AS601245 (1,3-Benzothiazol-2-yl (2-[[2-(3-pyridinyl) ethyl] amino]-4 pyrimidinyl) Acetonitrile): A c-Jun NH₂-Terminal Protein Kinase Inhibitor with Neuroprotective Properties

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ABSTRACT

Recent evidence suggests that activation of the c-Jun NH₂-terminal protein kinase (JNK) signal transduction pathway may play a role in ischemia-induced cell death. Thus, preventing the activation of JNK, or c-Jun phosphorylation could be neuroprotective. In the current study, we report that a small molecule, AS601245 (1,3-benzothiazol-2-yl (2-[[2-(3-pyridinyl) ethyl] amino]-4 pyrimidinyl) acetonitrile), which has been shown to inhibit the JNK signaling pathway, promotes cell survival after cerebral ischemia. In vivo, AS601245 (40, 60, and 80 mg/kg) administered i.p. provided significant protection against the delayed loss of hippocampal CA1 neurons in a gerbil model of transient global ischemia. This effect is mediated by JNK inhibition and therefore by c-Jun expression and phosphorylation. A significant neuroprotective effect of AS601245 administered either by i.p. injection (6, 18, and 60 mg/kg) or as i.v. bolus (1 mg/kg) followed by an i.v. infusion (0.6 mg/kg/h) was also observed in rats after focal cerebral ischemia. These data suggest that the use of JNK inhibitors such as AS601245 may be a relevant strategy in the therapy of ischemic insults.

c-Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein kinases that are characterized as proline-directed serine-threonine-protein kinases. JNK is activated in response to a number of extracellular stimuli, including inflammatory cytokines (Minden et al., 1994; Sluss et al., 1994), UV irradiation, heat shock, and ischemia (Davis, 1999; Kyriakis and Avruch, 2001). Upon activation by phosphorylation of threonine and tyrosine residues, JNK phosphorylates c-Jun (on serine residues 63 and 73), which heterodimerizes with Fos proteins to form the transcription factor activator protein 1 (AP-1). In complex with other DNA binding proteins, AP-1 regulates the transcription of numerous genes, including cytokines [interleukin-2 and tumor necrosis factor (TNF)-α], growth factors, inflammatory enzymes, and matrix metalloproteinases. Three JNK genes (JNK-1, -2, and -3) have been identified. JNK1 and JNK2 have a broad tissue distribution, whereas JNK3 seems primarily localized to neuronal tissues, testis, and cardiac myocytes. JNK has been shown to be involved in the immune response (Weiss et al., 2000), cancer (Kennedy and Davis, 2003), and apoptosis (Xia et al., 1995; Lin, 2003).

Several in vivo studies have reported activation of the JNK signaling pathway after cerebral ischemia (for reviews, see Nozaki et al., 2001; Irving and Bamford, 2002). Sugino et al. (2000) suggested that neuronal death induced by ischemia and ischemia/reperfusion may be in part mediated by activation of the JNK signaling pathway. A brief period of cerebral ischemia in gerbil causes neuronal death in the CA1 region of hippocampus several days after the ischemic insults. This phenomenon is known as “delayed neuronal death” (DND) (Kirino, 1982) and is supposed to be apoptotic (Nitatori et al., 1995), although the mechanisms are not completely clarified. It is interesting to note that increased JNK phosphorylation has been reported 15 min after global ischemia in the gerbil (Ferrer et al., 1997; Sugino et al., 2000; Tsuji et al., 2000),
and increased phospho-c-Jun levels were detected in the hippocampus after 15 min of global ischemia in rats (Gillardon et al., 1999; Hu et al., 2000). Increased activity of the JNK signaling pathways was also observed after focal ischemia (Herdegen et al., 1998; Hayashi et al., 2000). Supporting the idea that neuronal death-induced by ischemia is in part due to JNK activation, Tsuji et al. (2000) have demonstrated that the neuroprotective effect of α-phenyl-N-tert-butylnitrate, a spin trap agent, on the CA1 region of hippocampus after global ischemia, is due to its ability to block JNK activation. In addition, Raymon et al. (2001) have shown neuroprotective effects of the JNK inhibitor SPC9766 in a model of stroke. Together, these observations indicate a potential role of the JNK signaling pathway in ischemic injury and the importance of inhibition of the JNK signaling pathway to reduce the injury.

In the present study, we report on a small molecule that acts as a novel ATP-competitive JNK inhibitor and significantly protects the brain from ischemic injury. 1,3-Benzothiazol-2-yl-{[2-(3-pyridinyl) ethyl] amino}-4 pyrimidinyl) acetonitrile (AS601245) belongs to a new class of benzothiazole acetonitrile derivatives, which proved to be structurally unique JNK inhibitors. We assessed the effects of AS601245 on neuronal death after cerebral ischemia in two in vivo models: a transient model in gerbils and a focal model in rats, using histological and immunohistochemistry evaluation approaches.

AS601245 provided significant protection against the delayed loss of hippocampal neurons in the gerbil model of global ischemia. A neuroprotective effect of AS601245 was also observed in rats after focal cerebral ischemia. Moreover, treatment with AS601245 decreased the c-Jun ischemia-induced activation, confirming the role of the JNK pathway in ischemic damage. Thus, inhibition of JNK by AS601245 could be a relevant strategy in the therapy of ischemic insult.

Materials and Methods

Reagents
Carboxymethyl-cellulose (CMC), Tween 20, Triton X-100, cresyl violet acetate, formaldehyde 37%, bovine serum albumin, 3.3′-diaminobenzidine, and (±)-MK-801 were purchased from Sigma-Aldrich (St. Louis, MO).

2-Methylbutane, methylcyclohexane, and entellan were purchased from Merck Biosciences (Darmstadt, Germany). Polyonal antibody directed against phospho-c-Jun (Ser73) was obtained from Cell Signaling (Hertfordshire, England). Vectastain avidin-biotinylated enzyme complex and biotinylated goat anti-rabbit secondary antibody were purchased from Vector Laboratories (Burlingame, CA).

Animals
Wistar rats and C57/HEN mice were purchased from Charles River France (L’arbresle, France). Mongolian gerbils were purchased from Elevage Janvier (Le Genest St. Isle, France). All animals were kept in a temperature-controlled (20 ± 1°C) and light/dark cycle-controlled animal room (lights on at 7:00 AM and off at 7:00 PM). Standard laboratory chow (UAR, Villeemoisson-sur-orge, France) and water were available ad libitum. The experimental protocol was designed in accordance with the declaration of Helsinki, which complies with the Switzerland code of practice for the care and use of animals for scientific purposes.

JNK3 Kinase Assay

GST-JNK3 (1 mg/ml) was incubated overnight at room temperature with 0.05 mg/ml of GST-JNK2, in a solution containing 200 μM ATP-γ-S, 1 mM dithiothreitol, 10 mM MgCl2, and 100 μM Na3VO4, followed by dialysis against 50 mM Tris-HCl, pH 8.0, 150 mM NaCl/5 mM dithiothreitol overnight at 4°C to get ride of the ATP-γ-S.

Rat JNK3 assays are performed in 96-well low binding Corning MTP plates: 0.5 μg of recombinant, preactivated GST-JNK3 was incubated with 1 μg of recombinant, biotinylated GST-c-Jun and 2 μM [32P]ATP (2 nCi/μl), in the presence or absence of compounds according to formula I and in a reaction volume of 50 μl containing 50 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 1 mM dithiothreitol, and 100 μM Na3VO4, for 120 min and at room temperature. The reaction was stopped by the addition of 200 μl of a solution containing 250 μg of streptavidin-coated SPA beads (Amersham Biosciences Inc., Otelfingen, Switzerland), 5 mM EDTA, 0.1% Triton X-100, and 50 μM ATP, in phosphate saline buffer and further incubation at room temperature for 60 min. After incubation, beads were sedimented by centrifugation at 1500g for 5 min, resuspended in 200 μl of phosphate-buffered saline (PBS) containing 5 mM EDTA, 0.1% Triton X-100, and 50 μM ATP and the radioactivity was measured in a scintillation beta counter, following further sedimentation of the beads by settling down for 60 min at room temperature. Similar method was used to demonstrate inhibition of JNK1 and JNK2.

Protein kinase selectivity assays were performed as described previously (Davies et al., 2000). Briefly, protein kinases were assayed for their ability to phosphorylate the appropriate peptide/protein substrates. Assays were performed using 10 μM ATP. IC50 values were determined in triplicate.

LPS-Induced TNF-α Release in Mice

Mice received an oral treatment with AS601245 (0.3, 1, 3, or 10 mg/kg in 0.5% CMC/0.25% Tween 20). Fifteen minutes later, endotoxins (O111:B4; 0.3 mg/kg; Sigma-Aldrich) were i.p. injected. Heparinized whole blood was collected by retro orbital puncture under isoflurane anesthesia. TNF-α was determined in plasma using an enzyme-linked immunosorbent assay kit (R&D Systems, Abingdon, UK) following the manufacturer’s instructions. Control animals received 0.5% CMC/0.25% Tween 20 (10 ml/kg) as vehicle.

Transient Global Ischemia Model (Two-Vessels Occlusion Gerbils)

Surgery. Gerbils weighting 60 to 80 g were anesthetized with 4% isoflurane (Baxter, Volketswil, Switzerland) in medical air, administered via face mask. The anesthesia was then maintained using 3% isoflurane until the end of surgery. Bilateral common carotid arteries were dissected and occluded with bulldog clamps for 5 min. In the sham-operated group, the same procedure was performed without carotid occlusion.

Histology. Seven days after the onset of occlusion, the animals were killed by decapitation. The brains were frozen at −20°C in 2-methylbutane and cut into 20-μm-thick sections in a Cryo-Cut (Microm HM 500 OM; Microm Laborgeräte GmbH, Walldorf, Germany). The sections were stained with cresyl violet acetate, and the lesion in the hippocampus was scored with a 5-point scale (Gronborg et al., 1999): 0, no loss of CA1 neurons; 1, weak damage of CA1 (CA1/Subiculum or CA1/CA3 border); 2, loss of CA1 neurons (<1/2); 3, loss of CA1 neurons (>1/2); and 4, total loss of CA1 neurons and expanding into other areas (CA3, dentate gyrus, cortex). The total score was obtained as the sum of scores in the right and left hemispheres.

Experiment 1. Gerbils (n = 6) were treated with AS601245 (40, 60, or 80 mg/kg i.p.) administered as pretreatment (1 h before and 24 h after 5 min of ischemia) or post-treatment (15 min and 24 h after 5 min of ischemia). Control animals received 0.9% saline (10 ml/kg i.p.).

Experiment 2. The second experiment, we tested only one dose of
AS601245 (80 mg/kg i.p.) administered at different times after the reperfusion: 15 min and 24 h, 1 h and 24 h, and 2 h and 24 h. Control animals received 0.9% saline (10 ml/kg i.p.).

**Immunohistochemistry.** Animals were perfused transcardially with cold 0.1 M PBS, followed by cold 4% buffered paraformaldehyde. Brains were removed and postfixed for 24 h at 4°C and then transferred to 20% sucrose (Fluka, Steinheim, Germany) in PBS at 4°C overnight. Frozen coronal sections (40 μm in thickness) of the brains were prepared using a Cryo-Cut. The sections were processed by the free-floating method. After washing three times in PBS-T (1% Triton X-100 in PBS), quenching endogenous peroxidase in 2% H2O2 (Fluka), and blocking with 30% bovine serum albumin, the sections were incubated overnight at 4°C with a polyclonal antibody against phospho-c-Jun (Ser73) (p-c-Jun) diluted in PBS-T. The sections were washed three times in PBS-T and incubated in secondary antibody according to the recommended dilutions for 1 h. After three washes with PBS-T, the sections were incubated for 30 min in the Vectastain Elite ABC reagent according to recommended procedures. The immunoreactivity was visualized with a 3,3′-diaminobenzidine substrate kit. Negative control sections received identical treatment except for the primary antibody. Each section was mounted on glass slides, air-dried, dehydrated in ascending ethanol series, immersed in methylocyclohexane, and cover slipped with entellan. The number of p-c-Jun-labeled cells in three consecutive sections was manually counted across the CA1 region of hippocampus, and mean values were calculated.

**Experiment 1.** The gerbils were divided into four groups (n = 6) and sacrificed 2, 4, 24, and 40 h after the reperfusion, respectively. Sham-operated animals were used as control group.

**Experiment 2.** Gerbils (n = 6) were treated with AS601245 (20, 40, or 60 mg/kg i.p.), injected 15 min and 5 h after the reperfusion, and sacrificed 24 h after 5 min of ischemia. Control animals (n = 6) received 0.9% saline (10 ml/kg i.p.).

**Transient Focal Ischemia Model (Middle Cerebral Artery Occlusion Rats)**

**Surgery.** Focal cerebral ischemia was induced with a method adapted from Nagasawa and Kogure (1989) and from Longa et al. (1989). Rats were anesthetized with 4 to 5% isoflurane in air. The blood flow from the ICA, anterior cerebral artery, and posterior cerebral artery was not occluded. The catheter was tied in place with a silk suture. A puncture was made in the CCA and a small catheter (Harvard Apparatus Inc., South Natick, MA) was gently advanced to the area of the catheter by heat leaving 1 cm of catheter protruding so it could be withdrawn to allow reperfusion. The skin incision was closed by using skin clips. The animals were placed in a cold environment until recovery from anesthesia. After 2 h of ischemia, reperfusion was performed by removing the suture until the tip cleared the ICA lumen. Rectal temperature was recorded (Physitemp type BAT-12; Harvard Apparatus Inc.) from ischemia onset and up to 5 h after reperfusion time.

**Histology.** The animals were sacrificed by decapitation 24 h after the onset of ischemia. Immediately after decapitation, the brains were removed and frozen at −20°C in 2-methylobutane. They were then cut into 20-μm-thick coronal sections by a Cryo-Cut at −19°C. One in every 40 sections (every 800 μm) from the frontal to the occipital cortex was used for histological quantification of the extent of the cerebral lesion. The sections were stained with cresyl violet. Each section was examined under a light microscope, and regional infarct areas were determined according to the presence of cells with morphological changes. Areas of neuronal injury or infarction were plotted using the software NIH Image (version 1.62; National Institutes of Health, Bethesda, MD) from the coronal sections of individual rats. Cortex and striatum volumes were calculated for each animal (sum of mean of two consecutive surfaces × thickness 0.8 mm).

**Experiment 1.** Rats were randomly divided into a vehicle-treated control group, a reference compound-treated group, and a drug-treated group. The reference compound-treated group received MK-801 (3 mg/kg i.p.) administered 1 h postischemia onset. The drug-treated group received AS601245 (6, 18, or 60 mg/kg) administered at the initiation of reperfusion and 5 h later. Control animals received 0.9% saline (10 ml/kg i.p.).

**Experiment 2.** Rats were randomly divided into three groups as described above. The reference compound-treated group received MK-801 (3 mg/kg i.p.) administered 1 h postischemia onset. The drug-treated group received an intravenous bolus of AS601245 (1 mg/kg) injected at the initiation of reperfusion followed by an intravenous infusion with a flow of 0.6 mg/kg/h during 22 h. Control animals received a bolus plus an intravenous infusion of 0.9% saline (10 ml/kg).

**Statistical Analysis**

Data obtained from experiments were expressed as the mean of independent experiments ± S.E.M. and analyzed using one-way analysis of variance followed by Dunnett’s t test (*p < 0.05, **p < 0.01, and ***p < 0.001).

**Results**

**Enzyme Assays.** AS601245 demonstrated a nonspecific inhibition of the three JNK human isoforms (hJNK1, hJNK2, and hJNK3) with an IC50 value of 150, 220, and 70 nM, respectively. AS601245 inhibits isolated hJNK3 in an ATP-competitive manner.

Selectivity of AS601245 was tested against a large panel of kinases (Table 1). It exhibited 10- to 20-fold selectivity over c-src, CDK2, and c-Raf and more than 50- to 100-fold selectivity over a range of Ser/Thr- and Tyr-protein kinases.

**LPS-Induced TNF-α Release in Vivo.** AS601245 was a potent inhibitor of LPS-induced TNF-α release in mice. Administered orally at 0.3, 1, 3, and 10 mg/kg, AS601245 de-
creased the TNF-α release in a dose-dependent manner (Table 2).

Neuroprotective Effects of AS601245 on Gerbil Brain after Global Cerebral Ischemia. As reported in the literature (Kirino, 1982), 5 min of bilateral common carotid arteries occlusion in Mongolian gerbil produces a delay and selective neuronal death in the hippocampus, which occurs from about 3 to 7 days, a phenomenon known as DND. To establish whether AS601245 showed any protective effect on DND in vivo, animals were treated with AS601245 or saline after global ischemia.

In animals receiving saline, a score of 6 was obtained, indicating that the ischemic insult has successfully produced a selective neuronal death in the CA1 and CA2 subfields of hippocampus. A clear reduction in the ischemic score compared with saline-treated animals was observed in animals treated with AS601245 (Fig. 1). Figure 1A shows that administration of AS601245 (40, 60, or 80 mg/kg i.p.) 1 h before and 24 h after the reperfusion (pretreatment), decreased the hippocampal damage in a dose-dependent manner (16, 74, or 83%, respectively). Additionally, when injected 15 min and 24 h after the reperfusion (post-treatment) at the same doses, AS601245 also decreased hippocampal damage by 29, 40, or 52%, respectively. AS601245 remained also active with 37% of inhibition (Fig. 1B) when the first administration (80 mg/kg i.p.) was given 1 h after the reperfusion.

Increase in c-Jun Phosphorylation after Global Cerebral Ischemia in Gerbil. A study was performed to establish the kinetics of JNK activity after 5 min of bilateral carotid arteries occlusion. An anti-phospho-c-Jun (S73) polyclonal antibody was used to label sections from hippocampus from gerbils, because it is known that the ability of c-Jun to activate gene transcription is potentiated by phosphorylation at serine S73 and to a lesser extent at S63 (Pulverer et al., 1991).

A low expression of phospho-c-Jun was found in sham-operated animals (Fig. 2B) but not in nonoperated animals (Fig. 2A). This is in agreement with the role of stress enzyme played by JNKs (Kyriakis and Avruch, 2001). The levels of phospho-c-Jun expression, however, peaks at approximately 3-fold above control levels 24 h after the reperfusion (Fig. 2E); thereafter, the level declines, and it is only marginally above control levels 40 h after reperfusion (Fig. 2F). The results are shown in Fig. 2G. In contrast, no significant increased phospho-c-Jun levels were observed in the CA3 region. To verify the binding specificity for phospho-c-Jun, sections were incubated with only the secondary antibody (no primary). No positive staining was found in the sections, indicating that the immunoreaction was selective for phospho-c-Jun in the experiments performed.

AS601245 Inhibits Ischemia-Induced c-Jun Phosphorylation. Animals were treated with AS601245 or saline, after 5 min of bilateral carotid occlusion to determine whether AS601245 treatment is able to decreased phospho-c-Jun activation induced by the ischemic insults. Vehicle-treated animals showed a significant induction of phospho-c-Jun (Fig. 3A). The administration of AS601245 (20, 40, and 60 mg/kg i.p.) caused a significant (p < 0.01) reduction in the number of phospho-c-Jun-stained neurons at the highest dose. The reduction seemed dose-dependent, with a maximum reduction obtained with a dose of 60 mg/kg (Fig. 3D).

Neuroprotective Effects of AS601245 on Rat Brain after Focal Cerebral Ischemia. In addition to the model of global ischemia, we investigated the potential neuroprotec-

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### Table 2

<table>
<thead>
<tr>
<th>Treatment, mg/kg p.o.</th>
<th>TNF-α pg/ml</th>
<th>Inhibition</th>
<th>p &lt; 0.01</th>
</tr>
</thead>
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<tr>
<td>Control LPS</td>
<td>3383 ± 105</td>
<td>88 ± 2</td>
<td>0.001</td>
</tr>
<tr>
<td>Dexmethylasone, 0.1</td>
<td>417 ± 67</td>
<td>27 ± 6</td>
<td>0.001</td>
</tr>
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<td>AS601245, 0.3</td>
<td>2483 ± 213</td>
<td>49 ± 4</td>
<td>0.001</td>
</tr>
<tr>
<td>AS601245, 1</td>
<td>2022 ± 50</td>
<td>49 ± 4</td>
<td>0.001</td>
</tr>
<tr>
<td>AS601245, 3</td>
<td>1719 ± 129</td>
<td>49 ± 4</td>
<td>0.001</td>
</tr>
<tr>
<td>AS601245, 10</td>
<td>1317 ± 73</td>
<td>61 ± 2</td>
<td>0.001</td>
</tr>
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Fig. 1. Effect of AS601245 on neuronal damage in the hippocampus region after a 5-min period of global ischemia in the gerbil. A, AS601245 was injected at the doses of 40, 60, and 80 mg/kg i.p., −1 h and +24 h (pretreatment) or +15 min and +24 h (post-treatment) after the reperfusion. B, AS601245 (80 mg/kg i.p.) was administered at different times after the reperfusion (+15 min and +24 h; +1 h and +24 h or +1 h and +24 h). The histological score was quantified 7 days after the reperfusion using a 4-point scale as described in detail under Materials and Methods. Values are means ± S.E.M. *, p < 0.05 and **, p < 0.01, compared with the control group.
tive properties of AS601245 in a transient model of focal cerebral ischemia in male rats. Two separate studies were performed. In both experiments vehicle-treated animals had lesions in the right hemisphere in the cortex and in the striatum. The cerebral hemisphere contralateral to the MCA occlusion showed no lesioned areas. Neuroprotection was assessed in animals treated with AS601245 or with MK-801, a noncompetitive N-methyl-D-aspartate antagonist known to exert significant neuroprotection in models of focal ischemia (Hatfield and McKernan, 1992).

In the first experiment, AS601245 was administered at the initiation and 5 h after the reperfusion. The severity of the infarct volume was reduced in both AS601245- and MK-801-treated groups compared with saline-treated group. AS601245 significantly reduced the size of the cortical lesions after MCA occlusion, by 27 and 52% when injected at the dose of 18 and 60 mg/kg i.p., respectively. No significant effect was observed when AS601245 was given at the dose of 6 mg/kg. MK-801 (3 mg/kg i.p.), given 1 h after the ischemia onset, significantly reduced the size of the cortical and striatal lesion (Fig. 4).

The neuroprotection was also assessed administering AS601245 as an i.v. bolus (1 mg/kg), at the initiation of the reperfusion, followed by an i.v. infusion (0.6 mg/kg/h), during 22 h after the onset of the ischemia. This first set of data confirmed the results obtained in the model of global ischemia. The i.v. infusion of AS601245 significantly reduced the cerebral infarct by 45% to the same range as observed with MK-801 (3 mg/kg i.p.) injected 1 h after the ischemia onset (Fig. 5).

**Discussion**

In the current study, we were able to demonstrate for the first time that a small molecule, AS601245, belonging to a novel class of JNK inhibitors, produced significant neuroprotection in two different experimental models of stroke-induced brain injury.

Experimental models of stroke have been developed in many species using different procedures (Ginsberg and Busto, 1989; Hossmann, 1998). Among the existing models, many suffer from poor reproducibility and standardization. Two models that are more satisfactory in this respect are the model of global transient ischemia in gerbils and the model of focal ischemia in rats. The potent neuroprotection of AS601245 was demonstrated by the use of these two different models of ischemic stroke.

First, we examined the effect of AS601245 in gerbils submitted to global ischemia. In this model, neurons of the CA1 region of the hippocampus have been shown to be highly vulnerable, and the global model of cerebral ischemia is the most popular for evaluating neuroprotective agents (Small and Buchan, 2000). In fact, in the Mongolian gerbil 5 min of bilateral carotid occlusion induced neuronal death mainly in the CA1 and CA2 pyramidal area of the hippocampus, because this species lacks posterior communication arteries necessary to complete the Circle of Willis. This area of neuronal degeneration can be evaluated in individual animals accurately and it is reproducible in all animals.

![Fig. 2. Phospho-c-Jun expression in the CA1 region of hippocampus after a 5-min period of global ischemia in the gerbil.](image-url)
Although significantly protective in this model, it seems that fairly high doses of AS601245 (60 and 80 mg/kg) are required to oppose ischemic insults when administered during the reperfusion phase, whereas a more efficient neuroprotection was observed before the ischemic insult. Various parameters may account for the lack of efficacy of the compound when administered at low doses (i.p. route). As already mentioned, activation of JNK and c-Jun phosphorylation in the brain occurs very rapidly during the occlusion phase and right at the start of the reperfusion phase. It is therefore important to have a rapid and sustained penetration of the compound into the brain to exert a rapid inhibition of c-Jun phosphorylation. Pharmacokinetic studies have revealed that compound AS601245 was demonstrating rapid and high plasma exposure by i.v. and i.p. (bioavailability 70%) route. Distribution to the brain is accounting for 10 to 15% of the total dose injected with absorption/elimination pattern following the one observed in plasma.

We therefore anticipated that the maintenance of a constant plasma concentration (~5-fold above the IC_{50} of AS601245) would maximize the efficacy of the JNK inhibitor. Consequently, it was decided to optimize the route of administration as well as the treatment setup at the stage of the preclinical model of middle cerebral artery occlusion in rats.

The mechanisms leading to the ischemic brain damage are complex and only partially understood. During ischemia, many factors are released, including cytokines, growth factors, and glutamate, all of which are found to activate the JNK signaling pathway.

It is well established that neuronal death induced by ischemia could be in part due to JNK activation and that inhibition of the JNK signaling pathway can significantly protect the brain from stroke injury (Sugino et al., 2000). AS601245 reduces ischemia injury in the gerbil model of global ischemia by blocking the activation of JNK, thereby by inhibiting the proapoptotic effects of its downstream effector c-Jun.

Global ischemia induces the appearance of phospho-c-Jun-positive cells in the CA1 and CA2 region of the hippocampus. It is interesting that no increase in phospho-c-Jun levels was detected in the CA3 region of hippocampus. Five minutes of
bilateral carotid occlusion in the gerbil produces a selective lesion in CA1 cells in the hippocampus, and there is a very little damage in the cortex and striatum (Kirino, 2000). Therefore, the absence of c-Jun activation in the CA3 region showed that the distribution of phospho-c-Jun correlates well with selective vulnerability of neurons to global ischemia. It is known that in some cases, artifactual staining (resulting from nonspecific antibody binding to similar antigenic determinants in the tissue) for c-Jun in dying cells exists because of cross-reactivity with caspase-dependent products or with products of autophagy. In our study, we selected the antibody and we included immunohistochemistry controls (see Materials and Methods) to avoid artifactual staining.

The ischemia-induced phospho-c-Jun expression was attenuated by AS601245, providing an explanation as to the putative mechanism through which AS601245 blocks the loss of hippocampal pyramidal cells.

It is well known that apoptosis contributes to the development of ischemic infarction both in global (Nitatori et al., 1995) and focal ischemia (Li et al., 1995; Yao et al., 2001). We found that administration of AS601245 protected CA1 pyramidal cells with a reduced number of terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive cells (unpublished observations). Recently reported data (Tournier et al., 2000; Lin, 2003) strongly suggest that JNK activation play a role in apoptosis. It is still controversial whether JNK is an intrinsic component of the apoptotic machinery or like Lin (2003) suggested, whether activated JNK inactivates suppressors of the apoptotic machinery. It would be interesting in future experiments to determine whether inhibition of JNK by AS601245 blocks ischemia-induced apoptosis through control of mitochondria release and/or caspases inactivation.

AS601245 has been evaluated in the preclinical model of focal ischemia in rats. AS601245 injected i.p. significantly reduced the ischemic damage as it did in gerbils, but low doses such as 18 mg/kg are still effective. The different dosing regimen may be in part due to the fact that different models result in different pathology. The focal model produces a necrotic core where neurons died and a salvageable penumbra where the neurons can be saved if appropriate interventions are taken. On the contrary, in the global model, only certain groups of cells are selectively injured (the CA1 hippocampal cells being the most vulnerable followed by cells in the striatum and cerebral cortex). Moreover, gerbils versus rats can have different thresholds for pharmacological agents or have different pharmacokinetic properties (metabolism and biodistribution in brain area).

The intravenous route of administration was selected to define the optimal doses to be used during preclinical studies and thus the neuroprotective effects of AS601245 were assessed by administration as an i.v. bolus followed by an i.v. infusion to maintain a constant plasma concentration all along the reperfusion phase. The reduction in infarct volume (50%) was similar to that obtained with the i.p. administration, but the doses used here are lower (14.2 mg/kg/22 h). A possible explanation is that with an i.v. infusion, we obtained a higher and constant brain concentration that is able to block stably the JNK activation.

Evidence has accumulated showing that the JNK signaling pathway is activated during focal ischemia (Herdegen et al., 1998; Hayashi et al., 2000). MCA occlusion provokes a strong expression of p-c-Jun that became detectable 3 h after the onset of ischemia and was maximal at 72 h. Although the mechanism of action of AS601245 has not been studied in the model of focal ischemia, these previous studies support the hypothesis that the pharmacological efficacy of AS601245 could be due to JNK inhibition.

An additional contribution to the neuroprotective effect of AS601245 against focal ischemia relied on its effect on TNF-α release. Focal cerebral ischemia in rats produces elevated levels of TNF-α in the ischemic brain region (Bertorelli et al.,
2001). Furthermore, TNF-α production in the brain has been demonstrated after exposure of the brain to endotoxins (Bredier et al., 1994). The toxic effects of TNF-α during focal ischemia may involve different mechanisms. It is well known that TNF-α increases capillary permeability and opens the blood-brain barrier (Meyeri et al., 1992). Moreover, it causes damage to myelin and oligodendrocytes and plays a role in the inflammatory processes (Robbins et al., 1987; Selmaj and Raine, 1988). The inflammatory response of cells to TNF-α is mediated by the regulation of gene expression by the AP-1 and nuclear factor-κB groups of transcription factors (Baud and Karin, 2001). Ventura et al. (2003) have shown that JNK-deficient cells exhibited defects in the regulation of the AP-1-related transcription factors and that this change was associated with marked defects in TNF-α-regulated gene expression.

Together, these data corroborate the hypothesis that the neuroprotection demonstrated by AS601245 in the two models of cerebral ischemia is due to its ability to block the JNK signaling pathway and suggest that AS601245 may be neuroprotective not only by acting on neurons but also by reducing the consequence of inflammation and apoptosis. Although we cannot exclude, due to the ATP nature of the inhibitor and the number of existing kinases, that inhibition of other signaling pathways might be involved, these results clearly demonstrate that the neuronal protection observed with AS601245 is related to JNK phosphorylation blockade.

In conclusion, we have demonstrated that a small molecule inhibiting JNK is able to protect neurons from ischemic injury, in two different models of cerebral ischemia. In addition, we demonstrated that the activation of the JNK signaling pathway could be in part responsible for the tissue injury after the ischemia insult, suggesting that the inhibition of JNK by AS601245 could be a relevant strategy for stroke therapy.

References


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