Imidazoline-Binding Domains on Monoamine Oxidase B and Subpopulations of Enzyme

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Accepted for publication November 13, 1999

This paper is available online at http://www.jpet.org

ABSTRACT

A series of phenoxy-substituted methylimidazoline derivatives were synthesized and used to define the ligand recognition properties of the imidazoline-binding domain (IBD) on monoamine oxidase (MAO-B) and its role in substrate processing. The rank order of potency for selected compounds in competitive binding studies with the imidazoline [3H]idazoxan was different from that in enzyme activity assays, suggesting that the IBD and the site involved in enzyme inhibition are distinct. IC50 values for inhibition of MAO-B activity by imidazoline/guanidinium ligands were one to two orders of magnitude greater than ligand concentrations that probably saturate the IBD, but were equal to the Kd values of these ligands in competitive binding assays with the reversible MAO-B inhibitor [3H]Ro 19-6327. In addition, the degree of enzyme inhibition by these ligands was similar in platelet and liver, tissues exhibiting 10-fold differences in the amount of the IBD-accessible enzyme subpopulation. These data suggested that the inhibitory effect of these compounds on MAO-B activity involved a secondary interaction with the enzyme domain recognizing the inhibitor Ro 19-6327 and does not involve interaction with the IBD. Subsequent radioligand-binding studies indicated that human liver MAO-B actually existed as two distinct populations that differed in the accessibility of their IBD. The relatively small amounts of MAO-B possessing an accessible IBD (~5% in human liver) precludes determination of the functional consequences of ligand binding to the IBD. This subpopulation of MAO-B may be selectively regulated or generated in different individuals or tissues and targeted by pharmacologically active compounds in a cell type-specific manner.

Imidazoline-binding proteins, imidazoline/guanidinium-receptive sites, or imidazoline receptors comprise a family of membrane-bound binding sites for a class of ligands that elicits diverse pharmacological effects in the body (Parini et al., 1996; Regunathan and Reis, 1996; Reis and Piletz, 1997; Eglen et al., 1998). These binding sites share the common properties of not recognizing endogenous agonists for known neurotransmitter/receptor signaling systems, but recognizing various compounds with imidazoline or guanidinium moieties. Such binding sites also interact with endogenous entities of defined and undefined structure (Atlas, 1994; Regunathan and Reis, 1996). Members of the family of imidazoline-binding proteins are subgrouped as I1, I2, and I3 sites. The I1 sites are commonly identified with the imidazolines [3H]clonidine or 125I-para- amino clonidine and I2 sites with the imidazolines [3H]idazoxan or [3H](2-benzofuranyl)-2-imidazoline ([3H]2BFI). I3 sites are classified based on the pharmacological profile of imidazolines that influence insulin secretion from pancreatic β-cells (Mourtada et al., 1997; Eglen et al., 1998). Two types of I2 imidazoline-binding proteins were identified as monoamine oxidase (MAO)-A and MAO-B (Raddatz et al., 1995; Tesson et al., 1995). Progress with the molecular characterization of the I1 and I3 sites is limited by their apparently low density and the lack of high-affinity site-selective probes for structural analysis.

The imidazoline-binding domain (IBD) on MAO-B was recently localized to amino acids K149–M222 of human MAO-B (Raddatz et al., 1997). The IBD on MAO-B is distinct from the...
domain interacting with mechanism-based enzyme inhibitors based on the following points. First, the binding of radiolabeled imidazolines to the IBD on the enzyme is not inhibited by the mechanism-based enzyme inhibitors pargyline and deprenyl (Raddatz et al., 1997). Second, different domains of MAO-B are covalently labeled by the imidazoline photoaffinity adduct \([^{125}I]\)AZIPI \((2-[3\text{-azido-}4\text{-iodophenoxo}]\text{methyl imidazoline})\) and the subtype selective inhibitor of MAO-B \([^3\text{H}]\)lazabemide (Cesura et al., 1996; Raddatz et al., 1997). Although the IBD is distinct from the region of the enzyme interacting with classical enzyme inhibitors, it encompasses a region involved in substrate processing/selectivity as indicated by studies with chimeric MAO-A/B enzymes and enzymes with site-directed mutations (Gottowik et al., 1995; Tsugeno et al., 1995; Tsugeno and Ito, 1997).

In MAO-B, a T158A conversion results in the complete loss of enzyme activity and an I199F conversion results in isofrom switching relative to substrate selectivity and inhibitor sensitivity. Thus, ligands interacting at this domain on the enzyme probably influence substrate selectivity or the efficiency of substrate processing. Of particular note is that the IBD is apparently masked in human platelets and there is currently no explanation for the limited accessibility of this domain (Raddatz et al., 1995).

Due to its central role in metabolizing endogenous neurotransmitters as well as xenobiotics, the therapeutic targeting of MAO has always been appealing. However, the enzyme is widely distributed and its inhibition often results in numerous side effects. Nevertheless, inhibitors of MAO are used in the management of certain mood disorders and occupy a significant place in the early management of Parkinson’s disease. Within this context, the identification of another drug/regulatory site on the enzyme that is distinct from the domain interacting with classical enzyme inhibitors and is possibly accessed in a cell-specific manner is significant in regards to both the etiology of specific diseases and their therapeutic management. As part of our continuing effort to define imidazoline-binding proteins, we synthesized and evaluated a series of structural analogs to define the ligand recognition properties of the IBD on MAO-B and its role in substrate processing. Subsequently, we determined the influence of tissue-specific accessibility of the IBD on ligand-mediated inhibition of MAO-B and the variability of the "IBD accessible" subpopulation of MAO-B in human tissues.

**Experimental Procedures**

**Materials**

\[^{14}\text{C}]\text{phenylethylamine (42 mCi/mmol),} \[^{14}\text{C}]\text{tyramine (42 mCi/mmol),} \[^{3}\text{H}]\text{pargyline (36 Ci/mmol),} \[^{3}\text{H}]\text{MK-912 (70 Ci/mmol) were purchased from DuPont-NE} (Boston, MA). \[^{3}\text{H}]\text{azadroxan (41 Ci/mmol) and} \[^{3}\text{H}]\text{BFB1 (50–70 Ci/mmol) were purchased from Am} 

ersham (Arlington Heights, IL). \[^{3}\text{H}]\text{Ro 19-6327 (17 Ci/mmol) were purchased from E} 

crosis, GA) Electrospray MS and tandem MS/MS experiments were carried out in a Finnigan LCQ instrument at the Mass Spectrometry Facility at the Medical University of South Carolina. Sample aliquots in water/methanol/acetic acid (47:47:6) were loaded into a pulled glass capillary. The capillary was inserted into a custom-built nanospray source and the spray was initiated by placing 1.5 kV on a wire inserted into the sample solution. A mass spectrum was acquired and the ion of interest was isolated for subsequent fragmentation via collisions with helium background gas to generate structure-specific fragmentation. The fragment ion masses were recorded by a second scan of the instrument providing an MS/MS spectrum. Typically, 10 to 20 scans were averaged to produce one spectrum.

**Compounds**

\[100, 101, 102, 138, 139, 141, 207, 286.\] The appropriate phenol (6 g) was treated with potassium carbonate (2 eq) and chloroacetonitrile (1.2 eq) in 100 ml of methyl isobutyl ketone and was heated at reflux for 16 h. The mixture was filtered and washed with acetone and evaporated. The residue, which solidified on drying under vacuum, was recrystallized from ethyl acetate/hexane. The arylxoyacetonic acid (5.8 g, 10 mmol) was then added to the mixture and the mixture stirred at 24°C for 2 h. Ethylene diamine dihydrochloride (1.3 g, 10 mmol) was then added to the mixture, and the mixture stirred at 24°C for 16 h. The reaction mixture was evaporated and the residue in water was basified to pH 12 with NaOH and was extracted with ethyl acetate, washed with brine, dried, and evaporated. The residue was recrystallized from methanol/ethyl acetate. For compounds 207, 208, 286, and 287, the amine group was first protected by BOC. For example, 4-hydroxyphenethylamine HCl (5 g, 29 mmol) was mixed with potassium carbonate (4 g, 29 mmol) in 50 ml of water and stirred at 24°C. To this, a solution of di-t-butylcarbonate (6.3 g, 29 mmol) in 25 ml of dioxane was added. The resulting mixture was stirred at 24°C for 48 h. Then the mixture was extracted with ethyl acetate, washed with brine, and evaporated to 6 g of dark oil that was used as such in reaction a in scheme 1 (Fig. 1). The BOC group was removed by treating the BOC derivatives with ethanolic N HCl (1:1, 50 ml) at 50°C for 16 h. Removal of the volatiles and recrystallization of the residue from methanol/ethyl acetate gave the free amine as the hydrochloride salt.

**Compound 205.** The arylxoyacetronic acid (3 g, 20 mmol) was dissolved in 50 ml of anhydrous methanol. Sodium methoxide in methanol (25% by weight, 0.6 ml) was added and the mixture was stirred at 24°C for 2 h. Ethylene diamine dihydrochloride (1.3 g, 10 mmol) was then added to the mixture, and the mixture stirred at 24°C for 16 h. The reaction mixture was evaporated and the residue in water was basified to pH 12 with NaOH and was extracted with ethyl acetate, washed with brine, dried, and evaporated. The residue was recrystallized from methanol/ethyl acetate. For compounds 207, 208, 286, and 287, the amine group was first protected by BOC. For example, 4-hydroxyphenethylamine HCl (5 g, 29 mmol) was mixed with potassium carbonate (4 g, 29 mmol) in 50 ml of water and stirred at 24°C. To this, a solution of di-t-butylcarbonate (6.3 g, 29 mmol) in 25 ml of dioxane was added. The resulting mixture was stirred at 24°C for 48 h. Then the mixture was extracted with ethyl acetate, washed with brine, and evaporated to 6 g of dark oil that was used as such in reaction a in scheme 1 (Fig. 1). The BOC group was removed by treating the BOC derivatives with ethanolic N HCl (1:1, 50 ml) at 50°C for 16 h. Removal of the volatiles and recrystallization of the residue from methanol/ethyl acetate gave the free amine as the hydrochloride salt.

**Synthesis of Phenoxy-Substituted Methylimidazolines**

Imidazoline derivatives were prepared by the reaction of commercially available phenols with chloroacetonitrile to give the arylxyoxacetanitritites (Fig. 1; Tables 1 and 2). The arylxyoxacetanitrites were then treated with sodium methoxide in methanol to produce the imidazolines, which were then condensed with ethylene diamine, propylene diamine, aminoacetaldedheyd dimethy acetal, or o-phenylene diamine to obtain the imidazoline, tetrahydropropimidine, imidazole, or benzimidazole, respectively. For the aminooethyl analog, the amino group of the 4-hydroxyphenethylamine was protected as an N-t-butoxycarbonyl (BOC) substituent before alkylation with chloroacetonitrile. The BOC moiety was cleaved by acid hydrolysis after the imidazoline ring was formed.

The final products were characterized by elemental analysis (C, H, N), liquid chromatography, 300 MHz proton NMR, and mass spectrometry (MS) (Tables 1 and 2). The 300 MHz proton NMR spectra were obtained with either Varian XL-300 or Bruker Avance 300 instruments. Microanalyses data were provided by Atlantic Microlab Inc. (Norcross, GA). Electrospray MS and tandem MS/MS experiments were carried out in a Finnigan LCQ instrument at the Mass Spectrometry Facility at the Medical University of South Carolina. Sample aliquots in water/methanol/acetic acid (47:47:6) were loaded into a pulled glass capillary. The capillary was inserted into a custom-built nanospray source and the spray was initiated by placing 1.5 kV on a wire inserted into the sample solution. A mass spectrum was acquired and the ion of interest was isolated for subsequent fragmentation via collisions with helium background gas to generate structure-specific fragmentation. The fragment ion masses were recorded by a second scan of the instrument providing an MS/MS spectrum. Typically, 10 to 20 scans were averaged to produce one spectrum.
The reaction mixture was evaporated. The residue in water was basified to pH 12 with NaOH and was extracted with ethyl acetate, washed with brine, dried, and evaporated. The residue was recrystallized from methanol/ethyl acetate.

**Compound 209.** The nitrile (14.0 g, 95.1 mmol) was dissolved in 7 ml of absolute ethanol and 50 ml of dry ether. Hydrogen chloride gas was bubbled through for 20 min. The precipitated imidate hydrochloride was filtered, washed with ether and dried to the imidate (84%). The imidate (3.0 g, 13 mmol), aminoacetaldehyde dimethyl acetal (2.0 ml, 18 mmol) and absolute ethanol (50 ml) were combined and heated at reflux for 16 h. The reaction mixture was evaporated, the residue was basified and extracted with ethyl acetate. The product was then converted into the HCl salt from ether/HCl and was recrystallized from methanol/ether.

**Compound 206.** The aryloxy acetonitrile (3.0 g, 20 mmol) was dissolved in 50 ml of anhydrous methanol. Sodium methoxide in methanol (1.2 ml, 25%) was added and the mixture was stirred at 24°C for 2 h. Then 3.6 g (20 mmol) of o-phenylenediamine dihydrochloride was added and the mixture stirred at 24°C for 16 h. The reaction mixture was evaporated. The residue in water was basified to pH 12 with NaOH and was extracted with ethyl acetate, washed with brine, dried, and evaporated. The residue was recrystallized from methanol/ethyl acetate.

**Compounds 119, 128, and 288.** A mixture of the amine (0.3 mmol) and fluorescein isothiocyanate (0.3 mmol) in 6 ml of dimethylformamide, 3.2 ml of 0.1 N sodium bicarbonate, and 0.8 ml of 0.1 N sodium carbonate was stirred at 24°C for 16 h. The mixture was diluted with water and the precipitated solid was purified by silica gel chromatography (ethyl acetate/isopropanol/water/ammonium hydroxide, 50:30:16:4) to give the fluorescein derivative.

**Membrane Preparations**

The expression of human α2-adrenergic receptor subtypes in human embryonic kidney (HEK) cells and preparation of HEK membranes were as described in Jasper et al. (1998). For preparation of rat kidney membranes, frozen rat kidneys were minced in a Waring Blender (3 × 5 s, setting: high) in 10 × w/v 0.32 M sucrose at 4°C. The homogenate was passed through two-ply cheesecloth and centrifuged (Sorvall RC5C-SS34 rotor) at 100g for 10 min at 4°C. The supernatant was collected and stored on ice. The pellet was resuspended in 0.32 M sucrose and centrifuged at 1000g for 10 min at 4°C, and the resulting supernatant was combined with the supernatant from the first centrifugation. The combined supernatants were centrifuged at 11,500g for 20 min at 4°C and the pellet was resuspended in 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA at 4°C to desired
protein concentration and aliquots were stored at −80°C until use. Outdated human platelets obtained from the Red Cross were collected by centrifugation at 1000 g for 15 min at 4°C, resuspended, and frozen in lysis buffer (5 mM Tris-HCl, pH 7.4, containing 5 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 5 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride). Platelets were lysed by freeze-thawing twice in lysis buffer and homogenized with a glass dounce. Freshly isolated adipocytes or frozen human liver, kidney, and brain were homogenized in lysis buffer by a glass dounce. Lysates from frozen tissue were filtered through two layers of gauze mesh. Membranes were prepared from the lysates following removal of the pellet resulting from centrifugation at 1000 g for 10 min at 4°C. The supernatant was centrifuged at 35,000 g for 20 min (Lazo et al., 1991). This fraction was washed twice in sucrose buffer, resuspended in membrane buffer (described above), and stored at −70°C for up to 2 months. For preparation of human liver or platelet membranes enriched in mitochondria, tissue was homogenized with a glass dounce in 0.25 M sucrose buffer containing 1 mM EDTA, 5 mM MgCl₂, 0.2 mM phenylmethylsulfonyl fluoride), rapidly frozen, and stored at −70°C for up to 2 months. For preparation of human liver or platelet membranes enriched in mitochondria, tissue was homogenized with a glass dounce in 0.25 M sucrose buffer containing 1 mM EDTA, 5 mM MgCl₂, 0.2 mM phenylmethylsulfonyl fluoride), rapidly frozen, and stored at −70°C for up to 2 months.

### Photoaffinity Labeling, Immunoblotting, and Radioligand Binding

Photoaffinity labeling of the IBDr 19-6327 was performed in the presence of 0.1 μM clorgyline to block interaction with MAO-A and nonspecific binding was determined in the presence of 10 μM cirazoline. Binding of [3H]Ro 19-6327 was performed in the presence of 0.1 μM clorgyline to block interaction with MAO-A and nonspecific binding was determined in the presence of 10 μM Ro 16-6491. For saturation-binding assays with [3H]2BFI, rat kidney membranes were resuspended in 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 150 mM NaCl, and 2 mM MgCl₂ at 25°C to a concentration of −3.5 mg/ml. [3H]2BFI-binding assays were conducted in a total volume of 250 μl (100 μl of membranes) in polystyrene 96-well plates. Nonspecific binding was defined by 10 μM idazoxan. In initial experiments, it was determined that specific binding of [3H]2BFI was not inhibited by the α₂-adrenoceptor (α₂-AR) antagonist yohimbine (1 μM). To determine affinity estimates in competition-binding studies, varying concentrations of test ligands were incubated with 1 nM [3H]2BFI. For α₂-AR-binding isotherms, membranes were incubated with 0.01 to 5 nM [3H]MK-912 in the presence or absence of the α₂-AR antagonist ICI119,854 (1 μM) for 60 min at room temperature. For radioligand displacement assays, varying concentrations of test ligand were incubated with 0.3 to 0.5 nM [3H]MK-912 (α₂a, α₂AR) or 0.03 to 0.05 nM [3H]HM-912 (α₂b-AR). For each drug evaluated in radioligand-binding assays, the IC₅₀ was determined by nonlinear curve-fitting (GraphPad, San Diego, CA) and equilibrium dissociation constant (Kd) calculated according to the method of Cheng and Prusoff (1973). Saturation-binding data were evaluated with the nonlinear curve-fitting program LIGAND and were best fit by a one-site model.

### MAO Activity Assay

MAO activity was determined by a modification of previously described methods (Fellman et al., 1969). [14C]phenylethylamine (10 μM, 14 mCi/mmol) or [14C]tyramine (100 μM, 42 mCi/mmol) was used as substrate. Under our incubation conditions, product formation from the substrate [14C]phenylethylamine was not altered by the addition of the selective MAO-A inhibitor clorgyline to the incubation mixture. Assays with [14C]tyramine were conducted in the presence of 1 μM cirazoline to inhibit MAO-A. Membranes (50 μg of protein) were diluted in sodium phosphate buffer (100 mM), pH 7.4. and preincubated with inhibitors for 10 min at 24°C before the addition of the indicated concentrations of substrates (total volume 100 μl). Ro 16-6491 (100 μM) was used to define specific activity. Reactions were incubated for 20 min at 37°C and stopped by addition of 1 ml of HCl (2 N) at 4°C. The reaction products were extracted into 1 ml of ethyl acetate/toluen 1:1 (v/v), and the radioactivity contained in an aliquot of the organic phase was measured in a Beckman LS

### Table 1: Elemental analysis, melting points, and yields of synthesized compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Calculated</th>
<th>Found</th>
<th>Melting Point</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>138</td>
<td>C₁₁H₁₆N₄O₄ · HCl · H₂O</td>
<td>C, 50.68; H, 6.57; N, 10.74</td>
<td>C, 50.78; H, 6.24; N, 10.78</td>
<td>90–92</td>
<td>55</td>
</tr>
<tr>
<td>100</td>
<td>C₁₁H₁₆N₄O₄ · HCl · 0.5H₂O</td>
<td>C, 51.71; H, 6.15; N, 15.14</td>
<td>C, 52.08; H, 6.15; N, 15.18</td>
<td>&gt;250</td>
<td>65</td>
</tr>
<tr>
<td>101</td>
<td>C₁₁H₁₆N₄O₄ · HCl · 0.5H₂O</td>
<td>C, 52.55; H, 4.96; N, 15.32</td>
<td>C, 52.43; H, 4.96; N, 15.37</td>
<td>&gt;250</td>
<td>70</td>
</tr>
<tr>
<td>139</td>
<td>C₁₁H₁₆N₄O₄ · HCl</td>
<td>C, 42.65; H, 6.67; N, 13.57</td>
<td>C, 42.75; H, 6.60; N, 13.57</td>
<td>237–239</td>
<td>60</td>
</tr>
<tr>
<td>102</td>
<td>C₁₁H₁₆N₄O₄ · 2HCl</td>
<td>C, 45.47; H, 5.51; N, 15.91; Cl, 26.84</td>
<td>C, 45.66; H, 5.72; N, 15.81; Cl, 26.72</td>
<td>235 (dec)</td>
<td>65</td>
</tr>
<tr>
<td>207</td>
<td>C₁₁H₁₆N₄O₄ · HCl</td>
<td>C, 57.38; H, 7.36; N, 11.81</td>
<td>C, 57.25; H, 7.34; N, 11.72</td>
<td>170–171</td>
<td>75</td>
</tr>
<tr>
<td>208</td>
<td>C₁₁H₁₆N₄O₄ · 2HCl</td>
<td>C, 49.47; H, 6.58; N, 14.43</td>
<td>C, 49.33; H, 6.53; N, 14.32</td>
<td>&gt;250</td>
<td>88</td>
</tr>
<tr>
<td>206</td>
<td>C₁₁H₁₆N₄O₄ · 0.5H₂O</td>
<td>C, 58.08; H, 7.95; N, 10.69</td>
<td>C, 57.91; H, 7.87; N, 10.67</td>
<td>138–139</td>
<td>96</td>
</tr>
<tr>
<td>209</td>
<td>C₁₁H₁₆N₄O₄ · 2HCl</td>
<td>C, 52.51; H, 7.24; N, 13.12</td>
<td>C, 52.60; H, 7.31; N, 13.08</td>
<td>&gt;250</td>
<td>85</td>
</tr>
<tr>
<td>208</td>
<td>C₁₁H₁₆N₄O₄ · 2HCl</td>
<td>C, 66.02; H, 5.07; N, 8.80; S, 5.04</td>
<td>C, 65.84; H, 5.25; N, 8.56; S, 4.48</td>
<td>153–154</td>
<td>45</td>
</tr>
<tr>
<td>128</td>
<td>C₁₁H₁₆N₄O₄ · S · 2H₂O</td>
<td>C, 60.38; H, 4.58; N, 9.09; S, 5.20</td>
<td>C, 60.26; H, 4.67; N, 8.50; S, 5.21</td>
<td>238–240</td>
<td>20</td>
</tr>
<tr>
<td>119</td>
<td>C₁₁H₁₆N₄O₄ · S · H₂O</td>
<td>C, 62.20; H, 4.38; N, 9.36; S, 5.36</td>
<td>C, 62.54; H, 4.30; N, 9.01; S, 5.16</td>
<td>265 (dec)</td>
<td>25</td>
</tr>
<tr>
<td>206</td>
<td>C₁₁H₁₆N₄O₄ · HCl</td>
<td>C, 59.87; H, 7.12; N, 11.64</td>
<td>C, 59.77; H, 7.15; N, 11.70</td>
<td>186–187</td>
<td>71</td>
</tr>
<tr>
<td>209</td>
<td>C₁₁H₁₆N₄O₄ · HCl</td>
<td>C, 58.60; H, 5.83; N, 12.47</td>
<td>C, 58.51; H, 5.84; N, 12.31</td>
<td>158–160</td>
<td>41</td>
</tr>
<tr>
<td>205</td>
<td>C₁₁H₁₆N₄O₄ · HCl</td>
<td>C, 65.57; H, 5.55; N, 10.2</td>
<td>C, 65.28; H, 5.55; N, 10.3</td>
<td>199–200</td>
<td>55</td>
</tr>
</tbody>
</table>
TABLE 3

Compound structure and affinities at 12 imidazoline binding sites and \( \alpha_2 \)-AR receptor subtypes

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_1 )</th>
<th>( R_2 )</th>
<th>( R_3 )</th>
<th>( X )</th>
<th>12 Human Liver</th>
<th>12 Rat Kidney</th>
<th>( \alpha_2 )-AR</th>
<th>( \alpha_3 )-AR</th>
<th>( \alpha_4 )-AR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIRAZOLINE</td>
<td>Cyclopropyl</td>
<td>H</td>
<td>H</td>
<td>Imidazoline</td>
<td>7.50 ± 0.20</td>
<td>8.86 ± 0.08</td>
<td>7.0 ± 0.04</td>
<td>5.95 ± 0.1</td>
<td>&lt;5.5</td>
</tr>
<tr>
<td>Group I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>H</td>
<td>OCH(_3)</td>
<td>H</td>
<td>Imidazoline</td>
<td>7.28 ± 0.11</td>
<td>6.75 ± 0.20</td>
<td>6.4 ± 0.09</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>100</td>
<td>H</td>
<td>NCHCOH(_3)</td>
<td>H</td>
<td>Imidazoline</td>
<td>5.68 ± 0.31</td>
<td>5.71 ± 0.53</td>
<td>&lt;5.5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>101</td>
<td>H</td>
<td>H</td>
<td>NCHCOH(_3)</td>
<td>Imidazoline</td>
<td>6.11 ± 0.09</td>
<td>&lt;5.5</td>
<td>5.4 ± 0.55</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>139</td>
<td>H</td>
<td>Cl</td>
<td>NO(_2)</td>
<td>Imidazoline</td>
<td>8.46 ± 0.21</td>
<td>7.94 ± 0.06</td>
<td>&lt;5.5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>141</td>
<td>CH(_3)</td>
<td>H</td>
<td>NH(_2)</td>
<td>Imidazoline</td>
<td>7.35 ± 0.05</td>
<td>6.88 ± 0.30</td>
<td>&lt;5.5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>102</td>
<td>H</td>
<td>H</td>
<td>NH(_2)</td>
<td>Imidazoline</td>
<td>6.17 ± 0.09</td>
<td>6.21 ± 0.23</td>
<td>6.3 ± 0.02</td>
<td>&lt;5.5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>207</td>
<td>H</td>
<td>(CH(_2))(_2)NH</td>
<td>H</td>
<td>Imidazoline</td>
<td>8.48 ± 0.01</td>
<td>6.64 ± 0.12</td>
<td>&lt;5</td>
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<td>208</td>
<td>H</td>
<td>(CH(_2))(_2)NH</td>
<td>H</td>
<td>Imidazoline</td>
<td>5.30 ± 0.10</td>
<td>&lt;5</td>
<td>&lt;5</td>
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<td>286</td>
<td>H</td>
<td>(CH(_2))(_2)NH</td>
<td>H</td>
<td>Imidazoline</td>
<td>7.09 ± 0.11</td>
<td>6.78 ± 0.23</td>
<td>6.6 ± 0.04</td>
<td>6.0 ± 0.02</td>
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<td>287</td>
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<td>(CH(_2))(_2)NH</td>
<td>H</td>
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<td>7.43 ± 0.32</td>
<td>6.29 ± 0.09</td>
<td>&lt;5</td>
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<td>288</td>
<td>H</td>
<td>(CH(_2))(_2)NH-fluorescein</td>
<td>H</td>
<td>Imidazoline</td>
<td>6.33 ± 0.03</td>
<td>&lt;5</td>
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<td>119</td>
<td>H</td>
<td>H</td>
<td>NH-fluorescein</td>
<td>Imidazoline</td>
<td>6.09 ± 0.13</td>
<td>&lt;5</td>
<td>&lt;5</td>
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<tr>
<td>205</td>
<td>CH(_3)</td>
<td>H</td>
<td>H</td>
<td>Tetryahydro- pyrimidine</td>
<td>8.10 ± 0.05</td>
<td>7.56 ± 0.11</td>
<td>&lt;5.5</td>
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<tr>
<td>209</td>
<td>CH(_3)</td>
<td>H</td>
<td>H</td>
<td>Imidazoline</td>
<td>6.79 ± 0.10</td>
<td>6.79 ± 0.10</td>
<td>&lt;5.5</td>
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<tr>
<td>261</td>
<td>CH(_3)</td>
<td>H</td>
<td>H</td>
<td>Benzimidazole</td>
<td>&lt;5</td>
<td>&lt;5</td>
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Affinities of ligands in radioligand binding assays presented as \( pK_a \) values ± S.E. Receptor densities (fmol/mg membrane protein): rat kidney = 161 ± 0.06, HEK \( \alpha_2 \)-AR = 1569 ± 0.4, HEK \( \alpha_3 \)-AR = 2060 ± 0.2, HEK \( \alpha_4 \)-AR = 1600 ± 0.3.
IBD on MAO-B had considerably more flexibility than the series of ligands at the four binding sites indicated that the expressing each of the three receptor subtypes.

assays with membranes prepared from HEK cells stably
tution-binding assays with the imidazolines [3H]idazoxan and
liver and rat kidney membranes were determined in compe-
gand recognition properties of the IBD of MAO-B in human
group involved alterations in the imidazoline ring itself. Li-
ivatization at a specific site on the phenyl ring, and the third
phenyl ring. The second group of compounds focused on der-
influence of altered electron distribution within the basic
The first group of compounds was designed to explore the
binding site for these ligands on
ture-activity relationships for the IBD on MAO-B versus the
accommodation by the IBD on MAO-B and to further define struc-
umidazoline derivatives to explore the limits of ligand accom-
m 6000 SE liquid scintillation spectrometer at ~95% efficiency. IC_{50}
values were determined by nonlinear curve-fitting (GraphPad).

Results

Analysis of IBD on MAO-B. We synthesized a series of
imidazoline derivatives to explore the limits of ligand accom-
modation by the IBD on MAO-B and to further define structure-activity relationships for the IBD on MAO-B versus the binding site for these ligands on α₂-ARs (Fig. 1; Tables 1–3). The first group of compounds was designed to explore the influence of altered electron distribution within the basic phenyl ring. The second group of compounds focused on derivatization at a specific site on the phenyl ring, and the third group involved alterations in the imidazoline ring itself. Ligand recognition properties of the IBD of MAO-B in human liver and rat kidney membranes were determined in competition-binding assays with the imidazolines [3H]idazoxan and [3H]2BFI (Table 3). The ability of this class of compounds to interact with α₂-ARs was determined in competition-binding assays with membranes prepared from HEK cells stably expressing each of the three receptor subtypes.

A comparison of the relative affinities exhibited by this series of ligands at the four binding sites indicated that the IBD on MAO-B had considerably more flexibility than the ligand-binding site on α₂-AR subtypes (Table 3). As indicated in Table 2, the IBD on MAO-B accommodated small substitutions on the phenyl ring and the relative affinities for group I compounds were determined by the electron withdrawing properties of the substituent. The affinities exhibited by the compounds tested progressively decreased with bulkier substitutions, as indicated by the relative affinities observed for the group II ligands. Surprisingly, the IBD could handle an enlargement of the imidazoline ring structure as indicated by the high affinity (pKᵢ = 8.1 ± 0.05 in human liver) exhibited by the tetrahydropyrimidine compound 205. Replacement of the tetrahydropyrimidine ring by an imidazole lowered ligand affinity by one log unit. A fused benzene/imidazole ring structure (compound 206) further reduced the affinity exhibited by this series of ligands (Table 3), indicating the importance of the highly basic imidazoline group for ligand recognition. The relative abilities of these compounds to interact with the IBD were similar in human liver and rat kidney with the exception of compound 207, which exhibited higher affinity in human liver membranes (Table 3). Within this series of compounds, 139, 207, and 205 exhibited the highest affinity for the IBD on MAO-B in human liver and these three compounds were not recognized by the three α₂-AR subtypes (Table 3).

Several imidazolines and guanidinium ligands inhibit MAO-B enzyme activity (Palaty and Cragoe, 1989; Carpéné et al., 1995; Tesson et al., 1995; Ozaita et al., 1997). Enzyme inhibition by imidazolines was observed with two distinct substrates and the maximal inhibition of enzyme activity by the imidazolines BU224 and cirazoline was comparable to that achieved with the MAO-B isform selective inhibitor Ro 16-6491 (Fig. 2). However, the concentrations of ligand required for enzyme inhibition are one to two orders of magnitude higher than those that would likely saturate the IBD based on saturation-binding isotherms and determination of affinity constants (Fig. 2) (Tesson et al., 1995). The inhibition of enzyme activity by the imidazoline/guanidinium compounds is complex and reported to be noncompetitive, mixed competitive/noncompetitive, allosteric, and competitive (Palaty and Cragoe, 1989; Tesson et al., 1995; Ozaita et al., 1997).

2 The imidazoline BU224 and the guanidinium guanabenz exhibit Kᵢ values of 1 to 10 nM for the IBD on MAO-B as determined in radioligand-binding studies with [3H]idazoxan or photoaffinity labeling with [125I]AZIP.
Although Ro 16-6491 is often discussed as a reversible, slow-binding enzyme inhibitor (Keller et al., 1987; Cesura et al., 1989), its mechanism of action is also poorly understood. In our assay system, the extent of inhibition of enzyme activity by Ro 16-6491 and the imidazoline/guanidinium compounds was not influenced by extended incubation times (Fig. 2C). To investigate the relationship between inhibition of enzyme activity and interaction of imidazoline compounds with the IBD on MAO-B, we performed two series of experiments. First, we determined the potency of various compounds for enzyme inhibition relative to their interaction with the IBD on MAO-B. Second, we evaluated the effect of selected imidazoline/guanidiniums on enzyme activity in human liver and platelet, two tissues exhibiting differences in the accessibility of the IBD (Raddatz et al., 1995).

We selected two subgroups of compounds from Table 1 that contained modifications of the phenyl (139, 208) or imidazoline ring (205, 206, 209) and compared the ability of these compounds to inhibit radioligand binding to the IBD versus their effects on MAO-B activity in human liver membranes (Fig. 3). For both series of compounds, the rank order of potency in competitive binding assays was not observed in enzyme activity assays, indicating that the two types of analysis do not reflect the interaction of these compounds with the same enzyme domain (Fig. 3; Table 3). Preincubation of these compounds with membrane preparations for up to 120 min before initiation of the reaction by addition of substrate did not increase their effectiveness as enzyme inhibitors (R.R. and S.M.L., unpublished data). The inhibition of enzyme activity observed at higher ligand concentrations probably involves interaction of the compounds with an enzyme domain distinct from the IBD. Ro 16-6491 and Ro 19-6327 are selective inhibitors of MAO-B and their site of interaction with the enzyme is apparently distinct from the K149–M222 IBD (Raddatz et al., 1997). We then asked if there was a relationship between the inhibition of enzyme activity observed with the higher concentrations of imidazoline/guanidinium ligands and their ability to interact with the enzyme domain binding the reversible enzyme inhibitor [3H]Ro 19-6327 (Cesura et al., 1989). In human liver, the IC_{50} values for inhibition of MAO-B activity and [3H]Ro 19-6327 binding by imidazoline/guanidinium ligands were similar (Fig. 4), suggesting that the inhibitory effect of these compounds on MAO-B activity involves a secondary, low-affinity interaction with the enzyme domain recognizing Ro 19-6327.

To further evaluate the functional role of the K149–M222 binding domain, we compared the effect of imidazoline/guanidinium compounds on enzyme activity in liver and platelets, tissues exhibiting differences in the apparent accessibility of the IBD on MAO-B as determined by photoaffinity labeling with the imidazoline photoaffinity adduct [125I]AZIPI (Raddatz et al., 1995). Inhibition of enzyme activity by the higher concentrations of imidazoline and guanidinium ligands in human liver also was observed in human platelet membranes (Fig. 5). The potency of such ligands at inhibiting enzyme activity was similar in both human liver and platelet membranes (platelet: BU224 IC_{50} = 25 μM ± 2.7, n = 3; guanabenz IC_{50} = 50 μM ± 28, n = 2 and liver: BU224 IC_{50} = 62 μM ± 4.4, n = 3; guanabenz IC_{50} = 64 μM ± 14, n = 3).

**Subpopulations of MAO-B.** The preceding results suggested that ligand occupation of the IBD at K149–M222 had no discernable effect on enzyme activity within the confines of this assay system. Alternatively, there may be a mixed population of MAO-B in human liver consisting of enzymes with or without an accessible, high-affinity IBD (K149–M222) that complicates data interpretation. To address this issue, we performed saturation-binding isotherms with the imidazoline [3H]idazoxan, commonly used to identify imidazoline-binding proteins, and the reversible MAO-B inhibitor [3H]Ro 19-6327 (Cesura et al., 1989) to determine the relative amounts of imidazoline-binding sites and MAO-B. Human liver and platelet mitochondria expressed MAO-B at densities of 18 ± 1.6 and 7.9 ± 0.92 pmol/mg protein, respectively [K_{d} (nM) = 40 ± 6.2 for liver; K_{d} (nM) = 31 ± 5.2 for platelet; n = 2], as determined by Scatchard analysis of saturation-binding isotherms with the MAO-B competitive inhibitor [3H]Ro 19-6327 (Fig. 6). Radioligand-binding studies identified two to three times greater density of MAO-B in liver than in platelet mitochondria, and these results are consistent with the relative MAO-B densities in these two tissues as determined by immunoblotting (R.R. and S.M.L., unpublished data). The densities of imidazoline-binding sites in liver and platelet mitochondria were 0.92 ± 0.05 and 0.03 pmol/mg protein, respectively [K_{d} = 6.6 nM ± 0.07 for liver; K_{d} = 7.1 nM ± 3.0 for platelet; n = 2], as determined by Scatchard analysis of saturation-binding isotherms with...
Saturation-binding studies with [3H]idazoxan were conducted in the presence of the \( \alpha_2 \)-AR antagonist rauwolscine to block radioligand binding to \( \alpha_2 \)-AR. Thus, in liver the IBD was accessible on \(~5\%\) of the total population of MAO-B, whereas in platelets the domain was accessible on only \(~0.5\%\) of the total population of MAO-B.

Discussion

The identification of MAO-B as an imidazoline-binding protein was initially suggested by the common intracellular location of the two entities, their similar/identical \( M_r \) coregulation of the two entities and the copurification of ligand binding and enzyme activity (Tesson et al., 1991; Limon et al., 1992; Lanier et al., 1993; Olmos et al., 1993; Sastre and Fig. 4. Inhibition of MAO activity and displacement of radiolabeled enzyme inhibitors by imidazoline/guanidinium compounds. Enzyme activity or binding of radiolabeled enzyme inhibitors was measured after preincubation of membranes with increasing concentrations of Ro 16-6491, BU224, or guanabenz. MAO-B enzyme activity was measured in liver membranes (50 \( \mu \)g of protein) preincubated with 0.1 \( \mu \)M clorgyline (MAO-A inhibition) with the substrate \([14^C]\)phenylethylamine (10 \( \mu \)M) as substrate \([14^C]\)phenylethylamine (10 \( \mu \)M) as described in Experimental Procedures. Nonspecific binding of [3H]Ro 19-6327 (10–15 nM) was defined with 100 \( \mu \)M Ro 16-6491 and represented \(~6\%\) of total binding. The data are expressed as the percentage of specific binding (7808 \( \pm \) 2155 dpm) or activity (121 \( \pm \) 5.4 pmol product/20 min) in the absence of inhibitors and represent the mean \( \pm \) S.D. of two or mean \( \pm \) S.E. of three experiments. Fig. 5. Inhibition of MAO-B activity by imidazolines/guanidiniums in platelet membranes. MAO-B activity was measured in platelet membranes (200 \( \mu \)g of protein) in the presence or absence of increasing concentrations of Ro 16-6491, BU224, or guanabenz with \([14^C]\)phenylethylamine (10 \( \mu \)M) as substrate. The data are expressed as the percentage of activity in the absence of inhibitors (132 \( \pm \) 12 pmol product/20 min) and represent the mean \( \pm \) S.E. of three experiments with duplicate determinations. IC\(_{50}\) values (\( \mu \)M) for enzyme inhibition: Ro 16-6491, 0.71 \( \pm \) 0.2; BU224, 62 \( \pm \) 4.4; guanabenz, 64 \( \pm \) 14.

[3H]Idazoxan binding over this concentration range of radioligand probably involves only the high-affinity IBD on MAO-B and not the secondary, low-affinity site discussed previously in this manuscript. These results indicated that only a subpopulation of MAO-B was able to bind imidazoline ligands with high affinity. Determining the functional consequences of occupation of the IBD will require the isolation of the subpopulation of enzyme with an accessible, high-affinity IBD.

The apparent heterogeneity in MAO-B is of particular note relative to both enzyme structure and potential therapeutic targeting of the enzyme in selected tissues, and we determined the accessibility of the IBD on MAO-B in different human tissues. The relative amounts of MAO-B in various tissues were determined by immunoblotting and/or radioligand-binding assays with the irreversible, mechanism-based MAO inhibitor [3H]pargyline (Fig. 7). With aliquots of membrane protein from the different tissues containing approximately equivalent amounts of MAO-B, the relative accessibility of the IBD on the enzyme was determined by photoaffinity labeling with the imidazoline [125I]AZIPI (Fig. 7). Accessible versus inaccessible IBD was defined by quantitative differences in the amount of photoincorporation among individual samples when equivalent amounts of enzyme were used for photolabeling (Raddatz et al., 1995). The accessibility of the IBD on MAO-B was greatest in liver and least in platelets. The rank order of tissues possessing an accessible IBD was liver > adipocytes > kidney > brain > platelets (Fig. 7). These results indicated that the proportion of MAO-B molecules possessing an accessible, high-affinity imidazoline-binding site is tissue-dependent.
Garcia-Sevilla, 1993). Partial amino acid sequencing of the purified imidazoline-binding protein and heterologous expression studies indicated that the purified protein was actually MAO-B (Tesson et al., 1995). Subsequently, the imidazoline-binding proteins identified by photoaffinity labeling with imidazoline-specific photoaffinity adducts in human liver and placenta were determined to be MAO-B and -A, respectively, by immunoprecipitation of the photolabeled proteins (Raddatz et al., 1995). MAO-A and -B are clearly important enzymes for neurotransmitter and xenobiotic metabolism and thus we were immediately presented with an interesting drug target for the class of imidazoline/guanidinium ligands. The high-affinity interaction of selected imidazoline/guanidinium compounds with MAO-A and -B suggests that some pharmacological effects of these compounds may involve altered levels of monoamine neurotransmitters via regulation of enzyme activity.

Imidazolines and guanidiniums both inhibit MAO-B activity in rat and human liver (Palaty and Cragoe, 1989; Carpené et al., 1995; Tesson et al., 1995; Ozaita et al., 1997), although relatively high concentrations of ligand are required for enzyme inhibition. Relative to the latter point, the suggestion of MAO-B heterogeneity based on differential accessibility of ligands to the IBD and its location relative to the enzyme domains targeted by currently available enzyme inhibitors (Raddatz et al., 1995, 1997; Cesura et al., 1996) is of particular interest. This is perhaps best illustrated by the data presented in Figs. 6 and 7 in the present manuscript, which indicate tissue-specific subpopulations of MAO-B defined by accessibility of the IBD. One major question at present is what actually distinguishes the putative subpopulations of MAO-B and this is a focus of several research laboratories. Subpopulations of enzyme may be defined by post-translational modifications or editing of the primary transcript. Alternatively, the IBD may actually be masked by endogenous substances that vary in concentration among tissues. The MAO-B gene consists of 15 exons (Bach et al., 1988), and multiple types of MAO-B could perhaps be generated as splice variants differing in the enzyme domain that recognizes imidazoline/guanidinium ligands. However, because the enzyme exhibits a similar $M_r$ in different tissues and within different individuals, alternatively spliced transcripts must involve small enzyme domains, and there is no evidence for such processing.

The apparent existence of subpopulations of MAO-B defined by accessible versus inaccessible IBDs is also of interest.
Fig. 7. Accessibility of the IBD on MAO-B in various tissues. Aliquots of membrane protein containing equivalent amounts of MAO-B were determined by immunoblotting (top left) and radioligand-binding assays (top right) with the covalent enzyme inhibitor [3H]pargyline. Equivalent amounts of MAO-B from these tissues were then photolabeled with [125I]AZIPI to determine the accessibility of the IBD (bottom). For identification of MAO-B by immunoblotting, membrane transfers of denaturing polyacrylamide gels of human liver (75 μg of protein), adipocyte (50 μg of protein), kidney (100 μg of protein), brain (125 μg of protein), and platelet (200 μg of protein) membranes were immunoblotted with antibodies recognizing MAO (1:10,000). The arrows in the top left gel indicate the migration of MAO-A (Mₐ ∼ 63,000, top band) and MAO-B (Mᵦ ∼ 55,000, bottom band). The amount of MAO-B in membrane aliquots identical with those in the top left gel was determined with [3H]pargyline (100 nM) in the presence of 0.1 μM clorgyline to inhibit interaction with MAO-A (top band). Nonspecific binding was determined by preincubating membranes in the presence of 100 μM pargyline and was 2 to 10% of total binding for all of the tissues. Bottom, autoradiograph of photoaffinity-labeled membrane proteins. Aliquots of human liver (150 μg of protein), adipocyte (100 μg of protein), kidney (200 μg of protein), brain (250 μg of protein), and platelet (400 μg of protein) membranes were photolabeled with 2 nM [125I]AZIPI in the absence or presence of 100 μM cirazoline. The arrow indicates the migration of proteins with Mᵦ ∼ 55,000.

Data are representative of two experiments. Adipocytes were obtained at the CHU Rangueil Hopital of Toulouse from normal females undergoing liposuction of upper thigh adipose tissue. Human kidney and liver samples were obtained from an 85-year-old male and 61-year-old female (metastatic adenocarcinoma to liver), respectively (UAB Comprehensive Cancer Center). The human brain sample was obtained from a caudate/accumbens/putamen punch (sample 2482, Massachusetts Brain Bank, McLean Hospital, male, age 79, no diagnosis, PMI - 21). The sequence of experiments generating the data presented in this figure was repeated twice with similar results.

relative to a possible pathogenic role of MAOs. Platelet MAO-B activity has been measured in a number of patient populations to determine a possible correlation of specific diseases (e.g., mood disorders, schizophrenia, Parkinson’s disease) with enzyme activity. Results from such studies are often conflicting and inconclusive and rarely do these studies include measurements of the amount of the enzyme, but rather simply evaluate enzyme activity. MAO activity also was reported to be modified in specific brain regions of smokers (Fowler et al., 1996a,b). However, again it is difficult to conclude if such changes in enzyme activity reflect changes in the amount of enzyme, its ability to bind inhibitor radioligands, or its ability to process substrate. Altered accessibility of the IBD may occur in pathophysiological situations and influence enzyme activity independent of differences in the total amount of enzyme. This possibility could be addressed by analysis of the relative accessibility of the IBD on MAO-B in these different patient populations either in platelet samples or possibly by the use of comparative positron emission tomography imaging with ligands that interact at the IBD or the enzyme domain recognized by the enzyme inhibitors deprenyl or Ro 19-6327.

A full understanding of the functional consequences of occupation of the IBD on enzyme activity will eventually require isolation of the subpopulation of MAO-B that contains an accessible domain for ligands of this chemical class. Several points indicate that the inhibition of MAO-B by imidazoline ligands observed in vitro in human liver membranes does not involve the high-affinity IBD, but rather involves the enzyme site identified by the reversible enzyme inhibitor Ro 19-6327. First, concentrations of imidazoline/guanidinium compounds that probably saturate the imidazoline-binding site do not inhibit enzyme activity (Carpéné et al., 1995; Tesson et al., 1995). Second, the IC₅₀ for inhibition of enzyme activity by imidazoline/guanidinium compounds is similar to the Kᵦ of these compounds in competition-binding assays with [3H]Ro19-6327 in human liver, human platelet, and rat liver membranes (Carpéné et al., 1995; Tesson et al., 1995; Ozaita et al., 1997). Third, only ∼5 to 10% of the MAO-B in human liver possesses an accessible IBD. Effects of ligands on this subpopulation of enzyme would not be detected in the background of a larger population of active enzyme with which imidazoline ligands could not interact. Fourth, enzyme activity is inhibited by imidazolines/guanidiniums with a similar potency in human liver and platelets in which the high-affinity IBD on MAO-B is differentially accessible. Occupation of the IBD on MAO-B in vivo may affect aspects of MAO function other than directly regulating...
enzyme activity such as FAD incorporation, protein stability, or transport of the enzyme to the mitochondrial outer membrane. The unique high-affinity ligands synthesized in the present study may be particularly useful for addressing such issues based on their selectivity and potential for further modification of the nonimidazoline-based structures.

Acknowledgments

Polyclonal antibodies used for immunoblot were a kind gift from Dr. Akio Ito (Kyushu University, Fukuoka, Japan). We thank Dr. Grayson Richards (Hoffmann-La Roche Ltd., Basel, Switzerland) for providing [3H]Ro 19-6327 and Dr. Singh (Division of Pediatrics, Medical University of South Carolina) for the liver human mitochondrial. We appreciate the kind consideration of Dr. Kevin Schey (Medical University of South Carolina) for analysis of the liver synthesized compounds by MS.

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