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Synthesis and biological evaluation of pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane derivatives as potential therapeutic agents in Parkinson's disease

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Abstract—In previous studies, the polycyclic cage amine 8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (NGP1-01) and a number of its derivatives showed positive effects in neuroprotection studies with MPTP, in vivo. In view of these findings, we examined these compounds for their effects on [^{3}H]dopamine ([^{3}H]DA) release and uptake inhibition in murine striatal synaptosomes, as well as for inhibition of baboon liver monoamine oxidase (MAO) B. In order to assess specificity, initial experiments focused on compounds that blocked dopamine uptake without causing appreciable release (<40% at 100 μM) of the transmitter. NGP1-01 blocked the uptake of [^{3}H]DA with an IC_{50} of 57 μM, while another compound, 8-phenylethyl-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane, blocked uptake at an IC_{50} value of 23 μM. These values were comparable to that of another polycyclic cage amine, amantadine (IC_{50}; 82 μ), that is used in parkinsonian therapy. Structure–activity relationships of this series of compounds support the importance of geometric and steric, rather than electronic effects, in determining biological activity. MAO-B inhibition for this group was weak, with less than 50% inhibition at 300 μM for any of the compounds in the series. The present study suggests that blockage of the dopamine transporter may underlie, at least in part, their neuroprotective effects against MPTP-induced parkinsonism. These compounds may be considered as potential lead compounds for Parkinson's Disease therapy.

1. Introduction

Parkinson's disease (PD) is a terminal, late-onset neurodegenerative disorder characterized by a progressive and relatively selective degeneration of dopaminergic neurons in the substantia nigra.[1] To date, most therapies for PD are symptomatic in nature, with no neuroprotective therapies currently approved for prevention of this chronic disease. Some therapeutic agents used in the early treatment of PD have, however, been shown to have neuroprotective activity in addition to their symptomatic effects, such as the dopamine agonist, pramipexole.[2]

Amongst several drug therapies used in the symptomatic treatment of PD patients is the polycyclic cage amine, amantadine (Fig. 1). Amantadine expresses its antiparkinsonian activity, at least in part, by increasing extracellular dopamine levels via re-uptake inhibition[3] or dopamine (DA) release.[4] Electrophysiological studies further indicated that amantadine binds to the phencyclidine (PCP) or MK-801 (dizocilpine) binding site located within the NMDA receptor/ion channel complex.[5] In addition, amantadine and its dimethyl derivative memantine have been shown to be neuroprotective.[5,6]

Early interest in the in vivo biological activities of polycyclic cage amines was raised when Oliver et al.[7,8] reported antagonism of reserpine-induced catatonia as well as reduction of oxotremorine-induced tremor and salivation in rats by novel pentacycloundecylamines and trishomocubanes, at doses comparable to those of

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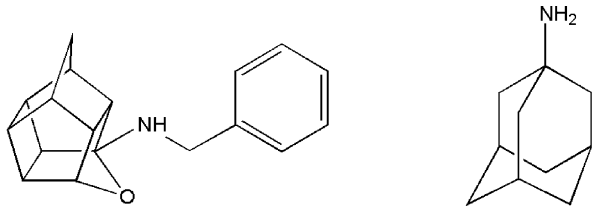


1 Amantadine

Figure 1. Structures of the prototypical cage amines NGP1-01 (**1**) and amantadine.

amantadine. These authors suggested that the polycyclic cage amines might be a new class of possible anti-parkinsonian agents due to anticataleptic and mild to weak anticholinergic activities.

L-type calcium channel antagonism has been reported by our laboratory for pentacycloundecylamines, in particular for the prototypical compound 8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (Fig. 1, **1**, NGP1-01).[9–13] Recently, we reported an in vivo pilot evaluation of a small series of pentacycloundecylamines in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) parkinsonian mouse model and found that one of the compounds, 8-phenylethyl-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane, tested positively as a neuroprotective agent.[14] MPTP is a protoxin that requires activation by means of monoamine oxidase (MAO) B to form the neurotoxic pyridinium species, 1-methyl-4-phenyl-pyridinium (MPP$^+$).[15] MPP$^+$ is then transported into dopaminergic neurons via the dopamine transporter (DAT), where it inhibits mitochondrial complex 1.[16,17] Excitotoxicity is also implicated in the MPTP model of neuronal cell death, and involves the over-stimulation of the *N*-methyl-D-aspartate (NMDA) receptor by glutamate, ultimately resulting in excess intracellular calcium and cell death.[18,19]

On the basis of this information, we have chosen to evaluate a series of polycyclic cage amines for activity in the dopamine system as part of an ongoing investigation into the therapeutic potential of novel pentacycloundecylamines. We are hoping to develop drugs that are dual neuroprotective and symptomatic therapy for neurodegenerative diseases such as PD. Since the DAT and MAO-B are key elements in the DA depletion seen in the MPTP mouse model, we investigated the pharmacology of the compounds in this study for interaction with the DAT and MAO-B in vitro, aiming to select compounds for future in vivo studies. Moreover, because our future plans include analysis of effects on the NMDA receptor and MPTP/C57 mouse model, we included MK-801 as an established NMDA antagonist in the present study.

2. Chemistry

Van der Schyf et al.[9–12] postulated that the cage-like pentacycloundecane skeleton mostly served as a bulk contributor to the calcium channel blocking activity of the parent compound, **1**. Structure–activity studies were combined with synthetic efforts to investigate the effects of different aromatic amine side chains as well as gaining insight into the effects of the polycyclic skeleton.

Synthesis of compounds **1**–**7** and **9** was conducted as previously described[12,13] and is shown in Scheme 1. The common starting diketone (**a**), was prepared according to the method of Cookson et al.[20] Reductive amination of **a** followed by Dean–Stark dehydration and reduction with $NaBH_4$ furnished **1**–**5**, and **9**. Syntheses of **6** and **7** were achieved following our previously reported procedure.[13] Transformation of **a** by a boron trifluoride etherate reaction with ethyl diacetate, followed by subsequent decarboxylation, yielded **11**, the starting ketone for **6**. Similarly, the starting ketone (**12**) of **7** was prepared by Baeyer–Villiger oxidation of the diketone (**a**). Final compounds **6** and **7** were prepared by reductive amination. Compound **8** was prepared by lithium aluminum hydride reduction of the oxime, which was obtained by condensation of the mono ketone **13** with hydroxyl amine.[7] Compound **8** was purified as the HCl salt.

3. Pharmacology

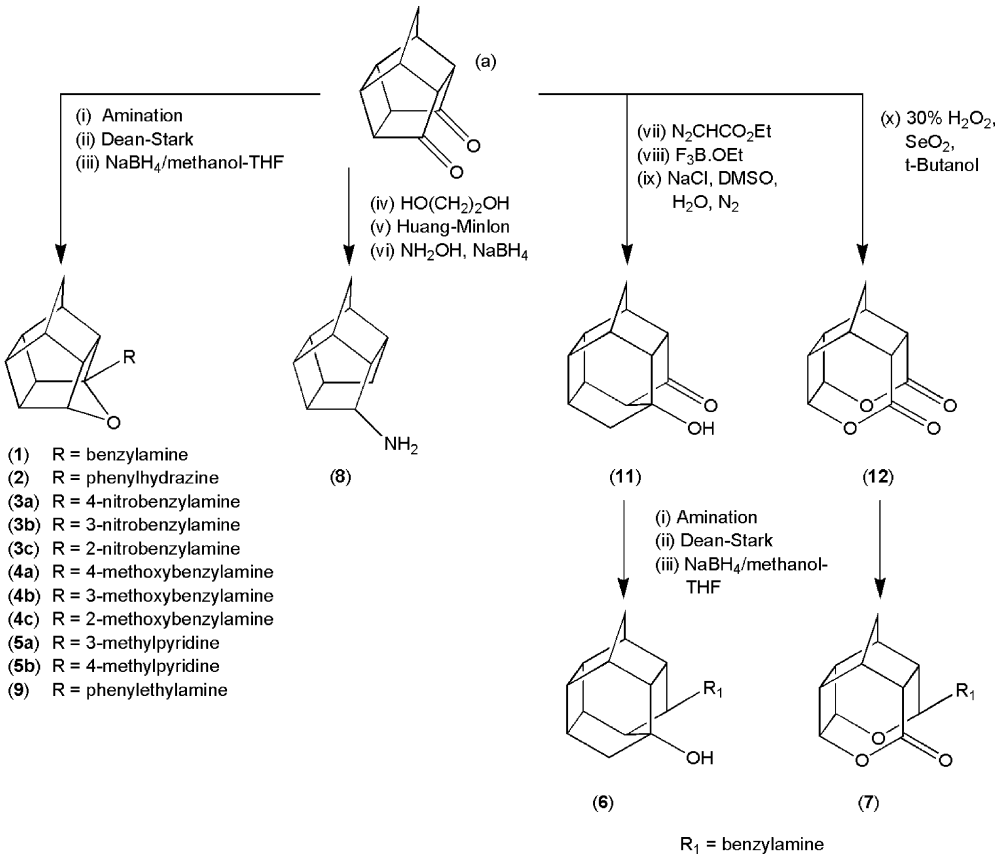
Release and uptake inhibition of [^{3}H]DA was carried out in murine striatal synaptosomes as described earlier.[21] Compounds were screened at 100 μM, except for GBR-12909 ([^{3}H]DA release, 10 μM; inhibition of [^{3}H]DA uptake, 2 μM). Initial experiments identified compounds that blocked [^{3}H]DA uptake without causing appreciable release ($\leq$40% at 100 μM), in order to ensure specificity. IC_{50} values for inhibiting [^{3}H]DA uptake were then determined for selected compounds. Inhibition of MAO-B was carried out in baboon liver MAO-B. The baboon liver contains MAO-B almost exclusively.[22] All compounds were screened initially in this assay at 100 μg/mL.

4. Molecular modeling

Molecular modelling to obtain a reasonable minimum energy conformation for each structure was performed by optimizing the structures with the Chemplus™ extension of Hyperchem® modeling software (Release 4.5 for Windows 1994, Hypercube Inc. Ontario, Canada) using MM$^+$ and AM1 with the Polak-Ribieré minimization procedure.[23] The lowest energy conformer found for compounds **3b** and **4b** was used in superpositioning and **9** was used to calculate its physicochemical properties, and to assist in estimating log BB (eq 1), the concentration of **9** in the central nervous system (CNS) following intraperitoneal treatment.

$$\text{Log BB} = 4.56796 - 0.52226(\log \text{Poct}) + 0.0052(\text{Energy}) + 0.05559(\text{SV}) - 0.33873(\text{MR}) \quad (1)$$

where log BB = log (Concentration$_{brain}$/Concentration$_{serum}$); Energy = minimum energy conformation; SV = solvent accessible molecular volume; and MR = molar refractivity.



Scheme 1. Synthesis of pentacycloundecylamine analogues.

5. Results and discussion

Initial screening studies determined the compounds' ability to release DA, and compounds with low activity were selected for further evaluation (Table 1). The compounds fell into three groups with respect to causing DA release; those that were able to elicit no more than 0–20% release of label (**1**, **2**, **3a**, **6**, and **7**), those that elicited 20–40% release of label (**3c**, **4a**, **c**, **5a**, **b**, **8**, **9**, memantine, and MK-801), and those that released >60% of label (**3b** and **4b**). Because these studies served as a screen only to eliminate potentially toxic compounds, each assay was replicated only twice.

For [^{3}H]DA release (Fig. 2, Table 1) it was apparent that replacement of the benzylamine moiety of **1** by phenylhydrazine (**2**) did not alter the release of [^{3}H]DA significantly, suggesting the importance of a linker atom spaced between the nitrogen and phenyl moieties in both these compounds. In addition, substitution on the aromatic ring seemed to influence the degree of [^{3}H]DA release. Introduction of a nitro group (**3a–c**) on the phenyl ring of **1** resulted in varying levels of [^{3}H]DA retention, with the rank order of release activity as *meta* > *ortho* > *para*. The *meta* nitro compound (**3b**) showed the least intracellular retention of [^{3}H]DA while the *para* substituted nitro compound showed at least twice the intracellular retention of [^{3}H]DA compared to *ortho*. For the methoxy compounds (**4a–c**), *meta* substitution yielded the most active compound in the whole series, while *para* and *ortho* compounds elicited lower, but equipotent release of [^{3}H]DA.

It would therefore seem that from the perspective of [^{3}H]DA release activity, addition of electron donating groups (e.g., methoxy) results in more active compounds when compared to electron withdrawing groups (e.g., nitro). However, electron donation seems to play only a small additive role, with steric considerations more important, since it was not the *para* compounds (which would be expected to elicit a stronger electron effect, than either of the other two positions on the phenyl ring), but the *meta* compounds that showed the highest activity (i.e., caused more release), when comparing the nitro and methoxy compounds. Both the nitro and the methoxy groups occupy the same spatial area, when **3b** and **4b** are superimposed. In addition, the oxygen of the methoxy group and one on the nitro group, are orientated in the same direction away from the cage (Fig. 3). The distance between these two oxygens is only 1.77 Å, which suggests that they are important for release of [^{3}H]DA, and that the orientation of the methoxy oxygen of **4b** may be more preferable than for either of the two oxygens of the nitro group of **3b**.

Further structure–activity relationships for [^{3}H]DA release are evident from results with additional molecules. When a nitrogen is included in the aromatic ring of **1** to produce the methylpyridine compounds (**5a** and **b**), release of [^{3}H]DA was only slightly less than that

Table 1. Summary of effects of cage amines and related compounds on dopamine release (at 100 μM), inhibition of dopamine transport (at 100 μM, with selected IC_{50} values), and inhibition of monoamine oxidase B activity (at 300 μM)

Compd	DA Release, % Control±S.E.M. ($n=2$)	DA Uptake, % Control±S.E.M. ($n=3$)	IC_{50}, DA Uptake, μM, ±S.E.M.	MAO-B Activity, % Control±S.E.M. ($n=2$)
1	88.00±23.00	42.66±1.76*	57±1.6	71.77±0.002*
2	79.50±16.50	54.66±9.17	n.d.	29.58±0.005*
3a	81.50±0.50	86.00±2.00	n.d.	70.16±0.016*
3b	30.00±12.00	25.66±6.69	n.d.	24.62±0.005*
3c	55.00±6.00	41.00±4.61	n.d.	34.95±0.009*
4a	61.00±1.00	43.00±7.09	n.d.	33.12±0.010*
4b	22.00±2.00*	7.00±1.73*	n.d.	35.74±0.004*
4c	57.50±3.50	33.00±2.30*	n.d.	77.97±0.021*
5a	71.50±8.50	66.00±1.00	n.d.	83.67±0.010*
5b	62.50±12.50	64.33±5.84	n.d.	88.82±0.003*
6	99.00±4.00	33.33±0.88*	55±1.2	88.33±0.011*
7	96.50±6.50	51.33±6.69*	199±1.1	97.64±0.016*
8	67.00±14.00	40.33±2.02*	n.d.	91.73±0.023*
9	76.00±3.00	18.66±0.88*	23±1.0	56.09±0.013*
Amantadine	72.50±3.50	30.00±6.55*	82±1.1	86.09±0.005*
Memantine	74.50±7.50	49.66±7.33*	>100	75.98±0.041*
MK-801	81.50±5.50	13.33±0.66*	18±1.1	92.64±0.017*
GBR 12909	69.57±0.32	6.00±2.51*	0.007±0.4[a]	n.d.
Deprenyl	n.d.	n.d.	n.d.	0.82±0.0002*
TMN	n.d.	n.d.	n.d.	4.40±0.002*

*Significantly different from control, $p<0.05$; n.d. = not determined.
[a] Reported IC_{50}.[24]

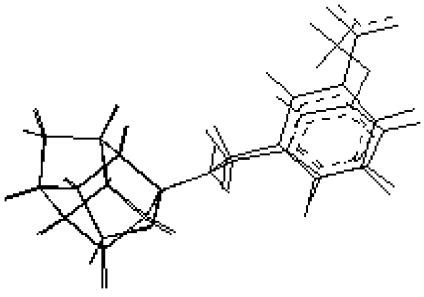


Figure 2. Superpositioning of **3b** and **4b** through the Chemplus extension of Hyperchem®.

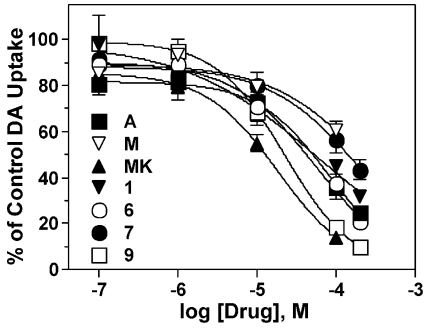


Figure 3. Dose–response relationships for the inhibition of [^{3}H]DA transport by selected compounds. Each data point represents the mean (±S.E.M.) of 3 determinations, each performed in triplicate. Lack of error bars indicates that the S.E.M. resides within the size of the symbol. Abbreviations are: Amantadine (A), Memantine (M) and MK-801 (MK).

found with **1**. This finding emphasizes the importance of aromaticity. Enlargement of the polycyclic cage (**6** and **7**) resulted in decreased release of [^{3}H]DA. In contrast, the primary amine **8**, afforded release of DA similar to that found with **1**. The three established NMDA receptor antagonists exhibited similar potency in releasing [^{3}H]DA, with amantadine and memantine slightly more active than MK-801. As was expected, the reference compound GBR-12909, a potent DAT inhibitor[24] was not very active in this assay.

With some exceptions, structure–activity relationships similar to those for the release of [^{3}H]DA were observed when the compounds were screened for inhibition of [^{3}H]DA uptake (Table 1). Substitution of the aromatic ring of **1** resulted in a more pronounced influence on [^{3}H]DA uptake inhibition than was seen for release of [^{3}H]DA. Substitution of the aromatic ring with nitro groups (**3a–c**) resulted in the *meta* compound **3b** being the most active uptake inhibitor in this group. The inhibition of [^{3}H]DA uptake by **3b** is over three times greater than that of its *para* analogue and one third more than that of the *ortho* compound **3c**. Substitution with methoxy groups on **1** (**4a–c**) resulted in the most active inhibitor of [^{3}H]DA uptake in this series, which was the *meta* compound, **4b**. Thus, as was seen for [^{3}H]DA release, the rank order of activity for both the nitro and methoxy compounds was found to be *meta* > *ortho* > *para*. We conclude similarly that the *meta* position was preferred for inhibition of [^{3}H]DA uptake, rather than the *ortho* and *para* positions, because of steric rather than electronic effects. The greater activity of *meta*-substituted compounds suggest an optimal binding pocket where this substituent fits into the DAT, compared to **1** with no substitution on the phenyl ring. The *para* position seems to be less favorable in this regard, especially with the nitro group. This finding might indicate a steric interaction that prevents the phenyl ring from forming a bond with the DAT. The greater activity of methoxy group substitution on the phenyl ring of **1** suggests an additional bond formation with the DAT, where a small electron donating effect of

the methoxy group increases hydrophobic/pi-pi bonding stabilization with **3b**, compared to **4b**.

Differences in inhibition of dopamine uptake were seen for the 'enlarged cages' in that compound **6** was more active than **7** (Table 1). These differences could be attributed to the hydroxyl group in **6** and the carbonyl group and ether ring linkages in **7,** suggesting that different associations are relevant for the two compounds in their interactions with the DAT. Removal of the side chain to yield the primary amine (**8**) resulted in slightly more activity for this compound than found for **1**, but less activity than found for amantadine. Increasing the distance between the polycyclic cage and the aromatic ring seemed to increase activity for compound **9**. The increased chain length instills more flexibility, which might improve fit at the site of interaction. Memantine exhibited less DA uptake inhibition than did amantadine and **8**. It was surprising that MK-801 exhibited such a potent inhibition of [^{3}H]DA uptake. The reference compound GBR-12909, a potent DAT inhibitor[24] was most active in this assay (Table 1). Binding studies done by us using the cocaine analogue WIN 35,248 suggested that these compounds bind to a different site on the DAT than WIN 35,248 (data not shown).

Initial experiments identified compounds that blocked DA uptake without causing appreciable release ($<40\%$ at 100 μM) of the neurotransmitter, in order to assess specificity. We are looking to develop neuroprotective drugs for PD, to be used as a daily preventative or as additional therapy with other compounds that treat PD symptomatically. Selectivity for DA uptake inhibition is desirable since excessive release of DA is thought to be neurotoxic.[25] DA uptake inhibition arguably may also be beneficial to patients in the early stages of PD, because decreasing DA uptake might not only result in increased synaptic and decreased intracellular DA concentrations, but also may help slow the progression of the disease. Moreover, in cases where endogenous or exogenous toxins use the DAT as a gateway (e.g., MPP^+), uptake inhibitors would be ideal neuroprotective compounds.

To this end, we undertook further dose-response studies (Fig. 3) with compounds **1**, **6**, **7**, **9**, as well as the reference compounds amantadine, memantine, and MK-801. Compounds **3b** and **4b** were excluded, because although potent in inhibition uptake assays, they are also potent releasers of DA. We focused on compounds that would be neuroprotective against endogenous or exogenous toxins, by selectively inhibiting DAT. The IC_{50} values ($\pm$SEM) of compounds **1**, **6,** and **9** were similar in magnitude to those measured for the reference compounds amantadine and MK-801 (Table 1). In contrast, a higher IC_{50} value was observed for **7** (Table 1), and memantine was similarly active ($IC_{50} > 100$ μM, Fig. 3). The shallower dose-response slopes of **1**, **6**, **7**, and memantine (Fig. 3) suggest that these compounds might be therapeutically more acceptable.[26] Steeper dose–response curves such as those found with **9** (Fig. 3) indicate a narrow index of therapeutic activity that might be compromised in vivo by individual differences in pharmacokinetics. It is therefore likely that compounds such as **1**, **6**, and **7** may be more viable for future in vivo studies. The slight difference in IC_{50} values between MK-801 and **9** may be explained by the steeper slope of the dose–response curve of MK-801 compared with **9** (Fig. 3).

The dose–response curves generated for **1**, **6**, **7**, amantadine, and memantine (Fig. 3) show incomplete blockade of [^{3}H]DA uptake ($\leq 80\%$). Possible explanations for this may be low drug penetration to the DAT, affinity differences, or enantiomeric preference for DAT binding with these compounds. In addition, multiple ligand acceptor sites have been reported to exist on the DAT,[26] and these may account for the differences in blocking activity. In contrast, **9** and MK-801 exhibited complete inhibition of [^{3}H]DA uptake.

The activity of **9** as an uptake inhibitor of DA in these experiments might offer an explanation for its neuroprotective activity as seen in the MPTP parkinsonian mouse model.[14] The degeneration of dopaminergic neurons by MPTP is initiated by accumulation, via the DAT, of the MAO-B generated pyridinium metabolite of MPTP, MPP^+. Blocking the DAT might underlie this neuroprotection in striatal neurons by preventing the action of agents or toxins similar to MPTP/MPP^+ that require access to dopaminergic cells through the DAT.[25,27] Based on this hypothesis, compounds **1** and **6** would additionally be ideal candidate compounds for screening in the MPTP parkinsonian mouse model.

Monoamine oxidases (MAOs) are thought to be involved in the etiology of PD.[28] For example, MPTP is converted by MAO-B to MPP^+, the active neurotoxin. Inhibition of this enzyme prevents the neurotoxicity of MPTP and increases the amount of free DA that is still present in the synaptic cleft.[29] We therefore screened the pentacycloundecylamines, the three NMDA receptor antagonists amantadine, memantine, and MK-801, and the two MAO-B inhibitors deprenyl (a mechanism-based irreversible inhibitor)[30] and 2,3,6-trimethyl-1,4-naphthoquinone (TMN, a reversible inhibitor)[15] in a robust MAO-B inhibition assay (Table 1). Although some of the compounds elicited marginal inhibition, none of the pentacycloundecylamines that were found to be selective DAT inhibitors, inhibited MAO-B in any significant way (inhibition $>50\%$) at therapeutically relevant concentrations. As expected, the known MAO-B inhibitors deprenyl and TMN resulted in complete ($95\%+$) inhibition of the enzyme (Table 1).

During the drug discovery process it is important to consider the pharmacokinetic properties of the drugs evaluated.[31] Due to the central nervous system site of action of the drugs screened in this study, it is important to mention that the pentacycloundecylamines are capable of crossing the blood–brain barrier. Permeation into the brain was observed in a study done by Zah et al.[32] These researchers developed a physicochemical prediction model from in vivo studies, to predict blood-brain distribution of pentacycloundecylamines. Using this predictive equation of Zah et al., and assuming ther-

apeutic serum levels are similar to that of amantadine (9 μM),[4] Eq 1 gives a predicted CNS concentration of 17 μM for **9**, which is higher than the therapeutic concentration range for amantadine (4–9.5 μM). This predicted concentration (17 μM) is near the IC_{50} value (23 μM) for DAT inhibition by compound **9**, and suggests that compound **9** is a good lead for further drug development as potential neuroprotective therapy in PD.

6. Conclusions

In this report, we describe the syntheses and in vitro biological studies (interaction with the DAT and the enzyme MAO-B) of a novel group of polycyclic cage amines, in order to identify promising compounds for future neuroprotection studies in the well known MPTP parkinsonian mouse model. The cage compounds proved to be effective inhibitors of dopamine uptake, with IC_{50} values comparable to that of amantadine. The most active compound (**9**) had an IC_{50} value of 23 μM. In structure–activity studies, we investigated both aromatic substitution as well as increased size of the cage moiety. All of the polycyclic cage amines showed greater activity for blocking DA uptake than for causing release of DA. For cage compounds with aromatic substituents, no selectivity between uptake inhibition and release of DA was observed. Increasing the cage moiety size enhanced selectivity for DA uptake inhibition. The distance between the aromatic moiety and the cage appears to play a vital role since **9** (phenylethylamine), the compound with the largest distance, was also the most active inhibitor in this series. Structure–activity relationships for this group of compounds therefore appear to be influenced by geometric and steric considerations rather than by electronic characteristics. Results from the in vitro assays suggest that inhibition of the DAT might underlie the neuroprotective effect of **9** in the MPTP parkinsonian mouse model seen in earlier work.[14] Inhibition of MAO-B activity by these compounds occurred in the high (several hundred) micromolar range and likely would not contribute any protection against MPTP. A primary goal of future PD therapy is the development of neuroprotective strategies that will retard or prevent neuronal death.[27] The spectrum of activity within the pentacycloundecylamines makes them candidates for future exploration as possible lead compounds for dual symptomatic and neuroprotective therapies against PD.

7. Experimental

7.1. Chemistry

All melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. The ^1H and ^{13}C spectra were recorded on a Varian VXR 300 spectrometer. Samples were dissolved in a deuterated solvent ($CDCl_3$) with tetramethylsilane (Me_4Si) as an internal standard. Infrared spectra were recorded as a neat film on KBr plates with a Shimadzu FT IR 4200 spectrophotometer. Mass spectra and HR-MS were recorded at 70 eV (EI) on a VG 7070E spectrometer. Elemental analyses were performed on a Perkin–Elmer model 240 instrument and data were all within acceptable limits (±0.04%). Amantadine (hydrochloride salt) and memantine (hydrochloride salt) were purchased form Sigma Chemical Co. (St. Louis, MO). GBR-12909 (hydrochloride salt) and (+)MK-801 (maleate) were purchased from Research Biochemicals (Natick, MA, USA). [^{3}H]Dopamine (20.3 Ci/mmol) was purchased from New England Nuclear (Wilmington, DE). *R*-deprenyl and TMN were gifts from Dr. Neal Castagnoli Jr. at the Harvey W. Peters Center, Department of Chemistry, Virginia Tech. Buffer constituents were obtained from other commercial sources.

7.1.1. General synthetic method for benzylamine phenylhydrazine, nitrobenzylamine, methoxybenzylamine, aminomethylpyridine, and phenylethylamine derivatives of pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane. Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (5 g, prepared according to the method of Cookson et al.)[20] was dissolved in tetrahydrofuran (THF, 50 mL) and cooled to ±5 °C in ice. An equimolar quantity of the desired amine was slowly added under stirring. The white precipitate that formed after about 10 min was filtered and washed with cold THF to render the hydroxylamine. This product was dehydrated in dry benzene under Dean–Stark conditions for 1 h or until no more water collected in the container. Evaporation yielded the Schiff base as a yellowish oil and reduction of this imine was accomplished with sodium borohydride in dry methanol (30 mL) and dry THF (150 mL) for 24 h at room temperature. The solvent was removed under reduced pressure and water (100 mL) was added. The mixture was extracted with dichloromethane (4×50 mL) and the combined organic fractions washed with water before being dried over magnesium sulfate. Evaporation yielded the desired products. Spectral characteristics for the compounds used in this study were previously reported.[13,14,32] Spectral parameters for the recently published[32] compound **9** are given below, with elemental analysis performed by Atlantic Microlab Inc. (Norcross, Georgia, USA).

7.1.2. 8-Phenylethyl-8,11-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]-undecane (9). Off white needles obtained by recrystallization from ethanol (yield 54.0%). $C_{19}H_{21}NO$; mp 49 °C; IR ν_{max} 3311, 2974, 1602, 1495; MS *m*/*z* 279 (M^+), 188, 131, 105, 91, 28; ^1H NMR ($CDCl_3$) δ 1.26 (t, 2H, *J* = 7.9, H-14a,4b), 1.70 (AB-q, 2H, *J* = 10.44, H-4a,4b), 2.36 (2×m, 6H, H-1,2,3,4,5,6), 2.7 (m, 2H, H-15), 4.59 (t, 1H, *J* = 5.3, H-11), 7.13-7.28 (m, 5H, H-16,17,18,19,20); ^{13}C NMR ($CDCl_3$) δ 37.46 (d, 1C), 41.46 (d, 1C), 41.86 (d, 1C), 43.20 (t, C-4), 44.43 (d, 1C), 44.75 (d, 1C), 44.90 (d, 1C), 54.70 (d, C-7 or 9), 55.33(d, C-7 or 9), 82.43 (d, C-11), 126.14 (d, C-18), 128.44 (d, C-17,19), 128.83 (d, C-16,20), 140.01 (s, C-15). Anal. calcd for $C_{19}H_{21}NO$: C, 81.68; H, 7.58; N, 5.01. Found: C, 81.54; H, 7.55; N, 5.06.

8. Biological methods

8.1. Animals

ICR male mice (20–26 g, 6–8 weeks old) were obtained from Harlan, Dublin, VA. All animal care and experimental

protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee in accordance with guidelines established by the United States Public Health Service and Virginia Tech.

8.2. Preparation of synaptosomes

Male ICR mice were sacrificed by cervical dislocation and the striata were rapidly removed. The striatal tissue was homogenized in 4 mL of sucrose buffer (0.32 M sucrose, 2 mM HEPES, pH 7.4). The homogenate was then centrifuged for 10 min at $1000\times g$. The supernatant was removed and centrifuged for 15 min at $10{,}000\times g$. The resulting pellets were washed once and resuspended in incubation buffer (125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, $CaCl_2.2H_2O$, 10 mM sucrose and 50 mM Tris–HCl, pargyline 0.05 mM, ascorbate 0.1 mM, pH 7.4).

8.3. Dopamine release studies

After preparation, the final pellet of synaptosomes was resuspended in incubation buffer containing 100 nM [^{3}H]DA and incubated for 5 min at 37 °C, and then re-centrifuged at $10{,}000\times g$ for 10 min. The supernatant was discarded and the pellets washed once with cold incubation buffer and resuspended. The synaptosomes were aliquoted into tubes, treated with drug, and incubated for 10 min at 37 °C. Test compounds (100 μM final concentration) were dissolved in DMSO, the final DMSO concentration in the incubations not to exceed 0.1%, with the controls receiving 0.1% DMSO alone. Synaptosomes then received 3 mL of wash buffer at 37 °C, filtered through 1 micron glass microfiber filters (Whatman GF/B) under vacuum and washed an additional 3 times with 37 °C buffer, followed by liquid scintillation counting to measure the radioactivity of [^{3}H]DA within the synaptosomes. For each compound screened, fresh striatal tissue was used.

8.4. Dopamine uptake inhibition studies

Test compounds (screened at 100 μM final concentration, dissolved in DMSO as described above) were incubated with synaptosomes in a heat block for 10 min. at 37 °C. Following the incubation, the synaptosomes were incubated with [^{3}H]DA for 2 min. Uptake was terminated by the addition of ice cold wash buffer, followed by vacuum filtration through 1 micron glass microfiber filters (Whatman GF/B). These filters were washed 4 times with 3 mL ice cold wash buffer, air dried, followed by liquid scintillation counting to measure the radioactivity of [^{3}H]DA within the synaptosomes. For each compound screened for DA uptake inhibition and each dose–response replicate, fresh striatal tissue was used.

8.5. MAO-B Inhibition

Intact mitochondria from baboon liver served as the source for MAO-B.[33] The mitochondrial fractions were prepared as described previously.[34] The mitochondrial homogenate was suspended in sodium phosphate buffer (100 mM, pH 7.4) containing 50% (w/v) glycerol. Protein was determined by the method of Bradford.[35] The inhibition of MAO-B by the test compounds (100 μg/mL) was evaluated by incubating the substrate (1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (MMTP; 0.25 mM)[21] with the mitochondrial homogenate (0.15 mg/mL). Test compounds were dissolved in DMSO and added to the buffered incubation mixture so that the final DMSO concentration did not exceed 4%. The final volume of the incubation mixtures was 500 μL (in sodium phosphate buffer, pH 7.4) and the samples were incubated at 37 °C for 15 min. The reaction was terminated by the addition of 20 μL perchloric acid (70% v/v), centrifuged ($16{,}000\times g$ for 8 min) and the concentration of the of the enzyme-generated metabolite of the substrate in the supernatant fractions determined spectrophotometrically at 420 nm.

8.6. Statistical analyses

Statistical significance for DA uptake and release studies was analyzed using Student's *t*-test (InStat™, GraphPad Software, San Diego, CA) to find statistically significant effects. Concentration–curve data were analyzed by non-linear least squares fit to a four parameter logistic equation, with resulting IC_{50} values and goodness of fit statistics calculated using Prism™ (GraphPad Software, San Diego, CA).

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