DMD Fast Forward. Published on January 31, 2008 as doi:10.1124/dmd.107.019406

Glucuronidation of tobacco-specific nitrosamines by UGT2B10

Gang Chen, Ryan Dellinger, Dongxiao Sun, Thomas E. Spratt, and Philip Lazarus

Cancer Prevention and Control (GC, PL) and Chemical Carcinogenesis (TES) Programs, Penn State Cancer Institute; Departments of Public Health Sciences (GC, PL), Pharmacology (RD, DS, PL) and Biochemistry and Molecular Biology (TES), Penn State University College of Medicine, Hershey, PA 17033

Running title: Glucuronidation of tobacco-specific nitrosamines

Corresponding author: Philip Lazarus, Ph.D., Penn State Cancer Institute, Penn State University College of Medicine, Rm. C3739D, MC-H069, 500 University Drive, Hershey, PA 17033. Tel: (717)-531-5734; Fax: (717) 531-0480; Email: plazarus@psu.edu

Number of text pages: 38

Number of Tables: 2

Number of Figures: 3

References: 30

Number of words in Abstract: 217;

Number of words in Introduction: 597;

Number of words in Discussion: 612.

Abbreviations: TSNA, tobacco-specific nitrosamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNN, N′-nitrosonornicotine; NAB, N′-nitrosoanabasine; NAT, N′-nitrosoanatabine; UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; HLM, human liver microsomes; NMR, nuclear magnetic resonance.

Abstract

NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol) is an important tobacco-specific nitrosamine (TSNA) in the etiology of tobacco-related cancers and *N*-glucuronidation is an important mechanism of NNAL detoxification. In the present study, an analysis of the UDP-glucuronosyltranferases (UGTs) responsible for the *N*-glucuronidation of the TSNAs *N*′-nitrosonornicotine, *N*′-nitrosoanabasine and *N*′-nitrosoanatabine was performed. Utilizing HEK293 cells over-expressing UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B11, 2B15, and 2B17, only UGTs 1A4 and 2B10 exhibited *N*-glucuronidating activity against these TSNAs. The K_M's for UGT2B10 were 15-22-fold lower than those of UGT1A4 against the three TSNAs, and were similar to those observed for microsomes prepared from human liver specimens. The overall activity of UGT2B10 was 3.6-27-fold higher than UGT1A4 against the three TSNAs as determined by V_{max}/K_M after normalization by levels of UGT2B10 versus UGT1A4 mRNA. Similarly high levels of activity were also observed for UGT2B10 against a fourth TSNA, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), exhibiting a 6.3-fold lower K_M and 3-fold higher normalized V_{max}/K_M than that observed for UGT1A4. Real-time PCR analysis demonstrated that UGT2B10 was expressed at a level that, on average, was 26% higher than that observed for UGT1A4 in a screening of normal liver tissue specimens from 20 individual subjects. These data suggest that UGT2B10 is likely the most active UGT isoform in human liver for the *N*-glucuronidation of TSNAs.

Introduction

TSNAs (Figure 1) are a family of carcinogens present in both tobacco smoke and smokeless tobacco products (Hecht and Hoffmann, 1989; Hecht, 1998; Nowell et al., 1999). The most abundant and potent TSNA is NNK. The major metabolic pathway for NNK is carbonyl reduction to NNAL (Carmella et al., 1993) which, like NNK, is a potent lung and pancreatic carcinogen in rodents (Rivenson et al., 1988; Hecht, 1998). It has been estimated that 39-100% of NNK is converted to NNAL in cigarette smokers (Carmella et al., 1993). Other abundant TSNAs include NNN, a potent esophageal carcinogen, NAB, a weak carcinogen, and NAT, which is not considered to be carcinogenic (Hecht, 1998). The estimated daily exposure to TSNAs is 20 μg in smokers and 68 μg in smokeless tobacco users (Hoffmann et al., 1994).

The 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 (Tephly and Burchell, 1990; Gueraud and Paris, 1998; Nowell et al., 1999; Ren et al., 2000). Based on protein structure and sequence homology, UGTs are classified into several families and subfamilies, each containing several highly homologous UGT genes. Glucuronidation by the UGT superfamily of enzymes is a major mode of metabolism of NNAL *in vivo*. Glucuronide conjugates of NNAL as well as other TSNAs including NNN, NAB, and NAT have been identified in the urine of tobacco smokers and smokeless tobacco users, accounting for an average of 59-90% of the total NNAL, NNN, NAB, or NAT in urine (Carmella et al., 2002; Stepanov and Hecht, 2005). Unlike NNAL which

causes lung tumors in rats (Rivenson et al., 1988), the glucuronide conjugate of NNAL (NNAL-Gluc) is non-tumorigenic after subcutaneous injection into A/J mice (Upadhyaya et al., 1999). It was also reported that skin fibroblasts from UGT family 1-deficient rats were more sensitive to NNK-mediated cytotoxicity (Kim and Wells, 1996). These data strongly suggest that glucuronidation is a major mode of detoxification of NNAL and potentially other TSNAs.

Glucuronidation of NNAL can occur at both the carbinol group (NNAL-*O*-Gluc) as well as the pyridine ring nitrogen (NNAL-*N*-Gluc), while glucuronidation of NNN, NAB, and NAT occurs exclusively on the nitrogen on the TSNA pyridine ring (Figure 1). Both NNAL-*N*-Gluc and NNAL-*O*-Gluc have been observed in the urine of smokers, with an average of 1:1 in the ratio of these two metabolites observed in individuals (Carmella et al., 2002). A ratio of 1.7:1 in NNAL-*N*-Gluc : NNAL-*O*-Gluc formation was observed *in vitro* in human liver microsomal (HLM) specimens (Wiener et al., 2004a). Previous studies have demonstrated that the hepatic UGTs 1A9 (Ren et al., 2000), 2B7 (Ren et al., 2000), and 2B17 (Lazarus et al., 2005) all specifically exhibit *O*-glucuronidating activity against NNAL while UGT1A4 exclusively catalyzes NNAL-*N*-glucuronide formation (Wiener et al., 2004a). Previous kinetic analysis demonstrated that the K_M of NNAL-*N*-Gluc formation catalyzed by UGT1A4 (15.5 mM) is much higher than that observed for HLM [0.31 mM; (Wiener et al., 2004a)], suggesting that UGT isoform(s) other than 1A4 may be involved in catalyzing NNAL-*N*-Gluc formation.

No studies examining the UGTs responsible for the glucuronidation of NNN, NAB, and NAT have as yet been performed, and several UGTs have not been previously screened for activity against NNAL. The goal of the present study was to fully

characterize the human UGT(s) responsible for the glucuronidation of these TSNAs. In this report, results are presented indicating that UGT2B10 is a major enzyme responsible for the glucuronidation and detoxification of TSNAs in humans.

Materials and Methods

Chemicals and materials. ^{14}C-UDPGA (200 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). The HPLC scintillation solution, Ecoscint Flow, was purchased from National Diagnostics (Atlanta, GA). TSNAs were purchased from Toronto Research Labs (Toronto, Canada), alamethicin, β-glucuronidase and bovine serum albumin from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagles medium (DMEM), Dulbecco's phosphate-buffered saline (minus calcium-chloride and magnesium-chloride), fetal bovine serum , penicillin-streptomycin, geneticin (G418), the Platinum® *Pfx* DNA polymerase and the pcDNA3.1/V5-His-TOPO mammalian expression vector were all obtained from Invitrogen (Carlsbad, CA). The Hotstar PCR kit, the RNeasy Mini and Midi kits, and the QIAEX® II gel extraction kit were all purchased from Qiagen (Valencia, CA). The BCA protein assay kit was purchased from Pierce (Rockford, IL). The UGT2B10, UGT2B11, UGT1A4 and GAPDH expression assay kits (TaqMan Gene Expression Assays, ID's HS02556282_s1, Hs01894900_gh, Hs0655285_s1, and Hs99999905_m1, respectively) were purchased from Applied Biosystems (Foster City, CA) while all restriction enzymes were purchased from New England Biolabs (Beverly, MA). PCR primers were purchased from IDT (Coralville, IA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA) unless specified otherwise.

Tissues. The normal human liver tissue specimens used for these studies have been described previously (Wiener et al., 2004a). Bovine liver was purchased from local supermarket. Liver microsomes were prepared through differential centrifugation as

previously described (Coughtrie et al., 1986) and stored (10-20 mg protein/mL) at –80 °C. Microsomal protein concentrations were measured using the BCA assay. Total RNA was extracted was extracted from cell lines using the RNeasy Midi kit from Qiagen as per manufacturer's protocols.

Generation of UGT over-expressing cell lines and cell homogenate preparation. Cells over-expressing wild-type UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17 have been described previously (Ren et al., 2000; Dellinger et al., 2006; Sun et al., 2006). Cell lines over-expressing UGT2B10 and UGT2B11 were generated as previously described (Dellinger et al., 2006) by reverse transcription (RT)-PCR using normal human liver total RNA as previously described. The sense and antisense primers used were: 5'-AAGGATGGCTCTGAAATGGACTA-3' (sense) and 5'-CCAGCTTCAAATCTCAGATATAACTAATCC-3' (antisense), corresponding to nucleotides -4 to +19 and +1620 to +1591 relative to the UGT2B10 translation start site, and 5'-TGCACCAGGATGACTCTGAAA-3' (sense) and 5'-CTTGCTGGAATAAACTGAAGTTGTCCT-3' (antisense), corresponding to -9 to +21 and +1654 to +1628, respectively, relative to the UGT2B11 translation start site (GenBank accession numbers NM_001075 and NM_001073 for UGTs 2B10 and 2B11, respectively). The sequence of the cloned UGT coding region was compared to that described in GenBank for both UGTs and were confirmed to be 100% homologous to the respective wild-type UGT sequence. HEK293 cell lines over-expressing UGT2B10 or UGT2B11 were generated by electroporation as previously described (Dellinger et al., 2006). Cell homogenates were prepared by re-suspending pelleted cells in Tris-buffered saline [25 mM Tris-HCl (pH 7.4), 138 mM NaCl, 2.7 mM KCl] and

subjecting them to 3 rounds of freeze-thaw prior to gentle homogenization. Total RNA was extracted using the RNeasy Mini kit from Qiagen as per manufacturer's protocols.

Glucuronidation Assays. Glucuronidation activities of HLM or cell homogenates from human UGT over-expressing cell lines toward TSNAs were determined after an initial incubation of HLM (250 μg protein) or cell homogenate (250 μg protein) with alamethicin (50 μg/mg protein) for 15 min in an ice bath. Incubations (50-μL) were subsequently performed at 37^{o}C in 50 mM Tris buffer (pH 7.5), 10 mM $MgCl_2$, 4 mM ^{14}C-UGPGA (1 μCi/50 μl reaction), and different concentration of substrate. Titrated stock concentrations of substrate (TSNAs) were used so that equivalent volumes of vehicle (100% methanol) were added to all incubations (1:100 v/v) To screen for activity for individual UGT-over-expressing cell lines, incubations were performed for up to 18 h, while 2 h incubations were performed for initial rate kinetic analyses, a time which was found to be within the linear range of product formation for all UGTs tested in this study (data not shown). Reactions were terminated by the addition of 50 μL of cold acetonitrile. Protein was then removed by centrifugation at 13,000 g for 10 min at 4^{o}C. The acetonitrile in the supernatant was evaporated in speedVac for 10 min.

Samples (50 μL) were analyzed for TSNA glucuronides by HPLC using a Beckman Coulter System Gold 126 Solvent Module HPLC system (Fullerton, CA) equipped with an automatic injector (model 508), a UV detector operated at 254 nm (model 166), and a radioactive flow detector with 1000 μL flow cell (INUS systems, Tampa, FL). HPLC was performed using a Synergi-Fusion-RP-80 4 μm column (4.6 mm X 250 mm, Phenomenx, Torrance, CA) and an Aquasil 5 μm C18 analytical column (4.6 mm $\times$ 250 mm, Thermo, Bellefonte, PA) in series. The gradient elution conditions

were as follows: starting with 100% buffer A (100 mM NH_4Ac, pH 5.0) for 5 min, a subsequent linear gradient to 78% buffer B (90% acetonitrile) over 20 min was performed and then maintained at 78% buffer B for 10 min. The elution flow rate was 1 mL/min, and the scintillation solution flow rate was 3 mL/min. The amount of *N*-glucuronide formed was calculated based on the ratio of the radioactivity of the *N*-glucuronide versus total radioactivity. TSNA-*N*-glucuronides *w*ere confirmed by 1M NaOH hydrolysis and sensitivity to β-glucuronidase as previously described (Upadhyaya et al., 1999). As controls, glucuronidation assays were regularly performed using HLM as a positive control for glucuronidation activity, and untransfected HEK293 cell homogenate protein as a negative control for glucuronidation activity. Experiments were always performed in triplicate in independent assays.

Analysis of TSNA glucuronides obtained from bovine liver microsomes. To obtain pure NNN, NAB, and NAT glucuronides for NMR analysis, bovine liver microsomes (5 mg protein/1 mL reaction) were used to catalyze TSNA glucuronidation reactions. The predicted glucuronide conjugates of NNN, NAB, and NAT were purified by HPLC using the same system as described above with a modified gradient program. For NNN, a gradient of 2% acetonitrile:98% buffer A was used for 10 min, increased to 78% buffer B in 20 min, and then maintained at 78% buffer B for 10 min. For NAB and NAT, 4% buffer B:96% buffer A was used for 15 min, increased to 78% B in 10 min, and then maintained in 78% for 10 min. TSNAs collections were de-salinated by a second purification using the gradient programs described above except that water was used instead of buffer A. Purity of the collected fraction was confirmed by HPLC analysis.

Collections were dried in a SpeedVac and the NMR spectra of unmodified and modified NNN, NAB and NAT were acquired with a 300 MHz Bruker spectrometer.

NAB (300 MHZ, D_2O), 8.35 (d, 1H, 2-*E*), 8.34 (m, 1H, 6-*E*), 8.28 (m, 1H 6-*Z*), 8.12 (d, 1H, 2-*Z*), 7.68 (dt, 1H, 4-*E*), 7.33 (dd, 1H, 5-*E*), 7.44 (dt, 1H, 4-*Z*), 7.28 (dd, 1H, 5-*Z*), 6.12 (m, 1H, 2'-*E*), 5.60 (m, 1H, 2'-*Z*), 4.58 (m, 1H, 6'-*E*), 4.0 (m, 1H, 6'-*E*), 3.66 (m, 1H, 6'-*Z*), 3.42 (m, 1H, 6'-*Z*), 2.4-2.2 (m, 4H, 3'-*E* and *Z*), 2.05 (m, 1H, 5'- (*E* or *Z*)), 1.8-1.4 (m, 7H 5'- *E* or *Z*,4'- *E* and *Z*).

NAB-Gluc (300 MHZ, D_2O), 8.92 (m, 2H, 2-*E* and 6-*E*), 8.65 (m, 1H, 6-*Z*), 8.54 (m, 1H, 2-*Z*), 8.22 (m, 2H, 4-*E* and *Z*), 8.03 (m, 2H, 5-*E* and *Z*), 6.18 (m, 1H, 2'-*E* or *Z*), 5.7 (m, 1H, 2'-*E* or *Z*), 5.65 (m, 2H, 1''), 3.94 (m, 1H, 6'-*E*), 3.8-3.4 (m, 8H, 2'', 3'', 4'', and 5''), 3 (m, 1H,), 2.25 (m, 1H, 3'-*E* or *Z*), 2.2 (m, 1H, 3'-*E* or *Z*), 2-1.5 (m, 8H, 4',5').

NAT (300 MHZ, D_2O), 8.36 (d, 1H, 2-*E*), 8.32 (dd, 1H, 6-*E*), 8.28 (dd, 0.2H, 5 6-*Z*), 8.24 (d, 0.25H, 2-*Z*), 7.69 (m, 1H, 4-*E*), 7.59 (m, 0.25H, 4-*Z*), 7.30 (dd, 1H, 5-*E*), 7.25 (dd, 0.25H, 5-*Z*), 6.31 (m, 0.25H, 5'-*Z*), 6.0 (t, 1H, 5'-*E*), 5.9 (m, 1H, 2'-*E*), 5.75 (m, 0.25H, 2'-*Z*), 5.58 (m, 1.25H, 4'-*E* and *Z*), 4.2 (m, 0.5H, 6'-*Z*), 3.49 (m, 2H, 6'-*E*), 2.84 (m, 1.25H, 3'-*E* and *Z*).

NAT-Gluc (300 MHZ, D_2O), 8.9 (m, 2H, 2 and 6), 8.5 (m, 1H, 4), 8.01 (m, 1H, 5), 6.21 (m, 1H, 5'), 5.9 (m, 2H, 2' and 4'), 5.64 (m, 1H, 1''), 3.9-3.4 (m, 4H, 2'',3'', 4'', and 5''), 3.05-2.85 (m, 2H, 3').

NNN (300 MHz, D_2O) 8.35 (m, 1.6H, 2-*E*), 8.33(m,1H, 6-*E*), 8.24(m, 1H, 6-*Z*), 8.16(m,1.6H, 2-*Z*), 7.64(dt, 1.6H, 4-*E*), 7.43(dt, 1H, 4-*Z*), 7.32(q, 1.6H, 5-*E*), 7.24(q, 1H, 5-*Z*), 5.52(t,1.6H,2′-*E*), 5.13(t,1H, 2′-*Z*), 4.5-4.3(m,2H, 5′-*Z*) 3.8-3.55(m, 3.2H,5′-*E*), 2.5-2.38(m,2H, 3′-E and Z), 2.1-2.0(m, 3.2H, 4'-*E* and *Z*).

NNN-Gluc (300 MHz, D_2O) 9.04(m,1H,2-*E*), 8.92(m,1H, 6-*E*), 8.83(m, 1H, 6-*Z*), 8.81(m,1H,2-*Z*), 8.50(m, 1H, 4-*E*), 8.27(m,1H, 4-*Z*), 8.04(m, 1H, 5-*E*), 7.95(m,1H, 5-*Z*), 5.7(m,1H, 2′-E), 5.65(m, 2H, 1”), 5.28(m,1H, 2′-Z), 4.55(m,1H, 5′-*Z*), 4-3.4(m, 9H, 5′-*E*, 2”, 3”, 4”, and 5”), 2.6(m, 2H, 3′), 2.0(m, 6H, 3′ and 4’).

Analysis of UGT2B10 versus UGT1A4 expression levels. To examine the relative expression of UGT2B10, UGT2B11 and UGT1A4 in newly-developed HEK293 over-expressing cell lines or in 20 human liver specimens, real-time PCR was performed using the TaqMan Gene Expression Assay kit from Applied Biosystems (ID’s Hs02556282_s1, Hs01894900 gh, and Hs0655285_s1 for UGTs 2B10, 2B11, and UGT1A4, respectively, and Hs99999905_m1 for GAPDH as a control) as per manufacturer’s protocols. Real-time PCR was performed using a 10-μL total reaction volume containing 40 ng of RT PCR cDNA using GAPDH as the normalizing ‘housekeeping’ gene for expression, with triplicates performed for each sample.

Statistical analysis. Kinetic constants were determined using Prism Version 4.01 software (GraphPad Software, San Diego, CA, USA).

Results

Characterization of NNN, NAB, and NAT glucuronides in HLM. As shown previously for NNAL (Ren et al., 2000; Wiener et al., 2004b), TSNA-glucuronide formation was observed for NNN, NAB and NAT in HLM as determined by HPLC analysis, with postulated glucuronide peaks observed at retention times of 17.8, 19.2, and 18.9 min, respectively (Figure 2). Collection of HPLC peaks predicted to correspond to the glucuronide conjugates of NNN, NAB and NAT were sensitive to treatments with either β-glucuronidase and 1M NaOH (data not shown), suggesting that these glucuronides corresponded to *N*-glucuronides as observed previously for NNAL (Wiener et al., 2004a). To better characterize the HPLC peaks corresponding to predicted TSNA glucuronides, the ^{1}H NMR spectra were obtained for the putative glucuronides. For these analyses, large microsomal preparations were made from bovine liver (30 g tissue) and used in assays identical to those performed for HLM as described above, with the HPLC peaks predicted to contain each of the TSNA-glucuronides collected and analyzed by NMR spectroscopy. Bovine liver was used since there was insufficient human liver available for synthesis of the large quantities (μg amounts) of TSNA glucuronides required for NMR analysis. Predicted TSNA-glucuronide HPLC peaks were observed for bovine liver microsomes with retention times and sensitivities to β-glucuronidase or 1M NaOH that were identical to that observed for HLM (data not shown). The NMR spectra of the nitrosamines show variable amounts of the E- and Z-isomers of the nitroso group; NNN had a 3:2 ratio while its corresponding glucuronide had a 1:1 ratio of the E and Z-isomers. Both NAB

and NAB-Gluc showed equal amounts of the *E*- and *Z*-isomers, while NAT had a 4:1 ratio of the *E*- and *Z*-isomers. The small amount of NAT-Gluc that was isolated did not allow the observation of the Z-isomer. The ^{1}H NMR spectra of NNN, NAB, and NAT were compared with the isolated glucuronides to determine the site of glucuronidation. The key changes in the NMR spectra resulting from the glucuronidation were in the chemical shifts of the pyridyl group. The NNN, NAB, and NAT pyridyl protons were shifted downfield 0.4 – 0.7 ppm in the glucuronides. This chemical shift difference is consistent with glucuronidation of the pyridyl nitrogen as previously demonstrated for NNK and NNN (Upadhyaya et al., 2001).

TSNA glucuronidation in UGT-overexpressing cells. Real-time PCR demonstrated that UGTs 2B10 and 2B11 mRNA were expressed at 0.075 ± 0.006- and 0.179 ± 0.028–fold the levels of GAPDH mRNA in the newly-constructed UGT2B10- and UGT2B11-over-expressing cells, respectively. Homogenates of these and previously-described UGT-over-expressing cell lines were used in assays to identify the specific UGTs that exhibited *N*-glucuronidation activity against TSNAs *in vitro* (Table 1). As described previously for NNAL (Wiener et al., 2004a), UGT1A4 was active against NNN, NAB, and NAT in the present study. In addition to UGT1A4, UGT2B10 was also found to be active against NNN, NAB and NAT. No other UGT screened in this study (including UGT2B11) exhibited detectable *N*-glucuronidating activity against any of these TSNAs. All of the HPLC peaks identified for UGT1A4 and UGT2B10 against these TSNAs exhibited the same retention times as that observed for the respective TSNA-*N*-glucuronide formed with HLM (or bovine liver microsomes) and exhibited sensitivity to β-glucuronidase as well as 1M NaOH (data not shown), suggesting *N*-

glucuronide formation for these enzymes. Since NNAL is *N*-glucuronidated like other TSNAs, homogenates from UGT2B10-overexpressing cells were also tested for activity against this TSNA. Similar to that observed previously for UGT1A4, UGT2B10 also exhibited *N*-glucuronidation activity against NNAL (Table 1). The retention time for the NNAL glucuronide peak observed for UGT2B10 homogenates was identical to that observed for UGT1A4 and exhibited sensitivity to β-glucuronidase as well as 1M NaOH (data not shown). No activity was observed for homogenates of UGT2B11-over-expressing cells against NNAL.

Kinetic analysis of NNAL, NNN, NAB and NAT glucuronidation by UGT1A4, UGT2B10 and HLM. Kinetic analysis was performed for UGT1A4, UGT2B10 and HLM against NNAL, NNN, NAB and NAT. Representative plots of glucuronidation rate versus substrate concentration are shown in Figure 3. As summarized in Table 2, UGT2B10 exhibited between 6- and 22-fold lower K_M's against NNN, NAB and NAT than UGT1A4. The K_M's of HLM were comparable to those observed with UGT2B10 against NAB and NAT, and was 3.7-fold lower than HLM against NNN and approximately 4-fold higher than HLM against NNAL.

As the V_{max} data reported in Table 2 were calculated based on total cell homogenate or HLM protein and were not normalized for UGT protein, direct comparisons of V_{max} were not performed between UGT-over-expressing cell lines or with HLM. However, real-time PCR of UGT2B10 versus UGT1A4 expression analysis was performed for their respective HEK293 UGT-over-expressing lines, demonstrating that UGT1A4 was expressed at the level of mRNA at a relative level that was 4-times higher than that observed for UGT2B10 in their respective over-expressing cell lines.

Using this ratio as a normalization factor, UGT2B10 exhibited 3-3.6-fold higher overall activity against NNAL and NNN than UGT1A4, and a 19-27-fold higher level of overall activity than UGT1A4 against NAB and NAT.

Assessment of UGT1A4 and UGT2B10 expression in human liver. To better assess the relative importance of UGT2B10 versus UGT1A4 in the *N*-glucuronidation of TSNAs in human liver, real-time PCR was performed using total RNA purified from 20 human liver specimens obtained from individual subjects. Relative to the expression of a house-keeping gene (GAPDH), the expression of UGT2B10 was 1.3-fold that observed for UGT1A4 for the 20 specimens. The range of expression of UGT2B10 and UGT1A4 between liver specimens was 14- and 13-fold, respectively. The relative expression of UGT2B10 verses UGT1A4 ranged from 0.16 to 5.8 for the 20 specimens analyzed, with 85% (n=17) of the samples analyzed exhibiting a UGT2B10:UGT1A4 ratio of >0.50 and 55% (n=11) exhibiting a ratio of >1.0 (results not shown).

Discussion

Glucuronidation is a major pathway for the elimination of NNAL and other TSNAs. Both the *O*- and *N*-glucuronide forms of NNAL-Gluc have been observed in the urine of smokers (Carmella et al., 1995; Richie et al., 1997; Carmella et al., 2002), never-smokers exposed to environmental tobacco smoke (Carmella et al., 1993; Parsons et al., 1998), and tobacco chewers (Murphy et al., 1994; Carmella et al., 2002), and both glucuronides were shown to be formed in HLM (Kim and Wells, 1996; Ren et al., 2000; Wiener et al., 2004a; Wiener et al., 2004b; Lazarus et al., 2005). Glucuronide conjugates comprise between 59-90% of the total NNAL, NNN, NAB, and NAT found in the urine of smokers (Carmella et al., 2002; Stepanov and Hecht, 2005). The importance of glucuronidation as a detoxification mechanism for NNAL and potentially other TSNAs is suggested by the fact that, in contrast to the relatively high tumorigenicity exhibited by NNAL, NNAL-Gluc is non-tumorigenic after subcutaneous injection into A/J mice (Upadhyaya et al., 1999), and skin fibroblasts from UGT family 1-deficient rats with decreased glucuronidation capacity were more sensitive to NNK-mediated cytotoxicity (Kim and Wells, 1996).

The present study is the first to examine the activity of specific UGT enzymes against the TSNAs NNN, NAT and NAB, with both UGT1A4 and UGT2B10 exhibiting glucuronidation activity against all TSNAs tested. While UGT1A4 was previously shown to exhibit *N*-glucuronidation activity against NNAL (Wiener et al., 2004a), the present study is also the first to demonstrate *N*-glucuronidation activity against NNAL for UGT2B10. The *N*-glucuronide formation activity exhibited by UGT2B10 against TSNAs

is consistent with the recent finding that UGT2B10 exhibits glucuronidation activity against both nicotine and cotinine (Chen et al., 2007). However, the glucuronidation activity observed for UGT2B10 against NNAL in the present study was not observed in a previous study (Wiener et al., 2004a). This may be due to differences in glucuronidation assay sensitivities, since several differences in the glucuronidation assay methodology employed between the two studies were identified, including the use of alamethecin, which increase glucuronidation activity 2-3 fold in *in vitro* assay systems (Fisher et al., 2000), in the current study.

The K_M exhibited by homogenates of UGT2B10-overexpressing cells was significantly lower than that observed for homogenates from UGT1A4-overexpressing cells against all TSNAs tested in the present study. This is similar to the differential kinetic pattern observed previously for UGT2B10 versus UGT1A4 against nicotine and cotinine (Chen et al., 2007; Kaivosaari et al., 2007). The mean relative mRNA expression level of UGT2B10 versus UGT1A4 was 1.3 in a series of normal human liver specimens, with 85% of the samples analyzed exhibiting a UGT2B10:UGT1A4 ratio of >0.50 and 55% exhibiting a ratio of >1.0. Since the overall activity of UGT2B10 in cell homogenate as determined by V_{max}/K_M after normalization for UGT expression levels was 3-27-fold higher for UGT2B10 than UGT1A4 against all TSNAs tested, these data suggest that UGT2B10 is the major *N*-glucuronidating enzyme for TSNAs in human liver and are consistent with the fact that the K_M of UGT2B10-overexpressing cell homogenates was similar to that observed for HLM against the same TSNAs.

The K_M observed for UGT2B10 against NNAL (3.6 mM) was comparable to that observed previously for the most active NNAL-*O*-glucuronidating enzyme, UGT2B17

[1.76 mM; (Lazarus et al., 2005)]. Previous studies have demonstrated that UGT2B10 is expressed at a 4-fold higher level than UGT2B17 in human liver (Nishimura and Naito, 2006). UGT2B10 is also well-expressed in target sites for tobacco-related cancers including lung (Strassburg et al., 1997; Turgeon et al., 2001; Nishimura and Naito, 2006). Together, these data suggest that UGT2B10 is a major enzyme responsible for the glucuronidation and detoxification of NNAL and potentially other TSNAs in humans.

References

Carmella SG, Akerkar S and Hecht SS (1993) Metabolites of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in smokers' urine. *Cancer Res* **53:**721-724.

Carmella SG, Akerkar SA, Richie JP, Jr. and Hecht SS (1995) Intraindividual and interindividual differences in metabolites of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in smokers' urine. *Cancer Epidemiol Biomarkers Prev* **4:**635-642.

Carmella SG, Le Ka KA, Upadhyaya P and Hecht SS (2002) Analysis of N- and O-glucuronides of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in human urine. *Chem Res Toxicol* **15:**545-550.

Chen G, Blevins-Primeau AS, Dellinger RW, Muscat JE and Lazarus P (2007) Glucuronidation of nicotine and cotinine by UGT2B10: loss of function by the UGT2B10 Codon 67 (Asp>Tyr) polymorphism. *Cancer Res* **67:**9024-9029.

Coughtrie MW, Burchell B and Bend JR (1986) A general assay for UDPglucuronosyltransferase activity using polar amino-cyano stationary phase HPLC and UDP[U-14C]glucuronic acid. *Anal Biochem* **159:**198-205.

Dellinger RW, Fang JL, Chen G, Weinberg R and Lazarus P (2006) Importance of UDP-glucuronosyltransferase 1A10 (UGT1A10) in the detoxification of polycyclic aromatic hydrocarbons: decreased glucuronidative activity of the UGT1A10139Lys isoform. *Drug Metab Dispos* **34:**943-949.

Fisher MB, Campanale K, Ackermann BL, VandenBranden M and Wrighton SA (2000) In vitro glucuronidation using human liver microsomes and the pore-forming peptide alamethicin. *Drug Metab Dispos* **28:**560-566.

Gueraud F and Paris A (1998) Glucuronidation: a dual control. *Gen Pharmacol* **31:**683-688.

Hecht SS (1998) Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. *Chem Res Toxicol* **11:**559-603.

Hecht SS and Hoffmann D (1989) The relevance of tobacco-specific nitrosamines to human cancer. *Cancer Surv* **8:**273-294.

Hoffmann D, Brunnemann KD, Prokopczyk B and Djordjevic MV (1994) Tobacco-specific N-nitrosamines and Areca-derived N-nitrosamines: chemistry, biochemistry, carcinogenicity, and relevance to humans. *J Toxicol Environ Health* **41:**1-52.

Kaivosaari S, Toivonen P, Hesse LM, Koskinen M, Court MH and Finel M (2007) Nicotine Glucuronidation and the Human UDP-Glucuronosyltransferase UGT2B10. *Mol Pharmacol* **72:**761-768.

Kim PM and Wells PG (1996) Genoprotection by UDP-glucuronosyltransferases in peroxidase-dependent, reactive oxygen species-mediated micronucleus initiation by the carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and benzo[a]pyrene. *Cancer Res* **56:**1526-1532.

Lazarus P, Zheng Y, Aaron Runkle E, Muscat JE and Wiener D (2005) Genotype-phenotype correlation between the polymorphic UGT2B17 gene deletion and

NNAL glucuronidation activities in human liver microsomes. *Pharmacogenet Genomics* **15:**769-778.

Murphy SE, Carmella SG, Idris AM and Hoffmann D (1994) Uptake and metabolism of carcinogenic levels of tobacco-specific nitrosamines by Sudanese snuff dippers. *Cancer Epidemiol Biomarkers Prev* **3:**423-428.

Nishimura M and Naito S (2006) Tissue-specific mRNA expression profiles of human phase I metabolizing enzymes except for cytochrome P450 and phase II metabolizing enzymes. *Drug Metab Pharmacokinet* **21:**357-374.

Nowell SA, Massengill JS, Williams S, Radominska-Pandya A, Tephly TR, Cheng Z, Strassburg CP, Tukey RH, MacLeod SL, Lang NP and Kadlubar FF (1999) Glucuronidation of 2-hydroxyamino-1-methyl-6-phenylimidazo[4, 5-b]pyridine by human microsomal UDP-glucuronosyltransferases: identification of specific UGT1A family isoforms involved. *Carcinogenesis* **20:**1107-1114.

Parsons WD, Carmella SG, Akerkar S, Bonilla LE and Hecht SS (1998) A metabolite of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in the urine of hospital workers exposed to environmental tobacco smoke. *Cancer Epidemiol Biomarkers Prev* **7:**257-260.

Ren Q, Murphy SE, Zheng Z and Lazarus P (2000) O-Glucuronidation of the lung carcinogen 4-(methylnitrosamino)-1- (3-pyridyl)-1-butanol (NNAL) by human UDP-glucuronosyltransferases 2B7 and 1A9. *Drug Metab Dispos* **28:**1352-1360.

Richie JP, Jr., Carmella SG, Muscat JE, Scott DG, Akerkar SA and Hecht SS (1997) Differences in the urinary metabolites of the tobacco-specific lung carcinogen 4-

(methylnitrosamino)-1-(3-pyridyl)-1-butanone in black and white smokers. *Cancer Epidemiol Biomarkers Prev* **6:**783-790.

Rivenson A, Hoffmann D, Prokopczyk B, Amin S and Hecht SS (1988) Induction of lung and exocrine pancreas tumors in F344 rats by tobacco-specific and Areca-derived N-nitrosamines. *Cancer Res* **48:**6912-6917.

Stepanov I and Hecht SS (2005) Tobacco-specific nitrosamines and their pyridine-N-glucuronides in the urine of smokers and smokeless tobacco users. *Cancer Epidemiol Biomarkers Prev* **14:**885-891.

Strassburg CP, Oldhafer K, Manns MP and Tukey RH (1997) Differential expression of the UGT1A locus in human liver, biliary, and gastric tissue: identification of UGT1A7 and UGT1A10 transcripts in extrahepatic tissue. *Mol Pharmacol* **52:**212-220.

Sun D, Chen G, Dellinger RW, Duncan K, Fang JL and Lazarus P (2006) Characterization of tamoxifen and 4-hydroxytamoxifen glucuronidation by human UGT1A4 variants. *Breast Cancer Res* **8:**R50.

Tephly TR and Burchell B (1990) UDP-glucuronosyltransferases: a family of detoxifying enzymes. *Trends Pharmacol Sci* **11:**276-279.

Turgeon D, Carrier JS, Levesque E, Hum DW and Belanger A (2001) Relative enzymatic activity, protein stability, and tissue distribution of human steroid-metabolizing UGT2B subfamily members. *Endocrinology* **142:**778-787.

Upadhyaya P, Kenney PM, Hochalter JB, Wang M and Hecht SS (1999) Tumorigenicity and metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol enantiomers and metabolites in the A/J mouse. *Carcinogenesis* **20:**1577-1582.

Upadhyaya P, McIntee EJ and Hecht SS (2001) Preparation of pyridine-N-glucuronides of tobacco-specific nitrosamines. *Chem Res Toxicol* **14:**555-561.

Wiener D, Doerge DR, Fang JL, Upadhyaya P and Lazarus P (2004a) Characterization of N-glucuronidation of the lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in human liver: importance of UDP-glucuronosyltransferase 1A4. *Drug Metab Dispos* **32:**72-79.

Wiener D, Fang JL, Dossett N and Lazarus P (2004b) Correlation between UDP-glucuronosyltransferase genotypes and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone glucuronidation phenotype in human liver microsomes. *Cancer Res* **64:**1190-1196.

Footnotes

These studies were supported by Public Health Service grants P01-CA68384 (Lazarus), R01-DE13158 (Lazarus), and R01-CA75074 (Spratt) from the National Institutes of Health, and two formula grant under the Pennsylvania Department of Health's Health Research Formula Funding Program (State of PA, Act 2001-77 – part of the PA Tobacco Settlement Legislation; to Lazarus, Spratt). We thank the Tissue Procurement Facility at the H. Lee Moffitt Cancer Center for the human tissues examined in these studies. We thank the Functional Genomics Core Facility at the Penn State College of Medicine for real-time PCR services.

Figure Legends

Figure 1. Schematic of TSNA structures.

Figure 2. HPLC analysis of NNAL-Gluc, NNN-Gluc, NAB-Gluc, and NAT-Gluc formation by human liver microsomes. Human liver microsomes (0.5 mg of protein) were incubated at 37°C for 2 h with 4 mM ^{14}C-UDPGA and 5 mM TSNA. HPLC was performed as described in the Materials and Methods. Panel **A**, NNAL; panel **B**, NNN; panel **C**, NAB; panel **D,** NAT. TSNA-glucuronide and UDPGA peaks are indicated.

Figure 3. Concentration curves for NNAL-, NNN-, NAB-, and NAT-*N*-glucuronide formation with homogenates from UGT1A4- and UGT2B10-overexpressing cells and HLM from one individual. Glucuronide formation assays were performed at 37°C for 2 h using 250 μg cell homogenate or HLM protein as described in Materials and Methods. Representative curves are shown – all kinetic analysis was performed in three independent experiments

DMD Fast Forward. Published on January 31, 2008 as DOI: 10.1124/dmd.107.019406
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. *N*-Glucuronide formation activity of individual UGT over-expressing HEK293 cell lines against TSNAs.

UGT	TSNA			
	NNN	NAB	NAT	NNAL
1A1	-[a]	-	-	-[b]
1A3	-	-	-	-[c]
1A4	+	+	+	+[c]
1A6	-	-	-	-[b]
1A7	-	-	-	-[b]
1A8	-	-	-	-[b]
1A9	-	-	-	-[b]
1A10	-	-	-	-[b]
2B4	-	-	-	-[b]
2B7	-	-	-	-[b]
2B10	+	+	+	+
2B11	-	-	-	-
2B15	-	-	-	-[b]
2B17	-	-	-	-[d]

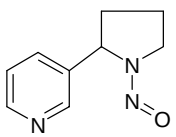
[a] not detected.
[b] No *N*-glucuronidation activity was observed against NNAL for the indicated UGTs in previous studies by Ren et al (2000; ref. 9) and [d] Lazarus et al (2005; ref. 15).
[c] UGT1A4 exhibited *N*-glucuronidation activity against NNAL in previous studies by [c] Wiener et al (2004; ref. 14) and in the present study.

Table 2. Kinetic analysis of *N*-glucuronidation in UGT1A4- and UGT2B10-overexpressing cell homogenates and HLMs of TSNAs.

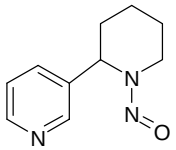
	TSNA	V_{max} (pmol·min^{-1}·mg total protein^{-1})	K_M (mM)	V_{max}/K_M (nL·min^{-1}·mg total protein^{-1})	normalized V_{max}/K_M UGT2B10:UGT1A4 ratio[a]
UGT1A4	NNAL	99 ± 27[b]	22.79 ± 3.83	4.35 ± 0.96	referent
	NNN	182 ± 43	26.24 ± 14.27	7.70 ± 2.13	referent
	NAB	166 ± 22	5.37 ± 1.61	32.1 ± 6.63	referent
	NAT	124 ± 4	3.31 ± 0.23	37.6 ± 2.31	referent
UGT2B10	NNAL	7.55 ± 0.37	3.62 ± 2.31	3.07 ± 2.13	3.0
	NNN	7.66 ± 1.63	1.18 ± 0.27	6.60 ± 1.08	3.6
	NAB	41.09 ± 11.28	0.28 ± 0.00	147 ± 42.0	19
	NAT	52.65 ± 14.99	0.22 ± 0.06	242 ± 2.86	27
HLM[c]	NNAL	259 ± 42	0.85 ± 0.42	348 ± 163	
	NNN	1,400 ± 60	3.17 ± 0.37	446 ± 61	
	NAB	2,476 ± 621	0.20 ± 0.03	12,806 ± 5,069	
	NAT	2,593 ± 457	0.16 ± 0.01	16,227 ± 2,852	

[a] V_{max}/K_M normalized by mRNA expression levels in UGT1A4- and UGT2B10-overexpressing HEK293 cell lines using GAPDH as an expression control.
[b] Data are expressed as the mean ± standard deviation of three independent experiments.
[c] HLM from one individual.

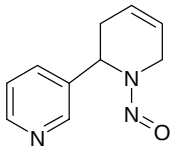
Figure 1



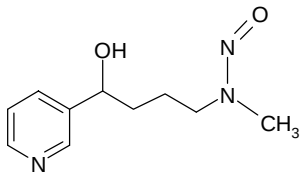
NNN



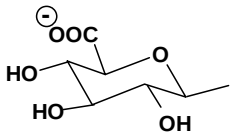
NAB



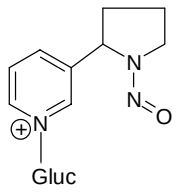
NAT



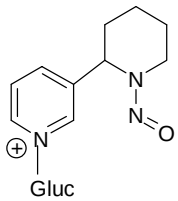
NNAL



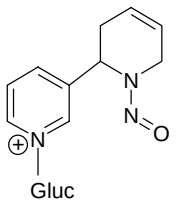
-Gluc



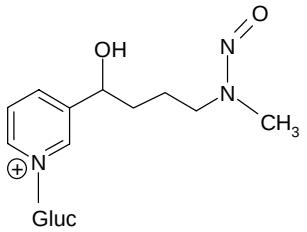
NNN-*N*-Gluc



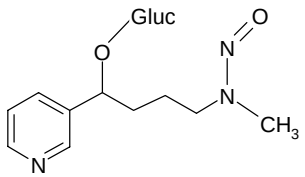
NAB-*N*-Gluc



NAT-*N*-Gluc



NNAL-*N*-Gluc



NNAL-*O*-Gluc

Figure 2

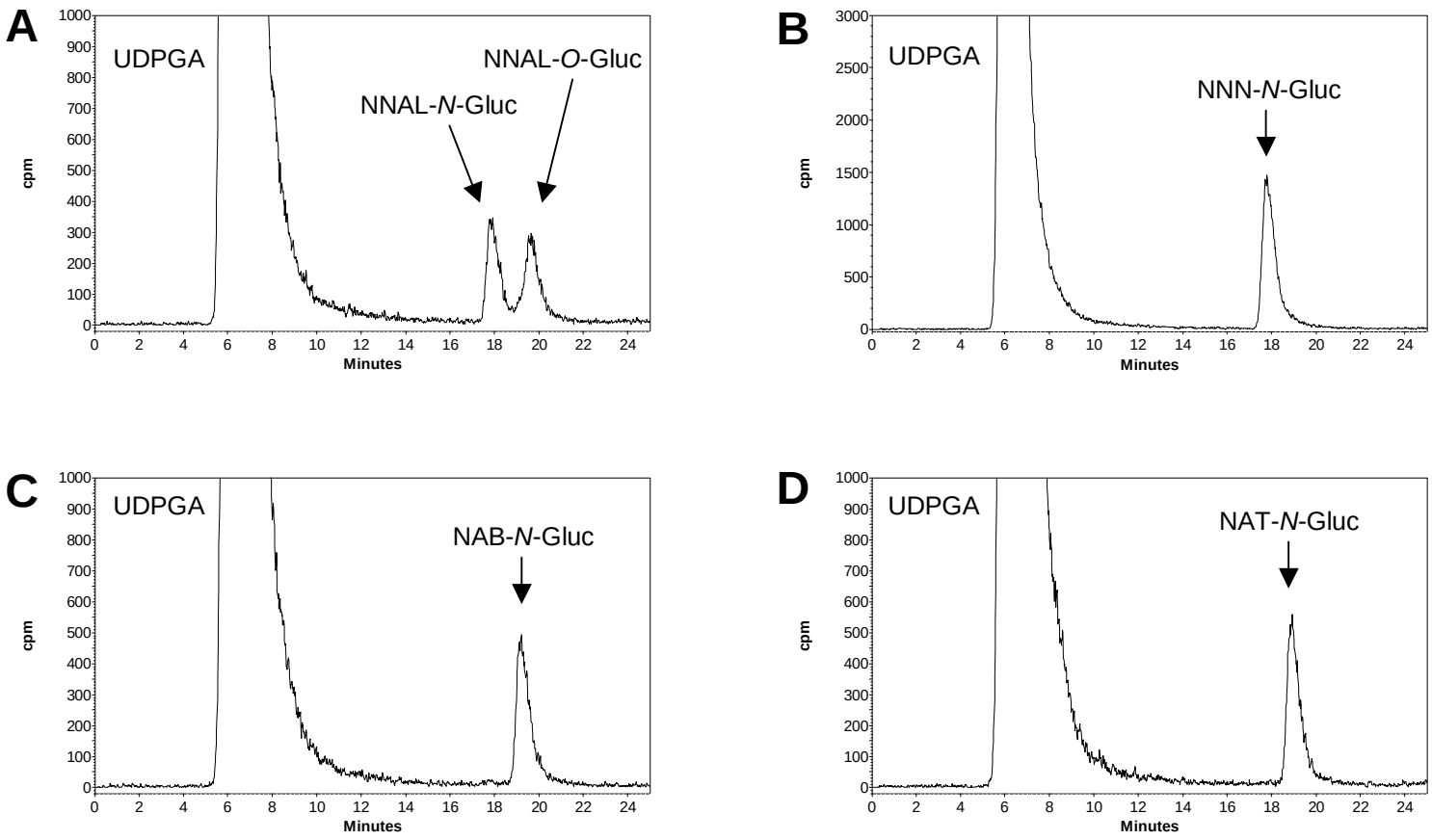


Figure 3