Metabolism of Bergamottin by Cytochromes P450 2B6 and 3A5

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ABSTRACT

Cytochromes P450 (P450) 2B6 and 3A5 are inactivated by bergamottin (BG). P450 2B6 metabolized BG primarily to M3 and M4 and one minor metabolite (M1). The metabolites were analyzed, and the data indicated that M1 was bergaptol, M3 was 5'-OH-BG, and M4 was a mixture of 6'- and 7'-OH-BG. Because 6'- and 7'-OH-BG were the primary metabolites, it suggested that P450 2B6 preferentially oxidized the geranyl-oxy chain of BG. Metabolism of BG by P450 3A5 resulted in three major metabolites: [bergaptol, M3 (5'-OH-BG), and M5 (2'-OH-BG)], and two minor metabolites [M2 (6',7'-dihydroxy-BG) and M4 (6'- and 7'-OH-BG)]. Because bergaptol was the most abundant metabolite formed, it suggested that P450 3A5 metabolized BG mainly by cleaving the geranyl-oxy chain. Molecular modeling studies confirmed that docking of BG in the P450 2B6 active site favors oxidation in the terminal region of the geranyl-oxy chain, whereas positioning the 2'-carbon of BG nearest the heme iron is preferred by P450 3A5. Glutathione (GSH)-BG conjugates were formed by both P450. Each enzyme predominantly formed conjugates with m/z values of 662. Tandem mass spectrometry analysis of the GSH conjugates indicated that the oxidation forming a reactive intermediate occurred on the furan moiety of BG, presumably through the initial formation of an epoxide at the furan double bond. The data indicate that oxidation of the geranyl-oxy chain resulted in the formation of stable metabolites of BG, whereas oxidation of the furan ring produced reactive intermediates that may be responsible for binding to and inactivating P450 2B6 and 3A4.

The polymorphic cytochromes P450 (P450) 3A5 and 2B6 are involved in the metabolism of many clinically important drugs used in chemotherapy, hormone therapy, suppression of immune responses, or as inhibitors of human immunodeficiency virus proteases (Ingelman-Sundberg, 2004). P450 3A5 is found primarily in liver, intestine, and lung and shares 85% amino acid sequence homology with the major human drug-metabolizing enzyme P450 3A4 (Wrighton et al., 1990; Ding and Kaminsky, 2003). The P450 3A family of enzymes is involved in the metabolism of at least 60% of drugs that are currently in clinical use (Wrighton et al., 1990). Recent reports indicate important clinical consequences for individuals expressing the polymorphic P450 3A5 in the metabolism of immune suppressants and statins (Hesselink et al., 2003; Kivistö et al., 2004).

Although the levels of P450 2B6 in liver are generally low (<2%) in the noninduced state, this isoform is responsible for the metabolism of >4% of drugs routinely used by patients such as buproprion, efaviranz, methadone, ifosfamide, and cyclophosphamide (Faucette et al., 2000; Huang et al., 2000; Ward et al., 2003; Gerber et al., 2004). BG, one of the major components in grapefruit juice, has been demonstrated to be, in part, responsible for the increased bioavailability of certain drugs in what has become known as the “grapefruit juice effect” (Bailey et al., 2000). BG and its hydroxylated product 6',7'-dihydroxy-BG (DHBG) are mechanism-based inactivators of P450 3A4 (He et al., 1998). The ability of BG and DHBG to inactivate P450 3A4 is thought to be the major reason for the grapefruit juice-induced drug interactions that have been observed clinically (Paine et al., 2004). Recently, we have reported that BG also inactivated P450 3A5 and...
2B6, further underscoring the potential problems associated with this component and its effect on the metabolism of clinically important drugs (Bumpus et al., 2005; Lin et al., 2005). Preliminary studies suggested that BG metabolism by P450 3A5 was more complex than that observed with P450 2B6. P450 3A5 generated five metabolites of BG that exhibited absorbencies at 310 nm, indicative of furanocoumarin-containing compounds. Two major metabolites were observed following metabolism of BG by P450 2B6.

Considerable information concerning the metabolism of furanocoumarins by P450 is available. 8-Methoxypsoralen has been shown to undergo similar in structure to BG except that BG contains a 5-geranyl-epoxy-furanocoumarins by P450 is available. 8-Methoxypsoralen is following metabolism of BG by P450 2B6. P450 3A5 was more complex than that observed with P450 2B6, further underscoring the potential problems associated

Materials and Methods

Chemicals. Glutathione, catalase, NADPH, 1-o-dilauroyl-phosphatidylcholine, 1-o-dioleoyl-sn-glycero-3-phosphatidylcholine, and 1-o-phosphatidylserine were purchased from Sigma-Aldrich (St. Louis, MO). DHBG was a gift from the Florida Department of Citrus (Lakeland, FL). 7-Ethoxy-4-(trifluoromethyl)coumarin was from Invitrogen (Carlsbad, CA). Accumbond solid-phase extraction C18 cartridges were from Agilent Technologies (Palo Alto, CA). All other chemicals and solvents were of the highest purity available from commercial sources.

Purification of Enzymes. Expression plasmids for P450 2B6 and 3A5 were generous gifts from Dr. James R.Halpert (Environmental Toxicology, University of Texas Medical Branch, Galveston, TX). The enzymes were expressed in Escherichia coli. Topp cells and purified using Ni²⁺-agarose affinity chromatography as described previously (Domanski et al., 2001; Scott et al., 2001). Reductase was expressed and purified as described by Hanna et al. (1998). Cytochrome b₅ was purified from liver microsomes obtained from Long-Evans rats as described previously (Lin et al., 2002).

Enzyme Assays. P450 2B6 (1 μM) was reconstituted with 2 μM reductase on ice for 45 min. The primary reaction mixture contained catalase (110 units), 10 mM GSH, 1 mM ascorbate, 10 μM BG, and 100 mM potassium phosphate buffer, pH 7.5, in a total volume of 1 ml. Ascorbate was included to stabilize any catechols that may be formed. P450 3A5 (1 μM) was reconstituted with 2 μM reductase, 1 μM cytochrome b₅, and 60 μg of a 1:1:1 mixture of L-o-dilauroyl-phosphatidylcholine, 1-o-dioleoyl-sn-glycero-3-phosphatidylcholine, and 1-o-phosphatidylserine on ice for 45 min. The P450 3A5 reaction mixture received 110 units of catalase, 30 mM MgCl₂, 0.5 mM EDTA, 10 mM GSH, and 1 mM ascorbate in a final volume of 1 ml of 50 mM HEPES buffer, pH 7.5, containing 20% glycerol. Each of the reconstitution mixtures was divided into two samples and incubated at 37°C for 5 min. One sample received 1 mM NADPH, and an equal volume of water was added to the control sample. The mixtures were then incubated for 30 min at 37°C.

HPLC Analysis. In the initial experiment, the samples were reconstituted and incubated with radiolabeled BG in the presence or absence of NADPH. After incubating the samples for 30 min at 37°C, the resulting metabolites were extracted as described below. For all subsequent metabolite analyses, the samples were incubated for 30 min at 37°C with unlabeled BG and then spiked with 10 μM internal standard 6-methoxypsoralen and immediately extracted twice with 2 to 3 ml of nitrogen-purged methylene chloride. The organic phases were pooled and dried under N₂ gas. The dried samples were resuspended in 100 μl of 50% solvent B (solvent A: 0.1% acetic acid in water; solvent B: 0.1% acetic acid in acetonitrile). The metabolites and BG were resolved on a C8 column (5 μm, 2.1 × 150 mm, Zorbax XDB; MAC-MOD, Chadds Ford, PA) equilibrated with 35% solvent B. Samples were applied and washed with 35% solvent B for 8 min. The percentage of solvent B was raised to 45% over the next 17 min followed by an increase in the percentage of solvent B to 80% by 55 min at a flow of 1 ml/min.

LC-ESI-MS/MS Analysis of Metabolites. The same column and gradient conditions were used as described for the HPLC analysis. The column effluent was split, and 0.3 ml/min was diverted into the LCQ mass analyzer (Thermo Electron Corporation, Waltham, MA). The ESI conditions were as follows: sheath gas, 90 arbitrary units; capillary temperature, 225°C; sheath gas, 55 arbitrary units; auxiliary gas, 15 arbitrary units; capillary temperature, 225°C; source voltage, 6 kV; source current, 5 μA; and tube lens offset, 51 V. Data were acquired in positive mode using the Excalibur software package (Thermo Electron Corporation) with one full scan from 100 to 1000 m/z followed by two data dependent scans of the second and third most intense ion. Alternatively, samples were analyzed in positive APCI mode using an LTQ mass analyzer equipped with a photodiode array detector (Thermo Electron Corporation) using the same chromatography conditions as described for the LCQ system. The analyzer conditions were optimized with BG, and the settings were as follows: APCI vaporizer temperature, 450°C; sheath gas, 55 arbitrary units; auxiliary gas, 15 arbitrary units; capillary temperature, 225°C; source voltage, 6 kV; source current, 5 μA; and tube lens offset, 51 V. Parent ion monitoring was carried out in positive APCI mode on a TSQ mass analyzer (Thermo Electron Corporation). The liquid chromatograph conditions were as described for the LCQ. The mass analyzer settings were as follows: sheath gas, 40 arbitrary units; auxiliary gas, 10 arbitrary
units; capillary temperature, 350°C; capillary offset, 35; discharge current, 4; and vaporizer temperature, 500°C.

**LC-ESI-MS Analysis of GSH Conjugates.** GSH conjugates were isolated after adding 60 μl of a 10% solution of trifluoroacetic acid in water to each 1 ml of sample and then applying the sample to a 1-ml Acquibond solid-phase extraction ODS C18 cartridge previously washed with 1 ml of methanol followed by 2 ml of water. After the samples were loaded, the cartridges were washed sequentially with 2 ml of water, 2 ml of methanol, and 0.3 ml of acetonitrile. The organic phases were pooled and dried under N2 gas. The dried samples were resuspended in 150 μl of 50% acetonitrile and 0.1% acetic acid. Samples were analyzed on a C18 reverse-phase column (3 μm, 100 × 4.6 mm, Luna; Phenomenex, Torrance, CA) using a gradient of 20 to 30% solvent B in 5 min followed by a linear increase to 40% solvent B by 15 min and to 90% solvent B by 30 min at a flow of 0.3 ml/min. The column effluent was directed into an LCQ mass analyzer. The ESI conditions were as follows: sheath gas, 90 arbitrary units; auxiliary gas, 30 arbitrary units; capillary temperature, 170°C; and spray voltage, 30 V. Data were acquired in positive mode using the Excalibur software package (Thermo Electron Corporation) with one full scan followed by two data-dependent scans of the most intense and the second most intense ions.

**NMR Analysis of Metabolites.** Approximately 20 to 30 nmol of each P450 was reconstituted with reductase and incubated with BG and NADPH as described above. The metabolites were extracted with nitrogen-purged CH2Cl2 and separated on a C8 reverse-phase column under the same conditions used for the HPLC analyses. Each injection contained the metabolites of BG derived from approximately 5 nmol of incubation mixture. M2 to M5 were collected manually and diluted with water to twice their initial volume. The metabolites were transferred into deuterated solvent by applying each of the diluted metabolite fractions to a 1-ml C8 SepPak solid-phase extraction cartridge (Waters, Milford, MA) previously hydrated with methanol and equilibrated with water. After each diluted metabolite was loaded onto the C8 cartridge, the cartridge was washed with 2 ml of D2O. The metabolites were then eluted with 1 ml of CD3OD and concentrated under N2 to approximately 300 μl. 1H NMR spectra were collected on a Bruker Avance DRX 500 NMR (Bruker Instruments, Billerica, MA). Additional 1H NMR spectra were collected after thoroughly drying the metabolite samples and resuspending them in CD3OD.

**Molecular Modeling of P450 2B6 and 3A5 and BG Docking Studies.** The recent solution of the crystal structures of P450 3A4 and 2B4 provide a basis for responsible construction of models of P450 2B6 and 3A5 based on high-sequence identity templates. P450 2B6 has a sequence identity of 72% with respect to 2B4, whereas P450 3A5 has a sequence identity of 80% with 3A4. Sequence alignments were performed using BLOSUM-30 scoring matrices and a gap penalty of 10 using ClustalW (Thompson and Higgins, 1994). Given the high-sequence identity and the lack of significant insertions of one sequence alignment with respect to the other, no manual realignment was necessary. Supplementary Figures 1 and 2 show the alignments with color coding of the secondary structural elements from the Dictionary of Protein Secondary Structure (Kabasch and Sander, 1983) scoring as reported in the crystal structures of P450 2B4 and 3A4 templates. Labels as to the positions of the α helices in previously reported analyses of bacterial P450 and P450 2C5 templates are indicated. Underlined regions indicate conserved secondary structural regions deduced from past alignments including bacterial and mammalian P450. It is clear that the precise regions of structurally conserved helices and β-sheets in P450 3A4 and 2B4 have a close correspondence with crystal structures of bacterial origins but deviate in the precise location and extent of those regions. The alignments were next used as input to Modeller (Sali and Blundell, 1993) to perform limited initial structural annealing with topological constraints. The model with the highest objective score (derived from both force field and topological constraints) was then used along with SCWRL to examine the optimal side chain conformation (rotamers) for residues not conserved between sequences (Mendes et al., 2001; Canutescu et al., 2003). In the construction of the P450 3A5 model, residues in the vicinity of the O2 binding cleft were not constrained despite the conservation across the P450 3A4/3A5 sequences. This is particularly significant given that variability has already been noted in this region in P450 2C5 (Blobaum et al., 2005) The KETE sequence (residues 281–284 in both P450 3A4 and 3A5) was constructed by conformational search and annealing of a short peptide fragment commensurate with the distance constraints of the insertion along with rotamer exploration to optimize side chain/side chain interactions. In addition, examination of the 1WOG and 1TNQ crystal structures of P450 3A4 by two different groups reveals that Arg212 may have variable conformations. The variable conformations of Arg212 in P450 3A4 may be a sensitive function of nature of substrate, manner of substrate entry in the binding cavity and bound waters. Lys212 in the LKF motif of P450 3A5 (Supplemental Table 1, sequence alignment of P450 3A4 and 3A5) is the corresponding amino acid to Arg212, and its conformation was explicitly explored to rotameric construction of nonconserved residues.

The initial models were then energy minimized using limited energy minimization (400 steps of steepest descents; 200 steps conjugate-gradient) merely to remove any bad contacts following rotamer exploration. Waters were then introduced using in-house software that places waters based on topological and energy metrics (Zhao et al., 1996). The resulting initial structure with modeled waters was then energy minimized for 150 steps of steepest descent minimization followed by 3000 steps of conjugate gradient minimization. The structure was then equilibrated at 300K for 600 ps using radial dielectric screening and a potential truncation of 12 Å. The final coordinates following molecular dynamic thermalization were then energy minimized for 2000 steps before use of the model in docking.

Prosa 2003 (Insoft Oy, Oulu, Finland) was used to assess the quality of the model/fold commensurate with sequence (Sippel, 1993). The P450 3A5 model following equilibration and annealing had a normalized Prosa score of 0.88 compared with the P450 3A4 (template of 0.94), whereas the normalized Z-score was 0.91 before annealing. The Prosa scores indicate that use of limited equilibration resulted in no serious degradation of model folds but does improve facets such as the hydrogen bonding in the region of the binding site before model use in docking and molecular dynamics (Park and Harris 2003). Inspection of Prosa plots revealed no region of the model with positive Prosa energies. The P450 2B6 model had a normalized Z-score of 0.88 compared with the P450 2B4 crystal structure Z-score of 0.82. The two models are seen to have normalized Z-scores comparable with the templates from which they were derived. The summary assessments shown in Table 1 in the Appendix indicate that the deviations from “standard” bond lengths/angles/dihedrals do not result in a model that has substantial regions of geometric violations compared with high-quality crystal structures. This is reflected in the Procheck scores, which by virtue of having values more than −0.5 are deemed adequate. Comparison values for all measures are also indicated for the templates from which the models were derived.

Energy based docking was performed using Autodock3 (Morris et al., 1998), ICM (Molsoft Inc., San Diego, CA), and Glide (Schrodinger Inc., Portland, OR) docking modules to verify that similar lowest energy docked configurations were obtained irrespective of differences in configuration sampling algorithms and potential function/scoring functions used. Autodock uses a classical coulomb + 6-9-12 type of interaction, and in this instance a charge model of the heme (resting state or oxyferryl/compound I) was derived from density functional theoretical calculations of the electrostatic potential. ICM uses an ECEPP/4 force field supplemented by van der Waals, hydrogen bonding, electrostatic, and hydrophobic ligand/receptor interaction terms. Glide makes use of a Chemscore-like function in addition to classical interaction terms. Glide uses a series of hierarchical filters based on a combination of scoring functions based on active
site and ligand shape, OPLS-AA potential function/receptor-flexible ligand Monte-Carlo docking followed by application of a Chemscore function (Friesner at al., 2004). Autodock3 protocols were as reported previously (Park and Harris, 2003), whereas in the case of ICM docking Monte Carlo based docking was performed into a region of the model within 12 Å of the model binding sites. Glide 4.02 docking was performed into a 12-Å box on the distal side of the heme. The lowest 100-energy configurations were saved from each docking study to assess the common low-energy compound I-BG interaction motifs. Molecular dynamics protocols for equilibration of docked configurations are as described previously (Park and Harris, 2003; Harris et al., 2004).

Results

HPLC Analysis of Metabolites of BG. P450 2B6 and 3A5 were incubated with BG that was radiolabeled on the C1 carbon of the geranyl-oxy chain. HPLC analysis of control samples incubated in the absence of NADPH revealed only one peak corresponding to BG that eluted at 50 min. This peak displayed an absorbance maximum at 310 nm indicative of the furanocoumarin moiety and also contained radioactivity due to the presence of the geranyl-oxy chain (data not shown). Incubation of BG with P450 2B6 in the presence of NADPH resulted in the appearance of two major metabolites that both exhibited absorbencies at 310 nm as well as radio-label and eluted at 34 min (M3) and 36 min (M4) (Fig. 1A). A minor third metabolite (M1) eluted at 10 min and was characterized by an absorbance at 310 nm indicative of an intact furanocoumarin moiety but an absence of counts, suggesting that this product was devoid of the geranyl-oxy chain. An additional smaller peak that also exhibited an absorbance at 310 nm without radioactivity eluted at 47 min. The BG metabolite profile obtained from P450 3A5 samples was more complex and exhibited five metabolites that eluted at 10 min (M1), 16 min (M2), 34 min (M3), 36 min (M4), and 39 min

<table>
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<tr>
<th>Table 1</th>
<th>Tandem mass spectral analysis of metabolites M1 to M5</th>
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<tr>
<td>Metabolite</td>
<td>Major Molecular Ions</td>
</tr>
<tr>
<td>M1, bergaptol</td>
<td><strong>203</strong>, 175, 159, 147, 131</td>
</tr>
<tr>
<td>M2, 6',7'-dihydroxy-BG</td>
<td>372, 354, 336, 203</td>
</tr>
<tr>
<td>M3, 5'-OH-BG</td>
<td>357, 341, 339, 215, 203</td>
</tr>
<tr>
<td>M4, 6'- and 7'-OH-BG</td>
<td>355, 337, 215, 203, 153, 135</td>
</tr>
<tr>
<td>M5, 2'-OH-BG</td>
<td>355, 339, 337, 273, 271, 255, 243, 215, 203, 153</td>
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*a* The parent molecular ion is displayed in bold; the top line shows the experimental product ions that were obtained with the metabolites after tandem mass analysis.

*b* The second line shows the ions obtained with the authentic standards when available.
(M5). The amount of M1 that was generated by P450 3A5 was approximately 5-fold higher than that obtained from incubations with P450 2B6. Again, no counts were associated with M1, suggesting that metabolism of BG had resulted in the removal of the labeled geranyl-oxy moiety. Previous studies that compared the retention times under the same HPLC conditions of the authentic standards bergaptol and DHBG with the BG metabolite elution profile suggested that M1 and M2 may be bergaptol and DHBG, respectively. However, conclusive evidence as to the identity of M1 to M5 had not been obtained at this point. With all of the samples incubated with radiolabeled BG, an additional peak was seen with an absorbance at 310 nm that eluted at 23 min. This peak was only present in samples incubated with radiolabeled BG and was not seen in samples incubated with nonradioactive BG in the presence of NADPH. Presumably this product was derived from metabolism of a radiolabeled breakdown product in the commercially obtained BG stock solution that occurred after long-term storage. Therefore, it was not analyzed further.

**LC-APCI-MS and MS/MS Analysis of Metabolites Generated by Incubating P450 2B6 with BG.** The data in Fig. 2A depict the HPLC chromatogram of metabolites from P450 2B6 samples incubated with BG in the presence of NADPH (Fig. 2A). The diode array spectra associated with the internal standard (6-methoxypsoralen), M3, M4, and BG are shown as insets to Fig. 2A. In the absence of NADPH, only BG and 6-methoxypsoralen were observed using diode array detection (data not shown). The total ion chromatogram traces for both the samples (incubated either in the absence or presence of NADPH) seemed similar and did not yield information as to the elution times of any metabolites (data not shown). If, however, an extracted ion chromatogram (XIC) with an [M+H] of 203 for the 5-hydroxypsoralen moiety was monitored, the chromatogram depicted in Fig. 2B was observed. In the absence of NADPH, only the peaks eluting at 18.3 min (6-methoxypsoralen; m/z 218) and at 50.2 min (BG; m/z 339) were detected (data not shown). Metabolites of BG with m/z of 355 (M4; 34.4 min) and m/z of 357 (M3; 33.1 min) were observed in the presence of NADPH (Fig. 2C). In addition to these species, another product with an m/z of 203, suggesting the removal of the geranyl-oxy chain, was seen at 9.7 min (M1). Most of the parent ions were also observed as species with an increase in mass of 41 that was derived from the acetonitrile in the solvent. Because the metabolites could only be detected in the XIC at [M+1] of 203, precursor ion monitoring was carried out on a triple
The spectra of M4 most closely fit the estimations at the possible structures for the M3 to M5 metabolites. A tandem mass spectrometry of M4 as well as the mass spectrum (presence of the m/z 203) indicated that the 5-hydroxypsoralen ring of this metabolite was intact, suggesting again that the hydroxylation occurred on the geranyl chain. However, the tandem mass spectrum of M3 was complex and indicated the presence of two different hydroxylated species. The major metabolite exhibited product ions of 203, 215, and 339. The second, albeit much lower abundant metabolite with the same m/z of 357.3 that coeluted with M3 displayed unique ions with an m/z of 221 and 235 that might be observed if the hydroxylation had occurred on the furanocoumarin moiety of BG (data not shown). M3 was also analyzed by 1H NMR in the same manner as M4. The furanocoumarin moiety of M3 was intact and the proton signals in the aromatic region for M4 corresponded to the expected number of protons for an unmodified furanocoumarin moiety. The signals arising from the 5'-protons at 2.09 ppm were missing. New peaks indicative of hydroxylation at the 5'-position were seen as multiplets arising from the 4'-protons in the 1.76 to 1.86 ppm region and a OH signal 3.95 ppm and a new multiplet at 3.7 to 3.9 ppm corresponding to the single 5'-proton. The spectra most closely resembled the predicted 1H NMR spectrum of 5'-OH-BG. Together, the data indicate that M3 is 5'-OH-BG. The coeluting minor metabolite that was observed by LC-MS/MS was probably too low to be detected above the background of the NMR spectra.

### LC-APCI-MS and MS/MS Analysis of Metabolites Generated by Incubating P450 2B6 and 3A5 with BG

A representative HPLC chromatogram depicting the elution profile of 6-methoxypsoralen, BG, and the BG-derived metabolites monitored at 310 nm from P450 2B6 incubated with BG in the presence of NADPH is shown in Fig. 3A. The photo diode array spectra between 250 and 340 nm of M1, M2, M3, and M5 are shown as insets in Fig. 3A. Because the total ion chromatograms of the samples incubated in the presence or absence of NADPH again were indistinguishable, the XIC with the [M + 1] at 203 was monitored (Fig. 3B). As was seen previously, BG with an m/z of 339 eluted at 50.2 min and the

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Furanocoumarin Ring ppm</th>
<th>Geranyl Chain ppm</th>
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<tr>
<td>BG</td>
<td>8.29 7.81 7.22 7.19 6.30</td>
<td>5.05 5.58 NO 2.09 2.09 5.06 NO 1.59 1.65 1.69 NO</td>
</tr>
<tr>
<td>M3, 5'-OH-BG</td>
<td>8.19 7.63 7.28 7.17 6.29</td>
<td>5.00 5.71 NO 1.76 1.86 3.8 5.14 NO 0.96 1.19 1.18 3.95</td>
</tr>
<tr>
<td>M4, 6'-OH-BG</td>
<td>8.29 7.81 7.25 7.16 6.31</td>
<td>5.05 5.60 NO 2.22 1.72 3.80 1.05 0.74 0.74 1.31 3.65</td>
</tr>
<tr>
<td>7-OH-BG</td>
<td>8.29 7.81 7.25 7.16 6.31</td>
<td>5.05 5.60 NO 2.39 1.48 1.05 NO 0.95 0.95 1.31 3.65</td>
</tr>
<tr>
<td>M5, 2'-OH-BG</td>
<td>8.32 7.63 7.23 7.00 6.35</td>
<td>4.52 4.22 3.88 1.7 2.00 2.02 5.06 NO 1.45 1.37 1.23 3.74</td>
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- NO: no proton signal associated.
- *d*: doublet; *s*: singlet; and *m*: multiplet.
internal standard 6-methoxypsoralen with an m/z of 218 eluted at 18.2 min. Metabolites with an m/z of 203 (M1), 373 (M2), 357 (M3), or 355 (for M4 and M5) eluted at 9.6, 20.3, 33.1, 34.4, and 38 min, respectively, and were only seen in samples incubated with NADPH. M1 eluted at the same retention time and displayed the same m/z of 203 and MS/MS pattern as the authentic standard bergaptol.

M2 eluted at the same retention time as the authentic standard 6',7'-DHBG and displayed an m/z of 373 consistent with this dihydroxylated product. The fragmentation patterns observed for M1 and M2 were similar to those of the authentic standards for bergaptol or DHBG and to the patterns predicted by the Mass Frontiers 3.0 software (Thermo Electron Corporation). The amount of M2 recovered was too low to analyze by 1H NMR.

M3 corresponded to 5'-OH-BG, the same M3 metabolite that was generated by P450 2B6, based on the HPLC retention time, the photodiode array spectrum, the XIC, the MS/MS fragmentation pattern, and the 1H NMR spectrum. An additional product that may correspond to a furan-hydroxylated species was again observed coeluting in the M3 peak but in at least a 10-fold lower abundance. M3 corresponded to the 6'-OH and 7'-OH metabolites of BG that were produced by P450 2B6, except that P450 3A5 generated less of these metabolites than P450 2B6.

M5, the second most abundant metabolite seen in the P450
Fig. 4. Energy-based docking of BG into models of P450 2B6 and 3A5: differential exposure of geranyl side chain atoms. A, energy-based docking distribution (Autodock3) for exposure of BG in the model of P450 2B6, indicating the frequency of exposure of geranyl side chain atoms within 4 Å of the oxyferryl (compound I) oxygen. Top, most frequently encountered configurations of BG in P450 2B6 to have exposure of the terminal atoms in the geranyl side chain with less exposure of the initial atoms at the 4 Å evaluation criteria. Bottom, structures show the lowest energy configuration of BG relative to the oxyferryl center without surrounding residues (left) and with nearest neighbor amino acid residues shown and BG shown in magenta (right). B, energy-based flexible ligand docking (Autodock3) of BG into the model for P450 3A5 with the heme in oxyferryl-heme form. Top left, histogram illustrating the frequency with which sites on the geranyl side chain were exposed within 4 Å of the oxyferryl (compound I) oxygen. Bottom left, one of the most frequently observed docked poses with the 1' and 2' centers exposed to the oxyferryl oxygen (indicated with an arrow). Right-most panel, space-filling illustration of a representative molecular dynamics configuration of BG interaction with compound I after 210 ps of molecular dynamics equilibration. C, docking of BG into the compound I heme model form of 3A5, using flexible-ligand/rigid protein docking (Glide 4.02), indicates that the configuration with 1'-exposure to compound I is "enriched" in P450 3A5 compared with P450 2B6.
3A5 incubation mixtures, exhibited a weak parent ion signal with an m/z of 355 to 357, consistent with the addition of one oxygen or OH. The photodiode array spectrum and the XIC indicated that the furanocoumarin ring system was unaffected and that the modification had occurred on the geranyl chain. MS/MS analysis of the fragmentation pattern suggested a modification at the 2'- or 3'-carbon of BG. ¹H NMR analysis of M5 was carried out as was done for M4 and M3, and the results confirmed that the furanocoumarin ring was not modified. The proton ratios and signals that were observed at 8.32, 7.63, 7.23, 7.00, and 6.35 ppm were similar to what was seen for intact BG. The most striking feature in the M5 spectrum was the absence of the 2'-signal at 5.58 ppm and the appearance of several multiplets in the 2 to 5 ppm region and the appearance of an OH signal at 3.74 ppm. With this sample, it was also possible to irradiate the OH peak at 3.74 ppm, which caused a triplet at 3.88 ppm corresponding to the 2'-proton to collapse, lending further evidence for M5 being a 2'-OH product of BG.

Interactions of BG Reversibly Bound to 3A5 and 2B6: Differential Exposure to Compound I. The models that were constructed using approaches described under Materials and Methods were next assessed to examine the degree to which low-energy configurations of BG in the binding site correlate with the observed products of BG metabolism by P450 3A4 and 2B6. Both geometric and electronic thermodynamics/electronic facets are important in the observed products in that it is both the low-energy-bound configurations and the energy landscape for competitive metabolism of multiple sites from each of those low-energy poses that determines the products formed.

ICM binding site locator was used to examine the binding site and closed cavity volumes near the substrate/inhibitor binding site of the models. 3A4(1TQN) had a binding site volume of 861 Å³, whereas 3A4(1WOG) had a binding site volume of 919 Å³, and P450 2B4 had a binding site volume of 594 Å³. The P450 3A5 model based on the 3A4(1TQN) structure had a binding site volume of 347 Å³, whereas P450 2B6 based on the P450 2B4 structure had a volume of 209 Å³. By any measure, qualitative and quantitative, the binding site of P450 3A5 is larger near the heme than that of P450 2B6. Moreover the architecture of the binding sites differs. This should, in principle, alter the product distribution and was in agreement with what was observed in the metabolism studies.

Figure 4A illustrates that when BG is docked in the active site of P450 2B6, the P450 2B6/BG binding site interactions that result in the lowest energy-bound configurations have the 4', 5', and 6'-carbons of the geranyl-oxy chain carbon atoms exposed to the oxyferryl oxygen of compound I (see the BG structure in Fig. 4A for the numbering assignments of the geranyl carbons). This orientation would therefore predict the preferential oxidation at the terminal end of the geranyl-oxy chain of BG and supports the experimental findings for the metabolism of BG to primarily 6',7'-epoxy-BG by P450 2B6. In contrast to P450 2B6, the P450 3A5/BG binding site interactions that result in the lowest energy-docked configurations of BG occur when the 1', 2', and 9'-carbons have the most favorable interactions with the compound I center (Fig. 4B). The results are again consistent with the experimental findings that indicated that with P450 3A5, the cleavage of the geranyl-oxy chain at the 1'-carbon to form bergapten is preferred, followed by oxidation of the geranyl chain to form M5 and M3 (Fig. 4C). Although, in general, the metabolism profile of a substrate is both a function of the configuration of the substrate (which sites are exposed to the active oxygen species) and kinetic factors (determining competitive transformation of sites simultaneously exposed), the docking results for BG in this study are consistent with the predominance of configuration factors in determining the differential profiles observed in the distribution of metabolites generated by P450 3A5 and 2B6.

LC-ESI-MS/MS Analysis of Glutathione Conjugates Obtained from Incubating P450 2B6 and 3A5 with BG in the Presence of GSH. For the P450 2B6 samples incubated with BG in the presence of NAPDP and GSH, one major GS conjugate of BG with a mass of 661 Da (M + 1; m/z of 662) was observed (Fig. 5). This adduct eluted at 25 min and was only seen in samples incubated with NADPH. The mass of the GS conjugate corresponded to the mass of BG plus the mass of glutathione plus one oxygen atom. MS/MS analysis of the 662 ion [M + 1] resulted in two major daughter ions with m/z of 526, and 308 (Fig. 6A). The structure most consistent with these fragmentation products is also shown in Fig. 6A. In addition to the monooxygenated GS conjugate of BG a minor secondary adduct with a mass of 678 Da corresponding to a GS conjugate of BG with two oxygen atoms was seen eluting at 16.9 min (data not shown). Again,
this ion was only present in samples incubated with NADPH. The appearance of the characteristic 526 and 308 ions in the MS/MS profile (Fig. 6B) of the doubly hydroxylated product suggests that it is structurally related to the monoxygenated GS conjugate of BG and that the second oxygen was introduced on the geranyl-oxy chain (Fig. 6B). Because P450 2B6 showed a preference for metabolism at the 6'- and 7'-carbon, it is conceivable that the 678 species arose from oxidation at that position, but oxidation at 5'-carbon may also be possible. The amount of GS conjugate that was formed was too low to further analyze these products by 1H NMR.

One major GS conjugate of BG with an m/z of 662 (Fig. 7A, inset) was also observed in P450 3A5 samples incubated with BG in the presence of NADPH and GSH. This adduct also eluted at 25 min and was only seen in samples incubated with NADPH and not in control samples (Fig. 7A; data not shown). The MS/MS analysis of the 662 ion [M + 1]+ revealed daughter ions with an m/z of 526, 355, and 308 (Fig. 7B). The structure that is most consistent with this observation is shown in the inset to Fig. 7B, identical to the structure shown in Fig. 6A. Two additional, albeit lower abundance GS conjugates of BG with masses of 678 Da [M + 1]+ were seen eluting at 16.9 and at 17.6 min (Fig. 8A). The MS/MS fragmentation patterns of these two products were similar to each other (Fig. 8B) and to the pattern seen with the deoxytetragenated product obtained with P450 2B6 (Fig. 7B). In each case, a prominent ion at m/z 526 was observed corresponding to a hydroxylated furanocoumarin moiety and oxidized glutathione, suggesting that the second oxidation event occurred on the geranyl-oxy chain. Whether the two products that were seen with P450 3A5 correspond to different isomers that were oxidized at the same position or to two different products with oxidation at the 6'- and 7'- or the 2'- and 3'-carbons of BG cannot be determined from this analysis. A previous report as well as observation in our laboratory suggests that these doublets could correspond to isomers of the same molecule (Shebley et al., 2006). However, the appearance of only one isomer at 16.9 min in the P450 2B6 samples suggests the possible formation of different GS conjugates and may be analogous to two of the possible sites of hydroxylation on the geranyl-oxy chain that were seen in the metabolites identified from P450 2B6 and 3A5. The structures believed to be
most consistent with the ion fragmentation pattern are shown in Fig. 8C.

**Discussion**

BG is a mechanism-based inactivator of P450 3A5 and 2B6 (Lin et al., 2005). A combination of metabolite analyses, characterization of GS conjugates of BG, and molecular modeling have been used in this report to elucidate differences in the routes of metabolism of BG by these two enzymes and to understand which reactive BG intermediates may be formed and could be responsible for the mechanism-based inactivation.

The chemical structures of BG and 6-methoxypsoralen share the same furanocoumarin ring structure, and this suggested that some of the routes of metabolism of these two compounds by P450 of the 2B family might be similar. Studies with P450 2B1 indicated that the metabolism of 8-methoxypsoralen resulted in the formation of a monooxygenated metabolite of the furanocoumarin ring, the formation of a dihydrodiol furanocoumarin, and the oxidation of the double bond of the furan ring to generate a monooxygenated reactive intermediate that was responsible for inactivation and could be trapped by GSH (Koenigs and Trager, 1998). Studies with various coumarins and their dihydrodiols indicated that hydroxylation of the furan ring double bond resulted in compounds that displayed a UV shift from approximately 310 to 325 nm (Koenigs and Trager, 1998). P450 2B6 metabolized BG to M4, M3, and M1 that could be observed by their UV absorbance at 310 nm, a molecular ion at m/z 203, and five characteristic 1H NMR signals at 8.29, 7.81, 7.22 6.31, and 6.28 ppm indicative of the presence of an intact furanocoumarin ring. The most abundant metabolite, M4, had a parent m/z of 355, suggesting the insertion of one oxygen atom into the geranyl-oxy chain of BG. MS/MS analysis was unable to conclusively determine the location of the oxygen addition. 1H NMR studies were carried out, and the data indicated that the M4 peak was actually composed of two different metabolites with hydroxylations at either the 6'- or the 7'-carbon on the BG geranyl-oxy chain. The difference in two mass units between the observed mass obtained by API-MS for M4 and the theoretical mass cannot be explained at this time. Similar studies with the remaining two metabolites generated by P450 2B6 indicated that M3 was hydroxylated on the 5'-carbon of BG. The minor product M1 was distinguished by an absence of associated radioactivity (i.e., removal of the geranyl-oxy chain containing the radiolabel at the 1'-carbon) and an m/z of 203 and coelution with the authentic standard bergaptol. The ratios of the metabolites suggest that metabolism of BG by P450 2B6 occurs primarily on the geranyl-oxy chain. This observation was underscored...
by docking studies of BG into a model of P450 2B6. The model indicated that the most favorable binding site for BG/2B6 interactions leads primarily to exposure of the terminal part of the geranyloxy chain and in particular the 5′-, 6′-, and 4′-carbons to the oxyferryl oxygen of compound I. These sites would be the most favorable sites of metabolism since they were the primary sites exposed within 4 Å of the active oxygen species. Previous studies with 6-methoxypsoralen indicated that hydroxylation of the furan double bond by P450 2B1 resulted in the formation of a dihydrodiol product (Koenigs and Trager, 1998). With P450 2B6 (and 3A5), only a very minor monohydroxylated furan product was observed eluting at almost the same time as the M3 metabolite. Either P450 2B6/3A5 do not generate the dihydrodiol species from BG or one of the hydroxyl groups could have been lost as water during sample analysis in the mass spectrometer.

In contrast, metabolism of BG that involves the production of the reactive intermediate responsible for the loss in enzymatic activity seems to result from the metabolism at the furan ring. This observation supports previous studies that indicated that inactivation of P450 enzymes by psoralsens required the presence of the furan ring double bond (Letteron et al., 1986; Cai et al., 1993). Tandem mass analysis of the glutathione conjugates of BG formed by P450 2B6 resulted in fragments that were most consistent with GS adduction to the furan ring analogous to what has been observed with 8-methoxypsoralen and P450 2B1 (Koenigs and Trager, 1998).

The HPLC profiles of the metabolites formed by incubating BG with P450 3A4 and 3A5 in the presence of NADPH were previously found to be identical (Lin et al., 2005). Although P450 3A4 seems to be the major P450 involved in the metabolism of BG, P450 3A5 may have additional and yet to be determined important physiological functions, because it is the only 3A family member found in the adrenal, prostate, and kidney and also shows differential levels of expression in children and African Americans (Kuehl et al., 2001). P450 3A5 metabolized BG to at least five different metabolites with bergaptol being the most abundant. Bergaptol could then in turn inactivate other P450 enzymes, such as P450 2B6 (Letteron et al., 1986). In addition to the 5′-OH-BG and 6′- and 7′-OH-BG seen with P450 2B6, a metabolite unique to P450 3A4/5 (M5) was observed. M5 exhibited a UV spectrum with an absorbance maximum at 306 nm, an m/z of 355 to 357, a prominent ion at m/z 203, and an 1H NMR spectrum in the aromatic region that was indicative of a monoxygenated...
BG molecule with an intact furanocoumarin ring structure. 

$^1$H NMR studies were required to locate the oxidation to the 2'-carbon on the geranyl-oxo chain of BG. The minor metabolite M2 was characterized by an absorbance maximum at 306 nm and an m/z of 371. The m/z 203 ion suggested the presence of an unmodified furanocoumarin ring system. The mass of this metabolite was most consistent with 6',7'-dihydroxy-BG. This result was supported by the observations that the authentic standard 6',7'-dihydroxy-BG exhibited the same LC, MS, and MS/MS characteristics. The observed metabolites were consistent with the BG-oxyferryl oxygen exposure probed by energy based docking of BG into a model of P450 3A5. The docking results indicated that in marked contrast to P450 2B6, the 1'-carbon in P450 3A5 was the most likely site for metabolism consistent with the formation of bergaptol as the primary metabolite generated by P450 3A5. The furan site was twice as probable as the 1'-site to be exposed to the oxyferryl center. Although the predicted metabolism is a function of both the propensity of exposure and the energy landscape of competitive metabolism of sites exposed to the active species center, the docking results are consistent with the observed metabolite distributions. Reactive intermediates that could be trapped by GSH were derived from oxidation of the double bond of the furan ring of BG. P450 3A5 generated at least two different GS conjugates of BG. The major conjugate exhibited an m/z of 662. The MS/MS fragmentation pattern of the GS conjugate of BG that was generated by P450 3A5 was identical to the one seen with P450 2B6 and indicated that GS was adducted to the furan ring of BG molecule. The most characteristic fragments were a 525 ion that could only arise from a GS adduct at the furanocoumarin ring and a 354 ion that was characteristic of a hydroxylated BG molecule. P450 3A5 also generated two additional GS conjugates of BG that 1) eluted approximately 5 min earlier than the 662 ion, 2) exhibited the same m/z of 678, and 3) eluted with approximately the same intensity within 1 min of each other. Tandem mass analysis of each of the ions indicated that GS was adducted to the furanocoumarin ring structure (M + 1; 525) and insertion of one oxygen atom at one of the geranyl-oxo double bonds (loss of M + 1 of 153 and M + 1 of 370).

In summary, these data show that BG is metabolized by P450 2B6 to two major and one minor metabolite and that the metabolite products are formed by oxidation on the geranyl-oxo chain. In contrast, metabolism that results in the formation of a reactive intermediate that may bind either to the heme or the apoprotein occurs on the furan ring of BG via oxygen insertion at the furan double bond, leading to the formation of a reactive epoxide (Fig. 9). Amino acid residues in the P450 2B6 active site that are nearest to the furan ring and may be exposed to a furan epoxide are Thr302, Leu363, and Ala298. P450 3A5 metabolism of BG generates five metabolites, primarily resulting in the removal of the geranyl-oxo chain and formation of bergaptol. Additional metabolism

Fig. 9. Proposed scheme for the metabolism of BG by P450 2B6 and 3A5.
of BG by P450 3A5 again involves hydroxylation on the geranyl-oxy moiety (Fig. 9). Initial molecular modeling studies indicate the manner in which differential binding site architecture in P450 isoforms subtly modulates preferred ligand/binding site configurations, resulting in differential product distributions. The reactive intermediate that may be responsible for the loss in P450 3A5 enzymatic activity was again formed from epoxidation of the furan moiety, Phe304, Ala305, and Thr309 are the residues closest to the furan ligand/binding site configurations, resulting in differential binding site properties. Studies indicate the manner in which differential binding site configurations of BG by P450 3A5 again involves hydroxylation on the geranyl-oxy chain.

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References


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