Delayed Treatment With AM-36, a Novel Neuroprotective Agent, Reduces Neuronal Damage After Endothelin-1–Induced Middle Cerebral Artery Occlusion in Conscious Rats

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Background and Purpose—AM-36 is a novel arylalkylpiperazine with combined antioxidant and Na\textsuperscript{+} channel blocking actions. Individually, these properties have been shown to confer neuroprotection in a variety of in vitro and in vivo animal models of stroke. Preliminary studies have shown that AM-36 is neuroprotective in vivo. The purpose of the present study was to assess the neuroprotective and behavioral outcome after delayed administration of AM-36 in an endothelin-1–induced, middle cerebral artery model of cerebral ischemia in conscious rats.

Methods—Conscious male hooded Wistar rats were subjected to middle cerebral artery occlusion by perivascular microinjection of endothelin-1 via a previously implanted cannula. AM-36 (6 mg/kg IP) or vehicle was administered intraperitoneally 30, 60, or 180 minutes after middle cerebral artery occlusion. Functional outcome was determined 24, 48, and 72 hours after stroke by neurological deficit score, motor performance, and sensory hemineglect tests. Rats were killed at 72 hours, and infarct area and volume were determined by histology and computerized image analysis.

Results—Endothelin-1–induced middle cerebral artery occlusion resulted in marked functional deficits and neuronal damage. AM-36 significantly reduced cortical damage when administration was delayed until 30, 60, or 180 minutes after stroke. Interestingly, neuronal damage was time-dependently reduced, with the greatest protection found when AM-36 was administered 180 minutes after stroke. Striatal damage was significantly reduced after treatment with AM-36 at 180 minutes after stroke. Functional outcome paralleled histopathology. Rota-rod performance, sensory hemineglect, and neurological deficit scores returned to preischemia levels in AM-36–treated rats by 72 hours after stroke when administration was delayed by 180 minutes after stroke.

Conclusions—AM-36 potently protects against both neuronal damage and functional deficits even when administered up to 180 minutes after induction of stroke. In fact, the greatest protection was found when administration was delayed by 180 minutes after stroke. The possible mechanisms of action of AM-36 are discussed. The present findings suggest that AM-36 may have great promise in the acute treatment of human stroke. (Stroke. 1999;30:2704-2712.)

Key Words: cerebral ischemia, focal endothelins neuroprotection rats

AM-36 is a novel arylalkylpiperazine with combined antioxidant and Na\textsuperscript{+} channel blocking activity within a single compound. The activity of AM-36 has been tested in in vitro assays, in which it was found to inhibit lipid peroxidation in the modified thiobarbituric acid reactive substances assay with activity comparable to that of known antioxidants such as 3,5-tert-butyl-4-hydroxytoluene (BHT), Trolox, and LY231617. In binding assays, AM-36 inhibits \[^{3}H\]batrachotoxinin binding to site 2 Na\textsuperscript{+} channels in rat brain homogenates. Furthermore, in cultured cerebellar granule cells, AM-36 potently inhibits veratridine-induced cell death with an IC\textsubscript{50} of 1.3 \(\mu\text{mol}/\text{L}\). We have recently demonstrated preliminary evidence that AM-36 is neuroprotective after acute stroke and believe that both its free radical scavenging and Na\textsuperscript{+} channel blocking activity contribute to its neuroprotective activity.

Individually, antioxidants and Na\textsuperscript{+} channel antagonists have been shown to be neuroprotective in animal models of cerebral ischemia. Substantial evidence suggests that the generation of reactive oxygen species during cerebral ischemia is an important contributor to neuronal damage. Studies have indicated dramatic increases in levels of oxygen radicals during reperfusion, which remain high for up to 2 hours and then increase again at 24 hours. Loss or blockade of...
endogenous antioxidants worsens ischemic injury, and mice overexpressing Cu/Zn superoxide dismutase are protected from ischemia. Antioxidants and inhibitors of lipid peroxidation attenuate neuronal damage in animal models of cerebral ischemia. Additionally, the spin trap reagent α-phenyl-tert-butylnitrone (PBN) reduced brain infarct volume in a middle cerebral artery (MCA) occlusion model of stroke when administered up to 3 hours after ischemia.

Blockade of Na⁺ channels has also been proposed as a possible neuroprotective mechanism by reducing energy expenditure in compromised tissue. Since a large part of the energy expenditure of excitable cells is used to maintain Na⁺ and K⁺ gradients across cell membranes, blockade of Na⁺ channels may constitute an effective neuroprotective mechanism. A number of experimental findings strongly suggest that administration of inhibitors of voltage-sensitive Na⁺ channels is beneficial even when it is delayed after stroke. This may constitute an important mechanism since a number of structurally unrelated neuroprotective drugs share the property of downmodulating Na⁺ channels. Compounds possessing Na⁺ channel blocking action such as lamotrigine, BW619C89, and riluzole are neuroprotective in MCA models of ischemia.

In view of the delay in the time taken to hospitalize and diagnose a stroke victim, it is now considered important to demonstrate significant neuroprotection after delayed administration of a compound. Hence, the purpose of the present study was to determine the effectiveness of the novel neuroprotective agent AM-36 when treatment commenced at various intervals after the onset of stroke. This study used the endothelin-1 (ET-1) model of MCA occlusion, which is less invasive than some other models, incorporates reperfusion, and has the advantage that rats are conscious during stroke. The use of anesthesia during stroke may confound experimental findings since it has been reported to be neuroprotective and to have interactions with other pharmacological agents (eg, MK801), and it may also potentially affect free radical production. Our present results indicate that AM-36 potently protects against neuronal damage and improves functional outcome after delayed administration.

Materials and Methods

Surgical Preparation

All procedures used in this study were performed in accordance with the Prevention of Cruelty to Animals Act 1986. Male hooded Wistar rats (weight, 280 to 320 g) were anesthetized with a 50:50 mixture of pentobarbital/methohexitol sodium in a volume of 0.6 mL (30 mg/kg and 30 mg/kg IP, respectively). According to the method of Sharkey and colleagues, a 23-gauge stainless steel guide cannula was stereotaxically implanted into the piriform cortex 2 mm dorsal to the right MCA. The stereotaxic coordinates were modified for this rat strain (0.2 mm anterior, −5.2 mm lateral, and −6.1 mm ventral, according to a stereotaxic atlas). The cannula was secured with dental acrylic cement, and 2 small screws were inserted into the skull. The scalp was closed with sutures. Animals were housed individually and allowed to recover for 3 days before induction of stroke.

Rats were divided into drug or vehicle groups before induction of stroke. Occlusion of the right MCA was induced in conscious rats by administration of ET-1 (120 pmol in 6 μL of saline over 6 minutes) via a 30-gauge injector that protruded 2 mm beyond the end of the previously implanted guide cannula. The injector was held in place by a poly tubing cuff, and the animal was placed in a clear Plexiglas box for observation during ET-1 injection. Stroke was characterized by counterclockwise circling, clenching, or failure to extend the contralateral forelimb. These behaviors occurred within 2 to 10 minutes of the beginning of the ET-1 injection. Rats that did not show any behavioral signs were deemed not to have had a stroke and were excluded from further study. Sham-injected rats underwent cannula implantation but did not receive any ET-1 injection. AM-36 hydrochloride was dissolved in water, and dose was expressed as the free base. The dose of AM-36 used here was chosen on the basis of preliminary investigations that evaluated 1.8 and 6 mg/kg IP. Whereas 1.8 mg/kg produced some neuroprotection, a dose of 6 mg/kg considerably reduced the size of the infarct. The first dose of AM-36 (6 mg/kg IP) or vehicle (water) was administered either 30, 60, or 180 minutes after the beginning of ET-1 injection. All rats then received the second and third doses at 24 and 48 hours after stroke. Hence, AM-36–treated rats received a total of 3×6 mg/kg IP. Rectal temperatures were taken with a thermistor probe before stroke and at 30- or 60-minute intervals for 3 hours after stroke and 2 hours after drug administration.

Assessment of Functional Outcome

All behavioral tests were conducted before any procedures (presurgery, day 1); after recovery from cannula implantation and immediately before ET-1–induced MCA occlusion (preischemia, day 4); and 24, 48, and 72 hours after ET-1–induced MCA occlusion (days 5, 6, and 7, respectively). Each rat acted as its own control.

Neurological abnormalities were evaluated with the use of a neurological deficit score based on detection of abnormal posture and hemiplegia, as described by Yamamoto and colleagues and De Ryck and colleagues. Abnormal posture was assessed by suspending rats by the tail and observing twisting of the thorax and extension of forelimbs. Presence of thorax twisting and absence of contralateral forepaw extension were scored at 1 each. Hemiplegia was evaluated by placing rats on a raised platform. When there is a deficit, the contralateral hindlimb slips off the edge of the platform (score = 1), and the contralateral forelimb slips off when the snout and whiskers lose contact with the surface (score = 1). Thus, when the scores were summed, the maximum neurological deficit score was 4. A score of 0 was considered normal.

Motor impairment was assessed with the use of the accelerating rota rod (Ugo Basile, model 7750). Rats were given 2 training sessions 10 minutes apart before surgery. Latency to fall off the rota rod was then determined before induction and 24, 48, and 72 hours after stroke. Rats not falling off within 5 minutes were given a maximum score of 300 seconds.

Sensory hemineglect was evaluated by a test developed by Schallert and Whishaw that measures sensitivity to simultaneous forelimb stimulation. This test is based on observations of behavior in humans with unilateral brain damage. If 2 stimuli are presented simultaneously, 1 on each side of the body, the contralateral stimulus appears to be masked ("extinguished"), and either remains undetected until the ipsilateral stimulus is removed or feels subjectively weaker. In rats, the test consists of placing adhesive tapes (Avery adhesive labels, 1-cm circles) on the distal-radial region of each wrist. Placement of the first tape was randomized between contralateral and ipsilateral limbs. The tape on both forepaws was touched simultaneously before the animal was placed in a Plexiglas cage, and both the latency to touch and the latency to remove each stimulus from the contralateral and ipsilateral forepaws were measured with a stopwatch. The test was terminated at 120 seconds if the tapes had not already been removed.

Spontaneous activity of individual rats was measured for 10 minutes at the same time each day with an Animex type S activity meter (FARAD Electronics). Rats were equilibrated in the apparatus for a period of 30 minutes before the first testing time on presurgery day 1.
Quantification of Ischemic Damage

Rats were decapitated 72 hours after ischemia; their brains were removed and frozen in liquid nitrogen and stored at \(-80^\circ C\). Coronal cryostat sections (18 μm thick) were cut at 8 predetermined planes throughout the brain from \(-3.2\) to \(6.8\) mm anterior to the interaural line. The sections were then fixed in paraformaldehyde vapor for 30 minutes at 60°C and then overnight at room temperature before they were stored at \(-80^\circ C\). Infarct area was measured in unstained sections with the use of our recently reported novel method,\textsuperscript{5} which employs an image analysis system (MCID M4 image analyzer, Imaging Research Inc) to trace the areas of damage in each brain section. Total infarct volume was calculated by integrating the cross-sectional area of damage at each stereotaxic level and the distances between the levels according to the method of Osbourne and colleagues.\textsuperscript{30}

Materials

AM-36 \([1-(2-(4-chlorophenyl)-2-hydroxy)ethyl-4-(3,5-bis(1,1-dimethyl)-4-hydroxyphenyl)methylpiperazine]\) was designed and synthesized in conjunction with AMRAD Operations Pty Ltd.\textsuperscript{1} ET-1 was obtained from the American Peptide Company, Inc.

Statistical Analyses

Values are presented as mean±SEM unless stated otherwise. Physiological data and infarct area data were analyzed by ANOVA followed by the Duncan test. Infarct volumes were compared by 1-way ANOVA followed by trend analysis. Neurological deficit scores were analyzed by Kruskal-Wallis nonparametric ANOVA and the Mann-Whitney test for analysis of individual differences. Rotarod performance and spontaneous activity were expressed as a percentage of presurgery performance for each rat and analyzed by

Table 1. Effects of AM-36 (3×6 mg/kg IP) or Vehicle at Different Times of Administration After Ischemia on Cortical and Striatal Infarct Volume

<table>
<thead>
<tr>
<th>Time After Stroke, min</th>
<th>Cortex</th>
<th></th>
<th>Striatum</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Vehicle Control</td>
<td>n</td>
<td>AM-36</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
<td>111.2±5.9</td>
<td>4</td>
<td>60.4±15.7*</td>
</tr>
<tr>
<td>60</td>
<td>6</td>
<td>152.9±38.9</td>
<td>5</td>
<td>43.9±7.3*</td>
</tr>
<tr>
<td>180</td>
<td>4</td>
<td>107.4±47.7</td>
<td>4</td>
<td>18.9±10.5*</td>
</tr>
</tbody>
</table>

Values are volume of infarction (mean±SEM), expressed in cubic millimeters.
*Significant compared with vehicle control (\(P<0.05\), Student’s \(t\) test).
or 180 minutes after MCA occlusion (Figure 1). AM-36

known to have had a stroke and were excluded from the study. Rats showing no neurological deficits were deemed

ANOVA for repeated measures, followed by Student-Newman-Keuls test. Sensory hemisegret data were analyzed by the Friedman repeated-measures test, and individual differences were analyzed by the Wilcoxon signed rank test or Mann-Whitney test. A value of

1.8
6
3.5*
25.0
2
6
4.9*
10.5
2
6
2
6
0.3 0.2
0.1 0.2
0.0 0.0

significant decrease in body weight was observed in AM-36–treated rats (mean ± SEM). This decrease was significant (F2,10 = 4.52, P = 0.029). Infarct volume was reduced by 45%, 71%, and 82% when drug administration began 30, 60, or 180 minutes after stroke induction, respectively. A similar trend was seen when striatal infarct volume was calculated with 0%, 35%, and 68% reductions when drug treatment began 30, 60, and 180 minutes after stroke, respectively. This trend was not quite statistically significant (F2,10 = 4.52, P = 0.059).

All rats, including sham-injected animals, lost weight after surgery (Table 2). Weight loss was greater in rats after ET–1–induced stroke. Rats treated with AM-36 at 30 minutes after stroke had significantly greater weight loss than sham-operated animals. Weight loss in rats given AM-36 at 60 or 180 minutes after stroke did not differ significantly from vehicle-treated controls. Core (rectal) temperatures, measured 3 hours after stroke and 1 and 2 hours after administration, did not differ significantly between treatment groups (Table 3).

Functional Assessment

Vehicle-treated control rats exhibited significantly higher neurological deficit scores than sham-injected controls (Table

Histopathological Analyses

Rats showed neurological deficits indicative of stroke (but not stress or vocalization) within 5 minutes of injection of ET-1, validating the correct placement of the cannula. These deficits included circling in the direction contralateral to the occlusion and failure to extend the contralateral forepaw. Some rats showed loss of righting reflex on the side contralateral to MCA occlusion. Rats showing no neurological deficits were deemed not to have had a stroke and were excluded from the study. Histopathology performed on the brains 72 hours after injection of ET-1 showed a pattern of damage similar to that found by Sharkey and colleagues18 and included consistent lesions in the parietal and insular cortex, variable degrees of damage in the frontal cortex, and infarction most often in the dorsolateral parietal and insular cortex, variable degrees of damage in the cortex but sometimes extending throughout the corpus striatum. Sham-injected rats showed only localized damage associated with the tract of the guide cannula.

Infarct area in the cortex was significantly reduced at several stereotaxic levels by AM-36 (3 × 6 mg/kg IP) when administration of the first dose was delayed by either 30, 60, or 180 minutes after MCA occlusion (Figure 1). AM-36 significantly reduced damage in striatum, but only when administration of the first dose was delayed by 180 minutes after the induction of stroke (Figure 1). Cortical infarct volume was significantly reduced by AM-36 irrespective of the time of administration after stroke induction (Table 1). There was a significant linear trend for the degree of protection to increase as the time delay in drug administration increased (F2,10 = 6.45, P = 0.029). Infarct volume was reduced by 45%, 71%, and 82% when drug administration began 30, 60, or 180 minutes after stroke induction, respectively. A similar trend was seen when striatal infarct volume was calculated with 0%, 35%, and 68% reductions when drug treatment began 30, 60, and 180 minutes after stroke, respectively. This trend was not quite statistically significant (F2,10 = 4.52, P = 0.059).

Results

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Functional Assessment

Vehicle-treated control rats exhibited significantly higher neurological deficit scores than sham-injected controls (Table

TABLE 2 | Change From Presurgery Body Weight in Sham-Injected, Vehicle-Treated, and AM-36–Treated Rats Measured After Surgery and 24, 48, and 72 Hours After Ischemia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Before Ischemia</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>8</td>
<td>3.6±2.0</td>
<td>−5.4±2.5</td>
<td>−6.1±3.8</td>
<td>−6.0±3.4</td>
<td></td>
</tr>
<tr>
<td>Vehicle control</td>
<td>11</td>
<td>0.1±1.8</td>
<td>−12.2±3.5*</td>
<td>−12.3±5.0*</td>
<td>−11.5±5.3*</td>
<td></td>
</tr>
<tr>
<td>AM-36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min after ET-1</td>
<td>4</td>
<td>−5.5±0.3</td>
<td>−25.0±9.3†</td>
<td>−29.8±11.7‡</td>
<td>−26.0±8.8‡</td>
<td></td>
</tr>
<tr>
<td>60 min after ET-1</td>
<td>5</td>
<td>−4.4±3.5</td>
<td>−15.8±3.1†</td>
<td>−12.4±2.3†</td>
<td>−12.2±3.3†</td>
<td></td>
</tr>
<tr>
<td>180 min after ET-1</td>
<td>4</td>
<td>−3.0±4.6</td>
<td>−10.5±4.9*</td>
<td>−10.5±4.6*</td>
<td>−13.3±4.9†</td>
<td></td>
</tr>
</tbody>
</table>

Values are change in body weight (mean ± SEM), expressed in grams.

*P<0.05, †P<0.01 compared with 0 hours (ie, before ET–1–induced stroke).

‡P<0.05 compared with sham (ANOVA followed by Duncan’s test).

TABLE 3 | Change in Core (Rectal) Temperatures Before and After Ischemia and Before and After AM-36 Administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time After Ischemia, min</th>
<th>Before AM-36*</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>37.8±0.2</td>
<td>0.3±0.4</td>
<td>0.6±0.3</td>
<td>0.4±0.4</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>37.2±0.3</td>
<td>0.2±0.3</td>
<td>0.3±0.2</td>
<td>−0.1±0.3</td>
</tr>
<tr>
<td>AM-36</td>
<td>38.4±0.3</td>
<td>−0.6±0.8</td>
<td>−0.4±0.4</td>
<td>−1.1±0.4</td>
</tr>
<tr>
<td>30 min after ET-1</td>
<td>37.0±0.1</td>
<td>0.3±0.6</td>
<td>−0.1±0.4</td>
<td>0.0±0.3</td>
</tr>
<tr>
<td>60 min after ET-1</td>
<td>38.1±0.3</td>
<td>0.2±0.3</td>
<td>−0.3±0.2</td>
<td>−0.7±0.2</td>
</tr>
</tbody>
</table>

Values are change in temperature (mean ± SEM) (°C), unless otherwise indicated (n=4–5 for AM-36–treated rats, n=8 for vehicle control rats, and n=11 for sham rats). There were no significant differences between treatments or between time intervals within treatments (P>0.05, ANOVA).

*Basal temperature.
Treatment with AM-36 at 30 minutes after stroke did not improve neurological outcome. Neurological deficit scores were significantly higher than those of vehicle controls in this group 24 hours after stroke but had improved at 72 hours. Rats treated with AM-36 at 60 or 180 minutes after stroke showed deficits at 24 hours. By 48 and 72 hours, neurological scores were improved and no longer significantly differed from those of sham-injected animals. Rats in the group treated with AM-36 at 180 minutes had statistically significantly better neurological deficit scores than vehicle-treated controls at 48 and 72 hours after stroke.

In the accelerating rota-rod test, each animal acted as its own control, and performance was compared with preischemia (0 hours after ischemia) results (Figure 2). Surgery had no effect on performance since there was no significant difference in presurgery and prestroke (postsurgery) performance in any of the treatment groups. In sham-injected rats, rota-rod performance did not significantly alter over time (Figure 2). Vehicle-treated control rats showed significant impairments in performance 24, 48, and 72 hours after stroke compared with prestroke performance (Figure 2). Rats treated with AM-36 at 30 minutes after stroke showed significant improvement at 24 and 48 hours compared with postsurgery scores. Variability was high at 72 hours, and no statistically significant differences were detected. When administration of AM-36 was delayed until 60 or 180 minutes after stroke, performance no longer differed from postsurgery performance when assessed at 24, 48, and 72 hours after stroke.

Spontaneous locomotor activity did not significantly differ between control, drug-treated, or sham-operated rats at any of the times tested (data not shown).

Latency to touch and remove an adhesive tape from the contralateral forepaw was consistently and significantly increased in control vehicle-treated rats (Figures 3 and 4). In comparison, there was no change compared with before stroke in the time taken to touch or remove a tape simulta-

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**Table 4. Neurological Deficit Score 24, 48, and 72 Hours After Ischemia in Sham and Vehicle-Treated Rats and After Delayed Administration of AM-36**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>8</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>11</td>
<td>2.3±0.3†</td>
<td>2.1±0.4‡</td>
<td>2.2±0.4‡</td>
</tr>
<tr>
<td>AM-36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 min after ischemia</td>
<td>4</td>
<td>3.8±0.3† §</td>
<td>3.5±0.3†</td>
<td>3.0±0.4†</td>
</tr>
<tr>
<td>60 min after ischemia</td>
<td>5</td>
<td>1.6±0.7*</td>
<td>0.8±0.1</td>
<td>1.0±0.8</td>
</tr>
<tr>
<td>180 min after ischemia</td>
<td>4</td>
<td>2.3±1.8†</td>
<td>0.5±0.3§</td>
<td>0.5±0.9§</td>
</tr>
</tbody>
</table>

Values are neurological deficit score (mean±SEM).

*P<0.05, †P<0.01, ‡P<0.001 compared with sham; §P<0.05 compared with vehicle control (Kruskal-Wallis nonparametric ANOVA and Mann-Whitney test for analysis of individual differences).

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**Figure 2.** Effects of delayed administration of AM-36 on rota-rod performance after MCA occlusion in rats. Vehicle (A) or AM-36 was administered 30 (B), 60 (C), or 180 minutes (D) after ET-1–induced stroke. Sham-injected rat data are shown in E. Performance before ET-1 injection (0 hours) is shown for comparison. Data are mean±SEM for rota-rod performance expressed as a percentage of presurgery performance for each individual rat. *P<0.05, **P<0.01 compared with 0 hours (ANOVA followed by Newman-Keuls test).
neously placed on the ipsilateral forepaw; that is, control vehicle-treated rats showed sensory hemineglect on the side contralateral to the MCA occlusion after ET-1–induced stroke. AM-36 had no significant effects on the increases in latency to touch or remove tapes when the first dose was administered 30 minutes after stroke. However, when the first dose was delayed until either 60 or 180 minutes after stroke, the difference in latency between contralateral and ipsilateral forepaws was reduced, and the latency to touch and remove tapes returned to presurgery times by 72 hours after stroke.

Discussion

This study demonstrates that the novel neuroprotective compound AM-36 greatly attenuates both cortical and striatal...
damage and also improves functional outcome after MCA occlusion in rats. Interestingly, the effects of this compound on both histological and functional outcome were most evident when administration of the first dose was delayed by 60 or 180 minutes after stroke. In fact, a significant linear trend for greater histological improvement with increased delay in time of administration of AM-36 was found with respect to cortical damage. A similar but not statistically significant trend was observed for striatal damage. However, a marked reduction in striatal damage was found in rats treated with AM-36 beginning 180 minutes after stroke. The striatum is generally considered to be the core of the ischemic lesion and previously has proved relatively refractory to neuroprotection.

Unlike some N-methyl-D-aspartate antagonists, which have a very narrow window of opportunity in focal ischemia models (e.g., MK801 is not effective if not administered within 30 minutes of stroke), Na+ channel antagonists and antioxidants appear to have a longer window of opportunity. For example, PBN reduced brain infarct volume in a rat MCA model of stroke when administered up to 3 hours after ischemia, and the Na+ channel antagonist lamotrigine was neuroprotective when administered 30 minutes or 24 hours after MCA occlusion in rats.

Studies have indicated dramatic increases in levels of oxygen radicals on reperfusion, with levels remaining high for 2 hours and then increasing again at 24 hours. A recent study in a photochemical-induced model of stroke in rats indicates that the period of hydroxyl radical formation most critical for brain damage occurs between 3 and 6 hours after ischemia. The present finding of a greater neuroprotective effect after delayed administration of AM-36 may reflect its greater effectiveness against higher free radical production at this time. Reperfusion most likely occurs after at least 3 hours in this model. ET-1 (120 pmol in 3 μL) resulted in blood flows of <25 mL/100 g per minute (<20% of normal), which were still evident in the striatum and sensory cortex at 3 hours after injection. Hence, it is possible that the greater effectiveness of this compound at 180 minutes may reflect the greater production of reactive oxygen species associated with reperfusion at this time. Alternatively, it could be speculated that maintenance of ionic homeostasis through the ability of AM-36 to block Na+ channels may reach a critical period at approximately 60 to 180 minutes. Previous studies showing effectiveness of delayed administration of Na+ channel antagonists support this notion.

Rectal temperatures did not differ between treatments groups after ischemia, and AM-36 administration had no effect on temperature, excluding the possibility that AM-36 exerted its effects by lowering body temperature. Cerebral blood flow was not measured in this study; however, studies in this laboratory have indicated that AM-36, at higher doses than those used in the present experiments, had no cardiovascular effects in conscious normotensive rats (J.K. Callaway, P.M. Beart, B. Jarrott, R.E. Widdop, unpublished data, 1999). While an effect of AM-36 on cerebral blood flow cannot be ruled out without direct measurement, it is unlikely that AM-36 would have a specific effect on other vascular beds.

Recent studies have emphasized the importance of demonstrating improvement in functional outcome as well as histological improvements when neuroprotective agents are assessed, since embolic stroke in humans leads to lesions and consequent behavioral deficits, including language and motor dysfunction. The importance of choosing functional tests that result in a correlation between functional outcome and histological outcome has been emphasized. The accelerating rota rod is a well-established procedure for testing coordination and balance aspects of motor performance in rats. Deficits in motor performance on the rota rod have been reported after both permanent and transient focal ischemia and are directly related to histological outcome 24 hours after occlusion. Deficits have been reported to still be apparent 2 months after focal cerebral ischemia in the rat. In the present study, significant deficits in performance were evident 24, 48, and 72 hours after ET-1–induced MCA occlusion. In contrast, performance was no longer different from prestroke levels in AM-36–treated rats (60- and 180-minute delayed administration groups) at 72 hours after stroke. This result parallels the trend for greater histological improvement with increased delay in time of administration of AM-36 and indicates protection against motor impairments induced by MCA occlusion.

Sensory hemineglect is a phenomenon that has been reported in humans during the course of recovery from stroke, in patients with damage to the striatum and surrounding white matter, and in patients with right parietal lobe infarction. If 2 stimuli are presented simultaneously, 1 on each side of the body, the contralateral stimulus appears to be masked (extinguished) and remains undetected until the ipsilateral stimulus is removed. Patients report that they can feel both stimuli, although the contralateral stimulus feels subjectively weaker than the ipsilateral stimulus. Sensory hemineglect was evaluated in the present study with a test developed for rats by Schallert and Whishaw. Vehicle-treated control rats showed a consistent sensory hemineglect on the side contralateral to the MCA occlusion, as indicated by an increased latency to both touch and remove a stimulus (an adhesive disk) placed on the contralateral forelimb. Latency to touch and remove a stimulus simultaneously placed on the ipsilateral side was unaffected by ischemia. Treatment with AM-36 either 60 or 180 minutes after stroke effectively removed the hemineglect observed in control rats and returned touch and remove latency to presurgery levels. This effect was most apparent at 48 and 72 hours after stroke. The lack of effect of AM-36 when administered 30 minutes after stroke may reflect the lack of protection found in the striatum. The greatest improvement in this test occurs in those groups that show the greatest reductions in volumes of infarction. Thus, the present results correlate well with histological outcome. Improvement in neurological deficit scores paralleled the findings in the hemineglect test. Vehicle-treated control rats showed significant neurological deficits that were reduced by delaying administration of AM-36 by 60 or 180 minutes but not 30 minutes after stroke.

Recent reports have shown that neurological deficit scores and rota-rod performance are sensitive indicators of behavioral deficits after stroke in experimental animals. Results
from the present study support these findings and provide further evidence that the sensory hemineglect test is a simple test capable of demonstrating a phenomenon in ischemic rats that is known to occur in human stroke patients. This test is also sensitive enough to demonstrate a neuroprotective effect of a compound. No differences in spontaneous activity were observed in sham-operated rats or rats subjected to cerebral ischemia with or without AM-36 treatment. Previous studies have also reported a lack of effect of ischemia on spontaneous activity.\textsuperscript{24} Nevertheless, this simple test serves to indicate that differences in ability in motor performance and sensory neglect tasks are unrelated to an effect of stroke or drug treatment on activity. Weight loss has previously been reported after ischemia and has been shown to increase with duration of ischemia.\textsuperscript{36} The greater weight loss compared with vehicle in rats treated with AM-36 at 30 minutes after stroke is difficult to explain since no differences between vehicle and 60-minute and 180-minute AM-36 groups was found. It is possible that the lack of functional improvements in this group of rats may be related to greater weight loss.

In conclusion, we have now shown that the novel neuroprotective compound AM-36 potently protects against both cortical and striatal neuronal damage in the ET\textsubscript{1}-induced MCA occlusion model of focal ischemia in conscious rats. The effectiveness of this compound increased with the delay in time after administration, a finding that may reflect increased free radical production associated with reperfusion. This compound also appears to provide some protection against motor impairments, sensory hemineglect, and neurological deficits. These findings with AM-36 when taken with the extent of neuroprotection noted, which also includes the normally refractory striatum, suggest that the bifunctional activity of the arylalkylpiperazine, incorporating both Na\textsuperscript{+} channel blockade and free radical scavenging activity, represents a unique strategy for the management of stroke injury.

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References


It is refreshing to see that some groups are concerned with the time window for therapeutic intervention as one of the major factors for successful intervention in ischemic stroke. Gone are the days when drugs that show neuroprotective effects when given before or a short period after MCA occlusion can be considered relevant for therapy of ischemic stroke. Several agents from this group have been evaluated clinically and failed. Other than unacceptable side effects, most of the clinical failures with new therapeutic agents are likely attributable to the fact that when the agent was administered, the targeted event that the therapeutic agent was designed to block had already taken place. If acute neuroprotection is ever to be realized successfully, agents that inhibit critical late-occurring pathophysiological events will have to be developed and administered within a few hours after the stroke.

In the accompanying article, Callaway et al succeeded in developing a novel neuroprotective agent, AM-36, that has a time window for therapeutic intervention of at least 3 hours. It is impressive that efficacy seems to improve as the interval between MCA occlusion and AM-36 administration increases. This feature is unlike most of the previously reported neuroprotective agents in studies of experimental stroke. Future studies will have to be conducted to determine the maximum time window for intervention with this compound. The proposed mechanism of action, Na⁺ channel blockade and antioxidant activity, may account for part of its efficacy, but activation of Na⁺ channels and generation of reactive oxygen species usually occur within the first 3 hours, and this is suggestive that other activities may have a role in the neuroprotective effect of AM-36. Other compounds, such as basic fibroblast growth factor¹ and the calpain inhibitor MDL-28,170, have been reported to have expanded time windows.² In experimental focal stroke, the time window for basic fibroblast growth factor was approximately 3 hours, whereas the time window for MDL-28,170 had an unusually long 6-hour time window for therapeutic intervention. Basic fibroblast growth factor is being evaluated clinically. However, it is surprising that MDL-28,170, with a 6-hour time window, is not being pursued further and has not been replicated experimentally. Thus, if acute neuroprotection from stroke is attainable, agents with expanded time windows, such as AM-36, will likely have an important role in achieving this goal.

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Delayed Treatment With AM-36, a Novel Neuroprotective Agent, Reduces Neuronal Damage After Endothelin-1–Induced Middle Cerebral Artery Occlusion in Conscious Rats
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