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Involvement of cytochrome P450 1A2 in the biotransformation of *trans*-resveratrol in human liver microsomes

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Abstract

This study was aimed at identifying the isoform(s) of human liver cytochrome P450 (CYP) involved in the hepatic biotransformation of *trans*-resveratrol (*trans*-3,5,4′-trihydroxystilbene). *Trans*-resveratrol metabolism was found to yield two major metabolites, piceatannol (3,5,3′,4′-tetrahydroxystilbene) and another tetrahydroxystilbene named M1. *Trans*-resveratrol was hydroxylated to give piceatannol and M1 with apparent K_m of 21 and 31 μM, respectively. Metabolic rates were in the range 14–101 pmol min^{-1} mg^{-1} protein for piceatannol and 29–161 pmol min^{-1} mg^{-1} protein for M1 in the 13 human liver microsomes tested. Using microsomal preparations from different human liver samples, piceatannol and M1 formation significantly correlated with ethoxy-resorufin-*O*-deethylation ($r^2 = 0.84$ and 0.88, respectively), phenacetin-*O*-deethylation ($r^2 = 0.92$ and 0.94) and immuno-quantified CYP1A2 ($r^2 = 0.85$ and 0.90). Formation of these metabolites was markedly inhibited by α-naphthoflavone and furafylline, two inhibitors of CYP1A2. Antibodies raised against CYP1A2 also inhibited the biotransformation of *trans*-resveratrol. In addition, the metabolism of *trans*-resveratrol into these two metabolites was catalyzed by recombinant human CYP1A1, CYP1A2 and CYP1B1. Our results provide evidence that in human liver, CYP1A2 plays a major role in the metabolism of *trans*-resveratrol into piceatannol and tetrahydroxystilbene M1.

Keywords: *Trans*-resveratrol; Piceatannol; Cytochrome P450; CYP1A1; CYP1A2; CYP1B1

1. Introduction

Trans-resveratrol (*trans*-3,5,4′-trihydroxystilbene), a phytoalexin present in mulberries, peanuts and grapes, is regularly consumed in the human diet. It is synthesized by grapes in response to fungal infections and is found in red wine at a concentration ranging from 1 to 10 μM [1]. *Trans*-resveratrol has been reported to have both anticarcinogenic and cardioprotective activities, which could be attributed to its antioxidant and anticoagulant properties [2]. This compound is not only a modulator of hepatic apolipoprotein and lipid biosynthesis [3] but also an inhibitor of platelet aggregation and eicosanoid production in human platelets and neutrophils [4]. It is also implicated in the inhibition of events associated with tumour initiation, promotion and progression [5]. According to recent findings, *trans*-resveratrol inhibits some CYPs, namely aromatic hydrocarbon-induced CYP1A1 expression and activity [6,7] as well as CYP1A2 and CYP1B1, which have been described as being crucial in tumour progression [8–11]. CYP3A4, an isoform predominantly expressed in colon and liver, and CYP2E1, involved in procarcinogen activation, are also inhibited by *trans*-resveratrol [10–13].

The molecular similarity of the phytoestrogen *trans*-resveratrol with estradiol and diethylstilbestrol (Fig. 1) raises the question of its hydroxylation by CYPs. According to a recent report [14], the cancer-preventive compound, *trans*-resveratrol, was converted into piceatannol, a compound with known anticancer activity, by an enzyme found in human tumours (CYP1B1). In contrast, another study found no metabolites of *trans*-resveratrol after incubation with human liver microsomes [15].

The aim of the present study was, therefore, to investigate *trans*-resveratrol metabolism in human liver microsomes. Several lines of evidence strongly suggest that the

Abbreviations: CYP, cytochrome P450; EROD, ethoxyresorufin-*O*-deethylase

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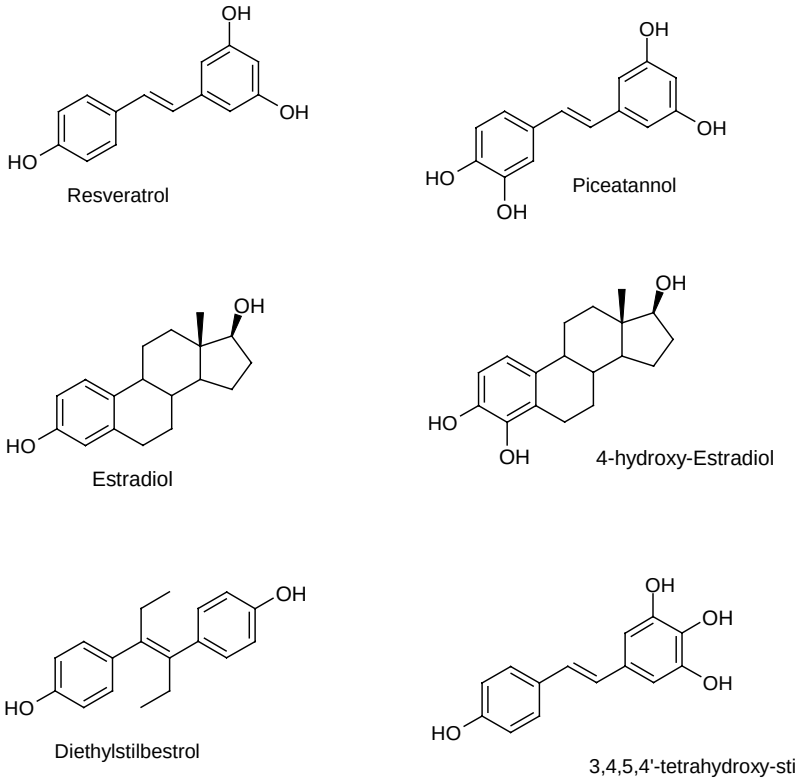

Fig. 1. Molecular structures of *trans*-resveratrol, piceatannol, 3,4,5,4′-tetrahydroxystilbene, estradiol, 4-hydroxy-estradiol and diethyl-stilbestrol.

human CYP1 enzyme family is the major one involved in the metabolism of *trans*-resveratrol into piceatannol.

2. Methods

2.1. Chemicals and reagents

Trans-resveratrol, *trans*-piceatannol, α-naphthoflavone, furafylline, estradiol and NADPH were purchased from Sigma Chemical Co. Ethoxyresorufin came from Interchim. Radiolabelled [^{14}C]-*trans*-resveratrol was synthesized as previously described [16].

2.2. Microsome samples and antibodies

Human liver microsomes were obtained either from Gentest Corporation (Woburn, MA, USA) (HG03, HG06, HG30, HG43, HG56, HG88, HG93, HG103, HG112, HK23, HK27) or from a microsome bank, which had been built up for many years within our laboratory [17], where they are stored at −80 °C until use (BR046 and BR052). Microsomes purchased from Gentest were available with immuno-quantitation of cytochromes P450 (1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5) and with complete catalytic assays of the major CYPs: phenacetin-*O*-deethylase (CYP1A2), coumarin 7-hydroxylase (CYP2A6), (*S*)-mephenytoin *N*-demethylase (CYP2B6), paclitaxel 6α-hydroxylase (CYP2C8), diclofenac 4′-hydroxylase (CYP2C9), (*S*)-mephenytoin 4′-hydroxylase (CYP2C19), bufuralol 1′-hydroxylase (CYP2D6), chlorzoxazone 6-hydroxylase (CYP2E1), testosterone 6β-hydroxylase (CYP3A4) and lauric acid 12-hydroxylase (CYP4A). All CYP assays were conducted at 0.8 mg mL^{-1} protein (except CYP3A4, which was at 0.5 mg mL^{-1}) with an NADPH-generating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate and 0.4 U mL^{-1} glucose 6-phosphate dehydrogenase), 3.3 mM $MgCl_2$, and incubated for 20 or 10 min (CYP2C8, CYP2C9, CYP3A4 and CYP4A). Potassium phosphate buffer (0.1 M, pH 7.4) was used for all CYP enzymes except CYP2B6 and CYP2C19 (0.05 M) and CYP2A6, CYP2C9 and CYP4A, which used 0.1 M Tris (pH 7.5). In addition, CYP1B1 contents were determined by Western blotting in all microsomes.

Monoclonal antibody for CYP1A2, polyclonal antibody for CYP1B1 (WB-1B1 kit) and insect cell microsomes (Supersomes) containing cDNA-expressed human CYP1A1, CYP1A2, CYP1B1, CYP3A4, CYP2C8, CYP2C9, CYP2E1 or CYP4A11 were purchased from Gentest Corporation. Microsomes with the cDNA-expressed CYP3A4 also contained cDNA-expressed NADPH cytochrome P450 reductase and cytochrome b5.

2.3. Determination of monooxygenase activities

2.3.1. Trans-resveratrol hydroxylation

Incubations were carried out in amber tubes. The standard incubation mixture contained 100 mM potassium

phosphate buffer (pH 7.6), 0.16 mg protein of microsomes from human liver (30–100 μg protein for recombinant CYPs), 1 mM ascorbate, and 200 μM *trans*-resveratrol dissolved in DMSO (at 0.2% v/v in final solvent concentration) in a total volume of 0.2 mL. After 2-min preincubation at 37 °C, the reaction was started by addition of 1 mM NADPH, and then stopped by addition of 105 μL acetic acid/methanol (5/100, v/v) after a 20-min incubation at 37 °C. After centrifugation of the reaction mixture, the supernatant was analyzed by HPLC. Samples (50 μL) were injected onto a Nucleosil C18 5 μm column (25 cm × 0.46 cm). The mobile phase A was water containing 0.5% acetic acid/acetonitrile (95/5, v/v) whereas 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 (L-6200A, Merck, Darmstadt, Germany). Eluates were detected by UV at 320 nm (spectrophotometer L-4250, Merck). Under these conditions, the retention times of piceatannol, tetrahydroxystilbene M1 and *trans*-resveratrol were 6.94, 7.47 and 10.12 min, respectively. Peaks were quantified by the integration microcomputer system from Kontron. Overall biotransformation was expressed as the percentage of substrate transformed into each UV-detected metabolite relative to the untransformed *trans*-resveratrol. Linearity of metabolite production was observed for various protein concentrations (0.13–0.6 mg) and incubation times (10–20 min). All assays were run in duplicate, and the differences were usually less than 10%.

To validate the measurement of piceatannol and M1 formed from *trans*-resveratrol, 11 microsomal samples were selected and 0.5 mg of protein were incubated with 200 μM radiolabelled *trans*-resveratrol (0.48 μCi μmol^{-1}) as described above. HPLC eluates were collected following UV detection, and their radioactivity was counted by liquid scintillation spectrometry.

Ethoxyresorufin-*O*-deethylation (EROD) was performed according to Burke et al. [18]. The reaction mixture was composed of 5 μM ethoxyresorufin, 0.15 mg of microsomal proteins and 100 mM Tris buffer (pH 7.6) in a final volume of 2 mL. The reaction was initiated by addition of 0.5 mM NADPH. The formation of resorufin was followed by fluorescence detection (excitation and emission wavelengths: 530 and 585 nm, respectively).

2.3.2. Western blot analysis of CYP1B1

Aliquots of human liver microsomal samples (50 μg) were separated by electrophoresis on 9% SDS polyacrylamide gels according to the method of Laemli [19] and then transferred electrophoretically to nitrocellulose sheets according to Towbin et al. [20]. Revelation of CYP1B1 was performed with polyclonal anti-CYP1B1 as first antibody followed by a second antibody conjugated to peroxidase and detected by enhanced chemiluminescence. cDNA-expressed CYP1B1 enzyme was used as standard.

2.4. Identification of trans-resveratrol metabolites

2.4.1. HPLC analysis

Trans-resveratrol was incubated with human liver microsomes (BR046 and BR052) as described above. Piceatannol and tetrahydroxystilbene M1 were detected either by UV (320 nm) or fluorescence with excitation and emission set at 310 and 405 nm, respectively (Spectra system FL 2000, Thermo Finnigan). UV and fluorescence spectra of both compounds were also assessed in microsomal metabolism samples.

2.4.2. GC–MS analysis

The aqueous microsomal samples were extracted with diethyl ether (2 mL). After centrifugation, the organic phase was dried under nitrogen as well as the standard *trans*-resveratrol and piceatannol solutions (50 μg in DMSO). Then, trimethylsilyl (TMS) derivatives were prepared as follows: 100 μL of a solution of BSTFA/TMCS (99/1, v/v) (Sigma Chemical Co.) was added to the dry residue, and then the mixture was left for 60 min at 60 °C, dried again under nitrogen and redissolved in 50 μL of hexane.

GC–MS analysis was performed on an HP-6890 gas chromatograph (Agilent Technologies) with an HP-5MS 5% phenyl-methylsiloxane capillary column (30 m × 0.32 mm i.d. × 0.25 μm film thickness). The injector temperature was 250 °C. The derivatized samples were separated with a GC oven temperature program 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 chromatograph was combined with an HP-5973N mass spectrometer operating in electron ionisation mode (70 eV).

2.5. Kinetics of trans-resveratrol metabolism

Human liver microsomes (BR046 and BR052, 0.16 mg protein) were incubated with *trans*-resveratrol at various concentrations (0, 25, 50, 100, 125, 200, 250 and 500 μM), as described above. K_m and V_{max} were assessed by use of non-linear regression analysis after Michaelis–Menten representation (rate of metabolite formation (pmol min^{-1} mg^{-1} protein) as a function of *trans*-resveratrol concentrations (μM) (SPSS, Sigma Plot).

2.6. Inhibition of trans-resveratrol metabolism by chemical compounds

Trans-resveratrol metabolism was inhibited by α-naphthoflavone or furafylline as described in [21]. Inhibition experiments started with a 10-min preincubation at 37 °C of 0.16 mg microsomal proteins (HG56, BR046 and BR052), 10 μM α-naphthoflavone or 5 μM furafylline, 100 mM phosphate buffer, pH 7.6, and 1 mM NADPH in a small volume (30 μL). The mixture was then diluted seven-fold to perform the catalytic assay with 200 μM

trans-resveratrol, 1 mM ascorbate and 1 mM NADPH in a 0.2 mL final volume. After a 20-min incubation, the reaction was stopped by addition of 105 μL acetic acid/methanol. Control experiments were carried out under the same conditions, but with neither α-naphthoflavone nor furafylline.

2.7. Immuno-inhibition assays

2.7.1. Immuno-inhibition of trans-resveratrol metabolism

An amount of 0.16 mg of human liver microsomes (HG30, HG43, HG56) was added to 40 μg monoclonal antibody for CYP1A2 and incubated for 20 min with 0.1 M potassium phosphate buffer (pH 7.6). The reaction was initiated by addition of 200 μM *trans*-resveratrol, 1 mM ascorbate, 1 mM NADPH and 0.1 M potassium phosphate buffer (pH 7.6) to get a 0.2 mL final volume.

2.7.2. Immuno-inhibition of EROD activity

An amount of 0.16 mg of human liver microsomes was added to 40 μg monoclonal antibody for CYP1A2 and incubated for 20 min with 0.1 M potassium phosphate buffer (pH 7.6). The reaction was started by addition of 5 μM ethoxyresorufin, 0.5 mM NADPH and 100 mM Tris buffer (pH 7.6) in a final volume of 2 mL.

2.8. Statistical analysis

Coefficients of determination (r^2) were calculated using the Spearman correlation analysis. They were considered as statistically significant when $P < 0.05$.

3. Results

3.1. Trans-resveratrol metabolism by human liver microsomes

Two main metabolites of *trans*-resveratrol, piceatannol and a still unconfirmed tetrahydroxystilbene denoted as M1, were generated further to its incubation with human liver microsomal preparations: they were separated within 10 min under our HPLC conditions (Fig. 2). Their formation depended on the presence of NADPH (Fig. 2B and C), which is in favour of cytochrome P450 involvement. It is worth noting the particularly good responsiveness of metabolite M1 in fluorescence detection as compared to M2 (piceatannol) (Fig. 2C and D). Incubation of microsomal samples with [^{14}C] *trans*-resveratrol confirmed that metabolite M1 and piceatannol were both generated from *trans*-resveratrol, and a highly significant correlation was observed between their rate of formation ($r^2 = 0.96$, $P < 0.001$, $n = 11$). Radioactivity and UV measurements were

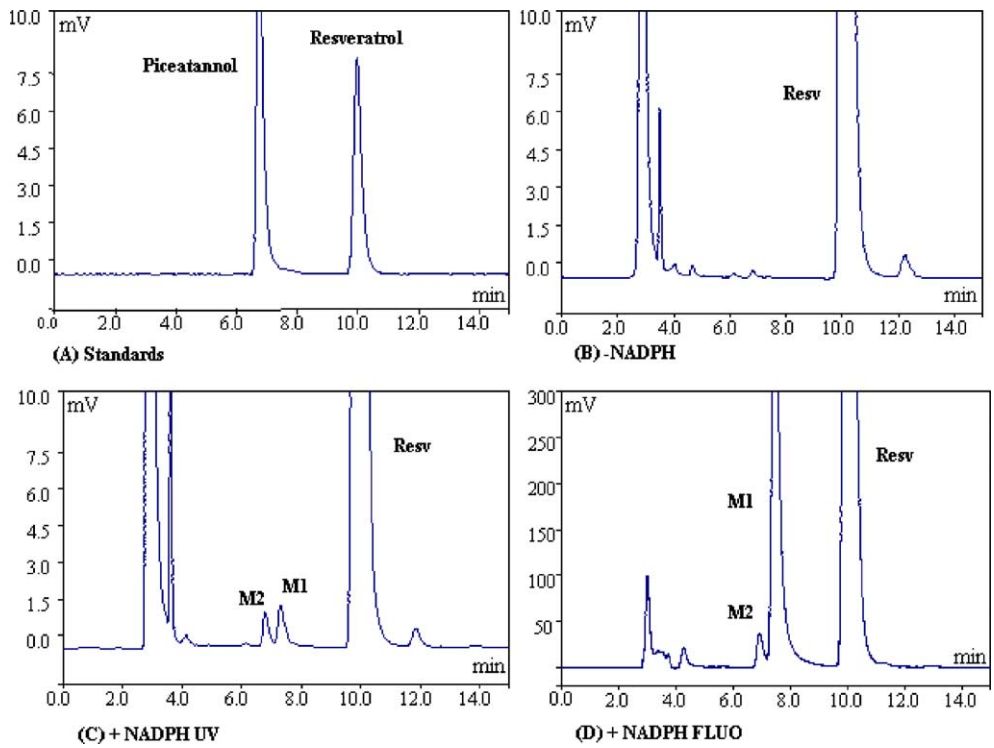

Fig. 2. HPLC chromatograms of *trans*-resveratrol metabolism. (A) Pure compounds; (B) chromatogram of incubation medium containing 200 μM *trans*-resveratrol, 0.16 mg of microsomal sample but no NADPH; (C) typical chromatogram of a sample incubated with 200 μM *trans*-resveratrol and 1 mM NADPH. Chromatograms (A–C) were carried out using UV detection and (D) using fluorescence. The following retention times were observed: piceatannol (M2) 6.94 min, *trans*-resveratrol 10.12 min and tetrahydroxystilbene (M1) 7.47 min.

also highly correlated for piceatannol or M1 ($r^2 = 0.88$ and 0.94, respectively; $P < 0.001$). Thus, UV quantification can safely be used for determination of *trans*-resveratrol hydroxylation in human liver microsomes.

3.2. Metabolites identification

Trans-piceatannol was identified by comparison with a standard, from its retention time, UV ($\lambda_{max} = 320$ nm) and fluorescence spectra ($\lambda_{excitation} = 340$ nm; $\lambda_{emission} = 420$ nm). The metabolite M1 differed from piceatannol by retention time, UV ($\lambda_{max} = 325$ nm) and fluorescence spectra ($\lambda_{excitation} = 350$ nm; $\lambda_{emission} = 390$ nm). In order to elucidate the structure of M1, GC–MS analysis was carried out after trimethylsilylation. The two trimethylsilyl derivatives (piceatannol and M1) displayed different retention times (Fig. 3B), but the same mass-to-charge ratio of 532 (Fig. 3C–E). However, the mass spectrum gave very little information on the structure of M1 because only weak fragmentation of the molecule was observed. The only helpful information from MS was the molecular weight of M1, which led us to assert that M1 was a tetrahydroxystilbene. We assumed that it could be *cis*-piceatannol because of the involvement of cytochromes P450 in the isomerization of some compounds like tamoxifen [22]. *Cis*-piceatannol can be produced further to UV exposure ($\lambda = 254$ nm) of *trans*-piceatannol for 10 min. Unfortunately, our hypothesis was not corroborated by HPLC and GC–MS analyses that gave different retention times for the unknown metabolite and *cis*-piceatannol (data not shown).

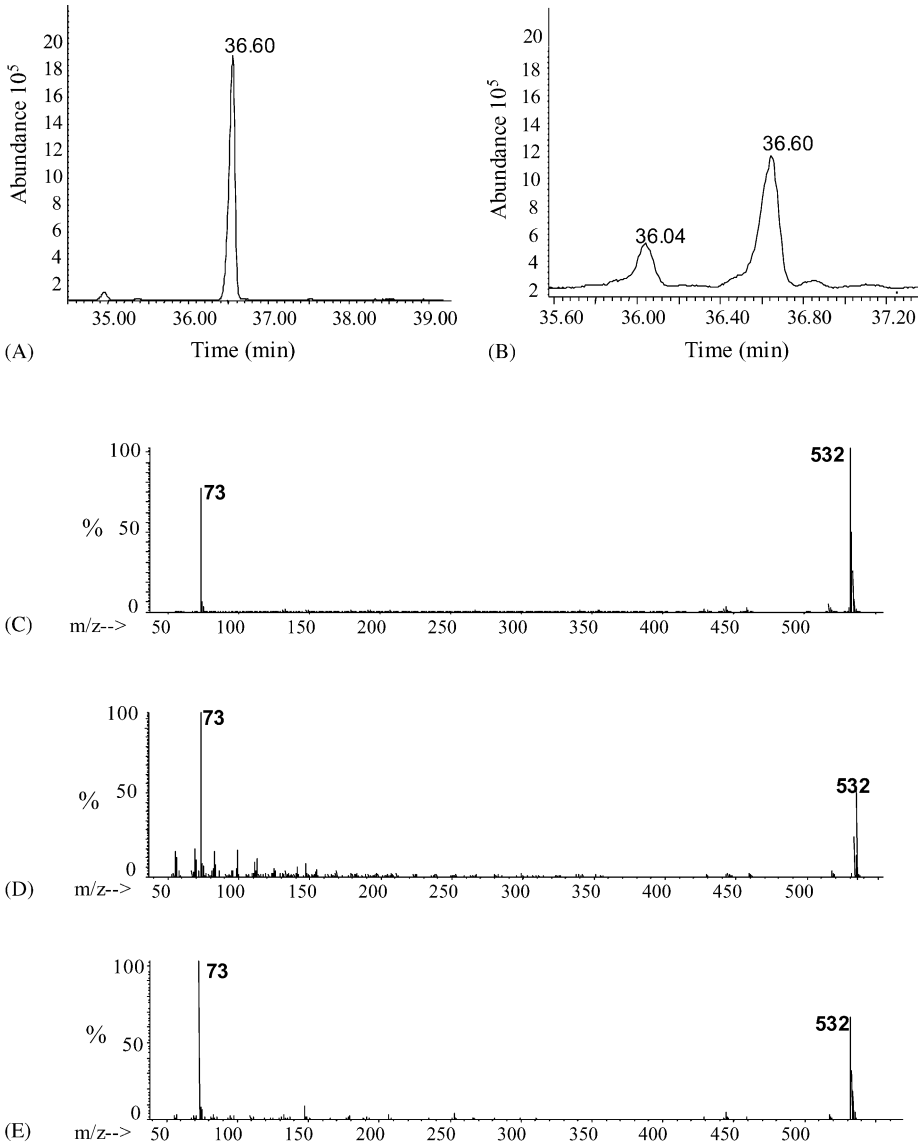

Fig. 3. GC–MS studies on the TMS-derivatised samples. (A) GC–MS elution profile of derivatised authentic piceatannol; and (B) GC–MS elution profile of the derivatised metabolism sample. The following retention times were observed for metabolites: piceatannol 36.60 min and tetrahydroxystilbene M1 36.04 min. (C) Mass spectrum of the derivatised piceatannol. (D) Mass spectrum of the metabolism sample peak that eluted at 36.60 min. (E) Mass spectrum of the metabolism sample peak that eluted at 36.04 min.

Table 1
Coefficient of determination (r^2) between catalytic activities and hydroxylated metabolites of *trans*-resveratrol measured by HPLC-UV in 11 human liver microsomes samples

Assays	Piceatannol	Tetrahydroxystilbene M1
Ethoxyresorufin-*O*-deethylase (CYP1A2)	0.84*	0.88*
Phenacetin-*O*-deethylase (CYP1A2)	0.92*	0.94*
Coumarin 7-hydroxylase (CYP2A6)	0.0002	0.0002
(*S*)-Mephenytoin *N*-demethylase (CYP2B6)	0.11	0.16
Paclitaxel 6α-hydroxylase (CYP2C8)	0.044	0.048
Diclofenac 4′-hydroxylase (CYP2C9)	0.0001	0
(*S*)-Mephenytoin 4′-hydroxylase (CYP2C19)	0.096	0.073
Bufuralol 1′-hydroxylase (CYP2D6)	0.026	0.026
Chlorzoxazone 6-hydroxylase (CYP2E1)	0.012	0.02
Testosterone 6β-hydroxylase (CYP3A4)	0.014	0.044
Lauric acid 12-hydroxylase (CYP4A)	0.21	0.15
Methyl *p*-tolyl sulfide oxidase (FMO)	0.22	0.17
Cytochrome *c* reductase (OR)	0.0004	0.01
Spectrophotometric (Cyt b5)	0.012	0.0036

* $P < 0.001$.

Therefore, we propose, according to Potter et al. [14], that M1 is the 3,4,5,4′-tetrahydroxystilbene (Fig. 1) because it is the most likely compound to be formed from a thermodynamic point of view, the para position of the aromatic ring being the most easily hydroxylated (data obtained from Hyperchem software, Hypercube Inc).

3.3. Kinetic parameters

The kinetics of *trans*-resveratrol metabolism to piceatannol and M1 were investigated in two different human microsomal samples (BR052 and BR046). K_m and V_{max} were calculated by non-linear regression analysis from the plots of formation of piceatannol and M1 against *trans*-resveratrol concentrations according to Michaelis and Menten. Apparent K_m was found to be 21 μM with a V_{max} of 86 pmol min^{-1} mg^{-1} microsomal protein for piceatannol and 31 μM with a V_{max} of 130 pmol min^{-1} mg^{-1} protein for M1. *Trans*-resveratrol hydroxylation ranged from 14 to 101 pmol min^{-1} mg^{-1} protein for piceatannol and 29–161 pmol min^{-1} mg^{-1} protein for M1 in the 13 human liver microsomes further to their incubation at a *trans*-trans-resveratrol concentration of 200 μM.

3.4. Correlation between trans-resveratrol metabolism and different monooxygenase activities in human liver microsomes

If two reactions are catalyzed by the same enzyme, then the rates should be correlated to each other when they are compared in a series of microsomal preparations containing varying levels of the enzyme, but the converse is not true. Table 1 shows that *trans*-resveratrol hydroxylation correlated significantly with two catalytic activities known as being supported by CYP1A2 enzyme, namely EROD activity (Fig. 4A and B) and phenacetin-*O*-deethylation. In no case, the r^2 value found for substrate marker of CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A or CYP4A exceeded 0.30, which means that no significant correlation was observed with *trans*-resveratrol metabolism for these CYPs. Because EROD activity and *trans*-resveratrol metabolism are also supported by CYP1B1 [14], it was verified by Western blotting using an antibody that detects CYP1B1 only that CYP1B1 was not present in our microsomal human liver samples (data not shown). This is in agreement with a recent work of Chang et al. [23] who found that

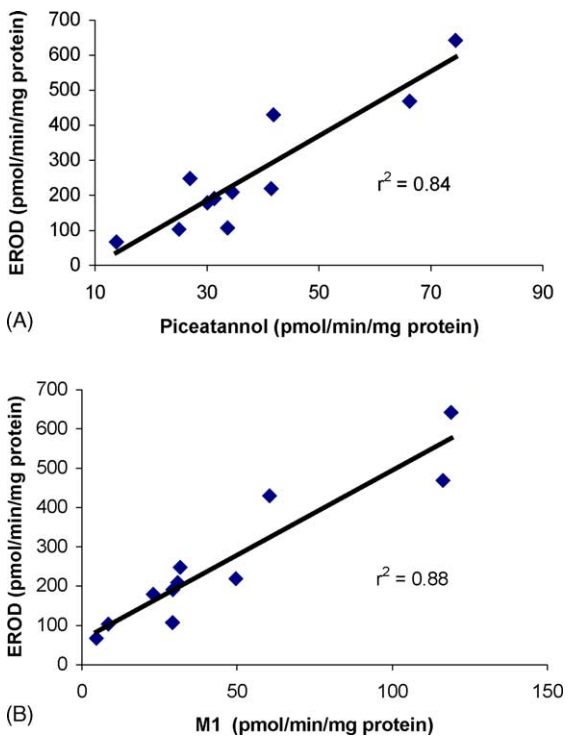

Fig. 4. Coefficient of determination (r^2) between (A) formation of piceatannol and EROD activity (CYP1A2), and (B) formation of M1 and EROD activity in human liver microsomes.

Table 2
Coefficient of determination (r^2) between immuno-quantified CYP and hydroxylated metabolites of *trans*-resveratrol measured by HPLC-UV in 11 human liver microsomes samples

Immuno-quantitated CYP	Piceatannol	Tetrahydroxystilbene M1
CYP1A2	0.85*	0.90*
CYP2A6	0.01	0.029
CYP2B6	0.0036	0.0004
CYP2C9	0.26	0.20
CYP2C19	0.0016	0.0004
CYP2D6	0.022	0.01
CYP2E1	0.0001	0.0064
CYP3A4	0.073	0.137
CYP3A5	0.09	0.04

* $P < 0.001$.

CYP1B1 protein was undetectable in human liver microsomes.

3.5. Correlation between trans-resveratrol metabolism and immuno-quantified CYP

Trans-resveratrol hydroxylation significantly correlated with the amounts of CYP1A2, immuno-detected by a monoclonal antibody anti-human CYP1A2: $r^2 = 0.85$ and $r^2 = 0.90$, and $P < 0.001$, for piceatannol and tetrahydroxystilbene M1, respectively. No significant correlation was found between *trans*-resveratrol metabolites and the amount of CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 or CYP3A5 (Table 2).

3.6. Inhibition of trans-resveratrol metabolism by different compounds

To further assess whether the different metabolic pathways of *trans*-resveratrol were catalyzed by CYP1A2, the effect of two chemical inhibitors of CYP1A2 (α-naphthoflavone and furafylline) was tested on *trans*-resveratrol metabolism in three different human samples. A strong inhibition of *trans*-resveratrol hydroxylation was observed when the microsomes were incubated with α-naphthoflavone and furafylline. Table 3 data show evidence that α-naphthoflavone and furafylline inhibited the formation of both piceatannol (89–96%) and tetrahydroxystilbene M1 (65–80%). One should note that the inhibition efficiency was alike in the three tested samples. In comparison, the inhibition of EROD activity by furafylline was 80% with the same microsomal samples (data not shown).

Table 4
Inhibition of formation of *trans*-resveratrol metabolites by monoclonal anti-CYP1A2 antibody

Inhibitor	Piceatannol pmol min^{-1} mg^{-1} (% inhibition)		Tetrahydroxystilbene M1 pmol min^{-1} mg^{-1} (% inhibition)	
	Control	Anti-CYP1A2	Control	Anti-CYP1A2
HG30	40.4	19.8 (51%)	44.9	23.3 (48%)
HG43	40.2	16.5 (59%)	54	14.0 (74%)
HG56	74.0	23.7 (68%)	119	33.3 (72%)

Each value represents the mean of duplicates.

3.7. Immuno-inhibition of trans-resveratrol metabolism

So as to confirm the involvement of CYP1A enzymes in *trans*-resveratrol metabolism, immuno-inhibition studies with anti-human CYP1A2 were carried out. We have shown that this antibody, at the dilution of 40 μg/0.16 mg protein, inhibits the formation of both piceatannol and metabolite M1 (Table 4). The extent of inhibition of *trans*-resveratrol biotransformation was similar in different samples (51–68% for piceatannol). In comparison, anti-human CYP1A2 exhibited at least 80% inhibitory effect on EROD activities (data not shown).

3.8. Metabolism of trans-resveratrol by recombinant human CYPs

To assess the formation of the two metabolites piceatannol M2 and tetrahydroxystilbene M1, *trans*-resveratrol was incubated with microsomal preparations of insect cells that had been genetically engineered for stable expression of human CYP1A1, CYP1A2, CYP1B1, CYP2C8, CYP2C9, CYP2E1, CYP3A4 and CYP4A11. Table 5 shows the results obtained, expressed as metabolic rate or turn-over. They suggest that at a substrate concentration of 200 μM, CYP1A2 is the major enzyme involved in piceatannol and M1 formation. The isoenzymes CYP1A1 and CYP1B1 metabolized *trans*-resveratrol into piceatannol and M1, but to a lesser extent. CYP2C8, CYP2C9, CYP3A4 and CYP4A11 did not metabolize *trans*-resver-

Table 3
Inhibition of formation of *trans*-resveratrol metabolites by α-naphthoflavone (10 μM), and furafylline (5 μM)

Inhibitor	Piceatannol pmol min^{-1} mg^{-1} (% inhibition)			Tetrahydroxystilbene M1 pmol min^{-1} mg^{-1} (% inhibition)		
	Control	α-Naphtho-flavone	Furafylline	Control	α-Naphtho-flavone	Furafylline
HG56	75	4.5 (94%)	8.2 (89%)	111	38.8 (65%)	28.9 (74%)
BR046	72	7.2 (90%)	6.5 (91%)	98	34.3 (65%)	30.4 (69%)
BR052	101	4.0 (96%)	11.1 (89%)	161	32.2 (80%)	56.3 (65%)

Each value represents the mean of duplicates.

Table 5
Trans-resveratrol metabolism by recombinant human CYP preparations

Recombinant CYP	Piceatannol		Tetrahydroxystilbene M1	
	Formation (pmol min^{-1} mg^{-1})	Turnover (pmol min^{-1} pmol CYP^{-1})	Formation (pmol min^{-1} mg^{-1})	Turnover (pmol min^{-1} pmol CYP^{-1})
1A1	25	0.33	33	0.25
1A2	148	0.68	208	0.96
1B1	11	0.07	65	0.40
3A4	N.D.	N.D.	N.D.	N.D.
2C8	N.D.	N.D.	N.D.	N.D.
2C9	N.D.	N.D.	N.D.	N.D.
2E1	4	0.03	4	0.03
4A11	N.D.	N.D.	N.D.	N.D.

N.D.: Not detectable. Each value represents the mean of duplicates.

atrol into piceatannol and M1 whereas CYP2E1 appeared to be a minor catalyst.

4. Discussion

Cytochromes P450 constitute a superfamily of hemoproteins that catalyze not only the biotransformation of a wide array of drugs and endogenous substances but also the bioactivation of many procarcinogens and toxins [24]. Consequently, specific CYP enzymes have been identified as potential targets for cancer chemoprevention [25]. One dietary compound under investigation as a chemoprevention agent is *trans*-resveratrol. This naturally occurring phytoalexin has attracted considerable attention because of its presence in dietary sources, such as grape, wine, peanuts and cranberries. The efficacy of orally administrated *trans*-resveratrol depends on its absorption, metabolism and tissue distribution [15]. However, only few authors have investigated *trans*-resveratrol bioavailability and metabolism [4,26–28]. None of these investigations have enabled a conclusion to be made about the metabolic profile of *trans*-resveratrol.

Indeed, two recent studies have focused on *trans*-resveratrol metabolism, but with contradictory results. According to Yu et al. [15], whatever the tested systems (human liver microsomes, rat and human hepatocytes, in vivo studies), no phase I metabolites of *trans*-resveratrol resultant from oxidation, reduction or hydrolysis were detected. It is noteworthy that the stability of piceatannol over time is poor because of its fast photooxidation in aqueous medium or at low concentrations [29]. This could explain the lack of metabolites found by these authors. However, Potter et al. [14] found two major metabolites of *trans*-resveratrol (M1, M2) and one minor metabolite (M3) following in vitro incubations of *trans*-resveratrol with microsomal preparations of human CYP1B1 from human lymphoblast or *Escherichia coli*-transfected cells. M2 was identified as piceatannol but M1 and M3 have not been conclusively identified due to the unavailability of authentic standards. M1 was suggested by these authors to be the 3,4,5,4′-tetrahydroxystilbene and M3 the 3,4,5,3′,4′-pentahydroxystilbene. However, no quantification of the rate of formation of these metabolites was reported and metabolic studies were performed only with microsomes from cells expressing human CYP1B1. These preliminary results led us to study more extensively the metabolism of *trans*-resveratrol by different CYPs in human liver microsomes or with recombinant CYPs.

Our study demonstrates the formation of two main metabolites in human liver microsomes, piceatannol and tetrahydroxystilbene M1. It also shows not only the P450-dependence of these two enzymatic reactions evidenced by their need of NADPH but also their inhibition by classical P450 inhibitors. Moreover, the use of [^{14}C]-*trans*-resveratrol confirmed that the two major metabolites were generated from *trans*-resveratrol and produced roughly in similar amounts. The characterisation of metabolites was performed by their chromatographic behaviors in HPLC, their UV and fluorescence spectra or GC–MS analysis. One of them, metabolite M2, was identified as piceatannol (3,4,3′,5′-tetrahydroxystilbene); on the other hand, the other one (M1) has not yet been conclusively identified due to the unavailability of an authentic standard. The proposed identity of M1 is the same as that proposed by Potter et al. [14] for the metabolite M1 generated from CYP1B1 *trans*-resveratrol metabolism in human lymphoblast microsomes. However, unlike the study of Potter et al. [14], we used UV spectrometry rather than fluorescence to detect *trans*-resveratrol metabolites because it allows their quantification even in the absence of authentic standards, assuming that a similar UV response is obtained for these two metabolites. This allowed us to calculate apparent K_m and V_{max} for each metabolite. They were found to be in the range 20–30 μM and 25–150 pmol min^{-1} mg^{-1} microsomal proteins, respectively.

Correlation of piceatannol and M1 rate of formation with specific activities of CYPs, selective chemical inhibition, immuno-inhibition and recombinant enzyme have been used to study *trans*-resveratrol metabolism. All together, our experimental data suggest a major role for CYP1A2 in the formation of the two tetrahydroxystilbenes

in human liver microsomes. Indeed, significant correlations were obtained with EROD and phenacetin-*O*-deethylation, which are both two CYP1A2-mediated activities, as well as with CYP1A2 protein content. The extensive inhibition obtained with α-naphthoflavone and furafylline, a mechanism-based inactivator of CYP1A2, or by antibodies directed against CYP1A2 provide further evidence of CYP1A2 involvement. Lastly, this assertion was strengthened by metabolic studies carried out on recombinant human CYP preparations. The highest catalytic activities were obtained for CYP1A2 and, to a lesser extent, for CYP1A1 and CYP1B1. One should note that CYP1A1 is essentially an extrahepatic enzyme and is expressed at only very low levels in human liver. On the other hand, CYP1B1 has been found in a wide variety of tumours, and it was not present in detectable amounts in our liver microsomes. Therefore, CYP1A2 appears to play a major role in the metabolism of *trans*-resveratrol into piceatannol and 3,4,5,4′-tetrahydroxystilbene in human liver. These results are consistent with the finding that *trans*-resveratrol is a mechanism-based inactivator of CYP1A2 [10]. *Trans*-resveratrol was also reported to be a mechanism-based inactivator of CYP3A4 when testosterone was used as substrate [12], but this was not confirmed when using 7-benzyloxy-4-trifluoromethylcoumarin [30]. As shown here, *trans*-resveratrol is not metabolized by CYP3A4 and thus should not be a mechanism-based inactivator of CYP3A4.

Piceatannol and M1 could greatly contribute to the chemopreventive activity of *trans*-resveratrol: their anticancer properties are expressed through the inhibition of tyrosine kinase for the piceatannol and by apoptosis induction in transformed cells (but not in their normal counterparts) for the 3,4,5,4′-tetrahydroxystilbene [31]. Therefore, *trans*-resveratrol could play the role of a pro-drug, which would be converted into anticancer drugs by CYP1A2 in the liver, CYP1A1 at the extra-hepatic level and CYP1B1 in tumours. However, the complexity of the interaction between *trans*-resveratrol and CYPs is increased by the capacity of the stilbene to inhibit CYP activities, another mechanism that may explain its chemopreventive effect. Indeed, different reports have described in vitro inhibition of human CYP1 enzymes by *trans*-resveratrol: inhibition of *CYP1A1* [6,7] or of *CYP1B1* [9] transcription, direct enzyme-selective inhibition of CYP1B1 and CYP1A1 [8,9,13], and mechanism-based inactivation of CYP1A2 [10].

5. Conclusion

Although many studies have implicated *trans*-resveratrol in disease prevention, information on its metabolism are incomplete or contradictory, and the structures of *trans*-resveratrol metabolites are debated. Our HPLC-UV and GC-MS data from human liver microsomes experiments confirm the formation of phase-I metabolites of *trans*-resveratrol and the involvement of CYP1A2 in the metabolism of *trans*-resveratrol. If piceatannol was clearly identified as one of the two major tetrahydroxystilbenes produced by human liver microsomes, further investigations are required to exactly identify the molecular structure of M1. In view of the in vitro CYP1 family biotransformation of *trans*-resveratrol and the mechanism-based inhibition of CYP1A2 by *trans*-resveratrol, further investigations should confirm the presence of phase-I metabolites in vivo as well as the modulations of CYP1A activity and expression following *trans*-resveratrol administration.

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