The Methoxylated Flavones Eupatorin and Cirsiliol Induce CYP1 Enzyme Expression in MCF7 Cells

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Flavonoids have often been associated with cancer prevention and activity of the human cytochrome P450 enzymes CYP1A1 and CYP1B1 with the occurrence of cancer. The flavones eupatorin (**1**) and cirsiliol (**2**) enhanced CYP1 enzyme activity in a concentration-dependent manner in MCF7 human breast adenocarcinoma cells. In the range of 0−2.5 μM, **2** caused a dose-dependent increase in CYP1B1 mRNA levels and an increase in CYP1A1 mRNA. Compound **1** caused an increase in CYP1A1 and CYP1B1 mRNA at higher doses (~5 μM). Both CYP1B1 and CYP1A1 catalyzed the conversion of **2** into an as yet unidentified compound. Application of the CYP1 family inhibitor, acacetin, significantly increased the IC_{50} value of **2** in MCF7 cells, but did not significantly affect the action of **1**. The data suggest that **2** induces CYP1 enzyme expression in cancer cells and is subsequently converted by CYP1B1 or CYP1A1 into an antiproliferative agent.

Flavonoids are a class of compounds abundantly present in fruits and vegetables. Over the past few decades, extensive investigation of this class of natural products has identified various effects on human cell physiology and gene expression.[1] The contribution of flavonoids in the prevention of cancer cannot be neglected; initial epidemiological studies and later more specific in vitro experimental studies have underpinned their beneficial action on multiple cancer-related biological pathways.[2] Several models have been proposed that may explain the role of flavonoids in the prevention of cancer; for example, these compounds may act as antioxidants or as phytoestrogens and interfere with estrogen metabolism, or they may affect cell signaling or the regulation of genes involved with carcinogenesis.[3−6] Particularly the effects on expression of genes coding for enzymes involved in estrogen metabolism, such as the cytochrome P450 CYP1 family, have been the focus of attention.[7−9]

Expression of the CYP1 genes is generally believed to be mediated by the aryl hydrocarbon receptor (AhR), which responds to a number of coal tar carcinogens, such as benzo[*a*]pyrene, and to potent tumor promoters such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).[10,11] Flavonoids may act as antagonists for the AhR and thus prevent a cascade that would eventually lead to tumor promotion.[12] In addition, several flavonoids were found to inhibit the activity of the CYP1 enzymes.[13−16] It has been postulated that the CYP1 enzymes play a role in tumor progression, either by activating procarcinogens or paradoxically by inactivation of anticancer drugs.[17]

Resveratrol, a naturally occurring phytoestrogen with a stilbene core structure, has proven to be a substrate of the enzyme CYP1B1, which is specifically expressed in tumors.[18,19] CYP1B1 was shown to catalyze the conversion of resveratrol into piceatannol, a potent tyrosine kinase inhibitor. It has been postulated that enzymes that are found specifically in human tumors can convert more dietary phytoestrogens into compounds that inhibit cell proliferation.[18,20,21]

The flavones eupatorin (**1**) and cirsiliol (**2**) (Figure 1) are present in the leaf extract of *Lantana montevidensis* Briq. (Verbenaceae), which showed antiproliferative activity against MK-1, HeLa, and B16F10 cells in vitro.[22] A recent study undertaken in our laboratory led to the identification of **1** as a CYP1-activated prodrug, which inhibits breast cancer cell proliferation, and of **2** as the main metabolite, responsible for this type of activity.[23] In the present study, the effects of **1** and **2** on CYP1 enzyme activity and on CYP1A1 and CYP1B1 gene expression in MCF7 cells were examined. A new hypothesis involving induction of CYP1 enzyme expression by natural flavonoids and subsequent activation in cancer cells is presented.

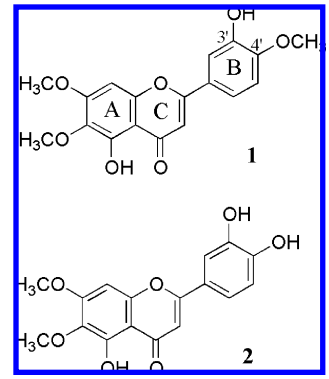

Figure 1. Structures of eupatorin (**1**) and cirsiliol (**2**).

Results and Discussion

The flavones eupatorin (**1**) and cirsiliol (**2**) induced CYP1 enzyme expression after 24 h in a concentration-dependent manner (Figure 2). Although the 7-ethoxyresorufin-*O*-deethylase assay (EROD) is mainly selective for CYP1A1, the contribution of CYP1B1 to the total activity cannot be excluded. The major induction was noticed at 2.5 μM. The increase in CYP1 activity in MCF7 cells caused by treatment of natural flavones was compared to TCDD, which was also used as a positive control. The enhancement caused by 2.5 μM **2** was nearly half that of 10 nM TCDD. The enhancement of EROD activity caused by treatment with 2.5 μM of the flavones was transient, and activity decreased after 24 h to reach minimal levels at 36 h (Figure 2).

We further investigated the effect of **1** and **2** on the transcription of CYP1A1 and CYP1B1 in MCF7 cells. PCR fragments for β-actin and both CYP1 genes were predicted from Döhr et al.[24] Compound **1** caused an increase in the mRNA levels of both CYP1A1 and CYP1B1 at higher concentrations (5 μM) (Figure 3A). At this concentration, CYP1B1 mRNA levels were higher than CYP1A1 (Figure 3B). No increase at concentrations lower than 5 μM was

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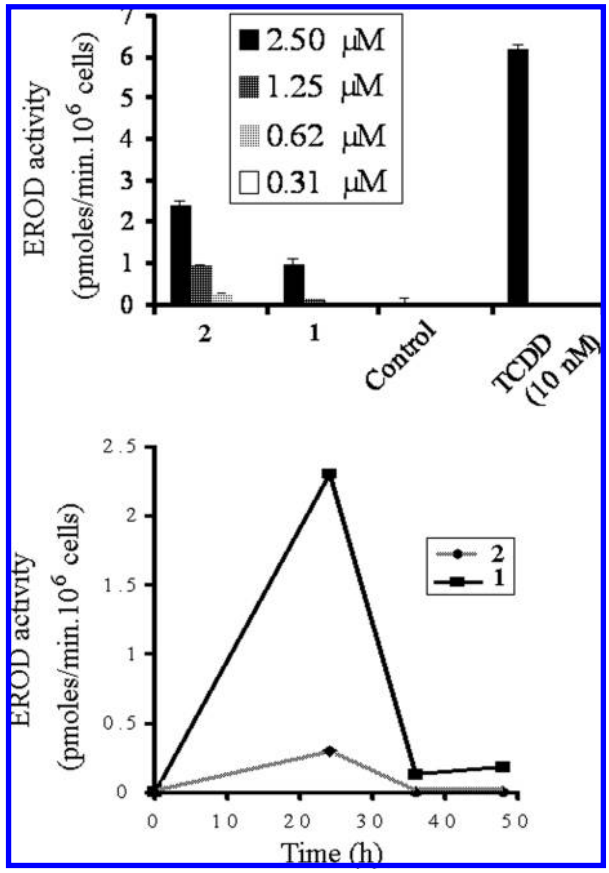

Figure 2. Effect of flavonoids on the expression of CYP1 family enzymes in MCF7 cells. Top: Dose−response effect after 24 h treatment. TCDD was used at 10 nM as positive control and 0.1% DMSO as negative control. The concentration range was 0.31, 0.625, 1.25, 2.5 μM. Bottom: Time profile of induced CYP1 activity by 2.5 μM of flavonoids. The cells were exposed to different concentrations of flavonoids for 24 h as described in the Experimental Section. Experiments were performed in three replicates.

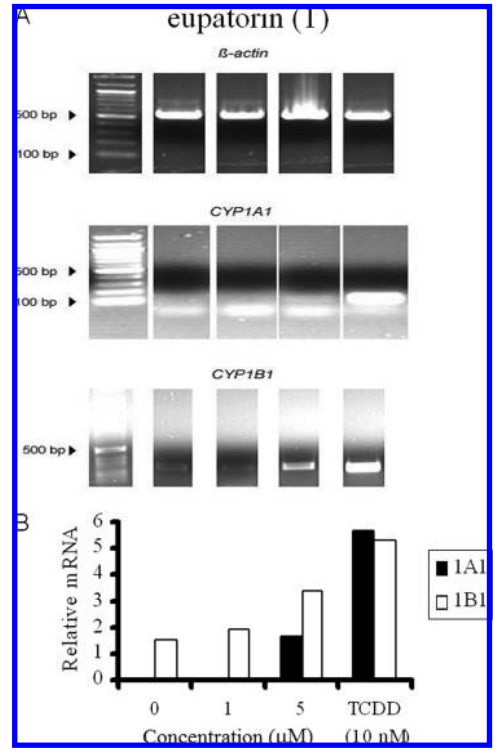

Figure 3. Effect of eupatorin (**1**) on the transcription of CYP1B1 and CYP1A1 in MCF7 cells. (A) RT-PCR analysis. MCF7 cells were treated with high concentrations of **1** for 24 h, and RT-PCR was performed as described in the Experimental Section. Top to bottom: β-actin, CYP1A1, CYP1B1; left to right: 0, 5 μM **1**. (B) Increase in CYP1A1 and CYP1B1 mRNA. The mRNA levels were normalized to β-actin using Quantity One software. Black bars: CYP1A1; white bars: CYP1B1.

noted. In turn, compound **2** caused a dose-dependent increase in the mRNA levels of CYP1B1 and an increase of CYP1A1 mRNA at 2.5 and 1.25 μM (Figure 4A). At the relatively high concentration of 5 μM, **2** did not induce transcription of CYP1A1 (Figure 4A), whereas the mRNA levels of CYP1B1 were comparable to those produced by the potent inducer TCDD (10 nM). At 1.25 and 2.5 μM **2**, CYP1A1 mRNA levels were nearly half of those caused by 10 nM TCDD induction (Figure 4B).

In an effort to quantify the antiproliferative activity of eupatorin (**1**) and cirsiliol (**2**), the viability of MCF7 cells was assessed after 96 h of treatment with various concentrations of these two flavones. The IC_{50} value for **2** (8 μM) rose significantly (to 15 μM, $p < 0.05$, Figure 5A) when the cells were co-incubated with 1.5 μM acacetin, which is a known CYP1 inhibitor.[25] The effect was more profound at 25% inhibition of cell proliferation (IC_{25} 0.5 μM), where a 10-fold difference was observed in the presence of the inhibitor (IC_{25} 5 μM). Compound **1** did not show a significant change in IC_{50} or IC_{25} value in the presence of acacetin (Figure 5B), even though the IC_{50} value was almost equal to that demonstrated for **2** ($\approx$7 μM). Acacetin alone at 1.5 μM did not affect the viability of MCF7 cells (Figure 5C).

Incubation with microsomes expressing recombinant human CYP1B1 or CYP1A1 caused a decrease in the concentration of **2** over a 20 min period (Figure 6). The decrease was not observed with control microsomes, expressing an empty vector. CYP1A1 metabolized **2** faster than CYP1B1 (Figure 6). The CYP1 enzymes converted **2** to a minor product with a shorter retention time in the HPLC assay used (data not shown).

The CYP1 enzymes have been shown to participate in the activation of various carcinogenic compounds, and as a result, their implication in cancer progression has been established by numerous studies.[19,26] In the past few years, various research groups have investigated the induction of CYP1A1 and CYP1B1 in cancer cells by a variety of environmental agents including natural products supplied in the diet.[10] The interaction of flavonoids with the CYP1 family of enzymes has mainly focused on the inhibition of the cancer-promoting effect of various chemicals (e.g., TCDD) through the AhR, as well as direct inhibition of the enzymes.[14,27] In the present study, an alternative cancer preventive mechanism of action for flavonoids is presented. We examined the effects of eupatorin (**1**) and cirsiliol (**2**) in inducing the expression of CYP1A1 and CYP1B1. Both compounds induced CYP1 expression in MCF7 cells in a dose-dependent manner. Compound **2** was a more potent inducer than **1**, as recorded by EROD activity and CYP1 mRNA levels.

Naturally occurring flavonoids are less active than the prototypic AhR ligand TCDD, and previous studies have demonstrated that their CYP1-inducing potential in MCF7 cells lies between 0.5 and 10 μM.[27,28] It was shown that in the range 0.5 to 10 μM, quercetin, a flavonoid with hydroxy substituents at the 3′ and 4′ positions of the B ring, increased CYP1A1 mRNA levels in MCF7 cells, whereas kaempferol, lacking one hydroxy group on the same ring, was completely inactive.[28] Incubation of MCF7 cells for 24 h with 5 μM diosmetin induced CYP1A1 expression and was shown to have the highest EROD activity at 2.5 μM within the same time period.[27] Diosmetin contains a methoxy group at the 4′ position of

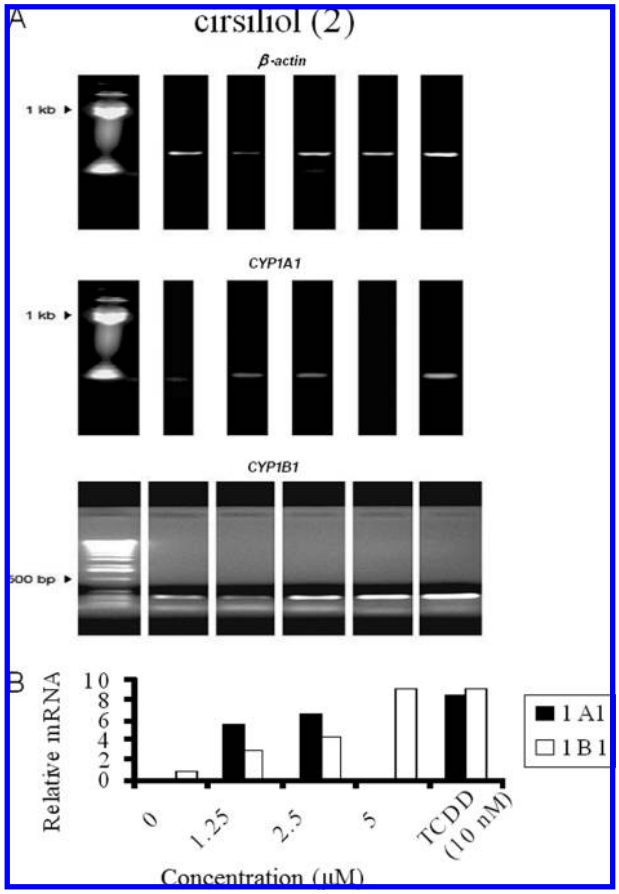

Figure 4. Cirsiliol (**2**) induction of CYP1A1 and CYP1B1 mRNA in MCF7 cells. (A) RT-PCR analysis. Compound **2** was incubated with MCF7 cells for 24 h, and RT-PCR was performed as described in the Experimental Section. Top to bottom: β-actin, CYP1A1, CYP1B1; left to right: 1 kb ladder, 0, 1.25, 2.5, 5 μM **2**, TCDD (10 nM). (B) Semiquantitative levels of transcription of CYP1A1 and CYP1B1. White bars: CYP1B1; black bars: CYP1A1. The gels and the graph are one trace of three individual experiments.

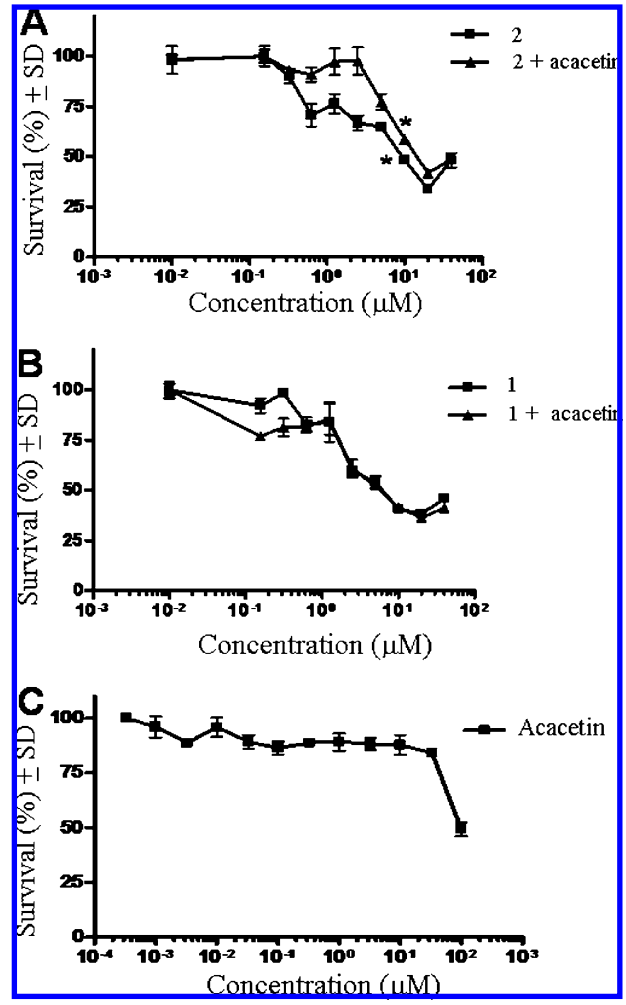

Figure 5. Cytotoxicity caused by flavonoids on MCF7 cells. Inhibition of MCF7 cellular proliferation by compound **1** or **2** in the presence or absence of the CYP1 family inhibitor acacetin. MCF7 cells were incubated for 96 h with the flavones in the presence or absence of 1.5 μM acacetin, and cell viability was measured using the MTT assay. Cytotoxicity caused by acacetin was tested by incubation of the cells with the compound in a separate experiment. Results are expressed as % survival ± SD for at least three independent determinations. *Results were significantly different $p < 0.05$. (A) **2**, (B) **1**, (C) acacetin.

the B ring; thus it seems that induction of the CYP1 family enzymes by flavonoids is affected mainly by substitutions on the B ring. Compound **2** differs from **1** by a hydroxy group at the 4′ position on the B ring (Figure 1), and therefore any difference observed in inducing activity must be due to this structural difference.

Having shown that treatment of MCF7 cells with eupatorin (**1**) or cirsiliol (**2**) increases the expression of CYP1A1 and CYP1B1 enzymes, we investigated whether these two flavones could induce their own activation to more antiproliferative species, through CYP1-mediated metabolism. Acacetin, a CYP1 family inhibitor, was used to block conversion of flavones by CYP1A1 or CYP1B1. Compound **1** did not show any major difference in MCF7 cell viability after cotreatment with acacetin, while **2** alone was significantly more toxic for MCF7 cells (especially when 25% of cellular inhibition was assessed) than when coadministered with acacetin. In addition, **2** was shown to be a substrate for CYP1 enzymes. CYP1A1 metabolized this compound faster than CYP1B1.

Previous studies have proposed that following flavonoid-induced gene expression: the increased activities of CYP1A1 and phase II enzymes contribute to the detoxification of polycyclic aromatic hydrocarbons (PAHs).[26,27] Furthermore, dietary flavonoids have been examined for their inhibition of CYP1 enzyme activity and CYP1A1-mediated PAH activation.[25,27−30] For example, diosmetin and galangin were shown to inhibit the formation of DNA adducts and DMBA-induced cytotoxicity in MCF7 cells.[28,29] In addition, flavonoids such as quercetin and 3′,4′-dimethoxyflavone have been reported as inhibitors of CYP1B1 activity and, thus, inhibitors of procarcinogen bioactivation.[13,31] Such a mechanism of action might apply to eupatorin (**1**) and cirsiliol (**2**) as well. However, we propose the novel idea that flavonoids can induce CYP1 genes and be substrates for CYP1 enzymes;that is, flavonoids might induce their own bioactivation. In our recent studies we have demonstrated that the flavones diosmetin and **1** are substrates for the enzymes CYP1A1 and CYP1B1.[23,32,33] Since diosmetin induces CYP1A1 in MCF7 cells, it can be assumed that it is metabolized by the same enzyme it induces. Herein, we provide additional evidence that flavonoids such as **2** can prevent cancer by inducing their metabolism through up-regulation of CYP1 enzyme expression in cancer cells. Our results demonstrate that this mechanism of action occurs in vitro at concentrations (2.5 μM) close to the physiological levels of circulating flavones in plasma.[34]

We have shown previously that eupatorin (**1**) is a CYP1 substrate, which causes inhibition of breast cancer growth at submicromolar concentrations.[23] In this study **1** exhibited a considerably low IC_{50} value in MCF7 cells, but this was not due to a CYP1-mediated metabolism. The most probable explanation is that the increase in CYP1 enzyme expression was not enough to yield the desirable

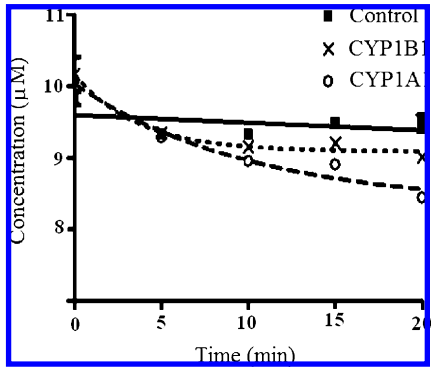

Figure 6. Disappearance of compound **2** catalyzed by CYP1B1 and CYP1A1. Compound **2** (10 μM) was incubated with control microsomes and microsomes expressing recombinant human CYP1B1 or CYP1A1, and samples were taken at 5 min intervals and analyzed by HPLC. Error bars represent min. and max. values of two independent experiments.

amount of metabolites, which can cause inhibition of cell proliferation at very low doses (<1 μM). In addition, CYP1 induction by **1** was decreased after 24 h to reach baseline levels at 36 h. In contrast to these findings, a strong bioactivation of the same compound was noticed previously, because CYP1A1 and CYP1B1 enzymes were expressed constitutively in MDA-MB 468 cells.[23]

A few studies have investigated previously the metabolism of flavonoids by various cytochrome P450s, including CYP1A1 and CYP1B1. It was shown that flavonoids such as hesperitin and galangin, either containing methoxy substituents or lacking functional group substitution in the B ring, can be hydroxylated or demethylated to produce more active products.[25,35,36] Similarly 7,3′,4′-trihydroxyisoflavone, the 3′-hydroxylated metabolite of the isoflavone daidzein, by CYP1 enzymes, was shown to increase cell death in MCF7 cells.[37] The results presented here provide more information on the cancer preventive effect of flavonoids, such as cirsiliol (**2**), which is attributed to CYP1 enzyme induction. Overall, our data show that flavonoids that increase CYP1 activity through induction of gene expression can be metabolized to more bioactive compounds by CYP1 enzymes.

Further studies are required to fully identify the products of cirsiliol (**2**) conversion by CYP1 enzymes and the activity of these in cancer cells. However, in this preliminary report we suggest that flavonoids can exert chemopreventive properties by induction of CYP1 genes and subsequent CYP1-catalyzed activation in cancer cells.

Experimental Section

Chemicals and Cell Culture Conditions. Eupatorin (**1**) and cirsiliol (**2**) were obtained from Lancaster (Heysham, UK). Acacetin, 7-ethoxyresorufin, and cell culture and molecular biology reagents were purchased from Sigma (Poole, UK). PCR primers were purchased from Invitrogen (Paisley, UK). HPLC grade water and methanol were from Fisher (Loughborough, UK). CYP1A1 and CYP1B1 supersomes were purchased from BD Biosciences (Oxford, UK). All other chemicals were of analytical grade. MCF7 cells were maintained in RPMI 1640 with phenol red. The medium also contained 2 mM glutamine and 10% (v/v) heat-inactivated fetal calf serum. The cells were grown at 37 °C, 5% CO_2 /95% air, with 100% humidity, and passaged using trypsin EDTA.

CYP1 Activity in MCF7 Cells. MCF7 cells were treated with test compound for the time and concentration indicated, and EROD activity was measured by incubation of the cells with 7-ethoxyresorufin (5 μM) and the phase II enzyme inhibitor salicylamide (1.5 mM) as described previously.[23] Following centrifugation (10 min at 3000 rpm) the supernatant was transferred to a 96-well plate and read using a Spectra Max M5/M5^e microplate reader (Molecular Devices, Sunnyvale, CA) with excitation and emission at 530 and 590 nm, respectively. Resorufin formation was measured by standard curves in each experiment.

RT-PCR and DNA Electrophoresis. MCF7 cells (0.4×10^5 cells/mL) were left to grow for 48 h and treated with compound for the time and concentration indicated. Total RNA was extracted using guanidinium-acid-phenol.[38] Reverse transcription and PCR were performed as described previously.[32] Amplifications were performed using a PTC-200 Peltier DNA thermal cycler (Biorad, Hemel Hempstead, UK), and DNA was visualized using a Biorad Molecular Imager FX (Biorad). Computer densitometry was carried by normalizing the relative amounts of CYP1A1 and CYP1B1 mRNA with β-actin mRNA (Quantity One software; Biorad).

MTT Assays. MCF7 cells (2×10^3) were plated in 100 μL of medium in 96-well flat-bottomed plates. After 24 h, **1** or **2** was added in quadruplicate to the wells to give a final concentration of not more than 0.1% (v/v) DMSO, alone and with 1.5 μM acacetin. The cells were then allowed to grow for 96 h at 37 °C. MTT (2 mg/mL in PBS) was then added to each well for 3 h. The formazan product generated by viable cells was solubilized with DMSO and the absorbance at 540 nm determined using a Spectra Max M5/M5^e microplate reader (Molecular Devices). Results are expressed as the percentage of 100% (control) proliferation, and IC_{50} values were calculated using Microsoft Excel. Dose ranges were in serial dilutions, e.g., 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, and 0.078 μM. Acacetin was tested alone in the following concentration range: 100, 30, 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, and 0.0003 μM.

Metabolism of Cirsiliol (2). Compound **2** (10 μM) was incubated with supersomes (20 pmol/mL CYP1B1, CYP1A1), NADPH (0.5 mM), $MgCl_2$ (0.5 mM), and phosphate buffer (20 mM) at a final volume of 100 μL. The reaction was carried out at 37 °C for 20 min, and samples were taken at 5 min intervals. On sampling, the reaction was terminated by the addition of 100 μL of a methanol−acetic acid mixture (100:1), and the incubates were centrifuged at 3500*g* for 20 min at 4 °C. The supernatant of each sample was extracted and analyzed at 37 °C on a Perkin-Elmer 200 HPLC (Perkin-Elmer, Wellesley) equipped with a Luna C18 4.6 × 150 mm 5 μm column (Phenomenex, Macclesfield, UK). Detection was by a Waters 200 UV detector (Waters, Manchester, UK) at 350 nm. Flow rate was 1 mL/min. Eluents were as follows: solvent A, water−acetonitrile−acetic acid (98.5:1:0.5), and solvent B, methanol−acetonitrile−acetic acid (95.5:4:0.5). A linear gradient program was used: 60% solvent A and 40% solvent B at time 0, 10% solvent A and 90% solvent B at time = 10 min.

Statistical Analysis. Results are expressed as means ± standard deviation for three independent experiments, unless indicated otherwise. Significant differences were determined using Student's *t*-test with 0.05 as the *p* threshold value.

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