Articles

Anticonvulsant Activity and Interactions with Neuronal Voltage-Dependent Sodium Channel of Analogues of Ameltolide

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Fifteen compounds related to ameltolide (LY 201116) were studied for (i) anticonvulsant potential in the maximal electroshock-induced seizures (MES) and the subcutaneous pentylenetetrazol (sc Ptz) tests in mice and rats and (ii) interactions with neuronal voltage-dependent sodium channels. Compounds were chosen ranging in anticonvulsant activity in mice from very active to inactive. The active compounds were defined as those protecting 50% of the animals at doses between 10 and 50 μmol/kg and inactive compounds as those protecting 50% of the animals at doses greater than 1 mmol/kg. The series studied included three *N*-(2,6-dimethylphenyl)benzamides (compounds **1**, **2** (ameltolide), and **3**), three *N*-(2,2,6,6-tetramethyl)-piperidinyl-4-benzamides (compounds **4**, **5**, **6**), one phenylthiourea (compound **7**), five *N*-(2,6-dimethylphenyl)phthalimides (compounds **8**, **9**, **10**, **13**, and **14**), two *N*-phenylphthalimide derivatives (compounds **11** and **12**), and one *N*-(2,2,6,6-tetramethyl)piperidinyl-4-phthalimide (compound **15**). Phenytoin (PHT) was employed as the reference prototype antiepileptic drug. After inital screening in mice, compounds **1**, **2**, **3**, **5**, **8**, **9**, **10**, **13**, and **14** were selected for further testing in rats. Anticonvulsant ED_{50}s (effective doses in at least 50% of animals tested) of compounds in the MES test were determined in rats dosed orally and amounted to 52 (**1**), 135 (**2**), 284 (**3**), 231 (**8**), 131 (**9**), 25 (**10**), 369 (**13**), 354 (**14**), and 121 (PHT) μmol/kg, compound **5** presenting with an ED_{50} value higher than 650 μmol/kg. In our hands, the apparent IC_{50}s (inhibitory concentrations 50) of compounds toward binding to rat brain synaptosomes of [^{3}H]batrachotoxinin-A-20α-benzoate were 0.25 (**1**), 0.97 (**2**), 0.35 (**3**), 25.8 (**5**), 161.3 (**8**), 183.5 (**9**), 0.11 (**10**), 1.86 (**13**), 47.8 (**14**), and 0.86 (PHT) μM. The relationship between the activity in the MES test and the capacity to interact in vitro with neuronal voltage-dependent sodium channels and the fact that the IC_{50} values obtained in the in vitro test are close to the brain concentrations at which anticonvulsant activities are reported to occur for ameltolide strongly suggest that the anticonvulsant properties of most compounds tested could be a direct result of their interaction with the neuronal voltage-dependent sodium channel.

Introduction

More than half of a century has elapsed since the anticonvulsant properties of phenytoin were first evidenced in laboratory animal models[1] with successful therapeutic administration in epileptic patients.[2,3] Phenytoin is still one of the most commonly used antiepileptic drugs (AEDs). Today, in addition to phenytoin, several AEDs are widely utilized in the treatment of the various forms of epilepsy.[3,4] Some of the other standard drugs include carbamazepine, phenobarbital, ethosuximide, valproic acid, and various benzodiazepines. In addition, several newer agents γ-vinyl-GABA,[4] felbamate,[5] gabapentin[6], lamotrigine,[7] and, most recently, topiramate,[8] are also employed. Further, a number of AEDs (i.e., tiagabine,[8] losigamone,[9] remacemide,[10] D-23129,[11] and oxcarbazepine[8]) are now in human clinical experimentation. In recent years, the field of AED development is quite dynamic, affording many promising research opportunities.

Our work is based on the drug ameltolide, a potent AED previously investigated by Eli Lilly, having originally emerged from the laboratories of Clark and coworkers.[12−18] This group of investigators isolated the promising 4-aminobenzamide pharmacophore which subsequently led to the fruitful design of several new and potent anticonvulsant compounds. Based on potency−structure relationships, we designed analogues related to ameltolide and phenytoin. This resulted in providing a family of *N*-phenylphthalimide compounds.[19−22] Preliminary studies pointed out that the

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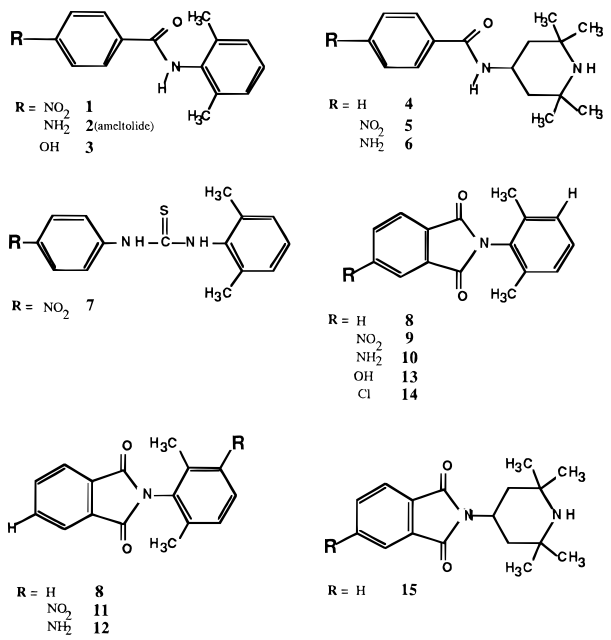

Figure 1. Compounds **1**−**15**.

N-phenylphthalimides exhibit anticonvulsant activity[16−19] similar to the profile of phenytoin, a prototype AED shown to interact with neuronal voltage-dependent sodium channels.[23,24]

In this paper, we report the anticonvulsant and neurotoxic properties of 15 compounds structurally related to ameltolide and their ability to interact with the voltage-dependent sodium channel from rat brain synaptosomal fractions as evidenced by inhibition of [^{3}H]batrachotoxinin-A-20α-benzoate binding. Compounds could not be chosen on the basis of SAR that would be discovered afterward as a result of [^{3}H]batrachotoxinin-A-20α-benzoate binding test data. Compounds were designed and synthesized after classical medicinal chemistry practices and were chosen on the basis of their level of anticonvulsant activity ranging from very active to moderately active and to inactive, i.e., with activity in the MES tests at 30, 100, and 300 mg/kg, respectively. We investigated whether a correlation could be obtained between interactions with the neuronal voltage-dependent sodium channel and activities in the maximal electroshock-induced seizures (MES) test. The view that the in vivo anticonvulsant activity of ameltolide (and several related compounds) is largely mediated by its interaction with the neuronal voltage-dependent sodium channel is supported by the present experimentation.

Chemistry

The structures of 15 compounds related to ameltolide (compound **2**) are shown in Figure 1. The series includes three *N*-(2,6-dimethylphenyl)benzamides (compounds **1**, **2**, and **3**), three *N*-(2,2,6,6-tetramethyl)-piperidinyl-4-benzamides (compounds **4**, **5**, and **6**), one phenylthiourea (compound **7**), five *N*-(2,6-dimethylphenyl)phthalimides (compounds **8**, **9**, **10**, **13**, and **14**), as well as two other *N*-phenylphthalimide derivatives (compounds **11** and **12**), and one *N*-(2,2,6,6-tetramethyl)-piperidinyl-4-phthalimide (compound **15**). Compounds were synthesized by standard methods. They included very active, moderately active, and inactive (protection offered in the MES test as defined above) anticonvulsant molecules. This large range of MES anticonvulsant activity was chosen with the view that the collection of a wide range of responses in the synaptosomal votage-dependent sodium channel binding test would aid in reaching conclusive SAR evaluations.

Table 1. Anticonvulsant and Neurotoxicity Screening Data in Mice Dosed Intraperitoneally with Ameltolide and Related Compounds[a]

	MES test		sc Ptz test		toxicity	
compd	30 min	4 h	30 min	4 h	30 min	4 h
1	+++	++	−	−	+	−
2	+++	++	−	−	−	−
3	+++	++	+++	−	−	−
4	−	−	−	−	−	−
5	+	+++	−	−	+	+
6	−	−	−	−	−	−
7	−	+++	−	−	−	−
8	++	−	−	−	−	−
9	++	++	−	+	−	+
10	+++	++	+++	−	++	++
11	−	−	−	−	−	−
12	−	+	−	−	−	−
13	++	++	−	−	−	−
14	++	++	−	−	+	−
15	−	−	−	−	−	−

[a] The anticonvulsant (MES and sc Ptz tests) and neurotoxicity activities were determined 30 min and 4 h after the administration of compounds. The symbols +++, ++, and + signify activity at 30, 100, and 300 mg/kg, respectively; − denotes no activity observed at 300 mg/kg. Toxicity was determined by the rotorod test. Abbreviations: MES, maximal electroshock-induced seizures; sc Ptz, subcutaneous pentylenetetrazol.

Results

Anticonvulsant and Neurotoxicity Screening Data in Mice Dosed Intraperitoneally (ip). Initial ip screening for anticonvulsant activity and toxicity in mice is documented in Table 1. Compounds **1**, **2**, and **3** showed activity in the MES test at 30 mg/kg. Compound **3** further exhibited activity in the subcutaneous pentylenetetrazol (sc Ptz) seizure test. Compounds **2** and **3** did not present with toxicity, whereas compound **1** was toxic at 300 mg/kg. Compounds **4** and **6** were devoid of both anticonvulsant activity and neurotoxicity. Compound **5** was active at 30 mg/kg in the MES but not in the sc Ptz seizure test, showing toxicity, however, at 300 mg/kg. Compound **7** was also active in the MES test without displaying activity in the sc Ptz seizure test or toxicity at the doses studied. Compound **8** was active only in the MES test at 100 mg/kg and did not exhibit toxicity at 300 mg/kg. Compounds **9** and **10** were active in the MES (at 100 and 30 mg/kg, respectively) and in the sc Ptz test (at 300 and 30 mg/kg, respectively), and exhibited toxicity at 300 and 100 mg/kg, respectively. Compounds **11** and **12** were inactive in the sc Ptz test and not toxic, the latter being active only at high doses (300 mg/kg) in the MES test. Compounds **13** and **14** were active in the MES test at 100 mg/kg but were inactive at all doses in the sc Ptz test, the latter compound being toxic at 300 mg/kg. Compound **15** failed to demonstrate any anticonvulsant protection or neurotoxicity at the various doses studied.

Anticonvulsant (anti-MES) Screening and Quantitative Evaluation Data in Rats Dosed Orally. From the initial ip mouse screening, compounds **1**, **2**, **3**, **5**, **8**, **9**, **10**, **13**, and **14** were selected for further oral

Table 2. Anticonvulsant (anti-MES) and Toxicity Screening Data in Rats Dosed Orally with Ameltolide Analogues[a]

compd	15 min	30 min	1 h	2 h	4 h	toxicity
1	++++	++++	++++	++++	++++	−
2	++++	++++	++++	++++	++++	−
3	−	+++	++	+	+	−
5	−	−	−	−	−	−
8	+++	+++	−	++	+++	−
9	+++	+++	++++	−	++++	−
10	++++	++++	++++	++++	++	−
13	−	−	+	−	+	+ (4 h)
14	−	−	−	+	++	−

[a] Rats were given a single dose of 30 mg compound/kg body weight and anticonvulsant activities determined in the maximal electroshock seizures (MES) test. No toxicity was observed at this dose except for compound **13** at the time point 4 h. Symbols are ++++, activity in 75−100% of administered animals; +++, in 50−75% of animals; ++, in 25−50% animals; +, 0−25% animals; and −, no activity or toxicity. At least four animals were utilized for each time point.

evaluation in rats. Anti-MES activities determined in rats treated with 30 mg/kg of the selected compounds are summarized in Table 2. No toxicity was observed at this dose for any of the compounds, except for compound **13** which showed toxicity in one of four animals tested. Compounds **1**, **2**, and **10** showed the best anticonvulsant activity. All animals dosed with these compounds showed protection at 5 time points from 15 min to 4 h. Intermediate anticonvulsant protection was offered by compounds **8** and **9**. Compound **3** presented with moderate activity, giving, however, a relatively good protection at the 30 min time point. No anticonvulsant protection was induced by compound **5**, whereas administration of compounds **13** and **14** led to a relatively weak anticonvulsant protection.

These screening data were confirmed by quantitative anticonvulsant evaluation of compounds in the MES test performed in rats dosed orally. Doses of compounds offering protection in 50% of test animals (i.e., ED_{50}s) were determined and are presented in Table 3. Mean ED_{50} values amounted to (in order of increasing values and decreasing efficacy of compounds) 25.3 (**10**), 52.3 (**1**), 120.7 (PHT), 130.6 (**9**), 135.2 (**2**), 230.8 (**8**), 284.1 (**3**), 354.0 (**14**), 369.0 (**13**), and more than 650 (**5**) μmol/Kg.

Quantitative Evaluation on Neuronal Sodium Channels. The competitions of selected compounds with the binding of [^{3}H]batrachotoxinin-A-20α-benzoate to the voltage-dependent sodium channels from rat brain synaptosomes were studied and compared with the displacement properties of phenytoin tested in the same experimental model. Apparent IC_{50}s offered in this binding test by the compounds are presented in Table 3. The mean apparent IC_{50} values were (in order of increasing values and decreasing efficacy of compounds) 0.14 (**10**), 0.25 (**1**), 0.35 (**3**), 0.86 (PHT), 0.97 (**2**), 1.86 (**13**), 25.8 (**5**), 47.8 (**14**), 161.3 (**8**), and 183.5 (**9**) μmol/L.

Table 3. Quantitative Evaluations of Selected Ameltolide Analogues in MES Tests and Batrachotoxin-Binding Experiments Performed in Rats[a]

compd	anticonvulsant ED_{50} (μmol/kg)	apparent IC_{50} (10^{-6} M)
1	52.3 ± 2.0	0.25 ± 0.05
2	135 ± 3	0.97 ± 0.14
3	284 ± 10	0.35 ± 0.06
5	>650	25.8 ± 5.0
8	231 ± 7	161 ± 14
9	131 ± 7	184 ± 20
10	25.3 ± 4.1	0.11 ± 0.03
13	369 ± 14	1.86 ± 0.13
14	354 ± 10	47.8 ± 7.3
PHT	121 ± 7	0.86 ± 0.07

[a] Experimental measurements were made in quadriplicate and results are mean values ± SD. Anticonvulsant ED_{50} values were determined in the MES test performed in rats dosed orally. Apparent IC_{50} were determined in the [^{3}H]batrachotoxinin-A-20-α-benzoate-binding test performed on rat brain synaptosomes. PHT: phenytoin.

Discussion

Interactions of anticonvulsant agents with the neuronal voltage-dependent sodium channel have been previously documented for phenytoin, carbamazepine,[23,25] ralitoline,[26] lamotrigine,[27] and U-54494A and metabolites.[28] Interaction of phenytoin with neuronal voltage-dependent sodium channels involves a site other than the [^{3}H]batrachotoxinin-A-20α-benzoate binding site[29] (structure and function studies of voltage-sensitive ion channels have been previously reviewed by Catterall[30]) but presenting with allosteric modulating properties toward the latter site.[24] The present work reports on the in vitro inhibition by 15 analogues related to ameltolide of batrachotoxin binding to the neuronal voltage-dependent sodium channel and the in vivo anticonvulsant activity of compounds in the MES test. Phenytoin (PHT) was utilized as a standard for which anti-MES activity was accounted for by interaction with Na channels. Anticonvulsant ED_{50} and apparent IC_{50} values of compounds may be linked through a semilogarithmic relationship involving half of the test molecules, i.e., PHT, compounds **1**, **2**, **10**, and **14**, and the relationship appears to fit the following equation:

$$ED_{50} = 130 \log \frac{IC_{50}}{9}$$

where ED_{50} is expressed as μmol/kg and (apparent) IC_{50} as 10^{-8} M values (Figure 2). Compared to this basal group of compounds (for which anti-MES activity may be considered as resulting from inhibition of sodium channels), compounds **3**, **13**, and **5**, on one hand, and compounds **8** and **9**, on the other hand, present with anticonvulsant ED_{50} values which are higher and lower, respectively, than would be expected on the basis of their ability to interact with Na channels (Figure 2). The relatively lower anticonvulsant potencies of compounds **3**, **13**, and **5** might be explained by low bioavailability of the compounds. The relatively higher anticonvulsant potencies of compounds **8** and **9** may be a result of relatively high bioavailability, mechanisms other than only their inhibition of sodium channels or their delivery as prodrug compounds. In this respect, **9** might give rise in vivo to **2**, and **8** to **13**, through oxidation of the nitro group to an amine and hydroxylation at the position 4 of the phthalimide pharmacophore, respectively. Plotting the EC_{50} and apparent IC_{50} data according to a linear instead of a semilogarithmic relationship does not change the general conclusions of our work, except that **13** instead of **14** should be considered in the basal group including **1**, **2** (ameltolide), **10**, and

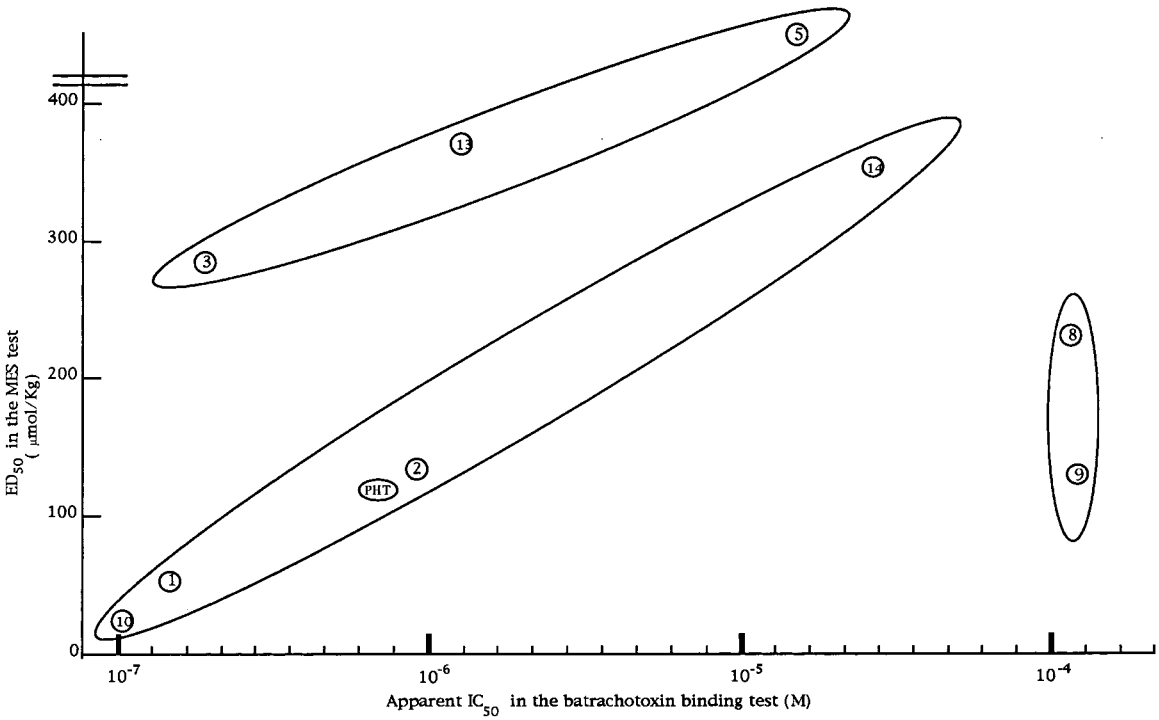

Figure 2. Relationships between anticonvulsant MES ED_{50} and batrachotoxin apparent IC_{50} values. Semilogarithmic plotting of data from Table 3 is illustrated. Other comments are in the text (Discussion). PHT: phenytoin.

PHT. In this view, at least on the basis of oral dosing, **14** would be considered more like **8** and **9**, and only compound **3** would present with anticonvulsant potency less than expected on the basis of observed activity in the BTX-binding test. It should be stressed that in mice dosed intraperitonally, **3** was highly active in the MES test and was endowed with an ED_{50} value of 24.8 μmol/kg versus 31.8, 10.8, 49.5, and 37.5 μmol/kg for compounds **1**, **2** (ameltolide), **10**, and PHT, respectively (data not shown). The low level of activity recorded for **3** in the oral rat MES test might be related to a low oral bioavailability or to rapid detoxifying conjugation in this animal species.

Our data supports the view that the antiepileptic activity of ameltolide largely documented in the literature[13,14,16,18,31−35] is the result of its interaction with the neuronal voltage-dependent sodium channel. A major element in favor of this view is given by the IC_{50} we recorded for ameltolide (0.9 μM), a concentration that is commonly observed in the brain of animals receiving this anticonvulsant agent. Potts et al.[32] reported in rats dosed orally with ameltolide (50 mg/kg) brain levels amounting to 5−7 μg/g tissue (approximated to 20 μM) 2 h after dosing and 2 μg/g tissue (approximated to 6 μM) 5 h after administration. Tested in the same experimental conditions, ameltolide (**2**) appears to be as potent as phenytoin (PHT) in interacting with the Na channels. The phthalimide (**10**) and nitro (**1**) counterparts of ameltolide are several times more potent than ameltolide and phenytoin in the binding test and oral MES test in rats. For this reason, we believe that these agents may represent promising new leads as potential anticonvulsants.

Experimental Section

Materials. Reagents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France; Bornem, Belgium). Solvents were obtained from Merck (Darmstadt, Germany). The [^{3}H]-batrachotoxinin-A-20α-benzoate was obtained from Amersham International plc, Buckinghamshire, England.

Analytical Procedures. Melting points (uncorrected) were determined in open capillary tubes on a Totolli apparatus. IR spectra were recorded on KBr disks using a Perkin-Elmer IR spectrophotometer (Grating infrared spectrophotometer, model 457). NMR spectra were measured on compounds in solution in DMSO-d_6 (or in CD_3OD) with tetramethylsilane as internal standard, at 300 MHz for ^{1}H and 75 MHz for ^{13}C, on a BRUCHER AM 300 apparatus. HPLC analyses of compounds were performed on a Lichrocart 125-R Licrospher RP 18 column with a flow rate of 1 mL/min of a methanol−water (65/35, v/v) mixture as elution solvent and with detection at 260 nm. Elemental analyses (C, H, N) were performed on a Carlo-Erba EA 1108 elemental analyzer. All compounds had IR and ^{1}H and ^{13}C NMR spectra consistent with their assigned structure. They were homogeneous in HPLC. Their microanalytical data were within ± 0.4% of the calculated figures.

Chemistry. 4-Nitro-*N*-(2,6-dimethylphenyl)benzamide (1). 4-Nitrobenzoyl chloride (5 g, 26.9 mmol) was added portionwise to 2,6-dimethylaniline (4.8 g, 40.4 mmol) dissolved in a mixture of dry THF (50 mL) and dry pyridine (5 mL). The reaction mixture was stirred for 3 h at room temperature and then quenched with 500 mL of cold 1 N HCl. The resulting precipitate was collected on a filter, washed with water, and recrystallized from absolute ethanol (5.60 g, 78% yield): mp 183−185 °C; ^{1}H NMR (DMSO-d_6) δ 2.22 (s, 6H, CH_3), 7.11−8.40 (m, 7H, aromatic), 10.15 (s, 1H, NH); ^{13}C NMR δ 18.29 (CH_3), 123.72−149.62 (aromatic C), 164.29 (C=O); IR (cm^{-1}) 3300, 1650 (amide), 1600 (phenyl), 1500−1300 (nitro).

4-Amino-*N*-(2,6-dimethylphenyl)benzamide (2). 4-Nitro-*N*-(2,6-dimethylphenyl)benzamide **(1)** (2 g, 8.13 mmol) was dissolved in cyclohexene (150 mL), and then 10% palladium on charcoal (250 mg) and 2-propanol (50 mL) were added. The mixture was heated under reflux for 8 h and then filtered. The solvent was evaporated in vacuo, and the residue was recrystallized from an ethanol−water (1/1, v:v) mixture (1.77 g, 91% yield). mp 210−212 °C. ^{1}H NMR (DMSO-d_6) δ 2.15 (s, 6H, CH_3), 6.59−7.76 (m, 7H, aromatic), 10.15 (s, 1H, NH); ^{13}C NMR δ 18.32 (CH_3), 112.84−132.01 (aromatic C), 165.81 (C=O); IR (cm^{-1}) 3450 (amino), 3300, 1650 (amide), 1600 (phenyl).

4-Hydroxy-*N*-(2,6-dimethylphenyl)benzamide (3). 4-Amino-*N*-(2,6-dimethylphenyl)benzamide (**2**) (0.35 g, 1.46 mol) was dissolved in a water−sulfuric acid solution (75/25, v:v) (20 mL). The resulting solution was cooled to 0−5 °C. A cold solution of $NaNO_2$ (0.18 g, 2.6 mmol in 0.4 mL of water) was added dropwise. The supernatant was added to a boiling sulfuric acid solution (1.65 mL of sulfuric acid and 1.5 mL of water). The reaction mixture was refluxed for 30 min. The resulting mixture was poured onto ice and stirred to give a precipitate which was filtered, washed with water, dried, and recrystallized from a hydrochloric acid−water solution (1/1, v:v) to give pure **3** (0.3 g, 75%): mp 233−235 °C; ^{1}H NMR (DMSO-d_6) δ 2.14 (s, 6H, CH_3), 6.63−7.08 (m, 7H, aromatic), 9.48 (s, 1H, NH); ^{13}C NMR δ 18.25 (CH_3), 115.07−135.80 (aromatic C), 164.81 (C═O); IR (cm^{-1}) 3350 (amino), 3250 (hydroxyl), 1650 (amide), 1620 (phenyl).

***N*-[4-(2,2,6,6-Tetramethyl)piperidin-1-yl]benzamide (4).** Using benzoyl chloride (5 g, 35.5 mmol) and 4-amino-(2,2,6,6-tetramethyl)piperidine (5 g, 31.99 mmol), compound **4** was synthesized in the same way as **1**. Compound **4** was recrystallized from acetone/ether (1/1, v:v) (6.91 g, 83% yield): mp 227−229 °C; ^{1}H NMR (CD_3OD-d_6) δ 1.56 (s, 6H, CH_3), 1.62 (s, 6H, CH_3), 1.80−2.17 (m, 5H, $CH(CH_2)_2$), 7.44−7.70 (m, 5H, aromatic), 9.50 (s, 1H, NH); ^{13}C NMR δ 25.14 (CH_3), 30.64 (CH_3), 41.78−58.81 (piperidine C), 128.42−135.41 (aromatic C), 169.81 (C═O); IR (cm^{-1}) 3440 (piperidine), 3400, 1650 (amide), 1610 (phenyl).

4-Nitro-*N*-[4-(2,2,6,6-tetramethyl)piperidin-1-yl]benzamide (5). Using 4-nitrobenzoyl chloride (5 g, 26.9 mmol) and 4-amino-(2,2,6,6-tetramethyl)piperidine (2.41 g, 15.42 mmol), compound **5** was synthesized in the same way as compounds **1** and **4**. Compound **5** was recrystallized from acetone/ether (1/1, v:v) (3.76 g, 80% yield): mp 262−264 °C; ^{1}H NMR (CD_3OD-d_6) δ 1.53 (s, 12H, CH_3), 1.77−2.10 (m, 5H, $CH(CH_2)_2$), 8.14−8.34 (m, 4H, aromatic), 9.03 (s, 1H, NH); ^{13}C NMR δ 24.14 (CH_3), 30.64 (CH_3), 40.59−58.74 (piperidine C), 124.51−151.05 (aromatic C), 167.53 (C═O); IR (cm^{-1}) 3500 (piperidine), 3300, 1650 (amide), 1600 (phenyl), 1500−1300 (nitro).

4-Amino-*N*-[4-(2,2,6,6-tetramethyl)piperidin-1-yl]benzamide (6). Using 4-nitro-*N*-[4-(2,2,6,6-tetramethyl)piperidin-1-yl]benzamide (**5**) (3 g, 9.81 mmol), compound **6** was synthesized in the same way as **2** and was recrystallized from ethanol−water (1/1, v:v) (1.60 g, 73% yield): mp 234−236 °C; ^{1}H NMR (CD_3OD-d_6) δ 1.46 (s, 6H, CH_3), 1.55 (s, 6H, CH_3), 1.60−2.10 (m, 5H, $CH(CH_2)_2$), 6.68−7.70 (m, 4H, aromatic), 9.03 (s, 1H, NH); ^{13}C NMR δ 15.86 (CH_3), 30.64 (CH_3), 41.78−58.81 (piperidine C), 105.34−144.16 (aromatic C); IR (cm^{-1}) 3440 (piperidine), 3350 (amino), 3300, 1650 (amide), 1620 (phenyl).

4-Nitrophenyl-2,6-dimethylphenylthiourea (7). 2,6-Dimethylaniline (3.04 g, 27.77 mmol) was solubilized in 3 mL ethanol, and the mixture was heated under reflux. Phenyl isothiocyanate (5 g, 27.75 mmol) dissolved in ethanol was added dropwise over 30 min, and the reaction was pursued for 1 h. The mixture was then poured onto ice, and the resulting precipitate was filtered and purified by column chromatography (silica gel, hexanes−ethyl acetate (3/1, v:v) as elution solvent) yielding pure **7** (4.90 g, 60% yield): mp 178−180 °C; ^{1}H NMR (DMSO-d_6) δ 2.21 (s, 6H, CH_3), 7.12−8.23 (m, 7H, aromatic), 9.49 (s, 2H, NH); ^{13}C NMR δ 18.12 (CH_3), 120.93−146.55 (aromatic C), 180.24 (C═S); IR (cm^{-1}) 1600 (phenyl), 1500 (C═S), 1300 (nitro).

***N*-(2,6-Dimethylphenyl)phthalimide (8).** A mixture of 2,6-dimethylaniline (3.43 g, 28.3 mmol) and phthalic anhydride (5 g, 32 mmol) in acetic acid (20 mL) was stirred and heated under reflux for 1 h. The solvent was evaporated in vacuo, and the residual material was recrystallized from methanol−water (1/1, v:v) yielding pure **8** (4.69 g, 66% yield): mp 206−207 °C; ^{1}H NMR (DMSO-d_6) δ 2.08 (s, 6H, CH_3), 7.20−8.13 (m, 6H, aromatic); ^{13}C NMR δ 17.44 (CH_3), 30.64 (CH_3), 123.72−136.58 (aromatic C), 166.27 (C═O); IR (cm^{-1}) 1780−1720 (imide), 1600 (phenyl).

4-Nitro-*N*-(2,6-dimethylphenyl)phthalimide (9). Starting from 2,6-dimethylaniline (3.43 g, 28.3 mmol) and 4-nitrophthalic anhydride (5.5 g, 28.3 mmol), compound **9** was synthesized in the same way as **8** and was recrystallized from methanol−water (1/1, v:v) (6.65 g, 79% yield): mp 176−179 °C; ^{1}H NMR (DMSO-d_6) δ 2.10 (s, 6H, CH_3), 7.24−8.73 (m, 6H, aromatic); ^{13}C NMR δ 17.48 (CH_3), 30.64 (CH_3), 118.63−151.70 (aromatic C), 164.29 (C═O); IR (cm^{-1}) 1730−1680 (imide), 1600 (phenyl), 1350 (nitro).

4-Amino-*N*-(2,6-dimethylphenyl)phthalimide (10). Using 4-nitro-*N*-(2,6-dimethylphenyl)phthalimide (**9**) (3 g, 10.1 mmol), compound **10** was synthesized in the same way as **2** and was recrystallized from ethanol−water (1/1, v:v) (2.02 g, 75% yield): mp 195−196 °C; ^{1}H NMR (DMSO-d_6) δ 2.10 (s, 6H, CH_3), 7.18−7.64 (m, 6H, aromatic); ^{13}C NMR δ 18.32 (CH_3), 112.84−132.02 (aromatic C), 165.81, 165.02 (C═O); IR (cm^{-1}) 3300 (amino), 1620 (phenyl).

***N*-(3-Nitro-2,6-dimethylphenyl)phthalimide (11).** Using 3-nitro-2,6-dimethylaniline (4.68 g, 28 mmol) and phthalic anhydride (5.5 g, 28 mmol), compound **11** was synthesized in the same way as **8** and was recrystallized from methanol−water (1/1, v:v) (6.40 g, 77% yield): mp 167−169 °C; ^{1}H NMR (DMSO-d_6) δ 2.29−2.32 (s, 6H, CH_3), 7.63−8.16 (m, 6H, aromatic); ^{13}C NMR δ 14.28 (CH_3, (p-NO_2)), 18.07 (CH_3, (o-NO_2)),124.04−148.61 (aromatic C); IR (cm^{-1}) 1780−1730 (imide), 1600 (phenyl), 1500−1300 (nitro).

***N*-(3-Amino-2,6-dimethylphenyl)phthalimide (12).** Using *N*-(3-nitro-2,6-dimethylphenyl)phthalimide (**11**) (3 g,10.1 mmol), compound **12** was synthesized in the same way as **2** and was recrystallized from ethanol−water (1/1, v:v) (2.23 g, 83% yield): mp 165−166 °C; ^{1}H NMR (DMSO-d_6) δ 2.09 (s, 3H, CH_3), 2.19 (s, 3H, CH_3), 7.53−8.06 (m, 6H, aromatic); ^{13}C NMR δ 14.10 (CH_3, (p-NO_2)), 18.07 (CH_3, (o-NO_2)), 124.04−148.61 (aromatic C); IR (cm^{-1}) 3300 (amino), 1780−1730 (imide), 1600 (phenyl).

4-Hydroxy-*N*-(2,6-dimethylphenyl)phthalimide (13). Using 4-amino-*N*-(2,6-dimethylphenyl)phthalimide (**10**), compound **13** was synthesized in the same way as **3** and was recrystallized from a hydrochloric acid−water solution (1/1, v:v) giving pure **13** (1.29 g, 97% yield): mp 170−172 °C; ^{1}H NMR (DMSO-d_6) δ 2.10 (s, 6H, CH_3), 7.24−8.74 (m, 6H, aromatic); ^{13}C NMR δ 17.49 (CH_3), 118.65−151.74 (aromatic C), 164.82, 165.09 (C═O); IR (cm^{-1}) 3100 (hydroxyl), 1780−1740 (imide), 1620 (phenyl).

4-Chloro-*N*-(2,6-dimethylphenyl)phthalimide (14). 4-Amino-*N*-(2,6-dimethylphenyl)phthalimide (**10**) (0.1 g, 0.45 mmol) was dissolved in a water−sulfuric acid solution (1/1, v:v) (10 mL). The temperature was lowered to 0−5 °C, and the mixture was stirred for 15 min. Cold $NaNO_2$ solution (0.24 g in 1 mL water) was added dropwise at 0−5 °C. The diazonium solution was then poured slowly in the cold solution of CuCl. When the temperature reached 15 °C, the reaction mixture was heated to 60 °C. The mixture was then filtered and washed with 0.1 N aqueous solution of $NaHCO_3$, with distilled water, and finally with a 0.1 N aqueous solution of H_3PO_4. Compound **14** was recrystallized from ethanol−water (9/1, v:v) (0.083 g, 65% yield): mp 192−194 °C; ^{1}H NMR (DMSO-d_6) δ 2.10 (s, 6H, CH_3), 7.24−8.81 (m, 6H, aromatic); ^{13}C NMR δ 17.63 (CH_3), 118.79−151.87 (aromatic C), 164.96, 165.22 (C═O); IR (cm^{-1}) 1730−1680 (imide), 1625 (phenyl), 1100 (C−Cl).

***N*-[4-(2,2,6,6-Tetramethyl)piperidinyl]phthalimide (15).** Using 4-amino-2,2,6,6-tetramethylpiperidine (5 g, 33.76 mmol) and phthalic anhydride (5 g, 32 mmol), compound **15** was synthesized in the same way as **8** and was recrystallized from methanol−water (1/1, v:v) (6.60 g, 72% yield): mp 276−278 °C; ^{1}H NMR (CD_3OD-d_6) δ 1.63 (s, 6H, CH_3), 1.67 (s, 6H, CH_3), 2.07−3.35 (m, 5H, $CH(CH_2)_2$), 7.78−7.92 (m, 5H, aromatic); ^{13}C NMR δ 24.72 (CH_3), 30.59 (CH_3), 30.59−58.87 (piperidine C), 124.18−135.57 (aromatic C), 169.27 (C═O); IR (cm^{-1}) 3520 (NH), 1780−1720 (imide), 1625 (phenyl).

In Vivo Experiments. Animals. Male albino mice (CF-1 strain, 18−25 g; Charles River, Wilmington, MA) and male albino rats (Sprague−Dawley, 100−150 g, Simonsen, Gilroy, CA) were used as experimental animals. The animals were

allowed free access to food (S/L Custom Lab Diet-7) and water, except when removed from their cages for the experimental procedures.

Anticonvulsant Tests. Animal seizure tests included one electrically and one chemically induced seizure episode test and were conducted according to the Anticonvulsant Drug Development (ADD) Program protocol.[36−38] The electrical test employed was the Maximal Electroshock Seizure (MES) pattern test. In this test, maximal seizures are elicited in mice by a 60-Hz alternating current of 50 mA delivered for 0.2 s via corneal electrodes. This amount of current is approximately 6 times the threshold and reveals the ability of the compound to prevent seizure spread. A drop of an anesthetic solution instilled in each eye prior to application of the electrodes ensures adequate electrical contact; it also reduces the incidence of fatalities to near zero. Maximal seizures are produced in virtually all normal mice. The maximal seizure typically consists of a short period of initial tonic flexion and a prolonged period of tonic extension (especially of the hind limbs), followed by terminal clonus. The typical seizure lasts approximately 22 s. Failure to extend the hind limbs to an angle with the trunk greater than 90° is defined as protection.

For the chemically induced convulsant test, pentylenetetrazol was dissolved in sufficient 0.9% sodium chloride solution to allow subcutaneous injections to mice and rats in volumes of 0.01 mL/g of body weight and 0.02 mL/10 g body weight, respectively. The animals given subcutaneous pentylenetetrazol (sc Ptz) were observed for at least 30 min for the presence or absence of a seizure.

Neurotoxicity Tests. The median minimal neurotoxic dose (TD_{50}) in mice was determined by the rotorod procedure. The mouse is placed on a 1-in. diameter knurled plastic rod rotating at 6 rpm. Unimpaired mice can easily remain on a rod rotating at this speed. Neurological deficit (e.g., ataxia, sedation, hyperexcitability) is indicated by the inability of the animal to maintain equilibrium on the rod for at least 1 min, in each of three concurrent trials. Neurological deficit in rats was indicated by ataxia, loss of placing response, and muscle tone.

In Vitro Experiments. Rat brain synaptosomal fractions were prepared essentially as previously described by Taumkun and Catterall.[39]

The binding assay with [^{3}H]batrachotoxinin-A-20α-benzoate was adapted from Willow and Catterall[23] and performed at 37 °C along with a 2-h incubation time in a medium at pH 7.4 (final volume, 0.6 mL), containing 130 mM choline chloride, 50 mM HEPES, 5.5 mM glucose, 5.4 mM KCl, 10 nM [^{3}H]batrachotoxinin-A-20α-benzoate, 20 mg/mL scorpion venom (this amount of venom was, in our hands, found to correspond to a concentration providing an optimal stimulation of the batrachotoxin binding [data not shown]), and rat brain synaptosome proteins (about 400 μg of protein per assay). The reaction was stopped by addition of 3 × 3 mL of a pH 7.4 solution containing 163 mM choline chloride, 5 mM HEPES, 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$. The contents of the tubes were then filtered on Whatman GF/B filters. Radioactivity retained on these filters was then measured.

Aspecific binding was measured by including 1.2 10^{-3} M veratridine in the incubation mixtures. Specific binding of [^{3}H]batrachotoxinin-A-20α-benzoate was obtained by subtraction from the total binding of the aspecific binding. Protein was measured according to the procedure described by Bradford.[40]

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