Bioactivation of a Novel 2-Methylindole-Containing Dual Chemoattractant Receptor-Homologous Molecule Expressed on T-Helper Type-2 Cells/\(\beta\)-Prostanoid Receptor Antagonist Leads to Mechanism-Based CYP3A Inactivation: Glutathione Adduct Characterization and Prediction of In Vivo Drug-Drug Interaction

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ABSTRACT:

The 2-methyl substituted indole, 2MI [2-(4-(2,4-dichlorophenylsulfonamido)-2-methyl-1\(H\)-indol-5-yloxy)-3-methoxyphenyl)acetic acid] is a potent dual inhibitor of 1) chemoattractant receptor-homologous molecule expressed on T-helper type-2 cells and 2) \(\beta\)-prostanoid receptor. During evaluation as a potential treatment for asthma and allergic rhinitis, 2MI was identified as a mechanism-based inactivator of CYP3A4 in vitro. The inactivation was shown to be irreversible by dialysis and accompanied by an NADPH-dependent increase in 2MI covalent binding to a 55- to 60-kDa microsomal protein, consistent with irreversible binding to CYP3A4. Two glutathione (GSH) adducts, G1 and G2, were identified in vitro, and the more abundant adduct (G1) was unambiguously determined via NMR to be GSH adducted to the 3-position of the 2-methylindole moiety. The potential for a clinical drug-drug interaction arising from mechanism-based inactivation of CYP3A4 by 2MI was predicted using a steady-state model, and a 4.3- to 7.5-fold increase in the exposure of midazolam was predicted at anticipated therapeutic concentrations. To better assess the potential for in vivo drug-drug interactions, the Sprague-Dawley rat was used as an in vivo model. An excellent in vitro-in vivo correlation was observed for the reduction in enzyme steady-state concentration (\(E_{ss\text{in}}/E_{ss\text{out}}\)) as well as the change in the exposure of a prototypical CYP3A substrate, indinavir (area under the curve (AUC) for indinavir/AUC). In summary, 2MI was identified as a potent mechanism-based inactivator of CYP3A4 and was predicted to elicit a clinically relevant drug-drug interaction in humans at an anticipated therapeutic concentration.

Prostaglandin D\(_2\), the major cyclooxygenase product formed in activated mast cells, is an important mediator of the inflammatory response associated with asthma. PGD\(_2\) activity is mediated through activation of chemoattractant receptor-homologous molecule expressed on T-helper type-2 cells and \(\beta\)-prostanoid receptor. The 2-substituted methylindole, 2MI [2-(4-(2,4-dichlorophenylsulfonamido)-2-methyl-1\(H\)-indol-5-yloxy)-3-methoxyphenyl)acetic acid] (Fig. 1) is a potent dual DP and CRTH2 antagonist and a potential treatment for asthma and allergic rhinitis. Preliminary absorption, distribution, metabolism, and excretion studies revealed that it was also a potent mechanism-based inhibitor of CYP3A4.

Mechanism-based inactivation (MBI) of CYP3A4 has been associated with clinically relevant drug-drug interactions (DDIs) (Zhou et al., 2005; Kalguurkar et al., 2007; Riley et al., 2007). Unlike competitive inhibition, metabolic activity after MBI is restored only after de novo enzyme synthesis (Silverman, 1995; Hollenberg, 2002), resulting in a prolonged reduction of enzyme activity. This irreversible inactivation occurs as the result of P450-mediated bioactivation of the parent compound to reactive species that are unable to leave the active site, leading to modifications of the enzyme either through tight

ABBRRIEVATIONS: CRTH2, chemoattractant receptor-homologous molecule expressed on T-helper type-2 cells; DP, \(\beta\)-prostanoid; 2MI, [2-(4-(2,4-dichlorophenylsulfonamido)-2-methyl-1\(H\)-indol-5-yloxy)-3-methoxyphenyl]acetic acid; MBI, mechanism-based inactivation; DDIs, drug-drug interactions; P450, cytochrome P450; HLM, human liver microsomes; RLM, rat liver microsomes; OH, hydroxy; ACN, acetonitrile; LC, liquid chromatography; MS/MS, tandem mass spectrometry; TAO, treloandomycin; ABT, 1-aminobenzoiazole; GSH, glutathione; PAGE, polyacrylamide gel electrophoresis; MRM, multiple reaction monitoring; HRP, horseradish peroxidase; SPE, solid-phase extraction; 1D, one dimensional; 2D, two dimensional; HMBC, heteronuclear multiple quantum correlation; AUC, area under the curve; AUCi, AUC in the presence of the inhibitor; MS, mass spectrometry; 3MI, 3-methylindole.
coordination with the prosthetic heme or covalent modification of P450 apoprotein and/or heme.

Recognizing the potential for 2MI to elicit an adverse DDI precipitating from MBI, the present studies were undertaken to 1) elucidate the putative reactive intermediate(s) and the mechanism of enzyme inactivation, 2) to predict the magnitude of in vivo DDIs based on in vitro inactivation kinetics using a steady-state model, and 3) to investigate the propensity for 2MI to elicit MBI and DDI using the Sprague-Dawley rat as an in vivo model.

Materials and Methods

Chemicals and Enzymes. 2MI was synthesized by the Amgen Medicinal Chemistry Department (Amgen, South San Francisco, CA). [35S]2MI (50 mCi/mmol, >99% purity) was synthesized by Moravek Biochemicals (Brea, CA). [1-14C]Naphthalene and horseradish peroxidase type XII were purchased from Sigma-Aldrich (St. Louis, MO). Pooled HLM, RLM, and recombinant CYP3A4-expressed microsomes (Supersomes, containing 960 pmol of cytochrome b₅/nmol of protein) were purchased from BD Biosciences (San Jose, CA). Deuterated 6β-OH-testosterone (6β-hydroxytestosterone-16,17-D3, >99% purity) was purchased from Cerilliant Corporation (Round Rock, TX). All other chemicals were obtained from commercial sources and were of the highest purity available.

Handling of and Treatment of Animals. Studies with rats were performed in accordance with the Institutional Animal Care and Use Committee of Amgen. Male Sprague-Dawley rats (250–300 g) were obtained from Charles River Laboratories (Wilmington, MA).

Microsomal Studies. To evaluate the potential for time-dependent P450 inactivation, incubations containing final concentrations of 2MI (10 μM), HLM, or RLM (1.0 mg/ml) in potassium phosphate buffer (100 mM, pH 7.4) were initiated with NADPH (1 mM). After initiation, aliquots of this primary incubation were transferred at 0, 5, 10, 15, and 30 min to a secondary incubation (at 1:10 dilution) containing a saturating concentration of the CYP3A substrate testosterone (200 μM), NADPH (1 mM), and phosphate buffer and incubated for an additional 20 min. Formation of 6β-OH-testosterone under these conditions was confirmed to be linear with protein concentration and incubation time (data not shown). The secondary reaction was terminated by addition of 1 volume of acetonitrile (ACN) containing deuterated 6β-OH-testosterone as the internal standard. The samples were centrifuged and CYP3A activity was determined by measuring 6β-OH-testosterone formation by an LC-MS/MS method as described previously (Walsky and Obach, 2004). To ascertain the contribution of CYP3A4, the preincubation was also performed in the presence of a competitive CYP3A4 inhibitor, ketocazole (1 μM). For assessment of MBI of other human P450 isoforms, incubation conditions were identical to those described previously and used the following substrates: 200 μM phenacetin (CYP1A2), 20 μM diclofenac (CYP2C9), and 50 μM bufuralol (CYP2D6). The respective reaction products were monitored (acetaminophen, 4′-OH-diclofenac, and 1-OH-bufuralol) by LC-MS/MS assay as described previously (Walsky and Obach, 2004).

Kinetic Studies. The kinetics of CYP3A inactivation were determined using a range of 2MI concentrations (0.1–100 μM). Nonspecific binding of 2MI to microsomal protein was determined to be <10%, and therefore nominal concentrations were used for kinetic calculations. Incubations were conducted as described above, with a 0- to 30-min preincubation followed by a secondary incubation. Data were plotted as the natural log (LN)-linear plot of the percentage of remaining CYP3A activity versus preincubation time, and observed initial inactivation rate constants (k_{obs}) were calculated from the pseudo-first-order kinetics slope. The maximum rate constant for enzyme inactivation (k_{max}) and the inactivation constant (I_{c}) were calculated (GraphPad Prism; GraphPad Software Inc., San Diego, CA) according to the following hyperbolic equation:

\[ k_{obs} = \frac{k_{max} - I_c}{I_c + f_0} \]

where k_{max} is the initial rate constant for CYP3A inactivation and f_0 is the initial inhibitor concentration (Waley, 1985; Silverman, 1995).

Partition Ratio. The partition ratio, the number of moles of product formed per mole of enzyme inactivated, was determined using recombinant CYP3A4 and the titration method (Silverman, 1995). In brief, 2MI (0–10 μM) was preincubated with a fixed amount of recombinant CYP3A4 (200 pmol/reaction) in the presence of NADPH (1 mM) in phosphate buffer for 60 min to ensure complete inactivation. The percentage of remaining CYP3A activity at each substrate concentration was plotted against the molar ratio of 2MI to CYP3A4 enzyme. The intercept between the linear regression line obtained at molar ratios less than 5 and the horizontal line obtained from saturating conditions were extrapolated to the x-axis to yield the turnover number. The partition ratio was calculated by subtracting a value of 1 from the turnover number.

Assessment of Reversibility. The reversibility of time-dependent CYP3A inhibition was assessed by dialysis. In brief, 2MI (20 μM), troleandomycin (TAO) (10 μM), or raloxifene (10 μM) was incubated with HLM (1 mg/ml) in the presence of NADPH (1 mM) in phosphate buffer for 30 min. After the incubation, an aliquot was removed for determination of the predialysis CYP3A activity. An aliquot (0.5 ml) of the remaining reaction mixture was transferred to a Slide-a-Lyzer (Pierce Biotechnology, Rockford, IL) dialysis cassette (10,000 mol. wt. cutoff) and dialyzed against 2 liters of phosphate buffer (0.1 M, pH 7.4) for approximately 22 h at 4°C. The buffer was replaced once during the dialysis at approximately 6 h of dialysis. Pre- and postdialysis CYP3A activity, after a 10-fold dilution, was determined by monitoring 6β-OH-testosterone as described above. CYP3A activities were then normalized to protein content, which was determined using a BCA assay kit (Pierce Biotechnology).

CO Binding and Spectrophotometric Studies. The effect of 2MI on carbon monoxide binding to reduced P450 was determined in HLM and RLM. 2MI (100 μM) was incubated with 1 mg/ml HLM or RLM in phosphate buffer in the presence and absence of NADPH. After 30 min, an aliquot was removed, reduced with sodium dithionite (2–5 mg), and equally divided between two 1-ml (volume), 1-cm path length cuvettes. After recording baseline absorbance, carbon monoxide was bubbled into the sample cuvette for 1 min, and the absorbance was scanned from 400 to 600 nm using a Cary UV spectrophotometer. Incubations containing 1-aminobenzotriazole (ABT) (5 mM) were used as a positive control.

Microsomal Covalent Binding Assay. Covalent binding to microsomal protein was assessed for [35S]2MI (20 μM) and the positive control [1,14C]naphthalene (20 μM). Incubations (0.2 ml) with HLM or RLM (1 mg/ml protein) were conducted in phosphate buffer for 1 h at 37°C and were performed in the absence of NADPH (used as a negative control) or in the presence of NADPH (1 mM) with or without GSH (10 mM). Covalently bound radioactive equivalents to protein were measured with a semiautomated protein washing procedure using a cell harvester (Semi-Automated Cell Harvester System; Brandel, Gaithersburg, MD) according to the method described by Day et al. (2005). After 1 h of incubation, aliquots (200 μl) of the incubations were taken and transferred to a second 96-well plate (2-ml well volume) containing 1 ml of acetone/well. The samples were vortexed (30 s) and washed using the Semi-Automated Cell Harvester System (48-Sample; Brandel). The precipitated protein was trapped onto Whatman glass fiber filter mat, washed with 80% methanol-water (2 ml/well, v/v), and treated further by 10 washes with 80% methanol-water (2 ml/well, v/v). The captured washed proteins on the glass fiber filter mat sections were transferred to another 96-well plate (2-ml
well volume) and treated with KOH (1 ml, 0.1 N) for 1 h in a heated water bath (95°C) with moderate horizontal shaking. The hydrolyzed samples were allowed to cool on ice (15 min), and aliquots (25 μl) were taken for protein concentration determination using the BCA protein assay (Pierce Biotechnology). Finally, aliquots (600 μl) of the hydrolyzed solutions were added to scintillation vials (6 ml) containing Pico-Fluor 40 liquid scintillant (5 ml; PerkinElmer Life and Analytical Sciences, Waltham, MA). Radioactivity was counted on a Tri-Carb 2800TR liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences). Covalent binding to protein is expressed as picomoles equivalents per milligram of protein.

SDS-PAGE. For SDS-PAGE analysis, aliquots (200 μl) from covalent binding incubations similar to those described above were quenched with 100% ACN (2 ml) and microcentrifuged (14,000g for 5 min). The supernatants were removed, and the pellets were washed by microcentrifugation three more times with ACN (2 ml) to remove all unbound radioactivity. The final supernatants were shown not to contain radioactivity when 0.5-ml aliquots were analyzed by scintillation counting as described above (data not shown). SDS-PAGE was performed using the Mini-PROTEAN 3 Cell electrophoresis system, in which approximately 50 μg of protein was loaded onto 10% Tris-HCl polyacrylamide gels (height 7.0 cm, width 8.3 cm, and thickness 1.0 mm) per well, and electrophoresis was conducted for 1 h at 120 V. The gels were stained for protein with Coomassie Blue R-250 for 2 min and destained with distilled water (30 min). The gels were dried using the model 3 gel dryer (1 h; Bio-Rad Laboratories, Hercules, CA) and then exposed to Fuji Film Imaging Plates (Fuji Film, Tokyo, Japan) for 1 month. Captured phosphorimages of radiolabeled proteins were analyzed by scintillation counting as described above (data not shown). SDS-PAGE analysis of protein samples was performed with HLM or RLM (1 mg/ml) and NADPH (1 mM) or 2MI at concentrations of 200 to 1000 ng/ml. After incubation for 15 min at 37°C, samples were centrifuged (3000 × g) for 5 min. The supernatants were removed and evaporated to dryness under a stream of nitrogen gas at room temperature, reconstituted with water-A CN (1:1), and then analyzed on an ABI 4000 QTrap LC-MS/MS system (Applied Biosystems). The LC system consisted of Shimadzu LC-10adVP pumps (Shimadzu Scientific Instruments, Columbia, MD) and a CTC PAL autosampler. Separation of the analytes was achieved using a linear gradient with a mobile phase flow rate of 0.6 ml/min (mobile phase A, aqueous 0.1% formic acid; and mobile phase B, ACN containing 0.1% formic acid) and a Luna C18 column (5 μm, 100 Å; Phenomenex, Torrance, CA) maintained at 50°C. The contents of the SPE cartridge were eluted serially three times, each with 25, 50, and 75% ACN water containing 0.1% formic acid. SPE fractions containing G1 were evaporated to dryness and reconstituted in 1 ml of ACN-water (1:1; v/v). Purification of the GSH adduct was achieved using a semipreparative HPLC system (Alliance 2695; Waters) in line with a diode array detector (996; Waters) and fraction collection (Foxy II; Teledyne-Isoeco, Lincoln, NE). Separation was achieved on a C18 [Luna C18(2), 5 μm, 100 Å; Phenomenex, Torrance, CA] column maintained at 40°C. The mobile phase consisted of 0.1% formic acid in water (solvent A) or methanol (solvent B) with the following linear gradient conditions: 0 to 3 min, 95% A; 3 to 33 min, 95 to 5% A; 33 to 38 min, 5% A; and 40 min, 95%. The flow rate was 4 ml/min. 2MI and pooled fractions containing G1 (retention time of 17.5 min) were combined and evaporated to dryness. The G1 isolate, approximately 600 μg, was reconstituted in 165 μl of deuterated d4-methanol and transferred to a 3-mm tube for NMR analysis.

NMR Analysis. NMR spectra of 2MI and G1 were acquired on a 600-MHz spectrometer equipped with a 5-mm cryoprobe (Bruker, Newark, DE). Complete spectral assignment was performed by analyzing the 1D 1H, 2D 1H/1H proton-proton total correlation spectroscopy, and 2D 1H/13C heteronuclear single quantum coherence and HMBC data sets. In addition, the isolated GSH adduct was analyzed by high-resolution mass spectrometry using an Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA).

Prediction of In Vivo Mechanism-Based Inactivation and the Magnitude of DDIs. The potential for 2MI to cause an increase in exposure (AUCi/AUC) of a coadministered “victim” drug was determined using a steady-state model (1) (Wang et al., 2004):

\[
\frac{AUC_i}{AUC} = \frac{f_{\text{degCPY3A}}}{k_{\text{deg}} + f_{\text{degCPY3A}}} + \frac{1}{k_{\text{deg}} \cdot (K_i + I_u)}
\]

where \(I_u\) is the unbound inhibitor concentration and \(F_G\) and \(F_{G1}\) are the intestinal wall availabilities in the absence and presence, respectively, of the inhibitor, respectively. For human predictions, a value of 0.000321 min⁻¹ was used as the P450 degradation rate constant, \(k_{\text{deg}}\) (Fromm et al., 1996), which has been demonstrated to yield good in vitro-in vivo correlation for known clinical DDIs (Eino1f, 2007). For the rat, a value of 0.000825 min⁻¹ was used for \(k_{\text{deg}}\) (Wattkins et al., 1986). For clinical DDI predictions with midazolam, a value of 0.89 was used for \(f_{\text{degCPY3A}}\) (Chen et al., 2006a) and a value of 0.57 was used for \(F_G\) (Thummel et al., 1996). For the rat, the interaction with the prototypical CYP3A substrate indinavir was predicted using an \(f_{\text{degCPY3A}}\) value of 1 (Chiba et al., 1995) and a value of 0.94 for \(F_G\) (Lin et al., 1999). The steady-state model was also used to predict the decrease in CYP3A2 steady-state concentration (\(E_{u,\text{ss}}/E_{u,\text{inf}}\)) after 2MI administration using eq. 2 (Mayhew et al., 2000):

\[
\frac{E_{u,\text{ss}}}{E_{u,\text{inf}}} = \frac{k_{\text{deg}}}{k_{\text{deg}} + k_{\text{deg CPY3A}}} + \frac{1}{K_i + I_u}
\]

where \(E_{u,\text{ss}}\) and \(E_{u,\text{inf}}\) are the enzyme steady-state concentrations in the presence and absence of the inhibitor, respectively, and \(I_u\) is the unbound inhibitor concentration.

Bioanalysis and Pharmacokinetics of 2MI in the Rat. Pharmacokinetic parameters were determined in the rat after 2MI administration (2 and 20 mg/kg p.o. administered in 1% Tween-1% methylcellulose suspension). Plasma samples were quenched 3:1 with acetonitrile containing internal standard, and after centrifugation the supernatant was transferred to a 96-well plate and analyzed by LC-MS/MS using electrospray ionization with MRM in positive ionization mode on an API 4000 triple quadrupole spectrometer (Applied Biosystems). The LC system consisted of Shimadzu LC-10AD VP pumps (Shimadzu Scientific Instruments, Columbia, MD) and a CTC PAL autosampler. Separation of the analytes was achieved using a linear gradient with a mobile phase flow rate of 0.5 ml/min (mobile phase A, aqueous 0.1% formic acid; and mobile phase B, ACN containing 0.1% formic acid) and a Luna C18 column (5 μm, dimensions 30 × 3 mm; Phenomenex). The initial mobile phase composition (95% A and 5% B) was held for 0.5 min, increased linearly over 1.5 min to 2% A and 98% B, held for an additional 1.2 min, and finally reequilibrated at the initial conditions for 1.2 min. The mass transition monitored was m/z 535 → 326. Pharmacokinetic parameters were calculated using standard noncompartmental methods (Watson LIMS; Thermo Fisher Scientific).

Measurement of Unbound Fraction. The unbound fraction of 2MI in plasma and microsomes was determined by an ultracentrifugation method. In brief, 2MI (200–1000 ng/ml) was added to plasma or hepatic microsomes (1 mg/ml) at 1000 ng/ml. After incubation for 15 min at 37°C, samples were centrifuged for 16 h at 355,000g, and unbound concentrations of 2MI were determined by an LC-MS/MS assay as described above.

In Vivo Mechanism-Based Inactivation of CYP3A and Interaction with Indinavir in Male Sprague-Dawley Rats. In vivo enzyme inactivation was determined by measuring hepatic microsomal CYP3A activity ex vivo in rats...
after oral gavage administration of 2MI (0, 2, or 20 mg/kg q.d. in 1% Tween-1% methylcellulose suspension) for 3 days. ABT (20 mg/kg q.d. for 3 days), a nonselective P450 mechanism-based inactivator, was used as a positive control (Murford et al., 1992; Streleitz et al., 2006). Four hours after the final dose, animals were sacrificed, and hepatic microsomes were prepared using standard methods (Fleischer and Kervina, 1974). The change in enzyme steady-state concentration (E*/Eo) was calculated by dividing the microsomal CYP3A activity from 2MI-treated animals by the corresponding activity measured in the vehicle control group.

In a separate study, the propensity for 2MI to increase the exposure of a CYP3A2 probe substrate in vivo was determined in rats. 2MI was administered orally (2 or 20 mg/kg q.d. in 1% Tween-1% methylcellulose suspension) for 3 days. Indinavir (10 mg/kg) was selected as a prototypical CYP3A substrate (Chiba et al., 1997) and was coadministered orally (10 mg/kg in 0.05 M citric acid) with the final dose of 2MI or vehicle control on the last day of the study. Plasma indinavir concentrations were determined by LC-MS/MS assay using electrospray ionization with MRM in positive ionization mode on an API 4000 triple quadrupole spectrometer. The mass transition monitored was m/z 614 → 421, and LC conditions were identical to those described above for 2MI. Indinavir pharmacokinetic parameters were calculated using standard noncompartmental analysis.

**Results**

**In Vitro Mechanism-Based Inactivation.** The effect of 2MI on the activities of several P450 isoforms evaluated in pooled HLM is summarized in Table 1. 2MI (10 μM, initial preincubation concentration) caused a time- and NADPH-dependent decrease in CYP3A4 activity and followed pseudo-first-order kinetics. In contrast, CYP1A2, CYP2C9, and CYP2D6 activities were not affected by 2MI, and inhibition of CYP3A4 activity was almost completely prevented by ketoconazole. In addition, CYP3A4 inactivation was not appreciably attenuated by the addition of GSH. In separate experiments, 2MI also did not cause competitive inhibition of CYP3A4, CYP2C9, or CYP2D6 with IC50 values >10 μM (data not shown). Figure 2 illustrates the effect of increasing concentrations of 2MI (0.1–100 μM) on the time-dependent decrease in CYP3A4 activity in HLM (Fig. 2A) and RLM (Fig. 2C). Kinetic parameters were determined by nonlinear regression of the k_inact versus 2MI concentration plot shown in Fig. 2, B (HLM) and D (RLM). In HLM, 2MI was a potent time-dependent inactivator with a calculated Ki of 18 μM and k_inact of 0.16 min⁻¹. In RLM, 2MI was less potent (Ki = 32 μM), but its maximal inactivation rate (k_inact = 0.83 min⁻¹) was 5-fold higher than that measured in HLM. With the use of recombinant human CYP3A4, the partition ratio (molar ratio of 2MI metabolized per CYP3A4 inactivated) was estimated to be approximately 5.4 (Fig. 3). The effect of dialysis on the inhibition of CYP3A4 activity in HLM after a 30-min preincubation with 2MI, raloxifene, or TAO is shown in Fig. 4. Dialysis was unable to restore CYP3A4 activity in incubations containing 2MI, and approximately 60% inhibition was observed both before and after dialysis. Raloxifene, which inactivates CYP3A4 through irreversible covalent binding (Chen et al., 2002), elicited CYP3A4 inhibition that was also not recovered after dialysis. In contrast, >85% activity was recovered in incubations containing TAO, which forms a reversible metabolite-intermediate complex with CYP3A4 (Pessayre et al., 1981). In separate experiments, 2MI was not able to decrease carbon monoxide binding to reduced P450 (recombinant CYP3A4) nor was there any evidence for metabolite-intermediate complex formation (i.e., characteristic absorbance at 455 nm) (data not shown).

**Table 1**

<table>
<thead>
<tr>
<th>Isozyme (Treatment)</th>
<th>Substrate</th>
<th>Remaining Activity %</th>
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<tr>
<td>CYP3A4 (-NADPH)</td>
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<tr>
<td>CYP3A4 (+NADPH)</td>
<td>Testosterone</td>
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<td>CYP3A4 (+NADPH, +1 μM ketoconazole)</td>
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<td>CYP3A4 (+NADPH, +5 mM GSH)</td>
<td>Testosterone</td>
<td>52.2</td>
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<tr>
<td>CYP2D6 (+NADPH)</td>
<td>Bufuralol</td>
<td>91.9</td>
</tr>
<tr>
<td>CYP2C9 (+NADPH)</td>
<td>Diclofenac</td>
<td>77.4</td>
</tr>
<tr>
<td>CYP1A2 (+NADPH)</td>
<td>Phenacetin</td>
<td>92.3</td>
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**Fig. 2.** Time- and concentration-dependent inactivation of CYP3A4 activity by 2MI in HLM (A and B) and RLM (C and D). Ki and k_inact were determined from nonlinear regression of the k_inact versus time plot (B and D). In human, Ki = 18 μM, k_inact = 0.16 min⁻¹; in rat, Ki = 32 μM, k_inact = 0.83 min⁻¹. LN, natural logarithm.
of [35S]2MI with RLM and HLM (Fig. 5A). In the presence of NADPH, a band of radioactivity representing a 55- to 60-kDa protein was observed, which is consistent with irreversible binding to one or more P450 isoforms (He et al., 1999). Incubations of [35S]2MI with recombinant CYP3A4 yielded a similar band of radioactivity representing a 55- to 60-kDa protein (Fig. 5B). A weak radioactive band representing binding to low molecular weight proteins (∼21 kDa) was also observed in the presence and absence of NADPH, and no further effort was directed to characterizing this band.

### In Vitro Metabolism of 2MI and GSH Adduct Formation

**In Vitro Metabolism of 2MI and GSH Adduct Formation.** In vitro turnover of 2MI in HLM in the presence of NADPH was low, with >90% parent compound remaining after 30 min. Two hydroxylated metabolites were detected via MRM-information dependent acquisition scan (data not shown). MS/MS fragmentation patterns suggested that the indole aromatic ring and the 3-methoxyphenyl ring were the sites of oxidation. No evidence for dihydrodiol formation was observed. When 2MI was incubated in RLM or HLM in the presence of NADPH and GSH, two GSH adducts (MH+ 840) designated as G1 and G2, were formed, and the collisionally induced product ion spectra are shown in Fig. 6. Incubations with HRP (1 mg/ml) in the presence of H2O2 and GSH also yielded two GSH adducts with fragmentation patterns and retention times identical to those observed in HLM and RLM. Therefore, it was concluded that G1 and G2 were formed in RLM, HLM, and HRP, and because of the near-complete conversion of 2MI to G1 by HRP, this system was used for scale-up and structural characterization.

**Structural Characterization of G1 and G2 Adducts.** G1. A full-scan MS spectrum obtained on the Q-trap instrument revealed a protonated ion at m/z 840, indicating a net addition of 305 Da (+GSH – 2H) to 2MI. MS/MS analysis (Fig. 6A) of the m/z 840 ion gave rise to ions characteristic of GSH adduction: m/z 711, neutral loss of pyroglutamic acid (−129 Da); and m/z 765, neutral loss of glycine (−75 Da) (Baillie and Davis, 1993). High-resolution MS and MSn data were also obtained on an Orbitrap mass spectrometer for G1 and were identical to the nominal-resolution data. High-resolution data for an ion at m/z 177 [observed m/z 177.04780 (C8H7N232S), calculated m/z 177.0481], −1.79 ppm error] provided specific evidence for the site of adduction of the glutathionyl sulfur to a carbon on the indole ring. Additional high-resolution MS/MS fragments and their assignments are detailed in Table 3.

1D proton and 2D 1H/13C HMBC NMR spectra of purified G1 are shown in Fig. 7, A and B, respectively. NMR data confirmed that GSH adduction had occurred at the 3-position (labeled C7 in Fig. 7A) of the indole moiety. The G1 NMR spectrum, compared with that of the parent 2MI, revealed that all protons from the parent molecule, with the exception of H7 at 6.41 ppm, were intact. In addition, GSH resonances from each of its nonexchangeable protons were also observed (Fig. 7A). The G1 rotating-frame Overhauser effect spectroscopy spectrum (data not shown) revealed a weak cross-peak from the methyl (H10 at 2.53 ppm) to one of the methylene protons (Hc1 at 3.29 ppm) on the GSH cysteine (data not shown). The HMBC spectrum from G1 (Fig. 7B) displayed correlations from Hc1 (3.09 and 3.29 ppm) and H10 (2.53 ppm) to C7 (at 98.6 ppm). Relevant 1H and 13C NMR data for 2MI and G1 are listed in Table 4.

G2. A full-scan MS spectrum obtained on the Q-trap instrument also revealed a protonated ion at m/z 840, indicating a net addition of 305 Da (+GSH – 2H) to 2MI. LC-MS/MS analysis (Fig. 6B) gave...
rise to fragments identical to those obtained from the G1 adduct. The presence of the m/z 177 ion, as observed in the G1 high-resolution MS/MS spectrum, indicated the site of GSH adduction was on the indole ring of G2. In addition, the similarity of G1 and G2 MS fragments favors the site of glutathione adduction to be on the aromatic ring instead of the 2-methylindole methyl carbon. However, no further attempt was made to isolate and characterize G2 because of its low abundance in all conditions tested.

Assessment of Drug-Drug Interaction Potential. Effect of 2MI on CYP3A2 steady-state concentration in rats. Figure 8 illustrates the effect of 2MI or ABT on ex vivo microsomal CYP3A2 activity. 2MI (20 mg/kg for 3 days) elicited a 62.4% decrease in CYP3A2 activity, which corresponds to a decrease in enzyme steady-state concentration ($E_{ss}/E_{0}$) of 0.376.

At the lower dose, a minimal decrease in CYP3A activity ($E_{ss}/E_{0}$ = 0.8) was observed. The nonselective P450 inhibitor ABT was used as a positive control and elicited an approximately 80% decrease in CYP3A2 activity ($E_{ss}/E_{0}$ = 0.18).

Predictions using the steady-state model and comparison with observed values. Table 5 summarizes the predicted changes in enzyme activity ($E_{ss}/E_{0}$) and the exposure (AUCi/AUC) of a prototypical CYP3A2 substrate, indinavir, using a steady-state model and the measured kinetic parameters ($K_{i}$ and $k_{inact}$) for 2MI in the rat. The experimentally observed values for $E_{ss}/E_{0}$ (determined from ex vivo microsomal CYP3A2 activity) and AUCi/AUC (determined by measuring indinavir AUC in the absence and presence of 2MI) are also summarized in Table 5. The in vitro-in vivo correlation was compared using three different values for the inhibitor concentration ($I_{u}$) for the prediction: the maximal systemic concentration ($C_{max}$), the 24-h average concentration ($C_{ave}$), and the concentration at the time of liver harvesting (4 h postdose, $C_{terminal}$). These values were determined after a single 2MI dose of 2 or 20 mg/kg and were corrected for the measured unbound fraction in plasma ($f_{u}$) in rats (0.005). Because of the high gut availability of indinavir ($F_{G}$ = 0.94) (Lin et al., 1999), the effect of intestinal CYP3A inhibition mediated by 2MI ($F_{G}$) was assumed to be minimal (i.e., $F_{G}/F_{C}$ = 1) when the change in AUC was predicted. At the higher dose group, a 65% decrease in CYP3A2 activity ($E_{ss}/E_{0}$ = 0.35) and a 2.65-fold increase in the AUC of indinavir was predicted when the unbound $C_{max}$ (60.8 nM) was used as the value for $I_{u}$. These predicted values (using unbound $C_{max}$)
correlated well with the experimentally observed in vivo values of 0.376 for \( E_{\text{ss}}/H_{11032} \) and 3.62 for AUCi/AUC in the rat. In contrast, both unbound \( C_{\text{terminal}} \) (1.77 nM) and \( C_{\text{ave}} \) (2.71 nM) underpredicted the change in CYP3A2 level (\( E_{\text{ss}}/H_{11005}/H_{11022} = 0.92 \)) and increase in indinavir exposure (AUCi/AUC = 1.0). At the lower dose group (2 mg/kg), unbound \( C_{\text{max}}, C_{\text{ave}}, \) and \( C_{\text{terminal}} \) values were less than 2 nM, and a minimal change in enzyme steady-state concentration or indinavir exposure was predicted, in agreement with the observed in vivo values.

**Predicted Human DDI.** The potential for 2MI to cause a DDI with the CYP3A4 substrate midazolam in humans was estimated using the in vitro kinetic parameters for \( K_I \) and \( k_{\text{inact}} \) and the steady-state model (eq. 2). Compared with indinavir, midazolam has lower gut availability (\( F_G = 0.57 \)) (Thummel et al., 1996), and thus inhibition of intestinal CYP3A4 could have a significant affect on the predicted change in AUC. Therefore, the predicted AUCi/AUC was calculated with and without an intestinal inhibition component. 2MI was predicted to cause a 4.3-fold increase in the AUC of midazolam when inhibition of intestinal CYP3A4 (\( F_G \) and \( F_{G}/H_{11032} \)) was ignored and a 7.5-fold increase
mechanism-based inactivation.

Covalently binds to the CYP3A active site, leading to irreversible adduction to CYP3A4 (He et al., 1999). Taken together, these data indicate that 2MI is bioactivated to a reactive intermediate that covalently binds to the CYP3A active site, leading to irreversible mechanism-based inactivation.

Each bar represents the mean ± S.D. from three determinations.

When complete intestinal CYP3A4 inhibition \((F'_C/F_G = 1/0.57)\) was assumed.

### Discussion

2MI was a selective, potent, and efficient mechanism-based inactivator of CYP3A4. Inhibition of CYP3A4 activity was time- and NADPH-dependent, followed pseudo-first order kinetics, and was irreversible after dialysis. GSH was unable to markedly attenuate CYP3A4 inhibition, and the low partition ratio (5.4) suggested that the reactive species involved in MBI may not appreciably leave the P450 active site. No spectrophotometric evidence for metabolite-intermediate formation or decreases in carbon monoxide binding to reduced P450 were observed, indicating that binding or interaction with the P450 active site did not involve perturbations of the heme moiety. The irreversible enzyme inactivation was accompanied by NADPH-dependent covalent binding of \([35S]2MI\) to microsomal protein, and SDS-PAGE analysis revealed that this binding was associated with a 55- to 60-kDa protein, consistent with irreversible addition to CYP3A4 (He et al., 1999). Taken together, these data indicate that 2MI is bioactivated to a reactive intermediate that covalently binds to the CYP3A active site, leading to irreversible mechanism-based inactivation.

Recognizing that P450-mediated bioactivation and subsequent MBI can lead to potentially adverse DDIs, subsequent efforts were focused on characterizing the reactive intermediate(s) formed during oxidative metabolism of 2MI. Two GSH adducts were detected, with identical MS/MS fragmentation patterns. The major adduct, \(G_1\), was determined unambiguously by NMR to comprise GSH linked, via a thioether bond, on the 3-position of the 2-methylindole ring. \(G_1\) was proposed to be formed via two possible mechanisms: A) through a 2,3-epoxy-2-methylindole intermediate (\(I_1\)) or B) through a 2-methylindole diimine intermediate (\(I_5\)), which is susceptible to Michael addition of GSH. Through pathway A, GSH preferentially attacks the less sterically hindered 3-position of the epoxide (\(I_1\)) and forms \(G_1\) predominantly after loss of a water molecule (Scheme 1, pathway A). Through pathway B, two successive 1-electron abstractions from 2MI and a loss of \(H^+\) yield a conjugated diimine (\(I_5\)), which is also susceptible to GSH addition at the 3-position of the indole moiety (Scheme 1, pathway B). After rearrangement of the epoxide (\(I_1\)), both proposed pathways A and B may proceed through the diimine (\(I_5\)).

A literature precedent for the proposed pathway A has been demonstrated for zomepirac and tolmetin. These 2-alkylpyrrole derivatives are bioactivated via an analogous mechanism involving an epoxide intermediate, and a GSH adduct has been identified with the glutathionyl moiety on the 3-position of the pyrrole ring (Chen et al., 2006b). With respect to pathway B, a mechanism involving two sequential 1-electron abstractions is consistent with the previously reported HRP-mediated bioactivation of zafrilukast (Kassahun et al., 2005). The efficient conversion of 2MI to \(G_1\) by HRP in the present study implies that this mechanism (B), proceeding through the diimine, may predominate for the peroxidase enzyme system. In contrast, for the P450-mediated bioactivation of 2MI, the relative contribution of the proposed pathway A or B is not clear and is further complicated by the potential interconversion of the diimine and epoxide. It is interesting to note that a diimine intermediate has been previously reported for the peroxidative metabolism of benzidine, followed by subsequent GSH and DNA adduct formation (Yamazoe et al., 1988; Lakshmi et al., 1994). In addition, unlike 2MI, bioactivation of 3-methylindole (3MI) proceeds through P450-mediated dehydrogenation to a highly reactive 3-methyleneindolenine intermediate capable of mechanism-based P450 inactivation and protein alkylation (Skiles and Yost, 1996; Skordos et al., 1998; Yan et al.,

**TABLE 4**

Summary of proton and carbon chemical shifts from affected portions for 2MI and GI

The labeling scheme is provided in Fig. 7A. NMR data were acquired in CD$_3$OD; the solvent signal appeared at \(\delta_{H} 3.32\) ppm, \(\delta_{C} 47.9\) ppm. Numbers in parenthesis are J couplings in Hertz.

<table>
<thead>
<tr>
<th>Label</th>
<th>2MI $^{1}H$</th>
<th>2MI $^{13}C$</th>
<th>GI $^{1}H$</th>
<th>GI $^{13}C$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ppm</td>
<td>ppm</td>
<td>ppm</td>
<td>ppm</td>
</tr>
<tr>
<td>1</td>
<td>7.07 (d, 8.7)</td>
<td>110.1</td>
<td>7.13 (d, 8.7)</td>
<td>110.6</td>
</tr>
<tr>
<td>2</td>
<td>6.42 (d, 8.7)</td>
<td>111.2</td>
<td>6.48 (d, 8.7)</td>
<td>112.1</td>
</tr>
<tr>
<td>3</td>
<td>144.7</td>
<td></td>
<td>146.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>133.8</td>
<td></td>
<td>133.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>129.0</td>
<td></td>
<td>127.0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>116.1</td>
<td></td>
<td>117.1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>6.41 (s)</td>
<td>98.8</td>
<td>3.09 (dd, 9.6, 13.5)</td>
<td>3.29 (buried peak)</td>
</tr>
<tr>
<td>8</td>
<td>136.9</td>
<td></td>
<td>4.36 (dd, 6.3)</td>
<td>54.2</td>
</tr>
<tr>
<td>10</td>
<td>2.44 (s)</td>
<td>12.1</td>
<td>2.52 (s)</td>
<td>10.6</td>
</tr>
</tbody>
</table>

$^{J}$ coupling notations: s, singlet; d, doublet; dd, doublet of a doublet; m, multiplet.
Both dehydrogenation and oxidative pathways participate in 3MI bioactivation, but the predominant mechanism is P450-isoform dependent (Yost, 2001). In the present study, formation of an analogous reactive species for 2MI [i.e., a 2-methyl-indolenine, or imine methide (I7)] (Scheme 1, pathway C) was not implied, because there was no evidence for GSH adduction on the 2-methyl carbon of the 2-methylindole ring. In contrast to the bioactivation of 3MI, the lack of evidence for the formation of a 2-methylene-indolenine suggests that dehydrogenation does not play an important role in the bioactivation of 2MI. To the best of our knowledge, the present study is the first report of 2-methylindole bioactivation leading to MBI.

Although more mechanistic studies are required, preliminary evidence suggests that a 2,3-epoxy-2-methylindole and/or a diimine is the potential reactive intermediate responsible for covalent adduction to the CYP3A active site.

### Table 5

Comparison of the predicted change in CYP3A enzyme steady-state concentration (E'/E_{ss}) and indinavir exposure (AUCi/AUC) with that observed in vivo in rats using different parameters for the inhibitor concentration (I_o) of 2MI.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Parameter Used for I_o</th>
<th>Unbound Concentration</th>
<th>Predicted</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nM</td>
<td>E'/E_{ss}</td>
<td>AUCi/AUC</td>
</tr>
<tr>
<td>2 mg/kg</td>
<td>C_{max}</td>
<td>2.32 ± 1.5</td>
<td>0.93 ± 0.04</td>
<td>1.07 ± 0.043</td>
</tr>
<tr>
<td></td>
<td>C_{ave}</td>
<td>0.15 ± 0.06</td>
<td>1.00 ± 0.002</td>
<td>1.00 ± 0.0017</td>
</tr>
<tr>
<td></td>
<td>C_{terminal}</td>
<td>0.24 ± 0.12</td>
<td>0.99 ± 0.004</td>
<td>1.01 ± 0.0034</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>C_{max}</td>
<td>60.8 ± 8.1</td>
<td>0.35 ± 0.029</td>
<td>2.65 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>C_{ave}</td>
<td>2.71 ± 0.36</td>
<td>0.92 ± 0.010</td>
<td>0.376 ± 0.026</td>
</tr>
<tr>
<td></td>
<td>C_{terminal}</td>
<td>1.77 ± 0.39</td>
<td>0.95 ± 0.011</td>
<td>1.08 ± 0.011</td>
</tr>
</tbody>
</table>

### Scheme 1

Proposed mechanism of CYP3A4-mediated bioactivation of 2MI.

Risk assessment for the likelihood of an in vivo DDI has become routinely implemented in the pharmaceutical industry when further advancement of a compound with an identified MBI liability is considered. One commonly implemented strategy uses a steady-state approach (Mayhew et al., 2000; Wang et al., 2004), which requires the following four key input parameters: 1) innate enzyme degradation rate constant (k_{deg}), 2) inhibitor potency (K_i), 3) maximal inactivation rate (k_{inact}), and 4) unbound concentration (I_o) of the inhibitor. This model has been shown retrospectively to be an effective tool for the prediction of in vivo DDI in humans (Galetin et al., 2006; Obach et al., 2007). It has also been successfully implemented preclinically to predict a 3-fold increase in the exposure of indinavir caused by MBI of CYP3A2 in the rat elicited by a novel melanocortin-4 receptor agonist (Tang et al., 2008). Although acceptable correlations have been established between the predicted and observed AUCi/AUC for a victim drug using the steady-state model, in vitro-in vivo correlation for the prediction of the change in enzyme steady-state concentration (E'/E_{ss}) is not routinely evaluated, due, in part, to the complexity of assessing in vivo enzyme levels.

In the present study, the steady-state model was used to predict both AUCi/AUC and E'/E_{ss} in the Sprague-Dawley rat. E'/E_{ss} was...
determined by comparing ex vivo CYP3A2 activity in hepatic microsomes prepared from rats pretreated with 2MI with that in rats receiving vehicle control. An excellent correlation was observed (within 10%) for the change in F<sub>1</sub>/E<sub>max</sub> when unbound systemic C<sub>max</sub> was used as the input I<sub>u</sub> concentration, which is consistent with reports indicating that the maximal systemic unbound concentration yields good prediction of AUCI/AUC (Obach et al., 2007). The model also accurately predicted a 3-fold increase in indinavir exposure, further emphasizing its relevance to in vivo MBI and DDIs mediated by 2MI.

The excellent in vitro-in vivo correlation increased the confidence for using the steady-state model for risk assessment of the likelihood of a clinically relevant DDI. Subsequently, at a therapeutically relevant 2MI concentration (225 nM), a 4.3- to 7.5-fold increase in the AUC of a midazolam was predicted. Because of the unfavorable risk for the potential of an in vivo DDI, strengthened by the excellent in vitro-in vivo correlation observed in the rat, further progression of 2-methylindole-containing compounds was de-prioritized, prompting investigation of alternative chemical series as dual CRTH2/DP antagonists. In summary, the 2-methylindole-containing compound 2MI was identified as a potent mechanism-based inactivator of CYP3A that binds covalently to the CYP3A apoprotein. A GSH adduct linked to the 3-position of the 2-methylindole moiety was fully characterized via MS and NMR. The putative reactive intermediate(s) were inferred to be a 2,3-epoxide and/or a conjugated diimine. The steady-state model was shown to accurately predict the interaction between 2MI and indinavir in vivo in the rat, and the molecule was predicted to cause a DDI at clinically relevant doses.

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References


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