Inhibition of Intestinal and Hepatic Glucuronidation of Mycophenolic Acid by *Ginkgo biloba* Extract and Flavonoids

Mohamed-Eslam F. Mohamed and Reginald F. Frye

Department of Pharmacotherapy and Translational Research, College of Pharmacy, University of Florida, Gainesville, Florida

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**ABSTRACT:**

Herb-drug interactions have received more attention in recent years because of the widespread popularity of herbal supplements. However, there are limited data on the effect of herbs on glucuronidation in humans. The goal of this work was to examine the effect of *Ginkgo biloba* extract and its main flavonoid and terpene lactone constituents on mycophenolic acid (MPA) 7-O-glucuronidation. Human liver (HLM) and intestinal (HIM) microsomes were incubated with MPA and *G. biloba* extract (unhydrolyzed or acid-hydrolyzed), quercetin, kaempferol, ginkgolide A, ginkgolide B, or bilobalide. MPA-7-O-glucuronide formation was inhibited in HLM and HIM incubations by unhydrolyzed [IC$_{50}$ = 84.3 (HLM) and 6.9 (HIM) µg/ml] and hydrolyzed [IC$_{50}$ = 20.9 (HLM) and 4.3 (HIM) µg/ml] *G. biloba* extracts, quercetin [IC$_{50}$ = 19.1 (HLM) and 5.8 (HIM) µM], and kaempferol [IC$_{50}$ = 23.1 (HLM) and 7.7 (HIM) µM]. Terpene lactones did not show inhibition of MPA glucuronidation. Quercetin was a mixed-type inhibitor in HLM and HIM incubations ($K_i$ = 11.3 (HLM) and 2.8 (HLM) µM), whereas kaempferol was a noncompetitive inhibitor in HLM ($K_i$ = 33.7 µM) and a mixed-type inhibitor in HIM ($K_i$ = 4.5 µM). These results indicate that *G. biloba* extract or quercetin- and kaempferol-rich supplements may inhibit intestinal and hepatic glucuronidation of MPA. Future studies are needed to evaluate the clinical significance of this interaction.

Herbal supplement use continues to increase around the globe, especially in populations looking for natural methods to promote health and wellness. In the United States, surveys estimate that 20% of the population use at least one herbal supplement (Bardia et al., 2007). This growing interest in herbal supplements is manifested by annual sales in the United States of more than $4 billion dollars (NBJ, 2008). Such public interest is met by concerns from health professionals regarding possible deleterious interactions of herbal supplements with conventional drugs. Herbal supplements are considered dietary supplements; hence, they are not routinely screened for interactions with drug-metabolizing enzymes (http://www.fda.gov). However, numerous in vitro, animal, and clinical studies and case reports provide evidence that herbal supplements can interact with conventional drugs, and this interaction may lead to serious adverse effects (Gardiner et al., 2008).

*G. biloba* is among the most popular herbal supplements used in the world. Its extract is available over the counter in the United States and is commonly prescribed in European countries for cerebral insufficiency (De Smet, 2005). Antioxidant effects as well as beneficial effects on memory and circulation have been attributed to *G. biloba* extract and its components. The primary active constituents of *G. biloba* are terpene lactones (ginkgolides and bilobalide) and flavone glycosides, which are hydrolyzed in vivo to flavone aglycones (e.g., quercetin and kaempferol) (Fig. 1A) (Chan et al., 2007). Several clinical and in vitro studies have investigated the effect of *G. biloba* on drug-metabolizing cytochrome P450 enzymes and transporters (Izzo and Ernst, 2009). In contrast, limited research has been conducted to investigate interactions of *G. biloba* and its components with conjugation pathways. In vitro studies have shown that quercetin and kaempferol inhibit sulfotransferase 1A1 (Eaton et al., 1996; Ghazali and Waring, 1999); meanwhile, information is lacking regarding effects of *G. biloba* on drug glucuronidation.

Glucuronidation constitutes the main pathway of conjugative metabolism for a wide variety of compounds (Ouzzine et al., 2003); substrates for UDP-glucuronosyltransferase enzymes (UGTs) include endogenous compounds, drugs, and many phytochemicals. Many flavonoids (e.g., quercetin and kaempferol) are substrates for UGT enzymes. Moreover, inhibitory effects of flavonoids on UGT1A enzymes have been reported in the literature (Williams et al., 2002; D’Andrea et al., 2005). For substrates metabolized mainly through glucuronidation, modulation of UGT activities can lead to significant effects on pharmacokinetics (Kiang et al., 2005).

Mycophenolic acid (MPA) is an immunosuppressive drug that acts by inhibiting the production of guanosine nucleotides in lymphocytes, ceasing their proliferation (Allison and Eugui, 2005). Therefore, it is used to prevent graft rejection in transplant recipients and to delay progression of autoimmune disorders (Heatwole and Ciafaloni, 2008). MPA is available as either a prodrug mofetil ester (CellCept; Roche, Nutley, NJ) or as an enteric-coated sodium salt (Myfortic; Novartis, East Hanover, NJ). Although both formulations have similar pharmacokinetic and efficacy profiles, absolute oral bioavailability of mycophenolate sodium is 72% compared with 94% for mycophenolate mofetil (Staatz and Tett, 2007). This difference is attributed to higher presystemic

**ABBREVIATIONS:** UGT, UDP glucuronosyltransferase; MPA, mycophenolic acid; MPAG, mycophenolic acid-7-O-glucuronide; HLM, human liver microsome(s); HIM, human intestinal microsome(s); UDPGA, UDP-glucuronic acid.
glucuronidation of MPA from the mycophenolate sodium formulation. After oral absorption, MPA is metabolized by UGTs to the major phenolic conjugate 7-O-MPA-glucuronide (MPAG) (Fig. 1b). In the liver, UGT1A9 is the main isoform catalyzing the formation of MPAG, whereas UGT1A7, UGT1A8, and UGT1A10 contribute to MPAG formation extrahepatically—mainly in the kidneys and intestine (Picard et al., 2005). MPA is a narrow therapeutic index drug with wide inter- and intraindividual variability and complex pharmacokinetics in transplant recipients (Staatz and Tett, 2007). Therefore, an alteration in MPA glucuronidation may cause changes in exposure to the immuno-suppressive drug and, consequently, undesired clinical outcomes. The aim of this study was to investigate the effect of G. biloba extract and its main components on MPAG formation in human intestinal and liver microsomes. The results demonstrate that G. biloba and its primary constituents have the ability to inhibit MPA glucuronidation in the intestine and liver.

Materials and Methods

Chemicals and Reagents. Mycophenolic acid (98%) and mycophenolic acid-7-O-glucuronide (98%) were purchased from Toronto Research Chemicals (North York, ON, Canada). Potassium phosphate dibasic, UDP-glucuronic acid, magnesium chloride, alamethicin, phenolphthalein-β-D-glucuronide, niflumic acid, and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile, methanol, quercetin dihydrate (99% purity), and kaempferol (90%) were purchased from Thermo Fisher Scientific (Waltham, MA). Ginkgolide A (95.1%), ginkgolide B (82.8%), and bilobalide (99.7%) were purchased from ChromaDex (Irvine, CA). Pooled human liver and intestinal microsomes were purchased from BD Biosciences Discovery Laboratory (Woburn, MA).

Herbal Extracts. G. biloba extract was provided by Finzelberg and Co. KG (Andernach, Germany) as dry powder. The extract was standardized by the supplier to contain 24% flavonglycosides, 6% terpene lactones, and <5 ppm ginkgolic acids using 60% acetone as the extraction solvent. Unhydrolyzed and neutralized with 2 N KOH. The acetone-rich extracts were serially diluted to working solutions were freshly prepared by dissolving 30 mg of the powder extract in 1 ml of either 60% acetone or 60% acetone-40% 5 N HCl for the unhydrolyzed and acid-hydrolyzed working solutions, respectively. The acid-treated extract was heated at 90°C for 1 h and neutralized with 2 N KOH. The acetone-rich extracts were serially diluted to prepare working solutions of G. biloba with concentrations of 0.05 to 5 mg/ml and acetone content of 10%.

Inhibition of MPA Glucuronidation Assay. The incubation conditions were optimized with respect to time of incubation and microsomal protein concentration. A typical 100-μl incubation mixture contained HLM or HIM (protein concentration, 0.16 mg/ml), alamethicin (100 μg/mg microsomal protein), MgCl2 (5 mM), MPA, and different concentrations of each test extract or test compound in 100 mM phosphate buffer, pH 7.4. Microsomes were preincubated on ice with alamethicin for 15 min to activate UGT enzymes. The reaction was started by adding UDPGA (1 mM) and placing incubation tubes in a water bath at 37°C for 30 min. The reaction was stopped by adding 300 μl of ice-cold acetonitrile and 20 μl of internal standard (0.5 mg/ml phenolphthalein-β-D-glucuronide). Tubes were vortex-mixed for 2 min and centrifuged for 10 min at 20,000g. The supernatant was diluted 12-fold with purified water, and 5 μl was injected into the high-performance liquid chromatography system. Incubations with herbal extracts and the corresponding controls contained 1% acetone. The HLM and HIM used in all experiments were from the same lot.

Screening experiments were conducted to generate IC50 values by incubating MPA at the estimated Kcat value in the presence of five concentrations of G. biloba unhydrolyzed and hydrolyzed extracts (final concentrations ranging from 5 to 500 μg/ml) or G. biloba individual components (final concentrations ranging from 1 to 100 μM). In addition to IC50 values, inhibitory potency was also expressed as the volume per dose index, which is defined as the volume in which one dose would be dissolved to obtain the corresponding IC50 concentration as described by Strandell et al. (2004). Comparison of this unit to physiological volumes facilitates an assessment of inhibitory potential.

A Ki value was determined if the IC50 value was lower than 100 μM. In such cases, MPA (60–600 μM with HLM or 30–600 μM with HIM) and a range of concentrations of individual ginkgo components (10–100 μM with HLM or 3–20 μM with HIM) were used for the construction of Dixon plots and estimation of Ki values.

Detection of MPA-7-O-Glucuronide. MPAG was determined by liquid chromatography-tandem mass spectrometry on a Thermo Finnigan Surveyor series high-performance liquid chromatography system connected to a TSQ Quantum triple quadrupole mass spectrometer (Thermo Fisher Scientific) using electrospray ionization, as described previously (Mohamed et al., 2008). Average assay within-day and between-day relative standard deviations were 5.2 and 6.9%, respectively, and accuracy was expressed as relative error was within 8%. In brief, 5 μl of each sample was injected on a reverse-phase Synergi Fusion-RP18 column (100 × 2 mm, 4 μm; Phenomenex, Torrance, CA). The two mobile phases consisted of (A) 1 mM acetic acid in deionized water and (B) 1 mM acetic acid in acetonitrile. Gradient elution at a flow rate of 0.22 ml/min was used with the following steps: at start of the run, 30% B
for 1 min, then increased to 90% B in 0.75 min, held at 90% B between 1.75 and 3.1 min, and from 3.6 to 6.5 min, the column was re-equilibrated at 30% B. Analysis was performed in the single reaction monitoring, negative ion mode using the mass transitions of m/z 495 → 319 and m/z 493 → 175 for MPAG and phenolphtalein-β-D-glucuronide, respectively. MPAG standard solutions were freshly prepared for each experiment with concentration ranges of 0.1 to 4 μM for HIM or 1 to 20 μM for HLM incubations.

Enzyme Kinetics Analysis. $K_i$ and $V_{\text{max}}$ were determined by nonlinear regression analysis of the MPAG formation data using eight different MPA concentrations (0.02–1 mM). Data points were fitted to the Michaelis-Menten model using Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

$IC_{50}$ values were determined similarly by nonlinear regression fitting of the inhibition data to the $IC_{50}$ equation (Copeland, 2005) using Prism 4.0. The $K_i$ values were determined by fitting competitive, noncompetitive, uncompetitive, and mixed-type inhibition models to the MPAG formation data (Copeland, 2005). The mode of inhibition was determined on the basis of visual inspection of the Dixon plot and the Akaike information criterion (Akaike, 1974) using SigmaPlot version 11 and enzyme kinetics Module version 1.3 (Systat Software, Inc., Chicago, IL).

Results

MPA-7-O-glucuronide formation was best explained by Michaelis-Menten kinetics. The $K_m$ and $V_{\text{max}}$ were 103.9 ± 19.5 μM and 2.6 ± 0.2 nmol/min/mg protein (mean ± S.E.M.), respectively, with pooled HIM, whereas with pooled HLM, these values were 67.2 ± 10.1 μM and 408.7 ± 17.1 pmol/min/mg protein, respectively. These values are similar to values reported previously (Shipkova et al., 2001; Miles et al., 2005; Chang et al., 2009).

Inhibition of MPA Glucuronidation by G. biloba. Both unhydrolyzed and acid-hydrolyzed G. biloba extracts inhibited MPA glucuronidation in pooled HIM and HLM (Fig. 2). MPA concentration was 100 μM for HLM incubations and 70 μM for HIM incubations. Results showed that unhydrolyzed and acid-hydrolyzed G. biloba extracts inhibited MPA glucuronidation in HLM with best-fit $IC_{50}$ values of 84.3 ± 11.6 and 20.9 ± 3.6 μg/ml, respectively. More potent inhibition of MPA glucuronidation was observed in HIM with $IC_{50}$ values of 6.8 ± 0.8 and 4.3 ± 1.2 μg/ml for the unhydrolyzed and acid-hydrolyzed extracts, respectively (Table 1). The volume/dose index values, calculated to estimate the clinical significance of the inhibition as described previously (Strandell et al., 2004), are shown in Table 1.

Effect of Ginkgo Compounds on MPA Glucuronidation. Ginkgo flavonoids (quercetin and kaempferol) and terpene lactones (ginkgolides A and B and bilobalide) were incubated with MPA to determine whether or not these compounds inhibit MPA glucuronidation. Ginkgo flavonoids but not terpene lactones showed inhibition with $IC_{50}$ values <100 μM (Table 1). Quercetin and kaempferol inhibited MPA glucuronidation in HLM with $IC_{50}$ values of 19.1 ± 1.3 and 23.1 ± 5.5 μM, respectively. In agreement with results from incubations with G. biloba extracts, inhibition of MPA glucuronidation was more potent in HIM, with $IC_{50}$ values of 5.8 ± 0.3 and 7.6 ± 0.6 μM for quercetin and kaempferol, respectively.

Inhibition Kinetics Analysis. To further characterize the inhibition of MPA glucuronidation by ginkgo flavonoids, enzyme inhibition kinetic experiments were performed. Based on the analysis of nonlinear regression of inhibition data and Dixon plots presented in Fig. 2, quercetin exhibited mixed-type inhibition against MPA glucuronidation in both HLM and HIM. Kaempferol exhibited noncompetitive inhibition in HLM and mixed-type inhibition in HIM. In HLM, $K_i$ values were 11.3 ± 1.7 and 33.6 ± 2.5 μM for quercetin and kaempferol, respectively (Table 2; Fig. 3A). Again, inhibitory potency of quercetin and kaempferol to MPA glucuronidation in HIM was 3-5 fold higher than in HLM.

TABLE 1

<table>
<thead>
<tr>
<th>Extract/Ginkgo Compound</th>
<th>HLM $IC_{50}$ Values</th>
<th>HIM $IC_{50}$ Values</th>
<th>Volume/Dose Index$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLM $IC_{50}$ Values</td>
<td>HIM $IC_{50}$ Values</td>
<td>HLM $IC_{50}$ Values</td>
</tr>
<tr>
<td>Unhydrolyzed G. biloba</td>
<td>84.3 ± 11.6 μg/ml</td>
<td>6.8 ± 0.8 μg/ml</td>
<td>14</td>
</tr>
<tr>
<td>Acid-hydrolyzed G. biloba</td>
<td>20.9 ± 3.6 μg/ml</td>
<td>4.3 ± 1.2 μg/ml</td>
<td>5.8</td>
</tr>
<tr>
<td>Quercetin</td>
<td>19.1 ± 1.3 μM</td>
<td>5.8 ± 0.3 μM</td>
<td>0.7</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>23.1 ± 5.5 μM</td>
<td>7.6 ± 0.6 μM</td>
<td>0.5</td>
</tr>
<tr>
<td>Ginkgolide A</td>
<td>&gt;100 μM</td>
<td>&gt;100 μM</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ginkgolide B</td>
<td>&gt;100 μM</td>
<td>&gt;100 μM</td>
<td>&lt;0.06</td>
</tr>
<tr>
<td>Bilobalide</td>
<td>&gt;100 μM</td>
<td>&gt;100 μM</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

$^a$ Volume/dose index was calculated by dividing the daily dose by the $IC_{50}$ value (Strandell et al., 2004). Daily dose was considered to be 120 mg G. biloba extract containing (% w/w) 10.75% quercetin, 8.75% kaempferol, 1.2% ginkgolide A, 0.48% ginkgolide B, and 2.94% bilobalide.
to 4-fold higher than that in HLM with $K_i$ values of 2.8 ± 0.4 and 4.5 ± 1.2 $\mu$M, respectively (Table 2; Fig. 3B).

### Discussion

Scientific and public interest in *G. biloba* has grown enormously in recent years because of its purported beneficial effects on memory and circulation (Bardia et al., 2007). *G. biloba* supplements have been widely used with little awareness of the potential for drug interactions with conventional drugs. Although *G. biloba* is considered generally safe, clinical studies and case reports have demonstrated that it can interact with conventional drugs and may lead to severe adverse effects (Hu et al., 2005; Kupiec and Raj, 2005). In the current study, *G. biloba* extract and flavone aglycones inhibited the UGT-mediated metabolism of mycophenolic acid in human intestinal and liver microsomes.

In intestinal microsomes, *G. biloba* extracts inhibited MPAG formation with IC$_{50}$ values of 4.3 and 6.8 $\mu$g/ml for acid-hydrolyzed and unhydrolyzed extracts, respectively. The clinical significance of this interaction can be postulated based on the recommended dose of *G. biloba* supplements and the fraction of MPA metabolized by intestinal enzymes. *G. biloba* extracts are usually taken at a dose of 120 to 240 mg/day. Therefore, IC$_{50}$-equivalent concentrations can be achieved in the intestine if a 120 mg of *G. biloba* dose is mixed with 18 to 28 liters of fluid (i.e., 6.7–4.3 mg/l) or if a 240-mg dose is mixed with 35 to 56 liters of fluid. Thus, based on estimates of intestinal volume that range from approximately 0.5 to 5 liters (Hellum et al., 2007), concentrations in the intestine after ingestion of a *G. biloba* supplement are expected to be much higher than IC$_{50}$ values; accordingly, inhibition of intestinal UGT enzymes in vivo is likely.

The potential for interaction is greater with enteric-coated mycophenolate sodium, because approximately 28% of the dose is eliminated through first-pass metabolism (Myfortic prescribing information: http://www.pharma.us.novartis.com/product/pi/pdf/myfortic.pdf). Inhibition of first-pass metabolism of MPA could result in higher systemic concentrations, an enhanced immunosuppressive effect, and an increased potential for side effects.

Incubations with HLM also showed inhibition of MPA glucuronidation by *G. biloba* extracts. In the liver, UGT1A9 selectively metabolizes MPA to MPAG (Picard et al., 2005); therefore, MPAG formation can be used as an in vitro UGT1A9 index reaction. An effect observed on MPAG formation is expected to reproduce with other UGT1A9 substrates like propofol. In vitro screening of *G. biloba* components for inhibition indicates that the observed inhibition can be attributed to *G. biloba* flavonoid components but not to terpene lactones. $K_i$ values for inhibition of hepatic MPA glucuronidation by quercetin and kaempferol were 11.3 and 33.6 $\mu$M, respectively.

To understand the clinical significance of this observation, adequate knowledge of the bioavailability and hepatic concentrations of the inhibitors is necessary. Quercetin and kaempferol are classified as flavonoids, which is a class of flavonoids ubiquitously found in plants, beverages, and dietary supplements, e.g., tea, onions, apples, red wine, St. John’s wort, and *G. biloba* (Nijveldt et al., 2001). A typical diet contains approximately 14 to 16 mg/day quercetin and 4 to 6 mg/day kaempferol according to dietary surveys in The Netherlands and United States (Hertog et al., 1993; Sampson et al., 2002); however, the intake can reach several hundred milligrams in dietary supplements and herbal products and several grams in anticancer therapy (Lamson and Brignall, 2000).

In contrast with kaempferol, a relatively large number of studies...
concerning the absorption of quercetin have been published. However, the extent to which quercetin reaches the liver remains largely unknown. Most studies were not able to detect free quercetin concentrations in plasma, and absorption was estimated from the quantities of quercetin and quercetin conjugates detected in the urine (0.3–1.4% of quercetin dose) (Scalbert and Williamson, 2000); thus, it was assumed that quercetin was poorly absorbed. However, an early study in healthy ileostomy subjects estimated quercetin absorption to be 17 to 52% of the orally ingested amount (Hollman et al., 1995). The authors reported that only 0.3% of the oral quercetin dose was recovered in urine and concluded that it might be possible that some quercetin accumulated in tissues and was released slowly over time. A recent study investigating tissue distribution of quercetin in pigs after long-term dietary supplementation reported that the total quercetin concentration in liver was 5- to 6-fold higher than that in plasma (Bieger et al., 2008). It is interesting to note that 93% of quercetin found in the liver was in the aglycone form. Therefore, further studies are needed to investigate whether long-term G. biloba or flavonoid-rich supplements may lead to accumulation of quercetin in human liver to levels that could inhibit mycophenolic acid glucuronidation.

Incubations with intestinal microsomes exhibited 3- to 12-fold more potent inhibition of MPAG formation than in liver microsomes by G. biloba extracts, quercetin, and kaempferol (Tables 1 and 2). This difference in inhibition potency can be explained by differentially expressed UGT enzymes in liver and intestine (Ohno and Nakajin, 2009) and the difference in catalytic activities toward MPAG glucuronidation between liver and intestine microsomes. In this study, microsomal intrinsic clearance (\(V_{\text{int}}/K_{\text{m}}\)) for MPAG formation was 4-fold higher by HLM compared with HLM (25.12 versus 6.08 \(\mu\)L/min/mg protein). This result is in accordance with previously reported values (Bowalgalha and Miners, 2001; Shipkova et al., 2001; Picard et al., 2005). In the intestine, UGT1A7, 1A8, 1A9, and 1A10 conjugate MPAG to MPAG with different affinities, whereas in the liver, MPAG is selectively formed by UGT1A9 (Picard et al., 2005). In addition, UGT1A10 exhibits a much lower catalytic activity toward MPAG glucuronidation than UGT1A8 and UGT1A9, whereas its expression in the intestine is 13- and 25-fold greater than that of UGT1A8 and UGT1A9, respectively (Picard et al., 2005; Ohno and Nakajin, 2009). Because of these differences, interactions may not always translate from liver to intestinal microsomes with the same magnitude. Therefore, use of intestinal microsomes to screen for interactions may be necessary for drugs metabolized by intestinal glucuronidation.

Two limitations are acknowledged for this study. First, the study does not rule out the possibility of induction of MPA metabolism by G. biloba. A recent study showed that G. biloba and its components induce cytochrome P450 enzymes, transporters, and UGT1A1 (Li et al., 2009). The effect of G. biloba on MPH-metabolizing enzymes in hepatocytes warrants further research. Second, the study did control for the possible inhibition of UGT activities by fatty acids released from the microsomal membrane, which may inhibit UGT1A9 and result in underestimation of inhibition potency (Rowland et al., 2008). Although the effect of released fatty acids on MPA glucuronidation has not been documented, it is possible that the actual potency of inhibition is greater than what we observed.

Based on our findings, G. biloba supplements taken concomitantly with mycophenolate sodium could lead to increased MPA exposure due to inhibition of presystemic glucuronidation. Therefore, patients should be advised to avoid G. biloba supplements while taking enteroic mycophenolate sodium—the form of MPA that is more subject to presystemic metabolism. Effect of G. biloba on MPA systemic metabolism cannot be predicted because of a lack of information on hepatic concentrations of quercetin and kaempferol but will probably be weaker than the presystemic inhibition. MPA is used in HLM as a probe of UGT1A9 activity because of selective formation of MPAG by UGT1A9. Therefore, the observed hepatic inhibition would be expected to extrapolate to other UGT1A9 substrates such as propofol and flavopiridol. The actual in vivo effect of this interaction should be verified in clinical studies.

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**References**


**Address correspondence to:** Dr. Reginald F. Frye, Associate Professor, Department of Pharmacotherapy and Translational Research, University of Florida, College of Pharmacy, P.O. Box 100486, Gainesville, FL 32610. E-mail: frye@cop.ufl.edu