Synthesis of Substituted Phenyl Diaziridines and Characterization as Mechanism-Based Inactivators of Human Cytochrome P450 2B6

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ABSTRACT:
The metabolism of arylhydrazines by cytochromes P450 (P450s) has previously been shown to yield aryl-iron complexes that inhibit P450 enzymes as a result of heme modification. These modifications of the heme have been used to probe the topology of the active site of several P450s. Therefore, diaziridines containing one or more substitutions on the phenyl ring were synthesized and evaluated as potential mechanism-based inactivators of P450 2B enzymes that could be used to elucidate the active site topology. Five of the six trifluoroaryldiaziridines tested selectively inactivated P450 2B6 in the reconstituted system in a time-, concentration-, and NADPH-dependent manner as measured using the 7-ethoxy-4-(trifluoromethyl)coumarin O-deethylation assay. The kinetic parameters for P450 2B6 inactivation by the five compounds were calculated. Analysis of the P450 heme from P450s inactivated by the five substituted diaziridines suggested that the activity loss was not due to heme destruction as measured by the reduced-CO spectrum or high-performance liquid chromatography of the P450 heme. Dialysis experiments indicated the irreversible nature of the inactivation and the reaction between the diaziridine compounds and the P450 enzyme. Interestingly, a thiomethyl-substituted phenyl diaziridine had no effect on the activity of P450 2B6 in the reconstituted system, but competitively inhibited the O-debenzylation activity of P450 3A4 with 7-benzyloxy-4-(trifluoromethyl)coumarin as substrate. Binding spectra suggest that this compound bound reversibly to P450 2B6, and preliminary results indicate that 3-[4-(methylthiophenyl)-3-(trifluoromethyl)diaziridine is metabolized by P450 2B6.

The membrane-bound mammalian cytochromes P450 (P450s) are heme proteins that play an important role in the metabolism of endogenous compounds as well as in the oxidation of myriad xenobiotics such as drugs and chemical carcinogens (Gonzalez, 1988; Porter and Coon, 1991). P450s catalyze a variety of reactions including aromatic and aliphatic hydroxylations, epoxidations, dealkylations, and dehydrogenations (Silverman, 1995). Some xenobiotics are subject to specific catalytic bioactivation events by P450 enzymes that result in the production of reactive intermediates that modify and inactivate the P450s. Several structurally distinct groups of mechanism-based inactivators of P450 have been examined including, but not limited to, acetylenes, isothiocyanates, and xanthates. Loss of P450 function has been documented to proceed through either covalent modification of the apoprotein or alkylation of the prosthetic heme group. The development of selective mechanism-based inactivators for P450 is critical for probing individual isozyme function and structure, and for potentially modulating P450 activity in a therapeutic setting (Kent et al., 2001; Coon, 2005).

Diaziridines are three-membered ring heterocyclic compounds that exhibit chemical and physiochemical characteristics analogous to both amines and hydrazines. Diaziridines have been used as precursors for the preparation of diazirines, photolabile groups that serve as robust photoaffinity reagents for receptor mapping studies. A report has also described the incorporation of diaziridine groups as part of structure-activity relationship studies (Hartmann et al., 2000). An earlier study showed that preincubation of 1,2-dimethyl-3-phenylhydrazine with rat liver microsomes resulted in the formation of methylamine, p-chlorobenzaldehyde, and p-chlorobenzylalcohol. The initial step in the metabolism of the diaziridine ring was believed to be a consequence of reduction of this ring (Hata and Watanabe, 1982). Studies have also shown that incubation of various cytochromes P450 including 1A1, 2B1, 2B2, and 2E1 with phenylhydrazines or phenyl diazene yielded inactive protein complexes with absorption maxima at 474 to 480 nm (Raag et al., 1990; Swanson et al., 1991). Phenylethylhydrazine, an antidepressive agent, has been shown to inhibit cytochrome P450 in rat microsomes by heme destruction (Tuck and Ortiz de Montellano, 1992). The inactivation of cytochrome P450 enzymes...
by hydrazines, olefins, and acetylenes has been shown to involve heme adduct formation (Muakkassah and Yang, 1981; Ortiz de Montellano et al., 1981; Blobaum et al., 2002).

Thus, in our attempts to design a new class of specific mechanism-based inhibitors for P450s, the diaziridine functional group containing two contiguous basic nitrogen atoms in a strained three-membered ring presented an attractive feature. A single electron transfer, occurring in the initial step of catalysis, may generate a reactive intermediate capable of inactivating the P450 (Scheme 1). We hypothesized that the prosthetic heme could possibly oxidize one of the equivalent diaziridine nitrogens to the corresponding amine radical cation, analogous to the oxidative catalytic mechanisms involving N-dealkylation reactions with P450 (Gonzalez, 1988; Silverman, 1995). Additional evidence for the practicality of using diaziridines as mechanism-based inactivators was found in a recent chemical model study that demonstrated that carbones can be generated directly from their respective diaziridines (Post and Morrison, 1996). The steps leading to carbene formation were believed to involve an initial single electron transfer from a diaziridine to the carbonyl triplet excited state to form an initial diaziridine radical cation. Homolytic bond scission followed by protonation and hydrogen atom abstraction (or e\(^-\), H\(^+\) loss) afforded cyclohexanecarbene (identified by trapping with methanol-d\(_4\) and N\(_2\)). Related bond scission reactions have been reported in the reaction of oxaziridines with ferrous sulfate. These chemical model studies support the hypothesis that diaziridine amine radical cation formation is both viable and favorable, and leads to highly reactive carbene or radical intermediates.

Thus, based on this hypothesis, six different diaziridines (1a-f, Fig. 1) with substitutions on the phenyl ring were synthesized and assessed for their potential to inhibit different P450 isoforms in vitro using purified P450s in a reconstituted system. This study shows that a number of these diaziridine compounds form reactive intermediates that act as mechanism-based inactivators and are selective for human P450 2B6. The study further indicates that diaziridines with para-alkoxide substitutions inactivated P450 2B6 in a mechanism-based manner.

Materials and Methods

Materials. All chemicals and reagents for the synthesis of compounds used in this study were purchased from Sigma-Aldrich (St. Louis, MO). Thin-layer chromatography was performed on Merck (Darmstadt, Germany) silica gel 60 F\(_{254}\) plates. Column chromatography was performed using standard grade Sorbent Technologies (Atlanta, GA) silica gel with a particle size of 32 to 63 \(\mu\)m. Mass spectral data were acquired using a Waters (Milford, MA) ZQ LCMS system (electrospray ionization positive mode), and HRMS data were acquired using a Micromass QTOF. Elemental analysis was performed using a PerkinElmer (Boston, MA) Series II 2400 CHNS/O Elemental Analyzer. \(^1\)H NMR spectra were recorded at 400 MHz on a Bruker (Newark, DE) Avance DXR 400, using CDCl\(_3\) as a solvent. The following compounds were synthesized as described previously: 4-methoxyphenyl series, 1a, 3a, 4a, 5a (Hatanaka et al., 1994); and 4-methyl thio series, 1d, 3d, 4d, 5d (Findlay et al., 1995).

Dilauroyl-1-\(\alpha\)-phosphatidylcholine, bovine serum albumin, NADPH, catalase, glutathione, sodium dithionite, and Hepes used in the assays were purchased from Sigma-Aldrich. BFC was obtained from BD Gentest (Woburn, MA). 7-EFC was obtained from Invitrogen (Carlsbad, CA). HPLC-grade acetonitrile was purchased from Mallinckrodt (Chesterfield, MO). Slide-A-Lyzer cassettes were obtained from Pierce Chemical (Rockford, IL).

General Procedure for the Preparation of Ketones (3a-f). Magnesium turnings (2.41 g, 0.1 mol), substituted bromobenzenes, 2a-f (0.1 mol), and anhydrous tetrahydrofuran (80 ml) were placed in a round-bottom flask. The mixture was heated to reflux, and refluxing was maintained until all the Mg was consumed. The mixture was cooled in an ice bath, and a solution of N-trifluoracetyl/piperidine (0.1 mol) in anhydrous tetrahydrofuran (25 ml) was added slowly to the Grignard reagent over a period of 0.5 h with stirring at 0°C. The reaction mixture was stirred for 2 h at ambient temperature, and the reaction was quenched by the addition of saturated aqueous ammonium chloride (10 ml). The precipitated solids were filtered, and the filtrate was dried over Na\(_2\)SO\(_4\) and evaporated in vacuo, and the residual oil was purified by column chromatography (Si gel eluting with hexanes/CH\(_2\)Cl\(_2\) (95:5) to give the product ketone.

2.2.2-Trifluoro-1-(4-methoxyphenyl)ethanone (3a). Yield, 14.6 g (72%); oil. \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 3.90 (s, 3H), 7.00 (d, 2H, \(J = 8.4\) Hz), 8.04 (d, 2H, \(J = 8.4\) Hz).

2.2.2-Trifluoro-1-(4-ethoxyphenyl)ethanone (3b). Yield, 15.2 g (70%); oil. \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 1.45 (t, 3H, \(J = 6.8\) Hz), 4.12 (q, 2H, \(J = 6.8\) Hz), 6.93 (d, 2H, \(J = 8.8\) Hz), 8.02 (d, 2H, \(J = 8.8\) Hz).

2.2.2-Trifluoro-1-(3,4-dimethoxyphenyl)ethanone (3c). Yield, 18.2 g (78%); oil. \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 3.95 (s, 3H), 3.99 (s, 3H), 6.95 (d, 2H, \(J = 8.6\) Hz), 7.56 (s, 1H), 7.72 (d, 2H, \(J = 8.4\) Hz).

2.2.2-Trifluoro-1-(4-methylthiophenyl)ethanone (3d). Yield, 14.3 g (65%); m.p. = 78°C. \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 3.95 (s, 3H), 3.99 (s, 3H), 6.95 (d, 2H, \(J = 8.6\) Hz), 7.56 (s, 1H), 7.72 (d, 2H, \(J = 8.4\) Hz).

2.2.2-Trifluoro-1-(3-methyl-4-methoxyphenyl)ethanone (3e). Yield, 14.8 g (68%); m.p. = 78°C. \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 2.26 (s, 3H), 3.94 (s, 3H), 6.91 (d, 1H, \(J = 8.4\) Hz), 7.87 (s, 1H), 7.94 (d, 1H, \(J = 8.4\) Hz).

2.2.2-Trifluoro-1-(3,4,5-trimethoxyphenyl)ethanone (3f). Yield, 21.1 g (80%); amorphous solid. \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 3.85 (bs, 9H), 6.58 (s, 2H).

General Procedure for the Synthesis of Oximes (4a-f). Hydroxylamine hydrochloride (0.125 mol), added to a solution of ketones 3a-f (0.05 mol) in absolute ethanol (25 ml) and dry pyridine (40 ml), was heated at 60°C for 8.0 h. After the solvent was removed, the remaining residue was dissolved in diethyl ether (40 ml) and washed with 1 N HCl. The organic layer was washed with water (50 times, 2 ml), and dried over Na\(_2\)SO\(_4\). After evaporation of the solvent, the crude oxime was purified by column chromatography (Si gel eluting with hexanes/CH\(_2\)Cl\(_2\) (90:10) to give white to off-white solids as mixtures of (E/Z) isomers.

2.2.2-Trifluoro-1-(4-methoxyphenyl)ethanone oxime (4a). Yield, 8.5 g (78%); m.p. 95–96°C (lit: 78–79°C). \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 6.85 (s, 3H), 6.99 (d, 2H, \(J = 8.4\) Hz), 7.54 (d, 1H, \(J = 8.4\) Hz), 8.42 (bs, 1H). HRMS: \(\text{m/z} [\text{M} + \text{H}]^+ = 220.0588\). (C\(_9\)H\(_9\)NO\(_2\) requires 220.0585).

2.2.2-Trifluoro-1-(4-ethoxyphenyl)ethanone oxime (4b). Yield, 9.0 g (78%); m.p. 94–95°C. \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 7.75 (t, 3H, \(J = 6.8\) Hz), 4.08 (q, 2H, \(J = 6.8\) Hz), 6.95 (d, 2H, \(J = 8.8\) Hz), 7.52 (d, 2H, \(J = 8.4\) Hz), 8.43 (bs, 1H). HRMS: \(\text{m/z} [\text{M} + \text{H}]^+ = 234.0734\). (C\(_9\)H\(_9\)FNO\(_2\) requires 234.0742).

2.2.2-Trifluoro-1-(3,4-dimethoxyphenyl)ethanone oxime (4c). Yield, 9.9 g
(80%); m.p. 132–134°C; ¹H NMR (CDCl₃): δ 3.89 (s, 3H), 3.92 (s, 3H), 6.95 (d, 1H, J = 8.0 Hz), 7.08 (s, 1H), 7.15 (d, 1H, J = 8.0 Hz), 8.37 (bs, 1H). HRMS: m/z [MH⁺] 250.0606. (C₁₁H₁₆F₃NO₄ requires 250.0609).

2,2,2-Trifluoro-1-[((4-methylthio)phenyl)ethanone oxime (4d). Yield, 7.2 g (62%); m.p. 78–80°C (lit. 64.5–77.5°C). ¹H NMR (CDCl₃): δ 2.47 (s, 3H), 7.30 (d, 2H, J = 8.4 Hz), 7.47 (d, 2H, J = 8.0 Hz), 8.60 (bs, 1H). HRMS: m/z [MH⁺] 236.0368. (C₁₁H₁₇NO₃F requires 236.0357).

2,2,2-Trifluoro-1-[((3-methyl-4-methoxyphenyl)ethanone oxime (4e). Yield, 7.5 g (65%); m.p. 96–99°C; ¹H NMR (CDCl₃): δ 2.25 (s, 3H), 3.87 (s, 3H), 6.88 (d, 1H, J = 8.4 Hz), 7.36 (s, 1H), 7.40 (d, 1H, J = 8.0 Hz), 8.79 (bs, 1H). HRMS: m/z [MH⁺] 234.0736. (C₁₀H₁₁F₃NO₂ requires 234.0742).

2,2,2-Trifluoro-1-[(3,4-dimethoxyphenyl)ethanone oxime (4f). Yield, 10.0 g (72%); m.p. 177–179°C; ¹H NMR (CDCl₃): δ 3.89 (s, 9H), 6.92 (d, 2H, J = 8.6 Hz), 7.00 (m, 1H), 8.42 (bs, 1H). HRMS: m/z [MH⁺] 280.0788. (C₁₀H₁₃F₃NO₃ requires 280.0797).

General Procedure for the Preparation of Tosylates (5a–f). To 40 ml of CH₂Cl₂ was added oximes 4a–f (0.05 mol), methylaminopyridine (0.01 mol), and p-toluenesulfonyl chloride (1.00 mol) in 10 ml of CH₂Cl₂. The mixture was allowed to evaporate. The remaining residue was dissolved in diethyl ether and washed with water and brine. Subsequent drying over Na₂SO₄ and evaporation yielded the crude products, which were purified by column chromatography (Si gel) (1–10% ethyl acetate, CHCl₃) to yield the final diaziridines.

3-(4-Methoxyphenyl)-3-(trifluoromethyl) diaziridine (1a). Yield, 0.15 g (72%); m.p. 72°C; (lit. 69–70°C). ¹H NMR (CDCl₃): δ 2.15 (s, 1H), 2.75 (bs, 1H), 3.85 (s, 3H), 6.95 (d, 2H, J = 8.0 Hz), 7.56 (d, 2H, J = 8.4 Hz). HRMS: m/z [MH⁺] 219.0753. (C₁₀H₁₂F₃NO requires 219.0745). Anal. Calcld for C₁₀H₁₁F₃NO: C, 55.93; H, 4.60; N, 11.90.

3-(4-Ethoxyphenyl)-3-(trifluoromethyl) diaziridine (1b). Yield, 0.17 g (76%); m.p. 66–67°C; ¹H NMR (CDCl₃): δ 1.14 (t, 3H, J = 6.8 Hz 2.15 (bs, 2H), 4.05 (q, 2H, J = 6.8 Hz), 6.91 (d, 2H, J = 8.4 Hz), 7.51 (d, 2H, J = 8.4 Hz). HRMS: m/z [MH⁺] 233.0906. (C₁₀H₁₁F₃NO requires 233.0920). Anal. Calcld for C₁₀H₁₁F₃NO: C, 51.73; H, 4.77; N, 12.06. Found: C, 51.59; H, 4.67; N, 11.81.

3-(4-Dimethoxyphenyl)-3-(trifluoromethyl) diaziridine (1c). Yield, 0.19 g (80%); m.p. 66–67°C; ¹H NMR (CDCl₃): δ 2.17 (bs, 1H), 2.74 (bs, 1H), 3.91 (s, 6H), 6.88 (d, 1H, J = 8.4 Hz), 7.10 (s, 1H), 7.19 (d, 1H, J = 8.4 Hz). HRMS: m/z [MH⁺] 249.0863. (C₁₀H₁₂F₃NO requires 249.0851). Anal. Calcld for C₁₀H₁₁F₃NO: C, 48.39; H, 4.47; N, 11.29. Found: C, 48.34; H, 4.37; N, 11.13.

3-(4-Methylthiophenyl)-3-(trifluoromethyl) diaziridine (1d). Yield, 0.14 g (62%); m.p. 107–109°C (lit. 105–107.5°C). ¹H NMR (CDCl₃): δ 2.18 (bs, 1H), 2.49 (s, 3H), 2.75 (bs, 1H), 7.26 (d, 2H, J = 8.4 Hz), 7.51 (d, 2H, J = 8.4 Hz). HRMS: m/z [MH⁺] 235.0508. (C₁₀H₁₁F₃NO requires 235.0517). Anal. Calcld for C₁₀H₁₀F₃NO: C, 46.15; H, 3.87; N, 11.96. Found: C, 46.30; H, 3.69; N, 11.90.

3-(4-Methyl-4-methoxyphenyl)-3-(trifluoromethyl) diaziridine (1e). Yield, 0.15 g (65%); yellow oil. ¹H NMR (CDCl₃): δ 2.15 (br, d, 3H, J = 6.8 Hz), 2.76 (br, d, 3H, J = 6.8 Hz), 3.85 (s, 3H), 6.82 (d, 1H, J = 6.8 Hz), 7.36 (m, 1H), 7.40 (d, 1H, J = 8.4 Hz). HRMS: m/z [MH⁺] 233.0910. (C₁₀H₁₀F₃NO requires 233.0920). Anal. Calcld for C₁₀H₁₀F₃NO: C, 51.73; H, 4.77; N, 12.06. Found: C, 52.21; H, 4.94; N, 11.25.

3-(4,5-Trimethoxyphenyl)-3-(trifluoromethyl) diaziridine (1f). Yield, 0.19 g (68%); m.p. 110–112°C; ¹H NMR (CDCl₃): δ 2.22 (br, d, 1H, J = 8.4 Hz), 2.76 (br, d, 1H, J = 8.4 Hz), 3.85 (s, 3H), 6.84 (s, 1H), 6.84 (s, 1H), 7.12 (d, 1H, J = 8.4 Hz). HRMS: m/z [MH⁺] 279.0967. (C₁₀H₁₂F₃NO requires 279.0957). Anal. Calcld for C₁₀H₁₁F₃NO: C, 47.49; H, 4.71; N, 10.07. Found: C, 47.49; H, 4.57; N, 9.93.

Purification of Enzymes. P450 NADPH-reductase from rat was expressed in Escherichia coli Top3 cells. Expression and purification was carried out as described by Hanna et al. (1998). P450 2B6 was expressed in JM109 cells and purified as described previously (Hanna et al., 2000). Expression and purification of P450s 2C9, 3A4, 2D6, and 2E1 was carried out according to previously published procedures (Gillam et al., 1993; Shimada et al., 1998).
of catalase. At the three time points indicated, 9-pmol aliquots of the enzyme were transferred into a secondary reaction mixture containing 1 mM NADPH, 50 μM FBC, 4 mM MgCl₂, and 40 μg/ml BSA in 200 mM potassium phosphate buffer (pH 7.4) and incubated for 10 min at 30°C with shaking. The reactions were quenched with a solution composed of 80% acetonitrile and 20% 0.5 M Tris. The BFC O-debenzylation activity was measured using fluorescence as indicated above with excitation and emission wavelengths of 409 and 530 nm, respectively.

**Inactivation of P450 2B6 by Trifluoroaryldiaziridines.** For the inactivation reactions, P450 2B6 (0.5 nmol) was reconstituted with reductase (1 nmol) at 4°C for 45 min. The reconstituted enzyme mixture was supplemented with the corresponding oximes (4a–f), which were converted to the tosyl oximes (5a–f) by reaction with p-toluenesulfonyl chloride. Reaction of the tosyl oximes with ammonia yielded diaziridines as the final products (1a–f). The diaziridines were characterized by analysis of the 1H and 13C NMR, in addition to mass spectrometry and elemental analysis. NMR figures of the compounds can be found in the Supplemental Data.

**Determination of Heme Loss and Irreversibility of the Inactivation.** Reconstitution and inactivation of P450 2B6 by the various trifluoroaryldiaziridines was carried out as described above. At 0 and 15 min after initiation of the inactivation reactions, an aliquot of each of the samples was assayed for 7-EFC O-deethyl- and 7-EFC O-debenzylation activity. A second aliquot (100 pmol) of each of the control (−inactivator, −NADPH), exposed (−inactivator, +NADPH), and inactivated samples (+inactivator, +NADPH) was then injected onto a C4 column (250 × 4.6 mm; Phenomenex, Torrance, CA) equilibrated with 30% acetonitrile, 0.1% trifluoroacetic acid. The components of the reconstituted system were resolved by linearly increasing the percentage of acetonitrile to 90% over a period of 30 min. The effluent was monitored at 405 nm for intact heme, and the areas under the heme peaks of the control, exposed, and inactivated samples were integrated and compared. Another portion of the primary reaction mixture was used to determine the reduced-CO spectra by transferring 100 pmol of the control, exposed, and inactivated samples into quench buffer containing 50 mM potassium phosphate, 40% glycerol, and 0.6% Tergitol. The samples were bubbled with CO, sodium dithionite was added, and the reduced-CO spectra were recorded between 400 and 500 nm on a DW2 UV/VIS spectrophotometer equipped with an OLIS operating system (On Line Instruments Systems, Bogart, GA). The remainder of each sample was dialyzed overnight in a Slide-A-Lyzer dialysis cassette at 4°C two times against 500 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol. Portions of the dialyzed samples were assayed for activity in the presence or absence of fresh reductase. The dialyzed samples were also assayed for reduced-CO spectra and for heme loss by HPLC.

**Partition Ratio.** The reconstituted mixture containing 0.5 μM P450 2B6 was incubated with 0 to 100 μM concentration range of the trifluoroaryldiaziridines (1a–f) and 1.2 mM NADPH. The reactions were allowed to go to completion by incubating for 30 min at 30°C. Aliquots of the reaction mixture were assayed for residual 7-EFC activity at 0 and 30 min as described above. Partition ratios were calculated as previously described (Kent et al., 2001).

**Results**

**Synthesis of Trifluoroaryldiaziridine Compounds.** Trifluoromethylaryl-diaziridines (1a–f) were synthesized from a series of commercially available aromatic bromides in a convenient four-step protocol as shown in Scheme 2. Treatment of the bromobenzenes (2a–f) with magnesium, followed by reaction with N-trifluoroacetylperipiperidine, afforded the trifluoromethyl ketones (3a–f). The reaction of the ketones (3a–f) with hydroxylamine yielded the trifluoromethyl ketones (3a–f), which were converted to the tosyl oximes (5a–f) by reaction with p-toluene sulfonic acid. Reaction of the tosyl oximes with ammonia yielded diaziridines as the final products (1a–f). The diaziridines were characterized by analysis of the 1H and 13C NMR, in addition to mass spectrometry and elemental analysis. NMR figures of the compounds can be found in the Supplemental Data.

**Effects of Trifluoroaryldiaziridines on Human Cytochromes P450.** Six phenyl diaziridines with substitutions on the phenyl ring were incubated at concentrations of 1, 10, and 100 μM with human cytochromes P450 2B6 (2B6, 2C9, 2D6, 2E1, and 3A4) in the reconstituted system. Time- and concentration-dependent loss was seen with five of the six phenyl diaziridines when incubated with P450 2B6 as measured by 7-EFC O-deethyl-activity. Table 1 shows the loss in the 7-EFC O-deethyl-activity after 16 min when 100 μM concentrations of the phenyl diaziridines were incubated with P450 2B6. The phenyl diaziridine with the 4-methyl thi substitution (1d) did not affect the 7-EFC O-deethyl-activity of P450 2B6. However, this diaziridine competitively inhibited P405 3A4A, exhibiting a 50% decrease in the BFC O-debenzylation activity at a concentration of 100 μM. Less than 10% loss in the 7-EFC O-deethyl-activity was seen with P450 2E1, 2D6, and 2C9 when incubated with any of the substituted phenyl diaziridines. Competitive inhibition of the BFC O-debenzylation activity of P450 3A4A was also seen when this enzyme was incubated with the dimethoxy substituted diaziridine (1e).

**Effect of Trifluoroaryldiaziridines on P450 2B6 Inactivation.** The kinetics for the inactivation of purified P450 2B6 by the substituted phenyl diaziridines in the reconstituted system were determined. At various time points, an aliquot of the primary reaction mixture was transferred into a secondary buffer containing EFC to measure the residual activity. Figure 2 shows the effect of 3-(trifluoromethyl)-3-methoxy-(4-methylphenyl)diaziridine (1e) on the 7-EFC O-deethyl-activity of P450 2B6 as a representative experiment. Addition of this compound to the P450 2B6 reconstituted system in the absence of NADPH reduced the 7-EFC O-deethyl-activity of P450 2B6 marginally, whereas the loss in activity ranged from 60 to 70% in the presence of NADPH when incubated with up to 50 μM concentrations of five of the substituted phenyl diaziridines. The loss in activity increased with time and with increasing concentration with all trifluoroaryldiaziridines (data not shown), except with the thiomethyl-substituted phenyl diaziridine (1d), which did not result in any inactivation of the enzyme. The time course studies demonstrated that the inactivation by the trifluoroaryldiaziridines resulted in pseudo-first
order losses in enzymatic activity. The inactivation rates also exhibited the expected concentration dependence with respect to the inactivator as shown in the inset of Fig. 2. The kinetic parameters for the five trifluoroaryldiaziridines are shown in Table 2 and were calculated from the double reciprocal plots of the rates of inactivation as a function of the inactivator concentration. The data represent the mean and standard error from three separate experiments. The inset shows a double reciprocal plot of the rate of inactivation of the 7-EFC O-deethylation activity as described under Materials and Methods. The kinetic constants represent the mean and standard error from three different experiments. The inset shows a double reciprocal plot of the rate of inactivation of the 7-EFC O-deethylation activity as a function of the inactivator concentration. The data represent the mean and standard error from three separate experiments.

### Determination of Heme Loss and Irreversibility

The loss in the 7-EFC O-deethylation activity of P450 2B6 upon exposure to the five trifluoroaryldiaziridines in the presence of NADPH was compared with the amount of heme remaining, and the results are shown in Table 3. The 7-EFC O-deethylation activity of P450 2B6 decreased by approximately 70% after incubation with 10 μM (3-(trifluoromethyl)-3-methoxy-(4-methylphenyl)diaziridine. The reaction mixtures were incubated in the reconstituted system with NADPH and 0.1 μM (L), 1 μM (∆), 5 μM ( ), 10 μM ( ) or 50 μM ( ) of substituted phenyl diaziridines. At the indicated time points, aliquots of the reaction mixture were assayed for residual activity as described under Materials and Methods. The inset shows a double reciprocal plot of the rate of inactivation of the 7-EFC O-deethylation activity as a function of the inactivator concentration. The data represent the mean and standard error from three separate experiments. The inset shows a double reciprocal plot of the rate of inactivation of the 7-EFC O-deethylation activity as a function of the inactivator concentration. The data represent the mean and standard error from three separate experiments.

### Table 1

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<tr>
<th>Substituted Phenyl Diaziridines</th>
<th>Activity Loss (% Control)</th>
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<tr>
<td>4-Methoxy (1a)</td>
<td>65%</td>
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<tr>
<td>4-Ethoxy (1b)</td>
<td>62%</td>
</tr>
<tr>
<td>3,4-Dimethoxy (1c)</td>
<td>70%</td>
</tr>
<tr>
<td>3-Methyl,4-methoxy (1e)</td>
<td>70%</td>
</tr>
<tr>
<td>3,4,5-Trimethoxy (1f)</td>
<td>70%</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Substituted Phenyl Diaziridines</th>
<th>Activity Remaining</th>
<th>P450 Remaining by Reduced CO</th>
<th>Heme Remaining by HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Methoxy (1a)</td>
<td>35 ± 6</td>
<td>87 ± 2</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>4-Ethoxy (1b)</td>
<td>55 ± 20</td>
<td>99 ± 11</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>3,4-Dimethoxy (1c)</td>
<td>45 ± 4</td>
<td>73 ± 7</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>3-Methyl,4-methoxy (1e)</td>
<td>30 ± 7</td>
<td>90 ± 4</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>3,4,5-Trimethoxy (1f)</td>
<td>39 ± 1</td>
<td>81 ± 2</td>
<td>64 ± 4</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Substituted Phenyl Diaziridines</th>
<th>Activity Remaining</th>
<th>P450 Remaining by Reduced CO</th>
<th>Heme Remaining by HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Methoxy (1a)</td>
<td>35 ± 6</td>
<td>87 ± 2</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>4-Ethoxy (1b)</td>
<td>55 ± 20</td>
<td>99 ± 11</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>3,4-Dimethoxy (1c)</td>
<td>45 ± 4</td>
<td>73 ± 7</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>3-Methyl,4-methoxy (1e)</td>
<td>30 ± 7</td>
<td>90 ± 4</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>3,4,5-Trimethoxy (1f)</td>
<td>39 ± 1</td>
<td>81 ± 2</td>
<td>64 ± 4</td>
</tr>
</tbody>
</table>

The 7-EFC O-deethylation activity of the trifluoroaryldiaziridine-inactivated P450 2B6 samples was not restored after overnight dialysis, suggesting that the inactivation was not due to competition but was irreversible under these conditions. Furthermore, no recovery of the P450 activity was observed after addition of fresh reductase to the inactivated samples, suggesting that the inactivation was due to covalent modification of the P450 and not the reductase (data not shown).

### Partition Ratio

The number of molecules of the various substituted phenyl diaziridines metabolized per molecule of P450 2B6 inactivated was determined as described under Materials and Methods. The reactions were allowed to go to completion (30 min) and the turnover number was estimated from a graph of the percentage of activity remaining as a function of the molar ratio of inactivator to P450 2B6 (Kent et al., 2001). The partition ratios for the five diaziridine compounds tested ranged from approximately 10 to 62 as shown in Table 4.
Partitions ratios for the inactivation of P450 2B6 by the substituted phenyldiaziridines

<table>
<thead>
<tr>
<th>Substituted Phenyl Diaziridines</th>
<th>Partition ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Methoxy (1a)</td>
<td>41</td>
</tr>
<tr>
<td>4-Ethoxy (1b)</td>
<td>62</td>
</tr>
<tr>
<td>3,4-Dimethoxy (1c)</td>
<td>9.6</td>
</tr>
<tr>
<td>3-Methyl,4-methoxy (1e)</td>
<td>29</td>
</tr>
<tr>
<td>3,4,5-Trimethoxy (1f)</td>
<td>45</td>
</tr>
</tbody>
</table>

Assay conditions are described under Materials and Methods. The partition ratio is determined by incubating various concentrations of the inactivator molecules with P450 2B6 in the reconstituted system and allowing the reaction to go to completion. The partition ratio was then calculated from the graph of the percentage of activity remaining as a function of the ratio of the inactivator to enzyme concentration (Kent et al., 2001). The numbers represent the average of the results from three experiments with each substituted phenyl diaziridine.

Discussion

We report here the synthesis of a new class of compounds containing a diaziridine moiety as a functional group and the evaluation of these compounds as mechanism-based inactivators of P450s. These compounds also have the potential to be used to target a variety of other oxidizing enzymes. It was anticipated that the heme-containing P450s would oxidize one of the diaziridine nitrogens to an amine radical cation leading to the formation of benzyl radicals. Radicals of type \( r = \text{benzyl} \), as shown in Scheme 3, have been implicated in the denaturation of hemoglobin by reacting with a cysteinyl thiol group and generation of a hemoglobin-thiyl free radical (Hb-S) (Maples et al., 1988). Also, with diazirines, it is possible that complexes could occur between the partially oxidized diaziridines and the heme center of the enzymes analogous to the reaction of alkyl diazidines with cytochrome P450, leading to the formation of polar porphyrin (Tuck and Ortiz de Montelano, 1992). It has also been shown that 1,2-disubstituted hydrazines lacking \( \alpha \)-hydrogens lead to stable azo derivatives that may then be further oxidized to their corresponding azoxy compounds (Lindeke, 1982). An accepted reaction mechanism for the \( N \)-oxidation of monosubstituted hydrazine is shown in Scheme 3. Starke et al. (1984) reported that hemoglobin exhibited monooxygenase activity in vitro with a variety of substrates in a system containing P450 reductase, NADPH, and O\(_2\), closely resembling the reactions catalyzed by P450s. These studies revealed a novel strategy to synthesize a new class of mechanism-based inhibitors for heme-containing enzymes using diaziridines. As described here, six substituted phenyl diaziridine compounds were synthesized and characterized using \(^1\)H and \(^13\)C NMR in addition to mass spectrometry and elemental analysis.

The six trifluoromethylaryl diaziridines were screened for their ability to inactivate a number of different recombinant human cytochromes P450 in the reconstituted system. The abilities of these compounds to inactivate various purified P450s in the reconstituted system are reported in Table 1. Five of the six trifluoromethylaryl diaziridines showed time-, concentration-, and NADPH-dependent inactivation of the 7-EFC O-deethylation activity of P450 2B6 in a reconstituted system. These five diaziridines were further characterized and found to be mechanism-based inactivators of P450 2B6 based on the following criteria: 1) losses in the 7-EFC O-deethylation activity of P450 2B6 were time-dependent; 2) the inactivations of 2B6 showed saturation kinetics with respect to the concentrations of the inactivators; 3) the inactivations were NADPH-dependent; 4) the inactivations were irreversible. A representative figure for the inactivation of P450 2B6 by one of the trifluoromethylaryl diaziridines is shown in Fig. 2. Similar results were obtained for the remaining trifluoromethylaryl diaziridine compounds except the 4-methyl thio compound (1d). The nucleophilic reagent, glutathione, did not significantly protect P450 2B6 from inactivation by trifluoroaryldiaziridines (data not shown) when added to the incubation mixture at a final concentration of 10 mM, suggesting that the enzyme was covalently modified by an electrophilic reactive intermediate formed from the inactivator before it could leave the active site.

The kinetic constants obtained for the 7-EFC inactivation of P450 2B6 by the trifluoroaryldiaziridines are summarized in Table 2. The concentration required for the half-maximal rate of inactivation (\( K_I \)) of P450 2B6 by 3-methyl,4-methoxy-substituted phenyl diaziridine (1e) was the lowest (1.7 \( \mu M \)) with respect to the other diaziridines. The range of \( K_I \) values varied by a factor of 4 and the \( K_{\text{inact}} \) values ranged from approximately 0.04 to 0.08 \( \text{min}^{-1} \), indicating that all five diaziridines are good mechanism-based inactivators. The partition ratio illustrates the efficiency of a particular inactivator for the enzyme. The partition ratio for the inactivation of 2B6 by the 3-methyl,4-methoxy compound (1e) was 29, approximately 3-fold higher than that observed for dimethoxy-substituted phenyl diaziridine (1e), indicating that the dimethoxy-substituted phenyl diaziridine was a more efficient inactivator for P450 2B6 compared with methyl substituted phenyl diaziridine (1e) or any of the other compounds tested (Table 4).

The five trifluoromethylaryl diaziridines that acted as mechanism-based inactivators of P450 2B6 did not inactivate P450s 2E1, 2C9, 2D6, or 3A4. Although the 4-methyl thio diaziridine (1d) did not lead to inactivation of P450 2B6, it competitively inhibited the BFC activity of P450 3A4. Thus, these data indicate that substitution of a thiomethyl for a methoxy at the ring position leads to a compound that has no in vitro inactivation activity. To investigate whether reversible binding of the 4-methyl thio diaziridine compound (1d) occurs in the enzyme active site without subsequent inactivation of the enzyme, spectral binding studies were performed. The spectral binding data revealed reversible binding of compound 1d to P450 2B6 (data not shown). Preliminary results also indicate that the compound is readily metabolized by P450 2B6. These data suggest that the methoxy group may play an important role in forming the reactive intermediate that covalently binds to the P450, resulting in inactivation.

To further understand the mechanism of inactivation, spectral and HPLC analyses were performed after the inactivation of 2B6 by the various diaziridines. The 7-EFC O-deethylation activity of P4502B6 in the reconstituted system decreased by approximately 60 to 70% with all five trifluoromethylaryl diaziridines after incubation with NADPH. In all cases, the degree of inactivation was always significantly larger than the loss of spectral P450, indicating that there is no significant modification in the native heme. Although HPLC analysis showed some losses in heme with all of the trifluoromethylaryl diaziridines, the losses in heme as measured by the pyridine hemochrome assay (data not shown) were minimal (less than 10%), suggesting that the loss ob-
erved with HPLC analysis could result from the acidic conditions used in the mobile phase that could have caused the inactivated protein to precipitate. These results suggested that the inactivation of P450 2B6 was not due to heme destruction but to covalent modification of the apoprotein. In addition, incubation of the trifluoroaryldiaziridines with recombinant P450 2B6 did not result in the appearance of a peak at 478 nm or a decrease in the native heme peak at 418 nm as would be expected for a phenyl-iron complex (data not shown) (Raag et al., 1990; Yamaguchi et al., 2004).

Extensive dialysis of P450 2B6 after inactivation by the five trifluoroaryldiaziridines did not result in recovery of any of the enzyme activity, suggesting that the reactive species formed during the inactivation covalently modified the enzyme. To verify that the loss in enzymatic activity resulted from covalent modification of P450 2B6 and not modification of reductase, fresh reductase was added to the dialyzed samples and the samples were checked for P450 catalytic activity. No gain in activity was observed, suggesting that the inactivation was due to modification of the P450 2B6 and not the NADPH-reductase.

In conclusion, these studies describe the synthesis of novel mechanism-based inhibitors for heme-containing enzymes and evaluate their ability to inhibit human cytochromes P450. The results show that several substituted phenyl diaziridines are relatively potent mechanism-based inactivators of human cytochrome P450 2B6 and that minor changes in the chemical structures of the compounds can have a significant influence on their ability to inactivate that P450. The most potent inhibitors were those derivatives that contained methoxy groups at the 4-position of the phenyl ring. Replacement of the methoxy moiety at position 4 with a methylsulide (1d) resulted in a compound that had no ability to act as a mechanism-based inactivator of P450 2B6. Spectral binding studies indicated that this was not due to an inability of this compound to bind to the P450 active site. Preliminary studies also indicated that the methylsulphide-substituted phenyl diaziridine (1d) was metabolized by P450 2B6. This result suggests that metabolism of the phenyl diaziridine to a reactive intermediate capable of inactivating P450 2B6 requires the presence of an alkoxide position 4 on the phenyl ring.

A preliminary mechanistic interpretation, consistent with the data presented, invokes the formation of a reactive metabolite that may be formed as a result of an initial hydroxylation event catalyzed by P450 2B6 (Scheme 4). Reaction of para-methoxy-substituted diaziridines with P450 2B6 would generate an N-hydroxy diaziridine, which we have not detected in the incubation mixtures, presumably because of its inherent reactivity. Although N-hydroxylation reactions of diaziridines have not been described in the literature, oxidation reactions of the counterpart acyclic hydrazines have been documented. Heterolytic bond scission of this initial oxidation product would result in the formation of a benzylic carbocation, further stabilized by electron-donating substituents (O-R groups) at the para position. Carbene formation concomitant with expulsion of nitrogen would afford the reactive species capable of enzyme inactivation (Scheme 4). Additional studies aimed at characterizing the metabolites formed and the reactive intermediates generated during the inactivation to better understand the mechanism of inactivation are in progress.

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References


