D-JNKI1, a Cell-Penetrating c-Jun-N-Terminal Kinase Inhibitor, Protects Against Cell Death in Severe Cerebral Ischemia

Lorenz Hirt, MD; Jérôme Badaut, PhD; Jonathan Thevenet; Cristina Granziera, MD; Luca Regli, MD; Fabienne Maurer, PhD; Christophe Bonny, PhD; Julien Bogoousslavsky, MD

**Background and Purpose**—In 2 models of severe ischemic injury, we have evaluated the neuroprotective action of D-JNKI1, a cell-penetrating and protease-resistant peptide selectively inhibiting the c-Jun-N-terminal kinase (JNK).

**Methods**—Hippocampal slices from newborn rats were subjected to oxygen (5%) and glucose (1 mmol/L) deprivation for 30 minutes. Cell death was evaluated with propidium iodide, and the evoked potential responses were recorded in the CA1 region after stimulation in CA3. Male ICR-CD1 mice were subjected to permanent endoluminal “suture” middle cerebral artery occlusion (MCAo). The lesion size was determined after 24 hours by triphenyl-tetrazolium chloride staining, and neurological scores and rotarod treadmill performance were used to evaluate the neurological outcome.

**Results**—In vitro, D-JNKI1 administration 6 hours after oxygen-glucose deprivation reduced cell death at 24 hours from 21%±8% (n=10) to 5%±3% (n=7, P<0.01). This protective effect was still seen at 48 hours, paralleled by an improved amplitude of the evoked potential response. In vivo, D-JNKI1 administration 3 hours after ischemia significantly reduced the infarct volume from 162±27 mm³ (n=14) to 85±27 mm³ (n=9, P<0.001). The functional outcome was also improved.

**Conclusions**—JNK inhibition prevents cell death induced by oxygen and glucose deprivation in hippocampal slice cultures in vitro and by permanent suture MCAo in vivo. D-JNKI1 is a powerful neuroprotectant in models of both mild and severe cerebral ischemia, with an extended therapeutic window. Further investigations are needed to identify the relevant JNK target(s) mediating ischemic neuronal death. (Stroke. 2004;35:1738-1743.)

**Key Words:** MAP kinase signaling system ■ gene products, tat ■ peptides ■ cerebral ischemia, focal ■ tissue culture
prevented. In vitro D-JNKI1 reduced neuronal death in neuronal cultures and in hippocampal slice cultures exposed to N-methyl-D-aspartate (NMDA), most likely by preventing the phosphorylation of JNK targets. A relevant contribution from a blood flow mediated mechanism could be ruled out also because intracerebroventricularly (ICV) injected D-JNKI1 did not alter regional cerebral blood flow.

At the preclinical stage, it is very important to carefully test a neuroprotective compound in more than one model, preferably with longer ischemia durations, in more than one species, and with determination of the window of therapeutic opportunity. We provide here further evidence of D-JNKI1's neuroprotective action and show, for the first time, that this compound provides strong protection in severe ischemia models in vitro in organotypic hippocampal slice cultures subjected to severe OGD with a 6-hour therapeutic window and in vivo in permanent suture MCAo in the mouse with a 3-hour therapeutic window.

Materials and Methods

All experiments were conducted in accordance with guidelines of the Swiss Veterinary Office.

Organotypic Slice Cultures

Organotypic slice cultures of the hippocampus were prepared as described from 10- to 12-day-old rats (OFA Sprague Dawley, IFA-CREDO, France). Coronal hippocampus sections (350 μm) were placed onto sterile porous membrane units (Millicell-CM, Millipore) in wells containing 1 mL of culture medium with 25% horse serum, 50% minimal essential medium supplemented with HEPES and sodium bicarbonate, 25% Hank's salt solution, L-glutamine 2 mM, and D-glucose 35 mM. Cultures were grown at 33°C, 100% humidity, 5% CO2, for 3 days, and then transferred to culture medium with 15% horse serum, containing D-glucose 5 mM, which was changed every 3 to 4 days.

Oxygen and Glucose Deprivation on Organotypic Slice Cultures

The OGD experiments were performed as described in serum-free "hypoglycemic" medium, DMEM (D5030, Sigma) supplemented with 1 mM L-glutamine and 2 mM D-glucose for 30 minutes at 37°C, and then transferred to culture medium with 15% horse serum, containing D-glucose 5 mM, which was changed every 3 to 4 days.

Assessment of the Electrical Recovery After OGD

Extracellular-evoked field potentials were recorded in the hippocampal CA1 region using a multi-electrode array system (Physiocard, Biocell). The CA1 region was subjected to a frequency of 1 stimulation every 2 minutes, at 30 minutes and 48 hours after OGD, on 11 to 14 different slices per condition. The slices were perfused (0.1 mL/min) with an electrolytically medium (NaCl, 126 mM; KCl, 2.5 mM; MgCl2·6 H2O, 1 mM; CaCl2·2 H2O, 2 mM; NaH2PO4, 3 mM; HEPES, 20 mM; and glucose, 5 mM). The maximum spike amplitudes of the repeated stimulations were averaged for each slice for the 30-minute recording period and expressed in mV.

Assessment of the Cell Death on Hippocampal Slices

Cell death was determined in CA1 using the fluorescent viability indicator propidium iodide (PI) on 7 to 10 different cultures containing 4 slices each. PI was applied at 50 μg/mL, 30 minutes before the measurements in each dish. PI fluorescence emission (excitation wavelength 568 nm) was measured 24 and 48 hours after ischemia using an epifluorescence microscope with a 5x lens coupled to a camera (Leica). PI images were acquired with standardized camera settings, and the optical density was measured with ScionImage software (Leica). After subtracting the background fluorescence (from control slices not subjected to OGD), the results were expressed as percentage of maximal cell death obtained by submerging slices in PBS for 24 hours. The cell death was averaged for the 4 slices of each culture.

Permanent Middle Cerebral Artery Occlusion

Male ICR-CD1 mice (Harlan, Netherlands, 22 to 34 g) were anesthetized and maintained under 1% halothane in 30% oxygen and 70% nitrous oxide with a face mask. Regional cerebral blood flow (rCBF) was measured in all animals by laser-Doppler flowmetry (Perimed) with a flexible probe fixed on the skull (1 mm posterior and 6 mm lateral from bregma) until 10 minutes after onset of ischemia. Ischemia was induced by introducing an 11 mm silicone-coated 8-0 filament from the common carotid artery into the internal carotid artery as described. Rectal temperature was maintained at 37°C ± 0.5°C with a control unit (FHS) during anesthesia; thereafter, animals were maintained in an incubator at 31°C. Two μL of either 20 μmol/L D-JNKI1 solution or vehicle (1 μL PBS, pH 7.4) were randomly injected intracerebroventricularly (0.9 mm laterally, 0.1 mm posteriorly, 3.1 mm deep from bregma) using a Hamilton syringe. For blood pressure measurements, the right common carotid artery was cannulated with a PE-10 catheter connected to a pressure transducer. The animals were euthanized after 24 hours. Infarction volumes were quantified by an observer blinded to the treatment group on 2 mm coronal sections stained with 2% 2,3,5-triphenyltetrazoliumchloride using ScionImage. Infarction volumes were calculated by summing the volumes of each section or, indirectly, using the following formula: (contralateral hemisphere − undamaged ipsilateral hemisphere) × contralateral hemisphere.

Neurological Deficits

Before euthanasia, the neurological deficit was rated by an observer blinded to the treatment group. No observable deficit was rated as 0 (O), failure to extend the forepaw, 1: circling, 2: and loss of circling or of righting reflex, 3: Intermittent circling was graded as 1.5.

Rotarod Test

Mice were placed on an accelerating rotating cylinder (UgoBasile), and latency to fall was recorded before 900 seconds; the better of 2 trials was selected. Mice were trained 3 days before ischemia and tested before euthanasia. Performances were expressed as a percentage of the performance of the day before ischemia.

JNK Assay

Samples (10 μg) of homogenates from ischemic hemispheres were incubated for 2.5 hours at 4°C with 1 μg of GST-c-Jun (amino acids 1 to 89) coupled to 10 μL glutathione agarose beads. Kinase reactions were initiated by the addition of 5 μCi of [γ32P]ATP; reaction products were separated on 10% denaturing gels, which were dried and autoradiographed.

Statistical Analysis

Data were expressed as mean ± SD. Statistical evaluation was performed using Instat (GraphPad). For 2 groups, data were compared with the Mann–Whitney test. Comparisons of the data of 3 groups were carried out by ANOVA followed by posthoc Tukey–Kramer multiple comparisons test, or by Kruskall–Wallis test followed by Dunn multiple comparison test in case of nonparametrical data.
neurological scores) or in case of small sample sizes. Probability values of ≤0.05 were considered significant.

**Results**

**D-JNKI1 Protects Organotypic Cultures Against OGD**

Organotypic slice cultures were subjected to hypoxia (5%) with hypoglycemia (1 mmol/L) for 30 minutes. Cell death was evaluated by PI staining and electrical recovery by measuring the amplitude of evoked field potentials. This approach showed that D-JNKI1 significantly reduced cell death after OGD (Figure 1), and that the surviving neurons kept functional synaptic connections (Figure 2). With D-JNKI1 administration 6 hours after OGD, we observed a strong reduction in cellular death from 21% ± 8% of total

![Figure 1](http://stroke.ahajournals.org/)

**Figure 1.** Oxygen glucose deprivation (OGD) on hippocampal slice cultures caused neuronal death of 24% ± 8% of neurons after 24 hours, evaluated by propidium iodide staining (A and E). Administration of D-JNKI1 6 hours after OGD reduced neuronal death to 4% ± 2% (P < 0.05, C and E). Administration of D-JNKI1 3 or 9 hours after OGD did not result in reduced neuronal death (B, D, and E). * indicates statistical significance. Scale bar: 500 μm.

![Figure 2](http://stroke.ahajournals.org/)

**Figure 2.** Forty-eight hours after OGD, hippocampal slices were placed on a microelectrode array (A). Extracellular-evoked potentials were recorded in the CA1 region (in blue, A) after stimulating in CA3 (electrodes S1, S2, in red; A). In control slices (B) there were large evoked potential amplitudes, compared with reduced amplitudes in vehicle-treated OGD slices (C). Treatment with D-JNKI1 6 hours after OGD partially rescued the evoked potential response (D). The mean-evoked potential amplitude was significantly (P < 0.05) greater in D-JNKI1–treated slices than in vehicle-treated slices (E). * indicates statistical significance.
There was no detectable cell death after exposing hippocampal cultures (4 slices per well) to 20 μmol/L, 66 μmol/L, 200 μmol/L, and 666 μmol/L of D-JNKI1 during the time frames of 24 hours (n=3), 48 hours (n=2), or 72 hours (n=1).

**D-JNKI1 Administration 3 Hours After Permanent MCAo Reduces the Infarct Volume and the Functional Impairment**

In permanent MCAo, leading to large lesions in this strain, the infarct volumes were reduced by D-JNKI1 administration 3 hours after ischemia, from 162 ± 27 mm³ (n=14) to 85 ± 27 mm³ (n=9; P<0.001, Figure 3A). Administration after 6 hours did not reduce the infarct volume (147 ± 58 mm³, n=4). In the group treated after 3 hours, there was a reduction in infarcted areas on coronal sections 3 (from 25.3 ± 4.5 mm² to 14.1 ± 7.3 mm², P<0.01), 4 (from 21.9 ± 9.0 mm² to 7.15 ± 7.3 mm², P<0.01), and 5 (from 10.3 ± 3.7 mm² to 2.0 ± 2.5 mm², P<0.05) (Figure 3B). In these mice, the indirect infarct volumes (corrected for swelling) were reduced from 54% ± 16% of contralateral hemisphere to 29% ± 7% (P<0.01).

D-JNKI1 administration 3 hours after MCAo improved neurological scores (reduced from median 2.0, minimum 1, maximum 3, to median 1.25, minimum 1, maximum 2,
Physiological Parameters

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<th>Vehicle (n=14)</th>
<th>D-JNKI1 Treated After 3 Hours (n=9)</th>
<th>D-JNKI1 Treated After 6 Hours (n=4)</th>
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<td>rCBF during ischemia, % of baseline</td>
<td>16±3</td>
<td>14±7</td>
<td>13±6</td>
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<tr>
<td>Weight, g</td>
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<td>28±4</td>
<td>27±2</td>
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<tr>
<td>Weight at death, g</td>
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<td>23±4</td>
<td>25±2</td>
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<tr>
<td>Temperature during ischemia, °C</td>
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<td>37.1±0.5</td>
<td>37.3±0.4</td>
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<td>Temperature at sacrifice, °C</td>
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<td>33.6±0.8</td>
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<tr>
<td>Mean arterial blood pressure, mm Hg</td>
<td>79±11</td>
<td>75±9</td>
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P<0.05; Figure 4A); as expected, administration 6 hours after MCAo did not improve scores. The Rotarod treadmill performance of vehicle-treated mice after permanent MCAo was reduced to 15%±10% (n=8) of the performance on the day before ischemia. Mice treated 3 hours after MCAo with D-JNKI1 performed better (72%±48%, n=6, P<0.01) than vehicle-treated ischemic animals, but those treated 6 hours after MCAo did not (32%±23%, n=6) (Figure 4B).

Our results, obtained in this model of severe focal cerebral ischemia, show a significant decrease in lesion size as well as an improved behavioral outcome.

There was no difference in body weight, rectal temperature (during surgery, at euthanasia), or rCBF between D-JNKI1-treated and vehicle-treated animals (Table). Mean arterial blood pressure was not different before or 30 minutes after ICV injection of D-JNKI1 (Table). As shown in Figure 5, the peptide (10 μmol/L) prevents the phosphorylation of the JNK substrate c-Jun.

Discussion

We provide new evidence of the broad neuroprotective action in ischemia, both in vitro and in vivo, of D-JNKI1, a peptide selectively preventing the interaction of JNK with its substrates.5 D-JNKI1 reduced the infarct volume by 47% with ICV administration 3 hours after permanent suture MCAo in the mouse. Importantlty, this substantial reduction in infarct volume was accompanied by an improved functional outcome, attested both by neurological scores and by the rotarod treadmill performance. The model was not suited to examine the infarct volumes at later time points because of the high mortality beyond 24 to 48 hours. As expected, the degree of protection was lower and the therapeutic window shorter than in the transient 30-minute MCAo in the mouse (>90%, 6 hours, ICV) and the permanent distal MCAo in the newborn rat (78%, 6 hours and 49%, 12 hours, IP), where the protection persisted at later time points.5 The degree of protection after permanent MCAo compares with the 36% reduction in infarct volume shown in the case of basic fibroblast growth factor administration after 2 hours in the rat,19 with the 42% reduction in cortical infarct seen with free radical trapping agent NXY-059 after 4 hours.20 A longer time window was reported by Iadecola and coworkers21,22 who showed that aminoguanidine, an inhibitor of the inducible nitric oxide synthase, given as late as 24 hours after permanent rat MCAo, reduced the neocortical infarct volume by 26% with an improved functional outcome. Interestingly, these authors observed that an earlier administration, 6 hours after MCAo, did not provide a significant neuroprotection.22 We made a similar observation in our in vitro paradigm where D-JNKI1 administration consistently reduced cellular death on hippocampal slice cultures with attenuated impairment of synaptic transmission when given 6 hours after OGD, but not when given after 3 hours. This observation raises the question of the kinetics of the interaction between JNK and its targets, suggesting that the phosphorylation of JNK substrates is detrimental, but not at every time point. It is noteworthy that D-JNKI1 is protective after reperfusion in the transient MCAo model, a situation that compares to a successful thrombolysis, and also in severe permanent ischemia,5 reflecting the common situation where thrombolysis could not be performed or did not result in repermeabilization. Again this shows the broad action of the compound. We also know that JNK is activated in the permanent suture MCAo as early as after 5 minutes,5 long before we administer the inhibitor. We do not, therefore, prevent phosphorylation of JNK targets before this time point. Our current opinion is that kinetics of availability and phosphorylation of JNK targets is critical. JNK has numerous cytoplasmic and nuclear targets, and which of them plays a relevant function in ischemic and cell death needs further investigation.

The present study shows that D-JNKI1 protects in 2 paradigms of severe ischemia. This is important, as other previously described neuroprotective agents, such as dextromethorphan, were shown to protect in transient but not in...
permanent MCAo. In clinical practice, strokes range from very mild to very severe, and it is important that a protection is obtained at all levels. It is noteworthy that D-JNKI1 has been shown to be protective after reperfusion in the transient MCAo model, a situation that compares to a successful thrombolysis, but also in severe permanent ischemia; thus, D-JNKI1’s action reflects the common situation where thrombolysis could not be performed or where thrombolysis did not result in repermeabilization, which again shows the broad action of the compound. Our results extend those of Borsello and coworkers and establish the neuroprotective action of D-JNKI1 in several models, with different experimental conditions ranging from mild to severe, with an extended therapeutic window in all cases, thus confirming that it is a promising compound to achieve neuroprotection in stroke.

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References

19. Li Q, Stephenson D. Postischemic administration of basic fibroblast growth factor improves sensorimotor function and reduces infarct size following permanent focal cerebral ischemia in the rat. Exp Neurol. 2002;177:531–537.
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