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Inhibition of Rat Liver Mitochondrial Monoamine Oxidase by Hydrazine–Thiazole Derivatives: Structure–Activity Relationships

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Abstract—The purpose of this research is to study the relationship between chemical structure and inhibitory activity of some hydrazine–thiazole derivatives on rat liver mitochondria monoamine oxidase (MAO). Forty-five compounds belonging to three series of hydrazine–thiazole derivatives, with either alkylic or arylic substituents in the thiazole ring, were tested. The highest inhibitory activity was observed with piperonyl derivatives **25** and **40**, which contain a 4-methyl group in the thiazole nucleus. The structure–activity relationship of MAO inhibitors was established in relation to hydrophobic, electronic and steric hindrance parameters. A mechanism of enzyme inhibition was proposed based on the calculation of HOMO energies.

Introduction

Monoamine oxidase (MAO, EC 1.4.3.4) is a ubiquitous flavoenzyme, which is particularly abundant in the liver and brain.[1] MAO is located in the outer mitochondrial membrane and catalyzes the oxidative deamination of several biogenic and exogenic amines.[2] There are two distinct isoforms of MAO (termed A and B)[3] which differ by their substrate and inhibitor specificity, tissue distribution and immunological characteristics.[4] Although the primary structure of the two isoenzymes is different,[5–7] the amino acids sequence at the covalently bound flavin site is identical.[8,9] Chemical modification studies suggest that there are two essential histidine and cysteine residues in the active site of the enzyme.[10,11]

In the central and peripheral nervous system, MAO catalyzes the metabolic transformation of amine neurotransmitters; whereas, in the liver, MAO functions as a detoxicating enzyme against foreign amine compounds that enter the body.[12] Numerous compounds, belonging to a great variety of substituted hydrazines, behave as MAO inhibitors.[13–15] A common structural feature of substrates and inhibitors is an amino or imino group, which is assumed to play an essential role in orientation and complex formation at the active site of the enzyme.[16] The presence of an aryl moiety is not an absolute requirement for enzyme inhibition; however, substitutions or modifications on the aryl ring influence the potency of inhibitors.[17] A general model for the interaction between MAO and substrates or inhibitors has been proposed. This model describes three binding sites on the enzyme: an amine binding site, a hydrophobic site and a nucleophilic site.[18] Variation in the hydrophobic or electronic characteristics of substituents are expected to influence the binding of inhibitors to the enzyme surface.[19]

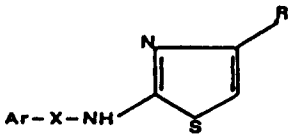
We have already described the synthesis of thiazol-2-yl–hydrazine derivatives and their potency as MAO inhibitors in rat brain mitochondria.[20] In addition, we have investigated, in lipid membrane models, the thermotropic behavior of these thiazole derivatives in relation to both their partition coefficient and MAOI (MAO inhibitory) activity, to evaluate the relationship between lipophilicity and potency of inhibitors.[21]

In this study, we have measured monoamine oxidase activity in the presence and absence of a series of thiazolyl–hydrazine derivatives containing alkyl and aryl substituents in the thiazole ring. The values obtained for the inhibitory activity of the thiazole derivatives were correlated to the hydrophobic characteristics of the compounds. The latter were evaluated on the basis of HOMO energy calculations.

Results and Discussion

The MAOI activities of the compounds examined are summarized in Table 1. Among the alkoxybenzoyl-thiazolhydrazines, the piperonyl and trimethoxyphenyl derivatives exhibited the highest MAOI activity. The presence of substituents in the fourth position of the thiazole ring potentiated the activity, which was higher in the case of phenyl (compounds **2** and **7**), or methoxyphenyl (compounds **4** and **9**) substituents. Compounds of the alkoxybenzylidene series were in general the most active; their activity was further

Table 1. *In vitro* MAOI activity on rat liver mitochondria of hydrazine–thiazole derivatives (10^{-4} M) by kynuramine fluorimetric assay (see Experimental)



Ar	R	X= - CO-NH -		X= - CH=N -		X= - CH_2 - NH -	
		Compound	inhibition %	Compound	inhibition %	Compound	inhibition %
[TRIMETHOXYPHENYL]							
3,4,5-$(OCH_3)_3$-C_6H_2	H	**1**	29.0 ± 0.07	**16**	37.6 ± 0.90	**31**	33.5 ± 1.14
3,4,5-$(OCH_3)_3$-C_6H_2	C_6H_5	**2**	62.1 ± 1.91	**17**	65.4 ± 0.19	**32**	43.8 ± 0.00
3,4,5-$(OCH_3)_3$-C_6H_2	C_6H_4-CH_3 (p)	**3**	56.1 ± 0.22	**18**	67.8 ±0.89	**33**	51.3 ± 1.85
3,4,5-$(OCH_3)_3$-C_6H_2	C_6H_4-OCH_3 (p)	**4**	68.2 ± 0.95	**19**	63.7 ± 0.37	**34**	45.4 ± 1.10
3,4,5-$(OCH_3)_3$-C_6H_2	CH_3	**5**	52.8 ± 3.99	**20**	76.1 ± 3.47	**35**	68.3 ± 0.44
[PIPERONYL]							
3,4-(O-CH_2-O)-C_6H_3	H	**6**	43.0 ± 0.42	**21**	20.5 ±0.63	**36**	38.4 ± 0.22
3,4-(O-CH_2-O)-C_6H_3	C_6H_5	**7**	65.6 ± 0.89	**22**	76.3 ± 1.46	**37**	52.0 ± 0.60
3,4-(O-CH_2-O)-C_6H_3	C_6H_4-CH_3 (p)	**8**	49.0 ± 0.89	**23**	21.8 ± 0.60	**38**	47.3 ± 1.24
3,4-(O-CH_2-O)-C_6H_3	C_6H_4-OCH_3 (p)	**9**	65.8 ± 0.38	**24**	29.3 ± 0.35	**39**	48.2 ± 1.22
3,4-(O-CH_2-O)-C_6H_3	CH_3	**10**	50.0 ± 0.15	**25**	97.6 ± 0.13	**40**	89.0 ± 0.54
[ETHYLSYRINGYL]							
3,5-$(OCH_3)_2$-4-OC_2H_5-C_6H_2	H	**11**	22.9 ± 2.64	**26**	36.7 ± 1.52	**41**	26.3 ± 1.10
3,5-$(OCH_3)_2$-4-OC_2H_5-C_6H_2	C_6H_5	**12**	54.5 ± 0.05	**27**	67.3 ± 1.30	**42**	65.3 ± 0.13
3,5-$(OCH_3)_2$-4-OC_2H_5-C_6H_2	C_6H_4-CH_3 (p)	**13**	37.4 ± 0.13	**28**	48.5 ± 0.85	**43**	60.7 ± 1.86
3,5-$(OCH_3)_2$-4-OC_2H_5-C_6H_2	C_6H_4-OCH_3 (p)	**14**	40.0 ± 0.57	**29**	53.2 ± 2.41	**44**	70.3 ± 1.22
3,5-$(OCH_3)_2$-4-OC_2H_5-C_6H_2	CH_3	**15**	36.0 ± 2.35	**30**	70.5 ± 0.17	**45**	47.4 ± 0.01

enhanced by the presence of a methyl group in the fourth position in the thiazole nucleus (compounds **20**, **25** and **30**). The highest inhibition value (97.6%), in this series was obtained with the piperonyl derivative **25**. Alkoxybenzyl derivatives showed the lowest MAOI activity. However, the introduction of a methyl group in the thiazole ring (compounds **35** and **40**), particularly with the piperonyl derivatives, substantially increased the activity.

Substituents in the fourth position of the thiazole ring (R) also influenced the activity, which appeared higher for methyl substituted in the alkoxybenzylidene series and for phenyl or methoxyphenyl substituted in the alkoxybenzoyl series. Some difference between these results and those we obtained on rat brain mitochondria[20] could be due to specificity of inhibitor molecules on two distinct isoforms of MAO which are present in different proportions in brain and liver rat mitochondria.

To further study the mechanism of MAO inhibition, we have selected the most potent compounds of the piperonyl series (**9**, **25** and **40**), which contain the methyl or *p*-methoxyphenyl substituent in the fourth position of the thiazole ring. MAOI activity was concentration-dependent (**9**, **25**, **40**) in the range of 10^{-8} to 10^{-2} M (Fig. 1). Apparent (IC_{50}) values relative to

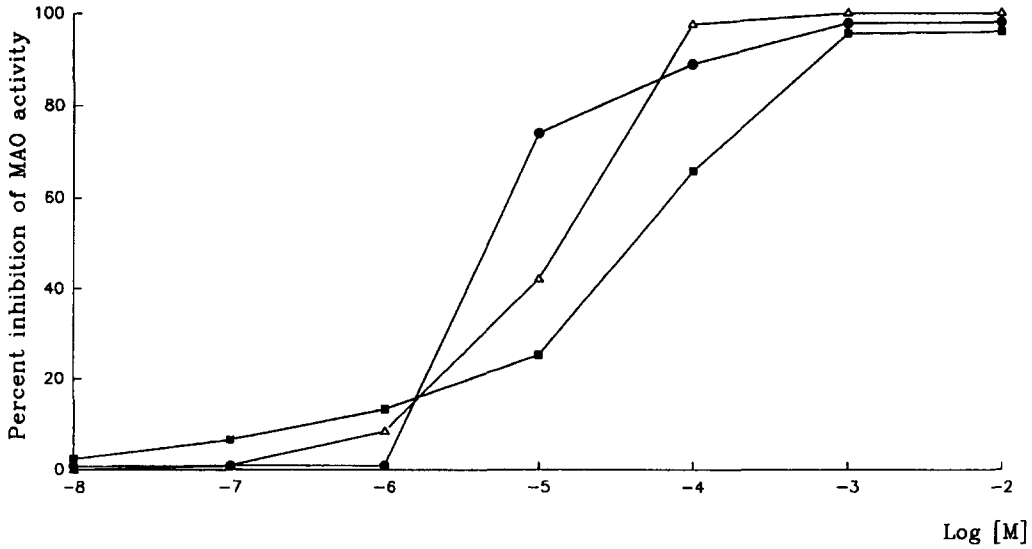


Figure 1. Concentration-dependent inhibition of MAO activity, in rat liver mitochondria, in the presence of different concentrations of compounds **9** (■), **25** (Δ), and **40** (●). Data are from a representative experiment performed in triplicate.

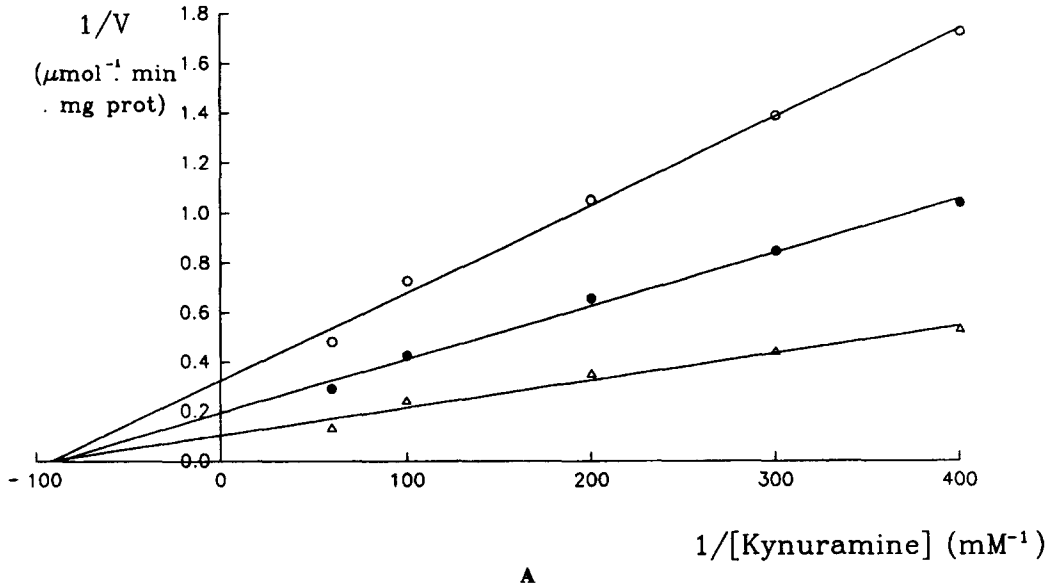
compounds **9**, **25** and **40**, were 57.4 ± 7.3, 13.2 ± 2.5 and 5.7 ± 1.2 μM, respectively. Kinetic analysis shows that compounds **25** and **40** influence the V_{max} without changing the K_m, which is typical of a noncompetitive type of inhibition (Fig. 2). In addition, to evaluate the specificity of inhibition, we have assayed compounds **9**, **20**, **25**, **40** or **44** in the presence of [^{14}C]-5-hydroxytryptamine (5-HT), or [^{14}C]-β-phenylethylamine (PEA), which are known substrates for MAO-A and -B, respectively. At 10^{-4} M concentration, compounds **20** and **25** selectively inhibited MAO-B, compound **9** specifically inhibited MAO-A, compounds **40** and **44** inhibited both MAO-A and -B (Table 2).

Structure–activity relationships

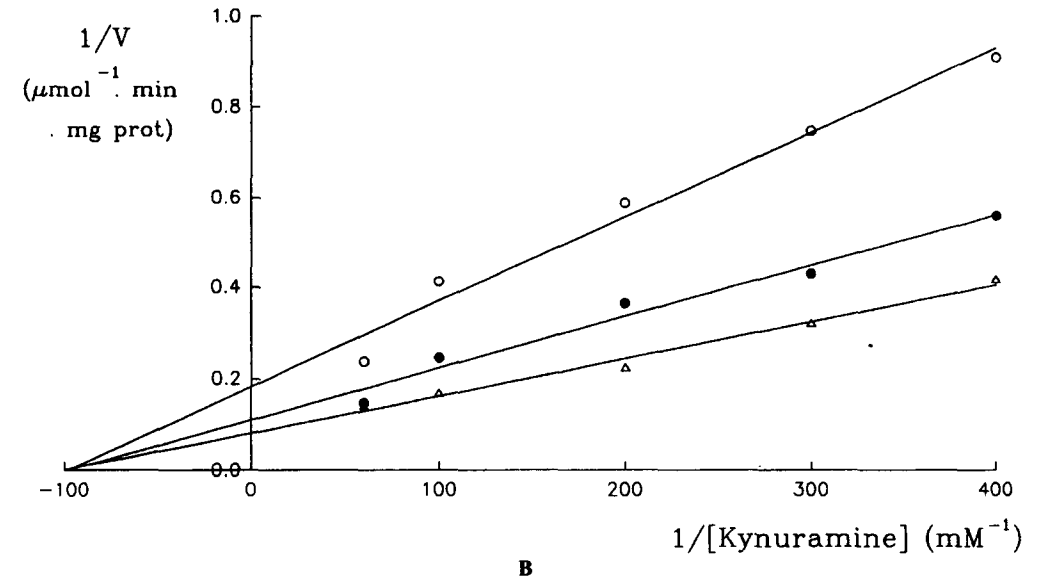
The analysis of the inhibition data reported in Table 1 reveals interesting effects of the substituents. All MAO inhibitors contained two side-groups, Ar and R, located at the edge of the main group. A major difference among the substituents lies in their size and hydrophobicity, while minor deviations are present in their electron-withdrawing or -donating power. Therefore, we

Table 2. Selectivity of the inhibitors (10^{-4} M) towards MAO types A and B in rat liver mitochondria, by radiochemical assay (see Experimental)

Inhibitor	MAO-A Inhibition %	MAO-B Inhibition %
9	29.5 ± 0.35	0
20	0	64.6 ± 0.19
25	0	84.6 ± 0.28
40	41.6 ± 0.57	60.2 ± 1.86
44	52.3 ± 0.60	13.2 ± 2.35



A



B

Figure 2. Lineweaver–Burk plot: MAO activity was measured, under standard condition, with 2.5, 3.0, 5.0, 10, 15 μM kynuramine in the absence (Δ) or presence of **25** (A) and **40** (B) at 10^{-5} M (●) and 10^{-4} M (○) concentration. The activity was expressed as μmol^{-1} min mg protein.

analyzed the biological effects in terms of the hydrophobic parameter, π, as reported in Figures 3A–C. Holding fixed the substituent Ar attached to the hydrazine group, we plotted the biological activity versus the parameter π of the R group linked to the thiazole ring. The parameters π were calculated according to Rekker's fragmental constants method,[22,23] whereas the biological activity parameter, expressed as per cent of inhibition, was measured as described in the Experimental section. A similar approach has been recently applied to the study of oxodiazolone derivatives, which exhibit MAOI activity.[24] Results indicated an increase in MAOI activity as a function of the lipophilic character of substituents in the aromatic ring. All curves exhibited a bell-shaped form where the MAOI activity increases with hydrophobicity, reaches a maximum and then decreases, suggesting two competing effects. While a drug's hydrophobicity is required to cross the mitochondrial membrane, too high a hydrophobicity of side groups could trap the substrate within the lipid membrane, impeding a rapid turnover and slowing down the overall reaction rate.

Another interesting point is the effect of the side group Ar (Table 1). The average biological activity (obtained as the mean value of the activity data of 15 different compounds) was higher with trimethoxyphenyl substituted, followed by piperonyl, and then ethyl-syringyl. This result correlates with the size of the substituents, but is not consistent with their hydrophobicity scale. These data suggest that steric hindrance of substituents linked to hydrazine residue may have a role at the enzyme's catalytic site.

Inhibition mechanism

An important point regarding the mechanism of inhibition is the reactivity difference, as related to the chemical structure of the hydrazinic groups -CO-NH-NH, -CH=N-NH-, or CH_2-NH-NH-, labeled as classes a, b and c, respectively.

It has been suggested that the first step of MAO-catalyzed amine oxidation is a one-electron transfer from the amino group to the flavine. This reaction would generate the amine radical cation, which can lose the proton to give a carbon radical.[25] On the other hand, several MAOIs seem to behave in a rather similar way, where the enzyme-inhibitor adduct is following an electron transfer from an amino group of the inhibitor to a basic enzyme's residue (flavine or, more likely, one of MAO's six cysteine residues[25]). Based on this mechanism, we should expect a good relationship between the energy of HOMO (Higher Occupied Molecular Orbital) of various amines (hydrazines) and their inhibitory power.

We calculated HOMO energies by a Quantum Mechanical program package (PM 3).[26–28] This computational tool is based on a Hartree–Fock Molecular Orbital (MO) procedure for rigorously calculating the electronic structure of medium-sized molecules. Useful parameters, such as atomic charge densities, dipole moments, energies of MO and ionization potentials, can be calculated. Moreover, automatic optimization of the energy versus geometrical parameters of the studied molecules gives very good estimates of bond distances and bond angles, together with the relative energies of possible conformations for molecules with internal degrees of freedom. The data obtained for the most stable molecular conformations with a full optimization of bond lengths and internal angles are Ar-CO-NH-NH-R: 9.5 eV, Ar-CH=N-NH-R: 9.1 eV, Ar-CH_2-NH-NH-R: 8.9 eV. These results are consistent with experimental data of photoionization spectroscopy of related compounds.[29] The differences of the HOMO energies among the hydrazines range up to 0.6 eV ($\approx$ 13.8 kcal mol^{-1}). Since the minimum energy conformation of free MAOIs may be totally irrelevant to the conformation of bound inhibitor, we calculated HOMO energies of several MAOI conformers. Differences among HOMO energies with respect to the most stable conformation are in the range of about $\pm$ 0.05 eV for most of the low-lying conformers. These results rule out the possibility that hydrazine HOMO energies are affected by inhibitor geometrical modification upon enzyme binding.

HOMO energies variations are significant but not exceedingly large because they are reflecting amine nitrogen basicity caused by modification of its chemical environment. Hence, we expect higher HOMO energy values associated with less active compounds as verified in the Ar-CO-NH-NH-R group (Fig. 3A). The small variation of electronic (HOMO energies) and hydrophobic parameters agrees with the comparable biological activity of all investigated compounds. To better understand the biological activity of our compounds, we have also considered the hydrophobicity of the hydrazinic residues a, b and c. Such a contribution has to be added to that of substituents previously calculated.

Since -CO-NH-NH- and -CH_2-NH-NH- groups are less hydrophobic than -CH=N-NH-, we expect in the plots of Figures 3A–C the maximum of -CH=N-NH-class to be shifted toward smaller π values in comparison with the maxima of remaining classes. The analysis of the data supports this hypothesis, although further experiments are needed to reach more quantitative conclusions.

The present work attempts to correlate the contributions of hydrophobic and electronic effects of a series of thiazole derivatives on the inhibition of monoamine oxidase enzyme activity. Electronic effects, as well as hydrophobicity, of the test compounds were modified by varying the Ar and R substituents attached to the hydrazine moiety or to the thiazole ring. These electron donating or withdrawing groups, however, generally modify also drug hydrophobicity, the optimum values being reached by the piperonyl derivatives which bear a 4-methyl group in the thiazole nucleus. However, the role of side group substituents in the mechanism of MAO inhibition could be studied in a way which would

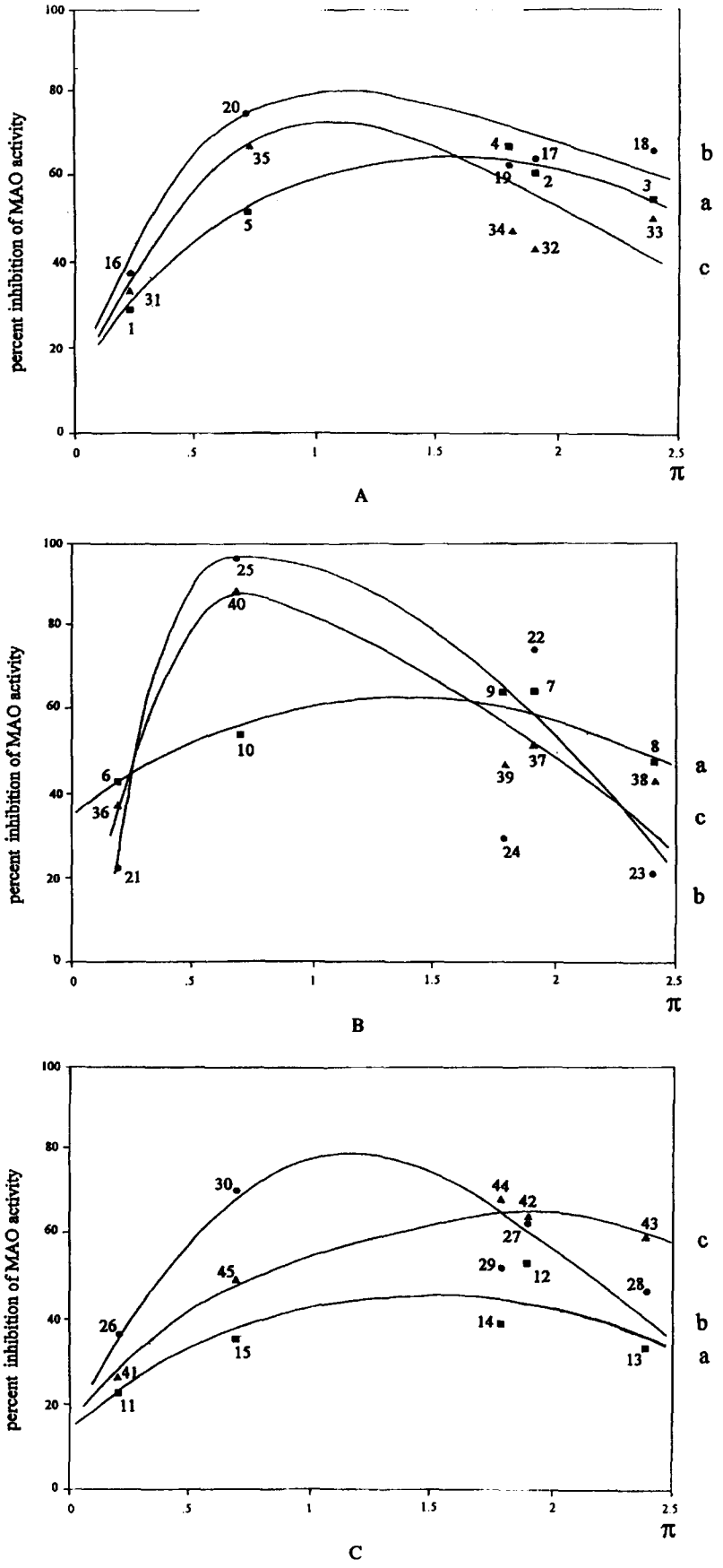


Figure 3. Plot of monoamine oxidase activity of inhibitors versus the hydrophobicity parameter π of substitutent R attached to thiazole ring. The letters A, B and C indicate the compounds containing trimethoxyphenyl, piperonyl and ethylsyringyl groups, respectively, linked to the hydrazine residue. The letters a, b and c refer to the benzoyl, benzylidene and benzyl series (see Table 1).

optimize the values associated with the physico-chemical parameters we have examined.

Experimental

Chemistry

The inhibitors of monoamine oxidase used in these experiments were synthesized in our laboratory. The three series of compounds are benzoyl-hydrazines, benzylidene-hydrazines, and benzyl-hydrazines. These compounds have the following alkoxyphenyl radicals (Ar) in the first position: 3,4,5-trimethoxyphenyl, 3,4-methylendioxyphenyl (piperonyl) and 3,5-dimethoxy-4-ethoxyphenyl (ethylsyringyl). In position 2 of the hydrazines, the thiazole nucleus is present, substituted or not by methyl or phenyl groups (R) in position 4. The structure of all the synthesized compounds was verified by elemental analysis and infrared, mass and ^{1}H NMR spectra.[20]

Chemicals

Kynuramine dihydrobromide and 4-hydroxyquinoline were purchased from Sigma Chemical Company (U.S.A.). The radioactive substrates, 5-hydroxy-tryptamine [side chain-2,^{14}C] tryptamine creatinine sulfate (57 μCi mmol^{-1}) and β-phenylethylamine [ethyl 1,^{14}C] hydrochloride (55 μCi mmol^{-1}) were obtained from Amersham Laboratories (Amersham, U.K.). The inhibitor molecules, slightly soluble in water, were dissolved in dimethylsulfoxide:H_2O (3:1, v/v). Other analytical grade chemicals were supplied by C. Erba Reagenti (Milan, Italy).

Mitochondrial preparation

Crude mitochondrial fraction was prepared from the liver of male and female albino rats, each weighing about 200–250 g. The animals were decapitated and their organs were rapidly removed. The tissue was homogenized in a Potter homogenizer with 9 volumes of ice-cold medium consisting of 220 mM mannitol, 70 mM sucrose, 0.1 mM EGTA and 5 mM Hepes buffer (pH 7.5). Mitochondria were isolated by sequential centrifugation. The homogenate was centrifuged twice at 800 *g* for 10 min at 4 °C; then resulting supernatant was centrifuged at 12,000 *g* for 20 min. The mitochondrial pellet was suspended (1:5, w/v) in 100 mM phosphate buffer (pH 7.4), fractionated in 0.5 mL samples and stored at –70 °C.

Protein determination

Protein content of mitochondrial preparations was determined according to the method of Lowry *et al.*[30] in the presence of 0.01% sodium dodecyl sulfate, using bovine serum albumin as standard.

Determination of MAO activity

MAO activity in hepatic mitochondrial fractions was determined by fluorimetric or radiochemical assay. The fluorimetric method was carried out as described by Morinan and Garratt.[31] Aliquots of 50 μL of mitochondrial suspension (800 μg of protein), 820 μL of 50 mM phosphate buffer (pH 7.4) and 100 μL of solubilizing solution (control) or inhibitor solution at the final concentration of 10^{-4} M, were preincubated at 37 °C for 5 min. The reaction was started by the addition of 30 μL of 3.07 mM kynuramine (final conc. 92 μM). The tubes were shaken for 15 min. After the addition of 300 μL of 0.4 M $HClO_4$, the tubes were mixed and centrifuged at 12,000 *g* for 30 s in a Hermle microcentrifuge to remove the precipitated proteins. The supernatant was transferred to test tubes containing 2 mL of 1 M NaOH and the fluorescence was measured against a blank at ex = 315 nm and em = 380 nm in a Perkin-Elmer LS5 spectrofluorimeter. The product concentration (4-hydroxyquinoline) was calculated from a standard curve (0.25–3.00 nmol). The MAO activity was calculated as nmol of 4-hydroxyquinoline formed mg^{-1} protein h^{-1} and expressed as per cent (± S.E.) inhibition of the respective control.

The MAO-A and -B activities were determined in the liver mitochondrial suspension by radiochemical assay (slightly modified from the assay by Mazouz *et al.*).[32] An aliquot of 10 μL of mitochondrial suspension (60 μg protein), 150 μL of 100 mM phosphate buffer (pH 7.4) and 20 μL of DMSO:H_2O (3:1, v/v), or the inhibitor diluted in DMSO:H_2O (3:1, v/v), were preincubated for 20 min at 37 °C in a shaking water bath. The reaction was started by adding 20 μL of [^{14}C]-5-hydroxy-tryptamine (5-HT) (20 μM, spec. act., 1.14 μCi μmol^{-1}) for the MAO-A assay or [^{14}C]-β-phenylethylamine (PEA) (80 μM, spec. act., 1.10 μCi μmol^{-1}) for the MAO-B assay. The total volume of the mixture was 200 μL. After incubating at 37 °C for either 40 min (MAO-A) or 10 min (MAO-B), the reaction was stopped by adding 200 μL of 2 N HCl. The radioactive product formed during the incubation was extracted with 1 mL toluene:acetate mixtures and an aliquot of 500 μL from the organic layer, obtained after centrifugation at 5000 *g* for 5 min. The product was then counted in a scintillation cocktail using a LS 5000 CE Beckman counter. The assay was routinely performed in triplicate. A blank and a control were run together for each series tested. All experiments were performed under conditions in which the product formation was linear with the amount of enzyme and the time of incubation. The MAO activity was expressed as per cent inhibition (± S.E.) as compared to the respective control sample.

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