Tangeretin inhibits the proliferation of human breast cancer cells via CYP1A1/CYP1B1 enzyme induction and CYP1A1/CYP1B1–mediated metabolism to the product 4′ hydroxy tangeretin

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\textbf{A R T I C L E  I N F O}

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\textbf{A B S T R A C T}

Tangeretin is a polymethoxylated flavone with multifaceted anticancer activity. In the present study, the metabolism of tangeretin was evaluated in the CYP1 expressing human breast cancer cell lines MCF7 and MDA–MB–468 and in the normal breast cell line MCF10A. Tangeretin was converted to 4′ OH tangeretin by recombinant CYP1 enzymes and by CYP1 enzymes expressed in MCF7 and MDA–MB–468 cells. This metabolite was absent in MCF10A cells that did not express CYP1 enzymes. Tangeretin exhibited submicromolar IC50 (0.25 \pm 0.15 \mu M) in MDA–MB–468 cells, whereas it was less active in MCF7 cells (39.3 \pm 1.5 \mu M) and completely inactive in MCF10A cells (> 100 \mu M). In MDA–MB–468 cells that were coincubated with the CYP1 inhibitor acacetin, an approximately 70-fold increase was noted in the IC50 (18 \pm 1.6 \mu M) of tangeretin. In the presence of the CYP1 inhibitor acacetin, the conversion of tangeretin to 4′ OH tangeretin was significantly reduced in MDA–MB–468 cells (2.55 \pm 0.19 \mu M vs. 6.33 \pm 0.12 \mu M). The mechanism of antiproliferative action involved cell cycle arrest at the G1 phase for MCF7 and MDA–MB–468 cells. Tangeretin was further shown to induce CYP1 enzyme activity and CYP1A1/CYP1B1 protein expression in MCF7 and MDA–MB–468 cells. These results suggest that tangeretin inhibits the proliferation of breast cancer cells via CYP1A1/ CYP1B1–mediated metabolism to the product 4′ hydroxy tangeretin.

1. Introduction

Flavonoids are polyphenolic molecules with multiple modes of biological action. Their cancer preventative and/or anticancer activity has been attributed to various different mechanisms including the induction of apoptosis, the modulation of cell signaling, the inhibition of the cell cycle and notably the process of mitosis, the induction and/or modulation of autophagy, the inhibition of invasion and metastasis and the interaction with Reactive Oxygen Species (ROS) and ROS signaling (Johnson and de Mejia, 2013; Qi et al., 2015; Hsieh et al., 2016; Cheng et al., 2015; Salmela et al., 2012; Sosa et al., 2013; Kim et al., 2015; Jo et al., 2015; Li et al., 2013; Doleckova et al., 2013). In addition to the aforementioned biological actions, the interactions of natural dietary flavonoids with xenobiotic phase I metabolizing enzymes, notably the cytochrome P450 CYP1 enzymes, have been investigated by recent studies (Androustopoulos et al., 2008, 2009a,b,c,d, 2010; Androustopoulos and Spandidos, 2013; Androustopoulos and Tsatsakis, 2014). CYP1 enzymes participate in the metabolic activation of pro–carcinogens to their ultimate carcinogenic derivatives, whereas recently an anti–inflammatory role of these enzymes was demonstrated in Cyp1a1/1a2/1b1 (−/−) C57BL/6J triple knockout mice compared with C57BL/6J wild-type mice, via the regulation of the metabolism of arachidonic acid lipid mediators of inflammation (Nebert and Dalton, 2006; Divanovic et al., 2013). With regard to dietary flavonoids, a cancer therapeutic effect of these compounds has been demonstrated in breast and liver cancer cells via their intracellular metabolism by CYP1 enzymes (Androustopoulos et al., 2008, 2009a,b,d, Androustopoulos and Spandidos, 2013). These compounds are oxidized to their corresponding hydroxylated derivatives via aromatic hydroxylation and/or demethylation reactions occurring at the B and/or A rings of the polyphenolic moiety (Androustopoulos et al., 2008, 2009a,b,d; Androustopoulos and Spandidos, 2013; Androustopoulos and Tsatsakis, 2014). The resulting metabolites inhibit cancer cell growth at equivalent and/or higher levels compared with the corresponding parent
compounds (Androutsopoulos et al., 2008, 2009a,b,d). It is important to note that synergistic effects resulting from the combined action of the parent flavonoid compound and the CYP1-metabolite have been documented, thus accounting for the overall antiproliferative activity (Androutsopoulos and Spandidos, 2013).

Tangeretin is a fully methoxylated flavone that is present in the peel of citrus fruits and has demonstrated anticancer activity via distinct mechanisms of action. Tangeretin causes G2/M arrest and induces apoptosis in glioma cells by modulating Phosphatase and tensin homolog (PTEN) protein, and the cell cycle regulating genes, namely D1 and cdc2, whereas in human meningioma cells this compound induces apoptosis by enhanced phosphorylation of glycogen synthase 3 β (GSK3β) and inhibition of the Wnt5/β-catenin pathway (Ma et al., 2016; Das et al., 2015). Tangeretin was further shown to inhibit breast cancer formation in a rat model of 7,12-dimethyl benz[a]anthracene (DMBA)–induced mammary carcinogenesis via p53/p21 upregulation and inhibition of metastasis by downregulation of MMP2, MMP9 and VEGF expression (Arivazhagan and Sorimuthu Pillai, 2014; Lakshmi and Subramanian, 2014). Despite these promising studies, the metabolism of tangeretin by CYP1 enzymes remains poorly defined. With the exception of a previous study by Walle and Walle that examined tangeretin metabolism by recombinant CYP enzymes and the hepatic liver S9 fraction, there is no evidence in the literature regarding the association of CYP1 enzymes and the cancer therapeutic effect of tangeretin (Walle and Walle, 2007). In the present study, the metabolism of tangeretin was investigated in the CYP1 expressing breast cancer cell lines MCF7 and MDA–MB–468 and the normal breast cell line MCF10A. The data suggest that tangeretin is metabolized to one major conversion product by a demethylation reaction at the B ring that in turn inhibits cancer cell growth.

2. Materials and methods

2.1. Chemicals and antibodies

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), 7-ethoxyresoruvin, resoruvin, α-naphthoflavone, acacetin, tissue culture reagents and media, Western blotting lysis buffer and DTT were purchased from Sigma Aldrich (St Louis, MO, USA). Tangeretin and 4’-OH tangeretin were purchased from Apin chemicals (Abingdon, UK). Western blotting reagents were from Bio-Rad (Berkeley, CA, USA). The polyclonal antibody for CYP1A1 was from Daichi Pure Chemicals (Gentest corporation, MA, USA), whereas the monoclonal antibodies for CYP1B1 from Auvation Limited (Glasgow, Scotland, UK), and 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 MCF10A cells were grown in DMEM:F12 with insulin (10 μg/ml), hydrocortisone (500 ng/ml), EGF (20 ng/ml), 10% FBS and 2 mM glutamine. The cells were grown in a humidified incubator at 37 °C in 5% CO2/95% air and passedaged every 3–4 days using trypsin EDTA (0.25% v/v).

2.3. Tangeretin metabolism

Recombinant CYP1 enzymes and control microsomes were incubated with 10 μM of tangeretin at 37 °C in the presence of NADPH (0.5 mM), MgCl2 (0.5 mM) and phosphate buffer (20 mM). The recombinant enzymes and the control microsomes were purchased from BD Biosciences (CYP1A1 + reductase supersomes catalogue number 456211, CYP1B1 + reductase supersomes catalogue number 456220, CYP1A2 + reductase supersomes catalogue number 456203 and insect cell control microsomes catalogue number 456200). Time points were obtained at 0, 5, 10, 15, 20 and 25 min intervals. The reactions were terminated by addition of equal volumes of methanol and acetic acid at a 100:1 ratio. The samples were centrifuged at 3500 g for 20 min at 4 °C and the supernatants were analyzed by reversed phase HPLC.

2.4. HPLC analysis

The methodology has been described in detail in previous publications (Androutsopoulos et al., 2009a, 2008). A Luna C18 4.6 × 150 mm 5 μm column was used and the mobile phase consisted of solvents A and B. Solvent A comprised 1% acetonitrile and 0.5% acetic acid in H2O and solvent B 4% acetonitrile and 0.5% acetic acid in CH3OH. 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 tangeretin concentration was monitored using a Waters Series 200 UV detector (Waters, Hertfordshire, UK) at 327 nm. The concentration of tangeretin was estimated by a calibration curve covering the range of 0.05–10 μM. The assay was carried out at 37 °C and the flow rate was 1 ml/min. The average recovery for tangeretin was 93% and the retention time using the aforementioned parameters was 17.9 min. The limit of detection of both compounds was estimated at 0.05 μM and the accuracy and precisions were in the range of 95–99% and 1.7–5% for tangeretin and 93–98% and 1.5–4.9% for TP1, respectively.

2.5. LC-MS analysis

Mass spectrometry analysis of tangeretin was conducted on an Agilent 1100 Series LC/MSD Trap XCT systems with a photodiode array detector. The separation of tangeretin was conducted using a Phenomenex Luna 5 μ C18 (250 × 4.6 mm) column and the following solvents: (A) water, (B) methanol and (C) acetonitrile. The initial conditions were as follows: 59% solvent A, 40% solvent B and 1% solvent C. The analytical column temperature was 40 °C and the flow rate was 0.8 ml/min. A total of 50 μL of sample solution was analyzed. A linear gradient was initiated immediately on the start of the analysis with solvent B rising to 90% and solvent C rising to 4% over 15 min. These solvent compositions were held for 2 min prior to the initial condition reset (using a flow rate of 0.8 ml/min). A re-equilibration time of 8 min was allowed between each sample analysis. The photodiode array detector was set at 330 nm. The LC flow was controlled from the photodiode array detector into the mass spectrometer without stream splitting. The MS determination was acquired in electrospray ionisation (ESI) positive mode. The molecular ions ([M + H]+) were detected in the range of 200–500 using an auto–MS mode. Nitrogen was used as the nebulizing gas at 60 psi and as drying gas with the flow rate of 10 L/min at 350 °C. The MS data were analyzed using LC/MSD Trap software 5.3. The Retention time (Rt) of tangeretin under these conditions was 17.9 min.

2.6. MTT assay

MCF7, MDA–MB–468 and MCF10A cells (1 × 10⁴ cells/ml) were seeded in 96-well plates and the antiproliferative effect of tangeretin was examined as described previously (Androutsopoulos et al., 2008). The inhibition experiments were conducted in the presence of 0.5 μM of the CYP1A1 inhibitor acacetin. Similar results were noted using higher concentrations of 1 μM of acacetin as well as for the CYP1A1 inhibitor α-naphthoflavone.
2.7. FACS analysis

MDA–MB–468, MCF7 and MCF10A were grown in T25 flasks at approximately 30% confluence. The cells were treated with tangeretin at concentrations of 10 μM in the presence and/or absence of CYP1 inhibitors (0.5–1 μM) for 24 and 48 h. The cells were subsequently washed with PBS, removed by trypsin/EDTA and centrifuged at 3500 rpm for 5 min. The cells were fixed in 70% ethanol for at least 24 h at −20 °C. The ethanol was removed and PBS containing PI (50 μg/ml) and RNase (100 μg/ml) was added to the cells that were incubated further for 30 min at 37 °C. Flow cytometry was conducted using a Beckman Coulter flow cytometer at PMT4 and at least 10,000 events were acquired for analysis. The Multicycle analysis 2.0 Software was used for the determination of the cell cycle corresponding to the samples.

2.8. Western blotting

MCF7, MDA–MB–468 and MCF10A cells were cultured in T25 flasks at a density of 5 × 10^5 cells/ml. The cells were washed once with PBS and lysed with 100 μl of lysis buffer that contained protease inhibitor cocktail and DL-dithiothreitol (DTT, 1 mM). The cells were subsequently sonicated on ice for 5 min and centrifuged at 13000 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 subsequently sonicated on ice for 5 min and centrifuged at 13000 rpm at 4 °C overnight. The membrane was incubated in 10% milk/0.05% TBST at room temperature for 1.5 h. The membrane was subsequently washed three times with 0.05% TBST and incubated with the secondary antibody against HRP (1:2000) diluted in 1% milk/0.05% TBST at room temperature for 1 h by continuous shaking in order to block the non-specific binding sites. The primary antibodies against CYP1A1, CYP1B1 and β-actin were added to the membrane at 1:800, 1:500 and 1:3000 dilutions, respectively at 4 °C overnight. The membrane was exposed to ECL reagents, and the expression profile of the proteins was developed on film.

2.9. EROD assay

MCF7 and/or MDA–MB–468 cells (2 × 10^5 cells/ml) were plated in 96–well plates. The cells were incubated at 37 °C, 5% CO2 for 24 h and subsequently tangeretin was added at a concentration range of 3, 10 and 30 μM, respectively. The induction of EROD activity was assayed at the 24 h time point. Following the end of the treatment period, the medium was aspirated and the cells were washed with PBS thrice. A total of 200 μl of medium containing 5 μM 7-ethoxyresorufin and 1.5 mM salicylamide were added to each well, and the plates were further incubated for 1 h. The reactions were terminated by addition of 150 μl of ice-cold methanol to the wells, followed by centrifugation at 3500 rpm at 4 °C for 15 min. Subsequently, 200 μl were transferred to a clear bottom black 96–well plate and the amount of resorufin produced was measured using a fluorescence plate reader with an excitation and emission wavelength of 530 nm and 590 nm, respectively. The data were analyzed, following subtraction of the blank fluorescence that contained resorufin alone from each sample reading. The concentration of resorufin was measured using a calibration curve. The units of enzyme activity were expressed as concentration of resorufin formed per time, per amount of cells.

Although 7-ethoxyresorufin O-deethylase is a marker of cytochrome P450 CYP1A1 activity (Burke and Mayer, 1974), the same substrate has affinity for CYP1A2 and CYP1B1 enzymes, yet to lesser extents. The concentrations of 3, 10 and 30 μM of tangeretin were selected based on previous studies conducted on flavonoids published by our group and others. The early studies of Ciolino have used a concentration range of 1–5 μM (Ciolino et al., 1998, 1999). Subsequent studies from our group have used the same or higher concentrations with those used by the study of Ciolino et al. for the structurally similar flavonoids cirsiliol, eupatorin and eupatorin-5-methyl ether (Androutsopoulos et al., 2009c; Androutsopoulos and Tsatsakis, 2014).

2.10. Statistical analysis

The data were presented as the mean of at least three independent measurements and were analyzed by the paired t-test, the unpaired t-test and the One-Way analysis of variance using GraphPadPrism. The error bars represent mean ± STDEV for at least n = 3 independent experiments.

3. Results

3.1. Recombinant CYP1 enzymes metabolize tangeretin

The metabolism of tangeretin by recombinant CYP1 enzymes was examined by LC–MS analysis. A main metabolite was formed, which was assigned as TP1. The mass spectrum of TP1 indicated the presence of an ion at 359.1, whereas the ion that corresponded to the parent compound tangeretin was 373.1 (Fig. 1B). The difference in the molecular weights of tangeretin and TP1 (373–359 = 14) was determined by their molecular ions, and suggested that a de-methoxylation occurred in the initial structure of tangeretin (Fig. 1A). The comparison with an authentic standard of 4’-OH tangeretin revealed that this compound had the same retention time as TP1, thus confirming the identity of this metabolite as 4’-OH tangeretin (Fig. 1A).

3.2. CYP1 enzymes metabolize tangeretin in MCF7 and MDA–MB–468 human breast cancer cells

The human breast cancer cell lines MCF7 and MDA–MB–468 have been employed in previous studies for the investigation of the CYP1-mediated metabolism and bioactivation of dietary flavonoids (Androutsopoulos et al., 2008). In the present study, the metabolism of tangeretin was examined in the aforementioned cell lines. A main metabolite that matched the retention time of TP1 was identified following incubation of tangeretin with MDA–MB–468 and/or MCF7 cells for 24 h (Fig. 2A). This metabolite was absent in MCF10A cell extracts (Fig. 2A, B). The reduction of the parent compound was higher in MDA–MB–468 compared with MCF7 cells following 24 h of incubation (Fig. 2A, B). The metabolism of tangeretin to TP1 was enhanced in MCF7 cells that were pretreated with the potent CYP1 inducer Tetra-chloro-dibenzo-p-dioxin (TCDD, 10 nM) for 24 h, whereas the metabolism of this compound in MDA–MB–468 cells (2 × 10^5 cells/ml) were plated in 96–well plates. The cells were incubated at 37 °C, 5% CO2 for 24 h and subsequently tangeretin was added at a concentration range of 3, 10 and 30 μM, respectively. The induction of EROD activity was assayed at the 24 h time point. Following the end of the treatment period, the medium was aspirated and the cells were washed with PBS thrice. A total of 200 μl of medium containing 5 μM 7-ethoxyresorufin and 1.5 mM salicylamide were added to each well, and the plates were further incubated for 1 h. The reactions were terminated by addition of 150 μl of ice-cold methanol to the wells, followed by centrifugation at 3500 rpm at 4 °C for 15 min. Subsequently, 200 μl were transferred to a clear bottom black 96–well plate and the amount of resorufin produced was measured using a fluorescence plate reader with an excitation and emission wavelength of 530 nm and 590 nm, respectively. The data were analyzed, following subtraction of the blank fluorescence that contained resorufin alone from each sample reading. The concentration of resorufin was measured using a calibration curve. The units of enzyme activity were expressed as concentration of resorufin formed per time, per amount of cells.

Although 7-ethoxyresorufin O-deethylase is a marker of cytochrome P450 CYP1A1 activity (Burke and Mayer, 1974), the same substrate has affinity for CYP1A2 and CYP1B1 enzymes, yet to lesser extents. The concentrations of 3, 10 and 30 μM of tangeretin were selected based on
3.3. Tangeretin is bioactivated in the CYP1–expressing cell lines MDA–MB–468 and MCF7

Previous studies have demonstrated that MDA–MB–468 cell constitutively express CYP1 enzymes compared with MCF10A cells that express little or no CYP1 protein, whereas MCF7 cells express minimal amounts of CYP1 enzyme levels that are increased following pretreatment with a CYP1 inducer (Androutsopoulos et al., 2008, 2009a). The present study utilized the aforementioned cell lines to investigate the antiproliferative activity of tangeretin. This compound was considerably active in MDA–MB–468 cells and indicated a submicromolar IC50 (0.25 ± 0.15 μM), whereas it demonstrated minimal antiproliferative activity in MCF10A cells (> 100 μM) (Fig. 4A). In MCF7 cells the IC50 of tangeretin (39.3 ± 1.5 μM, Fig. 4B) was considerably higher compared with MDA–MB–468 cells, whereas pretreatment of the cells with TCDD (10 nM) for 24 h decreased the overall IC50 (9.7 ± 0.5 μM, Fig. 4B). The results were significantly different between the two different types of treatments (MCF7 ± TCDD) (Fig. 4B).

The cytotoxicity of tangeretin in MDA–MB–468 cells was investigated in the presence of the CYP1 inhibitors acacetin and α-napthoflavone (Fig. 4C). The IC50 of tangeretin was considerably increased from 0.25 ± 0.15 μM to 18 ± 1.6 μM in the presence of the CYP1 inhibitor acacetin (Fig. 4C). Similar results were noted for the CYP1 inhibitor α-napthoflavone. The cytotoxicity of the metabolite TP1 was evaluated in the 3 breast cell lines and it was found that this compound was more active in MDA–MB–468 compared with MCF7 and MCF10A cells (Fig. 4D). The IC50 values of TP1 for the three cell lines were as follows: MCF7 (18.4 ± 0.7 μM), MDA-MB-468 (2.6 ± 0.3 μM) and MCF10A (68.6 ± 2.7 μM) (Fig. 4D).

In addition, metabolic studies revealed that in the presence of the CYP1 inhibitors the conversion of 4′OH tangeretin from tangeretin was substantially reduced both in MDA–MB–468 and MCF7 cells (Fig. 4E, F). Specifically, an approximately 3-fold decrease (2.55 ± 0.19 μM vs. 6.33 ± 0.12 μM) was noted for the conversion of TP1 by tangeretin, in the presence of the CYP1 inhibitors compared with the incubation of the parent compound with MDA–MB–468 cells alone (Fig. 4F).

The antiproliferative activity of tangeretin in the 2 human breast cancer cell lines and the normal breast cell line was further investigated by flow cytometry analysis. The cells were treated with 10 μM of tangeretin. This concentration (10 μM) has been used in our previous studies conducted on structurally similar flavonoids, such as diosmetin and eupatorin (Androutsopoulos et al., 2008, 2009b; Androutsopoulos and Spandidos, 2013). This is usually an approximation based on the evidence that a compound with submicromolar IC50 (e.g. 0.5 μM) at the 96 h exposure period will require a substantially higher concentration for a shorter time of exposure with the cells, such as 24 h. In addition, 10 μM is the standard concentration used in the metabolic studies. The concentration of 0.5 μM for acacetin has also been previously employed in the study of eupatorin (Androutsopoulos et al., 2008).

Treatment of MDA–MB–468 cells with tangeretin (10 μM) for 24 and 48 h resulted in a significant increase of the population of cells in
the G1 phase (Fig. 5A–D, K, P < 0.05). This increase was accompanied by a concomitant significant decrease in the percentage of cells in the S phase in the tangeretin–treated (Fig. 5A–D, K, P < 0.05) samples. The effect of tangeretin in MDA–MB–468 cells was partially reversible as in the presence of the CYP1 inhibitor acacetin, a significant decrease in the population of the cells at G1 phase was noted compared with the single treatment of tangeretin (Fig. 5A, B, D, K, P < 0.05).

MCF7 cells appeared less sensitive to tangeretin treatment and although significant differences were noted between the control and the treated samples, these were somewhat lower in magnitude compared to those noted in MDA–MB–468 cells (Fig. 5E–G, L). In contrast to the breast cancer cell lines, MCF10A cells were unaffected by tangeretin treatment (10 μM) at either 24 and/or 48 h (Fig. 5H–J, M).

3.4. Tangeretin induces CYP1 enzyme expression in MCF7 and MDA–MB–468 cells

The initial experiments in MCF7 cells revealed metabolism of tangeretin in the absence of TCDD pretreatment to the conversion product 4′ OH tangeretin (Fig. 2A). In addition, the previous study on nobiletin by Surichan and colleagues demonstrated that this structurally similar polymethoxylavone was capable of inducing its own metabolism in MCF7 cells via induction of CYP1 enzyme expression (Surichan et al., 2012). Based on this evidence, the induction of CYP1 enzyme expression by tangeretin was investigated in MCF7 and MDA–MB–468 cells following treatment with this compound for 24 h. CYP1 enzyme activity was significantly increased in MCF7 and MDA–MB–468 cells following treatment with 3, 10 and 30 μM of tangeretin (Fig. 6A, B). In MCF7 cells, CYP1 enzyme activity indicated a substantial increase at 3 μM that peaked at 10 and 30 μM of compound treatment compared with control cells (Fig. 6B). In contrast to MCF7, MDA–MB–468 cells exhibited a basal expression of CYP1 enzyme activity that was increased considerably following treatment with 3, 10 and 30 μM of tangeretin (Fig. 6A). This increase was similar among all treatments, as opposed to MCF7 cells where a dose response was noted for the concentration range 0–10 μM (Fig. 6B). The maximum activity in MDA–MB–468 cells was nearly half of that noted for 10 nM of TCDD alone (152 ± 3.7 vs. 225 ± 12.1 pmol/min/10^6 cells, Fig. 6A). A similar trend was noted...
for MCF7 cells, with the exception that the induction caused by TCDD was considerably higher compared with that caused by 30 μM of tangeretin treatment (53 ± 1.4 vs. 21 ± 1.1 pmol/min/10^6 cells, Fig. 6B). At the highest concentration of 30 μM of tangeretin, an approximately 3-fold and 20-fold increase in CYP1 enzyme activity was observed compared with control cells, for MDA–MB–468 and MCF7 cells, respectively (152 ± 3.7 vs. 55 ± 5.4 pmol/min/10^6 cells, 21 ± 1.1 vs. 0.9 ± 0.05 pmol/min/10^6 cells Fig. 6A, B).

The induction of CYP1 enzyme expression by tangeretin was further monitored by western immunoblotting. Tangeretin induced CYP1A1 protein in a dose dependent manner in MDA–MB–468 and MCF7 cells (3–30 μM) (Fig. 6C, D). The induction of CYP1A1 protein at 3 μM of tangeretin treatment was lesser in MCF7 compared with MDA–MB–468 cells, whereas the induction of CYP1A1 in MDA–MB–468 cells was approximately half of that noted for TCDD, as opposed to MCF7 cells (Fig. 6C, D). With regard to MCF7 cells, the induction of CYP1A1 by
Fig. 4. The bioactivation of tangeretin in the CYP1 expressing cell lines MDA–MB–468 and MCF7. Cell viability was measured using the MTT assay as determined in the materials and methods section. The experiments were conducted at least 3 independent times and error bars indicate STDEV of the mean. Cell survival plots indicating the IC50 determination of tangeretin in (A) MDA–MB–468 and MCF10A cells and (B) MCF7 cells and MCF7 cells pretreated for 24 with 10 nM TCDD, and (C) MDA–MB–468 and MCF10A cells in the presence of the CYP1 inhibitor acacetin (0.5 μM). Similar results were obtained when the CYP1 inhibitor α-napthoflavone was used. (D) The inhibition of cell viability in MDA–MB–468, MCF10A and MCF7 cells by the tangeretin–metabolite TP1. (E) The metabolism of tangeretin in MDA–MB–468 cells in the presence and/or absence of the CYP1 inhibitor acacetin (0.5 μM). HPLC chromatogram of MDA–MB–468 cell incubation with tangeretin, in the presence and/or absence of acacetin (0.5 μM) for 24 h. (F) Quantification of the concentration of the compounds tangeretin and TP1 in MDA–MB–468 cells, in the presence and/or absence of the CYP1 inhibitor acacetin (0.5 μM).
tangeretin was lower than half of that noted for TCDD (Fig. 6D). In contrast to CYP1A1, tangeretin induced CYP1B1 protein expression at minimal levels in MDA-MB-468 cells compared with DMSO control samples (Fig. 6C).

4. Discussion

Cytochrome P450s are enzymes that can activate a wide diversity of xenobiotics (Jiang et al., 2017; Vogel et al., 2017; Russo et al., 2017). In the present study, the interactions of cytochrome P450 CYP1 enzymes with tangeretin were investigated. CYP1 enzymes metabolized tangeretin to the conversion product TP1 that was identified as 4′OH tangeretin. In addition, tangeretin was metabolized to 4′OH tangeretin in the human breast cancer cell lines MDA-MB-468 and MCF7, whereas this conversion was absent in the normal breast cell line MCF10A. 4′OH tangeretin exhibited higher antiproliferative activity compared with tangeretin that resulted in the bioactivation of tangeretin in the CYP1 expressing breast cancer cell lines, as demonstrated by metabolic, cell cycle and cytotoxicity studies in the presence of the CYP1 inhibitor acacetin (0.5 μM) (E) control MCF7 cells (F) tangeretin-treated (24 h) MCF7 cells (G) tangeretin-treated (48 h) MCF7 cells (H) control MCF10A cells (I) tangeretin-treated (24 h) MCF10A cells (J) tangeretin-treated (48 h) MCF10A cells. Distribution of the cells in each phase of the cell cycle. (K) MDA-MB-468 cells (L) MCF7 cells (M) MCF10A cells. The experiments were conducted at least 3 independent times and error bars indicate STDEV of the mean. * indicate significant differences in the cell population of each phase between the control and the treated samples (P < 0.05). * indicate significant differences in the cell population of each phase between the tangeretin-treated samples in the presence and/or absence of the CYP1 inhibitor acacetin (0.5 μM) (P < 0.05).

The metabolism of tangeretin by CYP1 enzymes was investigated concisely in an early study conducted by Walle and Walle in 2007 (Walle and Walle, 2007). The authors examined the metabolism of a range of methoxylated flavones, including mono-, di-, tri- and fully methoxylated compounds, by human liver microsomes and recombinant CYP3A4, CYP2C9, CYP2D6 and CYP1A1/CYP1A2 enzymes (Walle and Walle, 2007). Oxidative demethylation was demonstrated as the rate-limiting metabolic reaction for fully methylated flavones, which is in agreement with the present study and our earlier report that investigated the metabolism of the fully methylated flavone nobiletin by CYP1 enzymes (Walle and Walle, 2007; Surichan et al., 2012). However, Walle and Walle did not examine the metabolism of tangeretin by CYP1B1 and did not identify the corresponding conversion products as opposed to the present study. In an earlier study conducted by Breinholt and colleagues, the identity of the CYP1A2, CYP3A4, CYP2D6 and CYP2C9-catalyzed metabolites of tangeretin was verified by LC/MS (Breinholt et al., 2002). The major metabolic routes involved one demethylation step at the 4′ position of the B ring and 2 demethylation steps at the 5 and 6 position of the A ring that yielded the corresponding metabolites 4′ hydroxy tangeretin and 5,6 dihydroxy tangeretin, respectively (Breinholt et al., 2002). In addition, the demethylation at the 4′ position of the B ring has been previously reported as the main CYP1–mediated metabolic reaction for the structurally similar flavones nobiletin and eupatorin (Surichan et al., 2012; 2013).
Androutsopoulos et al., 2008). Although the present study demonstrated that the major conversion product of CYP1A1 and CYP1B1 metabolism of tangeretin was 4′ hydroxy tangeretin, the presence of additional metabolites was not evident notably in the human breast cancer cell lines MCF7 and MDA–MB–468. The possible explanations for this outcome include the reduced levels of CYP1A1/CYP1B1 enzyme activity compared with the recombinant CYP1 enzymes.

The metabolism of tangeretin to 4′ hydroxy tangeretin enhanced the antiproliferative activity of the parent compound, as determined by cell viability and cell metabolism studies. Furthermore, it is important to note that the mechanism of action involved G1 arrest of MDA–MB–468 cells that was reversed in the presence of the CYP1 inhibitors acacetin and α-naphthoflavone. The data are in agreement with previous reports that demonstrated cytostatic effects of tangeretin in colon cancer, breast cancer and yeast cells (Pan et al., 2002; Morley et al., 2007; Chong et al., 2013; Arivazhagan and Sorimuthu Pillai, 2014). Induction of G1 arrest in combination with upregulation of p53/p21 and downregulation of p–Rb and cyclin D1 were caused by tangeretin treatment in rat breast cancer cells and human colon cancer cells, respectively (Arivazhagan and Sorimuthu Pillai, 2014; Pan et al., 2002). Moreover, induction of apoptosis, inhibition of VEGF activity and downregulation of MMP–2 and MMP–9 are additional modes of action that have been reported for tangeretin (He et al., 2015; Charoensinphon et al., 2013; Arivazhagan and Sorimuthu Pillai, 2014). The present study provides additional evidence regarding the antiproliferative activity of the CYP1–metabolite 4′ hydroxy tangeretin. This novel mechanism of action that includes CYP1–mediated bioactivation of tangeretin to 4′ hydroxy tangeretin may apply for other types of cancer cells, such as colon cancer cells that express active CYP1 enzymes.

With regard to the potency of tangeretin in inhibiting the proliferation of human breast cancer cells, this compound exhibited high activity in MDA–MB–468 compared with MCF7 cells. By contrast, MCF10A cells were notably unaffected as the IC50 of tangeretin noted in this cell line was higher than 100 μM. Tangeretin exhibited an activation factor of approximately 72-fold in MDA–MB–468 cells compared with MDA–MB–468 cells that were treated with tangeretin and CYP1 inhibitors (18 μM/0.25 μM). A similar trend has been previously demonstrated for the flavonoids sinensetin, genkwanin and eupatorin in our earlier reports (Androutsopoulos et al., 2009d, 2008). These compounds exhibited IC50 values for MDA–MB–468 cells of 0.2, 1.6 and 0.5 μM, respectively, whereas in MCF10A cells they were considerably less active (65, 75 and 50 μM, respectively). In the presence of the CYP1 inhibitor acacetin, the IC50 values of sinensetin, eupatorin and genkwanin were considerably increased (from 0.2 to 13.5 μM for sinensetin, from 0.5 to 15 μM for eupatorin and from 1.6 to 12.5 μM for genkwanin) (Androutsopoulos et al., 2009d, 2008). The data reported in the present study are in agreement with the aforementioned studies and suggest that the degree of methoxylation in the flavone moiety enhances the antiproliferative activity of these compounds in MDA–MB–468 cells due to cytochrome P450 CYP1–metabolism. This is verified by the observation that sinensetin and tangeretin that are both penta–methoxylated flavones exhibited submicromolar IC50 values with similar activation factors in the presence of CYP1 inhibitors (68-fold vs. 72-fold). In addition, MCF7 cells are less sensitive to the CYP1-activating effect of tangeretin and dietary flavonoids, as CYP1 activity is reduced following removal of TCDD, whereas basal activity of CYP1 enzymes in MCF7 cells is a lot lower than that in MDA–MB–468 cells (Androutsopoulos et al., 2008, 2009a). Furthermore, MCF7 cells are less sensitive compared with MDA–MB–468 cells to the anti-proliferative action of flavonoids and their metabolites (Androutsopoulos et al., 2009a,b; Wilsher et al., 2017).

Fig. 6. Tangeretin induces CYP1 activity and CYP1A1/CYP1B1 protein in human breast cancer cells. Tangeretin (3, 10, 30 μM) was incubated with MCF7 and MDA–MB–468 cells for 24 h and CYP1 activity was measured by the EROD assay as described in materials and methods. (A) MDA–MB–468 cells (B) MCF7 cells (C) Western immunoblotting of CYP1A1/CYP1B1 protein expression in MDA–MB–468 cells pretreated with Tangeretin (3, 10, 30 μM) for 24 h and (D) Western immunoblotting of CYP1A1/CYP1B1 protein expression in MCF7 cells pretreated with tangeretin (3, 10, 30 μM) for 24 h. TCDD was used as a positive control at 10 nM. The experiments were conducted at least 3 independent times and error bars indicate STDEV of the mean.
and CYP1A enzyme activity via direct binding to the AhR in MCF7 breast cancer cells (Colino et al., 1999, 1998). It was suggested that the lipophilicity of these natural compounds that is determined by their methoxy substitutions affected their binding to the AhR and consequently the induction of CYP1 enzyme activity. It is important to note that the induction of CYP1B1 protein by tangeretin is shown for the first time as previous studies were mainly focused on the interactions of CYP1A1 and CYP1A enzymes with dietary flavonones (Canivenc-Lavier et al., 1996; Colino et al., 1999, 1998).

In conclusion, the present study investigated the anticancer effects of tangeretin with regard to CYP1-mediated metabolism, CYP1–enzyme induction and inhibition of cellular proliferation of human breast cancer cells. The data demonstrate that tangeretin is activated in human breast cancer cells due to CYP1-mediated conversion to the metabolite TP1 that in turn results in G1 arrest of the cells (Fig. 7). These effects were absent in normal breast cells that do not express CYP1 enzymes. The findings suggest that tangeretin could possess therapeutic implications in breast cancer cells that express active CYP1 enzymes (Fig. 7). The clinical response of a patient to tangeretin and similar CYP1-activated prodrugs will depend on his/her endogenous levels of CYP1 expression. This is a prerequisite regarding the successful applications in breast cancer cells that express active CYP1 enzymes and CYP1A2-activated prodrugs will depend on his/her endogenous levels of CYP1 expression. This is a prerequisite regarding the successful applications in breast cancer cells that express active CYP1 enzymes as previous studies were mainly focused on the interactions of CYP1A1 and CYP1A enzymes with dietary flavonones (Canivenc-Lavier et al., 1996; Colino et al., 1999, 1998).

In conclusion, tangeretin may be considered as a selective CYP1-activated natural product that can be further evaluated for its cancer therapeutic efficacy in vivo. It is important to note that tangeretin can be used in vivo at concentrations as high as 50 mg/kg (Arivazhagan and Sorimuthu Pillai, 2014). Tangeretin has been shown to be effective in inhibiting TNBS-induced colitis and TNBS-induced body loss at a dose of 20 mg/kg in C57BL/6 mice (Eun et al., 2017), whereas it exerted no significant toxicity in vivo at a concentration of 30 mg/kg following administration to nude mice for 28 days (Zhang et al., 2015). Consequently, high doses of tangeretin may possess therapeutic activity against breast cancer.

Conflict of interest
None declared.

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