Substituted Imidazole of 5-Fluoro-2-[4-[(2-phenyl-1H-imidazol-5-yl)methyl]-1-piperazinyl]pyrimidine Inactivates Cytochrome P450 2D6 by Protein Adduction


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Running title: Inactivation of CYP2D6 by SCH 66712

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Abbreviations used: SCH 66712, 5-Fluoro-2-[4-[(2-phenyl-1H-imidazol-5-yl)methyl]-1-piperazinyl]pyrimidine; CYP, cytochrome P450 enzyme; HPLC, high performance liquid chromatography; ESI, electrospray ionization; LC, liquid chromatography; MS, mass spectrometry; m/z, mass to charge ratio; CID, collision induced dissociation; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; EMTPP, (1-[(2-ethyl-4-methyl-1H-imidazol-5-yl)-methyl]-4-[4-(trifluoromethyl)-2-pyridinyl]piperazine; tBPA, 4-tert-butylphenylacetylene; GSH, glutathione; NAC, N-acetyl cysteine.
SCH 66712 (5-fluoro-2-[4-[(2-phenyl-1H-imidazol5-yl)methyl]-1-piperazinyl]pyrimidine) is a potent mechanism-based inactivator of human cytochrome P450 2D6 (CYP2D6) that displays Type I binding spectra with $K_S$ of $0.39 \pm 0.10 \mu M$. The partition ratio is ~3 indicating potent inactivation that addition of exogenous nucleophiles does not prevent. Within 15 minutes of incubation with SCH 66712 and NADPH, ~90% of CYP2D6 activity is lost with only ~20% loss in ability to bind CO and ~25% loss of native heme over the same time. The stoichiometry of binding to the protein was 1.2:1. SDS-PAGE with Western blotting and autoradiography analyses of CYP2D6 following incubations with radiolabeled SCH 66712 further support the presence of a protein adduct. Metabolites of SCH 66712 detected by MS indicate that the phenyl group on the imidazole ring of SCH 66712 is one site of oxidation by CYP2D6 and could lead to methylene quinone formation. Three other metabolites were also observed. For understanding the metabolic pathway that leads to CYP2D6 inactivation, metabolism studies with CYP2C9 and CYP2C19 were carried out since neither of these enzymes is significantly inhibited by SCH 66712. The metabolites formed by CYP2C9 and CYP2C19 are the same as those seen with CYP2D6, though in different abundance. Modeling studies with CYP2D6 revealed potential roles of various active site residues in the oxidation of SCH 66712 and inactivation of CYP2D6 and showed that the phenyl group of SCH 66712 is positioned at 2.2 Å from the heme iron.
Introduction

Cytochrome P450s (CYPs) are a family of heme-containing enzymes ubiquitous in nature. In humans there are fifty-seven CYP enzymes responsible for the metabolism of a variety of substrates including lipids, hormones, vitamins, and exogenous small molecules including pharmaceuticals (Guengerich, 2003). The basic reaction of CYP enzymes is a mono-oxygenation of substrate through activation of molecular oxygen with electrons provided by P450 NADPH reductase, though a variety of products are possible due to various rearrangements (Guengerich, 2003).

A direct consequence of the capacity of CYPs to accommodate a wide range of drug substrates is that they are susceptible to inhibition by many agents (Ortiz de Montellano and Correia, 1983; Correia and Ortiz de Montellano, 2005). A fairly common mechanism of enzyme inhibition related to drug-drug interactions is mechanism-based inhibition (Ortiz de Montellano and Correia, 1983; Correia and Ortiz de Montellano, 2005). In the strict definition of a mechanism-based inhibitor, a substrate (inhibitor) is transformed by the enzyme in the usual way to a reactive intermediate (usually electrophilic) that has a finite half-life (Ortiz de Montellano and Correia, 1983; Guengerich and Shimada, 1991; Correia and Ortiz de Montellano, 2005). The inhibition of the CYP is NADPH-, time-, and concentration-dependent. The reactive intermediate can partition between reaction with the enzyme (at the active site) to inactivate the enzyme or undergo a different transformation to yield a stable (released) product (Ortiz de Montellano and Correia, 1983; Silverman, 1988; Guengerich and Shimada, 1991; Correia and Ortiz de Montellano, 2005). Mechanism-based inactivation of CYPs has been shown to occur by several mechanisms including covalent binding of the inhibitor to the heme or to the protein at specific
amino acids (or both), or covalent linking of modified heme to the protein (Ortiz de Montellano and Correia, 1983; Guengerich and Shimada, 1991; Correia and Ortiz de Montellano, 2005).

CYP2D6 is responsible for the metabolism of approximately 25-30% of commercially available pharmaceutical drugs including several antidepressants (Guengerich, 2003). The only P450 that metabolizes more clinically relevant drugs is CYP3A4, an enzyme that accounts for 30% of the P450 content in liver; CYP2D6 accounts for on average 5% of total P450 in liver (Shimada et al., 1994; Guengerich, 2005). CYP2D6 notably metabolizes drugs containing a basic nitrogen and a planar aromatic ring (Wolff et al., 1985; Islam et al., 1991; Koymans et al., 1992; Rowland et al., 2006). These moieties are common in drugs with narrow therapeutic indexes targeted at central nervous system or cardiovascular disorders (Rowland et al., 2006).

CYP2D6 is an important drug-metabolizing enzyme of wide interest due to its ability to metabolize a large number of drugs and its multiple polymorphic states. Individuals with poor metabolizer phenotype are susceptible to a variety of drug-induced side effects, some of which have severe clinical outcomes. Irreversible inactivation of CYP2D6 by mechanism-based inhibitors can lead to drug-induced loss of CYP activity and thus produce a poor-metabolizer type of response in individuals that are not poor metabolizers. Thus, understanding of the inactivation of CYP2D6 by mechanism-based inhibitors is of clinical interest. Beyond clinical understanding of inactivation of CYP2D6, mechanism-based inhibitors also can be used to understand the basic biochemistry of the CYP2D6 enzyme mechanism and catalysis.

SCH 66712 was the first described mechanism-based inhibitor of CYP2D6 (Palamanda et al., 2001). It was under development as an antagonist of human dopamine receptor D4 but was later abandoned (Palamanda et al., 2001). While previously SCH 66712 was shown to be a mechanism-based inhibitor of CYP2D6, the mechanism for inactivation was not determined
(Palamanda et al., 2001). Therefore, the current study was initiated in order to investigate the mechanism of this inhibition.
Materials and Methods

Chemicals. SCH 66712 was obtained from Schering-Plough Research Institute and was reconstituted in water for use in assays described below. Radiolabeled SCH 66712 (\(^{3}\)H and \(^{14}\)C) were synthesized by Schering-Plough Research Institute (Kenilworth, NJ). \(^{14}\)C-SCH 66712 had a specific activity of 55 mCi/mmol and a purity of ≥ 99% as determined by HPLC. \(^{3}\)H-SCH 66712 had a specific activity of 25.5 Ci/mmol. Ultra-pure solvents (water, ACN, and methanol) for MS were purchased from EMD Chemicals, Inc. (Gibbstown, NJ). All other solvents were HPLC grade and purchased from Sigma-Aldrich (St. Louis, MO). Glutathione was purchased from Cayman Chemicals (Ann Arbor, MI). Potassium phosphate, N-acetylcysteine, NADPH, L-\(\alpha\)-dilauroyl-phosphatidylcholine phospholipids, ACN, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP\(^+\), bufuralol, 1’-hydroxybufuralol, catalase, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Enzymes. Human CYP2D6 with P450 reductase (Supersomes™) were purchased from BD-Gentest (Woburn, MA) and used for all experiments except spectral analysis and binding titrations and hemochrome assays. For spectral analysis and binding titrations and hemochrome assays described below, recombinant human CYP2D6 and recombinant P450 NADPH-reductase were purified from \(E.\) \(coli\) as previously described and were a generous gift from Dr. F. P. Guengerich (Vanderbilt University, Nashville, TN) (Gillam et al., 1995).

Spectral Binding Titrations. Spectral binding titration studies were carried out with recombinant purified CYP2D6 (1 µM) in 100 mM potassium phosphate buffer, pH 7.4. The enzyme was evenly divided between two cuvettes and the experiments were performed at room temperature by titrating in aliquots of SCH66712 (0.05 – 16 µM) into the sample cuvette with the solvent control added to the reference cuvette. A baseline of the reference cuvette was
recorded (350-500 nm) on a Cary-300 dual-beam spectrophotometer (Varian, Walnut Creek, CA). Ligand was subsequently added and the spectra were recorded (350-500 nm) after each addition. The solvent was added to the reference cuvette. The difference in absorbance between the wavelength maximum and minimum was plotted versus the concentration of SCH 66712 and the data were analyzed by nonlinear regression methods with KaleidaGraph software (Synergy Software, Reading, PA). The dissociation constant, $K_s$, was determined using the following quadratic velocity equation or tight-binding equation: 

$$[CYP2D6\cdot SCH\ 66712] = 0.5(K_s + E_t + S) - [0.25(K_s + E_t + S)^2 - E_t S_t]^{1/2}$$

where $S$ represents substrate concentration, $E$ is the total enzyme concentration, and $K_s$ is the spectral dissociation constant for the reaction $CYP2D6 + SCH\ 66712 \rightleftharpoons CYP2D6\cdot SCH\ 66712$.

**Enzyme Assays and Inactivation.** A primary reaction mixture (inhibition assay) containing 16 $\mu$M SCH 66712 and 20 pmol CYP2D6 Supersomes in 100 mM potassium phosphate buffer, pH 7.4, (final volume 200 $\mu$L) was pre-incubated in a 37°C shaking water bath. After three minutes, reactions were initiated by the addition of NADPH (1 mM final). Aliquots of the primary reaction (10 $\mu$L) were removed at 0, 2, 5, 10, 15, 30, and 60 minutes and added to a secondary reaction (activity assay) containing 1 mM NADPH and 100 $\mu$M bufuralol in 100 mM potassium phosphate buffer, pH 7.4, in a final volume of 200 $\mu$L. Each secondary reaction was incubated for ten minutes at 37 °C in a shaking water bath before being quenched with 15 $\mu$L of 70% perchloric acid. Reaction mixtures were centrifuged (2000 x g, 5 min) to remove the precipitated enzyme and the recovered supernatants were directly injected onto HPLC for analysis. The formation of 1’-hydroxybufuralol product was quantified by HPLC with a Waters Symmetry 300, C$_{18}$, 5 $\mu$m column (4.6 x 250 mm) on a 515 HPLC pump system with a 474 fluorescence detector controlled with Empower software (Waters, Milford, MA). The detector
was set at an excitation wavelength of 252 nm and an emission wavelength of 302 nm. The mobile phase consisted of 30% acetonitrile and 70% 1 mM perchloric acid in water. All analyses were carried out at ambient temperature with a flow rate of 1 mL/min (Hanna et al., 2001a; Hanna et al., 2001b).

**Hemochrome Assays.** Native heme in CYP2D6 was determined using the methods of Omura and Sato (Omura and Sato, 1964). Primary reactions containing 1 µM recombinant purified CYP2D6, 2 µM recombinant purified P450 NADPH-reductase, 30 µM freshly sonicated L-α-dilauroyl-phosphatidylcholine phospholipids, 100 mM potassium phosphate buffer (pH 7.4), 400 units/mL catalase (8 µg/mL), 80 units/mL superoxide dismutase (0.18 µM), and 16 µM SCH 66712 were incubated for 0–40 minutes with NADPH-generating system (5 mM glucose 6-phosphate, 0.5 mM NADP⁺, and 0.5 units/mL glucose-6-phosphate dehydrogenase). Controls lacked either SCH 66712 or NADPH-generating system. Incubations were quenched by addition of solubilizing buffer [100 mM potassium phosphate (pH 7.4) containing 1.0 mM EDTA, 20% glycerol (v/v), 0.50% sodium cholate (w/v), and 0.40% Emulgen 913 (w/v)] and placed on ice. To quenched samples was added 22% pyridine (v/v), and 0.01 N NaOH (final concentrations). The samples were immediately mixed by inversion and divided between two cuvettes. A baseline spectrum was recorded from 520 to 620 nm. Following the addition of 1-2 mg of NaS₂O₄ to the sample cuvette, the spectrum was measured between 520 and 620 nm. The change in absorbance at 557 nm relative to that at 575 nm was used to determine the heme content using 32.4 mM⁻¹cm⁻¹ as the molar absorptivity.

**Native Heme Analysis by HPLC.** Reaction mixtures containing 16 µM SCH 66712 and 50 pmol CYP2D6 Supersomes in 100 mM potassium phosphate buffer, pH 7.4, (final volume 200 µL) were pre-incubated in a 37°C shaking water bath. After three minutes, the reactions
were initiated by the addition of NADPH (1 mM final); an equivalent volume of water was added to the control. After 0, 2, 5, 10, 15, and 40 min, the reactions were quenched by the addition of 10 µL of acetonitrile and samples were placed on ice. For heme adduct analysis, incubation mixtures were injected onto a PROTO 300, C4, 5 µm, 2.1 x 250 mm column connected to a Waters Alliance e2695 HPLC system and the mobile phase was a gradient elution with initial conditions of 70% A (0.1% TFA in H2O): 30% B (0.05% TFA in ACN) that was ramped linearly to 20% A over 30 min, and then returned to the initial conditions. Heme was monitored using a Waters model 2487 dual wavelength UV/Vis detector at 405 nm. Heme eluted at approx 22.5 min.

**Partition Ratio.** Primary reaction mixtures containing 0-15 µM SCH 66712, 20 pmol CYP2D6 Supersomes in 100 mM potassium phosphate buffer, pH 7.4, (final volume of 100 µL) were pre-incubated in a 37 °C shaking water bath. After five minutes the primary reactions were initiated with the addition of NADPH (1 mM, final) and incubated at 37 °C. To allow the inactivation to go to completion (Silverman, 1988), inactivation essays were incubated for 60 minutes. Then, aliquots of 10 µL were removed and added to secondary reaction mixtures containing 100 µM bufuralol and 1 mM NADPH in 100 mM potassium phosphate buffer, pH 7.4, in a final volume of 200 µL. The secondary mixtures (in triplicate) were incubated for 10 min at 37 °C and quenched with 15 µL 70% perchloric acid. Reaction mixtures were centrifuged (2000 x g, 5 min) to remove the precipitated enzyme and aliquots of the recovered supernatants were directly injected onto HPLC for analysis as described above.

**Spectral Analysis.** P450 reduced CO difference spectra were measured using the methods of Omura and Sato (Omura and Sato, 1964). Primary reactions containing 1 µM recombinant purified CYP2D6, 2 µM recombinant purified P450 NADPH-reductase, 30 µM
freshly sonicated L-α-dilauroyl-phosphatidylcholine phospholipids, 100 mM potassium phosphate buffer, pH 7.4, 400 units/mL catalase (8 µg/mL), 80 units/mL superoxide dismutase (0.18 µM), and 16 µM SCH 66712 were incubated for 0–25 minutes with NADPH-generating system. Incubations were terminated by the addition of with ice-cold quenching buffer [100 mM potassium phosphate buffer (pH 7.4) containing 1.0 mM EDTA, 20% glycerol (v/v), 0.50% sodium cholate (w/v), and 0.40% Emulgen 913 (w/v)]. Incubations without SCH 66712 or without NADPH generating system were carried out as controls. Quenched samples were divided into two cuvettes and a baseline spectrum from 400 to 500 nm was recorded using a Cary-300 dual-beam spectrophotometer (Varian, Walnut Creek, CA). To the sample cuvette only, CO was bubbled for 1 min and then ~1-2 mg of Na₂S₂O₄ was added to both the reference and sample cuvettes. The spectrum between 400 and 500 nm was recorded. The change in absorbance at 450 nm relative to that at 475 nm was used to determine the P450 content using 91 mM⁻¹cm⁻¹ as the molar absorptivity (Omura and Sato, 1964).

**LC-ESI-MS Analysis of CYP2D6.** Reactions containing CYP2D6 Supersomes (50 pmols) and SCH 66712 (100 µM) in 100 mM potassium phosphate buffer (pH 7.4) were preincubated for 3 min at 30 °C in a shaking water bath. Reactions were initiated by the addition of NADPH (1 mM); control incubations received an equal volume of water (final reaction volume 200 µL). Incubations were continued at 30 °C for 10, 20, and 40 minutes. Reactions were terminated by placing samples on ice. The same experiments were also done at 37 °C. An aliquot of each reaction (20 µL, 5 pmol) was directly injected on to a reversed-phase PROTO 300 C₄ column, 5 µm, 2.1 x 250 mm and chromatographic separation was carried out using an Alliance Waters 2690 (Waters, Milford, MA) HPLC system. The solvent system consisted of A (0.1 % formic acid in water) and B (0.1% formic acid in ACN). A flow rate of 0.2 mL/min was
used. After an initial 20 min hold at 90% A, a linear gradient of 90% A to 5% A over 35 min was applied for resolving of protein components followed by a 10 min hold at 5% A. The column effluent starting at 20 min was directed into an LXQ mass analyzer (Thermo Fischer Scientific, Waltham, MA) operated in the positive ion mode and using the Xcalibur software package. The system had been optimized with horse heart myoglobin. The ESI conditions were sheath gas, 20 arbitrary units; auxiliary gas, 9 arbitrary units; spray voltage, 5 kV; capillary temperature, 275 °C; capillary voltage, 48 V; and tube lens offset, 120 V. The molecular masses of CYP2D6 were determined by deconvolution of the apoprotein charge envelopes using ProMass software (Novatia, LLC, Mammouth Junction, NJ).

Covalent Binding of SCH 66712 to CYP2D6. For SDS-PAGE analysis, CYP2D6 Supersomes (100 pmol/mL), control and inactivated samples, in the presence of 14C-labeled or 3H-labeled SCH66712 were resolved on a 10% polyacrylamide gel and then transferred to a nitrocellulose membrane. Blots were blocked and then treated with anti-human CYP2D6 antibodies. CYP2D6 was detected by chemiluminescence using a secondary antibody with horse-radish peroxidase activity. Following Western blot analysis, the nitrocellulose membrane was subjected to autoradiography.

Stoichiometry of Binding. Stoichiometry of binding was determined using the method of Chan et al. (Chan et al., 1993). Briefly, reactions containing CYP2D6 Supersomes (0.1 nmol, 0.2 µM final) and 14C-labeled-SCH 66712 (16 µM) in 100 mM potassium phosphate buffer (pH 7.4) were preincubated for 3 min at 37 °C in a shaking water bath. Reactions were initiated by the addition of NADPH (1 mM) or the addition of an equal volume of water for the control (final reaction volume 500 µL). Incubations were continued at 37 °C for 60 minutes. A 5-fold volume (2.5 mL) of 5% sulfuric acid in methanol was added to quench the reactions and the mixtures...
were vortexed for 10 s. The protein pellet obtained after centrifugation at 1800 xg for 10 min was washed a total of 4 times or until the count was less than 300 cpm/0.5 mL for the supernatant. The final protein pellet was dissolved in 0.6 mL of 1 N NaOH, and 0.1 mL of the resulting sample was analyzed using a Beckman LS 6000 scintillation counter.

**Metabolism of SCH 66712.** A mixture of CYP2D6 Supersomes (500 pmol), 100 mM potassium phosphate buffer (pH 7.4), SCH 66712 (100 μM), freshly prepared glutathione (10 mM) or freshly prepared N-acetyl cysteine (10 mM) was preincubated for 3 min at 37 °C before addition of 150 μL of the NADPH generating system. The total reaction volume was 1 mL. After 40 min of incubation in a water bath, all samples were placed on ice and quenched with 60 μL of 1% TFA. All samples were then applied to a 1 mL C 18 solid phase extraction column (Agilent Technologies, Santa Clara, CA) preconditioned by washing with 1 mL of methanol followed by 1 mL of water. After the sample was loaded, the column was washed with 1 mL of water then 2 mL of methanol, and 300 μL of acetonitrile. All fractions were collected. The methanol and acetonitrile fractions were combined and dried under a stream of nitrogen to a final volume of approximately 50 μL. The sample was then resuspended in 200 μL solvent A that was composed of 90% water, 10% methanol, and 0.05% TFA for LC-MS analysis. A 20 μL aliquot of the sample was injected onto a Kinetex C 18 (2.6 μm, 2.1 x 100 mm) column (Phenomenex, Torrance, CA) for chromatographic separation using an Alliance 2690 HPLC system (Waters, Milford, MA) with a solvent system composed of solvent mixtures A (90% water, 10% methanol, and 0.05% TFA) and B (90% acetonitrile, 10% methanol and 0.05% TFA). After 5 min at the initial conditions (5% B), separation of metabolites was achieved by a linear gradient of 5% B to 50% B over 20 minutes then holding at 50% B for 1 min before returning to the initial conditions. The flow rate was 0.1 mL/min. The column was allowed to re-
equilibrate for 15 min at initial conditions (95% A/5% B) prior to the next injection. Mass spectrometry was performed using a Thermo LXQ (Thermo Scientific, West Palm Beach, FL) mass spectrometer with an ESI source in a positive ion mode. The ESI conditions were sheath gas set at 27 arbitrary units, the auxiliary gas was set at 5 arbitrary units, the spray voltage was set at 5 kV, and the capillary temperature was set at 250 °C.

**Molecular Modeling and Docking Simulations.** AutoDock 4.0 was employed to perform docking simulations and molecular models (http://autodock.scripps.edu; Morris et al., 1998; Huey et al., 2007). The protein structures used in these studies were CYP2D6 (PDB ID: 2F9Q) and CYP2C9 (PDB ID: 1R9O). Ligands and solvent molecules were removed, but the heme was retained. The Fe atom of the heme was assigned a charge of +3. The PDB of 2D6 was modified in Swiss-Deep View at position 374 to the reference amino acid (M374V). Residues within 5 Å of the heme iron were identified and set as flexible residues for computations. For each protein structure, charges were calculated by the Gasteiger-Marsili method. The 3D structures of the ligands for docking studies were built in Spartan 4.0 (Wavefunction, Inc., Irvine, CA) with all hydrogen atoms added and energy minimization. The grid maps were calculated using AutoGrid. The dimensions of the grid box were set to 40 x 40 x 40 Å and the grid spacing was set to 0.375 Å. Docking was performed using the Lamarckian genetic algorithm. Each docking experiment was performed 100 times, yielding 100 docked conformations. The consensus binding postures of the molecules were obtained by visual inspection and docking scores.
Results

**Inactivation of CYP2D6 by SCH 66712.** Binding analysis with SCH 66712 (Figure 1) and CYP2D6 showed Type I binding spectra characteristic of substrate binding. The $K_s$ was determined to be 0.39 ± 0.10 µM (Figure 2). Treatment of CYP2D6 with SCH 66712 in the presence of NADPH led to a ~90% loss of enzyme activity as evidenced by reduced bufuralol hydroxylation within less than 15 minutes (Figure 3). The same results were found when dextromethorphan was used as a substrate (data not shown). At the same time, the loss of ability to bind CO in reduced-CO difference assays was ~25% and the loss of native heme in hemochrome assays was ~15% (Figure 3, and Supplemental Figure 1). Loss of native heme and CO-binding ability correlated fairly well, though the extent of the loss of CYP activity was much higher (Figure 3). Furthermore, the control hemochrome assays that lacked SCH 66712 or NADPH showed nearly the same degree of loss of hemochrome formation over time as the reaction containing both SCH 66712 and NADPH (Supplemental Figure 2). Inactivation reactions with 2 mM potassium cyanide as an imminum scavenger showed the same rate of inactivation as reactions without cyanide (Supplemental Figure 3). In concordance with this finding, previous studies have shown that other exogenous nucleophiles including glutathione and reactive oxygen species scavengers including superoxide dismutase and mannitol had no affect on the inactivation (Palamanda et al., 2001).

**Analysis of Heme.** Hemochrome and spectral assays (Figure 3) suggested that heme adduction was not a significant contributor to CYP2D6 inactivation. However, due to the nature of hemochrome colorimetric assays, it is possible that a heme adduct could be formed but still detected as native heme in the hemochrome assay above. To further examine possible heme adduction, HPLC analysis of heme using UV/Vis detection was carried out. The results show an
~25% decrease in native heme content upon incubation with SCH 66712 and NADPH compared to the control reaction that did not contain NADPH (Figure 4). This is consistent with the loss of CO-binding ability (Figure 3) and a slightly greater loss of native heme than found in hemochrome assays (Figure 4). Furthermore, MS analysis of the heme showed only $m/z$ 616 with no peaks at potential adducted masses (data not shown). Taken together, these findings suggest that heme adduct formation is unlikely.

**Determination of the Partition Ratio.** The number of molecules of SCH 66712 metabolized per molecule of inactivated CYP2D6, i.e. the partition ratio, was determined by incubation of CYP2D6 with various concentrations of SCH 66712 over 60 minutes to allow the inactivation to progress until essentially complete. The percentage of the activity remaining was plotted as a function of the molar ratio of SCH 66712 to CYP2D6. The turnover number (partition ratio +1) was estimated from the intercept of the linear regression line obtained from the lower ratios of SCH 66712 to CYP2D6 with the straight line derived from the higher ratios of SCH 66712 to 2D6 as described previously (Silverman, 1988). With this method, the turnover number was 4, and consequently the partition ratio was 3 (Figure 5).

**Covalent Binding of SCH 66712 to CYP2D6.** SDS-PAGE with Western blotting and autoradiography analyses of CYP2D6 from Supersomes following incubation with radiolabeled SCH 66712 suggested the presence of a protein adduct (Figure 6). Both $^3$H- and $^{14}$C-labeled SCH 66712 bound to CYP2D6 in the presence of NADPH, but not in the absence of NADPH (Figure 6).

Attempts to identify adducted CYP2D6 by ESI-LC-MS were inconclusive due to lowered ionization of CYP2D6 upon inactivation. CYP2D6 (from Supersomes) in the presence of SCH 66712 but absence of NADPH produced reasonable MS that allowed for deconvolution of the
parent protein with mass of 55781 Da (Supplemental Figure 4). However, upon incubation with NADPH, ionization was greatly reduced and no protein adduct was identified (Supplemental Figure 4). The normalized level in the MS of CYP2D6 following inactivation by SCH 66712 was decreased by over an order of magnitude (Supplemental Figure 4), consistent with protein adduction and the loss of enzyme ionization. Varying temperature (37 or 30 °C) and duration of incubation (10, 20, or 40 min) had no affect on improving ionization. We confirmed our experimental method by the use of CYP2B4 inactivation by 4-tert-butylphenylacetylene (tBPA) as a positive control since tBPA is an inactivator known to form an adduct with CYP2B4 apoprotein. ESI-LC-MS analysis and deconvolution of CYP2B4 inactivation showed a characteristic increase in protein mass by 174 amu, consistent with protein adduct formation and previous findings (Zhang et al., 2009; Supplemental Figure 5).

**Stoichiometry of Binding.** The stoichiometry of binding of SCH 66712 to CYP2D6 was determined using scintillation counting and $^{14}$C-radiolabeled SCH 66712. Inactivation reactions were treated with 5% sulfuric acid in methanol and protein precipitated as described in the Materials and Methods and as previously described (Chan et al, 1993). While there was some non-NADPH-dependent binding apparent in samples, the difference between samples that received NADPH and those that did not was ~1.2 nmol SCH 66712 bound/nmol CP2D6 (Table I). Therefore, NADPH-dependent binding indicated a roughly 1.2:1 stoichiometry between SCH 66712 and CYP2D6 (Table I). Since heme was not present in the protein precipitate following acid washes, this stoichiometry is indicative of protein adduction.

**Metabolism of SCH 66712.** Four SCH 66712 metabolites were observed with molecular ion at $m/z$ 355 in samples incubated with CYP2D6 Supersomes and NADPH (Figure 7). The elution times for these products were 17, 19, 24, and 25 min. The metabolites were present in the
same abundance when GSH or NAC were included in the reactions, though no GSH or NAC conjugates were observed (both GSH and NAC in reduced forms were observed; data not shown). The \(m/z\) 355 products were not observed in control (no NADPH) reactions. The masses of these products are all consistent with mono-oxygenation of SCH 66712. CID fragmentation (MS\(^2\)) of the mono-oxygenated product at 17 min gave primarily two peaks, \(m/z\) 173 and \(m/z\) 183 (splitting of the molecule at the methylene group; Figure 7 and Supplemental Figure 6). This is consistent with addition of oxygen to the side of the molecule containing the phenyl and imidazole rings. Further CID fragmentation (MS\(^3\)) of \(m/z\) 173 gave \(m/z\) 119 and \(m/z\) 146, and CID of \(m/z\) 146 (MS\(^4\)) gave \(m/z\) 119. The \(m/z\) 146 fragment is consistent with loss of an HCN fragment from the imidazole ring while the \(m/z\) 119 fragment is consistent with loss of C\(_3\)H\(_5\)N from the imidazole ring and methyl substituent (Figure 7 and Supplemental Figure 6). Both results suggest mono-oxygenation of the phenyl ring; this is consistent with predictions from molecular modeling experiments (vide infra). Due to low abundance of \(m/z\) 183, we were unable to observe fragmentation of that peak though \(m/z\) 183 is consistent with unmodified SCH 66712 (Figure 8). The presence of di-oxygenated, dehydrogenated, ring-opened, or N-dealkylated SCH 66712 metabolites were not observed or supported by MS\(^n\) data.

The \(m/z\) 355 peaks eluting at 19, 24, and 25 minutes (Figure 7D and E) likely represent mono-oxygenation at different sites of the SCH 66712 molecule. The major fragments observed were at \(m/z\) 157 and \(m/z\) 199 (Figure 7D) consistent with mono-oxygenation on the side of the molecule with the piperazine and fluorinated-heteroaromatic rings and might include hydroxylation, N-oxide formation, or piperazine-ring oxidation consistent with what has been reported in the literature for other piperazine containing compounds (Miraglia et al., 2010; Kamel et al., 2010). CID analysis (MS\(^3\)) did not confirm specific site modifications, but suggest
the modification is on the piperazine ring (Supplemental Figure 6). Due to low abundance of fragments, further fragmentation experiments were not possible.

In comparison, fragmentation of the parent SCH 66712 ([M+H]+ with m/z 339) yielded primarily two peaks, m/z 157 (16 amu less than the m/z 173 observed in the metabolite peak at 17 min) and m/z 183 (16 amu less than the m/z 199 observed in the metabolite peak at 19, 24, and 25 minutes) (Figure 8). Further fragmentation (MS3) of m/z 157 gave m/z 103 and m/z 130 – both 16 amu less than that of the SCH 66712 metabolite.

When CYP2C9 and CYP2C19 were used in metabolism studies with SCH 66712, the same metabolites with molecular ion of m/z 355 were observed (Figure 7A, inset; data for CYP2C19 are not shown but were identical to CYP2C9). The fragmentation patterns noted for each metabolite were also the same as observed with CYP2D6 (Figure 7). The metabolites were also present when GSH or NAC were included in the reactions, though no GSH or NAC conjugates were observed. However, the distribution of each of the metabolites is different with much less formation of the peak at 17 min with CYP2C9 and CYP2C19. The peak at 17 min represents monooxygenation of SCH 66712 most likely on the phenyl ring. The other three metabolites observed – all of which represent mono-oxygenation on SCH 66712 at the piperazine or fluorinated heteroaromatic ring, are present in similar abundance in reactions with CYP2C9 and CYP2C19 as was observed with CYP2D6 (Figure 7A, inset). This result of oxygenation by CYP2C9 and CYP2C19 on SCH 66712 at the piperazine or fluorinated heterocyclic aromatic rings is also consistent with molecular docking experiments (vide infra). No metabolites of SCH 66712 were observed from incubations with CYP3A4.

**Molecular Modeling.** A series of molecular modeling studies were performed to better understand the metabolism of SCH 66712 that would lead to inactivation of CYP2D6. Initial
Docking experiments showed that the phenyl group of SCH 66712 is positioned closest to the heme iron of CYP2D6 (Figure 9A). This is consistent with MS metabolite studies that indicated mono-oxygenation on the phenyl end of SCH 66712 by CYP2D6 produces the most abundant metabolite (Figure 7A, inset). The phenyl group is ~2.1 Å from the heme iron, within reasonable distance for metabolism.

Previous studies have identified the active site residues of CYP2D6 associated with ligand binding and orientation as Asp301, Glu216, Phe483, and Phe120 (McLaughlin et al., 2005; Rowland et al., 2006; Ito et al., 2008; Marechal et al., 2008). In the docking model, Phe120 showed π–π stacking with the fluorinated heterocyclic aromatic ring as well as edge-to-edge interaction with the phenyl group of SCH 66712 (distances of 3.2 Å and 2.7 Å, respectively). Glu216 is within hydrogen bonding distance to a nitrogen in the fluorinated heterocyclic ring of SCH 66712 (~3.5 Å). The closest nucleophile identified as a potential target for inactivation by an electrophilic SCH 66712 metabolic intermediate was Thr309, an amino acid positioned at the juncture of the phenyl and imidazole rings of SCH 66712 and ~2.6 Å from the phenyl ring (Figure 9B).

In contrast, when SCH 66712 was modeled with CYP2C9 (structure by Wester et al., 2004) the binding orientation was seen most commonly in opposite orientation - namely the fluorinated heterocyclic aromatic ring was pointing toward the heme with a distance of ~5 Å to the heme iron from the ring center (Supplemental Figure 7). However, a horizontal binding orientation was also frequently observed in simulations with CYP2C9 – an orientation not observed with CYP2D6 (Supplemental Figure 7). The horizontal binding orientation may be more consistent with observed SCH 66712 metabolites that result from mono-oxygenation of either end of SCH 66712 (Figure 7). In the metabolism studies both CYP2C9 and CYP2C19...
produced metabolites of SCH 66712 that are more commonly mono-oxygenated on the piperazine or fluorinated heterocyclic aromatic ring end of the molecule (Figure 8) consistent with the molecular simulation models.

Discussion

SCH 66712 was the first reported mechanism-based inhibitor of CYP2D6 (Palamanda et al., 2001). Inactivation of CYP2D6 is potent with ~90% loss of enzyme activity within ~15 minutes and a low partition ratio of ~3 (Figures 3 and 5). Within the same incubation period, the loss of native heme and CO-binding ability are not reduced as effectively (Figure 3 and 4). These results support the role of SCH 66712 as an inactivator of CYP2D6 by adduct formation with the apoprotein rather than with the heme.

LC-ESI-MS of microsomal proteins (Supersomes) yielded a deconvoluted mass of CYP2D6 of 55781 Da, consistent with reported values (Supplemental Figure 4). However, when CYP2D6 was inactivated by SCH 66712 in the presence of NADPH, we were unable to determine a definitive mass increase for the apoprotein due to loss of ionizable CYP or aggregation after adduction or both. The inability to detect the adducted mass of CYP2D6 following inactivation is not uncommon and there are other reports in the literature that have observed similar results with CYP2B6, CYP3A4, and others (Bateman et al., 2004; Kent et al., 2006). The use of radiolabeled SCH 66712 in the inactivation assays allowed for the detection of radiolabeled CYP2D6 using SDS-PAGE with audioradiography and Western blotting supporting adduct formation (Figure 6). Using radiochemical analysis and scintillation counting, the stoichiometry of binding was determined to be ~1.2 further supporting protein adduction (Table I).
Metabolism of SCH 66712 by CYP2D6 produced mono-oxygenated metabolites (m/z 355) (Figure 7). No di-oxygenated, dehydrogenated, ring-opened, or N-dealkylated metabolites were observed from reactions with CYP2D6, CYP2C9 or CYP2C19. The m/z 355 metabolite eluting at 17 min is present in more abundant quantities in reactions with CYP2D6 than in those with CYP2C9 or CYP2C19, while the other three metabolites are roughly present in the same amounts (Figure 7A, inset). Furthermore, the metabolite at 17 min is the only one that represents modification of SCH 66712 at the end of the molecule with the phenyl and imidazole groups (Figure 7 B and C). Thus, the metabolite eluted at 17 min could be the one that leads to the inactivation of CYP2D6. CID analysis of this metabolite compared to the parent SCH 66712 supports mono-oxygenation of the phenyl ring, most certainly a hydroxylation on the aromatic ring. If the hydroxylation were to occur in the para position relative to the imidazole ring, a methylene quinone derived electrophile capable of inactivating the protein via the adduction on the imidazole or the phenyl ring could arise (Figure 10). Quinones are known to be involved in enzyme inactivation as reported for CYP3A4 by raolxifene (Chen et al. 2002).

Given the very low partition ratio of the inactivation and that previous studies indicated no protection from inactivation of CYP2D6 by SCH 66712 upon addition of exogenous nucleophiles (Palamanda et al., 2001), it is perhaps not surprising that we were unable in the current study to trap any reactive intermediates of SCH 66712 by use of GSH or NAC. GSH and NAC both have soft nucleophile cysteine residues. Since there was no detection of adduction of these nucleophiles in the incubations of SCH 66712 with CYP2D6 and NADPH, a different type of nucleophile (a hard nucleophile) might be the target for inactivation by SCH 66712 or these nucleophiles may simply not have access to the inactivator.
Molecular docking experiments were used to predict the modes of SCH 66712 binding to CYP2D6 and CYP2C9 and to rationalize experimental observations of inhibition in one case (with CYP2D6) versus little inhibition in the other (with CYP2C9), and the more abundant formation of a metabolite representing hydroxylation of the phenyl ring (with CYP2D6). SCH 66712 is positioned in the active site of CYP2D6 such that the phenyl group is 2.2 Å from the heme iron. Conversely, with CYP2C9 the fluorinated heterocyclic aromatic ring of SCH 66712 is closest to the heme iron as well as a low energy binding conformation that is more horizontal (Supplemental Figure 7). The active site of CYP2D6 contains Thr309, a possible nucleophilic target of a reactive electrophile (Figure 9). Use of a Thr309 mutant of CYP2D6 would allow for further investigation of this potential site of inactivation. At this time, the identity of amino acid targets for the any of the mechanism-based inhibitors of CYP2D6 are unknown.

Interactions between aromatic moieties, i.e. π-π interactions, play a crucial role in binding and substrate conformation in enzyme active sites and lead to increased inhibitory potential of mechanism-based inhibitors (Sridhar et al., 2010). Specifically, modeling research with polyaromatic hydrocarbon inhibitors of CYPs has shown that face-to-face and edge-to-edge orientations are unfavorable and may be preliminary steps leading to mechanism-based inhibition (versus edge-to-face or misaligned stacking orientations that are favorable orientations not as strongly associated with mechanism-based inhibition) (Sridhar et al., 2010). Our modeling simulations of binding show SCH 66712 interacting with Phe120 via π face-to-face and edge-to-edge interactions at distances of 3.2 Å and 2.7 Å, respectively (Figure 9). These distances are well within the range of previously reported binding distances for π-π interactions that can be associated with potent inhibition (Sridhar et al., 2010). Thus, the modeling studies shown here
are consistent with the hypothesis that face-to-face and edge-to-edge interactions could be preliminary steps in mechanism-based inhibition.

Since SCH 66712 was first reported as a mechanism-based inhibitor of CYP2D6, a few other compounds have been reported to be mechanism-based inhibitors including a related compound, EMTPP, that was intentionally studied based on its similarity to SCH 66712 (Hutzler et al., 2004). For EMTPP, the methylene carbon of the ethyl substituent on the imidazole group is the site of oxidation (structure of EMTPP in Supplemental Figure 8; Hutzler et al., 2004). For SCH 66712, there is a phenyl group at this location that is the site of oxidation. The $K_i$ values for inhibition of CYP2D6 (from Supersomes) by SCH 66712 and by EMTPP are 0.55 µM and 5.5 µM, respectively, consistent with much more potent inhibition of CYP2D6 by SCH 66712 (Palamanda et al., 2001; Hutzler et al., 2004). Likewise, the $k_i$ for inhibition by SCH 66712 is 0.32 min$^{-1}$ while it is lower for inhibition by EMTPP at 0.09 min$^{-1}$. The partition ratios further differentiate these compounds with a value of ~3 for SCH 66712 and of ~99 for EMTPP. The difference in partition ratio values might also explain the fact that few metabolites of SCH 66712 could be found upon inactivation while reactions with EMTPP produced at least four abundant metabolites in addition to the adducted CYP2D6 (Hutzler et al., 2004).

Molecular modeling studies shown here with CYP2D6 and EMTPP show that EMTPP, like SCH 66712, binds with the fluorinated ring away from the heme iron with a distance of 2.9 Å from the site of oxidation to the heme iron (Supplemental Figure 8). The distance between the heme iron atom and the proposed site of oxidation in SCH 66712 is 2.1 Å. For other known P450 mechanism based inhibitors, modeling studies show the moiety of the inhibitor that is activated for protein adduction pointing toward the heme iron. This includes inhibitors such as tBPA with CYPs 2B1 and 2B4 (Zhang et al., 2009; Lin et al., 2010), mifepristone and raloxifene with
CYP3A4 (Zhang et al., 2009; Moore et al., 2010), as well as inhibitors that inactivate CYPs by heme alkylation such as gemfibrozil glucuronide with CYP2C8 (Baer et al., 2009) and others (Sridhar et al., 2010).

In conclusion, the inactivation of CYP2D6 by SCH 66712 is very potent with production of few metabolites. SCH 66712 causes inactivation likely by apoprotein adduction that we postulate would be at Thr309 based on modeling studies. Finally, the current study supports the notion that some substituted imidazole groups may tend to give rise to inactivation of CYP2D6 and should be examined for mechanism-based inactivation early in the drug discovery process.
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Supporting Information Available. This material is available at xxxx.
Authorship Contributions

Participated in research design: Nagy, Mocny, Diffenderfer, Hsi, Palamanda, Nomeir, Furge

Conducted experiments: Nagy, Mocny, Diffenderfer, Hsi, Butler, Arthur, Fletke, Palamanda, Furge

Contributed new reagents: Palamanda, Nomeir

Performed data analysis: Furge, Nagy, Mocny, Diffenderfer, Hsi, Arthur

Wrote or contributed to the writing of the manuscript: Furge, Nagy, Diffenderfer, Nomeir

Other: Furge acquired funding for the research
References


Footnotes:

a.
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b.
This work was presented in part at the American Society for Biochemistry and Molecular Biology annual meeting, 2010, Anaheim, California.

c.
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d.
none.
Figure Legends

**Figure 1.** Structure of SCH 66712. SCH 66712 contains functional groups typically associated with substrates for CYP2D6 including basic nitrogens and planar aromatic rings. The asterisk (*) indicates the location of the $^{14}$C-radiolabel.

**Figure 2.** Spectral binding titration of SCH 66712. (A) Purified CYP2D6 (1 µM) was divided into each of two cuvettes and a baseline was set. Aliquots of SCH 66712 in H$_2$O were added to the sample cuvette and equal volumes of H$_2$O were added to the reference cuvette. (B) Plot of $\Delta A_{430-395}$ (from panel A) vs. concentration of SCH 66712. $K_s$ was determined to be 0.39 ± 0.10 µM.

**Figure 3.** Effect of SCH 66712 on CYP2D6 catalytic activity, reduced CO-difference spectra, and native heme content. CYP2D6 was treated with SCH 66712 (16 µM) in the presence of NADPH. Residual hydroxybufuralol activity (●) was determined at the times indicated as described in the Materials and Methods. Reduced CO difference spectra (○) were collected at 0, 10, and 40 minutes for inactivation of CYP2D6 by SCH 66712. Residual native heme (■) was determined by a hemochrome assay at 0, 10, 20, and 40 minutes for the inactivation of CYP2D6 by SCH 66712. The percent of control indicates relative catalytic activity, CO-binding, or native heme remaining as compared to time zero.

**Figure 4.** Analysis of native heme. CYP2D6 was treated with SCH 66712 (16 µM) in the presence of absence of NADPH for 0, 2, 5, 10, 15, and 40 min (only data for the 40 min
incubation are shown). Heme content was analyzed by HPLC using absorbance at 405 nm. Native heme eluted at 22.5 min. After 40 min incubation with SCH 66712 and NADPH (right panel) there was a 25% decrease in native heme compared to time zero (left panel).

**Figure 5.** Loss of CYP2D6 activity as a function of the ratio of SCH 66712 to CYP2D6. CYP2D6 was incubated with varying concentrations of SCH 66712 for 60 minutes to allow for complete inactivation. The partition ratio was estimated to be ~3.

**Figure 6.** Covalent Binding of SCH 66712 to CYP2D6. Labeled SCH 66712 was incubated with Supersomes (100 pmol/mL) for 10 min at 37 °C in the presence or absence of 1 mM NADPH. Samples were chilled on ice following the incubation. For SDS-PAGE, 20 µL aliquots were removed, mixed with 20 µL of gel loading buffer, and boiled for 3 min prior to loading on the 10% acrylamide gel. For analysis, 2 pmol of 2D6 protein were loaded on the gel. Protein was transferred gel to nitrocellulose paper for Western blotting. (A.) Western blot with anti-CYP 2D6 antibodies. Chemiluminiscence was used for detection. (B). Audioradiography of gel in panel A.

**Figure 7.** Analysis of SCH 66712 metabolites formed by CYP2D6 and CYP 2C9 in the presence of NADPH. CYP2D6 or CYP2C9 was incubated with SCH 66712 and NADPH and the resulting reaction mixtures were prepared for MS as described in the Materials and Methods. (A) Mass spectrum of the molecular ion eluting at 17 min and extracted ion chromatogram of the molecular ion [M+H]+ with m/z of 355 (inset). (B) MS/MS of the mono-oxygenated SCH 66712 metabolite eluting at 17 min with m/z 355. (C) The MS³ of the m/z 173 ion from panel B. (D) MS/MS of the mono-oxygenated SCH 66712 metabolite eluting at 19 min with m/z 355. (E) The
MS³ of the m/z 199 ion from panel D. (F) MS² fragmentation patterns giving rise to observed mass spectra in Panels B and D. Further analysis of fragmentation patterns given in Supplemental Figure 6. The data shown are representative of multiple analyses and results with CYP2C9 were identical to CYP2C19 (data not shown).

Figure 8. Mass spectral analysis of SCH 66712. (A) Mass spectrum of the molecular ion eluting at 21 min and extracted ion chromatogram of the molecular [M+H]+ ion with m/z of 339 (inset). (B) MS/MS of SCH 66712. (C) The MS³ of the m/z 157 ion from panel B. (D) MS² fragmentation pattern of SCH 66712.

Figure 9. Molecular modeling of SCH 66712 bound to CYP2D6. AutoDock was used to model binding of SCH 66712 to the active site of CYP2D6 as described in the Materials and Methods. (A) Docking simulation of SCH 66712 with CYP2D6 is shown. The phenyl moiety of SCH 66712 faces the heme iron. Helix I is also shown. (B) Active site amino acids Phe120, Phe483, Asp301, Glu216, and Thr309 are shown. The distance from Thr309 oxygen to the phenyl ring of SCH 66712 is 2.6 Å. The heme is shown in red.

Figure 10. Proposed metabolism and inactivation pathway for SCH 66712 by CYP2D6. p-Hydroxylation of the phenyl ring can lead to the formation of a methylene quinone. Collapse of the quinone with the capture of a nucleophile on the phenyl or the imidazole ring may lead to protein-adduct formation.
Table I. Covalent Binding of SCH 66712 to CYP2D6

<table>
<thead>
<tr>
<th></th>
<th>NADPH dependent</th>
<th>- NADPH</th>
<th>+ NADPH</th>
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</thead>
<tbody>
<tr>
<td>Precipitation assay (n=3)</td>
<td>1.20</td>
<td>1.95</td>
<td>3.15</td>
</tr>
</tbody>
</table>
Figure 4

The graph shows two peaks labeled 'control' and 'with NADPH'. The x-axis represents time in minutes (22 to 23) and the y-axis represents relative absorbance.
Figure 6

A.  
NADPH:  
-   +   -   + 

Western blot

B.  
NADPH:  
-   +   -   + 

Autoradiogram
Figure 7

A

CYP2D6

CYP2C9

RT = 17 min

RT = 19 min

B

D

MS²

MS³

C

E

F

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 8

A

B

C

D

\( m/z \)

Relative Abundance

Relative Abundance

Relative Abundance

Relative Abundance

157

183

103

130

157

157

183

F
Figure 10

Potential quinone formation:

m/z 355

Quinone

or

X-protein adduct

where X = S or N