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Inhibition of monoamine oxidase A and B activities by imidazol(ine)/guanidine drugs, nature of the interaction and distinction from I_2-imidazoline receptors in rat liver

Andrés Ozaita, Gabriel Olmos, M. Assumpció Boronat, *José Miguel Lizcano, *Mercedes Unzeta & [1]Jesús A. García-Sevilla

Laboratory of Neuropharmacology, Department of Biology, University of the Balearic Islands, E-07071 Palma de Mallorca, Spain, and *Medical Biochemistry Unit, Department of Biochemistry and Molecular Biology, Autonomous University of Barcelona, E-08193 Bellaterra, Catalonia, Spain

1 I_2-Imidazoline sites ([^{3}H]-idazoxan binding) have been identified on monoamine oxidase (MAO) and proposed to modulate the activity of the enzyme through an allosteric inhibitory mechanism (Tesson *et al*., 1995). The main aim of this study was to assess the inhibitory effects and nature of the inhibition of imidazol(ine)/guanidine drugs on rat liver MAO-A and MAO-B isoforms and to compare their inhibitory potencies with their affinities for the sites labelled by [^{3}H]-clonidine in the same tissue.

2 Competition for [^{3}H]-clonidine binding in rat liver mitochondrial fractions by imidazol(ine)/guanidine compounds revealed that the pharmacological profile of the interaction (2-styryl-2-imidazoline, LSL 61112 > idazoxan > 2-benzofuranyl-2-imidazoline, 2-BFI = cirazoline > guanabenz > oxymetazoline > > clonidine) was typical of that for I_2-sites.

3 Clonidine inhibited rat liver MAO-A and MAO-B activities with very low potency (IC_{50}s: 700 μM and 6 mM, respectively) and displayed the typical pattern of competitive enzyme inhibition (Lineweaver-Burk plots: increased K_m and unchanged V_{max} values). Other imidazol(ine)/guanidine drugs also were weak MAO inhibitors with the exception of guanabenz, 2-BFI and cirazoline on MAO-A (IC_{50}s: 4–11 μM) and 2-benzofuranyl-2-imidazol (LSL 60101) on MAO-B (IC_{50}: 16 μM). Idazoxan was a full inhibitor, although with rather low potency, on both MAO-A and MAO-B isoenzymes (IC_{50}s: 280 μM and 624 μM, respectively). Kinetic analyses of MAO-A inhibition by these drugs revealed that the interactions were competitive. For the same drugs acting on MAO-B the interactions were of the mixed type inhibition (increased K_m and decreased V_{max} values), although the greater inhibitory effects on the apparent value of V_{max}/K_m than on the V_{max} value indicated that the competitive element of the MAO-B inhibition predominated.

4 Competition for [^{3}H]-Ro 41-1049 binding to MAO-A or [^{3}H]-Ro 19-6327 binding to MAO-B in rat liver mitochondrial fractions by imidazol(ine)/guanidine compounds revealed that the drug inhibition constants (K_i values) were similar to the IC_{50} values displayed for the inhibition of MAO-A or MAO-B activities. In fact, very good correlations were obtained when the affinities of drugs at MAO-A or MAO-B catalytic sites were correlated with their potencies in inhibiting MAO-A ($r = 0.92$) or MAO-B ($r = 0.99$) activity. This further suggested a direct drug interaction with the catalytic sites of MAO-A and MAO-B isoforms.

5 No significant correlations were found when the potencies of imidazol(ine)/guanidine drugs at the high affinity site (pK_{iH}, nanomolar range) or the low-affinity site (pK_{iL}, micromolar range) of I_2-imidazoline receptors labelled with [^{3}H]-clonidine were correlated with the pIC_{50} values of the same drugs for inhibition of MAO-A or MAO-B activity. These discrepancies indicated that I_2-imidazoline receptors are not directly related to the site of action of these drugs on MAO activity in rat liver mitochondrial fractions.

6 Although these studies cannot exclude the presence of additional binding sites on MAO that do not affect the activity of the enzyme, they would suggest that I_2-imidazoline receptors represent molecular species that are distinct from MAO.

Keywords: MAO-A and MAO-B isoforms; imidazol(ine)/guanidine drugs; competitive inhibition; mixed inhibition; I_2-imidazoline receptors; [^{3}H]-clonidine

Introduction

The presence of non-adrenoceptor sites for imidazol(ine)/guanidine drugs in various tissues of several species is now well established (for a review see Bousquet, 1995; French, 1995; Reis *et al*., 1995; Regunathan & Reis, 1996). Based on the rank order of affinity for different ligands, imidazoline receptors have been classified into two main types: the I_1- and the I_2-imidazoline receptors (Michel & Insel, 1989; Ernsberger, 1992). The differential recognition of I_2-imidazoline receptors by the guanidide compound amiloride generated a further subtyping of these sites as amiloride-sensitive (I_{2A}-imidazoline receptors) and amiloride-insensitive (I_{2B}-imidazoline receptors) (Diamant *et al*., 1992; Miralles *et al*., 1993; Regunathan *et al*., 1993; Olmos *et al*., 1996).

Since the demonstration that I_2-imidazoline receptors are enriched in mitochondrial outer membrane fractions (Tesson & Parini, 1991), several studies have provided evidence that these receptors are related to the enzyme monoamine oxidase (MAO): (1) the irreversible MAO inhibitor clorgyline displays

[1]Author for correspondence at: Lab Neurofarmacologia, Departament de Biologia, Universitat de les Illes Balears, Cra. Valldemossa Km 7.5, E-07071 Palma de Mallorca, Spain.

high affinity for I_{2B}-imidazoline receptors and also irreversibly binds to this subtype but not to the I_{2A}-subtype (Olmos *et al*., 1993; 1996); (2) chronic treatment with various irreversible MAO inhibitors down-regulates I_2-imidazoline receptor density in the rat brain (Olmos *et al*., 1993; Alemany *et al*., 1995b); (3) in rat and human brains the regional distribution of I_2-imidazoline receptors correlates well with that of MAO-B, but not of MAO-A, and the densities of I_2- and MAO-B sites, but not MAO-A sites, increase in human brain during the process of ageing (Sastre & García-Sevilla, 1993; 1997); (4) I_2-imidazoline receptors and the two MAO isoforms are co-purified by using different chromatographic procedures and they are co-expressed in yeast after heterologous expression of the cDNA for both MAO isoforms (Tesson *et al*., 1995) and (5) photolabelled I_2-imidazoline binding proteins can be immunoprecipitated with monoclonal antibodies to MAO-A and MAO-B isoforms (Raddatz *et al*., 1995).

Moreover, the recent finding that some imidazoline drugs can inhibit MAO activity (Carpéné *et al*., 1995; Tesson *et al*., 1995) and that cirazoline appeared to be a noncompetitive inhibitor of the enzyme (Tesson *et al*., 1995) led to the proposal that these drugs interact with a site that is different from the MAO catalytic site and that the I_2-imidazoline binding site represents a previously unknown regulatory site located on this enzyme (Tesson *et al*., 1995; Parini *et al*., 1996).

In this context, the present study was designed (1) to investigate if [^{3}H]-clonidine, an imidazoline drug able to inhibit basal MAO activity in rabbit kidney and rat liver (Tesson *et al*., 1995; Raasch *et al*., 1996), could also label this enzyme in rat liver mitochondrial-enriched fractions; (2) to assess the effects, rank order of potency and, specifically, the nature of the inhibition of rat liver MAO-A and MAO-B activities by clonidine and a wide variety of imidazol(ine)/guanidine drugs, and (3) to compare the potency of these drugs in inhibiting MAO-A and MAO-B activities with their affinities for the catalytic sites of MAO isoforms and for the sites labelled by [^{3}H]-clonidine in the rat liver.

Methods

Preparation of rat liver mitochondrial-enriched fractions for radioligand binding experiments

Male Sprague-Dawley rats (200–250 g) were used. The rats received a standard diet with water freely available and were housed at 20±2°C with a 12 h light/dark cycle. The animals were decapitated and portions of liver were rapidly removed into ice-cold Tris-sucrose buffer (50 mM Tris-HCl; 250 mM sucrose; 1 mM $MgCl_2$; pH 7.4) and frozen at −80°C until required. After thawing, the tissue samples were homogenized (Ultra-Turrax) in 5 ml of ice-cold Tris-sucrose buffer. The homogenates were centrifuged at 1,100 *g* for 10 min and the supernatants were then recentrifuged at 11,000 *g* for 10 min. The resulting pellet containing mitochondria was washed twice with 2 ml of fresh incubation buffer (50 mM Tris-HCl, pH 7.4). The final pellet was resuspended in an appropriate volume of this buffer for [^{3}H]-clonidine binding assays or in 50 mM Tris-HCl, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4 for [^{3}H]-Ro 41-1049 and [^{3}H]-Ro 19-6327 binding assays. Final protein content was 300–500 μg ml^{-1}. Protein was determined by the method of Lowry *et al*. (1951), with bovine serum albumin as the standard.

[^{3}H]-clonidine, [^{3}H]-Ro 41-1049 and [^{3}H]-Ro 19-6327 binding assays

Total [^{3}H]-clonidine binding was measured in 0.5 ml aliquots (50 mM Tris-HCl, pH 7.5) of mitochondrial fractions which were incubated with shaking for 30 min at 25°C. When 10^{-6} M (−)-adrenaline was included in the assay, it did not affect total [^{3}H]-clonidine binding, suggesting that the radioligand does not label α_2-adrenoceptors in this tissue, as described for [^{3}H]-idazoxan (Zonnenschein *et al*., 1990; Alemany *et al*., 1995b). Therefore, total [^{3}H]-clonidine binding was determined without (−)-adrenaline in the assay. Nonspecific binding was determined with 10^{-4} M naphazoline (Olmos *et al*., 1992). For drug competition studies, mitochondrial fractions were incubated with [^{3}H]-clonidine (10^{-8} M) in the absence or presence of various concentrations of the competing drugs (10^{-10} M to 10^{-3} M; 15 concentrations). Total binding was determined as above and plotted as a function of the drug concentration.

[^{3}H]-Ro 41-1049 and [^{3}H]-Ro 19-6327 binding to MAO-A or MAO-B, respectively, were assessed as described previously (Olmos *et al*., 1993; Sastre & García-Sevilla, 1993). Mitochondrial fractions were resuspended in fresh incubation buffer (see above) and 0.5 ml aliquots were used in drug competition studies. Mitochondrial fractions were incubated for 60 min at 37°C with [^{3}H]-Ro 41-1049 (4×10^{-9} M) for binding to MAO-A or for 90 min at 20°C with [^{3}H]-Ro 19-6327 (2×10^{-9} M) for binding to MAO-B and in the absence or presence of various concentrations of the competing drugs (10^{-10} M to 10^{-3} M; 15 concentrations). Nonspecific binding was determined in the presence of 10^{-6} M clorgyline or 10^{-4} M L-deprenyl for [^{3}H]-Ro 41-1049 and [^{3}H]-Ro 19-6327 binding, respectively.

Incubations were terminated by diluting the samples with 5 ml of ice-cold Tris incubation buffer (4°C). Bound and free radioligands were separated by vacuum filtration through Whatman GF/C glass fibre filters which had been presoaked with 1% polyethylenimine (Bruns *et al*., 1983), by use of a Brandel 48R cell harvester (Biomedical Research & Development Laboratories, U.S.A.). Then the filters were rinsed twice with 5 ml of incubation buffer, air-dried, transferred to minivials containing 5 ml of OptiPhase 'HiSafe' II cocktail (LKB, U.K.) and counted for radioactivity by liquid scintillation spectrometry at 57% efficiency (Packard model 1900 TR).

Analyses of binding data

Analyses of competition experiments (K_i, inhibition constant) as well as the fitting of data to the appropriate binding models were performed by computer-assisted nonlinear regression by use of the EBDA-LIGAND (Munson & Rodbard, 1980; McPherson, 1985) programmes. All experiments were initially analysed assuming a one-site model of radioligand binding and then assuming a two-site binding model. The selection between the different binding models was made statistically by the extra sum of squares principle (F test) as outlined by Munson and Rodbard (1980). The more complex model was accepted if the P values resulting from the F test was less than 0.05.

Rat liver mitochondrial MAO preparations for inhibitory and kinetic experiments

In rat liver homogenates, two-thirds of MAO activity is present in mitochondria. Rat liver mitochondria were prepared from Sprague-Dawley rats (200–250 g) fasted overnight. Livers were homogenized (1:10, w/v) in 50 mM KH_2PO_4 buffer, pH 7.2, containing 0.25 M sucrose, by means of a Dounce homogenizer. Mitochondria were prepared by a standard differential centrifugation method (Gómez *et al*., 1988). Briefly, the homogenate was filtered through gauze and centrifuged at 600 *g* for 10 min. The supernatant was centrifuged again under the same conditions and the resulting supernatant was centrifuged at 8,000 *g* for 10 min. The final pellet containing mitochondria was washed twice, resuspended to a protein concentration of 8 mg ml^{-1} in 50 mM KH_2PO_4 buffer, pH 7.2, and stored at −20°C until the assays (Gómez *et al*., 1988). Protein was determined by the method of Hartree (1972), with bovine serum albumin as the standard.

MAO-A and MAO-B assays

MAO from rat liver mitochondria appears to contain equal amounts of the two known isoforms of the enzyme (Youdim *et al*., 1988). MAO-A and MAO-B activities were determined

radiochemically at 37°C as described previously (Fowler & Tipton, 1981; Gómez *et al*., 1988), with either 100 μM [^{14}C]-5-hydroxytryptamine (5-HT) or 20 μM [^{14}C]-2-β-phenylethylamine (PEA) as substrates, respectively. At these concentrations 5-HT and PEA have been shown to behave as specific substrates for the A and B isoforms of MAO, respectively (Johnston, 1968; Fowler & Tipton, 1981). The reaction was carried out in a final volume of 225 μl of a 50 mM KH_2PO_4 buffer, pH 7.2, containing 100 μg of protein (crude enzyme) and 25 μl of radioactivity labelled substrate (final concentrations: 100 μM for 5-HT and 20 μM for PEA). The incubation times were 10 min for both substrates and then the reaction was stopped by the addition of 100 μl of 2 M citric acid. The reaction products (i.e., the corresponding aldehydes of 5-HT and PEA after oxidative deamination) were extracted into toluene/ethyl acetate (1:1, v/v) containing 0.6% (w/v) 2,5-diphenyloxazole and the radioactivity of the extract in the organic phase was determined by liquid scintillation counting. Both MAO assays were performed over times where product formation was shown to proceed linearly under the conditions used, and where the initial rate was a linear function of enzyme concentration.

Inhibition of MAO-A and MAO-B basal activities and determination of enzyme kinetic parameters

Inhibition curves for different drugs were assessed against MAO-A and MAO-B activities. The reaction mixture containing the crude enzyme and various drug concentrations (10^{-10} M to 10^{-2} M; 8–9 concentrations) was stabilized for 5 min at 37°C. Then, the drug inhibitory effect was determined by measuring radiochemically for 10 min the enzyme activity against 5-HT or PEA remaining at each drug concentration. Control samples, in which the inhibitor was replaced by an identical volume of water, were run through the same procedure to ensure that no significant loss of activity occurred during this period of time. The remaining MAO-A and MAO-B activities were expressed as percentages of control basal activity and plotted as a function of the drug concentration. For each inhibitor the IC_{50} value, defined as the drug concentration necessary to give 50% enzyme inhibition, was calculated by nonlinear regression by use of the GraFit programme (Leatherbarrow, 1990).

Steady-state kinetic constants (K_m, Michaelis constant and V_{max}, maximum velocity) were determined from studies of the effects of substrate concentration on the initial velocity of MAO-A or MAO-B activity in the absence and presence of

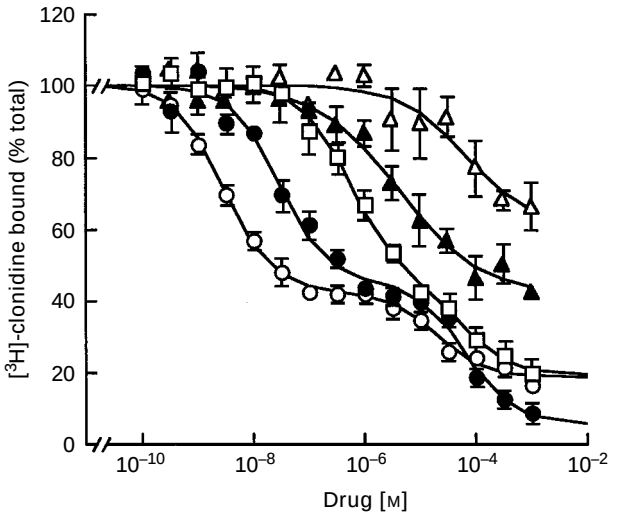

Figure 1 Inhibition of [^{3}H]-clonidine binding (10^{-8} M) in rat liver membranes by various imidazol(ine)/guanidine compounds: (○) idazoxan, (●) guanabenz, (□) clonidine, (▲) LSL 60101 and (△) agmatine. Total control binding was about 12,000 d.p.m. Computer-assisted curve fitting (EBDA-LIGAND programmes) demonstrated that, except for agmatine and LSL 60101, a two site fit was significantly better than a one site fit ($P<0.001$; F test). Data shown are mean with vertical lines showing s.d. or s.e.mean of 2 (agmatine) to 6 experiments. See Table 1 for K_i values and other details.

Table 1 Affinity for I_2 imidazoline receptors (labelled by [^{3}H]-clonidine binding) and inhibition of monoamine oxidase (MAO-A and MAO-B) activity of various imidazol(ine)/guanidine drugs and MAO inhibitors in rat liver mitochondrial fractions

	I_2-Imidazoline receptors [^{3}H]-clonidine binding		*Monoamine oxidase (MAO) activity*	
			MAO-A	*MAO-B*
Drug class	K_{iH} (nM)	K_{iL} (μM)	IC_{50} (μM)	IC_{50} (μM)
Imidazol(ine)/guanidines				
LSL 61122	0.1	5.6	100	32
Idazoxan	2.2	25	280	624
2-BFI	3.8	46	11	23
Cirazoline	3.8	494	11	31
Guanabenz	30	58	4	40
Oxymetazoline	58	16	530	1,380
Clonidine	757	64	700	6,000
LSL 60101	4,100	–	58	16
Agmatine	55,000	–	> 10,000	> 10,000
MAO inhibitors				
Clorgyline	492	66	0.03	8
Ro 41-1049	150,000	–	0.13	31
Ro 19-6327	2,100	–	640	0.02
Ro 16-6491	1,600	–	85	0.12
Chlordimeform	28	27	16	34
Isatin	–	> 1,000	18	2

For the binding experiments, rat liver mitochondrial fractions were incubated at 25°C for 30 min with [^{3}H]-clonidine (10^{-8} M) in the absence or presence of the competing drugs (10^{-11} M to 10^{-3} M). Binding parameters (K_{iH} and K_{iL}) were determined directly by simultaneous analysis of 2 (agmatine) to 6 independent experiments for each drug by use of the EBDA-LIGAND programmes. For most drugs computer-assisted curve fitting demonstrated that a two-site fit was significantly better than a one-site binding model ($P<0.001$, F test). The pharmacological profile obtained was typical of that of I_2-imidazoline receptors (see Figure 2). Inhibition of MAO-A and MAO-B activities in rat liver mitochondrial fractions were determined radiochemically at 37°C for 10 min with the specific substrates [^{14}C]-5-hydroxytryptamine (100 μM) for MAO-A and [^{14}C]-2-β-phenylethylamine (20 μM) for MAO-B in the absence and presence of the inhibitory drugs (10^{-10} M to 10^{-2} M). For each inhibitor the IC_{50} value was calculated by simultaneous analysis of 3 independent experiments per drug by use of the GraFit programme.

different drug concentrations. Both enzyme isoforms gave good fits to Michaelian type curves by nonlinear regression analysis. For each inhibitor, K_m (μM) and V_{max} (nmol min^{-1} mg^{-1} protein) values were also estimated, for illustrative purposes (Figures 3, 5 and 6), from Lineweaver-Burk plots (substrate concentration^{-1} versus velocity^{-1}) which in all cases resulted in straight line plots, with linear regression correlation coefficients very close to unity. Dixon plots (inhibitor concentration versus velocity^{-1}) were also used to assess further the nature of the interaction. Kinetic constants for MAO-A were assessed with five different concentrations of 5-HT (50, 100, 200, 300 and 500 μM) after total inhibition of MAO-B activity in crude membrane preparations by preincubation for 60 min at 37°C with 10^{-7} M L-deprenyl. After this incubation, the free inhibitor was removed by centrifugation at 4°C and 20,000 *g* for 15 min. The pellet was washed three times more by resuspension and centrifugation before it was finally resuspended in the incubation buffer as above. Similarly, kinetic constants for MAO-B were determined with five different concentrations of PEA (2.5, 5, 10, 25 and 50 μM) after total inhibition of MAO-A activity in samples that had been preincubated with 10^{-7} M clorgyline for 60 min at 37°C and washed as above. These concentrations of L-deprenyl and clorgyline were found to inhibit the activity of one isoform of the enzyme completely without significantly affecting the activity of the other (Gómez *et al*., 1986). Under these experimental conditions, the ranges of the kinetic parameters of MAO-A towards 5-HT as substrate (K_m: 145–160 μM; V_{max}: 7.7–8.4 nmol min^{-1} mg^{-1} protein) and of MAO-B with PEA as substrate (K_m: 4–7 μM; V_{max}: 5.1–5.9 nmol min^{-1} mg^{-1} protein) were similar to those obtained previously (Gómez *et al*., 1986).

Statistics

Results are expressed as mean ± s.d. or s.e.mean. Pearson's correlation coefficients were calculated by the method of least squares and used to test for possible associations between variables. The level of significance was chosen as $P = 0.05$. All tests were two-tailed.

Drugs

[^{3}H]-clonidine (specific activity 65.5 Ci $mmol^{-1}$) was purchased from New England Nuclear/Du Pont (U.S.A.). [^{3}H]-Ro 41-1049 [*N*-(2-aminoethyl)-5-(*m*-fluorophenyl)-4-thiazole carboxamide HCl] (specific activity, 30.8 Ci $mmol^{-1}$) and [^{3}H]-Ro 19-6327 (lazabemide) (*N*-(2-aminoethyl)-5-chloro-2-pyridine carboxamide HCl) (specific activity 20.2 Ci $mmol^{-1}$) were generous gifts from Dr G.J. Richards (Hoffmann-La Roche Ltd., Switzerland). For the binding assays, appropriate amounts of the stock solutions were diluted with distilled and purified water (Milli-Q) containing 2.5 mM HCl and 6% ethanol. [^{14}C]-5-HT (specific activity 500 μCi $mmol^{-1}$) and [^{14}C]-PEA (specific activity 2.5 mCi $mmol^{-1}$) were purchased from Amersham International plc (U.K.) and New England Nuclear/Du Pont, respectively.

Other drugs (and their sources) included: agmatine sulphate (Aldrich Chemical Co., U.S.A.); 2-BFI (2-(2-benzofuranyl)-2-imidazoline) or RX801077 (synthesized by Dr Plá as LSL 61103 at S.A. Lasa Laboratories, Spain); chlordimeform HCl (Ciba-Geigy, Switzerland); cirazoline HCl (Synthélabo Recherche, France); clonidine HCl (Sigma Chemical Co., U.S.A.); clorgyline HCl (Sigma); L-deprenyl HCl (Research Biochemicals International, U.S.A.); guanabenz base (Sigma); idazoxan HCl (synthesized by Dr F. Geijo at S.A. Lasa Laboratorios); isatin (indole-2,3-dione) base (Sigma); LSL 60101 [2-(2-benzofuranyl)imidazole HCl] and LSL 61122 [2-styryl-2-imidazoline HCl, valldemossine] (synthesized by Dr. F. Geijo at S.A. Lasa Laboratorios); oxymetazoline HCl (Sigma); Ro 41-1049; Ro 19-6327 and Ro 16-6491 [N-(2-aminoethyl-*p*-chlorobenzamide] HCl (F. Hoffman-La Roche Ltd.). Other reagents were obtained from Sigma.

Results

Pharmacological characterization of non-adrenoceptor [^{3}H]-clonidine binding sites in rat liver

[^{3}H]-clonidine binding to rat liver mitochondrial fractions was essentially to non-adrenoceptor sites because (−)-adrenaline (10^{-6} M) did not displace any significant portion of the binding. Specific binding of [^{3}H]-clonidine 10^{-8} M amounted to 12,000 d.p.m. Competition experiments for [^{3}H]-clonidine binding by selected imidazol(ine)/guanidine compounds and MAO inhibitors were performed to assess the pharmacological profile of the interactions. The compounds LSL 61122, idazoxan, 2-BFI, cirazoline, guanabenz, oxymetazoline and clonidine displayed biphasic curves (Hill coefficients lesser than unity) (Figure 1) and computer analysis resolved the binding of [^{3}H]-clonidine into two components (Figure 1 and Table 1). The high affinity component for these imidazol(ine)/guanidine drugs ($K_{iH} \sim 0.1-760$ nM) represented 60–70% of total [^{3}H]-clonidine binding. The low affinity component of [^{3}H]-clonidine binding displayed inhibition constants in the micromolar range ($K_{iL} \sim 6-500$ μM) for these compounds. The imidazole LSL 60101 and the guanidine agmatine, a proposed endogenous ligand for imidazoline receptors, displayed moderate and very low affinity, respectively, for [^{3}H]-clonidine binding, and at 1 mM they only displaced about 60% and 30%, respectively, of total radioligand binding and from only one site (Figure 1 and Table 1).

The irreversible and highly selective MAO-A inhibitor clorgyline and chlordimeform, a reversible and non-selective MAO inhibitor, fully inhibited [^{3}H]-clonidine binding to rat liver mitochondrial fractions (Table 1). These MAO inhibitors also displaced biphasic curves. Chlordimeform was more potent in competing for the high affinity component than clor-

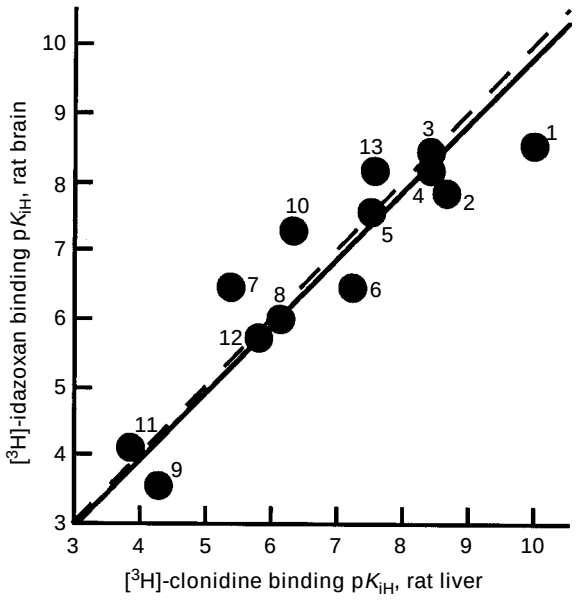

Figure 2 Correlation between the high affinity inhibition constants (expressed as pK_{iH} values, i.e., the negative logarithm of the inhibition constant for the high affinity site) for various drugs against [^{3}H]-clonidine binding in rat liver membranes and for I_2-imidazoline receptors labelled with [^{3}H]-idazoxan in rat brain membranes (data taken from Miralles *et al*., 1993; Alemany *et al*., 1995a,b; Olmos *et al*., 1996). The identification of drugs is as follows: (1) LSL 61122, (2) idazoxan, (3) cirazoline, (4) 2-BFI, (5) guanabenz, (6) oxymetazoline, (7) LSL 60101, (8) clonidine, (9) agmatine, (10) clorgyline, (11) Ro 41-1049, (12) Ro 16-6491, (13) chlordimeform. See Table 1 for K_{iH} values for [^{3}H]-clonidine binding and other details. The dotted line represents the line of identity. The solid line represents the regression of the correlation. The data were best described by the expression: $y = 1.16 + 0.82\times$ ($r = 0.92$, $P < 0.001$).

gyline (Table 1). Other reversible and highly selective MAO-A inhibitors such as Ro 41-1049 or MAO-B inhibitors such as Ro 19-6327 (lazabemide) and Ro 16-6491 displayed monophasic curves when competing against [^{3}H]-clonidine binding in mitochondrial fractions, with K_i values in the micromolar range. The endogenous MAO inhibitor isatin (indole-2,3-dione) (Glover *et al*., 1988) was ineffective at displacing [^{3}H]-clonidine binding to rat liver mitochondrial fractions (7% inhibition at 1 mM).

A highly significant correlation was obtained ($r=0.92$; $P<0.001$) when the potencies of all the drugs tested at the high affinity site of [^{3}H]-clonidine binding in rat liver mitochondrial fractions were compared with the potencies of the same drugs at the high affinity site of rat brain I_2-imidazoline receptors labelled with [^{3}H]-idazoxan (Figure 2). This correlation analysis clearly indicated that at least a high proportion (60–70%) of [^{3}H]-clonidine binding in rat liver corresponds to the I_2-imidazoline receptors.

Inhibition of rat liver MAO-A and MAO-B activities by clonidine and nature of the interaction

I_2-imidazoline binding sites have been proposed to regulate the activity of the enzyme MAO. To determine if [^{3}H]-clonidine binding to rat liver I_2-imidazoline receptors is related to an interaction of the radioligand with MAO, the effects of clonidine on MAO activity (potency and nature of the interaction) in rat liver mitochondria were assessed in comparison with specific MAO inhibitors. As expected, sufficiently high concentrations of the MAO inhibitors tested fully inhibited the MAO activity and showed the known pattern of selectivity for MAO-A (clorgyline, Ro 41-1049) and MAO-B (Ro 19-6327, Ro 16-6491) or non-selectivity (chlordimeform, isatin) (Table 1).

The imidazoline clonidine inhibited rat liver MAO-A activity in a monophasic manner with a very low potency (IC_{50}: 700 μM). It was an even poorer inhibitor of the MAO-B

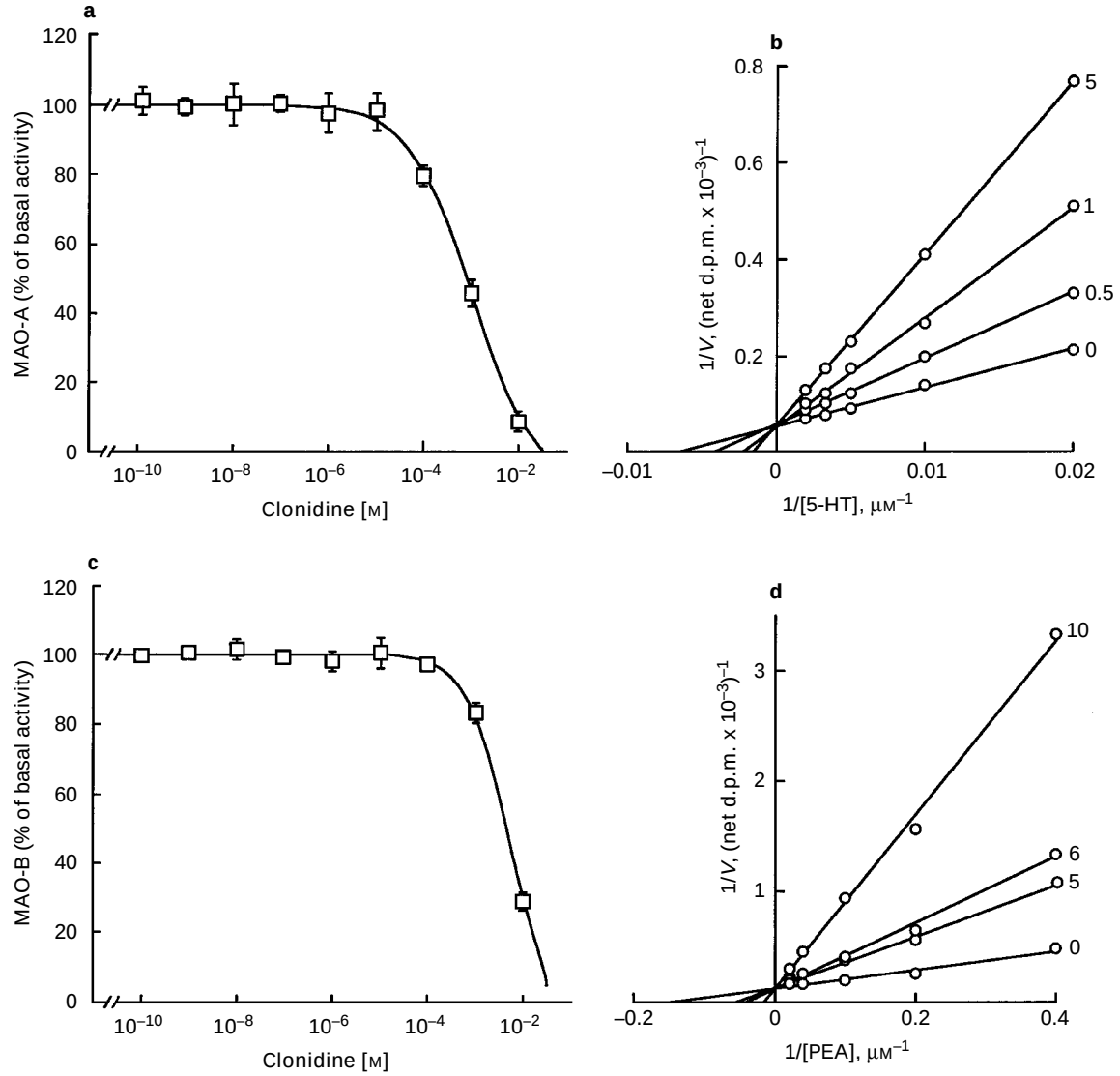

Figure 3 (a,c) Inhibition of basal MAO-A (a) and MAO-B (c) activities by clonidine in rat liver. Data are expressed as % of basal MAO activity (MAO-A: 7.7 nmol 5-HT min^{-1} mg^{-1} protein, MAO-B: 5.2 nmol PEA min^{-1} mg^{-1} protein). See Table 1 for IC_{50} values and other details. Data shown are the mean with vertical lines indicating s.e.mean of three independent experiments each in duplicate. (b,d) Kinetic of inhibition of MAO-A (b) and MAO-B (d) activities by clonidine. Lineweaver-Burk plots (substrate concentration^{-1} versus velocity^{-1}) of enzyme activity measured in the presence of increasing concentrations of the substrate (b) 5-HT for MAO-A (50, 100, 200, 300 and 500 μM) or (d) PEA for MAO-B (2.5, 5, 10, 25 and 50 μM) and increasing concentrations of the inhibitor clonidine (0.5 to 10 mM). The intercept of the extrapolated lines with the ordinates gave the reciprocal of the maximum rate (V_{max}), whereas dissociation constants (K_m) were calculated from the intercepts with the abscissa scale. Each plot is from one representative experiment which was repeated twice with similar results. V_{max} values for MAO-A (b) were: 7.7, 7.3, 7.3 and 7.3 nmol 5-HT min^{-1} mg^{-1} protein, and for MAO-B (d): 5.1, 5.0, 5.2 and 4.9 nmol PEA min^{-1} mg^{-1} protein. The corresponding K_m values were (b): 160, 226, 361 and 597 μM, and (d): 4, 11, 15 and 17 μM.

activity (IC_{50}: 6 mM) (Table 1). Studies on the effects of clonidine on the initial rates of substrate oxidation by MAO-A and MAO-B, shown as Lineweaver-Burk plots in Figure 3, indicated the inhibition to be competitive with respect to the amine substrate. Dixon plots of the inhibition data (not shown) were also consistent with simple competitive inhibition and gave K_i values of 510 ± 95 μM ($n=3$) and 2.5 ± 0.5 mM ($n=3$) for MAO-A and MAO-B, respectively. Such competitive inhibition would be consistent with clonidine binding to the active sites of the enzymes, although the possibility that the compound binds to a distinct site and exerts its effects through a conformational change cannot be excluded.

Inhibition of rat liver MAO-A and MAO-B activities by various imidazol(ine)/guanidine drugs and nature of the interactions

Among the drugs tested, guanabenz, 2-BFI and cirazoline were relatively potent in inhibiting MAO-A activity (IC_{50}s: 4 to 11 μM) and complete inhibition was achieved in the presence of 1 mM of each of these drugs (Table 1 and Figure 4). Other imidazol(ine) compounds such as LSL 60101, LSL 61122, idazoxan and oxymetazoline displayed only moderate to low potencies as inhibitors of MAO-A (IC_{50}s: 58 to 530 μM). Agmatine was a very poor inhibitor of MAO-A activity, with only about 13% inhibition being observed at a concentration of 10 mM, and its inhibition kinetics were not studied further (Table 1 and Figure 4).

When the kinetics of the interactions of these drugs (except agmatine) with the MAO-A isoform were assessed, Lineweaver-Burk plots indicated that the interaction was competitive (i.e., apparent increases in K_m values with no changes in V_{max} values in the presence of the inhibitor and therefore decreased V_{max}/K_m ratios) (Figure 5). Moreover, Dixon plots of MAO-A kinetics for guanabenz and cirazoline confirmed the competitive nature of the interaction for these drugs (data not shown). These results suggested that imidazol(ine)/guanidine compounds inhibited MAO-A activity by interacting with the catalytic site of the enzyme (see above comment for clonidine).

The effects of the same drugs also were evaluated on rat liver mitochondrial MAO-B activity. The compound LSL 60101 was the most potent inhibitor (IC_{50}: 16 μM) (Table 1 and Figure 4). Other drugs such as 2-BFI, cirazoline, LSL 61122 and guanabenz also fully inhibited the MAO-B activity but with lower potencies (IC_{50}s: 23 to 40 μM) (Table 1 and Figure 4). Idazoxan and oxymetazoline displayed low potencies in inhibiting MAO-B activity (IC_{50}s: 624 μM and 1,380 μM). Again, agmatine was a very poor inhibitor of MAO-B activity, with only a 17% inhibition being observed at a concentration of 10 mM, and its inhibition kinetics were not studied further (Table 1 and Figure 4).

Although clonidine behaved as a simple competitive inhibitor of MAO-B (see above) all the other drugs studied behaved as linear mixed inhibitors of this enzyme with respect to 2-phenylethylamine (Figure 6, Lineweaver-Burk plots: increases in K_m and decreases in V_{max} values). Dixon plots of MAO-B kinetics for guanabenz, cirazoline and LSL 60101 further suggested the mixed nature of the interaction for these drugs (data not shown). In no case did the Hill coefficient for inhibition differ significantly from unity, indicating the absence of cooperativity.

Inhibition of [^{3}H]-Ro 41-1049 and [^{3}H]-Ro 19-6327 binding to rat liver mitochondrial fractions by various imidazol(ine)/guanidine drugs

[^{3}H]-Ro 41-1049 and [^{3}H]-Ro 19-6327 are highly selective and reversible inhibitors of MAO-A and MAO-B isoforms, respectively (Cesura *et al*., 1989; 1990). Competition experiments against these radioligands with the same imidazol(ine)/guanidine drugs were performed to assess whether these drugs could also inhibit the binding of the radioligands to the catalytic site of MAO, and then to relate their binding potencies with their potencies in inhibiting MAO-A or MAO-B activity.

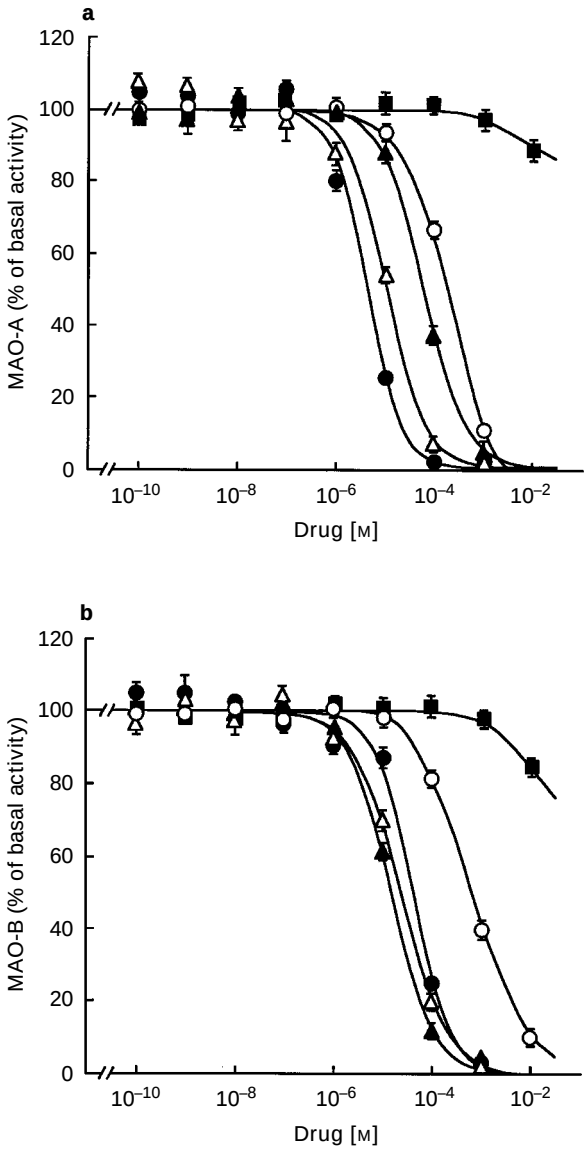

Figure 4 Inhibition of basal MAO-A (a) and MAO-B (b) activities by various imidazol(ine)/guanidine compounds in rat liver: (●) guanabenz, (△) 2-BFI, (▲) LSL 60101, (○) idazoxan and (■) agmatine. Data are expressed as % of basal MAO activity (MAO-A: 7.7 nmol 5-HT min^{-1} mg^{-1} protein, MAO-B: 5.2 nmol PEA min^{-1} mg^{-1} protein). Data shown are mean and vertical lines indicate s.e.mean of three independent experiments each in duplicate. See Table 1 for IC_{50} values and other details.

All the drugs tested (guanabenz, 2-BFL, LSL 60101, idazoxan and clonidine) displayed monophasic curves (Hill coefficients near unity) and fully inhibited the total binding of both radioligands to MAO-A or MAO-B isoforms in the rat liver mitochondrial fractions (Figure 7). Drug inhibition constants (MAO-A, K_{is}: 55 nM to 143 μM; MAO-B, K_{is}: 5 to 1,800 μM) were similar to the IC_{50} values displayed for the inhibition of MAO activity (MAO-A, IC_{50}s: 4 to 700 μM; MAO-B, IC_{50}s: 23 to 6,000 μM) (Figure 7 and Table 1). In fact, very good correlations were obtained when the potencies of the drugs in competing with [^{3}H]-Ro 41-1049 binding to MAO-A or [^{3}H]-Ro 19-6327 binding to MAO-B were correlated with the potencies of the same drugs in inhibiting MAO-A ($r=0.92$, $P=0.03$) or MAO-B ($r=0.99$, $P=0.001$) activity (Figure 8).

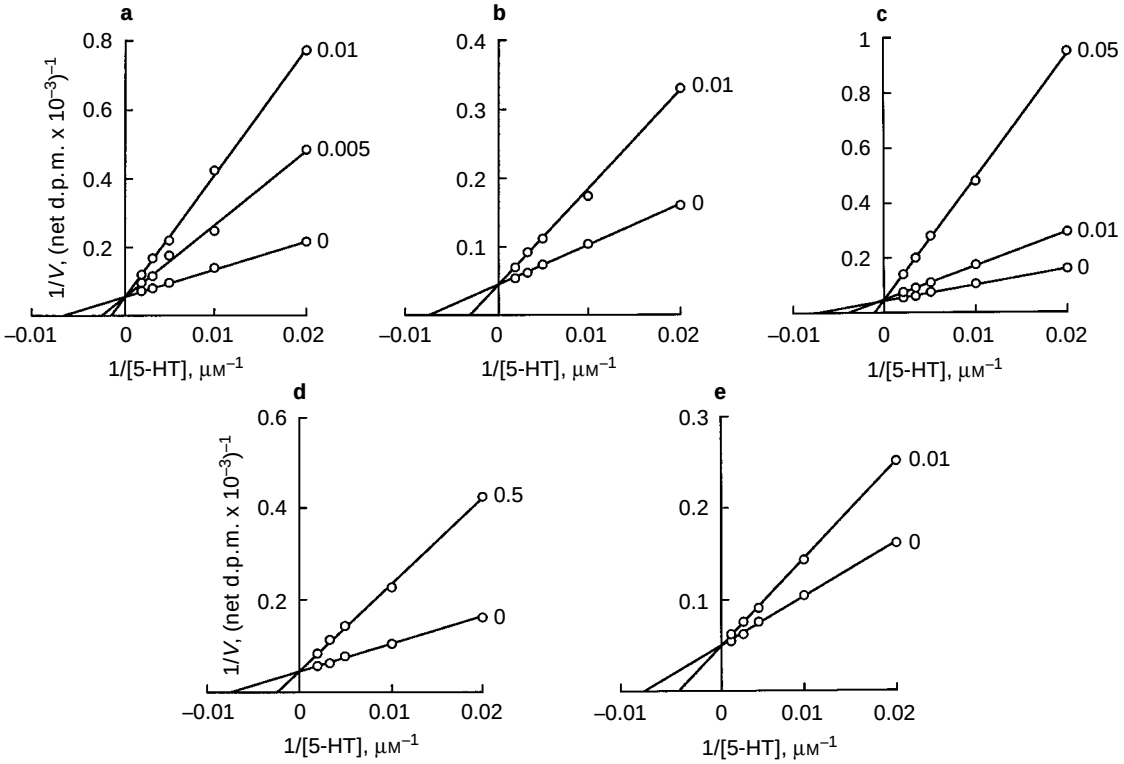

Figure 5 Kinetics of inhibition of MAO-A activity by various imidazol(ine)/guanidine compounds: (a) guanabenz, (b) 2-BFI, (c) cirazoline, (d) idazoxan and (e) LSL 60101. Lineweaver-Burk plots (substrate concentration^{-1} versus velocity^{-1}) of enzyme activity measured in the presence of increasing concentrations of the specific substrate 5-HT (50, 100, 200, 300 and 500 μM) and increasing concentrations of the inhibitors (0.005 to 0.5 mM). The intercept of the extrapolated lines with the ordinates gave the reciprocal of the maximum rate (V_{max}), whereas dissociation constants (K_m) were calculated from the intercepts with the abscissa scale. Each plot is from one representative experiment which was repeated with similar results. V_{max} (nmol 5-HT min^{-1} mg^{-1} protein) values were: 7.7, 7.7 and 8.1 (guanabenz), 8.4 and 8.2 (2-BFI), 8.4, 8.3 and 8.2 (cirazoline), 8.4 and 8.3 (idazoxan) and 8.4 and 8.3 (LSL 60101). The corresponding K_m (μM) values were: 160, 404 and 707 (guanabenz), 145 and 320 (2-BFI), 145, 254 and 237 (cirazoline), 145 and 449 (idazoxan) and 145 and 237 (LSL 60101).

Relationship between drug affinity for I_2-imidazoline receptors and drug potency in inhibiting MAO activity

In order to assess relationships between drug affinity (K_i values) for I_2-imidazoline receptors labelled with [^{3}H]-clonidine and drug potency (IC_{50} values) in inhibiting MAO-A and MAO-B activities in the rat liver mitochondria, the pK_i (pK_{iH} or pK_{iL}) and pIC_{50} values for the various imidazol(ine)/guanidine drugs were plotted (Figure 9). There were no significant correlations between the enzyme inhibitory and binding parameters either when only the data corresponding to these drugs were considered ($r = 0.14$ to 0.55; Figure 9) or when all the data including the various MAO inhibitors were considered as a whole ($r = 0.03$ to 0.11; data not shown). The discrepancies between the rank order of drug affinity and/or potency on [^{3}H]-clonidine binding and MAO inhibition, further suggested that [^{3}H]-clonidine binding sites (I_2-imidazoline receptors) are not directly related to the site of action of imidazol(ine)/guanidine drugs on MAO activity in rat liver mitochondrial fractions.

Discussion

Identification of I_2-imidazoline receptors by [^{3}H]-clonidine binding in rat liver

Clonidine, but not idazoxan, has been shown to inhibit MAO activity (Tesson *et al*., 1995; Carpéné *et al*., 1995). Because radiolabelled inhibitors of certain enzymes have been used to identify these complex proteins, [^{3}H]-clonidine was used to see if it could label MAO in rat liver, a tissue expressing high levels of both MAO-A and MAO-B (Saura *et al*., 1992). Total [^{3}H]-clonidine binding in rat liver was fully displaceable by several imidazol(ine)/guanidine drugs and some MAO inhibitors but not by (−)-adrenaline, excluding binding to α_2-adrenoceptors. Most drugs tested and the MAO inhibitors clorgyline and chlordimeform displayed biphasic curves against [^{3}H]-clonidine binding. Similar results have been shown for [^{3}H]-clonidine binding to I_1-imidazoline receptors in bovine adrenomedullary membranes (Molderings *et al*., 1993) and to putative σ_2 sites in rat stomach (Molderings *et al*., 1995). However, the rank order of affinity of competing drugs in rat liver against [^{3}H]-clonidine binding was different to that found in the above tissues. Since the K_i value of clonidine for the high-affinity binding site in rat liver was close to the micromolar range ($K_i = 0.8$ μM), this site is unlikely to represent I_1-imidazoline receptors for which K_i values of 15 and 55 nM have been obtained (Piletz & Sletten, 1993; Molderings *et al*., 1993). Moreover, the guanidine guanabenz displayed high affinity against [^{3}H]-clonidine binding in rat liver ($K_{iH} = 30$ nM) but it had low affinity ($K_i = 2{,}538$ nM) against [^{3}H]-clonidine binding to σ_2 sites in rat stomach (Molderings *et al*., 1995). Finally, a very good correlation was found when the pK_i values of imidazol(ine)/guanidine drugs were compared with the same drug values against [^{3}H]-idazoxan binding to I_2-imidazoline receptors in rat brain (Figure 2), indicating that [^{3}H]-clonidine binding to rat liver mitochondrial-enriched fractions corresponds to I_2-imidazoline receptors, previously labelled in this tissue with [^{3}H]-idazoxan (Zonneschein *et al*., 1990; Alemany *et al*., 1995b). The relevant question was then addressed as to whether the rank order of potency for I_2-imidazoline receptors of imidazol(ine)/guanidine drugs and MAO inhibitors was related to that for the inhibition of MAO-A and MAO-B ac-

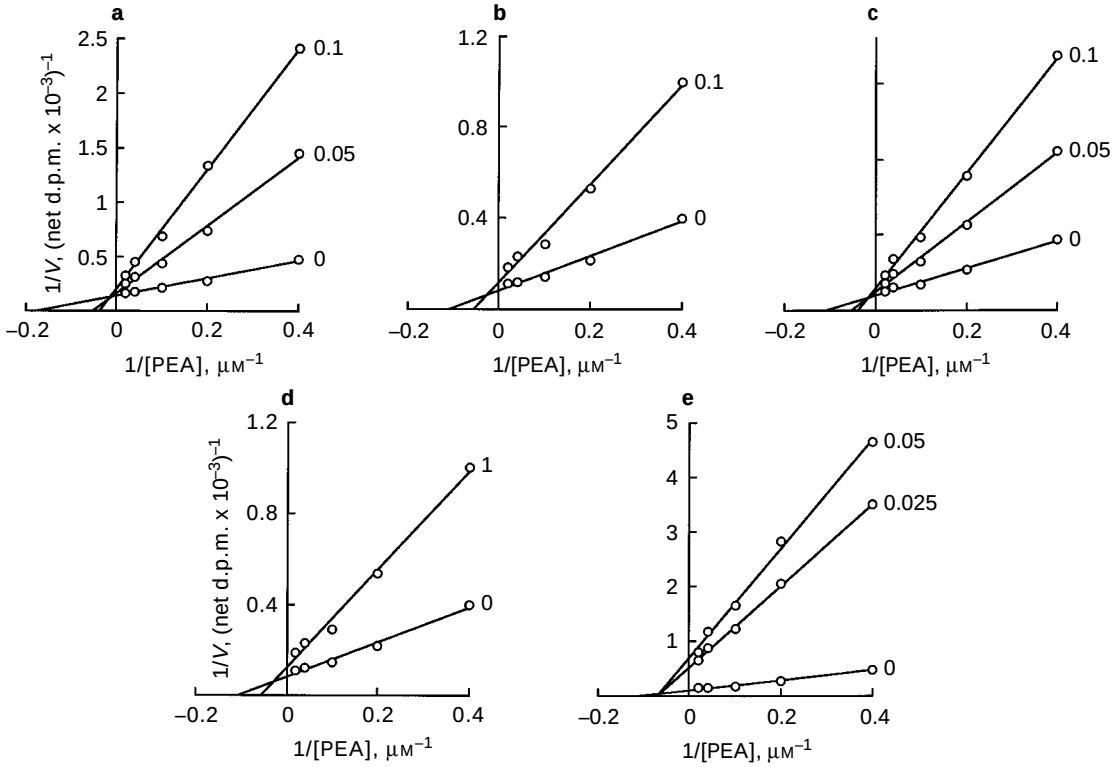

Figure 6 Kinetics of inhibition of MAO-B activity by various imidazol(ine)/guanidine compounds: (a) guanabenz, (b) 2-BFI, (c) cirazoline, (d) idazoxan and (e) LSL 60101. Lineweaver-Burk plots (substrate concentration^{-1} versus velocity^{-1}) of enzyme activity measured in the presence of increasing concentrations of the specific substrate PEA (2.5, 5, 10, 25 and 50 μM) and increasing concentrations of the inhibitors (0.025 to 1 mM). The intercept of the extrapolated lines with the ordinates gave the reciprocal of the maximum rate (V_{max}), whereas dissociation constants (K_m) were calculated from the intercepts with the abscissa scale. Each plot is from one representative experiment which was repeated with similar results. V_{max} (nmol PEA min^{-1} mg^{-1} protein) values were: 5.2, 4.0 and 3.5 (guanabenz), 5.9 and 3.0 (2-BFI), 5.8, 4.5 and 3.7 (cirazoline), 5.9 and 3.7 (idazoxan) and 5.6, 1.9 and 1.7 (LSL 60101). The corresponding K_m (μM) values were: 4, 13 and 22 (guanabenz), 6 and 15 (2-BFI), 6, 16 and 22 (cirazoline), 6 and 11 (idazoxan) and 7, 15 and 22 (LSL 60101).

tivities in rat liver. The results showed that this was not the case.

Inhibition of MAO activity by imidazol(ine)/guanidine drugs

Carpéné *et al*. (1995) first demonstrated that, at relatively high concentrations, various imidazol(ine) drugs (cirazoline, 2-BFI, 1-methyl-5-n-heptyl-imidazol or S15674; IC_{50}s: 20–50 μM), but not idazoxan and 2-n-heptyl-imidazoline or S15430 (up to 1 mM), fully inhibited the MAO activity in rat liver membranes. Furthermore, in transformed yeast expressing MAO-A and in rabbit renal cortex membranes, cirazoline, guanabenz, clonidine and moxonidine were able to inhibit MAO activity to various extents (40–95%) (Tesson *et al*., 1995). Data from other studies further indicated that imidazol(ine)/guanidine drugs can inhibit MAO activity in rabbit kidney *in vitro* (MacKinnon *et al*., 1995) and in rat liver *in vitro* and *in vivo* (Raasch *et al*., 1996).

The results of the present study confirm and extend these previous findings demonstrating the ability of various imidazol(ine)/guanidine drugs to inhibit MAO activity. The procedures used in the majority of the earlier studies did not discriminate between the MAO-A and MAO-B isoforms. Thus, MAO activity was measured with the non-selective substrate [^{14}C]-tyramine in rat liver (Carpéné *et al*., 1995) and in rabbit kidney (Tesson *et al*., 1995), two tissues co-expressing MAO-A and MAO-B isoforms (Youdim *et al*., 1988; Saura *et al*., 1992; Limon-Boulez *et al*., 1996). In contrast, the experimental conditions of the present study, use of the selective substrates 5-HT for MAO-A and PEA for MAO-B and the selective MAO inhibitors clorgyline and L-deprenyl, allowed the inhibitory effects and nature of the interactions of imidazol(ine)/guanidine drugs on the two MAO isoforms to be evaluated separately.

In general, imidazol(ine)/guanidine drugs were found to be weak MAO inhibitors with the exception of guanabenz, 2-BFI and cirazoline on MAO-A (IC_{50}s: 4–11 μM) and LSL 60101 on MAO-B (IC_{50}: 16 μM). The results of earlier studies showing incomplete inhibition of MAO by the prototypical I_2-imidazoline receptor ligand idazoxan (Carpéné *et al*., 1995; MacKinnon *et al*., 1995) might suggest the inhibition by this compound to be partial. However, the present studies revealed this compound to be a full inhibitor, although with rather low potency, on both MAO-A and MAO-B enzymes. Clonidine, the ligand used in the present study to identify I_2-imidazoline receptors in rat liver was a very weak inhibitor of both MAO isoforms in this tissue (IC_{50}s: 700 μM for MAO-A and 6,000 μM for MAO-B). Agmatine, a putative endogenous ligand of I_2-imidazoline receptors (Li *et al*., 1994) was a very weak inhibitor of both MAO-A and MAO-B activities. Moreover, these imidazol(ine)/guanidine drugs showed little discrimination between the two MAO isoforms. Cirazoline (10 fold) and clonidine (8.5 fold) were more selective for MAO-A, whereas LSL 60101 (3.6 fold) and LSL 61112 (3 fold) were more selective for MAO-B.

In vivo inhibition of MAO activity with some of these drugs (e.g. 2-BFI) has recently been obtained (Raasch *et al*., 1996) and treatment of rats with LSL 60101 (30 mg kg^{-1} for 60 min) has been shown to increase the levels of dopamine (61%, $n=7$, $P<0.01$) and 5-HT (40%, $n=7$, $P<0.01$) in the cerebral cortex (Artiga *et al*., 1994), which is consistent with the pattern of MAO-B and MAO-A inhibition for LSL 60101 obtained in the present study (Table 1). Other studies have also shown that

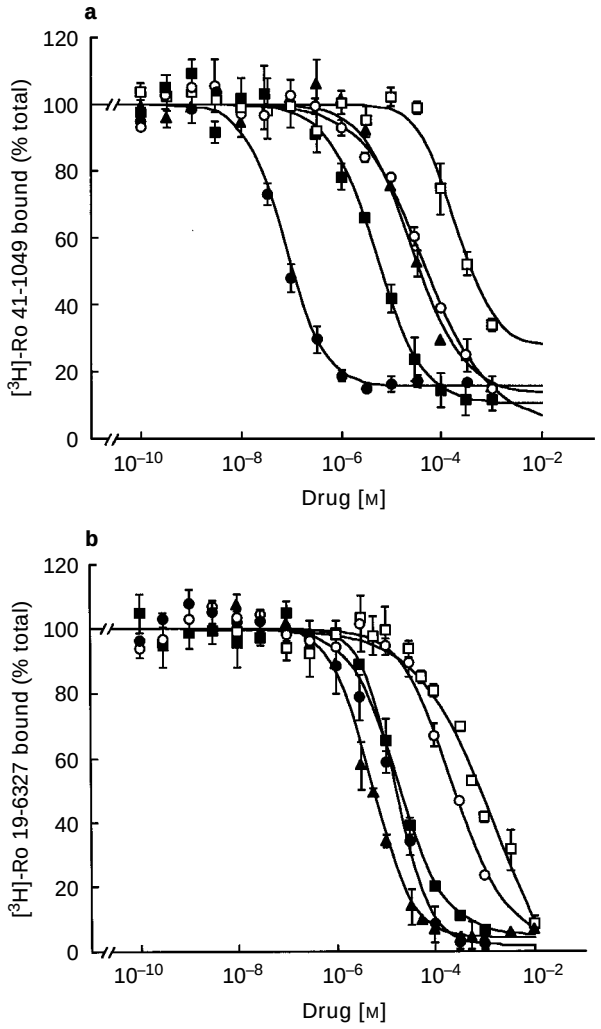

Figure 7 Inhibition of [^{3}H]-Ro 41-1049 binding to MAO-A (a) and [^{3}H]-Ro 19-6327 binding to MAO-B (b) in rat liver membranes by various imidazol(ine)/guanidine compounds: (●) guanabenz, (■) 2-BFI, (▲) LSL 60101, (○) idazoxan and (□) clonidine. Liver membranes were incubated with [^{3}H]-Ro 41-1049 (4×10^{-9} M) at 37°C for 60 min or with [^{3}H]-Ro 19-6327 (2×10^{-9} M) at 20°C for 90 min in the absence or presence of various concentrations of the competing drugs. Total control binding was 13,500 d.p.m. for [^{3}H]-Ro 41-1049 and 6,000 d.p.m. for [^{3}H]-Ro 19-6327. Data shown are mean and vertical lines indicate s.d. of two independent experiments per drug. Drug K_i values were calculated by simultaneous non-linear analysis of the two experiments with the GraFit programme. Against [^{3}H]-Ro 41-1049: guanabenz, $K_i=55$ nM; 2-BFI, $K_i=3.8$ μM; LSL 60101, $K_i=20$ μM; idazoxan, $K_i=36$ μM and clonidine, $K_i=143$ μM. Against [^{3}H]-Ro 19-6327: guanabenz, $K_i=14$ μM; 2-BFI, $K_i=15$ μM; LSL 60101, $K_i=5$ μM; idazoxan, $K_i=198$ μM and clonidine, $K_i=1{,}800$ μM.

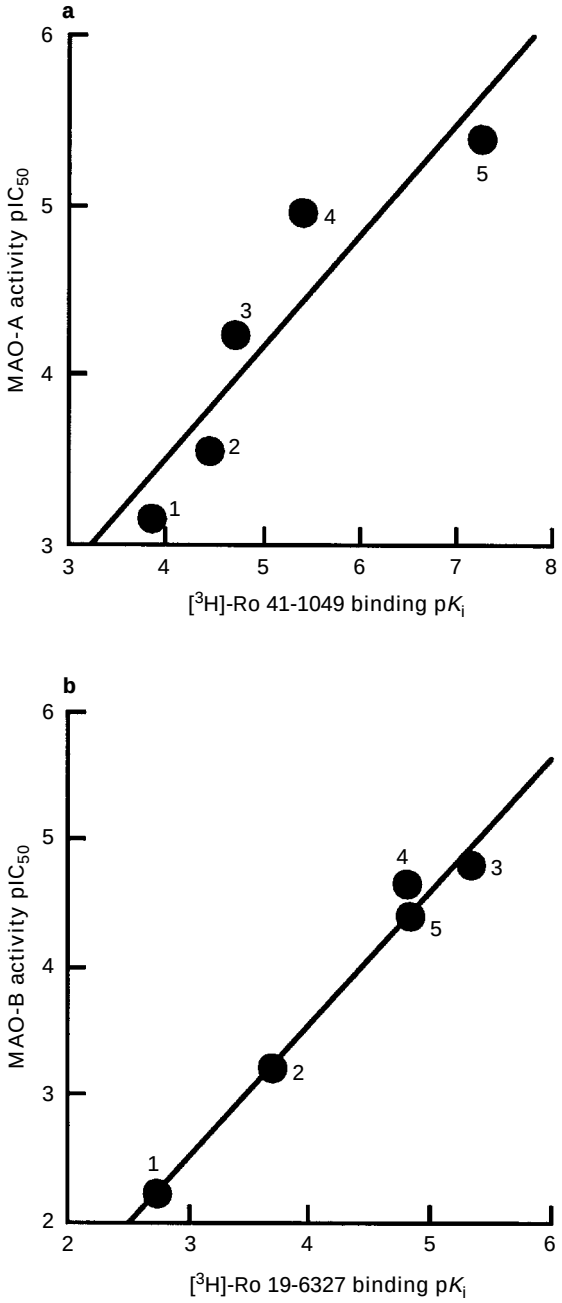

Figure 8 Correlations between the inhibition constants (expressed as pK_i values, i.e., the negative logarithm of the inhibition constant) of various drugs against [^{3}H]-Ro 41-1049 binding to MAO-A (a) or [^{3}H]-Ro 19-6327 binding to MAO-B (b) and the potencies of the same drugs (expressed as pIC_{50} values) for inhibition of MAO-A (a) or MAO-B (b) activity in rat liver. See Table 1 and Figure 7 for IC_{50} and K_i values, respectively. The identification of drugs is as follows: (1) clonidine, (2) idazoxan, (3) LSL 60101, (4) 2-BFI and (5) guanabenz. The solid line represents the regression of the correlation. The data were best described by the expressions: (a) $y=0.89+0.66\times$ ($r=0.92$; $P=0.03$) and (b) $y=0.61+1.04\times$ ($r=0.99$; $P=0.001$).

treatment of rats with the imidazolines RX 821029 (1,3-benzodioxanyl-2-imidazoline) and 2-BFI (5–20 mg kg^{-1}) caused increases in the extracellular levels of noradrenaline in the frontal cortex *in vivo* (Lalies & Nutt, 1993; 1995), which may also reflect inhibition of MAO activity in the brain. These results indicate that *in vivo* inhibition of MAO activity can be achieved with some of these imidazol(ine)/guanidine compounds and this could in turn explain some biological effects of these drugs on specific neurotransmitter systems.

Nature of MAO inhibition by imidazol(ine)/guanidine drugs: competitive for MAO-A and mixed for MAO-B

The mitochondrial enzyme MAO is involved in the metabolic pathways of various neurotransmitters (5-HT, noradrenaline, dopamine) and exogenous amines. It catalyses a bisubstrate reaction in which the amine is oxidatively deaminated in the presence of molecular oxygen. The oxidation of primary amines by both MAO-A and MAO-B isoforms is known to occur via a double-displacement, 'ping-pong' mechanism, involving a reduction of the covalently bound flavin-adenine dinucleotide cofactor (prosthetic group) by the amine substrate and its subsequent reoxidation by oxygen (Houslay & Tipton, 1973).

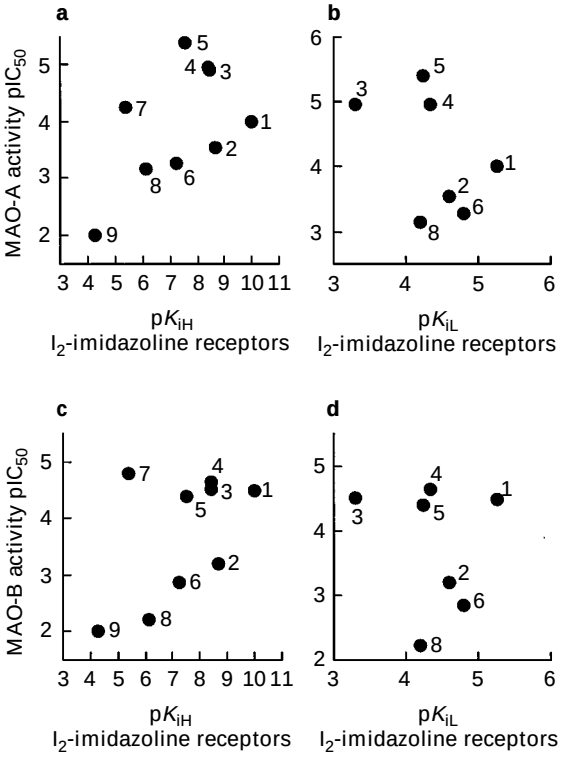

Figure 9 Relationship between the inhibition constants (expressed as pK_{iH} or pK_{iL} values, i.e., the negative logarithm of the inhibition constant for the high or low affinity site) of various drugs against [^{3}H]-clonidine binding to I_2-imidazoline receptors and the potencies of the same drugs (expressed as pIC_{50}) for inhibition of MAO-A (a,b) or MAO-B (c,d) activity in rat liver. See Table 1 for K_{iH}, K_{iL} and IC_{50} values. The identification of drugs is as follows: (1) LSL 61122, (2) idazoxan, (3) cirazoline, (4) 2-BFI, (5) guanabenz, (6) oxymetazoline, (7) LSL 60101, (8) clonidine and (9) agmatine. No significant correlations were found between variables. (a) $r=0.55$, $P=0.12$; (b) $r=0.46$, $P=0.30$; (c) $r=0.53$, $P=0.14$; (d) $r=0.14$, $P=0.77$.

Recently, I_2-binding sites ([^{3}H]-idazoxan binding) have been identified on both MAO-A and MAO-B isoforms (Raddatz *et al*., 1995; Tesson *et al*., 1995; Parini *et al*., 1996). Moreover and because of the different tissue sensitivity to amiloride, a guanidide able to discriminate between I_{2A}- and I_{2B}-subtypes, it has been proposed that I_{2A}-sites are located on MAO-A and I_{2B}-sites on MAO-B (Parini *et al*., 1996). These I_2-binding sites do not appear to be located within the enzyme catalytic site, or the prosthetic group, or the binding domain of classical MAO inhibitors (Carpéné *et al*., 1995; Raddatz *et al*., 1995; Tesson *et al*., 1995; Limon-Boulez *et al*., 1996). Therefore, I_2-binding sites on MAO-A and MAO-B were proposed to represent previously unknown regulatory sites able to modulate the activity of MAO through an allosteric inhibitory mechanism (for a review see Parini *et al*., 1996).

Such a mechanism was explicitly proposed to explain the nature of the inhibition of MAO activity by certain imidazol(ine)/guanidine drugs (Tesson *et al*., 1995) (see above). In that study I_2-imidazoline receptor ligands (cirazoline and guanabenz) were found to be more potent than I_1-ligands (clonidine and moxonidine) in inhibiting MAO activity in transformed yeast expressing MAO-A and rabbit renal cortical membranes co-expressing MAO-A/B. Furthermore, cirazoline acted as a noncompetitive MAO inhibitor in rabbit kidney membranes (Lineweaver-Burk plots: decreased V_{max} values with unchanged K_m values) (Tesson *et al*., 1995). The demonstration of such a noncompetitive mechanism was regarded as being crucial for the proposition of an allosteric regulatory site on MAO. However, unfortunately the nature of the drug/enzyme interaction was assessed only for cirazoline and only in rabbit kidney (mixture of MAO-A/B activities) (Tesson *et al*., 1995). In the study of Carpéné *et al*. (1995), the inhibition of MAO activity in rat liver (mixture of MAO-A/B activities under the assay conditions used) by the imidazol S15674 also appeared to be of noncompetitive nature (i.e. the reductions of V_{max} values, 24–71%, were similar to those of V_{max}/K_m ratios, 35–73%), but that induced by 2-BFI was clearly a mixed type inhibition (Lineweaver-Burk plots: decreased V_{max} values and increased K_m values). However, the calculated decreases on V_{max}/K_m ratios (19–76%) were greater than those on V_{max} values (8–31%) which suggests that enzyme inhibition by 2-BFI was predominantly of the competitive type (see Price & Stevens, 1989).

The results of the present studies show that all the drugs tested were linear competitive inhibitors of MAO-A (Figures 3 and 5). Although competitive inhibition may mean a compound binding to a site that is distinct from the active site of an enzyme and exerting its effects through a conformational change, the simplest explanation for the present finding would be direct competition through binding to the same site as the substrate. Such an explanation would also be consistent with the correlation between displacement of the specific MAO inhibitor Ro 41-1049 and MAO inhibition (see Figure 8a). Thus, the present results indicate that, in the case of MAO-A, there is no need to propose an allosteric inhibitory site on the enzyme (Parini *et al*., 1996) to account for the behaviour of these compounds.

With the exception of clonidine, which behaved as a simple competitive inhibitor, the inhibition of MAO-B by these compounds was found to be mixed (Figures 3 and 6). The results obtained with 2-BFI agree well with those previously found by Carpéné *et al*. (1995). The greater inhibitory effects of imidazol(ine)/guanidine drugs on the apparent value of V_{max}/K_m than on the apparent V_{max} value indicates that the competitive element of the MAO-B inhibition by these drugs predominates (see Price and Stevens, 1989), which is consistent with the affinities of the drugs for the catalytic site of the enzyme (see Figure 8b). It is noteworthy that none of the eight drugs studied in the present work gave the simple non-competitive inhibition that was interpreted in terms of allosteric interactions by Tesson *et al*. (1995) and Parini *et al*. (1996). However, at present the possibility that the mixed inhibition of MAO-B by these drugs might result from simple competitive inhibition by binding to the active site plus inhibition through allosteric interactions cannot be excluded.

Mixed inhibition in a reaction mechanism involving two substrates (amine and oxygen) and three products (ammonia, aldehyde and hydrogen peroxide) can occur in a variety of ways (see Tipton 1996), the simplest of which would be direct binding of the drug to the active sites of both the free enzyme and the reduced form liberated as an intermediate in the 'ping-pong' mechanism (see Houslay & Tipton, 1973). Thus, as was the case for MAO-A, there is no necessity for invoking allosteric interactions to explain such behaviour.

Lack of relationship between recognition of I_2-imidazoline receptors and inhibition of MAO

If an I_2-allosteric site were to exist on MAO (Parini *et al*., 1996), a similar drug structure-activity relationship would be expected when comparing drug potency at the postulated allosteric I_2-site and potency of the same drugs in inhibiting MAO activity. However, no significant correlations were found when the potencies of imidazol(ine)/guanidine drugs at the high affinity site (pK_{iH}, nanomolar range) or the low-affinity site (pK_{iL} micromolar range) of I_2-imidazoline receptors labelled with [^{3}H]-clonidine were correlated with the pIC_{50} values of the same drugs for inhibition of MAO-A or MAO-B activities (Figure 9). Moreover, for the drugs clonidine, idazoxan, LSL 60101, 2-BFI and guanabenz, multiple regression analyses between potency (pK_{iH} or pK_{iL}) on I_2-imidazoline receptors, potency (pIC_{50}) on MAO-A or MAO-B activities and affinity (pK_i) for the catalytic site of MAO-A or MAO-B

also resulted in nonsignificant correlations ($r = 0.06$ to 0.59; $P < 0.05$), which reinforced the lack of association between drug interaction with I_2-imidazoline receptors (dependent variable) and MAO activity (independent variables).

Although these studies cannot exclude the presence of additional binding sites on MAO that do not affect the activity of enzyme, several proteins with apparent molecular weights in the range 25–30 kDa, which is very different from those of either MAO-A or MAO-B (Bach *et al*., 1988), have been implicated in idazoxan binding (Escribá *et al*., 1994; 1996) or have been photolabelled with an imidazoline probe (Lanier *et al*., 1995) in rat liver. Thus these results and those obtained in the present study would suggest that the I_2-imidazoline receptors represent molecular species that are distinct from MAO.

Together the results indicate that most imidazol(ine)/guanidine drugs are weak inhibitors of MAO-A and MAO-B isoforms and that the nature of the interactions is most probably through competitive/catalytic site related mechanisms. Therefore, I_2-imidazoline binding sites in rat liver do not appear to represent allosteric inhibitory sites on MAO, as suggested previously (Tesson *et al*., 1995; Parini *et al*., 1996). The function, if any, of I_2-imidazoline sites on MAO (Tesson *et al*., 1995) remains to be determined.

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