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Altered Metabolism of Polycyclic Aromatic Hydrocarbons by UDP-Glycosyltransferase 3A2 Missense Variants

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Abstract

The UDP-glycosyltransferase (UGT) family of enzymes are important in the metabolism of a variety of exogenous substances including polycyclic aromatic hydrocarbons (PAHs), a potent class of environmental carcinogens. As compared to the majority of UGT enzymes, which utilize UDP-glucuronic acid as a cosubstrate, UGT3A2 utilizes alternative cosubstrates (UDP-glucose and UDP-xylose). UGT3A2 is expressed in aerodigestive tract tissues and was highly active against multiple PAHs with both cosubstrates. The goal of the present study was to assess the functional effects of UGT3A2 missense variants (MAF = 0.005) on PAH metabolism and the utilization of cosubstrates. The glycosylation activity ($V_{\max}/K_m$) of all variants against simple PAHs using both cosubstrates was significantly ($P < 0.05$) decreased by 42–100% when compared to wild-type UGT3A2. When utilizing UDP-glucose, the variant isoforms exhibited up to a 362-fold decrease in $V_{\max}/K_m$ when compared to wild-type UGT3A2, with a 3.1- to 14-fold decrease for D140N, A344T, and S435Y, a 24- and 43-fold decrease for A436T and R445C,

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The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.chemrestox.0c00233>.

Table S1. UGT3A2 variants identified from NCBI SNP and ENSEMBL Variation. Table S2. Primers for UGT3A2 genetic variants.

Figure S1. Western blot analysis of wt and variant UGT3A2 proteins in HEK293 overexpressing cell lines. Figure S2. Rate of wt UGT3A2 vs variants against B(a)P-7,8-diol (PDF)

respectively, and a 147- and 362-fold decrease for Y474C and Y74N, respectively. When utilizing UDP-xylose, the variants exhibited up to a 4.0-fold decrease in $V_{\max}/K_m$ when compared to wild-type UGT3A2; Y74N did not exhibit activity, and Y474C did not reach saturation ($K_m > 4000 \mu\text{M}$). Additionally, both wild-type and variant UGT3A2 exhibited a significant ($P < 0.05$) difference in their utilization of UDP-glucose vs UDP-xylose as cosubstrates using 1-OH-pyrene as substrate. These data suggest that UGT3A2 missense variants decrease the detoxification of PAHs, potentially resulting in altered individual risk for PAH-related cancers.

Graphical Abstract

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a class of carcinogens that occur in the environment as both natural products and pollutants, but are mainly known to occur through the incomplete combustion of organic compounds including tobacco, wood, coal, and many food sources.^{1,2} PAHs are activated by cytochromes P450 to form carcinogenic

metabolites, which are detoxified primarily by the UDP-glycosyltransferase (UGT) family of enzymes.³⁻¹⁰

In a recent study, UGT3A2 was shown to be highly expressed in aerodigestive tract tissues and was highly active against PAHs.¹¹ UGT3A2 is unique from other UGT isoforms because it uses the alternative UDP-sugars, UDP-glucose and UDP-xylose, as cosubstrates, whereas enzymes from the UGT1A, UGT2A and UGT2B subfamilies utilize UDP-glucuronic acid.^{12,13} Kinetic parameters have been examined for UDP-glucuronic acid for the UGT1A subfamily of enzymes, exhibiting K_m values ranging from 52 μM to 1256 μM for a variety of substrates.¹⁴⁻¹⁸ The kinetics of UDP-glucose and UDP-xylose by UGT3A2 have not been previously examined.

Rare variants [minor allele frequencies (MAF) < 0.01] have been shown to play an important role in risk for certain diseases, whereas more common variants (MAF > 0.05) usually have a smaller effect and low frequency variants (MAF = 0.01–0.05) have been shown to exhibit a more intermediate effect.¹⁹ Autism, mental retardation, epilepsy, and schizophrenia have been shown to be influenced by rare structural variants. For drug metabolizing enzymes, the relevance of rare genetic variants are highly gene-and drug-specific.²⁰ For example, rare variants comprise ~18% of deleterious CYP2C9 alleles and are predicted to contribute significantly to the interindividual variability of warfarin pharmacokinetics. In contrast, the relative importance of rare variants is expected to be lower for the metabolism of simvastatin, voriconazole, and olanzapine, for which rare variants only contribute between 1.6% and 8.7% of the variation in key metabolic and/or transport processes.

Several low prevalence variants (MAF of 0.005–0.020), are observed for UGT3A2, but no studies have examined their functional role on UGT3A2 activity. UGT3A2 was shown to exhibit high affinity against PAHs, as compared to other UGTs.¹¹ Therefore, it is important to investigate the role genetic variations may have in altering the detoxification of PAHs from the human body. The goal of the present study was to assess the functional effect of UGT3A2 missense variants in the metabolism of PAHs and determine the utilization of the alternative cosubstrates (UDP-glucose and UDP-xylose) by wild-type (wt) vs UGT3A2 variants.

MATERIALS AND METHODS

Chemicals and Materials.

Lipofectamine 3000, PureLink Genomic DNA Mini Kits, Invitrolon PVDF membranes, and Novex ECL Chemiluminescent Substrate Reagent Kits were obtained from Invitrogen (Carlsbad, CA). Mutagenic UGT3A2 primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA) while the QuikChange II XL Site-Directed Mutagenesis Kit was purchased from Agilent (Santa Clara, CA). The GeneJet Plasmid Mini and Midi Kit, Pierce BCA Protein Assay Kits, and SuperSignal West Femto Maximum Sensitivity Substrate were purchased from Thermo Scientific (Waltham, MA). Dulbecco's Modified Eagles Medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS), and geneticin were purchased from Gibco (Grand Island, NY). Premium grade fetal bovine serum

(FBS) was purchased from Seradigm (Radnor, PA), and ChromatoPur bovine albumin was purchased from MB Biomedicals (Santa Ana, CA).

The UGT3A2 antibody was purchased from Santa Cruz Biotechnology (Dallas, TX) while donkey anti-goat IgG horseradish peroxidase (HRP) conjugate was purchased from Thermo Fisher Scientific (Rockford, IL). The calnexin antibody was purchased from Cell Signaling Technology (Danvers, MA), and the Pierce goat anti-rabbit IgG peroxidase conjugated antibody was purchased from Thermo Scientific (Waltham, MA). UDP-glucose was purchased from Abcam (Cambridge, MA), and UDP-xylose was purchased from Carbosource Services (Athens, GA). 1-OH-pyrene, alamethicin, and ampicillin were purchased from Sigma-Aldrich (St. Louis, MO). 1-OH-benzo(a)pyrene [B(a)P], 7-OH-B(a)P, and 9-OH-B(a)P were purchased from MRI Global (Kansas City, MO). 3-OH-B(a)P and *trans*-7,8-dihydroxy-7,8-dihydro-B(a)P [B(a)P-7,8-diol] were purchased from Toronto Research Chemicals (North York, ON, Canada). High performance liquid chromatography (HPLC) grade ammonium acetate, Optima acetonitrile, and all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Identification of UGT3A2 Missense Variants.

Functional missense variants for UGT3A2 were identified using NCBI Variation Viewer using filters to search the dbSNP Source database (release 142; accessed on March 14, 2016) from the 1000 Genomes Project Phase 3 or from other projects in Ensembl Variation. Seven missense variants (Y74N: rs2197514, D140N: rs147471382, A344T: rs2591714, S435Y: rs61729692, A436T: rs61729693, R445C: rs149386304, and Y474C: rs150627093) were identified with a MAF of >0.005 in the human population. Data on MAFs for the UGT3A2 missense variants grouped into super populations (European, EUR; African, AFR; East Asian, EAS; Admixed American, AMR) can be found in Table S1.

Generation of UGT3A2-Overexpressing Cell Lines.

The wt UGT3A2- overexpressing HEK293 cell line has been described previously.¹¹ UGT3A2 missense variants were created by site-directed mutagenesis of the pcDNA3.1/V5-His-TOPO/wt UGT3A2 plasmid using the QuikChange II XL Site-Directed Mutagenesis Kit with mutagenic primers (Table S2). Lipofectamine 3000 was used to transfect 10 μ g of pcDNA3.1/V5-His-TOPO/UGT3A2 missense variants into the HEK293 parent cell line. For each variant, three individual colonies as well as pooled cells were grown to determine the cell line that exhibited the highest level of expression. Individual colonies were selected using cloning rings, with resuspended cells subsequently grown in six-well plates. Remaining pooled cells were allowed to continue to grow in a 100 mm dish. The HEK293 parent cell line was authenticated by ATCC using short-tandem repeat polymorphisms analysis in December 2017. Stable cell lines were established in DMEM supplemented with 10% FBS and 700 μ g/mL of geneticin. Pooled cell lines were used for activity assays for the D140N, A344T, and S435Y variants, and individual colonies were used for Y74N, A436T, R445C, and Y474C variants. Genomic DNA was extracted from the stable cell lines using the PureLink genomic DNA mini kit, and Sanger sequencing was used to confirm the presence and identity of the UGT3A2 missense variants.

Analysis of Protein Expression for UGT3A2-Overexpressing Cell Lines.

Whole cell homogenates and microsomal fractions were prepared from UGT3A2-overexpressing cell lines through differential centrifugation, utilizing methods described previously.¹¹ Western blot analysis was performed using 20 μ g of total microsomal protein using a 10% SDS-polyacrylamide gel and subsequent transfer to an Invitrolon PVDF membrane. The membrane was blocked with a 5% solution of ChromatoPur bovine albumin in TBST and probed with goat polyclonal UGT3A2 antibody (1:1000 dilution) followed by a donkey antigoat secondary antibody (1:2500 dilution). Loading variability was monitored with the calnexin antibody (1:1000 dilution), using the goat antirabbit secondary antibody (1:2000 dilution). Immunocomplexes were visualized using either the SuperSignal West Femto Maximum Sensitivity Substrate or the Novex ECL Chemiluminescent Kit (25 or 210 s exposure times, respectively) following manufacturer's protocols. Densitometry analysis was performed to determine the relative expression for the wt UGT3A2 and missense variants using the ImageJ software (<https://imagej.nih.gov/ij/>; National Institutes of Health, Bethesda, MD). The relative level of UGT3A2 variant expression was calculated based on the relative band intensities of the wt compared to the missense variants (all normalized to calnexin) and was used in subsequent calculations of the relative $V_{\max}$ obtained in glycosylation activity assays.

Glycosylation Activity of UGT3A2 Missense Variants against PAHs.

Glycosylation assays using microsomes from the UGT3A2 missense variants overexpressed in the HEK293 cell line were performed with alternative cosubstrates as described previously for wt UGT3A2.¹¹ Briefly, pooled cell microsomes (total protein 0.26 to 54 μ g) were incubated with alamethicin (50 μ g/mg total protein) for 15 min on ice. Glycosylation reactions for the simple PAHs [1-OH-pyrene, 1-OH-B(a)P, 3-OH-B(a)P, 7-OH-B(a)P, and 9-OH-B(a)P] were performed in a final reaction volume of 25 μ L at 37 °C containing 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, and 4 mM UDP-glucose or UDP-xylose. PAHs were added within the range of 0.25 to 1600 μ M. Reactions were performed for 45 min and terminated by the addition of 25 μ L of cold acetonitrile; reactions were linear for up to 2 h for all glycosylation reactions performed in this study. Reaction mixtures were centrifuged for 10 min at 16 100g, and the supernatant was collected for UPLC analysis.

Glycoside metabolites of the simple PAHs were quantified using an ACQUITY UPLC System with a PDA Detector (Waters, Milford, MA) utilizing an ACQUITY UPLC BEH C18 column (1.7 μ m, 2.1 $\times$ 100 mm) (Waters) at a constant temperature of 25 °C with an injection volume of 5 μ L. The flow rate was maintained at 0.4 mL/min, and a gradient of solution A [5 mM NH₄OAc (pH 5.0), 10% acetonitrile] and solution B (100% acetonitrile) was used to elute the glycoside and substrate. The chromatographic conditions used to detect the glycosylation of 1-OH-pyrene was 10% solution B for 2 min, a linear gradient to 75% solution B from 2 to 4 min, and then to 100% solution B from 4 to 6 min and re-equilibrium to the initial condition from 6 to 7.5 min. A similar gradient was used for the glycosides of other substrates, but the initial ratio of solution A to solution B varied slightly, with 1-OH-B(a)P, 3-OH-B(a)P, 7-OH-B(a)P, and 9-OH-B(a)P having an initial condition of 85% A and 15% B. The UV absorbance for each substrate and glycoside pair were 240 nm for

the detection of 1-OH-pyrene, and 305 nm for the detection of 1-OH-B(a)P, 3-OH-B(a)P, 7-OH-B(a)P, and 9-OH-B(a)P.

Glycosylation reactions for B(a)P-7,8-diol, the more complex PAH, was performed in a final reaction volume of 25 μL at 37 °C with 50 mM Tris-HCl (pH 7.4), 10 mM MgCl_2 , and 4 mM UDP-glucose or UDP-xylose, using a single point assay with 800 μM B(a)P-7,8-diol. Reactions were terminated after 120 min by the addition of 25 μL of cold acetonitrile. Reaction mixtures were centrifuged for 10 min at 16 100g, and the supernatant was collected for UPLC analysis. Glycoside metabolite formation for B(a)P-7,8-diol was quantified using the ACQUITY UPLC I-Class PLUS System with a PDA e λ Detector (Waters) utilizing an ACQUITY UPLC BEH C18 column (1.7 μm , 2.1 $\times$ 100 mm) (Waters) at a constant temperature of 30 °C with an injection volume of 5 μL . The flow rate was maintained at 0.4 mL/min, and a gradient of solution A and solution B was used to elute the glycoside and substrate from the column. The gradient had an initial condition of 85% A and 15% B with the same total run time as described for the simple PAHs. The wavelength was set to scan between 200 to 300 nm.

The area under the curve for the substrates and glycoside peaks were determined using the MassLynx software and quantified by the ratio of glycoside compared to unconjugated substrate. Calculation of the pmol amount of product is based on the area of the (glycoside peak)/(glycoside + substrate peaks) $\times$ the known substrate concentration in the incubation. For glycosylation rate determinations, each substrate was optimized for both time and protein concentration to ensure substrate utilization was less than 10%. The total protein used in the activity assays was normalized by the relative protein expression of the UGT3A2 variants as determined by Western blot analysis and was used in subsequent calculations of the relative V_{max} . Triplicate experiments were performed for all analysis, with kinetic parameters (V_{max} and K_m) calculated independently (using GraphPad Prism 7) and subsequently averaged ($\pm$ standard deviation).

Glycosylation Activity of UGT3A2-Overexpressing Cell Lines against UDP-Sugars.

A determination of the kinetic parameters for UDP-glucose and UDP-xylose was performed for wt UGT3A2 and the missense variants using 16 μM of 1-OH-pyrene as the cosubstrate. Microsomes (total protein 1.0 to 36 μg) were incubated with alamethicin (50 $\mu\text{g}/\text{mg}$ total protein) for 15 min on ice. Glycosylation reactions were performed in a final reaction volume of 25 μL at 37 °C with 50 mM Tris-HCl (pH 7.4), 10 mM MgCl_2 , 16 μM 1-OH-pyrene, and UDP-sugars added within the range of 31 to 8000 μM , depending on the UGT3A2-overexpressing cell line and the UDP-sugar examined. Reactions were terminated after 45 min by the addition of 25 μL of cold acetonitrile, centrifuged for 10 min at 16 100g, and the supernatant collected for UPLC analysis.

Glycosylated metabolite formation was quantified using the ACQUITY UPLC I-Class PLUS System with a PDA e λ Detector (Waters) utilizing an ACQUITY UPLC BEH C18 column (1.7 μm , 2.1 $\times$ 100 mm) (Waters) at a constant temperature of 30 °C with an injection volume of 5 μL . The flow rate was maintained at 0.4 mL/min, and the gradient was the same as described above for 1-OH-pyrene with the total run time of 7.5 min. The wavelength was

set to scan between 200 to 300 nm. The quantification, glycosylation rate determinations, and kinetic parameters were performed as described above.

Homology Modeling and Structural Analysis.

A high-quality homology model for human UGT3A2 was retrieved from the SWISS-MODEL Repository (<http://swissmodel.expasy.org/>) using the search term “ugt3a2”. UniProtKB accession number Q3SY77, corresponding to the human UGT3A2 sequence, was selected, and model coordinates were downloaded. The model of UGT3A2 was built by standard SWISS-MODEL Repository procedures based on available coordinates for UGT76G1 from *Stevia rebaudiana*, PDB ID 6INH.^{21,22} All structural analyses were performed using PyMOL. Sequence comparisons between UGT3A2 and UGT76G1 were performed in Clustal Omega.²³

Statistical analysis.

A two-tailed *t* test was used to compare the kinetics (K_m , V_{max} , and V_{max}/K_m) of glycoside formation for the UGT3A2 variants as compared to wt UGT3A2, as well as the kinetics of glycoside formation for the wt and variant UGT3A2 isoforms using UDP-glucose vs UDP-xylose as cosubstrates. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Glycosylation Activity of UGT3A2 Variants against PAHs.

To examine the functional effects of missense UGT3A2 variants on PAH activity, variants were created by site-directed mutagenesis of wt UGT3A2 and overexpressed in the HEK293 cell line. As shown by Western blot analysis of pooled cells for each variant as well as the wt UGT3A2 using a UGT3A2-specific antibody, the expression of the three UGT3A2 variants (D140N, A344T, and S435Y) were similar to that observed for wt UGT3A2 (Figure S1, panel A). The cell lines overexpressing the individual colonies from the Y74N, A436T, R445C, and Y474C variants exhibited between 11- to 21-fold decreases in their level of expression as compared to the wt UGT3A2-overexpressing cell line. Individual colonies for the Y74N-, A436T-, R445C-, and Y474C-overexpressing variants demonstrated only slightly improved expression for each variant as compared to pooled cells (shown for the R445C and Y474C variants in Figure S1, panel B). Repeated overexpression attempts to increase the expression of these four variants resulted in similar levels of expression for each variant (results not shown).

The kinetic analysis of UGT3A2 missense variants against PAHs is shown in Table 1 using UDP-glucose, and in Table 2 using UDP-xylose. Utilizing 1-OH-pyrene as a prototypical PAH substrate, the D140N variant exhibited the same K_m (3.3 μ M) with a slightly decreased (1.7-fold) V_{max}/K_m as compared to wt UGT3A2. Modest effects on UGT3A2 activity were observed for the A344T and S435Y variants with UDP-glucose as the cosubstrate, exhibiting 2.1- and 2.8-fold increased K_m values, respectively, and 2.2-fold decreased V_{max}/K_m values as compared to wt UGT3A2. Similar modest effects were observed for the R445C and A436T variants, which exhibited 2.6- and 4.5-fold increased K_m values and 3.9- and 5.2-fold decreased V_{max}/K_m values, respectively. The variants that exhibited the least glycosylation

activity against 1-OH-pyrene were Y74N and Y474C, both exhibiting approximately 12-fold decreased $V_{\max}/K_m$ values as compared to wt UGT3A2.

Of the monohydroxylated B(a)P substrates examined in this study [including 1-OH-B(a)P, 3-OH-B(a)P, 7-OH-B(a)P, and 9-OH-B(a)P], 7-OH-B(a)P was the only substrate that all of the UGT3A2 variants exhibited detectable levels of glycosylation activity against using UDP-glucose as the cosubstrate. Similar to that observed for 1-OH-pyrene, the D140N variant exhibited a modest 3.3-fold decrease in $V_{\max}/K_m$ against 7-OH-B(a)P with UDP-glucose as the cosubstrate as compared to wt UGT3A2. More pronounced effects were observed for the A344T and S435Y variants, with A344T and S435Y exhibiting 3.0- and 9.1-fold increased K_m values, respectively, compared to wt UGT3A2. The A344T variant exhibited a 3.5-fold decreased $V_{\max}/K_m$, which was comparable to that observed for the D140N variant, while the S435Y variant exhibited a 6.2-fold increase in $V_{\max}/K_m$. The Y74N, R445C, and Y474C variants all exhibited similar K_m values, which were approximately 11-fold higher than that observed for wt UGT3A2; the $V_{\max}/K_m$ values for these variants were 40- to 243-fold lower than that observed for wt UGT3A2. Similar to that observed for 1-OH-pyrene, the A436T variant exhibited the highest K_m (474 μM) against 7-OH-B(a)P with UDP-glucose as the cosubstrate; this variant again exhibited low overall glycosylation with UDP-glucose as cosubstrate ($V_{\max}/K_m = 2.3 \mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$). Similar patterns of modest to pronounced decreases in glycosylation activity were observed for all UGT3A2 variants tested against 1-OH-B(a)P, 3-OH-B(a)P, and 9-OH-B(a)P, exhibiting between a 42% and 100% reduction in $V_{\max}/K_m$ for all variants against these substrates using UDP-glucose as cosubstrate as compared to wt UGT3A2 (Table 1). Due to the very low or nondetectable levels of glycosylation activity with UDP-glucose as cosubstrate, kinetic values could not be obtained for the Y74N and Y474C variants against 1-OH-B(a)P, 3-OH-B(a)P, and 9-OH-B(a)P, and 3-OH-B(a)P and 9-OH-B(a)P, respectively.

The pattern of decreased glycosylation activity for UGT3A2 variants was similar when using UDP-xylose as the cosubstrate (Table 2). With 1-OH-pyrene as substrate, the A344T, D140N, and S435Y variants all exhibited a 1.7- to 4.5-fold increase in K_m and an approximately 3.5-fold decrease in $V_{\max}/K_m$ as compared to wt UGT3A2. A436T exhibited the highest K_m (26 μM), which was 22-fold higher than wt UGT3A2, and a 16-fold decrease in $V_{\max}/K_m$ as compared to wt. The R445C and Y474C variants exhibited the least activity ($V_{\max}/K_m = 6.2$ and $4.8 \mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, respectively) with an approximately 10-fold increase in K_m as compared to wt UGT3A2. The Y74N variant exhibited very low activity against 1-OH-pyrene with UDP-xylose as cosubstrate, not reaching saturation in kinetic assays ($K_m > 1600 \mu\text{M}$, $V_{\max} > 290 \text{ pmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$).

The overall pattern of decreased glycosylation activity with UDP-xylose as cosubstrate was also observed with 3-OH-B(a)P as substrate, with the Y74N and Y474C variants exhibiting detectable levels of glycosylation activity only with UDP-xylose as cosubstrate (Table 2). UGT3A2 variants also exhibited very similar patterns of glycosylation activity for the other monohydroxylated B(a)P substrates [1-OH-B(a)P, 7-OH-B(a)P, and 9-OH-B(a)P] when UDP-xylose was used as the cosubstrate, exhibiting between a 62% to 100% reduction in $V_{\max}/K_m$ when compared to wt UGT3A2. Very low levels of substrate affinity ($K_m > 1600 \mu\text{M}$) were observed for several variants against several monohydroxylated B(a)P

substrates including Y74N [1-OH-pyrene, 1-OH-B(a)P, 7-OH-B(a)P, 9-OH-B(a)P], A436T [9-OH-B(a)P], R445C [9-OH-B(a)P], and Y474C [1-OH-B(a)P, 7-OH-B(a)P, 9-OH-B(a)P]. Given that wt UGT3A2 activity is known to be significantly lower against complex PAHs, single point activity assays were used instead of kinetic analysis to determine the effects of UGT3A2 variants on glycosylation activity against B(a)P-7,8-diol.¹¹ For UDP-glucose, the rate for D140N, A344T, and S435Y variants decreased by 87% to 97% when compared to wt UGT3A2 (Figure S2, panel A). Similar results were observed when using UDP-xylose as the cosubstrate, with the rate for D140N, A344T, and S435Y variants decreased by 50% and 83% when compared to wt UGT3A2 (Figure S2, panel B). No glycosylation activity was observed for the Y74N, A436T, R445C, and Y474C variants using both cosubstrates.

Glycosylation Activity of UGT3A2 Variants against UDP-Glucose and UDP-Xylose.

Analysis of the kinetic properties of UGT3A2 variants on UDP-glucose and UDP-xylose were performed using a fixed concentration (16 μM) of 1-OH-pyrene as substrate while increasing the concentration of each UDP-sugar until saturation. As shown in Table 3, wt UGT3A2 exhibited a significant ($P < 0.05$) difference between the utilization of the UDP-sugars, with a 3.4-fold decrease in K_m (214 μM vs 728 μM) and 3.4-fold increase in $V_{\max}/K_m$ (4.7 $\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ vs 1.4 $\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) observed for UDP-glucose as compared to UDP-xylose. In contrast, there was little difference between the utilization of the UDP-sugars for most variants. Large differences in kinetics were observed for the UGT3A2 variants against UDP-glucose vs UDP-xylose when compared to wt UGT3A2 (Table 3). When comparing the $V_{\max}/K_m$ for variants vs that observed for wt UGT3A2, the seven variants were between 3.1- and 362-fold lower when UDP-glucose was the cosubstrate. When utilizing UDP-xylose, the $V_{\max}/K_m$ was significantly ($P < 0.05$) decreased for all variants except for A344T ($P = 0.31$), exhibiting between a 34% to 100% reduction in activity when compared to wt UGT3A2. The variants that could not be statistically compared to wt were Y74N (no activity) and Y474C ($K_m > 4000 \mu\text{M}$).

Homology Model and Variant Position Analysis.

The UGT3A2 homology model included 442 of the 523 amino acids (residues 21 to 463) of the full-length sequence, suggesting that the N- and C-termini were not able to be modeled effectively. This unmodeled region includes one of the UGT3A2 variants, Y474C. All other variant positions were included in the homology model. As expected, the UGT3A2 model and the UGT76G1 template are highly similar in their overall backbone structure (Figure 1, panel A), with a root-mean-square deviation (RMSD) of α -carbon positions of just 0.204 $\text{\AA}^2$. The sequence identity between UGT3A2 and the UGT76G1 template is 23.7% as calculated by Clustal Omega, lending further support to an expected similarity between the two proteins.

All six variants included in the homology model were located and their predicted environments studied further (Figure 1, panel B). Y74 and D140 are in similar regions of the structure (Figure 1, panel C). Y74 provides both hydrogen-bonding support to the active site helix from S34 to H49 through its hydroxyl group. The positive end of this helix's dipole is oriented toward the predicted position of the UDP phosphates in the active site. Y74 also forms hydrophobic interactions with the neighboring β -sheet through its phenyl ring. D140

is farther away from the active site, positioned on a loop at the N-terminus of one strand of the sheet supported by Y74. A344 is located on a loop far from the active site, making no immediately apparent contribution to active site structure or stability.

A435 and S436 are located near the N-terminus of the helix that spans Y433 to R445 (Figure 1, panel D). A435 occupies a hydrophobic pocket at the C-terminus of the D393-K404 helix. The N-terminal end of this helix points into the UGT3A2 active site. S436 is oriented toward the surface of the protein and makes no immediate contacts to other amino acids. R445 is at the C-terminus of the same helix as A435 and S436 (Figure 1, panel E) and provides support for the negative dipole of the helix that spans N373 to H381. This helix would provide significant contact to the UDP-glucose cosubstrate.

DISCUSSION

The role of the UGT3A subfamily in overall metabolism has been understudied when compared to members of the UGT1A, UGT2A, and UGT2B subfamilies. UGT3A2 was recently shown to be expressed in aerodigestive tract tissues and is highly active against PAHs, a class of potent environmental carcinogens.¹¹ The present study is the first to examine the role of UGT3A2 variants in PAH metabolism. All the variants examined in this study exhibited significantly reduced function when compared to wt UGT3A2. Four of the variants (Y74N, A436T, R445C, and Y474C) show less than 26% of wt activity against the substrates tested. The remaining three variants (D140N, A344T, and S435Y) do retain some activity against all substrates with both cosubstrates, but at a significantly reduced rate.

All the UGT3A2 variants examined in this study showed reduced or complete lack of function with all substrates tested. However, when using 1-OH-pyrene as a substrate, K_m and V_{max} values could be calculated for every variant, indicating at least some level of enzymatic function in vitro. The D140N variant was by far the most similar to wt UGT3A2. It is located in the N-terminal region and represents a conservative amino acid substitution of aspartate to asparagine, resulting in no significant changes to the binding affinities and slightly lower overall activity against all PAHs tested, as befits its position distant from the active site. Similarly, the A344T variant showed decreased activity and increased K_m values against most substrates, at values that were significant but not strikingly different than wt values. This variant substitutes a slightly bulkier and more polar side chain in threonine for the methyl-only alanine side chain. On the basis of homology modeling, this variant is located far from the active site. Four variants are in a similar region of the C-terminal domain, with S435Y, A436T, and R445C predicted to all be on the same surface helix, with Y474C in a disordered region unable to be modeled. The S435Y variant retains the most UGT3A2 activity of the three placed on the model, with catalytic efficiency ratios similar to the A344T variant. Due to its position on the surface of the modeled enzyme, this is not a surprising finding. An S435Y substitution placing a largely hydrophobic residue on the surface might destabilize the overall structure slightly, but it would not be expected to significantly affect the active site. The A436T variant exhibited much less overall enzymatic activity, suggesting that this substitution is more deleterious and that the 436 position plays a more significant role in enzyme function. Given that A436 occupies a hydrophobic pocket that might help stabilize an active site helix, the substitution of a bulkier, slightly polar

residue could disrupt the active site. The final variant predicted to be on this α helix, R445C, is a more severe change, a substitution of a large, positively charged arginine side chain for the small reactive side chain in cysteine. This substitution also appears to have a drastic effect on substrate binding and enzymatic activity as is seen from the large and significant deviations in K_m and V_{max} values calculated for this variant. Like A436, R445 appears to help position and stabilize a helix important to the structure of the active site, possibly through charge interactions with the helix dipole, which cysteine would not be able to provide.

The remaining variants, Y74N and Y474C, are both practically nonfunctional with every PAH tested except for 1-OH-pyrene. The Y74N variant is in the N-terminal region of the UGT3A2 enzyme and homology modeling places it in proximity to the active site, potentially involved in coordinating a catalytic α helix. Tyrosine is a large, hydrophobic amino acid and the substitution of a small, hydrophilic moiety from asparagine in its place may destabilize this region, resulting in the significantly lower activity and extremely high K_m values calculated for this variant. Similarly, the Y474C variant shows very little enzymatic activity. This residue does not appear on a homology model, suggesting that it might be found in a disordered region of the structure. However, a tyrosine to cysteine substitution is a drastic substitution that could significantly affect tertiary or quaternary structure.

As can be seen in Figure S1, the level of UGT3A2 expression of the variant cell lines is quite variable, ranging from 1.2-fold (D140N) to 0.048-fold (A436T) of wt UGT3A2. This variable level of expression may also be a reflection of the amino acid substitutions described above. The four cell lines which showed particularly low expression were also those which showed the smallest catalytic efficiencies. Despite multiple attempts, the expression levels of these variants were not able to be significantly improved, suggesting that the amino acid substitutions in these variants may also be affecting enzyme structure or stability at the tertiary or quaternary level. Further experiments will be needed to explore this possibility.

Utilization of UDP sugar cosubstrates by both wt UGT3A2 and variants were explored by kinetic analysis using a fixed concentration (16 μM) of 1-OH-pyrene as substrate with increasing concentrations of UDP-glucose and UDP-xylose as the cosubstrate. Wt UGT3A2 exhibited a significantly lower K_m value (3.4-fold) for UDP-glucose (214 μM) compared to UDP-xylose (728 μM). These values are within the range for the kinetics of UDP-glucuronic acid with the UGT1A enzymes (50–1256 μM) using a fixed concentration for various substrates.¹⁴⁻¹⁸ UGT1A4 was the only enzyme that used 1-OH-pyrene to determine the kinetics of UDP-glucuronic acid, which exhibited a K_m of 201 μM , similar to wt UGT3A2 for UDP-glucose.¹⁴ For UDP-xylose, wt UGT3A2 exhibited a similar K_m when compared to the K_m of UGT1A8 for UDP-glucuronic acid (875 μM) using entacapone. When comparing the kinetics of the UDP-sugars, wt UGT3A2 exhibited a significant difference ($P < 0.05$) with a 3.4-fold higher V_{max}/K_m (4.7 $\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ vs 1.4 $\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) for UDP-glucose as compared to that observed for UDP-xylose. The effect was seen in the K_m because the V_{max} was similar (997 vs 992 $\text{pmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$). These data suggest that UDP-glucose binds with a higher affinity and is therefore more efficiently used as a

cosubstrate. In general, the variants exhibit the same pattern, with most showing a slight preference for UDP-glucose as the more efficient cosubstrate.

Previous literature examining UGT3A2 variants is extremely limited, consisting of a single literature review predicting the potential effect on UGT3A2 function.²⁴ The present study confirmed that the missense variants do indeed have structural and functional effects on the UGT3A2 enzyme. All of the variants tested in this study have reduced or deficient UGT3A2 glycosylation activity against PAHs, which may potentially play an important role in the variability of an individual to effectively detoxify the PAHs present in tobacco. The Y74N, A436T, R445C, and Y474C variants exhibited the most drastic effect due to low expression and negligible glycosylation against PAHs.

Relatively few variants in genes encoding metabolizing enzymes that are important in carcinogen metabolism have been linked with increased aerodigestive tract cancer risk in genome wide association (GWA) studies. This may be due to the fact that few are primarily expressed in aerodigestive tract tissues, the point of contact with tobacco specific carcinogens, including the PAHs.²⁵⁻²⁷ In contrast, UGT3A2 is well-expressed in tobacco target tissues including trachea, larynx, lung, and esophagus.¹¹ In addition, when compared to other UGTs which also exhibit activity against PAHs, UGT3A2 was shown to have higher or comparable activity against 1-OH-pyrene, 1-OH-B(a)P, 3-OH-B(a)P, 7-OH-B(a)P, 9-OH-B(a)-P, and B(a)P-7,8-diol.⁵⁻¹¹ Given the low prevalence of UGT3A2 allelic variants in the population, it is not surprising that UGT3A2 has not been linked to increases in aerodigestive tract cancers in GWA studies. Studies of large populations (>100 000 subjects) should be performed to better examine whether the presence of one of the functional UGT3A2 variants examined in this study impacts aerodigestive cancer risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structural superposition of UGT3A2 model and UGT76G1 template and position of modeled variants. (A) Structural superposition of UGT3A2 model and UGT76G1 template. UGT76G1 template structure is shown in blue, and UGT3A2 model is shown in gray. (B) Position of modeled variants. UGT76G1 template structure is shown in blue, and UGT3A2 model is shown in gray. Each of the six modeled variant side chains are shown rendered as sticks, with carbon in gray, nitrogen in blue, oxygen in red, and phosphorus in orange. Substrate and UDP cosubstrate from the UGT76G1 structure are shown with carbons in light blue to indicate the predicted position of the UGT3A2 active site. (C) Positions of Y74 and D140 are indicated. (D) Positions of S435 and A436 are indicated. (E) Position of R445 is indicated.

Table 1.

Kinetic Analysis of PAH Glycosylation by UGT3A2 Variants Using UDP-Glucose^a

	WT ^d	Y74N	D140N	A344T	S435Y	A436T	R445C	Y474C
				1-OH-Pyrene				
K_m	3.3 ± 0.2	14 ± 7	3.3 ± 0.7	6.8 ± 2.1 ^e	9.4 ± 2.6 ^e	15 ± 1 ^e	8.6 ± 1.4 ^e	7.6 ± 1.3 ^e
V_{max}	1305 ± 118	425 ± 92 ^e	754 ± 178 ^e	1337 ± 179	1592 ± 150	1179 ± 1117	867 ± 120 ^e	215 ± 15 ^e
V_{max}/K_m ^c	396 ± 52	33 ± 8 ^e	230 ± 23 ^e	180 ± 27 ^e	178 ± 29 ^e	76 ± 2 ^e	101 ± 8 ^e	29 ± 5 ^e
V_{max}/K_m ratio	1.0	0.083	0.58	0.45	0.45	0.19	0.26	0.073
				1-OH-B(a)P				
K_m	11 ± 2	>1600	11 ± 3	38 ± 19 ^e	25 ± 1 ^e	397 ± 108 ^e	113 ± 21 ^e	94 ± 17 ^e
V_{max}	2035 ± 333	>2438	836 ± 124 ^e	915 ± 177 ^e	847 ± 49 ^e	3976 ± 1039	661 ± 137 ^e	344 ± 28 ^e
V_{max}/K_m ^c	202 ± 54	NC	81 ± 9 ^e	35 ± 3 ^e	34 ± 0 ^e	10 ± 0 ^e	6.0 ± 1.5 ^e	2.5 ± 0.4 ^e
V_{max}/K_m ratio	1.0	NC	0.40	0.17	0.17	0.050	0.030	0.012
				3-OH-B(a)P				
K_m	7.5 ± 0.9	NA	10 ± 2	26 ± 10	11 ± 0 ^e	121 ± 18 ^e	89 ± 9 ^e	NA
V_{max}	1734 ± 181	NA	986 ± 121 ^e	1140 ± 356	703 ± 96 ^e	1988 ± 191	684 ± 162 ^e	NA
V_{max}/K_m ^c	238 ± 51	NA	97 ± 6 ^e	45 ± 7 ^e	62 ± 7 ^e	17 ± 0 ^e	7.6 ± 1.5 ^e	NA
V_{max}/K_m ratio	1.0	NA	0.41	0.19	0.26	0.071	0.032	NA
				7-OH-B(a)P				
K_m	7.8 ± 1.9	86 ± 27 ^e	11 ± 2	22 ± 4 ^e	71 ± 4 ^e	474 ± 46 ^e	81 ± 18 ^e	83 ± 37 ^e
V_{max}	430 ± 67	18 ± 1 ^e	171 ± 44 ^e	330 ± 104	632 ± 46 ^e	1065 ± 28 ^e	110 ± 15 ^e	41 ± 9 ^e
V_{max}/K_m ^c	56 ± 6	0.23 ± 0.08 ^e	17 ± 6 ^e	16 ± 6 ^e	9 ± 0 ^e	2.3 ± 0.2 ^e	1.4 ± 0.1 ^e	0.57 ± 0.17 ^e
V_{max}/K_m ratio	1.0	0.0041	0.30	0.29	0.16	0.041	0.025	0.010
				9-OH-B(a)P				
K_m	12 ± 0	>1600	13 ± 5	41 ± 9 ^e	35 ± 5 ^e	398 ± 75 ^e	279 ± 73 ^e	>1600
V_{max}	984 ± 85	>425	444 ± 149 ^e	655 ± 121 ^e	727 ± 185	2049 ± 304 ^e	423 ± 29 ^e	>1225

	WT ^d	Y74N	D140N	A344T	S435Y	A436T	R445C	Y474C
$V_{\max}/K_m$ ^c	84 ± 5	NC	36 ± 3 ^e	16 ± 0 ^e	20 ± 3 ^e	5.2 ± 0.2 ^e	1.6 ± 0.3 ^e	NC
$V_{\max}/K_m$ ratio	1.0	NC	0.43	0.19	0.24	0.062	0.019	NC

^a Kinetic parameters of UGT3A2 variants against PAHs were determined using 4 mM UDP-glucose. Data are expressed as the mean ± SD of three independent experiments. K_m (μ M), $V_{\max}$ (μ mol·min⁻¹·mg⁻¹), $V_{\max}/K_m$ (μ L·min⁻¹·mg⁻¹).

^b $V_{\max}$ was calculated per total microsomal protein, normalized to relative UGT3A2 expression.

^c $V_{\max}/K_m$ ratio shows the relative activity for each variant versus wild-type UGT3A2.

^d Results for wild-type UGT3A2 were published previously.¹¹

^e $P < 0.05$ for each variant vs corresponding value for wild-type UGT3A2. NC, not calculated because it did not reach saturation. NA, no activity.

Table 2.

Kinetic Analysis of PAH Glycosylation by UGT3A2 Variants Using UDP-Xylose^a

	WT ^d	Y74N	D140N	A344T	S435Y	A436T	R445C	Y474C
1-OH-Pyrene								
K_m	1.2 ± 0.2	>1600	2.0 ± 0.5	5.4 ± 0.8 ^e	3.8 ± 0.7 ^e	26 ± 2 ^e	11 ± 7	12 ± 4 ^e
V_{max}	998 ± 204	>290	474 ± 75 ^e	1444 ± 121	790 ± 79	1640 ± 197 ^e	46 ± 7 ^e	54 ± 11 ^e
V_{max}/K_m	840 ± 254	NC	264 ± 86 ^e	274 ± 49 ^e	216 ± 46 ^e	52 ± 28 ^e	6.2 ± 2.9 ^e	4.8 ± 1.0 ^e
V_{max}/K_m ratio	1.0	NC	0.31	0.33	0.26	0.062	0.0074	0.0057
1-OH-B(a)P								
K_m	6.1 ± 1.5	>1600	9.6 ± 1.4	15 ± 2 ^e	52 ± 39 ^e	444 ± 179 ^e	48 ± 11 ^e	>1600
V_{max}	1135 ± 117	>175	404 ± 68 ^e	737 ± 82 ^e	807 ± 298	1673 ± 228 ^e	131 ± 51 ^e	>464
V_{max}/K_m	199 ± 53	NC	44 ± 12 ^e	50 ± 9 ^e	21 ± 7 ^e	4.4 ± 1.6 ^e	3.0 ± 1.6 ^e	NC
V_{max}/K_m ratio	1.0	NC	0.22	0.25	0.11	0.022	0.015	NC
3-OH-B(a)P								
K_m	72 ± 2.9	49 ± 26	5.5 ± 1.0	10 ± 2	16 ± 3	80 ± 11 ^e	19 ± 11	138 ± 28 ^e
V_{max}	2525 ± 599	63 ± 27 ^e	679 ± 136 ^e	966 ± 149 ^e	942 ± 174 ^e	1437 ± 252	163 ± 53 ^e	129 ± 17 ^e
V_{max}/K_m	389 ± 98	1.5 ± 0.3 ^e	126 ± 24 ^e	95 ± 2 ^e	61 ± 5 ^e	18 ± 3 ^e	11 ± 5 ^e	1.0 ± 0.1 ^e
V_{max}/K_m ratio	1.0	0.0039	0.32	0.24	0.16	0.046	0.028	0.0026
7-OH-B(a)P								
K_m	8.5 ± 0.5	>1600	3.7 ± 0.2 ^e	63 ± 22 ^e	29 ± 7 ^e	321 ± 75 ^e	36 ± 6 ^e	>1600
V_{max}	683 ± 227	>72	124 ± 11 ^e	813 ± 143	362 ± 92	719 ± 190	81 ± 7 ^e	>80
V_{max}/K_m	80 ± 26	NC	34 ± 5	14 ± 3	13 ± 5	2.2 ± 0.1 ^e	2.3 ± 0.3 ^e	NC
V_{max}/K_m ratio	1.0	NC	0.43	0.18	0.16	0.028	0.029	NC
9-OH-B(a)P								
K_m	9.6 ± 0.9	>1600	13 ± 6	23 ± 10	65 ± 28 ^e	>1600	>1600	>1600
V_{max}	1227 ± 107	>3220	553 ± 84 ^e	536 ± 116 ^e	1300 ± 358	>3664	>2212	>3739

	WT ^d	Y74N	D140N	A344T	S435Y	A436T	R445C	Y474C
$V_{\max}/K_m$ ^c	129 ± 10	NC	49 ± 14 ^e	26 ± 7 ^e	22 ± 5 ^e	NC	NC	NC
$V_{\max}/K_m$ ratio	1.0	NC	0.38	0.20	0.17	NC	NC	NC

^aKinetic parameters of UGT3A2 variants against PAHs were determined using 4 mM UDP-glucose. Data are expressed as the mean ± SD of three independent experiments. K_m (μM), $V_{\max}$ (pmol·min⁻¹·mg⁻¹), $V_{\max}/K_m$ (μL·min⁻¹·mg⁻¹).

^b $V_{\max}$ was calculated per total microsomal protein, normalized to relative UGT3A2 expression.

^c $V_{\max}/K_m$ ratio shows the relative activity for each variant versus wild-type UGT3A2.

^dResults for wild-type UGT3A2 were published previously.¹¹

^e $P < 0.05$ for each variant vs corresponding value for wild-type UGT3A2. NC, not calculated because it did not reach saturation. NA, no activity.

Table 3.

Kinetic Analysis of UGT3A2 Variants for UDP-Sugars Using 1-OH-Pyrene^a

	UDP-glucose			UDP-xylose			UDP-glucose/UDP-xylose	
	K_m (μ M)	V_{max}^b (pmol·min ⁻¹ ·mg ⁻¹)	V_{max}/K_m (μ L·min ⁻¹ ·mg ⁻¹)	K_m (μ M)	V_{max}^b (pmol·min ⁻¹ ·mg ⁻¹)	V_{max}/K_m (μ L·min ⁻¹ ·mg ⁻¹)	V_{max}/K_m	V_{max}/K_m ratio ^c
UGT3A2								
WT	214 ± 40	997 ± 131	4.7 ± 0.3	728 ± 169 ^d	992 ± 316	1.4 ± 0.4 ^d		3.4
Y74N	3359 ± 414	43 ± 7	0.013 ± 0.001	NA	NA	NA		NC
D140N	774 ± 5	253 ± 53	0.33 ± 0.07	1050 ± 517	321 ± 111	0.35 ± 0.11		0.94
A344T	597 ± 163	836 ± 84	1.5 ± 0.4	489 ± 26	455 ± 27 ^d	0.93 ± 0.01		1.6
S435Y	1284 ± 356	707 ± 233	0.55 ± 0.07	1708 ± 203	655 ± 217	0.47 ± 0.11		1.2
A436T	1008 ± 60	197 ± 16	0.20 ± 0.01	1072 ± 311	129 ± 38 ^d	0.13 ± 0.04 ^d		1.5
R445C	1322 ± 250	140 ± 14	0.11 ± 0.02	2946 ± 1334	159 ± 50 ^d	0.061 ± 0.018 ^d		1.8
Y474C	3133 ± 604	95 ± 13	0.032 ± 0.010	>4000	>24	NC		NC

^aKinetic parameters of UGT3A2 variants for UDP-sugars were determined using 16 μ M 1-OH-pyrene. Data are expressed as the mean ± SD of three independent experiments. K_m , V_{max} , V_{max}/K_m represent the mean ± SD of three independent experiments

^b V_{max} was calculated per total microsomal protein, normalized to relative UGT3A2 expression.

^c V_{max}/K_m ratio shows the relative activity for UDP-glucose versus UDP-xylose.

^d $P < 0.05$ for UDP-xylose vs corresponding value for UDP-glucose. NC, not calculated because it did not reach saturation. NA, no activity.