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Preliminary communication

Synthesis and anticonvulsant activity of some 4-nitro-*N*-phenylbenzamides

V Bailleux[1], L Vallée[1], JP Nuyts[1], G Hamoir[2], JH Poupaert[2], JP Stables[3], J Vamecq[1]*

[1]*North France Center for the Study of Childhood Epilepsy, Service du Professeur Jean-Pierre Nuyts, Centre Hospitalier & Universitaire de Lille, Hôpital B, 59037 Lille Cedex, France;*
[2]*Department of Medicinal Chemistry, School of Pharmacy, University of Louvain, B-1200 Brussels, Belgium;*
[3]*Preclinical Pharmacological Section, Epilepsy Branch, DCDND, NINDS, National Institutes of Health, Bethesda, MD 20892-9020, USA*

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Summary — A short series of 4-nitro-*N*-phenylbenzamides was synthesized and evaluated for anticonvulsant properties and neurotoxicity. In mice dosed intraperitoneally, three of the four 4-nitro-*N*-phenylbenzamides were efficient in the maximal electroshock-induced seizure (MES) test, especially *N*-(2,6-dimethylphenyl)-4-nitrobenzamide (ED_{50} value in the MES test = 31.8 μmol/kg, TD_{50} = 166.9 μmol/kg, protective index [PI] = 5.2) and *N*-(2-chloro-6-methylphenyl)-4-nitrobenzamide (ED_{50} value in the MES test = 90.3 μmol/kg, TD_{50} = 1.068 μmol/kg, PI = 11.8). The latter 4-nitro-*N*-phenylbenzamide was also found to be active against seizures induced by subcutaneous pentylenetetrazole (sc Ptz) and was selected for further evaluation in rats dosed orally. In these conditions, *N*-(2-chloro-6-methylphenyl)-4-nitrobenzamide was found to be, in the MES test, three times more active than phenytoin and 4-amino-*N*-(2,6-dimethylphenyl)benzamide, two potent anti-MES agents.

4-nitro-*N*-phenylbenzamide / electroshock / pentylenetetrazole / seizure / neurotoxicity

Introduction

During the last decade, consistent advances in the design of novel anticonvulsant agents have been obtained through the work of Clark and his colleagues. Several families of molecules have been tested, including the 4-aminobenzamide [1], 4-aminobenzanilide (*ie* 4-amino-*N*-phenylbenzamide) [2], 2- and 3-aminobenzanilide [3] and 4-amino-*N*-(1-phenylethyl)benzamide [4] series. From these experiments, the 4-amino-*N*-(2,6-dimethylphenyl)benzamide (4-ADMPB) (fig 1) was selected as a highly efficient anticonvulsant agent, offering protection against maximal electroshock-induced seizures (MES). After oral administration to rats, this compound undergoes *N*-acetylation with a short half-life (15 min) [5]. Considering the metabolic disposition of 4-aminobenzamide compounds, the use of 4-nitrobenzamides appears to be potentialy interesting, even if these compounds were previously judged by Clark's group to be essentially inactive in anticonvulsant tests [2]. Reevaluating the anticonvulsant activity of some 4-nitro-*N*-phenylbenzamides, we have found that in the MES test they are less active than 4-ADMPB in mice dosed intraperitoneally, partially confirming the view of Clark and his colleagues. Nevertheless, and interestingly enough, one of the 4-nitro-*N*-phenylbenzamides tested, *N*-(2-chloro-6-methylphenyl)-4-nitrobenzamide, is several times more active in the MES test than phenytoin and 4-ADMPB in rats dosed orally. This observation underlines that 4-nitro-*N*-phenylbenzamide derivatives are not only intermediates in chemical synthesis of the anticonvulsant 4-amino-*N*-phenylbenzamides but may also be anticonvulsant agents themselves, a property which might have previously been overlooked.

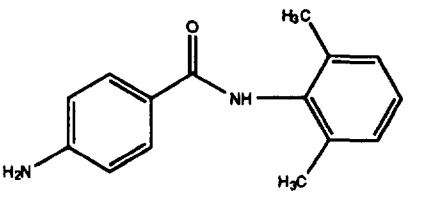
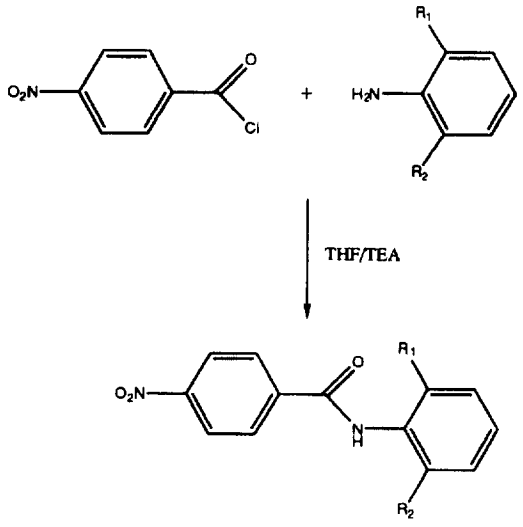


Fig 1. Chemical structure of 4-amino-*N*-(2,6-dimethylphenyl)-benzamide (4-ADMPB, LY201116).

*Correspondence and reprints

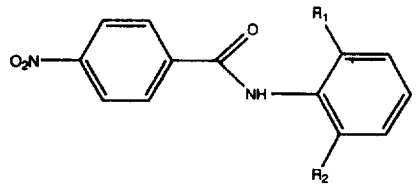


Scheme 1. Synthesis of the 4-nitro-*N*-phenylbenzamides. R_1 = F, CH_3, C_2H_5 and R_2 = F, CH_3, Cl, C_2H_5.

Chemistry

A short series of 4-nitro-*N*-phenylbenzamides (namely, *N*-(2,6-difluorophenyl)-4-nitrobenzamide **1**, *N*-(2,6-dimethylphenyl)-4-nitrobenzamide **2**, *N*-(2-chloro-6-methylphenyl)-4-nitrobenzamide **3** and *N*-(2,6-diethylphenyl)-4-nitrobenzamide **4** was synthesized according to the method outlined in scheme 1. These compounds were prepared by reaction of 4-nitrobenzoyl chloride with the required aniline derivatives in solution in a mixture of tetrahydrofuran and triethylamine. These four derivatives of 4-nitro-*N*-phenylbenzamide are presented in table I, whereas table II reports the NMR data obtained for these compounds.

Table I. Derivatives of 4-nitro-*N*-phenylbenzamide[a].



Compound	R_1	R_2	*Mp (°C)*	*Yield (%)*	*Formula*
1	F	F	229–232	56	$C_{13}H_8F_2N_2O_3$
2	CH_3	CH_3	194–197	55	$C_{15}H_{14}N_2O_3$
3	CH_3	Cl	177–179	50	$C_{14}H_{11}ClN_2O_3$
4	C_2H_5	C_2H_5	249–252	59	$C_{17}H_{18}N_2O_3$

[a]The infrared and nuclear magnetic resonance (^1H) spectra were consistent with structural assignments.

Table II. Physicochemical properties of 4-nitro-*N*-phenylbenzamide derivatives[a].

Compound	*δ ^1H-NMR*	*δ ^{13}C-NMR*
1	7.23–8.45 (m, 7H, H_{arom}), 10.61 (s, 1H, -NH)	111.75–159.63 (C_{arom}), 163.83 (C=O)
2	2.22 (s, 3H, $-CH_3$), 7.12–8.41 (m, 7H, H_{arom}), 10.15 (s, 1H, -NH)	18.29 ($-CH_3$), 123.72–149.62 (C_{arom}), 164.29 (C=O)
3	1.95 (s, 3H, $-CH_3$), 6.95–8.11 (m, 7H, H_{arom}), 10.11 (s, 1H, -NH)	23.23 ($-CH_3$), 128.80–154.41 (C_{arom}), 168.73 (C=O)
4	1.14 (t, 3H, $-CH_2-CH_3$), 2.58 (q, 2H, $-CH_2-CH_3$), 7.1–8.41 (m, 7H, H_{arom}), 10.14 (s, 1H, -NH)	14.44 ($-CH_2-CH_3$), 24.40 ($-CH_2-CH_3$), 123.69–149.18 (C_{arom}), 164.24 (C=O)

[a]Analyses (CHN) for compounds **1–4** were within ±0.4% of theoretical values.

Pharmacology

Initial anticonvulsant evaluation of the 4-nitro-*N*-phenylbenzamide derivatives in mice and rats was conducted by following the Anticonvulsant Drug Development (ADD) program protocol [6, 7]. The profile of anticonvulsant activity was established by one electrical and two chemical tests. The electrical test employed was the MES pattern test. The chemical tests employed were the subcutaneous pentylenetetrazole (sc Ptz) seizure threshold test and the intravenous pentylenetetrazole (iv Ptz) seizure threshold test.

Results

Except for compound **I**, all the 4-nitro-*N*-phenylbenzamides screened had anticonvulsant properties. More complete data were obtained from quantitative evalu-

ation in mice dosed intraperitoneally. Results of this quantitative test, along with literature data on 4-ADMPB [8] and phenytoin [7], are reported in table III. In the 4-nitro-*N*-phenylbenzamide series, **2** was the most active compound against MES, with an ED_{50} of 31.8 μmol/kg, and also the most neurotoxic, with a TD_{50} of 166.9 μmol/kg. Compound **3** gave an ED_{50} in the MES test and a TD_{50} of 90.3 and 1068 μmol/kg, respectively, resulting in a protective index (PI) of 11.8. This compound displayed an ED_{50} value of 598 μmol/kg in the sc Ptz test, yielding a PI of 1.79. With an ED_{50} of 233.4 μmol/kg in the MES test and a TD_{50} greater than 1676 μmol/kg, compound **4** was less active and toxic than **2** and **3**.

In the MES test, 4-ADMPB is the most active of the compounds presented in table III with an ED_{50} of 10.8 μmol/kg. Compound **2** is as active as phenytoin which exhibits an ED_{50} of 37.7 μmol/kg. Although, compound **3** is three times less potent than **2**, it has the highest PI in the MES test, exceeding that of 4-ADMPB and phenytoin. At non-toxic doses, **3** is the only compound active against sc Ptz-induced seizures.

From the results obtained in mice, **3** was selected for quantitative evaluation of anticonvulsant activities and neurotoxicity in rats dosed orally. These data are presented in table IV and compared with literature data on 4-ADMPB [8] and phenytoin [7]. With an ED_{50} of 39.8 μmol/kg, compound **3** is three times more potent than 4-ADMPB or phenytoin in the MES test. The TD_{50} and the PI (MES) of **3** were over 1720 μmol/kg and 43.2, respectively. These characteristics compare rather favorably with those obtained for 4-ADMPB (PI = 14.1 in the MES test); phenytoin exhibits a PI > 100. Compound **3** was totally inactive against sc Ptz-induced seizures at the dose of 860 μmol/kg.

The effects of intraperitoneal administration of **3** and phenytoin to mice on the threshold for minimal seizures induced by the timed intravenous infusion of Ptz are detailed in table V (for a general consideration of the iv Ptz test, see reference [9]). Two doses approximating the ED_{50} in the MES test and TD_{50} were tested. Compound **3** did not significantly ($p > 0.05$) modify the threshold for minimal seizures

Table III. Quantitative anticonvulsant activity and neurotoxicity in mice dosed intraperitoneally.

Compound	*TPE*[a] *(h)*	*TD_{50}*[b]	*ED_{50} MES*[c]	*ED_{50} sc Ptz*
2	1/4, 1/4	166.9 (127.4–219.8)	31.8 (25.1–39.6) [5.25]	> 185[d] [< 0.9]
3	2, 1	1068 (725–1502)	90.3 (59.4–125.5) [11.8]	598 (390.2–927.4) [1.79]
4	2, 1	> 1676[e]	233.4 (177.5–347.1) [> 7.2]	> 1341[f]
4-ADMPB[g]	1/2, 1/2	62.4 (55.3–70.3)	10.8 (9.2–12.9) [5.8]	> 83.2[d] [< 0.75]
Phenytoin[h]	2, 2	259.6 (208.1–285.8)	37.7 (32.2–41.2) [6.9]	> 1190[d] [< 0.22]

[a]Time to peak effect. The first value is for the rotorod test; the second is for the anticonvulsant tests. [b]TD_{50} (per kg body weight, μmol/kg) dose eliciting evidence of minimal neurological toxicity in 50% of animals; 95% confidence interval, in parentheses. [c]ED_{50} (per kg body weight, μmol/kg) PI, in brackets; PI = median minimal neurotoxic dose/median effective dose (TD_{50}/ED_{50}) for anticonvulsant test. [d]No protection up to the dose shown. [e]Two mice in eight exhibited neurotoxicity at 1676 μmol/kg. [f]Four mice in eight protected at 1341 μmol/kg. [g]Data from reference [8]. [h]Data from reference [7].

Table IV. Quantitative anticonvulsant activity and neurotoxicity in rats dosed orally.

Compound	*TPE*[a] *(h)*	*TD_{50}*	*ED_{50} MES*[d,e]	*ED_{50} sc Ptz*[f]
3	1/4 → 24, 2[b]	> 1720[c]	39.8 (27.1–54) [> 43.2]	> 860
4-ADMBP[g]	2, 1	1910 (1546–2281)	135.2 (121.9–150.2) [14.1]	> 2081 [< 0.92]
Phenytoin[h]	1/2, 4	> 11892	118.1 (86.8–154.2) [> 100]	> 3170

Abbreviations are as in table III; TD_{50} and ED_{50} are given in micromoles per kilogram of body weight (μmol/kg). [a]Time to peak effect. The first value is for the neurotoxicity test; the second is for the anticonvulsant tests. [b]In the neurotoxicity assay, all doses were tested at 1/4 h through 24 h. [c]No ataxia up to the dose shown. [d]95% confidence interval in parentheses. [e]PI in brackets. [f]No protection up to the dose shown. [g]Data from reference [8]. [h]Data from reference [7].

Table V. Effect of intraperitoneally administered **3** and phenytoin on the threshold for minimal seizures induced by the timed iv infusion of Ptz in mice.

Compound	*Intraperitoneal dose (μmol/kg)*	*Approximate equivalent*[a]	*Duration of test (h)*	*Ptz (mg/kg ± se)*	
				First twitch	*Clonus*
3	0[b]	–	1	32.8 ± 0.9	39.9 ± 1.1
	89.4	MES ED_{50}	1	32.6 ± 0.8	39.0 ± 1.9
	1066	TD_{50}	1	36.5 ± 1.6	44.8 ± 0.8[c]
Phenytoin	0[b]	–	2	33.6 ± 0.6	42.6 ± 1.5
	25.8	MES ED_{50}	2	35.5 ± 1.6	42.5 ± 2.1
	170.5	TD_{50}	2	28.2 ± 1.9[d,e]	50.1 ± 3.1[d,e]

[a]Approximate equivalent of the dose of tested compound. [b]Solvent control. [c]Significantly different from solvent control, $p < 0.01$. [d]Significantly different from solvent control, $p < 0.05$. [e]None of the animals exhibited true first twitch or clonus; onset of spasticity was taken as the endpoint for first twitch and onset of continuous seizure activity the endpoint for clonus. All animals had continuous seizures and one died. All the specific pharmacological tests whose results are included in this table were conducted by the Epilepsy Branch of the NIH (USA).

induced by iv Ptz, except for an increased delay before onset of clonus at the dose corresponding to its TD_{50} ($p < 0.01$). With 170.5 μmol/kg of phenytoin, neither true first twitch nor clonus really occurred, but the time before onset of spasticity and the time before appearance of continuous seizure activity were both increased. All animals dosed with the approximate TD_{50} of phenytoin exhibited continuous seizure activity.

Preliminary experiments were conducted on **3** and 4-ADMPB in order to check their ability to inhibit the binding of tritiated batrachotoxin B to neuronal sodium channels. At a concentration of 500 μM, **3** and 4-ADMPB provided inhibitions of 65 and 83%, respectively, while at a 250 μM concentration, inhibitions of 44 and 58%, respectively, were recorded. These results indicate that compound **3** is an efficient modifier at the sodium channel.

Discussion

Anticonvulsant properties of 4-nitro-*N*-phenylbenzamides are evidenced through the present work. In mice dosed intraperitoneally, compounds **2–4** mainly display anticonvulsant activity against MES. The protection in the MES test is related to the nature of the groups recovered in positions 2 and 6 of the *N*-phenyl ring of 4-nitro-*N*-phenylbenzamides in the order 2,6-dimethyl > 2-chloro-6-methyl > 2,6-diethyl > 2,6-difluoro. The anti-MES properties of 4-nitro-*N*-phenylbenzamides indicate that these compounds inhibit spread of the seizure. As is the case for 4-amino-*N*-phenylbenzamide derivatives [2], the 2,6-dimethyl substitution of the *N*-phenyl ring appears to confer optimal anticonvulsant properties to the 4-nitro-*N*-phenylbenzamide series towards MES in mice dosed intraperitoneally. Compound **2** has this substitution pattern and corresponds to the 4-nitro version of 4-ADMPB. It is possible that in previous studies the anticonvulsant properties of **2** were not considered because of the highly potent anti-MES activity of its chemically derived 4-ADMPB in mice.

Compounds **3** and **4** are original molecules whose anticonvulsant properties in the MES test have not been previously described. Interestingly enough, compound **3** is also active in the sc Ptz test at least in mice. Whether this activity could be related to an increase of the seizure threshold has been directly evaluated by checking the effect of intraperitoneal administration of **3** on the threshold for minimal seizures induced by the timed infusion of Ptz in mice. At the ED_{50} dose value obtained in the MES test, **3** does not significantly modify the seizure threshold (see table V).

At this stage, it is unknown whether 4-nitro-*N*-phenylbenzamides are active by themselves or through reduction to the corresponding 4-amino-*N*-phenylbenzamides, compounds whose anticonvulsant properties were extensively documented in the past. Indeed, metabolic reduction of the 4-nitro group to a 4-amino group remains a plausible event. The hypothesis that **2** is a prodrug for 4-ADMPB may not be ruled out at this stage. Future appropriate metabolic studies on the nitro derivatives will be helpful in elucidating this aspect.

Compound **3**, like phenytoin, is a potent anticonvulsant, mainly active against MES. Furthermore, several drug-binding, ion-flux and electrophysiological studies have demonstrated that the antiepileptic phenytoin produces a voltage-dependent block of sodium channels in mammalian neurons at therapeutically relevant concentrations [10–14]. As evidenced by preliminary experiments on neuronal sodium channels, this appears to be the case for **3**, supporting the view that this 4-nitro-*N*-phenylbenzamide derivative has an intrinsic anticonvulsant potential. At this stage, it is unknown whether **3** may serve as a prodrug for the corresponding 4-amino-*N*-phenylbenzamide, since the *in vivo* reduction of the former to the latter remains to be elucidated.

Experimental protocols

Chemistry

Melting points were determined using an Electrothermal melting point apparatus with open glass capillaries. ^{1}H- and ^{13}C-NMR spectra were measured in $CDCl_3$ or DMSO-d_6 on a Bruker AC-300 spectrometer with tetramethylsilane as an internal standard. HPLC chromatograms were performed with a Spectra Physics chromatograph (Spectra system P2000), fitted with an RP-18 (5 μm) column. The elution solvent was methanol/water mixture (65:35, v/v) with a flow rate of 1 ml/min. The detection wavelength was 260 nm. In these conditions, all compounds were found to be chromatographically homogeneous. 4-Nitrobenzoyl chloride, 2,6-difluoroaniline, 2,6-dimethylaniline, 2-chloro-6-methylaniline and 2,6-diethylaniline were products of Aldrich Chemie.

4-Nitro-N-phenylbenzamides

In a 250 ml round-bottomed flask equipped with a magnetic stirrer, an addition funnel and an ice bath, 10 g of the required aniline were dissolved in 50 ml tetrahydrofuran and 10 ml triethylamine. A solution of 4-nitrobenzoyl chloride (1.5-fold molar excess) in 50 ml tetrahydrofuran was added dropwise. The reaction mixture was then allowed to warm to room temperature and left for 5 h under magnetic stirring. The solution was diluted with 1 l of 1 N hydrochloric acid. A precipitate appeared and was filtered through a Büchner funnel and washed twice with 1 l of water. The crude 4-nitro-*N*-phenylbenzamide was recrystallized from 95% ethanol.

Pharmacology

Male albino mice (CF-1 strain, 18–25 g) and male albino rats (Sprague–Dawley, 100–150 g) were used in the experiment. The 4-nitro-*N*-phenylbenzamides were suspended in 0.5% methylcellulose/water mixture and they were administered either intraperitoneally to mice or orally to rats.

MES were elicited by a 60 Hz alternating current of 50 mA (mice) or 150 mA (rats) 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 sc Ptz seizure threshold test was conducted by administering 85 mg/kg (mice) or 70 mg/kg (rats) Ptz dissolved in 0.9% sodium chloride solution in the posterior midline of the animals. A minimal time 30 min subsequent to sc administration of Ptz was used for seizure detection. Protection was referred to as the failure to observe an episode of clonic spasms of at least 5 s duration during this time period.

The iv Ptz seizure threshold test is a timed iv infusion of 0.5% Ptz, 0.9% sodium chloride, 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-Nitro-*N*-(2-chloro-6-methylphenyl)benzamide and phenytoin were administered in a 0.5% methylcellulose/water mixture or 0.9% sodium chloride solution, respectively. These solvents were used as controls in the timed infusion of the Ptz test. Groups of ten mice were used in this test. The time periods 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 set of conditions. A significant increase from control in the time to first twitch or clonus indicates that the tested compound increases seizure threshold, whereas a decrease of these time periods results from the ability of tested compounds to lower the threshold. The mean time of each control and treated animal was converted to mg/kg of Ptz. 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. The mouse was placed on a 1 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 1 min in each of three trials. In rats, neurological deficit was indicated by ataxia and loss of placing response and muscle tone.

The doses of drug 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 by means of a computer program using probit analysis. Concentrations of 4-nitro-*N*-phenylbenzamides are expressed in the text as μmol/kg; 1 μmol of compounds **2**, **3** and **4** corresponds to 0.270, 0.291 and 0.298 mg/kg of these compounds, respectively.

Binding of tritiated batrachotoxin B to neuronal sodium channels was measured as described elsewhere [14, 15].

Acknowledgments

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