Bioorganic & Medicinal Chemistry 23 (2015) 4489–4500

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Pentamidine analogs as inhibitors of [^{3}H]MK-801 and [^{3}H]ifenprodil binding to rat brain NMDA receptors

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ARTICLE INFO

Article history:
Received 6 October 2014
Revised 5 June 2015
Accepted 5 June 2015
Available online 14 June 2015

Keywords:
Pentamidine
NMDA receptor
Ifenprodil
Polyamine
Bisbenzamidine
Alkylation
Coupling
Radioligand binding
Rat brain
Molecular docking

ABSTRACT

The anti-protozoal drug pentamidine is active against opportunistic *Pneumocystis* pneumonia, but in addition has several other biological targets, including the NMDA receptor (NR). Here we describe the inhibitory potencies of 76 pentamidine analogs at 2 binding sites of the NR, the channel binding site labeled with [^{3}H]MK-801 and the [^{3}H]ifenprodil binding site. Most analogs acted weaker at the ifenprodil than at the channel site. The spermine-sensitivity of NR inhibition by the majority of the compounds was reminiscent of other long-chain dicationic NR blockers. The potency of the parent compound as NR blocker was increased by modifying the heteroatoms in the bridge connecting the 2 benzamidine moieties and also by integrating the bridge into a seven-membered ring. Docking of the 45 most spermine-sensitive bisbenzamidines to a recently described acidic interface between the N-terminal domains of GluN1 and GluN2B mediating polyamine stimulation of the NR revealed the domain contributed by GluN1 as the most relevant target.

1. Introduction

Pentamidine (**1**), a bisbenzamidine with two aromatic nuclei and an unbranched saturated five-carbon chain inserted between 2 phenolic ether oxygens (Chart 1), introduced as a trypanocidal agent,[1] gained importance with the HIV pandemic as one of the most effective agents against opportunistic *Pneumocystis* pneumonia.[2] Despite successes of antiretroviral therapy, still 20% of HIV-infected patients suffer from neurocognitive disorders, most likely caused by mediators like quinolinic acid released by invading glia.[3,4] By virtue of its dicationic elongated structure, **1** is predestined to fit into the DNA minor groove,[5,6] an interaction possibly underlying its chemotherapeutic properties. However, **1** has several other biological targets in addition, including the NMDA receptor (NR),[7] the imidazoline I_2 binding site,[8] acid-sensing ion channels,[9] and the potassium channel $K_{IR}2.1$,[10] as recently reviewed.[11] The NR is one of the most common excitatory neurotransmitter receptors,[12] activated by glutamic acid; pathologically increased interstitial quinolinic acid might lead to neurotoxicity by acting as agonist at this receptor.[13] Pentamidine has been shown to block the NR at low micromolar concentration, but barely reaches the brain after systemic application.[14] The conformationally restricted pentamidine analog *N*,*N*′-bis(*p*-amidinophenyl)homopiperazine (**24**; Chart 1) inhibits more potently than pentamidine the growth of *Pneumocystis carinii* in culture[15] and the NR on rat brain membranes.[16] Inhibition of the NR by **1** and **24** is sensitive to polyamines,[7,17] suggesting the involvement of the polyamine regulatory site of the NR.[18] The endogenous polyamines spermidine and spermine increase the opening frequency of the

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http://dx.doi.org/10.1016/j.bmc.2015.06.012
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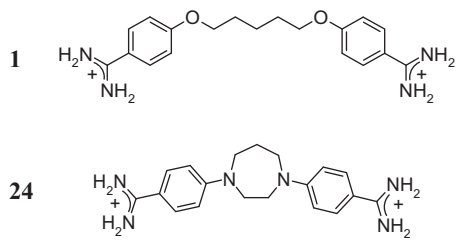


Chart 1.

NR-associated ion channel by allowing the closure of an interface in the extracellular N-terminal domain between subunits GluN1 and GluN2B; this is brought about by neutralization of negative surface charges, which in the absence of polyamines repel each other and keep the interface open.[18] Here, we present inhibitory potencies of several pentamidine analogs at the NR on rat brain membranes, with particular reference to the partial reversal of this inhibition by the polyamine spermine (polyamine sensitivity). While antimicrobial activities of these compounds have been evaluated in vitro, most of them have never been tested at the NR. We test these compounds as inhibitors of specific binding of the channel ligand [^{3}H]MK-801,[19] in absence and in presence of 30 μM spermine, and several of them also as inhibitors of [^{3}H]ifenprodil binding.[20] Also ifenprodil binds to the GluN1/2B interface, but somewhat more externally.[21] The interaction is mainly mediated by H-bonds and hydrophobic interactions, not by charge neutralization. MK-801 is an open-channel blocker that, upon opening of the NR-associated channel, gains access to a site deeply located close to the narrow restriction of the passage way; specific binding of non-saturating concentrations of [^{3}H]MK-801 can be taken as indicator for the accessibility and opening frequency of the channel.[19] Finally, we present first attempts to dock most of these compounds to a recently described binding pocket shared by the NR subunits GluN1 and GluN2B.[22]

2. Syntheses

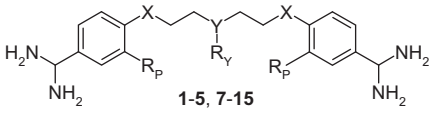
All structures are shown in Tables 1–4. The syntheses of several pentamidine analogs with heteroatom variation in the bridge have been described[23–28] (see Table S1 in the Supporting information for assignment). The syntheses of **9** and **11** and of their precursors **9a** and **11a** are described here for the first time (using established procedures). In short, 2 equiv of the appropriate 4-fluorobenzonitrile were subjected to nucleophilic displacement by 1 equiv of a diamino spacer (Scheme 1). The spacers were 1,5-diaminopentane and bis(2-aminoethyl) ether, respectively, and yielded the terminal nitrogens for the bridge. The resulting bisbenzonitriles **9a** and **11a** were converted in a sealed flask with ethanolic HCl to the iminoesters and thereafter, again in a sealed flask, under alkaline conditions to the respective bisbenzamidines **9** and **11** (*Procedure A*).[23]

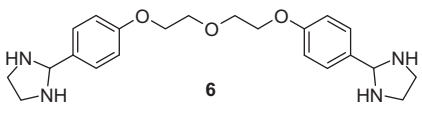
For the synthesis of **15a**, the nitrile precursor of **15**, the terminal heteroatoms in the bridge were (as in pentamidine) oxygens; this time, the leaving halogens were on the spacer *N*,*N*-bis(2-chloroethyl)-*p*-toluenesulfonamide, and the bridge oxygens were introduced with the benzonitrile (Scheme 2).

The piperazine and homopiperazine derivatives are known compounds;[15,29,30] also several of the open chain analogs have been described;[31] **40**, **43** and **44** are described here for the first time and were prepared via the bisbenzonitriles in 3 steps. The first one was equivalent to step one in Scheme 1 (with DMF as the dry solvent). As difunctional spacers we used *N*,*N*′-dimethylpropylenediamine, 1,7-diaminoheptane and 1,8-diaminooctane, respectively. The obtained bisbenzonitriles were converted into the corresponding bisbenzamidines via the bisbenzamidoximes (*Procedure B*).[31]

Most of the remaining compounds are known[32–38] (see Table S1). The dinitrile precursor **71a** for the terthiophene derivative **71** was prepared by Pd-catalyzed coupling of 2 equiv 5-bromo-2-cyanothiophene to 1 equiv 2,5-bis(trimethylstannyl)thiophene (Scheme 3) as described for the addition of 2 phenyl residues to a central thiophene.[39] The 3,3′-bipyridine bisbenzonitrile **76a** was prepared in 2 steps, starting with Pd-catalyzed coupling of

Table 1
Inhibition of [^{3}H]MK-801 and [^{3}H]ifenprodil binding to rat cortical membranes by pentamidine analogs with heteroatom variations in the bridge

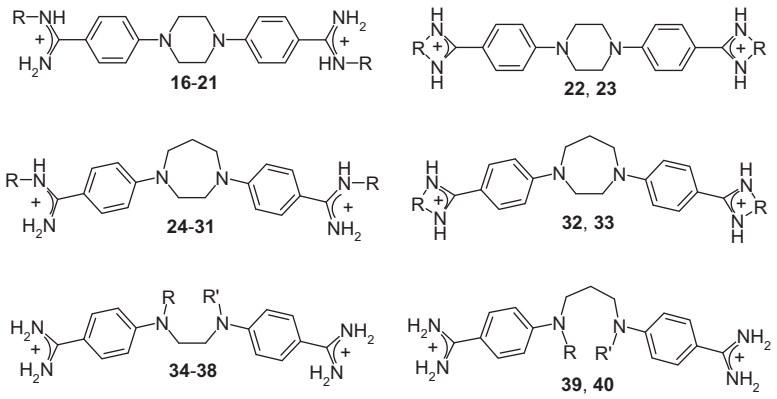




	X	YR_Y	R_P	[^{3}H]MK-801			[^{3}H]ifenprodil
				IC_{50} ± SD (*n*) (μM)		Ratio	IC_{50} ± SD (*n*)
				No spm	30 μM spm		No spm
Arcaine	—	—	—	4.60 ± 1.71 (4)	196 (2)	33.6 (2)	24.4 (2)
N12N	—	—	—	7.05 ± 2.38 (11)	245 ± 30 (5)	32.9 ± 6.3 (5)	960 (2)
N4T8N	—	—	—	0.52 ± 0.32 (46)	6.10 ± 1.43 (24)	15.1 ± 7.1 (24)	49.3 (2)
1	O	CH_2	—	2.68 ± 0.65 (9)	24.9 ± 5.7 (7)	9.1 ± 2.5 (7)	32.5 (2)
2	O	O	—	3.41 ± 0.52 (4)	148 ± 22 (3)	40.6 ± 4.2 (3)	63.8 (2)
3	O	O	OMe	10.6 ± 3.9 (4)	343 ± 27 (3)	33.1 ± 11.7 (3)	43.7 (2)
4	O	S	—	2.20 ± 0.32 (3)	29.1 (2)	12.3 (2)	19.2 (2)
5	S	O	—	1.22 ± 0.23 (3)	23.8 ± 2.7 (3)	20.2 ± 6.1 (3)	n.d.
6	O	O	—	317 ± 20 (3)	785 (2)	2.59 (2)	n.d.
7	NH	CH_2	—	3.10 ± 0.11 (3)	42.3 ± 1.9 (3)	13.7 ± 0.6 (3)	20.6 ± 5.4 (3)
8	NMe	CH_2	—	0.56 ± 0.08 (4)	5.62 ± 2.88 (4)	9.2 ± 3.5 (4)	16.6 (2)
9	NH	CH_2	OMe	2.88 (2)	20.7 (2)	7.2 (2)	4.54 ± 1.43 (3)
10	NH	O	—	0.85 ± 0.23 (7)	38.5 ± 10.4 (6)	50.6 ± 19.7 (6)	11.4 ± 3.7 (3)
11	NMe	O	—	0.76 ± 0.15 (4)	12.5 ± 1.8 (3)	15.2 ± 2.2 (3)	21.9 ± 5.2 (3)
12	O	NH^+Me	—	21.2 ± 5.1 (3)	143 (2)	7.65 (2)	n.d.
13	O	N-Bs	—	0.23 ± 0.06 (4)	2.13 ± 0.17 (3)	10.4 ± 1.56 (3)	1.47 ± 0.55 (3)
14	O	NTs	—	0.24 ± 0.08 (5)	1.32 ± 0.23 (4)	6.28 ± 1.18 (4)	0.41 ± 0.13 (3)
15	O	NTs	OMe	3.77 ± 1.37 (4)	28.7 ± 4.4 (3)	8.11 ± 2.91 (3)	0.84 ± 0.48 (4)

Number of experiments (*n*) in parentheses; if *n* = 2, no SD is indicated (see Supporting information for single values); spm, spermine; n.d., not determined. The column 'ratio' lists the mean ratios IC_{50} (30 μM spm)/IC_{50} (no spm). N12N, diaminododecane; N4T8N, 5-(4-aminobutyl)-2-thiopheneoctanamine;[41] Bs, benzenesulfonyl.

Table 2
Inhibition of [^{3}H]MK-801 and [^{3}H]ifenprodil binding to rat cortical membranes by pentamidine analogs with piperazine and homopiperazine bridge (including open-chain isosters)



	R	R′	[^{3}H]MK-801			[^{3}H]ifenprodil
			IC_{50} ± SD (n) (μM)		Ratio	IC_{50} ± SD (n)
			No spm	30 μM spm		No spm
16	H	—	3.08 ± 0.59 (4)	70.7 (2)	26.2 (2)	17.0 (2)
17	*i*-Pr	—	21.4 ± 5.2 (4)	544 ± 34 (3)	32.9 ± 5.8 (3)	n.d.
18	*c*-Pr	—	19.4 ± 7.4 (4)	466 (2)	21.8 (2)	n.d.
19	*n*-Pr	—	15.9 ± 4.9 (4)	371 (2)	21.8 (2)	n.d.
20	*n*-Bu	—	10.3 ± 3.6 (5)	139 ± 32 (4)	16.0 ± 3.2 (4)	n.d.
21	*c*-Pent	—	9.25 ± 3.85 (5)	159 ± 59 (4)	19.3 ± 6.4 (4)	n.d.
22	$(CH_2)_2$	—	6.43 ± 1.64 (4)	106 ± 20 (3)	15.1 ± 4.1 (3)	n.d.
23	$(CH_2)_3$	—	23.8 ± 8.9 (5)	264 ± 26 (3)	13.6 ± 5.2 (3)	n.d.
24	H	—	0.25 ± 0.07 (14)	5.47 ± 1.42 (6)	20.4 ± 7.3 (6)	19.1 (2)
25	*i*-Pr	—	17.7 ± 4.8 (4)	389 ± 85 (3)	25.3 ± 10.5 (3)	n.d.
26	*c*-Pr	—	11.2 ± 2.9 (4)	212 ± 32 (4)	19.9 ± 5.7 (4)	n.d.
27	*t*-Bu	—	34.6 ± 4.1 (3)	562 (2)	16.8 (2)	n.d.
28	*n*-Bu	—	9.94 ± 3.72 (3)	103 ± 3 (3)	11.2 ± 3.3 (3)	n.d.
29	*c*-Pent	—	8.13 ± 0.29 (3)	85.7 ± 7.8 (3)	10.6 ± 1.2 (3)	n.d.
30	2-HO-Et	—	9.44 ± 2.70 (6)	324 ± 34 (5)	35.7 ± 6.9 (5)	n.d.
31	3-HO-Pr	—	41.2 ± 13.2 (4)	427 (2)	9.72 (2)	n.d.
32	$(CH_2)_2$	—	3.03 ± 0.21 (3)	92.2 (2)	30.1 (2)	n.d.
33	$(CH_2)_3$	—	12.8 ± 4.3 (3)	143 ± 15 (3)	11.7 ± 2.5 (3)	n.d.
34	H	H	2.01 ± 0.76 (6)	50.7 ± 13.8 (5)	30.2 ± 10.4 (5)	0.76 ± 0.05 (3)
35	Me	H	3.18 ± 0.60 (3)	85.3 (2)	30.2 (2)	7.14 (2)
36	Et	H	2.61 ± 0.55 (4)	48.1 ± 6.5 (3)	19.3 ± 7.8 (3)	13.0 (2)
37	Me	Me	2.71 ± 0.41 (3)	54.5 (2)	20.9 (2)	16.9 (2)
38	Et	Et	1.14 ± 0.19 (3)	13.8 (2)	11.8 (2)	9.16 (2)
39	H	H	10.0 ± 2.3 (3)	148 (2)	17.0 (2)	77.7 (2)
40	Me	Me	1.84 ± 0.65 (4)	24.7 ± 0.8 (3)	14.7 ± 5.3 (3)	31.2 (2)

Definitions as in caption to Table 1; *c*-pent, cyclopentyl.

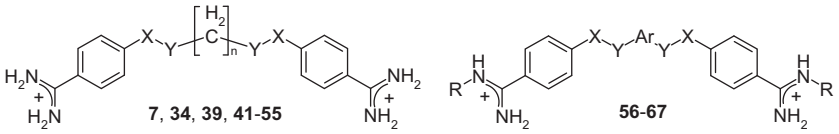
2,5-dibromopyridine to 4-cyanophenylboronic acid (Scheme 4) as described for the synthesis of *para*-terphenyl.[36] The resulting bromo nitrile **76b** was dimerized (once more under Pd catalysis) to the bisbenzonitrile **76a**. Both bisbenzonitriles **71a** and **76a** were finally converted to the respective bisbenzamidines **71** and **76** by LHMDS in THF (*Procedure C*).[36]

3. Results and discussion

3.1. Variation of bridge heteroatoms

The polyamine-sensitivity of the NR is one of the main physiological parameters evaluated in this study. We define this sensitivity as the ratio of two IC_{50}s; the IC_{50} of a polyamine-sensitive inhibitor of [^{3}H]MK-801 binding will be higher (weaker) in the presence of the polyamine (here 30 μM spermine) than in its absence. Replacing in the connecting chain of **1** the middle methylene group by oxygen (**2**; sometimes called 'γ-oxa-pentamidine'), but not by sulfur (**4**), converted a moderately spermine-sensitive inhibitor into a highly spermine-sensitive one (in the absence of added spermine the potencies of **1**, **2** and **4** were similar). Changing the 2 distal oxygens in the highly spermine-sensitive **2** to sulfurs yielded with **5** a compound more potent and more spermine-sensitive than pentamidine (although not reaching to the spermine-sensitivity of **2**). Introducing nitrogens instead of O or S had even more pronounced consequences. A nitrogen atom in the center of the pentamidine bridge introduces a positive charge at physiological pH, while at the distal positions nitrogen has no such consequences (due to the neighboring aromatic nuclei). Indeed, **12** with central nitrogen acted much weaker than pentamidine, while at the two distal positions (**7**) N did not change at all the properties of pentamidine (inhibition of the NR by this compound has already been described[40]). Again, also in this molecule an oxygen in the center (**10**) conferred very high sensitivity to spermine (as in **2**), but this time also the absolute potency was increased (by a factor 4.0). Remarkably, the potency of **7** (so to say 'diaza-pentamidine') was increased considerably (by a factor 5.5) by substituting the Ns with methyl groups (giving **8**; without changing sensitivity to spermine). The high potency of **10**, with O in the middle and Ns vicinal to the aromatic rings, was not further

Table 3
Inhibition of [^{3}H]MK-801 and [^{3}H]ifenprodil binding to rat cortical membranes by pentamidine analogs with aliphatic linkers of increasing length, and with aromatic linkers



	X	Y	*n*	R	Ar	[^{3}H]MK-801			[^{3}H]ifenprodil
						IC_{50} ± SD (*n*) (μM)		Ratio	IC_{50} ± SD (*n*)
						No spm	30 μM spm		No spm
34	NH	CH_2	0	—	—	2.01 ± 0.76 (6)	50.7 ± 13.8 (5)	30.2 ± 10.4 (5)	0.76 ± 0.05 (3)
39	NH	CH_2	1	—	—	10.0 ± 2.3 (3)	148 (2)	17.0 (2)	77.7 (2)
41	NH	CH_2	2	—	—	4.55 ± 1.74 (3)	84.2 (2)	17.2 (2)	n.d.
7	NH	CH_2	3	—	—	3.10 ± 0.11 (3)	42.3 ± 1.9 (3)	13.7 ± 0.6 (3)	20.6 ± 5.4 (3)
42	NH	CH_2	4	—	—	4.37 ± 0.32 (3)	23.9 (2)	5.26 (2)	n.d.
43	NH	CH_2	5	—	—	4.67 ± 0.38 (3)	25.4 (2)	5.37 (2)	n.d.
44	NH	CH_2	6	—	—	7.26 (2)	31.1 (2)	4.37 (2)	n.d.
45	NH	CO	3	—	—	230 ± 97 (4)	675 ± 131 (3)	2.81 ± 0.76 (3)	54.5, 65.6 (2)
46	NH	CO	4	—	—	165 ± 68 (3)	437 (2)	3.46 (2)	n.d.
47	NH	CO	5	—	—	>1000	>1000	n.d.	n.d.
48	NH	CO	6	—	—	22.9 ± 6.7 (4)	170 ± 15 (3)	7.25 ± 1.95 (3)	n.d.
49	NH	CO	7	—	—	26.5 ± 2.6 (3)	111 ± 13 (3)	4.25 ± 0.94 (3)	n.d.
50	NH	CO	8	—	—	15.7 ± 2.2 (3)	62.6 ± 4.8 (3)	4.07 ± 0.96 (3)	n.d.
51	NH	CO	10	—	—	9.63 ± 1.45 (3)	27.7 ± 6.9 (3)	2.98 ± 0.58 (3)	n.d.
52	CO	NH	3	—	—	>1000	>1000	n.d.	n.d.
53	CO	NH	4	—	—	>1000	>1000	n.d.	n.d.
54	CO	NH	5	—	—	688 (2)	>1000	n.d.	n.d.
55	CO	NH	6	—	—	266 (2)	1208 (2)	4.59 (2)	n.d.
56	CH_2	O	—	H	1,2-Ph	2.19 ± 0.85 (5)	18.4 ± 3.2 (4)	10.1 ± 3.2 (4)	7.29 ± 2.85 (4)
57	O	CH_2	—	H	1,2-Ph	1.46 ± 0.60 (4)	5.54 ± 1.23 (3)	4.00 ± 0.93 (3)	1.42, 2.05 (2)
58	O	CH_2	—	H	1,3-Ph	0.79 ± 0.32 (4)	6.70 ± 0.89 (4)	9.04 ± 1.93 (4)	3.97 ± 1.59 (3)
59	O	CH_2	—	H	1,4-Ph	5.24 ± 1.12 (4)	25.7 ± 0.9 (3)	5.25 ± 1.29 (3)	10.8, 16.8 (2)
60	O	CO	—	H	1,4-Ph	10.8 ± 1.7 (3)	46.6 (2)	3.98 (2)	38.6, 27.0 (2)
61	NH	CO	—	H	1,3-Ph	42.3 ± 6.8 (4)	36.7 (2)	0.97 (2)	n.d.
62	NH	CO	—	H	1,4-Ph	53.9 ± 2.4 (3)	52.9 (2)	0.96 (2)	n.d.
63	NH	CO	—	H	2,5-Fur	152 ± 10 (3)	153 ± 7 (3)	1.01 ± 0.02 (3)	n.d.
64	NH	CO	—	*i*-Pr	2,5-Fur	10.6 ± 1.3 (3)	174 ± 45 (3)	16.9 ± 6.0 (3)	n.d.
65	NH	CO	—	H	2,6-Pyr	58.2 (2)	70.9 (2)	1.22 (2)	>100
66	NH	CO	—	*i*-Pr	2,6-Pyr	6.94 ± 1.22 (3)	74.9 ± 13.5 (3)	10.8 ± 1.2 (3)	50.1, 49.4 (2)
67	CO	NH	—	H	2,6-Pyr	125 (2)	233 (2)	1.85 (2)	n.d.

Definitions as in caption to Table 1; Fur, furandiyl; Pyr, pyridinediyl.

increased by N-methylation (**11**), and the extreme spermine sensitivity of **10** was reset to 'normal' by this modification.

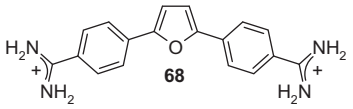
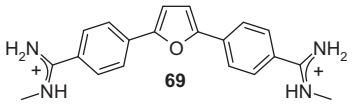
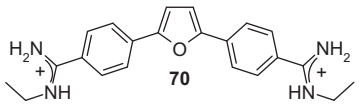
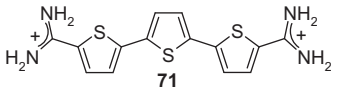
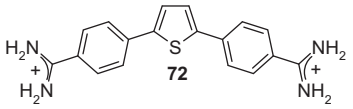
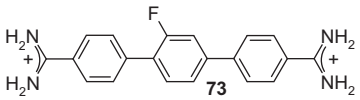
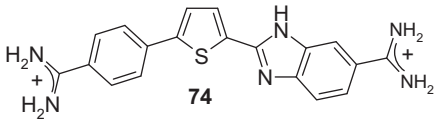
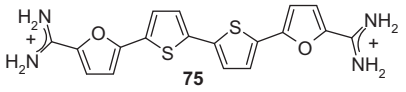
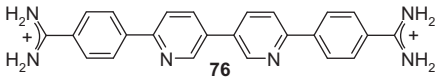
Already these first observations demonstrate that absolute potencies and spermine-sensitivities of pentamidine congeners follow separate SARs. Substitution with methoxy at the aryl nuclei had minor or unfavorable influence (**3** weaker than **2**; **9** similar to **7**; **15** weaker than **14**), but aromatic substituents at a central N were always favorable, most likely because they take away the central positive charge. This latter strategy resulted in the most potent compounds of this series (**13**, **14**), with spermine-sensitivities equivalent to or slightly weaker than that of pentamidine. Since the loss in potency after central introduction of N was not only equalized, but even reversed to a considerable gain, the aromatic residue may find a partner for favorable interaction in this middle part of the pharmacophore, as already suggested by previous studies.[41–43] Imidazoline instead of amidine substituents (as in **6**) were not tolerated at this target, as already shown for other pentamidine derivatives.[44]

3.2. Piperazine and homopiperazine as bridge

N,*N*′-Bis(*p*-amidinophenyl)homopiperazine (**24**)[15] may be seen as related to the diaza-analogs **7** and **8** described above, constraining the bridge between the 2 Ns into a strictly defined ring. All homopiperazine- and piperazine-bridged bisbenzamidines inhibited the NR with similar spermine-sensitivities (most of them slightly above the sensitivity of **1**), but the prototypic **24** clearly stands out from the rest by its absolute potency: none of the 17 analogs in Table 2 (**16**–**23**, **25**–**33**) came close to this value (not even by a factor 10). All modifications, be it by switching from a seven- to a six-membered ring, be it by adding alkyl substituents to the terminal amidino groups or by joining these groups to five- or six-membered rings, resulted in a clear loss of biological activity. Remarkably, the advantage of the prototypic homopiperazine **24** (IC_{50} 0.25 μM) over the prototypic piperazine **16** (IC_{50} 3.08 μM) disappeared upon amidine alkylation (IC_{50}s of all compounds around 10 μM). This is in contrast to favorable effects of amidine alkylation in pentamidine analogs with furan- and pyridine dicarboxamide bridges (see below, Table 3, **63**–**66**).

Breaking up the successful homopiperazine ring sets back the high potency of **24** to the 'normal' level of pentamidine (**34** with short –CH_2CH_2– bridge) or even below (**39** with long –$CH_2CH_2CH_2$– bridge), while keeping the somewhat increased sensitivity to spermine. This is surprising, since formally the longer propyl bridge is closer to the pentyl bridge of pentamidine than the shorter ethyl bridge. Apparently, the 5 carbons chain in pentamidine forms a rather sharp loop, bringing the 2 benzamidine moieties as close as in **34** and closer than in **39**. The potency of **34** was not increased by providing methyl or ethyl substituents to the ethyl bridge: **35**–**37** exhibited all pentamidine-like potencies (only the diethyl derivative **38** with IC_{50} 1.14 μM exceeded somewhat this potency). On the other hand, the rather poor

Table 4
Inhibition of [^{3}H]MK-801 binding to rat cortical membranes by pentamidine analogs with additional aromatic rings

	IC_{50} ± SD (n) (μM)		Ratio
	no spm	30 μM spm	
 **68**	4.23 ± 2.47 (4)	7.48 ± 2.51 (4)	2.37 ± 1.69 (4)
 **69**	6.97 ± 2.15 (3)	24.0 ± 19.2 (3)	3.47 ± 2.20 (3)
 **70**	12.2 (2)	30.7 (2)	2.56 (2)
 **71**	5.34 (2)	6.76 (2)	1.50 (2)
 **72**	6.68 ± 1.25 (4)	17.7 ± 6.3 (4)	2.61 ± 0.61 (4)
 **73**	9.9 ± 4.4 (3)	34.2 (2)	4.63 (2)
 **74**	3.21 (2)	13.4 (2)	4.14 (2)
 **75**	5.01 ± 3.28 (3)	11.7 (2)	4.26 (2)
 **76**	26.8 ± 13.1 (3)	30.0 ± 6.7 (3)	1.24 ± 0.38 (3)

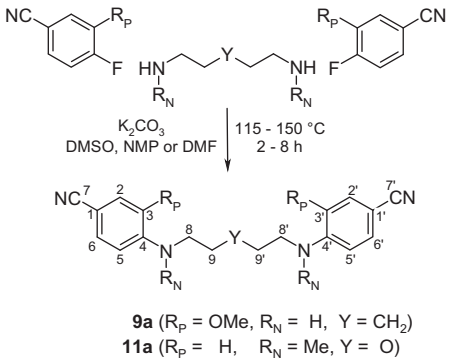
Definitions as in caption to Table 1.

performance of the propyl bridge (in **39** with IC_{50} 10 μM) was raised to the pentamidine level by methyl substituents (in **40** with IC_{50} 1.84 μM). These results suggest a double role of the bridge, tuning the extension and orientation of the molecule and providing itself favorable attachment points. It might be worth to explore larger substituents, given the success of central aromatic substituents in the first series (**13** and **14**).

3.3. Bridges of increasing length

The shorter chain homologs of pentamidine (butamidine and propamidine) have been tested as inhibitors of [^{3}H]MK-801 binding to rat brain membranes,[40] returning the IC_{50}s 2.59, 17.7 and 5.54 μM for 5, 4 and 3 methylene groups between the oxygens, respectively. Also in our diaza-analogs we observed two optima, one with an ethyl and another with a pentyl bridge (**34** and **7**; Table 3).

We also tested the influence of chain length with two other types of bridges. In the first one, the carbons vicinal to the nitrogens were carbonyl groups, changing the chain geometry at this position from sp3 (tetraedric) to sp2 (planar), with dramatic consequences. Although the dianilide **45** was equivalent in total bridge length to pentamidine, it turned out 100-fold weaker, with practically no sensitivity to spermine; the same was true for **46**, with butyl between the two amide groups (–NH–CO–butyl–CO–NH–).



Scheme 1. Preparation of the dinitrile precursors of **9** and **11**. Atom numbers in accordance with NMR data.

The next homolog **47** was still weaker (IC_{50} >1 mM). Surprisingly, activity returned in the higher homologs **48**–**51**, even with some indication for spermine sensitivity (**48**). Potencies were very weak, if the amide groups were inverted (–CO–NH–butyl–NH–CO–, **53**), and also here the weakest compounds were those of middle size. We conclude that the chain linking the two benzamidine moieties should either be short or should exhibit enough degrees of freedom

for folding to adopt the critical short distance between the end charges. With rigid segments like amide bonds, such a distance appears difficult to achieve.

3.4. Aryl inserted into the bridge

Finally, we explored the influence of aromatic nuclei inserted into the bridge linking the two benzamidine moieties. This kind of modification was not only well tolerated, but even increased the potency, if the geometry of pentamidine was maintained as in **58** (IC_{50} 0.79 μM). In this compound, still a continuous chain of 5 carbon atoms separated the 2 oxygens. Also a chain of 4 carbon atoms as in **57** was compatible with high potency (IC_{50} 1.46 μM), but only with quite low spermine sensitivity. Interestingly, this sensitivity was restored simply by switching atom order from –O–C–(1,2-Ph)–C–O– (**57**) to –C–O–(1,2-Ph)–O–C– (**56**), with no loss in potency. Inserting the phenyl nucleus in 1,4 orientation (**59**) was less favorable.

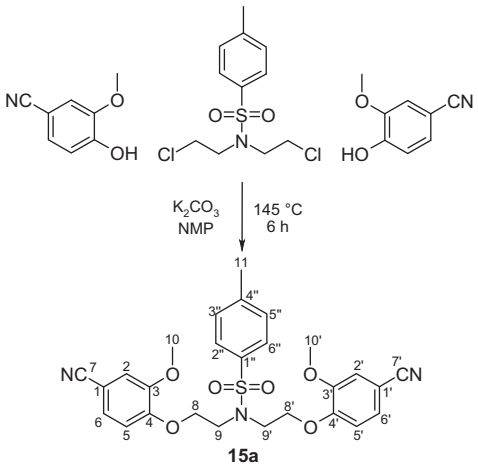
Still weaker compounds resulted from insertions of an aromatic group between two amide bonds, nevertheless returning some surprising SARs. While two ester bonds on both sides of a central phenyl nucleus (with the alcoholic oxygens at the outer positions; **60**) were tolerated almost as well as the ether bonds in **59**, amide bonds (with the nitrogens at the outer positions; **61** and **62**) led to a strong drop in potency and to the complete loss of spermine sensitivity. Replacing the phenyl nucleus between these amide bonds by a furan nucleus (**63**) lowered these figures even more, but to our surprise potency returned (IC_{50} 10.6 μM) upon alkylation of the distal amidine groups (**64**), with parallel restoration of spermine sensitivity. An analogous phenomenon was observed after insertion of a pyridine nucleus: weak potency without (**65**), but appreciable potency with amidine alkylation (**66**; IC_{50} 6.94 μM), also here with remarkable restoration of sensitivity to spermine. It should be taken into consideration, however, that these alkylations achieved medium potencies starting from a rather weak level (similar potencies had been achieved by alkylations of **16** and **24**—Table 2—starting from a much higher level).

If aryl nuclei were inserted without interspersed flexible linkers, compounds were obtained with absolute potencies (Table 4) either similar to pentamidine (**68**, **71**, **74**, **75**) or slightly weaker (**69**, **70**, **72**, **73**, **76**). In all these compounds, however, sensitivity to spermine was practically absent. In the bioisosteric sequence **71**, **72** and **73**, potencies seem to weaken with increasing distance between the distal charges (IC_{50}s 5.3 < 6.7 < 9.9 μM). The low potency of the tetracyclic **76** may be due to the still longer distance between them. Although **75** was also a tetracyclic compound, here the chain of five-membered rings would allow for some adjustment of length, possibly explaining the higher potency of this compound. Such flexibility cannot explain the relatively high potency of the benzimidazole-containing **74**, one of the more extended molecules in this series; here, the particular imidazole signature may have contributed favorably to receptor interaction.

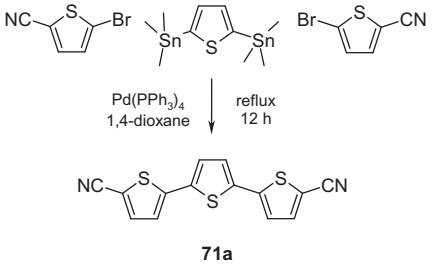
3.5. Inhibition of [^{3}H]ifenprodil binding

Polyamines activate the NR at an interface between the subunits GluN1 and GluN2B.[18,21] This site is formed by acidic amino acid residues contributed by both extra-cellular N-terminal domains; their neutralization by polyamines is hypothesized to result in the close apposition of these domains, facilitating the activation of the NR by agonists. A second site located more externally mediates inhibition of the NR by ifenprodil.[21] No high affinity ligands have been described binding directly to the first site; however, the outer site can be labeled with [^{3}H]ifenprodil. Compounds can usually be tested directly for their affinity to this site via their potency to displace this radioligand from neuronal membranes, with the precaution that indirect influences of polyamines on [^{3}H]ifenprodil binding have been demonstrated.[45]

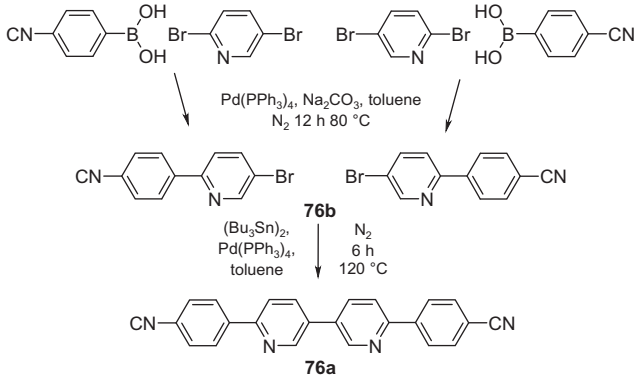
Some of our test compounds were quite potent displacers of [^{3}H]ifenprodil (last column in Tables 1–3). The most potent was **14**, a pentamidine with tosylated N in the middle of the bridge (IC_{50} 0.41 μM), that was also a potent displacer of [^{3}H]MK-801 (0.24 μM). Quite potent too were **15** (the methoxy derivative of **14**; 0.84 μM), **13** (also a congener of **14**; 1.47 μM), **34** (the diaza analog with the shortest bridge; 0.76 μM), and **57** (with phenyl in the bridge; 1.74 μM). Of all 31 compounds tested at both sites, only 3 were more potent at the 'IF-site', the other 28 more potent at the 'MK-site' (9 of them even more than 10-fold). Affinities of 31 compounds tested at both sites (including 3 prototypic polyamine



Scheme 2. Preparation of the dinitrile precursor of **15**. Atom numbers in accordance with NMR data.



Scheme 3. Preparation of the dinitrile precursor of **71**.



Scheme 4. Preparation of the dinitrile precursor of **76**.

inverse agonists) were significantly correlated to each other (Fig. 1A). The correlation line had no unity slope; high affinity compounds were more similar at both sites, but low affinity compounds more dissimilar. On the other hand, affinity at the IF-site was no predictor of spermine-sensitivity at the MK-site (Fig. 1B).

3.6. Modeling of potential sites of action

In an attempt to identify the possible molecular target mediating spermine-sensitive inhibition of the NR by bisbenzamidines, we performed docking calculations for the 45 most spermine-sensitive bisbenzamidines (see Supporting information, Fig. S1) at a site proposed to mediate polyamine-stimulation of the NR.[18,21,22] The endogenous polyamines spermidine and spermine (and also Mg^{2+}) by virtue of their positive charges neutralize surface charges on the N-terminal domains of subunits GluN1 and GluN2B. In the non-stimulated state (*Low P_o* mode; P_o stands for *open probability*), these negative surface charges keep the GluN1/2B domains apart from each other. Polyamines induce the *High P_o* mode by inserting themselves between the domains, leading them together. Antagonists interfering with this mechanism can be expected to stabilize the *Low P_o* mode, by binding to only one of the 2 surfaces (without leading them together). Although stimulation of the NR by polyamines exhibits parallels to stimulation by increased pH, not only GluN1/2B, but also GluN1/2A and GluN1/2D combinations are subject to proton inhibition.[46] Systematic examination of mutated receptors did not result in correlations between spermine and proton sensitivities,[18] and also in our own studies,[42,47] inhibition by similar dicationic compound families was differently influenced by spermine and pH. Thus, stimulation by polyamines seems to depend on the pattern of specific acidic amino acid residues on the domain surfaces, rather than on the general neutralization of a local acidic milieu. Bisbenzamidines were docked separately to the putative polyamine domains either of GluN1 or of GluN2B, departing from the whole GluN1/N2B complex in the *Low P_o* mode, i.e. with the domains separated from each other. We obtained highly significant ($r^2 = 0.42$; $p<0.001$) correlation for GluN1 (Fig. 2A), but not for GluN2B (Fig. 2B). No correlation was obtained with the domains close to each other (not shown). We conclude that more than 40% of the variability in the potency of these bisbenzamidines is explained by their interaction with targets on the polyamine domain of GluN1 in the *Low P_o* mode. A still higher correlation may have been obtained at pure GluN1/2B heterodimeric NRs; the majority of native NRs as used by us, however, seem to consist of GluN1/2A/2B heterotrimers,[48] with consequences for the GluN1/2B interface.[49] The GluN1/2B selective antagonist ifenprodil blocks with high affinity only 23% of NRs in our native membranes prepared from rat frontoparietal cortex and hippocampus.[50] Nevertheless, we tried to dock the same

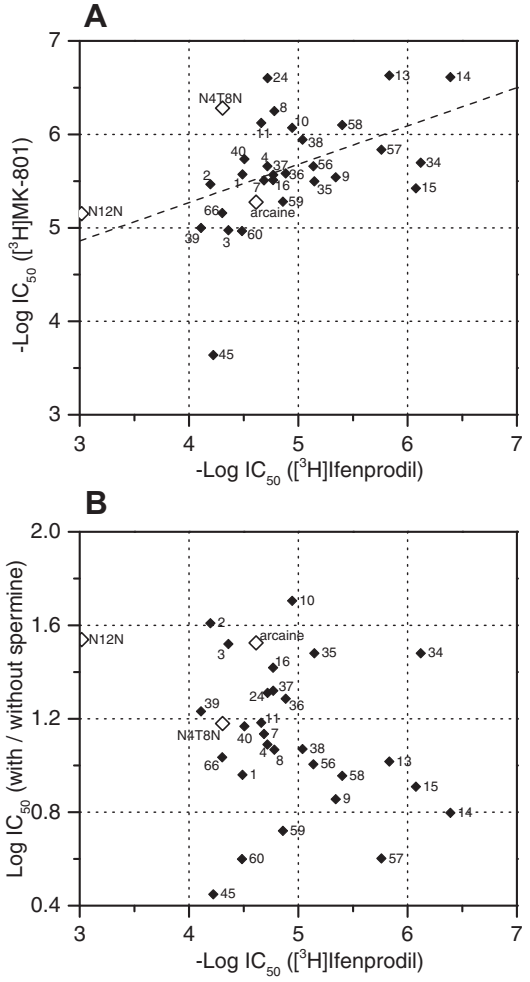


Figure 1. Inhibition by 31 dicationic compounds at the ifenprodil site of the NR correlates with inhibition at the channel binding site (A, interrupted line; $r^2 = 0.22$, $p<0.01$), but not with the sensitivity of this inhibition to 30 μM spermine (B; $r^2 = 0.08$, n.s.); data from Tables 1–3.

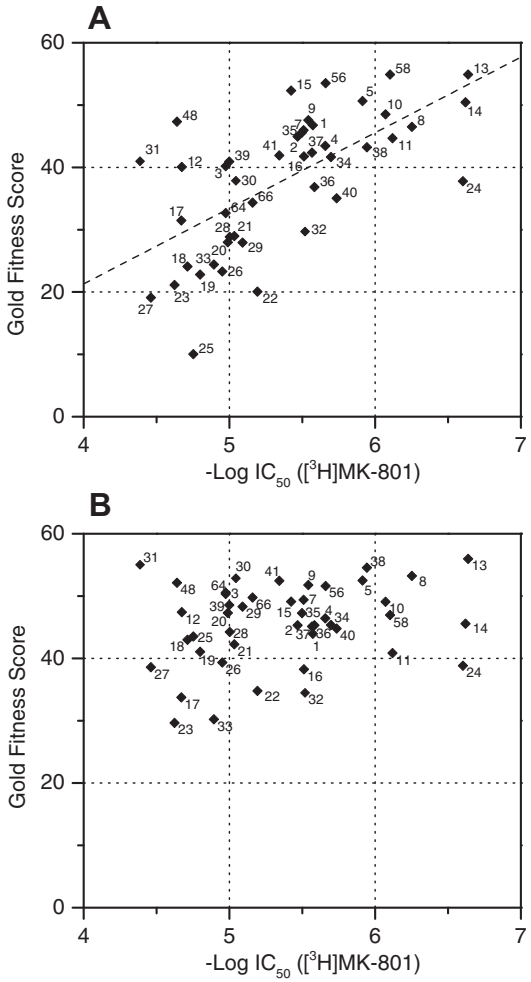


Figure 2. Relationship between goodness of fit to a polyamine site modeled at the GluN1/2B interface and potency to inhibit the binding of [^{3}H]MK-801 to native rat brain membranes. (A) Docking to GluN1, $r^2 = 0.42$. (B) Docking to GluN2B, $r^2 = 0.06$.

compounds also to the ifenprodil site proposed more externally at the GluN1/2B interface.[21] The obtained correlation ($r^2 = 0.18$; Fig. S2B), while less pronounced than for the 'polyamine site' on GluN1, was significant ($p < 0.01$). Thus, also this site may have contributed to the variability of inhibitory potency; however, for none of these compounds we observed partial inhibition of [^{3}H]MK-801 binding as typical for inhibition of [^{3}H]MK-801 binding by ifenprodil, and in general binding of [^{3}H]MK-801 was more potently inhibited than binding of [^{3}H]ifenprodil (Fig. 1A). To some extent, significant docking of bisbenzamidines also to the IF-site may be explained by mutual influences between spermine and ifenprodil at native NRs.[44]

Finally, we created an overlay of pentamidine (**1**) with 8 of the most potent analogs (**5**, **8**, **10**, **11**, **13**, **14**, **38**, **58**), docked to the amino acid residues on GluN1 allegedly mediating stimulation by polyamines (Fig. 3). Bisbenzamidines with flexible linker (as **1** itself) were able to partially wrap the helix α5 containing amino acid residues E185, E186 and E181, the amidine groups approaching the negatively charged ω-carboxylic groups. On the other hand, given the high number of acidic amino acid residues at this interface, several other modes of interaction may be possible as well, and not just the one suggested in the figure. The homopiperazine-bridged compound **24** did not superimpose with these compounds (not shown); although of high potency, it did not yield high docking scores in this model. Also other bisbenzamidines with inflexible bridge (**22**, **25**) yielded Gold Fitness scores below expectation (see Fig. 2A).

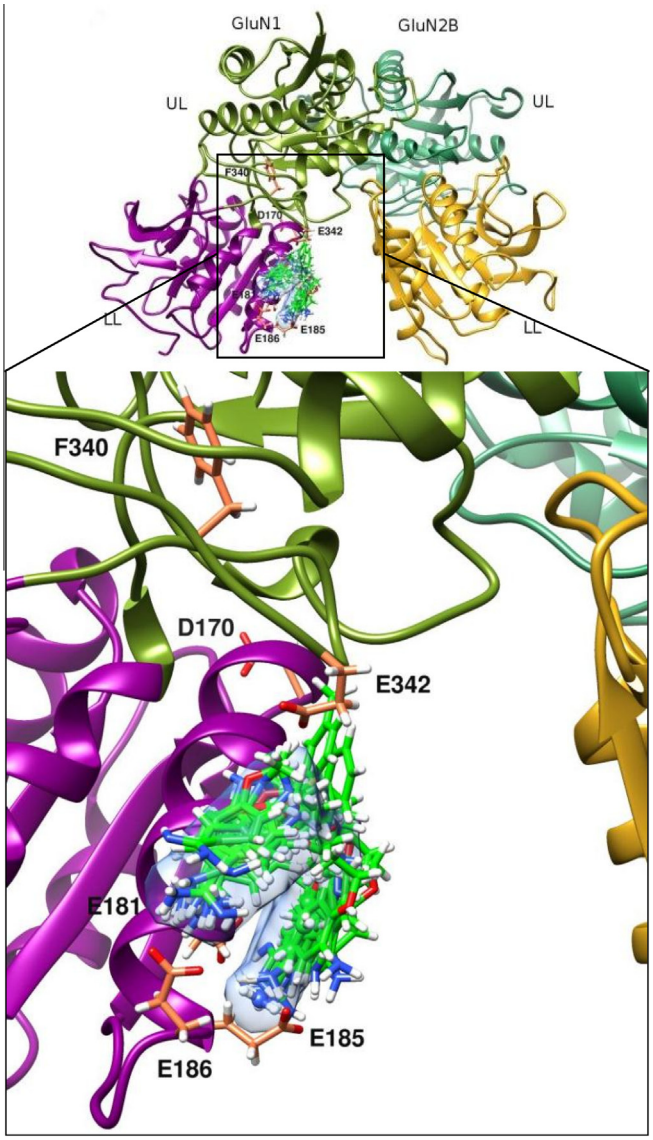


Figure 3. Binding mode of an overlay of **1** with the most potent analogs (**5**, **8**, **10**, **11**, **13**, **14**, **38**, and **58**—with omission of **24**) to the spermine binding site of GluN1 in the *Low* P_o mode. Six amino acid residues of GluN1 known to influence spermine stimulation and the bisbenzamidines are represented as sticks with carbon atoms in coral and green, respectively. Surface representation of **1** is colored light blue.

3.7. Competition or no competition?

Already Reynolds and Aizenman,[7] the discoverers of pentamidine as NR antagonist, demonstrated that inhibition of [^{3}H]MK-801 binding by pentamidine was counteracted by the endogenous polyamine spermidine (the shorter-chain homolog of spermine). Since in their hands pentamidine did not only shift der spermidine dose/response curve to the right, but also reduced maximum stimulation, they excluded competitive interaction. Similar data were produced by us for pentamidine and 2 congeners, compounds **10** and **13** (Fig. 1S, supplementary information) in 'competition' with spermine. Earlier studies by two of us (MLB, PR)[50] had demonstrated that interaction between dicationic long-chain inhibitors as diaminododecane (N12N) and pentamidine with spermine-stimulation of [^{3}H]MK-801 binding to native rat membranes was not pronounced enough to be explained by direct competition alone. Also another observation argues against simple direct competition: While polyamine-stimulation of NRs is limited to NRs with GluN2B subunit,[18] the prototypic inverse polyamine agonist arcaine inhibits also NRs without GluN2B.[51] It also remains to be explained why 'polyamine inverse agonists' reduce [^{3}H]MK-801 binding in well washed neuronal membranes (with no effective amounts of the endogenous polyamines spermidine and spermine left). Do they even then drive open to the *Low* P_o mode the GluN1/N2 interface, and against which 'polyamine-like' agent? Evidently, spermine and spermidine on the one side and dicationic inhibitors on the other side have additional effects. We interpret our docking data as indication that a considerable part of NR inhibition by bisbenzamidines' is mediated by targets at the GluN1/2B interface also mediating stimulation by spermine. This includes competition, but leaves ample room for other activities of both compound families.

4. Conclusions

Motivated by the high potency of the homopiperazine-bridged bisbenzamidine **24** as polyamine-sensitive NR inhibitor, we undertook a detailed SAR study with 76 pentamidine analogs prepared in 5 different synthetic laboratories. We identified 9 spermine-sensitive inhibitors with higher potency than **1** (see Fig. S4). Four of them (**5**, **8**, **10**, **11**) were isosteric to **1**, with heteroatom variations in the bridge. Three others had aromatic rings inserted in (**58**) or attached to the bridge (**13**, **14**), confirming earlier observations pointing to a role for such aromatic rings in target interaction.[41–43] Among analogs with shorter bridge, only **38** with 2 ethyl substituents was more potent than **1** (ANOVA, $p < 0.05$). Ironically, the lead homopiperazine **24** stayed the only high-potency bisbenzamidine of this structural type; none of the other homopiperazines came close. In addition, highly significant docking to the GluN1 part of the polyamine-sensitive GluN1/2B interface did not include **24**: the docking score for **24** was even lower than that obtained for **1**. Thus to provide a theoretical explanation for the particular behavior of **24**, we still need more refined docking models based on the molecular landscape of the real (and still unknown) polyamine-sensitive GluN1/2B interface in heterotrimeric NRs constituting the majority in native rat hippocampal and cortical neurons.[49]

5. Experimental section

All chemicals were purchased from major chemical suppliers as high or highest purity grade and used without further purification. Melting points were determined with an Electrothermal 9001 Digital Melting Point. ^{1}H NMR spectra in solution were recorded with a Bruker Avance DMX 400 or with Varian 300 V NMR S, and chemical shifts δ (ppm) in solutions were referenced to TMS (the solvent is given with the spectral data). Atom numbers are given in Schemes 1 and 2. Elemental analyses or HRMS are provided for new compounds.

5.1. New syntheses

The bisbenzamidines **9**, **11**, **15**, **40**, **43**, **44**, **71** and **76** were prepared as described below. For the conversion of the precursor bisbenzonitriles into bisbenzamidine hydrochlorides, 3 different procedures were used:

5.1.1. Procedure A

The appropriate dinitrile (5 mmol) was stirred in a sealed flask with a saturated solution of HCl in anhydrous ethanol (25–50 mL) for 20–40 h at rt. The solvent was evaporated in vacuo (or dry diethyl ether was added until a solid was precipitated). The solid residue was filtered, washed with diethyl ether and dried under vacuum over anhydrous $CaCl_2$ for 2–6 h. The crude iminoester obtained in high yield was added to a saturated solution of NH_3 in anhydrous ethanol (25–50 mL). The mixture was stirred at rt for 24–48 h in a sealed vessel. The solvent was evaporated in vacuo, and the solid residue was mixed with 10% aq NaOH (10 mL). The precipitate of free amidine was filtered, washed with water, and dried under reduced pressure over anhydrous $CaCl_2$. The white microcrystalline solid was suspended in anhydrous ethanolic HCl (10 mL) and boiled for a few min to obtain the appropriate dihydrochloride salt.

5.1.2. Procedure B

A mixture of hydroxylamine hydrochloride (4.15 g, 60 mmol) and sodium methoxide (3.24 g, 60 mmol) in DMF (15 mL) was heated at 50 °C for 1 h. The precipitate was discarded and the bisbenzonitrile (5 mmol) was added to the filtrate. The so-obtained mixture was heated at 50 °C for 24 h. The reaction medium was then poured into water. The obtained bisbenzamidoxime was collected by filtration and thoroughly washed with water, ethanol, and finally ether. In the next step, a mixture of the bisbenzamidoxime (3 mmol), ammonium formate (1.80 g, 30 mmol), and 10% Pd on carbon (0.3 g) in acetic acid (15 mL) was heated under reflux for 2 h. After cooling the mixture was filtered on a cake of alumina and the filtrate was concentrated under reduced pressure. Addition of a solution of HCl in isopropanol (6 M, 15 mL) afforded a precipitate that was washed with water and ethanol to yield the desired bisbenzamidine (dihydrochloride).

5.1.3. Procedure C

The dinitrile (1 mmol) was allowed to stir overnight in a 1 M solution of LHMDS in THF (20 mL) as described for the conversion of the dinitrile of *para*-terphenyl to the respective bisamidine.[36]

5.1.4. Syntheses of 9, 11, and 15

5.1.4.1. 9a 1,5-Bis(1-cyano-3-methoxyphenylamino)pentane 3-Methoxy-4-fluorobenzonitrile (3.02 g, 20 mmol), 1,5-diaminopentane dihydrochloride (1.75 g, 10 mmol) and anhydrous K_2CO_3 (11.04 g, 80 mmol) were stirred together at 150 °C in NMP (30 mL) for 3 h (progress of the reaction was followed by TLC). The hot reaction mixture was poured into ice water (400 mL). The obtained solid was filtered, washed with water and dried. Crystallization from EtOH gave 0.92 g of yellow solid **9a** (yield 24%), mp 153–155 °C. ^{1}H NMR (299.87 MHz, $CDCl_3$) δ: 1.53–1.55 (m, 2H, Y = CH_2), 1.72 (quintet, J = 7.2 Hz, 4H, 9- and 9′-CH_2), 3.19 (t, J = 6.9 Hz, 4H, 8- and 8′-CH_2), 3.85 (s, 6H, 2× R_P = OCH_3), 4.75 (br s, 2H, 2× R_N = NH), 6.50 (d, J = 8.1 Hz, 2H, H-5,-5′), 6.89 (br s, 2H, H-2,-2′), 7.18 (br d, J = 8.1 Hz, 2H, H-6,-6′). For $C_{21}H_{24}N_4O_2$ (364.45 g/mol) calcd: C, 69.21; H, 6.64; N, 15.37; found: C, 68.92; H, 6.91; N, 15.03.

5.1.4.2. 9 1,5-Bis(1-amidino-3-methoxyphenylamino)pentane dihydrochloride. This diamidine was obtained by *Procedure A* from the bisbenzonitrile **9a**. The solvent was evaporated in vacuo. Crude product was dissolved in water and then precipitated by addition of dry acetone. Precipitated solid was filtered, washed with acetone and dried to give **9** as a yellow solid (yield 37%), mp 283.5–284.5 °C. ^{1}H NMR (299.87 MHz, DMSO-d_6) δ: 1.38–1.40 (m, 2H, Y = CH_2), 1.59 (quintet, J = 7.2 Hz, 4H, 9- and 9′-CH_2), 3.18 (m, 4H, 8- and 8′-CH_2), 3.87 (s, 6H, 2× R_P = OCH_3), 6.01 (br s, 2H, 2× R_N = NH), 6.64 (d, J = 8.4 Hz, 2H, H-5,-5′), 7.33 (d, J = 1.5 Hz, 2H, H-2,-2′), 7.46 (dd, J_1 = 1.5 Hz, J_2 = 8.4 Hz, 2H, H-6,-6′), 8.56 (br s, 4H, NH_2), 8.90 (br s, 4H, NH_2). For $C_{21}H_{30}N_6O_2{\cdot}2HCl{\cdot}1½H_2O$ (498.46 g/mol) calcd: C, 50.60; H, 7.02; N, 16.85; found: C, 50.43; H, 7.05; N, 16.48.

5.1.4.3. 11a 1,5-Bis[(1-cyanophenyl)N-methylamino]-3-oxapentane. The bisbenzonitrile **11a** was obtained from 2 equiv 4-fluorobenzonitrile and 1 equiv 2,2′-methylaminodiethyl ether with K_2CO_3 in DMF (Scheme 1) as a white solid, mp 88.2–89.4 °C. ^{1}H NMR (299.87 MHz, $CDCl_3$) δ: 2.98 (s, 6H, 2× R_N = CH_3), 3.53–3.62 (m, 8H, 8-, 8′, 9 and 9′-CH_2), 6.62–6.65 (m, 4H, H-5,-3,-5′,-3′), 7.42–7.45 (m, 4H, H-6,-2,-6′,-2′). HRMS (ESI-ToF) M+Na $C_{20}H_{22}N_4O$+Na, calcd: m/z 357.1691; found: m/z 357.1697.

5.1.4.4. 11 1,5-Bis[(1-amidinophenyl)N-methylamino]-3-oxapentane. This diamidine was obtained by *Procedure A* from the dinitrile **11a**. The white solid was filtered off and dried at 80 °C for 1 h to obtain 0.356 g of **11** (yield 90%) mp 276–279 °C (dec). ^{1}H NMR (299.87 MHz, DMSO-d_6) δ: 2.98 (s, 6H, 2× R_N = CH_3), 3.57–3.60 (m, 8H, H-8,-9,-8′,-9′), 6.79–6.82 (m, 4H, H-5,-3,-5′,-3′), 7.70–7.73 (m, 4H, H-6,-2,-6′,-2′), 8.48 (br s, 4H, NH_2), 8.87 (br s, 4H, NH_2). For $C_{20}H_{28}N_6O{\cdot}2HCl$ (441.40 g/mol) calcd: C, 54.42; H, 6.85, N 19.04; found: C, 54.28; H, 6.96; N, 18.89.

5.1.4.5. 15a *N,N*-Bis[2-(1-cyano-3-methoxyphenoxy)ethyl]-4-methylbenzenesulfonamide. 3-Methoxy-4-hydroxybenzonitrile (1.49 g, 10 mmol) and *N,N*-bis(2-chloroethyl)-4-methylbenzenesulfonamide (1.48 g, 5 mmol) in NMP (20 mL) were stirred at 145 °C together with anhydrous K_2CO_3 (2.76 g, 20 mmol) (progress of the reaction was followed by TLC). After 6 h the reaction mixture was poured into ice water (300 mL). The obtained solid was filtered, washed with water and dried. The crude product was dissolved in hot EtOH and filtered hot. The precipitate was purified by column chromatography (Merck Silica gel 60, 230–400 mesh ASTM, eluent CH_2Cl_2:MeOH—98:2) to afford 1.31 g of **15a** (yield 50%), mp 177–179 °C. ^{1}H NMR (400.13 MHz, $CDCl_3$) δ: 2.34 (s, 3H, 11-CH_3), 3.61 (t, J = 6.0 Hz, 4H, 9- and 9′-CH_2), 3.69 (s, 6H, 10- and 10′-OCH_3), 4.24 (t, J = 6.0 Hz, 4H, 8- and 8′-CH_2), 6.82 (d, J = 8.4 Hz, 2H, H-5,-5′), 6.98 (d, J = 2 Hz, 2H, H-2,-2′), 7.16–7.21 (m, 4H, H-6,-6′,-3″,-5″), 7.64–7.66 (m, 2H, H-2″,-6″). For $C_{27}H_{27}N_3O_6S$ (521.60 g/mol) calcd: C, 62.17; H, 5.22; N, 8.06; S, 6.15; found: C, 62.03; H, 5.47; N, 7.86; S, 6.32.

5.1.4.6. 15 *N,N*-Bis[2-(1-amidino-3-methoxyphenoxy)ethyl]-4-methylbenzenesulfonamide dihydrochloride. This diamidine was obtained by *Procedure A* from the bisbenzonitrile **15a**. The solvent was evaporated in vacuo to give **15** as a yellow solid (yield 86%), mp 233–234 °C. ^{1}H NMR (299.87 MHz, DMSO-d_6) δ: 2.36

(s, 3H, 11-CH_3), 3.65 (t, J = 5.7 Hz, 4H, 9- and 9′-CH_2), 3.80 (s, 6H, 10- and 10′-OCH_3), 4.27 (t, J = 5.7 Hz, 4H, 8- and 8′-CH_2), 7.11–7.14 (m, 2H, H-5,-5′), 7.36–7.39 (m, 2H, H-3″,-5″), 7.53–7.55 (m, 4H, H-6,-2,-6′,-2′), 7.75–7.78 (m, 2H, H-2″,-6″), 9.12 (br s, 4H, NH_2), 9.39 (br s, 4H, NH_2). For $C_{27}H_{33}N_5O_6S{\cdot}2HCl{\cdot}H_2O$ (646.60 g/mol) calcd: C, 50.16; H, 5.77; N, 10.83; S, 4.96; found: C, 50.36; H, 5.94; N, 10.67, S, 4.80.

5.1.5. Syntheses of 40, 43, and 44

40, **43** and **44** were prepared via the bisbenzonitriles **40a**, **43a** and **44a**. For their synthesis, 4-fluorobenzonitrile (4.84 g, 40 mmol), the appropriate diamine (20 mmol), and K_2CO_3 (4.52 g, 40 mmol) in DMF (25 mL) were heated under reflux for 8 h. The mixture was then poured into water. The desired bisbenzonitrile was collected by filtration and thoroughly washed with water, ethanol, and finally ether. The bisbenzonitriles were converted to the bisbenzamidines by *Procedure B*.

5.1.5.1. 40a 1,3-Bis[(1-cyanophenyl)N-methylamino]propane. This bisbenzonitrile (75%) was obtained from the appropriate diamine *N*,*N*′-dimethylpropylenediamine as a white solid, mp 139–140 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.92 (m, 2H, CH_2), 3.01 (s, 6H, *N*-CH_3), 3.43 (t, J = 7 Hz, 4H, *N*-CH_2), 6.62 (d, J = 9 Hz, 4H, H-5,-3, H-5′,-3′), 7.46 (d, J = 9 Hz, 4H, H-6,-2, H-6′,-2′). IR: 2945, 2909, 2893, 2206, 1602, 1520 cm^{-1}. HRMS (ESI-ToF) MH^+ $C_{19}H_{21}N_4$, calcd: m/z 305.1766; found: m/z 305.1767.

5.1.5.2. 40 1,3-Bis[(1-amidinophenyl)N-methylamino]propane dihydrochloride. From the nitrile precursor **40a**, *Procedure B* yielded the desired bisbenzamidine **40** (55%) as a white solid, mp >300 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.73 (m, 2H, CH_2), 3.00 (s, 6H, *N*-CH_3), 3.51 (t, J = 7 Hz, 4H, *N*-CH_2), 6.82 (d, J = 9 Hz, 4H, H-5,-3,-5′,-3′), 7.81 (d, J = 9 Hz, 4H, H-6,-2,-6′,-2′), 8.85 (br s, 4H, NH_2), 9.09 (br s, 4H, NH_2). IR: 3130, 3040, 2970, 1682, 1609, 1487, 1393 cm^{-1}. HRMS (ESI-ToF) MH^+ $C_{19}H_{27}N_6$, calcd: m/z 339.2297; found: m/z 339.2300.

5.1.5.3. 43a 1,7-Bis[(1-cyanophenyl)amino]heptane. This bisbenzonitrile (85%) was obtained from the appropriate diamine 1,7-diaminoheptane as a white solid, mp 136–137 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.40–1.60 (br m, 10H, CH_2), 3.15 (t, J = 7 Hz, 4H, *N*-CH_2), 4.13 (br s, 2H, NH), 6.53 (d, J = 9 Hz, 4H, H-5,-3, H-5′,-3′), 7.41 (d, J = 9 Hz, 4H, H-6,-2, H-6′,-2′). IR: 3384, 2937, 2875, 2853, 2198, 1603, 1524 cm^{-1}. HRMS (ESI-ToF) MH^+ $C_{21}H_{25}N_4$, calcd: m/z 333.2079; found: m/z 333.2070.

5.1.5.4. 43 1,7-Bis[(1-amidinophenyl)amino]heptane dihydrochloride. From the nitrile precursor **43a**, *Procedure B* yielded the desired bisbenzamidine **43** (40%) as a white solid, mp >270–272 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.30–1.60 (br m, 10H, CH_2), 3.08 (t, J = 7 Hz, 4H, *N*-CH_2), 6.65 (d, J = 9 Hz, 4H, H-5,-3,-5′,-3′), 7.67 (d, J = 9 Hz, 4H, H-6,-2,-6′,-2′), 8.68 (br s, 4H, NH_2), 8.90 (br s, 4H, NH_2). IR: 3298, 3091, 2931, 2842, 1660, 1606, 1352 cm^{-1}. HRMS (ESI-ToF) MH^+ $C_{21}H_{31}N_6$, calcd: m/z 367.2610; found: m/z 367.2607.

5.1.5.5. 44a 1,8-Bis[(1-cyanophenyl)amino]octane. This bisbenzonitrile (80%) was obtained from the appropriate diamine 1,8-diaminooctane as a white solid, mp 152–153 °C.^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.37–1.62 (br m, 12H, CH_2), 3.14 (t, J = 7 Hz, 4H, *N*-CH_2), 4.14 (br s, 2H, NH), 6.53 (d, J = 9 Hz, 4H, H-5,-3, H-5′,-3′), 7.41 (d, J = 9 Hz, 4H, H-6,-2, H-6′,-2′). IR: 3383, 2927, 2852, 2203, 1605, 1526 cm^{-1}. HRMS (ESI-ToF) MH^+ $C_{22}H_{27}N_4$, calcd: m/z 347.2236; found: m/z 347.2224.

5.1.5.6. 44 1,8-Bis[(1-amidinophenyl)amino]octane dihydrochloride. From the nitrile precursor **44a**, *Procedure B* yielded the desired bisbenzamidine **44** (25%) as a white solid, mp >263–267 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.30–1.60 (br m, 12H, CH_2), 3.08 (t, J = 7 Hz, 4H, *N*-CH_2), 6.64 (d, J = 9 Hz, 4H, H-5,-3,-5′,-3′), 7.67 (d, J = 9 Hz, 4H, H-6,-2,-6′,-2′), 8.68 (br s, 4H, NH_2), 8.89 (br s, 4H, NH_2). IR: 3291, 3112, 2927, 2851, 1637, 1608, 1494, 1464, 1351 cm^{-1}. HRMS (ESI-ToF) MH^+ $C_{22}H_{33}N_6$, calcd: m/z 381.2767; found: m/z 381.2759.

5.1.6. Syntheses of 71 and 76

The bisbenzamidines with 3 (**71**) and 4 aromatic nuclei in a row (**76**) were prepared by first synthesizing, by Pd-catalyzed condensation, their dinitrile precursors **71a** and **76a**, respectively, and by converting them to the desired bisbenzamidines by *Procedure C*.

5.1.6.1. 71a 5,5″-Dicyano-2,2′:5′,2″-terthiophene. To a round-bottom flask were added 3.76 g (20 mmol) 5-bromo-2-cyanothiophene in 70 mL 1,4-dioxane, 0.46 g (2 mol %) $Pd(PPh_3)_4$ and 4.12 g (10 mmol) 2,5-bis(trimethylstannyl) thiophene. The mixture was allowed to reflux overnight. The solvent was removed under reduced pressure. Hexane was added to the residue and the precipitate was filtered. The solid was dissolved in 150 mL CH_2Cl_2 and washed with 5% NaF. The organic layer was dried over Na_2SO_4. The solvent was evaporated, the product was dried under reduced pressure at 50 °C (12 h) yielding **71a** as orange needles (2.10 g, 70%); mp 206–7 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.94 (d, 2H, J = 4 Hz), 7.58 (s, 2H), 7.53 (d, 2H, J = 4 Hz). MS (FAB): m/z 298 (M^+). Anal. calcd for $C_{14}H_6N_4S_3$: C, 51.51; H, 1.85; N, 17.16; found: C, 51.44; H, 2.12; N, 17.27.

5.1.6.2. 71 5,5″-Diamidino-2,2′:5′,2″-terthiophene dihydrochloride. This diamidine was obtained from the dinitrile **71a** by *Procedure C* as a brown solid (0.31 g, 76%); mp >320 °C (dec). ^{1}H NMR (400 MHz, DMSO-d_6/50 °C) δ: 9.66 (br s, 4H), 9.16 (br s, 4H), 8.11 (d, 2H, J = 4.2 Hz), 7.60 (d, 2H, J = 4.2 Hz), 7.57 (s, 2H). MS (FAB): m/z 333 (M^++1). Anal. calcd for $C_{14}H_{12}N_4S_3{\cdot}2HCl{\cdot}¼H_2O$: C, 41.02; H, 3.56; N, 13.66; found: C, 41.01; H, 3.62; N, 13.55.

5.1.6.3. 76b 4-(5-Bromopyridin-2-yl)benzonitrile. 2,5-Dibromopyridine (1.18 g, 5 mmol) was coupled to 4-cyanophenylboronic acid (0.88 g, 6 mmol) with $Pd(PPh_3)_4$ (0.231 g, 0.2 mmol) as described for linking together the dinitrile of *para*-terphenyl.[36] Finally, 0.9 g **76b** (70%) was obtained: mp 174–175 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.90–8.03 (m, 3H), 8.14–8.24 (m, 3H), 8.80 (s, 1H). MS (m/z, rel. int.); 259 (M^+, 100), 179 (70), 152 (55).

5.1.6.4. 76a 4,4′-[(3,3′Bipyridine)-6,6′-diyl]dibenzonitrile Homocoupling of the above bromo nitrile **76b** (0.78 g, 3 mmol) with $Pd(PPh_3)_4$ (0.231 g, 0.2 mmol) and hexa-*n*-butylditin (1.04 g, 1.8 mmol) was achieved as described for other ring systems.[52] After washing the precipitate with ether, 0.82 g **76a** was obtained (77%): mp >300 °C (dec). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.96 (d, J = 8.1 Hz, 4H), 8.20–8.37 (m, 8H), 9.18 (s, 2H). MS (m/z, rel int.); 358 (M^+, 100), 330 (5), 256 (10), 179 (70).

5.1.6.5. 76 4,4′-[(3,3′Bipyridine)-6,6′-diyl]dibenzamidine tetrahydrochloride. This diamidine was obtained from the dinitrile **76a** by *Procedure C* with 81% yield: mp >300 °C (dec). ^{1}H NMR (400 MHz, D_2O/DMSO-d_6) δ: 8.00 (s, 4H), 8.23–8.43 (m, 8H), 9.15 (s, 2H). MS (m/z, rel int.); 393 (M^++1, 95), 376 (8), 359 (5), 197 (100). Anal. calcd for $C_{24}H_{20}N_6{\cdot}4HCl{\cdot}2½H_2O$: C, 49.41; H, 4.99; N, 14.40; found: C, 49.18; H, 4.67; N, 14.22.

5.2. [^{3}H]MK-801 binding

For binding experiments with [^{3}H]MK-801, membranes were prepared from rat frontoparietal cortex and hippocampus in 50 mM Tris acetate buffer (pH 7.0) as described.[41] [^{3}H]MK-801 (5 nM) was bound in glass vials at 23 °C for 3 h in 10 mM Tris acetate buffer (pH 7.0) in presence of 10 μM glutamate and glycine to membranes corresponding to about 1 mg wet tissue per vial (triplicates). Under these conditions, the affinity of the radioligand to its binding site was 13.3 nM. Test compounds were tested at 3–4 widely spaced concentrations achieving inhibitions from 10% to 90% of specific binding. Non-specific binding (NB) was obtained in presence of 100 μM (*S*)-ketamine. Bound radioligand was separated by filtration over glass fiber filters pre-soaked in 0.3% polyethyleneimine (washing buffer had rt). Filters were shaken with toluene-based scintillation cocktail for 30 min and counted in a betacounter. Sensitivity of inhibition of [^{3}H]MK-801 binding to 30 μM spermine was defined as ratio of IC_{50}s, one obtained in presence, the other in absence of 30 μM spermine.

5.3. [^{3}H]Ifenprodil binding

The same membrane preparation was also used for [^{3}H]ifenprodil binding experiments. [^{3}H]Ifenprodil (5 nM) was bound in glass vials (triplicates) as described.[20] In brief, incubations were conducted for 2 h on ice in 50 mM Tris·HCl (pH 7.4) in presence of 2 μM GBR 12909 (to block σ-sites). Under these conditions, the affinity of the radioligand to its binding site was 42 nM. Test compounds were tested at 3–4 widely spaced concentrations achieving inhibitions from 10% to 90% of specific binding. NB was obtained with 10 μM Ro 25-6981. Bound radioligand was separated by filtration over glass fiber filters pre-soaked in 0.3% PEI (washing buffer was ice cold; background rises fast if PEI solution is exhausted by repeated use). Filters were shaken with toluene-based scintillation cocktail for 30 min and counted in a betacounter.

5.4. Inhibition data analysis

Inhibition of [^{3}H]MK-801 and of [^{3}H]ifenprodil binding by test compounds was evaluated by fitting the results to the function $y = A * IC_{50}{}^h/(IC_{50}{}^h + x^h) + NB$ (*A*, specific binding in absence of inhibitor; *x*, inhibitor concentration; *h*, Hill coefficient) by iterative non-linear curve fitting[53] using OriginPro 9.0.

5.5. Modeling

We observed molecular level interactions between the NR and 45 bisbenzamidine analogs (those with the highest spermine-sensitivities, Fig. S1) by docking them to putative spermine and ifenprodil binding sites of the NR. NR heterodimers GluN1/2B can adopt *High* P_o and *Low* P_o modes;[18] P_o stands for *open probability*). In the *High* P_o mode, the acidic domains of the lower lobes of GluN1 and GluN2B are in close apposition, whereas in the *Low* P_o mode, these domains are far apart as seen in the crystal of NR in complex with ifenprodil. Initial coordinates for the *Low* P_o mode from the species *Rattus norvegicus* were obtained from the protein data bank (*3Q41.pdb* and *3QEL.pdb* for GluN1-1a and GluN2B, respectively).[21] We also took into account the recently published crystal structures of whole length GluN1/2B heterodimeric channels;[54,55] we superimposed the 2014 to the 2011 structures and saw only very slight deviations. We stayed with the older structure because of its better resolution. Docking was calculated separately either to the acidic domain of GluN1 (contributed by amino acid residues D170, E181, E185, E186, F340, and E342) or to the acidic domain of GluN2B (amino acid residues E191, W197, E198, E201, D206, D210 and D213[18,21,22]), with the conformation in the *Low* P_o mode. In addition, these compounds were also docked to the ifenprodil binding site, again in the *Low* P_o mode as in the crystal structure. Initial structures of all compounds selected for docking were generated using the builder module of *Maestro* (Schrodinger, LLC, New York) followed by energy minimization using the *OPLS 2005* force field. Compounds were docked using the *GOLD* docking software[56] which is based on a genetic algorithm. This method allows partial flexibility of the protein and full range of ligand conformational flexibility. The default docking parameters were used for all the calculations, and the dockings produced with the *ChemPLP* scoring functions were rescored and re-ranked using *GoldScore*.

Acknowledgements

The following students contributed to the binding data: Tanja Wernle, Franziska Bender, Natalie Caspari, Dagmar Pretsch, Stephan Holzer, Andreas Pribitzer, Marie-Lena Müller, Katharina Blutsch, Turac Karaca, Julia Hnat. Computer Modeling was supported by the NIH-National Institute on Minority Health and Health Disparities (NIMHD) Grant No. 2G12MD007595, and by the Louisiana Cancer Research Consortium.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.06.012.

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