Chemoenzymatic Synthesis, Characterization, and Scale-Up of Milk Thistle Flavonolignan Glucuronides^S

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ABSTRACT

Plant-based therapeutics, including herbal products, continue to represent a growing facet of the contemporary health care market. Mechanistic descriptions of the pharmacokinetics and pharmacodynamics of constituents composing these products remain nascent, particularly for metabolites produced following herbal product ingestion. Generation and characterization of authentic metabolite standards are essential to improve the quantitative mechanistic understanding of herbal product disposition in both *in vitro* and *in vivo* systems. Using the model herbal product, milk thistle, the objective of this work was to biosynthesize multimilligram quantities of glucuronides of select constituents (flavonolignans) to fill multiple knowledge gaps in the understanding of herbal product disposition and action. A partnership between clinical pharmacology and natural products chemistry expertise was leveraged to optimize reaction conditions for efficient glucuronide formation and

evaluate alternate enzyme and reagent sources to improve cost effectiveness. Optimized reaction conditions used at least one-fourth the amount of microsomal protein (from bovine liver) and cofactor (UDP glucuronic acid) compared with typical conditions using human-derived subcellular fractions, providing substantial cost savings. Glucuronidation was flavonolignan-dependent. Silybin A, silybin B, isosilybin A, and isosilybin B generated five, four, four, and three monoglucuronides, respectively. Large-scale synthesis (40 mg of starting material) generated three glucuronides of silybin A: silybin A-7-*O*- β -*D*-glucuronide (15.7 mg), silybin A-5-*O*- β -*D*-glucuronide (1.6 mg), and silybin A-4'-*O*- β -*D*-glucuronide (11.1 mg). This optimized, cost-efficient method lays the foundation for a systematic approach to synthesize and characterize herbal product constituent glucuronides, enabling an improved understanding of mechanisms underlying herbal product disposition and action.

Introduction

Plant-based therapeutics, including herbal products, have existed for thousands of years, predating recorded history. Today, herbal products represent an ever-flourishing facet of the contemporary health care market, which has grown steadily in the United States since passage of the Dietary Supplement Health and Education Act in 1994 (Lindstrom et al., 2014). Despite millennia of usage, mechanistic descriptions of the pharmacokinetics and pharmacodynamics of constituents composing these products remain nascent.

The top-selling herbal product, milk thistle [*Silybum marianum* (L.) Gaertn. (Asteraceae)], is an extensively studied hepatoprotectant, as well as a perpetrator of herb-drug interactions (Wellington and Jarvis,

2001; Gurley et al., 2006, 2012; Abenavoli et al., 2010; Brantley et al., 2010, 2013, 2014a,b; Loguercio and Festi, 2011; Gurley, 2012; Polyak et al., 2013a,b; Gufford et al., 2014a, 2015). Despite considerable investigation, quantitative data describing mechanisms underlying the disposition of milk thistle constituents remain elusive and are virtually nonexistent when considering herbal constituent metabolites generated *in vivo*. Failing to account for the disposition and possible bioactivity of these metabolites may be a source of frequently cited *in vitro*–*in vivo* disconnects when translating preclinical “hits” to clinical application (Won et al., 2010, 2012). Generation and characterization of authentic metabolite standards of these constituents are essential to improve the quantitative mechanistic understanding of their disposition in both *in vitro* and *in vivo* systems.

Synthesis of metabolite standards of herbal products poses challenges beyond those with drug metabolites (Walker et al., 2011, 2014; Di and Obach, 2015). For example, because herbal products are plant-derived, product variation can occur at multiple stages of production, ranging from plant growth and sourcing to harvest, drying, and manufacturing (Won et al., 2010, 2012; Brantley et al., 2014a; Gufford et al., 2014b). These challenges in consistency necessitate careful consideration of key individual constituents prior to synthesis of metabolite standards. In addition, the supply of individual constituents to use as starting materials for the generation of metabolite standards can range from limited to nonexistent, a challenge not frequently encountered when synthesizing a metabolite standard of a conventional

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ABBREVIATIONS: BLM, bovine liver microsome; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; HLM, human liver microsome; HMBC, heteronuclear multiple-bond correlation; HPLC, high-performance liquid chromatography; MS, mass spectrometry; UDPGA, UDP glucuronic acid; UPLC, ultra-performance liquid chromatography.

small-molecule pharmaceutical. Addressing these additional hurdles requires collaboration between the divergent yet complementary skill sets of clinical pharmacologists and natural products chemists. This partnership can unravel the complicated composition and pharmacology of herbal products to improve the understanding of the disposition and action of these products.

Chemoenzymatic generation of major β -glucuronides of the flavonolignans silybin A and silybin B, the primary constituents of the semipurified milk thistle extract silibinin, has been reported (Kren et al., 2000; Han et al., 2004; Jančová et al., 2011; Kren et al., 2013; Charrier et al., 2014). However, approaches to enhance the efficiency of these procedures remain unexplored. Moreover, the crude milk thistle extract silymarin contains at least seven flavonolignan diastereoisomers, including isosilybin A and isosilybin B, which also undergo extensive glucuronidation. Challenges in isolating sufficient quantities of individual parent flavonolignans from silymarin have likely hindered generation of glucuronide metabolites for these structurally related constituents. Methods to isolate gram quantities of pure flavonolignans, including silybin A, silybin B, isosilybin A, and isosilybin B, have been developed (Graf et al., 2007; Monti et al., 2010). Chemical synthesis of glucuronide conjugates could be pursued but would require synthesis of an extensive library of orthogonally protected precursors of each flavonolignan (Stachulski and Meng, 2013; Zhang et al., 2013). Chemoenzymatic processes using isolated constituents as starting materials, followed by purification and structural characterization using natural products chemistry protocols, can be used to efficiently generate flavonolignan glucuronides on a multimilligram scale.

The cost-effective availability of authentic glucuronide metabolites of herbal product constituents will facilitate evaluation of pharmacologic activity, quantitative assessment of metabolic clearance pathways, direct quantification in biologic matrices to determine systemic exposure, and development of additional assays to describe herbal product constituent disposition. Using milk thistle as the model herbal product, the objective of this work was to generate glucuronides of select flavonolignans to fill multiple knowledge gaps in the understanding of herbal product disposition and action. The aims were to 1) enhance efficiency by optimizing reaction conditions for maximal glucuronide formation and 2) evaluate alternate enzyme and reagent sources to improve cost effectiveness. The results form the foundation for a systematic approach to synthesize and characterize herbal product metabolites (Fig. 1).

Materials and Methods

Materials and Chemicals. Human liver microsomes (HLMs; pooled from 50 donors, mixed gender) and bovine liver microsomes (BLMs; pooled from two donors, mixed gender, >12 months old) and bovine liver S9 fraction (pooled from two donors, mixed gender, >12 months old) were purchased from Xenotech, LLC (Lenexa, KS). Bovine serum albumin (BSA), MgCl_2 , silibinin, and UDP-glucuronic acid (UDPGA) were purchased from Sigma-Aldrich (St. Louis, MO). Alamethicin was purchased from Cayman Chemical Company (Ann Arbor, MI). The individual milk thistle flavonolignans were purified as described in detail previously (Graf et al., 2007) and were >97% pure as determined by ultra-performance liquid chromatography (UPLC) (Napolitano et al., 2013). Methanol [liquid chromatography/mass spectrometry (MS) grade], ethanol, Tris-HCl, Tris base, and formic acid were purchased from Fisher Scientific (Waltham, MA). Strata-X 33- μm polymeric reversed-phase solid phase extraction columns (60 mg of medium each) were purchased from Phenomenex (Torrance, CA). Acetone- d_6 was purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA).

Initial Optimization of Conditions. Glucuronidation reaction conditions evaluating a range of microsomal and UDPGA concentrations were examined and compared with typical conditions (Walsky et al., 2012; Walker et al., 2014) to optimize yield and cost. A series of 12 different conditions (Table 1) were examined at six different time points over a 24-hour period for silybin A, silybin

Fig. 1. Schematic of proposed approach to generate and characterize authentic herbal product glucuronides. PD, pharmacodynamics; PK, pharmacokinetics.

B, isosilybin A, and isosilybin B. Conditions examined included enzyme source (BLMs versus S9 fraction), enzyme concentration (1, 0.5, and 0.25 mg/ml for BLMs), and UDPGA concentration relative to flavonolignan concentration (10-fold and 2-fold molar excess). Optimization of alamethicin concentration and source were evaluated as potential cost-saving measures using a series of reactions with varying alamethicin concentration and an in-house alamethicin source (alamethicin F50, which was isolated from a filamentous fungal culture, MSX70741, as detailed previously) (Ayers et al., 2012).

TABLE 1
Comparison between typical and screened reaction conditions to optimize cost, efficiency, and yield of milk thistle glucuronides

Reagent	Typical Condition ^a	Screened Condition(s)
Tris-HCl (pH 7.4) (mM) ^b	100	100
MgCl ₂ (mM) ^b	5	5
BSA (% w/v) ^b	0.5	0.5
Alamethicin (μM) ^b	25	25
Silybin A (mM) ^b	0.4	0.4
UDPGA (mM)	4.0	4.0, 0.8
Enzyme source	HLMs	BLMs, bovine S9 fraction
Enzyme source concentration (mg/ml protein)	1.00	1.00, 0.50, 0.25 (BLMs); 2.00, 1.00, 0.50 (S9)

^aWalker et al. (2014).

^bConcentration was not varied to limit experimental complexity.

A master mixture of MgCl₂, BSA, and alamethicin was prepared in Tris-HCl buffer (100 mM, pH 7.4) at 3 times the final reaction concentration. This mixture was split into two aliquots, and UDPGA was added at either 10- or 2-fold molar excess. Each of these mixtures was split into four aliquots, and the four flavonolignans were added separately from dimethylsulfoxide (DMSO) stock solutions (19.28 mg/ml). Each flavonolignan-containing mixture was divided further into six microcentrifuge tubes. Three different volumes of BLMs (2 mg/ml) or bovine S9 (4 mg/ml) were added, depending on the desired concentration, with Tris-HCl buffer added to produce a final reaction volume of 0.65 ml. The 48 mixtures were incubated at 37°C, after which 100-μl aliquots were removed and quenched with 300 μl of ice-cold MeOH at each time point (0, 2, 4, 8, 12, and 24 hours), generating 288 samples, which were stored at 4°C until analysis.

Samples were prepared for UPLC-MS analysis by centrifugation (15,000g × 2 minutes), and 60 μl of the supernatant was removed and mixed with 90 μl of H₂O in a 96-well plate (final concentration of 30% MeOH and 40 μM total flavonolignans). The resultant mixture (3-μl injection volume) was separated using an Acquity UPLC HSS C18 (1.8 μm, 50 × 2.1 mm) column with an Acquity UPLC system (Waters, Milford, MA) coupled to a Q Exactive Plus high-resolution electrospray ionization mass spectrometer (Thermo Fisher Scientific, San Jose, CA) operated in positive mode.

Large-Scale Reaction of Silybin A and Purification of Glucuronides. After determining the optimal reaction conditions for silybin A (0.8 mM UDPGA, 0.25 mg/ml BLMs, 37°C × 12 hours), a large-scale reaction was initiated. Tris-HCl (204 ml, 100 mM, pH 7.4), MgCl₂·6H₂O (212 mg, 5 mM), UDPGA (108 mg, 0.8 mM), BSA (1.05 g, 0.5% w/v), silybin A (40 mg in 1.7 ml of DMSO, 0.4 mM), alamethicin (8 mg in 0.4 ml of DMSO, 38 μg/ml, 19.5 μM), and BLMs (2.5 ml of 20 mg/ml, 0.25 mg/ml) were combined to yield a total reaction volume of 209 ml (1.0% DMSO final). The reaction was incubated at 37°C in a rotary incubator at 100 rpm to prevent settling of the BLMs during the incubation and was terminated after 12 hours by adding 3 volumes (630 ml) of ice-cold MeOH and stored at 4°C. The resulting mixture was filtered [qualitative grade Whatman filter paper (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) in a Büchner funnel], and the supernatant was evaporated to a volume of ~20 ml. Wash solution (10% MeOH) was added to yield a volume of ~50 ml (5% MeOH). The reaction concentrate was purified further using six 60-mg Strata-X cartridges Phenomenex, loading 1 ml at a time over eight successive purification cycles. Before the first cycle, each cartridge was

conditioned with 4 ml of 100% MeOH and equilibrated with 3 ml of 10% MeOH. For each cycle, the cartridges were pre-equilibrated with 3 ml of 10% MeOH, loaded with 1.0 ml of the reaction concentrate, and washed with 3 ml of 10% MeOH; the combined load and wash fractions were designated pool 1. The sample-loaded cartridges were eluted with 3 ml of 50% MeOH, generating pool 2, then eluted with 4 ml of 100% MeOH, generating pool 3. Each of the pools was dried under vacuum, yielding 4707 mg of pool 1, 51 mg of pool 2, and 24 mg of pool 3.

Pool 2 (a white solid), containing the desired reaction products, was dissolved in 500 μl of 4:1 DMSO:dioxane (producing a fluorescent yellow-green solution) and purified in two successive separations (250 μl each) by preparative reverse-phase high-performance liquid chromatography (HPLC) using a Gemini-NX C18 column (5 μm, 250 × 21 mm) with a gradient from 20:80 to 40:60 CH₃CN:H₂O (0.1% formic acid) over 30 minutes at a flow rate of 21.2 ml/min with peak elution monitored at 288 nm (Phenomenex, Torrance, CA). Separations were accomplished using a Varian ProStar HPLC system (Varian, Palo Alto, CA) equipped with ProStar 210 pumps and a ProStar 335 photodiode array detector and Galaxie Chromatography Workstation (version 1.9.3.2; Varian).

Characterization and Structural Confirmation. All NMR experiments were conducted in acetone-*d*₆ using either an Agilent 700 NMR spectrometer equipped with a cryoprobe (700 and 175 MHz for ¹H and ¹³C, respectively; Agilent Technologies, Santa Clara, CA) or a JEOL ECS-400 NMR spectrometer (399.78 MHz for ¹H and 100.53 MHz for ¹³C; JEOL, Tokyo, Japan) equipped with an auto tune 5 mm field gradient tunable Royal probe (NM-03810RO5/UPG). High-resolution electrospray ionization mass spectrometry data were collected using an electrospray ionization source coupled to a Q Exactive Plus system (Thermo Fisher Scientific, San Jose, CA) in positive and negative ionization modes via a liquid chromatography/autosampler system composed of an Acquity UPLC system (Waters).

Comparison of BLM- versus HLM-Generated Glucuronides. Flavonolignan glucuronidation in BLMs was compared with HLMs using the previously determined optimal reaction conditions for silybin A in BLMs (Table 2). A mixture of MgCl₂, BSA, alamethicin, and UDPGA was prepared in Tris-HCl buffer (100 mM, pH 7.4) at 2.5 times the final reaction concentration and split into four aliquots. The four flavonolignans were added separately from DMSO stock solutions (19.28 mg/ml) and divided to yield duplicate microcentrifuge tubes. BLMs or HLMs (0.424 mg/ml) in Tris-HCl buffer were added to produce a final

TABLE 2
Summary of optimal reaction conditions and projected cost savings to generate milk thistle flavonolignan glucuronides

Compound	Optimized Conditions				Typical Conditions ^a
	Silybin A	Silybin B	Isosilybin A	Isosilybin B	N/A
Enzyme source	BLMs	S9	BLMs	BLMs	HLMs
Enzyme concentration (mg/ml)	0.25	0.25	0.25	0.25	1.0
UDPGA (mM)	0.8	0.8	0.8	0.8	4
Time (h)	8	8	4	12	N/A
Cost (U.S. dollars) ^b	480	310	460	480	2400

N/A, not applicable.

^aWalker et al. (2014).

^bBased on a 25-mg scale reaction.

reaction volume of 0.65 ml. Final concentrations of all reaction ingredients were the same as those described earlier for the large-scale reaction of silybin A. The eight mixtures were incubated at 37°C, after which 100- μ l aliquots were removed and quenched with 300 μ l of ice-cold MeOH at each time point (0, 2, 4, 8, 12, and 24 hours), generating 48 samples, which were stored at 4°C until analysis. Samples were prepared for and analyzed by UPLC-MS as described earlier.

Results

Synthesis of Authentic Glucuronides of Herbal Products Is Achievable. Milk thistle flavonolignans demonstrated compound-dependent glucuronidation (Figs. 2 and 3); typically, three to five glucuronidated products were observed by mass spectrometry for each reaction. Silybin A generated five monoglucuronides, silybin B generated four monoglucuronides, isosilybin A generated four monoglucuronides, and isosilybin B generated three monoglucuronides

Fig. 2. Optimization of incubation time, UDPGA concentration, and BLM concentration. Silybin A (A), silybin B (B), isosilybin A (C), or isosilybin B (D) (0.4 mM) was incubated with 1 (black), 0.5 (dashed), or 0.25 (crimson) mg/ml BLMs with 4 (upper) or 0.8 (lower) mM UDPGA. Disappearance of flavonolignan starting material (left column) and appearance of glucuronides (right column) were monitored over time. Glucuronide data are presented as the sum of all monoglucuronide products formed at each time point.

Fig. 3. Optimization of incubation time, UDPGA concentration, and S9 concentration. Silybin A (A), silybin B (B), isosilybin A (C), or isosilybin B (D) (0.4 mM) was incubated with 2 (gray), 1 (dashed), or 0.5 (black) mg/ml S9 with 4 (upper) or 0.8 (lower) mM UDPGA. Disappearance of flavonolignan starting material (left column) and appearance of glucuronides (right column) were monitored over time. Flavonolignan disappearance and glucuronide formation data using BLMs (0.25 mg/ml) are included as a comparison with microsomal systems (crimson). Glucuronide data are presented as the sum of all monoglucuronide products formed at each time point.

(Figs. 2 and 3). Diglucuronide flavonolignans were detected only in trace amounts. Optimization and scaling up of incubation procedures were focused on monoglucuronide formation due to the paucity of diglucuronide conjugates. Optimal incubation times based upon maximal product formation (0.8 mM UDPGA) were 8, 8, 4, and 12 hours for silybin A, silybin B, isosilybin A, and isosilybin B, respectively (Figs. 2 and 3). Twofold molar excess of UDPGA and 0.25 mg/ml protein was optimal for all flavonolignans. Only silybin B was conjugated more rapidly by S9 than BLMs, representing a potential cost-savings measure for generating silybin B glucuronides.

BLMs Represent a Cost-Effective and Efficient System for Generating Glucuronides. Optimized reaction conditions required 4-fold less enzyme and 5-fold less UDPGA cofactor compared with standard conditions (Table 1). Use of BLMs and optimized reaction conditions yielded substantial (at least 5-fold) cost savings compared

Fig. 4. Analysis of the large-scale reaction for silybin A. (A) Purification of silybin A glucuronides. Preparative HPLC UV trace (288 nm) of the reaction mixture applied to a Gemini-NX C18 column eluted using a 20–40% gradient of $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (0.1% formic acid). Isolated silybin A glucuronides (1–3) are indicated in the chromatogram. (B) Stacked plots showing the selected ion chromatogram (left), UV absorbance at 288 nm (middle), and UV spectrum from 190 to 500 nm (right) of isolated compounds silybin A-7-*O*- β -D-glucuronide (1), silybin A-5-*O*- β -D-glucuronide (2), and silybin A-4'-*O*- β -D-glucuronide (3).

with standard reaction conditions using human-derived subcellular fractions (Table 2). Further cost savings can be realized using an alternate alamethicin source [i.e., alamethicin F50 from fungal culture MSX70741 (Ayers et al., 2012)] for synthetic reactions (~\$150 for a 25-mg scale reaction). Relatively low protein concentrations of optimized conditions would be expected to reduce compound loss to nonspecific binding and simplify purification procedures.

Large-Scale Generation of Glucuronides Facilitates Structural Characterization. Large-scale synthesis generated three glucuronides of silybin A, which were purified by preparative HPLC (Fig. 4A). Compound 1 eluted at 13.5 minutes (15.7 mg), compound 2 at 15.1 minutes (1.6 mg), and compound 3 at 18.5 minutes (11.1 mg); the weights indicate the amounts isolated based on a reaction that started with 40 mg of silybin A. The purity of the glucuronides was measured by UPLC-MS, and the UV data were indicative of the conjugated core of flavonolignans (Fig. 4B). The structures of the three silybin A glucuronides (compounds 1–3) were assigned primarily using ^1H , ^{13}C , correlation spectroscopy, heteronuclear single-quantum correlation, and heteronuclear multiple-bond correlation (HMBC) NMR data

(Fig. 5; Table 3); the data for the major product (compound 1) are discussed later, and the other glucuronides (compounds 2 and 3) were assigned in an analogous manner (Supplemental Figs. S1–S6).

The high-resolution electrospray ionization mass spectrometry (Fig. 6), ^{13}C NMR (Table 3), and heteronuclear single-quantum correlation data for the major product of the silybin A glucuronidation reaction (compound 1 in Figs. 4–7) indicated a molecular formula of $\text{C}_{31}\text{H}_{30}\text{O}_{16}$, suggesting an index of hydrogen deficiency of 17. The ^1H NMR spectrum (Supplemental Fig. S1) of compound 1 displayed signals for a methoxy group, eight aromatic protons, nine oxymethines, and a pair of nonequivalent protons for the oxymethylene group, whereas the ^{13}C NMR spectrum revealed 31 carbons [20 sp^2 (eight protonated) and 11 sp^3 (nine tertiary, one secondary, and one primary)]. Two 1,2,4-trisubstituted aromatic rings were identified based on ^1H - ^1H coupling constants ($^2J_{\text{HH}} = 8.3$ Hz and $^4J_{\text{HH}} = 1.8$ Hz), and the corresponding ^{13}C NMR shifts (δ_{C} 144.7, 145.2, 148.5, and 148.0 for C-3', C-4', C-3'', and C-4'', respectively) required that they each be 1,2-dioxygenated. An additional tetrasubstituted aromatic ring, which was 1,3,5-trioxygenated, was also observed. Finally, two carbonyl carbons (δ_{C} 199.0 and 170.0 for C-4 and C-7''', respectively) accounted for the remaining sp^2 carbons.

Fig. 5. Selected HMBC spectrum of compound 1 (400/100 MHz) in acetone- d_6 . The HMBC correlation from H-1''' to C-7 supports the assigned site of attachment of the glucuronic acid to silybin A.

The structure of the silybin A portion of compound 1 was established by one-dimensional and two-dimensional NMR data and through comparisons to the literature (Lee and Liu, 2003). The structural data from the ^1H NMR spectrum of compound 1 (Supplemental Fig. S1; Table 3) were consistent with the characteristics of a 5,7-dioxygenated flavanonol by the signals at δ_{H} 6.20 (1H, s, H-6), 6.20 (1H, s, H-8), 5.18 (1H, d, $J = 11.7$ Hz, H-2), and 4.75 (1H, d, $J = 11.7$ Hz, H-3). The aromatic proton signals at δ_{H} 7.17 (1H, d, $J = 1.8$ Hz, H-2'), 6.97 (1H, d, $J = 8.3$ Hz, H-5'), and 7.10 (1H, dd, $J = 8.3, 1.8$ Hz, H-6') could be attributed to the B-ring unit of silybin A. The aromatic proton signals at δ_{H} 7.15 (1H, d, $J = 1.8$ Hz, H-2''), 6.88 (1H, d, $J = 8.3$ Hz, H-5''), and 6.98 (1H, dd, $J = 8.3, 1.8$ Hz, H-6'') could be attributed to the E-ring unit of silybin A. The proton signals at δ_{H} 5.01 (1H, d, $J = 8.3$ Hz, H-7'''), δ_{H} 4.17 (1H, ddd, $J = 8.3, 4.4, 2.4$ Hz, H-8'''), δ_{H} 3.76 (1H, dd, $J = 12.4, 2.4$ Hz, H-9''a), and δ_{H} 3.52 (1H, dd, $J = 12.4, 4.4$ Hz, H-9''b) along with the aromatic proton signals for the E-ring unit could be attributed to the coniferyl alcohol moiety of silybin A.

Additional proton (5 oxymethines) and carbon signals in the spectra of compound 1 indicated condensation of silybin A with a glucuronic moiety. The anomeric H-1''' of the glucuronide portion of compound 1 was readily identifiable due to its chemical shift, coupling pattern, and coupling constant (δ_{H} 5.27, d, 7.8 Hz). The two main structural units of compound 1 were linked on the basis of an HMBC correlation of H-1''' to C-7 (Figs. 5 and 6; Supplemental Fig. S2). The presence of a sharp peak at approximately δ_{H} 11.5, due to intramolecular H-bonding between the phenolic proton at C-5 to the C-4 carbonyl, further verified the linkage at the C-7 position.

The structures of compounds 2 and 3 were elucidated in a similar manner (Fig. 6; Supplemental Figs. S3–S6; Table 3). With respect to the characterization of flavonolignans by NMR, in general (Napolitano et al., 2013), this study varied in one major aspect. Based on earlier research that derivatized the flavonolignans (Sy-Cordero et al., 2013; Althagafy et al., 2013), the phenol moieties were hypothesized to be glucuronidated readily; indeed, other researchers have reported this observation (Kren et al., 2000; Jančová et al., 2011; Křen et al., 2013; Charrier et al., 2014). Determining the position of glucuronidation required an HMBC correlation from the anomeric proton back to the carbon attached to the phenol (Fig. 6). However, such a correlation would be somewhat inconclusive if the phenolic protons at the nonglucuronidated positions were not observed, which was a challenge

at the 5 position due to chelation with the carbonyl and proton exchange in protic solvents, e.g., MeOH- d_4 . Thus, all NMR spectra were acquired in acetone- d_6 such that the phenolic protons could be observed readily. For compounds 2 and 3, the ^{13}C NMR data (Supplemental Figs. S3 and S5; Table 3) displayed 31 signals, which were six more than silybin A due to the resonances from the glucuronic acid motif at C-1''' through C-7'''. The anomeric C-1''' appeared at approximately δ_{C} 102 and the anomeric H-1''' at approximately δ_{H} 5.0 as a doublet with a coupling constant at approximately 7 Hz, characteristic of the β -anomer (Pearson et al., 2005; Walker et al., 2007). Data from the HMBC experiments (Supplemental Figs. S4 and S6) demonstrated the connectivity of the glucuronide moiety, where correlations were observed between H-1''' to C-5 and H-1''' to C-4'' for compounds 2 and 3, respectively.

BLMs Generate Human-Relevant Glucuronides with Enhanced Efficiency. Compared with HLMs, BLMs converted all four flavonolignans to their respective glucuronides more rapidly and completely (Fig. 8). Silybin A and silybin B glucuronides were produced similarly by both BLMs and HLMs. The predominant glucuronides of isosilybin A and isosilybin B generated using BLMs (elution time 3.1 minutes) were opposite of the predominant glucuronides generated using HLMs (elution time 2.8 minutes) (Fig. 8). Overall, BLMs generated glucuronide profiles similar to those generated by HLMs (Fig. 8).

Discussion

Partnerships between clinical pharmacologists and natural products chemists with divergent yet complementary skill sets are imperative to advance the understanding of herbal product constituent disposition and pharmacology. Complete characterization of herbal product metabolites will enable identification of sites of metabolic lability that could be targeted to optimize pharmacokinetics of therapeutically promising constituents. These studies would enable structure-activity relationships that lay the groundwork for application of rational drug design concepts to compounds of natural origin.

Metabolite characterization can identify the potential for bioactivation and toxicity of a molecule that may occur in vivo. Identifying and characterizing metabolites with appreciable human exposure is critical to a complete understanding of the pharmacology of a particular herbal product. Minor metabolites may also be important to overall activity, as they can be unique in both disposition and action. Individual

TABLE 3
NMR spectroscopic data for silybin A glucuronides (1–3) in acetone-*d*₆

Values of δ are in ppm.

Position	Silybin A-7- <i>O</i> - β -D-glucuronide 1 ^a			Silybin A-5- <i>O</i> - β -D-glucuronide 2 ^{b,c}			Silybin A-4''- <i>O</i> - β -D-glucuronide 3 ^a		
	δ_C	δ_H	HMBC	δ_C	δ_H	HMBC	δ_C	δ_H	HMBC
	<i>Multiplicity, J in Hz</i>			<i>Multiplicity, J in Hz</i>			<i>Multiplicity, J in Hz</i>		
2	84.2	5.18 d 11.7	3, 4, 8a, 1', 2'	83.2	5.02 d 11.6	3, 4, 1', 2', 6'	84.1	5.11 d 11.5	3, 4, 8a, 1', 2', 6'
3	73.1	4.75 d 11.7	2, 4, 1'	73.8	4.42 d 11.6	2, 4, 1'	73.0	4.69 d 11.5	2, 4, 1'
4	199.0			192.8			198.2		
4a	103.0			104.2			101.5		
5	164.3			161.3			165.0		
6	97.8	6.20 s	4, 8, 4a, 8a	100.6	6.84 bs	5, 7, 8, 4a	97.1	6.00 d 1.8	4a, 5, 7, 8
7	166.6			166.8			167.8		
8	96.4	6.20 s	5, 6, 7	98.8	6.03 d 1.8	6, 7, 4a, 8a	96.0	5.96 d 1.8	4, 4a, 6, 7, 8a
8a	163.8			165.0			164.0		
1'	130.9			131.1			131.1		
2'	117.5	7.17 d 1.8	2, 4', 6'	117.1	7.11 d 1.9	2, 4'	117.4	7.17 d 1.8	2, 4', 6'
3'	144.7			144.4			144.5		
4'	145.2			144.8			145.1		
5'	117.4	6.97 d 8.3	1', 2', 3'	117.3	6.96 d 8.3	1', 3'	117.4	6.97 d 8.3	1', 3'
6'	122.2	7.10 dd 8.3, 1.8	2, 2', 4'	121.9	6.91 dd 8.3, 1.9	4'	122.2	7.1 dd 8.3, 1.8	2, 4', 5'
1''	129.1			128.9			132.6		
2''	111.8	7.15 d 1.8	4'', 6'', 7''	111.9	7.11 d 1.9	4'', 6'', 7''	113.0	7.2 d 1.8	4'', 6'', 7''
3''	148.5			148.5			150.9		
3''-OMe	56.3	3.87 s	3''	56.2	3.83 s	3''	56.4	3.86 s	3''
4''	148.0			147.8			147.8		
5''	115.7	6.88 d 8.3	1'', 2'', 3''	115.7	6.86 d 8.1	1'', 3''	117.4	7.23 d 8.3	1'', 3''
6''	121.6	6.98 dd 8.3, 1.8	2'', 3'', 7''	121.3	6.93 dd 8.1, 1.8	2'', 4'', 7''	121.0	7.06 dd 8.3, 1.8	2'', 4'', 7''
7''	77.2	5.01 d 8.3	3', 2'', 6'', 8'', 9''	77.0	4.95 d 8.1	1'', 2'', 6'', 8''	76.9	5.06 d 7.8	1'', 2'', 6'', 8''
8''	79.5	4.17 ddd	7''	79.2	4.14 ddd	7''	79.4	4.18 ddd 7.8, 3.8, 1.9	7''
		8.3, 4.4, 2.4			8.1, 4.4, 2.6				
9''a	61.8	3.76 dd	7'', 8''	61.4	3.68 dd	7''	61.7	3.77 dd	7'', 8''
9''b		12.4, 2.4	4'		12.2, 2.6	7''		12.4, 1.9	
		3.52 dd			3.48 dd			3.52 dd	
		12.4, 4.4			12.2, 4.4			12.4, 3.8	
1'''	100.6	5.27 d	7, 5'''	104.2	4.78 d	5	102.1	5.16 d	4''
		7.8			7.8			7.3	
2'''	74.0	3.53 dd	1''', 3'''	73.7	3.50 m	3'''	74.4	3.59 dd	1''', 3'''
		9.4, 7.8						8.9, 7.3	
3'''	76.9	3.61 dd	1''', 2''', 4'''	77.0	3.56 m	4'''	77.2	3.60 dd	2'''
		9.4, 9.0						9.4, 8.9	
4'''	72.4	3.71 dd	3''', 7'''	73.9	3.60 m	3''', 1'''	72.6	3.71 dd	3'''
		9.6, 9.0						9.6, 9.4	
5'''	76.0	4.23 d	1''', 3''', 4''', 7'''	—	—		76.1	4.09 d	1''', 3''', 4''', 7'''
		9.6						9.6	
7'''	170.0			174.8			170.1		

^a¹H NMR at 400 MHz and ¹³C NMR at 100 MHz.

^b¹H NMR at 700 MHz and ¹³C NMR at 175 MHz.

^cNMR acquired in 10% D₂O in acetone-*d*₆.

constituents within complex herbal product mixtures can be differentially metabolized. Milk thistle flavonolignans, although structurally similar (i.e., constitutional isomers), exemplify this consideration, as evidenced by the in vitro depletion curves demonstrating compound-dependent metabolism in BLMs (Figs. 2 and 3) and differential metabolism in BLMs compared with HLMs (Fig. 8). Although evaluation of isolated constituents can simplify investigation, the possibility exists that one constituent may modulate metabolism of another both in vitro and in vivo. This possibility underscores the importance of evaluating both individual constituents and complex mixtures to ascertain the metabolic fates in each scenario; such experiments would be exceedingly difficult without the availability of authentic metabolite standards.

Typical chemoenzymatic glucuronidation reactions include a 10-fold molar excess of UDPGA and ≥ 1 mg/ml of microsomal protein. Saturating UDPGA concentrations (5–10 mM) are routinely used when recovering in vitro kinetic parameters using HLMs ($K_m \sim 0.2$ –1.3 mM) (Court et al., 2001) to eliminate UDPGA availability as a potential rate-limiting step. Application of these relatively high concentrations to

synthetic reactions assumes that excess cofactor and enzyme enhance yield. The current work demonstrated that using less of these reagents for chemoenzymatic synthesis is an effective cost-saving measure, and that excess enzyme and reagents can reduce yields via what appear to be reverse catalases. Saccharolactone, a β -glucuronidase inhibitor, is often added to microsomal incubations to prevent enzymatic degradation of glucuronides. However, hepatic microsomal preparations are not expected to be substantially impacted by adding saccharolactone (Oleson and Court, 2008), and degradation of glucuronides under optimized conditions was minimal (Fig. 2). Consequently, saccharolactone was not included in the optimized reaction. Detergents could be explored as an additional cost-savings measure to replace alamethicin, but the impact of detergent selection and concentration on glucuronide yield would need to be evaluated (Soars et al., 2003). Albumin was added to all microsomal incubations based upon reports of substrate-dependent decreases in K_m and increases in V_{max} (Rowland et al., 2008; Manevski et al., 2011, 2013; Gill et al., 2012; Walsky et al., 2012), which are believed to reflect binding to inhibitory fatty acids (Rowland et al., 2007). Selection of additives that can modulate microsomal UDP-glucuronosyl transferase

Fig. 6. Stacked plots showing the high-resolution electrospray ionization mass spectrometry for positive (left) and negative (right) ionization modes of isolated compounds silybin A-7-*O*-β-D-glucuronide (1), silybin A-5-*O*-β-D-glucuronide (2), and silybin A-4''-*O*-β-D-glucuronide (3). Values in parentheses represent the difference between the measured versus calculated mass for the indicated molecular formula; values within 5 ppm are considered valid.

activity is an important consideration that can be addressed in microscale optimization reactions prior to scaling up synthetic procedures.

Generation of flavonolignan glucuronides on the desired scale initially appeared to be overtly cost-prohibitive based on literature protocols. However, relatively inexpensive microscale optimization reduced the total costs by at least an order of magnitude, making these previously cost-prohibitive experiments (thousands of U.S. dollars per reaction) feasible (Table 2). Cost-effective generation of large (multi-milligram) quantities are needed to support detailed investigation of the kinetics and pharmacology of herbal product metabolites across multiple laboratories. However, cost savings could be realized with microscale (<1 mg) incubations followed by quantitative NMR to generate small, yet useful, quantities of metabolites using approaches similar to those described for the synthesis of drug metabolites (Walker et al., 2011, 2014). Flavonolignan cost was not factored into total cost estimates, as these compounds are isolated in house, precluding meaningful comparisons to alternate herbal product constituents that can vary widely in cost and availability. Synthetic processes using isolated constituent starting materials enhance optimization of specific reaction conditions and simplify metabolite purification, leading to enhanced yield. Enhanced efficiency is crucial to reduce waste of limited individual constituents that may take weeks to months to isolate and purify in quantities sufficient for metabolite synthesis. Deconvolution of complex parent and metabolite mixtures encountered in both in vitro and clinical samples can be overcome partially using isolated constituents to simplify the diversity of generated metabolites in a particular system.

Bioanalysis of herbal product glucuronides historically has relied on indirect methods using enzymatic (e.g., β-glucuronidase) incubations to cleave the conjugates, followed by analysis of the parent compound. Indirect quantification methods increase sample preparation time and

frequently are plagued by poor accuracy and reproducibility resulting from incomplete enzymatic hydrolysis and instability of metabolites under enzymatic conditions. Moreover, simultaneous cleavage of multiple glucuronides present in a given system precludes quantitative

Fig. 7. Structures and diagnostic HMBC correlations of silybin A glucuronides (1–3).

Fig. 8. Comparison of BLM- versus HLM-generated glucuronides. Silybin A (A), silybin B (B), isosilybin A (C), or isosilybin B (D) (0.4 mM) was incubated with 0.25 mg/ml BLMs (crimson) or HLMs (black) with 0.8 mM UDPGA. (Left panel) Disappearance of flavonolignan starting material (dashed lines) and appearance of glucuronides (solid lines) were monitored over time. Glucuronide data are presented as the sum of all monoglucuronide products formed at each time point. (Right panel) UPLC-MS chromatograms of flavonolignan glucuronides (659 m/z) generated following 12-hour incubation with BLMs (upper) or HLMs (lower).

description of individual glucuronides. A lack of commercially available glucuronide standards necessitates chemoenzymatic synthesis and purification of glucuronide metabolites for analytical development and

application. The reaction scales described in the current work are capable of supporting bioanalytical method development, metabolism inquiries, and pharmacology evaluations.

In summary, techniques to efficiently and cost-effectively generate and purify authentic glucuronide metabolites of a model herbal product have been developed (Fig. 1). This approach was bolstered by a partnership between clinical pharmacology and natural products chemistry expertise. These techniques are essential to improve the understanding of mechanisms underlying herbal product disposition and action. Authentic metabolites can be used to provide quantitative description of metabolite formation rates, exposure, and activity. Quantitative description of herbal product disposition and action could lead to improved translation of natural product discoveries from bench to bedside.

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Authorship Contributions

Participated in research design: Gufford, Graf, Oberlies, Paine.

Conducted experiments: Gufford, Graf, Paguigan.

Contributed new reagents or analytic tools: Graf, Oberlies.

Performed data analysis: Gufford, Graf, Paguigan, Oberlies, Paine.

Wrote or contributed to the writing of the manuscript: Gufford, Graf, Paguigan, Oberlies, Paine.

References

- Abenavoli L, Capasso R, Milic N, and Capasso F (2010) Milk thistle in liver diseases: past, present, future. *Phytother Res* **24**:1423–1432.
- Althagafy HS, Graf TN, Sy-Cordero AA, Gufford BT, Paine MF, Wagoner J, Polyak SJ, Croatt MP, and Oberlies NH (2013) Semisynthesis, cytotoxicity, antiviral activity, and drug interaction liability of 7-O-methylated analogues of flavonolignans from milk thistle. *Bioorg Med Chem* **21**:3919–3926.
- Ayers S, Ehrmann BM, Adcock AF, Kroll DJ, Carache de Blanco EJ, Shen Q, Swanson SM, Falkinham JO, 3rd, Wani MC, and Mitchell SM, et al. (2012) Peptaibols from two unidentified fungi of the order Hypocreales with cytotoxic, antibiotic, and anthelmintic activities. *J Pept Sci* **18**:500–510.
- Brantley SJ, Argikar AA, Lin YS, Nagar S, and Paine MF (2014a) Herb-drug interactions: challenges and opportunities for improved predictions. *Drug Metab Dispos* **42**:301–317.
- Brantley SJ, Graf TN, Oberlies NH, and Paine MF (2013) A systematic approach to evaluate herb-drug interaction mechanisms: investigation of milk thistle extracts and eight isolated constituents as CYP3A inhibitors. *Drug Metab Dispos* **41**:1662–1670.
- Brantley SJ, Gufford BT, Dua R, Fedduk DJ, Graf TN, Scarlett YV, Frederick KS, Fisher MB, Oberlies NH, and Paine MF (2014b) Physiologically based pharmacokinetic modeling framework for quantitative prediction of an herb-drug interaction. *CPT Pharmacometrics Syst Pharmacol* **3**:e107.
- Brantley SJ, Oberlies NH, Kroll DJ, and Paine MF (2010) Two flavonolignans from milk thistle (*Silybum marianum*) inhibit CYP2C9-mediated warfarin metabolism at clinically achievable concentrations. *J Pharmacol Exp Ther* **332**:1081–1087.
- Charrier C, Azerad R, Marhol P, Purchartová K, Kuzma M, and Křen V (2014) Preparation of silybin phase II metabolites: Streptomyces catalyzed glucuronidation. *J Mol Catal, B Enzym* **102**:167–173.
- Court MH, Duan SX, von Moltke LL, Greenblatt DJ, Patten CJ, Miners JO, and Mackenzie PI (2001) Interindividual variability in acetaminophen glucuronidation by human liver microsomes: identification of relevant acetaminophen UDP-glucuronosyltransferase isoforms. *J Pharmacol Exp Ther* **299**:998–1006.
- Di L and Obach RS (2015) Addressing the challenges of low clearance in drug research. *AAPS J* **17**:352–357.
- Gill KL, Houston JB, and Galetin A (2012) Characterization of in vitro glucuronidation clearance of a range of drugs in human kidney microsomes: comparison with liver and intestinal glucuronidation and impact of albumin. *Drug Metab Dispos* **40**:825–835.
- Graf TN, Wani MC, Agarwal R, Kroll DJ, and Oberlies NH (2007) Gram-scale purification of flavonolignan diastereoisomers from *Silybum marianum* (Milk Thistle) extract in support of preclinical in vivo studies for prostate cancer chemoprevention. *Planta Med* **73**:1495–1501.
- Gufford BT, Chen G, Lazarus P, Graf TN, Oberlies NH, and Paine MF (2014a) Identification of diet-derived constituents as potent inhibitors of intestinal glucuronidation. *Drug Metab Dispos* **42**:1675–1683.
- Gufford BT, Chen G, Vergara AG, Lazarus P, Oberlies NH, and Paine MF (2015) Milk Thistle Constituents Inhibit Raloxifene Intestinal Glucuronidation: A Potential Clinically Relevant Natural Product-Drug Interaction. *Drug Metab Dispos* **43**:1353–1359.
- Gufford BT, Lazarus P, Oberlies NH, and Paine MF (2014b) Predicting pharmacokinetic herb-drug interactions: overcoming hurdles that extend beyond drug-drug interactions. *AAPS Newsmagazine* **April** **2014**:19–22.
- Gurley BJ (2012) Pharmacokinetic herb-drug interactions (part 1): origins, mechanisms, and the impact of botanical dietary supplements. *Planta Med* **78**:1478–1489.
- Gurley BJ, Barone GW, Williams DK, Carrier J, Breen P, Yates CR, Song PF, Hubbard MA, Tong Y, and Cheboyina S (2006) Effect of milk thistle (*Silybum marianum*) and black cohosh (*Cimicifuga racemosa*) supplementation on digoxin pharmacokinetics in humans. *Drug Metab Dispos* **34**:69–74.
- Gurley BJ, Fifer EK, and Gardner Z (2012) Pharmacokinetic herb-drug interactions (part 2): drug interactions involving popular botanical dietary supplements and their clinical relevance. *Planta Med* **78**:1490–1514.
- Han YH, Lou HX, Ren DM, Sun LR, Ma B, and Ji M (2004) Stereoselective metabolism of silybin diastereoisomers in the glucuronidation process. *J Pharm Biomed Anal* **34**:1071–1078.
- Jancová P, Siller M, Anzenbacherová E, Křen V, Anzenbacher P, and Šimánek V (2011) Evidence for differences in regioselective and stereoselective glucuronidation of silybin diastereoisomers from milk thistle (*Silybum marianum*) by human UDP-glucuronosyltransferases. *Xenobiotica* **41**:743–751.
- Křen V, Marhol P, Purchartová K, Gabrielová E, and Modrianský M (2013) Biotransformation of silybin and its congeners. *Curr Drug Metab* **14**:1009–1021.
- Křen V, Ulrichová J, Kosina P, Stevenson D, Sedmera P, Příkrylová V, Halada P, and Šimánek V (2000) Chemoenzymatic preparation of silybin beta-glucuronides and their biological evaluation. *Drug Metab Dispos* **28**:1513–1517.
- Lee DY and Liu Y (2003) Molecular structure and stereochemistry of silybin A, silybin B, isosilybin A, and isosilybin B. Isolated from *Silybum marianum* (milk thistle). *J Nat Prod* **66**:1171–1174.
- Lindstrom A, Ooyen C, Lynch M, Blumenthal M, and Kawa K (2014) Sales of herbal dietary supplements increase by 7.9% in 2013, marking a decade of rising sales: turmeric supplements climb to top ranking in natural channel. *HerbalGram* **103**:52–56.
- Loguercio C and Festi D (2011) Silybin and the liver: from basic research to clinical practice. *World J Gastroenterol* **17**:2288–2301.
- Manevski N, Moreolo PS, Yli-Kauhaluoma J, and Finel M (2011) Bovine serum albumin decreases Km values of human UDP-glucuronosyltransferases 1A9 and 2B7 and increases Vmax values of UGT1A9. *Drug Metab Dispos* **39**:2117–2129.
- Manevski N, Troberg J, Svaluto-Moreolo P, Dziedzic K, Yli-Kauhaluoma J, and Finel M (2013) Albumin stimulates the activity of the human UDP-glucuronosyltransferases 1A7, 1A8, 1A10, 2A1 and 2B15, but the effects are enzyme and substrate dependent. *PLoS One* **8**:e54767.
- Monti D, Gazák R, Marhol P, Biedermann D, Purchartová K, Fedrigo M, Riva S, and Křen V (2010) Enzymatic kinetic resolution of silybin diastereoisomers. *J Nat Prod* **73**:613–619.
- Napolitano JG, Lankin DC, Graf TN, Friesen JB, Chen SN, McAlpine JB, Oberlies NH, and Pauli GF (2013) HIFSA fingerprinting applied to isomers with near-identical NMR spectra: the silybin/isosilybin case. *J Org Chem* **78**:2827–2839.
- Oleson L and Court MH (2008) Effect of the beta-glucuronidase inhibitor saccharolactone on glucuronidation by human tissue microsomes and recombinant UDP-glucuronosyltransferases. *J Pharm Pharmacol* **60**:1175–1182.
- Pearson AG, Kiefel MJ, Ferro V, and von Itzstein M (2005) Towards the synthesis of aryl glucuronides as potential heparanase probes. An interesting outcome in the glycosidation of glucuronic acid with 4-hydroxycinnamic acid. *Carbohydr Res* **340**:2077–2085.
- Polyak SJ, Ferenci P, and Pawlowsky JM (2013a) Hepatoprotective and antiviral functions of silymarin components in hepatitis C virus infection. *Hepatology* **57**:1262–1271.
- Polyak SJ, Oberlies NH, Pécheur EI, Dahari H, Ferenci P, and Pawlowsky JM (2013b) Silymarin for HCV infection. *Antivir Ther* **18**:141–147.
- Rowland A, Gaganis P, Elliot DJ, Mackenzie PI, Knights KM, and Miners JO (2007) Binding of inhibitory fatty acids is responsible for the enhancement of UDP-glucuronosyltransferase 2B7 activity by albumin: implications for in vitro-in vivo extrapolation. *J Pharmacol Exp Ther* **321**:137–147.
- Rowland A, Knights KM, Mackenzie PI, and Miners JO (2008) The “albumin effect” and drug glucuronidation: bovine serum albumin and fatty acid-free human serum albumin enhance the glucuronidation of UDP-glucuronosyltransferase (UGT) 1A9 substrates but not UGT1A1 and UGT1A6 activities. *Drug Metab Dispos* **36**:1056–1062.
- Soars MG, Ring BJ, and Wrigton SA (2003) The effect of incubation conditions on the enzyme kinetics of udp-glucuronosyltransferases. *Drug Metab Dispos* **31**:762–767.
- Stachulski AV and Meng X (2013) Glucuronides from metabolites to medicines: a survey of the in vivo generation, chemical synthesis and properties of glucuronides. *Nat Prod Rep* **30**:806–848.
- Sy-Cordero AA, Graf TN, Runyon SP, Wani MC, Kroll DJ, Agarwal R, Brantley SJ, Paine MF, Polyak SJ, and Oberlies NH (2013) Enhanced bioactivity of silybin B methylation products. *Bioorg Med Chem* **21**:742–747.
- Walker GS, Atherton J, Bauman J, Kohl C, Lam W, Reilly M, Lou Z, and Mutlib A (2007) Determination of degradation pathways and kinetics of acyl glucuronides by NMR spectroscopy. *Chem Res Toxicol* **20**:876–886.
- Walker GS, Bauman JN, Ryder TF, Smith EB, Spracklin DK, and Obach RS (2014) Biosynthesis of drug metabolites and quantitation using NMR spectroscopy for use in pharmacologic and drug metabolism studies. *Drug Metab Dispos* **42**:1627–1639.
- Walker GS, Ryder TF, Sharma R, Smith EB, and Freund A (2011) Validation of isolated metabolites from drug metabolism studies as analytical standards by quantitative NMR. *Drug Metab Dispos* **39**:433–440.
- Walsky RL, Bauman JN, Bourcier K, Giddens G, Lapham K, Negahban A, Ryder TF, Obach RS, Hyland R, and Goosen TC (2012) Optimized assays for human UDP-glucuronosyltransferase (UGT) activities: altered alamethicin concentration and utility to screen for UGT inhibitors. *Drug Metab Dispos* **40**:1051–1065.
- Wellington K and Jarvis B (2001) Silymarin: a review of its clinical properties in the management of hepatic disorders. *BioDrugs* **15**:465–489.
- Won CS, Oberlies NH, and Paine MF (2010) Influence of dietary substances on intestinal drug metabolism and transport. *Curr Drug Metab* **11**:778–792.
- Won CS, Oberlies NH, and Paine MF (2012) Mechanisms underlying food-drug interactions: inhibition of intestinal metabolism and transport. *Pharmacol Ther* **136**:186–201.
- Zhang M, Jagdmann GE, Jr, Van Zandt M, Sheeler R, Beckett P, and Schroeter H (2013) Chemical synthesis and characterization of epicatechin glucuronides and sulfates: bioanalytical standards for epicatechin metabolite identification. *J Nat Prod* **76**:157–169.

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