The Competitive NMDA Antagonist MDL-100,453 Reduces Infarct Size After Experimental Stroke

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Background and Purpose: The competitive N-methyl-D-aspartate antagonist MDL-100,453 was used to determine whether a neuroprotective effect is demonstrable when the drug is administered beginning 30 minutes after the initiation of focal ischemia and whether the effect is related to blood levels of the drug.

Methods: Forty-eight Sprague-Dawley rats were randomly assigned to one of four intravenous treatment categories: a bolus of 100 mg/kg MDL-100,453 followed by a saline infusion for 24 hours, isotonic saline as a bolus and 100 mg/kg per 24 hours of MDL-100,453 as an infusion over 24 hours, active drug in the bolus and 24-hour infusion, and control treatment of an isotonic saline bolus and infusion. Focal cerebral ischemia was induced by the intraluminal suture, middle cerebral artery occlusion method. The drug infusion was accomplished by an osmotic minipump implanted under the skin and attached to the jugular vein, which delivered drug or vehicle over a period of 24 hours. Infarct volume was calculated using 2,3,5-triphenyltetrazolium chloride staining after 24 hours of middle cerebral artery occlusion.

Results: Infarct volume of animals that received the MDL-100,453 bolus injection followed by MDL-100,453 infusion was significantly smaller than that of controls (P<.01). A significant effect of infusion on the reduction of extent of infarct size was also demonstrated (P=.015). Moreover, a statistically significant inverse correlation was demonstrated between the infarct volume and blood levels of MDL-100,453 at 60 minutes and 120 minutes after injection (r = −.33 and r = −.49, respectively).

Conclusions: We demonstrated a significant neuroprotective effect of MDL-100,453 when treatment was initiated 30 minutes after ischemia began and was maintained for 24 hours.

Key Words: cerebral ischemia • N-methyl-D-aspartate • neuroprotection • rats

In response to an ischemic/hypoxic insult, extracellular concentrations of excitatory amino acids such as glutamate and aspartate are increased due to both enhanced release and impaired uptake.1-4 Elevated levels of extracellular excitatory amino acids cause excessive neuronal stimulation of postsynaptic receptors. The N-methyl-D-aspartate (NMDA) receptor type is one of the most extensively studied subtypes of postsynaptic ionotropic receptors. Several lines of evidence suggest that activation of the NMDA-type receptor in the acute stage of ischemia leads to substantial entry of ionic calcium into the neuron through an ion channel linked to the receptor.5 This results in progressive irreversible neuronal damage by calcium-activated enzyme systems.6-8 NMDA antagonists have been shown to effectively reduce ischemic lesions in animal stroke models, and some are currently being investigated in clinical trials.9,10 MDL-100,453 ([R]-4-oxo-5-phosphononorvaline) is a new competitive antagonist of glutamate at its recognition site on the NMDA receptor complex.11 We investigated the neuroprotective effects of MDL-100,453 administered 30 minutes after the start of focal ischemia and determined whether this neuroprotective effect is related to blood levels of the drug.

Materials and Methods: Nonfasted male Sprague-Dawley rats weighing 278 to 350 g were anesthetized for 3 hours with intraperitoneal chloral hydrate (400 mg/kg body weight). The left femoral artery and vein were cannulated with PE-50 polyethylene tubing for continuous monitoring of arterial blood pressure and blood sampling for analysis of blood gases and drug concentrations. Rectal temperature was monitored and kept at 37°C by a heating lamp positioned 20 cm above the animal.

We used the intraluminal suture, middle cerebral artery (MCA) occlusion model, which we described in detail previously.12 Briefly, an intraluminal oceluder, 4-0 monofilament nylon suture with its tip rounded by flame heating, was introduced through the ligated right common carotid artery (CCA) into the internal carotid artery, then gently advanced intracranially, approximately 17 mm from the CCA bifurcation. With this procedure, the suture occludes unilaterally the proximal anterior cerebral artery, distal internal carotid artery, and the MCA and origins of the posterior communicating artery.13-15 After the onset of focal cerebral ischemia, the right jugular vein was cannulated with PE-60 polyethylene tubing, and a bolus of 0.2 mL of placebo or drug solution (100 mg/kg) was injected over a 2-minute period, 30 minutes after the vascular occlusion. Then the catheter was connected to an osmotic minipump (Model 2001D, Alza Corp) that was implanted under the skin for continuous infusion of the drug over 24 hours. The pump infused 9 μL of the drug solution or placebo for 24 hours, isotonic saline as a bolus and 100 mg/kg per 24 hours of MDL-100,453 as an infusion over 24 hours, active drug in the bolus and 24-hour infusion, and control treatment of an isotonic saline bolus and infusion. Focal cerebral ischemia was induced by the intraluminal suture, middle cerebral artery occlusion method. The drug infusion was accomplished by an osmotic minipump implanted under the skin and attached to the jugular vein, which delivered drug or vehicle over a period of 24 hours. Infarct volume was calculated using 2,3,5-triphenyltetrazolium chloride staining after 24 hours of middle cerebral artery occlusion.

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per hour adjusted by body weight to deliver 4.2 mg/kg per hour of MDL-100,453. The drug concentration in plasma was determined from serial blood drawings (250 μL at each time point, performed just before the bolus injection and at 5, 15, 30, 60, and 120 minutes and 24 hours after the bolus injection) using reverse-phase high-performance liquid chromatography to separate fluorescently tagged amino acids (Waters AccQTag, Millipore). Arterial samples, 300 μL each, were drawn before and 1.5 hours after the MCA occlusion for blood gas analysis. At each blood collection, the same amount of saline was infused. The total loss of blood was approximately 2 mL.

After completion of the 2-hour blood sampling, femoral catheters were removed, and the rats were permitted to recover from the anesthesia. Animals were then allowed free access to food and water. Twenty-four hours after the occlusion, animals were evaluated for evidence of convulsions, tremors, abnormal behavior, and other neurological abnormalities. The animals then were reanesthetized with 500 mg/kg chloral hydrate. Arterial blood at 24 hours was sampled for drug levels by cardiac puncture. The animals were decapitated, and the brains were quickly removed. We inspected the brains to confirm that the MCA orifice was completely occluded by the intraluminal suture and that no subarachnoid hemorrhage had occurred. The brains were sectioned coronally at 2-mm intervals, incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C, and fixed by immersion in 10% buffered formalin solution. Six brain sections per animal were stained with TTC and photographed with a 35-mm camera mounted on an operating microscope. The unstained area was defined as infarcted tissue and measured with the aid of a digitizer (Sigma-Scan v3.10, Jandel Scientific), as was the total right and left hemispheric area for each coronal section. We calculated the corrected infarct volume to compensate for the effect of brain edema.\(^\text{16,17}\) Corrected infarct volume was calculated by multiplying the corrected infarct area by slice thickness. Corrected infarct area was calculated by the following equation: corrected infarct area equals left hemisphere area minus (right hemisphere area minus infarct area).

In a pilot study to determine the adequacy of the minipumps to deliver adequate blood levels of MDL-100,453, we measured the drug concentration levels in plasma during infusion of MDL-100,453 from the osmotic minipump at a rate of 100 mg/kg per 24 hours. In three nonischemic rats, the right jugular vein was cannulated and connected to an osmotic minipump containing 220 μL of MDL-100,453 solution (100 mg/kg), which was implanted under the skin. Blood samples, 250 μL each, were serially drawn at 2, 4, 8, 20, and 24 hours after connecting the minipump.

Another three animals were used to examine the effect of MDL-100,453 on brain temperature. A small copper-constantan thermocouple, 0.003-inch diameter (type IT-23, Physitemp Instruments Inc.), was inserted into the right caudate putamen and fixed to the frontoparietal bone using dental cement. The body temperature was monitored with a rectal probe (T type thermocouple, OMEGA Engineering Inc.) and maintained at 37°C with a heating lamp. After baseline temperature measurements, 100 mg/kg MDL-100,453 was injected intravenously as a bolus, and temperature changes in the brain were continuously monitored over 2 hours using a scanning thermocouple thermometer with 0.1°C resolution (Cole-Parmer Instrument Co.). Temperatures at 15, 30, 60, 90, and 120 minutes after drug injection were compared. After the experiment, the precise location of the thermocouples into the caudate putamen was confirmed by inspection of coronally cut brain slices in all animals.

For the main experiment, animals were randomly assigned before surgery to one of the following four treatment groups. The total number of animals to be studied was predetermined before surgery to one of the following four treatment groups. The total number of animals to be studied was predetermined before surgery to one of the following four treatment groups.

- The first group received a bolus injection of saline followed by an infusion of isotonic saline for 24 hours (S-S group). The second group received a bolus injection of saline followed by an infusion of isotonic saline for 24 hours (S-M group).
- The third group received a bolus injection of 100 mg/kg MDL-100,453 followed by an infusion of saline for 24 hours (M-S group).
- The fourth group received a bolus injection of 100 mg/kg MDL-100,453 followed by an infusion of 100 mg/kg per 24 hours of MDL-100,453 (M-M group). The compound displays a K value of 109 mmol/L for its site of action, measured using [\(1^H\)]-4-(3-phosphonopropyl)-2-piperazine-carboxylic acid binding, and greater than 100-fold selectivity versus other neuroreceptors. It is a potent antagonist of NMDA-mediated biochemical responses, including NMDA-induced elevation of cerebellar cyclic GMP content where it shows an IC\(_{50}\) value of 7 μmol/L and kinetics consistent with competitive inhibition. MDL-100,453 displays rapid (<5 minutes) penetration of the central nervous system after intravenous administration as judged by its time of peak effect in anticonvulsant models (ED\(_{95}\)=8.1 μg/kg versus maximal electroshock in rats; time peak effect is <5 minutes [J.H. Kehne, unpublished data, November 1993]).

MDL-100,453 is acidic due to both the phosphonate and the carboxylic acid groups present in the molecule (Fig 1). This necessitated neutralization of the drug substance with a base to form a concentrated aqueous solution. Drug solutions were prepared by the following method. All solutions were adjusted proportionately according to body weight. For a 300-g rat, 682 μL of 1.0N NaOH was added to 150 mg MDL-100,453. The pH was adjusted to approximately neutral (pH 6 to 8) by adding additional 1.0N NaOH solution if necessary, and the volume was completed to 1 mL by adding saline. We used 0.2 mL of this solution for the intravenous injections. The solution (220 μL) was also used for filling the osmotic minipump reservoir, and the delivery rate was 9 μL per hour. The osmotic minipump was incubated in 0.9% saline for at least 3 hours at 37°C before implantation.

A total of 59 rats including 11 discarded animals were used in this study. The total number of rats for the data analysis was 48. The following were reasons for exclusion: (1) subarachnoid hemorrhage (n=4), (2) the intraluminal suture did not occlude the MCA orifice at postmortem inspection (n=4), and (3) TTC vital staining was not available because of early death within 24 hours (n=3). Overdose of anesthetics was presumed to be the reason for the early death; the 3 dead animals were from the S-M, M-S, and M-M groups, respectively.

After acquiring all data, the randomization code was broken. Incorrect assignment to groups was noted for 2 rats. Therefore, the number of animals in each group was not equal. The numbers of animals in the S-S, S-M, M-S, and M-M groups were 12, 12, 11, and 13, respectively.

For parametric variables, ANOVA and post hoc analysis using the least significant difference were applied to determine the statistical significance of differences between groups. Linear regression analysis was performed to correlate the corrected infarct volume with blood level of MDL-100,453. Val-
The differences of mean values of infarct volume among the four groups were statistically significant (ANOVA, F=2.953, P=.0428); post hoc analysis demonstrated that the infarct volume in the M-M group was significantly smaller than that of the S-S and M-S groups (P<.01 and P<.05). The percentage of reduction of infarct volume was calculated as 46.4% between the S-S and M-M groups. Effects of bolus and infusion were analyzed by a two-factor ANOVA. The effect of bolus and interaction between the effects of bolus and infusion were not statistically significant (F=1.643, P=.21; F=0.46, P=.50). However, the effect of infusion was statistically significant (F=6.40, P=.015). As shown in Fig 2, the mean corrected TTC-infarcted areas (in square millimeters) in the M-M group demonstrated the smallest values for all brain slices; in particular, differences between the S-S and M-M groups were significant in the slices 4 and 6 mm caudal from the frontal pole.

Fig 2 shows the time course for the blood concentration of MDL-100,453 in each group. The mean blood concentrations of MDL-100,453 in the S-S and S-M groups were significantly lower than those of the M-S and M-M groups. Comparing the M-S and M-M groups, the mean blood concentration of the latter group showed significantly higher values at 120 minutes and 24 hours after injection (ANOVA, P<.05). Calculation of correlation coefficients between infarct volume and blood concentration levels of MDL-100,453 at each time point demonstrated statistically significant inverse correlations at 60 and 120 minutes after injection. Correlation coefficients at these time points were r = −.33 (P=.02) and r = −.49 (P=.0005). Fig 4 is a scattergram in which corrected infarcted volumes in each group were plotted against the MDL-100,453 blood level 120 minutes after injection.

### Table 1. Physiological Parameters: Blood Gas Analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>S-S Group</th>
<th>S-M Group</th>
<th>M-S Group</th>
<th>M-M Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>pH</td>
<td>7.341±0.01</td>
<td>7.361±0.006</td>
<td>7.321±0.014</td>
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<tr>
<td></td>
<td>PCO₂</td>
<td>45.3±1.2</td>
<td>45.8±1.0</td>
<td>47.8±1.3</td>
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<tr>
<td></td>
<td>PO₂</td>
<td>89.7±2.0</td>
<td>94.1±2.7</td>
<td>90.8±2.4</td>
</tr>
<tr>
<td></td>
<td>HCO₃⁻</td>
<td>24.5±0.7</td>
<td>23.36±0.6</td>
<td>24.6±0.6</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MCAO indicates middle cerebral artery occlusion; S, saline; and M, MDL-100,453.

### Table 2. Physiological Parameter: Mean Blood Pressure

<table>
<thead>
<tr>
<th>Time</th>
<th>S-S Group</th>
<th>S-M Group</th>
<th>M-S Group</th>
<th>M-M Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>89.5±5.5</td>
<td>90.8±4.5</td>
<td>88.4±2.9</td>
<td>89.4±5.5</td>
</tr>
<tr>
<td>At MCAO</td>
<td>86.8±4.9</td>
<td>90.4±5.1</td>
<td>86.4±4.2</td>
<td>88.5±4.1</td>
</tr>
<tr>
<td>30 Min (IV)</td>
<td>96.1±3.5</td>
<td>106.7±3.9</td>
<td>89.8±4.4</td>
<td>103.1±3.1</td>
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<tr>
<td>60 Min</td>
<td>88.3±4.9</td>
<td>88.4±3.1</td>
<td>82.9±3.9</td>
<td>88.6±2.9</td>
</tr>
<tr>
<td>90 Min</td>
<td>83.5±4.1</td>
<td>92.2±3.5</td>
<td>87.1±4.5</td>
<td>88.9±4.3</td>
</tr>
<tr>
<td>150 Min</td>
<td>84.8±4.0</td>
<td>96.5±3.9</td>
<td>89.5±5.9</td>
<td>92.7±3.6</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MCAO indicates middle cerebral artery occlusion; S, saline; and M, MDL-100,453.

**Results**

In the pilot studies, the drug concentration levels (micromoles per liter) at 2, 4, 8, 20, and 24 hours after connecting the osmotic minipump were 35.7±1.2, 92.2±8.0, 77.3±8.5, 71.3±4.1, and 127.3±51.6, respectively (n=3). Brain temperature was not significantly affected by the injection of MDL-100,453. At baseline, 15, 30, 60, 90, and 120 minutes after injection, the temperatures were 36.6±0.03°C, 36.5±0.03°C, and 36.6±0.03°C, respectively (n=3).

The physiological parameters in the four groups are shown in Tables 1 and 2. In each group, PaCO₂ after MCA occlusion showed slightly lower values than baseline, and a transient elevation of mean blood pressure was noted at 30 minutes after MCA occlusion. These changes were statistically significant and thought to be responses to the brain ischemia. The differences of mean values for each blood gas parameter and mean blood pressure were not statistically significant among the four groups.

At 24 hours after MCA occlusion, no rat showed convulsions, tremors, circling behavior, or ataxia. A depressed level of consciousness was observed in some animals that had large infarcts on postmortem in all four groups.

Corrected infarct volume of the four groups was 194.4±10.7 mm³ (S-S), 150.6±29.4 mm³ (S-M), 180.1±23.8 mm³ (M-S), and 104.2±25.7 mm³ (M-M). The differences of mean values of infarct volume among the four groups were statistically significant (ANOVA, F=2.953, P=.0428); post hoc analysis demonstrated that the infarct volume in the M-M group was significantly smaller than that of the S-S and M-S groups (P<.01 and P<.05). The percentage of reduction of infarct volume was calculated as 46.4% between the S-S and M-M groups. Effects of bolus and infusion were analyzed by a two-factor ANOVA. The effect of bolus and interaction between the effects of bolus and infusion were not statistically significant (F=1.643, P=.21; F=0.46, P=.50). However, the effect of infusion was statistically significant (F=6.40, P=.015). As shown in Fig 2, the mean corrected TTC-infarcted areas (in square millimeters) in the M-M group demonstrated the smallest values for all brain slices; in particular, differences between the S-S and M-M groups were significant in the slices 4 and 6 mm caudal from the frontal pole.

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Discussion

MDL-100,453 is a competitive NMDA antagonist that, with in vitro biochemical assays, appears very similar to other competitive NMDA antagonists. However, in animal models it has several properties that make this an interesting and differentiated approach for neuroprotection. First, MDL-100,453 acts rapidly, achieving peak anticonvulsant effects as early as 2 minutes after intravenous administration. In contrast, CPPene (D(-)(E)-4-(3-phosphonoprop-2-enyl)piperazine-2-carboxylic acid) and CGP 37,849 (DL-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid), also competitive NMDA antagonists being developed as neuroprotectants, exhibit a delayed pharmacologic effect after intravenous administration (at least 1 hour). This is a significant liability given our assumptions about the "window of opportunity" for such an agent to ameliorate ischemic stroke. Second, MDL-100,453 has a favorable therapeutic index with at least an eightfold separation on intravenous doses producing neurological impairment versus doses demonstrating anticonvulsant effects. Finally, MDL-100,453 has been shown to be free of cardiovascular side effects in both anesthetized and conscious dogs (P.R. Kastner, unpublished data, November 1991).

In the clinical setting, this type of neuroprotective drug will be administered after the onset of stroke. It is important that a significant neuroprotective effect of MDL-100,453 was demonstrated with postischemic administration. A statistically significant difference of the mean corrected infarct volume was demonstrated between the group receiving an initial bolus of MDL-100,453 followed by a 24-hour infusion and the group receiving placebo. Some NMDA antagonists such as MK-801 reduce brain temperature. Such a brain temperature reduction itself can reduce the extent of ischemic neuronal injury. We demonstrated that MDL-100,453 did not have direct effects on brain temperature. The significant reduction of infarct size induced by MDL-100,453 therefore cannot be attributed to effects on brain temperature.

Although the mean blood concentration levels up to 60 minutes after the initiation of the treatment were not different in the MDL-100,453 bolus and placebo infusion group when compared with the combined MDL-100,453 bolus and infusion group, a statistically significant reduction of infarct volume was not demonstrated in the former group. Infarct volume was inversely correlated with the blood level of MDL-100,453 at 60 minutes and 120 minutes after treatment initiation. Using this paradigm in nonischemic control animals, steady-state blood levels of MDL-100,453 were only observed 4 hours after infusion began in animals that did not receive an initial bolus of the drug. These results imply that the maintenance of adequate blood levels for at least several hours after stroke onset is an important factor in maintaining the neuroprotective effect of MDL-100,453 acutely after the onset of focal ischemia.

Most noncompetitive NMDA antagonists, including MK-801, are lipophilic and are able to penetrate the blood-brain barrier after systemic administration. In contrast, the blood-brain barrier penetration of competitive
NMN antagonists such as AP-7 (2-amino-7-phosphonoheptanoic acid), AP-5 (2-amino-5-phosphonoheptanoic acid), and CGS19755 (cis-4-phosphonophenyl-piperidino-2-carboxylic acid) is relatively poor. The rate of penetration of MDL-100,453 into the central nervous system through the blood-brain barrier in the acute stage of ischaemia has not been determined, but our results suggest that substantial penetration occurs because of its obvious neuroprotective effects, which were correlated with plasma drug concentrations during the first few hours of therapy.

The behavioral observations suggest that MDL-100,453 is relatively well tolerated by rats, and behavioral studies in other species are proceeding. Neuronal vacuolization was observed with the noncompetitive NMDA antagonist MK-801, although this vacuolization effect appears to be transient. The potential vacuolization effect of MDL-100,453 has not yet been evaluated.

Our results suggest that MDL-100,453 can substantially reduce infarct volume in a rat stroke model that produces a large infarction. The drug is well tolerated and should be further investigated as a potential cytoprotective agent for human ischaemic stroke.

Acknowledgments

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References


Editorial Comment

Glutamate normally functions as an excitatory neurotransmitter in brain, but glutamate can also be cytotoxic if released in excessive amounts. During hypoxic and/or ischemic insults, excessive release of glutamate
The competitive NMDA antagonist MDL-100,453 reduces infarct size after experimental stroke.
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