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# THIAZOLE DERIVATIVES AS INHIBITORS OF PURIFIED BOVINE LIVER MITOCHONDRIAL MONOAMINE OXIDASE-B: STRUCTURE–ACTIVITY RELATIONSHIPS AND THEORETICAL STUDY

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Structure–activity relationships were performed on a new series of thiazole derivatives which selectively inactivate monoamine oxidase-B (MAO-B), purified from mitochondrial beef liver. All of the synthesized and tested compounds showed non-competitive inhibition, suggesting the formation of a stable adduct between the tertiary amine function, linked to the thiazolyl derivatives and the active site of the enzyme. The mechanism of MAO-B inhibition is discussed in terms of the Ionization Potential of the amine nitrogen atom and the conformational flexibility of the inhibitors.

*Keywords:* Structure–activity relationships; Thiazolyl derivatives; Monoamine oxidase inhibition; Conformational flexibility

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## INTRODUCTION

Monoamine oxidases A and B are two isozymic forms of the mitochondrial membrane bound flavoenzymes which catalyze the carbon oxidation of a variety of amines, including neurotransmitters such as dopamine and serotonin. Numerous studies on structure–activity relationships (SAR) have been carried out on monoamine oxidases (MAO) utilizing various tissues as enzyme source and a variety of inhibitors. Most of these studies were carried out on homogenates or mitochondrial crude fractions containing mixtures of the two isozymic forms. These two isoforms differ by their substrates and inhibitor specificities and are expressed at different levels in various tissues, making the interpretations of the results very difficult.[1–8] In previous papers[9,10] we reported the structure–activity relationships of a new series of hydrazinothiazoles and their "open" analogs thiosemicarbazide derivatives, which exhibited inhibitory activity on MAO-B rat liver mitochondria. Correlation of MAO inhibitor (MAOI) activity with electronic, hydrophobic, and steric properties of the inhibitors, evaluated by molecular orbital energies, calorimetric analysis and molar volume calculations, suggested that all these parameters are relevant for the "second hit" irreversible mechanism in the enzyme–inhibitor interactions. Non-covalent interactions can play an important role as anchoring points to a complementary flat surface of the enzyme so that the complex formation becomes easier.[11] A recent paper on the MAO-B inhibitory activity of acylhydrazone derivatives, as "open" analogs of oxadiazolones, clearly supports the hypothesis that the electronic properties of the molecule must be conserved for fitting the enzyme active site and stabilize the interaction in terms of affinity and/or selectivity.[12] Moreover, it is well known that acylhydrazides became selective MAO-B inhibitors by methyl or aromatic substitution if a suitable bioactive conformation can be reached, so focusing on the pivotal role of lipophilic substituents when placed in a suitable lead skeleton.[13]

In the present work, we have analyzed the inhibitory activity of a new series of thiazole derivatives on MAO-B isoform, purified from beef liver mitochondria. The purpose was to contribute to a better understanding of the enzyme active site and particularly to further investigate if a rigid heterocycle, or simply its open analog, with similar electronic properties, are important for the bioactivity.

Recently, the mechanism-based inhibition has been developed as a rational approach for the design of selective enzyme inhibitors and particular attention has been focused on the new MAO-B inhibitors by virtue of their potential as therapeutic agents.[14–17]

The rational selection of the proposed chemical series was further supported by PASS (Prediction of Activity Spectra for Substance), a computer approach for biological activity prediction based on the compounds structural formula.[18–20]

## EXPERIMENTAL

### Chemistry

Aminoketone-thiazolyl derivatives (**1–4**), were synthesized using the modified Mannich reaction according to the method previously described.[21–23]

Following the reported methods, 2-(aminoacetamido or propionamido)-thiazolyl derivatives (**5–11**) were synthesized by reaction of the chloroacetamido derivatives with the pertinent amines. The chloroacetamido derivatives were in turn prepared by reaction of the 2-aminothiazoles with chloroacetyl chloride.[24,25]

### Mitochondrial Preparation

Mitochondrial fraction was prepared from beef liver removed immediately after sacrifice and kept in an ice-cold solution of 0.9%NaCl. Tissue was homogenized in a Waring blender at 4°C in a 10-fold wet weight to medium containing 0.33 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 15 mM Tris-HCl, pH 7.4. The homogenate was filtered through cheesecloth, re-homogenized with a teflon/glass tissue grinder and centrifuged at $800\times g$ for 10 min. The supernatant was centrifuged at $12{,}000\times g$ for 20 min to obtain a pellet that contained the crude mitochondrial fraction.

### MAO-B Purification

The pellet was re-suspended in buffer A consisting of 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 1.5% Triton X-100, pH 7.4, for solubilizing the membrane proteins. The mixture was stirred slowly for 1 h at 0°C and frozen overnight at −20°C. The thawed suspension was centrifuged at $100{,}000\times g$ for 30 min hence the supernatant was made up to 40% saturation with solid ammonium sulphate and the precipitate, collected after centrifugation at $20{,}000\times g$ for 30 min, was re-suspended in a small volume of buffer A without Triton. The solution was dialyzed overnight against the same buffer A with 0.1 M sucrose added. The solubilized proteins (3–4 mg in 1 ml) were

applied to an Amberlite X A-2 column to eliminate the detergent and the eluate, concentrated to a final volume of 2 ml. This solution was applied to a Sephadex G-200 column pre-equilibrated and eluted with 5 mM Tris-HCl, 1 mM DTT, 0.1 mM EDTA, pH 8.0. Fractions (1 ml) were collected on a LKB 2112 Redirac fraction collector and the protein was detected at 280 nm by Pharmacia-LKB optical Unit-U1.

### Enzyme Assay

MAO-B activity was measured by the spectrofluorimetric method of Morinan and Garratt, as described in a previous work,[10] by using aliquots of 25 µl of purified enzyme (65 µg of protein) and 100 µl of inhibitor solution at different concentrations. The MAO-B activity was calculated as nmol of product (4-hydroxiquinoline) formed mg protein$^{-1}$ h$^{-1}$ and expressed as percentage (± S.E) inhibition of the respective control (Table I). All assays were repeated three times.

### Time-courses of MAO-B Inhibition

The MAO-B activity of purified enzyme was determined under standard conditions after various periods of preincubation (3, 5, 7, 10 min) at 37°C in the absence or presence of inhibitor (**2** and **9**) at $10^{-4}$ M (Figure 1).

The time-course of the inhibition was also carried out at various periods of incubation (15, 30, 45, 60 min) after preincubation of samples for 5 min at 37°C (Figure 2).

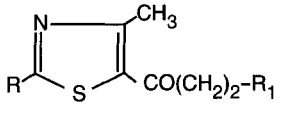
### $IC_{50}$ Calculations

The $IC_{50}$ value for each inhibitor was obtained graphically from a log concentration *vs* MAO-B inhibition plot in the range of six different inhibitor concentrations ($10^{-2}$–$10^{-7}$ M) selected in the pseudolinear region of the inhibition curve (Table II).

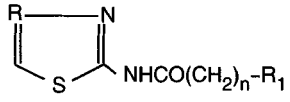
### Dialysis Experiments

Aliquots of 1 ml of purified enzyme (65 µg of protein) were incubated at 37°C in the absence or presence of inhibitors **2** and **9** ($10^{-4}$ M) or deprenyl (25 µM). The samples were then dialyzed for 24 h at 4°C against 1 litre of 10 mM phosphate buffer, pH 7.2. MAO-B activity was assayed under

TABLE I Inhibition of MAO-B activity by thiazolyl derivatives (**1–11**) at $10\,M^{-4}$ concentration:




A*




B*

| *Compound* | *R* | $R_1$ | *% Inhibition* | *PASS prediction % inhibition* |
|---|---|---|---|---|
| **1** | $CH_3NH$ | $N(C_2H_5)_2$ | 55.2 (0.18) | 52 |
| **2** | $CH_3C_6H_4$ | $N(C_2H_5)_2$ | 88.5 (0.49) | 51 |
| **3** | $C_6H_5$ |  | 72.0 (0.15) | 45 |
| **4** | $C_6H_5$ |  | 89.0 (0.97) | 48 |
| **5** | H | $-N(CH_3)_2$ | 21.6 (0.09) | 36 |
| **6** | $CH_3$ |  | 28.7 (0.12) | 33 |
| **7** | $CH_3$ |  | 26.0 (0.09) | 47 |
| **8** | $CH_3$ |  | 30.4 (0.27) | 33 |
| **9** | $C_6H_5$ |  | 50.0 (0.35) | 42 |
| **10** | $CH_3O–C_6H_5$ |  | 43.5 (0.1) | 41 |
| **11** | $CH_3O–C_6H_5$** |  | 43.2 (0.35) | 33 |

*Compounds A: **1–4**; Compounds B: **5–11**. **$n=2$; values represent the mean of three measurements (±SE).

standard conditions and expressed as % inhibition of the respective control (Table III).

## Quantum Mechanical Calculations

The molecular structure of MAOIs was optimized by the PM3 method[26–28] which is based on the classical Hartree–Fock procedure for the calculation of Molecular Orbitals (MOs). PM3 is a very efficient program which does

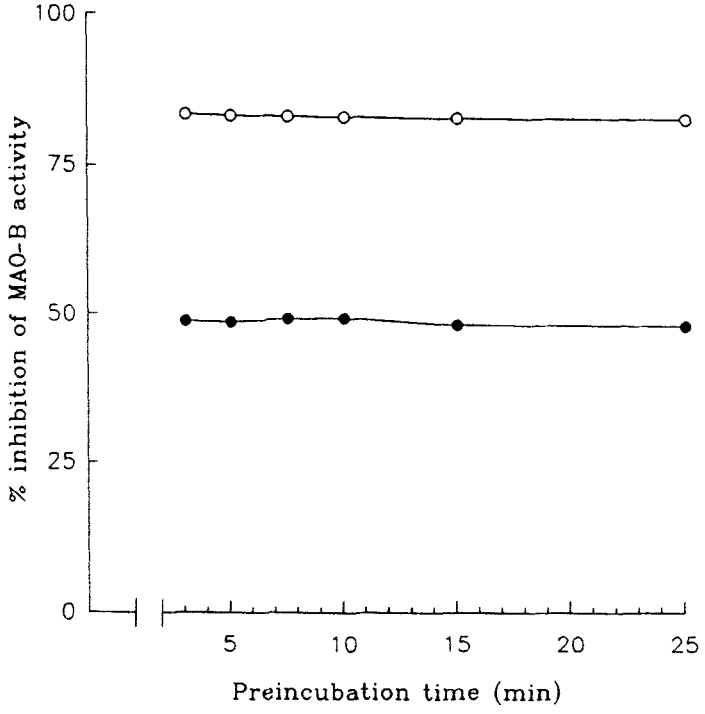



FIGURE 1 Time-courses of MAO-B inhibition by compounds **2** (○) and **9** (●). Data represent means of three separate experiments.

not require a lot of computational time for medium sized molecules of biochemical and pharmacological interest. This allows for a full optimization of the molecular geometry together with a careful investigation of the different conformations which coexist in the case of flexible molecules like those investigated here. The most stable conformations (in the range of 5 kcal/mol) were also calculated. For each of the most stable conformations, atomic charge density, dipole moment of the whole molecule, energy of the MOs and their localization/delocalization were calculated too. Particular attention was paid on the MOs energies to provide a direct measure of the electron transfer probability. In fact, according to the Koopman's theorem, the Ionization Potential (I.P.) of a MO is related to its energy by: $(\text{I.P.})_k = (-\text{MO})_k^i$ where $(-\text{MO})_k^i$ is the MO energy of a generic $k$th orbital for a molecule lying in the $i$th conformation. The mean I.P.s, averaged over the most stable molecular conformations of the inhibitors, were calculated following the procedure described by us in a previous paper.[29]

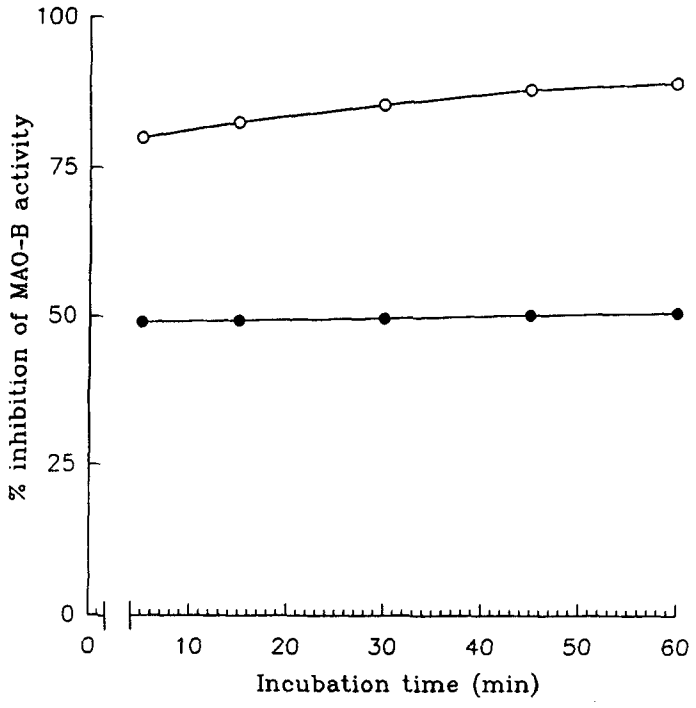
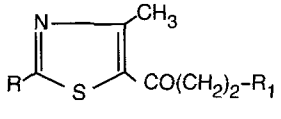
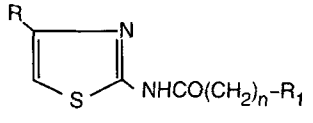



FIGURE 2 Time-courses of MAO-B inhibition by compounds **2** (○) and **9** (●). Data represent means of three separate experiments.

TABLE II Activity and selectivity of thiazolyl derivatives inhibitors (**1–4**) and (**9–11**):




A*




B*

| *Compound* | $IC_{50}$ (μM) |
|---|---|
| **1** | 82.3 |
| **2** | 7.5 |
| **3** | 43.1 |
| **4** | 7.8 |
| **9** | 90.2 |
| **10** | 411 |
| **11** | 313 |

*Compounds A: **1–4**; Compounds B: **9–11**.

TABLE III MAOI activity of deprenyl (25 nM) and of compounds **2**, **9** ($10^{-4}$ M), before and after dialysis (24 h, at 4°C) of the incubation samples

| *Compound* | *Inhibition %** | |
|---|---|---|
| | *Before dialysis* | *After dialysis* |
| Control | 0 | 0 |
| **2** | 90.8 (0.90) | 83.3 (0.50) |
| **9** | 49.2 (0.34) | 46.5 (0.12) |
| Deprenyl | 65.3 (0.18) | 64.0 (0.14) |

* ± S.D. in parentheses; $n = 3$.

## RESULTS AND DISCUSSION

MAOI activity of two series of thiazolyl derivatives and the pertinent PASS are summarized in Table I. The selection of the subject compounds was supported by the PASS. Analysis of the results indicates that the most active compounds **2**, **3**, **4** are those presenting a methylamino group or an aryl ring in the 2-position of the thiazole ring, leading to the suggestion that these groups act as anchoring points by additional hydrophobic and/or charge transfer interactions so stabilizing the enzyme–inhibitor complex. The replacement of an aryl residue with a methylamino group decreases the inhibitory activity of these compounds.

The first series (A) includes compounds **1–4** which contains a C4 methyl group and different alkyl or aryl substituents on the C2 of the thiazole ring. The tertiary amine function is linked, as the side chain, to the propanone residue in the C5 position of the thiazole ring.

The second series (B) includes compounds **5–11** which present alkyl or aryl substituents on the C4 and an acetamide or propionamide residue on the position 2 linked to the terminal amine function. Compounds **9–11**, bearing the phenyl or aryl substituent on the 4-position, display the highest MAOI activity, but they are less active in comparison with the compounds of the first series (A). The replacement of the aromatic substituent by a methyl group causes a clear drop (about 50%) in inhibitory activity.

### Mechanism of MAO Inhibition

Different methods were used to investigate the inhibition mechanism. MAOI activity of the selected inhibitors was concentration-dependent in the range of $10^{-7}$–$10^{-2}$ M (Figure 3). Unfortunately, the solubility limit ($10^{-2}$ M) of compounds **5–11** precluded the possibility of determining the complete inhibition of the enzyme activity (Figure 4). Apparent $IC_{50}$ values,

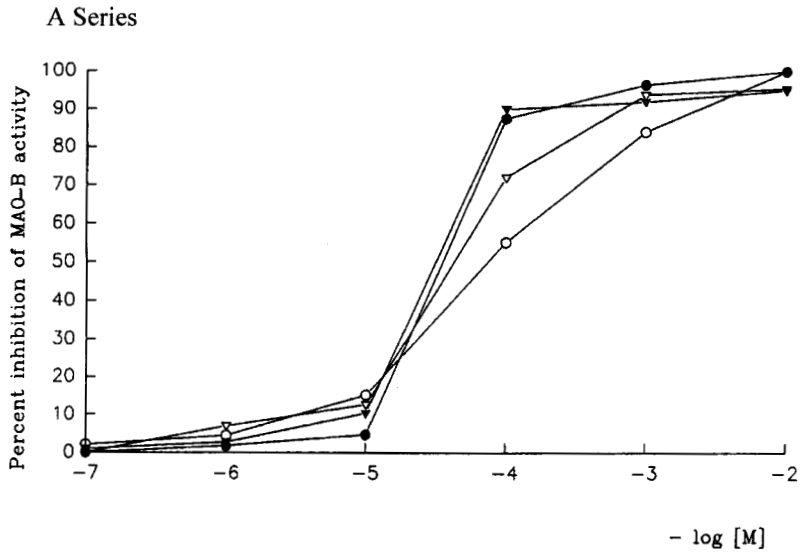



FIGURE 3 Inhibition of MAO-B activity in the presence of different concentrations of compounds **1** (○), **2** (●), **3** (▽) and **4** (▼). Data are from representative experiments performed in triplicate.

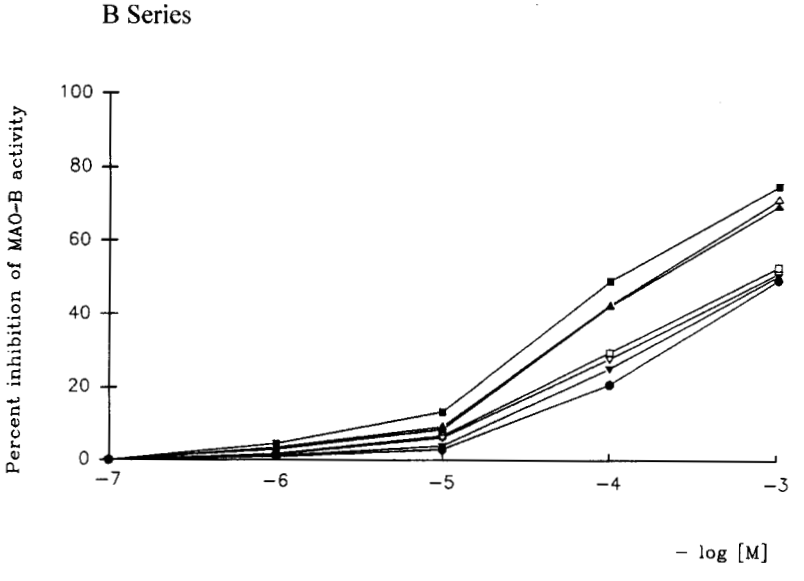



FIGURE 4 Inhibition of MAO-B activity in the presence of different concentrations of compounds **5** (●), **6** (▽), **7** (▼), **8** (□), **9** (■), **10** (△) and **11** (▲). Data are from representative experiments performed in triplicate.

graphically obtained from the respective MAO-B inhibition curves, indicated that the most potent inhibitors had $IC_{50}$ values of 7.5–7.8 μM (Table II). For this reason all the kinetic experiments were performed at $10^{-4}$ M concentration of inhibitors.

The time courses of MAO-B inhibition showed that a prolonged preincubation (up to 25 min), of compounds **2** and **9** with purified enzyme did not potentiate inhibition, suggesting that the binding process is very fast (Figure 1). The dependency on the incubation time by the same compounds was evaluated. Prolonged incubation slightly potentiates the inhibition, suggesting a strong interaction of the inhibitors with the enzyme (Figure 2).

The reversibility tests demonstrate that the inhibitory activity is fully conserved after a prolonged dialysis (24 h at 4°C) of the enzyme in the presence of inhibitors **2** and **9**. This result is similar to that observed for deprenyl[30] which is a well known irreversible inhibitor of MAO-B (Table III).

To further investigate the MAO-B inhibition mechanism the most potent inhibitors were selected. Kinetic analysis carried out by using Lineweaver–Burk reciprocal plot shows that $K_m$ remains constant whereas the $V_{max}$ decrease parallels that of the inhibitor concentration. This pattern is typical of a simple non-competitive inhibition where the enzyme–inhibitor complex is very stable, owing to possible formation of a covalent MAO-B inhibitor adduct (Figures 5 and 6).

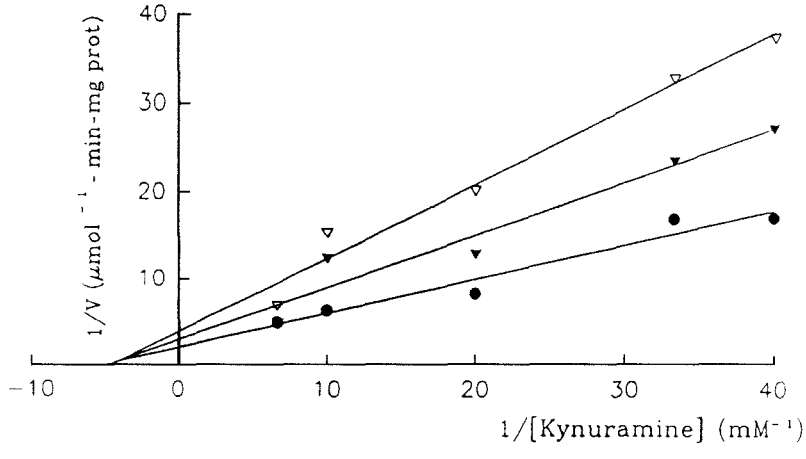



FIGURE 5 Lineaweaver–Burk plot. MAO-B activity was measured, under standard condition, with 2.5, 3, 5, 10 and 15 μM kynuramine in the absence (●) or presence of **2** at $10^{-5}$ M (▼) and $10^{-4}$ M (▽) concentrations.

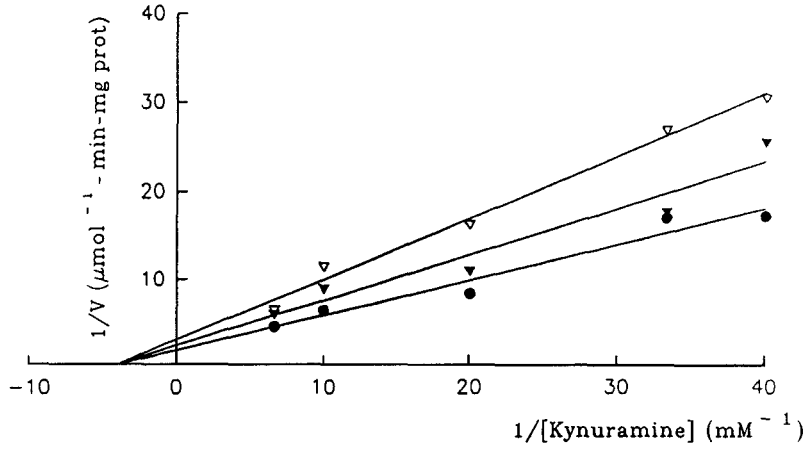



FIGURE 6 Lineaweaver–Burk plot. MAO-B activity was measured, under standard condition, with 2.5, 3, 5, 10 and 15 μM kynuramine in the absence (•) or presence of **9** at $10^{-5}$ M (▼) and $10^{-4}$ M (▽) concentrations.

To ascertain if the purified preparation of MAO-B contains MAO-A activity, experiments were performed in the absence or presence of clorgyline (20 mM) that produces complete inhibition of MAO-A activity. The results indicate that the enzyme inhibition was independent of the presence of clorgyline, suggesting the absence of MAO-A activity in this preparation.

From the analysis of the inhibition results some observations arise. The common structural feature of the thiazolyl inhibitors is the presence of the heterocyclic aromatic nucleus which is spaced by a carbon chain from the tertiary amine function.

The mechanism of MAO inhibition can be explained by the formation of a stable covalent adduct between the oxidizable nitrogen atom of the inhibitor and an active nucleophilic binding site in the enzyme. The enhanced stability of the covalent adduct was attributed to the stabilizing effect of the $sp^3$ hybridized carbon atom by the electron-withdrawing ability of the properly located acetamide/propanone group linked to the thiazole backbone. This hypothesis has been proposed, with the aid of chemical models, to explain the selective irreversible inhibition of MAO-B by oxazolidinone derivatives and milacemide analogs.[31–33]

The presence of an alkyl or aryl moiety on the thiazole nucleus can modulate the potency of the inhibitors and appears to be a relevant factor for a possible interaction of the inhibitor with a complementary hydrophobic site of the enzyme. In addition, nucleophilic attack of the inhibitor on the two

large electrophilic zones formed by the carbonyls of the flavin moiety can occur.

## Quantum Mechanical Calculation Results

As previously discussed, the most likely mechanism of MAOI inactivation mechanism by tertiary amines is the formation of a stable covalent adduct between the amino nitrogen atom and the active nucleophilic binding site of the enzyme. The electron transfer from the nitrogen lone pair to the enzyme should be inversely related to the I.P. of the inhibitor, a parameter which nowadays can be correctly calculated by modern quantum mechanical packages as reported in the Experimental section. Therefore, the aim of our calculations was to ascertain a possible relationship between the biological activity of MAOIs and the electron transfer probability determined by the I.P. Two difficulties arise when this procedure is applied to the classes of the investigated compounds: (a) the I.P.s of the amine lone pair is not the lowest I.P. of the whole molecule. Other MOs e.g. those delocalized over the aromatic or heterocyclic rings, sometimes have lower I.P.s. We believe that these MOs are not involved in the subsequent formation of stable MAOI–enzyme adducts. This conjecture is supported by the lack of any relationship between this lowest I.P. and the biological activity of the investigated molecules, while there is a consistent relationship between the I.P. of the amine *nitrogen* atom and the biological response, as briefly reported below; (b) the inhibitors under study, in particular compounds **1–4**, are very flexible molecules and possess several conformations of comparable energy, having low interconversion barriers among the different conformers.

The average I.P. value of each molecule was obtained by properly weighting the role of the different conformers through a quantum mechanical calculation of their relative conformational energies.

The main results are shown in Figure 7. Here we report the percentage inhibition by compounds **5–11** *vs* the conformational averaged I.P., relative to the MAOI Molecular Orbital localized on the tertiary amine lone pair. A sharp decrease in the inhibitory activity on increasing the nitrogen lone I.P. pair is observed, a result which is consistent with the hypothesis that the rate-determining step for MAO inhibition is electron transfer from the tertiary amine to the catalytic site of the enzyme. Interestingly, compounds **1–4** have a stronger activity than compounds **5–11**.

The small set of compounds **1–4** and the similiar I.P. values (about 9.2–9.3 eV) prevented construction of a I.P. *vs* biological activity plot similar to that reported in Figure 7. However, the higher activity for the class

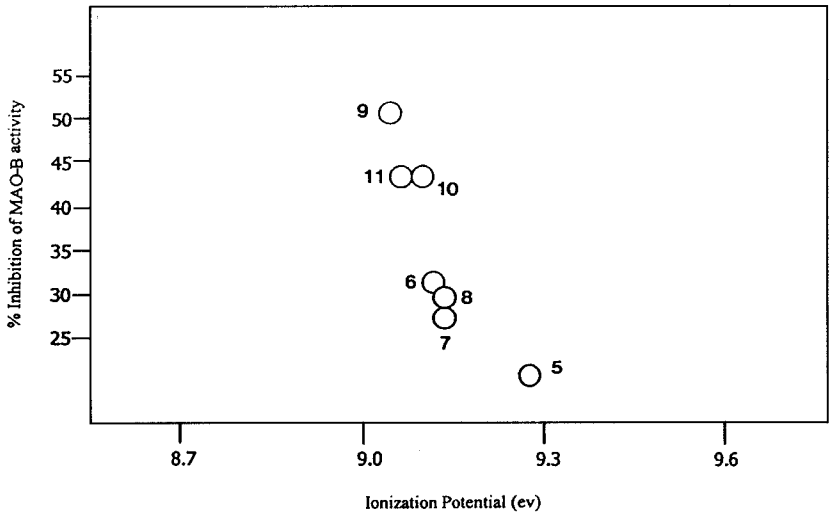



FIGURE 7 The conformational averaged I.P. values of inhibitors **5–11**.

**1–4** could be tentatively explained in terms of higher conformational flexibility which allows for a better fitting of the inhibitor inside the catalytic pocket of the enzyme. In fact, compounds **1–4** have about 24 low energy conformations in a range lower than 5 kcal/mol. Furthermore, the interconversion barriers among the different conformers are in the range of 3 kcal/mol allowing for an easy and fast interconversion of the different conformers. By contrast, compounds **5–11** have a slightly smaller number of low energy conformers (about 20) and, more important, higher interconversion barriers (6–8 kcal/mol) which prevent an efficient rearranging of the inhibitor inside the catalytic pocket.

We also investigated the possible role of the electron density on the tertiary amine nitrogen. In fact, a high electron density makes easier both the electron transfer to the enzyme binding site as well as a stronger electrostatic attraction which, stabilizes the enzyme–inhibitor complex furtherly. Analysis of the quantum mechanical results shows very small variations in the electron density of the amino nitrogen atom in the tested compounds (5.06–5.07 electrons per atom) apart from the structure of the substituents.

In conclusion, the structure–activity relationships concerning the non-competitive inhibition of some thiazole derivatives on purified mitochondrial MAO-B reported in this paper, suggest that a key feature for the enzyme inhibitory activity by these and related compounds should be an

electron-rich functional group such as the amino nitrogen. The substituents on the thiazole backbone can modulate the potency of the inhibitors by their possible interaction with a complementary active site of the enzyme. Moreover, thermodynamic considerations regarding the conformational flexibility of the inhibitors could tentatively explain their high biological activity in terms of a better fitting of the inhibitor inside the enzyme catalytic pocket.

Further synthetic and biological studies along with 3D QSAR approaches are on-going and will be reported in future papers.

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