Silybin Is Metabolized by Cytochrome P450 2C8 in Vitro

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ABSTRACT:
Silybin (a flavonolignan, the main component of silymarin, an extract from the seeds of *Silybum marianum*) has been used to date mostly as a hepatoprotectant. However, it also has other interesting activities, e.g., anticancer and hypocholesterolemic effects. It is also known that silybin can inhibit the activities of the cytochrome P450 enzymes. In this study, a weak interaction of silybin with human microsomal CYP2E1, 2A6, 2B6, 2C19, and 2D6 (IC₅₀ > 250 μM) was found; a moderate inhibition was observed for CYP1A2 and 2C8. The most prominent inhibition effect was found with CYP3A4 and CYP2C9 (IC₅₀ ≤ 50 μM). Using mass spectrometry detection, production of O-demethylated products and hydroxylated derivatives of silybin was detected. The effect of different P450 inhibitors on the formation of O-demethylated products was also studied. In particular, a relatively specific inhibitor of CYP2C8 (quercetin) markedly inhibited the formation of this metabolite. With the help of recombinant enzymes (bactosomes), it was confirmed that the CYP2C8 enzyme is responsible for the reaction leading to O-demethylated silybin.

Silybin (also known as silibinin, CAS 22888-70-6) is a flavonolignan and an active component of silymarin, an extract from *Silybum marianum* (milk thistle) seeds (Gazák et al., 2007). Its hepatoprotective effects have been known for hundreds of years; novel studies indicate that the molecular basis of this effect is its antioxidative and radical scavenging property (Flora et al., 1998; Gazák et al., 2007). Recent discoveries of its other activities (chemopreventive, anticancer, and neuroprotective effects) are responsible for an increasing number of articles in peer-reviewed journals devoted to this natural compound. An interference of silybin with cell cycle-regulating pathways is expected to be the mechanism underlying the majority of the effects described (Singh and Agarwal, 2006).

Although silybin (Fig. 1) is generally considered to be safe with only few adverse effects (involving mostly gastrointestinal discomfort), a possibility of drug interactions based on metabolism mediated by cytochromes P450 (P450) was recently investigated. Nifedipine oxidation, one of the major CYP3A4 activities, was found to be inhibited by silybin in the micromolar range (Beckmann-Knopp et al., 2000; Zuber et al., 2002; Sridar et al., 2004). Also, CYP2D6 and CYP2C9 as well as glucuronidation activities were found to be inhibited. These facts have led to studies on the potential influence of silybin on pharmacokinetics of a typical CYP3A4 substrate, indinavir (DiCenzo et al., 2003; Mills et al., 2005). The differences in the area under the curve of indinavir due to concomitant application of silybin (i.e., the silybin-containing extract) were found not to be significant. The possibility of a direct interaction of silybin with P450 enzymes and its conversion to metabolites by human liver was further studied; the presence of O-demethylated and hydroxylated derivatives of silybin was detected by HPLC-electrospray ionization-ion trap mass spectrometry (Gunaratna and Zhang, 2003). However, no systematic study on the metabolism of silybin by individual P450 enzymes with an identification of a particular P450 form responsible for the formation of metabolite(s) is available.

This work is a detailed investigation of the role of individual human liver microsomal P450 enzymes in the metabolism of silybin 1) by inhibition of prototypical microsomal P450 activities by silybin, 2) by inhibition of silybin metabolism by known substrates or inhibitors of specific P450 forms, and finally 3) by confirmation of the involvement of CYP2C8 in the formation of O-demethylated product, the main metabolite of silybin.

Materials and Methods

**Chemicals.** Silybin was a gift from Ivax-CR a.s. (Opava, Czech Republic). Chloroxazole, 6-hydroxyclochloroxazole, diclofenac, 4-hydroxyclofenac, bufuralol, 6-hydroxybufuralol, and 6β-hydroxytestosterone were supplied by Ultrafine Chemicals (Salford, UK). P450-Glo substrate for determination of P450 activities by luminescence spectrometry was the product of Promega (Madison, WI) obtained through East Port (Prague, Czech Republic). 7-Ethoxy-4-(trifluoromethyl)coumarin was supplied by Fluka (Buchs, Switzerland). All other chemicals were purchased from Sigma-Aldrich CZ (Prague, Czech Republic).

**Microsomes and Recombinant Enzymes.** Pooled human liver microsomes were purchased from Advancell (Barcelona, Spain). Microsomes were obtained in accordance with ethical regulations of the country of origin (Spain). They were from 10 donors (5 males and 5 females) with a protein content of 38.4 mg/ml: CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2E1, and CYP3A4.
enzyme activities are accessible at the Advancell website (http://www.advancell.net, batch reference no. 102091201). Bactosomes (bacterial membrane fractions from Escherichia coli) containing recombiant human cytochromes P450 enzymes (CYP1A2, 2C8, 2C9, 3A4, 2D6, 2B6, and 2E1) coexpressed with human NADPH-cytochrome P450 reductase, were purchased from Cypex (Dundee, UK).

**P450 Activities.** Individual P450 activities were measured according to established protocols. The following microsomal P450 activities were tested: CYP3A4, testosterone 6β-hydroxylation (Guengerich et al., 1986); CYP2C9, diclofenac 4'-hydroxylation (Crespi et al., 1998a); CYP2E1, chlorozoxazone 6-hydroxylation (Lucas et al., 1996); CYP1A2, 7-ethoxyresorufin O-deethylation (Chang and Waxman, 1998); CYP2D6, bufuralol 1'-hydroxylation (Crepi et al., 1998b); CYP2A6, coumarin 7-hydroxylation (Waxman and Chang, 1998); CYP2B6, 7-ethoxy-4-(trifluoromethyl)coumarin O-decylation (Damento et al., 2004); CYP2C19, S-mephenytoin 4'-hydroxylation (http://www.cypex.co.uk/intro.htm, Cypex 2C19 QC assays); and CYP2C8, luciferin-methyl ester demethylation (Promega Technical Bulletin no.325, http://www.promega.com). For determination of metabolites formed from specific substrates, an HPLC system (Class VP; Shimadzu, Kyoto, Japan) with UV condition with 65% of mobile phase B (acetonitrile); 1 mM MgCl₂, and 250 pmol of microsomal CYP, in a total volume of 1 ml. After 10 min of preincubation at 37°C, bubbling through the reaction mixture for 1 min. Then silybin was added as 20 μM in reaction mixture were 0, 10, 50, 100, 150, 200, and 400 μM silybin. The reaction was terminated by the addition of 1 ml of ethyl acetate. Samples were centrifuged at 5000 g for 10 min to take out the precipitated protein. The supernatant was evaporated under a stream of nitrogen.

**Identification of Silybin Metabolites by μLC/MS.** Residue obtained after incubation with microsomes or with bactosomes was dissolved in acetonitrile (50 μl) and further diluted by 200 μl of mobile phase A (see below). μLC/MS analyses were performed using a CapLC XE system (Waters, Milford, MA), a C₁₈ column Gemini (150 mm × 300 μm i.d.; Phenomenex, Torrance, CA) at a mobile phase flow rate of 6 μl/min. The elution was realized at the isocratic condition with 65% of mobile phase A (5.7 mM acetic acid + 5% acetonitrile) and 35% of mobile phase B (acetonitrile); 1 μl of sample solution was injected using an autosampler. Accurate mass measurement and MS/MS experiments were performed to confirm the identity of metabolites on a quadrupole time of flight mass spectrometer (Waters Micromass Q-Tof Premier Mass Spectrometer). Optimized parameters of electrospray were capillary voltage -2.8 kV (negative mode), sampling cone 45 V, source temperature 80°C, desolvation temperature 180°C, cone gas flow 50 liters/h, and desolvation gas flow 400
Inhibition by silybin expressed as IC₅₀ or Kᵢ ± S.E.M.

<table>
<thead>
<tr>
<th>P450 Enzyme</th>
<th>Substrate</th>
<th>Substrate Conc</th>
<th>Silybin IC₅₀</th>
<th>Silybin Kᵢ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Ethoxyresorufin</td>
<td>2.4</td>
<td>2.5</td>
<td>220 ± 31</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin</td>
<td>8.2</td>
<td>10</td>
<td>N.A.</td>
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<tr>
<td>CYP2B6</td>
<td>7-Ethoxy-4-(trifluoromethyl)coumarin</td>
<td>14.1</td>
<td>15</td>
<td>N.A.</td>
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<tr>
<td>CYP2C8</td>
<td>Luciferin-methyl ester</td>
<td>N.D.</td>
<td>150</td>
<td>≥250</td>
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<tr>
<td>CYP2C9</td>
<td>Diclofenac</td>
<td>15.9</td>
<td>16</td>
<td>34.1 ± 11</td>
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<tr>
<td>CYP2C9</td>
<td>S-Mephenytoin</td>
<td>81.0</td>
<td>80</td>
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<tr>
<td>CYP2D6</td>
<td>Bufuralol</td>
<td>25.3</td>
<td>25</td>
<td>≥250</td>
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<td>CYP2E1</td>
<td>Chloroxazone</td>
<td>52.0</td>
<td>50</td>
<td>N.A.</td>
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<tr>
<td>CYP3A4</td>
<td>Testosterone</td>
<td>77.5</td>
<td>100</td>
<td>49.8 ± 14</td>
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</tbody>
</table>

N.A., not accessible; N.D., not determined.

Discussion

The results of the inhibition of prototypic activities of individual microsomal CYP enzymes by silybin revealed that there are at least four P450 activities influenced by the presence of this compound, namely, those of CYP1A2, 2C8, 2C9, and 3A4 (Fig. 2). In the earlier literature, data showing a significant inhibition of CYP3A4 and CYP2C9 were published (Beckmann-Knopp et al., 2000; Zuber et al., 2002; Srirad et al., 2004). The character of inhibition was also studied using standard Dixon plots (results not shown), which indicated the presence of a noncompetitive inhibition of CYP2C9 activity and a competitive mechanism of inhibition of CYP3A4 activity. When the inhibition was more pronounced, IC₅₀ and Kᵢ values were also determined (Table 1). The results obtained here with the CYP3A4 and CYP2C9 are in agreement with the previous ones; on the other hand, they do not confirm the results of a certain degree of inhibition obtained also with CYP2E1 and CYP2D6 (Beckmann-Knopp et al., 2000; Zuber et al., 2002). Taken together, the results are indicative of an interaction between silybin and at least two microsomal P450 enzymes.

Incubation of Silybin with Recombinant Enzymes (Bactosomes). Although the inhibition experiments described in the preceding paragraph indicated a possible involvement of several P450 forms in an interaction with silybin and, hence, a possibility of their ability to form silybin metabolite(s), experiments using bacterial membrane fractions from E. coli containing recombinant human cytochrome P450 enzymes (CYP1A2, 2C8, 2C9, 3A4, 2D6, 2B6, and 2E1) coexpressed with human NADPH-cytochrome P450 reductase (bactosomes) confirmed (Fig. 6) a significant role only for CYP2C8 (and a minor contribution of CYP3A4, results not shown) in the formation of the main metabolite of silybin, i.e., of its O-demethylated derivative by HPLC/MS.

Results

Inhibition of Specific Activities in Human Liver Microsomes by Silybin. The results of in vitro inhibition of nine CYP enzymes by silybin in microsomal fraction are given in Fig. 2. Silybin displayed a weak or no interaction with CYP2E1, 2A6, 2B6, 2C19, and 2D6 (IC₅₀ ≥ 250 μM); a moderate inhibition was observed for CYP1A2 and CYP2C8. The most prominent inhibition effect was found with CYP3A4 and CYP2C9 (IC₅₀ = 49.8 and 34.1 μM) (Table 1).

Identification of Metabolites by μLC/MS Analysis. Incubation of silybin with human liver microsomes confirmed the formation of metabolites of silybin (Gunaratna and Zhang, 2003). The major metabolite was identified by μLC/MS analysis as O-demethylated silybin; the minor ones were silybin mono- and dihydroxy derivatives. Figure 3 shows chromatograms for selected m/z values (silybin m/z = 481; O-demethylated metabolite m/z = 467; monohydroxy m/z = 497 and dihydroxy m/z = 513) and fragmentation spectra of quasimolecular ions of the metabolites mentioned. It is evident that modification of a molecule of silybin occurs in two different positions for the monohydroxy metabolite (two peaks for m/z = 497 with retention times of 4.49 and 5.31 min, respectively). It has to be mentioned that chromatographic peaks in retention times of silybin (6.7–6.8 min) correspond to products of modification of silybin in the ion source. This fact was verified by an analysis of standard (unmodified) silybin, and it underlines the necessity of chromatographic separation. Identity of metabolites was verified by interpretation of MS/MS spectra and by accurate mass measurements. Experimental masses of metabolites were in good agreement with the calculated ones. Their relative errors were 0.6 ppm or less.

Effect of Carbon Monoxide and of the Specific P450 Inhibitors on the Formation of Metabolites of Silybin. To prove the role of cytochromes P450 in the metabolism of silybin, inhibition of this reaction by carbon monoxide was examined. Results indicated a considerable reduction (by 80%) of the main metabolite level in the reaction mixture, suggesting a role for a P450-mediated mechanism of metabolite formation (Fig. 4).

To find which P450 form is responsible for the formation of silybin metabolite(s), the effect of different inhibitors of particular P450 activities was studied. An overview of inhibitors used together with their effect on the formation of O-demethylated silybin is shown in Fig. 5. Among the inhibitors used, quercetin (a relatively specific inhibitor of CYP2C8) (Walsky et al., 2005) appeared to be the most potent inhibitor, causing 80% inhibition of O-demethylated silybin formation.

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The results of the inhibition of prototypic activities of individual microsomal CYP enzymes by silybin revealed that there are at least four P450 activities influenced by the presence of this compound, namely, those of CYP1A2, 2C8, 2C9, and 3A4 (Fig. 2). In the earlier literature, data showing a significant inhibition of CYP3A4 and CYP2C9 were published (Beckmann-Knopp et al., 2000; Zuber et al., 2002; Srirad et al., 2004). The character of inhibition was also studied using standard Dixon plots (results not shown), which indicated the presence of a noncompetitive inhibition of CYP2C9 activity and of a competitive mechanism of inhibition of CYP3A4 activity. When the inhibition was more pronounced, IC₅₀ and Kᵢ values were also determined (Table 1). The results obtained here with the CYP3A4 and CYP2C9 are in agreement with the previous ones; on the other hand, they do not confirm the results of a certain degree of inhibition obtained also with CYP2E1 and CYP2D6 (Beckmann-Knopp et al., 2000; Zuber et al., 2002). Taken together, the results are indicative of an interaction between silybin and at least two microsomal P450 enzymes.

Silybin was shown recently to yield several metabolites when incubated with human liver microsomes (Gunaratna and Zhang, 2003). The main metabolite was found to be the O-demethylated product; the mono- and dihydroxy silybins were the minor metabolites. In this work, the formation of these metabolites in human liver microsomes has been confirmed (Fig. 3); as the next step, we were interested in the identification of the particular P450 form involved in the formation of the main metabolite.

Carbon monoxide is known to bind strongly to the heme iron of all cytochromes P450, yielding a complex that is unable to bind molecular oxygen and perform the catalytic reaction (Cooper et al., 1977). The results (Fig. 4) have shown a clear inhibition of the formation of the main metabolite of silybin, i.e., of its O-demethylated derivative by HPLC/MS.
**FIG. 3.** Metabolites of silybin identified by μLCMS analysis. The major metabolite was identified as O-demethylated silybin; the minor ones were silybin mono and dihydroxy derivatives.

**FIG. 4.** HPLC analysis of silybin metabolites generated by human hepatic microsomes before and after treatment with carbon monoxide. M1 to M3 are metabolites of silybin. mAU, milli-absorbance units.

**FIG. 5.** Effect of selective inhibitors of P450 activities on the rate of formation of demethylated silybin by human hepatic microsomes. Furafylline, sulfaphenazole, triacetyloleandomycin (TAO), diethylthiocarbamate (DEDC), quinidine, 8-methoxypsoralen, 7-pentoxyresorufin, S-mephenytoin, and quercetin were used to inhibit the respective P450 activities. Experiments were performed in duplicate with results expressed as means; as a rule, the data obtained did not differ more than 5%.
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