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INHIBITORY EFFECTS OF PENTAMIDINE ANALOGUES ON SPERMINE STIMULATED LIGAND BINDING TO THE NMDA RECEPTOR COMPLEX

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Abstract: SAR studies of butamidine derivatives with the NMDA receptor complex revealed that hydrophobic rather than hydrophilic linkers between the amidinophenyl groups promote NMDA antagonism. Conformation of the linker did not influence binding in the absence of bis-methoxy substituents *ortho* to the linker. Like pentamidine, the compounds are noncompetitive NMDA receptor antagonists. © 1997 Elsevier Science Ltd.

NMDA (*N*-methyl-D-aspartate) receptors have been implicated in a number of physiological and pathophysiological processes in the mammalian central nervous system. These ligand-gated channels appear to be involved in neurodegeneration associated with cerebral ischemia, Alzheimer's disease, human immunodeficiency syndrome (HIV-1) associated dementia, and Huntington's disease.[1] It has been demonstrated that polyamines such as spermine (*N,N'*-bis(3-aminopropyl)-1,4-butanediamine) and spermidine [*N*-(3-aminopropyl)-1,4-butanediamine] display multiple effects at NMDA receptors. For instance polyamines enhance the binding of channel ligands such as [^{3}H]MK-801 to brain membranes in the presence of low levels of glutamate and glycine.[2-4] They elevate the apparent affinity of glycine at strychnine-insensitive glycine receptors[5,6] and enhance the potency of glycine to increase NMDA-mediated currents.[7] At higher concentrations, however, these endogenous amines precipitate a voltage-dependent blockade of NMDA receptors and inhibit the binding of [^{3}H]MK-801.[3,4,7] Despite their multiple actions, polyamines have been implicated in the neuropathology associated with excessive activation of NMDA receptors. For instance, an increase in polyamines has been observed following mechanical brain trauma or middle cerebral artery occlusion;[8] it has been demonstrated in vitro[9] and in vivo[10] that inhibitors of polyamine biosynthesis can reduce NMDA-mediated neurotoxicity. Additionally, NMDA antagonists acting at polyamine binding sites can reduce ischemic brain injury[6] and block NMDA mediated neurotoxicity in vitro.[11]

The structure activity relationship (SAR) of the polyamine binding site has been studied with linear and heteroalkyl polyamine analogues as well as nonpolyamine-like aliphatic and aromatic cationic compounds. Romano and Williams[12] using a series of mono-, di-, and triamines proposed a model for the polyamine recognition site on the NMDA receptor complex. Reynolds[13] have demonstrated that the spermidine analogue, 1,5-(diethylamino)piperidine, is a specific activator of the NMDA receptor-associated polyamine site. Berger et al.[14] have reported that long chain 1, ω-diamines with 10, 12, or 14 methylene groups between the terminal amino groups can reduce the binding of [^{3}H]MK-801 to rat hippocampal membranes in a spermine-sensitive way.

Reynolds and Aizenman[15] have shown that pentamidine is an NMDA antagonist and protects neurons in vitro from NMDA toxicity. These workers and their collaborators also synthesized structural analogues of pentamidine with reduced toxicity against neurons in culture, but maintained potency as NMDA antagonists.[16] Although they observed a decrease in the apparent potency of spermidine in stimulating [^{3}H]MK-801 binding under the influence of pentamidine, they also observed a decrease in the maximal effect of the polyamine and therefore rejected the hypothesis that pentamidine interacts in a competitive way with polyamines at the NMDA receptor complex. To further investigate the SAR of the polyamine binding site, we studied the interaction of novel pentamidine related dicationic compounds with the NMDA receptor complex using the NMDA channel blocker MK-801 as radioligand.

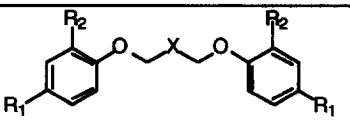
Methods. The pentamidine analogues used in this study were synthesized in our laboratory as previously reported.[17] The compounds were characterized by proton NMR, and elemental analysis. Pentamidine was purchased from Sigma.

Membrane Preparation: The CA1/dentate gyrus part of rat hippocampi (male Wistar rats, ages 3 - 6 months) was dissected from unfrozen brains and homogenized in 50 mM Tris acetate (pH 7.0) with a glass/teflon Potter-type homogenizer.[18] After centrifugation at 35,000 x *g* the pellet was washed three times by resuspension in fresh buffer and recentrifugation, including a treatment with 0.02% Triton X-100 (for 10 min at 37 °C) after the second resuspension. The final suspension was stored as aliquots at -80 °C.

Binding Assays: An aliquot of the above preparation was thawed and centrifuged. Assays were performed in polypropylene vials in triplicates. Each vial contained membranes of 1 mg original tissue (approximately 30 µg protein) in a final volume of 1 mL. In most assays, the incubation buffer was 50 mM Tris acetate (pH 7.0), but in some experiments a lower buffer concentration was used (20 mM). The hippocampal membranes were incubated at 24 °C for 2 h (3 h in the case of the lower buffer concentration) with 5 nM [^{3}H]MK-801 (20-23 Ci/mMol) and 1 μM glutamic acid and glycine. Nonspecific binding was obtained by replacing glutamic acid and glycine by their respective antagonists, D-2-amino-5-phosphonovaleric acid (10 μM) and 5,7-dichlorokynurenic acid (1 μM). The assay was terminated by rapid filtration via glass fiber filters (GF/C, Whatman) preincubated for 1 h in 0.3% aqueous polyethylenimine. Radioactivity was extracted by shaking the filters for 1 h in toluene based scintillation cocktail (Rotiscint 11) and quantified by liquid scintillation counting. The IC_{50} values and the Hill coefficients (n_H) of the test compounds were obtained by computerized curve fitting of the displacement curves to the function $y = B_o \cdot IC_{50}^{nH} / (IC_{50}^{nH} + [I]^{nH}) + NB$, where B_o is the specific binding of the radioligand in the absence of inhibitors, [I] is the concentration of the inhibitor, and NB is the nonspecific binding.

Results and Discussion. Under our usual assay conditions (50 mM Tris acetate buffer), pentamidine inhibited the specific binding of the NMDA channel ligand ([^{3}H]MK-801) to rat hippocampal membranes with an $IC_{50} = 3.1$ μM and with a Hill coefficient close to unity (means of 3 determinations, Table 1). In the presence of 100 μM spermine, the inhibitory potency of pentamidine was weakened by a factor 9.5 (last column in Table 1). Since spermine, in experiments performed in parallel (not shown), stimulated the specific binding of [^{3}H]MK-801 with EC_{50} values between 2 and 5 μM, a concentration of 100 μM was 20- to 50-fold the EC_{50} of spermine. If

Table 1. Effect of Spermine and Two Buffer Concentrations on the Inhibition of [^{3}H]MK-801 Binding to the NMDA Receptor Complex by Pentamidine and Its Derivatives.



Compound	R_1	R_2	X	Buffer[a,b]	IC_{50} (μM)[c]	n_H[c,d]	Increase in IC_{50} by 100 μM spermine
Pentamidine (1)		H	-$(CH_2)_5$-	A: B:	3.06 ± 0.59 (1.31, 1.74)	0.93 ± 0.06 (1.17, 1.15)	9.5 ± 2.7 (30.7, 21.4)
Butamidine (2)		H	-CH_2CH_2-	A: B:	5.18 ± 0.94 (2.64, 3.01)	0.99 ± 0.09 (1.11, 1.36)	10.2 ± 1.1 (28.8, 35.0)
3a		H	- CH=CH - (*Cis*)	A: B:	4.26 ± 0.48 (1.80, 1.48)	1.13 ± 0.10 (1.39, 1.38)	11.9 ± 2.6 (29.1, 38.2)
3b		H	- CH=CH - (*Trans*)	A: B:	3.81 ± 0.38 (2.48, 1.94)	0.71 ± 0.06 (0.98, 0.88)	9.5 ± 2.7 (21.7, 27.8)
4		H		A: B:	(66, 66) (24.8, 22.6)	(1.23, 1.08) (1.04, 1.09)	(9.3, 8.1) (28.4, 22.4)
5a		H	- CH=CH - (*Cis*)	A: B:	26.7 ± 8.8 (18.8, 15.9)	0.99 ± 0.07 (1.21, nd[e])	5.48 ± 1.13 (9.9, 12.1)
5b		H	- CH=CH - (*Trans*)	A: B:	(38, 37) (13.1, 13.8)	(0.98, 0.81) (0.95, 0.87)	(4.14, 4.88) (15.3, 12.7)
6a		OCH_3	- CH=CH - (*Cis*)	A: B:	(32.6, 49.0) (26.5, 23.3)	(1.33, 1.15) (1.13, 1.29)	(4.62, 3.43) (7.8, 8.6)
6b		OCH_3	- CH=CH - (*Trans*)	A: B:	(63, 83) (35, 33)	(1.09, 1.16) (1.04, 0.96)	(4.24, 3.79) (12.7, 13.8)
7a		OCH_3	- CH=CH - (*Cis*)	A: B:	(95, 73) (50, 62)	(2.14, 1.00) (1.21, 1.46)	(1.72, 2.87) (4.7, 4.5)
7b		OCH_3	- CH=CH - (*Trans*)	A: B:	(31.0, 26.4) (12.0, 12.6)	(1.28, 1.21) (1.19, 1.19)	(2.87, 4.15) (14.3, 12.2)

[a]Buffer A is 50 mM Tris acetate, pH 7.0. [b]Buffer B is 20 mM Tris acetate, pH 7.0. [c]In the cases where only two determinations were made both single results are reported. However, where three determinations were made, mean values ± S.D. are reported. [d]n_H is Hill coefficient. [e]nd = not determined. Computer analysis was performed with $n_H = 1$.

competitive interaction between the agonist, spermine, and the 'inverse agonist' pentamidine at a polyamine regulatory site of the NMDA receptor complex was the only mechanism of action involved, the IC_{50} of pentamidine should have been shifted by 100 μM spermine by approximately 20- to 50-fold. The observed shift, however, was less pronounced. Under lower ionic strength conditions (20 mM Tris acetate), the inhibitory potency of pentamidine was slightly higher ('buffer B' in Table 1), and 100 μM spermine weakened this inhibition by a mean factor of 26. Again, this factor was considerably lower than expected for purely competitive interaction since, under these buffer conditions, specific binding of [^{3}H]MK-801 was more sensitive to stimulation by spermine ($EC_{50} \approx 1$ μM).

Similar results were obtained with the shorter chain homologue, butamidine (**2**), and its geometric isomers **3a** and **3b**. The compounds inhibited specific [^{3}H]MK-801 binding at low micromolar concentrations and were slightly more potent under lower ionic strength conditions with Hill coefficients around unity. Spermine weakened their potencies considerably but not to the extent predicted for purely competitive interaction at a polyamine sensitive site. These findings suggest that the exact length and conformation of the linker between the amidinophenyl groups is not essential for polyamine-sensitive NMDA antagonism. The conformation of the compounds was, however, of some significance in the antimicrobial activity and general toxicity of the compounds.[17,19] Generally, bis-methoxy substitution *ortho* to the linker decreased the anti-NMDA activity of the compounds. For instance, compound **3a** without the methoxy substituents was over 20 times more potent than its methoxy substituted counterpart **7a**. This effect was more pronounced in compounds with amidino groups than those with imidazolino groups. The detrimental effects of methoxy substitution has also been reported for pentamidine.[16] Interestingly, the conformational effects of the linker on NMDA activity was dependant on the presence or absence of methoxy substituents ortho to the linker. Compounds **7a** and **7b** with methoxy substituents *ortho* to the 2-butene bridge were the only compounds in which the geometry of the linker influenced activity. The *trans*-isomer **7b** was more potent and more sensitive towards spermine in its inhibition of [^{3}H]MK-801 binding (especially under lower ionic strength conditions, 'B' in Table 1) than its *cis* counterpart **7a**. This trend was also observed (though less convincingly) by the spermine sensitivities of the *ortho*-methoxy substituted imidazoline derivatives **6a** and **6b**. In the absence of the methoxy substituents, conformation is of no significance in the NMDA antagonism of the compounds as seen with geometric isomers **3a** and **3b** and also with isomers **5a** and **5b**. Hydrophobicity of the linker is of greater importance than geometry. Introduction of hydrophilic residues (2,3-diol) into the linker as in compound **4** strongly reduced its anti-NMDA activity. Interestingly, such a modification also reduced the antimicrobial activity of the compound.[19] It is noteworthy that even the NMDA-antagonism of this weakly active compound was weakened by spermine to approximately the same extent as that of the amidino derivatives without the methoxy substituents (compounds **1**, **2**, **3a**, and **3b**) discussed above. Generally, replacement of the amidines with imidazolines, and/or introduction of methoxy residues *ortho* to the linker, resulted in a pronounced decrease in anti-NMDA potency. These modifications also reduced the influence of spermine by at least a factor of two compared to the amidino compounds without methoxy substituents. Berger et al.[20] have observed that long chain aliphatic diamines lose potency and spermine sensitivity upon *N*-methylation. Taken together, these results suggest that the presence of a terminal unsubstituted amino group(s) is a prerequisite for polyamine sensitive NMDA antagonism.

A spermine sensitivity considerably lower than that predicted on grounds of a purely competitive mechanism is not only observed for the NMDA antagonism by pentamidine,[13] but also for several other so called 'inverse

polyamine agonists'.[14,21] The superficial impression of competition between polyamines like spermine and spermidine and extended dicationic strongly basic inhibitors may result from the fact that both classes of compounds use the same (or very similar) mechanisms to influence the opening frequency of the NMDA receptor associated ion channel, thereby counteracting each other. Even if there appears to be no direct competition for a common site of action, SAR studies on polyamine sensitive NMDA antagonism of pentamidine analogues may provide fundamental insights into the mechanism by which polyamines increase the opening frequency of NMDA channels. Irrespective of the exact mechanisms involved, antimicrobial agents like pentamidine, used to treat opportunistic infections in AIDS patients, which also block NMDA receptors at low micromolar concentrations, are of considerable interest since neurological deficits in the late stages of AIDS have been related to high levels of the excitotoxin, quinolinic acid,[22] which is a compound that acts as an agonist at NMDA receptors.[23]

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References

1. Choi, D. W. *Neuron* **1988**, *1*, 623.
2. Ransom, R.; Stec, N. *J. Neurochem.* **1988**, *51*, 830.
3. Williams, K.; Romano, C.; Molinoff, P. B. *Mol. Pharmacol.* **1989**, *36*, 575.
4. Marvizon, J. C.; Baudry, M. *Eur. J. Pharmacol.* **1994**, *269*, 165.
5. Ransom, R. W.; Deschenes N. L. *Synapse* **1990**, *5*, 294.
6. Sacaan, A. I.; Johnson, K. M. *Mol. Pharmacol.* **1989**, *36*, 836.
7. Benveniste, M.; Mayer, M. L. *J. Physiol. (London)* **1993**, *464*, 131.
8. Carter, C. J.; Lloyd, K. G.; Zivkovic, B.; Scatton, B. *J. Pharmacol. Exp. Ther.* **1990**, *253*, 475.
9. Markwell, M. A. K.; Berger, S. P.; Paul, S. M. *Eur. J. Pharmacol.* **1990**, *182*, 607.
10. Kish, S. J.; Wilson, J. M.; Fletcher, P. J. *Eur. J. Pharmacol.* **1991**, *209*, 101.
11. Skolnick, P.; Boje K.; Miller R.; Pennington, M.; Maccecchini, M.-L. *J. Neurochem.* **1992**, *59*, 1516.
12. Romano, C.; Williams, K. *Mol. Pharmacol.* **1992**, *41*, 785.
13. Reynolds, I. J. *Mol. Pharmacol.* **1992**, *41*, 989.
14. Berger, M. L.; Seifriz, I.; Letschnig, M.; Schodl, C.; Noe, C. R. *Neuroscience Lett.* **1992**, *142*, 85.
15. Reynolds, I. J.; Aizenman, E. *J. Neurosci.* **1992**, *12*, 970.
16. Reynolds, I. J.; Zeleski, D. M.; Rothermund, K. D.; Hartnett, K. A.; Tidwell, R. R.; Aizenman, E. *Eur. J. Pharmacol.* **1993**, *244*, 175.
17. Donkor, I. O.; Tidwell, R. R.; Jones, S. K. *J. Med. Chem.* **1994**, *37*, 4554.
18. Berger, M. L.; Charton, G.; Ben-Ari, Y. *J. Neurochem.* **1986**, *47*, 720.

19. Donkor, I. O.; Queener, S. F.; Dalton, J. T. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1967.

20. Berger, M. L.; Schadto, O.; Noe, C. R. *Unpublished results.*

21. Berger, M. L.; Rebernik, P. *Unpublished results.*

22. Heyes, M. P.; Brew, B. J.; Martin, A.; Price, R. W.; Salazar, A. M.; Sidtis, J. J. *Ann. Neurol.* **1991**, *29*, 202.

23. McLennan, H. *Neurosci. Lett.* **1984**, *46*, 157.

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