Mechanism-based Inactivation of Human Cytochrome P450 3A4 by Two Piperazine-containing Compounds

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Abbreviations used: SCH 66712, 5-fluoro-2-[4-[(2-phenyl-1H-imidazol-5-yl)methyl]-1-piperazinyl]pyrimidine; EMTPP, (1-[(2-ethyl-4-methyl-1H-imidazol-5-yl)-methyl]-4-[4-(trifluoromethyl)-2-pyridinyl]piperazine; 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; TFA, trifluoroacetic acid; GSH, glutathione; NAC, N-acetyl cysteine.
Abstract

Human cytochrome P450 3A4 (CYP3A4) is responsible for the metabolism of more than half of pharmaceutical drugs and inactivation of CYP3A4 can lead to adverse drug-drug interactions. The substituted imidazole compounds, 5-fluoro-2-[(2-phenyl-1H-imidazol-5-yl)methyl]-1-piperazinyl]pyrimidine (SCH 66712) and 1-[(2-ethyl-4-methyl-1H-imidazol-5-yl)methyl]-4-[(trifluoromethyl)-2-pyridinyl]piperazine (EMTPP), have been previously identified as mechanism based inactivators (MBI) of CYP2D6. The present study shows that both SCH 66712 and EMTPP are also MBIs of CYP3A4. Inhibition of CYP3A4 by SCH 66712 and EMTPP was determined to be concentration-, time- and NADPH-dependent. In addition, inactivation of CYP3A4 by SCH 66712 was shown to be unaffected by the presence of electrophile scavengers. SCH 66712 displays type I binding to CYP3A4 with a spectral binding constant \((K_s)\) of \(42.9 \pm 2.9 \mu M\). The partition ratios for SCH 66712 and EMTPP were 11 and 94, respectively. Whole protein mass spectrum analysis revealed 1:1 binding stoichiometry of SCH 66712 and EMTPP to CYP3A4 and mass increase consistent with adduction by the inactivators without addition of oxygen. Heme adduction was not apparent. Multiple mono-oxygenation products with each inactivator were observed; no other products were apparent. These are the first MBIs to be shown to be potent inactivators of both CYP2D6 and CYP3A4.
Introduction

Cytochrome P450 enzymes (CYPs) are a family of heme-containing proteins involved in the metabolism of small endogenous and exogenous compounds in humans (Guengerich, 2003). One major class of substrates for CYPs is pharmaceutical drugs. Among drug metabolizing CYPs, CYP3A4 participates in the metabolism of up to 50% of marketed drugs and has high protein expression in liver (~30% of total CYP content) and small intestine (Guengerich, 2003). CYP2D6, another less abundant drug metabolizing CYP, metabolizes ~15% of pharmaceuticals and is expressed at much lower levels (~2-5% of total CYP content; Guengerich, 2003). Together, CYP3A4 and CYP2D6 metabolize nearly three quarters of pharmaceutical drugs. Substrates for CYP2D6 tend to contain basic nitrogens and aromatic rings and include many pharmaceuticals with narrow therapeutic indices such as anti-hypertensives and psychoactive drugs (Guengerich, 2005). CYP3A4 has a broader substrate specificity and metabolizes drugs from many diverse groups including anti-microbials, androgens, anti-cancer agents, anti-HIV agents, and plant alkaloids (e.g. St. John’s Wort; Guengerich, 2005). Given their important roles in drug metabolism, inhibition or inactivation of CYP3A4 or CYP2D6 can lead to adverse drug events, particularly in cases of polypharmacy.

Structurally, both CYP3A4 and CYP2D6 show the characteristic P450-fold with helixes A – J, some beta sheets, and the heme iron hexa-coordinated to the porphyrin ring nitrogens, the proximal cysteine sulfur, and water on the distal side (in the absence of substrate). One striking difference, however, is the binding cavity size. CYP3A4 has approximately more than double the binding cavity size of CYP2D6 (~1560 Å³ vs ~540
Å³, respectively; Ekroos and Sjogren, 2006; Rowland et al., 2006). The difference in binding cavity has been offered as an explanation for CYP3A4 greater substrate promiscuity as well as observed cooperativity (Ekroos and Sjogren, 2006).

One disadvantage of broad substrate specificity is susceptibility of CYPs to inhibition by covalent inactivation by compounds being metabolized (Correia and Ortiz de Montellano, 2005). This type of inhibition is known as mechanism-based inactivation (MBI) and the loss of enzyme activity can be by covalent adduction of the protein, the heme, or by cross reaction of heme and protein (Hollenberg et al., 2008). Since inactivation is not reversible, MBIs are an important class of inhibitors to consider when studying drug-drug interactions involving CYPs. There are many known MBIs of CYP3A4 including raloxifene, bergamottin, lapatinib, 4-ipomeanol, mifepristone, 17α-ethynylestradiol, erythromycin, and others (He et al., 1998; Chen et al., 2002; Lin et al., 2002; Alvarez-Diez, 2004; Baer et al., 2007; Yukinaga et al., 2007; Teng et al., 2010; reviewed in Zhou et al., 2005). Many of these inactivators form covalent adducts with CYP3A4 at amino acids, e.g. raloxifene and AMG 487 adduct at Cys239 while bergamottin adducts at Gln273 (Baer et al., 2007; Henne et al., 2012; Lin et al., 2012).

In contrast to CYP3A4, for CYP2D6 only a few inactivators are established and of those only two are known protein adductors – SCH 66712 and EMTPPP (Fig. 1) (Hutzler et al., 2004; Nagy et al., 2011; Livezey et al., 2012). Amino acid sites of adduction on CYP2D6 are still unknown. SCH 66712 was discovered as a human dopamine receptor D4 antagonist but, due to inactivation of CYP2D6, it was dropped as a lead compound (Palamanda et al., 2001). Initial studies of SCH 66712 interaction with CYPs suggested that CYP3A4 would also be susceptible to inactivation by SCH 66712.
(Palamanda et al., 2001). Given that EMTPP, which has no known pharmaceutical activity, is structurally similar to SCH 66712 and has previously been shown to inactivate CYP2D6, we considered that it might be an inactivator of CYP3A4 as well. Therefore the current study sought to evaluate possible inactivation of CYP3A4 by these two related CYP2D6 inactivators. To our knowledge, there are no compounds reported to be dual potent inactivators of both CYP3A4 and CYP2D6.
Materials and Methods

Chemicals. SCH 66712 was obtained from Schering-Plough Research Institute (now Merck & Co., Inc.) and reconstituted in water for use in assays described below. Ultra-pure solvents (water, ACN, and methanol) for MS were purchased from EMD Chemicals, Inc. (Gibbstown, NJ). EMTPP was purchased from Interchim, Inc. (San Pedro, CA). 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-α-dilauroyl-phosphocholine phospholipids, ACN, testosterone, 6β-hydroxytestosterone, catalase, TiCl₃, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Enzymes. Recombinant human CYP3A4 was used for spectral analysis and binding titrations, and whole protein mass spectrometry after purification from E. coli as previously described (Gillam et al., 1993; Gillam et al., 1995). Recombinant P450 NADPH-reductase and rabbit cytochrome b₅ were purified from E. coli as previously described (Shen et al., 1989; Holmans et al., 1994) and were a generous gift from Dr. F. P. Guengerich, Vanderbilt University, Nashville, TN (used for reconstitution with purified CYP3A4 as described below). For all other experiments, human CYP3A4 co-expressed with P450 reductase and cytochrome b₅ (Supersomes™) were used (BD-Gentest, Woburn, MA).

Spectral Binding Titrations. Spectral binding titrations studies were carried out with recombinant purified CYP3A4 (1 μM) in potassium phosphate buffer (100 mM, pH 7.4). The solution was evenly divided between two cuvettes and the experiments were performed at room temperature using a Cary 300 dual-beam spectrophotometer (Varian,
Inc., Walnut Creek, CA). A baseline correction was recorded (350-500 nm). SCH 66712 (1-300 µM) was then titrated into the sample cuvette and the equivalent volume of water was added to the reference cuvette; the spectra were recorded (350-500 nm) after each addition. The difference between the absorbance maximum and minimum was plotted against SCH 66712 concentration, and the data were analyzed by a nonlinear regression using KaleidaGraph (Synergy Software, Reading, PA). The dissociation constant, $K_s$, was determined using the following quadratic velocity equation (Eqn. 1): 

$$[CYP3A4 \cdot SCH 66712] = 0.5 (K_s + E_t + S_t) - 0.25 (K_s + E_t + S_t)^2 - E_t S_t^{1/2},$$

where $S_t$ represents substrate concentration, $E_t$ is the total enzyme concentration, and $K_s$ is the spectral dissociation constant for the reaction $CYP3A4 + SCH 66712 \leftrightarrow [CYP3A4\cdot SCH 66712]$.

**Time-Dependent Inactivation.** Primary reaction mixtures containing SCH 66712 (16 µM) or EMTPP (15 µM), CYP3A4 Supersomes (20 pmol), and potassium phosphate buffer (100 mM, pH 7.4 in a final volume of 100 µL) were preincubated in a 37 ºC shaking water bath for 3 min and then all reactions except control were initiated with NADPH (1 mM). Aliquots (10 µL) of the primary reaction were transferred after 0 to 30 min incubations to secondary reactions (in triplicate) containing NADPH (1 mM), testosterone (100 µM) and potassium phosphate buffer (100 mM, pH 7.4; final volume 200 µL). Secondary reactions were incubated for 10 min then quenched with dichloromethane (800 µL) and vortexed. Product extraction was as previously reported (Sohl et al., 2009). Briefly, a 400 µL aliquot of 0.3 M NaCl solution was added to the sample, the samples were vortexed, and then centrifuged for 10 min at 3000 xg. The bottom organic layer of the sample was moved to a small glass vial and the upper aqueous layer disposed. The organic layer was evaporated under a nitrogen stream. The
extracted testosterone and 6β-hydroxytestosterone were dissolved in 35 µL methanol. A 10 µL aliquot of sample was directly injected onto a Waters Symmetry C18 column (5 µm, 3.9 x 150 mm) connected to a Waters Alliance e2695 HPLC system with flow rate of 0.8 ml/min. The mobile phase was a gradient elution with initial conditions of 78% A [95% ammonium acetate (10 mM), 5% ACN] and 22% B [1% ammonium acetate (10 mM), 99% ACN]. After 5 min at initial conditions, a linear gradient of 78% A to 64% A over 7 min was initiated followed by a hold for 8 min at 64% A. The gradient finished with a final decrease to 20% A over 8 min. After an additional 8 min at 20% A, the system was returned to the initial conditions. 6β-hydroxytestosterone and testosterone eluted at approximately 6 and 18 min, respectively. The ratio of 6β-hydroxytestosterone to testosterone was quantified by HPLC and converted to percent remaining activity by comparing each sample ratio to the 0 min control. Percent remaining activity was plotted against primary reaction incubation time.

Concentration-Dependent Inactivation. Primary reaction mixtures with a final volume of 100 µL containing varying concentrations of SCH 66712 (0-40 µM) or EMTPP (0-100 µM), CYP3A4 Supersomes (20 pmols), and potassium phosphate buffer (100 mM, pH 7.4) were pre-incubated in a 37 °C shaking water bath for 3 min and then all reactions except control were initiated with NADPH (1 mM). After 20 min, 10 µL aliquots of the primary reactions were transferred to the secondary reactions (in triplicate) and the samples were incubated, extracted, and analyzed as described above.

Trapping Agents. Primary reaction mixtures containing SCH 66712 (16 µM), CYP3A4 Supersomes (20 pmols), and potassium phosphate buffer (100 mM, pH 7.4; 100 µL final volume) were prepared as follows: with or without NADPH (1 mM final), with
NADPH and GSH (10 mM final), with NADPH and NAC (10 mM final), with NADPH and SOD (1.0 unit/µL final), with NADPH and catalase (0.05 µg/µL final), and with NADPH and KCN (2 mM final). Primary reactions were pre-incubated for 3 min and then initiated with NADPH (1 mM), except the no NADPH control reaction that received an equal volume of water. Aliquots (10 µL) of the initiated primary reaction were removed and added to the secondary reactions at 0 and 12 min of incubation (final volume 200 µL) and samples were treated, extracted, and analyzed as described above. The effect of each variable on inhibition was determined by comparing the percent remaining activity after 12 min of the no NADPH control with the NADPH reactions.

**Partition Ratio.** Primary reaction mixtures contained CYP3A4 Supersomes (20 pmols), potassium phosphate buffer (100 mM, pH 7.4), and varying concentrations of SCH 66712 (0-40 µM) or EMTPP (0-80 µM) in a final volume of 100 µL. The primary reaction mixtures were pre-incubated in a shaking water bath at 37 ºC for 3 min and then all primary reactions except the control were initiated with NADPH (1 mM). The control received an equal volume of water. The primary reactions were then incubated for 30 min to ensure inactivation was complete. Aliquots of 10 µL were added to the secondary reactions and the samples were incubated, extracted, and analyzed as described above. The ratio of 6β-hydroxytestosterone to testosterone was converted to percent remaining activity by comparing each sample to the zero concentration control. From a plot of the percent remaining activity versus [inactivator]/[CYP3A4], the partition ratio was calculated by the method of Silverman using the intersection of the linear regression of the steeper slope of the high ratios with the x-axis (Silverman, 1988).
Determination of $K_I$ and $k_{\text{inact}}$. Primary reactions with a final volume of 100 µL containing varying concentrations of SCH 66712 (0-16 µM) or EMTPP (0-40 µM), CYP3A4 Supersomes (20 pmols), and potassium phosphate buffer (100 mM, pH 7.4) were pre-incubated in a 37 °C shaking water bath for 3 min and then all reactions were initiated with NADPH (1 mM). Aliquots (10 µL) of the primary reaction were transferred after 0 to 30 min incubations (0, 1, 2, and 5 min in reactions with SCH 66712 and 0, 10, 20, and 30 min in reactions with EMTPP) to the secondary reactions (in triplicate) and the samples were incubated, extracted, and analyzed as described above. The log of % remaining activity was plotted against incubation time for each concentration. The initial rates of inactivation ($k_{\text{obs}}$) were then plotted against the concentration of inactivator and fit by non-linear regression \[ k_{\text{obs}} = \frac{(k_{\text{inact}}[\text{inactivator}])}{(K_I + [\text{inactivator}])} \] to determine $k_{\text{inact}}$ and $K_I$ using KaleidaGraph (Synergy Software, Reading, PA).

Native Heme Analysis by HPLC. Analysis of heme was as previously described with the following modifications (Nagy et al., 2011). Four reaction mixtures were used: controls containing no SCH 66712 and no NADPH, no SCH 66712 with NADPH (1 mM, final), or SCH 66712 (16 µM) without NADPH and one experimental with SCH 66712 (16 µM) with NADPH (1 mM, final). Each reaction included CYP3A4 Supersomes (20 pmols) in potassium phosphate buffer (100 mM, pH 7.4; final volume 80 µL). Reactions were pre-incubated in a 37 °C shaking water bath for 3 min prior to initiation by the addition of NADPH (1 mM final); an equal volume of water was added to the no NADPH controls. After 0, 3, 5, 10, and 15 min, the reactions were quenched by the addition of 10 µL of acetonitrile and the samples were placed on ice. For heme adduct analysis, incubation mixtures were injected onto a PROTO 300, C4, 5 mm, 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) and 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 ~22.5 min.

**LC-ESI-MS Analysis of CYP3A4.** Purified, recombinant CYP3A4 (100 pmols; 1 µM) was reconstituted with reductase (3 µM), cytochrome b5 (2 µM), and freshly sonicated L-α-dilauroyl-phosphocholine phospholipids (30 µM) at room temperature for 10 min. Then potassium phosphate buffer (pH 7.4, 100 mM) was added along with SCH 66712 (100 µM in water) or EMTPP (75 µM in methanol not exceeding 1% final v/v of solvent). Control reactions without SCH 66712 or EMTPP received water or methanol, respectively. Reactions were incubated for an additional 3 min at 37 °C and then initiated by the addition of NADPH (1 mM) or water in the no NADPH controls. Final reaction volumes were 100 µL. After 15 min (reactions with SCH 66712) or 30 min (reactions with EMTPP) samples were immediately analyzed by LC-ESI-MS. Reaction of CYP3A4 with iodoacetamide was performed as a validation for the assay as previously described (Baer et al., 2007).

For LC-ESI-MS analysis, an aliquot of each reaction (20 µL, 20 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 HPLC system (Waters, Milford, MA). The solvent system consisted of A (0.1 % TFA in water) and B (0.1% TFA in ACN). A flow rate of 0.2 ml/min was used. After an initial 5 min hold at 100% A, a linear gradient of 100% A to 10% A over 35 min was applied for resolution of
protein components followed by a 10 min hold at 10% A and then returned to the initial conditions. The column effluent starting at 10 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 CYP3A4 were determined by deconvolution of the apoprotein charge envelopes using ProMass software (Novatia, LLC, Mamouth Junction, NJ).

Metabolite Analysis. We previously reported the metabolites of SCH 66712 formed by CYPs 2D6, 2C9, and 2C19 (Nagy et al., 2011). In similar metabolism experiments with CYP3A4, mono-oxygenation of SCH 66712 at four different positions was apparent by the presence of four distinct m/z 355 ions in the mass spectral analysis (M+1). To determine if mono-oxygenation was on carbon or nitrogen atoms, titanium trichloride (TiCl₃) was used to selectively reduce any hydroxylamines as previously described (Seto and Guengerich, 1993; Kulanthaivel et al., 2004; Livezey et al., 2014). Since the metabolites formed by CYP3A4 and by CYP2D6 were the same, the metabolites as formed by CYP2D6 were used in the TiCl₃ experiments due to the more uniform distribution of products (as compared to CYP3A4) and therefore ease of comparison of changes upon HCl or TiCl₃ treatment (vide infra).

Briefly, reaction mixtures containing SCH 66712 (100 μM) and CYP2D6 Supersomes (150 pmols) in potassium phosphate buffer (100 mM, pH 7.4; final volume 600 μL) were initiated by the addition of NADPH (1 mM). Reactions were incubated at
37 °C for 30 min and then analyzed. One aliquot (200 µL) of the reaction mixture was treated with 30 µL of a solution of TiCl₃ (~10 wt. % in 20-30 wt. % HCl). For controls, one aliquot (200 µL) was treated with HCl alone (25 wt %) and another aliquot (200 µL) was left untreated. Samples were left at room temperature for 1 hr to allow for TiCl₃ reduction of hydroxylamines to the parent amines. A control sample not treated with TiCl₃ or HCl was placed on ice for 1 h. All samples were then centrifuged and the supernatant was analyzed by LC-ESI-MS as described previously (Nagy et al., 2011; Livezey et al., 2014).

Metabolites of EMTPP formed by CYP3A4 were determined by LC-ESI-MS using the methods described previously with SCH 66712 with modification (Nagy et al., 2011). Briefly, CYP3A4 Supersomes were incubated for 45 min with EMTPP (100 µM) and NADPH (1 mM) in potassium phosphate (pH 7.4, 100 mM) and final volume of 100 µL. Reactions were terminated with 30 µL ACN. Samples were centrifuged and the supernatant (20 µL) injected directly on to a Kinetix C18 (2.5 µm, 2.1 x100 mm) column (Phenomenex, Torrance, CA) for chromatographic separation and MS analysis using conditions previously described (Nagy et al., 2011). TiCl₃ treatment of EMTPP metabolites as formed by CYP3A4 was carried out as described above with SCH 66712 metabolites.

**Site of Metabolism Predictions.** The software programs SMARTCyp (Rydberg et al., 2010; Rydberg and Olsen, 2012) and RS-Predictor (Zaretzki et al., 2012) were used for prediction of sites of metabolism on SCH 66712 by CYP3A4 and by other CYPs.

**Molecular Modeling and Docking Simulations.** AutoDock Vina was employed for docking simulations and molecular modeling (http://autodock.scripps.edu; Morris et
al., 1998; Huey et al., 2007). The protein structure used in these studies was CYP3A4 complexed with ritonavir (PDB ID: 3NXU) (Sevrioukova and Poulos, 2010). Ritonavir and solvent molecules were removed, but the heme was retained. A water molecule was placed 1.7 Å from the heme iron using COOT (Emsley and Cowtan, 2004) to simulate the electrostatics of Compound I in the mechanism of P450 catalysis (Shahrokh et al., 2012). 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 dimensions of the grid box were set to 18 x 18 x 18 Å and the grid spacing was set to 1.0 Å. The consensus binding postures of the molecules were obtained by visual inspection and docking scores.
Results

**Inactivators.** SCH 66712 and EMTPP are structurally similar molecules both containing piperazine rings and substituted-imidazole rings as well as heteroaromatic rings with fluorine substituents in the *para* position. While SCH 66712 is a human dopamine receptor antagonist, no pharmaceutical activity has been noted for EMTPP (Fig. 1).

**Spectral Binding with SCH 66712.** SCH 66712 displayed Type I substrate binding upon titration with CYP3A4 (Fig. 2A). Fit of the binding data with the quadratic equation yielded a $K_s$ of $42.9 \pm 2.9 \mu\text{M}$ (Fig. 2B).

**Time- and Concentration-Dependent Inactivation of CYP3A4 by SCH 66712 and EMTPP.** Treatment of CYP3A4 in time- and dose-dependent assays with either SCH 66712 or EMTPP resulted in the loss of CYP3A4 ability to 6β-hydroxylate testosterone (Fig. 3). Increasing the concentrations of SCH 66712 resulted in greater loss of CYP3A4 activity (Fig. 3A). Time-dependent assays with SCH 66712 resulted in a rapid loss of formation of 6β-hydroxytestosterone with nearly complete inactivation (~90%) within the first 5 min (Fig. 3B). Addition of exogenous nucleophiles including glutathione, NAC, cyanide, and reactive oxygen species scavengers such as superoxide dismutase and catalase did not protect CYP3A4 from inactivation by SCH 66712 (Table 1).

Treatment of CYP3A4 with the structurally similar compound EMTPP also lead to a concentration- and time-dependent inactivation, though the inactivation was weaker with ~80% loss of activity after ~20 min (Fig. 3C and 3D). Addition of NAC did not protect CYP3A4 from inactivation by EMTPP; reactions with NADPH had 18% of the
activity of control reactions after 12 min while reactions with NADPH and NAC had 19% of the control activity. Also, an NAC conjugate of EMTPP (m/z 515) was observed in low abundance by MS in reactions with CYP3A4 and NADPH as reported previously in reactions with CYP2D6 (data not shown and Hutzler et al., 2004).

**Determination of $K_1$ and $k_{\text{inact}}$ for SCH 66712 and EMTPP.** Given that both SCH 66712 and EMTPP act as inactivators, Kitz-Wilson kinetic analysis for the determination of $K_1$ and $k_{\text{inact}}$ was performed. Time course data (Fig. 4A and 4C) were used to estimate initial rate constants ($k_{\text{obs}}$) for CYP3A4 inactivation. Non-linear regression analysis of $k_{\text{obs}}$ and concentration of inactivators were used to determine $K_1$ and $k_{\text{inact}}$ (Fig. 4B and 4D). Values for $K_1$ and $k_{\text{inact}}$ with SCH 66712 were $1.6 \pm 0.7$ μM and $0.211 \pm 0.024$ min$^{-1}$, respectively, while $K_1$ and $k_{\text{inact}}$ with EMTPP were $11.8 \pm 2.6$ μM and $0.044 \pm 0.004$ min$^{-1}$, respectively. Efficiency of inactivation by SCH 66712 was 0.013 μM$^{-1}$min$^{-1}$ and was lower at 0.0037 μM$^{-1}$min$^{-1}$ with EMTPP. Kinetic constants are shown in Table II.

**Partition Ratio for SCH 66712 and EMTPP.** The number of molecules of SCH 66712 or EMTPP metabolized per molecule of 3A4 inactivated, i.e. the partition ratio, was determined by incubation of CYP3A4 with various concentrations of SCH 66712 or EMTPP over 30 min 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 inactivator to CYP3A4. The turnover number (partition ratio +1) was estimated from the intercept of the linear regression line obtained from the lower ratios of inactivator to CYP3A4 as described previously (Silverman, 1988). With this method, the turnover
number for SCH 66712 was 12, and the partition ratio was 11 (Fig. 5). The less potent inactivator EMTP showed a partition ratio of 94.

Analysis of Heme. Heme adduction was examined by HPLC analysis of heme at 405 nm. Incubation of CYP3A4 with SCH 66712 in the presence of NADPH generated only <10% loss of native heme as compared to controls with no SCH 66712 or no NADPH (Supplemental Fig. 1). Furthermore, MS analysis of the heme showed only \( m/z \) 616 with no peaks at potential adducted masses (data not shown).

Covalent Binding of Inactivators to CYP3A4. Given the lack of heme adducts, CYP3A4 apoprotein was analyzed for the presence of protein adducts. CYP3A4 was treated with SCH 66712 or EMTPP and analyzed by LC/MS as described in the Methods. Chromatograms showed clear separation of cytochrome \( b_5 \), clipped reductase, reductase, CYP3A4, and lipids (Fig. 6A,D,G). Deconvoluted masses of clipped reductase, reductase, and lipids were as expected at 70,363 Da, 77,727 Da, and 622/1243 Da, respectively (data not shown).

In the absence of both NADPH and SCH 66712, the CYP3A4 mass spectrum deconvoluted to 56,952 Da (Fig. 6B,C). Addition of NADPH increased the noise and lowered the signal abundance by an order of magnitude in the mass spectrum (Fig. 6E), but showed a consistent CYP3A4 deconvoluted mass of 56,958 Da (Fig. 6F). The presence of SCH 66712 and NADPH also resulted in more noise and lower signal in the mass spectrum (Fig. 6H). Deconvolution of the SCH 66712 treated sample, however, resulted in the appearance of an adducted protein mass at 57,284 Da as well as the non-adducted protein peak with mass of 56,957 Da (Fig. 6I). The difference between the parent CYP3A4 peak and adduced peak was 327 Da, approximately the mass of one SCH
66712 molecule (338 Da). The experiment was repeated three times with the difference in adducted mass peak varying between 327 to 348 Da with an average difference of 338 Da; these values are consistent with adduction by one SCH 66712 molecule. These differences are within the mass accuracy of the LXQ instrument combined with the limits of the ProMass deconvolution software and are similar to differences seen by other groups (Regal et al., 2000; Jushchyshyn et al., 2003; Bateman et al., 2004; Hutzler et al., 2004; Lin et al., 2005; Lin et al., 2009; Zhang et al., 2011; Lin et al., 2012). EMTPP also adducted CYP3A4 with mass consistent with mono-adduction by EMTPP (353 Da; data not shown). These results also support a 1:1 binding stoichiometry between CYP3A4 and SCH 66712 or EMTPP.

As a validation assay of the mass spectrum and deconvolution data, CYP3A4 was treated with the cysteine alkylating agent iodoacetamide that can form zero, one, or two alkyl adducts on solvent accessible cysteine residues in CYP3A4 (Baer et al., 2007). Treatment of CYP3A4 with iodoacetamide was consistent with the formation of two adducts as indicated by the difference of 117 Da in deconvoluted mass of CYP3A4 in the absence (56,953 Da) and presence (57,070 Da) of iodoacetamide (Supplemental Fig. 2).

**Metabolism of SCH 66712.** Previous studies in our group identified four mono-oxygenated metabolites of SCH 66712 in the presence of NADPH with molecular ion at m/z 355. The four products were formed in varying proportions by CYP2D6, CYP2C9, CYP2C19, and CYP3A4 (Nagy et al., 2011; Supplemental Fig. 3A, 4A, and unpublished data). No other metabolites were detected (Nagy et al., 2011 and unpublished data). CID fragmentation showed that one of the four products, peak 1, was mono-oxygenated on the phenyl ring end of the molecule (Supplemental Fig. 3B and Nagy et al., 2011). The other
three products were shown through CID to be oxygenated on the piperazine or the heteroaromatic ring (Supplemental Fig. 3 and Nagy et al., 2011).

Since both carbon and nitrogen oxygenation are possible products of metabolism, in the present study we treated the metabolites formed with TiCl₃ to further identify the sites of metabolism. TiCl₃ reverses N-hydroxylations, but not C-hydroxylations (Seto and Guengerich, 1993; Kulanthaivel et al., 2004). Treatment of the four metabolites with TiCl₃ resulted in specific loss of only one product peak (peak 3) in the mass spectrometry indicating that one site of metabolism is a nitrogen on the heteroaromatic ring end of the molecule (Supplemental Fig. 4). Product peak 2 was lost in control reactions treated with HCl alone and must be acid liable (other groups have seen similar issues upon acid treatment, e.g. Kulanthaivel et al., 2004). The mass spectra of product peaks 1 and 4 were unaffected by the addition of HCl or TiCl₃ and are presumed therefore to be carbon hydroxylations.

MSⁿ data and TiCl₃ data were combined with SMARTCyp and RS Predictor predictions of sites of metabolism on SCH 66712 (Supplemental Table 1). The prediction software was more accurate with CYP2D6 sites of metabolism than for CYP3A4 though both software programs performed similarly.

**Metabolism of EMTPP.** Incubation of CYP3A4 with EMTPP and NADPH resulted in the formation of eight mono-oxygenated product peaks with molecular ion m/z at 370 (Supplemental Fig. 5). Four of the metabolites, product peaks 4, 5, 7, and 8, represent mono-oxygenation on the imidazole end of the molecule while the other four product peaks, 1, 2, 3, and 6, represent mono-oxygenation on the piperazine/heteroaromatic end of the molecule (Supplemental Fig. 5). MS³ combined
with TiCl₃ analysis confirmed mono-oxygenation at some sites including on the ethyl substituent of the imidazole ring, mono-oxygenation on the imidazole ring (analysis by TiCl₃ treatment was inclusive as to C- or N-hydroxylation), and mono-oxygenation on the N of the heteroaromatic ring (Supplemental Table 1 and data not shown). No dehydrogenation or other metabolites were observed. Previous analysis of metabolism of EMTPP by CYP2D6 showed only two mono-oxygenation products (m/z 370) and one dehydrogenation product (m/z 351) (Hutzler et al., 2004).

**Molecular Modeling.** A series of molecular modeling studies were performed to better understand the metabolism of SCH 66712 and EMTPP that would lead to inactivation of CYP3A4.

With SCH 66712 the lowest energy and most common docking poses were with the phenyl ring of SCH 66712 in stacking geometry with Phe304 (Supplemental Fig. 6A). This placed the imidazole group and methylene closest to the heme iron at a distance of ~4.6 Å. An additional orientation observed was with the heteroaromatic ring parallel to the heme group at ~3.3 Å above the heme. In this configuration, the phenyl group pointed to the phenylalanine cluster in the roof the active site of CYP3A4 at ~4.0 Å from Phe213 and Phe215 (Supplemental Fig. 6A). Binding free energies for SCH 66712 docking ranged from -9.4 kcal/mol to -8.3 kcal/mol.

Docking experiments with EMTPP resulted in lowest energy conformations with the -CF₃ group pointing toward Phe304 and placement of the 6-membered heteroaromatic ring closest to the heme iron at ~4.4 Å (Supplemental Fig. 6B). In the other major orientation, the ethyl group of the imidazole ring was pointing toward Phe304 and the methylene group connecting the imidazole and piperazine rings was closest to the heme.
iron at ~4.2 Å. Stacking interactions between phenylalanine residues in CYP3A4 and aromatic rings in EMTPP were not observed in any of the docking results (data not shown). Binding free energies for EMTPP docking were weaker and ranged from -8.2 kcal/mol to -7.1 kcal/mol.
Discussion

The inactivation of CYP3A4 by SCH 66712 and EMTPP was concentration-, time-, and NADPH-dependent. Inactivation by SCH 66712 proceeded with $k_{\text{inact}}$ of 0.211 min$^{-1}$ and $K_1$ of 1.60 µM and overall efficiency of inactivation of 0.013 µM$^{-1}$min$^{-1}$. EMTPP was a weaker inactivator with $k_{\text{inact}}$ of 0.044 min$^{-1}$ and $K_1$ of 11.8 µM and overall efficiency of inactivation of 0.0037 µM$^{-1}$min$^{-1}$. The partition ratios for inactivation of CYP3A4 by SCH 66712 and EMTPP were 11 and 94, respectively, confirming that SCH 66712 is a more potent inactivator. Mass spectral analysis of CYP3A4 showed 1:1 binding stoichiometry for both SCH 66712 and EMTPP with no heme modifications. These results support CYP3A4 apoprotein adduction by both inactivators.

Metabolism of SCH 66712 by CYP3A4 produced the same metabolites as CYP2D6 as we previously reported (Nagy et al., 2011). However, the proportions of products produced were different. With CYP3A4 and SCH 66712, product peak 4, mono-oxygenation on the piperazine/heteroaromatic ring end of the molecule, was in highest abundance (Supplemental Fig. 3). Using TiCl$_3$ treatment of the SCH 66712 metabolites, product peak 4 was confirmed to be a carbon hydroxylation, not a nitrogen hydroxylation, since TiCl$_3$ reverses nitrogen hydroxylations (Seto and Guengerich, 1993; Kulanthaivel et al., 2004; Livezey et al., 2014) (Supplemental Fig. 4).

Compared to CYP2D6, CYP3A4 produced many additional mono-oxygenation products of EMTPP (Supplemental Fig. 5 and Hutzler et al., 2004). Sites of metabolism were confirmed on each major functional group including the ethyl group, imidazole ring, piperazine ring, and heteroaromatic ring (Supplemental Table 1 and Supplemental Fig. 5).
For both SCH 66712 and EMTPP, observed docking orientations in the active site of CYP3A4 were consistent with the multiple sites of metabolism observed in MS experiments. Both inactivators are small substrates for CYP3A4 and there was considerable room for docking of the inactivators within the active site. Interaction between the phenyl ring of SCH 66712 and Phe304 perhaps could provide a stabilizing interaction that would lead to fewer products formed, as observed in metabolite assays (Supplemental Fig. 3A). In metabolite assays the major SCH 66712 product formed by CYP3A4 was a C-mono-oxygenation on the piperazine/heteroaromatic ring end of SCH 66712 - the end of the molecule that would be positioned best for metabolism based on the docking experiment (Supplemental Fig. 6A). Conversely, EMTPP did not form significant stabilizing interactions that could hold EMTPP in a particular orientation for metabolism explaining possibly the large variety of mono-oxygenation products formed from EMTPP by CYP3A4. Furthermore, the binding free energies from docking experiments were less favorable for EMTPP than for SCH 66712.

There are many MBIs of CYP3A4 reported in the literature, but only a few of CYP2D6 including only two known apoprotein adductors – SCH 66712 and EMTPP. Inactivation of CYP2D6 by SCH 66712 was more potent than inactivation of CYP3A4 with partition ratio of 3 and \( k_{\text{inact}} \) and \( K_I \) of 0.032 min\(^{-1}\) and 0.55 µM, respectively (Nagy et al., 2011). However, inactivation by EMTPP was similar for both CYP2D6 and CYP3A4 (partition ratios of 99 and 94 for CYP2D6 and CYP3A4, respectively, and \( k_{\text{inact}} \) and \( K_I \) values of 0.09 min\(^{-1}\) and 5.5 µM for CYP2D6 versus 0.044 min\(^{-1}\) and 11.8 µM for CYP3A4). CYP2D6 inactivation by SCH 66712 and EMTPP also proceeded with 1:1
binding stoichiometries and no protection from inactivation by free radical scavengers (Palamanda et al., 2001; Hutzler et al., 2004; Nagy et al., 2011).

SCH 66712 and EMTPP are structurally related with piperazine rings, substituted imidazoles, and fluorinated heteroaromatic rings. To our knowledge, there have been no reports of potent MBI of CYP2D6 and CYP3A4 by the same inactivator [though there are conflicting reports regarding inactivation by tamoxifen (Sridar et al., 2002; Zhao et al., 2002)]. Part of the lack of dual inactivators of both CYP3A4 and CYP2D6 is the difference in the presence of potential requisite nucleophile(s) in the active site. For instance, Vandenbrink et al. (Vandenbrink et al., 2012) have shown that while raloxifene is a potent inactivator of CYP3A4, it does not inactivate CYP2D6. They further showed by structural analysis that CYP2D6 lacks cysteine residues in the quadrant most likely to interact with raloxifene and other soft-electrophile inactivators. It seems likely that SCH 66712 and EMTPP are not adducting at a cysteine since both 2D6 and 3A4 experience inactivation.

We previously proposed that a quinone could be the reactive electrophile in the mechanism of CYP2D6 inactivation by SCH 66712 (Nagy et al., 2011). However, we were unable to isolate a thiol-adduct in the presence of NAC or GSH with SCH 66712. In contrast, we were able to observe an NAC-EMTPP conjugate in reactions with CYP3A4 as did Hutzler et. al with CYP2D6. Hutzler et al. showed by NMR that the electrophile of EMTPP that reacted with NAC had formed on the methylene group of the ethyl substituent of the imidazole ring (Hutzler et al., 2004). However, they were unable to identify the amino acid target in CYP2D6.
In our molecular models, and those of others, it is not clear that a thiol, e.g. cysteine, would be available in CYP2D6 for inactivation on the distal side (Vandenbrink et al., 2012). And, it may be that the ethyl substituent of EMTPP is not the source of the reactive electrophile that leads to inactivation since several other metabolites were also formed by reactions at other sites in EMTPP and in SCH 66712 by both CYP2D6 and CYP3A4. Furthermore, in activity assays in the present study with CYP3A4, addition of NAC did not prevent inactivation by EMTPP though an NAC-EMTPP conjugate was captured by MS. No conjugates of SCH 66712 were observed in reactions with CYP3A4 or previous studies with CYP2D6 (Nagy et al., 2011). Future studies aimed at identifying the exact structural characteristic(s) of SCH 66712 (and EMTPP) that plays a role in the covalent binding of SCH66712 to CYP3A4 and CYP2D6, and the specific location of the covalent adduction to the CYPs, will provide great insight to drug design. Comparing the similarities and differences between inactivation of CYP2D6 and CYP3A4 by SCH66712 will additionally provide insight into enzyme function and specificity.

Conclusion

The inactivation of CYP3A4 and CYP2D6 is of great clinical significance due to the fact that together they are responsible for the metabolism of 70% of all pharmaceutical drugs currently on the market. Mechanism-based inactivation of P450s is of particular interest due to its irreversible nature and dependence on enzyme catalysis. SCH 66712 and EMTPP have been previously identified as a MBI of CYP2D6 and the current study shows SCH 66712 and EMTPP to be inactivators of CYP3A4 as well.
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Authorship Contributions

Participated in research design: Bolles, Fujiwara, Nomeir, Furge

Conducted experiments: Bolles, Fujiwara, Briggs, Furge

Contributed new reagents or analytic tools: Nomeir

Performed data analysis: Bolles, Fujiwara, Furge

Wrote or contributed to the writing of the manuscript: Bolles, Fujiwara, Briggs, Nomeir, Furge
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and rat cytochrome \( b_5 \) and studies on their mechanism of function. *Arch Biochem Biophys* **312**:554-565.


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heme destruction, and covalent binding to protein in P450s 2B6 and 3A5. *J Pharmacol Exp Ther* **313**:154-164.


Palamanda JR, Casciano CN, Norton LA, Clement RP, Favreau LV, Lin C and Nomeir AA (2001) Mechanism-based inactivation of CYP2D6 by 5-fluoro-2-[4-[(2-


Footnotes:

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b. This work was presented in part at the American Society for Biochemistry and Molecular Biology annual meeting, 2014, San Diego, California.

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d. Amanda K. Bolles and Rina Fujiwara contributed equally to this work.
Figure Legends

Figure 1. Structure of SCH 66712 and EMTPP. Both compounds contain substituted imidazole rings and piperazine rings.

Figure 2. Spectral binding titration of SCH 66712 with CYP3A4. (A) Purified CYP3A4 (1 µM) was divided into each of two cuvettes and a baseline was set. Aliquots of SCH 66712 in H₂O were added to the sample cuvette and equal volumes of H₂O were added to the reference cuvette. The increase in absorbance at lower wavelengths is due to addition of SCH 66712 that has a λ_max of 270 nm. (B) Plot of ΔA_{430-395} (from panel A) vs. concentration of SCH 66712. K_s was determined to be 42.9 ± 2.9 µM.

Figure 3. Time- and concentration-dependent inactivation of CYP3A4 by SCH 66712 and EMTPP. (A) Concentration-dependent inactivation by SCH 66712 in 20 min reactions. (B) Time-dependent inactivation by SCH 66712 (16 µM). (C) Concentration-dependent inactivation by EMTPP in 20 min reactions. (D) Time-dependent inactivation by EMTPP (20 µM).

Figure 4. Determination of K_I and k_inact for the inactivation of CYP3A4 by SCH 66712 and EMTPP. (A) Inactivation of CYP3A4 by SCH 66712 (0, 2, 4, 8, and 16 µM) (B) K_I and k_inact for the inactivation of CYP3A4 by SCH 66712 were 1.6 ± 0.7 µM and 0.211 ± 0.024 min⁻¹, respectively, as determined using Kitz-Wilson analysis and non-linear regression. Efficiency of inactivation, k_inact/K_I was 0.013 µM⁻¹min⁻¹. (C) Inactivation of
CYP3A4 by EMTPP (0, 5, 10, 20, and 40 µM). (D) $K_I$ and $k_{\text{inact}}$ for the inactivation of CYP3A4 by EMTPP were $11.8 \pm 2.6$ µM and $0.044 \pm 0.004$ min$^{-1}$, respectively, as determined using Kitz-Wilson analysis and non-linear regression. Efficiency of inactivation, $k_{\text{inact}}/K_I$ was 0.0037 µM$^{-1}$min$^{-1}$.

**Figure 5.** Partition Ratio for SCH 66712 Inactivation of CYP3A4. CYP3A4 was incubated with varying concentrations of SCH 66712 (A) or EMTPP (B) for 30 min to allow for complete inactivation. With SCH 66712, the turnover number was 12 and the partition ratio was estimated to be 11. With EMTPP, the turnover number was 95 and the partition ratio was estimated to be 94.

**Figure 6.** LC-ESI-MS analysis of CYP3A4 incubated with SCH 66712. (A) Chromatogram of reaction with no NADPH and no SCH 66712. (B) ESI-MS of recombinant CYP3A4 from panel A. (C) Deconvolution of MS in panel B yielded mass of 56,953 Da for CYP3A4. (D) Chromatogram of reaction with NADPH but without SCH 66712. (E) ESI-MS of recombinant CYP3A4 from panel D. The addition of NADPH resulted in loss of signal and increased background noise. (F) Deconvolution of MS in panel E yielded mass of 56,958 Da for CYP3A4. (G) Chromatogram of reaction with NADPH and with SCH 66712. (H) ESI-MS of recombinant CYP3A4 from panel G. (I) Deconvolution of MS in panel H yielded mass of 56,957 Da for non-adducted CYP3A4 and mass of 57,284 Da for adducted CYP3A4. The mass increase for the adducted form is consistent with mono-adduction of CYP3A4 by SCH 66712.
Chromatograms (A), (D), and (G) show separation of reconstituted system by HPLC and elution of clipped reductase, reductase, CYP3A4, and lipids.
Table I. Inactivation of CYP3A4 by SCH 66712 is not prevented by trapping agents.

<table>
<thead>
<tr>
<th>Assay Componentsa</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>No NADPH – Control, SCH 66712</td>
<td>100</td>
</tr>
<tr>
<td>NADPH, SCH 66712</td>
<td>15</td>
</tr>
<tr>
<td>NADPH, SCH 66712, GSH (10 mM)</td>
<td>13</td>
</tr>
<tr>
<td>NADPH, SCH 66712, NAC (10 mM)</td>
<td>18</td>
</tr>
<tr>
<td>NADPH, SCH 66712, SOD (1 U/µL)</td>
<td>10</td>
</tr>
<tr>
<td>NADPH, SCH 66712, catalase (0.05 µg/µL)</td>
<td>9</td>
</tr>
<tr>
<td>NADPH, SCH 66712, cyanide (2 mM)</td>
<td>15</td>
</tr>
</tbody>
</table>

aAll reactions contained 16 µM SCH 66712 and were carried out for 12 min.
Table II. Kinetic constants for inactivation of CYP3A4 by SCH 66712 and EMTPP.

<table>
<thead>
<tr>
<th>Inactivator</th>
<th>$K_i$</th>
<th>$k_{\text{inact}}$</th>
<th>$k_{\text{inact}} / K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCH 66712</td>
<td>1.6 ± 0.7</td>
<td>0.211 ± 0.024</td>
<td>0.013</td>
</tr>
<tr>
<td>EMTPP</td>
<td>11.8 ± 2.6</td>
<td>0.044 ± 0.004</td>
<td>0.0037</td>
</tr>
</tbody>
</table>
Figure 3

A. % Remaining Activity vs. [SCH 66712], μM

B. % Remaining Activity vs. Time, min

C. % Remaining Activity vs. [EMTTP], μM

D. % Remaining Activity vs. Time, min
Figure 4

A: Log % Remaining Activity vs. Time, min for SCH 66712.

B: $k_{obs}$ (min$^{-1}$) vs. [SCH 66712], μM.

C: Log % Remaining Activity vs. Time, min for EMTPP.

D: $k_{obs}$ (min$^{-1}$) vs. [EMTPP], μM.
Figure 6

No NADPH
No SCH 66712

A clipped reductase
reductase
3A4
lipids

Time, min

Abundance (x10^6)

RT: 34.73-34.96

B

Abundance (x10^4)

m/z

600 800 1000 1200 1400 1600 1800 2000

C 56,952 Da

Intensity (x10^5)

Mass

56000 56500 57000 57500 58000

D clipped reductase
reductase
3A4
lipids

Time, min

Abundance (x10^6)

RT: 34.64-34.94

E

Abundance (x10^3)

m/z

600 800 1000 1200 1400 1600 1800 2000

F 56,958 Da

Intensity (x10^4)

Mass

56000 56500 57000 57500 58000

G clipped reductase
reductase
3A4
lipids

Time, min

Abundance (x10^6)

RT: 34.63-34.81

H

Abundance (x10^3)

m/z

600 800 1000 1200 1400 1600 1800 2000

I 56,957 Da

Intensity (x10^4)

Mass

56000 56500 57000 57500 58000

57,284 Da