The Neuroprotective Effect of the Novel AMPA Receptor Antagonist PD152247 (PNQX) in Temporary Focal Ischemia in the Rat

Gerald P. Schielke, PhD; Nancy C. Kupina, BS; Peter A. Boxer, PhD; Christopher F. Bigge, PhD; Devin F. Welty, PhD

Background and Purpose—Evidence suggests that glutamate contributes to ischemic brain damage through activation of the \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor. We tested the novel, selective AMPA receptor antagonist PD152247 (PNQX) in a model of temporary focal ischemia to determine the dose-response relationship and to investigate the contribution of drug-induced hypothermia to the neuroprotective action of AMPA receptor antagonists.

Methods—Temporary focal cerebral ischemia was induced in Sprague-Dawley rats by occluding the middle cerebral artery and both carotid arteries for 3 hours. Body temperature was monitored by telemetry. PNQX was administered intraperitoneally or by intravenous infusion with various doses for 6 hours. Lesion volume was determined after 3 days by stereological methods.

Results—PNQX reduced the lesion volume by 51% after intraperitoneal administration. The intravenous dose-response study demonstrated that the lowest effective dose of PNQX was 1.0 mg/kg per hour, which corresponded to a steady state plasma level of 685 ng/mL. Neuroprotection was demonstrated at PNQX plasma concentrations that did not lower body temperature over the entire course of the experiment.

Conclusions—AMPA receptor activation plays an important role in the development of ischemic brain damage. Thus, novel AMPA receptor antagonists may be useful for the treatment of stroke in humans. (Stroke. 1999;30:1472-1477.)

Key Words: cerebral ischemia, focal \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor, glutamate antagonists, neuroprotection, middle cerebral artery occlusion, rats

Elevation of extracellular glutamate after cerebral ischemia is thought to play a major role in the pathophysiological processes leading to death of ischemic brain tissue. Early studies demonstrated that glutamate killed neurons in culture by a calcium-dependent process that was blocked by selective antagonists of the \( N \)-methyl-D-aspartate (NMDA) subtype of glutamate receptors.\(^1,2\) \( \alpha \)-Ami no-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), the ligand for a distinct non-NMDA glutamate receptor subtype, also results in cell death in cultured neurons with a slower time course.\(^3\) The observation that extracellular brain glutamate levels, measured by microdialysis, are increased in ischemic tissue\(^4,5\) suggested that the excitotoxicity demonstrated in culture may be relevant to ischemic cell death in vivo. Subsequently, it was demonstrated that blockers of the NMDA receptor reduce brain damage in several animal models of cerebral ischemia.\(^6\)–\(^10\)

More recently, several antagonists of the AMPA subtype of the glutamate receptor have been discovered and shown to be neuroprotective in a variety of models of cerebral ischemia.\(^11\) 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), a quinoxalininedione, was the first selective, competitive AMPA antagonist demonstrated to be effective in reducing ischemic brain damage.\(^12\) Unlike NMDA receptor antagonists, the AMPA receptor antagonist NBQX reduces selective neuronal death after global forebrain ischemia.\(^13\)–\(^15\) In models of focal ischemia, AMPA antagonists including NBQX\(^16\)–\(^19\) and more recently discovered antagonists LY215490,\(^20\) YM90K,\(^21\) YM872,\(^22\) and GYKI52466\(^23\) have been reported to reduce the volume of necrotic tissue. Additional evidence that AMPA receptor activation plays a role in ischemia is the recent finding that transgenic mice that...
overexpress the AMPA receptor subunit GluR2-flip have increased ischemic brain damage.24

Recently, a novel quinoxalinedione PD152247 (PNQX) was discovered.25 PNQX binds with high affinity to the AMPA receptor (IC50, 90 nmol/L) and with modest affinity to the kainate receptor and the glycine site of the NMDA receptor. PNQX antagonizes effects of AMPA in AMPA-induced cytotoxicity in primary neuronal cultures and protects against AMPA and maximal electroshock-induced seizures in mice (unpublished data, Boxer and Probert, 1999).

The present study was performed to determine whether PNQX is neuroprotective in a model of temporary focal ischemia. The first rationale for this study is that evaluation of novel AMPA antagonists with varying profiles of in vitro and in vivo activity and water solubility is important because the solubility-related renal toxicity of NBQX18 has prevented its clinical testing. Second, since it has been suggested that drug-induced hypothermia is responsible for the neuroprotective effects of AMPA antagonists,26 the possible contribution of hypothermia to the neuroprotective action of PNQX was addressed. Finally, the relationship between plasma concentrations of PNQX and the degree of neuroprotection in this model was evaluated.

Materials and Methods
All procedures were within the guidelines of the Institutional Animal Care and Use Committee of Parke-Davis.

Surgery
Male Sprague-Dawley rats (Charles River Laboratories), weighing 275 to 325 g, were anesthetized with 2% isoflurane (Anaquest) balanced with air, oxygen and allowed to breathe spontaneously. Rats receiving intraperitoneal treatments had femoral vein catheters (Micro-Renathane, Braintree Scientific) implanted before middle cerebral artery occlusion (MCAO). Both common carotid arteries were isolated through a small neck incision and occluded with vascular clips (Micro-Renathane, Braintree Scientific) just before MCAO. A vertical incision was made between the left eye and ear, the temporoparietal muscle was partially excised, and a craniotomy was performed at the site where the middle cerebral artery (MCA) meets the rhinal fissure. The dura over the MCA was opened and reflected to allow occlusion of the vessel with a Sundt AVM Micro Clip No. 1 (Codman) at the point where it meets the rhinal fissure. Anesthesia was discontinued until the time of reperfusion. All vascular clips were removed after 3 hours of ischemia, and reperfusion of the MCA was verified by examination of the site of occlusion. An absorbable gelatin sponge (Gelfoam, Upjohn) was placed in the area of excised muscle and incisions sutured with 4-0 silk. Rats receiving intravenous infusions (study 2) were returned to their restrainers for the remaining 2 hours of infusion. After drug or vehicle administration, rats were placed in plastic isolator cages and allowed free access to food and water. Rats were killed 3 days after initiation of ischemia, and their brains were removed and frozen in isopentane over dry ice at −40°C and stored at −80°C.

Temperature Monitoring
Rats receiving intraperitoneal treatments (study 1) were placed in isolators after MCAO and kept warm with a heating pad for 5 hours. In study 2, body temperature was maintained at 37.5°C during surgery, drug infusion, and recovery (6 hours) by individual feedback controllers and heating pads. In both studies, after the rats were returned to their cages, core body temperatures were monitored by telemetry with the use of a temperature-sensing radio transmitter (Data Sciences International) previously placed in the peritoneal cavity. The radio signal was converted to body temperature (Dataquest IV 2.10, Data Sciences International) and recorded every minute for 72 hours. Temperatures were averaged over 1-hour intervals for analysis and plotted at 5-hour intervals for illustration.

Drug Delivery
In study 1, drug or vehicle was administered by 3 intraperitoneal injections given at 0.5-hour intervals beginning 30 minutes after initiation of ischemia. The dose of PNQX was 30 mg/kg × 3 injections (total of 90 mg/kg) (concentration, 15 mg/mL; volume, 2 mL/kg per injection). PNQX was suspended in water, and pH was adjusted with methanesulfonic acid. In study 2, drug or vehicle was administered by continuous intravenous infusion (3 mL/kg per hour) for 5 hours, beginning 15 minutes after the initiation of ischemia. PNQX was dissolved in water with a small amount of 85% lactic acid; the pH was adjusted to 4.0 with sodium hydroxide and filtered before administration. Rats were randomly assigned to 1 of 5 groups: PNQX at 5.0, 2.5, 1.0, or 0.1 mg/kg per hour or vehicle.

Histology
Brains were sectioned at −17°C on a cryomicrotome. Twenty-micrometer coronal sections were collected at 600-μm intervals and stained with hematoxylin and eosin. Lesion volume was measured with the aid of the stereological software package GRID (Olympus, Denmark AS). A grid of points was randomly superimposed over a video image of each tissue section, and the number of points overlying an area of interest was counted. Total lesion volume was estimated by the equation V = t × A(p) × ΣP, where V is lesion volume, t is the distance between sections analyzed (1200 μm), A(p) is the surface area associated with 1 grid point, and ΣP is the total number of grid points associated with an area of interest in all of the sections examined.

PNQX Analytical Methods
Plasma PNQX levels were determined by UV high-performance liquid chromatography. In brief, matrix standards, quality control samples, and unknown samples were mixed with internal standard, diluted with 0.1 mol/L sodium phosphate buffer (dibasic), pH 6.0, and extracted through C18 solid-phase cartridges with 1% trifluoroacetic acid in acetonitrile. Samples were then evaporate to dryness and reconstituted with mobile phase and injected onto a reverse-phase C3 column. The mobile phase consisted of 90% 20 mmol/L sodium phosphate (dibasic), pH 2.75, and 10% methanol. Detection was by ultraviolet absorption at 210 nm, and quantitation was by peak height ratios.

Statistical Methods
Data are expressed as mean ± SD. Lesion volumes were compared by ANOVA and a Tukey-Kramer multiple comparisons test. Temperature data from study 1 were analyzed with 1-way ANOVA to compare control with the drug-treated animals at each time point. In study 2, ANOVA with linear contrasts to test for a relationship between dose and temperature was performed. P<0.05 after correction for multiple comparisons was considered significant.

Results
Effect of PNQX on Infarct Volume
In this model of temporary focal ischemia, the histological lesion, defined by the area of pallor at 72 hours, is large and primarily limited to the cortical tissue. In study 1, rats were treated intraperitoneally with PNQX (30 mg/kg at 30, 60, and 90 minutes after MCAO), a dosing paradigm used in the original studies with NBQX. The drug-treated group had a mean lesion volume 51% smaller than vehicle-treated rats (287±110 versus 142±87 mm3; P<0.001) (Figure 1, top). When lesion volumes were calculated by the indirect method,27 28 a similar reduction was seen (171±72 versus 73±57 mm3; P<0.001), demonstrating that the effect of

...
PNQX on stroke size was not due solely to a reduction in brain swelling. There was a modest (1°C to 1.25°C) reduction in body temperature, which was statistically significant between 4 and 19 hours (P < 0.05) after reperfusion in the PNQX-treated rats (Figure 1, bottom).

Relation of PNQX Plasma Concentration to Infarct Reduction

To determine the relationship between plasma levels of PNQX and its neuroprotective action, a 5-hour intravenous dose-response study was performed. Rats treated with PNQX at 5, 2.5, or 1 mg/kg per hour but not 0.1 mg/kg per hour had significantly smaller lesions than vehicle-treated controls (Figure 2, top). Figure 2 (bottom) illustrates that there was a slight but statistically significant reduction in body temperature (0.5°C; between 2 and 6 hours; P < 0.05) in rats receiving 5 mg/kg per hour PNQX. At the other neuroprotective doses of PNQX (2.5 and 1 mg/kg), body temperatures were not reduced compared with vehicle-treated rats at any time over the entire course of the experiment. Thus, in this reperfusion model of focal ischemia, the neuroprotective action of PNQX is not due to hypothermia. The plasma concentration of PNQX at the end of the 5-hour infusion was 685 ± 534 ng/mL at the lowest neuroprotective dose (1.0 mg/kg per hour), and values for the other groups were roughly proportional to dose. The Table shows the concentrations of PNQX in the plasma

Neuroprotective Effect of PNQX and Associated Steady State Plasma Levels

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Lesion Volume, mm³</th>
<th>Percent Protection</th>
<th>Plasma Concentration, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>259 ± 77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mg (kg · h)</td>
<td>232 ± 69</td>
<td>10</td>
<td>79 ± 80</td>
</tr>
<tr>
<td>1.0 mg (kg · h)</td>
<td>169 ± 40</td>
<td>35</td>
<td>685 ± 534</td>
</tr>
<tr>
<td>2.5 mg (kg · h)</td>
<td>110 ± 88</td>
<td>58</td>
<td>1471 ± 844</td>
</tr>
<tr>
<td>5.0 mg (kg · h)</td>
<td>150 ± 84</td>
<td>42</td>
<td>2634 ± 866</td>
</tr>
</tbody>
</table>

Values other than percentages are mean ± SD.
sampled at the end of the 5-hour infusion, mean lesion volumes, and percent protection at each dose.

**Discussion**

This study demonstrates that treatment with the AMPA receptor antagonist PNQX results in a dose-dependent reduction in cortical infarction in a rodent model of transient focal ischemia. Importantly, PNQX is neuroprotective at plasma drug concentrations that do not lower body temperature.

Previous studies with both competitive antagonists and noncompetitive allosteric modulators of the AMPA receptor have established the neuroprotective potential of blocking the action of this ionotropic glutamate receptor. The quinoxalinedione NBQX has been shown to reduce lesion size in both permanent and temporary rat focal ischemia models. Additionally, evidence is accumulating that repeated waves of depolarization in the periphery of the ischemic zone can amplify ischemic neuronal damage in both focal and global models. In a study of global forebrain ischemia in the gerbil, NBQX given at 30 mg/kg (×3) caused mild (1°C to 1.5°C) hypothermia that persisted for days. When the postischemic temperature was controlled, neuroprotection was lost. In focal ischemia in rats, mild to moderate hypothermia (30°C to 32°C) reduces lesion volume when the body temperature is lowered during the ischemia (in permanent MCAO), as well as during the reperfusion phase (in temporary MCAO). Typically, studies that have evaluated the neuroprotective effects of AMPA receptor antagonists (see above) used body temperature in cortical cell culture. J Neurosci. 1987;7:357–368.

PNQX is neuroprotective at plasma concentrations that do not produce hypothermia. This finding supports the concept that selective AMPA receptor antagonists will be useful for the treatment of stroke in humans.

**Acknowledgments**

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**References**

This study reports that the new AMPA receptor inhibitor PNQX, administered in the initial postischemic period, reduces cerebral ischemic injury in a rat model of transient focal cerebral ischemia. Importantly, the effect occurs at doses that do not induce hypothermia. The data provide additional evidence in support of the idea that AMPA receptor inhibitors are promising candidates for the treatment of human stroke.

The mechanisms of the protective effect of PNQX remain unclear. Although PNQX is likely to act by blocking AMPA receptors, the cellular mechanisms linking AMPA receptor blockade to neuroprotection have not been defined. Central to the glutamate hypothesis of cerebral ischemic damage is that activation of glutamate receptors increases intracellular calcium, which in turn initiates a series of cytoplasmic and nuclear events leading to tissue damage.\(^1,2\) Unlike NMDA receptors, however, most AMPA receptors are not permeable to calcium in the normal state. Yet the toxicity mediated through AMPA receptors seems to be calcium dependent.\(^3,4\)

One possibility is that ischemia, through downregulation of the GluR2 subunit of the receptor, leads to formation of calcium-permeable AMPA receptors (eg, Reference 5). This attractive hypothesis, however, needs to be tested experimentally in this particular model.

There are a number of practical issues that still remain to be addressed. First, the “time window” after ischemia in which PNQX administration is still effective remains to be defined. In the accompanying study PNQX was administered 15 or 30 minutes after induction of ischemia, whereas most stroke patients reach emergency rooms several hours after the onset of symptoms. Therefore, it would be important to study the effect of PNQX with longer intervals between induction of ischemia and onset of treatment. Second, it remains to be determined whether PNQX is also effective in species phylogenetically closer to humans. Many treatment modalities reduce stroke volume in rodents but not in higher species.\(^6\) Therefore, demonstration of efficacy in other species would increase the likelihood that the drug will be effective in humans. Third, it remains to be determined whether effective concentrations of PNQX can be safely reached in humans. These issues will have to be addressed in future studies. Overall, the well-controlled study of Schielke et al provides new and interesting data that, due to their potential clinical applicability, are likely to generate a great deal of interest.

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References
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