Special Section on Natural Products: Experimental Approaches to Elucidate Disposition Mechanisms and Predict Pharmacokinetic Drug Interactions

Modulation of Major Human Liver Microsomal Cytochromes P450 by Component Alkaloids of Goldenseal: Time-Dependent Inhibition and Allosteric Effects

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ABSTRACT

Botanical and other natural products (NPs) are often coconsumed with prescription medications, presenting a risk for cytochrome P450 (P450)-mediated NP-drug interactions. The NP goldenseal (Hydrastis canadensis) has exhibited antimicrobial activities in vitro attributed to isoquinoline alkaloids contained in the plant, primarily berberine, (-)-β-hydrastine, and to a lesser extent, hydrastinine. These alkaloids contain methylenedioxyphenyl rings, structural alerts with potential to inactivate P450s through formation of metabolic intermediate complexes. Time-dependent inhibition experiments were conducted to evaluate their ability to inhibit major P450 activities in human liver microsomes by using a cocktail of isozyme-specific substrate probes. Berberine inhibited CYP2D6 (dextromethorphan O-demethylation; \( K_I = 2.7 \mu M, k_{inact} = 0.065 \) minute\(^{-1} \)) and CYP3A4/5 (midazolam 1'-hydroxylation; \( K_I = 14.8 \mu M, k_{inact} = 0.019 \) minute\(^{-1} \)); (-)-β-hydrastine inhibited CYP2C9 (diclofenac 4'-hydroxylation; \( K_I = 49 \mu M, k_{inact} = 0.036 \) minute\(^{-1} \)), CYP2D6 (\( K_I > 250 \mu M, k_{inact} > 0.06 \) minute\(^{-1} \)), and CYP3A4/5 (\( K_I = 28 \mu M, k_{inact} = 0.056 \) minute\(^{-1} \)); and hydrastinine inhibited CYP2D6 (\( K_I = 37 \mu M, k_{inact} = 0.049 \) minute\(^{-1} \)) activity. Berberine additionally exhibited allosteric effects on midazolam hydroxylation, showing both positive and negative heterotopic cooperativity. Experiments with recombinant isozymes showed that berberine activated midazolam 1'-hydroxylation by CYP3A5, lowering \( K_{m(app)} \) but showed mixed inhibition and negative cooperativity toward this reaction when catalyzed by CYP3A4. Berberine inactivated CYP3A4 at a much faster rate than CYP3A5 and was a noncompetitive inhibitor of midazolam 4-hydroxylation by CYP3A4 but a strong mixed inhibitor of the CYP3A5 catalyzed reaction. These complex kinetics should be considered when extrapolating the risk for NP-drug interactions involving goldenseal.

SIGNIFICANCE STATEMENT

Robust kinetic parameters were determined for the reversible and time-dependent inhibition of CYP2C9, CYP2D6, and CYP3A4/5 activities in human liver microsomes by major component isoquinoline alkaloids contained in the botanical natural product goldenseal. The alkaloid berberine also exhibited opposing, isozyme-specific allosteric effects on midazolam hydroxylation mediated by recombinant CYP3A4 (inhibition) and CYP3A5 (activation). These data will inform the development of a physiologically based pharmacokinetic model that can be used to predict potential clinically relevant goldenseal-drug interactions.
alkaloids in goldenseal are berberine and (−)-β-hydrastine. The plant also contains a number of minor alkaloids, including hydrastinine, whose concentration in commercial extracts is highly dependent on the method of preparation (Weber et al., 2003; Ettefagh et al., 2011; Hermann and von Richter, 2012). Goldenseal appears unique among hydrastine-containing plants in that it produces only the (−)-enantiomer, whereas other plants produce primarily (+)-β-hydrastine (Gupta et al., 2015).

Several clinical studies have highlighted the potential for goldenseal extracts (GSEs) to precipitate NP-drug interactions by inhibiting cytochrome P450 (P450) enzymes. Pretreatment of human volunteers with a GSE (1 g by mouth 3× daily for 14 or 28 days) significantly inhibited both CYP2D6 and CYP3A4/5 activities (40%–50% decrease in metabolite/parent ratios) and had negligible effects on CYP2E1 and CYP1A2 activities (Gurley et al., 2005, 2008a,b). Berberine (200 mg by mouth 3× daily for 12 days) also inhibited CYP3A4/5-mediated cyclosporin A clearance (34.5% increase in area under the plasma concentration versus time curve) in renal transplant recipients (Wu et al., 2005). Finally, berberine pretreatment (300 mg by mouth 3× daily for 14 days) followed by administration of an oral cocktail of P450 substrate probes resulted in a 10-fold, 2-fold, and 1.6-fold increase in the parent/metabolite ratio for dextromethorphan (CYP2D6), losartan (CYP2C9), and midazolam (MDZ; CYP3A4/5), respectively (Guo et al., 2012).

In vitro experiments have evaluated the ability of GSE and/or one of the major alkaloids to reversibly inhibit human liver P450s. One GSE exhibited the lowest IC₅₀ (0.03% of full extract) for inhibition of CYP3A4 activity among 21 herbal extracts tested (Budzinski et al., 2000), and other studies reported strong inhibition (>50%) by a GSE of CYP3A4 and CYP2D6 activities. There are conflicting reports as to whether goldenseal inhibits CYP1A2, 2C9, 2C19, or 2E1 activities in vitro. Berberine generally has been shown to be a weak to moderate inhibitor of the CYP1As as well as CYP2C8, 2D6, 2E1, and 3A4, and (−)-β-hydrastine has shown to be a weak to moderate inhibitor of 2C8, 2C9, 2D6, 2E1, and 3A4 (Chatterjee and Franklin, 2003; Foster et al., 2003; Etheridge et al., 2007; Raner et al., 2007; Lo et al., 2013).

The objective of the present study was to fully define the mode and kinetics of inhibition of the major liver P450s CYP2C9, 2D6, and 3A4/5 by the two major GSE component alkaloids, berberine and (−)-β-hydrastine, and the minor alkaloid, hydrastinine, which appears to be the third most abundant alkaloid present in some commercial extracts (Chatterjee and Franklin, 2003; Weber et al., 2003). Through comprehensive kinetic studies, robust parameters were recovered for both the reversible inhibition and TDI of CYP2C9, 2D6, and 3A4/5 by these three alkaloids. A comprehensive analysis of the unique kinetic allosteric interactions of berberine with CYP3A4 and CYP3A5 provided further insight into these complex NP-drug interactions. The kinetic parameters generated can be used to develop physiologically based pharmacokinetic (PBPK) models to predict the risk of goldenseal-drug interactions involving susceptible object drugs, aid in the design of clinical interaction studies, and help inform clinicians and consumers about managing these potentially adverse NP-drug interactions.

Materials and Methods

General Reagents

MDZ and 1′-OH-MDZ were obtained as 1 mg/ml and 100 μg/ml methanolic solutions, respectively, from Cerilliant (Round Rock, TX). Diclofenac sodium ABBREVIATIONS: FDA, U.S. Food and Drug Administration; fUBM, fraction unbound in human liver microsomes; GSE, goldenseal extract; HLM, human liver microsome; KP, potassium phosphate; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MDZ, midazolam; Ml, metabolic intermediate; m/z, mass to charge ratio; NMp, natural product; P450, cytochrome P450; PBPK, physiologically based pharmacokinetic; TDI, time-dependent inhibition; UPLC, ultraperformance liquid chromatography.
salt, berberine chloride, 1'-OH-MDZ-\(d_4\), dextrophan tartrate, dextrophan tartrate-\(d_3\), and 4'-hydroxydiclofenac-\(d_4\) were purchased from Toronto Research Chemicals, Inc. (North York, ON); dextromethorphan was procured from LKT Laboratories, Inc. (St. Paul, MN). Hydroxystyrine, (\(\beta\)-)hydroxystyrine, 4-OH-MDZ, and all other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All inhibitors and substrates tested in these studies were deemed to be \(\geq 95\%\) pure. Solvents were purchased from J.T. Baker, Inc. (Phillipsburg, NJ) or Fischer Scientific (Springfield, NJ). Recombinant CYP3A4 and CYP3A5 Supersomes, coexpressed in insect cells with cytochrome P450 4C11, 2C19, and 3A4 and cytochrome b\(_5\), were purchased from Corning Life Sciences, Inc. (Corning, NY). Pooled human liver microsomes (HLMs) of mixed sex were either prepared in-house from eight individual livers as reported previously (Sadeque et al., 1992; McDonald et al., 2015) or were purchased from Corning Life Sciences (Ultrapool HLM150) specifically for use in the berberine-MDZ allostery experiments.

**IC\(_{50}\) Shift Experiments for the Inhibition of P450 Activity by Berberine, (\(\beta\)-)Hydroxystyrine, or Hydroxystyrine**

**Substrate Cocktail Assay.** IC\(_{50}\) shift experiments were carried out using a validated substrate cocktail method (McDonald et al., 2015). Wells 1–24 and 25–48 of a 96-well plate contained identical solutions of inhibitor (added from 12 different 200\(\mu\)M concentrated stock solutions, yielding final incubation inhibitor concentrations ranging from 0 to 500 \(\mu\)M) and pooled HLMs (final concentration, 0.25 mg/ml microsomal protein) in 100 mM potassium phosphate (KP, buffer), pH 7.4. After a 3-minute equilibration at 37°C in a water bath, 2.5 \(\mu\)M NADPH (wells 1–24; final concentration, 1 mM) or buffer without NADPH (wells 25–48) were added; final incubation volumes were 250 \(\mu\)l. The plate was incubated at 37°C for 30 minutes and then 196 \(\mu\)l were removed from each well and were added to a second plate containing 2 \(\mu\)l of a concentrated 3-substrate cocktail stock (prepared with 50\% aqueous methanol; final concentrations: 4 \(\mu\)M diclofenac, 4 \(\mu\)M dextromethorphan, and 2 \(\mu\)M MDZ) per well, plus 2 \(\mu\)l of either buffer alone (wells 1–24) or buffer with NADPH (1 mM final concentration, wells 25–48). This plate was incubated for 5 minutes at 37°C prior to quenching with 20 \(\mu\)l ice-cold 15\% aqueous ZnSO\(_4\). Ten micromoles each of 1'-OH-MDZ-\(d_4\) and dextrophan-\(d_3\) and 20 pmol of 4'-hydroxydiclofenac-\(d_4\) were added as internal standards; then, precipitates were removed by centrifugation, and the supernatants were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (described below).

**MDZ Assay.** These experiments used the same methodology outlined above for the cocktail assay with the following exceptions: 1) either CYP3A4 or CYP3A5 Supersomes (3 pmol per incubation reaction) were substituted for HLMs as the enzyme source; and 2) rather than using a substrate cocktail solution, a 100\% concentrated stock solution containing MDZ (final substrate concentration of either 2 \(\mu\)M for the (\(\beta\)-)hydroxystyrine experiments or 1 \(\mu\)M for the berberine experiments) was added to the second 96-well plate; and 3) 1'-OH-MDZ-\(d_3\) (10 pmol) was added to each reaction product as an internal standard. Results were plotted as percent enzyme activity remaining versus preincubation time, and slopes were determined for each inhibitor concentration by linear regression analysis. \(K_I\) and \(k_{	ext{max}}\) values were determined from plots of inverse slope (\(\lambda\)) versus inhibitor concentration.

**Allosterism Experiments with Berberine and CYP3A4/5 Enzymes**

The allosteric effect of berberine on MDZ metabolism (mediated by either HLMs or CYP3A4 or CYP3A5 Supersomes) was monitored at eight different substrate concentrations (0–250 \(\mu\)M MDZ) and five different inhibitor concentrations (0–100 \(\mu\)M berberine). Solutions of berberine and MDZ in 100 mM KP, buffer, pH 7.4, containing either 0.25 mg/ml HLMs or 2.5 pmol of CYP3A4 Supersomes, were pre-equilibrated in a 96-well plate at 37°C/50 rpm in a water bath for 3 minutes prior to initiating the reactions with the addition of a stock solution of NADPH (prewarmed to 37°C, 1 mM final concentration). After a 3-minute incubation, reactions were quenched with the addition of 20 \(\mu\)l 15\% aqueous ZnSO\(_4\), after which 10 pmol each of 1'-OH-MDZ-\(d_4\), dextrophan-\(d_3\), and 20 pmol of 4'-hydroxydiclofenac-\(d_4\) were added as internal standards. Protein precipitate was removed by centrifugation, and the supernatants were analyzed by LC-MS/MS.

**LC-MS/MS Protocol.** LC-MS/MS analyses were conducted on the same Xevo TQ-S Mass Spectrometer (Waters) and ACQUITY UPLC system (Waters) described above for the substrate cocktail assay. The Xevo instrument was operated in positive electrospray ionization mode at the same source and desolvation temperatures, and the following mass transitions were monitored in separate ion channels: \(m/z\) 342 \(\rightarrow\) 324 (1'-OH-MDZ-\(d_4\) and 1'-OH-MDZ-\(d_3\), \(m/z\) 346 \(\rightarrow\) 328 (1'-OH-MDZ-\(d_4\), and \(m/z\) 342 \(\rightarrow\) 234 (4-OH-MDZ). The cone voltage was set to 30 V for all analytes, with collision energies of 35 eV for 1'-OH-MDZ (labeled and unlabeled) and 20 eV for 4-OH-MDZ.

MDZ hydroxylation products from berberine coinubations were separated on an ACQUITY BEH Phenyl, 1.7 \(\mu\)m, 2.1 \(\times\) 150 mm UPLC column (Waters) using a binary solvent gradient (solvent A = 0.1\% aqueous formic acid, and solvent B = acetonitrile) at a constant flow rate of 0.35 ml/min and a column temperature of 50°C. An isocratic gradient of 25\% B was maintained for 5.5 minutes and then increased linearly to 95\% B over 0.5 minutes, at which time the gradient was maintained for 1 minute prior to column re-equilibration. Under these conditions, 4-OH-MDZ, 1'-OH-MDZ-\(d_4\), 1'-OH-MDZ-\(d_3\), MDZ, and berberine had retention times of approximately 3.7, 4.6, 4.7, 5.4, and 6.3 minutes, respectively. Metabolites were quantified through comparison of their peak area ratios (relative to the 1'-OH-MDZ-\(d_4\) peak area) to calibration curves, prepared from synthetic metabolite standards, using linear regression analysis. Limits of quantification for both metabolites were 20 fmol injected on the column.

**Data Analysis**

Mass spectral data analyses were conducted using Windows-based Micromass MassLynx NT software, version 4.1. GraphPad Prism v7 (GraphPad Software, San Diego, CA) was used to estimate all kinetic parameters using reaction velocities calculated as the mean of duplicate technical replicates. Unless otherwise stated, at least three biologic replicates were conducted for all experiments. Goodness-of-fit of the various metabolic/inhibition equations (Michaelis-Menten, IC\(_{50}\) determination,
Complex Effects of Goldenseal Alkaloids on P450s

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TABLE 1
Results of IC50 shift experiments showing TDI of CYP2C9, CYP2D6, and CYP3A4/5 activities in HLMs by goldenseal component alkaloids

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Probe Substrate</th>
<th>Inhibited Enzyme</th>
<th>IC50 (µM)</th>
<th>Shift Ratio a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-) NADPH</td>
<td>(+) NADPH</td>
</tr>
<tr>
<td>Berberine</td>
<td>Dextromethorphan</td>
<td>CYP2D6</td>
<td>9.9 ± 1.3</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Berberine</td>
<td>MDZ</td>
<td>CYP3A4/5</td>
<td>120 ± 30</td>
<td>31 ± 3.2</td>
</tr>
<tr>
<td>(-)-β-Hydrastine</td>
<td>Dextromethorphan</td>
<td>CYP2D6</td>
<td>270 ± 21</td>
<td>80 ± 18</td>
</tr>
<tr>
<td>(-)-β-Hydrastine</td>
<td>MDZ</td>
<td>CYP3A4/5</td>
<td>58 ± 7.6</td>
<td>9.9 ± 1.5</td>
</tr>
<tr>
<td>Hydrastinine</td>
<td>Dextromethorphan</td>
<td>CYP2D6</td>
<td>65 ± 21</td>
<td>8.4 ± 1.1</td>
</tr>
<tr>
<td>Tienilic Acid</td>
<td>Diclofenac</td>
<td>CYP2C9</td>
<td>2.8 ± 0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>Dextromethorphan</td>
<td>CYP2D6</td>
<td>0.9 ± 0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>MDZ</td>
<td>CYP3A4/5</td>
<td>61</td>
<td>1.5</td>
</tr>
</tbody>
</table>

aPrior to the addition of substrate, HLMs were preincubated with inhibitor for 30 minutes in the presence (+) or absence (−) of NADPH cofactor.

bShift ratio = IC50 (−) NADPH/IC50 (+) NADPH.

Reversible inhibition, TDI, and substrate inhibition were evaluated based on visual inspection, the extra sum-of-squares F test, Akaike information criterion, and randomness of the residuals. Tabulated data are presented as means ± S.D.

Preliminary In Vitro-In Vivo Prediction of Goldenseal-Drug Interactions

A preliminary risk assessment of potential clinical goldenseal-drug interactions due to inhibition of hepatic CYP2C9, CYP2D6, or CYP3A4 activity was made using basic static models (eqs. 1 and 2) (U.S. Food and Drug Administration, 2020). Specifically, the ratios of intrinsic clearance values (R) for a probe drug in the absence to presence of an interacting reversible or time-dependent inhibitor were estimated. Equation 1 predicts the change in clearance because of reversible inhibition:

\[ R_1 = 1 + \left( \frac{f_{\text{u,HLM}}}{K_a} \right) \]

where \( f_{\text{u,HLM}} \) (maximum unbound plasma concentration × fraction unbound in plasma) is the maximum unbound plasma concentration of the inhibitor (goldenseal alkaloid), and \( K_a \) = \( K_i \times f_{\text{u,HLM}} \) is the experimentally determined reversible inhibition constant (conservatively estimated as one-half of the IC50 value determined in the absence of NADPH in the preincubation step, Table 1) corrected for nonspecific binding to HLMs (\( \theta_{\text{HLM}} \)). The fraction unbound in plasma and \( f_{\text{u,HLM}} \) (at 0.25 mg/ml microsomal protein) were predicted using GastroPlus (v9.7, Simulations Plus, Lancaster, CA); values for berberine were 0.75 and 1.0, respectively, and those for hydrastine were 0.10 and 0.79, respectively. \( f_{\text{u,HLM}} \) values were obtained from the literature (1.75 and 470 nM for berberine and hydrastine, respectively; Gupta et al., 2009). Equation 2 predicts the change in probe drug clearance because of TDI:

\[ R_2 = \frac{k_{\text{obs}} + k_{\text{inact}}}{k_{\text{deg}}} \left( \frac{k_{\text{obs}}}{k_{\text{obs}} + 50^*f_{\text{u,HLM}}} \right) \]

where \( k_{\text{obs}} \) and \( k_{\text{inact}} \) are the TDI-biding (corrected for nonspecific binding to HLMs) and inactivation rate constants (Table 2), and \( k_{\text{deg}} \) is the degradation rate constant for a given P450. For hepatic CYP2C9, CYP2D6, and CYP3A4, \( k_{\text{deg}} \) has been estimated as 0.00026, 0.000226, and 0.000321 minute⁻¹, respectively (Obach et al., 2007).

Results

IC50 Shift Experiments: Inhibition of CYP2C9, CYP2D6, and CYP3A4/5 Activities in HLMs by Goldenseal Isoquinoline Alkaloids

Substrate Cocktail Assay. Berberine, (-)-β-hydrastine, and hydrastine were tested by IC50 shift assay as time-dependent inhibitors of CYP2C9, CYP2D6, and CYP3A4/5 activities in HLMs using a cocktail of specific substrate probes. Additionally, tienilic acid (CYP2C9), paroxetine (CYP2D6), and troleandomycin (CYP3A4) were tested as positive controls for clinically relevant isozyme-specific TDI (Bertelsen et al., 2003; Zhao et al., 2005; McGinnity et al., 2006).

Each isoquinoline alkaloid exhibited an IC50 shift ratio >1.5 (the predefined cutoff for TDI) for at least one of the P450 activities tested. Berberine had no effect on CYP2C9-mediated diclofenac-4'-hydroxylation (data not shown) but was a relatively strong time-dependent inhibitor of CYP2D6-mediated dextromethorphan O-demethylation (Fig. 2A) and showed both reversible activation and TDI of CYP3A4/5-mediated MDZ-1'-hydroxylation (Fig. 2B). (-)-β-Hydrastine inhibited CYP2C9 (Fig. 2C), CYP2D6 (Fig. 2D), and CYP3A4/5 (Fig. 2E) activities in a time-dependent manner. Hydrastine had no effect on CYP2C9 or CYP2A4/5 activities (data not shown) but was a moderate time-dependent inhibitor of CYP2D6 (Fig. 2F; Table 1).

Cytchrome P450 Kinetic Inactivation by Goldenseal Isoquinoline Alkaloids in HLMs (Determination of \( K_i \) and \( k_{\text{inact}} \))

TDI experiments were conducted to determine the kinetics of inactivation of CYP2C9, CYP2D6, and CYP3A4/5 activities in

TABLE 2
Kinetic parameters for the inactivation of CYP2C9, CYP2D6, and CYP3A4/5 in HLMs by goldenseal component alkaloids

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibited Enzyme</th>
<th>( K_i ) (µM)</th>
<th>( k_{\text{inact}} ) (min⁻¹)</th>
<th>( \frac{k_{\text{inact}}}{K_i} ) (mM⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berberine</td>
<td>CYP2D6</td>
<td>2.68 ± 0.26</td>
<td>0.065 ± 0.006</td>
<td>24.3</td>
</tr>
<tr>
<td>Berberine</td>
<td>CYP3A4/5</td>
<td>14.8 ± 2.6</td>
<td>0.019 ± 0.005</td>
<td>1.3</td>
</tr>
<tr>
<td>(-)-β-Hydrastine</td>
<td>CYP2C9</td>
<td>49 ± 16</td>
<td>0.036 ± 0.007</td>
<td>0.7</td>
</tr>
<tr>
<td>(-)-β-Hydrastine</td>
<td>CYP2D6</td>
<td>&gt;250</td>
<td>0.056 ± 0.005</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>(-)-β-Hydrastine</td>
<td>CYP3A4/5</td>
<td>28 ± 12</td>
<td>0.056 ± 0.005</td>
<td>2.0</td>
</tr>
<tr>
<td>Hydrastinine</td>
<td>CYP2D6</td>
<td>37 ± 13</td>
<td>0.049 ± 0.009</td>
<td>3.8</td>
</tr>
</tbody>
</table>

*Reported \( k_{\text{inact}}/K_i \) values (mM⁻¹ min⁻¹) for the TDI of specific P450 activities, in either HLMs (paroxetine, troleandomycin) or human hepatocytes (tienilic acid), for clinically relevant time-dependent inhibitors: tienilic acid (CYP2C9) = 25; paroxetine (CYP2D6) = 35; and troleandomycin (CYP3A4/5) = 13.3 (Bertelsen et al., 2003; Zhao et al., 2005; McGinnity et al., 2006).
HLMs by each alkaloid. The same isozyme-specific substrate probes used in the IC50 shift substrate cocktail experiments were used at 10× their reported $K_m$ values (McDonald et al., 2015). (−)-β-Hydrastine was a weak to moderate inactivator of the three enzyme activities (Fig. 3; Table 2). Berberine had no effect on CYP2C9 activity (data not shown), was a relatively strong inactivator of CYP2D6, and demonstrated both weak reversible activation and modest TDI of CYP3A4/5 activity (Table 2). Hydrastinine showed moderate TDI of CYP2D6 (Table 2) and had no effect on either CYP2C9 or CYP3A4/5 activity (data not shown).

**Preliminary Hepatic P450-Mediated Goldenseal-Drug Interaction Predictions**

The risk of each alkaloid to precipitate clinical pharmacokinetic interactions with drugs metabolized by hepatic CYP2C9, CYP2D6, and CYP3A4/5 was predicted using basic static equations. As reversible inhibitors, the $R_1$ value for all alkaloids involving all P450 substrates was

**Fig. 2.** Results from IC50 shift experiments showing TDI of (A) CYP2D6-mediated dextromethorphan O-demethylation by berberine, (B) CYP3A4/5-mediated MDZ 1'-hydroxylation by berberine, (C) CYP2C9-mediated diclofenac 4'-hydroxylation by (−)-β-hydrastine, (D) CYP2D6-mediated dextromethorphan O-demethylation by (−)-β-hydrastine, (E) CYP3A4/5-mediated MDZ 1'-hydroxylation by (−)-β-hydrastine, and (F) CYP2D6-mediated dextromethorphan O-demethylation by hydrastinine in HLMs. Mean IC50 values for each set of experiments (repeated in triplicate) are shown in Table 1.

**Fig. 3.** P450 inactivation kinetics showing inhibition of (A) CYP2C9-mediated diclofenac 4'-hydroxylation by (−)-β-hydrastine, (B) CYP2D6-mediated dextromethorphan O-demethylation by (−)-β-hydrastine, (C) CYP3A4/5-mediated MDZ 1'-hydroxylation by (−)-β-hydrastine, (D) CYP2D6-mediated dextromethorphan O-demethylation by berberine, (E) CYP3A4/5-mediated MDZ 1'-hydroxylation by berberine, and (F) CYP2D6-mediated dextromethorphan O-demethylation by hydrastinine in HLMs as a function of enzyme/NADPH preincubation time. $K_I$ and $k_{inact}$ values (see Table 2) were determined from plots (shown as insets in graphs (A–F)) of inverse slope (A), for the individual lines in each graph, vs. inhibitor concentration. Berb, berberine.
VMax with the CYP3A4 Supersome incubations, an activation effect was observed because of a lowering of K_i, apparent for MDZ (Table 5).

In the absence of effector, the kinetics of 4-OH-MDZ formation in both HLMs and CYP3A4 Supersomes were described best by a simple unienzyme Michaelis-Menten model, showing a similar K_m for MDZ (20–25 μM); upon addition of berberine, noncompetitive inhibition of MDZ 4-hydroxylation was observed. MDZ 4-hydroxylation by CYP3A5 Supersomes also were described best by a simple unienzyme Michaelis-Menten kinetic model, exhibiting a K_i and V_max that were both approximately 50% of those for recombinant CYP3A4 (Tables 2–4). When berberine was added to incubations containing CYP3A5 Supersomes, strong mixed inhibition of MDZ 4-hydroxylation was observed (Fig. 5).

In the absence of effector, CYP3A4 Supersomes produced a 1'-OH/4-OH-MDZ ratio of 7–9 at the lowest MDZ concentration tested, CYP3A5 Supersomes produced a ratio of 35–40, and HLMs produced a ratio of 10–12. The addition of berberine (up to 100 μM) resulted in a modest increase (1.35–1.6-fold) in the maximum metabolite ratio observed for CYP3A4-mediated MDZ hydroxylation while causing an increase of up to 30-fold in the CYP3A5-mediated metabolite ratio and an increase of 3- to 4-fold in the HLMs-mediated metabolite ratio (Fig. 6).

IC_{50} Shift Experiments with CYP3A4/5 Supersomes: Inhibition of MDZ 1'-Hydroxylation by Berberine and (−)-β-Hydrastine

Additional studies were conducted to determine the relative ability of berberine and (−)-β-hydrastine to inactivate recombinant CYP3A4 and CYP3A5 separately by using MDZ as a probe substrate. The final MDZ concentration in the incubation mixtures was either 2 μM (the reported K_i for CYP3A4) for experiments involving (−)-β-hydrastine or 20 μM when berberine was used as the inhibitor. In the

### TABLE 3

<table>
<thead>
<tr>
<th>Berberine (μM)</th>
<th>1'-Hydroxy MDZ</th>
<th>4-Hydroxy MDZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_m (mM)</td>
<td>V_max (pmol/min/mg)</td>
</tr>
<tr>
<td>0</td>
<td>2.29 ± 0.29</td>
<td>2110 ± 380</td>
</tr>
<tr>
<td>5</td>
<td>1.63 ± 0.41</td>
<td>2080 ± 380</td>
</tr>
<tr>
<td>25</td>
<td>1.58 ± 0.12</td>
<td>2280 ± 330</td>
</tr>
<tr>
<td>50</td>
<td>1.22 ± 0.18</td>
<td>2010 ± 220</td>
</tr>
<tr>
<td>100</td>
<td>1.06 ± 0.15</td>
<td>1880 ± 120</td>
</tr>
</tbody>
</table>

Values denote means ± S.D. of four separate experiments. K_m and K_i are given in micromolar; V_max is in picomoles per minute per milligram microsomal protein; V_max/K_m is in microliters per minute per milligram protein.

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Fig. 4. Plots showing allosteric effects of berberine on the kinetics of MDZ 1'-hydroxylation mediated by HLMs or CYP3A4 or CYP3A5 Supersomes. With both HLMs and CYP3A4 Supersomes, the kinetics were consistent with a substrate inhibition model, with an inhibitory allosteric effect exhibited with the addition of berberine (i.e., decreased K_i). There also appears to be some mixed inhibition of CYP3A4 at high berberine concentrations. In contrast, the kinetics of MDZ 1'-hydroxylation by CYP3A5 were described best by a simple, unienzyme Michaelis-Menten model. Berberine activated MDZ 1'-hydroxylation in both HLMs and CYP3A5 Supersomes by lowering K_m(app) (Tables 2–4).
latter case, the higher concentration of substrate used was an attempt to minimize the observed activation effect of berberine on CYP3A5-mediated MDZ 1′-hydroxylation.

There was essentially no reversible inhibition of CYP3A5-mediated MDZ 1′-hydroxylation by berberine (up to 500 μM) and only weak to moderate TDI (shifted IC50 = 83 μM; shift ratio >6). Berberine was a weak reversible inhibitor of CYP3A4 activity (IC50 ~30 μM) but a stronger time-dependent inhibitor of this enzyme (shifted IC50 = 9.9 μM; shift ratio ~50) (Supplemental Fig. 1). By contrast, there was no observable difference for the TDI of CYP3A4 versus CYP3A5 by (−)β-hydrastine (reversible IC50 ~30 μM; shifted IC50 = 2.5 μM; shift ratio = 12 for both isozymes) (Supplemental Fig. 2).

Discussion

Botanical NP usage continues to increase worldwide as a means to self-treat various illnesses and/or supplement prescribed pharmacotherapeutic regimens (Paine and Roe, 2018; Smith et al., 2019). Coconsuming NPs with prescribed medications can increase the risk of adverse NP-drug interactions, which can lead to suboptimal therapeutic outcomes. Mechanisms of pharmacokinetic NP-drug interactions include inhibition of the P450s, which are responsible for the metabolic elimination of numerous clinically used drugs, including those with a narrow therapeutic window (Guengerich, 2008; Zanger and Schwab, 2013). Goldenseal is a widely used NP that contains several isoquinoline alkaloids, the most prominent of which are berberine and (−)β-hydrastine (Weber et al., 2003). Despite clinical studies demonstrating goldenseal or berberine to precipitate pharmacokinetic interactions with the probe substrates MDZ (CYP3A4/5), debrisoquine (CYP2D6), dextromethorphan (CYP2D6), and losartan (CYP2C9) (Gurley et al., 2008a,b; Guo et al., 2012), a comprehensive characterization of the inhibition kinetics of major isoquinoline alkaloids has not been reported. Robust kinetic parameters are needed to develop PBPK models, which can be used to predict the magnitude and time course of potential clinical interactions with critical object drugs (i.e., drugs with narrow therapeutic windows) as well as the risk of these interactions in vulnerable populations (e.g., elderly, hepatically impaired, renally impaired, pregnant women). Based on these knowledge gaps, the reversible inhibition and TDI kinetics of berberine, (−)β-hydrastine, and hydrastinine against CYP2C9, CYP2D6, and CYP3A4/5 activities were determined using HLMs.

These isoquinoline alkaloids were predominantly weak reversible inhibitors of the P450 activities tested (at probe substrate concentrations equal to the respective Km values), generating IC50 values in the absence of a NADPH preincubation step of >50 μM (a lone exception was berberine, which was a moderate reversible inhibitor of CYP2D6 activity in HLMs, IC50 = 9.9 μM) (Table 1). Gupta et al. (2009) reported maximum human plasma concentrations of 1.75 and 470 nM, respectively, for berberine and hydrastane after administration of a single 2.14 g oral dose of a GSE containing 132 mg berberine (6.2% of extract) and 77 mg hydrastine (3.6% of extract).

Although no standard dosing protocol exists for GSEs, recommended doses generally range from 250 to 1000 mg 3x daily. The alkaloid concentrations contained in the extract used in the Gupta study were within the expected ranges determined for a selection of commercial GSEs (Brown and Roman, 2008). Using these reported Km values for berberine and hydrastane, we conclude that reversible inhibition is an unlikely mechanism of the reported clinical P450-mediated goldenseal-drug interactions at the level of the liver. However, reversible inhibition of CYP3A4/5 by either alkaloid at the level of the intestine cannot be ruled out based on estimated luminal concentrations ranging from 0.8 to 1.6 mM (dose/0.25 l per FDA guidance) (U.S. Food and Drug Administration, 2020).

Based on these observations, any clinical NP-drug interaction caused by GSE via inhibition of hepatic CYP2C9, CYP2D6, or CYP3A4/5 metabolism is likely due to TDI of enzyme activity by one or more of the component GSE alkaloids. All of these alkaloids contain a methylene-dioxophenyl ring, a known structural alert for the inactivation of P450 enzymes primarily via formation of metabolic intermediate (MI) complexes (Taxak et al., 2013). MI complexes typically exhibit a distinct

### TABLE 4

<table>
<thead>
<tr>
<th>Berberine (μM)</th>
<th>1′-Hydroxy MDZ</th>
<th></th>
<th>4-Hydroxy MDZ</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km</td>
<td>Vmax/Km</td>
<td>Ks</td>
<td>Km</td>
</tr>
<tr>
<td>0</td>
<td>1.38 ± 0.22</td>
<td>28.0 ± 4.6</td>
<td>20.7 ± 4.1</td>
<td>900 ± 250</td>
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<tr>
<td>5</td>
<td>1.28 ± 0.25</td>
<td>31.3 ± 3.7</td>
<td>24.7 ± 2.6</td>
<td>610 ± 190</td>
</tr>
<tr>
<td>25</td>
<td>1.34 ± 0.20</td>
<td>24.7 ± 4.0</td>
<td>18.6 ± 2.9</td>
<td>560 ± 120</td>
</tr>
<tr>
<td>50</td>
<td>1.63 ± 0.39</td>
<td>23.4 ± 2.6</td>
<td>15.2 ± 4.9</td>
<td>420 ± 160</td>
</tr>
<tr>
<td>100</td>
<td>2.04 ± 0.55</td>
<td>21.4 ± 2.4</td>
<td>11.0 ± 2.7</td>
<td>470 ± 70</td>
</tr>
</tbody>
</table>

### TABLE 5

<table>
<thead>
<tr>
<th>Berberine (μM)</th>
<th>1′-Hydroxy MDZ</th>
<th></th>
<th>4-Hydroxy MDZ</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km</td>
<td>Vmax/Km</td>
<td>Ks</td>
<td>Km</td>
</tr>
<tr>
<td>0</td>
<td>1.55 ± 0.18</td>
<td>59.3 ± 12.5</td>
<td>38.5 ± 12.9</td>
<td>12.6 ± 2.7</td>
</tr>
<tr>
<td>5</td>
<td>1.55 ± 0.50</td>
<td>58.7 ± 8.9</td>
<td>41.6 ± 19.3</td>
<td>20.5 ± 4.1</td>
</tr>
<tr>
<td>25</td>
<td>1.11 ± 0.10</td>
<td>58.2 ± 14.3</td>
<td>51.5 ± 9.7</td>
<td>39.4 ± 4.9</td>
</tr>
<tr>
<td>50</td>
<td>1.01 ± 0.15</td>
<td>56.3 ± 12.1</td>
<td>54.7 ± 10.8</td>
<td>60.5 ± 5.8</td>
</tr>
<tr>
<td>100</td>
<td>0.91 ± 0.11</td>
<td>53.9 ± 11.5</td>
<td>58.9 ± 15.4</td>
<td>100 ± 28</td>
</tr>
</tbody>
</table>
Berberine not only showed TDI but also reversibly activated CYP3A4 but activated this reaction [lowering $K_{i(app)}$, when catalyzed by CYP3A5. Berberine’s allosteric effects on MDZ 1’-hydroxylation in HLMs were an amalgamation of the individual effects observed for recombinant CYP3A4 and CYP3A5, exhibiting positive and negative heterotropic cooperativity via lowering of both $K_{m(app)}$ and $K_{i(app)}$. Berberine acted as a noncompetitive inhibitor of MDZ 4-hydroxylation by both HLMs and recombinant CYP3A4 but was a mixed inhibitor of this reaction when catalyzed by CYP3A5. The berberine effect on CYP3A4 kinetics for this reaction was dominant in HLMs because, based on relative intrinsic clearance rates (Tables 4 and 5), the alkaloid was a stronger inhibitor of MDZ 4-hydroxylation by CYP3A5.

The differences in MDZ metabolic kinetics between CYP3A4 and CYP3A5 resulted in large variability in the maximum MDZ 1’-OH/4-OH ratios produced by the two isozymes. In the absence of effector, CYP3A4 produced a maximum observed metabolite ratio of $\sim$8, whereas that produced by CYP3A5 was $\sim$5-fold higher (the ratio decreased substantively as MDZ concentrations approached and surpassed the $K_{m}$ for 4-hydroxylation). Adding berberine increased the ratio produced by all three enzyme sources, albeit to different degrees. Because berberine inhibited CYP3A4-mediated MDZ hydroxylation at both the 1’ and 4 positions, a relatively modest increase ($\sim$1.4-fold) was observed in the maximum metabolite ratio. In contrast, berberine not only activated CYP3A5-mediated MDZ 1’-hydroxylation but also more strongly inhibited MDZ 4-hydroxylation. This synergistic effect led to more than a 30-fold increase in the observed ratio produced by CYP3A5. As expected, the 3- to 4-fold increase in MDZ metabolite ratio effected by berberine in HLMs (which contain both CYP3A4 and CYP3A5) lay between the values observed for the individual isozymes. In a similar

**Fig. 5.** The kinetics of MDZ 4-hydroxylation by HLMs or by recombinant CYP3A4 or CYP3A5 Supersomes were described best by a simple unienzyme Michaelis-Menten model. Berberine acted as a noncompetitive inhibitor of CYP3A4 and HLM-mediated MDZ 4-hydroxylation as well as a mixed inhibitor of this reaction when catalyzed by CYP3A5 (Tables 2–4).

**Fig. 6.** Effect of berberine on the metabolite ratio for MDZ hydroxylation (i.e., 1’-OH/4-OH-MDZ) produced by HLMs or CYP3A4 or CYP3A5 Supersomes. Metabolite ratios were highest at the lowest concentrations of substrate tested and decreased markedly at concentrations exceeding the $K_{m}$ for MDZ 4-hydroxylation ($\sim$12–25 $\mu$M). When berberine was added as effector, an increase in the maximum metabolite ratio was observed with all three enzyme sources; however, this increase was modest for CYP3A4 (1.35–1.6-fold at 100 $\mu$M berberine) compared with CYP3A5 ($\sim$30-fold), whereas the 3.5-fold increase in the maximum metabolite ratio produced by berberine in HLMs lay in between these values.
The experiment, fluconazole decreased the maximum MDZ metabolite ratio produced by recombinant CYP3A4 by >50%, as it more strongly inhibited hydroxylation at the 1’ position of MDZ compared with the 4 position (Yang et al., 2012).

These allosteric data highlight a potential issue with using MDZ 1’-hydroxylation as a probe to monitor the effect of berberine or GSE on CYP3A4/5 activity in vivo. Because berberine is a stronger time-dependent inhibitor of CYP3A4 (IC50 shift ratio ~50) compared with CYP3A5 (shift ratio ~6), the possibility exists, especially after prolonged treatment with the alkaloid, that activation of CYP3A5-mediated MDZ 1’-hydroxylation could mask some degree of CYP3A4 inactivation. Therefore, rMDZ is used as an in vivo CYP3A4/5 probe in clinical studies involving GSE or berberine, monitoring MDZ 4-hydroxylation should be considered, as an increase over control in the MDZ 1’OH/4-OH metabolite ratio would suggest an increased involvement of CYP3A5 in MDZ metabolism.


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