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Inhibition of Monoamine Oxidase B by Selective Adenosine A_{2A} Receptor Antagonists

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Abstract—Adenosine receptor antagonists that are selective for the A_{2A} receptor subtype (A_{2A} antagonists) are under investigation as possible therapeutic agents for the symptomatic treatment of the motor deficits associated with Parkinson's disease (PD). Results of recent studies in the MPTP mouse model of PD suggest that A_{2A} antagonists may possess neuroprotective properties. Since monoamine oxidase B (MAO-B) inhibitors also enhance motor function and reduce MPTP neurotoxicity, we have examined the MAO-B inhibiting properties of several A_{2A} antagonists and structurally related compounds in an effort to determine if inhibition of MAO-B may contribute to the observed neuroprotection. The results of these studies have established that all of the (*E*)-8-styrylxanthinyl derived A_{2A} antagonists examined display significant MAO-B inhibitory properties in vitro with K_i values in the low μM to nM range. Included in this series is (*E*)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methylxanthine (KW-6002), a potent A_{2A} antagonist and neuroprotective agent that is in clinical trials. The results of these studies suggest that MAO-B inhibition may contribute to the neuroprotective potential of A_{2A} receptor antagonists such as KW-6002 and open the possibility of designing dual targeting drugs that may have enhanced therapeutic potential in the treatment of PD.

Introduction

Idiopathic Parkinson's disease (PD) is a neurodegenerative disorder characterized pathologically by a marked loss of dopaminergic nigrostriatal neurons and clinically by disabling movement disorders. At present the mainstay for the treatment of PD relies on dopamine replacement therapy with the dopamine precursor L-DOPA.[1] Although this approach provides considerable symptomatic relief in the early stages of this disease, advanced PD may be associated with poor quality of life and early death.[2,3] Drugs that target the mechanism of neuronal cell death and therefore delay or even halt the progression of this disease may offer improved therapeutic approaches for the treatment of PD.

Attempts to develop neuroprotective agents have focused on identifying compounds that protect against the degenerative processes associated with exposure to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [(MPTP, (**1**)]. The MPTP-induced losses of nigrostriatal neurons[4] in the human produce a syndrome that is neurochemically, behaviorally and pathologically similar to that observed in patients diagnosed with PD. The toxic effects of MPTP are reported to be mediated by the pyridinium species MPP^+ (**3**),[5] a mitochondrial toxin.[6,7] MPP^+ is formed via the MAO-B catalyzed oxidation of the parent tetrahydropyridinyl protoxin which generates the unstable dihydropyridinium intermediate $MPDP^+$ (**2**). A second 2-electron oxidation yields MPP^+ (Fig. 1).[8,9]

Early studies established that (*R*)-deprenyl (**4**), an irreversible MAO-B inhibitor and clinically useful antiparkinsonian agent, is neuroprotective in MPTP-treated animals.[10] Other inactivators and competitive inhibitors of MAO-B exhibit similar effects.[11–14] Although this neuroprotection may be linked to the blockade of the metabolic bioactivation of MPTP, the neuroprotective properties of (*R*)-deprenyl in MPTP animal models,[10,14] also appear to involve unknown pathways that are independent of the inhibition of MPP^+ formation.[15–17]

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Antagonists of adenosine receptors (A_{2A} antagonists) are reported to ameliorate motor deficits in MPTP-treated animals[18–21] and therefore may provide symptomatic relief in patients diagnosed with PD. Some A_{2A} antagonists also display neuroprotective properties in animal models of PD. We previously demonstrated that the nonselective A_1/A_{2A} antagonist caffeine protects against the MPTP induced nigrostriatal neurotoxicity in the mouse model of PD.[22] Results of more recent studies have documented that the potent and selective A_{2A} antagonist (*E*)-8-(3-chlorostyryl)caffeine [CSC (**5b**)][23,24] also is neuroprotective in the MPTP mouse model.[25] This protection, however, may be dependent in part on the inhibition of the MAO-B catalyzed bioactivation of MPTP since CSC was also found to be a potent and selective competitive MAO-B inhibitor with a K_i value of 100 nM in mouse brain mitochondrial preparations.[25] The combination of the neuroprotective and MAO-B inhibiting properties of CSC has prompted us to examine the MAO-B inhibiting properties of other A_{2A} antagonists and related compounds to determine if such dual actions are a common feature of these types of heterocyclic systems.

The majority of the reported A_{2A} antagonists are 1,3-disubstituted xanthinyl analogues bearing an (*E*)-8-styryl moiety modified on the phenyl ring. We have examined the MAO-B inhibiting properties of 16 known (*E*)-8-styrylxanthinyl A_{2A} antagonists (**5a–5h** and **11a–11h**) including (*E*)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methylxanthine [KW-6002 (**5a**)], a compound which is undergoing clinical trials as a novel, non-dopaminergic agent for the treatment of PD.[19,20] Furthermore, KW-6002 is reported to protect against the dopaminergic neurotoxicity of MPTP in mice and of 6-hydroxydopamine in rats.[21,22] We also have examined the photochemical isomerization of some members of this series in order to determine if the resulting *cis* isomers (specifically the *cis* isomer **6a** of KW-6002) might mediate MAO-B inhibition. As part of a limited SAR analysis, we have determined the MAO-B inhibiting properties of the two xanthinyl analogues **7a** and **7b** in which the styryl double bond has been reduced, and three (*E*)-2-styrylbenzimidazolyl analogues (**8a–8c**) (Fig. 2).

Results and Discussion

Chemistry

The majority of the compounds examined here have been reported previously as cited in the Experimental

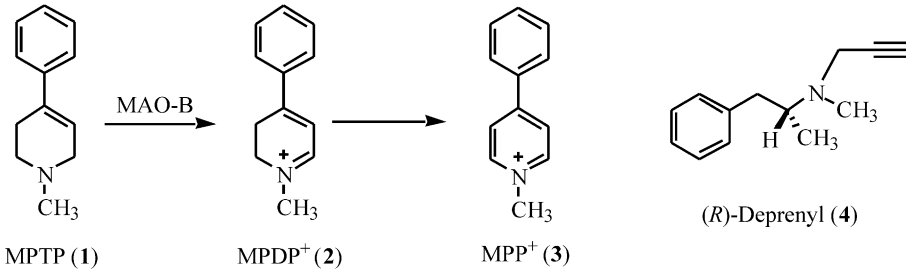


Figure 1. The MAO-B dependent bioactivation pathway for MPTP (**1**) via $MPDP^+$ (**2**) to MPP^+ (**3**) and the structure of the MAO-B inactivator (*R*)-deprenyl (**4**).

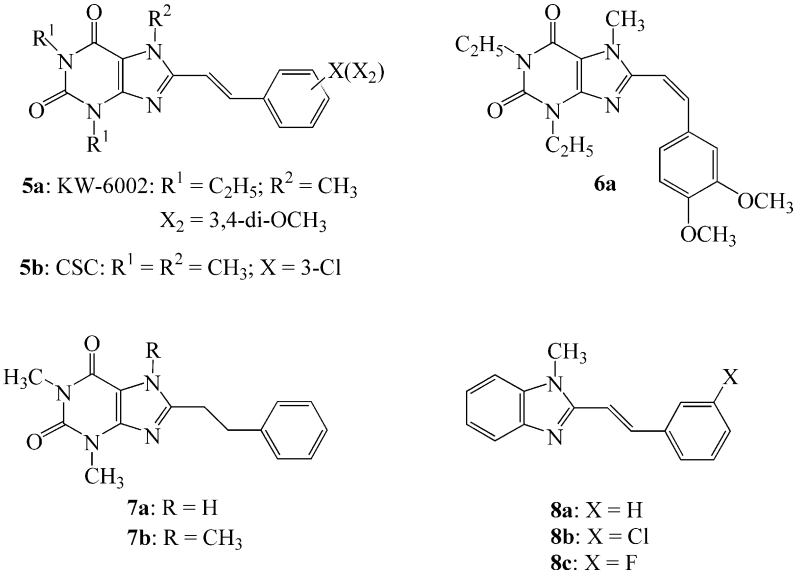


Figure 2. Structures of compounds discussed in the text.

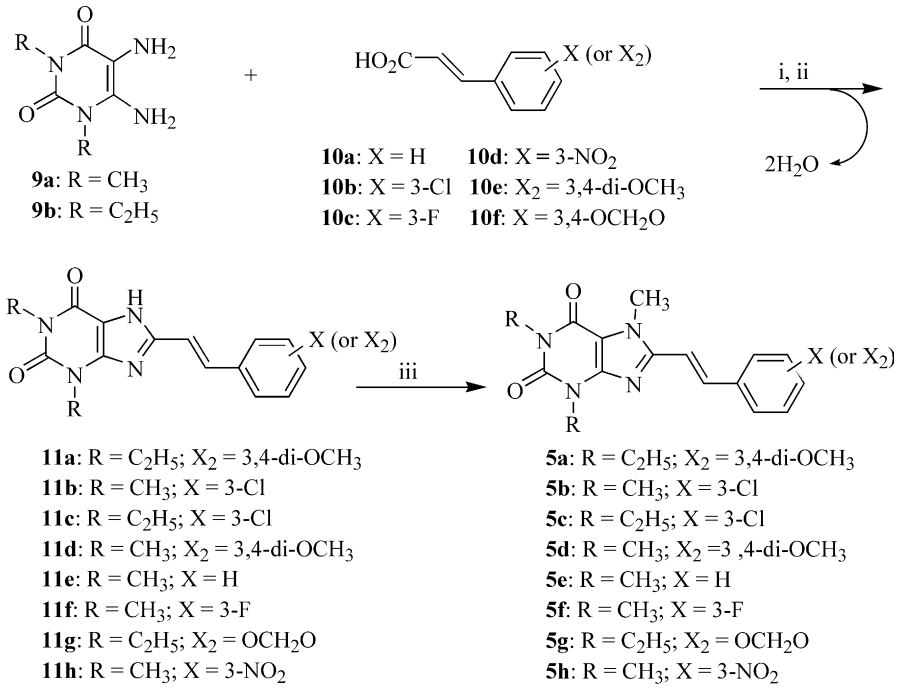


Figure 3. Synthetic pathway to (*E*)-8-styrylxanthinyl analogues **11a–11h** and **5a–5h**. Key: (i) EDAC, dioxane–H_2O; (ii) NaOH (aq), reflux; (iii) CH_3I, K_2CO_3, DMF.

Procedures. The (*E*)-8-styrylxanthinyl analogues (Fig. 3) were prepared by acylation of 1,3-dimethyl- (**9a**) or 1,3-diethyl- (**9b**) 5,6-diaminouracil[26] with commercially available *trans*-cinnamic acid (**10a**) or 3-chloro- (**10b**), 3-fluoro- (**10c**), 3-nitro- (**10d**), 3,4-dimethoxy- (**10e**) or 3,4-methylenedioxy- (**10f**) *trans*-cinnamic acid in the presence of a carbodiimide reagent (EDAC). The resulting crude amides (most likely as mixtures of 5- and 6- acylated derivatives) underwent cyclization when heated with base to give the corresponding 1,3-disubstituted (*E*)-8-styrylxanthinyl derivatives **11a–11h**.[23,27] Treatment of **11a–11h** with iodomethane led to the desired 7-methylated analogues **5a–5h**. The structures and purity of the compounds reported previously were verified by comparing melting points, mass spectrometric and ^{1}H NMR properties with the corresponding literature values. The trans geometry of the styryl derivatives was confirmed by proton-proton coupling constants ($\geq$15 Hz) of the olefinic proton signals. The same protocol was followed for the synthesis of the previously unknown 8-(2-phenylethyl)xanthinyl derivatives **7a** and **7b**. In this case, 1,3-dimethyl-5,6-diaminouracil (**9a**) was treated with 3-phenylpropanoic acid rather than cinnamic acid.

The *cis* isomer of KW-6002 [(*Z*)-KW-6002 (**6a**)] was prepared by exposing dilute solutions of KW-6002 to light followed by sequential recrystallizations to separate the *cis* from the trans isomer (see Experimental Procedures). An olefinic proton-proton coupling constant of 12.4 Hz was consistent with the *cis* configuration. This coupling constant corresponded with values reported for related (*Z*)-8-styrylxanthinyl derivatives.[28,29] These studies confirm speculation in the literature that the *cis* isomer is the principal photochemical product formed when solutions of KW-6002 are irradiated with light.[30]

In an effort to determine the relative importance of the xanthinyl moiety in the MAO-B inhibition properties of (*E*)-8-styrylxanthinyl analogues, we synthesized a series of (*E*)-2-styrylbenzimidazolyl derivatives (**8a–8c**). The key intermediate, 2-methyl-1H-benzimidazole (**13**), was prepared in high yield according to Phillips[31] by condensing *o*-phenylenediamine (**12**) with acetic acid. For the preparation of the (*E*)-2-styrylbenzimadazolyl analogues we used the protocol as described by Dubey et al.[32] 2-Methylbenzimidazole was treated with commercially available benzaldehyde (**14a**) or 3-chloro- (**14b**) or 3-fluoro- (**14c**) benzaldehyde at high temperatures to give the corresponding substituted (*E*)-2-styryl-1H-benzimidazoles **15a–15c** which could be isolated from the reaction mixtures as their oxalate salts. Treatment of 15a-15c with an equivalent of iodomethane resulted in the desired (*E*)-1-methyl-2-styrylbenzimidazoles **8a–8c**. In the presence of two equivalents of iodomethane the formation of the (*E*)-1,3-dimethylbenzimidazolium species **16** as its iodide salt was observed (Fig. 4). The structures were verified by comparing melting points, mass spectrometric and ^{1}H NMR properties with the corresponding literature values where applicable. The *trans* geometry of the styryl moiety was confirmed by proton-proton coupling constants ($J\geq$15 Hz) of the olefinic proton signals.

Photochemistry

We have examined the photochemical isomerization of certain members of the (*E*)-8-styrylxanthinyl series in order to determine if the resulting *cis* isomers (specifically the *cis* isomer **6a** of KW-6002) might mediate MAO-B inhibition (Fig. 5). In all experiments ambient laboratory light was used. Based on earlier reports that certain (*E*)-8-styrylxanthines[28,29] isomerize to the

corresponding *cis* isomers when exposed to light, it was suggested that KW-6002 may undergo a similar photochemical transformation.[30] As determined by HPLC analysis (see Experimental), when dilute solutions of KW-6002 were exposed to light the concentration of the trans isomer decreased as a function of exposure time. Loss of the *trans* isomer was accompanied by the appearance of a second peak with a slightly shorter retention time. In the case of KW-6002, the compound representing this second peak was identified as the *cis* isomer **6a** since it displayed identical absorption spectral features and retention time as those observed with the fully characterized sample of **6a** described above. In accordance with the literature,[28] dilute solutions of **5a** isomerized more rapidly than did more concentrated solutions. A 5 μM solution of KW-6002 reached the photostationary state (8.3% *trans* and 91.7% *cis*) after approximately 150 min of light exposure while a 50 μM solution reached the same equilibrium composition only after 360 min. A 500-μM solution of KW-6002 did not reach equilibrium even after 900 min of light exposure. This isomerization reaction was stoichiometric — the disappearance of the *trans* isomer was balanced by the appearance of the *cis* isomer (Fig. 6). The effect of light

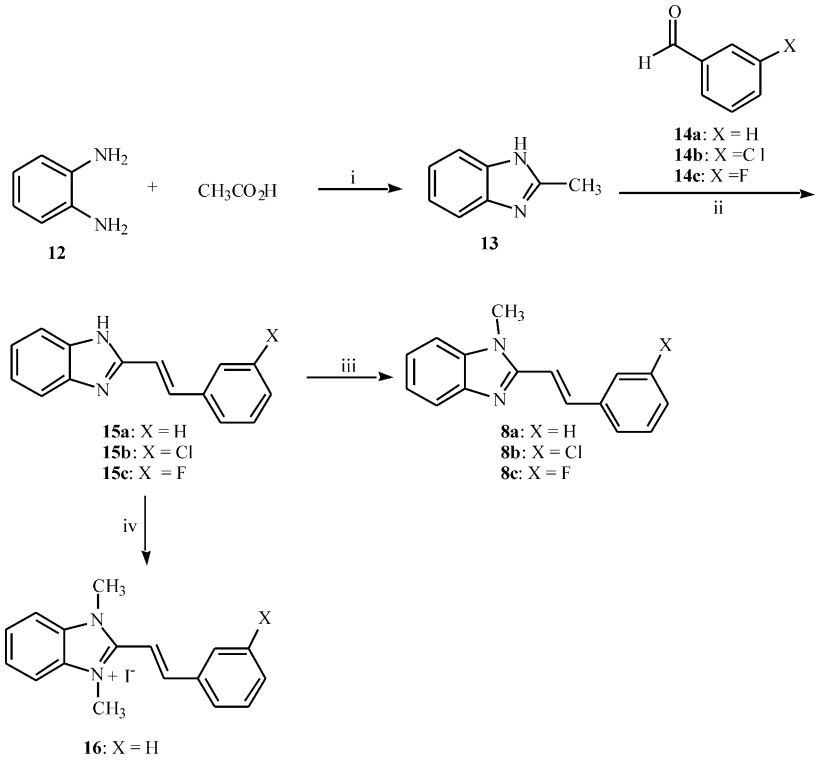
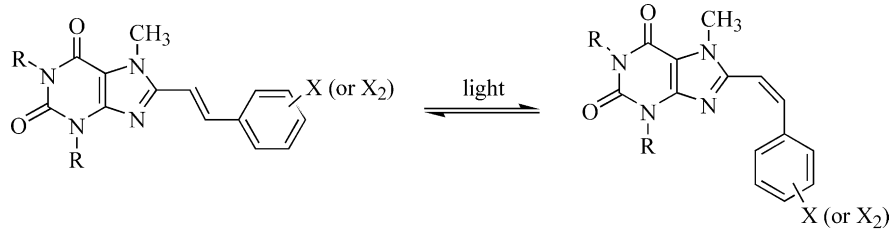


Figure 4. Synthetic pathway to substituted (*E*)-2-styrylbenzimidazolyl analogues **8a–8c** and **16**: (i) 4 N HCl, reflux 1 h; (ii) 180 °C, 24 h; (iii) CH_3I (1 equiv), K_2CO_3, DMF, rt; (iv) CH_3I (2 equiv), K_2CO_3, DMF, 100 °C.



5a: R = C_2H_5; X_2 = 3,4-di-OCH_3
5b: R = CH_3; X = 3-Cl
5c: R = C_2H_5; X = 3-Cl
5d: R = CH_3; X_2 = 3,4-di-OCH_3
5e: R = CH_3; X = H
5f: R = CH_3; X = 3-F
5g: R = C_2H_5; X_2 = OCH_2O
5h: R = CH_3; X = 3-NO_2

6a: R = C_2H_5; X_2 = 3,4-di-OCH_3
6b: R = CH_3; X = 3-Cl
6c: R = C_2H_5; X = 3-Cl
6d: R = CH_3; X_2 = 3,4-di-OCH_3
6e: R = CH_3; X = H
6f: R = CH_3; X = 3-F
6g: R = C_2H_5; X_2 = OCH_2O
6h: R = CH_3; X = 3-NO_2

Figure 5. Photo-induced isomerization of (*E*)-8-styryl-7-methylxanthinyl derivatives.

on the *cis* isomer of KW-6002 was also investigated. When **6a** (5 μM) was exposed to light the photostationary state (10.1% *trans* and 89.9% *cis*) was reached after approximately 150 min.

We also observed rapid photochemical isomerizations with compounds **5d** and **5g**. Analogue **5d** (4.5 μM) isomerized to the corresponding *cis* isomer with the photostationary state (12.7% *trans*) being achieved in approximately 150 min. Similarly **5g** (5 μM solution) reached the photostationary state (13.8% *trans*) after about 100 min. The rates of *cis* to *trans* isomerization of (*E*)-8-styryl-7-methylxanthines bearing electron withdrawing groups on the phenyl ring (**5b**, **5c**, **5f** and **5h**) were much slower than the other analogues (**5a**, **5d**, **5e**, **5g**). For example, the photostationary state of a 5 μM solution of **5b** had not been achieved after 4 h of continuous light exposure.

(*E*)-8-Styryl-7-methylxanthines bearing electron withdrawing groups on the phenyl ring (**5b**, **5c**, **5f** and **5h**) have λ_{max} values of 354 nm and molar extinction coefficients ranging from 28,000 to 32,000 whereas the other analogues have λ_{max} values near 362 nm and molar extinction coefficients greater than 41,000. This greater efficiency of light absorption of these analogues may account for their more rapid rates of photo-induced isomerization.

Since all our in vitro MAO-B assays required that the test compounds be in solution, we were concerned that those compounds containing olefinic bonds may isomerize under the assay conditions and in this way compromise the outcome of the experiments. We found that significant amounts of the *trans* isomer (**5a**) of KW-6002 were converted to the *cis* isomer (**6a**) during a 45-min incubation period. When appropriate measures were taken to protect these samples from light, the amounts of **6a** detected in the samples significantly decreased. For example when 5, 10 and 20 μM solutions of **5a** were incubated for 45 min without shielding from light, 80.4±5.4, 87.6±1.7 and 90.7±0.6% of the initial concentration of **5a**, respectively, were detected in the samples. In each of these solutions the presence of the *cis* isomer was also observed. In contrast, after shielding these incubations by covering the incubation tubes with aluminum foil, the amounts of **5a** remaining in the incubation mixtures were >99% of the initial concentration. We conclude that, when appropriate measures are taken to shield incubations from light, the photochemical isomerization of the *trans* to the *cis* alkene is negligible and therefore the MAO-B properties of KW-6002 and other (*E*)-8-styrylxanthinyl derivatives are independent of the formation of the *cis* isomers.

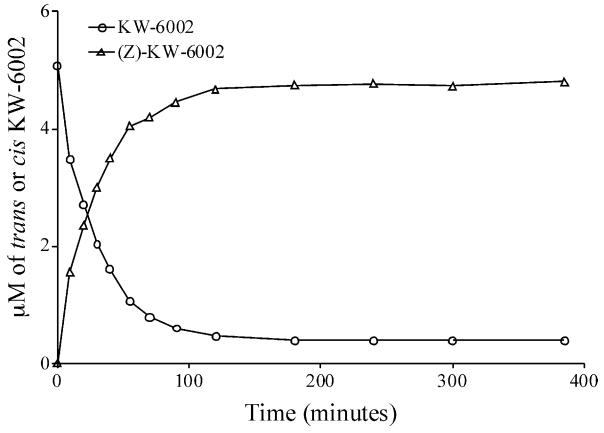


Figure 6. A graphical representation of the isomerization of KW-6002 (circles) to its corresponding *cis* isomer (triangles) when a 5-μM solution was exposed to light for various periods of time.

Enzymology

The K_i values for the competitive inhibition of MAO-B were determined in most instances by measuring the extent by which various concentrations of the test compounds slowed the rate of the α-carbon oxidation of 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine [MMTP (**17**)] to the corresponding dihydropyridinium metabolite, the 1-methyl-4-(1-methylpyrrol-2-yl)-2,3-dihydropyridinium species $MMDP^+$ (**18**) (Fig. 7).[33] $MMDP^+$ was monitored spectrophotometrically at 420 nm, a wavelength that was distant from the chromophores of both the substrate and the A_{2A} antagonists investigated in this study. The chemical stability of **18** obviated the need to monitor for the formation of the 1-methyl-4-(1-methylpyrrol-2-yl)pyridinium species MMP^+ (**19**).[34]

In certain cases, the limited solubility of the antagonists in the incubation mixture impacted negatively on our ability to determine the K_i values accurately using this spectrophotometric assay. For these compounds, concentrations that bracketed the K_i value and that were within the solubility range did not result in enough inhibition of MAO-B to enable accurate measurements. In these cases a reverse-phase HPLC assay was used to measure the time dependent formation of $MMDP^+$. This approach allowed us to estimate K_i values for inhibitors for which the limits of solubility and the K_i values are spaced closely together. We also confirmed that $MMDP^+$ does not undergo any detectable oxidation to the MMP^+ (**19**) in our in vitro assay because LC tracings of incubation mixtures failed to yield peaks that corresponded in retention time to synthetic MMP^+.[34]

The MAO-B inhibitory properties of KW-6002 (**5a**) and CSC (**5b**) also were investigated using MPTP (**1**) instead of MMTP as enzyme substrate since MPTP has been studied extensively as the proneurotoxin that undergoes bioactivation by MAO-B. Since MPTP is the biologically more relevant substrate, it was included in this study to compare with data generated with MMTP, our substrate of choice for screening of MAO activity. To make quantitative estimations of the oxidation of MPTP by MAO-B, the concentrations of both its α-carbon oxidation product $MPDP^+$ (**2**) and the subsequent 2-electron oxidation product MPP^+ (**3**) were determined by reversed phase HPLC analysis as described in the Experimental Procedures.

For screening of MAO-B inhibitory activity we used baboon liver mitochondria as enzyme source because it

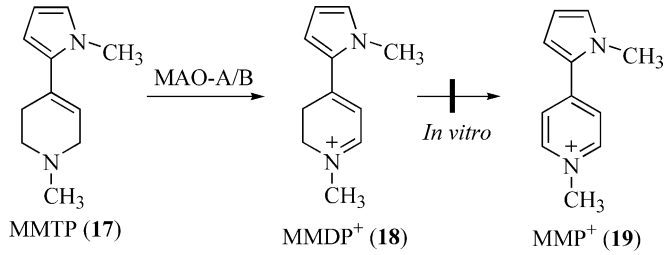


Figure 7. The MAO catalyzed oxidation of MMTP (**17**) to the corresponding dihydropyridinium species (**18**). The further oxidation of **18** to the pyridinium species **19** is not observed.

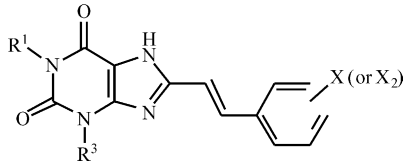
exhibits high MAO-B activity while MAO-A activity is negligible.[34] For select compounds C57BL/6 mouse brain mitochondria also served as a source for MAO-B since the C57BL/6 mouse is a well established model of MPTP-induced parkinsonism.[35,36] Finally, in some experiments human liver mitochondria also served as MAO-B source to determine if the baboon and human derived inhibition values are comparable.

(*E*)-8-Styrylxanthinyl derivatives

The K_i values for the inhibition of MAO-B by the (*E*)-8-styrylxanthinyl derivatives are presented in Tables 1 and 2. As illustrated by example with KW-6002 (Fig. 8), the plots we obtained in all cases were classical for competitive inhibition. While caffeine was found to be a very weak inhibitor of MAO-B, all of the other 8-substituted xanthinyl analogues were moderate to potent competitive inhibitors of MAO-B. CSC (**5b**) was confirmed to be an exceptionally potent inhibitor with K_i values of 70 and 235 nM in baboon and human liver mitochondria, respectively. The reported K_i value in mouse brain mitochondria is 100 nM.[25] The reported K_i value for the selective displacement of the preferred A_{2A} ligand ^{3}H-CGS 21680 from the A_{2A} receptor for CSC is 54 nM.[23] Consequently, the affinity of CSC for the enzyme and the receptor are comparable. Two other compounds (**5f** and **5h**) also proved to be potent inhibitors of MAO-B with K_i values in the nM range. Like CSC, these compounds are (*E*)-8-styrylcaffeinyl analogues bearing an electronegative group at the C-3 position of the styryl ring.

Structural modifications of CSC led to diminished MAO-B inhibitory activity. For example, replacement of the 1,3-dimethyl groups of the xanthinyl moiety with ethyl groups impacted negatively on the potency of MAO-B inhibition (compare **5b** vs **5c**). Within this series, the 7-*N*-methylxanthinyl analogues (Table 2) were more potent inhibitors than the corresponding 7*H*-xanthinyl (Table 1) analogues (for example **5a** vs **11a**, **5b** vs **11b** and **5e** vs **11e**). As documented in Table 3, saturation of the styryl double bond also led to reduced activity (**7a** vs **11e** and **7b** vs **5e**). The styryl moiety was essential for inhibition since caffeine proved to be a very weak inhibitor. Also, the trans geometry is required

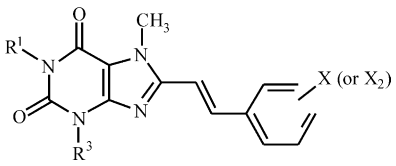
Table 1. The K_i values for the inhibition of MAO-B by various (*E*)-8-styryl-7*H*-xanthinyl derivatives (the reported K_i values for antagonism of the A_{2A} receptor are also listed)



Compd	R^1/R^3	X or X_2	K_i value	
			MAO-B (μM)	A_{2A} (nM)
11a	Ethyl	3,4-Dimethoxy	63[a]	23[37]
11b	Methyl	3-Chloro	1.5[a]; 8.0[b]	Not reported
11c	Ethyl	3-Chloro	Not determined[c]	Not reported
11d	Methyl	3,4-Dimethoxy	6[a]	1100[23]
11e	Methyl	H	31[a]	291[23]
11f	Methyl	3-fluoro	1.9[a]	516[23]
11g	Ethyl	3,4-Methylenedioxy	2.5[a]	15[23]
11h	Methy	3-Nitro	1.7[a]; 9[b]	438[23]

[a]Baboon liver mitochondria.
[b]Human liver mitochondria.
[c]Due to limited solubility in the incubation mixture the K_i-value could not be obtained for this compound.

Table 2. The K_i values for the inhibition of MAO-B by various (*E*)-8-styryl-7-methylxanthinyl derivatives (the reported K_i values for antagonism of the A_{2A} receptor are also listed)



Compd	R^1/R^3	X or X_2	K_i value	
			MAO-B (μM)	A_{2A} (nM)
5a	Ethyl	3,4-Dimethoxy	28[a]; 21[b]; 17[b,c]	2.2[30]
5b	Methyl	3-Chloro	0.07[a]; 0.1[b,c]; 0.235[d]	54[23]; 36[24]
5c	Ethyl	3-Chloro	3[a] ; 30[d]	Not reported
5d	Methyl	3,4-Dimethoxy	2.7[a]; 11	18[24]; 197[23]
5e	Methyl	H	3[a]; 3	94[23]
5f	Methyl	3-Fluoro	0.4[a]	83[23]
5g	Ethyl	3,4-Methylenedioxy	8[a]	6.1[37]
5h	Methyl	3-Nitro	0.16[a]	195[23]

[a]Baboon liver mitochondria.
[b]C57BL/6 mouse brain mitochondria.
[c]MPTP was used as substrate instead of MMTP.
[d]Human liver mitochondria.

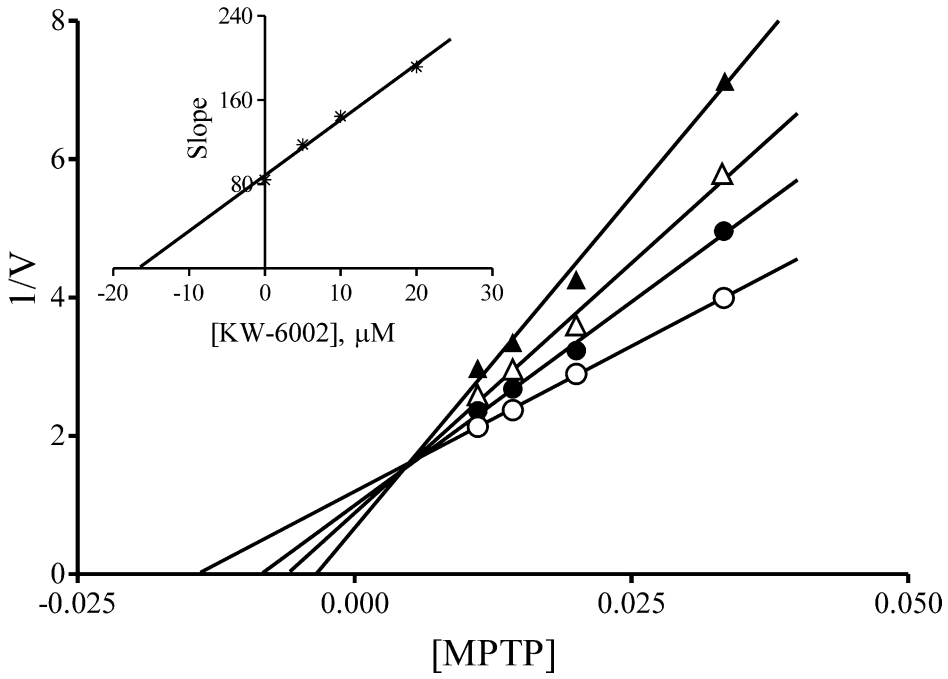
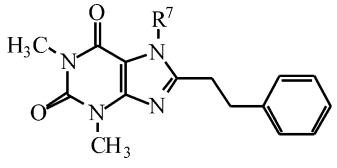


Figure 8. Lineweaver–Burke plots of the rates of the mouse brain MAO-B catalyzed oxidation of MPTP carried out in the absence (open circles) and presence of various concentrations of KW-6002 (filled circles, 5 μM; open triangles, 10 μM; filled triangles, 20 μM). The secondary plot of the slopes of the Lineweaver–Burke plots versus the concentrations of KW-6002 (inset) gave a K_i value of 17 μM.

because the *cis* isomer of KW-6002 was inactive as an MAO-B inhibitor.

KW-6002 (**5a**) was found to be a moderate MAO-B inhibitor with K_i values of 21 and 28 μM, respectively, in mouse brain and baboon liver mitochondria. A comparable K_i value (17 μM) was obtained when MPTP instead of MMTP served as substrate for mouse brain MAO-B (Fig. 8). With a reported K_i value of 2.2 nM[30] for the selective displacement of ^{3}H-CGS 21680 from the A_{2A} receptor, this compound binds much more tightly to the A_{2A} receptor compared to its binding to the active site of MAO-B. The extent to which MAO-B inhibition may contribute to the neuroprotective properties of KW-6002 is not clear at this time. Observations by Ikeda et al.[21] argue against a role for MAO-B inhibition since neuroprotective doses of KW-6002 do not alter MPP^+ levels in the striatum when measured 1–6 h after MPTP administration. This does not exclude inhibition of MAO as a possible contributor to the neuroprotective mechanism since the neuroprotective properties of the potent MAO-B mechanism-based inactivator (*R*)-deprenyl in the MPTP animal model also appear to involve pathways that are independent of the inhibition of MPP^+ formation.[15,16]

Table 3. The K_i values for the inhibition of MAO-B by 8-(2-phenylethyl)xanthines **7a** and **7b**



Compd	R^7	K_i value (μM)
7a	H	220[a]; 183[b]
7b	Methyl	30[a]; 36[b]

[a]Human liver mitochondria.
[b]Baboon liver mitochondria.

(*E*)-2-Styrylbenzimidazolyl derivatives

In an effort to determine the relative importance of the xanthinyl moiety on the MAO-B inhibition properties of (*E*)-8-styrylxanthinyl analogues, we synthesized a series of (*E*)-2-styrylbenzimidazolyl derivatives (**8a–8c**). Since the xanthinyl and benzimidazolyl moieties are both planar structures, the latter could provide a useful system to investigate the role of the xanthinyl moiety versus the requirement for planarity in MAO-B inhibition. The literature supports the idea that planarity is important in MAO-B inhibition since a wide variety of planar, heterocyclic systems have been found to be competitive inhibitors of MAO-B.[38–40]

The K_i values for the inhibition of MAO-B by the (*E*)-2-styrylbenzimidazolyl derivatives are presented in Table 4. All derivatives exhibited MAO-B inhibitory activity and the Lineweaver–Burke plots obtained were classical for competitive inhibition.[41] We observed that the (*E*)-1-methyl-2-styrylbenzimidazolyl analogues (**8a–8c**) consistently were more potent MAO-B inhibitors than their corresponding 1*H* analogues (**15a–15c**). Of all the compounds tested, (*E*)-1-methyl-2-(3-chlorostyryl)-benzimidazole (**8b**) proved to be the most potent inhibitor with a K_i value of 1.4 μM. The inhibitor potency of this compound is 20 times less than what was observed for the corresponding xanthinyl analogue CSC

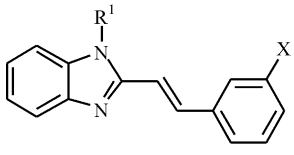
(**5b**). Also the K_i value for the inhibition of MAO-B by **8c** (2.6 μM) was significantly lower than the corresponding value for the (*E*)-8-(3-fluorostyryl)xanthinyl analogue **5f** (0.4 μM). We conclude that, although the planar character of the benzimidazolyl ring system contributes significantly to the competitive inhibition of MAO-B, the xanthinyl moiety is a better system for the development of exceptionally potent MAO-B inhibitors.

The 3-Cl (**5b** and **8b**) and 3-fluoro (**5f** and **8c**) analogues of the (*E*)-1-methyl-2-styrylbenzimidazolyl series and (*E*)-8-styryl-7-methylxanthinyl series, respectively, were considerably more potent MAO-B inhibitors than were the corresponding 3-H analogues **5e** and **8a**. This observation reinforces the importance of an electron withdrawing substituent on the styryl moiety for MAO-B inhibition and further suggests that the (*E*)-8-styrylxanthinyl and (*E*)-2-styrylbenzimidazolyl derivatives may interact in a similar manner with MAO-B. The positively charged (*E*)-1,3-dimethyl-2-styrylbenzimidazolium species **16** was found to be a weak inhibitor (K_i value of 85 μM with baboon liver mitochondria). This is in accordance with the reported hydrophobic nature of the active site.[42]

Conclusions

All of the (*E*)-8-styrylxanthinyl derived A_{2A} antagonists examined in this study exhibited significant inhibiting activity towards MAO-B. This behavior suggests the possibility of developing compounds that may provide enhanced antiparkinsonian activity since both A_{2A} antagonism and MAO-B inhibition may be linked to neuroprotective mechanisms as well as symptomatic enhancement of motor function. (*E*)-8-styrylcaffeine derived structures with electron withdrawing groups on C-3 of the styryl ring are worthy of further investigations since they are particularly potent inhibitors of MAO-B and antagonists of the A_{2A} receptor.

Table 4. The K_i values for the inhibition of MAO-B by (*E*)-2-styrylbenzimidazolyl derivatives



Compd	R^1	X	K_i values[a] (μM) MAO-B
15a	H	H	53
15b	H	Chloro	3.5
15c	H	Fluoro	5.3
8a	Methyl	H	17
8b	Methyl	Chloro	1.4
8c	Methyl	Fluoro	2.6

[a]The MAO-B source in all instances was baboon liver mitochondria and the enzyme substrate MMTP.

Experimental

***Caution**: Procedures for the safe handling of neurotoxic compounds such as MPTP were followed as described previously.*[43]

Chemistry

Materials and instrumentation. Chemicals and reagents not described elsewhere were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). MPTP HCl (**1**),[44] $MPDP^+$ ClO_4^- (**2**),[9] MPP^+ I^- (**3**),[45] MMTP HCl (**17**),[33] and 1-methyl-4-(1-methylpyrrol-2-yl)pyridinium iodide, MMP^+ I^- (**19**)[33] were synthesized according to literature procedures. Caffeine was purchased from Sigma (St. Louis, MO, USA). Melting points (mp) were obtained on a Thomas-Hoover melting point apparatus and are uncorrected. NMR spectra were recorded at 360 MHz on a Bruker AM 360 (Aspect 3000) instrument. Chemical shifts for ^1H are expressed in parts per million (δ) relative to the signal from tetramethylsilane added to an appropriate deuterated solvent ($CDCl_3$, CD_3CN or DMSO-d_6). Direct insertion probe electron ionization mass spectra (EIMS) were obtained on a VG Quattro I mass spectrometer. HPLC analyses were performed on a Hewlett Packard 1100HPLC system equipped with a UV–Vis diode array (DA) detector, a Rheodyne 7725I injector, a Zorbax SB-C8 analytical column (4.6×250 mm, 5 μm) and an in-line pre-column filter (2 μm; Upchurch Scientific Inc., Oak Harbor, WA, USA). Milli-Q water was obtained from a Waters Milli-Q UV Plus system (Millipore, Bedford, MA, USA). Microanalyses were performed by Atlantic Microlab, Inc., Norcross, GA, USA.

Preparation of 1,3-dialkyl-5,6-diaminouracil (9a,b)

The syntheses of 1,3-dimethyl-5,6-diaminouracil (**9a**) and its corresponding hydrochloric acid salt were achieved as described previously.[26] The melting points of the free base and hydrochloride salt were found to be 214 °C (lit. mp 209 °C) and >260 °C (lit. mp >260 °C), respectively.[26] Using the same procedure, 1,3-diethyl-5,6-diaminouracil (**9b**) was prepared from 1,3-diethyluracil. The hydrochloride salt was recrystallized from ethanol to yield white crystals: mp >260 °C; ^1H NMR (DMSO-d_6) δ 9.47 (s, 3H), 7.78 (s, 2H), 3.92 (q, 2H, J=6.8 Hz), 3.80 (q, 2H, J=6.8 Hz), 1.12 (t, 3H, J=7.2 Hz), 1.06 (t, 3H, J=7.2 Hz). Anal. calcd for $C_8H_{14}N_4O_2$·HCl: C, 40.93; H, 6.44; N, 23.87. Found: C, 41.23; H, 6.58; N, 23.76.

Preparation of (*E*)-8-styrylxanthines (11a–11h). The preparation of **11a–11g** was accomplished using the procedure described by Suzuki et al.[27] or Jacobson et al.[23] The melting points of compounds reported previously were as follows: **11d** mp >270 °C (from DMF), lit. mp >295 °C;[23] **11e** mp >270 °C (from DMF), lit. mp >280 °C;[23] **11f** mp >260 °C (from DMF), lit. mp >310 °C.;[23] **11h** mp >300 °C (from DMF), lit. mp >300 °C.[23] The characterizations of **11a**, **11c** and **11g**, which have been reported only in the patent literature,[37] and of **11b** (previously unreported), are summarized below.

(*E*)-1,3-Diethyl-8-(3,4-dimethoxystyryl)xanthine (11a). This was obtained from **9b** in 35% yield: mp 267 °C (from DMF), lit. mp 268.8–269.1;[37] ^{1}H NMR (DMSO-d_6) δ 13.45 (s, 1H, brs), 7.59 (d, 1H, J = 16.2 Hz), 7.26 (d, 1H, J = 1.4 Hz), 7.13 (dd, 1H, J = 1.4, 8.3 Hz), 7.00 (1H, d, J = 8.3 Hz), 6.95 (d, 1H J = 16.2 Hz), 4.06 (q, 2H, J = 6.8 Hz), 3.94 (q, 2H, J = 6.8 Hz), 3.83 (s, 3H), 3.79 (s, 3H), 1.26 (t, 3H, J = 7.2 Hz), 1.14 (t, 3H, J = 7.2 Hz); EIMS m/z 370 ($M^{\cdot+}$). Anal. calcd for $C_{19}H_{22}N_4O_4$: C, 61.61; H, 5.99; N, 15.13. Found: C, 61.72; H, 6.04; N, 15.16.

(*E*)-1,3-Dimethyl-8-(3-chlorostyryl)xanthine (11b). This was obtained in 74.6% yield from **9a**: mp >300 °C (from DMF); ^{1}H NMR (DMSO-d_6) δ 7.69 (s, 1H), 7.63–7.55 (m, 2H), 7.47–7.38 (m, 2H), 7.08 (d, 1H, J = 6.5 Hz), 4.11 (s, 1H,), 3.47 (s, 3H), 3.25 (s, 3H); EIMS m/z 316 ($M^{\cdot+}$). Anal. calcd for $C_{15}H_{13}N_4O_2Cl$: C, 56.88; H, 4.14; N, 17.69. Found: C, 56.96; H, 4.19; N, 17.69.

(*E*)-1,3-Diethyl-8-(3-chlorostyryl)xanthine (11c). This was obtained in 27% yield: mp >270 °C (from DMF), lit. mp >280 °C;[37] ^{1}H NMR (DMSO-d_6) δ 13.51 (s, 1H, brs), 7.72 (s, 1H), 7.64–7.59 (m, 2H), 7.45–7.42 (m, 2H), 7.12 (d, 1H, J = 16.4 Hz), 4.07 (q, 2H, J = 6.8 Hz), 3.94 (q, 2H, J = 6.8 Hz), 1.26 (t, 3H, J = 6.8 Hz), 1.14 (t, 3H, J = 6.8 Hz); EIMS m/z 344 ($M^{\cdot+}$). Anal. calcd for $C_{17}H_{17}N_4O_2Cl$: C, 59.22; H, 4.97; N, 16.25. Found: C, 59.35; H, 4.98; N, 16.38.

(*E*)-1,3-Diethyl-8-(3,4-methylenedioxystyryl)xanthine (11g). This was synthesized from **9b** in 30% yield: mp >260 °C (from DMF), lit. mp >275 °C;[37] ^{1}H NMR (DMSO-d_6) δ 13.50 (s, 1H, brs), 7.57 (d, 1H, J = 16.5 Hz), 7.31 (s, 1H), 7.08 (d, 1H, J = 8.2 Hz), 6.95 (d, 1H, J = 8.9 Hz), 6.90 (d, 1H, J = 16.5 Hz), 6.08 (s, 2H), 4.06 (q, 2H, J = 7.2 Hz), 3.94 (q, 2H, J = 7.2 Hz), 1.25 (t, 3H, J = 6.8 Hz), 1.14 (t, 3H, J = 6.8 Hz); EIMS m/z 354 ($M^{\cdot+}$). Anal. calcd for $C_{18}H_{18}N_4O_4$: C, 61.01; H, 5.12; N, 15.81. Found: C, 61.01; H, 5.25; N, 15.95.

Preparation of (*E*)-7-methyl-8-styrylxanthines (5a–5h). The preparations of **5a–5g** were accomplished using the procedure described by Suzuki et al.[27] For previously reported compounds the melting points were as follows: **5a**: mp 191–192 °C (from ethanol), lit. mp 190.4–191.3 °C;[27] **5b**: mp 205 °C (from methanol:ethyl acetate, 9:1), lit. mp 205 °C;[23] **5d**: mp 234 °C (from chloroform:ethanol, 1:1), lit. mp 230–232 °C[23] and 236–238 °C;[24] **5e**: mp 219 °C (from ethyl acetate), lit. mp 220–222 °C;[23]; **5f**: mp 208–209 °C (from ethanol), lit. mp 208–209 °C.[23] Analogue **5h** was prepared according to the procedure described by Jacobson et al.:[23] mp 305 °C (from DMF), lit. mp 306–308 °C.[23] The characterizations of **5c** and **5g**, which are reported only in the patent literature,[37] are as follows.

(*E*)-1,3-Diethyl-8-(3-chlorostyryl)-7-methylxanthine (5c). was obtained from **11c** in 87.6% yield. This compound is reported in the literature as a dihydrate: mp 134.0–134.4 °C.[37] We obtained an anhydrous form from ethanol (mp 178–179 °C) and a monohydrate from ethanol: water 4:1 (mp 154–155 °C): ^{1}H NMR ($CDCl_3$) δ 7.71 (d, 1H, J = 15.5 Hz), 7.55 (s, 1H), 7.45–7.27 (m, 3H), 6.89 (d, 1H, J = 15.8 Hz), 4.17 (q, 2H, J = 6.8 Hz), 4.05 (q, 2H, J = 6.8 Hz), 4.04 (s, 3H), 1.35 (t, 3H, J = 6.8 Hz), 1.23 (t, 3H, J = 6.8 Hz); EIMS, m/z 358 ($M^{\cdot+}$). Anal. calcd for $C_{18}H_{19}N_4O_2Cl$: C, 60.25; H, 5.34; N, 15.61. Found: C, 59.96; H, 5.36; N, 15.41. Anal. calcd for $C_{18}H_{19}N_4O_2Cl{\cdot}H_2O$: C, 57.37; H, 5.62; N, 14.87. Found: C, 57.40; H, 5.56; N, 14.82.

(*E*)-1,3-Diethyl-8-(3,4-methylenedioxystyryl)-7-methylxanthine (5g). was obtained from **11g** in 86% yield: mp 219–220 °C (from toluene/*n*-hexane 3:1), lit. mp 219.4–219.6 °C;[37] ^{1}H NMR ($CDCl_3$) δ 7.68 (d, 1H, J = 15.9 Hz), 7.07 (d, 1H J = 1.8 Hz), 7.03 (dd, 1H J = 1.6, 8.1 Hz), 6.81 (d, 1H, J = 7.9 Hz), 6.70 (d, 1H, J = 15.7 Hz), 5.99 (s, 2H), 4.18 (q, 2H, J = 7.2 Hz), 4.06 (q, 2H, J = 7.2 Hz), 4.02 (s, 3H), 1.36 (t, 3H, J = 7.2 Hz), 1.24 (t, 3H, J = 7.2 Hz); EIMS m/z 368 ($M^{\cdot+}$). Anal. calcd for $C_{19}H_{20}N_4O_4$: C, 61.95; H, 5.47; N, 15.21. Found: C, 62.00; H, 5.49; N, 15.22.

1,3-Dimethyl-8-(2-phenylethyl)xanthine (7a). To a solution of **9a** (7 mmol) and 1-ethyl-2-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDAC, 10.3 mmol) in 80 mL dioxane/H_2O (1:1) was added 3-phenylpropionic acid (7.6 mmol). The pH was adjusted to 5.0 with 2 N aqueous hydrochloric acid and stirring was continued for an additional 2 h by which time the reaction was complete according to TLC analysis. The reaction mixture was neutralized with 1 N aqueous sodium hydroxide and cooled to 0 °C. After the addition of 65 mL distilled water a precipitate formed which was collected by filtration. The crude product in 20 mL aqueous sodium hydroxide (1 N)/dioxane (1:1) was heated under reflux for 25 min. The solution was cooled to 0 °C and acidified to a pH of 4 with 4 N aqueous hydrochloric acid. The resulting precipitate (**7a**) was collected by filtration and recrystallized from ethyl acetate to give the pure product in 41% yield: mp 259 °C; ^{1}H NMR ($CDCl_3$) δ 12.44 (s, 1H, brs), 7.23–7.10 (m, 5H), 3.64 (s, 3H), 3.42 (s, 3H), 3.14 (m, 4H); EIMS, m/z 284 ($M^{\cdot+}$). Anal. calcd for $C_{15}H_{16}N_4O_2$: C, 63.37; H, 5.67; N, 19.71. Found: C, 63.19; H, 5.55; N, 19.61.

1,3-Dimethyl-8-(2-phenylethyl)-7-methylxanthine (7b). To a stirred solution of **7a** (2.11 mmol) in 35 mL DMF was added potassium carbonate (5.35 mmol) followed by iodomethane (4.22 mmol). The reaction was complete according to alumina TLC analysis (ethyl acetate/chloroform, 8:3) after 2 h at which time the insoluble materials were removed by filtration. The addition of 3 volumes of water led to a precipitate that was collected by filtration. Compound **7b** (37% yield from methanol) melted at 154 °C: ^{1}H NMR ($CDCl_3$) δ 7.29–7.71 (m, 5H), 3.59 (s, 3H), 3.57 (s, 3H), 3.36 (s, 3H), 3.03 (m, 4H); EIMS, m/z 298 ($M^{\cdot+}$). Anal. calcd for $C_{16}H_{18}N_4O_2$: C, 64.41; H, 6.08; N, 18.78. Found: C, 64.43; H, 6.05; N, 18.84.

(*Z*)-1,3-Diethyl-8-(3,4-dimethoxystyryl)-7-methylxanthine (6a). The isomerization of **5a** (1.3 mM in acetone) to **6a** when exposed to light was monitored at 360 nm using reversed-phase HPLC (75% acetonitrile/25% water; flow rate 1 mL/min) and UV–vis diode array detection (see Materials and Instrumentation). The retention

times of **5a** and **6a** were found to be 3.8 and 4.2 min, respectively. After 2 weeks, the peak height ratio of **5a** and **6a** was found to be approximately 1:1. This ratio corresponds to a concentration ratio of approximately 25% *trans* (**5a**) to 75% *cis* (**6a**). The acetone was removed under reduced pressure and the residue was crystallized from methanol to give the essentially pure *trans* isomer **5a**. The mother liquor was evaporated to dryness and the residue was crystallized from hot acetonitrile. The resulting light yellow crystals (16% yield) were found to consist of at least 98.5% of the *cis* isomer **6a**: mp 134–135 °C; ^{1}H NMR (CD_3CN) δ 7.14–7.16 (m, 2H), 6.88–6.93 (m, 2H), 6.29 (d, 1H J = 12.4 Hz), 4.04 (q, 2H, J = 7.2 Hz), 4.98 (q, 2H, J = 6.8 Hz), 3.80 (s, 3H), 3.75 (s, 3H), 3.66 (s, 3H), 1.23 (t, 3H, J = 6.8 Hz), 1.17 (t, 3H, J = 6.8 Hz); EIMS, m/z 384 ($M^{\cdot+}$). Anal. calcd for $C_{20}H_{24}N_4O_4$: C, 62.49; H, 6.29; N, 14.57. Found: C, 62.58; H, 6.42; N, 14.69.

2-Methyl-*1H*-benzimidazole (13). This was prepared from *o*-phenylenediamine (**12**) according to the method of Philips:[31] mp 176 °C (from water), lit. mp 176 °C.[31]

2-Styryl-*1H*-benzimidazole derivatives (15a–15c). These were prepared from **13** according to a previously described method:[32] **15a**: mp 202 °C (from toluene), lit. mp 203–205 °C[32] and 201 °C;[46] **15b**: yield 68.2%; mp 226–228 °C (from acetone); ^{1}H NMR (DMSO-d_6) δ 12.73 (s, 1H, brs), 7.76 (s, 1H), 7.69–7.55 (m, 4H), 7.46–7.30 (m, 3H), 7.195 (m, 2H); EIMS m/z 254 ($M^{\cdot+}$). Anal. calcd for $C_{15}H_{11}N_2Cl$: C, 70.73; H, 4.35; N, 10.99. Found: C, 70.56; H, 4.34; N, 10.86; **15c**: yield 59.8%; mp 205–212 °C (from chloroform); ^{1}H NMR (DMSO-d_6) δ 12.67 (s, 1H, brs), 7.67 (d, 1H J = 16.6 Hz), 7.59–7.46 (m, 5H), 7.31 (d, 1H, J = 16.6 Hz), 7.22–7.17 (m, 3H); EIMS m/z 238 ($M^{\cdot+}$). Anal. calcd for $C_{15}H_{11}N_2F$: C, 70.73; H, 4.35; N, 10.99. Found: C, 70.56; H, 4.34; N, 10.86.

2-Styryl-1-methylbenzimidazole derivatives (8a–8c). These were prepared in the following manner: The styryl derivative **15** (5.12 mmol) was dissolved in DMF (35 mL) containing potassium carbonate (12.95 mmol). The mixture was cooled in a dry ice/acetone bath while iodomethane (5.12 mmol) was added in a drop-wise manner. The reaction was allowed to return to room temperature and stirred for an additional 3 h at which time the reaction mixture was filtered to remove the potassium carbonate. The DMF was removed under reduced pressure and the residue in 40 mL chloroform was washed with water (3×30 mL). The organic phase was dried over anhydrous magnesium sulfate, filtered and removed under reduced pressure to give **8a**: mp 119–121 °C (from toluene), lit. mp 113 °C.[46] In a similar way, analogue **8b** was obtained in 84% yield: mp 101–104 °C (from methanol); ^{1}H NMR (DMSO-d_6) δ 7.94 (s, 1H), 7.83 (d, 1H, J = 16.1 Hz), 7.69–7.64 (m, 2H), 7.57 (d, 1H, J = 15.7 Hz), 7.49–7.35 (m, 3H), 7.22 (m, 2H), 3.91 (s, 3H); EIMS m/z 268 ($M^{\cdot+}$). Anal. calcd for $C_{16}H_{13}N_2Cl$: C, 71.51; H, 4.88; N, 10.42. Found: C, 71.56; H, 4.90; N, 10.51; Analogue **8c** was obtained in a similar manner in 81.7% yield: mp 85–102 °C (from chloroform); ^{1}H NMR (DMSO-d_6) δ 7.85 (d, 1H, J = 15.5 Hz), 7.76 (m, 1H), 7.60 (m, 4H), 7.47 (m, 1H), 7.22 (m, 3H), 3.94 (s, 3H); EIMS m/z 252 ($M^{\cdot+}$). Although the spectral data were in accord with the proposed structure **8c**, the microanalytical values suggested the presence of the solvent in the analytical sample. The unsatisfactory elemental analysis and broad melting range may be due to the poor quality of the crystals obtained with chloroform as solvent. Anal. calcd for $C_{16}H_{13}N_2F$: C, 76.17; H, 5.19; N, 11.10. Calcd. For $C_{16}H_{13}N_2F{\cdot}0.1CHCl_3$: C,73.13; H, 4.96; N, 10.59. Found: C, 73.27; H, 5.35; N, 10.66.

(*E*)-1,3-Dimethyl-2-styrylbenzimidazolium iodide (16). Compound **16** was prepared as follows: **15a** (3.41 mmol) was dissolved in 15 mL DMF containing potassium carbonate (8.64 mmol). After the addition of iodomethane (8.82 mmol) the reaction was stirred for 2 h at 100 °C. The reaction mixture was cooled to room temperature and the potassium carbonate was removed by filtration. After removal of the solvent under reduced pressure, the residue was recrystallized from DMF to give **16** in 56.6% yield: mp 282–286 °C, lit. mp 235 °C;[46] ^{1}H NMR (DMSO-d_6) δ 8.07 (m, 2H), 7.96 (m, 2H), 7.85 (d, 1H, J = 6.9 Hz), 7.70 (m, 2H), 7.61–7.52 (m, 4H), 4.16 (s, 6H). Anal. calcd for $C_{17}H_{17}N_2I$: C, 54.27; H, 4.55; N, 7.44. Found: C, 54.17; H, 4.58; N, 7.32.

Studies on the isomerization of (*E*)-8-styrylxanthinyl derivatives. Solutions (5–500 μM) of (*E*)-8-styryl-7-methylxanthinyl derivatives **5a–5h** were prepared in acetonitrile/water (50:50) in clear glass vials and exposed to light. At various time intervals 200 μL of these solutions were injected into the HPLC system as described in Materials and Instrumentation. The mobile phase consisted of 75% acetonitrile and 25% Milli-Q water at a flow rate of 1 mL/min. The effluent was monitored at 360 nm. Upon light exposure the concentration of each *trans* isomer decreased with the concomitant appearance of a second peak with a shorter retention time from that of the *trans* isomer. For KW-6002 (**5a**) this second peak was positively identified as the *cis* isomer (**6a**) by comparing the absorption spectrum and retention time with those of the synthetically prepared *cis* ifsomer (see above for preparation). The λ_{max} value of the **5a** (362 nm) was greater than that of **6a** (345 nm); the retention times were 4.2 and 3.8 min, respectively. This trend was observed for all of the (*E*)-8-styryl-7-methylxanthinyl derivatives (**5b–h**) studied — the newly formed peaks had shorter retention times and lower λ_{max} values than those of the corresponding parent compound. Based on these observations, we have concluded that these peaks represent the *cis* isomers for all compounds examined. Quantitative measurements (performed in triplicate) of the trans isomers (**5a–5h**) were made by comparing peak heights of the samples with those of linear calibration curves [0.5–7.0 μM in acetonitrile/water (1:1)] prepared with the synthetic standards.

Enzymology

In vitro inhibition studies with MAO-B. Intact mitochondria prepared from baboon liver, human liver and C57BL/6 mouse (Harlan Sprague Dawley, Dublin, VA,

USA) brain served as MAO-B sources as discussed in the Results and Discussion section. Mitochondria were prepared as described by Salach and Weyler[47] and stored in aliquots containing the equivalent of 5–12 mg protein at −70 °C in Eppendorf. Before use, the original mitochondrial isolate was suspended in 200 μL of sodium phosphate buffer (100 mM, pH 7.4 containing 50% glycerol, w/v) and the protein concentration was determined by the method of Bradford.[48] For most of the inhibition studies on MAO-B we utilized the MAO-A/MAO-B mixed substrate MMTP.[33] Human liver (0.3 mg protein/mL) and mouse brain (0.3 mg protein/mL) mitochondrial preparations were preincubated for 15 min with 3.3×10^{-8} M clorgyline, to inactivate MAO-A. Baboon liver mitochondria, which express no MAO-A activity,[34] were studied in the absence of clorgyline. The MAO-B inhibition characteristics of KW-6002 (**5a**) and CSC (**5b**) also were investigated using the MAO-B selective substrate MPTP.[49] All incubation mixtures (500 μL final volume in sodium phosphate buffer, pH 7.4) contained either MPTP (30–90 μM [K_m values range from 40 μM (mouse brain)[11] to 65 μM (human liver)[34] to 87 μM (baboon liver)[34]]) or MMTP (30–120 μM [K_m values range from 32 μM (mouse brain)[34] to 52 μM (human liver)[34] to 61 μM (baboon liver)[34]]), the mitochondrial homogenate (final concentration 0.15 mg protein/mL) and the appropriate concentrations of the test compounds which bracketed their K_i value. The test compounds were dissolved in dimethyl sulfoxide (DMSO) at concentrations which, following addition to the incubation mixture, gave a final concentration of 4% (v/v) DMSO. DMSO concentrations higher than 4% have been reported to inhibit MAO-B activity. The samples were incubated at 37 °C and the incubation times were 15 min (MMTP) or 45 min (MPTP), time periods for which dihydropyridinium metabolite formation are reported to be linear.[34] All samples were protected from light by covering the incubation tubes (1.5 mL microcentrifuge tubes) with aluminum foil. The reactions were terminated by the addition of 20 μL 70% perchloric acid and the samples were centrifuged at 16,000*g* for 5 min. The supernatant fractions were removed and assayed for the MAO-B generated dihydropyridinium α-oxidation products of MPTP or MMTP. Different analytical procedures were used to determine these rates as described below.

MPTP (1) as substrate. To measure the MAO-B generated dihydropyridinium species $MPDP^+$ (**2**) and its subsequent oxidation product MPP^+ (**3**), a modification of an HPLC method described earlier was used.[11] The mobile phase consisted of 80% Milli-Q water [containing 0.6% (v/v) glacial acetic acid and 1% (v/v) triethylamine] and 20% acetonitrile at a flow rate of 1 mL/min. A volume of 200 μL of the supernatant fraction was injected into the HPLC system. Quantitative estimations of these species were achieved with the aid of calibration curves prepared over the linear concentration ranges of interest ($MPDP^+$: 0.8–3.0 μM; MPP^+: 0.2–0.8 μM). The sum of the concentrations of $MPDP^+$ (5.34 min) and MPP^+ (5.08 min) were used to determine the initial rate (V) of the MAO-B catalyzed oxidation of MPTP. The Lineweaver–Burke plots of 1/[rate of $MPDP^+$ plus MPP^+ formation (1/V)] versus 1/[MPTP] with increasing concentrations of the A_{2A} antagonist were constructed for KW-6002 (Fig. 8) and CSC. The K_i value ($-x$ when $y=0$) was estimated from a secondary plot in which the values of the slopes obtained from the Lineweaver–Burke plots were graphed as a function of the concentration of the test compound.

MMTP (17) as substrate. The concentration of $MMDP^+$ (**18**), the MAO-B generated oxidation product of MMTP, was determined either spectrophotometrically or by HPLC analysis. In the former case, the absorption of $MMDP^+$ was monitored at a wavelength of 420 nm using a Beckman DU-7400 spectrophotometer ($\varepsilon = 25{,}000\ M^{-1}$).[34] The HPLC analyses were performed using a mobile phase consisting of 70% Milli-Q water [containing 0.6% (v/v) glacial acetic acid and 1% (v/v) triethylamine], 20% methanol and 10% acetonitrile. A volume of 200 μL of the supernatant fraction was injected into the HPLC system. The $MMDP^+$ eluted at 4.57 min. The rates (peak heights at 420 nm/mg protein-min) of the MAO-B catalyzed oxidation of MMTP to $MMDP^+$ were estimated by measuring the peak height of $MMDP^+$ formed. From these data Lineweaver–Burke plots of 1/[rate of $MMDP^+$ formation (1/V)] versus 1/[MMTP] were constructed and the K_i value was calculated as described above.

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