Inhibition of 7-Alkoxyresorufin O-Dealkylation Activities of Recombinant Human CYP1A1 and CYP1B1 by Resveratrol

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ABSTRACT : Resveratrol is known to have potent cancer chemopreventive activity against tumorigenesis caused by 7,12-dimethylbenz[α]anthracene (DMBA) which is known to be oxidized to reactive products by cytochrome P450 1B1 (CYP1B1). The effects of resveratrol on the activity of recombinant human P450 1 family enzymes, expressed in *Escherichia coli* membranes with human NADPH-P450 reductase, were determined by measuring alkoxyresorufin O-dealkylation activity, e.g., ethoxyresorufin O-deethylation (EROD) CYP1A1, methoxyresorufin O-demethylation (MROD), CYP1A2, benzyloxyresorufin-O-debenzylation (BROD), CYP1B1. Resveratrol inhibited CYP1B1 and CYP1A1 activities in a dose-dependent manner with IC_{50} values of 59 and 10 μM for EROD activity and 1.8 and 30 μM for BROD activity, respectively. Resveratrol had only weak inhibitory effect on CYP1A2 activity (IC_{50} values of 0.44 mM for EROD and >2 mM for MROD). Furthermore, resveratrol did not affect NADPH-P450 reductase activity significantly. Resveratrol inhibited the CYP1B1-dependent EROD activity with a K_i of 28 μM in a non-competitive type manner. These results suggest that resveratrol-derived inhibition of CYP1B1 and CYP1A1 activities may contribute to the suppression of DMBA inducible tumorigenesis observed in extrahepatic tissues.

Keywords: Resveratrol, recombinant human family 1 cytochrome P450s, 7-alkoxyresorufin O-dealkylations

Introduction

Resveratrol (Fig. 1) is a phytoalexin and is consumed widely as a component of vegetables and fruits such as mulberries and grapes (Soleas *et al.*, 1997). In this connection, epidemiological studies have shown that a moderate consumption cf wine (especially red wine) has a protective effect against the risk of coronary heart disease (so-called French paradox) (Soleas *et al.*, 1997; Renaud and DeLorgeril, 1992). This protective effect of red wine against the coronary heart disease has been attributed to the antioxidant and anticoagulation properties of resveratrol (Pace-Asciak *et al.*, 1995; Fauconneau *et al.*, 1997). Furthermore, resveratrol has been shown to inhibit the cellular events associated with tumor development caused by aryl hydrocarbon DMBA (Jang *et al.*, 1997). Carcinogenic aryl hydrocarbons are known to undergo metabolic activation to genotoxic metabolites by the family 1 and other P450 enzymes. P450 enzymes are a superfamily of hemoproteins that catalyze the biotransformation of not only a wide array of drugs and endogenous substances, but also the bioactivation of many procarcinogens and toxins

(Guengerich, 1988). In human, the family 1 P450s include CYP1A1, CYP1A2, and CYP1B1, which are involved in the oxidation of a large number of aryl hydrocarbon procarcinogens to their reactive electrophilic intermediates which can interact with cellular nucleophiles and trigger chemical carcinogenesis (Guengericll and Shimada 1998; Shimada *et al.*, 1998). The ability of human family 1 P450s to metabolically activate PAH carcinogens indicated that these enzymes might be pivotal in chemical carcinogenesis (Guengerich, 1988; Guengericll and Shimada 1998; Shimada *et al.*, 1998). CYP1B1 null mice were resistant to DMBA-induced lymphomas. Embryonic fibroblast cells that express CYP1B1 have been shown to metabolize DMBA effectively, and DMBA was shown to produce toxic effect on these
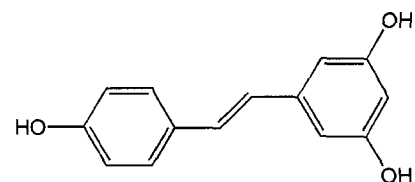
Fig. 1. Chemical structure of resveratrol (*trans*-3,4,5-trihydroxystilbene).

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cells. CYP1B1-null cells showed no significant DMBA metabolism and were resistant to DMBA mediated toxicity (Buters *et al.*, 1999).

Family 1 P450 enzymes are expressed in various human tissues. CYP1A2 is expressed primarily in liver, and CYP1A1 and CYP1B1 are expressed mainly in extrahepatic tissues. Among these human family 1 P450 enzymes, CYP1A1 and CYP1B1 are considered to be the most important in PAH-derived tumor initiation at extrahepatic tissues. In contrast, CYP1A1 appears not to be expressed constitutively in any human tissues but is inducible by dioxins, and PAHs in almost all tissues. CYP1B1 is expressed constitutively in steroidogenic tissues (e.g., adrenal, ovary and testes) and is inducible by adrenocorticotropin, cAMP, peptide hormones, and aryl hydrocarbon receptor ligands (Sutter *et al.*, 1994). CYP1B1 is also expressed in steroid-responsive tissue of mesodermal origin, e.g., uterus, breast and prostate. Thus, potent inhibitors of CYP1B1 may be good candidates as chemopreventive agents for arylhydrocarbon-inducible cancers in extrahepatic tissues such as breast, ovary, and lung (Shimada *et al.*, 1998; Buters *et al.*, 1999).

In this study, the inhibitory effects of resveratrol on the activities of recombinant human family 1 P450s (CYP1B1, CYP1A1, and CYP1A2) were investigated using bacterial membranes with a bicistronic expression of recombinant hNPR. We demonstrate that resveratrol preferentially inhibits human CYP1B1 and CYP1A1 activities.

Materials and Methods

Chemicals

7-Alkoxyresorufins (ethoxyresorufin, benzyloxyresorufin, and methoxyresorufin), resorufin, DMSO, δ-aminolevulinic acid, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO, USA). IPTG was obtained from Calbiochem (La Jolla, CA, USA). Resveratrol was kindly provided by Dr. Y.J. Surh (Seoul National University, Korea). Bactotryptone, bactopeptone, yeast extract and bactoagar were obtained from Difco-Becton Dickinson (Sparks, MD, USA). Other chemicals were of highest grade available.

Expression of recombinant human P450s.

Bicistronic plasmids containing human CYP1B1, CYP1A1 or CYP1A2 with hNPR cDNAs were expressed as described previously (Parikh *et al.*, 1997; Shimada *et al.*, 1998). Briefly, these bicistronic plasmids were introduced into *Escherichia coli* DH5α cells by electrophoration. A single ampicillin-resistant colony of transformed cells was picked up and grown overnight in LB medium containing 100 μg ampicillin/mL until saturation. Subsequently, a 10 mL aliquot was inoculated

into each liter of modified TB media (Shimada *et al.*, 1998) and grown further for 36 h at 28°C with shaking (220 rpm). Membrane fractions containing recombinant human family 1 P450s and hNPR were prepared from the cultured bacteria as described previously (Shimada *et al.*, 1998) and suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% (v/v) glycerol.

Enzyme assays

7-Alkoxyresorufin O-dealkylation activities of the isolated bacterial membranes and human liver microsomes (donated by Dr. Y.N. Cha of Inha University, Inchon, Korea) were determined by using ethoxyresorufin (EROD), methoxyresorufin (MROD) and benzyloxyresorufin (BROD) as model substrates. The enzyme reaction mixture (0.5 mL) contained 100 mM potassium phosphate buffer (pH 7.4), *E. coli* membranes (25-50 pmol P450) or human liver microsomes (0.2 mg protein), and 5 μM 7-alkoxyresorufin. The reaction mixture was preincubated at 37°C for 3 min and the oxidation reaction was initiated by adding 0.2 mM NADPH. The enzyme reaction was stopped by addition of 2 mL of ice-cold methanol. The production of resorufin from various 7-alkoxyresorufins was determined fluorometrically by using a Perkin-Elmer LS3 spectrofluorometer set at 535 nm (excitation) and 585 nm (emission) (Chang and Waxman, 1998). The content of P450 in the isolated *E. coli* membranes was determined by the spectral methods of Omura and Sato (Omura and Sato, 1964) using a Cary 300 Bio UV-visible spectrophotometer (Beckman, Australia) at ambient temperature and calculated by using an extinction coefficient of $\Delta\varepsilon_{450-490} = 91$ mM^{-1}cm^{-1}. Inhibitory effect of resveratrol on NADPH-driven cytochrome c reduction (NPR) rate was measured according to the method described by Yasukochi and Masters (Yasukochi and Masters, 1976) using an extinction coefficient of $\Delta\varepsilon_{550} = 21$ mM^{-1}cm^{-1}.

Data analysis

The IC$_{50}$ values of resveratrol on O-dealkylation activities were calculated using a non-linear regression analysis method (SigmaPlot, SPSS Inc, USA). The inhibitory mode of resveratrol on CYP1B1 catalyzed EROD activity was determined by employing Lineweaver-Burk plots. All results shown were obtained from at least 3 separate experiments.

Results

Comparision of O-dealkylation activities obtained with various recombinant human family 1 P450 enzymes using several 7-alkoxyresorufins

Specific enzyme activities of *E. coli* membranes containing

family 1 human P450s (CYP1A1, CYP1A2, or CYP1B1) and hNPR catalyzing EROD were compared (Table 1). The highest EROD activity per nmol P450 was obtained with CYP1A1, moderate activity by CYP1B1, and lowest activity with CYP1A2, as expected (Shimada *et al.*, 1998; Langouet *et al.*, 2000). When the substrate specificity of family 1 P450 dependent O-dealkylation was compared using several 7-alkoxyresorufins (ethoxyresorufin, methxoyresorufin, benzoxyresorufin), CYP1A1 had the highest specific activity for EROD, CYP1A2 had the highest activity for MROD, and CYP1B1 had the highest activity for BROD. CYP1B1 and CYP1A2 had extremely low MROD and BROD activities, respectively (Table 2).

Effects of resveratrol on family 1 P450s-dependent O-dealkylation activities

Among the O-dealkylation activities catalyzed by three human family 1 P450s, the EROD activity catalyzed by CYP1B1 was most strongly inhibited by resveratrol (CYP1B1 IC_{50} = 8.7 μM, CYP1A1 IC_{50} = 50 μM, CYP1A2 IC_{50} = 442 μM) (Fig. 2, Table 3). When we used benzyloxyresorufin or methoxyresorufin as substrates, the rank order of resveratrol IC_{50} values was inverted from that observed for EROD. The IC_{50} value of resveratrol for CYP1B1-catalyzed BROD was 42 μM and that for the CYP1A1 catalyzed BROD was 2.0 μM (Fig. 4). Resveratrol inhibited CYP1A1-catalyzed MROD activity with an IC_{50} value of 6.0 μM and

CYP1A2-catalyzed MROD with $IC_{50} > 2000$ μM (Fig. 3, Table 3). In comparing the IC_{50} values of resveratrol on O-dealkylation activities of recombinant human family 1 P450s, resveratrol appears to inhibit the activities of the CYP1B1 and CYP1A1 equally but with 10- to 100-fold less potency for CYP1A2 catalyzed O-dealkylation activities.
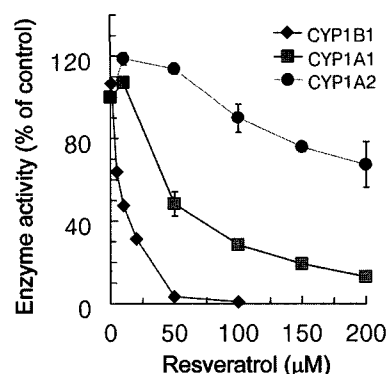
Fig. 2. Inhibitory effects of resveratrol on EROD activity catalyzed by human family 1 P450s expressed in *E. coli* membranes (for control activity see Table 2). Each point represents the mean ± S.D. n = 3.
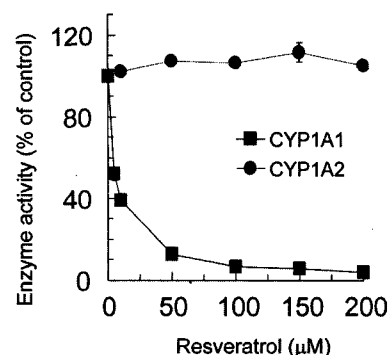
Fig. 3. Inhibitory effects of resveratrol on MROD activity catalyzed by CYP1A1 and CYP1A2 expressed in *E. coli* membranes (for control activity see Table 2). Each point represents the mean ± S.D., n = 3.

Table 1. Kinetic analysis of 7-ethoxyresorufin O-deethylation (EROD) catalyzed by *E. coli* membranes expressing human family 1 P450 enzymes and NADPH cytochrome P450 reductase

	K_m (μM)	V_{max} (nmol/min/nmol P450)
CYP1A1	4.8 ± 0.5	28.4 ± 2.1
CYP1A2	3.8 ± 0.2	2.9 ± 0.7
CYP1B1	4.4 ± 0.8	9.5 ± 1.1

Values indicate means ± S.D., obtained from triplicate independent determinations with 7-ethoxyresorufin concentrations ranging between 0.5~10 μM and using a non-linear kinetic analysis.

Table 2. Comparison of O-dealkylation activities of *E. coli* membranes expressing human family 1 P450 enzymes and hNPR using various 7-alkoxyresorufins

|	EROD (nmol/min/nmol P450)	MROD (nmol/min/nmol P450)	BROD (nmol/min/nmol P450)
CYP1A1	18.1 ± 0.58	2.38 ± 0.09	1.44 ± 0.09
CYP1A2	2.16 ± 0.10	9.90 ± 0.20	0.04 ± 0.01
CYP1B1	6.67 ± 0.15	0.10 ± 0.01	4.86 ± 0.11

Enzyme activities were determined as described in Materials and Methods section.
The values indicate means ± S.D., n = 3.

Table 3. Comparison of reveratrol IC_{50} values for inhibiting O-dealkylation activities of *E. coli* membranes expressing human family 1 P450 enzymes and hNPR

	IC_{50} of Resveratrol (μM)[*]		
	EROD	MROD	BROD
CYP1A1	59	5.9	1.8
CYP1A2	442	>> 2000	N.D.
CYP1B1	9.7	N.D.	29.6

[*]Each value represents the average of three independent experiments.
N.D.: not determinable
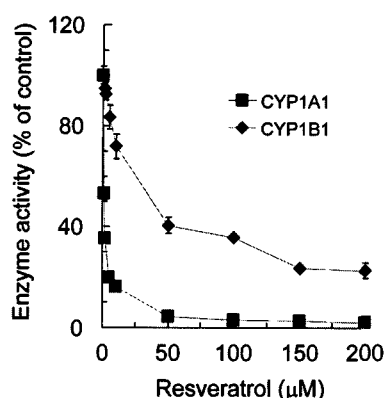
Fig. 4. Inhibitory effects of resveratrol on BROD activity catalyzed by CYP1B1 and CYP1A1 expressed in *E. coli* membranes (for control activity see Table 2). Each point represents the mean ± S.D., n = 3.
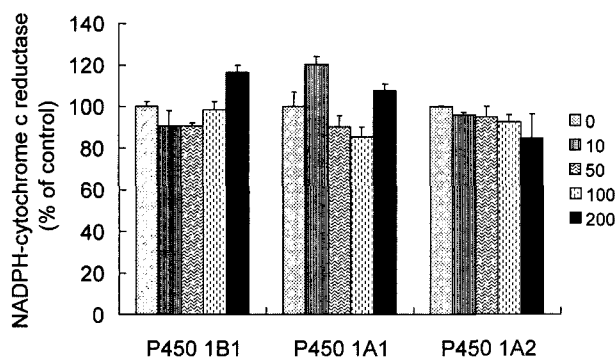
Fig. 5. Effects of resveratrol on NADPH-cytochrome c reductase activity catalyzed by *E. coli* membranes co-expressing human P450 and hNPR. Each value represents the mean ± S.D., n = 3.
Zero or 10, 50, 100, or 200 µM resveratrol was added to the reaction mixture. The control values were 71 ± 1.6, 51 ± 1.1 and 68 ± 1.5 pmol/mg protein for CYP1A1, CYP1A2 and CYP1B1, respectively.

Effects of resveratrol on hNPR activity

Human NADPH-cytochrome c reductase activity (in bacterial membranes expressed together with each of the three human family 1 P450s) was not significantly inhibited by 200 µM resveratrol (Fig. 5).

### Pattern of inhibition of human CYP1B1 activity by resveratrol

Resveratrol demonstrated a non-competitive inhibition of CYP1B1 catalyzed EROD activity, showing an increased apparent K_m value with a decreased the V_{max} and with an apparent K_i of 28 µM.

Inhibitory effects of resveratrol on human liver microsomal O-dealkylation activities

Resveratrol did not have a strong inhibitory effect on
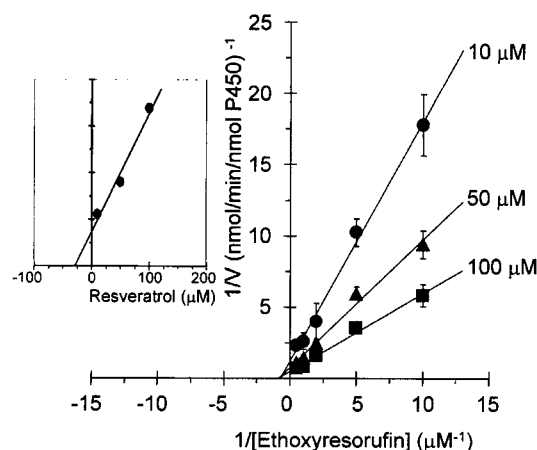
Fig. 6. Inhibition of EROD activity catalyzed by CYP1B1 expressed in *E. coli* membrane by resveratrol at concentrations of 10, 50, 100µM. Experiments were performed as described in Materials and Methods. The data are plotted on a Lineweaver-Burk double-reciprocal plot. Inset, replot of the slope obtained by linear regression of the data from the Lineweaver-Burk plot, with derivation of K_i. Each point represents the mean ± S.D., n = 6
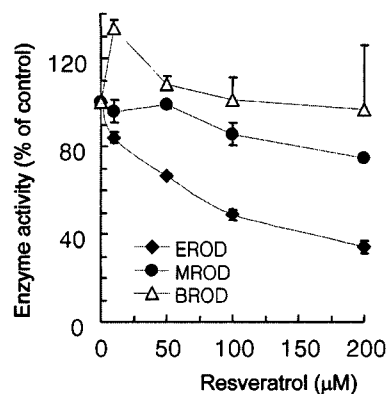
Fig. 7. Inhibitory effect of resveratrol on human liver microsomal 7-alkoxyresorufin O-dealkylation activity. Results are expressed as percentages of control activity obtained in the absence of resveratrol. Each point represents the mean of three independent experiments. The control values were 113 ± 6, 164 ± 10, and 8 ± 1 pmol product/min/mg protein for EROD, MROD and BROD, respectively.

MROD (IC_{50} value 1.2 mM) and BROD (IC_{50} value 1.3 mM) activities of human liver microsomes. However, human liver microsomal EROD activity was moderately inhibited by resveratrol (IC_{50} value = 97 µM) (Fig. 7).

Discussion

Resveratrol is one of phytoalexin compounds that are produced by plants in response to environmental stresses caused by exposure to ultraviolet light and ozone or

pathogenic attack by fungal infection (Soleas *et al.*, 1997). Since Jang *et al.* (Jang *et al.*, 1997) reported that resveratrol inhibits cellular events associated with tumor initiation, promotion and progression, considerable attention has been focused on the resveratrol as a naturally occurring cancer chemopreventive substance.

Resulting from much of the early works on the oxidative metabolism of carcinogenic polycyclic aromatic hydrocarbons and related chemicals, it is now understood that the three human P450s (CYP1A1, CYP1A2 and CYP1B1) belonging to family 1 are involved in chemical carcinogenesis (Buters *et al.*, 1999; Sutter *et al.*, 1994). In humans, amino acid sequence of CYP1A1 and CYP1A2 share 80% identity between them and are ~40% identical with CYP1B1. Recently, resveratrol has been shown to decrease the numbers of tumors induced by DMBA in the two stage mouse skin cancer model (Jang *et al.*, 1997) and to inhibit the mutagenic responses in *Salmonella typhimurium* TM677 strain induced by DMBA in a dose-dependent manner (Shamon *et al.*, 1994). Furthermore, susceptibility to DMBA-induced lymphoma formation has been reported to depend on the presence of CYP1B1, a member of family I P450s (Sutter *et al.*, 1994). Thus, we determined the effect of resveratrol in modulating the family 1 P450s by employing bacterial membranes co-expressing human P450s and hNPR (Clolino and yeh 1999). Bacterial membranes obtained from this expression system has equimolar concentration of P450 and hNPR, increased co-localization of the enzyme two proteins and has been demonstrated to have full P450 activity (Chun *et al.*, 1999). By employing this expression system, Chun et al (Chun *et al*, 1999) reported that resveratrol selectively inhibits the CYP1A1 activity in a mixed type inhibition. Resveratrol has also been shown to inhibit both the expression of CYP1A1 by preventing activation of Ah receptor required for transcription of CYP1A1 mRNA, and the CYP1A1 activity induced by the arylhydrocarbon (Clolino and Yeh, 1999; Ciolino *et al.*, 1998; Casper *et al.*, 1999). In addition to CYP1A1, effect of resveratrol on CYP1B1 has also been the subject of considerable interest due to its ability to oxidized 17β-estradiol, polycyclic hydrocarbons and other chemical carcinogens (Buters *et al.*, 1999; Hecht *et al.*, 1999; Shamon *et al.*, 1994; Sharma *et al.*, 1994; Lu and Serrero, 1999).

Thus, as an initial step to compare the ability of reveratrol modulating the activities of human family 1 P450s, we determined the substrate specificity of individual family 1 P450s using various alkoxyresorufines. As the results shown in Table 1 indicate, although the affinities (K_m values) of 7-ethoxyresorufin toward family I P450s were similar, the EROD activities (V_{max} value) were widely different. Per nmol of P450, EROD activity was the highest for CYP1A1,

moderate for CYP1B1 and the lowest for CYP1A2. However, when different substrates like methoxylrosorufin (MROD) and benzyloxyresorufin (BROD) were employed, the maximal MROD activity was obtained by CYP1A2 and the maximal BROD activity by CYP1B1 (Table 2). In any case, resveratrol inhibited all three 7-O-dealkylase activities with different potencies (Table 3) without affecting the NADPH-cytochrome c reductase activity (Fig. 5). Thus, resveratrol had potent inhibitory effects on the CYP1B1 catalyzed EROD, and the CYP1A1 catalyzed MROD and BROD activities (Fig. 3, 4, Table 3). Although Chun *et al* (Chun *et al.*, 1999) reported that resveratrol is one of the most selective and strong inhibitors of human CYP1A1 activity, as the P450 is not expressed constitutively, resveratrol may provide protection against arylhydrocarbon inducible chemical carcinogenesis mediated only by the previously induced extrahepatic CYP1A1. Alternatively, as resveratrol inhibited the CYP1B1 catalyzed EROD activities in *E. coli* membranes (Table 3) and human liver microsomes (Fig. 7), resveratrol may provide protection against chemical carcinogens medicated by the constitutively expressed CYP1B1 in extrahepatic tissues.

Fresh grape skin contains about 50 to 100 µg of resveratrol per gram, and the concentration in red wine is in the range of 1~10 mg/liter (Siemann and Creasy, 1992). Although the concentration of resveratrol in human blood has not been measured previously, concentration of resveratrol in the liver and kidney tissues of rats fed a single oral dose of red wine (4 ml containing 26 µg of resveratrol) was maintained at 10^{-4} M for more than 2 hr. At 2 hr, the plasma concentration of resveratrol in the rat was 10^{-5} M and the resveratrol concentration in the cardiac tissue remained at 10^{-5} M for 4 h (Bertelli *et al.*, 1996) indicating a rapid absorption but slow metabolism of resveratrol. If the same relatively slow metabolism of resveratrol occurs in humans, the micromolar concentration necessary to inhibit CYP1B1 and 1A1 should be easily attained.

In conclusion, we report that resveratrol strongly inhibited human CYP1A1 and 1B1 activities but, not that of CYP1A2 activity. As resveratrol is considered to be a normal of constituent human diet and does not cause excessive toxicity, resveratrol may serve as a powerful candidate for prevention of extrahepatic cancers caused by polycyclic aromatic hydrocarbons.

Acknowledgements

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