Correlation between the UDP-Glucuronosyltransferase (*UGT1A1*) TATAA Box Polymorphism and Carcinogen Detoxification Phenotype: Significantly Decreased Glucuronidating Activity against Benzo(*a*)pyrene-7,8-dihydrodiol(−) in Liver Microsomes from Subjects with the *UGT1A1*28* Variant

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Abstract

Of the hepatic UDP-glucuronosyltransferases (UGTs), only UGT1A1 and UGT1A9 exhibit activity against benzo(*a*)pyrene-*trans*-7*R*,8*R*-dihydrodiol [BPD(−)], precursor to the highly mutagenic *anti*-(+)-benzo(*a*)pyrene-7*R*,8*S*-dihydrodiol-9*S*,10*R*-epoxide. The *UGT1A1*28* allelic variant contains an additional (TA) dinucleotide repeat in the "TATAA" box [$(TA)_6$>$(TA)_7$] of the *UGT1A1* promoter that has been linked to decreased expression of the *UGT1A1* gene and decreased bilirubin conjugation, leading to the relatively nondebilitating condition known as Gilbert's syndrome. To determine whether the *UGT1A1* TATAA box polymorphism may play a role in the overall glucuronidation of BPD(−) in humans, we compared *UGT1A1* TATAA box genotype with BPD(−) glucuronidating activity in normal liver microsomes. Significant decreases in UGT1A1 protein ($P < 0.005$) and bilirubin conjugation activity ($P < 0.001$) were observed in liver microsomes from subjects homozygous for the *UGT1A1*28* allelic variant compared with subjects homozygous for the wild-type *UGT1A1*1* allele. Significant decreases in BPD(−) glucuronidation activity ($P < 0.02$) were observed in subjects with the *UGT1A1*(28/*28*) genotype compared with subjects having the wild-type *UGT1A1*(**1/*1*) genotype in assays of liver microsomes that included 0.1 mM α-naphthylamine, a competitive inhibitor of UGT1A9 and not UGT1A1. Similar phenotype:genotype correlations were observed when we compared subjects with the *UGT1A1*(**28/*28*) genotype with subjects having the *UGT1A1*(**1/*28*) genotype. In assays with α-naphthylamine, the K_m of liver microsomes against BPD(−) was similar to that reported for UGT1A1-overexpressing baculosomes (319 μM *versus* 290 μM; Fang *et al.*, Cancer Res., *62:* 1978–1986, 2002). These data suggest that the *UGT1A1* TATAA box polymorphism plays a role in an individual's overall ability to detoxify benzo(*a*)pyrene and in cancer risk.**

Introduction

The UDP-glucuronosyltransferase (UGT) superfamily of enzymes catalyze the glucuronidation of various compounds, including endogenous compounds such as bilirubin and steroid hormones, as well as xenobiotics, including drugs and environmental carcinogens (1–4). On the basis of structural as well as sequence homology, UGTs are classified into several families and subfamilies, each containing several highly homologous *UGT* genes (5). The entire *UGT1A* family is derived from a single locus in chromosome 2 coding for nine functional proteins that differ only in their amino terminus as a result of alternate splicing of independent exon 1 regions to a shared carboxy terminus encoded by exons 2–5 (6). In contrast to the *UGT1A* family, the *UGT2B* family is composed of several independent genes, all located on chromosome 4 (7–11).

In addition to being the major enzyme involved in the metabolism and detoxification of bilirubin (12), UGT1A1 is one of several UGTs that glucuronidate metabolites of tobacco carcinogens, including benzo(*a*)pyrene (BaP). Of the hepatic UGTs, only UGT1A1 and UGT1A9 exhibit significant activity against benzo(*a*)pyrene-*trans*-7*R*,8*R*-dihydrodiol [BPD(−)] (13), precursor to the highly mutagenic *anti*-(+)-BaP-7*R*,8*S*-dihydrodiol-9*S*,10*R*-epoxide. UGTs 1A7, 1A8, and 1A10 also exhibit glucuronidating activity against BPD(−) (13), but these are extrahepatic enzymes located primarily in the alimentary tract (14–18).

The *UGT1A1*28* allelic variant contains an additional (TA) dinucleotide repeat in the "TATAA" box [$(TA)_6$>$(TA)_7$] of the *UGT1A1* promoter that has been linked to decreased expression of the *UGT1A1* gene (19, 20) and decreased bilirubin conjugation (19, 21, 22), leading to a nondebilitating condition known as Gilbert's syndrome (19, 21–23). To determine whether the *UGT1A1* TATAA box polymorphism potentially

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plays a role in differential glucuronidation and detoxification of carcinogens such as BPD(−), subjects were screened comparing *UGT1A1* TATAA box genotype with normal liver UGT1A1 expression and BPD(−) glucuronidating activity in normal liver microsomes. Significant correlations between *UGT1A1* genotype and both UGT1A1 expression and BPD(−) glucuronidating activity were demonstrated, implicating this polymorphism as a potentially important risk factor for BaP-induced carcinogenesis.

Materials and Methods

Chemicals and Materials. BPD(−) was obtained from the National Cancer Institute Chemical Carcinogen Repository (synthesized and characterized at Midwest Research Institute, Kansas City, MO), dissolved in DMSO, and stored protected from light at −70°C. Bilirubin was purchased from Fluka Chemicals (Ronkonkoma, NY). UDP-glucuronic acid (UDPGA); D,L-2-lysophosphatidyl choline palmital C16:0; α-naphthylamine; *Escherichia coli* β-glucuronidase; and monoclonal anti-β-actin antibody were purchased from Sigma (St. Louis, MO). ^{14}C-labeled UDPGA (specific activity, 326 Ci/mol), [γ-^{32}P]-ATP (specific activity, 3000 Ci/mmol), and [α-^{35}S]-dATP (specific activity, 1250 Ci/mmol) were obtained from NEN Life Scientific Products (Boston, MA). *Taq* DNA polymerase was purchased from Boehringer Mannheim (Indianapolis, IN), a 10-bp DNA ladder was purchased from Invitrogen (Carlsbad, CA), the QIAEX II gel extraction kit was purchased from Qiagen (Valencia, CA), and the UGT1A1 Western blotting kit and baculosomes overexpressing UGT1A1 or UGT1A9 were purchased from Gentest (Woburn, MA). HPLC-grade solvents were provided by various suppliers and used after filtration. All other chemicals were of analytical grade and used without further purification.

Tissues. Genomic DNA was extracted from adjacent pathologically confirmed normal liver specimens from 95 Caucasian individuals undergoing surgery for excision of hepatocellular carcinoma at the H. Lee Moffitt Cancer Center. All DNA specimens were provided by the Tissue Procurement Facility at the H. Lee Moffitt Cancer Center, and demographic data were provided for all subjects. Normal liver specimens from 60 of these 95 normal human liver samples were obtained from the same facility and were confirmed to be histologically normal by the H. Lee Moffitt Pathology Department. Tissue microsomes were prepared from the 60 normal liver specimens through differential centrifugation as described previously (24) and were stored (10–20 mg protein/ml) at −70°C in 100-μl aliquots, with total protein concentrations measured with the BCA assay (Pierce Corporation, Rockford, IL).

All protocols involving the analysis of tissue specimens were approved by the Institutional Review Board at the University of South Florida and were in accordance with assurances filed with and approved by the United States Department of Health and Human Services. Assurances were given by the Tissue Procurement Facility at the H. Lee Moffitt Cancer Center that all samples were isolated and quick-frozen at −70°C within 2 h postsurgery.

***UGT1A1* PCR Amplifications and Genotyping Analysis.** The region spanning the *UGT1A1* TATAA box promoter element was PCR-amplified (PCR product, 98–100 bp) as described previously (25) with sense (1A1S, 5′-GTCACGTGACACAGTCAAAC-3′) and antisense (1A1AS, 5′-TTTGCTCCTGCCAGAGGTT-3′) primers corresponding to nucleotides −104 through −85 and −25 through −7, respectively, relative to the translation start site in *UGT1A1* exon 1 (GenBank accession no. AF297093). PCR amplifications were routinely performed in a 50-μl reaction volume containing 150 ng of purified genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each of deoxynucleotide trisphosphate, 20 pmol of both sense and antisense *UGT1A1* primers, 5 μCi [α-^{35}S]-dATP, and 2.5 units of *Taq* DNA polymerase. The reaction mixture underwent the following incubations in a GeneAmp 9700 thermocycler (Perkin-Elmer Corp., Foster City, CA): 1 cycle of 94°C for 5 min; 41 cycles of 94°C for 40 s, 61°C for 40 s, and 72°C for 30 s; followed by a final cycle of 10 min at 72°C. Four μL of the final PCR product were mixed with in 4 μl of 95% formamide-20 mM EDTA (pH 8.0)-0.05% bromphenol blue-0.05% xylene cyanole FF and denatured by heating at 95°C for 5 min before loading on a 6% denaturing polyacrylamide gel. Samples were subjected to electrophoresis at 50 V/cm for 4 h; gels were dried and subsequently exposed for autoradiography for 1 week. Bands of either 98 or 100 bp, representing the six or seven (TA) repeats of *UGT1A1*1* or *UGT1A1*28*, respectively, were visualized, and their size were determined by comparison with a 5′-end ^{32}P-labeled 10-bp DNA ladder.

Representative samples exhibiting each of the *UGT1A1* TATAA box genotype patterns observed by electrophoresis were examined by dideoxy sequencing, with PCR products purified after electrophoresis in 2% agarose using the QIAEX II gel extraction kit and sequenced at the DNA Sequencing Facility in the Department of Genetics at the University of Pennsylvania Medical Center using the same sense and antisense primers as were used for PCR amplification. Because the prevalences of non-*UGT1A1*28* variant alleles are low in the population (26, 27), all *UGT1A1* genotyping for the Gilbert's syndrome polymorphism that exhibited a pattern consistent with the wild-type $A(TA)_6TAA$ promoter region were considered to be *UGT1A1*1*.

Analysis of UGT1A1 Protein Levels. UGT1A1 protein levels were determined with the UGT1A1 Western blotting kit provided by Gentest, whereas β-actin protein levels were assayed with the monoclonal anti-β-actin antibody provided by Sigma. Western blots were performed with 20 μg of liver microsomal protein by standard procedures (28). UGT1A1 levels were quantitated against a known amount of human UGT1A1 protein (10 ng; supplied in the Western blotting kit provided by Gentest) by densitometric analysis of Western blots, with quantitation made relative to the levels of β-actin observed in each lane (also quantitated by densitometric analysis of Western blots).

Analysis of Bilirubin Glucuronidating Activity. Bilirubin glucuronidation was assayed in a 100-μl reaction volume using 0.25 mg of liver microsomes incubated for 1 h at 37°C in 0.7 mM bilirubin, 4 mM UDPGA, 10 mM $MgCl_2$, 50 mM sodium citrate (pH 7.4), and D,L-2-lysophosphatidyl choline palmital C16:0 (10 μg/100 μg of protein). Where indicated, α-naphthylamine (0.01–1 mM) was added to individual reactions. Reactions were terminated by the addition of an equal volume (100 μl) of 2% ascorbic acid in ethanol. Precipitates were removed by centrifugation (5 min at 14,000 × *g*), and supernatants were filtered (0.22 μm) and analyzed for bilirubin glucuronides by high-performance liquid chromatography (HPLC) using a Waters Associates binary pump (model 1525) HPLC system equipped with a dual λ absorbance detector operated at 450 nm, an automatic injector (model WISP 710B) and a β-RAM radioactive flow detector (IN/US, Tampa, FL). Samples were injected onto a Waters μBondapak (3.9 × 300

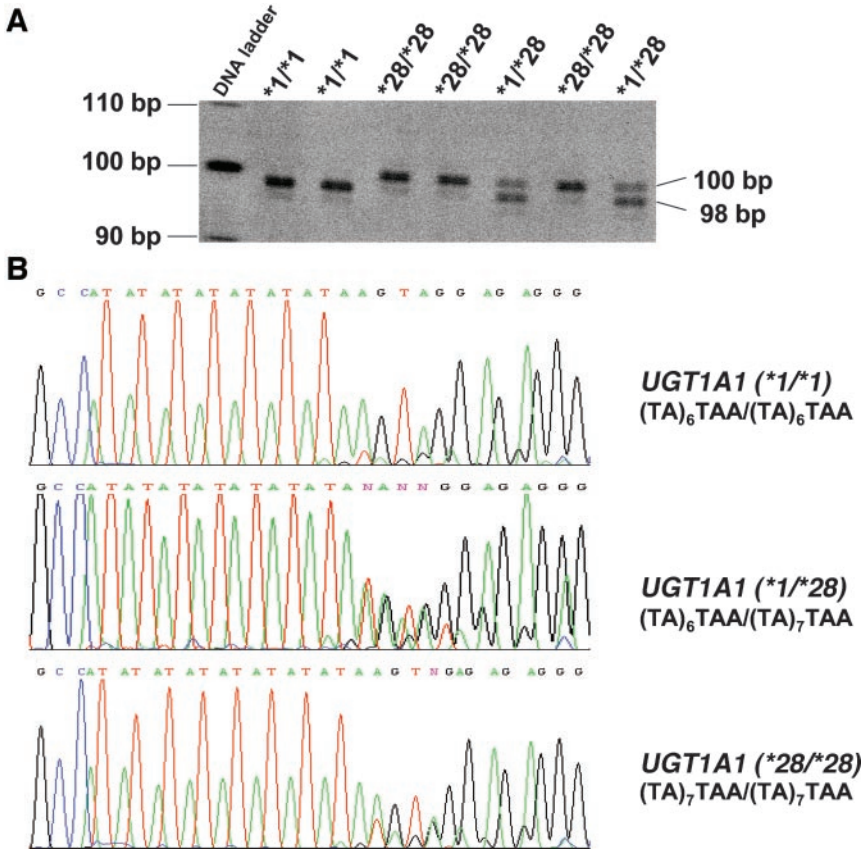

Fig. 1. *A*, representative results of *UDP-glucuronosyltransferase 1A1* (*UGT1A1*) TATAA box genotyping. Shown is a representative autoradiograph of ^{35}S-labeled PCR fragments of the *UGT1A1* promoter encompassing the *UGT1A1* TATAA box with normal liver genomic DNA as template. *UGT1A1* genotypes are indicated *above* each lane, with PCR fragment sizes of 98 bp (indicative of the *UGT1A1*1* allele) and 100 bp (indicative of the *UGT1A1*28* allele) shown on the *right*. DNA marker band sizes are shown on the *left*. *B*, representative results of *UGT1A1* TATAA box sequencing analysis. Shown are representative sequencing results of the TATAA box within the *UGT1A1* promoter for PCR fragments amplified from liver genomic DNA from subjects with each of the three potential *UGT1A1* TATAA box genotypes. *UGT1A1* genotypes and their respective TATAA box composition are shown on the *right*.

mm) 5 μ C_{18} column. HPLC separations were performed with the following linear gradient conditions: 0–20 min, 10–90% solvent A (0.01% trifluoroacetic acid in acetonitrile); 20–25 min, 90–100% A, where solvent A was diluted in solvent B (0.01% trifluoroacetic acid in water). The HPLC flow rate was 1 ml/min, whereas the scintillation fluid flow rate was 4 ml/min. The column was routinely washed with 100% A for 15 min and equilibrated after every HPLC run with 10% A for at least 20 min. Absorbance (450 nm) and ^{14}C-labeled peaks corresponding to glucuronide conjugates of bilirubin were tentatively identified by relative retention times, and then confirmed by sensitivity to *E. coli* β-glucuronidase treatment (1000 units; overnight incubation at 37°C), using HPLC as described above.

Analysis of BPD(−) Glucuronidating Activity. BPD(−) glucuronidation assays were performed essentially as described previously (13), with liver microsomes (0.25 mg of protein) or baculosomes (0.2 mg of protein) incubated with 1 mM BPD(−) and 4 mM UDPGA in 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), and D,L-2-lysophosphatidyl choline palmital C16:0 (10μg/100 μg of protein) in a total reaction volume of 100 μl for 1 h at 37°C. Where indicated, α-naphthylamine (0.05–0.5 mM) was added to individual reactions. All reactions were initiated by the addition of UDPGA and terminated by the addition of an equal volume of ice-cold acetonitrile. Precipitates were removed by centrifugation (5 min at 14,000 × *g*), and supernatants were filtered (0.22 μm) and analyzed for glucuronidated BPD(−) isomers by HPLC as described previously (13). For reactions including α-naphthylamine, HPLC was performed with the following linear gradient conditions: 0–40 min, 20% solvent C (100% acetonitrile); 40–60 min, 20–30% C; 60–65 min, 30% C, where solvent C was diluted in solvent D (20 mM ammonium acetate, pH 6.5).

Kinetic analysis in liver microsomes was performed as described above using 0.05–1 mM BPD(−) and 0.1 mM α-naphthylamine at a maximal incubation time (1 h) when the rate of BPD(−) glucuronide formation was still linear. The K_m values for the glucuronidation of BPD(−) by human liver microsomes were calculated after linear regression analysis of Lineweaver–Burk plots.

Glucuronidation activities against both BPD(−) and bilirubin were calculated based on radioflow detection and quantification of dpm within glucuronidated BPD(−)- or bilirubin-specific HPLC peaks as determined with the IN/US radioactivity detection program.

Statistical Analyses. The two-tailed Student's *t* test was used to compare UGT1A1 protein levels as well as bilirubin and BPD(−) glucuronidating activities in subjects with different *UGT1A1* genotypes.

Results

To screen whether the *UGT1A1* TATAA box [$(TA)_6$>$(TA)_7$] polymorphism plays a role in the glucuronidation and detoxification of BPD(−) in humans, we screened a cohort of 95 subjects from whom pathologically normal liver specimens could potentially be obtained for *UGT1A1* genotype. This cohort was 59% male with a mean age of 63 years. Efficient separation of wild-type (98-bp) *versus* polymorphic (100-bp) PCR amplimers was obtained with the denaturing gel system

Table 1 Genotypes and allelic prevalence of the *UGT1A1*[a] TATAA box promoter polymorphism

	n	Prevalence
UGT1A1 genotype		
**1/*1*	36	0.38
**1/*28*	48	0.50
**28/*28*	11	0.12
UGT1A1 allele		
**1*	120	0.63
**28*	70	0.37

[a] *UGT1A1*, UDP-glucuronosyl transferase 1A1.

used for *UGT1A1* genotyping (Fig. 1*A*). Sequencing analysis of individual PCR amplimers confirmed data obtained from genotyping analysis in all samples examined (Fig. 1*B*). As shown in Table 1, 12% of these subjects had the homozygous polymorphic *UGT1A1*(**28/*28*) genotype, which was similar to the genotype prevalence observed previously for Caucasians (26). The prevalence of the *UGT1A1*28* allele was 0.37 in this cohort, and it followed Hardy–Weinberg equilibrium.

The *UGT1A1*28* allelic variant has been previously linked to decreased expression of the *UGT1A1* gene and decreased bilirubin conjugation (19–23). To demonstrate a potential link between *UGT1A1* TATAA box genotype and carcinogen-metabolizing phenotype, we obtained liver specimens from 60 of the 95 subjects screened for *UGT1A1* genotype. This included liver specimens from 24 subjects with the homozygous wild-type *UGT1A1*(**1/*1*) genotype, 27 subjects with the heterozygous *UGT1A1*(**1/*28*) genotype, and 9 subjects with the homozygous polymorphic *UGT1A1*(**28/*28*) genotype. Microsomal fractions were prepared for all liver specimens and used for all biochemical analysis. As shown in Fig. 2*A*, Western blotting for the UGT1A1 enzyme using an anti-UGT1A1-specific antibody demonstrated lower levels of microsomal UGT1A1 protein in liver microsomes from subjects who exhibited either the homozygous polymorphic *UGT1A1*(**28/*28*) genotype or the heterozygous polymorphic *UGT1A1*(**1/*28*) genotype compared with subjects exhibiting the homozygous wild-type *UGT1A1*(**1/*1*) genotype. After screening of all 60 microsomal specimens, microsomes from *UGT1A1*(**1/*1*) subjects exhibited a significant ($P < 0.005$) 2-fold higher level of UGT1A1 protein (normalized relative to the levels of liver microsomal β-actin) compared with microsomes from subjects with the *UGT1A1*(**28/*28*) genotype (Fig. 2*B*). A similar increase of 1.7-fold ($P < 0.05$) was observed when we compared liver microsomes from subjects with the heterozygous *UGT1A1*(**1/*28*) genotype with microsomes from subjects with the *UGT1A1*(**28/*28*) genotype. No decrease in UGT1A1 protein was observed when we compared subjects who were heterozygous for the *UGT1A1*28* variant with individuals who were homozygous for *UGT1A1*1*.

To assess the effect of genotype-associated decreases in UGT1A1 protein levels on liver glucuronidation activity of a UGT1A1 substrate, we assessed the rates of bilirubin glucuronide formation in the same 60 liver microsomal specimens by HPLC. Several peaks corresponding to potential bilirubin glucuronides were observed in this HPLC assay (Fig. 3*A*). Bilirubin glucuronides were confirmed by sensitivity to β-glucuronidase (Fig. 3*B*). Consistent with the results for actual UGT1A1 protein levels, we observed significantly lower rates of bilirubin glucuronide formation for liver microsomes from subjects with the homozygous polymorphic *UGT1A1*(**28/*28*) genotype compared with subjects with either the wild-type *UGT1A1*(**1/*1*) ($P < 0.001$) or heterozygous *UGT1A1*(**1/*28*) ($P < 0.002$) genotypes (Fig. 4*A*). Similar results were obtained when data were analyzed per mg of total liver microsome protein (Fig. 4*A*) or when values were normalized to levels of microsomal β-actin (results not shown).

Efficient separation of BPD(−) diastereomers was obtained by HPLC in assays with liver microsomes (Fig. 3*C*) as well as UGT1A1-overexpressing baculosomes (Fig. 3*D*). BPD(−) glucuronides were confirmed by sensitivity to β-glucuronidase (Fig. 3*E*); based on known retention times for BPD(−) glucuronide diastereomers (13), peak 1 corresponded to BPD-7*R*-Gluc (retention time, 25.5 min), whereas peak 2 corresponded to BPD-8*R*-Gluc (retention time, 26.5 min). As observed previously for BPD(−) in liver microsomes (13), peak 1 was higher than peak 2 for all liver microsomes tested, suggesting that on exposure to BPD(−), BPD-7*R*-Gluc is preferentially formed in human liver regardless of potential variations in UGT expression or genotype. The BPD(−) glucuronide

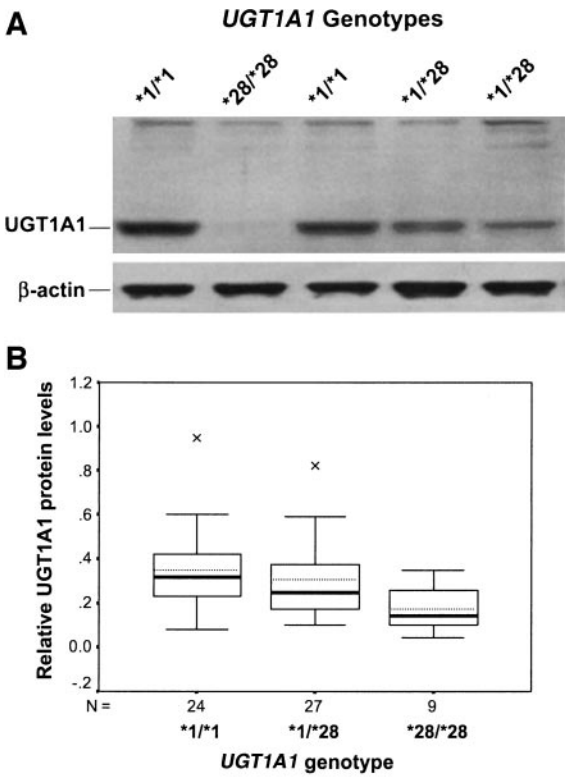

Fig. 2. *A*, Western blot of UDP-glucuronosyltransferase 1A1 (UGT1A1) protein. Shown is a representative Western blot of liver microsomal UGT1A1 protein from subjects with different *UGT1A1* genotypes. β-Actin was used as an internal control for protein loading for all gels. *UGT1A1* genotypes are shown *above* all lanes. *B*, box plots comparing UGT1A1 protein levels in liver microsomes from subjects with *UGT1A1*(**1/*1*), *UGT1A1*(**1/*28*), and *UGT1A1*(**28/*28*) genotypes. The number of subjects from each group are shown on the *X axis*. *Top* of each *box*, 75th percentile; the *bottom* of each *box*, 25th percentile; *solid center line*, 50th percentile; *dashed center line*, mean; ×, extreme values; *error bars*, furthest observations from the median that are not extreme values. UGT1A1 protein was determined by relative densitometric analysis of Western blots relative to that observed for 10 ng of human UGT1A1 standard (Gentest), with all liver microsomal UGT1A1 protein levels normalized for β-actin (also determined by densitometric analysis of Western blots). The levels of UGT1A1 protein in human liver microsomes from subjects with the *UGT1A1*(**28/*28*) genotype were significantly lower than the levels observed for liver microsomes from subjects with either the *UGT1A1*(**1/*1*) ($P < 0.005$) or *UGT1A1*(**1/*28*) ($P < 0.05$) genotype.

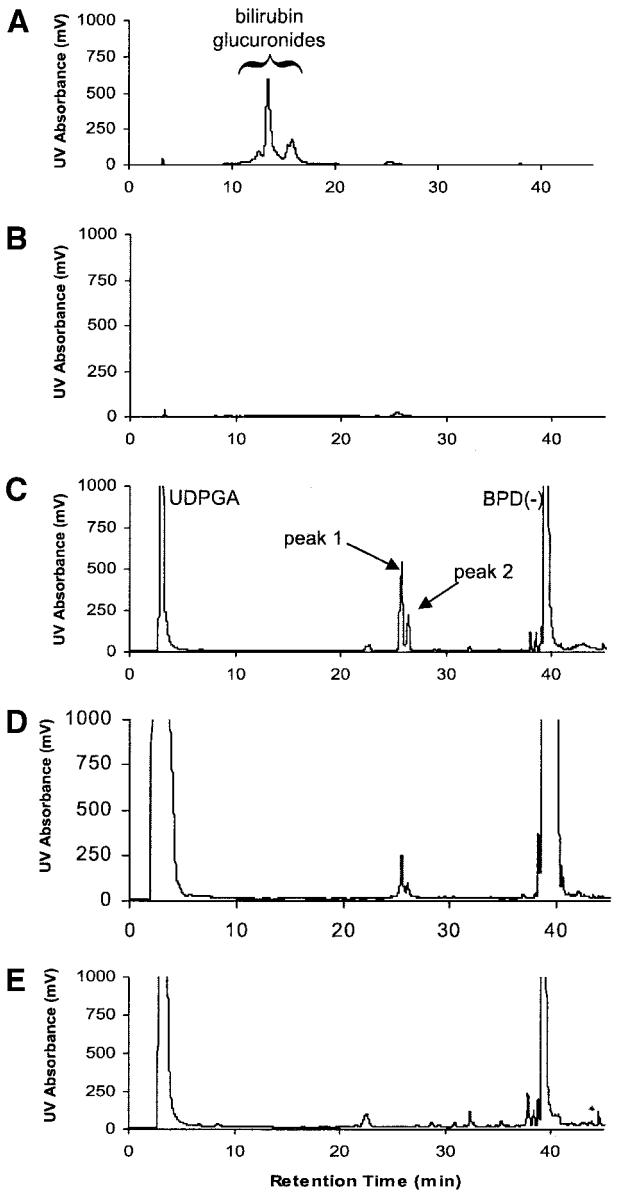

Fig. 3. HPLC analysis of bilirubin and benzo(*a*)pyrene-*trans*-7*R*,8*R*-dihydrodiol(−) [BPD(−)] glucuronide formation by human liver microsomes and UDP-glucuronosyltransferase 1A1 (UGT1A1)-overexpressing baculosomes. Human liver microsomes (0.25 mg of protein) or baculosomes (0.2 mg of protein) were incubated at 37°C for 1 h with either 0.7 mM bilirubin or 1 mM BPD(−) and 4 mM UDP-glucuronic acid (UDPGA) as described in "Materials and Methods." Glucuronide formation is shown by UV detection (450 and 254 nm for bilirubin and BPD(−) glucuronides, respectively). *A* and *B*, incubations performed with bilirubin; *C-E*, incubations performed with BPD(−). *A–C* and *E*, incubations performed with human liver microsomes; *D*, incubation performed with UGT1A1-overexpressing baculosomes formation. *B* and *E*, assays including β-glucuronidase as described in "Materials and Methods." Peaks corresponding to UDPGA, BPD(−), bilirubin glucuronides, and BPD(−) glucuronides (*peaks 1* and *2*) are indicated by *arrows*.

isomer pattern of BPD-7*R*-Gluc > BPD-8*R*-Gluc for human liver microsomes (Fig. 3*C*) is similar to that observed for both UGT1A1- (Fig. 3*D*) and UGT1A9-overexpressing baculosomes (13).

After screening for BPD(−) glucuronidation phenotype in all 60 liver microsomes, similar levels of BPD(−) glucuronide formation were observed in subjects with different *UGT1A1* TATAA box genotypes (Fig. 4*B*). Unlike that observed for bilirubin glucuronidation, no significant differences in BPD(−) glucuronide formation were observed when we compared subjects with the *UGT1A1*(**28/*28*) genotype with either the *UGT1A1*(**1/*1*) or *UGT1A1*(**1/*28*) genotypes. Because UGT1A1 and UGT1A9 are the only two hepatic UGT enzymes that exhibit significant activity against BPD(−) (13), attempts were made to eliminate UGT1A9-induced BPD(−) glucuronidating activity as a potential confounder in these assays by identifying a substrate that could inhibit UGT1A9 but not UGT1A1. Of the compounds tested, α-naphthylamine inhibited BPD(−) glucuronide formation in UGT1A9-overexpressing baculosomes but not in baculosomes overexpressing UGT1A1, with the inhibition of UGT1A9 activity against BPD(−) approaching 97% in assays with 0.5 mM α-naphthylamine (Fig. 5, *A* and *B*). In addition, no significant inhibition of bilirubin glucuronide conjugation was observed in liver microsomes at α-naphthylamine concentrations up to 0.1 mM (Fig. 5*C*), which is equivalent to approximately 9 times the k_i for α-naphthylamine inhibition of BPD(−) glucuronide formation in UGT1A9-overexpressing baculosomes (11.3 ± 4.0 μM). Efficient separation of α-naphthylamine from BPD(−) glucuronide diastereomers was observed in the HPLC assays used, with α-naphthylamine eluting at 56 min and BPD-7*R*-Gluc and BPD-8*R*-Gluc eluting at 65 and 66 min, respectively (Fig. 6).

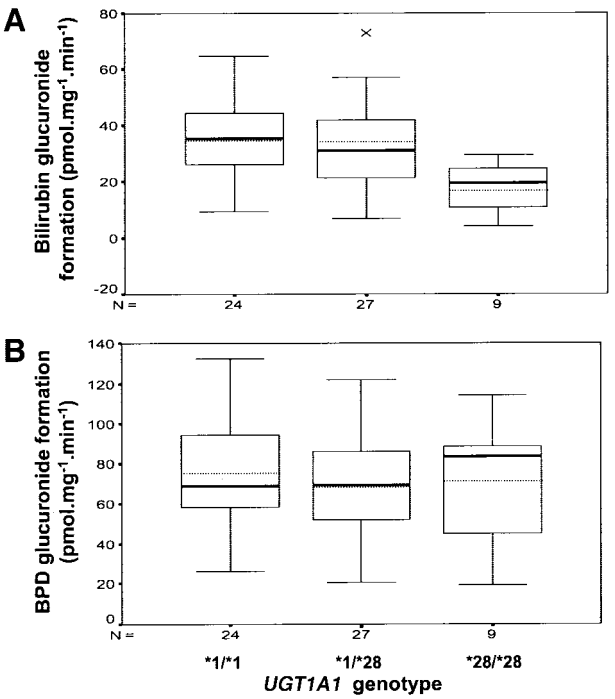

Fig. 4. Box plots comparing levels of bilirubin glucuronide (*A*) and BPD glucuronide (*B*) formation in liver microsomes from subjects with UDP-glucuronosyltransferase 1A1 [*UGT1A1*(**1/*1*)], *UGT1A1*(**1/*28*), and *UGT1A1*(**28/*28*) genotypes. The box plots are defined as the legend for in Fig. 2*B*. The levels of bilirubin glucuronide formation in human liver microsomes from subjects with the *UGT1A1*(**28/*28*) genotype were significantly lower than those observed for liver microsomes from subjects with either the *UGT1A1*(**1/*28*) ($P < 0.002$) or *UGT1A1*(**1/*1*) ($P < 0.001$) genotypes. There was no statistical difference in BPD glucuronide levels in human liver microsomes from subjects with different *UGT1A1* genotypes.

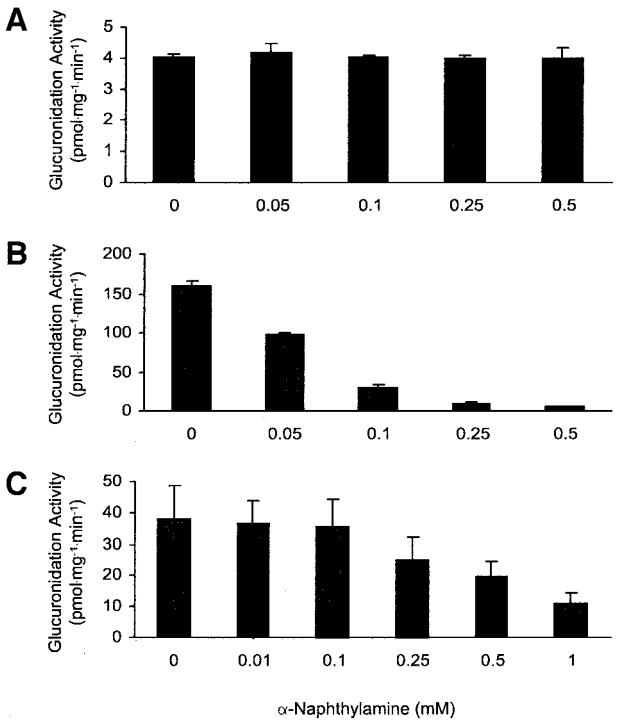

Fig. 5. Effects of α-naphthylamine on UDP-glucuronosyltransferase 1A1 (UGT1A1), UGT1A9, and human liver microsome-induced glucuronidation. Shown are bar graphs of the relative glucuronidation activities of UGT1A1- or UGT1A9-overexpressing baculosomes (0.2 mg of protein) or human liver microsomes (0.25 mg of protein) against 1 mM benzo(*a*)pyrene-*trans*-7*R*,8*R*-dihydrodiol(−) [BPD(−)] or 0.7 mM bilirubin in assays with 0.01–1 mM α-naphthylamine as described in the "Materials and Methods." *A*, UGT1A1-overexpressing baculosomes with 1 mM BPD(−); *B*, UGT1A9-overexpressing baculosomes with 1 mM BPD(−); *C*, human liver microsomes with 0.7 mM bilirubin.

Together, these data suggest that 0.1 mM α-naphthylamine can be used as a selective inhibitor of UGT1A9-induced glucuronidation of BPD(−) *in vitro* in liver microsomes.

Decreased BPD-glucuronide formation (3–6-fold) was observed when 0.1 mM α-naphthylamine was added to BPD glucuronidation assays of human liver microsomes (Figs. 4*B* and 7), a decrease that was significant regardless of *UGT1A1* genotype ($P < 0.001$ for all genotypes). When 0.1 mM α-naphthylamine was added to glucuronidation assays of liver microsomes, differences in the levels of BPD(−) glucuronide formation were observed for subjects with different *UGT1A1* TATAA box genotypes (Fig. 7). We observed a significantly lower rate of BPD(−) glucuronide formation in subjects with the homozygous polymorphic *UGT1A1*(**28/*28*) genotype compared with subjects who were either homozygous ($P < 0.02$) or heterozygous ($P < 0.02$) for the *UGT1A1*1* allele. Similar results were obtained when data were analyzed per mg of total liver microsome protein (Fig. 7) or when results were normalized to levels of microsomal β-actin (results not shown). No difference in BPD(−) glucuronidation phenotype was observed in heterozygous subjects compared with subjects with the wild-type *UGT1A1*(**1/*1*) genotype. The K_m of human liver microsomes for BPD(−) in assays with α-naphthylamine (0.1 mM) was 319 ± 52 μM and was similar in subjects with various *UGT1A1* genotypes (**1/*1*, **1/*28*, or **28/*28*; results not shown).

Discussion

In addition to being the major enzyme involved in the metabolism and detoxification of bilirubin, UGT1A1 is one of several UGTs that glucuronidate carcinogens, including metabolites of BaP. The UGTs 1A1, 1A7, 1A8, 1A9, 1A10, and 2B7 all exhibit activity against the racemic form of BPD and, with the exception of UGT2B7, the BPD(−) isomer (13), precursor to the highly mutagenic *anti*-(+)-BaP-7*R*,8*S*-dihydrodiol-9*S*,10*R*-epoxide. Of the BPD(−)-glucuronidating UGTs, only UGT1A1 and UGT1A9 are expressed in liver (18), which indicates that both of these enzymes are the sole hepatic glucuronidators of this potent carcinogenic precursor. The mutual importance of both enzymes in the hepatic metabolism and detoxification of BPD(−) is supported by the fact that the ratio of the two glucuronide isomers formed from BPD(−), BPD-7*R*-Gluc: BPD-8*R*-Gluc, is similar for both enzymes in *in vitro* assays with BPD(−) as substrate, with both enzymes exhibiting a K_m in the 0.2–0.3 mM range (13). In addition, the BPD-7*R*-Gluc: BPD-8*R*-Gluc ratio observed with both enzymes individually (1:0.3–1:0.4) is similar to that observed with liver microsomes (present study and Ref. 13). When we used α-naphthylamine,

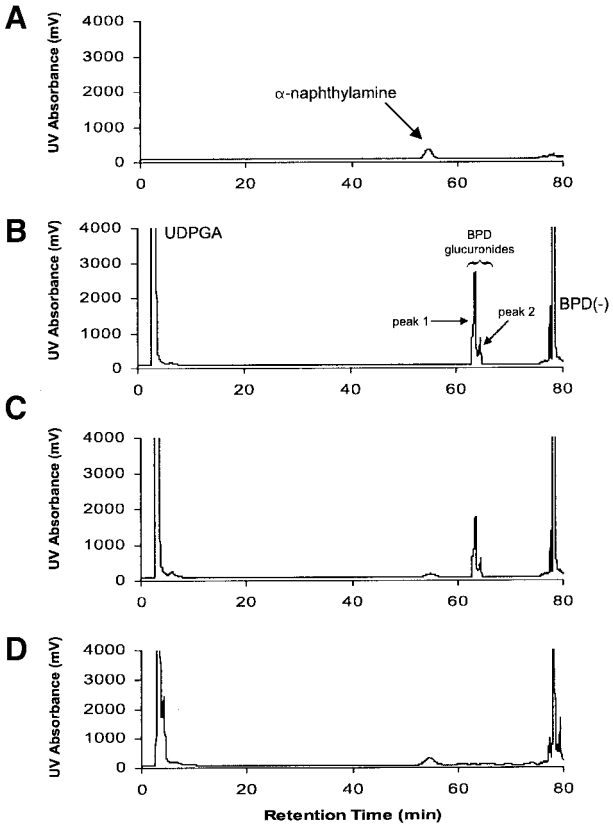

Fig. 6. High-performance liquid chromatographic analysis of benzo(*a*)pyrene-*trans*-7*R*,8*R*-dihydrodiol(−) [BPD(−)] glucuronide formation by human liver microsomes in the presence of α-naphthylamine. Human liver microsomes (0.25 mg of protein) were incubated with 1 mM BPD(−) or 0.1 mM α-naphthylamine as described in "Materials and Methods." Glucuronide formation is shown by UV detection (254 nm). *A*, 0.1 mM α-naphthylamine alone; *B*, glucuronidation assays performed with 1 mM BPD(−) without 0.1 mM α-naphthylamine; *C*, glucuronidation assays performed with 1 mM BPD(−) plus 0.1 mM α-naphthylamine; *D*, glucuronidation assays performed with 1 mM BPD(−) plus 0.1 mM α-naphthylamine in the presence of 1,000 units of β-glucuronidase. Peaks corresponding to α-naphthylamine, UDP-glucuronic acid (*UDPGA*), BPD(−), and BPD glucuronides are indicated by *arrows*.

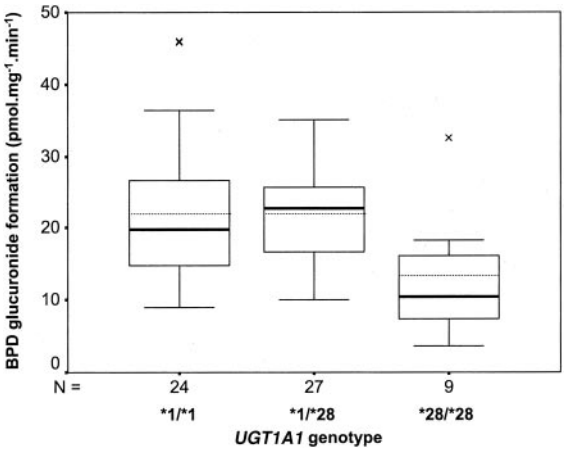

Fig. 7. Box plots comparing levels of benzo(*a*)pyrene-*trans*-7*R*,8*R*-dihydrodiol (BPD) glucuronide formation in liver microsomes from subjects with *UDP-glucuronosyltransferase 1A1* [*UGT1A1*(**1/*1*)], *UGT1A1*(**1/*28*), and *UGT1A1*(**28/*28*) genotypes in assays containing 0.1 mM α-naphthylamine. The box plots are defined as in the legend for Fig. 2*B*. The levels of BPD glucuronide formation in human liver microsomes from subjects with the *UGT1A1*(**28/*28*) genotype were significantly lower than those observed for liver microsomes from subjects with either the *UGT1A1*(**1/*28*) ($P < 0.02$) or *UGT1A1*(**1/*1*) ($P < 0.02$) genotype.

an inhibitor of UGT1A9-induced glucuronidation of BPD(−), results from the present study suggest that ~30% of BPD(−) glucuronidation is catalyzed by UGT1A1, whereas ~70% is catalyzed by UGT1A9 in subjects homozygous for the wild-type *UGT1A1*1* allele, a fact that is consistent with both enzymes playing major roles in the hepatic glucuronidation of BPD(−).

The studies described here are the first to directly examine glucuronidation activity against a major carcinogen in liver specimens from a large group of subjects. In assays focusing solely on UGT1A1-induced BPD(−) glucuronidation, significantly decreased levels of BPD(−) glucuronide formation were observed in liver microsomes from subjects with the *UGT1A1*(**28/*28*) genotype compared with subjects with the homozygous wild-type *UGT1A1*(**1/*1*) or heterozygous *UGT1A1*(**1/*28*) genotypes. These results are consistent with the decreased expression observed *in vitro* with a reporter gene transcriptionally regulated by the *UGT1A1* promoter with an additional (TA) dinucleotide repeat in its TATAA box [$(TA)_6$>$(TA)_7$; Refs. 19, 20]. The results of the present study also conclusively demonstrate that livers from subjects who are homozygous for the *UGT1A1*28* allele exhibit significantly lower liver microsomal UGT1A1 protein and decreased liver microsomal glucuronidating activity against bilirubin. These results are consistent with the decreased levels of bilirubin glucuronide formation observed in liver microsomes from subjects homozygous for the variant TATAA box genotype in previous studies (29) and the decreased levels of bilirubin glucuronide observed in serum from subjects homozygous for the variant TATAA box genotype (19, 20) and in subjects clinically diagnosed with Gilbert's syndrome (19, 21–23).

Together, these data suggest that the *UGT1A1* TATAA box polymorphism is associated with a decreased overall ability to glucuronidate an important metabolite [BPD(−)] within the BaP carcinogenic pathway. This suggests that individuals with the variant *UGT1A1*(**28/*28*) genotype may be less able to detoxify BaP (and potentially other carcinogens metabolized by UGT1A1) than those who are wild-type for UGT1A1, further suggesting that individuals with the variant *UGT1A1*(**28/*28*) genotype are at increased risk for certain cancers. Although the association between BPD(−) glucuronide formation and *UGT1A1* genotype was not observed in assays where hepatic UGT1A9 activity was not inhibited, this was likely because hepatic UGT1A9-induced activity comprised a majority (~70%) of the total BPD(−) glucuronidation observed in human liver microsomes. The fact that significant differences in genotype-associated UGT1A1-induced BPD(−) glucuronide formation were not detectable when UGT1A9-associated activities were not inhibited is consistent with the possibility that small but significant long-term decreases in the overall ability to detoxify a procarcinogen such BPD(−) may play an important role in cancer susceptibility. A potential role for *UGT1A1* genotype in cancer risk has been examined in studies of breast cancer (30, 31), but studies have as yet to be performed for organs/tissues that are targets for BaP exposure (*e.g.*, tobacco-related cancers). The effect of *UGT1A1* genotype on cancer risk may be most pronounced for those organs/tissues where there is exposure to BaP and where UGT1A1 is expressed. Although previous studies have shown no significant levels of expression of UGT1A1 in either the aerodigestive tract or lung (17), a hepatic *UGT1A1* genotype-associated effect on overall BPD(−) detoxification and cancer risk cannot be excluded for these organ/tissue sites. Further studies examining *UGT1A1* genotype in relation to susceptibility for tobacco-related cancers (as well as other cancers where exposure to BaP is a risk factor) will be necessary to better evaluate the role of *UGT1A1* genotype on cancer risk.

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