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Non-N-Methyl-D-Aspartate Receptor Antagonism by 3-N-Substituted 2,3-Benzodiazepines: Relationship to Anticonvulsant Activity

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ABSTRACT

Block of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) and kainate currents by GYKI 52466 [1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine], a noncompetitive non-N-methyl-D-aspartate (AMPA/kainate) receptor antagonist, and two 3-N-substituted 3,4-reduced GYKI 52466 analogs was assessed in whole cell voltage-clamp recordings from cultured rat hippocampal neurons. In addition, the activity of the analogs was determined in the maximal electroshock seizure test and for protection against kainate-induced seizures in mice. The analogs of GYKI 52466 tested were the 3-N-methylcarbamyl [GYKI 53655; 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine] and the 3-N-acetyl [GYKI 53405; 1-(4-aminophenyl)-3-acetyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine]. GYKI 53655 produced a concentration-dependent inhibition of AMPA- and kainate-induced currents with IC_{50} values of 1.1 and 1.5 μM, respectively; the corresponding values for GYKI 53405 were 3.8 and 5.0 μM. As blockers of AMPA currents, the analogs were 8- and 2.3-fold, respectively, more potent than the parent GYKI 52466. Kinetic analyses indicated increased association rates for the two 3-N-substituted analogs ($2.5–2.6 \times 10^5$ M^{-1} sec^{-1}) compared with GYKI 52466 (1.6×10^5 M^{-1} sec^{-1}). The dissociation rates of GYKI 52466, GYKI 53405 and GYKI 53655 were inversely correlated with increasing blocking potency (2.9, 1.7 and 0.6 sec^{-1}, respectively). Thus, the increased affinity of the 3-N-substituted analogs relates to their increased binding and decreased unbinding rates. In anticonvulsant testing *in vivo*, GYKI 53655 and GYKI 53405 had ED_{50} values against kainate (32 mg/kg s.c.) seizures of 4.6 and 7.5 mg/kg i.p., compared with 8.4 mg/kg for GYKI 52466. The corresponding values in the maximal electoshock seizure test were 4.6 and 5.9 mg/kg, compared with 11.8 mg/kg for GYKI 52466. The rank order of potencies of the three compounds *in vivo* corresponds with their *in vitro* potencies, supporting the view that the anticonvulsant activity is related to blockade of non-N-methyl-D-aspartate receptors.

Selective non-NMDA (AMPA/kainate) excitatory amino acid receptor antagonists exhibit anticonvulsant and neuroprotective activity, suggesting a role for such compounds in the treatment of epilepsy and other neurological disorders believed to be associated with excessive activation of excitatory amino acid receptors. The first such agents to be developed were competitive antagonists acting at the agonist (glutamate) recognition site on AMPA/kainate receptors (Sheardown *et al.*, 1990). More recently, the 2,3-benzodiazepine GYKI 52466 [1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine] has been identified as a potent and selective AMPA/kainate antagonist (Tarnawa *et al.*, 1990; Ouardouz and Durand, 1991; Jones *et al.*, 1992). GYKI 52466 antagonism of AMPA/kainate receptor responses cannot be overcome by increasing the concentration of agonist, indicating that block occurs in a noncompetitive (allosteric) fashion (Donevan and Rogawski, 1993). Like competitive AMPA/kainate antagonists, GYKI 52466 has anticonvulsant activity in a variety of animal seizure models (Chapman *et al.*, 1991; Smith *et al.*, 1991; Yamaguchi *et al.*, 1993) and is neuroprotective in focal and global ischemia (Le Peillet *et al.*, 1992; Smith and Meldrum, 1992; Zorumski *et al.*, 1993; May and Robison, 1993).

Tarnawa and colleagues (1993) have investigated a large series of GYKI 52466 analogs for *in vitro* and *in vivo* biological activity. Introduction of certain substituents at position 3 of the benzodiazepine ring of GYKI 52466 enhanced potency. The most potent analogs described were the 3-N-methylcarbamyl, GYKI 53655 [1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine], and the 3-N-acetyl, GYKI 53405

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ABBREVIATIONS: NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; MES, maximal electroshock seizure; TI, therapeutic index; NIH, National Institutes of Health.

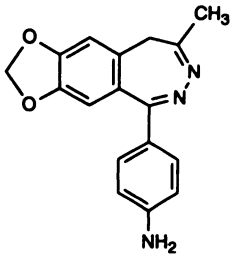
[1-(4-aminophenyl)-3-acetyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine; fig. 1]. In the present study we examined the blocking activity of these two analogs against AMPA- and kainate-induced currents in whole cell recordings from cultured rat hippocampal neurons. In addition, we compared the relative potencies of the compounds in this *in vitro* assay with their protective activities against kainate-induced seizures in mice and in the MES test, a model with high predictive value for clinically relevant antiepileptic activity (Rogawski and Porter, 1990). Finally, we assessed the propensity of the analogs to induce neurological impairment by using a simple test of motor toxicity.

Methods

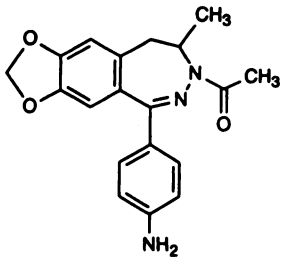
Electrophysiology

Cell culture. Hippocampal neurons from 19-day-old Sprague-Dawley rat (Taconic, Germantown, NY) embryos were grown in primary culture as described previously (Subramaniam *et al.*, 1994) and were used 5 to 10 days after plating.

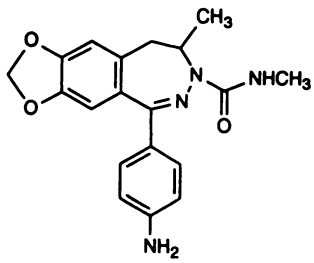
Whole-cell recording. Recordings were carried out at room temperature (23°C) in bathing solution containing (in millimolar): NaCl, 140; KCl, 5; $CaCl_2$, 2; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10. The bathing solution also contained 1 μM tetrodotoxin and 1 μM strychnine. Experiments were conducted in the absence of added glycine and contained 2 mM Mg^{++} to prevent activation of NMDA receptor channels. Whole-cell voltage clamp recordings were obtained with an Axopatch 200 amplifier (Axon Instruments, Burlingame, CA) using patch electrodes (2–4 megohm) filled with an intracellular solution containing (in millimolar): CsCl, 145; $MgCl_2$, 2; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5; $CaCl_2$, 0.1; and ethylene glycol bis-(β-aminoethyl ether)-N,N′-tetraacetic acid, 1. The holding potential was maintained at −60 mV. Voltages corresponding to the membrane currents were acquired in digital form by using the pClamp or Axotape software packages (Axon Instruments). NFIT (Island Products, Galveston, TX) was used for nonlinear curve fitting. Data are presented as the mean ± S.E.M.; *n* is the number of cells tested.



GYKI 52466



GYKI 53405



GYKI 53655

Fig. 1. Structures of 2,3-benzodiazepine analogs.

Drug perfusion. Drugs were applied by using a seven-barrel, rapid perfusion system in which all of the solutions exited *via* a common ~600 μm (inside diameter) orifice situated within 400 μm from the cell surface (see Donevan and Rogawski, 1993). The solution exchange time constant at the tip of a recording electrode (situated 300 μm away) was less than 5 msec. One barrel contained bathing solution whereas the other barrels contained agonist and antagonists either alone or in combination.

Anticonvulsant Testing

Animals. Male NIH Swiss mice (25–30 g) were obtained from the NIH Animal Program (Bethesda, MD). Animals were allowed to acclimatize with free access to food and water for a 24-hr period before testing. All procedures were carried out in strict compliance with the NIH Guide for the Care and Use of Laboratory Animals under a protocol approved by the NIH Animal Use Committee.

MES test. Fifteen minutes after i.p. injection of the 2,3-benzodiazepine, the animals were subjected to a 0.2 sec, 60 Hz, 50 mA electrical stimulus delivered with corneal electrodes (5 mm diameter stainless-steel balls) wetted with 0.9% saline. Animals failing to show tonic hindlimb extension were scored as protected. The interval between i.p. drug injection and testing was chosen based upon the time of peak effect of GYKI 52466 in mice as reported by Chapman *et al.* (1991).

Kainate seizure tests. Kainate was administered at a dose of 32 mg/kg s.c. (previously determined ED_{97} value) 15 min after i.p. injection of antagonist. Animals showing 5 sec or more of clonic activity were scored as not protected. The observation period was 60 min.

Motor toxicity test. Evaluation for motor toxicity was carried out by using a modification of the horizontal screen test described by Coughenour *et al.* (1977) which determines the animal's ability to support its own body weight by grasping a grid. Untrained mice were placed on a horizontally oriented grid (consisting of parallel 1.5 mm diameter rods separated by 1 cm) and the grid was inverted. Animals that fell from the grid within 5 sec were scored positive.

Drugs and solutions. Kainate was administered in a volume of 0.9% saline equaling 1% of the animal's body weight (0.01 ml/g). Stock solutions of GYKI 53655 and GYKI 5340 were prepared in 100% DMSO at a concentration of 10 mg/ml. The stocks were diluted in 0.9% saline so that the concentration of DMSO was < 35–40% and administered in a volume of 0.01 ml/g body weight. This concentration of DMSO failed to cause seizure protection or motor toxicity. GYKI 52466 was from Dr. Istvàn Tarnawa, Institute for Drug Research (Budapest, Hungary) and the 3-N-substituted analogs were from Dr. J. David Leander, Eli Lilly and Co. (Indianapolis, IN). AMPA was from Tocris Neuramin (Essex, UK). All other drugs were from Sigma Chemical Co. (St. Louis, MO).

Data analysis. ED_{50} values (doses protective in 50% of animals) and their 95% CL were determined by log-probit analysis by using the Litchfield-Wilcoxon method. The dose causing motor impairment in 50% of animals is referred to as the TD_{50}. The TI, a measure of the relative toxicity, was calculated as the ratio TD_{50}/MES ED_{50}.

Results

Whole-cell voltage clamp recording. Application of 100 μM AMPA to cultured hippocampal neurons under voltage clamp induced a rapidly desensitizing inward current response, followed by a steady plateau response that persisted at constant amplitude for > 30 sec. As illustrated in figure 2A, 3 μM GYKI 53655, GYKI 53405 and GYKI 52466 induced a reversible inhibition of the AMPA current. The degree of block produced by each of the analogs increased in a concentration-dependent fashion as shown in figure 2B, which plots data derived from a series of experiments similar to those illustrated in figure 2A. The analogs also blocked currents

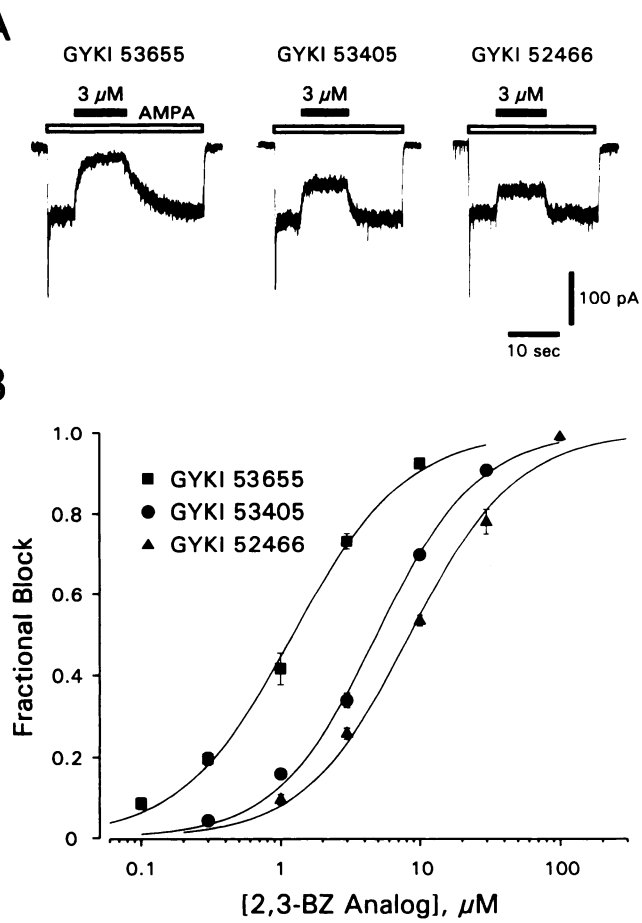


Fig. 2. Block of AMPA-evoked inward current responses by 2,3-benzodiazepine (2,3-BZ) analogs. A, representative traces showing block of steady-state current induced by 100 μM AMPA in a cultured hippocampal neuron by three 2,3-benzodiazepine analogs at a concentration of 3 μM. Holding potential, −60 mV. B, concentration-block isotherms for the three analogs. Each point represents mean ± S.E.M. of data from four to nine cells. Where not shown, error bars were smaller than the size of the symbols. The data were fit to the logistic equation $B = 1/\{1 + (IC_{50}/[D])^{n_H}\}$ where B is the fractional block, [D] is the 2,3-benzodiazepine concentration, IC_{50} is the concentration resulting in 50% block and n_H is the steepness of fit. IC_{50} values are given in table 1; n_H = 1.1 to 1.2.

evoked by 100 μM kainate (see fig. 3A). Concentration-response curves derived from experiments examining the inhibition of 100 μM kainate currents by GYKI 53655 and GYKI 53405 demonstrated similar blocking potencies as against AMPA responses. The IC_{50} values for block of AMPA and kainate currents are given in table 1.

The rate constants for binding and unbinding of the 2,3-benzodiazepines were determined in experiments examining the block of 100 μM kainate currents (fig. 3). The apparent rate for the onset of block (k_{app}) was calculated as the reciprocal of the time constant of the best single exponential fit to the current trajectory following onset of antagonist application. As illustrated in figure 3B, k_{app} increased in a linear fashion with increasing concentration of each of the antagonists. Assuming a 1:1 bimolecular binding reaction between the drugs and their acceptor sites, $k_{app} = k_1 [D] + k_{-1}$, where k_1 and k_{-1} are the forward and reverse rate constants, respectively, and $[D]$ is the concentration of the 2,3-benzodiazepine. k_1 and k_{-1} values were determined from the best fit straight lines to the k_{app} values for the analogs and the results are tabulated in table 1 along with the values for GYKI 52466 determined in a previous study (Donevan and Rogawski, 1993). The kinetically determined dissociation constants (calculated as $K_D = k_{-1}/k_1$) for each of the analogs compared favorably with the IC_{50} values determined from the concentration-response isotherms obtained for block of the steady-state response to kainate (see table 1). Figure 3C plots the off rates (k_{off}) determined from best single exponential fits to the trajectories of kainate current responses after termination of antagonist applications. For all three analogs, the slopes of the best straight line fits to the concentration-rate data were not significantly different from that of a horizontal line, indicating the expected lack of dependence of unbinding rate on antagonist concentration. The unbinding rates determined from the recovery data were 3.4 ± 0.3, 1.6 ± 0.1 and 0.37 ± 0.04 sec^{-1} for GYKI 52466, GYKI 53405 and GYKI 53655, respectively, and, as expected, were not significantly different from those derived from the extrapolation of the fits to the k_{app} data (table 1).

Similar kinetic experiments were performed for the GYKI 53655 block of steady-state currents evoked by 100 μM AMPA. The rate constant values determined from measurements of k_{app} in four cells were $k_1 = 2.0 \pm 0.1 \times 10^5$ M^{-1} sec^{-1} and $k_{-1} = 0.46 \pm 0.04$ sec^{-1}, which are comparable to the values for block of kainate currents (table 1).

Anticonvulsant and motor toxicity testing. GYKI 53655 and GYKI 53405 produced a dose-dependent protection against kainate-induced seizures and were also protective against hindlimb extension in the MES test (fig. 4). ED_{50} values in the two seizure models are presented in table 2. At doses within the same range as those that produced seizure protection, the analogs also induced motor impairment as assessed with the horizontal screen test (fig. 4; table 2). Animals exhibiting motor toxicity showed decreased activity levels and unsteady gait, but no other gross behavioral disturbances. The analogs failed to cause lethality even at the highest dose tested (17.3 mg/kg).

Discussion

Previous studies have demonstrated that the 2,3-benzodiazepine GYKI 52466 is a highly selective, noncompetitive AMPA/kainate receptor antagonist that appears to act *via* a novel allosteric site on the receptor complex (Donevan and Rogawski, 1993; Zorumski *et al.*, 1993). GYKI 52466 has anticonvulsant activity in a variety of animal seizure models (Chapman *et al.*, 1991; Smith *et al.*, 1991; Yamaguchi *et al.*, 1993). Because GYKI 52466 does not affect NMDA, γ-aminobutyric $acid_A$ or other known neurotransmitter receptor systems (Tarnawa *et al.*, 1990; Donevan and Rogawski, 1993), it is presumed that this anticonvulsant activity is due to AMPA/kainate receptor blocking activity. The results of the present study strongly support this concept. We compared the AMPA and kainate blocking potencies of GYKI 52466 with two 3-N-substituted, 3,4-reduced GYKI 52466 analogs. The rank order of potencies of the three 2,3-benzodiazepines (GYKI 53655 > GYKI 53405 > GYKI 52466) was identical to their ranking in the two anticonvulsant models and also in the motor toxicity test, indicating that activity in the *in vivo* test systems is likely due to AMPA/kainate receptor blocking activity. Additional support for this idea is pro-

TABLE 1

Equilibrium potencies and rate constants for block of AMPA and kainate currents by 2,3-benzodiazepine analogs

IC_{50} values for block of AMPA currents were determined from the fits to the data in figure 2B; values for block of kainate currents were obtained from a similar series of experiments. Kinetic values were determined for block of kainate currents. k_{-1} was determined by extrapolation of k_{app} values (see "Results"). Numbers in parentheses, numbers of cells tested; k_{-1} means are all significantly different (one-way analysis of variance; $P < .01$); mean k_1 for GYKI 52466 is significantly different from values for GYKI 53405 and GYKI 53655 ($P < .01$).

Analog	AMPA Block (IC_{50})	Kainate Block (IC_{50})	k_{-1}	k_1	K_D (k_{-1}/k_1)
	μM	μM	sec^{-1}	$\times 10^5\ M^{-1}\ sec^{-1}$	μM
GYKI 52466	8.8 ± 0.5 (5)	8.2 ± 0.5 (6)	2.9 ± 0.2 (7)	1.6 ± 0.1 (7)	18
GYKI 53405	3.8 ± 0.1 (5)	5.0 ± 0.3 (6)	1.7 ± 0.1 (4)	2.6 ± 0.2 (4)	6.9
GYKI 53655	1.1 ± 0.1 (6)	1.5 ± 0.1 (7)	0.6 ± 0.1 (3)	2.5 ± 0.1 (5)	2.4

vided by the demonstration that concentrations of GYKI 52466 which are effective in blocking AMPA and kainate-evoked currents *in vitro* are similar to brain levels obtained after i.p. administration of an anticonvulsant dose of the drug (Tarnawa *et al.*, 1990).

Kinetic experiments revealed that the increase in potency of the 3-N-substituted analogs resulted both from an increase in binding rate (k_1) and a decrease in the unbinding rate (k_{-1}) in comparison with the parent GYKI 52466. The two 3-N-substituted analogs had similar k_1 values, which were roughly 60% greater than that of GYKI 52466, indicating that the substitutions in position 3 result in molecules with a decreased energy barrier for binding.

GYKI 53405 was roughly twice as potent as GYKI 52466 in blocking AMPA and kainate-evoked currents and also was about twice as potent in protecting against MES-induced seizures. On the other hand, GYKI 53655 had 8-fold greater potency *in vitro* but was only twice as potent as GYKI 52466 in the seizure models. The less than expected potency of GYKI 53655 *in vivo* may reflect its lower bioavailability.

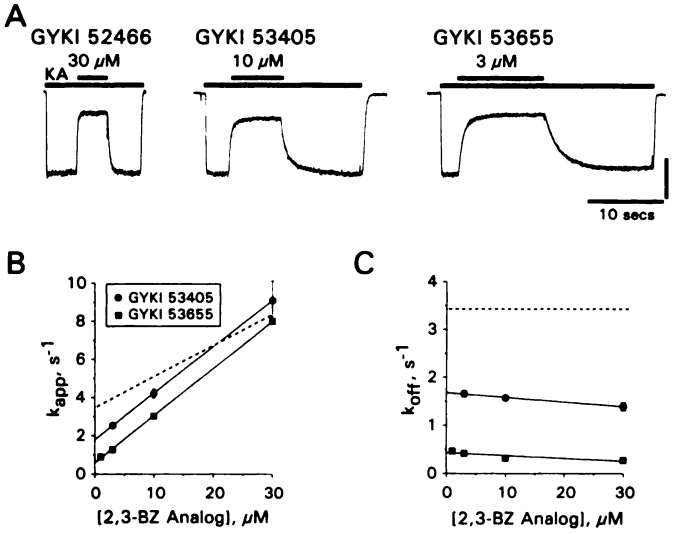


Fig. 3. Kinetic analysis of 2,3-benzodiazepine (2,3-BZ) analog block of kainate currents. A, block of 100 μM kainate currents by GYKI 52466 (30 μM), GYKI 53405 (10 μM) and GYKI 53655 (3 μM). The currents have been scaled to similar amplitudes; the scale bar represents 750, 350 and 650 pA for the *left*, *middle* and *right* traces, respectively. B, reciprocal time constant of onset of block (k_{app}) values at various concentrations of GKYI 53405 and GYKI 53655. The time constants were determined from the best single exponential fits to the current traces beginning upon onset of blocker application. Each point represents the mean ± S.E.M of data from experiments with three to five cells. When error bars are not shown they were smaller than the size of the symbols. The best fit straight lines to the data are shown. – – –, the best fit line to data obtained in a series of experiments with GYKI 52466 as reported previously (Donevan and Rogawski, 1993). C, reciprocal time constant of recovery (k_{off}) values obtained from best single exponential fits to current trace beginning at termination of the blocker application (same experiments as in B). The slopes are not significantly different from a flat line (test for parallelism). – – –, data for GYKI 52466 as reported previously. Kinetic parameters are given in table 1.

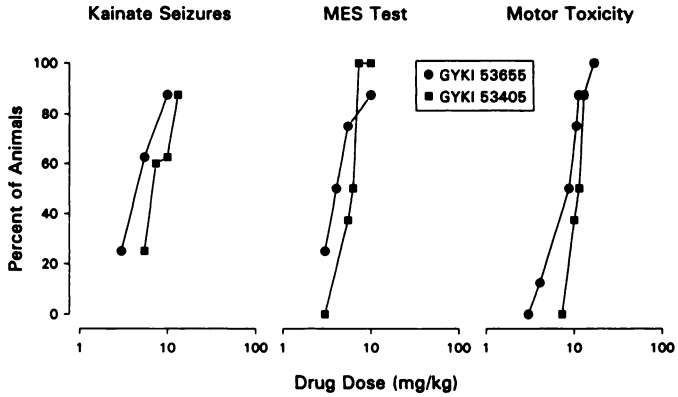


Fig. 4. Dose-response curves for anticonvulsant activity and motor toxicity of GYKI 53655 and GYKI 53405. Testing was carried out as described under "Methods." Each data point represents eight mice.

TABLE 2

Protection against maximal electroshock (MES) and kainate seizures and induction of motor impairment by 2,3-benzodiazepine analogs

ED_{50} and TD_{50} values were determined by log probit analysis of data presented in figure 4. Data for GYKI 52466 are from Yamaguchi *et al.* (1993).

Analog	MES Test (ED_{50})	Kainate Seizures (ED_{50})	Motor Impairment (TD_{50})	TI (TD_{50}/ MES ED_{50})
		mg/kg i.p.		
GYKI 52466	11.8 (9.7–14.3)	8.4 (5.7–12.4)	13.2 (11.2–15.6)	1.1
GYKI 53405	5.9 (4.7–7.3)	7.5 (5.4–10.2)	10.8 (9.6–12.3)	1.8
GYKI 53655	4.6 (3.4–6.3)	4.6 (2.9–7.3)	8.7 (7.2–10.6)	1.9

Although 2,3-benzodiazepine AMPA/kainate receptor antagonists are highly effective anticonvulsants in animal seizure models, the practical utility of this class of compounds in epilepsy therapy may be limited by toxicity. We reported previously that GYKI 52466 has a TI value near 1 (Yamaguchi *et al.*, 1993; see table 2). The TI of the 3-N-substituted analogs was nearly double this value, but still falls short of that exhibited by some widely used antiepileptic compounds (Rogawski and Porter, 1990). Recently, it has been demonstrated that low, nontoxic doses of AMPA/kainate receptor

antagonists can potentiate the anticonvulsant effects of conventional antiepileptic agents (Zarnowski *et al.*, 1993) and also NMDA antagonists (Löscher *et al.*, 1993). However, whether combined use of 2,3-benzodiazepine analogs with other anticonvulsants would result in improved efficacy with acceptable toxicity remains to be determined.

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