Inactivation of Purified Human Recombinant Monoamine Oxidases A and B by Rasagiline and Its Analogues

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The inactivation of purified human recombinant monoamine oxidases (MAO) A and B by rasagiline [*N*-propargyl-1(*R*)-aminoindan] and four of its analogues [*N*-propargyl-1(*S*)-aminoindan (*S*-PAI), 6-hydroxy-*N*-propargyl-1(*R*)-aminoindan (*R*-HPAI), *N*-methyl-*N*-propargyl-1(*R*)-aminoindan (*R*-MPAI), and 6-(*N*-methyl-*N*-ethyl carbamoyloxy)-*N*-propargyl-1(*R*)-aminoindan (*R*-CPAI)] has been investigated. All compounds tested, with the exception of *R*-CPAI, form stoichiometric *N*(5) flavocyanine adducts with the FAD moiety of either enzyme. No H_2O_2 is produced during either MAO A or MAO B inactivation, which demonstrates that covalent addition occurs in a single turnover. Rasagiline has the highest specificity for MAO B, as demonstrated by a 100-fold higher inhibition potency (k_{inact}/K_i) compared to MAO A, with the remaining compounds exhibiting lower isozyme specificities. MAO B and MAO A are more selective for the *R*-enantiomer (rasagiline) compared to the *S*-enantiomer (*S*-PAI) by 2500-fold and 17-fold, respectively. Differences in UV/vis and CD spectral data of the complexes of the studied compounds with both MAO A and MAO B are interpreted in light of crystallographic data of complexes of MAO B with rasagiline and its analogues (Binda, C.; et al. *J. Med. Chem.* **2004**, *47*, 1767−1774.

Introduction

Monoamine oxidase (MAO) A and MAO B are 70% identical, ~60 kDa outer mitochondrial membrane bound flavoenzymes responsible for oxidative deamination of neurotransmitters and dietary amines.[1] Inhibitors of MAO A are clinically used antidepressants,[2] whereas MAO B inhibitors are used for the treatment of Parkinson's disease.[3] However, nonselective, irreversible MAO inhibitors have serious pharmacological side effects ("cheese effect") induced by intake of foods rich in arylalkylamines (cheese, chocolate, and wine) that are typically metabolized by MAO A in the gastrointestinal tract.[4] Development of MAO B or organ-specific drugs is an important goal in order to maximize the beneficial effects of therapy and to minimize its side effects.

The inhibition of rat and human brain MAO A and MAO B by rasagiline and a large number of its analogues has been reported.[5−7] 6-(*N*-Methyl-*N*-ethylcarbamoyloxy)-*N*-propargyl-1(*R*)-aminoindan (*R*-CPAI, TV3326) is under development for the treatment of Alzheimer's disease. It combines the *N*-methyl-*N*-ethylcarbamoyloxy pharmacophore to inhibit acetylcholinesterase, with a propargylamine moiety that adds neuroprotective and MAO inhibitory properties.[8] The latter is based on rasagiline (*N*-propargyl-1(*R*)-aminoindan), which has recently successfully completed phase III clinical trials for the treatment of Parkinson's disease. *R*-CPAI itself is not a potent MAO inhibitor, but one of its metabolites, 6-hydroxy-*N*-propargyl-1(*R*)-aminoindan (*R*-HPAI), is a nonselective inhibitor of both MAO A and B in vitro. As a result, *R*-CPAI is a unique brain-selective irreversible MAO inhibitor, thus avoiding the cheese effect.[9] *N*-Propargyl-1(*S*)-aminoindan (*S*-PAI, TVP1022) and *N*-methyl-*N*-propargyl-1(*R*)-aminoindan (*R*-MPAI) are additional analogues of rasagiline with interesting pharmacological properties.[7]

With the development of expression systems that permit the production of large quantities of purified human MAO A and MAO B,[10,11] a careful study of the interaction of these MAO inhibitors with the purified enzymes would be valuable as a supplement to the structural studies on MAO B.[12] In this paper we report results of kinetic and spectroscopic analyses of the inhibition reaction of purified human recombinant MAO A and MAO B by rasagiline and four of its derivatives. The results of these biochemical studies are interpreted in light of structural information obtained from crystallographic studies of MAO B inactivated with rasagiline and its analogues.[12]

Results

UV/Vis Spectroscopy. When purified human recombinant MAO A or MAO B is incubated with rasagiline or its analogues (Scheme 1), the UV/vis absorption spectra of the enzymes are irreversibly altered (Figure 1). The flavin absorbance at 450 nm is bleached, and

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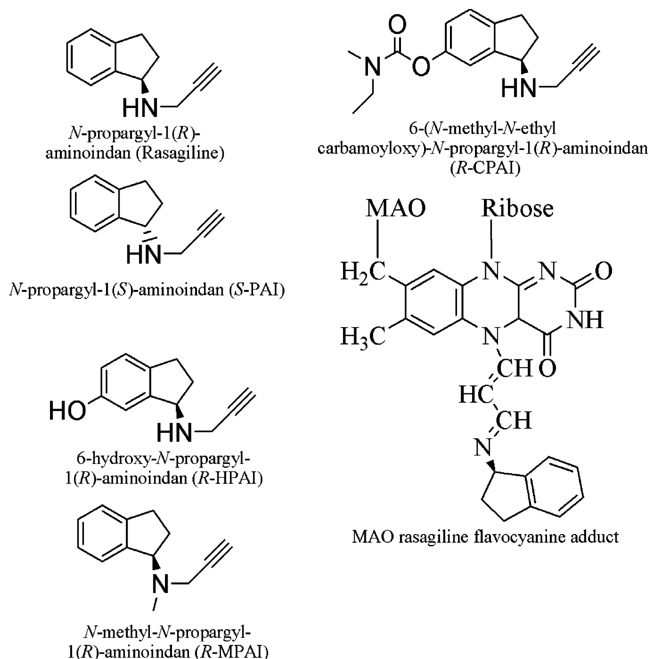
Scheme 1. Structures of Rasagiline and Its Analogues



absorption properties corresponding to the formation of flavocyanine adducts (Scheme 1) appear, with the observed absorption maxima for all compounds tested ranging from 411 to 418 nm (Figure 1). The extinction coefficients for the observed adducts range from 17 000 to 21 000 M^{-1} cm^{-1} for MAO B and from 21 000 to 26 000 M^{-1} cm^{-1} for MAO A. Any small residual absorbance at 450 nm (Figure 1) is due to incomplete inactivation of the enzymes and/or due to the presence of small amounts of inactive enzyme. These spectral changes are similar to those reported for the clorgyline and pargyline flavocyanine adducts of human MAO A and MAO B, respectively.[10,11] The *S*-PAI and *R*-HPAI adducts with MAO B show broader absorption peaks with additional maxima at 395 nm. These spectral properties are suggested to be due to exciton splitting originating from interactions between the aminoindan ring of the flavocyanine adducts with active site tyrosyl residues (Tyr326 for *S*-PAI and Tyr435 for *R*-HPAI), as seen in crystal structures of MAO B inhibitor complexes.[12]

Table 1. Summary of ESI-MS Analysis of Rasagiline and *S*-PAI MAO A and MAO B Flavocyanine Adducts

	MAO A mass		MAO B mass	
	obsd	calcd (1×)[a]	obsd	calcd (1×)[a]
rasagiline	60674 ± 6	60683	59621 ± 23	59646
S-PAI	60680 ± 12	60683	59638 ± 12	59646

[a] MAO A and MAO B masses were calculated assuming the presence of only one rasagiline or *S*-PAI flavocyanine adduct per enzyme molecule.

Electrospray Ionization Mass Spectrometry (ESI-MS) Analysis. The reaction stoichiometries of rasagiline analogues with MAO A and MAO B were determined by ESI-MS analysis of the inactivated enzymes (data not shown). Both MAO A and MAO B form covalent adducts with rasagiline or with *S*-PAI in a 1:1 molar stoichiometry as summarized in Table 1. The observed masses of enzymes inactivated with rasagiline and *S*-PAI are in agreement with the covalent addition of a single molecule of rasagiline or *S*-PAI to MAO A and MAO B, respectively. Precursor ion (PO_3^-) scanning MS analysis of isolated FAD-containing peptides shows that both rasagiline and *S*-PAI are covalently attached to the FAD-containing peptides of MAO A and MAO B (data not shown). The major species (m/z = 719.3) corresponds to the doubly negatively charged form of the modified FAD peptides in all experiments (M_w = 1440.6 Da, calculated M_w = 1440.3 Da). The mass difference between the unmodified FAD-containing peptide and the peptide modified with rasagiline or *S*-PAI (172.2 Da) is consistent with the formation of the *N*(5)-flavocyanine adduct (Scheme 1) as proposed by Maycock et al.[13] and shown for the acetylenic inhibitor pargyline.[14] These results are also consistent with the differences in the absorption spectra of the native and inactivated enzymes (see above). Similar experiments were performed with *R*-MPAI-inactivated MAO A and MAO B (data not shown), where a major species (m/z = 726.1) corresponds to a doubly negatively charged modified FAD peptide (M_w = 1455.2 Da, calculated M_w = 1455.3 Da). Considerable amounts of unmodified FAD-containing peptide (m/z = 633.5), presumably arising from hydrolytic cleavage of the rasagiline chain followed by air oxidation during denaturation and proteolysis, are also observed in all experiments.[15] An additional species (m/z = 660.4), most likely

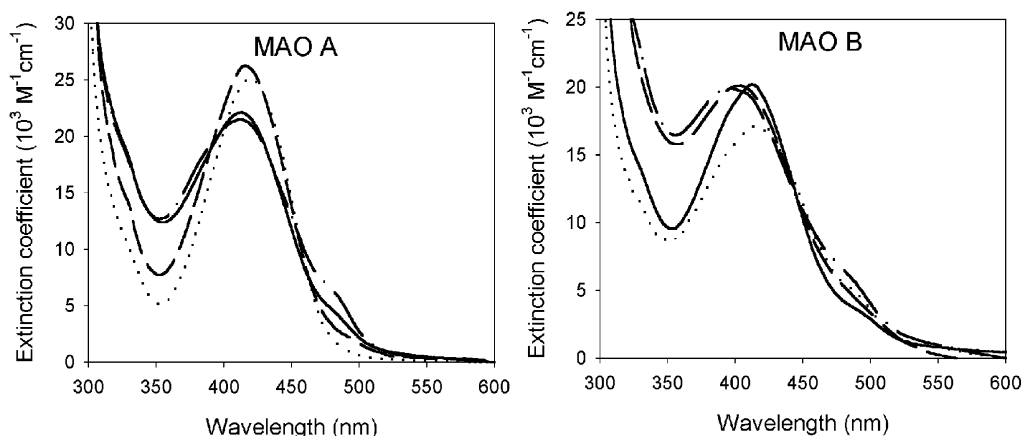


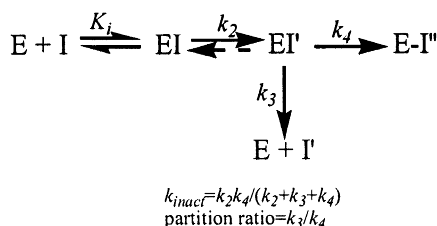
Figure 1. UV/vis spectra of flavocyanine adducts of MAO A and MAO B inactivated with rasagiline and its analogues. Both MAO A (left panel) and MAO B (right panel) were incubated with excess rasagiline (solid line), *S*-PAI (dash−dot line), *R*-HPAI (dashed line), and *R*-MPAI (dotted line). UV/vis spectra corresponding to the endpoints of inactivation reactions are shown.

Table 2. Inhibition Data of Purified Human Recombinant MAO A and MAO B by Rasagiline and Its Analogues

	K_i (μM)		IC_{50} (μM)		inactivation[a] k_{inact} (10^{-3} min^{-1})		A_{450} decay[a] k_{inact} (10^{-3} min^{-1})		A_{415} rise[a] k_{inact} (10^{-3} min^{-1})	
	MAO A[b]	MAO B[c]	MAO A	MAO B	MAO A[b]	MAO B[c]	MAO A	MAO B	MAO A	MAO B
rasagiline	9.7	0.7	0.7[e]	0.014[e]	7.2	53.3	12.4	57.8	6.7	53.3
S-PAI	112	127	17.7[e]	28.7[e]	4.1	3.2	5.8	7.5	2.4	1.4
R-HPAI	1.9	17	0.3[f]	0.2[f]	1.9	15.8	1.9	17.8	1.6	11.9
R-MPAI	4.9[d]	0.6[d]	0.003[g]	0.01[g]	3,500	3,500	3,500	2,300	1,400	1,400
R-CPAI	227	213	85[f]	120[f]	ND[h,i]	ND[h,i]	ND[i]	ND[i]	ND[i]	ND[i]

[a] Apparent rates of inactivation were measured at 15 °C in 50 mM potassium phosphate, 0.8% octyl glucoside, pH 7.5. For MAO A (10 μM) inactivation, 100 μM *R*-MPAI, 1 mM rasagiline and *R*-HPAI, and 10 mM *S*-PAI and *R*-CPAI were used. For MAO B (10 μM) inactivation, 100 μM rasagiline and *R*-MPAI, 1 mM *S*-PAI and *R*-HPAI, and 10 mM *R*-CPAI were used. [b] MAO A activity was measured using a kynuramine assay[10] at 25 °C. [c] MAO B activity was measured using a benzylamine assay[11] at 25 °C. [d] Since partial inactivation of both enzymes was observed during the time course of kinetic assays when *R*-MPAI was used as an inhibitor, K_i values for *R*-MPAI were estimated on the basis of the initial rates (linear portion of the UV traces) using low concentrations of *R*-MPAI. [e] Human brain MAO.[16] [f] From rat brain.[7] [g] From rat brain.[6] [h] Both enzymes precipitated upon 20 h of incubation with 10 mM *R*-CPAI. [i] ND: not determined.

Scheme 2. Kinetic Description of Mechanism-Based Inactivation of Enzymes[a]



[a] E, enzyme; I, inhibitor; EI, initial enzyme inhibitor complex; EI′, enzyme complex with reduced inhibitor; I′, reduced inhibitor; E-I″, enzyme inactivated by reduced inhibitor.

arising from hydrolysis of the rasagiline *N*(10) imine bond, was also observed in all experiments, in agreement with previous observations[13] although lower amounts were present in the case of *R*-MPAI, consistent with the expected slower rate of hydrolysis of tertiary imines compared to secondary imines.

Kinetic Analysis. Since the formation of the flavocyanine adduct of MAO A and MAO B with rasagiline and its analogues, with the exception of *R*-MPAI (Scheme 1), proceeds slowly, the inhibition constants for these compounds could be determined by competitive substrate inhibition (Scheme 2, Table 2). All compounds studied were found to competitively inhibit the catalytic activity of both MAO A and MAO B. *R*-HPAI exhibits the highest affinity toward MAO A (K_i = 1.9 μM) followed by rasagiline (K_i = 9.7 μM), *S*-PAI (K_i = 112 μM), and *R*-CPAI (K_i = 227 μM). In contrast, rasagiline exhibits the highest affinity toward MAO B (K_i = 0.7 μM) followed by *R*-HPAI (K_i = 17 μM), *S*-PAI (K_i = 127 μM), and *R*-CPAI (K_i = 213 μM). The K_i values for *R*-MPAI were estimated on the basis of initial rates (linear portions of UV traces) obtained with low concentrations of *R*-MPAI (Table 2). N-methylation of rasagiline does not have a major effect on initial binding of *R*-MPAI to either enzyme. Hydroxylation of the 6 position of the indan ring leads to a 5-fold increase in binding affinity to MAO A and a 20-fold decrease in binding affinity to MAO B. Thus, *R*-HPAI is more selective toward MAO A, whereas rasagiline is more selective toward MAO B. The *R* configuration in position 1 of the indan ring (rasagiline) results in 100-fold higher affinity for MAO B and 10-fold higher affinity for MAO A compared to the *S* configuration in the same position (*S*-PAI). Both *S*-PAI and *R*-CPAI are weak inhibitors with similar affinities for either enzyme.

Table 3. Inhibition Potencies for MAO A and B Inhibition by Rasagiline Analogues

	inactivation k_{inact}/K_i (10^3 M^{-1} min^{-1})		A_{450} decay k_{inact}/K_i (10^3 M^{-1} min^{-1})		A_{415} rise k_{inact}/K_i (10^3 M^{-1} min^{-1})	
	MAO A	MAO B	MAO A	MAO B	MAO A	MAO B
rasagiline	0.7	76.2	1.3	82.5	0.7	76.2
S-PAI	0.04	0.03	0.05	0.06	0.02	0.01
R-HPAI	1.0	0.9	1.0	1.0	0.8	0.7
R-MPAI	714	5833	714	3833	285	2333

The rates of inactivation (k_{inact}, Scheme 2) of both enzymes by rasagiline analogues were estimated by following absorbance changes at 450 nm (flavin bleaching) and 415 nm (flavocyanine adduct formation) and by activity decay as a function of time (Figure 2, Table 2). *R*-MPAI inactivates both enzymes with the fastest rate. The relative rates of MAO B inactivation are faster than those for MAO A with the exception of *S*-PAI, which inactivates MAO A more quickly than MAO B. These results are consistent with the results of previous studies with rat brain homogenate MAO A and MAO B preparations that showed that *N*-methyl-substituted derivatives are more effective but less specific inhibitors of both enzymes in comparison with their unsubstituted analogues.[7] The inhibition potencies (k_{inact}/K_i) for all studied compounds are listed in Table 3. *S*-PAI is the least effective inhibitor for both enzymes with 2500-fold lower inhibition potency for MAO B when compared to rasagiline; both the weaker binding affinity (~100-fold) and slower rate of inactivation (~25-fold) contribute to the reduced level of *S*-PAI inhibition. The *R*-enantiomer is also a better inhibitor of MAO A (17-fold higher inhibition potency, 10-fold reduction of binding affinity, and 2-fold slower rates of inactivation) relative to the *S*-enantiomer. The remaining compounds do not show specificity for either MAO A or MAO B.

It is noteworthy that the rate of formation of the flavocyanine adduct is slower by a factor of 2 compared to that of flavin bleaching during inactivation of MAO A by *S*-PAI and *R*-MPAI (Table 2). Likewise, the rates of flavin bleaching (measured at 450 nm) during inactivation of MAO B with *S*-PAI and *R*-MPAI are respectively 5-fold and 2-fold faster than the rate of flavocyanine adduct formation (measured at 415 nm). These data suggest that flavin reduction occurs prior to flavocyanine adduct formation.

The published IC_{50} values[6,7,16] (Table 2) for these inhibitors correlate better with inhibition potencies

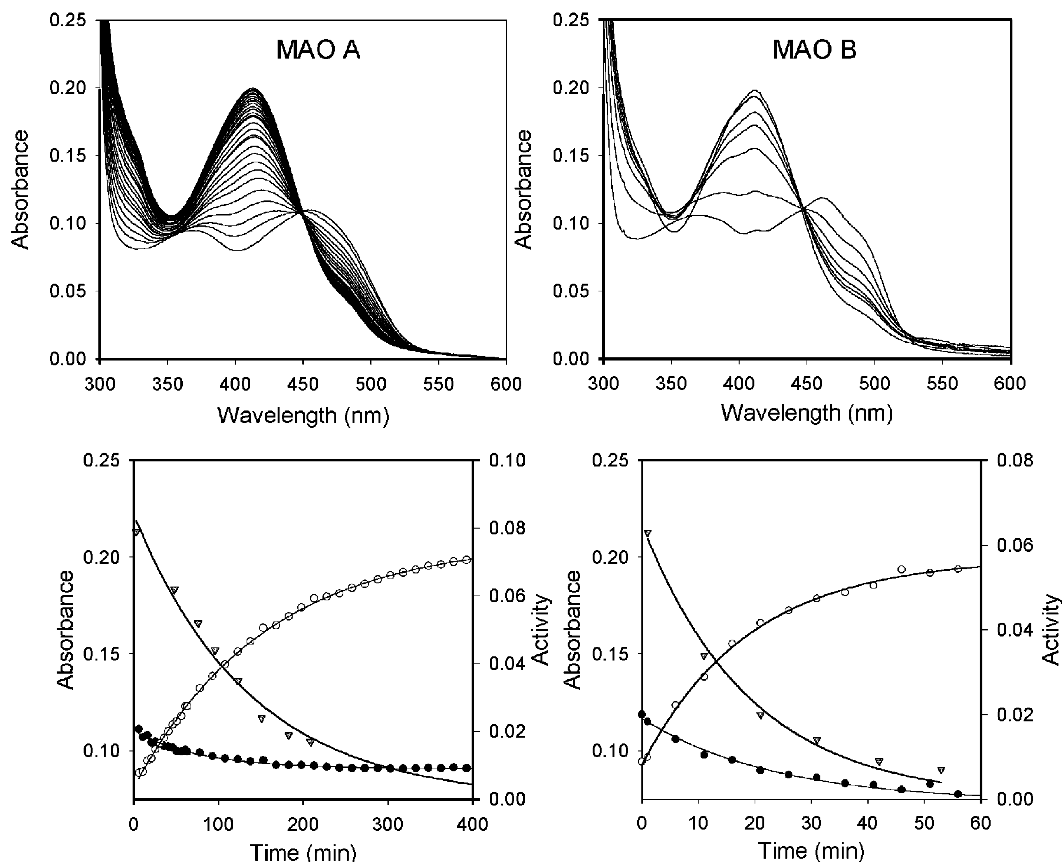


Figure 2. Time course of rasagiline inactivation of MAO A and MAO B. MAO A was incubated with 100-fold excess of rasagiline at 15 °C, and the resulting UV/vis spectrum was recorded every 15 min (top-left panel). MAO B was incubated with 10-fold excess of rasagiline at 15 °C, and the resulting UV/vis spectrum was recorded every 10 min (top-right panel). Reduction of flavin (at 455 nm, filled circles), formation of flavocyanine adduct (at 415 nm, empty circles), and remaining activity (triangles) for both enzymes are plotted as a function of time (bottom panels). Solid lines represent single-exponential fits of these time dependencies.

expressed in terms of k_{inact}/K_i than with either K_i or k_{inact} alone, suggesting that both binding affinities and inactivation rates need to be considered in inhibitor design. These data also suggest that for some of the slower inhibitors (low k_{inact}) preincubation times longer than 60 min period typically used for IC_{50} measurements[5,7,16] are required.

The efficiencies of a number of mechanism-based inhibitors of enzymes are related to the partition ratio (i.e., the ratio of product release to inactivation) as shown in the mechanism of Scheme 2.[17] To test whether such a partition ratio is relevant to the inhibition of MAO A and MAO B by rasagiline and its analogues, the production of hydrogen peroxide during the inhibition reaction was monitored to detect any catalytic turnovers. No hydrogen peroxide production was detected (data not shown) with either MAO A or MAO B on incubation with any of the analogues tested. These data demonstrate that no catalytic turnover is necessary for MAO inactivation by these compounds, in agreement with the observation that stoichiometric amounts of *R*-MPAI are sufficient for full inactivation of either MAO A or MAO B (data not shown). The partition ratio for this reaction therefore approaches zero. These observations are consistent with previous reports showing that pargyline (also a propargylamine) inhibits MAO B in stoichiometric amounts even under anaerobic conditions.[18,19]

Circular Dichroism (CD) Spectroscopy. To compare conformational rigidities of rasagiline and its analogues bound to the active site of MAO A and MAO B, the near-UV circular dichroism spectra of the inactivated enzymes were acquired (Figure 3). In all cases, any differences in flavocyanine optical activities are expected to be due to the asymmetric enzyme environment because no additional optically active centers are introduced. The CD spectrum of the *R*-MPAI flavocyanine adduct of MAO B is similar to that of the pargyline adduct. Both spectra show major negative dichroic peaks at 330 nm and broad, low-intensity negative dichroic peaks at 405 nm. The CD spectrum of the *R*-MPAI adduct is more intense relative to the CD spectrum of the pargyline adduct. Although the CD spectra of rasagiline and *S*-PAI adducts with MAO B showed the same two negative dichroic peaks, the relative intensities of the peaks were reversed and their positions were slightly shifted in the *S*-PAI adduct CD spectrum (345 and 398 nm) compared to that of the rasagiline adduct (335 and 408 nm). The overall intensity of the *S*-PAI adduct CD spectrum is higher (1.5-fold) than that of the rasagiline adduct. Similar results are observed for MAO A although there are no shifts in the peak positions of the *S*-PAI adduct CD spectrum and the overall intensities of the CD spectra of rasagiline and *S*-PAI are lower than in those observed with MAO B. The intensities of the CD spectra of clorgyline and pargyline adducts of MAO A and MAO B, respectively, are comparable. The intensities of CD spectra of rasagiline and *S*-PAI adducts with MAO B suggest that the *S*-enantiomer flavocyanine adduct conformational

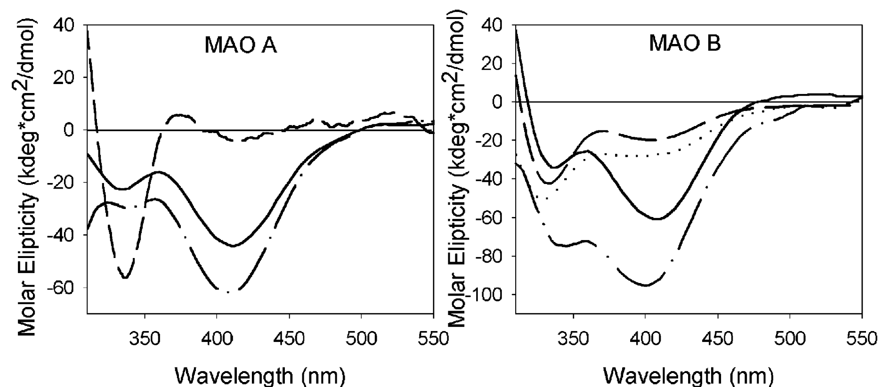


Figure 3. Near-UV and visible CD spectra of rasagiline–MAO flavocyanine adducts. CD spectra of MAO A inactivated with *S*-PAI (dash–dot line), rasagiline (solid line), and clorgyline (dashed line) are shown in the left panel. CD spectra of MAO B inactivated with *S*-PAI (dash–dot line), rasagiline (solid line), pargyline (dashed line), and *R*-MPAI (dotted line) are shown in the right panel.

environment is more rigid (higher intensity) than the environment of the *R*-enantiomer. These data are best explained by a close range interaction of the aminoindan ring of the *S*-PAI with Tyr326, as seen in the crystal structure of the *S*-PAI MAO B complex.[12] The dipole–dipole interaction between Tyr326 and the aminoindan ring of *S*-PAI is suggested to be responsible for the shifts in the positions of CD spectral peaks for the *S*-enantiomer.[20]

Discussion

The agreement between the results obtained with purified human enzymes in this study and the earlier results obtained with rat and human brain mitochondrial membrane bound enzymes[6,7,16] provides further evidence that the structures of monoamine oxidase A and B in solution and in the outer mitochondrial membrane are similar, consistent with a previous report.[21]

Since the structural data for MAO B complexes with these inhibitors are available,[12] attempts were made to correlate the kinetic data with the structural data for MAO B and to predict differences between the MAO A and MAO B active site structures by comparing the kinetic data for the two enzymes. In particular, major differences between rasagiline and *S*-PAI inhibition of MAO B correlate with the different orientation of the aminoindan ring in the *S*-enantiomer relative to the *R*-enantiomer. The side chain of Tyr326 in the active site of MAO B has to move ~1 Å in order to accommodate the *S*-enantiomer,[12] which results in a 20-fold decrease in k_{inact} for the *S*-enantiomer as well as changes in the UV/vis and CD spectra (Figures 1 and 3). In contrast, there is only a ~2-fold decrease in k_{inact} for the *S*-enantiomer and no significant changes in the UV/vis and CD spectra between the *R*- and *S*-enantiomers for MAO A, suggesting that no major rearrangement of the active site is necessary and that there are no close range dipole–dipole interactions with the aminoindan ring of *S*-PAI. This view is supported by the fact that Tyr326 in MAO B corresponds to Ile335 in MAO A. This substitution results in more flexibility for the bound aminoindan ring with no possibility for dipole–dipole interactions because the Ile side chain is not aromatic.

A comparison of crystallographic and kinetic data also has implications for the interaction with *R*-HPAI. This compound has a 10-fold higher binding affinity toward MAO A compared to MAO B but a 10-fold lower inactivation rate resulting in approximately equal inhibition potencies. The hydroxy group of this compound is hydrogen-bonded to Cys172 and Tyr435 in MAO B.[12] Cys172 in MAO B corresponds to Asn182 in MAO A. The ability of Asn to form a strong H bond with the hydroxyl of the aminoindan ring could explain the increase in binding affinity for *R*-HPAI in MAO A. Asn is also a larger amino acid than Cys, which is likely to position the propargyl chain of the inhibitor in an orientation that is not as favorable for oxidation by enzyme-bound FAD. The strong binding affinity of MAO A toward 6-HPAI is of interest because this compound is very similar to serotonin (a MAO A specific substrate), which has the hydroxyl group in the same position on its indole ring. The aminoethyl group of serotonin is smaller than the propargylamine group of *R*-HPAI. When Tyr444 was replaced with Phe in MAO A[22] (Tyr435 in MAO B), the side chain of which is H-bonded with the hydroxyl group of *R*-HPAI,[12] a loss of MAO A affinity for serotonin was observed, further suggesting the similarities between serotonin and *R*-HPAI interactions with MAO A.

Conclusions

Data for the inhibition of purified recombinant MAO A and MAO B by rasagiline and its four analogues correlate well with previous data in the literature on the inhibition of mitochondrial monoamine oxidases from human and rat tissues by this class of inhibitors. Analyses of these inhibition data from well-defined systems (purified enzymes, in contrast to crude tissue homogenates) allow for a more detailed description of inhibitor binding, reaction rates, and spectroscopic properties of these clinically relevant inhibitors. A comparison of kinetic and spectroscopic data obtained for both enzymes with the X-ray crystallographic data for MAO B confirms striking differences between MAO A and MAO B active sites, as reflected in the different potencies of these specific inhibitors. These striking differences are related to amino acid substitutions between MAO A and MAO B and need to be considered for development of isoenzyme-specific inhibitors.

Materials and Methods

Rasagiline and its analogues were synthesized at TEVA Pharmaceuticals. All other reagents used were purchased from Sigma-Aldrich. Human recombinant MAO A and MAO B were expressed in *Pichia pastoris* and purified as described previously.[10,11] Both enzymes (1−2 mg) were desalted from a glycerol stock solution using a G-25 (fine) Sephadex column (1 cm × 20 cm, Sigma) in 50 mM potassium phosphate buffer, pH 7.5, containing 0.8% (w/v) octyl glucoside before use.

MAO A and MAO B Activity Measurements. MAO A activity was determined spectrophotometrically (316 nm) using kynuramine as substrate in 50 mM potassium phosphate buffer, pH 7.5, containing 0.5% (w/v) reduced Triton X-100 at 25 °C. MAO B activity was determined spectrophotometrically (250 nm) using benzylamine as substrate in 50 mM hepes buffer, pH 7.5, containing 0.5% (w/v) reduced Triton X-100 at 25 °C.

K_i Determinations. Competitive K_i values for both enzymes were determined by measuring initial rates of substrate oxidation in the presence of varying concentrations of inhibitor (six different substrate concentrations and four different inhibitor concentrations were used; all assays were performed in duplicate). Apparent K_m values for each inhibitor concentration (slopes of double reciprocal plots) were plotted as a function of inhibitor concentration, and the K_i values were determined.

Reaction Rates Determination (k_{inact}). Enzymes were incubated with an inhibitor in a quartz cuvette (0.5 or 1.0 mL) inserted in a Cary 50 UV/vis spectrophotometer (Varian) in 50 mM potassium phosphate buffer, pH 7.5, containing 0.8% (w/v) octyl glucoside. The temperature was held at 15 °C using a Peltier device. Spectral scans were taken at defined time intervals (depending on the reaction rate). Aliquots were withdrawn at specified times and tested for enzyme activity as described above. The apparent rate constants were determined from single-exponential fits of time dependence of absorbance changes at 450 nm (oxidized flavin), 415 nm (flavocyanine adduct), and remaining catalytic activities. The inhibitor potency (efficiency) can be expressed as k_{inact}/K_i, similar to the catalytic efficiency for substrate that is expressed as k_{cat}/K_m.[17,23]

Hydrogen Peroxide Detection. Formation of hydrogen peroxide during MAO A inactivation by rasagiline, *R*-MPAI, and *S*-PAI as well as MAO B inactivation by *R*-MPAI was measured spectrophotometrically ($\epsilon_{498} = 4654\ M^{-1}\ cm^{-1}$) using a peroxidase coupled assay as described previously.[24] Spectral scans of MAO A and MAO B that were inactivated by rasagiline and its analogues were compared to spectral scans performed in the presence of peroxidase buffer (final concentrations: 200 μM vanillic acid, 100 μM 4-aminoantipyrine, 0.8 U/mL horseradish peroxidase in 50 mM potassium phosphate, pH 7.5).

Electrospray Mass Spectrometry Analysis. Electrospray ionization mass spectrometry (ESI-MS) analyses of MAO inhibitor adducts were performed after microbore HPLC desalting as described earlier.[25,10,11] To determine the site for covalent inhibitor binding, enzyme−inhibitor adducts (300 μg) were precipitated on ice with 5% (w/v) trichloroacetic acid, washed with ice-cold water, and resuspended in 0.1 M ammonium bicarbonate. Chymotrypsin (10 μg) and trypsin (10 μg) were added, and the sample was digested in the dark for 15 h at 37 °C. A C2 reversed-phase solid-phase extraction column (Alltech) was equilibrated with 1 mL of 50 mM ammonium bicarbonate prior to sample introduction. The column was washed with 0.5 mL of 50 mM ammonium bicarbonate. The flavin peptide was eluted with 25% aqueous methanol (v/v), concentrated in speedvac, and analyzed by ESI precursor ion (PO_3^-) scanning MS as described earlier.[11,25]

CD Spectroscopy. CD spectra were measured using a model 62DS circular dichroism spectrophotometer (Aviv Associates, Lakewood, NJ). All spectra were collected between 300 and 600 nm with 1.0 nm step and 1 s dwell time using a 1 cm path length cuvette (∼1 mL sample, 10 μM), and five scans were averaged to enhance the signal-to-noise ratio. The final spectra were smoothed using a negative exponential smoothing algorithm.

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