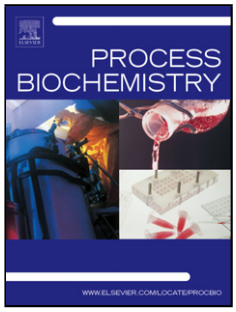

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Inhibitions of monoamine oxidases and acetylcholinesterase by 1-methyl, 5-phenyl substituted thiosemicarbazones: Synthesis, biochemical, and computational investigations

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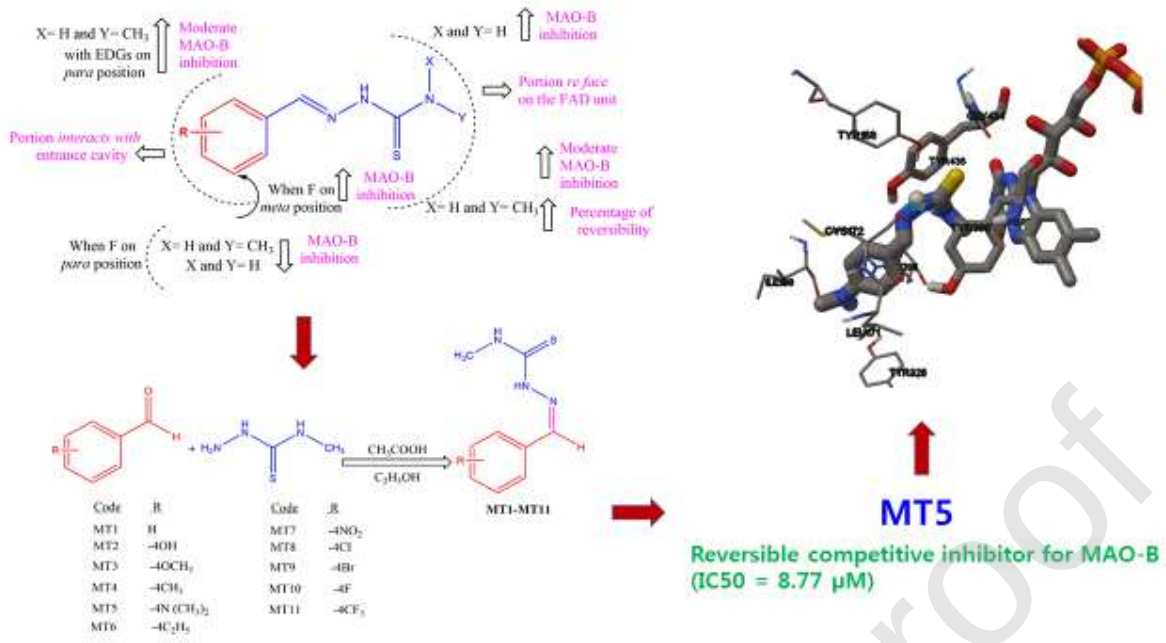
Graphical abstract



Highlights

- Eleven methylthiosemicarbazones were prepared and characterised.
- Inhibitory activities against monoamine oxidase (MAO) and acetylcholinesterase were evaluated.
- Compound **MT5** was non-toxic, and reversible competitive inhibitor for MAO-B (IC_{50} = 8.77 µM).
- **MT5** binding was stabilized by hydrogen bonding to Cys172 and π–π hydrophobic interaction with Tyr326 of MAO-B.
- **MT5** may be considered a potential compound for the treatment of neurodegenerative disorders.

ABSTRACT

A series of eleven 1-methyl, 5-phenyl substituted thiosemicarbazones (**MT1-MT11**) with the phenyl ring substitutions were prepared and investigated for their inhibitory activities against monoamine oxidases (MAOs) and acetylcholinesterase (AChE). [4-(dimethylamino) phenyl]methylidene}-*N*-methylhydrazine-1-carbothioamide (**MT5)** inhibited MAO-B potently with an IC_{50} of 8.77 µM. Potencies for MAO-B increased in the order $–N(CH_3)_2$ in **MT5** > $-OCH_3$ in **MT3** > -Br in **MT9**. Most of the 11 compounds weakly inhibited AChE by < 30% at 10 µM. **MT5** competitively inhibited MAO-B and K_i value was 6.58 ± 0.064 µM. Reversibility experiments showed **MT5** also reversibly inhibited MAO-B. MTT assays revealed that **MT5** and **MT3** were non-toxic to normal VERO cell lines with IC_{50} values of 191.96 and 187.04 µg/mL, respectively. From the molecular docking, **MT5** binding was found to be stabilized by hydrogen bonding to the non-bonding electron of the terminal N-methyl group with Cys172 (binding energy = -7.01 kcal/mol) of MAO-B. The molecular dynamics further predicted that **MT5** had a major π–π hydrophobic interaction with Tyr326 of MAO-B, suggesting that it plays an important role in the stabilization of protein-ligand interaction.These results documents that **MT5** is a moderately selective, reversible, and competitive inhibitor of MAO-B with low cytotoxic profile.

Keywords: Imines, Monoamine oxidase-B, Monoamine oxidase-A, Acetylcholinesterase, Kinetics, Reversibility

1. Introduction

Monoamine oxidase (MAO) A and B are flavoenzymes containing flavin adenine dinucleotide (FAD) and are primarily responsible for the degradation of biogenic amines [1]. This degradation involves oxidative deamination into corresponding aldehydes, hydrogen peroxide, and ammonia using molecular oxygen as electron acceptor [2]. Non-physiologic levels of biogenic amines can lead to diverse conditions such as depression, anxiety, and migraine and neurodegenerative diseases like Alzheimer's disease (AD) and Parkinson's disease (PD) [3-6]. Therefore, the medicinal chemists have been prompted to design selective MAO isoform-based inhibitors that are effective for a wide range of central nervous system (CNS) pathologies. MAO-A inhibitors like moclobemide and clorgyline are currently considered a third-line therapeutic defense, particularly for the treatment of mental disorders, like depression and anxiety [7-9]. On the other hand, MAO-B inhibitors like selegiline, safinamide and lazabemide are considered adjuvant treatments for AD and PD (**Fig. 1**) [10].

Recently, many different scaffolds, such as chalcones, coumarins, chromones, isatin derivatives, thiazolidindiones, xanthines, thiazoles base hydrazones, and analogues of food and drugs administration (FDA) approved drugs have been shown to significantly inhibit MAOs [11-18], and considerable research attempts have been tried on the discovery and development of hydrazone/thiosemicarbazone-based MAO inhibitors (MAOIs) [19-24]. Recently, our group synthesized a series of 5-phenyl substituted thiosemicarbazones and found that the majority

potently inhibited MAOs in the micromolar range and had moderate acetylcholinesterase inhibitory profiles [25]. In the present study, we tried to enhance the lipophilicity of 5-phenyl substituted thiosemicarbazones by introducing a methyl group on the terminal amino of the thiosemicarbazone group and subsequently evaluated the possible use of these new adducts for the treatments of neurodegenerative diseases. Here, we describe the synthesis of eleven 1-methyl, 5-phenyl thiosemicarbazones, their MAO and acetylcholinesterase inhibitory activities, the kinetics of their inhibitions using Lineweaver–Burk (LB) plots, reversibilities, and cytotoxic profiles on normal cell lines. Finally, the lead molecule as determined using *in vitro* results, was investigated to molecular docking analysis to investigate the nature of its interaction with MAO-B.

2. Materials and methods

2.1. Synthesis

Methylthiosemicarbazide (0.01 M) and an appropriate substituted benzaldehyde (0.01 M) in 1-2 mL acetic acid was mixed with stirring for 2-3 h. The resultant was refluxed for 6–7 h and treated on crushed ice. Solid obtained was thoroughly washed with water until the acid catalyst was free, filtered, and crystallized from ethanol [26].

2.2 Enzyme assays

MAO activities were determined by continuous methods as described previously [27, 28]. Shortly, recombinant human MAO-A or B were used, and kynuramine (0.06 mM) or benzylamine (0.3 mM), respectively, as substrates. Substrate concentrations were 1.5 × and 1.4 × K_m, respectively, and their K_m values were 0.041 mM and 0.21 mM, respectively. AChE

activities were measured using and *Electrophorus electricus* Type VI-S in a mixture of 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as a color development reagent and 0.5 mM acetylthiocholine iodide (ACTI) as a substrate [29].

2.3. Mode of inhibitions and kinetics

The inhibitory potencies of **MT1-11** on MAO-A, MAO-B, and AChE were assayed at a concentration of 10 μM, and then IC_{50} values were determined for compounds that inhibited MAO-B by >50%, by measuring residual activities at 7 concentrations between ~ 0.1 × and 10 × IC_{50}. The values were calculated using Graph pad PRISM 5 (Graphpad, San Diego, CA) with duplicated data. MAO-A inhibitory activities were then assayed [30, 31]. Reversibilities were performed by the dialysis method, and kinetic parameters were analyzed at five substrates and three inhibitor concentrations using Lineweaver-Burk plots on [4-(dimethylamino) phenyl]methylidene}-*N*-methylhydrazine-1-carbothioamide (**MT5**), which most potently inhibited MAO-B, as previously described [32, 33].

2.4. Reversibility studies

The inhibitory reversibilities of compound **MT5** were determined by dialysis after preincubating it with MAO-B for 30 min [34, 35]. The concentration used was ; **MT5** 18.0 μM, lazabemide as a reversible MAO-B inhibitor (positive control) at 0.12 μM, and pargyline as an irreversible MAO-B inhibitor (negative control) at 0.040 μM. The relative activities of dialyzed (A_D) and non-dialyzed (A_U) samples were compared to determine reversibility patterns [36].

2.5. Cytotoxicity

Tubes containing VERO cells (African green monkey kidney cells) were centrifuged and

adjusted to 1.0 X 10^5 cells/ml. Cultured cells were then diluted using Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS). Diluted suspensions (100 µl) were added to the wells of 96-well microplates. After incubation for 24 h, cells were centrifuged and pellets were diluted with 100 µl of maintenance medium. Plates were then incubated in a CO_2 atmosphere (5%) for 48 h at 37°C, during which cells were examined every 24 h under a microscope. Cells were then treated with 20 µl of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (2 mg/ml). The formazan crystals formed were dissolved by gently adding 100 µl of a DMSO/isopropanol mixture per well and absorbances were immediately measured using a microplate reader at 540 nm. Dose-response curves were used to determine the concentrations required to inhibit cell growth by 50% [37].

2.6. Molecular docking

The molecular docking study of **MT5** was carried out with the AUTODOCK4.2 version [38]. Energy minimization of the edited protein 2V5Z (hMAO-B) was performed using Swiss-Pdb viewer (4.1.0) [39, 40]. The energy minimization of the ligand was prepared using the PRODRG webserver [41]. The protocols of grid and docking parameters were as previously described [42-45].

2.7. Molecular dynamics

The molecular dynamics (MD) simulations were carried out using Desmond simulation package of Schrodinger LLC. The lead molecule **MT5** against MAO-B using the aqueous solvent system was initially prepared for the protein-ligand complex using the Desmond system builder panel [46]. The simulation conditions were 50 ns at 300 K, and 1.01325 bar pressure

with 1000 frames for entire protein-ligand simulations, for protein-ligand interactions and stability trajectory analysis.

3. Results

3.1. Chemistry

The target compounds were synthesized by acid catalyzed nucleophilic addition between various phenyl substituted aldehydes and methylthiosemicarbazide in the presence of ethanol [47]. The synthetic route is outlined in the scheme 1. The synthesized candidates were ascertained by ^{1}H-NMR and Mass spectroscopy. In ^{1}H-NMR spectra, a sharp singlet upfield observed between 3.24-3.38 is represented to the terminal N-CH_3 group. The singlet peaks observed between 7.64-7.95 are represented to the azomethine (-CH=N-) proton. The secondary amino (-NH-) group nearer to the thiocarbamoyl unit (HN-N-C=S) is observed in the range between 9.06–10.54. The mass spectra of all 1-methyl, 5-phenyl substituted thiosemicarbazones indicated intensive molecular ions, supporting the targeted compound structures. The spectral characterization is deposited in the supplementary material.

3.2. Inhibitory activities on MAO enzymes and AChE

MT5 inhibited MAO-B potently with an IC_{50} value of 8.77 µM, and whereas the other 10 compounds had IC_{50} values of > 10 µM (Table 1). Potencies for MAO-B increased in the order –$N(CH_3)_2$ in **MT5** > -OCH_3 in **MT3** > -Br in **MT9**. Other substituents had slight effects on MAO-B. The weak inhibition was observed for **MT1**, which contained an unsubstituted phenyl. Most of the compounds weakly inhibited AChE by < 30% at 10 µM.

3.3. Kinetics of MAO-B inhibition

LB plots showed that **MT5** competitively inhibited MAO-B (Fig. 2A). K_i value was determined to be 6.58 ± 0.064 µM from the secondary plots (Fig. 2B). These results suggested **MT5** competed with the substrate at the active site of the enzyme and that it selectively and competitively inhibited MAO-B.

3.4. Reversibility studies

Inhibition of MAO-B by **MT5** recovered from 21.6 (A_U) to 96.4% (A_D) after dialysis (**Fig. 3**), which was comparable to that standard lazabemide, a reversible inhibitor, from 19.3 to 94.6%. On the other hand, MAO-B inhibition by pargyline, an irreversible inhibitor, was not recovered by dialysis (from 37.8 to 36.8%). These results showed **MT5** reversibly inhibited MAO-B.

3.5. Cytotoxicity evaluation

The biocompatibilities of **MT5** and **MT3** were evaluated using an MTT assay and normal VERO cell lines. Both compounds achieved > 89% cell viability after treatment at 100 µg/mL. MTT assays revealed **MT5** and **MT3** were relatively non-toxic to normal VERO cell lines with IC_{50} values of 191.96 and 187.04 µg/mL, respectively.

3.6. Molecular docking

Molecular docking study was performed to investigate the interaction between **MT5** and hMAO-B co-crystallized with native ligand safinamide (PDB code: 2V5Z). The experimental inhibitory binding constant (K_i) 6.58 µM was found to agree well with the calculated inhibitory binding constant of 7.32 µM, obtained from 50 molecular docking simulations. Energies associated with the binding of **MT5** in the inhibitor binding cavity (IBC) of MAO-B are

provided in Table 2. Binding of **MT5** to MAO-B was found to be stabilized by hydrogen bonding with the non-bonding electrons of terminal N-methyl group with Cys172 (2.012Å). The binding pose of **MT5** is depicted in the **Fig. 4**.

3.7. Molecular dynamics

The simulation study of **MT5** was followed by MD simulations using Desmond package. As per RMSD (root mean square deviations) analysis, the protein C-alpha and ligand were stable in the range 1.7–2.4 Å for a long duration of simulation 50 ns time without any major fluctuations. This suggested that the complexes were energetically compatible (**Fig. 6A**). The interaction patterns between the **MT5** and protein was categorized as hydrogen bonds, hydrophobic, ionic and water bridges (**Fig. 6B**). The stacked bar charts were normalized over the course of the trajectory. The results documented that the interaction fraction value with Tyr326 was 0.6. This clearly recommended that 60% simulation time from the 50 ns was maintained by the Tyr326 hydrophobic interaction with the ligand. The other hydrophobic networks were with amino acids such as Phe168, Tyr188, Ile199, Ile316, Tyr 398 and Tyr435.

4. Discussion

In-line with structure activity relations (SARs) regarding the effect of electron-accepting or -donating groups at the *para* position of the phenyl of 1-methyl, 5-phenyl thiosemicarbazones, inhibitory activities improved when electron donating groups such as methoxyl, hydroxyl, dimethylamino, methyl, or ethyl were introduced on the *para* position of the phenyl ring. Several previous studies have concluded that the presence of electron donating groups on the phenyl group promotes selective MAO-B inhibition [48-51]. As regards halogen

substitution, chlorine or bromine improved MAO-B inhibitory activity more than fluorine substitution (residual activities were 63% and 62% at 10.0 μM, respectively). Fluorine substitution resulted in weak MAO-A and MAO-B inhibitions with residual activities of 96% and 78% at 10.0 μM, respectively. Interestingly, the substitution of three fluorine atoms in **MT11** at *para p*osition of phenyl ring (trifluoromethyl) moderately inhibited MAO-B (residual activity 63% at 10.0 μM).

MT1-11 weakly inhibited MAO-A (all IC_{50} values > 10.0 μM), which concurs with previous studies on aryl thiosemicarbazones that methyl substitution on the terminal amino group has little impact on MAO-A or B inhibitors. These results suggest that the presence of a small lipophilic unit on the terminal amino group reduces inhibitory activities for MAO-A, B, and AChE, and that the presence of lipophilic groups on the phenyl system of 1-methyl, 5-phenyl thiosemicarbazones greatly influences on MAO-A and MAO-B inhibitions. The common SAR principles of aryl methylthiosemicarbazones /thiosemicarbazones, as elucidated by our previous studies, are summarized in **Fig. 6**. Interestingly, of the 11 synthesized compounds, **MT7**, which possessed an electron withdrawing nitro group inhibited AChE most (residual activity 63% at 10.0 μM).

Kinetic studies were carried out on **MT5**, which inhibited most potently MAO-B in the present study. A set of LB plots was created in the presence or absence of compound **MT5** at four concentrations totally. The plots showed that lines were linear and intersected the y-axis, indicating **MT5** interacts competitively with the active site of hMAO-B. The reversibility studies of MAO–B inhibition by **MT5** was inspected by dialysis, and compared with the reversibilities of a reversible and an irreversible MAO-B inhibitor, i.e., lazabemide and paragyline, respectively. Percent recoveries suggested that **MT5** is a better reversible MAO-B

inhibitor than lazabemide. Reversible inhibitors have considerable therapeutic advantages over irreversible inhibitors due to less target disruption, shorter action durations, and better pharmacokinetic properties [52, 53].

The binding mode of **MT5** was investigated by AutoDock4.2 simulation. Architecturally MAO-B has two cavities, that is, an "entrance cavity" and a "substrate cavity" [54]. These two cavities are fused or separated by Ile199, which acts as a gating residue [55], and the fusing and binding of cavities normally depends on the nature of the inhibitor [56-58]. Docking studies showed that the side chain thiomethylsemicarbazone unit was anchored towards the FAD unit of the MAO-B substrate cavity and that the *para* substituted dimethylamino group on the phenyl group was efficiently positioned in the entrance cavity of MAO-B (**Fig. 7**). The electron donating dimethyl amino group enhances lipophilic character and might enhance binding between **MT5** and the MAO-B. The MD further predicted that the lead molecule **MT5** had a major π–π hydrophobic interaction with Tyr326, suggesting that it plays an important role in the stabilization of protein-ligand interaction.

5. Conclusions

Eleven 1-methyl, 5-phenyl thiosemicarbazones were synthesized and investigated for their MAO and AChE inhibitory profiles. Inhibitions were dependent on the natures of substituents on the phenyl ring at para substitution. Substituents with an electron-donating group such as the dimethylamino group effectively inhibited MAO-B. Our kinetic and reversibility studies revealed that **MT5** competitively and reversibly inhibited MAO-B, and our cytotoxicity study showed **MT5** is non-toxic to normal VERO cell lines. Reassuringly, calculated inhibition by docking simulation and experimentally determined inhibition agreed well. The hypothetical

binding mode of the **MT5** in the IBC of MAO-B is also well established. Summarizing, the study shows **MT5** effectively, competitively and reversibly inhibits MAO-B and has a low cytotoxic profile and suggests **MT5** may be the forerunner of a new class of compounds with therapeutic potential for the treatment of various neurodegenerative disorders.

Author statement

The manuscript is not submitted anywhere and the revision is submitted

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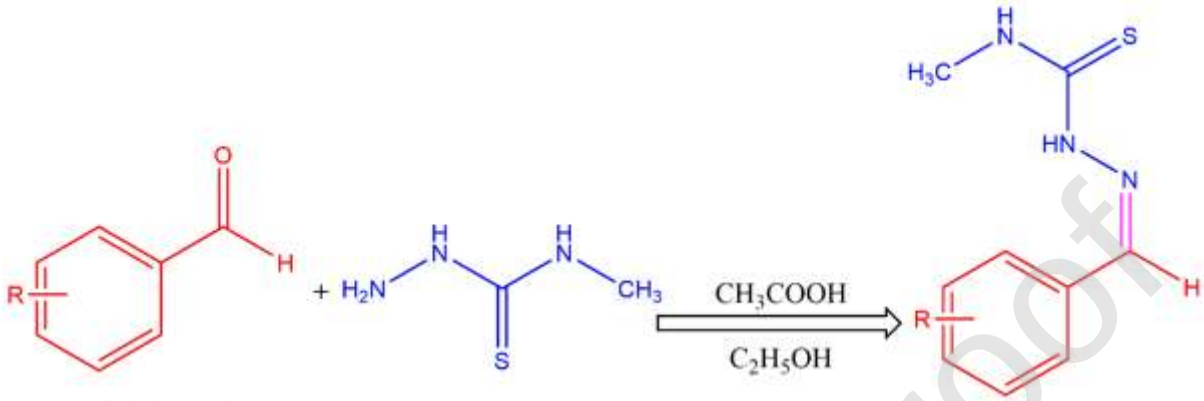


Code	R	Code	R
MT1	H	MT7	-4NO_2
MT2	-4OH	MT8	-4Cl
MT3	-4OCH_3	MT9	-4Br
MT4	-4CH_3	MT10	-4F
MT5	-4N (CH_3)$_2$	MT11	-4CF_3
MT6	-4C_2H_5		

Scheme 1. Synthetic route used to produce 1-methyl, 5-phenyl thiosemicarbazones (**MT1-MT11**).

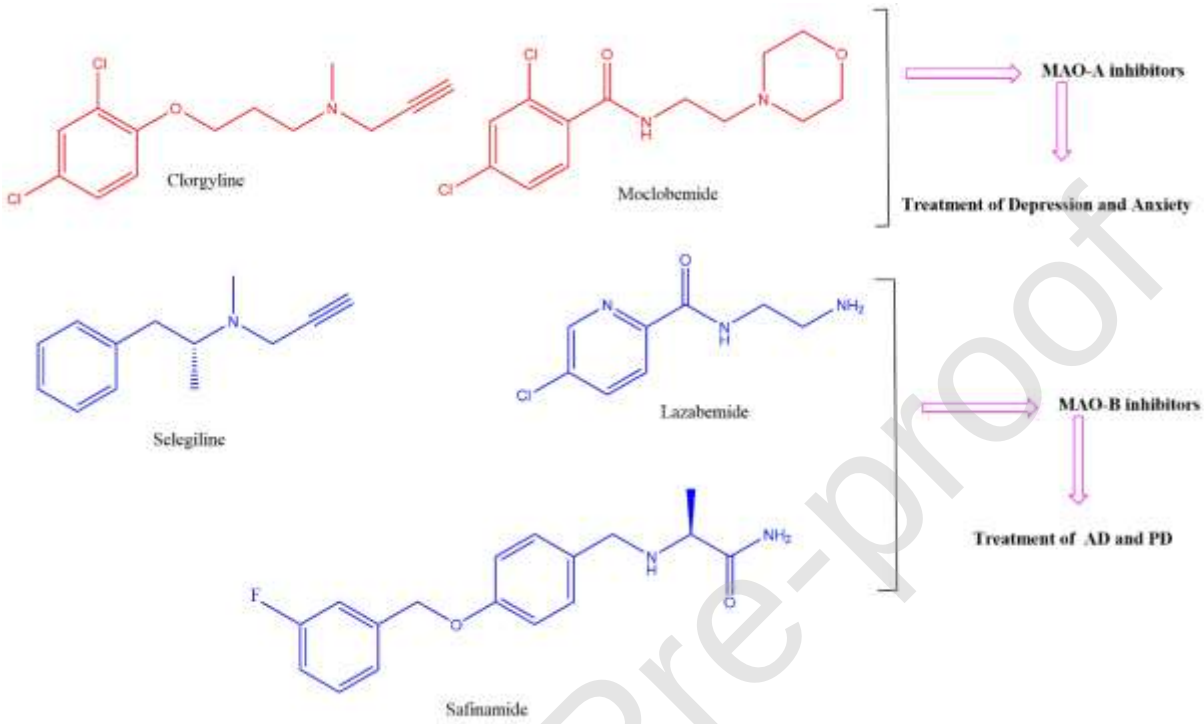
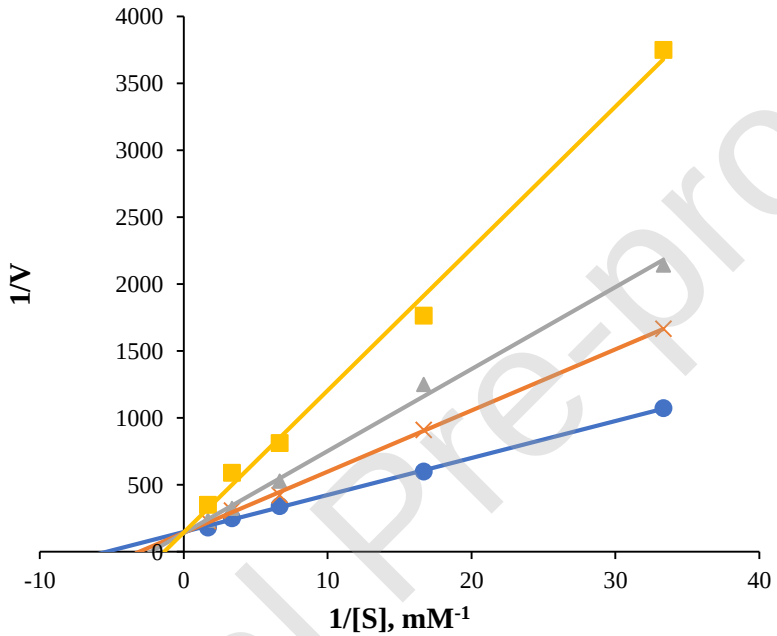


Fig. 1. Structures of known MAO-A and -B inhibitors and their uses.

(A)



(B)

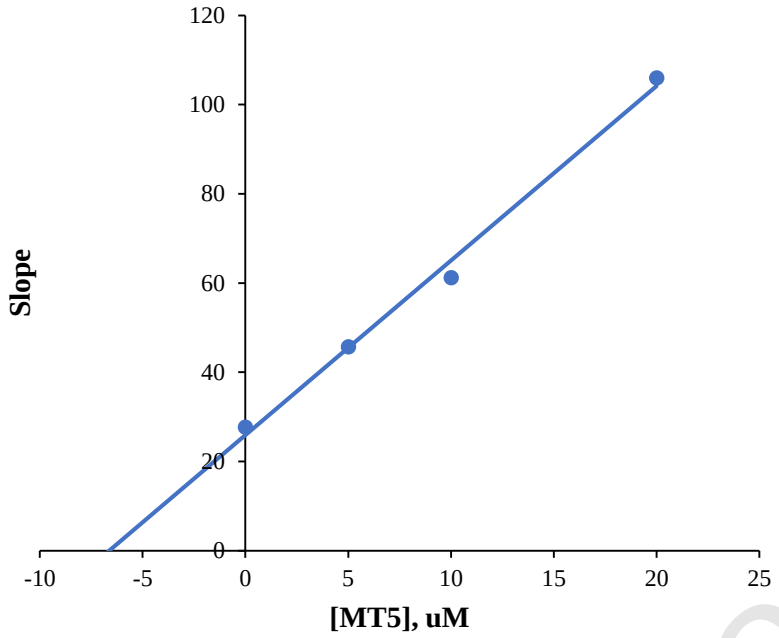


Fig. 2. Lineweaver-Burk plots of MAO-B inhibition by [4-(dimethylamino) phenyl]methylidene}-*N*-methylhydrazine-1-carbothioamide (**MT5**) (**A**), and a secondary plot (**B**) of slopes vs. inhibitor concentrations. [**MT5**]: 5, 10, and 20 µM.

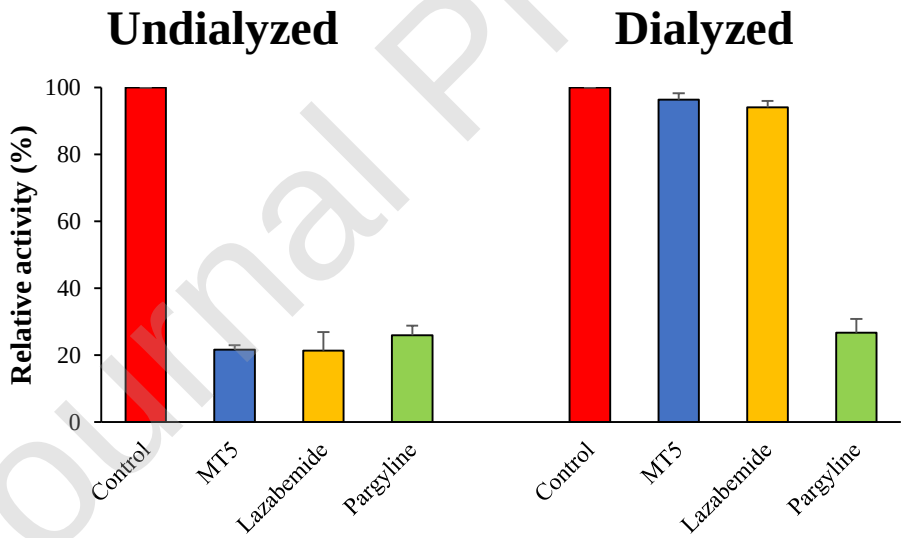


Fig. 3. Reversibility tests of MAO-B inhibition **MT5** as determined by dialysis method.

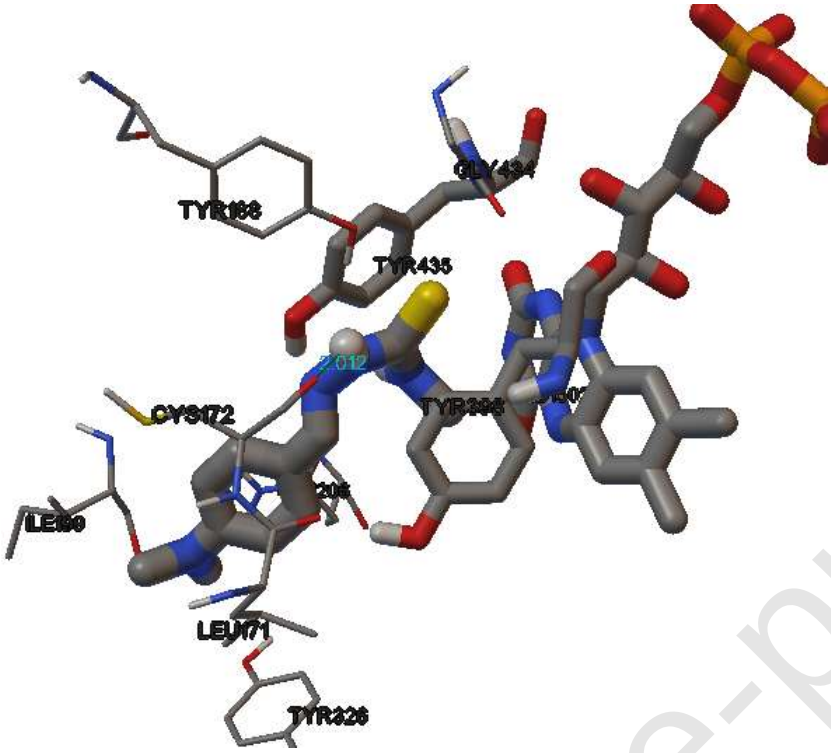


Fig. 4. Hypothetical binding mode of **MT5** in the active site of MAO-B. A hydrogen bonding with the non-bonding electrons of terminal N-methyl group with Cys172 (2.012Å) was shown.

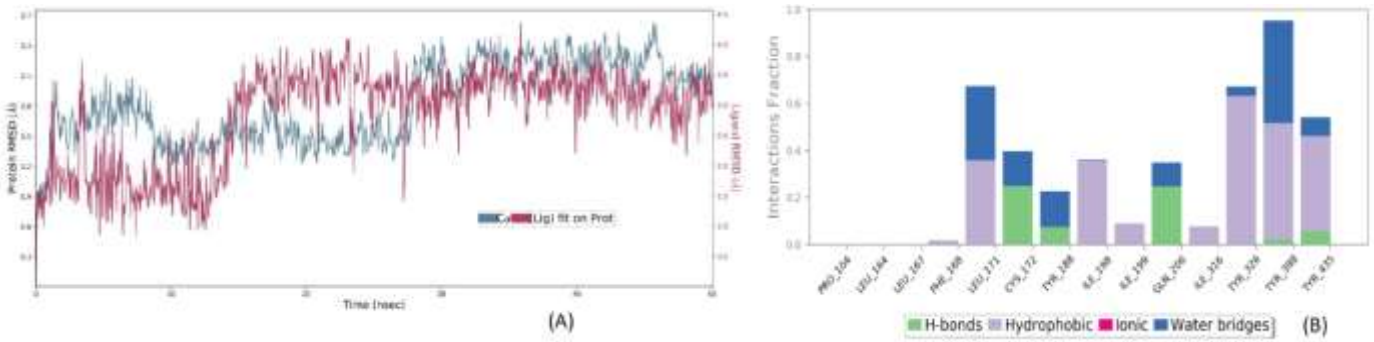


Fig. 5 (A) Protein-ligand root mean square deviation (RMSD); (B) Desmond-molecular dynamics (MD) calculated protein−ligand contacts.

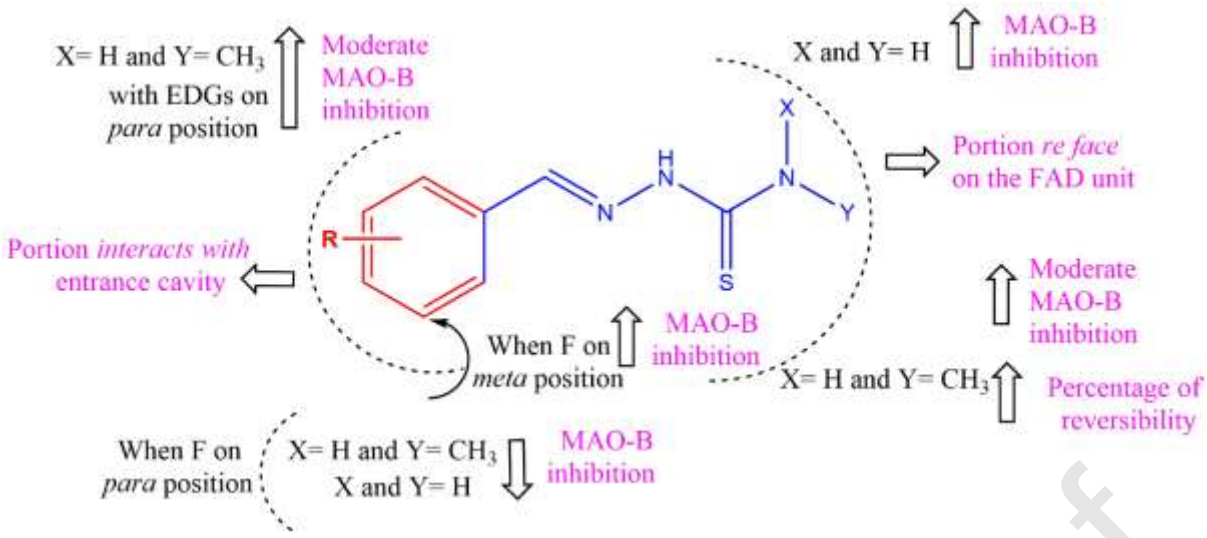


Fig. 6. Molecular recognition and structure activity relationships (SARs) of the aryl methylthiosemicarbazones/thiosemicarbazones at the active site of MAO-B.

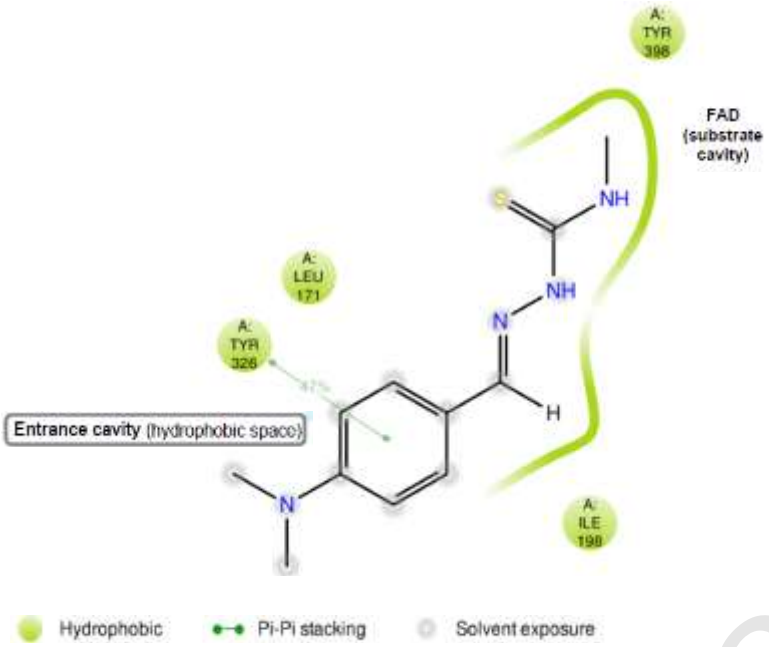


Fig. 7. A schematic of ligand atom interactions with protein residues. Simulation time in the selected trajectory: 50 ns.

Table 1

Inhibition of recombinant human MAO-A, -B, and AChE by 1-methyl, 5-phenyl substituted thiosemicarbazones (**MT1-11**)[a]

Compounds	Residual activity at 10 µM (%)			IC_{50} (µM)			SI[b]
	MAO-A	MAO-B	AChE	MAO-A	MAO-B	AChE	
MT 1	91.3 ± 8.84	89.7 ± 5.81	88.5 ± 9.77	-	-		-
MT 2	77.5 ± 2.36	62.3 ± 3.87	81.6 ± 2.79	-	-		-
MT 3	84.2 ± 1.18	60.2 ± 2.44	80.3 ± 5.58	> 40	11.8 ± 0.061		> 3.39
MT 4	98.3 ± 1.20	60.4 ± 1.20	83.2 ± 0.001	-	-		-
MT 5	55.5 ± 0.60	48.9 ± 2.28	75.2 ± 2.47	15.6 ± 0.11	8.77 ± 0.12		1.78
MT 6	76.3 ± 7.19	61.0 ± 3.43	80.8 ± 1.48	-	-		-
MT 7	97.2 ± 1.72	73.8 ± 3.50	68.5 ± 3.56	-	-		-
MT 8	85.0 ± 2.87	63.4 ± 1.40	82.6 ± 3.05	-	-		-
MT 9	80.1 ± 4.02	62.4 ± 1.55	80.5 ± 2.64	23.4 ± 0.082	14.8 ± 0.16		1.58
MT 10	95.6 ± 4.96	77.8 ± 1.43	91.7 ± 7.50	-	-		-
MT 11	84.2 ± 3.72	63.1 ± 0.71	79.3 ± 4.29	-	-		-
Toloxatone				1.08 ± 0.025	-	-	
Lazabemide				-	0.063 ± 0.015	-	
Clorgyline				0.0070 ± 0.00070	-	-	
Pargyline				-	0.028 ± 0.0043	-	
Tacrine				-	-	0.27 ± 0.019	

[a] Values are expressed as the means ± standard deviations of duplicate experiments.
[b] Selectivity index (SI) values = IC_{50} of MAO-A / IC_{50} of MAO-B

Table 2

The component binding energies and calculated/experimental inhibition constant (K_i) values of **MT5** towards MAO-B

Binding Energy (kcal/mol)	**Intermol energy (kcal/mol)**	**Vdw-hb-desolv-energy (kcal/mol)**	**Electrostatic energy (kcal/mol)**	**Total internal (kcal/mol)**	**Torsional energy (kcal/mol)**	**Unbound Energy (kcal/mol)**	**Calculated K_i (μM)**	**Experimental K_i (μM)**
-7.01	-8.50	-8.48	-0.01	0.32	1.49	-0.32	7.32	6.58