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MLC901, a Traditional Chinese Medicine protects the brain against global ischemia

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ABSTRACT

Global ischemia leads to damage in the hippocampal CA1 region and is associated with behavioral deficits. NeuroAid (MLC601 and MLC901), a Traditional Chinese Medicine is used in China for patients after stroke. We have investigated here the effects of MLC901 on brain injury and deficits after global ischemia in the rat. Global ischemia induced by four-vessel occlusion resulted in degeneration of CA1 neurons. MLC901 (0.074 mg/ml) prevented both necrosis and apoptosis of neurons up to 3 h after ischemia. These positive MLC901 effects were associated with a decrease in Bax expression and in levels of the lipid peroxidation product malondialdehyde. Using the PI3-kinase inhibitor LY294002 we also demonstrated the critical role of the Akt pathway in MLC901-mediated neuroprotection. MLC901 enhanced neurogenesis. Furthermore, MLC901 improved functional recovery of rats after global ischemia as assessed by the Morris water maze. In this test MLC901 reduced the increase in escape latency and in swim distance induced by ischemia. MLC901 also improved post-ischemic grip strength. If observations made with rats can be extended to humans, then MLC901 will represent a novel therapeutic strategy after cardiac arrest with a clinically interesting time window of protection.

1. Introduction

Global cerebral ischemia is a clinical outcome occurring as a consequence of cardiac arrest, reversible severe hypotension or other situations that deprive the brain of oxygen and glucose. Each year, estimated 350,000 people undergo sudden cardiac arrest in Europe (Herlitz et al., 1999). Recovery without residual neurologic damage after global ischemia is rare. Besides the dramatic clinical aspects of the disease, subsequent neurological injuries represent a considerable financial burden in medical and rehabilitation expenses and lost productivity. Hypothermia has been described as the only therapy which can improve outcome after cardiac arrest (Nolan et al., 2003; Sterz et al., 2006; Zhao et al., 2007). The need for new therapeutic strategies is imperative (Ginsberg, 2008).

The pyramidal neurons of the CA1 region in the hippocampus are among the cells most vulnerable to loss of blood supply to the brain in humans and rodents (Pulsinelli and Brierley, 1979). Cell death occurs days after the ischemic insult, a phenomenon termed delayed neuronal death, which is associated with severe behavioral impairments. Global ischemia when it is not lethal is often followed by some types of recovery. A large part of the recovery after ischemia is associated with the capacity of the brain to induce spontaneous adult neurogenesis, which generates functional neurons in subventricular and subgranular zones of the hippocampus (Alvarez-Buylla and Lim, 2004; Eriksson et al., 1998; Sharp et al., 2002; Di Filippo et al., 2008; Zhang et al., 2008). However, brain self-repair by neuronal replacement from endogenous precursors is insufficient and functional recovery remains incomplete. Amplification of this self-repair mechanism could be a promising strategy for developing restorative therapies for global ischemia.

Interestingly, NeuroAid (MLC601) is a Traditional Chinese Medicine (TCM), which was first registered by the Sino Food and Drug Administration in 2001 after being evaluated in clinical trials in China as a drug to facilitate recovery after stroke (Chen et al., 2009). It combines 9 herbal and 5 animal components. In a previous study, we demonstrated in a rodent model of focal ischemia that MLC601 and also MLC901, which is a simplified version with only the nine herbal components, improved survival, protected the brain from ischemic injury and drastically decreased functional deficits (Heurteaux et al., 2010). MLC601/MLC901 also

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prevented neuronal death in an *in vitro* model of excitotoxicity using cultures of cortical neurons exposed to glutamate. In addition, MLC601/MLC901 treatments have been shown to induce neurogenesis, promote cell proliferation and stimulate the development of a dense axonal and dendritic network. In the present work we investigated the therapeutic effectiveness of MLC901 on brain injury and motor deficits induced by global ischemia (four-vessel occlusion model) in rats. We also deciphered some of mechanisms associated with the effects of this TCM by analyzing MLC901 actions on neuronal cell death (necrosis and apoptosis), lipid peroxidation, phosphinositide-3-kinase/Akt pathway and neurogenesis.

2. Materials and methods

2.1. Animals

All experiments were performed on male Wistar rats (250 g) from Charles River Laboratories (France) and used according to policies on the care and use of laboratory animals of European Communities Council Directive (86/609/EEC). The local Ethics Committee approved the experiments (protocol numbers NCA/2006/10-1 and NCA/2006/10-2). All efforts were made to minimize animal suffering and reduce the number of animals used. The animals were housed under controlled laboratory conditions with a 12-h dark–light cycle, a temperature of 21 ± 2 °C, and a humidity of 60–70% for at least one week prior to drug treatment or surgery. Rats had free access to standard rodent diet and tap water.

2.2. Experimental design

The study was carried out on rats divided into three groups. (i) Sham group, (ii) Ischemic group + vehicle and (iii) Ischemic group + MLC901. MLC901 was provided by Moleac (Singapore). The composition of MLC901/capsule was the following: 0.57 g Radix astragali, 0.114 g Radix salvia miltiorrhizae, 0.114 g Radix paeoniae rubra, 0.114 g Rhizoma chuanxiong, 0.114 g Radix angelicae sinensis, 0.114 g *Carthamus tinctorius*, 0.114 g *Prunus persica*, 0.114 g Radix polygalae, 0.114 g Rhizoma acori tatarinowii. The TCM was diluted in saline (as vehicle) at the concentration of 74 mg/ml (Stock solution) and incubated under agitation for 1 h at 37 °C. The solution was then filtered with 0.22 μm filter. Rats were intraperitonally injected with a single dose of MLC901 (500 μl/rat) 30 min, 1, 2, 3 or 6 h after ischemia followed by one injection per day for three or seven days after reperfusion. The MLC901 intraperitoneal dose used has been selected based on the concentrations used in humans (oral administration: 4 capsules three times a day (Chen et al., 2009)) and reported to the rat according to the formula for dose translation based on BSA (Body Surface Area) from animal to human (Reagan-Shaw et al., 2008). In the neurogenesis and behavioral experiments where the reperfusion time exceeded one week MLC901 was administered in drinking water at the concentration of 10 mg/ml. Researchers, who carried out the ischemic surgery and measures of brain injury were blinded in regard to the treatment code.

2.3. Induction of the transient global ischemia

Forebrain ischemia was performed by four-vessel occlusion (Pulsinelli and Brierley, 1979). In brief, rats were anesthetized by inhalation of 2% halothane mixed with 30% oxygen and 70% nitrous oxide. Core temperature was monitored with a rectal probe. Core temperature was registered from the onset of anesthesia to 1 h reperfusion. Body temperature was maintained at 37 °C with a heating blanket during and in the hours following surgery. Heart rate, blood pressure and percutaneous arterial oxygen saturation were monitored. No difference in physiological parameters was seen between the different groups (data not shown). Animals were placed in a Kopf stereotaxic frame and the vertebral arteries were irreversibly occluded by electrocoagulation. The common carotid arteries were then exposed and a polyester non absorbable suture (Ethicon) was looped around the carotid arteries. On the following day, the animals were ventilated with 1% halothane and miniature aneurysm clips were attached to occlude both carotid arteries. Anesthesia was then disconnected and carotid arteries were clamped for 20 min. Rats lost their righting reflex during ischemia. Following ischemia, reperfusion was achieved by declamping the arteries. Negative controls were sham-operated rats. Each animal was anesthetized, the carotid arteries were isolated, but they were not clamped.

2.4. Tissue preparation and analysis of neuronal density (Cresyl violet and Fluoro-Jade B stainings)

One, 3 or 7 days following ischemia, animals were anesthetized, brains quickly extracted and fresh frozen in isopentane at −40 °C. Coronal sections (10 μm) were cut on a cryostat (Leica) and post-fixed by immersion in 4% paraformaldehyde/10^{-2} M phosphate-buffered saline (PBS) for 30 min. Slides were then dehydrated in ethanol baths (50, 70 and 100%), air-dried and stored at −20 °C until use. Cresyl Violet, a dye that stains the Nissl bodies in the stellate somas of viable neurons was used to analyze the cell survival in the different groups. For each brain studied (n = 6 per time point and treatment), two sections were placed on 3-aminopropylethoxysilane-coated slides, and 10 slides (randomly chosen) per rat were used in each stage of analysis. Slides were dipped into a solution of 1% Cresyl Violet in 0.25% acetic acid for 3 min, rinsed, dehydrated and mounted with Entellan. A neuropathologist, blind to experimental conditions, performed the histological assessment using light microscopy. The neuronal density of the hippocampal CA1 subfield was determined 7 days post-ischemia on cresyl-violet stained sections of the dorsal hippocampus corresponding to brain sections located between 3.14 and 4.16 mm posterior to bregma (Pulsinelli and Brierley, 1979). The total linear length of CA1 sector was measured by means of a digitizer. The number of living neurons in the stratum pyramidale within the CA1 subfield was counted using a Leica Aristoplan photomicroscope at a magnification of 400X. Neurons that had shrunken cell bodies with surrounding empty spaces were excluded. The neuronal density of CA1 sector, i.e. the number of intact pyramidal cells per 1 mm linear length of the CA1 stratum pyramidale observed in each 10-μm section was quantified. Thus, a mean value for each hippocampal CA1 substructure was obtained from 10 bilateral measurements on two sections per slide and 10 slides per rat, for the six animals in each of the experimental groups. The neuronal density for a given animal represents the average of both right and left hippocampal neuronal cell densities. Neuronal density values are expressed as mean ± S.D. Data analysis was performed by two-factor (experimental condition and brain region) analysis of variance followed by Tukey's w test for multiple comparisons.

For assessment of neuronal degeneration, we used staining with Fluoro-Jade B, a polyanionic fluorescein derivative which sensitively and specifically binds to degenerating neurons (both apoptotic and necrotic). Due to its high affinity it is an excellent marker for detecting degenerating neurons. Fluoro-Jade B staining was performed 7 days following ischemia according to the protocol described by Schmued et al (Schmued et al., 1997).

2.5. TUNEL staining

Terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate-biotin nick-end labeling (TUNEL) staining was performed 3 days following ischemia by using the *In Situ* Cell Death Detection Kit (Roche, Basel, Switzerland), which allows to detect the DNA fragmentation of apoptotic cells in ischemic brain tissue. Coronal 10 μm frozen sections were rehydrated in ethanol (95, 70 and 50%) followed by PBS and bathed in 0.3% hydrogen peroxide/methanol to inactivate endogenous peroxidase. Sections were then permeabilized in 0.3% Tween/PBS and washed twice in PBS. Labeling of 3-OH terminal DNA fragments was then performed at 37 °C for 2 h by using the TUNEL reaction mixture according to the manufacturer's protocol. Positive control was obtained by pre-incubation of a section with DNAse I (20 μg/ml) for 15 min at 37 °C before incubation with biotinylated dUTP. Quantitative analyses *i.e* counting of the number of TUNEL-positive cells/0.5 mm^2 were performed in the hippocampal CA1 subfield. TUNEL-positive cells displayed a brown staining within the nucleus in the apoptotic cells.

2.6. Immunohistochemistry

Frozen brain sections (25 μm thick) were immersed in 0.3% H_2O_2/methanol for 10 min, permeabilized in 0.1% Triton/PBS for 10 min and blocked with 3% goat serum/PBS for 2 h at room temperature. After a PBS rinse sections were incubated with the primary antibody overnight. The antibodies used were the rabbit polyclonal Bax antibody (Santa Cruz, SC493, diluted 1/300) or the rabbit polyclonal Akt (1:1000, Cell Signaling Technology, USA), phospho-Ser-473-Akt (p-Akt) (1:1000, Cell Signaling Technology, USA). After the primary incubation and three rinses in PBS, sections were then incubated in biotinylated horse anti-rabbit IgG (Jackson ImmunoResearch, diluted 1/15,000) for 2 h at room temperature. Bax expression was visualized by 3,3-diaminobenzidine (DAB) staining using VectaStain ABC kit (Biovalley). All sections were washed and mounted with Entellan. In the experiments with the PI3-kinase inhibitor LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1benzopyran-4-one), we intracerebroventricularly injected this drug (5 μl bregma, dorsoventral, −3.6 mm below the cortical surface, medio-lateral, 1.4 mm from bregma, antero-posterior,−0.8 mm from bregma) at the dose of 50 nmol in 25% dimethyl sulfoxide in PBS 1 h before ischemia.

2.7. Western blotting

Whole-cell protein extraction was performed using standard procedures. Samples were obtained from ischemic hippocampi (vehicle- and MLC901-treated) and from sham-operated controls. (n = 3 per group). Fresh brain tissue was cut into pieces after 24 h of reperfusion and homogenized in four volumes of cold lysis buffer (20 nmol/l Tris pH: 7.5, 137 mmol/l NaCl, 2 mmol/l EDTA, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail) on ice. The homogenates were centrifuged at 12,000 g for 30 min at 4 °C. The supernatant was stored at −70 °C until further use. Protein concentrations were measured using conventional Bradford's method. Fifty microgram proteins from each experimental group were applied to 10% SDS

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PAGE gels and electrophoresed for 1 h at 100 mA. Proteins were transferred onto a PVDF membrane in blotting buffer (156 mmol/l Tris, 1 mol/l glycine, PBS) for 90 min at 80 mA and blocked with 5% skim milk (Regilait) in PBS for 2 h at room temperature. The blotted membrane was then incubated with the rabbit polyclonal primary antibody against Akt (1:1000, Cell Signaling Technology, USA), phospho-Ser-473-Akt (p-Akt) (1:1000, Cell Signaling Technology, USA) for 2 h at room temperature. Western blots were incubated with horseradish-peroxidase conjugated anti-rabbit IgG (Jackson ImmunoResearch, diluted 1/15,000) for 1 h at room temperature and revealed using DAB kit. To control for sample loading, stripped membranes were rehybridized with a β-actin antibody (1:2000, Proteintech Group, USA) as internal control. Films with specific bands were scanned and quantified using an imaging densitometer. The optical densities of specific bands were analyzed with QUANTITY ONE software (Bio-Rad).

2.8. Analysis of in vivo neurogenesis on brain sections

BrdU treatment consisted of 4 injections (75 mg/kg, *i.p.* each, 2 h interval) (Heurteaux et al., 2010). Brains were removed at 24 h after the last injection. Serial sections of paraformaldehyde-perfused-brains were cut (40 μm) throughout the entire hippocampus on a vibratome (Leica). Every sixth section throughout the hippocampus was processed for immunohistochemistry using a monoclonal mouse anti-BrdU (1/200; BD Biosciences, Le Pont de Claix, France). For BrdU chromogenic immunodetection, sections were then incubated for 1 h in biotin-conjugated species-specific secondary antibodies (diluted 1:100, Vector Laboratories), followed by a peroxydase–avidin complex solution according to the manufacturer's protocol. The peroxydase activity of immune complexes was visualized with DAB staining using VectaStain ABC kit (Vector Laboratories). BrdU-labeled cells of granular and subgranular layers were counted in each section at 400X under a light microscope by a blind experimenter. The phenotype of BrdU-positive cells was determined using fluorescent double-labeling with the following antibodies and dilutions: anti-sheep BrdU (1:200, Interchim, Montluçon, France), anti-goat DCX (1/200, Santa Cruz Laboratories, Heidelberg, Germany), anti-mouse NeuN (neuron specific nuclear protein, 1/250, Millipore, St Quentin en Yvelines, france), GFAP (Glial Fibrillary Acidic Protein, 1/250, Dako cytomaytion, Trappes, France) and secondary antibodies conjugated with Alexa Fluor 488 or 594 (1/1000; Molecular Probes, Leiden, Netherlands). Confocal microscopy observations were performed with a Laser Scanning Confocal Microscope (TCS SP, Leica, Rueil Malmaison, France).

2.9. Determination of lipid peroxidation level

Malondialdehyde (MDA) levels in hippocampal tissue were measured by a fluorescence assay (Lipid Peroxidation Kit, Euromedex, France) (Nguemeni et al., 2010). Briefly, the hippocampi were extracted in butylated hydroxytoluene and homogenized in cold 0.1 M phosphate buffer (pH 7.4) to make a 10% homogenate. Then, the homogenate was centrifuged for 30 min at 3000 g at 4 °C, and 100 μl of supernatant was added to a reaction mixture containing 100 μl of 8.1% sodium dodecyl sulphate, 750 μl of 20%acetic acid (pH 3.5), 750 μl of 0.8% thiobarbituric acid (TBA) and 300 μl distilled water. Samples were then boiled for 1 h at 95 °C and centrifuged at 4000 g for 10 min. In the TBA reaction, MDA or MDA-like substances and TBA react with the production of a pink pigment having an absorption maximum at 532 nm. Absorbance of the supernatant was measured with a spectrophotometer ($UVmc^2$) at 532 nm. Results were expressed as nanomoles of MDA per mg of protein.

2.10. Behavioral testing

2.10.1. Morris water maze test

Spatial learning and memory were tested one and two weeks after ischemia. The circular water maze provides a measure of spatial memory in that animals must locate a submerged platform in a water pool using extra-maze visual location cues (Olsen et al., 1994). A 110-cm-diameter, 50-cm-high circular swimming pool was filled to a depth of 30 cm with water at 25 ± 1 °C. Three walls of the test room were covered with large posters that provided differential spatial cues. The position of the experimenter was constant following placement of the rat into the maze. A second experimenter (not visible to the animal) measured the latency and path distance displayed by each animal to find the submerged platform (diameter 10 cm) by using a commercially videotracking system (Smart, Bioseb, France). The platform was submerged 1.5 cm below the water surface and placed 25 cm from the edge of the tank equidistant from the edges of the quadrant. Water was made opaque with 1 L of shimmed milk and 100 mg of phenol red. The experiment included 2 phases:

Phase I: *Acquisition*. Acquisition training consisted of 4 days of conditioning with 4 trials per day, one and two weeks after ischemia. Each trial entailed placing the animal into the water facing the wall of the pool within one of 4 quadrants NW, NE, SW, SE and tracking its swimming path and duration until it found the platform. If a rat did not find the platform within 60 s, it was guided to the platform. After reaching the platform, rats were allowed to stay there for 30 s.

Phase II: *Probe trials*. On Day 12 and 19, the platform was removed and rats were allowed to swim for 60 s as during acquisition training. The times spent immediately at the former location of the platform and within the quadrant where the pedestal had been located were measured for each of 4 trials. All trials video were recorded and analyzed with a computer image analyzer (SMART, Bioseb).

2.10.2. Grip strength test

Muscle strength and neuromuscular integration relating to the grasping reflex in the forepaws was studied using a grip strength test. One day before surgery and different days after surgery (days 2, 4 and 7), rats were tested using a grip-strength-meter (BIOSEB®, Chaville, France). Animals maintained by their tail gripped with their forelimbs a grid (10 cm × 10 cm) linked to a high precision force sensor. The apparatus automatically records the highest pull force. Rats were tested on three successive daily trials, the mean of all three readings was computed and used for statistical analyses.

2.11. Statistical analysis

All data were expressed as mean values ± S.E.M. One sample corresponded to one animal. The tissues were not pooled. Statistical analysis of differences between groups was performed by using unpaired *t*-test or ANOVA ($n > 30$). When $n < 30$ the non-parametric Kruskal–Wallis *H*-test was used to compare the rank sums of the behavioral parameters. Where *H* ratios were significant, statistical analyses were extended. Pairwise comparisons were then made using a series of Mann–Whitney *U* post-hoc tests with the level of significance set at $P < 0.05$.

3. Results

3.1. Neuroprotective effect of MLC901 on necrotic and apoptotic cell death induced by global ischemia

To assay the potential neuroprotective effects of MLC901 against global ischemia, we carried out a dose-effect response in the rat 4 four-vessel occlusion model. Animals received an intraperitoneal MLC901 injection 30 min post-ischemia followed by one injection per day for seven days after reperfusion at the concentrations/ml of 74, 7.4, 0.74 or 0.074 mg/ml ($n = 6$ per experimental group). The two lower doses of MLC901 injected (0.74 and 0.074 mg/ml) induced a 100% survival rate of rats as compared to vehicle-treated rats (20% of mortality) (Fig. 1A). In contrast, with the highest doses of MLC901 (74 and 7.4 mg/ml), the animals displayed seizures in the 72 h following ischemia leading to a respective mortality of 30 and 90% of mortality (Fig. 1A).

Transient forebrain ischemia (20 min) causes selective degeneration of hippocampal CA1 pyramidal neurons. Counting of the neuronal density in the CA1 region confirmed that global ischemia led to a significant decrease in the number of CA1 pyramidal neurons as compared to Sham rats ($^{***}P < 0.001$) (Fig. 1B). Only 24.5% of CA1 pyramidal cells were preserved. In contrast, MCL901 treatment with the dose of 0.74 or 0.074 mg/ml significantly decreased the neuronal loss induced by ischemia with a pyramidal cell survival of 98 and 84%, respectively ($^{**}P < 0.01$). Based on these results, we chose the dose of 0.074 mg/ml of MLC901 as the reference treatment to analyze the MLC901 time window of protection. We administrated MLC901 at different times after reperfusion (0.5, 1, 2, 3 or 6 h post-ischemia followed by one injection per day until sacrifice, $n = 5$ rats per group). MLC901 treatment provided a significant protection up to 3 h of reperfusion. When treatment was delayed to 3 h after the end of ischemia, the number of survived CA1 neurons decreased but remained significantly higher as compared to vehicle-treated rats ($^{*}P < 0.05$, Fig. 1C). However, the protection was lost after 6 h post-ischemia. Representative photomicrographs of the hippocampal CA1 region on Cresyl violet- or Fluoro-Jade B-stained brain sections highlighted the neuronal degeneration induced by global ischemia and the potent protective effect of MLC901 treatment seven days after reperfusion. Cresyl violet staining revealed that the number of healthy neurons decreased in the CA1 area of vehicle-treated rats 7 days after reperfusion as compared to sham-operated group (Fig. 1D, left side). Fluoro-Jade B staining demonstrated a strong neuronal degeneration which was markedly increased 7 days post-ischemia (Fig. 1D,

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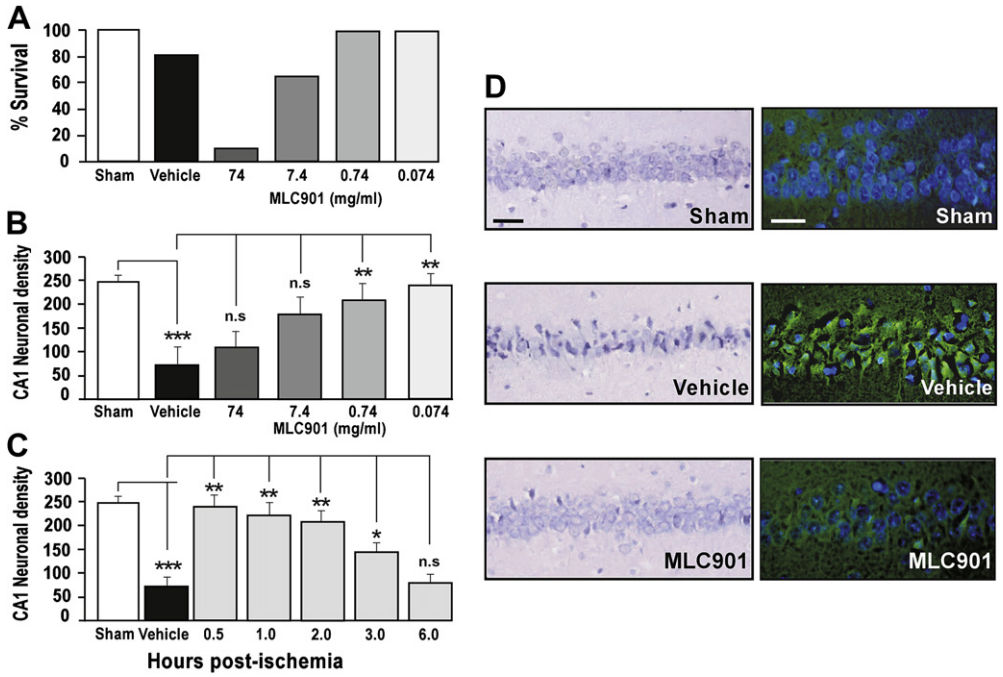

Fig. 1. MLC901 protects hippocampal CA1 neurons against ischemic injury in rat. (A–B): Dose–response effect of a MLC901 post-treatment on survival rate (A) and neuronal CA1 density (per 1 mm linear length) (B) in rats subjected to 20 min global ischemia and killed 7 days after reperfusion. Animals received an intraperitoneal MLC901 injection 30 min post-ischemia followed by one injection per day for seven days after reperfusion at the concentrations/ml of 74, 7.4, 0.74 or 0.074 mg/ml. (**C**) Time window of protection of a MLC treatment at the concentration of 0.074 mg/ml on the neuronal CA1 density after 7 days of reperfusion. ($n = 6$ per experimental group, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus vehicle ischemic group). Data are mean ± SEM assessed by ANOVA and post-hoc Tukey's *w* test. (D) Representative photographs of Cresyl violet-stained hippocampal CA1 subfield from rats killed 7 days after global ischemia corresponding to results from Fig. 1B.

right side). After a treatment with 0.074 mg/ml of MLC901, ischemia-induced delayed neuronal death, visualized by cell shrinkage (Cresyl violet staining) and neuronal degeneration (Fluoro-Jade B staining) in the CA1 substructure was significantly reduced as compared to the ischemic group (Fig. 1D). To validate MLC901 as a promising neuroprotective drug relevant in clinic we retained the most efficient dose of 0.074 mg/ml as the reference treatment injected 3 h post-ischemia and once a day during 3 or 7 days after ischemia for all further studies. The time window of 3 h post-ischemia is close to the maximal time of emergency intervention after ischemia generally admitted by clinicians. Consequently it was essential to test with this specific delay the effects of this treatment on the functional recovery and associated mechanisms (apoptosis, lipid peroxidation, phosphinositide-3-kinase/Akt pathway and neurogenesis).

Apoptosis was detected using TUNEL method which allows *in situ* labeling of DNA breaks in cell nuclei. TUNEL method revealed massive DNA fragmentation three days after ischemia in the vehicle-treated rats as compared to sham-operated animals (Fig. 2A and B). No TUNEL-positive cells, that are characterized by shrunken cytoplasm and dark nuclei were found in the CA1 region of the control group. In ischemic animals, most of morphologically damaged neurons were also positive for TUNEL staining. MLC901 treatment significantly decreased by 49.4% the number of TUNEL-positive cells as compared to vehicle-treated rats ($^{**}P < 0.01$, Fig. 2B). Expression of Bax protein, known to have a role in promoting apoptosis pathways, is regarded as a diagnostic marker of neurons undergoing apoptotic cell death (Chan, 2004). For Bax Western blotting, we used the polyclonal anti-Bax antibody, which revealed a single major band at 21 kDa, the size predicted for the Bax α protein (Fig. 2C–D). In protein hippocampal extracts of sham-operated rats, the level of Bax expression was poorly detectable. In contrast, global ischemia induced a high level of Bax expression three days after the ischemic insult. Compared with the control group, we observed a 2.55-fold increase in Bax expression from ischemic vehicle protein extracts. The injection of MLC901, 3 hours post-ischemia followed by one injection per day for three days after reperfusion led to a significant decrease (1.92-fold) of Bax expression (Fig. 2C–D). Bax immunostaining performed on brain sections of MLC901-treated rats subjected to 20 min ischemia and observed three days after reperfusion confirmed the results obtained using Western blot analysis (Fig. 2E–F). Compared to ischemic rats, where a strong induction was observed in perikarya and dendrites of the CA1 area, Bax immunostaining following MLC901 injections was low and comparable to its basal level observed in control CA1 substructure. Quantification of Bax-positive cells confirmed the results of Western blot analysis (Fig. 2F).

3.2. Critical role of AKT signaling pathway in MLC901-mediated neuroprotection

The serine–threonine kinase, Akt (protein kinase B) is a central mediator in signal transduction pathways involved in cell survival (Franke et al., 1997). Phosphorylation of the Ser473 residue is required for Akt activity. After phosphorylation, Akt functions through phosphorylation and inhibition of different substrates, including glycogen synthase kinase 3β (GSK3β), Bad, caspase 9 or forkhead transcription factors (Franke et al., 1997).

By using Western blot analysis and immunohistochemistry with a specific antibody against phospho-Akt (Ser473) and Akt, we tested whether MLC901 is able to activate the Akt pathway 8 h after ischemia ($n = 6$ per group). Bands of phospho-Akt (p-Akt) and Akt were observed at 60 kDa in the whole-cell fractions from hippocampi of vehicle- or MLC901-treated rats (Fig. 3A). Immunoreactivity of Akt showed no significant change after reperfusion in all experimental groups (Fig. 3A). Immunoreactivity of p-Akt showed a significant increase 8 h after reperfusion (Fig. 3A, $^{*}P < 0.05$). MLC901 treatment

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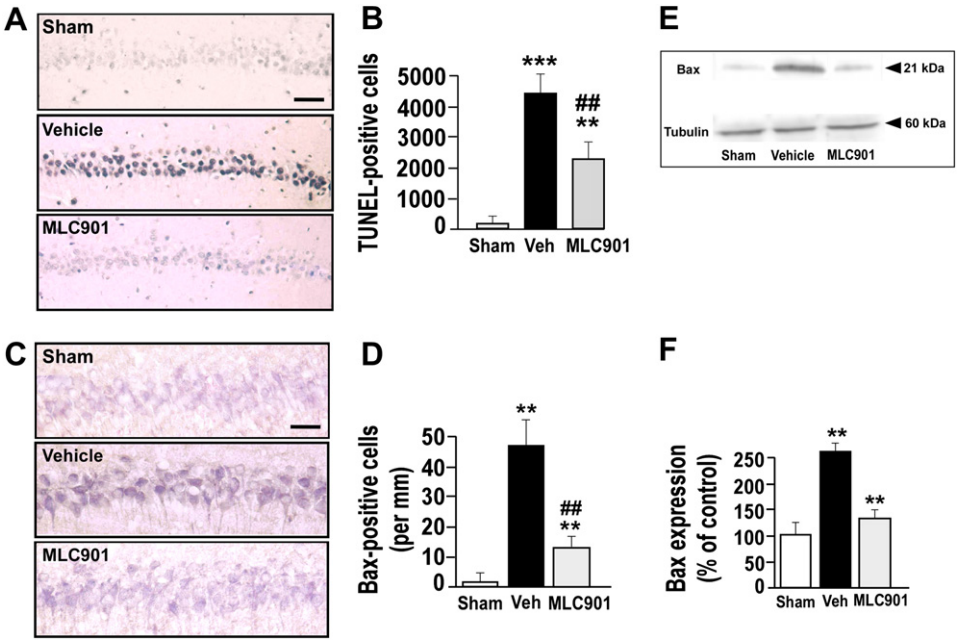

Fig. 2. MLC901 protects hippocampal CA1 neurons against DNA fragmentation and Bax expression induced by global ischemia. (A) Representative photomicrographs highlighting in the CA1 substructure the decrease in DNA fragmentation assessed by TUNEL method after a MLC901 post-treatment and 3 days of reperfusion. A dose of 0.074 mg/ml MLC901 was *i.p* injected 3 h post-ischemia and once a day during 3 days after reperfusion (B) Histograms representing the number of TUNEL-positive cells per hippocampus 72 h after reperfusion ($n = 6$ per experimental group, $^{***}P < 0.001$ and $^{**}P < 0.01$ versus sham-operated group, $^{\#\#}P < 0.01$ versus vehicle ischemic group). (C) Western blotting analysis of Bax protein in the rat hippocampi isolated from sham-operated, ischemic vehicle- or MLC901 -treated brains 3 days after ischemia. A dose of 0.074 mg/ml MLC901 was *i.p* injected 3 h post-ischemia and once a day during 3 days after reperfusion. Tubulin was used as internal control for the loading of protein level. (D) Quantitation of Bax expression obtained in Western blotting in the 3 experimental groups. Values are expressed as a percentage of control. Data are mean ± SEM assessed by ANOVA and post-hoc Tukey's *w* test, $^{**}P < 0.01$ versus sham-operated group, $^{\#\#}P < 0.05$ versus vehicle group. Data are representative of 3 separate experiments ($n = 4$ per group). (E) Representative photomicrographs of the immunohistochemical Bax expression in CA1 subfield after a MLC901 post-treatment and 3 days of reperfusion. (F) Histograms representing the number of Bax-positive cells/mm in the CA1 sector ($^{**}P < 0.01$ versus sham-operated group, $^{\#\#}P < 0.01$ versus vehicle ischemic group).

significantly increased the p-Akt level by 2.25-fold compared with the vehicle-treated group ($^{**}P < 0.01$; Fig. 3A). The immunohistochemical study confirmed the results described in the Western blot analysis. A slight immunolabelling of p-Akt was observed in the hippocampal CA1 region of sham-operated nonischemic rats (Fig. 3B). Immunostaining of p-Akt was significantly increased at 8 h after ischemia (Fig. 3B–C). Akt is a downstream effector of PI3-kinase. To more directly analyze the MLC901 effect of Akt in MLC901-induced survival, we intracerebroventricularly injected the PI3-kinase inhibitor LY294002 1 h before ischemia. After 8 h of reperfusion, p-Akt was statistically decreased in the LY294002-injected animals compared with the vehicle-injected animals ($^{*}P < 0.05$, Fig. 3A–C). In parallel, the injection of LY294002 induced a drastic decrease of the neuroprotective MLC901 effect (Fig. 3D, $^{\#\#\#}P < 0.001$). These results indicated that Akt plays an important role in the MLC901-mediated neuroprotection against CA1 brain injury induced by global ischemia.

3.3. Decrease of lipid peroxidation by MLC901 as an indicator of reduced oxidative stress

The accumulation of malondialdehyde (MDA), a lipid peroxidation by-product that has been often used as an indicator of cellular oxidation status, is significantly increased in many neurological diseases including brain ischemia/reperfusion. Oxidation of polyunsaturated fatty acids by HO_{2-} or OH_{-} leads to the formation of lipid peroxides and their enzymatic or chemical degradation results in the MDA generation. Since MDA is a potent neurotoxic agent, its accumulation is likely critical in the pathology of brain ischemia (Nguemeni et al., 2010). MDA assay ($n = 6$ per group) revealed that lipid peroxidation in hippocampi of ischemic vehicle-treated rats at 4 h post-ischemia increased 5.5-fold compared to the sham-operated group (Fig. 4). Administration of MLC901 3 h after reperfusion significantly reduced lipid peroxidation levels observed in the ischemic group ($^{**}P < 0.01$). These results indicated that MLC901 functions as an antioxidant.

3.4. Stimulation of neurogenesis by MLC901 in the hippocampal subgranular zone of rats

Adult hippocampal stem and progenitor cells are located at the border between the hilus and the inner margins of the upper and lower blade of the dentate granular cell layer (subgranular zone, SGZ). Ischemic injury stimulates neurogenesis in SGZ (Nakatomi et al., 2002). However brain self-repair by the neuronal replacement from endogenous precursors is insufficient and functional recovery is incomplete. To test the MLC901 potential in enhancing hippocampal neurogenesis, the incorporation of BrdU (5-bromo-2′-deoxyuridine, a DNA synthesis marker) in dividing progenitor cells, corresponding to the production of newborn neurons was analyzed 7 and 21 days after reperfusion ($n = 6$ per group). One week after ischemia, the number of BrdU-positive cells increased approximately 4-fold in the ischemic vehicle group, compared with that in the sham-operated group (Fig. 5A–B, $^{**}P < 0.01$). BrdU-labeled cells were located exclusively in the SGZ in clusters. MLC901 treatment administered 3 h post-ischemia followed by one injection per day for 7 days after reperfusion highly improved the increase in the number of BrdU-positive cells after ischemia as compared to ischemic vehicle-treated animals ($^{\#}P < 0.05$) (Fig. 5A–B). The phenotype of BrdU-positive cells in the SGZ was analyzed by double-labeling with doublecortin (DCX), a microtubule-associated protein that is specifically expressed in migrating

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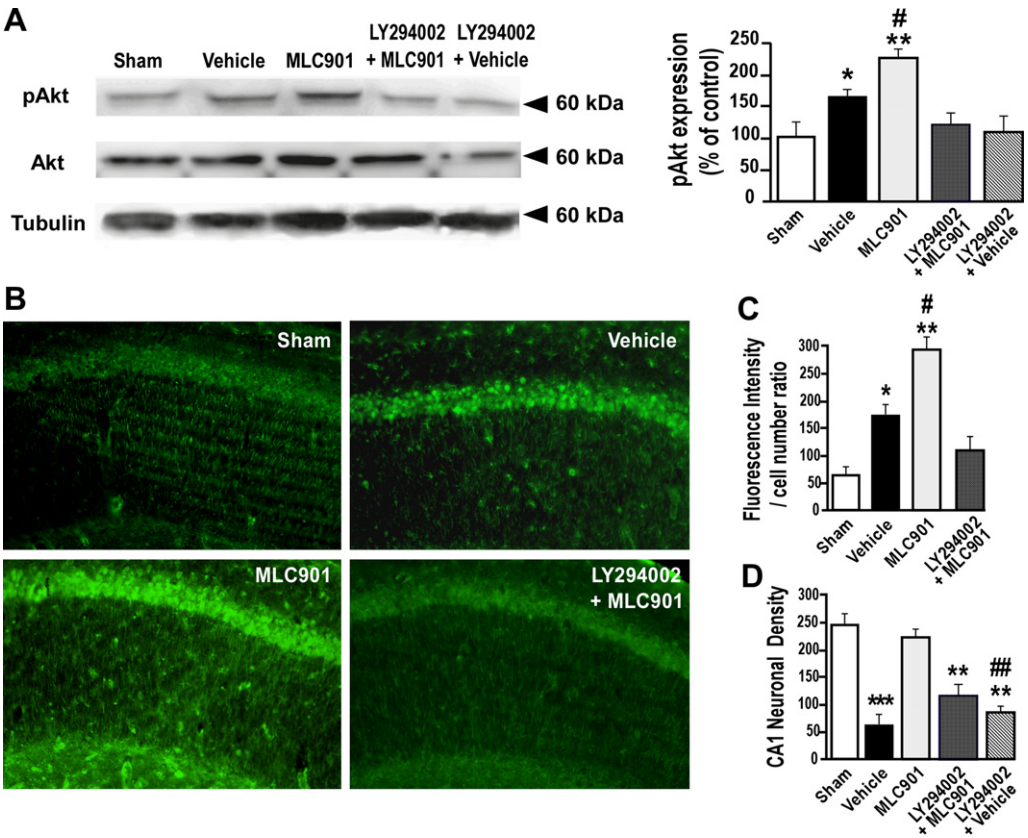

Fig. 3. Critical role of AKT signaling pathway in MLC901-mediated neuroprotection. (A) Western blotting analysis of phospho-Akt (p-Akt, serine-473) and Akt in the hippocampus samples at 8 h after global ischemia (left panel) and its quantitation (right panel). Phospho-Akt at serine-473 (top panel), Akt (middle panel) and tubulin (bottom panel) from the sham-operated (lane 1), ischemic vehicle- (lane 2) and ischemic MLC901-treated brains (lane 3). A dose of 0.074 mg/ml MLC901 was *i.p* injected 3 h post-ischemia. In lanes 4 and 5, LY294002, a PI3-kinase inhibitor was intracerebroventricularly injected at the dose of 50 nmol 1 h before ischemia in vehicle- and MLC901-treated rats, respectively. The bands of p-Akt and Akt were observed at 60 kDa in the whole-cell fraction from rat brains. Tubulin (60 kDa) was used as internal control for the loading of protein level. In right panel, histograms representing relative p-Akt expression obtained in Western blotting in the 5 experimental groups. Values are expressed as a percentage of control. Data are mean ± SEM assessed by ANOVA and post-hoc Tukey's *w* test, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus sham-operated group, $^{\#}P < 0.05$ versus ischemic vehicle group. Data are representative of 3 separate experiments ($n = 4$ per group). (B) Representative photomicrographs of the immunohistochemical p-Akt expression in CA1 subfield of sham-operated, ischemic vehicle-, ischemic MLC901- or ischemic LY294002 + MLC901-treated rats and 8 h of reperfusion. (C) Quantitation of p-Akt signal intensity in immunostained CA1 neurons observed in epifluorescence microscopy. Data are expressed as ratio of mean fluorescence intensity in AU (arbitrary unit x1000) to number of labeled cells ± SEM of three experiments. ($^{*}P < 0.05$ and $^{**}P < 0.01$ versus sham-operated group, $^{\#}P < 0.05$ versus ischemic vehicle group.). (D) Histograms representing the CA1 neuronal density (per 1 mm linear length) after the injection of the LY294002 inhibitor analyzed 7 days after reperfusion ($n = 6$ per experimental group, $^{***}P < 0.001$ and $^{**}P < 0.01$ versus sham-operated group, $^{\#\#}P < 0.01$ versus ischemic MLC901-treated group).

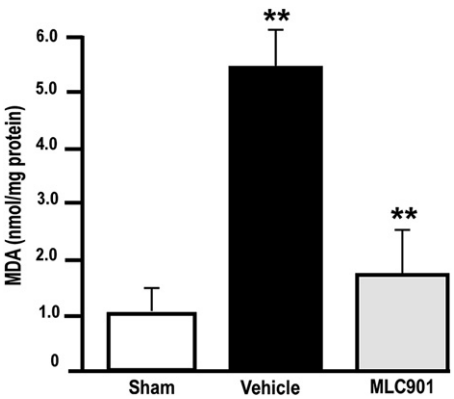

Fig. 4. Decrease of hippocampal MDA concentration by MLC901 as an indicator of reduced oxidative stress after 4 h of reperfusion. MLC901-treated rats were *i.p.* injected with a dose of 0.074 mg/ml 3 h post-ischemia. Data are mean ± SEM in nmol/mg protein MDA assessed by ANOVA and post-hoc Tukey's *w* test, $^{**}P < 0.01$ versus sham-operated group. Data are representative of 3 separate experiments ($n = 6$ per group).

neuronal precursors and in areas of continuous neurogenesis in adult brain for neurons (Fig. 5C). At post-ischemic day 21, the number of BrdU-positive cells in MLC901-treated ischemic group was still much larger than that in the ischemic group ($^{\#}P < 0.01$, Fig. 5B). Many BrdU-labeled cells were found in the granular cell layer, and some were still in the SGZ (data not shown). The survival rate of BrdU-positive cells in sham-operated rats was 91.5% whereas that in ischemic rats was 47.7% at post-ischemic day 21. Interestingly, MLC901 reversed the ischemia-induced decrease in the survival rate (76.6% in MLC901-treated ischemic rats) (Fig. 5B). At post-ischemic day 21, fluorescent double labeling for BrdU and NeuN (a mature neuronal marker) showed that about 60% of BrdU-labeled cells represented the neuronal phenotype in the three groups. The BrdU-positive cells did not show immunofluorescence for GFAP astroglial marker (data not shown).

3.5. Improvement of benefits in behavioral function recovery by MLC901

In the circular water maze, Fig. 6A–B shows the mean latencies to find the submerged platform for each group (Sham-operated,

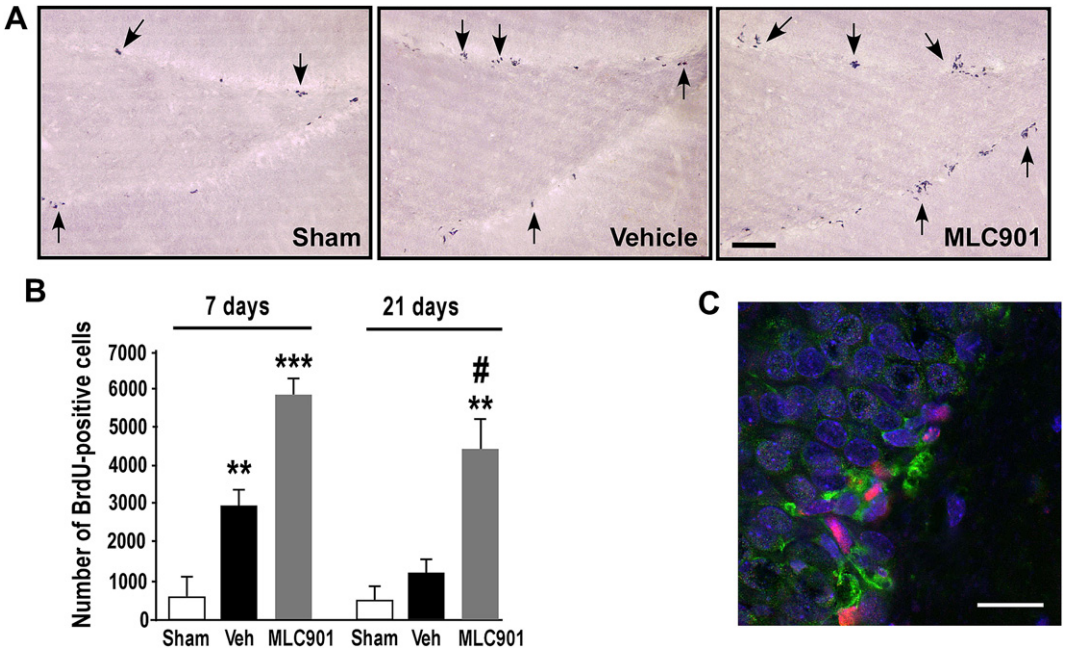

Fig. 5. MLC901 induces neurogenesis. (A) Representative photomicrographs of BrdU peroxidase-staining (arrows) in hippocampal dentate gyrus from sham-operated, ischemic vehicle- or MLC901-treated rats 7 days after reperfusion. (B) Quantitation of BrdU-positive cells per hippocampus of 3 experimental groups at 7 and 21 days following the last BrdU injection. A dose of 0.074 mg/ml MLC901 was *i.p* injected 3 h post-ischemia and once a day during 7 days after reperfusion. For the next two weeks animals were then under MLC901 administered in drinking water (10 mg/ml). Data are expressed as mean ± SEM. They were collected in three independent experiments from $n = 6$ per group, 8 sections per group, 10 fields per section, chosen randomly ($^{**}P < 0.01$, $^{***}P < 0.001$ versus sham-operated group, $^{\#}P < 0.05$ versus ischemic vehicle-treated rats). (C) Double-labeling of BrdU-labeled neurons (in red) with DCX neuronal precursor marker in green 7 days following the last BrdU injection. Scale bar, 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Vehicle and MLC901) during the four phases of acquisition 7 and 14 days after ischemia. The results indicated that the ischemic rats treated with MLC901 at 3 h after ischemia and once a day during the next two weeks performed equivalently to the sham-operated animals. In contrast the ischemic rats treated with vehicle performed poorly on this task. Specifically, there were differences in latencies among the groups during acquisition ($H_{2,12} = 14.503$, $^{***}P = 0.0007$). There was also a Day effect during acquisition ($H_{2,12} = 18.67$, $^{***}P = 0.0003$), indicating improved performance by all groups over days of acquisition training. There was no interaction effect for Treatment X Days ($H_{6,12} = 2.079$, $P = 0.912$). Sham-operated and ischemic animals that received MLC901 revealed significantly shorter latencies to find the pedestal than ischemic rats only treated with vehicle ($^{*}P < 0.05$). This difference persisted such that on Day 12 and 19, the MLC901-treated group displayed the shortest mean latency, followed by the sham-operated rats. In contrast the vehicle-treated rats required significantly greater time to locate the platform. Fig. 6C presents representative performances by one animal from each group on Day 4 of acquisition one week after ischemia (corresponding to Day 12 post-ischemia). Clearly, the swim pattern of sham-operated rats revealed a direct route to the platform, while the vehicle-treated animals were engaged in a complex search pattern for much of the trial. Throughout all escape trials the swim speed of vehicle-treated ischemic rats did not differ from that of sham-operated rats. Treatment with MLC901 did not affect the swim speed (data not shown). During the Probe testing on Day 5 one and two weeks after ischemia, the MLC901-treated group showed a pattern of greater time spent in the quadrant (where the platform was previously located) as compared to the vehicle-treated animals (Fig. 6D). There were significant differences among the groups in the second week after ischemia ($H_{2,18} = 6.53$, $^{*}P = 0.033$). The vehicle-treated group significantly displayed less time on target than the sham-operated or MLC901-treated rats ($^{*}P < 0.05$). The two latter groups did not differ. During probe trial of the second week after ischemia, the MLC901-treated group showed the greatest mean swim distance in the target quadrant in search of the platform as compared to vehicle-treated rats ($H_{2,18} = 6.45$, $^{*}P = 0.038$, Fig. 6E). There were no significant differences between sham and MLC901 groups for the two parameters measured (quadrant time and quadrant distance). Probe testing did not indicate a significant "Time after ischemia" effect ($H_{2,18} = 5.13$, $P = 0.069$) indicating the rapid protective effect of MLC901 treatment on the recovery of animals after ischemia.

In the grip strength test, sham-operated and ischemic rats did not show a significant difference in muscle strength before surgery ($H_{2,24} = 1.56$, $P = 0.546$, Fig. 6F). Compared to pre-surgery and sham-operated levels, vehicle-treated ischemic rats showed a significant strength force decrease from the first session (Day 3 following ischemia, $H_{2,24} = 20.48$, $^{*}P < 0.05$), an effect that was still present on the last two sessions (Day 10 and 15 post-ischemia, $H_{2,24} = 20.2$, $^{*}P < 0.05$ and $H_{2,24} = 16$, $^{*}P < 0.05$, respectively). However, in MLC901-treated animals, we observed a progressive and significant improvement of the strength force, which returned to that of pre-surgery and sham-operated levels as early as 10 days after reperfusion (Fig. 6F, $H_{2,24} = 20.2$, $^{\#\#\#}P < 0.001$ versus vehicle at Day 10 and $H_{2,24} = 16$, $^{\#\#\#}P < 0.001$ versus vehicle at Day 15; Fig. 6F).

4. Discussion

The classical development of drugs for neuroprotection and/or repair in ischemic situations normally goes from molecular targets to animal models and to clinical trials in humans. Things are different with Traditional Chinese Medicine, that has long been administered in humans but that often needs a scientific rationale for its putative mode of action. NeuroAid (MLC601) has been first

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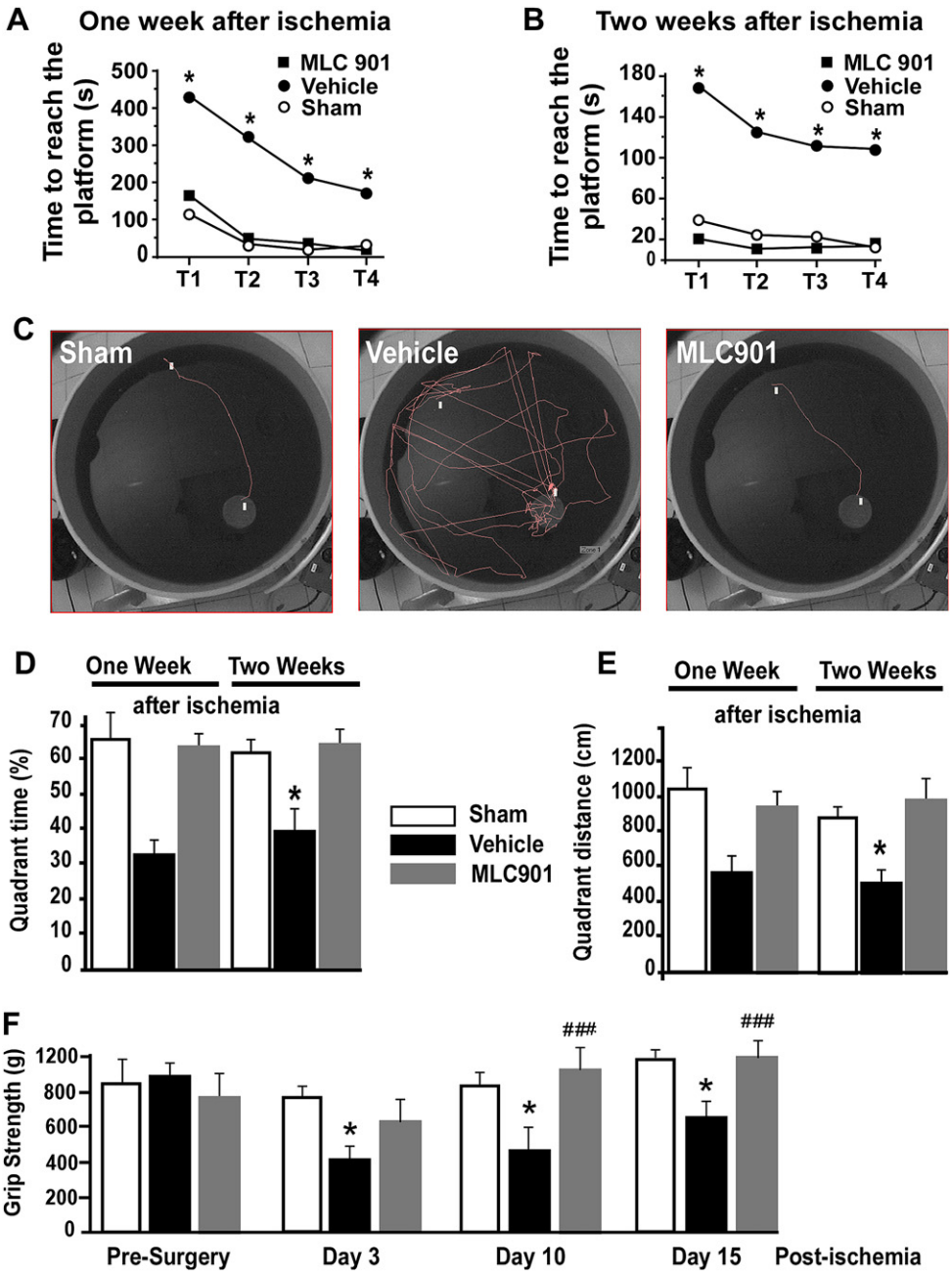

Fig. 6. MLC901 decreases the cognitive and motor deficits induced by global ischemia.(A–B) Escape latency in the Morris water maze task in the acquisition trials (T1–T4) one (A) and two weeks (B) after ischemia. *$P < 0.05$ versus sham and MLC901 groups. (C) Representative examples of search patterns in the circular water maze by members of each group on day 4 of acquisition. (D–E) Percentage of time spent (D) and swim distance (E) in the quadrant of the former platform position in and during the probe trial of sham-operated controls (Sh), ischemic rats treated with vehicle (Ve) or MLC901 after one and two weeks of reperfusion. Values are mean ± SEM ($n = 6$ per group). *$P < 0.05$ versus sham controls, Mann–Whitney *U* test. F: Muscle strength (g) observed in sham-operated controls, ischemic rats treated with vehicle or MLC901 during the grip strength test performed 3, 10 and 15 days after global ischemia. Values are mean ± SEM ($n = 8$ per group). *$P < 0.05$ versus sham controls, ###$P < 0.001$ versus vehicle-treated rats, (Mann–Whitney *U* test). In both tests, animals were injected 3 h after ischemia and once a day during the first week. The next week rats received the MLC901 treatment in drinking water (10 mg/ml).

tested in clinical trials conducted in Asia. These trials as well as clinical reports have demonstrated a high level of safety (Gan et al., 2008; Young et al., 2010) and efficiency (Gan et al., 2008; Siow, 2008) in improving cerebral blood flow velocity (Shahripour et al., in press) and stroke rehabilitation, even when taken several months after stroke onset (Chen et al., 2009). A multicenter, randomized, double-blind placebo-controlled study to investigate CHInese Medicine MLC601 Efficacy on Stroke recovery (CHIMES) is ongoing in Asia (Venketasubramanian et al., 2009). MLC601 treatment is already presented in several countries in Asia and in the Middle East for stroke patients. However, there is still a need for a deeper scientific demonstration of effects of this TCM. It is the reason why our laboratory first started to test the protective effects of MLC601 and its simplified formula MLC901 on a mouse model of focal ischemia close to the human disease. Consistent with observations of MLC601 efficiency in humans, pharmacological data obtained in mice have established that MLC601 and MLC901 prevented death of threatened neuronal tissues, decreased cognitive deficits and improved functional outcomes by restoring neuronal circuits (Heurteaux et al., 2010).

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This work is an extension of our previous studies (Heurteaux et al., 2010). Here we demonstrated that MLC901 protects the brain against the deleterious effects induced by transient global ischemia in an animal model which mimics cardiac arrest and cardiopulmonary resuscitation in humans.

After cardiac arrest, recovery without residual neurologic damage is rare. Patients who do not wake up immediately following cardiac arrest have suffered severe neurologic impairment from global ischemia. After cardiac arrest with no blood flow for more than 5 min the generation of free radicals, together with other mediators during reperfusion creates chemical cascades that result in cerebral injury (Doyle et al., 2008). At present, there is no therapy with documented efficiency in preventing brain damage induced by cardiac arrest. The survival of patients who suffer cardiac arrest depends on a chain including early access to emergency medical services, early cardiopulmonary resuscitation, early defibrillation, optimal emergency care and optimal post-resuscitation care. Over the past decade, mild hypothermia has been advocated as the best treatment to improve neurologic outcome after cardiac arrest (Nolan et al., 2003; Sterz et al., 2006). Two multicenter clinical trials in Europe and in Australia showed that post-ischemic cooling to 32–34 °C over a period of 24 h reduced the 6-month mortality and improved neurological outcome in about half of patients when treatment was begun within 2 h of cardiac arrest (Nolan et al., 2003; Sterz et al., 2006). It has been reported that hypothermia reduced brain metabolism, oxygen consumption and blood flow, ROS production, inflammation and apoptosis (Busto et al., 1989a, 1989b; Safar et al., 1996).

The purpose of this work was to analyze whether and how MLC901 would be able to induce a long-lasting brain protection. Such a protection could be self-sufficient or could be used to relay the beneficial effects of hypothermia, which is early used after cardiac arrest.

Transient global ischemia causes severe damage to pyramidal neurons of CA1 region and results in neurological impairment (Pulsinelli and Brierley, 1979). Pyramidal neuronal death in CA1 region pursues a protracted time-course after global ischemia, thus providing an opportunity for therapeutic interventions designed to protect these most vulnerable cells. Here, we demonstrated in rats that MLC901 when administered in post-treatment for 7 days strongly reduces delayed neuron death in the CA1 field after one week of reperfusion. Then, to demonstrate that neuronal networks remain functional in ischemic rats treated with MLC901, we used several types of behavioral tests. In line with first clinical trials that have shown promising results of MLC601 efficiency on the functional recovery after stroke (Chen et al., 2009), we first observed that MLC901 improves rat motor performances measured in the grip strength test, considered as an useful operant conditioning procedure to assess long-lasting sensorimotor deficits after ischemia. Performances obtained in this test showed that MLC901 improves post-ischemic grip strength. Because ischemia-induced damage in the hippocampus is generally considered as responsible for the profound impairment of spatial memory tasks, we then used the Morris water maze which is the most sensitive behavioral task available to measure such damage. The present study confirmed that 20 min four-vessel occlusion results in a significant impairment in spatial learning and memory, associated with CA1 hippocampal damage. In the vehicle-treated rats the swim distance was longer than that of sham-operated animals, whereas the swim speed was not altered. These results indicate that the increase in escape latency is not a result of a motor impairment but rather reflects an impairment in spatial orientation. The spatial bias of vehicle-treated ischemic rats is confirmed by the reduction in the time spent in the quadrant of the former platform position during the probe trial. The observed diminution of indicators (escape latency, distance, quadrant time) of learning and memory impairment after global ischemia/reperfusion injury is consistent with previous reports (Block and Schwarz, 1998; Olsen et al., 1994). Interestingly, this particular test demonstrates that treatments with MLC901 lead to a marked decrease of deficits in spatial learning induced by global ischemia, which is revealed by a reduction in escape latency and swim distance during the phase of acquisition in MLC901-treated rats two weeks after ischemia. The deficit in spatial memory induced by ischemia is reduced by MLC901, as indicated by the increase of the time spent in the quadrant of the former pedestal position.

MLC901 offers a time window of protection compatible with the clinical situation, since MLC901 provides protection from global ischemia in the rat when given as late as 3 h after ischemia.

Ischemic injury is associated with progressive DNA fragmentation in CA1 pyramidal neurons. At 72 h post-reperfusion, an intense TUNEL labeling was observed in this damaged structure. Ischemia-induced DNA fragmentation contains not only internucleosomal fragmentation but also random breakdown products often associated with necrosis. TUNEL method labels all types of DNA fragments and does not discriminate accurately between apoptotic and necrotic cells (Charriaut-Marlangue et al., 1996; Unal-Cevik et al., 2004) and therefore is mostly considered as a marker of damaged cells. In that context, we observed that MLC901 drastically reduces the number of TUNEL-positive cells in CA1 after 72 h reperfusion.

Programmed cell death and apoptosis are regulated by members of the Bcl-2 family, including Bcl-2, which promotes cell survival and Bax, which promotes cell death. Bax, which is a potent proapoptotic molecule triggering activation of terminal caspases, is upregulated following ischemia (Chan, 2004). Oligomerization of Bax facilitates its insertion into the outer mitochondrial membrane triggering cytochrome *c* release that promotes apoptosis (Chan, 2004). Both reduction of TUNEL labeling and Bax reactivity in CA1 pyramidal neurons suggest that the neuroprotection induced by MLC901 involves a decrease of apoptotic pathways occurring in this area.

Recent evidence suggests that elevated levels of ROS not only initiate the destruction of cell membranes and other cellular structures by activating lipid peroxidation and altering membrane phospholipids, but also disturb mitochondrial function, inducing the opening of the mitochondrial permeability transition pore and the release of cytochrome *c* to activate apoptotic pathways (Chan, 1996, 2004). Agents that decrease oxidative stress may therefore reduce ischemia/reperfusion-induced neuronal death. The production of ROS such as superoxide radicals, hydroxyl radicals and hydrogen peroxide is increased after global ischemia (Chan, 1996, 2004). In the present study, we indeed showed that the level of peroxidation caused by free radical release in the hippocampus, as assessed by MDA concentration (a stable metabolite of the ROS-mediated lipid peroxidation cascade) is enhanced in the vehicle-treated ischemic group. This result is consistent with other studies which have reported an early and a delayed increase in ROS generation and level of lipid peroxidation in the hippocampus after global ischemia (Friberg et al., 2002). The new result is that global ischemia-induced MDA production in hippocampus is largely decreased by a treatment with MLC901. The neuroprotective effect of MLC901 may then be attributed, at least in part, to its capacity to attenuate oxidative stress and lipid peroxidation.

There is increasing evidence that Akt activation is one of the principal factors to prevent apoptosis and that the Akt survival pathway plays a crucial role in neuronal survival after cerebral ischemia (Noshita et al., 2001). The current study showed that it is the activation of the Akt pathway after MLC901 treatment that significantly rescues cells from delayed neuronal cell death after global ischemia in the hippocampal CA1 region. Akt activation is

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PI3-kinase dependent. The PI3-kinase inhibitor LY294002 injected before ischemia significantly prevents the neuroprotective action of MLC901. The Akt pathway has also been shown previously to be critical in neuroprotection against ischemia provided by pre-conditioning, erythropoietin or BDNF (Brunet et al., 2001).

We then studied the effect of MLC901 treatment on neurogenesis. First, in agreement with previous studies (Nakatomi et al., 2002), we have shown that there is a significant increase of newborn cells in the dentate gyrus after 20 min global ischemia. However, it is known that this endogenous regenerative capacity of adult neural progenitors induced by ischemia is limited, with a capacity of replacing only a small fraction of neurons or with a limitation in the generation of new mature neurons (Bjorklund and Lindvall, 2000; Zhang et al., 2008). Again, after MLC901 treatment we observed an enhanced neurogenesis and again it was tempting to speculate that this process could markedly enhance the latent regenerative potential of the adult CNS and could improve the recovery of memory function in rodents but also in humans after global ischemia. Both recovery of learning and memory following ischemia and improved cognitive function induced by MLC901 correlate with dentate neurogenesis. The reduction of cognitive deficits is also probably related to an increased synaptogenesis as it has been previously reported in the model of focal ischemia (Heurteaux et al., 2010).

This work using the rat model of four-vessel occlusion is the first preclinical investigation demonstrating that MLC901 protects against the hippocampal damages induced by global ischemia and ameliorates cognitive function. It provides scientific support for the clinical use of MLC901 after cardiac arrest, where mild hypothermia applied to comatose survivors remains the only therapy available for improving the neurologic outcome of patients.

Disclosure/conflict of interest

This work is supported by a CNRS/MOLEAC contract. ML is vice-president for research of Moleac.

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