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The effects of AMPA receptor antagonists in models of stroke and neurodegeneration

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Abstract

Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists have been shown to have neuroprotective effects in stroke models and although clinical trials with some agents are still ongoing, published results have not been favourable. We therefore wished to compare the effects of GYKI 52466, GYKI 53405, EGIS-8332 and EGIS-10608, non-competitive AMPA receptor antagonists with homophthalazine chemical structures, in standard animal stroke models with effects in a neurodegenerative model — excitoxicity in newborn mice. All compounds inhibited the *S*-AMPA-induced spreading depression in the chicken retina, in vitro, and were potent anticonvulsants against maximal electroshock in mice, in vivo. The AMPA receptor antagonists prevented domoate-induced cell death of motoneurons, in vitro, and reduced infarct size in a dose-dependent manner in the permanent middle cerebral artery occlusion model in mice, in vivo. In newborn mice (P5, histopathology at P10), local injection of the AMPA receptor agonist S-bromo-willardiine at day 5 after birth induced cortical damage and white matter damage, which was reduced in a dose-dependent manner by the AMPA receptor antagonists. EGIS 10608 was a very powerful receptor antagonist of white matter damage. In contrast, GYKI 52466 did not antagonize cortical and white matter damage induced by ibotenic acid. These models allow quantification of the effects of AMPA receptor antagonists in vitro and in vivo.

Keywords: AMPA receptor antagonist; Newborn mouse; Stroke; Interleukin-1beta; Motoneuron; GYKI 52466; GYKI 53405; EGIS-8332; EGIS-10608

1. Introduction

Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists have been shown to be antiepileptic, and neuroprotective in a variety of tests of cerebral ischaemia, or of neurodegeneration. Even though drug therapy for acute stroke has been found to be difficult, AMPA receptor antagonists may still show promise (Szénasi and Harsing, 2004). Talampanel and YM872 (Kawasaki-Yatsugi et al., 2000) have proceeded to phase II/III clinical trials and proof of their clinical effectiveness is awaited. AMPA receptor antagonists are of two major classes: competitive agents, frequently quinoxalinediones, structurally related to 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)quinoxaline (NBQX) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and non-competitive agents, usually homophthalazines, based on the original compound, GYKI 52466 (Tarnawa et al., 1992; Donevan and Rogawski, 1993; Harsing et al., 2000). We developed two new homophthalazine analogues, EGIS-8332 and EGIS-10608. EGIS-8332 has been shown to be a potent AMPA receptor antagonist in electrophysiological experiments (Kapus et al., 2003), and both drugs have favourable pharmacokinetic and toxicological profiles with a prolonged duration of action compared with first generation compounds.

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However, a controversy has arisen about the neuroprotective value of selective AMPA receptor antagonists. The competitive receptor antagonists NBQX and CNQX have been shown recently to increase gamma-aminobutyric acid (GABA) transmission in the cerebellum by a non-AMPA dependent mechanism (Brickley et al., 2001) and to depolarise hippocampal interneurones (Maccaferri and Dingledine, 2002), implying that these agents may not be selective. Indeed, NBQX may also act at kainate receptors (Wilding and Huettner, 1996). Early reports indicated that GYKI 52466 was neuroprotective (Buchan et al., 1991a,b; Le Peillet et al., 1992; Smith and Meldrum, 1992; Vizi et al., 1996; Gyertyan et al., 1999), but these effects are accompanied with a hypothermic effect and the dosing schedules were complicated, requiring pretreatment (Block et al., 1996). Menniti et al. (2003) have shown that CP-465,022, a non-competitive, but selective, AMPA receptor antagonist, reached the brain but was neuroprotective in neither focal nor global models. O'Neill et al. (1998) reported that decahydroisoquinoline AMPA receptor antagonists had neuroprotective effects in the gerbil model of global ischaemia, but that these effects were not correlated with effects at AMPA or kainate receptors. Lack of correlation was also found between AMPA receptor antagonist activity of 2,3-benzodiazepines and their neuroprotective effects in gerbils (Kapus et al., 2003). Furthermore, one clinical trial in stroke was stopped, because of adverse effects, and transient worsening of the clinical condition, with the AMPA receptor antagonist ZK200775 (Elting et al., 2002). However, there are simple confounding factors in the experimental design of these trials, which can negate the outcome of clinical trials in stroke (Spedding, 2002). Testing the effects of AMPA receptor antagonists in neurodegenerative disease may be interesting, if compounds can be made with slow pharmacokinetics to avoid the deleterious effects associated with high peak to trough plasma variation. However, neurodegenerative models are normally labour intensive and have low throughput. We therefore wished to assess the effects of the drugs in a model which has reasonably high throughput.

Thus the goal of the present work was to compare the neuroprotective effects of the new homophthalazine non-competitive AMPA receptor antagonists, EGIS-8338 and EGIS-10608, which have good pharmacokinetic profiles, with the standard homophthalazine compounds, in a range of in vitro and in vivo models. In order to characterize the potency of AMPA receptor antagonists, prolongation of the latency of spreading depression caused by AMPA in chicken retina was measured in vitro (Sheardown, 1993) and protection against maximal electroshock-induced seizures was evaluated in mice, in vivo (Löscher and Hönack, 1994; Vizi et al., 1996; Szabados et al., 2001; De Sarro et al., 2003). Efficacy as neuroprotective agents was studied in an in vitro model of domoate-induced cell death of motoneurons, using highly purified motoneurons from rats (Henderson et al., 1995). As an animal model of human stroke, permanent middle cerebral artery occlusion in the mouse was used to determine the antiischaemic efficacy of the compounds (Karkoutly et al., 1990). Potential usefulness for the treatment of neurodegenerative disorders was based on the extent of attenuation of cortical plate and white matter lesions brought about by S-bromo-willardiine, an AMPA receptor agonist, in newborn mice. In this model neopallial injection of S-bromo-willardiine induced a focal neuronal death affecting all cortical layers and a periventricular white matter cystic lesion: the mechanism of cell death has been extensively investigated (Marret et al., 1995; Dommergues et al., 2000; Gressens et al., 1997, 1999; Tahraoui et al., 2001; Laudenbach et al., 2001; Largeron et al., 2001; Husson et al., 2002).

2. Materials and methods

2.1. Drugs used in this study

(*S*)-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ((*S*)-AMPA), domoic acid, S-bromo-willardiine, ibotenic acid (Tocris), 2,2,2,-tribromoethanol, 2,3,4-triphenyltetrazolium chloride, (Fluka), pentobarbital (Sanofi), interleukin-1beta (Serotec). EGIS-8332, (±)-7-acetyl-5-(4-amino-phenyl)-7,8-dihydro-8-cyano-8-methyl-9*H*-1,3-dioxolo-(4,5-*h*)-2,3-benzodiazepine; GYKI 53405, (±)-7-acetyl-5-(4-amino-phenyl)-7,8-dihydro-8-methyl-9*H*-1,3-dioxolo-(4,5-*h*)-2,3 benzo–diazepine (EGIS-8998); GYKI 52466, (±)-5-(4-amino-phenyl)-8-methyl-9*H*-1,3-dioxolo-(4,5-*h*)-2,3-benzodiazepine (EGIS 8159); EGIS-10608, (±)-7-acetyl-5-(4-amino-3-methyl-phenyl)-7,8-dihydro-8-methyl-9*H*-1,3-dioxolo-(4,5-*h*)-2,3-benzodiazepine (Fig. 1); NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)quinoxaline. All homophthalazines and NBQX were synthesised at the Division of

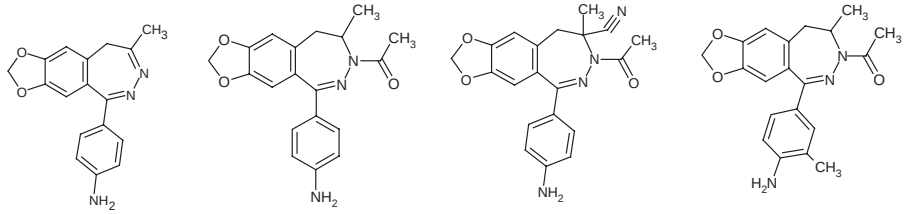

Fig. 1. The chemical structures of AMPA receptor antagonists, GYKI 52466, GYKI 53405, EGIS-8332 and EGIS-10608.

Chemical Research of EGIS Pharmaceuticals Ltd. All other chemicals were of analytical grade.

2.2. Spreading depression in the chicken retina

Chickens (Shaver Redbrow, 4–7 days old) were anaesthetized with diethyl ether and decapitated. The eyes were enucleated and cut along the equatorial plane. The anterior part and the vitreous body were removed and the posterior part of the eyes were placed in Ringer solution (NaCl 100 mM, KCI 3 mM, $CaCl_2$ 1 mM, $MgSO_4$ 1 mM, $NaHCO_3$ 30 mM, NaH_2PO_4 1 mM, glucose 10 mM, pH 7.4), saturated with carbogen (95% O_2 and 5% CO_2). Experiments were carried out at room temperature (24 °C). After a 60-min recovery period eyecups were transferred to Ringer solution containing 5 μM *S*-AMPA. Latency of *S*-AMPA-induced spreading depression was measured. After further incubation (30 min) in drug-free Ringer solution, retinas were placed to Ringer containing the test compound and were incubated for 15 min. Spreading depression was then induced again in the presence of the receptor antagonist. Following 60 min incubation in drug-free Ringer, spreading depression was measured in order to assess the degree of recovery from any drug effect.

Thirty seconds increase in spreading depression latency compared to control was considered as 100% inhibition (Sheardown, 1993). IC_{50} values were calculated using sigmoidal curve fitting (GraphPad Prism 1.03).

2.3. Maximal electroshock test

The experiments were performed in separate groups of male NMRI mice (Charles River Hungary Ltd.) weighing 20–25 g according to Swinyard et al. (1952). Every group consisted of 10 animals treated intraperitoneally with either various doses of the test compounds or vehicle (control group). Thirty minutes later, a 50 Hz, 40 mA, 0.4 s electroshock was applied through corneal electrodes to mice. The number of animals showing tonic extensor convulsion in the hind legs (positive reaction) was registered, percent inhibition was calculated and ED_{50} values were determined by the method of Litchfield and Wilcoxon (1949).

2.4. Motoneurons: purification and culture

Motoneurons were purified using a combination of density gradient centrifugation and immuno-purification, as described previously (Henderson et al., 1995; Raoul et al., 1999). Spinal cords were dissected from day E14.5 Sprague Dawley rat embryos (Elevage Janvier, France). The largest cells were isolated by centrifugation on a 6.5% (w/v) metrizamide density gradient. The immunoaffinity purification step performed previously by immunopanning (Henderson et al., 1995) was replaced by a cell-sorting step using microbeads (Arce et al., 1999). Cells were incubated with a mouse antibody (anti-rat p75 antibody [Immunoglobulin192]. Subsequently motoneurons were incubated with magnetic microbeads conjugated to anti-mouse secondary antibodies, thus allowing the purification of motoneurons on separating columns (Miltenyi Biotech, Inc.). The cells were then centrifuged through a bovine serum albumine cushion and resuspended in complete medium (neurobasal medium supplemented with B27, Life Technologies, 2% horse serum, 25 μM 2-mercaptoethanol). Cells were plated onto 384-well dishes coated with polyornithine and laminin in complete medium using a fully automatised system (Trophos). The final volume was 100 μl per well in the presence of 1 ng/ml brain-derived neurotrophic factor (BDNF, R&D Systems) (Sendtner et al., 1992; Yan et al., 1992).

2.5. Assay of glutamate toxicity

After four days of culture (time for the upregulation of AMPA-kainate glutamate receptors as the neurones mature) half of the medium was removed and replaced by a freshly made medium containing compounds. One hour later the cells were treated with domoic acid (10 μM final). Domoic acid, an AMPA-kainate receptor agonist, was used instead of glutamate as it elicits non-desensitizing responses at AMPA receptors (Debonnel et al., 1990). The concentration of domoic acid was optimised from an extensive series of preliminary experiments. Three controls were included (all with BDNF): no treatment, domoic acid treatment (10 μM final), domoic acid plus NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulphonamide disodium, 10 μM final concentration). NBQX, a specific AMPA/kainate receptor antagonist (Zeman and Lodge, 1992; Sheardown, 1993) was used as an internal control. Eight replicates were performed for each concentration and for all controls in three experiments. Fresh solutions were made from powdered stocks before each experiment. The compounds were weighed and dissolved, diluted to twice their final concentration, and then 50 μl was added per well. An equal volume of the appropriate diluent was added to the controls.

2.6. Assay of motoneuron survival

The number of surviving motoneurons was counted 2 days later. Live cells were counted by an automated image analyzer (Trophos) after labelling with a vital dye, calcein-AM (Fluka). Results were analysed using analysis of variance (ANOVA) followed by Dunnett's test.

2.7. Middle cerebral artery occlusion in mice

Focal cortical ischaemia was produced by electrocoagulation of the left middle cerebral artery according to the method of Welsh et al. (1987). Male NMRI mice (30–35 g, Charles River Hungary Ltd.) were anaesthetized with 2,2,2-tribromoethanol (500 mg/kg i.p., 10 ml/kg). An incision was made on the left temporo-parietal region of the head between the orbit and the ear. The temporal muscle was incised and reflected forward. A small burr hole was drilled into the lateral outer surface of the skull just over the middle cerebral artery. The stem of the middle cerebral artery was occluded by electrocoagulation. All compounds were administered intraperitoneally 30 min after middle cerebral artery occlusion. Two days later the animals were deeply anaesthetized with pentobarbital (100 mg/kg i.p., 10 ml/kg), perfused through the heart with 4% solution of 2,3,5-triphenyltetrazolium chloride. The animals were decapitated and the brains were removed and placed in saline containing 8% formalin for at least 24 h. The necrotic (non-stained) area was determined by means of an image analysing system (DigiCell for Windows 4.0). Results were expressed as means ± S.E.M. for the different treatment groups and statistical significance was assessed using ANOVA followed by Duncan's test.

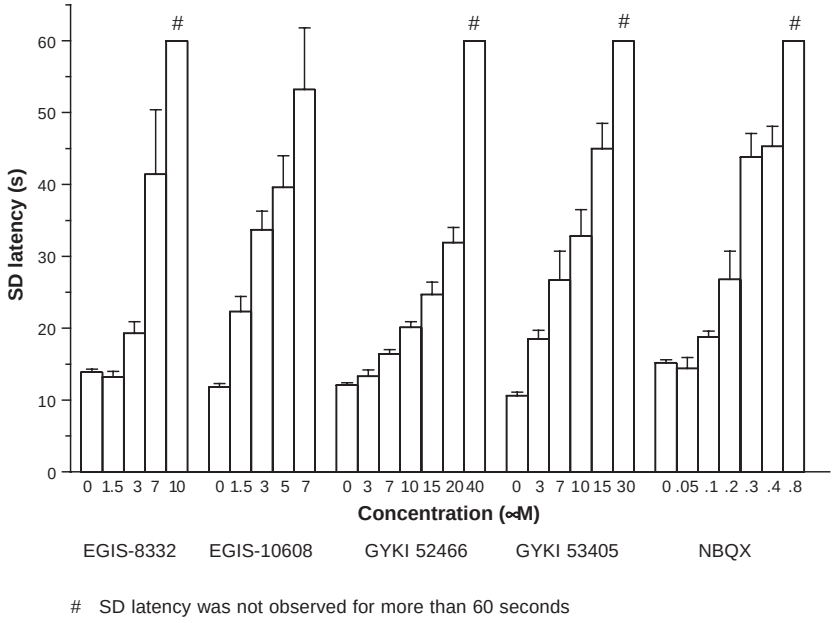

Fig. 2. AMPA receptor antagonists concentration-dependently blocked spreading depression (evoked by 5 μM *S*-AMPA), which resulted in an increase in spreading depression latency in chicken retina. Each column represents means ± S.E.M. ($n = 6-8$). Spreading depression latency >60 s was shown as 60 s and was considered as 100% inhibition of spreading depression.

2.8. Neuroprotection in newborn mice

At postnatal day (P) 5, Swiss mouse pups were anaesthetized for intracerebral (i.c.) and i.p. injections. I.c. injections were performed using a 26-gauge needle mounted on a calibrated microdispenser. The needle was inserted 2 mm under the external surface of scalp skin in the frontoparietal area of the right hemisphere, 2 mm from the midline in the lateral-medial plane and 3 mm (in the rostro-caudal plane) from the junction between sagittal and lambdoid sutures. Two 1 μl boluses were injected at a 30-s interval. Fifteen micrograms S-bromo-willardiine diluted in phosphate-buffered saline (PBS), or 10 μg ibotenate, diluted in PBS containing 0.02% acetic acid, were injected i.c.

In a first set of experiments, immediately following i.c. injection of the excitotoxin, 1, 3 or 10 mg/kg GYKI 52466, GYKI 53405, EGIS-8332 or EGIS-10608, diluted in 5 μl PBS containing 10% dimethylsulfoxide (DMSO), were administered i.p. Controls received i.p. PBS-10% DMSO alone.

In a second set of experiments, pups were pre-treated with recombinant mouse interleukin-1β prior to the excitotoxic challenge. Forty nanograms interleukin-1β were diluted in 5 μl PBS which were injected i.p. twice a day (between 8 and 10 AM and again between 6 and 8 PM) on days P1 to P4 and once a day (between 8 and 10 AM) on P5. Controls received i.p. PBS alone. On P5, 2 h following the last injection of interleukin-1β or PBS, i.c. injection of ibotenate or S-bromo-willardiine was performed.

Five days later, pups were sacrificed and brains fixed in formalin. Coronal serial sections, 15 μm thick, were cut and every third section was stained with cresyl-violet. Brain were completely and serially sectioned from the frontal pole to the occipital lobes permitting an accurate and reproducible determination of the maximal sagittal fronto-occipital diameter of both the cortical plate and white matter lesions. This diameter was used as a reliable and reproducible index of the lesion size (Marret et al., 1995; Gressens et al., 1997; Husson et al., 2002). Statistical analyses were performed with Student's *t*-test or with one-way ANOVA. When group interaction was found to be significant, a Dunnett's multiple comparison test was performed. Results were expressed as means ± S.E.M.

All experimental protocols and procedures complied with guidelines of the INSERM and local ethical committees.

3. Results

3.1. Spreading depression in the chicken retina

AMPA induced a depolarisation of neurones in the chicken retina, which was visible by the change in the luminosity of the tissue. Depolarisation, followed by the depressive state of the

Table 1
Inhibitory effects of AMPA receptor antagonists on spreading depression in the chicken retina and in maximal electroshock induced seizures model in mice

Compound	Spreading depression	Maximal electroshock
	IC_{50} (μM)	ED_{50} (mg/kg i.p.)
	(mean ± S.E.M.)	(95% confidence intervals)
EGIS-8332	5.3 ± 0.6	5.3 (4.03–7.03)
EGIS-10608	3.1 ± 0.3	4.0 (3.21–5.11)
GYKI 52466	16.6 ± 0.8	7.2 (6.50–7.88)
GYKI 53405	7.0 ± 0.6	3.7 (2.98–4.72)
NBQX	0.2 ± 0.01	47.5 (38.91–57.99)

In the MES test the compounds were injected intraperitoneally 30 min before testing, ED_{50} values were calculated by the method of Litchfield and Wilcoxon.

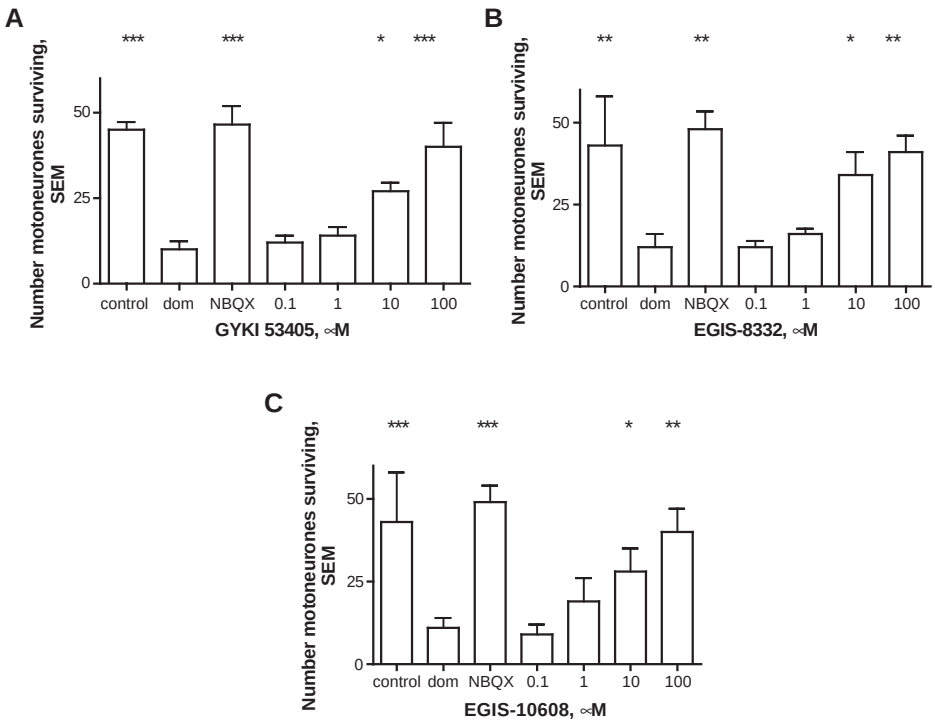

Fig. 3. Domoate (DOM, 10 μM)—induced death of motoneurons maintained in tissue culture, and protection by NBQX (10 μM), GYKI 53405, EGIS-8332, EGIS-10608. Vertical bars represent S.E.M., $n=3$.

neurones spread over the retina. All the AMPA receptor antagonists delayed the latency of spreading depression in a highly concentration-dependent manner (Fig. 2). Under the present conditions, NBQX was the most potent compound and ten-fold higher concentrations of the most potent homophthalazine (EGIS-10608) were needed to antagonize spreading depression. GYKI 52466 was the least effective compound (see data in Table 1).

3.2. Maximal electroshock test (MES)

The compounds protected mice against extensor seizures in the maximal electroshock test in a dose-dependent manner. The ED_{50} values for the inhibition of tonic extensor convulsions are summarised in Table 1. The non-competitive AMPA receptor antagonists (EGIS-8332, EGIS-10608, GYKI 52466, GYKI 53405) inhibited seizures in mice with a similar effectiveness

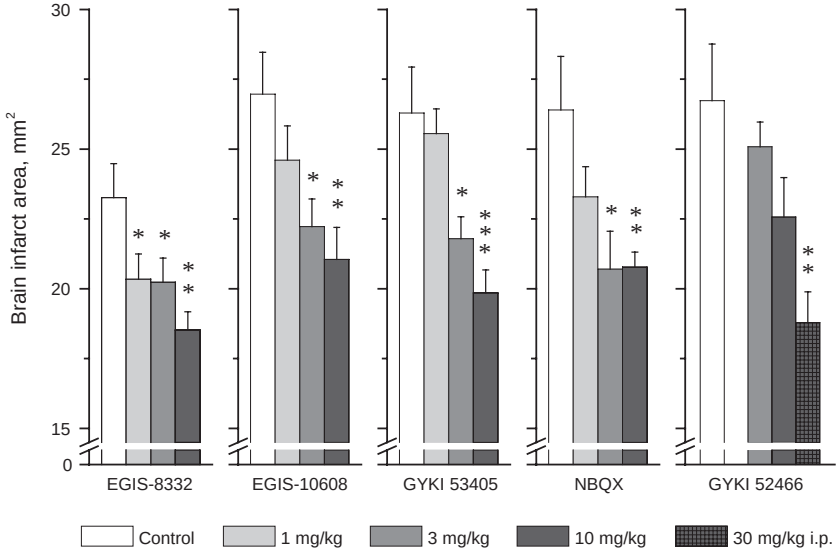

Fig. 4. Effects of EGIS-8332, EGIS-10608 and reference compounds (GYKI 52466, GYKI 53405, NBQX) in middle cerebral artery occlusion model in mice. The compounds were injected i.p. 30 min after the middle cerebral artery occlusion. Brain infarct area expressed as millimeter square is the mean ± S.E.M., $n=4-8$, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

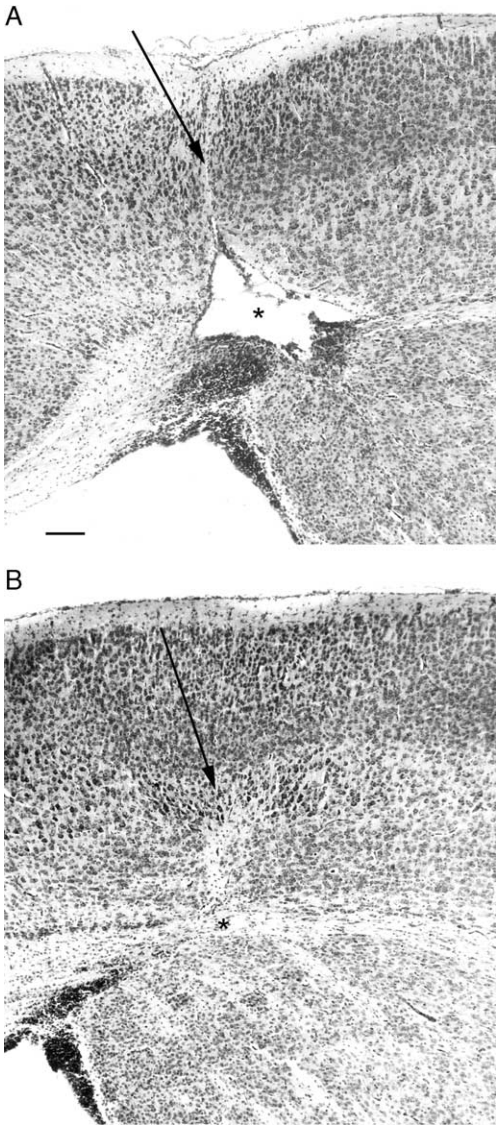

Fig. 5. AMPA receptor antagonists prevent S-bromo-willardiine-induced neuronal death and white matter cysts. Cresyl violet-stained sections showing typical brain lesions (arrows point to neuronal cell death and * indicate white matter cystic lesions) induced by S-bromo-willardiine injected at postnatal day 5 and studied at the age of postnatal day 10. Brains from pups injected with i.c. S-bromo-willardiine and i.p. PBS (phosphate-buffered saline, A) or with i.c. S-bromo-willardiine and i.p. EGIS-10608 (1 mg/kg i.p.) (B). Bar: 70 μm.

while NBQX, the competitive receptor antagonist, was much less potent. The anticonvulsant ED_{50} values of EGIS-8332 and EGIS-10608 were between those of GYKI 52466 and GYKI 53405.

3.3. Motoneuron survival

Motoneurons were maintained in a stable condition in a media containing BDNF. However, BDNF was essential for survival of the neurones because deprivation of BDNF induced cell death. In the presence of BDNF, domoate (10 μM) induced cell death (Fig. 3). Neuronal damage was entirely abolished with coadministration of NBQX (10 μM). Domoate-induced cell death was antagonized by GYKI 53405 (EGIS-8998) and by EGIS-8332 and EGIS-10608 in a concentration-dependent manner (Fig. 3A–C). Relatively high concentrations of the AMPA receptor antagonists were necessary (10 μM) for protective effects in this model.

3.4. Focal cerebral ischaemia — permanent middle cerebral artery occlusion in mice

All the AMPA receptor antagonists reduced brain cortical infarct surface in a dose-dependent manner (Fig. 4). In this model, the effectiveness of the AMPA receptor antagonists as neuroprotective agents can be best characterized by their minimal effective dose. EGIS-8332 showed the most effective neuroprotective activity in this model (minimal effective dose <1 mg/kg i.p.) when it was administered 30 min after middle cerebral artery occlusion. The antiischaemic activity of EGIS-10608 was similar to those of reference non-competitive AMPA receptor antagonist GYKI 53405 and competitive AMPA receptor antago-

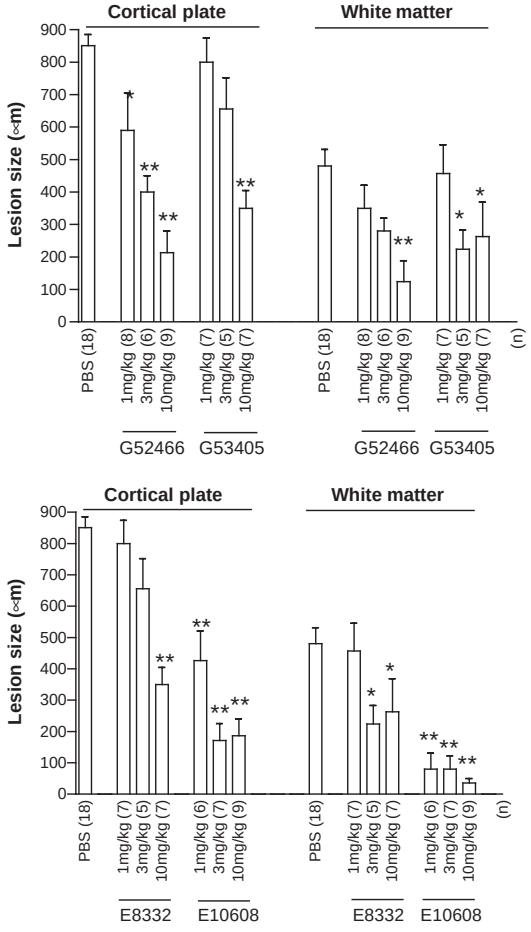

Fig. 6. AMPA receptor antagonists-induced neuroprotection in a newborn model of excitotoxic brain lesions produced by focal i.c. injection of S-bromo-willardiine. Bar represents the mean length of the neocortical lesion in the sagittal fronto-occipital axis ± S.E.M. PBS (phosphate-buffered saline), control animals co-injected with i.c. S-bromo-willardiine and i.p. PBS; all the other experimental groups were co-treated with i.c. S-bromo-willardiine and i.p. with the indicated drug (GYKI 52466, GYKI 53405, EGIS-8332, EGIS-10608). Asterisks indicate difference from control (*P < 0.05, **P < 0.01 in ANOVA with Dunnett's multiple comparison test).

nist NBQX (minimal effective dose=3 mg/kg i.p., respectively) while GYKI 52466 was less active in this test (minimal effective dose=30 mg/kg i.p.).

3.5. Neuroprotection in newborn mouse pups

At the neuropathological level, neopallial injection of S-bromo-willardiine induced a focal neuronal death affecting all cortical layers and a periventricular white matter cystic lesion, as reported in previous studies (Marret et al., 1995; Dommergues et al., 2000; Gressens et al., 1997, 1999; Tahraoui et al., 2001; Laudenbach et al., 2001; Largeron et al., 2001; Husson et al., 2002) (Fig. 5).

Co-treatment with S-bromo-willardiine and GYKI 52466, GYKI 53405, EGIS-8332 or EGIS-10608 (10 mg/kg i.p. at day 5, at the same time as administration of S-bromo-willardiine) significantly protected both the white matter and the cortical plate lesions against the insult (Fig. 6). The analysis of the dose–response curves revealed several differences between the AMPA receptor antagonists. EGIS-10608 exhibited a significant neuroprotective effect at all tested doses with a reduction of the cortical plate and white matter lesions up to 78% and 93%, respectively. The neuroprotective profile of GYKI 52466 on the cortical plate lesion was quite similar to EGIS-10608 (up to 75% reduction of the lesion size) but the neuroprotection of the white matter was less important (up to 75% reduction of the lesion size) and limited to the highest tested dose. GYKI 53405 and EGIS-8832 displayed comparable neuroprotective profiles, with a significant protection of the cortical plate observed at 10 mg/kg (59% reduction of the lesion size for both drugs) and a moderate neuroprotection of the white matter at 3 and 10 mg/kg (~45% reduction of the lesion size).

In contrast, co-treatment with ibotenate and GYKI 52466 (10 mg/kg i.p. at day 5, at the same time as administration of ibotenate) did not induce any detectable neuroprotective effect (Fig. 7).

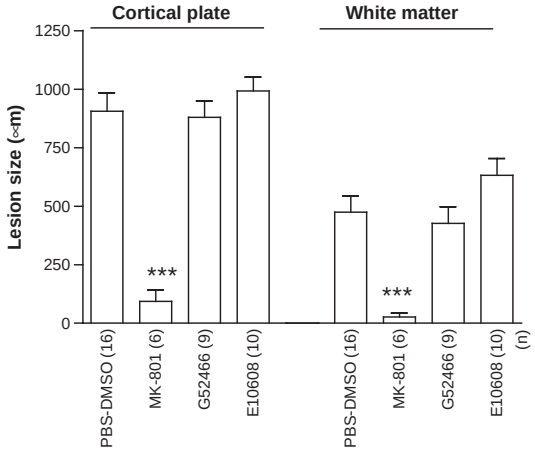

Fig. 7. Neither GYKI 54266 nor EGIS 10608 did not prevent neonatal excitotoxic brain lesions produced by focal i.c. injection of ibotenate, whereas MK 801 (1 mg/kg i.p.) was protective. Bars represent the mean length of the neocortical lesion in the sagittal fronto-occipital axis±S.E.M. PBS (phosphate-buffered saline), control animals injected with i.c. ibotenate and i.p. PBS; MK 801, animals injected with i.c. ibotenate and i.p. MK 801 (1 mg/kg i.p.); GYKI 52466, animals injected with i.c. ibotenate and i.p. GYKI 52466 (10 mg/kg); E 10608, animals injected with i.c. ibotenate and i.p. EGIS 10608 (10 mg/kg i.p.)—the number of animals are in parentheses.

4. Discussion

NBQX and the homophtalazine AMPA receptor antagonists dose-dependently blocked kainate-induced spreading depression in isolated chicken retinas. Ionotropic glutamate receptors are crucial in the mediating of excitatory transmission in retinal spreading depression (Sheardown, 1993). Activity of AMPA receptor antagonists tested in spreading depression were found to be well correlated with those collected from electrophysiological experiments (Kapus et al., 2003; Vizi et al., 1996). Thus, retinal spreading depression seems to be a suitable model for testing of AMPA receptor antagonist activity in vitro.

The AMPA receptor antagonists have been widely investigated in experimental seizure models, where they provide effective protection against convulsions evoked by chemical agents such as AMPA (Chapman et al., 1991; De Sarro et al., 2003), or kainate (De Sarro et al., 2003), and audiogenic stimulation (Chapman et al., 1991; De Sarro et al., 2003; Szabados et al., 2001). The present study demonstrated the anticonvulsant efficacies of both non-competitive AMPA receptor antagonists (EGIS-8332, EGIS-10608, GYKI 52466, GYKI 53405) and competitive AMPA receptor antagonist (NBQX) in maximal electroshock test in mice. All compounds were found to protect against maximal electroshock seizures. Some investigations were published about the anticonvulsive property of reference GYKI compounds or NBQX (Chapman et al., 1991; De Sarro et al., 2003; Szabados et al., 2001; Vizi et al., 1996). The current results for GYKI 52466 and GYKI 53405 are similar to those reported in a previous study (Szabados et al., 2001). The anticonvulsant effect of non-competitive AMPA receptor antagonists (EGIS-8332, EGIS-10608, GYKI 52466, GYKI 53405) were similar to each other but surpassed by one order of magnitude that of NBQX, a competitive AMPA receptor antagonist. EGIS-8332 and EGIS-10608 showed very active anticonvulsive activities in this test.

Models of focal cerebral ischaemia have been used extensively to study both the pathophysiology of brain ischaemia and to determine the neuroprotective potential of various pharmacologic agents. These animal models of human stroke produce a widespread loss of cortical and striatal neurones within the vascular territory supplied by the middle cerebral artery (Tamura et al., 1981). In the mouse model, the significant correlation between infarct volume and infarct area demonstrates that the infarct area after middle cerebral artery occlusion represents a sensitive, reliable, rapid and easy way to estimate the effects of compounds against ischaemic brain damage (Karkoutly et al., 1990). Our data indicated that both competitive and non-competitive AMPA receptor antagonists reduced infarct area in mouse middle cerebral artery model. In this model the competitive AMPA receptor antagonist NBQX reduced both infarct area and volume when it was administered 3×30 mg/kg i.p., 60, 70 and 85 min after surgery, while GYKI 52466 did not show neuroprotective

effect in 30 mg/kg i.p., applied immediately after occlusion (Seltz and Turski, 1994). In the present study NBQX reduced the value of the infarct area in 3 mg/kg i.p. dose when it was given only once 30 min following middle cerebral artery occlusion in the mouse. GYKI 52466 was able to diminish the infarct area after middle cerebral artery occlusion in 30 mg/kg i.p., administered 30 min after surgery. EGIS-8332 and EGIS-10608 were also shown to reduce the neuronal damage, the neuroprotective effect of EGIS-8332 was most effective in this test while the effect of EGIS-10608 was similar to that of reference GYKI 53405. Thus the compounds were all effective in models of stroke, albeit with different potencies.

The AMPA receptor antagonists were all active in inhibiting domoate-induced neurodegeneration of motoneurons, in a concentration-dependent manner. The interest of this test is that motoneurons of very high purity, obtained by using a combination of density gradient centrifugation and immuno-purification (Henderson et al., 1995; Raoul et al., 1999) could be maintained in 384 well plates in a stable condition for high throughput screening. BDNF was essential for survival of the neurones. In the presence of BDNF, domoate (10 μM) induced cell death, prevented by NBQX (10 μM). In preliminary experiments we had determined that NBQX appeared to have competitive interactions with domoate, in that changing domoate levels and NBQX levels in parallel, over a ten-fold range (1–10 μM), resulted in the same degree of cell death. The homophthalazine receptor antagonists were also effective.

Neurodegeneration models in vivo are, almost by definition, of low throughput, thus we wished to use this opportunity to fully characterize, for AMPA receptor antagonists, a high throughput neurodegeneration model: excitotoxic lesions in newborn mice. This model has been extensively characterized for other classes of drugs (Marret et al., 1995; Dommergues et al., 2000; Gressens et al., 1997, 1999; Tahraoui et al., 2001; Largeron et al., 2001; Laudenbach et al., 2001). Brain damage is induced with intracerebral administration of S-bromo-willardiine or ibotenate. S-bromo-willardiine acts on AMPA and kainate receptors while ibotenate acts on *N*-methyl-D-aspartate (NMDA) and metabotropic receptors. Injection of S-bromo-willardiine or ibotenate on postnatal day 5 (P5) induces neuronal death leading to cortical brain lesions, which resemble those observed in full-term human infants. Furthermore, injections into periventricular white matter induce cystic lesions that mimicked several aspects of human cystic periventricular leukomalacia, which is observed most frequently in premature human infants (Marret et al., 1995; Dommergues et al., 2000; Gressens et al., 1997, 1999; Tahraoui et al., 2001; Laudenbach et al., 2001; Largeron et al., 2001; Husson et al., 2002). The ibotenate-induced lesions are completely abolished by co-treatment with an NMDA receptor antagonist (Marret et al., 1995; Tahraoui et al., 2001) and are exacerbated by pre-treatment with pro-inflammatory cytokines such as interleukin-1β (Dommergues et al., 2000). The S-bromo-willardiine-induced lesion of the white matter involves both NMDA and AMPA-kainate receptors, while the cortical plate lesion observed in this model is purely mediated by AMPA-kainate receptors (Tahraoui et al., 2001). Both cortical plate and white matter lesions induced by S-bromo-willardiine can be reduced by antioxidants (Largeron et al., 2001). Thus, this is a useful model, in comparison with standard tests.

The tested AMPA receptor antagonists displayed a dose-dependent and significant neuroprotection against brain lesions induced by S-bromo-willardiine injected into the cortex or white matter of 5-day old mice pups, but as expected, GYKI 52466 was ineffective on brain lesions induced by the NMDA receptor antagonist, ibotenate, indicating the selective effects of these drugs against the AMPA receptor agonist, S-bromo-willardiine. EGIS-10608 induced the greatest degree of neuroprotection against S-bromo-willardiine-mediated damage, GYKI 52466 induced an intermediate degree of neuroprotection while EGIS-8332 and GYKI 53405 were the least active. EGIS-10608 was remarkably potent against the lesions in white matter, causing almost full protection at low doses. This is of interest in that it was reported that AMPA receptor antagonists could be effective in protecting both grey and white matter from damage caused by transient focal ischaemia. In this respect, massive activation of brain macrophages has been shown to take place during the early stages of ibotenate-induced lesions in newborn mice (Tahraoui et al., 2001). This macrophage activation is linked to the transient expression of NMDA on periventricular white matter macrophages in the neonatal period (Tahraoui et al., 2001). Further supporting the hypothesis of a pathophysiological role of brain macrophages, inhibitors of macrophage activation such as minocycline or strategies aiming at macrophage depletion are neuroprotective against ibotenate-induced lesions (Dommergues et al., 2003). In contrast, intracerebral injection of non-desensitising AMPA receptor agonists induces death of immature oligodendrocytes in the periventricular white matter (Follett et al., 2000).

As mentioned above, ibotenate and S-bromo-willardiine-induced brain lesions involve different glutamate receptors and, therefore, different cellular and molecular mechanisms (Marret et al., 1995; Tahraoui et al., 2001). Negative modulators cause protective effects only against AMPA-mediated neurodegeneration, whereas positive modulators (AMPAkines) are effective against both ibotenate (NMDA) and S-bromo-willardiine-induced brain lesions by increasing the production of neurotrophins, particularly brain-derived neurotrophic factor (BDNF, Dicou et al., 2003). The neuroprotection induced by the AMPA receptor antagonists against S-bromo-willardiine-induced brain lesions was not via BDNF-induced mechanisms (Gressens, unpublished). Thus the model is also useful in that both positive and

negative allosteric modulators of AMPA receptors cause neuroprotection, but by different mechanisms.

In conclusion, the new AMPA receptor antagonists, EGIS-8332 and EGIS-10608, are as active or more active as standard compounds in a wide range of tests for neuroprotective activity.

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