INFLUENCE OF LIPOPHILICITY ON THE INTERACTIONS OF N-ALKYL-4-PHENYL-1,2,3,6-
TETRAHYDROPYRIDINES AND THEIR POSITIVELY CHARGED N-ALKYL-4-
PHENYLPYRIDINIUM METABOLITES WITH CYTOCHROME P450 2D6

AMIT S. KALGUTKAR, SUE ZHOU, ODETTE A. FAHMI, AND TIMOTHY J. TAYLOR

Pharmacokinetics, Dynamics, and Metabolism Department (A.S.K., S.Z., T.J.T.) and Candidate Enhancement Group (O.A.F.), Pfizer Global Research & Development, Groton, Connecticut

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ABSTRACT:

The relationship between lipophilicity and CYP2D6 affinity of cyclic tertiary (N-alkyl-4-phenyl-1,2,3,6-tetrahydropyridines) and quaternary (N-alkyl-4-phenylpyridinium) amines was examined. The 1,2,3,6-tetrahydropyridine scaffold was chosen due to its common occurrence in the structures of CYP2D6 ligands such as the Parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and the dehydrated haloperidol metabolite N-[4-(4-fluorophenyl)-4-oxobutyl]-1-[4-(4-chlorophenyl)-1,2,3,6-tetrahydropyridine (HPTP). Likewise, the pyridinium framework is found in and 4-(4-oxobutyl)-4-(4-chlorophenyl)-1,2,3,6-tetrahydropyridine (HPP). The relationship between lipophilicity and CYP2D6 inhibition by pyridiniums and 1,2,3,6-tetrahydropyridines was limited to straight chain N-alkyl analogs, since certain N-alkylaryl analogs of lower lipophilicity were better CYP2D6 inhibitors. CYP2D6 substrate properties of straight chain N-alkyltetrahydropyridines were also governed by lipophilicity, and N-heptyl-4-phenyl-1,2,3,6-tetrahydropyridine was the optimal substrate (K_{mapp} = 0.63 \mu M). Metabolism studies indicated that the N-heptyl analog underwent monohydroxylation on the aromatic ring and on the N-heptyl group suggesting that 1,2,3,6-tetrahydropyridines can bind in more than one conformation in the CYP2D6 active site. Increased lipophilicity of haloperidol metabolites did not correlate with inhibitory potency since the more lipophilic HPTP metabolite was less potent as an inhibitor than reduced-haloperidol and reduced-HPTP. Furthermore, HPTP and reduced-HPTP, of comparable lipophilicity to the N-heptyltetrahydropyridine analog were inactive as CYP2D6 substrates. This observation suggests that steric constraints rather than lipophilicity are responsible for the lack of CYP2D6 substrate properties of cyclic tertiary amines tethered to bulky N-substituents. This phenomenon appears to be a common theme among several cyclic tertiary amine-containing anti-depressants and should be taken into consideration when designing central nervous system agents devoid of CYP2D6 substrate properties.

Although cytochrome P450 2D6 (CYP2D6) is a minor component (~2%) of total cytochrome P450 content in human liver, a significant proportion (~30–40%) of drugs currently in clinical use are metabolized by CYP2D6. Furthermore, the genetic polymorphism associated with CYP2D6-mediated metabolism in humans has heightened awareness of the potential adverse drug reactions following impaired clearance of CYP2D6 substrates in individuals designated as poor metabolizers. Therefore, several efforts have been directed to better understand structure-function relationships of CYP2D6 substrates/inhibitors.

Despite overall structural diversity, most tightly bound CYP2D6 ligands usually contain a protonated basic amine nitrogen thought to be essential for electrostatic interactions with an active site Asp residue (Ellis et al., 1995; Mackman et al., 1996; Lewis et al., 1997; de Groot et al., 1999a,b; Hanna et al., 2001). Binding of substrate is generally followed by oxidation 5 to 7 Å from this interaction (Modi et al., 1996; Lin et al., 1997; Wu et al., 1997; Bach et al., 1999; Miller et al., 2001). Based on the findings from numerous quantitative structure-activity relationship (QSAR) studies on structurally diverse CYP2D6 ligands (Ferrari et al., 1991; Koymans et al., 1992; Strobl et al., 1993; Venhorst et al., 2000; Upthagrove and Nelson, 2001), lipophilicity and amine basicity have emerged as crucial determinants of binding. The observation that more basic amines possess a higher

1 Abbreviations used are: QSAR, quantitative structure-activity relationship; MPTP, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP \(^{\pm}\), N-methyl-4-phenylpyridinium; PTP, 4-phenyl-1,2,3,6-tetrahydropyridine; HP, haloperidol; 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-4-piperidinol; HPTP, N-[4-(4-fluorophenyl)-4-oxobutyl]-1-[4-(4-chlorophenyl)-1,2,3,6-tetrahydropyridine; HP\(^{+}\), 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]pyridinium; (±)-RHP, (±)-4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-hydroxybutyl]-4-piperidinol; (±)-RHPTP, (±)-4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-hydroxybutyl]-1,2,3,6-tetrahydropyridine; CID, collision-induced dissociation; LC/MS/MS, liquid chromatography/tandem mass spectrometry; cLog P, calculated log \(P\); \(R_t\), retention time; SAR, structure-activity relationship; DMSO, dimethyl sulfoxide.
CYP2D6 affinity is also consistent with the proposal involving the formation of a “tighter” ion-pair with Asp301 (Upthagrove and Nelson, 2001).

With relatively few exceptions (Wu et al., 1997), most of the QSAR studies performed to date have been limited to secondary amine CYP2D6 ligands, and therefore important physiochemical properties governing CYP2D6 affinity of cyclic tertiary and quaternary amines remain poorly understood. In the present study, the correlation between lipophilicity and CYP2D6 affinity of cyclic tertiary and quaternary amines was examined for a series of N-substituted-4-phenyl-1,2,3,6-tetrahydropyridines and positively charged N-substituted-phenylpyridiniums, respectively. The N-substituted-4-phenyl-1,2,3,6-tetrahydropyridine scaffold was chosen based on the reasonably good and selective CYP2D6 substrate properties of the cyclic tertiary amine-containing Parkinsonian neurotoxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, see Table 2) (Kalugtak et al., 2001). Besides the monoamine oxidase-B-mediated bioactivation of MPTP to the positively charged mitochondrial neurotoxin N-methyl-4-phenylpyridinium (MPP\(^+\), see Table 1), CYP2D6 metabolizes MPTP to the corresponding non-oxidative N-4-(4′-hydroxyphenyl)-N-methyl-1,2,3,6-tetrahydropyridine and 4-phenyl-1,2,3,6-tetrahydropyridine (PTP) metabolites (Coleman et al., 1996; Modi et al., 1997).

An additional reasoning behind the proposed QSAR analysis on 1,2,3,6-tetrahydropyridines and pyridiniums was due to their presence in the structures of the haloperidol (HP) metabolites N-4-[4-(4-fluorophenyl)-4-oxobutyl]-1-[4-(4-chlorophenyl)-1,2,3,6-tetrahydropyridine (HPTP, see Table 3) and 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]pyridinium (HPP\(^+\), see Table 1), respectively. Interestingly, HPP\(^+\) and MPP\(^+\) are potent CYP2D6 inhibitors (Tindale et al., 1991; Pan et al., 1997; Shin et al., 2000) but not substrates (Usuki et al., 1996; Fang et al., 2001). In this context, the lack of CYP2D6 inhibition by the less lipophilic MPTP and MPP\(^+\) analogs (A. Kalugtak, unpublished observations) suggests that lipophilicity is an important contributor toward inhibition by 1,2,3,6-tetrahydropyridines and pyridiniums. Based on the finding that the less lipophilic MPTP, but not HPTP, is a CYP2D6 substrate, the role of lipophilicity as a determinant of CYP2D6 substrate properties of 1,2,3,6-tetrahydropyridines is less clear.

Thus, we evaluated the importance of lipophilicity on the CYP2D6 inhibitory/substrate properties for a series of MPTP and MPP\(^+\) analogs containing N-alkyl substituents of increasing chain length against thebufuralol-1′-hydroxylase activity of the enzyme. For comparative purposes, these studies were also extended to include HP and its metabolites including HPTP and MPP\(^+\) as well as the reduced HP and reduced HPTP metabolites (±)-4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-hydroxybutyl]-1-piperidinol [(±)-RHP] and (±)-4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-hydroxybutyl]-1,2,3,6-tetrahydropyridine [(±)-RHPTP], respectively, that are known inhibitors of the CYP2D6-catalyzed dextromethorphan O-demethylation reaction (Shin et al., 2000).

### Experimental Procedures

**Caution.** MPTP is a known neurotoxicant, and therefore compounds of this class should be handled using disposable gloves in a properly ventilated hood. Detailed procedures for the safe handling of MPTP have been reported (Markey and Schmif, 1986).

**Chemistry.** Chemical yields are unoptimized-specific examples of one preparation. All chemicals and solvents used in synthesis were purchased from Aldrich (Milwaukee, WI). 1H NMR spectra in DMSO-d\(_6\) were recorded on a Varian Unity M-400 MHz spectrometer (Varian Medical Systems, Palo Alto, CA); chemical shifts are expressed in parts per million (ppm, δ) calibrated to the deuterium lock signal for DMSO-d\(_6\), t-spin multiplicities are given as s (singlet), d (doublet), bd (broad doublet), dd (doublet of doubles), t (triplet), q (quartet), and m (multiplet). Coupling constants (\(J\)) are given in hertz (Hz). Positive ion electrospray ionization and collision-induced dissociation (CID) mass spectra were obtained on a Sciex API model 2000 liquid chromatography/tandem mass spectrometer (LC/MS/MS) triple quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada). MPP\(^+\) \(\cdot\) 1H, MPP\(^+\) \(\cdot\) COOH\(_2\), N-propyl-4-phenylpyridinium iodide (2 \(\cdot\) 1), and N-propyl-4-phenyl-1,2,3,6-tetrahydropyridine oxalate salt (12 \(\cdot\) COOH\(_2\)) were synthesized as described previously (Kalugtak and Castagnoli, 1992). HP was obtained from Sigma-Aldrich (St. Louis, MO) whereas reduced HP, HPTP, reduced HPTP, and HPP\(^+\) were synthesized via procedures outlined in previously published reports (Usuki et al., 1996). cLog P for the test compounds was calculated from the Hansch and Leo’s Pomona College Medicinal Chemistry Project MedChem software (clog P 4.0) distributed by BioByte Corp. (Claremont, CA) whereas \(\text{pK}_a\)'s for tetrahydropyridines were estimated from the ACDLogD program (Advanced Chemistry Development Inc., Toronto, ON, Canada). Experimental and calculated log P values were essentially identical for MPTP (2.71 versus 2.74) and N-benzyl-4-phenyl-1,2,3,6-tetrahydropyridine (4.0 versus 4.5) (Altomare et al., 1992).

**General Procedure for the Preparation of N-Alkyl- or N-Arylalkyl-4-phenylpyridinium Salts.** A reaction mixture containing 4-phenylpyridine (1. 6.45 mmol), alkyl- or arylalkyl halide (25 mmol) in anhydrous acetone (10 ml) was stirred overnight at room temperature, during which time the crude pyridinium salt separated out of the reaction mixture. In some instances, ethyl ether was added to the solution to precipitate the pyridinium salt. Recrystallization from acetone/ethyl ether gave the desired product as a crystalline solid.

\(N\)-3-(Hydroxypropyl)-4-pyridinium iodide (3 \(\cdot\) 1) was obtained as pale yellow solid (0.40 g, 18%). 1H NMR (DMSO-d\(_6\)) \(\delta\) 9.06–9.07 (d, 2H, J = 7.2 Hz, ArH), 8.49–8.51 (d, 2H, J = 6.8 Hz, ArH), 8.04–8.07 (m, 2H, ArH), 7.60–7.64 (m, 3H, ArH), 4.62–4.65 (t, 2H, J = 6.8 Hz, CH\(_2\)), 3.44–3.47 (t, 2H, J = 6.8 Hz, CH\(_2\)), 2.04–2.10 (m, 2H, CH\(_2\)).

\(N\)-Pentyl-4-phenylpyridinium iodide (4 \(\cdot\) 1) was obtained as pale yellow solid (1.47 g, 65%). 1H NMR (DMSO-d\(_6\)) \(\delta\) 9.08–9.10 (d, 2H, J = 7.2 Hz, ArH), 8.49–8.51 (d, 2H, J = 6.8 Hz, ArH), 8.05–8.07 (m, 2H, ArH), 7.60–7.64 (m, 3H, ArH), 4.53–4.57 (t, 2H, J = 7.2 Hz, CH\(_2\)), 1.87–1.95 (m, 2H, CH\(_2\)), 1.22–1.33 (m, 2H, CH\(_2\)), 0.82–0.86 (m, 3H, J = 7.2 Hz, CH\(_3\)).

LC/MS/MS analysis revealed a single peak [retention time (\(R_t\)) = 12.2 min] with a protonated molecular ion at 226 and a base fragment ion at 156.

\(N\)-Heptyl-4-phenylpyridinium iodide (5 \(\cdot\) 1) was obtained as pale yellow solid (0.29 g, 24%). 1H NMR (DMSO-d\(_6\)) \(\delta\) 9.07–9.09 (d, 2H, J = 7.2 Hz, ArH), 8.49–8.51 (d, 2H, J = 8.0 Hz, ArH), 8.04–8.07 (m, 2H, ArH), 7.60–7.64 (m, 3H, ArH), 4.53–4.57 (t, 2H, J = 7.2 Hz, CH\(_2\)), 1.88–1.92 (m, 2H, CH\(_2\)), 1.22–1.27 (m, 6H, CH\(_3\)), 0.80–0.84 (t, 3H, J = 7.2 Hz, CH\(_3\)).
LC/MS/MS analysis revealed a single peak ($R_t = 14.8$ min) with a protonated molecular ion (MH$^+$) at 258 and a base fragment ion at 128.

Oxalate Salt of N-Octyl-4-phenyl-1,2,3,6-tetrahydropyridine [16 • (COOH)$_2$] was obtained as a crystalline white solid in 81% yield. 1H NMR (DMSO-d$_6$) δ 7.26–7.46 (m, 5H, ArH), 6.15 (s, 1H, C5H), 3.75 (2H, CH$_2$), 3.32–3.35 (m, 2H, CH$_2$), 2.70 (m, 2H, CH$_2$), 1.63 (m, 2H, CH$_2$), 1.24–1.26 (m, 8H, CH$_2$), 0.82–0.85 (t, 3H, J = 7.2 Hz, CH$_3$). LC/MS/MS analysis revealed a single peak ($R_t = 13.9$ min) with a protonated molecular ion (MH$^+$) at 258 and a base fragment ion at 128.

Oxalate Salt of N-Octyl-4-phenyl-1,2,3,6-tetrahydropyridine [16 • (COOH)$_2$] was obtained as a crystalline white solid in 81% yield. 1H NMR (DMSO-d$_6$) δ 7.76–7.46 (m, 5H, ArH), 6.15 (s, 1H, C5H), 3.75 (2H, CH$_2$), 3.32–3.35 (m, 2H, CH$_2$), 2.99–3.03 (m, 2H, CH$_2$), 2.70 (m, 2H, CH$_2$), 1.61–1.68 (m, 2H, CH$_2$), 1.32–1.24 (m, 4H, CH$_2$), 0.84–0.87 (t, 3H, J = 7.2 Hz, CH$_3$). LC/MS/MS analysis revealed a single peak ($R_t = 12.5$ min) with a protonated molecular ion (MH$^+$) at 230 and a base fragment ion at 100.

Oxalate Salt of N-Heptyl-4-phenyl-1,2,3,6-tetrahydropyridine [15 • (COOH)$_2$] was obtained as a crystalline white solid in 93% yield. 1H NMR (DMSO-d$_6$) δ 7.26–7.46 (m, 5H, ArH), 6.15 (s, 1H, C5H), 3.73 (2H, CH$_2$), 3.30–3.35 (m, 2H, CH$_2$), 3.03 (m, 2H, CH$_2$), 2.70 (m, 2H, CH$_2$), 1.63 (m, 2H, CH$_2$), 1.24–1.26 (m, 8H, CH$_2$), 0.82–0.85 (t, 3H, J = 7.2 Hz, CH$_3$). LC/MS/MS analysis revealed a single peak ($R_t = 16.7$ min) with a protonated molecular ion at 275 and a base fragment ion at 60.

Enzymology. Recombinant human CYP2D6 was expressed in-house using a baculovirus/S9 cell expression system. NADPH was purchased from Sigma-Aldrich, whereas (-)-bufuralol and 1'-hydroxy(-)-bufuralol were obtained from BD Gentest Corp. (Woburn, MA).

Inhibition of Bufrural ○ 1'-Hydroxybufuraldehyde Activity in Human Recombinant CYP2D6. In all experiments, the pyridinium salts and their corresponding tetrahydropyridine derivatives were dissolved and diluted serially in methanol. The final concentration of these compounds ranged from 0.05 to 100 µM and that of methanol was less than 0.1% in 200 µl of reaction
volume. Each inhibition study was performed in triplicate. Incubation mixtures (200 μl) contained (±)-bufuralol (10 μM) and human recombinant CYP2D6 (10 nM) in 0.1 M phosphate buffer (pH 7.4). The reaction mixtures were prewarmed at 37°C for 2 min before adding NADPH (1.3 mM), then incubated for 15 min. Reactions were stopped by the addition of 0.4 ml of acetonitrile containing metyrapone (1 μM) as internal standard, and samples were centrifuged at 3000g for 15 min. 1'-Hydroxybufuralol formation was linear with respect to protein concentration and time. 1'-Hydroxybufuralol formation was monitored on a Sciex API model 3000 LC/MS/MS triple quadrupole mass spectrometer with positive ion mode at the ionspray interface temperature of 150°C. The LC gradient was programmed as follows: solvent A to solvent B ratio was held at 100:0 (v/v) for 3 min and then adjusted from 100:0 (v/v) to 10:90 (v/v) for 20 min and then from 10:90 (v/v) to 100:0 (v/v) from 20 to 25 min. The column was re-equilibrated for 5 min prior to the next analytical run. Postcolumn flow was split such that mobile phase was introduced into the mass spectrometer via an ion spray interface at a rate of 50 μl/min. The remaining flow was diverted to the UV detector positioned in line to provide simultaneous UV detection (λ = 254 nm) and total ion chromatogram. Ionization was conducted in the positive ion mode at the ionspray interface temperature of 150°C and using nitrogen for nebulizing and heating gas. 1'-Hydroxybufuralol and metyrapone were analyzed using multiple reaction monitoring at mass ranges m/z 278 → 186 and m/z 268 → 116, respectively. For 1'-hydroxybufuralol, this reaction follows the protonated parent mass MH+ → 278 to its corresponding collision-induced dissociated fragment at m/z 186, which corresponds to dehydration and loss of the N-isopropylamine moiety. IC50 values were determined by fitting the data in Deltagraph (version 4.5; SPSS Science Inc., Chicago, IL).

**Results**

**Chemistry.** All of the N-substituted-4-aryl-1,2,3,6-tetrahydropyridine derivatives were prepared by sodium borohydride reduction of the corresponding N-substituted-4-arylpyridinium intermediates, which in turn were obtained by quaternization of commercially available 4-phenylpyridine with alkyl halides (Fig. 1). N-Substituted-4-aryl-1,2,3,6-tetrahydropyridine derivatives were isolated and characterized as their corresponding oxalate salts. N-oxidation of N-heptyl-4-phenyl-1,2,3,6-tetrahydropyridine (15) free base with m-chloroperbenzoic acid afforded the corresponding N-oxide 22 that was isolated and characterized as the corresponding m-chlorobenzoate salt (see Fig. 1).

**Inhibition of (±)-Bufuralol → (±)-1'-Hydroxybufuralol Activity in Human Recombinant CYP2D6**

**IC50 values for inhibition of CYP2D6-mediated (±)-bufuralol hydroxilation by N- and C-4-substituted pyridiniums and 1,2,3,6-tetrahydropyridines were measured in triplicate using recombinant enzyme and are displayed in Table 1 (pyridiniums), Table 2 (1,2,3,6-tetrahydropyridines), and Table 3 (HP...
and its metabolites), along with the corresponding clog P and pKa values where applicable. Plots of clog P versus log[IC_{50}] for pyridiniums (Fig. 2, panel A) and 1,2,3,6-tetrahydropyridines (Fig. 2; panel B) are also shown. Under the present experimental conditions, the specific CYP2D6 inhibitor quinidine inhibited bufuralol-1'-hydroxylase activity with an IC_{50} value of 0.05 μM, consistent with that reported in previous publications (Mankowski, 1999).

**CYP2D6 Inhibition by N-Alkyl- and N-Arylalkyl-4-phenylpyridiniums.** The CYP2D6 inhibitory potency of HPP^+, the pyridinium metabolite of HP, against (+)-bufuralol-1'-hydroxylase activity was ~6-fold greater than that reported for the inhibition of the corresponding CYP2D6-mediated dextromethorphan O-demethylation [IC_{50}(bufuralol-1'-hydroxylase) = 2 μM; IC_{50}(dextromethorphan O-demethylation = 0.34 μM) (Shin et al., 2000). In contrast, the comparatively less lipophilic MPP^+ did not inhibit the bufuralol-1'-hydroxylase activity of CYP2D6 at the concentration range employed (IC_{50} > 100 μM).

Chain length extension of the N-methyl group in MPP^+ to higher alkyl homologues revealed a substantial increase in CYP2D6 inhibitory potency. For example, compared with MPP^+, N-propyl-4-phenylpyridinium (2) was a much better CYP2D6 inhibitor with an IC_{50} value of ~25 μM. Introduction of a terminal polar hydroxyl group, however, was detrimental for CYP2D6 inhibition by 2 as observed with the N-(3-hydroxypropyl)-4-phenylpyridinium derivative 3 (IC_{50} > 100 μM). Further increases in lipophilicity via extensions of the alkyl chain length in 2 resulted in extremely potent CYP2D6 inhibitors with IC_{50} values in the low micromolar range and 4-phenyl-N-undecylpyridinium (8) was the most potent inhibitor with an IC_{50} value of 0.1 μM. Furthermore, the undecyl chain appeared to be optimal for CYP2D6 inhibition by N-alkyl-4-phenylpyridiniums since the more lipophilic N-tetradecylpyridinium (9) was ~3-fold less potent compared with the undecyl derivative. In contrast to the excellent correlation between lipophilicity and CYP2D6 inhibitory potency in the straight chain N-alkyl-4-phenylpyridinium analogs, the N-phenthethyl-4-phenylpyridinium analog 11 of identical lipophilicity as the N-pentylpyridinium analog 4 was ~12-fold more potent as a CYP2D6 inhibitor [N-phenethylpyridinium (clog P = −0.2) IC_{50} = 0.6 μM; N-propylpyridinium (clog P = −0.2) IC_{50} = 7 μM].

**CYP2D6 Inhibition by N-Alkyl- and N-Arylalkyl-4-phenyl-1,2,3,6-tetrahydropyridines.** The good correlation between lipophilicity and inhibitory potency observed with the straight chain N-alkyl-4-phenylpyridiniums was maintained only with the short → medium straight chain N-alkyl-4-phenyl-1,2,3,6-tetrahydropyridines (Table 2). Thus, less lipophilic tetrahydropyridines such as MPTP and PTP as well as the neutral 4-phenylpyridine did not inhibit CYP2D6, but replacement of the N-methyl group in MPTP with a N-propyl substituent (compound 12) resulted in a significant increase in potency (MPTP, IC_{50} > 100 μM; N-propyltetrahydropyridine (12), IC_{50} = 10 μM). Unlike N-alkyl-4-phenylpyridinium SAR, where introduction of a terminal polar hydroxyl group was detrimental for inhibition, the

### Table 2

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<th>Compound</th>
<th>R</th>
<th>cLog P</th>
<th>pKa</th>
<th>IC_{50}</th>
<th>K_{m}</th>
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<td></td>
<td>μM</td>
<td>μM</td>
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<tr>
<td>4-Phenylpyridine</td>
<td>2.53</td>
<td>5.45 &gt;100</td>
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<td>9.76 &gt;100</td>
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<td>MPTP</td>
<td>CH_{3}</td>
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<td>Octyl (16)</td>
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* Calculated from Project MedChem software (cLog P 4.0) distributed by Biobyte.

* Calculated from ACDLogD Suite and pKa software (Advanced Chemistry Development Inc.).

K_{m} were determined using heterologously expressed CYP2D6 and are average of three separate incubations.

### Table 3

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<tr>
<th>Compound</th>
<th>cLog P</th>
<th>pKa</th>
<th>IC_{50}</th>
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<td>μM</td>
<td>μM</td>
<td>μM</td>
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<td>Haloperidol (HP)</td>
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</table>

* IC_{50} were determined using heterologously expressed CYP2D6 and are average of three separate incubations.
corresponding \(N\)-(3-hydroxypropyl)-4-phenyl-1,2,3,6-tetrahydropyridine derivative 13 retained the inhibitory potency of 12. That tetrahydropyridine 13 inhibited CYP2D6 also was somewhat surprising considering that its lipophilicity was comparable with MPTP and PTP, which were inactive as inhibitors. Although increasing the alkyl chain length in 12 also resulted in increased inhibition, the optimal alkyl chain length in the tetrahydropyridine series comprised of 5 carbons as opposed to 11 carbon atoms in the pyridinium series and further increases in alkyl chain length did not appear to significantly influence inhibition.

**CYP2D6 Inhibition by Haloperidol and its Metabolites.** Consistent with previous reports on the inhibition of the CYP2D6-catalyzed dextromethorphan \(O\)-demethylation reaction (Shin et al., 2000), HP and several of its metabolites including \((\pm)\)-RHP, HPTP, and \((\pm)\)-RHPTP also inhibited CYP2D6 catalyzed bufuralol-1'-hydroxylation pathway (Table 3). The \(IC_{50}\) values for the inhibition of the bufuralol-1'-hydroxylase activity by HP (clog \(P = 3.85\)), \((\pm)\)-RHP (clog \(P = 3.28\)), HPTP (clog \(P = 5.54\)), and \((\pm)\)-RHPTP (clog \(P = 4.81\)) were 6.5, 0.4, 5.7, and 0.08 \(\mu M\), respectively (see Table 3), whereas those for the inhibition of dextromethorphan \(O\)-demethylation by HP, \((\pm)\)-RHP, and \((\pm)\)-RHPTP were 5.7, 0.89, and 1.34 \(\mu M\), respectively. Interestingly, RHPTP was \(\sim 17\)-fold more potent as an inhibitor of bufuralol-1'-hydroxylation when compared with its inhibitory effects against the dextromethorphan \(O\)-demethylase activity of the enzyme. Furthermore, increased lipophilicity of HP metabolites did not necessarily correlate with inhibitory potency since the more lipophilic HP metabolite, HPTP, was less potent as a CYP2D6 inhibitor than the relatively less lipophilic \((\pm)\)-RHP and \((\pm)\)-RHPTP metabolites. Also of some interest were the findings that the reduced HP and HPTP metabolites were \(\sim 6.0\) and \(71\)-fold more potent than the corresponding oxidized compounds (HP and HPTP).

**Effect of Lipophilicity on the CYP2D6 Substrate Properties of \(N\)-Alkyl-4-phenyl-1,2,3,6-tetrahydropyridines.** The effect of lipophilicity on the CYP2D6 substrate properties of several \(N\)-alkyl-4-phenyl-1,2,3,6-tetrahydropyridines was also assessed. The exercise was only limited to \(N\)-alkyl substituents of increasing chain length (increased clog \(P\)) and the \(N\)-phenethyl-4-phenyl-1,2,3,6-tetrahydropyridine analog 21 that had demonstrated potent CYP2D6 inhibition.

Since authentic standards of all metabolites were unavailable, the apparent \(K_m\) values for \(N\)-alkyl/\(N\)-arylalkyl-4-phenyl-1,2,3,6-tetrahydropyridine derivatives as CYP2D6 substrates were assessed using the substrate depletion methodology (extension of the in vitro \(t_{1/2}\) method traditionally used in intrinsic clearance predictions) (Obach and Rech-Hagen, 2002). Recombinant CYP2D6 was incubated with the candidate 1,2,3,6-tetrahydroxyridine (0–100 \(\mu M\)) in the presence of NADPH, and aliquots were analyzed for substrate loss versus time. The percentage of the 1,2,3,6-tetrahydroxyridine remaining versus time at each substrate concentration was fitted to first order decay functions to determine initial substrate depletion rate constants (\(k_{\text{deg}}\)). In theory, as the substrate concentration exceeds \(K_m\), the depletion rate constant declines, and the inflection point of this relationship represents the apparent \(K_m\) value.

Using the substrate depletion approach, the apparent \(K_m\) value for the CYP2D6 catalyzed \((\pm)\)-bufuralol-1'-hydroxylation was within 2-fold of the value determined using the traditional product formation approach (\(K_m\) substrate depletion = 9 \(\mu M\); \(K_m\) product formation = 5 \(\mu M\)). 4-Phenylpyridine and PTP were stable in CYP2D6, and the apparent \(K_m\) for MPTP as a CYP2D6 substrate in our study (70 \(\mu M\)) was similar to those previously reported (Coleman et al., 1996; Gilham et al., 1997). As observed in the SAR analysis for inhibition, lipophilicity was also a major determinant for CYP2D6 substrate properties of the straight chain \(N\)-alkyltetrahydropyridine derivatives, since the apparent \(K_m\) values decreased with increasing alkyl chain length, and the \(N\)-heptyl chain length was optimal for CYP2D6 substrate properties (see Table 2). Consistent with previous reports (Usuki et al., 1996; Fang et al., 2001), HP and its metabolites namely RHP, HPTP, and RHPTP were stable toward oxidation by CYP2D6.

**Structural Elucidation of Metabolites Derived from the CYP2D6-Catalyzed Oxidation of \(N\)-Alkyl-4-phenyl-1,2,3,6-Tetrahydropyridines and Pyridiniums.** \(N\)-Alkyl-4-phenyl-1,2,3,6-tetrahydropyridines. Attempts were also made to identify the metabolites obtained following CYP2D6-mediated oxidation of \(N\)-alkyl-4-phenyl-1,2,3,6-tetrahydropyridines (Table 4). Consistent with previous reports (Modi et al., 1997), CYP2D6-catalyzed oxidation of MPTP resulted in the formation of PTP (\(MH^+ = 160\), \(R_t = 9.40\) min) and \(N\)-4-(4'-hydroxyphenyl)-\(N\)-methyl-1,2,3,6-tetrahydropyridine (\(MH^+ = 161\), \(R_t = 9.40\) min).
TABLE 4
Metabolite identification following the CYP2D6 mediated oxidation of candidate N-alkyl-4-phenyl-1,2,3,6-tetrahydropyridines

<table>
<thead>
<tr>
<th>Tetrahydropyridine # (R)</th>
<th>Metabolic Pathways &amp; R_t (min)</th>
<th>Diagnostic Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>MPTP (CH3)</td>
<td>9.4</td>
<td>6.06</td>
</tr>
<tr>
<td>12 ((CH2)_2CH3)</td>
<td>10.0</td>
<td>11.0</td>
</tr>
<tr>
<td>14 ((CH2)_3CH3)</td>
<td>11.0</td>
<td>13.3</td>
</tr>
<tr>
<td>15 ((CH2)2CH3)</td>
<td>13.3</td>
<td>14.3</td>
</tr>
<tr>
<td>16 ((CH2)3CH3)</td>
<td>12.3</td>
<td>15.2</td>
</tr>
<tr>
<td>17 ((CH2)4CH3)</td>
<td>15.2</td>
<td>17.0</td>
</tr>
<tr>
<td>18 ((CH2)5CH3)</td>
<td>16.0</td>
<td>19.3</td>
</tr>
</tbody>
</table>

* R_t corresponds to the retention time of the individual metabolites.
* In the case of the pyridinium metabolites, authentic standards were available.
* The protonated iminium fragments are also common to the parent tetrahydropyridine.

= 190, R_t = 6.06 min), the structures of which were confirmed via CID of the respective protonated molecular ions and by comparison of the LC R_t with those of the synthetic standards. Likewise, LC/MS/MS analysis of the CYP2D6/N-propyl derivative (12, MH+ = 202, R_t = 10.4 min) mixture indicated that the protonated molecular mass of the more polar metabolite (MH+ = 218, R_t = 8.47 min) was 16 mass units higher than the parent tetrahydropyridine. The CID spectrum of parent and metabolite produced a common base fragment at mL_C + H+ = 72 consistent with the molecular mass of the protonated N-propylluminium ion suggesting that parahydroxylation had most likely occurred on the C-4 aromatic ring in 12 as was observed with MPTP and in rat liver microsomal incubations with the related N-cyclopropyl-4-phenyl(1,2,3,6)-tetrahydropyridine analog (Kuttab et al., 2001). The structure of the less polar metabolite corresponded to PTP. Besides aromatic hydroxylation and N-demethylation, trace quantities of N-propyl-4-phenylpyridinium (M+ = 190, mL_C + H+ = 156; R_t = 9.84 min) were also discernible in the incubation mixture.

CYP2D6-mediated oxidation of 1,2,3,6-tetrahydropyridines 14-19 also afforded two predominant polar metabolites in all cases (see Table 4). In all examples, the base peak in the CID spectrum of the less polar metabolite (for R_t see Table 4) was the protonated N-alkyliminium ion, common to the parent tetrahydropyridines as well. Thus, by analogy with MPTP and 12, we propose the structure of each of these metabolites to be the corresponding N-4(4’-hydroxyphenyl)-N-alkyl-1,2,3,6-tetrahydropyridine (see Table 4, pathway C). In contrast to MPTP and 12, long-chain tetrahydropyridines did not undergo the characteristic N-dealkylation reaction leading to PTP. Instead, a second hydroxylated metabolite was observed in all examples suggesting that hydroxylation had occurred at a site different from the aromatic ring and which led to a metabolite of greater polarity. The CID spectrum of these relatively more polar-hydroxylated metabolites (see Table 4 and Fig. 3) revealed the addition of 16 mass units to the base fragment of the protonated N-alkyliminium ions followed by the loss of 18 mass units (H_2O) suggesting that hydroxylation had occurred on the long-chain N-alkyl substituent or on the nitrogen atom leading to the formation of the corresponding N-oxides. The general unstable nature of carbinolamines ruled out hydroxylation on the endo- and exocyclic carbons α to the nitrogen atom.

N-oxidation was ruled out in the case of the N-heptyl derivative 15 since the authentic standard of N-heptyl-4-phenyl-1,2,3,6-tetrahydropyridine-N-oxide (22) eluted at an R_t different from the observed metabolite (synthetic N-oxide 22, R_t = 16.7 min; metabolite R_t = 11.0 min). Although the CID spectra indicated the fragment ion at mL_C + H+ = 144 as common to both the authentic N-oxide standard and the observed metabolite, the base fragment ions were different. Thus, the protonated base peak in the CID spectrum of the authentic N-oxide standard was 60, whereas that in the corresponding CID spectrum of the metabolite was 144 (see Fig. 3). Thus, these results suggest that hydroxylation had occurred on the heptyl side chain. Positional assignment of hydroxylation was not possible from the available mass spectral data and will require additional NMR analysis. Finally, as observed with 12, CYP2D6-mediated oxidation of all of the long-chain N-alkyltetrahydropyridines also resulted in the formation of trace levels of the respective pyridinium metabolites, the structures of which were confirmed via comparison of the LC/MS/MS properties with the authentic standards.

N-Alkyl-4-phenylpyridiniums. Interestingly, at high concentrations (200 μM), qualitative LC/MS/MS analysis of some of the lipophilic long-chain N-alkyl-4-phenylpyridinium derivatives (e.g., heptyl, octyl, nonyl, undecyl, and tetradecyl) also demonstrated the formation of trace levels of monohydroxylated metabolites following incubation with NADPH-supplemented recombinant CYP2D6. Thus, LC/MS/MS analysis of incubation mixtures revealed the formation of a single polar metabolite with molecular weight 16 mass units higher than the respective masses of the parent compounds. The CID spectrum indicated a base fragment at MH+ = 156 consistent with the molecular mass of protonated 4-phenylpyridine [4-((C6H5)CH3)C6H4N+] suggesting that CYP2D6-mediated hydroxylation of the long-chain N-alkylpyridiniums had exclusively occurred on the long-chain alkyl substituent as observed with the corresponding long-chain alkyl tetrahydropyridines.
Discussion

The present study clearly establishes the importance of lipophilicity in influencing CYP2D6 inhibition by quaternary pyridinium derivatives. Results from the QSAR analysis indicate that the hydrophilic nature of MPP\(/\text{H}11001\)/H11001\(N\)-methyl group is mainly responsible for the lack of CYP2D6 inhibition, since chain length extension of its \(N\)-methyl group to higher alkyl homologues substantially increases inhibitory potency. As the size of the \(N\)-alkyl group increases so does lipid solubility that results in increased cell penetration and allows access to the active site of the enzyme. QSAR studies revealed that a critical chain length, equivalent to a minimal degree of lipophilicity (clog \(P\)/H11350\)0), is essential for CYP2D6 inhibition and is further supported by a reasonably good correlation between the log IC\(_{50}\) values and the calculated lipophilicities of \(N\)-alkyl-4-phenylpyridiniums as well as the positively charged pyridinium metabolite of haloperidol (Fig. 2). An outlier in this correlation was the \(N\)-phenethyl-4-phenylpyridinium analog 11 of identical lipophilicity as the \(N\)-pentyl-4-phenylpyridinium analog 4 but ~12-fold more potent as a CYP2D6 inhibitor, suggesting that subtle differences exist in the active site binding of straight chain \(N\)-alkyl- and \(N\)-arylalkylpyridiniums. Detailed QSAR studies comparing CYP2D6 inhibition by a series of \(N\)-alkyl and \(N\)-arylalkylpyridiniums of similar lipophilicity should help in resolving this discrepancy. Overall, the identification of lipophilicity as an important physiochemical parameter for CYP2D6 inhibition is consistent with previous reports on secondary amine-containing \(\beta\)-adrenergic blockers (Ferrari et al., 1991) and \(N\)-alkylamine-substituted warfarin \(O\)-methyl ethers (Venhorst et al., 2000), which also indicated strong positive correlations between increased lipophilicity and low IC\(_{50}\) values for CYP2D6 inhibition.

The excellent correlation between lipophilicity and CYP2D6 inhibitory potency of the straight chain \(N\)-alkyl-4-phenylpyridiniums was also maintained with the straight chain \(N\)-alkyl-4-phenyl-1,2,3,6-tetrahydropyridine-N-oxide metabolite of \(N\)-hydroxyheptyl-4-phenyl-1,2,3,6-tetrahydropyridine, which was catalyzed by the CYP2D6-catalyzed oxidation of \(N\)-heptyl-4-phenyl-1,2,3,6-tetrahydropyridine, and parent \(N\)-heptyl-4-phenyl-1,2,3,6-tetrahydropyridine. Collectively, these findings suggest that \(N\)-alkyl side chains in pyridiniums and 1,2,3,6-tetrahydropyridines must bind in different regions of the protein, a feature that could be explored via specific SAR studies on heteroatom-containing \(N\)-alkyl substituents in pyridiniums.
Nefazodone: \( R = C \log \text{P} = 5.1 \)

Trazodone: \( R = C \log \text{P} = 3.2 \)

Buspirone: \( R = C \log \text{P} = 1.22 \)

Gepirone: \( R = C \log \text{P} = 0.83 \)

**Fig. 4.** Examples of cyclic tertiary amines that are inactive as CYP2D6 substrates.

Although, the lipophilicities of HPTP and reduced HPTP were comparable with the N-heptyltetrahydropyridine derivative, the HP metabolites were inactive as CYP2D6 substrates. This observation suggests that steric constraints rather than lipophilicity are responsible for the lack of CYP2D6 substrate properties of cyclic tertiary amines tethered to bulky N-arylalkyl substituents such as those found in HP and its metabolites. This phenomenon appears to be a common theme among cyclic tertiary amine-containing anti-depressants such as trazodone (Rotzinger et al., 1998), nefazodone (von Molitke et al., 1999), buspirone (Lilja et al., 1998), and gepirone (von Molitke et al., 1998) (Fig. 4). All of these drugs contain bulky N-alkyl substituents and are inactive as CYP2D6 substrates. Like HP and its metabolites, these compounds are exclusively metabolized by CYP3A4. Whether this property could be exploited as a general strategy for designing central nervous system agents devoid of CYP2D6 substrate properties remains to be explored.

Finally, metabolite identification studies indicated that N-alkyl-4-phenyl-1,2,3,6-tetrahydropyridines underwent monohydroxylations on the aromatic ring and on the N-alkyl substituent. Given the clear preference of CYP2D6 for aromatic hydroxylation (5–7 Å from the basic amine nitrogen), hydroxylation on the N-alkyl substituent (medium to long-chain N-alkyltetrahydropyridines) or N-demethylation (MPTP) seems to be a paradox, because it suggests that 1,2,3,6-

aminones and 1,2,3,6-tetrahydropyridines. Furthermore, the observation that N-(3-hydroxypropyl)tetrahydropyridine inhibits CYP2D6 is surprising, considering that its lipophilicity is comparable with MPTP and PTP that are inactive as CYP2D6 inhibitors. Thus, it appears that, in addition to lipophilicity, CYP2D6 inhibition is also influenced by the nature of the N-alkyl substituent within the 1,2,3,6-tetrahydropyridines. Collectively, this is reflected in a poor correlation between lipophilicity and inhibitory potency of this class of cyclic tertiary amines. An additional example of this behavior is evident upon comparison of the CYP2D6 inhibitory properties of HP and HPTP versus those of reduced HP and reduced HPTP. The less lipophilic-reduced compounds were ~6.0- and 71-fold more potent than the corresponding oxidized ones suggesting that presence of a hydrogen bond donor (OH group) instead of hydrogen bond acceptor (C = O) adjacent to the 4-fluorophenyl ring increases inhibitory potency. Active site docking of candidate pyridiniums and 1,2,3,6-tetrahydropyridines in the CYP2D6 homology model, especially within the lipophilic pocket, that has been predicted to bind alkyl substituents in 4-N-alkylaminomethyl-7-methoxy coumarinyl derivatives (Venhorst et al., 2000), amphetamines (de Groot et al., 1999a), and the H1 receptor antagonist terfenadine (Jones et al., 1998), may help resolve some of these issues.

An interesting observation was also noted in the course of inhibition studies with HP metabolites in that the IC_{50} values for inhibition of the bufuralol-1'-hydroxylase activity of CYP2D6 by (±)-RHPTP and HPP\textsuperscript{+} were ~16- and 6-fold higher than previously reported for inhibition of the dextromethorphan O-demethylase activity. In contrast, IC_{50} values for CYP2D6 inhibition of HP and (±)-RHPTP were substrate-independent. This finding raises the possibility that CYP2D6 inhibition is substrate-dependent in a manner similar to that reported for the CYP3A4 isozyme (Wang et al., 2000; Schrag and Wienkers, 2001). Comparing the inhibitory potencies of CYP2D6 inhibitors against diverse CYP2D6 substrates can confirm this hypothesis.

As observed in the course of the inhibition studies, increasing the length of the N-alkyl substituent in MPTP vastly improved the CYP2D6 substrate properties of the resulting analogs such that N-heptyl-4-phenyl-1,2,3,6-tetrahydropyridine demonstrated a >100-fold decrease in \( K_m \) compared with the parent. It is interesting to point out that in many cases, 1,2,3,6-tetrahydropyridines were better CYP2D6 inhibitors than substrates as reflected by the significantly higher \( K_m \) compared with the IC_{50} values. Similar observations have been noted with other CYP2D6 substrates and inhibitors including the antiemetic metoclopramide, which is a better inhibitor (IC_{50} = 1 \mu M) than substrate (\( K_m = 68 \mu M \)) (Desta et al., 2002). A potential explanation for this anomaly could be that 1,2,3,6-tetrahydropyridines inhibit CYP2D6 in a partially competitive fashion as opposed to purely competitive inhibition that generally results in similar inhibition (\( K_i \)) and Michaelis-Menten (\( K_m \)) constants. To verify this proposal, detailed kinetic characterization of CYP2D6 inhibition by 1,2,3,6-tetrahydropyridines and pyridiniums is currently underway.
tetrahydrodipyrindines can bind in the completely opposite or “upside-down” orientation in a single active site model. An alternate explanation for CYP2D6-mediated N-dealkylation/N-alkylhydroxylation reactions is that the protonated amine binds to Asp301 but is rapidly deprotonated to yield an uncharged amine for single electron oxidation (Grace et al., 1994). Our preliminary observation on the weak CYP2D6 substrate properties of the long-chain N-alkylpyridinum compounds suggests that additional SAR on the N- and C-4 substituent could potentially provide a good quaternary amine-based CYP2D6 substrate, which should prove valuable in investigating these proposals.

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