Bioactivation of the citrus flavonoid nobiletin by CYP1 enzymes in MCF7 breast adenocarcinoma cells

Somchaiya Surichan a, Vasilis P. Androutsopoulos b,⇑, Stavros Sifakis c, Eleni Koutala b, Aristidis Tsatsakis b, Randolph R.J. Arroo a, Michael R. Boardera

a De Montfort University, Leicester School of Pharmacy, Leicester LE1 9BH, UK
b University of Crete, Centre for Toxicology, Medical School, Heraklion 71003, Greece
c University of Crete, Department of Gynecology and Obstetrics, Medical School, Heraklion 71003, Greece

ARTICLE INFO

Article history:
Received 1 June 2012
Accepted 19 June 2012
Available online 26 June 2012

Keywords:
Nobiletin
Citrus flavonoid
Antiproliferation
MCF7 cells
CYP1-mediated metabolism
CYP1 expression

Abstract

Recent studies have demonstrated cytochrome P450 CYP1-mediated metabolism and CYP1-enzyme induction by naturally occurring flavonoids in cancer cell line models. The arising metabolites often exhibit higher activity than the parent compound. In the present study we investigated the CYP1-mediated metabolism of the citrus polymethoxyflavone nobiletin by recombinant CYP1 enzymes and MCF7 breast adenocarcinoma cells. Incubation of nobiletin in MCF7 cells produced one main metabolite (NM1) resulting from O-demethylation in either A or B rings of the flavone moiety. Among the three CYP1 isoforms, CYP1A1 exhibited the highest rate of metabolism of nobiletin in recombinant CYP1 microsomal enzymes. The intracellular CYP1-mediated bioconversion of the flavone was reduced in the presence of the CYP1A1 and CYP1B1-selective inhibitors α-naphthoflavone and acacetin. In addition nobiletin induced CYP1 enzyme activity, CYP1A1 protein and CYP1B1 mRNA levels in MCF7 cells at a concentration dependent manner. MTT assays in MCF7 cells further revealed that nobiletin exhibited significantly lower IC50 (44 µM) compared to cells treated with nobiletin and CYP1A1 inhibitor (69 µM). FACS analysis demonstrated cell cycle block at G1 phase that was attenuated in the presence of CYP1A1 inhibitor. Taken together the data suggests that the dietary flavonoid nobiletin induces its own metabolism and in turn enhances its cytostatic effect in MCF7 breast adenocarcinoma cells, via CYP1A1 and CYP1B1 upregulation.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Cytochrome P450s are haem-containing enzymes that promote the oxidative metabolism of various drugs, xenobiotics and endogenous substrates. The most common reactions catalyzed by cytochrome P450s are hydroxylation and dealkylation reactions that occur on a structurally diverse number of functional groups (aromatic groups, aliphatic chains). The CYP1 family of cytochrome P450s consists of the extrahepatic members CYP1A1 and CYP1B1 and the third member CYP1A2 that is present in the liver (Androutsopoulos et al., 2009c). CYP1 family enzymes metabolize amongst other compounds nitrosoamines, polycyclic aromatic hydrocarbons (PAHs), oestrogens, prostaglandins and polyphenolics (Androutsopoulos et al., 2009c; Choudhary et al., 2004). Oxidative metabolism of CYP1 substrates often results in activation of the metabolite compared to the corresponding parent compound. Thus procarcinogens such as nitrosoamines and PAHs are converted by CYP1 enzymes to carcinogenic metabolites, whereas some endogenous substrates are metabolized to biologically active cell mediators such as the conversion of arachidonic acid to hydroxyeicosatetraenoic and epoxygenicosatetraenoic fatty acids by CYP1B1 (Shimada and Fujii-Kuriyama, 2004; Nebert and Dalton 2006; Choudhary et al., 2004).

With respect to flavonoid–polyphenols and CYP1 enzymes interactions two recent hypotheses have been established over the last decade. Flavonoids are phytochemicals with anticancer activities and may be considered as inhibitors of cytochrome P450 CYP1 enzymes that block the metabolism of several procarcinogens to their ultimate carcinogenic products, or as CYP1 substrates undergoing selective bioactivation to cell cycle inhibiting metabolites in tumor cells (Kim et al., 2010; Zhang et al., 2008; Delaney et al., 2002; Doostdar et al., 2000; Chang et al., 2006; Potter et al., 2002; Androutsopoulos et al., 2010; Arroo et al., 2009). Based on the structural diversity of each polyphenolic moiety, in terms of hydroxyl or methoxy substitutions, certain flavonoids...
may present with simultaneous inhibitor- and substrate-like features (Androutsopoulos et al., 2010; Androutsopoulos et al., 2011). For example acacetin or 4’methoxy, 5,7 dihydroxy flavone inhibits the CYP1A1-mediated dealkylation of 7-ethoxyresorufin strongly, while it is metabolized to the flavone apigenin by CYP1A1 weakly (Androutsopoulos et al., 2011). Generally flavonoids with multiple hydroxyl groups are considered more effective CYP1 inhibitors, whereas flavonoids with multiple methoxy groups are thought to exhibit higher rate of CYP1 metabolism (Androutsopoulos et al., 2010). Although the interactions of polyhydroxylated and monomethoxyxylated flavonoids with CYP1 enzymes, in terms of CYP1-inhibition or CYP1-metabolism have been well defined (Doostdar et al., 2000; Androutsopoulos et al., 2008; Androutsopoulos et al., 2009c), the metabolism of fully methylated flavonoids by CYP1A1 and CYP1B1 remains poorly understood. Fully methylated flavonoids such as the compounds sinisetin, nobiletin and tangeretin constitute a major part of the daily diet, as they are present in various citrus fruits (Stuetz et al., 2010). In addition their high lipophilic nature improves their bioaccumulation and half-life in vivo, by reducing phase II metabolism occurring at free hydroxyl groups (Walle, 2007). A recent study undertaken by our group investigated the metabolism of the citrus flavonoid tangeretin by recombinant CYP1 enzymes and rat hepatocytes stimulated by EGF (Cheng et al., 2011). Tangeretin was converted to the metabolite 4-hydroxy tangeretin by a single demethylation step and this resulted in inhibition of phosphorylation of p70S6K (Cheng et al., 2011). In the present study we sought to explore the metabolism of the citrus polymethoxyflavone nobiletin, by recombinant human CYP1 enzymes. The latter differs to tangeretin by a single CYP1 enzymatic activity was determined using the EROD assay, as previously described (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009d). MCF7 cells (3 x 10^6 cells/well) were plated out in a 96-well plate and incubated at 37°C 5%CO2, 95% air and were passaged using trypsin-EDTA, as described previously (Androutsopoulos et al., 2009a). Cultured cells were routinely passaged every 3–4 days.

2.2. Cell culture

MCF7 were maintained in RPMI-1640 containing 10% (v/v) heat inactivated (56°C for 45 min to inactivate complement) fetal calf serum at 37°C, 5%CO2,95% air and were passaged using trypsin-EDTA, as described previously (Androutsopoulos et al., 2009a). Cultured cells were routinely passaged every 3–4 days.

2.3. Metabolism in MCF7 cells

Nobiletin (10 μM) was added in MCF7 cells, cultured in 775 flasks that were pre-treated with either DMSO (0.1%) or 10 nM TCDD for 24 h. Samples were taken repassage from media and cells and compounds with their metabolites were extracted with the addition of methanol. The concentration of nobiletin was measured at specific time points (3, 6, 9, 24 h) from both media and cellular extracts. Media aliquots were mixed with 1% acetic acid in methanol solution in 1:1 ratio and centrifuged for 15 min at 13,000 rpm at 4°C to remove proteins and cellular debris. The supernatants were collected and analyzed with HPLC.

The cells were washed 3 times with PBS and lysed with 1% acetic acid in methanol/water solution at 4°C. The cells were freeze-thawed for at least 3 times at −20°C and pelletted thoroughly. The resulting liquid-sample was centrifuged for 15 min at 13,000 rpm at 4°C to remove proteins and cellular debris. The supernatants were collected and analyzed with HPLC. Controls were prepared by incubation of compound without cells.

2.4. Incubation with recombinant CYP1 enzymes

Citrus flavonoids (10 μM) were incubated with CYP microsomes under the following conditions: CYP (20 pmol mL−1), NADPH (0.5 mM), and MgCl2 (0.5 mM) in phosphate buffer (pH 7.4, 20 mM) at 37°C in a humidified incubator (Androutsopoulos et al., 2008). Samples (100 μL) were taken at 0, 5, 10, 15, 20, 25 min and the reaction terminated immediately by addition of an equal volume of ice-cold methanol. Following centrifugation (13,000 rpm, 4 min, 4°C), the supernatants were analysed by HPLC or LC–MS. Controls were prepared by incubation of nobiletin with control microsomes containing empty vector or incubations with CYP1 enzymes without NADPH.

2.5. Quantification of compounds

Denatured, pooled microsomes were spiked with compounds (stock 100 μM in DMSO) to give a final calibration concentration of 1–10 μM, then the calibration standards were extracted by methanol and centrifuged (similar as incubated samples) before analysis using HPLC. Calibration curves were linear in the concentration range used with R2 > 0.995.

2.6. HPLC and LC–MS analysis

Analysis of nobiletin was conducted on a Perkin Elmer HPLC series 200 analytical system using a method adapted from previous studies (Androutsopoulos et al., 2008). Briefly the following analytical conditions were used: Phenomenex Luna 5 μC18 (250 x 4.6 mm) analytical column at 37°C, flow rate of 1 ml/min, UV detection at wavelengths of 330 nm. The mobile phase consisted of 30% solvent A (0.5% acetic acid and 1% acetonitrile in water) and 70% solvent B (0.5% acetic acid and 4% acetonitrile in methanol). A linear gradient was initiated immediately on the start of analysis with solvent B rising to 90% over 10 min and held at 90% for 2 min before returning to the initial conditions (using). A re-equilibration time of 8 min was allowed between each sample analysis. The retention time of nobiletin was 16.90 min. Mass spectrometry analysis of nobiletin was conducted on an Agilent 1100 Series HPLC with a photodiode array detector and a MSD Trap XCT system. The LC conditions were the same as described above. The photodiode array detector was set to 330 nm. The LC flow was directed from photodiode array detector into the mass spectrometer without stream splitting. Nitrogen was used as the nebulizing gas at 60 psi and as drying gas with the flow rate of 10L/min at 350°C. Mass spectra were recorded in electrospray ionisation (ESI) positive mode. Molecular ion ([M + H]^+) was detected in the range of 200–500 using an auto-MS mode. The MS data were analysed using LC/MS Trap software 5.3.

2.7. EROD assay

CYP1 enzymatic activity was determined using the EROD assay, as previously described (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009d). MCF7 cells (3 x 10^6 cells/ml) were plated out in a 96-well plate and incubated at 37°C 5%CO2 for 24 h. Nobiletin was added at a final concentration range of 0.001–100 μM, for 24 h. The medium was then removed and the cells washed once with 200 μL of PBS. 200 μL of medium containing 5 μM ethoxyresorufin and 1.5 mM salicylamide were added to each well and the plates were further incubated for 1 h. The reaction was terminated by addition of equal volumes of methanol to the wells. The samples were centrifuged at 3,500 rpm at 4°C for 15 min. The resorufin produced was measured using a SpectraMax fluorescence plate reader with an excitation and emission wavelength at 530 and 590 nm.

2.8. Western blotting

MCF7 were plated out in 6-well plates at a density of 3 x 10^6 cells/ml and incubated at 37°C for 24 h in the presence of flavonoids. The medium was removed and the cells were lysed with 100 μL of lysis buffer (Sigma, Poole) containing protease inhibitor cocktail and DI-dithiothreitol (DTT, 1 mM), sonicated on ice for 5 min and finally centrifuged at 13,000 rpm at 4°C for 10 min. Protein concentration was estimated at 0.7 mg/ml for each sample and the protein lysate was mixed with sample buffer at 1:1 ratio. Samples were heated at 100°C for 5 min and then loaded on an acrylamide gel, at a ratio of 10% for the resolving and 5% 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 (polyvinylidene fluoride) membrane. The membrane was incubated in 10% milk/0.05% TBST (Tris-Rorate Tween 20) at room temperature for 1 h by continuous shaking and further with primary antibody for CYP1A detection, diluted in 5% milk/0.05% TBST at 4°C overnight. The membrane was washed three times with 0.05% TBST and incubated with secondary antibody against HRP.
diluted in 5% milk/0.05% TBST at room temperature for 1.5 h. The membrane was finally exposed with ECL (chemiluminescence) reagents and the protein profile developed on a film.

2.9. RT-PCR

MCF7 cells were seeded in 725 flasks and treated with nobiletin for 24 h at a concentration range of 1–10 μM. Total RNA was extracted using Trizol. Briefly 1 ml of Trizol reagent was added to each flask and the resulting sample was mixed with 200 μl of chloroform and centrifuged at 14,000 rpm for 15 min at 4 °C. The top layer containing the RNA was removed, mixed with an equal volume of ice-cold isopropanol and stored at −20 °C overnight. The next morning the samples were centrifuged at 13,000 rpm for 5 min. Following centrifugation the supernatant was removed and the resulting pellet containing the RNA was further precipitated with 25 μl of 1 M H3O. Total RNA was checked for purity by spectrophotometry and gel electrophoresis. cDNA was synthesized using a Promega kit and PCR was carried out as described before (Androutsopoulos et al., 2009a).

2.10. MIT assay

MCF7 cells were plated in 96-well flat bottomed plates and treated with nobiletin at a concentration range of 0.001–100 μM and left for 96 h. Cell viability was measured spectrophotometrically using MTT as a substrate, as described in previous studies (Döhr et al., 1995).

2.11. FACS analysis

MCF7 cells were cultured in T75 flasks. The cells were serum starved for 24 h and then nobiletin was added for 48 h at 75 or 100 μM. The cells were washed once with PBS, trypsinized and collected in a sterile endopod. The pellet was washed again with PBS and fixed in 70% ethanol overnight. Propidium iodide was added to the cellular pellet at a concentration of 50 μg/ml containing RNase (100 μg/ml). Fluorescence intensity was measured in a Beckman Coulter flow cytometer.

2.12. Statistical considerations

Data are presented as the average of at least four independent measurements and analysed by paired t-test and one-way ANOVA with Dunnett’s post test as indicated, using GraphPadPrism.

3. Results

3.1. Nobiletin intracellular metabolism in MCF7 cells

The MCF7 human breast adenocarcinoma cell line has been employed in our previous studies to assess in vitro metabolism of dietary flavonoids by CYP1A1 and CYP1B1 (Androutsopoulos et al., 2009a). In order to address whether MCF7 cells metabolize nobiletin in vitro the compound was incubated for 24 h and the samples analyzed by HPLC. Incubation of MCF7 cells with nobiletin generated one major metabolic product assigned NM1 (Fig. 1A).

In order to confirm that the metabolic product of nobiletin incubation with MCF7 cells occurred via CYP1-metabolism, we examined the metabolism of nobiletin in TCDD-treated and non treated and MCF7 cells at shorter time points. Induction of CYP1 enzyme activity has been reported at 12 h in TCDD-treated MCF7 cells (Döhr et al., 1995). Therefore we would expect metabolism of nobiletin at incubation time points shorter than 24 h. In TCDD-treated MCF7 cells, the metabolite NM1 was detected after incubation with the compound for at least 1 h (Fig. 1C). In un-induced MCF7 cells, the metabolite was present after incubation for at least 6 and 9 h with nobiletin (Fig. 1B). NM1 had the same retention time in TCDD-induced and un-induced MCF7 cells, indicating that it occurred via CYP1-mediated bioconversion.

The metabolism of nobiletin in un-induced MCF7 cells was further confirmed with the use of specific CYP1 inhibitors. In the presence of the CYP1B1-selective inhibitor, acacetin (1 μM), nobiletin produced the metabolite NM1 at approximately 50% smaller quantities, whereas in the presence of the CYP1A1-selective inhibitor α-napthoflavone 20% of the total NM1 was obtained (Fig. 2A). Thus nobiletin metabolism in MCF7 cells is CYP1A1-selective.

3.2. Oxidative metabolism of nobiletin in MCF7 cells occurs by demethylation reactions

The metabolism of nobiletin was further investigated in MCF7 cells by LC–MS methodologies. LC–MS analysis revealed that the mass to charge ([M + H]+) ratio of nobiletin was 403.1 (Fig. 3). One major metabolite with a mass to charge ratio of 389.1 was identified, which corresponded to NM1 retention time in MCF7 cell lysates that were treated with nobiletin for 24 h (Fig. 3). Given that the mass of nobiletin is 402 and the mass of NM1 388 the conversion of NM1 occurs via a single demethylation step (–OCH3 to –OH).

Metabolism of nobiletin by recombinant CYP1 enzymes revealed that among the three CYP1 isoforms CYP1A1 had the highest rate of nobiletin metabolism during the 30 min period. The total concentration of nobiletin was depleted within 30 min of incubation with CYP1A1 (Fig. 2B). CYP1B1 was the weakest metabolizer, whereas CYP1A2 was of intermediate efficacy (Fig. 2B). The disappearance of nobiletin following incubation with CYP1B1 for 30 min was not statistically significant (Fig. 2B). However, the mass spectrum confirmed the presence of NM1, yet to negligible amounts (data not shown).

3.3. Nobiletin induces CYP1 activity and CYP1A1 protein in MCF7 cells

Methoxylated flavones such as eupatorin and cirsiliol were shown to increase CYP1 enzyme activity and mRNA levels in MCF7 cells (Androutsopoulos et al., 2009d). In the present study we reasoned that nobiletin, carrying several methoxy groups, will also be capable of inducing CYP1 enzyme activity. Given that HPLC and LC–MS metabolic profile analysis, showed the presence of NM1 in MCF7 cells, a nobiletin CYP1-catalyzed conversion product, we investigated further the CYP1-induction mediated by nobiletin by EROD assays and Western immunoblotting. Treatment of MCF7 cells with 10 μM of nobiletin resulted in a time dependent increase in EROD activity. The maximum induction of 10 μM of the flavone was noted at 24 h, whereas a significant change in CYP1 activity was evident after 6 h incubation with the compound (Fig. 4B). Nobiletin was nearly 2-fold less active than 10 nM of TCDD (Fig. 4A). Moreover, dose-response EROD assays were performed, to identify the concentration required for maximum induction at the 24 h period. EROD activity of MCF7 cells peaked at 30 μM of nobiletin (Fig. 5A). The IC50 (concentration required for 50% induction) calculated from sigmoidal-dose response curves that were fitted to the data points obtained, was ~1 μM for nobiletin (Fig. 5B).

EROD activity is indicative of all three CYP1 isoforms (CYP1A1, 1B1 and 1A2). As a result, Western immunoblotting was employed to confirm that CYP1A1 is the major contributor among the other two isoforms involved in the induction process. Probing of MCF7 cell lysates that were pretreated with 10 μM of nobiletin for 3, 6, 9 and 24 h, with a primary CYP1A1 antibody, led to an increase in CYP1A1 protein, as it was previously seen with EROD activity (Fig. 6A). The maximum increase in CYP1A1 protein was noted at 24 h, whereas the concentration required for peak-induction was 30 μM (Fig. 6A). Of note is that at the latter concentration nobiletin exhibited equal capacity in inducing CYP1A1 protein with 10 nM of the potent inducer TCDD.

3.4. Nobiletin induces CYP1B1 mRNA levels in MCF7 cells

The effect of nobiletin on the expression of the second CYP1 isoform CYP1B1 was further examined by RT–PCR in MCF7 cells. Nobi-
letin was shown to increase CYP1B1 mRNA levels at concentrations of 5 and 10 μM (Fig. 6B and C). This effect was comparable to the induction caused by the potent inducer TCDD (Fig. 6B). The ligand activated factor ARNT was not affected by such treatment (Fig. 6B). The mRNA levels of the receptor AhR in MCF7 cells were very low and barely detectable, compared to the mRNA levels of CYP1B1 where a stronger signal was obtained by RT-PCR (data not shown).

3.5. Nobiletin antiproliferative effect in MCF7 cells

Flavonoids containing one or more methoxy groups (e.g. eupatorin, diosmetin) were shown to undergo selective bioactivation in tumor cell lines by CYP1 enzymes (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009a; Androutsopoulos et al., 2009b). To further test the hypothesis that CYP1A1 metabolism of nobiletin in MCF7 cells may affect cell proliferation the cytotoxic and cytostatic profile of the latter was investigated by MTT assay and FACS analysis. The compound exhibited an IC₅₀ of 44 μM in MCF7 cells, and of 69 μM in the presence of the CYP1-inhibitor α-napthoflavone (Table 1). This effect was confirmed by FACS analysis. Treatment of MCF7 cells with 100 μM of nobiletin for 48 hrs produced a G1 block in the cell cycle that was accompanied with a reduction of the percentage of cells in S and G2/M phases (Fig. 7B). In the presence of the CYP1A1 inhibitor α-napthoflavone a weak reduction in the percentage of G1 arrested cells (approximately 3–4%) was obtained, whereas when a lower concentration of 75 μM of nobiletin was used this effect was more profound (Fig. 7B). At 75 μM of nobiletin 75% of MCF7 cells were arrested at G1 after 48 h, while when α-napthalflavone was present this effect was reduced to 65% (Fig. 7A and B). Similarly an increase in the percentage of cells in G2/M phase was obtained in the presence of the inhibitor, compared to MCF7 cells treated with 75 μM nobiletin alone (Fig. 7B). HPLC analysis revealed that at the latter concentration (75 μM) almost one third of parent nobiletin was metabolized, whereas in the presence of α-napthalflavone this conversion was substantially reduced (Fig. 7C).

4. Discussion

Nobiletin is a polymethoxylflavone, found in the peel of Citrus plants with well documented cancer chemopreventive activity.
Fig. 2. Metabolism of nobiletin in MCF7 cells in the presence of CYP1 inhibitors and in recombinant CYP1 microsomes. (A) Nobiletin (10 μM) was incubated for 24 h in the absence of any inhibitor and in the presence of 1 μM acacetin and 1 μM α-napthoflavone and the results analyzed by HPLC. (B) Disappearance of nobiletin after incubation with recombinant CYP1 microsomes for 30 min. The concentration of nobiletin was measured by HPLC as described in Methods. Results are expressed as mean ± STDEV for n = 3 determinations.

Fig. 3. Metabolism of nobiletin (10 μM) by MCF7 cells as analyzed by LC–MS. Black trace: medium without cells. Dark grey trace: MCF7 cells 24 h-incubate.
Numerous studies have reported the ability of the latter compound to inhibit proliferation of cancer cells and initiation of carcinogenesis in various in vitro and in vivo models. The mechanism of action involves inhibition of azoxymethane-induced colon and large bowel carcinogenesis (Miyamoto et al., 2010; Suzuki et al., 2004), inhibition of peritoneal dissemination of human gastric carcinoma (Minagawa et al., 2001) and anti-invasive activity through down-regulation of MMP expression and MEK inhibition, inhibition of MEK1/2 activation of PKC\(^\text{bII/e-JNK}\) pathway (Miyata et al., 2004) and induction of apoptosis via downregulation of Bcl-2 and upregulation of Bax and p53 (Luo et al., 2008). Despite the vast majority of studies that focused on the chemopreventative ability of nobiletin, its interaction with cytochrome P450 CYP1 enzymes remains poorly understood. We report for the first time that nobiletin undergoes oxidation by CYP1 enzymes, by inducing its own metabolism through upregulation of CYP1 enzyme expression in MCF7 human breast adenocarcinoma cells.

The metabolism of nobiletin by CYP1 enzymes in MCF7 cells has not been demonstrated to date. However metabolism of structurally similar flavonoids in cancer cell lines has been documented earlier. For example, the monomethoxylated flavone diosmetin is converted to the flavone luteolin, in MCF7 cells pretreated with TCDD (Androutsopoulos et al., 2009a). Oxidative metabolism of hydroxylated isoflavones was demonstrated in MCF7 cells by CYP1A1 and CYP1B1 (Atherton et al., 2006). Moreover, the flavones eupatorin and genkwanin are demethylated to cirsiliol and apigenin respectively, in MDA-MB 468 cells, whereas the monomethoxylated flavone acacetin is demethylated to the flavone apigenin by recombinant CYP1 enzymes (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009e; Androutsopoulos et al., 2011). The polymethoxylated flavone tangeretin has further been shown to undergo metabolic oxidation in vivo (Nielsen et al., 2000).

The results presented indicate that CYP1-bioconversion of parent nobiletin can enhance the cytostatic effect of this compound in MCF7 cells, as demonstrated by FACS analysis. Generally nobiletin is considered as a flavonoid with weak cytostatic properties towards progression of breast and colon cancer cells (Morley et al., 2007). Morley and coworkers reported that nobiletin induces G1 arrest in MCF7 cells at high concentrations varying from 60 to 200 \(\mu\)M (Morley et al., 2007). The results presented herein are in agreement with these findings. Although the concentrations required for nobiletin to achieve its therapeutic effect are relatively high, the optimum pharmacokinetic properties of fully methoxylated flavonoids ensure that the concentrations utilized in vitro are also achievable in vivo (Walle, 2007). For examples in hamsters that were fed a 1% tangeretin/nobiletin diet the total parent flavonoid levels reached the concentration of 16–67 \(\mu\)M in liver and 21 \(\mu\)M in plasma, following 35 days of feeding (Kurowska and Manthey, 2004). In addition methoxylated flavonoids are expected to reach 100-fold higher concentration levels in plasma compared to hydroxylated flavonoids that exhibit approximately 0.2 \(\mu\)M plasma concentrations (Walle, 2007). A recent study has underlined that the plasma concentration of flavonoids can increase significantly after daily supplementation for a prolonged period (Heinz et al., 2010). Moreover our data demonstrated that CYP1 enzymes can enhance further the cytostatic action of nobiletin, as in the presence of the CYP1A1 inhibitor \(\alpha\)-naphthoflavone G1 arrest caused by nobiletin was partially attenuated. Thus it is possible that nobiletin can exert tumor suppressing growth effects via CYP1-metabolism after a prolonged daily dietary intake.

**Fig. 4.** Induction of EROD activity in MCF7 cells by nobiletin. (A) Nobiletin (10 \(\mu\)M) was incubated with MCF7 cells for 24 hrs and EROD activity measured by fluorescence as described in Methods. TCDD (10 nM) was incubated in separate sample for 24 h. (B) EROD activity of nobiletin (10 \(\mu\)M) treated MCF7 cells at different time points. Results are presented as mean ± STDEV for n = 4 determinations. *Statistically different than control (<0.001).

**Fig. 5.** Dose response curves of EROD induction in MCF7 cells by 24 h treatment of nobiletin. (A) EROD activity as expressed in pmol/min/10^6 cells, calculated by resorufin and MTT standard curves. (B) Estimation of EROD IC\(_{50}\) (concentration required for 50% induction) by nobiletin. Data points were fitted to sigmoidal dose response curves (Prism software). Results are indicative of n = 4 independent determinations.
A recent study reported on the biological activity of 4'-demethylnobiletin in enhancing PKA/ERK/CREB signaling (Al Rahim et al., 2009). 4'-demethylnobiletin is the active metabolite of nobiletin identified in rats and mice and is responsible for phosphorylation of ERK and CREB thus enhancing CRE-mediated transcription by activating a PKA/MEK/ERK pathway, in a similar manner as nobiletin, in hippocampal neurons (Al Rahim et al., 2009). More importantly, 3',4'-didimethyl-nobiletin which is the major metabolite of nobiletin identified in mouse urine, possesses markedly stronger antitumorigenic effect than nobiletin (Lai et al., 2008). 3',4'-didimethyl-nobiletin was recently shown to exert a protective effect against TPA-mediated epithelial carcinogenesis via downregulation of inflammatory and proliferative pathways such as PI3K/Akt/IkB, MAPK, PKC signaling pathways, as well as STAT3, NFkB and AP-1 transcription factors (Lai et al., 2008). Taken together such studies indicate the important role of bioactive oxidative metabolism of nobiletin that results to 4'-O-demethylated or other hydroxylated products. The active metabolites increase the pharmacological properties of the corresponding parent flavonoid-compounds (Al Rahim et al., 2009; Lai et al., 2008). CYP1A1 and CYP1B1 enzymes are believed to play a critical role in these metabolic pathways (Androutsopoulos et al., 2010).

Several naturally occurring flavonoids have been reported as AhR agonists. Such compounds are capable of inducing CYP1A1 in cancer cell line models through the AhR-ARNT-DRE axis (Androutsopoulos et al., 2009c). Previous work in the late 90s by Ciolino and colleagues focused on the CYP1A1 inducing capacity of hydroxylated flavonoids and the mono-methoxylated flavone diosmetin (Ciolino et al., 1998; Ciolino et al., 1999; Ciolino and Yeh, 1999). Recent results from our group indicate that polymethoxylated flavones such as eupatorin can induce CYP1A1 and CYP1B1 enzymes in MCF7 cells (Androutsopoulos et al., 2009d). It is noteworthy at 30 μM nobiletin induced CYP1A1 expression at comparable levels to those produced by 10 nM of the potent CYP1 inducer, TCDD. In addition it must be emphasized that some flavonoids such as genistein are capable of downregulating the expression of MMP-2, MMP-9, MT-MMP 1, 2 and 3 genes in human breast cancer cells (Kousidou et al., 2006). This causes inhibition of invasion properties of the cells as the above proteins are involved in the degradation of all extracellular matrix macromolecules of the supporting stroma and consequently in the invasive and metastatic potential of the cells (Kousidou et al., 2005). The effect is possibly attributed to inhibition of tyrosine kinase activity as anti-metastatic activity towards breast cancer cells has been noted for the tyrosine kinase inhibitor STI-571 (Roussidis et al., 2004). CYP1-mediated metabolism of nobiletin to NM1 in breast cancer cells may produce anti-

![Fig. 6. Induction of CYP1 enzymes by nobiletin in MCF7 cells. (A) Dose- and time- response of nobiletin treatment of MCF7 cells on CYP1A1 protein induction. CYP1A1 microsome standard was used as positive control and MCF7 cell lysate treated with 0.1% DMSO for 24 h as negative. TCDD was added at a concentration of 10 nM for 24 h. Nobiletin was incubated at different concentration points for 24 h and for different time points at 10 μM. Western immunoblotting was carried out as described in Methods. (B) Concentration-response of nobiletin treatment of MCF7 cells on CYP1B1 and ARNT. RT-PCR was performed as described in Methods. (C) Relative mRNA levels of CYP1B1 and ARNT following nobiletin treatment at 0, 1, 5 and 10 μM for 24 h. Results are expressed as mean ± STDEV for n = 3 determinations. *Statistically different than control (<0.05.).](image)

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cytotoxicity of nobiletin in MCF7 cells in the presence and absence of CYP1A1 inhibitor α-naphthoflavone. MCF7 cells were incubated with nobiletin for 96 h and cell viability measured as described in Methods. α-naphthoflavone was used as a CYP1A1 inhibitor at 1 μM. Results are expressed as mean ± STDEV for at least n = 4 determinations. IC50s are in μM.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nobiletin</td>
<td>Nobiletin + α-naphthoflavone</td>
</tr>
<tr>
<td>IC50</td>
<td>44 ± 3.1 μM</td>
</tr>
</tbody>
</table>

* Statistically different than control, i.e. nobiletin without any inhibitor (<0.05).
invasive effects additional to cytostatic effects. This is a point worthy of further investigation in future studies.

Natural dietary flavonoids have been investigated mainly in terms of their inhibitory effects on CYP1-procarcinogen activation and CYP1-mediated transcription. However there is growing evidence that such compounds undergo selective metabolism to antiproliferative agents by the extrahepatic enzymes CYP1A1 and CYP1B1. The results presented in this study reinforce these findings and provide a novel mechanism of antitumor action of the citrus flavone nobiletin, which is based on CYP1 enzyme induction and CYP1-mediated metabolism.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This study was sponsored by Research and Development Institute, The Government Pharmaceutical and the Prefecture of Heraklion Grant Code ELKE 2600.

References


