Comparison of the AMPA Antagonist Action of New 2,3-Benzodiazepines *in Vitro* and Their Neuroprotective Effects *in Vivo*

Gábor Kapus,[1] Szabolcs Kertész,[1] Gábor Gigler,[1] Annamária Simó,[1] Miklos Vegh,[1] József Barkóczy,[1] László G. Hársing,[1] Géza Szabó,[1] and György Lévay[1,2]

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Purpose. AMPA receptor-mediated excitotoxicity is thought to be a critical process in diseases accompanied by neuronal cell loss following a hypoxic/anoxic state of the central nervous system. It has been suggested that blockade of AMPA receptors might result in significant protection of neurons against cellular damage. For testing the hypothesis, *in vitro* efficacy and *in vivo* neuroprotective action of new 2,3-benzodiazepine (2,3BDZ) AMPA antagonists have been compared.

Methods. 2,3BDZs were tested on kainate-evoked whole-cell currents in cultured neurons as well as on population spikes (PS) in rat hippocampal slices. Data were correlated with those obtained from the spreading depression (SD) experiments in chicken retina. Compounds were also examined in the gerbil bilateral carotid occlusion model (BCO), where percentage decrease of ischemia-related hypermotility (HM), impaired spatial memory (SA), and hypoxia-induced hippocampal CA1 neuronal cell death (CA1) were evaluated.

Results. Certain structural modifications of classical 2,3BDZs resulted in increased *in vitro* activity and improved *in vivo* efficacy. In particular, the halogen-substituted compounds EGIS-9879 and EGIS-9883 showed the highest neuroprotective efficacy (84% and 47% protection in CA1, 71% and 82% decrease in HM, respectively; 4×5 mg/kg i.p.) in BCO. PS and SD were correlated to the decrease of neuronal loss in the CA1 area. Lack of significant correlation was found between PS and CA1 ($r = 0.437$, $p = 0.079$) or SD and CA1 ($r = 0.380$, $p = 0.146$).

Conclusions. Several new 2,3BDZ AMPA receptor antagonists have been synthesized at EGIS Pharmaceuticals characterized by remarkable *in vitro* and corresponding *in vivo* neuroprotective properties.

KEY WORDS: AMPA antagonists; 2,3-benzodiazepines; EGIS-9879; cerebral ischemia; neuroprotection.

INTRODUCTION

Excitotoxicity conveyed by cerebral glutamate receptors is one of the major factors of neuronal loss observable after brain insults such as stroke, hypoxic/anoxic states, or physical brain injures (1–3). The cytotoxic events responsible for cellular damage are mediated by several subtypes of excitatory amino acid receptors, one of which is the ionotropic AMPA receptor (4,5). Neuroprotective properties of competitive (i.e., NBQX) and noncompetitive (2,3-benzodiazepines) AMPA antagonists were proven *in vitro* and in several *in vivo* models of stroke and ischemia (6–10). Although NBQX is more effective in the blockade of AMPA receptors *in vitro* (5,13), its *in vivo* potency is lower than that of the 2,3-benzodiazepine GYKI 52466 or its successors, GYKI 53405 and GYKI 53655 (8,9,11). Demonstration of significant efficacy of the latter compounds in global and/or focal cerebral ischemia models has raised interest in developing new structural analogues with improved pharmacologic profiles. Therefore, 15 new 2,3-benzodiazepines have been synthesized at EGIS Pharmaceuticals. To determine the structure–activity relationships of the compounds, their pharmacologic properties were characterized using *in vitro* systems and *in vivo* ischemia models.

For *in vitro* testing, the effects of 2,3-benzodiazepines were examined on synaptic transmission in rat hippocampal slices as well as in patch-clamp experiments (PC) in cultured neurons (12). AMPA antagonist effects were also shown in spreading depression experiments carried out on isolated chicken retinas (13). For *in vivo* testing, a widely used model of global cerebral ischemia, transient bilateral carotid artery occlusion (BCO) in Mongolian gerbils, was used, and two behavioral variables were measured. Impact of AMPA antagonists on increased motility level of gerbils in the Y-maze was measured. Ischemia-induced hyperactive state in gerbils (hypermotility, HM) has been considered to be a functional correlate of hippocampal damage (15). Because the hippocampus plays a crucial role in spatial memory, we examined the effect of the compounds on memory functions by measuring spontaneous alternation (SA) behavior (sequential visits of three arms of the Y-maze). Neuroprotective effects were evaluated *ex vivo* on the basis of histologic changes by estimation of neuronal cell death in the hippocampal CA1 region (14).

The aim of our study was to compare the *in vitro* efficacy and the *in vivo* neuroprotective activity of recently synthesized 2,3-benzodiazepines (Table I) by examination of behavioral (HM, SA) and histologic changes (hippocampal CA1 neuronal cell loss) caused by BCO in gerbils and the inhibition of hippocampal population spikes (PS), spreading depression (SD), and kainate-induced AMPA receptor-mediated whole-cell currents.

MATERIALS AND METHODS

Chemicals and Reagents

S-AMPA, kainate, and NBQX were obtained from Tocris. GYKI 52466 (Table I, ***1***), GYKI 53405 (***2***), and GYKI 53655 (***3***) as well as EGIS compounds (***4–18***) were synthesized at EGIS Pharmaceuticals. All other chemicals were purchased from Sigma. In *in vitro* experiments 2,3-benzodiazepines were dissolved at 5–100 mM in DMSO and further diluted with the bathing solution to the final concentrations (always below 0.1%). In *in vivo* experiments tested drugs

[1] EGIS Pharmaceuticals Ltd., H-1475 Budapest 10, POB 100, Hungary

[2] To whom correspondence should be addressed (email: pharmacology.rd@egis.hu).

ABBREVIATIONS: AMPA, α-amino-3-hydroxy-5-methyl-4-izoxazolepropionic acid; BCO, bilateral carotid occlusion; DMSO, dimethylsulfoxide; EMEM, Eagle Minimal Essential Medium; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylendioxy-5H-2,3-benzodiazepine; GYKI 53405, 1-(4-aminophenyl)-3-acetyl-4-methyl-3,4-dihydro-7,8-methylendioxy-5H-2,3-benzodiazepine; GYKI 53655, 1-(4-aminophenyl)-3N-methylcarbamoyl-4-methyl-3,4-dihydro-7,8-methylendioxy-5H-2,3-benzodiazepine; HM, hypermotility; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline; PS, population spike; SA, spontaneous alternation; SD, spreading depression.

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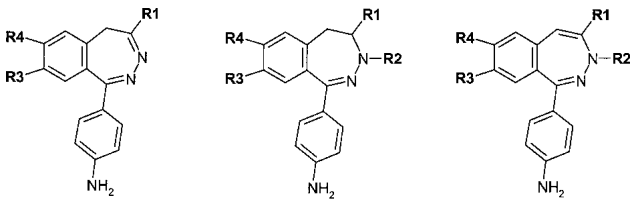
Table I. Chemical Structures of 2,3-Benzodiazepines



1, 4 *2, 3, 5, 7–18* *6*

No.	R1	R2	R3	R4
GYKI 52466 (***1***)	CH_3		- OCH_2O -	
GYKI 53405 (***2***)	CH_3	$COCH_3$	- OCH_2O -	
GYKI 53655 (***3***)	CH_3	$CONHCH_3$	- OCH_2O -	
4	CH_2OCOCH_3		- OCH_2O -	
5	CH_3, CN	$COCH_3$	- OCH_2O -	
6	CH_3	$CONHCH_3$	- OCH_2O -	
7	CH_3	$CONHC_3H_5$	- OCH_2O -	
8	CH_3	$CONHOCH_3$	- OCH_2O -	
9	CH_3	$COCH_3$	Cl	
10	CH_3	$CONH_2$	Cl	
11	CH_3	$CONHCH_2CH_3$	Cl	
12	CH_3	$COCH_3$	Br	
13	CH_3	$COCH_2CH_3$	Br	
14	CH_3	$CONHCH_3$	Br	
15	CH_3	$COCH_3$	Cl	Cl
16	CH_3	$CONH_2$	Cl	Cl
17	CH_3	$CONHCH_3$	Cl	Cl
18	CH_3, CN	$COC_2H_4NC_5H_8OHC_6H$	- OCH_2O -	

Note: NBQX [6-nitro-7-sulfamoylbenzo(f)quinoxaline-2,3-dione] is a competitive antagonist of AMPA receptor.

were dissolved in 100 μL 5 M HCl and then diluted with distilled water, and pH was adjusted to 3.0–4.0 with 1 M NaOH. NBQX was first dissolved in 1 M NaOH then diluted with distilled water (pH was adjusted to 7.5–8.0 with 5 M HCl).

Retinal Spreading Depression

Chicks (4–7 days old, Shaver Redbrow) were decapitated under ether anesthesia. Eyes were dissected, enucleated, and sectioned equatorially. After removal of the vitreous body, the posterior eyecup was immersed in a Ringer solution (composition: 100 mM NaCl, 3 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 30 mM $NaHCO_3$, 1 mM NaH_2PO_4, 10 mM glucose, pH 7.4) (13). The solution was saturated with 95% O_2 and 5% CO_2 and maintained at room temperature. The eyecups were left in Petri dishes for at least 60 min to recover from preparation. Retinal SD was triggered by 5 μM S-AMPA, and a change in the luminosity of the tissue could easily be observed. The latency for SD to start was measured. After further incubation in normal Ringer solution, the retinas were transferred to the same type of solution containing the test compounds as well and incubated for 15 min. SD was then stimulated by AMPA in the presence of antagonists. Retinas were then returned to drug-free solution. Sixty minutes later SD was elicited again to assess the degree of recovery from drug effects. A 30-s increase in the latency compared to control time was considered to be 100% inhibition of SD (13). The drug effects were expressed as percentage inhibition. IC_{50} values were calculated by sigmoid curve fitting (GraphPad 1.03).

Hippocampal Field Potential Recordings

Hippocampal slices were prepared from male Wistar rats (120–160 g) under anesthesia. Brain was removed after decapitation, hippocampi were excised and placed into aerated (95% O_2/5% CO_2), ice-cold solution (124 mM NaCl, 3.5 mM KCl, 30 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose; pH 7.4). Transverse slices (400 μm) were prepared and then kept in the same solution at 27°C for at least 1 h before transfer into an interface-type recording chamber (flow rate was 2 ml/min, temperature was set to 33 ± 0.5°C). Bipolar electrode (Ni/Cr) was placed into the stratum radiatum of the CA2 area to stimulate Schaffer collateral commissural fibers. Slices were continuously stimulated (0.2-ms square voltage pulses at 0.05 Hz), and the evoked field potentials were recorded by the recording glass micropipette (R_e = 2–5 MΩ) positioned in the pyramidal cell body layer of CA1 area. The amplitude of the population spikes (PSs) was expressed as the difference between the negative and the following positive peak of field potentials. The stimulation intensity was set to elicit about 80% of the maximal PS amplitude. Field potentials were amplified and filtered by a CyberAmp 320 amplifier (Axon Instruments), digitized (Digidata 1200A, Axon Instruments), recorded, and analyzed by a custom-made computer program. IC_{50} values were calculated using sigmoid curve fitting to logistic equation (Origin 4.1).

Patch-Clamp Recordings

Primary culture of telencephalon cells from 16-day-old embryos of Sprague-Dawley rats were prepared and grown according to Kovács and Szabó (16). Briefly, cells were plated onto 13-mm glass plates precoated with poly-L-lysine and cultured under humidified 5% CO_2/95% O_2 air atmosphere in EMEM containing 4 mM L-glutamine, 12 mM D-glucose, 25 mM KCl, 10% heat inactivated serum and antibiotics. KCl was omitted from the culture medium from day 7. Medium was changed at the second and seventh days. The overgrowth of glia cells was diminished by the 24-h-long application of cytosine arabinoside (10 μM on day 6).

Whole-cell patch-clamp studies were carried out on cells clamped at −70 mV at room temperature, 8–13 days after plating. The bathing solution contained 140 mM NaCl, 5 mM KCl, 10 mM Hepes, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM D-glucose, pH 7.3. A solution containing 140 mM KCl, 4 mM NaCl, 10 mM Hepes, 5 mM EGTA, 0.5 mM $CaCl_2$, pH 7.2 was used to fill microelectrodes. Osmolarity was set to 295 mOsm. Perfusion with external solution (containing 0.25 mM kainate, antagonists, or in combination) was led into a common 0.7 mm i.d. borosilicate glass tube ending 1 mm from the cell under study, and the flow was driven by gravity at a rate of 2 ml per min. Patch electrodes at resistance 2.7–3.2 MΩ attached to a CV-4 headstage were connected to an Axopatch-1D amplifier in voltage-clamp mode. Kainate-induced whole-cell inward currents were recorded at −70 mV holding potential. Percentage change of the steady-state current was evaluated. Voltage control and data acquisition were achieved by the Digidata 1200 board and Clampex version 7.0 (all from Axon Instruments, California). IC_{50} values were calculated by Sigmaplot for Windows 4.1.

Bilateral Carotid Occlusion in Gerbils

Male Mongolian gerbils (60-70 g) were subjected to global ischemia via bilateral common carotid artery occlusion (BCO) for 3 min under ether anesthesia. During surgery, the body temperature of the animals was kept at the individual preoperative level using a heating pad and a heating lamp. The compounds were administered either 4 × 5 or 4 × 15 mg/kg intraperitoneally (i.p.) 30, 45, 60 and 75 min after reperfusion.

Four days after surgery, spontaneous alternation behavior (SA) and locomotor activity (hypermotility, HM) of gerbils were measured in a symmetric Y-maze (arms were 40 cm long, 10 cm wide with 21.5 cm high walls) (9). The sequence of entries into the three arms was recorded for 5 min. Total number of arm entries has been used as motor activity measurements (9). Spontaneous alternation was registered when the animal entered the least recently visited arm. Entries into one of the other two arms (return to the last or formerly visited arms) were considered as errors in spontaneous alternation. Total number of arm entries and percentage of spontaneous alternations were determined for each animal. Percentage changes compared to the sham-operated group were calculated for both behavioral variables in each group subjected to BCO. Differences among groups were statistically evaluated by Kruskal-Wallis ANOVA followed by Mann-Whitney *U*-test.

After behavioral testing, the animals were deeply anesthetized with pentobarbital (60 mg/kg i.p.) and perfused through the heart by a fixative solution containing 0.1% glutaraldehyde, 4% paraformaldehyde, and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The brains were postfixed in the same fixative solution overnight, and 60-μm-thick coronal sections were prepared.

Coronal sections of brains were stained by silver impregnation according to the procedure of Gallyas *et al.* (17). Investigators were blinded to experimental conditions while assessing cell loss in the hippocampal CA1 region (CA1). Neuronal damage was scored from 0 to 6 as follows: 0, undamaged; 1, <10%; 2, 10–30%; 3, 30–50%; 4, 50–70%; 5, 70–90%; and 6, 90–100% cell loss. In case of asymmetric damage in the two sides, the rating was assigned to the higher score. Baseline values of BCO controls varied between 4.5 and 5.5. Neurons were considered to be damaged irreversibly when they were shrunken and strongly argylophilic, whereas somata of intact neurons appeared dark yellow. Data were statistically analyzed by Mann-Whitney *U*-test (Statistica 4.5, StatSoft).

Experimental Animals

Before decapitation, experimental animals were anesthetized with ether, and all efforts were made to avoid any suffering of the animals. We acted according to the guidelines of the Institutional Committee for Animal Welfare (EGIS Pharmaceuticals Ltd., Budapest), which is in accordance with the Principles of Laboratory Animal Care (NIH #85-23, revised 1985).

RESULTS

Effects of AMPA Antagonists *In Vitro*

Inhibitory effects of the prototype 2,3-benzodiazepine compound GYKI 52466 (Table I, ***1***) on population spikes (PS) in hippocampal slices, latency of spreading depression (SD) in the chicken retina, as well as on kainate-induced currents (PC) are illustrated (Fig. 1). Concentration-dependent effects of GYKI 52466, as an example, are shown in Fig. 2.

Results of the chemical modifications of 2,3-benzodiazepines (displayed in Table I) are summarized in Table II. We found that introduction of a cyanide group at R1 position (***5*** vs. ***2***) and the substitution on the N3 position of the diazepine ring (R2) increased the *in vitro* efficacy of the compounds (***2, 3,*** and ***7, 8*** from the new compounds). When a methylenedioxy moiety (R3, R4) was changed to halogen atoms (Cl or Br), compounds retained their activity. Dichloro derivatives were two or three times more potent compared to the effects of chloro (***15*** and ***16*** vs. ***9*** and ***10***) or bromo derivatives (***17*** vs. ***14***). In contrast, long-side-chain substitution (R1 at compound ***4*** or R2 at compound ***18***) decreased the effects of ***1*** and ***5***.

Data from the various *in vitro* models were correlated to each other (Fig. 3). Both the inhibition of PS in hippocampal slices and SD in the chicken retina significantly correlated with the inhibitory effects of the compounds on whole-cell currents in patch-clamp recordings (correlations are $r = 0.931$, $p < 0.007$, $n = 6$ and $r = 0.972$, $p < 0.001$, $n = 6$; respectively). Furthermore, inhibition of PS amplitude was

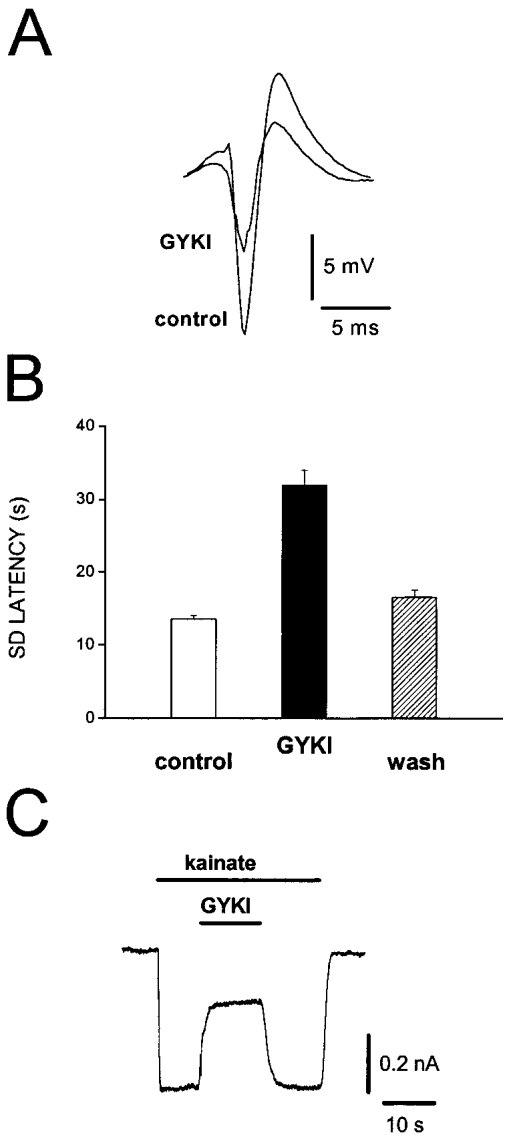


Fig. 1. *In vitro* effects of GYKI 52466. (A) Inhibition of population spikes (PS) recorded from the CA1 region of rat hippocampus (representative recordings, 20 μM GYKI 52466). (B) 20 μM GYKI 52466 increased the latency of spreading depression (SD) evoked by 5 μM S-AMPA in isolated chicken retina. (C) Effects of GYKI 52466 in whole-cell voltage-clamp experiments (identical recording, 250 μM kainate, 20 μM GYKI 52466).

found to significantly correlate with the blockade of retinal SD ($r = 0.893$, $p < 0.001$, $n = 19$ and $r = 0.907$, $p < 0.013$, $n = 6$ for IC_{50} values).

Effects of AMPA Antagonists In Vivo

As shown in Table II, most of the 2,3-benzodiazepines tested significantly ($p < 0.05$) blocked the ischemia-induced hypermotility (HM), and some of them markedly prevented the deterioration of spatial memory (SA) of the animals following transient carotid occlusion (see data of compounds ***1, 3, 5, 7***, and ***15***). Complete protection of SA was obtained when compound ***7*** was used. In most cases, histologic evaluation showed significant reduction in the rate of the neuronal damage in the hippocampal CA1 region following 2,3-

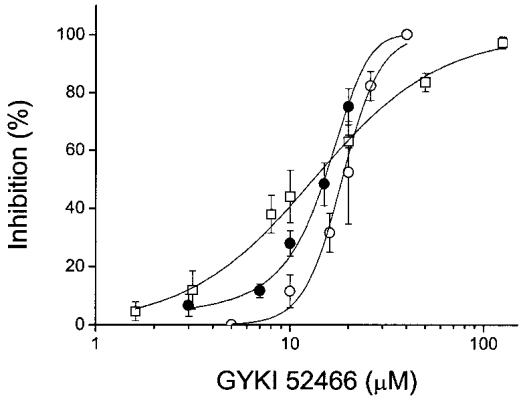


Fig. 2. GYKI 52466 concentration-dependently blocked the induction of PS amplitude (○, $n = 5$), retinal SD (●, $n = 6$), and whole-cell currents (□, $n = 4–7$). Mean ± SEM was expressed as percentage reduction of the control.

benzodiazepine administration. Neuroprotective effects of the prototype 2,3-benzodiazepine ***1*** and its derivatives GYKI 53405 (***2***) and GYKI 53655 (***3***) were dose-dependent (tested doses were 4 × 5 and 4 × 15 mg/kg) (Table II).

Introduction of a double bond at the C4–C5 position (***6*** vs. ***3***) decreased the neuroprotective effect (inhibition of cell loss at CA1 changed from 100% to 34%). On the contrary, saturation of the double bond at N3–C4 and substitution of the N atom at N3 position resulted in a marked increase in antiischemic activity (***2*** and ***3*** compared to ***1***). Compounds were effective in HM and CA1 at the lower repeated dose (4 × 5 mg/kg) when the R3–R4 methylenedioxy moiety was modified to halogen atoms (***9–17*** in Table II). Particularly, the bromo substituent ***12*** and the dichloro derivative ***15*** exerted considerable protection against CA1 cell death (47% and 84%, respectively) and HM (82% and 71%, respectively). The *in vitro* inactive compound ***18*** showed no effect on *in vivo* parameters (HM, CA1).

Correlation Between *In Vitro* and *In Vivo* Efficacy of AMPA Antagonists

Although most of the modifications in the molecular structure of GYKI 52466 (***1***) resulted in marked increase both in the *in vitro* and *in vivo* efficacy of 2,3-benzodiazepines, statistically significant correlation has not been established between efficacy of the compounds on hippocampal synaptic activity (population spike, PS) and histologic (CA1) consequences of BCO in gerbils ($r = 0.437$, $p = 0.079$, $n = 17$) (Fig. 4A). Retinal spreading depression (SD) showed no correlation with the blockade of the hippocampal effect of BCO in gerbils either ($r = 0.380$, $p = 0.146$, $n = 16$) (Fig. 4B).

DISCUSSION

In the present study, the efficacy of new 2,3-benzodiazepines (noncompetitive AMPA antagonists) was investigated in different *in vitro* tests and in one *in vivo* ischemia model comparing their AMPA antagonist properties and neuroprotective potency.

It has been demonstrated that most of the indicated structural changes of the prototype 2,3-benzodiazepine GYKI 52466 (***1***) enhanced the *in vitro* efficacy in rat hippocampus and chicken retina (Table II). Data of some prominent AMPA antagonists obtained from these tests (IC_{50} values) were found to be well correlated with those collected from

Table II. Structure–Activity Relationship of 2,3-Benzodiazepines

No.	Patch Clamp IC_{50} (μM)	Hippocampal PS		Retinal SD		BCO in Gerbils (% Inhibition)					
		IC_{50} (μM)	10 μM (% inh.)	IC_{50} (μM)	5 μM (% inh.)	CA1		HM		SA	
						4 × 5	4 × 15	4 × 5	4 × 15	4 × 5	4 × 15
1	13.7 ± 1.9	20.1 ± 1.1	12 ± 6	16.6 ± 0.8	12 ± 3	0	49[b]	39	87[b]	0	51[b]
2	7.4 ± 1.9	17.8 ± 1.0	12 ± 3	7.0 ± 0.6	22 ± 2	33	61[b]	20	72[b]	0	0
3	2.3 ± 0.1	2.3 ± 0.2	100 ± 0	1.4 ± 0.08	(100)	52[b]	100[c]	59[b]	—	53[b]	—
NBQX	0.2 ± 0.1	0.5 ± 0.05	(100)	0.2 ± 0.01	(100)	—	—	—	—	—	—
4	—	—	0 ± 5	21.7 ± 0.7	—	—	23	—	22	—	—
5	7.6 ± 0.3	10.0 ± 0.8	66 ± 10	5.3 ± 0.6	55 ± 7	—	58[b]	—	63[b]	—	6[b]
6	—	—	100 ± 0	1.7 ± 0.1	100 ± 0	—	34[b]	—	58[b]	—	21
7	2.5 ± 0.6	2.1 ± 0.04	100 ± 0	1.1 ± 0.07	(100)	—	35[b]	—	79[b]	—	100[b]
8	—	—	95 ± 4	1.1 ± 0.05	100 ± 0	—	53[b]	—	76[b]	—	29
9	—	—	22 ± 6	11.4 ± 0.4	9 ± 3	63[b]	—	0	—	0	—
10	—	—	45 ± 15	8.7 ± 0.4	13 ± 2	36	—	9	—	18	—
11	—	—	44 ± 7	7.7 ± 0.5	28 ± 2	100[d]	—	—	—	—	—
12	—	—	60 ± 4	8.7 ± 0.4	12 ± 5	47[b]	—	82[b]	—	40	—
13	—	—	85 ± 13	5.9 ± 0.3	40 ± 4	41[b]	—	45	—	0	—
14	—	—	99 ± 2	3.4 ± 0.2	77 ± 8	36	—	43[b]	—	25	—
15	—	—	61 ± 3	3.1 ± 0.2	80 ± 5	84[b]	—	71[b]	—	64[b]	—
16	—	—	100 ± 0	2.5 ± 0.1	95 ± 2	60[b]	—	65	—	—	—
17	—	—	98 ± 2	1.3 ± 0.07	(100)	33	—	54[b]	—	11	—
18	—	>50	—	>50	—	—	2	—	1	—	—

Effects of AMPA antagonists on kainate-indued current in whole-cell patch clamp (n = 4–7), on hippocampal synaptic activity (PS, population spike, n = 4–6) in rats and retinal spreading depression (SD) in chickens (n = 6–8). All *in vitro* data are means ± SEM. In bilateral carotid occlusion (BCO) test in gerbils, compounds were administered at 5 or 15 mg/kg i.p. four times after reperfusion (CA1, neuronal cell loss in hippocampal CA1 region; HM, ischemia-induced hypermotility; SA, deterioration of spontaneous alternation; n = 10). In PS test all drugs (except ***18***) completely blocked synaptic activity at 50 μM.

[a] $p < 0.05$, ANOVA followed by Duncan's test; [b] $p < 0.05$, Kruskal-Wallis ANOVA (one-way analysis of variance) followed by Mann-Whitney *U*-test; (100) means complete blockade of SD at 3 μM (or in the case of NBQX at 0.8 μM); [c] 3/6 exit; [d] 3/4 exit.

whole-cell patch-clamp experiments, which is the most adequate model for measuring direct effects on AMPA receptor-mediated currents. However, it cannot be excluded that other effects than AMPA receptor antagonism may also play role in the inhibitory action of 2,3-benzodiazepines in the retinal (22) and hippocampal model (26).

Although some new derivatives (e.g., ***7*** and ***8***) were one order of magnitude stronger in terms of AMPA antagonism when compared to the prototype compound GYKI 52466 (***1***), their *in vitro* inhibitory effects were less pronounced than that of the competitive AMPA receptor blocker NBQX. Similarly to the results of Ábrahám *et al.* (18), it has been shown that halogen atom substitutions in the benzene ring preserve the AMPA antagonistic character of the original compounds (see data in Table II). Although chloro or bromo substitutions retained or somewhat decreased the *in vitro* activity of the

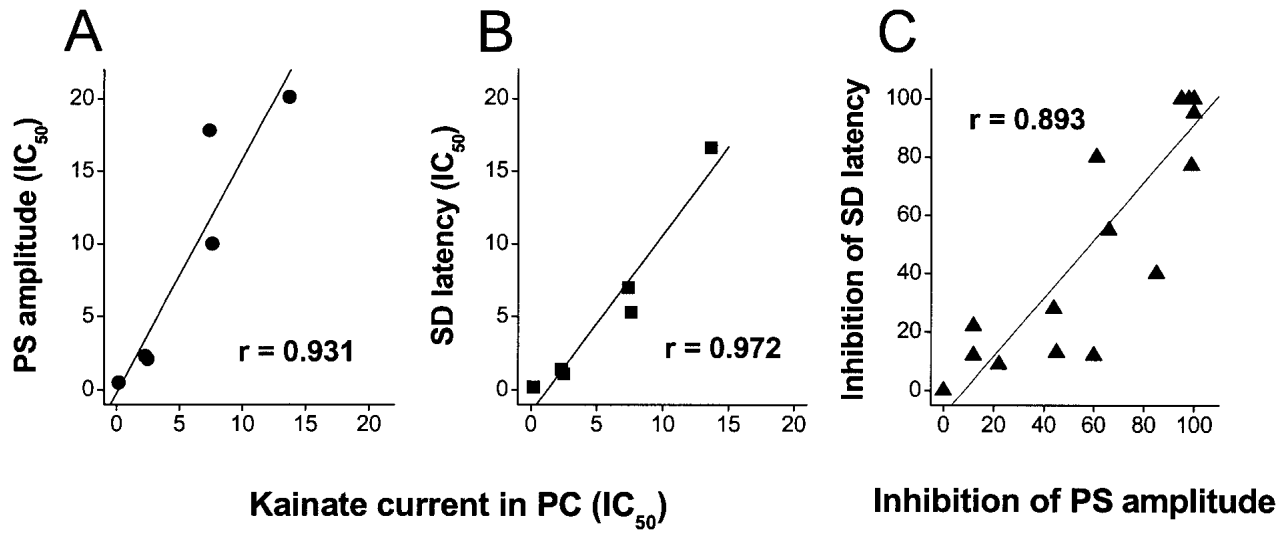


Fig. 3. Correlation between the *in vitro* effects of AMPA receptor antagonists. Inhibition of hippocampal PS amplitude (A) as well as latency of chicken retinal SD (B) were correlated to the blockade of kainate-induced whole-cell currents in PC measurements (IC_{50} values, n = 6). (C) Percentage inhibition of PS amplitude and SD latency was compared (n = 19).

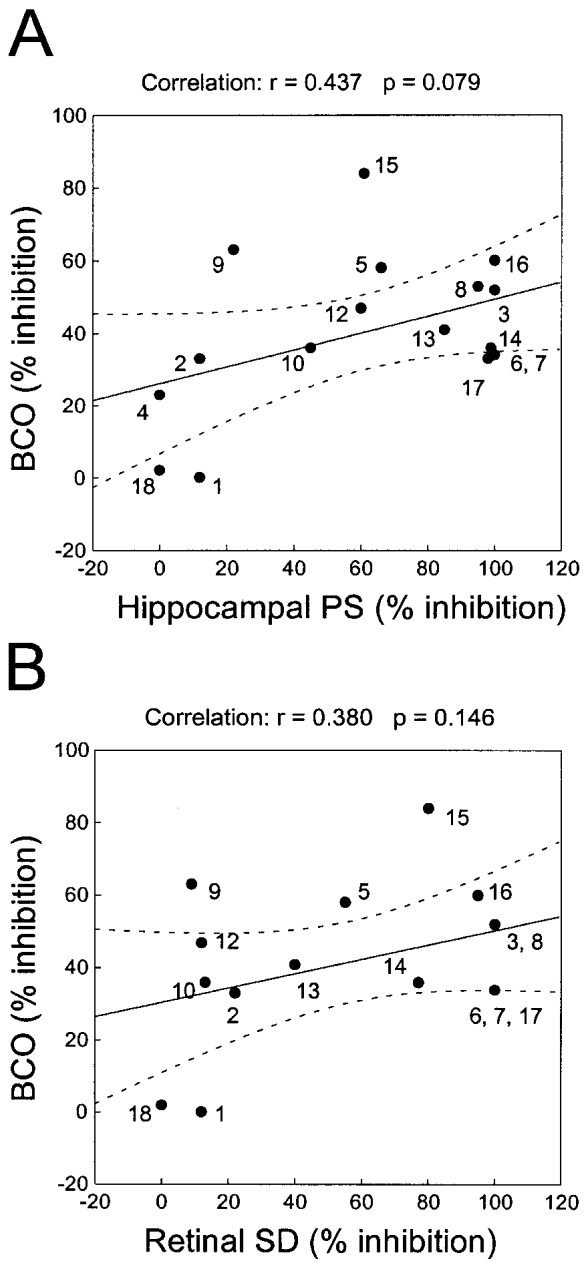


Fig. 4. Correlation between the *in vitro* and *in vivo* effects of 2,3-benzodiazepine AMPA antagonists. (A) Inhibition of PS amplitude (10 μM) compared to neuroprotective effects in the gerbil bilateral carotid occlusion model (BCO) (4 × 5 or 4 × 15 mg/kg i.p., n = 16). (B) Inhibition of retinal spreading depression (SD) (5 μM) against effects in hippocampal CA1 in BCO test (n = 16).

compounds, we found decreased *in vivo* efficacy (***14*** and ***17*** *vs.* ***3***), and oppositely, chemical modification of compound ***2*** resulted in significant increase in the *in vivo* neuroprotective effect in gerbil BCO model (CA1 data of ***9*** and ***12***).

In the case of 7,8-dichloro derivative ***15*** (EGIS-9879), increased *in vitro* efficacy was accompanied by significantly reduced hypermotility in the gerbil after BCO (increase in motility is a well-established phenomenon in gerbils after a stroke episode, see Ref. 19) and a marked protection of the animals from the deterioration of memory function (SA). In addition, EGIS-9879 caused a considerable attenuation of the ischemia-induced damage in CA1. The 8-bromo derivative ***12*** (EGIS-9883) dramatically decreased hypermotility; however, it was less potent than ***9*** against CA1 cell loss.

Earlier we have shown a moderate correlation between the CA1 score and hypermotility ($r = 0.631$, $p < 0.001$, $n = 16$), although the CA1 score did not correlate with the change in SA ($r = -0.32$, $p = 0.069$, $n = 17$) (20). Lack of correlation between the *in vivo* data is conspicuous at compound ***9***. Lack of the effect on the behavioral parameters (SA and HM) can be explained by the relatively weak *in vitro* efficacy. However, the surprisingly high protection rate in CA1 (63%) supports our hypothesis that factors other than AMPA antagonism may participate in the preventing effects of 2,3-benzodizepines on neuronal cell loss in the hippocampus.

It has been widely demonstrated that the blockade of AMPA receptors is effective in various stroke models (14,21,22), and AMPA antagonists have been regarded as potential neuroprotective agents (21,23). However, it was not obvious that stronger *in vitro* AMPA antagonist potency of a compound corresponds to higher antiischemic activity. For example, treatment with the very potent competitive blocker NBQX resulted in significant antiischemic effects only at relatively high doses in the gerbil BCO model (9).

In order to answer the question whether higher *in vitro* potency predicts more pronounced neuroprotection, we compared the AMPA antagonist efficacy of the new 2,3-benzodiazepines *in vitro* and their neuroprotective effects *in vivo*. Despite that structural changes of the compounds resulted in increased or decreased *in vitro* activity, we found that these changes were not consequently mirrored in the BCO model. Although weak correlation was established between the percentage inhibition of the amplitude of population spikes (PS) and neuronal cell loss in the CA1 area in the gerbil model, it was not statistically significant. Also, the lack of significant correlation was found between the blockade of spreading depression (SD) and CA1 cellular loss.

One explanation of the differences between *in vivo* and *in vitro* efficacy of 2,3-benzodiazepines might be that sensitivity of neurons in different brain regions (i.e., hippocampus, cortex) or in the retina are possible as a result of different cellular patterns of AMPA receptor subunits (24,25). This must be true when *in vitro* experiments are concerned in different species (25,26); however, we could not establish significant differences between the results obtained from our *in vitro* tests. It is also possible that certain metabolic or kinetic factors considerably modify the activity or bioavailability of the compounds. It was found that compound ***1*** and its two derivatives blocked sound-induced seizures in mice with different kinetics (10) that might be related to differential metabolism of the compounds. Different pharmacokinetic properties of 2,3-benzodiazepines were described in rats (27) and in various species (28). In addition, patch-clamp experiments showed that AMPA current blockade of the 7-chloro or 7,8-dichloro derivatives developed slowly, and the recovery from the blockade took several seconds, suggesting considerably slower binding and unbinding kinetics than those of the original compound. Moreover, capability of compounds in crossing the blood–brain barrier might be different and may represent an important factor for their *in vivo* efficacy. It is also presumable that in addition to the AMPA antagonist effects 2,3-benzodiazepines possess further antiischemic potential via different mechanisms. It is known that compound ***1*** at high concentrations inhibited voltage-gated Na^+ channels (29). In

addition, inhibition of excitatory amino acid release by 2,3-benzodiazepines can also be an important component of their neuroprotective actions (30,31). Different hypothermic effects of 2,3-benzodiazepines may also underlie the differences between their *in vivo* effects. Gyertyán *et al.* (9) reported that compound ***1*** proved to be superior to NBQX in the gerbil BCO model that might be attributed to the considerable hypothermia in its neuroprotective dose.

We conclude, that the *in vitro* models used seem to be suitable for primary pharmacologic screening of AMPA receptor antagonist activity. However, these tests mainly provide qualitative prediction of the neuroprotective efficacy of the compounds. Beside the direct effects of 2,3-benzodiazepines on the AMPA receptor-mediated neuronal damage, several other factors might considerably influence the *in vivo* cerebral antiischemic potential of these compounds. It cannot be excluded that, in addition to their short-term effects on AMPA receptors, 2,3-benzodiazepines possess modulatory effects on the delayed mechanisms of neuronal damage (32) such as excitotoxicity, peri-infarct depolarisation, inflammation or apoptosis.

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