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رمنگوند بر به سبک reversible hMAO-B inhibitors CH₃ NH-S-

Highlights

- Synthesis and *in vitro* MAO inhibitory activity of new 2-thiazolylhydrazones
- hMAO-B selectivity was also corroborated by molecular modelling studies
- Thiazole substitution is important for the activity of this scaffold
- They were endowed with a reversible mechanism of enzyme inhibition

Exploring 4-substituted-2-thiazolylhydrazones from 2-, 3-, and 4acetylpyridine as selective and reversible hMAO-B inhibitors

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Abstract

A series of 4-substituted-2-thiazolylhydrazone derivatives have been synthesized and tested *in vitro* for their human monoamine oxidase (hMAO) A and B inhibitory activity. Our findings confirmed that the substitution at C4 of the thiazole ring was important to obtain highly potent and selective hMAO-B inhibitors with IC₅₀ values in the nanomolar range. Moreover, these derivatives were endowed with a reversible mechanism of enzyme inhibition. Molecular modelling studies were performed to rationalize the recognition of all inhibitors with respect to hMAO-A and -B isoforms.

1. Introduction

Monoamine oxidase metabolizes monoamine neurotransmitters and dietary amines by oxidative deamination, and thus plays an important role in regulating emotional and other brain functions. Substrate oxidation is accompanied by the production of potentially harmful species such as H_2O_2 , which may contribute to cellular degeneration. There are two isoforms, MAO-A and MAO-B,

distinguished by their different substrate and inhibitor specificities and tissue distribution [1]. Immunohistochemical studies revealed that hMAO-A is the main isoform in the gastrointestinal tract, where it regulates the metabolism of tyramine, and in catecholaminergic neurons in the brain. Since hMAO-A inhibition is associated with the risk for hypertensive crisis, selective hMAO-B inhibitors are more frequently employed to modulate central monoamine levels [2]. In fact, hMAO-B is responsible for >80% of the MAO activity in the brain, where it metabolizes dopamine and other amines in serotonergic and histaminergic neurons and astrocytes [1]. This enzymatic activity was shown to increase with age, a process associated with gliosis. Additionally, hMAO-B activity is significantly higher in the brains of patients with Alzheimer's and Parkinson's disease (PD) [3]. Human MAO-B inhibitors may also have neuroprotective and neurorestorative properties. Although these effects may be the result of the inhibition of the formation of neurotoxic by-products derived from the MAO catalytic cycle, evidence suggests that mechanisms not related to MAO inhibition may be involved [4]. Several large-scale, randomized placebo-controlled clinical trials (DATATOP, TEMPO, ADAGIO) have demonstrated that treatment with selective hMAO-B inhibitors leads to a symptomatic amelioration of early PD with disease-modifying capabilities in the clinical settings [5,6].

In the course of our research [7], we have reported on the synthesis and MAO inhibitory activities of a large number of 2-thiazolylhydrazone derivatives. A 3D-QSAR study [8] indicated that the 2-thiazolylhydrazone moiety, in itself, contributes significantly towards the inhibitory activity against hMAO-B. We were particularly interested in also examining the effects that different substituents at C2, C4, and C5 of the thiazole nucleus have on MAO inhibitory activity and selectivity. On the basis of the information extrapolated from the interaction pattern among these isoforms and their inhibitors [9], we placed various moieties on these positions (aliphatic, cycloaliphatic, and aromatic groups) and examined the MAO inhibitory properties of the resulting compounds. The best moieties attached to the hydrazonic nitrogen were found to be derived from 2-, 3-, and 4-acetylpyridine interacting with the substrate-

orienting Tyr326 in hMAO-B active site. Substituents on the phenyl ring at C4 of the thiazole ring influenced the activity as demonstrated by the introduction of several groups (NO₂, CN, CH₃, OCH₃) or halogens (F, Cl) in the ortho, meta, and para positions [10-16], while the introduction of more sterically hindered moieties (coumarin and naphthalene) led to a decreased hMAO inhibitory activity [12,17]. The C5 of the thiazole ring must be unsubstituted [17]. Encouraged by these results, in this paper, we designed a series of 2-thiazolylhydrazone derivatives, in which the ethylpyridine moiety on the hydrazonic nitrogen was retained for all homologues. The aim of this study was to explore how the steric hindrance and the electronic properties of the substituent at C4 position (methyl, ethyl ester, and phenyl) affect hMAO-A and hMAO-B inhibitory activity and selectivity. For this purpose IC₅₀ values for the inhibition of the MAO enzymes were measured. Moreover, the time-dependence of inhibition of the series of 2-thiazolylhydrazone derivatives was also investigated and the K_i values determined for selected compounds.

2. Chemistry

4-Substituted-2-thiazolylhydrazone derivatives (1-9) were synthesized in high yields (81-99%) as reported in our previous communications (**Table 1**) [18]. 2-, 3-, and 4-Acetylpyridine reacted directly with thiosemicarbazide in ethanol in the presence of catalytic amounts of acetic acid at room temperature, and the obtained thiosemicarbazones were subsequently converted into 4-substituted-2thiazolylhydrazones by reaction with the appropriate α -haloketone or α -haloketoester under the same conditions (Hantzsch reaction). All the synthesized products were washed with petroleum ether and diethyl ether and purified by chromatography (ethyl acetate/petroleum ether) before characterization by spectroscopic methods and elemental analysis.

3. **Results and discussion**

The hMAO inhibitory properties of the pyridine derivatives (**1**-**9**) were investigated using commercially available (Sigma-Aldrich) microsomes from insect cells containing recombinant hMAO-A and hMAO-

B with kynuramine as the substrate [19]. All inhibition data (IC_{50} values determined with kynuramine at \sim 1 x K_m) are presented in **Table 1**. Six out of nine derivatives were found to be relatively weak hMAO-A inhibitors. With the exception of compound 4, all compounds displayed selective inhibition of the hMAO-B isoform with the most potent MAO-B inhibitors identified in the present study, 5, 8, and 9, exhibiting binding affinities that are much higher for human MAO-B than for MAO-A. Once again, the best results have been obtained with compounds deriving from 3- and 4-acetylpyridine and bearing a phenyl ring or an ethyl ester group at C4 of the thiazole nucleus. To determine if the inhibition of hMAO-B by the test compounds is time-dependent, 5, 8, and 9 at concentrations approximately equal to twofold their respective IC₅₀ values, were preincubated with hMAO-B for 0, 15, 30, and 60 min and the residual enzyme activities were subsequently determined. As shown in Fig. 1, the enzyme activities are not reduced with increased preincubation time which indicates that these inhibitors are not timedependent inhibitors of hMAO-B over the period evaluated. Since irreversible inhibitors display a timedependent reduction of enzyme activity, it can be concluded that these derivatives act as reversible human MAO-B inhibitors (the only possible exception is compound 5 which may possess a slow-tight binding component towards hMAO-A). To provide further evidence that compounds 5, 8 and 9 are reversible MAO-B inhibitors, a set of Lineweaver-Burk plots were constructed for the inhibition of MAO-B by each inhibitor. As shown in Fig. 2, the plots obtained for 8 and 9 are linear and intersect on the y-axis. This is indicative of competitive inhibition and is further support for a reversible interaction of these inhibitors with MAO-B. The plots obtained for compound 5 intersect to the right of the y-axis, which suggests a partial mixed inhibition mechanism. The deviation from strict competitive pattern can also arise from the failure of the MAOs to fulfill the assumptions of the Michaelis-Menten equation in the presence of inhibitor 5. Intersection to the right of the y-axis may also be suggestive of either nonlinear plots or differential binding of the inhibitor to different forms of the enzyme [20]. Further investigation is necessary to clarify this point.

In order to derive structure-activity relationship information, the recognition of the 2-thiazolylhydrazone derivatives by hMAO-A and -B was investigated by means of docking experiments. With the aim to evaluate the stability of the enzyme-inhibitor complexes and possible induced fit phenomena, the best scored configuration of each ligand within both isozymes was further examined *via* molecular dynamics simulations. To facilitate discussion of the results, our inhibitors were classified, on the basis of the R substituent, in three groups: (I) R= methyl, (II) R= ethyl ester, and (III) R= phenyl ring. The results showed that the isoform selectivity can be attributed to hydrogen bonds between the 2thiazolylhydrazone moiety and the hMAO-B Cys172 (Asn181 in hMAO-A). Additional productive interactions with two other hMAO-B exclusive residues (Ile199 and Tyr326) also contributed to complex stabilization. In hMAO-A the corresponding residues are Phe208 and Ile335 (Fig. 3). Residues Ile199 and Tyr326 determine, to a large degree, the shape and size of the hMAO-B binding site, and allow the placement of the ligands close to the hMAO-B Cys172 side chain. In hMAO-A, the Phe208 residue, which is sterically larger than Ile199 in hMAO-B, does not permit binding of the inhibitors in the proximity of Asn181. Instead the 2-thiazolyl moiety of this scaffold occupies space in a lipophilic cage opened by Ile335. This area is not accessible in the hMAO-B active site due to the presence of the Tyr326 at this position [21]. All inhibitors exhibited similar orientations in both hMAOs active sites and their docking complexes were not significantly modified by molecular dynamics simulations. The pyridine rings of the inhibitors were close to the FAD cofactor, while the R moiety was located towards the entrance of the active site. The only exception was the group I inhibitors. In hMAO-A these inhibitors exhibited an opposite orientation with the R group extending towards the FAD and, consequently, the pyridine ring extending towards the access of the cleft. Also, the group I inhibitors do not undergo hydrogen bonding to Cys172 in hMAO-B. Since this interaction makes a remarkable thermodynamic contribution to complex stabilization, group I inhibitors are comparatively weaker hMAO-B inhibitors.

We also investigated the role of the R moiety in the binding of the inhibitors to the MAO enzymes. Visual inspection of the most stable enzyme-inhibitor complexes revealed that, in both MAO-A and -B, small groups, such as the methyl, do not interact with the enzyme as strongly as ethyl ester or phenyl. As already described, in hMAO-A, group I inhibitors exhibit a reversed binding orientation, which places the methyl moiety towards the FAD where it can establish relatively few Van der Waals contacts to the cofactor, Tyr407, and Tyr444. In hMAO-B, the methyl group of group I inhibitors is located at the entrance cavity and undergoes Van der Waals contacts to Leu171, Tyr326, and Ile199. The balance of these two effects makes compound 4, the only active derivative of group I, and at variance with the other compounds, more selective with respect to isoform A. The ethyl ester moiety (group II) exhibits more interactions with both enzyme isoforms. The steric hindrance and the electronic properties of the ethyl ester prevent the reversed orientation observed with the group I in hMAO-A. Although these compounds display similar binding orientations to both MAO-A and -B active sites, the R moiety forms a larger interaction network in hMAO-B. In hMAO-A, the ethyl ester moiety of group II inhibitors is located at the entrance gorge interacting to Leu97, Ala111, Val210, Gln215, Leu325, and Leu337, while in hMAO-B productive contacts are formed with Phe103, Pro104, Trp119, Leu164, Leu167, Phe168, Ile199, and Ile316. The binding modes of the group III molecules (R= phenyl) were similar to those of the group II inhibitors: the phenyl ring occupied the same areas as those occupied by the ethyl ester substituent, but its conformational rigidity reduced the number of interacting residues in both enzymes. The phenyl moiety, however, overcame such a disadvantage in terms of complex stability by forming stronger T-shape π - π interactions to hMAO-A Phe208 and hMAO-B Tyr326. It may thus be concluded that both the, ethyl ester and phenyl moieties contribute equally to complex stabilization, and this degree of stabilization is greater than that obtained with the methyl group.

With the aim to rationalize the different MAO inhibitory potencies of the compounds containing the 2-, 3-, and 4-pyridine moieties, the molecular interactions of these moieties with MAO-A and -B were

investigated. The active site affinities were found to be dependent on the position of the nitrogen atom in the pyridine ring: in general, the highest affinity was observed for the 3- or 4-pyridine analogues while the lowest affinity was observed for the 2-pyridine analogues. The only exception was observed for the group III inhibitors for which the 2-pyridine derivative interacted slightly better with hMAO-A than the 3-pyridine derivative. Among the group III inhibitors, the 4-pyridine (9) was still the best inhibitor. This behavior may be attributed to the strong contribution of the R moiety (phenyl) that reduced the influence of the pyridine. Visual inspection of all complexes revealed that pyridine rings can establish one hydrogen bond to the side chains of hMAO-A Tyr197 and hMAO-B Tyr188 (Fig. 3). Such a productive contribution was geometrically prevented for 2-pyridine derivatives. The importance of the pyridine ring confirmed our previous results which suggested that this part of the molecule interacts with the "aromatic cage" (towards the flavin coenzyme), thereby stabilizing the inhibitor within the MAO enzyme active site. In conclusion, this study confirms the role of the 2thiazolylhydrazone scaffold to selectively recognize hMAO-B. Insertion of phenyl or ethyl ester substituent at position R allowed for additional productive interactions and led to an enhancement in hMAO-B inhibition potency. MAO-B selectivity was further enhanced by the introduction of 3-/4ethylpyridine moieties.

4. Experimental protocol

4.1 Chemistry

Starting materials and reagents used in the synthetic procedures were obtained from commercial suppliers and were used without purification. Melting points (mp) were determined by the capillary method on an FP62 apparatus (Mettler-Toledo) and are uncorrected. ¹H and ¹³C NMR spectra were recorded on 400 MHz Bruker spectrometer using DMSO- d_6 as the solvent. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane (TMS) which was added to the deuterated solvent. Coupling constants J are expressed in hertz (Hz). IR spectra were registered on a

PerkinElmer FTIR Spectrometer Spectrum 1000 in anhydrous potassium bromide (KBr). Elemental analyses for C, H, and N were determined with a Perkin-Elmer 240 B microanalyzer and the analytical results were \geq 95% purity for all compounds. All reactions were monitored by TLC performed on 0.2 mm thick silica gel plates (60 F₂₅₄ Merck). Preparative flash column chromatography was carried out on silica gel (230-400 mesh, G60 Merck). Organic solutions were dried over anhydrous sodium sulfate. Concentration and evaporation of the solvent after reaction or extraction was carried out on a rotary evaporator (Büchi Rotavapor). Fluorescence spectrophotometry was conducted with a Varian Cary Eclipse fluorescence spectrophotometer. Microsomes from insect cells containing recombinant human MAO-A and -B (5 mg/mL) and kynuramine 2HBr were obtained from Sigma-Aldrich.

4.2 General procedure for the synthesis of derivatives 1-9.

The appropriate 2-, 3-, or 4-acetylpyridine (50 mmol) was dissolved in 50 mL of ethanol and magnetically stirred with an equimolar quantity of thiosemicarbazide for 24 h at room temperature with catalytic amounts of acetic acid. The desired thiosemicarbazone was a coloured powder which precipitated from reaction mixture. It was filtered, washed with adequate solvents (*n*-hexane, petroleum ether, and diethyl ether), and dried under vacuum. The prepared thiosemicarbazone (50 mmol) reacted with equimolar amounts of the corresponding α -haloketone or α -haloketoester (50 mmol) dissolved in ethanol (50 mL) under magnetic stirring at room temperature. The reaction was monitored until completion by TLC. The resulting 2-thiazolylhydrazone derivative was filtered, washed with petroleum ether and diethyl ether, and, if necessary, purified by chromatography using ethyl acetate/*n*-hexane (3/1) as mobile phase, to give all compounds in high yields (81-99%). Some compounds (**2**, **5**, **8**) have been already synthesized and characterized by us in a previous study [18].

Characterization data for new compounds:

4.2.1. 4-Methyl-2-(2-(1-(pyridin-2-yl)ethylidene)hydrazinyl)thiazole (1).

Dark yellow solid, yield 99%; mp 231-233 °C; IR cm⁻¹ (KBr): 3107 (NH), 3055 (C_{sp2}-H), 2912 (C_{sp3}-H), 1607 (C=C), 1555 (C=N); ¹H NMR (DMSO-*d*₆): 2.28 (s, 3H, CH₃), 2.43 (s, 3H, CH₃), 6.66 (s, 1H, C₅H-thiazole), 7.72-7.73 (t, 1H, Ar), 8.27-8.28 (m, 1H, Ar), 8.31-8.33 (m, 1H, Ar), 8.72-8.73 (d, 1H, Ar), 10.78 (bs, 1H, NH, D₂O exch.); ¹³C NMR (DMSO-*d*₆): 12.14, 17.86, 107.25, 124.32, 128.87, 130.65, 131.99, 135.54, 144.45, 146.46, 158.78, 170.41. *Anal.* Calcd for C₁₁H₁₂N₄S: C, 56.87; H, 5.21; N, 24.12. Found: C, 56.99; H, 5.09; N, 24.01.

4.2.2. 4-Phenyl-2-(2-(1-(pyridin-2-yl)ethylidene)hydrazinyl)thiazole (3).

Yellow solid, yield 97%; mp 252-254 °C; IR cm⁻¹ (KBr): 3135 (NH), 3093 (C_{sp2} -H), 2924 (C_{sp3} -H), 1617 (C=C), 1534 (C=N); ¹H NMR (DMSO-*d*₆): 2.43 (s, 3H, CH₃), 7.33 (s, 1H, C₅H-thiazole), 7.40-7.44 (m, 3H, Ar), 7.62-7.63 (m, 1H, Ar), 7.87-7.89 (m, 2H, Ar), 8.17-8.18 (m, 2H, Ar), 8.67-8.68 (m, 1H, Ar), 11.85 (bs, 1H, NH, D₂O exch.); ¹³C NMR (DMSO-*d*₆): 14.35, 105.34, 126.74, 128.11, 129.01, 134.25, 136.85, 140.77, 142.74, 160.54, 169.15. *Anal.* Calcd for C₁₆H₁₄N₄S: C, 65.28; H, 4.79; N, 19.03. Found: C, 65.13; H, 5.00; N, 19.19.

4.2.3. 4-Methyl-2-(2-(1-(pyridin-3-yl)ethylidene)hydrazinyl)thiazole (4).

Dark red solid, yield 99%; mp 114-116 °C; IR cm⁻¹ (KBr): 3117 (NH), 3026 (C_{sp2} -H), 2924 (C_{sp3} -H), 1619 (C=C), 1548 (C=N); ¹H NMR (DMSO-*d*₆): 2.24 (s, 3H, CH₃), 2.45 (s, 3H, CH₃), 6.55 (s, 1H, C₅H-thiazole), 7.95-7.98 (t, 1H, Ar), 8.80-8.82 (m, 2H, Ar), 9.26 (s, 1H, Ar), 10.70 (bs, 1H, NH, D₂O exch.); ¹³C NMR (DMSO-*d*₆): 12.02, 17.45, 107.44, 124.23, 128.74, 130.11, 131.78, 135.99, 144.56, 146.74, 158.36, 170.44. *Anal*. Calcd for C₁₁H₁₂N₄S: C, 56.87; H, 5.21; N, 24.12. Found: C, 56.99; H, 5.07; N, 24.01.

4.2.4. 4-Phenyl-2-(2-(1-(pyridin-3-yl)ethylidene)hydrazinyl)thiazole (6).

Yellow solid, yield 90%; mp 256-257 °C; IR cm⁻¹ (KBr): 3105 (NH), 3035 (C_{sp2}-H), 2926 (C_{sp3}-H), 1610 (C=C), 1533 (C=N); ¹H NMR (DMSO-*d*₆): 2.40 (s, 3H, CH₃), 7.32 (s, 1H, C₅H-thiazole), 7.39-7.44 (m, 3H, Ar), 7.87-7.91 (m, 3H, Ar), 8.60-8.62 (m, 1H, Ar), 8.77-8.78 (m, 1H, Ar), 9.07 (s, 1H, Ar),

11.71 (bs, 1H, NH, D₂O exch.); ¹³C NMR (DMSO-d₆): 14.22, 105.18, 126.03, 126.91, 128.14, 129.12, 134.86, 136.96, 140.76, 142.74, 169.64. *Anal.* Calcd for C₁₆H₁₄N₄S: C, 65.28; H, 4.79; N, 19.03. Found: C, 65.04; H, 4.56; N, 18.87.

4.2.5. 4-Methyl-2-(2-(1-(pyridin-4-yl)ethylidene)hydrazinyl)thiazole (7).

Red solid, yield 98%; mp 247-249 °C; IR cm⁻¹ (KBr): 3163 (NH), 3057 (C_{sp2}-H), 2914 (C_{sp3}-H), 1620 (C=C), 1552 (C=N); ¹H NMR (DMSO- d_6): 2.18 (s, 3H, CH₃), 2.35 (s, 3H, CH₃), 6.43 (s, 1H, C₅H-thiazole), 8.17-8.18 (m, 2H, Ar), 8.78-8.80 (m, 2H, Ar), 10.72 (bs, 1H, NH, D₂O exch.); ¹³C NMR (DMSO- d_6): 12.74, 17.23, 107.45, 124.55, 128.47, 130.36, 131.25, 135.89, 144.77, 146.74, 158.62, 170.37. *Anal.* Calcd for C₁₁H₁₂N₄S: C, 56.87; H, 5.21; N, 24.12. Found: C, 56.75; H, 5.01; N, 24.28. 4.2.6. 4-Phenyl-2-(2-(1-(pyridin-4-yl)ethylidene)hydrazinyl)thiazole (**9**).

Yellow solid, yield 95%; mp 246-248 °C; IR cm⁻¹ (KBr): 3114 (NH), 3025 (C_{sp2} -H), 2923 (C_{sp3} -H), 1636 (C=C), 1525 (C=N); ¹H NMR (DMSO-*d*₆): 2.36 (s, 3H, CH₃), 7.34 (s, 1H, C₅H-thiazole), 7.89-7.91 (m, 1H, Ar), 8.14-8.16 (m, 1H, Ar), 8.37 (bs, 2H, Ar), 8.61 (s, 1H, Ar), 8.80 (bs, 2H, Ar), 10.67 (bs, 1H, NH, D₂O exch.); ¹³C NMR (DMSO-*d*₆): 14.35, 105.44, 126.02, 128.10, 129.78, 134.56, 136.64, 140.63, 142.88, 160.76, 169.03. *Anal.* Calcd for C₁₆H₁₄N₄S: C, 65.28; H, 4.79; N, 19.03. Found: C, 65.47; H, 4.93; N, 19.25.

4.3. hMAO-A and -B inhibition studies

As enzyme sources, commercially available (Sigma-Aldrich) microsomes from insect cells, containing recombinant human MAO-A and -B, were employed. The enzyme activity measurements were based on the MAO-A or MAO-B (0.0075 mg protein/mL) catalyzed oxidation of kynuramine to 4-hydroxyquinoline. The incubations were conducted in 500 μ L potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl 20.2 mM) in the presence of various concentrations of the test inhibitors (0-100 μ M) and 4% DMSO as the cosolvent. For MAO-A activity measurements the reactions contained 45 μ M kynuramine while for the for MAO-B activity measurements the kynuramine concentration was

30 µM. Following a 20 min incubation at 37 °C, the reactions were terminated by the addition of 400 µL NaOH (2N) and 1000 μ L water. The reactions were centrifuged for 10 minutes at 16,000 g and concentration measurements of 4-hydroxyquinoline in the supernatants of the samples were carried out spectrofluorometrically at excitation and emission wavelengths of 310 nm and 400 nm, respectively. The quantitative estimations were made with the aid of a linear calibration curve constructed with known amounts (0.047-1.56 µM) of the authentic metabolite dissolved in 500 µL potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl 20.2 mM). To each calibration standard, volumes of 400 µL NaOH (2N) and 1000 µL water were added. The necessary control samples were included to confirm that the test inhibitors do not fluoresce or quench the fluorescence of 4-hydroxyquinoline under these assay conditions. IC₅₀ values were calculated by plotting the initial rate of kynuramine oxidation versus the logarithm of the inhibitor concentration to obtain a sigmoidal dose-response curve. For each sigmoidal curve, six different inhibitor concentrations spanning at least 3 orders of a magnitude were used. These values were selected to bracket the IC₅₀ values of the test inhibitors and to properly define the upper and lower plateaus of the sigmoidal curve. The inhibitor concentrations were also selected so that they are equally spaced on a logarithmic scale. These kinetic data were fitted to the one site competition model incorporated into the Prism software package (GraphPad). Each IC₅₀ value was measured using six single inhibitor concentrations as described above. Three such experiments were performed for each compound yielding three IC₅₀ values. The mean and standard deviation (SD) of these three IC_{50} values are reported in the results section.

4.3.1. Time-dependent studies

This study shows that the test pyridine derivatives act as moderate to potent hMAO-B inhibitors while possessing little or weak hMAO-A inhibition potential. To determine whether the inhibitors interact reversibly or irreversibly with hMAO-B, time-dependent inhibition studies were carried out with three selected inhibitors (**5**, **8**, and **9**). They were preincubated with recombinant human MAO-B (0.03 mg

protein/mL) for periods of 0, 15, 30, 60 min at 37 °C in potassium phosphate buffer (100 mM, pH 7.4, made isotonic with KCl 20.2 mM) at concentrations which are approximately 2 fold the measured IC₅₀ values for the inhibition of human MAO-B (**Table 1**). The reactions were diluted twofold with the addition of kynuramine (30 μ M), and then incubated for a further 20 min at 37 °C. After dilution, the final volumes of these incubations were 500 μ L, the concentrations of the test inhibitors were approximately equal to the IC₅₀ values for the inhibition of hMAO-B by the respective inhibitors, and the enzyme concentration was 0.015 mg protein/mL. The reactions were terminated with 200 μ L NaOH (2N) and a volume of 1200 μ L distilled water and the rates of formation of 4-hydroxyquinoline were measured and quantified as described above. All measurements were carried out in triplicate and are expressed as mean ± SD.

4.3.2. Lineweaver-Burk plots

A set consisting of four Lineweaver-Burk plots were constructed for the inhibition of human MAO-B by compounds **5**, **8** and **9**. For this purpose, the rates of the hMAO-B-catalyzed oxidation of kynuramine were measured in the absence and presence of three different concentrations of the test compounds. The hMAO-B incubations and rate measurements were carried out as described above. The reactions contained kynuramine (15-90 μ M), the test inhibitors, and hMAO-B (0.015 mg protein/mL). DMSO (4%) was added as cosolvent to all incubations. Linear regression analysis was performed using GraphPad Prism 5. K_i values were estimated from the x-axis intercepts (-K_i) of replots of the slopes of the Lineweaver–Burk plots versus inhibitor concentration.

4.4. Molecular modelling

The 3D models of our compounds were built in E configuration by means of the Maestro GUI [22] and were energy optimized using the OPLS-AA force field [23] coupled to the GB/SA [24] water implicit solvation model as implemented in MacroModel software [25]. High resolution Protein Data Bank [26] (PDB) crystallographic structures, deposited with codes 2Z5X [9a] and 2V60 [27], were adopted as receptor models of hMAO-A and hMAO-B, respectively. Taking into account the limits of the PDB file

format, both structures were manipulated to be used in our docking: missing atoms and FAD bond order were fixed, hydrogen atoms were added and co-crystallized water solvent molecules and ligands, harmine and 7-(3-chlorobenzyloxy)-4-carboxaldehyde-coumarin, for 2Z5X and 2V60 respectively, were removed. The recognition with respect to both targets was investigated by means of docking experiments carried out using the flexible algorithm of Glide software [28]. The binding site, of both targets, was defined by a regular box of about 110,000 Å³ centred onto the FAD N5 atom. The docking complexes scoring was performed using the default Glide XP function. The best docking scored complex of each inhibitor into both targets was submitted to 1.2 ns of molecular dynamics (MD) simulations, at 300 K, carried out with Desmond package [29]. The final MD structures were considered in our binding mode analysis.

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Table 1.

Synthetic route and hMAO inhibition data of compounds 1-9. IC₅₀ values were determined with kynuramine at ~1 x K_m.



Compound	Pyridine isomer	R	IC ₅₀ hMAO-A (µM)	IC ₅₀ hMAO-B (µM)
1	2-acetylpyridine	CH ₃	No inhibition	No inhibition
2	2-acetylpyridine	COOEt	No inhibition	No inhibition
3	2-acetylpyridine	Ph	16.6 ± 2.01	3.84 ± 0.133
4	3-acetylpyridine	CH ₃	6.910 ± 0.227	13.633 ± 0.870
5	3-acetylpyridine	COOEt	6.571 ± 0.296	0.0722 ± 0.0057
6	3-acetylpyridine	Ph	21.3 ± 0.88	0.944 ± 0.075
7	4-acetylpyridine	CH ₃	No inhibition	No inhibition
8	4-acetylpyridine	COOEt	6.630 ± 0.667	0.1274 ± 0.0028
9	4-acetylpyridine	Ph	2.67 ± 0.082	0.013 ± 0.0012

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17



Fig. 1. The time-dependency of hMAO-A (left) and hMAO-B (right) inhibition by compounds **5** (top, 13.14 μ M for hMAO-A and 0.144 μ M for hMAO-B), **8** (middle, 13.26 μ M for hMAO-A and 0.255 μ M for hMAO-B), and **9** (bottom, 5.33 μ M for hMAO-A and 0.026 μ M for hMAO-B). The test inhibitors were preincubated with the hMAO isoforms for various periods of times (0-60 min) and the residual enzyme catalytic rates (nmol 4-hydroxyquinoline formed/min/mg protein) were measured.



Fig. 2. Lineweaver-Burk plots of the oxidation of kynuramine by recombinant human MAO-B. The plots were constructed in the absence (asterisk) and presence of various concentrations of compounds **5** (top), **8** (middle) and **9** (bottom). The inhibitor concentrations employed were 0.25 x IC₅₀ (open squares), 0.5 x IC₅₀ (filled circles) and 1 x IC₅₀ (open circles). K_i values were estimated from the x-axis intercepts ($-K_i$) of replots of the slopes of the Lineweaver–Burk plots versus inhibitor concentration ($r^2 > 0.993$). These values are 0.048 µM, 0.048 µM and 0.011 µM for compounds **5**, **8** and **9**, respectively.



Fig. 3. Docking best poses of 9 in a) hMAO-A and b) hMAO-B and 4 in c) hMAO-A and d) hMAO-B. Interacting residues and ligands are reported in CPK and green carbon coloured sticks respectively. FAD cofactor is displayed in spacefill. Yellow dotted lines indicate hydrogen bonds.