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CYTOCHROME P450 CYP1 METABOLISM OF HYDROXYLATED FLAVONES AND FLAVONOLS: SELECTIVE BIOACTIVATION OF LUTEOLIN IN BREAST CANCER CELLS

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Abstract

Natural flavonoids with methoxy substitutions are metabolized by CYP1 enzymes to yield the corresponding demethylated products. The present study aimed to characterize the metabolism and further antiproliferative activity of the hydroxylated flavonoids apigenin, luteolin, scutellarein, kaempferol and quercetin in CYP1 recombinant enzymes and in the CYP1 expressing cell lines MCF7 and MDA–MB–468, respectively. Apigenin was converted to luteolin and scutellarein, whereas kaempferol was metabolized only to quercetin by recombinant CYP1 enzymes. Luteolin metabolism yielded 6 hydroxyluteolin only by recombinant CYP1B1, whereas CYP1A1 and CYP1A2 were not capable of metabolizing this compound. Molecular modeling demonstrated that CYP1B1 favored the A ring orientation of apigenin and luteolin to the heme group compared with CYP1A1. The IC50 of the compounds luteolin, scutellarein and 6 hydroxyluteolin was significantly lower in MDA–MB–468, MCF7 and MCF10A cells compared with that of apigenin. Similarly, the IC50 of quercetin in MDA–MB–468 cells was significantly lower compared with that of kaempferol. The most potent compound was luteolin in MDA–MB–468 cells (IC50= 2±0.3 μM). In the presence of the CYP1-inhibitors α-napthoflavone and/or acacetin, luteolin activation was lessened. Taken collectively, the data demonstrate that the metabolism of hydroxylated flavonoids by cytochrome P450 CYP1 enzymes, notably CYP1A1 and CYP1B1, can enhance their antiproliferative activity in breast cancer cells. In addition, this antiproliferative activity is attributed to the combined action of the parent compound and the corresponding CYP1 metabolites.

Abbreviations

CYP1, cytochrome P450 CYP1B1, CYP1A1 and CYP1A2; HPLC, high pressure liquid chromatography; IC50, 50% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

Keywords

Flavonoids, apigenin, luteolin, kaempferol, quercetin, cytochrome P450 CYP1 enzymes, antiproliferation, breast cancer

1. Introduction

Breast cancer is one of the major causes of cancer–related deaths accounting for 540,000 deaths each year worldwide (Lopes et al., 2017). The treatment options for breast cancer include surgery, radiotherapy, hormone therapy and chemotherapy. A major obstacle that is encountered with conventional breast cancer therapy is the recurrence of the tumor progression. In addition, the use of certain chemotherapeutic drugs, notably the alkylating agent carboplatin, the antimetabolite 5–fluorouracil and the antimitotic agents paclitaxel and docetaxel, confers limited specificity for the tumor site and consequently severe side effects to the healthy tissue. Hence, in the past two decades considerable effort has been made to the research and development of new drugs with enhanced specificity and improved efficacy for the treatment of breast cancer. In addition, the use of complementary medical treatment namely, phytotherapeutic natural products and nutritional supplements, for women with a previous history of breast cancer has considerably increased (Lopes et al., 2017; Margină, et al., Fenga et al., 2016).

Flavonoids comprise a class of natural polyphenolic molecules that have demonstrated a major role in the prevention of cancer. These compounds are ubiquitous in plant–based food products, such as fruits, vegetables and tea extracts and in medicinal plants. Their anticancer activity has been attributed to the inhibition of DNA damage, the inhibition of cell cycle associated proteins, the induction of apoptosis, the modulation of cell signaling pathways involved in proliferation and in the inhibition of carcinogenic metabolite formation (George et al., 2017; Curti et al., 2017; Kerimi et al., 2017; Clementino et al., 2017). The hydroxylated flavonoids apigenin, luteolin, quercetin and kaempferol are the main constituents of various dietary products and beverages and have been the focus of extensive research over the

last years. Apigenin exerts anticancer effects through the modulation of various pathways namely, apoptosis, ROS and DNA damage and repair (Salmani et al., 2017). Luteolin is a flavone that differs from apigenin by a single hydroxyl group at the 3´ position of the B ring. This compound is found in various common dietary sources notably artichoke, chamomile and olive oil (Garcia–Gonzalez et al. 2010; Kato et al., 2008; Pandino et al., 2010). Luteolin has demonstrated anticancer activity in cancer cell lines and *in vivo* models (Seelinger et al., 2008). This type of activity has been attributed to activation of apoptosis, cell cycle arrest and inhibition of tumor invasion (Seelinger et al., 2008). Moreover, quercetin that contains an extra hydroxyl group at the 3 position of the C ring compared with luteolin, has been examined as a putative anticancer agent in ovarian and melanoma cancers via similar modes of action (Parvaresh et al., 2016; Harris et al., 2016)

The metabolism of xenobiotics is facilitated by specific phase–I and phase–II drug metabolizing enzymes (Tsatsakis et al., 2009; Tsatsakis et al., 2011). Previous studies conducted by our group have demonstrated that methoxylated flavonoids, such as diosmetin and eupatorin, are demethylated to their corresponding hydroxylated derivatives by recombinant cytochrome P450 CYP1A1, CYP1B1 and CYP1A2 enzymes and by MCF7 and MDA–MB–468 breast adenocarcinoma cellular extracts (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009a). The cytochrome P450 enzymes CYP1A1 and CYP1B1 have been implicated in carcinogenesis and their upregulation in cancer cells has been used for the development of novel chemotherapeutic drugs (Patterson and Murray 2002; Gribben et al., 2005). Extrahepatic CYPs, notably CYP1B1 and CYP1A1, may be utilized to target tumor cells by the use of selective antibodies, or the activation of prodrugs that are inactive in tissues or cells that do not express these enzymes (Gribben et al., 2005; Loaiza–

Pérez et al., 2004). Our previous reports suggest that several dietary flavonoids are substrates for CYP1 enzymes and may exhibit cancer–therapeutic applications, provided their conversion products inhibit tumor cell growth (Androutsopoulos et al., 2010).

In the present study, the CYP1–mediated metabolism of hydroxylated flavonoids namely, apigenin, luteolin, quercetin and kaempferol was investigated in recombinant CYP1 enzymes. In addition, their antiproliferative activity was assessed in the CYP1 expressing cell lines MCF7 and MDA–MB–468. The data demonstrate that bioactivation of hydroxylated dietary flavonoids by CYP1 enzymes is possible and that the number of hydroxyl groups affects the inhibition of cellular proliferation in breast cancer cells.

2. Materials and Methods

2.1 Chemicals and antibodies

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), α-napthoflavone, acacetin, apigenin, luteolin, kaempferol, quercetin, tissue culture reagents and media, Western blotting lysis buffer and DTT were purchased from Sigma Aldrich (St Louis, MO, USA). Scutellarein was purchased from Extrasynthese (Lyon, France) and 6-OH luteolin was synthesized as described in previous methodologies (Androutsopoulos et al., 2009c). Western blotting reagents were from Bio-rad (Berkeley, CA, USA). The polyclonal antibody for CYP1B1 was from Gentest (BD Biosciences, CA, USA), whereas the monoclonal for β-actin from Cell signaling (Leiden, Netherlands). Secondary antibodies for western blotting were from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2 Cell culture

MCF7 and MDA–MB–468 cells were maintained in RPMI with glutamine (2 mM) containing 10% heat-inactivated FBS and penicillin/streptomycin. MDA–MB–468 cells were grown in RPMI without phenol red, whereas the growth medium of MCF10A cells included DMEM:F12 with insulin (10 μg/ml), hydrocortisone (500 ng/ml) EGF (20 ng/ml), in addition to 10% FBS and 2 mM of glutamine. The cells were grown in a humidified incubator at 37 °C in 5% CO_2 /95% air and passaged using trypsin EDTA (0.25% v/v), every 3 to 4 days.

2.3 Flavonoid metabolism

Recombinant CYP1 enzymes and control microsomes that were purchased from Gentest (BD Biosciences, CA, USA) were incubated with 10 μM of flavonoids (apigenin, luteolin, kaempferol and quercetin). The reactions were carried out at 37 °C in a humidified incubator in the presence of NADPH (0.5 mM), $MgCl_2$ (0.5 mM) and phosphate buffer (20 mM). Time points were obtained at 0, 5, 10, 15, 20 and 25 min intervals. The reactions were terminated by addition of equal volumes of a solution containing methanol and acetic acid at a 100:1 ratio. The samples were centrifuged at 3,500 g for 20 min at 4 °C and the supernatants were analyzed by reversed phase HPLC. The co–elution studies included a 25 min sample incubate that was spiked with a low concentration of an authentic standard (0.2–1 μM) of the putative metabolite.

2.4 HPLC analysis

The methodology has been described in detail in previous publications (Androutsopoulos et al., 2009a, Androutsopoulos et al., 2008). A Luna C18 4.6 x 150

mm 5 µ column was used with a mobile phase that contained solvents A and B. Solvent A comprised 1% acetonitrile and 0.5% acetic acid in H_2O and solvent B 4% acetonitrile and 0.5% acetic acid in CH_3OH. The following gradient was used: 60% solvent A and 40% solvent B at time=0 min and 10% solvent A and 90% solvent B at time=10 min. The final conditions were maintained for 1 min and the composition of the solvents was adjusted to the initial conditions with 8 min remaining for column equilibration after each run. The detection of the flavonoid concentration was was monitored using a Waters Series 200 UV detector (Waters, Hertfordshire, UK). Luteolin, 6 OH luteolin, kaempferol and quercetin were detected at 360 nm, whereas apigenin and scutellarein were detected at 350 nm. The concentration of the flavonoids was estimated by a calibration curve covering the concentration range of 0.05–10 µM for each compound. The assay was carried out at 37 °C and the flow rate was 1 ml/min. The average recoveries for apigenin, luteolin, scutellarein, 6 OH luteolin, kaempferol and quercetin were estimated at 95, 91, 93, 87, 84 and 81%, respectively.

2.5 Kinetics of 6 OH luteolin formation

CYP1B1 recombinant microsomes (10 pmol/ml) were incubated with a concentration range of luteolin namely, 0.03-10 µM (10, 7, 5, 3, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03 µM) for 10 min. The optimum CYP1B1 concentration for linearity of product formation was estimated at 5 pmol/ml. The concentrations of 6 OH luteolin were estimated by a calibration curve covering the range 0.01 to 10 µM. Apparent Km and Vmax values were calculated using Graph Pad Prism (version 4.03). Intrinsic clearance (Cl) was calculated from the ratio Vmax/Km.

2.6 MTT assay

MCF7, MDA–MB–468 and MCF10A cells (1 x 10^4 cells/ml) were seeded in 96-well plates and the antiproliferative effect of the flavonoids was assayed as described previously (Androutsopoulos et al., 2008). Inhibition experiments were conducted in the presence of 0.5-1 μM α-napthoflavone and/or acacetin.

2.7 Western blotting

MCF7, MDA–MB–468 and MCF10A cells were cultured in T25 flasks at a density of 5×10^5 cells/ml. Following medium withdrawal and washing of the cells with PBS, the cells were lysed with 100 μl of lysis buffer that contained protease inhibitor cocktail and DL-dithiothreitol (DTT, 1 mM). The cells were sonicated on ice for 5 min and centrifuged at 13,000 rpm at 4 °C for 15 min. The protein concentration required for the experiment was adjusted to 0.7 mg/ml for each sample, and the protein extract was mixed with sample buffer that contained 5% mercaptoethanol at a 1:1 ratio. The samples were heated at 100 °C for 5 min and then loaded on an acrylamide gel containing 10% acrylamide for the resolving gel and 5% acrylamide for the stacking gel. Electrophoresis was carried out for 1 h at 120 V and the proteins were transferred by wet blotting to a PVDF membrane. The membrane was incubated in 10% milk/0.05% TBST at room temperature for 1 h by continuous shaking. The primary antibodies against CYP1B1 and β-actin were added to the membrane at 1:500 and 1: 3,000 dilutions, respectively at 4 °C overnight. The membrane was subsequently washed three times with 0.05% TBST and incubated with the secondary antibody against HRP diluted in 1% milk/0.05% TBST at room temperature for 1.5 h. The membrane was finally exposed to ECL reagents, and the expression profile of the proteins was developed on film.

2.8 Molecular modeling

For the docking procedure, the crystal structures of CYP1A1 (pdb code: 4I8V)(Walsh et al., 2013), CYP1B1 (pdb code: 3PM0)(Wang et al., 2011) and CYP1A2 (pdb code: 2HI4)(Sansen et al. 2007) were obtained from the RSCB protein data bank. The enzymes were energy minimized using the conjugate gradient algorithm in the CHARMm forcefield for 500 steps to remove any conformational strain. The AutoDockTools program (Morris et al., 2009) was used to prepare the corresponding PDBQT-protein file, which included partial charges and solvation parameters for all the atoms. For all three enzymes, the heme Fe (II) ion formal charge was set to +2. The compounds were flexibly docked into the binding sites of each CYP1 using AutoDock Vina (Trott et al., 2010). The proteins were held rigid during the docking process, while the ligands were allowed to be flexible. Docking simulations in both cases were performed using a grid box with dimensions of 23x23x23 Å, a search space of 10 binding modes and the exhaustiveness parameter of 10.

2.9 Statistical analysis

The data were presented as the mean of at least three independent measurements and were analyzed by the paired and unpaired t-tests using GraphPadPrism. Error bars represent mean ± STDEV for at least n=3 determinations.

3. Results

3.1 CYP1 enzymes metabolize hydroxylated flavonoids

Previous studies have demonstrated that CYP1 enzymes can demethylate mono–methoxylated and/or poly–methoxylated flavonoids to their corresponding hydroxylated derivatives (Androutsopoulos et al., 2008, Androutsopoulos et al.,

2009a, Androutsopoulos et al., 2009b, Androutsopoulos et al., 2009c). In the present study, the hypothesis that this metabolic pattern occurs in hydroxylated flavonoids was examined. CYP1 enzymes metabolized the hydroxylated flavonoids apigenin, and kaempferol and CYP1B1 metabolized the flavonoid luteolin over a 25 min incubation period compared with control microsomes containing empty vector, whereas quercetin metabolism by CYP1 enzymes did not yield any conversion products (Fig. 1, data not shown). The flavonoids apigenin and kaempferol that contained a single hydroxyl group on the B ring were more extensively metabolized by CYP1 enzymes compared with the flavonoid luteolin that contained 2 hydroxyl groups on the B ring (Fig. 1). CYP1A1 and CYP1A2 metabolized markedly apigenin and kaempferol compared with CYP1B1, whereas CYP1B1 was the sole catalyst of luteolin metabolism by CYP1 enzymes (Fig. 1). Notably, the concentration of apigenin was decreased from 8.8±0.3 μM to 5.8±0.2 μM following metabolism by CYP1A1, whereas CYP1B1 reduced the concentration of luteolin from 9.5±0.2 μM to 7.4±0.5 μM (Fig. 1) over a 25 min incubation period.

3.2 CYP1 enzymes catalyze hydroxylation reactions at the 3′ position of the B ring and the 6 position of the A ring of flavonoids

Co–elution experiments were carried out with authentic standards corresponding to the putative CYP1–hydroxylated metabolites. Apigenin metabolism by CYP1A1 yielded one major and one minor metabolite that were identified as luteolin and scutellarein (6 OH apigenin), respectively (Fig. 2A). The same metabolites were present by CYP1B1 and CYP1A2 metabolism of apigenin (Fig. 2A, data not shown). In contrast to CYP1A1, CYP1B1 yielded higher and lower amounts of scutellarein and luteolin, respectively (Fig. 2A). The metabolism of luteolin by CYP1A1 and

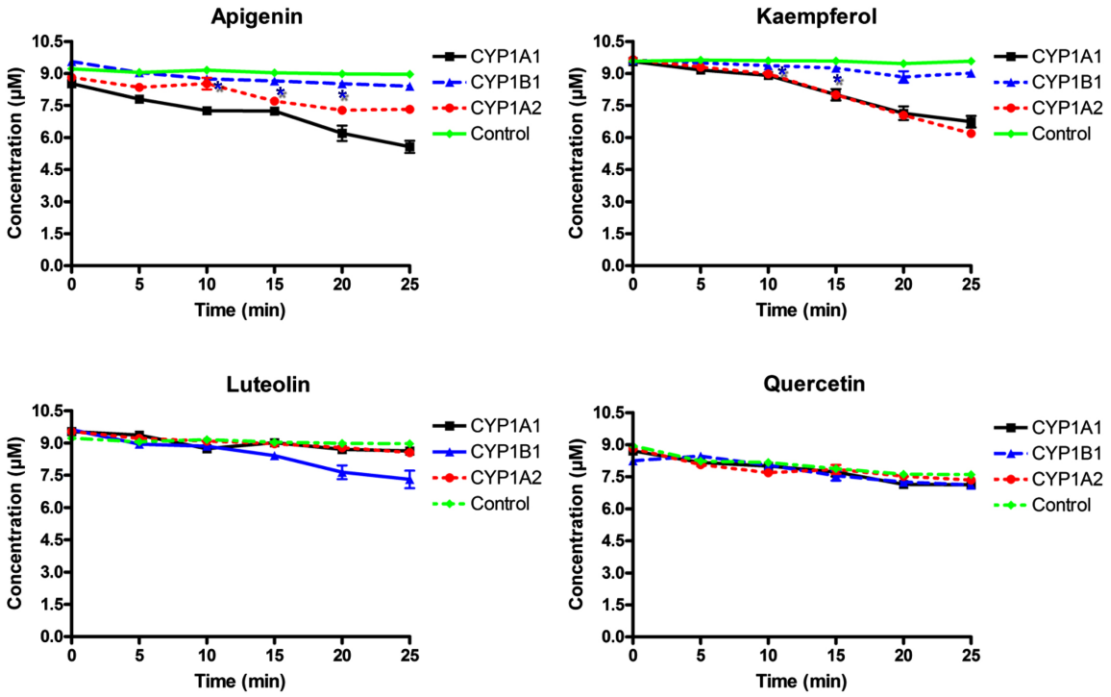

Figure 1. *Cytochrome P450 CYP1–mediated metabolism of hydroxylated flavonoids. Recombinant CYP1 enzymes were incubated with hydroxylated flavonoids (10 μM) and samples were collected at 0, 5, 10, 15, 20 and 25 min time intervals as described in Material and Methods. The experiments were conducted at least 3 times and error bars indicate STDEV of the mean. * indicate significant differences for the time points 10, 15 and 20 min of CYP1B1 samples compared to control samples (P<0.05).*

CYP1A2 did not yield any metabolites, whereas only one metabolite was present following CYP1B1 incubation with luteolin (Fig. 2B,C). The retention time of the authentic standard of the compound 5,7,3′,4′,5′ pentahydroxy flavone did not match the retention time of the CYP1B1 metabolite of luteolin, indicating that CYP1B1 does not hydroxylate luteolin at the 5′ position of the B ring (Fig. 2B,C). This metabolite was identified as 6–OH luteolin (Fig. 2B,C). The metabolism of kaempferol by CYP1 enzymes yielded one metabolite that was identified as quercetin (Fig. 2D). The extent of metabolism followed the order CYP1A1>CYP1A2>CYP1B1 (Fig. 2D). In addition, an analysis of the metabolic turnover of the conversion products of the

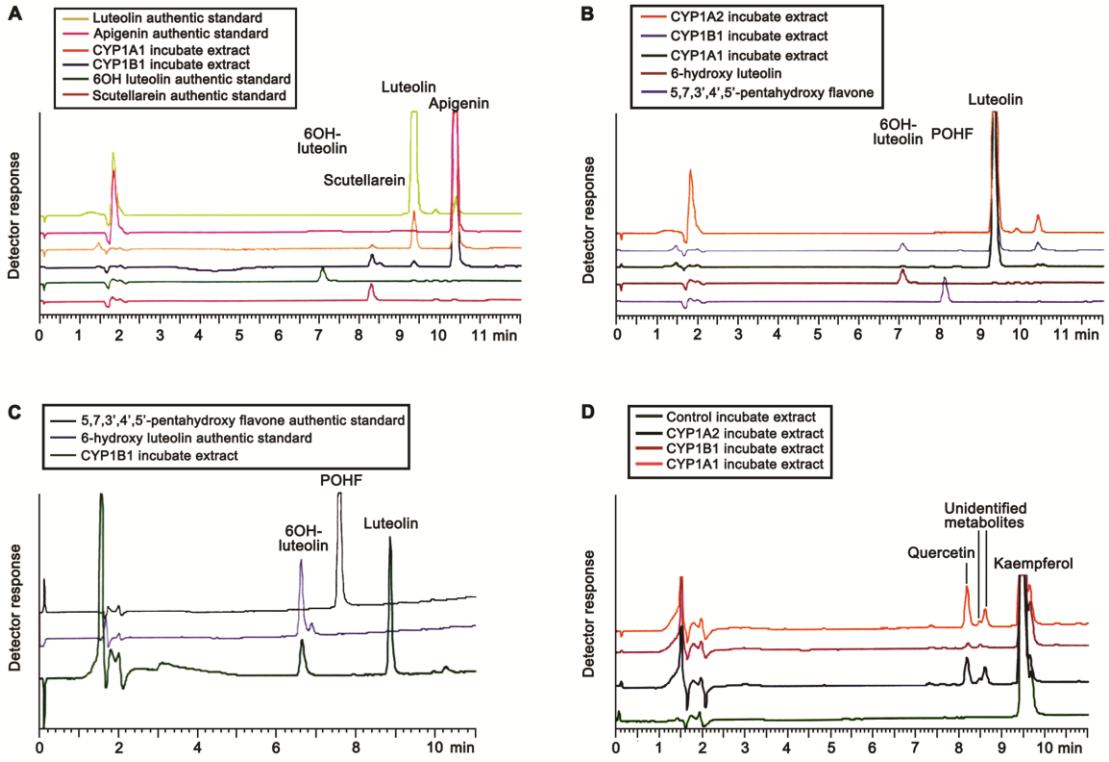

Figure 2. *HPLC analysis of hydroxylated flavonoids following incubation with CYP1 enzymes. (A) The metabolism of apigenin (10 μM) by CYP1 enzymes. Retention time of apigenin, luteolin, scutellarein and 6 OH luteolin authentic standards and of apigenin CYP1A1 and CYP1B1 incubates, as separated by the HPLC methodology used in the study. (B) The metabolism of luteolin (10 μM) by CYP1 enzymes. Retention time of luteolin, 6 OH luteolin and 5,7,3',4',5' pentahydroxy flavone authentic standards and of luteolin CYP1A1, CYP1B1 and CYP1A2 incubates, as separated by the HPLC methodology used in the study. (C) Identification of 6 OH luteolin as the CYP1B1 metabolite of luteolin. The metabolic profile of a CYP1B1 incubate of luteolin (2 μM) was compared with the authentic standards 6 OH luteolin and 5,7,3',4',5' pentahydroxy flavone in terms of retention time. (D) The metabolism of kaempferol (10 μM) by CYP1 enzymes. Retention time of kaempferol and quercetin as demonstrated by CYP1A1, CYP1B1 and CYP1A2 incubates, based on the HPLC methodology used in the study.*

CYP1–mediated metabolism of hydroxylated flavonoids was conducted. The highest amount of metabolite was formed by the CYP1A1–mediated conversion of apigenin to luteolin, followed by the formation of 6 OH luteolin by CYP1B1 and the formation of scutellarein from apigenin by CYP1B1 (Fig. 3A,B). The catalysis of kaempferol to

quercetin resulted in lesser amounts of product formation with regard to CYP1A1 and CYP1A2 compared with the corresponding CYP1–catalyzed metabolism of apigenin (Fig. 3B).

3.3 The cytotoxicity of the hydxroxylated metabolites of CYP1 enzymes is increased compared to their corresponding parent compounds in breast cancer cell lines

The CYP1 expressing cell lines MDA–MB–468 and MCF7 have been previously employed in order to assess the metabolism and further antiproliferative activity of dietary flavonoids (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009a). In the present study the expression of CYP1B1 was further confirmed in the aforementioned cell lines by Western immunoblotting. Higher levels of CYP1B1 expression were noted in MDA–MB–468 compared with MCF7 cells, whereas in MCF10A cell the expression of this enzyme was negligible (Fig. 4A). The expression of CYP1B1 in MCF7 cells was increased following induction of the cells for 24 h with 10 nM of the potent CYP1 inducer TCDD (tetrachloro-dibenzo-p-dioxin) (Fig. 4A).

The compounds apigenin, luteolin, scutellarein and 6OH luteolin exhibited higher antiproliferative activity in MDA–MB–468 compared with MCF10A cells in terms of IC50 values (Fig. 4B). Luteolin was the most potent compound in MDA–MB–468 cells (IC50 = 2±0.3 μM), followed by 6OH luteolin, scutellarein and apigenin (Fig. 4B). In MCF10A cells, 6OH luteolin was the most active compound with an IC50 of approximately 30 μM (Fig. 4B). In MCF7 cells, 6OH luteolin retained the maximun potency of growth inhibition followed by scutellarein, luteolin and apigenin (Fig. 4E). Following induction of the cells with TCDD for 24 h, the cytotoxicity of apigenin and

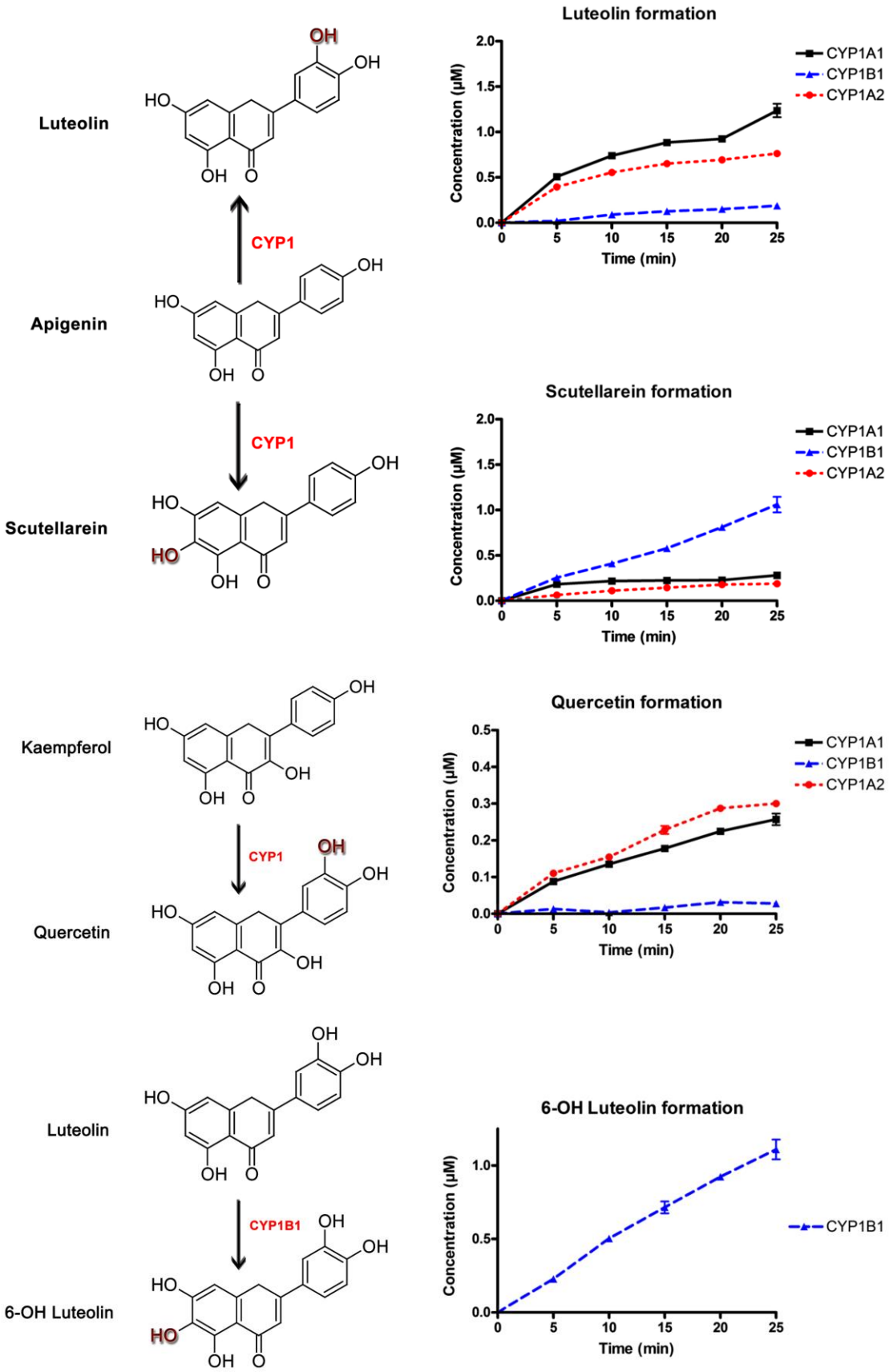
Luteolin
CYP1
Apigenin
CYP1
Scutellarein
Kaempferol
CYP1
Quercetin
Luteolin
CYP1B1
6-OH Luteolin
Luteolin formation
Scutellarein formation
Quercetin formation
6-OH Luteolin formation
Concentration (μM)
Time (min)
CYP1A1
CYP1B1
CYP1A2

Figure 3. *CYP1-mediated formation of flavonoid metabolites. A. The formation of scutellarein and luteolin by the CYP1–mediated metabolism of apigenin. Apigenin (10 μM) was incubated with recombinant CYP1 enzymes and the formation of the metabolites scutellarein and luteolin was detected by HPLC, as described in the materials and methods section. B. The formation of quercetin from kaempferol (10 μM) was catalyzed by all 3 CYP1 enzymes, whereas the formation of 6 OH luteolin from luteolin (10 μM) only by CYP1B1. Error bars indicate STDEV of the mean for n=3 experiments.*

luteolin was slightly increased (26.2±0.5 μM vs. 29.7±1.5μM and 22±0.36 μM vs. 24±0.31 μM, $P<0.05$), whereas the IC50 values of the remaining compounds were unaffected (Fig. 4E). Similar findings were observed for the flavonols quercetin and kaempferol with regard to inhibition of MCF7 cell proliferation (data not shown). The parent compound kaempferol was less active than the metabolite quercetin in MDA–MB–468 and MCF10A cells (Fig. 4D). Quercetin exhibited the lowest IC50 from the 2 flavonols examined in MDA–MB–468 cells (Fig. 4D, 13.7±0.8 μM).

Luteolin exhibited the lowest IC50 in MDA–MB–468 cells and yielded one metabolite that was produced solely by the enzyme CYP1B1. In order to test whether CYP1B1 metabolism was selective for luteolin in MDA–MB–468 cells *in vitro*, the cytotoxicity of this compound was estimated in the presence of the CYP1 inhibitors acacetin and α–napthoflavone. The incubation of luteolin with 1 μM of CYP1 inhibitors reversed the IC50 noted in MDA–MB–468 cells (2±0.3 μM) to 15±2.3 μM (Fig. 4C), which indicated an approximate 7.5 fold selectivity of the MDA–MB–468 cell line for luteolin (Fig. 4C). The IC50 noted in the presence of the CYP1 inhibitors for the MDA–MB–468 cells was comparable to that noted in MCF10A cells that were incubated with luteolin (30.4±5.4 μM) (Fig. 4C). It is important to note that the bioactivation of luteolin in MDA–MB–468 cells was attributed to CYP1B1

expression as demonstrated by HPLC analysis, western immunoblotting and CYP1 inhibition cytotoxicity assays.

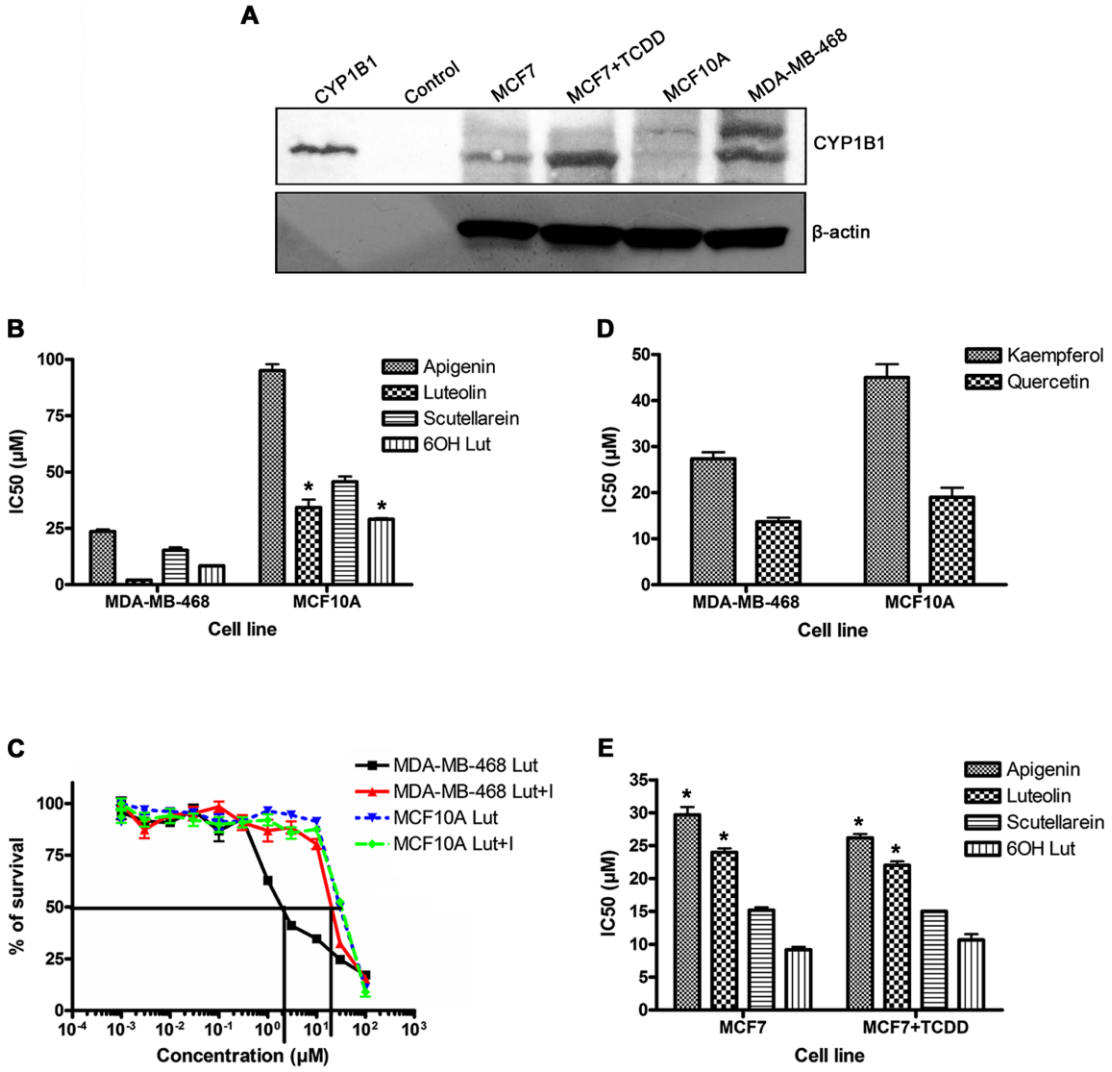

Figure 4. *The bioactivation of dietary flavonoids in the CYP1 expressing cell lines MDA–MB–468 and MCF7. A. The expression of CYP1B1 protein in MDA–MB–468, MCF10A and MCF7 cells. Protein extracts were obtained from the aforementioned cell lines and western blotting was conducted as described in Materials and Methods. MCF7 cells were pre–treated with 10 nM TCDD in order to induce the expression of CYP1B1. β–actin was used as a loading control for the samples derived from the human cell lines. Control microsomes (0.5 mg/ml) (Gentest, BD Biosciences) that were derived from recombinant insect cells with an empty vector were used as a negative control, whereas microsomes (Gentest, BD Biosciences) derived from recombinant insect cells expressing CYP1B1 (0.5*

*mg/ml) were used as a positive control. B. The IC50 of the flavones apigenin, luteolin, scutellarein and 6 OH luteolin was estimated in MDA–MB–468 and MCF10A cell by MTT assay toxicity curves, as described in the Materials and Methods. * indicate significant differences for the IC50 values of 6 OH luteolin and luteolin in MCF10A cells (P<0.05). C. Luteolin is selectively bioactivated in MDA–MB–468 breast cancer cells by CYP1B1. Luteolin was incubated at a concentration range covering 0.001-100 μM for 96 hrs, in the presence (+ I) or absence of the CYP1B1/CYP1A1-inhibitors acacetin and/or α-napthoflavone (0.5–1 μM) and cell viability was measured using the MTT assay, as described in Materials and Methods. Error bars indicate STDEV of the mean for n = 3 experiments. D. The IC50 of the flavonols kaempferol and quercetin was estimated in MDA–MB–468 and MCF10A cell by MTT assay toxicity curves, as described in the Materials and Methods. E. The inhibition of cellular proliferation of MCF7 cells with and without pre–treatment with TCDD (10 nM) by the dietary flavones apigenin, luteolin, scutellarein and 6 OH luteolin. The IC50 for each compound was determined by MTT assay toxicity curves, as described in the Materials and Methods. * indicate significant differences for the IC50 values of apigenin and luteolin in non–induced MCF7 cells and MCF7 cells that were pre–induced with 10 nM of TCDD (P<0.05).*

3.4 CYP1B1 exhibits optimum metabolic turnover of luteolin 6 hydroxylation

The kinetics of the formation of 6 OH luteolin from the CYP1B1-catalyzed metabolism of luteolin were investigated. The data indicated that the formation of 6 OH luteolin followed Michaelis–Menten kinetics. The apparent Km and Vmax values of the formation of 6 OH luteolin by CYP1B1 were estimated at 0.12±0.02 μM and 7.4±0.20 pmol min^{-1} pmol CYP^{-1}, respectively (Figure 5). The intrinsic clearance value (Cl) was calculated by the ratio Vmax/Km and was estimated at 61.44±10.66 (x 10^{-3}) ml min^{-1} pmol CYP^{-1} (Figure 5). Based on previous data published on the demethylation of diosmetin by CYP1B1 at the 4′ position (Km=0.18±0.01, Vmax=3.05±0.5 pmol min^{-1} pmol CYP^{-1} Cl=16.28±1.38 (x 10^{-3}) ml min^{-1} pmol CYP^{-1}), the hydroxylation of luteolin at the 6 position was a more catalytically favorable

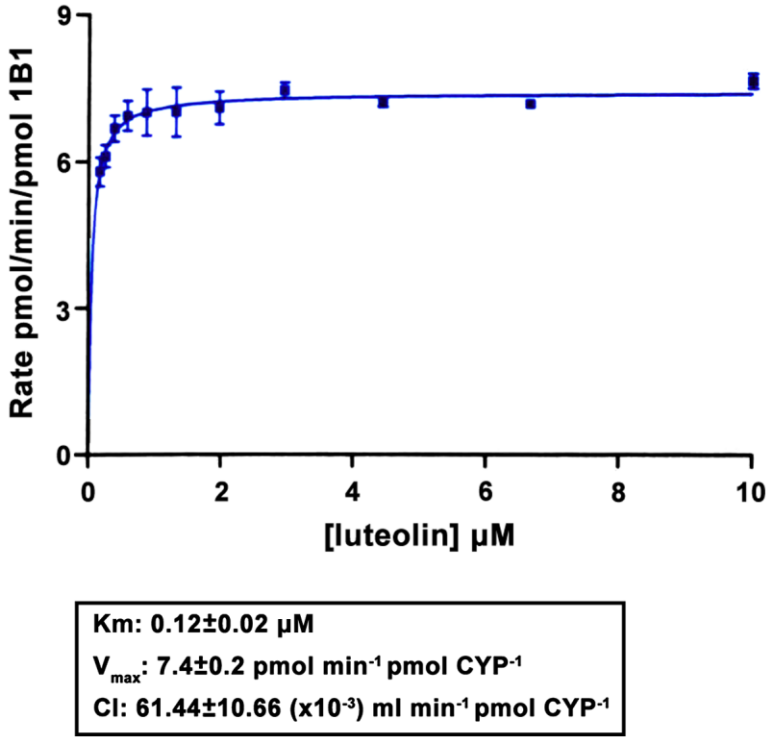

Figure 5. *Michaelis–Menten kinetics of 6 OH luteolin formation by CYP1B1. Km, Vmax and intrinsic clearance values for the production of 6 OH luteolin by CYP1B1. The incubations were conducted as described in Materials and methods for 10 min using 10 pmol/min of CYP1B1 enzyme. The amount of 6 OH luteolin was detected by UV absorption at 360 nm and converted into units of catalytic activity. Michaelis–Menten kinetic parameters were estimated using Graph Pad Prism (version 4.03). The error bars represent the mean SD for n = 3 independent determinations.*

reaction that required less substrate concentration in order to achieve the maximum formation of the product 6 OH luteolin (Androutsopoulos et al., 2009a).

3.5 Molecular interactions of the flavonoids apigenin, luteolin and kaempferol with the heme group of CYP1 enzymes

Apigenin, luteolin and kaempferol were docked to the heme binding site of CYP1B1 and CYP1A1 in order to assess their preferable binding positions with regard to the heme group. The favorable orientations were evaluated using the dock scoring

Table 1. *Docking scores of the two distinct poses of flavonoids (rings B and A towards the heme group) obtained from the molecular docking calculations. The values represent kcal/mol.*

CYP1A1		
Flavonoid ring	**B**	**A**
apigenin	-11.5	-10.9
luteolin	-11.2	-10.1
kaempferol	-11.5	-11.1

CYP1B1		
Flavonoid ring	**B**	**A**
apigenin	-11.7	-11.8
luteolin	-11	-11
kaempferol	-12	-11.9

CYP1A2		
Flavonoid ring	**B**	**A**
apigenin	-10.6	-10.5
luteolin	-10.1	-9.4
kaempferol	-10.5	-10.4

function (Table 1). The residues of the enzyme CYP1A1 that were involved in the binding of the flavonoid molecules were mainly F123, N222, F224, L254 and F258. N222 appeared to bind with hydrogen bonds in all cases with regard to the orientation of apigenin and luteolin to the heme group. Docking of apigenin indicated a preferable orientation of the B ring (-11.5 kcal/mol) instead of the ring A (-10.9) towards the heme group with the formation of a hydrogen bond between N222 and 7 –OH and 4′ –OH, respectively (Fig. 6A,B, Table 1). With regard to luteolin, the binding of ring B to the heme group revealed favorable docking scores (-11.2

kcal/mol) (Fig. 6C, Table 1) compared with the corresponding docking scores for ring A (-10.1 kcal/mol) (Fig. 6D, Table 1).

The residues of the enzyme CYP1B1 that were involved in the binding of the flavonoids to the heme group were notably F134, N228, F231, N265, F268 and D333. Apigenin was predicted to bind to the heme group with similar docking scores with regard to the A (-11.8 kcal/mol) and B rings (-11.7 kcal/mol) (Fig. 7A, B). In both cases, N228 was predicted to form a hydrogen bond with the carboxyl groups located in positions 4′ and 7 respectively. Similarly, both docking positions of luteolin exhibited the same binding scores (Table 1) for CYP1B1. For the A ring orientation towards heme, apart from the hydrogen bond between N228 and 4′ –OH, also 3′ –OH forms a hydrogen bond with the side chain of N265 (Fig. 7C). With regard to the B ring orientation, except from N228 and N265, the residue D333 was predicted to form a hydrogen bond with 3′–OH, in addition to the interactions with the residues N228 and N265 (Fig. 7D). However, the binding score was not increased probably due to additional non–favorable interactions.

4. Discussion

In the current study, evidence is presented regarding the CYP1–mediated metabolism of hydroxylated flavonoids. CYP1 enzymes were shown to catalyze hydroxylation reactions at the 3′ and 6 position of the B and A rings of hydroxylated flavonoids, respectively. With regard to B ring hydroxylation, CYP1A1 is more metabolically active compared with the remaining 2 CYP1 enzymes, whereas with regard to hydroxylation at the 6 position of the A ring CYP1B1 is the optimal catalyst. Furthermore, the addition of extra hydroxyl groups on the A and/or B rings of flavonoids enhances their antiproliferative activity with regard to breast cancer cells,

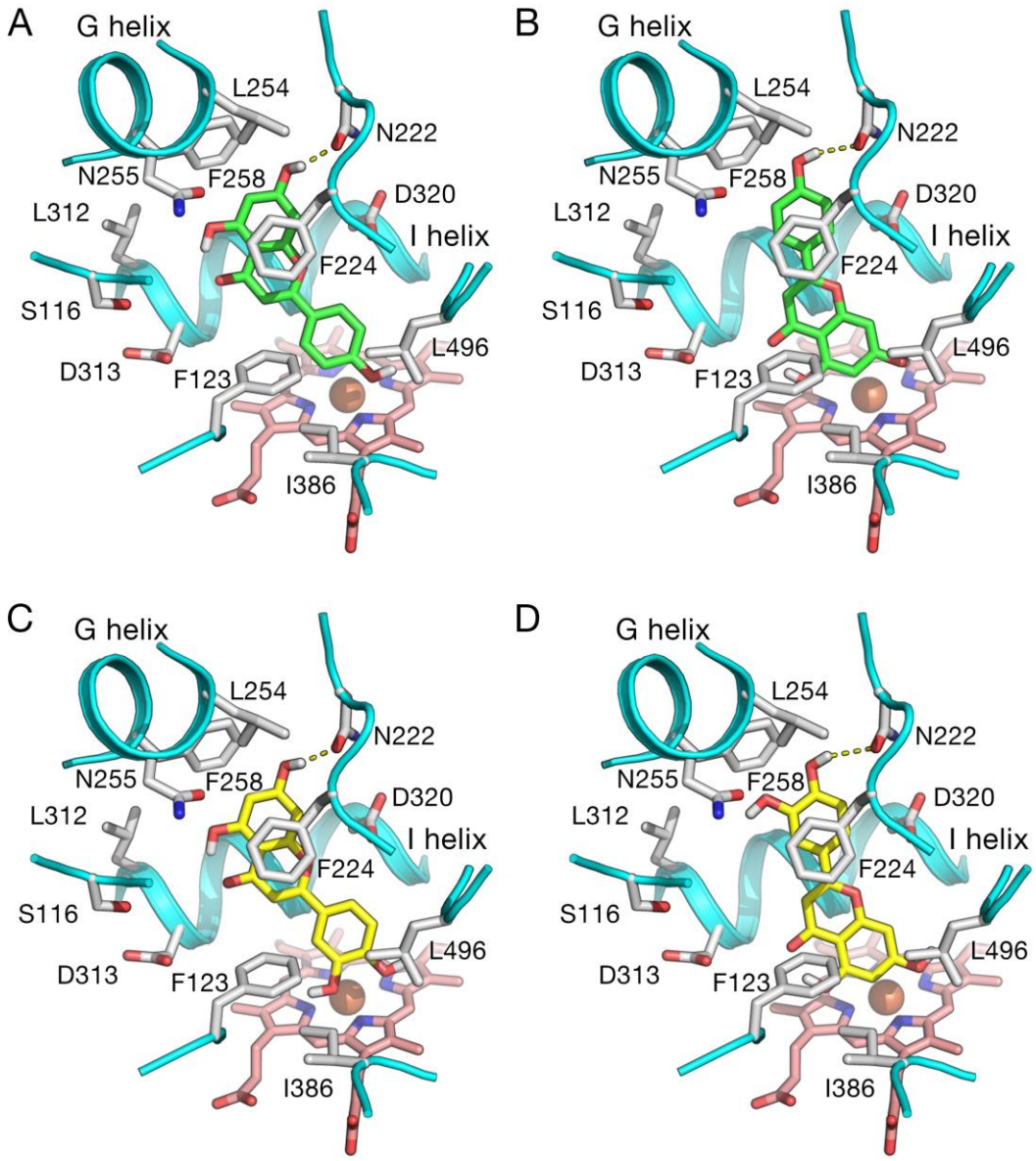

Figure 6. *Human CYP1A1 active site models depicting the molecular interactions of the flavonoids apigenin and luteolin with the heme group of the cytochrome P450 enzyme. The substrates apigenin and luteolin (green and yellow sticks) and the main amino acids (cyan sticks) that comprise the active site cavity are shown. Oxygen and nitrogen atoms are colored red and blue, respectively. (A) Apigenin with the B ring orientation. (B) Apigenin with the A ring orientation. (C) Luteolin with the B ring orientation. (D) Luteolin with the A ring orientation.*

as 6 OH luteolin was considerably more potent than apigenin (approximately 3.5–fold activation) in inhibiting proliferation of MCF7 and MDA–MB–468 cells. CYP1B1 is considered to play a significant role in the metabolic activation of luteolin in breast cancer MDA–MB–468 cells, as demonstrated by the IC50 of luteolin in the presence of the CYP1 inhibitors acacetin and/or α–napthoflavone. The hydroxylation of luteolin at the 6 position of the A ring is an important structural feature that determines the antiproliferative activity of luteolin in breast cancer cells.

Dietary flavonoids have been considered the hallmark molecules for cancer prevention. Long term in depth research on the bioactivity of dietary flavonoids has been exhaustively conducted during the last 30 years. Although significant advances have been made with regard to their mechanism of action, a common model that links the core structure of flavones and flavonols with the degree of their antiproliferative action is yet to be established. This is notably due to the diversity of their mode of anticancer action. Certain flavonoids have been characterized as apoptosis inducers, whereas others as cell cycle inhibitors, cell signaling modulators and/or antioxidants (Priyadarsini et al., 2010; Bitis et al., 2010; Cheng et al., 2011; Curti et al., 2017; Seelinger et al., 2008). In the present study, a unifying model is presented where the inhibition of breast cancer cellular proliferation caused by structurally related flavonoids is dependent on their CYP1–mediated metabolism. The data presented are in agreement with the previous studies conducted by our group regarding the bioactivation of the methoxylated flavonoids eupatorin, cirsiliol, diosmetin and genkwanin by CYP1 enzymes in breast cancer cells (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009a; Androutsopoulos et al., 2009b; Androutsopoulos et al., 2009c). It is important to note that the number of hydroxyl groups in the flavonoid structure of the metabolites enhanced the total antiproliferative activity compared with

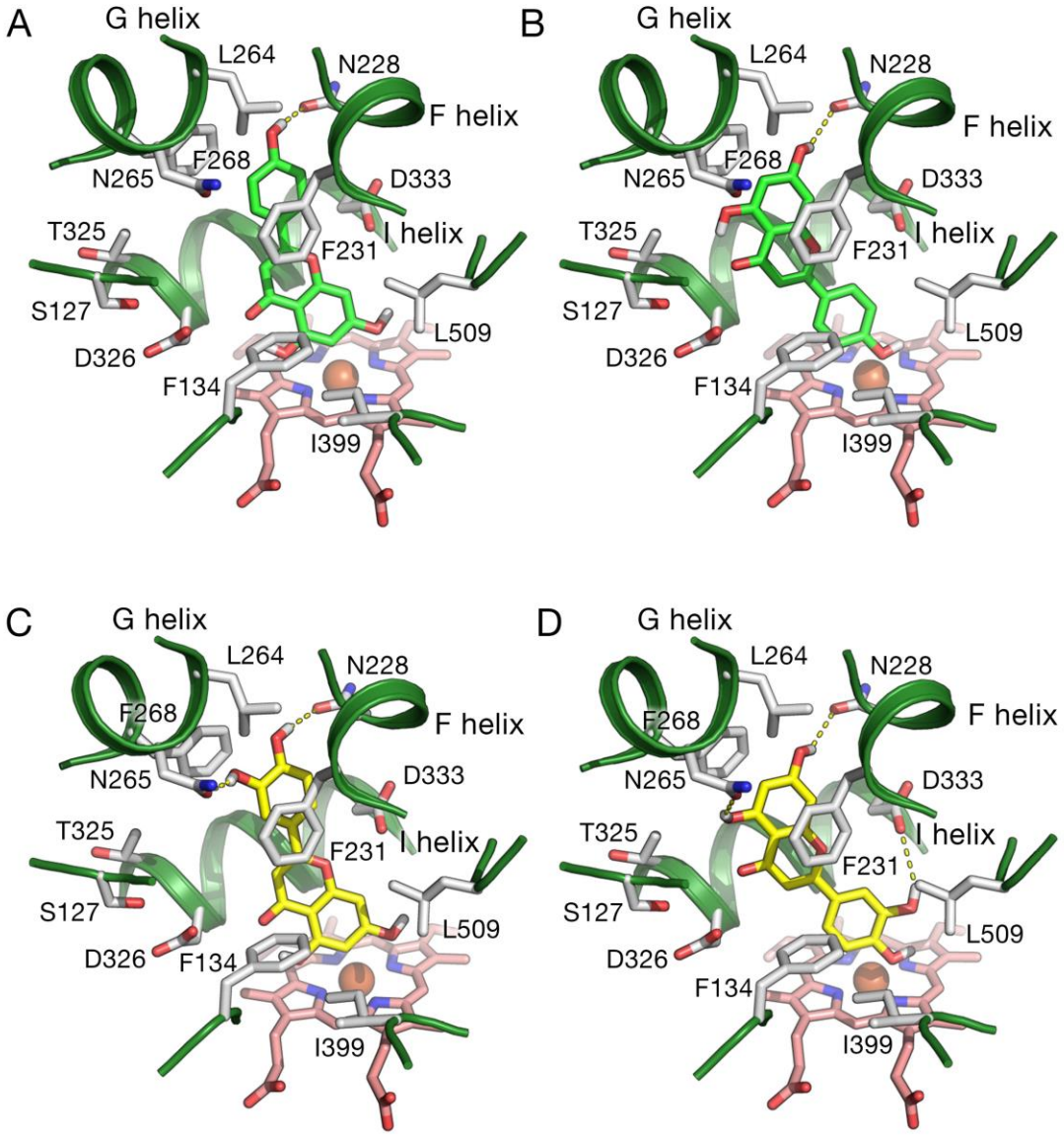

Figure 7. *Human CYP1B1 active site models depicting the molecular interactions of the flavonoids apigenin and luteolin with the heme group of the cytochrome P450 enzyme. The substrates apigenin and luteolin (green and yellow sticks) and the main amino acids (cyan sticks) that comprise the active site cavity are shown. Oxygen and nitrogen atoms are colored red and blue, respectively. (A) Apigenin with the A ring orientation. (B) Apigenin with the B ring orientation. (C) Luteolin with the A ring orientation. (D) Luteolin with the B ring orientation.*

that noted for the parent compounds (Androutsopoulos et al., 2009c), which is in concordance with the results presented in the current study. For example, genkwanin (parent compound) was less active than apigenin (metabolite) and scutellarein (metabolite), whereas diosmetin (parent compound) was less active than luteolin (metabolite) (Androutsopoulos et al., 2009c; Androutsopoulos et al., 2009a; Androutsopoulos et al., 2009b). Similarly, the present study demonstrated that apigenin (parent compound) was less active than scutellarein (metabolite) and kaempferol (parent compound) than quercetin (metabolite) in inhibiting the proliferation of MDA−MB−468 and MCF7 cells.

Although the chemopreventive function of the flavonoids apigenin, luteolin, kaempferol and quercetin has been extensively investigated, evidence regarding the anticancer activity of the flavonoids 6 OH luteolin and scutellarein is limited. This is possibly due to the limited knowledge concerning the relative abundance of these compounds in dietary products and food. It is well known that apigenin and luteolin, are the main constituents of parsley, celery, green peppers and chamomile, whereas kaempferol and quercetin are notably found in apples and onions. However, the sources of 6 OH luteolin and scutellarein that have been identified to date are mostly plant species that are used for traditional medicinal use and are absent from the daily diet (Uehara et al., 2015; Nalewajko-Sieliwoniuk et al., 2015; de Beer et al., 2011). The sole food source that has been reported for 6 OH luteolin and scutellarein is Mexican oregano (Lin et al., 2007). With regard to their biological activity, several reports have suggested antioxidant, antimutagenic and antiproliferative modes of action (de Oliveira et al., 2013; Peng et al., 2003; Gordo et al., 2012; Nagao et al., 2002). It is important to note that luteolin was more potent than apigenin in HeLa cervical carcinoma and B16F10 melanoma cells, whereas 6 OH luteolin was more

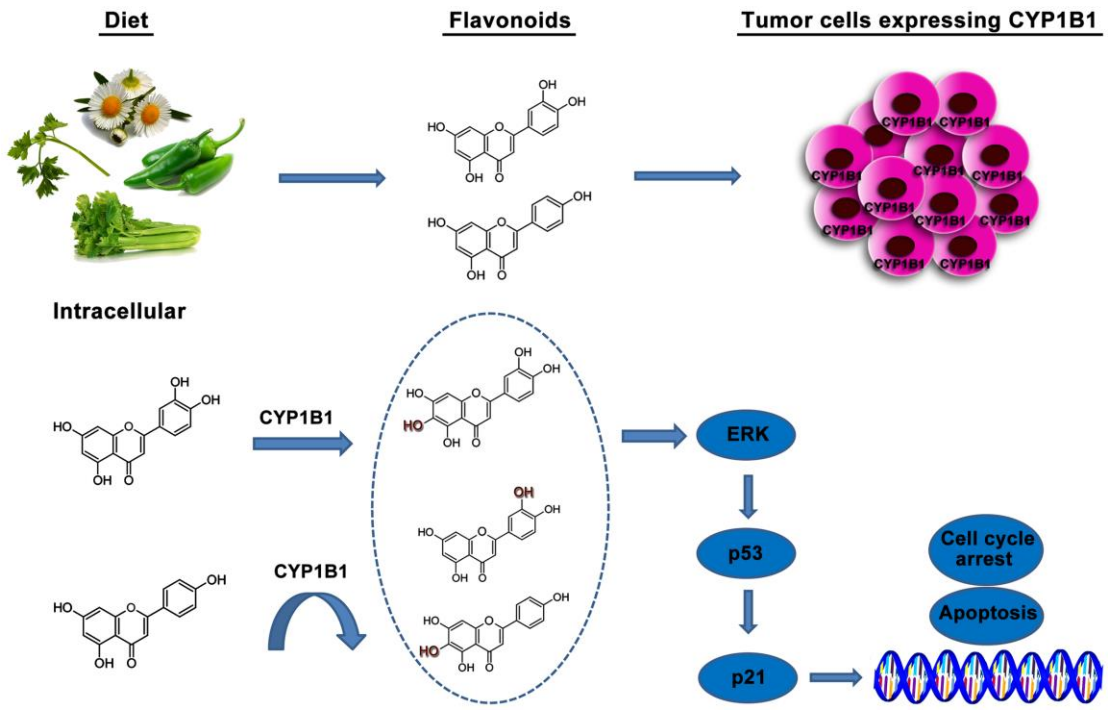

Figure 8. *Putative metabolic pathway indicating the bioactivation of luteolin and structurally related dietary flavonoids by CYP1B1 in cancer cells. The mechanism of action may be attributed to induction of cell cycle arrest and apoptosis by modulation of cell signaling, as previously reported (Androutsopoulos and Spandidos 2013; Zhang et al., 2009; Meng et al., 2017; Chen et al., 2017; Sonoki et al., 2017).*

potent than luteolin only in B16F10 cells (Nagao et al., 2002). This suggests that hydroxylation reactions at the A and B ring of flavones may partially enhance the antiproliferative activity of the parent compounds, which is in concordance with the present study.

The interactions of dietary flavonoids with CYP1 enzymes have notably been investigated in terms of inhibition studies. The majority of the literature has explored the capacity of these compounds to inhibit CYP1 enzyme activity by direct and/or indirect assays (Schwarz et al., 2005; Schwarz et al., 2003; Takemura et al., 2010; Doodstdar et al., 2000; Ciolino et al., 1998; Ciolino et al., 1999; Zhai et al., 1998).

The metabolism of hydroxylated flavonoids has been investigated to a lesser extent. It was reported in an early study that the flavonols galangin (3′,5,7 trihydroxyflavone) and kaempferide were both hydroxylated and demethylated at the 4′ position of the B ring, respectively in order to yield the metabolite kaempferol (Otake and Walle 2002). This type of bioconversion (galangin to kaempferol) has been shown to occur *in vitro* in Caco–2 cells that were pre–induced with TCDD (Hamada et al., 2010). In addition, the conversion of luteolin by apigenin has been demonstrated by Breinholt and colleagues only for human CYP1A2 (Breinholt et al. 2002). The present findings corroborate the aforementioned studies and highlight that aromatic hydroxylation reactions can be catalyzed by CYP1 enzymes to the pre–substituted B ring of dietary flavonoids. Hydroxyl groups can be added to the 4′ position of the unsubstituted ring of galangin to yield kaempferol, which in turn can be metabolized to quercetin. Similarly, hydroxylation at the 6 position of the A ring was demonstrated to occur for apigenin and luteolin in order to yield scutellarein and 6 OH luteolin, respectively. It is important to note that CYP1B1 is shown for the first time to play a significant role in the catalysis of these reactions, since earlier studies were notably focused on CYP1A1 and CYP1A2.

In addition to metabolism studies, docking calculations depicted a general preference of the B ring orientation towards the heme group for CYP1A1, although this trend was not noted for CYP1B1 and/or CYP1A2 (with the exception of luteolin). Furthermore, the B ring orientation towards the heme group of CYP1A1 was favored compared with the A ring for the hydroxylation of apigenin, which was in agreement with the metabolism studies. With regard to CYP1B1, similar scores were noted for the docking of the A and B ring orientations of luteolin to the heme group, indicating no preference for a ring group towards hydroxylation, as demonstrated by the docking

calculations. It is important to note that the energy levels required for the docking of the A ring orientations of both apigenin and luteolin to the heme group of CYP1B1, were higher compared to those noted to the heme group of CYP1A1 (Table 1), which indicates that CYP1B1 favors the A ring orientation compared with CYP1A1. This finding is in agreement with the data derived from the metabolism studies that demonstrated favorable metabolism of apigenin and luteolin by CYP1B1 to the 6 hydroxylated derivatives namely, scutellarein and 6 OH luteolin (A ring orientation) compared with CYP1A1 that demonstrated lesser or no metabolism at the 6 position of the A ring. Moreover, luteolin was not metabolized by CYP1A1 and CYP1A2 indicating that it acts more as a inhibitor rather than a substrate for these enzymes.

The bioactivation of luteolin in MDA–MB–468 cells was attributed to metabolism of this compound by CYP1B1, as in the presence of the CYP1B1/CYP1A1 inhibitor α-napthoflavone and/or acacetin, the inhibition of cellular proliferation by luteolin was lessened. A similar trend has been previously demonstrated for the flavonoids genkwanin and eupatorin in our earlier reports (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009c). These compounds exhibited IC50s for MDA–MB–468 cells of 1.6 and 0.5 μM, respectively. The potent inhibition of cellular proliferation was reversed by cotreatment with the CYP1B1 inhibitor acacetin. The IC50 values in the presence of acacetin were increased to 15 μM for eupatorin and to 12.5 μM for genkwanin (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009c). Luteolin exhibited a similar activation threshold (approximately 7.5–fold activation in MDA–MB–468 cells). However, although eupatorin and genkwanin were metabolized by all three CYP1 enzymes, luteolin was solely metabolized by CYP1B1, indicating that CYP1B1 was notably responsible for the bioactivation of this compound in MDA–MB–468 cells. CYP1B1 has previously been reported as a

putative cancer therapeutic target due to the selective overexpression of this enzyme in tumor cells (McFadyen et al., 1999; McFadyen et al., 2004; Murray et al., 1997; Androutsopoulos et al., 2013; Spyrou et al., 2014). However, with regard to the detection of CYP1B1 enzymatic activity in human tumors, a paucity of studies has been conducted and the evidence presented is limited in order to address the accurate determination of CYP1B1 activity levels in tumors (McFadyen et al., 2004; Androutsopoulos et al., 2013; Spyrou et al., 2014). Despite these inconsistencies, the present study reveals that luteolin is an optimum substrate for CYP1B1, as demonstrated by its low Km and high intrinscic clearance. Consequently, the formation of 6 OH luteolin in breast cancer cells will theoretically require a very small amount of initial substrate concentration that can be achieved by a minimum CYP1B1 activity level. Thus, it could be deduced that luteolin could exhibit significant therapeutic applications in human breast cancer via the selective metabolism to 6 OH luteolin by CYP1B1.

It is important to note that the bioactivation of dietary flavonoids in MDA–MB–468 and MCF7 breast cancer cells cannot be solely attributed to the production of one single CYP1–metabolite that corresponds to each parent compound (i.e. luteolin from apigenin, scutellarein from apigenin). It is more likely that a combination effect of the CYP1–flavonoid metabolites with their corresponding parent compounds occurs. For example, the bioactivation of apigenin is possibly attributed to the concomitant antiproliferative action of apigenin, luteolin and scutellarein. A similar finding has been previously reported for the conversion of luteolin from diosmetin by CYP1A1 in liver cancer HepG2 cells (Androutsopoulos and Spandidos 2013). This study indicated a synergistic mode of action between the two compounds with regard to the inhibition of HepG2 cell proliferation (Androutsopoulos and Spandidos 2013).

Furthermore, the mechanism of action of the CYP1–mediated metabolites has been attributed to induction of apoptosis, activation of cell signaling proteins, induction of cell cycle inhibitors and inhibition of topoisomerase–I catalyzed DNA relegation (Zhang et al. 2009; Kandaswami et al., 1999; Androutsopoulos and Spandidos 2013; Boege et al., 1996). It is likely that these biological processes occur in breast cancer cells that express active CYP1B1 (Figure 6).

In conclusion, the present study investigated the anticancer effects of hydroxylated flavones and flavonols with regard to CYP1–mediated metabolism and inhibition of cellular proliferation of human breast cancer cells. The data demonstrate that hydroxylation of dietary flavonoids by CYP1 enzymes can enhance their antiproliferative activity in human breast cancer cells. The overall effect is possibly attributed to the combined action of the parent compound and the corresponding metabolites. Moreover, the flavonoid luteolin is characterized as a selective CYP1B1–activated prodrug that originates from dietary sources and can exert significant therapeutic effects in breast cancer cells that express active CYP1B1. Current research is in progress to examine the *in vivo* therapeutic effects of luteolin and to design semi-synthetic flavonoids that aim to enhance markedly (up to 100-fold) the cytotoxic action of their corresponding metabolites thus providing more evidence on the use of cytochrome P450s in cancer therapy.

Conflict of interest

None declared.

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