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LU 73068, a new non-NMDA and glycine/NMDA receptor antagonist: pharmacological characterization and comparison with NBQX and L-701,324 in the kindling model of epilepsy

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1 The aim of this study was to assess whether a drug which combines an antagonistic action at both NMDA and non-NMDA receptors offers advantages for treatment of epileptic seizures compared to drugs which antagonize only one of these ionotropic glutamate receptors.

2 The novel glutamate receptor antagonist LU 73068 (4,5-dihydro-1-methyl-4-oxo-7-trifluoromethyl-imidazo[1,2*a*]quinoxaline-2-carbonic acid) binds with high affinity to both the glycine site of the NMDA receptor (K_i 185 nM) and to the AMPA receptor (K_i 158 nM). Furthermore, binding experiments with recombinant kainate receptor subunits showed that LU 73068 binds to several of these subunits, particularly to rGluR7 (K_i 104 nM) and rGluR5 (K_i 271 nM). In comparison, the prototype non-NMDA receptor antagonist NBQX (2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo[f]quinoxaline) binds with high affinity to AMPA receptors only.

3 Both NBQX and LU 73068 were about equieffective after i.p. injection in mice to block lethal convulsions induced by AMPA or NMDA.

4 In the rat amygdala kindling model of temporal lobe epilepsy, LU 73068 dose-dependently increased the focal seizure threshold (afterdischarge threshold, ADT). When rats were stimulated with a current 20% above the individual control ADT, LU 73068 completely blocked seizures with an ED_{50} of 4.9 mg kg^{-1}.

5 Up to 20 mg kg^{-1}, only moderate adverse effects, e.g. slight ataxia, were observed.

6 NBQX, 10 mg kg^{-1}, and the glycine/NMDA site antagonist L-701,324 (7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-quinoline-2(1H)one), 2.5 or 5 mg kg^{-1}, exerted no anticonvulsant effects in kindled rats when administered alone, but combined treatment with both drugs resulted in a significant ADT increase.

7 The data indicate that combination of glycine/NMDA and non-NMDA receptor antagonism in a single drug is an effective means of developing a potent and effective anticonvulsant agent.

Keywords: Seizures; glutamate; AMPA; kainate; neuroprotection; anticonvulsant

Introduction

In view of the role of excitatory amino acid transmitters such as glutamate in the initiation of seizures and their propagation, development of glutamate receptor antagonists has long been thought to be a valuable strategy in the search for new effective anticonvulsant drugs (Dingledine *et al*., 1990; Meldrum, 1992; Löscher & Schmidt, 1994; Löscher, 1998b,c). Previously, most attention has been directed to drugs blocking the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors, mainly because NMDA antagonists were readily available, including some which crossed the blood-brain-barrier (BBB), but more recent evidence indicates potential anticonvulsant efficacy of BBB-permeable non-NMDA (AMPA/kainate) receptor antagonists as well (Lees, 1996). First clinical trials with competitive and uncompetitive NMDA antagonists in epileptic patients were disappointing because of lack of anticonvulsant efficacy and the occurrence of severe adverse effects, such as motor impairment and confusion (Löscher & Schmidt, 1994). The only animal model that predicted the unfavourable clinical effects of NMDA antagonists was the kindling model of temporal lobe epilepsy (*c.f.* Löscher, 1998a), in which NMDA antagonists were not effective anticonvulsants but induced more severe adverse effects than in nonkindled animals, probably because epileptogenesis as induced by kindling altered the pharmacology of these drugs (Löscher & Hönack, 1991; Löscher, 1998c). In contrast to NMDA antagonists, non-NMDA antagonists such as NBQX (2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo[f]quinoxaline; Figure 1) are potent anticonvulsants in the kindling model in the absence of marked adverse effects (Löscher *et al*., 1993; Löscher, 1998c). Furthermore, we found that addition of a low dose of a competitive or uncompetitive NMDA receptor antagonist to NBQX profoundly increases the anticonvulsant potency of the treatment without concomitantly increasing severity of adverse effects, suggesting a synergistic interaction between NMDA and non-NMDA antagonists (Löscher *et al*., 1993; Löscher & Hönack, 1994). Based on these data we proposed that a drug which combines an antagonistic action at both NMDA and non-NMDA receptors might be an interesting candidate for antiepileptic drug development (Löscher, 1998c).

The NMDA receptor-ion channel complex contains multiple regulatory sites, including the glutamate/NMDA recognition site, a co-agonist glycine site, and a site within the ion channel, all of which are targets for pharmacologically modulating NMDA receptor function (Cotman *et al*., 1995). In contrast to drugs binding at the glutamate/NMDA

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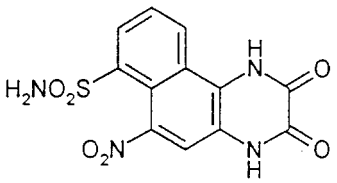
recognition site (competitive NMDA antagonists) and the 'phencyclidine site' within the ion channel (uncompetitive NMDA antagonists), drugs blocking the glycine co-agonist site are thought to offer several advantages in terms of risk-benefit ratio (Carter, 1992). In the present study, we describe a novel drug, LU 73068 (4,5-dihydro-1-methyl-4-oxo-7-trifluoromethyl-imidazo[1,2*a*]quinoxaline-2-carbonic acid; Figure 1), which acts as an antagonist at both the glycine/NMDA site and at non-NMDA receptors. This compound was selected from a large series of newly synthesized BBB-permeable quinoxaline derivatives acting predominantly on non-NMDA receptors, and exhibited the highest neuroprotective potency of all compounds of this series tested so far. Here we provide pharmacological characteristics of this novel compound with special emphasis on its effects in the kindling model. In order to evaluate whether a combination of non-NMDA receptor antagonism and glycine/NMDA receptor antagonism leads to a synergistic interaction in this model, we compared the effects of LU 73068 with those of NBQX and the novel glycine/NMDA antagonist L-701,324 (7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-quinoline-2(1H)one; Bristow *et al.*, 1996), both given alone or in combination.

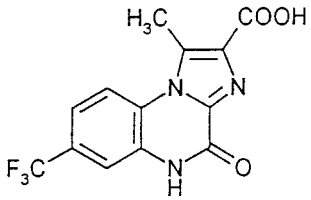
Methods

Drugs

LU 73068 and NBQX were provided by BASF (Ludwigshafen, Germany). L-701,324 was kindly provided by Merck, Sharp and Dohme (Harlow, U.K.). For *in vivo* experiments, LU 73068 was dissolved in 0.075 M Tris buffer (pH about 8.5). NBQX was dissolved in water as its sodium salt (prepared by adding equimolar amounts of NaOH). L-701,324 was dissolved in polyethylene glycol (PEG 400), diluted with water and alkalinized with NaOH (final concentration of PEG 400 about 20%; final pH 9-10). All doses or drug concentrations refer to the free drug forms. Controls received the respective vehicle (Tris, saline or PEG) injections. Injection volumes were 2–3 ml kg^{-1} in rats and 10 ml kg^{-1} in mice.



NBQX



LU 73068

Figure 1 Chemical structure of LU 73068 and, for comparison, NBQX.

Binding experiments with LU 73068 and NBQX

For the determination of affinities for kainate-receptor subunits binding assays were performed using membranes from HEK293 cells transfected with the particular receptor subunits. Affinities for AMPA-receptors, high affinity kainate-receptors, the glycine site of the NMDA-receptor, and the inhibition of use-dependent binding of ^{3}H-MK-801 (^{3}H-dizocilpine) were investigated using native receptors from rat brain. The experimental protocols used for AMPA, high affinity kainate and glycine binding assays were essentially similar to the assays described by Honoré *et al.* (1982, 1986) and Marvizón (1988). The assays for kainate receptor subunits were slight modifications of the protocol used by Honoré *et al.* (1986).

In short, the membrane preparations were performed as follows: HEK 293 cells expressing human KA2 receptor subunits (hKA2), rat GluR5 subunits (rGluR5), rat GluR6 subunits (rGluR6) or rat GluR7 subunits (rGluR7) were cultured in RPMI Glutamax dialysed with 10% FCS containing 1% antibiotic/antimycotic (from Gibco, containing penicillin, streptomycin and amphotericin) and G418 (400, 600 or 800 μg ml^{-1}). Cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C, detached with trypsin solution (0.05% trypsin, 0.0004% EDTA, 0.02% EGTA, 2.682 mM KCl, 1.47 mM KH_2PO_4, 6.46 mM Na_2HPO_4, 136.89 mM NaCl) and centrifuged at $250 \times g$ at room temperature. Thereafter approximately 1×10^7 cells ml^{-1} were resuspended in ice cold low salt buffer (5 mM Tris pH 7.4, 10% glycerol) and incubated for 30 min at 4°C. The membranes were then washed twice (by resuspension and centrifugation) and frozen at −70°C until required for use.

For the AMPA and high affinity kainate binding assays as well as for the use-dependent binding of ^{3}H-MK-801 membranes were prepared from rat forebrains. Forebrains were homogenized in about 15 volumes of preparation buffer (30 mM Tris pH 7.4, 0.5 mM Na_4EDTA) with the aid of an Ultra-Turrax (2×15 s). The homogenate was centrifuged at $48{,}000 \times g$ for 20 min. The supernatant was discarded and the membranes contained in the pellet were washed three times by resuspending in preparation buffer and centrifuging at $48{,}000 \times g$ (20 min each time). After the third washing, the membranes were resuspended in 15 volumes of preparation buffer, followed by incubation for 30 min at 37°C. The membranes were then washed twice (by resuspension and centrifugation) and frozen at −70°C until required for use. For the NMDA/glycine binding assay membranes from rat hippocampus were prepared. Hippocampi were homogenized in 15 volumes preparation buffer (50 mM Tris pH 7.4, 10 mM Na_4EDTA) with the aid of a Potter-Elvehjem homogenizer (500 r.p.m.). The homogenate was centrifuged at $48{,}000 \times g$ for 20 min. The supernatant was discarded and the membranes contained in the pellet were washed twice by resuspending in the buffer and centrifuging at $48{,}000 \times g$ (20 min each time). In the next step the membranes were frozen in liquid nitrogen and immediately thereafter thawed at 37°C. The membranes were washed once and incubated for 37°C for 15 min. After washing the membranes four times (by resuspension and centrifugation) they were frozen at −70°C until required for use.

Binding assays: For all assays the membranes were washed by centrifugation (20 min, $48{,}000 \times g$) and resuspension. The incubation buffer for the AMPA binding assay consisted of 50 mM Tris-HCl pH 7.1, 100 mM KSCN, 2.5 mM $CaCl_2$, for the NMDA/glycine binding assay of 50 mM Tris pH 7.4, 10 mM $MgCl_2$, and for the ^{3}H-MK-801 binding assay of 50 mM Tris pH 7.4, 0.1 mM EDTA. For all other assays

50 mM Tris-HCl pH 7.4 was used as buffer. All assays with the exception of the ^{3}H-MK-801 binding (final assay volume: 0.5 ml) were performed in a final volume of 1 ml. The following radioligands were used: 1.5 nM ^{3}H-AMPA (40–70 Ci mmol^{-1} for the AMPA assay, 10 nM ^{3}H-glycine (14.8 Ci mmol^{-1}) for NMDA/glycine, 1 nM ^{3}H-kainate (58 Ci mmol^{-1}) for high affinity kainate, 1 nM ^{3}H-kainate for the hKA2, 3 nM ^{3}H-kainate for rGluR5 assay, 1.5 nM ^{3}H-kainate for rGluR6, 3 nM ^{3}H-kainate for rGluR7, and 1.7 nM ^{3}H-MK-801 (23.9 Ci mmol^{-1}) for the use-dependent binding of ^{3}H-MK-801. The unspecific binding was determined by 1 mM glycine in the glycine assay, by 10 μM glutamate in the high affinity kainate assay, and 10 μM (unlabelled) MK-801 in the ^{3}H-MK-801 binding assay. In the other assays 1 mM glutamate was used. The amount of membranes in the assays was equivalent to 0.25 mg protein for the AMPA assay, 0.1 mg protein for NMDA/glycine, 0.25 mg protein for high affinity kainate, 0.125 mg protein for ^{3}H-MK-801 binding, 0.1 Mio cells for hKA2, 0.2 Mio cells for rGluR5, 0.1 Mio cells for rGluR6 and 0.1 Mio cells for rGluR7. In the case of ^{3}H-MK-801 binding, membranes and the compounds were incubated for 20 min (at 30°C) in the absence of radioligand. Thereafter, ^{3}H-MK-801 was added and the samples were incubated for further 30 min at 30°C. For all tests, samples were incubated for 60 min at 4°C. Incubation was stopped by filtration through Whatman GF/B-filters with 5–9 ml washes of cold incubation buffer. The radioactivity on the filters was determined by liquid scintillation counting (Packard Tri-Carb, counting efficiency: 35–50%). The mean values for the binding of the radioligands in the absence of inhibitors were 2500 d.p.m. for AMPA, 2200 d.p.m. for high affinity kainate, 2800 d.p.m. for NMDA/glycine, 1900 d.p.m. for hKA2, 1300 d.p.m. for rGluR5, 3400 d.p.m. for rGluR6, 1600 d.p.m. for rGluR7, and 600–2500 d.p.m. (depending on the presence of NMDA and glycine) for the ^{3}H-MK-801 binding.

Estimation of K_i-values: For each compound and particular binding assay 2–3 displacement experiments were performed with each concentration tested in triplicates. IC_{50} values were determined by simultaneous fitting of the data from 2–3 displacement experiments using the equation $\%B = \%B_{max}/[1 + (L/IC_{50})^n]$, where %B is the percentage of specific binding, IC_{50} the concentration of ligand resulting in 50% inhibition of specific binding, L the concentration of the ligand and n the Hill coefficient. IC_{50}-values were converted into K_i-values using the equation of Cheng & Prusoff $K_i = IC_{50}/[1 + (L/K_D)]$, where L is the concentration of the radioligand and K_D the dissociation constant of the radioligand. K_D values for the different radioligands were: 10 nM for AMPA, 460 nM for NMDA/glycine, 2.8 nM for kainate/high affinity, 1.5 nM for hKA2, 200 nM for rGluR5, 47 nM for rGluR6, 22 nM for rGluR7. For each compound and receptor assay the mean K_i value and the 95% confidence interval were calculated.

Prevention of AMPA- and NMDA-induced lethal convulsions in the mouse

The *in vivo* activity of LU 73068 and NBQX was determined as protective effect against AMPA- and NMDA-induced convulsions in the mouse.

Male NMRI mice (Janvier, Le Genest-Saint-Isle, France) of 28–30 g were pretreated (10 ml kg^{-1}) with the test drug (dissolved in water by adding NaOH) or corresponding vehicle. Sixty min [NBQX: 5 min] later, the glutamate receptor agonist was injected intracerebroventricularly (i.c.v.). Ten μl of an 0.035% NMDA [Sigma Chemicals Co., St. Louis MO, U.S.A.] solution or of an 0.075% AMPA [Tocris Neuramin Ltd., Essex U.K.] solution were injected (1–2 s), corresponding to a total amount of 3.5 μg NMDA and 7.5 μg AMPA, respectively, and the mice were observed for the following 3 min. Severe convulsions started shortly after the injection of the glutamate receptor agonist and resulted in death of vehicle treated control animals within approximately 1 min.

For statistical analysis, death within the 3-min-observation period was used as parameter, and inhibition of death as criterion of drug effect. The relationship between the log doses (mg kg^{-1}) and the relative rate of protection – compared to the vehicle controls [nearly 100% mortality] – was assessed. Probit analysis was used to determine the ED_{50} (mg kg^{-1}; with 95% confidence interval), i.e. the dose which prevents death in 50% of the animals.

Anticonvulsant and adverse effects in the kindling model

Experiments were performed on 210–230 g female Wistar rats (Harlan Winkelmann, Borchen, Germany). A bipolar electrode was stereotaxically implanted into the basolateral nucleus of the right amygdala as previously described (Ebert *et al*., 1995). After a postoperative period of 2 weeks, constant current stimulations (500 μA, 1 ms, monophasic square-wave pulses, 50/s for 1 s) were delivered to the amygdala at intervals of 1 day until ten sequential fully kindled (i.e. focal and secondarily generalized clonic) seizures were elicited. For evaluation of anticonvulsant drug effects on focal seizures, the afterdischarge threshold (ADT), i.e. the most sensitive measure of anticonvulsant activity against focal seizure activity in kindled rats, was recorded after kindling acquisition (with an interval of at least 4 days after the tenth stage five seizure) using an ascending stairstep procedure (Freeman & Jarvis, 1981). The initial current intensity was 10 μA, and the current intensity was increased in steps of about 20% of the previous current at intervals of 1 min until an afterdischarge of at least 3 s duration was elicited. In addition to ADT, the following parameters of kindled seizures were measured at ADT current: Seizure severity was classified according to Racine (1972): (1) immobility, eye closure, twitching of vibrissae, sniffing, facial clonus; (2) head nodding associated with more severe facial clonus; (3) clonus of one forelimb; (4) rearing, often accompanied by bilateral forelimb clonus; (5) rearing with loss of balance and falling accompanied by generalized clonic seizures. Seizure duration was the duration of limbic (stage 1–2) and/or motor seizures (stage 3–5). Afterdischarge duration was the total time of spikes in the EEG recorded from the site of stimulation. The effects of LU 73068, NBQX, and L-701,324 on ADT and severity and duration of seizures recorded at ADT were determined in groups of 6–9 fully kindled rats after i.p. drug injection (injection volume 2–3 ml kg^{-1}). The control ADT was determined 2–3 days prior to and after each drug treatment, and the next drug experiment was only undertaken if the post-drug ADT was not significantly different from the pre-drug ADT. For control determinations, rats received i.p. injection of vehicle with the same pretreatment time as in the respective drug experiment. For all drug experiments, at least 4 days were interposed between two drug injections in the same group of rats in order to avoid alterations in drug potency due to cumulation or tolerance. Significance of differences between seizure readings in the same group of rats (e.g. the difference between control and drug trial) was calculated by the Wilcoxon signed-rank test for paired replicates. ED_{50} for prevention of seizures at stimulation with 20% above individual predrug control ADT was calculated by the method of Litchfield & Wilcoxon (1949).

For examination of behavioural drug effects, the animals were removed from their home cages and placed singly in plastic cages. The animals were continuously observed for alterations in behaviour after i.p. drug injection up to the time of amygdala stimulation. For comparative evaluation of experiments, behavioural alterations determined immediately before ADT determination were used. Control experiments with vehicle (saline) injection were done in the same way. For all observations, rigorous observational protocols described elsewhere were used (Löscher & Hönack, 1992). Hyper- or hypolocomotion, head weaving (swaying movements of the head and upper torso from side to side for at least one complete cycle; i.e. left-right-left), stereotyped sniffing, biting, licking or grooming, reciprocal forepaw treading ('piano playing'), stereotyped rearing, reduction of normal rearing, hyperexcitability (as indicated by increased reactions to noise or handling), tremor, abduction of hind limbs, reduction of righting reflexes, flat body posture, circling, Straub tail and piloerection were scored using a ranked intensity scale where 0 = absent, 1 = equivocal, 2 = present and 3 = intense. Ataxia was scored using a six point rating system as described previously (Hönack & Löscher, 1995). In addition to rating motor impairment by observational scores, impaired motor function was quantitated by the rotarod test as described previously (Hönack & Löscher, 1995). Significance of differences between behavioural scores in the same group of rats (e.g. the difference between control and drug trial) was calculated by the Wilcoxon signed-rank test for paired replicates.

In all experiments, rectal body temperature was recorded by an electronic thermometer immediately before as well as 15 and 28 min after drug or vehicle administration. Significance of difference to predrug values in the same group of rats was determined by Student's *t*-test for paired data.

All rats were habituated to the various manipulations prior to onset of the drug experiments. Vehicle injection did not induce any behavioural alterations or rotarod failures, but sometimes significantly increased body temperature, most likely due to the stress associated with handling of the animals.

Results

Binding experiments

As NBQX, LU 73068 bound to native AMPA receptors with high affinity, but markedly differed in its glutamate receptor binding profile from NBQX (Table 1). Thus, while NBQX did not bind to the glycine/NMDA site, LU 73068 bound to this site with a similar high affinity (K_i 185 nM) than to native AMPA receptors (K_i 158 nM). Furthermore, while NBQX did essentially not bind to kainate receptors at relevant concentrations, LU 73068 exhibited relevant affinity to recombinant kainate receptor subtypes, particularly rGluR7 and rGluR5, in addition to AMPA receptors (Table 1).

IC_{50} values for the inhibition by LU 73068 of the use dependent binding of ^{3}H-MK-801 to membranes from rat forebrain are shown in Table 2. In the absence of added NMDA and glycine, LU 73068 displaced ^{3}H-MK-801 binding with an IC_{50} of 170 nM. However, this effect was lost when NMDA and glycine or glycine alone were added to the assay, while NMDA alone had only a moderate effect on the IC_{50} of LU 73068 (Table 2).

The use dependent binding of ^{3}H-MK-801 reflects the activity state of the receptor since ^{3}H-MK-801 binds only to the open state. The inhibition of the use-dependent ^{3}H-MK-801 bindings provides evidence for the antagonistic properties of LU 73068 at the NMDA receptor complex. The finding that the IC_{50} of the inhibition curve was shifted by glycine but only marginally by NMDA strongly indicates that LU 73068 antagonises the receptor *via* the glycine binding site while it does not bind to the glutamate binding site. It also does not bind to the phencyclidine/MK-801 site within the channel since

Table 1 K_i values [nM] for native and recombinant nonNMDA-receptors and the glycine site of NMDA receptors

	Native receptors from rat brain						
	AMPA	*NMDA/gly*	*kainate/high aff.*	*Recombinant kainate-receptor subunits expressed by HEK293 cells*			
	Rat forebrain	*Rat hippocampus*	*Rat forebrain*	*hKA2*	*rGluR5*	*rGluR6*	*rGluR7*
LU 73068	158	185	5580	n.d.**	271	551	104
	(107–208)	(62–309)	(3640–7530)		(148–394)	(471–631)	(62–146)
NBQX	112*	>20000	3480	18380	857	2200	3340
	(90–135)		(2750–4200)	(11900–24800)	(218–1500)	(1420–2980)	(2110–4570)

Affinities for AMPA-receptors, high affinity kainate-receptors and the glycine site of the NMDA-receptor were investigated using membrane preparations from rat brain. The affinities for kainate-receptor subunits were determined using membranes from HEK293 cells expressing the particular receptor subunits. For the determination of K_i values 2–3 individual displacement experiments were performed for each receptor and compound, respectively. IC_{50} values were calculated by simultaneous fitting of the Hill equation to the inhibition data from 2–3 individual experiments. IC_{50} values were transformed into the K_i values using the equation of Cheng & Prusoff. Data represent the mean K_i values and the 95% confidence intervals. *Data obtained from 5 individual displacement experiments. **n.d.: not determined.

Table 2 IC_{50} values for the inhibition of the use dependent binding of ^{3}H-MK801 to membranes from rat forebrain by LU 73068

−NMDA *−glycine*	*+0.1 mM NMDA* *+1 mM glycine*	*−NMDA* *+1 mM glycine*	*+0.1 mM NMDA* *−glycine*
170 nM (134–205 nM)	>3000 nM	>3000 nM	360 nM (315–400 nM)

For the determination of IC_{50}-values 3 individual displacement experiments were performed for each combination of NMDA and glycine. IC_{50}-values were calculated by simultaneous fitting of the Hill equation to the inhibition curves of the 3 individual experiments. Data represent the mean IC_{50} values and the 95% confidence intervals.

the binding to this site should not be inhibited by the addition of glycine as observed in the experiments.

Prevention of AMPA- and NMDA-induced lethal convulsion in the mouse

Time-course studies with i.p. drug injection at different times (5, 15 or 60 min) before i.c.v. application of AMPA or NMDA showed that maximum effects of NBQX and LU 73068 were obtained at 5 (NBQX) and 60 (LU 73068) min, respectively (not illustrated). At time of peak effect, both NBQX and LU 73068 were about equally effective to block AMPA- or NMDA-induced lethal convulsions, but LU 73068 was about twice as potent as NBQX in this respect (Table 3). ED_{50}s of LU 73068 were about 9 mg kg^{-1} i.p. against both excitatory amino acids.

Table 3 EAA-antagonism *in vivo*

	Prevention of lethal convulsions in the mouse induced by	
	NMDA	*AMPA*
Substance	ED_{50} (95% conf. lim.) [mg kg^{-1} i.p.]	
LU 73068	9.0 (4.5/18)	9.1 (5.7/15)
NBQX [5 min]	18.0 (12/29)	17.0 (6.6/42)

In unanesthetized mice, by intracerebroventricular injection of 10 µl of an 0.035% NMDA and of an 0.075% AMPA solution, respectively, severe convulsions were induced which resulted in death of vehicle treated control animals within 3 min. Test drugs were given i.p. 60 min [NBQX: 5 min] prior to the EAA-agonist; n/dose: 8–12. Using probit analysis, as mean effective dose (ED_{50} with 95% confidence interval) the dosage [mg kg^{-1} i.p.] is calculated which protects 50% of the animals from exitus.

Anticonvulsant and adverse effects in the kindling model

In the kindling model, LU 73068 dose-dependently increased the focal seizure threshold (ADT), effects being significant at 10 and 20 mg kg^{-1} when ADT was recorded 15 min after drug administration (Figure 2). At 20 mg kg^{-1}, ADT was increased by 230% above predrug control. When ADT was determined 30 min after injection of 20 mg kg^{-1}, ADT increased by 280% above control (not illustrated). Seizure parameters (seizure severity, seizure duration, afterdischarge duration) recorded at ADT were not altered by LU 73068 (Figure 2) except a significant reduction of seizure severity at 20 mg kg^{-1}, which was seen both 15 and 30 min after drug injection. Increase of dosage to 30 mg kg^{-1} led to marked adverse effects, i.e. pronounced ataxia and sedation and difficulty of breathing, so that only few animals were treated and no seizure parameters were recorded at this dose. At the lower doses, there were almost no rotarod failures and only moderate ataxia (Figure 3), so that doses up to 20 mg kg^{-1} were well tolerated by the kindled rats. Locomotion was not affected by 5 mg kg^{-1}; 10 and 20 mg kg^{-1} induced moderate hypolocomotion with average scores of 0.9 and 1.3, recorded 15 min after drug injection. No stereotyped behaviours were seen at any dose of LU 73068. Body temperature was not affected by 5 mg kg^{-1}, but significantly ($P<0.05$) reduced by 10 (from 38.93 ± 0.12 to

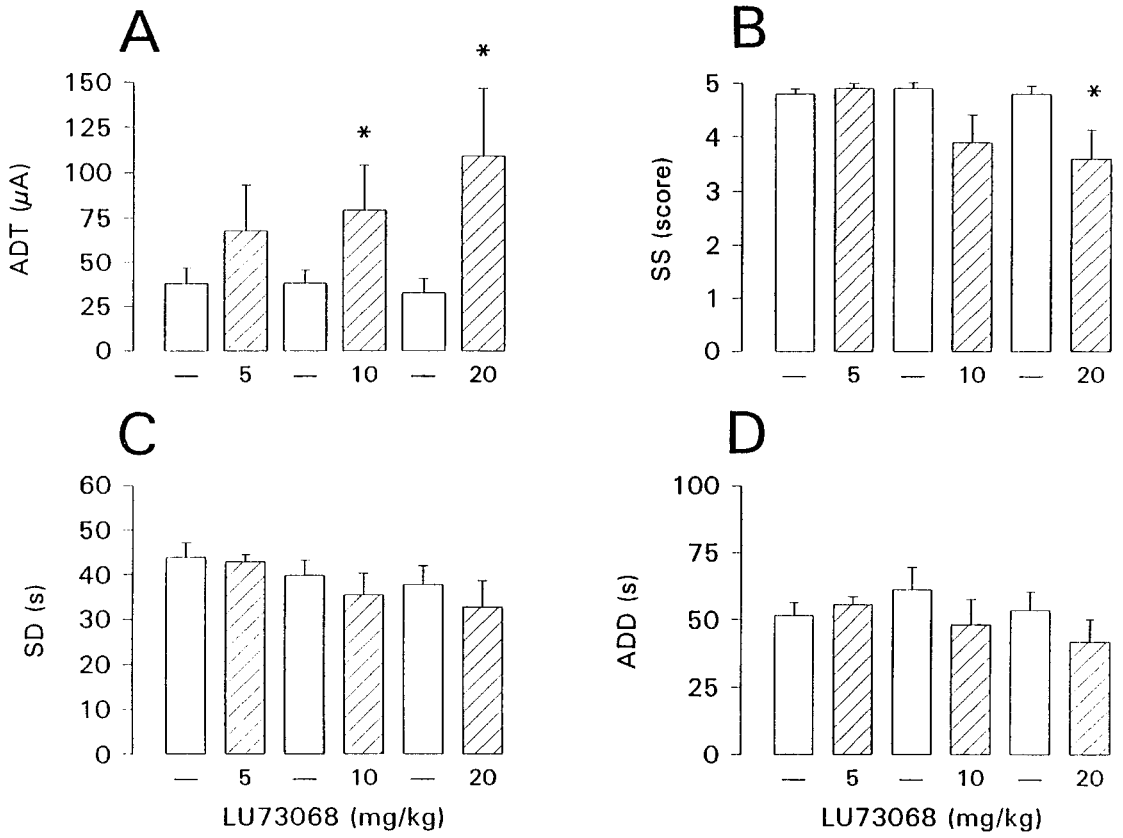


Figure 2 Effect of LU 73068 on focal seizure threshold (ADT) and seizure parameters recorded at ADT in fully kindled rats. All data were recorded 15 min after i.p. drug injection. Data are means + s.e.mean of 8–9 rats per dose. Predrug control recordings were performed 2–3 days before each drug trial. Vehicle (Tris) was injected 15 min prior to control recordings. Significance of differences between drug trial and the individual predrug control trial is indicated by asterisk (P at least <0.05).

37.83±0.23°C) and 20 (from 39.09±0.1 to 37.32±0.17°C) mg kg^{-1}, respectively. Injection of vehicle alone did not induce any adverse effects on behaviour or body temperature (not illustrated).

In addition to the actual ADT and seizure parameters recorded at ADT under treatment with LU 73068 as shown in Figure 2, we calculated the number of animals which were totally protected from seizures when ADT determination would have been stopped 20% above the individual predrug control ADT of each rat at each dose of LU 73068. By this type of determination of anticonvulsant efficacy, 4 of 8 rats would have been protected from focal and secondarily generalized seizures at 5 mg kg^{-1}, 6 of 9 rats at 10 mg kg^{-1}, and 7 of 9 rats at 20 mg kg^{-1}, yielding an ED_{50} of 4.9 (confidence limits 1.8–12.9) mg kg^{-1}.

Based on previous experiments of our group with NBQX and L-701,324 in kindled rats (Löscher *et al*., 1993; Ebert *et al*., 1997), we administered both drugs at doses that were ineffective to increase ADT when administered alone (Figures 4 and 5). When NBQX, 10 mg kg^{-1}, and L-701,324, 2.5 mg kg^{-1}, were administered together there was a tendency to increased ADT which, however, was not significant (Figure 4). Furthermore, seizure duration was significantly reduced by the combination, but not by the drugs injected in this experiment alone (Figure 4). When protection of rats was calculated for stimulation at 20% above individual predrug control ADT, 80% of rats would have been protected against seizures by the combination of NBQX and L-701,324.

When the dose of L-701,324 was increased to 5 mg kg^{-1}, again the glycine/NMDA antagonist was not effective when given alone (Figure 5), but combination with NBQX resulted in a marked (130% above control) and significant increase in ADT (Figure 5). For comparison, administration of a higher dose (15 mg kg^{-1}) of NBQX alone resulted in a significant ADT increase of 100% above predrug control (not illustrated). In addition to markedly increasing ADT, the combination profoundly decreased seizure severity. Six of seven rats treated with the combination would have been protected from seizures if ADT determination would have been performed only at 20% above individual predrug control ADT.

While both NBQX and L-701,324 were devoid of any marked effects on motor function when injected alone, the combination tended to induce more marked ataxia and rotarod failures (Figure 3). Almost no sedation was seen in any experiment with the two drugs alone or in combination nor were any stereotyped behaviours observed. Body temperature was decreased by more than 1°C at the combination of 10 mg kg^{-1} NBQX and 2.5 mg kg^{-1} L-701,324 (from 38.92±0.07 to 37.68±0.22°C), but not by the combination of 10 mg kg^{-1} NBQX and 5 mg kg^{-1} L-701,324.

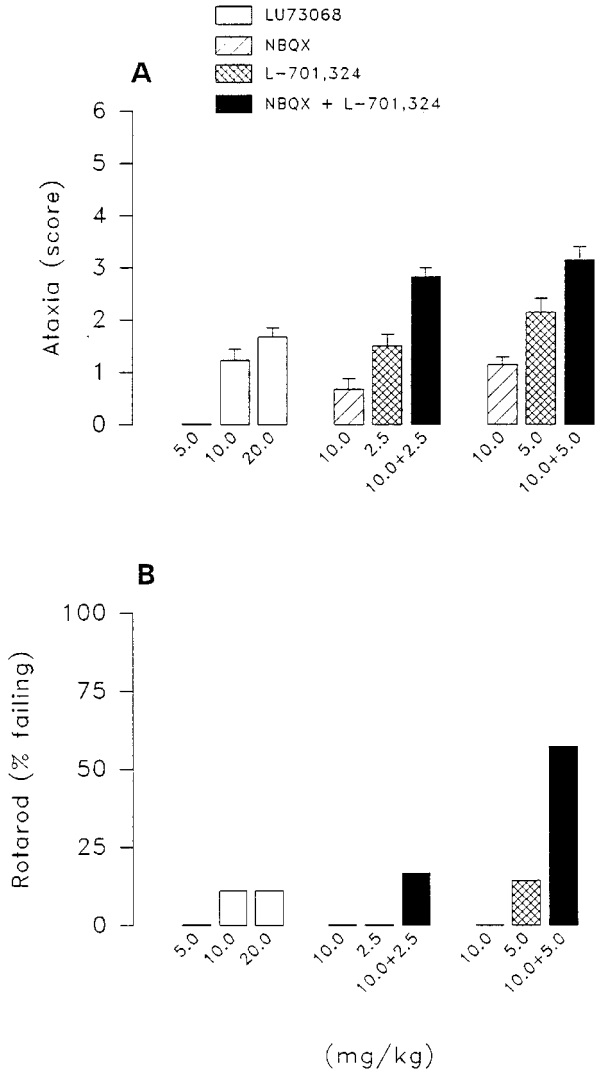


Figure 3 Motor impairment induced by LU 73068, NBQX, L-701, 324, or combinations of NBQX and L-701,324 in fully kindled rats. Motor impairment is shown by ataxia scores in the open field and rotarod failures. Absence of bars indicates that no ataxia or rotarod failures were observed. Data are mean+s.e.mean of 6–9 rats per experiment. Pretreatment times were 15 min in case of LU 73068 and 30 min in case of NBQX, L-701,324, and the drug combination. The data on NBQX are from 2 different experiments.

Discussion

The novel glutamate receptor antagonist LU 73068, which binds to both non-NMDA and glycine/NMDA receptors, proved to be an effective anticonvulsant in the kindling model of epilepsy at doses that were almost devoid of adverse effects on motor function or behaviour. In contrast, a combination of the AMPA antagonist NBQX with the glycine/NMDA antagonist L-701,324 resulted in a potentiation of both anticonvulsant and adverse effects. This may implicate that the favourable profile of LU 73068 in the kindling model was related to additional mechanisms which contributed to the overall activity of the novel compound. In this respect it is interesting that LU 73068 not only binds with high affinity to AMPA receptors but also exhibits considerable affinity to some kainate receptor subunits, particularly rGluR7 and rGluR5. With respect to GluR5, it is important to note that recently a selective antagonist for the GluR5 subtype of kainate receptors, LY294486 ((3SR, 4aRS, 6SR, 8aRS)-6-((((1H-tetrazol-5-yl)methyl)oxy)methyl)-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid), has been described (Clarke *et al*., 1997), but this compound has not yet been tested in seizure models. From their findings that kainate receptors, comprised of or containing the GluR5 subtype of kainate receptor, regulate synaptic inhibition in the hippocampus, Clarke *et al*. (1997) suggested that GluR5 should provide a selective target for antiepileptic drugs.

In contrast to LU 73068, the novel systemically available glycine/NMDA antagonist L-701,324 is not an effective anticonvulsant in the kindling model, even when administered in doses that induce marked motor impairment (Ebert *et al*., 1997). On the other hand, NBQX was shown to significantly increase ADT at doses which are below the dose range of this

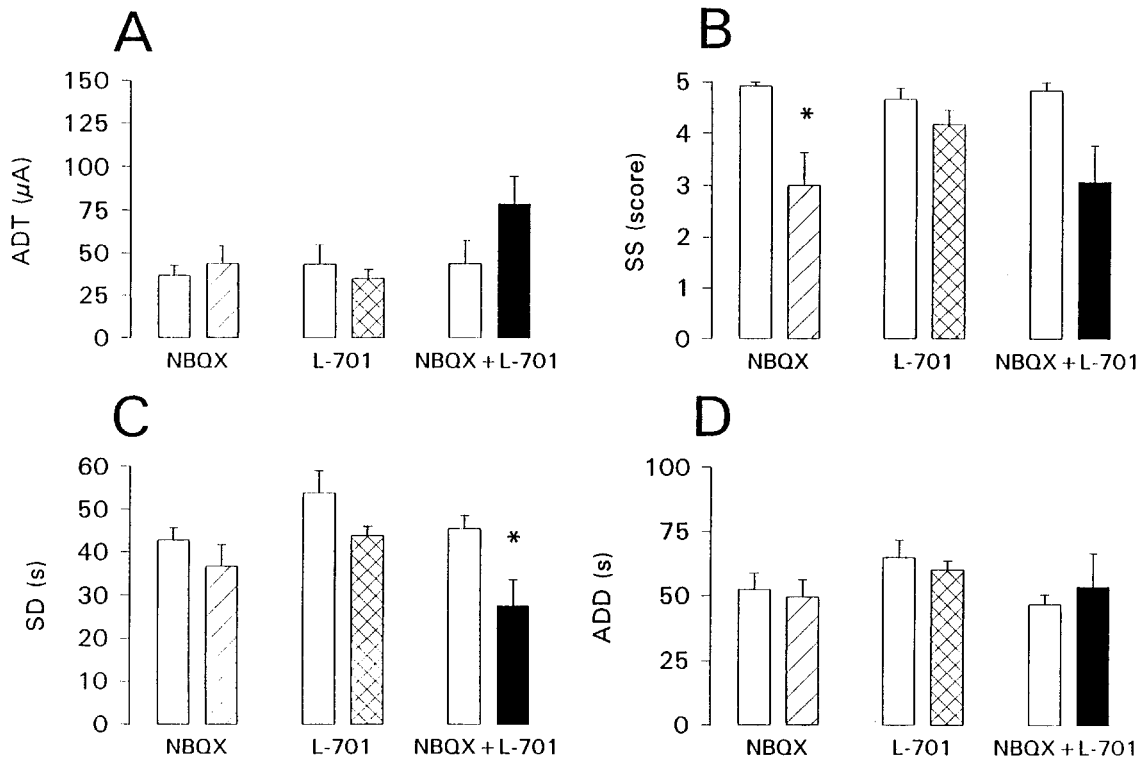


Figure 4 Effect of 10 mg kg^{-1} NBQX, 2.5 mg kg^{-1} L-701,324, or combined injection of both drugs on focal seizure threshold (ADT) and seizure parameters recorded at ADT in fully kindled rats. All data were recorded 30 min after i.p. drug injection. Data are means + s.e.mean of 6 rats per dose; the same 6 rats were used for all 3 experiments shown to allow direct comparison of data. Predrug control recordings (open bars) were performed 2–3 days before each drug trial. Vehicle was injected 30 min prior to control recordings. Significance of differences between drug trial and the individual predrug control trial is indicated by asterisk (P at least <0.05).

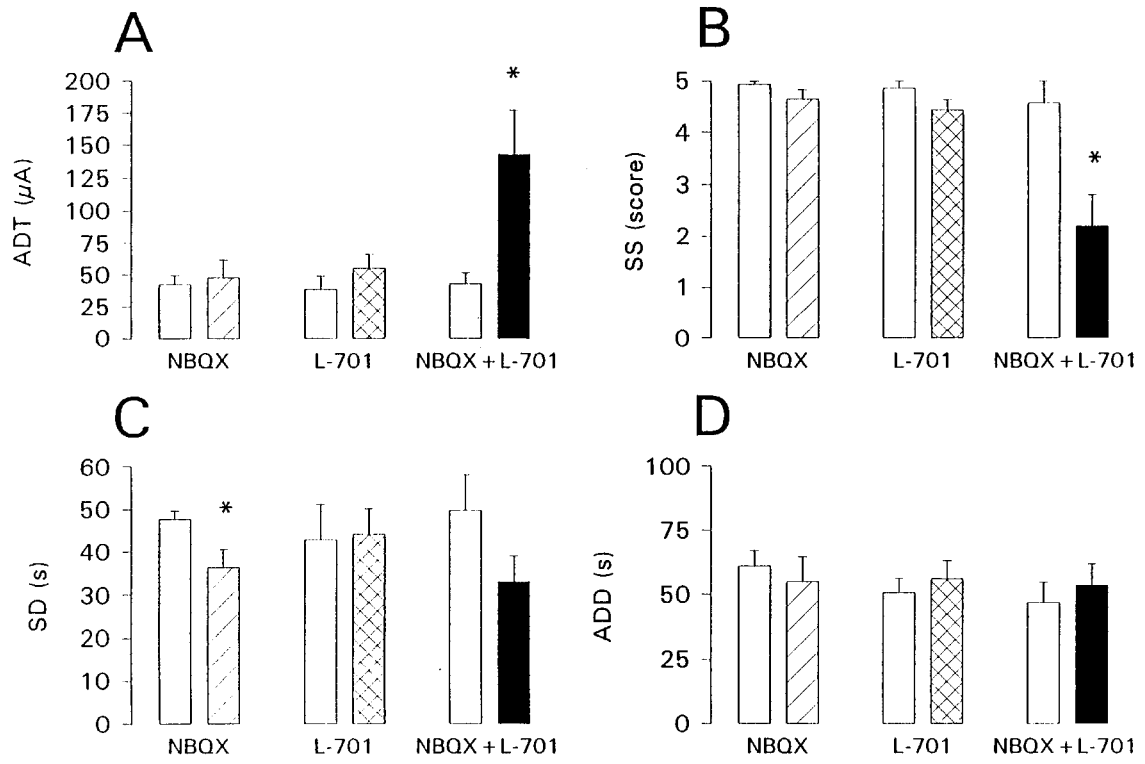


Figure 5 Effect of 10 mg kg^{-1} NBQX, 5 mg kg^{-1} L-701,324, or combined injection of both drugs on focal seizure threshold (ADT) and seizure parameters recorded at ADT in fully kindled rats. All data were recorded 30 min after i.p. drug injection. Data are means + s.e.mean of 7 rats per dose; the same 7 rats were used for all 3 experiments shown to allow direct comparison of data. Predrug control recordings (open bars) were performed 2–3 days before each drug trial. Vehicle was injected 30 min prior to control recordings. Significance of differences between drug trial and the individual predrug control trial is indicated by asterisk (P at least <0.05).

compound that is associated with marked adverse effects (Löscher *et al*., 1993). Very low doses of competitive and uncompetitive NMDA receptor antagonists, which had no effect on ADT by themselves even when administered at much higher doses, potentiated the anticonvulsant effect of NBQX in a synergistic manner without concomitantly leading to increased severity of adverse effects (Löscher *et al*., 1993; Löscher & Hönack, 1994). The present data extend these previous observations and show that NBQX's anticonvulsant potency is also enhanced by a NMDA receptor antagonist that acts *via* the glycine co-agonist site, but this synergistic interaction is associated with increased motor impairment.

Theoretically, the increased anticonvulsant and adverse effects after combined treatment with NBQX and L-701,324 could be simply due to a pharmacokinetic interaction. However, the pharmacokinetics of NBQX in rats (Dalgaard *et al*., 1994) and the fact that pharmacodynamic effects of NBQX were potentiated by different categories of NMDA receptor antagonists (Nellgard & Wieloch, 1992; Löscher *et al*., 1993; Foutz *et al*., 1994; Lippert *et al*., 1994; Löscher & Hönack, 1994; McManigle *et al*., 1994) makes such simple explanation unlikely.

Why is a combination of non-NMDA and NMDA antagonism more potent and effective in an epilepsy model than administration of a drug with one of these mechanisms? In fact, different categories of NMDA antagonists are ineffective to provide any anticonvulsant effect on focal kindled seizures (Löscher, 1998c). While NMDA receptors seem to play a role in the induction of kindling, they do not account for permanent increases in seizure susceptibility in kindled rats, and their role in the expression of seizures in fully kindled rats is not certain (Löscher, 1998c). In contrast, non-NMDA receptor antagonists are potent anticonvulsants in fully kindled rats (Löscher, 1998c). In experiments on the role of NMDA and AMPA receptors in seizures and burst-firing in the hippocampus, it was shown that synaptic activation of AMPA receptors triggers burst initiation, whereas NMDA receptors become active after the initial AMPA receptor-meditated depolarization has caused Mg^{2+} to dissociate from the NMDA channel (Dingledine *et al*., 1990). Both NMDA and AMPA receptors contribute to seizure elaboration (Dingledine *et al*., 1990), and the functional interaction of the two glutamate receptor subtypes may be due to the different parallel ion entry mechanisms activated by these receptors. Thus, pharmacological strategies inhibiting both glutamate receptor subtypes might be particularly promising, which is substantiated by the present experiments.

A combination of non-NMDA and NMDA antagonism not only results in a synergistic potentiation of anticonvulsant effects (Löscher *et al*., 1993; present data), but also in an over-additive potentiation of neuroprotective action (*c.f.* Lees, 1996), which may explain that LU 73068 proved clearly more potent and effective than pure non-NMDA antagonists in a rat model of focal ischaemia (Szabo, unpublished experiments). The over-additive neuroprotective effect of combinations of non-NMDA and NMDA antagonists has been explained by blockade of interacting ways of calcium entry (Lippert *et al*., 1994), which could also be relevant for synergistic anticonvulsant effects of such combinations. However, more recently it was shown that in cats severe respiratory depression leading to apneusis results from combinations of NBQX and the NMDA antagonist MK-801, both administered in subneuroprotective doses (Foutz *et al*., 1994; McManigle *et al*., 1994). Respiratory depression was also seen in the present rat experiments with LU 73068, but only at doses higher than those needed to afford significant anticonvulsant effects in the kindling model.

Interestingly, the data on prevention of lethal convulsions in the mouse induced by i.c.v. injection of NMDA or AMPA showed that NBQX was equally effective against NMDA and AMPA, suggesting that AMPA receptor blockade is able to block these effects whether initiated by either receptor agonist. As reported previously (e.g. Chizh *et al*., 1994), the effect of NBQX was very short-lasting, being maximal at 5 min and totally lost at 60 min following i.p. administration. Indeed, in rodents half-recovery times of 15 min were reported for NBQX (Chizh *et al*., 1994), corresponding to the short half-life of this drug (Dalgaard *et al*., 1994). From the data with NBQX on NMDA and AMPA-induced convulsions, one might expect that LU 73068, i.e. a drug combining non-NMDA and glycine/NMDA receptor antagonist properties, would be more potent against NMDA-induced convulsions versus those induced by AMPA, but this was clearly not the case. However, it should be noted that we recently showed that, in contrast to NBQX, novel non-NMDA receptor antagonists with high selectivity for AMPA and kainate receptors were more potent against AMPA than against NMDA-induced convulsions in mice (Löscher *et al*., 1998). Thus, based on these recent data, the equieffective action of LU 73068 on AMPA and NMDA-induced convulsions would be in line with its additional NMDA receptor antagonist properties.

In conclusion, the new quinoxaline derivative LU 73068 is an effective anticonvulsant in the kindling model of temporal lobe epilepsy. Similar to clinically established anticonvulsant drugs (Löscher, 1998c), this novel compound increases ADT, i.e. directly protects against focal seizure activity, and, at higher doses, reduces seizure severity recorded at ADT, suggesting that the compound also inhibits propagation of seizure activity from the focus. When kindled rats would have been stimulated with a fixed current 20% above individual ADT, LU 73068 would have completely protected against both focal and secondarily generalized seizures with an ED_{50} of about 5 mg kg^{-1}, which is among the most potent compounds tested in the kindling model as yet (Löscher, 1998c). A major disadvantage of combining antagonism at both NMDA and non-NMDA receptors is respiratory depression, which was also seen at a high dose (six times above anticonvulsant ED_{50}) of LU 73068. Thus, although combining NMDA and non-NMDA receptor antagonism in a single drug might be a promising strategy for antiepileptic and neuroprotective drug development, the ultimate success of this strategy will depend on whether it is possible to sufficiently separate the therapeutically relevant actions from undesired adverse effects on motor and respiratory function.

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