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cis-cyclopropylamines as mechanism-based inhibitors of monoamine oxidases

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Cyclopropylamines, inhibitors of monoamine oxidases (MAO) and lysine-specific demethylase (LSD1), provide a useful structural scaffold for the design of mechanism-based inhibitors for treatment of depression and cancer. For new compounds with the less common *cis* relationship and with an alkoxy substituent at the 2-position of the cyclopropyl ring, the apparent affinity determined from docking experiments revealed little difference between the enantiomers. Using the racemate, kinetic parameters for the reversible and irreversible inhibition of MAO were determined. No inhibition of LSD1 was observed. For reversible inhibition, most compounds gave high IC50 values with MAO A, but sub-micromolar values with MAO B. After pre-incubation of the cyclopropylamine with the enzyme, the inhibition was irreversible for both MAO A and MAO B, and the activity was not restored by dilution. Spectral changes during inactivation of MAO A included bleaching at 456 nm and an increased absorbance at 400 nm, consistent with flavin modification. These derivatives are MAO B-selective irreversible inhibitors that do not show inhibition of LSD1. The best inhibitor was *cis*-*N*-benzyl-2-methoxycyclopropylamine, with an IC_{50} of 5 nM for MAO B and 170 nM for MAO A after 30 min pre-incubation. This *cis*-cyclopropylamine is over 20-fold more effective than tranylcypromine, so may be studied as a lead for selective inhibitors of MAO B that do not inhibit LSD1.

Introduction

Interest in cyclopropylamine chemistry was revived when tranylcypromine (TCP; *trans*-2-phenylcyclopropan-1-amine) was identified as an irreversible inhibitor of lysine-specific demethylase (LSD1), one of the key demethylase enzymes in epigenetic gene regulation [1,2]. TCP is a mechanism-based inactivator of monoamine oxidases (MAO), and has been used in the treatment of depression for decades [3–5]. TCP inactivates MAO B by forming a C4a adduct with the flavin cofactor, whereas LSD1 forms an N5 adduct [5,6]. These adducts are formed after oxidation of TCP by the enzyme, and each may arise via a C4a–N5 cyclic structure [6–8]. A new series of 1-substituted cyclopropylamine derivatives with improved affinity for LSD1 formed various adducts depending on the derivative, at C4a, N5, or bridging both, probably via a radical mechanism [9]. The inactivation is irreversible, and thus new protein synthesis is required for restoration of activity in the cell.

The potential usefulness of cyclopropylamine inhibitors of MAO and LSD1 for treatment of depression [10–12] and cancer [4,13–17], and the need for selective

Abbreviations
LSD1, lysine-specific (histone) demethylase (also known as KDM1A); MAO, monoamine oxidase; TCP, tranylcypromine.

inhibition of the targets, have prompted the synthesis and evaluation of new inhibitors such as *trans*-1-substituted derivatives [9]. For the MAO enzymes, more derivatives of *trans* isomers have been studied, but *cis*-2-phenylcyclopropylamine is only slightly less effective than the *trans* isomer [18–20]. Enantiomeric selectivity is also a concern. On MAO B, (*1R,2S*)-(–)-TCP was 20-fold more effective as a competitive inhibitor, but *cis*-2-phenylcyclopropylamine showed no enantiomeric selectivity [4,5,21]. LSD1 showed no enantiomeric selectivity for TCP [5,22], but the two enantiomers of a 1-substituted cyclopropylamine resulted in different adducts [9].

Here we describe inhibition of the two forms of MAO by selected *cis* isomers of primary and secondary cyclopropylamines with an alkoxy group at the 2-position of the cyclopropyl ring, replacing the more common phenyl substitution [23]. The *trans* compound TCP, which was already well-established as a drug before the full impact of the existence of two forms of MAO was appreciated [21,24,25], is included as a reference compound. We show that *cis*-cyclopropylamine, like TCP, forms a covalent adduct with the flavin in MAO A and MAO B. Docking studies, performed to explore enantiomer binding in MAO A and MAO B, also revealed occupancy of the imidazoline (I2) site [26–28] in the entrance cavity of MAO B.

Results

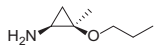
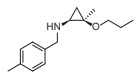
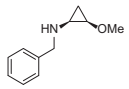
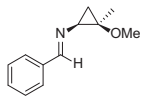
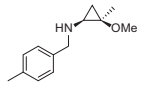
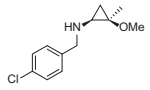
Absence of inhibition of LSD1

The *cis*-cyclopropylamine compounds, synthesized as described previously [23], were tested against LSD1 for which TCP is an established inhibitor. In the LSD1 enzyme assay [22], the compounds were inactive at the maximum tested concentration of 25 μM.

Molecular modelling with MAO to explore enantiomeric selectivity

Reversible binding may be predicted by docking, so this was used to guide the selection of previously synthesized compounds [23] used in this study, and then to explore whether the enantiomers bind differently to MAO A and MAO B (Table 1). Molecular modelling [29] was performed to determine binding energies and estimate K_i values for the *cis*-cyclopropylamines with MAO A and MAO B (Table S1). Theoretical K_i values

Table 1. Experimental IC_{50} values and predicted K_i values for reversible inhibition of MAO by *cis*-cyclopropylamines. The experimental IC_{50} values were obtained using racemic mixtures. The selectivity for MAO B was calculated as the ratio between the IC_{50} values for MAO A and those for MAO B. The K_i values were calculated for the (1*S,2R*) enantiomer using AutoDock 4 [30].

		Experimental IC_{50} (μM)			K_i value (μM)	
Compound		MAO A	MAO B	Selectivity	MAO A	MAO B
1		> 300	3.70	> 81	2010	674
2		> 300	0.78	> 385	691	445
3		52.4	24.5	2.1	4.65	2.88
4		21.1	0.17	124	89.0	48.8
5		> 400	4.61	> 87	61.7	14.4
6		115	64.0	1.8	17.4	7.81
7		71.3	33.4	2.1	16.0	6.37
Tranylcypromine		23.6	4.02	5.9	91.0	105

for both enantiomers of all seven compounds were obtained by docking the compounds into MAO A (PDB ID 2Z5X) and MAO B (PDB ID 2V5Z) using AutoDock 4 [30] (as shown in Table 1) and AutoDock Vina [31]. Both programs gave concordant values for the binding energies, with essentially no difference between the (1*R*,2*S*) and (1*S*,2*R*) enantiomers (Table S1). The aromatic group improves binding energy, and the *para*-methyl group gives compounds **3** and **6** better affinity than compound **4** in this theoretical ranking. The *para*-chloro compound (**7**) gives values similar to the *para*-methyl compound.

Based on the lack of enantiomeric differences, racemic *cis*-cyclopropylamines were used for the experimental work.

Reversible binding: experimental IC_{50} values

For MAO A and B, the reversible interaction was measured as the IC_{50} value. Under the assay conditions used here, the IC_{50} values with MAO A are proportional to K_i and reflect the initial reversible binding of the inhibitor to MAO A [32]. For MAO B, the IC_{50} is influenced by the oxidative half-reaction as well as the reductive half-reaction because the rates of reduction and re-oxidation of the flavin in the steady state are similar. In practice, the affinity of the inhibitor for the reduced form of MAO B becomes significant, such that the experimental IC_{50} is influenced by more factors than is the true K_i for binding to a single (oxidized) form of MAO B [32]. The reversible inhibition of MAO A by these *cis*-cyclopropylamine compounds is very poor, as indicated by the high IC_{50} values for all compounds except compound **4** (Table 1). In contrast, the IC_{50} values are micromolar for compounds **1**, **2**, **4** and **5** with MAO B (Table 1), demonstrating that the selectivity of reversible binding for MAO B is as good as the standard drug TCP (for **3**, **6** and **7**), or better than TCP (for **1**, **2**, **4** and **5**).

The K_i values obtained from docking calculations qualitatively predict the experimental values for reversible binding for MAO A in these assays, which were carefully designed to reflect the initial reversible binding to the active site. With the exception of compound **4**, the theoretical K_i values for MAO A shown in Table 1 agree with the order of potency observed for the experimental IC_{50} values. In contrast, for MAO B, compounds **3**, **6** and **7** give poor experimental IC_{50} values compared to the predicted affinity (Table 1), presumably for the kinetic reasons explained above. In general, the output from AutoDock 4 [30] predicted a selectivity for MAO B over MAO A that was much smaller than found experimentally.

IC_{50} values for irreversible binding

All compounds showed a time-dependent increase in inhibition (decreased IC_{50}) due to irreversible inactivation, as demonstrated by the lack of restoration of activity after dilution. Table 2 gives the IC_{50} values after a 30 min pre-incubation of the inhibitor with the enzyme.

The selectivity ratios calculated from the 30 min IC_{50} values indicate that compounds **6** and **7** act equally on MAO A and B. The other compounds (**1**–**5**) are better inactivators of MAO B than of MAO A, and thus are more selective than TCP (Table 2). The most effective inactivator is *N*-benzyl-2-methoxycyclopropylamine (**4**), with an IC_{50} of 5 nM against MAO B, 15-fold more potent than TCP and 10-fold more selective for MAO B. Comparing the selectivity at 0 and 30 min, those for compounds **1**, **2**, **5**, **6** and **7** do not change, but compound **3** is more selective at 30 min whereas compound **4** has a lower selectivity for MAO B at 30 min. Compounds that show unchanged, more and less selectivity (**1**, **3**, **4** and **6**) were studied in detail to investigate whether the rate constant for inactivation (k_{inact}) may account for the differences.

Kinetic parameters for inactivation of MAO A and MAO B by *cis*-cyclopropylamines

After pre-incubation with the enzyme, inhibition by all four selected compounds (**1**, **3**, **4** and **6**) was irreversible, and the activity was not restored by dilution into excess substrate. The kinetic parameters for the mechanism-based irreversible inactivation of MAO A, termed K_I (the concentration of inhibitor that produces half-maximal inactivation) and k_{inact} (the maximum rate of inactivation) [33], were determined from the time course of

Table 2. Irreversible inhibition of MAO after 30 min. The IC_{50} values are means ± SD from a three-parameter fit to at least 20 experimental values. The selectivity values were calculated as the ratio between the IC_{50} values for MAO A and those for MAO B.

Compound (racemic)	IC_{50} (μM)		Selectivity
	MAO A	MAO B	
1	6.12 ± 0.03	0.084 ± 0.05	73
2	21.6 ± 0.5	0.029 ± 0.002	745
3	10.8 ± 5.0	0.104 ± 0.017	104
4	0.175 ± 0.068	0.00470 ± 0.00005	37
5	3.00 ± 0.30	0.120 ± 0.004	25
6	2.55 ± 0.22	3.03 ± 0.20	0.8
7	1.75 ± 0.20	1.49 ± 0.22	1.1
Tranylcypromine	0.237 ± 0.061	0.0735 ± 0.0049	3.2
Clorgyline	0.00039	0.013	0.03
Deprenyl	0.635	0.00029	2190

Table 3. Parameters for inactivation by TCP and by 2-substituted *cis*-analogs. The selectivity values were calculated as the ratio between the k_{inact}/K_I values for MAO A and those for MAO B.

	MAO A			MAO B			
Compound	K_I (μM)	k_{inact} (min^{-1})	k_{inact}/K_I ($min \cdot mM^{-1}$)	K_I (μM)	k_{inact} (min^{-1})	k_{inact}/K_I ($min \cdot mM^{-1}$)	Selectivity
1	58.9 ± 7.4	0.167 ± 0.010	2.84	0.90 ± 0.18	0.016 ± 0.001	18	6.33
3	30.4 ± 9.5	0.028 ± 0.003	0.92	4.5 ± 0.6	0.037 ± 0.002	8.22	8.93
4	0.123 ± 0.051	0.052 ± 0.012	440	0.065 ± 0.012	0.104 ± 0.005	1600	3.64
6	16.6 ± 3.2	0.030 ± 0.003	1.81	17.5 ± 5.6	0.058 ± 0.007	3.31	1.83
TCP	7.7 ± 1.0	0.776 ± 0.034	101	3.8 ± 0.6	0.263 ± 0.005	69	0.68

inactivation, and are shown in Table 3. The K_I values for MAO A are all poor, with the exception of compound **4** (Table 3). The K_I values for MAO B indicate better discrimination of the structural variations in the compounds, with values of 0.07 μM for compound **4**, 0.9 μM for compound **1**, 5 μM for compound **3**, and 17 μM for compound **6**, presumably as a result of its narrower substrate cavity [34].

For MAO A, 2-methoxy-2-methylcyclopropylamine (**1**) gives the fastest rate of inactivation (k_{inact} = 0.17 min^{-1}), perhaps because its small size facilitates the correct orientation for its oxidation. Compounds **3**, **4** and **6** all inactivate MAO A at slower rates. Compounds **4** and **1** inactivate MAO A without generation of detectable H_2O_2. For MAO B, compound **1** gives the slowest inactivation (k_{inact} = 0.016 min^{-1}), whereas compound **4** gives the fastest (k_{inact} = 0.104 min^{-1}), but both generate H_2O_2 during pre-incubation with MAO B, indicating less tight coupling between oxidation and adduct formation.

The specificity constants (k_{inact}/K_I) provide a comparison of the efficiency of inactivation by each compound, and were used to calculate the selectivity for MAO B compared to MAO A (Table 3). The specificity constants for inactivation show that all compounds inactivate MAO B more efficiently than MAO A. Compared to TCP, compound **4** more effectively inactivates MAO A (four times better) and MAO B (> 20 times better). Compound **4** is also five times more selective for MAO B. The rate of inactivation (k_{inact}) by compound **4** is considerably lower than that by TCP (15-fold in MAO A and more than twofold in MAO B), but the low K_I values for compound **4**, particularly for MAO B (65 nM), offset the lower rates.

Characterization of the adduct formed between MAO A and compound 4

Covalent adducts with the N5 group of the FAD moiety of MAO, such as those formed after inactivation by clorgyline or deprenyl, are characterized by a distinctive change in the spectrum of MAO that differs from that seen for the C4a adduct [35–38]. The spectral changes that occur during adduct formation between MAO A and compound **4** were studied (Fig. 1). The MAO A flavin absorbance at 456 nm was bleached, indicating at

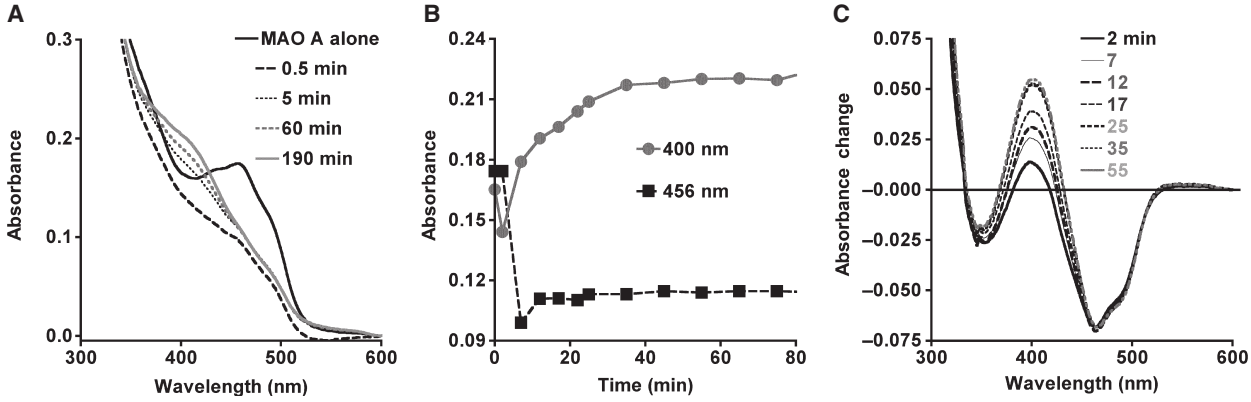


Fig. 1. MAO A inactivation by compound **4**: spectral changes during adduct formation. The original spectra (A) and the time course (B) show rapid reduction of MAO A (18 μM) by compound **4** (20 μM). Adduct formation at 400 nm proceeds more slowly than reduction of the flavin at 456 nm, as shown in (B) and (C). In (C), the spectrum for MAO A alone has been subtracted from that for the MAO A + **4** mixture. The incubation shown resulted in approximately 90% inactivation; a second addition of compound **4** was required for complete inactivation.

least partial reduction of the flavin (Fig. 1A). The absorbance at 400 nm increased, but this increase lagged behind the rapid flavin reduction (Fig. 1B,C). This suggests a slower chemical step for adduct formation after reduction of the flavin.

The spectral change during inactivation of MAO A by compound **4** has some similarity to that for N5 modification by clorgyline, but it has a less intense absorbance increase at 400 nm rather than the large 415 nm increase seen for N5 propargyl adducts with MAO A. However, the flavin remains reduced after denaturation with urea, suggesting that it is a stable adduct, unlike the labile adducts for *trans*-cyclopropylamines that are assumed to be at C4a [39], for which re-oxidation of the flavin is obvious after urea denaturation.

Small *cis*-cyclopropylamines occupy multiple positions in the active sites

With the exception of compound **4**, all the *cis*-cyclopropylamines are poor inactivators of MAO compared to TCP. Molecular modelling was used to compare how these small molecules interacted with the active sites of the two enzymes. Multiple poses were found for each compound at various locations in the active site and with varying orientations, as illustrated for selected enantiomers in Fig. 2. Interestingly, in MAO B, poses with energy minima for the smallest compound **1** are found in the entrance cavity, mid-cavity and near the flavin. The latter location (as shown in Fig. 2, top right) near the N5 of the flavin is required in order to inactivate MAO B. The amino acids surrounding the (1*R*,2*S*) enantiomer of compound **1** near the flavin are shown in Fig. 3A. The entrance-cavity pose (Fig. 3B) was found in only two of the ten runs for the (1*R*,2*S*) enantiomer of compound **1**, and gave an energy of -3.74 kcal·mol^{-1}, and in only one run for the (1*S*,2*R*) enantiomer of compound **1**, with an energy of -4.33 kcal·mol^{-1}. This entrance-cavity location (Fig. 3B) is similar to that of 2-(2-benzofuranyl)-2-imidazoline (2-BFI) bound in the imidazoline I_2 site of MAO B, which has been characterized in binding

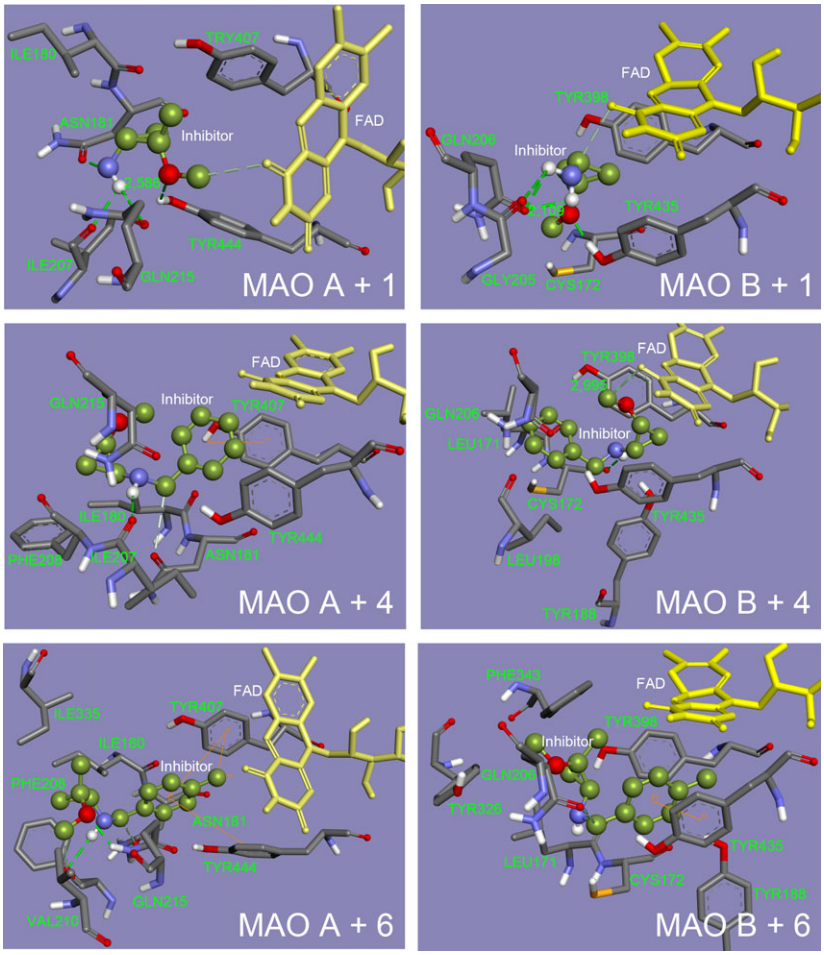


Fig. 2. Docking poses for *cis*-cyclopropylamines in the active sites of MAO A (PDB ID 2Z5X) and MAO B (PDB ID 2V5Z). Docking simulations were performed using AutoDock 4 [30] (carbons in green) and AutoDock Vina [31] (carbons in white); visualization was performed using PyMOL. Compound **1** with MAO A (top left) was 1*R*,2*S*; for all the others, the enantiomer was 1*S*,2*R*. Optimum poses were defined by the steric position necessary for interaction between the flavin N5 and the inhibitor.

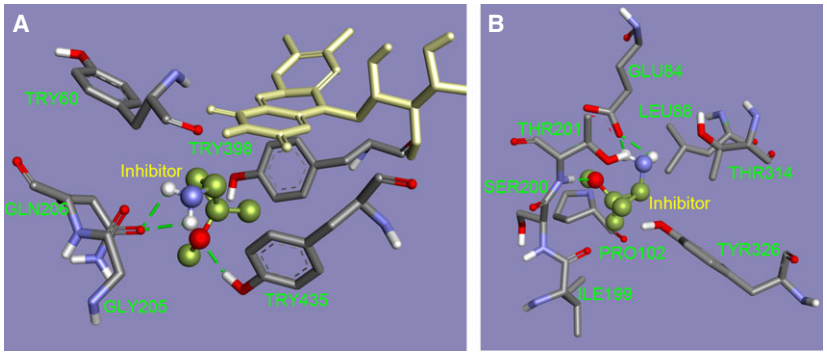


Fig. 3. Amino acids surrounding the (1*R*,2*S*) enantiomer of compound **1** either near the flavin or in the imidazoline I_2 site of MAO B. Docking of compound **1** (carbons in green) to MAO B (PDB ID 2V5Z) was performed using AutoDock 4 [30] (10 runs). Various poses were found: (A) bound near the flavin (yellow); (B) bound near the I_2 site within the entrance cavity (in two of ten poses). Hydrogen bonding interactions are shown as green dashes.

studies and demonstrated by crystallography [26,27]. However, unlike the I_2 ligands, which have nanomolar affinity, the predicted K_i for binding of compound **1** at this location is in the micromolar range.

The low probability of binding close to the flavin may also explain the low rate of inactivation (Table 3: k_{inact} is 0.016 min^{-1} for compound **1** with MAO B compared to 0.263 min^{-1} for TCP). The introduction of a benzyl substituent attached to the nitrogen improves the affinity for MAO A but not for MAO B, and increases the rate of inactivation (from 0.016 min^{-1} for compound **1** to 0.104 min^{-1} for compound **4**) for MAO B but not for MAO A (Table 3).

Discussion

The inhibition of MAOs by cyclopropylamines is well established, and is exemplified by the clinical drug tranylcypromine (TCP). In TCP, the cyclopropylamine has a *trans* relationship. The phenyl substituent is considered to facilitate ring opening of the cyclopropyl ring by stabilizing radical-type intermediates [40,41]. This has led to considerable interest in *trans*-substituted tranylcypromine analogues as MAO inhibitors, as well as inhibitors of the recently identified epigenetic enzyme LSD1. Here, we have investigated novel cyclopropylamines with the less common *cis* relationship. Furthermore, our compounds do not contain a phenyl ring as the cyclopropane substituent, but instead have an intervening alkoxy group. These new cyclopropylamine derivatives were found to be inactive against LSD1 at concentrations of 25 μM. For MAO, although the initial binding is micromolar, these *cis*-cyclopropylamines inhibit MAO A and MAO B irreversibly at sub-micromolar levels, making them selective for MAO without an effect on LSD1. The best inhibition was observed with MAO B. Compound **4** is > 20 times more effective than TCP, so this di-substituted cyclopropylamine (secondary amine) may be studied as a lead compound for selective inhibitors of MAO B that do not inhibit LSD1.

Both the primary amines (compounds **1** and **2**) and the secondary amines (compounds **3**–**7**) inactivate both MAO isoenzymes, confirming that *cis*-cyclopropylamines interact with MAO to produce reactive products that form a covalent bond to the flavin. The spectrum obtained with MAO A during inactivation and the stability of the adduct formed even after unfolding suggest that the modification by *N*-benzyl-2-methoxycyclopropylamine (**4**) may have occurred at the N5 of the flavin. Although the crystal structure of MAO B after TCP inactivation shows C4a modification, the structure of LSD1 shows that TCP modifies the N5 of the flavin [5]. Recent crystal structures have revealed that some 1-substituted cyclopropylamines formed different adducts with LSD1 at C4a, N5, or bridging both, probably via a radical mechanism [9]. Others have also described the formation of a cyclic N5 and C4a adduct [6–8,13,22,42,43], so perhaps both are possible even if only one form crystallizes. The spectrum of the adduct is not definitively that of an N5 adduct such as is formed with clorgyline or deprenyl [35,44], so only the stability [39] favours this interpretation for *cis*-cyclopropylamine (**4**) . This study does not address the structure of the adduct nor the mechanism of adduct formation, but the lack of H_2O_2 production during inactivation of MAO A suggests that the radical mechanism proposed by others must be considered [9,45].

In conclusion, *cis*-*N*-benzyl-2-methoxycyclopropylamine (compound **4**) is an irreversible MAO inhibitor with an IC_{50} of 5 nM for MAO B, 170 nM for MAO A, and no activity on LSD1.

Experimental procedures

Compounds

cis-isomers of primary and secondary cyclopropylamines with an alkoxy group at the 2-position of the cyclopropyl ring replacing the more common phenyl substitution were synthesized as previously described [23].

Enzyme activity

Initial activity for membrane-bound MAO (Sigma-Aldrich, St Louis, MO) was determined from the production of hydrogen peroxide, measured using horseradish peroxidase to couple hydrogen peroxide formation to the production of fluorescent compound, resorufin [46–48]. For the reversible inhibition, IC_{50} values were determined from the rates obtained with varied inhibitor concentrations in the presence of 2.5 × K_M substrate concentration with the enzyme added last. Under the conditions used, the K_M for tyramine with MAO A was 0.4 mM and that with MAO B was 0.16 mM. Data are expressed as means standard deviation (SD) obtained by fitting the data (at least 20 points) to the appropriate three-parameter equation using GraphPad PRISM version 4 (GraphPad Software, La Jolla, CA, USA; www.graphpad.com). At least two separate determinations were made for each value reported.

The IC_{50} values for the irreversible inactivation of MAO A and MAO B were determined from the activity (assayed as above) remaining after 30 min of incubation of the enzyme and inhibitor. Inactivation parameters (K_I and k_{inact}) were determined as described previously [33,38].

Molecular docking

Molecular models of the *cis*-cyclopropylamine inhibitors were built and optimized using ArgusLab 4.0.1 (ArgusLab, Seattle, WA, USA; http://www.arguslab.com/). Protein structures for MAO A (PDB ID 2Z5X) and MAO B (PDB ID 2V5Z) were minimized using Accelrys 6.0 (Biovia, San Diego, CA, USA) with a CHARMM force field and simulated annealing. All .pdbqt, .gpf and .dpf files were created using AutoDockTools software [30] (http://autodock.scripps.edu/), using a Lamarckian genetic algorithm, Docking was achieved using AutoDock 4 [30] and AutoDock Vina [31] (http://autodock.scripps.edu/). All comparisons were performed using PyMOL (https://www.pymol.org/).

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Author contributions

RRR, AG, SM and TM planned the experiments, TM, KY, MTB and RRR performed the experiments, TM, RRR, KY, SM and AG analyzed the data, ES, NDK and SM contributed essential reagents, and RRR wrote the paper with the assistance of all authors.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1. Binding energy and predicted K_i values for both enantiomers of the *cis*-cyclopropylamines with MAO A and MAO B.