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NOVEL BISBENZAMIDINES AND BISBENZIMIDAZOLINES AS NONCOMPETITIVE NMDA RECEPTOR ANTAGONISTS

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Abstract: A series of novel bisbenzamidines and bisbenzimidazolines with different linkers connecting the aromatic groups was tested in vitro for NMDA receptor antagonist activity. IC_{50} values for these compounds ranged from 1.2 to >200 μM. The bisbenzamidine with a homopiperazine ring as the central linker was found to be the most potent NMDA receptor antagonist among all the pentamidine analogues tested so far.

The N-methyl-D-aspartate (NMDA) receptor has been demonstrated to be involved in a wide variety of physiological and pathophysiological processes in the central nervous system (CNS), which include memory and learning[1] and epileptogenesis,[2] neurodegeneration associated with cerebral ischemia, stroke, Alzheimer's disease and Huntington's disease.[1] Studies have also shown that human immunodeficiency virus-1 (HIV-1) associated dementia eventually develop in more than 60% of patients with AIDS.[3,4] These neurological alterations are believed to be associated with the pathological changes in neural and non-neuronal cells observed in postmortem specimens,[5] and loss of cortical neurons.[6,7] It has been demonstrated that the HIV-1 coat protein (gp120)-induced increase in intracellular Ca^{2+} and neurotoxicity could be blocked and prevented by the NMDA receptor antagonists such as D-(-)-2-amino-5-phosophonovalerate (D-APV) and dizocilpine (MK-801).[8] These studies suggest that development of effective NMDA receptor antagonists could lead to useful therapeutic agents for these CNS diseases and for preventing or delaying the onset of dementia in AIDS patients.

Many polyamine compounds such as spermine and spermidine analogues have been reported to bind to the NMDA receptor.[9-12] Recently, Reynolds et al.[13,14] reported that pentamidine and its analogues were effective NMDA receptor antagonists and protected neurons in vitro from NMDA-induced neurotoxicity. Donkor and Berger[15] studied the inhibitory effects of a series of butamidine analogues on spermine-associated NMDA receptor complex and found that hydrophobic linkers between the benzamidine groups enhanced NMDA receptor antagonism. We report here the structure–activity relationships of a series of novel pentamidine analogues with different cyclic linkers separating the benzamidine or benzimidazoline groups as noncompetitive NMDA receptor antagonists.

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Materials and Methods

Compounds **1–13**, shown in Table 1, were synthesized and characterized by ^{1}H NMR and elemental analyses as previously described.[16,17]

Receptor Binding Assays: [^{3}H]Dizocilpine binding assays were performed in well washed rat brain membranes as previously described.[18,19] Assays contained 50–100 μg protein, 0.5 nM [^{3}H]dizocilpine, 100 μM glutamate, 30 μM glycine and appropriate test compounds in a final volume of 1 ml 10 mM HEPES-buffered salt solution (HBSS) pH 7.4. Assays were incubated for 2 h at room temperature, which represents nonequilibrium conditions that are optimal for measuring drug interactions with the NMDA receptor. Assays were terminated by vacuum filtration and radioactivity was determined by liquid scintillation counting. Data were analyzed and IC_{50} values were determined using Prism (GraphPad Software, San Diego, CA). The results are listed in Table 1.

Intracellular Ca^{2+} Measurements: The $[Ca^{2+}]_i$ measurements were recorded in primary cultures of neurons that were obtained from rat fetal forebrain and grown on coverslips.[13] The neurons were rinsed and loaded with fura-2 by incubation in HBSS containing 5 μM fura-2 and 5 mg/mL BSA for 45 min at 37 °C. The coverslips were mounted in a chamber and placed on the stage of the inverted microscope for fluorescence recording. The experiments were conducted in Mg^{2+}-free buffer. NMDA (30 μM) and glycine (10 μM) were added to the superfusate to obtain responses. Receptors were stimulated with the NMDA and glycine for 15 s and a control response was recorded to which concentration response curves were compared. Then the neurons were incubated with each concentration of compound **11** for 30 s prior to the NMDA and glycine stimulation in the presence of the compound. The results are shown in Table 2.

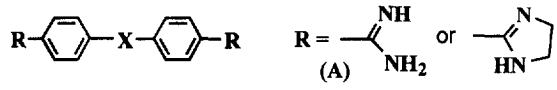
Toxicity Assays: Coverslips were washed twice with sterile HBSS and flipped face up. After a 10-min pretreatment in HBSS at 37 °C, cultured cells were incubated in the presence of 100 μM glutamate, 1 μM glycine and compound **11** for 10 min at 37 °C. Neurons were rinsed in HBSS and returned to Minimum Essential Media (MEM) with penicillin (24 U/mL) and streptomycin (24 μg/mL) for 24 h. Viability was assayed by measuring calcein fluorescence with the Cytofluor II plate reader. After a 45-minute incubation in HBSS containing 5 μM Calcein AM and 5 mg/mL bovine serum albumin, samples were excited with 485 nm light and emission at 530 nm was measured. The fluorescence values measured were background-corrected by subtraction of the fluorescence measured from a sample of calcein AM incubated in the absence of cells. The results are shown in Table 3.

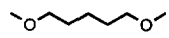
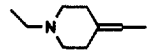
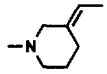
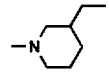
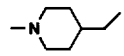
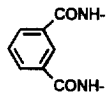
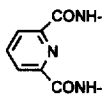
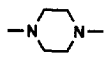
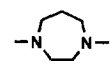
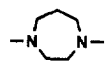
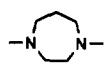
Results and Discussion

NMDA receptor antagonist effects of pentamidine analogues: The effect of structurally dissimilar cyclic linkers (such as piperidine, piperazine, homopiperazine, pyridine and phenyl) on the NMDA receptor antagonism of the bisbenzamidines and bisbenzimidazolines was investigated. Table 1 lists the chemical structures of the novel bisbenzamidines and bisbenzimidazolines (compounds **1–12**), along with pentamidine and bis(4-cyanophenyl) homopiperazine **13**, and their inhibitory effects (IC_{50}s) on [^{3}H]dizocilpine binding to the NMDA receptor complex in rat brain membranes. Nine of the twelve compounds (**2–6** and **9–12**) were effective inhibitors of [^{3}H]dizocilpine binding to the NMDA receptor. The central linker of these compounds dramatically influenced their inhibitory potencies. For instance, bisbenzamidine **11** with a homopiperazine linker was about 168- and 55-fold more potent than **7** (with a meta-phenyldicarboxamide linker) and **8** (with a meta-pyridinyldicarboxamide linker), respectively. It is also noteworthy that compound **11** emerged as the most potent NMDA receptor antagonist among all the pentamidine analogues tested so far. Its IC_{50} value of 1.19 μM is two-fold greater than that of pentamidine (2.59 μM).[13] Within the piperidine series (**1–4**), positioning the imidazolino benzyl group meta or para to the piperidine ring nitrogen did not significantly affect NMDA receptor inhibition (**2** or **3** vs **4**). However, substitution of the imidazolino phenyl group on the piperidine ring nitrogen with an imidazolino benzyl functionality, thus increasing the distance between the two terminal cationic groups, resulted in about 6-fold decrease in inhibitory activity (**1** vs **4**). Taken together, these observations suggest that the distance between the two terminal cationic groups and the shape of the molecule influence the NMDA receptor inhibitory potencies of these compounds. Generally, amidines were better inhibitors than the corresponding imidazolines (**5** vs **6**, **9** vs **10**, and **11** vs **12**) although the differences were modest. In order to determine the importance of the cationic groups, compound **13** with the nitrile as the terminal groups was tested. It showed weak inhibitory activity and was about 38-fold less potent than the corresponding diamidine **11**, suggesting that amidine or imidazoline groups are required for potent inhibition of the NMDA receptor complex. The Hill slopes of the concentration-response curves for all the compounds were greater than unity, indicating that these compounds are non-competitive inhibitors of [^{3}H]dizocilpine binding. Similar observations with other pentamidine analogues have been reported.[14]

Inhibition of Intracellular Ca^{2+} increases by compound 11: Uncontrolled and excess accumulation of intracellular Ca^{2+} has emerged as the common cause in the cytotoxic processes, including HIV-1 associated dementia. The increase in $[Ca^{2+}]_i$ could be blocked by NMDA receptor antagonists.[8] The most potent compound **11** in this series was tested for its effectiveness in blocking the increase in $[Ca^{2+}]_i$. Indeed, **11** inhibited the NMDA (30 μM)- and glycine (10 μM)-induced increases in $[Ca^{2+}]_i$ in cultured forebrain neurons

Table 1. NMDA receptor antagonist potency of pentamidine analogues



Compound	X	R	IC_{50} (μM)[a]
Pentamidine		A	2.59[b]
1		I	35.6 (30.4–41.6)
2		I	8.35 (6.71–10.3)
3		I	7.84 (5.94–10.3)
4		I	6.29 (4.89–8.09)
5		A	11.7 (9.67–14.1)
6		I	16.5 (13.2–20.6)
7		A	>200
8		A	66.0 (53.1–82.0)
9		A	5.32 (3.96–7.14)
10		I	18.2 (15.6–21.3)
11		A	1.19 (0.96–1.48)
12		I	4.99 (4.09–6.08)
13		CN	45.8 (38.7–54.3)

[a]Inhibition (IC_{50}) of [^{3}H]dizocilpine binding to NMDA receptor complex in rat brain membranes. The IC_{50} values were obtained from 10 concentrations of each compound, run in triplicate. The 95% confidence limits are listed in parenthesis. [b]Data taken from reference 13.

from fetal rats. As shown in Table 2, 0.1 μM of **11** had no effect on the increase. But, at 1.0 μM and 10.0 μM, **11** significantly inhibited the $[Ca^{2+}]_i$ increases (33 and 62% inhibition, respectively).

Table 2. Inhibitory effect of compound **11** on NMDA/glycine induced $[Ca^{2+}]_i$ increase

Stimulation	$[Ca^{2+}]_i$ increase, % control
Control (30 μM NMDA + 10 μM glycine)	100.00 ± 0.00
11 (0.1 μM) + Control	111.26 ± 7.13
11 (1.0 μM) + Control	* 67.04 ± 2.55
11 (10.0 μM) + Control	** 37.58 ± 3.02

* $P < 0.01$; ** $P < 0.001$ when compared to control. The data represents the mean ± SEM of three determinations.

Neuroprotective effects of compound 11: Pentamidine was shown to be an effective NMDA receptor antagonist with neuroprotective properties. However, the drug also caused direct toxicity to cultures of embryonic rat brain at higher concentrations (30 μM).[14] It was also demonstrated that the neuroprotective actions were separable from the direct toxic effects for several pentamidine analogues with NMDA receptor antagonist activity.[14] This finding is significant because it allows the development of useful drugs with a wider margin of safety. The protective property of **11** against glutamate-induced neurotoxicity was therefore tested. When administrated alone without glutamate, no direct toxic effects to the cortical neurons were observed for 10 μM or 100 μM **11**. On the other hand, 100 μM glutamate destroyed about 40% of the neurons in vitro 24 h later after a 10 min exposure to the toxin. Addition of 10 or 100 μM of **11** totally prevented the loss of these neurons (Table 3). This indicated that **11** protected cortical neurons from glutamate-induced toxicity without causing any direct toxic effects.

Table 3. Protective effect of **11** on cortical neurons from glutamate-induced toxicity

Stimulation	Viability, % control
Control (HBSS)	100 ± 0
Glutamate (100 μM)	* 60 ± 5
11 (10 μM)	100 ± 13
11 (10 μM) + Glutamate (100 μM)	99 ± 7
11 (100 μM)	87 ± 10
11 (100 μM) + Glutamate (100 μM)	98 ± 10

* $P < 0.05$ when compared to control. The data represents the mean ± SEM of three determinations.

In summary, we have shown that a series of novel bisbenzamidines and bisbenzimidazolines are effective NMDA receptor antagonists. Nine of the thirteen compounds tested are potent inhibitors of [^{3}H]dizocilpine binding to the NMDA receptor. The central linker in these compounds plays a significant role in their NMDA receptor antagonist potencies. The homopiperazine ring was the best linker in this series of compounds. Compound **11** with a homopiperazine linker was found to be the most potent pentamidine-like NMDA receptor antagonist tested so far. Compound **11** also inhibited the increase in $[Ca^{2+}]_i$ induced by NMDA and glycine, and effectively protected cortical neurons in vitro from the toxicity induced by glutamate without causing any direct toxic effects to the neurons.

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