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Nonselective Inhibition of Monoamine Oxidases A and B by Activators of Soluble Guanylate Cyclase[1]

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Abstract—Several activators of soluble guanylate cyclase were investigated as potential inhibitors of rat liver mitochondrial monoamine oxidases (MAO) A and B. They all fitted into the previously designed "molds" of substrate–inhibitor binding sites of these enzymes. However, only two of them, NO donors (7-nitro-benzotetrazine-1,3-dioxide (7-NBTDO) and benzodifuroxan), caused nonselective inhibition of MAO A and MAO B with IC_{50} values of 1.3-1.6 and 6.3-6.8 μM, respectively. The inhibitory effect on both MAO A and MAO B was reduced by mitochondria wash suggesting reversible mode of the enzyme inhibition. There was no correlation between potency of MAO inhibition and activation of human platelet soluble guanylate cyclase. The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) had no effect on the manifestation of MAO inhibition by benzodifuroxan and 7-NBTDO; however, at 50 μM concentration carboxy-PTIO caused potent inhibition of MAO A with minor effect on MAO B activity. The data suggest that nonselective inhibition of MAO A and MAO B by benzodifuroxan and 7-NBTDO can be attributed to the properties of the chemical structures of these compounds. The results of the present study demonstrate a real possibility for the development of a new generation of effective reversible nonselective MAO inhibitors exhibiting equal inhibitory activity with respect to both MAO A and MAO B.

Key words: monoamine oxidase, reversible inhibitors of monoamine oxidase, nonselective inhibition of MAO A and MAO B

Monoamine oxidase (MAO; EC 1.4.3.4) catalyzes oxidative deamination of monoamine neurotransmitters in the central nervous system and peripheral tissues [1-3]. Altered MAO activity observed in numerous neuropsychiatric disorders and possibility of their correction by MAO inhibitors have made this enzyme a very popular target of basic and clinical studies [1-3].

In mammals MAO exists in two forms, MAO-A and MAO-B, which are encoded by different genes and differ in sensitivity to acetylenic inhibitors (clorgyline and deprenyl, respectively) and by preferential substrate oxidation (serotonin and phenylethylamine, respectively) [1, 2]. Selective MAO A inhibitors are employed clinically as antidepressants, whereas selective MAO B inhibitor is used for the treatment of Parkinson's disease [4, 5].

In spite of a long history of clinical use of several generations of MAO inhibitors [6, 7], irreversible inhibitors of the first generation (e.g., iproniazide) nonselectively inhibiting both MAOs are still considered as the most effective preparations [8]. Their use is restricted because of serious side effects and adverse interactions with foods and beverages containing amines such as tyramine [6, 8]. So, long-term strategy in the development of effective MAO-inhibiting drugs has focused on design of reversible and selective inhibitors of MAO A and MAO B [7, 8]. The behavior of such selective and reversible MAO inhibitors differs in several respects from those of the irreversible inhibitors. The rate of recovery from the effects of a reversible inhibitor will be dependent on the rate at which it is eliminated from the tissues, since removal of the free drug will result in its dissociating from the enzyme [8].

The reversibility and relatively rapid recovery rate, therefore, gives a much lower risk of hypertensive interactions provoked by accumulating (including dietary) monoamines. Increased concentrations of monoamines (especially dietary tyramine accumulated during absorption in the intestine) displace the reversible inhibitor from the active site of MAO, and the reactivated enzyme will catalyze their biological degradation (and therefore reduce the possibility of development of hypertensive reaction) [8].

[1] Dedicated to 75th birthday of Professor Vladimir Gorkin, unquestioned leader in monoamine oxidase research in Russian Science.

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Recently, the idea of the development of a new generation of MAO inhibitors which would effectively and nonselectively inhibit both MAO A and MAO B has become more and more popular [4, 8, 9]. Its realization would provide reversible MAO inhibitors as effective as iproniazide, but lacking side-effects typical for the first generation of MAO inhibitors. However, obvious spatial differences of the substrate binding region of active sites of MAO A and MAO B seriously complicate this problem. The evaluation of three dimensional sizes of the substrate binding region of these enzymes by means of overlay of selective competitive inhibitors of MAO A and MAO B [10-12] revealed (Fig. 1) that the substrate binding region of MAO A is "wider" and "longer" than that of MAO B. Recent X-ray analysis of the MAO B–irreversible inhibitor complex [13] also revealed the existence of a narrow canal limiting access of large inhibitors to the active site (Fig. 2). So it is reasonable to suggest that compounds which may be accommodated in the active site of MAO B at least will not meet steric barriers in the process of binding at the active site of MAO A.

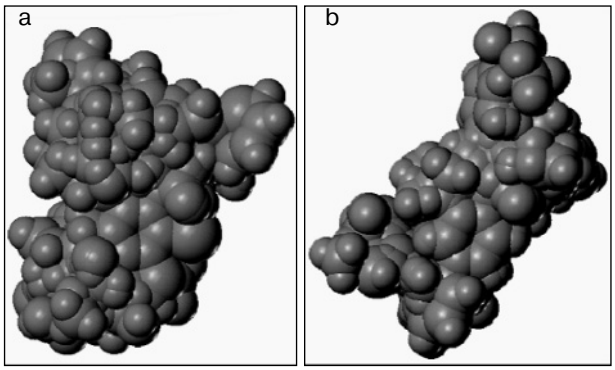


Fig. 1. Models of MAO A (a) and MAO B (b) which allow evaluation of the shapes and sizes of the substrate binding regions of these enzymes were obtained by overlay of known competitive inhibitors of MAO A and MAO B [10, 11].

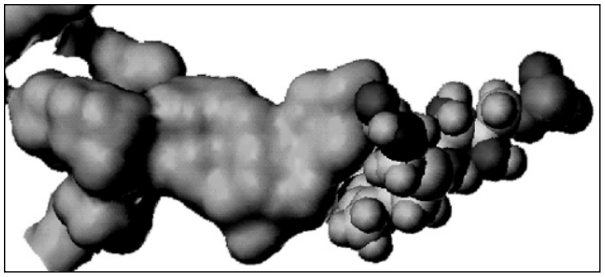


Fig. 2. Molecular surface of the active site of MAO B after removal of the irreversible mechanism based inhibitor, pargyline. Extended substrate binding region of active site of MAO B (canal) adjacent to the flavin cofactor shown as spheres of van der Waals radii is on the left.

Recently, studying regulatory properties of isatin (indole-2,3-dione), endogenous inhibitor of MAO B and atrial natriuretic peptide stimulated guanylate cyclase, we found that this compound may also attenuate activation of soluble guanylate cyclase by nitric oxide [14]. Taking into consideration literature data on the increase of nitric oxide formation in brain vessels and tissue induced by deprenyl [15], the existence of regulatory interrelationship between enzymes involved in metabolism of biogenic amines (MAO) and those realizing signals of nitric oxide (NO-stimulated soluble guanylate cyclase) becomes increasingly evident. The latter suggests that some activators of guanylate cyclase might inhibit MAO. So, in this study we investigated several activators of guanylate cyclase as potential inhibitors of MAO A and MAO B. The stimulatory effect of three of them on guanylate cyclase activity is attributed to nitric oxide release [16, 17]. The data provide experimental evidence of simultaneous equipotent reversible inhibition of both MAOs by some activators of soluble guanylate cyclase. Their structures may be used in the development of the new generation of reversible nonselective MAO inhibitors.

MATERIALS AND METHODS

Guanylate cyclase activators—7-nitro-benzotetrazine-1,3-dioxide (7-NBTDO), benzofuroxan, benzodifuroxan, and benzotrifuroxan—were synthesized as described earlier [16, 17].

Rat liver mitochondria isolated by the conventional method of differential centrifugation [18] were used as the source of MAO A and MAO B. Isolated mitochondria were washed and suspended in 50 mM phosphate buffer, pH 7.4, and stored at –20°C. MAO activity was assayed radiometrically by accumulation of ^{14}C-labeled aldehydes formed during enzymatic deamination of ^{14}C-labeled amines [9, 18]. [^{14}C]5-Hydroxytryptamine creatinine sulfate (100 μM) and [^{14}C]2-phenylethylamine (5 μM) (Amersham, England) were used as substrates of MAO A and MAO B, respectively. The enzyme activities were determined after preincubation of mitochondria with inhibitors for 30 min at 37°C using a range of concentrations 10^{-9}-10^{-4} M. (Such approach is widely used during pilot analysis of potential MAO inhibitors [19].) Compounds were initially dissolved in dimethyl sulfoxide at 10 mM concentration; subsequent dilutions were prepared using distilled water. Control samples contained an equivalent volume of the solvent containing the same amount of dimethyl sulfoxide as in the samples containing the tested inhibitors.

In parallel experiments, these compounds were tested as activators of human platelet guanylate cyclase that was isolated as described previously [14, 16-17]. Experimental conditions and composition of assay medium were as described in previous reports [14, 16-17].

The computer modeling was run on an Origin 200 server (Silicon Graphics, Inc.) using SYBYL 6.8 software (TRIPOS, Inc.) [20]. Three-dimensional models of the tested molecules were optimized using Tripos standard field forces. Partial atomic charges were calculated using semi-empirical quantum-mechanical AM1 method and by the Gasteiger–Huckel method.

Docking procedure was carried out using an original program of geometrical docking DockSearch developed at the Institute of Biomedical Chemistry, Russian Academy of Medical Sciences [21]. The program had the following parameters: angle of ligand rotation along each axis was 15°, resolution of three-dimensional approximation lattice was 0.2 Å, probe sphere radius and atom sphere radius were 1.4 and 1.5 Å, respectively, standard deviation of distances between ligand atoms in complexes within one cluster was 0.25 Å.

Spatial structure of MAO B (pdb1GOS) was obtained from the PDB database of spatial structures of macromolecules [22]. After removal of covalently bound irreversible inhibitor pargyline, protein structure was optimized using Tripos standard field force.

Optimization of possible position for ligand binding in the active site of MAO B was carried out by energy minimization of the enzyme–inhibitor complex using the LeapFrog module of SYBYL software.

RESULTS AND DISCUSSION

Among the four compounds tested only benzofuroxan did not inhibit MAO A and MAO B (Fig. 3). Benzotrifuroxan selectively inhibited MAO B. Benzodifuroxan and 7-NBTDO caused nonselective inhibition of MAO A and MAO B (Fig. 3, Table 1). However, in the case of 7-NBTDO IC_{50} values (concentration required for 50% inhibition of enzyme activity) were 4-5 times lower than those obtained with benzodifuroxan. A single wash of mitochondria pretreated with 7-NBTDO and benzodifuroxan reduced inhibition of both enzymes. This suggests reversible mode of inhibition of both MAO A and MAO B by these compounds (Table 2).

Three of the four compounds stimulated activity of soluble guanylate cyclase with nearly the same efficacy (Table 1). It was previously recognized that they can generate NO and activate guanylate cyclase only in the presence of thiols [16, 17]. However, direct evidence for the NO-dependence of activation of this enzyme was obtained only for one of them: addition of an NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO), significantly reduced activation of soluble guanylate cyclase by 7-NBTDO [17].

Since the reaction medium for assay of MAO A and MAO B activity did not contain exogenously added thiols the possibility of NO-dependent inhibition of MAO A and MAO B by these compounds seemed unlikely. It should be noted that the NO effect on mitochondria is usually associated with NO-dependence of reactive oxy-

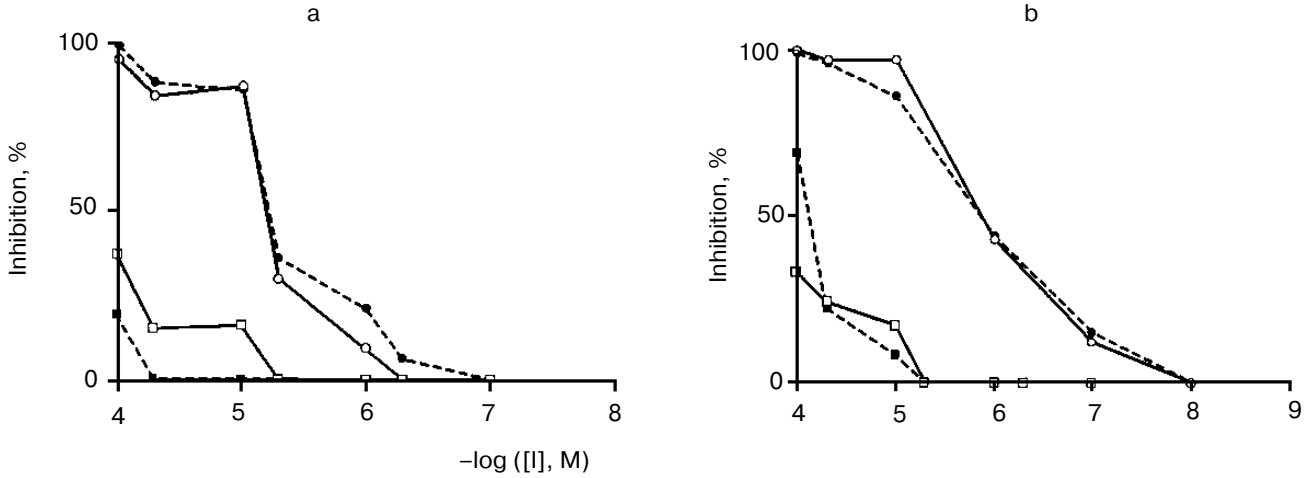
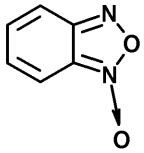
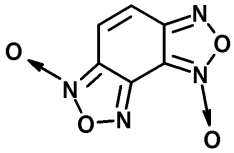
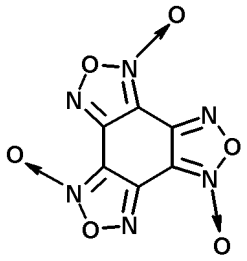
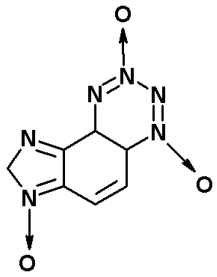


Fig. 3. Effect of benzofuroxan (□■) and benzodifuroxan (○●) (a), benzotrifuroxan (□■) and 7-NBTDO (○●) (b) on the activity of MAO A (solid line, open symbols) and MAO B (dashed line, closed symbols). Data represent mean of 3-5 experiments. In all cases standard error did not exceed 10%.

Table 1. Effect of benzofuroxan, benzodifuroxan, benzotrifuroxan, and 7-NBTDO on the activity of soluble guanylate cyclase from human platelets and rat liver mitochondrial MAO A and MAO B

Compound	Guanylate cyclase activation*, %	Inhibition (IC_{50}), μM	
		MAO A	MAO B
Benzofuroxan 	200 ± 20	>100	>>100
Benzodifuroxan 	**1130 ± 40**	**6.8 ± 0.3**	**6.3 ± 0.9**
Benzotrifuroxan 	1100 ± 50	>100	63 ± 9.4
7-NBTDO 	**1450 ± 60**	**66 ± 6.0**	**70 ± 8.0**

* Guanylate cyclase activation was studied using 10 μM concentration of the compounds tested. Basal activity of this enzyme was 96 ± 10 pmol/min per mg protein. Compounds causing nonselective inhibition of MAO A and MAO B are shown in bold. Data represent mean ± SEM of 3-5 independent experiments.

gen species [23], which may oxidize SH-groups of MAO [24, 25]. However, oxidation of SH-groups of membrane-bound MAO A and MAO B never caused total inactivation of these enzymes. Oxidized MAOs could not be reactivated by washing the mitochondria in the absence of reducing agents (e.g., dithiothreitol) [24, 25]. These data argue against possible NO-dependent oxidative modification of MAO A and MAO B as the mechanism responsible for inhibition of these enzymes during their incubation with benzodifuroxan and 7-NBTDO. The presence of the NO scavenger, 50 μM carboxy-PTIO, did not influence MAO B inhibition by benzodifuroxan and 7-NBTDO (Fig. 4). In the case of MAO A, 50 μM carboxy-PTIO caused independent inhibition of this enzyme

Table 2. Effect of benzodifuroxan (A) and 7-NBTDO (B) on the activity of mitochondrial MAO A and MAO B

Experiment	Effect	MAO A	MAO B	*p*
A	Without wash	**87 ± 5%**	**86 ± 2%**	> 0.3
	After wash	**68 ± 2%**	**78 ± 1%**	< 0.01
	p	< 0.01	< 0.01	
B	Without wash	**86 ± 4%**	**97 ± 3%**	> 0.2
	After wash	**35 ± 2%**	**43 ± 3%**	= 0.05
	p	< 0.001	< 0.001	

Note: Rat liver mitochondria (2 mg/ml) were incubated in 50 mM phosphate buffer, pH 7.4, for 30 min at 37°C in the presence or absence of 10 μM benzodifuroxan (A) or 7-NBTDO (B). The incubation was stopped by adding 10-fold excess of cold phosphate buffer. Activity of MAO A and MAO B was assayed after mitochondria sedimentation and resuspension in the same buffer. Results are expressed as percent of inhibition. Control samples were treated in the same way as experimental samples but in the absence of the inhibitors. Data represent mean ± SEM of 3-5 experiments.

Table 3. Energy values (kcal/mole) for ligand binding at the active site of MAO B

Compound	LeapFrog	Electrostatic component	Van der Waals forces
Benzofuroxan	−14.01	−1.27	2.43
Benzodifuroxan	−19.47	10.20	−16.88
Benzotrifuroxan	−13.66	−11.02	−12.14
7-NBTDO	−16.32	−14.34	−18.99

Note: Molecules were docked using DockSearch software. Primary selection of hypothetical complexes was carried out using the criterion of 70% complementarity between ligands and the active site surface. Subsequent optimization of mutual ligand positioning at the active site of MAO B was carried out using LeapFrog software. Data represent mean of energetic characteristics of the optimal complexes. The LeapFrog column reflects the value of binding energy of the most optimal complexes in the LeapFrog software. Electrostatic component and van der Waals forces were evaluated by the standard method of SYBYL software $E_{bn} = E_{complex} - E_{protein} - E_{ligand}$.

which was comparable with the effect of benzodifuroxan and 7-NBTDO. However, the effects of these inhibitors and carboxy-PTIO were not additive.

All these data suggest that differences in the effectiveness of MAO A and MAO B inhibition by benzodifuroxan and 7-NBTDO reflect differences in their structures.

Docking of these compounds into the active site cavity revealed that all of them can be accommodated by the active site of MAO B and model of active site of MAO A. For subsequent evaluation of the possibility of forming

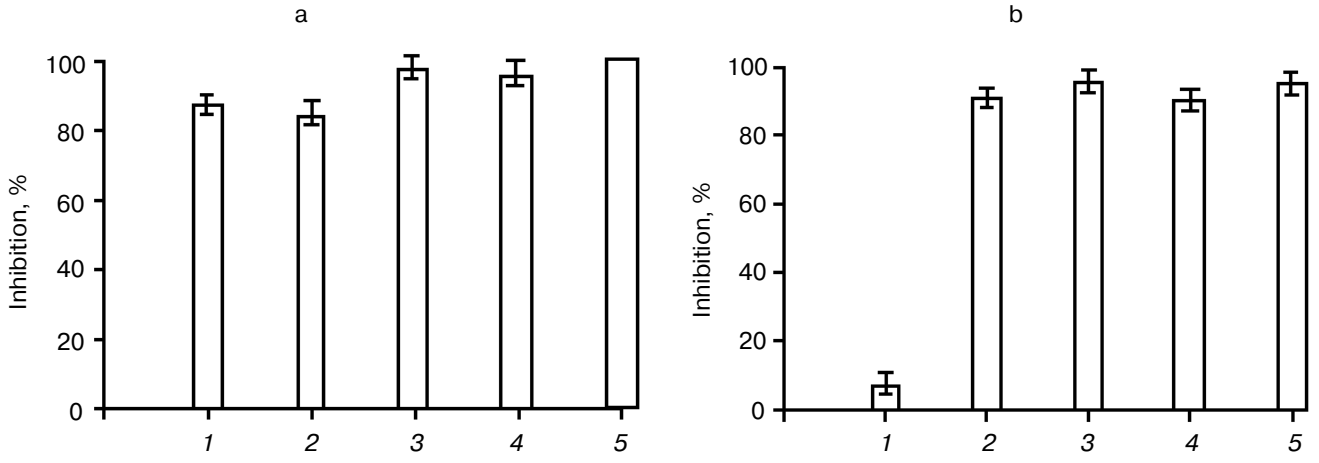


Fig. 4. Effect of carboxy-PTIO on inhibition of MAO A (a) and MAO B (b) by benzodifuroxan and 7-NBTDO: *1*) 50 μM carboxy-PTIO; *2*) 10 μM benzodifuroxan; *3*) 50 μM carboxy-PTIO + 10 μM benzodifuroxan; *4*) 10 μM 7-NBTDO; *5*) 50 μM carboxy-PTIO + 10 μM 7-NBTDO. Activity of MAO A and MAO B in control was 0.80 ± 0.12 and 0.70 ± 0.08 nmol/min per mg of protein, respectively.

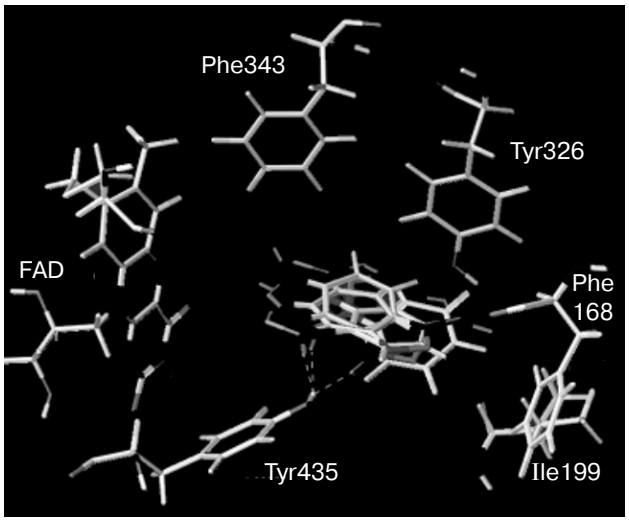


Fig. 5. Putative position of inhibitors in the active site cavity of MAO B. Dashed lines show possible hydrogen bonds between Tyr435 and benzodifuroxan, benzotrifuroxan, and 7-NBTDO. Benzofuroxan may form a hydrogen bond with Glu206 (not shown). The figure shows that all these compounds are positioned in the same region of the active site of MAO B. White color shows carbon atoms, white gray color designates hydrogen atoms, dark gray and black colors show oxygen and nitrogen atoms, respectively. Structures of these compounds were shown in Table 1. Isomers with minimal internal energy were used for modeling.

the MAO B–inhibitor complex, we calculated binding energies for these ligands. Data of Table 3 clearly demonstrate that the most potent inhibitors are characterized by lower calculated binding energy and the most effective inhibitor of MAO B, 7-NBTDO, has the lowest binding energy. All compounds were positioned in the upper site of the canal and blocked the entrance into it (Fig. 5).

Due to lack of information on spatial organization of MAO A, such calculations were made only for MAO B.

During recent decades many rather effective and highly selective tightly bound inhibitors of either MAO A or MAO B have been found; they effectively operate within the range of IC_{50} values of 10^{-5}-10^{-8} M [26]. However, until now nonselective inhibitors of MAO A and MAO B acting within this range of IC_{50} values remained unknown. Analyzing MAO inhibitory activity of benzyloxycarbonyl derivatives of ethylene diamine (EDA), we found that the 3,4-dichloro-benzyloxycarbonyl-EDA derivative caused nonselective inhibition of MAO A and MAO B with IC_{50} of 22 μM [9]. The results of the present report suggest that a "threshold" of sensitivity of both MAOs to nonselective reversible inhibition may be reduced to $IC_{50} < 1.0$ μM. This is a real precondition for the development of a new generation of MAO inhibitors which can cause simultaneous equipotent reversible inhibition of both MAO A and MAO B.

Previously developed pharmacophore models of selective inhibitors of MAO A and MAO B share one common structural element, an aromatic ring [27]. The presence of a nitro-group in the aromatic ring of some compounds decreases effectiveness of MAO A inhibition and increases inhibition of MAO B [9, 28]. It is possible that introduction of a nitro-group and/or other substituent with similar electron-acceptor properties into some selective MAO A inhibitors, which can also be accommodated in the active site of MAO B, may increase effectiveness of nonselective inhibition of both MAOs up to the pharmacologically acceptable level of $IC_{50} \sim 0.1$ μM.

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