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Metabolism of the soyabean isoflavone daidzein by CYP1A2 and the extra-hepatic CYPs 1A1 and 1B1 affects biological activity

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ABSTRACT

Metabolism of the isoflavones daidzein and genistein, which may protect against some cancers, was studied using human liver microsomes and recombinant CYP isoforms. The detection of three, more polar metabolites of each isoflavone by RP-HPLC required NADPH, consistent with CYP-mediated metabolism. For different liver preparations, metabolite generation from daidzein showed a significant linear correlation with metabolite generation from genistein, indicating metabolism by the same CYP(s). The lowest rate of metabolism of both isoflavones was by the preparation with the lowest CYP1A2 activity. Metabolite peak areas were substantially and significantly reduced by the CYP1A2 inhibitor furafylline and to a lesser extent by the CYP2E1 inhibitor 4-methylpyrazole. Recombinant CYP1A2, but not CYP2E1, generated the metabolites of daidzein and genistein and recombinant CYP1A1 and CYP1B1, expressed at sites including the breast and prostate, were also active. The effects of two CYP-derived metabolites of daidzein, 6,7,4′-trihydroxyisoflavone and 7,3′,4′-trihydroxyisoflavone, were studied in the MCF-7 human breast cancer cell line at a concentration (50 μM) at which daidzein induces an antiproliferative response. 7,3′,4′-Trihydroxyisoflavone reduced total cell numbers to a greater extent than 6,7,4′-trihydroxyisoflavone or daidzein and increased cell death. Together, these data demonstrate proof of principle that CYP-mediated metabolism of daidzein can be an activation pathway. We conclude that CYP1A2 makes the major contribution to the hepatic metabolism of both daidzein and genistein and along with metabolism at sites of hormone-dependent tumours may enhance a cancer-protective effect of daidzein if sufficiently high concentrations are reached in target tissues.

1. Introduction

The isoflavones are a related group of plant compounds which, because of their structural similarity to the endogenous human steroid hormone 17-β-oestradiol, are known also as phytoestrogens. There is substantial epidemiological evidence linking the typical Asian diet, of which the isoflavone-rich soyabean forms a staple, to lower incidence of hormone-dependent cancers including cancers of the breast (reviewed in [1]) and prostate (reviewed in [2,3]) and also bowel cancer (reviewed in [2]). Effects of immigration and dietary change on breast cancer risk substantiate evidence based on geographical

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variation in incidence (reviewed in [1]). Further evidence for anti-cancer properties of the isoflavones comes from studies on the effects of dietary soyabean and/or isoflavones in animal models (reviewed in [4,5]). For example, the incidence of azoxymethane-induced colon tumours in female mice was lower in animals fed a diet including soyabean protein plus isoflavones than in mice in which the diet included soyabean protein without isoflavones [6]. An antimetastatic effect of dietary genistein in mice was demonstrated recently as reduced lung tumour burden following surgical removal of a primary mammary tumour [7]. Studies in cell line models also demonstrate numerous biological activities of the isoflavones, many of them commensurate with anti-cancer properties (reviewed in [4,5]). It is notable that antiproliferative effects of isoflavones on breast cancer cell lines have been reported widely (e.g. [8,9]), but these are at concentrations in the 10–100 μM range, in excess of those generally observed in human plasma. The latter are typically 0.1–1 μM (e.g. [10–12]), although concentrations as high as 25 μM have been achieved after supplementation [13]. In fact, in vitro studies generally indicate a proliferative response of breast cancer cell lines to isoflavones when supplied at concentrations similar to those observed in human plasma (e.g. [14–16]). A possible explanation for such apparent discrepancy between the epidemiological and/or in vivo evidence and in vitro effects of the isoflavones may be that isoflavones, being lipophilic molecules, are concentrated in breast tissue so that levels inhibitory to cell proliferation can be achieved. Documented technical difficulties associated with measurement [10] may account for the current lack of knowledge about isoflavone concentrations reached in breast tissue.

Studies in in vitro systems, which probably have the greatest potential to yield a mechanistic understanding of anti-cancer effects of the isoflavones, typically focus on the activity of the aglycone forms of the most abundant soyabean isoflavones, daidzein and genistein. Hydroxylated metabolites of both daidzein and genistein can be detected in the urine of human subjects following soyabean consumption [17], but their biological activity has received limited attention and effects at concentrations equivalent to those of the parent compounds at which antiproliferative effects on breast cancer cell lines are observed have not, to our knowledge, been reported.

To establish the implications of isoflavone metabolism with respect to interindividual variability in potential beneficial effects, and also to assess the possibility of extra-hepatic metabolism and drug–diet interactions, it is important to identify the CYPs that contribute to isoflavone metabolism. Since CYPs are present in tissues as multiple isoforms with overlapping and promiscuous substrate specificities, demonstrating that a specific isoform makes an important contribution to the metabolism of a substrate in vivo requires a number of complementary approaches. These approaches include demonstrating catalytic competency of the specific isozyme in isolation, demonstrating that metabolism by microsomes is inhibited by isozyme-specific chemical inhibitors or antibodies and establishing correlation between rates of microsomal metabolism with that of marker substrates or with levels of enzyme expression at the protein level. For genistein, evidence using all of these approaches indicates that CYP1A2 makes the major contribution to metabolism by the human liver [18–20]. For daidzein, specific inhibition of CYP1A2 has been shown to reduce the rate of formation of the major hydroxylated metabolites [21], but it has yet to be demonstrated that rates of metabolism correlate with levels of enzyme expression in the human liver and studies demonstrating the catalytic competency of individual CYPs are lacking.

The findings of the present study add to the body of accumulating data that human CYP1A2 makes the major contribution to the hepatic metabolism of both daidzein and genistein, and identify the potential of the extra-hepatic CYPs 1A1 and 1B1 to contribute to localised metabolism of both isoflavones. Further, the findings provide proof of principle that CYP-derived isoflavone metabolites can have biological activities that are enhanced in comparison with the parent compound.

2. Materials and methods

2.1. Materials

Genistein and daidzein were from LC Laboratories (Woburn, MA). Monohydroxylated metabolites of daidzein—6,7,4′-trihydroxyisoflavone (M1) and 7,3′,4′-trihydroxyisoflavone (M2)—were from the Indofine Chemical Company (Hillsborough, NJ). Uridine 5′-diphosphoglucuronic acid (trisodium salt), NADPH, quinidine and 4-methylpyrazole were from the Sigma–Aldrich Company Ltd. (Poole, Dorset). Ketoconazole and furafylline were a gift from Prof. F.C. Campbell, Queen's University, Belfast. Gentest Supersomes (microsomes from Sf9 insect cells co-expressing human NADPH cytochrome P450 reductase and specific CYPs from recombinant baculovirus) were purchased from BD Biosciences (Cowley, Oxford). Human liver microsomes characterised for specific CYP activities were as previously described [22]. Media and reagents for cell culture were from Simga (Poole, Dorset).

2.2. CYP-mediated metabolism of daidzein and genistein

Daidzein or genistein (100 μM, added from a stock of 100 mM in DMSO) were incubated with human liver microsomes (0.5 mg protein/incubation for 30 min) or Supersomes (160 pmol of baculovirus-expressed CYP for 120 min) at 37 °C with 1 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 400 μl. Chemical inhibitors of specific CYPs were added from 200× stock solutions made up in methanol (furafylline (final concentration 50 μM); 4-methylpyrazole (final concentration 100 μM); ketoconazole (final concentration 1 μM)) or DMSO (quinidine (final concentration 5 μM)). Incubations were terminated by placing on ice and adding 50 μl of 5% perchloric acid and 400 μl of acetonitrile. After an additional 5 min incubation on ice the samples were centrifuged for 5 min at maximum speed in a microfuge. Supernatants were analysed on a Kontron HPLC system with detection at 260 nm using a C_{18} reverse-phase column (Phenomenex, Macclesfield, Cheshire) with an isocratic mobile phase comprising 0.18% TFA in water (solvent A) and 0.15% TFA in acetonitrile (solvent B) mixed in ratios 60A:40B for daidzein and 55A:45B for genistein.

2.3. *Effects of daidzein and monohydroxylated metabolites on MCF-7 cells*

The human breast cancer cell line MCF-7 was maintained at 37 °C in an environment of 5% CO_2/95% air in Eagle-modified essential medium (EMEM) with Glutamax and Earle's salts, containing 10% (v/v) foetal calf serum and 1% (v/v) non-essential amino acids. Medium was replaced every 2–3 days. Cells were seeded at approximately 1 million cells per 25 cm^2 flask and passaged at a ratio of 1:4 when confluent (approximately 7 days post-seeding). For passage, medium bathing the cells was discarded and the cells were washed twice in PBS before adding a solution of 0.05% trypsin and 0.2% EDTA in Earle's balanced salt solution (1 ml per 25 cm^2 flask) and incubating at 37 °C until cells became detached. Trypsin was neutralised by the addition of 1 ml of culture medium and the suspension was centrifuged at 1000 × *g* for 5 min. The cell pellet was resuspended in fresh culture medium. Treatment with daidzein and metabolites was 24 h after seeding at a density of 5×10^5 cells per 25 cm^2 flask. Medium was replaced with EMEM with Glutamax and Earle's salts, containing 2% (v/v) foetal calf serum and 1% (v/v) non-essential amino acids plus the test compound added at 50 μM from a stock solution of 100 mM in DMSO. An equivalent volume (0.05%, v/v) of vehicle only was added to control flasks. Cells were maintained in this medium, which was replaced daily, for 72 h at 37 °C in an environment of 5% CO_2/95% air. To determine total cell counts in the floating population, media were removed and centrifuged at 1000 × *g* for 5 min and the cell pellet resuspended in approximately 200 μl complete medium (exact volume determined) before counting using a haemocytometer. Adherent cells were removed by trypsinisation as described above and resuspended in 1 ml complete medium before counting. Cells in the floating population were counted after staining with acridine orange (1.9 μg/μl) and ethidium bromide (1.9 μg/μl) and were detected by fluorescence microscopy. Cells were scored as live if appearing green with no membrane blebbing. Cells appearing green and showing clear membrane blebbing were scored as non-necrotic and those appearing orange were scored as necrotic. A minimum of 100 cells were scored visually for each sample.

3. Results

3.1. *Common isozymes are responsible for the majority of CYP-mediated hydroxylation of both daidzein and genistein in human liver*

The NADPH-dependent metabolism of daidzein (7,4′-dihydroxyisoflavone) and genistein (5,7,4′-trihydroxyisoflavone) was studied in microsome preparations from four different human livers, previously characterised with respect to the rate with which they metabolised known substrates of specific CYPs [22], and in commercially-available microsome preparations from recombinant baculovirus-transduced Sf9 insect cells (Gentest Supersomes). For both compounds, three major peaks with reduced retention time compared with the parent compound were observed on HPLC analysis following the incubation of human liver microsomes with 100 μM substrate (Fig. 1). The appearance of these metabolites was NADPH-dependent. No metabolites were observed when this co-factor was omitted from the incubation buffer, commensurate with catalysis by CYP. Hydroxylated metabolites of daidzein and genistein, other than 7,3′,4′-trihydroxyisoflavone and 6,7,4′-trihydroxyisoflavone, were not available for use as standards so peak areas were quantified relative to standard curves based on the respective parent compounds. Standard curves were linear over the full range of concentrations used (0–10 μM). Mean intraday coefficients of variation, based on the

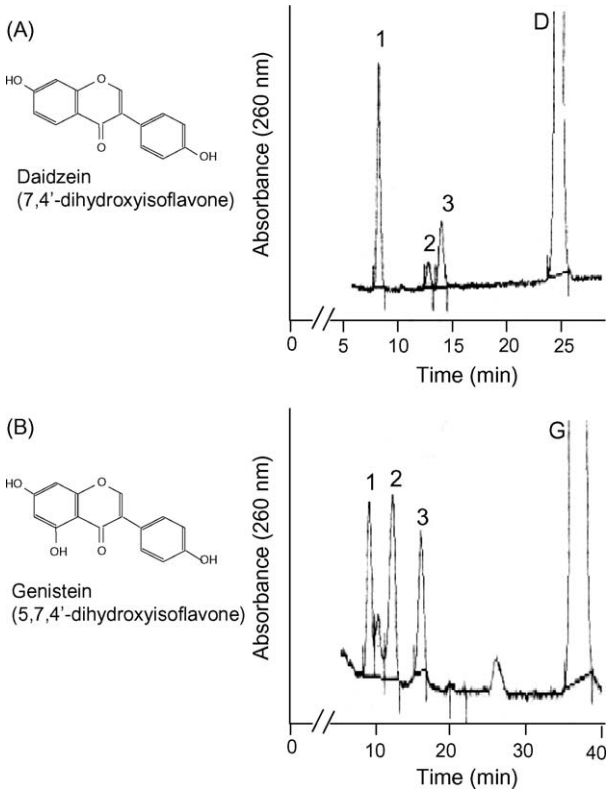

Fig. 1 – Typical HPLC traces showing metabolites of daidzein and genistein generated by human liver microsomes in the presence of NADPH. Panel A shows a trace following incubation with daidzein. Panel B shows a trace following incubation with genistein. Microsomes were incubated at a concentration of 1.25 mg protein/ml with 100 μM substrate for 30 min at 37 °C and products were separated by RP-HPLC on a C18 column using a mobile phase comprising 0.18% TFA in water (solvent A) and 0.15% TFA in acetonitrile (solvent B) mixed in ratios 60A:40B for daidzein and 55A:45B for genistein. The structures of the parent compounds are shown and metabolite peaks designated 1, 2 and 3 for each, along with peaks for the parent compounds (D: daidzein; G: genistein), are labelled. The peak in Panel B eluting at around 27 min was observed in the absence of microsomes and also when reactions were stopped at time 0 min and so is assumed to be a contaminant of the genistein preparation.

agreement on duplicate standards across the full concentration range, were 4.6% for daidzein and 3.4% for genistein. Interday coefficients of variation based on the slope of repeated standard curves were 7.6% for daidzein and 25.1% for genistein. Mean intraday coefficients of variation for the generation and quantification of the metabolites, based on the agreement of independent, duplicate measures using three preparations of human liver microsomes were 4.5%, 15.5% and 10.0% for daidzein metabolite peaks 1, 2 and 3, respectively, and 8.3%, 5.8% and 5.7% for genistein metabolite peaks 1, 2 and 3, respectively. Mean interday coefficients of variation for the generation and quantification of metabolites of daidzein, based on four different Supersome preparations which generated metabolites were 28.5%, 44.3% and 15.8% for peaks 1, 2 and 3, respectively. For all microsome preparations, the major metabolite of daidzein was that with the lowest retention time (peak 1; Fig. 1, Panel A and Fig. 2, Panel A) but for genistein the relative levels of the major metabolites varied between different microsome preparations (Fig. 1, Panel B and Fig. 2, Panel B). Peak 2 generated in incubations with daidzein co-eluted with 7,3′,4′-trihydroxyisoflavone and peak 3 co-eluted with 6,7,4′-trihydroxyisoflavone. The relative proportions of each metabolite generated by each of the four human liver microsome preparations are shown in Fig. 2 (Panels A and B for daidzein and genistein, respectively). The sum of the three major metabolites generated from daidzein and genistein by each of the four human liver microsome preparations showed a significant linear correlation ($P < 0.05$; Fig. 2, Panel C), indicating that the same CYP(s) is/are primarily responsible for the metabolism of both isoflavones. Of the four human liver microsomal preparations used in these experiments, that with the lowest measured rate of ethoxyresorufin O-dealkylation, indicating CYP1A2 activity, generated the metabolites of daidzein and genistein in the lowest quantities (Fig. 2, Panel C).

3.2. *Inhibition of specific CYP activities and incubations with single isozymes indicate the involvement of CYP1A2, CYP1A1 and CYP1B1 in the hydroxylation of daidzein and genistein*

The effect of inhibitors of specific CYP activities on the generation of the metabolites of daidzein and genistein was determined using microsomes prepared from three different human livers which expressed high levels of CYP1A2, CYP2E1 or CYP3A4. The generation of all three major metabolites of both isoflavones was reduced significantly by the inclusion of 50 μM furafylline (CYP1A2 inhibitor) or 100 μM 4-methylpyrazole (CYP2E1 inhibitor) in the incubation buffer, but not by 1 μM ketoconazole (CYP3A4 inhibitor) or 5 μM quinidine (CYP2D6 inhibitor) (Fig. 3). To investigate further the potential for specific CYP isoforms, including those expressed extrahepatically, to contribute to the metabolism of daidzein and genistein, a range of commercially-available microsome preparations from recombinant baculovirus-transduced Sf9 insect cells (Gentest Supersomes) were screened for their ability to generate these more polar metabolites. Microsomes expressing CYP1A2 catalysed the formation of metabolites of both daidzein and genistein (Fig. 4), consistent with the ability of the CYP1A2 inhibitor furafylline to reduce the formation of these metabolites. CYP2E1 was found to be inactive in incubations with daidzein or genistein (Fig. 4) but the microsome preparation was known to generate 13.7 pmol 4-nitrocatechol/pmol P450/min by the hydroxylation of 200 μM

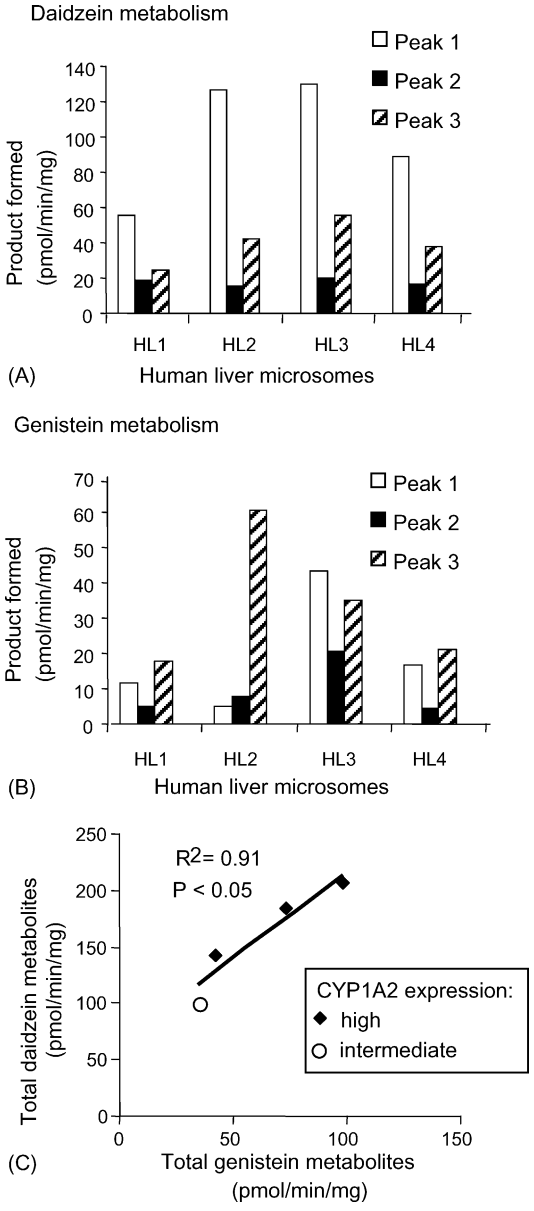

Fig. 2 – The CYP-mediated metabolism of daidzein and genistein by human liver microsomes. Panel A shows the rate of formation of the three major metabolites of daidzein and Panel B shows the rate of formation of the three major metabolites of genistein, identified as three major peaks on analysis by RP-HPLC, by preparations of microsomes from livers from four different human donors. Panel C shows the relationship between the rate of formation of the sum of all three major metabolites of daidzein and genistein by the four human liver microsome preparations and indicates the relative level of CYP1A2 expression as determined by ethoxyresorufin O-dealkylation. Data are the means of two independent measures for each microsome preparation.

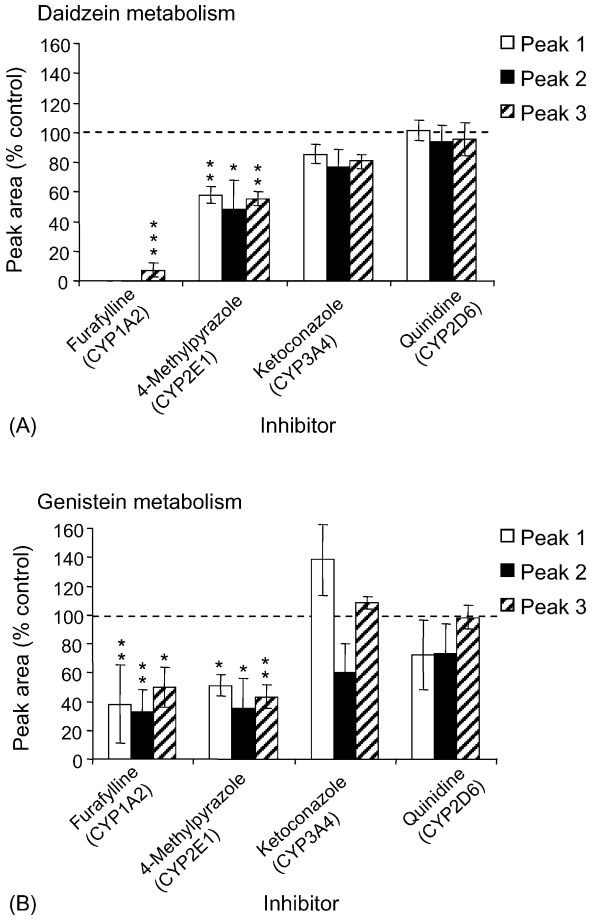

Fig. 3 – The effect of chemical inhibitors of specific CYP activities on the metabolism of daidzein and genistein by human liver microsomes. Panel A shows data for daidzein. Panel B shows data for genistein. Measurements were carried out in duplicate on three separate preparations of human liver microsomes. Each microsome preparation was analysed on a separate day in a single experiment, including all inhibitors tested. Substrate was supplied at a concentration of 100 μM and incubations were for 30 min at 37 °C. Inhibitor concentrations were: furafylline—50 μM; 4-methylpyrazole—100 μM; ketoconazole—1 μM; quinidine—5 μM. Data are expressed as mean ± S.E.M. ($n = 3$) as a percentage of the corresponding metabolite peak area, identified by RP-HPLC, for a control incubation containing vehicle only. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control by one-way ANOVA followed by Dunnett's post-test.

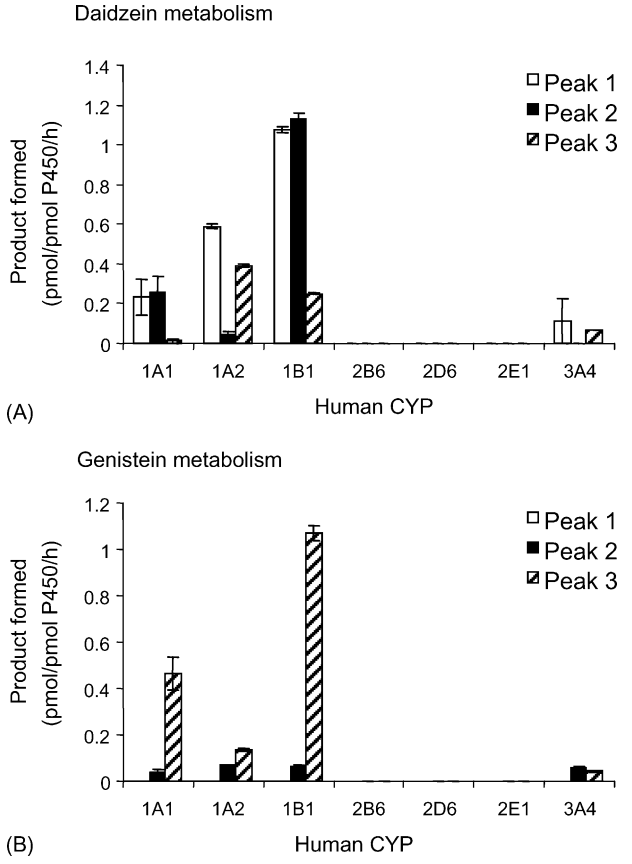

Fig. 4 – The metabolism of daidzein and genistein by recombinant human CYPs, supplied as Supersomes. Panel A shows data for daidzein. Panel B shows data for genistein. Substrate was supplied at a concentration of 100 μM and incubations were for 120 min at 37 °C. Activity of the full panel of CYPs was measured in a single experiment for each compound. Data are expressed as mean ± S.E.M. ($n = 3$).

p-nitrophenol, confirming that active CYP1E1 was present. This rate was over two-fold greater than that measured for another CYP2E1 preparation (5.7 pmol/pmol P450/min), obtained independently from the same supplier. The hepatic isoform CYP3A4 also generated metabolites of both isoflavones at comparatively low levels but CYP2B6 and CYP2D6 were found to be inactive (Fig. 4). The extra-hepatic enzymes CYP1A1 and CYP1B1 were found to be catalytically active with either daidzein or genistein as substrate, with CYP1B1 showing a particularly high level of activity (Fig. 4).

3.3. *CYP-mediated hydroxylation of daidzein affects antiproliferative activity in the MCF-7 breast cancer cell line*

Having confirmed the identity of CYPs involved in the metabolism of genistein and daidzein, we sought to determine the effect of metabolism on the biological activity of daidzein with respect to its antiproliferative effect on human breast cancer cells, using the MCF-7 cell line model. The effect of 50 μM daidzein on both total cell numbers and number of dead cells in the floating population was compared with that of two monohydroxylated CYP-generated metabolites—6,7,4′-trihydroxyisoflavone (M1) and 7,3′,4′-trihydroxyisoflavone (M2). After 72 h exposure, all three compounds reduced significantly total cell numbers compared with control, with the effect of 7,3′,4′-trihydroxyisoflavone being significantly greater than that of either daidzein or 6,7,4′-trihydroxyisoflavone (Fig. 5, Panel B). The data also indicate that CYP metabolism potentiated the effect of daidzein on cell death. The parent compound and 6,7,4′-trihydroxyisoflavone, at 50 μM, had no statistically significant effect, compared with control, on the number of dead cells in the floating population, expressed as a percentage

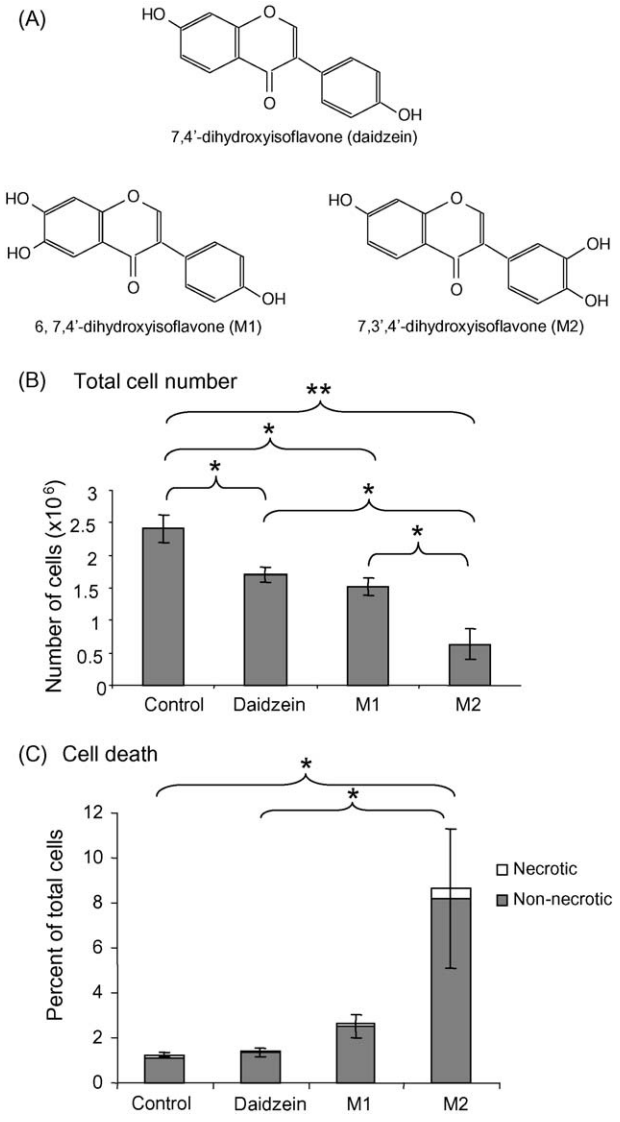

Fig. 5 – The effects of daidzein and its CYP-derived metabolites 6,7,4′-trihydroxyisoflavone (M1) and 7,3′,4′-trihydroxyisoflavone (M2) on cell growth and death in the MCF-7 human breast cancer cell line. Panel A shows the structure of daidzein and the two monohydroxylated metabolites studied. Panel B shows total cell counts. Panel C shows the number of dead cells in the floating population scored visually after staining with ethidium bromide and acridine orange, expressed as a percentage of total cell number. Cells were exposed to the test compounds at a concentration of 50 μM for 72 h. Error bars and indications of statistically significant differences in Panel C relate to the total dead cell population. Data are expressed as mean ± S.E.M. ($n = 3$). $^{*}P < 0.05$ by one-way ANOVA followed by Bonferroni's multiple comparisons test.

of total cell number, but 50 μM 7,3′,4′-trihydroxyisoflavone increased significantly the number of dead cells observed after 72 h exposure (Fig. 5, Panel C). This increase in cell death observed with 7,3′,4′-trihydroxyisoflavone was also significant for the population scored as non-necrotic but not for the population scored as necrotic. Viable cells in the floating population accounted for less than 0.1% of total cell numbers.

4. Discussion

The three major metabolites of both daidzein and genistein identified in this study, generated by human liver microsomes and also by single, recombinant CYPs, had a retention time on RP-HPLC analysis that was reduced compared with the parent compound, demonstrating they were more polar and therefore consistent with them being hydroxylated metabolites. The fact that the appearance of the metabolites in the incubation mixture required the presence of NADPH is consistent with them being metabolites generated by the catalytic activity of CYP. The present study did not identify directly the metabolites corresponding to these peaks, but identification of CYP-derived metabolites of both daidzein and genistein has been the focus of published work which also identified three major peaks for both compounds by HPLC with peak areas for daidzein metabolites of the same relative magnitudes as those observed in the present study (i.e. peak 1 > peak 3 > peak 2) [17] and assigned these as 7,8,4′-trihydroxyisoflavone, 7,3′,4′-trihydroxyisoflavone and 6,7,4′-trihydroxyisoflavone for daidzein metabolites eluting as peaks 1, 2 and 3, respectively, and 5,7,8,4′-tetrahydroxyisoflavone, 5,6,7,4′-tetrahydroxyisoflavone and 5,7,3′,4′-tetrahydroxyisoflavone (orobol) for genistein metabolites eluting as peaks 1, 2 and 3, respectively. Furthermore, we demonstrated that the daidzein metabolites 7,3′,4′-trihydroxyisoflavone and 6,7,4′-trihydroxyisoflavone co-eluted with daidzein metabolite peaks 2 and 3, respectively, giving further confidence to this peak assignation. Together, our data demonstrate that the same isozymes are predominantly responsible for the CYP-dependent metabolism of both daidzein and genistein by human liver (demonstrated by a linear correlation between rates of metabolism for microsomes prepared from four independent human livers) and that CYP1A2 makes a major contribution to the metabolism of both daidzein and genistein in the human liver. The latter conclusion is substantiated by a number of observations. Firstly, of four livers with different rates of NADPH-dependent daidzein and genistein metabolism, one which had lower CYP1A2 activity than the other three metabolised both compounds at the lowest rate. Secondly, the CYP1A2 inhibitor furafylline reduced the rate of NADPH-dependent metabolism of daidzein (to levels that were almost undetectable) and genistein (by approximately 50%) by human liver microsomes. Thirdly, recombinant CYP1A2 expressed in a preparation of microsomes from a recombinant baculovirus-transduced Sf9 insect cell line catalysed the metabolism of both isoflavones. A correlation between rates of CYP-mediated genistein hydroxylation and CYP1A2 activity was previously reported [19], as was catalytic activity of recombinant CYP1A2 with genistein as substrate [19,20]. In addition, an anti-CYP1A2 monoclonal antibody [19] and also the CYP1A2 inhibitor fluvoxamine [20] inhibited the CYP-mediated metabolism of genistein by human liver microsomes. Our finding that the CYP1A2 inhibitor furafylline also had this effect adds to the evidence that human CYP1A2 plays

a major role in the hepatic metabolism of genistein. To our knowledge, this is the first study to report daidzein metabolism by recombinant human CYP1A2 and also the first to demonstrate a correlation between the rate of daidzein metabolism and CYP1A2 expression in human liver, but it was previously reported that furafylline and an anti-CYP1A2 monoclonal antibody reduced the rate of daidzein monohydroxylation by human liver microsomes [21].

Our observation that the CYP2E1 inhibitor 4-methylpyrazole inhibited significantly the CYP-mediated metabolism of both daidzein and genistein by human liver microsomes is commensurate with the involvement of this isoform in the metabolism of both isoflavones in human livers which express high levels of this isoform and is, to our knowledge, the first evidence to demonstrate a link between inhibition of CYP2E1 activity and reduced daidzein metabolism. Our observation that Supersomes expressing CYP2E1 generated no detectable metabolite with either daidzein or genistein as substrate is, however, at variance with these findings and also with the findings of Roberts-Kirchoff et al. [18] who detected metabolites of genistein using CYP2E1 Supersomes from the same supplier, albeit at very low levels. Catalytic activity of the preparation of CYP2E1 Supersomes used in the present study was confirmed by measuring the conversion of *p*-nitrophenol to 4-nitrocatechol and was over two-fold greater than for an equivalent CYP2E1 Supersome preparation obtained from the same supplier. This variation in activity indicates the difficulty in comparing results obtained from different laboratories, particularly when rates of metabolism are low. Other evidence indicating that CYP2E1 may not be important in the metabolism of daidzein, but which is at variance with the inhibition of daidzein metabolism observed using the CYP2E1 inhibitor 4-methylpyrazole, is the observation that the CYP2E1 inhibitor diethyldithiocarbamate did not reduce the rate of CYP-mediated daidzein hydroxylation by human liver microsomes [21]. A monoclonal antibody to CYP2E1 slightly, though significantly, reduced the metabolism of genistein by human liver microsomes and that rate of metabolism by microsomes from a bank of human livers was very weakly correlated with CYP2E1 activity [19] indicating a role for CYP2E1 in the metabolism of genistein. The balance of evidence based on other published studies and data reported here indicates that CYP2E1 plays a role in the hepatic metabolism of both daidzein and genistein but that its contribution is minor compared with that of CYP1A2. The contribution of CYP1E2 to the metabolism of both isoflavones may be proportionally greater in individuals expressing relatively high levels of this isoform.

We found no indication that any other hepatic CYP studied is important in the metabolism of daidzein or genistein in the human liver. Inhibitors active against CYP3A4 (ketoconazole) and CYP2D6 (quinidine) had no significant effect on the rate of daidzein or genistein metabolism by human liver microsomes, in agreement with the observation that monoclonal antibodies inhibitory against these two isoforms had no effect on genistein metabolism [19] and that neither the alternative CYP3A4 inhibitor troleandomycin nor quinidine affected daidzein metabolism by human liver microsomes [21]. In agreement, we detected no metabolism of either daidzein or genistein using recombinant CYP2D6. Published studies on genistein metabolism include data both in agreement [18] and at variance [19] with the low rate of metabolism of both isoflavones we detected using Supersomes expressing CYP3A4, but it appears that this activity does not translate into any significant contribution from this isozyme to metabolism in the human liver.

We detected high levels of activity with either daidzein or genistein as substrate using Supersomes expressing the extrahepatic CYPs 1A1 and particularly 1B1. As discussed above, our own direct measurements on the activity of different CYP2E1 preparations and also comparison of data derived using recombinant CYPs as Supersomes in different studies, which measure different relative rates of genistein metabolism [18,19], indicates that such comparative data is not robust. Unlike the present study, neither of two published studies focusing exclusively on genistein metabolism [18,19] found CYP1B1 to be the most active isoform. Nonetheless, the data indicate that genistein and, for the first time, daidzein can be metabolised extrahepatically, including in tissues such as breast and prostate which express these isoforms [23–25].

Our observation that daidzein at 50 μM, reduced the apparent growth of MCF-7 cells over a period of 72 h is in agreement with other reports of effects of daidzein at similar concentrations [8,9], but was not, under our experimental conditions, due to an increase in cell death so may have resulted from a reduced rate of cell division. Daidzein 6-hydroxylation, to generate 6,7,4′-trihydroxyisoflavone (M1), had no effect on the ability of daidzein to reduce cell numbers, but 3′-hydroxylation, to generate 7,3′,4′-trihydroxyisoflavone (M2), potentiated this observed response. The reduction in total cell numbers observed with 7,3′,4′-trihydroxyisoflavone may have been due in part to the observed increase in cell death compared with control conditions or with daidzein, but such a response can account for only a fraction of the observed total reduction in cell numbers, indicating an antiproliferative effect. The enhancement of antiproliferative activity and increased rate of cell death observed in the presence of 7,3′,4′-trihydroxyisoflavone demonstrates proof of principle that metabolism of daidzein by CYP can increase biological activity. An earlier study [16] reported attenuated, rather than enhanced, biological activity of these metabolites compared with daidzein in MCF-7 cells, but these observations were made at concentrations in the range 0.1–10 μM, where daidzein induced cell proliferation. Uncertainty about concentrations of isoflavones achieved in breast tissue, discussed previously, and the agreement between epidemiological and/or in vivo evidence and in vitro effects of isoflavones on breast cancer cell lines at higher concentrations, as used in the present study, highlights the possibility that daidzein metabolism in vivo may enhance its biological effects relevant to the prevention of breast cancer.

In conclusion, the data presented here on the CYP-mediated metabolism of the isoflavones daidzein and genistein and on the effect of this metabolism on selected aspects of the biological activity of daidzein in vitro indicate that metabolism in liver, predominantly by CYP1A2, and also extra-hepatic metabolism, including at sites of hormone-dependent tumours by isoforms including CYP1A1 and CYP1B1, may enhance anti-cancer activity if sufficiently high concentrations are achieved in target tissues.

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