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Prominent Stereoselectivity of NNAL Glucuronidation in Upper Aerodigestive Tract Tissues

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Abstract

Tobacco specific nitrosamines (TSNAs) are among the most potent carcinogens found in cigarettes and smokeless tobacco products. Decreases in TSNA detoxification, particularly 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), have been associated with tobacco-related cancer incidence. NNK is metabolized by carbonyl reduction to its major carcinogenic metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which is detoxified by glucuronidation at the nitrogen within the pyridine ring or at the chiral alcohol to form four glucuronide products: (*R*)-NNAL-*O*-Gluc, (*S*)-NNAL-*O*-Gluc, (*R*)-NNAL-*N*-Gluc, (*S*)-NNAL-*N*-Gluc. Stereoselective NNAL-Gluc formation and the relative expression of NNAL-glucuronidating UGTs (1A4, 1A9, 1A10, 2B7, 2B10, 2B17) were analyzed in 39 tissue specimens from the upper aerodigestive tract (esophagus ($n = 13$), floor of mouth ($n = 4$), larynx ($n = 9$), tongue ($n = 7$), and tonsil ($n = 6$)). All pooled tissue types preferentially formed (*R*)-NNAL-*O*-Gluc in the presence of racemic-NNAL; only esophagus exhibited any detectable formation of (*S*)-NNAL-*O*-Gluc. For every tissue type examined, UGT1A10 exhibited the highest relative expression levels among the NNAL-*O*-glucuronidating UGTs, ranging from 36% (tonsil) to 49% (esophagus), followed by UGT1A9 > UGT2B7 > UGT2B17. UGT1A10 also exhibited similar or higher levels of expression as compared to both NNAL-*N*-glucuronidating UGTs, 1A4 and 2B10. In a screening of cells expressing individual UGT enzymes, all NNAL glucuronidating UGTs exhibited some level of stereospecific preference for individual NNAL enantiomers, with UGTs 1A10 and 2B17 forming primarily (*R*)-NNAL-*O*-Gluc. These data suggest that UGTs 1A10 and 2B17 may be important enzymes in the detoxification of TSNAs like NNK in tissues of the upper aerodigestive tract.

Graphical Abstract

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The authors declare no competing financial interest.

INTRODUCTION

Tobacco use has been linked to lung cancer for over a century and remains the leading cause of preventable premature death in adults worldwide.^{1,2} In the United States, tobacco smokers have a mortality rate three times higher than individuals who have never smoked.³ Cigarette smoking is highly associated with cancers in the airway such as lung and laryngeal cancers,^{4–7} while smokeless tobacco use has been associated with cancers of the mouth and throat such as oral and esophageal cancers.^{8,9}

A major class of carcinogens in both tobacco smoke and smokeless tobacco products are the tobacco-specific nitrosamines (TSNAs).^{10–13} This class of carcinogens includes 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a compound classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC).¹⁴ NNK is rapidly metabolized in the body to the (*R*)- and (*S*)-enantiomers of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by carbonyl reduction.^{15–17} NNAL can undergo the same CYP-mediated α -hydroxylation pathways as NNK to form reactive intermediates which form DNA adducts.^{18–23} It is thought that NNK carcinogenicity is largely manifested via the NNAL formation pathway, with 14–100% of the NNK dose metabolized to NNAL.^{15,24–28} By measuring NNK in mainstream smoke vs urinary NNAL in smokers, it was estimated that 39–100% of NNK was converted to NNAL systemically in smokers.²⁶ NNK exposure in smokeless tobacco users was measured in saliva and it was estimated that 14–17% of NNK was converted to NNAL within the oral cavity.²⁸ Additionally, it has been shown that NNAL comprised 82–92% of total NNK metabolites in human lung tissue.²⁷ NNK and NNAL have been extensively studied for carcinogenicity in animal models and have been shown to methylate and pyridyloxobutylate DNA after metabolic activation by cytochrome P450 enzymes in oral and lung tissues,^{29–33} suggesting that NNK and NNAL are strong oral and lung carcinogens.

There are seven enzymes known to metabolize NNK to NNAL: hydroxysteroid dehydrogenases (HSD) 11 β 1 and 17 β 12, carbonyl reductase type 1 (CBR1), and aldo-keto reductases (AKR) 1C1, 1C2, 1C3, 1C4, and 1B10.^{34–37} (*R*)-NNAL is preferentially formed by HSD17 β 12, while the remaining enzymes primarily form (*S*)-NNAL.³⁷ Like NNK, both (*R*)- and (*S*)-NNAL are very potent carcinogens in rodents,^{15–17} with (*S*)-NNAL exhibiting higher carcinogenic potential than (*R*)-NNAL.^{25,27,38,39}

The major mode of detoxification of NNAL is by glucuronidation via the UDP-glucuronosyltransferase (UGT) enzymes. It has been found that (*S*)-NNAL is stereoselectively retained in rat lung and has a higher tumorigenicity than (*R*)-NNAL, and that (*R*)-NNAL exhibits a higher rate of glucuronidation in rats^{17,31–33} and the A/J mouse.^{17,30} However, studies indicate that (*S*)-NNAL may be stereoselectively retained in smokeless tobacco users⁴⁰ yet it exhibits a higher rate of glucuronidation in the patas monkey.⁴¹ It is not clear if either NNAL enantiomer has higher carcinogenic potential in humans.

In contrast to the relatively high tumorigenicity exhibited by both (*R*)- and (*S*)-NNAL, NNAL-glucuronide (Gluc) was found to be nontumorigenic.³⁰ NNAL can be glucuronidated at the hydroxy group (NNAL-*O*-Gluc) or at the nitrogen within the pyridine ring (NNAL-*N*-Gluc). Since NNAL has a chiral center, there are four glucuronide products that can be formed; (*R*)-NNAL-*O*-Gluc, (*S*)-NNAL-*O*-Gluc, (*R*)-NNAL-*N*-Gluc, (*S*)-NNAL-*N*-Gluc. Each of these NNK metabolites have been identified in smoker's urine directly, with NNAL-*N*-Gluc ((*R*)-NNAL-*N*-Gluc + (*S*)-NNAL-*N*-Gluc), NNAL-*O*-Gluc ((*R*)-NNAL-*O*-Gluc + (*S*)-NNAL-*O*-Gluc), and free NNAL accounting for 22–23%, 48–50%, and 27–31% of urinary NNK metabolites, respectively.^{42,43} Few studies have yet explored the tissue-specific glucuronidation of NNAL and, to the best of our knowledge, no studies have directly compared the tissue-specific expression of all six NNAL glucuronidating enzymes in the upper aerodigestive tract. The goal of the present study was to characterize the (*R*)- and (*S*)-NNAL clearance capacity of upper aerodigestive tract tissues as well as to determine which UGTs may be driving NNAL clearance in these tobacco-target tissues.

MATERIALS AND METHODS

Chemicals and Materials.

Racemic (*rac*)-NNAL (#M325740), NNAL-*N*-Gluc (M325745), NNAL-*O*-Gluc (M325720), and the internal standards (IS) NNAL-*N*-Gluc-*d*₃ (M325747), and NNAL-*O*-Gluc-*d*₅ (M325722) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). UDP glucuronic acid (UDPGA), alamethicin, methanol (MeOH) and isopropanol were purchased from Sigma (St Louis, MO), and Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), TaqMan Fast Advanced Master Mix, TaqMan Gene Expression Assays, Pure Link RNA extraction kit, TriZol reagent, SuperScript VILO cDNA synthesis kit, Geneticin, and penicillin-streptomycin were all purchased from Life Technologies (Carlsbad, CA). Pooled human liver microsomes (HLM) and pooled human intestinal microsomes (HIM) were purchased from XenoTech (Kansas City, KS) while the Pierce BCA protein assay kit, ammonium acetate and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ).

Tissue Specimens.

Human tissue specimens ($n = 39$) were procured from the Cooperative Human Tissue Network (CHTN) or the National Disease Research Institute (NDRI; esophagus, $n = 13$; floor of mouth, $n = 4$; larynx, $n = 9$; tonsil, $n = 6$; tongue $n = 7$). All tissues were normal tissue harvested during surgery or post-mortem and were flash frozen in liquid nitrogen upon procurement. Tissue specimens were received via dry ice shipment and were stored at -80°C . Tissues were concurrently prepared for both protein and RNA extractions while specimens were still frozen on dry ice.

Cell Lines and Tissue Protein Fractions.

HEK293 cells expressing UGT1A4, UGT1A9, UGT1A10, UGT2B7, UGT2B10, and UGT2B17 have been described previously.^{44–46} The parent HEK293 cell line used in this study was obtained prior to 2000, and most recently authenticated by the American Type Culture Collection utilizing short tandem repeat analysis in December 2017. The individual HEK293 UGT expressing cell lines were verified to contain the UGT clone of interest by Sanger sequencing in August 2016 by Genewiz, LLC. HEK293 cell lines were grown to 80% confluence in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin, and maintained in 400 $\mu\text{g/mL}$ of Geneticin in a humidified incubator atmosphere of 5% CO_2 at 37°C . For the preparation of cell microsomal fractions, cells were suspended in phosphate-buffered saline (PBS) and subjected to five rounds of rapid freeze/thaw before gentle homogenization. The cell homogenate was centrifuged at $9000g$ for 30 min at 4°C . The supernatant was then centrifuged at $105\,000g$ for 60 min at 4°C . The microsomal pellet was suspended in PBS and stored at -80°C . Total microsomal protein concentrations were determined using the Pierce BCA protein assay.

Each tissue specimen was homogenized in a TissueLyser (Qiagen) for 45 s at 30 Hz with 500 μL of PBS per 100 μg of tissue. Lysed tissue was then centrifuged for 30 min at $9000g$ and the supernatant (S9) was stored at -80°C . Total S9 protein concentrations were determined using the Pierce BCA protein assay. Activity assays were performed for each of the specimens independently as well as for pooled tissue specimens using equivalent protein amounts for each of the independent or pooled specimens.

NNAL Glucuronidation Assay.

The activity of (*R*)- and (*S*)-NNAL-*N*-Gluc, as well as (*R*)- and (*S*)-NNAL-*O*-Gluc formation for tissue S9 fractions and UGT-expressing cell microsomes were determined using the following conditions: after preincubation with alamethicin (50 $\mu\text{g/mg}$ total protein) for 10 min on ice, UGT-expressing cell microsomes (15–20 μg total protein), pooled aerodigestive tract tissue S9 fractions (8–12 μg total protein), or purchased HLM and HIM (20 μg) were incubated (20 μL , final volume) in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl_2 , 4 mM UDPGA, and each NNAL enantiomer (UGT overexpressing cell microsomes only, 0.5–16 mM) or *rac*-NNAL (1 mM or 4 mM) at 37°C for 1 h. As described previously, glucuronidation reactions were rate linear for up to 2 h.⁴⁷ Reactions were terminated by the addition of an equal volume of cold methanol and spiked with 2 μL of deuterated NNAL-*N*-Gluc and NNAL-*O*-Gluc internal standard mix. The precipitate was removed by

centrifugation at 16 000*g* for 10 min at 4 °C and the organic solvent in supernatant was removed by centrifugation under vacuum at room temperature for 20 min prior to transfer to glass vials for liquid chromatography mass spectrometry (LC-MS) analysis. All reactions were performed in triplicate.

LC-MS Analysis.

LC separation of NNAL-*N*-Gluc and *O*-Gluc was achieved using an Acquity H class ultrahigh pressure liquid chromatograph (UPLC; Waters) equipped with an auto sampler (model FPN). (*R*)-NNAL-*O*-Gluc, and (*S*)-NNAL-*O*-Gluc were separated from each other using a method optimized from a previous publication.⁴⁸ Briefly, NNAL glucuronides were analyzed with an HSS T3 1.8 μ m column (2.1 $\times$ 100 mm; Acquity, Waters, Milford, MA) at 30 °C with gradient elution at 0.35 mL/min using the following conditions: 0.5 min with 99% buffer A (5 mM ammonium formate with 0.01% formic acid) and 1% buffer B (100% MeOH), followed by a linear gradient for 3.0 min to 20% buffer B, and a subsequent linear gradient for 1.0 min to 95% buffer B. The column was washed with a 1.0 min linear gradient to 1% buffer B and re-equilibrated for 1.0 min in 1% buffer B.

For analysis of kinetics and tissue S9 fraction NNAL-*O*-Gluc activity, the Waters Xevo TQD tandem MS equipped with a Zspray electrospray ionization interface was operated in the positive ion mode, with capillary voltage at 0.6 kV. Nitrogen was used as both the cone gas and desolvation gas at 50 and 800 L/h, respectively. Ultrapure argon was used for collision-induced dissociation. The desolvation temperature and the ion source temperature were 500 and 150 °C, respectively. The cone voltage was 20 V each and the collision energy was 20 V for both NNAL-*O*-Gluc diastereomers.

(*R*)- and (*S*)-NNAL-*N*-Gluc were separated independently using the same UPLC system with gradient elution at a 0.2 mL/min flow rate using the following conditions: a 1 min linear gradient of 100% buffer A (5 mM ammonium formate with 0.01% formic acid) to 99% buffer A and 1% buffer B (100% acetonitrile), a subsequent isocratic gradient of 99% buffer A for 9 min, followed by a linear gradient for 8 min to 97% buffer A. After an additional 8 min at 97% buffer A, column was then cleaned with 95% buffer B and re-equilibrated to initial conditions before the next sample injection.

For the analysis of separated (*R*)-NNAL-*N*-Gluc and (*S*)-NNAL-*N*-Gluc, a Waters Xevo G2-S QToF MS was used for increased resolution. The MS was operated in positive electrospray ionization MS/MS sensitive mode, with capillary voltage at 0.6 kV. Nitrogen was used for both cone and desolvation gases at 50 L/h and 800 L/h, respectively. Ultrapure argon was used as the collision gas with a flow rate of 0.1 L/h for collision-induced dissociation. The source temperature was 120 °C, desolvation gas temperature was 500 °C. The dwell time for each ion was 0.1 s. The cone voltage 25 V and the collision energy was 20 V.

For the detection of all NNAL glucuronides, MS was operated in the multiple reaction monitoring mode (MRM). The ion related parameters for each transition were monitored as follows: NNAL-*N*-Gluc, MS transition of 386.2 *m/z* > 180.1 *m/z* (IS: 389.2 *m/z* > 183.1 *m/z*) and NNAL-*O*-Gluc, MS transition of 386.2 *m/z* > 162.1 *m/z* (IS: 391.2 *m/z* > 167.1 *m/z*). MS transitions and LC retention times for each molecule were compared to

purchased NNAL-*O*-Gluc and NNAL-*N*-Gluc standards (Toronto Research Chemicals) for each metabolite. NNAL-*O*-Gluc and NNAL-*N*-Gluc formation were quantified by dividing their peak areas by the peak areas for deuterated NNAL-*O*-Gluc and NNAL-*N*-Gluc internal standards, respectively, and quantified against a standard curve made from purchased NNAL-*O*-Gluc and NNAL-*N*-Gluc of known quantity.

Determination of UGT Relative Expression Levels.

All tissues were homogenized for 45 s at 30 Hz with 500 μ L of TriZol per 25 μ g of tissue. RNA was then extracted from tissue homogenates using a Pure Link RNA kit. RNA concentrations were determined on a Thermo Scientific Nano Drop 2000 using a 25 ng RNA equivalent of cDNA as template. Expression levels of each UGT mRNA was normalized to the expression of ribosomal protein lateral stock P0 (RPLP0). Quadruplicate qPCR was performed for each tissue sample using a 10 μ L final spectrophotometer assay volume. Reverse transcription was performed using the SuperScript VILO cDNA synthesis kit with 280 ng of starting RNA per reaction.

qPCR was carried out using a 10 μ L reaction volume containing 5 μ L of TaqMan Master Mix, 4.5 μ L diluted cDNA, and 0.5 μ L of UGT specific TaqMan Gene Expression Assay for UGTs 1A4 (Hs01655285_s1), 1A9 (Hs02516855_sh), 1A10 (Hs02516990_s1), 2B7 (Hs00426592_m1), 2B10 (Hs04195423_s1), or 2B17 (Hs00854486_sh). Assays were performed using the Bio-Rad CFX384 Real-Time System under the following conditions: 1 cycle at 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative expression was calculated using the C_t method. C_t was calculated as the target gene C_t minus the C_t of the control gene (RPLP0). Relative expression within each tissue type was determined with the eq 2^{-C_t} . As previously described, UGT genes that amplified with a mean $C_t > 35$ cycles were determined to be below the limit of quantification (BLQ).⁴⁹

Data Analysis.

Kinetic parameters were determined using Prism version 7.0 (GraphPad Software, San Diego, CA). When calculating the mean relative expression values, BLQ transcripts were included in the analysis as zero expression.

RESULTS

Stereospecificity of NNAL-Gluc formation in human upper aerodigestive tract tissues was examined in pooled S9 fractions, assayed as described above in reactions that contained 1 mM *rac*-NNAL. All S9 fractions from upper aerodigestive tract tissues, as well as commercially purchased HIM preferentially formed (*R*)-NNAL-*O*-Gluc over (*S*)-NNAL-*O*-Gluc; only commercial liver microsomes preferentially formed (*S*)-NNAL-*O*-Gluc (Figure 1). Pooled HIM and esophageal S9 fractions favored the formation of (*R*)-NNAL-*O*-Gluc but also exhibited significant levels of (*S*)-NNAL-*O*-Gluc formation, with it comprising 31 and 19% of the total NNAL-*O*-Gluc formed in HIM and esophageal microsomes, respectively. Pooled S9 fractions from floor of mouth, larynx, tongue, and tonsil exhibited no detectable levels of (*S*)-NNAL-*O*-Gluc formation. None of the pooled S9 fractions from any of the tissues examined exhibited detectable levels of NNAL-*N*-Gluc formation except

for HLM (data not shown). Similar results were obtained when examining each of the corresponding microsomal fractions from each of the specimens individually, with each of the larynx, tongue and tonsil specimens exhibiting no detectable level of (*S*)-NNAL-*O*-Gluc formation. Similar to that observed in the pooled floor of mouth ($n = 4$) and esophageal ($n = 12$) microsomal samples, each of the microsomes from the independent specimens exhibited higher levels of (*R*)-NNAL-*O*-Gluc vs (*S*)-NNAL-*O*-Gluc formation, with (*S*)-NNAL-*O*-Gluc formation reaching as high as 45% of total NNAL-*O*-Gluc in a single specimen for each site (data not shown).

Stereoselective NNAL-*O*-Gluc formation was examined by LC-MS for UGT enzymes known to form NNAL-*O*-Gluc using microsomes from HEK293-overexpressing cell lines (UGTs 1A9, 1A10, 2B7, or 2B17) incubated with up to 4 mM *rac*-NNAL.^{48,50} Representative chromatograms for each cell line are shown in Figure 2A. UGT1A9 exhibited the lowest level of stereospecificity, with ~65% of total NNAL-*O*-Gluc formation being (*R*)-NNAL-*O*-Gluc. UGTs 2B7 and 1A10 exhibited the highest levels of stereospecificity, each forming ~5% (*S*)- or (*R*)-NNAL-*O*-Gluc, respectively. UGT2B17 exhibited stereoselectivity for the formation of (*R*)-NNAL-*O*-Gluc with ~10% of the total NNAL-*O*-Gluc formation being (*S*)-NNAL-*O*-Gluc.

A method was developed to separate and identify (*R*)-NNAL-*N*-Gluc and (*S*)-NNAL-*N*-Gluc formed by UGT2B10 and UGT1A4. Microsomes from HEK293 cell lines overexpressing each UGT were incubated with 4 mM *rac*-NNAL or 2 mM of each NNAL enantiomer, as described above. Separation of the NNAL-*N*-Gluc diastereomers was achieved but with overlap between the minor rotamer (*R*)-NNAL-*N*-Gluc peak and the main rotamer peak of (*S*)-NNAL-*N*-Gluc (Figure 2B). Because these rotamers are created by the rotation of the nitroso group that can be sterically hindered by proximity to the chiral alcohol group, these compounds cannot be isolated from each other. The retention time of the main (*R*)-NNAL-*N*-Gluc peak was 5.6 min and was identified by the retention time of the peak formed when (*R*)-NNAL was incubated with each UGT. The retention time of the main (*S*)-NNAL-*N*-Gluc peak was 5.9 min and was identified by matching with the retention time of the peak formed when (*S*)-NNAL was incubated with each UGT. UGT1A4 appears to exhibit a slight preference for the formation of (*S*)-NNAL-*N*-Gluc when incubated with *rac*-NNAL while UGT2B10 appears to have a slight preference for the formation of (*R*)-NNAL-*N*-Gluc with the same assay.

The UGT stereospecificity against NNAL was further characterized by kinetic analysis. Microsomes from cells overexpressing NNAL-*O*-glucuronidating enzymes (UGTs 1A9, 1A10, 2B7, and 2B17) were incubated with the (*R*)- and (*S*)-NNAL enantiomers separately, over a concentration range from 0.5 to 16 mM NNAL (Figure 3). Comparing kinetic curves for (*R*)- and (*S*)-NNAL, UGTs 1A10 and 2B17 exhibited a clear difference in the rates of (*R*)-NNAL-*O*-Gluc vs (*S*)-NNAL-*O*-Gluc formation, clearly favoring the (*R*)- NNAL enantiomer as substrate (Figure 3A). While accurate kinetic values could not be determined for UGT1A10, there was a 1.9-fold lower K_M and a 11-fold higher V_{max}/K_M observed for UGT2B17 for (*R*)-NNAL-*O*-Gluc vs (*S*)-NNAL-*O*-Gluc formation (Table 1). UGT2B7 also exhibited a clear difference in the rate of formation for each NNAL-*O*-Gluc diastereomer, clearly favoring (*S*)-NNAL as substrate (Figure 3A). This is consistent with the >3.3-fold

lower K_M observed for UGT2B7 for (*S*)-NNAL-*O*-Gluc vs (*R*)-NNAL-*O*-Gluc formation (Table 1). While UGT1A9 exhibited marginal specificity for (*R*)-NNAL-*O*-Gluc formation when incubated with rac-NNAL (Figure 2A), UGT1A9 indicated a marginally faster rate of NNAL-*O*-Gluc formation with pure (*S*)-NNAL as substrate (Figure 3A), a pattern consistent with its marginal 1.5-fold lower K_M and 1.6-fold higher V_{max}/K_M for (*S*)-NNAL-*O*-Gluc vs (*R*)-NNAL-*O*-Gluc formation (Table 1).

Cell microsomes overexpressing the two enzymes that form NNAL-*N*-Gluc (UGTs 1A4 and 2B10) were also incubated with (*R*)- and (*S*)-NNAL in separate reactions. UGTs 1A4 and 2B10 exhibited stereospecificity, with UGT1A4 exhibiting a faster rate of (*S*)-NNAL-*N*-Gluc formation and UGT2B10 exhibiting a faster rate of (*R*)-NNAL-*N*-Gluc formation (Figure 3B). These data are consistent with the 2.3-fold higher V_{max}/K_M observed for (*S*)-NNAL-*N*-Gluc formation by UGT1A4 and the 2.4-fold higher V_{max}/K_M observed for (*R*)-NNAL-*N*-Gluc formation by UGT2B10 (Table 1).

The interindividual level of expression of each UGT with known NNAL activity was measured by qPCR in several aerodigestive tract tissues including esophagus, floor of mouth, larynx, tongue, and tonsil (Figure 4). Previous studies using the same UGT expression assays as used in the present study demonstrated minimal differences in expression efficiencies.⁴⁹ No expression of any gene, including the housekeeping RPLP0 gene, was observed for three specimens (one each of esophagus, larynx, and tongue), and these specimens were excluded from the data set. Any UGT gene that amplified with a mean Ct > 35 cycles was determined to be below the limit of quantification (BLQ) but were included in the calculations of mean relative expression as exhibiting zero gene expression. Each UGT was stratified by relative nonzero expression in each tissue tested for both NNAL-*O*-Gluc (Figure 4A) and NNAL-*N*-Gluc forming enzymes (Figure 4B). UGTs 1A9 and 1A10 exhibited the highest interindividual differences in expression in esophagus and tongue with a 100-fold difference in expression between the lowest and highest expressing samples, while floor of mouth and tonsil exhibited the least variation in expression between specimens with up to a 10-fold difference in relative expression between samples for both sites. UGTs 2B7 and 2B17 exhibited the largest interindividual expression differences in larynx and tongue with a 100-fold difference in expression between the highest and lowest expressing samples. Similar to UGTs 1A9 and 1A10, UGTs 2B7 and 2B17 exhibited the lowest interindividual expression differences in floor of mouth and tonsil.

The most highly expressed NNAL-*O*-glucuronidating UGT in upper aerodigestive tract tissues was UGT1A10 (Table 2). UGT1A10 exhibited mean values that were between 1.6- and 6.6-fold higher than the mean values of expression in esophagus, 1.3 and 8.6-fold higher than floor of mouth, 2.5- and 4.4-fold higher than larynx, 1.2- and 4-fold higher than tongue, and 1.3- and 3-fold higher than tonsil, as compared to the other *O*-glucuronidating UGTs. UGT1A9 was the second most highly expressed *O*-glucuronidating UGT, followed by UGT2B7 and UGT2B17, in all tissues examined. For the NNAL-*N*-glucuronidating UGTs, 1A4 and 2B10 exhibited similar levels of expression in all aerodigestive tract tissues examined except floor of mouth, where UGT1A4 exhibited 2.3-fold higher levels of expression as compared to UGT2B10 (Table 2). In addition, the expression of UGTs 1A4 and 2B10 was either similar to or less than UGT1A10 in all tissues examined.

DISCUSSION

Glucuronidation is the primary detoxification pathway for TSNAs. Previous studies into the stereospecificity of NNAL glucuronidation focused primarily on hepatic UGTs and systemic NNAL clearance.^{42,48} The present study focuses on understanding the stereospecific glucuronidation of NNAL in aerodigestive tract tissues, which are targets for tobacco-induced cancer.⁸ One of the major findings from the present study is that all of the aerodigestive tract tissues tested exhibited a strong preference for (*R*)-NNAL-*O*-Gluc formation. S9 fractions from tissues within the oral cavity (floor of mouth, tongue, tonsil) and airways (larynx) exhibited low levels of (*S*)-NNAL-*O*-Gluc formation capacity (<5%). While HIM and the S9 fraction from esophagus exhibited higher ratios of (*S*)-NNAL-*O*-Gluc:(*R*)-NNAL-*O*-Gluc formation capacity than other aerodigestive tract tissues, the levels of (*S*)-NNAL-*O*-Gluc formation were still far lower than the formation of (*R*)-NNAL-*O*-Gluc in both cases. This preference for (*R*)-NNAL-*O*-Gluc formation contrasts with that observed in HLM where (*S*)-NNAL-*O*-Gluc formation is preferentially formed.

Data from the present study suggest that the differences in stereoselectivity for (*R*)-NNAL-*O*-Gluc formation (over (*S*)-NNAL-*O*-Gluc formation) in aerodigestive tract tissues vs liver may be due to differences in the expression of the UGTs expressed within these tissues. Each of the four enzymes that mediate the formation of NNAL-*O*-Gluc exhibit a distinctive stereoselective glucuronide formation profile when incubated with *rac*-NNAL. The pattern of stereoselectivity observed for each enzyme upon incubation with *rac*-NNAL was also observed at each concentration tested for the separate NNAL enantiomers. As observed in previous studies, UGT1A9 exhibited the least stereospecificity for NNAL enantiomers.⁴⁸ UGTs 1A10 and 2B17 each exhibited a strong preference for (*R*)-NNAL, with UGT2B7 being the only UGT to exhibit a strong preference for (*S*)-NNAL.^{42,48} The kinetic parameters for each UGT isoform were similar to previously published values for (*R*)- and (*S*)-NNAL,⁴⁸ except for UGTs 1A10 and 1A4, which did not have previously reported values. UGT1A10 exhibited a much higher rate of formation for (*R*)-NNAL-*O*-Gluc at every concentration tested, but did not yield kinetic values within the Michaelis–Menten kinetic equation at the substrate levels tested.

This study is the first to develop a direct separation method for the detection of (*R*)- and (*S*)-NNAL-*N*-Gluc. UGT2B10 and UGT1A4 exhibit activity with *rac*-NNAL similar to the stereopreferences exhibited by the enantio-specific kinetic assays with preferences for (*R*)- and (*S*)-NNAL respectively. The separation method resulted in some overlap between the minor peak of (*R*)-NNAL-*N*-Gluc and the major peak of (*S*)-NNAL-*N*-Gluc, which limits the ability of this method to quantify the levels of each *N*-Gluc diastereomer. A qualitative assessment indicated that the pattern of (*R*)- and (*S*)-NNAL-*N*-Gluc formation from assays with *rac*-NNAL match the differences observed in the kinetic analysis performed for UGT2B10 and UGT1A4 with each NNAL enantiomer separately, where UGT2B10 exhibits a preference for (*R*)-NNAL and UGT1A4 exhibits a preference for (*S*)-NNAL. Previous *in vivo* studies have indicated that UGT2B10 is responsible for >90% of NNAL-*N*-Gluc formation.⁴² These data indicate that the NNAL-*N*-Gluc identified *in vivo* may primarily be the (*R*)-NNAL-*N*-Gluc form. Interestingly, none of the aerodigestive tract tissues tested in the present study exhibited detectable levels of NNAL-*N*-Gluc when incubated with

rac-NNAL. Since UGT2B10 and UGT1A4 expression was observed in each of these tissue types, the lack of NNAL-*N*-Gluc formation in upper aerodigestive tract tissues may be due to limitations in assay sensitivity. Alternatively, the $V_{\max}$ values described for the UGT-expressing microsomes in this study are per mg of total protein, not per UGT protein. Therefore, they are not a reflection of actual $V_{\max}$ differences between individual enzymes and are useful only when comparing kinetic values of the same UGT for different substrates. It is possible, therefore, that UGTs 2B10 and 1A4 exhibit significantly lower actual $V_{\max}$ values as compared to the NNAL-*O*-glucuronidating UGTs (including UGT1A10 and UGT2B17), resulting in limited upper aerodigestive tract tissue NNAL-*N*-Gluc formation capacity even though both UGTs 2B10 and 1A4 are expressed in these tissues.

UGT1A10 has often been overlooked for contribution to NNAL metabolism because it is the only extra-hepatic enzyme with NNAL glucuronidation activity.⁵¹ Previous studies indicate that UGT2B7 is the highest expressed of the NNAL glucuronidating UGTs in liver tissue,⁴⁹ and is likely to be driving the higher formation of (*S*)-NNAL-*O*-Gluc (compared to (*R*)-NNAL-*O*-Gluc) observed in HLM. The data from the present study show that UGT1A10 was the most highly expressed NNAL-glucuronidating UGT in tissues of the upper aerodigestive tract. UGT1A10 comprised nearly half of the total NNAL-*O*-Gluc forming UGT genes in esophagus, floor of mouth, and larynx, and nearly 40% of the total in tongue and tonsil. The high stereospecificity exhibited by UGT1A10 for (*R*)-NNAL-*O*-Gluc formation is consistent with the similar stereospecificity exhibited for (*R*)-NNAL-*O*-Gluc formation in aerodigestive tract tissues. The other UGT with high stereospecificity for (*R*)-NNAL-*O*-Gluc formation, UGT2B17, is expressed at 3.2- to 8.6-fold lower levels than UGT1A10 in the aerodigestive tract tissues examined. While the K_M was higher for UGT1A10 than UGT2B17 for (*R*)-NNAL, an assessment of differences in $V_{\max}$ between enzymes could not be examined due to reaction rates being calculated per mg of total microsomal protein for each UGT-over-expressing cell line.

The low levels of (*S*)-NNAL-*O*-Gluc formation (relative to (*R*)-NNAL-*O*-Gluc formation) in upper aerodigestive tract tissues is consistent with the low levels of UGT2B7 expression in these tissues. UGT2B7 is the only enzyme that exhibits high stereospecific activity for (*S*)-NNAL, and although NNAL glucuronidation has not been observed in assays with lung tissue, (*R*)-NNAL glucuronidation may also form at a higher rate (relative to (*S*)-NNAL-*O*-Gluc) in lung as UGT2B17 has been shown to be higher expressed than UGT2B7 in lung tissue.⁴⁹ It is interesting that UGT1A9 exhibits relatively high levels of expression in upper aerodigestive tract tissues. However, it exhibits a relatively high K_M against both NNAL enantiomers, and its $V_{\max}$ against both NNAL enantiomers could not be compared with the other UGT enzymes. The fact that relatively low levels of (*S*)-NNAL-*O*-Gluc formation was observed in upper aerodigestive tract tissues suggests a limited role for both UGTs 1A9 and 2B7 in the glucuronidation of NNAL in these tissues.

The data presented in this study suggests that UGTs 1A10 and 2B17 may be important enzymes in the detoxification of NNAL, and therefore NNK, in upper aerodigestive tract tissues. In addition to detoxifying NNAL, UGT1A10 exhibits glucuronidating activity against the carcinogenic polycyclic aromatic hydrocarbons found in tobacco smoke.^{45,52} Additionally, UGT1A10 polymorphisms have been identified as independent risk factors for

upper aerodigestive tract cancer in smokers.⁵³ Therefore, UGT1A10 may be an important enzyme in the detoxification of several tobacco carcinogens in upper aerodigestive tract tissues. Limitations of the present study include small sample sizes for each tissue type that does not allow for age, sex, or race comparisons. Future studies should include larger sample sizes to further explore the impact of *UGT1A10* and *UGT2B17* gene polymorphisms on the rate of detoxification in upper aerodigestive tract tissues.

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Figure 1.

Representative traces of LC-MS analysis of NNAL-*O*-Gluc formation by pooled human tissue specimens. Pooled human tissue S9 fractions (8–12 ug total protein) and commercial human liver microsomes (20 ug total protein) were incubated with 1 mM of *rac*-NNAL and 4 mM UPGA cosubstrate as described in the Materials and Methods. The *y*-axis is an intensity scale for each panel while the *x*-axis is time (min).

Figure 2.

Representative traces of LC-MS analysis of NNAL-*O*-Gluc and NNAL-*N*-Gluc formation from *rac*-NNAL. UGT-overexpressing cell microsomes (15–20 ug total protein) were incubated for 1 h at 37 °C with 4 mM UDPGA and substrate *rac*-NNAL, (*S*)-NNAL, or (*R*)-NNAL as described in the Materials and Methods. Concentrations of *rac*-NNAL were at 4 mM or at the K_M for each enzyme, whichever was smaller. The *y*-axis are scaled arbitrary units where 100% is the highest peak in each assay. A, (*S*)- and (*R*)-NNAL-*O*-Gluc peaks are single peaks that contain the NNAL-*O*-Gluc rotamers. B, (*S*)- and (*R*)-NNAL-*N*-Gluc peaks (peaks 2 and 4, and 1 and 3, respectively) are the separate NNAL-*N*-Gluc rotamers. From the *rac*-NNAL assay, the shoulder (peak 3) on the (*S*)-NNAL-*N*-Gluc peak (peak 2) was attributed to one of the (*R*)-NNAL-*N*-Gluc rotamers by retention times matched to assays with (*R*)- and (*S*)-NNAL. The *y*-axis is an intensity scale for each panel while the *x*-axis is time (min).

Figure 3.

Representative concentration curves for the formation of NNAL-*O*-Gluc and NNAL-*N*-Gluc by individual UGT enzymes. A, NNAL-*O*-Gluc formation by UGTs 1A9, 1A10, 2B7, and 2B17. B, NNAL-*N*-Gluc formation by UGTs 1A4 and 2B10. Glucuronide formation assays were performed with 15–20 μg of UGT-overexpressing microsomal protein and 0.5–16 mM of either (*R*)-NNAL (●) or (*S*)-NNAL (○) incubated at 37 °C for 1 h.

Figure 4.

Relative expression levels of UGT genes in upper aerodigestive tract tissues. UGT mRNA expression levels were calculated in 36 normal tissue specimens: esophagus ($n = 12$), floor of mouth ($n = 4$), larynx ($n = 8$), tongue ($n = 6$), and tonsil ($n = 6$). Expression was calculated as arbitrary units relative to the housekeeping gene RPLP0 via the C_t method, with each point representing the 2^{-C_t} for each UGT in each specimen and separated by NNAL-*O*-Gluc forming UGTs (panel A) and NNAL-*N*-Gluc forming UGTs (panel B). Specimens with UGT expression below the limit of quantification (mean $C_t > 35$ cycles) are not shown (UGT1A10, $n = 1$; UGT2B7, $n = 9$; UGT2B17, $n = 7$). The y -axis is relative expression (arbitrary units).

Table 1.

Michaelis-Menton Kinetic Values of UGT-Expressing Microsomes with Each NNAL Enantiomer^{a,b}

		NNAL- <i>O</i> -Gluc			NNAL- <i>N</i> -Gluc		
		UGT1A9	UGT1A10	UGT2B7	UGT2B17	UGT1A4	UGT2B10
(R)-NNAL	K_M ^c	11 ± 5.0	>16	>16	1.8 ± 0.40	9.0 ± 4.7	4.3 ± 2.5
	V_{max} ^d	13 ± 3.2	ND	ND	3.0 ± 0.20	5.5 ± 1.4	6.3 ± 1.5
	V_{max}/K_M ^e	0.58 ± 0.49	ND	ND	1.7 ± 1.0	0.61 ± 0.30	0.99 ± 0.62
(S)-NNAL	K_M ^c	7.3 ± 4.5	>16	4.9 ± 2.6	3.4 ± 1.7	15 ± 6.0	4.0 ± 1.4
	V_{max} ^d	16 ± 4.6	ND	4.0 ± 1.1	0.50 ± 0.10	21 ± 5.0	1.6 ± 0.20
	V_{max}/K_M ^e	0.94 ± 0.88	ND	1.9 ± 0.54	0.16 ± 0.11	1.4 ± 0.81	0.41 ± 0.057

^aSubstrate concentration ranges tested were 0.5–16 mM.^bAll values are expressed as mean ± SD for three independent experiments. ND, indicates unsaturated curves where values could not be determined.^c K_M values are in mM concentrations.^d V_{max} are in pmol·min⁻¹·mg total protein.^e V_{max}/K_M are in min⁻¹·mg total protein⁻¹·nL.

Table 2.

Mean Relative UGT Expression in Human Tissue Specimens.^a

		NNAL-O-Gluc				NNAL-N-Gluc			
		UGT1A9	UGT1A10	UGT2B7	UGT2B17	UGT1A4	UGT2B10		
esophagus	mean	1.16 × 10 ⁻⁰²	1.87 × 10 ⁻⁰²	4.81 × 10 ⁻⁰³	2.91 × 10 ⁻⁰³	7.64 × 10 ⁻⁰³	9.00 × 10 ⁻⁰³		
	SE	6.45 × 10 ⁻⁰³	8.75 × 10 ⁻⁰³	1.52 × 10 ⁻⁰³	1.58 × 10 ⁻⁰³	2.75 × 10 ⁻⁰³	5.53 × 10 ⁻⁰³		
	% total	30.5%	49.2%	12.7%	7.7%	45.9%	54.1%		
	# BLQ	0 of 12	0 of 12	3 of 12	2 of 12	0 of 12	0 of 12		
floor of mouth	mean	9.18 × 10 ⁻⁰³	1.16 × 10 ⁻⁰²	3.06 × 10 ⁻⁰³	1.43 × 10 ⁻⁰³	1.10 × 10 ⁻⁰²	4.67 × 10 ⁻⁰³		
	SE	3.63 × 10 ⁻⁰³	3.35 × 10 ⁻⁰³	1.78 × 10 ⁻⁰³	7.57 × 10 ⁻⁰⁴	3.41 × 10 ⁻⁰³	2.20 × 10 ⁻⁰³		
	% total	36.3%	46.0%	12.1%	5.7%	70.3%	29.7%		
	# BLQ	0 of 4	0 of 4	2 of 4	1 of 4	0 of 4	0 of 4		
larynx	mean	2.12 × 10 ⁻⁰²	5.26 × 10 ⁻⁰²	1.65 × 10 ⁻⁰²	1.62 × 10 ⁻⁰²	1.51 × 10 ⁻⁰²	1.18 × 10 ⁻⁰²		
	SE	1.07 × 10 ⁻⁰²	1.74 × 10 ⁻⁰²	1.25 × 10 ⁻⁰²	1.36 × 10 ⁻⁰²	5.72 × 10 ⁻⁰³	8.66 × 10 ⁻⁰³		
	% total	19.9%	49.4%	15.5%	15.2%	56.3%	43.7%		
	# BLQ	0 of 8	0 of 8	2 of 8	2 of 8	0 of 8	0 of 8		
tongue	mean	1.68 × 10 ⁻⁰²	2.18 × 10 ⁻⁰²	1.24 × 10 ⁻⁰²	5.02 × 10 ⁻⁰³	1.86 × 10 ⁻⁰²	1.15 × 10 ⁻⁰²		
	SE	1.14 × 10 ⁻⁰²	1.48 × 10 ⁻⁰²	7.46 × 10 ⁻⁰³	3.04 × 10 ⁻⁰³	1.35 × 10 ⁻⁰²	7.01 × 10 ⁻⁰³		
	% total	30.0%	38.9%	22.1%	9.0%	61.7%	38.3%		
	# BLQ	0 of 6	1 of 6	1 of 6	0 of 6	1 of 6	1 of 6		
tonsil	mean	4.35 × 10 ⁻⁰³	5.40 × 10 ⁻⁰³	3.47 × 10 ⁻⁰³	1.68 × 10 ⁻⁰³	4.30 × 10 ⁻⁰³	3.19 × 10 ⁻⁰³		
	SE	1.12 × 10 ⁻⁰³	1.29 × 10 ⁻⁰³	9.61 × 10 ⁻⁰⁴	6.90 × 10 ⁻⁰⁴	9.48 × 10 ⁻⁰⁴	7.83 × 10 ⁻⁰⁴		
	% total	29.2%	36.2%	23.3%	11.3%	57.4%	42.6%		
	# BLQ	0 of 6	0 of 6	1 of 6	1 of 6	0 of 6	0 of 6		

^aMean relative expression (arbitrary units) was calculated to include UGTs with no expression.

^bThe % total for the expression of each UGT was calculated separately for NNAL-O-Gluc and NNAL-N-Gluc enzymes. BLQ, below limit of quantification (mean Ct > 35 cycles).