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MECHANISM-BASED INACTIVATION OF CYP2D6 BY 5-FLUORO-2-[4-[(2-PHENYL-1*H*-IMIDAZOL-5-YL)METHYL]-1-PIPERAZINYL]PYRIMIDINE

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ABSTRACT:

SCH 66712 [5-fluoro-2-[4-[(2-phenyl-1*H*-imidazol-5-yl)methyl]-1-piperazinyl]pyrimidine] caused a time- and NADPH-dependent loss of CYP2D6 activity. The inactivation of human liver (HL) microsomal dextromethorphan *O*-demethylase activity, a prototype marker for CYP2D6, was characterized by a K_I of 4.8 μM and a maximal rate constant of inactivation (k_{inact}) of 0.14 min^{-1}. The inactivation of the recombinant CYP2D6 in Supersomes (r-CYP2D6) was characterized by a K_I of 0.55 μM and a k_{inact} of 0.32 min^{-1}. Extensive dialysis of the SCH 66712-inhibited enzyme failed to restore the activity to control levels (dialyzed reaction mixture lacking SCH 66712) for both HL microsomes and r-CYP2D6. Addition of glutathione, superoxide dismutase, or mannitol to the reaction mixture failed to protect CYP2D6 against SCH 66712-NADPH-catalyzed inactivation. Addition of quinidine, a reversible inhibitor of CYP2D6, to a preincubation mixture consisting of SCH 66712, HL microsomes, or Supersomes and NADPH partially protected CYP2D6 from inactivation. SCH 66712 also inhibited HL microsomal CYP3A4, CYP2C9, and CYP2C19; however, the concentrations required to inhibit those isoforms were 5- to 10-fold higher than those required to inhibit CYP2D6. These results demonstrate that SCH 66712 is a potent and fairly selective mechanism-based inhibitor of CYP2D6.

Human liver (HL[1]) cytochrome P450 isoforms (P450s) are known to participate in the oxidative metabolism of xenobiotics. Notably, CYP2D6 and CYP3A4 collectively metabolize approximately 80% of commercially available drugs. The potential of new chemical entities that are prepared for evaluation as potential drug candidates to inhibit HL P450s, especially CYP3A4 and CYP2D6, is now routinely evaluated in the pharmaceutical industry during the discovery stage (Lin and Lu, 1997; Palamanda et al., 1998; Chu et al., 2000; White 2000). Significant inhibition of P450s such as CYP3A4 and CYP2D6 may lead to adverse drug-drug interactions.

Inhibition of P450s due to the inactivation of the enzyme via the formation of an intermediate species that binds tightly to the enzyme (irreversible) has been documented and is referred to as mechanism-based inhibition (Silverman, 1988). Examples include furafylline inhibition of CYP1A2 (Kunze and Trager, 1993), menthofuran inhibition of CYP2A6 (Khojasteh-Bakht et al., 1998), tienilic acid inhibition of CYP2C9 (Lopez-Garcia et al., 1994; Jean et al., 1996), halothane inhibition of CYP2E1 (Madan and Parkinson, 1996), and gestodene inhibition of CYP3A4 (Guengerich, 1990). Mechanism-based inactivators of animal P450s have also been well documented (Hopkins et al., 1992; Crowley and Hollenberg, 1995; Roberts et al., 1995; Sharma et al., 1996; Foroozesh et al., 1997). However, to our knowledge, potent and selective mechanism-based inhibitors of HL polymorphic CYP2D6 and CYP2C19 have not been reported.

SCH 66712 [5-fluoro-2-[4-[(2-phenyl-1*H*-imidazol-5-yl)methyl]-1-piperazinyl]pyrimidine; Fig. 1] is a potent and selective antagonist of the human D4 dopamine receptor. In a preliminary screen using human liver microsomes, the results suggested that SCH 66712 was a mechanism-based inhibitor for CYP2D6. Therefore, further studies were conducted to characterize this inhibition in both HL microsomes and cDNA-expressed recombinant CYP2D6 (Supersomes).

Materials and Methods

Reagents. Testosterone, its metabolite 6β-hydroxytestosterone, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Dextromethorphan, tolbutamide, *S*-mephenytoin, and levallorphan were purchased from RBI/Sigma (Natick, MA). Supersomes (CYP2D6) were obtained from GENTEST (Woburn, MA). Individual HL microsomes (four males and four females) were supplied by International Institute for the Advancement of Medicine (Exton, PA), pooled, aliquotted, and stored frozen at −80°C. SCH 66712 was provided by Schering-Plough Research Institute (Kenilworth, NJ).

Inhibition of CYP3A4, -2C9, -2C19, and -2D6 by SCH 66712. *Coincubation.* SCH 66712, at various concentrations (0.05–16 μM for CYP2D6 and 0.4–16 μM for other P450s), was directly incubated with HL microsomes and NADPH in the presence of probe substrates of various P450s for 10 min at 37°C (within the linear range). The final reaction mixture consisted of microsomes (0.4 mg/ml), SCH 66712, substrate at the approximate K_m for each reaction [50 μM testosterone (CYP3A4), 100 μM tolbutamide (CYP2C9), 100 μM *S*-mephenytoin (CYP2C19), or 16 μM dextromethorphan (CYP2D6)], methanol (between 1–2% concentration for the various assays), and 1 mM NADPH in 200 μl of either 100 mM potassium phosphate buffer, pH 7.4, or 100 mM Tris-acetate buffer, pH 7.4 (tolbutamide assay only). The reaction was terminated by the addition of 100 μl of methanol (for the testosterone assay)

[1] Abbreviations used are: HL, human liver; SCH 66712, 5-fluoro-2-[4-[(2-phenyl-1*H*-imidazol-5-yl)methyl]-1-piperazinyl]pyrimidine; k_{inact}, maximal rate constant of inactivation; P450, cytochrome P450; HPLC, high-performance liquid chromatography; GSH, glutathione.

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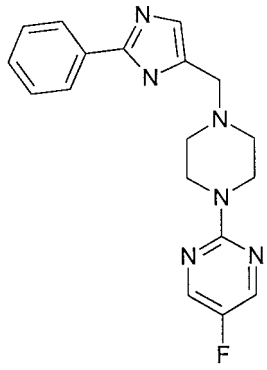

FIG. 1. *Chemical structure of SCH 66712*

or 15 μl of 70% perchloric acid (for the tolbutamide, *S*-mephenytoin, and dextromethorphan assays), vortexing, and chilling the incubation mixtures on ice. The chilled incubation mixtures were centrifuged, and the clear supernatants were analyzed by HPLC.

Preincubation. SCH 66712, at the same concentrations described under coincubation, was preincubated with microsomes and NADPH for 10 min at 37°C. The preincubation reaction mixture consisted of either 100 mM potassium phosphate buffer, pH 7.4, or 100 mM Tris-acetate buffer, pH 7.4 (tolbutamide assay only), containing 0.5% methanol, liver microsomes (0.4 mg/ml), SCH 66712 at various concentrations, and 1 mM NADPH. Following preincubation, the substrates for the various assays were added to the incubation mixtures at concentrations identical to those specified under the coincubation experiment. The final methanol concentration was between 1 and 2% to accommodate various substrates. The reaction was terminated after 10 min of incubation, and the samples were prepared for analysis as outlined above.

CYP2D6 Inactivation Studies. SCH 66712 at 0, 2, 4, 8, and 16 μM final concentrations was preincubated with 4 mg/ml of HL microsomal protein, or alternatively, SCH 66712 at 0, 0.13, 0.25, 0.5, and 1 μM final concentrations was preincubated with 20 pmol of r-CYP2D6 at 37°C in the presence of 1 mM NADPH in a total volume of 100 μl of a 100 mM potassium phosphate buffer, pH 7.4 (inactivation assay). At selected time intervals (0, 5, and 10 min for HL microsomes and 0, 1.25, 2.5, 5, and 10 min for r-CYP2D6 in Supersomes), 20-μl aliquots of the incubation mixture were transferred to tubes containing 180 μl of a 100 mM potassium phosphate buffer, pH 7.4, containing dextromethorphan and NADPH (activity assay). By diluting the contents of the inactivation mixture 10-fold, the activity assay was designed to minimize the direct inhibition of CYP2D6 by SCH 66712. The dilution also helped to quench the inactivation reaction at the desired time point of incubation. The final concentrations of the components of the activity assay were dextromethorphan (16 μM, the approximate K_m for the assay), NADPH (1 mM), HL microsomal protein (0.4 mg/ml), or r-CYP2D6 (2.0 pmol) and the remaining SCH 66712 diluted 10-fold. Dextromethorphan activity assays typically lasted 10 min (within the linear range). Each reaction was terminated by the addition of 15 μl of 70% perchloric acid, and the samples were immediately chilled on ice. A volume of 20 μl of an aqueous solution of 15 μM levallorphan (internal standard) was added to each sample, followed by vortexing and centrifugation at 2000*g* for 5 min. The clear supernatant was injected into the HPLC system for the analysis of dextrorphan, the CYP2D6-mediated metabolite of dextromethorphan.

HPLC Assays. *Dextromethorphan* O*-demethylation (2D6).* Dextrorphan formation was quantified using reversed phase HPLC. The system consisted of a Waters 710 B autoinjector, a Hitachi L-7100 pump, and a Shimadzu RF535 fluorescence detector. The stationary phase was a 3-μm Microsorb MV phenyl column (10 cm × 4.6-mm i.d.). The mobile phase consisted of a mixture of 30% acetonitrile, 1% acetic acid, and 0.05% triethylamine in water delivered at 0.75 ml/min. Dextrorphan, levallorphan, and dextromethorphan were eluted isocratically and detected fluorometrically (excitation and emission wave-

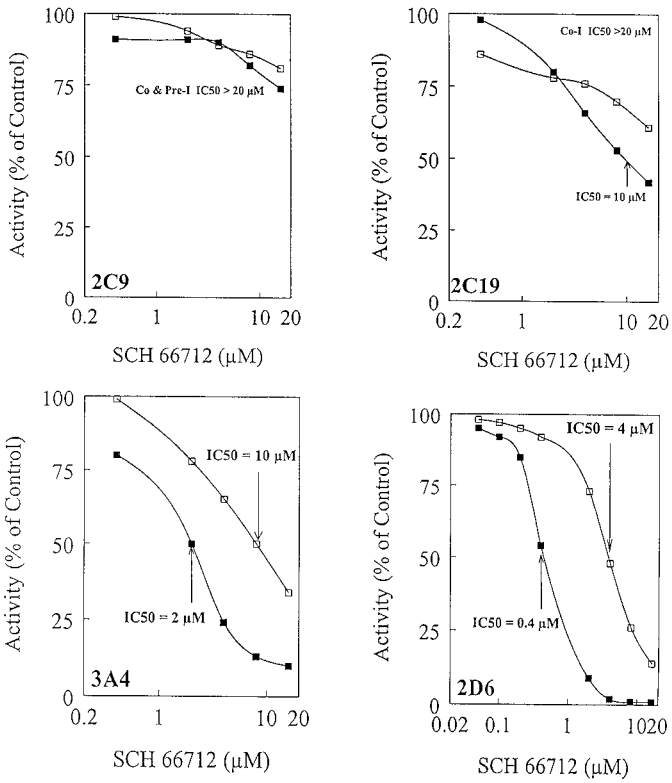

FIG. 2. *Inhibition of CYP3A4, -2C9, -2C19, and -2D6 in HL microsomes by SCH 66712.*

IC_{50} curves following coincubation (□) and preincubation (■) with SCH 66712.

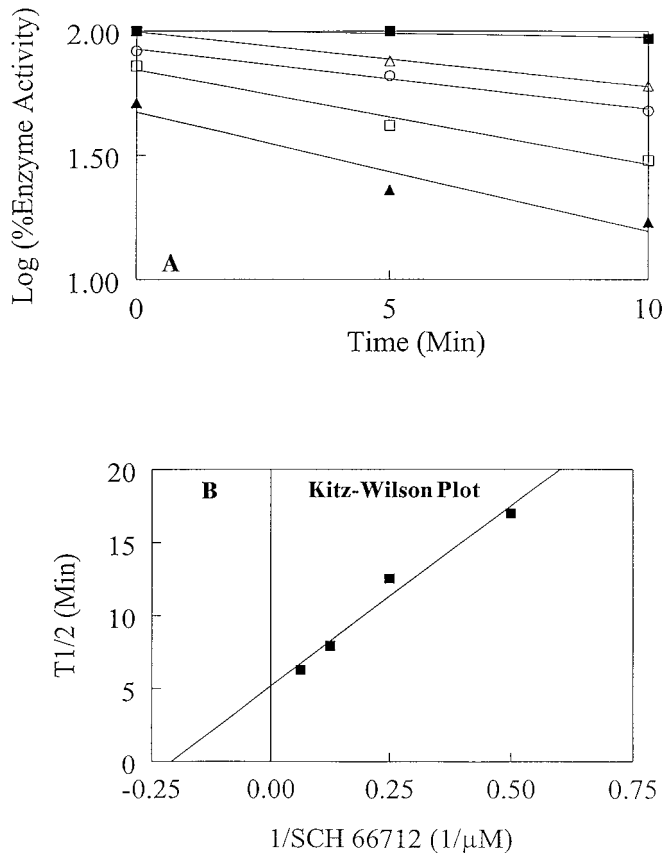

FIG. 3. *Inactivation of CYP2D6 in HL microsomes by SCH 66712.*

A, time- and concentration-dependent inactivation of dextromethorphan *O*-demethylase activity; B, corresponding Kitz-Wilson plot. ■, 0 μM; △, 2 μM; ○, 4 μM; □, 8 μM; ▲, 16 μM.

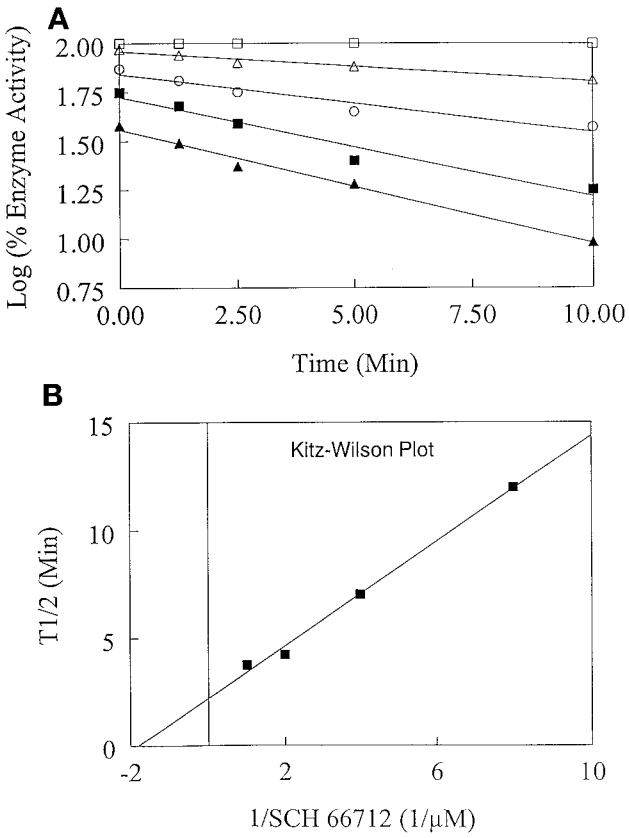

FIG. 4. *Inactivation of r-CYP2D6 in Supersomes by SCH 66712.*

A, time- and concentration-dependent inactivation of dextromethorphan *O*-demethylase; B, corresponding Kitz-Wilson plot. □, 0 μM; △, 0.13 μM; ○, 0.25 μM; ■, 0.5 μM; ▲, 1 μM.

lengths where 285 and 310 nm, respectively). The retention times for dextrorphan, levallorphan, and dextromethorphan were 4.5, 7, and 12 min, respectively (Palamanda et al., 1998).

Tolbutamide hydroxylation (2C9). Hydroxytolbutamide was quantified using reversed phased HPLC according to the method described by Miners et al. (1988) with the modifications reported by Palamanda et al. (2000). The stationary phase was a Zorbax-CN 4.6-mm × 15-cm column. The mobile phase consisted of 22% acetonitrile and 78% 10 mM sodium acetate, pH 4.3, delivered at a flow rate of 1.2 ml/min. The analytes were quantified by UV absorbance at 230 nm. The retention times for hydroxytolbutamide, chlorpropamide (internal standard for the analysis), and tolbutamide were 4, 8, and 11.5 min, respectively.

Testosterone 6β-hydroxylation (3A4). 6β-Hydroxytestosterone was quantified using reversed phase HPLC. The stationary phase was a 5 μM Supelcosil LC-18, 15-cm × 4.6-mm column. The mobile phase consisted of a mixture of 35% methanol, 9% tetrahydrofuran, and 56% water at a flow rate of 1 ml/min. Testosterone and its primary CYP3A4 metabolite 6β-hydroxytestosterone were detected by UV absorbance at 245 nm. The retention times for 6β-hydroxytestosterone and testosterone were 5 and 16 min, respectively.

S-*Mephenytoin hydroxylation (2C19).* The stationary phase was a 5-μm Beckman ODS Ultrasphere, 7.5-cm × 4.6-mm C_{18} column. The mobile phase consisted of 20% acetonitrile in water, which was eluted at 1.5 ml/min. 4′-Hydroxy *S*-mephenytoin was detected by UV absorbance at 230 nm. The retention time for 4′-hydroxy *S*-mephenytoin was 4 min.

Effect of NADPH and Trapping Agents on the Inhibition of CYP2D6 by SCH 66712. The effect of NADPH on CYP2D6 inactivation was determined by preincubating SCH 66712 with either HL microsomes or r-CYP2D6 for 10 min at 37°C in the presence and absence of 1 mM NADPH. The effects of the nucleophile-trapping agent glutathione (2 mM), and the scavengers for reactive oxygen species superoxide dismutase (1000 units/ml) and mannitol (1 mM), were evaluated by including each component individually in the inactivation assay.

Dialysis. This experiment was conducted to evaluate the effect of dialysis on the regeneration of inhibited CYP2D6 activity. Incubations were conducted, as described for the CYP2D6 inactivation assay, for 10 min at 37°C in 500 μl of a 100 mM potassium phosphate buffer, pH 7.4. Twenty-microliter aliquots were removed and immediately assayed for dextromethorphan *O*-demethylase activity. The remaining reaction mixture was dialyzed against 100 mM potassium phosphate buffer, pH 7.4 (2 × 100 ml) for 6 h (buffer replaced after 3 h) at 4°C in Spectra/por cellulose ester sterile dispodialyzer with molecular mass cut-off of 10,000 Da. After 6 h of dialysis, dextromethorphan *O*-demethylase activity was again measured. A reference dialysis was carried out in the absence of SCH 66712. No apparent changes in volumes of the dialysis mixtures were evident after the 6 h of dialysis.

Calculation of Kinetic Constants. The slopes obtained from linear regression of log percentage remaining activity versus time plots at each concentration were determined. The first order inactivation constant (k) at each inactivator concentration was obtained by multiplying the slope obtained from the linear regression analysis by 2.303. The $t_{1/2}$ of the inactivation reaction was determined ($t_{1/2} = 0.693/k$) at each inactivator concentration. The $t_{1/2}$ values were plotted on the y-axis versus the reciprocal of the inactivator concentrations on the x-axis (Kitz-Wilson plots). The k_{inact} and K_I values were determined from the y and the x intercepts of the Kitz-Wilson plots, respectively.

Statistics. Each experiment was carried out in triplicate. Data are reported as means of three separate determinations and expressed as a percentage of the control with percentage of coefficient of variation (%CV).

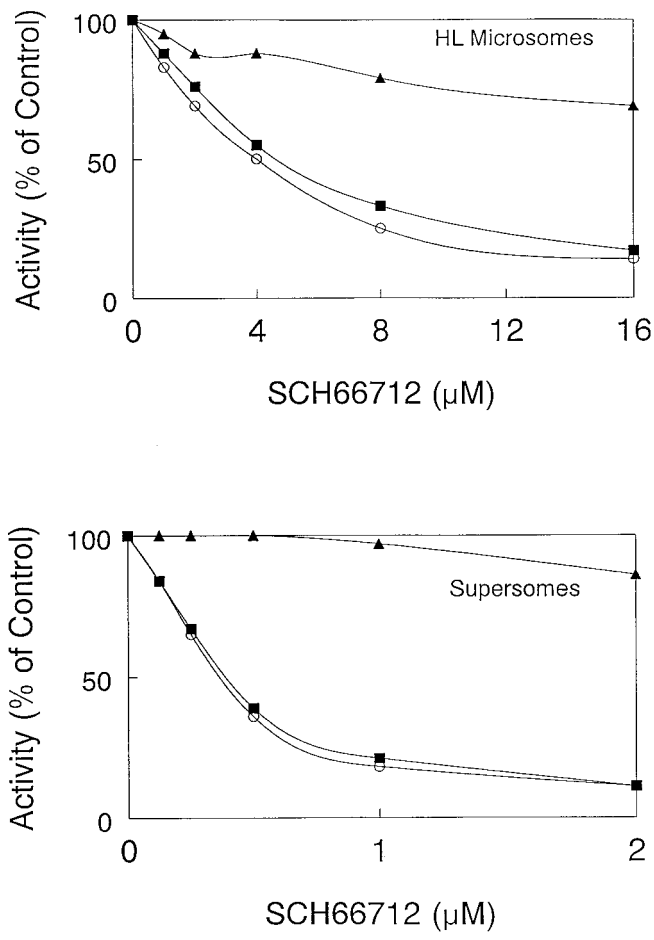

FIG. 5. *Requirement of NADPH for the SCH 66712-mediated CYP2D6 inactivation, and the failure of glutathione to protect against inactivation in HL microsomes and Supersomes.*

■, +GSH+NADPH; ▲, −GSH−NADPH; ○, −GSH+NADPH.

TABLE 1

Effect of glutathione and scavengers of reactive oxygen species on the inactivation of CYP2D6 by SCH 66712 in HL microsomes and Supersomes

Human liver microsomes and Supersomes were preincubated with 4 μM SCH 66712 for 10 min at 37°C. Control preparations were preincubated with NADPH but lacked SCH 66712. Aliquots were removed and diluted 10-fold to detemine dextromethorphan *O*-demethylase activity.

Incubation Conditions	CYP2D6 Activity	
	HL Microsomes	Supersomes
	% of Control[a]	
+NADPH	100	100
SCH 66712 + NADPH	60 (10)	8 (17)
+Glutathione (2 mM)	55 (8)	7 (9)
+Superoxide dismutase (1000 units/ml)	66 (23)	7 (9)
+Mannitol (1 mM)	52 (2)	7 (4)

[a] Data are means (%CV) of three separate determinations.

TABLE 2

Effect of dialysis on CYP2D6 dextromethorphan O-demethylase activity

The inactivation assay was conducted for 10 min at 37°C. After a 10-fold dilution, CYP2D6 activity wsa determined before and after dialysis. There were 15 and 24% loss of CYP2D6 activity in Supersomes and HL microsomes, respectively, after 6 h of dialysis in the absence of any other reagents.

Inactivation Assay Components	CYP2D6 Activity	
	Before Dialysis	After 6 h of Dialysis at 4°C
	% of Control[a]	
Supersomes (control)	100 (6)	100 (5)
Supersomes + NADPH	91 (5)	67 (3)
Supersomes + NADPH + 1% methanol	105 (3)	77 (3)
Supersomes + NADPH + SCH 66712 (2 μM)	10 (21)	8 (11)
Supersomes + SCH 66712 (2 μM)	83 (2)	98 (4)
HL microsomes (control)	100 (8)	100 (2)
HL microsomes + NADPH	94 (5)	65 (6)
HL microsomes + NADPH + 1% methanol	104 (2)	69 (12)
HL microsomes + NADPH + SCH 66712 (8 μM)	30 (4)	23 (9)
HL microsomes + SCH 66712 (8 μM)	79 (4)	105 (1)

[a] Data are means (%CV) of three separate determinations.

Results

Figure 2 shows the inhibition profile of SCH 66712 for the major drug-metabolizing P450s in HL microsomes. The IC_{50} values of SCH 66712 for CYP2C9, -2C19, -3A4, and -2D6 following coincubation were >20, >20, 10, and 4 μM, respectively. Following preincubation, the IC_{50} values for the same isoforms were >20, 10, 2, and 0.4 μM, respectively. The results of this preliminary experiment suggested that SCH 66712 is a potent mechanism/metabolism-based inhibitor of CYP2D6. Therefore, subsequent experiments were carried out to characterize this inhibition.

Figures 3A and 4A show the inactivation profiles of HL CYP2D6 and r-CYP2D6, respectively, at various concentrations of SCH 66712. The inactivation was time- and concentration-dependent in both systems (Figs. 3A and 4A). The inactivation of r-CYP2D6 proceeded more rapidly (Fig. 4A) compared with HL CYP2D6 (Fig. 3A). In addition to the time-dependent loss of enzyme activity, Figs. 3A and 4A also show a pronounced component of reversible inhibition which increases with increasing SCH 66712 concentration. Kitz-Wilson plots (Figs. 3B and 4B) indicated that the maximal rate constant of inactivation (k_{inact}) was 0.14 min^{-1} for HL CYP2D6 and approximately 2-fold higher for r-CYP2D6 (0.32 min^{-1}). The inactivator concentration required for half-maximal inactivation (K_I) was 4.8 μM for HL CYP2D6 and approximately 9-fold lower for the r-CYP2D6 (0.55 μM).

The inactivation of CYP2D6 by SCH 66712 was demonstrated to be NADPH-dependent in both HL microsomes and Supersomes (Fig. 5). Addition of GSH to the inactivation reaction mixture did not prevent or slow the inactivation (Fig. 5; Table 1). The scavengers of the reactive oxygen species mannitol and superoxide dismutase did not prevent the inactivation of CYP2D6 (Table 1).

Table 2 shows the results of the dialysis experiments. There was a 90 and 70% loss of CYP2D6 enzyme activity in Supersomes and HL microsomes, respectively, following incubation with SCH 66712 and NADPH. Extensive dialysis of the inhibited incubation mixtures failed to restore the lost activity (Table 2). In the absence of NADPH in the inactivation assay, SCH 66712 inhibited only 17 and 21% of CYP2D6 activity in the Supersomes and HL microsomes prior to dialysis (Table 2). This inhibition of CYP2D6 that occurred despite a 10-fold dilution of the reaction mixture prior to the dextromethorphan activity assay is presumably due to the intrinsic ability of SCH 66712 to inhibit CYP2D6 by a reversible mechanism, which had been demonstrated in a different experiment (Figs. 3A and 4A). Subsequent dialysis of this mixture completely restored the enzyme activity to control values (reaction mixtures lacking SCH 66712). The dialysis experiment also demonstrated the underlying requirement of NADPH for the inactivation, indicating the involvement of a catalytic step(s) in the inactivation of CYP2D6.

One of the major characteristics of mechanism-based inhibition is that reversible inhibitors slow down the inactivation of the enzyme by competing for the binding site (Silverman, 1988). Quinidine, a potent prototype reversible inhibitor of CYP2D6, was used in these experiments. In the absence of quinidine, SCH 66712 inhibited 81 and 92% of CYP2D6 activity compared with control in HL microsomes and Supersomes, respectively, following incubation in the presence of NADPH. Addition of quinidine (200 nM) to the inactivation reaction diminished the inactivation of CYP2D6 to 40% of control in both HL microsomes and Supersomes (Table 3), despite the potential of quinidine itself to inhibit slightly the dextromethorphan *O*-demethylation reaction following a 10-fold dilution to 20 nM in the activity assay.

Discussion

SCH 66712 is a potent mechanism-based inactivator of CYP2D6. This inactivation can be classified as mechanism-based (Silverman, 1988) because of the following observations. First, SCH 66712 inactivated CYP2D6 in a time-dependent manner (Figs. 3 and 4). Second, the inactivation proceeded via a catalytic step(s) as indicated by the requirement for NADPH (Fig. 5). Third, the loss of activity was irreversible, as the activity could not be recovered by extensive dialysis (Table 2). Fourth, the inactivation was not protected by the nucleophile glutathione (Fig. 5; Table 1). The failure of GSH to protect CYP2D6 suggested that the inactivation was probably due to modification at the active site by a SCH 66712 reactive metabolite(s), which remained at the active site after its formation. Fifth, quinidine, a prototype reversible CYP2D6 inhibitor, partially protected the enzyme from inactivation, presumably by competing with SCH 66712 for the binding site of CYP2D6 (Table 3). Finally, the inactivation did not appear to be due to the generation of a reactive oxygen species, since superoxide dismutase and mannitol failed to ameliorate the CYP2D6 inactivation (Table 1). Importantly, in addition to mecha-

TABLE 3

Protection of CYP2D6 enzyme activity by quinidine

Human liver microsomes or Supersomes were preincubated for 10 min at 37°C (inactivation assay). The reaction mixtures were diluted 10-fold, and the dextromethorphan *O*-demethylase activity was determined.

Inactivation Assay Components	Activity
	% of Control[a]
HL microsomes (4 mg/ml) + NADPH (1 mM) (control)	100 (3.0)
HL microsomes + NADPH + SCH 66712 (16 μM)	19 (1.3)
HL microsomes + NADPH + SCH 66712 (16 μM) + Quinidine (200 nM)	40 (3.0)
Supersomes (20 pmol of CYP2D6) + NADPH (control)	100 (4.0)
Supersomes + NADPH + SCH 66712 (4 μM)	8 (4.0)
Supersomes + NADPH + SCH 66712 (4 μM) + Quinidine (200 nM)	40 (31)

[a] Data are means (%CV) of three separate determinations.

nism-based inactivation, SCH 66712 showed a pronounced component of reversible inhibition of CYP2D6 (Figs. 3A and 4A).

SCH 66712 is a relatively efficient inactivator of CYP2D6, characterized by a K_I of 4.8 μM and a k_{inact} of 0.14 min^{-1} in HL microsomes (Fig. 3B). The corresponding K_I of 0.55 μM and k_{inact} of 0.32 min^{-1} for r-CYP2D6 established SCH 66712 as a rapid inactivator of this important polymorphic cytochrome P450 isoform. Interestingly, the rate of inactivation of r-CYP2D6 by SCH 66712 is approximately 2 times faster and occurred at lower concentrations compared with HL CYP2D6. Although the exact reason for this difference is not fully understood, the fact that SCH 66712 has the ability to bind to other P450s in addition to CYP2D6 could explain the observed difference. It is reasonable to assume that SCH 66712 may be metabolized by or bound to P450s other than CYP2D6 in HL microsomes, thereby reducing the concentration of SCH 66712 available for CYP2D6. Since Supersomes contain CYP2D6 and no other P450s, metabolism of SCH 66712 by CYP2D6 leading to inactivation of this enzyme is probably more efficient in the absence of other P450s.

The results of this study demonstrate that SCH 66712 is a potent mechanism-based inhibitor of CYP2D6. This adds to the growing list of mechanism-based inhibitors of P450s that have been documented.

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