GENERATION OF THE HUMAN METABOLITE PICEATANNOL
FROM THE ANTI-CANCER PREVENTIVE AGENT RESVERATROL BY
BACTERIAL CYTOCHROME P450 BM3

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Running title: Oxidation of resveratrol catalyzed by bacterial P450 BM3

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Abbreviations used: P450 or CYP, cytochrome P450; CPR, NADPH-P450 reductase; CYP102A1, Cytochrome P450 BM3; IPTG, isopropyl-β-D-thiogalactopyranoside; resveratrol, 3,4′,5-trihydroxystilbene; piceatannol, 3,5,3′,4′-tetrahydroxystilbene; TTN, total turnover number
Abstract

Recently, the wild-type and mutant forms of cytochrome P450 BM3 (CYP102A1) from Bacillus megaterium were found to metabolize various drugs through reactions similar to those catalyzed by human P450 enzymes. Therefore, it was suggested that CYP102A1 can be used to produce large quantities of the metabolites of human P450-catalyzed reactions. *trans*-Resveratrol, an anti-cancer preventive agent, is oxidized by human P450 1A2 to produce two major metabolites, piceatannol and another hydroxylated product. In this report, we show that the oxidation of *trans*-resveratrol, a human P450 1A2 substrate, is catalyzed by wild-type and a set of CYP102A1 mutants. One major hydroxylated product, piceatannol, was produced as a result of the hydroxylation reaction. Other hydroxylated products were not produced. Piceatannol formation was confirmed by HPLC and GC-MS by comparing the metabolite to the authentic piceatannol compound. These results demonstrate that CYP102A1 mutants can be used to produce piceatannol, a human metabolite of resveratrol.
Introduction

Resveratrol (3,4’,5-trihydroxystilbene) (Fig. 1) is a phytoalexin found in a wide variety of dietary sources including grapes, plums and peanuts. It exhibits pleiotropic health-beneficial effects including anti-oxidant, anti-inflammatory, cardioprotective and anti-tumor activities (Kundu and Surh, 2008; Pirola and Fröjdö, 2008; Athar et al., 2007). Currently, numerous preclinical findings suggest resveratrol as a promising part of nature's arsenal for cancer prevention and treatment. As a potential anti-cancer agent, resveratrol has been shown to inhibit or retard the growth of various cancer cells in culture and implanted tumors in vivo. The compound significantly inhibits experimental tumorigenesis in a wide range of animal models. The biological activities of resveratrol are found to relate to its ability to modulate various targets and signaling pathways. Resveratrol exists as cis- and trans-isomers. trans-Resveratrol is the preferred steric form and is relatively stable.

Piceatannol (3,5,3’,4’-tetrahydroxystilbene) (Fig. 1) is a polyphenol found in grapes and other plants. It is known as a protein kinase inhibitor that modifies multiple cellular targets, exerting immunosuppressive and antitumorigenic activities in several cell lines. Piceatannol has been shown to exert various pharmacological effects on
immune and cancer cells (Kim et al., 2008b and references therein). In humans, piceatannol is produced as a major metabolite of resveratrol by CYP1B1 and CYP1A2 (Potter et al., 2002; Piver et al., 2004). In addition, the metabolism of trans-resveratrol into two major metabolites, piceatannol (3,5,3′,4′-tetrahydroxystilbene) and another tetrahydroxystilbene, is catalyzed by recombinant human CYP1A1, CYP1A2 and CYP1B1 (Piver et al., 2004). It is also known that trans-resveratrol can inhibit reactions catalyzed by human CYP1A1 and CYP1A2 (Chun et al., 1999).

If pro-drugs are converted to biologically ‘active metabolites’ by human liver P450s during the drug development process (Johnson et al., 2004), large quantities of the pure metabolites are required to understand the drug’s efficacy, toxic effect, and pharmacokinetics. Because the pure metabolites may be difficult to synthesize, an alternative is to use P450s to generate the metabolites of drugs or drug candidates. Although hepatic microsomes can be a source of human P450s, their limited availability makes their use in preparative-scale metabolite synthesis impractical. Some human enzymes can also be obtained by expression in recombinant hosts (Yun et al., 2006). Metabolite preparation has been demonstrated using human P450s expressed in Escherichia coli and in insect cells (Parikh et al., 1997; Rushmore et al., 2000; Vail et al., 2005), but these systems are costly and have low productivities due to limited
stabilities and slow reaction rates (Guengerich et al., 1996). An alternative approach to preparing the human metabolites is to use an engineered bacterial P450 that has the desired catalytic activities.

Several mutants of *Bacillus megaterium* P450 BM3 (CYP102A1) generated through rational design or directed evolution could oxidize several human P450 substrates to produce authentic metabolites with higher activities (Kim et al., 2008a; Otey et al., 2005; Stjernschantz et al., 2008; Yun et al., 2007 and references therein). These recent advances suggest that CYP102A1 can be developed as biocatalysts for drug discovery and synthesis (Bernhardt, 2006; Di Nardo et al., 2007). Very recently, it was reported that some selected mutations enabled the CYP102A1 enzyme to catalyze \(O\)-deethylation and 3-hydroxylation of 7-ethoxycoumarin, which are the same reactions catalyzed by human P450s (Kim et al., 2008a).

In this study, we tested whether CYP102A1 mutants could be used to produce piceatannol, the major human metabolite of *trans*-resveratrol. Piceatannol is more expensive than *trans*-resveratrol, its substrate. Some of the tested mutations enabled the CYP102A1 enzyme to catalyze the hydroxylation of *trans*-resveratrol to generate its human metabolite, piceatannol.
Materials and Methods

**Chemicals.** *trans*-Resveratrol, piceatannol, and NADPH were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Other chemicals were of the highest grade commercially available.

**Construction of BM3 mutants by site-directed mutagenesis.** Seventeen different site-directed mutants of CYP102A1 were prepared as described (Kim et al., 2008a and references therein). The CYP102A1 mutants used in this study were selected based on earlier work showing their increased catalytic activity toward several human substrates. Each mutant bears the amino acid substitution(s) relative to wild-type CYP102A1, as summarized at Table 1. (Kim et al., 2008a)

**Expression and purification of CYP102A1 mutants.** Wild-type and mutants of CYP102A1 were expressed in *Escherichia coli* and purified as described (Kim et al., 2008a). The CYP102A1 concentrations were determined from CO-difference spectra as described by Omura and Sato (1964) using $\varepsilon = 91$ mM/cm. For all of the wild-type and mutated enzymes, a typical culture yielded 300 to 700 nM P450. The expression level of CYP102A1 wild-type and mutants was typically in the range of 1.0 – 2.0 nmol P450/mg cytosolic protein.
Hydroxylation of trans-resveratrol. Typical steady-state reactions for the trans-resveratrol hydroxylation included 50 pmol CYP102A1 in 0.25 ml of 100 mM potassium phosphate buffer (pH 7.4) containing final concentration of 100 µM trans-resveratrol. To determine the kinetic parameters of several CYP102A1 mutants, we used 2 - 500 µM of trans-resveratrol. An aliquot of a NADPH-generating system was used to initiate reactions (final concentrations: 10 mM glucose 6-phosphate, 0.5 mM NADP+, and 1 IU yeast glucose 6-phosphate per ml). A stock solution of trans-resveratrol (20 mM) was prepared in DMSO and diluted into the enzyme reactions with a final organic solvent concentration of < 1% (v/v).

The reaction mixtures (final volume of 0.25 ml) were incubated for 10 min at 37 °C and terminated with 0.50 ml of ice-cold ethyl acetate. After centrifugation of the reaction mixture, organic phases were evaporated under a nitrogen gas. The product formation was analyzed by HPLC as described previously (Piver et al. 2004). Samples (30 µl) were injected onto a Gemini C18 column (4.6 mm x 150mm, 5 µm, Phenomenex, Torrance, CA). The mobile phase A was water containing 0.5% acetic acid/acetonitrile (95/5, v/v), and the mobile phase B was acetonitrile/0.5% acetic acid (95/5, v/v); the mobile phase A/B (75/25, v/v) was delivered at a flow rate of 1 ml·min⁻¹ by a gradient pump (LC-20AD, Shimadzu, Kyoto, Japan). Eluates were detected by UV at 320 nm.
To determine the total turnover numbers (TTNs) of several CYP102A1 mutants, 100 µM of trans-resveratrol was used. The reaction was initiated by the addition of the NADPH-generating system, incubated for 1 and 2 h at 30 °C. The formation rate of piceatannol was determined by HPLC as described above.

The kinetic parameters ($K_m$ and $k_{cat}$) were determined using nonlinear regression analysis with GraphPad PRISM software (GraphPad, San Diego, CA, USA). The data were fit to the standard Michaelis-Menten equation: $v = \frac{k_{cat}[E][S]}{([S] + K_m)}$, where the velocity of the reaction is a function of the turnover ($k_{cat}$), which is the rate-limiting step, the enzyme concentration ([E]), substrate concentration ([S]), and the Michaelis constant ($K_m$).

**GC-MS analysis.** For the identification of the trans-resveratrol metabolite produced by the CYP102A1 mutants, the GC-MS analysis compared the GC-profile and fragmentation patterns of the authentic compounds, piceatannol and trans-resveratrol. The oxidation reaction of trans-resveratrol by CYP102A1 mutants was done as described above. The aqueous samples were extracted with ethyl acetate. After centrifugation, the organic phase, standard trans-resveratrol and authentic piceatannol solution (10 mM in ethanol) were each dried under nitrogen. Then
trimethylsilyl (TMS) derivatives were prepared as follows: 100 µl of a solution of BSTFA/TMCS (99/1, v/v) (Supelco) was added to the dry residue and the mixture was left for 60 min at 60 °C.

GC-MS analysis was performed on a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) with an Rtx-5 column (5% diphenyl/95% dimethyl polysiloxane capillary column) (30 m × 0.32 mm i.d. × 0.25 µm film thickness). The injector temperature was 250 °C. The derivatized samples of resveratrol and piceatannol were separated by GC under GC oven conditions of 60 °C for 5 min, followed by an increase of 50 °C· min⁻¹ up to 200 °C and then 2 °C· min⁻¹ to 300 °C. The gas chromatography was combined with a GCMS-QP2010 Shimazu mass spectrometer operating in electron ionization mode (70 eV) (Piver et al. 2004).
Results and Discussion

Oxidation of trans-resveratrol by P450 BM3 wild-type and its mutants. We examined whether CYP102A1 can oxidize trans-resveratrol. First, the ability of wild-type and a set of P450 BM3 mutants to oxidize trans-resveratrol was measured at a fixed substrate concentration (100 μM). The metabolites were analyzed by HPLC and compared to those of human CYP1A2 (Table 1, Fig. 2). While human CYP1A2 oxidized resveratrol to produce two major metabolites, CYP102A1 mutants produced only one major metabolite. In the case of the human metabolites, one is piceatannol and the other is also hydroxylated product, as reported previously (Piver et al., 2004). Wild-type and catalytically active mutants of CYP102A1 produced only one major product, which had a retention time that exactly matched the piceatannol standard, but not the other hydroxylated product. The turnover numbers for the entire set of the 17 mutants for the trans-resveratrol oxidation (piceatannol formation) varied over a wide range. CYP102A1 wild-type showed a much lower catalytic activity (0.22 min⁻¹) (Table 1). Mutants #1 through #7 did not show apparent activities (<0.03 min⁻¹). Mutants #8 through #17 showed higher activities than that of CYP102A1 wild-type. In the case of mutant #13, its turnover number (4.0 min⁻¹) was 18-fold higher than that of wild-type.
The identities of the major metabolite and substrate were verified by comparing the results of HPLC (Fig. 2) and GC/MS (Supplementary Data Fig. S1 and S2) with standard compounds.

The production of piceatannol by CYP102A1 mutants was confirmed by GC-MS analysis after derivatization of the reaction mixture. The retention time and fragmentation pattern of the CYP102A1 metabolite were exactly matched to those of the piceatannol standard.

trans-Resveratrol proved to be a good substrate for CYP102A1 enzymes, with high turnover numbers (up to 4.0 min⁻¹ in the case of mutant #13). Although piceatannol and another hydroxylated product were found to be the major two metabolites of human liver microsomes (Piver et al., 2004), all of the CYP102A1 enzymes including wild-type and active mutants showed only one hydroxylated product, piceatannol (Fig. 2). The human P450 1A2, the major enzyme for the hydroxylation reactions of resveratrol in human liver, also shows a preference for the 3’-hydroxylation reaction over the hydroxylation reaction at another position (Fig. 2B and Supplementary Data Fig. S3). However, unlike the human P450 enzyme, wild-type and active mutants of CYP102A1 catalyzed only the 3’-hydroxylation reaction, but not the hydroxylation reaction at
another site. This preference is likely due to the orientation of the substrate in the active site.

**Kinetic parameters of resveratrol oxidation by CYP102A1 mutants.** Four high-activity and three low-activity mutants were chosen and used to measure kinetic parameters for the 3’-hydroxylation of resveratrol (Table 2). Some mutants of CYP102A1 did not show any or appreciable activities to determine reliable kinetic parameters (Table 1). Only mutant #13 showed significantly elevated $k_{\text{cat}}$ values for the 3’-hydroxylation reaction, which had the highest $k_{\text{cat}}$ values of 6.7 min$^{-1}$. Mutant #11 showed values of only 0.12 min$^{-1}$. The overall range of $K_m$ values was from 2.7 to 66 $\mu$M. The mutants displayed 56-fold and 24-fold variations in $k_{\text{cat}}$ and $K_m$ values for the 3’-hydroxylation reactions, respectively (Table 2). The catalytic efficiencies ($k_{\text{cat}}/K_m$) varied ~190-fold. In the case of human CYP1A2, the usual kinetic parameters could not be determined.

When the total turnover numbers (TTNs; mol product/mol catalyst) of the CYP102A1 mutants were determined, the overall range was 7 to 97 (Supplementary Data Fig. S4). Mutant #13 showed the highest activity, which was about 10-fold higher than that of human CYP1A2 with a 1-h incubation. Meanwhile, the wild-type
CYP102A1 enzyme did not show apparent catalytic activity towards resveratrol (<1.0 nmol product/nmol P450). After the reaction mixtures were incubated for 1 h and 2 h, the amount of the metabolite, piceatannol, was measured by HPLC. When the incubation was performed for 2 h, the overall activity increased slightly compared to that of the 1-h incubation.

This result seems due to the instability of the enzymes, inhibition of the P450 enzymes by the metabolite, or instability of the metabolite. In the case of human CYP1A2, no product remained after a 2-h incubation, although some amount of the product was shown after 1 h.

**Stability of CYP102A1 mutants measured by CO-difference spectra.** It is known that the metabolite of resveratrol is a potent inhibitor against human CYPs, 1A1, 1A2, and 1B1 (Chun et al., 1999, 2001). Resveratrol acts as a substrate and inhibitor to human CYP1A2 at the same time (Fairman et al., 2007; Piver et al., 2004). Interestingly, piceatannol, the major metabolite of resveratrol, is also known as a potent inhibitor to human CYP1A2 (Mikstacka et al., 2006).

During the reaction of resveratrol oxidation by P450 in the presence of NADPH, the stability of P450 enzymes was examined by measuring the CO-difference spectra of
reaction aliquots at the indicated time. The stabilities of the mutants were quite different from one another, depending on the type of mutant. Mutants #10, #11, #14, and #15 showed the highest stability among the tested mutants. This result might be related to that of the TTN experiments. The TTNs for the 2-h incubation increased 15~80% compared to those of the 1-h incubation (Supplementary Data Fig. S4).

Mutant #13 with the highest activity among the tested mutants showed the lowest stability. After a 6-min incubation, only 20% of the intact P450 remained under the tested condition (Supplementary Data Fig. S5). This instability might be related to the high rate of production of piceatannol, which is known as a potent inhibitor of human CYP1A2 (Mikstacka et al., 2006). Although mutant #10 showed moderately high activity, its stability was quite different from that of mutant #13. After a 30-min incubation, over 70% of mutant #10 was still intact, although over 90% of mutant #13 were destroyed. Only one amino acid is different between the two mutants (mutant #10, R47L/F87V/L188Q; mutant #13, R47L/L86I/F87V/L188Q). At present, the structural differences between the two mutants are not known.

Several mutant forms of CYP102A1 were found to metabolize various drugs of human P450 enzymes (Yun et al., 2007 and references therein). Structural
rationalization to the mutations of CYP102A1 should be useful to make desired activities of the enzyme. In addition to the directed evolution, the use of rational design for predicting protein structure and calculating the precise molecular interactions between the substrate and active site of the enzyme can be successfully applied to the engineering of CYP102A1 to specific a medium chain p-nitrophenoxycarboxylic acid (Li et al., 2001). Recently computational methods combined with experimental techniques including molecular dynamics simulations, resonance Raman and UV-VIS spectroscopy, as well as coupling efficiency and substrate-binding experiments, could be successfully applied to the engineering of CYP102A1 to metabolize human CYP2D6 substrates, 3,4-methylenedioxymethylamphetamine and dextromethorphan (Stjernschantz et al., 2008).

The production of piceatannol by chemical synthesis has been reported (Kim et al., 2008b). However, an alternative to chemical synthesis of piceatannol is to use CYP102A1 enzymes to generate the metabolites of resveratrol. From the viewpoint of “White Biotechnology” as part of a sustainable “Green Biotechnology” (Stottmeister et al., 2005), the production of piceatannol via an enzyme reaction should be more effective and cleaner.
In summary, this work with a set of CYP102A1 mutants and *trans*-resveratrol, a human P450 substrate, revealed that bacterial CYP102A1 enzymes catalyze the same reaction as human CYP1A2. The oxidation of resveratrol, a human CYP1A2 substrate, is catalyzed by wild-type and some mutants of CYP102A1. One major hydroxylated product, piceatannol, was produced as a result of a hydroxylation reaction. Other hydroxylated products were not produced. Piceatannol formation was confirmed by HPLC and GC-MS by comparing the metabolite to the authentic piceatannol compound. Thus, the CYP102A1 mutants efficiently produce piceatannol, an authentic human metabolite of resveratrol.
References


Li QS, Schwaneberg U, Fischer M, Schmitt J, Pleiss J, Lutz-Wahl S, Schmid RD.


FOOTNOTES

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Legends for Figures

**Fig. 1.** Molecular structures of *trans*-resveratrol and piceatannol. The conversion of *trans*-resveratrol to piceatannol is catalyzed by P450 enzyme in the presence of NADPH.

**Fig. 2.** HPLC chromatograms of resveratrol metabolites produced by CYP102A1 mutants (C: Mutants #10, #13, #14, and #15) and human CYP1A2 (B). Peaks were identified by comparing the retention times with those of standards, authentic piceatannol and resveratrol (A). The peaks of the substrate and two major products (piceatannol and other hydroxylated product) are indicated. UV absorbance was monitored at 320 nm.
**TABLE 1**

*Rates of piceatannol formation by various CYP102A1 mutants*

Assays were performed using 100 µM trans-resveratrol as described in the “Materials and Methods.” Values are the mean ± SD of triplicate determinations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mutated amino acid residues</th>
<th>Piceatannol nmol product/min/nmol P450</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>Mutant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>F87A</td>
<td>0.021 ± 0.001</td>
</tr>
<tr>
<td>#2</td>
<td>A264G</td>
<td>N.D.</td>
</tr>
<tr>
<td>#3</td>
<td>F87A/A264G</td>
<td>N.D.</td>
</tr>
<tr>
<td>#4</td>
<td>R47L/Y51F</td>
<td>0.027 ± 0.001</td>
</tr>
<tr>
<td>#5</td>
<td>R47L/Y51F/A264G</td>
<td>N.D.</td>
</tr>
<tr>
<td>#6</td>
<td>R47L/Y51F/F87A</td>
<td>N.D.</td>
</tr>
<tr>
<td>#7</td>
<td>R47L/Y51F/F87A/A264G</td>
<td>N.D.</td>
</tr>
<tr>
<td>#8</td>
<td>A74G/F87V/L188Q</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>#9</td>
<td>R47L/L86I/L188Q</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>#10</td>
<td>R47L/F87V/L188Q</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>#11</td>
<td>R47L/F87V/L188Q/E267V</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td>#12</td>
<td>R47L/L86I/L188Q/E267V</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>#13</td>
<td>R47L/L86I/F87V/L188Q</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>#14</td>
<td>R47L/F87V/E143G/L188Q/E267V</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>#15</td>
<td>R47L/E64G/F87V/E143G/L188Q/E267V</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>#16</td>
<td>R47L/F81I/F87V/E143G/L188Q/E267V</td>
<td>0.97 ± 0.1</td>
</tr>
<tr>
<td>#17</td>
<td>R47L/E64G/F81I/F87V/E143G/L188Q/E267V</td>
<td>1.8 ± 0.1</td>
</tr>
</tbody>
</table>

N.D., not detectable (the rate of product formation was less than 0.001 nmol product/min/nmol P450)
In the case of human CYP1A2, the usual kinetic parameters could not be obtained due to disappearance of the metabolite.

<table>
<thead>
<tr>
<th>Mutant #</th>
<th>(k_{\text{cat}}) (min(^{-1}))</th>
<th>(K_m) (µM)</th>
<th>(k_{\text{cat}}/K_m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#9</td>
<td>0.20 ± 0.02</td>
<td>66 ± 14</td>
<td>0.0030 ± 0.0007</td>
</tr>
<tr>
<td>#10</td>
<td>1.1 ± 0.1</td>
<td>30 ± 13</td>
<td>0.037 ± 0.016</td>
</tr>
<tr>
<td>#11</td>
<td>0.12 ± 0.01</td>
<td>2.7 ± 0.7</td>
<td>0.044 ± 0.012</td>
</tr>
<tr>
<td>#12</td>
<td>0.13 ± 0.01</td>
<td>54 ± 11</td>
<td>0.0024 ± 0.0005</td>
</tr>
<tr>
<td>#13</td>
<td>6.7 ± 0.3</td>
<td>15 ± 3</td>
<td>0.45 ± 0.09</td>
</tr>
<tr>
<td>#14</td>
<td>0.58 ± 0.04</td>
<td>13 ± 3</td>
<td>0.046 ± 0.011</td>
</tr>
<tr>
<td>#15</td>
<td>0.54 ± 0.02</td>
<td>16 ± 2</td>
<td>0.038 ± 0.005</td>
</tr>
</tbody>
</table>
FIGURE 1

trans-Resveratrol

Piceatannol