Published in final edited form as:
Mol Pharm. 2007 ; 4(6): 883–894. doi:10.1021/mp700135a.

Disposition of Flavonoids via Enteric Recycling: Enzyme Stability Affects Characterization of Prunetin Glucuronidation across Species, Organs, and UGT Isoforms

Tiby B. Joseph, **Stephen W.J. Wang**, **Xing Liu**, **Kaustubh H. Kulkarni**, **Jingrong Wang**, **Haiyan Xu**, and **Ming Hu**

Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, 1441 Moursund Street, Houston, TX 77030

Abstract

We characterized the in vitro glucuronidation of prunetin, a prodrug of genistein that is a highly active cancer prevention agent. Metabolism studies were conducted using expressed human UGT isoforms and microsomes/S9 fractions prepared from intestine and liver of rodents and humans. The results indicated that human intestinal microsomes were more efficient than liver microsomes in glucuronidating prunetin, but rates of metabolism were dependent on time of incubation at 37°C. Human liver and intestinal microsomes mainly produced metabolite 1 (prunetin-5-O-glucuronide) and metabolite 2 (prunetin-4'-O-glucuronide), respectively. Using 12 human UGT isoforms, we showed that UGT1A7, UGT1A8 and 1A9 were mainly responsible for the formation of metabolite 1 whereas UGT1A1, UGT1A8 and UGT1A10 were mainly responsible for the formation of metabolite 2. This isoform specific metabolism was consistent with earlier results obtained using human liver and intestinal microsomes, as the former (liver) is UGT1A9-rich whereas the latter is UGT1A10-rich. Surprisingly, we found that thermo stability of the microsomes were isoform- and organ-dependent. For example, human liver microsomal UGT activities were much more heat (37° C) stable than intestinal microsomal UGT activities, consistent with the finding that human UGT1A9 is much more thermo stable than human UGT1A10 and UGT1A8. The organ-specific thermo stability profiles were also evident in rat microsomes and mouse S9 fractions, even though human intestinal glucuronidation of prunetin differ significantly from its rodent intestinal glucuronidation. In conclusion, prunetin glucuronidation is species-, organ- and UGT-isoform dependent, all of which may be impacted by thermo stability of specific UGT isoforms involved in the metabolism.

INTRODUCTION

Glucuronidation is the major metabolic pathway for the disposition of flavonoids in humans and in experimental animals such as rodents. Catalyzed by the UDP-glucuronosyltransferases, an important superfamily of enzymes, glucuronidation pathway is responsible for metabolic clearance of drugs (e.g. SN-38) [1], phytochemicals (e.g., genistein) [2], carcinogens (e.g., PhIP) [3], and endogenous substances (e.g., serotonin) [4]. Therefore, many investigators have determined if one or several expressed UGT isoforms are responsible for the metabolism of a particular drug because of need to predict metabolic interactions and pharmacogenetics that can impact a drug's safety or efficacy or both.

Address correspondence to: Ming Hu, Ph.D., 1441 Moursund Street, Department of Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX 77204, Tel: (713)-795-8320, E-mail: mhu@uh.edu.

This research was supported by NIH CA87779 of the National Cancer Institute to MH. TJ was supported in part by a University of Houston Summer Undergraduate Research Fellowship (2006). SW was supported by a USP Fellowship for 2005–2007.

In general, the characterization of a compound's glucuronidation pathway involves the use of multiple UGT isoforms, 1 to 3 concentrations of substrates, 1 incubation time, and microsomes prepared from different organs. This is based on a survey of published literature, where characterization is usually done using one incubation time and mostly one concentration, but sometimes 2 and seldom three concentrations [5–12]. Therefore, one of the purposes of the present study is to test the validity of this approach and to establish a viable approach useful for the characterization of other isoflavones and flavones.

We have chosen prunetin as the model isoflavone for several reasons. First, prunetin, an isoflavone that is also a naturally occurring methylated prodrug of genistein, is biologically active [13] and present in various plants including Kudzu roots [14]. Second, in rats, liver metabolism of prunetin was the slowest among the six isoflavones (genistein, daidzein, glycitein, biochanin A, formononetin, and prunetin) studied [15], but the intestinal metabolism of prunetin was not [15]. Third, there appears to be species-specific effects as the metabolism of prunetin in human intestinal Caco-2 cell lysates also appeared to be the slowest among the same six isoflavones [16]. Therefore, the use of prunetin as a model compound affords us the opportunity to fully characterize all facets of a flavonoid's glucuronidation: incubation time, concentration, species, organ, and UGT isoforms.

In addition, because prunetin is metabolized into two glucuronidated compounds in human microsomes in our preliminary studies, additional characterization studies are conducted to determine if formation of a particular metabolite is (expressed UGT) isoform-specific and whether isoform-specific metabolism could predict formation of these two metabolites in human liver and intestinal microsomes. This is especially important for the characterization of flavonoid metabolism as vast majority of flavonoids have more than one phenolic hydroxyl group.

MATERIALS AND METHODS

Materials

Expressed human UGT isoforms (Supersomes), pooled female human liver, jejunum, and ileum microsomes were purchased from BD Biosciences (Woburn, MA). Prunetin was purchased from Indofine Chemical (Summerville, NJ). β-D-Glucuronidase without sulfatase (catalog no. G7396), uridine diphosphoglucuronic acid, alamethicin, D-saccharic-1,4-lactone monohydrate, magnesium chloride, and Hanks' balanced salt solution (HBSS, powder form) were purchased from Sigma-Aldrich (St Louis, MO). All other materials (typically analytical grade or better) were used as received.

Enzymatic Activity of Expressed UGTs

The incubation procedures for measuring UGTs activities using microsomes were as follows: 1) mix microsomes (final concentration ≈0.05 mg protein/ml), magnesium chloride (0.88 mM), saccharolactone (4.4 mM), and alamethicin (0.022 mg/ml); different concentrations of substrates in a 50 mM potassium phosphate buffer (pH 7.4); and uridine diphosphoglucuronic acid (3.5 mM, add last); 2) incubate the mixture (final volume=200μl) at 37°C for pre-determined periods of time (e.g., 1 hr); and 3) stop the reaction by the addition of 50 μl of 94% acetonitrile/6% glacial acetic acid containing 100 μM testosterone as an internal standard. This is essentially the same as our published procedures[15, 17].

Hydrolysis of Prunetin Glucuronides

To confirm the presence of prunetin glucuronides, metabolite samples were divided into two parts; one of which was added water and then analyzed directly via UPLC (see below) whereas the other was analyzed after β-D-glucuronidase was added to hydrolyze O-glucuronides. The

difference between amounts of aglycones found in these two samples was usually regarded as the amounts of metabolites formed. Matching the decreased peak areas of the metabolites and the increased peak areas of aglycone after the hydrolysis is used to confirm the presence of prunetin O-glucuronides. Moreover, development of the relationship between a decrease in metabolite peak area and an increase in aglycone peak area is used to quantitate metabolite concentration in the reaction mixture, as was done previously [18].

UPLC Analysis of Prunetin and its Glucuronides

We analyzed prunetin and prunetin glucuronides by using: system, Waters Acquity UPLC with photodiode array detector and Empower software; column, BEH C18, 1.7 um, 2.1 × 50mm; mobile phase B, 90% acetonitrile in water, mobile phase A, 10% acetonitrile in aqueous buffer (0.06 % $C_6H_{15}N$ and 0.045% HCOOH); gradient, 0 to 0.3 min, 0% B, flow rate = 1ml/min, 0.3 to 1.80, 0–50% B, flow rate = 0.925 ml/min, 1.80 to 2.10, 50–100% B, flow rate = 0.925ml/min, 2.10 to 2.40, 100% B, flow rate = 0.925, 2.40 to 2.50, 100%-0% B, flow rate = 1 ml/min; wavelength, 254 nm; and injection volume, 10μl. The retention times were 1.01 min for metabolite 1, 1.23 min for metabolite 2, 1.16 min for metabolite 3, 1.62 min for testosterone (internal standard or IS), and 1.70 min for prunetin, respectively. Representative analysis of the metabolic samples is shown in Fig.1A.

LC-MS/MS Analysis of Prunetin and its Glucuronides

Prunetin and its glucuronides were separated by the same UPLC system but using slightly different chromatographic conditions because of mass spectrometer requirements. Here, mobile phase A was an ammonium acetate buffer (pH 7.5) and mobile phase B was 100% acetonitrile with the gradient as follows; 0 to 2.0 min, 10–35% B, 2.0 to 3.0 min, 35–70% B, 3.2 to 3.5 min, 70-10% B, 3.5 to 3.7 min, 10% B. The flow rate was 0.5 ml/min. The effluent was introduced into an API 3200 Qtrap triple quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) equipped with a TurboIonSpray™ source. The mass spectrometer was operated in the negative ion mode to perform the analysis of prunetin and prunetin glucuronides. The main working parameters for the mass spectrometers were set as follows: ion-spray voltage, −4.5 kV; ion source temperature, 400°C; the nebulizer gas (gas1), nitrogen, 40 psi; turbo gas (gas2), nitrogen, 40 psi; curtain gas, nitrogen, 20 psi. Prunetin and prunetin glucuronides were identified by MS full scan and MS2 full scan modes (Fig.1B).

Purification of Metabolite 2 and Its Structural Identification via NMR

A large amount of metabolite 2 was produced using human intestinal microsomes. The prepared metabolite 2 solution was concentrated using a C-18 solid phase extraction procedure, where the co-enzyme and other water soluble components were washed off first. The compound was eluted by using 40% acetonitrile in water, whereas the microsomal proteins were retained on the column. The metabolite 2 solution was then lyophilized to remove water and organic solvent. The resulting residue was dissolved in D-methanol and used for NMR spectrum acquisition. ^{1}H NMR was recorded on a Bruker Avance 800 MHz NMR spectrometer. Coupling constants are given in Hz, and chemical shifts are represented in δ (ppm).

Thermal Stability of Human UGTs

We first incubate the expressed UGTs at 37°C for various time periods (1, 4, 8, or 24 hr) with everything needed for glucuronidation reaction to proceed except the substrate. The substrate was then added to the mixture after 1, 4, 8, or 24 hr to allow the reaction to proceed for 1 hr as would normally done for any studies that measure glucuronidation rates. The rates were then determined by measuring amount of metabolites formed. In addition, we also added DTT (final

concentration in reaction mixture = 5 mM), a strong antioxidant, to the reaction mixture to determine if DTT could mitigate the thermal instability of the UGTs.

Statistical Analysis

One-way ANOVA or Student's T-tests was used to analyze the data. The prior level of significance was set at 5%, or $p<0.05$.

RESULTS

Prunetin Glucuronidation in Human Liver, Jejunum, and Ileum Microsomes

Microsomal glucuronidation of prunetin was site-/organ-dependent. At 40 μM, glucuronidation was faster in intestinal microsomes than liver microsomes, in that approximately 90% was metabolized by intestinal microsomes whereas approximately 12% of prunetin was metabolized by liver microsomes in 8 hrs. Intestinal glucuronidation of prunetin mainly formed metabolite 2 whereas the liver glucuronidation mainly formed metabolite 1. Specifically, the rate of formation of metabolite 2 was 72 times higher in the jejunal microsomes (2.20±0.10 nmol/min/mg protein), and 65 times higher in the ileal microsomes (1.99±0.10 nmol/min/mg protein) than that in the liver microsomes (0.030±0.001 nmol/min/mg protein) (Fig.2A). On the other hand, the formation rates of metabolite 1 using liver microsomes (0.073 ±0.001 nmol/min/mg protein) was about 7 times higher than using intestinal microsomes (0.010 ±0.001 nmol/min/mg protein). Taken together, the rate of metabolite 2 formation in jejunal microsomes were 30 times higher than the rate of metabolite 1 formation in liver microsomes.

We also determined the effect of concentration change on prunetin metabolism. At 2.5 μM, approximately 34% of prunetin was metabolized in 8 hr by liver microsomes and approximately 99% by intestinal microsomes. The formation of metabolite 1 was only detectable in liver microsomes at a rate of 0.040±0.002 nmol/min/mg protein, where the formation of metabolite 2 was at least 15 times higher in jejunal and ileal microsomes (0.156±0.01 nmol/min/mg protein) than in liver microsomes (0.010±0.001 nmol/min/mg protein) (Fig.2B). The formation rate by intestinal microsomes was underestimated at 2.5 μM since more than 98% of the substrate has been exhausted at 8 hr.

We also determined how prolongation of incubation time changed metabolism and the results indicated that after 24 hr incubation, 30% prunetin (concentration=40 μM) was metabolized by liver microsomes whereas 100% was metabolized by the intestinal microsomes. For 2.5 μM, the rates of intestinal microsomal metabolism were not quite meaningful since substrate was nearly exhausted by 8 hr, but liver microsomal metabolic rates were still meaningful as the results showed an approximately 70% substrate exhaustion at 24 hr. In addition, the retention time of two metabolite peaks did not change, and no additional metabolite peak was visible at longer incubation time, suggesting the absence of di-glucuronide formation as aglycone was exhausted.

Identification of Prunetin Metabolites

There are only two O-glucuronides identified in metabolism studies conducted using human microsomes. These two glucuronides have the same molecular weight (m/z=459 in negative ion mode, Fig.1B), corresponding to two phenolic groups available for O-glucuronidation in prunetin. Therefore, we only need to purify one of the metabolites to identify the structures of both. M2 was selected for purification because glucuronidation of prunetin in human intestinal microsomes produced this metabolite almost exclusively (>98% of the glucuronides). The structure of the main metabolite (M2) was identified by MS and NMR spectra. The positive ESI/MS spectrum showed that M2 and M1 have the same molecular weight as the monoglucuronide derivative of prunetin (m/z = 459) (Fig.1B). Its ^{1}H NMR suggested the

presence of a β-D-glucuronopyranosyl moiety in M2 as evidenced by the anomeric proton at δ 4.86 (1H, d, *J*=7.2 Hz). By comparing the ^{1}H NMR of M2 with those of parent compound, downfield shift of H-2′6′ ($\Delta\delta^{H}$ = + 0.27 ppm) and of H-3′5′ ($\Delta\delta^{H}$= + 0.12 ppm) was observed, indicating that the glucuronyl group is located at C-4′. This conclusion is further evidenced by the disappearance of the signal of 4′-OH (D_2O exchanged) at δ_H 9.62 (D_2O exchanged) in ^{1}H NMR of M2. Based on the above spectroscopic evidence, M2 was identified as prunetin 4′-*O*-β-D-glucuronide.

Metabolism of Prunetin by Expressed Human UGT Isoforms

Prunetin glucuronidation rates were measured using 12 expressed human UGT isoforms since drastically different patterns of prunetin metabolism in the human intestinal and liver microsomes (as shown earlier) were likely the results of organ-specific expression of UGT isoforms.

To investigate which UGT isoforms may be responsible, we first used one incubation time and a high substrate concentration to determine the contribution of UGT isoforms as has been done previously [5–9]. At a high concentration of prunetin (40 μM) and short incubation period (1 hr), UGTs 1A7, 1A8 and 1A9 produced significant amount of metabolite 1 and the averaged rates of glucuronidation were 0.14±0.0123, 0.24±0.0058, and 0.25±0.0109 nmol/min/mg of protein, respectively (Fig.3A). Among other tested UGT isoforms, only UGT1A3 and UGT1A10 made minor amounts of metabolite 1 (5-O-glucuronide), whereas other UGT1A and UGT2B subfamily members (UGT2B4, 2B7, 2B14 and 2B17) did not (Fig.3A). On the other hand, UGTs 1A1, 1A8, and 1A10 produced significant amounts of metabolite 2 (4'-O-glucuronide) and the glucuronidation rates were 0.04±0.001, 0.11±0.01, and 0.06±0.002 nmol/min/mg of protein, respectively (Fig.3B). Lastly, UGT2B4 and UGT2B7 produced minor but measurable amounts of prunetin metabolite 2 (4'-O-glucuronide) whereas other isoforms did not (Fig.3B).

Effect of Concentration Change on Prunetin Metabolism by UGT Isoforms

Some investigators have argued for the use of more than one (mostly two) substrate concentrations to probe isoform-specific metabolism [10–12], probably because different UGT isoforms will likely have different Km and Vmax values, resulting in different rates at different concentrations. Therefore, we also examined the UGT isoform-specific metabolism profiles using more physiological relevant concentration of 12.5 and 2.5 μM, again for the same short incubation time of 1 hour. At 12.5 μM, metabolite 1 (5-O-glucuronide) was formed by UGTs 1A7, 1A8, and 1A9 at rates of 0.17, 0.50±0.03, and 0.41±0.02 nmol/min/mg protein, respectively (Fig.3C); whereas metabolite 2 (4'-O-glucuronide) was formed by UGTs 1A1, 1A8, and 1A10 at rates of 0.04±0.003, 0.06±0.01, and 0.22±0.02 nmol/min/mg protein, respectively (Fig.3D). At this concentration, several isoforms produced minor amounts of metabolites 1 and 2 (Fig.3C and Fig.3D). At 2.5 μM, UGTs 1A7, 1A8, and 1A9 were still the main isoforms that made significant amounts of metabolite 1 (5-O-glucuronide) at rates of 0.11 ±0.01, 0.08±0.003, and 0.21±0.01 nmol/min/mg of protein, respectively; whereas metabolite 2 (4'-O-glucuronide) was formed in significant amounts by 1A1, 1A8, and 1A10 at rates of 0.02±0.004, 0.01±0.003, and 0.12±0.01 nmol/min/mg protein, respectively. Two isoforms (UGT1A1 and 2B4) made minor contribution to the formation of metabolite 1 whereas other isoforms did not make detectable contribution to the formation of either metabolite. Looking at the isoform-specific metabolism profiles of metabolite 1 (Figs.3A, 3C, and 3E), dominant isoforms changed with substrate concentration. This change in dominant isoforms was more obvious for metabolite 2 (see Figs.3B, 3D and 3F).

Effect of Incubation Time on Metabolite Formation

We were interested in determining the effect of time on metabolite formation for four reasons. First of all, metabolism of some isoflavones followed autoactivation[15, 19]. Second, metabolite could be a di-glucuronide, and if it is, its formation may continue to increase even if the substrate is depleted after a long incubation time as di-glucuronide can be formed from mono-glucuronide. Third, as a glucuronide is accumulated in the reaction mixture, it may inhibit the function of a particular isoform, resulting in the change of isoform specific metabolite profile. The latter was observed in intact Caco-2 cell metabolism of raloxifene, where the pattern of metabolism was different in cell lysate versus intact cells as result of longer incubation time [20]. Last, certain UGT isoform may be heat sensitive.

At 40 μM, prunetin (the substrate of reaction) was not exhausted, with a maximal of 70% being metabolized by UGT1A8 at 24 hr. The rates of metabolite 1 (5-O-glucuronide) formation measured at 1, 4, 8 and 24 hr following the incubation were fairly constant with a statistically significant ($p<0.01$) but small change of the most active form from UGT1A8 and UGT1A9 to UGT1A9 only (Fig.4A, 4C, 4E and 4G). On the other hand, the formation rates of metabolite 2 (4'-O-glucuronide) at 1, 4, 8 and 24 incubation hr were fluctuating substantially and the main isoform changed from UGT1A8 (at 1 hr) to UGT1A10 (for 4 hr or longer) (Fig.4B, 4D, 4F, 4H). This is not due to substrate exhaustion since only 15% of substrate was metabolized at 4 hr using the present experimental condition.

At 2.5 μM, which is a more physiological concentration, the substrate prunetin was 100% metabolized by UGT1A9 in less than 24 hr since more than 40% of prunetin was metabolized by UGT1A7 (49%) and UGT1A9 (45%) at 4 hr, with even higher percentages by 8 hr. The isoform specific metabolic profile for metabolite 1 (5-O-glucuronide) formation changed from UGT1A9 dominant to UGT1A7 and UGT1A9 dominant, and then back to UGT1A9 dominant with significant contribution from UGT1A8 (Fig.5A, 5C, 5E, 5G). The isoform specific metabolic profile for metabolite 2 was consistently dominated by UGT1A10 (Fig.5B, 5D, 5F, 5H). In addition, many minor contributing isoforms were found at a longer reaction time including UGT1A3, and UGT2B7.

Thermal Stability of Expressed UGTs

We were somewhat surprised by the fact that there was a strong dependence of the UGT "isoform-specific metabolic profiling" on the length of incubation. Since the incubation was conducted at 37°C, a series of experiments were performed to determine the stability of expressed isoforms. The results indicated that intestinal human UGT isoforms such as UGT1A8 and UGT1A10 were not stable, whereas liver UGT isoform UGT1A9 and ubiquitous UGT1A1 were relatively stable (Fig.6A & 6B). As a result, the liver UGTs was more stable than intestinal UGTs (Fig.6A & 6B). Addition of DTT to the incubation media did not stabilize UGTs, and in fact it inhibits the activities of UGT1A8 or UGT1A10, two thermo labile isoforms. Mixing of thermal unstable UGT isoforms with thermal stable UGT isoforms also did not stabilize the thermal labile UGTs (Fig.6C).

Comparison of Human and Rat Metabolism of Prunetin

We also determined glucuronidation of prunetin in rat liver and jejunal microsomes, using the same concentration(s) of microsomal protein (0.05 mg/ml in final reaction mixture) and prunetin (40 and 2.5 μM), and the same incubation time (8 and 24 hr) for the purpose of comparing these results with those obtained using human intestinal and liver microsomes. The results indicated that rat glucuronidation only share **limited similarities** in that metabolite 1 (5-O-glucuronide) was the main metabolite in the rat liver microsome as well, but only at low concentration of 2.5 μM (Fig.7). In addition, intestinal microsomes were more sensitive to length of incubation time than liver microsomes, although formation rates of metabolite 3 in

the rat intestinal microsomes were not heat sensitive. Other characteristics appeared to be quite different. Among them, metabolite 1 is no longer the main metabolite in the rat liver microsomes at the higher concentration (40 μM), whereas it was in human liver microsomes. Metabolite 1 is the main metabolite in rat intestine (Fig.7) even though human intestine almost did not produce it (Fig.2). We then performed β-O-D-glucuronidase hydrolysis experiment to determine which peaks will decrease or disappear following O-glucuronide hydrolysis experiments (Fig.8). Because both M1 and M2 peaks disappeared following β-O-D-glucuronidase treatment and have the same retention time and molecular weight (in MS/MS) as the human microsomes generated O-glucuronides, M1 was determined to be 5-O-β-D-glucuronide and M2 4'-O-β-D-glucuronide. In addition to these two O-glucuronides, a third glucuronidated metabolite (metabolite 3) appeared in the rat only. Since M3 was not hydrolysable using the β-O-D-glucuronidase, M3 is not a β-O-D-glucuronide but perhaps a C-glucuronide, which is consistent with its molecular weight of 441 (m/z) (Fig.1B). The position of this C-glucuronide is likely to be C-8, since prunetin has a naturally occurring C-8 glucoside: puerarin, derived from the plant Kudzu. This C-glucuronide is rather rare and has not been previously reported for UGT glucuronidation of flavonoids in mammals based on a PubMed search (conducted on September 4, 2007) using the keywords "c-glucuronide flavonoid" or "c-glucuronide flavone" or "c-glucuronide isoflavone."

Mouse Intestinal and Liver Metabolism of Prunetin

We also determined glucuronidation of prunetin in mouse liver and jejunal S9 fractions, using the same prunetin concentration (40 and 2.5 μM), and the same incubation time (8 and 24 hr) for the purpose of comparing these results with those obtained previously using rats and humans. The results indicated that mouse S9 fractions only formed 2 metabolites (Fig.9), similar to humans (Fig.2). The mouse intestinal S9 fraction metabolizes prunetin faster than liver microsomes, into two metabolites. Metabolite 1 is the major metabolite in mouse intestinal S9 fraction but metabolite 2 is the major metabolite in mouse liver S9 fraction. In addition, apparent rates of metabolism decreased as the incubation time increased at high prunetin concentration where only a small percentage (<30%) of prunetin is metabolized.

DISCUSSION

In our continued effort to characterize the intestinal disposition of flavonoids, we have begun in this paper to determine how human subcellular fractions such as microsomes and expressed human UGTs metabolize isoflavones. Earlier, we have conducted comprehensive studies of six isoflavones using rat intestinal and liver microsomes[15]. Therefore, the present work represented first systematic and comprehensive characterization of prunetin glucuronidation in human liver and intestinal microsomes and in expressed human UGT isoforms. We also contrasted the metabolism of prunetin in human liver and intestinal microsomes to that of rat or mouse liver and intestinal microsomes/S9 fractions. In addition to these two sets of conventional studies, we also performed pioneering study of how thermo stability of selected UGT isoforms and human intestinal and liver UGTs affected prunetin metabolism.

One of the results of our conventional studies was that intestinal microsomes produced metabolite 2 (4'-O-glucuronide) at rates substantially faster than metabolite 1 (5-O-glucuronide) by intestinal or liver microsomes. Based on distribution pattern of human UGT1As, we expected that prunetin would be mainly metabolized into metabolite 2 by UGT1A8 and 1A10 since they are mainly expressed in the intestine [21]. We expected that prunetin would be metabolized into metabolite 1 by UGT1A9 and UGT1A1 since they are abundantly expressed in the liver [22]. The results of our study confirmed our expectations, and were consistent with the results of an earlier study showing more rapid glucuronidation of genistein (but not daidzein) in human colon microsomes than in human liver microsomes [2].

We then performed additional studies to characterize the isoform specific metabolism of prunetin using so called “isoform-specific metabolism profiling” technique, which is the determination of UGT isoform(s) responsible for the glucuronidation of a specific substrate to form a specific metabolite. This is often performed because of the need to identify possible drug interaction potentials and to search out single nucleotide polymorphism of a UGT isoform that may impact the metabolism of a therapeutically important drug. It is also done to find out whether we can predict organ-specific metabolism based on the isoform distribution patterns.

We found that UGT “isoform-specific metabolism profiles” were metabolite- and substrate concentration-dependent (Fig.3), which was quite consistent with what other investigators have found using other UGT substrates. However, we were surprised that incubation time impacted the UGT “isoform-specific metabolism profiling” (Fig.4) since this was not expected as long as the concentration of prunetin (40 μM) was sufficient to sustain the reaction rate. This is a chance discovery since our initial aim was to use long incubation time to determine if there is a di-glucuronide during phase II reactions. In other words, we expected di-glucuronide peak to increase as the aglycone was exhausted, but this did not happen. What did happen was that the rates of metabolism decreased when incubation time increased, even if substrate concentrations were high enough to sustain constant reaction rates.

This led us to determine why reaction rates decreased as a function of incubation time, in that the longer the incubation time, the slower the rates of metabolism, which is especially evident in the intestinal microsomes. We hypothesized that this could be the result of isoform-specific thermo instability. Previously, SULT1A1 was found to be more heat stable than many other SULT isoform[23, 24].

The results of our studies showed that certain isoforms such as UGT1A9 and UGT1A1 were substantially more thermo stable than UGT1A10 and UGT1A8 (Fig.6). Interesting, the thermo labile isoforms appear to be mostly intestinal isoforms such as UGT1A10. In fact, UGT enzyme activities are more thermo stable in liver than in intestinal microsomes of humans. Using the same criteria, it appears that UGT activities in liver microsomes of rodents are also more thermo stable than those in the intestinal microsomes (Fig.7 and Fig 9).

At present, we do not know why certain UGT isoforms are heat labile whereas the other isoforms are heat resistant. Oxidation did not appear to be the reason, since the use of a strong antioxidant (5 mM DTT) failed to stabilize these isoforms. Mixing of heat-stable and heat-labile UGT isoforms also did not stabilize the heat-labile isoforms. Further studies are also needed to identify the mechanism by which heat deactivates these intestinal UGT isoforms but not the liver UGT isoforms.

Lastly, we also compared the similarities and differences in UGT metabolism of prunetin between humans and rodents (Fig.10). Our studies indicated that the intestinal microsomes were much more active than liver microsomes in all three species, a rare similarity. However, metabolite 1 was the predominant metabolite formed in both rat liver and intestinal microsomes, where metabolite 2 was the dominant human intestinal metabolite. Based on human and rat isoform expression pattern, we could assign this difference to the lack of UGT1A10 in rats [25]. In contrast, high expression levels of UGT1A1 and UGT1A7 in the rat liver and intestine [26] but not in the human liver or intestine (e.g., expression of UGT1A7 limited to the stomach in humans) probably explain why rat liver microsomes appeared to be more active than humans. It also explains why metabolite 1 was always the major metabolite in both organs. There was little difference in pattern of metabolism and type of metabolites formed between rat and mouse metabolism of prunetin. A major difference is the presence of C-glucuronide in rat intestinal microsomes but not in the intestinal or liver subcellular fractions

of mice (Fig.8). C-Glucuronide of prunetin was also not found in studies conducted using human microsomes.

In conclusion, UGT-mediated metabolism of prunetin was organ-, concentration-, and UGT isoform-specific, all of which may be impacted by the heat stability of UGTs involved in the glucuronidation. Because UGT isoforms have differential heat stability, more studies are needed to carefully characterize heat stability of various isoforms so that we can better measure UGT activities under various conditions. Lastly, our results suggest we should conduct "isoform-specific metabolism profiling" study at two or more concentrations and for at least two time periods. If those conditions provide consistent results, then no additional studies are required to complete the profiling study. Cares must be taken to interpret the results when only one concentration and one time are used for the characterization.

References

1. Ando Y, Hasegawa Y. Clinical pharmacogenetics of irinotecan (CPT-11). Drug Metab Rev 2005;37 (3):565–574. [PubMed: 16257834]
2. Doerge DR, Chang HC, Churchwell MI, Holder CL. Analysis of soy isoflavone conjugation in vitro and in human blood using liquid chromatography-mass spectrometry. Drug Metab Dispos 2000;28(3): 298–307. [PubMed: 10681374]
3. Malfatti MA, Felton JS. Human UDP-glucuronosyltransferase 1A1 is the primary enzyme responsible for the N-glucuronidation of N-hydroxy-PhIP in vitro. Chem Res Toxicol 2004;17(8):1137–1144. [PubMed: 15310245]
4. Krishnaswamy S, Duan SX, Von Moltke LL, Greenblatt DJ, Sudmeier JL, Bachovchin WW, Court MH. Serotonin (5-hydroxytryptamine) glucuronidation in vitro: assay development, human liver microsome activities and species differences. Xenobiotica 2003;33(2):169–180. [PubMed: 12623759]
5. Yamanaka H, Nakajima M, Katoh M, Kanoh A, Tamura O, Ishibashi H, Yokoi T. Trans-3'-hydroxycotinine O- and N-glucuronidations in human liver microsomes. Drug Metab Dispos 2005;33 (1):23–30. [PubMed: 15470160]
6. Kaji H, Kume T. Identification of human UDP-glucuronosyltransferase isoform(s) responsible for the glucuronidation of 2-(4-chlorophenyl)- 5-(2-furyl)-4-oxazoleacetic acid (TA-1801A). Drug Metab Pharmacokinet 2005;20(3):212–218. [PubMed: 15988124]
7. Mano Y, Usui T, Kamimura H. Identification of human UDP-glucuronosyltransferase responsible for the glucuronidation of niflumic acid in human liver. Pharm Res 2006;23(7):1502–1508. [PubMed: 16783480]
8. Murai T, Samata N, Iwabuchi H, Ikeda T. Human UDP-glucuronosyltransferase, UGT1A8, glucuronidates dihydrotestosterone to a monoglucuronide and further to a structurally novel diglucuronide. Drug Metab Dispos 2006;34(7):1102–1108. [PubMed: 16595710]
9. Brill SS, Furimsky AM, Ho MN, Furniss MJ, Li Y, Green AG, Bradford WW, Green CE, Kapetanovic IM, Iyer LV. Glucuronidation of trans-resveratrol by human liver and intestinal microsomes and UGT isoforms. J Pharm Pharmacol 2006;58(4):469–479. [PubMed: 16597364]
10. Mutlib AE, Goosen TC, Bauman JN, Williams JA, Kulkarni S, Kostrubsky S. Kinetics of acetaminophen glucuronidation by UDP-glucuronosyltransferases 1A1, 1A6, 1A9 and 2B15. Potential implications in acetaminophen-induced hepatotoxicity. Chem Res Toxicol 2006;19(5): 701–709. [PubMed: 16696573]
11. Bowalgaha K, Elliot DJ, Mackenzie PI, Knights KM, Swedmark S, Miners JO. S-Naproxen and desmethylnaproxen glucuronidation by human liver microsomes and recombinant human UDP-glucuronosyltransferases (UGT): role of UGT2B7 in the elimination of naproxen. Br J Clin Pharmacol 2005;60(4):423–433. [PubMed: 16187975]
12. Foti RS, Fisher MB. Assessment of UDP-glucuronosyltransferase catalyzed formation of ethyl glucuronide in human liver microsomes and recombinant UGTs. Forensic Sci Int 2005;153(2–3): 109–116. [PubMed: 16139098]
13. Keung WM. Biochemical studies of a new class of alcohol dehydrogenase inhibitors from Radix puerariae. Alcohol Clin Exp Res 1993;17(6):1254–1260. [PubMed: 8116840]

14. Delmonte P, Perry J, Rader JI. Determination of isoflavones in dietary supplements containing soy, Red Clover and kudzu: extraction followed by basic or acid hydrolysis. J Chromatogr A 2006;1107 (1–2):59–69. [PubMed: 16413562]
15. Wang SW, Chen J, Jia X, Tam VH, Hu M. Disposition of flavonoids via enteric recycling: structural effects and lack of correlations between in vitro and in situ metabolic properties. Drug Metab Dispos 2006;34(11):1837–1848. [PubMed: 16882763]
16. Chen J, Lin H, Hu M. Absorption and metabolism of genistein and its five isoflavone analogs in the human intestinal Caco-2 model. Cancer Chemother Pharmacol 2005;55(2):159–169. [PubMed: 15455178]
17. Chen J, Lin H, Hu M. Metabolism of flavonoids via enteric recycling: role of intestinal disposition. J Pharmacol Exp Ther 2003;304(3):1228–1235. [PubMed: 12604700]
18. Liu Y, Hu M. Absorption and metabolism of flavonoids in the caco-2 cell culture model and a perused rat intestinal model. Drug Metab Dispos 2002;30(4):370–377. [PubMed: 11901089]
19. Chen J, Wang S, Jia X, Bajimaya S, Tam V, Hu M. Disposition of Flavonoids via Recycling: Comparison of Intestinal versus Hepatic Disposition. Drug Metab Dispos 2005;33:1777–1784. [PubMed: 16120792]
20. Jeong EJ, Lin H, Hu M. Disposition mechanisms of raloxifene in the human intestinal Caco-2 model. J Pharmacol Exp Ther 2004;310(1):376–385. [PubMed: 15020665]
21. Radominska-Pandya A, Little JM, Pandya JT, Tephly TR, King CD, Barone GW, Raufman JP. UDP-glucuronosyltransferases in human intestinal mucosa. Biochim Biophys Acta 1998;1394(2–3):199–208. [PubMed: 9795217]
22. Kiang TK, Ensom MH, Chang TK. UDP-glucuronosyltransferases and clinical drug-drug interactions. Pharmacol Ther 2005;106(1):97–132. [PubMed: 15781124]
23. Kudlacek PE, Clemens DL, Anderson RJ. Characterization of recombinant human liver thermolabile phenol sulfotransferase with minoxidil as the substrate. Biochem Biophys Res Commun 1995;210 (2):363–369. [PubMed: 7755612]
24. Li X, Clemens DL, Cole JR, Anderson RJ. Characterization of human liver thermostable phenol sulfotransferase (SULT1A1) allozymes with 3,3x00027;,5-triiodothyronine as the substrate. J Endocrinol 2001;171(3):525–532. [PubMed: 11739018]
25. Emi Y, Ikushiro S, Iyanagi T. Drug-responsive and tissue-specific alternative expression of multiple first exons in rat UDP-glucuronosyltransferase family 1 (UGT1) gene complex. J Biochem (Tokyo) 1995;117(2):392–399. [PubMed: 7608130]
26. Shelby MK, Cherrington NJ, Vansell NR, Klaassen CD. Tissue mRNA expression of the rat UDP-glucuronosyltransferase gene family. Drug Metab Dispos 2003;31(3):326–333. [PubMed: 12584160]

ABBREVIATIONS

UGT, UDP-glucuronosyltransferases; UDPGA, uridine diphosphoglucuronic acid; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DTT, dithiothreitol.

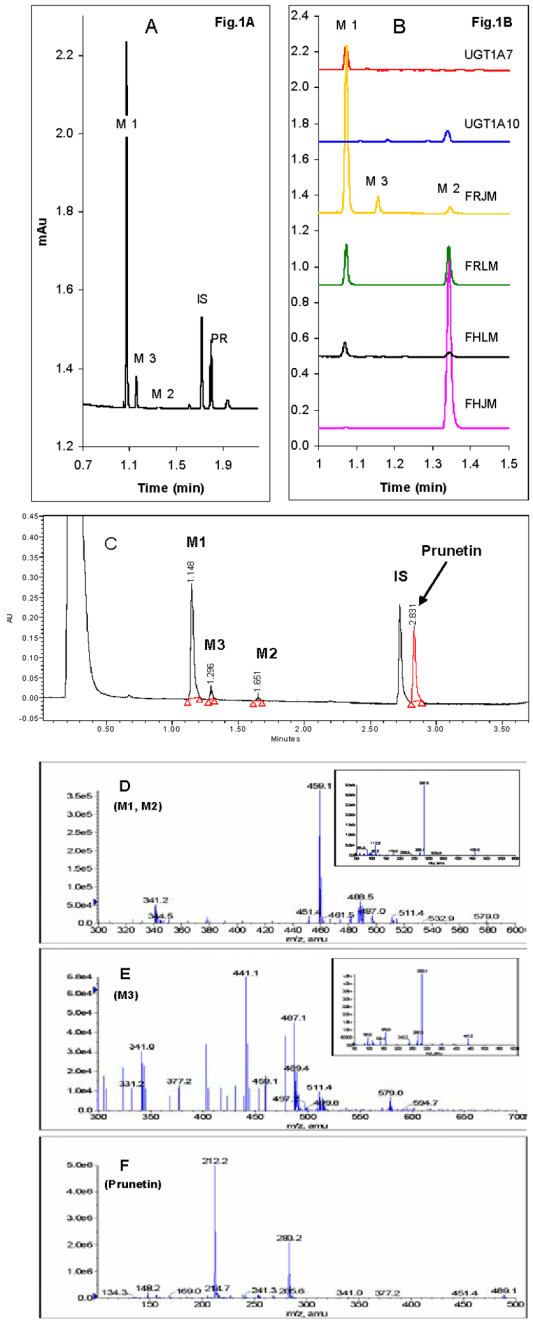

Fig. 1.

Ultra performance LC (UPLC) chromatograms of prunetin and prunetin metabolites following UV absorbance (Fig.1A, Fig.1B, Fig.1C) or MS/MS spectral analysis (Fig.1D–1F). The UPLC methods were described in the "Method" section. Fig.1A shows the UPLC trace of 40 μM of prunetin metabolism by female rat jejunal microsome (FRJM), with peak of metabolite 1 (M1), metabolite 2 (M2), metabolite 3 (M3), internal standard (IS), and prunetin (PR) labeled. Fig. 1B shows metabolites 1–3 (if present) of 40 μM of prunetin in female human jejunal microsomes (FHJM), female human liver microsomes (FHLM), female rat liver microsomes (FRLM), female rat jejunal microsomes (FRJM), expressed human UGT1A10 (UGT1A10), and expressed human UGT1A7 (UGT1A7). Notice the distinct pattern of metabolite formation.

Fig.1C shows an alternative UPLC tracing using UV absorbance, which shows prunetin and its three glucuronidated metabolites. Fig.1D–1F shows the MS3 spectra of prunetin *O*-glucuronide (Fig.1D), prunetin *C*-glucuronide (Fig.1E) and prunetin (Fig.1F), respectively. The small windows in Fig.1D and Fig.1E show the MS2 full scan for the corresponding metabolite.

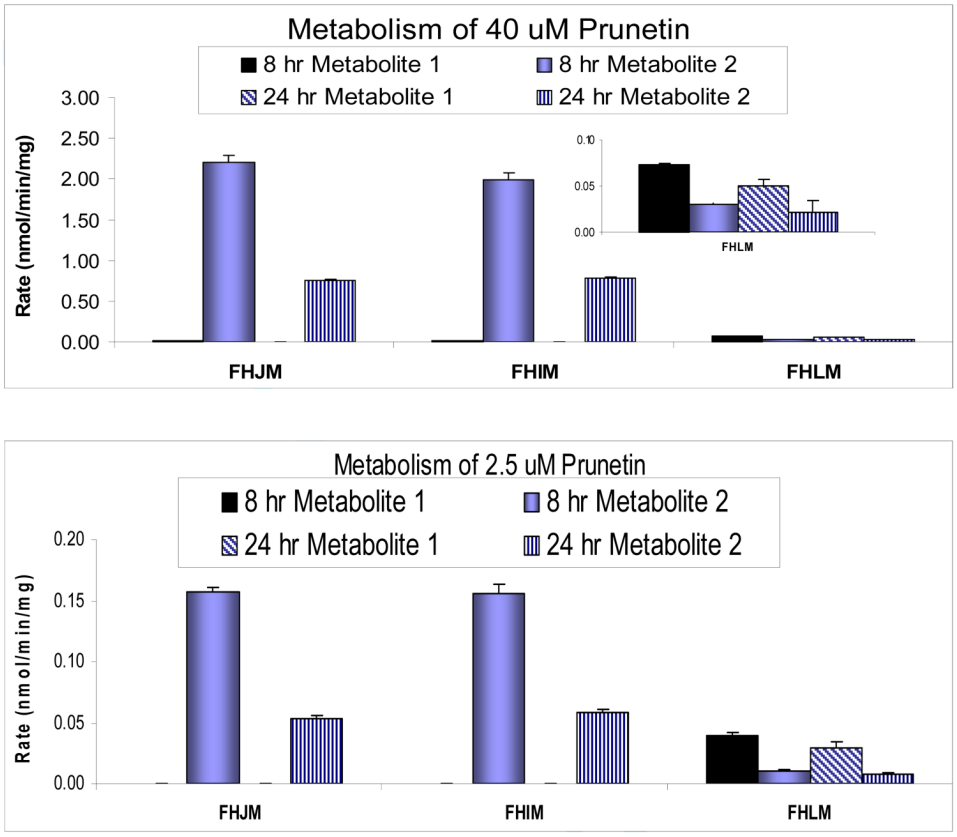

Fig. 2.

Metabolism of prunetin by female human jejunal microsomes (FHJM), female human ileal microsomes (FHIM) and female human liver microsomes (FHLM) at two concentrations (40 or 2.5 μM) and two incubation time (8 and 24 hr). The experiments were conducted at 37°C for two incubation times and substrate concentrations and the amounts of metabolite 1 and metabolite 2 were measured using UPLC (Table 1). Each bar is the average of three determinations and the error bars are the standard deviations of the mean.

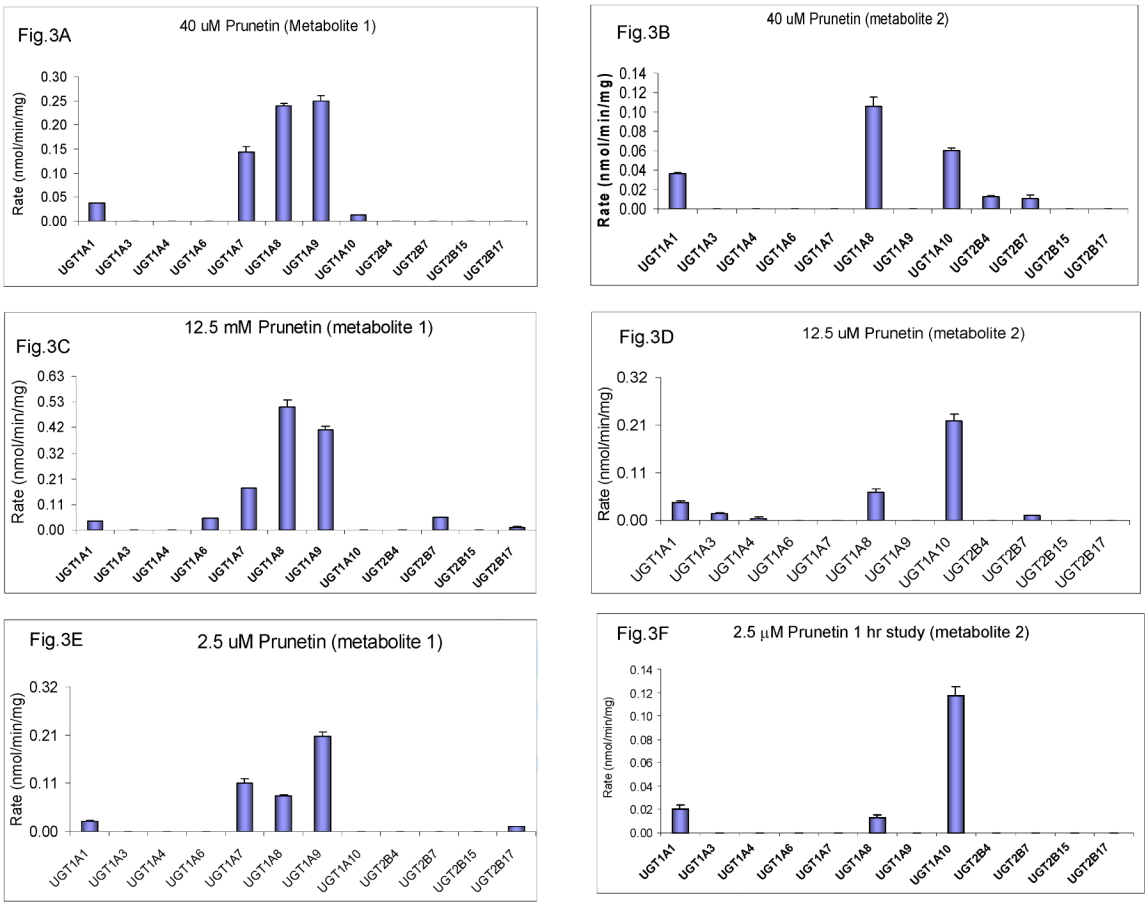

Fig. 3.

Effects of concentration on the isoform-specific metabolism of prunetin. The experiments were conducted at 37°C for 1 hr and the amounts of metabolite 1 and metabolite 2 were measured using UPLC. Each bar is the average of three determinations and the error bars are the standard deviations of the mean.

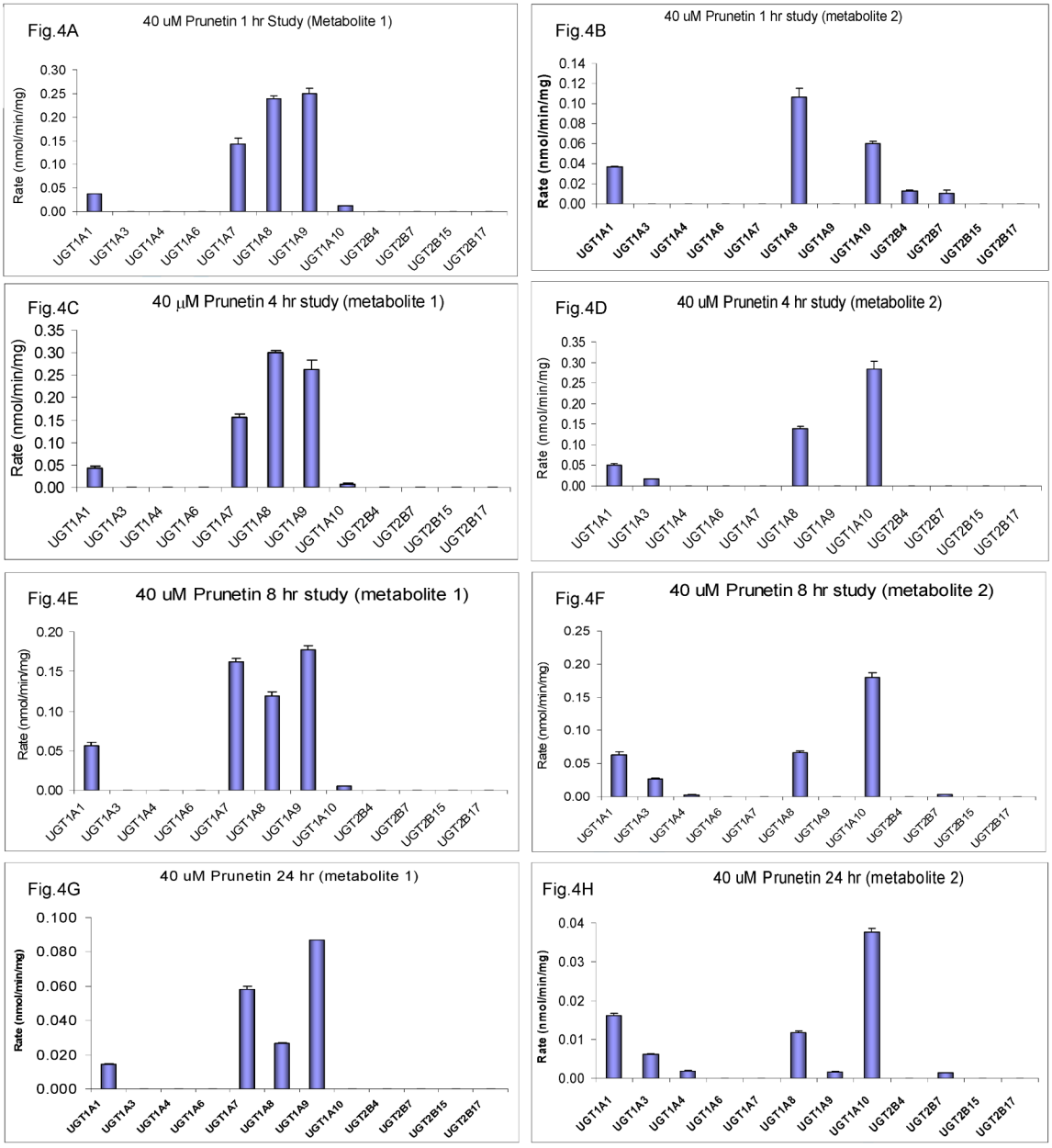

Fig. 4.

Effects of incubation time on the isoform-specific metabolism of prunetin at 40 μM. The experiments were conducted at 37°C for various times (1, 4, 8, or 24 hr) and the amounts of metabolite 1 and metabolite 2 were measured using UPLC. Each bar is the average of three determinations and the error bars are the standard deviations of the mean.

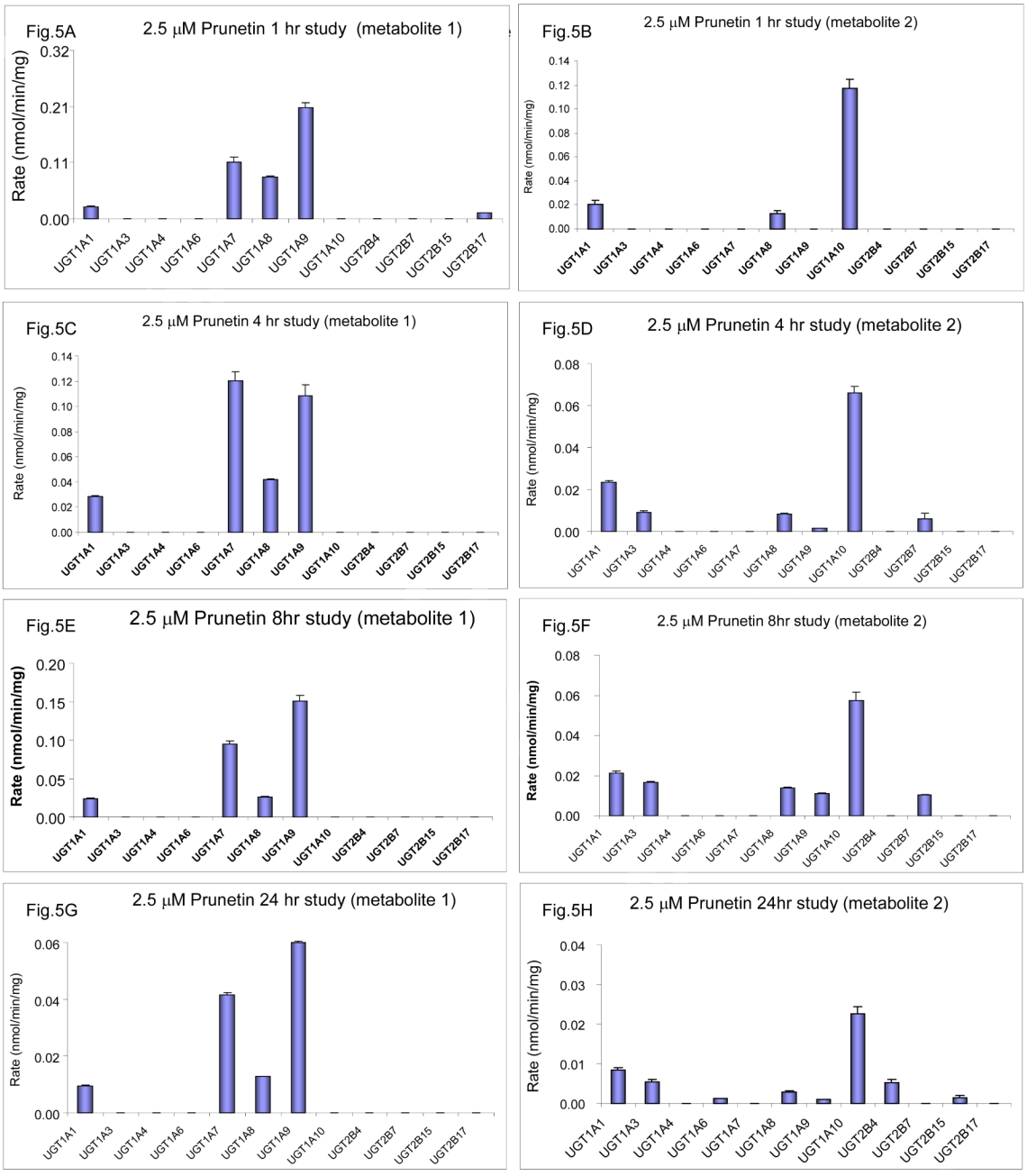

Fig. 5.

Effects of incubation time on the isoform-specific metabolism of prunetin at 2.5 μM. The experiments were conducted at 37°C for various time and the amounts of metabolite 1 and metabolite 2 were measured using UPLC (Table 1). Each bar is the average of three determinations and the error bars are the standard deviations of the mean.

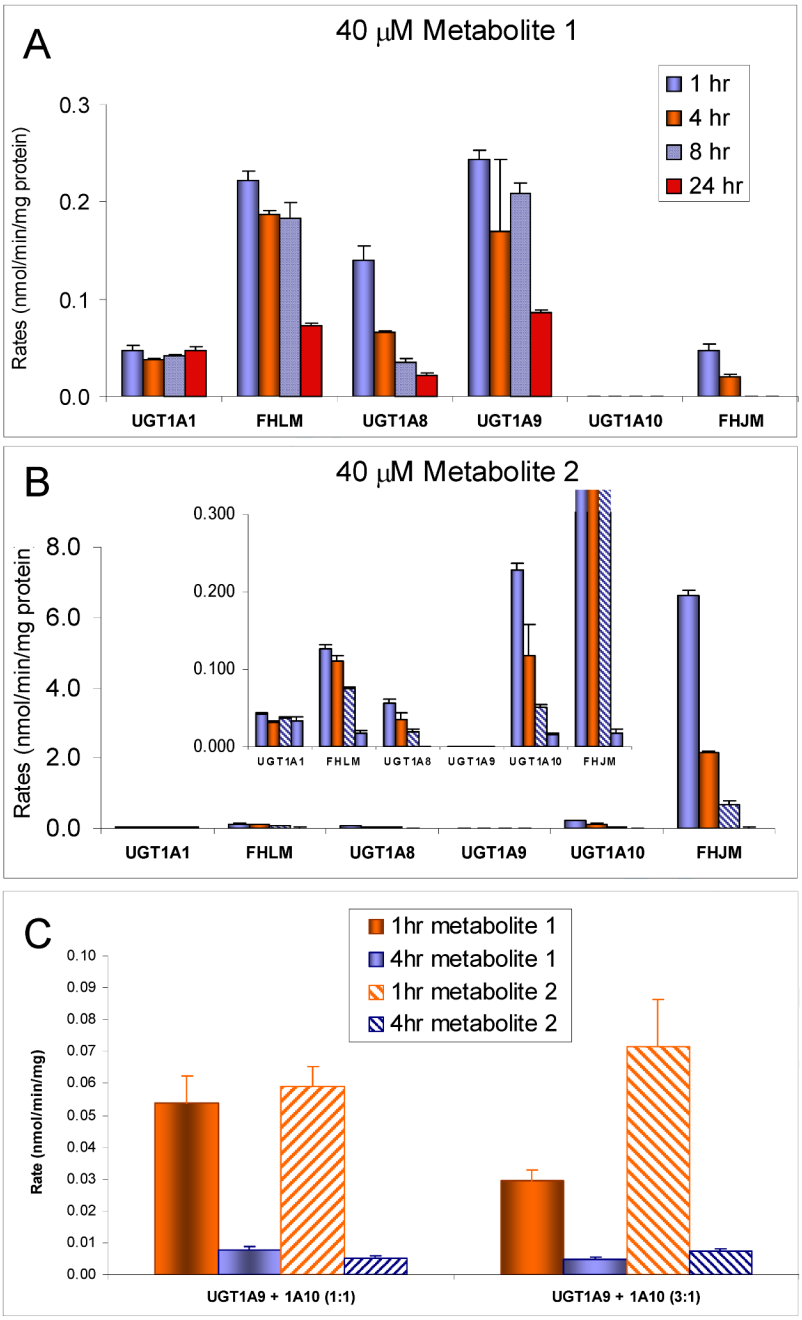

Fig. 6.

Testing of thermal stability of UGT activities using human microsomes and expressed UGT microsomes. The abilities of UGT to form metabolite 1 or metabolite 2 were followed as a function of pre-incubation time (or the length of time where microsomes were incubated with everything but substrate). The concentration used was 40 μM and the actual reaction time was 1 hr. Each bar is the average of three determinations and the error bars are the standard deviations of the mean.

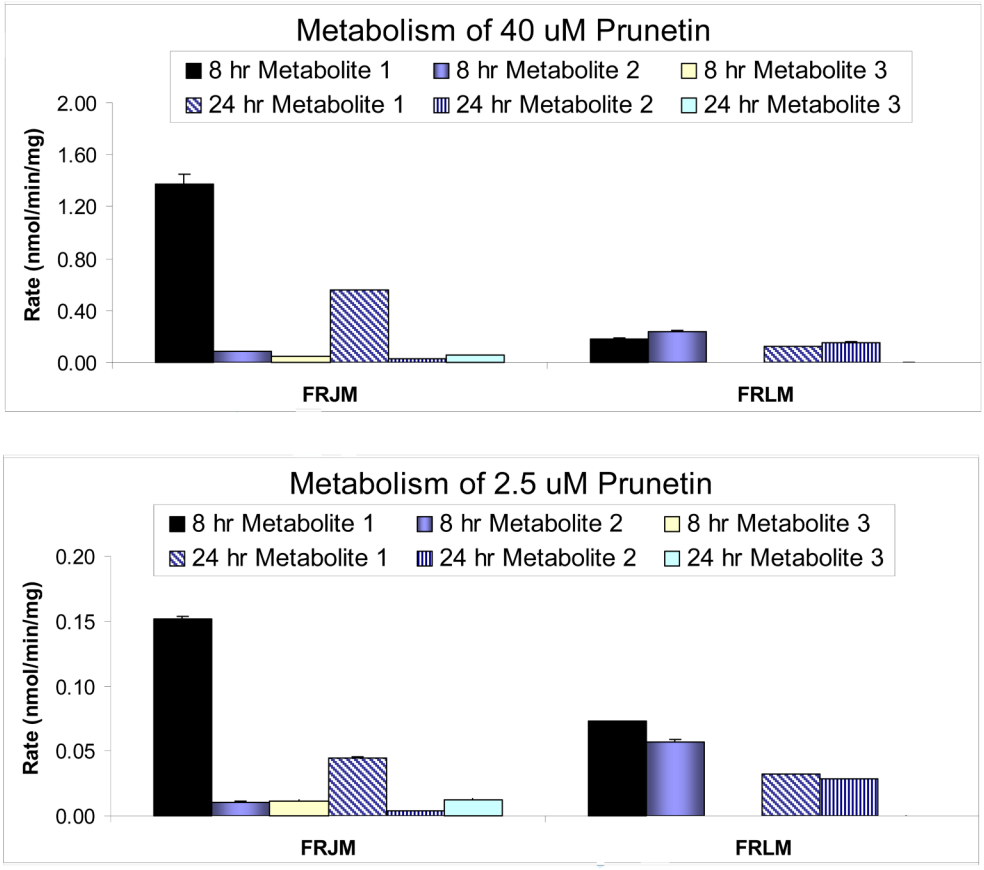

Fig. 7.

Metabolism of prunetin by female rat jejunal microsomes (FRJM), and female rat liver microsomes (FRLM) at two concentrations (40 or 2.5 μM) and two incubation time (8 and 24 hr). The experiments were conducted at 37°C and the amounts of metabolite 1, metabolite 2 and metabolite 3 were measured using UPLC. Each bar is the average of three determinations and the error bars are the standard deviations of the mean.

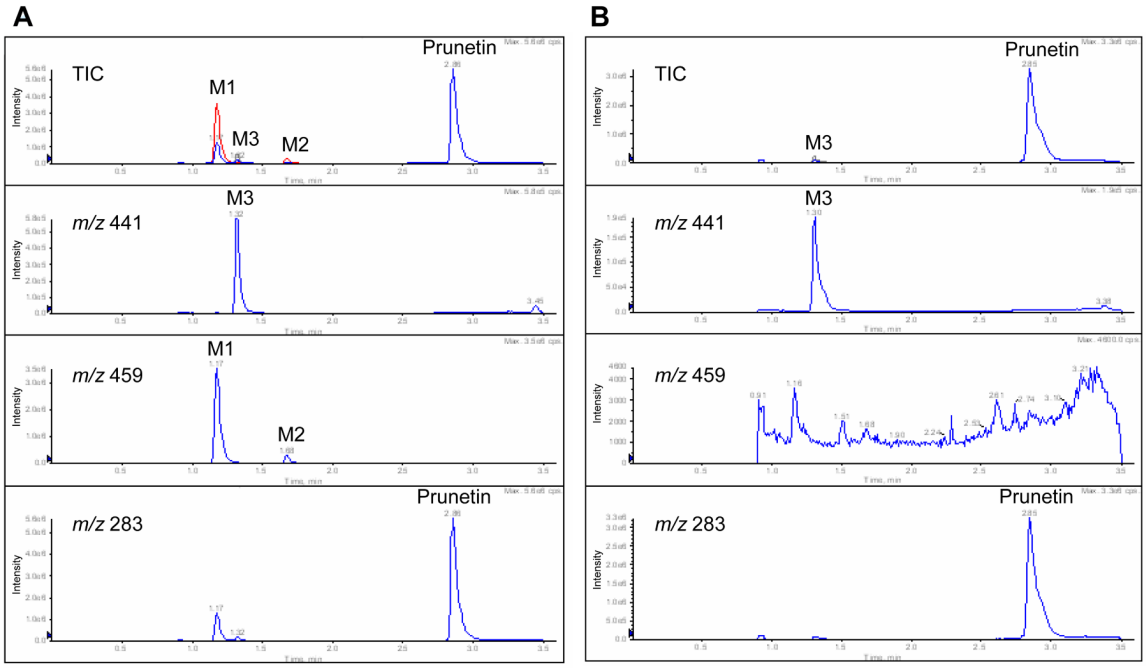

Fig. 8.

Total anion scan (TIC) or selective anion scan (m/z 441, m/z459, and m/z 283) of rat microsomal metabolism samples containing prunetin and three of its glucuronides before (Panel A) and after (Panel B) hydrolysis with β-D-glucuronidases (24 hrs). Clearly shown in this figure is that C-glucuronide (m/z 441) is not hydrolysable by β-D-glucuronidases. There was some cross talk between prunetin (m/z 283) and its glucuronides as these glucuronides are not very stable during the ionization process.

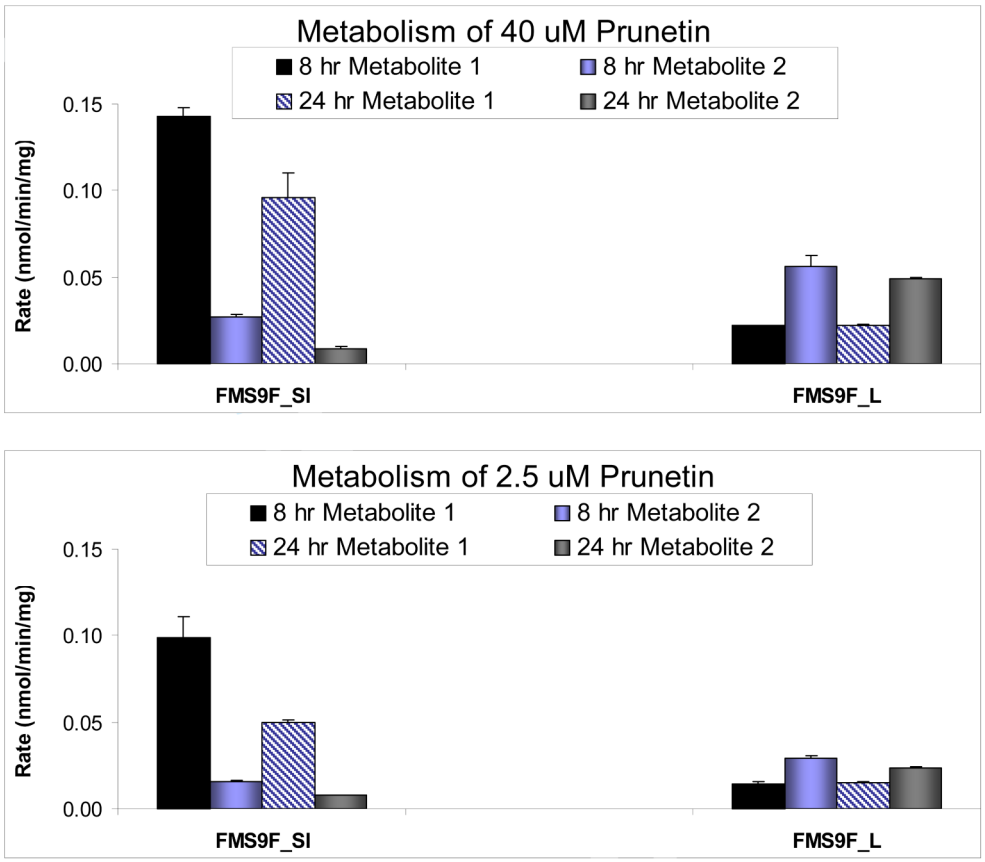

Fig. 9.

Metabolism of prunetin by female mouse jejunal S9 fraction (FMS9F_SI), and female mouse liver S9 fraction (FMS9F_L) at two concentrations (40 or 2.5 μM) and two incubation time (8 and 24 hr). The experiments were conducted at 37°C and the amounts of metabolite 1 and metabolite 2 were measured using UPLC. Each bar is the average of three determinations and the error bars are the standard deviations of the mean.

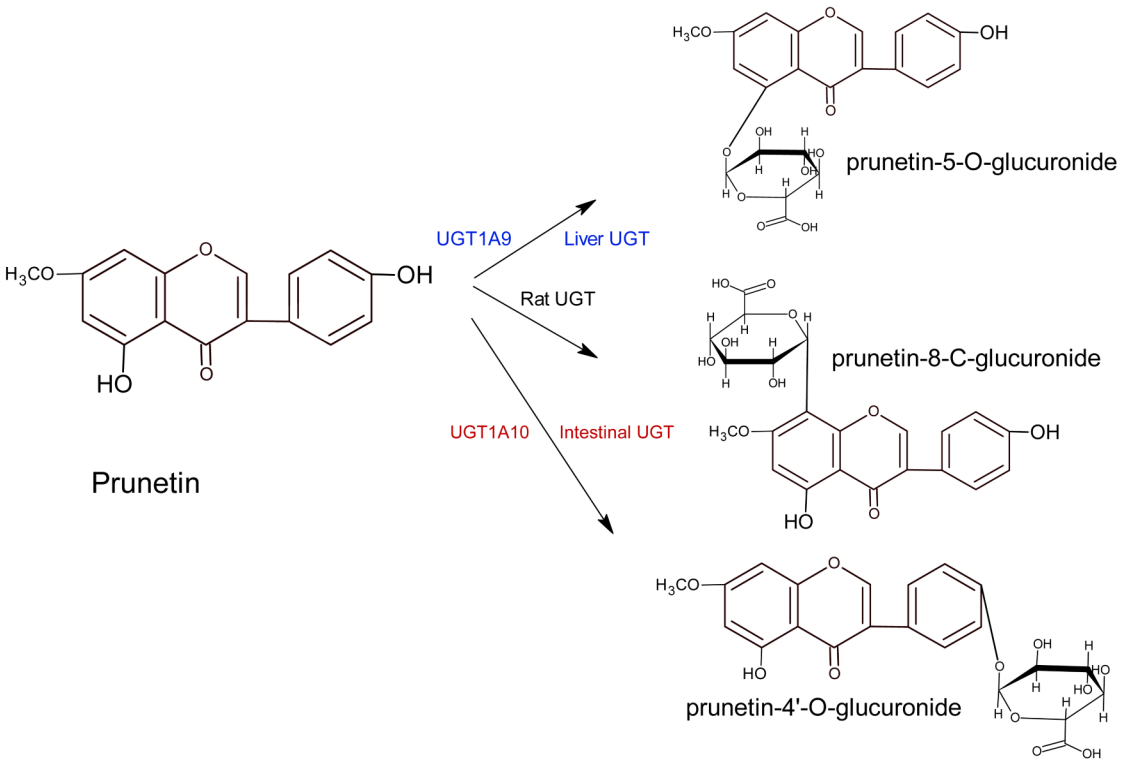

Fig. 10.
Metabolic pathways of prunetin in intestine and liver of rodents and humans. Position of C-glucuronide remained to be confirmed using NMR.