CP-465,022, a Selective Noncompetitive AMPA Receptor Antagonist, Blocks AMPA Receptors but Is Not Neuroprotective In Vivo

Frank S. Menniti, PhD; Alistair M. Buchan, MD, FRCP; Bertrand L. Chenard, PhD; Donald J. Critchett, MS; Alan H. Ganong, PhD; Victor Guanowsky, MA; Patricia A. Seymour, PhD; Willard M. Welch, PhD

Background and Purpose—α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor inhibition has been hypothesized to provide neuroprotective efficacy after cerebral ischemia on the basis of the activity in experimental ischemia models of a variety of compounds with varying selectivity for AMPA over other glutamate receptor subtypes. CP-465,022 is a new, potent, and selective noncompetitive AMPA receptor antagonist. The present study investigated the ability of this compound to reduce neuronal loss after experimental cerebral ischemia to probe the neuroprotective potential of AMPA receptor inhibition.

Methods—To demonstrate that CP-465,022 gains access to the brain, the effects of systemic administration of CP-465,022 were investigated on AMPA receptor–mediated electrophysiological responses in hippocampus and on chemically induced seizures in rats. The compound was then investigated for neuroprotective efficacy in rat global and focal ischemia models at doses demonstrated to be maximally effective in the electrophysiology and seizure models.

Results—CP-465,022 potently and efficaciously inhibited AMPA receptor–mediated hippocampal synaptic transmission and the induction of seizures. However, at comparable doses, CP-465,022 failed to prevent CA1 neuron loss after brief global ischemia or to reduce infarct volume after temporary middle cerebral artery occlusion.

Conclusions—Given the high selectivity of CP-465,022 for AMPA over kainate and N-methyl-d-aspartate subtypes of glutamate receptors, the lack of neuroprotective efficacy of the compound calls into question the neuroprotective efficacy of AMPA receptor inhibition after ischemia. (Stroke. 2003;34:171-176.)

Key Words: excitatory amino acid antagonists ■ receptors, AMPA ■ receptors, glutamate ■ stroke

The hypothesis that overactivation of glutamate receptors causes neuronal death has fueled extensive efforts to identify agents that inhibit glutamate receptors as potential neuroprotectants. While the initial focus was on compounds that inhibit N-methyl-d-aspartate (NMDA) receptors, there has also been interest in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists. This interest stems largely from studies with the prototype AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline (NBQX) and structurally related analogues such as 6-(1H-imidazol-1-yl)-7-nitro-2,3(1H,4H)-quinoxalinedione hydrochloride (YM-90K) and [1,2,3,4-tetrahydro-7-morpholinyl-2,3-dioxo-6-(trifluoromethyl)quinoxalin-1-yl]methane phosphonate (ZK-700,775). These compounds effectively reduce the neuronal loss caused by ischemia and traumatic brain injury under a variety of experimental conditions. Significantly, the quinoxalinediones appear to offer 2 advantages over the NMDA receptor antagonists. First, these compounds, but not NMDA receptor antagonists, prevent the slowly developing loss of hippocampal CA1 pyramidal neurons caused by brief, severe (global) forebrain ischemia. Second, the neuroprotective effects of the quinoxalinediones are realized with longer delays after the initiation of ischemia compared with the NMDA receptor antagonists. Unfortunately, to date, the quinoxalinediones have proved unsuitable for clinical use because of poor pharmaceutical properties. Thus, new compounds are needed that match the pharmacological profile of the quinoxalinediones while possessing improved physiochemical properties.

The efficacy of the quinoxalinediones has been attributed to AMPA receptor inhibition because NBQX was initially believed to be specific for AMPA receptors on the basis of radioligand binding. However, it is now appreciated that NBQX has significant functional inhibitory activity at both kainate and AMPA receptors. Thus, the contribution of AMPA receptor inhibition to the neuroprotective efficacy of the quinoxalinediones is not clear. The present study was undertaken to test specifically whether AMPA receptor inhibition alone provides broad neuroprotective efficacy.
relatively long treatment window, similar to what is observed with the quinoxalinediones. These studies use CP-465,022, a recently identified compound demonstrated to be a potent noncompetitive AMPA-specific receptor antagonist in vitro systems. In the present study CP-465,022 is shown to readily gain access to the brain after systemic administration to block AMPA receptor activity. However, CP-465,022 fails to reduce the loss of CA1 hippocampal neurons observed after brief global ischemia or infarct size after middle cerebral artery occlusion (MCAO) in rats. The implications of these results for the hypothesized role of AMPA receptors in acute neurodegenerative conditions are discussed.

Materials and Methods

Chemicals
The preparation of CP-465,022 [(S)-3-(2-chlorophenyl)-2-[2-(6-diethylaminomethyl-pyridin-2-yl)-vinyl]-6-fluoro-3H-quinoxalin-4-one] has been described. The resolved S atropisomer was used unless otherwise noted. YM-90K was synthesized at Pfizer Global Research and Development as previously described. CP-465,022 and YM-90K were dissolved in 10% Captisol (sulfobutylether β-cyclodextrin, licensed by Pfizer Global Research and Development from the University of Kansas).

Animals
Male CD rats (Charles River Laboratories, Raleigh, NC) served as subjects for the anticonvulsant, motor activity, and electrophysiology studies, under conditions approved by the Pfizer Institutional Animal Care and Use Committee. Male Wistar rats (Charles River Laboratories, Montreal, Quebec, Canada) and SHIZ rats were used for the local and focal ischemia studies, respectively. These procedures were in accordance with the Canadian Council on Animal Care Guidelines.

CA1 Synaptic Transmission
The effects of compounds were examined on population spike amplitude in the CA1 region of the hippocampus evoked by constant voltage stimulation of the Schaeffer collateral/commissural pathway in the contralateral hemisphere. Animals anesthetized with urethane (1.5 g/kg) were placed in a stereotaxic apparatus (body temperature maintained at 37°C). A bipolar, glass-insulated (50-μm tip) stainless steel microelectrode was lowered to the level of the CA3 cell group (stereotaxic coordinates: −4.3 anteroposterior, −4.2 lateral, −4.0 to −4.3 vertical), and a recording electrode (glass pipette, ~4-μm tip, ~1.5 Ω) was placed in the contralateral CA1 cell group (−4.3 anteroposterior, −2.0 lateral, −2.0 vertical). Single bipolar voltage pulses (100 μs) were applied to establish a stimulus intensity resulting in CA1 population spikes of at least 8 mV. Stimulus intensity was then reduced to a voltage that produced approximately 50% of the maximum amplitude population spike. A stimulation rate of 0.05 Hz was used for the recording session. After evoked CA1 potential had remained stable for 20 minutes, compounds were administered intravenously (IV) via the femoral vein or subcutaneously (SC). Population spike amplitude was measured as the voltage change from peak to peak.

Plasma Levels of CP-465,022
Cannulas were inserted into the jugular vein of fasted rats (n=5) under ketamine/xylazine (70:30) anesthesia. The following day, animals were administered 10 mg/kg SC of racemic CP-465,022. Plasma samples were obtained before dosing and at 0.25 to 6 hours afterward. Plasma concentrations of CP-465,022 were determined by high-performance liquid chromatography with UV detection.

Pentylenetetrazole-Induced Seizures
Compounds were administered 30 or 60 minutes before pentylenetetrazole (100 mg/kg IP). Rats were then observed for 30 minutes, and latencies to clonic seizures, tonic seizures, and lethality were recorded. Data were analyzed with Kruskal-Wallis ANOVA followed by Mann-Whitney U tests.

Spontaneous Locomotor Activity
Rats were placed into chambers (30 cm³) equipped with photocells housed in sound-attenuating cabinets. Compounds were administered to unhabituated animals immediately before placement into chambers, and horizontal (crossovers) and vertical (rears) locomotion was recorded for 12 hours. Data were analyzed with ANOVA followed by Dunnett’s t tests for multiple comparisons with a control.

Global Ischemia
Rats were subjected to brief global cerebral ischemia as previously described. Under 1% to 2% halothane anesthesia, both common carotid arteries were isolated, and a ligature was gently placed around each vessel. The vertebral arteries were electrocauterized, and a ligature was passed ventral to the cervical and paravertebral muscles but dorsal to the trachea, esophagus, external jugular veins, and common carotid arteries. On the following day, aneurysm clips were put on carotid arteries, resulting in occlusion of the 4 major vessels supplying the cerebrum (4-vessel occlusion). Loss of consciousness was seen within 10 to 15 seconds. The ligature surrounding the paravertebral musculature was then tightened to prevent the opening of collateral blood flow channels. Body temperature was maintained at 37.5°C throughout the ischemic period. After 10 minutes of ischemia, animals were checked for unresponsiveness and dilated pupils. The aneurysm clips were removed, the vessels were inspected for patency, and the wound was closed with a surgical clip. Immediately after reperfusion and 4 hours afterward, animals were administered CP-465,022 or vehicle by subcutaneous injection. Animals were allowed to survive for 7 days, after which the animals were perfusion-fixed with 4% buffered formaldehyde. The number of normal and abnormal (dead) CA1 neurons was counted, and results were expressed as the percentage of dead neurons, as previously described.

Focal Ischemia
Rats were subjected to temporary MCAO as previously described. The right common carotid artery was first isolated and permanently occluded. The right MCA was then exposed by a subtemporal approach and occluded with clips. Ninety minutes after occlusion and an additional 4 hours afterward, animals were administered CP-465,022 or vehicle by subcutaneous injection. Occlusion was maintained for an additional 30 minutes after the first compound administration (for a total of 2 hours of occlusion), and then the clips were removed. Temperature was maintained at 37°C throughout surgery and treatment. After survival for 22 hours, animals were killed by decapitation, and the brains were rapidly removed and frozen. Coronal brain sections (20 μm thick) were cut at 500-μm intervals, fixed in 90% ethanol, and stained with hematoxylin and eosin. Infarcted areas for each section were traced, and a total infarct volume was calculated by summation of infarct area in sequential sections and multiplication by the interval thickness between sections.

Results

AMPA Receptor–Mediated Synaptic Transmission In Vivo
Stimuli applied to the Schaeffer collateral/commissural pathway evoked a reproducible population spike in the contralateral CA1 region of approximately 3 mV (Figure 1A). This response is mediated by AMPA and NMDA receptors on the CA1 neurons, as has been established in hippocampal slice
preparations. CP-465,022 efficaciously and reversibly decreased the amplitude of the evoked population spike (Figure 1A). When administered as a 1-minute intravenous infusion, this effect was dose dependent (Figure 1B). Maximal inhibition was at 1 mg/kg, which reversed within approximately 30 minutes. After subcutaneous administration, inhibition was maintained for much longer periods (Figure 1C). Maximum inhibition was observed at 15 mg/kg SC of racemic CP-465,022 (equivalent to 7.5 mg/kg of the resolved S enantiomer); at higher doses, lethality was sometimes observed. Subcutaneous administration of the quinoxalinedione AMPA receptor antagonist YM-90K also reversibly decreased the amplitude of the evoked population spike. At a maximally effective dose of 56 mg/kg SC (Figure 1C), the magnitude of inhibition was similar to that caused by CP-465,022. However, inhibition by YM-90K developed more quickly and was of shorter duration than for CP-465,022.

**Plasma Levels of CP-465,022**

Plasma levels of racemic CP-465,022 after subcutaneous administration of 10 mg/kg are indicated in Figure 2. Levels reached a peak within approximately 30 minutes of dosing and then declined slowly with a half-life of approximately 4 hours. Qualitative observation indicated that the animals became ataxic within 30 minutes of dosing and remained so for approximately 4 hours.

**Pentylenetetrazole-Induced Seizures**

Administration of pentylenetetrazole (100 mg/kg IP) to rats results in a characteristic syndrome of clonic seizures followed by tonic seizures and lethality within 30 minutes. CP-465,022 administered subcutaneously 60 minutes before pentylenetetrazole dose-dependently increased the latency to and decreased the incidence of pentylenetetrazole-induced clonic seizures, tonic seizures, and lethality (Figure 3, Table). Complete protection was observed across all 3 measures at 10 mg/kg. Efficacy at this dose was observed with pretreatment times up to 4 hours (data not shown). YM-90K also increased the latency to and increased the incidence of pentylenetetrazole-induced seizures and lethality, albeit with less potency than CP-465,022 (Table). A dose of YM-90K that completely inhibited tonic seizures and lethality when given 30 minutes before pentylenetetrazole (56 mg/kg SC) was ineffective if given 2 hours before pentylenetetrazole (data not shown).

**Locomotor Activity**

CP-465,022 dose dependently decreased horizontal and vertical locomotor activity, with ED_{50} values of 11.9 and 6.6 mg/kg, respectively (Table). YM-90K produced similar effects but with less potency (Table).

**Global Ischemia**

Ten minutes of global cerebral ischemia results in a substantial loss of hippocampal CA1 pyramidal neurons after 7 days. The ability of CP-465,022 to prevent this neuronal loss was assessed at 2 dose levels. In 1 group, CP-465,022...
Summary of the Anticonvulsant and Motor Activity Effects of CP-465,022

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<th>CP-465,022</th>
<th>YM-90K</th>
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<tbody>
<tr>
<td>Inhibition of PTZ-induced clonic seizures</td>
<td>4.0 ± 1.2</td>
<td>68.1</td>
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<tr>
<td>Inhibition of PTZ-induced tonic seizures</td>
<td>1.0 ± 1.5</td>
<td>3.2</td>
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<tr>
<td>Inhibition of crossovers</td>
<td>11.9 ± 1.2</td>
<td>&gt;32</td>
</tr>
<tr>
<td>Inhibition of rears</td>
<td>6.6 ± 1.1</td>
<td>&gt;32</td>
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Compounds were administered subcutaneously as described in Materials and Methods. All values are the ED50 in mg/kg. Each ED50 value represents the geometric mean ± SEM in mg/kg subcutaneously from three (CP-465,022) or one (YM-90K) experiments with n = 8–10 animals/group.

was administered at 5 mg/kg SC at the time of reperfusion and at 2 mg/kg SC 4 hours later. A second group was similarly dosed but with 10 and 4 mg/kg of CP-465,022. The comparator group received vehicle injections. After 7 days, the brief global ischemia caused a loss of 81% of CA1 neurons in the vehicle-treated group (Figure 4). CP-465,022 at either of the doses tested failed to reduce the observed neuronal loss.

Focal Ischemia

The neuroprotective effect of CP-465,022 was also examined in a rat temporary MCAO model. Animals were subjected to unilateral MCAO and after 90 minutes were administered 5 mg/kg SC of CP-465,022 or vehicle. After 30 minutes, artery clips were removed, and, after an additional 3.5 hours, animals received a second administration of CP-465,022 at 2 mg/kg SC or vehicle. Body temperatures were maintained at 37°C for 6 hours after the initiation of occlusion, and CP-465,022 had minimal effects on other physiological parameters during this period. Twenty-four hours after the initiation of occlusion, the volume of cortical infarction was 141 ± 46 mm³ (mean ± SD) in vehicle-treated animals (Figure 4). CP-465,022 caused a small decrease in infarct volume (to 124 ± 49 mm³), but this was not significantly different from the vehicle control group. A second group of 4 animals received a higher dose of CP-465,022 (10 mg/kg SC) under identical conditions; however, these animals all died within 1 hour of compound administration.

Discussion

Numerous studies have demonstrated that the quinoxalinedione class of non-NMDA receptor antagonists is efficacious, with a relatively long treatment window, for preventing neuron loss after cerebral ischemia in a variety of experimental systems. This profile has made these compounds attractive candidates for treatment of stroke in humans. However, to date, poor pharmaceutical properties have prevented clinical development. One approach to the continued pursuit of such a therapy is to identify the key pharmacological activity(s) that accounts for the neuroprotective efficacy of the quinoxalinediones and then to incorporate this activity(s) into pharmaceutically more acceptable molecules. It was originally hypothesized that the efficacy of the quinoxalinediones results from AMPA receptor inhibition. However, it is now clear that NBQX and presumably other quinoxalinediones are functional inhibitors of both AMPA and kainate receptors, qualifying the original hypothesis. The aim of the present study was then to clarify the relevant pharmacology by investigating the neuroprotective efficacy of highly selective AMPA receptor antagonists. Two well-studied models of cerebral ischemia, rat 4-vessel (global) ischemia and rat temporary MCAO, were used in which a number of the quinoxalinediones have been demonstrated to be efficacious. In fact, one of the present authors has demonstrated in several studies the efficacy and long treatment window of NBQX in these models, as used in the present study.

CP-465,022 was used as the pharmacological probe. Previous in vitro studies demonstrate that CP-465,022 is a potent and highly selective AMPA receptor antagonist. Inhibition is not competitive with glutamate binding but results from interaction with an allosteric binding site labeled with [3H]CP-526,427 (see Menniti et al), in contrast to the quinoxalinediones. The present results demonstrate that CP-465,022 readily enters the central nervous system after peripheral administration and inhibits AMPA receptors. CP-465,022 administered intravenously or subcutaneously inhibits AMPA receptor–mediated synaptic responses at the Schaeffer collateral/CA1 synapse in hippocampus. Notably, synaptic inhibition persists after subcutaneous administration of 7.5 mg/kg for 4 hours. This time course of inhibition agrees closely with the plasma residence time of the compound after administration by this route. AMPA receptor antagonists of various chemical classes and mechanisms of action have previously been found to be anticonvulsant. In the
present study CP-465,022 dose dependently inhibited pentylenetetrazole-induced seizures and lethality in rats. At 10 mg/kg SC, full efficacy was maintained for at least 4 hours. AMPA receptor antagonists have also been found to produce a number of central nervous system depressant–like effects, including ataxia and respiratory depression. In the present study CP-465,022 dose dependently inhibited locomotor activity in rats. This effect occurred at a dose slightly higher than that efficacious in the seizure model.

The data summarized above indicate that CP-465,022 readily gains access to and inhibits central AMPA receptors after systemic administration. The activity of CP-465,022 was qualitatively similar to that of the quinoxalinedione YM-90K, which was also assessed in the aforementioned assays. YM-90K was chosen as the comparator because of the wealth of relevant published literature and because of the relative ease of synthesis in the quantity needed for these in vivo studies. Similar to CP-465,022, YM-90K also inhibited synaptic transmission, pentylenetetrazole-induced seizures and lethality, and locomotor activity. However, CP-465,022 was 2.5-fold more potent than YM-90K across the various measures and displayed a considerably longer pharmacodynamic half-life.

In light of the demonstrable inhibition of AMPA receptors by CP-465,022 after subcutaneous administration, the neuroprotective efficacy of the compound was assessed with this route of administration. The dosing regimens (5 and 2 mg/kg SC or 10 and 4 mg/kg SC, with doses separated by 4 hours) were chosen on the basis of the following considerations. The 5 mg/kg dose was above the ED₉₀ for inhibition of tonic and clonic seizures in rats, whereas the 10 mg/kg dose was associated with complete seizure inhibition and significant ataxia. Furthermore, these doses of CP-465,022 bracket the dose of compound found to maximally inhibit synaptic transmission at the CA1/Schaeffer collateral synapse in vivo. Administration of the second lower dose after 4 hours was used to ensure sustained, relatively constant plasma levels of the compound for >6 hours. This target duration of exposure was based on the published data for efficacious dosing regimens with the quinoxalinediones. Both NBQX and YM-90K are reported to have very short plasma half-lives (half-lives of ~30 minutes⁷–⁸). This is reflected in the short pharmacodynamic half-life of these compounds in vivo, as is widely noted in the literature and observed with YM-90K in the present studies. Two dosing schemes are generally used with these compounds in ischemia studies: (1) 3 intraperitoneal or subcutaneous doses given at 15- or 30-minute intervals or (2) intravenous infusions of 3 to 6 hours. Given the short half-lives of these compounds, the literature data are most parsimoniously interpreted to indicate that exposures of 3 to 6 hours are sufficient for neuroprotection. Finally, initiation of CP-465,022 dosing immediately on reperfusion after 4-vessel occlusion or 90 minutes after the initiation of MCAO was chosen because the quinoxalinediones have clearly demonstrated efficacy within these time windows. Thus, the dosing regimens for CP-465,022 were expected to produce near-maximal levels of AMPA receptor inhibition in time frames consistent with that demonstrated to be efficacious for the quinoxalinediones.

In the present study brief global ischemia after permanent occlusion of the vertebral arteries and 10-minute occlusion of the carotid arteries in rats results in nearly complete loss of CA1 pyramidal neurons after 7 days, as previously reported. However, CP-465,022 administered immediately after reperfusion at either of the doses tested did not reduce CA1 neuronal loss after brief global ischemia. MCAO in rats for 2 hours results in development of a significant area of cortical and striatal infarction within 24 hours, again as previously reported. When administered subcutaneously at 5 mg/kg 90 minutes after the start of occlusion and at 2 mg/kg 4 hours later, CP-465,022–treated animals had slightly smaller infarct volumes than vehicle-treated animals at 24 hours; however, this difference (12%) was not statistically significant. A small number of animals were administered 10 mg/kg CP-465,022 at 90 minutes after the start of the occlusion, but these animals did not survive. Thus, CP-465,022 apparently lacks significant neuroprotective efficacy against acute ischemia-induced neuronal loss. This is in contrast to the robust efficacy for the quinoxalinediones. Possible reasons for this difference are considered below.

It seems unlikely that the lack of efficacy of CP-465,022 in the ischemia models is the result of inadequate level or duration of exposure for the aforementioned reasons. However, the quinoxalinediones are known to precipitate in the body after systemic administration. Thus, these compounds may accumulate, resulting in unexpectedly sustained exposure. This possibility must be weighed against the observed short pharmacodynamic half-life of these compounds reported in the literature and as observed for YM-90K in the present study. It is also difficult to rationalize the difference in neuroprotective efficacy between CP-465,022 and the quinoxalinediones on the basis of the difference in mechanism of receptor inhibition, since CP-465,022 and YM-90K had qualitatively similar profiles of activity in the synaptic transmission, seizure, and motor function assays in the present study.

It also must be considered that the difference in neuroprotective efficacy between CP-465,022 and the quinoxalinediones is due to the difference in glutamate receptor specificity. CP-465,022 is highly specific for AMPA over NMDA and kainate receptors in in vitro functional models, whereas NBQX is nearly equally potent for inhibition of both receptors when measured in functional assays. The present results with CP-465,022 suggest that AMPA receptor inhibition alone is insufficient for neuroprotective efficacy. There are only limited published data with AMPA receptor antagonists other than the quinoxalinediones to evaluate this hypothesis further. Neuroprotective effects have been reported for the prototypical noncompetitive AMPA receptor antagonist 4-(8-methyl-9H-1,3-dioxo-6,7-diazacyclohepta[5,6]phenylamylamine (GYKI-52,466) in focal and global ischemia models. However, Buchan et al found that this latter compound was effective in preventing focal but not global ischemia when administered after cerebral ischemia. Block et al found this compound to reduce the effects of global ischemia only when administered before occlusion, in clear contrast to the relatively long time window after the initiation of ischemia in which NBQX provided neuroprotection. Schoepp
et al.\textsuperscript{25} have also reported the activity of a series of decahydroisoquinoline competitive AMPA receptor antagonists. This class of compounds also was found to have affinity for receptors containing the GluR5 subunit of kainate receptor.\textsuperscript{26} In a very interesting study, the neuroprotective activity of a series of decahydroisoquinolines was compared with affinities for both AMPA and kainate receptors.\textsuperscript{27} It was found that reduction in CA1 neuronal loss after brief global ischemia in gerbil did not correlate well with activity at either of these glutamate receptor subtypes.

In summary, the results presented here suggest that AMPA receptor inhibition alone may not be sufficient to account for the robust efficacy profile of the quinoxalinediones against ischemia-induced neuronal damage. On the other hand, the similar profiles of central nervous system depressant activity observed between CP-465,022 and YM-90K indicate that AMPA receptor inhibition does account for the central nervous system depressant activity of the quinoxalinediones. If AMPA receptor inhibition is not obligatory for neuroprotective efficacy, then it may be possible to identify a glutamate receptor target that provides neuroprotection at a reduced side effect burden. CP-465,022 provides an important new tool in pursuing this line of research.

**Acknowledgment**

This work was supported by Pfizer Inc.

**References**

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Stroke. 2003;34:171-176; originally published online December 12, 2002; doi: 10.1161/01.STR.0000048216.90221.9C
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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