMe	H	OH	OH	H
Genkwanin	OH	H	OMe	H	H	OH	H
Eupatorin-5-methyl ether	OMe	OMe	OMe	H	OH	OMe	H
Tangeretin	OMe	OMe	OMe	OMe	H	OMe	H
Nobiletin	OMe	OMe	OMe	OMe	OMe	OMe	H
Sinensetin	OMe	OMe	OMe	H	OMe	OMe	H
Flavonols							
Kaempferol	OH	H	OH	H	H	OH	H
Kaempferide	OH	H	OH	H	H	OMe	
Quercetin	OH	H	OH	H	OH	OH	H
Tamarixetin	OH	H	OH	H	OH	OMe	H
Myricetin	OH	H	OH	H	OH	OH	OH
Quercetagetin	OH	OH	OH	H	OH	OH	H

A variant of the anti-estrogen model assumes that dietary phytoestrogens interfere with the biosynthesis of steroid hormones, thus reducing the amount of endogenous hormones and depriving hormone-related cancers—breast, prostate, endometrium, testis, ovary, thyroid, and osteosarcoma—(Henderson and Feigelson 2000) of an essential growth factor. The dietary flavones luteolin and apigenin have been shown to effectively inhibit activity of CYP19 or aromatase (Rice et al. 2006; van Meeuwen et al. 2007; Li et al. 2011), the enzyme responsible for the aromatization of androgens into estrogens. In addition, these flavones downregulate CYP19 transcription (Rice et al. 2006), possibly by affecting signalling transduction pathways involving JNK and ERK (Li et al. 2011). Thus, these compounds may play a role in regulating the levels of oestrogen formed in the human body. The levels necessary for effective inhibition of CYP19 activity can be achieved in the blood serum, but can only be sustained by regular intake of food supplements. It is probably best to take a cautionary approach with phytoestrogen-rich food supplements since their mode of action is not fully understood; some may act as oestrogen agonists and

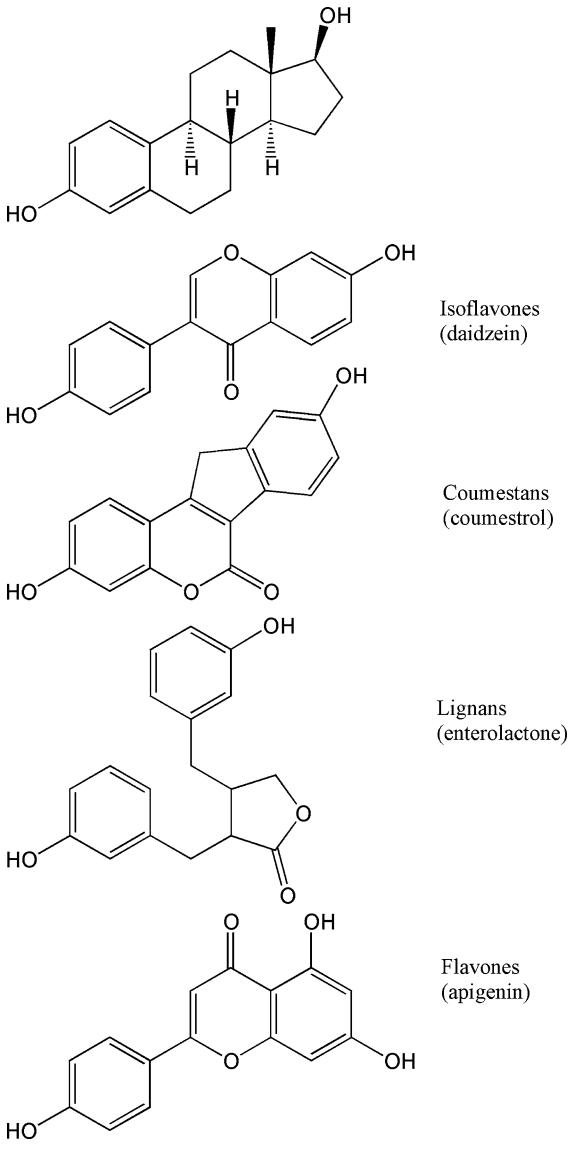


Fig. 1 Structural similarity between estradiol (top) and several phytoestrogens

actually stimulate cell proliferation (van Meeuwen et al. 2007).

The mechanism by which endogenous estrogens exert their carcinogenic effects involves the regio-selective oxidation of 17 β-estradiol (E2), and subsequent formation of estrogen-DNA adducts. This pathway is regulated by phase I detoxification enzymes, mainly cytochromes P450 (CYPs) 1A1 and 1B1. In contrast to most cytochromes P450, CYP1A1 and CYP1B1 are expressed in various tissues rather than just in the liver. The extrahepatic CYPs are known to be activated by phytoestrogens, and thought to play a role in xenobiotic metabolism throughout the body (Ito et al. 2007; Androutsopoulos et al. 2009b; Surichan et al. 2012).

CYP1A1 predominantly converts E2 to non-carcinogenic 2-hydroxy-E2, while CYP1B1 predominantly catalyzes the hydroxylation of the C4-position of 17 β-estradiol and thus forms the carcinogenic 4-hydroxy-E2 (4-OH-E2). CYP1B1 is highly expressed, and consequently 4-OH-E2 is predominantly detected, in estrogen dependent neoplastic tissues (Murray et al. 1997). The overexpression of CYP1B1 is considered an early marker of carcinogenesis (Shi et al. 2009). Once 4-OH-E2 is formed, it is further oxidized to quinones and semi-quinones, which then form DNA adducts, resulting in mutagenic lesions. Dietary phytoestrogens may hamper this process of carcinogenesis by inhibiting the activity of CYP1B1 (Doostdar et al. 2000), and thus preventing the formation of 4-hydroxy-E2. The EROD assay determines the activity of CYP1 enzymes, by monitoring the *O*-dealkylation of ethoxyresorufin. The assay was used to show that flavones inhibit the activity of CYP1 enzymes (Table 2), which could have been expected considering the structural similarity of flavones and estrogen (Fig. 1). Notably, flavones with hydroxy substitutions at C-5 and C-7, such as chrysin and luteolin are potent inhibitors. These A-ring substitutions are possibly required for docking the flavone molecule to the active sites of the CYP1 enzymes. Hence, 6-hydroxy substitution decreased the inhibitory activity and methoxy substitution at C-5 completely abolished it. Inhibition of the activity of CYP1A1 and CYP1B1 could plausibly explain why some phytoestrogens have cancer preventive properties. But the story does not end here, because the phytoesterogens are also substrates for the CYP1 enzymes in their own right, and some products of CYP1-catalysed conversions of phytoestrogens directly affect cell proliferation (Arroo et al. 2008, 2009; Martineti et al. 2010).

Flavone bioconversions that are typically catalysed by CYP1-enzymes include the 3′-hydroxylation of the B-ring, and the demethylation of a 4′-methoxy substituent (Breinholt et al. 2002). However, in vitro assays with microsomes carrying recombinant human CYP1s have indicated that further dealkylations can also be catalysed by these enzymes (Androutsopoulos et al. 2009a, c; Surichan et al. 2012).

Table 2 The effect of dietary and synthetic (6-OH luteolin) flavonoids on the EROD activity of human CYP1 enzymes

Compound	CYP1B1	CYP1A1	CYP1A2
Quercetin	0.7 ± 0.3	7	12 ± 3.5
Myricetin	0.9 ± 0.2	4	13 ± 0.7
Kaempferol	1.3 ± 0.2	8 ± 1.4	9 ± 2.1
Apigenin	2	4.2	6
Luteolin	1.8 ± 0.4	10 ± 0.7	15 ± 1.4
6-OH luteolin	7.7 ± 2	>25	>25
Diosmetin	0.5 ± 0.1	1.2 ± 0.3	18 ± 4
Baicalein	7 ± 1.1	20	20
Chrysin	0.5 ± 0.1	0.5 ± 0.1	2
Eupatorin	1.1 ± 0.1	2.3 ± 0.1	25
Eupatorin-5-methyl ether	21 ± 1	6.8 ± 0.4	>25
Genkwanin	>25	>25	>25
Cirsiliol	>25	>25	>25

CYP1 (CYP1A1, CYP1A2, CYP1B1) microsomes (5 pmol/ml) were incubated with various concentrations of flavonoids (25, 2.5, 0.25, 0.025, 0.0025 μM). Results are expressed as IC_{50}, calculated from percentage of inhibition of EROD activity compared to control. Each value corresponds to mean ± SD for n = 4 determinations. IC_{50} are in μM

Since the substitution patterns are important features of biologically active metabolites, it is important to recognise parent compound substitutions prior to CYP1-mediated biotransformations, e.g. luteolin can be considered an apigenin derivative, quercetin can be derived from either kaempferol or tamarixetin (Breinholt et al. 2002). The anticancer activities of the product flavones of CYP1-mediated conversions tend to be greater than those of the substrate flavones.

Structure activity relationship for anticancer flavones

Structural features present in flavones which have been demonstrated key for inhibition of cell proliferation include a 3′,4′-dihydroxy (catechol) B-ring and a 5,7-dihydroxy A-ring (Duarte Silva et al. 2000). As a further rule, polyhydroxy flavones seem to be particularly active. Quercetagetin (3,5,6,7,3′,4′-hexahydroxyflavone) is reported to be extremely effective in vitro against several tumour cell lines, including P-388 (murine lumphocytic leukemia), A-549 (human lung carcinoma), MCF-7 (human breast adenocarcinoma), HT-29 (human colon adenocarcinoma), and KB (human nasopharynx carcinoma) cells (Ferreira et al. 2010). This polyhydroxyflavone also demonstrate potent and selective inhibition (IC_{50} = 0.34 μM) of the serine-threonine kinase PIM1, which is implicated in the development of leukemia, lymphoma, and prostate cancer (Holder et al. 2007). Myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone) was able to inhibit the viability and proliferation of T24 cells in a dose- and time-dependent manner, promoting cell cycle arrest at G2/M by down-regulation of cyclin B1 and cyclin-dependent kinase cdc2 (Sun et al. 2012). Myricetin-induced apoptosis correlates with the modulation of Bcl-2 family proteins and activation of the caspase-3. Kaempferol and, more so, quercetin (3,5,7,3′,4′-pentahydroxyflavone) were shown to inhibit growth of OVCAR-3 (ovarian cancer cells) in a dose-dependent manner (Luo et al. 2008), possibly by inhibiting expression of vascular endothelial growth factor (VEGF). The flavanonol taxifolin and the flavanone glycoside naringin, which were screened in the same experiment showed little inhibitory effect, possibly due to the absence of a double bond between C2 and C3 in the C-ring, which makes that their B ring is nearly perpendicular to the rest of the molecule, as opposed to a planar molecule like kaempferol. The C2–C3 double bond in flavones and flavonols has also been shown to be required for strong CYP1 inhibition; the planar structure increases their similarity with estradiol (Bentrem et al. 2003), and improves the enzyme docking ability of the flavonoid molecule (Hodek et al. 2002; Takemura et al. 2010).

Thus, from in vitro experiments it appears that polyhydroxy flavones could be potential candidates for anticancer drugs, that either work by inhibition of extrahepatic CYP1-enzymes—thus preventing the formation of the carcinogenic 4-OH-E2, or directly affecting cell division through interfering with cell signalling processes.

Metabolism of flavones

The promising biological effects of polyhydroxy flavones in vitro are not consistently replicated in vivo. This is mainly due to their low oral bioavailability. The major factors regulating the bioavailability of polyphenolic compounds, e.g. polyhydroxy

flavones, are the conjugation reactions of phase II metabolism, and efflux transporters. In general, the bioavailability of flavonoids depends on their chemical form in foods (generally glycosides), hydrophobicity, susceptibility to degradation, the microbial flora of the consumer, and the food matrix.

Saliva has some impact on the stability of green tea catechins. (Tsuchiya et al. 1997). For example, catechin esterase activity in human saliva results in the degradation of the flavanol gallate ester epigallocatechin gallate (EGCG) to epigallocatechin (EGC), which is then absorbed through the oral mucosa (Yang et al. 1999). However, other flavonoids like procyanidin oligomers are not modified in the mouth or in the esophagus before accessing to the stomach (Spencer et al. 2001). Only in the acidic stomach environment, procyanidins usually rapidly decompose into epicatechin monomeric, dimeric, and other oligomeric units (Porter 2002) although cocoa procyanidins proved remarkably stable (Rios et al. 2002).

Going from stomach to jejunum, the pH rises from 2 to 7. Flavonoids have been shown to be absorbed in the small intestine (Spencer et al. 1999; Andlauer et al. 2000; Gee et al. 2000). For flavonoid glycosides, deglycosylation represents an important step for the delivery of flavonoids from the intestinal lumen into the circulation. There are two ways through which the absorption of flavonoids glycosides may occur: one involves the active uptake of the glucoside by the sodium-dependent glucose transporter (SGLT1) with subsequent deglycosylation within the enterocyte by cytosolic β-glucosidase (EC 3.1.1.21), the other way is via luminal hydrolysis of the glucoside by lactase phlorizin hydrolase (LPH, EC 3.2.1.108) and absorption by passive diffusion of the released aglycone.

Important parameters determining the site of deglycosylation and transport across the intestinal enterocytes are the nature of the aglycone moiety of flavonoids and the nature and position of the sugar moiety (Day et al. 2003). Different studies have shown that flavonoids undergo intestinal first-pass phase II metabolism conversions during their transfer from the lumen of the small intestine to the mesenteric circulation (Cheng et al. 1999; Rice-Evans 2001; Carbonaro and Grant 2005).

Glucuronidation is reported to be a metabolic pathway for different flavonoids, such as galangin, kaempferol, baicalein, and other polyphenols and the reaction is catalysed by different uridine 5′-diphospho-glucuronosyltransferases (UGTs) (Otake et al. 2002; Zhang et al. 2007a). Glucuronides of polyhydroxyflavones such as quercetin and kaempferol represent the main metabolic products detected in human systemic circulation after their oral administration (Janisch et al. 2004; Dupont et al. 2004).

Flavones, including chrysin, apigenin, luteolin and baicalein are metabolized by the intestinal UGT (EC 2.4.1.17) (Ng et al. 2005), and then absorbed as glucuronide (Zhang et al. 2005). Glucuronidation of quercetin and luteolin occurs preferentially at 7-, 3-, 3′- or 4′-hydroxyl groups in human liver and intestine microsomes (Boersma et al. 2002). In addition, quercetin-3-glucuronide, quercetin-3′-sulfate and isorhamnetin-3-glucuronide have been found to be the main metabolic products detected in blood, after oral administration of quercetin to humans (Janisch et al. 2004). Some flavones like chrysin, apigenin, luteolin, and diosmetin are able to induce UGTs, thus determining an increase of the extent of first-pass metabolism of flavonoids (Walle et al. 2000; Galijatovic et al. 2001).

The extent of glucuronidation depends on flavonoid structure: flavonoids with a substituted hydroxyl group on the B-ring (i.e. hesperetin) are less predisposed to glucuronidation, whereas flavonoids with a 3′,4′-ortho-dihydroxy (or catechol) B-ring are more easily metabolized into glucuronides (Shimoi et al. 1998; Spencer et al. 1999). Analoguous patterns of metabolism occur in the ileum, although in general, glucuronidation takes place to a lesser extent due to lower levels of phase I and II enzymes present in the ileum compared with the jejunum (Spencer et al. 1999).

Another metabolic reaction occurring in human intestine is sulfonation, mediated by different sulfotransferases such as SULT1A3 and SULT1A2. Though, in humans, sulfonation of the flavone baicalein occurs mainly in the liver (Zhang et al. 2007b). In intestinal cells, UGTs and SULTs catalyse the conjugation of the flavanone hesperetin into its glucuronidated and sulfonated metabolites (Brand et al. 2010).

Colon bacteria posess different deconjugating enzymes able to release flavonoid aglycones from their glycosides and glucuronides, e.g. β-D-glucuronidases, β-D-glucosidases and α-L-rhamnosidases (MacDonald et al. 1983; Bokkenheuser and Winter 1988; Aura et al. 2002). However, many flavonoids only transiently exist as aglycones since intestinal microbiota is able to metabolize the flavonol-*O*-

glycosides (Hein et al. 2008). Then the released aglycones are completely metabolized to their corresponding degradation products, e.g. 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, and phloroglucinol.

Flavonoid neohesperidosides and rutinosides are poorly absorbed in the small intestine and thus reach the colon where they are metabolised by colonic microbiota. Rutinosides are probably absorbed only in colon, after bacterial hydrolysis (Manach et al. 2003). The kinetics of the appearance of hesperidin (hesperitin-7-*O*-rutinoside) and narirutin (naringenin-7-*O*-rutinoside) in plasma show these Citrus flavanones are absorbed from the distal part of the intestine.

Fully methylated flavonoids have been reported to show increased intestinal absorption (Wen and Walle 1999), and are not substrates for phase II metabolism. Whereas unmethylated flavones, like 7-hydroxyflavone, 7,4′-dihydroxyflavone, chrysin (5,7-dihydroxyflavone), and apigenin (5,7,4′-trihydroxyflavone), are rapidly eliminated from the body due to extensive glucuronidation and/or sulfation, the corresponding methylated flavones, i.e. 7-methoxyflavone, 7,4′-dimethoxyflavone, 5,7-dimethoxyflavone, and 5,7,4′-trimethoxyflavone, are more resistant to hepatic metabolism and have a fivefold–eightfold higher apparent permeability of apical to basolateral flux, in the in vitro model of intestinal absorption of Caco-2 cells grown in porous inserts.

CYP1-activation of polymethoxylated flavones

Polymethoxy flavones exist almost exclusively in genus *Citrus*, particularly in the peels, and several epidemiological studies have linked citrus consumption with a reduced cancer risk (Benavente-Garcia 2008; Xiao et al. 2009; Foschi et al. 2010; Ho et al. 2012; Grosso et al. 2013; Song and Bae 2013).

Conversion of polymethoxylated flavones, such as tangeretin and nobiletin, into partially demethylated metabolites has been shown to occur in vivo (Kurowska and Manthey 2004; Manthey et al. 2011), and conjugated forms of these metabolites appear in the blood serum at later time points. However, the rate of conjugation and subsequent excretion of the metabolites is much slower than for polyhydroxylated flavones.

In tumour cells, or even cells in earlier stages of carcinogenesis (initiated cells, preneoplasia), where CYP1s are expressed, polymethoxy flavones will be substrates for these enzymes. In vitro experiments with microsomes expressing recombinant human CYP1 (Walle and Walle 2007; Androutsopoulos et al. 2010) have shown that the enzymes catalyse conversion of polymethoxy flavones into a range of products. The main bioconversions that have been recognised are 3′-hydroxylation of an already C4′ substituted B-ring, and 4′-*O*-demethylation of the same ring (Breinholt et al. 2002), but further conversions have been shown to occur (Androutsopoulos et al. 2008, 2009a; Cheng et al. 2011). Analysis of conversion products of the polymethoxy flavones tangeretin and nobiletin by LC–MS indicated that these further CYP1-catalysed bioconversions resulted in more polar compounds which were characterised by a loss of 14 mass units, which is consistent with additional *O*-demethylations (Surichan unpublished data). At present, it remains a matter of speculation which methoxy substituents on the flavones are converted to hydroxyls, but they putatively include those on C7 (Tsuji et al. 2006; Walle and Walle 2007; Androutsopoulos et al. 2009c). Demethylation of C3′ of nobiletin has been proposed (Koga et al. 2007), but is disputed (Arroo et al. 2009). Further, in an already C5–C7-substituted A-ring, the C6 can be hydroxylated (Androutsopoulos et al. 2009c, 2011), a reaction analogous to the CYP1-catalysed C4 hydroxylation of the stilbenoid resveratrol (Potter et al. 2002). Furthermore, CYP1 enzymes can catalyse C4′-hydroxylation of a further unsubstituted B-ring in flavones (Otake and Walle 2002).

Genkwanin (4′,5-dihydroxy-7-methoxyflavone) has been found to induce terminal differen-tiation of HL-60 leukemic cells, and was considered a natural chemopreventive agent (Suh et al. 1995). More recent work showed that this flavone is also a substrate for CYP1 mediated 7-*O*-demethylation, resulting in the formation of apigenin (Androutsopoulos et al. 2009c) which is a flavone with well-documented anticancer properties (Shukla and Gupta 2010; Leonardi et al. 2010; Zhong et al. 2010; Mamadalieva et al. 2011; Wang et al. 2011). In turn, apigenin is a substrate for further CYP1-mediated conversion to yield luteolin (Breinholt et al. 2002), another active anticancer flavonoid (Seelinger et al. 2008; Wang et al. 2012; Xie et al. 2012; Pandurangan et al. 2013; George et al. 2013).

The triglycoside of kaempferide (3,5,7-trihydroxy-4′-methoxyflavone) inhibits the proliferation of an

engineered human colon cancer cell line that was engineered to overexpress estrogen receptor β. However, proliferation of native colon cancer cells was also inhibited, and it was concluded that the process was not mediated by ligand binding dependent estrogen receptor activation (Martineti et al. 2010). The kaempferide triglycoside caused G_1 arrest in HCT8 cells, which are now known to express CYP1A1 (Volkova et al. 2013). Thus, the observed activity might be interpreted as another example of CYP-1 mediated flavone activation; CYP1A1-mediated conversion of the kaempferide aglycone would give kaempferol, and subsequently quercetin. The latter flavonol possesses all the typical features necessary for inhibition of cell proliferation.

Several flavones have been shown to induce the expression of CYP1A1 and CYP1B1 (Androutsopoulos et al. 2009b; Chatuphonprasert et al. 2010; Surichan et al. 2012), which may further indicate a specific role for these enzymes in the bioconversion of dietary phytoestrogens.

Expression of CYP1A1 and CYP1B1 is generally seen as harmful and part of the carcinogenesis process (Murray et al. 1997; Cheung et al. 1999; McFadyen et al. 2001; Androutsopoulos et al. 2013; Rodriguez and Potter 2013). Thus, inhibition of CYP1 activity by flavonoids has been used to explain inhibition of tumour cell proliferation by this group of compounds (Takemura et al. 2013). An alternative explanation is that the CYP1 enzymes are expressed in cells where normal the normal cell cycle is already compromised, i.e. they are a result rather than the cause of carcinogenesis. Their role could be beneficial rather than harmful because they activate flavonoids into agents that cause cytostasis or apoptosis. In this model, proliferation of cells that express CYP1s would be strongly inhibited whereas growth and development of other cells would hardly be affected. Following this line of argument, the cytochromes P450 CYP1A1 and CYP1B1 have been chosen as potential targets for cancer therapy through selective activation of non-cytotoxic prodrugs (Bruno and Njar 2007; Callero et al. 2012; Sutherland et al. 2013). We propose that the same model may apply to flavones and other phytoestrogens that are substrates for the CYP1 enzymes and have long been associated with cancer prevention.

Perhaps the most striking example of the CYP1-mediated activation of polymethoxy flavones comes from the demonstration that breast cancer cell lines which express CYP1A1 and CYP1B1 are 20 times more sensitive to eupatorin than cell lines that do not express these enzymes. Eupatorin causes G2/M arrest in CYP1-expressing breast cancer cells, but does not inhibit the cell cycle of breast cells that do not express CYP1s (Androutsopoulos et al. 2008). This was the first study that demonstrated a cancer therapeutic effect of a naturally occurring flavonoid with *selective inhibition* of tumour cell proliferation, based on CYP1A1/CYP1B1 mediated metabolism. Most other studies have shown a cancer therapeutic effect of flavonoids directly via cell cycle arrest, or induction of apoptosis. However, those compounds would affect normal cells as well as tumour cells, with very little selectivity.

Further studies on CYP1-mediated bio-activation of methoxylated flavones showed a similar pattern (Androutsopoulos et al. 2009a, c; Androutsopoulos and Tsatsakis 2013; Surichan et al. 2012), CYP1-activated flavones are stronger cytostatics than their parent compounds. The CYP1-mediated bioconversions of polymethoxy flavones do not result in a single product, but rather in a cocktail of flavones with varying hydroxylation patterns (Androutsopoulos et al. 2010). It is not always clear which of the metabolites is responsible for the anti-proliferative effect. The possibility of a synergistic mode of anti-proliferative action should also be considered (Androutsopoulos and Spandidos 2013).

When CYP1-expressing cells were grown in the presence of different concentrations polymethoxy flavones, the MTT cell viability assay consistently gives a biphasic curve (Fig. 2), i.e. at nM concentrations polymethoxy flavones inhibit cell proliferation in a dose-dependent fashion, but at μM concentrations the inhibitory effect lessened (Androutsopoulos et al. 2009a; Surichan et al. unpublished data). Considering that the CYP1 enzymes can catalyse several conversions, an explanation for the biphasic curve may be that at low substrate concentrations CYP1s catalyse a number of subsequent conversions of polymethoxy flavone substrates, resulting in formation of an as yet unidentified product that arrests cell proliferation. At higher concentrations, CYP1s catalyse the initial conversions of the polymethoxy flavone substrates (notably 4′-demethylation), but then the initial products that are not converted further because, now substrates in their own right, they have to compete with the original

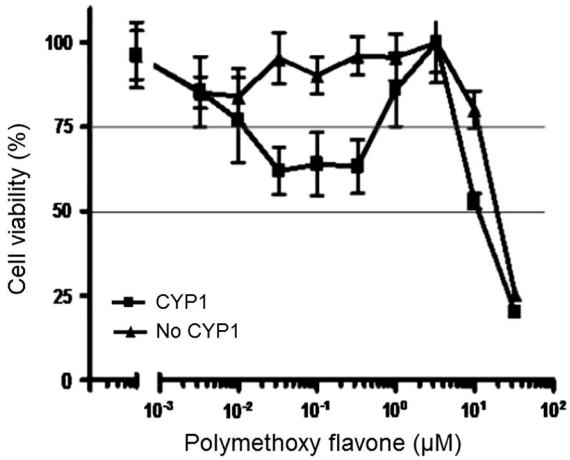


Fig. 2 Cell viability curves for cells expressing CYP1 enzymes, and for cells that do not express CYP1s, cultured in vitro in the presence of polymethoxy flavones

polymethoxy substrate for the active site on the CYP1 enzyme. Thus, high concentrations of polymethoxy flavones may prevent formation of further conversion products through competitive inhibition.

Much work is required to provide unambiguous proof that the CYP1-activation model is valid in vivo, since most of the results are derived from in vitro experiments. In addition it remains unclear whether this generalization can cover a vast range of polymethoxy flavones, as each compound follows a similar yet distinct pattern of CYP1-mediated conversions. For example nobiletin metabolism by CYP1A1 yields mainly 4′-hydroxy nobiletin (Koga et al. 2011) whereas eupatorin metabolism by CYP1A1 mainly cirsiliol (Androutsopoulos et al. 2008). Although the pattern of metabolism is similar (4′-demethylation) the two main products are structurally different and consequently possess dissimilar biological activities. At present, it remains a matter of some speculation which metabolic products of the polymethoxy flavones are mainly responsible for the anti-proliferative action. However, based on the structure/activity relationships that have been found through in vitro studies with polyhydroxy flavones, some educated guesses can be made.

CYP-activated flavones and cell signalling

Polymethoxylated flavones derived from citrus peel—e.g. tangeretin, nobiletin, and sinensetin—effectively inhibit proliferation of a range of human tumour cell lines in a dose- and time-dependent manner (Pan et al. 2002; Morley et al. 2007; Du and Chen 2010; Nemoto et al. 2013). In addition, polymethoxyflavones have been shown to inhibit proliferation and migration of lens epithelial cells and thus prevent posterior capsular opacification, the most frequent complication after extracapsular cataract surgery (Miyata et al. 2013). However, the concentrations at which the polymethoxy flavones exert their activity in vitro tend to be quite high (>10 μM, up to 100 μM), i.e. at levels which are not likely to be sustained for long in blood plasma. Nevertheless, at these high levels, the compounds seem to act as cytostatics rather than as cytotoxins; following removal of the flavonoids, cells regain their capacity to proliferate and cycle (Morley et al. 2007). Arguably, the capacity to quickly return to normal function would remain more intact in normal tissues than it would in tumour tissue.

A metabolite of nobiletin, 3′,4′-dihydroxy-5,6,7-trimethoxyflavone (DTF), has been shown to induce activation of JNK and ERK (MAPK) and thus modulate the PI3K/Akt signalling in the PC12 rat pheochromocytoma cell line. Both nobiletin and DTF activated the ERK, JNK, and Akt pathways in PC12 cells, but whereas the effect of nobiletin was modest, DTF led to a rapid phosphorylation which increased with time (Su et al. 2012).

In HepG2 liver cancer cells, diosmetin was demethylated to luteolin with a concomitant G_2/M arrest of the cell cycle. The cell cycle arrest was accompanied by up-regulation of the cell cycle inhibitors p21, p53 and an activation of the MAPK kinases ERK and JNK. The overall inhibition of cellular proliferation was attributed to the synergistic action of diosmetin and luteolin (Androutsopoulos and Spandidos 2013). Similarly, CYP1-catalysed demethylation of eupatorin-5-methyl ether in MCF7 breast cancer cells resulted in up-regulation of the cell cycle inhibitor p21 and induction of JNK total levels and p-JNK to a lesser extent resulting in G_1 arrest (Androutsopoulos and Tsatsakis 2013).

The polymethoxyflavone tangeretin and its CYP-1 mediated demethylation product, tangeretin, 4′-hydroxy-5,6,7,8-tetramethoxyflavone (4′-OH-TMF), both caused G_1 arrest in EGF-stimulated hepatocytes, as indicated by a concentration dependent inhibition of [^{3}H]thymidine incorporation into DNA (Cheng et al. 2011). It was also shown that 4′-OH-TMF, but not

tangeretin, inhibited p70S6K-phosphorylation in EGF-stimulated hepatocytes, while neither tangeretin nor 4′-OH-TMF affected phosphorylation of Akt, GSK, or ERK.

Conclusion

The term 'phytoestrogens' is a blanket term for plant compounds that interact with human estrogen receptors; the compounds are grouped together through their pharmacological action. Chemically, the group includes isoflavones, lignans, coumestans, and flavones. This review concentrates on the latter group of compounds which are a common ingredient of fruits and vegetables and have been linked with cancer prevention. Flavones share some structural features with 17β-estradiol, and interact not only with estrogen receptors, but also with enzymes that metabolise estrogens, e.g. the cytochromes P450 enzymes CYP1A1 and CYP1B1 which are overexpressed in various tumour tissues.

Polyhydroxy flavones have been shown to effectively inhibit cell proliferation in vitro, through interaction with cell signalling processes. However, bioavailability of polyhydroxy flavones is poor because they are rapidly removed from the body by phase II metabolism. This makes that they show very little effect in vivo. In contrast, dietary polymethoxy flavones can circulate in the body for a substantial time and in micromole concentrations, but they have relatively little immediate effect on cell physiology. Only when polymethoxy flavones become substrates for the tumour-specific cytochromes P450 CYP1A1 and CYP1B1, they are *O*-demethylated to yield polyhydroxy flavones which have long been known to effectively inhibit cell proliferation. Thus, the polymethoxy flavones can be seen as prodrugs that are activated by CYP1 enzymes to yield cytostatic polyhydroxy flavones. The activation selectively occurs inside cells that overexpress CYP1-enzymes; no cytostatic compounds are formed in normal cells.

The hepatic enzyme CYP1A2 catalyses bioconversions similar to those catalysed by CYP1A1 and CYP1B1, albeit at a more limited rate, and would thus inhibit cell division in the liver. However, in a healthy liver there is very little cell proliferation anyway—only following partial hepatectomy do hepatocytes enter the cell cycle, an event dependent in part on stimulation with epidermal growth factor. Thus, CYP1-activation of polymethoxy flavones presents a credible model that may explain how dietary phytoestrogens can suppress tumour development at the earliest stages of carcinogenesis without affecting normal cell physiology.

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