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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 λ_{exc} 530 nm and λ_{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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