Characterization of UGTs Active against SAHA and Association between SAHA Glucuronidation Activity Phenotype with UGT Genotype

Renee M. Balliet,1,2 Gang Chen,1,3 Carla J. Gallagher,1,3 Ryan W. Dellinger,1,2 Dongxiao Sun,1,2 and Philip Lazarus1,2,3

1Cancer Control and Population Sciences, Penn State Cancer Institute, and Departments of 1Pharmacology and 2Public Health Sciences, Penn State University College of Medicine, Hershey, Pennsylvania

Abstract
Suberoylanilide hydroxamic acid (SAHA) is a histone deacetylase inhibitor used in the treatment of cutaneous T-cell lymphoma and in clinical trials for treatment of multiple other cancers. A major mode of SAHA metabolism is by glucuronidation via the UDP-glucuronosyltransferase (UGT) family of enzymes. To characterize the UGTs active against SAHA, homogenates from HEK293 cell lines overexpressing UGT wild-type or variant UGT were used. The hepatic UGTs 2B17 and 1A9 and the extrahepatic UGTs 1A8 and 1A10 exhibited the highest overall activity against SAHA as determined by $V_{\text{max}}/K_M$ (16 ± 6.5, 7.1 ± 2.2, 33 ± 6.3, and 24 ± 2.4 nL·min$^{-1}$·μg UGT protein$^{-1}$, respectively), with UGT2B17 exhibiting the lowest $K_M$ (300 μmol/L) against SAHA of any UGT in vitro. Whereas the UGT1A8p.Ala173Gly variant exhibited a 3-fold ($P < 0.005$) decrease in glucuronidation activity against SAHA compared with wild-type UGT1A8, the UGT1A8p.Cys277Tyr variant exhibited no detectable glucuronidation activity; a similar lack of detectable glucuronidation activity was observed for the UGT1A10p.Gly139Lys variant. To analyze the effects of the UGT2B17 gene deletion variant (UGT2B17*2) on SAHA glucuronidation phenotype, human liver microsomes (HLM) were analyzed for glucuronidation activity against SAHA and compared with UGT2B17 genotype. HLM from subjects homozygous for UGT2B17*2 exhibited a 43% ($P < 0.01$) decrease in glucuronidation activity and a 73% ($P < 0.002$) increase in $K_M$ compared with HLMs from subjects homozygous for the wild-type UGT2B17*1 allele. Overall, these results suggest that several UGTs play an important role in the metabolism of SAHA and that UGT2B17-null individuals could potentially exhibit altered SAHA clearance rates with differences in overall response.

Introduction
Histone deacetylases (HDAC) are a family of enzymes that have been identified as a promising target to reverse aberrant epigenetic states associated with cancer via regulation of levels of histone and nonhistone protein acetylation and gene expression (1). Several classes of HDAC inhibitors have been developed to target HDACs, with hydroxamic acid–derived compounds found to be effective and potent pan-HDAC isoform inhibitors (2). The Food and Drug Administration–approved hydroxamic acid HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA; Zolinza; Merck), is currently prescribed to treat cutaneous T-cell lymphoma and is also being tested in several phase I to III clinical trials for the treatment of a variety of other cancers including breast, lung, and colon (3, 4). Although SAHA is commonly administered at a conventional oral dose of 400 mg daily, the dose may be reduced to 300 mg daily or 300 mg 5 days a week in certain patients due to increased toxicity including diarrhea, fatigue, nausea, and anorexia (3, 5). Pharmacokinetic studies have identified two inactive metabolites in patients treated with SAHA, SAHA-glucuronide and 4-anilino-4-oxobutanoic acid (Fig. 1; refs. 6, 7). In serum from patients treated with SAHA, SAHA-glucuronide and 4-anilino-4-oxobutanoic acid were on average 3- to 4-fold and 10- to 13-fold higher, respectively, than that of the parent compound (5). Because the apparent terminal half-life of SAHA-O-glucuronide was shown to be similar (~1.8 hours) to that of SAHA itself but much longer (~6–9 hours) for 4-anilino-4-oxobutanoic acid (7), the overall contribution of glucuronidation to SAHA metabolism is significant.

The UDP-glucuronosyltransferase (UGT) superfamily of enzymes catalyze the glucuronidation of a variety of endogenous compounds such as bilirubin and steroid hormones, as well as xenobiotics such as drugs and environmental carcinogens (8–10). Based on structural and amino acid sequence homology, UGTs are classified into several families and subfamilies (11). The UGT2B family members are derived from independent genes located on chromosome 4. The entire UGT1A family is derived from a single gene locus on chromosome 2, coding for 9 functional proteins that differ only in their amino-terminus as a result of alternate splicing of independent exons 1 regions to a shared carboxy terminus encoded by exons 2 to 5 (8, 12). These independent exon 1A regions are responsible for the wide range of substrate specificity shown by the UGT1A family of enzymes, whereas the common region coded by exons 2 to 5 is involved in UDP glucuronic acid binding (10). The specific enzymes responsible for the glucuronidation of SAHA have as yet to be identified.

Polymorphisms in drug metabolizing enzymes have been shown to alter drug efficiency and/or toxicity leading to interindividual differences in drug response (13, 14). Polymorphisms have been identified for many of the UGT genes and several studies have examined their potential role in risk for several cancer types (15–17). Specifically, the UGT2B17 gene deletion polymorphism has been shown to be associated with decreased carcinogen detoxification and increased risk for lung adenocarcinoma (18). UGTs also have a role in modifying response to chemotherapeutic
agents (19). For example, the UGT1A1*28 allele was associated with decreased glucuronidation activity in liver microsomes against SN-38, the major metabolite of irinotecan (20), and irinotecan-treated patients homozygous for the UGT1A1*28 allele exhibit higher levels of its active metabolite (SN-38; ref. 21) and exhibit significant toxicity (19). Similarly, the UGT2B7p.His268Tyr variant was recently associated with decreased liver microsomal glucuronidation activity against major active tamoxifen metabolites, which may be linked to altered patient response to tamoxifen (22); in some cases, however, the UGT2B7p.His268Tyr variant was shown to have no effect or exhibit increased activity against certain substrates (e.g., morphine; ref. 23). As glucuronidation is an important mode of SAHA metabolism in vivo, it is possible that variant UGTs may contribute to differences in SAHA toxicity and potentially in overall patient response to SAHA. The goals of the present study were to fully characterize the human UGT(s) responsible for the glucuronidation of SAHA and to determine whether genetic variations in the major SAHA-glucuronidating enzymes potentially contribute to altered metabolism of SAHA.

Materials and Methods

Chemicals and materials. SAHA was synthesized in the Organic Synthesis Core at Penn State University College of Medicine. Alamethicin, β-glucuronidase, and bovine serum albumin were purchased from Sigma-Aldrich. DMEM, Dulbecco’s PBS (minus calcium chloride and magnesium chloride), fetal bovine serum, penicillin-streptomycin, genetin (G418), the Platinum PCR DNA polymerase, and the pcDNA3.1/V5-His-TOPO mammalian expression vector were all obtained from Invitrogen. The Hotstar PCR kit, the RNeasy Mini and Midi kits, and the QIAEX II gel extraction kit were all purchased from Qiagen. The BCA protein assay kit was purchased from Pierce. All restriction enzymes were purchased from New England Biolabs. PCR primers were purchased from IDT. All other chemicals were purchased from Fisher Scientific unless specified otherwise.

Tissues. The normal human liver tissue specimens used for these studies have been described previously (24). Colon tissue was obtained from the Penn State College of Medicine Tissue Bank. Human liver microsomes (HLM) were prepared through differential centrifugation as previously described (25) and stored (10–20 mg microsomal protein/mL) at −80 °C. Microsomal protein concentrations were measured using the BCA assay. Colon homogenate was prepared as described below for cell line homogenates.

UGT overexpressing cell lines and cell homogenate preparation. Four new UGT-overexpressing cell lines were generated for the experiments outlined in this study. UGT1A5 cDNA was amplified from the UGT1A5 pENTR223.1 vector (Open Biosystems) using primers UGT1A5 (sense, 5′-TGGATGGCCACAGACTGAGTTCC-3′) and UGT1A5AS (antisense, 5′-TTTCTCAATGGGTCTTGG-3′), corresponding to nucleotides 4 to +22 and +1584 to +1608, respectively, relative to the UGT1A5 translation start site. The PCR product encompassing the UGT1A5 coding region (1612 bp) was subcloned into the pcDNA3.1/V5-His-TOPO mammalian expression vector, and conformation of insertion orientation and sequence was performed using 2 vector primers and 2 internal UGT1A5 primers (5′-CACACCTTTCCCTCTGCG-3′ and 5′-AGCTACATTAATGCTTGG-3′, corresponding to nucleotides +452 to +472 and +876 to +896, respectively, relative to the UGT1A5 translation start site) to be previous described (26). The cloned UGT1A5 insert was compared with the sequence described in Genbank and was confirmed to be 100% homologous to the wild-type UGT1A5 sequence.

Site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) was performed for the UGT1A7*2 and UGT1A7*4 alleles [encoding UGT1A7p.(Asn129Lys) + (Arg131Lys) and UGT1A7p.Trp208Arg variant, respectively] using a previously synthesized wild-type UGT1A7 clone (27). The UGT1A7*3 allele [encoding the UGT1A7p.(Asn129Lys) + (Arg131Lys) + (Trp208Arg) variant] was made using the newly synthesized UGT1A7*2 clone as template. Primer set UGT1A7-129/131S (sense, 5′-GGAACCACATCATGCACTTG-3′ and UGT1A7-208AS (antisense, 5′-CCCTTAACTTTCTCCTCCTTG-3′), corresponding to nucleotides +371 to +422 relative to the UGT1A7 translation start site, were used to generate constructs that encode for the amino acid changes at UGT1A7 codons 129 (Asn>Lys) and 131 (Arg>Lys); Primer set UGT1A7-208S (5′-CAAGTGCATGATGTGGTTCC-3′ and UGT1A7-129/131AS (antisense, 5′-CCCTTAACTTTCTCCTCCTTG-3′), corresponding to nucleotides +371 to +422 relative to the UGT1A7 translation start site, were used to generate constructs that encode for the amino acid change at codon 208 (Trp>Arg). The italicized base for each primer indicates the bp change. Site-directed mutagenesis PCR amplification and construct confirmation were performed as previously described (26) with newly generated UGT-overexpressing constructs compared with sequences described in Genbank—all were confirmed to be 100% homologous to their respective allele.

Each construct was used to generate individual UGT-overexpressing human embryonic kidney fibroblast HEK293 cell lines by standard
electroproportionation techniques as previously described (26). Overexpressing cell lines were selected and monitored for UGT expression via Western blotting analysis and levels of UGT1A protein were determined as described previously by our laboratory (26, 27).

All of the additional cell lines overexpressing the UGT1A and UGT2B isoforms analyzed in this study have been described previously (9, 26, 29). UGT1A-overexpressing cell lines were grown in DMEM to 80% confluence as described above before preparation of cell homogenates by resuspending pelleted cells in TBS [25 mmol/L Tris base, 138 mmol/L NaCl, and 2.7 mmol/L KCl (pH 7.4)] and subjecting them to three rounds of freeze-thaw before gentle homogenization. Cells were stored at −80°C in 100-μL aliquots. Total homogenate protein concentrations were measured using the BCA protein assay.

**Western blot analysis.** UGT protein levels were determined by Western blot analysis for all UGT-overexpressing cell lines examined in this study as previously described (26). For UGT1A-overexpressing cells, the UGT1A antibody from Gentest was used and a UGT1A standard of known concentration (Gentest) was used for normalization; for UGT2B-overexpressing cells, a previously described UGT2B-specific antibody was used and a UGT2B7 standard of known concentration (Gentest) was used for normalization (26). Relative UGT protein levels were expressed as the mean of three independent experiments, and all activity assays were normalized relative to UGT expression in the respective UGT-overexpressing cell line.

**Glucuronidation assays.** Glucuronidation activities of HLM or homologs from human UGT-overexpressing cell lines or human colon against SAHA were determined after an initial incubation of colon homogenate (200 μg protein), cell line homogenate (400 μg protein for an initial screen for activity or 40 μg protein for kinetic analysis), or HLM (20 μg protein) with alamethicin (50 μg/mg protein) for 15 min in an ice bath similar to that described previously (24, 27, 30). Incubations (40 μL for cell homogenate and 20 μL for HLM and colon homogenate) were subsequently performed at 37°C in 50 mmol/L Tris buffer (pH 7.5), 10 mmol/L MgCl2, 4 mmol/L UDPGA, and 65 μmol/L to 8 mmol/L of substrate. Screening assays for glucuronidation activity in UGT-overexpressing cell lines were performed using a 1-h incubation, and reactions were terminated by the addition of the same volume as the initial reaction of cold acetonitrile. Reactions were centrifuged at 13,000 g for 10 min at 4°C, and supernatants were collected and then diluted 5-fold using water/acetonitrile (1:1). Glucuronidation assays (1–10 μL) were analyzed for SAHA glucuronidation formation using a Waters ACQUITY UPLC System as previously described (26) with a gradient of 5.6% buffer B (100% acetonitrile) to 72% buffer B over 3 min, followed by 94.4% buffer A [10 mmol/L ammonium acetate (pH 5.0) and 10% acetonitrile]; a linear gradient to 72% buffer B over 3 min was performed. The flow rate was maintained at 0.3 mL/min. The amount of glucuronide formed was determined based on the ratio of SAHA-glucuronide versus unconjugated SAHA after calculating the area under the curve for the SAHA and SAHA-glucuronide peaks using the known amount of SAHA to each reaction as the reference. SAHA-glucuronide was confirmed by sensitivity to β-glucuronidase as previously described (31) and mass spectrometry (described below). As controls, glucuronidation assays were performed using HLM as a positive control for glucuronidation activity and untransfected HEK293 cell homogenate protein as a negative control for glucuronidation activity. Three independent experiments were always performed for kinetic analysis of UGT-overexpressing cell homogenates, with all assays within each experiment performed in triplicate; two independent experiments were performed for rate determination assays and three independent experiments were performed for kinetic analysis for HLM specimens.

**Mass spectrometry.** Triple-quadrupole tandem mass spectrometric detection was performed using a ACQUITY SQD (Waters Corp.) with electrospray ionization interface and a ultra performance liquid chromatography (UPLC) system consisting of a binary gradient pump, an auto sampler (4°C), and a column oven (35°C). A 100 × 2.1 mm i.d. Acquity UPLC Beth Ch18 column with 1.7 μm particles (Waters) and a 0.2 μm prefiter installed before the column were used for this analysis. UPLC was operated under the same conditions as described above. The mass spectrometer operated in positive mode was set up to scan the daughter ion of m/z 414.1. The optimized mass spectrometry parameters used were as follows: capillary voltage, 0.57 kV; cone voltage, 30 V; collision energy, 15 V; source temperature, 450°C; and desolvation temperature, 140°C. Nitrogen was used as the desolvation and cone gas with a flow rate of 760 L/h. Argon was used as the collision gas at a flow rate of 0.1 mL/min. Data acquisition and analysis were performed using the MassLynx NT 4.1 software with QuanLynx program (Waters Corp.).

**cDNA synthesis and UGT2B17 real-time expression assays and deletion genotyping.** To analyze UGT2B17 expression levels in liver specimens, real-time PCR was performed using the TaqMan Gene Expression Assay kit from Applied Biosystems [ID: Hs00854486_s1 for UGT2B17 and Hs99999904_m1 for peptideylprolyl isomerase A (cyclophilin A; PPIA) as the housekeeping gene control]. Total RNA was isolated using a standard Trizol protocol (24, 32) from each of the normal liver tissues examined in this analysis, and 5 μg of total RNA was reverse-transcribed in a final volume of 20 μL containing 1 × reverse transcription-PCR buffer, 0.5 mmol/L of each deoxynucleotide triphosphate, 40 units of recombinant RNase inhibitor, 200 units of SuperScript II reverse transcriptase, and 1 μL of oligo (dT)25-38 (500 μg/mL). Samples were incubated at 42°C for 60 min, and reverse transcriptase was inactivated by heating at 65°C for 5 min and cooling to 4°C.

Real-time PCRs were performed in a volume of 10 μL containing equal amounts of cDNA (20 ng) from each liver specimen. PPIA was used as the normalizing "housekeeping" gene for expression because this gene was shown to exhibit the least variability of any housekeeping gene tested using the TaqMan hsp90 endogenous control plate (Applied Biosystems) using total RNA from a series of six of the tested liver specimens described above.4 PCRs were carried out in 384-well thin-well PCR plates covered with optically clear sealing film (Applied Biosystems). Amplification, detection, and data analysis were performed using the ABI 7900HT sequence detection system and SDS 2.2.2 software using standard settings provided by ABI (Applied Biosystems). Results were expressed using the comparative threshold method following the recommendations of the manufacturer (Applied Biosystems). The threshold cycle number (CT) value for UGT2B17 was normalized against PPIA and calculated as ΔCT = CT_{UGT2B17} − CT_{PPIA}. Relative UGT2B17 mRNA expression was expressed as fold of UGT2B17 versus reference: F = 2^{−ΔΔCT}. HLM genotyping for UGT2B17 has been previously described (18).

**Statistical analysis.** Michaelis-Menten kinetic constants were determined using Prism Version 5 software (GraphPad Software). The Student's t test (two sided) was used for comparing rates and kinetic values of glucuronidation formation for the UGT1A8 isoforms against SAHA. The rate of SAHA-glucuronidation formation in the 100 liver microsomal samples was normally distributed. Student’s t test (two sided) was used to compare the levels of SAHA-Glc formation in subjects with at least one intact UGT2B17 allele (*1/*1 or *1/*2) versus subjects that were UGT2B17-null (*2/*2). Linear Regression was used to compare UGT2B17 expression to HLM activity.

**Results**

**Characterization of the UGTs responsible for SAHA glucuronidation.** Although the SAHA glucuronidase has been identified in the serum and urine of patients treated with SAHA, it has yet to be determined which UGT enzymes are responsible for this activity (6, 7, 33). To identify the UGTs responsible for the glucuronidation of SAHA, cell lines overexpressing "wild-type" UGTs were examined. As shown, in glucuronidation assays with UGT1A17-overexpressing cell homogenate (Fig. 2A), homogenate prepared from colon tissue (B), or with HLM (C), the unconjugated SAHA peak was observed at a retention time of 2.85 minutes and a postulated glucuronide peak was observed with a retention time of 2.59 minutes. The postulated glucuronide peak was sensitive to treatment with β-glucuronidase (D), and similar to that observed previously for

4 Unpublished results.
other compounds (34), this glucuronide peak was not alkali-sensitive (data not shown), suggesting that this glucuronide was an O-glucuronide of SAHA. The mass spectrum for the SAHA-O-glucuronide observed at 2.59 minutes [with protonated molecules (M+H)+ of 441] showed a clear daughter ion at m/z 265 corresponding to the (M+H)+ of SAHA (Fig. 2E), a pattern that was identical to the previously published ion spectra for SAHA-glucuronide (35).

To screen for activity against SAHA, 400 μg of UGT-overexpressing cell homogenate was used in in vitro glucuronidation activity assays. Of the UGTs tested, UGTs 1A3, 1A7, 1A8, 1A9, 1A10, and 2B17 exhibited high levels of glucuronidation against SAHA. Although UGTs 1A1, 1A4, 1A6, and 2B7 exhibited detectable levels of glucuronidation against SAHA, their K_m's were all above 8 mmol/L (accurate K_m could not be measured). No detectable glucuronidation activity against SAHA was observed in our in vitro glucuronidation assays for UGTs 1A5, 2B4, 2B10, 2B11, and 2B15. All of the peaks identified by UPLC for UGTs active against SAHA exhibited the same retention time (2.59 minutes; data not shown) as that observed for UGT1A7 or HLM (see Fig. 2).

Kinetic analysis was performed for those UGTs (1A3, 1A7, 1A8, 1A9, 1A10, and 2B17) for whom accurate kinetic data could be calculated. The kinetic plots for this screening are illustrated in Fig. 3. As shown in Table 1, UGTs 2B17, 1A8, and 1A10 were considerably more active against SAHA compared with any other UGT family member as determined by V_max/K_m. UGT2B17 exhibited the lowest K_m (300 μmol/L) of all the UGTs screened, with the UGT exhibiting the closest K_m being 3-fold higher (UGT1A10) than that observed for UGT2B17. UGT2B17 exhibited a 2.3- and 9.4-fold higher level of glucuronidation activity compared with the other 2 active hepatic UGTs (1A9 and 1A3, respectively) as determined by V_max/K_m.

In vitro characterization of effects on SAHA glucuronidation by missense polymorphic variants of active UGTs. To address potential interindividual differences in glucuronidation rates against SAHA, kinetic analysis of missense variants of the most active UGTs, 1A7, 1A8, and 1A10, were performed. UGT1A7 has 3 common (>2% prevalence in Caucasians, African Americans, or Asians) amino acid–changing polymorphisms that confer four known variant alleles: wild-type UGT1A7*1, UGT1A7*2, UGT1A7*3, and UGT1A7*4 (36). UGT1A8 has two known variant alleles, UGT1A8*2 and UGT1A8*3, which contain amino acid changes encoding UGT1A8p.Ala173Gly and UGT1A8p.Cys277Tyr, respectively (37). UGT1A10 has a variant allele, UGT1A10*2, that contains an amino acid change encoding UGT1A10p.Gly139Lys (38). There are no known common missense variants for UGT2B17.

As shown in Table 2, the UGT1A8p.Ala173Gly variant exhibited a 3-fold (P < 0.005) decrease in glucuronidation activity compared with the UGT1A8 wild-type; no detectable glucuronidation activity was observed for the UGT1A8p.Cys277Tyr variant using up to 8 mmol/L SAHA. Similarly, no glucuronidation activity against SAHA was observed for the UGT1A10p.Gly139Lys variant. However,
both the UGT1A8p.Cys277Tyr and UGT1A10p.Gly139Lys variants did exhibit detectable levels of activity against 4-methylumbelliferone (data not shown), indicating that the UGT1A8- and UGT1A10-variant cell homogenates used in these glucuronidation assays were active. No significant difference in glucuronidation activity was observed against SAHA for any of the UGT1A7 variants.

UGT2B17 genotype and characterization of SAHA glucuronidation in HLM. Data from recent studies have shown the

<table>
<thead>
<tr>
<th>UGT</th>
<th>$K_M$ (mmol/L)</th>
<th>$V_{max}$ (pmol-min$^{-1}$µg$^{-1}$)$^*$</th>
<th>$V_{max}/K_M$ (nL-min$^{-1}$µg$^{-1}$)$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A3</td>
<td>3.4 ± 0.8</td>
<td>6.0 ± 1.8</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>UGT1A7</td>
<td>2.7 ± 0.6</td>
<td>10 ± 0.4</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>UGT1A8</td>
<td>1.9 ± 0.1</td>
<td>62 ± 7.6</td>
<td>33 ± 6.3</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>1.6 ± 0.5</td>
<td>11 ± 1.3</td>
<td>7.1 ± 2.2</td>
</tr>
<tr>
<td>UGT1A10</td>
<td>1.0 ± 0.1</td>
<td>24 ± 2.7</td>
<td>24 ± 2.4</td>
</tr>
<tr>
<td>UGT2B17</td>
<td>0.3 ± 0.1</td>
<td>3.7 ± 0.7</td>
<td>16 ± 6.5</td>
</tr>
</tbody>
</table>

NOTE: All reactions were performed using 40 µg of UGT-overexpressing cell homogenate and incubated for 1 h at 37°C. Kinetic data are reported as mean ± SD for three independent experiments.

$^*$ $V_{max}$ values are adjusted per µg of the corresponding UGT protein as determined by Western blot analysis.

Figure 3. Representative concentration curves for SAHA-glucuronide formation. Representative concentration curves for homogenates from wild-type UGT-overexpressing cell lines and HLM. SAHA-glucuronide formation assays were performed for 1 h at 37°C using 40 µg total cell homogenate protein or 20 µg HLM total protein.
presence of a prevalent polymorphic deletion of the entire UGT2B17 gene (39, 40) that was associated with reduced glucuronidation activity against tobacco carcinogens in assays of HLM (32) and in the levels of glucuronidated tobacco carcinogens in the urine of smokers (18). To examine the effect of the UGT2B17 whole-gene deletion on SAHA glucuronidation activity in human liver, HLMs were prepared from normal liver tissue from 100 independent subjects, which were screened for SAHA glucuronidation activity using 200 μmol/L SAHA (the apparent K_M for UGT2B17 in vitro) and correlated with UGT2B17 genotype by realtime PCR using genomic DNA purified from the same liver specimens. As shown in Fig. 4A, a 45% (P = 0.01) decrease in SAHA-glucuronidation formation was observed in HLM from subjects with the UGT2B17 (2/*2) genotype compared with HLM with at least one intact UGT2B17 allele. Similar levels of SAHA-glucuronidation formation activity were observed for HLM from subjects with one or two intact UGT2B17 alleles. Similar to the pattern of SAHA-glucuronidation formation activities observed in HLM, a significant (P < 0.0001) decrease in UGT2B17 expression was observed in HLM from subjects with the UGT2B17 (2/*2) genotype compared with subjects with at least one intact UGT2B17 allele, and no difference in UGT2B17 expression was observed in HLM from subjects with one versus two intact UGT2B17 alleles (Fig. 4B). A significant (R^2 = 0.30; P < 0.0001) correlation was observed between SAHA-glucuronidation formation in HLM and UGT2B17 expression in the same HLM.

To determine if the association observed between UGT2B17 genotype and HLM glucuronidation activity against SAHA was also manifested as a change in kinetics, kinetic analysis was performed for all of the 13 HLM specimens from subjects exhibiting the UGT2B17 (2/*2) genotype examined in this HLM series, and 13 randomly chosen HLMs from subjects exhibiting the wild-type UGT2B17 (1/*1) genotype. Similar to the pattern observed in the HLM glucuronidation screening assay (described above), a significant (P = 0.0018) 75% increase in K_M was observed for UGT2B17 (2/*2) HLMs (2.1 mmol/L) compared with HLMs from UGT2B17 (1/*1) subjects (1.2 mmol/L; Fig. 4C). No change in V_max was observed in HLMs from different UGT2B17 genotype subgroups.

Discussion

Pharmacokinetic studies have identified two inactive metabolites in patients treated with SAHA, SAHA-glucuronide and 4-anilino-4-oxobutanoic acid (Fig. 1; refs. 6, 7). Although the specific enzymes responsible for the formation of 4-anilino-4-oxobutanoic acid has not as yet been investigated, results from the present analysis show that UGT2B17 is a major hepatic enzyme involved in the glucuronidation of SAHA. The apparent K_M exhibited by UGT2B17 against SAHA was 5- and 11-fold lower than that observed for the other active hepatic UGTs, 1A9 and 1A3, respectively. Based on a comparison of the levels of SAHA-glucuronidation formation in HLM from UGT2B17-deleted subjects [exhibiting the UGT2B17 (2/*2) genotype] versus HLM from subjects wild-type for UGT2B17, ~45% of hepatic SAHA glucuronidation is from UGT2B17. In addition, the K_M is significantly increased (75%; P = 0.0018) in HLM from UGT2B17 (2/*2) subjects compared with HLM from UGT2B17 (1/*1) subjects. This is consistent with the higher level of activity observed for UGT2B17 relative to the other hepatic SAHA-glucuronidating enzymes UGT1A9 and UGT1A3. The increase in K_M from 1.2 to 2.1 mmol/L in HLMs from subjects homozygous for the wild-type UGT2B17 (1/*1) genotype versus subjects homozygous for the UGT2B17 (2/*2) deletion genotype is also clearly consistent with the apparent K_M observed in vitro for UGTs 2B17, 1A9, and 1A3, and are consistent with the fact that all three UGTs are expressed in liver and are contributing to SAHA glucuronidation. Interestingly, the K_M values obtained in vitro for the UGT-overexpressing cell lines (outlined in Table 1) are highly consistent with the K_M values obtained in HLM from subjects with intact UGT2B17 versus HLM from subjects deleted for UGT2B17, indicating a high degree of integrity in the HEK293 cell line system for in vitro analysis of glucuronidation kinetics for individual UGTs. The fact that 45% of overall glucuronidation activity is still observed in HLM from UGT2B17-deleted subjects is consistent with a role for UGTs 1A9 and, potentially, 1A3, being important in hepatic SAHA glucuronidation.

The extrahepatic enzymes UGT1A8 and 1A1 also exhibited high levels of activity against SAHA in vitro, suggesting that they too may play an important role in SAHA glucuronidation in vivo. This effect could potentially be most important in terms of target tissues where these enzymes are expressed and for which SAHA is being used as a chemotherapeutic target including lung, breast, colon, small intestine, and tissues of the aerodigestive tract (17, 29, 41, 42). As colon is a high expresser of extrahepatic UGTs (41, 43), the significant levels of glucuronidation activity against

### Table 2. Kinetic analysis of SAHA-glucuronide formation by UGT1A variants

<table>
<thead>
<tr>
<th>UGT allele</th>
<th>Amino acid change</th>
<th>K_M (mmol/L)</th>
<th>V_max (pmol·min⁻¹·μg⁻¹)*</th>
<th>V_max/K_M (nL·min⁻¹·μg⁻¹)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A7*1</td>
<td></td>
<td>2.7 ± 0.6</td>
<td>10 ± 0.4</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>UGT1A7*2</td>
<td>(Asn129Lys)-(Arg131Lys)</td>
<td>2.5 ± 0.3</td>
<td>12 ± 2.5</td>
<td>5.0 ± 1.6</td>
</tr>
<tr>
<td>UGT1A7*3</td>
<td>(Asn129Lys)-(Arg131Lys)-(Trp208Arg)</td>
<td>2.3 ± 0.3</td>
<td>15 ± 4.0</td>
<td>6.7 ± 2.0</td>
</tr>
<tr>
<td>UGT1A7*4</td>
<td>Trp208Arg</td>
<td>2.2 ± 0.5</td>
<td>12 ± 4.4</td>
<td>5.4 ± 1.9</td>
</tr>
<tr>
<td>UGT1A8*1</td>
<td></td>
<td>1.9 ± 0.1</td>
<td>62 ± 7.6</td>
<td>33 ± 6.3</td>
</tr>
<tr>
<td>UGT1A8*2</td>
<td>Ala173Gly</td>
<td>3.0 ± 0.7</td>
<td>34 ± 2.6</td>
<td>12 ± 2.2</td>
</tr>
<tr>
<td>UGT1A8*3</td>
<td>Cys277Tyr</td>
<td>No detectable activity</td>
<td>No detectable activity</td>
<td>No detectable activity</td>
</tr>
<tr>
<td>UGT1A10*1</td>
<td></td>
<td>1.0 ± 0.1</td>
<td>24 ± 2.7</td>
<td>24 ± 2.4</td>
</tr>
<tr>
<td>UGT1A10*2</td>
<td>Gly193Lys</td>
<td>No detectable activity</td>
<td>No detectable activity</td>
<td>No detectable activity</td>
</tr>
</tbody>
</table>

NOTE: All reactions were performed using 40 μg of UGT-overexpressing cell homogenate and incubated for 1 h at 37°C. Kinetic data are reported as mean ± SD for three independent experiments. *V_max values are adjusted per μg of the corresponding UGT protein as determined by Western blot analysis.
SAHA exhibited by colon homogenate in this study is consistent with a potential role for UGTs 1A8 and/or 1A10 in SAHA glucuronidation in extrahepatic tissues. This suggests that functional single nucleotide polymorphisms in these enzymes may potentially be important in interindividual variability in target tissue SAHA glucuronidation and in SAHA absorption within the colon/intestinal tract.

Variants of UGTs have previously been shown to alter drug response and toxicity (13, 44). The *UGT1A1*/*28 allele, which is associated with decreased *UGT1A1* expression (45), has been linked to decreased glucuronidation of SN-38, the major active metabolite of Irinotecan, in HLM and in the levels of urinary SN-38-glucuronide in patients treated with Irinotecan (19, 20, 46, 47). These decreases in excretion have been linked to increased Irinotecan toxicity (19, 46, 48), and testing for the *UGT1A1*/*28 allele is a FDA-approved genetic screening test for patients who may be considered for treatment with this agent (49). Similarly, UGT2B10 is the major *N*-glucuronidating enzyme of nicotine, and the *UGT2B10*/*2 allele, which is associated with inactivation of UGT2B10 due to a Asp>Tyr amino acid change at codon 67, was shown to be linked to a null activity phenotype against nicotine in HLM (50).

Variation in patient response to SAHA has been clinically observed (3, 5, 51). Recent studies have indicated that resistance to HDAC inhibitors such as SAHA may be multifactorial (52), and that a pharmacogenetic mechanism may be responsible for the differences in overall response to SAHA treatment (33, 52). Results from the present study showed significant alterations in SAHA glucuronidation associated with variants in UGTs active against SAHA. Although active against other substrates, the *UGT1A8p.Cys277Tyr* and *UGT1A10p.Gly139Lys* variants were shown to be inactive against SAHA in *in vitro* experiments using homogenates from cell lines overexpressing these variants. Although the prevalence of their corresponding alleles are relatively low in the population (<3% in Caucasians; refs. 37, 38), their presence could potentially have high penetrance in terms of target tissue metabolism of SAHA. The *UGT1A8p.Ala173Gly* variant was shown to be inactive against SAHA in *in vitro* experiments with wild-type *UGT1A8*; although less penetrant in *in vitro* experiments shown to be inactive against SAHA in *in vitro* experiments using homogenates from cell lines overexpressing these variants. Although the prevalence of their corresponding alleles are relatively low in the population (<3% in Caucasians; refs. 37, 38), their presence could potentially have high penetrance in terms of target tissue metabolism of SAHA. The *UGT1A8p.Cys277Tyr* and *UGT1A10p.Gly139Lys* variants were shown to be inactive against SAHA in *in vitro* experiments with wild-type *UGT1A8*; although less penetrant than the *UGT1A8p.Cys277Tyr* and *UGT1A10p.Gly139Lys* variants, it is more common (~28% prevalence in Caucasians; ref. 37) and could affect larger segments of the population.

Perhaps most important is the significant decrease in SAHA-glucuronide formation observed in HLM with the *UGT2B17*-null genotype. The 45% decrease in SAHA-glucuronide formation observed in HLM from *UGT2B17*-null subjects was similar to the 50% decrease in glucuronidation activity observed in HLM from subjects homozygous for the *UGT1A1*/*28 allele against SN-38, the major metabolite of Irinotecan (48). Irinotecan-treated patients with this *UGT1A1* genotype exhibit higher SN-38 levels (21) and significant toxicity, a pattern that contributed to this genotype being a FDA-approved genetic test for Irinotecan treatment (19). Therefore, the similar alteration in glucuronidation activity against SAHA observed in HLM from subjects with the *UGT2B17*-null genotype suggests that a similarly significant effect on overall levels of circulating SAHA and subsequent toxicity or overall patient response could be observed in *UGT2B17*-null patients treated with SAHA.

*UGT2B17* exhibits high levels of activity against endogenous compounds including androgens as well as many xenobiotics including various types of compounds including carcinogens.
counemzins, anthraquinones, flavonoids, nonsteroidal anti-inflammatory drugs, monoterpenoids, and phenols (53). The UGT2B17 gene deletion could potentially have a significant clinical effect on the metabolism of these as well as other UGT2B17 substrates. For example, urine from smokers homozygous for the UGT2B17*2 allele exhibit a significant reduction in the ratio of NNAL-glucuronide/NNAL, a pattern that was linked to increased risk for lung adenocarcinoma (18). The UGT2B17 deletion variant has also been associated with altered levels of glucuronides of androstanediol, testosterone, estradiol, and dihydrotestosterone in human serum compared with serum from UGT2B17 (∗1/*1) or (∗1/*2) subjects (54, 55). These data are consistent with a clinical effect by the UGT2B17 deletion and a potential effect on SAHA metabolism in vivo. It should be emphasized that characterization of the pathway that produces 4-anilino-4-oxobutanoic acid must be similarly performed to fully evaluate the UGT2B17 deletion on overall SAHA metabolism.

No difference in SAHA-glucuronide formation was observed in HLM from subjects with either one or two intact copies of the UGT2B17 gene. This corresponded with the finding that no difference in UGT2B17 expression was observed in the same liver specimens as determined by real-time PCR. Studies are under way to examine the mechanisms underlying this pattern of UGT2B17 expression.

together, the data presented in this report suggest that the presence of genetic variation in active UGTs, including UGT2B17, are associated with alterations in SAHA metabolism. Studies are currently on going to determine whether these differences are potentially associated with observed differences in overall SAHA toxicity or patient response to SAHA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 10/28/08; revised 1/2/09; accepted 1/21/09. Published OnlineFirst 3/24/09.

Grant support: Public Health Service grant R01-DE13158 (P. Lazarus) from the National Institute for the Study of SAHA, the Tissue Procurement Facilities at the Lee L. Howitt Cancer Center and Penn State College of Medicine for tissue procurement, and the Functional Genomics Core Facility at the Penn State University College of Medicine for real-time PCR services.

References


8. UGT1A10139Lys isoform. Drug Metab Dispos 2006;34:96–596.


15. Lee L. Howitt Cancer Center and Penn State College of Medicine for tissue procurement, and the Functional Genomics Core Facility at the Penn State University College of Medicine for real-time PCR services.


22. Hofseth LJ, Burchell B, Bend JR, et al.ブラウザが分からず、正常なテキストが表示できません。


Characterization of UGTs Active against SAHA and Association between SAHA Glucuronidation Activity Phenotype with UGT Genotype

Renee M. Balliet, Gang Chen, Carla J. Gallagher, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-08-4143

Cited articles
This article cites 55 articles, 26 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/7/2981.full.html#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
/content/69/7/2981.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.