

Research Article

Characterization of Raloxifene Glucuronidation: Potential Role of UGT1A8 Genotype on Raloxifene Metabolism *In Vivo*Dongxiao Sun¹, Nathan R Jones¹, Andrea Manni², and Philip Lazarus^{1,3}

Abstract

Raloxifene is a second-generation selective estrogen receptor modulator used for the prevention and treatment of osteoporosis and the prevention of breast cancer in postmenopausal women. Raloxifene is extensively metabolized by glucuronidation to form raloxifene-6-glucuronide (ral-6-Gluc) and raloxifene-4'-glucuronide (ral-4'-Gluc). The goal of the present study was to determine whether functional polymorphisms in active UGTs could play a role in altered raloxifene glucuronidation *in vivo*. Using homogenates from HEK293 UGT-overexpressing cell lines, raloxifene was shown to be glucuronidated primarily by the hepatic UGTs 1A1 and 1A9 and the extra-hepatic UGTs 1A8 and 1A10; no detectable raloxifene glucuronidation activity was found for UGT2B enzymes. Functional UGT1A1 transcriptional promoter genotypes were significantly ($P_{\text{trend}} = 0.005$) associated with ral-6-Gluc formation in human liver microsomes, and, consistent with the decreased raloxifene glucuronidation activities observed *in vitro* with cell lines overexpressing UGT1A8 variants, the UGT1A8*2 variant was significantly ($P = 0.023$) correlated with total raloxifene glucuronide formation in human jejunum homogenates. While ral-4'-Gluc exhibited 1:100th the anti-estrogenic activity of raloxifene itself as measured by binding to the estrogen receptor, raloxifene glucuronides comprised about 99% of the circulating raloxifene dose in raloxifene-treated subjects, with ral-4'-Gluc comprising ~70% of raloxifene glucuronides. Plasma ral-6-Gluc ($P_{\text{trend}} = 0.0025$), ral-4'-Gluc ($P_{\text{trend}} = 0.001$), and total raloxifene glucuronides ($P_{\text{trend}} = 0.001$) were increased in raloxifene-treated subjects who were predicted slow metabolizers [UGT1A8 (*1/*3)] versus intermediate metabolizers [UGT1A8 (*1/*1) or UGT1A8 (*1/*2)] versus fast metabolizers [UGT1A8 (*2/*2)]. These data suggest that raloxifene metabolism may be dependent on UGT1A8 genotype and that UGT1A8 genotype may play an important role in overall response to raloxifene. *Cancer Prev Res*; 6(7); 719–30. ©2013 AACR.

Introduction

There was an estimated 226,870 subjects who developed breast cancer and 39,510 deaths arising from this disease in the United States in 2012 (1). Much of the treatment for estrogen receptor-positive (ER+) breast cancer in postmenopausal women has targeted the blocking of the ER-binding activity of estrogen or reducing estrogen synthesis. Tamoxifen, a first-generation selective estrogen receptor modulator (SERM), has been used for treatment and chemoprevention of breast cancer for over 25 years, but its long-term use is associated with rare but serious adverse effects (2). Raloxifene is a second-generation SERM approved by the U.S. Food and Drug Administration for the treatment and pre-

vention of osteoporosis and the chemoprevention of invasive breast cancer in postmenopausal women (3). Raloxifene acts as an estrogen agonist in bone and liver to increase bone mineral density and decreases low-density lipoprotein (LDL) cholesterol (4) and exhibits strong anti-estrogen effects in breast and uterus (5). Recent clinical trials showed that raloxifene significantly reduced the incidence of breast cancer in high-risk women although not as effectively as tamoxifen (38% vs. 50%, respectively; ref. 6). However, in contrast to tamoxifen, raloxifene does not cause endometrial proliferation (7). Although not as serious as those associated with tamoxifen, adverse effects associated with raloxifene include hot flashes, vaginal dryness, and leg cramps and thromboembolic events such as deep venous thrombosis, pulmonary emboli, and retinal vein thrombosis (8).

Up to 60% of the raloxifene dose is absorbed rapidly after oral administration (8–11), but there is less than 2% bioavailability due mainly to extensive *in vivo* glucuronidation (8–11). Raloxifene is primarily excreted in feces, with less than 0.2% excreted as unchanged raloxifene and less than 6% eliminated as urinary glucuronide conjugates. In addition to the hepatic metabolism of raloxifene, several studies suggest that the intestine may play an important role

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in raloxifene metabolism (12–16). Previous studies have shown the presence of two raloxifene glucuronides in the plasma of women taking raloxifene, raloxifene-6- β -glucuronide (ral-6-Gluc) and raloxifene-4'- β -glucuronide (ral-4'-Gluc), with a plasma ral-4'-Gluc:ral-6-Gluc ratio of ~about 8:1. Unconjugated raloxifene comprises less than 1% in human plasma (9–11, 17).

Previous studies characterizing the family 1A UDP-glucuronosyltransferase (UGT) enzymes involved in the glucuronidation of raloxifene showed that the hepatic UGTs 1A1 and 1A9 and the extrahepatic UGTs 1A8 and 1A10 were active against raloxifene (14). A recent study suggested that the UGT1A1*28 allelic variant, which contains an A (TA)₇TAA in the TATAA box of the UGT1A1 transcriptional promoter and is associated with decreased expression of the UGT1A1 gene (18), is associated with altered raloxifene pharmacokinetics (19). No studies have as yet been conducted examining the role of genotypes in other active UGTs on raloxifene glucuronidation phenotype. The goal of the present study was to fully characterize the glucuronidating activity of individual UGT1A and UGT2B enzymes against raloxifene and to compare the overall glucuronidating activity of variant active UGTs versus their wild-type counterparts both *in vitro* and *in vivo*.

Materials and Methods

Chemicals and materials

Raloxifene, UDP-glucuronic acid (UDPGA), alamethicin, β -glucuronidase, β -actin, and bovine serum albumin were purchased from Sigma-Aldrich. Ral-6-Gluc, ral-4'-Gluc, raloxifene-d4, ral-6-Gluc-d4, and ral-4'-Gluc-d4 were purchased from Toronto Research Chemical. Dulbecco's modified Eagle's medium (DMEM), Dulbecco's PBS (minus calcium chloride and magnesium chloride), FBS, penicillin/streptomycin, and Geneticin (G-418) were purchased from Invitrogen. The BCA protein assay kit was purchased from Pierce Chemical.

Tissues and cell lines

A description of the normal human liver tissue specimens used for these studies and the methods used for liver microsomal preparation and protein quantification was provided previously (20). Normal jejunum tissues ($n = 46$) were purchased from Sun Health Research Institute (Sun City, AZ) and were obtained from non-cancer subjects between 2.5 and 4 hours postmortem and flash-frozen at -70°C . Jejunum homogenates were prepared by tissue homogenization in Tris-buffered saline at 4°C and stored in 100 μL aliquot (10–20 mg protein/mL) at -80°C until use. gDNA was extracted from liver and jejunum specimens using a Qiagen DNeasy Blood & Tissue extraction kit. All protocols involving the collection and analysis of tissue specimens were approved by the Institutional Review Board at Penn State University and were in accordance with assurances filed with and approved by the United States Department of Health and Human Services.

HEK293 cells were originally obtained from American Type Culture Collection and were stably transfected with

individual wild-type and variant UGTs as has been previously described (21–26). To authenticate the cell lines, overexpressed UGTs were directly sequenced using intron-spanning primers, and all of the UGT-overexpressing cell lines and homogenates used in these studies were tested for glucuronidation activity by glucuronidation assays/ultrapressure liquid chromatography (UPLC) detection against known test substrates within 3 months before a given experiment as previously described (21–24).

Plasma samples

Plasma samples were obtained from subjects entered into a clinical trial conducted at Penn State University College of Medicine examining the combined effects of raloxifene on biomarkers of hormone-independent breast cancer (27). All subjects were postmenopausal women with a breast density in excess of 25% and without a history of thromboembolic disorders and cardiovascular disease. Blood samples were collected from subjects within the 2 raloxifene-only treatment groups (30 or 60 mg daily doses) immediately before commencement of raloxifene treatment (time "0") and at one or more time points (6, 12, 18, and 24 months) after the commencement of treatment. All subjects provided written consent and agreed to their tissues being used for genetic studies. Bloods were fractionated by centrifugation at $1,200 \times g$ at 4°C for 5 minutes, and plasma and lymphocyte fractions were stored in 1 mL aliquots at -80°C until analysis or gDNA extraction as described above.

Glucuronidation assays

Glucuronidation activity assays were conducted essentially as previously described (24) for human liver microsomes (HLM; 15 μg), human jejunum homogenates (HJH; 10 μg), or homogenates from human UGT1A and UGT2B-overexpressing cells (2–100 μg). Glucuronidation rate assays were conducted in duplicate for HLM ($n = 105$) and HJH ($n = 46$) using 1 to 2 $\mu\text{mol/L}$ raloxifene. For kinetic analysis, 0.0625 to 256 $\mu\text{mol/L}$ raloxifene were used for assays with UGT-overexpressing cell homogenates, 3 randomly chosen HLM, and 3 randomly chosen HJH and was conducted in triplicate in independent assays. All kinetic data for the analysis of UGT-overexpressing cell homogenates were analyzed after normalizing relative to UGT protein levels expressed in each of the overexpressing cell lines, conducted by Western blot analysis as described previously (24, 28).

Raloxifene glucuronidation was analyzed using a Waters ACQUITY ultrapressure liquid chromatography UV detector (UPLC/UV) system with a 1.7- μm ACQUITY UPLC BEH C18 analytical column (2.1 \times 50 mm^2 , Waters, Ireland) in series with a 0.2- μm Waters assay frit filter (2.1 mm). The gradient elution was conducted using a flow rate of 0.5 mL/min with 5% acetonitrile and 95% buffer A (5 mmol/L ammonium acetate, pH 5.0) for 1 minute, a subsequent linear gradient to 100% acetonitrile over 5 minutes, and then maintained at 100% acetonitrile for 2 minutes. The wavelength for determination of raloxifene and its

glucuronides was 274 nm. Raloxifene-glucuronides (ral-6-Gluc and ral-4'-Gluc) were confirmed by their stability in 1 mol/L NaOH and sensitivity to the treatment of β -glucuronidase. In addition, confirmation of raloxifene glucuronide formation was conducted by loading up to 5 μ L of incubation product onto an UPLC identical to that described above in tandem with a Waters TQD triple quadrupole MS system. By using a positive mode, the parent compound $[M + H]^+$ peak and their corresponding glucuronide $[M\text{-Gluc} + H]^+$ peaks were characterized.

Determination of raloxifene metabolites in plasma

Stock solutions of raloxifene, ral-6-Gluc, ral-4'-Gluc, and their deuterated internal standards were prepared in dimethyl sulfoxide (DMSO). Raloxifene, ral-6-Gluc, and ral-4'-Gluc were combined into a standard stock solution and used to make a standard working solution from 25 ng/mL to 25 μ g/mL for raloxifene and 100 ng/mL to 100 μ g/mL for ral-6-Gluc and ral-4'-Gluc. Deuterated internal standards were combined with final concentrations of 5 μ g/mL for raloxifene-d4, 20 μ g/mL for ral-6-Gluc-d4, and 20 μ g/mL for ral-4'-Gluc-d4 and were kept at -20°C before use.

Standard curves were constructed by plotting the ratio of analyte peak area to peak area of the corresponding internal standard versus analyte concentration for at least 7 analyte concentrations. The standard working solution and deuterated internal standard were spiked into plasma from untreated women and mixed (125 μ L) with extraction solution (375 μ L, containing 49.9:49.9:0.2 methanol:acetonitrile:formic acid) to precipitate out proteins and extract raloxifene metabolite standards. After vortexing and subsequent centrifugation at $13,000 \times g$ for 20 minutes at 4°C , the supernatant was dried and the residue was reconstituted in 125 μ L of reconstitution solution (50.0:49.9:0.1 acetonitrile:H₂O:formic acid) to make a final concentration of 0.32 to 320 ng/mL for raloxifene, 1.28 to 1,280 ng/mL for both ral-6-Gluc and ral-4'-Gluc, 60 ng/mL for raloxifene-d4, and 240 ng/mL for both ral-6-Gluc-d4 and ral-4'-Gluc-d4.

The plasma from raloxifene-treated subjects was preconcentrated 3.2-fold before loading onto the UPLC/tandem mass spectrometry (MS-MS) system. After spiking of deuterated internal standards into plasma (320 μ L) from raloxifene-treated subjects, samples were mixed with extraction solution (960 μ L) and processed as described above for plasma specimens from untreated subjects using 100 μ L of reconstitution solution to keep analyte concentrations within the range of the calibration curve. The calculated concentrations from standard curves were divided by 3.2 to reflect the final raloxifene metabolites levels in plasma from raloxifene-treated subjects.

Calibration standards as well as the plasma sample extracts from each subject were analyzed by UPLC/MS-MS. Quantification of raloxifene, ral-6-Gluc, and ral-4'-Gluc was conducted using MRM of the transitions of m/z 474.2 \rightarrow 112.2 for raloxifene, m/z 650.5 \rightarrow 474.3 for ral-6-Gluc and ral-4'-Gluc, m/z 478.2 \rightarrow 116.2 for raloxifene-d4, and m/z 654.5 \rightarrow 478.3 for ral-6-Gluc-d4 and ral-4'-Gluc-d4. The ral-6-Gluc and ral-4'-Gluc were distinguished by matching the

retention time with the commercial standards in the chromatograms. The optimized MS conditions were positive ionization mode, capillary voltage 3.0 kV, cone voltage 30 V, collision voltage 30 V, source temperature 150°C , and desolvation temperature 350°C . Nitrogen was used as the desolvation and cone gas with the flow rate at 760 and 50 L/h, respectively. Argon was used as the collision gas at a flow rate of 0.1 L/h. The dwell time for each ion was 0.01 second. All data were quantified by MassLynx NT 4.1 software with QuanLynx program (Waters Corp.).

UGT genotyping

gDNA from human liver, human jejunum, and human lymphocytes was used to genotype the UGT1A1*28 allele and the UGT1A8 codons 173 (Ala > Gly; rs1042597) and 277 (Cys > Tyr; rs17863762) SNPs. For the UGT1A1 TATAA box polymorphism genotype, DNA was PCR-amplified as previously described (29) using sense and antisense primers: 5'-GAGTATGAAATTCCAGCCAGTTCAAC-3' and 5'-TCCACTGGGATCAACAGTATCTT-3' (corresponding to -224 to -198 and $+107$ to $+85$ relative to the UGT1A1 ATG translation start site, respectively), resulting in an amplicon of 331 bp with the TATAA box polymorphism near the middle of the amplicon. After running on a 1.0% agarose gel and extraction using a Qiagen gel extraction kit, purified PCR products were sequenced using an ABI Hitachi 3730XL DNA Analyzer, with sequencing confirmed using both the forward and reverse amplification primers described above. Sequencing results were confirmed by visual inspection of the TATAA box chromatogram peaks. The UGT1A1*28 allele was in Hardy-Weinberg equilibrium and indicated an allelic frequency of 35% in subjects from whom HLM samples were obtained and 34% in subjects taking raloxifene from whom plasma raloxifene metabolites were analyzed.

The UGT1A8 coding single-nucleotide polymorphisms (SNP) were genotyped by real-time PCR using ABI TaqMan Drug Metabolism Genotyping Assays (C_11742072_10 for rs1042597 and C_34418788_20 for rs17863762) according to manufacturer's protocols. The three UGT1A8 alleles were in Hardy-Weinberg equilibrium with allelic frequencies of 76.2% for UGT1A8*1, 20.2% for UGT1A8*2, and 3.6% for UGT1A8*3 in subjects from whom HJH samples were obtained and 71.3%, 26.4%, and 2.3%, respectively, in subjects taking raloxifene from whom plasma raloxifene metabolites were analyzed.

UGT mRNA expression in human jejunum

RNA was extracted from all 46 jejunum specimens using the Qiagen RNeasy Mini Kit according to the manufacturer's protocol. After digestion with DNase I digestion, RNA concentrations were determined using a Nanodrop ND-1000 spectrophotometer. RNA purity was assessed by absorbance ratios A_{260}/A_{280} (>1.9) and A_{260}/A_{230} (>1.8). RNA integrity was determined using an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano chips, and all 46 jejunum samples used in this study had an RIN >4.0 with clearly visible 28S and 18S rRNA bands. Reverse

transcription (RT) real-time PCR was carried out for 5 randomly selected jejunum RNA specimens as previously described (30) to assess the relative expression levels of UGTs 1A1, 1A8, 1A9, and 1A10. Real-time PCR was carried out using a 25 ng RNA equivalent of cDNA, and expression levels were normalized to the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. Quadruplicate real-time PCRs were carried out for each cDNA sample analyzed using a 10 μ L final reaction volume according to manufacturer's protocols (assay IDs: UGT1A1, Hs02511055_s1; UGT1A8, Hs01592482_m1; UGT1A10, Hs02516990_s1; UGT1A9, Hs02516855_sH; GAPDH, Hs99999905_m1). Reactions were conducted in a 384-well plate using the ABI 7900 HT Sequence Detection System under the following conditions: 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative quantification (RQ) of UGT1A expression was calculated using the $\Delta\Delta C_t$ method as previously described (30).

Estrogen receptor binding assay

Competitive binding assays of raloxifene, ral-6-Gluc, and ral-4'-Gluc with the estrogen receptor (ER) were conducted essentially as previously described (31) by incubating the cytosolic fraction of MCF-7 cells (500 μ g total protein) with 10^{-9} mol/L 3 H-labeled-estradiol (E2) and between 10^{-11} and 10^{-6} mol/L of competitor (raloxifene, ral-6-Gluc, or ral-4'-Gluc). Data were expressed as the percentage of specific binding of 3 H-E2 for the ER when competitor was not present. The relative binding affinity (RBA) for each test compound was calculated as IC_{50} which was normalized to that of E2.

Statistical analysis

The Student *t* test (2-sided) was used for comparing kinetic values of glucuronidation formation for UGT wild-type versus variant overexpressing cell lines and for comparing raloxifene Gluc formation rates in HLM and HJH between 2 different genotypes. The ANOVA trend test was used to examine the overall effect of UGT genotypes on raloxifene glucuronide formation in HLM and HJH, whereas the Jonckheere-Terpstra trend test was used to examine the overall effect of UGT genotypes on raloxifene glucuronide levels in human plasma. Kinetic constants were determined using the Michaelis-Menten Model in GraphPad Prism 5 software.

Results

Previous studies have shown that HLM catalyzes the formation of two glucuronides of raloxifene, raloxifene-6-Gluc and raloxifene-4'-Gluc (14, 32). Similar to that observed in previous studies (14, 32), 2 major peaks with retention times of 2.70 and 2.97 minutes were observed by UPLC/MS-MS in *in vitro* raloxifene glucuronidation assays with HLM (Fig. 1A) and HJH (Fig. 1B). The retention time of the 2 peaks was the same as that of purchased ral-6-Gluc (peak 1) and ral-4'-Gluc (peak 2) standards (Fig. 1C) and were confirmed to be *O*-glucuronides of raloxifene by their

insensitivity to alkali but sensitivity to β -glucuronidase treatment (results not shown). Ral-6-Gluc and ral-4'-Gluc were also observed in plasma samples from subjects treated with raloxifene (Fig. 1D), exhibiting the same retention times as the plasma-spiked ral-6-Gluc-d4 and ral-4'-Gluc-d4 internal standards (results not shown), the same retention times and MS/MS spectrum as the ral-6-Gluc and ral-4'-Gluc standards, and the *in vitro* assays with HLM and HJH described above. A third peak detected in plasma from raloxifene-treated subjects exhibited a retention time of 3.61 minutes (Fig. 1E), which was the same as that observed for the raloxifene standard (peak 3; Fig. 1F). Similar to previous studies (19, 32, 33), a ral-6,4'-diGluc was not observed in the plasma of subjects treated with raloxifene or *in vitro* with HLM or HJH. Using MS/MS daughter scan mode, the mass spectrum of both peaks showed a $[M + H]^+$ peak at m/z 650 for raloxifene-*O*-glucuronide, a $[M + H]^+$ peak at m/z 474 for raloxifene after loss of the glucuronide acid moiety (molecular weight = 176 g/mol; Fig. 1G), and a $[M + H]^+$ peak at m/z 474 for raloxifene and a major daughter fragment at m/z 112.2 (Fig. 1H).

UGTs 1A1, 1A3, 1A7, 1A8, and 1A9 catalyzed both ral-6-Gluc and ral-4'-Gluc formation, whereas UGT1A10 specifically catalyzed the formation of ral-4'-Gluc (Table 1). Representative kinetic analysis curves for ral-6-Gluc and ral-4'-Gluc formation are shown for UGTs 1A1 (Fig. 2A) and 1A8 (Fig. 2B) against raloxifene. After normalizing for UGT1A protein expression as determined by Western blot analysis (34), the order of ral-6-Gluc formation based on V_{max}/K_M was UGT 1A8 > 1A1 > 1A7 \approx 1A9 > 1A3, whereas the order of ral-4'-Gluc formation based on V_{max}/K_M was UGT 1A10 > 1A8 > 1A9 > 1A1 > 1A7 > 1A3. In addition to UGTs 1A4 and 1A6, none of the UGT2B enzymes screened in this analysis exhibited detectable levels of raloxifene glucuronide formation.

Of the hepatic UGT enzymes active against raloxifene, UGT1A1 was the most active UGT for ral-6-Gluc formation and was the second-most active UGT for ral-4'-Gluc formation (Table 1). The UGT1A1*28 allele is a common variant (\sim 30% frequency in Caucasians) that encodes an A (TA)₇TAA repeat in the TATAA box of the UGT1A1 promoter region instead of the more common A(TA)₆TAA repeat encoded by the wild-type UGT1A1*1 allele, leading to lower UGT1A1 expression (18) and a decreased glucuronidation phenotype against a variety of endogenous and exogenous compounds (29, 35, 36). To investigate the possible relationship between raloxifene glucuronidation phenotype and this polymorphism, a series of 105 HLMs were examined *in vitro*. Using a concentration of raloxifene (2 μ mol/L) that was close to the K_M of 8 μ mol/L for ral-6-Gluc formation and 1.5 μ mol/L for ral-4'-Gluc formation for 3 randomly chosen HLMs (data not shown), there was a 16- and 43-fold range in formation observed for ral-6-Gluc and ral-4'-Gluc, respectively, in the 105 HLM specimens. When stratifying the HLM by UGT1A1 genotype, ral-6-Gluc formation was significantly ($P_{trend} = 0.005$) decreased with increasing numbers of the UGT1A1*28 allele (Fig. 3A), with significant decreases observed in UGT1A1 (*1/*28)

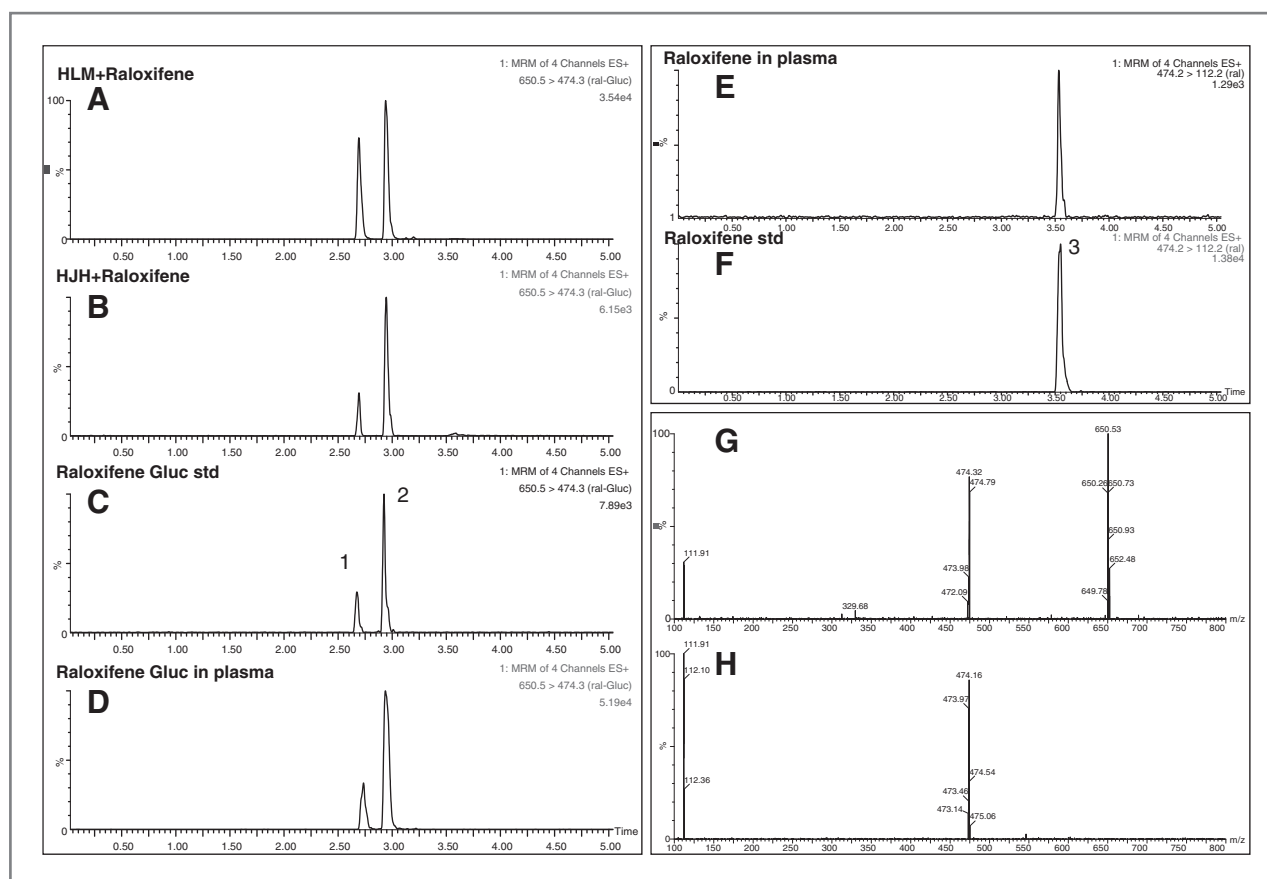


Figure 1. UPLC/MS-MS analysis of raloxifene glucuronides. Shown are MRM analysis of raloxifene glucuronides formed by HLM (A) and HJH (B), the purchased ral-6-Gluc/ral-4'-Gluc (C), raloxifene glucuronides observed in the plasma of raloxifene-treated patients (D), raloxifene in the plasma of raloxifene-treated patients (E), and raloxifene (F) standards. UPLC/MS-MS of purchased standards was conducted in a standard glucuronidation assay without a protein source (e.g., cell homogenate, HLM, or HJH) added. Peak 1, ral-6-Gluc; peak 2, ral-4'-Gluc; peak 3, raloxifene. MS daughter scan spectrum for raloxifene glucuronide and raloxifene are shown in G and H, respectively.

(26%; $P = 0.004$; $n = 49$) and (*28/*28) (39%; $P = 0.01$; $n = 11$) HLMs as compared with HLM with the UGT1A1 (*1/*1) genotype ($n = 45$). Significant differences in the levels of ral-4'-Gluc formation were not observed for HLM after stratification by UGT1A1 genotype (Fig. 3A). No significant differences in K_M for the formation of ral-4'-Gluc were observed in HLM with the UGT1A1 (*28/*28) or (*1/*28) genotypes versus UGT1A1 (*1/*1) HLM (3 HLM examined per genotype group; results not shown).

Of the extrahepatic UGT enzymes shown to be active against raloxifene *in vitro*, UGTs 1A8 and 1A7 exhibit missense SNPs with prevalences of more than 3% in the population. Two coding region SNPs resulting in Ala to Gly at codon 173 (encoded by the UGT1A8*2 allele) and Cys to Tyr at codon 277 (encoded by the UGT1A8*3 allele) are present in the UGT1A8 gene [prevalences of 0.24 and 0.036, respectively, in Caucasians according to HapMap (37)]. In an *in vitro* analysis of UGT1A8-overexpressing HEK293 cell homogenates, the UGT1A8^{173Gly/277Cys} variant (encoded by the UGT1A8*2 allele) exhibited a significantly lower K_M ($P < 0.005$) and higher overall activity as determined by V_{max}/K_M ($P < 0.01$) for ral-6-Gluc formation, as compared

with wild-type UGT1A8^{173Ala/277Cys} (Table 1). While this variant also exhibited a significantly lower K_M ($P < 0.005$) for ral-4'-Gluc formation, a similar V_{max}/K_M was observed. No detectable glucuronidation activity was observed for the UGT1A8^{173Ala/277Tyr} variant (encoded by the UGT1A8*3 allele) against raloxifene *in vitro*. No difference in glucuronidation activity was observed against raloxifene for any of the UGT1A7 variants [UGT1A7*1, UGT1A7*2, UGT1A7*3, and UGT1A7*4 (refs. 26, 38)] in an *in vitro* analysis of UGT1A7-overexpressing HEK293 cell homogenates (results not shown).

Previous reports have shown that small intestine is an important contributor to raloxifene glucuronidation and clearance *in vivo* (13, 14, 39) and that several UGTs including UGT1A8 are well-expressed in tissues of the digestive tract (40). However, previous studies examining the expression of intestinal UGTs have been relatively nonquantitative (41). Using real-time PCR, UGT1A1 was shown to be expressed at the highest levels in jejunum (Fig. 3B). While UGT1A8 was expressed in jejunum at levels that were about 2-fold lower than UGT1A1, UGT1A8 was expressed at levels that were 10-fold higher than UGT1A10. The mRNA

Table 1. Kinetic analysis of the glucuronidation activity of UGTs against raloxifene

UGT ^a	Raloxifene-6-Gluc			Raloxifene-4'-Gluc		
	V_{max} , pmol/min/ μ g	K_M , μ mol/L	V_{max}/K_M , μ L/min/ μ g	V_{max} , pmol/min/ μ g	K_M , μ mol/L	V_{max}/K_M , μ L/min/ μ g
1A1	20 \pm 3	8.9 \pm 1.9	2.2 \pm 0.2	11 \pm 2	12 \pm 3	0.95 \pm 0.05
1A3	0.41 \pm 0.02	21 \pm 1	0.019 \pm 0.002	0.48 \pm 0.04	3.1 \pm 0.3	0.15 \pm 0.02
1A7	1.9 \pm 0.2	13 \pm 3	0.16 \pm 0.02	6.9 \pm 0.4	22 \pm 2	0.32 \pm 0.03
1A8 ^{173Ala/277Cys}	7.0 \pm 1.6	0.31 \pm 0.05	23 \pm 4	19 \pm 5	2.4 \pm 0.4	7.9 \pm 1.0
1A8 ^{173Gly/277Cys}	6.1 \pm 1.5	0.08 \pm 0.04 ^b	80 \pm 18 ^c	4.1 \pm 1.1 ^c	0.76 \pm 0.11 ^b	5.3 \pm 0.9
1A8 ^{173Ala/277Tyr}	No activity detected					
1A9	3.4 \pm 0.5	22 \pm 6	0.16 \pm 0.03	8.3 \pm 0.9	4.5 \pm 0.8	1.9 \pm 0.1
1A10	No activity detected					

^aNo raloxifene glucuronide formation was observed for homogenates from cells overexpressing UGTs 1A4, 1A6, 2B4, 2B7, 2B10, 2B11, 2B15, or 2B17.

^bA significant ($P < 0.005$; ^c $P < 0.01$) difference was observed for 1A8^{173Gly/277Cys}- versus 1A8^{173Ala/277Cys}-overexpressing cell homogenates.

expression level of UGT1A9 was not quantifiable in all 5 jejunum specimens analyzed.

As UGT1A8 was among the 2 most active UGTs against raloxifene and was shown to exhibit high levels of relative expression in human jejunum, a potential role for UGT1A8 genotypes on raloxifene glucuronidation phenotype was examined using a series of 46 HJH specimens. A concentration of 0.8 μ mol/L raloxifene was used for HJH glucuronidation activity assays, which approximated the K_M of

0.75 μ mol/L for the major raloxifene metabolite in HJH, ral-4'-Gluc (as measured by kinetic analysis of 3 randomly chosen HJH; data not shown). There was a 64-fold range in formation observed for total raloxifene glucuronide formation in the 46 HJH specimens. When stratifying the HJH specimens by UGT1A8 codon 173 genotype, a significant ($P = 0.018$) 1.8-fold increase in total raloxifene glucuronide formation was observed in HJH from subjects with the UGT1A8 (*2/*2) genotype ($n = 3$) as compared with

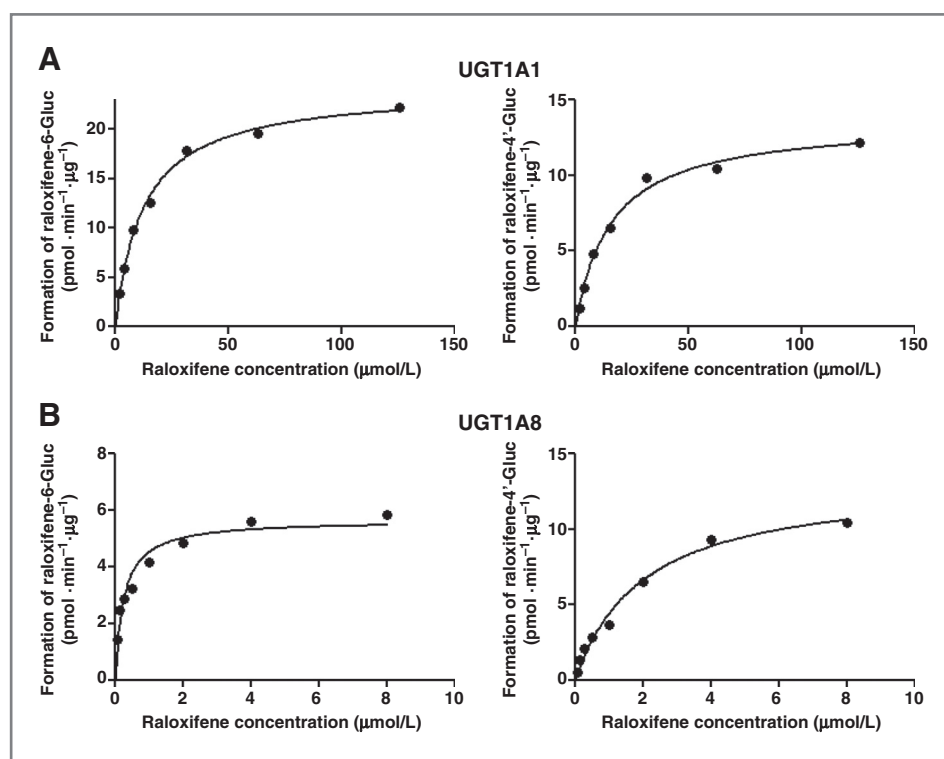


Figure 2. Representative plots for raloxifene glucuronidation kinetics by individual UGTs. Shown are representative concentration curves for ral-Gluc formation by homogenates from UGT1A1 (A) and UGT1A8 (B) overexpressing cell lines. Left, concentration curves for ral-6-Gluc formation; right, concentration curves for ral-4'-Gluc formation.

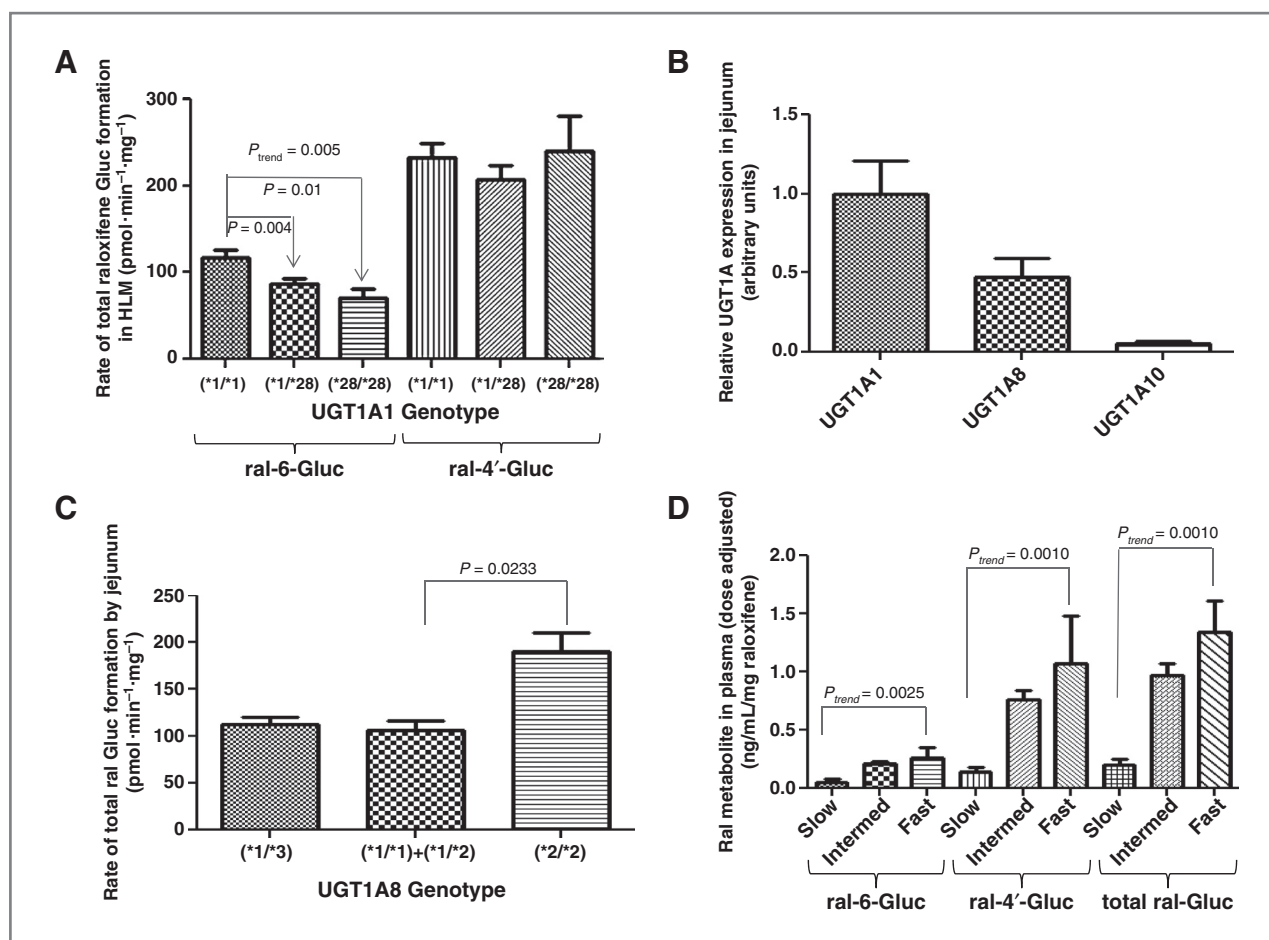


Figure 3. Importance of UGT1A genotypes in raloxifene glucuronide formation in human tissues and in plasma samples from raloxifene (ral)-treated subjects. Glucuronidation activity assays were conducted by incubation of raloxifene with HLM or HJH, and raloxifene glucuronides were analyzed by UPLC or UPLC/MS-MS as described in the Materials and Methods. The relative abundance of UGTs 1A1, 1A8, and 1A10 mRNAs in the jejunum was measured in 5 individual jejunum specimens by quantitative PCR using the $\Delta\Delta C_t$ method. A, rate of raloxifene glucuronide formation in HLM stratified by UGT1A1 genotype. B, relative expression levels of UGT1A mRNA in jejunum. C, rate of total raloxifene glucuronide formation in HJH stratified by UGT1A8 genotype. D, levels of raloxifene glucuronides in plasma stratified by UGT1A8 genotype. Subjects with the UGT1A8 (*1/*3) genotype were defined as slow raloxifene metabolizers (slow), subjects with either the UGT1A8 (*1/*1) or UGT1A8 (*1/*2) genotypes were defined as intermediate raloxifene metabolizers (intermed), and subjects with the UGT1A8 (*2/*2) genotype were defined as fast raloxifene metabolizers (fast). The Student *t* test was used to compare raloxifene gluc formation in HLM from subjects with UGT1A1 (*1/*28) or (*28/*28) genotypes with the wild-type UGT1A1 (*1/*1) and to compare total raloxifene glucuronide formation in HJH from subjects with UGT1A8 (*2/*2) genotype with UGT1A8 (*1/*1 + *1/*2) genotypes. The ANOVA trend test was used to examine the overall effect of UGT1A1 genotype on rate of ral-6-Gluc and ral-4'-Gluc formation in HLM. The Jonckheere-Terpstra trend test was used to examine the overall effect of UGT1A8 genotype on ral-6-Gluc, ral-4'-Gluc, and total raloxifene glucuronide levels in plasma from women treated with raloxifene. Samples from subjects treated by 60 and 30 mg raloxifene daily were combined after plasma raloxifene and glucuronide levels were adjusted for raloxifene dose (mg) for each subject.

subjects with at least one UGT1A8*1 allele ($n = 40$; Fig. 3C). No difference in total raloxifene glucuronide formation was observed in HJH specimens that exhibited the UGT1A8 (*1/*3) genotype [$n = 3$; there were no specimens with the UGT1A8 (*3/*3) genotype]. There was a near-significant ($P = 0.058$) decrease in K_M for ral-4'-Gluc formation in HJH with the UGT1A8 (*2/*2) genotype ($0.46 \pm 0.11 \mu\text{mol/L}$) than UGT1A8 (*1/*1) HJH ($0.75 \pm 0.16 \mu\text{mol/L}$); the K_M of UGT1A8 (*1/*3) HJH ($0.80 \pm 0.39 \mu\text{mol/L}$) was similar to that observed for HJH with the UGT1A8 (*1/*1) genotype.

The levels of raloxifene and its glucuronides were determined simultaneously in the plasma of subjects treated with either 30 or 60 mg/d raloxifene. Validation of the UPLC/

MS-MS analytical method used for this analysis showed high assay recovery of a range of plasma raloxifene/raloxifene glucuronide levels (88%–110%). The quantification limit (signal/noise > 10) was 0.08 ng/mL for raloxifene, 0.625 ng/mL for ral-6-Gluc, and 0.78 ng/mL for ral-4'-Gluc. The intra- and interday precision (CV), respectively, for raloxifene were 9.4% and 12.3% at 0.32 ng/mL, 4.1% and 6.5% at 2.5 ng/mL, and 0.6% and 1.4% at 160 ng/mL. For ral-6-Gluc, the CV were 8.0% and 9.6% at 1.28 ng/mL, 6.2% and 7.0% at 20 ng/mL, and 1.6% and 4.1% at 640 ng/mL. For ral-4'-Gluc, the CV were 3.8% and 8.8% at 1.28 ng/mL, 2.7% and 4.0% at 20 ng/mL, and 1.3% and 2.3% at 640 ng/mL. The levels of plasma raloxifene and its metabolites

Table 2. Raloxifene metabolite profiles in plasma from subjects treated with raloxifene

Treatment group	ral-4'-Gluc (ng/mL plasma)	ral-6-Gluc (ng/mL plasma)	ral-4'-Gluc/ ral-6-Gluc	Raloxifene (ng/mL plasma)	Total ral-Gluc/ raloxifene
Raloxifene 30 mg (<i>n</i> = 39)	22 (2.7–95)	6.3 (0–26)	3.3	0.28 (0.12–0.58)	114
Raloxifene 60 mg (<i>n</i> = 42)	50 (5.9–188)	15 (2.6–59)	3.8	0.58 (0.18–2.3)	133

showed extensive variability in bloods drawn from patients coming into the breast cancer clinic for all time points examined (6-, 12-, 18-, and 24-month blood draws following initiation of the trial). However, the range of raloxifene metabolite levels was similar for all time points. For example, the range of the plasma ral-4'-Gluc for subjects from the 30 mg daily treatment group was 2.7 to 95 ng/mL for the month 6 visit (*n* = 35), 3.3 to 96 ng/mL for the month 12 visit (*n* = 27), 5.1 to 66 ng/mL for the month 18 visit (*n* = 14), and 13 to 89 ng/mL for the month 24 visit (*n* = 3). A similar pattern was observed for ral-4'-Gluc for the 60-mg daily treatment group as well as ral-6-Gluc for both treatment groups (results not shown).

Using bloods drawn from the first available visiting time for each raloxifene-treated subject, there was extensive metabolism to ral-6-Gluc and ral-4'-Gluc, with unchanged raloxifene comprising only 0.98% and 0.88% of the total plasma raloxifene metabolite profile (raloxifene + ral-6-Gluc + ral-4'-Gluc) in subjects taking either 30 or 60 mg/d raloxifene, respectively (Table 2). The level of ral-4'-Gluc was about 3- to 4-fold higher than that of ral-6-Gluc in both groups. There were 26- and 23-fold differences in the level of plasma ral-6-Gluc, 35- and 32-fold differences in the level of plasma ral-4'-Gluc, and 4.8- and 13-fold differences in the level of plasma raloxifene between subjects from the 30 and 60 mg/d treatment groups, respectively. While the mean ratios of both plasma ral-4'-Gluc/ral-6-Gluc and total plasma ral-Gluc/raloxifene were similar between groups, the

levels of ral-6-Gluc, ral-4'-Gluc, and raloxifene increased by 2.4-, 2.3-, and 2.1-fold, respectively, in subjects from the 60 mg/d group as compared with subjects from the 30 mg/d group. Similar raloxifene metabolite profiles were observed when examining bloods drawn from subjects at other time points or when using an average of all time points (results not shown).

On the basis of the cell line data obtained in this study, it was predicted that individuals with a UGT1A8*3 allele would exhibit lower raloxifene glucuronidation capacities, whereas subjects with a UGT1A8*2 allele would exhibit higher raloxifene glucuronidation capacities. When stratifying by UGT1A8 genotype, we found essentially no difference in the levels of plasma ral-6-Gluc, ral-4'-Gluc, or raloxifene in subjects who were either UGT1A8 (*1/*2) versus UGT1A8 (*1/*1) (results not shown), so they were combined into one group. To best compare the levels of plasma raloxifene and its glucuronides in the two treatment groups, the ratios of ral-6-Gluc/raloxifene, ral-4'-Gluc/raloxifene, and total raloxifene glucuronide/raloxifene were examined in subjects after stratifying by UGT1A8 genotype. As the two raloxifene glucuronides comprise more than 99% of the total plasma raloxifene metabolites in subjects taking raloxifene, other ratios including total ral-Gluc/ (total ral-Gluc + raloxifene) were not informative. As shown in Table 3, increases in the ratios of ral-6-Gluc/raloxifene, ral-4'-Gluc/raloxifene, and total ral-Gluc/raloxifene were observed for plasma specimens from subjects

Table 3. Raloxifene metabolites in plasma stratified by UGT1A8 genotype

Group	Metabolite	UGT1A8 (*1/*3) ^a	UGT1A8 (*1/*1)+(*1/*2) ^a	UGT1A8 (*2/*2) ^a
60 mg raloxifene	Raloxifene-6-Gluc/raloxifene	13 (1)	31 ± 27 (36)	43 ± 21 (5)
	Raloxifene-4'-Gluc/raloxifene	42	94 ± 74	146 ± 62
	Total raloxifene-Gluc/raloxifene	55	125 ± 96	189 ± 77
30 mg raloxifene ^b	Raloxifene-6-Gluc/raloxifene	10 ± 14 (2)	27 ± 24 (37)	
	Raloxifene-4'-Gluc/raloxifene	18 ± 9	93 ± 97	
	Total raloxifene-Gluc/raloxifene	27 ± 23	120 ± 120	
Combined treatment groups	Raloxifene-6-Gluc/raloxifene ^c	11 ± 10 (3)	29 ± 26 (73)	43 ± 21 (5)
	Raloxifene-4'-Gluc/raloxifene ^d	26 ± 15	93 ± 86	146 ± 62
	Total raloxifene-Gluc/raloxifene ^e	37 ± 23	123 ± 101	189 ± 77

^aValues are ng/mL. Numbers in parenthesis represent the number of subjects analyzed in each group.

^bThere were no subjects with the UGT1A8 (*2/*2) genotype in this group.

^c*P*_{trend} = 0.020.

^d*P*_{trend} = 0.003.

^e*P*_{trend} = 0.005.

who were UGT1A8 (*1/*3) versus subjects who were either UGT1A8 (*1/*1) or UGT1A8 (*1/*2). A similar pattern was observed when comparing plasma ral-6-Gluc/raloxifene, ral-4'-Gluc/raloxifene, and total ral-Gluc/raloxifene ratios from subjects who were either UGT1A8 (*1/*1) or UGT1A8 (*1/*2) versus subjects who were UGT1A8 (*2/*2). This trend was significant ($P_{\text{trend}} = 0.020$ for ral-6-Gluc/raloxifene, $P_{\text{trend}} = 0.003$ for ral-4'-Gluc/raloxifene, and $P_{\text{trend}} = 0.005$ for total ral-Gluc/raloxifene) when subjects from both treatment groups were combined. The levels of dose-adjusted plasma ral-6-Gluc, ral-4'-Gluc, and total ral-Gluc in the combined group were significantly ($P_{\text{trend}} = 0.0025$, 0.001 , and 0.001 , respectively) increased in predicted slow metabolizers [UGT1A8 (*1/*3)] versus intermediate metabolizers [UGT1A8 (*1/*1) or UGT1A8 (*1/*2)] versus fast metabolizers [UGT1A8 (*2/*2); Fig. 3D]. No difference in the levels of ral-6-Gluc or ral-4'-Gluc were observed in plasma from subjects with either the UGT1A1 (*1/*1) ($n = 34$), UGT1A1 (*1/*28) ($n = 39$), or UGT1A1 (*28/*28) ($n = 9$) genotypes (results not shown). Unfortunately, there was insufficient power to examine combined UGT1A1/UGT1A8 genotypes versus plasma raloxifene metabolites in this study.

The mechanism of raloxifene action for the prevention of breast cancer is to compete with estrogen for binding to the ER to prevent the stimulation of proliferation of breast cancer cells. To examine the relative binding affinity to the ER of the two raloxifene glucuronides versus raloxifene, cytosolic fractions of MCF-7 cells were used as an ER source as previously described (31). The IC_{50} for raloxifene, ral-6-Gluc, and ral-4'-Gluc was $(4.0 \pm 3.5) \times 10^{-10}$ mol/L, $(2.9 \pm 0.8) \times 10^{-7}$ mol/L, and $(3.7 \pm 1.9) \times 10^{-8}$ mol/L, respectively.

Discussion

While raloxifene does not undergo significant P450-dependent oxidation (10), it is extensively glucuronidated by first-pass metabolism. Similar to that observed in previous studies (14), several UGT1A enzymes were found to exhibit raloxifene glucuronidating activity in the current study, with UGTs 1A1 and 1A9 the most active hepatic UGTs, and the extrahepatic UGTs 1A8 and 1A10 exhibiting the highest levels of activity of any UGT screened in this study. UGT1A8 exhibited the highest overall activity for ral-6-Gluc formation and the second highest activity for ral-4'-Gluc formation, and UGT1A10 exhibited the lowest K_M and highest overall activity for ral-4'-Gluc formation. The K_M values reported in the current study are 25- and 19-fold lower for ral-6-Gluc and ral-4'-Gluc formation by UGT1A8 and 23-fold lower for ral-4'-Gluc formation by UGT1A10, compared with previous studies (14), discrepancies that are likely due to differences in assay conditions. The K_M values for UGT1A1 against raloxifene were about 10 $\mu\text{mol/L}$ for both ral-6-Gluc and ral-4'-Gluc formation in our study, whereas the K_M for UGT1A1 could not be determined in previous studies due to solubility limitations as indicated in that study (14). UGTs 1A3 and 1A7 were also shown to be active in the present study. While this previous study did not

test the activity of UGT1A3, no glucuronidation activity was previously observed for UGT1A7. This is likely due to the fact that UGT-overexpressing baculosomes, which have been found to exhibit significant differences in substrate specificities as compared with UGT-overexpressing human cell homogenates (22), were used in this previous study. While the present study is the first to examine UGT2B enzyme activities against raloxifene, none were found to be active.

Previous studies have shown that the level of UGT1A1 in human liver is 2-fold higher than that of UGT1A9 (30). There was only a ~ 2.5 -fold difference in the K_M values for ral-6-Gluc and ral-4'-Gluc formation between enzymes, suggesting that both UGTs 1A1 and 1A9 may be important in the hepatic glucuronidation of raloxifene. However, while UGT1A1 exhibits 2- and 10-fold higher levels of expression in jejunum than UGTs 1A8 and 1A10, respectively, it exhibits a ~ 29 -fold higher K_M and a ~ 10 -fold lower V_{max}/K_M for ral-6-Gluc formation as compared with UGT1A8, and a ~ 5 - and 57-fold higher K_M and a ~ 8 - and 80-fold lower V_{max}/K_M for ral-4'-Gluc formation activity as compared with UGTs 1A8 and 1A10, respectively, suggesting a lesser role for UGT1A1 in jejunum raloxifene glucuronidation activity. The barely detectable level of expression for UGT1A9 in the small intestine in the present study is consistent with that observed previously (42) and suggests a minimal role for UGT1A9 in raloxifene glucuronidation in this tissue. Given the very low activity of UGTs 1A3 and 1A7 against raloxifene, it is likely that these UGTs play only a marginal role in raloxifene glucuronidation in either liver or jejunum.

Previous studies have shown that the UGT1A1*28 allele is associated with altered glucuronidation activity against a variety of endogenous and exogenous substrates (29, 35, 36). The association observed between UGT1A1 genotype and HLM raloxifene glucuronidation in the present study is consistent with the likely importance of UGT1A1 in overall hepatic raloxifene glucuronidation activity. The fact that this was observed specifically for the formation of ral-6-Gluc is consistent with UGT1A1 cell homogenates exhibiting the highest overall activity of any hepatic UGT for this metabolite. The fact that this pattern was not observed for hepatic ral-4'-Gluc formation is consistent with UGT1A9 playing a more important role in the formation of this metabolite given the higher V_{max}/K_M exhibited by UGT1A9 versus UGT1A1 *in vitro*. The fact that no differences in raloxifene glucuronide K_M values were observed in HLM stratified by UGT1A1 genotypes is consistent with UGTs 1A9 and 1A1 exhibiting similar K_M values for both raloxifene metabolites *in vitro*. The fact that no association was observed between HJH raloxifene glucuronidation activities and UGT1A1 genotype is consistent with UGT1A1 playing a more minor role in jejunum raloxifene glucuronidation. As high-prevalence coding SNPs are not observed for UGT1A9 (43), a similar hepatic phenotype-genotype study was not conducted for this enzyme.

The vast majority of circulating raloxifene in the plasma of subjects treated with raloxifene was in the form of a

glucuronide conjugate, with unchanged raloxifene comprising approximately 1% of total plasma raloxifene in subjects treated with either 30 or 60 mg raloxifene/d. Considerable variation in raloxifene glucuronide levels were observed in plasma between individuals taking raloxifene. Previous studies focusing on the role of the UGT1A1*28 allele on raloxifene glucuronidation gave conflicting results (19, 44, 45). The fact that UGT1A1 genotype did not contribute to variation in plasma raloxifene glucuronide levels *in vivo* in the present study suggests that, despite it contributing to ral-6-Gluc formation variation in HLM, potential effects by the UGT1A1*28 allele were overcome *in vivo* by glucuronidation contributions of other UGTs in both liver (UGT1A9) and jejunum (UGTs 1A8 and 1A10).

In previous studies, the polymorphic variants of UGT1A8 at codons 173 and 277 have been associated with altered glucuronidation activity (46) and cancer risk (47). A functional effect by these variants was also observed in the present study. UGT1A8 genotype was significantly correlated with raloxifene glucuronide formation in HJH *in vitro* and plasma raloxifene glucuronide levels in subjects treated with raloxifene, with the plasma levels of both ral-6-Gluc and ral-4'-Gluc significantly lower in subjects with the predicted UGT1A8 slow metabolizer genotype as compared with subjects with intermediate or fast metabolizer genotypes. These data were also consistent with the results from cell lines overexpressing UGT1A8 variants showing functional effects by UGT1A8 variants on raloxifene glucuronidation capacity. The fact that no association was observed between HJH raloxifene glucuronidation and the UGT1A8*3 allele may have been due to low power due to low UGT1A8*3 prevalence (allelic frequency = 2.2%) and the fact that all of the HJH specimens with an UGT1A8*3 allele examined in this study were from subjects who were heterozygous (*1/*3) for that allele. As high-prevalence coding SNPs are not observed for UGTs 1A9 (43) or 1A10 (48), a similar phenotype-genotype study in jejunum was not conducted for these enzymes. Therefore, in addition to suggesting that UGT1A8 coding SNPs may play an important role in the glucuronidation of raloxifene *in vivo*, these data support an important role for jejunum in overall raloxifene metabolism.

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Ral-4'-Gluc was the major form of raloxifene metabolite observed in the plasma of subjects treated with raloxifene. The ER-binding affinity of ral-4'-Gluc was shown to exhibit an IC₅₀ value that was approximately 100-fold less than raloxifene itself. However, as more than 70% of total circulating plasma raloxifene is in the form of ral-4'-Gluc and because the levels of ral-4'-Gluc are more than 70-fold higher than parent unconjugated raloxifene, variations in the level of ral-4'-Gluc could potentially have an important effect on overall patient response to raloxifene. Therefore, UGT1A8 genotype could potentially impact overall patient response to raloxifene by altering circulating levels of raloxifene glucuronides, particularly the levels of ral-4'-Gluc. Clinical studies with a larger population size will be required to further examine the role of UGT1A8 genotype on the preventive effect of raloxifene for breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: D. Sun, N.R. Jones, P. Lazarus

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.R. Jones, A. Manni, P. Lazarus

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Sun, N.R. Jones, P. Lazarus

Writing, review, and/or revision of the manuscript: D. Sun, N.R. Jones, A. Manni, P. Lazarus

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Sun

Study supervision: A. Manni, P. Lazarus

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