Nobiletin bioactivation in MDA-MB-468 breast cancer cells by cytochrome P450 CYP1 enzymes

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ABSTRACT

Nobiletin is a fully methoxylated flavone that has demonstrated anticancer activity via multiple modes of action. In the present study, the metabolism and further antiproliferative activity of nobiletin was evaluated in the CYP1 expressing human breast cancer cell line MDA–MB–468 and the normal breast cell line MCF10A. Nobiletin was metabolized in MDA–MB–468 cells to a single-demethylated derivative assigned NP1. This metabolite was absent in MCF10A cells that did not express CYP1 enzymes. Nobiletin exhibited submicromolar IC50 (0.1 ± 0.04 μM) in MDA–MB–468 cells, whereas it was considerably less active in MCF10A cells (40 μM). In MDA–MB–468 cells that were coincubated with the CYP1 inhibitor acacetin, an approximately 300-fold increase was noted in the IC50 (30 ± 2.4 μM) of nobiletin. In the presence of the CYP1 inhibitor acacetin, the conversion of nobiletin to NP1 was significantly reduced in MDA–MB–468 cells. Furthermore, a significant increase was noted in the population of the cells at the G1 phase, following treatment with nobiletin (10 μM) for 24 h compared with the control cells treated with DMSO (0.1%) alone (55.9 ± 0.14 vs. 45.6 ± 1.96), whereas the cell cycle of MCF10A cells was not significantly altered under the same treatment conditions. Taken collectively, the results suggest that nobiletin is selectively bioactivated in MDA–MB–468 breast cancer cells via metabolism by the cytochrome P450 CYP1 family of enzymes.

1. Introduction

Flavonoids comprise a class of natural polyphenolic compounds with multiple anticancer properties (Zafar et al., 2017; Marrelli et al., 2015). Nobiletin is a flavonoid, which is a major constituent of citrus fruits, including oranges and tangerines (Kim et al., 2016; Ho and Kuo, 2014). Nobiletin can be found in the plants of the genus Citrus, and is particularly more abundant in the peel compared to the other edible parts of the fruit (Manthey and Grohmann, 2001). This compound has been examined extensively due to its potential cancer preventative activity. Nobiletin caused G2/M arrest in Hs578T breast cancer cells via metabolism by the cytochrome P450 CYP1 family of enzymes.

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

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MCF7 breast cancer cells (Surichan et al., 2012). The interactions of structurally similar dietary flavonoids with CYP1 enzymes have been investigated by recent studies and a cancer therapeutic effect of these compounds has been demonstrated in breast and liver cancer cells via their intracellular metabolism (Androutsopoulos et al., 2008, 2009a, 2009b, 2009c, 2009d, 2010; Androutsopoulos and Spandidos, 2013; Androutsopoulos and Tsatsakis, 2014). Flavonoids are oxidized to their corresponding hydroxylated derivatives via aromatic hydroxylation and/or demethylation reactions occurring on the B and/or A rings of the polyphenolic moiety and the resulting metabolites inhibit cancer cell growth at equivalent and/or higher levels compared with the corresponding parent compounds (Androutsopoulos et al., 2008, 2009a, 2009b, 2009d; Androutsopoulos and Spandidos, 2013; Androutsopoulos and Tsatsakis, 2014). Nobiletin was previously shown to induce CYP1 enzyme activity and expression in MCF7 breast cancer cells that in turn caused metabolism of this compound to a major metabolite by a one step demethylation reaction (Surichan et al., 2012).

In the present study, the metabolism of nobiletin was investigated in the CYP1 expressing breast cancer cell line MDA–MB–468 that expresses constitutively CYP1 enzymes and the normal breast cell line MCF10A that is devoid of CYP1 expression. The data indicated that nobiletin was metabolized to the same major conversion product as previously described that resulted in potent inhibition of breast 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), α-naphthoflavone, acacetin, tissue culture reagents and tissue culture media were purchased from Sigma Aldrich (St Louis, MO, USA). Nobiletin was purchased from Extrasynthese (Lyon, France).

2.2. Cell culture

Triple negative MDA–MB–468 cells were maintained in RPMI without phenol red in the presence of 10% FBS and 2 mM glutamine. MCF10A cells were a kind gift from Dr Paul Butler (De Montfort University, UK). The cells were kept in liquid nitrogen at passage 2 and stored in cryovials. The cells were thawed and cultured accordingly. Passage 3 cells were stored at −80°C for subsequent experiments. The cells were cultured until passage 10 and then replaced by passage 3 cells. MCF10A cells were grown in DMEM:F12 that contained the aforementioned supplements and additionally insulin (10 μg/ml), hydrocortisone (500 ng/ml) and EGF (20 ng/ml). The cells were grown in a humidified incubator at 37°C in 5% CO2/95% air and passaged every 3–4 days using trypsin/EDTA (0.25% v/v).

2.3. Metabolic studies

MDA–MB–468 and/or MCF10A cells were incubated with 10 μM of nobiletin for 24 h or longer time points. At 24 h or 96 h time points, cell aliquots were obtained and the extraction of nobiletin and its metabolite(s) was conducted by addition of equal volumes of 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.

2.4. HPLC analysis

The methodology has been described in detail in previous publications (Androutsopoulos et al., 2008, 2009a). The separation was conducted on a Luna C18 4.6 × 150 mm 5 μm column with a mobile phase consisting of solvents A and B. Solvent A comprised 1% acetonitrile and 0.5% acetic acid in H2O and solvent B comprised 4% acetonitrile and 0.5% acetic acid in CH3OH. The separation was conducted using gradient mixture of the solvents A and B as follows: 60% solvent A and 40% solvent B at time = 0 min and 10% solvent A and 90% solvent B at time = 10 min. The composition of the solvents was adjusted to the initial conditions with 8 min remaining for column equilibration after each run. The detection of nobiletin concentration was monitored using a Waters Series 200 UV detector (Waters, Hertfordshire, UK) at 327 nm. The concentration of nobiletin 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 nobiletin was 90% and the retention time using the aforementioned parameters was 16.8 min.

2.5. LC-MS analysis

Mass spectrometry analysis of nobiletin was conducted on an Agilent 1100 Series LC/MSD Trap XCT systems with a photodiode array detector as described previously (Surichan et al., 2012). The LC parameters were the same as those used for the HPLC 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. 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. Molecular ions ([M+H]+) were detected in the range of 200–500 using an auto–MS mode. The MS determination was acquired in electropray ionisation (ESI) positive mode. The MS data were analyzed using LC/MSD Trap software 5.3. The Retention time (Rt) of nobiletin under these conditions was 16.8 min.

2.6. MTT assay

MDA–MB–468 and MCF10A cells (1 × 10⁴ cells/ml) were seeded in 96–well plates and the antiproliferative effect of nobiletin was examined as described previously (Androutsopoulos et al., 2008). Inhibition experiments were conducted in the presence of 0.5–1 μM α-naphthoflavone and/or acacetin.

2.7. FACS analysis

MDA–MB–468 and MCF10A cells were grown in T25 flasks and were treated with nobiletin at a concentration of 10 μM in the presence and/or absence of CYP1 inhibitors (0.5–1 μM) for 24 h. The cells were subsequently washed with PBS, removed by trypsin/EDTA and centrifuged at 3,500 rpm for 5 min. The cells were fixed in 70% ethanol for at least 24 h at −20°C. The cells were incubated with PI (50 μg/ml) and RNase (100 μg/ml) 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. 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. Error bars represent mean ± STDEV for at least n = 3 determinations.

3. Results

3.1. CYP1 enzymes metabolize nobiletin in MDA–MB–468 human breast cancer cells

The previous study conducted by our research group investigated the metabolism of nobiletin in MCF7 cells and/or MCF7 cells that were pre-induced with the potent CYP1 inducer TCDD (Surichan et al., 2012). In the present study, the investigation of nobiletin was carried out in
MDA–MB–468 cells that express constitutively CYP1 enzymes in the absence of an inducer (Androutsopoulos et al., 2008; Wilsher et al., 2017). Incubation of nobiletin for 24 h with MDA–MB–468 cells resulted in an apparent decrease in the concentration of this compound, while a main metabolite denoted NP1 was present (Fig. 1A). This metabolite exhibited a retention time of 15.8 min (Fig. 1A). In contrast to MDA–MB–468 cells, the incubation of nobiletin with MCF10A normal breast cells for 24 h revealed neither a significant decrease in the concentration of nobiletin, nor the production of the metabolite NP1 (Fig. 1A and B). The concentration of nobiletin was significantly reduced in MDA–MB–468 cells at the 24 h time point, whereas no significant change was noted for MCF10A cells (40 μM) (Fig. 3A). This resulted in an activation factor of approximately 400 (IC50normal/IC50tumor) exhibiting favorable selectivity towards the breast cancer cells.

The cytotoxicity of nobiletin in MDA–MB–468 cells was further investigated in the presence of the CYP1 inhibitors acacetin and α-napthoflavone (Fig. 3B). The IC50 of nobiletin was considerably increased from 0.1 ± 0.04 μM to 30 ± 2.4 μM in the presence of the CYP1 inhibitor acacetin (Fig. 3B). Similar results were noted for the use of α-napthoflavone as a CYP1 inhibitor. Furthermore, metabolic studies revealed that in the presence of the CYP1 inhibitors the conversion of NP1 from nobiletin was significantly reduced in MDA–MB–468 cells (Fig. 3C and D). The concentration of the parent compound nobiletin was reduced to a smaller extent in the presence of the CYP1 inhibitor NP1 + I cells compared with the cells that were treated with nobiletin alone for 24 h (168,921 ± 19,619 peak area vs. 397,680 ± 27,437 peak area) (Fig. 3D).

3.2. Nobiletin is bioactivated in the CYP1–expressing cell line MDA–MB–468

Previous studies have demonstrated that MDA–MB–468 cell constitutively express CYP1 enzymes compared with MCF10A cells that express little or no CYP1 protein (Androutsopoulos et al., 2008; Wilsher et al., 2017). The cytotoxicity of nobiletin was tested in the aforementioned two cell lines. This compound was considerably active in MDA–MB–468 cells and indicated a submicromolar IC50 (0.1 ± 0.04 μM), whereas it demonstrated limited toxicity in MCF10A cells (40 μM) (Fig. 3A). This resulted in an activation factor of approximately 400 (IC50normal/IC50tumor) exhibiting favorable selectivity towards the breast cancer cells.

The cytotoxicity of nobiletin in MDA–MB–468 cells was further investigated in the presence of the CYP1 inhibitors acacetin and α-napthoflavone (Fig. 3B). The IC50 of nobiletin was considerably increased from 0.1 ± 0.04 μM to 30 ± 2.4 μM in the presence of the CYP1 inhibitor acacetin (Fig. 3B). Similar results were noted for the use of α-napthoflavone as a CYP1 inhibitor. Furthermore, metabolic studies revealed that in the presence of the CYP1 inhibitors the conversion of NP1 from nobiletin was significantly reduced in MDA–MB–468 cells (Fig. 3C and D). The concentration of the parent compound nobiletin was reduced to a smaller extent in the presence of the CYP1 inhibitor NP1 + I cells compared with the cells that were treated with nobiletin alone for 24 h (168,921 ± 19,619 peak area vs. 397,680 ± 27,437 peak area) (Fig. 3D).
3.3. Nobiletin induces G1 arrest in MDA–MB–468 cells

In order to further explore the mechanism of the antiproliferative action of nobiletin in MDA–MB–468 breast cancer cells, flow cytometry analysis was employed following treatment of the cells with 10 μM of nobiletin for 24 h. Nobiletin caused G1 arrest in the cell cycle of MDA–MB–468 cells that was attenuated in the presence of the CYP1 inhibitor acacetin (Fig. 4A and B). A significant increase was noted in the population of the cells at the G1 phase, following treatment with nobiletin (10 μM) for 24 h compared with the control.
cells treated with DMSO (0.1%) alone (55.9 ± 0.14 vs. 45.6 ± 1.96, Fig. 4A and B). In the presence of the CYP1 inhibitor acacetin and the compound nobiletin, the increase in the cell population at the G1 phase was smaller and non-significant compared with the control cells (49.03 ± 0.97 vs. 45.6 ± 1.96, Fig. 4A and B). In contrast to MDA-MB-468 cells, MCF10A cells were not affected by nobiletin treatment (10 μM) for 24 h (Fig. 4C and D). Specifically, the population of MCF10A cells was not significantly altered following treatment with nobiletin for 48 h compared with the control cells (89.4 ± 1.3 vs. 88.6 ± 1.2, Fig. 4D).

4. Discussion

In the present study, the anticancer activity of nobiletin was investigated in the CYP1 expressing cell line MDA-MB-468 in terms of cytochrome P450 CYP1 metabolism. Nobiletin was metabolized to the conversion product NP1 by a single demethylation reaction in the breast cancer cell line, whereas no metabolism was noted in the normal breast cell line MCF10A. In addition, nobiletin exhibited sub-micromolar IC50 in MDA-MB-468 cells that was increased in the presence of CYP1 inhibitors, whereas it was considerably less active in...
MCF10A cells. The mechanism of action involved inhibition of cell cycle progression at the G1 phase of the cell cycle of MDA–MB–468 cells, while the data further demonstrated that in the presence of the CYP1 inhibitor acacetin, the conversion of nobiletin to NP1 was significantly reduced. The present study suggests that nobiletin is selectively bioactivated in MDA–MB–468 breast cancer cells via metabolism by the cytochrome P450 CYP1 family of enzymes. The metabolism of nobiletin by CYP1 enzymes was previously described by our research group in recombinant enzymes and in MCF7 breast adenocarcinoma cells (Surichan et al., 2012). Nobiletin metabolism by CYP1 enzymes resulted in the production of the major metabolite NP1 that resulted from a single demethylation step in the structure of nobiletin (Surichan et al., 2012). Additional metabolites were reported only in the recombinant enzyme assay, whereas in MCF7 cells the metabolism of nobiletin involved solely the production of NP1 (Surichan et al., 2012). In the current analysis, the metabolite NP1 was evident in the MDA–MB–468 cellular samples. However, in contrast to MCF7 cells that are inducible in the presence of a CYP1 inducer, MDA–MB–468 cells express constitutively active CYP1 enzymes (Androutsopoulos et al., 2009a; Androutsopoulos et al., 2008, 2009c; Surichan et al., 2012; Androutsopoulos and Tsatsakis, 2014; Wilsher et al., 2017). Therefore the metabolism of nobiletin in MDA–MB–468 cells was evident at shorter time points compared with that in the MCF7 cell line that requires induction of cytochrome CYP1 enzymes by the activation of the AhR (Surichan et al., 2012).

The identity of NP1 remains unknown, although it may be speculated that it is the 4′-demethylated derivative of nobiletin. Previous studies conducted on structurally similar flavones have demonstrated that the 4′-methoxy position of the B ring is a predominant site of metabolism of the flavones by the CYP1 family enzymes, notably CYP1A1 (Androutsopoulos et al., 2008; Androutsopoulos et al., 2009a, 2011; Androutsopoulos and Spandidos, 2013). Furthermore, the reports by Koga and co–workers have suggested that nobiletin can be metabolized by human liver microsomes that include CYP1A2 and to a lesser extent CYP1A1, to the 4′,6 and 7 positions of the flavone moiety (Koga et al., 2007, 2011). The 6 and 7 hydroxylated metabolites originated...
mainly by CYP3A metabolism, as in the presence of the CYP3A inhibitors ketoconazole and troleandomycin their formation was attenuated (Koga et al., 2011). By contrast, the production of the 4′-hydroxylated derivative of nobiletin was attenuated in the presence of the CYP1A2 inhibitor furafylline and the CYP1A1 inhibitor α-naphthoflavone, indicating that this conversion was notably catalyzed by the CYP1A family of enzymes (Koga et al., 2011). This finding agrees with the current report, as MDA–MB–468 cells have been shown to express active CYP1A1 (Androutsopoulos et al., 2008). Moreover, the contribution of CYP1B1 in the formation of NP1 cannot be ruled out, since this enzyme is expressed in MDA–MB–468 cells (Wilsher et al., 2017).

The inhibition of MDA–MB–468 cellular proliferation by nobiletin metabolism to NP1 was mediated via G1 phase cell cycle arrest. This effect was not noted in MCF10A cells. Fully methoxylated flavones such as tangeretin and nobiletin have demonstrated cell cycle arrest at the G1 phase of the cell cycle in colon and breast cancer cells (Pan et al., 2002; Wu et al., 2017; Chen et al., 2014) via a mechanism involving downregulation of cyclin–dependent kinases 2 and 4 activities and upregulation of the CDK inhibitors p21 and p27, as well as induction of apoptosis in combination with atorvastatin (Pan et al., 2002; Wu et al., 2017). It is important to note that the metabolite of nobiletin 4′-de-methyl nobiletin and/or 4′ hydroxyl nobiletin was capable of inducing G0/G1 arrest in combination with atorvastatin in HT–29 colon adenocarcinoma cells (Wu et al., 2017). With regard to breast cancer, nobiletin exhibited dose–time–dependent antitumor activity against 3 different subtypes of breast cancer cell lines, namely, MDA–MB–468, SK–BR–3 and MCF7, with the greatest inhibition observed against the MDA–MB–468 cell line (Chen et al., 2014). This type of activity was mediated via induction of cell-cycle arrest at the G0/G1 phase by suppressing ERK1/2 activity, with concomitant cyclin–D1 suppression and p21 up-regulation (Chen et al., 2014). In MDA–MB–468 cells, nobiletin further inhibited the activity of AKT and downstream mTOR (Chen et al., 2014). These data are in agreement with the present study and may explain the mechanism of the antiproliferative effect of nobiletin in MDA–MB–468 cells, as a result of a combined action of nobiletin and the metabolite NP1.

With regard to the potency of nobiletin in inhibiting the proliferation of human breast cancer cells, this compound exhibited high activity in MDA–MB–468 compared with MCF7 cells (Surichan et al., 2012). By contrast, MCF10A cells were notably unaffected as the IC50 of nobiletin noted in this cell line was 40 μM. Nobiletin exhibited an activation factor of approximately 300–fold in MDA–MB–468 cells compared with MDA–MB–468 cells that were treated with nobiletin and CYP1 inhibitors (30 μM/0.1 μM). A similar pattern was previously noted for the flavonoids sinenisten, genkwanin and eupatorin with IC50 values for MDA–MB–468 and MCF10A cells of 0.2, 1.6 and 0.5 μM and 65, 75 and 50 μM, respectively (Androutsopoulos et al., 2008, 2009d). In the presence of the CYP1 inhibitor acetamin, the IC50 values of sinenisten, eupatorin and genkwanin were considerably increased (from 0.2 to 13.5 μM for sinenisten, from 0.5 to 15 μM for eupatorin and from 1.6 to 12.5 μM for genkwanin) (Androutsopoulos et al., 2008, 2009d). The present report is in concordance with the aforementioned studies that suggest that the degree of methoxylonation 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 sinenisten and nobiletin that are penta– and hexa–methoxylated flavones respectively, exhibited submicromolar IC50 values compared to fully hydroxylated flavones such as luteolin and apigenin that indicated higher IC50 values (2 and 22 μM, respectively) (Androutsopoulos et al., 2009d; Wilsher et al., 2017).

In conclusion, the present study investigated the anticancer effects of nobiletin with regard to CYP1–mediated metabolism in human breast cancer cells. The data demonstrate that nobiletin is activated in human breast cancer cells due to CYP1–mediated conversion to the metabolite NP1 that in turn results in G1 arrest of the cells. These effects were absent in normal breast cells that did not express CYP1 enzymes. The findings suggest that nobiletin may be considered as a selective CYP1–activated natural product that can be further evaluated for its cancer therapeutic efficacy in vivo, in human cancer cells that express active CYP1 enzymes.

Conflict of interest
None declared.

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