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Synthesis, molecular modeling, and in vitro screening of monoamine oxidase inhibitory activities of some novel hydrazone derivatives

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Abstract Thirteen 2-[2-(5-methyl-2-benzoxazolinone-3-yl) acetyl]-3/4/5-substituted benzylidenehydrazine derivatives were synthesized by reacting 2-(5-methyl-2-benzoxazolinone-3-yl)acetylhydrazine and substituted benzaldehydes in neutral and acid/base catalyzed conditions, and a comparison was made in terms of their yields and reaction times. The structures of all compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR, mass spectral data, and elemental analyses. All the compounds were investigated for their ability to selectively inhibit MAO isoforms by in vitro tests and were found to inhibit recombinant human MAO-B selectively and reversibly in a competitive manner. Among the compounds examined, compound **16** was found to be more selective than selegiline, a known MAO-B inhibitor, in respect to the K_i values experimentally found. Additionally, compounds **9** and **15** showed moderate MAO-B inhibitor activity. The interaction of compounds with MAO isoforms was investigated by molecular docking studies using recently published crystallographic models of MAO-A and MAO-B. The results obtained from the docking studies were found to be in good agreement with the experimental values.

Keywords Hydrazone · 5-methyl-2-benzoxazolinone · Human monoamine oxidase B inhibitors · Molecular docking

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Introduction

It is common knowledge that both isoforms of monoamine oxidase, A and B (MAO, EC 1.4.3.4), play a key role in the metabolism of neurotransmitters and are important for the treatment of psychiatric and neurological diseases. In particular, the relevance of MAO-B in the pathogenesis of Parkinson's disease (PD) and the therapeutic potential of MAO-B selective inhibitors in this pathology has been pointed out (Youdim et al. 2006; Elmer and Bertoni 2008). Moreover, interest in the B isoform of MAO has grown since the detection of increased MAO-B levels in a number of neurodegenerative disorders such as Alzheimer's disease, Huntington's chorea, and amyotrophic lateral sclerosis (Saura et al. 1994; Kumar et al. 2003; Distinto et al. 2012).

Lately, hydrazone derivatives have taken on greater significance owing to their application in pharmaceutical chemistry (Turan Zitouni et al. 2011). Hydrazide–hydrazones compounds are not only intermediates but they are also very effective organic compounds in their own right (Rollas and Küçükgüzel 2007). The biological activity associated with these compounds was attributed to the presence of the (–CONHN=CH–) moiety. Consequently, several hydrazide–hydrazone derivatives have displayed a broad spectrum of biological activities such as antimicrobial (Vicini et al. 2002;

Küçükgüzel et al. 2003; Salgın-Gökşen et al. 2007; Rasras et al. 2010; Turan Zitouni et al. 2011), antituberculosis (Küçükgüzel et al. 2003; Koçyiğit-Kaymakçıoğlu et al. 2006; Vavrikova et al. 2011), analgesic and anti-inflammatory (Sondhi et al. 2006; Salgın-Gökşen et al. 2007; Moldovan et al. 2011), anticonvulsant (Küçükgüzel et al. 2003; Kulandasamy et al. 2009a, b, antitumor (Iradyan et al. 2008; Hassan et al. 2011; Mohareb et al. 2011), antidepressant (Oliveira et al. 2011), and monoamine oxidase inhibitory (Chimenti et al. 2007, 2008, 2010a, b; MacKenzie et al. 2008). However, the MAO inhibition trait of this group started with the serendipitous finding of antidepressant effects in patients treated with iproniazid, a hydrazide-based antitubercular agent. Subsequently, numerous substituted hydrazines were studied as MAO inhibitors. The common structural feature of inhibitors and substrate in these studies usually contains an amino or imino group, which seems to play an essential role in orientation and complex formation at the active site of the enzyme.

In the course of our research, we have reported the synthesis and inhibitory activity of a series of cyclic hydrazine derivatives named pyrazoline and hexahydroindazole (Gökhan-Kelekçi et al. 2007, 2009a, b). Continuing our efforts to synthesize various bioactive molecules, we combined 5-methyl-2-benzoxazolinone with substituted benzaldehydes to obtain hydrazone molecules and investigated the eventual role of the hydrazone subunit on selective MAO inhibitor activities. Furthermore, we performed a computational study on the most potent inhibitor (compound **16**) in order to rationalize enzyme recognition with respect to hMAO-A and hMAO-B.

Materials and methods

Chemistry

All chemicals and solvents used in the present study were purchased from Merck A.G., Aldrich Chemical. Melting points were determined with a Thomas Hoover Capillary Melting Point Apparatus and were uncorrected. Infrared (IR) spectra were obtained with a Bruker Vector 22 IR (Opus Spectroscopic Software Version 2.0) spectrometer and the results were expressed in wave number (cm^{-1}). ^1H NMR spectrums were recorded on a Bruker 400 MHz UltraShield spectrometer using dimethylsulfoxide (DMSO-d_6) with chemical shifts reported as δ (ppm) from TMS. Mass spectrums were obtained via an electron impact technique using a Direct Insertion Probe and Agilent 5973-Network Mass Selective Dedector at 70 eV or in ESI-API-positive ion mode on a Waters Micromass ZQ ESCI Multi-Mode Ionization Mass spectrometer in methanol. Elemental analyses (C, H, N) were performed on an LECO CHNS 932 analyzer at the laboratory of Ankara University. The purity of the compounds was assessed by TLC on silicagel $HF_{254+366}$ (E. Merck, Darmstadt, Germany).

General procedure for the preparation of 2-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-3/4/5-substituted benzylidenehydrazine derivatives (4–16)

Method A: (Neutral) A solution of 2-(5-methyl-2-benzoxazolinone-3-yl)acetylhydrazine (1 mmol) in 40 mL ethanol was refluxed with substituted benzaldehyde (1 mmol) for 30 h. The reaction mixture was then cooled and the solid precipitated was recrystallized from appropriate solvents.

Method B: (Acid) A solution of 2-(5-methyl-2-benzoxazolinone-3-yl)acetylhydrazine (1 mmol) in 40 mL ethanol was refluxed with substituted benzaldehyde (1 mmol) in the presence of a catalytic amount of conc. HCl (4 drops) for 3–4 h. The reaction mixture was then cooled and the solid precipitated was recrystallized from appropriate solvents.

Method C: (Base) A solution of 2-(5-methyl-2-benzoxazolinone-3-yl)acetylhydrazine (1 mmol) in 40 mL ethanol was refluxed with substituted benzaldehyde (1 mmol) in the presence of a catalytic amount of triethylamine (4 drops) for 15–16 h. The reaction mixture was then cooled and the solid precipitated was recrystallized from appropriate solvents.

Biochemistry

Chemicals

hMAO-A (recombinant, expressed in baculovirus infected BTI insect cells), hMAO-B (recombinant, expressed in baculovirus infected BTI insect cells), *R*-(–)-deprenyl hydrochloride, resorufin, dimethyl sulfoxide, and other chemicals were purchased from Sigma-Aldrich TM (Germany). Moclobemide was donated (Roche Pharmaceuticals, Germany). The Amplex®-Red MAO Assay Kit (Molecular Probes, USA) contained benzylamine, *p*-tyramine, Clorgyline (MAO-A inhibitor), Pargyline (MAO-B inhibitor), and horseradish peroxidase.

Determination of inhibitory activities of the compounds on human MAO-A and -B

The activities of hMAO-A and hMAO-B (using *p*-tyramine as common substrate for both isoforms) were found to be 185.6 ± 9.50 and 153.2 ± 9.55 pmol/mg/min for hMAO-A and -B, respectively ($n = 3$). The interactions of the synthesized compounds with hMAO isoforms were determined by a fluorimetric method described and modified previously (Anderson et al. 1993; Yáñez et al. 2006;

Chimenti et al. 2008). The production of H_2O_2 catalyzed by MAO isoforms was detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex®-Red reagent), a non-fluorescent, highly sensitive, and stable probe that reacts with H_2O_2 in the presence of horseradish peroxidase to produce the fluorescent product resorufin.

Kinetic experiments

Newly synthesized compounds were dissolved in dimethyl sulfoxide, with a maximum concentration of 1 %, and used in the concentration range of 1–100 μM. Kinetic data for interaction of the enzyme with the compounds were determined using the Microsoft Excel package program. Lineweaver–Burk plots were used to estimate the inhibition constant (K_i) of the inhibitors. SI (K_i(MAO-A)/K_i(MAO-B)) was also calculated. The protein was determined according to the Bradford method (Bradford 1976), in which bovine serum albumin was used as a standard.

Reversibility experiments

Reversibility of the MAO inhibition with novel derivatives was evaluated by a centrifugation-ultrafiltration method (Chimenti et al. 2010c). In brief, adequate amounts of the recombinant hMAO-A or B were incubated together with a single concentration of the newly synthesized compounds or the reference inhibitors in a sodium phosphate buffer (0.05 M, pH 7.4) for 15 min at 37 °C. After this incubation period, an aliquot was stored at 4 °C and used for the measurement of MAO-A and -B activity. The remaining incubated sample was placed in an Ultrafree-0.5 centrifugal tube (Millipore, USA) with a 30 kDa Biomax membrane in the middle of the tube and centrifuged at 9,000×*g* for 20 min at 4 °C. The enzyme retained in the 30 kDa membrane was resuspended in a sodium phosphate buffer at 4 °C and centrifuged again two successive times. After the third centrifugation, the enzyme retained in the membrane was resuspended in sodium phosphate buffer (300 mL) and an aliquot of this suspension was used for MAO-A and -B activity determination.

Molecular docking studies

The crystal structures of MAO-A and MAO-B were extracted from the Protein Data Bank (PDB) [(http://www.rcsb.org, for MAO-A pdb code: 2Z5X; human monoamine oxidase in complex with harmine, resolution 2.2 Å (Son et al. 2008) and for MAO-B pdb code: 2V5Z; human MAO-B in complex with inhibitor safinamide, resolution 1.6 Å (Binda et al. 2007))]. Each structure was cleaned of all water molecules and inhibitors as well as all non-interacting ions before being used in the docking studies. The initial oxidized form of the FAD was used in all docking studies. For MAO-A and MAO-B, one of the two subunits was taken as the target structure. Using a fast Dreiding-like force field, each protein's geometry was first optimized and then submitted to the "Clean Geometry" toolkit of Discovery Studio (Accelrys, Inc.) for a more complete check. Missing hydrogen atoms were added based on the protonation state of the titratable residues at a pH of 7.4. Ionic strength was set to 0.145 and the dielectric constant was set to 10. The ADT 1.5.4 (AutoDock Tools) (Morris et al. 2009) graphical user interface program was employed to setup the enzymes for docking.

Ligand setups and docking

The 3D structures of ligand molecules were built, optimized at (PM3) level and saved in pdb format. The ADT package was also used here to generate the docking input files of ligands. AutoDock 4.2 (Huey et al. 2007; Morris et al. 2009) was employed for all dockings; and the detailed docking procedure has been given elsewhere (Yelekçi et al. 2007).

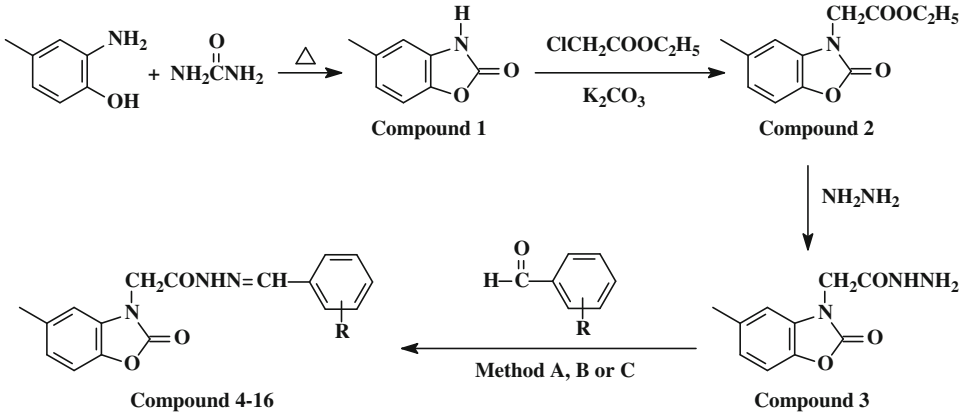
Results and discussion

Chemistry

In this study, the synthesis of 2-[2-(5-methyl-2-benzoxazolinone-3-yl)acetyl]-3/4/5-substituted benzylidenehydrazine derivatives (Compounds **4–16**) are described. The reaction of various substituted benzaldehydes with 2-(5-methyl-2-benzoxazolinone-3-yl)acetylhydrazine in neutral or acid/base catalyzed conditions provided the title compounds and these were evaluated for their MAO-inhibitory activities by in vitro tests.

The synthesis pathway leading to the title compounds is given in Scheme 1. The starting material, 5-methyl-2-benzoxazolinone **1**, was synthesized per the methods in the literature using 5-methyl-2-hydroxyaniline and urea (Close et al. 1949). Ester **2** was prepared in good yield by alkylation in acetone of potassium salt of 5-methyl-2-benzoxazolinone **1** with adequate α-halogenoester (Milcent et al. 1996). Treatment of ester **2** in ethanol with hydrazine hydrate at reflux gave hydrazide derivative **3**. The reaction could then proceed via a nucleophilic attack of hydrazine hydrate at the ester function of **2**, which produced a 89 % yield (Salgın-Gökşen et al. 2007). The reaction of 2-(5-methyl-2-benzoxazolinone-3-yl)-acetylhydrazine with various benzaldehydes in neutral or with a catalytic amount of HCl or triethylamine in ethanol gave the corresponding hydrazones **4–16** in very good yields.

Scheme 1 Synthetic pathway of compounds 4–16



R= **4**: 3-Br; **5**: 3-F; **6**: 3-Cl; **7**: 3-CF_3; **8**: 3-NO_2; **9**: 4-Br; **10**: 4-F; **11**: 4-OH; **12**: 4-CH_3; **13**: 4-CF_3; **14**: 4-NO_2; **15**: 3,5-diCl; **16**: 3,4,5-triOCH_3

It is known that acid or base catalysis accelerates the transformation to the hydrazones more than a neutral reaction state. In this study, it was also seen that yields in the acid catalyzed reaction were generally higher than the others and reaction time was shorter compared to neutral and base catalysis reaction.

The structures of the newly synthesized compounds were confirmed by microanalyses, IR, ^{1}H NMR, ^{13}C NMR, and mass spectrums (Table 1). The elemental analyses' results were within ±0.4 % of the theoretical values. ^{13}C NMR was performed for compound **9**. The IR spectra of compounds **4–16** exhibited a peak at 1683–1674 cm^{-1} due to a carbonyl function derived from the hydrazone structure beside the C=O stretching band of a 2-benzoxazolinone ring at 1790–1735 cm^{-1}. In the ^{1}H NMR spectra of hydrazones, the absence of the NH_2 absorptions of the hydrazide ($\delta = 4.3$ ppm) and the presence of new resonances assigned to the –CH=proton of N=CH provided evidence for hydrazone formation.

It is known that hydrazones may exist as geometrical isomers in respect to the C=N double bonds and as conformers about a C(O)–N bond (Fig. 1) (Palla et al. 1986; Himmelreich et al. 1993; Wyrzykiewicz et al. 2000). Unfortunately, we do not have direct evidence for a particular configuration in this series of derivatives, but careful examination of the pattern of the methynic group CH=N in the ^{1}H NMR spectra indicates the presence of a major configurational isomer, preliminary considered as *E* isomer, with a basis in the observed chemical shift. According to this, in the ^{1}H NMR spectra of the compounds **4–16** in DMSO-d_6, two sets of signals each belonging to the CH_2 and =CH group of *cis* and *trans* conformers were observed between 4.58–4.67/8.12–8.36 and 5.00–5.11/7.95–8.18 ppm, respectively. One of the most remarkable differences regarding the chemical shifts of the corresponding protons in the two isomeric forms is the resonance signal of the NH proton: i.e, δ (NH) = 14–15 ppm for the *Z*-form and δ (NH) = 9–12 ppm for the *E* form. NH protons of the compounds **4–16** showed a broad singlet varying between δ 11.62 and 12.13 ppm, exchangeable with D_2O, indicating that the hydrazone form is the preferred *E*-configuration.

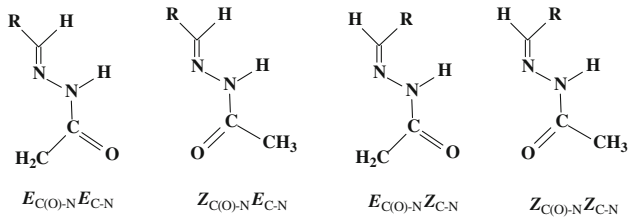
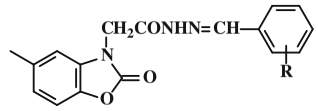


Fig. 1 Structures of the $E_{C=N}/Z_{C=N}$ isomers and $E_{C(O)–N}/Z_{C(O)–N}$ amide conformers of acetylhydrazones

The characteristic peaks were observed in the mass spectra of the compounds. The ions produced under ESI showed a characteristic $[M + Na]^+$ ion peak as the base signal for compounds **5**, **6**, **8**, **10**, **15**. The principal mass fragmentation pathways of N-substituted hydrazones of substituted benzaldehydes show key similarities but also differences in abundances of the important ions. Molecular ion signals for two of the compounds were base signal (Compounds **13** and **16**). Fragments resulting from the loss of O≡CNHN=CH–C_6H_4–R ions from the hydrazone derivatives were observed for compounds **4**, **7**, **14** as a base signal. In addition, β cleavage of the benzoxazolinone ring causing ejection of the benzoxazolinone methyl group was also observed. The structure of O≡CNHN=CH–C_6H_4–R ions were observed for compounds **11**, **12** at $m/z = 163$ and 161 as a base peak. Characteristic M + 2 and M + 4 isotope peaks were observed in the mass spectra of the compounds, which had bromine and chloride ions at a different level of intensity depending on the halogen numbers.

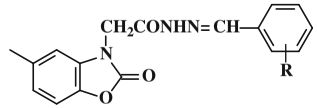
Table 1 Some characteristic and spectroscopic data of the synthesized compounds (**4–16**



Compounds	Melting point (°C)	IR ν (cm^{-1})	^{1}H NMR (DMSO-d_6) δ ppm (*J* in Hz)	Mass *m/z*
4	246–247	3,344 (N–H), 2,975 (C–H), 1,780, 1,681 (C=O)	2.31 (s, 3H, –C*H*$_3$), 4.62 and 5.05 (s, 2H, –N–C*H*$_2$–CO), 6.92–7.98 (m, 7H, aromatic-H), 8.01 and 8.19 (s, 1H, N=C*H*), 11.90 (bs, 1H, –CO–N*H*–N=)	387 ($M^{+\cdot}$), 389 241, 239, 227, 225, 206, 189, 163 (100 %), 162, 150, 149, 134, 118, 107, 91, 77.
5	233–234	3,077, 2,931 (C–H), 1,769, 1,682 (C=O)	2.33 (s, 3H, –C*H*$_3$), 4.63 and 5.07 (s, 2H, –N–C*H*$_2$–CO), 6.94–7.65 (m, 7H, aromatic-H), 8.06 and 8.24 (s, 1H, N=C*H*)	366, 351, 350 (100 %), 328.
6	245–247	3,064, 2,941 (C–H), 1,781, 1,681 (C=O)	2.33 (s, 3H, –C*H*$_3$), 4.64 and 5.08 (s, 2H, –N–C*H*$_2$–CO), 6.94–7.88 (m, 7H, aromatic-H), 8.04 and 8.22 (s, 1H, N=C*H*), 11.97 (bs, 1H, –CO–N*H*–N=)	384, 382, 368, 366 (100 %), 346, 344.
7	255–256,5 (dec.)	3,072, 2,944 (C–H), 1,778, 1,679 (C=O)	2.33 (s, 3H, –C*H*$_3$), 4.66 and 5.10 (s, 2H, –N–C*H*$_2$–CO), 6.95–8.14 (m, 7H, aromatic-H), 8.16 and 8.34 (s, 1H, N=C*H*), 12.01 (bs, 1H, –CO–N*H*–N=)	377 ($M^{+\cdot}$), 358, 229, 215, 206, 189, 163 (100 %), 162, 150, 149, 145, 134, 118, 107, 91, 77.
8	>270 (dec.)	3,063, 2,949 (C–H), 1,772, 1,681 (C=O)	2.31 (s, 3H, –C*H*$_3$), 4.65 and 5.09 (s, 2H, –N–C*H*$_2$–CO), 6.93–8.56 (m, 7H, aromatic-H), 8.18 and 8.36 (s, 1H, N=C*H*), 12.01 (bs, 1H, –CO–N*H*–N=)	354 ($M^{+\cdot}$), 341, 329, 311 (100 %), 267, 206, 189, 163, 162, 149, 134, 123, 118, 107, 91, 77.
9*	–	–	–	–
10	246–247,5	3,070, 2,942 (C–H), 1,776, 1,681 (C=O)	2.32 (s, 3H, –C*H*$_3$), 4.62 and 5.05 (s, 2H, –N–C*H*$_2$–CO), 6.94–7.85 (m, 7H, aromatic-H), 8.06 and 8.23 (s, 1H, N=C*H*), 11.84 (bs, 1H, –CO–N*H*–N=)	366, 351, 350 (100 %), 328.
11	261–263 (dec.)	3,204 (N–H), 3,096 (C–H), 1,735, 1,682 (C=O)	2.32 (s, 3H, –C*H*$_3$), 4.58 and 5.00 (s, 2H, –N–C*H*$_2$–CO), 6.81–7.59 (m, 7H, aromatic-H), 7.95 and 8.12 (s, 1H, N=C*H*), 9.96 (bs, 1H, O*H*), 11.62 (bs, 1H, –CO–N*H*–N=)	325 ($M^{+\cdot}$), 206, 189, 176, 163 (100 %), 162, 149, 136, 135, 134, 120, 118, 91, 77.
12[a]	–	–	–	–
13	243–243,5	3,064, 2,946 (C–H), 1,770, 1,682 (C=O)	2.31 (s, 3H, –C*H*$_3$), 4.64 and 5.07 (s, 2H, –N–C*H*$_2$–CO), 6.92–7.98 (m, 7H, aromatic-H.), 8.12 and 8.30 (s, 1H, N=C*H*), 12.02 (bs, 1H, –CO–N*H*–N=)	377 ($M^{+\cdot}$, 100 %), 358, 229, 215, 206, 189, 172, 163, 162, 150, 149, 145, 134, 118, 107, 91, 77.
14	266–268 (dec.)	3,071, 2,942 (C–H), 1,776, 1,677 (C=O)	2.33 (s, 3H, –C*H*$_3$), 4.67 and 5.11 (s, 2H, –N–C*H*$_2$–CO), 6.94–7.27 (m, 3H, 5-metil-2-benz.-H), 8.04 (d, 2H, aromatic-H), 8.30 (d, 2H, aromatic-H), 8.17 and 8.35 (s, 1H, N=C*H*), 12.13 (bs, 1H, –CO–N*H*–N=)	354 ($M^{+\cdot}$), 324, 206, 192, 189, 163, 162 (100 %), 149, 134, 118, 107, 91, 77.
15	260–262 (dec.)	3,062, 2,947 (C–H), 1,783, 1,674 (C=O)	2.33 (s, 3H, –C*H*$_3$), 4.65 and 5.10 (s, 2H, –N–C*H*$_2$–CO), 6.94–7.85 (m, 6H, aromatic-H), 8.02 and 8.20 (s, 1H, N=C*H*)	404, 402, 400 (100 %), 380, 378 ($M^{+\cdot}$), 233.
16	245,5–246,5	3,198 (N–H), 3,057, 2,978, 2,940, 2,824 (C–H), 1,790, 1,677 (C=O)	2.32 (s, 3H, –C*H*$_3$), 3.70 (s, 3H, –OC*H*$_3$), 3.83 (s, 6H, –OC*H*$_3$ and –OC*H*$_3$), 4.62 and 5.06 (s, 2H, –N–C*H*$_2$–CO), 6.93–7.26 (m, 5H, aromatic-H), 7.96 and 8.15 (s, 1H, N=C*H*), 11.84 (bs, 1H, –CO–N*H*–N=)	399 ($M^{+\cdot}$, 100 %), 400, 252, 237, 206, 195, 193, 163, 162, 150, 134, 118, 91, 77.

[a] Salgın-Gökşen et al. (2007)

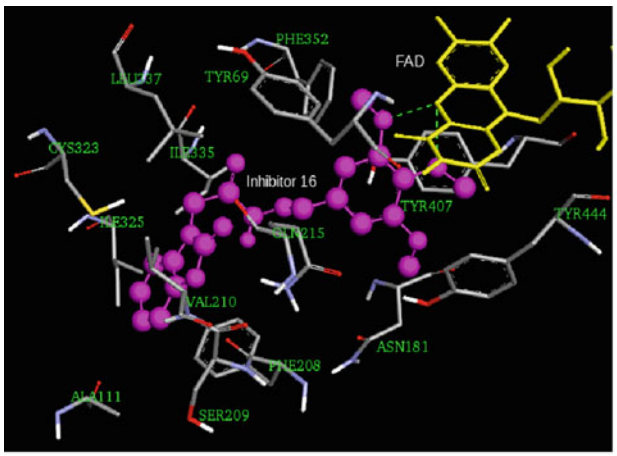
Table 2 Calculated and experimental K_i values corresponding to the inhibition of rat liver MAO isoforms by the newly synthesized *hydrazone* derivatives



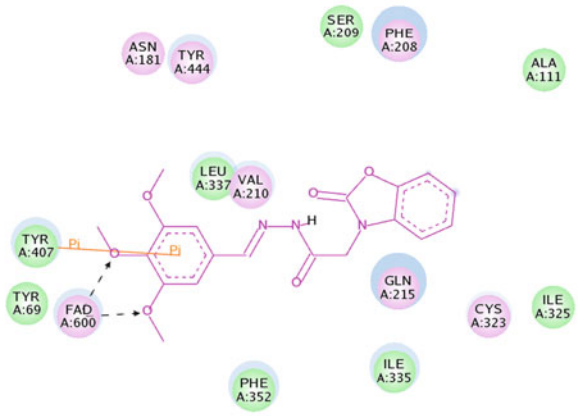
Compounds	Calculated K_i value for MAO-A (nM)	Calculated K_i value for MAO-B (nM)	Calculated SI[a]	Experimental K_i value for MAO-A (nM)[a]	Experimental K_i value for MAO-B (nM)[a]	Experimental SI[b]	Selectivity inhibition type, reversibility
4	26.17	11.96	2.19	60.8 ± 4	35.4 ± 2	1.71	MAO-B competitive, reversible
5	360.31	90.06	4.00	390.9 ± 19	79.8 ± 5	4.90	MAO-B competitive, reversible
6	41.29	10.39	3.97	612.8 ± 40	70.1 ± 4	8.74	MAO-B competitive, reversible
7	96.19	23.43	4.11	195.1 ± 8	50.9 ± 2	3.83	MAO-B competitive, reversible
8	151.25	40.19	3.76	320.6 ± 26	130.4 ± 12	2.46	MAO-B competitive, reversible
9	784.81	11.12	70.58	1,000 ± 0.1	60.0 ± 5	16.66	MAO-B competitive, reversible
10	106.38	26.20	4.06	455.3 ± 29	129 ± 10	3.53	MAO-B competitive, reversible
11	231.36	63.43	3.65	880.3 ± 65	291 ± 20	3.03	MAO-B competitive, reversible
12	134.92	32.21	4.19	450 ± 33.3	233.5 ± 18	1.93	MAO-B competitive, reversible
13	266.04	48.11	5.53	1,850 ± 0.1	890.2 ± 56	2.08	MAO-B competitive, reversible
14	1290.00	27.63	46.69	455.3 ± 31	260.3 ± 17	1.75	MAO-B competitive, reversible
15	69.60	3.48	20.00	705.4 ± 55	60 ± 4	11.78	MAO-B competitive, reversible
16	1210.00	17.46	69.30	3,560 ± 0.2	24.2 ± 3	147.11	MAO-B competitive, reversible
Selegiline				2,060 ± 9	30.3 ± 0.1	67.88	MAO-B competitive, irreversible
Moclobemide				8.9 ± 0.3	1,080 ± 3	0.008	MAO-A competitive, reversible

[a] K_i values were determined from kinetic experiments in which *p*-tyramine (substrate) was used at 500 μM to measure MAO-A and 2.5 mM to measure MAO-B. Pargyline or clorgyline were added at 0.50 μM to determine the isoenzymes MAO-A and -B. Newly synthesized compounds and the known inhibitors (moclobemide as selective MAO-A inhibitor, and selegiline as selective MAO-B inhibitor) were preincubated with the homogenates for 60 min at 37 °C. Each value represents the mean ± SEM of three independent experiments

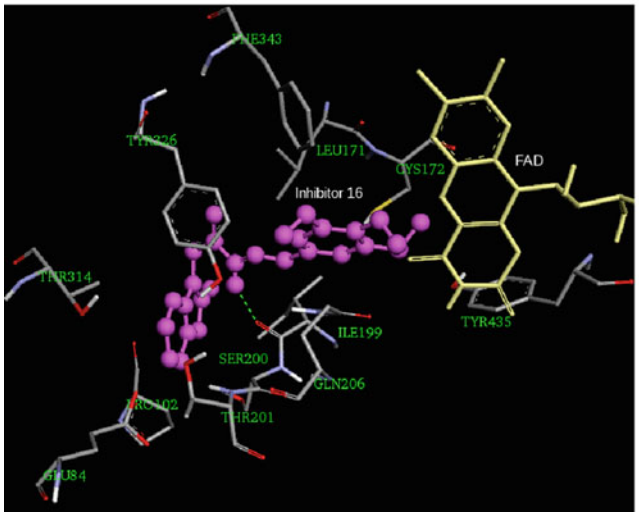
[b] Selectivity index. It was calculated as K_i (MAO-A)/K_i (MAO-B)



MAO-A 3-D



MAO- 2-D



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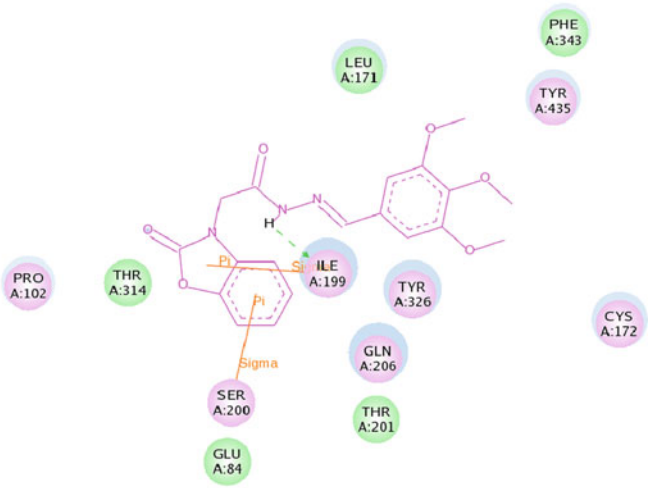

MAO-B 2-D

Fig. 2 Docked poses of compound **16** in MAO-A and MAO-B active sites in 3D and 2D, respectively. Amino acid side chains are shown as sticks, the inhibitor is shown as a ball and stick (*magenta*), and the cofactor FAD is depicted as a *yellow stick*. Residues involved in hydrogen bonding or polar interactions are represented by *magenta-colored circles*, and residues involved in vdW and hydrophobic interactions are shown by *green circles* in all 2D figures

Biochemistry

It has been suggested that the hydrazone moiety in the parent structure is responsible for the MAO inhibitory activity of the newly synthesized compounds. According to our experimental data, all the compounds (**4**–**16**) inhibited MAO-B selectively. The mode of inhibition was found to be competitive and reversible for all hydrazone derivatives tested. In respect to the K_i values experimentally found (Table 2), compounds **4** and **16**, which carry a bromide substituent at the third position on the phenyl ring and methoxy substituents at the third, fourth, and fifth positions of phenyl ring, respectively, were found to be highly potent MAO-B inhibitors with K_i values of 35.44 and 24.20 nM, respectively. MAO-A/MAO-B selectivities of compound **4** (bearing a bromide group) and **16** (bearing a trimethoxy group) were found to be 1.71 and 147.11, respectively, while MAO-A/MAO-B selectivity of selegiline, the known selective MAO-B inhibitor, was calculated to be 67.88.

Compounds **9** and **15**, which contain bromide at the fourth position and chloride groups at the third and fifth positions of the phenyl ring respectively also inhibited MAO-B selectively and reversibly in a competitive manner. These two molecules (compounds **9** and **15**) have very similar K_i values for MAO-B, which is approximately 60 nM. The MAO-A/MAO-B selectivities of compounds **9** and **15** were calculated from the experimental data as 16.66 and 11.78, and as 70.58 and 20, from the docking studies,

respectively. These two compounds therefore are also potent MAO-B inhibitors among the novel compounds studied in respect to both calculated and experimental data (Table 2).

Compounds **5** and **6** bearing a substituent at their third position (fluoride and chloride, respectively) also inhibited MAO-B selectively and reversibly in a competitive manner with experimental SI values of 4.9 and 8.74 and calculated (by docking studies) SI values of 4 and 3.97, respectively.

Among all novel derivatives studied, compound **16** was found to be the most potent MAO-B inhibitor with an experimental K_i value of 24.20 ± 2.93 nM. This new compound was more potent than selegiline, the well known MAO-B inhibitor (K_i value was determined as 30.35 ± 0.12).

It was suggested that by having a substitution at the phenyl ring, especially at the third position (compounds 4, 5, 6, 7, 8, 15, 16) strengthen the MAO-B inhibitory activity of newly synthesized hydrazone derivatives compared to those having a substitution at the fourth position of phenyl ring (compounds 9–14). Among compounds 4, 5, 6, 7, 8, 15, and 16, the compound carrying the methoxy group at the third, fourth, and fifth positions of phenyl ring (compound 16) was superior in potency in comparison to the rest. It was also suggested that bromide substitution at the third position (compound 4) is more effective in terms of MAO-B inhibitory activity than fluoride (compound 5) or chloride (compound 6) substitutions at the same position. Furthermore, having a bromide substitution at the fourth position of the phenyl ring (compound 9) leads to stronger MAO-B inhibition than compounds 5 and 6 which carry fluoride or chloride, respectively, at their third position of the phenyl ring.

Molecular docking studies

In order to see the detailed interactions of the docked poses of the inhibitors, compound **16** was selected. The binding modes for compound **16** (Fig. 2) in the MAO-A and MAO-B active site cavities are shown in the below images. Careful analysis of the MAO-A binding pose of compound **16** revealed that this compound is located in the vicinity of the hydrophobic packet which is composed of the TYR444, TYR407, and FAD cofactor. The 3,4,5-trimethoxy benzene ring of compound **16** makes two important polar interactions and one π–π interaction with the FAD cofactor. ASN181, SER209, PHE208, ALA111, ILE325, CYS323, GLN215, ILE335, LEU337, PHE352, and TYR69 contribute to the other significant attractions. The last two images in Fig. 2 show the poses of **16** in the active side of MAO-B in 3D and 2D depictions, respectively. The benzoxazolinone ring of **16** is attracted tightly between the SER200 and ILE199, making two σ–π interactions. One important hydrogen bond occurs between the amide hydrogen of **16** and the ILE199 backbone carbonyl moiety. On the other hand, the 3,4,5-trimethoxy benzene ring of compound **16** was strongly held by three polar attractions with TYR435, CYS172, and TYR326. The selectivity and potency of compound **16** on MAO-B compared to MAO-A can be noted in the above poses in MAO-A and MAO-B. The experimental data given in Table 2 are in agreement with these observations. All of the docking results may suggest computationally why the MAO-B inhibitory potency of compound **16** (K_i = 17.46 nM) is much better and more selective in comparison to MAO-A (K_i = 1210.00 nM).

Conclusion

The results presented here show that newly synthesized hydrazone derivatives may be promising candidates as potent anti-Alzheimer's/anti-parkinson agents. At the same time, this study indicates a significant correlation between the docking results and experimental ones. However, further experiments are necessary to fully elucidate the binding characteristics of the novel compounds to MAO isoforms.

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