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'-methoxy-stilbene) exhibited a potent and selective inhibition of human P450 1A1 with an IC₅₀ 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 Kᵢ and kᵦᵢcientation 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.

Human cytochrome P450 (P450 1A1) is a well known aryl hydrocarbon hydroxylase and is involved in the metabolic activation of procarcinogens of the poly cyclic aromatic hydrocarbons. In humans, P450 1A1 shares 80% amino acid sequence identity with P450 1A2 and about 40% with P450 1B1 (Jaiswal et al., 1985; Quattrochi et al., 1985; Shimada et al., 1998b). The substrate specificities of these enzymes can often overlap. Although P450 1A1 is mainly expressed in human lung, placenta, and lymphocytes, P450 1A2 is one of the major P450s in human liver. P450 1B1 is normally expressed in human fibroblasts and steroidogenic tissues. To distinguish the activities of these P450s and to determine the mechanism of reactions of the enzymes, selective inhibitors are required. Selective inhibitors may be good chemopreventive agent candidates for cancer because these P450s are related to tumor initiation.

Resveratrol has been studied to determine its role as a cancer chemopreventive agent and shown to inhibit tumor formation, acting through cyclooxygenase inhibition, blockage of free-radical formation, and induction of quinone reductase (Jang et al., 1999). Recently we reported that resveratrol can inhibit human P450 1A1 in a concentration-dependent manner (Chun et al., 1999). Resveratrol showed 50-fold selectivity in its inhibition of P450 1A1 over P450 1A2. However, the IC₅₀ value for 1A1 inhibition (23 μM) by resveratrol is not very low compared with other known P450 1A inhibitors such as α-naphthoflavone and 7-hydroxyflavone (Shimada et al., 1998b; Zhai et al., 1998). To find more potent and selective P450 1A1 inhibitors among resveratrol analogs, several hydroxystilbene compounds obtained from natural sources were evaluated for selective inhibition of P450 1A1 activity (Shin et al., 1998). To examine these compounds, recombinant systems in which human P450s 1A1, 1A2, or 1B1 was expressed along with human NADPH-P450 reductase in bacterial membranes were used (Parikh et al., 1997; Josephy et al., 1998; Shimada et al., 1998a). Of the compounds tested, rhapontigenin showed potent and selective inhibition of P450 1A1 over 1A2 or 1B1. Kinetic analyses were performed to determine the mechanism of P450 1A1 inhibition, and we suggest that rhapontigenin is a mechanism-based inactivator of human P450 1A1.

Experimental Procedures

Materials. The hydroxystilbenes used were isolated from herbal extracts or obtained from chemical modification (Table 1) (Ryu et al., 1988). Rhapontigenin, rhaponticin, and 3,5-dihydroxy-4'-methoxystilbene were purified from Rheum undulatum Linne. Oxysresveratrol was isolated from Morus alba Linne. Resveratrol was isolated from Veratrum album var. grandiflorum Maxim., and 3',4',5-trimethoxystilbene was obtained by methylation of resveratrol. Piceid was isolated from Polygonum cuspidatum Sieb. et Zucc., and 3,4'-dimethoxy-5-hydroxystilbene was obtained by methylation of piceid followed by the acid hydrolysis. Human liver samples were kindly provided by Dr. F. Peter Guengerich (Vanderbilt University, Nashville, TN). Ethoxyresorufin, resorufin, ethoxycoumarin, hydroxycoumarin, dimethyl sulfoxide, thiamine, IPTG,
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 commercially available.

**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 (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 spectrofluorometer 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 mM 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. Bufuralol 1′-hydroxylation activity was determined as previously described (Yamazaki et al., 1994). Incubation mixtures consisted of membranes (100 nM P450 2D6) and bufuralol (0.4 mM) in a final volume of 0.1 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM NADPH. Incubations were carried out at 37°C for 20 min and were terminated by addition of 10 μl of 60% (v/v) HClO₄. The mixtures were centrifuged at 3000g for 5 min, and aliquots of the supernatant were separated using a 150 × 4.6-mm steel C₁₈ Nucleosil column eluted with 45% (v/v) acetonitrile/55% 20 mM NaClO₄, pH 2.5. The hydroxylated metabolites of bufuralol were detected with a fluorescence detector (excitation = 252 nm; emission = 302 nm).

Palmitelx 6α-hydroxylase activity was used as a catalytic marker for recombinant human P450 2C8 (Rahman et al., 1994). Reaction mixtures (0.2 ml of total volume) contained 100 mM potassium phosphate buffer (pH 7.4), 10 μM palmitelx, membranes (50 nM P450 2C8), and 1 mM NADPH. Incubations were performed in a shaking water bath for 20 min at 37°C and terminated by addition of 50 μl of ice-cold acetonitrile. The samples were centrifuged at 12,000g for 5 min, and aliquots of the supernatant were analyzed using a 150 × 4.6-mm steel C₁₈ Nucleosil column with a linear gradient of 45% of 10% (v/v) methanol and 55% of 100% (v/v) methanol to 35% of 10% (v/v) methanol and 65% of 100% (v/v) methanol over 20 min, then for a further 5 min with 55% of 10% (v/v) methanol and 65% of 100% (v/v) methanol with ultraviolet detection at 254 nm.

Diclofenac 4′-hydroxylation was determined for the recombinant human P450 2C9 specific activity (Leemann et al., 1992). Incubation mixture (0.2 ml of total volume) consisted of 100 mM Tris-HCl buffer (pH 7.5), 100 μM diclofenac, membranes (50 nM P450 2C9), and 1 mM NADPH. Incubations were carried out at 37°C for 20 min and were terminated by addition of 50 μl of a mixture of 94% acetonitrile/6% acetic acid. The reaction mixtures were centrifuged at 12,000g for 5 min and the supernatant was analyzed using a 150 × 4.6-mm steel C₁₈ Nucleosil column with a linear gradient from 70% of 30% (v/v) acetonitrile containing 2 mM perchloric acid and 30% of 100% (v/v) methanol to 100% of 100% (v/v) methanol over 20 min. The flow rate was 1.0 ml/min, and the production of 4′-hydroxydiclofenac was measured with ultraviolet detection at 280 nm.

Rates of NADPH-cytochrome c reduction were 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 ethoxyresorufin, and 120 μM NADPH. The mixtures were further incubated at 37°C for 20 min.

### TABLE 1

Structure of hydroxystilbenes used in this study

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhapontigenin</td>
<td>OH</td>
</tr>
<tr>
<td>3,5-Dihydroxy 4′-methoxy stilbene</td>
<td>OH</td>
</tr>
<tr>
<td>Oxysterolverat</td>
<td>OH</td>
</tr>
<tr>
<td>3,4′-Dimethoxy 5′-hydroxystilbene</td>
<td>OCH₃</td>
</tr>
<tr>
<td>Piceid</td>
<td>O-Glucose</td>
</tr>
<tr>
<td>Rhapontocin</td>
<td>O-Glucose</td>
</tr>
</tbody>
</table>

![Structure of hydroxystilbenes used in this study](image-url)
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, with time- and concentration-dependent, and required NADPH. Trapping agents such as 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 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 tumor initiators. For example, oxyresveratrol potently inhibited dopa oxidase activity of tyrosinase (Shin et al., 1998a). Rhapontigenin significantly inhibited the release of β-hexamidine from cultured RBL-2H3 cells (Cheong et al., 1999). These hydroxystilbenes also suppressed ovine 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 dithiothreitol (Fig. 5).

**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'-dihydroxy-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'-dihydroxy-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 significantly more activity versus time (Fig. 4A). A replot of the data in Fig. 4A showed that Kᵢ and kᵢᵦ 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).

**Table 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (μM)</th>
<th>Ratio (IA2/IA1)</th>
<th>Ratio (IB1/IA1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhampectin</td>
<td>0.4</td>
<td>160</td>
<td>9</td>
</tr>
<tr>
<td>3,5-Dihydroxy 4'-methoxystilbene</td>
<td>0.4</td>
<td>35</td>
<td>1</td>
</tr>
<tr>
<td>Oxyresveratol</td>
<td>15</td>
<td>150</td>
<td>34</td>
</tr>
<tr>
<td>3,4'-Dihydroxy-5-hydroxystilbene</td>
<td>0.1</td>
<td>8</td>
<td>0.1</td>
</tr>
<tr>
<td>3,4',5'-Trimethoxystilbene</td>
<td>0.6</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Piceid</td>
<td>480</td>
<td>&gt;2000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Rhapontigenin</td>
<td>200</td>
<td>166</td>
<td>145</td>
</tr>
</tbody>
</table>

**[Rhapontigenin], μM**

Membranes 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-ethoxyresorcinol O-deethylation by P450 2E1 (●), nifedipine oxidation by P450 3A4 (▲), bufuralol 1'-hydroxylation by P450 2D6 (▲), pachytauxol 6α-hydroxylation activity by P450 2C8 (●), 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
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 used for EROD assay. Each data point represents the mean ± range of duplicate determinations.

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References


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