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Structure–activity relationships of pentacycloundecylamines at the *N*-methyl-D-aspartate receptor

Werner J. Geldenhuys,[a,b] Sarel F. Malan,[c] Jeffrey R. Bloomquist[d] and Cornelis J. Van der Schyf[a,b,c,*]

[a]*Department of Pharmaceutical Sciences, Northeastern Ohio Universities College of Pharmacy, Rootstown, OH 44272, USA*
[b]*Department of Pharmaceutical Sciences, Texas Tech University School of Pharmacy, Amarillo, TX 79106, USA*
[c]*Pharmaceutical Chemistry, North-West University, Potchefstroom 2520, South Africa*
[d]*Neurotoxicology Laboratory, Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA*

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Abstract—Prompted by our interest in neuroprotective agents with multiple mechanisms of action, we assessed the structure–activity relationship of a series of pentacycloundecylamine derivatives previously shown to have both L-type calcium channel blocking activity and *N*-methyl-D-aspartate receptor (NMDAR) antagonistic activity. We utilized a functional assay to measure NMDAR channel block using $^{45}Ca^{2+}$ influx into synaptoneurosomes. The cage amine 8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}. 0^{3,10}.0^{5,9}]undecane (NPG1-01) proved to be the most potent experimental compound with an IC_{50} of 2.98 μM, while 8-amino-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane had the next most potent IC_{50} of 4.06 μM. Increasing the polycyclic cage size of NGP1-01 from a pentacycloundecane to a tridecane cage structure, but retaining the *N*-benzyl moiety decreased potency 10-fold, indicating a limitation on the volume of the cage that can be accommodated in the channel binding site. In the presence of NGP1-01, NMDA/glycine-induced maximal $^{45}Ca^{2+}$ influx was attenuated by 34% with an insignificant effect on agonist potency. These results are consistent with uncompetitive antagonism for this group of compounds. Radioligand binding studies with [^{3}H]MK-801 or [^{3}H]TCP showed little or no displacement of these ligands by pentacycloundecylamines, suggesting that the latter compounds bind to a unique site in the NMDAR channel. The pentacycloundecylamines tested represent a novel group of NMDAR antagonists that have potential as therapeutic agents for neurodegenerative diseases including Parkinson's and Alzheimer's disease.

1. Introduction

The *N*-methyl-D-aspartate receptor (NMDAR) has been suggested as a drug target through its involvement in neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD).[1] Overstimulation of the NMDAR by an excess of the endogenous neurotransmitter glutamate during pathological conditions leads to excessive influx of calcium into neuronal cells resulting in cell death, a process known as excitotoxicity. Calcium entry through L-type calcium channels also contributes to calcium overload and mitochondrial disruption that lead to the recruitment or release of mediators responsible for the activation of an apoptotic cascade and ultimately, in cell death.[2]

Non- and uncompetitive NMDAR antagonists show promise as neuroprotective agents by preventing excessive influx of calcium into neuronal cells.[3–6] Potent NMDAR antagonists such as MK-801 and PCP bind to the PCP binding site located within the ion-channel pore of the NMDA receptor.[7] This antagonism is use-dependent in that the PCP site is only accessible when the ion channel pore is in an open, or activated state. The block is accelerated by increases in open channel probability. This phenomenon implies that compounds with pronounced use-dependency will have greater affinity for the brain region where overstimulation occurs.[8] Once bound, the blocker can be trapped by channel closure. Recovery from the trapped blocked state is

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* Corresponding author at present address: Department of Pharmaceutical Sciences, Northeastern Ohio Universities College of Pharmacy, Rootstown, OH 44272-0095, USA. Tel.: +1 330 325 6467; fax: +1 330 325 5930; e-mail: cvanders@neoucom.edu

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generally slow.[7] Block produced by MK-801 or PCP is difficult to reverse and MK-801 exhibits a very long duration of action.[9] Unfortunately, attempted clinical use of these long duration antagonists had become associated with adverse CNS side effects including hallucinations,[10] memory impairment,[11] and neuronal vacuolization.[8] However, low-affinity use-dependent channel blockers (i.e., antagonists with a K_i above 200 nM) are usually not associated with these unacceptable side effects and are well tolerated.[12,13]

Polycyclic cages are an interesting and highly promising group of compounds that are receiving intense scrutiny as potential chemical scaffolds for the development of new drugs.[14] One example of this class, the adamantyl cage amine, memantine, is a low-affinity, clinically well-tolerated uncompetitive NMDAR antagonist.[12] The reason for memantine being better tolerated as opposed to MK-801 is that memantine exhibits rapid and strong voltage-dependent blocking kinetics. Partial trapping and release of memantine from NMDAR channels favor the occurrence of strong block only during sustained receptor stimulation, which may occur during brain trauma and stroke, with more limited effects expected on normal synaptic transmission.[7] Memantine is used clinically to treat PD and AD[12] and has recently been approved by the FDA under the trade name Namenda® for use in moderate to severe AD.[15] Memantine is therefore an ideal lead compound in the development of agents with a favorable therapeutic index to treat excitotoxic NMDAR channel mediated neurodegenerative diseases.

We recently reported neuroprotective activity in an in vivo pilot study of a small series of pentacycloundecylamines in the MPTP parkinsonian mouse model.[16] Based on this information, and the striking structural similarities between the pentacycloundecylamines and adamantyl compounds such as memantine (Figs. 1 and 2), we reasoned that the polycyclic cage amine NGP1-01, an L-type calcium channel blocker,[17,18] (Fig. 1; **1**) might also be able to mimic the NMDAR antagonism of memantine. This hypothesis was recently confirmed in a study in which NGP1-01 significantly inhibited depolarization-induced calcium influx in cortical neurons preloaded with fura-2 AM, with a potency similar to that of nimodipine, and also inhibited NMDA-induced calcium influx with a potency only slightly less than that of memantine.[19] Furthermore, in vivo microdialysis experiments—in which choline release in brain during NMDA infusion was monitored as a measure of excitotoxic membrane breakdown—showed that NGP1-01 (40 mg/kg, ip) reduced NMDA-induced choline release by 31% ($p < 0.01$).[19] These results demonstrated that NGP1-01 simultaneously blocks both major neuronal calcium channels and traverses the blood–brain barrier.[19] Such a mechanism may be contributory to the neuroprotective activity seen with other pentacycloundecylamine compounds.[16]

In the current study, we utilized ligand-stimulated $^{45}Ca^{2+}$ influx into synaptoneurosomes to measure NMDAR channel block by a series of structurally related polycyclic cage compounds. Further, in order to gain insight into the site of interaction of these compounds inside the NMDA channel, we used radioligand binding studies with either [^{3}H]MK-801 or [^{3}H]TCP, and measured the displacement of these blockers by the same series of compounds.

2. Results

Effects of the pentacycloundecylamines on background (no agonists), or NMDA/Gly-mediated ^{45}Ca influx into synaptoneurosomes are shown in Figure 3. Significant inhibition of NMDA-mediated ^{45}Ca influx was measured for compounds **6** and **8**, while complete inhibition ($\leqslant$100% of control) was attained by compounds **1**, **3b**, **3c**, **4b**, **9**, and the reference compounds memantine and MK-801. Compounds **2**, **3a**, **4a**, **4c**, **5a**, **5b**, **7**, and the reference compound amantadine weakly blocked NMDA-mediated ^{45}Ca influx into synaptoneurosomes. Because the pentacycloundecylamines are reported to be L-type calcium cannel blockers,[14,17,18,20] we evaluated the effect of the pentacycloundecylamines on the background ^{45}Ca influx as well by screening the compounds in the absence of NMDA/Gly. All the pentacycloundecylamines showed only modest inhibition of background calcium influx, with only MK-801 having a statistically significant antagonist effect (Fig. 3).

Dose–response curves were performed on selected compounds to determine IC_{50} values, Hill slopes, and goodness of fit or r^2 values (Table 1). All fittings of dose–response relationships to a sigmoidal curve were good, with r^2 values of no less than 0.94, and typically near 0.99. MK-801 was the most potent compound, having an IC_{50} value near 1 μM in this assay. The other compounds fell into three potency ranges. Memantine was of 3-fold lower potency, similar to compounds **1** and **8**. Other compounds that were less active (IC_{50}s ranging between 20 and 40 μM), with the following rank order of potency, were: **4b** = **9** > **3b** = **6** = **3c**. Hill slope values showed a trend related to inhibitory potency (Table 1). The Hill slopes for compounds with high-affinity as NMDA antagonists (MK-801, memantine, and **1**) were near unity, with lower potency compounds having greater Hill slope values.

Paired agonist dose–response curves for NMDA/Gly in the absence and presence of compound **1** are shown in Figure 4. NMDA significantly ($p < 0.05$) increased the amount of ^{45}Ca taken up by synaptoneurosomes. The threshold for NMDA-stimulated influx into synaptoneurosomes was approximately 10 μM and a maximal effect was observed at 1 mM. Higher concentrations of NMDA resulted in decreased uptake of ^{45}Ca (data not shown). Compound **1** was selected as representative of this group, as well as being one of the most potent polycyclic cage amines, to explore the mechanism of action. The maximal effect of NMDA-mediated ^{45}Ca influx was depressed in the presence of **1** (2 μM) from 113.4% to 108.9% of control. The NMDA curve was shifted slightly to the right in the presence of **1**, although there were no statistical significance between the EC_{50} of the

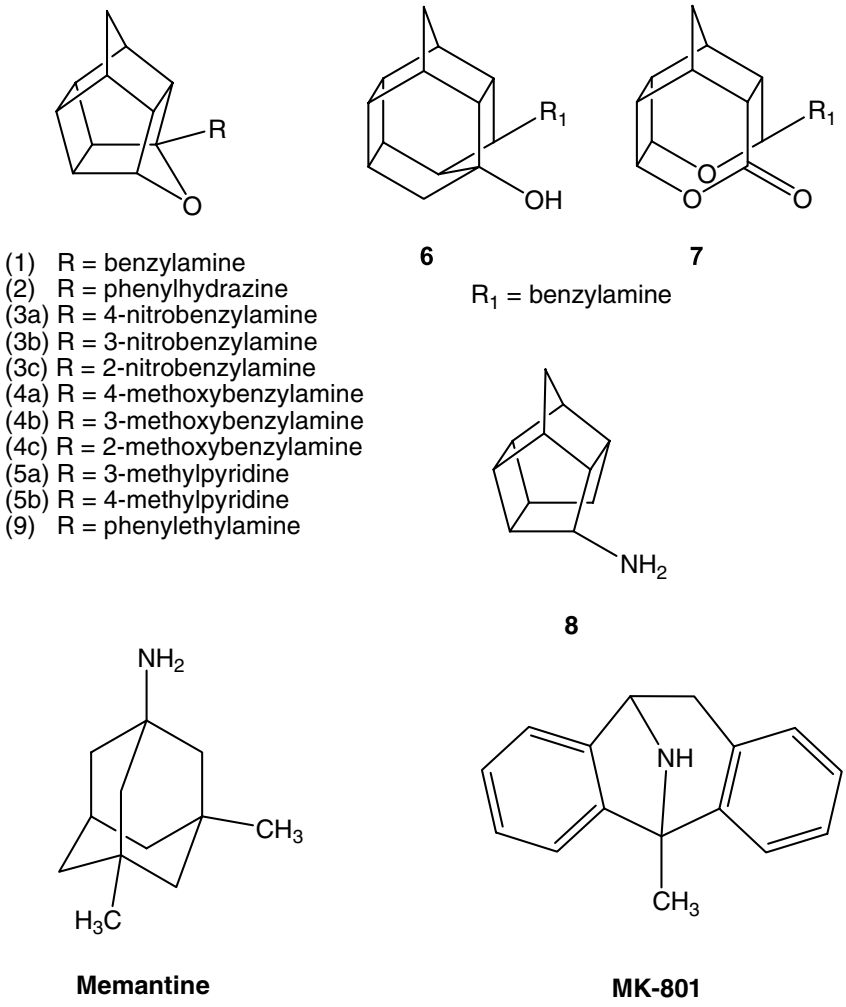

Figure 1. Structures of pentacycloundecylamines used in the current study, as well as the reference compounds memantine and MK-801.

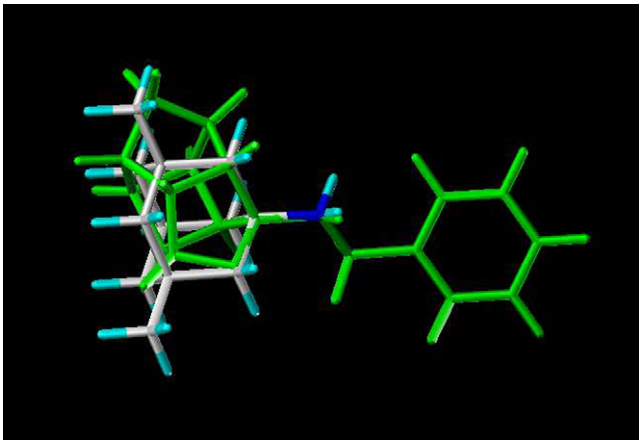

Figure 2. Structural similarity between minimum energy conformations (semi-empirical AM1 calculations through Hyperchem®) of NGP1-01 (**1**; green) and memantine (atom colors). RMS fitting of **1** on memantine was <0.1 Å.

NMDA dose–response curve in the presence (68 μM) and absence (49 μM) of **1**.

Figure 5 shows the results of binding studies done with [^{3}H]MK-801 and [^{3}H]TCP. The reference compounds memantine and MK-801 displaced 90% and 73% of [^{3}H]MK-801 binding, respectively. In contrast, none of the pentacycloundecylamines tested (**1**, **8**, and **9**) displaced [^{3}H]MK-801 binding significantly at concentrations up to 100 μM. Memantine and MK-801 displaced [^{3}H]TCP binding markedly (67% and 65%, respectively). Compounds **1** and **9** displaced [^{3}H]TCP weakly (20% and 44%, respectively), but were significantly different ($p < 0.05$, *t*-test) from control and each other, while **8** caused no displacement of [^{3}H]TCP. In addition, no displacement by **1** and **8** was found for [^{3}H]glutamate binding to synaptoneurosomes (data not shown).

3. Discussion

Daniell[26] described an assay with utility for screening a series of compounds using NMDA-mediated uptake of $^{45}Ca^{2+}$ into murine microsacs. The $^{45}Ca^{2+}$ flux assay used in this study provided a convenient method for measuring the effects of putative antagonists on NMDA receptor function while mitigating the contribution from L-type calcium channels. $^{45}Ca^{2+}$ uptake stimulated by NMDA/Gly was sensitive to block by the classical

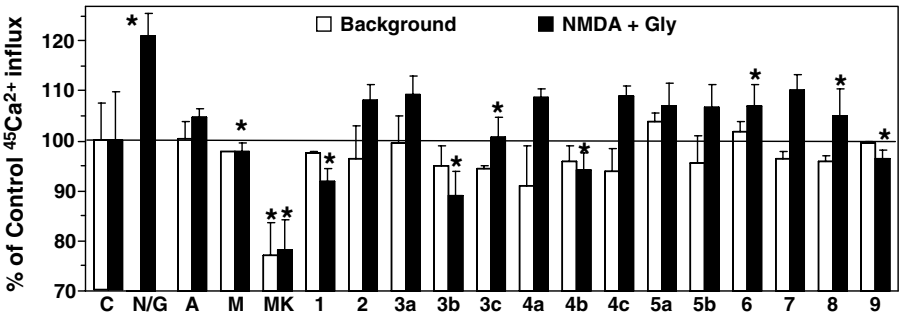

Figure 3. Screening of pentacycloundecylamines (100 μM) for antagonism of background or NMDA + Gly-mediated ^{45}Ca influx into synaptoneurosomes. Each bar represents mean percentage of control values ± SEM. Abbreviations are: Control (C), NMDA/Gly (N/G), amantadine (A), memantine (M) and MK-801 (MK). Statistical analysis (see Section 4.4) was performed on raw data, with asterisks signifying significant inhibitory effect ($p < 0.05$) when compared to NMDA + Gly (if black bar is labeled) or vehicle-treated background (if white bar is labeled).

Table 1. IC_{50} values for selected compounds calculated from dose–response studies

Compound	IC_{50} ± SEM (μM)	Hill slope	r^2
1	2.98 ± 1.0	−0.89	0.99
3b	32.78 ± 1.2	−4.62	0.99
3c	37.62 + 1.0	−3.05	0.99
4b	20.65 ± 1.1	−1.97	0.99
6	36.22 ± 1.2	−1.21	0.99
8	4.06 ± 1.1	−0.40	0.94
9	23.5 ± 1.5	−1.74	0.97
MK-801	1.23 ± 1.4	−1.09	0.99
Memantine	3.05 ± 1.7	−1.03	0.99

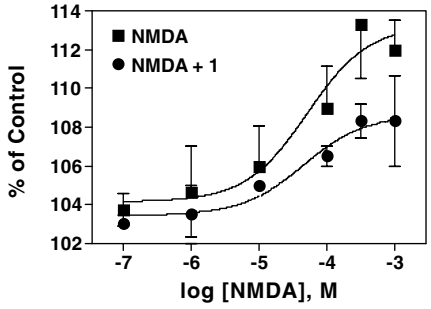

Figure 4. Concentration–response curve of NMDA in the absence and presence of **1** (2 μM). Gly was kept constant at 100 μM. Symbols are mean % of controls ± SEM of three determinations, performed three times (see Section 4.4). Where not visible, the error bars are contained within the size of the symbol.

antagonist MK-801 and had an agonist potency (EC_{50} = 49 μM) for NMDA channels similar to that reported for mouse hippocampal microsacs[26] (30 μM), and comparable to values found in patch clamp experiments.[27]

When run in the presence of 10 μM nitrendipine, only MK-801 showed a statistically significant inhibition of background $^{45}Ca^{2+}$ uptake, suggesting that the pentacycloundecylamines did not interfere with background calcium fluxes and that such action did not contribute to their observed effects. Specific inhibitory effects of nitrendipine on NMDA receptors were reported in murine cerebellar granule cells,[28] as well as inhibition by

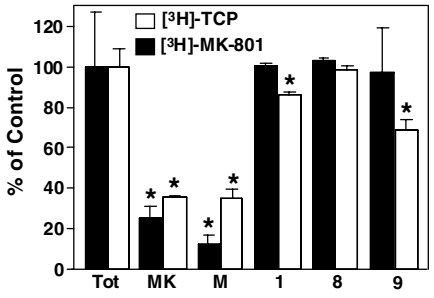

Figure 5. Competition binding of compounds **1**, **8**, and **9** with [^{3}H]MK-801 and [^{3}H]TCP. Values are means (pmol/mg protein) ± SEM of 2–5 separate experiments. Abbreviations are: Total binding (Tot) or binding in the presence of cold MK-801 (MK), memantine (M), or compounds **1**, **8**, and **9**, all at 100 μM. Statistical significance (see Section 4.4) compared to the total binding in a *t*-test, $^*p < 0.05$.

nitrendipine of MK-801 binding,[24] an action not shared by other L-type channel blockers, such as verapamil. Accordingly, we ran additional $^{45}Ca^{2+}$ uptake studies in the absence or presence of 10 μM nitrendipine or verapamil. We observed little or no effect of either compound on NMDA/Gly-stimulated $^{45}Ca^{2+}$ uptake compared to agonists alone (data not shown). Thus, we conclude that the reported effects of nitrendipine on NMDA receptors had little impact on the results of the present study.

Among the established antagonists tested, MK-801 was by far the most potent antagonist of NMDA/Gly-specific $^{45}Ca^{2+}$ uptake, followed by memantine, and then amantadine, a rank order of potency that accurately agrees with previous studies.[29,30] The potency of MK-801 in the present study (IC_{50} ca. 1 μM) is greater than that observed by Daniell,[26] who reported that 100 μM MK-801 was required to block 50% of $^{45}Ca^{2+}$ uptake stimulated NMDA/Gly in mouse hippocampal microsacs. We note that Daniell[26] used only a single centrifugation step for harvesting synaptoneurosomes, whereas we found that two such steps were required for obtaining consistent levels of background $^{45}Ca^{2+}$ uptake that did not increase through time and affect measurement of antagonist activity (data not shown). This reported

difference in technique may account for the differences in observed potency of MK-801 between our method and that of Daniell[26] (ca. 100-fold). The IC_{50} value of MK-801 was somewhat higher (ca. 1 μM) in our assay than was observed in previous studies, where the IC_{50} values for blocking responses to NMDA in rat brain slices were 75 nM[31] and 400 nM.[32] On the other hand, the IC_{50} value of memantine (3 μM) did correlate well with previous studies, which ranged from 7.6 to 0.54 μM.[29,30] This finding possibly indicates that crude synaptoneurosome preparations may blunt the sensitivity of lipophilic compounds with nanomolar binding affinities, but have utility in identifying compounds within the micromolar range. Whole brain synaptoneurosomes as used here contain all endogenous subtypes of NMDA receptors present in the CNS. Accordingly, the EC_{50} values we report represent a composite affinity affected by both the sensitivity of subtypes present and their relative abundance. Thus, although potencies measured in synaptoneurosomes should reflect in vivo conditions, it provides less precise potency results compared to isolated single neuron electrophysiology or genetically engineered cells expressing a single NMDA receptor subtype.

Screening studies on the pentacycloundecylamines indicated that these compounds are effective antagonists of NMDA-mediated $^{45}Ca^{2+}$ influx into synaptoneurosomes. Structure–activity relationships for the pentacycloundecylamines appear to be determined primarily by geometric factors, with a small influence of electronic effects (Fig. 3 and Table 1). The polycyclic cage amine seems to be the most important pharmacophoric element contained within the pentacycloundecylamine structure required to interact with the NMDA receptor. The increased potency of **1** compared to that of **8** (Table 1) suggests that the phenyl ring in **1** adds to the interaction and therefore increases NMDA antagonism for pentacycloundecylamines, although this was not the case for the methylpyridine compounds, **5a**–**b**. The bridgehead atom, linking C-8 and C-11, does not seem to be important for an interaction with the NMDA receptor/ion channel complex, when comparing **8** to **1**. However, the number of linker atoms between the amino and phenyl rings does appear to play an important role, since there was an 8-fold decrease in potency (Table 1) with an increase in chain length from methyl (C_1, **1**) to ethyl (C_2, **9**).

Lower potencies were observed for compounds with substitutions on the phenyl ring (Table 1). It may be argued that such moieties might be too bulky for a proposed 'phenyl pocket', with steric hindrance causing attenuated affinity and therefore reduced NMDA antagonism. However, this proposed steric interaction seems to be more tolerant toward substitution at the meta position because para-substituted compounds were observed to have the lowest activity among compounds with phenyl ring substituents (Fig. 3). Electronic effects impact these phenyl substituents as well, since a lower IC_{50} value (Table 1) was observed for the electron-donating methoxy moiety as opposed to the electron-withdrawing effect of the nitro moiety. The donation of electrons makes the phenyl ring more lipophilic and would increase affinity for an aromatic π–π or hydrophobic interaction.

Compounds **6** and **7** were synthesized to investigate the effect of increased cage size, and both were of reduced effectiveness compared to **1** and **8**. Research done by Sobolevsky et al.[33] indicated that the affinity of adamantyl amines for the NMDA channel diminished with an increase in molecular size and improved with an increase in hydrophobicity. The larger cage moiety contained in compound **6** would be consistent with an experimental IC_{50} value 12-fold greater than that of **1**, although the orientation of the phenyl side chain likely also may be an issue. Figure 6 shows the root mean square fitting of **1** and **6**, indicating the orientation of the side chains compared with one another. In compound **6** the side chain is flexed closer to the cage than is the case for **1**. This orientation may be the result of intramolecular hydrogen bonding, disallowing the atoms involved to form interactions with the NMDA ion channel.

log *P* calculations predict a lower log *P* value for **6** (1.78) than for **1** (2.57). It is expected that the larger cage would increase the hydrophobicity, but the hydroxyl group in **6** apparently compensates for the increase in cage size in this compound. Figure 7 shows the MOLCAD surface areas of **1** and **6**, indicating hydrogen bonding areas. There appear to be additional hydrogen bonding regions present for **6** than found for **1**, which would account for the lower calculated log *P* value of **6**, and resultant lower activity with the NMDA channel, compared to **1**. This result is in agreement with findings by Sobolevsky et al.[33] which predict diminished affinity for the NMDA channel to be associated with a decrease in hydrophobicity. Such a decrease in hydrophobicity, in conjunction with its increased molecular size, could explain the lower potency of **6** compared to **1**.

Compound **1** reduced the maximal uptake of $^{45}Ca^{2+}$ stimulated by NMDA/Gly with little effect on agonist potency (Fig. 4). This finding strongly suggests that uncompetitive antagonism may be the mechanism of action for the pentacycloundecylamines. A review[7] of

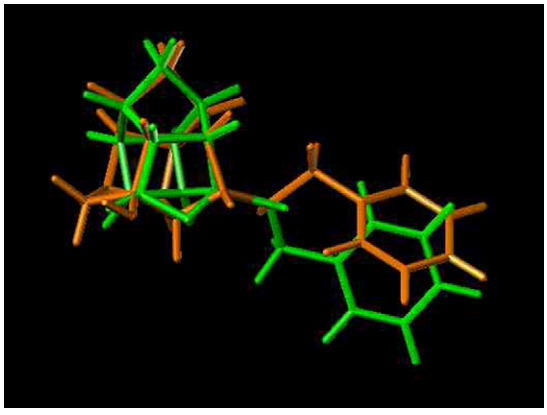

Figure 6. Overlay of **6** (orange) on **1** (green), indicating the energy-minimized positions of the phenyl side chains relative to the cage.

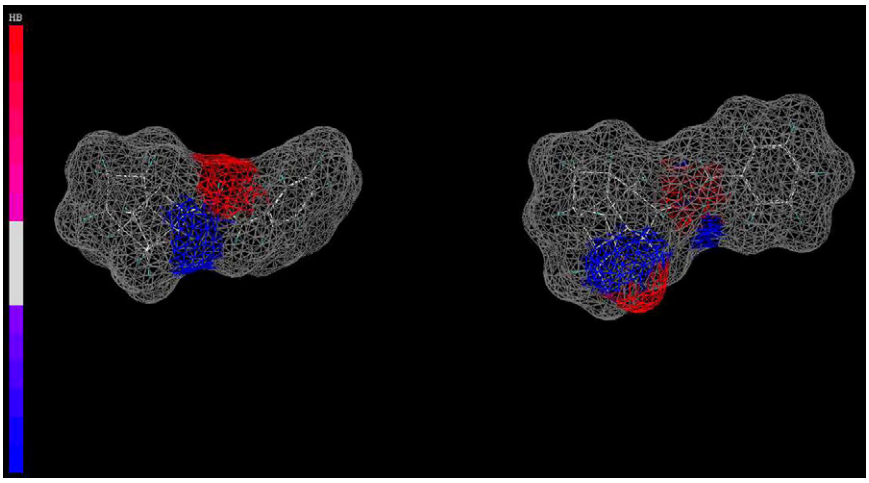

Figure 7. MOLCAD surface maps of **1** (left) and **6** (right) indicating hydrogen bonding regions. Color code indicates blue as high capacity for H-acceptor areas and red high capacity for H-donating areas.

earlier studies with memantine and amantadine defined their mechanism of action as open channel block of the NMDA receptor. Electrophysiological studies will be required to confirm a similar action for the pentacycloundecylamines. Hill slope values for high-affinity NMDAR antagonists (MK-801, memantine, and **1**) were near unity, suggesting that one molecule is required per channel to block the NMDA-mediated influx of $^{45}Ca^{2+}$. Higher Hill slope values suggest a positive cooperativity for such compounds.[34]

Binding studies with [^{3}H]MK-801 and [^{3}H]TCP were used as a tool to identify the binding site of the pentacycloundecylamines (Fig. 5). Compounds **1**, **8**, and **9** were selected for these assays, due to their potent antagonism of NMDA-stimulated $^{45}Ca^{2+}$ influx into murine synaptoneurosomes. Since memantine and the pentacycloundecylamines used in this study share a similar polycyclic cage amine template,[14] we speculate that these compounds would interact with the PCP binding site within the ion channel of the NMDA receptor. In addition, compound **1** exhibited uncompetitive antagonism in the functional $^{45}Ca^{2+}$ flux assay, which further suggests a mode of action similar to MK-801, PCP, and memantine.[7] Surprisingly, the binding studies with [^{3}H]MK801 and [^{3}H]TCP indicated that these compounds do not bind to the MK-801 binding site and only weakly to the TCP binding site, even at 100 μM. Thus, it seems that the pentacycloundecylamines interact with a unique binding site, different to that reported for MK-801 or PCP, in the NMDA receptor/ion channel complex. We are currently investigating the binding sites with specific focus on determining the K_i values for these compounds and we hope to report these data soon.

One hypothesis for the functional antagonism of the cage compounds (with the exception of **8**) would be to suggest that the phenyl ring undergoes a π–π type aromatic interaction with (an) aromatic amino acid(s) located at the entrance of the NMDA receptor channel. Such an interaction would allow the molecule to be 'anchored' in such a way that the cage can descend into the channel lumen to a depth allowed by the 'spacer' between the nitrogen and the phenyl ring. In support of this hypothesis, the significantly enhanced ability of **9** to displace [^{3}H]TCP binding may therefore be a result of its longer ethyl linkage, compared to the methyl spacer in **1** and **8** (Fig. 1), allowing the cage moiety to explore deeper into the channel to reach the proximity of the PCP binding site. The blocking action of compound **8**, devoid of alkyl substitution on the amino groups (compared to **1**), may be different from that of compounds that contain a phenyl side chain; therefore the mechanism of this compound requires further study. We note that compound **8** alone among all the compounds tested had a Hill slope value of <1 for blocking NMDA receptor-dependent calcium uptake (Table 1), suggesting negative cooperativity.[34]

The binding characteristics reported in this study are similar to those found for a set of azapentacycloundecylamines studied by Kassiou et al.[35] These authors investigated sigma receptor and PCP binding for a set of pentacycloundecylamines based on an earlier report suggesting that amantadine interacts with the sigma binding site in human frontal cortex.[36] The compounds investigated by this group included aza-bridgehead compounds, in contrast to the oxa-bridgehead compounds used in the present study. These investigators found high selectivity for sigma binding sites, and no cross-reactivity with the PCP site (K_i > 10 μM).[35] Although our binding data suggest very limited interaction with the PCP binding site, the pentacycloundecylamines studied here were functional antagonists of the NMDA receptor, findings that highlight the importance of functional assay screens.

In conclusion, the pentacycloundecylamines in the present study represent a novel group of NMDA receptor antagonists. The uncompetitive nature of NMDA receptor antagonism, in addition to their use-dependent L-type calcium channel blocking effects, suggests that these compounds may be useful as dual mechanism neuroprotective agents[37] in neurodegenerative disorders.[38]

Further studies are underway to elucidate the exact mechanism and site of interaction that result in the NMDA receptor/ion channel complex block achieved by the pentacycloundecylamines tested in this study.

4. Materials and methods

4.1. Chemicals and reagents

Amantadine and memantine were purchased as the hydrochloride salts from Sigma Chemical Co. (St. Louis, MO). (+)MK-801 (as the maleate salt) was purchased from Research Biochemicals (Natick, MA, USA). $^{45}CaCl_2$ (12 mCi/ml) was purchased from ICN Biochemicals CA, (USA). [^{3}H]MK-801 (17.1 Ci/mmol) and [^{3}H]TCP (45.2 Ci/mmol) were purchased form Perkin-Elmer (Boston, MA, USA). Buffer constituents were obtained from Sigma Chemical Co. (St. Louis, MO). The pentacycloundecylamines used in the current study (Fig. 1) were prepared as reported previously by our laboratory[18,20] with the exception of **8** and **9**, which were prepared according to the method detailed by Oliver et al.[21]; and Geldenhuys et al.,[16] and Zah et al.,[22] respectively.

4.2. Calcium flux assay

Synaptoneurosomes where prepared as described previously[23] with minor modifications. Whole brains of male ICR mice (Harlan) were dissected out on ice and coarsely minced with scissors. The tissue was homogenized in magnesium-free incubation buffer by hand using a Dounce homogenizer. The incubation buffer contained 118 mM NaCl, 4.7 mM KCl, 0.1 mM $CaCl_2$, 20 mM Hepes, and 30.9 mM glucose. The tissue was then centrifuged for 15 min at 928*g* and 0 °C. After centrifugation, the pellet was resuspended by hand in fresh incubation buffer using a Pasteur pipette and then centrifuged again as described above. We found that two centrifugation steps were absolutely required to achieve consistent levels of background $^{45}Ca^{2+}$ uptake, as well as a reliable estimate of antagonist activity (data not shown). The final pellets were resuspended in a volume of incubation buffer adequate for experimental use. The tissue suspension containing synaptoneurosomes was then exposed to drug dissolved in DMSO, with the final concentration of DMSO not exceeding 0.1% in the final solution. Because the pentacycloundecylamines have previously been reported as L-type calcium cannel blockers[14,17,18,20] we added 10 μM of the dihydropyridine L-type calcium channel blocker nitrendipine to all the incubations as a precautionary action to minimize flux through these channels. Control incubates received DMSO in addition to nitrendipine. All drug treatments were preincubated for 10 min at room temperature. Stock solutions of NMDA and Gly were prepared in incubation buffer. Treated synaptoneurosomes were then exposed to incubation buffer containing $^{45}CaCl_2$ (3 μM final concentration) and NMDA/Gly for 5 s. The final concentrations of NMDA and Gly were 100 μM each for the screening assays, and for the NMDA dose–response curves the concentration of NMDA varied, while Gly was kept constant at 100 μM. Flux was terminated by the addition of 4 ml ice-cold wash buffer. The wash buffer contained 118 mM cholinechloride, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.18 mM $MgSO_4$, 20 mM Hepes, and 27 mM glucose. The incubate was poured onto a Whatman GF/C glass fiber filter under vacuum and then washed twice with 4 ml of wash buffer. The filters were then air dried, scintillation cocktail added, and the amount of trapped $^{45}Ca^{2+}$ measured by liquid scintillation counting. Treatments were repeated two to four times on different tissue preparations with three determinations in each replicate.

4.3. Radioligand binding assay

Procedures similar to those reported in published studies were used to measure [^{3}H]MK-801[24] and [^{3}H]TCP[25] binding. Murine synaptoneurosomes were prepared as described for the $^{45}Ca^{2+}$ functional assay. The reaction mixture consisted of 380 μL of membrane preparation typically containing 0.38 mg protein, and [^{3}H]MK-801 (5 nM) or [^{3}H]TCP (5 nM) as radioligand. Unlabeled MK-801 and other test compounds were applied at 100 μM final concentration and were dissolved in DMSO. The addition of unlabeled MK-801 served to assess non-specific binding. The reaction was initiated by the addition of 20 μL incubation buffer containing NMDA (100 μM) and Gly (100 μM) to the membrane preparation. Incubation of the mixture was for 1 h at 25 °C. The reaction was terminated by the addition of ice-cold wash buffer and filtration on Whatman GF/B filters. Filters were pretreated with polyethyleneimine (0.05%) for [^{3}H]TCP binding.[25] Following air drying, the filters were placed into scintillation liquid and radioactivity retained on the filters was determined by liquid scintillation counting.

4.4. Data analysis

Calcium flux and radioligand binding data were analyzed with InStat 2.0 and plotted with Prism 2.0 software (both from GraphPad Software, San Diego CA, USA). Concentration–response data were analyzed by nonlinear least squares curve fitting, with IC_{50} values for blockers or EC_{50} values for NMDA/Gly calculated using Prism™. The bottom value for the antagonism dose–response curves was held constant at 100% of control, as explained in the results section, to obtain Figure 4. Student's *t*-tests were performed on mean responses of test compound versus controls in the screening studies and on curve fit parameters in the dose–response studies. A $p < 0.05$ was considered statistically significant.

4.5. Molecular modeling

Molecular modeling of minimum energy conformations and volume for each structure (memantine and **1**) were obtained by optimizing the drawn structures with the Chemplus™ extension of Hyperchem 7.0 modeling software (Hypercube Inc., Gainesville, Florida, USA) using the semi-empirical AM1 approach. Low energy conformers of **1** and memantine were used for the fitting (Fig. 2). For the MOLCAD surface area analysis, the structures of **1** and **6**, used in the study, were built using

SYBYL 6.91 molecular modeling software (Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA) on a Silicon Graphics Octane computer (SGI, Mountain View, California). Both structures were energy minimized using the standard Tripos molecular mechanics force field. Gasteiger-Hückel charges were applied, with a 0.005 kcal/mol energy gradient convergence criterion.

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