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# 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 IC<sub>50</sub> ( $0.1 \pm 0.04 \mu\text{M}$ ) in MDA-MB-468 cells, whereas it was considerably less active in MCF10A cells ( $40 \mu\text{M}$ ). 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 ( $10 \mu\text{M}$ ) for 24 h compared with the control cells treated with DMSO (0.1%) alone ( $55.9 \pm 0.14$  vs.  $45.6 \pm 1.96$ ), 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 CYP1 family of enzymes.

**Abbreviations**

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

**Keywords**

Flavonoids, nobiletin, cytochrome P450 CYP1 enzymes, antiproliferation, breast cancer

## 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 tangerins (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 downregulation of the Akt and MAPK cell signaling pathways (Borah et al., 2017), whereas it further induced autophagy in human gastric cancer SNU-16 cells via upregulation of the glucose regulated protein GRP78 and the ER-stress related proteins IRE1- $\alpha$ , ATF-4 and CHOP (Moon and Cho, 2016). In addition, the effects of nobiletin on the metastatic spread of cancer cells have been investigated and it was reported that this compound could downregulate the enzymatic activity of MMP-9 by inhibiting the PI3K/Akt pathway and prevent the TGF- $\beta$  triggered migration and epithelial to mesenchymal transition via modulation of the Akt/GSK3 $\beta$ / $\beta$ -catenin signaling (Miyata et al., 2017; Zhang et al., 2017). Moreover, nobiletin inhibited tumor angiogenesis in MCF7 breast cancer cells via Src, FAK and STAT3 signaling and in ovarian cancer cells by inhibition of VEGF, Akt, HIF-1 $\alpha$  and NF- $\kappa$ B secretion, while it concomitantly decreased invasion, motility and migration via MMP-2 and MMP-9 downregulation in U2OS osteosarcoma cells (Sp et al., 2017; Chen et al., 2015; Cheng et al., 2016). *In vivo*, nobiletin demonstrated inhibition of colitis-associated colon carcinogenesis in azoxymethane/dextran sulfate sodium-treated mice via decreased expression of iNOS

and increased Nrf2 enzyme expression, whereas it inhibited the growth of H22 transplantable hepatocellular tumor (Wu et al., 2017; Ma et al., 2014).

In addition to the extensive profiling of nobiletin, in terms of inhibition of metastasis, invasion and proliferation, a previous study conducted by our research group demonstrated selective bioactivation of this compound by the cytochrome P450 CYP1 family enzymes in 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; Androutsopoulos et al., 2009a; Androutsopoulos et al., 2009b; Androutsopoulos et al., 2009c; Androutsopoulos et al., 2009d; Androutsopoulos et al., 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; Androutsopoulos et al., 2009a; Androutsopoulos et al., 2009b; Androutsopoulos et al., 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),  $\alpha$ -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 grown in DMEM:F12 that contained the aforementioned supplements and additionally insulin (10  $\mu$ g/ml), hydrocortisone (500 ng/ml) and EGF (20 ng/ml). The cells were grown in a humidified incubator at 37 °C in 5% CO<sub>2</sub> /95% air and passaged every 3 to 4 days using trypsin EDTA (0.25% v/v).

### *2.3 Metabolic studies*

MDA-MB-468 and/or MCF10A cells were incubated with 10  $\mu$ M 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., 2009a, Androutsopoulos et al., 2008). The separation was conducted on a Luna C18 4.6 x 150 mm 5  $\mu$  column with a mobile phase consisting of solvents A and B. Solvent A comprised 1% acetonitrile and 0.5% acetic acid in H<sub>2</sub>O and solvent B 4% acetonitrile and 0.5% acetic acid in CH<sub>3</sub>OH. 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  $\mu$ M. The assay was carried out at 37 °C and the flow rate was 1 ml/min. The average recovery for tangeretin 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 electrospray 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 ( $1 \times 10^4$  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  $\mu$ M  $\alpha$ -naphthoflavone and/or acacetin.

### *2.7 FACS analysis*

MDA-MB-468 and MCF10A were grown in T25 flasks and were treated with nobiletin at a concentration of 10  $\mu$ M in the presence and/or absence of CYP1 inhibitors (0.5–1  $\mu$ M) 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  $\mu$ g/ml) and RNase (100  $\mu$ g/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  $\pm$  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,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 (Fig. 1B). The metabolism of this compound in MDA-MB-468 cells was evident from 1 h of incubation and gradually increased at the 3, 6 and 9 h time points, respectively (Fig. 1C, D). LC-MS was further used in order to add insight in the identification of the

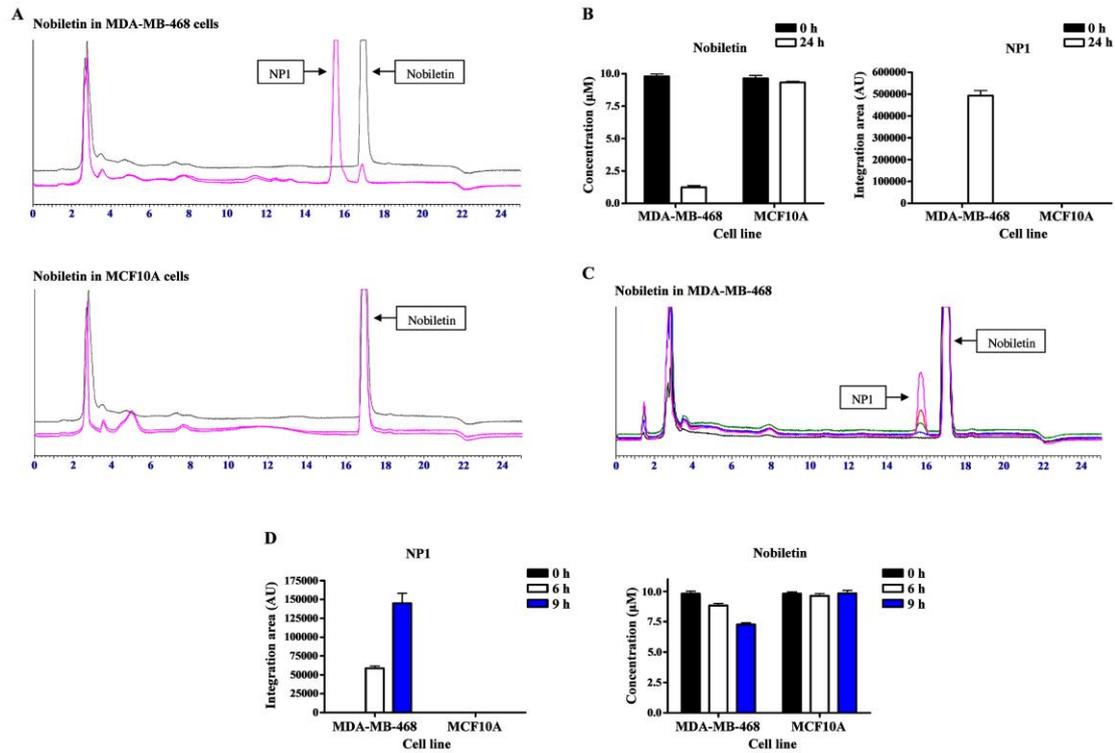


Figure 1. HPLC analysis of nobiletin following incubation with the human breast cell lines MDA–MB–468 and MCF10A. A starting concentration of 10  $\mu\text{M}$  was used and the metabolism was investigated at 0, 3, 6, 9 and 24 h time points by HPLC as described in materials and methods. The experiments were conducted at least 3 times and error bars indicate STDEV of the mean. (A) HPLC chromatograms of nobiletin in the 2 breast cell lines at 0 (black color) and 24 h (red color) time points. (B) Measurement of the concentration of the parent compound and the metabolite NP1 in the 2 cell lines. Due to the unavailability of NP1 authentic standard the concentration of this compound was expressed in AU. (C) HPLC chromatograms of nobiletin in MDA–MB–468 cells at 0 (black color), 1 (blue color), 3 (green color), 6 (red color) and 9 (orange color) h time points. (D) Quantification of the concentration of nobiletin and the metabolite NP1 at the 6 and 9 h time points.

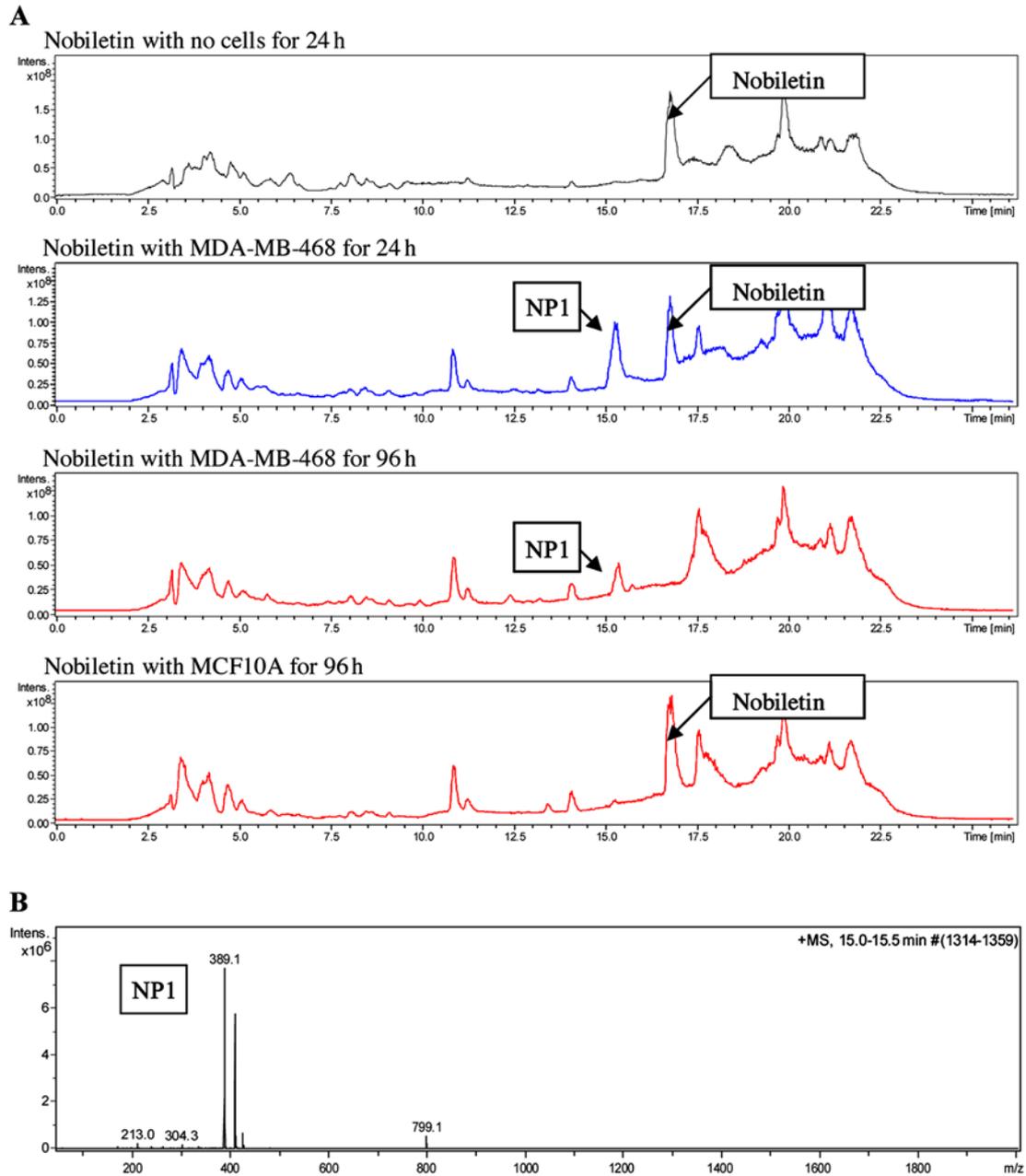


Figure 2. LC-MS analysis of the metabolism of nobiletin in MDA-MB-468 and MCF10A cells. (A) LC chromatograms of the incubation of nobiletin with the MDA-MB-468 and MCF10A cell lines at various time points (24, 96 h). (B) Mass spectrum of NP1 and nobiletin in the cell line incubates.

primary metabolite of nobiletin. The LC chromatogram of the assay matched that noted from the HPLC experiments with regard to the retention time of the compounds, whereas the MS analysis revealed that NP1 exhibited a molecular mass of 389 (Fig. 2A,B).

### *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 2 cell lines. This compound was considerably active in MDA-MB-468 cells and indicated a submicromolar IC<sub>50</sub> ( $0.1 \pm 0.04 \mu\text{M}$ ), whereas it demonstrated limited toxicity in MCF10A cells ( $40 \mu\text{M}$ ) (Fig. 3A). This resulted in an activation factor of approximately 400 ( $\text{IC}_{50_{\text{normal}}}/\text{IC}_{50_{\text{tumor}}}$ ) 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  $\alpha$ -naphthoflavone (Fig. 3B). The IC<sub>50</sub> of nobiletin was considerably increased from  $0.1 \pm 0.04 \mu\text{M}$  to  $30 \pm 2.4 \mu\text{M}$  in the presence of the CYP1 inhibitor acacetin (Fig. 3B). Similar results were noted for the use of  $\alpha$ -naphthoflavone 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, D). The concentration of the parent compound nobiletin was reduced to a smaller extent in the presence of the CYP1 inhibitor ( $3.94 \pm 0.41 \mu\text{M}$  vs.  $1.78 \pm 0.34 \mu\text{M}$ ), whereas a significant decline in the concentration of the metabolite NP1 was noted in MDA-MB-468+I cells

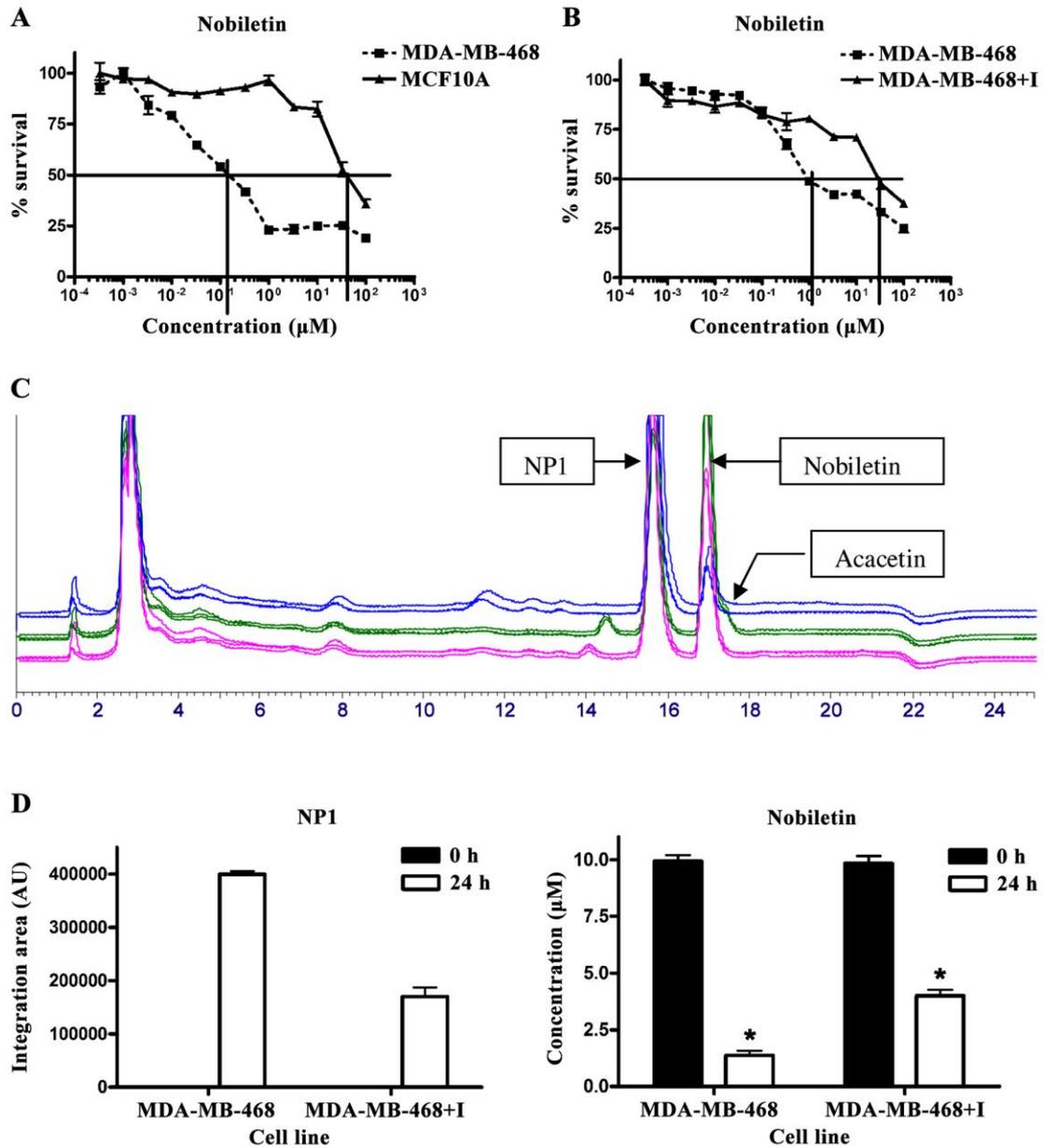


Figure 3. The bioactivation of tangeretin in the CYP1 expressing cell line MDA-MB-468. Cell viability was measured using the MTT assay as determined in the materials and methods section. The experiments were conducted at least 3 times and error bars indicate STDEV of the mean. Cell survival plots indicating the IC<sub>50</sub> determination of nobiletin in (A) MDA-MB-468 and MCF10A cells and (B) MDA-MB-468 and MCF10A cells in the presence and/or absence of the CYP1 inhibitor acacetin (0.5  $\mu\text{M}$ ). Similar results were obtained when the CYP1 inhibitor  $\alpha$ -naphthoflavone was used. (C) The metabolism of nobiletin in MDA-MB-468 cells in the presence and/or absence of the CYP1 inhibitor acacetin (0.5  $\mu\text{M}$ ). HPLC chromatogram of MDA-MB-468 cell incubation with tangeretin, in the presence and/or absence of acacetin (0.5  $\mu\text{M}$ ) for 24 h. (D) Quantification of the concentration of the compounds nobiletin and NP1 in MDA-MB-468 cells, in the presence and/or absence of the CYP1 inhibitor acacetin (0.5  $\mu\text{M}$ ). NP1 concentration was expressed as AU, since its authentic standard was not available.

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,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,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,B). In contrast to MDA–MB–468 cells, MCF10A cells were not affected by nobiletin treatment (10 µM) for 24 h (Fig. 4C,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).

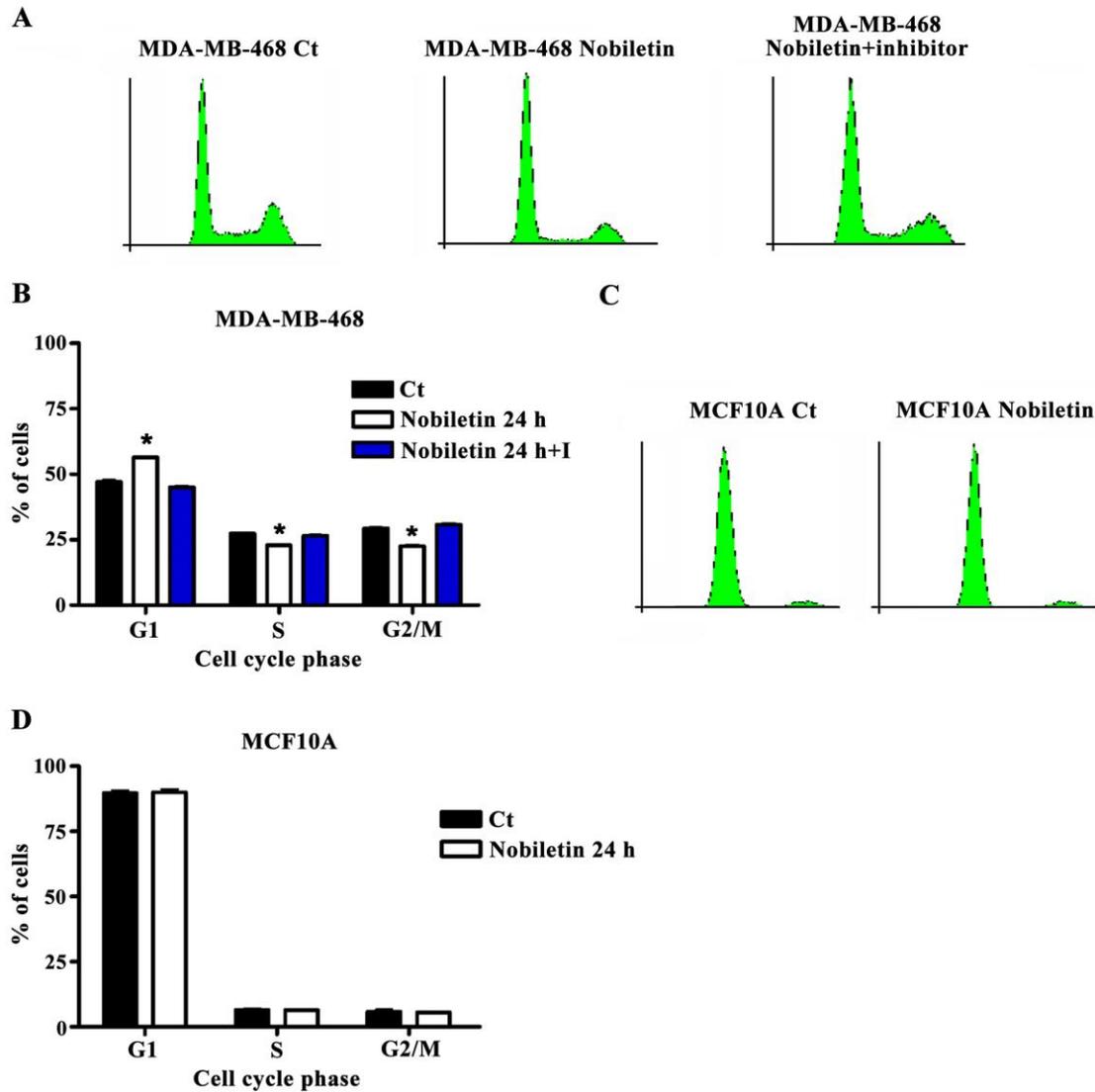


Figure 4. Cell cycle analysis of nobiletin in the 2 human breast cell lines MDA-MB-468 and MCF10A at 24 h. Cell cycle histograms of (A) control MDA-MB-468 cells, nobiletin-treated (24 h) MDA-MB-468 cells, nobiletin-treated (24 h) MDA-MB-468 cells in the presence of the CYP1 inhibitor acacetin (0.5  $\mu$ M). (B) Distribution of the MDA-MB-468 cells in each phase of the cell cycle. (C) Cell cycle histograms of control MCF10A cells and nobiletin-treated MCF10A cells. (D) Distribution of the MCF10A cells in each phase of the cell cycle. The experiments were conducted at least 3 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 ( $P < 0.05$ ).

#### 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 submicromolar IC<sub>50</sub> 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., 2009c; Surichan et al., 2012; Androutsopoulos and Tsatsakis, 2014; Androutsopoulos et al., 2008, 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; Androutsopoulos and Spandidos 2013; Androutsopoulos et al., 2011). 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 position of the flavones moiety (Koga et al., 2011; Koga et al., 2007). 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  $\alpha$ –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'-demethyl 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- and 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  $\mu$ M. 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  $\mu$ M/0.1  $\mu$ M). A similar pattern was previously noted for the flavonoids

sinensetin, genkwanin and eupatorin with IC<sub>50</sub> values for MDA–MB–468 and MCF10A cells of 0.2, 1.6 and 0.5  $\mu$ M and 65, 75 and 50  $\mu$ M, respectively (Androutsopoulos et al., 2009d; Androutsopoulos et al., 2008). In the presence of the CYP1 inhibitor acacetin, the IC<sub>50</sub> values of sinensetin, eupatorin and genkwanin were considerably increased (from 0.2 to 13.5  $\mu$ M for sinensetin, from 0.5 to 15  $\mu$ M for eupatorin and from 1.6 to 12.5  $\mu$ M for genkwanin) (Androutsopoulos et al., 2009d; Androutsopoulos et al., 2008). The present report is in concordance with the aforementioned studies that 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 nobiletin that are penta– and hexa–methoxylated flavones respectively, exhibited submicromolar IC<sub>50</sub> values compared to fully hydroxylated flavones such as luteolin and apigenin that indicated higher IC<sub>50</sub> values (2 and 22  $\mu$ M, 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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