Title: Microsomal quercetin glucuronidation in rat small intestine depends on age and segment

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Running Title: Quercetin glucuronidation in senescent rat

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ABBREVIATIONS: AIC, Akaike’s Information Criterion; CL_{int}, intrinsic clearance; ELT-3, Erythroid-Like Transcription factor-3; Nrf2, nuclear factor erythroid 2 (NF-E2)-related factor 2; PXR, pregnane X receptor; Q, quercetin; SI, small intestine; TBST, Tris-Buffer-Saline-Tween; M1, uncharacterized quercetin glucuronide; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.
ABSTRACT

UDP-glucuronosyltransferase (UGT) activity toward the flavonoid quercetin and UGT protein were characterized in 3 equidistant small intestine (SI) segments from 4, 12, 18, and 28 mo male F344 rats, n=8/age using villin to control for enterocyte content. SI microsomal intrinsic clearance of quercetin were increased 3- to 9-fold from 4 mo in the proximal and distal SI at 12 and 18 mo. Similarly, at 30 µM quercetin, SI microsomal glucuronidation activity was increased with age, 4.8- and 3.9-fold greater at 18 mo than 4 mo. Quercetin UGT regioselectivity was not changed by age. The distal SI preferentially catalyzed glucuronidation at the 7-position, whereas the proximal SI produced the greatest proportion of 4’- and 3’-conjugates. Enterocyte UGT content in different SI segments was not consistently changed with age. In the proximal SI, UGT1A increased 64 and 150 % at 12 and 18 mo and UGT1A1, UGT1A7, and UGT1A8 were also increased at 12 and 18 mo. However, age-related changes in expression were inconsistent in the medial and distal segments. Microsomal rates of quercetin glucuronidation and UGT expression were positively correlated with UGT1A1 content for all pooled samples (R = 0.467) and at each age (R= 0.538 to 0.598). UGT1A7 was positively correlated with total, 7-O- and 3-O- quercetin glucuronidation at 18 mo. Thus, age-related differences in UGT quercetin glucuronidation depend upon intestinal segment and are more pronounced in the proximal and distal segments, and may be partially related to UGT1A1 and UGT1A7 content.
INTRODUCTION

Quercetin, a flavonol, displays protective effects against chronic diseases in animal models and clinical trials through multiple mechanisms, e.g., antioxidation and anti-inflammation (Bischoff, 2008; Boots et al., 2008). Dietary or supplemental quercetin has low oral bioavailability and is extensively glucuronidated, sulfated, and methylated in intestine and liver with large inter-individual variations (Mullen et al., 2006; Moon et al., 2008). These phase II enzyme conjugations facilitate quercetin efflux back to intestinal lumen before its systematic distribution and plays a role in its limited bioavailability (van der Woude et al., 2004). Following quercetin consumption, the aglycone is not detectable in rat or human plasma (Graf et al., 2006; Mullen et al., 2006). In non-GI tissues of rats, quercetin is present mainly as glucuronide and sulfate conjugates, with less than 10% in the aglycone form (de Boer et al., 2005; Graf et al., 2006). Thus, flavonoid phase II metabolites appear to be critical to their biological activities after their consumption.

Previously we reported that in rats, advanced age modifies hepatic microsomal quercetin glucuronidation regioselectivity and isoflavone metabolism kinetics (Bolling et al., 2010). Similarly, Handler and Brian (Handler and Brian, 1997) also reported that $K_m$ and $V_{max}$ values for hepatic microsomal glucuronidation of 4-nitrocatechol were increased in senescent Sprague Dawley rats. Changes to first-pass glucuronidation regioselectivity or capacity with advanced age could affect bio-efficacy or bioavailability of flavonoids in elderly. Since older populations are at higher risk for chronic diseases, more work is needed to characterize age-related changes in flavonoid metabolism since they are commonly consumed in the form of foods and supplements.
Intestinal abundance of UGT has been partially characterized in humans and rodents, but the effect of advanced age on UGT abundance is unknown. *Ugt1A1, 1A3, 1A4, 1A6, 1A10, 2B4, 2B7, 2B10, and 2B15* mRNA have been detected in human small intestine (Strassburg et al., 2000). In Sprague-Dawley rats, the orthologs *Ugt1A1, 1A2, 1A3, 1A5, 1A6, 1A7, 1A8, 2B6, 2B8, 2B12, and 2B3* mRNA have also been reported and respond differentially to pregnane X receptor (PXR) and nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) ligands (Shelby et al., 2003; Shelby and Klaassen, 2006). UGT expression is also dependent on small intestine segment. Rat UGT1A1 protein increased along the descent of the small intestine, while UGT1A7 protein decreased (Miles et al., 2006). Quercetin glucuronidation has been partially characterized using some recombinant human UGT isoforms (Boersma et al., 2002; Chen et al., 2008). However, the cumulative contribution of all individual UGT isoforms to glucuronidation activity and regioselectivity toward quercetin and other polyphenols has not been characterized. Therefore, the aim of the present study was to characterize the effect of age on glucuronidation activity and regioselectivity toward quercetin in the small intestine of F344 rats.

**MATERIALS AND METHODS**

**Chemicals and supplies.** Acetonitrile and methanol were HPLC grade from Fisher Scientific (Thermo Fisher Scientific, Rockford, IL). Immunoblotting loading, running, Tris-Buffer-Saline-Tween (TBST), and transfer buffers were from Boston Bioproducts (Boston, MA). Pre-cast SDS-acrylamide gels, PVDF membrane, and transfer paper were from BioRad (Hercules, CA). The primary antibodies toward rodent UGT1A (C19), UGT1A1 (V-19), UGT1A5 (F-19), UGT1A6 (D-20), UGT1A7 (E-15), UGT1A8 (T-17), and villin (H-60), secondary antibodies, and luminol reagent were from Santa Cruz Biotechnology (Santa Cruz, CA). Human UGT1A1
was from BD Biosciences (San Jose, CA). Quercetin, daidzein, UDP-glucuronic acid, alamethicin, and all other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

**Tissue preparation.** Male Fischer F344 rats aged 4, 12, 18 and 28 mo (n = 8/group) were obtained from the Aging Rodent Colony of the NIA/NIH (Bethesda, MD). Animals were acclimated at the Jean Mayer USDA HNRCA for two weeks, and handled according to an approved IACUC protocol. During the acclimation period, rats were housed with a 12:12 h light:dark cycle and fed a standard chow ad libitum. Following anesthesia using Aerrane™, animals were sacrificed by terminal exsanguinations. The gastrointestinal system was removed and rinsed in ice-cold saline. The small intestine (SI) length was measured and divided into 3 equidistant segments. Small intestine segments were cut lengthwise and rinsed with saline, and mucosa was collected by scrapping with a glass slide on an ice-cold surface. Mucosa samples were snap frozen in liquid nitrogen and stored at -80°C until analyses.

Animals had body masses of 317 ± 28 (4 mo), 360 ± 22 (12 mo), 367 ± 37 (18 mo) and 342 ± 50 g (28 mo). SI lengths were 99.1 ± 8.6 (4 mo), 103.4 ± 8.8 (12 mo), 105.7 ± 2.8 (18 mo) and 104.1 ± 9.8 cm (28 mo).

Mucosa samples were homogenized in 10 volumes of sucrose buffer (50 mM Tris-HCl, 0.25 M sucrose, pH 7.5) in an ice bath, using a Tekmar Tissumizer, SDT-1810 (Spokane, WA) at 85% power for 2 min. Microsomal and cytosolic fractions in the resulting homogenate were prepared as previously described (Bolling et al., 2010). Microsomes were resuspended in 0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol. Protein concentrations in microsomal and cytosolic fractions were determined by a Pierce BCA kit (Thermo Fisher
and then adjusted to 5 and 1 mg/mL, respectively. Aliquots were stored at -80°C until use.

**Kinetics and individual rate of glucuronidation.** The glucuronidation kinetics of rat SI microsomes was determined as previously described with slight modifications (Bolling et al., 2010). Microsomal protein (0.2 mg/mL, final concentration) was pre-incubated with alamethicin (0.25 µg/mL) at 37°C for 5 min in microcentrifuge tubes containing quercetin previously dried under nitrogen gas. To initiate the reaction, a cofactor solution of UDP-glucuronic acid (5 mM, final concentration), magnesium chloride (5 mM, final concentration), and potassium phosphate buffer, pH 7.5 (0.05 mM, final concentration) was added in a final assay volume of 0.1 mL. After incubation for selected durations at 37°C, the reaction was terminated with 0.1 mL ice-cold methanol containing 33 µM daidzein as an internal standard. After centrifugation at 14,000 x g for 5 min, 180 µL of supernatant was dried under a stream of purified nitrogen gas at room temperature and stored at -20°C until HPLC analysis.

SI mucosa microsomes were pooled by age and segment for linearity and kinetic analysis. When incubating with 30 µM quercetin for 30 min, total glucuronide formation was linear from 0.05 to 0.4 mg microsomal protein/mL in 4 and 28 mo pooled samples, and with 300 µM quercetin, from 0.05 to 0.5 mg microsomal protein/mL. Similarly, at 0.25 mg microsomal protein/mL, total glucuronide formation was linear from 0 to 60 min at both quercetin concentrations. The kinetics of quercetin glucuronide formation for each age and SI segment was determined in duplicate using pooled microsomes at 0.2 mg/mL and a 30 min incubation period with concentrations ranging from 4.7–300 µM quercetin. We attempted to achieve linearity with shorter incubation periods and less protein content to assay within direct range of Km values.
However, assay conditions were not linear or resulted in quercetin glucuronide peaks below the limit of quantification.

Following kinetic analysis, the quercetin glucuronidation rate of individual microsomal samples from each age and SI segments was determined in triplicate, using 30 and 300 µM quercetin, 0.2 mg microsomal protein/mL, and 30 min incubation.

**HPLC analyses of quercetin glucuronides.** The quercetin glucuronidation products were analyzed using a HPLC method as previously described (Boersma et al., 2002; Bolling et al., 2010). Reconstituted products were injected into a Thermo-Finnigan Surveyor HPLC (Thermo Fisher Scientific, San Jose, CA) equipped with an autosampler, UV detector, and a Phenomenex Synergi 10 µm Hydro-RP80 250 x 4.6 mm column (Torrance, CA). A gradient of 20 mM phosphate buffer at pH 2.0 and acetonitrile were used to separate quercetin glucuronides (Bolling et al., 2010). Quercetin glucuronides were quantified by integrating the area under the curve of peaks at 340 nm, and normalizing to the concentration of the internal standard daidzein that was integrated at 250 nm. Concentrations were determined using standard curves of quercetin, and daidzein subjected to the glucuronidation protocol with the absence of UDPGA. Quercetin was employed for quantification of quercetin glucuronides based on the assumption that quercetin glucuronides display the same UV extinction coefficient as quercetin aglycone. The limit of quantification for quercetin was 1 µM.

The four principal quercetin glucuronides produced by rat intestine microsomes were identified as previously described by Boersma et al. (Boersma et al., 2002) and van der Woude et al. (van der Woude et al., 2004) with retention times (Rt) of 7-O-glucuronide at 13.4 min, 3-O-glucuronide at 13.8 min, 4′-O-glucuronide at 16.6 min, and 3′-O-glucuronide a 17.1 min. An additional quercetin glucuronide product was observed at 12.4 min, and designated M1.
**Western blotting.** To further investigate the observed age-related differences in UGT activity toward quercetin, UGT protein content in microsomal samples from 4 and 28 mo were measured using western blotting. Aliquots of microsomal and cytosolic protein were separated on a 4-20% SDS-acrylamide gel using electrophoresis at 0.06 Amps in Tris-glycine-SDS running buffer. Gels were transferred to PVDF membranes using semi-dry transfer (BioRad). Membranes were then blocked for 1 hr at room temperature using 5% nonfat dried milk in TBST buffer, rinsed with TBST, then incubated at 4 °C overnight with primary antibody in 5% nonfat dried milk in TBST buffer. UGT1A, 1A1, 1A5, 1A6, 1A7, and 1A8 antibodies were incubated at 1:200 dilutions for microsomal protein, and villin antibody was incubated at 1:150 dilutions for cytosolic protein. After incubation with primary antibodies, membranes were allowed to return to room temperature, rinsed with TBST, and incubated with secondary anti-goat (UGT) and antirabbit (villin) IgG-HRP antibodies at a 1:2500 dilution at room temperature for 1 hr. Membranes were rinsed with TBST, incubated with luminol reagent for 3 min, and then exposed to film (X-OMAT Blue XB, Kodak, Rochester, NY) for 3 min (UGT1A), 35 min (UGT1A1, 1A8, villin) or overnight (UGT 1A5, 1A6, 1A7) and developed in a darkroom. Denisitometry analysis was performed using BioRad GS-710 scanner and Quantity One version 4.1.0 software (Biorad).

Human UGT1A1 (for UGT1A) or samples with abundant UGT or villin content were used to create standard curves for immunoblotting. Villin is indicative of enterocyte content of tissue preparations, and UGT protein content was expressed as UGT density/villin density.

**Data analysis.** All results are expressed as mean ± standard error. The best-fit kinetic models were selected by examining Michaelis-Menton, Lineweaver-Burke, Eadie-Hofstee plots and by an F-test and comparison of Akaike's information criterion (AIC) values, a measure of the goodness of fit of an estimated statistical model, using GraphPad Prism v5.01 (GraphPad.
Software, San Diego, CA). The Michaelis-Menten model (Eq. 1) and the uncompetitive substrate inhibition model (Eq. 2) were used to model data where $V$ is the velocity, $S$ is substrate concentration, $V_{max}$ is the maximum velocity, $K_m$ is the substrate concentration at 50% $V_{max}$, and $K_s$ is the substrate inhibition constant. Kinetic parameters are expressed as the estimate and standard error of the model.

\[
V = \frac{V_{max} \times S}{K_m + S} \quad \text{(Eq. 1)}
\]

\[
V = \frac{V_{max} \times S}{K_m + S \times (1 + S/K_s)} \quad \text{(Eq. 2)}
\]

Logarithmic transformation of data was applied prior to statistical analysis, to normalize unequal variance. Two-way ANOVA was used to test the effect of age and SI segment on quercetin glucuronidation, UGT immunoblotting, and villin concentration, while one-way ANOVA was used for analysis of regioselectivity. When $P$ values were $\leq 0.05$, post-hoc analysis was performed using Tukey’s honestly significant difference test. Pearson’s correlation analysis was used to determine relationships between rates of glucuronidation and UGT protein expression. Statistical analyses were performed using GraphPad Prism 5.01 (GraphPad Software).

RESULTS

**Determination of villin.** Villin is a cytoskeleton protein, and was used to control for enterocyte content of tissue preparations. Villin was present in the cytosol (Supplemental Figure 1), but not the microsomal fraction. Villin was unchanged with age in the proximal and distal SI, but 28 mo was 115% greater than 4 mo in the medial SI ($P = 0.0003$) (Figure 1). Similar to a previous study (Iiizumi et al., 2007), the villin content of mucosa declined along the length of the SI in 4 and 28 mo rats ($P = 0.0003$). Since villin content apparently varied by preparation, we
subsequently normalized UGT activity and immunoblots to villin content. Data that were not normalized to villin are presented in Supplemental Table 1, Supplemental Figure 2, and Supplemental Figure 3.

**Microsomal quercetin glucuronidation kinetics.**

*Production of quercetin glucuronide isoforms.* Quercetin was glucuronidated by intestinal microsomes at the 7-, 3-, 4’-, and 3’-hydroxyl position, and to a lesser extent to an uncharacterized glucuronide specified as M1.

*Kinetic modeling of quercetin glucuronidation.* Pooled SI microsomal UGT activity toward quercetin, as the sum of quercetin metabolites, was better modeled by Michaelis-Menten rather than uncompetitive substrate inhibition kinetics in most SI segments and ages (Figure 2) based on inspection of Lineweaver-Burke and Eadie-Hofstee plots. Total quercetin-glucuronidation by the medial SI at 4 and 28 mo and distal SI at 28 mo were better modeled by uncompetitive substrate inhibition than Michaelis-Menten kinetics based on AIC values (Supplemental Table 2). Similarly, kinetics of quercetin glucuronide isomer production in the medial and distal SI at 28 mo were also better modeled by uncompetitive substrate inhibition. Visual inspections of the plots revealed only slight differences between fitted curves, so for comparative purposes, both kinetic models are presented in Supplemental Table 2 and Supplemental Table 3.

Kₘ values for the 7-O-glucuronide, 3-O-glucuronide, and the 4’-O-glucuronide at 4 and 12 mo in the proximal small intestine were below the lowest concentration employed in the glucuronidation assay, so these results should be interpreted with caution.

*Proximal SI quercetin glucuronidation kinetics.* In the proximal SI, age-related changes in microsomal quercetin glucuronidation kinetics as the sum of glucuronide isoforms were apparent beginning at 12 mo, but were most profound at 18 mo, with a 13-fold increase in Vₘₐₓ from 4 mo
(Figure 2, Supplemental Table 2). In the proximal SI, $\text{CL}_{\text{int}}$ increased by 3- and 9-fold in 12 and 18 mo as compared to 4 mo (Supplemental Table 2). $K_m$ values increased by 50% gradually from 4 to 28 mo, but were offset by larger increases in $V_{\text{max}}$ values at 12 and 18 mo. Increases in $\text{CL}_{\text{int}}$ at 12 and 18 mo followed a similar trend for the 7-, 3-, 4’,- and 3’-O-quercetin-glucuronides (Table 1). $K_m$ values peaked at 28 mo for 7-glucuronide and at 18 mo for the 3- and 4’-glucuronides, and at 12 mo for the 3’-glucuronide. $V_{\text{max}}$ values for 7-, 3-, 4’, and 3’-glucuronides declined from 18 to 28 mo, but were still 1.7- to 2.8-fold higher than 4 mo.

**Medial SI quercetin glucuronidation kinetics.** Age-related changes in the medial SI $V_{\text{max}}$ values for UGT activity, as a sum of all quercetin glucuronides, were not as pronounced as in the proximal and distal SI segments (Figure 2). In the medial SI, total quercetin $\text{CL}_{\text{int}}$ values increased 1.4-fold at 12 mo, were similar at 18 mo, but declined at 28 mo (Supplemental Table 2). The decline in total quercetin $\text{CL}_{\text{int}}$ from 18 to 28 mo was mainly due to a 68% decrease in $V_{\text{max}}$. $V_{\text{max}}$ values for the 7-, 3-, 4’, and 3’-glucuronides declined by a similar magnitude to total quercetin glucuronides (Table 1). Total quercetin $K_m$ values at 12 mo were 50% of 4 mo but were restored at 28 mo. For quercetin glucuronide isomers, similar changes in $K_m$ values were observed (Table 1).

**Distal SI quercetin glucuronidation kinetics.** In the distal SI, total quercetin $V_{\text{max}}$ values increased by 3- and 3.5- fold at 12 and 18 mo as compared to 4 mo, while $K_m$ values were increased at least 42% from 12 to 28 mo (Supplemental Table 2). The $K_m$ values of 7-, 3-, and 4’-glucuronides peaked at 12 mo, but declined to 28 mo, whereas the 3’-O-glucuronide peaked at 28 mo (Table 1). In contrast, $V_{\text{max}}$ values for 7-, 3-, 4’, and 3’-glucuronides peaked at 18 mo, with increases from 2.6- to 3.8-fold from 4 mo.
Comparative SI segment quercetin glucuronidation kinetics. Villin-adjusted total quercetin CL_{int} values increased from the proximal to distal SI at each age, due to increases in V_{max} values (Supplemental Table 2). Likewise, total quercetin K_{m} values increased stepwise from the proximal to distal SI in 12, 18, and 28 mo rats, but declined by 7% from the medial to distal segment at 4 mo. The pattern of proximal > medial > distal SI in V_{max}, K_{m} and CL_{int} values was apparent for most quercetin-glucuronide isoforms at each age (Table 1). However, K_{m} values peaked in the medial SI for the 4’-glucuronide at 4 and 28 mo, and the 3’-glucuronide at 4 mo. The K_{m} value for the 7-glucuronide was also lowest in the medial SI as 12 mo only. Thus, age-related changes in quercetin glucuronidation kinetics in SI were dependent upon segment and most pronounced at 12 and 18 mo.

Quercetin glucuronidation at 30 and 300 µM. Following characterization of pooled microsomal kinetics, rates of quercetin glucuronidation were determined in SI segments from each rat at 30 µM and 300 µM quercetin. These substrate concentrations were in range of K_{m} values and 10 times of K_{m} values, respectively, and were linear with respect to time and protein concentration for rate of quercetin glucuronidation. Using this approach, significant age and segment-related differences in villin-adjusted quercetin-UGT activity (as the sum of all metabolites) were observed at 30 µM quercetin (P < 0.0001) (Figure 3). In all SI segments, rates of quercetin glucuronidation were increased at 12 and 18 mo relative to 4 mo. The greatest increases were observed in the proximal and distal SI at 18 mo, where total quercetin glucuronidation rates increased by 4.8- and 3.9-fold, respectively. The formation of 7-, 3-, 3’-, and 4’- glucuronide isomers increased with age in each SI segment, and followed a nearly identical trend with total quercetin glucuronidation at 30 µM (Figure 4). In the proximal SI, activity at 18 mo was 4.3- to 5.5-fold greater than 4 mo for each glucuronide isoform. In the
medial and distal SI, increases were evident beginning at 12 mo, where activity was 40 to 79% greater than 4 mo. In each segment, the changes in activity with age were of similar magnitude among isoforms, as there was not a significant interaction ($P = 1.0$).

Significant age-related increases in rates of total quercetin glucuronidation were also observed at 300 µM quercetin ($P = 0.0013$, Supplemental Figure 4). As at 30 µM, the formation of different quercetin-glucuronide isoforms mirrored total quercetin UGT activity within SI segments (Supplemental Figure 5).

At each age and quercetin concentration, rates of villin-adjusted glucuronidation increased by descending segment of the SI. For example, at 4 mo and 30 µM quercetin, the ratio of UGT activity for proximal:medial:distal SI segments was 1:7:12, whereas at 12 mo, this ratio was 1:7:23, and was 1:2:6 at 28 mo. Thus, age had a significant impact on relative enterocyte quercetin glucuronidation rates between SI segments.

**Tissue regioselectivity of quercetin UGT activity.** In all SI segments, there was not a significant interaction between age and formation of quercetin-glucuronide isoforms. Thus, data was pooled by age to examine the effect of SI segment on UGT regioselectivity. The relative microsomal rates of formation of the four identified quercetin-glucuronide isomers changed along the length of the SI at 30 and 300 µM quercetin, but those of M1 were unchanged (Figure 5). From proximal to distal SI, UGT selectivity toward the 7- and 3- hydroxyl group of quercetin increased, whereas selectivity decreased toward the 4’- and 3’- hydroxyl group. Changes in UGT selectivity toward the 7- and 4’- hydroxyl groups of quercetin from proximal to distal SI were the most extreme, by an increase from 29 to 51% and decline of 11 to 27%, respectively.

**Enterocyte UGT expression.** Upon observing age-related increases in enterocyte UGT activity, we further examined UGT protein expression in SI samples by semi-quantitative western blot.
UGT1A5 and UGT1A6 proteins were not sufficiently abundant in SI microsomal samples for further screening. Standard curves of human UGT1A1 (for UGT1A) or samples with abundant UGT content were used to calibrate data (Figure 6).

In contrast to quercetin glucuronidation kinetics and activities, enterocyte UGT protein content was not consistently affected by age (Figure 7). For all antibodies, increases in proximal SI UGT content at 12 and 18 mo from 4 mo were evident, but not in other segments. For UGT1A, there was a significant interaction between age and segment (P =0.0012). Enterocyte UGT1A protein was increased by 64 and 150% at 12 and 18 mo from 4 mo in the proximal SI, but decreased by 48 to 82% at the same time points in the medial and distal SI. At each age, enterocyte UGT1A protein was larger in the proximal SI than the medial and distal segments. In 4 mo rats, enterocyte UGT1A1 and UGT1A7 content increased from proximal to distal SI segment, but not at other ages. Notwithstanding, distal UGT1A1 and 1A7 content was greater than in proximal or medial segments. UGT1A8 protein was significantly different by segment (P = 0.0008), with an interaction between age and segment (P = 0.0034) as medial SI content was the greatest at 4 mo, while in the distal segment, rats at 28 mo had 50% greater content relative to 4, 12, and 18 mo.

**Correlation of microsomal UGT content with quercetin glucuronidation.** Correlation of microsomal UGT content with quercetin glucuronidation was performed to provide insight into age-related increases in UGT activity and changes in SI UGT regioselectivity (Supplemental Table 4). Independent of age or SI segment, UGT1A1 protein was positively correlated with total glucuronidation rates at 30 and 300 µM quercetin (P <0.001) with r-values of 0.467 and 0.447, respectively. In contrast, UGT1A and UGT1A8 content was significantly negatively correlated (P <0.001) with total glucuronidation rates at both 30 and 300 µM quercetin with r-
values from -0.305 to -0.412. Independent of age, UGT1A1 was positively correlated with 30 µM quercetin activity in the medial SI only with r-values of 0.404 to 0.475. In contrast, at 300 µM quercetin, UGT1A1 was only correlated in distal SI for total quercetin glucuronidation and 7- and 3- glucuronide rates with r-values of 0.398 to 0.467. Independent of SI segment, UGT1A1 was correlated with total quercetin glucuronidation rates with r-values from 0.538 to 0.589 at each age. Since pooling SI segment data resulted in stronger correlations (r-values of 0.385 to 0.614) than pooling ages (r-values of 0.005 to 0.475), despite smaller sample size (n = 24 vs. n = 32), UGT1A1 content may partly explain age-related differences in quercetin glucuronidation more so than SI segment differences.

Correlations of isoform-specific glucuronidation rates to UGT content were not largely different that to the sum of glucuronide isoforms. However, for UGT1A1 activity, formation of 4’- glucuronide (r = 0.320) was more weakly correlated than other isoforms (r > 0.424). UGT1A7 was significantly correlated (r = 0.431) with the 3’- isoform production from 30 µM quercetin in the medial SI, whereas other isoforms were not. However, this relationship was not evident at 300 µM quercetin.

**DISCUSSION**

Advanced age is known to affect intestinal function, including enterocyte structure and nutrient absorption (Drozdowski and Thomson, 2006). Yet little is known about age-related differences in intestinal xenobiotic metabolism enzymes. Therefore, we used quercetin, a dietary flavonoid that is extensively glucuronidated upon absorption, to evaluate small intestine (SI) microsomal enterocyte UGT activity and protein abundance in young adult to senescent male Fisher F344 rats.
Our observation that senescent rats had increased SI microsomal quercetin glucuronidation rates and intrinsic clearance values contrasts with the few animal studies of SI xenobiotic metabolism in advanced age. Phenolphthalein glucuronidation was unchanged with advanced age in intestinal tissues of male rats up to 30 mo (Borghoff and Birnbaum, 1985). However, the authors noted that the low specific activity of phenolphthalein glucuronidation in tissues may have lacked the necessary sensitivity to the observed age-related changes. Warrington et al., found that CYP3A and p-glycoprotein expression were unchanged in male Fisher 344 rat intestine, although NADPH reductase expression declined (Warrington, 2004a; Warrington, 2004b). To our knowledge, this study is the first report of age-related changes to enterocyte glucuronidation capacity or UGT content in rodents.

The use of villin as a control for enterocyte abundance in tissue preparation may have facilitated these observations. Villin is constitutively expressed in enterocytes and localized at the brush-border (West et al., 1988). It has been used in other studies to normalize drug-metabolizing enzymes and transporters to enterocyte content of tissue preparations (Lown et al., 1994; Johnson et al., 2001, Gibbs et al., 1999). We could find no published studies that suggest there are changes in villin content with age. Thus, it is not clear if our observation is truly an age-dependent difference in enterocyte content of villin or the result of differences in villin-containing enterocytes relative to other cells in the preparations.

The changes in small intestine quercetin glucuronidation rates and kinetics were accompanied by changes in UGT expression. UGT1A1 and UGT1A7 were positively correlated to rates of SI quercetin glucuronidation, particularly when stratified with age. Interestingly, Brand et al. (2010) concluded that UGT1A1 and UGT1A7 were the predominant isoforms responsible for the microsomal glucuronidation of the flavanone hesperetin in rat liver and
intestine. The significance of the negative correlations of UGT1A and UGT1A8 to quercetin glucuronidation rates is unclear and may suggest differential regulation of total UGT1A and UGT1A1 or UGT1A7. It should also be noted that substrate specificity of the primary antibodies toward UGT has not been extensively validated.

Previous studies of recombinant human UGT have characterized the differential activity of UGT isoforms toward quercetin. UGT1A3 and 1A9 from Sf9 cells have similar $K_m$ values of ~40 µM, but $V_{max}$ values of 2 and 10 nmol/min/mg, respectively (Chen et al., 2008). Basu et al. (2003) also found that the specific activity for 16 h incubations of UGT from COS kidney cells with quercetin were greatest for UGT1A1, 1A6, 1A7, and 1A10, and lower for UGT1A8 and 1A9. In contrast, 30 min incubations with 100 µM quercetin demonstrated that UGT1A1, UGT1A3, UGT1A8, 1A9, and 2B7 had greater rates of glucuronidation than UGT1A4, 1A6, 1A10, and 2B15 (Boersma et al., 2002). Differences in $V_{max}$ and $K_m$ values between UGT isoforms could lead to their increased or decreased glucuronidation activity toward substrates assessed at different test concentrations (Tang et al., 2009). In this study, correlations of UGT content to glucuronidation at 30 and 300 µM quercetin were generally similar. Notably, independent of age, UGT1A1 was positively correlated with 300 µM glucuronidation in the distal SI only, while 30 µM was correlated to the medial SI only. Comprehensive kinetic profiles of rat recombinant UGT isoforms toward quercetin are necessary to predict the contributions of UGT isoforms to age-related changes in quercetin glucuronidation.

The mechanism for the age-related changes in enterocyte UGT content and capacity needs further definition. Plausibly, increased oxidative stress or inflammation along with advance of aging might induce UGT expression. The intestine-specific transcription factor caudal-related homeodomain protein 2 (Cdx2) and hepatocyte nuclear factor (HNF1) activate promoters for
UGT1A8, 1A9, and 1A10 in human Caco-2 cells (Gregory et al., 2004). In *Drosophila*, both oxidative stress and age up-regulated intestinal caudal expression via the NFκB pathway (Choi, 2008). In contrast, pharynx *ugt-9* gene expression is down-regulated in normal aging in *C. elegans* through Erythroid-Like Transcription factor-3 (ELT-3), but up-regulated through ELT-3 upon exposure to oxidative stress through paraquat (Budovskaya et al., 2008). Pregnane X receptor can modulate *Ugt1A2* mRNA expression in duodenum of rats (Shelby and Klaassen, 2006), but did not vary with age in the mouse liver (Echchgadda et al., 2004). Long-lived mice were found to have increased hepatic UGT1A9 mRNA expression regulated by bile acids through farnesoid X receptor (FXR) (Amador-Noguez et al., 2007), but the impact of FXR on SI UGT expression has not been defined. Thus, while oxidative stress associated with aging may differentially regulate UGT isoforms, more work is needed to elucidate the pathways responsible for differences in enterocyte UGT in rats of older age.

While human studies of intestinal glucuronidation in the elderly are lacking, *in vitro* results from liver-bank studies indicate advanced age makes little to no difference in hepatic microsomal glucuronidation of valproate (Argikar and Remmel, 2008), S-oxazepam, trifluoperazine, serotonin, propofol, zidovudine (Court, 2010), and 4-methylumbelliferone (Parkinson et al., 2004). In rats, hepatic microsomal glucuronidation of acetaminophen was also unchanged with advanced age (Sweeny and Weiner, 1985; Woodhouse and Herd, 1993). However, we observed modest changes in quercetin and genistein microsomal glucuronidation in livers of F344 rats of advanced age (Bolling et al., 2010), and increased steady-state tissue concentrations of genistein in older Sprague Dawley rats (Chen and Bakheit, 2006). Age-related diseases could also modify UGT activity, as arthritic rats had decreased hepatic microsomal rates
of \( p \)-nitrophenol glucuronidation but not bilirubin, and reduced \( V_{\text{max}} \) values of R- and S-
ketoprofen glucuronidation (Meunier and Verbeeck, 1999).

We and others have reported that glucuronidation in small intestine plays a key role in
quercetin metabolism in rats (Graf et al., 2006; Mullen et al., 2008). Our results further confirm
the importance of this organ for first-pass metabolism of flavonoids through glucuronidation.
Prolonged quercetin feeding to F344 rats resulted in mainly mono-, di-, or mixed glucuronides or
quercetin in intestinal tissue (Graf et al., 2006). In Sprague-Dawley rats, the primary metabolites
of quercetin-4'-glucoside in intestinal tissue were also glucuronides (Mullen et al., 2008). We
also found that SI microsomal quercetin \( \text{CL}_{\text{int}} \) was similar to the liver of F344 rats of our
previous study (Bolling et al., 2010). However, glucuronidation capacity of human intestinal
microsomes could be more than 1-fold larger than rats (Boersma et al., 2002).

Species- and organ-specific UGT regioselectivity likely impacts flavonoid bioefficacy, as the
position of quercetin glucuronidation affects its bioactions, e.g., inhibition of lipoxygenase and
xanthine oxidase (Day et al., 2000). Quercetin glucuronidation regioselectivity \textit{in vivo} has not
been quantitatively assessed, in part due to the lack of suitable standards. Our \textit{in vitro} study
showed that regioselectivity of microsomal quercetin glucuronidation in SI is segment-
dependent, possibly due to differential expression of isoforms. For example, human recombinant
UGT1A1 catalyzed mainly the 3'-hydroxyl quercetin glucuronidation, while UGT1A6
preferentially catalyzed the 4'- and 7-hydroxyl moieties (Boersma et al., 2002). In addition, such
regioselectivity of UGT isoforms is dependent flavonoid subfamilies, e.g., human recombinant
UGT1A1 mainly catalyzed 7-hydroxyl position of the flavanone hesperetin and UGT1A7
selectively glucuronidated the 3'-hydroxyl position (Brand et al., 2010). Further, the
regioselectivity of flavonoid glucuronidation in intestine is species dependent, with 7% quercetin
conjugated in 7-hydroxy moiety in human and 41% at this position in rats (Boersma et al., 2002). Differences between quercetin glucuronide profiles in rodents and humans likely derive from regulatory and nucleotide differences in UGT isoforms (Mackenzie et al., 2005). Interestingly, 7-quercetin-glucuronide isoforms was not found in human plasma (Day et al., 2001) despite production of 7-O-glucuronide in human liver microsomes (Boersma et al., 2002).

The potential impact of age-related changes in SI UGT expression and quercetin glucuronidation on in vivo bioavailability is unknown. Mizuma (2009) reported that extensive intestinal glucuronidation of raloxifene accounts for its low oral bioavailability. Quercetin likewise has low oral bioavailability (Moon et al., 2008) which may arise from its extensive intestinal glucuronidation. Wang et al. (2006) found that rat intestinal microsomal CL_{int}, V_{max}, and K_{m} values for glucuronidation of the isoflavones were unrelated to rates of glucuronidation in an intestinal perfusion model. Data from clinical trials of flavonoid metabolism using a wide range of ages are lacking, but interestingly, in a study of 129 subjects consuming orange juice, urinary excretion of hesperetin was decreased with advanced age, equating to a 4% decrease between the ages of 20 and 80 years (Brett et al., 2008). Although the mechanism of the decrease in urinary excretion remains to be explored, age could be considered as a potential, albeit weak, modifier of flavonoid metabolism.

In conclusion, age-related differences in UGT quercetin glucuronidation activity in male Fisher F344 rats depend upon intestinal segment. The rates of villin-adjusted SI microsomal quercetin glucuronidation were increased from 4 to 12 to 28 mo, accompanied by increases in K_{m} and V_{max} values. Age did not alter UGT regionselectivity toward quercetin in SI while differential expression of UGT isoforms between segments could enable the formation of divergent glucuronide metabolite profiles. Given that only UGT1A1 and UGT1A7 content were
positively correlated with age-specific quercetin glucuronidation, age could potentially affect expression of intestinal UGT isoforms. Since UGT isoforms displayed varied substrate specificity, our finding with quercetin is not readily extrapolatable to other UGT substrates. Thus, more work is needed characterize the degree by which advanced age affects intestinal glucuronidation of other xenobiotics.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Blumberg, Bolling, Chen, Court

Conducted experiments: Bolling, Chen

Contributed new reagents or analytic tools: Court

Performed data analysis: Bolling, Chen, Court.

Wrote or contributed to the writing of the manuscript: Blumberg, Bolling, Chen, Court
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Mizuma T (2009) Intestinal glucuronidation metabolism may have a greater impact on oral bioavailability than hepatic glucuronidation metabolism in humans: A study with raloxifene, substrate for UGT1A1, 1A8, 1A9, and 1A10. *International Journal of Pharmaceutics* **378**:140-141.


FOOTNOTES

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LEGENDS FOR FIGURES

**Figure 1.** Relative cytosolic villin content of tissue preparations at different ages and rat small intestine (SI) segment. Data is mean ± SEM, n = 8/age, log-transformed data was analyzed by 2-way ANOVA. $P < 0.0003$ for section and age.

**Figure 2.** Age-related changes in villin-adjusted kinetics of quercetin glucuronidation, as sum of all metabolites, by pooled microsomal protein fractions from A.) proximal, B.) medial, and C.) distal small intestine (SI) segments of male F344 rats. Solid lines indicate a Michaelis-Menten model and dashed lines indicate an uncompetitive inhibition model were best fits for data as specified in methods section.

**Figure 3.** Villin-adjusted rates of microsomal glucuronidation of 30 µM quercetin by SI segment and age with 0.2 mg microsomal protein/mL and 30-min incubation. Data is mean of the sum of metabolites ± SEM, n = 8/age. Log transformed data was analyzed by 2-way ANOVA and $P < 0.0001$ for age and segment, and $P = 0.3050$ for interaction between age and segment.

**Figure 4.** Villin-adjusted rates of glucuronidation of 30 µM quercetin (Q) by microsomal fractions collected from mucosa of small intestine (SI) segment with 0.2 mg microsomal protein/mL and 30-min incubation. Data are mean ± SEM of n = 8/age. Log-transformed data were analyzed by 2-way ANOVA.

**Figure 5.** Regioselectivity 30 µM quercetin in small intestine is independent of age. Data is mean ± SEM, n = 32.
Figure 6. Standard dilution curves and representative Western blots. A. UGT1A using recombinant human UGT1A1 for a standard curve, and 37.5 µg/well of rat proximal SI microsomal protein at different ages. B. UGT1A1 using mixtures of rat SI microsomal samples with high and low responses at 37.5 µg/well, and 37.5 µg/well distal SI microsomes from 4 and 28 mo rats. C. UGT1A7 using dilutions of a rat SI microsomal samples with a high response, and 37.5 µg/well of proximal SI microsomal protein from 4 and 28 mo rats. D. UGT1A8 using dilutions of a rat SI microsomal sample with a high response, and 37.5 µg/well of distal SI microsomal protein from 4 and 28 mo rats.

Figure 7. A.) UGT1A, B.) UGT1A1, C.) UGT1A7, and D.) UGT1A8 enterocyte protein content [(UGT density)/(villin density)] assessed by Western blotting of equidistant small intestine mucosa from 4, 12, 18, and 28 mo rats. Data were analyzed by 2-way ANOVA, n = 8/age, using log-transformed values. P values for UGT1A were 0.3064 for age, <0.0001 for segment, and 0.0012 for their interaction; for UGT1A1 were 0.0763 for age, <0.0001 for segment, and 0.0799 for their interaction; for UGT1A7 were <0.0001 for age, <0.0001 for segment, and 0.2668 for their interaction; for UGT1A8 were 0.9585 for age, 0.0008 for segment, and 0.0034 for their interaction. Representative Western blots and semi-quantitative standard curves for each antibody are presented in Figure 6.
Table 1. Villin-adjusted kinetics of pooled microsomal quercetin glucuronidation in the small intestine of male F344 rats.

<table>
<thead>
<tr>
<th>Isomer</th>
<th>Segment</th>
<th>Age</th>
<th>(V_{\text{max}}) (nmol/min/mg)/villin</th>
<th>(K_m) (µM)</th>
<th>(CL_{\text{int}}) (mL/min/mg)/villin</th>
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<td>7-(O)-gluc</td>
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<td></td>
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<td></td>
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<td>1.37 ±0.07</td>
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<td>20.4 ±0.5</td>
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<td>5.81 ±0.18</td>
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Data is modeled by Michaelis-Menten kinetics and is expressed as the estimate ± standard error of duplicate determinations. Abbreviations: gluc, glucuronide.
Figure 1.
Figure 2.
Figure 3.
Figure 4.

A. Proximal SI, 30 μM Quercetin

- Age: $P < 0.0001$
- Isoform: $P < 0.0001$
- Interaction: $P = 0.9970$

B. Medial SI, 30 μM Quercetin

- Age: $P = 0.0044$
- Isoform: $P < 0.0001$
- Interaction: $P = 1.000$

C. Distal SI, 30 μM Quercetin

- Age: $P < 0.0001$
- Isoform: $P < 0.0001$
- Interaction: $P = 1.000$
Figure 5.
Figure 6.

A. UGT1A
Protein (µg): 0.5 1 2 3 4 5 10 15 18
64 kDa
Age (mo): 4 4 4 4 28 28 28 28
64 kDa

B. UGT1A1
% Sample: 100 90 75 60 45
64 kDa
Age (mo): 4 4 4 4 28 28 28 28
64 kDa

C. UGT1A7
Protein (µg): 75 56 37.5 19
64 kDa
Age (mo): 4 4 4 4 28 28 28 28
64 kDa

D. UGT1A8
Protein (µg): 80 40 20 10 5 2 1 0.2
64 kDa
Age (mo): 4 4 4 4 28 28 28 28
64 kDa
Figure 7.