CHARACTERIZATION OF N-GLUCURONIDATION OF THE LUNG CARCINOGEN
4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANOL (NNK) IN HUMAN LIVER:
IMPORTANCE OF UDP-GLUCURONOSYLTRANSFERASE 1A4

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ABSTRACT:

The nicotine-derived tobacco-specific nitrosamine, 4-(methylnitrosa-
mino)-1-(3-pyridyl)-1-butanol (NNK), is one of the most potent and
abundant procarcinogens found in tobacco and tobacco smoke and is considered to be a causative agent for several tob-
acco-related cancers. Glucuronidation of the major metabolite of
NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), has
been implicated as an important mechanism for NNK detoxifica-
tion. To characterize NNAL N-glucuronidation in humans, high-pressure liquid chromatography was used to detect
glucuronides of NNAL formed in human liver micro-
somes in vitro. In addition to peaks corresponding to the
O-glucuronides of NNAL (NNAL-O-Gluc), a second series of peaks were
observed in human liver microsomes that were identified by liquid
chromatography-mass spectrometry to be NNAL N-glucuronides
(NDAL-N-Gluc). Microsomes prepared from liver specimens from
individual subjects (n = 42) exhibited substantial variability in the
levels of NNAL-N-Gluc (49-fold variability) and NNAL-O-Gluc (49-
fold variability) formed in vitro. This variability was likely not due to
differences in tissue quality, as substantial variability (5-fold) was
also observed in the ratio of NNAL-N-Gluc/NNAL-O-Gluc forma-
tion, with a mean ratio of 1.7 in the 42 specimens. Liver micro-
somes from smokers (n = 14) exhibited no significant difference in
the levels of either NNAL-N-Gluc or NNAL-O-Gluc formation, or in
the ratio of NNAL-N-Gluc/NNAL-O-Gluc formation, as compared
with liver microsomes from never smokers (n = 28). Overex-
pressed UDP-glucuronosyltransferase (UGT) 1A4 exhibited signif-
ificant levels of N-glucuronidating activity (Vmax/Km = 3.11 μl ·
min⁻¹ · g⁻¹) in vitro; no NNAL-N-glucuronide formation was detected for the 11 other overexpressed UGT enzymes tested in
these studies. These results demonstrate the importance of N-
glucuronidation in the metabolism of NNAL and the role of UGT1A4
in this pathway.

The nicotine-derived tobacco-specific nitrosamine NNK¹ is one of
the most potent and abundant procarcinogens found in tobacco and
tobacco smoke (Hecht and Hoffmann, 1989; Hecht, 1998). NNK
levels in tobacco smoke are 3 to 15 times higher than the levels of
another major potent carcinogen in tobacco smoke, benzo[a]pyrene
(Adams et al., 1987). NNK induces predominantly lung adenocarci-
nomas in rodents independent of the route of administration (Hecht,
1998). In the Fischer 344 rat, NNK induces pancreatic tumors (Riv-
enson et al., 1988) and, when applied together with the related
tobacco-specific nitrosamine, N'-nitrosonornicotine, oral cavity tu-
ors (Hecht et al., 1986). The cumulative dose of 1.8 mg of NNK/kg
of body weight required to produce lung tumors in rodents (Belinsky
et al., 1990) is similar to the cumulative lifetime dose of 1.6 mg of
NNK/kg of body weight for the average American smoking two packs
of cigarettes a day for 40 years (Hecht and Hoffmann, 1989; Hecht,
1998). NNK is therefore considered to be a likely causative agent for
several tobacco-related cancers in humans, including lung, oral cavity,
and pancreas (Rivenson et al., 1988; Hecht, 1998).

The major metabolic pathway of NNK in most tissues is car-
boxyl reduction to NNAL. NNK reduction to NNAL occurs in
rodents, monkeys, and humans (Carmella et al., 1993; Hecht et al.,
1993; Hecht, 1998), and it is estimated that between 39 and 100%
of the NNK dose is converted to NNAL in smokers (Carmella et al.,
1993). NNAL is activated via pathways similar to those ob-
erved for NNK and, like NNK, is a potent lung and pancreatic
carcinogen in rodents (Rivenson et al., 1988; Hecht, 1998). Previ-

¹ Abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; UGT, UDP-glucuronosyltransferase; NNAL-N-Gluc, β-N-[4-(methylnitrosamino)-1-(3-pyridyl)-1-but-1-yl]-α-D-glucosiduronic acid; NNAL-O-Gluc, β-O-[4-(methylnitrosamino)-1-(3-pyridyl)-1-but-1-yl]-α-D-glucosiduronic acid; UDPGA, UDP-glucuronic acid; HPLC, high-pressure liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry.
ous studies have shown that NNAL is also metabolized to its glucuronide, NNAL-Gluc (see Fig. 1) (Morse et al., 1990; Carmella et al., 1993; Hecht et al., 1993; Hecht, 1998; Ren et al., 2000). Although the formation of NNAL is not a detoxification pathway for NNK, the glucuronidation of NNAL appears to be an important mechanism for NNK detoxification. This is supported by the fact that the glucuronidation of R-NNAL was significantly greater than S-NNAL after injection into A/J mice, a pattern consistent with the higher tumorigenicity exhibited by S-NNAL in the same experiments (Upadhyaya et al., 1999, 2000). In contrast to the relatively high tumorigenicity exhibited by both R- and S-NNAL, NNAL-Gluc is nontumorigenic after subcutaneous injection into A/J mice (Upadhyaya et al., 1999). In addition, Kim and Wells (1996) reported that skin fibroblasts from UGT family 1-deficient rats were more sensitive to NNK-mediated cytotoxicity.

Glucuronidation can occur at both the carbinol group of NNAL (NNAL-O-Gluc) (Morse et al., 1990; Carmella et al., 1993; Hecht et al., 1993; Hecht, 1998; Ren et al., 2000) and on the pyridine nitrogen on NNAL’s ring (NNAL-N-Gluc) (Carmella et al., 2002; see Fig. 1). Although NNAL-O-Gluc formation in human tissues is well characterized and was found to be mediated in a stereospecific manner by UGTs 1A9 and 2B7 (Ren et al., 2000), the identification of NNAL-N-Gluc in human urine has only recently been identified (Carmella et al., 2002). The relative ratio of NNAL-N-Gluc/NNAL-O-Gluc formation in the urine of smokers was observed to be approximately 1.0 (Carmella et al., 2002), implicating NNAL-N-Gluc formation as an important detoxification mechanism for NNAL and NNK. Significant interindividual variability in the ratios of both NNAL-Gluc/NNAL (Carmella et al., 1995) and NNAL-N-Gluc/NNAL-O-Gluc (Carmella et al., 2002) was observed in the urine of smokers and snuff users, suggesting that individuals may differ greatly in their ability to detoxify NNK and to form different NNAL glucuronides. In addition, the ratio of NNAL-N-Gluc/NNAL-O-Gluc was significantly higher in the urine of smokers compared with the urine of snuff users (Carmella et al., 2002). The goal of the present study was to better characterize NNAL-N-glucuronide formation in human liver and to identify the major UGT(s) responsible for this metabolic pathway.

Materials and Methods

Chemicals and Materials. UDPGA, D,L-2-lysophosphatidyl choline palmito-
tial C16:0, and β-glucuronidase were purchased from Sigma-Aldrich (St. Louis, MO). NNAL was purchased from Toronto Research Labs (Toronto, ON, Canada), and 14C-UDPGA (specific activity: 300 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Dulbecco’s modified Eagle’s medium, fetal bovine serum, trypsin-EDTA, geneticin, and Dulbecco’s phosphate-buffered saline (minus calcium and magnesium) were obtained from Invitrogen (Carlsbad, CA). Baculoses preparations overexpressing UGTs 1A3, 1A7, and 1A10 were purchased from PanVera Corp. (Madison, WI), and baculosomes overexpressing UGTs 1A4 and 1A9 were purchased from BD Biosciences (San Jose, CA).

Tissues. Normal human liver, lung, and tonsil tissue specimens were provided by the Tissue Procurement Facility at the H. Lee Moffitt Cancer Center from individuals (n = 42) undergoing surgery for resection of hepatocellular carcinoma. Tissue samples were quick-frozen at −70°C within 2 h after surgery. Micromeres for all tissues were prepared through differential centrifugation as previously described (Coughtrie et al., 1987) and were stored (10−20 mg of protein/ml) at −70°C, with total protein concentrations measured using the BCA protein assay (Pierce Chemical, Rockford, IL). Demographic data including smoking habits were collected by medical chart review for corresponding individuals from whom liver specimens were obtained. Tissues were obtained only from subjects deemed “smoking-history informative” and who were identified as never, moderate, or heavy smokers on the patient medical charts; tissues from subjects with less definitive smoking history descriptions or from “light” smokers were not included in this analysis. None of the “smoking-history informative” subjects reviewed for this study were tobacco or snuff chewers as determined by analysis of chart data. All subjects were Caucasian, 65% were female, and the average age of these subjects was 52 years. All protocols involving the analysis of tissue specimens were approved by the institutional review board at the University of South Florida and in accordance with assurances filed with and approved by the United States Department of Health and Human Services.

Cell Lines and Cell Homogenate Preparation. HK293 (human embryonic kidney fibroblast) cells and HK293 cell lines overexpressing the human UGTs 1A1, 1A8, 2B4, 2B10, 2B7, or 2B15, as well as the rat UGT2B1, have been previously described (Pritchard et al., 1994; Coffman et al., 1995; 1998; Green et al., 1994; King et al., 1997; Cheng et al., 1998; Ren et al., 2000). V79 (Chinese hamster fibroblast) cells and V79 cells overexpressing UGT1A6 were kindly provided by Brian Burchell (University of Dundee, Scotland, UK) (Ebner and Burchell, 1993).
All V79 and HK293 cell lines were grown to 80% confluence in Dulbecco’s modified Eagle's medium supplemented with 4.5 mM glucose, 10 mM HEPES, 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin, and maintained in 700 μg/ml of Geneticin for selection of UGT overexpression, in a humidified incubator in an atmosphere of 5% CO₂. For the preparation of cell homogenates, cells were suspended in Tris-buffered saline (25 mM Tris base, 138 mM NaCl, 2.7 mM KCl, pH 7.4) and subjected to three rounds of freeze/thaw before gentle homogenization. Cell homogenates (5–30 mg/ml) were stored at −70°C in 100-μl aliquots. Total cell homogenate protein concentrations were determined using the BCA protein assay as described above.

**NNAL-Glucuronidation Assays.** The rate of NNAL glucuronidation by tissue microsomes was determined after a preincubation with N,N′-2-lysophosphatidyl choline palmital C16:0 (0.2 mg/mg of protein) for 10 min at 4°C using the following conditions: microsomes (0.25 mg/ml) were incubated (100–000 μl aliquots) in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, N,N′-2-lysophosphatidyl choline palmital C16:0 (0.2 mg/mg of protein), under saturating conditions of both 14C-UDPGA (4 mM; 1–2 μCi/100 μl reaction volume) and NNAL (5 mM) at 37°C for up to 2 h. Reactions were terminated by the addition of 1/10 volume of 0.3 M Ba(OH)₂/0.3 N ZnSO₄ on ice. The precipitate was removed by centrifugation, and the supernatant was subjected to solid-phase extraction on an Oasis HLB 3 cc reverse-phase cartridge (Waters, Milford, MA) activated with acetonitrile and equilibrated with buffer A (50 mM ammonium acetate; pH 7.0). After loading onto the cartridge, the sample was washed with 1 ml of buffer A and eluted with 0.5 ml of acetonitrile. The acetonitrile was evaporated, the resulting sample diluted to 110 μl with water, and the sample was analyzed for glucurononated NNAL metabolites by HPLC with radioflow detection using the following system: a Waters dual-pump (model 510) HPLC system (Waters), equipped with an automatic injector (WISP model 710B), a UV detector operated at 254 nm (model 440), and a radioflow detector (INUS Systems, Fairfield, NJ). For the purposes of discriminating between different NNAL-N-Gluc isomers, HPLC was performed using a Spherisorb ODS1 5-μm C18 column (4.6 mm × 250 mm; Thermo Hypersil, Keystone Scientific Operations, Bellefonte, PA) with gradient elution at 1 ml/min using the following conditions: 10 min with 100% buffer B (0.2 M ammonium acetate; pH 7.0), a linear gradient for 10 min to 15% buffer C (100% methanol), a subsequent linear gradient to 35% buffer C for 20 min, and a final linear gradient to 100% buffer C for 5 min. The column was washed for 15 min with 100% buffer C and regenerated for 15 min with 100% buffer B.

For all other experiments in human liver microsomes, cell homogenates, and baculocytosis, NNAL-N-Gluc formation was assayed using a 5-μm Aquasil C18 column (4.6 mm × 250 mm, Thermo Hypersil, Keystone Scientific Operations) with gradient elution at 1 ml/min using the following conditions: 5 min with 100% buffer A, a linear gradient for 10 min to 30% buffer C, a subsequent linear gradient to 50% buffer C for 10 min, and a final linear gradient to 100% buffer B for 5 min. The column was washed for 10 min with 100% buffer C and regenerated for 15 min with 100% buffer A. Although usage of the Aquasil column resulted in decreased separation of individual peaks corresponding to NNAL-N-Gluc isomers as compared with the Spherisorb ODS1 column, it allowed for more accurate quantification of total NNAL-N-Gluc formation and was more useful in UGT-overexpressing cell line homogenate or baculosome activity-screening assays. In addition, the Aquasil C18 column is specifically designed to withstand prolonged exposure to aqueous conditions while maintaining retention of polar compounds (a problem encountered with the Spherisorb column), a characteristic useful for the analysis of total NNAL-N-Gluc formation for a large number of samples. For quantification of NNAL-N-Gluc and NNAL-O-Gluc formation in liver microsomes, assays were repeated for randomly selected samples (n = 17) to assure the validity and reproducibility of our assay system.

Where indicated, 14C-NNAL-O-Gluc was coinjected (5000–7000 dpm) with the reaction mixture and monitored by radioflow detection. 14C-NNAL-O-Gluc was prepared by HPLC purification from glucuronidation assays with UGT2B1-overexpressing cell homogenates as described previously (Ren et al., 1999). 14C-NNAL-Gluc peaks were tentatively identified by relative retention time and confirmed by sensitivity to Escherichia coli β-glucuronidase treat-

ment as previously described (Ren et al., 1999, 2000). The parent HK293 or V79 cell lines served as negative controls for in vitro glucuronidation reactions. Kinetic analysis for UGT1A4-induced glucuronidation of NNAL was performed as described above using 0.6 mg of baculosomal protein, 0.05 to 20 mM NNAL, and 2 mM 14C-UDPGA (2 μCi/100 μl reaction volume) at 37°C for 2 h.

**LC-MS/MS Analysis of NNAL Glucuronides.** The LC separation was performed using a Spherisorb ODS1 column under conditions described above for the discrimination of NNAL-N-Gluc isomers by HPLC. The column effluent was split such that 20% entered the mass spectrometer and 80% went to an AD20 UV detector (Dionex Corp., Sunnyvale, CA) set at 254 nm. Mass spectra were acquired on a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization interface. The desolvation temperature was 400°C, and the ion source temperature was 120°C. Nitrogen was used as the cone gas and desolvation gas at 100 and 750 liter/h, respectively. Full-scan positive and negative ion mass spectra were acquired simultaneously over a mass range of 100 to 450 with a cone voltage of 40 V. Subsequently, product ion mass spectra were acquired for m/z 386 in positive ion mode and m/z 384 in negative ion mode. The product ion spectra were acquired simultaneously using a collision gas cell pressure of 1.6 × 10⁻³ mbar and collision energy of 30 eV with a cone voltage of 40 V.

**Statistical Analysis.** The Student’s t test (two-tailed) was used for all comparative analyses. The correlation coefficient (r²) from linear regression analysis of Lineeweaver-Burk plots from three independent experiments was used to determine the Kₗ and Vₘ₉ₙ of UGT1A4-induced glucuronidation of NNAL.

**Results.**

The N-glucuronide of NNAL was previously identified in human urine specimens from smokers and tobacco chewers at levels similar to those observed for its O-glucuronide (Carmella et al., 2002). We hypothesized that since NNAL-N-Gluc is more polar than NNAL-O-Gluc (Carmella et al., 2002), NNAL-N-Gluc would be poorly retained on the C18 column previously used to identify glucuronide formation in assays of liver microsomes (Ren et al., 1999, 2000), with NNAL-N-Gluc peaks potentially masked by the highly polar UDPGA peak at early HPLC retention times. To analyze whether NNAL-N-Gluc is formed in human liver, an HPLC method was developed to distinguish between NNAL-N- and O-glucuronides. In addition to the polar UDPGA peak (retention time of ~4 min), two distinct peak regions containing four subpeaks (peak region 1; retention times between 14–21 min) and two subpeaks (peak region 2; retention times between 26–30 min) were resolved by HPLC analysis of NNAL-glucuronidation assays with human liver microsomes using a Spherisorb ODS1 HPLC column (see Materials and Methods) (Fig. 2A). The ratio of peak region 1/peak region 2 for this liver specimen was 1.3. The retention time of peak region 1 was identical to that observed for synthesized NNAL-N-Gluc standard (Upadhyaya et al., 2002) assayed in the same HPLC system (Fig. 2B). Only peak region 2 was observed by HPLC analysis of NNAL-glucuronide formation assays using cell homogenates prepared from rat UGT2B1-overexpressing HK293 cells (Fig. 2C), a UGT shown previously to form NNAL-O-Gluc (Ren et al., 1999). Both of these peak regions formed from human liver microsomes were sensitive to treatment with β-glucuronidase (Fig. 2D). These data suggest that peak region 2 was comprised of isomers of NNAL-O-glucuronides, whereas peak region 1 was potentially comprised of isomers of NNAL-N-glucuronides. Neither peak region 1 nor peak region 2 was observed in assays of lung or tonsil microsomes (using up to 1 mg of microsomal protein; results not shown).

To determine whether the four HPLC peaks of peak region 1 contained isomers of NNAL-N-glucuronides, LC-MS/MS was performed. The product ion mass chromatograms and spectra (Figs. 3 and 4, respectively) are shown for NNAL glucuronidases formed in vitro assays with human liver microsomes. Analysis of NNAL glucuronidases using positive ion electrospray tandem mass spectrometry in the

A large degree of variability in total urinary NNAL-Gluc and urinary NNAL-N-Gluc/NNAL-O-Gluc ratios were observed between individual subjects in previous studies (Carmella et al., 2002). To determine whether variability in NNAL-N-Gluc formation exists for human liver specimens from individual subjects, NNAL-glucuronidation assays were performed for microsomes prepared from liver specimens from 42 different subjects. Substantial variability in the ratio of NNAL-N-Gluc/NNAL-O-Gluc was observed, with a ratio range of 0.65 to 3.21 (Table 1). The mean (± S.D.) NNAL-N-Gluc/NNAL-O-Gluc ratio for the 42 specimens was 1.70 ± 0.58. Substantial variability was also observed between liver microsomal specimens from individual subjects in terms of absolute levels of both NNAL-N-Gluc (49-fold) and NNAL-O-Gluc (49-fold) formation, with a range of 0.48 to 23.3 and 0.27 to 13.4 pmol/mg microsomal protein/min for NNAL-N-Gluc and NNAL-O-Gluc, respectively (Table 1).

Recent studies suggest that variability in levels of NNAL glucuronidation may be linked to subject smoking habits, with higher ratios of urinary NNAL-N-Gluc/NNAL-O-Gluc observed in smokers compared with tobacco chewers (Carmella et al., 2002). To assess whether smoking influences NNAL-N-Gluc/NNAL-O-Gluc ratios in vitro assays of individual liver microsomal specimens, attempts were made to correlate NNAL-N-Gluc/NNAL-O-Gluc ratios with the smoking histories of the same subjects. Since this was a retrospective analysis of banked liver specimens, smoking data for individual subjects could only be collected by a review of associated medical chart data (see Materials and Methods). Of the tissue bank normal liver specimens from subjects with smoking history-informative chart data, 28 were from subjects identified as never smokers, 12 were from subjects who were identified as moderate smokers, and two were identified as heavy smokers. Although there was significant variability in the ratio of NNAL-N-Gluc/NNAL-O-Gluc for liver microsomes from individual subjects within smoking groups (range of 0.65–3.21 in never smokers and 0.68–2.52 in smokers), no significant association was observed between in vitro NNAL-N-Gluc/NNAL-O-Gluc ratios and corresponding subject smoking history (Table 2). The mean NNAL-N-Gluc/NNAL-O-Gluc ratio for liver microsomes from moderate-heavy smokers was 1.77, whereas the ratio was 1.67 for liver microsomes from never smokers. No increase in the mean NNAL-N-Gluc/NNAL-O-Gluc ratio was observed for liver microsomes from the two heavy-smoking subjects compared with liver microsomes from never smokers. Similarly, no correlation was observed between subject smoking history and absolute levels of NNAL-N-Gluc or NNAL-O-Gluc formation in human liver microsomes (Table 2).
Incubations of human liver microsomes with NNAL were separated using the Spherisorb ODS1 column and HPLC conditions described under Materials and Methods and analyzed using electrospray tandem mass spectrometry in positive and negative ion modes. A, total negative ions from m/z 384 showing NNAL-O-glucuronides. B, reconstructed m/z 149 (positive) ions from m/z 386 highlighting NNAL-N-glucuronides. C, total positive ions from m/z 386 showing all NNAL glucuronides.

Product ion mass spectra were recorded from the LC-MS/MS acquisition shown in Fig. 3 using positive and negative ion modes. A, negative precursor ion m/z 384, 20.31 min peak, for NNAL-O-glucuronide; B, positive precursor ion m/z 386, 20.31 min peak, for NNAL-O-glucuronide; and C, positive precursor ion m/z 386, 18.36 min peak, for NNAL-N-glucuronide.
5B), indicating that NNAL-N-glucuronides were formed by UGT1A4.

To identify whether any previously cloned human UGTs exhibited N-glucuronidating activity against NNAL, we performed a comprehensive screening of known human UGTs. All UGT-overexpressing cell lines or baculosomes used in the present studies were active against previously characterized aglycones (results not shown) (Fang et al., 2002). As shown in Fig. 5A, a peak was observed in assays using UGT1A4 baculosomes that was similar in retention time (14.2 min) to peak region 1 in assays using human liver microsomes (Fig. 5B), indicating that NNAL-N-glucuronides were formed by UGT1A4.

Of the 12 human UGT-overexpressing cell lines or baculosomes screened in this study, only baculosomes overexpressing UGT1A4 formed NNAL-N-Gluc (Table 3); no NNAL-glucuronidating activity was observed for UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A10, 2B4, 2B10, or 2B15. Similar activity was observed for microsomes prepared from UGT1A4-overexpressing cell lines (provided by Tom Tephly, University of Iowa; results not shown). As described in previous studies (Ren et al., 2000), both UGTs 1A9 and 2B7 formed NNAL-O-Gluc (peak 2), but no NNAL-N-Gluc (peak 1) formation was observed for either enzyme (Fig. 4, C and D; Table 3). The estimated $K_m$ for the N-glucuronidation of NNAL by UGT1A4 was 15.5 mM, with a $V_{max}/K_m$ of 3.11 pmol·min$^{-1}$·mg protein$^{-1}$. For human liver microsomes, the estimated $K_m$ for the N-glucuronidation of NNAL was 0.31 ± 0.08 mM, with a $V_{max}/K_m$ of 220 pmol·min$^{-1}$·g$^{-1}$.

**Discussion**

N-glucuronidation has been shown to be a major detoxification pathway for the elimination of a variety of xenobiotics (Green and Tephly, 1996, 1998; Hawes, 1998; Breyer-Pfaff et al., 2000; Kaivosaaari et al., 2002). The N-glucuronide form of NNAL has been synthesized in vitro (Upadhyaya et al., 2002) and has been identified and quantified in human urine (Carmella et al., 2002).
an HPLC method developed for the effective quantification of both NNAL-\(N\)- and -\(O\)-glucuronides, results from the present study demonstrate for the first time that NNAL-\(N\)-Gluc can be produced by human liver. The overall ratio of NNAL-\(N\)-Gluc/NNAL-\(O\)-Gluc of 1.7 in liver microsomes from 42 individual subjects confirms previous studies reported for urinary NNAL glucuronides (Carmella et al., 2002), indicating that \(N\)-glucuronidation is an important mechanism for the metabolism of NNAL in vivo and contributes as much if not more than -\(O\)-glucuronidation to the overall glucuronidation of NNAL. Interestingly, no NNAL-\(N\)-glucuronidating activity was observed in target tissues of tobacco-induced cancers including lung and tonsil. This is consistent with the fact that UGT1A4 was not found to be expressed in these tissues (Zheng et al., 2002) and suggests that detoxification of NNAL by \(N\)-glucuronidation may be mediated systemically in liver and potentially other nontobacco-targeted organs.

Several peaks corresponding to NNAL-\(N\)-Gluc were observed by HPLC and LC-MS/MS analysis of NNAL-glucuronidating assays of human liver microsomes and UGT1A4-overexpressing baculosomes. It is likely that these isomers are derived from the \((R)\) and \((S)\) isomers of NNAL (Upadhyaya et al., 2002), as no additional chiral center is formed by the glucuronidation of NNAL at the nitrogen position of its pyridine ring. However, the exact configuration of these isomers could not be elucidated by nuclear magnetic resonance analysis because sufficient quantities of purified NNAL-glucuronide isomers could not be obtained.

Of the UGTs tested in the present study, the only UGT exhibiting \(N\)-glucuronidation activity against NNAL was UGT1A4. This activity is consistent with UGT1A4’s activity spectrum to produce \(N\)-glucuronidated metabolites with other compounds (Green and Tephly, 1996, 1998; Breyer-Pfaff et al., 2000; Kaivosaari et al., 2002; Nakajima et al., 2002). Although no NNAL-\(N\)-glucuronidating activity was observed for UGT1A9 and UGT2B7, similar to that observed in previous studies (Ren et al., 2000) both enzymes exhibited NNAL-\(O\)-glucuronidating activity. Since all three NNAL-glucuronidating UGTs are expressed in human liver (Strassburg et al., 1997; Ren et al., 2000), it is likely that the three enzymes contribute significantly to the overall glucuronidation of NNAL and to the detoxification of this potent carcinogen.

The high \(K_m\) associated with UGT1A4-induced glucuronidation of NNAL brings into question the physiological importance of this enzyme in individuals exposed to NNAL (i.e., smokers, tobacco chewers), where blood NNAL concentrations are in the nanomolar range (Hecht et al., 1999, 2002). The observed \(K_m\) for the \(N\)-glucuronidation of NNAL in human liver microsomes was substantially lower than that observed for UGT1A4 baculosomes. However, high \(K_m\) values were also observed for the two \(O\)-glucuronidating enzymes UGT2B7 (9.3 mM) and UGT1A9 (23.1 mM) (Ren et al., 2000). This pattern of high \(K_m\) values for UGTs overexpressed in vitro may be due to the fact that artificial systems such as overexpressing cell lines and baculosomes are suboptimal for the examination of UGT activity relationships, particularly as they compare with a more in vivo experimental system such as liver microsomes. The observed \(K_m\) for

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UGT1A4 bacularsomes may therefore not be an absolute representa-
ion of the physiological importance of this UGT against a specific
substrate such as NNAL. An alternative scenario could be that other
unidentified or previously untasted hepatic UGTs [including
UGT2B17 (Beaulieu et al., 1996) and UGT2B28 (Levesque et al.,
2001)] may exhibit N- (or O-) glucuronidating activity against NNAL
that may significantly impact the \( K_m \) values calculated for liver
microsomes. This possibility requires further study.

Although the levels of urinary NNAL-N-Gluc/NNAL-O-Gluc ratios
were associated with smoking in vivo (Carmella et al., 2002), a similar
association was not found when comparing in vivo smoking exposure
with in vitro results of the NNAL-glucuronidation assays of microsomes
from livers from the same subjects as described in the present studies.
This could be due to the fact that the in vivo exposure (i.e., subject
smoking) was not controlled experimentally. Since this was a retrospec-
tive study of liver specimens obtained from a tissue bank, the possibility
exists that many of the subjects from whom liver specimens were
obtained and were designated as smokers in medical chart data were not
necessarily smoking within the required time frame that could manifest a
smoking-induced effect on the expression/activity of glucuronidating
enzymes. In addition, potential confounders could be the subjective
nature of medical chart data and the lack of experimental parameters for
the definition of smokers versus never smokers.

One potential mechanism by which smoking-induced alterations in
glucuronidation expression/activity may be mediated is via p-aminoo-
hippurate induction of the aryl hydroxylase receptor pathway. How-
ever, no upstream aryl hydroxylase receptor-binding elements (con-
sensus sequence = CACGCA) were identified in the UGT1A4
promoter region as determined by a National Center for Biotechnol-
ogy Information (National Institutes of Health) Pairwise Blast search
studies using cell line models examining the effects of specific p-
aminohippurates on NNAL-glucuronidating enzyme expression/activity
are required to better evaluate the role of tobacco smoke induction of
NNAL-N-glucuronidation pathways.

In conclusion, the results from the present study demonstrate that
NNAL-N-Gluc is a major hepatic glucuronide of NNAL in humans
and that the hepatic enzyme, UGT1A4, is a likely major contributor
to this pathway. The mechanism underlying the significant interindi-
vidual variation in NNAL glucuronide formation is presently unclear
and could include variations in UGT1A4 expression or the presence of
activity-altering UGT1A4 gene polymorphisms. Experiments exami-
nining these possibilities are currently being performed.

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ing tissue specimens, corresponding genomic DNA, and medical chart
data from the same subjects.

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