ABSTRACT:

Methoxylated flavones were recently shown to be promising cancer chemopreventive agents. Their high metabolic stability compared with the hydroxylated analogs was shown in our laboratory using the human hepatic S9 fraction with cofactors for glucuronidation, sulfation, and oxidation. In the present study, the resistance of methoxylated flavones toward oxidative metabolism was investigated with human liver microsomes and recombinant cytochrome P450 (P450) isoforms. Among 15 methoxylated flavones investigated, the two partially methylated compounds, tectochrysin and kaempferide, were among the most susceptible to monosomal oxidation (Cl\text{int} 283 and 82 ml/min/kg). Of the fully methylated compounds, 5,7-dimethoxyflavone and 5-methoxyflavone were the most stable (Cl\text{int} 13 and 18 ml/min/kg, respectively), whereas 4'-methoxyflavone, 3'-methoxyflavone, 5,4'-dimethoxyflavone, and 7,3'-dimethoxyflavone were the least stable (Cl\text{int} 161, 140, 119, and 92 ml/min/kg, respectively), emphasizing the importance of the positions of the methoxy substituents in the flavone ring system. Among the five P450 isoforms tested, CYP1A1 showed the highest rate of metabolism of fully methylated compounds, followed by CYP1A2 and CYP3A4. CYP2C9 and CYP2D6 gave minimal disappearance of the parent compound. Finally, in incubations with hepatic S9 fraction with cofactors for oxidation and both conjugation reactions, partially methylated flavones, as expected, were much less metabolically stable than fully methylated flavones, confirming that oxidative demethylation is the rate-limiting metabolic reaction for fully methylated flavones only. In summary, the rate of oxidative metabolism of methoxylated flavones, mainly involving CYP1A1 and CYP1A2, varied widely, even between compounds with very similar structures.

Promising biological effects of dietary flavonoids and other polyphenols as chemopreventive agents against cancer, cardiovascular disease, and other diseases have been shown in cell culture studies (Middleton et al., 2000; Williams et al., 2004; Walle, 2007). However, in vivo, in particular in humans, using moderate, clinically relevant doses, these effects have not been replicated. This is mainly because of very low oral bioavailability, as has been shown in clinical studies of, for example, chrysirin (5,7-dihydroxyflavone) (Walle et al., 2001a) and quercetin (3,5,7,3',4'-pentahydroxyflavone) (Walle et al., 2004; Manach and Donovan, 2004; Williamson and Manach, 2005). This poor bioavailability of dietary polyphenols is highly dependent on their free hydroxyl groups, making them susceptible in particular to glucuronidation and sulfation and much less so to oxidation (Otake et al., 2002), which commonly, but not always, removes the biological activity of these compounds. In contrast, the methoxylated flavones are much more resistant to hepatic metabolism and have higher intestinal absorption through Caco-2 cell monolayers compared with their unmethylated analogs (Wen and Walle, 2006a,b). Among the few in vivo disposition studies of the methoxylated flavones, nobletin (5,6,7,8,3',4'-hexamethoxyflavone) reached significant tissue levels after p.o. administration to rats (Murakami et al., 2002), whereas the coadministered unmethylated flavone luteolin (5,7,3',4'-tetrahydroxyflavone) gave much lower tissue levels. Similarly, in the rat, 5,7-dimethoxyflavone (5,7-DMF) reached high tissue levels (Walle et al., 2007), whereas coadministered chrysin was undetectable. Tangertin (5,6,7,8,4'-pentamethoxyflavone) administered in the feed to hamsters was absorbed but extensively metabolized (Kurowska and Manthey, 2004).

Whereas the antioxidative properties of flavonoids with free phenolic groups have been considered necessary for effects (Rice-Evans, 2001), recent studies have shown that some of the methoxylated flavones, including the citrus flavonoids nobletin and tangeretin, have potent anti-proliferative activities (Pan et al., 2002; Morley et al., 2007; Walle et al., 2007), inhibit the bioactivation of benzo[a]pyrene by CYP1A1 and 1B1 and thereby cancer initiation in various cell lines (Wen and Walle, 2005; Wen et al., 2005; Tsuji and Walle, 2006; Walle and Walle, 2007), and are inhibitors of aromatase (Ta and Walle, 2007), an important target in hormone-sensitive cancers.

The present study focused on the oxidative metabolism of a number of methoxylated flavones (Fig. 1) by human liver microsomes and pure P450 isoforms to try to elucidate whether there are particular structural features that confer resistance or susceptibility to metabolism. This should be important when selecting methoxylated flavones for testing as potential chemopreventive agents in human disease.

Materials and Methods

Materials. 5-MF, 7-MF, 3'-MF, 4'-MF, 5,7-DMF, 5,3'-DMF, 5,4'-DMF, 7,3'-DMF, 7,4'-DMF, 3',4'-DMF, 5,7,4'-trimethoxyflavone (5,7,4'-TMF), silenestin (5,6,7,3',4'-pentamethoxyflavone), tectochrysin (5-hydroxy-7-MF), rutin, and quercetin.

ABBREVIATIONS: DMF, dimethoxyflavone; MF, methoxyflavone; TMF, trimethoxyflavone; UDPGA, UDP-gluconic acid; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; HPLC, high-performance liquid chromatography.
5-MF  
7-MF  
3'-MF  
4'-MF  
5,7-DMF  
5,3'-DMF  
5,4'-DMF  
7,3'-DMF  
5,7,4'-TMF  
Sinensetin  
Tangeretin  
Tectochrysin  
Kaempferide

Substituent
3  5  6  7  8  3'  4'

4'-hydroxy-3'-MF, and kaempferide (3,5,7-trihydroxy-4'-MF) (chemical purities 97–98%) were purchased from Indofine Chemical Co. (Somerville, NJ). Tangeretin (5,6,7,8,4'-pentamethoxyflavone) was obtained from ChromaDex (Irvine, CA). Chrysin (5,7-dihydroxyflavone), apigenin (5,7,4'-trihydroxyflavone) (purities >95%), NADPH, and UDP-glucuronic acid (UDPGA) were obtained from Sigma-Aldrich (St. Louis, MO). Ultra pure (purity >99%) 3'-phosphohydroxylflavone-5'-phosphosulfate (PAPS) was obtained from Sigma-Aldrich (St. Louis, MO). Human recombinant cytochrome P450 (P450) 1A1, 1A2, 2C9, 2D6, and 3A4 assays (baculovirus insect cell-expressed enzymes with human P450 reductase), pooled human liver microsomes from 30 donors, and pooled human liver S9 fractions from 15 donors were purchased from BD Gentest (Woburn, MA). Other chemicals and reagents were obtained from Fisher Scientific Co. (Pittsburgh, PA).

The oxidative elimination half-lives of the flavones were calculated as previously described (Wen and Walle, 2006b and references therein), assuming 45 mg of microsomes/g of liver and 20 g of liver/kg b.w.t.: \( t_{1/2} = \frac{0.693}{\text{Cl}_{\text{int}}} \times (\text{incubation volume (ml)/microsomal protein (mg)}) \times 45 \times 20.\)

Results

Metabolic Stability of Methoxylated Flavones in Pooled Human Liver Microsomes. The metabolic stability of 15 flavones with one to five methoxy substituents (Fig. 1) was determined using pooled human liver microsomes. A flavone concentration of 5 \( \mu \text{M} \) was used with a microsomal concentration of 0.5 mg of protein (130 pmol of total P450)/ml conditions that gave essentially linear rates of elimination (Fig. 2). The half-lives of elimination and intrinsic clearances for all 15 compounds are summarized in Table 1. Among the fully methoxylated flavones, 5,7,DMF and 5,7,4'-DMF and 7,4'-DMF also were relatively stable (Clint 13 and 18 ml/min/kg). Sinensetin, 5,7,4'-TMF, 3',4'-DMF, and sinensetin, 326 nm for 5,7,4'-DMF, 338 nm for 7,5'-DMF, 7,4'-DMF, 3,4'-DMF, and sinensetin, 326 nm for 5,7,4'-TMF and tangeretin, and 370 nm for kaempferide. Tectochrysin analyses used 70% methanol/0.3% trifluoroacetic acid and 268-nm detection. Quantitation was achieved by comparing the detected peak areas with those of the synthetic standards.

Data Analysis. The oxidative elimination half-lives of the flavones were calculated from the slope of the line obtained by linear regression of the percentage flavone remaining versus incubation time. The correlation coefficients in all the cases were 0.99 or better. The intrinsic clearance (Cl_{int}) of the flavones was calculated as previously described (Wen and Walle, 2006b and references therein), assuming 45 mg of microsomes/g of liver and 20 g of liver/kg b.w.t.: \( t_{1/2} = \frac{0.693}{\text{Cl}_{\text{int}}} \times (\text{incubation volume (ml)/microsomal protein (mg)}) \times 45 \times 20.\)

Table 1. Half-lives of elimination and intrinsic clearances for all 15 compounds is summarized in Table 1. Among the fully methoxylated flavones, 5,7,DMF and 5,7,4'-DMF and 7,4'-DMF also were relatively stable (Clint 13 and 18 ml/min/kg). Sinensetin, 5,7,4'-TMF, 3',4'-DMF, and 7,4'-DMF also were relatively stable (Clint 28–44 ml/min/kg). Tectochrysin with one free hydroxyl group was much less stable than any of the fully methoxylated flavones, with complete disappearance of the parent compound already after 20 min (Clint 283 ml/min/kg). 4'-MF and 3'-MF also were highly susceptible to oxidation (Clint 161 and 140 ml/min/kg, respectively). 5,4'-DFM, 7,3'-DMF, kaempferide (a partially methylated flavone), 7-MF, and tangeretin were relatively unstable with intrinsic clearances of 72 to 119 ml/min/kg.

Metabolic Stability of Methoxylated Flavones with P450 Isoforms. The contribution of individual P450 isoforms to the metabolism of four of the fully methoxylated flavones, 5,7-DMF, 3',4'-DMF, 5,7,4'-TMF, and tangeretin (5 \( \mu \text{M} \)) was determined using the same incubation conditions with the liver microsomes. The P450 isoforms tested were CYP1A2, CYP2C9, CYP2D6, and CYP3A4, which have previ-
Mean values are shown (n = 3). The S.E.M. in most cases are smaller than the symbols.

### TABLE 1

<table>
<thead>
<tr>
<th>Flavone</th>
<th>Half-life</th>
<th>Clₜₜ</th>
<th>N/M/mg/kg</th>
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<tr>
<td>5,7-DMF</td>
<td>97.1</td>
<td>12.8</td>
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<tr>
<td>5-MF</td>
<td>68.2</td>
<td>18.3</td>
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</tr>
<tr>
<td>Sinensetin</td>
<td>44.8</td>
<td>27.8</td>
<td></td>
</tr>
<tr>
<td>5,7,4'-TMF</td>
<td>33.3</td>
<td>37.4</td>
<td></td>
</tr>
<tr>
<td>3',4'-DMF</td>
<td>30.9</td>
<td>40.3</td>
<td></td>
</tr>
<tr>
<td>7,4'-DMF</td>
<td>28.6</td>
<td>43.6</td>
<td></td>
</tr>
<tr>
<td>5,3'-DMF</td>
<td>20.8</td>
<td>60.0</td>
<td></td>
</tr>
<tr>
<td>Tangeretin</td>
<td>17.4</td>
<td>71.7</td>
<td></td>
</tr>
<tr>
<td>7-MF</td>
<td>15.7</td>
<td>79.4</td>
<td></td>
</tr>
<tr>
<td>Kaempferide</td>
<td>15.2</td>
<td>82.0</td>
<td></td>
</tr>
<tr>
<td>7,3'-DMF</td>
<td>13.6</td>
<td>91.7</td>
<td></td>
</tr>
<tr>
<td>5,4'-DMF</td>
<td>10.5</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>3'-MF</td>
<td>8.9</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>4'-MF</td>
<td>7.8</td>
<td>161</td>
<td></td>
</tr>
<tr>
<td>Tectochrysin</td>
<td>4.4</td>
<td>283</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Time-dependent metabolic depletion of selected methoxylated flavones with human liver microsomes. The flavones (5 μM) were incubated with pooled human liver microsomes (500 μg of protein/ml) and NADPH for 0 to 30 min. The data are expressed as percent of parent compound remaining compared with 0 min. Mean values are shown (n = 3). The S.E.M. in most cases are smaller than the symbols.

Enzyme concentrations. Tangeretin was metabolized by CYP1A1 and CYP1A2 to two compounds, where the major CYP1A2 metabolite has been previously identified as the 4'- demethylated compound (Breinholt et al., 2003). CYP3A4 gave rise to an additional metabolite, previously identified as 6,4'-demethylated tangeretin (Breinholt et al., 2003). CYP1A1 and CYP1A2 gave rise to the same 5,7,4'-TMF metabolites, with the more polar metabolite increasing at higher enzyme concentrations at the expense of the less polar compound, suggesting stepwise demethylation reactions. CYP3A4 gave a small peak with longer retention time.

**Metabolic Stability of Methoxylated Flavones in Pooled Human Liver S9 Fraction.** To assess the relative importance of oxidation versus glucuronidation and sulfation in fully compared with partially methylated flavones, four flavones were incubated with human liver S9 fraction in the presence of cofactors for all three reactions, as previously described (Wen and Walle, 2006b). Two fully methylated flavones, 5,7,4'-TMF and 5,7-DMF, were completely stable during a 30-min incubation, whereas tectochrysin with one free phenolic group and kaempferide with three free phenolic groups were rapidly metabolized with 32 and 6% remaining unchanged at the end of the incubation (Fig. 4).

### Discussion

Although this study did not allow any firm conclusions regarding structural requirements for resistance to oxidative metabolism, the finding that all four of the MF showing the highest resistance to microsomal oxidation contained a methoxy group in the 5-position indicates that the 5-methoxy group confers resistance to metabolism. The difference in stability between 5-MF and 7-MF was quite dramatic. The instability of 7-MF appeared to be either increased or negated by additional methoxy substituents, varying from the highly stable 5,7-DMF to the least stable, 7,3'-DMF. The number of methoxy groups does not appear to have any effect on the susceptibility to oxidation because 5-MF, 5,7-DMF, and sinensetin with 1, 2, and 5 methoxy groups, respectively, were quite stable, whereas 7-MF, 7,3'-DMF, and tangeretin with the same numbers of methoxy groups were much less stable. The two flavones with a single methoxy substituent in the B-ring (i.e., 3'-MF and 4'-MF) were the methoxylflavones most prone to oxidation. The two tested flavones with both hydroxy and methoxy groups (i.e., kaempferide and especially tectochrysin) were more susceptible to oxidation than most of the fully methylated flavones.

In a metabolically competent system with the liver S9 fraction and cofactors for conjugative and oxidative metabolism, the stability of the fully methylated flavones is vastly improved compared with the partially methylated compounds tectochrysin and particularly kaempferide with three free hydroxy groups (Fig. 4). A previous study showed that also 7-MF and 7,4'-DMF are metabolized to less than 5% using identical experimental conditions (Wen and Walle, 2006a). The partially methylated flavones, which include a vast number of natural compounds, thus have two strikes against them with respect to oral bioavailability; in addition to intestinal and hepatic conjugation they are also more susceptible to oxidation.

The finding that CYP1A2 and CYP3A4 and to a very minor extent CYP2D6 contribute to the metabolism of fully methoxylated flavones agrees with a previous study of tangeretin (Breinholt et al., 2003). However, we found that CYP1A1 was also quite active, metabolizing the four flavones tested at the same rate as or faster than CYP1A2. Whereas CYP3A4 is very abundantly expressed in both the liver (Shimada et al., 1994) and the intestine (Paine et al., 2006) and CYP1A2 is abundant in the liver (Shimada et al., 1994), most studies have found low levels of the mainly extrahepatic CYP1A1 isofrom in the liver (Drahushuk et al., 1998; Stiborová et al., 2005). CYP1A1 is highly inducible by polyaro-
matic hydrocarbons, such as benzo[a]pyrene, both in vitro and in vivo (Harrigan et al., 2006) and could potentially contribute to the metabolism of the methoxylflavones in extrahepatic tissues, especially in smokers. However, because of their relative expression in the tissues, CYP3A4 likely is the most important isozyme for intestinal metabolism and CYP1A2 for hepatic metabolism.

The high resistance of 5,7-DMF to microsomal oxidative metabolism shown here is consistent with previous observations showing an intrinsic clearance of 13 ml/min/mg protein (i.e., similar to commonly used drugs) (Wen and Walle, 2006b). It is also consistent with high resistance of this methoxylflavone to metabolism by the human liver S9 fraction and a much greater net transport across the intestinal Caco-2 cell monolayer (Wen and Walle, 2006a). Most important, it is also consistent with high oral bioavailability and tissue accumulation in the rat (Walle et al., 2007) in vivo. In addition, similar high tissue accumulation of 5,7-DMF was recently observed in a small fish model (Tsuji et al., 2006).

Many of the most stable methylated flavonoids studied here can be found in a variety of plant species. 5,7-DMF is abundant in the leaves of a Malaysian Piper species (Ahmad et al., 1997) and has been found in several herbs, and 5,7,4′-TMF, like sinemetin and tangeretin, is a citrus flavonoid, present also in other plants used in folk medicine (Jaipetch et al., 1983; Yenjai et al., 2004). 5,7-DMF and 3′,4′-DMF have already been described as having potent cancer chemopreventive properties at the cancer initiation stage (Wen and Walle, 2005; Wen et al., 2005; Tsuji and Walle, 2006). At the cancer promotion stage, the inhibition of oral cancer cell proliferation is much more potent with 5,7-DMF and 5,7,4′-DMF than with the unmethylated analogs chrysin and apigenin (Walle et al., 2007). In addition, 7-MF and 7,4′-DMF were very recently found to be potent aromatase inhibitors (Ta and Walle, 2007).

In summary, the present study shows that the susceptibility to oxidative metabolism, as mediated by CYP1A1, CYP1A2, and CYP3A4 and perhaps other isoforms, varies widely among the fully methylated flavones. However, fully methylated flavones have dramatically higher metabolic stability than those of the unmethylated or partially methylated forms. These findings together with observations of potent biological activities provide promise of efficient in vivo chemopreventive activities of such dietary compounds.

References


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