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Dual inhibition of monoamine oxidase B and antagonism of the adenosine A_{2A} receptor by (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeine analogues

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ABSTRACT

The adenosine A_{2A} receptor has emerged as an attractive target for the treatment of Parkinson's disease (PD). Evidence suggests that antagonists of the A_{2A} receptor (A_{2A} antagonists) may be neuroprotective and may help to alleviate the symptoms of PD. We have reported recently that several members of the (*E*)-8-styrylcaffeine class of A_{2A} antagonists also are potent inhibitors of monoamine oxidase B (MAO-B). Since MAO-B inhibitors are known to possess anti-parkinsonian properties, dual-target-directed drugs that block both MAO-B and A_{2A} receptors may have enhanced value in the management of PD. In an attempt to explore this concept further we have prepared three additional classes of C-8 substituted caffeinyl analogues. The 8-phenyl- and 8-benzylcaffeinyl analogues exhibited relatively weak MAO-B inhibition potencies while selected (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues were found to be exceptionally potent reversible MAO-B inhibitors with enzyme–inhibitor dissociation constants (K_i values) ranging from 17 to 149 nM. Furthermore, these (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeines acted as potent A_{2A} antagonists with K_i values ranging from 59 to 153 nM. We conclude that the (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeines are a promising candidate class of dual-acting compounds.

1. Introduction

Currently, the therapy of Parkinson's disease (PD) is largely focused on dopamine replacement strategies with the dopamine precursor levodopa and dopamine agonist drugs.[1] Although these strategies are highly effective in controlling the early stages of the disease, long-term treatment is associated with drug-related complications such as a loss of drug efficacy, the onset of dyskinesias and the occurrence of psychosis and depression.[2,3] The inadequacies of dopamine replacement therapy have prompted the search for alternative drug targets. The adenosine A_{2A} receptor has emerged as one such target and antagonists of this receptor (A_{2A} antagonists) are considered promising agents for the symptomatic treatment of PD.[4] Additionally, evidence suggests that A_{2A} antagonists may slow the course of the disease by protecting against the underlying neurodegenerative processes[5,6] and may prevent the development of dyskinesias that are normally associated with levodopa treatment.[7] Furthermore, since the symptomatic relief conferred by A_{2A} antagonists are additive to the effect produced by dopamine replacement therapy, it may be possible to reduce the dose of the dopaminergic drugs and therefore the occurrence of side effects.[2,8] A_{2A} antagonists are therefore a promising adjunctive to dopamine replacement therapy.[9]

A particularly well-characterized A_{2A} antagonist, (*E*)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methylxanthine (KW-6002; **1**) (Scheme 1), is currently undergoing clinical trials for the treatment of the motor symptoms associated with PD.[10] Another A_{2A} antagonist and structural analogue of KW-6002, (*E*)-8-(3-chlorostyryl)caffeine (CSC; **2b**) (Scheme 1), is frequently used when examining the in vivo pharmacological effects of A_{2A} antagonists.[11,12] We have previously reported that CSC is also a potent reversible inhibitor of monoamine oxidase B (MAO-B) with an enzyme–inhibitor dissociation constant (K_i value) of 128 nM.[5,13,14] Inhibitors of MAO-B also are considered to be useful for the treatment of age-related neurodegenerative diseases such as Alzheimer's disease and PD.[15,16] Since MAO-B appears to be predominantly responsible for dopamine metabolism in the basal ganglia,[17,18] inhibition of this enzyme in the brain may conserve the depleted supply of dopamine. MAO-B inhibitors are used in combination with levodopa as dopamine replacement therapy in patients diagnosed with early PD.[19] MAO-B inhibitors have been shown to elevate dopamine levels in the striatum of primates treated with levodopa.[20] Furthermore, for each mole of dopamine oxidized by MAO-B, one mole of hydrogen peroxide (H_2O_2) is produced. H_2O_2 may interact with free iron to form highly reactive hydroxyl radicals that may contribute to neurodegenerative processes.[21] Inhibition of MAO-B, therefore, may also exert a protective effect by reducing H_2O_2 production in the brain.[22] These effects of MAO-B inhibitors are especially relevant when considering that the brain shows an age-related increase in MAO-B activity.[23,24] This increase

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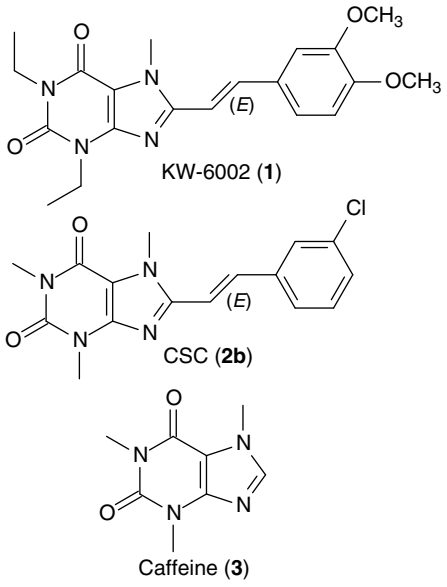


Scheme 1. The structures of the adenosine A_{2A} receptor antagonists KW-6002 (**1**), CSC (**2b**), and caffeine (**3**).

may be attributed to glial cell proliferation, since central MAO-B is predominantly located in glial cells.[25] In the aged parkinsonian brain, inhibition of MAO-B may, therefore, counter the effects of increased MAO-B activity and protect against further neurodegeneration.[26]

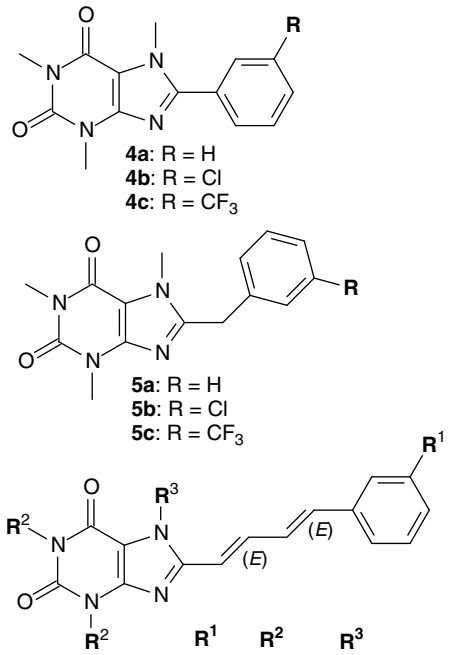
Based on these observations, dual-target-directed drugs, compounds that inhibit MAO-B and antagonize A_{2A} receptors, may have enhanced value in the management of PD. Using CSC as a lead compound we have, in the present study, attempted to identify additional dual-acting compounds, possibly with enhanced MAO-B inhibition and A_{2A} antagonism potencies. Such compounds may deepen our understanding of the structural requirements of C-8 substituted caffeinyl analogues to act as dual inhibitors of MAO-B and antagonists of the A_{2A} receptor. The (*E*)-styryl group at C-8 appears to be critical for the dual-action of CSC since caffeine (**3**) is only a moderate A_{2A} antagonist (K_i = 22 μM)[12] and a weak MAO-B inhibitor.[5] In this study, we further investigate the importance of the (*E*)-styryl functional group for dual-action by preparing and evaluating three additional classes of C-8 substituted caffeinyl analogues. These are the 8-phenylcaffeinyl analogues **4a**–**c**, the 8-benzylcaffeinyl analogues **5a**–**c** and the (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a**–**d** (Scheme 2). The particularly potent series of (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a**–**d** was expanded to include the ethyl homologs, **6e**–**g**. The MAO-B inhibition properties of the test compounds were first investigated, and the most potent inhibitors were then further evaluated for binding at the A_{2A} receptor.

As part of this effort we have measured both the K_i and IC_{50} values (concentration of inhibitor producing 50% inhibition) for the inhibition of MAO-B of a subset of the test compounds. The results have provided an opportunity to examine the validity of the relationship between K_i and IC_{50} of a competitive inhibitor of a mono-substrate reaction that is described by the Cheng-Prusoff equation, $K_i = IC_{50}/(1 + [S]/K_m)$,[27] by comparing experimentally determined K_i values to those calculated from the IC_{50} values.[28]

2. Results

2.1. Chemistry

The C-8 substituted caffeinyl analogues **4a**–**c**, **5a**–**c**, and **6a**–**g** (Scheme 2) were prepared in high yield according to the procedure

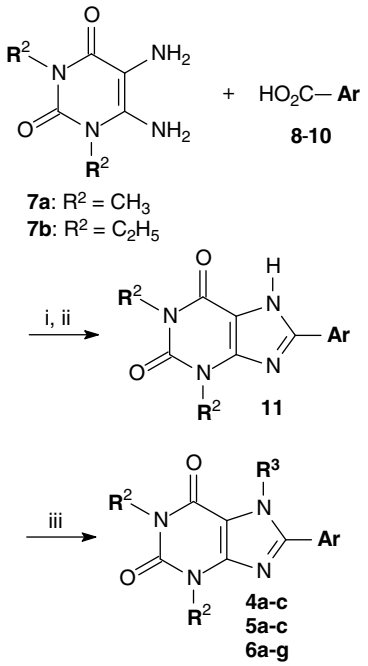


	R^1	R^2	R^3
6a:	H	CH_3	CH_3
6b:	Cl	CH_3	CH_3
6c:	Br	CH_3	CH_3
6d:	F	CH_3	CH_3
6e:	H	CH_3	C_2H_5
6f:	H	C_2H_5	CH_3
6g:	H	C_2H_5	C_2H_5

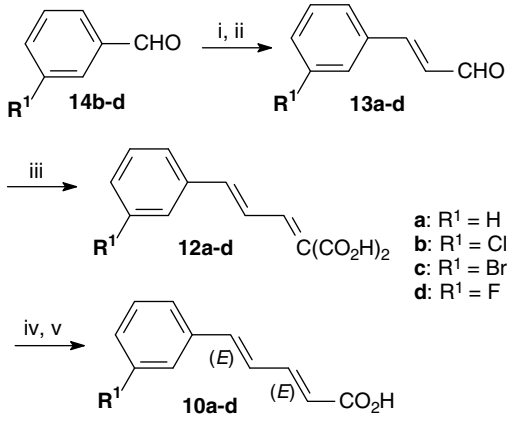
Scheme 2. The structures of the C-8 substituted caffeinyl analogues that were investigated in the present study: 8-phenylcaffeinyl analogues **4a**–**c**, 8-benzylcaffeinyl analogues **5a**–**c**, and (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a**–**g**.

previously reported for the preparation of (*E*)-8-styrylcaffeinyl analogues.[29,14] The key starting materials for the procedure, 1,3-dimethyl- (**7a**) and 1,3-diethyl-5,6-diaminouracil (**7b**),[30] were allowed to react with the appropriate carboxylic acid in the presence of the carbodiimide activating reagent *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) (Scheme 3). The commercially available benzoic acids **8a**–**c**, phenylacetic acids **9a**–**c**, and newly synthesized (*E*,*E*)-5-phenyl-2,4-pentadienoic acids **10a**–**d** (see below) were used for the preparation of **4a**–**c**, **5a**–**c**, and **6a**–**g**, respectively. The resulting amidyl intermediates underwent ring closure when heated under reflux in aqueous sodium hydroxide to yield the corresponding 1,3-dialkyl-8-sustituted-7*H*-xanthinyl analogues (**11**). Without further purification, the crude xanthinyl intermediates were selectively 7N-alkylated with an excess of iodomethane (**4a**–**c**, **5a**–**c**, **6a**–**d**, and **6f**) or iodoethane (**6e** and **6g**) and potassium carbonate to yield the target compounds **4**–**6**. Following crystallization from a suitable solvent, the structures and purity of all compounds were verified by mass spectrometry, ^{1}H NMR and ^{13}C NMR. The *trans*–*trans* geometry about the conjugated ethenyl π-bonds of **6a**–**g** was confirmed by proton–proton coupling constants in the range of 14.6–15.5 Hz for the olefinic proton signals.

The (*E*,*E*)-5-phenyl-2,4-pentadienoic acids (**10a**–**d**) required for the preparation of **6a**–**g** were conveniently prepared by allowing the appropriately substituted cinnamylidenemalonic acid (**12a**–**d**) to react with refluxing acetic anhydride and acetic acid (Scheme 4).[31] A solution of the resulting crude 5-phenyl-2,4-pentadienoic acids in chloroform containing a crystal of iodine was exposed to ambient light for 5 h. This photochemical reaction converts the allo-styryl-acrylic acid into the desired *trans*–*trans* geometry.[31]



Scheme 3. Synthetic pathway to the C-8 substituted caffeinyl analogues **4a**–**c**, **5a**–**c**, and **6a**–**g**. Reagents and conditions: (i) EDAC, dioxane/H_2O; (ii) NaOH (aq), reflux; (iii) CH_3I or C_2H_5I, K_2CO_3, DMF.



Scheme 4. Synthetic pathway to the (*E*,*E*)-5-phenyl-2,4-pentadienoic acids (**10a**–**d**). Reagents and conditions: (i) NaOH, CH_3CHO; (ii) Ac_2O, 120 °C; (iii) $CH_2(CO_2H)_2$, pyridine, 100 °C; (iv) Ac_2O, CH_3CO_2H, reflux; (v) I_2, light.

Following recrystallization from benzene, **10a**–**d** were obtained in good yield and with a high degree of purity. The required cinnamylidenemalonic acids (**12a**–**d**) were in turn prepared in high yield from the corresponding cinnamaldehydes (**13a**–**d**) and malonic acid in pyridine.[32] Except for cinnamaldehyde (**13a**), which is commercially available, the other substituted cinnamaldehydes (**13b**–**d**) were prepared by reacting the corresponding benzaldehydes (**14b**–**d**) with acetaldehyde under basic conditions.[33,34] The resulting cinnamaldehydes were purified by neutral aluminum oxide column chromatography.

2.2. MAO-B inhibition studies

Enzyme activity measurements were based on the MAO-B catalyzed oxidation of 1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (MMTP) to the corresponding dihydropyridinium metabolite ($MMDP^+$).[35] $MMDP^+$ absorbs light maximally at a wavelength of 420 nm. Since neither MMTP nor the test inhibitors absorb light at this wavelength, it is possible to measure the rates of substrate oxidation spectrophotometrically. We have employed baboon liver mitochondrial preparations as the enzyme source. Even though MMTP is a MAO-A/B mixed substrate, its oxidation by baboon liver mitochondria is exclusively attributed to the action of MAO-B since baboon liver tissue exhibits a high degree of MAO-B catalytic activity while MAO-A activity is negligible.[35] Also, the interaction of reversible inhibitors with MAO-B obtained from baboon liver tissue appears to be similar to the interaction with the human form of the enzyme, since inhibitors such as CSC are approximately equipotent with both enzyme sources.[14] The incubation time of the enzyme catalyzed reaction was 10 min since the rate of MMTP oxidation was found to be linear (Fig. 1) for at least 10 min at all substrate concentrations (30–120 μM) used in the inhibition studies. From Lineweaver–Burke plots generated from data obtained in the absence of inhibitor, we have estimated the K_m value for the oxidation of MMTP by baboon liver MAO-B to be 68.3 ± 1.60 μM, a value consistent with the reported value of 60.8 μM.[35] This K_m was used in the studies where K_i values for the inhibition of MAO-B were calculated from the corresponding IC_{50} values (see below).

All of the C-8 substituted caffeinyl analogues tested were found to be inhibitors of MAO-B. As demonstrated with (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeine (**6a**) (Fig. 2), the lines of the Lineweaver–Burke plots intersected at the *y*-axis, indicating the mode of inhibition to be competitive. Competitive inhibition has also been observed with (*E*)-8-styrylcaffeinyl analogues.[13,14,36] The enzyme–inhibitor dissociation constants (K_i values) for the inhibition of MAO-B by the test compounds are presented in Tables 1–3. The 8-phenylcaffeinyl analogues **4a**–**c** (Table 1) and 8-benzylcaffeinyl analogues **5a**–**c** (Table 2) were found to be relatively weak inhibitors with K_i values ranging from 36.0 to 97.6 μM. Compounds **4c** and **5a** were especially weak inhibitors with only 24.0% and 18.0% inhibition at a concentration of 1000 μM. In contrast, the (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a**–**d** (Table 3) were exceptionally potent MAO-B inhibitors. The most potent inhibitor was (*E*,*E*)-8-[4-(3-bromophenyl)butadien-1-yl]caffeine (**6c**) with a K_i value of 17.2 nM, approximately 3.6 times more potent than that of the corresponding (*E*)-8-(3-bromostyryl)caffeinyl analogue **2c** (Table 4).[36] The second most potent inhibitor was (*E*,*E*)-8-[4-(3-chlorophenyl)butadien-1-yl]caffeine (**6b**) with a K_i value of 42.1 nM. This is approximately 2.5 times more potent than that of the corresponding (*E*)-8-(3-chlorostyryl)caffeinyl analogue

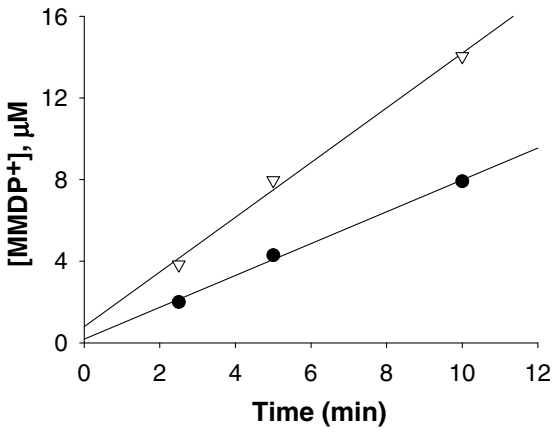


Figure 1. Linearity in the oxidation of MMTP by baboon liver MAO-B (0.15 mg protein/mL of the mitochondrial preparation). The concentration of $MMDP^+$ produced was measured spectrophotometrically following termination of the enzyme catalyzed reaction at time points of 2.5, 5, and 10 min. The concentrations of MMTP used in this study were 30 μM (filled circles) and 120 μM (open triangles).

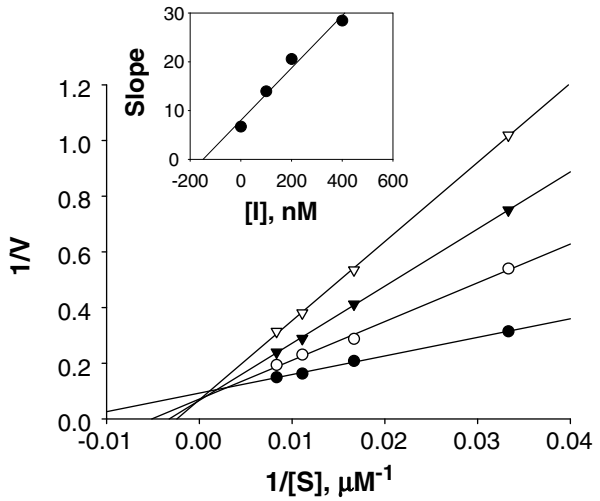
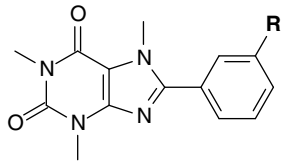


Figure 2. Lineweaver–Burke plots of the oxidation of MMTP by baboon liver MAO-B in the absence (filled circles) and presence of various concentrations of **6a** (open circles, 0.1 μM; filled triangles, 0.2 μM; open triangles, 0.4 μM). The concentration of the baboon liver mitochondrial preparation was 0.15 mg protein/mL, and the rates are expressed as nmol of $MMDP^+$ formed/mg protein/min. The inset is the replot of the slopes versus the inhibitor concentrations.

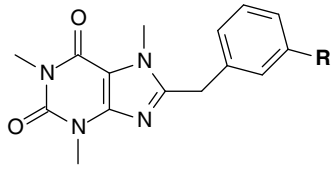
Table 1
The K_i values for the inhibition of MAO-B by 8-phenylcaffeinyl analogues **4a–c**



	R	K_i value[a] (μM)
4a	H	86.2
4b	Cl	36.0
4c	CF_3	24.0%[b]

[a] The enzyme source used was baboon liver mitochondrial MAO-B.
[b] Percentage inhibition at an inhibitor concentration of 1000 μM. Due to limited solubility in the aqueous incubation solvent, higher concentrations were not tested.

Table 2
The K_i values for the inhibition of MAO-B by 8-benzylcaffeinyl analogues **5a–c**



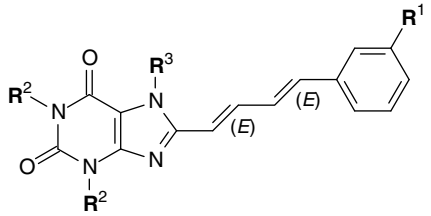
	R	K_i value[a] (μM)
5a	H	18.0%[b]
5b	Cl	54.6
5c	CF_3	97.6

[a] The enzyme source used was baboon liver mitochondrial MAO-B.
[b] Percentage inhibition at an inhibitor concentration of 1000 μM. Due to limited solubility in the aqueous incubation solvent, higher concentrations were not tested.

CSC (**2b**) that has a reported K_i value for the inhibition of baboon liver MAO-B of 128 nM.[14] The K_i value for CSC determined in this study is 80.6 nM (Table 4). This trend also exists for the other (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a** and **6d** with the most dramatic difference in activities found with **6a** (K_i = 148.6 nM) that is 19 times more potent than (*E*)-8-styrylcaffeine (**2a**) with a reported K_i value of 2864 nM.[14] In view of the exceptional MAO-B inhibition potencies of **6a–d**, we have expanded the series to include the ethyl homologs **6e–g**. These compounds provided an opportunity to examine the effects that homologation at positions 1, 3, and 7 of the caffeinyl ring would have on MAO-B inhibition potency and A_{2A} antagonism activity. As shown in Table 3, **6e–g** proved to be much weaker MAO-B inhibitors than the corresponding methyl analogues **6a–d**. For example, the K_i value for the inhibition of MAO-B by **6e** (1712 nM) was over 10 times higher than the corresponding value for **6a**. Similarly, at a relatively high concentration of 30 μM, **6f** and **6g** exhibited only 32.7% and 14.6% MAO-B inhibition, respectively.

For the (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a–e** we have also measured the IC_{50} values for the inhibition of

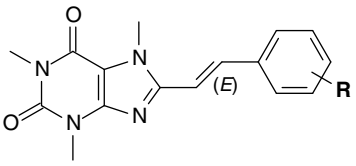
Table 3
The K_i and IC_{50} values for the inhibition of MAO-B by (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a–g**



	R1	R^2	R^3	Exp. K_i[a] (nM)	Exp. IC_{50}[a,b] (nM)	Calcd K_i[c] (nM)
6a	H	CH_3	CH_3	148.6	383 ± 5.65	221.1
6b	Cl	CH_3	CH_3	42.1	96.3 ± 22.2	55.6
6c	Br	CH_3	CH_3	17.2	37.9 ± 7.42	21.9
6d	F	CH_3	CH_3	46.4	89.0 ± 6.20	51.4
6e	H	CH_3	C_2H_5	1712	2371 ± 42.5	1369
6f	H	C_2H_5	CH_3	—	32.7%[d]	—
6g	H	C_2H_5	C_2H_5	—	14.6%[d]	—

[a] The enzyme source used was baboon liver mitochondrial MAO-B.
[b] The IC_{50} values were experimentally determined by fitting the rate data to the one site competition model incorporated into the Prism software package.
[c] The K_i values were calculated from the experimental IC_{50} values according to the equation by Cheng and Prusoff: $K_i = IC_{50}/(1 + [S]/K_m)$ with [S] = 50 μM and K_m (MMTP) = 68.3 ± 1.60 μM.[27]
[d] Percentage inhibition at an inhibitor concentration of 30 μM. Due to limited solubility in the aqueous incubation solvent, higher concentrations were not tested, and K_i values were not determined.

Table 4
The K_i and IC_{50} values for the inhibition of MAO-B by (*E*)-8-styrylcaffeinyl analogues **2a–d**



	R	Exp. K_i[a] (nM)	Exp. IC_{50}[a,b] (nM)	Calcd K_i[c] (nM)
2a	H	2864[d]	Not determined[e]	—
2b	3-Cl	80.6 ± 1.96; 128[d]	146 ± 1.42	84.3
2c	3-Br	62.7 ± 2.73; 83[f]	107 ± 4.59	61.8
2d	3,4-Cl2	18.9 ± 0.89; 36[f]	28.4 ± 1.07	16.4

[a] The enzyme source used was baboon liver mitochondrial MAO-B.
[b] The IC_{50} values were experimentally determined by fitting the rate data to the one site competition model incorporated into the Prism software package.
[c] The K_i values were calculated from the experimental IC_{50} values according to the equation by Cheng and Prusoff: $K_i = IC_{50}/(1 + [S]/K_m)$ with [S] = 50 μM and K_m (MMTP) = 68.3 ± 1.60 μM.[27]
[d] Value obtained from Ref. 14.
[e] For weak inhibitors limits of aqueous solubility prevents accurate IC_{50} determinations.
[f] Value obtained from Ref. 36.

MAO-B. An example of the data routinely obtained for the IC_{50} determinations is illustrated by (*E*,*E*)-8-[4-(3-chlorophenyl)butadien-1-yl]caffeine (**6b**) (Fig. 3). Considering that the substrate concentration used for the IC_{50} determinations was 50 μM and the K_m value of MMTP oxidation by baboon liver mitochondrial MAO-B is 68.3 ± 1.60 μM, we have also calculated the K_i values from the measured IC_{50} values. As mentioned in the introduction, the relationship between K_i and IC_{50} of a competitive inhibitor of a monosubstrate reaction is described by the Cheng–Prusoff equation: $K_i = IC_{50}/(1 + [S]/K_m)$.[27] As shown in Table 3, the calculated K_i values closely approximate those that were experimentally determined, and the differences are within the range expected for experimental error. For example, the K_i value for the reversible interaction of **6a** with MAO-B was measured as 148.6 nM, while the value calculated from the IC_{50} was found to be 221.1 nM. The same trend is observed for (*E*)-8-styrylcaffeinyl analogues **2b**–**d** (Table 4) where the experimentally determined K_i value of CSC (**2b**), for example, was 80.6 nM while the value calculated from the corresponding IC_{50} was 84.3 nM. It should be noted that the accuracy of an IC_{50} determination is dependent upon adequately defining the sigmoid curve (obtained from plotting the MAO-B catalyzed MMTP oxidation rate versus the logarithm of the inhibitor concentration) at both low and high inhibitor concentrations. For relatively weak inhibitors such as **2a**, the limit of solubility in the aqueous incubation solvent prevents the definition of the curve at higher inhibitor concentrations (maximal inhibition) and an accurate IC_{50} determination is therefore not possible.

2.3. Adenosine A_{2A} receptor antagonism studies

The (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a**–**g** were selected for further evaluation as potential antagonists of the adenosine A_{2A} receptor. The potencies by which the test compounds antagonize A_{2A} receptors were determined by the radioligand binding procedure described in lit.[37] Binding to the A_{2A} receptors was evaluated with *N*-[^{3}H]ethyladenosin-5′-uronamide ([^{3}H]NECA) in rat striatal membranes in the presence of N^6-cyclopentyladenosine (CPA) to minimize the adenosine A_1 receptor binding component of [^{3}H]NECA. This procedure is frequently used to identify compounds that exhibit high binding affinity and selectivity to A_{2A} receptors.[11,12,29] As positive controls we included the known A_{2A} antagonists CSC (**2b**) and KW-6002 (**1**). As reported in Table 5, a K_i value of 30.2 ± 5.40 nM was observed for CSC. This value corresponds well with the lit. values of 36 ± 6 nM[12] and 54 ± 19 nM.[11] The K_i value obtained for KW-6002 (**1**) was 4.46 ± 1.13 nM that compares favorably with the lit. value of 2.2 ± 0.34 nM.[10]

As shown in Table 5 all of the (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues (**6a**–**g**) tested were found to be potent antagonists of the A_{2A} receptor. The dose–inhibition curves for the test compounds versus [^{3}H]NECA that were routinely observed are illustrated by example with **6f** (Fig. 4). This analogue was found to be the most potent antagonist with a K_i value of 2.74 ± 0.35 nM. Since **6f** is approximately 55 times more potent than the corresponding caffeinyl analogue **6a**, 1,3-diethyl substitution of the xanthinyl ring leads to enhanced A_{2A} antagonism potency compared to 1,3-dimethyl substitution.

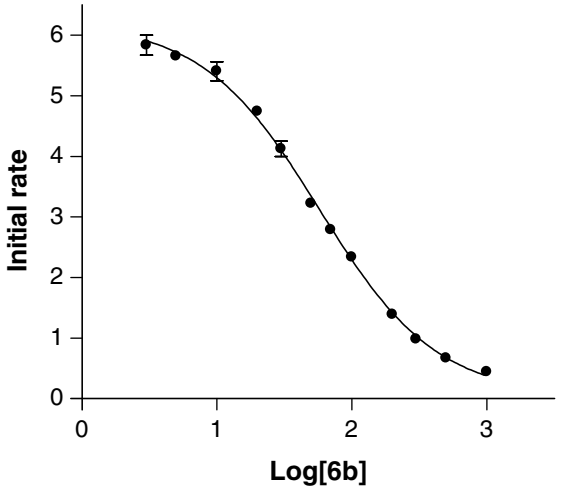
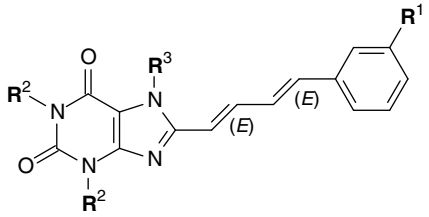


Figure 3. The sigmoidal dose–response curve of the initial rates of oxidation of MMTP versus the logarithm of concentration of inhibitor **6b** (expressed in nM). The concentration of the baboon liver mitochondrial preparation was 0.15 mg protein/mL; the rates are expressed as nmol $MPDP^+$ formed/mg protein/min; the concentration of MMTP used was 50 μM. The determinations were carried out in duplicate and the values are expressed as mean ± SEM.

3. Discussion

We have recently reported that several (*E*)-8-styrylcaffeines act as potent reversible inhibitors of MAO-B.[13,14,36] (*E*)-8-Styrylcaffeines have also been shown to be antagonists of adenosine A_{2A} receptors.[11,12,29] In the present study, using (*E*)-8-styrylcaffeine as the lead compound, we have attempted to identify additional dual-target-directed compounds, possibly with enhanced MAO-B inhibition and A_{2A} antagonism potencies. This study also served to elucidate the structural requirements of C-8 substituted caffeinyl analogues to act as dual inhibitors of MAO-B and antagonists of the adenosine A_{2A} receptor. Since caffeine (**3**) is a weak inhibitor of MAO-B[5] and only a moderately potent adenosine A_{2A} antagonist,[12] it can be concluded that the (*E*)-styryl group at C-8 plays an important role in the dual-action of (*E*)-8-styrylcaffeines. In this study we have prepared three additional classes of C-8 substituted caffeinyl analogues **4**, **5**, and **6**. The series of (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a**–**d** was expanded to include the ethyl homologs **6e**–**g** that provided additional insight into the ste-

Table 5
The K_i values for the competitive inhibition of [^{3}H]NECA binding to rat striatal adenosine A_{2A} receptors by selected caffeinyl analogues



	R^1	R^2	R^3	K_i value[a] (nM)
6a	H	CH_3	CH_3	153 ± 5.40
6b	Cl	CH_3	CH_3	104 ± 1.50
6c	Br	CH_3	CH_3	59.1 ± 15.8
6d	F	CH_3	CH_3	114 ± 14.2
6e	H	CH_3	C_2H_5	13.5 ± 4.87
6f	H	C_2H_5	CH_3	2.74 ± 0.35
6g	H	C_2H_5	C_2H_5	7.73 ± 2.80
1	KW-6002			4.46 ± 1.13; (2.2)[b]
2b	CSC			30.2 ± 5.40; (36)[c]; (54)[d]

[a] The K_i values for the competitive inhibition of [^{3}H]NECA (K_d = 15.3 nM) binding was calculated from the corresponding IC_{50} values according to the Cheng–Prusoff equation.[27] The IC_{50} values were in turn determined by fitting the data, using nonlinear least-squares regression analysis, to the one site competition model incorporated into the Prism software package.
[b] Value obtained from Ref. 10.
[c] Value obtained from Ref. 12.
[d] Value obtained from Ref. 11.

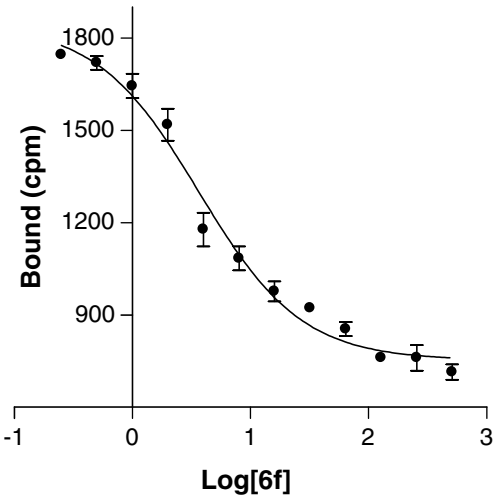


Figure 4. The sigmoidal dose–response curve for the inhibition of [^{3}H]NECA binding to rat striatal A_{2A} receptors by antagonist **6f** (expressed in nM). The IC_{50} value was determined by fitting the data, using nonlinear least-squares regression analysis, to the one site competition model incorporated into the Prism software package (GraphPad Software Inc.). The K_i value (2.74 nM) for the competitive inhibition of [^{3}H]NECA (K_d = 15.3 nM) binding was calculated with the Cheng–Prusoff equation. [27] In this experiment the non-saturable, nonspecific binding was 623 cpm. The determinations were carried out in duplicate and the values are expressed as mean ± SEM.

ric features associated with the potencies of MAO-B inhibition and A_{2A} antagonism.

The results of MAO-B inhibition studies have shown that the 8-phenylcaffeinyl (**4a**–**c**) and 8-benzylcaffeinyl (**5a**–**c**) analogues were weak inhibitors of MAO-B while the (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a**–**d** were found to be exceptionally potent inhibitors. For example, the (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogue **6b** substituted with chlorine at C-3 of the phenyl ring (K_i = 42.1 nM) was approximately 855 and 1269 times more potent as an MAO-B inhibitor than was the corresponding C-3 chlorine substituted 8-phenylcaffeinyl analogue **4b** (K_i = 36.0 μM) and 8-benzylcaffeinyl analogue **5b** (K_i = 54.6 μM), respectively. The (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a**–**d** were also more potent inhibitors than the corresponding (*E*)-8-styrylcaffeinyl analogues **2a**–**c**. For example, chloro substituted analogue **6b** (K_i = 42.1 nM) was approximately 1.9 times more potent than CSC (**2b**) (K_i = 80.6 nM) while the unsubstituted (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogue **6a** (K_i = 148.6 nM) was almost 20 times more potent than the corresponding unsubstituted (*E*)-8-styrylcaffeinyl analogue **2a** (K_i = 2864 nM). Compounds **6e**–**g** were found to be relatively weak inhibitors of MAO-B indicating that ethyl substitution at positions 1, 3, and 7 of the caffeinyl ring has a negative effect on the potency of MAO-B inhibition. This result suggests that 1, 3, and 7 methyl substitution is probably optimal for the design of xanthine-based reversible MAO-B inhibitors. In the case of (*E*)-8-styrylcaffeines, 1,3-diethyl substitution of the caffeine ring has also been shown to reduce the MAO-B inhibition potency compared to 1,3-dimethyl substitution.[13]

Since the series of (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues was found to include exceptionally potent reversible MAO-B inhibitors, they were selected for further evaluation as potential antagonists of the adenosine A_{2A} receptor. All the (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeinyl analogues **6a**–**g** were found to be relatively potent A_{2A} antagonists. In contrast to its effect on MAO-B inhibition potency, ethyl substitution at positions 1, 3, and 7 of the caffeinyl ring had a positive effect on the potency of A_{2A} antagonism. For example, the 1,3-diethyl analogue **6f** (K_i = 2.74 nM) was approximately 55 times more potent than the corresponding 1,3-dimethyl analogue **6a** (K_i = 153 nM). Although functional antagonism has not been demonstrated, it is reasonable to assume caffeine derived structures would act in an antagonistic manner since caffeine analogues are well-known antagonists of the adenosine A_{2A} receptor.

We conclude that the (*E*,*E*)-8-(4-phenylbutadien-1-yl)caffeines, **6a**–**d**, are promising lead compounds for the development of dual-target-directed compounds that inhibit MAO-B and antagonize A_{2A} receptors. Although 1,3-diethyl substitution of the caffeinyl ring leads to enhanced potency of A_{2A} antagonism, the opposite effect was observed on MAO-B inhibition potency.

In the second part of this study it was shown that for potent inhibitors, the K_i values for competitive interaction with MAO-B could be accurately calculated from the corresponding IC_{50} values using the Cheng–Prusoff equation. This would facilitate the comparison of IC_{50} and K_i values from different lit. sources and the employment of the inhibitors in QSAR studies.

4. Experimental

Caution: MMTP is a structural analogue of the nigrostriatal neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and should be handled using disposable gloves and protective eyewear. Procedures for the safe handling of MPTP have been described previously.[38]

4.1. Chemicals and instrumentation

All starting materials, unless mentioned elsewhere, were obtained from Sigma–Aldrich and were used without purification. The oxalate salt of MMTP,[39] KW-6002 (**1**),[13,29] compounds **2b**–**2d**,[14,36] 1,3-dimethyl- (**7a**) and 1,3-diethyl-5,6-diaminouracil (**7b**)[30] were prepared according to previously reported procedures. Because of chemical instability, compounds **7a–b** were used within 24 h of preparation. Proton and carbon NMR spectra were recorded on a Varian Gemini 300 spectrometer. Proton (^{1}H) spectra were recorded in $CDCl_3$ or DMSO-d_6 at a frequency of 300 MHz and carbon (^{13}C) spectra at 75 MHz. Chemical shifts are reported in parts per million (δ) downfield from the signal of tetramethylsilane added to the deuterated solvent. Spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), or m (multiplet) and the coupling constants (*J*) are given in hertz (Hz). Direct insertion electron impact ionization (EIMS) and high resolution mass spectra (HRMS) were obtained on a VG 7070E mass spectrometer. Melting points (mp) were determined on a Stuart SMP10 melting point apparatus and are uncorrected. UV–vis spectra were recorded on a Shimadzu UV-2100 double-beam spectrophotometer. Thin layer chromatography (TLC) was carried out with neutral aluminum oxide 60 (Merck) containing UV_{254} fluorescent indicator. [^{3}H]NECA was obtained from Amersham (specific activity 25 Ci/mmol), while adenosine deaminase (type X from calf spleen) and CPA were from Sigma–Aldrich. Counting of radio activities was performed using a Packard Tri-Carb 2100 TR liquid scintillation counter.

4.2. General procedure for the synthesis of (*E*,*E*)-5-phenyl-2,4-pentadienoic acids (10a–d)

Compounds **10a**–**d** were prepared from the corresponding cinnamylidenemalonic acids (**12a**–**d**)[32] according to the previously described procedure.[31] Cinnamylidenemalonic acids (**12a**–**d**) (1 g) were allowed to reflux for 60 min with acetic anhydride (5 mL) and acetic acid (3 mL). The reaction was cooled to room temperature and poured into 100 mL water. After 3 h, the resulting precipitate was collected by filtration. The crude product and a crystal of iodine were dissolved in 20 mL $CHCl_3$ and incubated in

ambient light for 5 h. The $CHCl_3$ was removed under reduced pressure and the residue was recrystallized from benzene. For previously described **10a–c**, the melting points were recorded as follows: **10a** 194 °C, lit. 178 °C[31]; **10b** 190 °C, lit. 173–174 °C[40]; **10c** 187 °C, lit. 179–180.[41]

4.2.1. (*E*,*E*)-5-(3-Fluorophenyl)-2,4-pentadienoic acid (10d)

The title compound was prepared from **12d** in a yield of 42%. Mp 164 °C; ^{1}H NMR (DMSO-d_6) δ 6.03 (d, 1H, J = 15.0 Hz), 7.10–7.45 (m, 7H) ^{13}C NMR (DMSO-d_6) δ 112.90, 113.16, 115.33, 123.18, 123.51, 128.09, 130.63, 130.74, 138.19, 143.66, 167.31; EIMS m/z 192 ($M^{\cdot+}$); HRMS calcd 192.05866; found: 192.05936.

4.3. General procedure for the synthesis of caffeinyl analogues (4a–c, 5a–c, and 6a–g)

The C-8 substituted caffeinyl analogues examined in this study were prepared according to the procedure described in lit.[29] 1,3-Dimethyl- (**7a**) or 1,3-diethyl-5,6-diaminouracil (**7b**) (3.50 mmol) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC; 5.11 mmol) were dissolved in 40 mL dioxane/H_2O (1:1) and the appropriate carboxylic acid [benzoic acids (**8a–c**), phenylacetic acids (**9a–c**), or (*E*,*E*)-5-phenyl-2,4-pentadienoic acids (**10a–d**), 3.81 mmol] was added. A suspension was obtained and the pH was adjusted to 5 with 2 M aqueous hydrochloric acid. The reaction mixture was stirred for an additional 2 h and then neutralized with 1 M aqueous sodium hydroxide. After cooling to 0 °C, the precipitate that formed was collected by filtration. A solution of this crude amide in 40 mL aqueous sodium hydroxide (1 M)/dioxane (1:1) was heated under reflux for 2 h, cooled to 0 °C and then acidified to a pH of 4 with 4 M aqueous hydrochloric acid. For the preparation of **4a–c**, **5a–c**, and **6a–e**, the resulting precipitate, the corresponding 1,3-dimethyl-8-substituted-7*H*-xanthinyl analogue (**11**), was collected by filtration and used in the subsequent reaction without further purification. For the preparation of **6f–g**, the resulting precipitate was removed via filtration and the filtrate was extracted to $CHCl_3$ (2× 100 mL). The organic phase was dried over anhydrous $MgSO_4$ and removed under reduced pressure to yield a yellow oily residue, the 1,3-diethyl-8-substituted-7*H*-xanthinyl analogues (**11**). To a stirred suspension of **11** (0.20 mmol) and potassium carbonate (0.50 mmol) in 5 mL DMF was added iodomethane (**4a–c**, **5a–c**, **6a–d**, and **6f**) or iodoethane (**6e** and **6g**) (0.40 mmol). Stirring was continued at 60 °C for 60 min, and the insoluble materials were removed by filtration. Sufficient water was added to the filtrate to precipitate the product (**4–6**) which was collected by filtration. Following crystallization from a mixture of methanol/ethyl acetate (9:1) (**4a–c**, **5a–c**, and **6a–e**) or ethanol (**6f–g**), analytically pure samples of the target compounds were obtained. For previously described **4a** and **5a**, we found the melting points to be 180 and 165 °C [from methanol/ethyl acetate (9:1)] while the reported melting points are 178 °C[12] and 161–163 °C,[42] respectively.

4.3.1. 8-(3-Chlorophenyl)caffeine (4b)

The title compound was prepared from 1,3-dimethyl-5,6-diaminouracil (**7a**), 3-chlorobenzoic acid (**8b**), and iodomethane in a yield of 91%: mp 202 °C; ^{1}H NMR ($CDCl_3$) δ 3.39 (s, 3H), 3.58 (s, 3H), 4.04 (s, 3H), 7.43–7.48 (m, 2H), 7.53–7.56 (m, 1H), 7.67–7.69 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 27.97, 29.72, 33.90, 108.73, 127.07, 129.28, 130.10, 130.42, 135.05, 148.16, 150.40, 151.60, 155.51; EIMS m/z 304 ($M^{\cdot+}$); HRMS calcd 304.07270; found: 304.07108.

4.3.2. 8-(3-Trifluoromethylphenyl)caffeine (4c)

The title compound was prepared from 1,3-dimethyl-5,6-diaminouracil (**7a**), 3-trifluoromethylbenzoic acid (**8c**), and iodomethane in a yield of 33.6%: mp 192 °C; ^{1}H NMR ($CDCl_3$) δ 3.39 (s, 3H), 3.59 (s, 3H), 4.05 (s, 3H), 7.61–7.67 (m, 1H), 7.73–7.77 (m, 1H), 7.84–7.88 (m, 1H), 7.95–7.96; ^{13}C NMR ($CDCl_3$) δ 27.97, 29.74, 33.87, 108.84, 121.74, 126.14 (q), 126.93 (q), 129.32, 129.46, 131.62 (q), 132.26, 148.20, 150.23, 151.59, 155.52; EIMS m/z 338 ($M^{\cdot+}$); HRMS calcd 338.09906; found: 338.09735.

4.3.3. 8-(3-Chlorobenzyl)caffeine (5b)

The title compound was prepared from 1,3-dimethyl-5,6-diaminouracil (**7a**), 3-chlorophenylacetic acid (**9b**) and iodomethane in a yield of 42%: mp 132 °C; ^{1}H NMR ($CDCl_3$) δ 3.36 (s, 3H), 3.56 (s, 3H), 3.79 (s, 3H), 4.10 (s, 2H), 7.02–7.05 (m, 1H), 7.14–7.16 (m, 1H), 7.21–7.24 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 27.83, 29.74, 31.00, 32.96, 107.85, 126.38, 127.54, 128.35, 130.16, 134.84, 137.00, 147.87, 151.20, 151.59, 155.30; EIMS m/z 319 ($M^{\cdot+}$); HRMS calcd 318.08835; found: 318.08702.

4.3.4. 8-(3-Trifluoromethylbenzyl)caffeine (5c)

The title compound was prepared from 1,3-dimethyl-5,6-diaminouracil (**7a**), 3-(trifluoromethyl)phenylacetic acid (**9c**), and iodomethane in a yield of 49%: mp 163 °C; ^{1}H NMR ($CDCl_3$) δ 3.36 (s, 3H), 3.55 (s, 3H), 3.81 (s, 3H), 4.18 (s, 2H), 7.34–7.52 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 27.83, 29.72, 31.00, 33.14, 107.86, 122.00, 124.23 (q), 125.08 (q), 125.61, 129.45, 131.37 (q), 131.62, 136.09, 147.90, 151.00, 151.60, 155.31; EIMS m/z 352 ($M^{\cdot+}$); HRMS calcd 352.11471; found: 352.11570.

4.3.5. (*E*,*E*)-8-(4-Phenylbutadien-1-yl)caffeine (6a)

The title compound was prepared from 1,3-dimethyl-5,6-diaminouracil (**7a**), (*E*,*E*)-5-phenyl-2,4-pentadienoic acid (**10a**), and iodomethane in a yield of 27%: mp 253 °C; ^{1}H NMR ($CDCl_3$) δ 3.37 (s, 3H), 3.58 (s, 3H), 3.96 (s, 3H), 6.44 (d, 1H, J = 15.0 Hz), 6.84–6.95 (m, 2H), 7.24–7.36 (m, 3H), 7.42–7.46 (m, 2H), 7.56 (dd, 1H, J = 15.0, 10.0 Hz); ^{13}C NMR ($CDCl_3$) δ 27.86, 29.66, 31.35, 107.82, 114.46, 126.90, 127.30, 128.65, 128.77, 136.40, 138.14, 138.50, 148.61, 149.98, 151.64, 155.13; EIMS m/z 322 ($M^{\cdot+}$); HRMS calcd 322.14298; found: 322.14186.

4.3.6. (*E*,*E*)-8-[4-(3-Chlorophenyl)butadien-1-yl]caffeine (6b)

The title compound was prepared from 1,3-dimethyl-5,6-diaminouracil (**7a**), (*E*,*E*)-5-(3-chlorophenyl)-2,4-pentadienoic acid (**10b**), and iodomethane in a yield of 10%: mp 249 °C; ^{1}H NMR ($CDCl_3$) δ 3.39 (s, 3H), 3.59 (s, 3H), 3.99 (s, 3H), 6.49 (d, 1H, J = 15.0 Hz), 6.80 (d, 1H, J = 15.5 Hz), 6.92–7.01 (m, 1H), 7.24–7.31 (m, 3H), 7.44 (d, 1H, J = 0.41 Hz), 7.55 (dd, 1H, J = 14.7, 10.9 Hz); ^{13}C NMR ($CDCl_3$) δ 27.92, 29.71, 31.42, 108.01, 115.47, 125.15, 126.61, 128.48, 128.67, 130.01, 134.85, 136.34, 137.87, 138.32, 148.65, 149.72, 151.68, 155.21; EIMS m/z 357 ($M^{\cdot+}$); HRMS calcd 356.10400; found: 356.10571.

4.3.7. (*E*,*E*)-8-[4-(3-Bromophenyl)butadien-1-yl]caffeine (6c)

The title compound was prepared from 1,3-dimethyl-5,6-diaminouracil (**7a**), (*E*,*E*)-5-(3-bromophenyl)-2,4-pentadienoic acid (**10c**), and iodomethane in a yield of 40%: mp 246 °C; ^{1}H NMR ($CDCl_3$) δ 3.38 (s, 3H), 3.58 (s, 3H), 3.98 (s, 3H), 6.49 (d, 1H, J = 15.0 Hz), 6.78 (d, 1H, J = 15.5 Hz), 6.95 (dd, 1H, J = 15.5, 10.9 Hz), 7.17–7.22 (m, 1H), 7.33–7.40 (m, 2H), 7.55 (dd, 1H, J = 14.6, 10.9 Hz), 7.59 (d, 1H, J = 1.8 Hz); ^{13}C NMR ($CDCl_3$) δ 27.91, 29.70, 31.40, 108.00, 115.50, 123.01, 125.58, 128.70, 129.53, 130.27, 131.38, 136.20, 137.82, 138.60, 148.63, 149.70, 151.67, 155.20; EIMS m/z 400, 402 ($M^{\cdot+}$); HRMS calcd 400.05349; found: 400.05193.

4.3.8. (*E*,*E*)-8-[4-(3-Fluorophenyl)butadien-1-yl]caffeine (6d)

The title compound was prepared from 1,3-dimethyl-5,6-diaminouracil (**7a**), (*E*,*E*)-5-(3-fluorophenyl)-2,4-pentadienoic acid

(**10d**), and iodomethane in a yield of 22.1%: mp 223 °C; ^{1}H NMR ($CDCl_3$) δ 3.22 (s, 3H), 3.44 (s, 3H), 3.95 (s, 3H), 6.87 (d, 1H, J = 15.0 Hz), 7.01 (d, 1H, J = 15.4 Hz), 7.03–7.15 (m, 1H), 7.26 (dd, 1H, J = 15.4, 11.0 Hz), 7.35–7.44 (m, 3H), 7.47 (dd, 1H, J = 14.6, 11.0 Hz); EIMS m/z 340 ($M^{\cdot+}$); HRMS calcd 340.13355; found: 340.13367.

4.3.9. (*E*,*E*)-1,3-Dimethyl-8-(4-phenylbutadien-1-yl)-7-ethylxanthine (6e)

The title compound was prepared from 1,3-dimethyl-5,6-diaminouracil (**7a**), (*E*,*E*)-5-phenyl-2,4-pentadienoic acid (**10a**), and iodoethane in a yield of 6.9%: mp 227 °C; ^{1}H NMR ($CDCl_3$) δ 1.46 (t, 3H, J = 7.2 Hz), 3.43 (s, 3H), 3.63 (s, 3H), 4.45 (q, 1H, J = 7.2 Hz), 6.50 (d, 1H, J = 14.9 Hz), 6.92 (d, 1H, J = 15.5 Hz), 7.01 (dd, 1H, J = 15.5, 11.0 Hz), 7.31 (t, 1H, J = 7.3 Hz), 7.38 (t, 2H, J = 7.4 Hz), 7.49 (d, 2H, J = 7.4 Hz), 7.63 (dd, 1H, J = 14.9, 10.9 Hz); ^{13}C NMR ($CDCl_3$) δ 16.65, 28.12, 29.93, 40.18, 107.76, 115.16, 127.73, 128.16, 129.48, 129.63, 137.30, 139.00, 139.40, 149.78, 150.10, 152.69, 155.77; EIMS m/z 336 ($M^{\cdot+}$); HRMS calcd 336.15863;found:336.15872.

4.3.10. (*E*,*E*)-1,3-Diethyl-8-(4-phenylbutadien-1-yl)-7-methylxanthine (6f)

The title compound was prepared from 1,3-diethyl-5,6-diaminouracil (**7b**), (*E*,*E*)-5-phenyl-2,4-pentadienoic acid (**10a**),and iodomethane in a yield of 24%: mp 156–157 °C; ^{1}H NMR ($CDCl_3$) δ 1.19 (t, 3H, J = 7.1 Hz), 1.30 (t, 3H, J = 7.0 Hz), 3.93 (s, 3H), 4.01 (q, 2H, J = 6.9 Hz), 4.13 (q, 2H, J = 7.0 Hz), 6.41(d, 1H, J = 15.0 Hz), 6.84 (d, 1H, J = 15.5 Hz), 6.92 (dd, 1H, J = 15.5, 11.2 Hz), 7.23 (m, 1H), 7.30 (t, 2H, J = 7.4 Hz), 7.40 (d, 2H, J = 7.8 Hz), 7.53 (dd, 1H, J = 14.9, 10.9 Hz); ^{13}C NMR ($CDCl_3$) δ 13.49, 29.90, 31.57, 36.60, 38.68, 108.76, 115.30, 127.73, 128.20, 129.48, 129.64, 137.33, 138.93, 139.38, 149.10, 150.90, 151.70, 156.00; EIMS m/z 350 ($M^{\cdot+}$); HRMS calcd 350.17428; found: 350.17277.

4.3.11. (*E*,*E*)-1,3-Diethyl-8-(4-phenylbutadien-1-yl)-7-ethylxanthine (6g)

The title compound was prepared from 1,3-diethyl-5,6-diaminouracil (**7b**), (*E*,*E*)-5-phenyl-2,4-pentadienoic acid (**10a**), and iodoethane in a yield of 21%: mp 177–178 °C; ^{1}H NMR ($CDCl_3$) δ 1.19 (t, 3H, J = 7.1 Hz), 1.31 (t, 3H, J = −7.0 Hz), 1.38 (t, 3H, J = 7.2 Hz), 4.01 (q, 2H, J = 7.0 Hz), 4.13 (q, 2H, J = 7.0 Hz), 4.36 (q, 2H, J = 7.2 Hz), 6.41 (d, 1H, J = 14.9 Hz), 6.84 (d, 1H, J = 15.5 Hz), 6.93 (dd, 1H, J = 15.5, 11.0 Hz), 7.22 (m, 1H), 7.29 (t, 2H, J = 7.6 Hz), 7.41 (d, 2H, J = 8.0 Hz), 7.55 (dd, 1H, J = 14.9, 11.0 Hz); ^{13}C NMR ($CDCl_3$) δ 13.47, 16.68, 29.89, 36.62, 38.64, 40.10, 108.00, 115.38, 127.70, 128.26, 129.43, 129.63, 137.37, 138.73, 139.23, 149.33, 149.98, 151.74, 155.57; EIMS m/z 364 ($M^{\cdot+}$); HRMS calcd 364.18993; found: 364.18904.

4.4. MAO-B inhibition studies

The mitochondrial fraction of baboon liver tissue was isolated as described previously[43] and stored at –70 °C. Following addition of an equal volume of sodium phosphate buffer (100 mM, pH 7.4) containing glycerol (50%, w/v) to the mitochondrial isolate, the protein concentration was determined by the method of Bradford using bovine serum albumin as reference standard.[44] MMTP (K_m = 68.3 ± 1.60 μM for baboon liver MAO-B),[35] served as substrate for the inhibition studies. The enzymatic reactions were prepared in sodium phosphate buffer (100 mM, pH 7.4) and contained MMTP (30–120 μM), the mitochondrial isolate (0.15 mg protein/mL) and various concentrations of the test inhibitors. The final volume of the incubations was 500 μL. For the IC_{50} determinations, a fixed substrate concentration of 50 μM was used and the inhibitor concentrations spanned at least three orders of a magnitude (3–1000 μM). The stock solutions of the inhibitors were prepared in DMSO and were added to the incubation mixtures to yield a final DMSO concentration of 4% (v/v). DMSO concentrations higher than 4% are reported to inhibit MAO-B.[45] The reactions were incubated at 37 °C for 10 min and then terminated by the addition of 10 μL perchloric acid (70%). The MAO-B catalyzed production of $MMDP^+$ was found to be linear for the first 10 min of incubation under these conditions. The samples were centrifuged at 16,000*g* for 10 min, and the concentrations of the MAO-B generated product, $MMDP^+$, were measured spectrophotometrically at 420 nm (ε = 25,000 M^{-1}) in the supernatant fractions.[35] For the K_i determinations, the initial rates of oxidation at four different substrate concentrations (30–120 μM) in the absence and presence of three different concentrations of the inhibitors were used to construct Lineweaver–Burke plots. The slopes of the Lineweaver–Burke plots were plotted versus the inhibitor concentration and the K_i value were determined from the abscissa intercept (intercept = $-K_i$). Linear regression analysis was performed using the SigmaPlot software package (Systat Software Inc.). Each K_i value reported here is representative of a single determination where the correlation coefficient (R^2 value) of the replot of the slopes versus the inhibitor concentrations was at least 0.98. The IC_{50} values were determined by plotting the initial rates of oxidation versus the logarithm of the inhibitor concentrations to obtain a sigmoidal dose–response curve. This kinetic data were fitted to the one site competition model incorporated into the Prism 4 software package (GraphPad Software Inc.). The IC_{50} values were determined in duplicate and are expressed as mean ± standard error of the mean (SEM).

4.5. Adenosine A_{2A} receptor antagonism studies

The adenosine A_{2A} receptor binding studies were carried out according the procedure described in lit.[37] The striata of male Sprague–Dawley rats (n = 40) were dissected, immediately frozen in liquid nitrogen and stored at −70 °C. The striata were thawed on ice, weighed and disrupted for 30 s with the aid of a Polytron homogenizer in 10 volumes of ice-cold 50 mM Tris·HCl (pH 7.7 at 25 °C). The resulting homogenate was centrifuged at 50,000*g* for 10 min and the pellet was resuspended in 10 volumes of ice-cold Tris·HCl, again with the aid of a Polytron homogenizer as above. The resulting suspension was recentrifuged and the pellet obtained was suspended in Tris·HCl to a volume of 5 mL/g original striatal weight. The striatal membranes were aliquoted into microcentrifuge tubes and stored at −70 °C. The incubations were carried out in 4 mL polypropylene tubes, previously coated with Sigmacote (Sigma–Aldrich) and were prepared in 1 mL Tris·HCl containing 5 mg of the original tissue weight striatal membranes, 4 nM [^{3}H]NECA, 50 nM CPA, 10 mM $MgCl_2$, 0.1 U/mL adenosine deaminase, and various concentrations of the test compounds. The stock solutions of the compounds to be tested as well as that of CPA were prepared in DMSO. On the day of the study, CPA and [^{3}H]NECA were diluted to concentrations of 500 nM and 40 nM, respectively. A membrane suspension was prepared that contained 5 mg/0.79 mL striatal membranes and sufficient amounts of $MgCl_2$ and adenosine deaminase to produce concentrations of 10 mM and 0.1 U/mL, respectively, in the final 1 mL incubation mixtures. The order of additions was as follows: test compound (10 μL), CPA (100 μL), [^{3}H]NECA (100 μL), and the membrane suspension (0.79 mL). The incubations were vortexed and incubated for 60 min at 25 °C in a shaking water bath. Halfway through the experiment, the incubations were vortexed again. The incubations were filtered through a prewetted 2.4 cm Whatman glass microfiber filter (grade GF/B) under reduced pressure. The tubes were washed with 4 mL ice-cold Tris·HCl and the filters were washed twice more with 4 mL ice-cold Tris·HCl. The damp filters were place in scintillation vials, 4 mL of

Filter-Count (Perkin-Elmer) was added and the vials were incubated overnight before being counted. The IC_{50} values were determined by plotting the count values versus the logarithm of the inhibitor concentrations to obtain a sigmoidal dose–response curve. This kinetic data were fitted to the one site competition model incorporated into the Prism 4 software package (GraphPad Software Inc.). The K_i values for the competitive inhibition of [^{3}H]NECA (K_d = 15.3 nM) binding by the test compounds were calculated according to the Cheng and Prusoff equation.[27] All incubations were carried out in duplicate and the K_i values are expressed as mean ± SEM. An estimate of the nonspecific binding was obtained from binding studies in the presence of 100 μM CPA.

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