Food and Chemical Toxicology 110 (2017) 383–394

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Cytochrome P450 CYP1 metabolism of hydroxylated flavones and flavonols: Selective bioactivation of luteolin in breast cancer cells

Nicola E. Wilsher[a], Randolph R. Arroo[a], Minos–Timotheos Matsoukas[b], Aristidis M. Tsatsakis[c], Demetrios A. Spandidos[d], Vasilis P. Androutsopoulos[c,d,*]

[a] De Montfort University, Leicester School of Pharmacy, The Gateway, Leicester LE1 9BH, UK
[b] Department of Pharmacy, University of Patras, Patras 26504, Greece
[c] Laboratory of Toxicology, University of Crete, Medical School, Voutes, Heraklion 71409, Crete, Greece
[d] Laboratory of Clinical Virology, University of Crete, Medical School, Voutes, Heraklion 71409, Crete, Greece

ARTICLE INFO

Keywords:
Flavonoids
Apigenin
Luteolin
Kaempferol
Quercetin
Cytochrome P450 CYP1 enzymes
Antiproliferation
Breast cancer

ABSTRACT

Natural flavonoids with methoxy substitutions are metabolized by CYP1 enzymes to yield the corresponding demethylated products. The present study aimed to characterize the metabolism and further antiproliferative activity of the hydroxylated flavonoids apigenin, luteolin, scutellarein, kaempferol and quercetin in CYP1 recombinant enzymes and in the CYP1 expressing cell lines MCF7 and MDA–MB–468, respectively. Apigenin was converted to luteolin and scutellarein, whereas kaempferol was metabolized only to quercetin by recombinant CYP1 enzymes. Luteolin metabolism yielded 6 hydroxyluteolin only by recombinant CYP1B1, whereas CYP1A1 and CYP1A2 were not capable of metabolizing this compound. Molecular modeling demonstrated that CYP1B1 favored the A ring orientation of apigenin and luteolin to the heme group compared with CYP1A1. The IC50 of the compounds luteolin, scutellarein and 6 hydroxyluteolin was significantly lower in MDA–MB–468, MCF7 and MCF10A cells compared with that of apigenin. Similarly, the IC50 of quercetin in MDA–MB–468 cells was significantly lower compared with that of kaempferol. The most potent compound was luteolin in MDA–MB–468 cells (IC50 = 2 ± 0.3 μM). In the presence of the CYP1-inhibitors α-napthoflavone and/or acacetin, luteolin activation was lessened. Taken collectively, the data demonstrate that the metabolism of hydroxylated flavonoids by cytochrome P450 CYP1 enzymes, notably CYP1A1 and CYP1B1, can enhance their antiproliferative activity in breast cancer cells. In addition, this antiproliferative activity is attributed to the combined action of the parent compound and the corresponding CYP1 metabolites.

1. Introduction

Breast cancer is one of the major causes of cancer–related deaths accounting for 540,000 deaths each year worldwide (Lopes et al., 2017). The treatment options for breast cancer include surgery, radiotherapy, hormone therapy and chemotherapy. A major obstacle that is encountered with conventional breast cancer therapy is the recurrence of the tumor progression. In addition, the use of certain chemotherapeutic drugs, notably the alkylating agent carboplatin, the antimetabolite 5–fluorouracil and the antimitotic agents paclitaxel and docetaxel, confers limited specificity for the tumor site and consequently severe side effects to the healthy tissue. Hence, in the past two decades considerable effort has been made to the research and development of new drugs with enhanced specificity and improved efficacy for the treatment of breast cancer. In addition, the use of complementary medical treatment namely, phytotherapeutic natural products and nutritional supplements, for women with a previous history of breast cancer has considerably increased (Lopes et al., 2017; Margină et al., 2015; Fenga et al., 2016).

Flavonoids comprise a class of natural polyphenolic molecules that have demonstrated a major role in the prevention of cancer. These compounds are ubiquitous in plant–based food products, such as fruits, vegetables and tea extracts and in medicinal plants. Their anticancer activity has been attributed to the inhibition of DNA damage, the inhibition of cell cycle associated proteins, the induction of apoptosis, the modulation of cell signaling pathways involved in proliferation and in the inhibition of carcinogenic metabolite formation (George et al., 2017; Curti et al., 2017; Kerimi and Williamson, 2017; Clementino

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

* Corresponding author. Laboratory of Toxicology, Laboratory of Clinical Virology, University of Crete, Voutes, Heraklion 71409, Crete, Greece.
E-mail address: androuts@uoc.gr (V.P. Androutsopoulos).

http://dx.doi.org/10.1016/j.fct.2017.10.051
Received 28 September 2017; Received in revised form 27 October 2017; Accepted 28 October 2017
Available online 31 October 2017
0278-6915/

et al., 2017). The hydroxylated flavonoids apigenin, luteolin, quercetin and kaempferol are the main constituents of various dietary products and beverages and have been the focus of extensive research over the last years. Apigenin exerts anticancer effects through the modulation of various pathways namely, apoptosis, ROS and DNA damage and repair (Salmani et al., 2017).

Luteolin is a flavone that differs from apigenin by a single hydroxyl group at the 3′ position of the B ring. This compound is found in various common dietary sources notably artichoke, chamomile and olive oil (Garcia–Gonzalez et al., 2010; Kato et al., 2008; Pandino et al., 2010). Luteolin has demonstrated anticancer activity in cancer cell lines and *in vivo* models (Seelinger et al., 2008). This type of activity has been attributed to activation of apoptosis, cell cycle arrest and inhibition of tumor invasion (Seelinger et al., 2008). Moreover, quercetin that contains an extra hydroxyl group at the 3 position of the C ring compared with luteolin, has been examined as a putative anticancer agent in ovarian and melanoma cancers via similar modes of action (Parvaresh et al., 2016; Harris et al., 2016)

The metabolism of xenobiotics is facilitated by specific phase–I and phase–II drug metabolizing enzymes (Tsatsakis et al., 2009, 2011). Previous studies conducted by our group have demonstrated that methoxylated flavonoids, such as diosmetin and eupatorin, are demethylated to their corresponding hydroxylated derivatives by recombinant cytochrome P450 CYP1A1, CYP1B1 and CYP1A2 enzymes and by MCF7 and MDA–MB–468 breast adenocarcinoma cellular extracts (Androutsopoulos et al., 2008, 2009a). The cytochrome P450 enzymes CYP1A1 and CYP1B1 have been implicated in carcinogenesis and their upregulation in cancer cells has been used for the development of novel chemotherapeutic drugs (Patterson and Murray, 2002; Gribben et al., 2005). Extrahepatic CYPs, notably CYP1B1 and CYP1A1, may be utilized to target tumor cells by the use of selective antibodies, or the activation of prodrugs that are inactive in tissues or cells that do not express these enzymes (Gribben et al., 2005; Loaiza-Pérez et al., 2004). Our previous reports suggest that several dietary flavonoids are substrates for CYP1 enzymes and may exhibit cancer–therapeutic applications, provided their conversion products inhibit tumor cell growth (Androutsopoulos et al., 2010).

In the present study, the CYP1–mediated metabolism of hydroxylated flavonoids namely, apigenin, luteolin, quercetin and kaempferol was investigated in recombinant CYP1 enzymes. In addition, their antiproliferative activity was assessed in the CYP1 expressing cell lines MCF7 and MDA–MB–468. The data demonstrate that bioactivation of hydroxylated dietary flavonoids by CYP1 enzymes is possible and that the number of hydroxyl groups affects the inhibition of cellular proliferation in breast cancer cells.

2. Materials and methods

2.1. Chemicals and antibodies

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), α-napthoflavone, acacetin, apigenin, luteolin, kaempferol, quercetin, tissue culture reagents and media, Western blotting lysis buffer and DTT were purchased from Sigma Aldrich (St Louis, MO, USA). Scutellarein was purchased from Extrasynthese (Lyon, France) and 6-OH luteolin was synthesized as described in previous methodologies (Androutsopoulos et al., 2009c). Western blotting reagents were from Biorad (Berkeley, CA, USA). The polyclonal antibody for CYP1B1 was from Gentest (BD Biosciences, CA, USA), whereas the monoclonal for β-actin from Cell signaling (Leiden, Netherlands). Secondary antibodies for western blotting were from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Cell culture

MCF7 and MDA–MB–468 cells were maintained in RPMI with glutamine (2 mM) containing 10% heat-inactivated FBS and penicillin/streptomycin. MDA–MB–468 cells were grown in RPMI without phenol red, whereas the growth medium of MCF10A cells included DMEM:F12 with insulin (10 μg/ml), hydrocortisone (500 ng/ml) and EGF (20 ng/ml), in addition to 10% FBS and 2 mM of glutamine. The cells were grown in a humidified incubator at 37 °C in 5% CO_2/95% air and passaged using trypsin EDTA (0.25% v/v), every 3–4 days.

2.3. Flavonoid metabolism

Recombinant CYP1 enzymes and control microsomes that were purchased from Gentest (BD Biosciences, CA, USA) were incubated with 10 μM of flavonoids (apigenin, luteolin, kaempferol and quercetin). The reactions were carried out at 37 °C in a humidified incubator in the presence of NADPH (0.5 mM), $MgCl_2$ (0.5 mM) and phosphate buffer (20 mM). Time points were obtained at 0, 5, 10, 15, 20 and 25 min intervals. The reactions were terminated by addition of equal volumes of a solution containing 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. The co–elution studies included a 25 min sample incubate that was spiked with a low concentration of an authentic standard (0.2–1 μM) of the putative metabolite.

2.4. HPLC analysis

The methodology has been described in detail in previous publications (Androutsopoulos et al., 2008, 2009a). A Luna C18 4.6 × 150 mm 5 μ column was used with a mobile phase that contained solvents A and B. Solvent A comprised 1% acetonitrile and 0.5% acetic acid in H_2O and solvent B 4% acetonitrile and 0.5% acetic acid in CH_3OH. The following gradient was used: 60% solvent A and 40% solvent B at time = 0 min and 10% solvent A and 90% solvent B at time = 10 min. The final conditions were maintained for 1 min and the composition of the solvents was adjusted to the initial conditions with 8 min remaining for column equilibration after each run. The detection of the flavonoid concentration was monitored using a Waters Series 200 UV detector (Waters, Hertfordshire, UK). Luteolin, 6 OH luteolin, kaempferol and quercetin were detected at 360 nm, whereas apigenin and scutellarein were detected at 350 nm. The concentration of the flavonoids was estimated by a calibration curve covering the concentration range of 0.05–10 μM for each compound. The assay was carried out at 37 °C and the flow rate was 1 ml/min. The average recoveries for apigenin, luteolin, scutellarein, 6 OH luteolin, kaempferol and quercetin were estimated at 95, 91, 93, 87, 84 and 81%, respectively.

2.5. Kinetics of 6 OH luteolin formation

CYP1B1 recombinant microsomes (10 pmol/ml) were incubated with a concentration range of luteolin namely, 0.03–10 μM (10, 7, 5, 3, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03 μM) for 10 min. The optimum CYP1B1 concentration for linearity of product formation was estimated at 5 pmol/ml. The concentrations of 6 OH luteolin were estimated by a calibration curve covering the range 0.01–10 μM. Apparent Km and Vmax values were calculated using Graph Pad Prism (version 4.03). Intrinsic clearance (Cl) was calculated from the ratio Vmax/Km.

2.6. MTT assay

MCF7, MDA–MB–468 and MCF10A cells (1×10^4 cells/ml) were seeded in 96-well plates and the antiproliferative effect of the flavonoids was assayed as described previously (Androutsopoulos et al., 2008). Inhibition experiments were conducted in the presence of 0.5–1 μM α-napthoflavone and/or acacetin.