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Inactivation of the Pure Antiestrogen Fulvestrant and Other Synthetic Estrogen Molecules by UDP-Glucuronosyltransferase 1A Enzymes Expressed in Breast Tissue

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ABSTRACT

Fulvestrant (Faslodex) is administered by intramuscular injection and is converted into ketone, sulfate, sulfone and glucuronide metabolites. Glucuronidation, catalyzed by 18 members of the UDP-glucuronosyltransferase (UGT) enzyme family, plays a major role in the elimination of natural estrogens. The present study was aimed at identifying and characterizing human UGT enzymes involved in the glucuronidation of this antiestrogen as well as other synthetic estrogen derivatives with aliphatic chains on the E_2 molecule. In contrast to E_2, which is conjugated by UGT1A1, -1A3, -1A8, -1A10, and -2B7, fulvestrant is glucuronidated by UGT1A1, -1A3, -1A4, and -1A8. The four UGT1A-fulvestrant conjugating enzymes glucuronidate this substrate at position 3, whereas only UGT1A8 also produces fulvestrant-17-glucuronide. For E_2, only UGT1A3 and UGT2B7 are capable to conjugate at 17-hydroxyposition. These observations indicate that addition of an aliphatic chain to the E_2 molecule modifies the specificity of the UGT enzymes toward the C_{18} molecules. To further investigate the specificity of these enzymes, a series of E_2 derivatives with aliphatic or phenyl chains at position 2, 7α, and 11β was also tested for its conjugation with human UGT enzymes. It was observed that, in addition to UGT1A3, UGT1A1 and UGT1A8 also played important roles for the glucuronidation of these compounds. This suggests that the basic structure of E_2 is one of the major determinants for the glucuronidation catalyzed by this group of enzymes. Considering the high level of UGT1A3 and -1A4 expression in the gastrointestinal tract and mammary gland, our results suggest that fulvestrant can be inactivated both in intestine and in its target tissue.

Since the introduction in the 1970s of tamoxifen as an endocrine agent for the treatment of breast cancer, the concept of endocrine therapy has been extensively developed, leading to the production of a long series of new potent therapeutic agents (Howell et al., 2004). The variety of these drugs, which possess different mechanisms of action, makes the cross-resistance less likely to occur. One of the current challenges is to identify the optimal use of these novel compounds. The pharmacological and pharmacokinetic properties of these drugs are closely associated with changes in their metabolism at both the systemic level and directly in the tumor (Guillemette et al., 2000). Therefore, it is of crucial importance to identify the metabolic factors, such as metabolizing enzymes, that can affect antiestrogen bioavailability.

Most xenobiotics and endobiotics are initially metabolized

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ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; HPLC, high-performance liquid chromatography; HEK, human embryonic kidney; PCR, polymerase chain reaction; UDPGA, uridine 5′-diphosphoglucuronic acid; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; EM-100, estra-1,3,5(10)-triene-7-undecanamide, *N*-butyl-3,17-dihydroxy-*N*-methyl-, (7α,17β); EM-139, estra-1,3,5(10)-triene-7-undecanamide, *N*-butyl-16-chloro-3,17-dihydroxy-*N*-methyl-, (7α,16α,17β); EM-652, acolbifene; EM-1959, estra-1,3,5(10)-triene-3,17-diol, 2-methyl-, (17β); EM-2036, estra-1,3,5(10)-triene-3,17-diol, 2-(2-propenyl)-, (17β); EM-4570, estra-1,3,5(10)-triene-3,17-diol, 11-methyl-, (11β,17β); EM-4834, estra-1,3,5(10)-triene-3,17-diol, 11-pentyl-, (11β,17β); EM-4887, estra-1,3,5(10)-triene-3,17-diol, 11-(2-phenylethyl)-, (11β,17β); EM-4896, estra-1,3,5(10)-triene-3,17-diol, 11-(3-phenylpropyl)-, (11β,17β); EM-5314, estra-1,3,5(10)-triene-3,17-diol, 7-methyl-, (7α,17β); EM-5463, estra-1,3,5(10)-triene-3,17-diol, 11-(8-hydroxyoctyl)-, (11β,17β); EM-5498, estra-1,3,5(10)-triene-3,17-diol, 11-(4-hydroxybutyl)-, (11β,17β); EM-5499, estra-1,3,5(10)-triene-3,17-diol, 11-(3-butenyl)-, (11β,17β); EM-4541, estra-1,3,5(10)-triene-3,17-diol, 11-phenyl-, (11β,17β); EM-4720, estra-1,3,5(10)-triene-3,17-diol, 11-(4-phenylbutyl)-, (11β,17β).

by the cytochrome P450 family of enzymes (phase I) to biologically inactive metabolites. However, conjugation by UDP-glucuronosyltransferase (UGT) enzymes also constitutes a major route for inactivation of active compounds and their metabolites while facilitating their elimination from the body as a result of increased water solubility (Armstrong and Cozza, 2003). To date, 18 human UGT proteins have been characterized, and based on their primary amino acid sequence homology, they were categorized into two major families, UGT1 and UGT2 (Mackenzie et al., 1997). The entire UGT1 family is derived from a single gene locus (*UGT1A*), located on chromosome 2 (2q37), coding for nine functional proteins (UGT1A1, UGT1A3–UGT1A10) and three pseudogenes (Gong et al., 2001). The physiological importance of UGT1 enzymes is demonstrated by mutations in the *UGT1A* gene that cause several diseases with varying degrees of hyperbilirubinemia (Crigler-Najjar types I and II and Gilbert's disease) (Burchell et al., 2000). Enzymes of the UGT2 family are further divided into two subfamilies, UGT2A and UGT2B, which are encoded by different genes clustered on chromosome 4q13–4q21.1 (Turgeon et al., 2000). UGT2A mRNAs are expressed in human olfactory epithelium, where the corresponding enzymes control the inactivation of odorant molecules (Lazard et al., 1991; Jedlitschky et al., 1999). Seven human UGT2B proteins have been characterized: UGT2B4, -2B7, -2B10, -2B11, -2B15, -2B17, and -2B28 (Belanger et al., 2003). Human UGTs conjugate a wide variety of endo- or xenobiotics (Green et al., 1998; Cheng et al., 1999; Turgeon et al., 2003a; Sabolovic et al., 2004); however, it is well demonstrated that UGT2B7 and several members of the family UGT1A are closely associated to the conjugation of estradiol and its hydroxylated and/or methylated metabolites (Lepine et al., 2004). It is noteworthy that previous studies also identified UGT1A enzymes as being responsible for the conjugation of nonsteroidal antiestrogens, such as hydroxytamoxifen, raloxifene, and EM-652 (Barbier et al., 2001; Kemp et al., 2002; Nishiyama et al., 2002).

Fulvestrant (Fig. 1) is a pure antiestrogen that antagonizes the hormone-dependent activation of estrogen receptors and has no reported agonistic effects (Wakeling et al., 1991; Robertson et al., 2001). Given the absence of cross-resistance to fulvestrant after endocrine therapies, it has been suggested that the antiestrogen should be administrated, in the sequences of endocrine agents, after progressing from first-line tamoxifen (Bross et al., 2002). This antiestrogen possesses the basic structure of estradiol with an aliphatic chain of 14 carbons at position 7α. Previous studies have demonstrated that fulvestrant metabolism in humans involves different metabolizing enzymes, including CYP3A4, UGT, and sulfotransferase. However, the relative contribution of these metabolic enzymes remains to be clearly established (Robertson and Harrison, 2004). It is interesting that this estradiol-type derivative cannot be taken orally because of its very low level of intestinal absorption and is thus administered intramuscularly (Robertson and Harrison, 2004). These observations indicate a powerful intestinal first-pass extraction of the drug and suggest that phase I (such as CYP3A4) or phase II (such as UGTs) may drastically inactivate fulvestrant in the intestine. Although the liver is considered the major tissue for glucuronidation, it is also well recognized that extrahepatic tissues, such as the intestine, also play a significant role for drug glucuronidation (Tukey and Strassburg, 2000; Strassburg et al., 2002). Indeed, several drugs are more efficiently glucuronidated in the human intestine than in the liver (van Heek et al., 2000). Because each UGT enzyme displays a distinct pattern of tissue distribution, identifying isoforms involved in the glucuronidation of a given molecule is required for a better understanding of its pharmacokinetic properties. In the present study, we have identified the major UGT isoform(s) responsible for the glucuronidation of fulvestrant, characterized the enzymatic properties, and, finally, investigated the glucuronidation of a series of estradiol derivatives to gain a better understanding of the structure-function relationship between estrogen substrates and the UGT enzymes.

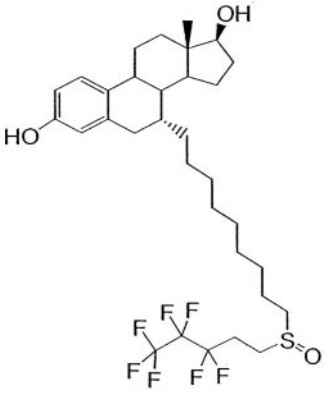


Fig. 1. Chemical structure of fulvestrant.

Materials and Methods

Chemicals. UDPGA and other molecular reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Estradiol, estrone, estradiol-3-glucuronide and estradiol-17-glucuronide were purchased from Steraloids Inc. (Wilton, NH). All synthetic estradiol derivatives were synthesized in the Medicinal Chemistry Division of our laboratory. Ammonium formate was from Aldrich Chemical (Milwaukee, WI), and high-performance liquid chromatography (HPLC)-grade methanol was provided by VWR Canlab (Montréal, QC, Canada). UGT1A1, UGT1A3–1A10, and UGT2B4, -2B7, -2B15, and -2B17 baculosomes were purchased from BD Biosciences (Mississauga, ON, Canada).

Cell Culture and Microsomal Preparations from HEK293. Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (Manassas, VA). The isolation of human UGT1A and UGT2B cDNAs and their stable expression in HEK293 cells have been described previously (Coffman et al., 1997; Albert et al., 1999; Levesque et al., 1999). Microsomes were obtained from stably transfected human UGT-expressing HEK293 cells cultured in Dulbecco's modified Eagle's medium (Cellgro, Herndon, VA) added with 5% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml sodium pyruvate, and 1 mg/ml G418 in a humidified incubator with an atmosphere of 5% CO_2 and with a temperature of 37°C. Cells were then sonicated and microsomal proteins were isolated by differential centrifugations. The crude cell extracts were centrifuged at 12,000*g* at 4°C for 20 min to remove nuclei, unbroken cells, and mitochondria. Supernatants were centrifuged at 105,000*g* for 60 min at 4°C to obtain the membrane fraction, which was homogenized in the microsome buffer (2.62 mM KH_2PO_4, 1.38 mM K_2HPO_4, 20% glycerol, and 1 mM EDTA) added with 1 mM dithiothreitol. Protein contents were determined using the Bradford's reagent, with bovine serum albumin for standard curves. Samples were divided into aliquots and kept at −80°C until glucuronidation assays and Western blot experiments.

Western Blot Experiments. To determine the level of UGT proteins in microsomes and baculosomes, Western blot analyses were conducted on preparations (20 μg), size-separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes. Immunoblots were performed by using the anti-human UGT1A common carboxyl-terminal region (amino acids 312–531) antiserum RC-71 (dilution 1/2000) (Albert et al., 1999; Gagne et al., 2002; Villeneuve et al., 2003) and the anti-human UGT2B antiserum EL-93 (dilution 1/2000) (Turgeon et al., 2003b) to quantify UGT1A and UGT2B proteins, respectively. An anti-rabbit IgG antibody conjugated with the horseradish peroxidase (Amersham Biosciences, Oakville, ON, Canada) was used as the secondary antibody (1:10,000 dilution). The resulting immunocomplexes were visualized using an enhanced chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA) and exposed on hyperfilm (Amersham Biosciences). The relative levels of UGT1 allozymes were determined by integrated optical density using BioImage programs visage 110S (Genomic Solution Inc., Ann Arbor, MI).

Expression Analyses of UGT1A1, -A3, -A4, and -A8 in Breast Tissues. Total RNA of breast tissues were obtained from Ambion (Austin, TX). Two cDNA preparations were synthesized using 1 μg of total RNA, random hexamers, and Superscript II cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. The real-time PCR reaction was performed using the ABI 7000 from Applied Biosystems (Foster City, CA) for the detection of RNA 18S, UGT1A1, -1A3, -1A4, and -1A8 with Taqman gene expression assay from Applied Biosystems (RNA 18S, Hs99999901_s1; UGT1A1, Hs01589938_m1; UGT1A3, Hs01592480_m1; UGT1A4, Hs01655285_s1; and UGT1A8, Hs01592482_m1). The real-time PCR reaction of 25 μl contained 7 μl of a dilution of the reverse transcription reaction, 1.25 μl of Taqman gene expression assay probe, and 12.5 μl of Master Mix 2× (Applied Biosystems). Conditions for PCR were 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 60 s.

Glucuronidation Assays. Glucuronidation assays were performed with 50 mM Tris-HCl, 10 mM $MgCl_2$, 8.5 mM saccharolactone, 10 μg/ml phosphatidylcholine, 1 mM UDPGA, 2.5 μg/ml pepstatin, 0.5 μg/ml leupeptin (all from Sigma Chemical), 2 μl of microsomal preparations of HEK293 (35–55 μg) or 2 μl of commercial microsomal preparations from baculosomes (5 μg), and 200 μM concentrations of the substrate in a total volume of 100 μl. For liver, jejunum, and ileum, 2 μl of microsomal preparations containing 20 μg were used. Assays were stopped with 100 μl of methanol containing 1 μg/ml estrone-glucuronide (internal standard), centrifuged 5 min at 14,000*g* and kept at −80°C until LC-MS/MS analysis. Screening assays with UGT and fulvestrant were performed for 16 h, and enzymes that demonstrated reactivity were reassayed for 1, 2, 4, 8, and 16 h to determine the linear period of time. Thereafter, all assay were performed for 1 h. For competition experiments, estradiol or estrone (50 μM) was incubated for 1 h with microsomes from HEK293 cells in the absence or the presence of increasing concentrations of fulvestrant (0.5–100 μM).

Mass Spectrometry Analysis. Each sample (50 μl) was diluted 1:1 with water, vortexed, and then transferred into a conical vial for injection into the mass spectrometer. The high-performance liquid chromatography system consisted of a mass spectrometer (model API 3000; PerkinElmer/MDS Sciex, Thornhill, ON, Canada) and was used to detect glucuronide conjugates formed. It was operated in the single-ion monitoring or in multiple reactions monitoring mode and equipped with an electrospray ionization interface in negative or positive ion mode and a high-performance liquid chromatography pump plus autosampler model 2690 (Waters, Milford, MA). Chromatographic separation was achieved with a 4.6-mm i.d. × 100-mm Luna C18 column (3.5-μm particle size) (Phenomenex, Torrance, CA). The mobile phase A was water 0.1% ammonium hydroxide and B was methanol 0.1% ammonium hydroxide. Ammonium hydroxide (trace metal grade) was purchased from Fisher Scientific (Nepean, ON, Canada). Separation was achieved using a linear gradient of 35–90% B in 6 min at a flow rate of 0.9 ml/min. Afterward, the column was re-equilibrated to initial conditions over 4 min. For EM-100-G, EM-139-G, and fulvestrant-G, samples were analyzed with a 4-6-mm i.d. × 100-mm Synergie RP Hydro column (4-μm particle size) (Phenomenex) and HPLC-MS system, as mentioned previously, whereas the mobile phase (solvent C, water + 1 mM formate ammonium; solvent D, MeOH + 1 mM formate ammonium) and ionization mode was used. Separation was achieved using a linear gradient of 60–95% D in 6.1 min at a flow rate of 0.9 ml/min. Afterward, the column was re-equilibrated to initial conditions over 2.9 min. To further characterize the kinetic parameters of fulvestrant glucuronidation, chromatographic separation of fulvestrant with a glucuronide at position 3 (fulvestrant-3G) and at position 17 (-17G) was achieved using the same column and MS system as mentioned previously, excepted that the linear gradient of 60–95% D was achieved in 11 min at a flow rate of 0.9 ml/min. Peak areas of glucuronides obtained were corrected for the concentration of UGT enzymes as determined by Western blot quantification and peak area of estrone-glucuronide, which was used as an internal standard. Fulvestrant was synthesized in our laboratory, and fulvestrant glucuronide at position 3, obtained from enzymatic assays using UGT1A3 enzyme, was purified onto HPLC with a 4.6-mm i.d. × 100-mm Synergie RP Hydro column (4-μm particle size) (Phenomenex). The fractions were evaporated under nitrogen and diluted in methanol, and the purity of the compound was confirmed by HPLC. An aliquot was treated with β-glucuronidase (Sigma Chemical) and the residue was quantified with a calibration curve of fulvestrant. The concentration of fulvestrant obtained for the aliquot digested by β-glucuronidase was then converted in a concentration of fulvestrant glucuronide. To quantify fulvestrant-17-glucuronide, enzymatic assays were performed with UGT1A8 and the fractions containing fulvestrant-3G and fulvestrant-17G were collected from HPLC using the linear gradient of 60–95% D in 11 min at a flow rate of 0.9 ml/min. Determination of fulvestrant-3G of an aliquot of this sample was performed using a standard curve containing fulvestrant-3G. Then, a second aliquot of the same sample was treated with β-glucuronidase (Sigma Chemical) and the amount of fulvestrant released from with β-glucuronidase was determined using a fulvestrant standard curve. The residual fulvestrant (fulvestrant-17G = total fulvestrant − fulvestrant-3G) present in the sample corresponded to fulvestrant-17G.

Enzyme Kinetics. Microsomal preparations were incubated in the presence of increasing concentrations of estradiol and fulvestrant varying from 0.5 to 200 μM for 1 h. Absolute glucuronidation activities were adjusted with same parameters as above and were expressed as relative glucuronidation activities in picomoles per minute per milligram of UGT protein level with the standard prepared in our laboratory. Visual inspection of fitted functions (V as a function of [S]) and Eadie-Hofstee plots (V as a function of V/[S]) was used to select the best-fit enzyme kinetic model (Venkatakrishnan et al., 2001). Analysis of data was performed using SigmaPlot 8.0 with Enzyme Kinetics 1.1 (SPSS Inc., Chicago, IL). For Michaelis-Menten and substrate inhibition models, the intrinsic clearance (Cl_{int}) estimation values were calculated as $Cl_{int} = V_{max}/K_m$. For sigmoid model, the maximal clearance was calculated as $CL_{max} = V_{max}/K_m \times ([n - 1]/[n \times (n - 1)^{1/n}])$ (Venkatakrishnan et al., 2001).

Results

Glucuronidation of Fulvestrant by Human UGT Enzymes. To determine which human UGT enzymes are involved in fulvestrant metabolism, glucurono-conjugation assays were performed by incubating fulvestrant for 16 h with microsomes from home-made UGT enzymes (UGT1A1, -1A3, -1A6, -1A7, -1A8, -1A9, -1A10, -2B4, -2B7, -2B10, -2B11, -2B15, -2B17, and -2B28), with subsequent analysis of fulvestrant glucuronide with LC-MS/MS. These assays revealed

the presence of a glucuronide conjugate when fulvestrant is incubated in the presence of UGT1A1, -1A3, -1A4, and -1A8, whereas all other UGT enzymes were not reactive with this substrate. Furthermore, time course analyses performed with UGT1A1, -1A3, -1A4, and -1A8 revealed that the formation of fulvestrant-glucuronide was linear for 4 h (data not shown). Therefore, a quantitative screening was performed with all UGT using this linear condition (Fig. 2). These experiments confirmed that UGT1A3 was the highest producer of fulvestrant-glucuronide, whereas UGT1A1, -1A4, and -1A8 also produced significant amounts of the glucuronide. Similar observations were made when fulvestrant was incubated with commercial UGT baculosomes (BD Biosciences, Mississauga, ON, Canada).

Identification of Fulvestrant Glucuronide. Incubation of fulvestrant with recombinant human UGT1A1, -1A3, and -1A4 resulted in a peak detected at *m/z* 783 with the same retention time of 7.16 min for all enzymes (Fig. 3). Tandem MS analysis of the *m/z* 783 gave a daughter ion at *m/z* 607, which corresponded to the protonated aglycone formed from cleavage of the glycosidic bond ($MH^+ - 176$). Then, the position of the glucoside residue at position 3 of the fulvestrant glucuronide was established with NMR data obtained from the highly purified polar product after incubation of fulvestrant with UGT1A3. Specifically, comparison of the aromatic region in NMR spectra of fulvestrant, fulvestrant-glucuronide, estradiol-3-glucuronide, and estradiol-17-glucuronide showed the presence of both a doublet at 6.9 ppm and a doublet at 6.85 ppm (methanol-d_4) which is associated with glucuronidation at position 3 of estradiol derivatives. These two signals were moved from 6.55 and 6.5 ppm to 6.9 and 6.85 ppm, respectively. For UGT1A8, two peaks at *m/z* 783 were detected, one at the retention time of 7.16 min (which corresponded to that observed for the incubations of UGT1A1, -1A3, and -1A4) and a second at retention time of 7.39 min (Fig. 3). This peak also gave a daughter ion at *m/z* 607, which corresponded to the protonated aglycone formed from cleavage of the glycosidic bond ($MH^+ - 176$), thus suggesting that UGT1A8 also forms fulvestrant-17G.

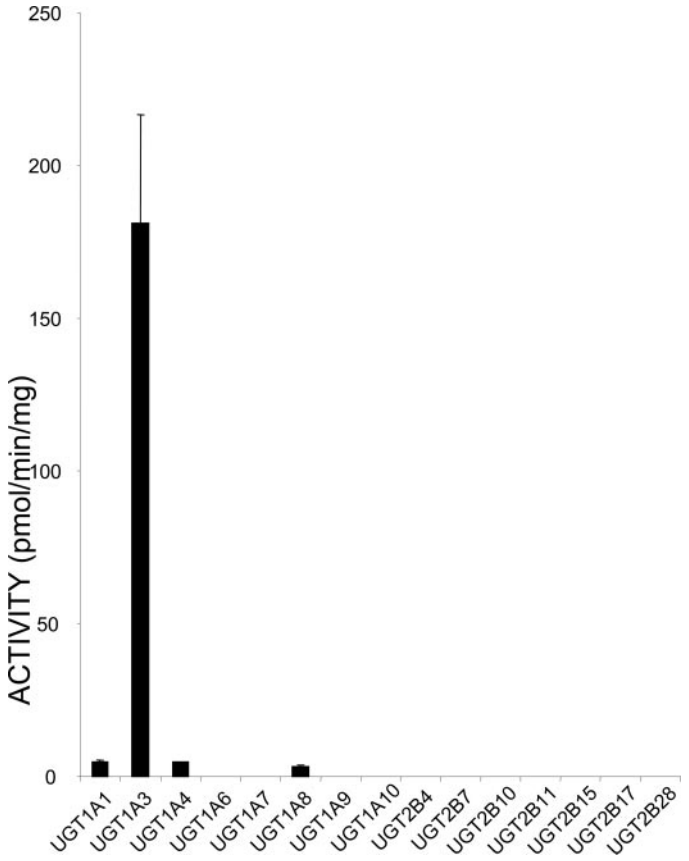


Fig. 2. Conversion of fulvestrant to a glucuronide derivative by microsomal proteins from HEK293 cells stably expressing human UGT proteins. To determine which UGT isoenzyme was involved in fulvestrant glucuronidation, microsomal proteins of HEK293 cells stably expressing human UGT enzymes were incubated in the presence of UDPGA (1 mM) and fulvestrant (50 μM) for 1 h at 37°C. Enzymatic reactions were stopped by adding methanol, and products were analyzed using LC-MS/MS method (see *Materials and Methods*). Values represent the mean (±S.D.) of activity corrected for the concentration of UGT enzymes as determined by Western blot quantification of two independent experiments.

Kinetics of Fulvestrant Glucuronidation. To further characterize the fulvestrant glucuronidation activity, kinetic analyses were performed using microsomes from UGT1A1-, UGT1A3-, UGT1A4-, and UGT1A8-HEK293 cells in the presence of substrate concentrations varying from 0.5 to 200 μM (Fig. 4, Table 1). UGT1A4 presented the highest affinity, with a calculated K_m value of 0.51 μM, which was significantly lower than that for UGT1A1 (1.9 μM) and UGT1A3 (5.6 μM). The K_m values for UGT1A8 were 19.1 and 25 μM for fulvestrant-3 and fulvestrant-17G, respectively. The apparent V_{max} for fulvestrant glucuronidation activity determined during kinetic experiments was corrected for the amount of protein contained in each extract as described previously (Lepine et al., 2004). The V_{max} value for UGT1A3 (217 pmol of fulvestrant-3G/min/mg of protein) was 10-fold higher than those for UGT1A1, -1A4, and -1A8, whereas a value of 0.74 pmol of fulvestrant-17G/min/mg of protein was observed for UGT1A8. The normalized V_{max} was used to determine the efficiency of glucuronidation (ratio V_{max}/K_m). UGT1A3 and UGT1A4 displayed the highest efficiency of fulvestrant glucuronidation, whereas UGT1A1 and UGT1A8 (for both glucuronides), by demonstrating only low V_{max} efficiency values, may be considered poor conjugators for this molecule (Table 1). It is noteworthy that UGT1A8 was 30-fold less efficient for the formation of fulvestrant-17G than for fulvestrant-3G.

Comparison of the glucuronidation parameters of UGT1A1, UGT1A3, and UGT1A8 for estradiol and the synthetic antiestrogen indicates that UGT1A3 shows a 6-fold lower affinity for conjugation at the 3-hydroxy position of estradiol compared with fulvestrant (Table 1). Likewise, the apparent V_{max} at which this enzyme forms estradiol-3-glucuronide is also much lower than that for fulvestrant, thus resulting in a glucuronidation efficiency for fulvestrant that was >300-fold higher than that of estradiol (39.06 and 0.125 μl/min/mg, respectively). UGT1A4 was more specific for fulvestrant glucuronidation because this enzyme does not conjugate estradiol (Table 1).

Expression of Fulvestrant-Conjugating Enzymes in Breast Tissue and Conjugating Activity of Fulvestrant in Liver, Jejunum, and Ileum. To determine the presence of the fulvestrant-conjugating enzymes in breast tissue, a real-time PCR reaction was performed on a commercial RNA sample. Our data revealed the presence of UGT1A3, -1A4, and -1A8 mRNA, and UGT1A4 was the most expressed (lower threshold cycle value). It is noteworthy that the

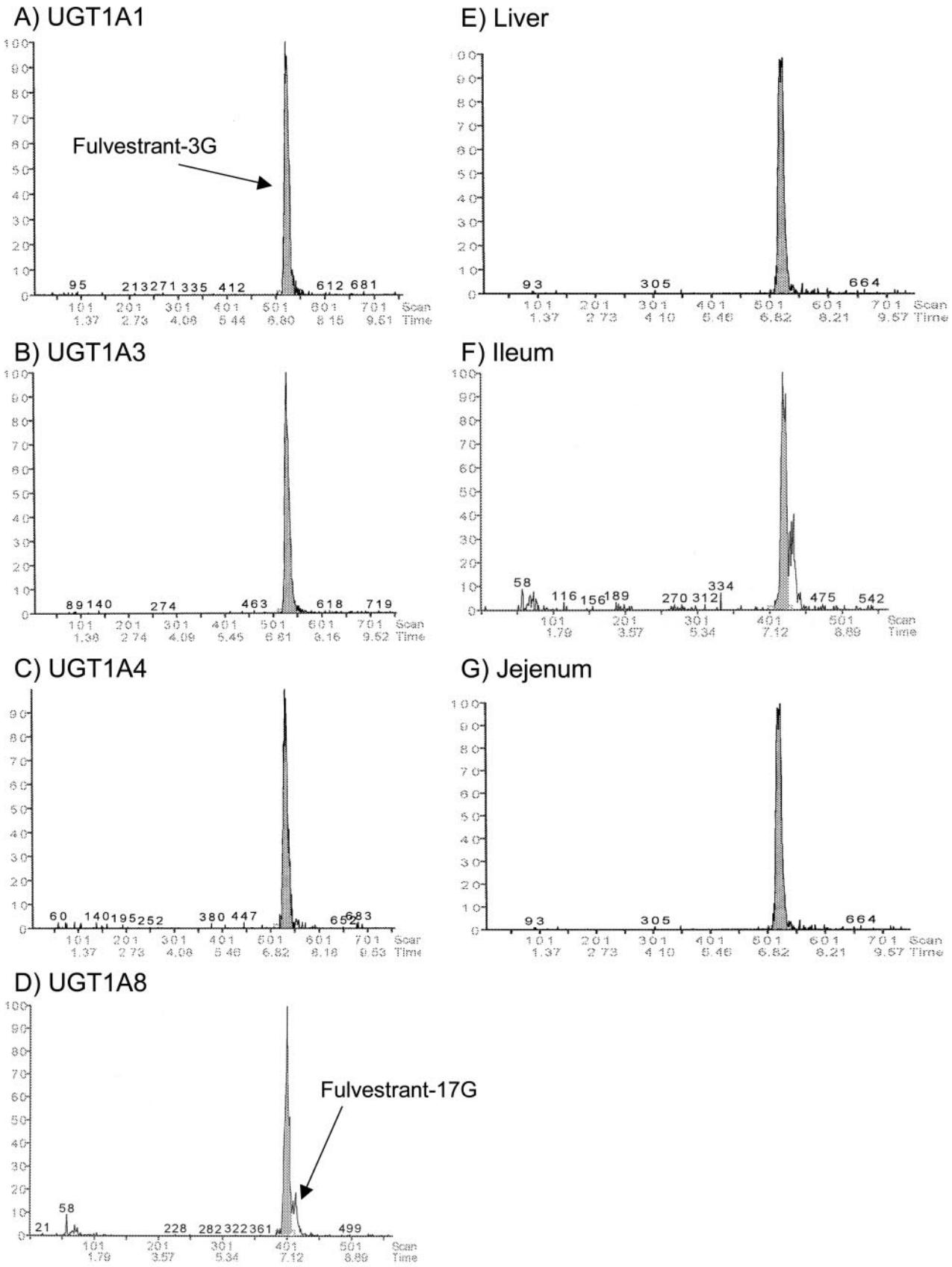


Fig. 3. Chromatographic profiles for the glucuronidation of fulvestrant by UGT1A1 (A), UGT1A3 (B), UGT1A4 (C), UGT1A8 (D), liver (E), ileum (F), and jejunum (G). Chromatographic resolution of the two glucuronide products detected at *m/z* 783 formed from fulvestrant. The MS was operated in the multiple-reactions monitoring mode with mass at *m/z* 783 and *m/z* 607. For fulvestrant, the two glucuronides formed were fulvestrant-3G, with a retention time of 7.16 min, and fulvestrant-17G, with a retention time of 7.39 min.

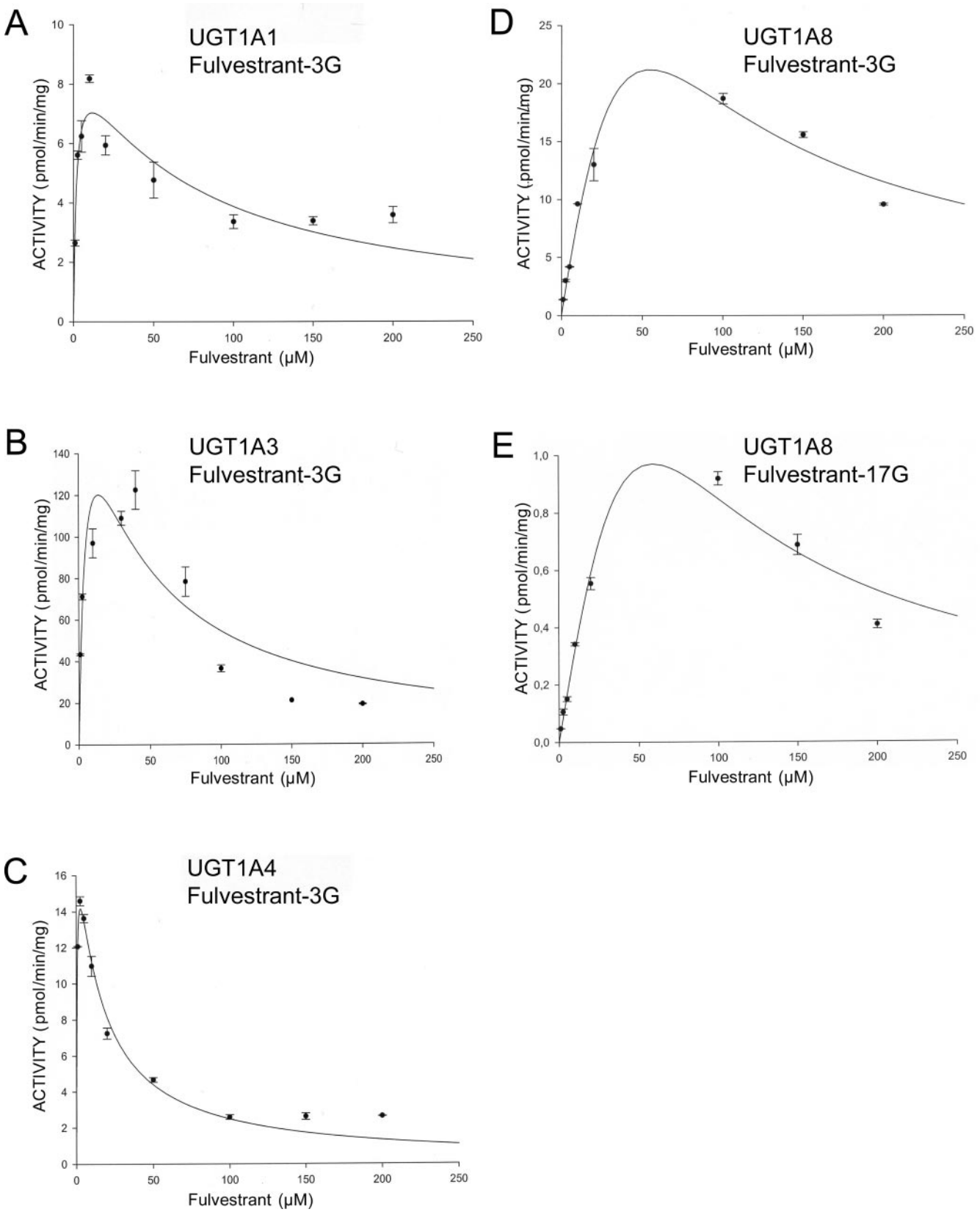


Fig. 4. Kinetic profiles for the glucuronidation of fulvestrant by UGT1A1 (A), UGT1A3 (B), UGT1A4 (C), and UGT1A8 (D and E). Microsomal fractions from UGT1A1- (A), UGT1A3- (B), UGT1A4- (C), and UGT1A8-HEK293 (D and E) cells were incubated in the presence of increasing concentrations of fulvestrant (0.5 to 200 μM) for 1 h. Absolute glucuronidation activities determined by LC-MS/MS were divided by the level of UGT protein assessed by Western blot and expressed as relative glucuronidation activities in picomoles per minute per milligram.

UGT1A1 mRNA was not detected at a quantifiable level (Table 2).

Liver, jejunum, and ileum are three tissues known to express UGT1A enzymes, particularly the four fulvestrant-conjugating enzymes (Strassburg et al., 1998). Then, kinetic analyses similar to those performed with UGT were performed using microsomes from these tissues. Glucuronidation of fulvestrant occurred with K_m values varying from 2.4 to 4.4 μM for the formation of fulvestrant-3G, whereas the V_{max} value of 339 pmol/min/mg of protein obtained with jejunum was more than 10-fold higher than that obtained with liver (29.1 pmol/min/mg of protein); finally, the ratio V_{max}/K_m displayed higher efficiency of fulvestrant conjugation by the jejunum compared with the liver (Fig. 5C). It is noteworthy that incubation with ileum microsomes resulted in formation of two forms of fulvestrant glucuronide, indicating the presence of UGT1A8 in this tissue. In addition, in this tissue, fulvestrant-17G was formed with low affinity and a 30-fold lower efficiency compared with fulvestrant-3G. Similar differences were also observed with UGT1A8, thus supporting an important role for this UGT in the glucuronidation of fulvestrant in ileum.

Fulvestrant Stimulates the UGT1A3-Dependent Glucuronidation of Estrone but Fails to Affect Estradiol Conjugation. Considering the major role of UGT1A3 in the conjugation of estrone and estradiol (Lepine et al., 2004), we investigated whether these metabolic processes may be affected in the presence of fulvestrant, the target tissue of which is mammary gland. These experiments were performed by incubating estrone or estradiol (50 μM) with microsomes from UGT1A3-HEK293 cells in the presence of increasing concentrations of fulvestrant (0–100 μM). We observed that formation of estrone glucuronide by UGT1A3 was increased dose dependently in the presence of fulvestrant (Fig. 6); the stimulatory effect occurred at low concentrations of fulvestrant to reach a maximal induction of 273% in the presence of 20 μM fulvestrant and remained stable at higher concentration of the antiestrogen (Fig. 6). By contrast, the formation of estradiol-3-glucuronide catalyzed by UGT1A3 was not affected in the presence of the antiestrogen.

Glucuronidation of Estrogen Derivatives by the Human UGT Enzymes. The results described above suggest that addition of an aliphatic chain to the estradiol molecule affects the specificity of UGT enzymes toward the C_{18} steroid molecules. Therefore, to further understand the structure-activity of UGT1A1, -1A3, -1A4, and -1A8 toward estrogen molecules, compounds having short or long aliphatic chains attached at position 2, 7α, or 11β of estradiol were tested by incubating human UGT microsomal enzymes. As shown in Table 3, UGT1A1, -1A3, and -1A8 conjugated almost all molecules tested, whereas UGT1A9 and UGT1A10 catalyzed the glucuronidation of only few substrates. EM-100, a synthetic steroid with a structure almost similar to that of fulvestrant was also conjugated by UGT1A4 but at a lower level than that of UGT1A1 and UGT1A8. Among members of the UGT2B subfamily, only UGT2B7 was able to conjugate substrates having an aliphatic chain at position 11β, except for EM-4570, which was glucuronidated by UGT2B4 (Table 3). Therefore, we focused our work on the glucuronidation activity of UGT1A1, UGT1A3, and UGT1A8 by performing quantitative analyses for 1 h.

In addition to fulvestrant, which possesses a sulfoxide chain with 14 carbon atoms at position 7α, we also investigated the glucuronidation of EM-5314, EM-100, and EM-139, three molecules having a methyl group or amide chain including 16 carbon atoms at position 7α, whereas EM-139 also possesses an additional chlorine group at position 16α (Fig. 7). We observed that, as for estradiol, the existence of only a methyl group at position 7α (e.g., as in compound EM-5314) resulted in the formation of two glucuronides, EM-5314-G1 and EM-5314-G2. The two glucuronide derivatives are identified based on their retention time on HPLC, although they were not further analyzed to determine the position of the glucuronide on the phenol molecule. UGT1A1 and UGT1A3 were thus the highest active conjugating enzymes of EM-5314 at position 1 but only UGT1A3 was capable of forming a second glucuronide derivative (EM-5314-G2). EM-100 and EM-139 formed only a unique glucuronide conjugate (Fig. 7), thus suggesting that the methyl group did not avoid conjugation at both sites of the steroid, whereas a longer chain of carbon atoms may selectively inhibit one site of glucuronidation but again with a predominant role of UGT1A3.

TABLE 1

Kinetic parameters for the glucuronidation of estradiol and fulvestrant by UGT1A isoenzymes

Kinetic studies were performed for 1 h using microsomal concentrations of proteins from HEK293 cells stably expressing human UGT1A1, -1A3, -1A4, and -1A8 proteins in the presence of UDPGA (2 mM) and concentrations of fulvestrant ranging from 0.5 to 200 μM. For estradiol, kinetic parameters were obtained from Lépine et al. (2004). Values represent the mean of two independent experiments ± S.D. Glucuronide conjugates were quantified using LC/MS-MS. V_{max} values were normalized for the variable level of UGT1A protein expression in stably expressing HEK293 cells. Each normalized V_{max} value was used to determine the glucuronidation efficiency (ratio V_{max}/K_m). K_m is expressed as micromolar, V_{max} as picomoles per minute per milligram of protein, and catalytic efficiency as microliters per minute per milligram of protein.

	K_m	V_{max}	Catalytic Efficiency	Kinetic Profile
Estradiol-3-glucuronide				
UGT1A1	23	93	2.0	S
UGT1A3	47	39	0.4	S
UGT1A4	N.A.	N.A.	N.A.	
UGT1A8	38	195	5.1	H
Estradiol-17-glucuronide				
UGT1A1	N.A.	N.A.	N.A.	
UGT1A3	35	13	0.2	S
UGT1A4	N.A.	N.A.	N.A.	
UGT1A8	N.A.	N.A.	N.A.	
Fulvestrant-3-glucuronide				
UGT1A1	1.9	9.30	4.89	I
UGT1A3	5.6	217.2	39.06	I
UGT1A4	0.5	19.50	36.79	I
UGT1A8	19	16.94	0.88	I
Fulvestrant-17-glucuronide				
UGT1A1	N.A.	N.A.	N.A.	
UGT1A3	N.A.	N.A.	N.A.	
UGT1A4	N.A.	N.A.	N.A.	
UGT1A8	25	0.74	0.03	I

N.A., no activity of glucuronidation; H, hyperbolic; S, sigmoid; I, substrate inhibition.

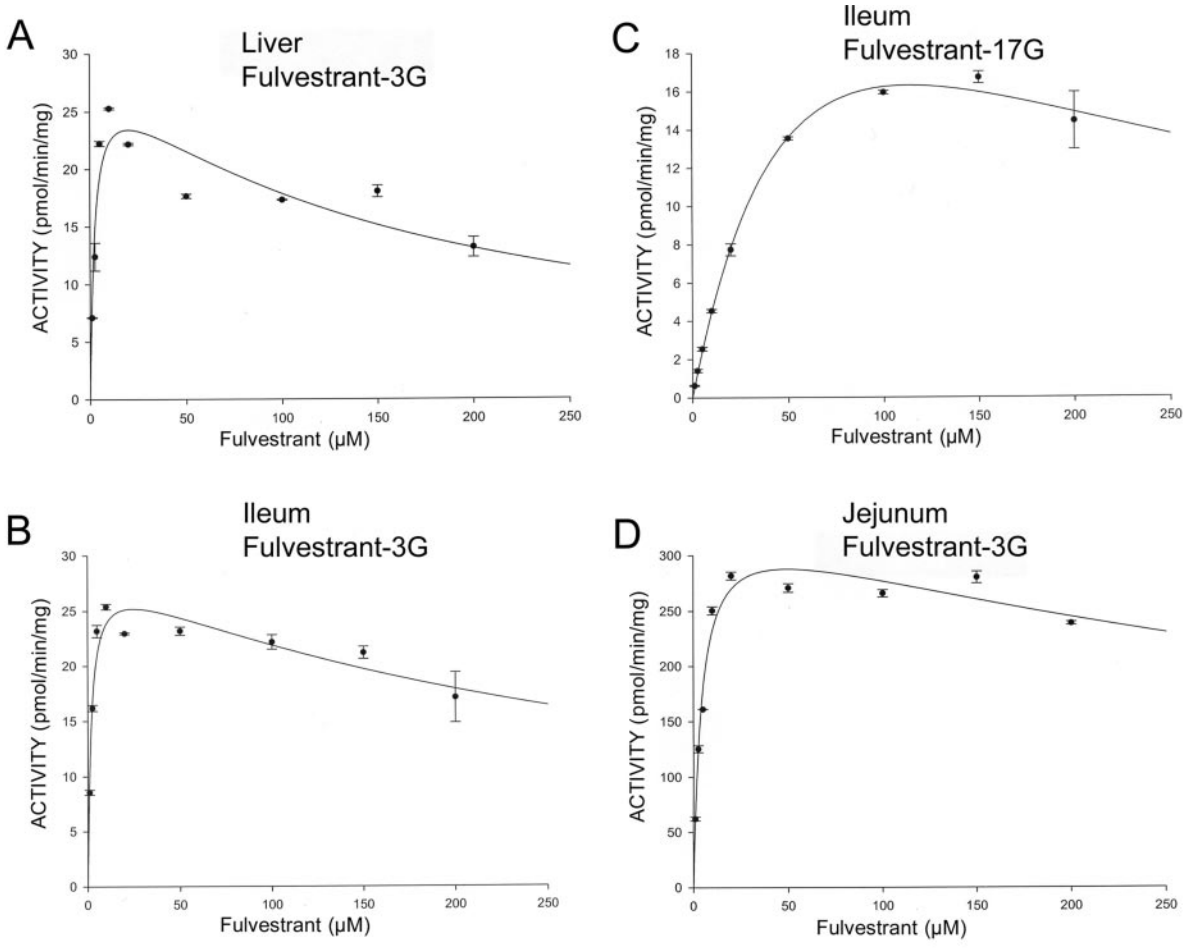
TABLE 2

Expression in breast tissues of UGT1A enzymes catalytically active on fulvestrant

	Threshold Cycle
UGT1A1	BLQ
UGT1A3	38.04
UGT1A4	37.09
UGT1A8	37.46

BLQ, below limit of quantification.

We also tested the effects of adding an aliphatic chain at position 11β of the estradiol molecule. For this series of experiments, estrogenic molecules having an aliphatic chain of one (EM-4570), four (EM-5499), or five (EM-4834) carbons were used (Fig. 8A). UGT1A1 was the major enzyme for the formation of EM-4570-G1, whereas the activity of the UGT1A1 enzyme was comparable with that of UGT1A3 and UGT1A8 for the conjugation of EM-5499 (four carbons) and completely inactive toward EM-4834 (five carbons) (Fig. 8A). This observation suggests that increasing the length of an



E

	Fulvestrant-3-glucuronide				Fulvestrant-17-glucuronide			
Tissue	K_m	V_{max}	Catalytic Efficiency	Kinetic Profile	K_m	V_{max}	Catalytic Efficiency	Kinetic Profile
Liver	2.4	29.1	8.38	I	NA	NA	NA	
Ileum	1.9	29	15.26	I	73.6	37.5	0.51	I
Jejunum	4.4	339	77.04	I	NA	NA	NA	

Fig. 5. Kinetic profiles for the glucuronidation of fulvestrant by liver (A), ileum (B and C), and jejunum (D) and their kinetic parameters (E). Microsomal fractions were incubated in the presence of increasing concentration of fulvestrant (0.5–200 μM) for 1 h as described under *Materials and Methods*. The formation of fulvestrant-3 and -17G was quantified by LC-MS/MS. Absolute glucuronidation activities were divided by the level of UGT1A protein assessed by Western blot and expressed as relative glucuronidation activities in picomoles per minute per milligram. E, each normalized V_{max} value was used to determine the glucuronidation efficiency (ratio V_{max}/K_m). I, substrate inhibition profile. K_m is expressed in micromolar, V_{max} in picomoles per minute per milligram of protein, and catalytic efficiency in microliters per minute per milligram of protein.

aliphatic chain at position 11β of the estradiol molecule has a marked influence on UGT1A1 activity. On the other hand, UGT1A3 was the predominant enzyme involved in the formation of EM-4570-G2 and EM-5499-G2, whereas its activity was preponderant for EM-4834-G1 formation and markedly low for EM-4834-G2, further indicating that the length of the aliphatic chain also affected UGT1A3 activity (Fig. 8A). UGT1A8 was the preponderant enzyme for EM-5499 conjugation but had relatively low activity for the two other compounds tested.

The effects of a phenyl group (EM-4541, EM-4887, EM-4896, and EM-4720) at position 11β were studied next (Fig. 8B). It is interesting that insertion of a phenyl group near the estradiol molecule (EM-4541) completely abolished the glucuronidation catalyzed by UGT1A enzymes (Fig. 8B), whereas UGT2B7 was the only enzyme tested that was capable of converting this molecule into a glucuronide derivative. However, when the number of carbon atoms inserted between the estrogen nucleus and the phenyl group was two or three (EM-4887 and EM-4896), the UGT1A1 and UGT1A3 enzymes progressively became active for the conjugation of the compound with the lowest retention time (Fig. 8B). We were surprised to find that UGT1A8 was the efficient enzyme catalyzing the formation of the second glucuronide of EM-4720. This molecule contained a phenyl group separated from the steroid nucleus by four carbons. These data demonstrate that the proximity of the phenyl group from the site of glucuronidation inhibits the activity of UGT1A enzymes.

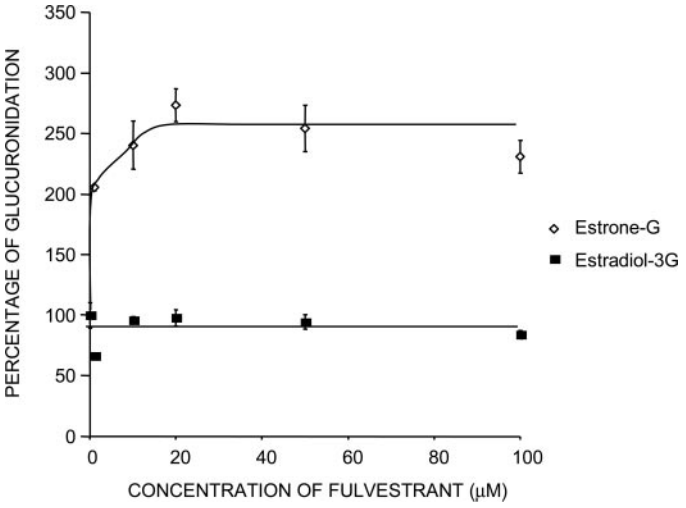


Fig. 6. Effects of increasing concentration of fulvestrant on the UGT1A3-dependent formation of estrone-glucuronide and estradiol-3-glucuronide. Microsomal proteins from UGT1A3-HEK293 cells were incubated in the presence of increasing concentrations of fulvestrant (0.5–100 μM) and with either estrone or estradiol (50 μM) for 1 h at 37°C, and the formation of estradiol-3-glucuronide (estradiol-3G) and estrone-glucuronide (estrone-G) was determined by LC-MS/MS. Values represent the percentage (mean ± S.D. of two independent experiments) of glucuronides formed in the presence of fulvestrant compared with the absence of fulvestrant.

We also tested whether the presence of a hydroxyl group (EM-5498 and EM-5463) at the end of the aliphatic chain might modify the profile of UGT enzymes involved in the glucuronidation reaction (Fig. 8C). The most remarkable observation is the formation of a third glucuronide, which is accompanied by a reduction of UGT1A8 activity for the overall glucuronidation of these compounds (EM-5498 and EM-5463). It is interesting that UGT1A3 was the most active UGT enzyme involved in the formation of glucuronide derivatives from these trihydroxylated molecules (Fig. 8C) followed by UGT1A1, which conjugated EM-5498 and EM-5463.

Finally, we investigated the effect of inserting an aliphatic chain at position 2 of the estradiol molecule on the glucuronidation activity catalyzed by human UGT1A1, -1A3, and -1A8 enzymes (Fig. 9). With EM-1959 and EM-2036, a similar pattern in the formation of two glucuronides for each molecule tested was observed with the UGT1A3 and UGT1A8 enzymes, whereas UGT1A1 formed only relatively low amounts of EM-1959-G1 and EM-2036-G1. Overall, the pattern of glucuronidation of these molecules with an aliphatic chain at position 2 near a possible site of glucuronidation, however, were comparable with that observed for estradiol (Fig. 9).

Discussion

Estradiol is glucuronidated at the 3-hydroxyl group by four members of the UGT1A subfamily (UGT1A1, -1A3, -1A8, and

TABLE 3

Screening of estrogen derivatives with aliphatic or phenyl chains at position 2, 7α, and 11β of the estradiol molecule.
All enzymes were catalytically active on eugenol except UGT2B10 and 2B11, which were catalytically active on 13-HODE.

Substrates	UGT														
	2B4	2B7	2B10	2B11	2B15	2B17	2B28	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10
Estradiol	+	+	−	−	−	+	−	+	+	−	−	−	+	−	+
Position 2															
EM-1959	−	−	−	−	−	−	−	+	+	−	−	−	+	−	+
EM-2036	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−
Position 7α															
Fulvestrant	−	−	−	−	−	−	−	+	+	+	−	−	+	+	+
EM-139	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−
EM-100	−	−	−	−	−	−	−	+	+	+	−	−	+	−	−
EM-5314	−	−	−	−	−	−	−	+	+	−	−	−	+	−	−
Position 11β															
EM-4541	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
EM-4570	+	−	−	−	−	−	−	+	+	−	−	−	+	−	−
EM-4720	−	+	−	−	−	−	−	−	−	−	−	−	+	−	−
EM-4834	−	+	−	−	−	−	−	−	−	−	−	−	+	+	−
EM-4887	−	+	−	−	−	−	−	+	+	−	−	−	+	−	+
EM-4896	−	+	−	−	−	−	−	+	+	−	−	−	+	+	−
EM-5463	−	+	−	−	−	−	−	+	+	−	−	−	+	−	−
EM-5498	−	+	−	−	−	−	−	+	−	−	−	−	+	−	−
EM-5499	−	+	−	−	−	−	−	+	+	−	−	−	+	−	−

-1A10) and at position 17 by UGT1A3 and UGT2B7. Previous studies have indicated that estrone and catecholestrogens with a hydroxyl group at position 2 or 4 are also conjugated by the same set of enzymes (Lepine et al., 2004). In the present study, we demonstrate that the antiestrogen fulvestrant, which possesses an estradiol-type structure in addition to a long aliphatic chain at position 7α, is efficiently conjugated at position 3 by UGT1A3 and -1A4. Two other UGT1A enzymes, UGT1A1 and -1A8, can also conjugate fulvestrant at this position but with minor impacts. Only UGT1A8 can also conjugate fulvestrant at position 17. UGT1A1, -1A3, and -1A8 presented a higher affinity for fulvestrant glucuronidation at position 3 compared with E_2, thus suggesting that fulvestrant is more easily glucuronidated. Furthermore, we observed the presence of UGT1A3, -1A4, and -1A8 mRNA in the breast tissue, which is the target organ of fulvestrant. Therefore, these results suggest that by glucuronidation of fulvestrant, UGT may alter this tissue's pharmacological response to the antiestrogen.

Examination of several estradiol derivatives with varying lengths and functional groups of a chain at position 2, 7α, or 11β shows that the estradiol-conjugating enzymes are generally implicated in the conjugation of the hydroxyl group at position 3 or 17, thus suggesting that the basic structure of estradiol is a major determinant for the specificity of this group of enzymes. However, for some substrates, such as fulvestrant, a particular enzyme may be predominantly implicated. The homology of less than 80% of UGT1A3 with other members of the UGT1A subfamily (except for UGT1A4) may be responsible for the high specificity toward fulvestrant. In the other hand, the UGT1A4 enzyme which is 93% homolog with UGT1A3 has a complete different pattern of substrate recognition, except for substrates possessing an amine nucleophile site (Green et al., 1998). Because UGT1A4 was unable to catalyze the glucuronide-conjugation of E_2 (Lepine et al., 2004), it is surprising to observe herein that the catalytic efficiencies (V_{max}/K_m) of UGT1A3 and -1A4 for fulvestrant are in the same range. This suggests that adding the chain at position 7α of E_2 favors the glucuronidation by UGT1A4 without affecting conjugation by UGT1A3.

Previous studies have demonstrated that oral delivery of fulvestrant using several formulation types could not provide significant blood concentrations, but it remains unclear whether it was caused by poor absorption or by a first-pass intestine metabolism of the synthetic compound (Robertson and Harrison, 2004). Nevertheless, our data suggest that, in addition to the hydroxylation reaction, the activity of UGT1A enzymes in the gastrointestinal tract, as reported by several authors (Desai et al., 2003), would probably contribute to rapidly conjugate the molecule when administered orally and may thus reduce its absorption. To overcome this problem, fulvestrant was developed with a slow-released intramuscular (i.m.) formulation, which allows long-term active serum concentrations (Robertson and Harrison, 2004). The limited available data on the metabolism of fulvestrant indicate that, after i.m. administration, the compound is metabolized by the liver to be eliminated through the bile with very few excretions into the urine. Further studies also demonstrated that CYP3A4 contributes to the catabolism of fulvestrant in addition to sulfation and glucuronidation (Robertson and Harrison, 2004).

The presence of UGT1A enzymes in tissues other than the liver (e.g., the breast) may also significantly contribute locally to the inactivation of fulvestrant. Indeed, recent studies strongly suggest that the local glucuronidation in steroid target tissues is important for the maintenance of steroid homeostasis, which depends on both synthesizing and inactivating enzymes (Guillemette et al., 2004). UGT1A3, -1A4, and -1A8 activity in breast tissue may thus have a negative impact on fulvestrant action, and the levels of circulating fulvestrant cannot reflect this. Therefore, it will be of interest to determine their levels of expression and/or activity in breast tumor tissue for the patients displaying poor response to fulvestrant after previous endocrine therapy or after long-

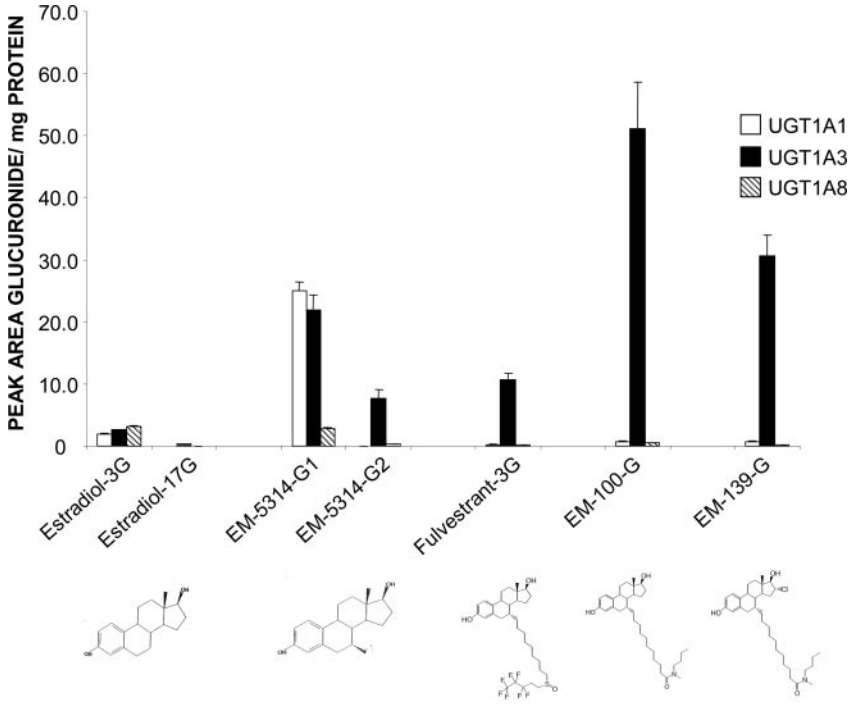


Fig. 7. Glucuronidation of estradiol derivatives having an aliphatic chain at position 7α. Microsomal proteins of HEK293 cells stably expressing human UGT enzymes were incubated in the presence of UDPGA (1 mM) and substrates (200 μM) for 1 h at 37°C. Enzymatic reactions were stopped by adding methanol, and products were analyzed by LC-MS/MS. For EM-5314, the two glucuronides formed were numbered depending of their retention time in HPLC. EM-5314-G1 had a retention time of 2.43 min; EM-5314-G2 had a retention time at 2.55 min. Values represent the mean (mean ± S.D.) of glucuronide area corrected for the concentration of UGT enzymes as determined by Western blot quantification and area of estrone-glucuronide, which was used as an internal standard of each UGT enzyme of two independent experiments.

term treatment with fulvestrant. Because little is known on the level of UGT1A expression in breast cells, it would also be important to determine factors that influence fulvestrant glucuronidation. For example, the conjugation of fulvestrant by one of the four recently isolated polymorphic forms of UGT1A3, which possess 3-fold higher activity on estrone than the wild type (Iwai et al., 2004), could be investigated because a lower concentration of fulvestrant in breast tissue may result from this mutation. In addition, previous studies aimed at defining the substrate specificity of UGT1A3 have demonstrated the conjugation of coumarins, flavonoids, anthraquinones, and small phenolic compounds in addition to estradiol and some catecholestrogens (Green et al., 1998). Coumarins and flavonoids are a large class of phenolic compounds that are present in many plants, including citrus fruits, berries, leafy vegetables, spices, and cereal grains (Cai et al., 2004). It is possible that important natural product interactions in the conjugation of fulvestrant result in a decrease glucuronidation of the antiestrogen. Because of the low toxicity of this antiestrogen, the natural diet products would favor higher concentrations of the antiestrogen and a benefit for the patients.

Our data also demonstrate that fulvestrant has no effect on estradiol glucuronidation, whereas a stimulation of estrone conjugation is observed. An activation of glucuronidation activity at low concentrations of substrates was also observed for LTB_4 conjugation by UGT2B7 at low concentrations of ADT, and a similar activation was also reported for estradiol conjugation at position 3 by UGT1A1 in the presence of several types of phenol substrates (Turgeon et al., 2003b). Although the mechanism of autoactivation associated with UGT1A enzymes is poorly understood, it may be possible that these enzymes act in a multimeric form, where the binding of one molecule may facilitate the conjugation of the second molecule at the other site (Soars et al., 2003). Nevertheless, an increased conjugation of estrogen would facilitate the antiestrogenic effect of fulvestrant by eliminating the endogenous carcinogenic substrate. Whereas this phenomenon of

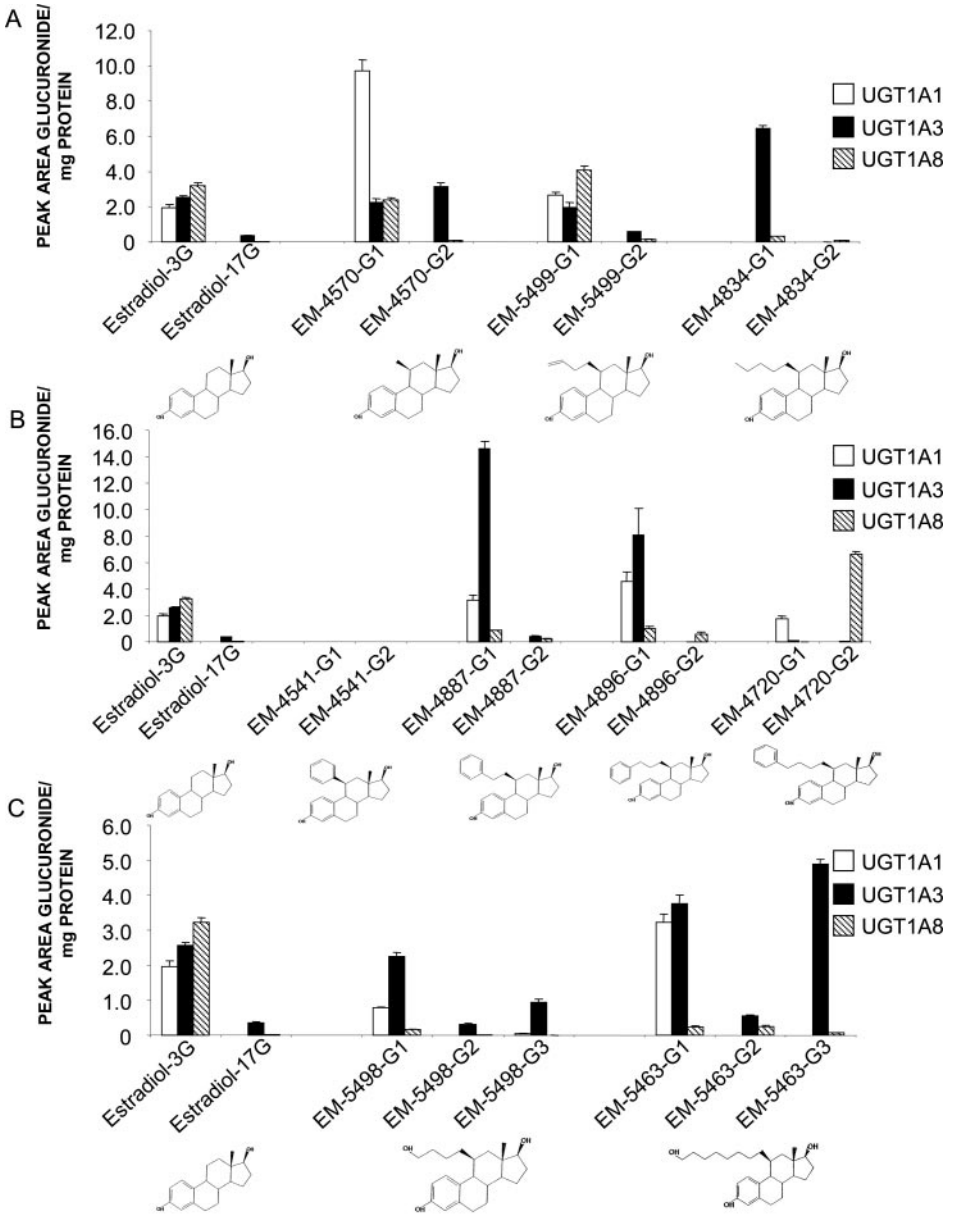


Fig. 8. Glucuronidation of estradiol derivatives having an aliphatic chain at position 11β. Microsomal proteins of HEK293 cells stably expressing human UGT enzymes were incubated in the presence of UDPGA (1 mM) and 200 μM concentrations of estradiol derivatives with an aliphatic chain at position 11β that is ended or not (A), a phenyl group (B), or a hydroxyl group (C) for 1 h at 37°C. Enzymatic reactions were stopped by adding methanol, and products were analyzed by LC-MS/MS. A, the retention times for the glucuronides 1 were 1.81, 3.16, and 3.23 min, whereas the glucuronides 2 had retention times of 2.14, 3.50, and 4.57 min for EM-4570, EM-5499, and EM-4834, respectively. B, for the glucuronides 1, the retention times were 3.08, 3.75, 4.00, and 4.46 min, whereas the retention times for the glucuronides 2 were 3.40, 4.05, 4.36, and 4.76 min for EM-4541, EM-4887, EM-4896, and EM-4720, respectively. C, the retention times for the glucuronides 1 were 2.17 and 3.85 min, the retention times for the glucuronides 2 were 2.61 and 4.16 min, and the retention times for the glucuronides 3 were 3.00 and 4.56 min for EM-5498 and EM-5463, respectively. Values represent the mean (±S.D.) of glucuronide area corrected for the concentration of UGT enzymes as determined by Western blot quantification and area of estrone-glucuronide, which was used as an internal standard of each UGT enzyme of two independent experiments.

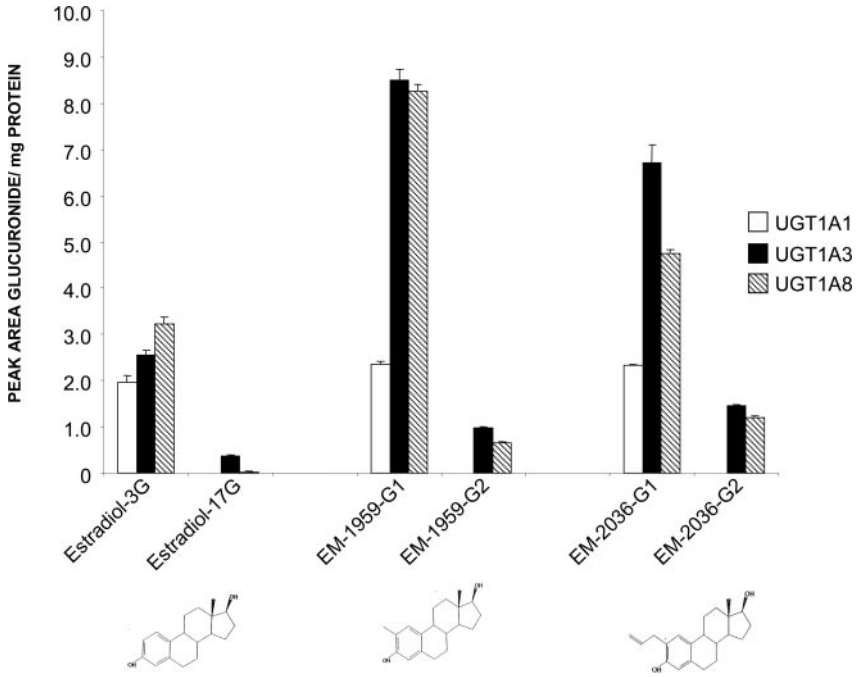


Fig. 9. Glucuronidation of estradiol derivatives having an aliphatic chain at position 2. Microsomal proteins of HEK293 cells stably expressing human UGT enzymes were incubated in the presence of 1 mM UDPGA and 200 μM concentrations of estradiol, EM-1959 or EM-2036 for 1 h at 37°C. Enzymatic reactions were stopped by adding methanol, and products were analyzed by LC-MS/MS. Retention times for the glucuronides 1 were 1.97 and 2.96 min, whereas the retention times for glucuronides 2 were 2.50 and 3.25 min for EM-1959 and EM-2036, respectively. Values represent the mean (± S.D.) of glucuronide area corrected for the concentration of UGT enzymes as determined by Western blot quantification and area of estrone-glucuronide, which was used as an internal standard of each UGT enzyme of two independent experiments.

autoactivation is unclear, it is reasonable to speculate that the concentration of estrone glucuronide could be increased in patients treated with fulvestrant.

The conjugating activity of the UGT1A isoforms toward several estradiol derivatives was investigated in this study to determine the effects of several types of aliphatic chains on the specificity of this enzymatic reaction. It is remarkable that the same set of enzymes is generally implicated in the conjugation of estradiol derivatives with aliphatic or phenyl chains at positions 2, 7α, and 11β, thus suggesting that the basic estradiol structure is a dominant factor for their specificity. We also observe that lengthening the chain at position 7α or 11β of E_2 reduces the conjugating activity of UGT1A1 and -1A8 without affecting UGT1A3. In addition, no marked difference in UGT enzyme specificity is noted for the glucuronidation of molecules with different ends on the chain at position 7α of the estradiol nucleus. In addition, when a chlorine group is at position 16α of EM-100, again the specificity of UGT1A3 is not significantly affected, thus further suggesting that the recognition of UGT1A3 for this type of substrate is strongly based on the estradiol molecule and the long aliphatic chain. It is also remarkable that a phenyl group at position 11β markedly inhibits the enzymatic activity for all enzymes investigated, whereas when a short aliphatic chain of two or three carbons is present between the phenyl group and the estrogen molecule, the activity of UGT1A1 and UGT1A3 is again observed. It is likely that the proximity of the bulky phenyl group at position 11β exerts major interference at the reaction site, whereas few carbons may help to make the phenyl more freely moving in the reaction site, thus further suggesting the importance of the intrinsic estradiol structure for the specificity of the UGT1A1, -1A3, and -1A8 enzymes. In agreement with several previous reports (Cheng et al., 1999; Turgeon et al., 2003b; Lepine et al., 2004), our data indicate that the UGT enzymes have significant overlap in specificity, but an individual isoform exhibits distinct substrate recognition.

In summary, knowledge of the enzymes involved in drug metabolism is important for rationalizing and optimizing dosage regiments. In the present study, we illustrate major metabolism of fulvestrant by UGT1A3 and UGT1A4, two UGT enzymes expressed in the gastrointestinal tract (Strassburg et al., 1998). This conjugating activity is likely to contribute to rapid inactivation of the drug when administered orally. In addition, our data strongly suggest that the basic structure of a steroid may be a major factor for the recognition of substrates by a particular UGT enzyme. These studies based on structure-function activity, using several substrates possessing the basic structure of the estradiol molecule, may help to further understand the involvement of specific UGT enzymes in the metabolism of a particular drug and contribute to better define in silico design of new drugs, as suggested previously (Miners et al., 2004).

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