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Bioactivation of the phytoestrogen diosmetin by CYP1 cytochromes P450

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ABSTRACT

Breast cancer is a major cause of death worldwide. Amongst the various forms of treatment chemoprevention is favoured and natural products such as the dietary flavonoids have been examined for their cancer preventative activity. In this study we investigated the anti-cancer activity of the flavonoid diosmetin, as a result of cytochrome P450 CYP1 metabolism. Diosmetin was metabolized to luteolin via an aromatic demethylation reaction on the B-ring from CYP1A1, CYP1B1 and the hepatic isozyme CYP1A2. CYP1A1 and CYP1A2 also produced additional unidentified metabolites. CYP1B1 showed the lowest apparent KM and CYP1A1 the highest apparent Kcat. Diosmetin was also metabolized to luteolin in estrogen receptor positive breast cell-line (MCF-7 cells) preinduced for 24 h with the potent CYP1 inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Treatment of MCF-7 cells with TCDD caused bioactivation of diosmetin enhancing its cytotoxicity. Taken together these data suggest that the flavonoid diosmetin is metabolised to the more active molecule luteolin by CYP1 family enzymes.

1. Introduction

Every year an estimated 11 million people are diagnosed with cancer (excluding skin cancers) and nearly 7 million people are recorded as dying from cancer. Inherited high susceptibility to cancer accounts for only a small proportion of cases; most adult cancers are caused by environmental factors. This means that most cancers are at least in theory preventable [1]. As a result there is a focus towards chemoprevention, and several research groups have focused on the identification of dietary constituents with cancer preventative properties [2,3].

Phytoestrogens are naturally occurring polyphenols – e.g. flavonoids, isoflavonoids, coumestans, stilbenes, lignans – with estrogenic activities, that have often been associated cancer prevention [4]. They have been shown to act as antioxidants [5] and affect cell signaling pathways [6,7]. Recently their interaction with cytochrome P450s and mainly the CYP1 family has been of particular interest. Phytoestrogens, notably flavonoids, are often regarded as inhibitors of CYP1A1 and CYP1B1 enzyme activity [8,9], thus blocking the activation of procarcinogens into carcinogens [10]. On the other hand, some phytoestrogens have been shown to be substrates for CYP1 enzymes, and are metabolized to more active products that inhibit tumor growth [11].

Diosmetin (Fig. 6) is a flavone, found in the legume *Acacia farnesiana* Willd. [12,13], and in olive (*Olea europaea* L.) leaves [14]. It has been shown to inhibit proliferation of human oral squamus carcinoma SCC-9 cells [15] and to inhibit CYP1A1-catalyzed activation of the procarcinogen 7,12 dimethylbenz[α] anthracene [16]. Doostdar and coworkers demonstrated by that diosmetin is a potent inhibitor of CYP1A1 and CYP1B1 enzyme activity, and suggested that the compound might protect against certain cancers by inhibiting the activation of procarcinogens [17]. However, whether or not diosmetin is a substrate for CYP1 enzymes has not yet been established.

Here, we report the metabolism of diosmetin by CYP1 enzymes, using enzyme and cell based assays. In addition, we identified the main metabolite, and show an increase in

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cytotoxicity of diosmetin for MCF-7 cells, after induction of CYP1 gene expression. The data suggest that diosmetin undergoes CYP1A1 and CYP1B1 bioactivation to metabolic products with cancer protective properties.

2. Materials and methods

2.1. Chemicals

Diosmetin was obtained from Indofine (Somerville, NJ, USA) and luteolin from Lancaster (Heysham, Lancashire, UK). Cytochrome P450 containing microsomes, obtained from insect cells expressing recombinant human CYP genes (Supersomes™) were purchased from BD Biosciences (Cowley, Oxford, UK). Water, acetonitrile, acetic acid and methanol were of HPLC grade and purchased from Fisher (Loughborough, UK). Cell culture reagents, PBS, MTT and salicylamide were purchased from Sigma (Poole, Dorset, UK).

2.2. Enzyme assay and HPLC analysis

Incubation mixtures (total volume 100 μl) contained Supersomes (20 pmol/ml of human cytochrome P450), NADPH (0.5 mM), $MgCl_2$ (0.5 mM), phosphate buffer (20 mM), and diosmetin (10 μM). The buffer was a mixture of disodium hydrogen orthophosphate (Na_2HPO_4) and potassium dihydrogen orthophosphate (KH_2PO_4) at a ratio of 1:1. The final pH was adjusted to 7.4 with NaOH (1 M). Following incubation at 37 °C, samples were taken at 5 min intervals until 20 min. Each reaction was terminated by the addition of equal volume of 1% acetic acid in methanol. The samples were centrifuged at 3500 *g* for 20 min at 4 °C and the supernatants analysed by reversed phase HPLC (Perkin-Elmer 200, Wellesley, USA). For co-elution studies, a 20 min CYP incubate of diosmetin was prepared as above and spiked with an authentic standard of the putative metabolite, luteolin. The resulting sample was analysed by HPLC and compared to unspiked incubates. A Luna 5 μ C_{18} 4.6 × 150 mm column (Phenomenex, Cheshire, UK) was used, with a mobile phase flow rate of 1 ml/min, at a temperature of 37 °C. The mobile phase consisted of: Solvent A (1% acetonitrile and 0.5% acetic acid in water), and solvent B (4% acetonitrile and 0.5% acetic acid in methanol). The following gradient program was used: 60% solvent A and 40% solvent B at time 0, 10% solvent A and 90% solvent B after 10 min. Final conditions were held for 1 min before returning to initial solvent conditions. Diosmetin and its metabolites were monitored by a Waters 200 UV detector (Waters, Hertfordshire, UK) at 360 nm. Diosmetin concentrations were estimated by comparison to a calibration curve covering the range 0.1 to 10 μM.

2.3. Kinetics of luteolin formation

CYP1A1, CYP1B1 and CYP1A2 microsomes (10 pmol/ml) were incubated with various concentrations (0.025, 0.25, 0.5, 1, 2, 4, 10 μM) of diosmetin for 10 min, as described above. Preliminary experiments had shown that 10 pmol/ml was the optimum enzyme concentration to ensure linearity of product formation. Luteolin concentrations were estimated by comparison to a calibration curve covering the range 0.01 to 10 μM. Apparent K_M and K_{cat} were calculated using Graphpad Prism software (version 4.03) nonlinear regression analysis (San Diego, USA).

2.4. Cell culture

MCF-7 cells were maintained in RPMI 1640 with phenol red (Sigma, Poole, UK), 2 mM glutamine (Sigma, Poole, UK), and 10% (v/v) heat inactivated fetal calf serum (Sigma, Poole, UK). No antibiotics were added to the culture medium. Contamination was checked for by microscopic investigation. The cells were grown at 37 °C, 5% CO_2/95% air with 100% humidity, and passaged using trypsin EDTA (0.25%) (Sigma, Poole, UK).

2.5. RT-PCR and DNA-gel electrophoresis

The method including primer sequences, reagent concentrations and cycling parameters was adapted from Döhr and colleagues [18]. MCF-7 cells, seeded at a density of 0.4 × 105 cells/ml, were left to grow for 48 h treated with compound for the time and concentration indicated. The cells were washed twice with PBS and total RNA was extracted using guanidinium-acid-phenol as described previously [19]. Reverse transcription was performed using a RT kit (Sigma, Poole, UK). RNA template of interest (0.005–0.25 μg/μl) was mixed with deoxynucleotide mix (500 μM of each dNTP) and anchored oligo(dT)23 and heated at 70 °C for 10 min. Samples were placed on ice with RNAse inhibitor and (1 U/μl) reverse transcriptase buffer 1×. Enhanced avian reverse transcriptase (1 U/μl) was added to start the reaction at 43 °C for 50 min. The first strand cDNA was amplified using parameters described previously [18]. PCR reactions were carried out containing cDNA (0.08 μg/μl), 1× *Taq* buffer, and 200 μM of each dNTP in the presence of 0.2 μM of each primer and 2.5 U *Taq* DNA polymerase. Amplifications were performed using a PTC-200 Peltier DNA thermal cycler (Biorad, Hertfordshire, UK). Following PCR, each sample was electrophorized and the DNA visualized using a Biorad Molecular Imager FX (Biorad, Hertfordshire, UK).

2.6. MTT cell proliferation assay

MCF-7 (2 × 103) cells were plated in 96-well flat-bottomed plates. After incubation for 4 h to allow maximum cell adherence, medium containing TCDD (Greyhound, Birkenhead, UK), or medium with DMSO as control were added to the wells to give final concentrations of 10 nM or 0.1% (v/v) DMSO, respectively. Following 24 h incubation, the medium was removed by aspiration, and diosmetin in medium was added at a concentration of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, or 33 μM. The cells were left to grow for 96 h. MTT (2 mg/ml in PBS) was then added to each well for 3 h and the formazan product generated by viable cells was solubilised with DMSO. Cell viability was measured from the absorbance at 540 nm using a Lab Systems Multiskan Plus plate reader (Labsystems, UK). Results were expressed as the percentage of 100% (control) proliferation and the IC_{50}