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The AMPA receptor/Na^+ channel blocker BIIR 561 CL is protective in a model of global cerebral ischaemia

Thomas Weiser [a], Marion Wienrich [a], Michael Brenner [b], René Kubiak [c], Gerhard Weckesser [c], Rainer Palluk [a,*]

[a] *Department of CNS Research, Boehringer Ingelheim Pharma KG, D-52218 Ingelheim, Germany*
[b] *Department of Medicinal Chemistry, Boehringer Ingelheim Pharma KG, Ingelheim, Germany*
[c] *Department of Research and Development Coordination, Boehringer Ingelheim Pharma KG, Ingelheim, Germany*

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Abstract

In this study, we investigated whether the novel neuroprotective compound dimethyl-{2-[2-(3-phenyl-[1,2,4]oxadiazol-5-yl)-phenoxy]-ethyl}-amine hydrochloride, BIIR 561 CL, a combined non-competitive antagonist of AMPA receptors and blocker of voltage-gated Na^+ channels, is protective in a rat model of severe global ischaemia. BIIR 561 CL administered immediately after 10 min of ischaemia (occlusion of both carotid arteries plus reduction of arterial blood pressure to 38–40 mm Hg) significantly reduced hippocampal damage at 4×26.8 mg/kg (subcutaneous injections). The competitive AMPA receptor antagonist 2,3-dihydro-6-nitro-7-sulfamoyl-benz(*F*)quinoxaline, NBQX, was used as a reference compound and was protective at 3×30 mg/kg (intraperitoneal and/or subcutaneous administration). BIIR 561 CL significantly reduced the ischaemia-induced premature mortality from 33.6% in the controls to 14.3%, whereas NBQX treatment had no statistically significant effect.

Thus, BIIR 561 CL could be shown to reduce hippocampal damage and premature mortality in a model of severe global ischaemia. A compound with these properties might be an interesting candidate for the treatment of disorders related to global cerebral ischaemia in man.

Keywords: AMPA receptor antagonist; Na^+ channel blocker; Ischaemia, global; Neuroprotection; BIIR 561 CL

1. Introduction

In a recent publication, we presented data on dimethyl-{2-[2-(3-phenyl-[1,2,4] oxadiazol-5-yl)-phenoxy]-ethyl}-amine hydrochloride, BIIR 561 CL, a novel development compound for the treatment of excitotoxicity-related disorders of the brain (Weiser et al., 1999). BIIR 561 CL is a compound with a dual mechanism of action: It is a non-competitive inhibitor of glutamate receptors of the AMPA subtype, as well as a blocker of voltage-gated Na^+ channels. Both of these mechanisms have been shown to be neuroprotective in models of global ischaemia. NBQX (2,3-dihydro-6-nitro-7-sulfamoyl-benz(*F*)quinoxaline), a competitive AMPA receptor antagonist, as well as some of its newer derivatives, reduced the brain damage induced by transient global ischaemia (Buchan et al., 1991; Diemer et al., 1992; Sheardown et al., 1993; Kawasaki-Yatsugi et al., 1997; Turski et al., 1998; O'Neill et al., 1998). Blockers of voltage-gated Na^+ channels, like riluzole, mexiletine, or even tetrodotoxin, also have neuroprotective effects in vivo (Malgouris et al., 1989; Stys and Lesiuk, 1996; Lysko et al., 1994). One can therefore hypothesize that a compound which combines these two mechanisms should be neuroprotective as well.

Therefore, we tested BIIR 561 CL in a rat model of severe global ischaemia. Our investigations targeted mainly two questions:

1. Does BIIR 561 CL reduce hippocampal damage in a severe rat model of global ischaemia?
2. Does the compound reduce mortality of the animals which were subjected to ischaemia?

The effects of BIIR 561 CL were compared with those of the well-described AMPA receptor antagonist NBQX.

* Corresponding author. Tel.: +49-613-277-3366; fax: +49-613-277-4593.
E-mail address: palluk@ing.boehringer-ingelheim.com (R. Palluk).

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2. Methods

2.1. Experimental procedures

Male Wistar rats weighing 330–400 g were used. The experiments were approved by the Bezirksregierung Rheinhessen-Pfalz. The animals were kept in groups of 5 in. Makrolon cages type 4 with softwood granulate bedding and had free access to tap water and standard pellet diet until the experiment began.

Transient global cerebral ischaemia was induced by occlusion of both common carotid arteries and hypobaric hypotension, similar to the method described by Dirnagl et al. (1993): The animals were anaesthetised with isoflurane (spontaneous respiration, initial concentration 2.5%, then 1.3% during surgery, reduction to approximately 0.5% as appropriate to maintain spontaneous respiration during ischaemia). During the whole experiment, the body temperature of the rat was maintained between 37°C and 38°C with a heating pad and a heating lamp.

The body was shaved below the costal margin in a belt-like manner. A mark was made with a pen 1.5 cm below the costal margin. The shaved belt was wetted with tylose gel and the lower body of the animal was placed in a plastic tube. The sealing between plastic tube and rat was achieved by a belt-like latex membrane which was fixed to the rat at the mark with a circular rubber ring.

A median incision was made in the neck. Both common carotid arteries were carefully exposed without lesioning the vagus nerves. The right carotid artery was distally ligated and proximally cannulated with a PE 50 tube for measuring blood pressure (Statham pressure transducer P 23 XL, Grass polygraph model 7 or 7C). Thus, the right carotid artery was permanently occluded. Cerebral ischaemia was induced for 10 min by evacuating the plastic tube by a membrane pump until the blood pressure measured in the carotid artery was 38–40 mm Hg and then clamping the left carotid artery with a small serrefine ($t = 0$ min). The cerebral perfusion was shown to be $< 5\%$ of normal in this model when the arterial blood pressure was adjusted to 55 mm Hg (Dirnagl et al., 1993).

After 10 min of ischaemia, the serrefine was removed, and the lower body suction was terminated by turning off the pump and opening the rubber ring. An arterial blood sample was taken from the cannulated carotid artery for blood gas analysis (ABL 600, Radiometer, Copenhagen). The proximal stump of the right carotid artery was ligated and the wound was sutured. The animals were kept for two further hours in a Makrolon cage type 3 under a heating lamp which was adjusted to keep the animals' rectal temperature between 37°C and 38°C.

The first administration of test compound or vehicle was immediately after the end of ischaemia ($t = 10$ min). Further administrations were done at $t = 40$, 70 and 130 min for BIIR 561 CL (all administrations s.c.) and at $t = 40$ and 70 min for NBQX (2,3-dihydro-6-nitro-7-sulfamoyl-benz(F)quinoxaline; all administrations i.p.). The latter was also administered in a different regimen at $t = -5$ min s.c. (for technical reasons; before beginning of ischaemia), $t = 10$ min i.p. (after end of ischaemia) and $t = 25$ min i.p. The dosing scheme of NBQX was based on published reports (maximum tolerated dose, e.g., Shimizu-Sasamata et al., 1996). The dosing scheme of BIIR 561 CL was chosen in a way to obtain a fast rise of the plasma level with single doses which did not induce unbearable side effects in conscious animals. The plasma half-life of BIIR 561 CL is 0.9 h, and the plasma level after the fourth injection was approx. 1 μg/ml (unpublished observations).

Furthermore, two sham-operated groups were included in the study, sham-I, in which both carotid arteries were clamped or cannulated as usual, but no lower body suction was applied, and sham-II in which the left carotid artery was not clamped for 10 min during the hypotension.

The animals were then kept in single cages with tap water and standard pellet diet ad libitum, and were daily weighed. The number of deaths within the first week was counted per treatment group for statistical evaluation. On the seventh day after ischaemia, the animals were sacrificed by exsanguination under isoflurane anaesthesia. The brain was rapidly removed and suspended in 7.5% formalin solution for at least 24 h. The relevant part of the brain (1 mm anterior of bregma–4 mm posterior of bregma) was bedded in a paraffin block. Coronal sections (7 μm) of the dorsal hippocampus were made and stained with cresyl violet, according to standard techniques. The section (approx. bregma—3.3, Paxinos and Watson, 1982) was selected for histological examination. The hippocampal regions CA1–CA4 were examined by light microscopy. The degree of the lesion was separately scored (0–4, in steps of 0.5) for both sides of the brain according to the cell loss in the hippocampal pyramidal cell layer as follows.

1. In approximately 1/3 of the CA1 region more than 50% of the cells were degenerated or pycnotic.
2. In approximately 2/3 of the CA1 region more than 50% of the cells were degenerated or pycnotic.
3. In the whole CA1 region more than 50% of the cells were degenerated or pycnotic.
4. Also in the CA2, CA3 and CA4 regions more than 50% of the cells were degenerated or pycnotic.

The scores of both sides were added (maximum lesion = 8) and this total score was used for statistical evaluation. The examination was independently performed twice, by the two investigators, in a non-blinded manner. If a different rating occurred, both investigators commonly re-evaluated the section and made a consensus decision on the score.

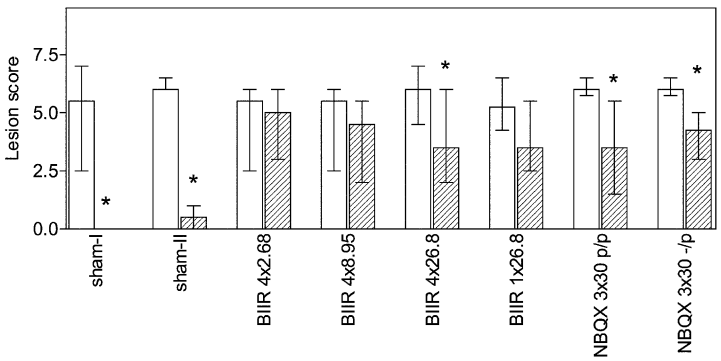

Fig. 1. Hippocampal lesions after global ischaemia. BIIR 561 CL (BIIR) dose-dependently reduced the lesion scores induced by 10 min of severe global ischaemia. The effect was statistically significant with 4×26.8 mg/kg. NBQX reduced the lesion scores when administered pre- and post-ischaemia (p/p), or only post-ischaemia (−/p). Both sham groups did not suffer hippocampal damage (sham-I = both carotid arteries clamped or cannulated, no lower body suction; sham-II = left carotid was not clamped, but lower body suction applied). Open bars refer to the scores of animals subjected to ischaemia with vehicle; hatched bars represent the verum or sham groups, respectively. Data are shown as medians with 25% and 75% quartiles. Statistical significance ($P < 0.05$) is indicated by asterisks; see text for details.

2.2. Statistical evaluation

Approximately 10 animals were used per treatment group. The data of repeated experiments were additionally pooled and statistically evaluated.

For the lesion scores, a non-parametric evaluation was considered appropriate. Therefore, the medians and the upper and lower quartiles were used for descriptive statistics. A two-tailed rank test for complete block designs (Haux et al., 1984) was applied to compare control and treated groups (level of significance = 0.05). Within the test, the different technicians were considered as the block variable. In the case of several comparisons to one control, P values were adjusted according to Bonferroni–Holm.

The numbers of premature deaths within the first week were analysed with the Pearson chi-square test for the groups with significant protection as judged by the lesion score. This was to exclude a pretended protection by potentially increased mortality in the treated groups (better survival of animals with small lesions): For the resulting 3×2-contingency table "treatment × status (death 'yes/no')", the treatment specific probabilities for 'death within the first week' were compared by the Pearson chi-square statistic with two degrees of freedom. In case that the hypothesis of equal death probabilities had to be rejected ($P < 0.05$) subsequently within a closed testing procedure, the three possible pairwise treatment comparisons were performed applying the Pearson chi-square test with one degree of freedom for 2×2-contingency tables.

To the other parameters measured (body temperature, arterial oxygen saturation (% sO_2) and arterial CO_2 partial pressure (pCO_2, mm Hg), only descriptive statistics were applied (arithmetic mean, standard deviation (S.D.)). The statistical analysis was carried out with the program SAS (SAS Institute, Cary, NC), version 6.11.

2.3. Compounds

BIIR 561 CL (synthesized at the Department of Medicinal Chemistry of Boehringer Ingelheim Pharma KG) was dissolved in demineralised water. NBQX (Tocris Cookson, Bristol, UK) was dissolved in demineralised water with addition of one equivalent lithium hydroxide (final pH 8–8.5). The subcutaneous injections were made in the

Table 1
Lesion scores in the single experiments
Unpooled data for the experiments performed repeatedly. BIIR 561 CL was administered via subcutaneous injections; NBQX was given s.c./i.p. (pre- and post-ischaemia; "pre"), or only i.p. (post ischaemia; "post"). For details of the statistical analysis, see text. P values < 0.05 were considered statistically significant.

Experiment	Median score (Control)	Interquartile range	n	Median score (Verum)	Interquartile range	n	P value
BIIR 561-I 4×26.8 mg/kg	6.50	3.00–7.00	13	3.25	2.00–5.75	16	0.0217
BIIR 561-II 4×26.8 mg/kg	6.50	6.00–7.50	10	3.50	1.50–6.00	11	0.0028
BIIR 561-III 4×26.8 mg/kg	7.00	5.50–8.00	11	5.25	1.00–6.00	10	0.0265
BIIR 561-IV 4×26.8 mg/kg	5.00	2.5–6.00	11	3.00	3.00–6.00	11	0.6490
NBQX-I 3×30 mg/kg pre	6.00	5.50–6.50	10	2.75	1.25–4.50	12	0.0060
NBQX-I 3×30 mg/kg post				4.25	3.00–5.00	10	0.0177
NBQX-II 3×30 mg/kg pre	6.00	6.00–6.50	10	4.75	2.50–6.00	10	0.0302
NBQX-II 3×30 mg/kg post				4.00	3.00–5.00	10	0.0372

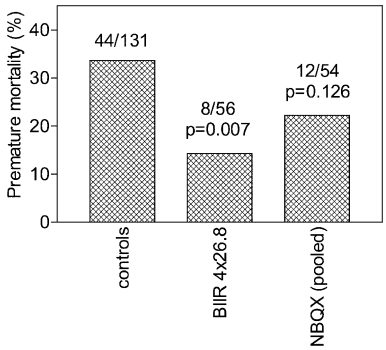

Fig. 2. Effects of BIIR 561 CL and NBQX on premature mortality after global ischaemia. Global ischaemia induced mortality in 33.6% of the control animals. BIIR 561 CL (BIIR, 4×26.8 mg/kg) significantly reduced premature mortality to 14.3%. NBQX (pooled data for all experiments) showed an insignificant trend towards reduction of mortality (22.2%). Figures on top of the bars give the numbers of prematurely died animals out of the total group size.

lower dorsal region. The injection volume was 0.1 ml/100 g body weight for BIIR 561 CL and 0.5 ml/100 g for NBQX. The control groups received either the respective vehicle (0.02 N LiOH for NBQX, pH 10.8) or no treatment (sham groups).

3. Results

In vehicle-treated animals, the median lesion scores obtained varied from 5.0 to 7.0 which indicates a considerable ischaemic hippocampal lesion under control conditions. No lesions were observed in the sham-I group, showing that bilateral carotid occlusion did not induce ischaemic damage in the rat hippocampus. In the sham-II group, some small to moderate lesions occurred, resulting in a median score of 0.5 (interquartile range 0.0–1.0), showing that if one carotid artery was occluded, the hypotension applied was severe enough to induce in some cases hippocampal lesions (Fig. 1).

The effects of BIIR 561 CL and the reference compound NBQX (2,3-dihydro-6-nitro-7-sulfamoyl-benz(*F*)-quinoxaline) on the hippocampal lesion score are shown in Fig. 1. BIIR 561 CL was tested in four separate experiments at a dose of 4×26.8 mg/kg s.c. Analysis of the pooled data showed that treatment with 4×26.8 mg/kg s.c. BIIR 561 CL reduced the median score by 42% from 6.0 to 3.5 ($P = 0.0001$).

Doses of 4×8.95 ($P = 0.2808$) and 4×2.68 mg/kg s.c. ($P = 0.5972$) did not exert significant protection under these experimental conditions.

A single dose of 26.8 mg/kg s.c. BIIR 561 CL tended to reduce the lesion score (from 5.3 in the controls to 3.5 in the treatment group; $P = 0.0534$).

NBQX (3×30 mg/kg) significantly reduced the hippocampal lesion score either when administered before (s.c./i.p.) or after (i.p.) ischaemia (Table 1). When the data from the two experiments with NBQX were evaluated after pooling, the effect appeared to be similar in size for both ways of administration (pre- and post-ischaemia treatment), and comparable to the effect achieved with BIIR 561 CL (4×26.8 mg/kg s.c.; Fig. 1). The data of the separate experiments are listed in Table 1.

We further assessed the number of animals which died prematurely during the survival period of 7 days in the dose groups with significant protection as judged by the lesion score. In the pooled control animals, the mortality was 33.6% which may indicate the severity of the ischaemic lesion. Treatment with 4×26.8 mg/kg s.c. BIIR 561 CL lowered the mortality to 14.3% ($P = 0.007$ vs.

Table 2
Body temperature and blood gasses before and after global ischaemia.

Treatment	*n*	Rectal temperature (°C)		Arterial sO_2(%)	Arterial pCO_2 (mm Hg)
		Before ischaemia	After ischaemia	After ischaemia	After ischaemia
Control	45	37.2 ± 0.4	37.9 ± 0.7	88.0 ± 14.0	43.6 ± 7.4
BIIR 561 CL 4 × 26.8 mg/kg s.c.	48	37.4 ± 0.4	37.7 ± 0.5	91.4 ± 4.8	43.6 ± 5.0
Control	10	37.6 ± 0.6	37.7 ± 0.9	87.3 ± 18.7	46.7 ± 8.3
BIIR 561 CL 4 × 2.68 mg/kg s.c.	10	37.5 ± 0.5	37.6 ± 0.7	92.3 ± 1.8	43.9 ± 1.9
BIIR 561 CL 4 × 8.96 mg/kg s.c.	10	37.6 ± 0.6	37.7 ± 0.9	87.3 ± 18.7	46.7 ± 8.3
Control	12	37.2 ± 0.4	38.2 ± 0.6	91.1 ± 4.5	42.1 ± 4.6
BIIR 561 CL 1 × 26.8 mg/kg s.c.	11	37.2 ± 0.4	37.9 ± 0.8	88.5 ± 16.2	44.8 ± 11.8
Control	21	37.5 ± 0.5	37.6 ± 0.6	91.1 ± 3.0	44.4 ± 4.0
NBQX 3 × 30 mg/kg s.c./i.p.	22	37.5 ± 0.6	37.5 ± 0.5	89.4 ± 5.2	44.1 ± 4.9
NBQX 3 × 30 mg/kg i.p.	22	37.4 ± 0.5	37.5 ± 0.4	92.6 ± 5.1	43.0 ± 3.0
Sham-I	10	37.4 ± 0.4	38.2 ± 0.6	n.d.	n.d.
Sham-II	10	37.4 ± 0.3	37.4 ± 0.4	93.1 ± 2.9	43.0 ± 4.2

Sham-I = both carotid arteries clamped or cannulated, no lower body suction.
Sham-II = left carotid was not clamped, but lower body suction applied.
NBQX experiments: s.c./i.p. = pre- and post-ischaemia treatment, i.p. = only post-ischaemia treatment.
n.d. = not determined.

control), whereas NBQX (3×30 mg/kg s.c./i.p. or i.p.) appeared to lower mortality to 22.2% ($P = 0.126$ vs. control; Fig. 2). None of the sham-operated animals died prematurely which indicates that the mortality was specific to the severe global ischaemia elicited under these experimental conditions and not due to vagal nerve lesion. In any case, it could be excluded that a potential increase in mortality (selection due to survival only of animals with smaller lesion size) in response to treatment with BIIR 561 CL or NBQX was the reason for the reductions in lesion score observed.

The results of the rectal body temperature measurements and the blood gas analyses are shown in Table 2. There was a slight, but generally observed increase in body temperature from "before ischaemia" to "after ischaemia" and some values somewhat exceeded the intended range. This slight hyperthermia might have aggravated the sequelae of global ischaemia in our model.

The mean values for arterial O_2 saturation (% sO_2) and carbon dioxide partial pressure (pCO_2, mm Hg) were in the range expected for spontaneously breathing rats anaesthetised with isoflurane.

4. Discussion

The aim of this study was to test whether the novel anticonvulsant and neuroprotectant BIIR 561 CL was similarly protective in a model of global ischaemia as the well described reference compound NBQX. The latter compound was administered at the maximum tolerated dose (Shimizu-Sasamata et al., 1996). BIIR 561 CL was dosed up to 4×26.8 mg/kg s.c., because up to this dosing the side effects were tolerable in conscious rats and mice.

The presented model of global ischaemia was intentionally tailored to yield a relatively high degree of premature mortality in the control groups. This was done to cover the diverse causes and sequelae of global ischaemia in the clinical situation. Thus, we could define two endpoints: (1) a possible reduction of the hippocampal lesion score by the drug treatment, and (2) the reduction of premature mortality.

The high hippocampal lesion scores and the considerable mortality in the control groups indicate that a severe global cerebral ischaemia was achieved under the experimental conditions of this study. The lesions appeared to be specific since no comparable changes were observed in the sham groups.

In a previous study, we reported the anticonvulsant effects of BIIR 561 CL after systemic administration, which demonstrates that the compound crosses the blood–brain barrier (Weiser et al., 1999).

When data from all groups treated with 4×26.8 mg/kg s.c. BIIR 561 CL after the end of ischaemia were evaluated in a pooled manner, the neuroprotection elicited by this compound was highly significant. The neuroprotective effect of a single administration of 26.8 mg/kg s.c. BIIR 561 CL only slightly missed the level of significance, no protective effects could be detected for the two lower doses under these experimental conditions. This may be interpreted as indicating that a rapid initial increase in the plasma levels of BIIR 561 CL is important, or that the achieved brain levels were not sufficient to provide significant neuroprotection.

In the groups receiving the dose of 4×26.8 mg/kg s.c. BIIR 561 CL, a significantly reduced premature mortality was observed. This indicates that BIIR 561 CL exerts a protective effect against the sequelae of ischaemia. Moreover, it suggests that the reduction in lesion scores was not an artifact caused by the premature death of animals (with severe lesions), and the resulting survival of animals with smaller lesions.

As expected from published results, NBQX also significantly reduced the hippocampal lesion score. The protective effect was comparable for pre- and post-lesion treatment. A trend for a reduction in premature mortality was seen, but this was not statistically significant with NBQX.

Antagonists of AMPA receptors were among the first compounds shown to be effective in models of global ischaemia. The first compound tested in a larger number of studies was NBQX. This compound provided neuroprotection in several models using rats or gerbils at doses comparable to the 30 mg/kg which we used in this study (Buchan et al., 1991; Diemer et al., 1992; Sheardown et al., 1993; Kawasaki-Yatsugi et al., 1997). Solubility problems of this compound led to the development of improved derivatives with similar receptor-binding profiles and neuroprotective properties, like YM90K (6-(1*H*-imidazol-1-yl)-7-nitro-2,3(1*H*,4*H*)-quinoxalinedione) or ZK200775 ([1, 2, 3, 4-tetrahydro-7-morpholinyl-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methylphosphonate; Kawasaki-Yatsugi et al., 1997; Turski et al., 1998). Na^+ channel blockade also can offer profound neuroprotection, as has been shown for riluzole or tetrodotoxin in gerbils (Malgouris et al., 1989; Lysko et al., 1994). In these studies, however, compound administration was started before the onset of ischaemia.

The neuroprotection provided by BIIR 561 CL was comparable to the effects of NBQX in our study. There was, however, a more pronounced reduction in premature mortality in the BIIR 561 CL-treated animals. One could hypothetize that the blockade of Na^+ channels by this compound has additional beneficial effects. Thus, a compound like BIIR 561 CL might be an interesting candidate for the treatment of disorders related to global cerebral ischaemia in man.

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