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Original article

1,2-Ethane *bis*-1-amino-4-benzamidine is active against several brain insult and seizure challenges through anti-NMDA mechanisms targeting the ^{3}H-TCP binding site and antioxidant action

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ABSTRACT

Five *bis*-benzamidines were screened towards murine magnesium deficiency-dependent audiogenic seizures, unravelling two compounds with efficacious doses 50 (ED_{50}) less than 10 mg/kg. They were also screened against maximal electroshock and subcutaneous pentylenetetrazole-induced seizures, and explored for superoxide -scavenging activity. 1,2-Ethane *bis*-1-amino-4-benzamidine (EBAB) was selected and evaluated in 6 Hz seizure test ($ED_{50} = 49$ mg/kg) and at 4 μg/kg in focal cerebral ibotenate poisoning in pups (sizes of both white and grey matter wounds were halved). EBAB was further tested on NMDA-induced seizures in mice ($ED_{50} = 6$ mg/kg) and on ^{3}H-TC -binding to a rodent cerebral preparation ($IC_{50} = 1.4$ μM). Taken as a whole, present data emphasise the suitability of *bis*-benzamidines as templates for designing brain protective compounds.

1. Introduction

Though search for new compounds exhibiting brain anticonvulsant and neuroprotective properties has literally exploded in the last decade, many promising neuroprotectants have seen their pharmacological development compromized [1,2]. Importantly, the compound needs to cross blood–brain barrier (BBB) at a sufficient rate to yield intracerebral concentrations compatible with efficacious pharmacological activity. In this respect, animal models of Alzheimer's and Parkinson's diseases, multiple sclerosis and stroke may rest on experimental conditions in which the disruption of the BBB is more rapid and/or severe than that developing in the corresponding human disorder. Obviously getting information on the pathogenesis, these animal models might be less suitable for evaluation of drugs in a way translatable to human patients. To overcome this issue, some laboratories have developed *in vitro* cellular models combining endothelial and astrocytic cells aimed at mimicking the BBB in a relatively predictive way [3]. An alternative approach is search for an animal model which would combine its suitability for neuroprotective evaluation along with a relative integrity of its BBB. In fact, the brain neurotoxicity challenge should be such that BBB would not be altered. This is not easy to obtain in practice because BBB injury is precisely an expected component of brain injury conditions.

Abbreviations: MES, maximal electroshock seizures; scPtz, subcutaneous pentylenetetrazol; EBAB, 1,2-Ethane *bis*-1-amino-4-benzamidine; ED_{50}, efficacious doses 50 (dose protecting 50% animals); IC_{50}, inhibitory concentration 50 (concentration inhibiting 50% of the binding); MDDAS, magnesium deficiency-dependent audiogenic seizures; BBB, blood–brain barrier; NMDA, *N*-methyl-D-aspartate; TCP, the thienyl analogue of phencyclidine, namely *N*-[1-(2-thienyl) cyclohexyl]-3,4-piperidine).

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Nutritional magnesium deprivation provides mice with brain toxicity including central hyperactivity and susceptibility to audiogenic seizures which develop into four successive phases (latency, wild running, convulsion and recovery phases) [4]. This magnesium deficiency-dependent audiogenic seizure (MDDAS) model is the resultant of two factors: susceptibility to audiogenic seizures (linked to the generalized oxidative/inflammatory state of magnesium deficiency) and development of seizures (triggered by an acoustic stimulus acting as a pro-convulsant factor). Influencing only one of these two factors is sufficient to prevent seizures; susceptibility to and development of which respond to neuroprotective (notably antioxidant) and anticonvulsant compounds, respectively, all acting here as anti-seizure compounds. Elucidation of underlying anticonvulsant or neuroprotective mechanisms requires additional compound testing which may include modulation of the audiogenic seizure phases mentioned above, specific anticonvulsant evaluation in classic animal seizure tests, and specific neuroprotective evaluation in well standardized brain injury models. This overall strategy previously emphasized original neuroprotective potentials of WEB2170 (an anti-PAF receptor antagonist) [4], FMOC-L-leucine (a PPARγ agonist) [5] and 1-methyl-2-[3-trifluoromethylphenyl]-4-mercapto-imidazole (a synthetic ovothiol analogue) [6], and confirmed neuroprotective potentialities of melatonin [4], ebselen and magnesium [7]. Importantly, compounds described as neuroprotective but inactive in the MDDAS model, rosiglitazone [5] and fenofibrate (the authors, unpublished data), represent compounds which do not cross the intact BBB at a substantial rate [8,9]. Integrity of the BBB in the MDDAS model was suggested by failure of systemic folic acid to induce seizures [4]. All of this emphasizes that MDDAS might be one of these *in vivo* models ensuring this rare combination of brain neurotoxicity and relative integrity of BBB mentioned above and, for this reason, might be helpful in detecting brain protective compounds potentially suitable for human neurodegenerative disorders. The present work was aimed at exploring whether one might include among such compounds those issued from a short series of five *bis*-benzamidines including compound **2**, 1,2-ethane *bis*-1-amino-4-benzamidine, referred throughout the text to as **EBAB**.

2. Chemistry

Compounds **2** to **5** were synthesised as indicated in Scheme 1. A first step resulted in the synthesis of *bis*-benzonitrile intermediates obtained by reaction of a diamine with 4-fluorobenzonitrile in boiling *N,N*-dimethylacetamide in the presence of a base. The subsequent two steps allowed the conversion of the nitrile functions into amidines, involving either the Pinner's reaction for asymetric compounds **4** and **5** (imidate synthesis from an alcohol in the presence of hydrochloric acid and further reaction with an amine) or formation followed by reduction of an amidoxime for the symetric compounds **2** and **3**. The synthesis route followed for compounds **4** and **5** was dependent on the solubility of the dinitrile in the presence of hydrochloric acid. The chemical synthesis of compound **1** was previously described [10,11].

3. Results

3.1. Anticonvulsant and neurotoxic evaluation of compounds

3.1.1. Audiogenic seizure test

The five compounds were evaluated in the MDDAS test. Only compounds **1** and **2** (**EBAB**) were active at non toxic doses. The efficacious doses 50 (ED_{50}) of these compounds (doses providing 50% animals with protection against audiogenic seizures) were 7.5 mg/kg and 6 mg/kg, respectively. In the 50% unprotected mice submitted to these doses, duration of the latency plus wild running as well as duration of seizures were reduced whereas duration of the recovery phase was not significantly affected (Table 1). These changes in the durations of phases distinguished clearly from those of reference compounds including GABAergic and sodium blocker prototype drugs previously tested in the audiogenic seizure model, suggesting distinct protective mechanisms for compounds **1** and **2** (**EBAB**).

3.1.2. MES, scPtz and rotarod tests

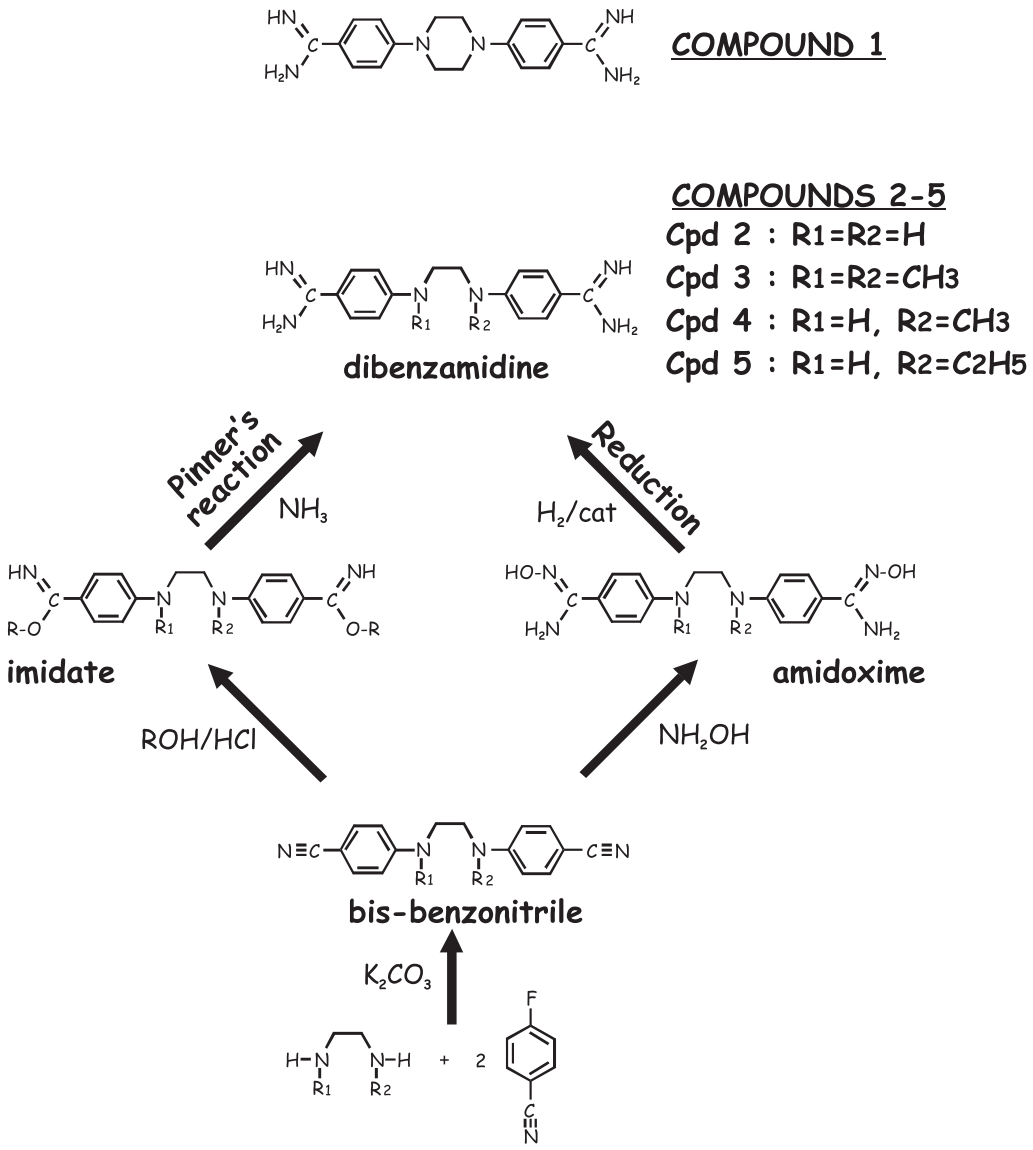
The series of five *bis*-benzamidines were further evaluated for anticonvulsant properties in classic animal seizure models including MES and scPtz tests (this subsection) and the 6 Hz seizure test (next subsection). Table 2 provides screening data for the activity of compounds in animals undergoing seizures induced by electroshock (MES test) or pentylenetetrazol (scPtz test), their minimal acute neurotoxicity being determined in the rotorod test. Compounds were administered to mice by the intraperitoneal route 30 min or 4 h before testing. Activity at these time points prompted further studies on administration performed 15 min and 1 h before anticonvulsant or toxic evaluation. At doses tested (30, 100 and/or 300 mg/kg), compounds were inactive in the MES test at the 30 min and 4 h time points and were toxic at the 30 min time point. At 30 mg/kg dose, compound **2** (**EBAB**) was not toxic, contrasting with toxicity observed for other compounds at that dose at the 15 min time point. The toxicity aggravated with increasing dose from 30 to 100 and 300 mg/kg. At the 4 h time point and administering the 30 mg/kg dose, minimal acute neurotoxicity was not, however, observed for compounds **2** (**EBAB**), **3**, **4** and **5** despite that toxicity was expressed by compound **1**. The former compounds were also not toxic at 100 mg/kg at this time point. Some activity was recorded in the scPtz test for compounds **2** (**EBAB**) and **3** (active at 300 mg/kg) and **5** (active at 100 mg/kg) at 30 min but not 4 h. Additional evaluation in the scPtz test at the 15 min and 1 h time points indicated that 100 mg/kg compound **2** (**EBAB**) was fully active at these two time points while 100 mg/kg compound **3** was active moderately at 1 h and inactive at the other studied time points.

3.1.3. The 6 Hz seizure test

Compounds **2** (**EBAB**) and **5** were selected for further evaluation in the 6 Hz seizure test. This selection especially of compound **2** (**EBAB**) took into account the potent activity expressed in the MDDAS test, which like the 6 Hz seizure test, is known also to respond to a wide range of anticonvulsant compounds. In contrast to compound **5** which was inactive at non toxic doses, compound **2** (**EBAB**) displayed a good activity in the 6 Hz seizure test, being here endowed with a ED_{50} of 48.95 mg/kg.

3.2. Antioxidant and neuroprotective evaluation of compounds

The differences observed between ED_{50} values of **EBAB** in the MDDAS test (6 mg/kg) and in the 6 Hz seizure test (49 mg/kg) suggested that in the former test part of anti-seizure activity might be accounted for by mechanisms uncovered by classic anti-seizure tests, suggesting alternative protective mechanisms. So, in this context, **EBAB** was evaluated for neuroprotective properties. In this respect, antioxidant and so-called neuroprotective mechanisms of **EBAB** were directly assessed in specific tests. These tests included *in vivo* evaluation of protection given by **EBAB** in the focal intracerebral ibotenate poisoning of rat immature brain and by *in vitro* measurement of antioxidant activities of compounds.



Scheme 1. Synthesis pathway for compounds **2** to **5.** A first step, common to the synthesis of compounds **2** to **5**, resulted in the formation of dinitriles (*bis*-benzonitriles). The second and third steps converted *bis*-benzonitriles to final compounds, involving either Pinner's reaction (imidate formation [left portion of the chemical pathway]) for compounds **4** and **5**, or amidoxime reduction [right intermediate] for compounds **2** and **3**. Compound **1** was synthesised as described in the literature (see Refs [10,11] in the text). The chemical names of depicted compounds are: piperazine-*N1,N4-bis*-benzamidine **(1)**, 1,2-ethane *bis*-1-amino-4-benzamidine (namely, 4,4′-(1,2-ethanediyldiimino)*bis*-(N-hydroxybenzenecarboximidamide)) **(2) (EBAB)**, 1,2-ethane *bis*-1-methylamino-4-benzamidine (namely, 4,4′-[1,2-ethanediyl (*N*,*N*′-dimethyl *bis*-nitrilo)]*bis*-(N-hydroxy benzenecarboximidamide)) **(3)**, 1-(1-amino-4-benzamidine)-2-(1-methylamino-4-benzamidine)-ethane (namely, 4,4′-(N-methyl 1,2-ethanediyldiimino)*bis*-(benzene-carboxymidamide) dihydrochloride) **(4)**, 1-(1-amino-4-benzamidine)-2-(1-ethylamino-4-benzamidine)-ethane (namely, 4,4′-(N-ethyl 1,2-ethanediyldiimino)*bis*-(benzene-carboximidamide) dihydrochloride) **(5)**.

3.2.1. Antioxidant evaluation

Compounds were evaluated for their ability to scavenge the superoxide anion radical by determining their capacity to inhibit rates of pyrogallol autooxidation, a reaction involving generation of the superoxide anion radical. Table 3 gives an account for this antioxidant evaluation and indicates that all five compounds were endowed with substantial scavenging properties towards superoxide with potencies of **1** > **3** > **2** **EBAB** > **5** > **4**. Interestingly, 4-aminobenzamidine, a motif recovered in the chemical formula of compounds, was quasi-devoid of the superoxide-scavenging activity.

3.2.2. Neuroprotective evaluation

EBAB was selected for direct evaluation of its protective activity towards brain wounds. It was evaluated at the dose of 4 μg/kg in a well standardized model designed for the evaluation of neuroprotective compounds, namely the ibotenate-induced focal cerebral injury model using pups. In this evaluation model, 4 μg/kg **EBAB** reduced significantly the size of lesions in both the cortical plate (i.e. gray matter) and white matter by 49% and 69%, respectively (Fig. 1). The protective effects of EBAB were of the same order of magnitude as those afforded by 0.1 mg/kg leptin, a well accepted neuroprotectant [12,13], the former being a little more active on white matter and the latter on grey matter (Fig. 1).

3.3. Molecular modelling studies

Molecular modelling of compounds **1** to **5** was carried out to visualize the tridimensional structure of their low energy conformation (Fig. 2). These conformations were compared with that of spermine, a compound which also exhibits protonated nitrogen ends (namely, $-NH_3^+$). The distance between the two protonated

Table 1
Modulation by compounds **1** and **2** (**EBAB**) ED_{50} doses of durations of phases in the MDDAS test.

	Duration of latency plus wild running periods	Duration of convulsions	Duration of recovery
Controls	7.2 ± 1.5	1.6 ± 0.4	50.3 ± 5.6
Compound **1** (7.5 mg/kg)	$6.05 \pm 1.45^{*}$	$0.79 \pm 0.24^{**}$	48.0 ± 3.2
Compound **2** (**EBAB**) (6 mg/kg)	$4.75 \pm 0.25^{**}$	$1.4 \pm 0.4^{*}$	50.3 ± 3.5
Qualitative modifications by ED_{50} of			
neuroprotective compounds devoid of intrinsic anticonvulsant properties	Increased	Increased	Unaffected or decreased
anticonvulsant compounds acting on voltage-gated sodium channels	Increased	Decreased	Decreased
GABAergic anticonvulsant compounds	Increased	Decreased	Decreased
ethosuximide	Unaffected	Unaffected	Unaffected

Adult OF1 mice receiving a magnesium-deficient diet (35-40 ppm for 27 days) were utilized. Drugs were given 1 hr before testing *via* the intraperitoneal route at a dose corresponding to their previously determined ED_{50} in the MDDAS testing (for additional experimental details and rationale, see Ref. [4]). Phase durations are expressed as seconds (mean values ± SEM, n = 5) in convulsing animals at drug dosing ensuring anticonvulsant protection in 50% and no protection in the other 50% of tested animals (those concerned by this table). Controls refer to untreated magnesium-deficient mice (mice given neither vehicle nor drug), the administration of the compound vehicle being without effect on the control values. The first three lanes of results are those experimentally obtained in the scope of this work. The data concerning the qualitative modification of durations of phases refer to previous works [4–6] and are given to allow a rapid comparison of phase modulation properties of compounds **1** and **2** (**EBAB**) with those of reference prototype antiepileptic drugs.
$^{*}p < 0.05$ and $^{**}p < 0.01$.

nitrogen ends of *bis*-benzamidine (namely, $=NH_2^+$) was measured for each compound, and compared with the distance between protonated nitrogen ends of spermine. This distance was comparable for **1**, **EBAB** and spermine (15.55, 16.09 and 16.13 Å, respectively), three compounds for which conformation was extended. By contrast, this distance was lower for compounds **3**, **4** and **5** (11.76, 12.70 and 12.21 Å, respectively) for which conformation was partially folded. Though bioactive conformations are not necessarily low energy conformations, **EBAB** might be, however, considered to represent a spermine analogue and in this respect was evaluated for its ability to interfere with the NMDA receptor function.

3.4. Direct and indirect exploration of the activity of EBAB towards the NMDA receptor

Both the magnesium deficiency [7] and the focal intracerebral ibotenate-poisoning challenge [14,15] are characterized, among other features, by an activation of the NMDA receptor. Polyamines towards which **EBAB** shares in common some structural analogies (see above) influences also the activity of the NMDA receptor [16]. For all these reasons, **EBAB** was evaluated for its ability to control features directly and indirectly related to the NMDA receptor.

3.4.1. Testing EBAB against NMDA-induced seizures

Whether **EBAB** might act, like polyamines, on the NMDA receptor calcium channel complex was studied by evaluating the protection given by **EBAB** against NMDA-induced seizures in mice. **EBAB** at 49 mg/kg was found to increase the threshold for NMDA seizures by more than 10%, and this finding prompted to evaluate the protection given by this compound against seizures induced in normally fed OF1 mice by 137 mg NMDA. In the absence of **EBAB**, 100% of mice given intraperitoneal NMDA (137 mg/kg) developed fatal seizures. Administration of **EBAB,** 1 hr prior to giving 137 mg/kg NMDA to animals, resulted in a protection against seizures, this protective activity being referred to the absence of seizure development in animals observed for a period of 4 hours following NMDA administration and the absence of death within 48 hours.

Table 2
Anticonvulsant and neurotoxicity screening data in mice dosed intraperitoneally with compounds.

Compounds	MES test		ScPtz test				Toxicity			
	30 min	4 h	30 min	15 min	1 h	4 h	30 min	15 min	1 h	4 h
1 30 mg	−	−	−			−	+++			++
2 (EBAB)										
30 mg	−	−	−			−	−			−
100 mg	−	−	−	++++	++++	−	++	+++	++++	−
300 mg	−	−	++++			−	++++			++
3 30 mg	−	−	−			−	+			−
100 mg	−	−	−	−	+	−	++	+++	++	−
300 mg	−	−	++++			−	++++			−
4 30 mg	−	−	−			−	+			−
100 mg	−	−	−			−	++++			−
300 mg							++++			
5 3 mg			−				−			
10 mg			−				−			
30 mg	−	−	+++			−	++			−
100 mg							++++			
300 mg							++++			

The anticonvulsant (MES and scPtz tests) and neurotoxicity activities were determined 30 min and 4 h (occasionally 15 min and 1 h) after the intraperitoneal administration of compounds to mice. Symbols are as follows: ++++, activity in 75–100% of administered animals; +++, in 50–75% of animals; ++, in 25–50% of animals; +, 0–25% of animals; and −, no activity or toxicity. Toxicity was determined by the rotorod test. Abbreviations are as follows: MES, maximal electroshock seizures; scPtz, subcutaneous pentylenetetrazol.

Table 3
Effect of compounds on pyrogallol autooxidation rates.

Experimental conditions	Inhibition of pyrogallol autooxidation rates
Vehicle	0%
Compound **1**	69.4%
Compound **2** (**EBAB**)	36.3%
Compound **3**	51.0%
Compound **4**	17.5%
Compound **5**	28.8%
4-aminobenzamidine	<5%

Pyrogallol autoxidation rates were measured at 420 nm as indicated in the experimental procedure. The assay medium contained pH 7.4 TRIS saline buffer (5 mM TRIS and 50 mM NaCl), 10 μL DMSO or 10 μL compound 50 mM stock solution prepared in DMSO (final concentration of compound in the cuve being 0.5 mM). Reaction was started by the addition of 0.75 mM pyrogallol. 4-Aminobenzamidine, a chemical motif present in each compound formula was also tested.
Each result corresponds to the mean of three separate experimental determinations. Inhibition of pyrogallol autooxidation was calculated using rates monitored during the first two minutes of the reaction and are expressed as percentage values in the table.

The ED_{50} exhibited by **EBAB** in the 137 mg/kg NMDA-induced seizure test was found to be 6 mg/kg. Compound **4** for which no activity was obtained in MDDAS, MES and scPtz tests, failed to provide animals with protection against NMDA-induced seizures at doses up to 50 mg/kg.

3.4.2. *Effect of EBAB on locomotor activity and body temperature*

Indirect evidence for a depression of the brain excitatory pathways was explored by determining the effect of **EBAB** on the locomotor activity and body temperature. For locomotor activity, mice were transferred individually in the actimeter and were allowed to explore for a 2 min period. Their spontaneous locomotor activity was then measured during 3 minutes. The number of crossings of the photocell activity meter was 121.8 ± 28.2 for control (untreated) mice ($n = 10$), and 13.3 ± 2.2, 90.5 ± 10.7 or 103.3 ± 20.4 for mice given 30, 10 and 5 mg/kg **EBAB** (1 hr before testing), respectively. Mean rectal temperature was lowered from 38 °C to 35.7 °C (30 mg/kg **EBAB**). The **EBAB** 30 mg/kg dose reduced spontaneous (actimeter test) but not forced (rotarod test) locomotor activity, a differential effect lost by a small increase in the administered dose (from 30 to 35 mg/kg) which altered both types of locomotor activities (data not shown).

3.4.3. *In vitro pharmacology of EBAB on the phencyclidine site of the NMDA receptor*

EBAB was evaluated for its ability to displace the binding of ^{3}H-TCP (a thienyl analogue of phencyclidine which targets the phencyclidine site of NMDA receptor and not that of sigma receptors) to rat brain structures. The IC_{50} value determined for **EBAB** was 1.4 10^{-6} M. The corresponding competition curve is shown in Fig. 3.

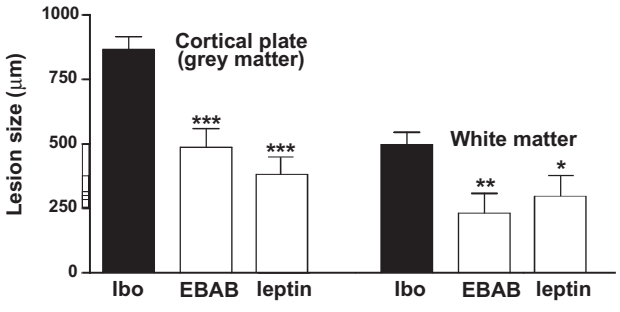


Fig. 1. Effect of **EBAB** on lesions induced by focal intracerebral injection of ibotenate to pups. **EBAB** was given to CNS ibotenate-treated pups at the dose of 4 μg/kg. Ibo represents the reference conditions (animals given ibotenate plus the vehicle) and leptin (0.1 mg/kg) is a standard neuroprotective compound. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (ANOVA, Bonferroni test).

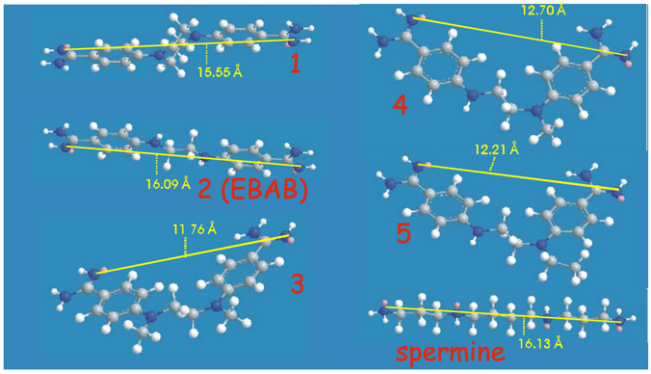


Fig. 2. Molecular modelling studies of low energy conformations of spermine and bisbenzamidine compounds **1** to **5.** Low energy conformations of compounds were determined using the Chem 3D Ultra version 9.0.1 de Cambridgesoft. Carbon, nitrogen and hydrogen atoms are represented by spheres coloured in grey, blue and white, respectively. Red spheres refer to protonation of nitrogen atoms, and distances between protonated nitrogens are indicated by a red line annotated in red characters. Note that compound **2** (**EBAB**) and spermine adopt both a low energy conformation which is linear and in which the distances between the protonated nitrogens placed at each extremity of the molecules are very close, 16.09 and 16.13 Å, respectively. The two compounds along with compound **1** adopt an extended conformation in contrast to compounds **3**, **4** and **5** which exhibit a folded conformation in which the distance between the protonated nitrogens is lower (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

3.4.4. *Evaluation of EBAB in the hot plate test*

The effect of **EBAB** on the phencyclidine site of NMDA receptor might be accounted for by an effect on the polyamine site of this receptor. Action on the last site (namely, antagonism of stimulatory effects of polyamines on the NMDA receptor) is known for other compounds to convey potent antinociceptive activity (for details, see Discussion). So **EBAB**, given 45 min before testing, was evaluated in the hot plate test. To correct for individual differences in base-line latencies, the nociceptive data (latencies) were converted to percentage maximum possible effect (% MPE) in accordance with

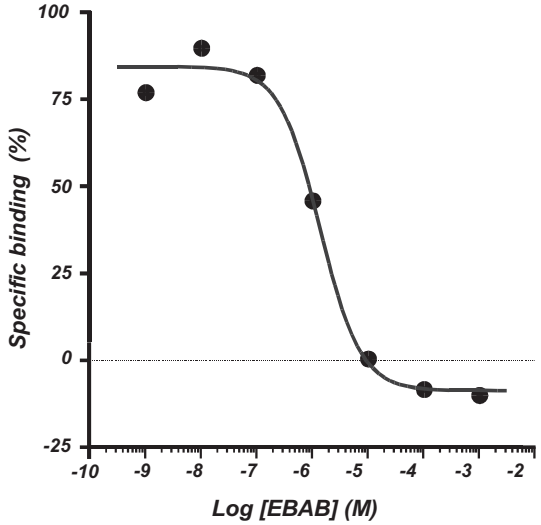


Fig. 3. Curve of competition by **EBAB** of binding of ^{3}H-TCP to a rat cerebral preparation. **EBAB** $IC_{50} = 1.4 \times 10^{-6}$ M ($nH = 1.2$).

Brady and Holtzman [17] (for calculation of %MPE values, see the Materials and methods section). **EBAB** provided protection against nociception in mice undergoing the hot plate test with % MPE at 30 mg and 60 mg/kg amounting to $31.17 \pm 14.52\%$ and $70.33 \pm 28.91\%$ (6 **EBAB**-treated versus 6 untreated mice for each drug dosing group), respectively.

4. Discussion

The amidine ($-C(-NH_2)=NH_2^+$) and benzamidine (-phenyl-C ($-NH_2$)=NH_2^+) moieties represent the pharmacophore of several pharmaceutical specialities such as propamidine (used to cure infectious corneal ulcers caused by *Acanthamoeba keratitis*), pentamidine (clinically prescribed in the treatment of *Pneumocystis pneumonia*, human african trypanosomiasis cause of sleeping sickness, human and animal leishmaniasis), hexamidine, an approved antiseptic found in many topical, ocular, and oral preparations [18,19] and a prodrug of furamidine which is in phase III development for the treatment of african trypanosomiasis [20]. Treatment of infectious diseases, including recent developments [21–23], represents thus a major indication of amidines and benzamidines. The present short series of *bis*-benzamidines were in this line, displaying also anti-infectious properties. On the basis of neuroprotective and anticonvulsant properties here documented, medical indications of benzamidines might be potentially extended to counteract diverse nutrition, chemical or disease-driven brain injuries.

At a mechanistic point of view, pentamidine analogues were previously shown to interact with the NMDA receptor calcium channel complex [24]. More precisely, pentamidine analogues were shown to interact with the spermine ligand binding site of this receptor channel complex [24], acting *via* a mechanism referred to as an inverse agonistic activity [25,26]. This is for instance the case of polyamines flanked with aromatic substituents that provide compounds with a more potent blocker activity towards NMDA receptor calcium channel complex [26]. In addition to these works, our data report *in vivo* efficiency of this class of compounds (arylamidines) towards the NMDA receptor. Therefore, a breakthrough of the present work is to demonstrate clearly that this type of compounds is effective *in vivo*, i.e. on the whole animals, indicating that compounds (or metabolites) are likely to permeate blood–brain barrier at substantial rates, a original feature which should promote their future pharmacological development in the field of disease-driven brain toxicity.

Exploration of NMDA receptor modulating properties of bis-benzamidines, though *a posteriori* logical, has been, however, the result of a methodical approach providing the MDDAS test with a central place in the strategy developed to characterise brain protective properties of our short series of compounds. In this respect, our data have been presented *grosso modo* in the chronological order in which they were collected. In agreement with the overall strategy appearing in the Introduction, MDDAS testing (protection against audiogenic seizures) was used to detect compounds of interest for drug development in the field of disease-driven brain injuries, guiding the pursuit of evaluation to assess the compound mode of action *via* additional experiments. They included a previously described alternative use of the MDDAS model (modulation by compound ED_{50} of phase durations) [4] and the testing in classic animal seizure tests (MES and scPtz tests). Because of the discrepancy between the potencies of compounds in MDDAS and other (MES,scPtz) tests, the selected compound, **EBAB**, was further evaluated in the 6 Hz test, a seizure test responsive to a wide spectrum of anticonvulsant activities [27]. Potency of **EBAB** in this test (ED_{50} value of 49 mg/kg) was here less in discrepancy with the evaluation in the MDDAS test ($ED_{50} = 6$ mg/kg). However, the residual discrepancy between the two evaluations has led us to determine whether it could not be attributed to alternative mechanisms, so-called neuroprotective properties adding to the classic anti-seizure hits (NIH testing) observed for the selected compound. For this reason, **EBAB** was evaluated (with success) for its activity in a standard test of cerebral injury (ibotenate-poisoned brain) classically employed for detecting neuroprotectants as well as in antioxidant assays. As a general rule, neuroprotectants and/or antioxidants even if devoid of intrinsic anti-seizure activity in classic tests appear to be active against audiogenic seizures associated with magnesium deficiency. Fig. 4 provides the reader with an illustrated account of this particular facet of the MDDAS model which combines neurotoxicity and seizure development.

The anti-NMDAergic activity of bis-benzamidines, here clearly shown for the selected compound **EBAB**, might account for most central nervous system (CNS) effects here reported. Magnesium-deficiency has been recently shown to be associated with a lowered threshold to NMDA-induced seizures [7], being consistent with a sensitivity of audiogenic seizures to **EBAB**. Ibotenate is a cyclic analogue of glutamate acting on glutamate receptors including metabotropic and NMDA receptors but neither alpha-3-amino-hydroxy-5-methyl-4-isoxazole (AMPA) nor kainate receptors [28]. Blunting the effect of ibotenate on the NMDA receptor by an anti-NMDA activity might explain the protection offered by **EBAB** against ibotenate poisoning. The activity of **EBAB** in the 6 Hz seizure test is also logical in view of the large spectrum of anti-convulsant mechanisms to which this model responds; anti-NMDA activity (such as that conveyed by **EBAB**) representing a known anticonvulsant mechanism as for instance inferred from the previous use of MK-801 [29–31]. Finally, indirect evidence for impact of **EBAB** on the NMDA receptor may have been also given by a reduction in locomotor activity and body temperature, and in pain. This antinociceptive effect of **EBAB** might be linked to a putative action at polyamine sites of the NMDA receptor, consistent with modulation of the binding at the phencyclidine site, as previously incriminated for drugs such as ifenprodil which antagonizes polyamine stimulatory sites located on NR2B-containing NMDA receptors and binding of ^{3}H-TCP to rat cerebral membranes and which exhibits antinociceptive properties [32,33 and references therein].

5. Conclusion

EBAB is a novel compound active against various brain neuro-insult models. Though other mechanisms may not be ruled out, **EBAB** antagonizes the NMDA receptor/calcium channel complex, via antagonism possibly of polyamine stimulatory sites and effectively of ^{3}H-TCP-binding site. This view is consistent with most of the CNS effects reported here for this compound.

6. Materials and methods

6.1. Materials

Reagents and solvents were purchased from Sigma–Aldrich (Bornem, Belgium).

6.2. Analytical procedures

Melting points (uncorrected) were determined in open capillary tubes on an Electrothermal apparatus. Carbons of the phenyl ring on the linker nitrogen bearing substituent R1 (see Scheme 1) were numbered from 1 to 6, carbon 1 bearing nitrile, amidine or amidoxime group. The 1′ to 6′ numbering was used similarly for the other phenyl ring. IR spectra were recorded in the ATR mode on

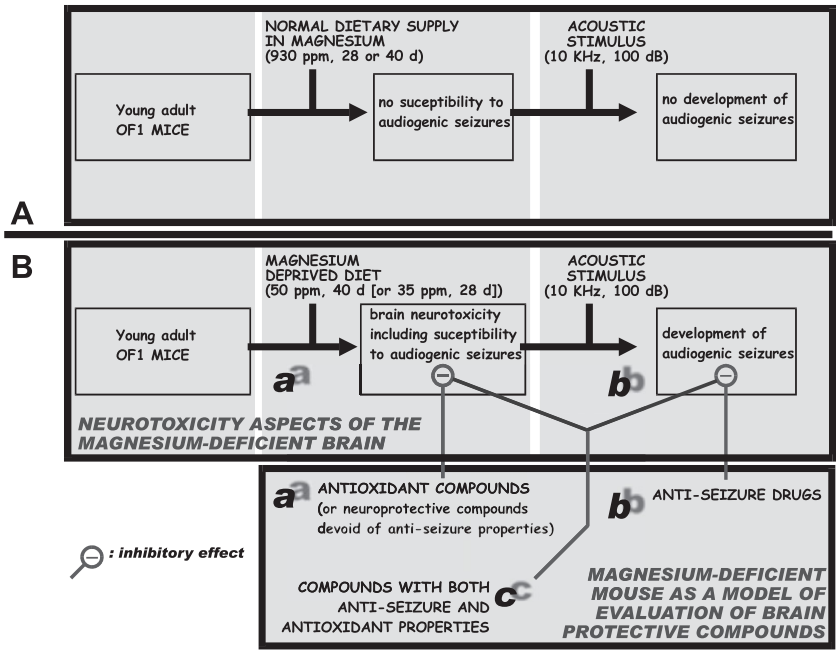


Fig. 4. Current understanding of magnesium deficiency in mice as a model emphasizing neurotoxicity aspects of magnesium-deficient brain and their suitability for evaluation of brain protective drugs. A. Normal diet supply in magnesium. B. Deficient diet supply in magnesium. a. The susceptibility to develop seizures is induced by magnesium deprivation and is likely to result from the pro-oxidant and pro-inflammatory state associated with magnesium deficiency (note that disturbances of neurotransmission are concomitantly induced). By removing this inflammatory/oxidative stress-driven susceptibility to audiogenic seizures, compounds endowed with antioxidant properties even if devoid of intrinsic anti-seizure activity are capable of protecting magnesium-deficient mice against audiogenic seizures. b. The development of audiogenic seizures is triggered by a well defined acoustic stimulus which here acts as a physicial convulsant stimulus like the chemical convulsant pentylenetetratzol and the electrical convulsant electroshock do in their respective tests. Anti-seizure drugs protect mice against audiogenic seizures through classic inhibition of seizures. c – Compounds combining anti-seizure and antioxidant properties are capable of interfering with both susceptibility to and development of audiogenic seizures.

a Perkin-Elmer Spectrum 100 spectrophotometer. NMR spectra were measured at 25 °C on compounds in solution in DMSO-d6 at 300 MHz for ^{1}H on a BRUKER AMX-300 apparatus. Chemical shifts are expressed relative to the resonance of $(CD_3)_2SO$ at ‰ 2.49 for ^{1}H NMR. All compounds had IR and ^{1}H NMR spectra consistent with their assigned structure. Their high resolution mass spectra were recorded on a Waters QtoF 2 spectrometer.

6.3. Chemistry, intermediary and final compounds

Compounds were synthesized according to the chemical pathway depicted in Scheme 1. General comments of this chemical pathway were given in the Chemistry section. Detailed aspects are the following.

6.3.1. Synthesis of the bis-benzonitriles (products of the first step of the chemical synthesis pathway, Scheme 1)

A mixture of the appropriate diamine (25 mmol), 4-fluorobenzonitrile (55 mmol), and potassium carbonate (50 mmol) in *N,N*-dimethylacetamide (25 ml) was heated under reflux for 9 h. After cooling, the reaction mixture was poured into cold water, the solid was filtered and successively washed with water and ethanol.

6.3.1.1. 4,4′-(N-ethyl 1,2-ethanediyldiimino)bis-benzonitrile. Synthesis was performed with 1-amino-2-ethylamino-ethane as the diamine reagent; yield 23%; mp 144–146 °C; ^{1}H RMN (DMSO d_6): δ (ppm): 7,6 (2H, d, $J = 9$ Hz, $H^{2\,(2')}$ and $H^{6\,(6')}$); 7,5 (2H, d, $J = 9$ Hz, $H^{2\,(2')}$ and $H^{6\,(6')}$); 6,8 (1H, s large, N-**H**); 6,8 (2H, d, $J = 9$ Hz, $H^{3\,(3')}$ and $H^{5(5')}$); 6,5 (2H, d, $J = 9$ Hz, $H^{3\,(3')}$ and $H^{5\,(5')}$); 3,5 (2H, t, $J = 6$ Hz, NH-C**H**$_2$); 3,4 (2H, q, $J = 7$ Hz, N–C**H**$_2$–CH_3); 3,3 (2H, t, $J = 6$ Hz, Et-N-C**H**$_2$); 1,1 (3H, t, $J = 7$ Hz, C**H**$_3$–CH_2); IR (KBr): ν (cm^{-1}): 3380 (N–H), 2211 (C≡N), 1602, 1522, 1178; HRMS (ESI-ToF) MH^+ $C_{18}H_{19}N_4$: experimental: *m/z* 291,1600; calculated: *m/z* 291,1610.

6.3.1.2. 4,4′-(N-methyl 1,2-ethanediyldiimino)bis-benzonitrile. Synthesis was performed with 1-amino-2-methylamino-ethane as the diamine reagent; yield 71%; mp 150–152 °C; ^{1}H RMN (DMSO d_6): δ (ppm): 7,5 (2H, d, $J = 8$ Hz, $H^{2\,(2')}$ and $H^{6\,(6')}$); 7,4 (2H, d, $J = 8$ Hz, $H^{2\,(2')}$ and $H^{6\,(6')}$); 6,8 (1H, s large, N–**H**); 6,8 (2H, d, $J = 8$ Hz, $H^{3\,(3')}$ and $H^{5\,(5')}$); 6,6 (2H, d, $J = 8$ Hz, $H^{3\,(3')}$ and $H^{5\,(5')}$); 3,6 (2H, t, $J = 6$ Hz, NH–C**H**$_2$); 3,3 (2H, t, $J = 6$ Hz, Met-N-C**H**$_2$); 3,0 (3H, s, C**H**$_3$–N–CH_2); IR (KBr): ν (cm^{-1}): 3388 (N–H), 2214 (C≡N), 1603, 1521, 1386, 1181; HRMS (ESI-ToF) MH^+ $C_{17}H_{17}N_4$: exp.: m/z 277,1453; Calc.: m/z 277,1453.

6.3.1.3. 4,4′-[1,2-ethanediyl (N,N′-dimethylbis-nitrilo)]bis-benzonitrile. Synthesis was performed with 1, 2-dimethylamino-ethane as the diamine reagent; yield 68%; mp 204–206 °C; ^{1}H RMN (DMSO d_6): δ (ppm): 7,5 (4H, d, $J = 8$ Hz, H^3, H^5, $H^{3'}$ and $H^{5'}$); 6,7 (4H, d, $J = 8$ Hz, H^2, H^6, $H^{2'}$ and $H^{6'}$); 3,6 (4H, s, CH_3–N–C**H**$_2$); 2,9 (6H, s, C**H**$_3$–N); IR (KBr): ν (cm^{-1}): 2208 (C≡N), 1604, 1520, 1387, 1177; HRMS (ESI-ToF) MH^+ $C_{18}H_{19}N_4$: exp.: m/z 291,1613; Calc.: m/z 291,1610.

6.3.1.4. 4,4′-(1,2-ethanediyldiimino)bis-(benzonitrile). Synthesis was performed with 1, 2-dimethylamino-ethane as the diamine reagent; yield 70%; mp 207–210 °C; ^{1}H RMN (DMSO d_6): δ (ppm): 7,5 (4H, d, $J = 9$ Hz, H^2, H^6, $H^{2'}$ and $H^{6'}$); 6,8 (2H, s large, N–**H**); 6,6 (4H, d, $J = 9$ Hz, H^3, H^5, $H^{3'}$ and $H^{5'}$); 3,3 (4H, s, C**H**$_2$–NH); IR (KBr): ν (cm^{-1}): 3368 (N–H); 2211 (C≡N); 1604, 1528, 1303, 1283; HRMS (ESI-ToF) MH^+ $C_{16}H_{15}N_4$: exp.: m/z 263,1296; Calc.: m/z 263,1297.

6.3.2. Synthesis of diamidines via the Pinner's reaction (second step of the chemical synthesis pathway relative to the asymmetric diamidine molecules, Scheme 1 and its legend)

A mixture of the appropriate *bis*-benzonitrile (3.4 mmol) and methanol (20 ml) in dichloromethane (200 ml) was saturated with

gaseous hydrochloric acid. After 4 days at room temperature, the solvents were evaporated under reduced pressure and the solid was washed with ether. The imidate was then treated with a methanolic solution of ammonia (7 N, 15 ml) and the mixture was heated under reflux for 1 h. After cooling the precipitate was filtered and washed with alcohol.

6.3.2.1. 4,4′-(N-ethyl1,2-ethanediyldiimino)bis-(benzene-carboximidamide) dihydrochloride. Synthesis was performed with 4,4′-(*N*-ethyl 1,2-ethanediyldiimino) *bis*-benzonitrile as the *bis*-benzonitrile reagent; yield 33%; mp > 300 °C; ^{1}H RMN (DMSO d_6): δ (ppm): 9,1 (2H, s, N—**H** amidine); 8,9 (2H, s, N—**H** amidine); 8,8 (2H, s, N—**H** amidine); 8,7 (2H, s, N—**H** amidine); 7,8 (2H, d, $J = 9$ Hz, $H^{2\,(2')}$ and $H^{6\,(6')}$); 7,7 (2H, d, $J = 9$ Hz, $H^{2\,(2')}$ and $H^{6\,(6')}$); 7,1 (1H, t large, N-**H**); 6,8 (2H, d, $J = 9$ Hz, $H^{3\,(3')}$ and $H^{5\,(5')}$); 6,7 (2H, d, $J = 9$ Hz, $H^{5\,(5')}$ and $H^{3\,(3')}$); 3,6 (2H, t, $J = 8$ Hz, C**H**$_2$—NH); 3,5 (2H, q, $J = 7$ Hz, CH_3—C**H**$_2$—N); 3,4 (2H, t, $J = 8$ Hz, Et-N-C**H**$_2$); 1,1 (3H, t, $J = 7$ Hz, C**H**$_3$—CH_2); IR (KBr): ν (cm^{-1}): 3317, 3136, 1663, 1605, 1494; HRMS (ESI-ToF) MH^+ $C_{18}H_{25}N_6$ exp.: m/z 325,2136; Calc.: m/z 325,2141.

6.3.2.2. 4,4′-(N-methyl-1,2-ethanediyldiimino)bis-(benzene-carboximidamide) dihydrochloride. Synthesis was performed with 4,4′-(N-methyl 1,2-ethanediyldiimino)*bis*-benzonitrile as the *bis*-benzonitrile reagent; yield 36%; mp > 300 °C; ^{1}H RMN (DMSO d_6) : δ (ppm) : 9,1 (2H, s, N—**H** amidine) ; 8,9 (2H, s, N—**H** amidine) ; 8,8 (2H, s, N-**H** amidine) ; 8,7 (2H, s, N-**H** amidine) ; 7,8 (2H, d, $J = 9$ Hz, $H^{2\,(2')}$ and $H^{6\,(6')}$) ; 7,7 (2H, d, $J = 9$ Hz, $H^{2\,(2')}$ and $H^{6\,(6')}$) ; 7,1 (1H, t large, N-**H**) ; 6,8 (2H, d, $J = 9$ Hz, $H^{3\,(3')}$ and $H^{5\,(5')}$) ; 6,7 (2H, d, $J = 9$ Hz, $H^{5\,(5')}$ and $H^{3\,(3')}$) ; 3,6 (2H, t, $J = 5$ Hz, C**H**$_2$—NH) ; 3,4 (2H, t, $J = 5$ Hz, Et-N-C**H**$_2$) ; 3,0 (3H, s, C**H**$_3$—N—CH_2); IR (KBr) : ν (cm^{-1}) : 3329, 3143, 1661, 1608, 1495; HRMS (ESI-ToF) MH^+ $C_{17}H_{23}N_6$: exp.: m/z 311,1970; Calc.: m/z 311,1984.

6.3.3. Synthesis of diamidines via the formation followed by reduction of amidoximes (second step of the chemical synthesis pathway relative to the symmetric diamidine molecules, Scheme 1 and its legend)

6.3.3.1. Formation of amidoximes. A mixture of hydroxylamine hydrochloride (40 mmol) and a methanolic solution of sodium methoxide (30%, 40 mmol) in methanol (25 ml) was heated under reflux for 30 min. After cooling the precipitate was filtered and the filtrate was transferred into a round-bottom flask. The appropriate *bis*-benzontrile (4 mmol) was added and the mixture was heated under reflux for 8 h. After cooling the precipitate was filtered and washed with methanol.

6.3.3.2. Reduction of amidoximes (diamidine formation). The so-obtained diamidoxime (2.2 mmol) was dissolved in acetic acid (15 ml). Ammonium formate (25 mmol) and Pd/C 10% (0.2 g) were successively added. The mixture was heated under reflux for 2 h. After cooling the mixture was filtered on Celite® and the filtrate was concentrated under reduced pressure. The solid was separated, poured into dichloromethane and precipitated as the dihydrochloride salt by addition of gaseous hydrochloric acid.

6.3.3.3. Formation of amidoximes. 4,4′-[1,2-ethanediyl (N,N′-dimethyl bis-nitrilo)]bis-(N-hydroxy benzenecarboximidamide) — Synthesis was performed with 4,4′-[1,2-ethanediyl (*N,N′*-dimethyl *bis*-nitrilo)]*bis*-benzonitrile as the *bis*-benzonitrile reagent; yield 90%; mp 198-202 °C; ^{1}H RMN (DMSO d_6): 9,3 (2H, s, O—**H**); 7,6 (4H, d, $J = 9$ Hz, H^2, H^6, $H^{2'}$and $H^{6'}$); 6,7 (4H, d, $J = 9$ Hz, H^3, H^5, $H^{3'}$and $H^{5'}$); 5,7 (4H, s, —N**H**$_2$); 3,6 (4H, s, CH_3—N—C**H**$_2$); 2,9 (6H, s, C**H**$_3$—N); IR (KBr) : ν (cm^{-1}) : 3496 (NH_2), 3374 (NH_2), 1665, 1609, 1527; HRMS (ESI-ToF) MH^+ $C_{18}H_{25}N_6O_2$: exp.: m/z 357,2033; calc. : m/z 357,2039.

4,4′-(1,2-ethanediyldiimino)bis-(N-hydroxybenzenecarboximidamide) — Synthesis was performed with 4,4′-(1,2-ethanediyldiimino) *bis*-(benzonitrile) as the *bis*-benzonitrile reagent; yield 87%; mp 206–209 °C; ^{1}H RMN (DMSO d_6) : 9,3 (2H, s, O—**H**) ; 7,5 (4H, d, $J = 9$ Hz, H^2, H^6, $H^{2'}$and $H^{6'}$) ; 6,6 (4H, d, $J = 9$ Hz, H^3, H^5, $H^{3'}$and $H^{5'}$) ; 5,9 (2H, s, N—**H**) ; 5,6 (4H, s, —N**H**$_2$) ; 3,2 (4H, s, NH—C**H**$_2$); IR (KBr) : ν (cm^{-1}) : 3489 (NH_2), 3379 (NH_2), 1661, 1608, 1529; HRMS (ESI-ToF) MH^+ $C_{16}H_{21}N_6O_2$: exp.: m/z 329,1728; Calc.: m/z 329,1732.

6.3.3.4. Reduction of amidoximes (diamidine formation). 4,4′-[1,2-ethanediyl(*N,N′*-dimethylbis-nitrilo)] bis-***(benzenecarboximidamide) dihydrochloride*** — Synthesis was performed by reduction of 4,4′-[1,2-ethanediyl (*N,N′*-dimethyl *bis*-nitrilo)]*bis*-(N-hydroxy benzene carboximidamide) as the amidoxime reagent; yield 68%; mp > 300 °C; ^{1}H RMN (DMSO d_6) : δ (ppm) : 8,9 (2H, s, N-**H** amidine) ; 8,6 (4H, s, N-**H** amidine) ; 7,7 (4H, d, $J = 8$ Hz, H^2, H^6, $H^{2'}$ and $H^{6'}$) ; 6,8 (4H, d, $J = 8$ Hz, H^3, H^5, $H^{3'}$ and $H^{5'}$) ; 3,7 (4H, s, CH_3-N-C**H**$_2$) ; 3,0 (6H, s, C**H**$_3$—N—CH_2); IR (KBr) : ν (cm^{-1}) : 3307, 3137, 1664, 1609, 1485; HRMS (ESI-ToF) MH^+ $C_{18}H_{25}N_6$: exp./m/z 325,2133; Calc.: m/z 325,2141.

4,4′-(1,2-ethanediyldiimino)bis-benzenecarboximidamide dihydrochloride — Synthesis was performed by reduction of 4,4′-(1,2-ethanediyldiimino)*bis*-(N-hydroxybenzenecarboximidamide) as the amidoxime reagent; yield 65%; mp > 300 °C; ^{1}H RMN (DMSO d_6) : δ (ppm) : 8,9 (2H, s, N—**H** amidine) ; 8,7 (4H, s, N—**H** amidine) ; 7,7 (4H, d, $J = 8$ Hz, H^2, H^6, $H^{2'}$ and $H^{6'}$) ; 7,1 (2H, s, N—**H**) ; 6,8 (4H, d, $J = 8$ Hz, H^3, H^5, $H^{3'}$ and $H^{5'}$) ; 3,4 (4H, s, CH_3—N—C**H**$_2$); IR (KBr) : ν (cm^{-1}) : 3296, 3211, 3122, 1657,1609, 1490, 1460; HRMS (ESI-ToF) MH^+ $C_{16}H_{21}N_6$: exp.: m/z 297,1829 ; Calc.: m/z 297,1828.

6.4. In vivo and in vitro experiments

6.4.1. Preparation and mode of administration of compounds

All tested compounds were administered *via* the intraperitoneal route. Compounds studied in the audiogenic seizure model were dissolved in a 10 μl dimethylsufoxide (DMSO)/10 μl polyethylene glycol 300 mixture and administered 1 h before testing. For the ibotenate poisoning experiments and *in vitro* assays, compounds were dissolved in DMSO. Compounds were dissolved in methylcellulose for evaluation in the MES, scPtz and 6 Hz seizure test at different time-points ranging from 15 min to 4 h.

6.4.2. In vivo evaluations on the animals

6.4.2.1. Audiogenic seizure susceptibility. This was induced in OF1 mice by magnesium deprivation in the diet as described previously [4], except that 35 ppm instead of 50 ppm magnesium content of the diet was used in order to induce the susceptibility in 100% animals in 28 days instead of 42 days in this mouse strain. The magnesium deficiency dependent audiogenic seizure (MDDAS) was triggered by an acoustic stimulus. It was studied as an animal model for yes/no anticonvulsant protection screening, for determination of the efficacious dose ED_{50} (compound dose preventing seizures in 50% tested animals) and its subsequent impact on the duration of the phases of audiogenic seizures in convulsing mice (latency plus wild running, seizure and recovery). Essentially for the audiogenic seizure testing [4], individual animals were placed in a 9-dm^3 volume test chamber (30, 20 and 15 cm for length, width and height, respectively) and exposed for 15 s to an auditory signal of 10 ± 0.1 kHz frequency and 100 ± 1 db intensity. This acoustic signal was produced by a signal generator and projected via a high frequency speaker mounted on the roof of the chamber. The noise level was measured close to the animal's ear by an external decibelmeter probe. Each audiogenic test used only one test chamber and each animal was subjected to only one audiogenic test during the experimentation. Tested animals were OF1 mice [3-month-old

(adult) mice] obtained from Janvier (Le Genest Saint Isle,France) and were randomly divided into groups of 20 mice per cage.

6.4.2.2. Focal intracerebral injection of ibotenate. In Swiss mouse pups and determination of the subsequent lesions in white and grey matters were performed with or without concomitant drug administration as previously described [14,15,28]. In these studies, Swiss mouse pups were anesthetized by isoflurane inhalation for intracerebral and intraperitoneal injections. Essentially for the stereotaxic intracerebral injections, 10 μg of ibotenate (Sigma, St. Louis, MO) were administered with a 26-gauge needle on a 50 μl Hamilton syringe mounted on a calibrated microdispenser. The needle was inserted 2 mm under the external surface of scalp skin in the frontoparietal area of the right hemisphere, 2 mm from the midline in the lateral-medial plane and 3 mm (in the rostro-caudal plane) from the junction between the sagittal and lambdoid sutures. Two 1 μl boluses were injected at 30-s intervals. The needle was left in place for an additional 30 s. Compound **2** (**EBAB**) powder were diluted in DMSO and injected i.p. at the dose of 4 μg/kg. Controls received i.p. DMSO. Five days later, the surviving pups were killed by decapitation and the brains were fixed in formalin. Paraffin normal serial sections, 15 μm thick, were cut and every third section was stained with cresyl-violet. These serial sections of the entire brain were made in the coronal plane. As explained previously, this permitted an accurate and reproducible determination of the maximal diameter of the lesion in the sagittal fronto-occipital axis (which is equal to the number of sections where the lesion was present multiplied by 15 μm). We used this measure as an index of the volume of the lesion.

6.4.2.3. Electrical and chemical seizure tests. This included the classic MES (50 Hz) and scPtz tests of the NIH [34] as we reported previously [4]. The induction of seizures by low frequency electrical stimulation, using the 6 Hz seizure model, was performed as described by Kaminski et al. [27]. NMDA-induced seizures were induced as previously indicated [7].

6.4.2.4. Minimal acute neurotoxicity. In adult mice was determined by the rotarod procedure in untreated animals and in treated animals at different time points after the tested compound was administered. The mouse was placed on a 1 inch diameter knurled plastic rod rotating at 6 rpm. Normally, unimpaired mice can easily remain on a rod rotating at this speed. Minimal acute neurotoxicity refers to neurological deficit (e.g. ataxia, sedation, hyperexcitability) indicated by the inability of the animal to maintain equilibrium on the rod for at least 1 min, in each of three concurrent trials.

6.4.2.5. Locomotor activity. Mice were transferred individually in an Apelex type 01-1668B actimeter (Bagneux, France). They were allowed to explore for a 2 min period. Their spontaneous locomotor activity was measured for another 3 minutes by the crossing of the photocell activity meter and automatically recorded. The experiment was carried out in a sound proof room between 9:00 and 13:00 to reduce the confounding influence of diurnal variation in motility.

6.4.2.6. Hot plate test. The degree of antinociception induced by **EBAB** (administered i.p. 45 min before testing) was quantified using the hot plate latency test [35]. The animals (OF1 mice) were placed on a hot plate maintained at a temperature 55 ± 1 °C. The latency for the appearance of pain reflexes (vocalization, paw lick, hindpaw flick or jump) in response to the thermal stimulus, were measured and the animals immediately removed from the hot plate, the maximal time of observance being fixed to 30 sec in order to prevent tissue damage to the mouse's paws. To correct for individual differences in base-line latencies, the nociceptive data (latencies) were converted to percentage maximum possible effect (% MPE) using the following formula [17]: % MPE = 100 x [(postdrug latency - predrug latency) divided by (maximum latency -predrug latency)], maximum latencies (100%) being equal to the maximal time of observance, i.e. 30 s.

6.4.2.7. Ethics. These experimental protocols and procedures complied with the European Communities Council Directives of 24 November 1996 (86/609/EEC).

6.4.3. In vitro evaluations

6.4.3.1. Superoxide dismutase-like activity of compounds. refers to their ability to scavenge the superoxide anion radical and was measured as the capacity of compounds to inhibit pyrogallol autooxidation, a process which involves superoxide anion radical as an intermediate. Pyrogallol autooxidation rates were monitored at 420 nm according to the procedure of Marklund and Marklund [36] as described previously [6]. Incubation mixture included saline pH 7.4 TRIS buffer (5 mM TRIS and 50 mM NaCl in final concentrations) with the vehicule DMSO (blank conditions) or with the compound dissolved in this vehicule (test conditions) before the reaction was started by the addition of 0.75 mM pyrogallol.

6.4.3.2. In vitro pharmacology: binding assay. The binding assay using ^{3}H-TCP as a ligand and rat cerebral cortex as a receptor/site source was adapted from the procedure described by Vignon et al. [37] and was performed by Cerep, an independent contract laboratory (Cerep, Bois l'Evêque, France), using 5 nM tritiated ligand and a 60 min incubation at 22 °C, in the absence or in the presence of various concentrations of the selected benzamidine (**EBAB**). The analysis and expression of the binding assay results were as follows. The specific ligand binding to the receptors was defined as the difference between the total binding and the nonspecific binding determined in the presence of an excess of unlabelled ligand. The results were expressed as a percent of control specific binding ((measured specific binding/control specific binding) x 100) obtained in the presence of **EBAB**.

The IC_{50} values (concentration causing a half-maximal inhibition of control specific binding) and Hill coefficients (*nH*) were determined by non-linear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting ($Y = D + [(A - D)/(1 + (C/C_{50})^{nH})]$, where Y = specific binding, D = minimum specific binding, A = maximum specific binding, C = compound concentration, $C_{50} = IC_{50}$, and nH = slope factor). This analysis was performed using a software developed at Cerep (Hill software) and validated by comparison with data generated by the commercial software SigmaPlot® 4.0 for Windows® (© 1997 by SPSS Inc.). The inhibition constants (K_i) were calculated using the Cheng Prusoff equation ($K_i = IC_{50}/(1 + (L/K_D))$, where L = concentration of radioligand in the assay, and K_D = affinity of the radioligand for the receptor).

6.4.4. Molecular modelling studies

Low energy conformations of compounds were determined using the Chem 3D Ultra version 9.0.1 of Cambridgesoft.

6.5. Statistical analysis

Quantitative data were expressed as the means ± S.E.Ms for each treatment group. Means were compared using Student's t test or ANOVA with Dunnett's or NewmaneKeul's multiple comparison of the means test (GraphPad Prism version 3.03 for Windows;

GraphPad Software Inc, San Diego, CA). For hot plate experiments, results were means ± SD calculated using Microsoft Excel®.

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DS and JJVE contributed to the design and chemical synthesis of compounds. PM, NP, PB and JPS contributed to the evaluation of compounds in the various animal seizure tests. PM and NP also performed the measurements relative to the locomotor activity, body temperature and hot plate test. PG investigated the effects of EBAB on a neonatal model of focally induced ibotenate poisoning of immature brain. JV performed biochemical assays. JV and JJVE contributed to the supervision and drafting of the manuscript.

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