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

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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

Stroke is a leading cause of disability and mortality in industrialized countries. Although thrombolysis is now widely used in selected ischemic stroke patients in the acute phase,1 neuroprotective approaches have so far failed to prove beneficial in clinical practice. This study demonstrates a significant reduction of cell death and of functional impairment by a cell-penetrating peptide, which selectively inhibits the c-Jun-N-terminal kinase (JNK) when given 6 or 3 hours, respectively, after ischemia and in 2 different models of severe ischemic injury: an oxygen and glucose deprivation (OGD) paradigm on rat organotypic slices and a severe stroke model in the mouse.

JNK, a mitogen-activated kinase, has been shown to play a role in excitotoxic neuronal death, as JNK3-deficient mice are resistant to kainate-induced seizures and apoptotic neuronal death in the hippocampus.2 JNK is activated in focal cerebral ischemia in the mouse and mediates neuronal death.3–5 Its numerous cytoplasmic and nuclear targets include transcription factors and proteins involved in apoptosis or in the cell cycle (for review see Mielle and Herdegen6). To investigate its role in cell death, a peptide inhibitor was designed to block the interaction between JNK and its targets:7 JNKI1 is a peptide containing a 20-aa sequence of the JNK binding domain of Islet-Brain-1 (IB-1)/JNK-interacting protein-1 (JIP-1), a scaffold protein, combined to a 10-aa TAT sequence of the HIV TAT protein, allowing intracellular translocation.7 JNKI1 is capable of inhibiting JNK in vitro in a very specific manner.5,7 Its D-retro-inverso form D-JNKI1 (made of D-amino acids in reversed sequence order) retains the capacity of JNK inhibition and of translocation, and has an extended activity as it is resistant to degradation by proteases.7 D-JNKI1 has recently been shown to provide protection in 2 models of mild focal cerebral ischemia: 30 minutes endoluminal (“suture”) middle cerebral artery occlusion (MCAo) in the mouse and permanent distal MCAo in young rats, with extended therapeutic windows.5 As the protection was still seen after 2 and 1 weeks, respectively, it was shown that neuronal death was not delayed but, indeed,

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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.5

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 mmol/L, and d-glucose 35 mmol/L. Cultures were grown at 33°C, 100% humidity, 5% CO2 for 4 days, and then transferred to culture medium with 15% horse serum, containing d-glucose 5 mmol/L, 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 mmol/L d-glucose and 2 mmol/L L-glutamine. This medium was equilibrated for 1 hour at 37°C in a humidified hypoxic chamber (COY) with a “hypoxic” atmosphere of 5% O2, 5% CO2, and 90% nitrogen, and the slices were transferred into this “buffered medium” and into the hypoxic chamber for 30 minutes. Control cultures were placed in DMEM medium supplemented with 5 mmol/L d-glucose and 2 mmol/L L-glutamine for 30 minutes at 37°C, in a humid normoxic atmosphere. For recovery, cultures were placed in culture medium at 33°C for 24 or 48 hours. D-JNKI1 in PBS (final concentration 47.1 ng/mL or 12 mmol/L) or an equal volume of PBS was added 3, 6, or 9 hours after OGD.

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) by 6 electrodes, after stimulating in the CA3 region at 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 electrophysiological medium (NaCl, 126 mmol/L; KCl, 2.5 mmol/L; MgCl2·6 H2O, 1 mmol/L; CaCl2·2H2O, 2 mmol/L; NaH2PO4, 3 mmol/L; HEPES, 20 mmol/L, and glucose, 5 mmol/L). 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 5× lens coupled to a camera (Leica). PI images were acquired with standardized camera settings, and the optical density was measured with ScionImage software (Scion Corp). 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 (Hurlan, 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×PBS, pH 7.4) were randomly injected intracerebroventriculally (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-triphenyl-tetrazoliumchloride 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, 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 tries 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 neurons.

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**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.** 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.
Forty-eight hours after OGD, we recorded the evoked potential (EP) in 6 different points localized in CA1 induced by electrical stimulation in CA313 (Figure 2A). In control slices without OGD, the EP amplitudes were 1.2 mV for the 6 electrodes (Figure 2B). In slices treated after 6 hours with D-JNKI1, the EP amplitudes were higher (1 mV, Figure 2C) than in vehicle-treated slices after OGD (0.4 mV, Figure 2D). Comparison of the mean EP amplitude from the 6 electrodes, with repeated stimulations during 30-minute time frames on 14 to 17 slices per group, showed reduction of amplitude in slices subjected to OGD (0.36 ± 0.19 mV, n = 14), compared to vehicle control slices (0.83 ± 0.29 mV, n = 11; P < 0.001, Figure 2E). Treatment with D-JNKI1 significantly attenuated the reduction of field potential amplitude (0.63 ± 0.22 mV, n = 12, P < 0.05), demonstrating an improved functional recovery by treatment with D-JNKI 6 hours after OGD.

**Toxicity of D-JNKI1 on Hippocampal Slice Cultures**

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,18 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,
The JNK substrate c-Jun (arrow) was phosphorylated in brains from 2 animals (A and B), 1 hour after permanent MCAo. Figure 5. JNK assay with activated JNK isolated from ischemic brains from 2 animals (A and B), 1 hour after permanent MCAo. The JNK substrate c-Jun (arrow) was phosphorylated in absence of inhibitory peptide (−). A 30-minute preincubation with 10 μmol/L inhibitory peptide (+) induced an approximately 4-fold inhibition.

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.6 D-JNKI1 reduced the infarct volume by 47% with ICV administration 3 hours after permanent suture MCAo in the mouse. Importantly, 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,6 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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