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MLC901 Favors Angiogenesis and Associated Recovery after Ischemic Stroke in Mice

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Key Words
Ischemic stroke · Angiogenesis · MLC601/901 · NeuroAid · Blood brain barrier · Neurological deficits · Recovery

Abstract
Background: There is increasing evidence that angiogenesis, through new blood vessel formation, results in improved collateral circulation and may impact the long-term recovery of patients. In this study, we first investigated the preventive action of a 5-week pretreatment of MLC901, an herbal extract preparation derived from Chinese medicine, against the deleterious effects of ischemic stroke and its effects on angiogenesis in a model of focal ischemia in mice. ***Methods:*** The stroke model was induced by 60 min of middle cerebral artery occlusion followed by reperfusion. MLC901 was administered in the drinking water of animals (6 g/l) for 5 weeks before ischemia and then during reperfusion. ***Results:*** MLC901 treatment increased the survival rate, reduced the cerebral infarct area and attenuated the blood brain barrier leakage as well as the neurologic dysfunction following ischemia and reperfusion. We provide evidence that MLC901 enhances endothelial cell proliferation and angiogenesis by increasing the number of neocortical vessels in the infarcted area. MLC901 regulates the expression of hypoxic inducible factor 1α and its downstream targets such as vascular endothelial growth factor and angiopoietins 1 and 2. This work also shows that erythropoietin is an important player in the enhancement of angiogenesis by MLC901. ***Conclusions:*** These results demonstrate therapeutic properties of MLC901, in addition to those previously described, in stimulating revascularization, neuroprotection and repair of the neurovascular unit after ischemic stroke.

Introduction

Although stroke is associated with high mortality and serious long-term disability, therapeutic options for clinical management remain quite limited. In the acute phase, thrombolytic reperfusion with recombinant tissue plasminogen activator is still only used in <10% of all ischemic stroke patients [1]. During the chronic phase after stroke, pharmacological treatment aimed at promoting repair and regeneration of brain tissue damaged by ischemia may provide unique opportunities to improve the clinical outcome of stroke patients. Recent developments have strongly suggested that induction of brain angiogenesis has positive effects in the long-term recovery of stroke

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patients [2, 3]. It is well known that stimulation of angiogenesis, which generates new vessels, aims to increase collateral circulation [4].

In humans, angiogenesis takes place 3 or 4 days after stroke. It is directly associated with neurogenesis since blood supply is necessary for the survival of new neurons [3]. In rodents, angiogenesis genes are upregulated within minutes of the onset of stroke and angiogenic proteins remain increased in the area of ischemia for days to weeks [5]. Thus, therapeutic angiogenesis is an approach of regenerative medicine that may help in improving the outcomes of patients after an ischemic stroke. Among the factors capable of modulating angiogenesis and vascular maturation/remodeling, a particular interest has been given to vascular endothelial growth factor (VEGF) and the angiopoietin/Tie receptor system [6–8].

MLC601 (NeuroAiD™) and MLC901 (NeuroAiD™ II) originated from Traditional Chinese Medicine have been demonstrated to have both neuroprotective and neurorestorative properties in preclinical models of stroke, global cerebral ischemia and traumatic brain injury [9]. Originally containing 9 herbal extracts and 5 non-herbal components in capsules, MLC601 was simplified to MLC901, which contains only the herbal extracts. Clinical studies to assess the benefit and safety of MLC601 in stroke patients using different clinical outcome have been performed [10–18]. The Chinese Medicine Neuroaid Efficacy on Stroke recovery (CHIMES) trial, which compared MLC601 with placebo in 1,099 patients with acute ischemic stroke of intermediate severity, reported a reduction in early recurrent vascular events and vascular deaths in post-stroke patients [11, 19, 20]. Interestingly, in the Philippine cohort of the CHIMES trial, which included more patients with predictors of poorer prognosis, functional and neurological outcomes were improved in favor of MLC601 [15]. Stratification of the entire CHIMES cohort by prognosis also showed that patients with 2 or more predictors of poorer outcome have better treatment effect with MLC601 than patients with single or no prognostic factor [21]. A recent extension study of the CHIMES trial (CHIMES-E) revealed that a 3-month treatment with MLC601 after stroke improves the functional outcome for up to 2 years among patients with cerebral infarction of intermediate severity [22]. All these clinical results are encouraging to pursue further exploration of NeuroAid as a new efficient therapeutic strategy against stroke. Two clinical trials with MLC901 are also ongoing for assessment in vascular cognitive impairment patients [23] and in New Zealand patients with traumatic brain injury (https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=364449).

Consistent with clinical observations of the benefit of MLC601 in humans, preclinical data in rodents have established that MLC601 and MLC901 prevent death of threatened neuronal tissues, decrease cognitive deficits and improve functional outcome by restoring neuronal circuits in models of ischemia and traumatic brain injury [24–26]. The efficacy of MLC601/MLC901 has also been demonstrated in in vitro models of excitotoxicity [24] and oxygen glucose deprivation [27]. MLC601 and MLC901 have been reported to display multiple effects in impeding the ischemic cascade propagating from the core to the penumbra as well as in stimulating proliferation and differentiation of new neural cells to repair. Activation of the Akt survival pathway and opening of K_{ATP} channels are important steps in the neuroprotective properties of MLC901 [9]. The ability of MLC901 to promote neurogenesis, neurite outgrowth and synaptogenesis coupled to BDNF stimulation is a strong argument for a positive effect of MLC901 on brain plasticity and restorative processes after stroke [9].

Because the neural and vascular cell cross-talk is important in brain repair, and since MLC901 has repeatedly been demonstrated to strongly stimulate neurogenesis, we decided to further investigate the in vivo effects of MLC901 on angiogenesis. For this, we analyzed the effects of a 5-week MLC901 pre-treatment and during reperfusion on the expression of angiogenesis-induced proteins such as VEGF and angiopoietin 1 (Ang1)/angiopoietin 2 (Ang2) in a model of transient middle cerebral artery occlusion (MCAO)-induced ischemic stroke in mice. We also investigated its mechanisms of action via regulation of key proteins such as hypoxic inducible factor 1α (Hif1α) and erythropoietin (EPO), which are known to mediate angiogenesis and cell survival after stroke.

Materials and Methods

Animals

Four-week-old C57BL/6 male mice (Janvier) were housed 5 per cage on inversed 12-hour light/dark cycle (light on at 08:00 p.m.) in an animal facility maintained at ambient temperature of 21 ± 1°C. They were provided with food and beverage ad libitum. All the experiments were performed in accordance with the policies on the care and use of laboratory animals of European Community laws (directive 2010/63/EU) and approved by the French Ministry of Higher Education and Scientific Research (approval number 01314.04). In this study, a total of 378 animals were used. The researchers who carried out the ischemic surgery and all post-stroke experiments were blinded to the treatment code.

Drug Treatment

MLC901 combines 9 herbal extracts equivalent to the following composition of raw herbs per capsule: 0.80 g Radix astragali, 0.16 g Radix salvia miltiorrhizae, 0.16 g Radix paeoniae rubra, 0.16 g Rhizoma chuanxiong, 0.16 g Radix angelicae sinensis, 0.16 g Carthamus tinctorius, 0.16 g Prunus persica, 0.16 g Radix polygalae [24] and 0.16 g Rhizoma acori tatarinowii. MLC901 (batch BN112 provided by Moleac, Singapore) was administered in drinking water (6 g/l) for 5 weeks before ischemia and during reperfusion until the sacrifice of animals at different times post ischemia depending on the type of experiment. The dosage used in this work has been determined according to the formula currently used for dose translation from animal to human studies [28]. Considering that a mouse drinks 5 ml per day and weights 25 g, the MLC901 dose ingested by mice at the concentration of 6 g/l was around 30 mg/day, which corresponds to a HED of 97 mg/kg and to the dose used in humans for clinical trials.

Ischemic Stroke Model (MCAO)

The ischemic stroke model (focal ischemia) was performed on 9 weeks mice by transient (60 min) left MCAO and reperfusion, using a 6–0 coated filament (Doccol, Redlands, Calif., USA) as previously described [24]. The regional cerebral blood flow was monitored by laser Doppler flowmetry (Perimed, Craponne, France) to control MCAO severity and reperfusion. Animals presenting with sustained CBF reduction >70% during ischemia or a severe brain hemorrhage after MCAO were excluded from the study (<1%). Mice received MLC901 during both pre-treatment for 5 weeks before the induction of stroke and post-treatment until the sacrifice of animals. The mortality rate was analyzed at 7 days after ischemia. The protocol and dose were selected based on our previous studies [24]. Sham-operation was performed by inserting the thread into the common carotid artery without advancing it to occlude MCA. The animals were allowed to regain full consciousness on a nursing cage during 24 h before returning to the home cage.

Neurological Deficits

Neurological deficits of mice were assessed 24 h post ischemia in a blinded fashion according to a scoring scale in a postural reflex test developed by Bederson et al. [29]: Grade 0 = no visible deficits, Grade 1 = forelimb flexion, Grade 2 = unidirectional circling when the animal is pulled by the tail, Grade 3 = circling and rolling movement, Grade 4 = decreased level of consciousness, Grade 5 = death. Mice with a neurological deficit above Grade 4 were excluded from the study.

Measurements of Infarct Volume, Brain Edema and BBB Leakage

To assess the infarct volume, mice were euthanized by decapitation at 24 h after reperfusion. Coronal frozen sections of brain (20 mm-thick) were stained using a solution of 1% cresyl violet in 0.25% acetic acid and mounted with Entellan. The striatal and cortical areas of infarction, outlined in light were measured on each section using a computer image analysis system and corrected for brain edema according to Golanov and Reis [30]. Infarct volume, expressed in mm^3, was calculated by a linear integration of the corrected lesions areas as previously described [24].

To measure blood brain barrier (BBB) leakage, the Evans blue was used as a BBB permeability tracer. Briefly, 2% Evans blue solution (4 ml/kg), dissolved in normal saline, was injected intravenously 4 h before the animals were euthanized. Mice were transcardially perfused with normal saline to wash away the remaining dye in the blood vessels. The brains were removed and coronal sections from bregma-1 to 1 mm were performed to visualize the blue staining at 24 h after MCAO.

To measure the vasogenic edema, animals were sacrificed 30 h after ischemia. The brains were divided into the ipsilateral hemisphere (ischemic side) and contralateral hemisphere. The ipsilateral hemisphere was weighed to obtain the wet weight and then dried at 110°C for 24 h. The percentage of brain water content in the ipsilateral hemisphere was calculated as follows: water content = (wet weight – dry weight)/wet weight.

Accelerated Rotarod Test

The rotarod test is used to assess motor coordination and balance alterations after ischemic brain injury in the rodent [31]. The rotarod apparatus consists of a striated rod (diameter 3 cm) subdivided into 5 areas (width 5 cm) by disks 25 cm in diameter. Mice (n = 20 per group) were conditioned to the accelerating rotarod (Ugo Basile, France) for 3 days before MCA occlusion. To this end, mice were first placed on the apparatus during 30 s with no rotation and thereafter for 2 min with a constant low speed (4 rpm). They were tested until they achieved a criterion of remaining on the rotating spindle for 1 min. This procedure was performed only the first day of training. After 10 min rest, each mouse then received a single baseline trial on the accelerating rotarod in which the spindle increased in speed from 4 to 40 rpm over a period of 6 min. The same protocol was applied at Days 3–1. The test trial was performed at different times after MCAO from day +2 to +15. The maximum duration the animals were able to walk on the rotarod before falling was measured (maximum value 6 min). Mice were tested over 3 daily trials in the accelerated condition (4–40 rpm). The daily mean value was taken for each mouse and used for statistical analysis.

Pole Test

Gross sensorimotor function (integration and coordination of movements as well as balance) was evaluated by ability of animals to move along the pole [32]. The mouse was placed head upward on the top of a vertical wooden rough-surfaced pole (diameter 1 cm, height 50 cm). Each mouse was habituated on the day before MCAO and then allowed to descend 5 times on a single session. The total time needed to turn completely head downward ('time-to-turn') and the time until the mouse reached the floor with its 4 paws ('time to come down') was recorded. The testing trial was carried out at day +3 after MCAO. Results were expressed as the mean of 5 trials.

Analysis of Brain Endothelial Cell Proliferation and Angiogenesis

Endothelial cell proliferation was identified by using BrdU labeling. BrdU treatment consisted of 4 injections (75 mg/kg, i.p. each, 2 h interval) [24]. Brains were removed at 24 h after the last injection. Serial sections of paraformaldehyde-perfused brains were cut (40 μm) throughout the brain at the level of the lesion on a vibratome (Leica). Every sixth section throughout the brain was processed for immunohistochemistry using a monoclonal sheep anti-BrdU (Interchim, 1/200). To specifically identify BrdU-reactive cells co-localized with endothelial cells, double immunofluorescence labeling of BrdU with CD31 (platelet-endothelial cell adhesion molecule), a specific marker for endothelial cells (Abcam, ab24590, 1/500) was performed. Secondary antibodies were con-

jugated with Alexa Fluor 488 or 594 (Molecular Probes, 1/1,000). Confocal microscopy observations were performed with a Laser Scanning Confocal Microscope (TCS SP, Leica, Rueil Malmaison, France). BrdU-immunostained sections were digitized using a ×40 objective via the ImageJ computer imaging analysis system. The number of endothelial and BrdU-labeled cells within a total of 10 vessels located in the ischemic border area was counted in each section by a blind experimenter. Data are presented as the percentage of number of the BrdU-immunoreactive cells within vessel/total endothelial cell number.

The capillary count was assessed from immunostaining for the endothelial-specific marker CD31 in the parietal cortex and striatum of each experimental group (n = 6 per each experimental group). CD31 labeling was characterized by a relatively strong staining of the vascular perimeters of the blood vessels. The capillary count was performed in 5 sets of 3 sections (10 μm thick) cut 150 μm apart corresponding to stereotaxic coordinates of plates 12–16 in a mouse brain atlas [33]. Images spanning the full depth of the parietal cortex and the entire area of the striatum were obtained, which were digitized and analyzed using the ImageJ computer imaging analysis system to determine the number of CD31-positive capillaries per field.

ELISA Assay (VEGF and Hif1α)

Ischemic brain tissue extracts (including core and penumbra) from MLC901- and vehicle-treated mice 1, 3 and 14 days after MCAO were dissected on ice and their wet weights were rapidly measured. The tissues (150 mg/ml) were homogenized in DMEM and centrifuged for 10 min at 10,000 g at 4°C. The brain extracts were then divided into 200 μl triplicate samples. VEGF and Hif1α ELISA kits (M0050 and M0687, Elabscience Biotechnology, Cliniscience) were used to determine the VEGF and Hif1α levels.

Western Blots

Whole-cell protein extraction was performed using standard procedures. Samples were obtained from ischemic hemispheres (vehicle- and MLC901-treated) and from sham-operated controls (n = 3 per group). Fresh brain tissue was cut into pieces after different times of reperfusion and homogenized in 4 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-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 different primary antibodies: rabbit polyclonal primary antibody against VEGF 165B (Abcam, ab90719, 1/100), rabbit monoclonal Hif1α, 51608, 1/2,000), rabbit polyclonal Ang1 (Abcam, ab134504, 1/500) and Ang2 (Abcam, ab8452, 1/500) for overnight at 4°C. 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 diaminobenzidine (DAB) kit. To control for sample loading, stripped membranes were re-hybridized with a β-actin antibody (1:2,000, Proteintech Group Inc., Chicago, Ill., 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). Western blots were duplicated with 3 independent sets.

Immunohistochemistry

Frozen brain sections (25 μm thick) were immersed in 0.3% H_2O_2/PBS 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 monoclonal or polyclonal antibodies used were the following: rabbit VEGF (Abcam, Ab46154, diluted 1/500), rabbit polyclonal Ang1 (Abcam, ab134504, 1/200) and Ang2 (Abcam, ab8452, 1/200), rabbit Hif1α (Abcam, ab51608, 1/200), rabbit EPO H-162 (Santa Cruz, sc-7956, 1/300), goat GFAP (Abcam, ab53554, 1/500), mouse CD31 (Abcam, ab24590, 1/500), sheep Von Willebrand factor (Abcam, ab11713, 1/100), mouse Iba1 (Abcam, ab15690, 1/1,000), mouse NeuN (Millipore, MAB377, 1/300). After the primary incubation and 3 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. Protein expression was visualized by 3,3-DAB staining using VectaStain ABC kit (Biovalley). All sections were washed and mounted with Entellan. For fluorescent labeling, sections were incubated with secondary antibodies conjugated with Alexa Fluor 488 and 594 (1/1,000, Molecular Probes) for 2 h. Sections were observed using confocal and/or epifluorescence microscopy. Confocal microscopy observations were performed using a Laser Scanning Confocal Microscope (TCS SP, Leica) equipped with a DMIRBE inverted microscope and an argon-krypton laser (laser excitation 488 nm, acquisition 500–600 nm every 10 nm). Signal specificity was assessed in negative control coverslips by omitting primary antibody. Images were acquired as single transcellular optical sections and averaged over at least 4 scans per frame. Epifluorescence microscopy images of protein labeling were captured with identical time of exposition after spectral correction of the autofluorescence background. Analysis of the fluorescence intensity was performed by using the NIH ImageJ software (http://rsbwebnih.gov/ij), which allowed to measure the fluorescent intensity levels of cells of each fluorescent image saved as a 16-bit TIFF file. Results are given as ratio of mean fluorescence intensity in AU (arbitrary unit)/number of labeled cells ± SEM of 3 experiments.

Statistical Analysis

Statistical values are presented as mean ± SEM. Statistical analysis of differences between groups was performed by using unpaired t test or analysis of variance. Where F ratios were significant, statistical analyses were extended and post-hoc comparisons made by using Tukey's test multiple comparison tests. In all analyses, the level of significance was set at $p < 0.05$.

Results

MLC901 Protects against Ischemic Brain Infarction and Reduces Functional Deficits

To evaluate the protective effects of MLC901 against stroke, we tested the preparation in the mouse model of

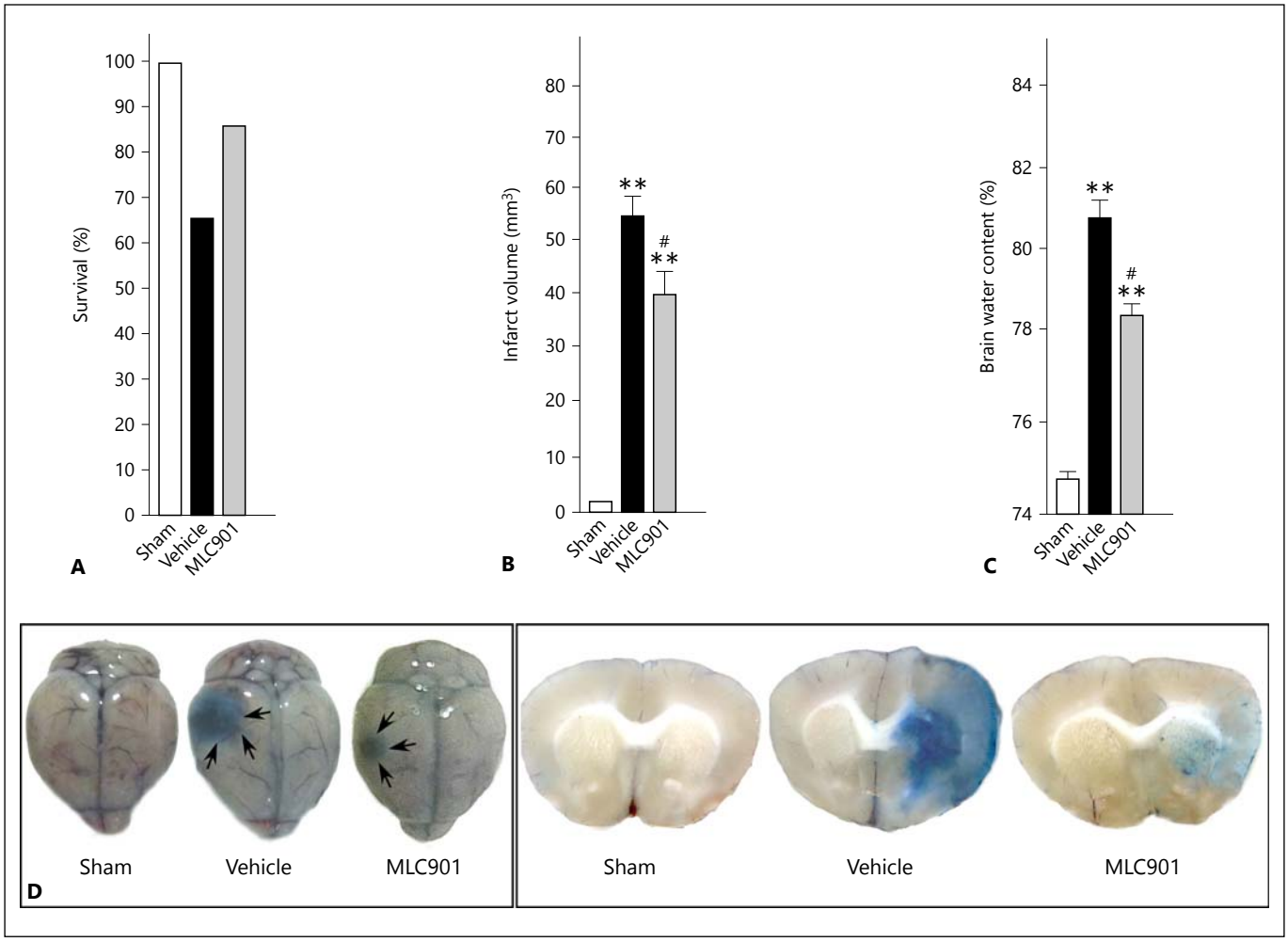

Fig. 1. Effects of MLC901 on survival rate, cerebral infarct volume, hemispheric swelling and BBB leakage induced by focal ischemia. **A** Percentage of survival rate 7 days post ischemia; **B** infarct volume (mm^3); **C** percentage of brain water content in ipsilateral cortical segments measured at 30 h post ischemia. Data are reported as mean ± SEM (n = 6 per experimental group, ** $p < 0.05$ vs. sham operated group and $^{\#}$ $p < 0.05$ vs. vehicle ischemic group). **D** Representative images of Evans blue extravasation in the brains (left panel) and brain sections (right panel) of mice submitted to focal ischemia and observed 30 h after MCAO. MLC901 was administered in the drinking water (6 g/l) for 5 weeks before MCAO and then until the sacrifice of animals performed at different time points after reperfusion (30 h (**B–D**) or 7 days (**A**), n = 10 per experimental group, mice were naïve for each experimental approach).

focal ischemia. Animals (n = 10 per experimental group) were first treated with MLC901 for 5 weeks before the induction of ischemia and then until the sacrifice of mice. There was no significant difference in the consumption of food and drinking solution between vehicle- and MLC901-treated groups (data not shown). Figure 1A shows that at 7 days post ischemia, MLC901 induced a marked reduction in mortality among MLC901-treated mice compared to vehicle-treated ischemic animals. MLC901 induced a survival rate of 85.2% as compared to 64.8% in the control group (fig. 1A).

Cerebral infarct and hemispheric swelling induced by MCAO were evaluated 30 h following ischemia (n = 10 per experimental group). Total infarct volume was decreased in the MLC901-treated mice as compared with vehicle-treated group (fig. 1B; 56.1 ± 0.3 vs. 39.8 ± 0.2; $p < 0.01$). The results of measured water content were consistent with infarct volume measurements, the MLC901-treated group showing significantly reduced brain edema when compared to vehicle group (fig. 1C; $p < 0.05$; n = 10 per experimental group). The brain water content significantly increased in the vehicle group with 80.9 ± 2.1%

compared with the saline-treated sham group (74.8 ± 0.2%). MLC901 significantly decreased the brain water content by 78.1 ± 1.1%, compared to the vehicle group (fig. 1C; $p < 0.05$).

BBB disruption is a critical event in the progression of ischemic stroke [34] and is induced at the onset of cerebral ischemia [35]. When BBB breakdown occurs, many serum proteins that are detrimental to neurons pass through the barrier, further worsening brain injury. To evaluate BBB permeability after ischemic injury, BBB leakage was measured 30 h after stroke using Evans blue extravasation (n = 10 per experimental group). Representative photographs of Evans blue stained ischemic brain sections demonstrated the beneficial effects of MLC901 on the BBB damage (fig. 1D). Evans blue extravasation was strongly reduced in MLC901-treated mouse brain compared with the vehicle controls.

Functional deficits are common neurological sequelae in patients with brain injuries induced by stroke and in animal models of stroke. The effects of MLC901 on neurological function were studied by using behavioral tests (n = 16 per experimental group). Ischemia-induced neurological deficits, assessed by neurological Bederson scores, were significantly attenuated in MLC901-treated mice at 30 h after MCAO (1.8 ± 0.5 vs. 3.25 ± 0.3 in vehicle group, $p < 0.05$; fig. 2A). The improvement of the neurological outcome was confirmed in the pole test on day +3 (fig. 2B). The 'time to turn' and 'time to come down' were strongly decreased in MLC901-treated mice as compared to those of vehicle-treated group. The positive effects of MLC901 on the motor outcomes were also observed in the test of the accelerated rotarod (fig. 2C). There was no significant difference in performance between pre-ischemia groups with or without MLC901 ($p > 0.5$). From day +2 to +9 after MCAO, vehicle-treated mice showed a strong decrease in their performances as compared to the corresponding pre-ischemia and sham groups ($p < 0.05$). MLC901-treated mice showed a significant improvement of their performances on the rotarod compared with the vehicle-treated ischemic group (fig. 2C; $p < 0.05$). At day 9 post ischemia, vehicle-treated mice still displayed a significant negative difference in the time they could spend on the rod compared to sham-operated and pre-ischemia groups ($p < 0.05$). Interestingly, as early as 3 days after MCAO, MLC901-treated mice tended to behave nearly as well as the sham group, revealing better performance of MLC901-treated ischemic animals in comparison to vehicle-treated ischemic mice in the rotarod assay.

MLC901 Stimulates Angiogenesis and Modulates the Expression of Angiogenesis-Regulated Molecules in Response to Focal Cerebral Ischemia

Angiogenesis is a potent process in stroke [3]. To detect angiogenesis that occurs in response to stroke in mouse brain and to study the effects of MLC901, the CD31-positive capillaries were counted in the perifocal area 14 days after MCAO (fig. 3A, B, n = 6 per experimental group). Treatment with MLC901 significantly increased the capillary density, vascular perimeters and BrdU-reactive endothelial cell numbers in the ischemic border, as compared with the vehicle-treated mice (fig. 3A, B). Double immunostaining BrdU/CD31 showed that BrdU-positive endothelial cells localized in the vessel (fig. 3C), indicating that MLC901 enhanced endothelial cell proliferation and angiogenesis by increasing the number of neocortical vessels invading the ipsilateral infarcted area. MLC901 did not induce angiogenesis in the contralateral hemisphere (data not shown).

VEGF and its receptors are central mediators of vasculogenesis and angiogenesis [6, 36]. They have been shown to be upregulated after MCAO in rodents [5, 37, 38]. Unlike VEGF, the angiopoietins (Ang1 and Ang2) exert their functions at later stages of vascular development, that is, during vascular remodeling and maturation [39]. To examine the contribution of VEGF and angiopoietins after stroke and analyze the effects of MLC901, we investigated the expression of these factors after MCAO in vehicle- and MLC901-treated mice at 24 h, 3 and 14 days post ischemia (n = 4 per experimental group and method used). Figure 4A shows that VEGF levels, detected in the ELISA assay, were transiently increased in vehicle-treated ischemic brains compared with sham group ($p < 0.05$) at 24 h and 3 days after ischemia and then had a tendency to return to basal levels at 14 days. Interestingly, MLC901 treatment exerted a dual effect on VEGF depending on the time kinetics after MCAO. One day after MCAO, MLC901 diminished the increase in VEGF levels seen in the vehicle group, while at 3 and 14 days, it induced a significant increase in levels of the growth factor compared with the vehicle group ($p < 0.05$). The results and subsequent analysis of the Western blots (fig. 4B) confirmed the data obtained by the ELISA method. Immunohistochemical staining (fig. 4C) revealed, at 24 h after MCAO, an intense VEGF immunoreactivity which was mainly localized in the cytoplasm of neurons in the peri-ischemic area of vehicle-treated mice (fig. 4Cb). VEGF immunostaining was weaker in the MLC901-treated animals than in the vehicle-treated group (fig. 4Cc). At 3 days post stroke, an increase of VEGF expression was observed si-

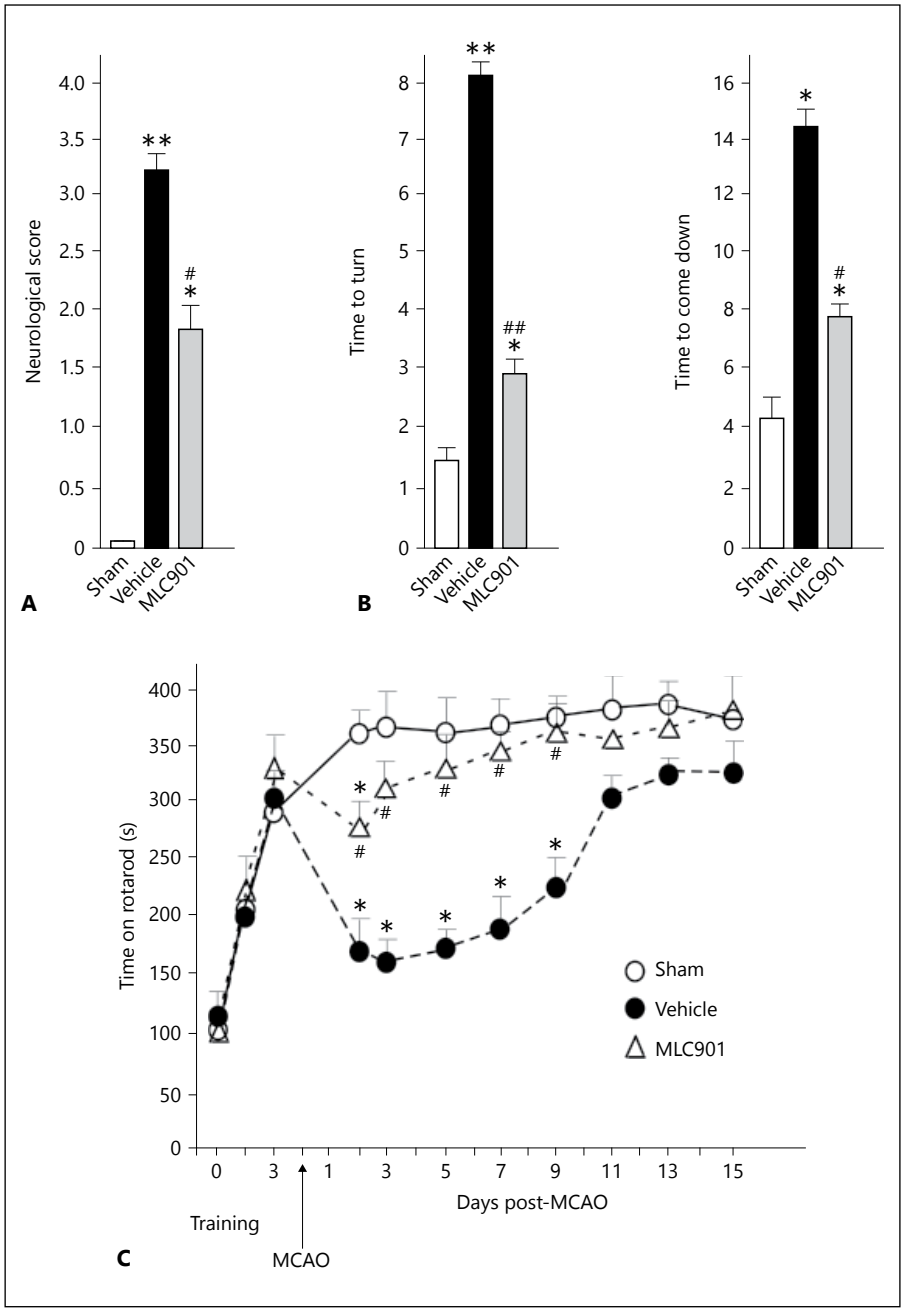

Fig. 2. MLC901 improved neurological function in injured mice submitted to behavioral tests. **A** Neurological deficits test (Bederson scoring) 30 h after MCAO. **B** Pole test performed 3 days after ischemia. **C** Rotarod test performed at different times after reperfusion. MLC901 was administered in the drinking water (6 g/l) for 5 weeks before MCAO and then until the sacrifice of animals (n = 10 per experimental group). Data are reported as mean ± SEM (* $p < 0.05$ vs. sham operated and pre-ischemia groups, # $p < 0.05$ vs. vehicle ischemic group, ** $p < 0.01$ vs. sham operated group, ## $p < 0.01$ vs. vehicle group, n = 16 per experimental group).

multaneously with the development of thickened endothelial cell capillaries, mainly in the MLC901-treated mice. MLC901 stimulated the growth of new vessels and compared to mice treated with vehicle, where VEGF was mainly expressed in neurons (fig. 4Ce), MLC901 enhanced earlier VEGF expression within endothelial cells and pericyte inter-digitations, identified by their particular morphology (fig. 4Cf–h). Double labeling experiments with VEGF/GFAP and VEGF/Iba1 revealed that VEGF was also expressed in microglial cells/macrophages (Iba1 marker; fig. 4Cj) but not in astrocytes (GFAP marker; fig. 4Ci). The upregulation of VEGF persisted up to 14 days in the peri-ischemic area (data not shown).

Because Ang1 and its natural antagonist Ang2 have been reported to be involved in the molecular mechanisms of angiogenesis [8, 39, 40], we investigated their expression in MCAO-induced ischemic brains of vehicle- and MLC901-treated mice (n = 4 per experimental group

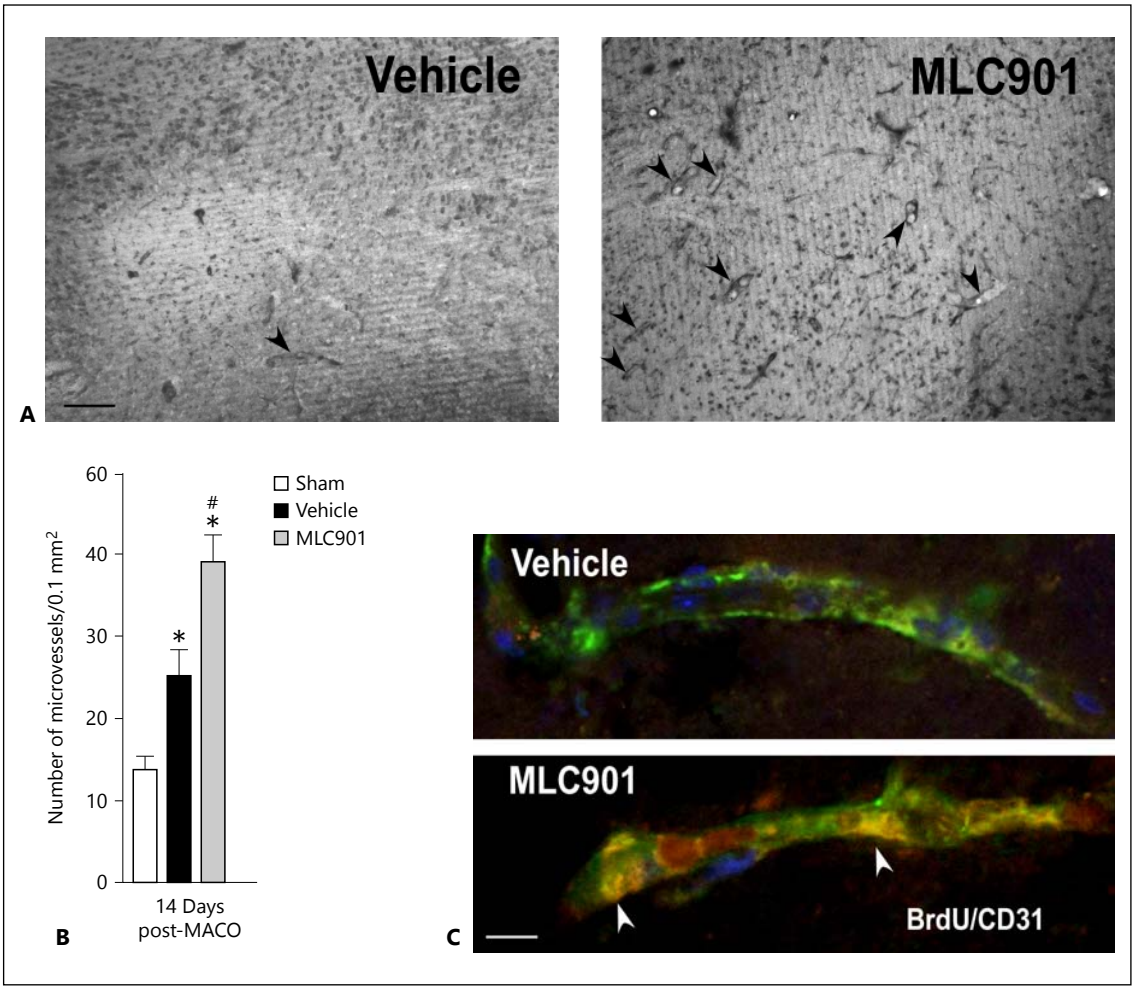

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Fig. 3. MLC901 increased the neovascularization in the infarcted zone 14 days after MCAO. **A** Representative photographs of CD31-positive cells, which identified the capillaries in brain sections of ischemic mice treated with vehicle (left panel) or MLC901 (right panel). Scale bar: 50 μm. The CD31 immunolabeling identified the capillaries. **B** Histograms showing microvessel counts per 0.1 mm^2 14 days after MCAO. Microvessels were counted from 10 fields of the ischemic penumbra under a microscope at 400× magnification. Data are mean ± SEM (* $p < 0.05$ vs. sham operated group and $^{\#}$ $p < 0.05$ vs. vehicle ischemic group, n = 6 per experimental group). **C** Double labeling of BrdU-labeled cells (red) with CD31 endothelial cell marker (green). Arrows indicate the BrdU/CD31 co-localization. Scale bar: 10 μm. MLC901 was administered in the drinking water (6 g/l) for 5 weeks before MCAO and then 14 days post MCAO.

and method used). Western blots showed that at 24 h post ischemia, Ang1 levels of vehicle-treated mice decreased compared with sham group, while the Ang2 expression increased (fig. 5A, B). Interestingly, MLC901 treatment reversed this situation by increasing Ang1 and decreasing Ang2 expression. However, in the vehicle group, an up-regulation of Ang1 and a downregulation of Ang2 protein levels were observed at 3 days and persisted for at least 14 days. Again, MLC901 reversed the effects of MCAO on these 2 proteins in the peri-focal area (fig. 5A, B). All these data seem to indicate that MLC901 has marked effects both on the changes of the early and delayed expressions of Ang1 and Ang2. Immunohistochemical staining confirmed the Western blot results and revealed that Ang1 and Ang2 were mainly expressed in neuron-like and glial-like cells in the first 24 h post ischemia and then in the microvessels and endothelial cells at 3 and 14 days after MCAO (fig. 5C).

A critical molecular pathway induced by hypoxia/ischemia is the activation of hypoxia-inducible factor 1, a transcriptional activator of genes encoding VEGF and other important mediators of angiogenesis [41]. Hif1α is

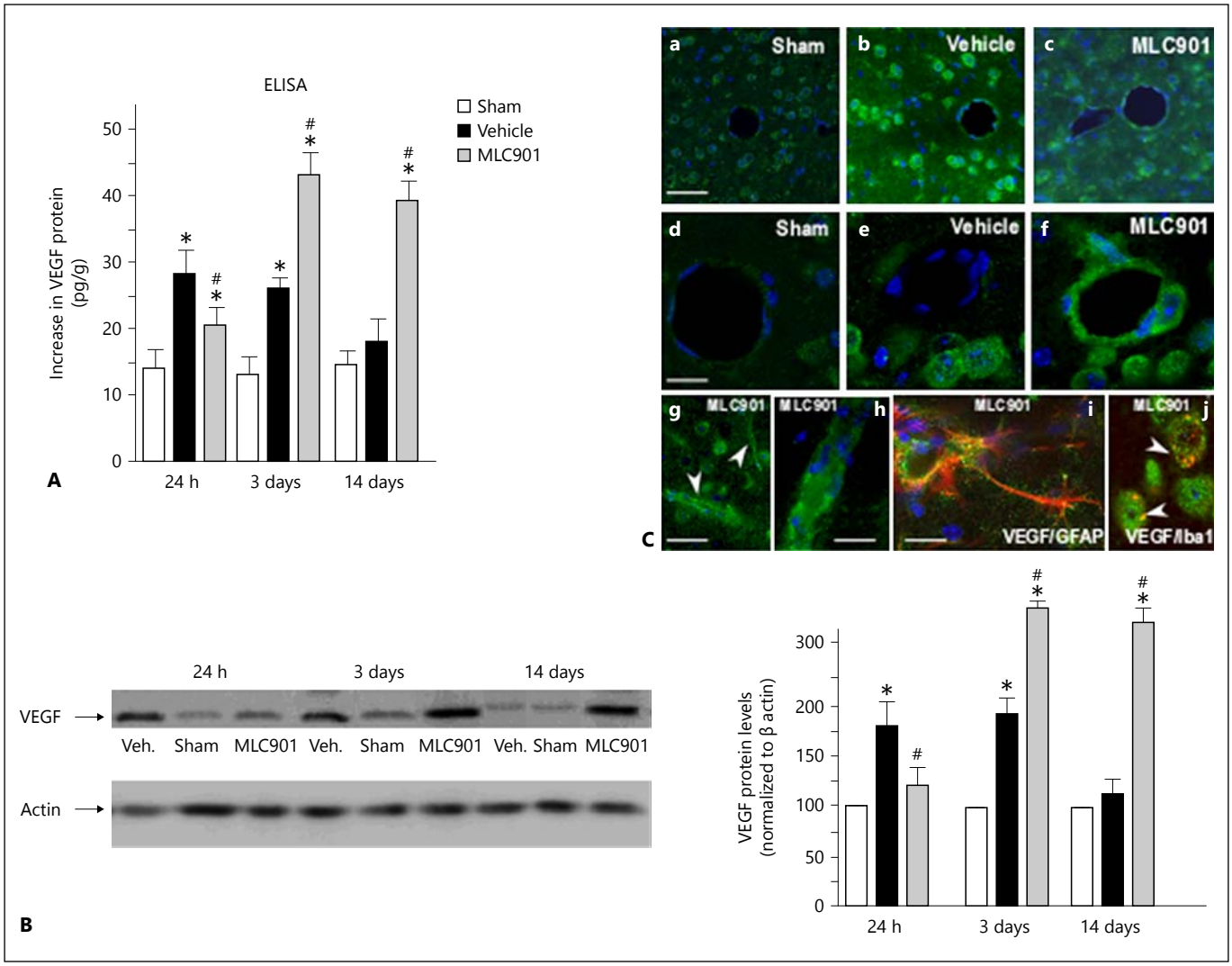

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Fig. 4. Effects of MLC901 on the increase in VEGF expression at different time points (24 h, 3 and 14 days) after ischemia. **A**, **B** Increase in VEGF protein levels obtained by ELISA assay (**A**) and Western blotting (**B**). In (**B**) β actin was used as internal control for the loading of protein level. Left: Western blot of VEGF expression (42 kDa) in injured cortical tissue. Right: quantitation of VEGF expression obtained in Western blots in the 3 experimental groups. Western blot and ELISA data are representative of 3 separate experiments (n = 4 per experimental group and method used). Values are expressed as a percentage of control. Data are mean ± SEM (* $p < 0.05$ vs. sham-operated group and # $p < 0.05$ vs. vehicle ischemic group). **C** Representative photomicrographs of VEGF immunolabeling in the ipsilateral injured cortex at 2 time points of reperfusion (24 h and 3 days, n = 4 per group). **C**a–c VEGF expression in the 3 experimental groups (sham, vehicle and MLC901) at 24 h post MCAO. Scale bar: 30 μm. **C**d–f VEGF expression in the 3 experimental groups (sham, vehicle and MLC901) at 3 days post MCAO. Scale bar 30 μm. **C**g–h VEGF expression in the capillary endothelium in MLC901-treated mice at 3 days post MCAO. Scale bar 60 and 15 μm. **C**i Double labeling of VEGF-labeled cells (green) with GFAP astrocyte marker (red) in MLC901 treated mice at 3 days post MCAO. Scale bar: 10 μm. **C**j Double labeling of VEGF-labeled cells (green) with Iba1 microglia marker (red) in MLC901-treated mice at 3 days post MCAO. Arrows indicate the VEGF/Iba1 co-localization.

the central transcriptional factor for the regulation of oxygen-associated genes in response to hypoxia [42]. Quantitative Western blotting and ELISA methods (n = 4 per experimental group and methods used) showed that cerebral ischemia induced by MCAO in vehicle-treated mice triggered a 2-fold increase of Hif1α protein levels in the ischemic hemisphere as compared with sham group at 3 days of recovery. The Hif1α levels remained elevated for at least 14 days (fig. 6A–C). Immunohistochemical analysis indicated that Hif1α accu-

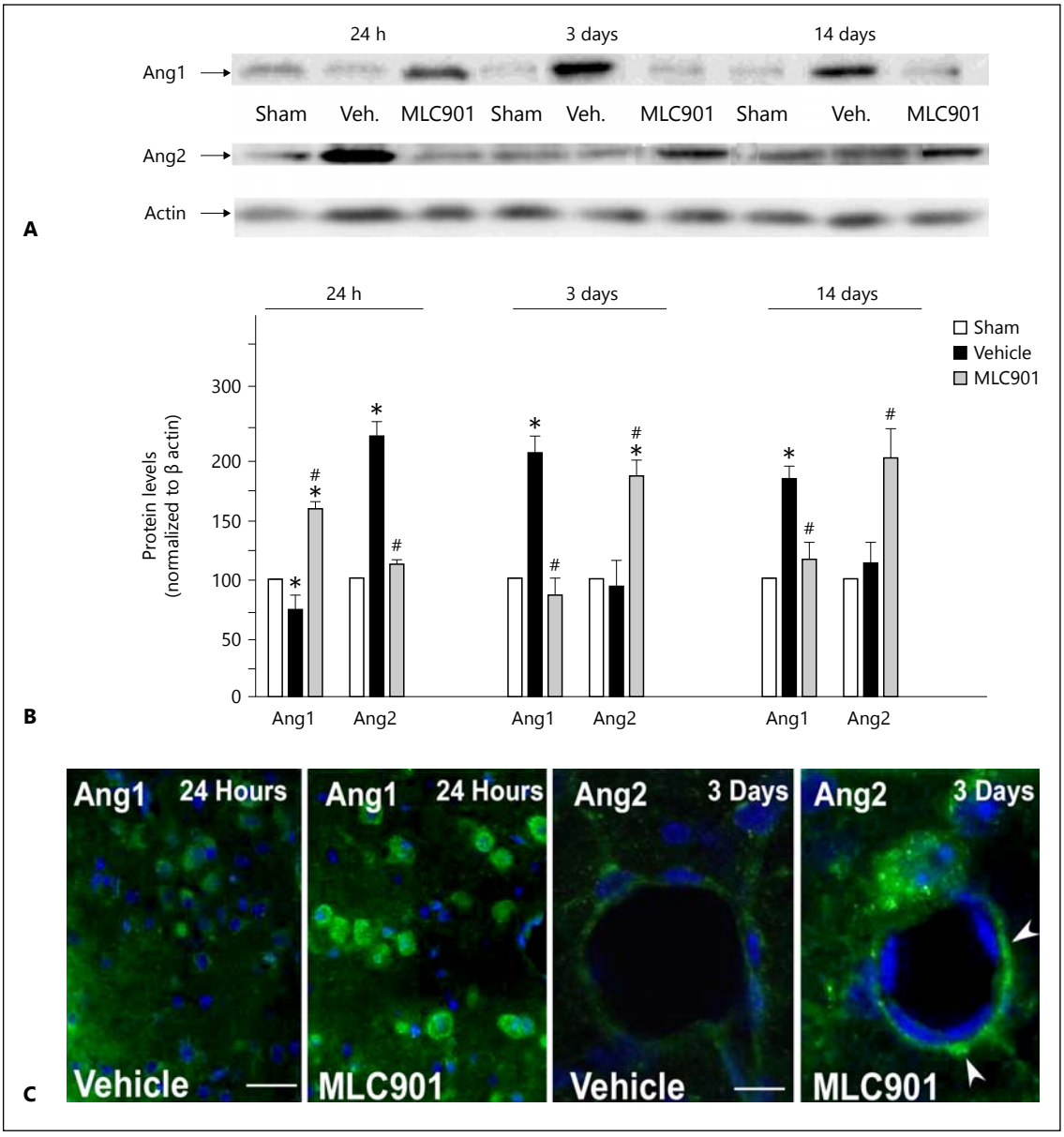

Fig. 5. Effects of MLC901 on the changes in Ang1 and Ang-2 expression at different time points (24 h, 3 and 14 days) after ischemia. **A** Western blots of Ang1 (57 kDa) and Ang-2 (73 kDa) in injured cortical tissue. β actin was used as internal control for the loading of protein level. **B** Quantitation of Western blots normalized to β actin. Data are representative of 3 separate experiments (n = 4 per experimental group). Values (mean ± SEM) are expressed as percentage of control (* $p < 0.05$ vs. sham-operated group and # $p < 0.05$ vs. vehicle ischemic group). **C** Representative photomicrographs of Ang1 and Ang2 immunolabeling in the ipsilateral injured cortex in vehicle- and MLC901-treated mice at 2 time points of reperfusion (24 h (for Ang1, scale bar: 30 μm) and 3 days (for Ang2, scale bar: 10 μm, n = 4 per experimental group). MLC901 increased Ang2 expression in the capillary endothelium at 3 days post MCAO. Scale bar 60 and 15 μm.

mulation was mainly restricted to neurons and astrocytes in the peri-infarct area (fig. 6D). As already observed for VEGF and angiopoietins, MLC901 particularly stimulated the late (14 days) upregulation of Hif1α (fig. 6D).

EPO, a hematopoietic growth factor, is a downstream target gene of HIF1. It increases oxygen availability during hypoxia/ischemia and is associated with neuroprotection following hypoxia ischemia in several preclinical models of stroke [43, 44]. Astrocytes and neurons are the

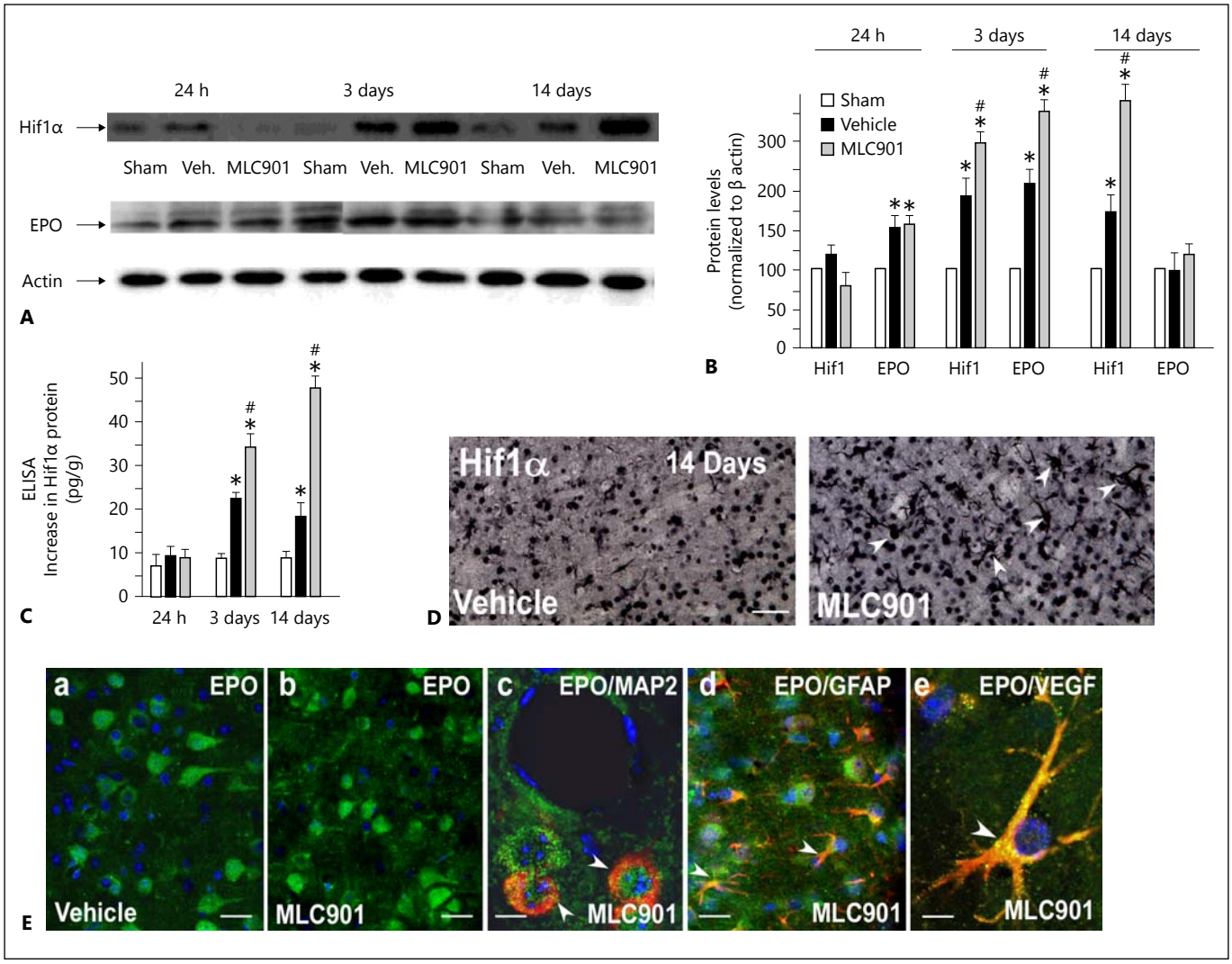

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Fig. 6. Effects of MLC901 on the changes in Hif1α and EPO expression at different time points (24 h, 3 and 14 days) after ischemia. **A** Western blotting analysis of Hif1α (93 kDa) and EPO (37 kDa) expression in injured cortical tissue (n = 4 per experimental group). Actin was used as internal control for the loading of protein level. **B** Quantitation of Western blots normalized to β actin. **C** ELISA assay showing the increase of Hif1α expression after ischemia. Data (mean ± SEM) of Hif1α and EPO protein levels are representative of 3 separate experiments (n = 4 per experimental group, * $p < 0.05$ vs. sham-operated group and # $p < 0.05$ vs. vehicle ischemic group). **D** Representative photomicrographs of the upregulation of Hif1α expression by MLC901 in neurons and astrocytes (arrows) of the ipsilateral injured cortex when compared to vehicle group after 14 days of reperfusion (n = 4 per experimental group, scale bar: 50 μm). **E** Representative photomicrographs of EPO immunolabeling in the ipsilateral injured cortex in vehicle- and MLC901-treated mice 3 days after MCAO (**E**a, b, scale bar: 20 μm). **E**c Double labeling of EPO-labeled cells (green) with MAP2 neuronal marker (red) in MLC901-treated mice at 3 days post MCAO. Scale bar: 10 μm. Arrows indicate the EPO/MAP2 co-localization. **E**d Double labeling of EPO-labeled cells (green) with GFAP astrocyte marker (red) in MLC901 treated mice at 3 days post MCAO. Scale bar: 20 μm. Arrows indicate the EPO/GFAP co-localization. **E**e Double labeling of EPO-labeled cells (green) with VEGF (red) in MLC901-treated mice at 3 days post MCAO. Scale bar: 10 μm. Arrows indicate the EPO/VEGF co-localization.

major sources of EPO in the brain [43]. Quantitative Western blots (n = 4 per experimental group) revealed that MCAO induced a modulation of EPO expression in the ipsilateral ischemic hemisphere in the vehicle-treated mice, which started in the first 24 h after ischemia and peaked after 3 days of recovery (fig. 6A, B). At 3 days post stroke, EPO contents in vehicle samples were 2-fold increased versus sham non-ischemic hemisphere ($p < 0.05$). MLC901 treatment stimulated by 1.75-fold the increase of EPO when compared to vehicle group ($p < 0.05$; fig. 6A,

B). The immunohistochemical study (n = 4 per experimental group) confirmed the upregulation of EPO after MCAO both in vehicle and MLC901 groups at 3 days post MCAO (fig. 6E). At 3 days after occlusion, in the peri-infarct region, an increase in the EPO immunoreactivity was observed in MLC901-treated mice when compared to vehicle group (fig. 6Ea, b). EPO immunolabeling was mainly apparent in cortical MAP2-positive neurons identified (fig. 6Ec) and GFAP-positive astrocytes (fig. 6Ed) surrounding the infarct (fig. 6Ed). It was often co-localized with VEGF around endothelial cells and microvessels (fig. 6Ee).

Discussion

In the present study, we demonstrated that MLC901 administered for 5 weeks before the induction of ischemia, and then during the entire time of recovery, significantly protected mice against brain injury after focal cerebral ischemia. MLC901 increased the survival of mice after MCAO, reduced the infarct volume and hemispheric swelling and attenuated BBB disruption. The current data also demonstrated that MLC901 treatment significantly improved neurological function assessed by rotarod and pole tests, which are a sensitive index for assessing motor impairment after focal ischemia and traumatic brain injury [45, 46]. In the rotarod test, mice exhibited marked motor impairment at 2 days after stroke, and partial recovery was detected in the vehicle-treated mice from 11th day, which is consistent with the fact that partial functional neurological recovery from a stroke occurs commonly [47]. As currently observed in this MCAO model, the vehicle-treated animals presented similar rotarod performances compared to sham animals after 2 weeks of recovery. However, MLC901-treated mice showed a significant early improvement of motor performances compared with vehicle-treated animals. This early MLC901-induced improvement in the rotarod test, known to primarily detect motor impairment in the ischemic cortex is a good indication of the therapeutic potential of this drug. These beneficial effects are also confirmed by the pole test. These data are in line with previous preclinical studies indicating beneficial effects of MLC901 on both the rate of recovery and on the functional outcome in different rodent models of ischemia and traumatic brain injury [9, 24–26].

All these beneficial effects of MLC901 on the early phase of cerebral ischemia cannot easily be explained with the more slowly working angiogenesis. The decrease of infarct volume, the suppression of brain swelling and the attenuation of neurological dysfunction are probably due to the early activation of neuroprotective K_{ATP} channels [27], the acute decrease of excitotoxicity [27] as well as the stimulation of the Akt survival pathway, the reduction of apoptosis and free radical generation [25], impeding the deleterious ischemic cascade propagation from the core to the penumbra. At this point, it should be reminded that MLC901 could also stimulate neurogenesis and synaptogenesis [24, 25] and increase BDNF production, which is expected to prevent brain damage and stimulate brain plasticity and hopefully subsequent neurorepair after brain injury. The results presented in this work have been obtained with a MLC901 treatment given 5 weeks before MCAO, but the efficacy of MLC901 given in post-treatment on acute cerebral ischemia has been previously demonstrated by our laboratory in rodents [9, 24–26] and confirmed in clinical trials [11, 19].

In the key restorative mechanisms in response to stroke, there is increasing evidences that angiogenesis, through new blood vessel formation, results in improved collateral circulation and may impact the long-term recovery of patients [2, 3, 6]. Indeed, the severe reduction of blood flow to the brain tissue results in a lack of oxygen and nutrient transportation, leading to brain hypoxia and cell death. To counteract these deleterious effects, compensatory mechanisms try to increase oxygen delivery to the injured region by induction of angiogenesis. Newly formed vessels would allow increased blood flow, thus increasing perfusion of the suffering ischemic area. Studies using human brain samples have suggested that active angiogenesis takes place at 3–4 days after stroke and indicated that the number of new vessels in ischemic penumbral regions appears to be correlated with survival [48–50]. A key objective in the management of effective treatment of cerebral ischemia is then to try to design therapies able to enhance both new blood vessels and neurogenesis particularly in an early period after stroke.

This work indicates that, in addition to stimulation of neurogenesis, that has been previously reported [9, 24–26], MLC901 also promotes angiogenesis in response to MCAO by modulating the expression of angiogenesis-related factors such as Hif1α, EPO, VEGF and Ang1/Ang2 (fig. 7). These factors are known to not only mediate the proliferation of endothelial cells, but also regulate vascular differentiation, regression and permeability.

During and after stroke, brain vasculature becomes leaky and unstable, and the normally impermeable BBB breaks down [34]. Then, endothelial cells begin to proliferate, and angiogenesis occurs. Expression studies have

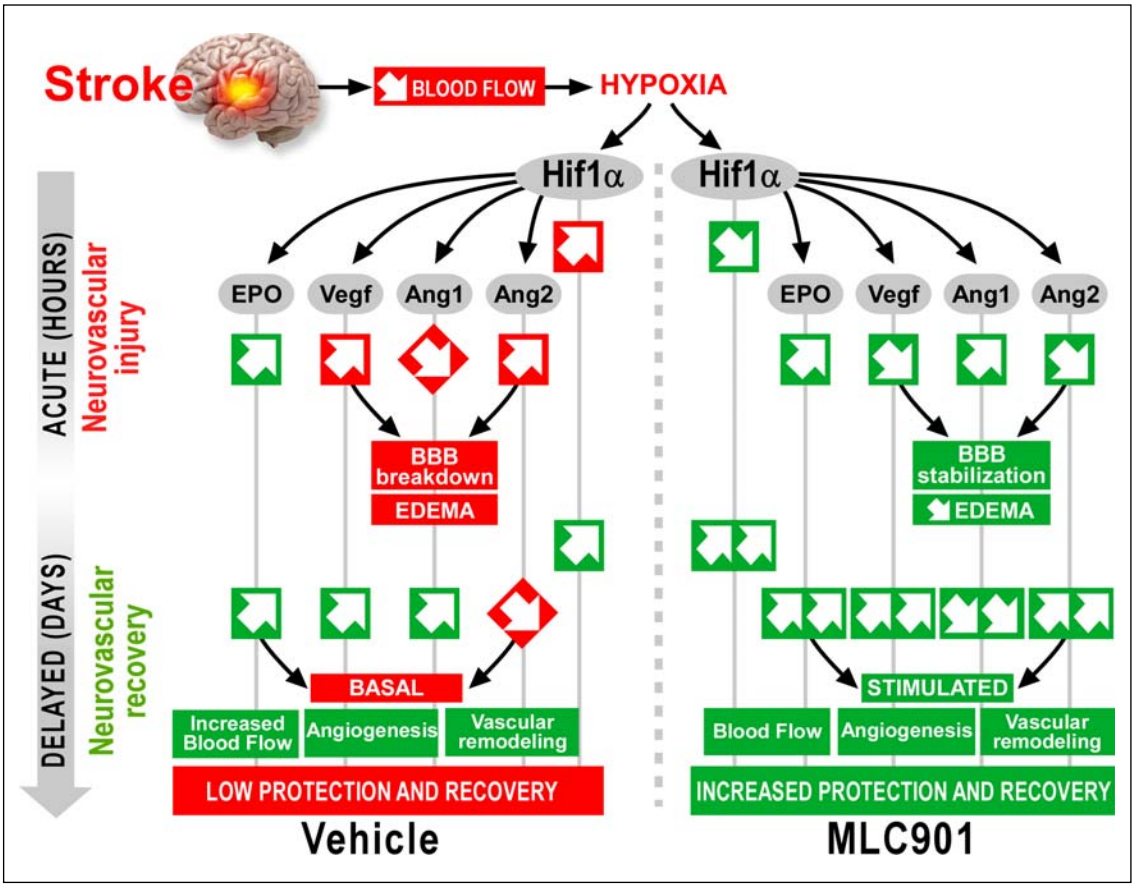

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Fig. 7. Schematic representation of the effects of MLC901 on the modulation of angiogenic factors in the acute (24 h) and delayed (3–14 days) phase after MCAO leading to increased neuroprotection and recovery by stimulation of angiogenesis and vascular modeling. Red and green colors indicate the deleterious and beneficial effects. Ascending and descending arrows indicate the up- or downregulation of angiogenic factors, respectively.

shown that the key vascular factors are regulated, during these processes, in a complex and coordinated manner [51]. Hypoxia is an important driving force for angiogenesis, which begins when cells within a tissue respond to hypoxia by increasing their VEGF production. Then, VEGF promotes adult angiogenesis for re-establishment of blood flow following ischemic damage [52]. This response is orchestrated at the molecular level by Hif1α, which is responsible for the transcriptional activation of the VEGF gene in hypoxic cells [41, 53]. This work demonstrated that (i) MLC901 strongly decreases BBB breakdown and also brain edema, which rapidly occurs after stroke injury, and (ii) MLC901 promotes endothelial cell proliferation in the ischemic border by regulating the expression of both Hif1α and VEGF. The early MLC901-induced downregulation of Hif1α and VEGF probably maintains BBB integrity preventing brain swelling and late upregulation of both Hif1α and VEGF is believed to be associated with angiogenesis and vascular remodeling. Indeed, an early administration of VEGF has been shown to increase vascular permeability and to induce brain edema, while late treatment with VEGF has been shown to improve histological as well as both sensorimotor and cognitive deficits and functional outcome [54]. These findings are also consistent with studies which have documented the biphasic nature of the Hif1α response [42]. It has been proposed that transient increases in Hif1α within the first 24 h after an injury contribute to cell death in part through the transcriptional upregulation of pro-apoptotic target genes, while at a late phase of activation, Hif1α induces the expression of pro-survival genes mainly implicated in angiogenesis [42, 54]. Thus, an increased expression of Hif1α would be detrimental in the acute stages of ischemia whereas it would be beneficial in the delayed/recovery stage of injury.

Ang1 and Ang2 also play an essential role in the stepwise process of angiogenesis including destabilization of

vessels, sprouting and branching of destabilized vessels, proliferation and migration of endothelial cells and stabilization of neomicrovessels [8, 39, 40]. Ang1 and Ang2 are the ligands for the endothelial specific receptor tyrosine kinase, Tie2. Ang1 induces the auto-phosphorylation of Tie-2 in cultured endothelial cells. It recruits pericytes and potentiates microvessel stabilization and maturation by supporting interactions between endothelial cells and pericytes. Ang1 is a strong anti-permeability factor that can reduce vascular leakage. Ang2 acts as a natural Ang1 antagonist. It promotes a process of vessel wall disassembly by blocking Ang1-induced Tie-2 phosphorylation in endothelial cells [55, 56]. Ang2 initiates extensive angiogenesis, resulting in pericyte drop-off and vascular destabilization and is considered as an important pro-angiogenic factor [39, 55]. This work shows that MLC901 has marked effects both on the changes of the early and delayed expressions of Ang1 and Ang2. These effects are probably related to the reduction of the BBB leakage and to the stimulation of angiogenesis, respectively. Indeed, Ang2 acts synergistically with VEGF. Consistent with previous studies [39], we observed in vehicle-treated mice that unlike Ang1, VEGF and Ang2 were significantly increased in the infarcted area during the early period of cerebral ischemia corresponding to the formation of brain edema. Interestingly, MLC901 treatment reversed the situation by increasing Ang1 and decreasing Ang2 and VEGF expressions in the early phase following ischemia. The early MLC901-induced modulation of these factors is probably linked, as already mentioned, to a decrease of BBB leakage and subsequent brain edema. Consistent with the previously discussed results, we have also observed that MLC901 also prevents the early increases in expression of Hif1α after the onset of ischemia, an increased expression that is known to upregulate VEGF and downregulate the expression of Ang1 [57, 58]. In addition, it turns out that, the days following MCAO, the upregulation of both Ang1 and VEGF and downregulation of Ang2 protein levels coincides with endothelial cell proliferation and neovascularization at the border of the infarct. Thus, MLC901, by modulating protein levels of the 3 angiogenic factors (VEGF, Ang1 and Ang2), appears to stimulate angiogenesis and the long-term vascular remodeling by stabilizing or enriching brain vasculature after ischemia.

EPO is the main identified target gene induced by Hif1α, the expression levels of which we have shown to be regulated by MLC901. On the other hand, exogenous EPO has been shown to be cerebroprotective [59, 60]. It is possible that the observed beneficial effects of MLC901 may be due to a stimulation of endogenous cerebral EPO production. This study has shown that MLC901 induced a continuous formation of EPO during the active evolution of the focal cerebral infarct. This highly suggests that the EPO system, known to be an endogenous defense system to protect the brain against damage consequent to a reduction of blood flow or hypoxia [43, 44] might well be involved in the processes of MLC901-induced neuroprotection and restructuring including angiogenesis after stroke.

Results presented in this work strengthen previous views that MLC901 is a therapeutic tool with multiple targets due to the many molecules that are present in the 9 herbal extracts of the cocktail. In the different herbs that are extracted to manufacture MLC901, we have shown that there are a number of identified molecules that are neurobeneficial. However, at present, none of these molecules, alone or in combination in vitro or in vivo, can reconstitute the overall properties of MLC901 in stroke indicating the complexity of the therapeutic cocktail.

The MLC901 mode of action clearly appears to be a potentiation of endogenous mechanisms that normally take place in the neurovascular unit after stroke to allow many of the stroke patients to recover, most of the time partly and rarely completely, their initial behavioral and motor activity [61]. We see MLC901 as a treatment that limits stroke damage and that accelerates the time course and increases the extent of the recovery process. Compared to majority of studies on pharmacological neuroprotection against stroke that are first tested in animal models and failed in clinical trials, MLC901 has the advantage of having already demonstrated clinical efficacy in stroke patients. Our preclinical studies provide further scientific basis to describe its mechanisms of action and help in future clinical trials.

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Disclosure Statement

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