3,5-Dihydro-4*H*-2,3-benzodiazepine-4-thiones: A New Class of AMPA Receptor Antagonists

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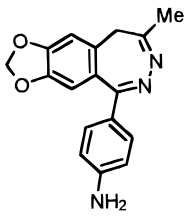
Synthesis and evaluation of anticonvulsant activity of a series of 2,3-benzodiazepin-4-ones (**2**) chemically related to 1-(4′-aminophenyl)-4-methyl-7,8-(methylenedioxy)-5*H*-2,3-benzodiazepine (**1**, GYKI 52466) have been reported in our recent publications. Compounds **2** manifested marked anticonvulsant properties acting as 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)-propionic acid (AMPA) receptor antagonists. In an attempt to better define the structure−activity relationships (SAR) and to obtain more potent and selective anticonvulsant agents, 1-aryl-3,5-dihydro-4*H*-2,3-benzodiazepine-4-thiones **3** were synthesized from the corresponding isosteres **2**. The evaluation is reported of their anticonvulsant effects, both in the audiogenic seizures test with DBA/2 mice and against the maximal electroshock- and pentylenetetrazole-induced seizures in Swiss mice. New derivatives **3** showed higher potency, less toxicity and longer-lasting anticonvulsant action than those of the parent compounds **2** in all tests employed. Analogous to derivatives **2**, new compounds **3** do not affect the benzodiazepine receptor (BZR) while they do antagonize AMPA-induced seizures; their anticonvulsant activity is reversed by pretreatment with aniracetam but not with flumazenil, thus suggesting a clear involvement of AMPA receptors. Electrophysiological data indicate a noncompetitive blocking mechanism at the AMPA receptor sites for **3i**, the most active of the series and over 5-fold more potent than **1**.

Introduction

Glutamate, the neurotransmitter at most of the excitatory synapses in the brain, activates a variety of receptor subtypes that can be broadly divided into ionotropic and metabotropic receptors. Ionotropic receptors mediate fast excitatory synaptic transmission and, on the basis of pharmacological and molecular biological studies, are named *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors.[3] The non-NMDA receptor group is further divided into 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA) and kainate subtypes.

There is strong evidence that both NMDA and non-NMDA receptors play an important role in acute neurodegeneration and in convulsant phenomena.[4] Hence, all subtypes of these receptors are potential drug targets for therapeutic intervention in a number of central nervous system (CNS) diseases.[5]

Several previous studies have indicated that the systemic administration of AMPA receptor antagonists may be useful in the treatment of epilepsy and cerebral ischemia[6−9] and that some 2,3-benzodiazepine derivatives such as 1-(4′-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (**1**, GYKI 52466) act as



1 (GYKI 52466)

highly selective noncompetitive antagonists at AMPA receptors.[10−13] They possess anticonvulsant properties in various seizure models but, contrary to the classical 1,4-benzodiazepines, lack sedative−hypnotic activity and do not bind to benzodiazepine receptors (BZR).[14]

In light of these pharmacological properties and as a part of a program on potential anticonvulsant agents,[15−17] our research group has been involved in designing new compounds structurally related to **1**.[1] After the first promising results and in an attempt to better define the structure−activity relationships (SAR), we designed and synthesized a wider series of 1-aryl-3,5-dihydro-4*H*-2,3-benzodiazepin-4-ones **2** bearing suitably selected substituents on the heptatomic ring.[2] By modification of the substitution pattern, we obtained new potent anticonvulsant agents which showed longer-lasting activity and less toxicity than those of compound **1**; moreover, they proved to be selective AMPA receptor antagonists.[2]

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With the goal of developing more potent and selective compounds and improving the definition of the structure−activity relationships, we now report the synthesis and biological activity of 1-aryl-3,5-dihydro-4*H*-2,3-benzodiazepine-4-thiones **3a**−**k**, a new class of benzodiazepine derivatives obtained from the corresponding isosteres **2a**−**k**, with the aim to explore the effects of the lipophilicity on the anticonvulsant activity.

All synthesized compounds were tested against sound-induced seizures in DBA/2 mice; this has been considered an excellent animal model for generalized epilepsy and for screening new anticonvulsant drugs.[18]

Compounds **3a** and **3i**, the most active of the series, were also evaluated both against pentylenetetrazole- and maximal electroshock-induced seizures in Swiss mice.

In addition, with the aim to confirm that their anticonvulsant activity is mediated by the affinity for AMPA receptor, we also evaluated **3a** and **3i** against AMPA-induced seizures with or without a concomitant treatment with aniracetam. Electrophysiological experiments were conducted in order to investigate the mode of inhibitory action at AMPA receptor sites, and furthermore, the time course of anticonvulsant activity was studied.

BZR affinity was assessed by the potencies of the 4*H*-2,3-benzodiazepine-4-thiones to inhibit [^{3}H]flumazenil binding in a cortical membrane preparation, or by the ability of flumazenil, a "neutral" BZR antagonist, to reverse their anticonvulsant activity.

The results obtained were compared to the findings previously reported[2] for compounds **2**, and the resulting modulation of anticonvulsant activity is described.

Chemistry

Compounds **2a**−**k** were prepared by a multistep synthetic pathway according to a method previously described.[2] The target 1-aryl-3,5-dihydro-4*H*-2,3-benzodiazepine-4-thiones **3a**−**k** were obtained in good yields by treatment with Lawesson's reagent of compounds **2a**−**k** (Scheme 1).

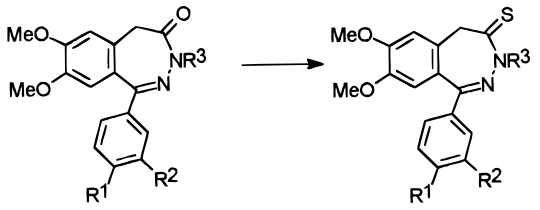
The structures of all synthesized compounds were determined by analytical and spectroscopic data (^{1}H NMR) and are reported in the Experimental Section.

Results and Discussion

1-Aryl-3,5-dihydro-4*H*-2,3-benzodiazepine-4-thiones **3a**−**k** were evaluated after intraperitoneal (ip) administration in DBA/2 mice, a strain genetically susceptible to sound-induced seizures,[18] and the results were compared with the anticonvulsant properties of the corresponding 4*H*-2,3-benzodiazepin-4-ones **2a**−**k** and **1**. Table 1 reports the median effective dose (ED_{50}) values required to prevent clonic and tonic phases of seizures. The rank order of anticonvulsant potency was as follows: **3i** > **2i** > **3j** > **3a** > **2j** > **3k** > **3f** > **2a** > **1** > **2f** > **2k** > **3c** > **2g** > **3b** > **3d** > **2b** > **3h** > **3g** > **2c**. The remaining compounds showed no activity.

The introduction of a thiocarbonyl group at the C-4 position of the heptatomic ring generally leads to new compounds **3** with anticonvulsant properties higher than those of the carbonyl isostere analogues **2**. In particular, 1-(4′-aminophenyl) derivative **3i** is 2.5-fold more active than the corresponding **2i**.

Scheme 1



2 a-k **3 a-k**

	R1	R2	R3
a	H	H	H
b	Cl	H	H
c	Br	H	H
d	NO_2	H	H
e	H	NO_2	H
f	H	H	Me
g	Br	H	Me
h	NO_2	H	Me
i	NH_2	H	H
j	H	NH_2	H
k	NH_2	H	Me

Table 1. Anticonvulsant Activity of Compounds **1**−**3** against Audiogenic Seizures in DBA/2 Mice and Relative Lipophilicity (R_m)

	ED_{50}, μmol/kg[a] (±95% confidence limits)		
compd	clonic phase	tonic phase	R_m
2a[b]	33.9(26.0−44.2)	31.8(24.8−40.6)	−0.244
3a	19.7(13.1−29.7)	15.0(8.60−26.1)	0.014
2b[b]	102(76.1−137)	75.3(60.7−93.2)	0.012
3b	82.4(35.2−193)	55.0(36.0−84.2)	0.244
2c[c]	110(79.3−151)	82.5(58.6−116)	0.035
3c	52.1(23.3−118)	33.3(15.2−73.1)	0.269
2d[c]	>120	>120	−0.113
3d	93.4(64.7−135)	69.8(51.4−94.8)	0.122
2e[c]	>120	>120	−0.135
3e	ND	ND	0.092
2f[c]	37.8(23.7−60.1)	26.7(14.7−48.2)	−0.003
3f	30.6(19.6−44.7)	24.7(14.3−42.6)	0.282
2g[c]	63.0(33.0−121)	38.0(20.0−72.0)	0.263
3g	105(52.0−194)	60.4(23.6−154)	0.575
2h	>120	>120	0.110
3h	103(73.6−144)	81.5(63.3−105)	0.333
2i[c]	15.0(9.01−24.0)	12.6(8.01−19.0)	−0.630
3i	6.30(2.60−15.4)	3.30(1.30−8.30)	−0.308
2j[c]	19.3(16.9−22.0)	18.3(16.0−20.8)	−0.542
3j	18.8(8.70−36.5)	9.10(3.70−22.3)	−0.259
2k[c]	50.2(34.6−73.0)	43.7(31.3−61.0)	−0.421
3k	29.8(21.4−41.4)	20.3(13.6−30.5)	−0.072
1	35.8(24.4−52.4)	25.3(16.0−40.0)	−0.308

[a] All data were calculated according to the method of Litchfield and Wilcoxon.[32] At least 32 animals were used to calculate each ED_{50} value. [b] Reference 1. [c] Reference 2. ND = not detectable.

Analogous to the **2** series, in the new thiocarbonyl derivatives **3**, the presence of a methyl group at N-3 led to compounds (i.e., **3f**, **3g**, **3h**, and **3k**) with anticonvulsant properties weaker than those of N-3 unsubstituted derivatives.

The different anticonvulsant potencies of the 1-aryl-3,5-dihydro-4*H*-2,3-benzodiazepine-4-thiones may be correlated to their relative lipophilicities (Table 1), which positively influence the cellular permeability and, therefore, the penetration through the blood−brain barrier.

Unexpectedly, the 3′-nitrosubstituted derivative **3e** induced clonic jerks in DBA/2 mice. There is no

Table 2. Anticonvulsant Activity against the MES- and PTZ-Induced Seizures in Swiss Mice of **2a**, **3a**, **2i**, **3i**, and **1**

	ED_{50}, μmol/kg (±95% confidence limits)[a]	
compd	MES	PTZ
2a[b]	35.8(28.6−44.7)	68.2(54.6−85.2)
3a	27.8(21.5−35.9)	33.7(18.4−61.6)
2i[c]	15.9(7.3−33.5)	22.6(11.7−43.8)
3i	7.75(3.89−15.4)	15.4(7.00−33.9)
1	35.7(29.3−43.4)	68.3(56.2−83.1)

[a] All data were calculated according to the method of Litchfield and Wilcoxon.[32] At least 32 animals were used to calculate each ED_{50} value. [b] Reference 1. [c] Reference 2.

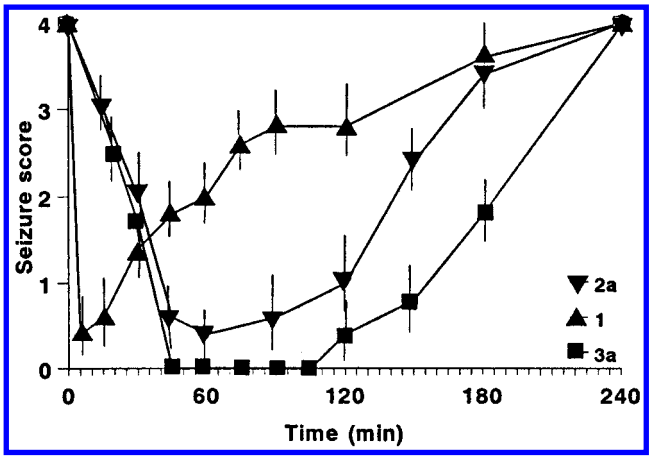


Figure 1. Anticonvulsant effects of **2a**, **3a**, and **1** (33 μmol/kg ip) plotted against audiogenic seizures in DBA/2 mice. The ordinate shows seizure score, the abscissa shows the time after intraperitoneal administration of drug in min. For the determination of each point 10 animals were used. The latter represents the mean seizure score (±SEM) against audiogenic seizures in DBA/2 mice.

explanation for this discrepancy, and one can only speculate that this may be due to a change in the interaction mode of **3e** which probably might act as an AMPA receptor agonist or bind to a different receptor site.

The most active derivatives of the series, **3a** and **3i**, their parent compounds, **2a** and **2i**, and **1** were further evaluated against seizures induced by maximal electroshock (MES) and the administration of pentylenetetrazole (PTZ), and the biological results are reported below.

As shown in Table 2, the tonic extension of the seizures induced by MES and the clonic phase of the seizures induced by PTZ were significantly reduced 45 min after ip administration of **2a**, **2i**, **3a**, **3i**, and **1** with the same rank order of anticonvulsant potency observed in the test of audiogenic seizures.

Following ip administration of some active compounds such as **2a** and **3a**, maximum protection was observed from 45 to 90 min for **2a** and from 45 to 120 min for compound **3a** with subsequent return to control seizure response at 180 min for derivative **2a** and at 240 min for compound **3a**, as shown in Figure 1. In contrast, **1** displayed maximum protection from 5 to 15 min followed by gradual return to control seizure response between 30 and 90 min (Figure 1).

The longer-lasting anticonvulsant activity of compounds **3a** could be explained taking into account also the influence of biotransformation reactions on molecular properties and the consequences of such changes.

Table 3. ED_{50} Values of **2a**, **3a**, **2i**, **3i**, and **1** against the Clonic and Tonic Seizures Induced by icv Injection of AMPA in DBA/2 Mice[a]

	ED_{50}, μmol/kg (±95% confidence limits)	
compd	clonic phase	tonic phase
2a[b]	66.0(45.9−94.9)	42.6(26.4−68.8)
3a	43.1(29.1−63.8)	31.1(19.3−50.2)
2i[c]	32.1(23.2−44.3)	25.0(16.5−30.0)
3i	17.1(7.70−38.0)	11.9(4.60−30.8)
1	57.5(43.5−76.0)	40.5(26.3−60.8)

[a] AMPA was administered icv at the CD_{97} for either clonus (9.7 nmol) or forelimb tonic extension (11.7) 30 min after injection of tested compounds. All data were calculated according to the method of Litchfield and Wilcoxon.[32] At least 32 animals were used to calculate each ED_{50} value. [b] Reference 1. [c] Reference 2.

Thiocarbonyl derivatives **3** could undergo biotransformation into the corresponding compounds **2** (as observed by preliminary HPLC studies on rat plasma) acting at first as unaltered and then as carbonyl isosters **2**.

The hypothesis that the derivatives of compounds **3** interact with non-NMDA receptors was also confirmed by suitable tests on some of them. Analogous to parent compounds **2**, **3a** and **3i** afforded effective protection against seizures induced by intracerebroventricular (icv) administration of AMPA, a chemical agent which stimulates the AMPA/kainate receptor complex (Table 3). The clonic and tonic phases of the AMPA-induced seizures were significantly reduced 30 min after ip administration of **3a**, **3i**, and **1** as shown in Figure 2.

In the present study we also demonstrated that aniracetam, a potentiator of AMPA effects,[19] markedly antagonized the anticonvulsant effects of **3a** and **3i** in DBA/2 mice (Table 4) with a pattern of activity similar to that of **1**, considered as AMPA/kainate receptor antagonist.[10c] Since it has been shown that aniracetam is a positive allosteric modulator of AMPA/kainate-selective glutamate receptors, we therefore suggest that, by analogy to **1**,[10d,11] **2a**, and **2i**,[2] also the 4*H*-2,3-benzodiazepine-4-thiones **3a** and **3i** described here might antagonize the AMPA/kainate receptor-mediated responses by an allosteric blocking mechanism.

In addition, the anticonvulsant properties of derivatives **3a** and **3i** were not significantly affected by the concomitant treatment with flumazenil (Table 5). The results obtained in vivo were corroborated by in vitro binding experiments in which the potency of the derivatives **3** as inhibitors of [^{3}H]flumazenil binding to membranes from cortex was evaluated, and no inhibition was observed (IC_{50} > 10 000 nM).

Moreover, binding experiments showed that compounds **3** failed to displace [^{3}H]spiperone from dopamine and 5-hydroxytryptamine$_1$ receptors, [^{3}H]ketanserin from 5-hydroxytryptamine$_2$ receptors, [^{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 ([^{3}H]MK-801) from NMDA receptors, [^{3}H]5,7-dichlorokynurenic acid ([^{3}H]5,7-DCKA) from the glycine site on NMDA receptors, [^{3}H]AMPA and [^{3}H]6-cyano-7-nitroquinoxaline-2,3-dione ([^{3}H]CNQX) from AMPA/kainate receptors, and a mixture of [^{3}H](1S,3R)ACPD and [^{3}H](1R,3S)ACPD from metabotropic glutamate receptors. Despite the lack of activity at the glycine site on NMDA receptors and dopamine, serotonin, noradrenaline, NMDA, and metabotropic glutamate binding sites an interaction at other sites involved in the

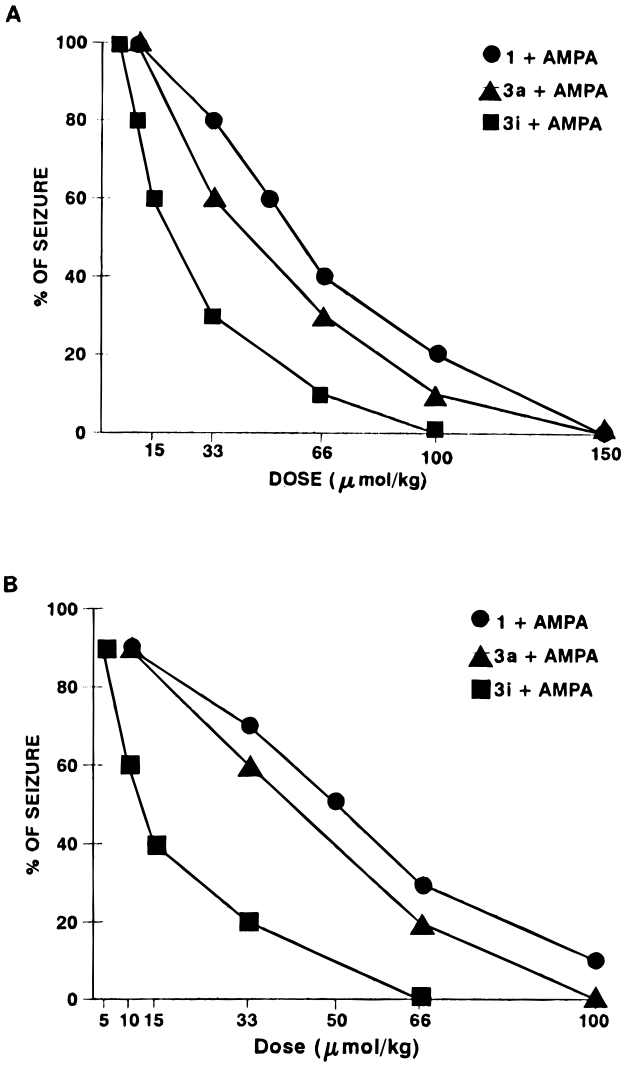


Figure 2. Anticonvulsant effects of **3a**, **3i**, and **1** plotted against seizures induced by AMPA in DBA/2 mice. The ordinate shows percent of response of clonic (A) or tonic (B) seizures, the abscissa shows the dose in μmol/kg ip. For the determination of each point, 10 animals were used.

Table 4. ED_{50} Values of **2a**, **3a**, **2i**, **3i**, and **1** against the Clonic and Tonic Phases of the Audiogenic Seizures after Pretreatment with Aniracetam in DBA/2 Mice[a]

	ED_{50}, μmol/kg (±95% confidence limits)	
compd	clonic phase	tonic phase
2a[b]	215(142−324)*	157(114−217)*
3a	98.0(64.4−149)*	69.1(42.4−113)*
2i[c]	65.4(44.5−96.2)*	58.2(43.4−77.9)*
3i	46.1(34.6−61.3)*	38.5(26.9−55.0)*
1	134(88.8−203)*	100(63.4−158)*

[a] All data were calculated according to the method of Litchfield and Wilcoxon.[32] At least 32 animals were used to calculate each ED_{50} value. 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 *$P < 0.01$. [b] Reference 1. [c] Reference 2.

generation or expression of seizures cannot presently be ruled out.

Indeed, the noncompetitive nature of the AMPA block exerted by the most potent derivative tested, **3i**, was confirmed in electrophysiological experiments performed in olfactory cortical brain slice neurons. In these tests, **3i**, similar to **2i** and **1**, consistently depressed the AMPA

Table 5. ED_{50} Values of **2a**, **3a**, **2i**, **3i**, and **1** against the Clonic and Tonic Phases of the Audiogenic Seizures after Concomitant Treatment with Flumazenil in DBA/2 Mice[a]

	Flumazenil,	ED_{50}, μmol/kg (± 95% confidence limits)	
compd	μmol	clonic phase	tonic phase
2a[b]	8.24	38.2(28.5−51.2)	35.8(26.2−49.0)
	24.72	50.9(36.9−70.1)	41.3(28.0−60.7)
3a	8.24	22.4(17.9−28.0)	13.6(7.40−25.2)
	24.72	19.3(16.9−22.0)	12.6(8.01−19.0)
2i[c]	8.24	14.1(10.1−19.9)	12.0(6.82−21.0)
	24.72	12.0(6.82−21.0)	10.2(7.61−13.7)
3i	8.24	6.90(4.70−10.2)	3.24(1.24−4.85)
	24.72	7.10(5.60−9.00)	32.2(2.71−4.57)
1	8.24	37.8(23.7−60.1)	26.7(14.7−48.2)
	24.72	39.5(29.6−53.7)	29.7(20.9−42.1)

[a] All data were calculated according to the method of Litchfield and Wilcoxon.[32] At least 32 animals were used to calculate each ED_{50} value. [b] Reference 1. [c] Reference 2.

dose−response relationship in a nonparallel manner, even at the highest agonist concentration used (5 μM). This noncompetitive mode of antagonism of AMPA responses by **3i** detected here (Figure 3), is in agreement both with previous in vitro data obtained in our laboratory with compound **2i** and **1**[2] and with other data observed from cultured hippocampal neurons,[10d,11b] cortical wedges,[12a] and hippocampal slices.[12b]

As shown in Figure 3, in the presence of a fixed concentration (50 μM) of **3i**, the peak amplitude of AMPA-induced inward currents was markedly reduced at each agonist dose level (∼75−85% suppression) with a clear noncompetitive-type depression of the AMPA dose−response curve. These data confirm that derivatives **2i**, **3i**, and **1** may all share a common noncompetitive mode of action at the AMPA receptor.

Although it has long been known that antagonists of the excitatory amino acids (EAA) acting on NMDA receptors, especially those which block ion channels [e.g., dizocilpine (MK-801)], can induce cognitive deficits and a variety of other neurological and behavioral side effects,[11a,20] there are studies showing that potent antagonists at the AMPA/kainate receptor have anticonvulsant effects at doses below those impairing behavior.[21]

The anticonvulsant activity of the title compounds was evident at doses which generally did not cause sedation and ataxia. The therapeutic index (TI) of the most active compounds **3a** and **3i** is 2.5−3 times that of compound **1** (Table 6).

In conclusion, the anticonvulsant activity profile and improved therapeutic index of some of the new 1-aryl-3,5-dihydro-4*H*-2,3-benzodiazepine-4-thiones **3** provide an interesting illustration of the research effort which is being made to discover new drugs interacting with AMPA receptors and possessing therapeutic potential with lower side effects. Extensive further modifications are in progress and will be used to challenge and refine our pharmacophore model.

Experimental Section

Chemistry. Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Elemental analyses (C, H, and N) were carried out on a Carlo Erba model 1106 elemental analyzer, 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

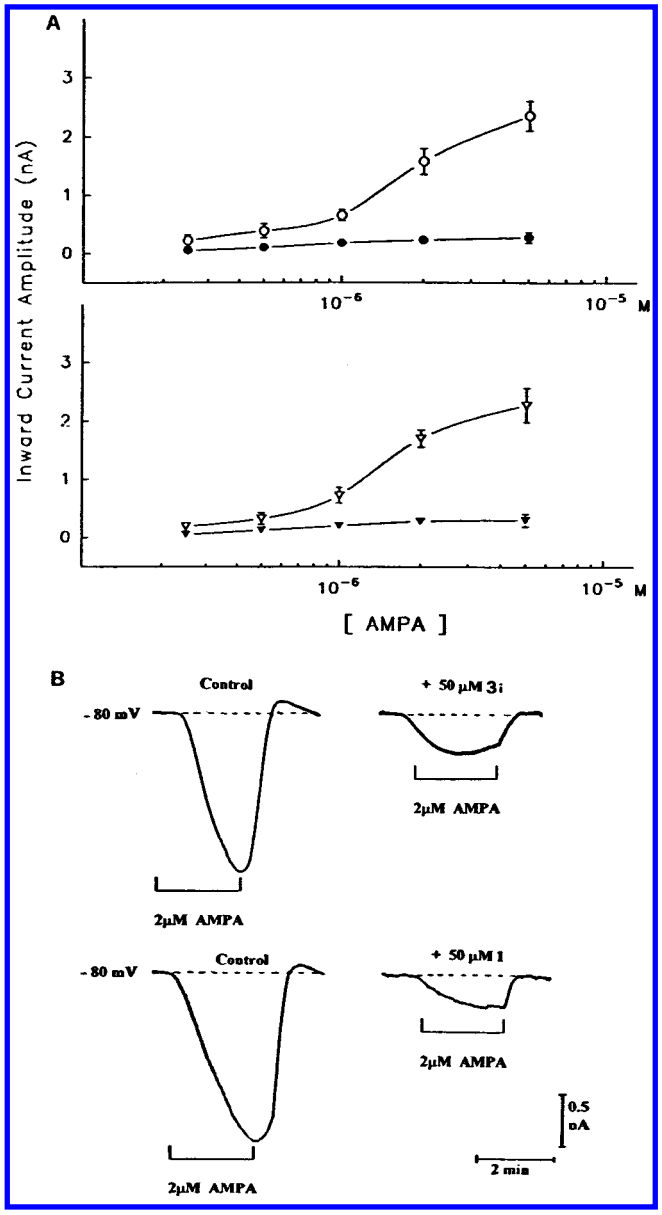


Figure 3. Noncompetitive-type antagonism of AMPA-induced inward currents by 2,3-benzodiazepine derivatives, recorded in guinea pig olfactory cortical neurons voltage-clamped at −80 mV (in the presence of 1 μM TTX). (A) Pooled dose−response curves to AMPA measured in the absence (○; $N = 4$) and presence (●; $N = 4$) of 50 μM compound **3i** (upper panel) or in the absence (△; $N = 3$) and presence (▼; $N = 3$) of 50 μM **1** (lower panel). Each point represents the mean (±SEM) plotted against the AMPA concentration (0.25−5 μM, log scale). Points without error bars had a SEM less than the symbol size. (B) Chart records showing inward currents activated by a 4-min bath application of AMPA (2 μM) in control and after a 15-min preincubation with 50 μM compound **3i** (upper panel) or 50 μM **1** (lower panel); note the similar degree of response depression induced by both compounds. The depression of 1, 2, and 5 μM AMPA mean currents by either antagonist was significant relative to control ($P < 0.05$, 0.01, and 0.001, respectively; Student's t test). Scale bar applies to all traces. Drug washout periods were recorded at 0.4 times the chart speed.

recorded in $CDCl_3$ on a Varian-Gemini-300 spectrometer. Chemical shifts were expressed in δ (ppm) relative to TMS as internal standard and coupling constants (J) in Hz. All exchangeable protons were confirmed by addition of D_2O.

General Procedure for the Synthesis of 1-Aryl-3,5-dihydro-7,8-dimethoxy-4*H*-2,3-benzodiazepine-4-thiones (3a−k). Lawesson's Reagent [2,4-bis(4-methoxyphenyl)-2,4-dithioxo-1,3,2,4-dithiadiphosphethane] (0.55 mmol) was added to a solution in dry toluene (150 mL) of the suitable 2,3-benzodiazepin-4-one derivative (**2a−k**, 1.00 mmol), which was prepared according to a procedure previously described.[2] The mixture was heated at 120 °C for 90 min, and the course of the reaction was monitored by TLC. The solution was then cooled at room temperature and filtered. The solvent was removed under reduced pressure, and the resulting oily yellow residue was treated with EtOH to provide compound **3**, which was recrystallized from EtOH (derivatives **3a−h**) or purified by column chromatography with diethyl ether/light petroleum (80/20) as eluant (derivatives **3j−k**).

Table 6. ED_{50} and TD_{50} Values (95% Confidence Limits) of Compounds **1**−**3** on Locomotion Assessed by Rotarod Test Following 30 min Pretreatment[a]

compd	ED_{50}, μmol/kg clonic phase	TD_{50}, μmol/kg locomotor deficit	TI[d] TD_{50}/ED_{50}
2a[b]	33.9(26.0−44.2)	142(87.3−231)	4.2
3a	19.7(13.1−29.7)	98.9(16.0−611)	5.02
2b[c]	102(76.1−137)	240(102−564)	2.3
3b	82.4(35.2−193)	193(169−220)	3.26
2c[c]	110(79.3−151)	226(116−437)	2.0
3c	52.1(23.3−119)	73.0(56.2−95.0)	1.4
2d[c]	>120	>150	ND
3d	93.4(64.7−135)	>150	ND
2f[c]	37.8(23.7−60.1)	154(104−227)	4.1
3f	30.6(19.6−44.7)	97.1(73.2−129)	3.17
2i[c]	15.0(9.01−24.0)	56.8(39.3−82.1)	3.8
3i	6.30(2.60−15.4)	42.6(26.4−68.8)	6.76
2j[c]	19.3(16.9−22.0)	51.5(34.1−77.8)	2.7
3j	18.8(8.10−36.5)	76.7(63.3−93.0)	4.08
2k[c]	50.2(34.6−73.0)	159(95.0−268)	3.2
3k	29.8(21.4−41.4)	130(85.4−197)	4.35
1	35.8(24.4−52.4)	76.1(47.5−122)	2.1

[a] All data are expressed as μmol/kg and were calculated according to the method of Litchfield and Wilcoxon.[32] At least 32 animals were used to calculate each ED_{50} and TD_{50} value. [b] Reference 1. [c] Reference 2. [d] TI = Therapeutic Index and represents the ratio between TD_{50} and ED_{50} (from the clonic phase of the audiogenic seizures); ND = not determined.

3,5-Dihydro-7,8-dimethoxy-1-phenyl-4*H*-2,3-benzodiazepine-4-thione (3a): yellow crystals, mp 166−169 °C, yield 65%; ^{1}H NMR 3.71 (s, 3H, OCH_3-8), 3.99 (s, 3H, OCH_3-7), 3.97 (bs, 2H, CH_2), 6.35 (s, 1H, H-9), 6.88 (s, 1H, H-6), 7.42−7.65 (m, 5H, Ar), 10.02 (bs, 1H, NH). Anal. ($C_{17}H_{16}N_2O_2S$) C, H, N.

1-(4′-Chlorophenyl)-3,5-dihydro-7,8-dimethoxy-4*H*-2,3-benzodiazepine-4-thione (3b): yellow crystals, mp 222−225 °C, yield 82%; ^{1}H NMR 3.73 (s, 3H, OCH_3-8), 3.99 (s, 3H, OCH_3-7), 3.91 (bs, 2H, CH_2), 6.59 (s, 1H, H-9), 6.87 (s, 1H, H-6), 7.40−7.60 (m, 4H, Ar), 10.13 (bs, 1H, NH). Anal. ($C_{17}H_{15}ClN_2O_2S$) C, H, N.

1-(4′-Bromophenyl)-3,5-dihydro-7,8-dimethoxy-4*H*-2,3-benzodiazepine-4-thione (3c): yellow crystals, mp 115−118 °C, yield 79%; ^{1}H NMR 3.73 (s, 3H, OCH_3-8), 3.99 (s, 3H, OCH_3-7), 3.91 (bs, 2H, CH_2), 6.59 (s, 1H, H-9), 6.87 (s, 1H, H-6), 7.50−7.60 (m, 4H, Ar), 9.89 (bs, 1H, NH). Anal. ($C_{17}H_{15}BrN_2O_2S$) C, H, N.

3,5-Dihydro-7,8-dimethoxy-1-(4′-nitrophenyl)-4*H*-2,3-benzodiazepine-4-thione (3d): yellow crystals, mp 228−230 °C, yield 65%, ^{1}H NMR 3.72 (s, 3H, OCH_3-8), 4.00 (s, 3H, OCH_3-7), 3.97 (bs, 2H, CH_2), 6.53 (s, 1H, H-9), 6.88 (s, 1H, H-6), 7.83−8.31 (m, 4H, Ar), 10.20 (bs, 1H, NH). Anal. ($C_{17}H_{15}N_3O_4S$) C, H, N.

3,5-Dihydro-7,8-dimethoxy-1-(3′-nitrophenyl)-4*H*-2,3-benzodiazepine-4-thione (3e): yellow crystals, mp 256−258 °C, Yield 57%; ^{1}H NMR 3.72 (s, 3H, OCH_3-8), 4.01 (s, 3H, OCH_3-7), 3.99 (bs, 2H, CH_2), 6.57 (s, 1H, H-9), 6.90 (s, 1H, H-6), 7.62−8.54 (m, 4H, Ar), 10.06 (bs, 1H, NH). Anal. ($C_{17}H_{15}N_3O_4S$) C, H, N.

3,5-Dihydro-7,8-dimethoxy-3-methyl-1-phenyl-4*H*-2,3-benzodiazepine-4-thione (3f): yellow crystals, mp 160−162 °C, Yield 75%; ^{1}H NMR 3.73 (s, 3H, OCH_3-8), 3.99 (s, 3H,

OCH_3-7), 3.73 and 4.18 (dd, 2H, $J = -12.6$, CH_2), 3.81 (s, 3H, NCH_3), 6.64 (s, 1H, H-9), 6.91 (s, 1H, H-6), 7.43−7.67 (m, 5H, Ar). Anal. ($C_{18}H_{18}N_2O_2S$) C, H, N.

1-(4′-Bromophenyl)-3,5-dihydro-3-methyl-7,8-dimethoxy-4*H*-2,3-benzodiazepine-4-thione (3g): yellow crystals, mp 168−170 °C, yield 66%; ^{1}H NMR 3.75 (s, 3H, OCH_3-8), 3.99 (s, 3H, OCH_3-7), 3.71 and 4.19 (dd, 2H, $J = -12.6$, CH_2), 3.80 (s, 3H, NCH_3), 6.61 (s, 1H, H-9), 6.90 (s, 1H, H-6), 7.52−7.62 (m, 4H, Ar). Anal. ($C_{18}H_{17}BrN_2O_2$) C, H, N.

3,5-Dihydro-7,8-dimethoxy-3-methyl-(4′-nitrophenyl)-4*H*-2,3-benzodiazepine-4-thione (3h): yellow crystals, mp 228−230 °C, yield 45%; ^{1}H NMR 3.73 (s, 3H, OCH_3-8), 4.00 (s, 3H, OCH_3-7), 3.72 and 4.09 (dd, 2H, $J = -12.4$, CH_2), 3.84 (s, 3H, NCH_3), 6.55 (s, 1H, H-9), 6.92 (s, 1H, H-6), 7.83−8.32 (m, 4H, Ar). Anal. ($C_{18}H_{17}N_3O_4S$) C, H, N.

1-(4′-Aminophenyl)-3,5-dihydro-7,8-dimethoxy-4*H*-2,3-benzodiazepine-4-thione (3i): yellow powder, mp 225−227 °C, yield 50%; ^{1}H NMR 3.75 (s, 3H, OCH_3-8), 3.99 (s, 3H, OCH_3-7), 3.96 (bs, 4H, NH_2 and CH_2), 6.68 (s, 1H, H-9), 6.72 (s, 1H, H-6), 6.69−7.46 (m, 4H, Ar), 9.80 (bs, 1H, NH). Anal. ($C_{17}H_{17}N_3O_2S$) C, H, N.

1-(3′-Aminophenyl)-3,5-dihydro-7,8-dimethoxy-4*H*-2,3-benzodiazepine-4-thione (3j): yellow powder, mp 128−130 °C, yield 58%; ^{1}H NMR 3.74 (s, 3H, OCH_3-8), 3.99 (s, 3H, OCH_3-7), 3.90 (bs, 4H, NH_2 and CH_2), 6.68 (s, 1H, H-9), 6.86 (s, 1H, H-6), 6.80−7.24 (m, 4H, Ar), 9.87 (bs, 1H, NH). Anal. ($C_{17}H_{17}N_3O_2S$) C, H, N.

1-(4′-Aminophenyl)-3,5-dihydro-7,8-dimethoxy-3-methyl-4*H*-2,3-benzodiazepine-4-thione (3k): yellow powder, mp 182−185 °C, yield 40%; ^{1}H NMR 3.75 (s, 3H, OCH_3-8), 3.98 (s, 3H, OCH_3-7), 3.71 and 4.07 (dd, 2H, $J = -12.4$, CH_2), 3.76 (s, 3H, NCH_3), 3.92 (bs, 2H, NH_2), 6.68 (s, 1H, H-9), 6.89 (s, 1H, H-6), 6.70−7.47 (m, 4H, Ar). Anal. ($C_{18}H_{19}N_3O_2S$) 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.[16a,22] 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. Audiogenic Seizures in DBA/2 Mice. All experiments were performed with DBA/2 mice which are genetically susceptible to sound-induced seizures.[23] DBA/2 mice (8−12 g; 22−25 days old) were purchased from Charles River (Calco, Como, Italy). Groups of 10 mice of either sex 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 50% dimethyl sulfoxide (DMSO) and 50% 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.[24] The time course of the anticonvulsant action of **2a**, **3a**, and **1** was determined following the administration of 33 μmol/kg of each benzodiazepine derivative to groups of 10 mice that were tested for sound-induced seizure responses at 5−240 min after drug administration.

Maximal Electroshock Seizure 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.[25] 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.

Pentylenetetrazole-Induced Seizures in Swiss Mice. Male Swiss mice (20−26 g, 42−48 days old) were purchased from Charles River (Calco, Como, Italy) and were pretreated with vehicle or drug 45 min before the subcutaneous (sc) administration of pentylenetetrazole. 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 50% DMSO and 50% 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 the PTZ seizure threshold.[26]

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:[27] 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.

Membrane Preparation and [^{3}H]Flumazenil and Other Binding Studies. Male SD/Rij rats (FRAR, S.Pietro al Natisone, UD, Italy) weighing 200−250 g were decapitated, and different brain areas were rapidly dissected on ice. Brain regions were homogenized in 20 mL of ice-cold 0.32 M sucrose, pH 7.4, by using a glass homogenizer with a Teflon pestle (10 up and down strokes). The homogenate was centrifuged at 1000*g* at 4 °C for 10 min, the P_1 pellet was discarded, and the supernatant was collected and recentrifuged at 20000*g* at 4 °C for 20 min. The resulting crude mitochondrial pellet (P_2) was resuspended in 20 mL of ice-cold distilled water and homogenized. The homogenate was centrifuged at 8000*g* at 4 °C for 20 min, the supernatant was collected and recentrifuged at 48000*g* at 4 °C for 20 min, and the final crude microsomal pellet (P_3) was frozen for at least 24 h. The pellet was resuspended in 10 mL of 50 mM Tris-HCl pH 7.4, centrifuged at 48000*g* at 4 °C for 20 min, and then resuspended in 10 vol of the same buffer for the standard binding assay. Aliquots of membrane suspensions (100 μL or 0.15 mg of protein) were added to the incubation medium containing 1 nmol of [^{3}H]flumazenil (specific activity 72.4 Ci/mmol) in a final volume of 1 mL of 50 mM Tris-HCl, 120 mM NaCl, and 5 mM KCl, pH 7.4. All compounds were dissolved in DMSO at a final concentration of 1%. Incubations were carried out for 60 min at 4 °C in triplicate, and nonspecific binding was measured in the presence of 10 μM diazepam. Reactions were stopped by the addition of 5 mL of ice-cold Tris-HCl followed by rapid filtration through Whatman GF/C glass fiber filters (Whatman Inc. Clifton, NJ) and two additional washes. The radioactivity trapped on the filters was counted after the addition of 8 mL of Filter Count (Packard) by liquid scintillation spectrometry. The experiments were run in triplicate with eight different concentrations of competing ligand. The possibility that compounds **3** bind to receptors other than BZR was studied in crude rat brain synaptic membranes according to established protocols[28] by using [^{3}H]spiperone, [^{3}H]ketanserin, [^{125}I]pindolol, [^{3}H]CPP, [^{3}H]MK-801, and [^{3}H]AMPA. In other binding studies aliquots of membrane suspension were

incubated at the appropriate temperature for 60 min with 50 nM [^{3}H]CNQX (specific activity, 18.3 Ci/mmol), 10 nM [^{3}H]-5,7-DCKA (specific activity, 18.3 Ci/mmol), or [^{3}H]ACPD (mixture of [^{3}H](1*S*,3*R*)ACPD and [^{3}H](1*R*,3*S*)ACPD, specific activity 30−50 Ci/mmol). Nonspecific binding was defined as the binding measured in the presence of 0.2 mM quisqualate, 0.1 mM d-serine, or 0.2 mM (1*S*,3*R*)ACPD, respectively.

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 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.[29] The ability of the mice to remain on the rotarod was tested 30 min after administration of various compounds.

Electrophysiology. Transverse slices of olfactory cortex (~450 μm thick) were obtained from adult guinea pigs (250−400 g, either sex), as previously described,[30] and stored in oxygenated Krebs solution at 32 °C for at least 30 min before being transferred to an immersion chamber for recordings. The composition of the Krebs fluid was (mM) as follows: NaCl, 118; KCl, 3; $CaCl_2$, 1.5; $NaHCO_3$, 25; $MgCl_2 \cdot 6H_2O$, 1; and D-glucose, 11 (bubbled with 95% O_2:5% CO_2, pH 7.4). Conventional intracellular recordings were obtained from the periamygdaloid area of the slices within the olfactory pyramidal cell layer II−III,[31] using glass microelectrodes (tip resistances 40−60 MΩ) filled with 4 M potassium acetate. Voltage clamp recordings were made at a holding membrane potential of −80 mV with the aid of a DAGAN 8100 sample-and-hold voltage clamp preamplifier (2−3 kHz switching frequency, 25% duty cycle). Sampled membrane currents (filtered at 30 Hz, low pass) and voltage were recorded on a Gould 2400 ink jet chart recorder. The following compounds were tested: AMPA, **3i**, and **1**. In addition, slices were continuously superfused with 1 μM TTX to block voltage-activated sodium currents and underlying repetitive firing at the peak of AMPA responses. AMPA and TTX were freshly prepared in Krebs solution, whereas compounds **3i** and **1** were predissolved in dimethyl sulfoxide (DMSO) to give 10 mM stock solutions and subsequently diluted in Krebs solution (containing 0.1−1% v/v DMSO) immediately prior to use. These concentrations of DMSO had no deleterious effects on neuronal membrane properties or AMPA-induced inward currents. All measurements were performed before, during, and after bath application of pharmacological agents so that each neuron served as its own control.

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 electroshock or pentyleneterazole was determined for each dose of compound administered, and dose−response curves were fitted using a computer program by the method of Litchfield and Wilcoxon.[32] The relative anticonvulsant activities were determined by comparison of respective ED_{50} values. The dose which induced 50% of mice to fall from the rotarod (TD_{50} values) was estimated using the method of Litchfield and Wilcoxon.[32] The relative activities were determined by comparison of respective TD_{50} values. For the binding experiments IC_{50} values for the displacement of [^{3}H]flumazenil or of other ligands were determined by the nonlinear curve-fitting program based on Ligand.[33] All data of electrophysiology are expressed as mean ± standard error of the mean (SEM). Statistical significance between control and test groups of data means was tested using a two-tailed Student's *t* test.

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