Synthesis and Anticonvulsant Activity of Novel and Potent 2,3-Benzodiazepine AMPA/Kainate Receptor Antagonists

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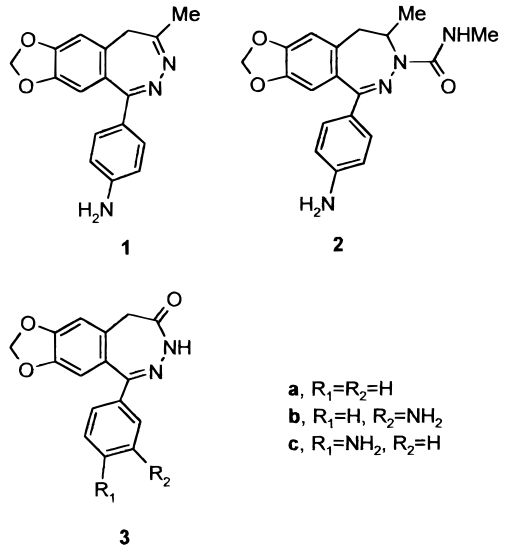
We have previously shown that 1-aryl-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-ones (**3**) possess marked anticonvulsant properties and antagonize seizures induced by 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) in analogy to the structurally related 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (**1**, GYKI 52466), a well-known noncompetitive AMPA/kainate receptor antagonist. We now report the synthesis of 3-(*N*-alkylcarbamoyl)-1-aryl-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-ones (**4a**−**h**) and 1-aryl-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepine-4-thiones (**5a**−**c**). The activity of all compounds, intraperitoneally (ip) injected, was evaluated against audiogenic seizures in DBA/2 mice and against seizures induced by maximal electroshock (MES) and pentylenetetrazole (PTZ) in Swiss mice. Some of the new compounds **4** and **5** showed remarkable anticonvulsant activity, and their toxicity, as evidenced by the rotarod test, is lower than that of **1**. The time course of anticonvulsant activity of derivatives **4b** and **5b,c** was studied and compared to that of **1** and **3b,c**. Compounds **4a,b** and **5a**−**c** antagonize seizures induced by AMPA and kainate (KA) and their anticonvulsant activity is reversed by pretreatment with aniracetam. Using the patch-clamp technique, the capability of derivatives **3c**, **4b**, and **5c** to antagonize KA-evoked currents in primary cultures of granule neurons was tested and compared with that of the parent compounds **1** and 1-(4-aminophenyl)-3,4-dihydro-4-methyl-3-methylcarbamoyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (**2**, GYKI 53655).

Introduction

Overstimulation of ionotropic glutamate receptors, *N*-methyl-D-aspartic acid (NMDA), 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA), and kainate (KA) receptors, is believed to be implicated in the development of several neurodegenerative disorders including epilepsy, stroke, and Alzheimer's disease.[1]

1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (**1**, GYKI 52466) (Chart 1), a 2,3-benzodiazepine derivative, is the prototype of selective noncompetitive AMPA receptor antagonists acting via an allosteric site on the receptor complex.[2] It possesses remarkable anticonvulsant properties[3] and behaves as a neuroprotective agent in focal and global ischemia.[4] A significant improvement in the pharmacological profile of GYKI 52466 was obtained by appending an alkylcarbamoyl group at N-3 of the benzodiazepine ring.[5] As a matter of fact, the 3-*N*-methylcarbamoyl derivative **2** (GYKI 53655, LY 300168) (Chart 1) emerged as the most potent compound among this series of derivatives.[6] Its ED_{50} value against KA-induced seizures and in the maximal electroshock seizure test is 2−3-fold higher than that of **1**. As a blocker of the AMPA and KA currents, **2** is 5−8-fold more potent than its parent compound **1**.

Chart 1



As part of a program aimed at identifying potent and selective AMPA receptor antagonists,[7,8] we have recently reported[9] an investigation on a series of 1-aryl-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-ones (**3**) where the iminohydrazone portion of **1** was replaced by an iminohydrazide moiety. In this context

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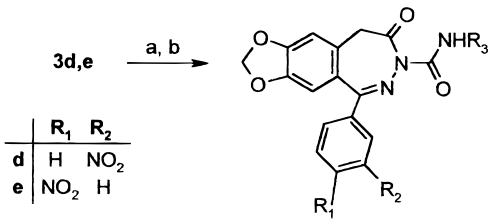
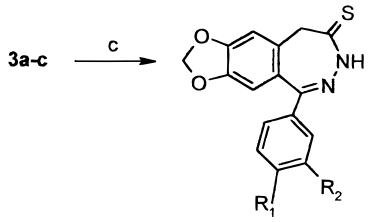
Scheme 1[a]



4a-h

	R_1	R_2	R_3
a	H	NH_2	Me
b	NH_2	H	Me
c	H	NH_2	Et
d	NH_2	H	Et
e	H	NH_2	Pr
f	NH_2	H	Pr
g	H	NH_2	Bu
h	NH_2	H	Bu



5a-c

	R_1	R_2
a	H	H
b	H	NH_2
c	NH_2	H

[a] (a) R_3NCO/Et_3N, CH_2Cl_2, rt, 36 h; (b) H_2/5% Pd−C, MeOH, rt, 3 h; (c) Lawesson's reagent, toluene, reflux, 2 h.

we noticed that 3-aminophenyl and 4-aminophenyl derivatives **3b**,**c** are roughly 2-fold more potent than both **1** and parent compound **3a**. As deduced from the rotarod test, compounds **3** are all characterized by a toxicity lower than that of **1**.

In analogy to what has been previously observed[5] in the series of GYKI 52466 analogues, where the introduction of an alkylcarbamoyl group appended at the N-3 position of the heterocyclic nucleus brought about an increase in potency, we prepared a series of 3-(*N*-alkylcarbamoyl)-1-aryl-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-ones (**4a**−**h**) (Scheme 1). Furthermore, since it has been reported[10] that the bioisosteric replacement of oxygen by sulfur in the carbonyl group of derivatives structurally related to **3** gave a significant improvement in the pharmacological profile, we synthesized some 1-aryl-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepine-4-thiones (**5a**−**c**). The new compounds (**4a**−**h** and **5a**−**c**) were evaluated for their anticonvulsant properties in DBA/2 mice, a strain genetically susceptible to sound-induced seizures, which has been considered an excellent animal model for generalized epilepsy and for screening new anticonvulsant drugs.[11] Derivatives **4a**−**h** and **5a**−**c** were also examined as antagonists against seizures induced either by maximal electroshock (MES) or by pentylenetetrazole (PTZ) in Swiss mice. We also assessed the propensity of all compounds to induce neurological impairment by using the rotarod test. The time course of anticonvulsant activity of **3b,c**, **4b**, and **5b,c** was studied and compared to that of reference compound **1**. The activity of **4a,b** and **5a**−**c** was also evaluated through their ability to antagonize seizures induced by the intracerebroventricular (icv) administration of AMPA or subcutaneous (sc) administration of KA. In addition, the ability of aniracetam (Ro 13-5057), a potentiator of the AMPA effects,[12] to reverse the anticonvulsant properties of these compounds was studied.

To investigate at the cellular level the mechanism of action of the in vivo active substances, we have used the patch-clamp technique in primary cultures of granule neurons. The capability of **3c**, **4b**, and **5c** to antagonize KA-evoked current was tested and compared with that of parent compounds **1** and **2**.

Chemistry

2,3-Benzodiazepines **3d,e**, used as starting material, were prepared via Friedel−Crafts acylation of methyl 3,4-methylenedioxyphenylacetate with 3- or 4-nitrobenzoic acid, following a methodology previously reported,[13] and subsequently reacted with an excess of hydrazine. These intermediates were then treated with an excess of the appropriate alkyl isocyanate in the presence of triethylamine to yield the corresponding *N*-3-alkylcarbamoyl derivatives, which were then transformed into final derivatives **4a**−**h** (Scheme 1) by a catalytic reduction of their nitro group. Compounds **3a**−**c** were prepared as previously reported[9] and converted into the corresponding thiocarbonyl derivatives **5a**−**c** by treatment with Lawesson's reagent at reflux in toluene (Scheme 1). The elemental analysis (C, H, N) and ^1H NMR spectral data of synthesized compounds are in full agreement with the proposed structures and are reported in the Experimental Section.

Results and Discussion

Novel 3-(*N*-alkylcarbamoyl)-1-aryl-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-ones (**4a**−**h**) and 1-aryl-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepine-4-thiones (**5a**−**c**) were tested for anticonvulsant activity against audiogenic seizures induced in DBA/2 mice, and the results are compared with those previously reported[9] for model compounds **1** and **3a**−**c** (Table 1). The compounds were administered intraperitoneally (ip) at various doses in the range of 3.3−200 μmol/kg and their anticonvulsant properties, expressed as ED_{50}, evaluated 30 min after injection. A number of the new derivatives are provided with remarkable anticonvulsant activity which is higher than that displayed by **1**. A survey of the results reported in Table 1 reveals that the same modification carried out on derivatives **3b,c** produces an increase in the anticonvulsant activity only in the case of methyl derivatives **4a,b**. A lengthening of the side chain brought about a decrease in activity in all the derivatives but **4g** in which activity remained unchanged. On the other hand, replacement of the carbonyl group of **3a**−**c** with a thiocarbonyl moiety was always productive causing an increase in potency. This result matches that previously reported for related compounds.[10] A conceivable explanation could be envis-

Table 1. Anticonvulsant Activity of Compounds **1** and **3**−**5** Against Audiogenic Seizures in DBA/2 Mice, TD_{50} Values on Locomotion Assessed by Rotarod Test, and Relative Lipophilicity (R_m)

compd	ED_{50}, μmol/kg[a] clonic phase	ED_{50}, μmol/kg[a] tonic phase	TD_{50}, μmol/kg,[a] locomotor deficit	TI,[b] TD_{50}/ED_{50}	R_m
1	35.8 (24.4−52.4)	25.3 (16.0−40.0)	76.1 (47.5−122)	2.1	−0.404
3a	43.3 (34.4−54.6)	40.6 (30.1−54.9)	159 (82.6−306)	3.7	−0.215
3b	18.0 (10.0−32.5)	12.7 (6.13−26.2)	101 (52.0−194)	5.6	−0.484
3c	15.4 (10.1−23.5)	10.9 (4.60−24.6)	99.1 (72.4−135)	4.5	−0.552
4a	13.6 (6.22−29.9)	11.2 (5.31−23.5)	48.9 (34.7−68.7)	3.6	−0.441
4b	12.4 (6.44−23.8)	8.70 (4.61−16.4)	48.6 (31.4−54.6)	3.9	−0.459
4c	44.9 (26.6−75.8)	32.1 (17.7−58.3)	126 (80.5−196)	2.8	−0.323
4d	35.0 (18.5−66.3)	23.8 (13.4−42.1)	134 (70.7−255)	3.8	−0.368
4e	85.3 (69.1−105)	63.8 (45.1−90.2)	>100	ND	−0.181
4f	69.4 (36.2−133)	49.2 (25.9−93.4)	182 (95.1−350)	2.6	−0.250
4g	18.1 (10.4−31.5)	12.7 (9.31−17.2)	56.2 (39.4−80.0)	3.1	−0.075
4h	38.7 (21.2−70.8)	32.6 (18.2−58.4)	108 (82.2−143)	2.8	−0.110
5a	23.8 (13.8−41.2)	18.8 (10.2−34.5)	97.1 (73.2−129)	4.1	−0.185
5b	16.7 (10.2−27.3)	8.88 (5.71−13.8)	53.7 (32.9−87.6)	3.2	−0.213
5c	11.8 (6.14−22.5)	5.09 (2.14−12.1)	39.8 (25.0−63.3)	3.4	−0.269

[a] All data were calculated according to the method of Litchfield and Wilcoxon;[27] 95% confidence limits are given in parentheses. At least 32 animals were used to calculate each ED_{50} and TD_{50} value. [b] TI, therapeutic index, represents the ratio between TD_{50} and ED_{50} (from the clonic phase of the audiogenic seizures); ND, not determined.

Table 2. Anticonvulsant Activity of Compounds **1** and **3**−**5** Against MES- and PTZ-Induced Seizures in Swiss Mice

compd	ED_{50}, μmol/kg[a] (±95% confidence limits) MES	PTZ
1	35.7 (29.3−43.4)	68.3 (56.2−83.1)
3a	42.6 (26.4−68.8)	78.0 (46.0−132)
3b	19.3 (16.9−22.0)	40.5 (22.9−71.7)
3c	32.1 (23.2−44.3)	71.8 (53.2−96.9)
4a	21.4 (12.6−36.3)	19.6 (7.70−49.6)
4b	18.6 (8.40−41.2)	16.3 (7.30−36.0)
4c	60.2 (19.5−185)	90.7 (72.2−114)
4d	52.5 (40.4−68.2)	68.2 (40.0−116)
4e	>100	>100
4f	76.5 (52.2−112)	>100
4g	26.6 (17.4−40.6)	36.4 (31.3−42.3)
4h	79.7 (36.5−174)	>100
5a	37.8 (23.7−60.1)	80.3 (47.9−134.6)
5b	30.9 (19.4−48.4)	40.2 (24.4−66.2)
5c	9.79 (8.09−15.7)	25.2 (15.2−43.5)

[a] All data were calculated according to the method of Litchfield and Wilcoxon.[27] At least 32 animals were used to calculate each ED_{50} value.

aged in an easier ip absorption and a more favorable diffusion across the blood−brain barrier. As a matter of fact, compounds **5a**−**c** are provided with a higher lipophilicity (expressed as R_m value) than parent derivatives **3a**−**c** (e.g. $R_m = -0.269$ for **5c** versus $R_m = -0.552$ for **3c**; Table 1). However, lipophilicity is not the only parameter able to affect the potency of the compounds since in the series of *N*-3-carbamoyl derivatives there is no correlation between lipophilicity and activity (Table 1).

All compounds were also tested against MES- and PTZ-induced seizures in Swiss mice. As shown in Table 2, the tonic extension and the clonic phase of the seizures induced by MES and PTZ, respectively, were significantly reduced 45 min after ip administration of the tested compounds. It is noteworthy that compounds **4b** and **5c**, the most active of the series, show in both tests activity 2−4-fold higher than that of the parent compound **3c**.

The time course of the anticonvulsant activity of **3b,c**, **4b**, and **5b,c** was also studied and compared to that of **1** (Table 3). Model compound **1** displayed its maximum protection at 15 min from ip administration and returned to control at 90 min. The same trend was observed in the 4-aminophenyl-substituted derivatives **3c** and **5c** which showed their peak effect at 15 min and returned to control seizure after 90−120 min from administration. Noteworthy, the *N*-3-methylcarbamoyl derivative **4b** showed its maximum protection between 15 and 90 min with a return to control after 120 min from administration. On the contrary the 3-aminophenyl-substituted derivatives **3b** and **5b** displayed their maximum protection in the range 30−45 min, and their effect disappeared after 90−120 min. As a consequence, if the comparison is referred to the data obtained after 15 min from administration, only derivatives **3c** and **5c** are more active than reference compound **1**, whereas if we compare the results obtained in the range 30−60 min, all five compounds are more active than **1** (e.g. $ED_{50} = 11.8$ μmol/kg for **5c** versus $ED_{50} = 35.8$ μmol/kg for **1**).

To correlate the anticonvulsant activity of novel compounds **4** and **5** with their affinity for AMPA receptors, an additional test, based on AMPA-induced seizures in DBA/2 mice, was performed (Table 4). As shown in Figures 1 and 2, the clonic and tonic phases of the seizures induced by icv administration of AMPA were significantly reduced 30 min after ip administration of **4a,b** and **5a**−**c** in analogy to **1**.

We also tested the influence of aniracetam, a potentiator of the AMPA effect,[12] on the anticonvulsant activity of derivatives **4a,b** and **5a**−**c** in DBA/2 mice (Table 4). An icv injection of aniracetam (50 nmol/mouse) on its own had no convulsant activity; nevertheless, the administration of aniracetam 60 min before the injection of the tested compounds reversed their anticonvulsant effects and shifted the dose−response curves to the right with a pattern of activity similar to that of **1** and **3**.

Furthermore, compounds **4a,b** and **5a**−**c** produced a dose-dependent protection against KA-induced seizures (Table 4). The ED_{50} values are higher than those needed to block audiogenic seizures (Table 1) and lower than or similar to those capable of protecting the animals against hind limb extension in the MES test (Table 2). In addition, although few mice exhibited a lowering in body temperature, a decrease in motor activity, and unsteady gait after administration of the highest doses,

Table 3. ED_{50} Values at Various Times following ip Administration of Compounds **1**, **3b**,**c**, **4b**, and **5b**,**c**

	ED_{50}, μmol/kg (±95% confidence limits),[a] clonic phase					
compd	15 min	30 min	45 min	60 min	90 min	120 min
1	10.8 (7.11−16.4)	35.8 (24.4−62.4)	37.3 (27.2−52.1)	39.5 (29.6−52.7)	>50	>50
3b	>50	18.0* (10.0−32.5)	19.1* (12.4−29.4)	26.3 (21.1−32.9)	47.1 (33.5−66.3)	>50
3c	7.55 (4.08−14.0)	15.4** (10.1−23.5)	18.7** (11.3−31.0)	21.3* (14.2−31.9)	>50	>50
4b	14.3 (7.54−27.0)	12.4** (6.44−23.8)	12.1** (7.28−20.2)	13.0** (7.40−22.8)	19.4** (7.96−47.5)	40.8 (13.2−126)
5b	32.6 (25.2−42.1)	16.7** (10.2−27.3)	15.2** (11.5−20.1)	22.4* (16.2−31.0)	45.2 (31.4−65.1)	>50
5c	5.90* (4.70−7.41)	11.8** (6.14−22.5)	13.4** (11.3−15.9)	19.6* (12.8−30.1)	45.0 (34.1−59.4)	>50

[a] Significant differences among compounds **1** and **3**−**5** were evaluated at the corresponding times and denoted as *$p < 0.05$ and **$p < 0.001$ using the method of Litchfield and Wilcoxon.[27]

Table 4. ED_{50} Values of **1** and **3**−**5** Against AMPA- and KA-Induced Seizures and Against Audiogenic Seizures after Pretreatment with Aniracetam in DBA/2 Mice

	ED_{50}, μmol/kg[a] (±95% confidence limits)				
	AMPA[b]			pretreatment with aniracetam[d]	
compd	clonic phase	tonic phase	KA[c]	clonic phase	tonic phase
1	57.5 (43.5−76.0)	40.5 (26.3−60.8)	27.8 (18.8−40.9)	134 (88.8−203)*	100 (63.4−158)*
3a	70.3 (58.7−84.1)	56.9 (40.9−79.2)	49.9 (29.6−84.0)	ND	ND
3b	29.2 (16.9−50.4)	23.8 (14.2−39.9)	23.2 (14.7−36.6)	55.0 (36.0−84.2)*	40.6 (30.1−54.9)*
3c	37.9 (27.3−52.8)	28.5 (20.6−39.4)	19.6 (7.74−49.6)	62.6 (44.7−87.7)*	39.6 (22.9−68.7)*
4a	23.4 (14.3−38.4)	14.2 (7.08−28.4)	11.5 (5.81−22.9)	44.9 (32.4−62.2)*	38.1 (28.2−58.5)*
4b	18.6 (6.44−53.6)	10.3 (5.27−20.1)	8.61 (4.85−15.3)	33.9 (23.1−49.8)*	31.0 (21.7−44.3)*
5a	46.3 (32.9−65.2)	35.4 (23.1−54.0)	32.8 (21.7−49.6)	85.9 (71.5−100)*	65.2 (43.1−98.6)*
5b	32.8 (26.5−40.6)	26.2 (19.3−34.2)	18.6 (6.44−53.6)	65.2 (43.1−98.6)*	85.9 (71.5−103)*
5c	15.6 (9.86−24.8)	11.5 (9.76−13.6)	15.6 (5.37−45.3)	31.4 (15.8−62.4)*	16.3 (6.87−38.7)*

[a] All data were calculated according to the method of Litchfield and Wilcoxon.[27] At least 32 animals were used to calculate each ED_{50} value. [b] AMPA was administered icv at the CD_{97} for either clonus (9.7 nmol) or forelimb tonic extension (11.7 nmol) 30 min after injection of tested compounds. [c] KA was administered sc at the CD_{97} (32 mg/kg) 15 min after injection of tested compounds. [d] Significant differences between ED_{50} values of the group treated with aniracetam + 2,3-benzodiazepine and the group treated with 2,3-benzodiazepine alone (Table 1) are denoted as *$p < 0.01$; ND, not determined.

the therapeutic index (TI) of all derivatives was always more favorable than that of compound **1** (Table 1).

Binding experiments, carried out on crude cortical synaptic membranes prepared from the rat brain, showed that derivatives **4b** and **5c** (100 μM) failed to displace [^{3}H]spiperone from dopamine and 5-hydroxytryptamine$_1$ receptors (5-HT$_1$); [^{3}H]ketanserin from 5-hydroxytryptamine$_2$ receptors (5-HT$_2$); [^{125}I]pindolol from β-adrenergic receptors; [^{3}H](*RS*)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid ([^{3}H]CPP) and [^{3}H]-dizocilpine from NMDA receptors; [^{3}H]5,7-dichlorokynurenic acid ([^{3}H]5,7-DCKA) from glycine site on NMDA receptors; [^{3}H]AMPA and [^{3}H]6-cyano-7-nitroquinoxaline-2,3-dione ([^{3}H]CNQX) from AMPA/KA receptors; mixture of [^{3}H](1*S*,3*R*)-ACPD and [^{3}H](1*R*,3*S*)-ACPD from metabotropic glutamate receptors. Due to the lack of activity at dopamine, serotonin, noradrenaline, NMDA, glycine site on NMDA, and metabotropic glutamate receptors, it is conceivable to assume that these new series of 2,3-benzodiazepines act as noncompetitive AMPA antagonists. Nevertheless, since no reliable ligand-binding assay has been reported for noncompetitive agonists and antagonists affecting the AMPA receptor complex,[14] our data do not exclude an interaction at other sites involved in the generation or expression of seizures.

Block of KA-evoked current by **3c**, **4b**, and **5c** was assessed in whole-cell voltage clamp recording from cerebellar granule neurons. KA elicits a current that is mediated by the activation of both AMPA and KA receptors. Application of 100 μM KA induced an inward current that is reduced by the coapplication of KA and 100 μM **4b** (Figure 3). The degree of block of the peak currents produced by each antagonist **3c**, **4b**, and **5c** (100 μM), expressed as the percent of reduction of the KA currents (100%), is reported in Table 5. On the basis of the structural similarity of **3c**, **4b**, and **5c** with GYKI 53655 (**2**), it is reasonable to assess that the reduction of the current is due to the selective block of the AMPA-mediated component, while the remaining fast desensitizing current results from the activation of the KA receptors. The extent of the current reduction was slightly higher for **3c** than for **4b** and **5c**. The higher activity in the in vivo test systems of **4b** and **5c** with respect to **3c** is likely due to their higher in vivo bioavailability. Since GYKI 52466 potency has been shown to be different at diverse AMPA receptor subtypes,[15] it is also possible that **4b** and **5c** show a preferential activity at the level of specific AMPA receptor subtypes involved in the genesis of seizure.

The anticonvulsant activity of compounds **4** and **5** was effective at doses which did not cause sedation and ataxia, in agreement with several studies which showed an anticonvulsant activity of AMPA receptor antagonists at doses that do not affect behavior and learning processes.[16] It is noteworthy that the present compounds **4** and **5** cause marginal motor impairment in the rotarod test at variance of **1**; some of them possess TI values approximately twice that of **1** (Table 1).

In conclusion, the present results indicate that re-

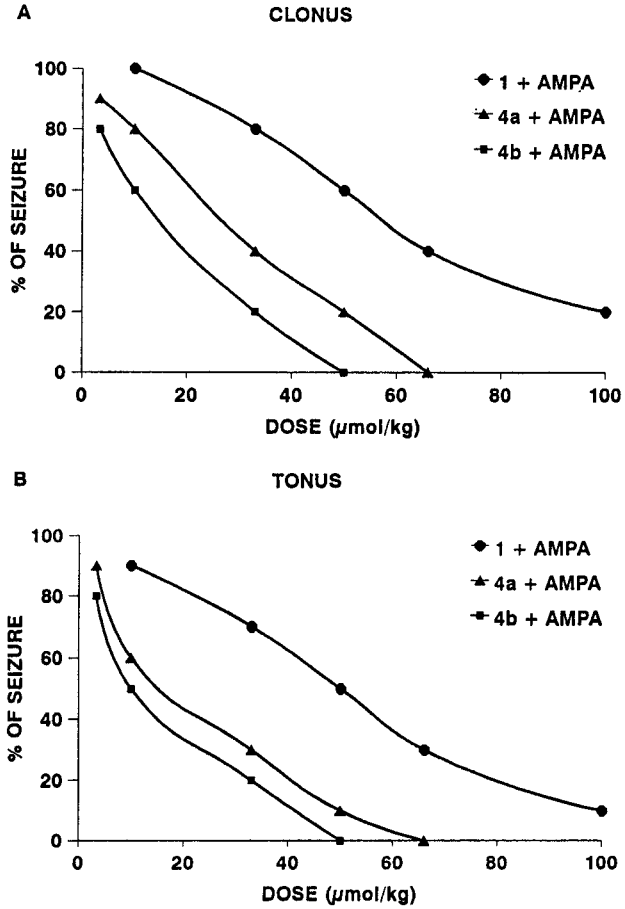


Figure 1. Anticonvulsant effects of **4a,b** and **1** against seizures induced by AMPA in DBA/2 mice. Ordinate shows % of response of clonic (A) or tonic (B) seizures; abscissa shows the dose in μmol/kg ip. For the determination of each point, 10 animals were used.

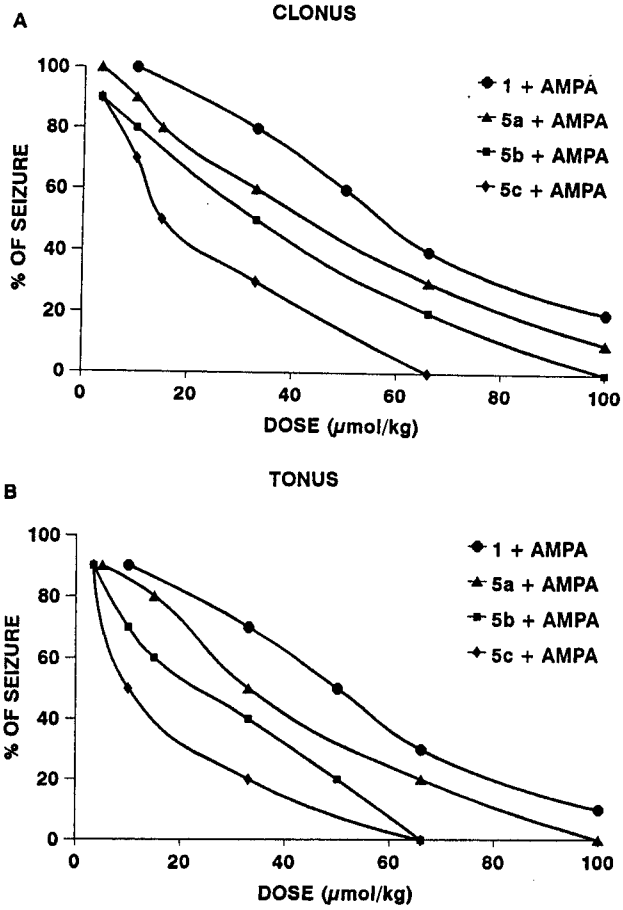


Figure 2. Anticonvulsant effects of **5a**−**c** and **1** against seizures induced by AMPA in DBA/2 mice. Ordinate shows % of response of clonic (A) or tonic (B) seizures; abscissa shows the dose in μmol/kg ip. For the determination of each point, 10 animals were used.

placement of the carbonyl group of 7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-ones with the bioisosteric thiocarbonyl moiety and introduction of a methylcarbamoyl group at N-3 bring about an increase in anticonvulsant potency. Derivative **4b**, in analogy to that observed with the analogues of GYKI 52466, shows ED_{50} values against MES- and KA-induced seizures 2−3-fold higher than parent compound **3c**. Nevertheless, such an increase was observed in the in vivo tests and not in the electrophysiological experiments. Interestingly, derivative **4b** is provided with a longer-lasting anticonvulsant activity and a lower toxicity. As a consequence, 1-(4-aminophenyl)-3,5-dihydro-3-methylcarbamoyl-7,8-methylenedioxy-4*H*-benzodiazepin-4-one (**4b**) may become a useful tool in the mapping of the AMPA/KA receptor complex.

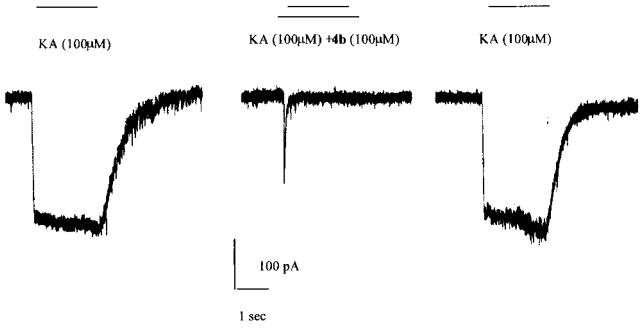


Figure 3. Representative trace of the inward current evoked by the application of 100 μM KA and its reduction in the presence of compound **4b** (100 μM). The cells were voltage-clamped at −60 mV. Bars show the duration of drug application.

Experimental Section

Chemistry. Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Elemental analyses were carried out on a Carlo Erba 1106 elemental analyzer for C, H, and N, and the results are within ±0.4% of the theoretical values. Merck silica gel 60 F_{254} plates were used for analytical TLC; column chromatography was performed on Merck silica gel 60 (70−230 mesh). ^{1}H NMR spectra were recorded in $CDCl_3$ by means of a Varian Gemini-300 spectrometer. Chemical shifts were expressed in δ (ppm) relative to TMS as internal standard, and coupling constants (*J*) are in Hz. All exchangeable protons were confirmed by addition of D_2O.

General Procedure for the Synthesis of 3-Alkylcarbamoyl-1-(3- or 4-aminophenyl)-3,5-dihydro-7,8-methylenedioxy-4*H*-benzodiazepin-4-ones 4a−h. To a solution of **3d** or **3e** (0.5 g, 1.54 mmol) in CH_2Cl_2 (100 mL) were added triethylamine (2 mL, 14.4 mmol) and the suitable isocyanate (7.7 mmol). The reaction mixture was stirred at room temperature for 36 h and then concentrated in vacuo. The resulting residue was purified by column chromatography with $CHCl_3$/EtOAc (70:30) as eluant. The subsequent hydrogenation was carried out at atmospheric pressure by adding 5% Pd/C (50 mg) to a methanol solution (40 mL) of the nitro derivative. The mixture was shaken under hydrogen for 3 h and the Pd/C was filtered out. The solvent was removed in vacuo and the

Table 5. Reduction of KA-Evoked Current Induced by **1**, **2**, **3c**, **4b**, and **5c**[a]

compd	% reduction[b] (mean ± SE)
1	73 ± 3
2	87 ± 3
3c	79 ± 4
4b	58 ± 4
5c	66 ± 6

[a] KA and compounds **1**, **2**, **3c**, **4b**, and **5c** were tested at 100 μM. [b] Each value is the mean ± SE of at least 10 cells.

resulting residue was purified by column chromatography with $CHCl_3$/MeOH (95:5) as eluant to give **4a**−**h**. All compounds were recrystallized from EtOAc.

1-(3-Aminophenyl)-3,5-dihydro-3-methylcarbamoyl-7,8-methylenedioxy-4*H*-benzodiazepin-4-one (4a): mp 137−140 °C (0.43 g, 80%); ^{1}H NMR 2.92 (d, 3H, J = 4.5, CH_3), 3.52 (s, 2H, CH_2-5), 3.82 (bs, 2H, NH_2), 6.05 (m, 2H, OCH_2O), 6.71 (s, 1H, H-9), 6.81 (dd, 1H, J = 1.6 and 8.0, H-4′), 6.86 (s, 1H, H-6), 6.88 (dd, 1H, J = 1.6 and 8.0, H-6′), 7.18 (t, 1H, J = 8.0, H-5′), 7.24 (s, 1H, H-2′), 8.62 (bs, 1H, NH). Anal. ($C_{18}H_{16}N_4O_4$) C, H, N.

1-(4-Aminophenyl)-3,5-dihydro-3-methylcarbamoyl-7,8-methylenedioxy-4*H*-benzodiazepin-4-one (4b): mp 140−143 °C (0.35 g, 65%); ^{1}H NMR 2.91 (d, 3H, J = 4.7, CH_3), 3.49 and 3.54 (dd, 2H, J = 12.4, CH_2-5), 3.98 (bs, 2H, NH_2), 6.05 (m, 2H, OCH_2O), 6.68 (d, 2H, J = 8.7, H-3′,5′), 6.73 (s, 1H, H-9), 6.86 (s, 1H, H-6), 7.56 (d, 2H, J = 8.7, H-2′,6′), 8.66 (bs, 1H, NH). Anal. ($C_{18}H_{16}N_4O_4$) C, H, N.

1-(3-Aminophenyl)-3,5-dihydro-3-ethylcarbamoyl-7,8-methylenedioxy-4*H*-benzodiazepin-4-one (4c): mp 110−112 °C (0.39 g, 70%); ^{1}H NMR 1.20 (t, 3H, J = 7.4, CH_3), 3.38 (m, 2H, CH_2), 3.51 (s, 2H, CH_2-5), 3.80 (bs, 2H, NH_2) 6.05 (m, 2H, OCH_2O), 6.71 (s, 1H, H-9), 6.88 (dd, 1H, J = 1.5 and 8.0, H-4′), 6.86 (s, 1H, H-6), 6.88 (dd, 1H, J = 1.5 and 8.0, H-6′), 7.18 (t, 1H, J = 8.0, H-5′), 7.25 (s, 1H, H-2′), 8.73 (bs, 1H, NH). Anal. ($C_{19}H_{18}N_4O_4$) C, H, N.

1-(4-Aminophenyl)-3,5-dihydro-3-ethylcarbamoyl-7,8-methylenedioxy-4*H*-benzodiazepin-4-one (4d): mp 111−114 °C (0.46 g, 81%); ^{1}H NMR 1.20 (t, 3H, J = 7.4, CH_3), 3.37 (m, 2H, CH_2), 3.48 and 3.53 (dd, 2H, J = 12.5, CH_2-5), 4.00 (bs, 2H, NH_2), 6.05 (m, 2H, OCH_2O), 6.68 (d, 2H, J = 8.4, H-3′,5′), 6.73 (s, 1H, H-9), 6.86 (s, 1H, H-6), 7.56 (d, 2H, J = 8.4, H-2′,6′), 8.69 (bs, 1H, NH). Anal. ($C_{19}H_{18}N_4O_4$) C, H, N.

1-(3-Aminophenyl)-3,5-dihydro-7,8-methylenedioxy-3-propylcarbamoyl-4*H*-benzodiazepin-4-one (4e): mp 116−118 °C (0.42 g, 72%); ^{1}H NMR 0.94 (t, 3H, J = 7.3, CH_3), 1.59 (m, 2H, CH_2), 3.30 (m, 2H, CH_2), 3.52 (s, 2H, CH_2-5), 3.80 (bs, 2H, NH_2), 6.04 (m, 2H, OCH_2O), 6.71 (s, 1H, H-9), 6.80 (dd, 1H, J = 1.4 and 7.9, H-4′), 6.86 (s, 1H, H-6), 6.88 (dd, 1H, J = 1.4 and 7.9, H-6′), 7.18 (t, 1H, J = 7.9, H-5′), 7.24 (s, 1H, H-2′), 8.73 (bs, 1H, NH). Anal. ($C_{20}H_{20}N_4O_4$) C, H, N.

1-(4-Aminophenyl)-3,5-dihydro-7,8-methylenedioxy-3-propylcarbamoyl-4*H*-benzodiazepin-4-one (4f): mp 135−137 °C (0.41 g, 70%); ^{1}H NMR 0.94 (t, 3H, J = 7.4, CH_3), 1.58 (m, 2H, CH_2), 3.27 (m, 2H, CH_2), 3.48 and 3.54 (dd, 2H, J = 12.4, CH_2-5), 3.97 (bs, 2H, NH_2), 6.05 (m, 2H, OCH_2O), 6.68 (d, 2H, J = 8.7, H-3′,5′), 6.74 (s, 1H, H-9), 6.86 (s, 1H, H-6), 7.56 (d, 2H, J = 8.7, H-2′,6′), 8.74 (bs, 1H, NH). Anal. ($C_{20}H_{20}N_4O_4$) C, H, N.

1-(3-Aminophenyl)-3-butylcarbamoyl-3,5-dihydro-7,8-methylenedioxy-4*H*-benzodiazepin-4-one (4g): mp 176−178 °C (0.39 g, 65%); ^{1}H NMR 0.93 (t, 3H, J = 7.4, CH_3), 1.35 (m, 2H, CH_2), 1.49 (m, 2H, CH_2), 3.34 (m, 2H, CH_2), 3.52 (s, 2H, CH_2-5), 3.90 (bs, 2H, NH_2), 6.05 (m, 2H, OCH_2O), 6.71 (s, 1H, H-9), 6.81 (dd, 1H, J = 1.5 and 8.0, H-4′), 6.86 (s, 1H, H-6), 6.88 (dd, 1H, J = 1.5 and 8.0, H-6′), 7.18 (t, 1H, J = 7.8, H-5′), 7.25 (s, 1H, H-2′), 8.73 (bs, 1H, NH). Anal. ($C_{21}H_{22}N_4O_4$) C, H, N.

1-(4-Aminophenyl)-3-butylcarbamoyl-3,5-dihydro-7,8-methylenedioxy-4*H*-benzodiazepin-4-one (4h): mp 173−175 °C (0.41 g, 67%); ^{1}H NMR 0.93 (t, 3H, J = 7.4, CH_3), 1.35 (m, 2H, CH_2), 1.39 (m, 2H, CH_2), 3.33 (m, 2H, CH_2), 3.48 and 3.53 (dd, 2H, J = 12.4, CH_2-5), 3.97 (bs, 2H, NH_2), 6.05 (m, 2H, OCH_2O), 6.68 (d, 2H, J = 8.7, H-3′,5′), 6.74 (s, 1H, H-9), 6.86 (s, 1H, H-6), 7.56 (d, 2H, J = 8.7, H-2′,6′), 8.72 (bs, 1H, NH). Anal. ($C_{21}H_{22}N_4O_4$) C, H, N.

General Procedure for the Synthesis of 1-Aryl-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepine-4-thiones 5a−c. A solution of **3a**−**c** (1 mmol) and Lawesson's reagent (0.22 g, 0.55 mmol) in dry toluene (50 mL) was heated to reflux for 2 h. The solution was then cooled at room temperature and filtered. The toluene was removed in vacuo, the residue was purified by column chromatography with EtOAc/CCl_4 (70:30) as eluant and recrystallized from EtOAc to give **5a**−**c**.

3,5-Dihydro-7,8-methylenedioxy-1-phenyl-4*H*-2,3-benzodiazepine-4-thione (5a): mp 201−203 °C (0.25 g, 85%); ^{1}H NMR 3.88 (bs, 2H, CH_2-5), 6.04 (s, 2H, OCH_2O), 6.61 (s, 1H, H-9), 6.88 (s, 1H, H-6), 7.41−7.63 (m, 5H, Ar), 9.91 (bs, 1H, NH). Anal. ($C_{16}H_{12}N_2O_2S$) C, H, N.

1-(3-Aminophenyl)-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepine-4-thione (5b): mp 122−124 °C (0.23 g, 75%); ^{1}H NMR 3.87 (bs, 2H, CH_2-5), 6.03 (s, 2H, OCH_2O), 6.64 (s, 1H, H-9), 6.81(d, 1H, J = 7.8, H-6′), 6.86 (s, 1H, H-6), 6.89 (d, 1H, J = 7.8, H-4′), 6.97 (s, 1H, H-2′), 7.20 (t, 1H, J = 7.8, H-5′), 8.45 (bs, 1H, NH). Anal. ($C_{16}H_{13}N_3O_2S$) C, H, N.

1-(4-Aminophenyl)-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepine-4-thione (5c): mp 138−140 °C (0.24 g, 77%); ^{1}H NMR 3.84 (bs, 2H, CH_2-5), 6.03 (s, 2H, OCH_2O), 6.68 (s, 1H, H-9), 6.86 (s, 1H, H-6), 6.68 (d, 2H, J = 8.3, H-3′,5′), 7.43 (d, 2H, J = 8.3, H-2′,6′), 9.91 (bs, 1H, NH). Anal. ($C_{16}H_{13}N_3O_2S$) C, H, N.

Lipophilicity Measurements. The relative lipophilicity (R_m) of the compounds was measured by reversed-phase high-performance thin-layer chromatography (RP-HPTLC) according to the method previously described.[17] Briefly, Whatman KC18F plates were used as the nonpolar stationary phase. The plates were dried at 105 °C for 1 h before use. The polar mobile phase was a 2:1 (v/v) mixture of acetone and water. Each compound was dissolved in $CHCl_3$ (3 mg/mL), and 1 μL of solution was applied onto the plate. The experiments were repeated five times with different disposition of the compounds on the plate. The R_f values were expressed as the mean values of the five determinations. The R_m values were calculated from the experimental R_f values according to the formula $R_m = \log[(1/R_f) - 1]$. Higher R_m values indicate higher lipophilicity.

Testing of Anticonvulsant Activity Against Audiogenic Seizures in DBA/2 Mice. All experiments were performed with DBA/2 mice which are genetically susceptible to sound-induced seizures.[18] Male DBA/2 mice (8−12 g, 22−25 days old) were purchased from Charles River (Calco, Como, Italy). Groups of 10 mice were exposed to auditory stimulation 30 min following administration of vehicle or each dose of drugs studied. The compounds were given ip (0.1 mL/10 g of body weight of the mouse) as a freshly prepared solution in 30% dimethyl sulfoxide (DMSO) and 70% sterile saline (0.9% NaCl). Individual mice were placed under a hemispheric Perspex dome (diameter 58 cm) and were allowed 60 s for habituation. Assessment of locomotor activity was also made during this time period. Auditory stimulation (12−16 kHz, 109 dB) was applied for 60 s or until tonic extension occurred and induced a sequential seizure response in control DBA/2 mice, consisting of an early wild running phase, followed by generalized myoclonus and tonic flexion and extension, sometimes followed by respiratory arrest. The control and drug-treated mice were scored for latency to and incidence of the different phases of the seizures.[19] The time course of the anticonvulsant action of **3** and **5** was determined following the administration of 33 μmol/kg of each benzodiazepine derivative to groups of 10 mice for each time. The animals were tested for sound-induced seizure responses at 5−180 min after drug administration.

MES Test in Swiss Mice. Electrical stimuli were applied via ear-clip electrodes to Swiss mice (rectangular constant current impulses, amplitude 50 mA, width 20 ms, frequency 35 Hz, duration 400 ms) according to the method of Swinyard

et al.[20] Abolition of tonic hindlimb extension after drug treatment was considered as the endpoint of protection. In general, the dose−response curves were estimated by testing four to five doses using 8−10 mice for each dose.

PTZ-Induced Seizures in Swiss Mice. Male Swiss mice (20−26 g, 42−48 days old) were purchased from Charles River (Calco, Como, Italy) and pretreated with vehicle or drug 45 min before sc administration of PTZ. For systemic injections, all tested compounds were given ip (0.1 mL/10 g of body weight of the mouse) as a freshly prepared solution in 30% DMSO and 70% sterile saline (0.9% NaCl). The convulsive dose 97 (CD_{97}) of PTZ (85 mg/kg) was applied, and the animals were observed for 30 min. A threshold convulsion was an episode of clonic spasms lasting for at least 5 s. The absence of this threshold convulsion over 30 min indicated that the tested substance had the ability to elevate PTZ seizure threshold.[21]

AMPA-Induced Seizures in DBA/2 Mice. Seizures were also induced by icv injection of AMPA. The CD_{50} of AMPA for clonus was 1.76 (1.06−3.07), while that for tonus was 2.90 (1.83−4.58) nmol. For icv injection, mice were anesthetized with diethyl ether, and injections were made in the left or right lateral ventricle (coordinates 1 mm posterior and 1 mm lateral to the bregma, depth 2.4 mm) using a 10-μL Hamilton microsyringe (type 701N) fitted with a nylon cuff on the needle as previously described;[22] injections of drugs by this procedure led to a uniform distribution throughout the ventricular system within 10 min. The animals were placed singly in a 30- × 30- × 30-cm box, and the observation time was 30 min after the administration of AMPA.

Pretreatment with Aniracetam. The icv microinjection of aniracetam was performed according to experimental procedures previously described for AMPA microinjection.[21] The dose of aniracetam (50 nmol icv) was administered 60 min before auditory stimulation or 30 min before each compound in DBA/2 mice.

KA-Induced Seizures in Swiss Mice. KA was administered sc at a dose of 32 mg/kg (previously determined CD_{97} value) 15 min after ip administration of the 2,3-benzodiazepine derivative. Animals showing 5 s or more of clonic activity were scored as not protected according to Donevan et al.[6] The period of observation was 60 min.

Electrophysiology. Primary cultures of cerebellar granule neurons were prepared from 7−8 days old Sprague−Dawley rats as previously described.[23] Briefly cells from cerebella were dispersed with trypsin (0.24 mg/mL) and plated at a density of 10^6 cells/mL on 35-mm Falcon dishes coated with poly-L-lysine (10 μg/mL). Cells were grown in basal Eagle's medium, supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 μg/mL gentamycin, and maintained at 37 °C in 5% CO_2. Cytosine arabinofuranoside (10 μM) was added to the cultures 24 h after plating to prevent astroglia proliferation.

Electrophysiological Recordings. Recordings were performed on single cerebellar granule neurons after 7 days in culture[23] using the voltage-clamp technique in the whole-cell configuration.[24] Electrodes were pulled from borosilicate glass on a vertical puller and had a resistance of 5−7 MΩ when filled with KCl internal solution. Currents were amplified with an Axopatch 1D amplifier, filtered at 5 kHz, and digitized at 10 kHz by using pClamp software. Intracellular solution contained (mM): KCl 140, $MgCl_2$ 3, EGTA 5, Hepes 5, ATP-Na 2, pH 7.3 with KOH. Cells were continuously perfused with the external solution (mM): NaCl 145, KCl 5, $CaCl_2$ 1, Hepes 5, glucose 5, sucrose 20, pH 7.4 with NaOH. KA was purchased from Sigma, GYKI 52466 was from RBI, and GYKI 53655 was a gift from Lilly Research Lab. GYKI 52466, GYKI 53655, **3c**, **4b**, and **5c** were dissolved in DMSO and diluted at the final concentration (<1%) in extracellular medium. KA was dissolved in the extracellular solution. All drugs were applied directly by gravity through a Y-tube perfusion system.[25]

Binding Studies. The binding affinities were obtained by the methods previously described by us.[8]

Effects on Motor Movements. Male Swiss mice (20−26 g, 48−54 days old) were purchased from Charles River (Calco, Como, Italy). Groups of 10 mice were trained to do coordinated motor movements continuously for 2 min on a rotarod, 3 cm in diameter, at 8 rpm (U. Basile, Comerio, Varese, Italy). Impairment of coordinated motor movements was defined as the inability of the mice to remain on the rotarod for a 2-min test period.[26] The ability of the mice to remain on the rotarod was tested 30 min after administration of various compounds.

Statistical Analysis. Statistical comparisons between groups of control and drug-treated animals were made using Fisher's exact probability test (incidence of the seizure phases) or ANOVA followed by post hoc Dunnett's *t*-test (rectal temperatures). The ED_{50} values of each phase of the audiogenic seizure or seizures induced by MES, PTZ, AMPA, or KA were determined for each dose of compound administered, and dose−response curves were fitted using a computer program by the method of Litchfield and Wilcoxon.[27] The relative anticonvulsant activities were determined by comparison of respective ED_{50} values. The TD_{50} values were estimated using the method of Litchfield and Wilcoxon.[27] The relative activities were determined by comparison of respective TD_{50} values. For the binding experiments IC_{50} values were determined by the nonlinear curve-fitting program based on Ligand.[28] Statistical significance between control and test groups of data means was tested using a two-tailed Student's *t*-test. Electrophysiological data were analyzed using the software Clampex (Axon Instrument). Results are expressed as mean ± SE. Origin (Microcal Software, Northampton, MA) was used for figure preparation and statistical analysis.

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