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Lubeluzole protects hippocampal neurons from excitotoxicity in vitro and reduces brain damage caused by ischemia

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Abstract

Previously reported effects of lubeluzole, such as inhibition of glutamate release, inhibition of nitric oxide (NO) synthesis and blockage of voltage-gated Na^+- and Ca^{2+}-ion channels, suggest a neuroprotective action of this drug. Here we report about the effects of lubeluzole and its *R*-isomer on glutamate-induced neuronal cell death in mixed hippocampal cultures. In addition, we studied the effect of lubeluzole in focal cerebral ischemia models in mice and rats. In hippocampal cultures exposed to 500 nM glutamate for 1 h, lubeluzole (0.1–100 nM), but not the *R*-isomer (1–100 nM), reduced the percentage of damaged neurons from $42 \pm 8\%$ to $18 \pm 7\%$ ($P < 0.01$). In mice and rats, lubeluzole reduced ischemic brain damage, when administered immediately after middle cerebral artery occlusion. Interestingly, the protective effect (reduction of the infarct volume in rats to 77% of control; $P < 0.01$) was also found when the lubeluzole treatment (2.5 mg/kg) was started 3 h after ischemia. Especially this latter effect suggests that lubeluzole will be a useful drug for stroke therapy. © 1998 Elsevier Science B.V.

Keywords: Lubeluzole; Excitotoxicity; Hippocampal cells; Glutamate; Cerebral ischemia; Middle cerebral artery occlusion

1. Introduction

Various biochemical events that occur as a result of a dramatically reduced supply of oxygen and glucose have been proposed to play a role in the development of cerebral infarction after ischemic insults (for references see Krieglstein and Oberpichler-Schwenk, 1994; Krieglstein, 1996). Many experimental studies in vitro and in vivo have demonstrated the neuroprotective potential of agents that interact with Ca^{2+}-channels, inhibit the release of excitatory amino acids or block their receptors or reduce the generation of free radicals or the synthesis of NO (O'Neill et al., 1995; Krieglstein, 1996). However, only a few of the experimentally investigated neuroprotective compounds were proven to be beneficial in clinical stroke trials and many of the effective compounds had severe side effects (Marshall and Mohr, 1993; Krieglstein, 1996; Lees and Anzal, 1996).

Lubeluzole, the *S*-isomer of a novel benzothiazole derivative, was investigated in clinical trials for the treatment of ischemic stroke. Previously reported effects of lubeluzole, such as inhibition of glutamate release, inhibition of glutamate-activated NO synthesis and blockage of voltage-gated Na^+- and Ca^{2+}-ion channels, suggest that it has neuroprotective potential (De Ryck et al., 1994, 1995, 1996; Scheller et al., 1995; Lesage et al., 1996). For the *R*-isomer of lubeluzole or the racemate the described effects were either moderate or not evident. The compound improved the neurological outcome in a model of photochemically induced thrombotic cerebral infarction in rats when administered within 6 h after the onset of ischemia, whereas the *R*-isomer of lubeluzole was inactive (De Ryck et al., 1994, 1996). However, to our knowledge there is no evidence, so far, that this improvement in neurological outcome by lubeluzole administered 3–6 h after the onset of the ischemic insult corresponds to a reduction in damage to brain tissue. In contrast, in a rat model of transient focal ischemia, lubeluzole reduced the infarct volume only when treatment was started immediately after the onset of ischemia (Aronowski et al., 1996a,b). A recent phase II clinical trial involving 232 patients with acute cerebral infarction showed that mortality could be reduced by intravenous infusion of lubeluzole (10 mg/d), whereas the higher dose (20 mg/d) was found to increase mortality although neurological variables were improved (Diener et

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al., 1996). Thus, it is of interest to further study acute and delayed administration of lubeluzole in experimental models of ischemia to provide support for the promising results obtained with lubeluzole in clinical stroke trials.

The aim of this study was to investigate the neuroprotective potency of lubeluzole and its *R*-isomer against L-glutamate-induced excitotoxicity in primary mixed hippocampal cultures containing astrocytes and neurons. Furthermore, we tested whether lubeluzole could protect brain tissue from ischemic damage when administered immediately and 3–4 h after permanent middle cerebral artery occlusion.

2. Materials and methods

2.1. Animals

Neonatal (P1) pups from Fischer-344 rats (Charles River, Sulzfeld) were used for preparing cultured cells, and male Long–Evans rats (Møllegaard) and male NMRI mice (Charles River, Sulzfeld) for ischemia experiments. The animals were maintained under controlled light and environmental conditions (12:12 h dark/light cycle, 23 ± 1°C, 55% relative humidity) and had free access to food (Altromin, Lage) and water.

2.2. Cell culture agents and other substances

Eagle's minimum essential medium (MEM) (containing 2 mM L-glutamine, 28 mM of glucose and 22 mM of sodium bicarbonate), Dulbecco's modified MEM (DMEM), Leibovitz L-15 medium (containing 2 mM L-glutamine), fetal calf serum and penicillin–neomycin–streptomycin solution were obtained from Gibco (Eggenstein). Sodium L-glutamate, cytosine-β-D-arabinofuranoside, papain, trypsin inhibitor and poly-L-lysine hydrobromide were purchased from Sigma (Deisenhofen).

Lubeluzole (*S*-4-(2-benzothiazolylmethylamino)-alpha-[(3,4-difluorophenoxy) methyl]-1-piperidine-ethanol) and its *R*-isomer are benzothiazole derivatives provided by Janssen–Cilag (Neuss).

2.3. Primary rat hippocampal cultures

Primary mixed hippocampal cultures containing neurons and astrocytes were prepared from neonatal (P1) Fisher-344 rats. The animals were decapitated under sterile conditions, and the hippocampi were isolated and incubated with papain (0.1% in L-15 medium) at 37°C for 20 min. The supernatant was removed and the tissue pieces were triturated in MEM containing 10% NU serum. The supernatant was transferred to another tube and the undispersed pieces were triturated again through a fire-polished glass pipette. The supernatants were combined, 4 ml 1% trypsin-inhibitor in MEM containing 1% bovine serum albumin (20°C) was added and the mixture was centrifuged (600 rpm, 20°C, 10 min). The cells were resuspended in MEM containing 10% NU serum and seeded onto poly-L-lysine-coated 35 mm culture dishes at 3×10^7 cells/dish. The cells were cultivated in MEM supplemented with 10% fetal calf serum, 20 U/ml penicillin, 20 μg/ml streptomycin, and were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After 2 d cytosine-β-D-arabinofuranoside (1 μM) was added to the medium for 24–36 h to minimize glial cell proliferation. The medium was changed every 3–4 d.

2.3.1. Glutamate neurotoxicity in rat hippocampal cultures

Excitotoxic injury was induced in rat hippocampal neurons maintained 14 d in vitro by the method of Koh and Choi (1988). The cultures were washed with serum-free MEM and then exposed to serum-free MEM containing 500 nM L-glutamate for 1 h. The glutamate containing medium was removed and replaced with serum-free MEM for 18 h. Control sister cultures that were not exposed to L-glutamate were also washed and then cultured with serum-free MEM. The percentage of damaged neurons was determined 18 h later by trypan blue exclusion. Cells stained with trypan blue were considered to be non-viable. The identification of neurons was based on standard morphological criteria such as phase bright fusiform and pyramidal cell bodies which extend one or more processes.

Lubeluzole (1–100 nM) or its *R*-enantiomer (1–100 nM) was dissolved in DMSO (0.01% final concentration in the medium) and added to the cell cultures. In a second series of experiments, the neuroprotective activity of lubeluzole was investigated at lower concentrations (0.01–100 nM) to define the no-effect level of the drug. The substances were present in the medium 1 h before until 18 h after excitotoxic injury. Control cultures received the vehicle only, except for a group of glutamate-free control cultures, which additionally received the *R*-isomer at a concentration of 100 nM.

2.4. Permanent focal cerebral ischemia in mice

Permanent middle cerebral artery occlusion was performed in male NMRI mice (14–16 animals per group) according to the method described by Welsh et al. (1987). Briefly, after the mice were anesthetized with tribromoethanol (600 mg/kg i.p.), a small hole was drilled in the skull to expose the middle cerebral artery. The stem of the middle cerebral artery and both branches were permanently occluded by electrocoagulation. Body temperature was maintained at 37 ± 1°C with a heating lamp during the surgical procedure. Afterwards, the mice were kept at an environmental temperature of 30°C for 2 h. For histological evaluation, the mice were anesthetized again with tribromoethanol and perfused intraperitoneally with a 1% solution of neutral red (0.5 ml) 2 d after middle cerebral artery occlusion.

The brains were removed and stored in fixative (4% formalin in phosphate buffer solution, pH 7.4) for 24 h. The tissue on the brain surface unstained by neutral red was measured as the infarcted surface area (in mm^2) by means of an image analyzing system (Kontron, Eching) according to Backhauß et al. (1992). Lubeluzole was administered intraperitoneally at doses of 0.31–2.5 mg/kg immediately after middle cerebral artery occlusion. In a second study doses of 0.08–0.31 mg/kg were administered to evaluate the no effect-level of lubeluzole. Control animals received the vehicle only.

2.5. Permanent focal cerebral ischemia in rats

Permanent middle cerebral artery occlusion was performed in male Long–Evans rats (Møllegaard) according to the method described by Tamura et al. (1981) with modifications as previously described by Semkova et al. (1996). The animals were anesthetized with halothane (1.5% in a mixture of O_2/N_2O 30:70). An incision was made through the left temporalis muscle perpendicular to a line between the external auditory canal and lateral canthus of the left eye. Under direct visualization with the surgical

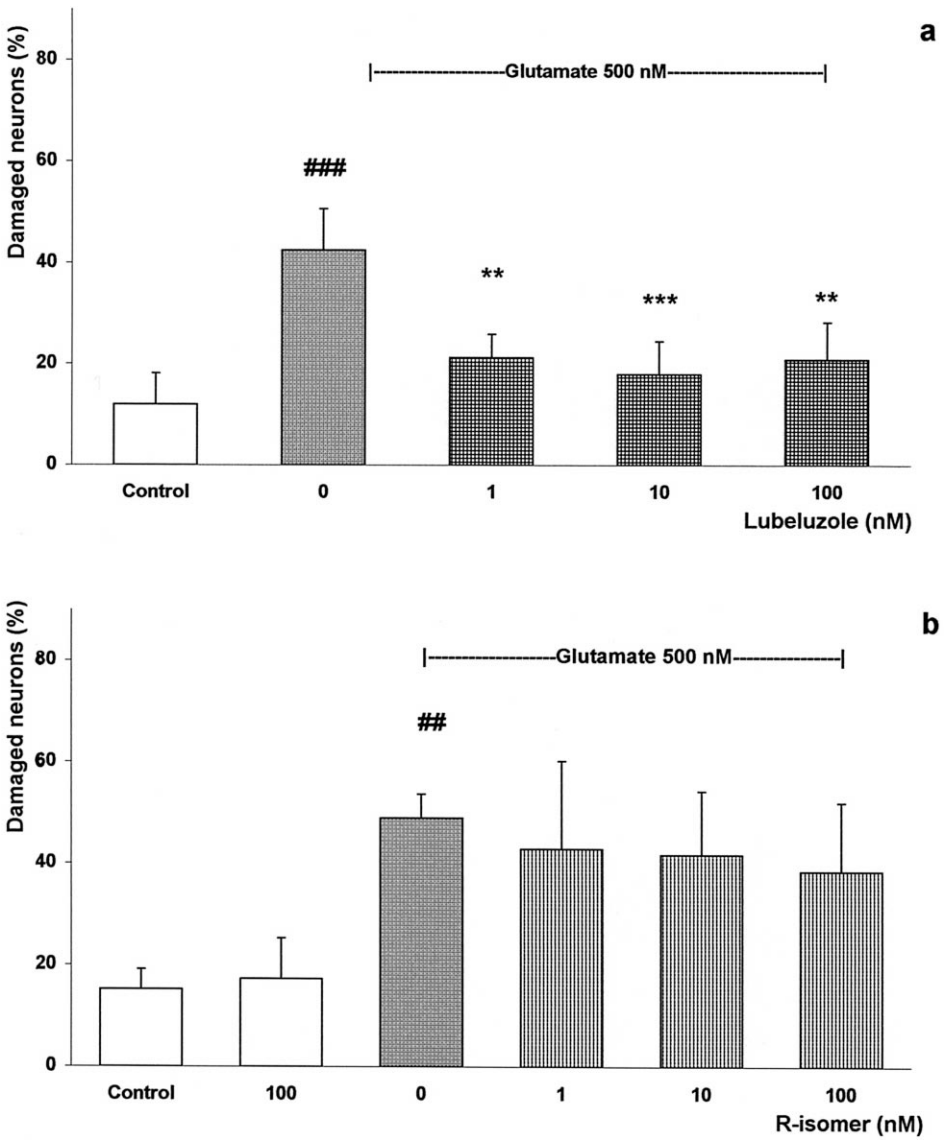

Fig. 1. Stereoselective protection of hippocampal neurons against excitotoxic damage by lubeluzole. Lubeluzole (a) or the *R*-enantiomer (b) was present in the medium of primary hippocampal cultures 1 h before until 18 h after a 1 h treatment with 500 nM L-glutamate. The substances were dissolved in dimethylsulfoxide (DMSO, 0.01% final concentration in the medium). Controls received 0.01% DMSO. The values are means ± S.D. from 5 experiments. Different from glutamate-free controls by Scheffé's test: $^{###}P < 0.001$; and from glutamate-exposed cells: $^{**}P < 0.01$, $^{***}P < 0.001$.

microscope, a burr hole was made with a hand-held drill to expose the left middle cerebral artery. After retraction of the dura the left middle cerebral artery was occluded by microbipolar electrocoagulation. After occlusion the incisions in the left temporal muscle and the skin were closed with the adhesive histoacryl (Braun–Dexon, Germany) to guarantee the function of the temporal muscle for the uptake of food after surgery. This modified model of middle cerebral artery occlusion produces exclusively cortical infarction. During surgery the body temperature was maintained at 37 ± 0.5°C by means of a heating pad. To prevent a decrease of body temperature, the animals were kept at an environmental temperature of 30°C up to 2 h after middle cerebral artery occlusion. Mean arterial blood pressure and plasma glucose concentration as well as arterial pH, p_{CO_2} and p_{O_2} were monitored (Corning 178, Corning, Germany) up to 30 min after the administration of lubeluzole was completed. Seven days after middle cerebral artery occlusion, the rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and decapitated. Brains were removed and frozen in 2-methylbutane precooled on dry ice to -20 to -30°C (Fluka, Switzerland). Transversal sections of 20 μm thickness were taken every 0.5 mm, using a cryomycrotome (Frigocut, Reichert–Jung, Germany), and stained with 0.5% cresyl violet to differentiate between intact and damaged brain tissue. Morphometric determination of the manually outlined surface (mm^2) was performed, using a computer-based image analyzing system (Kontron, Germany). The infarct volume (mm^3) was calculated from the infarct area of each section and the distance between succeeding sections.

2.5.1. Experimental groups in the rat model of permanent middle cerebral artery occlusion

In the first experiment half of the dose of lubeluzole (0.63–2.5 mg/kg) was administered i.p. immediately after middle cerebral artery occlusion followed by infusion of the second half of the dose over 1 h into the tail vein of the rats (8 animals per group). In a second series of ischemia experiments (11–13 animals per group) lubeluzole (2.5 mg/kg) was administered 3 h after the onset of ischemia (1.25 mg/kg i.p. +1.25 mg/kg i.v. over 1 h). In all experiments the vehicle (saline, 0.9%; pH 5.5) was administered to control animals.

2.6. Statistics

All values are given as means ± S.D. For the in vitro data one-way analyses of variance combined with Scheffé's test were used for multiple comparisons. Homogeneity of errors was determined by using Bartlett's test. Student's *t*-test (for the 3 h post-occlusion experiment) and Duncan's analysis of variance were used for the in vivo data.

3. Results

3.1. Effect of lubeluzole on glutamate toxicity in rat hippocampal cultures

The neuroprotective effect of lubeluzole and its *R*-enantiomer against glutamate-induced excitotoxic damage was

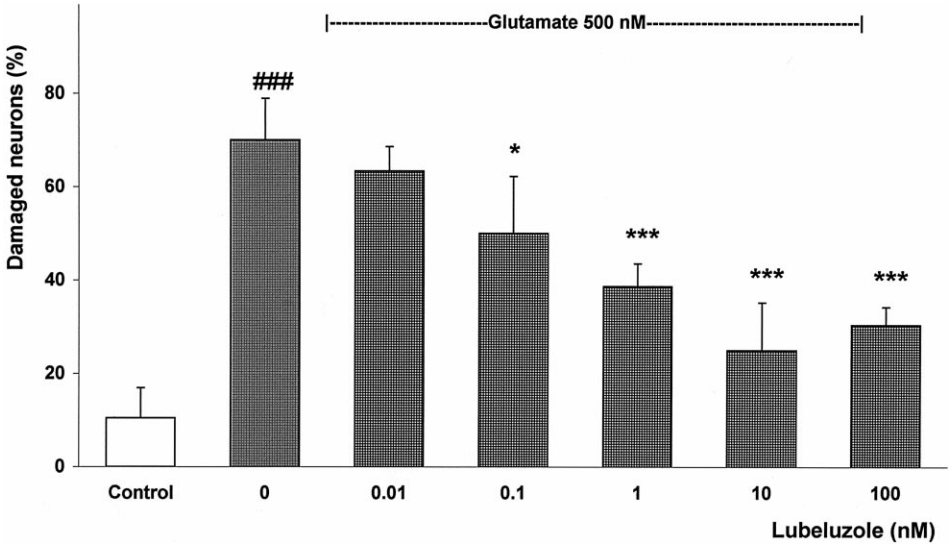

Fig. 2. Lubeluzole protects hippocampal neurons against excitotoxic damage. Lubeluzole was present in the medium of 12 d old primary hippocampal cultures 1 h before until 18 h after a 1 h treatment with 500 nM L-glutamate. Lubeluzole was dissolved in dimethylsulfoxide (DMSO 0.01% final concentration in the medium). Controls received 0.01% DMSO. The values are means ± S.D. from 5 experiments. Different from glutamate-free controls by Scheffé's test: $^{###}P < 0.001$; and from glutamate-exposed cells: $^{*}P < 0.05$, $^{***}P < 0.001$.

tested. L-Glutamate (500 nM for 1 h) induced pronounced neuronal cell death in primary hippocampal cultures. In glutamate-treated cultures the number of neurites was decreased and some neurons had disintegrated into debris. Eighteen hours after exposure to glutamate 42.4 ± 8.2% (from 5 experiments) of the neurons had lost their membrane integrity and were stained with trypan blue. Lubeluzole (1–100 nM; 5 experiments each), added to the medium from 1 h before until 18 h after the induction of damage, significantly reduced the proportion of damaged hippocampal neurons to approximately 20% (Fig. 1a). A second series of in vitro experiments was carried out with lubeluzole (0.01–100 nM) to characterize the lowest concentration of the drug that provided protection against glutamate toxicity. Lubeluzole significantly reduced the percentage of damaged neurons already at a concentration of 0.1 nM (Fig. 2).

3.2. Effect of the R-isomer of lubeluzole on glutamate toxicity in rat hippocampal cultures

We tested whether the *R*-isomer of lubeluzole could protect hippocampal neurons from glutamate-induced excitotoxic damage as well. As shown in Fig. 1b the *R*-isomer of lubeluzole (100 nM) did not increase the number of damaged neurons when added to glutamate-free control

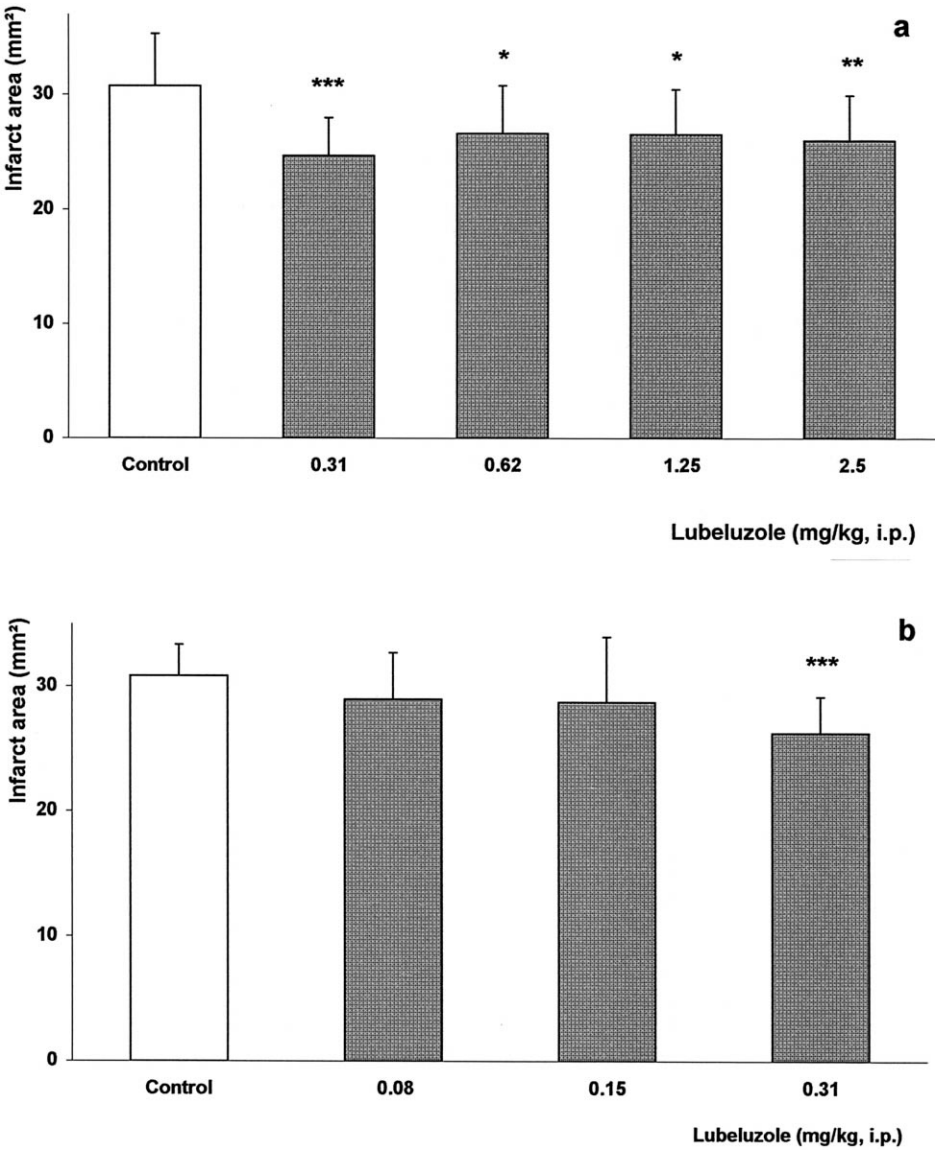

Fig. 3. Lubeluzole decreases the infarct area in a mouse model of focal cerebral ischemia. Permanent middle cerebral artery occlusion was performed in male NMRI mice. Forty-eight hours after middle cerebral artery occlusion neutral red was injected intraperitoneally to stain the brain tissue. The unstained region on the brain surface was measured as the infarct surface area (in mm^2) by means of an image analyzing system. The animals (14–16 per group) received a single intraperitoneal injection of lubeluzole at doses of 0.31–2.5 mg/kg (a) and 0.08–0.31 mg/kg (b), respectively, immediately after occlusion of the middle cerebral artery. Controls received the vehicle only. Data are means ± S.D. Different from control: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ by analysis of variance with subsequent Duncan's test.

cultures for 20 h. L-Glutamate (500 nM for 1 h) damaged approximately 50% of the neurons, as measured 18 h later ($P < 0.01$; 5 experiments). The *R*-isomer of lubeluzole, present in the culture medium 1 h before until 18 h after exposure to L-glutamate, failed to protect hippocampal neurons from excitotoxic injury (Fig. 1b).

3.3. Focal cerebral ischemia in mice

Single doses of lubeluzole (0.31–2.5 mg/kg) injected intraperitoneally immediately after middle cerebral artery occlusion significantly reduced the infarct area on the mouse brain surface (Fig. 3a). In the second series of experiments the lowest protective dose of lubeluzole was determined. Doses of lubeluzole lower than 0.31 mg/kg did not reduce the infarct area (Fig. 3b).

3.4. Physiological variables

Mean arterial blood pressure and blood glucose levels were not found to be changed by drug administration when measured up to 30 min after the end of the lubeluzole infusion in rats (Fig. 4). The values of arterial pH, p_{CO_2} and p_{O_2}, monitored at the same time, were not influenced by treatment with lubeluzole either (data not shown).

3.5. Focal cerebral ischemia in rats

In the first series of experiments half of the dose of lubeluzole (0.63, 1.25 and 2.5 mg/kg) was administered intraperitoneally immediately after the onset of ischemia followed by 1 h infusion of the second half of the dose into a tail vein of the animals. Lubeluzole (0.63 and 2.5

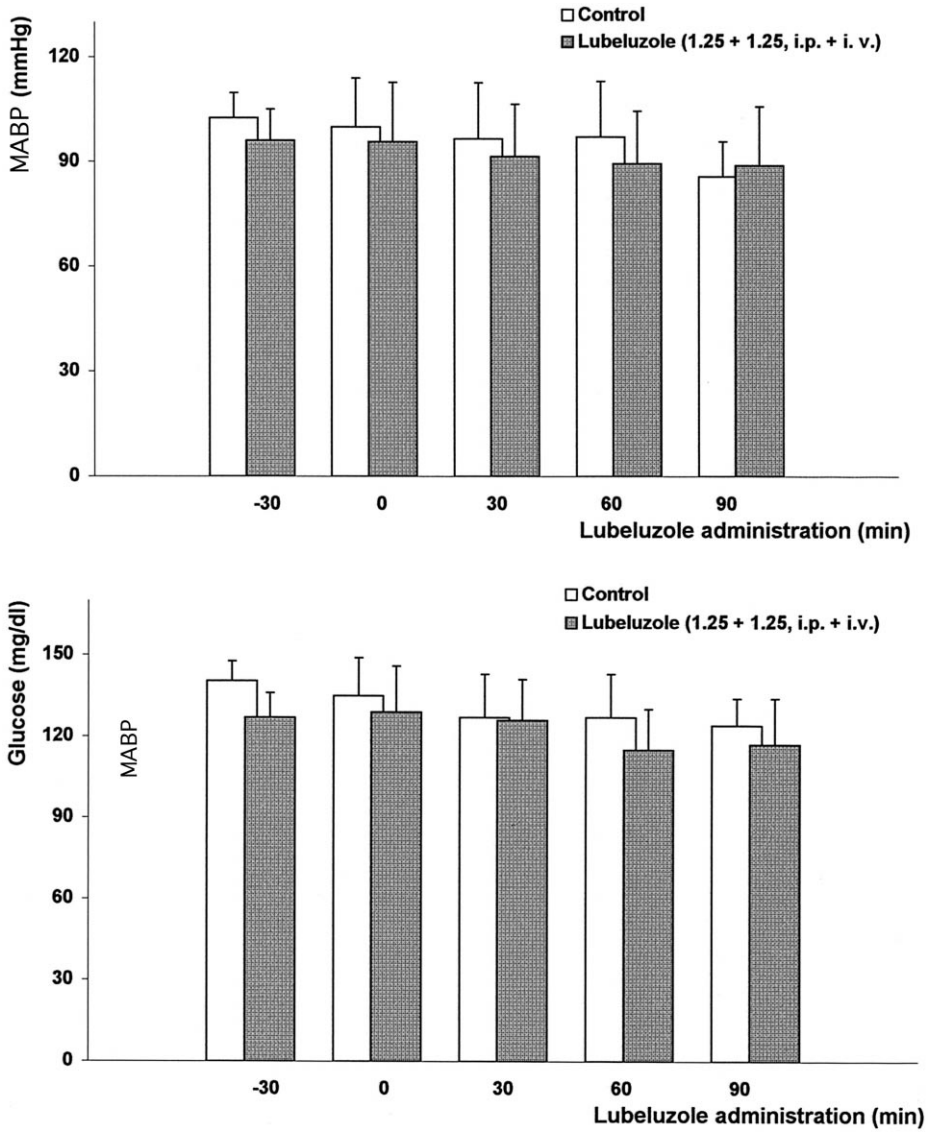

Fig. 4. Physiological variables. Half of the dose of lubeluzole was administered to Long–Evans rats intraperitoneally, followed by infusion of the second half into a tail vein over 1 h. 0: start of lubeluzole infusion; 60: end of lubeluzole infusion. MABP = Mean arterial blood pressure. Values are given as means ± S.D. from 10 animals.

Table 1
Lubeluzole reduces the infarct volume after permanent middle cerebral artery occlusion in the rat

Treatment	Infarct volume (mm^3)	*n*
Vehicle	95.8 ± 22.5	7
Lubeluzole 0.63 mg/kg	60.9 ± 20.7[a]	7
Lubeluzole 1.25 mg/kg	62.0 ± 35. 9	8
Lubeluzole 2.5 mg/kg	63.3 ± 24.6[a]	8

Half of the dose of lubeluzole (0.31–1.25 mg/kg) was administered intraperitoneally immediately after middle cerebral artery occlusion as a bolus injection and the second half was infused over 1 h into a tail vein. Seven days after surgery, the brains were removed, and the infarct volume was calculated. Values are given as means ± S.D. for 7–8 animals. Statistics were performed by analysis of variance with subsequent Duncan's test.
[a] $P < 0.05$, different from control.

mg/kg) reduced the infarct volume to approximately 70% of control levels (Table 1). The reduction of the infarct volume in the animals treated with 1.25 mg/kg immediately after middle cerebral artery occlusion was not found to be statistically significant, because of the high standard deviation in this group. Two animals, one in the control group and one in the group treated with 0.63 mg/kg lubeluzole, had to be excluded because they showed no infarction 7 d after middle cerebral artery occlusion.

In the second series of in vivo experiments the administration of lubeluzole (2.5 mg/kg) was started 3 h after middle cerebral artery occlusion. The infarct volume in the drug-treated group (12 animals) was found to be reduced to 93.7 ± 21.9 mm^3 compared to 120.4 ± 14 mm^3 measured in the control group (10 animals) (Fig. 5). In each group, one animal died during the infusion procedure 3 h after the onset of ischemia.

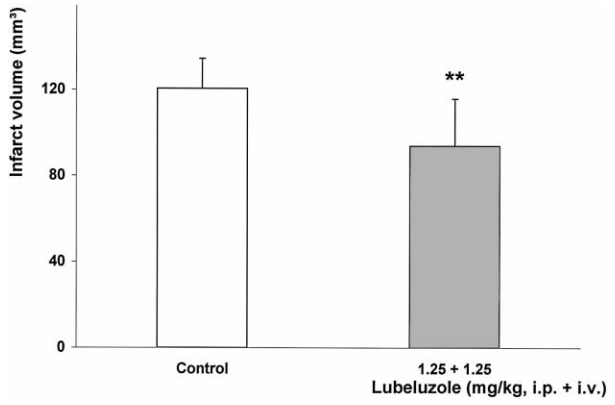

Fig. 5. Neuroprotective effects of lubeluzole given 3 h after permanent middle cerebral artery occlusion in the rat. Lubeluzole (2.5 mg/kg) was administered in two parts: a bolus injection (1.25 mg/kg i.p.) 3 h after middle cerebral artery occlusion was followed by infusion (i.v.) of the second half (1.25 mg/kg) over 1 h. Male Long–Evans rats were used in all experiments. Seven days after surgery, the brains were removed and coronal slices of 20 μm were taken every 500 μm. The infarct volume was calculated from the infarct area of each section and the distance between succeeding slices. Values are given as means ± S.D. for 10–12 animals. Different from control by Student's *t*-test: ** $P < 0.01$.

4. Discussion

For the treatment of acute ischemic stroke, there is a need for substances that protect brain tissue from ischemic damage when administered within 3–6 h after the onset of ischemia. It has been proposed that agents capable of interfering with one or more crucial steps in the damaging biochemical cascade caused by ischemia could be used as neuroprotectants for the treatment of stroke (Marshall and Mohr, 1993; Krieglstein, 1996). Lubeluzole, the *S*-isomer of a benzothiazole derivative, has been reported to reduce mortality after acute ischemic stroke in humans (Diener et al., 1996). In a rat model of photochemically induced cerebral ischemia, lubeluzole improved neurologic functions when the treatment was started within 6 h after the onset of the insult. The mechanism of action of lubeluzole is unclear, but there is evidence that the compound has several potentially neuroprotective effects, such as its ability to block voltage-gated Na^+- or Ca^{2+}-ion channels or to modulate the signal transduction pathway of NO.

In the present study, we demonstrated that lubeluzole protected cultured hippocampal neurons stereoselectively against glutamate induced excitotoxic injury. In a second series of experiments, the lowest neuroprotective concentration of lubeluzole was found to be 0.1 nM. The glutamate-induced neuronal cell death in the controls in this second in vitro study (Fig. 2, percentage of damaged neurons: 70%) was more pronounced than in the first series of experiments (Fig. 1, percentage of damaged neurons: 40%). However, in both series of experiments a comparable neuroprotective effect of lubeluzole at 1–100 nM was demonstrated (Fig. 1a and Fig. 2). The different sensitivity to glutamate exposure of control cultures in the two in vitro studies clearly indicates that it is very important to use sister cultures from the same preparation for statistical comparisons. As demonstrated in the sister cultures in the first series of experiments, the percentage of damaged neurons after glutamate exposure was very similar in control groups and in groups treated with *R*-isomer (Fig. 1a and b).

Based on the results of the in vitro studies, we tested lubeluzole in models of cerebral ischemia in mice and rats. We could demonstrate that in mice lubeluzole protected brain tissue against ischemic damage when it was administered immediately after the onset of ischemia. In rats, this protective effect against ischemic brain damage was confirmed with similar doses of lubeluzole. Only at a dose of 1.25 mg/kg was the infarct volume (60.0 mm^3) found not to be significantly different from control (95.8 mm^3; $P = 0.066$ by analysis of variance with subsequent Duncan's test), most likely because of the high standard deviation in this group (Table 1). However, the results of this study indicate that the protective effects of lubeluzole demonstrated in vitro and in mice were also confirmed in the rat model of middle cerebral artery occlusion, as two of the doses (0.63 and 2.5 mg/kg) investigated in rats produced

a significant reduction in the infarct volume (Table 1). Interestingly, the protective effect in rats was found even when the administration of lubeluzole (2.5 mg/kg) was started 3 h after permanent occlusion of the middle cerebral artery. It has been reported for other neuroprotective agents, such as the Ca^{2+}-channel blocker nimodipine, that there is a time window of about 3 h, as determined by their protective activity in models of permanent focal cerebral ischemia. Additionally, clinical stroke studies investigating either neuroprotective compounds or reperfusion therapy revealed a rather small therapeutic time window of 3 h after the insult. Recently, lubeluzole has been investigated in a phase II trial during which treatment with lubeluzole was started within 6 h after the onset of stroke (Diener et al., 1996). In addition, there is now evidence from our results that lubeluzole is a useful drug for acute stroke therapy, because a single infusion from 3 to 4 h after middle cerebral artery occlusion prevented brain tissue from sustaining ischemic damage.

Our in vitro data support the hypothesis that the cerebroprotective action of lubeluzole is based on interference with key mechanisms of the biochemical cascade that leads to irreversible damage of brain tissue in the ischemic region (De Ryck et al., 1996). The excessive release of excitatory amino acids, such as L-glutamate and L-aspartate, followed by elevation of intracellular Ca^{2+} concentration, accumulation of free radicals and NO synthesis has been postulated to contribute to the neurodegeneration that occurs after ischemic insults and trauma (Simon et al., 1984; Krieglstein, 1996). Furthermore, among other events excitotoxicity has been suggested to play a role in the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease, which are characterized by a progressive loss of neurons (Greenamyre and Young, 1989, Olney et al., 1990). In the present study, we could show that lubeluzole (0.1–100 nM) protected neurons from glutamate-induced damage in mixed cultures of hippocampal neurons and astrocytes. In contrast, the *R*-isomer failed to show any neuroprotective effect at any dose used. This supports the hypothesis that the neuroprotective activity of lubeluzole is stereoselective. It has been reported that the IC_{50} of the *R*-isomer for neuroprotection in pure neuronal cultures is nine times higher than that of lubeluzole (Lesage et al., 1996). Furthermore, in vivo lubeluzole but not the *R*-isomer improved the neurological parameters in a photothrombosis model of cerebral ischemia (De Ryck et al., 1996).

The inhibition of glutamate release and the inhibition of glutamate-induced NO synthesis are two reported effects that suggest a neuroprotective potency of the drug against excitotoxicity (O'Neill et al., 1995). The mechanism of action of lubeluzole is apparently not dependent upon NMDA receptor blockade, as the compound was not found to bind to any of the known binding sites of the NMDA receptor complex (De Ryck et al., 1996). Furthermore, the drug did not block the glutamate-induced increase in the intracellular free Ca^{2+} concentration (Lesage et al., 1994). Thus, the mechanism of action of lubeluzole against glutamate-induced excitotoxic injury is more likely a modulation of the ischemic cascade at a point below glutamate release. It has been reported that the neuroprotective activity of lubeluzole in a neuronal culture system after prolonged pretreatment over 7 d was accompanied by a reduction in neuronal NO synthase. Based on these findings, the authors suggested that the protective effect was based on regulation of gene expression which resulted in a reduced generation of the neurotoxic radical NO (Lesage et al., 1996). In contrast, in our study lubeluzole was protective after acute pretreatment. In addition, the present results from the models of permanent focal ischemia showed that treatment with lubeluzole protected brain tissue even when lubeluzole administration was delayed up to 3 h after the onset of ischemia. Thus, it seems to be unlikely that the effect of prolonged pretreatment with lubeluzole on neuronal NO synthase activity is also responsible for the in vitro or in vivo effects presented in this study. Additionally, it has been reported very recently that lubeluzole, but not the *R*-isomer, is protective against the toxic effects of NO in vitro and a protective window of 6 h after NO exposure was suggested for the reversal of NO toxicity (Maiese et al., 1997). These findings are in line with the improvement in sensorimotor function elicited by lubeluzole administered up to 6 h after the onset of ischemia in a model of photochemically induced stroke. In the same study the *R*-isomer of lubeluzole was not active at all (De Ryck et al., 1995, 1996).

In contrast to our findings, lubeluzole failed to reduce the infarct volume in a model of transient focal ischemia when administered more than 30 min after the onset of ischemia (Aronowski et al., 1996b). These authors measured the infarct volume 24 h after the lesion while in our study the infarct volume was measured 7 d after ischemia. However, there is evidence that, especially after transient focal ischemia, an infarct develops slowly and the time of development depends on the duration of the ischemic period (Du et al., 1995). Delayed cell death, characterized as apoptosis, was considered to be the dominant reason for cell loss in the border zone of the cerebral infarction after 30 min of transient focal ischemia, in contrast to models of permanent middle cerebral artery occlusion where necrosis leads to a faster development of the infarct (Du et al., 1995; Linnik et al., 1993). Therefore, it is likely that neuroprotectants like lubeluzole could show very different results concerning the time window of neuroprotective activity in different models of focal cerebral ischemia.

In conclusion, the present study indicates that lubeluzole, but not the *R*-isomer, is capable of protecting neurons against excitotoxic injury in vitro and protects rat cortical tissue from ischemic damage after middle cerebral artery occlusion. Interestingly, lubeluzole even showed protective activity when administered 3 h after the onset of

ischemia in vivo, suggesting that this compound could be useful for the treatment of acute cerebral infarction.

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