MECHANISM-BASED INHIBITION OF HUMAN CYTOCHROME P450 1A1
BY RHAPONTIGENIN

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ABSTRACT:

Recently we reported that resveratrol (trans-3,4',5-trihydroxystilbene) showed selective inhibition of recombinant human cytochrome P450 1A1 (P450 1A1) in a concentration-dependent manner. The inhibition of recombinant human P450 1A1, 1A2, or 1B1 by various hydroxystilbene compounds having a similar structure to resveratrol was investigated using bacterial membranes from a human P450/NADPH-P450 reductase bicistronic expression system to find new candidates for cancer chemopreventive agents. Of seven compounds tested, rhapontigenin (3,3',5-trihydroxy-4'-methoxystilbene) exhibited a potent and selective inhibition of human P450 1A1 with an IC$_{50}$ value of 0.4 μM. Rhapontigenin showed 400-fold selectivity for P450 1A1 over P450 1A2 and 23-fold selectivity for P450 1A1 over P450 1B1. Rhapontigenin did not show any significant inhibition of ethoxyresorufin O-deethylation (EROD) activity in human liver microsomes, the other human P450s such as P450 2E1, P450 3A4, P450 2D6, P450 2C8, and P450 2C9, or human NADPH-P450 reductase. We have further investigated the inhibition kinetics of P450 1A1 by rhapontigenin. Rhapontigenin inhibited EROD activity of expressed human P450 1A1 in a competitive manner. The loss of EROD activity was time- and concentration-dependent. The values for $K_i$ and $k_{acivation}$ were 0.09 μM and 0.06 min$^{-1}$, respectively. The loss was not blocked by the trapping agents glutathione, N-acetylcysteine, or dithiothreitol. These results suggest that rhapontigenin is a potent mechanism-based inactivator of human P450 1A1 and may be considered as a good candidate for a cancer chemopreventive agent in humans.
δ-aminolevulinic acid, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Nifedipine and nifedipine metabolite were from RBI/Sigma (Natick, MA). Bactopeptone, yeast extract, and bacto-agar were obtained from Difco Lab. (Detroit, MI). Human P450 2C8 microsomes were purchased from GENTEST (Woburn, MA). Other chemicals were of the highest grade commercially available.

**Recombinant Human P450s.** Coexpression (bicistronic) plasmids for human P450s (1A1, 1A2, 1B1, 2E1, 3A4, 2C9, or 2D6), and NADPH-P450 reductase were transformed into *Escherichia coli* DH5α (Parikh et al., 1997). A single ampicillin-resistant colony of transformed cells was selected and grown in overnight culture to saturation at 37°C in Luria-Bertani medium containing 100 µg of ampicillin ml−1. A 10-ml aliquot was inoculated to isole and Terrific Broth containing 0.2% bactopeptone (w/v), 100 µg of ampicillin ml−1, 1.0 mM thiamine, trace elements, 0.5 mM ethoxycoumarin, and 1.0 mM IPTG. The cultures were grown at 30°C with shaking at 200 rpm for 24 h. Membrane fractions were prepared by differential centrifugation from bacteria and suspended in 10 mM Tris–HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% (v/v) glycerol (Guengerich et al., 1994). Protein concentrations were estimated using the bicinchoninic acid method according to the supplier’s recommendations (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard. The isolated microsomes were stored at −80°C.

**Enzyme Assays.** EROD activity was determined for the measurement of P450 1A1, 1A2, or 1B1 activities (Burke et al., 1985). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.4), 2 mg of bovine serum albumin ml−1, 10 µM dicoumarol, human liver microsomes, or *E. coli* membranes (5 nM P450 1A1, 10 nM P450 1A2, or 10 nM P450 1B1, respectively), and 2 µM ethoxyresorufin. The reaction mixtures were preincubated at 37°C for 3 min, and the reaction was initiated by addition of 120 µM NADPH. Incubations were performed in a shaking water bath for 20 min at 37°C and terminated by addition of 1 ml of methanol. The formation of resorufin was determined fluorometrically with a PerkinElmer LS 5 spectrophurometer (with excitation and emission wavelengths of 550 and 585 nm, respectively) (PerkinElmer, Norwalk, CT).

P450 content of cells and membranes was quantitated by the spectrophotometric method of Omura and Sato (1964) using an extinction coefficient of 91 mM−1 cm−1 with a Shimadzu UV-160A spectrophotometer at ambient temperature (Shimadzu, Kyoto, Japan). For the measurement of P450 2E1 activity, O-deethylation of 7-ethoxycoumarin was determined fluorometrically according to methods described previously (Yamazaki et al., 1996). Reaction mixtures contained 0.1 M potassium phosphate (pH 7.4), 2 mM ethoxycoumarin, *E. coli* membranes (40 nM P450 2E1), and 1 mM NADPH. Incubations were performed in a shaking water bath for 20 min at 37°C and terminated by addition of 25 µl of 10% (v/v) trichloroacetic acid and 1 ml of CH3Cl. The mixture was vortex mixed and extracted with 30 mM sodium borate (pH 9.0). Concentrations of hydroxycoumarin were determined by a PerkinElmer LS 5 spectrophurometer with excitation and emission wavelengths of 370 and 450 nm, respectively.

Nifedipine oxidation was determined by high-performance liquid chromatography as a measure of recombinant human P450 3A4 activity using previously described methodology (Guengerich et al., 1986). Reaction mixtures contained 50 mM HEPES (pH 7.4), 30 mM MgCl2, 0.2 mM nifedipine, *E. coli* membranes (40 nM P450 3A4), and 1 mM NADPH. Incubations were performed in a shaking water bath for 20 min at 37°C and terminated by addition of 2 ml of CH3Cl, and 100 µl of 1 M Na2CO3, (pH 10.5) containing 2 M NaCl. The samples were centrifuged at 3000g for 10 min, and 1.4 ml of each of the lower organic layers were dried under a N2 stream. Determination of a nifedipine metabolite was performed using a 150 × 4.6-mm steel C18 Nucleosil column with ultraviolet detection at 254 nm. The flow rate was 1.0 ml/min, and the mobile phase was 64% methanol. Under these conditions, retention times of nifedipine metabolite and nifedipine were 6.9 and 9.1 min, respectively. The detection limit of a nifedipine metabolite was ~50 pmol with a signal-to-noise ratio of 3:1.

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
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<tr>
<td>Rhapontigenin</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>OCH3</td>
<td>OH</td>
</tr>
<tr>
<td>3,5-Dihydroxy 4'-methoxythibene</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
<td>OCH3</td>
<td>H</td>
</tr>
<tr>
<td>Oxysoverolat</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>3,4'-Dimethoxy 5'-hydroxysilbene</td>
<td>OCH3</td>
<td>OCH3</td>
<td>H</td>
<td>OCH3</td>
<td>H</td>
</tr>
<tr>
<td>Piceid</td>
<td>O-Glucose</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Rhaponticin</td>
<td>O-Glucose</td>
<td>OH</td>
<td>H</td>
<td>OCH3</td>
<td>OH</td>
</tr>
</tbody>
</table>

The rate of NADPH-cytochrome *c* reduction was measured using an extinction coefficient of 21 mM−1 cm−1 (Yasukoshi and Masters, 1976). The intra- and interday variation coefficients did not exceed 10% in any of the assays.

**NADPH Dependence of Inhibition.** Bacterial membranes containing human P450 1A1 and NADPH-P450 reductase were preincubated in 0.1 M potassium phosphate buffer (pH 7.4) containing hydroxystilbene at 37°C for 10 min in the presence or absence of 1 mM NADPH (Chun et al., 1999). At various times during the preincubation, an aliquot of the preincubation mixture was diluted 10-fold into the reaction mixture containing 0.1 M potassium phosphate (pH 7.4), 2 mg/ml bovine serum albumin, 0.2 µM ethoxycoumarin, and 120 µM NADPH. The mixtures were further incubated at 37°C for 20 min.
The product of 7-ethoxyresorufin was monitored fluorometrically as described above.

**Data Analysis.** Kinetic parameters from individual experiments were calculated using a nonlinear regression analysis program (Prism, GraphPad Software, San Diego, CA).

**Results**

**Inhibition of EROD activity by Hydroxystilbenes.** To examine their effects on the activity of human P450 1A1, 1A2, and 1B1, seven hydroxystilbene compounds were compared with respect to inhibition of EROD activities (Table 2). Rhapontigenin was found to be a potent inhibitor of P450 1A1, with an IC₅₀ value of 0.4 μM (Fig. 1; Table 2). Similar studies showed that rhapontigenin also inhibits P450 1A2 and 1B1, but the IC₅₀ values were much higher (i.e., 160 μM for P450 1A2 and 9 μM for P450 1B1). Thus, rhapontigenin exhibited 400-fold greater selective inhibition of P450 1A1 over 1A2 and 23-fold greater inhibition of P450 1A1 over 1B1. As shown in Table 2, 3,4'-dimethoxy-5-hydroxystilbene and 3,5-dihydroxy-4'-methoxystilbene were also more potent inhibitors of P450 1A1 than of P450 1A2, but the selectivity between P450 1A1 and 1B1 was much lower than that of rhapontigenin. The most potent P450 1A1 inhibitor of all hydroxystilbenes we examined was 3,4'-dimethoxy-5-hydroxystilbene. 3,4',5'-Trimethoxystilbene also strongly inhibited P450 1A1, 1A2, and 1B1 with IC₅₀ values of 0.6, 0.6, and 0.4 μM for 1A1, 1A2, and 1B1, respectively, but no selectivity was shown. The IC₅₀ of oxyresveratrol for P450 1A1 was about 15 μM. The hydroxystilbenes containing a glucosyl moiety at the R1 position, such as piceid or rhaponticin, had little inhibitory effect on P450s. The bulky and polar glucosyl moiety may block the accessibility of the chemicals into the active sites of P450s.

Rhapontigenin showed very weak inhibition of the catalytic activities of the other human P450s, such as P450 2E1, 3A4, 2D6, 2C8, and 2C9 (Fig. 1). The EROD activity in human liver microsomes and tissues coexpressing human P450s and NADPH-P450 reductase were incubated with rhapontigenin for 20 min at 37°C in the presence of NADPH. Assays included EROD by P450 1A1 (○), P450 1A2 (●), or 1B1 (▲), 7-ethoxycoymarin O-deethylation by P450 2E1 (▲), nifedipine oxidation by P450 3A4 (△), bufuralol 1'-hydroxylation by P450 2D6 (○), pachitaxel 6α-hydroxylase activity by P450 2C8 (ϕ), or diclofenac 4'-hydroxylation by P450 2C9 (□) in the presence of the indicated concentrations of rhapontigenin. Each data point represents the mean ± S.E.M. of three experiments.

The data in Fig. 4A showed that Kᵢ and kᵢnactivation were 0.09 μM and 0.06 min⁻¹, respectively (Fig. 4B). To determine the possibility that the reactive species could escape from the P450 active site and bind to nucleophilic sites in the vicinity of the active site, the effect of trapping agents such as glutathione, N-acetylcysteine, or dithiothreitol on the inactivation was studied. Rhapontigenin did not protect against P450 1A1 inactivation in the presence of 2 mM glutathione, N-acetylcysteine, or dithiothreitol (Fig. 5).

**Discussion**

Tumor initiation begins when DNA in cells is damaged by exposure to carcinogens. If this damage is not repaired, it can lead to genetic mutations (Hurting et al., 1999). Metabolic activation of procarcinogens is often catalyzed by P450 enzymes through oxidation. Enzymes such as P450 1A1, 1A2, or 1B1 of the human P450 1 subfamily are responsible for much of the metabolism of procarcinogens and regarded as the target enzymes for blocking tumor initiation (Shimada et al., 1989, 1996; Guengerich and Shimada, 1991). Therefore, specific P450 inhibitors or inactivators could be beneficial for preventing tumor formation.

Recently, several hydroxystilbene compounds isolated from herbal sources including *Morus alba* or *Rheum undulatum* were identified as having various pharmacological effects. For example, oxyresveratrol potently inhibited dopa oxidase activity of tyrosinase (Shin et al., 1998a). Rhapontigenin significantly inhibited the release of β-hexamidase from cultured RBL-2H3 cells (Cheong et al., 1999). These hydroxystilbenes also suppressed ovoine cyclooxygenase-1 activity (Shin et al., 1998b).

In this study we demonstrated that rhapontigenin is a potent mechanism-based inactivator of P450 1A1 as well as a competitive inhibitor. Rhapontigenin inactivation of P450 1A1 followed pseudo-first order kinetics, was time- and concentration-dependent, and required NADPH. Trapping agents such as glutathione, N-acetylcysteine, or

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**Table 2**

*Inhibition of human P450 1A1, 1A2, and 1B1 by various hydroxystilbene compounds*

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P450 1A1</td>
</tr>
<tr>
<td>Rhapontigenin</td>
<td>0.4</td>
</tr>
<tr>
<td>3,5-Dihydroxy 4'-methoxystilbene</td>
<td>0.4</td>
</tr>
<tr>
<td>Oxycysemarin</td>
<td>15</td>
</tr>
<tr>
<td>3,4'-Dimethoxy-5-hydroxystilbene</td>
<td>0.1</td>
</tr>
<tr>
<td>3,4,5'-Trimethoxystilbene</td>
<td>0.6</td>
</tr>
<tr>
<td>Piceid</td>
<td>480</td>
</tr>
<tr>
<td>Rhaponticin</td>
<td>200</td>
</tr>
</tbody>
</table>

**Fig. 1.** Effects of rhapontigenin on the P450 1A1, 1A2, 1B1, 2E1, 3A4, 2D6, 2C8, or 2C9 activities in the P450-expression systems.

Membranes coexpressing human P450s and NADPH-P450 reductase were incubated with rhapontigenin for 20 min at 37°C in the presence of NADPH. Membranes were assayed for EROD by P450 1A1 (○), P450 1A2 (●), or 1B1 (▲), and 7-ethoxycoymarin O-deethylation by P450 2E1 (▲), nifedipine oxidation by P450 3A4 (△), bufuralol 1'-hydroxylation by P450 2D6 (○), pachitaxel 6α-hydroxylase activity by P450 2C8 (ϕ), or diclofenac 4'-hydroxylation by P450 2C9 (□) in the presence of the indicated concentrations of rhapontigenin. Each data point represents the mean ± S.E.M. of three experiments.
dithiothreitol could not block the inactivation of P450 1A1 by raphontigenin.

Several chemopreventive agents are known as a mechanism-based inactivators of P450s, such as isothiocyanates (P450 2E1) and oltipraz (P450 1A2) (Kent et al., 1998; Moreno et al., 1999; Langouët et al., 2000). The two main mechanisms for the chemopreventive action are 1) inhibition of phase I enzymes such as the P450 enzymes and 2) induction of phase II enzymes such as glutathione S-transferase and quinone reductase. We have previously demonstrated that human P450 1A1 activity was selectively inhibited by resveratrol (Chun et al., 1999). Resveratrol seems to be a promising cancer chemopreventive agent because it inhibits phase I enzymes and induces phase II enzymes. However, the inhibition of P450 1A1 by resveratrol is relatively weak and not mechanism-based. Furthermore, resveratrol could not discriminate in inhibiting the activities of P450s 1A1 and 1B1 (unpublished results). We suggest that raphontigenin is a better candidate for cancer chemopreventive agents because its inhibitory potential of P450 1A1 is much stronger, selective, and mechanism-based. We are studying the effects of raphontigenin on the expression of phase II enzymes in cultured cells.

A number of the known inhibitors of the different P450 enzymes are mechanism-based (Yun et al., 1992; Wrighton et al., 1993; Roberts et al., 1995; Heyn et al., 1996; Hickman et al., 1998). Many selective P450 1A1 inhibitors have been reported. For example, 2-(1-propynyl)phenanthrene showed 70-fold greater inhibition of P450 1A1 than of 1A2 (Shimada et al., 1998b), and 7-hydroxyflavone exhibited 6-fold greater selectivity in its inhibition of P450 1A1 over P450 1A2 (Zhai et al., 1998). As we showed previously, resveratrol is also selective for inhibiting P450 1A1 (Chun et al., 1999). These
inhibitors are used for labeling the P450 4 active site and identifying critical amino acid(s) of P450s. The 400-fold greater selectivity of rhapontigenin in the inhibition of P450 1A1 over P450 1A2 is of interest. Thus far, rhapontigenin is one of the most selective inhibitors of P450 1A1 from natural sources. We propose that rhapontigenin also will be a useful compound for characterizing P450 1A1 active sites because of its strong selectivity.

The precise mechanism by which rhapontigenin is transformed by P450 1A1 into an inactivating intermediate is not yet known. We have considered the possibility that activated oxygen bound to heme may react with the allyl moiety of the stilbene structure, and the intermediate species could then react with protein or heme nucleophiles in a process that destroys heme function or binding (Ortiz de Montellano 1983; Hammons et al., 1989; Guengerich, 1990).

In summary, we report for the first time that rhapontigenin is a potent mechanism-based inactivator of human P450 1A1. Because rhapontigenin is isolated from the oriental medicinal plant Rheum undulatum, its potent and selective inhibitory effect on P450 1A1 takes on additional advantages as a chemopreventive agent. Future studies will focus on the potential of rhapontigenin as a strong chemopreventive agent in vivo.

Acknowledgment. We thank Dr. F. P. Guengerich for providing us with the bacterial biocistronic expression plasmids and for helpful suggestions.

References

FIG. 5. Effects of the trapping agents on the inactivation of P450 1A1 by rhapontigenin.

The E. coli membranes expressing human P450 1A1 and NADPH-P450 reductase were preincubated for 10 min with NADPH (1 mM), rhapontigenin (5 μM), and the indicated trapping agents (2 mM) or water. At the end of the incubation, aliquots were assayed for EROD activity. Each data point represents the mean ± range of duplicate determinations.