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Disposition of Flavonoids via Enteric Recycling: UGT1As Deficiency in Gunn Rats Is Compensated by Increases in UGT2Bs Activities

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JPET#147371

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JPET#147371

List of Non-Standard Abbreviations: ANOVA, Analysis of Variance; HPLC, High-Performance Liquid Chromatography; UGT, Uridine 5'-diphosphate glucuronosyltransferases; SN-38, 7-Ethyl-10-Hydroxycamptothecin; MRP2, Multidrug Resistance-Associated Protein 2; MRP3, Multidrug Resistance-Associated Protein 3

JPET#147371

ABSTRACT

Flavonoids have poor bioavailabilities largely because of metabolism via UDP-glucuronosyltransferases (UGT). This study aims to further understand the functions of UGT in metabolizing genistein and apigenin, two compounds metabolized more extensively in the gut than the liver. Since Gunn rats are deficient in UGT1As, we determined if this deficiency would result in less flavonoid glucuronidation, using rat intestinal perfusion model and microsomes prepared from rat liver and intestine. In yeast-expressed rat UGT isoforms, rat UGT1A isoforms (especially UGT1A7) were mainly responsible for flavonoid metabolism. In perfusion studies, the two flavonoids were rapidly absorbed at comparable rates, but the intestinal excretions of glucuronides in Gunn rats as compared to Wistar rats were not only comparable for genistein but were higher ($p < 0.05$) for apigenin, suggesting up-regulation of UGT isoforms in Gunn rats. To determine the possible compensatory UGT isoforms, we first verified that UGT1A activities were significantly lower ($p < 0.05$) in Gunn rats by using UGT1A-specific probes SN-38 and prunetin. It was then demonstrated using UGT2B probes testosterone, ezetimibe, and indomethacin that UGT2B activities were usually significantly higher in Gunn rats. Also, testosterone was metabolized much faster in liver microsomes than in intestinal microsomes, and in microsomes prepared from Gunn rats than Wistar rats. In conclusion, flavonoids are efficiently metabolized by UGT1A-deficient Gunn rats because of compensatory up-regulation of intestinal UGT2Bs and hepatic anion efflux transporters, which increases their disposition and limits their oral bioavailabilities.

JPET#147371

INTRODUCTION

Flavonoids hold great promises as agents to improve human health because epidemiological studies have shown protective effects associated with flavonoid consumption against coronary heart diseases (Arts and Hollman, 2005), stroke (Keli et al., 1996), and lung and colorectal cancers (Hirvonen et al., 2001; Bobe et al., 2008). Currently, several clinical trials are ongoing to test the anticancer efficacy of soy isoflavones and green tea, both of which contain flavonoids as the active ingredients (Crowell, 2005). A major impediment to the development of flavonoids is their poor bioavailabilities, which are usually about 3-5% (Coldham et al., 2002). Poor bioavailabilities make it more difficult to test flavonoids in clinical setting because it requires a large number of subjects as exposure levels are going to be highly variable across the population. Poor bioavailabilities also make it more challenging to get sufficient amount of flavonoids into humans from dietary sources (e.g., fruits and vegetables), or in the form of a pill (e.g., soy isoflavone tablets).

Improving our understanding of the processes that govern flavonoid bioavailabilities, hence, is of great significance if we were to identify means to improve flavonoid bioavailabilities.

Researchers from several laboratories including ours have been studying these processes and shown that flavonoid aglycones are well absorbed, but absorbed flavonoids are rapidly metabolized via phase II conjugation, resulting in poor bioavailabilities (Busby et al., 2002; Chen et al., 2003; Hu et al., 2003; Setchell et al., 2003; Walle, 2004; Chen et al., 2005a). Because flavonoid conjugation is predominantly carried out by UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs), the metabolites formed are highly hydrophilic organic anions

JPET#147371

(Chen et al., 2003; Chen et al., 2005a; Jeong et al., 2005c). These organic anions are then eliminated by active efflux transporters because of their inability to passively diffuse across the cell membranes (Liu and Hu, 2007).

A coupling mechanism where UGTs and efflux transporters (i.e., MRP2) work in concert is considered to be necessary to enable rapid elimination of flavonoid conjugates (Jeong et al., 2005b). This coupling mechanism enables efficient metabolism of flavonoids, resulting in poor oral bioavailabilities. It also allows efficient enteric and enterohepatic recycling, which allow the excreted flavonoid glucuronides (sulfates) to return as aglycones following their hydrolysis by microflora, thereby increasing their apparent half-lives. The enterohepatic recycling has long been documented as the mechanism responsible for the apparent inconsistency of low bioavailabilities (3-5%) but long (3-7 hrs) apparent half-lives of flavonoids (Cassidy, 2006; Moon et al., 2006). Our laboratory has shown recently that enteric recycling contributes to and further enhances the flavonoid recycling (Chen et al., 2003; Chen et al., 2005b; Jeong et al., 2005a; Wang et al., 2006).

Earlier studies from our laboratory shown that glucuronides not sulfates (usually not detected in rats) were the predominant metabolites of flavonoids such as genistein and apigenin, and that rat intestinal UGTs play a more important role than liver UGTs (Chen et al., 2003; Chen et al., 2005b; Wang et al., 2006). However, it was unknown which rat UGT isoforms were responsible for the metabolism of flavonoids, and if deficiency in major rat UGT isoforms will result in changes in metabolic phenotypes. The latter is important if we aim to increase flavonoid bioavailabilities, since less flavonoid metabolism in Gunn rats would mean that it is feasible to

JPET#147371

use selective chemical inhibitors of UGT1As to increase flavonoid bioavailabilities. It is also important since certain UGT1A polymorphism could affect the potential beneficial effects of flavonoids, since UGT polymorphism was shown to affect toxicities and efficacies of SN-38 as well as androgen signaling (Ando and Hasegawa, 2005; Chouinard et al., 2008).

The current study: 1) determined the consequences of UGT1As deficiency (as in Gunn rats) in the overall glucuronidation of flavonoids; and 2) identified mechanisms that may compensate the functional absence of UGT1As in Gunn rats.

JPET#147371

METHODS

Materials. Genistein and apigenin were purchased from Indofine Chemicals (Somerville, NJ). Testosterone, indomethacin, β -glucuronidase with (catalog no. G1512) or without sulfatase (catalog no. G7396), uridine diphosphoglucuronic acid, alamethicin, D-saccharic-1,4-lactone monohydrate, magnesium chloride, Tris, 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 without further purification.

Animals. Male Gunn rats (10-11 weeks old, verified as homozygous (j/j) by the supplier) and their controls (i.e., the Wistar rats) weighing between 300 and 324 g were purchased from Harlan Laboratories (Madison, WI). Gunn rats are deficient in UGT1As because of a recessive gene (Chowdhury et al., 1993). The rats were fed with Teklad F6 rodent diet (W) from Harlan Laboratories (Madison, WI). The rats were fasted overnight before the day of the experiment. No flavonoids were found in perfusate obtained by perfusing blank pH 7.4 HBSS buffer through a segment of jejunum, indicating minimal presence of dietary flavonoids in the intestine.

Rat intestinal microsomes and liver microsomes preparation. Rat intestinal microsomes and male rat liver microsomes were prepared as described (Chen et al., 2003). The resulting microsomes were suspended in 250 mM sucrose solution, and separated into microcentrifuge tubes, and stored at -80°C until use.

JPET#147371

Measurement of protein concentration. Protein concentrations of microsomes were determined by a protein assay kit (Catalog # 500-0006, Bio-Rad, Hercules, CA) using bovine serum albumin as the standard.

Expression of UDP-Glucuronosyltransferase Isoforms in Yeast AH22 Cells. The procedures for the preparation of UGT isoforms were described previously and same yeast cell lysates were used in the present study (Daidoji et al., 2005). All UGT isoforms expressed by yeast microsomes were confirmed using Western blots (not shown) and subsequently used for glucuronidation reactions using procedures described below.

Measurement of UGT activities using microsomes. The incubation procedures for measuring UGT activities using microsomes were as follows: 1) mix microsomes (final concentration \approx 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=200 μ l) at 37°C for 30 or 60 min; and 3) stop the reaction by addition of 50 μ l of 94% acetonitrile/6% glacial acetic acid containing 100 μ M testosterone as the internal standard.

Sample extraction. To confirm the formation of a particular conjugate, samples were selectively extracted with methylene chloride to remove >90% of aglycones. The resulting sample was then divided into two parts, one of which was analyzed after incubating with water (same volume as the enzyme) and the other was analyzed after hydrolysis by glucuronidase. The difference in amount of aglycones found in these two samples was the amount of metabolite formed. The

JPET#147371

relationship between the peak areas of the metabolites before hydrolysis and the peak areas of aglycones after the hydrolysis is used to establish the conversion factor used to quantify the amounts of isoflavone glucuronides as described previously (Liu and Hu, 2002).

Animal surgery. The procedures were approved by the University of Houston's Institutional Animal Care and Uses Committee. The intestinal surgical procedures were modified from our previous publications (Hu et al., 1998), in that four segments of the intestine were cannulated simultaneously (a "four-site model") along with a bile duct cannulation (Liu and Hu, 2002; Chen et al., 2003; Wang et al., 2006). The circulation to the liver and intestine was not disrupted in this model. To keep the temperature of the perfusate constant, the inlet cannulate was insulated and kept warm by a 37°C circulating water bath.

Transport and metabolism experiments in perfused rat intestinal model. This is a single-pass perfusion method. Four segments of the intestine (duodenum, upper jejunum, terminal ileum, and colon) were perfused simultaneously with a perfusate containing the compound of interest using an infusion pump (Model PHD2000; Harvard Apparatus, Cambridge, MA) at a flow rate of 0.191 ml/min. After a 30-min washout period, which was usually sufficient to achieve the steady-state absorption, four samples were collected from the outlet cannulae every 30 min. Bile samples (about 0.4 ml) were collected before perfusion started and every 30 min afterward. After perfusion, the length of the intestine was measured as described previously (Hu et al., 1988; Hu et al., 1998). The outlet concentrations of test compounds in the perfusate were determined by HPLC. Bile samples (without detectable aglycones) were diluted (1:10) with

JPET#147371

buffer, added glucuronidase + sulfatase, and reacted for 2 h to release the aglycones for HPLC measurement.

HPLC analysis of flavonoids/isoflavonoids and their conjugates. The conditions for analyzing genistein, apigenin, testosterone, indomethacin and ezetimibe and their conjugates were as follows: system, Agilent 1090 with diode array detector and ChemStation; column, Aqua (Phenomenex, Gilroy, CA), 5 μm , 150 \times 0.45 cm; mobile phase A, water (0.04% H_3PO_4 and 0.09% triethylamine or $\text{C}_6\text{H}_{15}\text{N}$, pH 6.0); mobile phase B, 100% acetonitrile; gradient, 0-3 min, 15% B, 3-15, 15 to 19% B, 15- 52, 19 to 41% B; wavelength, 254 nm (for the isoflavones and the internal standard); and injection volume, 200 μl . There was a 4-min interval between the end of the run and the next injection to allow the column to be reequilibrated with 15% mobile phase B. The detection limit is 0.25 μM , and the tested linear range is 0.5-100 μM . The precision is typically better than 5% and accuracy better than 10%.

UPLC analysis of flavonoids/isoflavonoids and their conjugates. Selected samples were also run on ultra-performance LC (UPLC) that was much more time efficient than HPLC. The conditions for analyzing isoflavones using UPLC were as follows: system, Waters AcQuity™ UPLC with photodiode array detector and Empower software; column, Acquity™ UPLC BEH C18 (Waters, Milford, MA), 1.7 μm , 2.1 \times 50 mm; mobile phase A, 100% acetonitrile, mobile phase B, aqueous solution (0.06% triethylamine and 0.045% formic acid:methanol 90:10); gradient, 0-0.3 min, 0% B, flow rate = 1ml/min, 0.3-1.80 min, 0-50% B, flow rate = 0.925 ml/min, 1.80-2.10 min, 50-100% B, flow rate = 0.925ml/min, 2.10-2.40 min, 100% B, flow rate = 0.925, 2.40-2.50, 100%-0% B, flow rate = 1ml/min; wavelength, 254 nm; and injection

JPET#147371

volume, 10 μ l. We have recently started to use UPLC method to measure the concentration of flavonoids. The detection limit is 0.2 μ M and the tested linear range is from 0.38-50 μ M. The precision is typically within 5% and accuracy 9%.

Data analysis. Amounts of isoflavones absorbed (M_{ab}), amounts of conjugated isoflavones excreted into the intestinal lumen (M_{gut}), amounts of conjugated isoflavones excreted via the bile (M_{bile}) and the percent absorbed and percent metabolized values were calculated as described (Chen et al., 2003; Wang et al., 2006). Briefly, M_{ab} was expressed as:

$$M_{ab} = Q \cdot \tau \cdot (CA_{in} - CA_{out}) \quad \text{Equation (1)}$$

where Q is the flow rate (ml/min), τ is the sampling interval (30 min), and CA_{in} and CA_{out} are the inlet and outlet concentrations of aglycones corrected for water flux, respectively. M_{gut} was expressed as:

$$M_{gut} = Q \cdot \tau \cdot CM_{out} \quad \text{Equation (2)}$$

where CM_{out} is the outlet concentrations (nmol/ml) of metabolites corrected for water flux.

And, M_{bile} was expressed as:

$$M_{bile} = V \cdot CM_{bile} \quad \text{Equation (3)}$$

JPET#147371

where CM_{bile} is the bile concentrations (nmol/ml) of metabolites, and V is the volume of bile collected over a 30 min time period.

% Absorbed and % Metabolized were calculated as:

$$\% \text{ Absorbed in the Intestine} = \frac{M_{ab}}{M_{total}} \times 100\% \quad \text{Equation (4)}$$

$$\% \text{ Metabolites Excreted in the Intestine} = \frac{M_{gut}}{M_{total}} \times 100\% \quad \text{Equation (5)}$$

where M_{total} is the total amount of compound perfused over a 30 min time period.

Rates of metabolism in intestinal or liver microsomes were expressed as amounts of metabolites formed per min per mg protein or nmol/min/mg. Detailed procedures and models used to determine the kinetic parameters were previously published (Chen et al., 2005b; Wang et al., 2006). Briefly, assuming the glucuronidation rates versus concentration curves follow simple Michaelis-Menten kinetics, equation (6) is used to derive K_m , V_{max} , and the intrinsic clearance (V_{max}/K_m) values.

$$V = \frac{V_{max} \times C}{K_m + C} \quad \text{Equation (6)}$$

JPET#147371

Statistical analysis. General linear model (or GLM) ANOVA, two sample T-tests, one-way ANOVA, and sub sequential Tukey-Kramer multiple comparison tests (Critical Value = 4.1987) were used to analyze the data (NCSS 2001). The prior level of significance was set at 5%, or $p < 0.05$.

JPET#147371

RESULTS

Metabolism of flavonoids by expressed rat UGT isoforms.

The main conjugation reaction of genistein and apigenin in rats is glucuronidation, which is expected to be catalyzed by multiple UGT isoforms (Doerge et al., 2000; Jia et al., 2004; Chen et al., 2005b; Wang et al., 2006).

Because microsomes prepared from both intestine and liver are composed of pooled UGT isoforms, it is impossible to determine the UGT isoform(s) responsible without specific chemical or macromolecular (monoclonal) inhibitors. Therefore, AH22 yeast-expressed rat UGT isoforms were used to determine which isoform(s) are responsible for flavonoid glucuronidation at 50 μ M. A high concentration of substrate is used because it is more likely to produce quantifiable amounts of glucuronides when glucuronidation reaction occurs.

The results indicated that glucuronidation of 50 μ M genistein and apigenin was largely carried out via UGT1A isoforms (Fig. 1). UGT1A7 was the main isoform responsible for the glucuronidation of 50 μ M genistein (1.47 ± 0.001 nmol/hr/mg) and apigenin (1.65 ± 0.002 nmol/hr/mg). For genistein, UGT1A7 rates was followed by UGT2B1, UGT1A6, UGT1A1 and UGT1A5 (0.21 ± 0.002 , 0.17 ± 0.06 , 0.083 ± 0.008 , and 0.017 ± 0.003 nmol/min/mg, respectively) (Fig. 1). For apigenin, the UGT1A7 rates were followed closely by UGT2B1 (0.69 ± 0.001 nmol/hr/mg), and then by UGT1A6, UGT1A5, UGT2B3 and UGT1A3 (0.06 ± 0.038 , 0.02 ± 0.002 , 0.004 ± 0.001 , and 0.002 ± 0.003 nmol/min/mg, respectively) (Fig. 1). One isoform (i.e., UGT2B12) did not metabolize these two flavonoids.

JPET#147371

The kinetic parameters of UGT1A7-catalyzed glucuronidation of genistein and apigenin were determined using a concentration range of 0.78 to 50 μ M, and glucuronidation kinetics of both compounds followed a simple Michaelis-Menten kinetics (not shown). Genistein displayed an intrinsic clearance (CL_{int}) value of 1.26 ml/min/mg, a V_{max} value of 4.188 nmol/hr/mg, and a K_m value of 3.318 μ M. Apigenin, on the other hand displayed a CL_{int} value of 8.67 ml/min/mg, a V_{max} value of 3.887 nmol/hr/mg, and a K_m value of 0.45 μ M.

Absorption of genistein and apigenin from different regions of the Wistar and Gunn rat

Intestine.

Given the ability of UGT1A isoforms to metabolize apigenin and genistein, it was of interest to investigate whether metabolism of these flavonoids would be different in rats lacking UGT1As. Perfusion studies were performed to compare the absorption of genistein and apigenin (10 μ M) in four regions of the intestine between Wistar and Gunn rats, since Gunn rats are derived from Wistar rats but lack UGT1A isoforms (Chowdhury et al., 1993).

In all four sites of the intestine (duodenum, jejunum, ileum and colon), significant amounts of genistein and apigenin were absorbed at generally comparable rates in these two rat strains (Fig. 2A).

Excretion of genistein and apigenin metabolites from different regions of the Wistar and Gunn rat intestine.

Luminal glucuronides but not sulfates were found in the outlet intestinal perfusate, and their concentrations were quantified as described in “Method” section. For genistein, only in the

JPET#147371

duodenum of Gunn rats was the excretion of metabolites significantly ($p < 0.05$) lower than that of Wistar control rats (Fig.2B). In the jejunum, ileum and colon, excretion rates between Gunn and Wistar control were comparable (Fig.2B). For apigenin, the intestinal excretion of its metabolites in Gunn rats was significantly ($p < 0.05$) higher than Wistar control for all three small intestinal segments (Fig.2B), although it was only marginally higher in the colon (35%, $p > 0.05$).

Biliary excretion of genistein and apigenin metabolites in Wistar and Gunn rats.

Amounts of genistein and apigenin metabolites excreted in the bile were measured after the initiation of perfusion, and samples were taken every 30 min from 60-150mins. Only glucuronides were quantifiable and sulfates were not found. For genistein, amounts of its glucuronides in Gunn rat bile were significantly ($p < 0.05$) lower than their wild-type Wistar rats (Fig. 3). This was consistent for the duration of the experiment (from 60 min sample time to 150 min sample time). For apigenin, the results were exactly the opposite. The amount of apigenin glucuronides excreted in the bile of Gunn rats was significantly ($p < 0.05$) higher than their Wistar control (Fig. 3), which was also consistent for the duration of the experiment.

Metabolism of genistein and apigenin by rat intestinal and liver microsomes.

We found saturable glucuronidation of genistein and apigenin in microsomes prepared from tissues of both Gunn and Wistar rats (Fig.4 and Fig.5). This was the only similarity between apigenin and genistein metabolism. For genistein, its glucuronidation in liver microsomes of both strains was nearly identical (Fig.4C and Table 1). In contrast, its glucuronidation in duodenal microsomes of Gunn rats was lower than Wistar rats, but its glucuronidation in jejunum microsomes of Gunn rats was higher than Wistar rats (Fig.4A and Fig.4B). For

JPET#147371

apigenin, the glucuronidation rates of apigenin in Gunn rat microsomes were generally higher than that in Wistar rat microsomes (Fig. 5), except for liver microsomes (Fig.5C). In liver microsomes prepared from Gunn rats, the glucuronidation rates were slower (than Wistar rat liver microsomes) at lower concentrations (<30 μM), but faster at higher concentrations (>35 μM) (Fig.5C). Kinetic parameters for the glucuronidation of flavonoids by intestinal and liver microsomes for both strains were listed in Table 1.

Metabolism of UGT1A probe substrates by rat intestinal and liver microsomes.

The above differences in glucuronidation between Gunn and Wistar control rats were unexpected in that glucuronidation was either lower (as in the case of genistein) or became elevated (as in the case of apigenin) in Gunn rats. Since both compounds may be metabolized by UGT2B, UGT1A specific substrates were used to verify that the UGT1A activities were significantly lower in Gunn rat microsomes. The two compounds we used were SN-38 and prunetin, both of which have been shown to be mainly the substrates of human UGT1As (Joseph et al, 2007; Paoluzzi et al, 2004). In addition, SN-38 glucuronide formation was slower in Gunn rats and expression of UGT1A1 gene in Gunn rats through gene therapy increased glucuronidation of SN-38 (Tallman et al, 2007).

Our results indicated that the metabolism of both of these compounds were significantly less ($p < 0.05$) in Gunn rat liver microsomes. For prunetin, this decrease in metabolism was clearly reflected in the decreased V_{max} values (Table 1), consistent with a lack of expression of UGT1As. For SN-38, this slower metabolism was shown at all three tested concentrations (Fig.6). Use of jejunal microsomes yielded results consistent with those derived from liver

JPET#147371

(Fig.6). A human UGT1A1 specific antibody was then used to probe the expression level of UGT1A in rats using the Western blot, and the results indicated a reduced expression of rat UGT1A in Gunn rats (Fig.7), consistent with the results of the kinetic studies shown above. Unfortunately, the antibody used is not highly reactive with rat UGT1A, and react weakly with human UGT2B7.

Metabolism of UGT2B probe substrates by rat intestinal and liver microsomes.

The results from earlier perfusion and microsome studies using Gunn and Wistar rats indicated the possibility of compensatory mechanisms by which alternative UGT enzyme isoforms have been up-regulated due to decreased UGT1A activities in Gunn rats.

To determine which UGT2B isoform(s) were responsible, the metabolic profile of testosterone (a common UGT2B1, 2B3 and 2B6 metabolic probe substrate) were determined using 2 concentrations and three different (tissue) microsomes. The results indicated that little or no metabolism occurred in the intestine (Fig. 8), but glucuronidation in liver microsomes of Gunn rats was rapid and much faster (10-13 folds) than Wistar rats at both 5 and 50 μ M concentrations.

The second UGT2B probe used was ezetimibe (or 1-(4-fluorophenyl)-3(R)-(Ghosal et al.)-4(S)-(4-hydroxyphenyl)-2-azetidinone) to confirm the testosterone glucuronidation results. Ezetimibe was shown to be metabolized by recombinant human UGT2B7 and UGT2B15 (Ghosal et al., 2004). At 5 μ M, Gunn rat duodenum and jejunum microsomes had a significantly ($p < 0.05$) higher ezetimibe glucuronidation rate than Wistar rat microsomes (Fig.8A). On the other hand,

JPET#147371

Gunn rat liver microsomes had a significantly ($p < 0.05$) lower glucuronidation rate (0.56 ± 0.11 nmol/min/mg) than Wistar rat microsomes (1.62 ± 0.11 nmol/min/mg). Metabolic patterns at $50 \mu\text{M}$ concentration observed for both rat strains (Fig.8B) were similar to the pattern observed at $5 \mu\text{M}$ (Fig.8A).

The third UGT2B probe used was indomethacin as it is a substrate for intestinal UGT2B7. At $5 \mu\text{M}$, Gunn rat jejunal microsomes metabolized indomethacin slower (33% less, $p > 0.05$) than Wistar rats (≈ 0.05 nmol/min/mg protein), its liver microsomes metabolizes indomethacin at comparable rates (≈ 0.09 nmol/min/mg protein) as Wistar rats, and its duodenal microsomes metabolized indomethacin at a faster rate (50%, $p < 0.05$) than Wistar rats. At $50 \mu\text{M}$, Gunn rat liver and jejunal microsomes metabolized indomethacin at a rate (≈ 0.35 nmol/min/mg protein) comparable to Wistar rats, where its duodenal microsomes metabolized indomethacin slightly slower (30% less or ≈ 0.025 nmol/min/mg protein, $p > 0.05$).

DISCUSSION

We have performed the present study using the UGT1A deficient Gunn rats to determine the roles of UGT1As in rendering poor oral bioavailabilities of flavonoids. The latter is an important problem since bioavailability challenges have limited the potentials of flavonoids as chemopreventive agents (Setchell et al., 2001; Yang et al., 2001).

We have chosen the Gunn rat model for the present study because it lacks expression of UGT1A, as the result of recessive genes. This animal model was used frequently to determine the consequence of UGT1A deficiency in vivo, which usually resulted in much slower excretion of glucuronides (Chowdhury et al., 1993; Watanabe et al., 2000). A recent search of PubMed using the keyword combo “Gunn rats” generated more than 800 hits, indicating that this animal model is well-received by biomedical researchers. The results of present study were therefore important since this would be the first report to show that activities of other UGT isoforms had changed (i.e., elevated) to compensate for UGT1A deficiency.

We observed consistent but unexpected results: similar or higher intestinal excretion of both flavonoid glucuronides and higher biliary excretion of apigenin glucuronides (Fig.2 and Fig.3). They were unexpected because absorption was similar in both rat strains (Fig.2). We expected much slower glucuronidation of apigenin and genistein because previous studies from various research groups have shown the importance of UGT1As for metabolism phenolic and polyphenolic compounds (Strassburg et al., 1997; Galijatovic et al., 2001; Malfatti and Felton, 2004; Tian et al., 2005). Our own results also did not predict this observation because both

JPET#147371

genistein and apigenin (at 50 μM) were mainly metabolized by UGT1As, especially UGT1A7 (Fig.1). Although UGT2B sub-family (specifically UGT2B1) participated in the glucuronidation, they appeared to be much less efficient than UGT1A7 (Fig.1). In addition, combined levels of UGT2B mRNAs were shown to be much lower (several folds) than combined levels of UGT1A mRNAs (Shelby et al., 2003).

To explain the observed excretion pattern, we first performed microsomal glucuronidation experiments, and the results suggested that glucuronidation of flavonoids in Gunn rat microsomes was usually faster than Wistar rat microsomes (Fig.4 and 5). The only exceptions were the duodenal glucuronidation of genistein and liver glucuronidation of apigenin at low concentrations (<25 μM) (Fig.4 and 5). Therefore, the slower intestinal excretion of genistein glucuronides in duodenum of Gunn rats and faster intestinal excretion of apigenin glucuronides in the duodenum and jejunum of Gunn rats observed in perfusion studies were likely caused by changes in relevant UGT activities in the gut. However, the same statement cannot be made for biliary excretion of flavonoid glucuronides (discussed later).

We then determined the possible mechanisms by which flavonoid glucuronidation became faster in the intestine of Gunn rats. To do this, we first verified that UGT1A activities in Gunn rat microsomes were indeed lower by using two UGT1A specific substrates, SN-38 and prunetin. The results confirmed significantly slower glucuronidation rates in Gunn rat intestinal microsomes (Fig.6 and Table 1). We then conducted glucuronidation studies using UGT2B-specific substrates testosterone, ezetimibe and indomethacin, and the results showed that their metabolism rates in Gunn rat intestinal microsomes were usually higher or the same (Fig.8).

JPET#147371

Taken together, these results suggested that higher UGT2B activities in Gunn rats, as the result of compensation for UGT1A deficiency, were the main reason for the observed pattern of flavonoid glucuronide excretion observed in the Gunn rat gut. Based on known expression pattern of 6 rat UGT2Bs (Shelby et al., 2003), the possible isoforms responsible for enhanced flavonoid glucuronidation in the gut are UGT2B6 and 2B8 since UGT2B3 and 2B12 did not metabolize either flavonoids (Fig.1) and UGT2B1 and 2B2 were not expressed in rat intestine (Shelby et al., 2003). Since UGT2B8 is only expressed in the duodenum (Shelby et al., 2003) and genistein was glucuronidated less in Gunn rat duodenum, it is probably not the isoform responsible. Therefore, UGT2B6 is the only known intestinal isoform that could have been up-regulated in the gut, although yet-to-defined new UGT2Bs or other UGTs could also be up-regulated. The latter was recognized here as a possible source of uncertainty because of limitations imposed by available methods.

We also determined the possible mechanisms by which excretion of flavonoid glucuronides in the bile of Gunn rats was lower (as for genistein) or higher (as for apigenin) than the control Wistar rats. The use of two UGT1A specific substrates, SN-38 and prunetin, in microsomal studies confirmed lower glucuronidation rates in Gunn rat liver microsomes (Fig.6 and Table 1). The latter was also consistent with the lower UGT1A expression in Western blot of Gunn rat liver microsomes (as opposed to Wistar rats) (Fig.7). However, the biliary excretion pattern of genistein glucuronides was not consistent with its microsomal reaction pattern, since a big drop in biliary excretion was not consistent with a lack of change in the microsomal rates of genistein metabolism between the two strains (Fig.4C). Biliary excretion pattern of apigenin glucuronides was also surprising, since glucuronidation rates of Gunn rat microsomes were only higher at

JPET#147371

concentrations greater than the maximal expected plasma level of 0.25 μM (Fig.5C). Therefore, in the liver, changes in excretion of flavonoids glucuronides were related not only to changes in UGT activities but also to changes to efflux transporter activities, although the exact transporters involved in the excretion remained to be determined. Previously, organic anion efflux transporter MRP3 (located at the bile canicular membrane) was shown to be up-regulated in Gunn rats (Ogawa et al., 2000), but it was unknown if MRP3 can selectively efflux apigenin glucuronides over genistein glucuronides. As for the UGT isoforms that were up-regulated, we believe that the higher than expected hepatic UGT activities against flavonoids in Gunn rats likely came from elevated UGT2B1 activities, since only UGT2B1 and 2B2 are highly expressed in liver (Shelby et al., 2003) but UGT2B2 was not a testosterone-conjugating enzyme (Tian et al., 2005). Lastly, glucuronidation of testosterone was higher only in Gunn rat liver microsomes (Fig.8). Additional evidence in support of our analysis is the fact that UGT2B8 was not expressed in liver (Shelby et al., 2003) and UGT2B3 and 2B12 were not active against flavonoids (Fig.1). However, we could not rule out the contribution of 2B6 isoforms as it is also expressed in liver, although an equal up-regulation of UGT2B6 at both intestine and liver would have resulted in much higher glucuronidation of testosterone in the Gunn rat intestinal microsomes, which did not occur. As stated before, unknown UGT2Bs and other UGTs could also contribute.

The results of these studies showed that UGT1A deficiency had limited negative impact on the intestinal excretion of flavonoid glucuronides, where only duodenal glucuronidation of genistein was significantly slowed. UGT1A deficiency in Gunn rats had variable impact on biliary conjugate excretion since only excretion of genistein glucuronides was lowered, whereas the

JPET#147371

excretion of apigenin glucuronides was elevated. Because intestinal excretion of flavonoid glucuronides usually changed with intestinal UGT activities whereas biliary excretion did not, hepatic efflux transporters probably played a more important role in affecting flavonoid disposition than intestinal efflux transporters. Taken together, we have demonstrated strong compensatory responses to UGT1A deficiency in Gunn rats. These responses have resulted in no suppression or even elevation in flavonoid disposition. Therefore, the enteric and enterohepatic recycling are highly robust *in vivo* processes that would be difficult to suppress. The inability to suppress the flavonoid disposition as demonstrated here will make it more difficult to increase the bioavailabilities of flavonoids via the use of UGT inhibitors. In other words, it would be more difficult to overcome the bioavailability challenges associated with flavonoids because viable *in vivo* mechanisms are ready to compensate for any functional deficiency in metabolism of flavonoids. Looking at this point positively would suggest that flavonoids are going to be safe in humans since only small amounts of these compounds are able to reach the systemic circulation.

In conclusion, our studies using expressed rat UGT isoforms indicate that UGT1As are responsible for the glucuronidation of flavonoids such as genistein and apigenin. Systematic investigation of glucuronidation in Gunn rats suggest that deficiency in UGT1As is compensated by up-regulation of intestinal UGT2Bs, and hepatic UGT2Bs and efflux transporters. The up-regulation of several UGT2Bs, likely including UGT2B6 in the gut and UGT2B1 in the liver, compensates effectively for UGT1A deficiencies in intestine and liver of Gunn rats.

JPET#147371

REFERENCES

- Ando Y and Hasegawa Y (2005) Clinical pharmacogenetics of irinotecan (CPT-11). *Drug Metab Rev* **37**:565-574.
- Arts IC and Hollman PC (2005) Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* **81**:317S-325S.
- Bobé G, Weinstein SJ, Albanes D, Hirvonen T, Ashby J, Taylor PR, Virtamo J and Stolzenberg-Solomon RZ (2008) Flavonoid intake and risk of pancreatic cancer in male smokers (Finland). *Cancer Epidemiol Biomarkers Prev* **17**:553-562.
- Busby MG, Jeffcoat AR, Bloedon LT, Koch MA, Black T, Dix KJ, Heizer WD, Thomas BF, Hill JM, Crowell JA and Zeisel SH (2002) Clinical characteristics and pharmacokinetics of purified soy isoflavones: single-dose administration to healthy men. *Am J Clin Nutr* **75**:126-136.
- Cassidy A (2006) Factors affecting the bioavailability of soy isoflavones in humans. *J AOAC Int* **89**:1182-1188.
- Chen J, Lin H and Hu M (2003) Metabolism of flavonoids via enteric recycling: role of intestinal disposition. *J Pharmacol Exp Ther* **304**:1228-1235.
- Chen J, Lin H and Hu M (2005a) Absorption and metabolism of genistein and its five isoflavone analogs in the human intestinal Caco-2 model. *Cancer Chemother Pharmacol* **55**:159-169.
- Chen J, Wang S, Jia X, Bajimaya S, Tam V and Hu M (2005b) Disposition of Flavonoids via Recycling: Comparison of Intestinal versus Hepatic Disposition. *Drug Metab Dispos* **33**:1777-1784.

JPET#147371

- Chouinard S, Yueh MF, Tukey RH, Giton F, Fiet J, Pelletier G, Barbier O and Belanger A (2008) Inactivation by UDP-glucuronosyltransferase enzymes: the end of androgen signaling. *J Steroid Biochem Mol Biol* **109**:247-253.
- Chowdhury JR, Kondapalli R and Chowdhury NR (1993) Gunn rat: a model for inherited deficiency of bilirubin glucuronidation. *Adv Vet Sci Comp Med* **37**:149-173.
- Coldham NG, Zhang AQ, Key P and Sauer MJ (2002) Absolute bioavailability of [14C] genistein in the rat; plasma pharmacokinetics of parent compound, genistein glucuronide and total radioactivity. *Eur J Drug Metab Pharmacokinet* **27**:249-258.
- Crowell JA (2005) The chemopreventive agent development research program in the Division of Cancer Prevention of the US National Cancer Institute: an overview. *Eur J Cancer* **41**:1889-1910.
- Daidoji T, Gozu K, Iwano H, Inoue H and Yokota H (2005) UDP-glucuronosyltransferase isoforms catalyzing glucuronidation of hydroxy-polychlorinated biphenyls in rat. *Drug Metab Dispos* **33**:1466-1476.
- Doerge DR, Chang HC, Churchwell MI and Holder CL (2000) Analysis of soy isoflavone conjugation in vitro and in human blood using liquid chromatography-mass spectrometry. *Drug Metab Dispos* **28**:298-307.
- Galijatovic A, Otake Y, Walle UK and Walle T (2001) Induction of UDP-glucuronosyltransferase UGT1A1 by the flavonoid chrysin in Caco-2 cells--potential role in carcinogen bioinactivation. *Pharm Res* **18**:374-379.
- Ghosal A, Hapangama N, Yuan Y, Achanfuo-Yeboah J, Iannucci R, Chowdhury S, Alton K, Patrick JE and Zbaida S (2004) Identification of human UDP-glucuronosyltransferase

JPET#147371

enzyme(s) responsible for the glucuronidation of ezetimibe (Zetia). *Drug Metab Dispos* **32**:314-320.

Hirvonen T, Pietinen P, Virtanen M, Ovaskainen ML, Hakkinen S, Albanes D and Virtamo J (2001) Intake of flavonols and flavones and risk of coronary heart disease in male smokers. *Epidemiology* **12**:62-67.

Hu M, Chen J and Lin H (2003) Metabolism of flavonoids via enteric recycling: mechanistic studies of disposition of apigenin in the Caco-2 cell culture model. *J Pharmacol Exp Ther* **307**:314-321.

Hu M, Roland K, Ge L, Chen J, Li Y, Tyle P and Roy S (1998) Determination of absorption characteristics of AG337, a novel thymidylate synthase inhibitor, using a perfused rat intestinal model. *J Pharm Sci* **87**:886-890.

Hu M, Sinko PJ, deMeere AL, Johnson DA and Amidon GL (1988) Membrane permeability parameters for some amino acids and beta-lactam antibiotics: application of the boundary layer approach. *J Theor Biol* **131**:107-114.

Jeong EJ, Jia X and Hu M (2005a) Disposition of formononetin via enteric recycling: metabolism and excretion in mouse intestinal perfusion and Caco-2 cell models. *Mol Pharm* **2**:319-328.

Jeong EJ, Liu X, Jia X, Chen J and Hu M (2005b) Coupling of conjugating enzymes and efflux transporters: impact on bioavailability and drug interactions. *Curr Drug Metab* **6**:455-468.

Jeong EJ, Liu Y, Lin H and Hu M (2005c) Species- and disposition model-dependent metabolism of raloxifene in gut and liver: role of ugt1a10. *Drug Metab Dispos* **33**:785-794.

JPET#147371

- Jia X, Chen J, Lin H and Hu M (2004) Disposition of flavonoids via enteric recycling: enzyme-transporter coupling affects metabolism of biochanin A and formononetin and excretion of their phase II conjugates. *J Pharmacol Exp Ther* **310**:1103-1113.
- Keli SO, Hertog MG, Feskens EJ and Kromhout D (1996) Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study. *Arch Intern Med* **156**:637-642.
- Liu Y and Hu M (2002) Absorption and metabolism of flavonoids in the caco-2 cell culture model and a perused rat intestinal model. *Drug Metab Dispos* **30**:370-377.
- Liu Z and Hu M (2007) Natural polyphenol disposition via coupled metabolic pathways. *Expert Opin Drug Metab Toxicol* **3**:389-406.
- Malfatti MA and Felton JS (2004) Human UDP-glucuronosyltransferase 1A1 is the primary enzyme responsible for the N-glucuronidation of N-hydroxy-PhIP in vitro. *Chem Res Toxicol* **17**:1137-1144.
- Moon YJ, Sagawa K, Frederick K, Zhang S and Morris ME (2006) Pharmacokinetics and bioavailability of the isoflavone biochanin A in rats. *Aaps J* **8**:E433-442.
- Ogawa K, Suzuki H, Hirohashi T, Ishikawa T, Meier PJ, Hirose K, Akizawa T, Yoshioka M and Sugiyama Y (2000) Characterization of inducible nature of MRP3 in rat liver. *Am J Physiol Gastrointest Liver Physiol* **278**:G438-446.
- Setchell KD, Brown NM, Desai PB, Zimmer-Nechimias L, Wolfe B, Jakate AS, Creutzinger V and Heubi JE (2003) Bioavailability, disposition, and dose-response effects of soy isoflavones when consumed by healthy women at physiologically typical dietary intakes. *J Nutr* **133**:1027-1035.
- Shelby MK, Cherrington NJ, Vansell NR and Klaassen CD (2003) Tissue mRNA expression of the rat UDP-glucuronosyltransferase gene family. *Drug Metab Dispos* **31**:326-333.

JPET#147371

- Strassburg CP, Oldhafer K, Manns MP and Tukey RH (1997) Differential expression of the UGT1A locus in human liver, biliary, and gastric tissue: identification of UGT1A7 and UGT1A10 transcripts in extrahepatic tissue. *Mol Pharmacol* **52**:212-220.
- Tallman MN, Miles KK, Kessler FK, Nielsen JN, Tian X, Ritter JK and Smith PC (2007) The contribution of intestinal UDP-glucuronosyltransferases in modulating 7-ethyl-10-hydroxy-camptothecin (SN-38)-induced gastrointestinal toxicity in rats. *J Pharmacol Exp Ther* **320**:29-37.
- Tian H, Ou J, Strom SC and Venkataramanan R (2005) Activity and expression of various isoforms of uridine diphosphate glucuronosyltransferase are differentially regulated during hepatic regeneration in rats. *Pharm Res* **22**:2007-2015.
- Walle T (2004) Absorption and metabolism of flavonoids. *Free Radic Biol Med* **36**:829-837.
- Wang SW, Chen J, Jia X, Tam VH and Hu M (2006) Disposition of flavonoids via enteric recycling: structural effects and lack of correlations between in vitro and in situ metabolic properties. *Drug Metab Dispos* **34**:1837-1848.
- Watanabe T, Furukawa T, Sharyo S, Ohashi Y, Yasuda M, Takaoka M and Manabe S (2000) Effect of troglitazone on the liver of a Gunn rat model of genetic enzyme polymorphism. *J Toxicol Sci* **25**:423-431.

JPET#147371

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JPET#147371

Legends for Figures

Figure 1. Metabolism of genistein (open bars) and apigenin (solid bars) using rat UGT isoforms expressed in yeast strain AH22. From left to right, UGT1A1, UGT1A3, UGT1A5, UGT1A6, UGT1A7, UGT2B1, UGT2B3 and UGT2B12 were tested with optimal incubation with either 50 μ M of genistein or apigenin (number of replicates, or $n=3$).

Figure 2. Disposition of genistein and apigenin in a four-site rat intestinal perfusion model (number of replicates, or $n=4$). Four segments of the intestine (i.e., duodenum, upper jejunum, terminal ileum, and colon) were perfused simultaneously at a flow rate of 0.191 ml/min using perfusate containing 10 μ M genistein in Wistar control (solid white columns) or Gunn rats (solid black columns), or using perfusate containing 10 μ M apigenin in Wistar control (light graded columns) or Gunn rats (dark diagonal slashed columns). Amounts absorbed (A) or excreted (B) were normalized to 10-cm intestinal length using Equations 1 to 3. At a flow rate of 0.191 ml/min, the amount perfused was 57.3 nmol/10cm/30 min for each segment or 229 nmol/30 min for all 4 intestinal segments. According, absorption of 27 nmol from one perfused segment represents 47% of the perfused amount. The arrow indicated a statistically significant difference between two strains of rats using the same flavonoid. Each column represents the average of four determinations and the error bar is the S.D.

Figure 3. Biliary excretion of flavonoid glucuronides in Wistar and Gunn rats. Biliary excretion samples (at 30 min sample interval) ($n=4$) were collected when conducting perfusion experiments using 10 μ M genistein in Wistar control (solid white columns) or Gunn rats (solid

JPET#147371

black columns), as well as using 10 μM apigenin in Wistar control rats (light graded columns) or Gunn rats (dark diagonal slashed columns) (number of replicates, or $n=4$). Significant difference ($p<0.05$) in biliary excretion between two rat strains using either genistein or apigenin was indicated with an arrow in the figure. Each column represents the average of four determinations and the error bar is the S.D.

Figure 4. Apparent glucuronidation rates of genistein in rat duodenal (A), jejunal (B) and liver (C) microsomes of Gunn and Wistar as a function of concentration ($n=3$). Rates of metabolism were determined from 0.625 to 50 μM , and reaction time was for 30 min. The metabolism rate is the average of three determinations and the error bar represents the standard deviation of the mean. The points were observed genistein glucuronide formation rates, and the curves were estimated based on fitted parameters using a simple Michaelis-Menten equation (Equation 6).

Figure 5. Apparent glucuronidation rates of apigenin in rat duodenal (A), jejunal (B), and liver (C) microsomes of Gunn and Wistar as a function of concentration ($n=3$). Rates of metabolism were determined from 0.625 to 50 μM , and reaction time was for 30 min. The metabolism rate is the average of three determinations and the error bar represents the standard deviation of the mean. The points were observed apigenin glucuronide formation rates, and the curves were estimated based on fitted parameters generated by a simple Michaelis-Menten equation (Equation 6).

Figure 6. Apparent glucuronidation rates of SN-38 in Gunn and Wistar rat liver and jejunal microsomes at three concentrations. SN-38 is a specific probe substrate for UGT1A1 and

JPET#147371

formation of SN-38 glucuronide was impaired in Gunn rats (Tallman et al., 2007). Higher concentration of SN-38 was not used since it is not soluble in the current reaction system. Each column bar represents an average of three determinations and the error bar represents the standard deviation of the mean.

Figure 7. Western blot analysis of UGT1A and UGT2B expression. Lane 1, 0.5 μ g expressed human UGT1A1; lane 2, 100 μ g yeast lysate expressing rat UGT1A7; lane 3, 10 μ g Wistar rat liver microsomes; lane 4, 20 μ g Wistar rat liver microsomes; lane 5 10 μ g Gunn rat liver microsomes; lane 6, 20 μ g Gunn rat liver microsomes; lane 7, defective; lane 8, 0.5 μ g expressed human UGT2B7. The antibody (Santa Cruz Biotechnology, Catalog # sc-25847) appears to be highly selective for the human UGT1A1 over human UGT2B7, although it reacts weakly with UGT2Bs. As such the antibody was expected to be selective with rat UGT1As over rat UGT2Bs.

Figure 8. Apparent glucuronidation rates of testosterone and ezetimibe at 5 μ M (A) and 50 μ M (B). The reaction was proceeded at an optimal incubation time of 1hr using duodenal, jejunal and liver microsomes prepared from Wistar and Gunn rats ($n=3$). Each column represents the average of three determinations and the error bar is the S.D.

Table 1. Apparent Kinetic Parameters of Genistein and Apigenin Glucuronidation in Different Microsomes*

Flavonoid	Species	Duodenum Kinetic Parameters			Jejunum Kinetic Parameters			Liver Kinetic Parameters		
		K_m (μM)	V_{\max} (nmol/min/ mg protein)	CL_{int} (mL/min /mg)	K_m (μM)	V_{\max} (nmol/min/ mg protein)	CL_{int} (mL/min /mg)	K_m (μM)	V_{\max} (nmol/min/ mg protein)	CL_{int} (mL/min/ mg)
Genistein	Wistar	2.5	8.1	3.2	4.5	3.4	0.76	11.1	3.1	0.28
	Gunn	4.6	7.3	1.6	2.4	4.1	1.8	15.3	3.8	0.25
Apigenin	Wistar	12.2	4.4	0.36	4.4	7.2	1.6	7.7	21.4	2.8
	Gunn	4.5	5.9	1.3	3.5	10.1	2.9	99.7	66.8	0.69
Prunetin	Wistar	-	-	-	0.48	4.3	8.9	0.51	0.56	0.91
	Gunn	-	-	-	0.81	2.2	2.7	0.77	0.19	0.25

*Data analyzed using simple Michaelis-Menten equation and nonlinear regression.

Figure 1

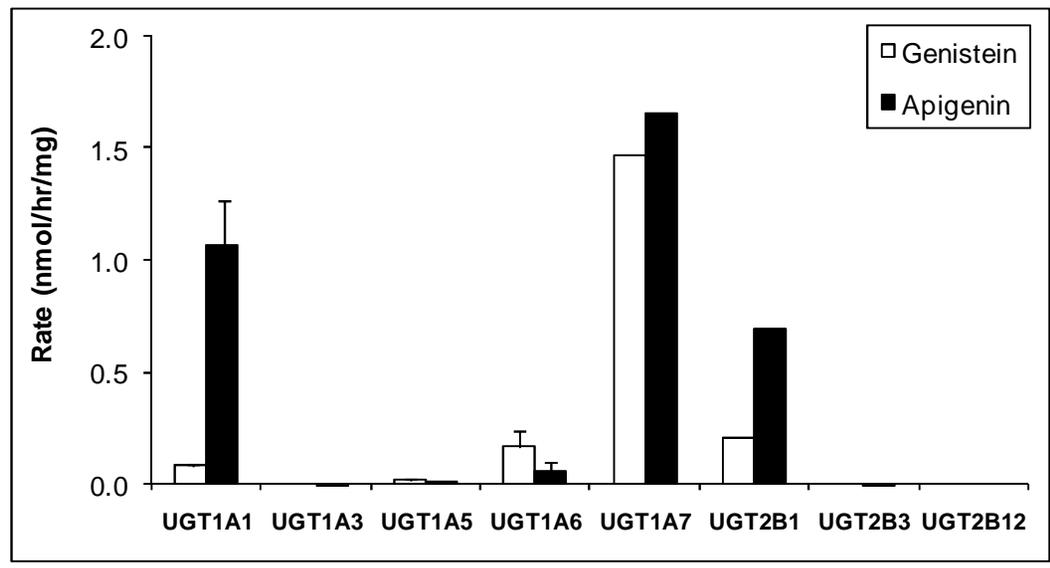


Figure 2

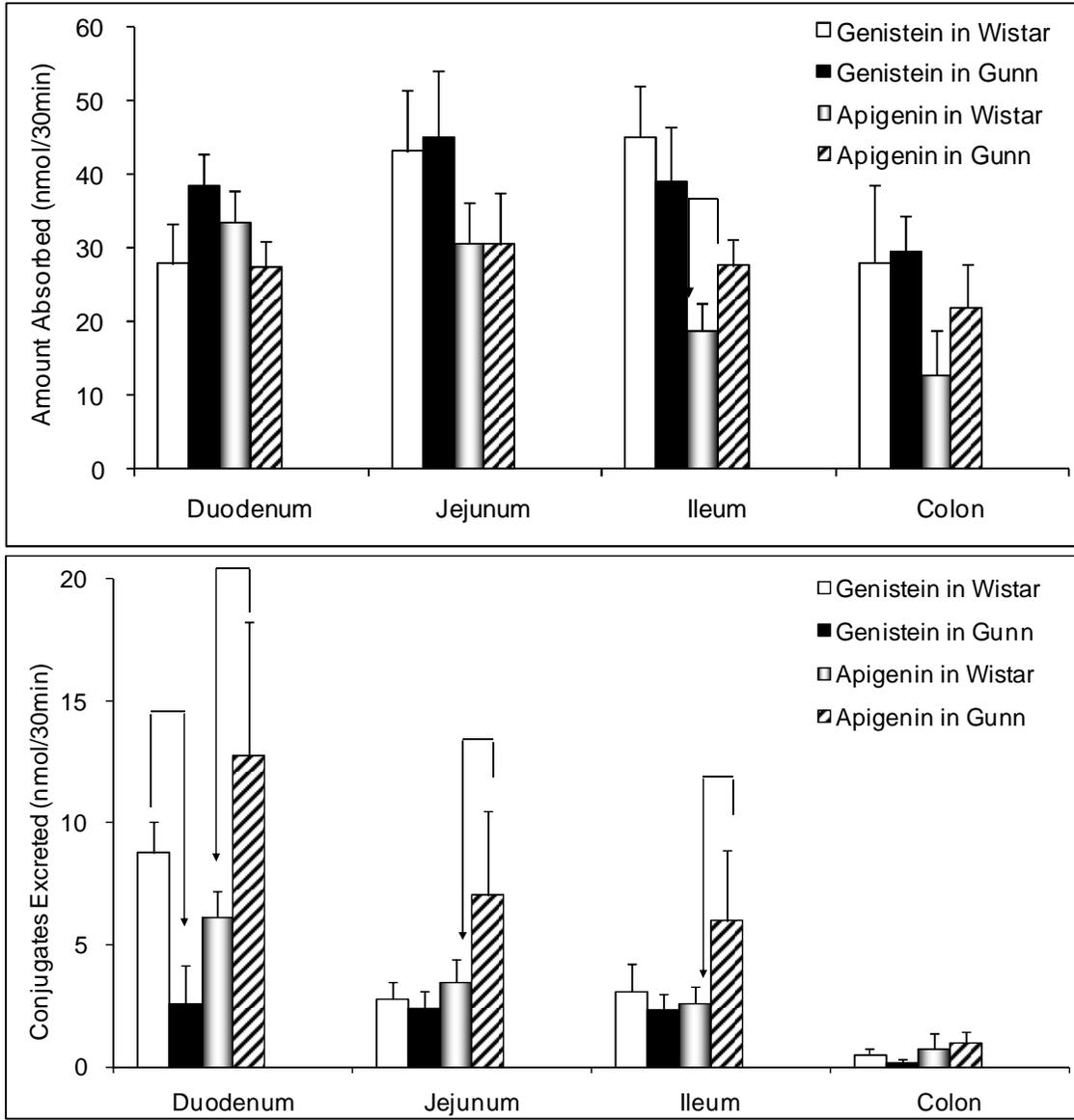


Figure 3

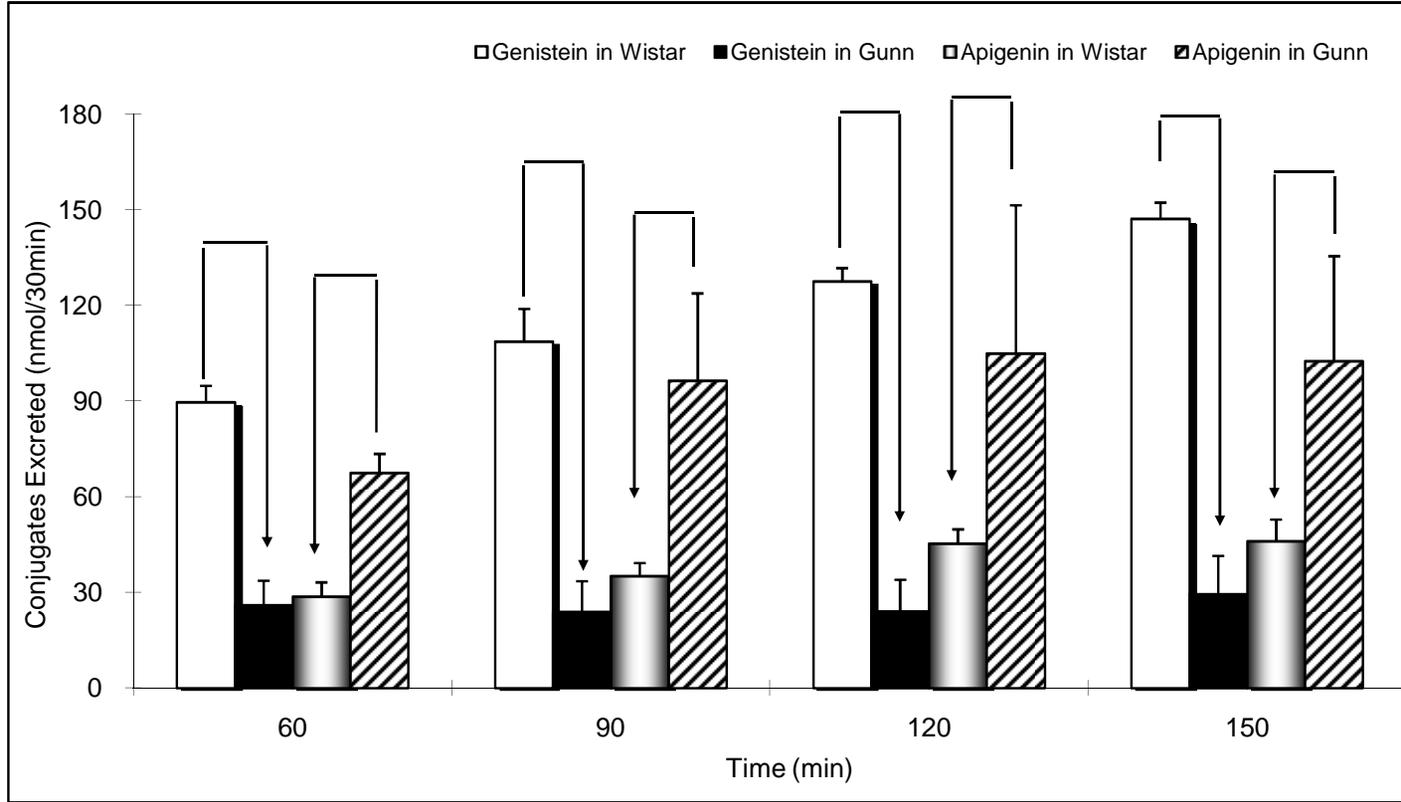


Fig.4

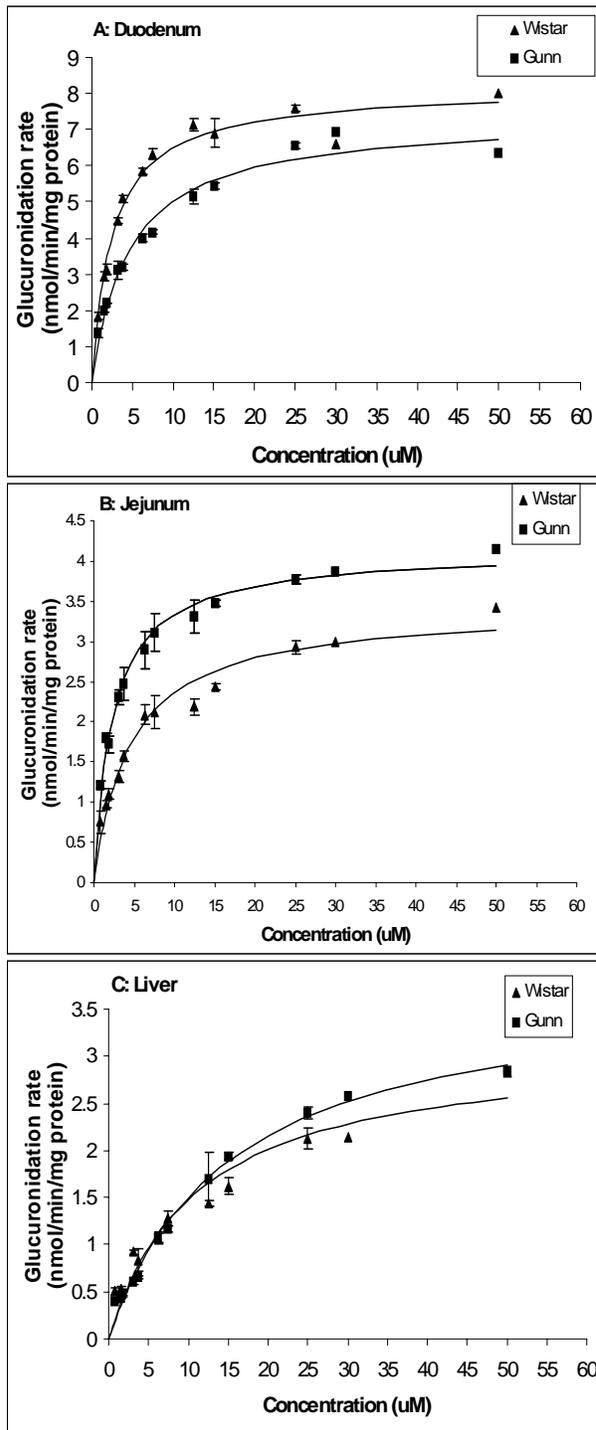


Fig.5

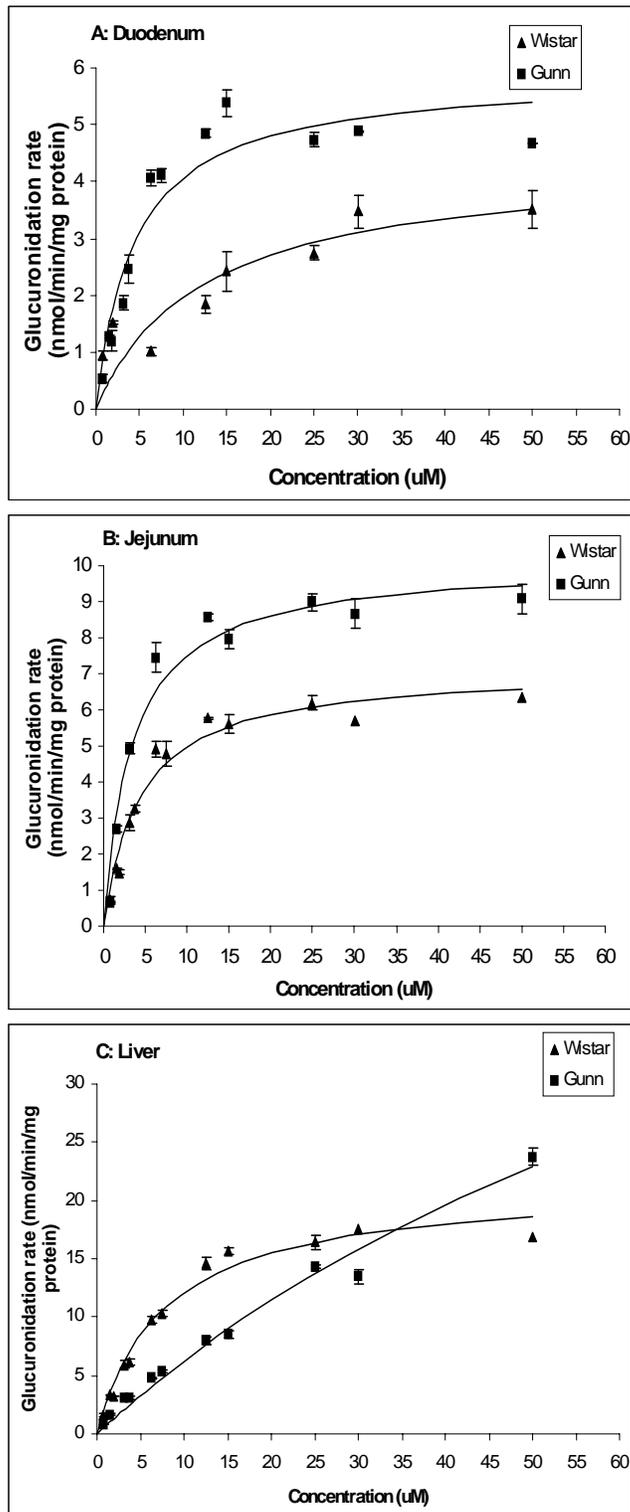
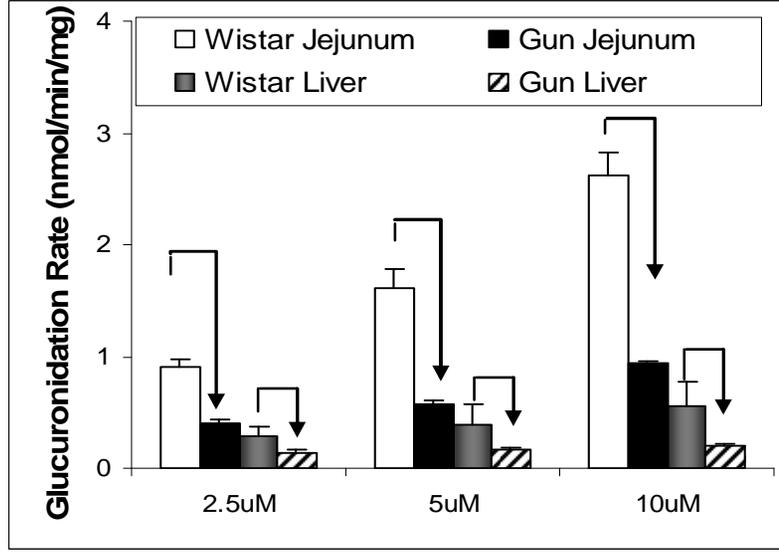


Figure 6



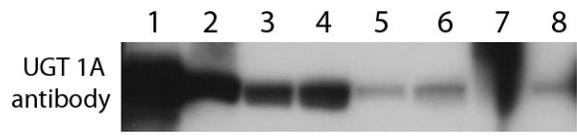


Figure 7

Figure 8

