

Glucuronidation of the Nonsteroidal Antiestrogen EM-652 (SCH 57068), by Human and Monkey Steroid Conjugating UDP-Glucuronosyltransferase Enzymes

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ABSTRACT

EM-652 (SCH 57068) is a new orally active antiestrogen that demonstrates pure antagonistic effects in the mammary gland and endometrium. *In vivo* studies have shown that EM-652 is primarily glucuronidated at the 7-hydroxy position in rats and that the metabolite is present in the plasma of female monkeys and human subjects after EM-800 (SCH 57050) or EM-652-HCl oral administration. Using hepatic microsomes from rat, monkey, and human, the formation of two EM-652 monoglucuronides at positions 4' and 7 was demonstrated by a liquid chromatographic tandem mass spectrometric method. Although no difference in EM-652 conjugation was observed between male and female monkey livers, an interindividual variation of hepatic EM-652 glucuronidation was shown with female human donors. Using microsomes preparations from

human embryonic kidney 293 cells stably expressing each of the 12 human and 11 monkey UGT enzymes cloned to date, the two EM-652-monoglucuronides were detected after incubation with microsomes containing human UGT1A1, UGT1A3, UGT1A8, UGT1A9, and monkey monUGT1A01, monUGT1A03, and monUGT1A09. Despite human UGT1A1 and monkey monUGT1A09 favored formation of EM-652-7-glucuronide, other active UGT1A enzymes formed both 4'- and 7-glucuronide derivatives in equal amounts. Kinetic analysis of EM-652 glucuronidation by these enzymes showed Michaelis constant (K_m) values between 36 and 302 μ M for EM-652-4'-glucuronide and 19 and 233 μ M for EM-652-7-glucuronide. The present results demonstrate the importance of UGT1A isoforms, mainly UGT1A1, for EM-652 metabolism in humans.

Breast cancer is the most frequent cancer in women. Estrogens, which act as specific ligands of estrogen nuclear receptors (ER) in target tissues, are well recognized to play a major role in the development and growth of this cancer (Davidson and Lippman, 1989; Landis et al., 1999). A logical approach for the treatment of estrogen-dependent cancer is the use of compounds that block the interaction of estrogens with their receptor. Although several steroidal and nonsteroidal drugs, such as ICI 182,780 and tamoxifen, are available, they present mixed agonist and antagonist activity. EM-652 is an orally active antiestrogen with pure antagonistic activity in the mammary gland and endometrium (Labrie et al., 1999). It demonstrated the most potent inhibitory effect on both ER α and ER β compared with any of the other antiestrogens and it was shown to be the best inhibitor of human breast cancer ZR-75-1, MCF-7, and T-47-D cell

growth (Simard et al., 1997; Labrie et al., 1999). Moreover, EM-800, which is a prodrug of EM-652, prevented the development of dimethylbenz[*a*]anthracene-induced mammary gland carcinoma in the rat (Luo et al., 1997) and also prevented bone loss in ovariectomized animals and decreased serum cholesterol and triglycerides (Labrie et al., 1999).

Studies have shown that after a single oral dose of either [14 C]EM-800 or [14 C]EM-652-HCl to female Sprague-Dawley rats, drug-derived radioactivity was rapidly absorbed and extensively metabolized (High et al., 1999). By 6 h postdose, 60 to 80% of plasma radioactivity was composed of metabolite(s) other than EM-652 or the pro-drug EM-800. Profiles of [14 C]EM-800-derived and [14 C]EM-652-HCl-derived radioactivity extracted from rat plasma both showed one principal metabolite and EM-652. The principal metabolite in rat plasma had a mass-to-charge ratio that corresponded to a monoglucuronide conjugate of EM-652 (High et al., 1999), with the glucuronide moiety most likely located on one of the two hydroxypositions (7 or 4') in the EM-652 molecule (Fig.

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ABBREVIATIONS: ER, estrogen receptor; LC-MS/MS, liquid chromatographic tandem mass spectrometric method; UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis.

1). The presence of EM-652-7-glucuronide was further confirmed, using a liquid chromatographic tandem mass spectrometric (LC-MS/MS) method, in plasma from female rats, monkeys and human subjects after a single oral dose of either EM-800 or EM-652-HCl (High et al., 1999).

Glucuronidation is catalyzed by a family of membrane bound enzymes, the UDP-glucuronosyltransferases (UGTs), which have an active site located in the lumen of the endoplasmic reticulum. This mechanism of cellular detoxification consists of the transfer of glucuronic acid from uridine diphosphoglucuronic acid (UDPGA) to excretable compounds (Mulder, 1992). In this S_N2 reaction, a nucleophile acceptor group on the substrate (hydroxyl, carboxyl, sulfhydryl, or amines) attacks an electrophilic C-1 atom of the glucuronic acid group. Glucuronidation generally terminates the biological and pharmacological activity of endobiotics or drugs (Mulder, 1992) and the products are more polar, less toxic, and more easily excretable from the body through the bile or urine.

So far, 61 different UGT enzymes have been isolated in

several mammalian species. UGT enzymes have been categorized into two families, UGT1 and UGT2, (Mackenzie et al., 1997). In humans, the UGT1 gene family is located on chromosome 2q37 where the gene locus contains 12 different versions of exons one and exons two to five, which are common to all UGT1As (Owens and Ritter, 1995). UGT2 proteins are subdivided into two subfamilies, UGT2A and UGT2B. UGT2A enzymes are expressed in the olfactory epithelium, whereas the UGT2B enzymes are expressed from distinct genes that share a similar organization composed of six exons and five introns (Monaghan et al., 1994, 1997; Beaulieu et al., 1997; Turgeon et al., 2000). UGT2B enzymes can glucuronidate xenobiotics such as eugenol, and their ability to conjugate steroid substrates is well established, especially for androgens. UGT1A enzymes are more specific for xenobiotics conjugation, whereas their activity on several endogenous substrates, such as bilirubin and estrogens, has been demonstrated (Ritter et al., 1991; Senafi et al., 1994; King et al., 1996; Albert et al., 1999; Radomska-Pandya et al., 1999).

The presence in the UGT family of genetic polymorphisms

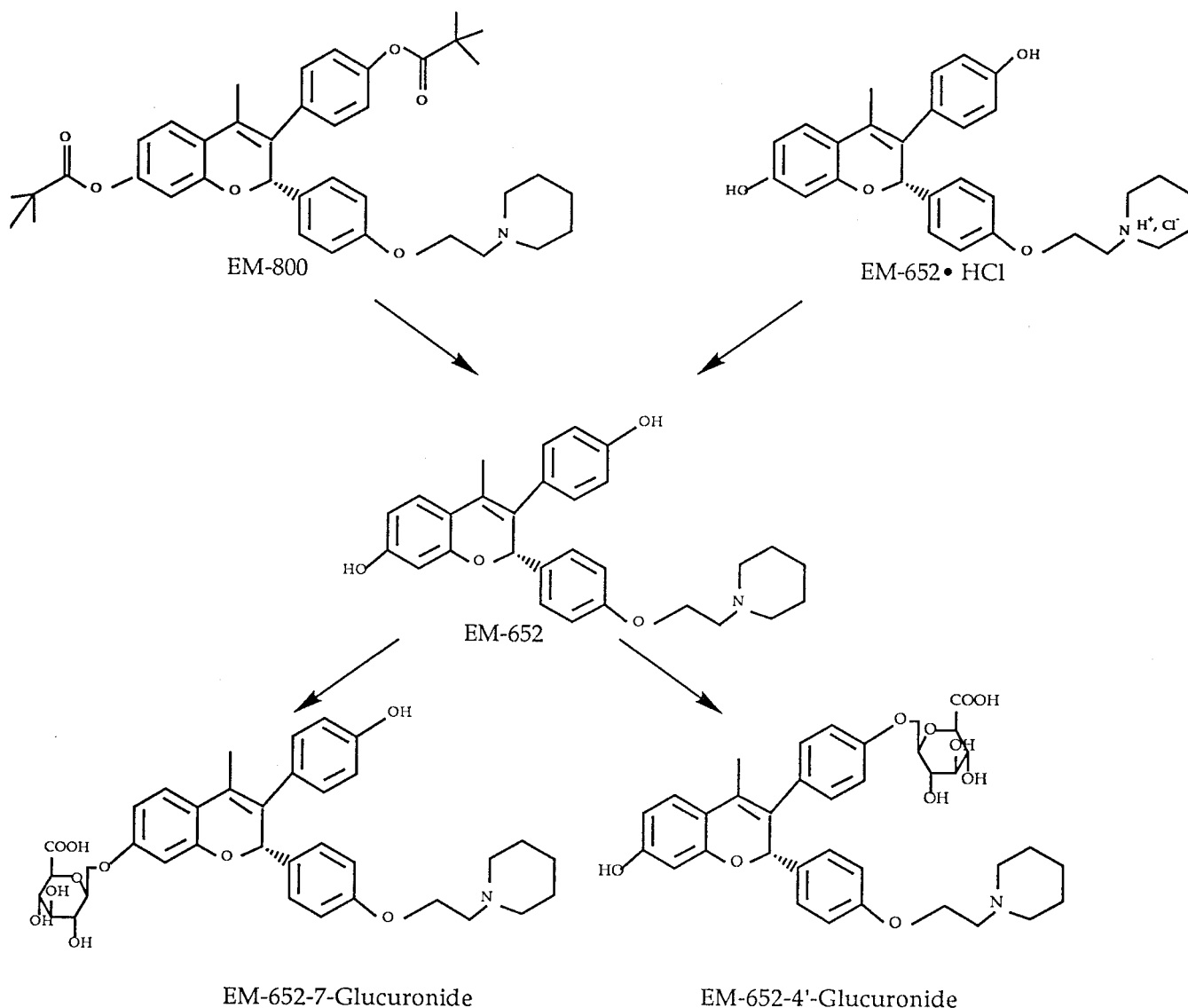


Fig. 1. Chemical structures of EM-800, EM-652-HCl, EM-652, EM-652-4'-glucuronide, and EM-652-7-glucuronide.

that could affect enzyme expression and activity has been described (Jansen, 1996; Lévesque et al., 1999). Thus, determination of the identity of the enzyme(s) involved in drug metabolism is important to further understand variation in drug effect and metabolism. Moreover, a specific UGT isoform could be involved in the metabolism of several xenobiotics; thus, determination of the enzyme involved in the metabolism of a specific drug could provide essential information about potential drug interactions.

The purpose of this study was to determine the identity of the human and monkey UGT enzyme(s) responsible for EM-652 glucuronidation. The *in vitro* metabolism of EM-652 by normal rat, monkey, and human liver microsomes was measured. In addition, human embryonic kidney (HEK) 293 cells expressing specific monkey and human UGT isoforms were evaluated for their reactivity with EM-652.

Experimental Procedures

Materials. UDP-glucuronic acid was obtained from Sigma Chemical Co. (St. Louis, MO). EM-652, EM-800, and EM-652-HCl were synthesized in the Medicinal Chemistry Division of our laboratory. The synthesis of EM-652 and its precursors was described previously (Gauthier et al., 1997). Human embryonic kidney 293 cells were obtained from the American Type Culture Collection (Manassas, VA). Human liver microsome preparations [from 1 pool ($n = 5$) and from eight different donors] were from female subjects and were purchased from the Human Cell Culture Center, Inc. (Laurel, MD).

UGT cDNA Isolation and HEK 293 Cell Stable Expression. The isolation of human and monkey UGT2B and UGT1A cDNAs and their stable expression in HEK 293 cells have been described previously (Jin et al., 1993; Beaulieu et al., 1996, 1997, 1998a,b; Bélanger et al., 1997; Coffman et al., 1997; Lévesque et al., 1997, 1999; Albert et al., 1999; Barbier et al., 1999a,b).

Quantification of EM-652, EM-652-4'-glucuronide and EM-652-7-glucuronide in Plasma Samples of Healthy Woman Volunteers following a Single Oral Dose (20 mg) of EM-652-HCl. Plasma samples were obtained from 18 healthy postmenopausal white women (age range, 43 to 64 years; mean, 54 years). Each patient received a single oral dose of 20 mg of EM-652-HCl in the morning, after breakfast. Plasma samples were collected 3 h after oral administration for subsequent analysis of EM-652, EM-652-4'-glucuronide, and EM-652-7-glucuronide. EM-652 derivatives were extracted from plasma samples by solid phase using C18 cartridges, and the extracts were separated by HPLC followed by quantification with a mass spectrometric detector (API300; PE Sciex, Toronto, Ontario, Canada).

Microsomal Protein Isolation from Rat and Monkey Liver and from UGT-Expressing HEK 293 Cells. Microsomal proteins were extracted from 500 mg of female ($n = 5$) and male ($n = 2$) monkey livers, of female Sprague-Dawley rats ($n = 6$) livers, or from 8×10^6 UGT-expressing HEK 293 cells. Immediately after necropsy, monkey and rat tissues were frozen in liquid nitrogen and kept at -80°C until isolation of microsomes. The UGT-expressing HEK 293 cells were collected and washed in 10 mM Tris-buffered saline containing 0.5 mM dithiothreitol. Then cells were concentrated in homogenization buffer by centrifugation at 14,000g for 5 min and frozen at -80°C until isolation of microsomes. Frozen tissue or cell samples were subsequently homogenized in 0.1 M K_2HPO_4 , 0.1 M KH_2PO_4 , pH 7.4, 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2.5 $\mu\text{g}/\text{ml}$ pepstatin, and 0.5 $\mu\text{g}/\text{ml}$ leupeptin using a potter-glas-col (Terre Haute, IN) type homogenizer with a Teflon pestle. The resulting homogenates were centrifuged at 12,000g, 4°C for 20 min. The supernatant was then centrifuged at 105,000g for 1 h at 4°C . The microsomal pellets were resuspended in homogenization buffer at a concentration of 10 mg of protein/ml and stored at -80°C .

Immunoblot Analysis. To ascertain the level of UGT1A protein expression in human liver, 10 μg of microsomal protein from nine hepatic microsome preparations were separated by 10% SDS-PAGE. The gel was transferred onto a nitrocellulose membrane and probed with the antihuman UGT1A common carboxyl terminus region (amino acids 312 to 351) antiserum RC-71 (1:2000 dilution), as reported previously (Albert et al., 1999). An antirabbit IgG horse antibody conjugated with peroxidase (Amersham Pharmacia Biotech, Oakville, Canada) was used as the second antibody. Resulting immunocomplexes were visualized using an enhanced chemiluminescence kit (Renaissance, Québec, Canada), exposed on hyperfilm for 15 s (Eastman Kodak Co., Rochester, NY), and quantified by BioImage Visage 110s (Genomic Solution Inc., Ann Arbor, MI).

The variable level of UGT protein expression in HEK 293-derived cell lines was determined by immunoblot analysis of microsomal proteins (10 μg) from cells stably expressing human UGT1A1, UGT1A3, UGT1A8, UGT1A9, and monkey monUGT1A01, monUGT1A03, and monUGT1A09, as described above. To ensure that equal amounts of protein from the stably transfected cells were loaded in each lane of the Western blot, the same blot was subsequently probed with an anti-calnexin CT antibody (1:1000 dilution; StressGen Biotech. Corp., Victoria, Canada) as control.

Glucuronidation Assay Using Microsomes Extracted from Tissues and UGT-HEK 293 Cells. EM-652 glucuronidation by rat, monkey, and human tissues, and by stably expressed human and monkey UGT enzymes, was assayed in the presence of 500 μM unlabeled UDPGA, 200 μM EM-652, and 40 μg of microsomal proteins in 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , 100 $\mu\text{g}/\text{ml}$ phosphatidylcholine, and 8.5 mM saccharolactone in a final volume of 100 μl . Reaction mixtures were incubated for 16 h at 37°C , and assays were terminated by the addition of methanol (100 μl). Samples were centrifuged at 13,000 g for two min to remove precipitated proteins and the supernatants were analyzed by a liquid chromatographic tandem mass spectrometric (LC-MS/MS) method. All preparations that demonstrated reactivity with EM-652 in this screening assay were subsequently reassayed in the same mix containing 500 μM EM-652 and 2 mM UDPGA, for 3 h at 37°C . Time-course experiments have demonstrated that under these saturating conditions, the reaction is linear for 4 h (data not shown). Glucuronidation activity was determined from three independent experiments.

Kinetic Analysis Using Microsomal Proteins. Kinetic analyses were performed for 3 h using microsomal protein (40 μg) from HEK 293 cells stably expressing UGT1A1, UGT1A3, UGT1A9, monUGT1A01, and monUGT1A09. The same conditions as for the glucuronidation assay with 2 mM UDPGA were used with EM-652 concentrations that ranged from 25 to 1000 μM . The values obtained by double reciprocal plots (Lineweaver-Burk) represent the mean of two independent experiments.

Results

Plasma Concentrations of EM-652 and its Metabolites in Healthy Human Volunteers. Each of a group of 18 postmenopausal women received a single oral dose of 20 mg of EM-652-HCl, and the plasma concentration in EM-652, EM-652-4'-glucuronide, and EM-652-7-glucuronide were determined. The main circulating metabolite detected was EM-652-7-glucuronide, whereas only minor amounts of EM-652-4'-glucuronide were measured (Fig. 2). Three hours after the single oral administration of EM-652-HCl (20 mg), circulating level of EM-652-4'-glucuronide, and EM-652-7-glucuronide were 0.95 ± 0.25 and 24.6 ± 3.4 ng/ml, respectively. Plasma concentration of EM-652 was 5.9 ± 0.8 ng/ml.

Glucuronidation of EM-652 by Human, Monkey and Rat Liver Microsomes. EM-652 was incubated with UDPGA (2 mM) and microsomal proteins isolated from liver of

female rat, male and female cynomolgus monkeys, and from female human donors. The incubation products were analyzed by LC-MS/MS and identified using EM-652, EM-652-4'-glucuronide, and EM-652-7-glucuronide standards (Fig. 3). The assays demonstrated that rat, monkey, and human liver microsomal proteins conjugated EM-652 into the two EM-652-monoglucuronides detected in vivo (Fig. 4). Other conjugation products (such as sulfono-conjugates) were not detected (data not shown). Interestingly, the pattern of EM-652 glucuronidation was similar in all three species with a predominant formation of EM-652-7-glucuronide. EM-652-monoglucuronide formation was higher in rat and monkey liver compared with human liver (Fig. 4). In addition, EM-652 glucuronidation was analyzed in monkey liver using 40 μg of hepatic microsomal proteins isolated from female ($n = 5$) and male ($n = 2$) cynomolgus monkeys. As shown in Fig. 4, both preparations had similar glucuronidation rates for either the 4'- and 7-hydroxyposition of the molecule.

Further characterization of the in vitro glucuronidation of EM-652 by human liver was conducted to analyze a possible interindividual variation of the conjugation process. The formation of EM-652-glucuronide was measured after incubation with nine hepatic microsome preparations, including a pool (from five different subjects) and microsomes isolated from eight different female human donors (Fig. 5A; Table 1). EM-652-glucuronide formation varied from 9.9 to 36.9 pmol/min/mg of protein for EM-652-4'-glucuronide and from 41.6 to 154.9 pmol/min/mg of protein for EM-652-7-glucuronide. Immunoblot analysis showed that the interindividual variation of EM-652 glucuronidation by hepatic microsome samples reflected the variation of their level of UGT1A protein expression (Fig. 5, B and C). For example, liver microsomes from patient 7, which exhibited the highest EM-652 glucuronidation activity, corresponded to the microsomal preparation that had the highest relative expression of UGT1A compared with the pool, which served as a reference (relative expression = 1). As observed with monkey liver, it is of interest to note that all human liver microsomes conjugated EM-652 predominantly at the 7-hydroxyl position (Table 1).

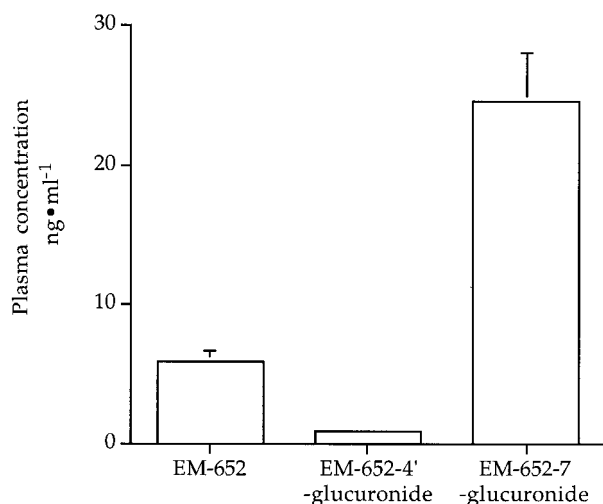


Fig. 2. Human plasma levels of EM-652 derivatives detected by LC-MS/MS. Plasma collected 3 h after a 20-mg oral dose of EM-652-HCl to 18 healthy postmenopausal women was analyzed for EM-652 and its glucuronide metabolites. Results represent the mean (ng/ml) of EM-652, EM-652-4'-glucuronide and EM-652-7-glucuronide \pm S.E.M.

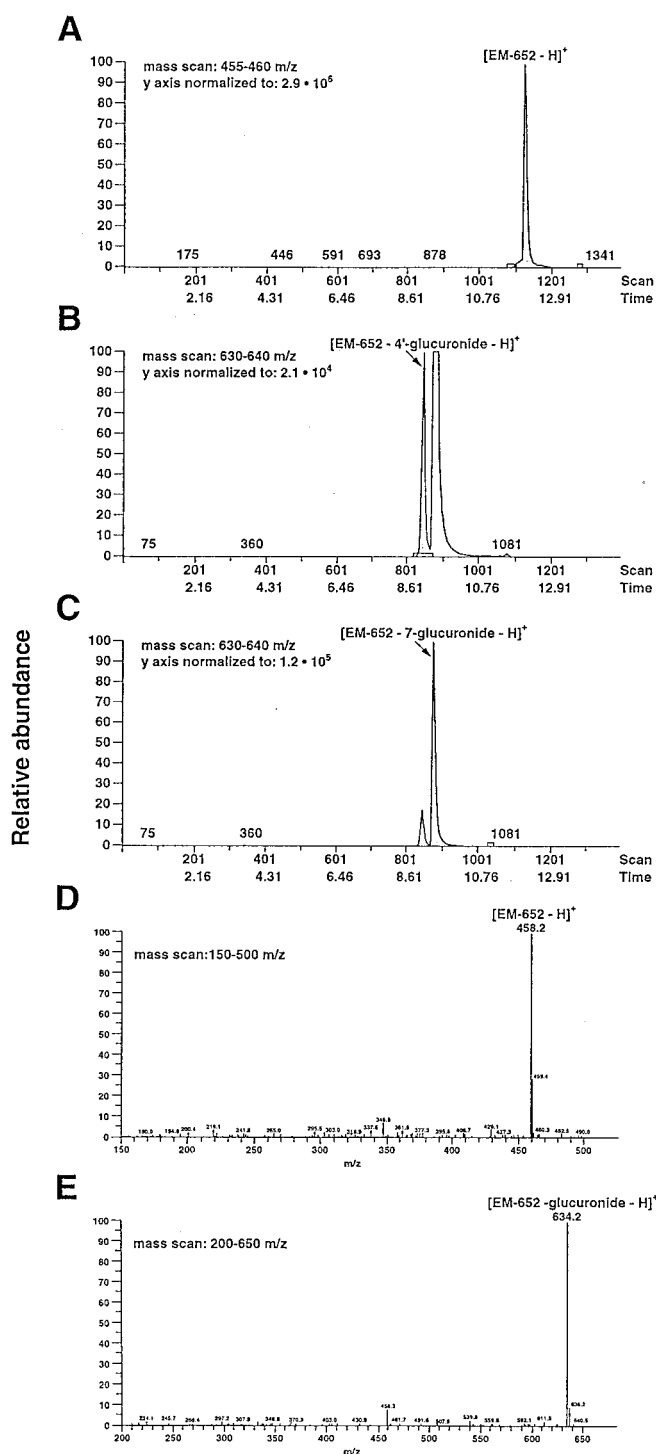


Fig. 3. LC-MS/MS spectra of EM-652, EM-652-4'-glucuronide and EM-652-7-glucuronide after incubation with human liver microsomal proteins. Microsomal protein (40 μg) from a pool of female human liver samples were incubated with 500 μM EM-652 and 2 mM UDPGA for 3 h at 37°C. The incubation products were analyzed by a liquid chromatographic tandem mass spectrometric (LC-MS/MS) method. A scan ranging between 455 and 460 m/z was performed to detect EM-652 (A), and additional scans from 630 to 640 m/z were performed to specifically detect the two EM-652-monoglucuronides (B and C). The molecular masses of the compounds detected in the scans were determined by mass spectrometry for EM-652 (D) and EM-652-glucuronide (E). Identification of the two monoglucuronides was obtained by comparing retention times of incubation products to those of EM-652-4'-glucuronide and EM-652-7-glucuronide standards. The y-axis represents the abundance of each compound, relative to the highest peak value set at 100.

Glucuronidation of EM-652 by Human and Monkey UGT Enzymes. To determine which human and cynomolgus monkey UGT enzymes are involved in EM-652 metabolism, glucuroconjugation assays were performed by incubating EM-652 with microsomal proteins extracted from HEK 293 cells that stably express human or monkey UGT enzymes. HEK 293 cells are devoid of endogenous UGT activity as demonstrated by incubation of EM-652 with microsomal proteins from untransfected HEK 293 cells, which did not produce EM-652-glucuronide (Fig. 6).

LC-MS/MS analysis demonstrated that several human and monkey UGTs could transform EM-652 into the two EM-652-monoglucuronides previously detected in vivo (Fig. 6). Human UGT1A1, UGT1A8, and UGT1A9, as well as their related monkey orthologs monUGT1A01, monUGT1A08, and monUGT1A09, catalyzed the glucuronidation of EM-652. Human UGT1A3, but not monUGT1A03, also converted EM-652 to EM-652-glucuronide. The relative amounts of EM-652-4'-glucuronide and EM-652-7-glucuronide produced by human UGT1A1 most closely matched the amounts of these metabolites detected in vivo, whereas human UGT1A3, UGT1A8, and UGT1A9 produced roughly equivalent amounts of the two monoglucuronides. In monkeys, monUGT1A09 produced EM-652-4'-glucuronide and EM-652-7-glucuronide in proportions similar to those observed in vivo, whereas monUGT1A01 and monUGT1A08 produced equivalent amounts of EM-652-4'-glucuronide and EM-652-7-glucuronide (Fig. 6). Interestingly, monkey UGT2B9 and UGT2B18 displayed an appreciable level of EM-652 conjugating activity, producing preferentially EM-652-4'-glucuronide over EM-652-7-glucuronide. However, none of the human UGT2B proteins tested produced EM-652-glucuronide. These experiments suggested that UGT1 enzymes are probably involved in the glucuronidation of EM-652 in vivo.

To verify if the difference observed in EM-652-glucuronida-

tion activity values with the UGT1 enzymes tested reflected differences in UGT protein levels, the microsomal protein extracts used in these experiments were size-separated by SDS-PAGE and UGT1 enzymes were detected using an antibody that recognizes all UGT1 enzymes (Fig. 7). Quantification of immunoreactive UGT1 proteins was determined as follows: staining obtained with the anti-UGT antibody (Fig. 7A) was normalized using the corresponding signal obtained with the anti-calnexin antibody (Fig. 7B). Then, the ratios of UGT/calnexin were compared in each stable cell line, using human UGT1A1 and monkey monUGT1A01 ratios as reference values (ratio = 1) (Fig. 7C). It was found that the microsomal extracts from human UGT1A8- and UGT1A9-expressing HEK 293 cells contained approximately 15 times more UGT proteins than the extracts from human UGT1A1- and UGT1A3-expressing HEK 293 cells (Fig. 7C). The EM-652-glucuronidation activity determined after 3 h of incubation was therefore corrected for the amount of protein contained in each extract. As shown in Table 2, UGT1A3 displayed the highest level of EM-652-conjugation activity, followed by UGT1A1. EM-652 glucuronidation activity of UGT1A8 and UGT1A9 appears minor compared with UGT1A1 and UGT1A3 activities. Similarly, microsomal extracts from monkey monUGT1A08- and monUGT1A09-expressing HEK 293 cells contained approximately 15 times more UGT proteins than the extracts from monUGT1A01-expressing HEK 293 cells (Fig. 7). After normalization, monUGT1A09 displayed the highest level of EM-652 conjugation activity, followed by monUGT1A01 and monUGT1A08 (Table 2).

To further characterize EM-652 glucuronidation activity by stably expressed human and monkey UGT1A enzymes, kinetic analyses were performed using microsomes from UGT1A1-, UGT1A3-, UGT1A9-, monUGT1A01-, and monUGT1A09-HEK 293 cells in the presence of EM-652 concentrations varying from

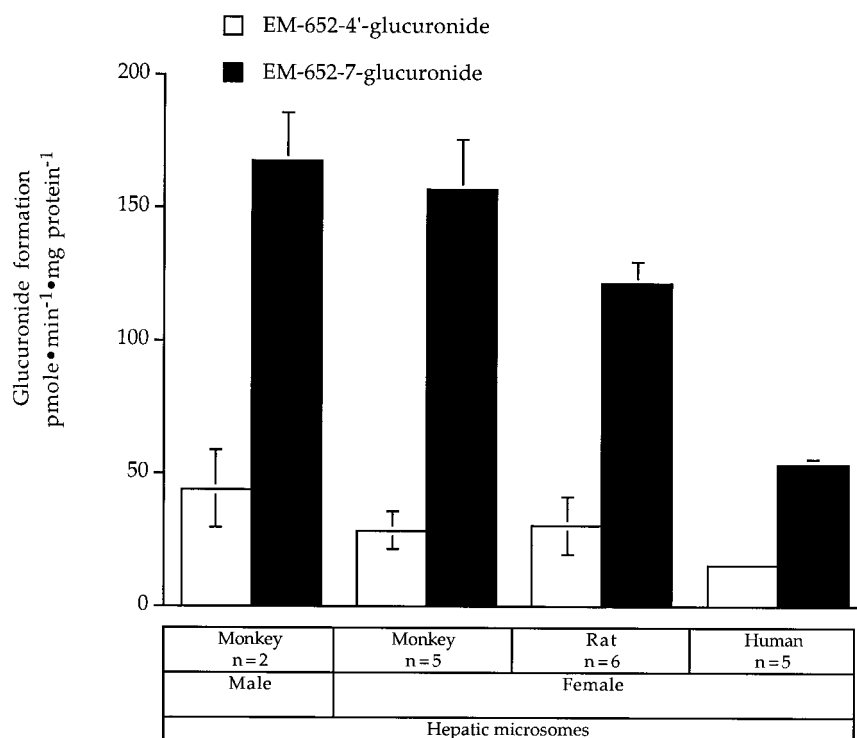


Fig. 4. Conversion of EM-652 to EM-652-4'-glucuronide and EM-652-7-glucuronide by rat (female), monkey (male and female), and human (female) liver. To detect the formation of EM-652 glucuronide metabolites by female rat liver, female and male monkey liver, and female human liver, 40 μ g of microsomal proteins extracted from these tissues were incubated in presence of UDPGA (2 mM) and EM-652 (500 μ M) for 3 h at 37°C. Reactions were stopped by adding methanol, and products were analyzed using a LC-MS/MS method. Values represent the mean of two independent experiments \pm S.D.

25 μM to 1000 μM (Table 3). In humans, UGT1A3 presented the highest affinity for both the 4'- and 7-hydroxypositions, with apparent K_m values of 36 and 19 μM , respectively. Interestingly, UGT1A1 and UGT1A9 displayed similar apparent K_m values for EM-652-4'- and EM-652-7-glucuronidation. In monkey, monUGT1A01 presented lower affinities for both hydroxyl positions of EM-652 than its human ortholog, and apparent K_m values of monUGT1A09 were similar to those of human UGT1A1 and UGT1A9 (Table 3).

The V_{max} for EM-652 glucuronidation activity determined during kinetic experiments was corrected for the amount of protein contained in each extract. The normalized V_{max} was used to determine the efficiency of glucuronidation (ratio V_{max}/K_m) (Table 3). In humans, UGT1A3 displayed the highest efficiencies of EM-652-conjugation for both hydroxyl positions, followed by UGT1A1. EM-652 glucuronidation effi-

ciency values for UGT1A9 and monUGT1A01 seem very low compared with UGT1A1, UGT1A3, and monUGT1A09 efficiencies. UGT1A1 and monUGT1A09 had 11.4- and 10-fold higher V_{max}/K_m ratio values for EM-652-7-glucuronide compared with EM-652-4'-glucuronide, respectively; however, UGT1A3, UGT1A9, and monUGT1A01 presented equal glucuronidation efficiencies for the two hydroxyl positions of EM-652.

Discussion

The present data identify the UGT enzymes responsible for the metabolism of EM-652, the active metabolite of EM-800 and EM-652-HCl. The *in vitro* glucuronidation of this drug was demonstrated by incubation with rat, monkey and human liver microsomes, and screening of UGT enzymes indicated that members of the UGT1A subfamily are involved in the metabolism of this antiestrogen.

In human volunteers, EM-652 is rapidly metabolized into more polar glucuronide products. Three hours after oral administration, approximately 20% of the circulating EM-652 derived material was EM-652 whereas the primary EM-652 metabolite, the 7-glucuronide, accounted for roughly 78%. Only trace amounts of the other monoglucuronide metabolite, EM-652-4'-glucuronide, were detected. In agreement with these observations, *in vitro* analysis showed that rat, monkey, and human liver microsomes conjugated EM-652 to two metabolites: EM-652-7-glucuronide and EM-652-4'-glucuronide. Rat and monkey microsomes displayed a higher level of glucuronidation than in the human. The similarity of EM-652 metabolism by monkey and human liver is consistent with previous phase I and phase II hepatic enzyme studies, which demonstrated only minor differences between cynomolgus monkey and human drug metabolism (Sharer et al., 1995).

The similarity of the *in vitro* pattern of EM-652 glucuronidation between rat and human liver microsomes was surprising because comparison of other drug glucuronidation in rat and human has demonstrated significant differences between the two species (Pless et al., 1999). For example, glucuronidation of the new antithrombotic thioxyloside occurred only on the 2-hydroxyl position when incubated with human liver microsomes, although rat microsomes were able to form glucuronide conjugates on either the 2-, 3-, or 4-hydroxyl position of the molecule (Pless et al., 1999). Analysis of enzymes involved in thioxyloside glucuronidation demonstrated that UGT1A and UGT2B proteins of both species can catalyze the glucuronidation of this drug (Pless et al., 1999). It has been well described that glucuronidation by UGT2B enzymes is significantly different between rat and human, as illustrated by the absence of circulating androgen-glucuronides in rat plasma (Guillemette et al., 1996).

In cynomolgus monkey, hepatic glucuronidation of EM-652 was independent of the gender of the animals. Although several studies have demonstrated that glucuronidation of endogenous substrates could be affected by the sex of the individual organisms (Pacifci et al., 1997; Albert et al., 2000), our results are consistent with observations concerning the disposition of exogenous substrates of UGT1A enzymes. For example, it was shown that race and sex had no effect on the plasma availability of SN-38-glucuronide, the

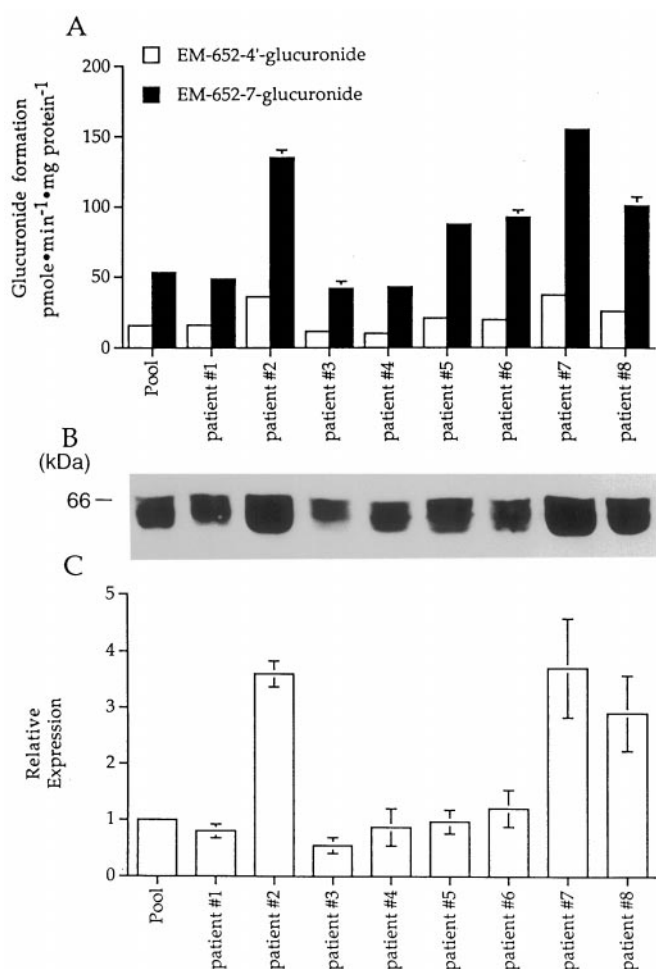


Fig. 5. Comparison of EM-652-glucuronide formation by a pool of human hepatic microsomes and eight different human liver microsomal preparations (A); expression of UGT1A proteins in these microsomal preparations (B); and relative expression of UGT1A enzymes in each preparation (C). The formation of EM-652 glucuronide metabolites by all liver microsomes was determined using 40 μg of microsomal proteins in the presence of UDPGA (2 mM) and EM-652 (500 μM) for 3 h at 37°C. Glucuronide derivatives of EM-652 were detected and quantified by LC-MS/MS. To evaluate the difference of UGT1A protein expression, 10 μg of these microsomal proteins were analyzed by Western blot experiments using the anti-UGT1A RC-71 antibody. Immunocomplexes detected by Western blot were quantified using a BioImage Visage 110s from Genomic Solution Inc., and the relative expression was calculated using the pool of microsomes as a reference value (relative expression = 1). Values represent the mean of two independent experiments \pm S.D.

metabolite of the topoisomerase inhibitor, irinotecan (Gupta et al., 1997).

It is well recognized that glucuronidation activity varies largely between individual animals (Little et al., 1999). In accordance with this fact, the present study demonstrates an interindividual variability in women hepatic glucuronidation of EM-652. It is of interest to note that the variation was correlated with the level of UGT1A protein expression in the human microsomal samples.

Determination of the specific isoforms involved in EM-652 glucuronidation showed that both human and monkey UGT1A1 (1A01), UGT1A8 (1A08), and UGT1A9 (1A09) and human UGT1A3 enzymes can catalyze the transfer of glucuronic acid to hydroxyl groups of the EM-652 molecule. Although UGT1A proteins contain the same carboxyl-terminal half, each enzyme possesses its own substrate specificity, which is associated with the variable amino-terminal half of the protein. For instance, the UGT1A1 enzyme was extensively studied for its important role in the metabolism of bilirubin; however, this enzyme is also known to be involved in the glucuronidation of phenolic compounds, estrogens, oripavine opioids, coumarins, flavonoids, retinoids, and anthraquinones (Senafi et al., 1994; King et al., 1996). UGT1A3 and UGT1A4 are important catabolic enzymes in the glucuronidation of tertiary amines to quaternary ammonium-linked glucuronides and of secondary and primary amines (Ebner and Burchell, 1993; Green et al., 1995). Expressed UGT1A3 catalyzes the glucuronidation of estrone, 2-hydroxyestrone, hydroxylated benzo[*a*]pyrene metabolites and 2-acetylaminofluorene metabolites (Green et al., 1998). Human UGT1A6 preferentially conjugates planar phenols, whereas UGT1A9 catalyzes the glucuronidation of bulky phenols, anthraquinones, flavonoids, certain aliphatic alcohols, nonsteroidal anti-inflammatory drugs, and catecholestrogens (Ebner and Burchell, 1993; Albert et al., 1999). Extrahepatic human UGT1A8 has been shown to glucuronidate phenolic compounds and benzo[*a*]pyrene (Mojarrabi and Mackenzie, 1998).

The present data show that EM-652 glucuronidation is catalyzed by enzymes that possess different substrate specificities. However, previous studies have shown an overlap of these specificities; opioid glucuronidation by stably expressed UGT1A3 is similar to that observed for human UGT1A1 (King et al., 1996). Both enzymes catalyze the glucuronidation of oripavine opioids with a higher efficiency than for morphine-derived opioids. In contrast, EM-652 glucuronidation is catalyzed by human UGT1A3 but not by UGT1A4, although the two proteins are 93% identical in amino acid sequence. In addition to EM-652 glucuronidation, some differences between these two enzymes, in terms of substrate specificity, have been shown (Green et al., 1998). Interestingly, the pattern of EM-652 glucuronidation by the four human and three monkey UGT1A isoforms demonstrates an important level of stereospecificity of UGT enzymes.

Of the four human UGT enzymes that were shown to catalyze the glucuronidation of EM-652, the UGT1A3 enzyme presents the highest normalized EM-652 glucuronidation activity (Table 2). In contrast, the 4-fold higher amount of EM-652-7-glucuronide versus EM-652-4'-glucuronide produced with human liver microsomes, and the similar pattern of glucuronide metabolites measured in human plasma, indicate that UGT1A1, which also preferentially glucuronidated the 7-hydroxy position, could be the major enzyme involved in the hepatic metabolism of EM-652 in vivo. However, comparison of normalized 7- versus 4'-glucuronidation efficiencies (ratio V_{max}/K_m) might be a better indicator of the UGT isoenzymes involved in EM-652 conjugation in vivo. It is interesting that, in humans, only UGT1A1 shows a 11.4-fold higher efficiency for EM-652-7-glucuronidation, compared with EM-652-4'-glucuronide. Indeed, human UGT1A9 presents almost equal efficiency values for EM-652-4'- and EM-652-7-glucuronidation. This suggests that it has a minor role in in vivo glucuronidation of EM-652. These results reinforce the role of UGT1A1 as the major UGT enzyme involved in EM-652 glucuronidation.

Although human and monkey liver displayed similar pat-

TABLE 1

Summary of female liver donor demographics and EM-652 glucuronidation activity.

The level of EM-652 glucuronidation by female liver was determined for each hydroxyl position of the molecule by incubating microsomal proteins of liver samples (40 mg) and EM-652 (500 mM) for 3 h in presence of UDP-glucuronic acid (2 mM). EM-652-4'-glucuronide (EM-652-4'-G) and EM-652-7-glucuronide (EM-652-7-G) formation was detected and quantified using LC/MS-MS analysis. Values represent the mean of two independent experiments \pm S.D.

Donor	Age	Smoker	Alcohol Consumption	Medicine	Glucuronide formation		Ratio EM-652-7-G/ EM-652-4'-G
					EM-652-4'-G	EM-652-7-G	
<i>pmol/min/mg of protein</i>							
Pool (<i>n</i> = 5)	N.D.	N.D.	N.D.	N.D.	15.6 \pm 0.3	53.0 \pm 2.2	3.4
Patient 1	59	No	Yes	Nifedipine Diabetes	15.8 \pm 0.3	48.4 \pm 1.5	3
Patient 2	65	Yes	No	Glibenclamide, warfarin Diabetes	36.0 \pm 2.3	134.9 \pm 5.4	3.7
Patient 3	52	Yes	No	Insulin, Paroxetine, Buspirone Diabetes	11.5 \pm 1.5	41.6 \pm 4.9	3.6
Patient 4	44	Yes	No	Insulin, sumatriptan succinate, isometheptine mucate/ dichlovalphenazome/acetaminophen, Sulax	9.9 \pm 0.1	42.6 \pm 1.2	4.3
Patient 5	42	No	No	Clodine	20.9 \pm 0.5	86.9 \pm 1.1	4.2
Patient 6	49	No	No	Conjugated estrogen, Oxybutynin, Trazodone	19.6 \pm 1.0	91.8 \pm 5.1	4.7
Patient 7	34	Yes	No	Anorexia Lanotil, Diazepam	36.9 \pm 1.6	154.9 \pm 4.9	4.2
Patient 8	53	No	No	Estrogen	25.7 \pm 1.5	100.2 \pm 6.5	3.9

N.D., not determined.

terms of EM-652-7-glucuronide versus EM-652-4'-glucuronide, kinetic studies demonstrated that human and monkey orthologs UGT1A1/monUGT1A01 and UGT1A9/monUGT1A09 had different efficiency ratios of 7:4' glucuronidation (Table 3). Although the efficiencies of EM-652-4' and -7-glucuronidation by monUGT1A01 are very low, those of monUGT1A09 are close to those of human UGT1A1. These results indicate that in monkey, monUGT1A09 plays a major role in hepatic glucuronidation of EM-652, thus suggesting the presence of a species difference in the isoforms responsible of EM-652 glucuronidation. In addition, the appreciable glucuronidation activity of monkey UGT2B enzymes and the absence of EM-652 conversion by human UGT2B reinforces this difference between human and monkey. Interestingly, comparison of human UGT1A9 and monkey monUGT1A09 substrate specificity demonstrates that these two proteins catalyze similar endogenous and exogenous conjugations, whereas only minor differences between the two orthologs were observed (Albert et al., 1999). Similar results were observed for the conjugation pattern of other drugs; for example, rat UGT1A6 catalyzes glucuronidation of thioxyloside, whereas human UGT1A6 is not reactive (Pless et al., 1999). In human, the glucuronidation of the same drug is catalyzed by UGT1A9 and UGT2B4 (Pless et al., 1999).

Kinetic studies performed with microsomal proteins revealed that all human and monkey UGT1A enzymes have a low affinity for EM-652, yielding apparent K_m values ranging from 19 to 302 μM (Table 3). MonUGT1A09 had a K_m value similar to those of human UGT1A1 and UGT1A9, whereas human UGT1A3 had a lower Michaelis constant. In addition

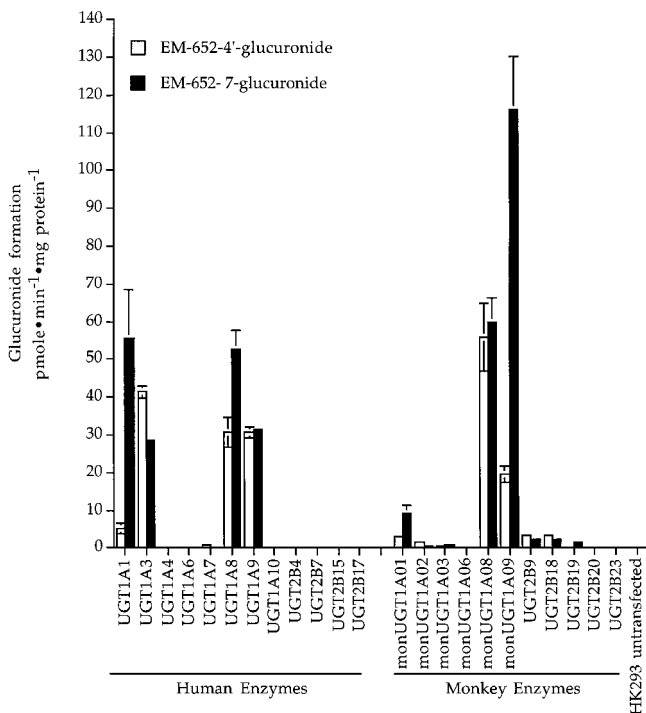


Fig. 6. Conversion of EM-652 to EM-652-4'-glucuronide and EM-652-7-glucuronide by microsomal proteins from HEK 293 cells stably expressing human and monkey UGT proteins. To determine which UGT isoenzyme was involved in EM-652 glucuronidation, microsomal proteins (40 μg) of untransfected HEK 293 cells and of HEK 293 cells stably expressing human and monkey UGT enzymes were incubated in presence of UDPGA (2 mM) and EM-652 (500 μM) for 3 h at 37°C. Enzymatic reactions were stopped by adding methanol, and products were analyzed by LC-MS/MS. Values represent the mean of three independent experiments \pm S.D..

to the exogenous compounds described above, these EM-652 conjugating UGT enzymes are involved in the glucuronidation of endogenous C18-steroids. For example, in human, estradiol-glucuronide is formed by both UGT1A1, UGT1A3 and UGT1A9, and estrone is glucuronidated by UGT1A1 and UGT1A9 (King et al., 1996; Albert et al., 1999; Gall et al., 1999). Nevertheless, the affinities of UGT1A isoforms for endogenous estrogens is in the micromolar range (up to 10-fold higher compared with affinity for EM-652) making improbable an interference between the glucuronidation of EM-652 and C18-steroids by a specific isoform (Albert et al., 1999).

Specific tissue distribution analysis of human UGT1A1 and UGT1A3 messenger RNAs demonstrated that both enzymes are expressed in various tissues, including the liver, stomach, bile duct, jejunum, and colon (Strassburg et al., 1998). Such data suggest that EM-652 glucuronidation could also occur in a wide variety of extrahepatic tissues. Interestingly, a recent study showed that UGT1A3 mRNA was expressed in jejunum from five patients, whereas UGT1A1

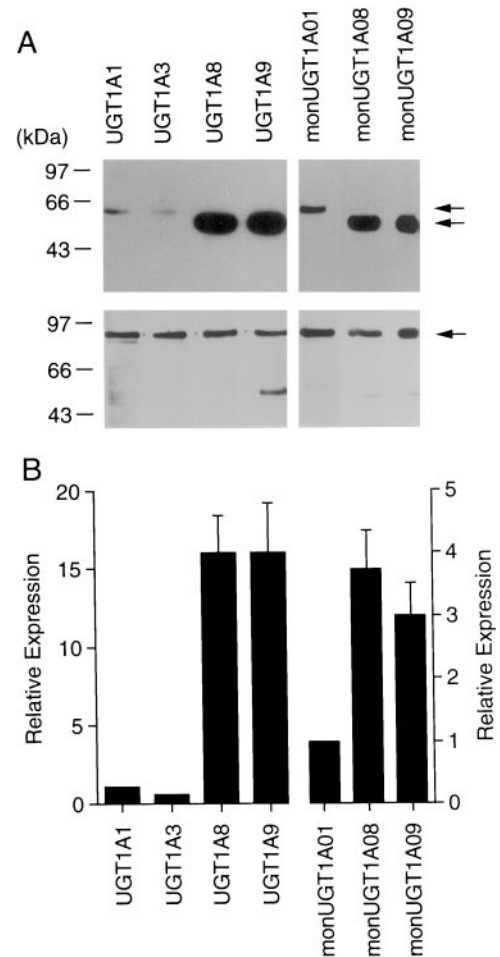


Fig. 7. Differential expression of UGT proteins in UGT1A-HEK 293 cell lines. A, microsomal proteins (10 μg) from UGT1A1, UGT1A3, UGT1A8, UGT1A9, and monkey monUGT1A01, monUGT1A08, monUGT1A09-expressing HEK 293 cells were separated by 10% SDS-PAGE, transferred onto nitrocellulose membrane and probed with the anti-UGT1A RC-71 antisera. The blot shown in A was subsequently probed with a polyclonal anti-calnexin antibody. B, relative expression levels determined after quantification using BioImage Visage 110s from Genomic Solution Inc. Values represent the mean of three independent blots \pm S.D.

TABLE 2

EM-652 glucuronidation by human UGT1A1, UGT1A3, UGT1A8, and UGT1A9 and by monkey monUGT1A01, monUGT1A08, and monUGT1A09 before and after normalization for level of protein expression.

The level of EM-652 glucuronidation by UGT enzymes was determined for each hydroxyl position of the molecule by incubating microsomal proteins of UGT-HEK293 cells and EM-652 for 3 h. Values were normalized for the level of protein expression in stably expressing HEK293 cells. Expression of protein in UGT1A1- and monUGT1A01-HEK293 cells defined as reference values (expression = 1). Values represent the mean of three independent experiments \pm S.D.

	Glucuronide Formation					
	EM-652-4'-G	Normalized	EM-652-7-G	Normalized	EM-652-G Total	Normalized
	<i>pmol/min/mg of protein</i>					
Human						
UGT1A1	5.3 \pm 1.5	5.3	55.7 \pm 12.7	55.7	61.0 \pm 14.2	61.0
UGT1A3	41.4 \pm 1.5	82.8	28.6 \pm 2.1	57.2	70.0 \pm 3.6	140.0
UGT1A8	30.7 \pm 3.9	1.9	52.8 \pm 3.9	3.3	83.5 \pm 7.8	5.2
UGT1A9	30.8 \pm 13.5	1.9	31.3 \pm 0.7	1.9	62.1 \pm 14.2	3.8
Monkey						
UGT1A01	3.2 \pm 0.2	3.2	9.0 \pm 2.2	9.0	12.2 \pm 2.4	12.2
UGT1A08	55.9 \pm 9.0	14.9	59.8 \pm 6.4	16	115.7 \pm 15.4	6.6
UGT1A09	19.7 \pm 2.2	15.5	116.2 \pm 14.2	78.0	135.9 \pm 16.4	93.5

TABLE 3

Kinetic parameters of EM-652 glucuronidation by microsomal proteins from stably expressed human UGT1A1, UGT1A3, UGT1A9, and monkey monUGT1A01 and monUGT1A09.

Kinetic studies were performed for 3 h using microsomal proteins from HEK293 cells stably expressing human and monkey UGT1A proteins (40 mg) in the presence of UDPGA (2 mM) and with EM-652 concentrations ranging from 25 to 1000 nM. Values obtained by double reciprocal plots (Lineweaver-Burk) represent the mean of two independent experiments \pm S.D. EM-652-4'-G and EM-652-7-G glucuronide (EM-652-7-G) formation was quantified using LC/MS-MS. V_{max} values were normalized for the variable level of UGT1A protein expression in stably expressing HEK293 cells (reference value determined for expression in UGT1A1- and monUGT1A01-HEK293 cells). Each normalized V_{max} value was used to determine the glucuronidation efficiency (ratio V_{max}/K_m).

		K_m (app.)	V_{max}	Normalized V_{max}	V_{max}/K_m
		μM	<i>pmol/min/mg of protein</i>		<i>$\mu l/min/mg of protein$</i>
Human					
UGT1A1	EM-652-4'-G	168 \pm 45	14.5 \pm 4.0	14.5	0.08
	EM-652-7-G	63 \pm 8	59.5 \pm 7.4	59.5	0.95
UGT1A3	EM-652-4'-G	36 \pm 3	33.3 \pm 3.0	47.6	1.31
	EM-652-7-G	19 \pm 4	21.1 \pm 4.4	30.2	1.58
UGT1A9	EM-652-4'-G	172 \pm 31	52.2 \pm 9.4	3.0	0.01
	EM-652-7-G	64 \pm 23	30.8 \pm 8.3	1.8	0.03
Monkey					
monUGT1A01	EM-652-4'-G	302 \pm 36	16.6 \pm 2.0	16.6	0.05
	EM-652-7-G	233 \pm 58	27.7 \pm 6.9	27.7	0.12
monUGT1A09	EM-652-4'-G	170 \pm 59	39.8 \pm 14.0	13.2	0.08
	EM-652-7-G	69 \pm 7	166.6 \pm 18.3	55.5	0.80

mRNA was found in only one of these tissues (Strassburg et al., 2000). In contrast, all human livers analyzed ($n = 16$) expressed both messengers (Strassburg et al., 2000). Thus, these data suggest that UGT1A3 could play an important role in intestinal conjugation of EM-652, whereas UGT1A1 would be a better UGT enzyme for the hepatic glucuronidation of this antiestrogen. Further investigation of the in vitro glucuronidation of EM-652 by human intestinal microsomes remains necessary to confirm this hypothesis.

Several mutations of the UGT1A gene are associated with pathologies such as the Crigler-Najjar Type I and II and Gilbert's syndromes. In Crigler-Najjar disease, mutations are located in exon 1 of UGT1A1 or in one of the common exons, whereas Gilbert's syndrome is an autosomal recessive disease that results from an abnormality in the promoter region at the 5' end of the UGT1A1 gene (Jansen, 1996). In all these pathologies, levels of serum bilirubin are elevated and the hepatic bilirubin UGT activity is decreased, but in Crigler-Najjar type II and Gilbert's syndromes, phenobarbital treatment lowers serum bilirubin (Jansen, 1996). The apparent physiological implication of the UGT1A1 enzyme in hepatic EM-652 glucuronidation implies a genetic predisposition to a lower metabolism of this drug by patients presenting low bilirubin UGT activity. In addition to phenobarbital, several drugs such as oltipraz and 3-methylcholanthrene can induce

the expression of UGT1A1 (Emi et al., 1996; Ritter et al., 1999). The expression of UGT1A1 mRNA was significantly more expressed in cases of hepatocellular carcinoma (Emi et al., 1996; Strassburg et al., 1997; Ritter et al., 1999). All these data indicate that metabolism of EM-652 could be significantly affected in patients with Gilbert's syndrome or with hepatocarcinoma, or in patients treated with drugs that increase the expression of UGT1A proteins. In addition, UGT1A enzymes are implicated in the metabolism of a wide variety of drugs and exogenous compounds; thus, the pharmacokinetic properties of EM-652 metabolism could be affected by potentially competing drugs. Although such possibilities remain to be tested, a defect of UGT1A1 activity could be compensated by UGT1A3 glucuronidation of EM-652.

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