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Glucuronidation of *trans*-resveratrol by human liver and intestinal microsomes and UGT isoforms

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Abstract

Resveratrol (*trans*-resveratrol, *trans*-3,5,4′-trihydroxystilbene) is a naturally occurring stilbene analogue found in high concentrations in red wine. There is considerable research interest to determine the therapeutic potential of resveratrol, as it has been shown to have tumour inhibitory and antioxidant properties. This study was performed to investigate the glucuronidation of resveratrol and possible drug interactions via glucuronidation. Two glucuronide conjugates, resveratrol 3-*O*-glucuronide and resveratrol 4′-*O*-glucuronide, were formed by human liver and intestinal microsomes. UGT1A1 and UGT1A9 were predominantly responsible for the formation of the 3-*O*-glucuronide ($K_m = 149\,\mu M$) and 4′-*O*-glucuronide ($K_m = 365\,\mu M$), respectively. The glucuronide conjugates were formed at higher levels (up to 10-fold) by intestinal rather than liver microsomes. Resveratrol was co-incubated with substrates of UGT1A1 (bilirubin and 7-ethyl-10-hydroxycamptothecin (SN-38)) and UGT1A9 (7-hydroxytrifluoromethyl coumarin (7-HFC)). No major changes were noted in bilirubin glucuronidation in the presence of resveratrol. Resveratrol significantly inhibited the glucuronidation of SN-38 ($K_i = 6.2 \pm 2.1\,\mu M$) and 7-HFC ($K_i = 0.6 \pm 0.2\,\mu M$). Hence, resveratrol has the potential to inhibit the glucuronidation of concomitantly administered therapeutic drugs or dietary components that are substrates of UGT1A1 and UGT1A9.

Introduction

Resveratrol (*trans*-resveratrol, *trans*-3,5,4′-trihydroxystilbene, Figure 1) is a naturally occurring stilbene analogue found in a variety of plant sources, such as grapes, mulberries and peanuts, and in particular is found in high concentrations in red wine (Kopp 1998). It has been shown to have a wide range of pharmacological properties, including antioxidant (Leonard et al 2003), lipid-lowering (Miura et al 2003) and cardioprotective activities (Kopp 1998) and the ability to decrease aortic accumulation of cholesterol (Zern et al 2003). Recently, there has been a major interest in the development of resveratrol in chemoprevention, as it is known to inhibit tumour cell growth and proliferation (Aziz et al 2003; Aggarwal et al 2004; Pezzuto 2004).

The metabolism of resveratrol is complex involving several pathways, with significant phase II conjugation to sulfates and glucuronides. Resveratrol undergoes sulfate conjugation to form resveratrol 3-*O*-sulfate in man (De Santi et al 2000b, c; Walle et al 2004). In addition, several other sulfate conjugates (4′-sulfate, 3,5-disulfate, 3,4′-disulfate, 3,4′,5-trisulfate) have been identified in rats (Wenzel et al 2005). Both *cis*- and *trans*-isomers of resveratrol are known to undergo glucuronide conjugation by uridine diphosphate glucuronosyltransferases (UGTs) to form two corresponding glucuronides (3-*O*-glucuronide and 4′-*O*-glucuronide) (Aumont et al 2001). It has been proposed that the biotransformation of resveratrol occurs in two steps, the first step being glucuronidation followed by sulfation, the second step, at higher doses of resveratrol (Wenzel et al 2005). The glucuronides of resveratrol undergo extensive enterohepatic circulation in a linked-rat model and contribute significantly to the bioavailability of resveratrol (Marier et al 2002). Several studies have investigated the CYP1A1/CYP1A2 and CYP1B1 inhibitory

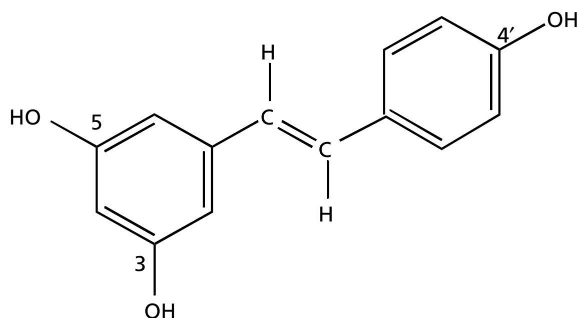

Figure 1 Chemical structure of resveratrol (*trans*-3,5,4′-trihydroxystilbene).

potential of resveratrol, since these enzymes mediate the activation of procarcinogens to carcinogenic compounds and is hence a proposed mechanism of chemopreventive action of resveratrol (Ciolino & Yeh 1999; Chang et al 2001; Piver et al 2001, 2004; Guengerich et al 2003; Yu et al 2003; Nath et al 2004). In-vitro studies have shown that resveratrol can be hydroxylated by CYP1B1 to piceatannol (3,3′,4′,5–tetrahydroxystilbene) which by itself has anticancer properties with tyrosine kinase inhibitory potential (Thakkar et al 1993; Potter et al 2002). Our previous studies (Nath et al 2004) as well as others (Piver et al 2004) have shown the involvement of CYP1A2 in the formation of piceatannol. We have shown also that piceatannol is formed at higher rates in human liver vs intestinal samples (Nath et al 2004). CYP metabolites of resveratrol were not found in a recent clinical study, which showed also the presence of a hydrogenated metabolite (dihydroresveratrol) along with its sulfate and glucuronide conjugates (Walle et al 2004).

This study has focused on the glucuronidation of *trans*-resveratrol, as there is limited information available on possible drug interactions of resveratrol via glucuronidation. Glucuronidation reactions involve the transfer of glucuronic acid to non-polar substrates by a multigene family of enzymes (UGTs), thereby generating hydrophilic glucuronides that are more easily eliminated. Two distinct families of UGTs have been identified: UGT1 and UGT2, each of which has been further classified into individual isoforms (Mackenzie et al 1997). This study includes a detailed characterization of the glucuronidation of *trans*-resveratrol by human liver and intestinal microsomes as well as cDNA expressed UGT isoforms. In addition, we have investigated the potential for drug interactions between resveratrol and substrates of key enzymes (UGT1A1 and UGT1A9) that have been found in this study to be involved in its glucuronidation.

Materials and Methods

Materials

Resveratrol (*trans*-resveratrol) was supplied by the National Cancer Institute. Uridine diphosphate glucuronic acid (UDPGA) and its radiolabelled form ([^{14}C]UDPGA), β-glucuronidase (bovine liver, Type B-1), dimethyl sulfoxide (DMSO), bilirubin, L-phosphatidyl choline, saccharolactone, glycine, alamethicin, 3,4,5-methoxycinnamic acid, 7-hydroxytrifluoromethyl coumarin glucuronide, camptothecin (CPT) and other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). 7-Hydroxytrifluoromethyl coumarin (7-HFC) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). 7-Ethyl-10-hydroxycamptothecin (SN-38) was kindly provided by Yakult Honsha Ltd (Tokyo, Japan).

Lymphoblast expressed human UGT isoforms (Supersomes), pooled human liver microsomes (HLM, prepared from 21 individual liver samples) and individual liver microsomes from seven donors were purchased from BD Biosciences (Bedford, MA, USA). Supersomes (BD Biosciences) from insect cells infected with the wild-type baculovirus were used as control. Liver microsomes from three individual donors were prepared from specimens obtained from the National Disease Research Interchange (NDRI) and Anatomic Gift Foundation. Pooled human intestinal microsomes (HIM, prepared from 10 individual intestinal samples) were purchased from In Vitro Technologies (Baltimore, MD, USA). All procedures with human liver and intestinal microsomes were approved by the Human Subjects Committee of SRI International.

Resveratrol glucuronidation assay

The glucuronidation of resveratrol was studied using pooled HLM and HIM, using methods modified from those described by Aumont et al (2001). The conditions of incubation such as incubation time and final concentrations of microsomal protein, substrate, organic solvent (DMSO) and UDPGA were selected from preliminary studies. The assay was linear with respect to the selected incubation times and microsomal protein concentrations. The final conditions of a typical incubation included the addition of UDPGA (3 mM) to a prewarmed mixture (37°C for 3 min) of microsomes (HLM 1 mg mL^{-1} and HIM 0.6 mg mL^{-1}) with resveratrol (500 μM, dissolved in DMSO, 0.5%, v/v), saccharolactone (5 mM) and $MgCl_2$ (10 mM) in phosphate-buffered saline (PBS, 1X, pH = 7.2) in a total volume of 250 μL. After 1-h incubation in a shaking water-bath (37°C), the reaction was stopped with the addition of 50 μL chilled 3.6 M HCl. The samples were centrifuged at 14 000 rev min^{-1} for 5 min at 4°C.

The supernatant (25 μL) from the incubations was mixed with 75 μL 3,4,5-methoxycinnamic acid (internal standard, IS, 0.2 mg mL^{-1}) and injected (25 μL) into a reverse-phase HPLC system with fluorescence detection at λ_{ex} = 330 nm and λ_{em} = 374 nm (Waters Corp, Milford, MA, USA) and Luna C18 column (4.6 × 250 mm, 5 μm particle size, Phenomenex Inc., Torrance, CA, USA). The isocratic mobile phase consisted of 76% 0.1% trifluoroacetic acid (TFA) in water and 24% 0.1% TFA in acetonitrile, used at a flow rate of 1 mL min^{-1}. The formation of resveratrol glucuronides was confirmed by treatment of

supernatants from incubation with β-glucuronidase enzyme (from bovine liver, Type B-1, 6000 U mL^{-1}) in 0.1 M sodium acetate buffer (pH = 4.5) for 4 h at 37°C. Control samples without β-glucuronidase were included. As pure standards of resveratrol glucuronides were unavailable, their formation has been expressed as the peak area ratio of the respective glucuronide to the IS, obtained in chromatograms from HPLC analysis.

To differentiate between the 4′-*O*-glucuronide and 3-*O*-glucuronide of resveratrol, proton NMR analysis (Varian Mercury 300 Spectrometer, Varian, Inc., Palo Alto, CA, USA) was performed on fractions collected from HPLC at the respective retention times, after evaporation to dryness and reconstitution in deuterated water.

Resveratrol metabolism by cDNA expressed UGT isoforms

Supersomes expressing specific UGT isoforms (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, UGT2B17), along with corresponding negative controls, were screened for resveratrol glucuronidation. Incubation conditions for glucuronidation were similar to those described above, with resveratrol and protein concentrations of 500 μM and 1 mg mL^{-1}, respectively.

Bilirubin glucuronidation assay

The formation of bilirubin mono- and diglucuronides was quantitated in pooled HLM with ^{14}C-labelled UDPGA, using methods described previously (Matern et al 1994; Iyer et al 1999). Resveratrol was prepared as a stock in DMSO (1%, v/v) and was added to the incubations to give final concentrations of 0.5–1000 μM in a reaction mix (100 μL) with HLM (0.5 mg mL^{-1}), bilirubin (0.2, 0.34, or 0.6 mM), saccharolactone (8.5 mM), L-phosphatidyl choline (0.75 mg mL^{-1}) and [^{14}C]UDPGA (5 mM), with an incubation time of 60 min at 37°C. Solvent control samples were included to account for any effect of DMSO on bilirubin glucuronidation. Blank samples without substrate (bilirubin) were included to correspond to each test sample. After extraction with ethyl acetate, the radioactivity in supernatants from incubation was measured using a liquid scintillation counter (Packard Tri-Carb 2200CA, Perkin Elmer Life Sciences, Downers Grove, IL, USA).

SN-38 glucuronidation assay

SN-38 glucuronidation assay was performed using methods described previously (Iyer et al 1998). Briefly, resveratrol at various concentrations (0.1–100 μM) was incubated with SN-38 (1, 5 or 7.5 μM), Supersomes expressing UGT1A1 (0.1 mg mL^{-1}), $MgCl_2$ (10 mM), and UDPGA (5 mM) in an incubation volume of 200 μL for 30 min at 37°C. SN-38 and resveratrol were dissolved in DMSO (0.5%, v/v). The reaction was stopped with the addition of methanol (400 μL) and the samples centrifuged at 14 000 rev min^{-1} for 5 min. The supernatants were analysed by reverse-phase HPLC with fluorescent detection (Waters Corp, Milford, MA, USA) at λ_{ex} = 355 nm and λ_{em} = 515 nm, using a Luna C18 column (4.6 × 250 mm, 5 μm, Phenomenex, Torrance, CA, USA). The mobile phase comprised 0.1 M potassium dihydrogen phosphate (KH_2PO_4) with 3 mM sodium heptane sulfonate (pH = 4.0) and acetonitrile (70:30). As authentic SN-38 glucuronide (SN-38G) was not available, the formation of SN-38G was expressed as the ratio of peak heights of SN-38G to the internal standard (camptothecin). SN-38G peaks were confirmed after treatment with β-glucuronidase and by comparison with previous results (Iyer et al 1998).

7-HFC glucuronidation assay

7-HFC (35, 50 and 75 μM) and resveratrol (0.5–750 μM in DMSO, 0.5%, v/v) were incubated with Supersomes expressing UGT1A9 (0.05 mg mL^{-1}), $MgCl_2$ (10 mM), alamethicin (0.025 mg mL^{-1}) and UDPGA (1 mM) in an incubation volume of 200 μL for 20 min at 37°C (www.bdbiosciences.com). The reaction was stopped with 100 μL chilled acetonitrile/acetic acid (94:6), centrifuged at 14 000 rev min^{-1} for 5 min, and supernatants were stored at −20°C until HPLC analysis. Thawed samples were evaporated to dryness, reconstituted in 200 μL 10% methanol in water and 60 μL injected into the HPLC system with UV detection at 325 nm (Waters Corp, Milford, MA, USA) and Luna C18 column (4.6 × 250 mm, 5 μM, Phenomenex, Torrance, CA, USA), set at 45°C. The mobile phase consisted of 10% methanol (A), 100% methanol (B) and 30% acetonitrile with 1 mM perchloric acid (C) used at 1 mL min^{-1} at the following gradient: T = 0 min: A = 80%, B = 10%, C = 10%; T = 15 min: A = 0%, B = 90%, C = 10%; T = 17 min: A = 0%, B = 90%, C = 10%; T = 18 min: A = 80%, B = 10%, C = 10%; T = 25 min: A = 80%, B = 10%, C = 10%.

Correlation between resveratrol and SN-38, bilirubin and 7-HFC glucuronidation using human liver microsomes

The glucuronidation of resveratrol was correlated with the glucuronidation of other substrates known to be conjugated by UGT1A1 (SN-38 and bilirubin) and UGT1A9 (7-HFC). Liver microsomes from ten donors were individually screened for resveratrol, SN-38, bilirubin and 7-HFC glucuronidation using methods described above with substrate concentrations of 500, 5, 340 and 50 μM, respectively. The glucuronidation rates of resveratrol in the ten liver samples were also used to get preliminary information on inter-individual variability in the formation of the two glucuronides.

Data and statistical analyses

Incubations for the various glucuronidation assays using pooled HLM and HIM, liver microsomes from ten individuals, and Supersomes expressing UGT isoforms were

performed in triplicate. The results were expressed as mean formation of respective glucuronide ± s.d. Unpaired *t*-test was used to test for significant differences in the formation of each resveratrol glucuronide between HIM and HLM (Primer of Biostatistics, Version 4.02 by S. A. Glantz, McGraw Hill, 1996). One-way analysis of variance followed by multiple comparisons with Student–Newman–Keuls test was used to compare the formation of each glucuronide between Supersomes expressing various UGT isoforms. Paired *t*-test was used to compare the formation of the two glucuronides within each tissue fraction, i.e. HLM, HIM, or Supersomes expressing specific UGT isoforms. Correlation analyses between glucuronidation of resveratrol and other UGT1A1 and UGT1A9 substrates were performed using correlation coefficients (r). $P < 0.05$ was considered significant.

The data for enzyme kinetic analysis were fit to Michaelis–Menten (hyperbolic) and Hill (sigmoidal) models. Standard parameters such as coefficient of determination (R^2), standard deviation of the parameter estimates and visual inspection were used to determine the quality of fit to a specific model. The apparent enzyme kinetic parameters of K_m and n (for Hill model) were calculated by nonlinear regression analysis (Enzyme Kinetics Module 1.1 of Sigma Plot 2001, version 7.101, SPSS Inc., Chicago, IL, USA). Inhibitory constant (K_i) values from the drug interaction studies with resveratrol were calculated using the raw data from all three replicates in each experiment using Dixon analysis (Enzyme Kinetics Module 1.1 of Sigma Plot 2001).

Results

Resveratrol glucuronidation in human liver and intestinal microsomes

The chromatographic profile obtained from a typical resveratrol glucuronidation assay using HLM indicated retention times (RT) of ~ 20 and 25 min for resveratrol and the IS, respectively. Two unknown peaks were observed at ~ 5 and 7 min. These were confirmed to correspond to glucuronides of resveratrol, as these peaks were absent in supernatants from control incubations without UDPGA and were reduced after treatment of incubation samples with β-glucuronidase. A similar chromatographic profile was obtained from glucuronidation experiments using HIM. The peak with RT ~ 7 min was identified as the 3-*O*-glucuronide of resveratrol, based on NMR data (^{1}H NMR (D_2O): δ 7.37 (d, 2H $J = 9.0$), 7.20 (d, 1H $J = 15.9$), 6.95 (d, 1H $J = 15.6$), 6.78 (d, 2H $J = 8.7$), 6.66 (t, 1H $J = 2.1$), 6.64 (t, 1H $J = 8.7$), and 6.35 (t, 1H $J = 2.1$) ppm) and by comparison with published reports (Aumont et al 2001; Learmonth 2003). The glucuronide peak that eluted at ~ 5 min was most likely the 4′-*O*-glucuronide of resveratrol, based upon information from published reports on in-vitro conjugation of resveratrol to two glucuronides (3-*O*-glucuronide and 4′-*O*-glucuronide) (Aumont et al 2001). However, the exact structure of this metabolite could not be

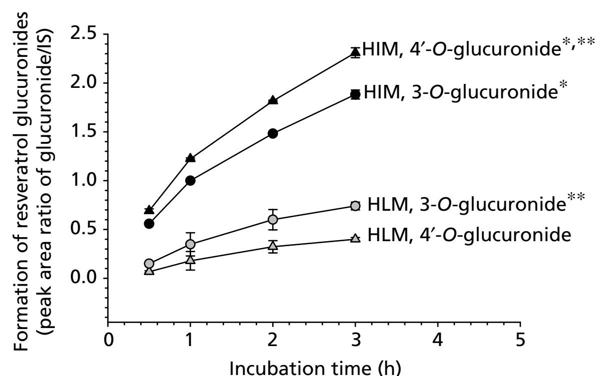

Figure 2 Resveratrol glucuronidation in human liver microsomes (HLM) and human intestinal microsomes (HIM). Microsomes ($1\,mg\,mL^{-1}$ pooled HLM and $0.6\,mg\,mL^{-1}$ pooled HIM) were incubated for various time periods, with resveratrol ($500\,\mu$M), $MgCl_2$ (10 mM) and UDPGA (3 mM) in PBS (pH = 7.2). Each data point represents the mean (± s.d.) value from three separate incubations of each sample. The error bars are smaller than the symbols for some points. *$P < 0.05$, greater formation of each glucuronide in HIM than HLM at all incubation times. **$P < 0.05$, greater formation of resveratrol 4′-*O*-glucuronide in HIM at all incubation times and that of 3-*O*-glucuronide in HLM at all incubation times (except 0.5 and 1 h for HLM).

definitively confirmed in this study, as sufficient amounts were not available for confirmation by NMR.

The formation of both resveratrol glucuronides was 3- to 10-fold greater in HIM as compared with HLM, consistently, at various incubation times ($P < 0.05$, Figure 2). A lower intestinal microsomal protein concentration ($0.6\,mg\,mL^{-1}$, when compared with $1\,mg\,mL^{-1}$ HLM) was used in these experiments to maintain the heights of glucuronide peaks in HPLC analysis within scale. There was a trend toward formation of higher levels of the 4′-*O*-glucuronide in intestinal tissue and those of the 3-*O*-glucuronide in liver tissue (Figure 2, $P < 0.05$ at all incubation times except 0.5 and 1 h for HLM).

Resveratrol glucuronidation by cDNA expressed UGT isoforms

Among the UGT isoforms screened, resveratrol 3-*O*-glucuronide was formed predominantly by UGT1A1 (peak area ratio of 3-*O*-glucuronide/IS ± s.d. of 1.18 ± 0.02, $P < 0.05$, Figure 3). UGT1A9 (0.30 ± 0.01) and UGT1A7 (0.32 ± 0.03) were also capable of resveratrol 3-*O*-glucuronide formation, which accounted for approximately one-quarter the activity observed with UGT1A1 ($P < 0.05$). In addition, UGT1A3, UGT1A6, UGT1A8 and UGT1A10 produced the 3-*O*-glucuronide, but at much lower levels than those produced by UGT1A1, UGT1A7 and UGT1A9 ($P < 0.05$, Figure 3). Resveratrol 4′-*O*-glucuronide was formed predominantly by UGT1A9 (peak area ratio of 4′-*O*-glucuronide/IS ± s.d. of 0.93 ± 0.07, $P < 0.05$, when compared with other UGT isoforms, Figure 3). Other isoforms such as UGT1A1, UGT1A3, UGT1A8, and UGT1A10 were also

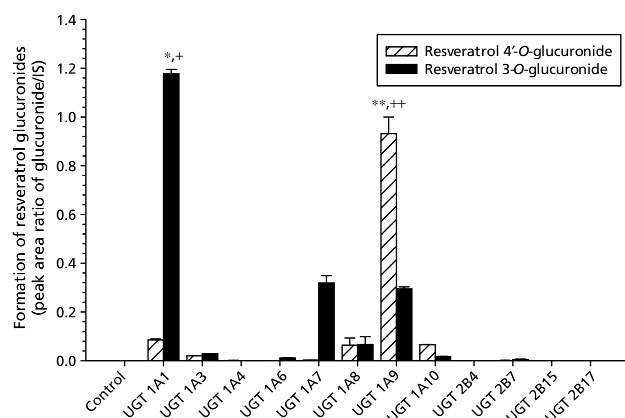

Figure 3 Glucuronidation of resveratrol by specific cDNA expressed human UGT isoforms. Supersomes expressing specific UGT isoforms ($1\,mg\,mL^{-1}$) were incubated for 1 h with resveratrol ($500\,\mu$M), $MgCl_2$ (10 mM) and UDPGA (3 mM) in PBS (pH = 7.2). Each bar represents the mean value from three separate incubations of each sample. The error bars are smaller than the fills for some bars. $^{*,**}P < 0.05$, greater resveratrol 3-*O*-glucuronide and 4′-*O*-glucuronide formation by UGT1A1 and UGT1A9, respectively, compared with other UGT isoforms. $^{+,++}P < 0.05$, greater formation of resveratrol 3-*O*-glucuonide than 4′-*O*-glucuronide by UGT1A1 and that of resveratrol 4′-*O*-glucuronide than 3-*O*-glucuonide by UGT1A9, respectively.

capable of conjugating resveratrol to its 4′-*O*-glucuronide, but to a minor extent ($P < 0.05$). Both glucuronides of resveratrol were absent in incubations with Supersomes expressing UGT1A4, as well as those with most UGT2B isoforms (UGT2B4, UGT2B15 and UGT2B17), with very low activity by UGT2B7 (Figure 3).

The kinetics for the formation of resveratrol 4′-*O*-glucuronide and resveratrol 3-*O*-glucuronide were studied using Supersomes expressing UGT1A9 and UGT1A1, as these isoforms were predominantly responsible for the formation of the two glucuronides, respectively. Atypical kinetics were observed for the formation of both glucuronides. The formation of resveratrol 4′-*O*-glucuronide by UGT1A9 was best described by the Hill equation ($R^2 = 0.99$, n = 2.7), resulting in $K_m = 365\,\mu$M. The formation of resveratrol 3-*O*-glucuronide by UGT1A1 also followed Hill kinetics ($R^2 = 0.97$, n = 2.2), with $K_m = 149\,\mu$M.

Correlation of resveratrol glucuronidation with SN-38, bilirubin and 7-HFC glucuronidation

To further understand the roles of UGT1A1 and UGT1A9 in the glucuronidation of resveratrol, the formation of resveratrol 3-*O*-glucuronide and 4′-*O*-glucuronide were correlated with bilirubin, SN-38 and 7-HFC glucuronidation, using microsomes from ten different human liver samples (Figure 4). Resveratrol 3-*O*-glucuronidation correlated significantly with SN-38 glucuronidation ($r = 0.92$, $P < 0.01$) (Figure 4A) while the correlation with bilirubin glucuronidation was minimal ($r = 0.40$, $P = 0.26$) (Figure 4B). Resveratrol 4′-*O*-glucuronidation did not correlate significantly with SN-38 glucuronidation ($r = 0.50$, $P = 0.14$) (Figure 4A) or bilirubin glucuronidation ($r = 0.22$, $P = 0.54$) (Figure 4B). There was a significant association of 7-HFC glucuronidation with resveratrol 4′-*O*-glucuronide formation ($r = 0.73$, $P = 0.01$) and a moderate correlation with resveratrol 3-*O*-glucuronide formation ($r = 0.60$, $P = 0.06$) (Figure 4C). The mean formation (peak area ratio of *O*-glucuronide/IS ± s.d.) of resveratrol 4′-*O*-glucuronide and resveratrol 3-*O*-glucuronide by liver microsomes from ten individuals was 0.16 ± 0.06 and 0.38 ± 0.21, with coefficients of variation (CV) of 41% and 55%, respectively.

Interaction of resveratrol with other substrates of UGT1A1 and UGT1A9

UGT1A1 (bilirubin and SN-38)

Incubation of resveratrol with bilirubin did not influence bilirubin glucuronidation in a consistent manner. There were no major changes in bilirubin glucuronidation at most resveratrol concentrations studied (Figure 5A). A trend toward increased glucuronidation (12 to 40%) was observed at very high concentrations of resveratrol (250 and $500\,\mu$M) that are pharmacologically unrealistic. The effect of resveratrol on UGT1A1 activity was also studied using SN-38 as a substrate. Resveratrol had a major concentration-dependent inhibitory effect on the formation of SN-38 glucuronide by UGT1A1, as shown in Figure 5B. A mixed type of inhibition ($R^2 = 0.98$) with a K_i value of $6.2 \pm 2.1\,\mu$M was indicated for this effect of resveratrol on SN-38 glucuronidation.

UGT1A9 (7-HFC)

Resveratrol inhibited UGT1A9 activity as it decreased 7-HFC glucuronidation by UGT1A9 in a concentration-dependent manner (Figure 5C). The inhibition curves were shifted to the right with increasing substrate (7-HFC) concentrations (35, 50 and $75\,\mu$M) in a parallel fashion. Dixon analysis of the data indicated a low K_i value of $0.6 \pm 0.2\,\mu$M and a mixed type of inhibition ($R^2 = 0.98$).

Discussion

Resveratrol is a phytoestrogen that has wide popularity in complementary and alternative medicine due to its cardioprotective and chemopreventive actions. It is found in high concentrations in red wine (2–40 μM) and may be a possible explanation for the “French paradox” (Kopp 1998), due to lower incidences of heart disease in the French population despite a diet high in fat content. Resveratrol is readily available as a nutritional supplement in health food stores and has the potential to be consumed on a regular basis, especially by people with or at high risk for heart disease or cancer. Besides, if resveratrol is approved for use as a chemopreventive agent, it is likely to be consumed on a chronic basis. Hence, it is important to understand the pathways of

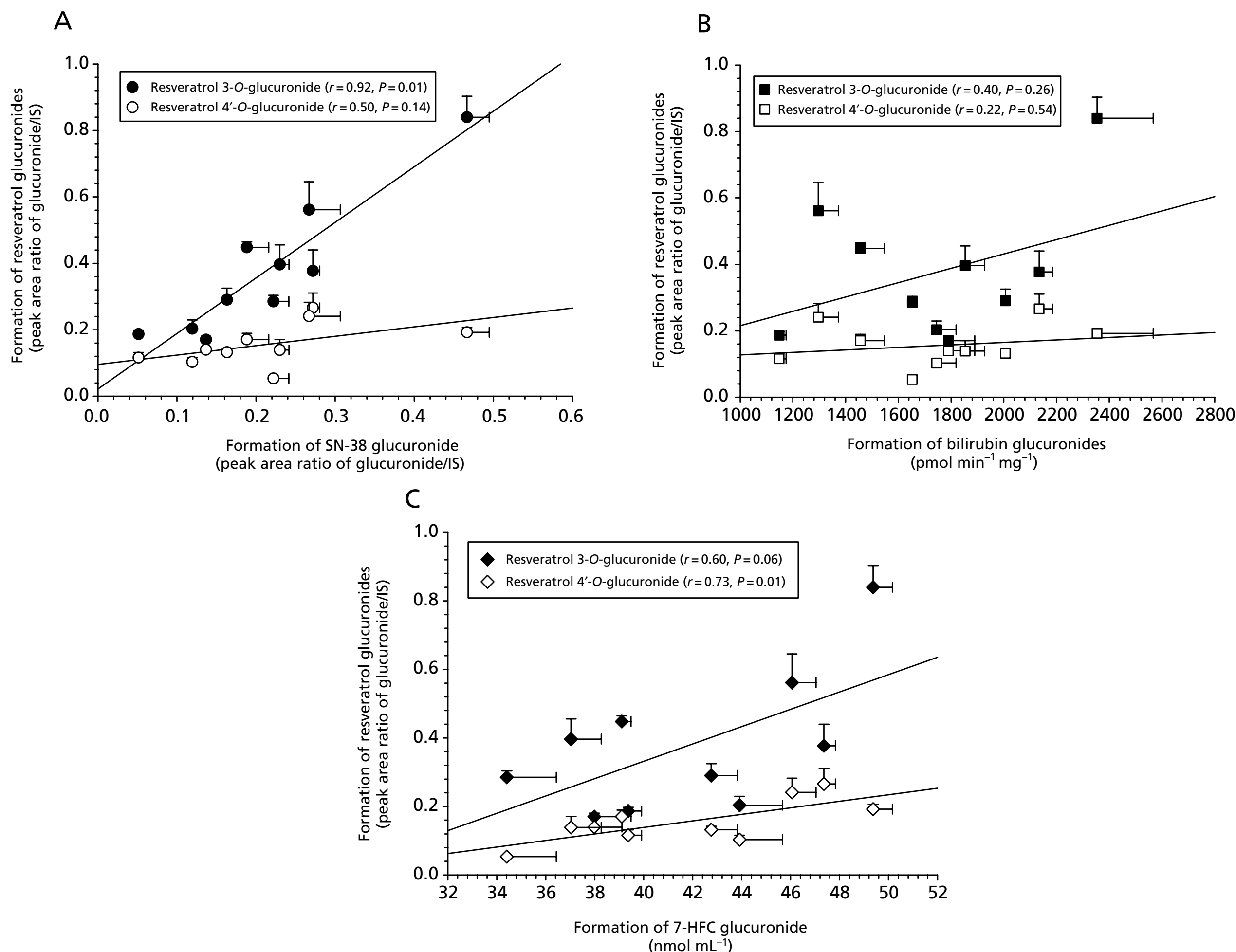

Figure 4 Correlation of resveratrol 3-*O*-glucuronidation and 4′-*O*-glucuronidation with glucuronidation of other substrates of UGT1A1 and UGT1A9: SN-38 (A), bilirubin (B) and 7-HFC (C) in individual (n = 10) human liver microsomes. Conditions of each assay include: HLM (1 mg mL^{-1}), resveratrol (500 μM), saccharolactone (5 mM) and $MgCl_2$ (10 mM) for 1 h (resveratrol glucuronidation); HLM (0.1 mg mL^{-1}), SN-38 (5 μM), $MgCl_2$ (10 mM), and UDPGA (5 mM) for 30 min (SN-38 glucuronidation); HLM (0.5 mg mL^{-1}), L-phosphatidyl choline (0.75 mg mL^{-1}), saccharolactone (8.5 mM), $MgCl_2$ (10 mM), [^{14}C]UDPGA (5 mM) and bilirubin (0.34 mM) for 1 h (bilirubin glucuronidation); and HLM (0.05 mg mL^{-1}), UDPGA (3 mM), 7-HFC (50 μM), $MgCl_2$ (10 mM), alamethicin (0.025 mg mL^{-1}) for 20 min (7-HFC glucuronidation). Each data point represents the mean value from three separate incubations of each sample. The error bars are smaller than the symbols for some points.

metabolism of resveratrol, including the specific enzymes involved, as well as the potential for interaction of resveratrol with other dietary constituents or therapeutic agents.

The relative importance of sulfation vs glucuronidation of resveratrol remains to be established. Both conjugation pathways are being studied extensively (De Santi et al 2000a, b, c; Aumont et al 2001; Marier et al 2002; Walle et al 2004; Wenzel et al 2005; Wenzel & Somoza 2005), especially since resveratrol mainly circulates in the body in its conjugated forms. A clinical study with a 25 mg oral dose of resveratrol in six subjects identified sulfate and glucuronide conjugates with rapid sulfation of resveratrol in two subjects (Walle et al 2004). It is likely that both conjugation pathways are of significance and may depend on the levels of resveratrol available, which in turn will depend on the dose of resveratrol. A recent study in rats suggested that the specific conjugation pathway of resveratrol may be dose-dependent, as it is likely that glucuronidation is the first step in the metabolism of resveratrol followed by sulfation, which may occur only after a certain level of resveratrol is reached (Wenzel et al 2005). Hence, it is important to investigate the metabolism of resveratrol in pharmacologically relevant doses in man, which remain to be determined.

This study has provided a detailed investigation of the glucuronidation of resveratrol metabolism by human liver and intestinal microsomes as well as the specific UGT isoforms responsible for the formation of resveratrol glucuronides. This study has demonstrated for the first time that human intestinal microsomes were capable of generating the two known glucuronide conjugates of resveratrol

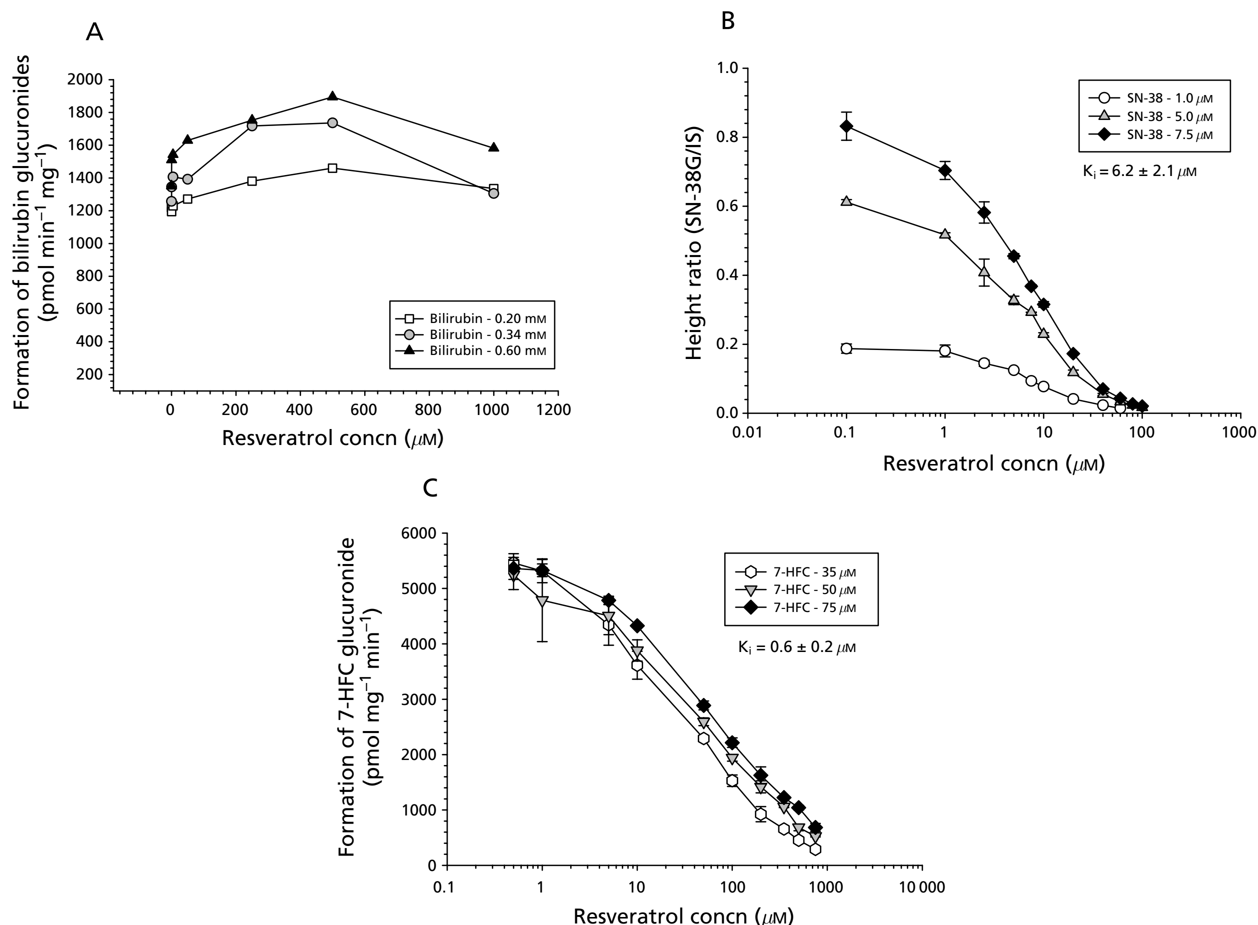

Figure 5 Interaction of resveratrol with substrates of UGT1A1 (bilirubin/A and SN-38/B) and UGT1A9 (7-HFC/C). Various concentrations of resveratrol were incubated with other ingredients using the following conditions: A, protein ($0.5\,mg\,mL^{-1}$), L-phosphatidyl choline ($0.75\,mg\,mL^{-1}$), saccharolactone (8.5 mM), $MgCl_2$ (10 mM), [^{14}C]UDPGA (5 mM) and bilirubin (0.2, 0.34 or 0.6 mM) for 1 h; B, protein ($0.1\,mg\,mL^{-1}$), SN-38 (1, 5 and 7.5 μM), $MgCl_2$ (10 mM), and UDPGA (5 mM) for 30 min; and C, protein ($0.05\,mg\,mL^{-1}$), UDPGA (3 mM), 7-HFC (35, 50, or 75 μM), $MgCl_2$ (10 mM), alamethicin ($0.025\,mg\,mL^{-1}$) for 20 min. Each data point represents the mean value from three separate incubations of each sample. The error bars are smaller than the symbols for some points.

at levels greater than those produced by human liver tissue. We have provided new information on the potential for drug interactions with resveratrol via glucuronidation. Our findings indicated that the interaction of resveratrol with other substrates of UGT1A1 and UGT1A9 could be of significance, given the poor in-vivo exposure of unchanged resveratrol and its extensive glucurono-conjugation in the gastrointestinal tract.

In these studies, the glucuronidation of resveratrol was evaluated using all currently available UGT isoforms, including UGT1A8 and UGT2B17 that had not been evaluated for this activity in earlier studies (Aumont et al 2001). Our results confirmed that resveratrol was conjugated to form two glucuronides i.e. resveratrol 3-*O*-glucuronide and 4′-*O*-glucuronide using human liver microsomes. UGT1A1 and UGT1A9 were mainly involved in resveratrol 3-*O*-glucuronidation and 4′-*O*-glucuronidation, respectively, which was in agreement with previous literature on resveratrol glucuronidation, including stereo specificity. The two isomers, *cis*- and *trans*-resveratrol, have been shown to be conjugated to 3-*O*-glucuronides by different UGT isoforms, UGT1A6 and UGT1A1, respectively (Aumont et al 2001). UGT1A7, UGT1A8, and UGT1A10 were also capable of resveratrol glucuronidation. These three UGT isoforms are known to be extra-hepatic (Strassburg et al 1997, 1998) and therefore may contribute to the increased glucuronidation of resveratrol observed in human intestinal microsomes. Most of the screened UGT2B isoforms were incapable of conjugating resveratrol. The atypical kinetic behaviour observed for resveratrol 3-*O*-glucuronidation by UGT1A1 and resveratrol 4′-*O*-glucuronidation by UGT1A9 suggested possible auto-activation. This has been reported with CYP3A4 substrates, and with glucuronidation, such as estradiol 3-glucuronidation by UGT1A1, paracetamol glucuronidation by UGT1A6 (Fisher et al 2000), and 1′-hydroxyestragole glucuronidation by UGT2B7 (Iyer et al 2003). The K_m value for resveratrol 3-*O*-glucuronide agreed with that (150 μM) reported by De Santi et al (2000a) for resveratrol glucuronidation in human liver samples. *Trans*-resveratrol was selected for all

these studies, as this conformation (4′-hydroxystyryl moiety) is required for in-vitro activity, along with the presence of the 4′-OH group (Matsuoka et al 2002), and is under development as a chemopreventive agent.

Significant inter-individual variability in resveratrol glucuronidation was observed in our studies with liver microsomes from ten different individuals. As the sample size was low, no major conclusions could be made about possible pharmacogenetic significance of resveratrol glucuronidation. However, such an influence cannot be ruled out, as UGT1A1 and UGT1A9 enzymes have been known to exist in polymorphic states. The presence of an extra TA repeat in the promoter sequence of *UGT1A1* gene results in decreased enzyme activity and has been shown to be a determinant of disposition and toxicity of therapeutic drugs such as irinotecan (Iyer et al 2002). With regard to UGT1A9, several single nucleotide polymorphisms and a one base insertion of thymidine in the promoter region of *UGT1A9* gene have been described (Girard et al 2004; Yamanaka et al 2004).

The involvement of UGT1A1 (and UGT1A9) in 3-*O*-glucuronidation of resveratrol was further substantiated by the fact that resveratrol 3-*O*-glucuronide formation correlated significantly with SN-38 glucuronidation. SN-38, the active metabolite of the anticancer agent, irinotecan, is conjugated predominantly by human UGT1A1 (Iyer et al 1998). Other hepatic UGT isoforms such as UGT1A9, UGT1A3 and UGT1A6 are known to mediate conjugation of SN-38 (Hanioka et al 2001; Tallman et al 2005). These isoforms were also found to conjugate resveratrol in this study and hence may have contributed to the significant correlation between the two phenotypes. UGT1A7 and UGT1A8, also known to conjugate SN-38 (Hanioka et al 2001; Tallman et al 2005) and resveratrol (this study), are extra-hepatic isoforms and may not have contributed to this correlation in HLM. Resveratrol 4′-*O*-glucuronide formation had only a moderate association with SN-38 glucuronidation and bilirubin glucuronidation, most likely due to UGT1A9 being the more predominant isoform conjugating resveratrol to its 4′-*O*-glucuronide than UGT1A1. The correlation between resveratrol 4′-*O*-glucuronidation and 7-HFC glucuronidation was significant possibly due to the involvement of UGT1A9 in both reactions. However, it is to be noted that 7-HFC is a substrate for several UGT isoforms except UGT1A4 (Kaku et al 2004), and hence this correlation does not substantiate the role of UGT1A9 in resveratrol 4′-*O*-glucuronidation to a significant degree. The results from these correlation studies need to be interpreted with caution as the sample size was small ($n = 10$).

The potential of resveratrol to exhibit drug interactions via glucuronidation was investigated by evaluating its ability to modulate the glucuronidation of compounds that are known to be substrates of UGT1A1 and UGT1A9. Resveratrol did not have a major influence on bilirubin glucuronidation under the conditions studied. Also, resveratrol 3-*O*-glucuronidation (predominantly mediated by UGT1A1) did not have a major association with bilirubin glucuronidation in the correlation studies. However, resveratrol inhibited SN-38 glucuronidation at low concentrations that may be pharmacologically relevant. Bilirubin is the endogenous substrate for UGT1A1 and is known to accumulate at high levels in genetic abnormalities in UGT1A1 (Crigler & Najjar 1952; Bosma et al 1995). It was surprising to find that resveratrol modulated the glucuronidation of SN-38 but not that of bilirubin. A similar finding has been reported for buprenorphine (a substrate for UGT1A1) and bilirubin, which was explained by the possible presence of distinct binding sites for the two compounds in UGT1A1 (Rios & Tephly 2002). Hence, it is likely that resveratrol and bilirubin bind to UGT1A1 at two separate active sites. A second explanation may be the involvement of UGT1A9 in SN-38 and resveratrol glucuronidation, but not in bilirubin glucuronidation (Bosma et al 1994). Resveratrol inhibited 7-HFC glucuronidation by cDNA expressed UGT1A9 significantly with an extremely low K_i of 0.6 μM. Hence, resveratrol may have the potential to modulate the glucuronidation of other substrates of UGT1A1 and UGT1A9. Besides SN-38, other known substrates of UGT1A1 include the oral contraceptive 17α-ethinylestradiol (Ebner et al 1993), opioids such as buprenorphine (Senafi et al 1994), anticancer agents such as etoposide (Watanabe et al 2003) and hormones such as thyroxine (Findlay et al 2000). UGT1A9 is also known to conjugate several pharmaceutical agents such as (*R*)-oxazepam (Court et al 2002), paracetamol (Court et al 2001) and the anticancer agent, flavopiridol (Ramirez et al 2002). UGT1A1 and UGT1A9 are involved in the metabolism of the dietary flavonoid quercetin (Boersma et al 2002), which has been shown to inhibit resveratrol glucuronidation (De Santi et al 2000a). Given the fact that UGT1A9 plays a significant role in the formation of both glucuronides of resveratrol, it is likely that this isoform is more important than UGT1A1 in the overall disposition of resveratrol.

There is limited information on the range of plasma concentrations or target tissue level of resveratrol that is chemopreventive or cardioprotective in man. A recent clinical study in a small number of volunteers ($n = 6$) showed a low bioavailability of resveratrol with plasma concentrations of free resveratrol only in the range of 21 nM after an oral dose of 25 mg (Walle et al 2004). An apparent lack of an association between chemopreventive concentrations of resveratrol determined in in-vitro studies and bioavailability of resveratrol in in-vivo studies has been discussed in many recent reports (Gescher & Steward 2003; Signorelli & Ghidoni 2005; Wenzel & Somoza 2005). It may be argued that resveratrol may not reach systemic concentrations significant enough to interact with the glucuronidation of other substances in man in the range of K_i values determined in this study for inhibition of UGT1A1 (6 μM) and UGT1A9 (0.6 μM). However, the gastrointestinal tract may be a potential site of interaction of resveratrol with other dietary components or chronic therapeutic agents, as resveratrol is known to accumulate in intestinal epithelial cells (Kaldas et al 2003). The oesophagus and digestive tract have been suggested to be major target sites for biological activity for resveratrol (Walle et al 2004; Wenzel & Somoza 2005)

as shown by its effectiveness in carcinogenesis models of colon and oesophageal tumours after oral administration (Tessitore et al 2000; Li et al 2002). Resveratrol is extensively metabolized by Caco-2 cells via glucuronidation and sulfation (Li et al 2003). Studies with an isolated rat small intestinal model have shown that resveratrol was transported across the jejunum as its glucuronide, which accounted for approximately 99% of resveratrol equivalents absorbed on the serosal side of the gut (Kuhnle et al 2000). Our studies have also demonstrated the glucuronidation of resveratrol by human intestinal microsomes, which was several-fold greater than that observed with liver microsomes. Both UGT1A1 and UGT1A9 are expressed in human intestine (Strassburg et al 1998). Hence, resveratrol could potentially exhibit clinically significant interactions with other therapeutic agents or dietary components that are also substrates for UGT1A1 and UGT1A9 and are orally administered. On the other hand, preliminary results from one study with resveratrol (500 mg) indicated a peak plasma concentration of 0.4 μM (unpublished data). Based on this study, a K_i value of 0.6 μM for UGT1A9 inhibitory activity could be within the expected pharmacological range for resveratrol.

Glucuronidation may also be an important determinant of pharmacological activity of resveratrol as it is possible that resveratrol glucuronides may get deconjugated at the target sites of action, thereby releasing the aglycone for eliciting biological activity. This possibility has been speculated with resveratrol glucuronides and sulfates (Kuhnle et al 2000; Walle et al 2004; Signorelli & Ghidoni 2005). Another contributing factor to the importance of glucuronidation of resveratrol is the significant enterohepatic circulation observed with resveratrol glucuronides (Marier et al 2002). Furthermore, a biologically active resveratrol glucuronide cannot be ruled out, as has been shown with morphine glucuronide which is several times (up to 360-fold) more potent as an analgesic than morphine (Frances et al 1992). Although the bioavailability of resveratrol itself is low in man, peak plasma levels of total resveratrol (combined with glucuronides and sulfates) up to 2 μM have been reported in man (Walle et al 2004). Hence, the extensive phase II metabolism of resveratrol to its glucuronide or sulfate conjugates may partly explain the disparity between the low circulating levels of resveratrol and proposed therapeutic efficacy of resveratrol.

Conclusions

We have demonstrated the roles of UGT1A1 and UGT1A9 in the glucuronidation of resveratrol to its 3-*O*-glucuronide and 4′-*O*-glucuronide. Both glucuronides were formed at higher levels in human intestinal samples than liver tissue, thereby emphasizing the significance of the intestine as an important site for the metabolism of resveratrol. Resveratrol inhibited the glucuronidation of other substrates of UGT1A1 and UGT1A9, and hence could potentially interact with the metabolism of other dietary components or therapeutic drugs concomitantly administered with resveratrol and that were also metabolized by these UGT isoforms. Although there are some open questions regarding the bioavailability of resveratrol, it is quite possible that resveratrol can modulate the metabolism of other compounds in the gastrointestinal tract, which is known to express measurable levels of UGT1A1 and UGT1A9. These findings have pharmacogenomic significance as UGT1A1 and UGT1A9 are known to exhibit polymorphisms.

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