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Novel methoxylated flavone inhibitors of cytochrome P450 1B1 in SCC-9 human oral cancer cells

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Abstract

Dietary polyphenols, including flavonoids, have been implied to have cancer preventive properties. Suggested mechanisms include inhibition of carcinogen-activating cytochrome P450 (CYP) transcription and activities. These studies have focused mainly on CYP1A1. However, CYP1B1 has recently been shown to be of particular importance in smoking-induced oral and oesophageal cancer. Previous observations in our laboratory demonstrated that methoxylated flavonoids may be effective inhibitors of CYP1A1 transcription and activity as well as being orally bioavailable. In this study, an initial screening of 19 methoxylated flavones, using the ethoxyresorufin de-ethylation assay in human oral squamous cell carcinoma SCC-9 cells pretreated with 1 μM benzo[*a*]pyrene, identified six strongly inhibitory compounds for further studies. The effect of these flavones on CYP1B1 mRNA expression was measured with quantitative branched DNA methodology. Four of the compounds – 3′,4′-dimethoxyflavone and 5,7,4′-trimethoxyflavone and, in particular, 7,3′-dimethoxyflavone and 7,4′-dimethoxyflavone – were potent inhibitors of CYP1B1 mRNA expression. Two of the more common unmethylated polyphenols – curcumin and quercetin – were also potent inhibitors. Whereas most unmethylated polyphenols, such as curcumin and quercetin, have very poor bioavailability, the high metabolic stability of the methoxylated flavones studied here suggests that these CYP1B1 inhibitors may also be effective in-vivo.

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Introduction

The cytochrome P450 (CYP) 1A1 and 1B1 isoforms are both important in the bioactivation and DNA binding of polyaromatic hydrocarbons, such as the tobacco-smoke carcinogen benzo[*a*]pyrene (BaP) (Kim et al 1998). In most tissues where BaP or similar compounds are believed to induce DNA damage, including lung tissue, CYP1A1 appears to be the predominant catalytically active enzyme, although CYP1B1 is also important (Kim et al 2004). However, recent studies have shown that both tobacco-smoke concentrate (Port et al 2004) and BaP (Wen & Walle 2005) induce CYP1B1 in human oral epithelial cells in a cell-selective way, with CYP1A1 being induced to a much lower level (Wen & Walle 2005; Walle et al 2006). In human oesophageal epithelial cells, DNA binding is entirely dependent on CYP1B1 activation (Walle et al 2007). CYP1B1 is also commonly overexpressed in tumour cells compared with normal cells (Murray et al 1997; Murray et al 2001), and is highly expressed in vascular smooth muscle cells, where it is potentially responsible for BaP-induced atherogenesis (Moorthy et al 2003).

Dietary polyphenols, including flavonoids, have been shown to be potent inhibitors of CYP1A1 and CYP1B1 by direct interactions with the proteins (Doostdar et al 2000; Henderson et al 2000; Guengerich et al 2003; Wen & Walle 2005; Wen et al 2005). However, an additional inhibitory effect on the expression of these isoforms at the transcriptional and/or translational level should give rise to a more sustained response, important when considering the potential utility of such flavonoids as cancer chemopreventive agents (Ciolino et al 1998b; Ciolino et al 1999). We recently demonstrated that 3′,4′-dimethoxyflavone (3′,4′-DMF), an aryl hydrocarbon receptor (AhR) antagonist in breast cancer cells (Lee and Safe 2000), is capable of inhibiting BaP-induced expression of CYP1B1 at both the mRNA and protein levels in oral epithelial squamous cell carcinoma SCC-9 cells (Wen & Walle 2005). This finding prompted a search for other methoxylated flavones capable of inhibiting BaP-induced CYP1B1 mRNA expression in oral epithelial cells. As pointed out by Port et al

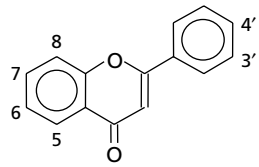

Figure 1 Common ring structure for the methoxylated flavones studied. The numbers indicate the positions where one or more hydrogen atoms are replaced by methoxy (CH_3O-) groups.

(2004), there are no such CYP1B1 inhibitors recognized today.

Our experimental approach was to treat human SCC-9 cells with a low concentration of BaP, which induces CYP1A and in particular CYP1B1 in these cells. Initially, we used the ethoxyresorufin de-ethylation (EROD) assay, a highly sensitive and rapid assay that monitors almost exclusively CYP1A1 activity (Shimada et al 1997). This assay had previously enabled us to identify another methoxylated flavone, 5,7-dimethoxyflavone (5,7-DMF), as an inhibitor of CYP1A1 expression and activity in Hep G2 cells (Wen et al 2005). Eighteen additional flavones, all methoxylated, were screened as potential inhibitors of CYP1A1. Figure 1 shows the common flavone ring structure for these compounds, the numbers indicating the positions of 1–5 methoxy groups. The most potent inhibitors were then evaluated for their inhibitory effects specifically on CYP1B1 mRNA expression in BaP-treated cells, mimicking tobacco-carcinogen-induced oral carcinogenesis. The rationale for this approach was that as both CYP1A1 and CYP1B1 are induced by BaP, CYP1A1 inhibitors may also inhibit CYP1B1. Some of the most abundant non-methoxylated dietary polyphenols were also investigated for their ability to inhibit CYP1B1 mRNA in these cells.

Materials and Methods

Materials

BaP, ellagic acid, epigallocatechin gallate (EGCG), curcumin (>80% curcumin; >98% curcuminoids) and quercetin were purchased from Sigma-Aldrich (St Louis, MO, USA). 5-, 7-, 3′- and 4′-Methoxyflavone (MF), 3,6-, 3,7-, 5,7-, 5,3′-, 5,4′-, 6,7-, 7,8-, 3′,4′-, 7,3′- and 7,4′-dimethoxyflavone (DMF), 5,7,4′- and 7,3′,4′-trimethoxyflavone (TMF), 5,7,3′,4′-tetramethoxyflavone (5,7,3′,4′-TeMF), 3,5,7,3′,4′-pentamethoxyflavone (3,5,7,3′,4′-PMF), sinensetin (5,6,7,3′,4′-PMF) and 5,7-dimethoxyflavanone (5,7-DMFan) were purchased from Indofine Chemical Co. (Hillsborough, NJ, USA). 5,7-DMFan is missing the 2–3 double bond from the flavone ring structure (Figure 1). 3,5,2′,4′-Tetramethoxy-trans-stilbene (TMS) was bought from Tocris (Ellisville, MO, USA) and tangeretin (5,6,7,8,4′-PMF) from ChromaDex (Santa Ana, CA, USA). All other chemicals were of analytical grade and were bought from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich.

Cell culture

Human oral (tongue) SCC-9 cells (Rheinwald & Beckett 1981) were obtained from ATCC (Rockville, MD, USA) and were maintained in DMEM/F12 medium (Mediatech, Fisher Scientific) with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO, USA), 0.4 μg mL^{-1} hydrocortisone and penicillin/streptomycin in a humidified 37°C incubator with 5% carbon dioxide. SCC-9 cells were used at passage 14–20.

Ethoxyresorufin de-ethylation (EROD) assay

SCC-9 cells were seeded in six-well plates. When the cells were confluent, they were treated with DMSO or 1 μM BaP, or 1 μM BaP plus 25 μM methoxyflavone in 3 mL complete medium for 24 h. Triplicate wells were used for each treatment. Following the treatments, the cells were washed with fresh medium to remove BaP and flavones, and incubated with 0.6 μM ethoxyresorufin in 3 mL medium for 2 h in the presence of salicylamide to inhibit conjugation enzymes (Ciolino et al 1998a). This inhibitor did not have any other detectable effects in our short-term assay, although it has been shown to affect AhR-mediated *CYP1* transcription in 24-h assays (MacDonald et al 2004). The formation of resorufin was measured in the cell culture medium by fluorometry with excitation at 530 nm and emission at 590 nm. The results were adjusted for cellular protein content (Lowry et al 1951) after saline rinse and digestion of the cells with 0.5 M NaOH.

CYP1B1 mRNA analysis

The cells were seeded in 96-well plates at a density of 5000 cells per well. Four days after seeding, the cells were treated for 24 h with 1 μM BaP or DMSO (0.2%, v/v), or 1 μM BaP plus 25 μM methoxyflavone or polyphenol. We used branched DNA (bDNA) signal amplification technology for the quantitative detection of CYP1B1 in cell lysates (Hartley & Klaassen 2000; Wen & Walle 2005; Wen et al 2005) with primer sets for human CYP1B1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (QuantiGene kits, Panomics, Inc., Fremont, CA, USA). The CYP1B1 and GAPDH primers (capture extenders and label extenders) target bases 574–1143 and 149–409 of the respective coding regions. The lysate from each well was divided: 90 μL was used for CYP1B1 analysis and 10 μL for GAPDH analysis. Briefly, the target mRNA was captured in coated microwells and amplified with branched oligonucleotide probes with covalently attached alkaline phosphatase. After addition of the chemilumigenic substrate, the luminescence, as measured with a plate reader, is directly proportional to the amount of target mRNA (Hartley & Klaassen 2000). Six wells were used for each treatment, with each sample normalized to its GAPDH mRNA content.

Data analysis

Effects of test compounds were compared with those of BaP alone using one-way analysis of variance with the Dunnett multiple comparisons post-hoc test using statistical analysis

software (InStat, GraphPad, San Diego, CA, USA). A P value below 0.05 was considered significant.

Results

The experimental model used in this study was cultured human oral SCC-9 cells treated with a low concentration (1 μM) of BaP for 24 h. As previously shown, this resulted in induction of CYP1B1 in particular, but also CYP1A1, as determined by both mRNA and protein measurements (Wen & Walle 2005).

As CYP1A1 induction can be monitored by the simple and sensitive EROD assay, this was used as a rapid initial screen of the effects of a variety of completely methoxylated flavones containing 1–5 methoxy groups. The flavone concentration used (25 μM) is a concentration that may be anticipated in the oral cavity and intestinal lumen after dietary intake of many flavonoids (Walgren et al 1998) and is below the concentrations of this type of flavone that might produce cell toxicity (Walle et al 2007). Interestingly, the response was highly variable, from further induction to potent inhibition (Table 1).

Table 1 Effects of methoxylated flavones on ethoxyresorufin deethylation (EROD) activity in SCC-9 cells after co-treatment with benzo[*a*]pyrene (BaP)

Polyphenol	Resorufin formation
DMSO	100 ± 15[a]
BaP alone	967 ± 133
BaP + 5-MF	722 ± 26
BaP + 7-MF	58 ± 4[a]
BaP + 3′-MF	275 ± 6[a]
BaP + 4′-MF	370 ± 25[a]
BaP + 3,6-DMF	740 ± 102
BaP + 3,7-DMF	592 ± 69
BaP + 5,7-DMF	103 ± 13[a]
BaP + 5,3′-DMF	466 ± 22[a]
BaP + 5,4′-DMF	151 ± 10[a]
BaP + 6,7-DMF	305 ± 23[a]
BaP + 7,8-DMF	263 ± 21[a]
BaP + 7,3′-DMF	68 ± 6[a]
BaP + 7,4′-DMF	35 ± 5[a]
BaP + 3′,4′-DMF	68 ± 2[a]
BaP + 5,7,4′-TMF	52 ± 4[a]
BaP + 7,3′,4′-TMF	300 ± 36[a]
BaP + 5,7,3′,4′-TeMF	1589 ± 214[b]
BaP + sinensetin	1200 ± 222
BaP + 5,7-DMFan	1134 ± 11

SCC-9 cells were treated with DMSO, or 1 μM BaP alone or with 25 μM flavone for 24 h. EROD activity was measured fluorometrically as the formation of resorufin in the culture medium. Mean basal EROD activity in SCC-9 cells was 0.008 pmol min^{-1} mg^{-1} protein after treatment with DMSO (vehicle control) and 0.0783 pmol min^{-1} mg^{-1} protein after BaP treatment. Values are percentage of control activity (DMSO) mean ± s.e.m; n ≥ 3. [a]Significantly lower than BaP alone; $P < 0.01$; [b]Significantly higher than BaP alone; $P < 0.01$. MF, methoxyflavone; DMF, dimethoxyflavone; TMF, trimethoxyflavone; TeMF, tetramethoxyflavone; DMFan, dimethoxyflavanone.

Most of the compounds showed some inhibition of BaP-induced activity. Six of the compounds (7-MF, 5,7-DMF, 7,3′-DMF, 7,4′-DMF, 3′,4′-DMF and 5,7,4′-TMF) decreased EROD activity down to, or even below, basal uninduced levels. In contrast, 5,7-DMFan (lacking the 2–3 double bond) and the polymethoxylated flavone sinensetin (5,6,7,3′,4′-PMF) had no significant effect and 5,7,3′,4′-TeMF was a further inducer.

The six most potent inhibitory flavones were evaluated for their ability to inhibit BaP-induced CYP1B1 mRNA expression in SCC-9 cells, using the bDNA assay under identical treatment conditions. Whereas 3′,4′-DMF and 5,7,4′-TMF reduced CYP1B1 mRNA expression to control values, 7,3′-DMF and 7,4′-DMF reduced the expression even more dramatically (Figure 2). On the other hand, 7-MF and 5,7-DMF, which greatly inhibited BaP-induced EROD activity, had no effect. Two pentamethoxyflavones, including tangeretin, also had no effect on expression of CYP1B1 mRNA.

For comparison, we also tested some of the most common and widely studied unmethylated dietary polyphenols as potential inhibitors of BaP-induced CYP1B1 mRNA expression in the same cell line. Both curcumin and quercetin were potent inhibitors (Figure 3). Both the major tea flavonoid EGCG and ellagic acid caused a slight further induction. As a negative control, TMS, the potent direct inhibitor of CYP1B1 protein, had no effect on CYP1B1 mRNA expression.

Because of the unexpected inhibition of CYP1B1 mRNA expression by curcumin and quercetin, we repeated these experiments with a range of concentrations (1–25 μM, Table 2). A clear concentration-dependent effect was observed for both polyphenols. Interestingly, the 1 μM

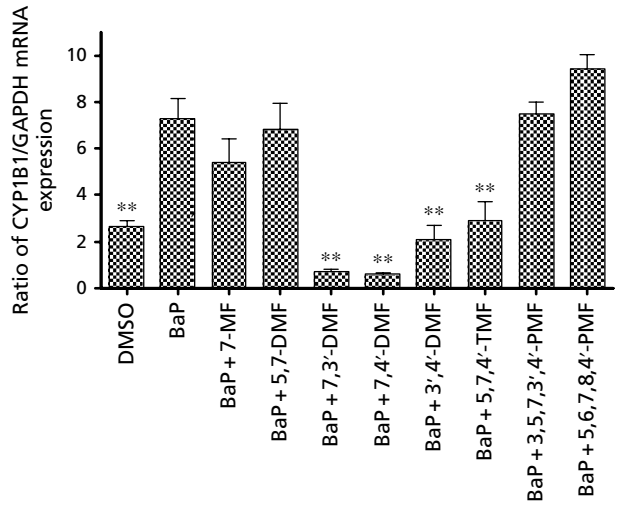

Figure 2 Effect of methylated flavones on CYP1B1 mRNA expression in SCC-9 cells. The cells were treated with DMSO, or 1 μM benzo[*a*]pyrene (BaP), alone or in combination with 25 μM flavones for 24 h. Cell lysates were analysed for CYP1B1 mRNA expression by a quantitative branched DNA assay. Values are mean ± s.e.m of six samples, each normalized to its glyceraldehyde-3-phosphate dehydrogenase (GAPDH) value. **$P < 0.01$ compared with BaP alone. MF, methoxyflavone; DMF, dimethoxyflavone; TMF, trimethoxyflavone; PMF, pentamethoxyflavone.

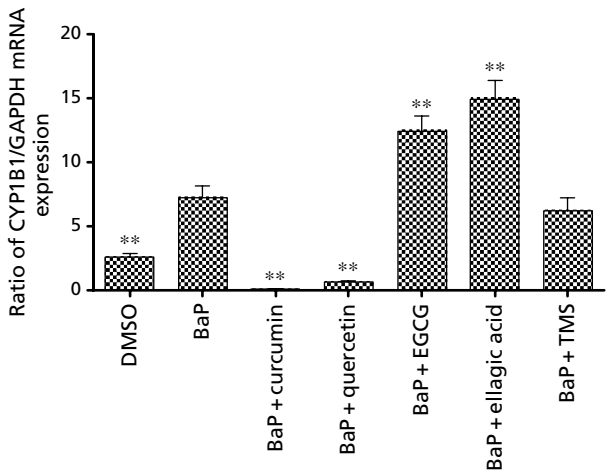

Figure 3 Effect of common dietary polyphenols on CYP1B1 mRNA expression in SCC-9 cells, as measured by the branched DNA assay. Cells were treated with DMSO or 1 μM benzo[*a*]pyrene (BaP), alone or in combination with 25 μM polyphenols for 24 h. Values are mean ± s.e.m. of six samples, each normalized to its glyceraldehyde-3-phosphate dehydrogenase (GAPDH) value. $^{**}P < 0.01$ compared with BaP alone. EGCG, epigallocatechin gallate; TMS, tetramethoxy-trans-stilbene.

Table 2 Effects of quercetin and curcumin on CYP1B1 mRNA expression in SCC-9 cells after co-treatment with benzo[*a*]pyrene (BaP)

Treatment	Ratio of CYP1B1/ GADPH mRNA expression
DMSO	2.6 ± 0.2*
BaP alone	12.3 ± 1.9
BaP + 1 μM quercetin	17.3 ± 1.3*
BaP + 5 μM quercetin	9.7 ± 0.6
BaP + 10 μM quercetin	5.0 ± 0.2*
BaP + 25 μM quercetin	2.7 ± 0.1*
DMSO	5.1 ± 0.5*
BaP alone	17.1 ± 0.6
BaP + 1 μM curcumin	15.8 ± 1.1*
BaP + 5 μM curcumin	10.1 ± 1.4*
BaP + 10 μM curcumin	4.7 ± 0.4*
BaP + 25 μM curcumin	1.1 ± 0.3*

SCC-9 cells were treated with DMSO or 1 μM BaP alone or in combination with 1–25 μM quercetin or curcumin for 24 h. Cell lysates were analysed for CYP1B1 mRNA expression by a quantitative branched DNA assay. Values are means ± s.e.m. of six samples, each normalized to its glyceraldehyde-3-phosphate dehydrogenase (GAPDH) value. $^{*}P < 0.05$ compared with BaP alone.

concentration of quercetin showed a slight but statistically significant stimulation compared with BaP alone.

Discussion

This study has demonstrated the ability of selected methoxylated flavones to inhibit BaP-induced CYP1B1 expression in human oral SCC-9 cells. As shown in a previous investigation (Wen & Walle 2005), CYP1B1 is by far the most important enzyme induced by BaP in oral epithelial cells, causing a dramatic increase in BaP–DNA covalent binding. At the same time, down-regulation of CYP1B1 mRNA by a methoxylated flavone, 3′,4′-DMF, was shown to reduce the BaP–DNA binding. Thus, the present study has extended the number of flavones with this beneficial effect from 3′,4′-DMF, a compound that is not expected to be present in the human diet, to a number of other methoxylated flavones, some of which are present in various plants and including some compounds consumed in modest amount in the normal diet.

The initial screening of 19 methoxylated flavones was done in the SCC-9 cells after co-treatment with a low concentration of BaP, which in this cell type induces CYP1B1 but also, although to a lesser extent, CYP1A1, both at the mRNA and protein levels (Wen & Walle 2005). In this screening, we used the EROD catalytic activity assay, which is highly selective for CYP1A1 (Shimada et al 1997; Wen & Walle 2006c). There is no such assay for CYP1B1. The result was a surprising variety of responses, ranging from a 2-fold further increase in the BaP inductive response to almost complete inhibition to the control (DMSO) level response (Table 1). Six of the compounds (7-MF, 5,7-DMF, 7,3′-DMF, 7,4′-DMF, 3′,4′-DMF and 5,7,4′-TMF) were thus effective inhibitors of BaP-induced CYP1A1 expression.

Assuming that CYP1B1 expression after BaP treatment would follow that of CYP1A1 catalytic activity, which is reasonable based on previous studies, we examined the ability of the methoxylated flavones to inhibit CYP1B1 mRNA expression with the same treatment protocol and cell line and using the bDNA assay, a quantitative and reproducible approach (Hartley & Klaassen 2000; Wen & Walle 2005; Wen et al 2005; Tsuji & Walle 2006). Four of the CYP1A1 inhibitors were also strong inhibitors of CYP1B1 mRNA (3′,4′-DMF, 5,7,4′-TMF and, especially, 7,3′-DMF and 7,4′-DMF; Figure 1). In spite of the potent inhibition of BaP-induced EROD activity by 7-MF and 5,7-DMF, mainly reflecting CYP1A1, these flavones had no effect on BaP-induced CYP1B1 mRNA. Although the induction of both CYP1A1 and CYP1B1 is most likely mediated by the AhR, the inhibitory effect of the methoxylated flavones on the expression of these enzymes may be via other transcription or signalling factors. Thus, the mechanism(s) by which the methoxyflavones affect the transcription of CYP1B1 is likely to be complex. 3′,4′-DMF has been shown to be an AhR antagonist in human breast cancer cells, directly binding to the receptor (Lee & Safe 2000). 5,7-DMF has been shown to inhibit CYP1A1 transcription in both Hep G2 (Wen et al 2005) and SCC-9 human cell lines (Wen & Walle 2005). However, in neither case was there evidence for any effect on AhR nuclear translocation (unpublished). As previously reported (Wen & Walle 2005), and as also seen in this study, 5,7-DMF had no effect on the transcription of CYP1B1 in the SCC-9 cells; however, it has been shown to affect CYP1B1 transcription in human oesophageal HET-1A cells (Wen & Walle 2006c). This demonstrates that CYP1B1 is regulated differently than CYP1A1 and that this is highly cell specific. As previously indicated, the effects of flavonoids as AhR agonists or antagonists is highly dependent on cell context (Zhang et al 2003; Harper et al 2006). Our present study has

now identified three further methoxyflavones as inhibitors of CYP1B1 transcription, based on mRNA measurements. The mechanism of this effect remains to be elucidated. It could be via AhR interaction, as with 3′,4′-DMF (Lee & Safe 2000), by effects on regulators of AhR, as indicated previously, or is likely to be via a combination of both. Thus, 3′,4′-DMF may also have effects on CYP1B1 transcription via mechanisms other than the AhR. Such regulatory targets may include hsp90, as shown for the tea flavonoid EGCG (Palermo et al 2005), or aryl hydrocarbon receptor nuclear translocator (ARNT), as shown for the yellow spice curcumin (Choi et al 2006). It could also involve promoter/enhancer hypomethylation of the CYP1B1 gene (Tokizane et al 2005).

It may thus be possible to find specific inhibitors for either CYP1A1 or CYP1B1. It is clear that the structural requirements for CYP1B1 mRNA inhibition are complex, depending on both the number of methoxy groups and their positions. This deserves more in-depth investigation.

Although the methoxylated flavones investigated here were synthetic compounds, most of the CYP1B1 inhibitors are present in modest quantities in plants suitable for human consumption. For example, 5,7,4′-TMF is a citrus flavonoid (Mizuno et al 1991) and is also present in other plants used in folk medicine (Jaipetch et al 1983; Yenjai et al 2004). 7,4′-DMF has been identified in fruits and leaves from neotropical nutmeg species (Cavalcante et al 1985; Santos et al 1996) as well as from propolis (Popravko et al 1969). Also, 5,7-DMF is a natural product (Ahmad et al 1997) that is highly abundant in pepper tree leaves. While none of the methoxylated flavones examined in this study is abundant in the human diet, mounting evidence of the protective properties of these flavones may increase use of their natural sources.

Although most dietary polyphenols have very poor bioavailability (Manach and Donovan 2004; Walle 2004), we have recently shown that the methoxylated flavones investigated here are highly resistant to human hepatic metabolism (Wen and Walle 2006a) and also have much improved intestinal transcellular absorption (Wen and Walle 2006b). This was recently confirmed in an in-vivo rat model, in which high concentrations of 5,7-DMF were found in plasma and tissues, but no detectable levels of the unmethylated analogue 5,7-dihydroxyflavone (chrysin) after oral co-administration of the two flavones (Walle et al 2007). The new inhibitors of this class may thus also be effective in-vivo.

The potent inhibitory effects of quercetin and curcumin were not expected, as both compounds have been shown to be mainly AhR agonists in MCF-7 breast cancer cells (Ciolino et al 1998a; Ciolino et al 1999). In contrast, EGCG, a weak CYP1B1 inducer in this study, has been shown to be mainly an AhR antagonist in an animal study (Palermo et al 2005). This difference in responses between studies may reflect cell context (Zhang et al 2003) (i.e. SCC-9 cells versus MCF-7 cells) or species differences, as the latter study was done with a rodent liver preparation. As quercetin, curcumin and EGCG, in contrast to the methoxyflavones, are potent antioxidants, the possibility that this property contributes to the effects of these polyphenols cannot be discounted, although antioxidant effects typically require higher concentrations than those required for CYP1B1 inhibition in this study. Our seemingly different results with these compounds may also be due to the fact that BaP-induced cells were used in our study in contrast to studies of uninduced cells or animals. Nevertheless, our observations indicate that our current knowledge of these polyphenols was not predictive of CYP1B1 mRNA inhibition, at least not in SCC-9 cells. However, the very poor oral bioavailability of quercetin (Walle 2004; Williamson and Manach 2005) and curcumin (Garcea et al 2004) would only make them useful in the digestive tract in-vivo.

Conclusions

In summary, this study establishes several methoxylated flavones, several of them found in plants, as potent inhibitors of BaP-induced CYP1B1 mRNA in human oral epithelial cells. The high metabolic resistance of these flavones suggests that they may have utility as chemopreventive agents in-vivo, possibly in cancer but also in cardiovascular disease.

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