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CYP1-mediated antiproliferative activity of dietary flavonoids in MDA-MB-468 breast cancer cells

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ABSTRACT

Among the different mechanisms proposed to explain the cancer-protecting effect of dietary flavonoids, substrate-like interactions with cytochrome P450 CYP1 enzymes have recently been explored. In the present study, the metabolism of the flavonoids chrysin, baicalein, scutellarein, sinensetin and genkwanin by recombinant CYP1A1, CYP1B1 and CYP1A2 enzymes, as well as their antiproliferative activity in MDA-MB-468 human breast adenocarcinoma and MCF-10A normal breast cell lines, were investigated. Baicalein and 6-hydroxyluteolin were the only conversion products of chrysin and scutellarein metabolism by CYP1 family enzymes, respectively, while baicalein itself was not metabolized further. Sinensetin and genkwanin produced a greater number of metabolites and were shown to inhibit strongly in vitro proliferation of MDA-MB-468 cells at submicromolar and micromolar concentrations, respectively, without essentially affecting the viability of MCF-10A cells. Cotreatment of the CYP1 family inhibitor acacetin reversed the antiproliferative activity noticed for the two flavones in MDA-MB-468 cells to 13 and 14 μM respectively. In contrast chrysin, baicalein and scutellarein inhibited proliferation of MDA-MB-468 cells to a lesser extent than sinensetin and genkwanin. The metabolism of genkwanin to apigenin and of chrysin to baicalein was favored by CYP1B1 and CYP1A1, respectively. Taken together the data suggests that CYP1 family enzymes enhance the antiproliferative activity of dietary flavonoids in breast cancer cells, through bioconversion to more active products.

1. Introduction

Cytochrome P450s are haem-containing enzymes, responsible for the metabolism of a vast number of xenobiotics as well as endogenous compounds. The first family consists of three members CYP1A1, CYP1A2 and CYP1B1. CYP1A2 is expressed predominantly in the liver, whereas CYP1B1 and CYP1A1 are found in extrahepatic tissues (Murray et al., 1997; McFadyen et al., 2004). Metabolism of xenobiotics by CYP1 family enzymes can elicit toxic responses, or contribute to the detoxification of potentially harmful compounds. For example it has been shown that CYP1A2 participates in the activation of the anti-androgenic drug Flutamide and the anthraquinone AQ4N, whereas recent evidence in Cyp1a1 (−/−) knockout mice, suggests that CYP1A1 contributes to the detoxification of the environmental carcinogen B[*a*]P (Shet et al., 1997; Patterson and Murray, 2002; Uno et al., 2004; Androutsopoulos et al., 2009c). With respect to environmental toxicology and chemoprevention the interactions of CYP1 family enzymes with flavonoids, a major class of natural products encountered frequently in the diet, have been well documented over the last decade. It has been proposed that flavonoids act either as CYP1 enzyme inhibitors and inhibitors of CYP1 gene-mediated transcriptional activation, thereby blocking any mutagenic effects produced by metabolism of environmental carcinogens, or as CYP1 substrates undergoing activation to antiproliferative agents, notably cell cycle inhibitors, in tissues or cells where CYP1 enzymes are expressed (Chun et al., 1999, 2001; Ciolino et al., 1998a,b; Ciolino and Yeh, 1999a,b; Ciolino et al., 1999; Androutsopoulos et al., 2008, 2009a,b, in press; Atherton et al., 2006; Potter et al., 2002).

Baicalein (5,6,7-trihydroxyflavone), chrysin (5,7-dihydroxyflavone) and scutellarein (5,6,7,4′-tetrahydroxyflavone) (Fig. 1) are bioactive flavonoids, containing hydroxyl groups, present in SCUTELLARIA, a traditional herbal remedy with potential anticancer activity (Parajuli et al., 2009). Baicalein is also a major component of sho-saiko-to, which is a Chinese medicine admin-

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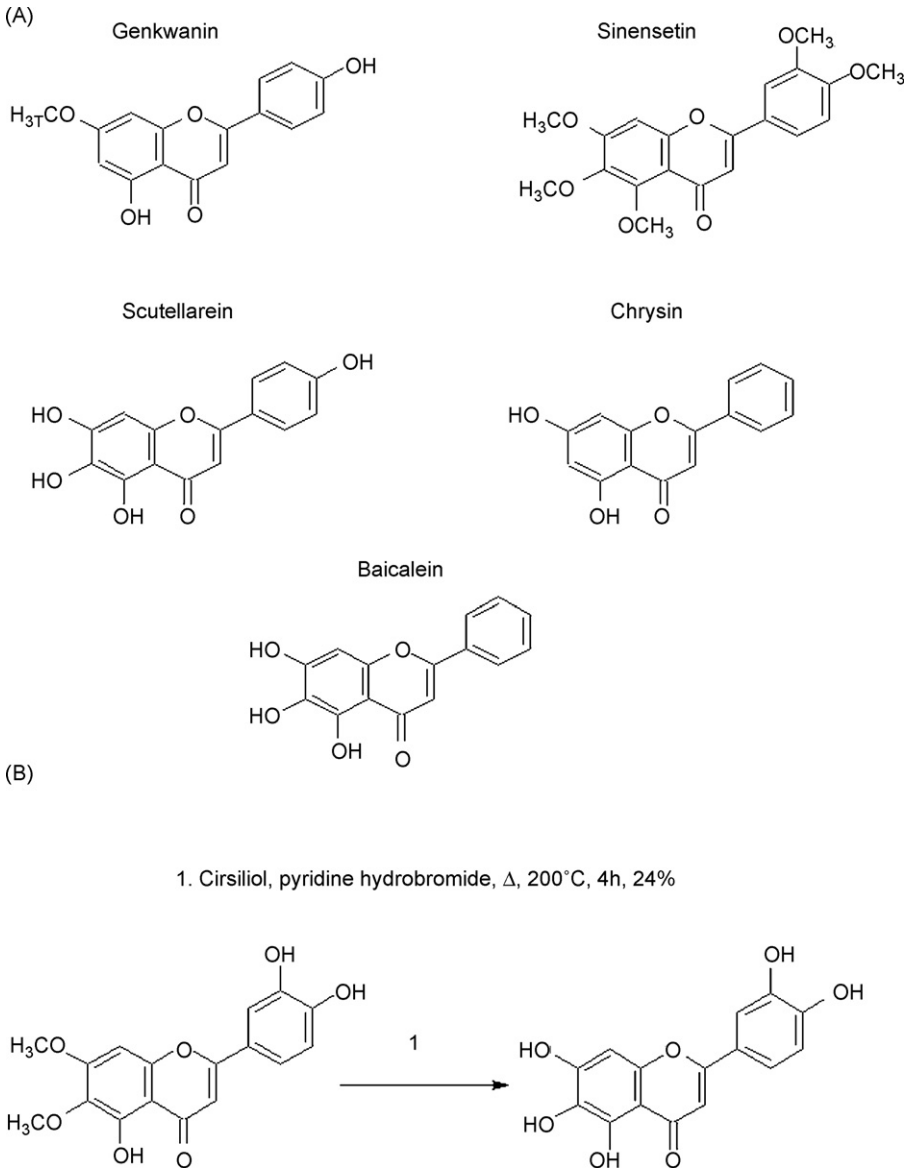

Fig. 1. (A) Chemical structures of the flavonoids investigated in the present study. (B) Schematic diagram indicating the synthetic route of 6-hydroxyluteolin (6OHL) from 6,7-dimethoxy 3′,4′,5-trihydroxy flavone (cirsiliol).

istered in Japan for the chemoprevention of hepatic fibrosis and carcinoma, whereas chrysin, a major constituent of propolis found in the regions of Sonora in Mexico, exhibits antiproliferative activity in cancer cell lines (Shimizu, 2000; Hernandez et al., 2007). Genkwanin (7-methoxy,5,4′-dihydroxyflavone) and sinensetin (3′,4′,5,6,7-pentamethoxyflavone) (Fig. 1) are flavonoids containing one and five methoxy groups, respectively, which are present in dietary products, as well as medicinal plants. Genkwanin is found in its aglycone form in the oil droplets produced by the plant *Origanum* × *intercedens*, which is considered as a high quality oregano spice, as well as in the traditional medicinal herbs *Artemisia afra* and *Combretum erythrophyllum* (Combretaceae), used as antimalarial remedies and for the treatment of abdominal pains and venereal diseases respectively (Bosabalidis et al., 1998; Kraft et al., 2003; Martini et al., 2004). Sinensetin is found in small amounts in the juice of mandarins and related citrus fruits and in higher amounts in the corresponding peel extracts (Nogata et al., 2003; Lu et al., 2006; Wang et al., 2008). Genkwanin induces terminal differentiation of HL-60 leukemic cells, while sinensetin is capable of inhibiting production of TNF-α and suppress in vivo natural killer cell activity, as well as inhibit mutagenic activity caused by furylfuramide, a well-known mutagen (Suh et al., 1995; Delaney et al., 2001; Miyazawa et al., 1999). However little is known regarding the exact mechanism by which such flavonoids exert their cancer-protecting activity.

Recent studies in our lab have identified the dietary flavonoids eupatorin, cirsiliol and diosmetin as CYP1-activated prodrugs, i.e. metabolism of these compounds by CYP1 enzymes results in further activation and enhances their antiproliferative activity (Androutsopoulos et al., 2008, 2009a,b, in press). In the present study, the hypothesis that structurally similar flavonoids, present in the diet, act through the same mechanism was examined. The metabolism of the flavones genkwanin, sinensetin, chrysin, baicalein and scutellarein (Fig. 1A) by CYP1 enzymes was investigated. We suggest that all flavones with the exception of baicalein are CYP1 substrates and that they are activated to more antiproliferative conversion products in MDA-MB-468 cells, through CYP1-mediated metabolism. Together with our previous findings the first preliminary evidence for a tumor-suppressing role of CYP1 enzymes is presented.

2. Materials and methods

2.1. Materials and reagents

Scutellarein and sinensetin were purchased from Indofine (Somerville, NJ, USA), whereas apigenin, genkwanin, baicalein and cirsiliol were from Lancaster (Heysham, Lancashire, UK). Acacetin and chrysin were obtained from Sigma (Poole, Dorset, UK). Microsomes containing recombinant human CYP1A1, CYP1B1 or CYP1A2 enzymes were purchased from BD Biosciences (Cowley, Oxford, UK). HPLC solvents were from Fisher (Loughborough, UK) and reagents for cell culture including MTT were from Sigma (Poole, Dorset, UK). Pyridine hydrobromide and the reagents and solvents for synthesis of 6-hydroxyluteolin (6OHL) were from Sigma (Poole, Dorset, UK).

2.2. Synthesis of 6-hydroxyluteolin (5,6,7,3′,4′-pentahydroxyflavone)

A mixture of cirsiliol (0.16 g, 0.48 mol) and pyridine hydrobromide (0.78 g, 4.8 mol) was heated to 200 °C for 4 h. The resulting yellow paste was allowed to cool to room temperature and the reaction was quenched with water (20 ml), and extracted with ethylacetate (2× 50 ml). The pooled organic extract was washed with water (2× 20 ml) and brine (2× 20 ml). The extract was then dried over anhydrous magnesium sulfate and concentrated *invacuo*. The crude product was recrystallised from the pentanol–hexane mixture to afford a yellow solid (0.035 g, 24%). ^{1}H NMR (DMSO) 6.55 (s, 1, H-3), 6.65 (s, 1, H-8), 6.9 (d, 1, H-6), 7.4 (s, 2, H-2, H-5), 8.75 (bs, 1), 9.40 (bs, 1), 9.88 (bs, 1), 10.40 (bs, 1). ^{13}C NMR (DMSO) 182.06, 163.80, 153.40, 149.79, 149.62, 147.25, 145.84, 129.25, 121.97, 118.95, 116.15, 113.43, 104.14, 102.44, 93.86, 32.34, 14.13. Mass spectrum *m/e* (M+1) 303; (M+Na) 325. R_f 0.18 (CH_2Cl_2/MeOH/AcOH; 8.5:1.5:0.1). The ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz super-conducting Bruker Spectrometer (Karlsruhe, Germany) at 30 °C. Infrared spectra were recorded in potassium bromide disks on a Perkin-Elmer 298 FTIR spectrophotometer (Massachusetts, USA). Melting points were determined on a Gallenkamp melting point apparatus (Leicestershire, UK). Thin layer chromatography (TLC) was performed on aluminium sheets pre-coated with silica gel 60f_{254} (Merck, Darmstadt, Germany) observed under UV light (450 nm). 6-Hydroxyluteolin: IR spectrum (max (KBr)/cm^{-1} 1655 (C=O).

2.3. Cell culture

MDA-MB-468 cells were maintained in RPMI 1640 without phenol red with 2 mM glutamine and 10% (v/v) heat-inactivated foetal calf serum. MCF-10A cells were maintained in DMEM/F12 Ham (1:1) containing glutamine (2 mM), 10% (v/v) heat-inactivated foetal calf serum as above, EGF (20 ng/ml), insulin (10 μg/ml) and hydrocortisone (500 ng/ml). Medium supplements for MCF-10A were adapted from Soule et al. (1990). The cells were grown at 37 °C, 5% CO_2/95% air and passaged using trypsin EDTA (0.25% v/v), every 2–3 days. MCF-10A is a non-tumorigenic epithelial cell line, which shows no sign of terminal differentiation or senescence (Soule et al., 1990). The doubling level of the cells was estimated to 18 h with a calibration curve.

2.4. RT-PCR

MDA-MB-468 and MCF-10A cells, seeded at a density of 0.4×10^5 cells/ml, were left to grow for 48 h. The cells were washed twice with PBS. Total RNA was extracted using guanidinium–acid–phenol, as shown by Chomczynski and Sacchi (1987). Total RNA was reverse-transcribed using an RT kit (Sigma, Poole, UK). A master mix containing RNA template of interest (0.005–0.25 μg/μl) with deoxynucleotides (500 μM of each dNTP) and anchored oligo(dT)$_{23}$ was heated at 70 °C for 10 min. Following incubation, samples were placed on ice with RNAse inhibitor and (1 U/μl) reverse transcriptase buffer 1×. Enhanced avian reverse transcriptase (1 U/μl) was added to start the reaction at 43 °C for 50 min. The first strand cDNA was amplified using primer sequences, reagent concentrations and cycling parameters, which were adapted from Dohr et al. (1995). PCR reactions were carried out containing cDNA (0.08 μg/μl), of 1× *Taq* buffer and 200 μM of each dNTP in the presence of 0.2 μM of each primer and 2.5 units *Taq* DNA polymerase. Amplifications were performed using a PTC-200 Peltier DNA thermal cycler (BioRad, Hertfordshire, UK). Following PCR, of each sample was electrophorized and the DNA visualized using a BioRad Molecular Imager FX (BioRad, Hertfordshire, UK).

2.5. EROD assay

7-Ethoxyresorufin-*O*-deethylase activity was measured in MDA-MB-468 and MCF-10A cells as described previously (Androutsopoulos et al., 2008). Background fluorescence produced by 7-ethoxyresorufin (ER) in cell-free wells, was subtracted from the fluorescence obtained in the medium samples. Fluorescence units were converted into concentration of resorufin formation with the aid of a resorufin standard curve. Typically 0.1–0.2 μM of resorufin was formed following incubation of MDA-MB-468 cells with ER for 60 min. This concentration value was divided by 60 min to give amount of product formed per time. The resulting value was further divided by the concentration of cells present in each sample (usually 0.8×10^6 cells/ml).

2.6. MTT assay

1×10^4 cells/ml were seeded in 96-well plates and the antiproliferative effect of the flavonoids on the two cell lines MDA-MB-468 and MCF-10A was assayed as described previously (Androutsopoulos et al., 2008). Inhibition experiments were performed only for genkwanin and sinensetin in the presence of 1.5 μM acacetin.

2.7. Flavonoid metabolism

Incubations (100 μl) contained CYP1 microsomes (20 pmol/ml of human cytochrome P450 expressed in insect cells), NADPH (0.5 mM), $MgCl_2$ (0.5 mM), phosphate buffer (20 mM), and flavonoid (genkwanin, sinensetin, scutellarein, chrysin or baicalein at 10 μM). Phosphate buffer was made by addition of equal volumes of disodium hydrogen orthophosphate (Na_2HPO_4) and potassium dihydrogen orthophosphate (KH_2PO_4) and the final pH was adjusted to 7.4. Following incubation at 37 °C, samples were taken at 5 min intervals until 20 min. Metabolism in MDA-MB-468 and MCF-10A cells was performed for genkwanin by incubation of the compound with the cells for 45 min in the presence of 1.5 mM salicylamide as described previously (Androutsopoulos et al., 2008). Each reaction was terminated by the addition of 100 μl of methanol:acetic acid mixture at a ratio of 100:1 respectively. Incubates were centrifuged at 3500 × *g* for 20 min at 4 °C and supernatants were analyzed by reverse phase HPLC.

2.8. HPLC analysis

A Luna C18 4.6 mm × 150 mm × 5 μm column was used with a mobile phase containing ratios of solvents A and B. Solvent A contained water, 1% acetonitrile and 0.5% acetic acid and solvent B contained methanol, 4% acetonitrile and 0.5% acetic acid. The following gradient program was used: 60% solvent A and 40% solvent B at time 0 and 10% solvent A and 90% solvent B at 10 min. Final conditions were maintained for 1 min prior to returning to the initial solvent conditions with 8 min remaining for column equilibration after each run. Detection of chrysin–baicalein, genkwanin–apigenin, sinensetin and scutellarein–6-hydroxyluteolin was monitored by a Waters Series 200 UV detector (Waters, Hertfordshire, UK) at 325, 350, 345 and 360 nm respectively. The concentration of the parent compounds was estimated by a calibration curve covering the concentration range of 0.1–10 μM .The assay was carried out at 37 °C and the flow rate was 1 ml min^{-1}.

2.9. Kinetics of apigenin and baicalein formation

Human CYP1A1, CYP1B1 and CYP1A2 (10 pmol/ml) were incubated with genkwanin or chrysin for 10 min at various concentrations (0.025, 0.25, 0.5, 1, 2, 4 and 10 μM). The formation of apigenin or baicalein was detected by HPLC as described above and the concentration estimated with a calibration curve. Apparent Km and Vmax values for the formation of apigenin were calculated using GraphPad Prism software (version 4.03) non-linear regression analysis (San Diego, USA).

2.10. Statistical considerations

Results are expressed as means ± standard deviation (SD) for $n = 3$ or $n = 4$ independent determinations, unless indicated otherwise. Paired *t*-test, unpaired *t*-test and ANOVA were used to determine statistical differences.

3. Results

3.1. CYP1 enzymes are differentially expressed in MDA-MB-468 as opposed to MCF-10A cells

We have previously used the breast adenocarcinoma cell line MDA-MB-468 as a model to test bioactivation of the natural product eupatorin by CYP1 enzymes and the normal breast cell line MCF-10A for a comparison (Androutsopoulos et al., 2008). In the present study we confirmed a differential overexpression of the two enzymes between the breast tumor and non-tumor cell lines, by RT-PCR and EROD activity assays. MDA-MB-468 cells expressed CYP1B1 and CYP1A1 at the mRNA and active protein levels, whereas negligible expression was noted in MCF-10A cells (Fig. 2).

3.2. CYP1 metabolism of dietary flavonoids increases their antiproliferative activity in breast cancer cells

Given that MDA-MB-468 cells express active CYP1 enzymes, the antiproliferative activity of putative CYP1 substrates with a flavone core structure, was investigated in this cell line. Genkwanin had an IC_{50} of 1.6 μM in MDA-MB-468 cells, whereas it appeared to

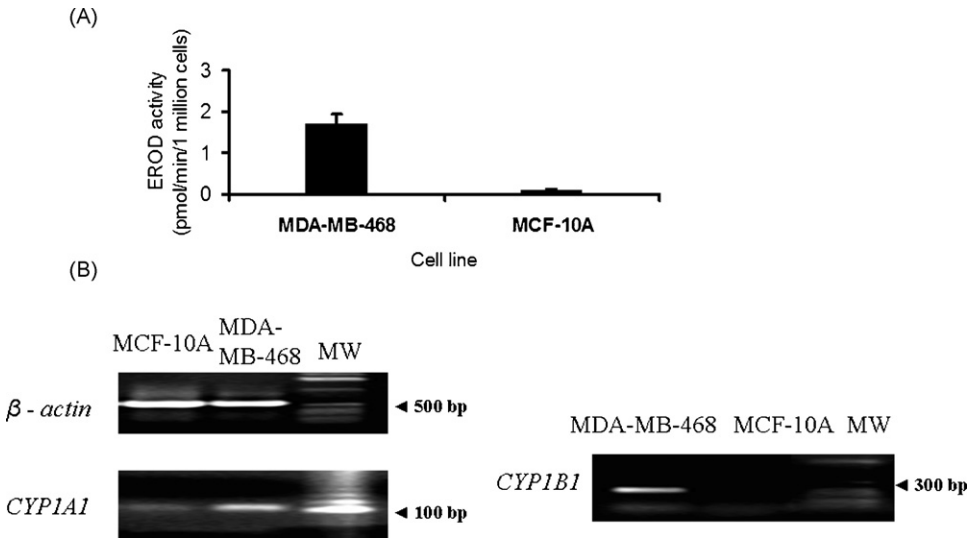

Fig. 2. Differential overexpression of CYP1A1 and CYP1B1 enzymes in MDA-MB-468 and MCF-10A cells. Total RNA was extracted using guanidinium–acid–phenol and RT-PCR was performed as described in Section 2. Activity assays were performed by addition of 7-ethoxyresorufin in cells and the fluorescence was measured at an emission λ of 530 nm and excitation λ of 590 nm. Error bars represent mean ± SD for $n = 3$ determinations.

be non-toxic in MCF-10A cells (IC_{50} = 75 μM) (Table 1). Sinensetin was somewhat more potent than genkwanin with overall IC_{50s} of 0.2 and 65 μM in MDA-MB-468 and MCF-10A cells respectively (Table 1). Co-treatment of either of these two flavonoids and the CYP1 family enzyme inhibitor acacetin reversed the IC_{50} observed in MDA-MB-468 cells to 13 and 14 μM respectively (Table 1). The antiproliferative effect of scutellarein, chrysin and baicalein in the two cell lines was also investigated. Scutellarein was more active in inhibiting proliferation of MDA-MB-468 (IC_{50} = 13 μM) than MCF-10A cells (IC_{50} = 42 μM) (Table 1). However, a shorter selectivity window was noted compared to genkwanin and sinensetin. Baicalein was more potent than chrysin and scutellarein in both cell lines and the IC_{50} noted in the tumorigenic cell line was statistically different than the one in the normal cell line (Table 1). No significant difference was observed in the IC_{50s} of chrysin between the breast tumor and non-tumor cell lines (Table 1).

3.3. CYP1 enzymes metabolize dietary flavonoids in vitro

Having shown a proof of principle concept of dietary flavonoid bioactivation in a cell-based model, their metabolism was further investigated in microsomes expressing recombinant CYP1A1, CYP1B1 or CYP1A2 enzymes. The concentration of genkwanin and sinensetin decreased considerably following incubation with CYP1 enzymes over a 20-min period, whereas a smaller decrease was noticed in the case of scutellarein (Table 2). The metabolism of chrysin by CYP1 enzymes was of intermediate efficacy compared to genkwanin and scutellarein (Table 2). CYP1A1 and CYP1A2 were generally faster metabolizers of flavonoids compared to CYP1B1, which appeared to metabolize the latter compounds weakly. Sinensetin rate of metabolism by CYP1B1 was not significantly different than the control incubations (Table 2). The flavonoid baicalein, was not metabolized by CYP1 family enzymes (data not shown).

3.4. CYP1 enzymes catalyze hydroxylations of flavonoids on aromatic rings with neighboring hydroxyl groups, or demethylation on pre-existing methoxy substituents

Following our HPLC analysis of flavonoid-CYP1-mediated metabolism we set out to identify possible conversion products on the basis of their structural similarity or identity with authentic standard solutions. Genkwanin and sinensetin produced various unidentified metabolites, while scutellarein and chrysin produced only one. The main metabolic product of genkwanin CYP1B1-catalyzed metabolism, assigned G_2, was apigenin (4′,5,7-trihydroxyflavone) (Fig. 3B). Additions of an apigenin standard

Table 1
Antiproliferative activity of flavonoids on MDA-MB-468 and MCF-10A cells.

Cell line compound	MDA-MB-468	MCF-10A	MDA-MB-468 + acacetin
Genkwanin	1.6 ± 0.5	75 ± 13	12.5 ± 5.6
Sinensetin	0.2 ± 0.02	65 ± 5	13.5 ± 3
Scutellarein	12.8 ± 1.5	41.8 ± 2.9	
Chrysin	20.5 ± 1*	22 ± 2.2*	
Baicalein	6.3 ± 0.5	10.8 ± 1.5	

Compounds were incubated with MDA-MB-468 and MCF-10A cells for 96 h and cell viability was measured with the MTT assay as described in Section 2. Acacetin was co-incubated in MDA-MB-468 cells with genkwanin or sinensetin at 1.5 μM and cell viability was measured as described previously. Values indicate IC_{50s} in μM. Error bars represent mean ± SD for $n = 4$ determinations. Statistical differences were determined by paired t test in the case of genkwanin and sinensetin in MDA-MB-468 cells in the presence and absence of acacetin and by unpaired t test for all five compounds, in order to compare differences between MDA-MB-468 and MCF-10A cells.

* Not significantly different from control with $p < 0.05$.

Table 2
Cytochrome P450 CYP1-mediated metabolism of dietary flavonoids.

	Control (%)	CYP1B1 (%)	CYP1A1 (%)	CYP1A2 (%)
Scutellarein	95.0 ± 0.9	91.1 ± 0.6	86.2 ± 1.1	86.0 ± 1.0
Sinensetin	92.2 ± 1.8	87.9 ± 2.1*	28.7 ± 5.9	54.4 ± 1.3
Genkwanin	98.4 ± 2.1	78.2 ± 0.6	65.0 ± 0.8	67.8 ± 1.4
Chrysin	98.6 ± 2.4	92.4 ± 1.0	82.9 ± 2.0	92.6 ± 0.9

Recombinant CYP1A1, CYP1B1 and CYP1A2 were incubated with flavonoids and NADPH (0.5 mM) at 37 °C for 20 min and samples were taken and further analyzed by HPLC. The concentration of the parent compound was estimated by the aid of a calibration curve covering the points 0.1, 0.5, 0.8, 1, 2, 4, 6, 8 and 10 μM. UV detection was monitored at 345, 330, 325 and 360 nm for genkwanin, sinensetin, chrysin and scutellarein. Percentages represent recovery of flavonoids after 20 min of incubation with insect cell microsomes containing human recombinant cytochromes P450. Control is incubation with insect cell microsomes free of human cytochromes. Error bars represent mean ± SD for $n = 3$ determinations. Statistical differences were determined by ANOVA.

* Not significantly different from control with $p < 0.05$.

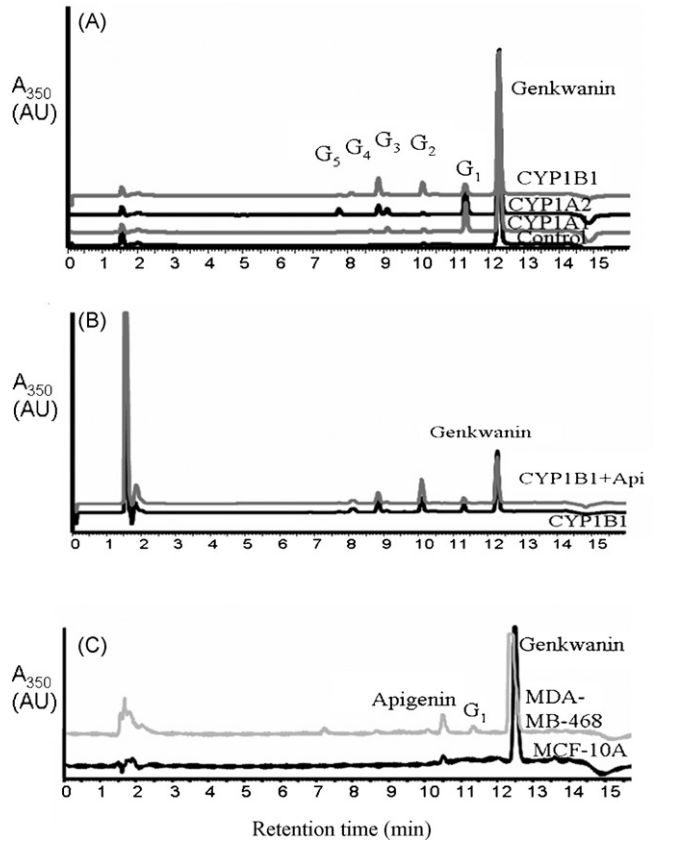

Fig. 3. The metabolism of genkwanin by CYP1 enzymes. Genkwanin (10 μM) was incubated with control microsomes and microsomes expressing recombinant human CYP1 enzymes and with the cell lines MDA-MB-468 and MCF-10A. (A) Metabolites formed after a 20-min incubation of genkwanin with CYP1 enzymes. Metabolites are assigned G_1, G_2, G_3, G_4 and G_5. (B) Identification of apigenin as the main conversion product of genkwanin metabolism by CYP1B1. A CYP1B1 incubate was spiked with 1 μM of apigenin and an increase in the total concentration of the metabolite was observed. (C) Metabolic products of genkwanin incubation with MDA-MB-468 and MCF-10A cells. Genkwanin and metabolites were extracted from medium samples by the addition of 100:1 methanol:acetic acid mixture.

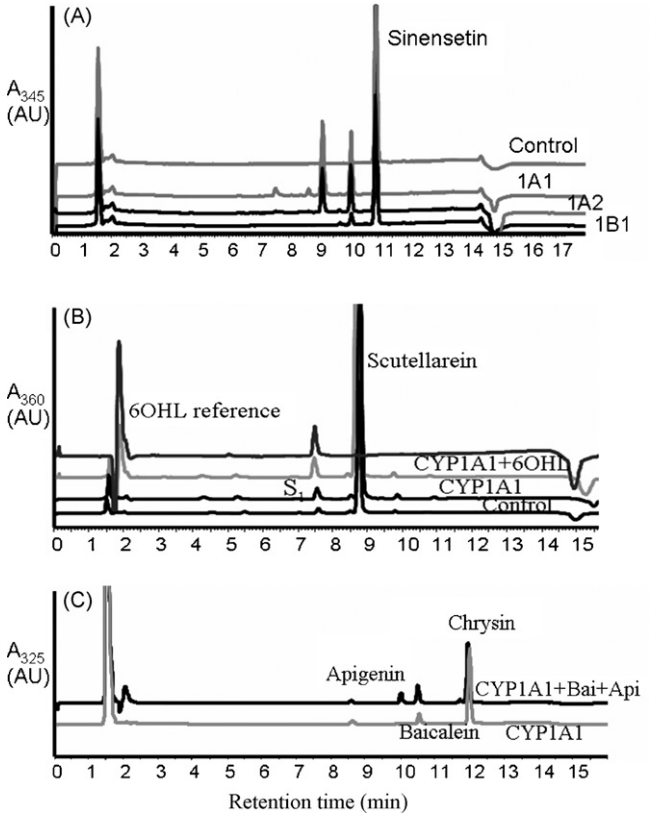

Fig. 4. The metabolism of sinensetin, scutellarein and chrysin by CYP1 enzymes. (A) Metabolites formed after a 20-min incubation of sinensetin with CYP1 enzymes. (B) Identification of the product S_1 of scutellarein metabolism by CYP1A1 by co-elution with a 6-hydroxyluteolin standard. A CYP1A1 incubate was spiked with 1 μM of 6OHL and an increase in the total concentration of the metabolite was observed. The same co-elution profile was observed for the other two members of the CYP1 family. (C) Identification of baicalein as the conversion product of chrysin metabolism by CYP1A1. Both apigenin and baicalein standards were added to a CYP1A1 incubate and an increase in the total concentration of baicalein was noted. The same co-elution pattern was observed for CYP1B1 and CYP1A2 (data not shown). Recovery for sinensetin, scutellarein-6OHL and chrysin-baicalein was 95%, 87% and 89% respectively.

to a CYP1A1 or CYP1B1 incubate resulted in an increase of the total concentration of the metabolite G_2. Apigenin was also present in MDA-MB-468 cell cultures, as a result of CYP1-catalyzed metabolism of genkwanin, whereas negligible conversion of genkwanin to apigenin was noted in MCF-10A cells (Fig. 3C). Apigenin was further tested for its ability to inhibit proliferation of MDA-MB-468 and MCF-10A cells and found 4-fold selective for the tumor cell line (data not shown). The metabolic products of sinensetin metabolism could not be identified, because no authentic standards corresponding to their structure were available (Fig. 4A). The conversion product S_1, resulting from the metabolism of scutellarein by CYP1B1 or CYP1A1, was identified as 6-hydroxyluteolin (6OHL) or 3′,4′,5,6,7-pentahydroxy flavone by co-elution (Fig. 4B). 6OHL was synthesized from commercially available 6,7-dimethoxy 3′,4′,5-trihydroxyflavone (Cirsiliol) (Fig. 1B). The compound was added to a CYP1 incubate as described above and an increase in the total concentration of S_1 was observed. Similarly, identification of CYP1-mediated conversion product of chrysin was performed by co-elution with an authentic standard (Fig. 4C). Both apigenin and baicalein were putative metabolites, and thus were added along with a CYP1 incubate. The analysis showed that baicalein and not apigenin was the only metabolite arising from CYP1 oxidation of chrysin (Fig. 4C).

3.5. CYP1A1 and CYP1B1 enzymes favor bioconversion of flavonoids to specific products

We studied the kinetics of CYP1-catalyzed formation of the identified conversion products, apigenin and baicalein. Our analysis showed that the two reactions follow the Michaelis–Menten equation. The CYP1 enzyme catalytic efficiencies were compared in terms of Km_{app}, $Vmax_{app}$ and intrinsic clearance ($Vmax_{app}/Km_{app}$). CYP1B1 had higher substrate turnover than either CYP1A1 or CYP1A2 and was more efficient in producing apigenin at lower concentrations of genkwanin (Fig. 5A). The opposite was observed for the formation of baicalein from chrysin (Fig. 5B). Thus CYP1B1 and CYP1A1 are shown to be better catalysts of apigenin and baicalein formation than the other members of the CYP1 family.

4. Discussion

The present study describes the metabolism of dietary flavonoids by the extrahepatic enzymes CYP1A1 and CYP1B1 and the third CYP1 isoform, CYP1A2, which is found in the liver, as well as their antiproliferative activity in breast cancer and normal breast cells. With the exception of baicalein, the flavonoids mentioned herein were substrates for CYP1 enzymes. Furthermore, chrysin and scutellarein were metabolized only to baicalein and

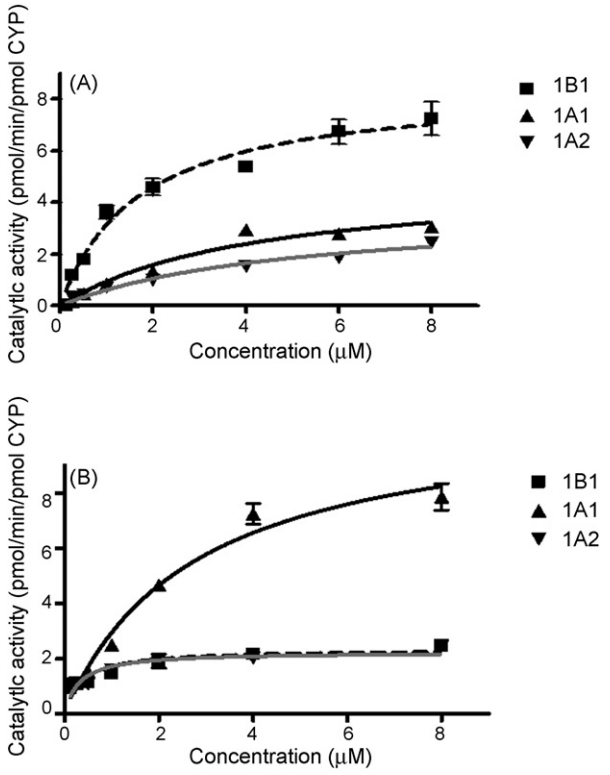

Fig. 5. Michaelis–Menten kinetics of the conversion of genkwanin to apigenin (A) and chrysin to baicalein (B) from CYP1 enzymes. Recombinant CYP1A1, CYP1B1 and CYP1A2 were incubated with either genkwanin or chrysin at a concentration of 10 pmol/ml and NADPH (0.5 mM) at 37 °C for 10 min at the concentration points 0.025, 0.25, 0.5, 1, 2, 4 and 10 μM. Samples were analyzed by HPLC and the formation of flavonoid metabolite detected as described in detail in Section 2. Recovery for genkwanin–apigenin was 90%. $Vmax_{app}$ and Km_{app} kinetic parameters were estimated by GraphPad Prism. Error bars represent the mean ± SD for $n = 3$ determinations. Intrinsic clearance values for the formation of apigenin from genkwanin for CYP1B1, CYP1A1 and CYP1A2 were 5 ± 0.3, 1 ± 0.2 and 0.7 ± 0.1 × 10^{-3} ml min^{-1} pmol of $enzyme^{-1}$, respectively, whereas for the formation of baicalein from chrysin the clearance for CYP1B1, CYP1A1 and CYP1A2 was 6.5 ± 1.4, 7.6 ± 1.8 and 4 ± 0.1 × 10^{-3} ml min^{-1} pmol of $enzyme^{-1}$, respectively.

6OHL, respectively, whereas genkwanin and sinensetin yielded a greater number of metabolites, with apigenin being one of the main conversion products in the case of genkwanin. All five compounds inhibited the proliferation of MDA-MB-468 and MCF-10A cells. Genkwanin and sinensetin were more sensitive in MDA-MB-468 breast cancer as opposed to MCF-10A normal breast cells, as a result of CYP1-enzyme mediated metabolism. The IC_{50} noted for the two compounds in MDA-MB-468 cells was increased with the addition of the CYP1 inhibitor acacetin, indicating that the antiproliferative effect was due to bioconversion of the flavones by endogenous CYP1 expression. In contrast, the flavonoids chrysin and baicalein showed similar IC_{50} in the two cell lines, whereas scutellarein was nearly 4 times more active in the cancerous cell line, which suggests that interaction with CYP1 enzymes did not affect considerably their antiproliferative activity.

In an effort to explain the tumor selectivity of genkwanin and sinensetin the possibility that the latter compounds have higher intracellular incorporation in breast cancer cells must be considered. Consequently the intracellular concentration of genkwanin was examined following treatment of the compound in MDA-MB-468 and MCF-10A cells. We found no difference in the concentration of genkwanin between the two cell lines (data not shown). Based on our previous observations and the fact the CYP1 family inhibitor reverses the IC_{50} of genkwanin and sinensetin from 1.6 and 0.2 to 13 and 14 μM respectively we conclude that intracellular incorporation of sinensetin and genkwanin is not affecting cell viability of tumor cells to a major extent as opposed to CYP1-mediated metabolism. The CYP1 family inhibitor acacetin cannot protect completely the cell viability of the cancerous cells for two reasons. First some metabolism escapes the action of the inhibitor and second the conversion products generated from CYP1 metabolism are compounds which affect cell signaling and thus are more sensitive in rapidly proliferating cells. The same effect has been observed for the flavone eupatorin (Androutsopoulos et al., 2008), which has a similar structure to sinensetin. Addition of acacetin cannot entirely return the cell cycle profile to the control (0.1% DMSO) population for these two reasons i.e. the IC_{50} noted when acacetin is added in MDA-MB-468 cells is always slightly smaller than the one seen in MCF-10A cells. In addition, most flavonoids are known to easily penetrate in vitro cultured cells. Bioavailability of flavonoids in vivo is usually affected by phase II detoxification in the small intestine and in the liver; i.e. some flavonoids that would be bioactive simply never reach the target cells (Arroo et al., 2009; Walle, 2007).

The investigation of the anticancer activity of the flavonoids examined in this study has been inconclusive by previous reports. Genkwanin has mainly been investigated in terms of antioxidant and antinflammatory activity even though a few studies have documented cytotoxicity of this compound against a panel of human cancer cell lines and induction of HL-60 leukemia cell differentiation (del Baño et al., 2003; Jones et al., 2007; Suh et al., 1995). Scutellarein also exhibits antioxidative activity and it inhibits hypoxia and high glucose-induced proliferation and VEGF expression of human retinal endothelial cells (Ferreres et al., 2006; Gao et al., 2008). Chrysin, baicalein and scutellarein can inhibit the proliferation of malignant glioma and breast carcinoma cells without affecting primary or non-malignant cells (Parajuli et al., 2009). In the present study, it is shown that aromatic hydroxylation reactions of dietary flavonoids, catalyzed by CYP1 enzymes, promoted further their antiproliferative activity towards breast cancer cells. It seemed that the more active compounds were those that produced a larger number of CYP1-metabolites, as scutellarein with only one metabolite was not as potent as the methoxylated flavonoids genkwanin and sinensetin. Surprisingly, the conversion products of CYP1-catalyzed metabolism of the flavonoids examined are also compounds with well documented anticancer activity, such as induction of apoptosis, G_2/M arrest, inhibition of topoisomerase I-catalyzed DNA religation, phosphorylation of p38 and downregulation of cyclin B1 and phosphorylated CDC2 (Zhang et al., 2008; Landis-Piwowar et al., 2008; Lee et al., 2008; Boege et al., 1996; Lepley and Pelling, 1997; Chao et al., 2007). Since apigenin was of intermediate efficacy in inhibiting proliferation of MDA-MB-468 cells (4-fold selectivity) the tumor specific action of genkwanin is attributed to all of the metabolites produced (notably G1).

All three CYP1 enzymes were found not to promote hydroxylation of either chrysin or baicalein on the B ring. In contrast hydroxylation was only possible at the 6 position of the A ring of chrysin, where neighboring hydroxyl groups at positions 5 and 7 were present. Otake investigated in a short report the 4′-demethylation and 4′-hydroxylation of the flavonoids kaempferide, galangin (flavonol equivalent of chrysin) and chrysin by CYP1A1 and CYP1A2 (Otake and Walle, 2002). In concordance with the results presented here neither CYP1A1 nor CYP1A2 promoted oxidation of chrysin to apigenin, whereas demethylation of kaempferide and oxidation of galangin to kaempferol was possible (Otake and Walle, 2002). Thus, it can be assumed that hydroxylation of flavones at the B ring requires pre-existing substitution as vacant 3′ and 4′ positions were not hydroxylated. As expected, scutellarein which contains a hydroxyl group at position 4′ of the B ring was further hydroxylated to the 3′ position to give 6-hydroxyluteolin. No other hydroxylation was possible on the A ring, as the 6 position is already occupied. A similar finding was observed for the

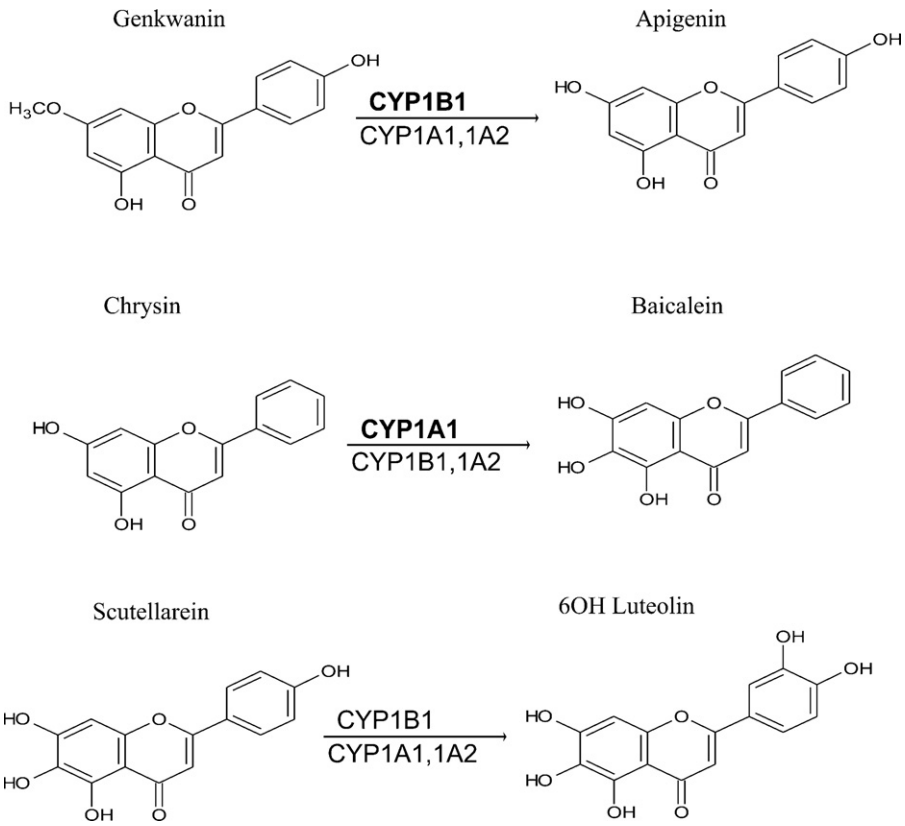

Fig. 6. Schematic conversions of the CYP1-catalyzed oxidations of the flavonoids genkwanin, chrysin and scutellarein. Bold letters indicate parent compounds and metabolites, as well as the predominant metabolic catalyst of each pathway.

conversion of luteolin from apigenin by recombinant CYP1A2 (Breinholt et al., 2002). Genkwanin, containing a methoxy group at position 7 was demethylated to apigenin. In the chromatographic analysis a number of unidentified metabolites, apart from apigenin, were found. These conversion products had shorter retention times than genkwanin as a result of further hydroxylation reactions on the B or the A ring, but their identity remains unknown since the authentic standards corresponding to their structure were not available. The structures of the flavonoids and their CYP1 conversion products are shown in Fig. 6.

Our results demonstrate that flavones containing methoxy groups, such as genkwanin and sinensetin were metabolized faster and to a larger number of conversion products than their hydroxylated analogues. Methoxylated flavonoids are an emerging class of dietary anticancer compounds, due to particularly desirable pharmacokinetic properties (Walle, 2007). Flavonoids with multiple hydroxyl groups, such as quercetin have been found to be eliminated rapidly in human cell lines, mainly due to oxidative degradation (Boulton et al., 1999). Analogues of chrysin such as 5,7-dimethoxy flavone exhibit higher stability in liver S9 microsomal fractions and pooled human liver microsomes (Walle and Walle, 2007). The results of a recent study showed that amongst the hepatic enzymes, CYP2C9, CYP2D6, CYP1A2 and CYP3A4, which largely contribute to phase I oxidation reactions occurring in the liver, CYP1A1 was found to have the highest rate of metabolism of fully methylated flavonoids, which is in agreement with our findings (Walle and Walle, 2007).

To our knowledge, this is one of the few studies in which the antiproliferative effect of the particular flavonoids in cancerous cells is examined, in terms of CYP1 enzyme oxidation. Our previous reports have underlined the importance of this process in increasing the antiproliferative activity of the natural flavones diosmetin and eupatorin in the tumor cell lines MCF-7 and MDA-MB-468 (Androutsopoulos et al., 2008, 2009a,b). A similar finding has been observed in the metabolism of the isoflavone daidzein by cultured MCF-7 cells, i.e. hydroxylation on the A ring enhances the pharmacological and cytotoxic properties of the parent compound (Atherton et al., 2006). Furthermore resveratrol, the stilbene analogue of apigenin, has been shown to undergo hydroxylation on the B ring by CYP1B1, which has shown a tumor-specific expression, to piceattanol, a tyrosine kinase inhibitor (Potter et al., 2002). Combined with our present findings these data reinforce the important role of CYP1A1 and CYP1B1 extrahepatic metabolism of dietary flavonoids in eliciting tumor-suppressing effects, in tissues expressing high levels of the latter enzymes. (Arroo et al., 2008, 2009).

The conversion of genkwanin to apigenin and of chrysin to baicalein is favoured by CYP1B1 and CYP1A1 respectively, as shown by the Michaelis–Menten kinetic analysis. CYP1B1 has been validated as a cancer therapeutic target and recently a CYP1B1 vaccine has successfully passed phase I clinical trials (Murray et al., 1997; Murray and McFadyen, 2005). A CYP1B1 selective substrate, provided from dietary sources offers great potential for the prevention or treatment of several cancer types which overexpress the enzyme. Genkwanin is shown by this preliminary data to possess a selectivity window for CYP1B1 catalyzed formation of apigenin, as opposed to the other two members of the CYP1 family. A significant drawback, however, is the non-specific activation by CYP1A2, since the latter is present predominantly in the liver, where extensive phase I metabolism of xenobiotics takes place. Similarly, chrysin conversion to baicalein is also catalyzed to a small extent by CYP1A2. Previous studies have demonstrated that although CYP-mediated metabolism of flavonoids is possible in human liver microsomes and recombinant CYPs, conjugation reactions are favored in vivo and in intact cells, such as human hepatocytes (Walle, 2004). This has been demonstrated for the flavonoid

galangin, where it was shown that the main metabolites in hepatocytes are two isomeric glucuronic acid conjugates and a sulphate conjugate (Otake et al., 2002). Based on these observations it can be assumed that flavonoids, such as chrysin and genkwanin will remain unaffected to some extent by hepatic CYP1A2 metabolism in vivo, thus raising the possibility of reaching extrahepatic sites, where CYP1B1 and CYP1A1 enzymes are expressed. In addition our findings show that at least a part of CYP1-catalyzed oxidation of genkwanin occurs in extrahepatic breast cancer cells, whereas negligible bioconversion is noted in normal breast cells. In this sense, genkwanin may be regarded as a candidate dietary anticancer compound, due to selective metabolism to apigenin by the tumor-specific enzyme CYP1B1.

Dietary flavonoids have been extensively studied in the last two decades as the core chemicals responsible for cancer prevention. Numerous studies have underpinned among other mechanisms of action, the inhibitory activity of flavonoids against the formation of carcinogenic intermediates and subsequently CYP1A1 and CYP1B1 enzymes. However it is only recently that substrate-like interactions with CYP1 enzymes have been explored. The structural similarities of these compounds with oestradiol, which is an endogenous CYP1A1 and CYP1B1 substrate, provide a novel mechanism of chemopreventative action based on the extrahepatic expression of CYP1 enzymes.

Conflict of interest

None

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