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Original article

Anticonvulsant and neurotoxicological properties of 4-amino-*N*-(2-ethylphenyl)benzamide, a potent ameltolide analogue

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Summary – A well documented study on the anticonvulsant properties of 4-amino-*N*-(2-ethylphenyl)benzamide (4-AEPB) is here provided. Initial screening in mice dosed intraperitoneally and rats dosed orally indicated that 4-AEPB is active against maximal electroshock-induced seizures (MES), but does not protect animals against subcutaneous pentylenetetrazole (sc Ptz)-induced seizures. Quantitative evaluation of anti-MES activity and neurotoxicity of 4-AEPB given intraperitoneally to mice provided ED_{50} and TD_{50} values amounting to 28.6 and 96.3 μmol/kg respectively, resulting in a protective index ($PI = TD_{50}/ED_{50}$) equal to 3.36. Further quantitative evaluation in rats dosed orally indicated that the respective ED_{50} and TD_{50} values for 4-AEPB were 29.8 and more than 1,530 μmol/kg, resulting in a very high PI value of over 51. Comparison of anticonvulsant properties and neurotoxicity of 4-AEPB with those previously reported in the literature for two 4-aminobenzamide derivatives, 4-amino-*N*-(2,6-dimethylphenyl)benzamide (or ameltolide, an antiepileptic drug prototype developed by Eli Lilly), and phenytoin, underlines the value of 4-AEPB for future pharmacological development. In this perspective, an additional favorable element is represented by the ability of 4-AEPB to increase the seizure threshold in the intravenous Ptz seizure threshold test in mice dosed intraperitoneally. Molecular modeling studies show that the translocation of one carbon unit in the isomerization of the 2,6-dimethylphenyl moiety of ameltolide to the 2-ethylphenyl counterpart succeeds in maintaining the conformational low energy presentation adopted by ameltolide, providing clues as to why the 4-AEPB here described is an anticonvulsant agent derived from the 4-aminobenzamide pharmacophore platform as potent as ameltolide.

4-aminobenzamide pharmacophore / 4-amino-*N*-(2-ethylphenyl)benzamide / anticonvulsant activity / antiepileptic drug / neurotoxicity / ameltolide / phenytoin

INTRODUCTION

Current research on new antiepileptic drugs has greatly benefited from the studies of Clark et al and those arising from them [4–8, 12]. This research group isolated the 4-aminobenzamide pharmacophore, which subsequently led to the design of a number of new and potent anticonvulsant agents [4–8, 12]. This *N*-phenylbenzamide platform has been shown to represent an important recognition template moiety of the receptor, stabilizing the voltage-dependent sodium channel in its inactivated state [2]. Among the various compounds originating from this approach, two lead structures have emerged, ie (*R,S*)-4-amino-*N*-(α-methylbenzyl)-benzamide (4-AαMBB) [12] and 4-amino-*N*-(2,6-dimethylphenyl)benzamide (4-ADMPB) [4]. The latter was developed by Eli Lilly as an antiepileptic compound under the name of ameltolide (LY201116) [11]. Both compounds received considerable attention because of their strong potency in the MES (maximal electroshock seizure) test.

In another issue of this journal, we recently described the anticonvulsant properties of a series of 4-amino-*N*-phenylphthalimides [1]. In these series, it was noticed that the 2-ethyl substitution at the *N*-phenyl moiety was met with considerable in vivo potency in the MES test [1]. It thus appeared worthwhile to study the effect on the an-

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ticonvulsant potential of the 2-ethyl substituent, also in the 4-aminobenzanilide series. The purpose of this study is therefore to report the anticonvulsant and neurotoxicological properties of 4-amino-*N*-(2-ethylphenyl)benzamide (4-AEPB) in comparison with established anticonvulsant benzamide analogues, ie, 4-amino-*N*-(2,6-dimethylphenyl)benzamide (4-ADMPB) and (*R,S*)-4-amino-*N*-(α-methylbenzyl)benzamide (4-AαMBB), and with the prototype antiepileptic drug phenytoin (fig 1).

MATERIALS AND METHODS

Chemistry

4-Nitro-*N*-(2-ethylphenyl)benzamide was prepared by interaction at room temperature of 4-nitrobenzoyl chloride and 2-ethylaniline in solution in tetrahydrofuran in the presence of triethylamine as catalyst. The resulting intermediate was precipitated by addition of water, filtered, dried and recrystallized from 95% ethanol. Reduction of the nitro group was performed using the couple cyclohexene/palladium on charcoal (10%) in 2-propanol. The resulting 4-amino-*N*-(2-ethylphenyl)benzamide compound was obtained in 57% yield after recrystallization from 95% ethanol (melting point: 165–167 °C, formula = $C_{15}H_{16}N_2O$). Synthesis of the other compounds has been previously described [4, 12].

Pharmacology

Male albino mice (CF-1 strain, 18–25 g) and male albino rats (Sprague–Dawley, 100–150 g) were used as experimental animals. 4-Amino-*N*-(2-ethylphenyl)benzamide was suspended in 0.5% methylcellulose/water mixture and was administered either intraperitoneally to mice or orally to rats.

Maximal electroshock seizures (MES) were elicited by a 60 Hz alternating current of 50 mA (mice) delivered for 0.2 s via corneal electrodes. A drop of 0.9% sodium chloride solution was instilled in each eye prior to application of electrodes. Abolition of the hindlimb tonic extension component of the seizure was defined as protection in the MES test.

The subcutaneous pentylenetetrazole seizure threshold test (sc Ptz) was conducted by administering 85 mg/kg (mice) or 70 mg/kg (rats) of pentylenetetrazole dissolved in 0.9% sodium chloride solution in the posterior midline of the animals. Animals were observed for 30 min following sc Ptz administration. Protection was referred to as an absence of clonic spasms of at least 5 s during the observation period.

The intravenous pentylenetetrazole seizure threshold test (iv Ptz) [13] is a timed intravenous infusion of 0.5% Ptz, 0.9% NaCl and 10 USP units/mL of sodium heparin infused in the tail vein of an unrestrained mouse at a constant rate of 0.37 mL/min. 4-Amino-*N*-(2-ethylphenyl)benzamide was administered in 0.5% methylcellulose/water mixture. This solvent was used as control in the timed infusion of the pentylenetetrazole

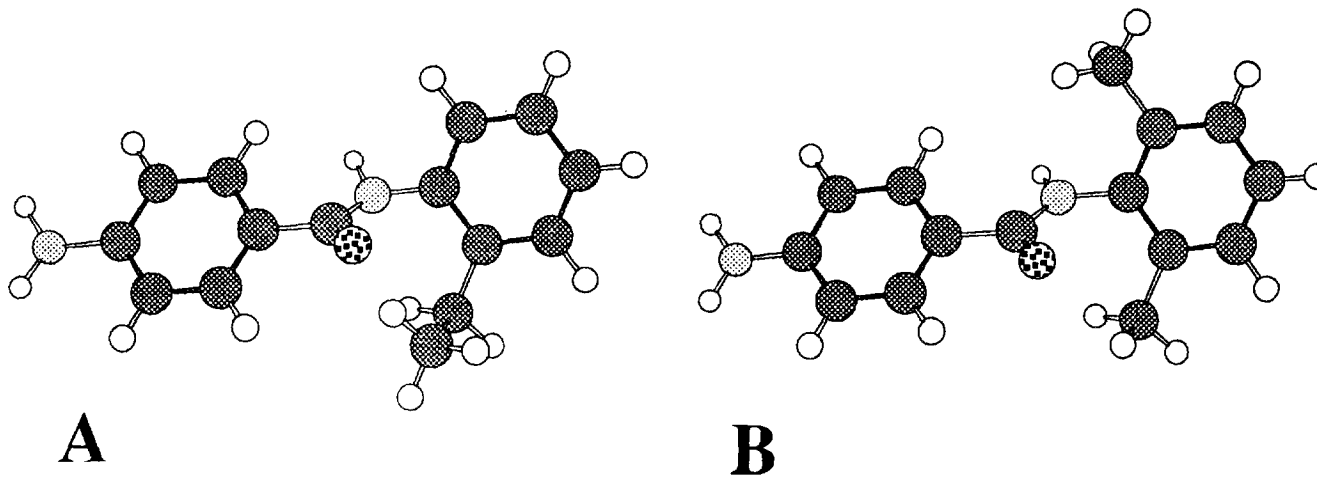

Fig 1. Molecular modeling study of low energy conformers of 4-amino-*N*-(2-ethylphenyl)benzamide (A) and 4-amino-*N*-(2,6-dimethyl)benzamide (B).

test. Groups of ten mice were utilized in this test. The time period in seconds from the start of infusion to the appearance of the first twitch and onset of clonus were recorded in animals for each experimental and control condition. A significant increase from control in the time to first twitch indicated that the tested compound increased the seizure threshold, whereas a decrease in these time periods denoted the ability of test compounds to lower the threshold. The mean time of each control and treated animal was converted to mg/kg of pentylenetetrazole, the mean and standard error (SE) of each group was calculated, and the significance of the difference (P value) determined.

Neurological deficit was measured in mice by the rotorod test, procedure. The mouse was placed on a one-inch diameter knurled plastic rod rotating at 6 rpm. Neurotoxicity was indicated by the inability of the animal to maintain equilibrium on the rod for at least one minute in each of three trials. In rats, neurological deficit was indicated by ataxia, loss of placing response and muscle tone.

The drug dose required to produce the desired endpoint in 50% of animals (ED_{50}) or minimal neurotoxicity in 50% of animals (TD_{50}) and the respective 95% confidence intervals were calculated via a computer program using probit analysis.

Molecular modeling studies

Molecular mechanics calculations were performed using the 3.1.2 version of Chem 3-D Plus (version released on October 22, 1993), this software containing a new implementation of Allingers's MM2 force field.

RESULTS

Anticonvulsant properties and neurotoxicity evaluation of 4-amino-*N*-(2-ethylphenyl)benzamide was conducted by following the Anticonvulsant Drug Development (ADD) Program protocol [9, 14].

Initial screening in mice dosed intraperitoneally revealed that 4-amino-*N*-(2-ethylphenyl)benzamide (4-AEPB) is active against MES at the dose of 10 and 100 mg/kg after 30 minutes and four hours, respectively. In the sc Ptz seizure threshold test, mice dosed 30 minutes previously with 4-AEPB showed continuous seizure activity at 100 mg/kg and died during test without seizure at 300 mg/kg. In the rotorod test, mice dosed with 100 mg/kg of 4-AEPB exhibited neurological deficit after 30 minutes. Moreover, mice with 300 mg/kg of 4-AEPB were anesthetized after 30 minutes and died after four hours.

Initial screening in rats dosed orally with the single dose of 50 mg/kg showed that 4-AEPB is an active anti-MES agent; four rats out of four were protected from 15 minutes to two hours. By using the same dose, rats did not exhibit any neurological deficit from 15 minutes to four hours.

Quantitative evaluation of anticonvulsant potencies and neurotoxicity of 4-AEPB was then performed in mice dosed intraperitoneally. The results of this study and the comparison with literature data on 4-AEPB, 4-AαMBB, and the prototype antiepileptic drug phenytoin are reported in table I. In the 4-aminobenzamide series, 4-

Table I. Quantative anticonvulsant and neurotoxic data in mice dosed intraperitoneally.

Compounds	*TPE[a] (h)*	*TD_{50} (h)*	*ED_{50} (MES)*	*ED_{50} (sc Ptz)*
4-AEPB	1/4, 1/4	96.3 (66.5–122.5)[b]	28.6 (26.4–31)[b] [3.36][c]	> 100[d] [< 0.96]
4-ADMPB	1/2, 1/2	62.4 (55.3–70.3)	10.8 (9.2–12.9) [5.8]	> 83.2[d] [< 0.75]
4-AαMBB	1, 1/2	711.6 (640.9–790.7)	74.9 (55.8–89.1) [9.5]	173.5 (161.5–191.4) [4.1]
Phenytoin	2.2	259.6 (208.1–285.8)	37.7 (32.2–41.2) [6.9]	> 1.190[d] [< 0.22]

TD_{50} and ED_{50} are given in μmoL/kg. [a] TPE: time to peak effect. The first value is for the rotorod test, the second is for the anticonvulsant test. [b] 95% confidence intervals given in parentheses. [c] Protective index, (PI) in brackets; PI = median minimal neurotoxic dose/median effective dose (TD_{50}/ED_{50}) for the anticonvulsant test. [d] No protection up to the dose shown. MES = maximal electroshock seizures; sc Ptz: subcutaneous pentylentetrazole seizure threshold test.

AEPB with an ED_{50} of 28.6 μmoL/kg showed an anti-MES activity intermediate between those reported for 4-ADMPB (ED_{50} = 10.8 μmoL/kg) and 4-Aαmbb (ED_{50} = 74.9 μmoL/kg). With a TD_{50} of 62.4 μmoL/kg, 4-ADMPB was the most toxic of this series; 4-AEPB exhibited the lowest protective index (PI = TD_{50}/ED_{50}), with a value equal to 3.36. With an ED_{50} of 173.5 μmoL/kg and a PI of 4.1, 4-Aαmbb was the only compound active against sc Ptz-induced seizures. Two mice out of eight dosed with 50 μmoL/kg of 4-AEPB died upon the convulsant test following an episode of continuous seizure activity. The anticonvulsant activity profile of 4-AEPB and phenytoin were identical, but 4-AEPB with a TD_{50} of 96.3 μmoL/kg appeared to be relatively more neurotoxic than phenytoin, in which the TD_{50} amounted to 259.6 μmoL/kg.

Further quantitative evaluation of anticonvulsant potencies and neurotoxicity of 4-AEPB in rats dosed orally are reported in table II. The results of this study are compared with literature data on 4-ADMPB, 4-Aαmbb and phenytoin. With an ED_{50} of 29.8 μmoL/kg, in the MES test 4-AEPB was two-fold more potent than 4-Aαmbb (ED_{50} = 67 μmoL/kg) and four-fold more potent than 4-ADMPB and phenytoin (ED_{50} = 135.2 and 118.1 μmoL/kg, respectively). Like phenytoin, the three 4-aminobenzamides were inactive at non-toxic doses against sc Ptz-induced seizures. 4-AEPB and phenytoin presented with relatively low neurotoxicity in rats dosed orally showing a TD_{50} superior to 1,530 and 11,982 μmol/kg, respectively. On the basis of their TD_{50} and anti-MES ED_{50} values, very high PIs were recorded: over 51 and 100 for 4-AEPB and phenytoin, respectively.

In the sc Ptz seizure threshold test we observed that some mice dosed intraperitoneally with 4-AEPB exhibited continuous seizure activity, sometimes leading to death. To check whether or not 4-AEPB could lower the seizure threshold, the effect of its intraperitoneal administration on the threshold for minimal seizures induced by the timed intravenous infusion of pentylenetetrazole in mice was studied (table III). At a dose corresponding to its anti-MES ED_{50} value, 4-AEPB significantly increased the delay before onset of the first twitch ($P < 0.05$) and clonus ($P < 0.01$), and thus raised the threshold for minimal seizures induced by iv Ptz. A similar significant ($P < 0.01$) increase in time before onset of first twitch and clonus was also induced by the TD_{50} for 4-AEPB.

Using a molecular modeling approach, initial models for 4-ADMPB and 4-AEPB were built using Chemdraw and automatic conversion of the 2-D coordinates to 3-D coordinates using Chem 3-D Plus. These initial conformers were energy-minimized, and then submitted to a molecular dynamics study using a target temperature of 310 °K. All other parameters were those already implemented in the software. Conformers were

Table II. Quantitative anticonvulsant and neurotoxic data in rats dosed orally.

Compounds	*TPE[a] (h)*	*TD_{50} (h)*	*ED_{50} (MES)*	*ED_{50} (sc Ptz)*
4-AEPB	1/4 → 24[b], 1/4	> 1.530[c]	29.8 (20.1–43.1)[d] [> 51][e]	> 765.7f
4-ADMPB	2, 1	1.910 (1.546–2.281)	135.2 (121.9–150.2) [14.1]	> 2.081[f] [< 0.92]
4-Aαmbb	2, 1	707.9 (541–857.3)	67 (62.8–72.8) [10.6]	> 1.248.4[g] [< 0.57]
Phenytoin	1/2, 4	> 11.892[c]	118.1 (86.8–154.2) [> 100]	> 3.170[f]

Abbreviations are as in table I. TD_{50} and ED_{50} are given in μmol/kg.
[a] TPE: time to peak effect. The first value is for neurotoxicity; the second is for the anticonvulsant test. [b] In the neurotoxicity assay, all doses were tested at 1/4 hour through 24 hours. [c] No ataxia up to the dose shown. [d] 95% confidence interval, in parentheses. [e] Protective index (PI) in square brackets. [f] No protection up to the dose shown. [g] Maximal protection of 50% at the dose shown.

Table III. Effects of intraperitoneally administered 4-AEPB on the threshold for minimal seizures induced by the timed intraveinous infusion of pentylenetetrazole in mice.

	First twitch		*Clonus*	
Dose ip (mg/kg)	*Time (s)*	*Ptz (mg/kg ± SE)*	*Time (s)*	*Ptz (mg/kg ± SE)*
Solv control	30.6	30.6 ± 0.8	34.3	34.3 ± 0.9
ED_{50} (MES)	34.0	34.3 ± 1.1[a]	39.8	40.2 ± 1.4[b]
TD_{50}	34.0	34.3 ± 0.9[b]	42.4	42.7 ± 1.0[b]

The test was performed 15 min after ip administration of 4-AEPB. [a] Significantly different from solvent control; $P < 0.05$. [b] Significantly different from solvent control; $P < 0.01$.

sampled every two pseconds. Each conformer was then energy-minimized. The conformer exhibiting the lowest steric energy was then resubmitted for a longer molecular dynamics study (150 pseconds). Conformers were again sampled every one psecond and energy-minimized. The conformer showing the lowest steric energy was chosen. The two molecular models shown in figure 2 are the result of these calculations. The dihedral angle formed by the planes of 4-aminophenyl and 2,6-dimethylphenyl groups was 124° for 4-ADMPB. The corresponding 2-ethyl analogue (4-AEPB) was characterized by a rather similar dihedral angle, ie 128° between the 4-aminophenyl and ethylphenyl planes. So the translocation of one carbon unit in the isomerization of the 2,6-dimethylphenyl moiety to the 2-ethylphenyl counterpart did not induce any significant conformational changes.

DISCUSSION AND CONCLUSION

4-Amino-*N*-(2,6-dimethylphenyl)benzamide was originally selected by Eli Lilly for clinical trials under the name of ameltolide (LY201116) [10, 11]. This compound emerged from a series of aminobenzamides originally developed by Clark [12]. These works established the paradigm that a 4-amino group along with a 2′,6′-disubstitution by alkyl groups provided optimal anticonvulsant properties.

Our initial work on the phthalimide compounds [1, 3] which can be regarded as rigidified analogs of the 4-aminobenzamide pharmacophore prompted us to revisit that paradigm in the 4-aminobenzanilide series. While the 2′-ethyl-subs-

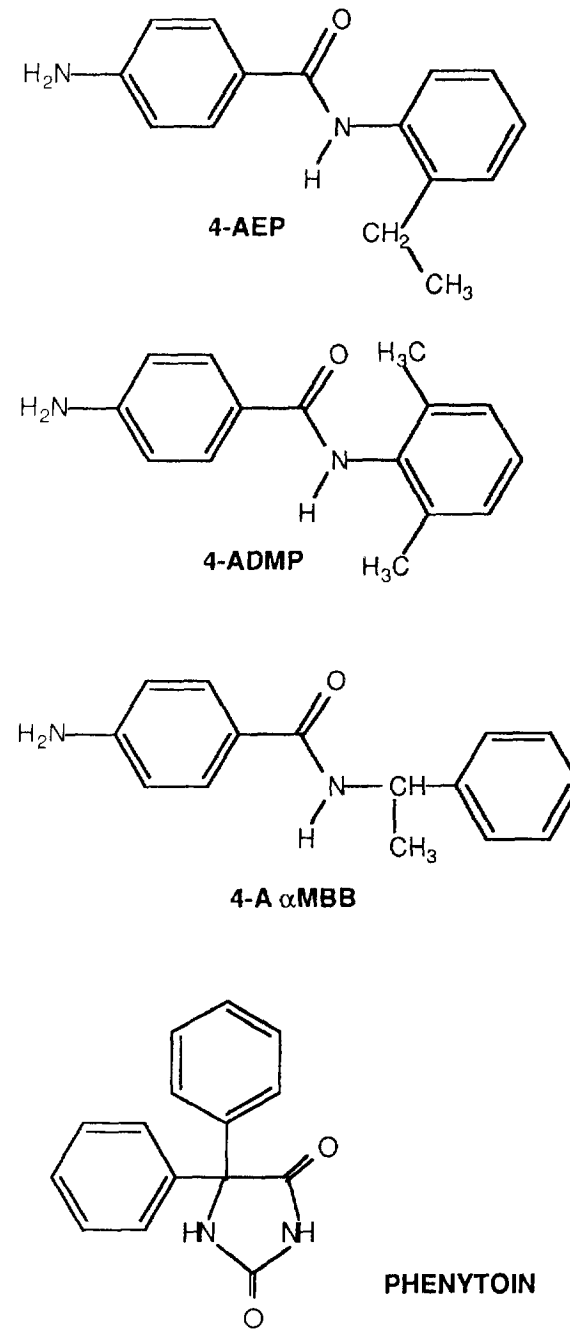

Fig 2. Chemical structures of 4-amino-*N*-(2-ethylphenyl)benzamide (4-AEPB); 4-amino-*N*-(2,6-dimethyl)benzamide (4-ADMPB); (*R,S*)-4-amino-*N*-(α-methylbenzyl)benzamide (4-AαMBB) and phenytoin.

tituted compounds either in the 4-aminobenzanilide (table I) and 4-aminophthalimide (data not shown) series were not superior to ameltolide in terms of efficacy and PI in mice after intraperitoneal administration, 4-AEPB reached a higher level of efficacy and security than ameltolide when administered per os to rats (table II). In the latter case, 4-AEPB was by far superior to phenytoin (table II).

In view of the present results, the paradigm based on the superiority of the 2′,6′-disubstitution does not appear to be definitively established. Conclusively, these data indicate that in the search for new antiepileptic drugs, 4-aminobenzamide as well as 4-aminophthalimide are very promising platforms. Substitutions other than the combined 2′,6′-positions should not be overlooked, and exploration of such alternative issues should be systematically pursued.

In this perspective, molecular modeling studies might represent a preliminary helpful step in revealing similarities in structural conformations of studied compounds, as we have pointed out in the case of 4-ADMPB and 4-AEPB.

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