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# Benzo[a]pyrene sensitizes MCF7 breast cancer cells to induction of G1 arrest by the natural flavonoid eupatorin-5-methyl ether, via activation of cell signaling proteins and CYP1-mediated metabolism

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## HIGHLIGHTS

- Eupatorin-5-methyl ether (E5M) induced CYP1 enzymes via translocation of the AhR to the nucleus.
- E5M inhibited proliferation of MCF7 cells more potently following pretreatment with BaP.
- E5M caused G1 arrest in MCF7 cells and upregulation of p21, JNK, p-JNK.
- Pretreatment of MCF7 cells with BaP potentiated the cytostatic effect caused by E5M.
- Induction of CYP1 enzymes sensitizes MCF7 cells to E5M antiproliferative activity.

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## ABSTRACT

Eupatorin-5-methyl ether (E5M) is a flavone containing 4 methoxy groups that is present in plants with medicinal activity, whereas luteolin (L) is a polyhydroxylated flavone commonly encountered in dietary products. In the present study we investigated the interaction of the two flavonoids with cytochrome P450 CYP1 enzymes in breast cancer MCF7 cells. Both compounds induced a dose dependent increase in CYP1A1 and CYP1B1 mRNA levels, as well as in EROD activity, a marker of CYP1 enzyme activity. Induction of cytochrome P450 CYP1 expression by E5M was accompanied by translocation of the ligand-activated transcription factor AhR to the nucleus, as demonstrated by confocal immunofluoresence. More importantly, although E5M was less active than L in inhibiting proliferation of MCF7 cells, when the cells were pretreated with the CYP1 inducer Benzo[a]pyrene (BaP) the potency of E5M was augmented. HPLC and LC–MS analysis revealed that E5M was metabolized to a major conversion product assigned E5M1 resulting from one step demethylation reaction in MCF7 cells whereas L metabolism by recombinant CYP1A1 did not reveal any metabolites. E5M1 production in BaP-induced MCF7 cells was attenuated in the presence of the CYP1A1 inhibitor α-napthoflavone. E5M further induced a dose dependent increase in the cell signaling proteins p21, JNK and p-JNK in MCF7 cells. This effect was enhanced in BaP pretreated cells and was associated with G1 arrest and a small percentage of apoptosis (3.5%). E5M antiproliferative effect in BaP pretreated cells was attenuated in the presence of the CYP1A1 inhibitor α-napthoflavone, as demonstrated by Western blotting and FACS analysis. Taken together the results demonstrate that BaP sensitizes MCF7 cells to E5M antiproliferative activity via enhanced induction of p21, JNK and p-JNK that in turn results by cytochrome P450 CYP1-mediated conversion to the metabolite E5M1.



## 1. Introduction

Cytochrome P450s are haem-containing enzymes that catalyze the metabolic activation of several carcinogens and exogenous substrates. The first family consists of three members CYP1A1, CYP1B1 and CYP1A2. CYP1A1 and CYP1B1 are located primarily in extrahepatic tissues, whereas CYP1A2 is expressed in the liver (Uno et al., 2009; Murray et al., 1997). The involvement of CYP1 family enzymes in carcinogenesis is well established. The latter enzymes promote activation of several pro-carcinogens to their reactive conversion products via hydroxylation reactions occurring at unsubstituted aromatic rings (Shimada and Fujii-Kuriyama, 2004; Androutsopoulos et al., 2009c). Induction of CYP1 expression is mediated through the Aryl hydrocarbon receptor (AhR) that translocates from the cytoplasm to the nucleus upon ligand-binding with a pro-carcinogenic compound such as Benzo[a]pyrene (BaP), or Dimethyl benzanthracene (DMBA) (Androutsopoulos et al., 2009c).

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Although the involvement of CYP1 enzymes in cancer progression is apparent, the hypothesis that their fundamental function is attributed solely to the activation of pro-carcinogens seems to be a misconception. CYP1B1 has been proven to play essential roles in the regulation of retinoid acid signaling, via metabolism of retinol to retinoic acid and retinal and consequently in the establishment of normal pattern and cellular specification of the vertebrate embryo (Chambers et al., 2007). In addition several reports suggest that CYP1 enzymes can be targeted for cancer therapy through the metabolism of pro-drugs to active metabolites with enhanced antitumor activities (Bruno and Njar, 2007; Callero et al., 2012; Ciolino et al., 2004). Research work in our laboratory has focused on the bioactivation of natural products by CYP1A1 and CYP1B1 enzymes in cancer cell line models. We have demonstrated that CYP1 enzymes are capable of metabolizing dietary flavonoids to structurally similar conversion products and thus enhance their potential to inhibit cell proliferation (Androutsopoulos et al., 2008, 2009a,b,e; Androutsopoulos and Spandidos, 2013). Metabolism occurs mainly to the B ring of the flavonoid structure either through a hydroxylation reaction at an unsubstituted position or via a demethylation reaction at a methoxy group (Androutsopoulos et al., 2010). Flavonoids with multiple methoxy groups possess better CYP1-substrate turnover rate, whereas flavonoids bearing multiple hydroxyl groups show reduced CYP1-catalyzed metabolism and are more effective inhibitors of CYP1 enzymes (Androutsopoulos et al., 2011).

Eupatorin-5-methyl ether (E5M) is a polymethoxylated flavonoid present in the plant Orthosiphon stamineus (Yam et al., 2010). Although previous reports demonstrated antitumor properties of this flavonoid the mechanism of action remains poorly understood (Laavola et al., 2012). Luteolin (L) is a polyhydroxylated flavonoid that is present in common dietary sources such as artichoke and chamomile and has documented anticancer activity through the inhibition of cell cycle arrest, induction of apoptosis and inhibition of CYP1 EROD activity (Pandino et al., 2010; Kato et al., 2008; Seelinger et al., 2008; Kim et al., 2005). In the present study the mechanism of antiproliferative action of the flavonoids E5M and L was investigated in the MCF7 breast adenocarcinoma cell line, with particular emphasis to the interaction of the compounds with cytochrome P450 CYP1A1 and CYP1B1 enzymes. We report that E5M is a substrate for CYP1A1 and is activated in MCF7 cells via exogenous stimulation with the synthetic CYP1 inducer Benzo[a]pyrene (BaP), whereas L does not undergo CYP1A1-catalyzed metabolism.

## 2. Materials and methods

### 2.1. Materials

L was purchased from CayMan (MI, USA), α-napthoflavone, 7-ethoxyresorufin, PI and MTT from Sigma–Aldrich (MO, USA), NADPH from Trevigen (Maryland, USA). Reagents for tissue culture were purchased from Biosera (East Sussex, UK), cDNA synthesis kit from Takara (Otsu, Japan) and Real time Master mix from Kappa Biosystems (Boston, USA). Recombinant CYP1A1 was purchased from BD Biosciences (CA, USA). Reagents for immunofluoresence were purchased from Chemicon (CA, USA). Antibodies for western blotting and immunofluoresence were from Santa Cruz (Heidelberg, Germany), Sigma–Aldrich (MO, USA) and Abnova (Taipei, Taiwan). E5M was a kind gift from Dr Randolph Arroo (De Montfort University, UK).

### 2.2. Cell culture

MCF7 cells were maintained in RPMI with glutamine supplemented with 10% FBS and 1% Pen/Strep in a humidified incubator at 37 °C with 95% $O_2$/5% $CO_2$. The cells were passaged routinely with trypsin/EDTA (0.25%, v/v) every 2-3 days.

### 2.3. EROD activity assay

MCF-7 cells were incubated with E5M, L or BaP and the assay carried out as described previously (Androutsopoulos et al., 2009d). Briefly cells were washed twice with PBS and 7-ethoxyresorufin was added at a final concentration of 5 μM in RPMI. The reaction was performed for 45 min at 37 °C and finally terminated with the addition of equal volumes of ice-cold methanol. The samples were centrifuged at 3500 rpm for 5 min and the supernatants analyzed at a fluorescence plate reader (FLx800 Biotech Instruments, USA) with $\lambda_{exc}$ 530 nm and $\lambda_{em}$ 590 nm.

### 2.4. RNA extraction and Real time PCR

MCF7 cells were incubated with flavonoids or BaP for 24 h and RNA was extracted using Trizol as described previously (Androutsopoulos and Spandidos, 2013). cDNA was constructed from total RNA preparations using a Takara kit and Real time PCR was carried out with mRNA specific primers for CYP1A1 and CYP1B1 (Ek et al., 2007). Annealing temperature was at 60 °C and each reaction was run for 40 cycles in the presence of Kappa SyBr Master mix, primers and RNase–DNase free water. Quantification was performed with the aid of standard curve for each gene of interest. Two housekeeping gene positive controls (GAPDH, β-actin) were used for mRNA transcript normalization. The primer sequences have been published in previous studies (Ek et al., 2007).

### 2.5. Enzyme assays and HPLC analysis

L (10 μM) was incubated with recombinant CYP1A1 for 40 min in the presence of NADPH, $MgCl_2$, and phosphate buffer ($NaHPO_4$ and $K_2HPO_4$) as described previously (Androutsopoulos et al., 2009a). The reaction was terminated with the addition of ice-cold methanol containing 1% acetic acid. The samples were centrifuged for 15 min at 13,000 rpm at 4 °C and the supernatants analyzed by HPLC using a Luna C18 5 μ column with UV detection at 360 nm. Separation was achieved with a gradient system using water/acetonitrile/acetic acid (98.5/1/0.5) and methanol/acetonitrile/acetic acid (95.5/4/0.5) solutions (Androutsopoulos et al., 2009a). For metabolism studies in cells, E5M and L were incubated with BaP pretreated or DMSO pretreated MCF7 cells for 24 h and media aliquots were mixed with ice cold methanol containing 1% acetic acid. The samples were centrifuged and analyzed by HPLC as described above.

### 2.6. LC–MS analysis

For mass spectrometric analysis the initial LC composition of solvents was retained with flow rate set at 1 ml/min. Detector voltage was set at 1.5 kV and $APCI^+$ detection was used. Ions selected for E5M and metabolite E5M1 detection were 359 and 345 that correspond to molecular weights of 358 and 344, respectively.

### 2.7. MTT assay

MCF7 cells were pretreated with DMSO or BaP for 24 h and incubated with E5M or L at a concentration range covering the points 40-0.0156 μM. The cells were left to grow for 96 h and viability was measured using the reduction of MTT to a blue formazan product, as a marker, as described previously (Androutsopoulos et al., 2008, 2009a). Absorbance was read at 540 nm using a UV–vis plate reader.

### 2.8. Confocal immunofluoresence

MCF7 cells were plated on coverslips in a 24 well plate at a density of 50,000 cells/well. The cells were treated with E5M or BaP for 48 h and then washed 3 times with PBS, and incubated with fixation solution (Chemicon, USA) for 10 min. The cells were washed again 3 times with PBS and further incubated for another 10 min with permeabilization solution (Chemicon, USA). The solution was removed and the cells were washed 3 times with PBS-1% FBS. Primary antibody for AhR was added at a dilution of 1:500 in PBS-1% FBS to the cells and left for 1 h on a rocker. The antibody was removed and the cells were washed with PBS-1% FBS 3 times. Secondary anti-mouse antibody conjugated with FITC was added at a 1:500 dilution in PBS-1% FBS and the coverslips were incubated in the dark for 1 h. Following 3 washes with PBS-1% FBS DAPI solution was added at a concentration of 100 ng/ml for 5 min to the cells. The solution was then removed and the cells were washed gently with PBS-1% FBS and stored at 4 °C until the day of the analysis. A Leica TCS SPE confocal microscope was used.

### 2.9. FACS analysis

MCF7 cells were pretreated with compounds for 24 h, washed once with PBS, trypsinized and fixed with 70% ethanol for 24 h at −20 °C. PI was added at a final concentration of 50 μg/ml with RNase A (100 μg/ml) and the cells were incubated for 30 min at 37 °C. DNA cell cycle analysis was performed at a Beckman Coulter cytometer and at least 10,000 events were acquired.

### 2.10. Western blotting

The assay was carried out as described previously (Androutsopoulos and Spandidos, 2013). Antibodies for p21, cyclin D1 and p-ERK were from SantaCruz (Santa Cruz, USA), while antibodies for JNK and p-JNK from UpState. Antibody for AhR was purchased from Abnova (Walnut, USA) while antibody for β-actin from

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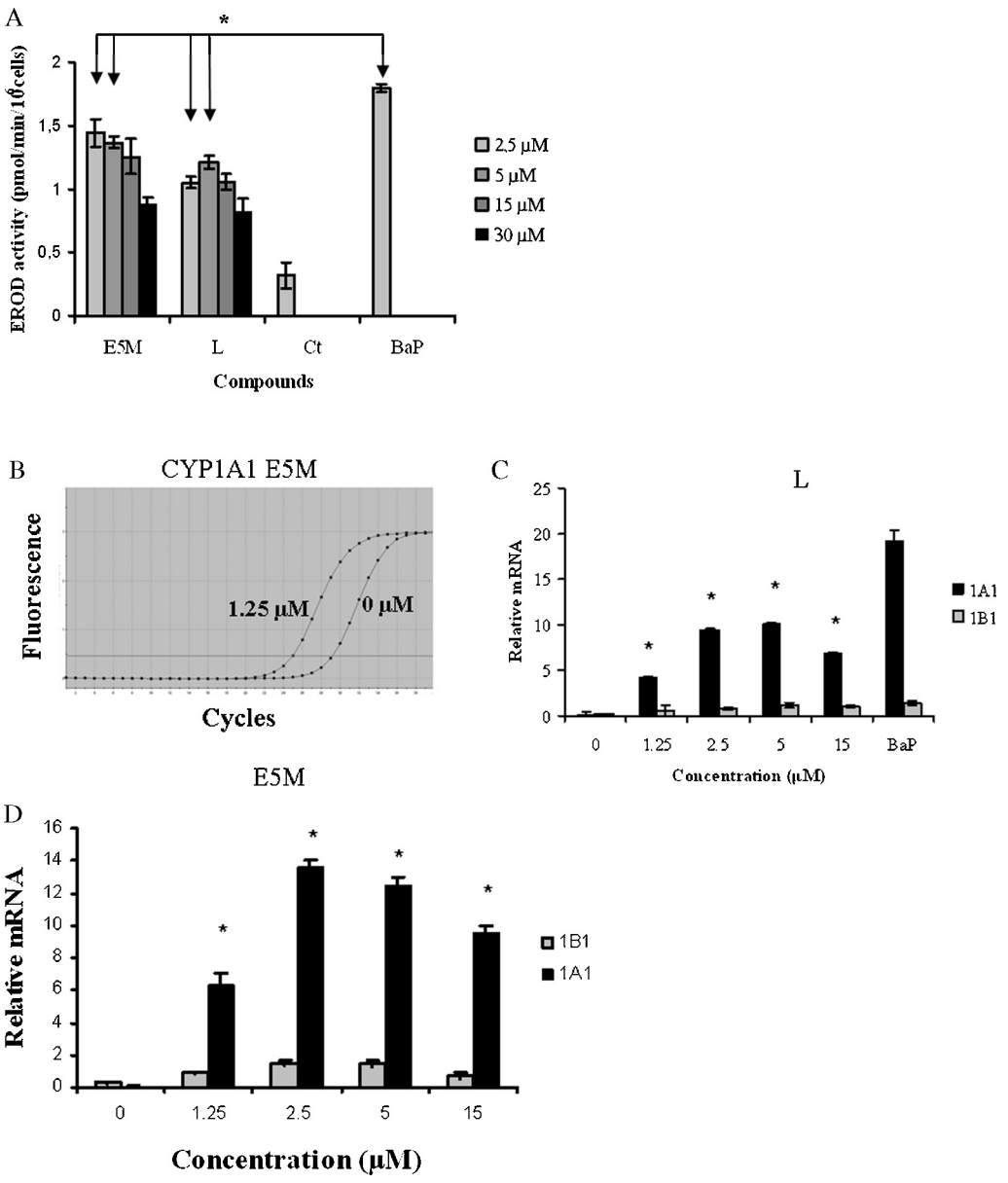


**Fig. 1.** Induction of cytochrome P450 CYP1 expression by E5M and L in breast adenocarcinoma MCF7 cells. (A) MCF7 cells were cultured in 96-well plates, treated for 24 h with E5M, L or BaP and EROD was assay carried out as described in Section 2. Statistical differences were obtained for 2.5 and 5 μM E5M treatment compared to 2.5 and 5 μM L treatment, as well as between E5M, L (2.5 and 5 μM) and BaP (5 μM) treatment (*statistically different $p < 0.05$). (B) Real time PCR amplification curves for CYP1A1 mRNA transcripts following treatment of cells with 2.5 μM E5M for 24 h. (C) Quantification of mRNA levels of CYP1A1 and CYP1B1 transcripts following L treatment. (D) Quantification of mRNA levels of CYP1A1 and CYP1B1 transcripts following E5M treatment. BaP (5 μM) was used as a positive control. Experiments were performed at least 3 times and error bars indicate STDEV of the mean. *$p < 0.05$ statistically significant differences between E5M and L CYP1A1 induction.

Sigma–Aldrich (Missouri, USA). Antibodies for p21, cyclin D1, JNK and p-JNK were used at 1:300 dilutions, whereas antibodies for AhR and β-actin were used at 1:500 dilutions. Secondary antibodies were from Santa Cruz (Santa Cruz, USA) and were used at 1:2000 dilutions.

### 2.11. Statistical considerations

Data are presented as mean ± STDEV for at least $n = 3$ determinations. Statistical differences were determined by paired *T*-test and one way Anova.

## 3. Results

### 3.1. E5M and L increase CYP1A1 expression in MCF7 cells

In a recent report we demonstrated that polymethoxyflavonoids, such as eupatorin induce CYP1A1 and CYP1B1 enzymes in MCF7 cells (Androutsopoulos et al., 2009d). To evaluate whether this interaction occurs in the case of E5M and L, MCF7 cells were exposed to a concentration range of 2.5-30 μM of each compound for 24 h and the CYP1A1 expression levels measured initially by EROD assay. L exhibited a dose dependent increase in EROD activity with the exception that at higher concentrations of 15 and 30 μM the activity declined (Fig. 1A). E5M showed an approximately 3-fold increase in CYP1 activity compared to control at 2.5 and 5 μM, whereas at 15 and 30 μM the activity was considerably lower (Fig. 1A). The induction of CYP1 activity caused by E5M at concentrations of 2.5 and 5 μM was higher than that caused by L and lower than that caused by 5 μM of the pro-carcinogen BaP (Fig. 1A, $p < 0.05$).

In order to identify which of the two isoforms was notably induced, qRT-PCR was employed to evaluate the mRNA expression

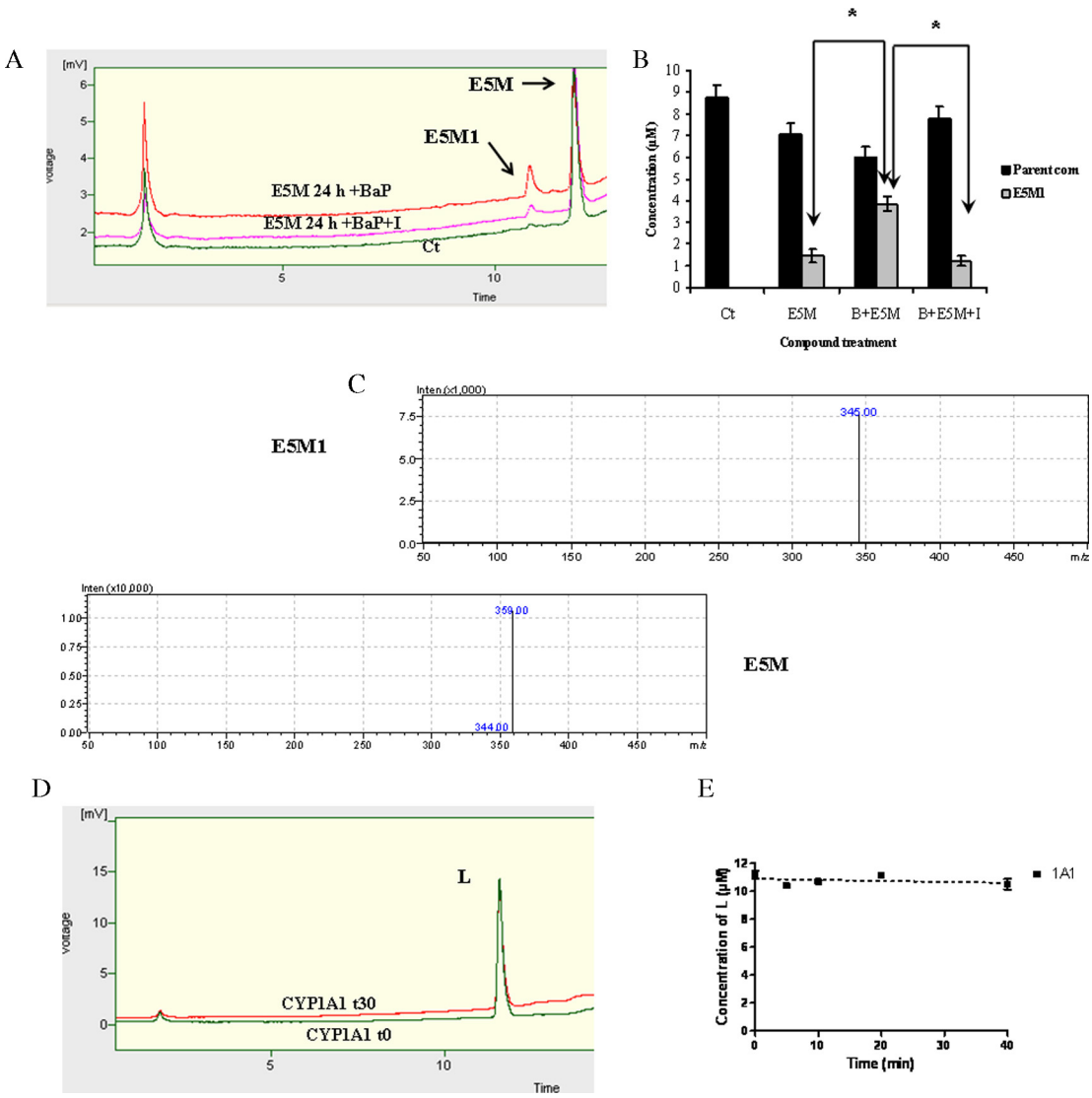


**Fig. 2.** The metabolism of E5M and L in MCF7 cells and recombinant CYP1A1. (A) HPLC chromatograms of E5M (10 μM) incubated with BaP pretreated MCF7 cells in the presence or absence of the CYP1A-inhibitor α-napthoflavone. Control samples included E5M incubated for 24 h with media without cells. (B) Bar chart depicting the concentration levels of the compound E5M and its metabolite E5M1 after 24 h incubation with MCF7 cells. The cells were pre-treated with BaP or DMSO, in the presence or absence of the CYP1A-inhibitor α-napthoflavone. α-napthoflavone was used at concentrations of 0.5-1 μM. (C) Mass spectrometry traces of E5M and the metabolite E5M1 in MCF7 cells. Error bars indicate STDEV of the mean for $n = 3$ determinations. (D) HPLC chromatograms of L metabolism by recombinant CYP1A1 at time points 0 and 30 min. L (10 μM) was incubated with recombinant CYP1A1 (20 pmol/ml) and analyzed by HPLC as described in Section 2. (E) Concentration time profile of L (10 μM) metabolism by CYP1A1 at time points 0, 5, 10, 20 and 40 min. *Statistically different compared to control ($p < 0.05$).

levels of CYP1A1 and CYP1B1 in MCF7 cells pretreated by E5M and L. The analysis revealed that CYP1A1 was the major isoform that was induced by E5M and L (Fig. 1B–D). E5M exhibited a higher degree of CYP1A1 mRNA transcript induction compared to L ($p < 0.05$) and lower than that obtained by 5 μM of BaP, whereas levels of CYP1B1 mRNA induction were comparable between E5M and L treatment of MCF7 cells (Fig. 1C and D).

### 3.2. E5M is metabolized by CYP1A1 in MCF7 cells to a demethylated conversion product

Based on the initial observation that CYP1 activity (mainly CYP1A1) is enhanced in MCF7 cells pretreated with E5M or L we hypothesized that CYP1 enzymes (mainly CYP1A1) may in turn metabolize the latter compounds to demethylated or hydroxylated conversion products. HPLC analysis of MCF7 cells pretreated with BaP for 24 h revealed the presence of an unidentified metabolite denoted E5M1 that eluted at a retention time of approximately 11 min (Fig. 2A). The latter metabolite was evident after incubation of untreated MCF7 cells with E5M for 24 h yet to smaller quantities (Fig. 2B). In order to prove that this metabolite was produced by CYP1A1 metabolism of E5M, the incubation was performed in the presence of the CYP1A1 inhibitor α-napthoflavone. The analysis showed that E5M conversion to E5M1 in the presence of α-napthoflavone was significantly reduced (Fig. 2A and B). To provide information regarding the identification of E5M1 Mass spectrometry was utilized. The LC–MS assay indicated that the mass to charge ratio of E5M was 359, corresponding to a molecular mass of 358, whereas the mass to charge ratio of E5M1 was 345 corresponding to a molecular mass of 344 (Fig. 2C). Given that the difference in the molecular weight of parent compound and metabolite is 14, the results indicate that E5M1 is the demethylated product of E5M (—$OCH_3$ to —OH).

The metabolism of the second flavonoid L was investigated by HPLC and LC–MS assays. Initial experiments did not show the presence of a metabolite in MCF7 cell cultures incubated with L (data

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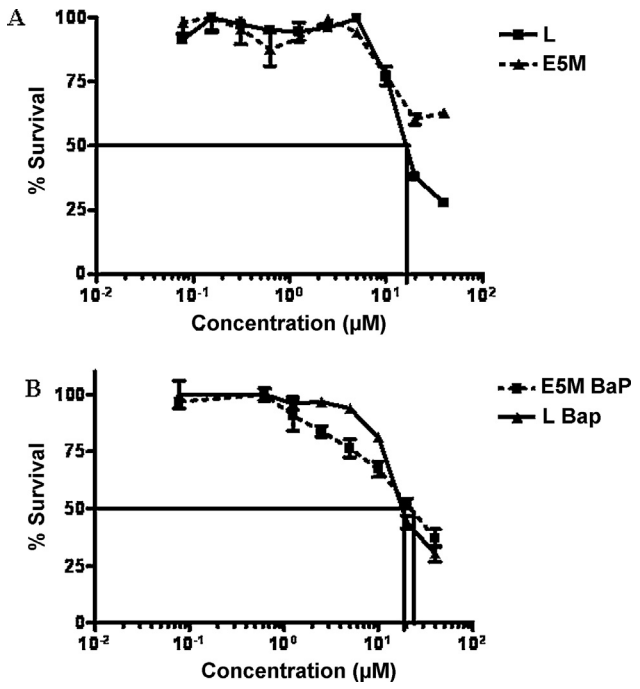


**Fig. 3.** The cytotoxicity of E5M and L in MCF7 cells pretreated with (A) DMSO or (B) BaP for 24 h. Flavonoids were incubated at a concentration range covering 0.078-40 μM for 96 h and cell viability was measured using the MTT assay, as described in Section 2. Error bars indicate STDEV of the mean for $n = 3$ experiments.

not shown). Similar results were obtained when the compound was incubated by recombinant CYP1A1 for 40 min (Fig. 2D). No metabolism occurred, while the concentration of L remained relatively constant throughout the incubation (Fig. 2E).

### 3.3. BaP enhances the antiproliferative effect caused by E5M but not L in MCF7 cells

Following the first line of evidence that E5M is a substrate for CYP1A1 in MCF7 cells, while L is not, we examined the antiproliferative effect of both compounds alone in MCF7 cells that were pretreated with either 0.1% DMSO (Ct) or BaP (5 μM) for 24 h. L was more toxic than E5M in DMSO pretreated cells with an IC50 of approximately 15 μM (Fig. 3A). However there was no difference observed between BaP treated and control cells with respect to 50% inhibition of cellular proliferation caused by L (Fig. 3A and B). In contrast, E5M exhibited an IC50 of 30 μM in BaP treated cells, whereas in control cells the IC50 was higher than 40 μM (Fig. 3A and B). More importantly we observed that the dose response survival curve of BaP pre-treated MCF7 cells caused by E5M, followed a decline proportional to increasing concentrations of the compound, as opposed to control cells where the survival declined only the highest concentrations of E5M (40 μM Fig. 3A and B). Consistent with this observation is the lower IC75 value (concentration required for 25% inhibition of cellular proliferation) of E5M in BaP pretreated cells compared to control cells (4 μM compared to 10 μM, Fig. 3A and B), indicating that BaP treatment enhances the cytotoxic effect of E5M in MCF7 cells. Based on these results further experiments were focused on E5M.

### 3.4. E5M induces AhR expression in MCF7 cells

To further investigate whether the induction of CYP1 activity by E5M is AhR-dependent, MCF7 cells were treated with E5M and the expression of AhR examined by Western immunoblotting and confocal immunofluoresence microscopy. E5M (5 μM) induced a comparable increase in total AhR levels in MCF7 cells after 24 h and 48 h treatment, compared to the synthetic CYP1 inducer BaP (Fig. 4B and C). Statistical analysis revealed significant differences between Ct and E5M or Ct and BaP treated cells, whereas non-significant differences were obtained between E5M and BaP with respect to induction of AhR protein levels (Fig. 4C). In addition confocal microscopy revealed that treatment of E5M with MCF7 cells for 48 h resulted in more intense AhR staining, whereas the protein was localized in the nucleus and cytoplasmic regions, as opposed to control cells where staining was present mainly in the cytoplasmic regions (Fig. 4A).

### 3.5. E5M induces G1 arrest and upregulation of cell signaling proteins in BaP pre-treated cells

To add further insight into the mechanism of action of E5M in MCF7 cells, flow cytometry was used to explore which phase of the cell cycle was predominantly affected by E5M treatment. BaP-pretreated cells that were further exposed to 10 μM of E5M showed an arrest at G1 phase (10% higher than control $p < 0.05$) with a simultaneous decrease in the number of cells progressing through S phase (approximately 10% less) (Fig. 5A and B). In contrast incubation of DMSO pre-treated cells with 10 μM E5M did not reveal a statistically significant increase in G1 phase ($p > 0.05$, Fig. 5B). No difference in the percentage of cells undergoing apoptosis was noted (Fig. 5A). When the cells were pretreated with BaP and further incubated with a higher concentration of E5M (20 μM) for 24 h a higher percentage of cells were arrested at G1 (20%), whereas the amount of cells that remained in S phase was negligible (Fig. 6B). The percentage of apoptotic cells was also increased to 3.5% compared to 1% noted in the case of 10 μM E5M treatment (Fig. 5B and 6B). More importantly, incubation of 20 μM of E5M with DMSO pre-treated cells resulted in a lower degree of G1 arrest (6%) and simultaneous S phase decrease, to that observed in BaP pre-treated cells, indicating that BaP enhances the cytostatic effect of E5M in MCF7 cells (Figs. 6B and 7B).

Western immunoblotting demonstrated that G1 arrest caused by E5M in BaP-pretreated cells was accompanied by upregulation of the cell cycle inhibitor $p21^{waf1/cip1}$, as well as the MAPK kinase JNK (Fig. 6A). The antibody for JNK recognizes total JNK expression that corresponds to JNK1 at 46 kDa and JNK2/3 at 54 kDa. E5M caused activation of JNK, as the levels of the phosphorylated form of this protein were increased following treatment with the compound (Fig. 6A). The expression levels of cyclin D1 were reduced which is consistent with G1 arrest. Importantly, the effects caused by E5M upon cell signaling protein levels were augmented in BaP pre-treated cells (Fig. 6A).

### 3.6. The cytostatic effect caused by E5M in BaP pretreated cells is CYP1A1-mediated and partially reversible

To further substantiate that CYP1A1 plays a major role in the activation of E5M, MCF7 cells were pretreated with BaP for 24 h and further incubated with E5M in the presence and absence of the CYP1A1 inhibitor α-napthoflavone. The latter compound reduced the cytostatic effect caused by E5M in BaP pretreated MCF7 cells, but failed to completely return the cell cycle to its original form (Fig. 7A and B). Furthermore cotreatment of E5M with α-napthoflavone resulted in attenuation of $p21^{waf1/cip1}$ induction in BaP pretreated cells (Fig. 7C), compared to E5M treatment alone (Fig. 6A). To prove that BaP does not sensitize MCF7 cells due to toxicity caused by the initial 24 h pretreatment, we incubated MCF7 cells with 5 μM E5M for 24 h to induce CYP1 enzymes and then for an additional 24 h with a fresh aliquot of 10 μM E5M. MCF7 cells pretreated with E5M (5 μM) and further incubated with fresh E5M

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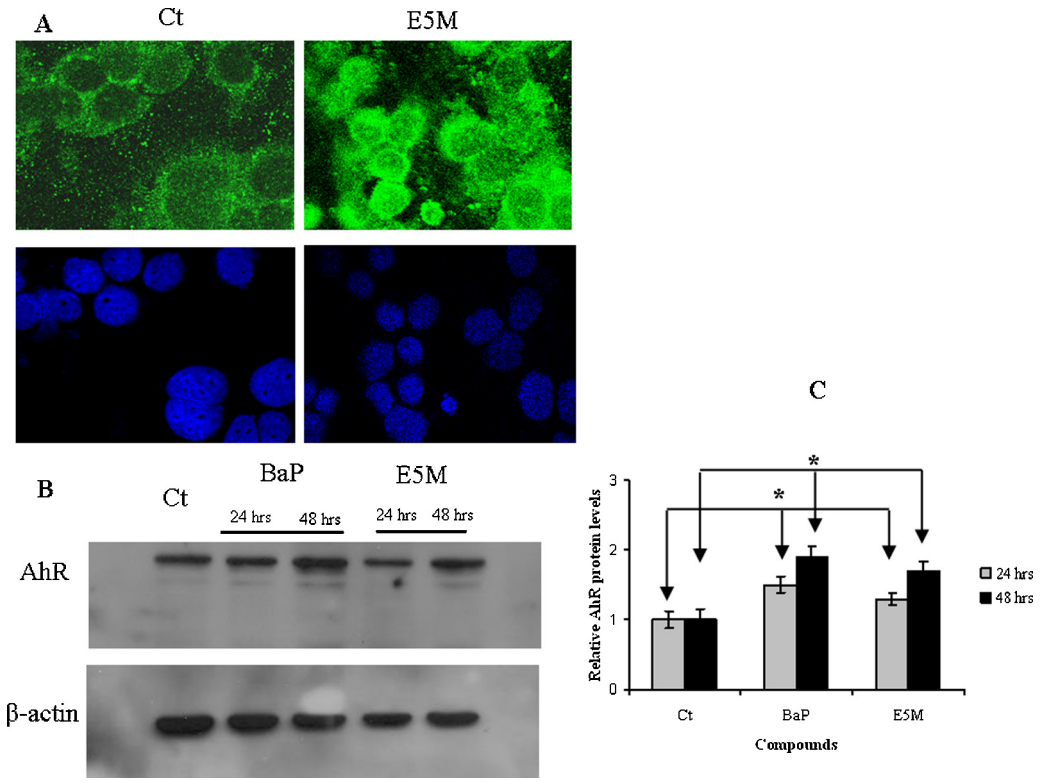


**Fig. 4.** E5M induces AhR expression and nuclear translocation in MCF7 cells. (A) MCF7 cells were incubated with E5M (5 μM) for 48 h and immunofluoresence was carried out as described in Section 2. DAPI staining (blue) indicates nuclei, whereas FITC staining (green) indicates expression of AhR. (B) Western blot analysis indicating expression of AhR in MCF7 cells incubated with E5M (5 μM) or BaP (5 μM) for 24 or 48 h. (C) Semiquantification of AhR protein expression following BaP or E5M treatment by computer densitometry. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(10 μM) for 24 h showed comparable G1 arrest to that noted by 5 μM of BaP pretreatment (Fig. 7B).

## 4. Discussion

Flavonoids are polyphenolic molecules with multiple types of bioactivity. Their potential use for the treatment of cancer as chemopreventive and cytostatic agents has been the focus a vast number of studies (Lee et al., 2009; Russo et al., 2012; Link et al., 2010). Flavonoids inhibit the proliferation of cancer cells through multiple pathways mainly affecting the cell cycle and the apoptotic cascade, whereas their chemopreventive effect is attributed to inhibition of pro-carcinogen activation (Lee et al., 2009; Chaudhary and Willet, 2006; Russo et al., 2012; Link et al., 2010; Moon et al., 2006).

Eupatorin-5-methyl ether (E5M) is a methoxylated flavonoid (Fig. 8A) present in indigenous plants with medicinal activity, such as Orthosiphon stamineus, whereas luteolin is a polyhydroxylated flavone (Fig. 8A) found in common dietary sources, notably chamomile, and artichoke (Yam et al., 2010; Pandino et al., 2010; Kato et al., 2008). Luteolin has demonstrated anticancer activity, mainly through activation of apoptosis and induction of cell cycle arrest, whereas evidence regarding the potential anticancer activity of E5M is limited (Laavola et al., 2012; Seelinger et al., 2008). However, the structurally similar flavonoid eupatorin, the 5-demethylated analog of E5M was recently reported as an antimitotic agent (Salmela et al., 2012). Moreover eupatorin is a substrate for cytochrome P450 CYP1 enzymes (mainly CYP1A1) and exerts its anticancer effects via CYP1-mediated metabolism (Androutsopoulos et al., 2008). In the present study we provide evidence regarding the antiproliferative action of the flavonoid E5M through a pathway that involves activation of CYP1 enzymes. E5M induced CYP1 enzyme activity via upregulation of the AhR in MCF7 cells and was subsequently converted to the demethylated product E5M1 by CYP1A1 (Fig. 8B). Moreover, the synthetic CYP1 inducer BaP was able to enhance inhibition of MCF7 cell proliferation caused by E5M, via increased production of the corresponding metabolite E5M1, suggesting that tumors that are exposed to CYP1 inducers can be sensitized to the antiproliferative action of E5M.

The interaction of flavonoids with the CYP1 family of enzymes has been an active area of research. Numerous studies have demonstrated the ability of dietary flavonoids to induce the activity of CYP1A1 in *in vitro* models that is mediated via translocation of the AhR to the nucleus (Ciolino et al., 1998, 1999; Ciolino and Yeh, 1999). However no study has examined the interaction of E5M with CYP1 enzymes. In the present study both E5M and L induced CYP1 enzyme activity. E5M was somewhat more potent than L. This may be in part due to the methoxylated nature of E5M as induction of cytochrome P450 CYP1 enzymes is favored by lipophillic compounds, e.g. TCDD and chlorinated biphenyls with three and four lateral chlorine substitutions (Kan et al., 2006). Hydrophobicity, molecular size and a stero-coplanar structure are important determinants for the binding of small molecules to the AhR (Ashida et al., 2000).

Furthermore E5M caused an increase in the protein levels of AhR in MCF7 cells similar to that observed by BaP and was accompanied by a translocation of the protein to the nucleus as demonstrated by confocal immunofluoresence. Although the interaction of this specific flavonoid (E5M) with AhR has not been demonstrated to date, structurally similar flavonoids are ligands for the Aryl hydrocarbon receptor thus inducing the transcription of the CYP1A1 gene (Ciolino et al., 1998, 1999). Ciolino and colleagues reported earlier that the flavonoid diosmetin that contains the 4′-$OCH_3$ structural group binds to the AhR and enhances CYP1A1 activity (Ciolino et al., 1998). In addition the polyhydroxy flavonoid quercetin mediates

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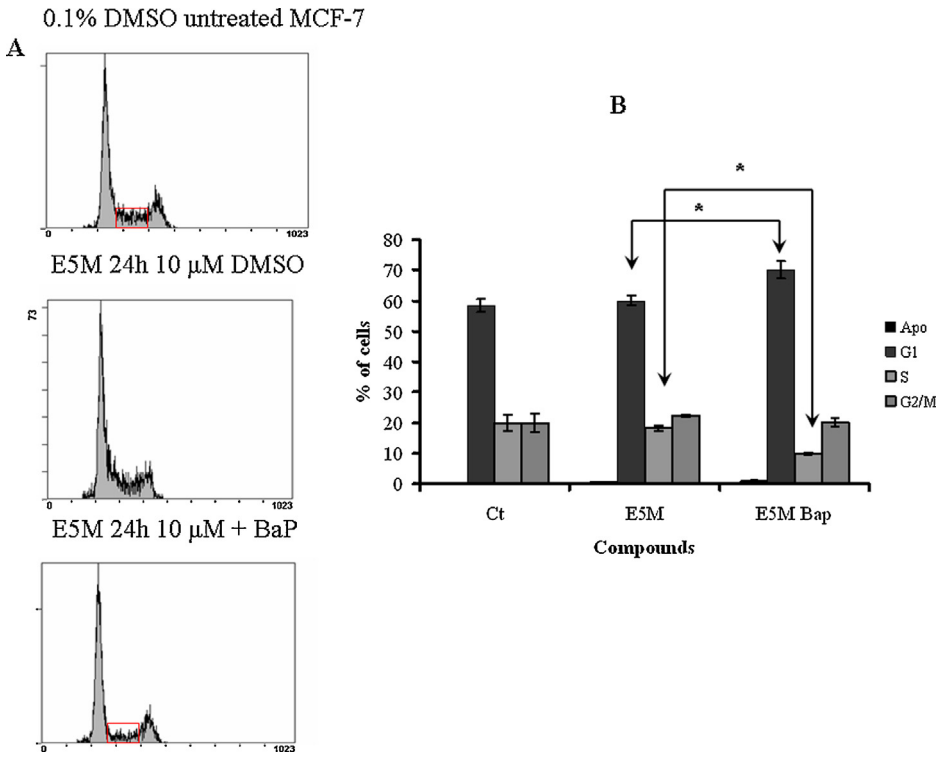


**Fig. 5.** Effect of E5M on cell cycle profile of MCF7 cells pretreated with BaP or DMSO for 24 h. The cells were treated with propidium iodide solution in PBS (50 μg/ml) containing RNase A (100 μg/ml) and fluorescence was measured using a Beckman Coulter flow cytometer as described in Section 2. (A) Cell cycle histogram showing the results of 24 h incubation of the cells with 0.1% DMSO (control) or 10 μM of E5M. BaP (5 μM) or DMSO (0.1%) was used for 24 h pretreatment of the cells to examine the effect of CYP1 induction on E5M antiproliferative activity. Red square indicates the percentage of cells in S phase following pretreatment with DMSO or BaP and subsequent treatment with E5M or DMSO. (B) Percentage of MCF7 (BaP or DMSO pretreated) cells in each phase of the cell cycle, following 24 h treatment of E5M (10 μM). Error bars are STDEV of the mean for at least $n = 3$ determinations. *Statistically different ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

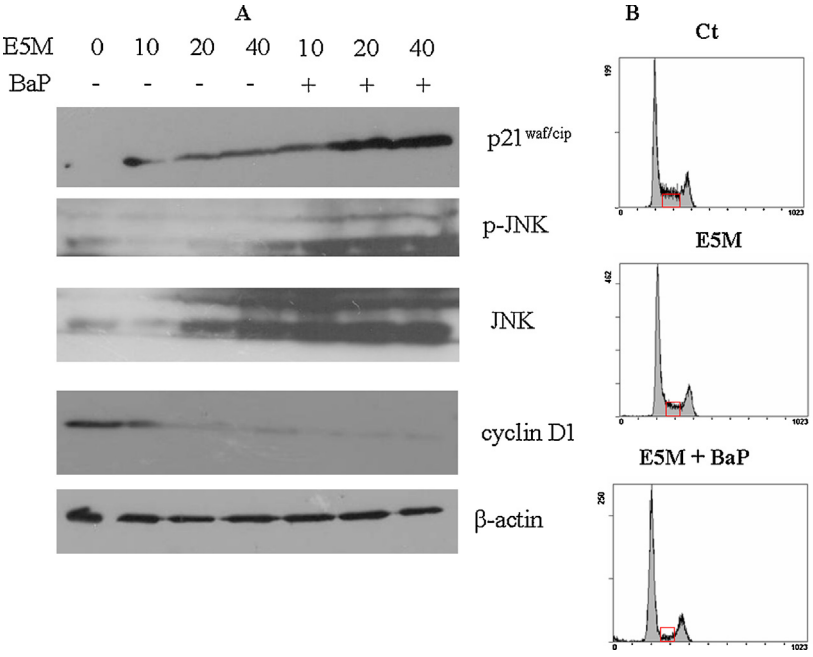


**Fig. 6.** BaP sensitizes MCF7 cells to G1 arrest caused by E5M. (A) Western blotting analysis of cell signaling proteins JNK, p-JNK, p21 and cyclin D1 on MCF7 cells pretreated for 24 h with 0.1% DMSO or 5 μM BaP. E5M was used at concentrations of 10, 20 and 40 μM. β-actin was used as a loading control. (B) Cell cycle histograms of E5M (20 μM) treatment on BaP (5 μM) or DMSO (0.1%) pretreated cells. Red square indicates the percentage of cells in S phase following pretreatment with DMSO or BaP and subsequent treatment with E5M or DMSO. Histograms are representative traces of $n = 3$ determinations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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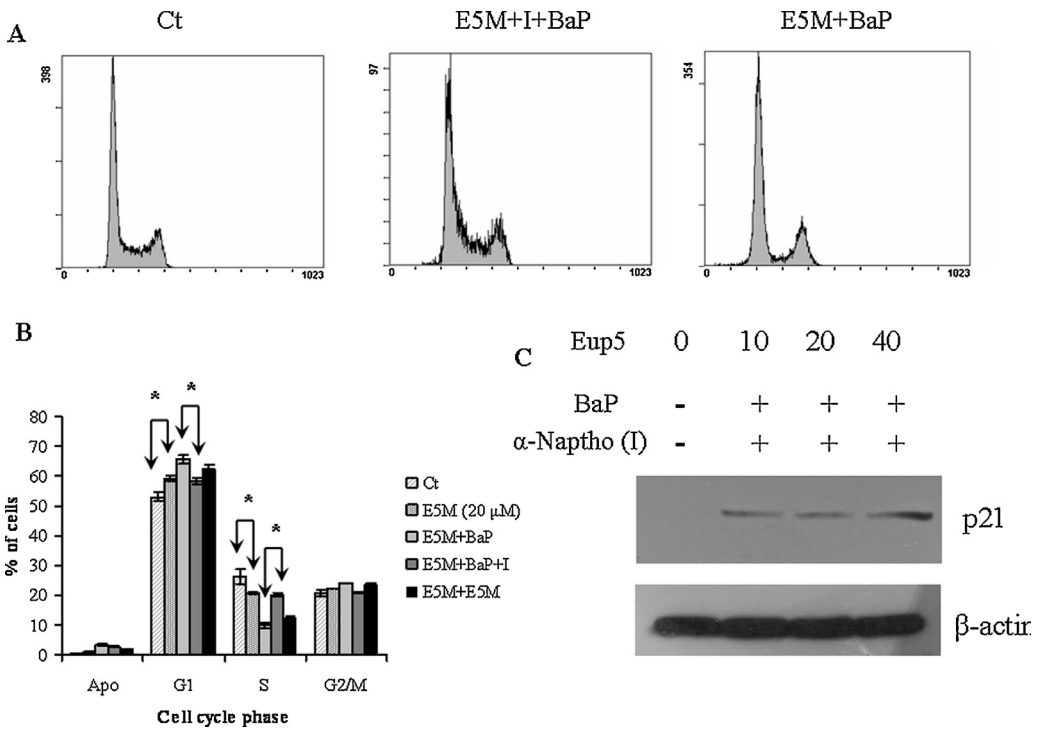


**Fig. 7.** Effects of E5M (20 μM) on cell cycle of BaP pretreated MCF7 cells in the presence (+I) of the CYP1A1 inhibitor α-napthoflavone. MCF7 cells were pretreated with BaP (5 μM) or E5M (5 μM) for 24 h and incubated with E5M (20 μM) in the presence or absence of α-napthoflavone (0.5 μM). (A) Cell cycle histograms of MCF7 cells pretreated with DMSO (0.1%) or BaP (5 μM) and further incubated with E5M (20 μM) in the presence or absence of α-napthoflavone (0.5 μM). (B) Percentages of cells in each phase of the cell cycle after treatment with compounds. (C) Western blot analysis of p21 protein expression in MCF7 cells pretreated with BaP for 24 h and further incubated with E5M in the presence of α-napthoflavone (0.5 μM). E5M was used at concentrations of 10, 20 and 40 μM. Error bars are STDEV of the mean for at least $n = 3$ determinations. *Statistically different compared to control ($p < 0.05$).

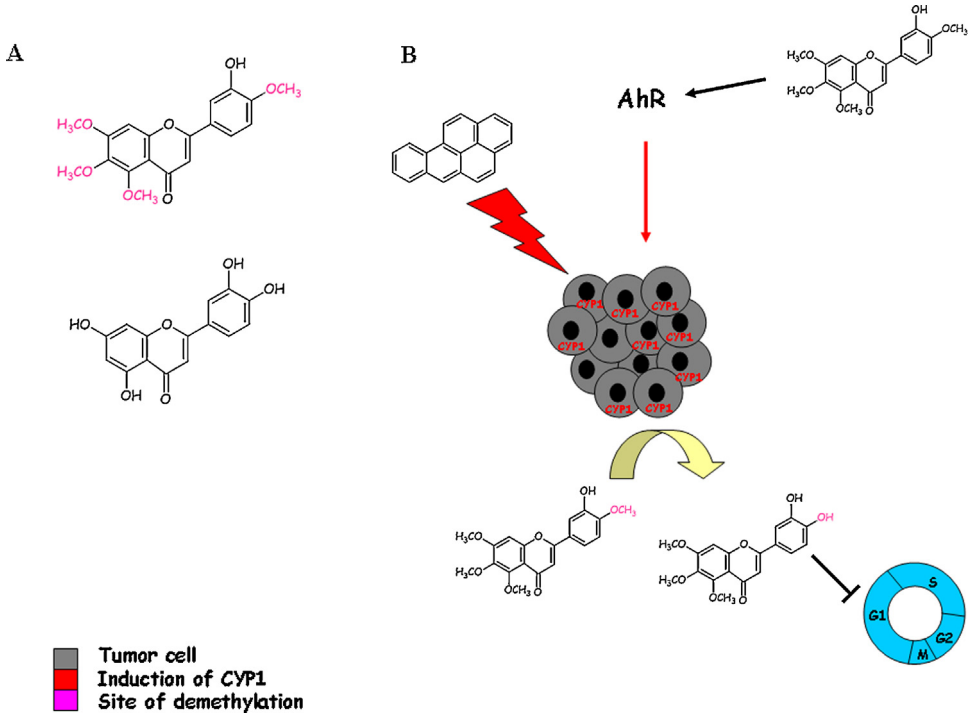


**Fig. 8.** (A) Chemical structures of E5M and L. (B) Molecular pathway indicating the cell cycle events and metabolic effects mediated by E5M in BaP pretreated MCF7 cells. Pink color indicates putative sites of demethylation of E5M.

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induction of CYP1A1 enzyme activity by binding to the AhR in MCF7 cells (Ciolino et al., 1999).

The metabolism of E5M and L by CYP1 enzymes in cancer cell lines has not been reported to date. Despite the vast majority of studies highlighting the inhibition of CYP1 enzymes by flavonoids and polyphenolics, evidence regarding the metabolism of such compounds in cancer cell lines is limited. Our previous reports have underlined the importance of the 4′-$OCH_3$ structural group as a major determinant for high CYP1A1 catalytic turnover (Androutsopoulos et al., 2008, 2009a,b). Generally flavonoids with multiple hydroxyl groups are considered effective CYP1A1 inhibitors whereas flavonoids with one or more methoxy groups exhibit high CYP1A1 metabolic rate (Androutsopoulos et al., 2010, 2011). This provides an explanation on the lack of metabolism of L by CYP1A1. In addition to the polyhydroxylated nature, the remaining vacant positions for hydroxylation are limited as the predominant routes of flavonoid metabolism by CYP1A1 involve 3′ or 4′ position of the B ring (Androutsopoulos et al., 2010, 2009e). The general concept suggests that demethylation or hydroxylation of a flavonoid by CYP1 enzymes produces a metabolite with similar or higher in potency antiproliferative activity with that of the parent compound (Androutsopoulos et al., 2008, 2009a,b,e; Androutsopoulos and Spandidos, 2013). The overall inhibition of cellular proliferation is increased as it results from the combined action of the parent compound and the metabolite (Androutsopoulos et al., 2008; Androutsopoulos and Spandidos, 2013). This has been documented for the flavonoids: diosmetin, eupatorin, cirsiliol, daidzein and genkwanin in the cancer cell lines MCF7, MDA-MB 468 and HepG2 (Androutsopoulos et al., 2008, 2009a,b,e; Androutsopoulos and Spandidos, 2013; Atherton et al., 2006). In addition the flavonoid fisetin was shown to undergo metabolic activation to the flavonoid geraldol in mice, whereas the corresponding metabolite was more cytotoxic than the parent compound in tumor cells *in vitro* (Touil et al., 2011). In the current study we observed that E5M failed to produce an IC50, although it was metabolized to E5M1 in MCF7 cells. This may seem contradictory to our previous studies where E5M and structurally similar flavonoids produced considerably low IC50s in the CYP1 expressing cell line MDA-MB 468 (Androutsopoulos et al., 2008, 2009b,e, 2011). There are two possible explanations for these observations: (1) MCF7 cells are less sensitive than MDA-MB 468 with respect to cytostatic effect caused by flavonoids. (2) The amount of metabolite formed from E5M by CYP1A1 is possibly lower than that produced by metabolism of E5M and similar flavonoids in the MDA-MB 468 cell line, where CYP1 enzymes are constitutively expressed. Obviously a combination of the above is also possible. Consistent with these hypotheses is the observation that upon pre-treatment of MCF7 cells with 5 μM of BaP the antiproliferative action of E5M was augmented. The effect was mainly attributed to conversion of E5M to E5M1 by CYP1A1 as in the presence of the CYP1A1 specific inhibitor the cytostatic effect was partially reversed and in BaP pre-treated cells the production of E5M1 was considerably higher than DMSO pre-treated control cells. Importantly we observed that in BaP-sensitized cells higher concentrations of E5M enhanced the antitumor activity of the latter, suggesting that the overall effect is based on the combined action of the parent compound and the metabolite.

E5M was capable of inducing a G1 arrest at 20 μM, whereas at 10 μM no significant change in the percentage of cells was observed compared to control cells containing no compound. Upon pre-treatment with BaP 20% and 10% of the cells were arrested at G1 at concentrations of 20 and 10 μM of E5M respectively. This was accompanied by upregulation of p21, JNK and p-JNK levels. Accordingly expression of cyclin D1 was reduced. Increased expression of p21 and decreased expression of cyclin D1 are common events that characterize blockage of the cell cycle at G1 phase (Heffeter et al., 2006; Deep et al., 2007). Cyclin D1 associates with CDK4-6 in order to phosphorylate Rb and facilitate the G1 to S phase progression, thus a block at G1 will result in reduction of cyclin D1 levels.

In addition, a similar cytostatic effect to that obtained for E5M has been observed for the structurally similar flavonoids tangeretin and nobiletin (Morley et al., 2007; Surichan et al., 2012). The latter compounds induced G1 arrest in MCF7, MDA-MB-435 and HT-29 cells (Morley et al., 2007; Surichan et al., 2012). Nobiletin differs to E5M in the presence of two methoxy groups at positions 3′ and 8, whereas tangeretin in the presence of a methoxy group at position 8 and the absence of a hydroxyl group at position 3′. It is important to note that substitutions of methoxy groups in place of hydroxyl groups or vacant positions of the flavonoid ring structure reduce the antiproliferative activity of flavonoid compounds, as tangeretin, nobiletin and E5M exhibit considerably high IC50s (>40 μM) in cancer cell lines, compared to hydroxylated flavonoids such as chrysin and luteolin (Morley et al., 2007; Surichan et al., 2012; Zhang et al., 2008). CYP1A1 is believed to play a critical role in the activation of polymethoxy flavonoids by metabolism of methoxy moieties to hydroxyl groups (Androutsopoulos et al., 2010). The results presented herein are in agreement with these findings as BaP-treated cells that showed elevated CYP1A1 levels appeared more sensitive to the cytostatic effect of E5M.

BaP is a chemical CYP1 inducer that belongs to the family of polycyclic aromatic hydrocarbons (PAHs). Environmental exposure to BaP occurs through combustion smoke and exhaust fumes while the compound is also present in coal tar and tobacco. In the present study we report that BaP is capable of enhancing the cytostatic effect caused by the flavonoid E5M upon MCF7 breast cancer cells, via induction of CYP1 enzymes (mainly CYP1A1) and subsequent conversion to the metabolite E5M1 (Fig. 8B). This is of particular importance as exposure to natural or synthetic CYP1 inducers, such as BaP, can enhance the activity of cytochrome P450 CYP1 enzymes in non-malignant or cancerous tissue. In this context the findings offer a novel mechanism of action of CYP1 enzymes that involves enhancement of the antitumor properties of E5M in cells exposed to CYP1 inducers.

## Conflict of interest

There are none conflict of interest to declare with respect to this article.

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