Restorative Neurology and Neuroscience 17 (2000) 45–59

Invited review

Phosphonate quinoxalinedione AMPA antagonists

Lechoslaw Turski*, Herbert H. Schneider, Roland Neuhaus, Fiona McDonald, Graham H. Jones, Boel Löfberg, Hermann Schweinfurth, Andreas Huth, Martin Krüger and Eckhard Ottow

Research Laboratories of Schering AG, Müllerstrasse 178, D-13342 Berlin, Germany

Received 17 July 1998; accepted 7 July 2000

Abstract

In the Western world, over 350,000 deaths and $30 billion in medical costs are attributed annually to stroke. Head and spinal cord trauma cause an estimated 250,000 deaths annually and result in medical costs of $15 billion. Although stroke and head/spinal cord trauma are leading causes of disability and death in humans, no adequate neuroprotective treatment is available. Glutamate antagonists derived from the quinoxalinedione scaffold are as drug candidates for neuroprotection in stroke and trauma. Quinoxalinedione derivatives such as 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline and 6-(1H-imidazol-1-yl)-7-nitro-2,3-(1H,4H)-quinoxalinedione failed clinical trials because of insolubility and resulting nephrotoxicity. Introduction of a phosphonate group into the quinoxalinedione skeleton improves solubility and leaves potency for the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor unchanged. Phosphonate quinoxalinedione derivatives ZK202000 and ZK200775 protected rodent brain against sequelae of permanent occlusion of the middle cerebral artery and head trauma. No major deleterious effects on motor coordination, cardiovascular, or respiratory systems were detected in doses required for neuroprotection. No psychotomimetic and no neurotoxic side effects, typical for N-methyl-D-aspartate antagonists, were observed following treatment with phosphonate quinoxalinediones.

Keywords: glutamate antagonists, anxiety, analgesia, muscle tone, seizures, stroke, traumatic brain injury, neuroprotection

1. Introduction

The amino acid L-glutamate acts in the mammalian brain at ionotropic receptors which gate cation channels and can be selectively activated by N-methyl-D-aspartate (NMDA), kainate, or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and at the metabotropic receptors, activated by *trans*-1-amino-cyclopentyl-1,3-dicarboxylate [35,50,61, 67]. Changes in the physiological function of glutamate are believed to contribute to the pathogenesis of acute (stroke, traumatic brain/spinal cord injury) and chronic neurodegenerative disorders (amyotrophic lateral sclerosis, Huntington's disease, parkinsonism, olivopontocerebellar degeneration, Alzheimer's disease), and to the etiology of major psychiatric and neurological disorders (anxiety, schizophrenia, drug dependence, epilepsy, spasticity, pain disorders) [1,3,11,15, 23,27,40,60,62,64].

Rescuing the brain from stroke and head trauma, or slow onset neurodegenerative disorders remains an unmet goal of contemporary medicine despite the wealth of research efforts towards the discovery of neuroprotective drugs [2,48]. NMDA antagonists, the first generation of glutamate antago-

* Present address: Solvay Pharmaceuticals, Research Laboratories, C.J. van Houtenlaan 36, 1381 CP Weesp, The Netherlands.
Correspondence to: Solvay Pharmaceuticals, Research Laboratories, C.J. van Houtenlaan 36, 1381 CP Weesp, The Netherlands;
Tel.: +31 294 479498, Fax: +31 294 477109;
E-mail: lechoslaw.turski@solvay.com.

nists designed for the treatment of acute onset neurodegeneration, have reached the clinics but the anticipated success in patients was limited due to unacceptable side effects [22]. The failures in clinical trials of NMDA antagonists raised questions about the feasibility of neuroprotective measures in stroke and trauma.

The lack of sufficiently selective and bioavailable non-NMDA antagonists has hampered progress towards the detection of AMPA receptor operated mechanisms for over two decades [29]. The advent of quinoxalinediones allowed the study of the mechanisms controlled by AMPA receptors in the brain and revealed their potential in neuroprotection [52]. Unfortunately, AMPA antagonists 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (NBQX) [52] and 6-(1H-imidazol-1-yl)-7-nitro-2,3-(1H,4H)-quinoxalinedione (YM90K) [54], failed clinical trials because of nephrotoxicity due to their limited solubility in water [22].

Achieving water-solubility for quinoxalinediones without loss of their selectivity and potency profiles has been a major challenge for medicinal chemistry in the past decade. Here we report the design of a novel class of water-soluble quinoxalinediones which retain high selectivity at the AMPA receptor and are effective neuroprotective drugs in rodent models of ischemia and head trauma.

2. Materials and methods

2.1. In vitro binding

The potency of quinoxalinediones in inhibiting the specific binding of ^{3}H-AMPA [26], ^{3}H-CNQX [24], ^{3}H-kainate [25], ^{3}H-CPP [42,43], ^{3}H-TCP [57] and ^{3}H-dihydrochlorokynurenate [12] was tested on rat cortical membranes.

2.2. Spreading depression

To assess the effect of quinoxalinediones on spreading depression triggered by glutamate agonists, posterior chambers of chicken eyes (3–6 day-old, Copenhagen Serum Institute, Copenhagen, Denmark; Laptec, Gödöllö, Hungary) were placed in O_2-saturated saline containing AMPA/quisqualate, kainate, NMDA, or glycine alone or with different concentrations of antagonists and the latency to initiation of spreading depression was measured [51].

2.3. Single-cell recordings in cultured hippocampal neurons

Electrophysiological studies were performed on primary cultures of hippocampal neurons obtained from 1–3 day-old Wistar rats (Schering AG, Berlin, Germany). Cells were cultured in Neurobasal™ medium (Gibco; Life Technologies, Karlsruhe, Germany) supplemented with 2 % B27 (Gibco), 5 % FCS, 1 % penicillin/streptomycin and 0.5 mM glutamine. Cytosine arabinoside (5 mM) was added after 4 days in culture to stop proliferation of non-neuronal cells. For the experiments, cells were used between days 7 and 16. Membrane currents were recorded at –60 mV in the whole cell patch clamp configuration [21]. For recording of kainate-induced currents, patch electrodes were filled with a solution containing (mM) CsCl 150, $MgCl_2$ 1, EGTA 10 and HEPES 10 (pH 7.4; resistance 2–5 MΩ). The bathing medium contained (mM) NaCl 150, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 2 and HEPES 10 (pH 7.4). For recording of NMDA-induced currents, Mg^{2+} was omitted and 3 μ*M* glycine added. Kainate, NMDA and quinoxalinediones were applied via a multichannel application system with a common outlet. NBQX and [[1,2,3,4-tetrahydro-7-(phenylethyl)-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methyl]phosphonate (ZK 202000) were dissolved in DMSO (0.3 %) and made up to the desired concentration with the bathing medium. [[1,2,3,4-tetrahydro-7-(4-morpholinyl)-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methyl]phosphonate (ZK 200775) was dissolved in bathing medium. The concentration-response curves and inhibition curves were fitted to the equation: $I = 100/(1 + (K/[drug])^n)$ with I: normalised current response in %, K: EC_{50} or IC_{50}, n: slope. Concentration-dependence of quinoxalinediones (up to 5 different concentrations applied to the same cell in ascending order) was studied by coapplication of kainate or NMDA at 100 μ*M*. The effect of quinoxalinediones (100 μ*M*) on the concentration-response curve for kainate was investigated by applying different concentrations of kainate to the cell.

2.4. Pharmacology

Anxiolytic activity was assessed in NMRI mice (Schering AG), 23–28 g, placed in the center of a chamber (23 × 18 × 30 cm) with a floor divided into four plates [56]. To confirm anxiolytic activity of quinoxalinediones, ZK200775 was tested in an elevated plus-maze in mice [44]. For determination of the threshold for clonic seizures, NMRI mice, 20–24 g, received an intracerebroventricular infusion of AMPA, kainate or NMDA until clonic seizures were triggered [58]. The motor coordination of mice was estimated in the rota-rod test [63]. For the analysis of anticonvulsant action, the effect of phosphonate quinoxalinediones and NBQX on seizures induced by pentylenetetrazol and maximal electroshock was examined in mice. The effect of quinoxalinediones on maximal electroshock seizures (tonic extension of the hindlimbs) was determined by means of electrical stimulation using auricular electrodes [28,65]. To explore the spectrum of anticonvulsant activity, the effect of ZK200775 on seizures induced by the chemoconvulsants bicuculline, picrotoxin, strychnine, 3-mercaptopropionate (Sigma, St. Louis, MO, USA), and methyl-4-ethyl-6,7-dimethoxy-9H-pyrido-(3,4-b)-indole-3-carboxylate (DMCM; Schering AG) was estimated in mice after i.p. or s.c. administration. Exploratory activity in non-habituated NMRI mice, 22–25 g, was estimated with a computerized Digiscan-16 monitoring system (AccuScan, Columbus, OH, USA). The distance in cm travelled by the mouse and detected by interruptions of the horizontal sensors was taken as a measure of exploratory activity [46]. Exploratory activity was monitored over 10 min beginning 30 min after i.v. administration of quinoxalinediones. Spinal reflexes were recorded in NMRI mice, 35–40 g, under α-chloralose (Merck, Darmstadt, Germany; 80 mg/kg i.p.)/urethane (Sigma; 400 mg/kg i.p.) anesthesia. For EMG recording of muscle- (M-) wave and

Hoffmann- (H-) reflex the tibial nerve was stimulated by means of single square shocks with 0.2 ms duration until the respective maximal response (M_{max} or H_{max}) was reached. For the recording of flexor reflexes, the tibial nerve was stimulated electrically (5 square shocks at 500 Hz, 0.2 ms duration) at 3.0 times the nerve threshold (T_n) [58]. For assessment of antinociceptive activity, NMRI mice (Møllegaard, Schönweide, Germany), 24–30 g, were placed on a copper plate (15 × 9 cm) at 58 °C. The latency for the mouse to react to the heat (either by licking its paws, lifting its paws, or by jumping) was recorded. The maximum reaction time was limited to 15 s. The body temperature in male NMRI mice, 25–30 g, or Wistar rats (Charles River, Sulzfeld, Germany), 100–120 g, was monitored by means of a rectal thermistor probe [66]. For the monitoring of arterial blood pressure and blood gases Fischer 344 rats (Charles River), 230–250 g, had catheters placed in the femoral and tail artery [4].

2.5. Focal ischemia in rodents

The permanent middle cerebral artery occlusion (MCAO) was established by means of microbipolar permanent coagulation in NMRI mice (Schering AG), 35–40 g, anaesthetized with tribromoethanol (Aldrich, Gillingham, UK), 600 mg/kg i.p., or in Fischer 344 rats (Charles River), 250–300 g, anesthetized with halothane [34,63]. ZK202000 was injected into mice i.p. at −1, 0, 1 and 2 h after MCAO or was infused i.v. at a dose of 3 mg/kg/h over 6 h beginning immediately after MCAO. ZK200775 was infused i.v. at a dose of 3 mg/kg/h for 6 h starting immediately after occlusion, while NBQX was administered to mice at a dose of 30 mg/kg i.p. 60, 70, and 85 min after MCAO. ZK200775 was also infused at doses of 0.1, 0.25, 0.75 and 1.5 mg/kg/h for 24 h in Fischer 344 rats, 250–390 g, subjected to MCAO by permanent electrocoagulation. ZK202000 was continuously infused i.v. in such rats for 24 h at doses of 0.1, 0.25 and 0.75 mg/kg/h. One day after MCAO the size of infarct in the brain was estimated stereologically by means of image analysis.

2.6. Head trauma

Fischer 344 rats (Charles River), 300–340 g, anesthetized with tribromoethanol, 260 mg/kg, i.p. were subjected to cortex contusion by means of a falling weight (20 g) with a force of 380 g·cm [4]. ZK202000 was continuously infused i.v. at doses of 0.025, 0.1, and 0.25 mg/kg/h over 24 h beginning 1 h after trauma. Three days after injury, neuronal loss in the hippocampus was assessed using an unbiased stereological disector technique to estimate the mean numerical density (N_v) in the CA3 subfields [4]. The differences in N_v of pyramidal neurons in the CA3 subfield between the damaged and the non-damaged side were analysed statistically by means of analysis of variance.

2.7. Dependence on phosphonate quinoxalinediones

To provide a continuous administration of ZK200775, 4 mg/kg/h over 14 days, NMRI mice (Schering AG), 20–24 g, were implanted i.p. with Alzet minipumps (Alza, Palo Alto, CA, USA). After 14 days, treatment with ZK200775 was abruptly terminated and withdrawal anxiety monitored using the computerized Digiscan-16 tracking system (AccuScan). Withdrawal seizures were monitored by electroencephalogram (EEG), and changes in muscle tone registered in EMG [58]. For long-term EEG monitoring, mice were stereotaxically implanted with bipolar twisted electrodes positioned in the dorsal hippocampus (AP 2.5; L 2.0; V 3.5) [38] under sodium pentobarbital (Ceva, Neuilly-sur-Seine, France), 50 mg/kg i.p., anaesthesia. Surface recordings were led from screws positioned bilaterally over the occipital cortex [46]. Video- and EEG monitoring started on the day following administration of ZK200775 and was continued for up to 21 days. Seizure recognition was performed on-line using a computerized detection program (Monitor 5.0; Stellate, Montreal, Canada).

2.8. Vacuoles and degeneration of neurons in the retrosplenial/cingulate cortex

NMDA antagonists may induce degeneration of sensitive neurons in the rat cingulate and retrosplenial cortex [41]. To determine whether quinoxalinediones induced neurodegeneration, neuronal density in the retrosplenial/cingulate cortex in Fischer 344 rats, 220–340 g, subjected to i.v. infusion of ZK202000, 0.25 mg/kg/h over 24 h, was assessed using an unbiased stereological disector technique [4].

2.9. Pharmacokinetics

Mice or rats were treated with ZK200775 i.v., either as a bolus injection or as a continuous infusion. In plasma, unchanged ZK200775 was analysed by means of normal-phase HPLC with UV-detection. In urine and faeces, ^{14}C-ZK200775 was determined by liquid scintillation counting. The AUC (area under the plasma concentration-time curve) was calculated by the trapezoidal rule using the equation $AUC = AUC_{0-t(n)} + C_{(n)}/\lambda_z$ where $C_{(n)}$ is the last value above the limit of quantitation, t(n) the corresponding time and λ_z the terminal elimination rate constant. The terminal elimination half-life was determined as $t_{1/2} = \ln 2/\lambda_z$. Total clearance was calculated as $CL = Dose/AUC$ or as $CL = R_0/C_{ss}$ where R_0 is the rate of infusion and C_{ss} the plasma concentration at steady-state. The volume of distribution was calculated as $V_z = CL/\lambda_z$.

3. Results

3.1. Structure activity relationships and synthesis of phosphonate quinoxalinediones

Unsubstituted quinoxaline-2,3-dione displaced AMPA from its binding sites in cortical membranes with an IC_{50} of 204 μM (Fig. 1a). The introduction of a CF_3 group in position 6 (Fig. 1b) increased potency at the AMPA binding site 16-fold giving an IC_{50} value of 13 μM. The methyl substitution of 6-trifluoromethyl-1,2,3,4-tetrahydroquinoxaline-2,3-dione at position 1 (Fig. 1c) enhanced the affinity at the AMPA binding site yielding an IC_{50} value of 9 μM and demonstrating

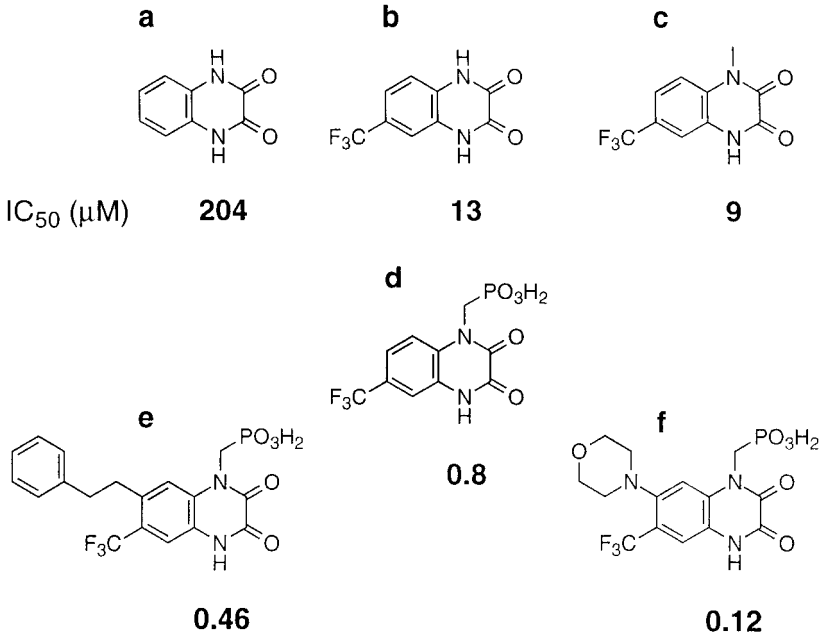

Fig. 1. Substitution patterns of quinoxalinediones leading to increase in water solubility and the preservation of *in vitro* binding affinity to the AMPA receptor. *Lower panel* shows *in vitro* binding affinity to AMPA receptors in rat cortical membranes.

a steric tolerance at this position. Surprisingly, further prolongation of the chain length at position 1 by replacing the methyl group with a methylphosphonate group (Fig. 1d), led to a 16-fold increase of AMPA receptor affinity compared to the predecessor compound (Fig. 1d) and a 250-fold increase in comparison with the unsubstituted parent quinoxaline-2,3-dione (Fig. 1a). Incorporation of electron-donating moieties, such as phenethyl or morpholino groups (Fig. 1e,f), in position 7 of the quinoxalinedione framework further increased AMPA receptor affinity 2 to 7-fold, in comparison with the predecessor compound, resulting in an IC_{50} of 0.46 μM for [[1,2,3,4-tetrahydro-7-(phenylethyl)-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methyl]phosphonate (ZK 202000) (Fig. 1e) and 0.12 μM for [[1,2,3,4-tetrahydro-7-(4-morpholinyl)-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methyl]phosphonate (ZK 200775) (Fig. 1f). In addition to a potency increase, the incorporation of a polar phosphonate group into the quinoxalinedione scaffold resulted in increased solubility in aqueous solutions reaching > 1 mg/ml at pH 7.35 for ZK202000 and > 25 mg/ml for ZK 200775.

ZK202000 and ZK200775 were synthesized by means of a stepwise regioselective nucleophilic displacement of the halogen atoms in 4,6-dichloronitro-3-(trifluoromethyl)-benzene (Fig. 2 and 3). Firstly, the chloride atom in the ortho position to the nitro group was displaced by aminomethanephosphonic acid leading to [[[5-chloro-2-nitro-4-(trifluoromethyl)phenyl]amino]methyl]phosphonic acid (Fig. 2 and 3). Secondly, in order to achieve ZK202000, esterification of the acid with triethyl orthoformate under acid catalysis yielded diethyl [[[5-chloro-2-nitro-4-(trifluoromethyl)phenyl]amino]methyl] phosphonate (Fig. 2). Nucleophilic displacement of the second chloride atom by a styryl moiety achieved through palladium coupling yielded (E)-diethyl [[[2-nitro-4-(trifluoromethyl)-5-(phenylethenyl) phenyl]amino]methyl]phosphonate (Fig. 2). Simultaneous reduction of the nitro group and the styryl double bond was accomplished by hydrogenation in ethanol using Raney-nickel as catalyst leading to diethyl [[[2-amino-4-(trifluoromethyl)-5-(phenylethenyl)phenyl]amino]methyl] phosphonate. In case of ZK200775, nucleophilic displacement of the second chloride atom by morpholine yielded [[[5-(4-morpholinyl)-2-nitro-4-(trifluoromethyl) phenyl]amino]methyl] phosphonic acid, which was subsequently converted to the corresponding diethyl phosphonate with triethyl orthoformate under acid catalysis (Fig. 3). Reduction of the nitro group was accomplished by heterogenous hydrogenation in ethanol using palladium on charcoal as a catalyst (Fig. 3). For completion of both syntheses, in a two step sequence the desired quinoxalinedione framework was built up by condensing the phenylenediamine with ethyl oxalyl chloride and subsequent ring closure by heating the crude product in ethanolic hydrochloric acid (Fig. 2 and 3). Ester cleavage with concentrated aqueous hydrochloric acid under heating resulted in [[1,2,3,4-tetrahydro-7-(phenylethyl)-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methyl]phosphonic acid (ZK 202000) (Fig. 2) or [[1,2,3,4-tetrahydro-7-(4-morpholinyl)-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl] methyl]phosphonic acid (ZK 200775) (Fig. 3). The overall yield of the 6 step synthesis was above 27 % for ZK202000 and above 40 % for ZK200775.

3.2. Receptor binding profiles of quinoxalinediones

ZK202000 had high affinity to ^{3}H-AMPA (IC_{50} 0.46 μM) and ^{3}H-CNQX (0.025 μM) binding sites. It was 7-fold less potent at ^{3}H-kainate (3.25 μM) and ^{3}H-CPP (3.37 μM) binding sites, and had no affinity at ^{3}H-TCP or ^{3}H-dichlorokynurenate sites (Table I). The receptor binding profile of ZK200775 showed high affinity to ^{3}H-AMPA

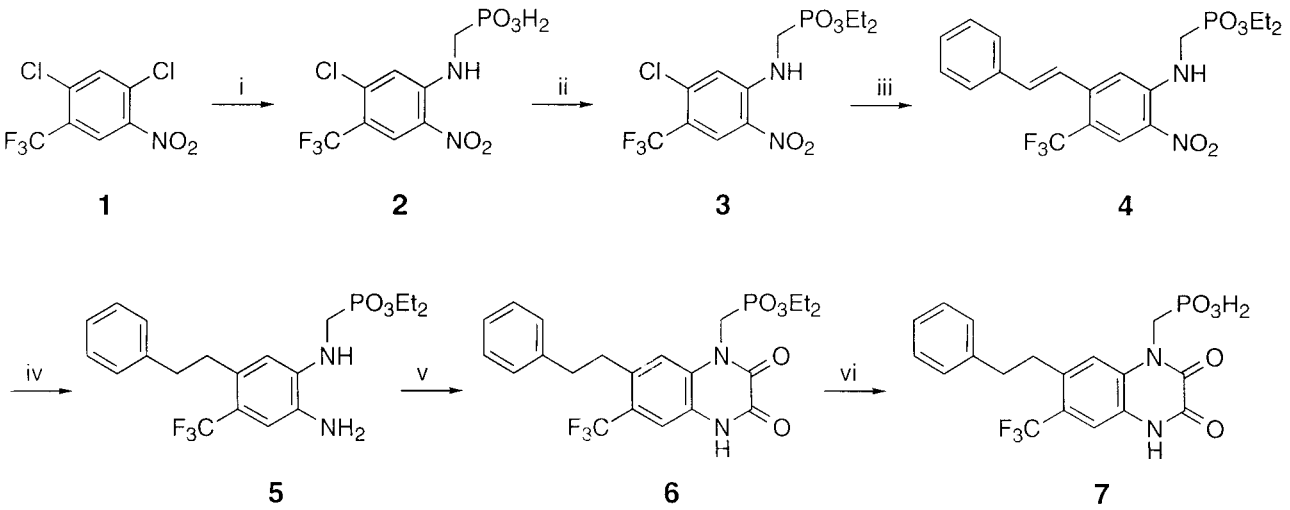

Fig. 2. Synthesis of ZK202000. ***Reaction conditions*** – **i:** aminomethanephosphonic acid, Na_2CO_3, H_2O, 120 °C; **ii:** $HC(OEt)_3$, pTs, 1.5 h, 70 °C; **iii:** $Pd(PPh_3)_4$, styrylboronic acid, toluene, Na_2CO_3, H_2O, EtOH; **iv:** EtOH, Ra-Ni, H_2, rt, 6h; **v:** step 1: tetrahydrofurane, triethylamine, ethyl oxalyl chloride, rt; step 2: ethanol, 1N HCl, 100 °C; **vi:** concentrated HCl, 120 °C, 8.5 h. (**1**) 4,6-dichloronitro-3-(trifluoromethyl)benzene; (**2**) [[[5-chloro-2-nitro-4-(trifluoromethyl)phenyl]amino]methyl]phosphonic acid; (**3**) diethyl [[[5-chloro-2-nitro-4-(trifluoromethyl)phenyl]amino]methyl]phosphonate; (**4**) (E)-diethyl [[[2-nitro-4-(trifluoromethyl)-5-(phenylethenyl)phenyl]amino]methyl]phosphonate; (**5**) diethyl [[[2-amino-4-(trifluoromethyl)-5-(phenylethyl)phenyl]amino]methyl]phosphonate; (**6**) diethyl [[1,2,3,4-tetrahydro-7-(phenylethyl)-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methyl]phosphonate; (**7**) [[1,2,3,4-tetrahydro-7-(phenylethyl)-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methyl]phosphonic acid (ZK 202000).

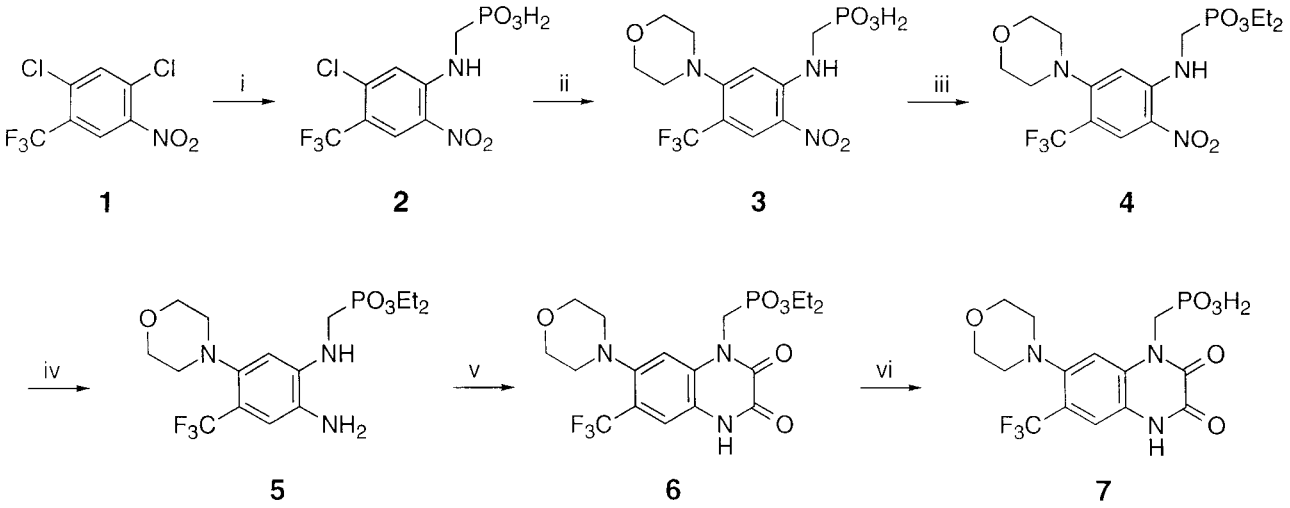

Fig. 3. Synthesis of ZK200775. ***Reaction conditions*** – **i:** aminomethanephosphonic acid, Na_2CO_3, H_2O, 120 °C; **ii:** morpholine, 120 °C; **iii:** $HC(OEt)_3$, pTs, 4h, 150 °C; **iv:** EtOH, Pd/C 10 %, H_2, rt, 3h; **v:** step 1: tetrahydrofurane, triethylamine, ethyl oxalyl chloride, rt; step 2: ethanol, 1N HCl, 100 °C; **vi:** concentrated HCl, 120 °C, 2h. (**1**) 4,6-dichloronitro-3-(trifluoromethyl)benzene; (**2**) [[[5-chloro-2-nitro-4-(trifluoromethyl)phenyl]amino]methyl]phosphonic acid; (**3**) [[[5-(4-morpholinyl)-2-nitro-4-(trifluoromethyl)phenyl]amino]methyl]phosphonic acid; (**4**) diethyl [[[5-(4-morpholinyl)-2-nitro-4-(trifluoromethyl)phenyl]amino]methyl]phosphonate; (**5**) diethyl [[[2-amino-5-(4-morpholinyl)-4-(trifluoromethyl)phenyl]amino]methyl]phosphonate; (**6**) diethyl [[1,2,3,4-tetrahydro-7-(4-morpholinyl)-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methyl]phosphonate; (**7**) [[1,2,3,4-tetrahydro-7-(4-morpholinyl)-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methyl]phosphonic acid (ZK 200775).

(0.12 μM) and ^{3}H-CNQX (0.032 μM) binding sites (Table I). It was 21-fold less potent at ^{3}H-kainate (2.5 μM) and had weak affinity to the binding sites within the NMDA receptor complex such as ^{3}H-CPP (2.80 μM), ^{3}H-TCP (> 10 μM), and ^{3}H-dichlorokynurenate (2.8 μM) sites (Table I). No affinity to non-glutamate receptor binding sites was detected up to concentrations of 20–100 μM in rat cortical membrane preparations for ZK202000 and ZK200775. The receptor binding profile of NBQX was similar to that of phosphonate quinoxalinediones with high affinity to ^{3}H-AMPA (0.15 μM) and ^{3}H-CNQX (0.016 μM). NBQX was 32-fold less potent at ^{3}H-kainate (4.8 μM) site and showed no affinity to binding sites within NMDA receptor complex (Table I).

3.3. Spreading depression

Functional *in vitro* assays using antagonism of AMPA/quisqualate-, kainate-, NMDA-, and glycine-induced

TABLE 1: *In vitro* binding affinity of ZK202000, ZK200775 and NBQX to glutamate receptors in rat cortical membranes

Glutamate receptor ligands IC_{50} *(μM)*	*^{3}H-AMPA*	*^{3}H-CNQX*	*^{3}H-KA*	*^{3}H-CPP*	*^{3}H-TCP*	*^{3}H-DCKA*
ZK202000	0.46 ± 0.040	0.025 ± 0.005	3.25 ± 0.69	3.37 ± 0.07	> 10	> 10
ZK200775	0.12 ± 0.090	0.032 ± 0.008	2.50 ± 0.20	2.80 ± 0.35	> 10	2.80 ± 0.5
NBQX	0.15 ± 0.014	0.016 ± 0.003	4.80 ± 0.47	> 10	> 10	> 10

Rat (Wistar; Schering AG, Berlin, Germany) frontoparietal cortex was homogenized in a buffer containing 30 mM Tris.HCl, 2.5 mM $CaCl_2$ (pH 7.1). The homogenate was centrifuged at 40,000 g for 15 min, the pellet washed (3×) and suspended in buffer. Incubations were performed in triplicates at 0 °C for 30 min and bound radioactivity separated by filtration through Whatman GF/C glass fiber filters. Non-specific binding was defined by the addition of 680 μM L-glutamate, except for TCP binding, where 3.5 μM phencyclidine was employed, and DCKA binding, where 1 mM glycine was employed. ZK202000, ZK200775 and NBQX were dissolved in ethanol and added in at least 4 concentrations. The IC_{50} values were calculated by non-linear regression analysis. Shown are means ± SEM of 3 determinations. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; KA, kainate; CPP, 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonate; TCP, 1-[1-(2-thienyl)cyclohexyl]-piperidine; DCKA, dichlorokynurenate.

TABLE 2: Effect of ZK202000, ZK200775 and NBQX on spreading depression in chicken retina

	Quisqualate/ AMPA	*Kainate*	*NMDA*	*Glycine*
ZK202000 IC_{50} (μM)	0.1 (0.08–0.12)	0.02 (0.018–0.022)	0.39 (0.30–0.51)	–
ZK200775 IC_{50} (μM)	0.2 (0.05–0.90)	0.076 (0.015–0.379)	13 (7–23)	18 (6.68–48.25)
NBQX IC_{50} (μM)	0.16 (0.14–0.18)	3.6 (2.9–5.1)	> 30	> 10

The posterior chamber of each chicken (3–6 day-old; Copenhagen Serum Institute, Copenhagen, Denmark and Laptec, Gödöllö, Hungary) eye was placed in O_2-saturated saline containing quisqualate (5 μM), AMPA (5 μM), kainate (5 μM), NMDA (100 μM), or glycine (100 μM) alone or with different concentrations of ZK202000 ranging from 0.0125 to 10 μM, ZK200775 ranging from 0.025 to 75 μM, and NBQX ranging from 0.01 to 30 μM. The occurrence of a white area (½ mm in diameter) was taken as the onset of spreading depression and the latency to it was measured. Cut-off time was set at 60 s. An increase in the latency by 30 s was considered to represent maximal inhibition of spreading depression. The drug effects were expressed as the percentage of maximum inhibition at a given concentration. IC_{50} values were calculated by means of linear regression analysis.

spreading depression in chicken retina revealed that ZK202000 and ZK200775 were highly potent AMPA/kainate antagonists (Table II). ZK202000 was 4-fold, whereas ZK200775 65-fold less potent against NMDA-induced spreading depression (Table II). NBQX showed similar selectivity and potency profile to ZK200775 in the spreading depression assay and gave IC_{50} values of 0.16, 3.6, > 10 and > 10 μM against quisqualate, kainate, NMDA and glycine (Table II).

3.4. Electrophysiology

Electrophysiological studies using whole cell patch clamp performed on primary cultures of rat hippocampal neurons revealed a high potency for phosphonate quinoxalinediones as competitive AMPA/kainate antagonists (Fig. 4 and 5). Currents evoked by kainate (non-desensitizing AMPA agonist at 100 μM) were inhibited by ZK202000 with an IC_{50} of 40 nM, by ZK200775 with an IC_{50} of 27 nM, and by NBQX with an IC_{50} of 26 nM (Fig. 5). Currents evoked by NMDA (100 μ*M*) in Mg^{2+}-free solutions were inhibited by ZK202000 with an IC_{50} of 3 μM and by ZK200775 with an IC_{50} of 40 μM, which are 75-fold or 1500-fold higher than those required to block kainate-induced currents. Currents evoked by NMDA were only slightly affected by NBQX in a concentration of 100 μM (up to 10 % inhibition).

3.5. Pharmacology of phosphonate quinoxalinediones

In a four-plate test, which measures anxiolytic activity, ZK200775 in doses of 0.3–3 mg/kg i.p. significantly enhanced punished locomotor activity in mice, leaving spontaneous locomotor activity in unpunished mice unchanged (Table III). ZK202000, 0.3–3 mg/kg i.p., did not affect punished locomotor activity in mice and did not suppress spontaneous locomotor activity in unpunished mice (Table III). NBQX was inactive in the four-plate test in mice up to the dose of 3 mg/kg i.p. (Table III), slightly enhanced punished locomotor activity in a dose of 10 mg/kg, and significantly supressed both punished and unpunished locomotor activity in a dose of 30 mg/kg i.p. In the elevated plus-maze test in mice, ZK200775 increased the number of entries into open arms (8.43 ± 1.39; n = 7 vs. 5.50 ± 0.48; n = 10) and significantly prolonged time spent by mice in open arms (97 ± 10.66 s vs. 54.50 ± 4.16 s; $P < 0.05$, Kruskal-Wallis test) 30 min after i.p. administration, confirming its anxiolytic activity. ZK202000 elevated the threshold for AMPA- and kainate-induced clonic seizures in mice with a $THRD_{50}$ of 5.61 (3.76–8.34) and 5.65 (1.69–8.77) mg/kg i.v., respectively, whereas the threshold for NMDA induced seizures was elevated at 33.88 (19.03–60.28) mg/kg. ZK200775 showed a similar profile of anticonvulsant activity but was 2–4 fold more potent than ZK202000 (Table IV). NBQX was 3–6 fold less potent than phosphonate quinoxalinediones (Table IV). ZK200775 and NBQX disturbed motor coordination in the rotating rod test with the respective ED_{50} values of 14.6 (12.1–17.6) and 6.63 (5.34–8.24) mg/kg i.v. (Table IV). Motor coordination in the rotating rod test was not affected by ZK202000 up to the dose of 40 mg/kg (Table IV). ZK200775 was more potent against pentylenetetrazol and electroshock seizures in mice than NBQX and ZK202000 (Table V). ZK200775 also displayed anticonvulsant activity in a variety

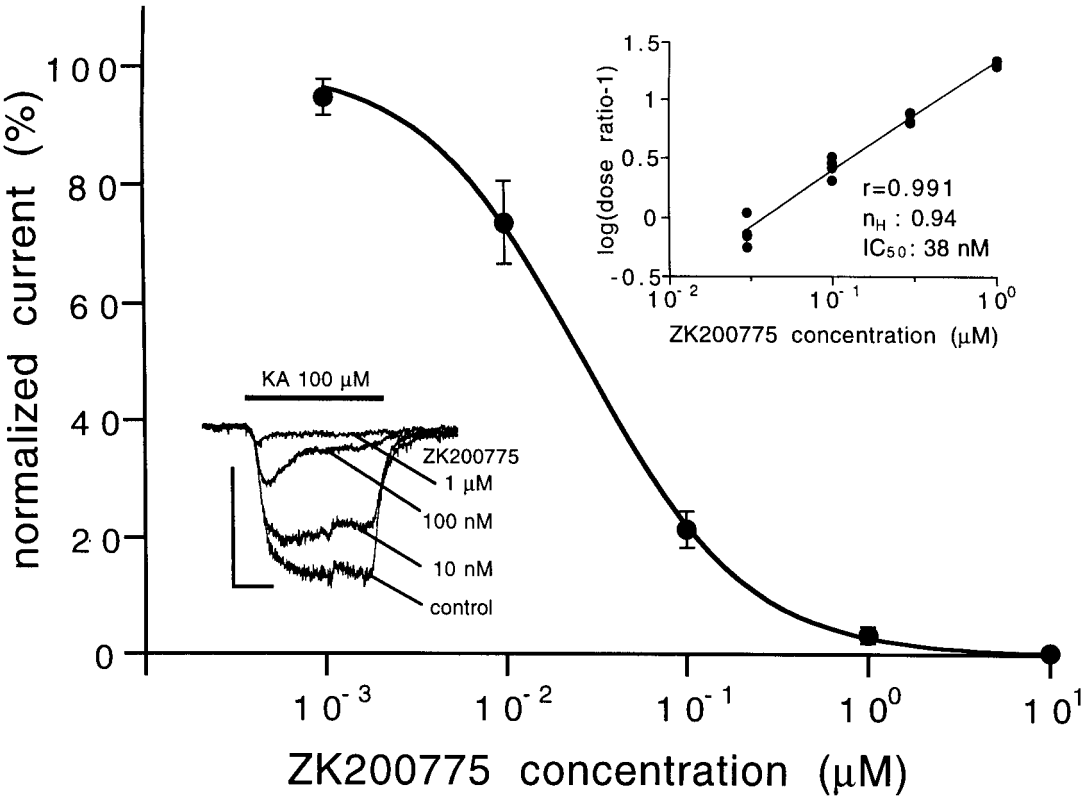

Fig. 4. Concentration-response effect of ZK200775 on kainate-induced currents in neonatal rat hippocampal neurons. Current amplitudes were leak-subtracted, normalized to control, and plotted against concentration of ZK200775. Data represent means ± SEM from 5–13 determinations. *Lower inset* shows original current traces demonstrating effect of ZK200775, 10 nM, 100 nM and 1 μM, on kainate-induced (100 μM) currents in a single cell. Vertical scale bar represents 10 pA, while horizontal scale bar represents 10 s. *Upper inset* shows Schild analysis of the antagonism by ZK200775 derived from concentration-response curves for kainate in the presence of increasing concentrations of ZK200775 (0.03, 0.1, 0.3 and 1 μM). Current amplitudes were leak-subtracted and related to the maximal current obtained at 10 mM kainate in the absence of the antagonist.

TABLE 3: Anxiolytic activity of phosphonate quinoxalinediones and NBQX in the four-plate test in mice

Four-plate test, mice	*Number of crossings (means ± SEM) (n)*							
Treatment	*Vehicle*		*0.3 mg/kg*		*1 mg/kg*		*3 mg/kg*	
	unpunished	*punished*	*unpunished*	*punished*	*unpunished*	*punished*	*unpunished*	*punished*
ZK202000 30 min	14.1 ± 0.7 (8)	6.6 ± 0.8 (8)	15.1 ± 0.7 (8)	7.0 ± 0.9 (8)	15.1 ± 0.9 (8)	7.5 ± 0.9 (8)	15.5 ± 1.0 (8)	7.5 ± 0.6 (8)
ZK200775 30 min	19.5 ± 1.3 (8)	7.4 ± 0.5 (8)	20.8 ± 1.9 (8)	9.6 ± 0.6* (8)	22.0 ± 1.8 (8)	11.1 ± 1.4* (7)	21.0 ± 1.6 (8)	12.9 ± 1.5* (8)

For assessment of anxiolytic activity, NMRI mice (Schering AG) were placed in the center of a chamber with the floor divided into four plates and, after 20 s of exploration, received a shock (1 mA, 60 ms) each time crossing from one plate to another. The number of crossings within 1 min under unpunished or punished conditions, 30 min after i.p. administration of ZK202000 or ZK200775, was taken as a measure of exploratory or anxiolytic activity. Analysis of variance revealed that ZK200775 [$F(3,30) = 4.8$, $P = 0.0083$] increased punished locomotor activity in mice, while ZK202000 was ineffective up to a dose of 3 mg/kg. n, number of mice. *$P < 0.05$ vs. crossings in vehicle-treated punished mice.

of chemical seizure models in mice (Table VI). It was preferentially active against tonic seizures induced by 3-mercaptopropionate, picrotoxin, bicuculline and strychnine and less so against clonic seizures (Table VI). ZK200775 reduced monosynaptic reflexes in urethane/chloralose anaesthetized mice at doses of 0.1 and 1 mg/kg i.v. between 5 and 30 min after administration, while polysynaptic reflexes remained unaffected (Table VII). ZK202000 at a dose of 1 mg/kg i.v. only briefly decreased monosynaptic reflexes in mice and had no effect on polysynaptic reflexes (Table VII). NBQX reduced both monosynaptic and polysynaptic reflexes in mice at doses of 1–3 mg/kg i.v. [65]. ZK202000 and ZK200775 induced analgesia in a hot-plate test in mice (Table VIII A,B). The potency for the analgesic effect of ZK200775 in the hot-plate test in mice was comparable to that of morphine sulphate (Table VIII D). NBQX did not induce analgesia in mice up to

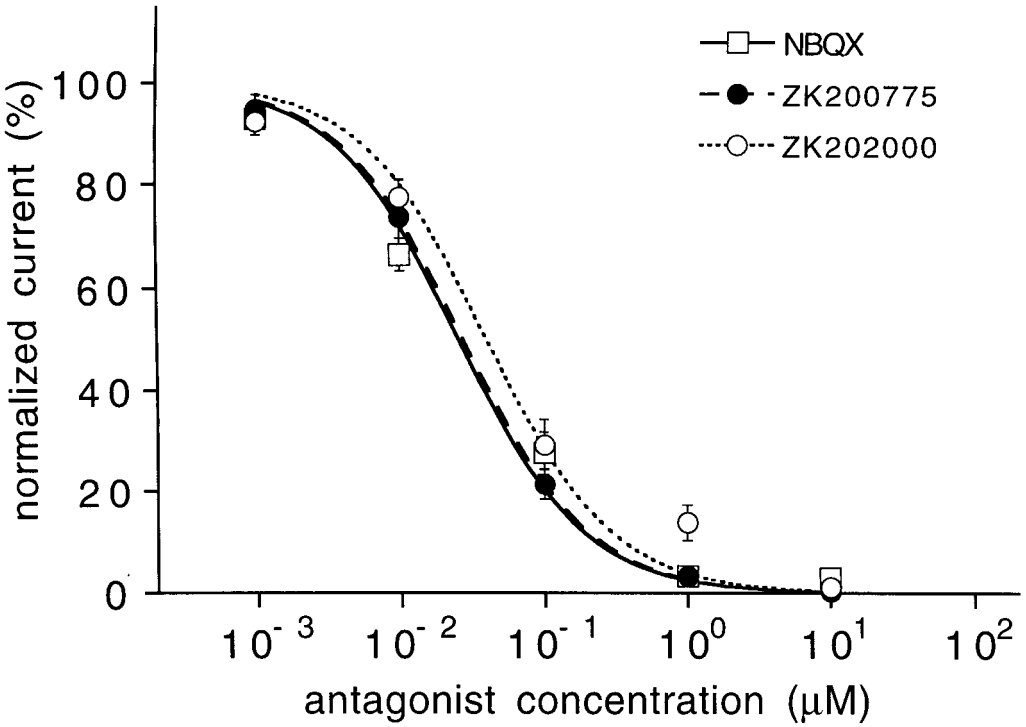

Fig. 5. Concentration response effects of ZK202000, ZK200775 and NBQX on kainate-induced currents in neonatal rat hippocampal neurons. Kainate was applied at a concentration of 100 µM. Current amplitudes were leak-subtracted, normalized to control, and plotted against antagonist concentration. Data represent means ± SEM from 5–13 determinations. IC_{50} values were 40, 27 and 25 nM for ZK202000, ZK200775 and NBQX, respectively.

TABLE 4. Effect of phosphonate quinoxalinediones and NBQX on threshold for clonic seizures induced by glutamate agonists in mice

	AMPA $THRD_{50}$ *(mg/kg)*	*Kainate* $THRD_{50}$ *(mg/kg)*	*NMDA* $THRD_{50}$ *(mg/kg)*	*RR* ED_{50} *(mg/kg)*
ZK202000	5.61 (3.76–8.34)	5.65 (1.69–8.77)	33.88 (19.03–60.28)	> 40
ZK200775	2.9 (1.7–4.6)	1.6 (1.3–2.0)	24.1 (21.9–26.5)	14.6 (12.1–17.6)
NBQX	16.23 (15.81–16.66)	7.59 (6.51–8.85)	60.41 (53.51–68.19)	6.63 (5.34–8.24)

For assessment of the threshold for clonic seizures, AMPA, kainate or NMDA, 1 nmol/5 µl, was infused continuously intracerebroventricularly into NMRI mice (Schering AG) at a rate of 5 µl/min. ZK202000, ZK200775 and NBQX were administered i.v. 5 min before the seizure test. The time in s to a clonic seizure was used as an endpoint determining susceptibility to convulsions. The $THRD_{50}$ (threshold dose) was calculated in mg/kg by means of regression analysis. For assessment of motor coordination, mice were placed on a rotating rod (RR; diameter, 3 cm; 6 revolutions/min; height, 15 cm) for 180 s or until they fell off the rotating rod 3 times during the maximum of 3 trials. The ED_{50} was calculated in mg/kg by means of regression analysis. Experimental groups consisted of 5–8 mice.

a dose of 20 mg/kg i.v. (Table VIII C). Exploratory activity in non-habituated mice was reduced by ZK202000 with an ED_{50} of 15.57 (11.75–20.64) mg/kg and by ZK200775 with an ED_{50} of 14.2 (7.8–25.9) mg/kg i.v. ZK202000 at doses of 1–30 mg/kg i.v. induced short-lasting (up to 10 min) hyperthermia (up to +0.5–1 °C) in rats. ZK200775 at a dose of 30 mg/kg i.v. induced initial short-lasting (up to 5 min) hyperthermia (up to +0.5 °C) and a late time-dependent (30–180 min) hypothermia in rats (up to −2.8 °C). ZK200775, 3 and 10 mg/kg, had no effect on body temperature of rats. In mice, only hypothermia was recorded after i.v. administration of ZK202000 at a dose of 30 mg/kg (up to −1.7 °C) and ZK200775 at a dose of 10 mg/kg (up to −2.7 °C). ZK202000, 10 mg/kg, and ZK200775, 3 mg/kg, did not induce any changes in body temperature in mice. In conscious rats, i.v. infusion of ZK202000 and ZK200775 at a dose of 3 mg/kg/h for 6 h, a treatment regimen required for neuroprotection in rodent permanent cerebral ischemia, had no effect on blood gases or haemodynamic status, inducing only moderate increases (< 10 %) in arterial blood pressure.

3.6. Middle cerebral artery occlusion in rodents

In mice subjected to focal ischemia, i.v. infusion of ZK202000 at a dose of 3 mg/kg/h for 6 h, starting immediately after permanent MCAO, reduced the infarct volume by 39 % (29.55 ± 2.69 mm^3; n = 10 vs. 18.13 ± 2.58 mm^3; n = 9,

TABLE 5. Effect of quinoxalinediones on pentylenetetrazol- and electroshock-induced convulsions in mice

Seizure models *(dose)*	***ZK202000*** ED_{50} *(mg/kg)*		*n*
	Clonic	*Tonic*	
Pentylenetetrazol (150 mg/kg)	> 75	52.01 (33.13–81.82)	20
MES (50 mA, 50 Hz, 0.2 s)	–	23.31 (17.39–31.19)	35
Seizure models *(dose)*	***ZK200775*** ED_{50} *(mg/kg)*		*n*
	Clonic	*Tonic*	
Pentylenetetrazol (150 mg/kg)	7.76 (3.53–13.40)	20.15 (10.99–28.77)	30
MES (50 mA, 50 Hz, 0.2 s)	–	13.41 (11.89–15.21)	24
Seizure models *(dose)*	***NBQX*** ED_{50} *(mg/kg)*		*n*
	Clonic	*Tonic*	
Pentylenetetrazol (150 mg/kg)	47.97 (40.02–57.53)	30.05 (23.29–38.84)	25
MES (50 mA, 50 Hz, 0.2 s)	–	11.83 (8.63–16.23)	26

For analysis of anticonvulsant action, the effect of ZK202000, ZK200775 and NBQX on the CD_{97} (convulsant dose) of pentylenetetrazol and on maximal electroshock-induced seizures (MES) was estimated in NMRI mice (Schering AG). Pentylenetetrazol was injected s.c. 30 min following s.c. administration of quinoxalinediones. MES was applied through auricular electrodes 5 min after i.v. administration of quinoxalinediones. The data are expressed as ED_{50} values (and their 95 % confidence limits) estimated from probit-log dosage regression curves. n, number of mice.

TABLE 6. Effect of ZK200775 on chemically-induced convulsions in mice

Seizure models *(dose)*	***ZK200775*** ED_{50} *(mg/kg)*		*n*
	Clonic	*Tonic*	
3-Mercaptopropionate (40 mg/kg i.p.)	3.56 (0.74–7.81)	0.42 (0.06–1.53)	25
DMCM (15 mg/kg i.p.)	8.25 (2.31 – 56.77)	–	20
Picrotoxin (10 mg/kg s.c.)	> 30	4.64 (3.19–6.74)	20
Bicuculline (5 mg/kg s.c.)	> 30	12.24 (7.15–18.79)	20
Strychnine (1.5 mg/kg s.c.)	> 30	14.35 (7.53–26.80)	20

For analysis of anticonvulsant action of ZK200775, the effect on the CD_{97} (convulsant dose) of chemoconvulsants was estimated in NMRI mice (Schering AG). The chemoconvulsants bicuculline, picrotoxin, strychnine nitrate, 3-mercaptopropionate, and methyl-4-ethyl-6,7-dimethoxy-9H-pyrido-(3,4-b)-indole-3-carboxylate (DMCM) were injected s.c. or i.p., 30 min following systemic (s.c.) administration of ZK200775. The data are expressed as ED_{50} values (and their 95 % confidence limits) estimated from probit-log dosage regression curves. n, number of mice.

$P < 0.01$), while i.v. infusion of ZK200775 at a dose of 3 mg/kg/h over 6 h, reduced the infarct volume by 25 % (24.73 ± 1.90 mm^3; n = 5 vs. 18.54 ± 0.85 mm^3; n = 6, $P < 0.05$). ZK202000, 4 × 3 mg/kg i.p., reduced the infarct volume in mice by 28 % (Table IX B), while NBQX, 3 × 30 mg/kg i.p., reduced the infarct volume by 21 % (23.23 ± 1.72 mm^3; n = 9 vs. 18.40 ± 1.27 mm^3; n = 12, $P < 0.05$). In rats subjected to permanent MCAO, ZK202000 reduced the infarct volume by 23 % at 0.25 mg/kg/h when infused i.v. over 24 h (Table IX A). Similarly, ZK200775 reduced the infarct volume by 23 % at 1.5 mg/kg/h when infused over 24 h (Table IX A).

3.7. Traumatic brain injury

Rats subjected to percussion trauma of the cortex showed damage in the hippocampal CA3 subfield. Morphometric analysis demonstrated that hippocampal damage was mitigated by treatment with ZK202000. Delayed treatment of rats with ZK202000 at a dose of 0.25 mg/kg/h over 24 h, beginning 1 h after trauma, increased the number of remaining pyramidal neurons in the hippocampal CA3 subfield from $0.039 \pm 0.008 \times 10^6$/mm^3 (n = 7), which represents 25 % of the numerical density of pyramidal cells on contralateral side, to $0.065 \pm 0.008 \times 10^6$/mm^3 (n = 7; $P < 0.05$, Student's t-test) (Table IX C). Similarly, NBQX and ZK200775 protected hippocampal CA3 subfield against delayed damage following the cortex trauma [4,63].

3.8. Dependence

Abrupt termination of long-term treatment with 4 mg/kg/h of ZK200775 over 14 days by means of i.p. implanted minipumps in mice did not result in development of signs of dependence such as withdrawal anxiety, increase in muscle tone, or seizures suggesting lack of abuse potential.

TABLE 7: Effect of phosphonate quinoxalinediones on spinal reflexes in mice

A *Hoffmann reflexes* *(monosynaptic)* *ZK202000*	*Electromyogram* *(% of pre-administration peak-to-peak amplitude; means ± SEM)* *(n)*				
Treatment *i.v.*	*Vehicle*	*0.001 mg/kg*	*0.01 mg/kg*	*0.1 mg/kg*	*1 mg/kg*
ZK202000 5 min	99.73 ± 6.68 (9)	82.43 ± 4.04 (9)	76.73 ± 8.91 (9)	68.44 ± 9.27 (9)	53.38 ± 9.38* (9)
ZK202000 15 min	98.21 ± 4.63 (9)	83.40 ± 5.38 (9)	87.54 ± 9.11 (9)	73.54 ± 9.38 (9)	73.49 ± 9.42 (9)
ZK202000 30 min	100.28 ± 10.31 (9)	95.83 ± 7.70 (9)	101.57 ± 9.39 (9)	85.29 ± 10.71 (9)	83.01 ± 10.01 (9)
B *Hoffmann reflexes* *(monosynaptic)* *ZK200775*	*Electromyogram* *(% of pre-administration peak-to-peak amplitude; means ± SEM)* *(n)*				
Treatment *i.v.*	*Vehicle*	*0.001 mg/kg*	*0.01 mg/kg*	*0.1 mg/kg*	*1 mg/kg*
ZK200775 5 min	99.94 ± 12.38 (8)	107.83 ± 12.80 (7)	87.43 ± 8.50 (9)	75.74 ± 7.50 (8)	63.67 ± 9.19* (8)
ZK200775 15 min	104.57 ± 13.45 (8)	95.14 ± 8.38 (7)	89.30 ± 4.88 (9)	68.53 ± 5.48* (8)	51.40 ± 8.78** (8)
ZK200775 30 min	103.18 ± 17.01 (7)	82.21 ± 7.67 (7)	69.18 ± 3.39 (9)	58.74 ± 7.68* (8)	55.39 ± 6.64* (8)

The spinal reflexes were recorded in NMRI mice (Schering AG) under α-chloralose/urethane anesthesia. For recording of H-reflexes (Hoffmann reflexes) the tibial nerve was stimulated with single square shocks, 0.2 ms duration at 1.2–1.6 times the nerve threshold. Electromyogram (EMG) recordings were made with a pair of skin clip surface electrodes from the plantar foot muscle. The magnitude of EMG was evaluated by measuring the peak-to-peak amplitude. For recording of flexor reflexes, the tibial nerve was stimulated electrically (5 square shocks at 500 Hz, 0.2 ms duration) at 3.0 times the nerve threshold. EMG recordings were made using a pair of wire electrodes inserted percutaneously into the ipsilateral tibial muscle (M. tibialis cranialis). The magnitude of flexor reflexes was evaluated by measuring the area bounded by the averaged response and the baseline. Twenty consecutive EMG responses were averaged digitally before (pre-administration value) and 5, 15 and 30 min after i.v. injection of ZK202000, ZK 200775 or vehicle. The EMG values measured after vehicle or drug application were expressed as a percentage of the pre-administration values. A one-way multivariate analysis of variance was performed to test equality of (dose) group mean profiles at times 5, 15 and 30 min after i.v. administration. Wilks' Lamba (transformed to F-statistics) was used to assess the significance of the differences in mean group profiles. MANOVA revealed that ZK200775 dose dependently suppressed Hoffmann-reflexes in mice, $F(12,85) = 2.59$; $P < 0.01$, while flexor (polysynaptic) reflexes remained unaffected, $F(12,90) = 1.54$, NS. ZK202000 did not affect Hoffmann- [$F(12,95) = 1.60$, NS] or flexor reflexes [$F(12,98) = 1.72$, NS] in mice in a dose-dependent manner. *$P < 0.05$; **$P < 0.01$ vs. vehicle-treated mice.

3.9. Effect of ZK202000 on morphology of retrosplenial/cingulate cortex in rats

Treatment of rats with ZK202000 at a dose of 0.25 mg/kg/h for 24 h did not affect the density of neurons in both the cingulate and retrosplenial cortex after 72 h (Table X).

3.10. Pharmacokinetics in rodents

Pharmacokinetic studies in mice after i.v. bolus administration of ZK200775 at a dose of 10 mg/kg revealed a total clearance (CL) of 0.44 l/h/kg, a volume of distribution (V_z) of 0.5 l/kg and a half-life ($t_{1/2}$) of 0.8 h. Pharmacokinetic studies in rats after continuous infusion of 2 mg/kg/h over 4 weeks showed a steady-state plasma concentration (C_{ss}) of 13.2 µM (5.4 mg/l), a total clearance (CL) of 0.37 l/h/kg, a volume of distribution (V_z) of 0.38 l/kg and a terminal half-life ($t_{1/2}$) of 0.72 h. After a 30 min i.v. infusion of ^{14}C-ZK200775 at a dose of 2 mg/kg/h, 45 % of the dose was excreted in the urine and 50 % in the feces. The *in vitro* plasma protein binding in rats amounted to 85 %.

4. Discussion

These studies clearly demonstrate that phosphonate quinoxalinediones are water-soluble, competitive and preferential AMPA/kainate antagonists. Potency and selectivity of ZK200775 *in vitro* are very similar to those of NBQX, whereas ZK202000 is slightly less selective. ZK200775 displayed anxiolytic, analgesic, anticonvulsant, muscle relaxant and sedative properties *in vivo*. Phosphonate quinoxalinediones and NBQX showed similar profiles of *in vivo* activity, but differed regarding the extent of sedative and muscle relaxant activity, e.g. ZK200775 and NBQX were more sedative and depressed spinal reflexes, while ZK202000 induced

TABLE 8. Effect of quinoxalinediones and morphine on latency to react in the mouse hot-plate test

A *Hot-plate test, ZK202000*			*Latency to react (s; means ± SEM) (n)*			
Treatment	*Vehicle*	*1 mg/kg*	*5 mg/kg*	*10 mg/kg*	*20 mg/kg*	*30 mg/kg*
ZK202000 i.v., 5 min	2.9 ± 0.1 (8)	3.1 ± 0.3 (8)	3.0 ± 0.2 (10)	3.0 ± 0.2 (10)	3.7 ± 0.3* (10)	4.2 ± 0.3*** (10)
ZK202000 s.c., 30 min	3.0 ± 0.2 (10)	3.2 ± 0.3 (10)	3.4 ± 0.2 (10)	4.0 ± 0.2* (10)	4.1 ± 0.2** (10)	3.9 ± 0.3** (10)

B *Hot-plate test, ZK200775*			*Latency to react (s; means ± SEM) (n)*			
Treatment	*Vehicle*	*1 mg/kg*	*3 mg/kg*	*5 mg/kg*	*10 mg/kg*	*20 mg/kg*
ZK200775 i.v., 5 min	2.7 ± 0.1 (10)	3.9 ± 0.2 (10)	4.6 ± 0.4* (10)	4.7 ± 0.2** (10)	6.6 ± 0.8*** (9)	8.5 ± 0.6*** (6)
ZK200775 s.c., 30 min	2.9 ± 0.2 (10)	3.6 ± 0.2 (10)	4.6 ± 1.4 (10)	6.1 ± 0.7*** (10)	9.5 ± 0.9*** (10)	14.4 ± 0.5*** (5)

C *Hot-plate test, NBQX*			*Latency to react (s; means ± SEM) (n)*			
Treatment	*Vehicle*	*1 mg/kg*	*3 mg/kg*	*10 mg/kg*	*20 mg/kg*	*30 mg/kg*
NBQX i.v., 5 min	2.7 ± 0.2 (8)	3.3 ± 0.1 (8)	2.6 ± 0.2 (3)	2.6 ± 0.2 (8)	3.9 ± 0.4 (6)	–
NBQX s.c., 30 min	3.1 ± 0.2 (8)	–	3.7 ± 0.3 (8)	3.5 ± 0.3 (8)	3.6 ± 0.2 (8)	3.6 ± 0.3 (8)

D *Hot-plate test, morphine*			*Latency to react (s; means ± SEM) (n)*			
Treatment	*Vehicle*	*1 mg/kg*	*3 mg/kg*	*5 mg/kg*	*10 mg/kg*	*20 mg/kg*
Morphine i.v., 5 min	2.9 ± 0.1 (8)	3.7 ± 0.2 (8)	7.1 ± 0.4*** (8)	9.5 ± 0.6*** (8)	11.8± 0.9*** (8)	12.6 ± 1.3*** (8)
Morphine s.c., 30 min	3.2 ± 0.2 (8)	3.8 ± 0.2 (8)	5.9 ± 0.5** (8)	6.2 ± 0.8*** (9)	8.3 ± 0.7*** (9)	10.1± 0.9*** (9)

NMRI mice (Møllegaard, Schönweide, Germany), were placed on a copper plate (15 × 9 cm) kept at 58 °C, 5 min after i.v. administration of vehicle or drugs. Each mouse was tested once only. The latency to react to the heat either by licking or lifting paws, or by jumping was recorded. The maximum observation time was limited to 15 s. *P < 0.05; **P < 0.01; ***P < 0.001 vs. latency to react in vehicle-treated mice, Student's t-test.

little sedation and hardly affected muscle tone or motor coordination.

Pharmacological observations in rodent models of ischemia and trauma suggest that AMPA antagonists may be beneficial in the clinic for the treatment of global ischemia (cardiac arrest), ischemic stroke, and trauma. The AMPA antagonists NBQX, YM90K, 1,4,7,8,9,10-hexahydro-9-methyl-6-nitropyrido[3,4-f]quinoxaline-2,3-dione (PNQX), and 6,7-dichloro-2-(1H)-oxoquinoline-3-phosphonate (S17625-2), (3RS,4aRS,6RS,8aRS)-6-(2-(1H-tetrazole-5-yl)ethyl)-decahydroisoquinoline-3-carboxylate (LY293558), and 1-(4-aminophenyl)-4-methyl-7,8-methylene-dioxy-5H-2,3-benzodiazepine (GYKI52466) are highly potent neuroprotectants in rodent models of global forebrain ischemia [8,16,17,30, 32,39,52,69]. The therapeutic time-window for AMPA antagonists in global ischemia models is approximately 24 h [33,53]. Neuroprotection in rodent and feline models of focal ischemia has been reported when therapy with AMPA antagonists was introduced up to 2–4 h after the middle cerebral artery occlusion [9,10,20,55,63]. In focal ischemia, AMPA antagonists were preferentially active against cortical and less so against striatal damage [7,10,19,20,31,49,54, 55,68].

In rodent models for traumatic brain injury, blockade of AMPA-mediated excitation by the antagonist NBQX protects against cortical damage when therapy is introduced before the impact and against delayed damage in the hippocampus when therapy is introduced up to 7 h after the insult [4]. Our current studies demonstrate that the extent of neuroprotective action achieved with phosphonate quinoxalinedi-

TABLE 9. Effect of phosphonate quinoxalinediones on brain injury in rodent models of ischemia and head trauma

A *Middle cerebral artery occlusion (MCAO)*	*Infarct volume (mm^3; means ± SEM) (n)*				
Treatment i.v.	*Vehicle*	*0.1 mg/kg/h*	*0.25 mg/kg/h*	*0.75 mg/kg/h*	*1.5 mg/kg/h*
ZK202000 Rat, 24 h	257.04 ± 10.08 (30)	224.35 ± 26.45 (6)	196.83 ± 12.09*** (18)	224.92 ± 13.95 (13)	–
ZK200775 Rat, 24 h	267.28 ± 13.81 (23)	249.95 ± 12.70 (21)	246.58 ± 17.28 (15)	262.84 ± 25.18 (6)	206.39 ± 21.14** (9)

B *Middle cerebral artery occlusion (MCAO)*	*Infarct volume (mm^3; means ± SEM) (n)*				
Treatment i.v.	*Vehicle*	*0.3 mg/kg/h*	*1 mg/kg/h*	*3 mg/kg/h*	*10 mg/kg/h*
ZK202000 Mouse,-1,0,1,2 h	42.81 ± 3.51 (10)	32.01 ± 2.87 (5)	34.21 ± 3.03 (6)	30.92 ± 3.37* (9)	34.64 ± 2.71 (12)
% protection	-	25.23	21.10	27.80	19.16

C *Rat traumatic brain injury, 3 d*	*Numerical density of pyramidal cells in the hippocampal CA3 subfield (N_v; means × $10^6/mm^3$ ± SEM) (n)*			
Treatment i.v.	*Vehicle*	*0.025 mg/kg/h*	*0.1 mg/kg/h*	*0.25 mg/kg/h*
ZK202000 Rat, 24 h	0.039 ± 0.008 (7)	0.057 ± 0.008 (7)	0.063 ± 0.014 (5)	0.065 ± 0.008* (7)

Body temperature of NMRI mice (Schering AG) or Fischer 344 rats (Charles River, Sulzfeld, Germany) was kept at 37.5 ± 0.5 °C. 24 h after ischemia the brains were stained with triphenyltetrazolium chloride and the infarct size was determined stereologically using image analysis. In rats, ZK202000 and ZK200775 were administered i.v. over 24 h beginning immediately after MCAO. In mice, ZK202000 was administered i.p. −1, 0, 1 and 2 h after MCAO. In trauma experiments, ZK202000 was infused i.v. over 24 h beginning 1 h after the head injury. Three days after traumatic brain injury, neuronal loss in the hippocampal subfield CA3 was assessed using an unbiased stereological disector technique (24). On the side contralateral to traumatic injury numerical density (N_v) of pyramidal cells in the CA3 subfield varied from 0.161 to 0.168 × $10^6/mm^3$. The differences in N_v of pyramidal cells in the CA3 subfield between the damaged and non-damaged side were analysed statistically by means of analysis of variance. *P < 0.05; **P < 0.01; ***P < 0.001 vs. vehicle-treated animals, Student's t-test.

TABLE 10. Numerical density of neurons in cingulate and retrosplenial cortex in rats subjected to i.v. infusion of ZK202000, 0.25 mg/kg/h, or vehicle for 24 h

ZK202000	*Numerical density of cells in the cingulate/retrosplenial cortex (N_v; means × $10^6/mm^3$ ± SEM) (n)*			
Treatment	*Vehicle*	*0.025 mg/kg/h*	*0.1 mg/kg/h*	*0.25 mg/kg/h*
Cingulate cortex	0.190 ± 0.004 (10)	0.183 ± 0.003 (9)	0.192 ± 0.003 (9)	0.190 ± 0.004 (10)
Retrosplenial cortex	0.324 ± 0.006 (10)	0.324 ± 0.006 (8)	0.331 ± 0.010 (9)	0.329 ± 0.008 (8)

To quantitatively assess neuronal loss in the retrosplenial and posterior cingulate cortex 3 days after the beginning of i.v. infusion of ZK202000 or vehicle in Fischer 344 rats (Charles River), an unbiased stereological disector technique [4] was used to estimate the mean numerical density (N_v). An unbiased counting frame (0.05 × 0.05 mm; disector height 0.01 mm) and a high-aperture (100×) objective were used for the sampling. The N_v for each field was determined with 9–12 disectors. Analysis of variance revealed no significant difference between vehicle- and ZK202000-treated rats: $F_{cing}(1,18) = 0.01$, NS; $F_{retro}(1,16) = 0.93$, NS.

ones in ischemia and trauma models is very similar to that of NBQX.

An important drawback of possible therapy with AMPA antagonists may be depression of respiratory and cardiovascular functions [13,45]. Combination of NMDA and AMPA antagonists, which may be required for treatment of several neurological disorders [6,7,34,47] is deleterious to respiration [18,37]. Furthermore, NBQX inhibits spontaneously occuring sympathetic nerve activity and reduces arterial blood pressure [13]. Possible interactions of AMPA antagonists with anesthetic and other sedative drugs potentially affecting respiratory functions should also be carefully considered [14,36]. A remarkable advantage of phosphonate quinoxalinediones over NBQX or YM90K and also NMDA antagonists is the favorable safety profile allowing for effective protection against ischemic and traumatic brain injury with only minimal systemic side effects. No major deleterious side effects on motor behavior, cardiovascular status or the respiratory system were detected in doses up to 3 mg/kg/h i.v. over 6 h and in doses up to 0.75–1.5 mg/kg/h i.v. over 24 h. Body temperature was affected by ZK202000 and ZK200775 only in doses higher than those required for neuroprotection in ischemia and trauma models.

Anxiolytic, analgesic and anticonvulsant effects, but no disturbances of motor coordination, were detected with phosphonate quinoxalinediones in doses necessary for neuroprotection. Furthermore, like other AMPA antagonists, phosphonate quinoxalinediones seem to be more suitable than NMDA antagonists for chronic use because of absence of psychotomimetic and psychostimulant properties [59]. In our studies, long-term use of phosphonate quinoxalinediones (up to 14 days) did not lead to development of dependence on ZK200775, and no withdrawal signs such as anxiety, seizures, or increase in muscle tone were seen in mice after abrupt termination of treatment with ZK200775.

Neuropathological changes (intracytoplasmic vacuoles) in the cingulate/retrosplenial cortex, a feature common among NMDA antagonists [41], are less likely to occur following treatment with AMPA antagonists [5]. Neurodegeneration in the retrosplenial/cingulate cortex was not observed in the rodent brain after treatment with ZK202000 and ZK200775.

In summary, structure-activity relationships for quinoxalinediones demonstrate that introduction of a phosphonate group into the quinoxalinedione scaffold increases the solubility in water and does not lead to loss of selectivity and potency at AMPA receptors. ZK202000 dissolves in water at > 1 mg/ml and ZK200775 at > 25 mg/ml at a pH of 7.35, while NBQX is insoluble. The pharmacokinetic properties of phosphonate quinoxalinediones, exemplified by ZK200775, are favorable and suggest that they can be safely given in a parenteral preparation. Phosphonate quinoxalinediones are neuroprotective in ischemia and trauma models in rodents and, in addition, show various other actions such as strong antinociceptive or anticonvulsant activity, which deserve further exploration. The side-effect profile of phosphonate quinoxalinediones suggests that nephrotoxicity, neurotoxicity, psychoses, respiratory depression, hypotension or hypothermia are not likely to occur during or after therapy with doses required for neuroprotection.

Acknowledgements

The unfailing technical assistance of K. Behn, P. Böttcher, K. Bressler, F. Fiske, M. Gieseler, H. Lam, R. Neumeister, W. Niemann, P. Pietzuch, E. Richter, J. Seidler, A. Seltz, O. Schmücker, C. Schneider, K. Zessin is gratefully acknowledged. The authors also thank Drs K. Höhlmann, B. Voet and R. Vonk for expert statistical advice.

References

[1] Baker, A.J., Moulton, R.J., MacMillan, V.H. and Shedden, P.M., Excitatory amino acids in cerebrospinal fluid following traumatic brain injury in humans, *J. Neurosurg.*, **79** (1993) 369–372.

[2] Barinaga, M., Finding new drugs to treat stroke, Science, **272** (1996) 664–666.

[3] Beal, M.F., Mechanisms of excitotoxicity in neurologic disease, *FASEB J.*, **6** (1992) 3338–3344.

[4] Bernert, H. and Turski, L., Traumatic brain damage prevented by the non-N-methyl-D-aspartate antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline, *Proc. Natl. Acad. Sci. USA*, **93** (1996) 5235–5240.

[5] Browne, S.E. and McCulloch, J., AMPA receptor antagonists and local cerebral glucose utilization in the rat, *Brain Res.*, **641** (1994) 10–20.

[6] Buchan, A., Do NMDA antagonists protect against cerebral ischemia: are clinical trials warranted? Cerebrovasc. *Brain Metab. Rev.*, **2** (1990) 1–16.

[7] Buchan, A.M., Lesiuk, H., Barnes, K.A., Li, H., Huang, Z.-G., Smith, K.E. and Xue, D., AMPA antagonists: do they hold more promise for clinical stroke trials than NMDA antagonists? *Stroke*, **24** (1993) I148–I152.

[8] Buchan, A.M., Li, H., Cho, S., and Pulsinelli, W.A., Blockade of the AMPA receptor prevents CA1 hippocampal injury following severe but transient forebrain ischemia in adult rats, *Neurosci. Lett.*, **132** (1991) 255–258.

[9] Buchan, A.M., Xue, D., Huang, Z.G., Smith, K.H. and Lesiuk, H., Delayed AMPA receptor blockade reduces cerebral infarction induced by focal ischemia, *Neuroreport*, **2** (1991) 473–476.

[10] Bullock, R., Graham, D.I., Swanson, S. and McCulloch, J., Neuroprotective effect of the AMPA receptor antagonist LY-293558 in focal cerebral ischemia in the cat, *J. Cereb. Blood Flow Metab.*, **14** (1994) 466–471.

[11] Bullock, R., Zauner, A., Woodward, J. and Young, H.F., Massive persistent release of excitatory amino acids following human occlusive stroke, *Stroke*, **26** (1995) 2187–2189.

[12] Canton, T., Doble, A., Miquet, J.M., Jimonet, P. and Blanchard, J.C., A rapid filtration assay for the glycine binding site on the NMDA receptor in rat cortical membranes using [^{3}H]-dichlorokynurenic acid, *J. Pharm. Pharmacol.*, **44** (1992) 812–816.

[13] Chen, K., Hernandez, Y.M., Dretchen, K.L. and Gillis, R.A., Intravenous NBQX inhibits spontaneously occurring sympathetic nerve activity and reduces blood pressure in cats, *Eur. J. Pharmacol.*, **252** (1994) 155–160.

[14] Dall, V., Orntoft, U., Schmidt, A. and Nordholm, L., Interaction of the competitive AMPA receptor antagonist NBQX with hexobarbital, *Pharmacol. Biochem. Behav.*, **46** (1993) 7376.

[15] Dávalos, A., Castillo, J., Serena, J. and Noya, M., Duration of glutamate release after acute ischemic stroke, *Stroke*, **28** (1997) 708–710.

[16] Desos, P., Lepagnol, J.M., Morain, P., Lestage, P. and Cordi, A.A., Structure-activity relationship in a series of 2(1H)-quinolones bearing different acidic function in the 3-position: 6,7-dichloro-(1H)-oxoquinoline-3-phosphonic acid, a new potent and selective AMPA/kainate antagonist with neuroprotective properties, *J. Med. Chem.,* **39** (1996) 197–206.

[17] Diemer, N.H., Jorgensen, M.B., Johansen, F.F., Sheardown, M. and Honoré, T., Protection against ischemic hippocampal CA1 damage in the rat with a new non-NMDA antagonist, NBQX, *Acta Neurol. Scand.*, **86** (1993) 45–49.

[18] Foutz, A.S., Pierrefiche, O. and Denavit-Saubrie, M., Combined blockade of NMDA and non-NMDA receptors produces respiratory arrest in the adultcat, *Neuroreport*, **5** (1994) 481–484.

[19] Gill, R. and Lodge, D., The neuroprotective effects of the decahydroisoquinoline, LY215490, a novel AMPA antagonist in focal ischaemia, *Neuropharmacology*, **33** (1994) 1529–1536.

[20] Gill, R., Nordholm, L. and Lodge, D., The neuroprotective actions of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) in a rat focal ischemia model, *Brain Res*., **580** (1992) 35–43.

[21] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J., Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches, *Plügers Arch.*, **391** (1981) 85–100.

[22] Herrling, P.L., *Excitatory amino acids – Clinical results with antagonists.* Academic, New York, 1997.

[23] Hollmann, M. and Heinemann, S., Cloned glutamate receptors, *Annu. Rev. Neurosci.*, **17** (1994) 31–108.

[24] Honoré, T., Drejer, J., Nielsen, E.O. and Nielsen, M., Non-NMDA glutamate receptor antagonists ^{3}H-CNQX binds with equal affinity to two agonist states of quisqualate receptors, *Biochem. Pharmacol.*, **38** (1989) 3207–3212.

[25] Honoré, T., Drejer, J. and Nielsen, M., Calcium discriminates two ^{3}H-kainate binding sites with different molecular target sizes in rat cortex, *Neurosci. Lett.*, **65** (1989) 47–52.

[26] Honoré, T., Drejer, J. and Nielsen, M., Chaotropic ions affect the conformation of quisqualate receptors in rat cortical membranes, *J. Neurochem*., **51** (1988) 457–461.

[27] Ikonomidou, C. and Turski, L., Neurodegenerative disorders: clues from glutamate and energy metabolism, *Crit. Rev. Neurobiol.*, **10** (1996) 239–263.

[28] Ikonomidou, C. and Turski, L., Pharmacology of the AMPA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline, *Ann. NY Acad. Sci.*, **827** (1997) 394–402.

[29] Ikonomidou, C. and Turski, L., Therapeutic approaches to neurologic disorders with excitatory amino acid antagonists. In M.F. Beal, N. Howell and I. Bodis-Wollner (Eds), *Mitochondria and free radicals in neurodegenerative diseases*, Wiley-Liss, New York, 1997, pp: 473–495.

[30] Kawasaki-Yatsugi, S., Yatsugi, S., Koshiya, K. and Shimizu-Sasamata, M., Neuroprotective effect of YM90K, an AMPA-receptor antagonist, against delayed neuronal death induced by transient global cerebral ischemia in gerbils and rats, *Jap. J. Pharmacol.*, **74** (1997) 253–260.

[31] Lepagnol, J.M., Roger, A.C., Cordi, A.A. and Lestage, P.J., Post-ischemic treatment with S 17625-2, a new AMPA receptor antagonist, protects the rat brain after permanent focal ischemia, *J. Cereb. Blood Flow Metab*., **15** (1995) S373.

[32] LePeillet, E., Arvin, B., Moncada, C. and Meldrum, B.S., The non-NMDA antagonists, NBQX and GYKI52466, protect against cortical and striatal cell loss following transient global ischaemia in the rat, *Brain Res*., **571** (1992) 115–120.

[33] Li, H. and Buchan, A.M., Treatment with an AMPA antagonist 12 hours following severe normothermic forebrain ischemia prevents CA1 neuronal injury, *J. Cereb. Blood Flow Metab*., **13** (1993) 933–939.

[34] Lippert, K., Welsch, M. and Krieglstein, J., Over-additive protective effect of dizocilpine and NBQX against neuronal damage, *Eur. J. Pharmacol.*, **253** (1994) 207–213.

[35] McBain, C.J. and Mayer, M., N-Methyl-D-aspartic acid receptor structure and function, *Physiol. Rev.*, **74** (1994) 723–760.

[36] McFarlane, C., Warner, D.S., Todd, M.M. and Nordholm, L., AMPA receptor competitive antagonism reduces halothane MAC in rats, *Anesthesiology*, **77** (1992) 1165–1170.

[37] McMaingle, J.E., Taveira, G., DaSilva, A.M., Dretchen, K. and Gillis, R.A., Potentiation of MK-801-induced breathing impairment by 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline, *Eur. J. Pharmacol.*, **252** (1994) 11–17.

[38] Montemurro, D.G. and Dukelow, R.H., *A stereotaxic atlas of the diencephalon and related structures of the mouse*, Futura, Mount Kisco, 1972.

[39] Nellgård, B. and Wieloch, T., Postischemic blockade of AMPA but not NMDA receptors mitigates neuronal damage in the rat brain following transient severe cerebral ischemia, *J. Cereb. Blood Flow Metab*., **12** (1992) 2–11.

[40] Olney, J.W. and Ikonomidou, C., Excitotoxic mechanisms operative in youth and old age. In A. Kozikowski (Ed*.), Neurobiology of the NMDA receptor*, VCH, New York, 1991, pp: 187–202.

[41] Olney, J.W., Labruyere, J. and Price, M.T., Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs, *Science*, **244** (1989) 1360–1362.

[42] Olverman, H.J., Jones, A.W. and Watkins, J.C., L-Glutamate has higher affinity than other amino acids for [^{3}H]-D-AP5 binding sites in rat brain membranes, *Nature*, **307** (1984) 460–462.

[43] Olverman, H.J., Monaghan, D.T., Cotman, C.W. and Watkins, J.C., [^{3}H]CPP, a new competitive ligand for NMDA receptors, *Eur. J. Pharmacol*., **131** (1986) 161–162.

[44] Pellow, S., Chopin, P.H., File, S.E. and Briley, M., Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat, *J. Neurosci. Method*s, **14** (1985) 149–167.

[45] Pierrefiche, O., Foutz, A.S., Champagnat, J. and Denavit-Saubrie, M., NMDA and non-NMDA receptors may play distinct roles in timing mechanisms and transmission in the feline respiratory network, *J. Physiol.*, **474** (1994) 509–523.

[46] Pinna, G., Galici, R., Schneider, H.H., Stephens, D.N. and Turski, L., Alprazolam dependence prevented by substituting with the β-carboline abecarnil, *Proc. Natl Acad. Sci. USA*, **94** (1997) 2719–2723.

[47] Pulsinelli, W., Pathophysiology of acute ischemic stroke, *Lancet*, **339** (1992) 533–536.

[48] Schehr, R.S., New treatments for acute stroke, *Nature Biotech.* **14** (1996) 1549–1554.

[49] Schielke, G.P., Kupina, N.C., Boxer, P.A., Cordon, J.J., Bigge, C.F. and Marcoux, F.W., Delayed treatment with the novel AMPA receptor antagonist, PD152247 (PNQX), reduces lesion size following focal ischemia and reperfusion in the rat, *J. Cereb. Blood Flow Metab.*, **15** (1995) S372.

[50] Seeburg, P.H., Bresink, I. and Turski, L*., Excitatory amino acids – From genes to therapy.* Springer, Heidelberg, 1998.

[51] Sheardown, M.J., The triggering of spreading depression in the chicken retina, *Brain Res*., **607** (1993) 189–194.

[52] Sheardown, M.J., Nielsen, E.O., Hansen, A.J., Jacobsen, P. and Honoré T., 2,3-Dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline: a neuroprotectant for cerebral ischemia, *Science*, **247** (1990) 571–574.

[53] Sheardown, M.J., Suzdak, P.D. and Nordholm, L., AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischemia, even when delayed 24 h, *Eur. J. Pharmacol.*, **236** (1993) 347–353.

[54] Shimizu-Sasamata, M., Kawasaki-Iatsugi, S., Okada, M., Sakamoto, S., Yatsugi, S.-I., Togami, J., Hatanaka, K.-I., Ohmori, J., Koshija, K., Usuda, S. and Murase, K., YM90K: pharmacological characterization as a selective and potent α-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate receptor antagonist, *J. Pharmacol. Exp. Ther.*, **276** (1996) 84–92.

[55] Smith, S. and Meldrum, B.S., Cerebroprotective effect of a non-N-methyl-D-aspartate antagonist, GYKI 52466, after focal ischemia in the rat, *Stroke*, **23** (1992) 861–864.

[56] Stephens, D.N. and Kehr, W., β-Carbolines can enhance or antagonise the effects of punishment in mice, *Psychopharmacology*, **85** (1985) 143–147.

[57] Stirling, J.M., Cross, A.J. and Green, A.R., The binding of [^{3}H]thienylcyclohexyl-piperidine ([^{3}H]TCP) to the NMDA-phencyclidine receptor complex, *Neuropharmacology*, **28** (1989) 1–7.

[58] Steppuhn, K.G. and Turski, L., Diazepam dependence prevented by glutamate antagonists, *Proc. Natl Acad. Sci. USA*, **90** (1993) 6889–6893.

[59] Swedberg, M.D., Jacobsen, P. and Honoré, T., Anticonvulsant, anxiolytic and discriminative effects of the AMPA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX), *J. Pharmacol. Exp. Ther.*, **274** (1995) 1113–1121.

[60] Takagi, K., Ginsberg, M.D., Globus, M.-Y.T., Dietrich, D., Martinez, E., Kraydieh, S. and Busto, R., Changes in amino acid neurotransmitters and cerebral blood flow in the ischemic penumbral region following middle cerebral artery occlusion in the rat: correlation with histopathology, *J. Cereb. Blood Flow Metab.*, **13** (1993) 575–585.

[61] Turski, L., N-Methyl-D-aspartate receptor complex: different sites for regulation and clinical consequences, *Drug Res.*, **40** (1990) 511–514.

[62] Turski, L., Bressler, K., Rettig, K.-J., Löschmann, P.-A. and Wachtel, H., Protection of substantia nigra from MPP^+ neurotoxicity by N-methyl-D-aspartate antagonists, *Nature*, **349** (1991) 414–418.

[63] Turski, L., Huth, A., Sheardown, M., McDonald, F., Neuhaus, R., Schneider, H.H., Dirnagl, U., Wiegand, F., Jacobsen, P. and Ottow, E., ZK200775: a phosphonate quinoxalinedione AMPA antagonist for neuroprotection in stroke and trauma, *Proc. Natl. Acad. Sci. USA,* **95** (1998) 10960–10965.

[64] Turski, L. and Ikonomidou, C., Glutamate, energy metabolism and neuropathology: relevance to neurodegenerative disorders. In E. Martignoni and G. Nappi (Eds.), *Parkinson's disease and extrapyramidal disorders*, Smith-Gordon, London, 1994, pp: 15–22.

[65] Turski, L., Jacobsen, P., Honoré, T. and Stephens, D.N., Relief of experimental spasticity and anxiolytic/anticonvulsant actions of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline, *J. Pharmacol. Exp. Ther.*, **260** (1992) 742–747.

[66] Turski, L. and Kleinrok, Z., Effect of kainic acid on body temperature in rats, *Psychopharmacology*, **71** (1980) 35–39.

[67] Watkins, J.W., Krogsgaard-Larsen, P. and Honoré T., Structure-activity relationship in the development of excitatory amino acid receptor agonists and competitive antagonists, *Trends Pharmacol. Sci.*, **11** (1990) 25–33.

[68] Xue, D., Huang, Z.-G., Barnes, K., Lesiuk, H., Smith, K.E. and Buchan, A.M., Delayed treatment with AMPA, but not NMDA, antagonists reduces neocortical infarction, *J. Cereb. Blood Flow Metab.*, **14** (1994) 251–259.

[69] Yatsugi, S., Kawasaki, S., Katoh, M., Takahashi, M., Koshiya, K. and Shimizu-Sasamata, M., (1993): Effects of a novel AMPA antagonist YM900 and other excitatory amino acid antagonists on the delayed neuronal death in the gerbil hippocampus, *J. Cereb. Blood Flow Metab.*, **13** (1993) S635.