1. **Neuroprotective and neuroproliferative activities of NeuroAid (MLC601, MLC901), a Chinese medicine, in vitro and in vivo.** Neuropharmacology. 2010
   
   The published results describe a significant neuroprotective effect of NeuroAid against an ischemic insult when given prior and/or after injury. It shows that NeuroAid: Supports neuroplasticity, Increases neurogenesis, Increases neurites outgrowth and synaptogenesis, Provides a better environment for post stroke recovery and decreases neurological functions impairments.

2. **NeuroAid, a traditional Chinese medicine, in poststroke recovery.** Stroke. 2009
   
   Report on clinical trials on 605 patients recruited between 2 weeks and 6 months after their stroke (initial stroke trials in China). Results: Patients on NeuroAid had 2.4 times more chances to achieve independence after one month of treatment, and showed a 25% higher recovery in the motor components.

   
   Case Report on 10 patients who received NeuroAid after ischemic stroke onset confirmed on imagery. Conducted at outpatient private clinic, Mount Alvernia Hospital in Singapore. NeuroAid as an add on to other medications including anti-platelet, warfarin, lipid-lowering, anti-hypertensive, diabetic and antidepressant medications.

   
   Description of CHIMES study, evaluation NeuroAid efficacy and safety when initiated at the acute stage of stroke. (72 H)

5. **A double-blind, placebo-controlled, randomized phase II pilot study to investigate the potential efficacy of the traditional Chinese medicine Neuroaid (MLC 601) in enhancing recovery after stroke (TIERS).** Cerebrovascular Diseases. 2009
   
   “Our aim was to investigate the efficacy of NeuroAid on motor recovery in ischemic stroke patients using rehabilitation endpoints in order to provide predictive information for further larger trials.”

   20 cases of patients who received 4 capsules of NeuroAid 3 times a day for 4 weeks, 20 other patient received placebo. Treatment was initiated less than one month post ischemic stroke. NeuroAid performed better in severe cases (+58% compared to the Placebo panel). Strong tendency of better recovery in posterior circulation infarction (POCI). 5 best patient responders having taken NeuroAid recovered 39% more than 5 best placebo responders.

6. **Safety Profile of MLC601 (NeuroAid ) in Acute Ischemic Stroke Patients: A Singaporean Substudy of the Chinese Medicine NeuroAid Efficacy on Stroke Recovery Study.** Cerebrovascular Diseases. 2010
   
   Safety profile of NeuroAid, 3 months treatment when initiated within 72 hours post cerebral infract. (CHIMES sub study). Will be published within the next months.

7. **NeuroAid does not modify hemostasis, hematology, and biochemistry in normal subjects and stroke patients.** Cerebrovascular Diseases. 2008.
   
   “NeuroAid does not significantly affect hematological, hemostasis, and biochemical, in normal and stroke patients. Clinical parameters and expected effect of aspirin are not altered by co-administration of the drug even when started and maintained at the early stage of acute stroke”
Neuroprotective and neuroproliferative activities of NeuroAid (MLC601, MLC901), a Chinese medicine, in vitro and in vivo

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ABSTRACT

Although stroke remains a leading cause of death and adult disability, numerous recent failures in clinical stroke trials have led to some pessimism in the field. Interestingly, NeuroAid (MLC601), a traditional medicine, particularly used in China, South East Asia and Middle East has been reported to have beneficial effects in patients, particularly in post-stroke complications. Here, we demonstrate in a rodent model of focal ischemia that NeuroAid II (MLC901) pre- and post-treatments up to 3 h after stroke improve survival, protect the brain from the ischemic injury and drastically decrease functional deficits. MLC601 and MLC901 also prevent neuronal death in an in vitro model of excitotoxicity using primary cultures of cortical neurons exposed to glutamate. In addition, MLC601/MLC901 treatments were shown to induce neurogenesis in rodent and human cells, promote cell proliferation as well as neurite outgrowth and stimulate the development of a dense axonal and dendritic network. MLC601 and MLC901 clearly represent a very interesting strategy for stroke treatment at different stages of the disease.

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1. Introduction

Stroke affects numerous people every year. When brain cells die, the function of the body parts they control is impaired or lost, causing paralysis, speech and sensory problems, memory and reasoning deficits, coma, and possibly death. Treatment for stroke is almost reduced to fibrinolysis, a therapy that unfortunately can be only used in a relatively low percentage of patients. Dozens of clinical trials have failed to show efficacy in humans for a variety of neuroprotective drugs (Ginsberg, 2008). In addition, there are no effective, clinically approved methods that promote restoration of CNS function, days, weeks or months after stroke. The need for new therapeutic strategies is high.

A slow but consistent recovery can be observed in the clinical practice over a period of weeks and months. Whereas the recovery in the first few days likely results from edema resolution and/or from reperfusion of the ischemic penumbra, a large part of the recovery afterwards is mainly due to brain which spontaneously recovers by the reorganization of surviving central nervous system elements in the damaged areas (Cramer, 2008). Neurogenesis and angiogenesis are key mechanisms of recovery after stroke (Zhang et al., 2008). The research of therapeutic agents able to stimulate proliferation, migration and differentiation of new neural cells that can replace those lost during a stroke episode is important for future.

Due to the complexity of stroke disease, there is increasing evidence that the search for a “magic drug” which specifically acts on a single target is exceeded and that combination therapies comprising more than one active ingredient can represent a better strategy against stroke. Interestingly, combination therapy has been advocated for >2500 years by prescriptions of formulae in traditional Chinese Medicine (TCM), that consist of several types of medicinal herbs, based on clinical experience. As recently shown for promyelocytic leukemia, Chinese herbal medicines can represent a new promising area in drug discovery (Wang et al., 2008). The aim of this work is to address the possible beneficial effects of MLC601 and MLC901 against stroke disease. MLC601 (NeuroAid, Molecap Pte. Ltd, Singapore) is a TCM which is used extensively in China to facilitate recovery after stroke (Chen et al., 2009). It combines 9 herbal (including Radix astragali, Radix salviae miltiorrhizae, Radix paonieae rubra, Rhizoma chuanxiong, Radix angelicae sinensis, Carthamus tinctorius, Prunus persica, Radix polygalae and Rhizoma acori tatarinowii) and 5 animal components (including Hirudo, Eupolyphaga seu stelephaga, Calculus bovisartefactus, Buthus
stroke patients (Gan et al., 2008; Siow, 2008). However, before this assessing immediate and long-term effects, alone or in combination with aspirin showed the safety of MLC601 in normal subjects and stroke patients (Gan et al., 2008; Siow, 2008). However, before this work was started, there was no scientific background for the use of this TCM against stroke. The purpose of this work is to analyze whether MLC601 and MLC901 have interesting neuroprotective and/or neurogenerative properties in vitro and in vivo assays that are normally used in Western medicine to develop new drugs, preclinically, before assaying them in humans. We report here the protective effects of MLC601 and MLC901 on neuronal and brain injuries as well as positive effects on functional recovery after ischemic stroke. We also demonstrate in vitro neuronal proliferation and neurite outgrowth as well as in vivo neurogenesis induced by MLC601/MLC901.

2. Materials and methods

2.1. Neuronal culture

Time-pregnant (E14)/C57Bl/6J mice were anesthetized with isoflurane followed by cervical dislocation. Fetuses were removed and placed in cold HBSS solution. Cerebral cortices were dissected in cold HBSS solution and the meninges were removed. Cortical samples were cut in small pieces and were gently triturated with a fire-polished glass Pasteur pipette in 8 ml HBSS solution. The mix was filtered (40 μm filter) and centrifuged at 800 rpm for 8 min. The supernatant was removed and the pellet was dissolved in 2 ml culture medium. Cells were plated on poly-lysine (Sigma–Aldrich Chimie, St Quentin Fallavier, France) coated 12 well (24 mm diameter) plates with glass coverslips (12 mm diameter) (CML, Nemours, France) at a density of 1 x 10^5 cells/well. Cultures were maintained at 37 °C in a humidified 5% CO2 atmosphere incubator in Neurobasal supplemented with B27, Glutamax, antimycotics and antibiotics for 16 days. Glial growth was suppressed by addition of 5-Fluoro-2-deoxyuridine (2 μM) and Uridine (2 μM) during the second day of culture. The degree of damage observed in the current in vitro system was similar to that previously reported in aging cultures of mouse cortical neurons (Lesuisse and Martin, 2002).

2.2. In vitro model of excitotoxicity

As model of excitotoxicity, we used glutamate at the concentration of 10 μM in magnesium-free glycine-supplemented PBS during 10 min, which induced significant damage as previously reported (Hartley et al., 1993). MLC601 or MLC901 was added at the concentration of 1 μM/ml, 4 days before and after treatment with glutamate. Control cells were incubated with vehicle alone. Cell survival and lactate dehydrogenase (LDH) release were estimated 5, 8 and 24 h after glutamate treatment (n = 3 cultures, 36 wells per experimental group). Results corresponded to the mean of three independent experiments with triplicate determination. Statistical analyses of cell viability and LDH results were assessed using one factor ANOVA test following by post-hoc test (P < 0.05).

2.4. Focal ischemia

2.4.1. Animals

All experiments were performed according to policies on the care and use of laboratory animals of European Community legislation. The local Ethics Committee approved the experiments (protocol numbers NCA/2008/6 and NCA/2008/10). All mice were made to minimize animal suffering and reduce the number of animals used. Adult male C57/B16 mice, weighing 22–26 g (7–9 weeks old) were used in this study. Animals 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. Mice had free access to standard rodent diet and tap water. The researchers, who carried out the ischemic surgery and measured infarct volumes were blinded in regard to the treatment code.

2.4.2. Model of focal ischemia

Ischemia was induced by occlusion of the left middle cerebral artery (MCA) using an intraluminal filament technique (Heurteaux et al., 2006a; Huang et al., 1994). After a midline neck incision was made, the left common and external carotid arteries were isolated and ligated with a 4–0 silk suture thread (Ethicon). A yasargil aneurysm clip (BMH31, Aesculap, Tuttlingen, Germany) was temporarily placed on the internal carotid artery. A 6–0 coated filament (Doccol, Redlands, CA, USA) was introduced through a small incision into the common carotid artery and 13 mm distal to the carotid bifurcation for occlusion of MCA origin. Animals were kept at 37 °C for 1 h, after which time the thread was carefully withdrawn to allow reperfusion of MCA territory. To control MCAO severity regional cerebral blood flow (rCBF) was determined by laser-Doppler flowmetry (Perimed) using a flexible 0.5 mm fiber optic extension to the master probe fixed on the intact skull over the ischemic cortex (2 mm posterior and 6 mm lateral from the bregma). Sham-operation was performed inserting the thread into the common carotid artery without advancing it to occlude MCA. Animals were allowed to regain full consciousness on a heating pad before returning to the cage.

2.4.3. Physiological parameters

General anesthesia was induced with 3% isoflurane and maintained with 1% isoflurane by means of an open face mask for each mouse. Mice were allowed to breathe spontaneously. A subset of animals (n = 5 per group) were monitored for physiological parameters including mean arterial blood pressure (MABP), rectal temperature, arterial blood gases and pH before, during and after ischemia. The right femoral artery was catheterized with PE-10 polyethylene tubing and connected to a blood pressure transducer (Harvard Apparatus) for continuous monitoring of MABP (mmHg). A heparinized blood sample (75 μl) was then obtained from the catheterized femoral artery. Blood PO2, PCO2 and pH were measured using an Acid–Base Laboratory system (ABL 555, Radiometer). Core temperature was continuously monitored with a thermometer (3-mm probe diameter; Harvard Apparatus), inserted into the rectum and maintained at physiological temperatures using a thermostatically controlled heating blanket (Harvard Apparatus). Core temperature was maintained before, during and 3 h after ischemia at physiological values by using the homeothermic blanket control.

2.4.4. Determination of infarct volume

Mice were sacrificed 24 h after reperfusion. To visualize the evolution and the extent of infarct volume by TTC (2,3,5-triphenyltetrazolium chloride) staining, brains were removed and sectioned into six 1 mm-thick coronal slices using a tissue chopper (Phynemp, France). Coronal slices were immediately immersed into 2% TTC (Sigma, France) for 20 min at room temperature in the dark followed by fixation in 4% paraformaldehdye solution overnight prior to analysis (Heurteaux et al., 2006a).
Areas of infarction, outlined in light, appeared in white on coronal TTC-stained slices. To confirm the extent of the cerebral lesion, cresyl violet staining on coronal frozen brain sections (10 μm-thick) was performed 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 (1995). Infarct volume, expressed in mm$^3$, was calculated by a linear integration of the corrected lesions areas as previously described (Heurteaux et al., 2006a).

2.5. Drug treatments

MLC601 (NeuroAid) and MLC901 (NeuroAid II) were provided by Moleac (Singapore). The composition of MLC601 (0.4 g per 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, 0.095 g Buthus martensi, 0.0665 Hirudo, 0.0665 g Eupolyphaga seu steleophaga, 0.0285 g Calculus bovisarticulatus, 0.0285 g Cornu saigae tataricae. In MLC901, Buthus martensi, Hirudo, Eupolyphaga seu steleophaga, Calculus bovisarticulatus and Cornu saigae tataricae have been removed. For in vitro experiments, the concentration used in each 24 mm well was 1 μg/ml. A capsule containing 400 mg MLC601 or MLC901 was diluted in 40 ml Neurobasal medium corresponding to a concentration of 10 mg/ml (Stock solution) at 37°C during 60 min. Cell treatment with MLC601 or MLC901 started at Day 3 of culture during 14 days (corresponding to 17 days of culture). For in vivo experiments, MLC901 pre-treatment was given in drinking water at the concentration of 6 mg/ml. One capsule of MLC901 was dissolved in 66 ml water under stirring with an agitator for 1 h at 37°C. The solution was then filtered with 0.22 μm filter. For in vivo post-treatment, mice were intraperitoneally injected with a single dose of 2 μg/ml MLC901 or MLC601 solution diluted in saline (as vehicle) in a total volume of 500 μl/mouse weighing 25 g at the onset of ischemia and 6 h after reperfusion (Post-treatment Onset) or 3 and 24 h following the end of ischemia (Post-Treatment 3H). The dose used for in vivo pre-treatment 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 mouse weight and its daily water intake. The dose used in the post-treatment corresponded to the doses used on cortical neurons in culture (see Results Section 3.1). Each treatment group had its own control. The flowchart illustrating the experimental design is given in Fig. 1.

2.6. Motor performance tests

To explore the functional recovery after ischemia, behavioral testing was performed 3 days following ischemia with the rotarod and the actimeter tests, which were monitored by one researcher blinded to mouse treatment code.

### 2.6.1. Accelerated rotarod

The rotarod test has been used to assess motor coordination and balance alterations after ischemic brain injury in the rodent (Rogers et al., 1997). 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 = 10 per group) were conditioned to the accelerating rotarod (Ugo Basile, France) for three days before MCA occlusion. To this end, mice were first...

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**Fig. 1.** Flowchart illustrating the different in vivo paradigms. In A, B and C, mice were subjected to a 60 min middle cerebral artery occlusion (MCAO). (A) MLC901 pre-treatment administered during 42 days (6 weeks) in drinking water (6 mg/ml) during 60 Min. (B) MLC901 Post-treatment: ONSET. MLC01 was intraperitoneally injected (1 μg per mouse) at the onset and 6 h after ischemia. (C) MLC901 Post-treatment: 3H. MLC901 or MLC601 was intraperitoneally injected (1 μg per mouse) 3 and 24 h after ischemia. (D) MLC901 pre-treatment and neurogenesis. MLC901 was administered during 42 days (6 weeks) in drinking water (6 mg/ml). 24 h later, mice received 4 BrdU intraperitoneal injections (75 μg/kg) at 2 h interval.

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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 (Day 0). After 10 min, each mouse was placed on a single base accelerating rotable in which the spindle increased in speed from 4 to 40 rpm over a period of 6 min. The same protocol was applied at Day –3, Day –2 and Day –1. The test trial was performed at Day –3 and Day –2 after MCA occlusion. The maximum duration the animals were able to walk on the rotarod before falling was measured (maximum value: 6 min). The trial was ended if the mice grabbed the device and spun around for 2 consecutive revolutions. Mice were tested over three daily trials in the accelerated condition (4–40 rpm). The daily mean value was taken for each mouse and used for statistical analysis.

2.6.2. Spontaneous locomotor activity
Mice were placed individually into an activity-monitoring system (Inetrometric, France), consisting of clear plexiglas cages each with 3 banks of photoelectric emitters and detectors. Total locomotor activity i.e. quantification of the total number of activity counts (photocell beam breaks) was recorded for 24 h. A locomotor activity test for 24 h was performed before ischemia surgery and three days after MCA occlusion. Total activity corresponded to different movements of animals: coming-and-going between the back and the front of the cage, climbing, and other movements in the back or the front of the cage (n = 10 per group).

2.7. Immunohistochemistry on cortical neurons in culture or brain sections
Cortical cells on coverslips or brain sections were fixed with 4% paraformaldehyde/PBS, permeabilized in 0.3% polyoxyethyleneboronic monolaurate (Tween 20, Sigma) for 10 min and blocked with 2.5% donkey serum/PBS for 2 h at room temperature. Cells or sections were incubated with anti-doublecortin (DCX) antibody (1/200, Santa Cruz SC-8066), a mouse anti-synaptogamin 1 (1/100, Stressgen, Euromedex), a rabbit anti-AP43 (1:100, Abcam Limited) or a rabbit anti-mature-BDNF (1/200, Chemicon International, Hampshire, UK) in 2% donkey serum/ phosphate buffer saline overnight (Heurtjeaux et al., 2006b). After 3 washes in phosphate buffer saline/PBS, cells or sections were incubated in anti-gold conjugated goat-antibody (FluoProbes) in 2% donkey serum for 2 h, washed three times in PBS for 5 min each. Then, neurons were incubated in Hoechst solution (3 μl in 10 ml, Sigma–Aldrich, Saint Quentin Fallavier, France) for 10 min to label cell nuclei. After 2 washes in PBS and 1 wash in water, coverslips or sections were dried and mounted on glass slides with Fluoroprep (Biomeda). Cells or sections were observed using confocal epifluorescence microscopy. Confocal microscopy observations were performed using a Laser Scanning Confocal Microscope (TCS SP, Leica) equipped with a DMRBE 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 four scans per field. Epifluorescence microscopy images of protein labelling 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 (ij4/1.48d, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA), which allowed for the fluorescence quantification of the levels of cells of each fluorescent image saved as a 16-bit TIFF file (n = 3 cultures, 12 wells per experimental group, total 15 fields per condition). Results are given as ratio of mean fluorescence intensity in AU (arbitrary unit)/number of labelled cells ± SEM of three experiments. The differentiated neurites of cortical neurons in culture were observed by DCX immunostaining at Day 14 of treatment. Neurite outgrowth was determined on epifluorescence microscopy by measuring total length of neurites in culture dishes at different times of treatment using a cell photo image and Neurite Tracer Image J software (Pool et al., 2008).

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). Brains were removed at 24 h after the last injection. Serial sections of paraffin-embedded brains were cut (40 μm) throughout the entire hippocampus on a vibratome (Leica). The rostral and caudal part of the hippocampal was processed for immunohistochemistry (Heurtjeaux et al., 2006b) using a monoclonal mouse anti-BrdU (1/200; BD Biosciences, Le Pont de Claux), For BrdU chromogenic immunodetection, sections were then incubated for 1 h in biotin-conjugated species–specific secondary antibodies (diluted1/100, Vector Laboratories), followed by a peroxidase–avidin complex solution according to the manufacturer’s protocol. The peroxidase activity of immune complexes was visualized with DAB staining using the Vectastain ABC kit (Vector Laboratories). BrdU-labeled cells of granular and subgranular layers were counted in each section (n = 8 mice per group, 8 sections per mouse, 3 independent experiments) at 400× under a light microscope by a blind experimenter. The percentage of BrdU-positive cells was determined using fluorescent double-labeling with the following antibodies and dilutions: anti-sheep BrdU (1/200, Interchim, Montlucon, 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 cytometry, 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). Counting of BrdU/DCX and BrdU/NeuN-positive cells were performed in five sections (n = 8 per group and 8 sections per mouse, 3 independent experiments).

2.9. Human embryonic stem cells (hESC) culture
To assess the effects of MLC901 on human cells, neural progenitors were derived from the SA601 (Cellartis AB, Sweden) embryonic stem cells (hESC) line. Neural rosettes were derived in DMEM–12 enriched with N2/B27, FGF2 (10 ng/ml) and B2GF (10 ng/ml). After 10 days of induction, rosettes were harvested, dissociated and cultured in non-adherent conditions to form large floating rosette clusters. Rosette clusters were gently dissociated and plated on polyornithin (15 μg/ml, Sigma)– laminin (15 μg/ml, Sigma)-coated dishes for enrichment of an adherently growing monolayer of neural precursors (NSC). These culture conditions generated a synchronized and homogenously nestin-positive NSC population that can be frozen and thawed for further differentiation. Cell density was adjusted as required before seeding on 96-well plates. MLC901 was added 6 h after seeding at concentra-

3. Results

3.1. MLC601 and MLC901 protect cortical neurons against death associated with aging in culture
Cortical cells were first exposed to three concentrations of MLC601: 0.1, 0.5 and 1.0 μg/ml from Day 1 until Day 14 of treatment. The doses used were first selected based on a previous study on the anti-inflammatory effects of Radix astragali which constitutes the major component of both MLC601 and MLC901 (Ryu et al., 2008). From these results we then conducted pilot studies using a wide range of MLC601/MLC901 concentrations to search the best protection (data not shown). Cell survival was studied at Day 8, 10 and 14. Fig. 2A shows the dose-response effect of MLC601 treatment. Until Day 8 there was no significant differences in neuronal protection on cells treated with MLC601 concentrations of 0.1–0.5–1.0 μg/ml as compared to control (P > 0.05) (n = 36 wells per group). The protection induced by 1 μg/ml MLC601 appeared at Day 10 of treatment (P < 0.05 versus control group). At Day 14 the concentration of 1 μg/ml MLC601 induced a significant increase (50%) in neuronal survival as compared to control (P < 0.01 versus control group). We then compared the protective effects of MLC601 and MLC901 treatments against neurodegeneration of cortical cells over time in culture by using cell viability and LDH measurements. At the concentration of 1 μg/ml, which corresponds to the best results obtained on cell viability with MLC601, both treatments induced, as soon as Day 10 of treatment, a significant increase in neuronal viability as compared to respective controls (P < 0.05, **P < 0.01) (Fig. 2B). The highest efficacy of both treatments was observed at Day 14 with ~48% increase of cell survival (**P < 0.01). There was no significant difference of efficacy between MLC601 and MLC901 at the different stages of culture (n = 36 wells per group) (Fig. 2B). It is well known that increased cell survival leads to cell death being associated with increased LDH release. Compared to respective controls both treatments significantly reduced the ratio LDH release/cell viability after 12 and 14 days of treatment (P < 0.05).
and **P < 0.01) (n = 36 wells per group). There was no significant difference of efficacy on LDH release between MLC601 and MLC901 treatments (Fig. 2C).

3.2. MLC601 and MLC901 protect against glutamate-induced cell death in cortical cultures

Cell injury was estimated by measuring the ratio LDH release/cell viability at various time points (5, 8 and 24 h) after glutamate treatment applied for 10 min in the medium of the primary cortical culture at the concentration of 10 μM. When cortical cells were exposed to 10 μM glutamate, no signs of cell death were observed in the first hours after the exposure. Then, cells died during the next several hours. Application of glutamate on cortical cells during 10 min induced a time-dependent increase in LDH release. Addition of either 1 μg/ml MLC601 or MLC901, 4 days before and after glutamate treatment significantly reduced the ratio LDH release/cell survival (Fig. 2D). For both TCM preparations, the protection was obtained as soon as 5 h and was maintained at 24 h after glutamate. MLC901-induced the best protective effect against excitotoxicity. At 24 h after glutamate treatment for 10 min, the ratio LDH/cell survival decreased from 2.98 to 1.65 with MLC601. It was further decreased to 0.85 with MLC901 (Fig. 2D). Compared to control, half of cortical neurons survived after MLC601/MLC901 treatments. However, MLC901 showed a significant efficacy against glutamate-induced cell death as compared to MLC601 (**P < 0.01).

3.3. Pre- and post-treatments with MLC901/MLC601 protect against ischemic brain injury in vivo

To assay the potential neuroprotective effects of MLC901/MLC601 in vivo, we tested the preparation in a mouse model of focal ischemia. Ischemia was induced by transient middle cerebral artery occlusion (MCAO) for 60 min (Huang et al., 1994). We first analyzed whether a pre-treatment of MLC901 could increase the rate of survival of mice subjected to ischemia and reduce the infarct volume. Animals were first treated with MLC901 administered in the drinking water (6 mg/ml) for 6 weeks before the induction of ischemia. This paradigm corresponds to Fig. 1A. There was no significant difference in the consumption of food and drinking solution between vehicle- and MLC901-treated groups (data not shown). MCAO (60 min) resulted in an infarct in the right MCA perfused region. Fig. 3A shows that a 6 week-pre-treatment of MLC901-induced a marked reduction of the mortality of treated animals, compared to control ischemic mice. MLC901 pre-treatment induced a survival rate of 82% as compared to 65.5% in the control group (Fig. 3A). Representative photographs of stained brain slices at 30 h following ischemia are shown in Fig. 3E. As indicated by the white area on TTC-stained brain slices, cerebral infarcts in MLC901-treated mice were reduced. The infarct spread into the dorsomedial cortex in the MCA region and the caudate-putamen was particularly inhibited by MLC901 pre-treatment (Fig. 3E). The beneficial MLC901 effect was confirmed by the quantitative assessment of total infarct volume on cresyl violet-stained brain sections 30 h following MCAO, which revealed...
a significant decrease as compared to sham and ischemic control (Fig. 3B, **P < 0.01 versus vehicle group). We then analyzed the potential protection against ischemic stroke induced by an acute MLC901 post-treatment. Mice were subjected to focal ischemia and intraperitoneally injected with a single dose of 1 μg of MLC901 solution at the onset of ischemia and again 6 h after reperfusion (Fig. 3C–E: Post-treatment Onset). This paradigm corresponds to Fig. 1B. Acute administration of MLC901-induced a survival rate of 90% compared to 69.5% in ischemic vehicle-treated mice (Fig. 3C). This treatment also drastically decreased cerebral infarction (Fig. 3D and E). MLC901 reduced the stroke volume by 47.2% (**P < 0.01) as compared to control ischemic mice at 30 h post-ischemia (Fig. 3D). To analyze whether the time window of protection would allow delayed administration of TCM after stroke, MLC901 was intraperitoneally administered at 3 h and then again at 24 h after MCAO (Post-treatment 3H). This treatment also provided an important protection (Fig. 3C–E). When given as late as 3 and 24 h after the end of ischemia (see paradigm Fig. 1C), the level of survival of MLC901-treated mice remained high (Fig. 3C) and their infarct size was significantly smaller than for vehicle-treated animals after 30 h of reperfusion (Fig. 3D and E. ***P < 0.001). MLC601 (1 μg, i.p.) injected at 3 and 24 h after MCAO (Post-treatment 3 H, see paradigm Fig. 1C) produced beneficial effects on mortality rate (Fig. 3C) and infarct volume (Fig. 3D and E. ***P < 0.01 versus vehicle group) comparable to those of MLC901 at the same dose and in the same time window (P > 0.05). Physiological parameters were carefully measured after 1 h in selected mice (n = 5) subjected to MCAO and treated with vehicle versus MLC901 (pre- or post-treatment). Overall, there was no difference in mean arterial blood pressure, P<sub>CO2</sub>, P<sub>O2</sub>, pH or rectal temperature after MLC901 administration compared with vehicle-treated animals (Table 1).

3.4. MLC901 pre-treatment protects against functional deficits induced by focal ischemia in vivo

To determine whether a MLC901 pre-treatment before ischemia could have a positive effect on functional recovery after stroke, mice were preventively treated with MLC901 administered in the drinking water (5 mg/ml) during 6 weeks before MCAO occlusion. Then, a first type of functional assessment was carried out 3 and 7 days after stroke using the accelerated rotarod test. There was no significant difference in performance between pre-ischemia groups with or without MLC901 (P > 0.5). Three days after MCAO, vehicle-treated mice showed a decrease in their performances as compared to the corresponding pre-ischemia and sham groups (***P < 0.001). MLC901-treated mice showed a very significant improvement of their performances on the rotarod compared with the vehicle-treated ischemic group (Fig. 4A, ***P < 0.001). At Day 7 post-ischemia, vehicle-treated mice still displayed a very significant negative difference in the time they could spend on the rod compared to sham-operated and pre-ischemia groups (***P < 0.001). Interestingly after 7 days, MLC901-treated mice tended to behave in the rotarod assay as well as mice in the pre-ischemia group and as well as, or nearly as well as, the sham group (±P < 0.01), again indicating the beneficial effect of MLC901 treatment. To determine whether the functional outcome assessed by measurement of the motor impairment was correlated to the volume of infarction, we quantified at Day 7 post-ischemia the brain damage of mice that underwent the behavioral tests. Fig. 4B shows that there is a significant correlation.

![Fig. 3. Pre- and post-treatments with MLC901/MLC601 significantly increased the cerebral protection in a model of focal ischemia in vivo. (A–B) Effect of MLC901 pre-treatment on survival rate (A) and infarct volume (B) in mice subjected to 1-h reversible MCAO and killed after 30 h of reperfusion (n = 23 per experimental group, ***P < 0.01 versus water ischemic group). MLC901 pre-treatment was given in drinking water (6 mg/ml) for 6 weeks before the induction of ischemia. (C) Survival rate of mice post-treated with MLC901 or MLC601 and killed 30 h post-MCAO. (D) Infarct volume after MLC901-post-treatment measured at 30 h post-ischemia (n = 15 per group, ***P < 0.01 or ***P < 0.001 versus respective vehicle-treated ischemic mice). Mice were subjected to focal ischemia and intraperitoneally injected with a single dose of 1 μg of MLC901 solution at the onset of ischemia and 6 h after reperfusion (Post-treatment called ONSET) or injected with MLC901 or MLC601 (1 μg) 3 h after the end of ischemia and 24 h after reperfusion (Post-treatment called 3H). (E) Representative photographs of brain infarction at cortical, hippocampal and striatal levels assessed in each experimental group on seried TTC-stained slices from mice killed 30 h after MCAO. For the survival rate, results are given in percentage of control, corresponding to sham group. For the infarct volume, data are expressed as means ± SEM.](image-url)
Mice were subjected to 60 min MCAO followed by reperfusion. With MLC901 pre-treatment animals were treated with MLC901 administered in the drinking water (6 mg/ml) for 6 weeks before the induction of ischemia. With MLC901 post-treatment (1H), mice were subjected to focal ischemia and intraperitoneally injected with a single dose of 1 μg/ml of MLC901 solution at the onset of ischemia and 6 h after reperfusion. MABP (mean arterial blood pressure in mm Hg) was measured before, during and 3 h after ischemia. 50 μl of blood were withdrawn during focal ischemia for blood gas determination (pH, P O2, P CO2). Recta core temperature (in °C) was controlled by using a homeothermic blanket control before, during and 3 h after ischemia.

\( r = 0.868 \) between performances on the accelerated rotarod and infarct volumes one week after stroke \( F(1,34) = 0.418, P = 0.0002 \). The spontaneous locomotor activity test confirmed the rotarod results. Again, when tested before MCAO, there was no difference between groups. However, a large difference in behavioral impairment appeared at three days after ischemia. The locomotor activity, including climbing was much higher in MLC901-treated mice than in corresponding vehicle-treated animals (Fig. 4C, \( P < 0.05, **P < 0.01 \)).

<table>
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<th>Parameters</th>
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<th>MLC901 post-treatment (1H)</th>
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<tr>
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<td>7.29 ± 0.01</td>
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<td>PaO2, mm Hg</td>
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<tr>
<td>MABP, mm Hg</td>
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<td>83 ± 6</td>
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<td></td>
<td>1 h MCAO</td>
<td>62 ± 5</td>
<td>59 ± 7</td>
</tr>
<tr>
<td></td>
<td>3 h reperfusion</td>
<td>75 ± 4</td>
<td>79 ± 6</td>
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<tr>
<td>Core temperature (°C)</td>
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3.5. MLC601/MLC901 treatment induces neurogenesis, neuroproliferation, and neurite outgrowth

A focal cerebral ischemia, induced by insertion of a filament in the MCA leads to damage of cortical and striatal brain areas. A “repair” of these damaged areas might be possible by activating endogenous stem cells. It is known that an increase of endogenous cell proliferation occurs in the subgranular zone (SGZ) of dentate gyrus after ischemia (Zhang et al., 2008) as it does after application of different factors such as growth factors (Chen et al., 2003; Sharp et al., 2002).

To determine whether MLC901 is able to promote basal neurogenesis, we analyzed incorporation of BrdU (5-bromo-2’-deoxyuridine, a DNA synthesis marker) in dividing progenitor cells, corresponding to the production of newborn neurons, in mice with a 6-week-treatment with MLC901 alone, not followed by an ischemic insult (see paradigm in Fig. 1D) and compared the results with untreated mice. Fig. 5A shows representative photographs of MLC01 effect on neurogenesis. There was a clear increase of the number of BrdU-labeled cells in the SGZ. MLC901 treatment in the drinking water for 6 weeks resulted in a 1.4-fold increase in the number of BrdU-labeled cells as compared to vehicle-treated animals (**P < 0.001). We then provided evidence that proliferating cells were immature neurons. The phenotype of BrdU-positive cells in the SGZ was analyzed by double-labelling with doublecortin (DCX) for neurons and Glial Fibrillary Acidic Protein (GFAP) for astroglia. DCX is a highly hydrophilic microtubule-associated protein that is specifically expressed in migrating neuronal precursors and in areas of continuous neurogenesis in adult brain (Couillard-Despres et al., 2005). Fig. 5B shows representative confocal microscopy images of dual labelling of BrdU and DCX at 24 h following the last injection of BrdU. In contrast, no co-localization of BrdU-positive cells with the astroglial marker GFAP was observed. Counting of BrdU-positive cells (i.e. number of new dividing cells) showed that at 24 h following the last injection of BrdU, 72 ± 9% expressed DCX, which identifies immature neurons, in MLC901-treated mice and only 45 ± 5% in the vehicle group (Fig. 5C, ***P < 0.001) Since newborn cells need about three weeks to differentiate into mature neurons, we then decided to investigate whether the large MLC901-induced increase in neuronal precursors observed at 24 h following the last BrdU injection would correlate with an increase in neuronal maturation as determined by the mature neuron marker NeuN three weeks after the last BrdU injection (Fig. 5B). At this time, counting of BrdU/NeuN+ cells showed that MLC901 pre-treatment induced a 2.1-fold increase in the number of mature neurons as compared to vehicle-treated mice (Fig. 5C, **P < 0.01).

Neurotrophic factors, and particularly BDNF influence neurogenesis. It is well known that BDNF-mediated pathways are involved in cell survival and plasticity (Aguado et al., 2003; Gorski et al., 2003; Lipsky and Marinii, 2007; Mattson, 2008). For this reason, we were curious to see whether MLC901 pre-treatment administered in drinking water for 6 weeks could trigger BDNF expression. Fig. 6A shows in vivo effects of MLC901 on BDNF protein levels in cortex sections. A quantitative analysis showed BDNF expression, that was increased 2.46 fold in the cortex of MLC901-treated mice as compared to vehicle group (Fig. 6B, **P < 0.001).

At this stage, because the TCM MLC601 has been administered to humans for a long time, it was important to see whether the neurogenic effects of MLC901 could be observed on human ESC-derived progenitors. After 2 days of culture, neural progenitors had displayed a 3-fold increase in number, with a plateau at 250 000 cells for the highest seeding densities. An increase in cell number induced by MLC901 was observed in low-density cultures, and was not observed at higher cell densities (Fig. 7A). Low-density cultures were characterized by spontaneous formation of radiating clusters of nestin-positive progenitors that evoked rosettes (Fig. 7B–D). The number of rosettes was significantly (**P < 0.05, **P < 0.01) increased by around 3-fold with all indicated concentrations of MLC901 (Fig. 7E).

A more systematic analysis of MLC601/MLC901 effects on neuronal proliferation and neurite outgrowth was then carried out following expression of DCX in the course of time in cultured cortical cells from embryonic mice. Cortical cultures were treated during 14 days and observed at Day 7 and 14 of treatment. In Fig. 8A, representative confocal images of DCX staining show that until Day 7, there is no difference in DCX expression between Vehicle group and cortical cells treated with 1 μg/ml MLC601 or MLC901. However, at Day 14, while DCX immunoreactivity stagnated in Vehicle group, there was a spectacular increase of DCX expression induced by MLC601/MLC901 treatment, highlighting the development of an important axonal and dendritic network. Quantification of the fluorescence intensity in each epifluorescence microscopy image confirmed the neuroproliferative effect of MLC901/601 (Fig. 8B).

To investigate whether MLC601/901 treatment could promote neurite outgrowth, we measured the total length of neurites in cultured cortical neurons at Day 14 of treatment. On culture day 1–3 neuronal cells started to aggregate into small clumps. From Day 4, neurons showed developing neurites with increased neurite numbers and size. An analysis of the length of neurites at different times of treatment indicated that neurite outgrowth of cortical cells treated with MLC901 or MLC601 is very significantly increased compared with that of vehicle-treated cells (Fig. 8C, **P < 0.01) with a maximum at 8 days of treatment. A similar neurite outgrowth promoting activity was observed for MLC901 and MLC601 (P > 0.05).

The effects of MLC601 and MLC901 on expression of the 43 kDa growth-associated protein GAP43 and synaptotagmin 1 at various time points of cortical cultures were also analyzed. GAP43 has an
important role in the regulation of neurite outgrowth, growth cone guidance and synaptic plasticity (Van Hooff et al., 1989; Aigner et al., 1995). Immunofluorescent staining of primary cortical neurons with an antibody against GAP43 revealed that this protein was distributed in cytoplasm, membrane and neurite extensions (Fig. 9A). GAP43 expression increased over time in culture both in control and TCM-treated neurons (Fig. 9A). MLC601 and MLC901-treated cortical neurons developed a denser neuritic network, with more frequent elongating neurites and branching, resulting in a relative overgrowth of GAP43 in neurite arborizations compared to vehicle-treated neurons at the same stage. The increase of neurite outgrowth already observed at Day 7 in living neurons was

**Fig. 4.** MLC901 pre-treatment improved functional deficits induced by focal ischemia in vivo. (A) MLC901 effect on accelerated rotarod performance (duration spent on rod in seconds) (n = 12 per experimental group, ***P < 0.001 versus vehicle-treated mice, ****P < 0.001 versus pre-ischemia and sham groups, $^5$P < 0.01 versus pre-ischemia and sham groups). (B) Correlation between histopathological outcome and motor function 7 days after MCAO. The correlation was obtained from the measure of infarct volumes in the 3 experimental groups (sham, vehicle and MLC901, n = 12 per group) and their respective performances on the accelerated rotarod test carried out at day 3 after ischemia. (C) MLC901 effect on spontaneous climbing activity during 24 h, performed 3 days after ischemia. Inset shows the total locomotor activity, including coming-and-going between the back and the front of the cage, climbing, and other movements in the back or the front of the cage (n = 12 per group, *P < 0.05, **P < 0.01, $$$P < 0.001 versus vehicle-treated mice). MLC901 was given in drinking water (6 mg/ml) for 6 weeks before the induction of ischemia.

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confirmed and amplified from Day 7 to Day 14. At Day 14, GAP43 was increased 2.2-fold in MLC601- and 2.5-fold MLC901-treated neurons as compared to control neurons (**P < 0.01). There was no significant difference between MLC601 and MLC901 (Fig. 9B).

Developing neurons are engaged in neurite outgrowth as well as the synthesis and transport of proteins involved in synaptic transmission. Synaptotagmin 1 is one of synaptic vesicle proteins having a critical role in synaptogenesis and synapse function (Jessell and Kandel, 1993; Sudhof, 1995). It therefore appeared of interest to study the effects of MLC601 and MLC901 treatments at Day 7 and 14 on synaptotagmin 1 expression in cortical neurons in culture. Fig. 10 shows again that cortical neurons underwent a well-defined program of differentiation, including expression of neurite extension and also synapse formation visualized by the expression of

![Image](image_url)

**Fig. 5.** MLC901 pre-treatment induced neurogenesis and cell proliferation. (A) Representative photomicrographs of BrdU peroxidase-staining (arrows) in dentate gyrus of mouse hippocampus treated for 6 weeks either with vehicle or MLC901. MLC901 treatment was given in drinking water (6 mg/ml). (B) Double-labelling of BrdU-labeled neurons either with DCX, or GFAP 24 h following the last BrdU injection and with NeuN (a neuronal marker) 3 weeks after BrdU. We showed a co-localization only with DCX (neuronal precursor marker in green and BrdU in red labelling), and not with GFAP (glial marker in red and BrdU in green labelling) at 24 h and a BrdU/NeuN co-localization at 3 weeks following BrdU injections (Scale bar, 25 μm). (C) Quantification of BrdU, BrdU/DCX and BrdU/NeuN-positive cells in dentate gyrus treated with vehicle or MLC901 at 24 h and 3 weeks following the last BrdU injection. Data are number of BrdU-positive cells in mouse hippocampus, expressed as mean ± SEM versus vehicle-injected mice. Data were collected in three independent experiments from n = 8 per group, 8 sections per group, 10 fields per section, chosen randomly (**P < 0.01, ***P < 0.001 versus vehicle-treated mice).

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**Fig. 6.** In vivo effect of MLC901 pre-treatment on BDNF protein levels in cortex sections. (A) Representative epifluorescence microscopy photographs of BDNF immunoexpression in cortical neurons in brain sections. (B) Quantification of BDNF signal intensity in immunostained neurons. Data are expressed as ratio of mean fluorescence intensity in AU (arbitrary unit × 1000) to number of labeled cells ± SEM of three experiments. Average fluorescence intensity was expressed from two independent experiments (n = 8 sections per experimental group, 15 fields per section and analyzed in triplicate) (**P < 0.01 versus vehicle group).
synaptotagmin 1, which increased with time in culture in the three experimental groups (Fig. 10A). While synaptotagmin 1 immunoreactivity was localized primarily to the soma with a diffuse staining throughout neuritic processes in 4 day-old cultures (data not shown), the staining profile became strikingly different at the 7 and 14 days of treatment with the appearance of intense punctuate staining along neuritic processes, which is characteristic of synaptic release sites in neurons (Fig. 10A). Quantitative analysis of fluorescence intensity in vehicle and MLC601/MLC901-treated cultures showed that both MLC601 and MLC901 treatments significantly increased the levels of synaptotagmin 1, by 1.9 and 2.2-fold respectively, as compared to control cultures (*P < 0.05, **P < 0.01). MLC901 appears to be slightly more potent than MLC601 (Fig. 10B, #P < 0.05).

4. Discussion

The development of neuroprotective and neurorestorative drugs is essential for the treatment or management of ischemic stroke.
Despite considerable recent progress defining cellular and molecular responses of brain to ischemia, there is no effective treatment for stroke patients besides fibrinolysis at hyper acute stage, and secondary prevention treatments to manage the well identified risk factors. Clinical use of potential neuroprotective treatments has been prevented owing to inefficiency or/and serious side effects caused by their interference with normal brain function (Ginsberg, 2008; Wahlgren and Ahmed, 2004). In an overall research of stroke therapies, whose goal is not only to salvage acutely threatened neuronal tissue but also to promote repair and restoration of function (Martinez-Vila and Irimia, 2005), we have focused our studies on MLC601/MLC901. MLC601 originates from traditional Chinese medicine and MLC901 is a simplified version of MLC601.

Traditional Chinese medicine is currently attracting a lot of interest (Wang et al., 2008), particularly in diseases that are not adequately treated with Western medicine. MLC601 is prescribed in several countries of Asia and Middle East. It can be used on top of usual medications, including anti-platelets or anticoagulants. It does not seem to have significant side effects (Gan et al., 2008). Recent trials of MLC601, analyzed in China and Singapore demonstrated beneficial effects on the recovery of independence and motor function after stroke (Chen et al., 2009; Siow, 2008). In regard to these encouraging results, a multicenter clinical trial, called CHIMES is ongoing in Asia (Venketasubramanian et al., 2009).

The purpose of this work was to analyze whether MLC601 and its simplified version MLC901 have any effects on neurogenesis, on the development of the axonal and dendritic network and in neuroprotection, with the idea that positive answers to these questions would also be a strong encouragement to pursue the development of clinical investigations. We demonstrate that MLC901 treatment, when administered in vivo in pre- or post-treatments improved animal survival as well as functional neurological recovery and decreases neurodegeneration without affecting physiological parameters. In this work, we used C57Bl/6J mice, a strain known to have an increased vulnerability to focal and global ischemia with a higher level of mortality in comparison to other strains such as DBA/2, MF1 and 129/Sv (Connolly et al., 1996; Fujii et al., 1997). The gain of an important cerebral protection with MLC901 in the suture model of focal ischemia is a strong argument in favor of MLC901 efficiency. Using cortical cells in 17 day-old culture (corresponding to 14 days of treatment), we observed that both MLC901 and MLC601 induced a strong protective effect against glutamate-induced cell death that...
was maintained 24 h after the excitotoxic injury. It is well known that excessive synaptic glutamate concentration produces excitotoxicity that leads to neuronal death in both global and focal ischemia (Choi, 1998; Obrenovitch et al., 2000). In our in vivo model of focal ischemia we clearly demonstrated that MLC601 and MLC901 significantly protected the brain against an ischemic insult. Both pre-treatments administered in drinking water and post-treatment administered intraperitoneally decreased the mortality rate as well as the infarct volume. Therefore, MLC601/MLC901 might be useful as a preventive therapy or as a postischemic treatment to reduce the damaging effects of stroke. MLC601/MLC901 has a time window of protection compatible with clinical trials, since MLC901 provided protection from focal ischemia in the mouse when given as late as 3 h after ischemia. It is interesting to note that MLC601 has the same kind of efficacy in vivo since MLC901 post-treatment up to 3 h after stroke also improved survival and protected the brain from ischemic injury. Until now, the majority of preclinical studies traditionally focused on the prevention of neuronal cell death and attempts to assess behavioral deficits arising from stroke were few, particularly in mice. The ability to demonstrate an improvement of function impaired by ischemia is as important as, and clinically more relevant, than a simple statement of lesion volume. At this stage, it was essential to show that MLC901 protection was accompanied in surviving animals by a decrease of behavioral deficits. In line with first clinical trials that have shown promising results of MLC601 efficiency on the functional recovery after stroke (Chen et al., 2009; Siow, 2008), this work shows that MLC901 improved motor performances measured in the accelerated rotarod and actimeter tests, considered as useful operant conditioning procedures to assess long-lasting deficits after stroke (Ferrara et al., 2009). We focused on the accelerated rotarod test, which provides quantitative, objective and reproducible measures of functional impairment of mice following an ischemic insult. We observed that ischemia-induced impairments with and without MLC901 are directly correlated with the infarct volume: The smaller the infarct volume after MLC901, the higher the level of performance in the rotarod test. The linear relationship between the histopathological outcome and the motor function provides convincing information concerning the use of MLC901 in stroke treatment. These results suggest that MLC901 both preserve damaged neurons and probably at least partially restores neuronal circuits with associated behavioral benefits.

Whereas recovery, in the first few days after stroke, results from edema resolution and/or from reperfusion of the ischemic penumbra, a large part of the recovery over periods of weeks or months is due mainly to brain plasticity, i.e. to reorganization of surviving central nervous system elements, probably including stem cells, in the damaged areas (Chopp et al., 2009). Neural plasticity probably involves modulation of signal transduction pathways and regulation...
of gene expression as well as neurogenesis, and synaptogenesis. After stroke, the brain uses its complement of neural plastic responses to reorganize, at least partially the cortical maps (Chopp et al., 2009; Di Filippo et al., 2008). Changes in cortical organization include an increase in the number and density of dendrites and synapses. Robust experimental evidence supports the hypothesis that neuronal aggregates adjacent to a lesion in the sensorimotor brain areas can take over progressively the function previously played by the damaged neurons (Zhang et al., 2004). This reorganization subtends clinical recovery of motor performance and sensorimotor integration after stroke. Brain functional imaging studies have shown that recovery from hemiplegic strokes is associated with a marked reorganization of the activation patterns of specific brain structures (Nelles et al., 1999). On the other hand, stroke is known to induce neuronal proliferation associated with directed migration of nascent neurons towards ischemic lesions (Jin et al., 2003). Experimental stroke in adult rodents has been shown to trigger neurogenesis in neuroproliferative zones such as the subgranular zone of dentate gyrus (Sharp et al., 2002). These stroke-activated endogenous neuronal progenitors can migrate into regions that do not normally exhibit neurogenesis in the adult. This raises the possibility that these cells may constitute a pool for the replacement of dead or dysfunctional cells after an ischemic episode. The newly born cells generated from the dentate gyrus develop into granule neurons and are capable of extending axonal projections to the CA3 area and integrating into functional circuits (Hastings and Gould, 1999; Markakis and Gage, 1999). Pharmacological agents able alone to promote basal neurogenesis and synaptogenesis are needed to amplify the intrinsic brain properties for neuroplasticity and subsequent neurological recovery after stroke. In preclinical studies, several potential therapeutic agents have been shown to promote functional outcome after stroke. Most of them are growth factors such as the vascular endothelial growth factor (VEGF), the basic fibroblast growth factor (bFGF), and the brain-derived neurotrophic factor (BDNF) (Chen and Chopp, 2006). Our data show that MLC901 treatment administered for 6 weeks in the drinking water (6 mg/ml) significantly increased the number of BrdU-positive cells in the SGZ, suggesting that this type of medicine could promote basal neurogenesis in the adult brain and play a role in neurologic function recovery of both motor and cognitive functions after stroke. In addition, we report in our in vitro experiments with cultured cortical cells that both MLC901 and MLC601 helped to develop a dense axonal and dendritic arborization illustrated by a large increase of DCX fluorescent labelling intensity as well as an enhanced neurite outgrowth. DCX protein is currently used as a classical marker for neurogenesis (Couillard-Despres et al., 2005). DCX appears to be important for the normal developmental...

![Fig. 10. Effects of MLC601/MLC901 treatment on in vitro Synaptotagmin 1 expression in cultured cortical neurons. Synaptotagmin 1 expression was analyzed after MLC601/MLC901 treatment (1 μg/ml) at Day 7 and 14 of treatment. (A) Representative photographs of Synaptotagmin 1 expression in epifluorescence microscopy on cortical neurons stained with anti-Synaptotagmin 1 antibody. Nuclei were stained with Hoecht 33342 (in blue). (C) Quantitation of Synaptotagmin 1 signal intensity in immunostained neurons. Data are expressed as ratio of mean fluorescence intensity in AU (arbitrary unit × 1000) to number of labeled cells ± SEM of three experiments. Average fluorescence intensity was expressed from three independent experiments (n = 12 wells, 15 fields per well for each experimental group and analyzed in triplicate). *P < 0.05, **P < 0.01 versus vehicle group, P < 0.05 versus MLC601 group, Mann–Whitney test.)

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migration of cortical neurons, because mutations in DCX in humans lead to syndromes characterized by migrationar arrest of these neurons and manifested clinically by subcortical lamarin heterotopias, mental retardation and seizures (des Portes et al., 1998). Increased DCX expression is then closely associated with neurogenic processes.

MLC901-induced neurogenic processes in cortical neurons have also been observed in hESC. MLC901 had a positive effect on the number of neural progenitors derived from hESC, indicating either a neuroprotective effect or an accentuation of proliferation. This effect was observed in low-density cultures, suggesting that cell contact may decrease the action of MLC901. In low-density cultures, hESC spontaneously reconstituted radiating clusters of cells similar to rosettes. Rosettes are radially organized columnar epithelial cells that typically form during differentiation of hESC towards the neural lineage (Elkabetz et al., 2008; Zhang et al., 2001). Progenitors within rosettes differentiate into neurons, astrocytes and oligodendrocytes through a sequence similar to the one observed during neurogenesis. Cell clustering within rosettes reproduces a developmental niche that influences proliferation and differentiation (Illes et al., 2009).

Our observations suggest that MLC901 contains key molecules able to create a neurogenic niche and enriched microenvironment to promote and amplify differentiation of neural.progenitors. In order to better decipher the effects of MLC901 and MLC601 on cortical plasticity, we also studied in vitro how they modulate the expression of GAP43, associated with neuronal neurite growth and synaptotagmin 1, associated with synaptic formation. GAP43 is a membrane-bound protein found in the growth cones of sprouting CNS axons (Aigner et al., 1995; Meiri et al., 1986; Oestreicher et al., 1997; Van Hooft et al., 1989). GAP43 regulates $\gamma_{o}$, a GTP-binding protein that transduces information from transmembrane receptors and that is a major component of the neuronal growth cone membrane (Strittmatter et al., 1990). By blocking GAP43 expression with antisense oligonucleotide probes, neurite outgrowth can be eliminated in cultured neurons (Zuber et al., 1989). Synaptotagmin 1 is one of presynaptic vesicle proteins having a critical role in synaptogenesis and synaptic function (Jessell and Kandel, 1993; Sudhof, 1995). Synaptotagmin 1 immunostaining is often used to estimate increases or decrees in synaptic numbers. This work reports a significant increase in density of both GAP43 and synaptotagmin 1 in cultured cortical neurons treated with MLC901 compared to vehicle-treated neurons. These data clearly indicate that neurite outgrowth followed by synaptogenesis in cortical neurons is increased by MLC901 treatment. While the specific mechanism responsible for MLC901-promoted expression of proteins involved in neurite growth and synaptogenesis is not yet elucidated, the up-regulation of such proteins classically associated with neuronal remodeling might well explain the enhanced behavioral recovery reported in stroke patients treated with this Chinese medicine (Chen et al., 2009; Siow, 2008) and also in our in vivo model of focal ischemia (this work).

One possible mechanism of MLC601/MLC901 effect includes its ability to stimulate BDNF secretion. BDNF is a growth factor which regulates neuronal survival and protect neurons from glutamate-induced damages (Mattson, 2008). BDNF has multiple effects on sustaining and evolving elements of brain plasticity including neurite outgrowth and differentiation (Aguado et al., 2003; Gorski et al., 2003; Volosin et al., 2006). BDNF induction is both spatially and temporally associated with recruitment of new neurons (Mattson, 2008). Our in vitro data show that MLC901 indeed increased BDNF expression in cortical neurons. On the other hand GAP43, whose expression is itself increased by MLC901 and MLC601 is known to be essential for the neurotrophic effects of BDNF (Gupta et al., 2009). BDNF is known to also induce anti-apoptotic mechanisms after stroke and reduce infarct size and secondary neuronal cell death (Schabitz et al., 2000; Zhang and Partridge, 2001). All these data suggest that BDNF may play a significant role in the many beneficial effects displayed by MLC901. In addition, Radix astragali, which is the major component of MLC901/MLC601 herb mixture, has been reported to scavenge active oxidants, and regulate the expression of cytokines such as TNF$\alpha$, IL-1a, IL-1b, IL-6 as well as the production of nitric oxide (NO), which are all involved in the pathophysiology of stroke (Lee et al., 2005). All these interesting effects induced by Radix astragali alone could of course contribute to the beneficial effects of MLC901/601 against stroke.

Brain injury following stroke results from the complex interplay of multiple pathways including excitotoxicity, acidosis, ionic balance, peri-infarct depolarization, oxidative stress, inflammation and apoptosis (Doyle et al., 2008). It is highly probable that MLC901/MLC601 have “multi-target” effects, which will have to be investigated in details in the near future. MLC901/MLC601 contains a complex mixture of natural molecules that are probably acting in an additive way or in synergy, as recently reported for another TCM treatment against promyelocytic leukemia (Wang et al., 2008).

This work represents the first preclinical study demonstrating neuroprotective and neuro proliferative effects of two natural preparations MLC601 and MLC901, which are already used for the treatment of patients against the deleterious effects of stroke, particularly in Asia. It provides scientific support for their clinical use (i) preventively in patients at high risks to have stroke, (ii) curatively for stroke patients, immediately after stroke, (iii) as it is the case at the present time (Chen et al., 2009) after some weeks or some months after stroke to increase chances to recover better neuro logical functions. Given the near absence of effective treatments today, the results of the multicenter CHIMES study, which is ongoing in Asia (Venketasubramanian et al., 2009) to more systematically test MLC601 efficacy in humans in a 72 h time window post-stroke onset will be awaited.

Acknowledgments

The authors are very grateful to Dr M. Vasseur for suggesting the project and to D. Picard (Moleac Singapore) for helpful and inspiring discussions concerning the present evaluation of the clinical effects of MLC601 and for both providing MLC601 and MLC901 capsules. This work is supported by the Centre National de la Recherche Scientifique (CNRS), the Fondation de la Recherche Médicale (FRM) and the Association Française contre les Myopathies (AFM).

References


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Danqi Piantang Jiaonang (DJ), a Traditional Chinese Medicine, in Poststroke Recovery

Christopher Chen, MD; N. Venketasubramanian, MD; Robert N. Gan, MD; Caroline Lambert, MD; David Picard, MSc; Bernard P.L. Chan, MD; Edwin Chan, PhD; Marie G. Bousser, MD; Shi Xuemin, MD

Background and Purpose—Stroke is a leading cause of death and disability worldwide. Despite improvements in acute stroke treatment, many patients only make a partial or poor recovery. Therefore, there is a need for treatments that would further improve outcome. Danqi Piantang Jiaonang (DJ; NeuroAid), a traditional Chinese medicine widely used in China to improve recovery after stroke, has been compared with another traditional Chinese medicine in 2 unpublished randomized clinical trials. The results of these studies were pooled and reanalyzed to assess efficacy and safety.

Methods—Six hundred five subjects were randomized in 2 randomized double-blinded, controlled trials to receive either DJ or Buchang Naoxintong Jiaonang. Subjects were treated for 1 month. Inclusion criteria were: (1) patients with recent (from 10 days to 6 months) ischemic stroke; (2) patients satisfying Western diagnostic standards for stroke and traditional Chinese medicine standards for diagnosis of apoplexy; and (3) Diagnostic Therapeutic Effects of Apoplexy score ≥10.

Results—The functional outcome, measured by the Comprehensive Function Score component of the Diagnostic Therapeutic Effects of Apoplexy scale, showed a statistically significant superiority of DJ over the control treatment group (relative risk, 2.4; 95% CI, 1.28 to 4.51; P=0.007). Tolerance was excellent in both groups.

Conclusions—The pooled analysis of 2 unpublished trials of DJ, a traditional Chinese medicine currently approved in China to improve neurological recovery after stroke, shows good tolerability and superiority of DJ over another traditional Chinese medicine also approved for stroke. A large double-blind randomized clinical trial is required to further assess the safety and efficacy of DJ. (Stroke. 2009;40:00-00.)

Key Words: cerebral infarct ■ randomized controlled trials ■ stroke recovery ■ traditional Chinese medicine
Patients were eligible if they (1) were between 18 and 70 years old; (2) had a DTER score of 10 to 13 was classified as mild, 14 to 26 as moderate, and 27 to 34 as severe. Study centers were requested to target recruitment of approximately 20% mild, 60% moderate, and 20% severe cases.

Inclusion Criteria
Patients with transient ischemic attacks, lacunar infarcts, or infarction of the basilar artery system were excluded from the study. Patients were also excluded from the study if they had other intracranial pathologies such as intracranial tumors, atrial fibrillation, other clinically significant systemic diseases, or were pregnant and lactating women.

Stratification and Randomization
In the 2 randomized trials, eligible patients were randomized after stratification according to whether their condition was mild, moderate, or severe, using the patients’ score on the DTER (Table 1). A DTER score of 10 to 13 was classified as mild, 14 to 26 as moderate, and 27 to 34 as severe. Study centers were requested to target recruitment of approximately 20% mild, 60% moderate, and 20% severe cases.

Randomization numbers were generated by computer. Randomization numbers were pregenerated and placed in sealed envelopes. A serial number was given to each envelope according to the sequence of allocation of the randomized number. Each envelope was then opened in sequence according to the admission sequence of the subjects at the respective study center. Subjects were randomized into treatment or control groups according to the randomized number in the envelope.

Subjects as well as investigators and pharmacists were blinded to the allocation. The password for the randomization envelope for each subject was kept by the sponsor and a designated researcher.

For simplicity, the BNJ treatment group is later referred to in this article as the “control group.”

Interventions
Subjects were randomized to receive either DJ or BNJ, which served as a control, in a 1:1 ratio in the first study of 200 subjects and in a 3:1 ratio in the second study of 405 subjects. BNJ was used as no placebo was allowed in accordance with the Chinese guidelines governing TCM clinical research.

DJ was developed by the No. 1 Hospital attached to the Tianjin TCM Institute for treating apoplexy with qi deficiency and blood stasis during the recovery phase. It consists of a dry extract of 14 components, the 2 main ingredients being Radix Astragalus (Huangqi) and Radix Salviae miltiorrhizae (Danchen). The respective raw materials were processed into a dry extract, which was then used to fill a hard gelatin capsule. Dextrin, an inert pharmaceutical excipient, was added to the dry extract to make up the weight of each capsule to 0.4 g.

Table 1. Diagnostic Therapeutic Effects of Apoplexy Scoring System

<table>
<thead>
<tr>
<th>1-Visual fields/eye symptoms</th>
<th>2-Facial movement/facial paralysis</th>
<th>3-Upper limb paralysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0=No visual loss</td>
<td>0=Normal facial movement, no asymmetry</td>
<td>0=No drift</td>
</tr>
<tr>
<td>1=Mild aphasia with unclear pronunciation</td>
<td>1=Partial facial paresis</td>
<td>1=Weakness in raising arm</td>
</tr>
<tr>
<td>2=Moderate aphasia with incomplete sentence</td>
<td>2=Complete facial paralysis</td>
<td>2=Ability to hold/raise the arm over the shoulder</td>
</tr>
<tr>
<td>3=Severe aphasia</td>
<td>3-Lower limb paralysis</td>
<td>3=Slight movement of the arm</td>
</tr>
<tr>
<td>0=No paralysis</td>
<td>0=No drift</td>
<td>6=No movement of the arm</td>
</tr>
<tr>
<td>1=Mild paralysis</td>
<td>1=Ability to raise/hold the leg by more than 45°</td>
<td>1=Weakness in raising arm</td>
</tr>
<tr>
<td>2=Partial paralysis</td>
<td>2=Inability to raise/hold the leg by more than 45°</td>
<td>2=Ability to hold/raise the arm over the shoulder</td>
</tr>
<tr>
<td>3=Complete paralysis</td>
<td>3=Horizontal movement only</td>
<td>4=Inability to hold/raise the arm over the shoulder</td>
</tr>
<tr>
<td>1=No paralysis</td>
<td>4=Slight movement or no movement</td>
<td>5=Slight movement of the arm</td>
</tr>
<tr>
<td>2=Partial paralysis</td>
<td>5=Complete paralysis</td>
<td>6=No movement of the arm</td>
</tr>
<tr>
<td>3=Complete paralysis</td>
<td>6=Confined to bed</td>
<td></td>
</tr>
</tbody>
</table>

Methods
Two randomized clinical trials comparing the efficacy and safety of DJ and BNJ, a TCM approved by the Sino Food and Drug Administration, in subjects with recent ischemic stroke, are included in this pooled analysis. Two hundred subjects were randomized in the first study and 405 in the second. Both studies had similar designs that are described subsequently.

Patients
Stroke in- or outpatients were recruited from 6 participating institutions (Heilongjiang and Changchun TCM Universities, Shanxi, Anhui, Henan, and Liaoning Traditional Chinese Medicine Institutes). The protocol was approved by the New Pharmaceuticals Examination Centre of the State Food and Drug Administration and by each individual institution’s ethics committee.

Inclusion Criteria
Patients were eligible if they (1) were between 18 and 70 years old; (2) were diagnosed with ischemic stroke according to Western medicine diagnosis standards in China; (3) met the requirements of the TCM standards for diagnosis of apoplexy; (4) had a Diagnostic Therapeutic Effects of Apoplexy (DTER) score ≥10 (Table 1) (5) were at the restoration stage according to TCM criteria (ie, between 15 days and 6 months after the onset of symptoms); and (6) provided signed informed consent.

The Western medicine diagnosis standards followed the “Key Points for Diagnosing Cerebrovascular Diseases” modified in the 4th National Cerebrovascular Disease Seminar by the China Medical Society in 1995. Details of the DTER scoring system are provided in Table 1.

Exclusion Criteria
Patients with transient ischemic attacks, lacunar infarcts, or infarction of the basilar artery system were excluded from the study. Patients were also excluded from the study if they had other intracranial pathologies such as intracranial tumors, atrial fibrillation, other clinically significant systemic diseases, or were pregnant and lactating women.

Tienjin Chinese Medicine Institute for treating apoplexy with qi deficiency and blood stasis during the recovery phase. It consists of a dry extract of 14 components, the 2 main ingredients being Radix Astragalus (Huangqi) and Radix Salviae miltiorrhizae (Danchen). The respective raw materials were processed into a dry extract, which was then used to fill a hard gelatin capsule. Dextrin, an inert pharmaceutical excipient, was added to the dry extract to make up the weight of each capsule to 0.4 g.

Downloaded from stroke.ahajournals.org by guest on January 22, 2009
BNJ was produced by the Xianyang Bunchang Medicines Co., Ltd. Both the investigational drug and the control drug were provided by Tianjin Shitian Medicines Co., Ltd. Subjects took 4 capsules after each meal, 3 times per day for 4 weeks.

Data Management
We compiled an electronic database consisting of data from individual subjects in the 2 eligible trials. Data included baseline characteristics, the allocated treatment medication, scores as defined by the DTER scoring system (Table 2) as well as adverse events and laboratory evaluations. Data were checked for completeness and internal consistency with subjects’ records.

Objectives and Outcome Measures
The 2 Chinese studies compared the efficacy of DJ as measured by the DTER scoring system with that of BNJ and also compare their safety profiles.

Likewise, the primary outcome measure of this pooled analysis was the improvement at 1 month in the comprehensive functions score. The neurological deficit score (obtained by adding the first 7 subscores of the DTER scoring system) and each of its individual 7 components were also analyzed. Safety was evaluated by the pooled analysis of serious and nonserious adverse events and of laboratory evaluations collected in the 2 randomized trials.

Statistical Methods
Because statistical analyses in the Chinese studies were performed on nonstandard outcome measures likely to be unfamiliar to Western-trained physicians, we have extracted data from these 2 randomized studies, pooled the data together, and reanalyzed using the random effects model.\(^{10}\) The comprehensive functions score was obtained by adding the first 7 subscores of the DTER scoring system (Table 1) and each of its individual 7 components were also analyzed. The trend in the pooled analyses was in favor of DJ, but the result was not statistically significant (weighted mean difference, 0.22; 95% CI, 0.11 to 0.33; P = 0.007).

The neurological deficit score was obtained by adding the first 7 subscores of the DTER and individual subscores—evaluating language function, facial paralysis, visual symptoms, upper and lower limb paralysis, upper and lower distal limb paralysis—were treated as continuous variables. Improvement in the DJ compared with the control group was quantified by the difference in mean scores.

Results
Recruitment and Subjects’ Flow
In the first study, 201 subjects were enrolled initially. One subject was subsequently excluded and not randomized due to the administration of concomitant medication. One hundred subjects were randomized to the DJ treatment group and 100 to the control group. In the second study, 405 subjects were enrolled, 300 subjects allocated to the DJ treatment group and 105 subjects to the control group. Thus, in total, 605 subjects were enrolled, 300 subjects allocated to the DJ treatment group and 205 subjects to the control group. No subjects were withdrawn or lost to follow-up.

Characteristics of Subjects
Baseline characteristics are indicated in Table 2. There was no difference at baseline between the DJ and control group in gender, age, time from stroke onset, or stroke severity.

Efficacy Results
The results of the pooled analysis are summarized subsequently.

Effects on Functional Outcomes
Functional outcome was assessed using the Comprehensive Function Score component of the DTER scale (Table 1). The scores were dichotomized into 2 categories: 0 versus 2 to 8. Both studies showed an advantage to DJ and the pooled analysis suggested that subjects receiving DJ were more likely to achieve a good functional outcome at 1 month than those randomized to the control treatment group (relative risk, 2.4; 95% CI, 1.28 to 4.51; P = 0.007; Figure 1).

Effects on Recovery of Neurological Deficits
The neurological deficit score was obtained by adding the first 7 subscores of the DTER (Table 1). The trend in the pooled analyses was in favor of DJ, but the result was not statistically significant (weighted mean difference, 0.22; 95% CI, −0.11 to 0.56; P = 0.18; Figure 2).

Effect on Motor Scores
The first 7 subscores were analyzed individually. Most of these separate motor function pooled analyses showed an advantage in those subjects randomized to the DJ treatment group compared with the control treatment group. Specifically, DJ statistically significantly decreased the scores at 1 month for the 2 domains of upper limb (weighted mean difference, −0.43; 95% CI, −0.73 to −0.12; P = 0.006) and distal lower limbs (weighted mean difference, −0.32; 95% CI, −0.59 to −0.06; P = 0.02) as compared with the active control. A numeric decrease in the score for lower limb, facial and distal upper limb functions was observed but not statistically significant. No significant effect was observed on visual and language functions.

Safety Results
The clinical trials reported no severe adverse events and only 2 cases of nausea and vomiting in subjects receiving DJ.

Blood cell count, renal function (blood and urine testing), and
liver function were measured and no abnormal changes were observed.

**Discussion**

The pooled analysis of 2 unpublished trials of DJ, a TCM currently approved in China to improve neurological recovery after stroke, shows the superiority of DJ over another TCM also approved for stroke. Functional outcome as measured by the Comprehensive Function Score component of the DTER scale showed a statistically significant superiority of DJ over the BNJ treatment group. There was also a trend in the pooled analyses in favor of DJ with respect to the neurological deficits score. Tolerance was excellent in both groups.

Although the use of DJ in post-stroke recovery appears promising, the data from the Chinese studies are not sufficient for an evidence-based medicine recommendation to change current prescribing or treatment practice. This is due to methodological inadequacies in the studies such as the use of TCM diagnostic criteria for stroke, the lack of placebo control, the broad time interval after onset of stroke, the short treatment period, and the use of outcome measure scales, which are different from those currently widely used in international stroke trials.

The use of BNJ as a control instead of placebo may impact the interpretation of the results. However, BNJ is an approved TCM for stroke, is widely used, and is well tolerated. Hence, it seems less likely that the effect of BNJ on stroke recovery was negative rather than neutral or positive. Another possible confounder may be the wide variation in the time to randomization. This may had led to a bias due to patients being at different stages of the natural recovery process.

In the Chinese studies, DJ exhibited a favorable safety profile; there were no serious adverse events recorded and only 2 cases of mild nausea and vomiting. This low rate of adverse events may be due to a combination of the fact that the patients were recruited during their recovery phase when their clinical condition had stabilized and to the method of collection of adverse events in China. However, such a low rate of adverse events again leads clinicians to suspect that the patients selected differ significantly from those recruited in stroke trials in general.

Traditional medicine is widely used globally in both developing and developed countries and is a rapidly growing health system and economic importance. Although providers of traditional medicine seek increased recognition and support, many Western-trained professionals have strong reservations about the benefits of traditional medicine. This conflict between “uncritical enthusiasm versus uninformed skepticism” can only be resolved by improving the evidence base from which reliable conclusions can be drawn on the efficacy and safety of traditional medicine. It is vital more efforts are made to identify promising treatments from traditional medicine in a scientifically credible format. Performing well-controlled randomized clinical trials is the only means to ensure that potentially beneficial practices are not neglected nor inadequately evaluated practices promoted.

Establishing whether potential stroke treatment from TCM can be effective and safe through well-designed clinical trials may be considered a priority because it may then open up the

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**Figure 1. Effect on functional outcome at 1 month.** Favorable functional outcome is defined as a score of 0 (able to take care of oneself and speak freely) versus any higher score according to the Standards for Evaluating the Diagnosis Therapeutic Effects of Apoplexy scoring system comprehensive function subscore (as defined in Table 1).

**Figure 2. Effect on neurological deficits at 1 month.** The neurological deficit score was obtained by adding the first 7 subscores of the Scoring system comprehensive function subscore (as defined in Table 1).
potential to develop improved treatments based on investigating the active ingredients and mechanisms of actions.

Conclusions
DJ, a TCM drug currently approved in China to improve stroke recovery, has been shown to be well tolerated in this pooled analysis of 2 trials. However, due to various methodological inadequacies, there is a need for a large Phase III double-blind randomized, placebo-controlled trial of DJ and other TCM for stroke recovery before such treatments can be recommended for general clinical use.

Sources of Funding
The clinical studies conducted in China were funded by Tianjin University of Traditional Chinese Medicine and its affiliates.

Disclosures
C.C., N.V., R.N.G., and B.P.L.C. have received a grant from the National Medical Research Council of Singapore to conduct a randomized double-blinded, placebo-controlled clinical trial of DJ in acute stroke. S.X. is a member of the Scientific Advisory Board and a shareholder of Moleac, which owns the commercial and intellectual property rights of DJ outside China. DP is a shareholder and an employee of Moleac until July 2006. S.X. led the development of DJ in China and is responsible for the integrity of the clinical data.

References
Neuroaid in Stroke Recovery

Charles Hua Chiang Siow
Mount Alvernia Hospital, Singapore, Singapore

Stroke is a leading cause of death and disability worldwide [1]. Many patients only make a partial or poor recovery after stroke, and the major burden of stroke is chronic disability [2]. To date, no effective treatment has been found for reducing stroke-induced disabilities.

Neuroaid originates from Traditional Chinese Medicine. It has been developed to aid post-stroke recovery, and has recently been approved in 7 countries, including Singapore.

Early trials of Neuroaid, performed in China on 605 patients in 2000, established its safety and demonstrated a positive effect on the recovery of independence and motor functions. Patients receiving Neuroaid were found to be 2.4 times more likely to achieve independence at 1 month after stroke than the control group [3, 4]. More recently, safety trials showed that Neuroaid, taken either alone or in combination with aspirin, does not modify hemostasis, hematology and biochemistry in normal subjects and stroke patients [6]. Additional double-blind randomized placebo-controlled trials are ongoing in Asia [5].

This is a case description of 10 patients who took Neuroaid after ischemic stroke onset. All patients were seen in a private clinic at Mount Alvernia Hospital in Singapore and in the Neurology Outpatient Clinic for subsequent follow-up.

Neuroaid was given as an add-on to other medications, including antiplatelet, anticoagulant (warfarin), lipid-lowering, antihypertensive, diabetic and antidepressant medications, which were used as the patient’s condition dictated (table 2). The Neuroaid dose received was 4 tablets, 3 times per day. Treatment was initiated between 1 week and 6 months after stroke, and given to each patient for 2–3 months.

Cases presented with neurological impairments affecting motor, balance, speech and visual functions. These were assessed during initial examination and confirmed with imagery (table 1).

The patients showed a good tolerability to the treatment. Only 1 mild adverse event was reported, with patient No. 4 reporting diarrhea after starting Neuroaid. Treatment was reduced, and then progressively increased to full dosage within a week.

On follow-up, all cases reported improvements over the period in which they received Neuroaid. There were 6 cases of patients showing full recovery, 3 cases of good or moderate recovery and 1 case of poor recovery. Significant improvements were recorded in motor, visual, speech and cognitive functions (table 1). Motor skills: the 8 patients with motor deficits improved in the strength of their upper and lower extremities, and their ability to walk; motor disabilities fully resolved in 6 patients. Balance: the 3 patients showing difficulties in their balance recovered. Vision: the diplopia and hemianopia in 5 patients resolved. Speech: 4 patients reported improvements in speech disabilities, including expressive aphasia and anomia; after 3 months, 2 had fully recovered from their speech impairments.

The impact of Neuroaid treatment cannot be differentiated from the contribution of natural recovery, med-
It is consistent with late-stage recovery data shown in early clinical trials. Although the exact mechanism is not well understood, initial laboratory studies suggest improvements in brain neuroplasticity and neuroprotection. Larger double-blind placebo-controlled studies will provide more comprehensive data on Neuroaid in the future.

### Table 1. Patient recovery

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Presentation at treatment initiation</th>
<th>MRI</th>
<th>Follow-up assessment</th>
<th>areas of improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>time since stroke</td>
<td></td>
<td></td>
<td>motor balance vision speech</td>
</tr>
<tr>
<td>1</td>
<td>1 week</td>
<td></td>
<td>full recovery (2 months)</td>
<td>✓ ✓</td>
</tr>
<tr>
<td>2</td>
<td>1 week</td>
<td></td>
<td>full recovery (7 months)</td>
<td>✓</td>
</tr>
<tr>
<td>3</td>
<td>2 weeks</td>
<td></td>
<td>full recovery (3 months)</td>
<td>✓ ✓</td>
</tr>
<tr>
<td>4</td>
<td>1 week</td>
<td></td>
<td>full recovery (3 months)</td>
<td>✓ ✓ ✓</td>
</tr>
<tr>
<td>5</td>
<td>1 week</td>
<td></td>
<td>full recovery (2 weeks)</td>
<td>✓ ✓ ✓</td>
</tr>
<tr>
<td>6</td>
<td>1 month</td>
<td></td>
<td>full recovery (3 months)</td>
<td>✓</td>
</tr>
<tr>
<td>7</td>
<td>1 month</td>
<td></td>
<td>only residual acalculia</td>
<td>✓ ✓ ✓</td>
</tr>
<tr>
<td>8</td>
<td>6 months</td>
<td></td>
<td>only residual mild aphasia</td>
<td>✓ ✓</td>
</tr>
<tr>
<td>9</td>
<td>1 week</td>
<td></td>
<td>residual motor deficit: strength</td>
<td>✓</td>
</tr>
<tr>
<td>10</td>
<td>1 week</td>
<td></td>
<td>strength 1/5 RUE, 2/5 RLE, 5/5 LUE-LLE, can turn over and make noises (3 months)</td>
<td>✓</td>
</tr>
</tbody>
</table>

RUE = Right upper extremities; RLE = right lower extremities; LLE = left lower extremities; LUE = left upper extremities.

It is interestingly noted that 3 patients started Neuroaid treatment at a later stage of stroke recovery. In particular, patient No. 8 started Neuroaid 6 months after reaching a plateau in his recovery, and after this continued to experience improvements in his speech and cognitive abilities. Another 2 patients (No. 6 and 7) started Neuroaid 1 month after their strokes, and both recovered significantly.

These findings support the safety of Neuroaid and its positive effect on the recovery of the post-stroke patient.
Table 2. Concomitant medications

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Gender</th>
<th>Age</th>
<th>Antiplatelet/Anticoagulant</th>
<th>Antihypertensive</th>
<th>Cholesterol Lowering</th>
<th>Antidiabetic</th>
<th>Antidepressant</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>50</td>
<td>Clopidogrel 75 mg M</td>
<td>Aspirin 300 mg M</td>
<td>Warfarin 4 mg M</td>
<td>Rosuvastatin 20 mg N</td>
<td>Sitagliptin 100 mg N</td>
<td>Melatonin 3 mg N</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>51</td>
<td>Dipyridamole 75 mg T</td>
<td>Nifedipine 30 mg M</td>
<td>Metformin 200 mg T</td>
<td>Lovastatin 20 mg N</td>
<td>Meloxicam 10 mg N</td>
<td>Sodium valproate 300 mg N</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>67</td>
<td>Dipyridamole 75 mg T</td>
<td>Perindopril 16 mg M</td>
<td>Candesartan 16 mg M</td>
<td>Rosuvastatin 20 mg N</td>
<td>Sitagliptin 100 mg N</td>
<td>Domperidone 20 mg</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>68</td>
<td>Dipyridamole 75 mg T</td>
<td>Nifedipine 30 mg M</td>
<td>Rosuvastatin 20 mg N</td>
<td>Metformin 200 mg T</td>
<td>Trandolapril 10 mg N</td>
<td>Domperidone 20 mg</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>69</td>
<td>Dipyridamole 75 mg T</td>
<td>Nifedipine 30 mg M</td>
<td>Rosuvastatin 20 mg N</td>
<td>Metformin 200 mg T</td>
<td>Trandolapril 10 mg N</td>
<td>Domperidone 20 mg</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>70</td>
<td>Dipyridamole 75 mg T</td>
<td>Nifedipine 30 mg M</td>
<td>Rosuvastatin 20 mg N</td>
<td>Metformin 200 mg T</td>
<td>Trandolapril 10 mg N</td>
<td>Domperidone 20 mg</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>68</td>
<td>Dipyridamole 75 mg T</td>
<td>Nifedipine 30 mg M</td>
<td>Rosuvastatin 20 mg N</td>
<td>Metformin 200 mg T</td>
<td>Trandolapril 10 mg N</td>
<td>Domperidone 20 mg</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>89</td>
<td>Dipyridamole 75 mg T</td>
<td>Nifedipine 30 mg M</td>
<td>Rosuvastatin 20 mg N</td>
<td>Metformin 200 mg T</td>
<td>Trandolapril 10 mg N</td>
<td>Domperidone 20 mg</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>76</td>
<td>Dipyridamole 75 mg T</td>
<td>Nifedipine 30 mg M</td>
<td>Rosuvastatin 20 mg N</td>
<td>Metformin 200 mg T</td>
<td>Trandolapril 10 mg N</td>
<td>Domperidone 20 mg</td>
</tr>
</tbody>
</table>

M = Morning; N = night; D = once a day; B = twice a day; T = thrice a day.

* Intake for patient No. 2 was D.

b Intake for patient No. 10 was B.

References

A double-blind, placebo-controlled, randomized, multicenter study to investigate CHInese Medicine Neuroaid Efficacy on Stroke recovery (CHIMES Study)


Rationale Traditional Chinese Medications (TCM) have been reported to have beneficial effects in stroke patients, but were not rigorously evaluated by GCP standards.

Aim This study tests the hypothesis that Neuroaid, a TCM widely used in China post-stroke, is superior to placebo in reducing neurological deficit and improving functional outcome in patients with acute cerebral infarction of an intermediate severity.

Design This is a multicenter, randomised, double-blind, placebo-controlled study of Neuroaid in ischemic stroke patients with National Institute of Health Stroke Scale (NIHSS) 6–14 treated within 48 h of stroke onset. Neuroaid or placebo is taken (4 capsules) 3 times daily for 3 months. Treatments are assigned using block randomization, stratified for centers, via a central web-randomization system. With a power of 90% and two-sided test of 5% type I error, a sample size is 874. Allowing for a drop-out rate of up to 20%, 1100 individuals should be enrolled in this study.

Study Outcomes The primary efficacy endpoint is the modified Rankin Scale (mRS) grades at 3 months. Secondary efficacy endpoints are the NIHSS score at 3 months; difference of NIHSS scores between baseline and 10 days, and between baseline and 3 months; difference of NIHSS sub-scores between baseline and 10 days, and between baseline and 3 months; mRS at 10 days, 1 month, and 3 months; Barthel index at 3 months; Mini Mental State Examination at 10 days and 3 months. Safety outcomes include complete blood count, renal and liver panels, and electrocardiogram.

Study registration: ClinicalTrials.gov identifier: NCT00554723.

Key words: acute stroke therapy, Asia, cerebral infarction, ischemic stroke, therapy, treatment

Introduction

Stroke is a major cause of death and disability in many countries of the world, placing a heavy burden on patients, families, healthcare systems, and economies (1). Only a few therapies have consistently reduced death and/or disability poststroke – intravenous thrombolysis with recombinant tissue plasminogen within 3 h of stroke onset (2), hemicraniectomy for malignant middle cerebral artery territory infarction (3), early administration of aspirin (4), and organized in-patient stroke care (5). The former is given to only a small proportion of stroke patients due to its many contraindications and short window of treatment opportunity, while the latter are generally more applicable but may not be widely practiced due to delayed patient arrival or organizational challenges. Despite these evidence-based interventions, recovery is still incomplete in many stroke patients. There is thus a real need for better treatments to enhance poststroke recovery.

Neuroaid is a Traditional Chinese Medicine (TCM) product, combining nine herbal components and five animal components, manufactured by Shitian Pharmaceuticals.
(Tianjin, China). It was certified as Good Manufacturing Practice compliant and registered under the Chinese name Danqi Piantan Jiaonang with the Sino-Food and Drug Administration (Sino-FDA) in August 2001 for the treatment of stroke recovery. Previous clinical studies performed in China under Chinese Standard Guidelines have shown that it increased stroke patients’ recovery from their neurological disability and functional outcome (6). It was found to be superior in reducing neurological deficit compared with another TCM, Buchang Naoyintong Jiaonang, which was previously shown to be at least as effective as citicoline (7, 8). It was found to be safe and well tolerated, without any effect on hemorrhagic or thrombotic risks or blood pressure (9).

But the China trials were not International Conference of Harmonisation/Good Clinical Practice (ICH/GCP) compliant (Sino-FDA did not require it), and used positive controls. The sample sizes were also small, had a wide window of recruitment after stroke onset, a short duration of treatment, and used nonstandard measurements of functional and neurological outcomes. A longer duration, well-conducted trial with large patient numbers is needed, performed in accordance with ICH/GCP guidelines.

We have thus planned this trial of Neuroaid to assess its efficacy in improving outcomes poststroke.

Study objective

To test the hypothesis that Neuroaid is superior to Placebo in reducing neurological deficit and improving the functional outcome in patients with cerebral infarction of an intermediate range of severity.

Methods

Design

CHInese Medicine Neuroaid Efficacy on Stroke (CHIMES) is a prospective, multicenter, randomized, placebo-controlled, double-blinded clinical trial of Neuroaid among patients with acute ischemic stroke. Participating centers are from Hong Kong, Philippines, Singapore, and Thailand. The study will be conducted according to ICH/GCP guidelines. Local ethics committee approval will be obtained before commencing the trial at a site.

Patient population

Inclusion criteria: A subject will be eligible for inclusion in the trial only if all the following criteria are fulfilled at baseline:

- age 21 years old and above;
- time window is <48 h after the onset of symptoms;
- on antiplatelet therapy;
- prestroke modified Rankin Scale (mRS) less than or equal to 1;
- intermediate severity range: 6 ≤ National Institute of Health Stroke Scale score (NIHSS) ≤ 14;
- cerebral infarction with compatible imaging on computed tomography (CT) scan or magnetic resonance imaging (MRI);
- females are eligible to participate in the trial if they are of non-child-bearing potential (i.e. physiologically incapable of becoming pregnant, including any female who is post-menopausal); and
- subject or his/her legally acceptable representative is willing to provide written informed consent.

Exclusion criteria: A subject will not be eligible for inclusion in the trial if any of the following criteria apply at baseline:

- received thrombolysis;
- evidence of intracerebral hemorrhage on brain CT scan or MRI;
- rapidly improving neurological deficit;
- definite indication for full-dose or long-term anticoagulation therapy;
- other significant nonischemic brain lesions that could affect function disability;
- coexisting systemic diseases: terminal cancer, renal failure (creatinine > 200 μmol/l, if known), cirrhosis, severe dementia, or psychosis; and
- participated in another clinical trial within the last 3 months.

Baseline measurements

CT scan or MRI must be performed to exclude hemorrhagic stroke. If the subject is eligible, the following variables will be recorded: normal, recent cerebral infarction, or others.

Information on the following variables will be collected at the baseline assessment (Table 1):

- demographic data: date of birth and gender;
- vital signs: temperature, blood pressure, pulse rate, body weight, and height;
- time from stroke onset;
- medical history: neurological, cardiovascular, endocrine, hematological, eyes, ear–nose–throat (ENT), peripheral vascular, respiratory, gastrointestinal, hepatic, renal, genito–urinary, dermatological, musculoskeletal, neoplasia, immune, and psychiatric;
- physical examination: general appearance, neurological, eyes, head and neck, ENT, heart, lungs, abdomen, lymph nodes, genito–urinary, extremities, skin, musculoskeletal, immune, and psychiatric;
- stroke history: date of previous stroke, type of stroke;
- risk factors: previous myocardial infarction, angina, hypertension, peripheral vascular disease, diabetes mellitus, hyperlipidemia, smoking history, and habitual drinking;
- previous and ongoing medications: name of drugs, route, dosage, frequency, date started, date stopped, and indication;
routine laboratory investigations i.e. complete blood count, blood urea, serum creatinine, serum uric acid, blood glucose, serum alkaline phosphatase, glutamate oxaloacetate transaminase (also called aspartate transaminase), glutamate pyruvate transaminase (also called alanine transaminase), serum bilirubin, serum electrolytes, total proteins with albumin and globulin, and electrocardiogram; NIHSS; mRS; and Mini-Mental State Examination (MMSE).

Randomization
Treatments will be assigned using block randomization, stratified for centers.
A web-based randomization/registration system will be provided by the Clinical Trials and Epidemiology Research Unit (CTERU), Singapore. When a subject meets the inclusion/exclusion criteria and gives written informed consent, the investigator will register this subject in the web system. A subject number will then be assigned to this subject by the system. In the case that the web system fails to operate, investigators will be asked to use the back-up envelope system and take the next subject number available at their respective site, complete the subject registration form, and fax the form to CTERU. Once the web system resumes functioning, investigators will need to register these subjects in the web system according to the information recorded in the subject registration form, before a new subject can be registered via web registration. CTERU will verify the web registration notification against the received registration fax.

Blinding
As this is a double-blind study, the following persons will be blinded:
- Patients;
- CHIMES Investigators and their study-related staff;
- CHIMES Society Members;
- Data and Safety Monitoring Board (DSMB) Members; and
- the CTERU Clinical Project Coordinator.

The following persons will not be blinded:
- CTERU statistician;
- statistician (CTERU) who will prepare the emergency envelopes (one envelope will refer to one randomized subject);
- Neuroaid/Placebo manufacturer or any independent staff appointed to be assigned to put the sticker/treatment identification number based on the randomization list; and
- DSMB statistician.

Treatment (Fig. 1)
Subjects are randomly assigned to receiving a 3-month course of either:
- Neuroaid or
- a matched placebo of Neuroaid.

Both involve the subject taking four capsules, three times daily.
If the subject is unable to swallow the capsules, the capsule contents are removed and diluted in water before serving by mouth or by a nasogastric tube.

Neuroaid is manufactured by Shitian Pharmaceuticals. It contains 0.4 g of medicinal extracts of plant and animal origins – animal origins include scorpion and leech (Table 2).

The placebo of Neuroaid will be manufactured by the same manufacturer as Neuroaid. Its composition will include four constituents sold as food in China and known to have no active effect (Table 3).

Neuroaid and placebo capsules have an identical appearance, smell, and taste.

All patients will receive standard stroke care, which will include antiplatelet therapy, control of vascular risk factors, and appropriate rehabilitation.

Antiplaletics used in the trial will depend on standard practice and the licensing situation in each participating study country. Any of the following five oral antiplatelet therapies will be allowed. A combination of two oral antiplatelets will also be allowed.
- Aspirin (acetylsalicylic acid, aspirin): the dosage is 75–300 mg per day;
- Clopidogrel Plavix®: the dosage is 75–300 mg per day;
- Ticlopidine Ticlid®: the dosage is 250 mg twice a day;

<table>
<thead>
<tr>
<th>Table 1 Schedule of trial-related activities</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td>Eligibility check</td>
</tr>
<tr>
<td>Informed consent</td>
</tr>
<tr>
<td>Demographic data</td>
</tr>
<tr>
<td>Vital signs</td>
</tr>
<tr>
<td>Time from stroke onset</td>
</tr>
<tr>
<td>Medical history</td>
</tr>
<tr>
<td>Physical examination</td>
</tr>
<tr>
<td>Neurological evaluation</td>
</tr>
<tr>
<td>Stroke history</td>
</tr>
<tr>
<td>Risk factors</td>
</tr>
<tr>
<td>Previous medication</td>
</tr>
<tr>
<td>Ongoing medication/change in concomitant medication</td>
</tr>
<tr>
<td>CT scan/MRI</td>
</tr>
<tr>
<td>Laboratory examination</td>
</tr>
<tr>
<td>NIHSS</td>
</tr>
<tr>
<td>Modified Rankin Score</td>
</tr>
<tr>
<td>Barthel index</td>
</tr>
<tr>
<td>Mini-Mental State Examination</td>
</tr>
<tr>
<td>Examination</td>
</tr>
<tr>
<td>Drug accountability and compliance</td>
</tr>
<tr>
<td>Adverse event</td>
</tr>
</tbody>
</table>

CT, computed tomography; MRI, magnetic resonance imaging; NIHSS, National Institute of Health Stroke Scale.
Eligibility confirmed
Informed Consent obtained

Randomization

Day 1 - Baseline
Initial Assessment 6≤NIHSS≤14, mRS, MMSE

10 days - Discharge
Assessment mRS, Barthel, MMSE, Safety, compliance

1 month
Phone assessment
Assessment mRS, Safety, compliance

3 months
Assessment NIHSS, mRS, Barthel, MMSE, Safety, compliance

Standard stroke care + matched-placebo for 3 months

Standard stroke care + Neuroaid for 3 months

Fig. 1 Flow diagram for CHinese Medicine Neuroaid Efficacy on Stroke.

Table 2 Composition of Neuroaid

<table>
<thead>
<tr>
<th>Ingredients (Latin name)</th>
<th>Content per capsule in mg (equivalent of raw abstracts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radix astragali</td>
<td>570</td>
</tr>
<tr>
<td>Radix salviae miltiorrhiza</td>
<td>114</td>
</tr>
<tr>
<td>Radix paeoniae rubra</td>
<td>114</td>
</tr>
<tr>
<td>Rhizoma chuanxiong</td>
<td>114</td>
</tr>
<tr>
<td>Radix angelicae sinensis</td>
<td>114</td>
</tr>
<tr>
<td>Carthamus tinctorius</td>
<td>114</td>
</tr>
<tr>
<td>Prunus persica</td>
<td>114</td>
</tr>
<tr>
<td>Radix polygalae</td>
<td>114</td>
</tr>
<tr>
<td>Rhizoma acori tatarinowii</td>
<td>114</td>
</tr>
<tr>
<td>Buthus martensi*</td>
<td>95</td>
</tr>
<tr>
<td>Hirudo*</td>
<td>66.5</td>
</tr>
<tr>
<td>Eupolyphaga seu steleophaga*</td>
<td>66.5</td>
</tr>
<tr>
<td>Calculus bovis artifactus*</td>
<td>28.5</td>
</tr>
<tr>
<td>Saigae tataricae cornu*</td>
<td>28.5</td>
</tr>
</tbody>
</table>

*Refers to ingredients of animal origin.

Table 3 Composition of placebo to Neuroaid

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Content per capsule in mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>227.27</td>
</tr>
<tr>
<td>Dried ripe fruit</td>
<td>45.45</td>
</tr>
<tr>
<td>Noodle fish</td>
<td>90.91</td>
</tr>
<tr>
<td>Citric acid</td>
<td>5.00</td>
</tr>
</tbody>
</table>

- heparins (including low-molecular-weight heparins) or heparinoids.
  Other concomitant medications will be allowed throughout the trial but should be recorded in the subjects’ Case Report Form during the study.

Trials' medication management

If a subject is not compliant to the treatment (< 80% of the treatment), they will not be withdrawn from the trial, but will still be included in the ‘Intention-to-treat’ analysis; however, they will be excluded from the ‘Per protocol’ analysis.

Primary outcomes

The primary efficacy end-point is the mRS grades at 3 months for all randomized subjects.
Secondary outcomes

The secondary efficacy end-point measures will be the recovery of the subjects as assessed by:

- NIHSS score response at 3 months (plus or minus 1 week);
- difference of NIHSS scores between baseline and 10 days (plus or minus 2 days), and between baseline and 3 months (plus or minus 1 week);
- difference of NIHSS sub-scores between baseline and 10 days (plus or minus 2 days) or discharge, and between baseline and 3 months (plus or minus 1 week);
- mRS response at 10 days (plus or minus 2 days), at 1 month (plus or minus 1 week), and at 3 months (plus or minus 1 week);
- Barthel index (BI) at 3 months (plus or minus 1 week); and
- MMSE at 10 days (plus or minus 2 days), and at 3 months (plus or minus 1 week).

DSMB

The purpose of a DSMB is to assess at intervals the progress of a clinical trial, the safety data, and the clinical efficacy end-points. The Board will then recommend to the Trial Steering Committee whether to continue, modify, or terminate the trial based on the data of either the present trial or from other studies on a similar drug or of a similar nature.

In particular, the DSMB will help:

- review the research protocol and plan for data and safety monitoring;
- ensure patient safety (i.e. to minimize the avoidable risk of subjects’ participation);
- ensure that the trial can be stopped as soon as a reliable conclusion can be drawn from the data;
- ensure continued scientific validity, and to ensure that the trial is stopped if it is unlikely to be able to answer the original question (for trial ‘futility’); and
- protect the confidentiality of the trial data and the results of monitoring.

Two interim efficacy analyses are scheduled. A first DSMB report shall be prepared after 20% or 220 patients have been recruited. The second DSMB report will be prepared after 60% or 660 patients have been recruited. The DSMB will have the option to request an additional report if considered necessary.

In order to set very stringent criteria for stopping the trial at the earliest interim analysis, ‘stopping guidelines’ based on O’Brien-Fleming’s method will be adopted, with the significance level for each analysis time-point being as follows:

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Interim 1</th>
<th>Interim 2</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Significance level</td>
<td>0-0006</td>
<td>0-0156</td>
<td>0-05</td>
</tr>
</tbody>
</table>

However, it should be emphasized that the DSMB will use these stopping criteria as guidelines to assist their decision making rather than to follow them dogmatically.

Sample size

Based on the distribution of mRS at 6 months obtained in the FISS-Tris study (10) and if we assume an average odds ratio (OR) of 1.5 for the Neuroaid group, we then obtain the distribution (Table 4). With a power of 90% and a two-sided test of a 5% type I error, a total sample size of 874 would be needed. To allow for a maximum drop-out rate of up to 20%, 1100 individuals should be enrolled in this study.

Statistical analyses

An ‘Intention-to-treat’ analysis will be carried out.

Primary efficacy outcomes – mRS grades at 3 months. Tabulation of frequency will be presented and the difference in the distribution of subjects within each range of mRS between the placebo group and the Neuroaid group will be tested by the Mann–Whitney U-test, with allowance for ties (3). This is equivalent to ordinal logistic regression using treatment groups as the independent variable, which then provides an estimate of the OR and the corresponding 95% confidence interval. Further, ordinal logistic regression allows the observed treatment difference to be adjusted by potentially prognostic factors.

Secondary efficacy outcomes

NIHSS score response at 3 months (plus or minus 1 week). Patients will be deemed to be responders if their NIHSS scores improve by five points or more from the NIHSS score at baseline and at 10 days. Tabulation of frequency for responders will be presented. The χ²-test will be performed and/or presented where appropriate.

- Difference of NIHSS scores between baseline and 10 days (plus or less 2 days), and between baseline and 3 months (plus or minus 1 week) between two groups. The two-sample t-test will be performed, with appropriate transformation carried out on NIHSS scores if necessary.
- Difference of NIHSS sub-scores (such as motor function) between baseline and 10 days (plus or minus 2 days) or discharge, and between baseline and 3 months (plus or minus 1 week) between two groups. The Mann–Whitney U-test, with allowance for ties, will be performed.

<table>
<thead>
<tr>
<th>Table 4 mRS scores from the FISS-Tris study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of patients in mRS at 6 months (%)</td>
</tr>
<tr>
<td>6 months</td>
</tr>
<tr>
<td>Placebo group (from FISS-Tris, n = 292)</td>
</tr>
<tr>
<td>Proposed Neuroaid group</td>
</tr>
<tr>
<td>Average proportion of Placebo and Neuroaid group</td>
</tr>
</tbody>
</table>

mRS, modified Rankin Scale.
• mRS response (1) at 10 days (plus or minus 2 days), at 1 month (plus or minus 1 week), and at 3 months. Patients will be deemed to be responders if their mRS is less than or equal to one. Tabulation of frequency for responders will be presented. The \( \chi^2 \)-test will be performed and OR will be presented where appropriate.
• mRS response (2) at 10 days (plus or minus 2 days), at 1 month (plus or minus 1 week), and at 3 months (plus or minus 1 week). Patients will be deemed to be responders if their mRS is less than or equal to two. Tabulation of frequency for responders will be presented. The \( \chi^2 \)-test will be performed and OR will be presented where appropriate.
• BI at 3 months (plus or minus 1 week) will be analyzed for both groups. The two-sample \( t \)-test will be performed.
• MMSE at 10 days and at 3 months (plus or minus 1 week). MMSE for both groups will be analyzed. The two-sample \( t \)-test will be performed.

Per protocol and sub-group analysis
Per protocol analysis will also be performed for primary and secondary efficacy outcomes on subjects who have not had any deviation from the protocol.

Sub-group analysis will be performed for the following sub-groups:
• time from stroke onset: therapeutic window \( \leq 24 \) h and therapeutic window \( > 24 \) h;
• NIHSS score: patients with \( 6 \leq \) NIHSS \( \leq 10 \) and patients with \( 10 < \) NIHSS \( \leq 14 \);
• type of cerebral infarction: patients presenting with lacunar infarction and patients with large artery occlusive disease;
• antiplatelet treatment received; and
• other prespecified sub-groups.

Study organization and funding
The study is headed by a Steering Committee, assisted by an independent DSMB. The CTERU will handle trial monitoring, data management, and storage as well as statistical analysis.

The study is funded internationally in part by a grant from the CHIMES Society, a nonprofit charity organization based in Singapore. Additional local funding in each center is listed in Table 5.

Conclusions
CHIMES will be the first multicenter, randomized, double-blinded, placebo-controlled trial assessing the efficacy and safety of a TCM in the management of acute ischemic stroke according to GCP guidelines. It will provide valuable information on the conduct of randomized-controlled trials involving TCMs and similar botanicals. If Neuroaid is efficacious, further studies may be needed to assess which component of Neuroaid is most efficacious. It will also spur GCP-based evaluations of other TCMs in stroke.

### Table 5 Funding sources per country/center

<table>
<thead>
<tr>
<th>National Research Council (Singapore)</th>
<th>CHIMES Society</th>
<th>Molecac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall coordination</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Singapore sites</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Philippines sites</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Thailand sites</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

CHIMES investigators


**DSMB members:** G.A. Donnan (chair), D. Machin, C. Tzourio


**Sites**

- **Philippines**
  - University of Santo Tomas Hospital, Manila – J.C. Navarro, A. Baroque, J. Lokin, M. Yumul
  - Jose R. Reyes Medical Center, Manila – H. Gan, J.C. Navarro
  - San Pedro Hospital, Davao City – A. Lao

- **Singapore**
  - Changi General Hospital – N. Venketasubramanian, R. Gan
  - National University Hospital – B.P.L. Chan
  - National Neuroscience Institute (Singapore General Hospital) – H.M. Chang
  - National Neuroscience Institute (Tan Tock Seng Hospital) – N. Venketasubramanian, R. Gan

**References**


A Double-Blind, Placebo-Controlled, Randomized Phase II Pilot Study to Investigate the Potential Efficacy of the Traditional Chinese Medicine Neuroaid (MLC 601) in Enhancing Recovery after Stroke (TIERS)

Keng He Kong\textsuperscript{a}  Seng Kwee Wee\textsuperscript{a}  Chwee Yin Ng\textsuperscript{a}  Karen Chua\textsuperscript{a}  Kay Fei Chan\textsuperscript{a}  N. Venketasubramanian\textsuperscript{b}  Christopher Chen\textsuperscript{c}

\textsuperscript{a}Tan Tock Seng Hospital Rehabilitation Centre, Ang Mo Kio Hospital, \textsuperscript{b}Division of Neurology, and \textsuperscript{c}Department of Pharmacology, National University Hospital of Singapore, Singapore

Key Words
Stroke  Neuroaid  Motor rehabilitation  Functional recovery  Clinical trial

Abstract

Background and Objective: Previous clinical studies have shown that Neuroaid (MLC 601) may be beneficial in post-stroke rehabilitation. Our aim was to investigate the efficacy of Neuroaid on motor recovery in ischemic stroke patients using rehabilitation endpoints in accordance with the International Conference on Harmonization/Good Clinical Practice guidelines, in order to provide predictive information for further larger trials. Methods: This is a phase II double-blind, placebo-controlled pilot study of 40 subjects admitted with a recent (less than 1 month) ischemic stroke. All subjects were given either Neuroaid or placebo, 4 capsules 3 times a day for 4 weeks. Fugl-Meyer Assessment (FMA), National Institutes of Health Stroke Scale and Functional Independence Measure scores were measured at initiation of the treatment, and at 4 and 8 weeks. Results: None of the outcomes was statistically significant between the two groups. However, FMA scores showed a positive trend for improvement with Neuroaid treatment over time. Subgroup analysis of subjects with posterior circulation infarction and severe stroke both showed a tendency for better recovery. Conclusion: Some positive trends were observed in the Neuroaid group. A larger multicenter trial focusing on severe stroke patients is needed to better evaluate the role of Neuroaid in aiding stroke recovery in rehabilitation.

Introduction

Standard treatment modalities in stroke rehabilitation are physiotherapy, occupational therapy, and speech therapy, in addition to skilled medical and nursing care. Despite intensive inpatient rehabilitation with these modalities in a stroke unit, 36% of acute stroke patients remain moderately to severely disabled at discharge [1]. There is thus a real need for better treatments to further improve the outcome of stroke rehabilitation.

Rehabilitation pharmacology refers to the use of medications in combination with rehabilitative training to improve functions. The two most commonly studied medications for this purpose are amphetamine and levodopa. Their mechanisms of action include increased noradrenergic and dopaminergic functions and facilitation of activity-dependent neuroplasticity [2–4]. However, the efficacy of these medications is still debatable [5, 6].
Neuroaid (MLC 601, Moleac Pte. Ltd, Singapore) is a traditional Chinese medicine which has been used extensively in China as a drug to facilitate recovery after stroke. It combines 9 herbal (radix astragali, radix salviae miltiorrhizae, radix paonieae rubra, rhizoma chuanxiong, radix angelicae sinensis, Carthamus tinctorius, Prunus persica, radix polygalae and rhizoma acori tatarinowii) and 5 animal (Hirudo, Eupolyphaga seu Steleophaga, calculus bovis artifactus, Buthus martensi and cornu saigae tatarinowii) components and was registered under the Chinese name of Danqi Piantan Jiaonang with the Sino-Food and Drug Administration in August 2001. It is manufactured by Shitian Pharmaceutical Industry in Tianjin, China, and was certified as good manufacturing practice compliant with the Sino-Food and Drug Administration.

Previous clinical studies performed in China have shown that Neuroaid enhances stroke patients’ recovery from their neurological disability and improves functional outcome and thus, may be beneficial in post-stroke rehabilitation trial in accordance with the International Conference on Harmonization/Good Clinical Practice guidelines and used positive controls. Furthermore, the outcome measures in these trials were not the standard scales used in modern-day stroke trials.

A previous study [7] suggested that Neuroaid’s effectiveness in improving stroke recovery may be related to its role in neuronal protection and plasticity. The safety of Neuroaid in hemostasis, hematology and biochemistry has been established in 3 clinical trials [8]. A case series of 10 patients in Singapore supported the results reported in the initial studies [9].

A large-scale academic randomized controlled trial is currently recruiting in South East Asia to evaluate the impact of 3-month treatment with Neuroaid and is assessing patients on neurological endpoints using the modified Rankin Scale and National Institutes of Health Stroke Scale (NIHSS) [10]. Rehabilitation studies typically use more detailed evaluation scales of the different components of recovery and rehabilitation.

As there have been no previous studies on Neuroaid conducted using rehabilitation endpoints, we decided to investigate the efficacy of Neuroaid in motor recovery in ischemic stroke patients admitted to an inpatient rehabilitation center within the setting of a post-stroke rehabilitation trial in accordance with the International Conference on Harmonization/Good Clinical Practice guidelines. The objective of this research was to obtain pilot data which will support the design of a larger, controlled trial in the future.

Methods

Study Design and Subjects
This was a single-center, double-blind, placebo-controlled, randomized phase II pilot study. The design was similar to the one used in the early trials [7]. The patients were recruited from the Tan Tock Seng Hospital rehabilitation center in Ang Mo Kio Hospital, Singapore within 1 month after ischemic stroke onset.

All subjects were randomized to a group A (Neuroaid, 4 capsules 3 times daily) or group B (placebo, 4 capsules 3 times daily) 1-month treatment according to a balanced randomization scheme of 1:1, based on a computer-generated randomization list prepared by an appointed staff. The components of the placebo included the following constituents: barley 227.27 mg, dried ripe fruit 45.45 mg, noodle fish 90.91 mg and citric acid 5.00 mg, and had an appearance, smell and taste similar to Neuroaid. Only the designated, unblinded, independent staff member, performing the subject randomization, was aware of the treatment allocation of group A and B treatment.

The inclusion and exclusion criteria are listed below.

Inclusion Criteria
(1) Adults between 21 and 80 years old
(2) Randomizable within 1 month after stroke onset
(3) Motor power of grade 1–4/5 on the Medical Research Council Scale in at least one limb
(4) Prestroke modified Rankin Scale score ≤1 [11]
(5) Cerebral infarction with compatible imaging on computed tomography scan or magnetic resonance imaging
(6) Female subjects are eligible to participate in the trial if they are of nonchildbearing potential (hysterectomy or postmenopausal)
(7) Written informed consent obtained from the subject or legal representative

Exclusion Criteria
(1) Recent thrombolysis treatment
(2) Evidence of intracerebral hemorrhage on brain computed tomography scan or magnetic resonance imaging
(3) Full-dose or long-term anticoagulation therapy
(4) Significant nonischemic brain lesions which could affect functional disability
(5) Coexisting systemic diseases: terminal cancer, renal failure (creatinine >200 μmol/l, if known), cirrhosis, severe dementia or psychosis
(6) History of previous stroke
(7) Participation in another clinical trial within the last 3 months
(8) Aphasia or any other cognitive disabilities which prevent cooperation with study instructions
(9) Hemoglobin level of <10 mg/dl on admission
(10) History of craniotomy or seizures

Outcome Measures
The primary efficacy endpoint was improvement of impairment of the affected upper and lower limb as assessed on the Fugl-Meyer Assessment (FMA) at 4 weeks. Previous studies on the responsiveness and validity of FMA [12] have shown that the FMA score is suitable to detect changes over time for patients after
stroke rehabilitation, and it may be a relatively sound measure of motor function for stroke patients.

The secondary endpoint measures were:
(a) functional status as assessed on the Functional Independence Measure Scale (FIM) [13] at 4 and 8 weeks;
(b) FMA scores and subscores at 4 and 8 weeks, and
(c) stroke severity scores and subscores as assessed on the NIHSS [14] at 4 and 8 weeks.

Patients were categorized into three categories at baseline according to their FMA score at initiation of the trial: severe (0–35), moderate (36–79), and mild (80–100) [15].

Other Tests
All the following tests were performed at baseline and at 4 weeks.

- Routine blood investigations: full blood count; renal function test; liver function tests; glucose, calcium, electrolytes, and uric acid
- Routine investigations on the urine: albumin and glucose
- Electrocardiogram

This study was approved by the Institutional Review Board.

Sample Size
This is primarily a pilot study. There have been few studies of drug intervention in subacute stroke patients using the FMA score as an outcome measure, on which we can base our expected treatment effect. The sample size was determined based on a priori power analysis [16]. At least 20 subjects for each group would be required to detect an effect size d of 0.80 given a significance level of 5% (1-tailed) and 80% power. This effect size was estimated based on the findings of 2 previous trials [17, 18] on the distributed constraint-induced therapy in which the effect size d was 1.39 and 0.75 on the FMA, respectively.

Statistical Analysis
Baseline variables were compared using a two-group t test for continuous variables (i.e., age) and a χ² test for categorical variables (i.e., sex or race, etc).

Intention-to-treat analysis was used. For efficacy variables, comparisons were made between the two groups at baseline, at 4 and at 8 weeks. The two-group t test was used separately for each comparison. In case of nonnormality, the nonparametric Mann-Whitney test was performed. Further repeated-measures analyses were conducted to analyze the interaction effect of natural recovery over time and Neuroaid efficacy using the linear model.

All the statistics tests were performed using the Statistical Package for Social Sciences version 17.

Results

Baseline Characteristics
A total of 40 subjects were recruited in this study. The active and control groups had similar baseline characteristics (table 1). In general, the patients were young-elderly, predominantly male, Chinese, with moderately severe stroke, recruited 2 weeks after stroke. The higher proportion of males and shorter time interval between stroke onset and recruitment in the placebo group compared to the active group were not statistically significant.

Patient Flowchart
All 40 subjects were included in the final analysis: 20 received Neuroaid, and the other 20 placebo (fig. 1). Thirty-two subjects completed the study, 15 in the Neuroaid group and 17 in the placebo group; 3 patients in the Neu-

<table>
<thead>
<tr>
<th>Table 1. Baseline participant characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristics</td>
</tr>
<tr>
<td>Demographics</td>
</tr>
<tr>
<td>Age, years</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Race</td>
</tr>
<tr>
<td>Chinese</td>
</tr>
<tr>
<td>Malay</td>
</tr>
<tr>
<td>Indians</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Medical history: risk factors</td>
</tr>
<tr>
<td>Hypertension</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>Score at baseline</td>
</tr>
<tr>
<td>Modified Rankin Scale</td>
</tr>
<tr>
<td>FMA</td>
</tr>
<tr>
<td>Stroke severity</td>
</tr>
<tr>
<td>Severe</td>
</tr>
<tr>
<td>Moderate</td>
</tr>
<tr>
<td>Mild</td>
</tr>
<tr>
<td>NIHSS</td>
</tr>
<tr>
<td>FIM</td>
</tr>
</tbody>
</table>

Values presented are either means ± SD or number of subjects in subgroups with percentages in parentheses. ACI = Anterior circulation infarct; LACI = lacunar infarction; POCI = posterior circulation infarct.
Potential Efficacy of the Traditional Chinese Medicine Neuroaid

Efficacy Results

None of the primary or secondary outcomes was statistically significant between the Neuroaid group and the control group, probably due to the small sample size. However, overall, at 8 weeks the FMA scores were higher in the Neuroaid group and the FIM scores were higher in the placebo group. The NIHSS scores were similar in both groups (table 2). Using repeated-measures tests, the treatment effect was not significant over time although the trend towards the Neuroaid treatment was seen at 8 weeks (p = 0.40) (fig. 2a).

Exploratory Analysis

The exploratory analysis was based on the FMA as it has been shown to be the relevant scale to detect changes over time for patients after stroke rehabilitation [12]. Additionally, as all the trends were increasing over time, we also focused our analysis on the scores at 8 weeks.

Subgroup Analysis

We observed that the Neuroaid group performed better than the placebo group when the severity of the stroke was high; this difference increased at the later stage of the study (+58% higher improvement at 8 weeks in the Neuroaid group in severe cases, p = 0.36) (table 3).

Additionally, we observed a very strong tendency of a better recovery in posterior circulation infarction (POCI) patients receiving Neuroaid both at 4 weeks and 8 weeks (respectively p = 0.15, p = 0.23) (table 3). Since the FMA scores at baseline differed in both groups (43.3 in the Neuroaid group vs. 82 in the placebo group), we compared the recovery of the POCI patients in the Neuroaid group and 1 in the placebo group were lost to follow-up at 8 weeks, 3 patients in the Neuroaid group and 2 in the placebo group were withdrawn for safety reasons.

Patient compliance information was available for 39 subjects. Of these, only 1 subject in the placebo group was reported to be noncompliant with the treatment regimen.

Fig. 1. Patient flowchart.
group with the recovery achieved by the overall population of the placebo group (43.3 for the POCI Neuroaid group vs. 48.4 for the placebo group at baseline) and also found it to be higher (23.75 vs. 12.5 at 4 weeks, p = 0.35; 26.25 vs. 14.5 at 8 weeks, p = 0.39).

Other characteristics at baseline were shown to have no influence on the results.

Best Responders

In order to generate testable hypotheses, we looked at the best responders. We found that the relative improvement of the patients compared to their score at baseline results showed that subjects on Neuroaid were more likely to achieve important recovery as the threshold increased (fig. 2a, 3). While 10 and 9 patients in the Neuroaid and placebo group, respectively, achieved at least 50% progress, 6 in the Neuroaid versus 2 in the placebo group achieved more than 150% progress (p = 0.24) (fig. 3). All the characteristics at baseline of these 8 patients were similar to the overall population except for the severity of their stroke (mean of 14.9 on the FMA at baseline).

More detailed analysis showed that at 8 weeks, the 15 patients with the lowest recovery in both groups had very similar improvement in scores. However, the scores of the 5 best-recovered patients in both groups diverged. These 5 patients were further analyzed and the subjects receiving Neuroaid showed a better recovery (+11% at 4 weeks and +39% at 8 weeks, p = 0.17) than the patients in the placebo group. This difference was not statistically significant but showed a trend towards significance over time. Using repeated-measures tests, similar conclusions could be drawn. While the treatment effect did not show significance over time, a tendency could be observed in the later trial period that Neuroaid enhances the recovery of patients (fig. 2b).

The FIM showed a higher score in the placebo group; however, this difference was nonsignificant. The FIM score is employed to test the functional abilities of stroke survivors and might not be relevant for this study focus.

Table 2. Outcome results showing improvement at 4 and 8 weeks

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Neuroaid group (n = 20)</th>
<th>Placebo (n = 20)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMA improvement (ref. baseline)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By 4 weeks</td>
<td>11.7 ± 14.6</td>
<td>12.5 ± 12.2</td>
<td>0.84</td>
</tr>
<tr>
<td>By 8 weeks</td>
<td>16.7 ± 19.6</td>
<td>14.5 ± 14.2</td>
<td>0.68</td>
</tr>
<tr>
<td>NIHSS improvement (ref. baseline)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By 4 weeks</td>
<td>-2 ± 1.9</td>
<td>-1.9 ± 2.5</td>
<td>0.89</td>
</tr>
<tr>
<td>By 8 weeks</td>
<td>-2.4 ± 2.0</td>
<td>-3 ± 3.3</td>
<td>0.49</td>
</tr>
<tr>
<td>FIM improvement (ref. baseline)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By 4 weeks</td>
<td>13.6 ± 11.9</td>
<td>19.95 ± 15.5</td>
<td>0.17</td>
</tr>
<tr>
<td>By 8 weeks</td>
<td>14.7 ± 11.5</td>
<td>22.6 ± 16.3</td>
<td>0.12</td>
</tr>
</tbody>
</table>

FMA, NIHSS and FIM scores measured at baseline, and at 4 and 8 weeks. The improvement was calculated by numerical difference between the score at baseline and the one at 4 or 8 weeks. Values presented are means ± SD.

Fig. 2. FMA outcome results: repeated-measures analyses (a: n = 40; b: n = 10, i.e. the 5 best-recovered in both groups), with means (standard deviations).
Further larger trials are needed to provide conclusive evidence.

**Safety Data**

A total of 15 subjects reported 16 adverse events (AEs) during the study: 7 subjects on Neuroaid had 8 AEs, while 8 subjects on placebo had 8 AEs. All AEs were mild (12/16) or moderate (4/16) in severity.

Four serious adverse events (SAEs) were reported in the Neuroaid group (jaundice, hypokalemia, seizures, and recurrent stroke) while 1 SAE was reported in the placebo group (perianal abscess). The SAEs were considered not to be related to the study medication. No deaths were reported.

Two patients in the Neuroaid group left the trial because of SAEs (jaundice and recurrent stroke) compared with 2 patients in the placebo group with AEs (rash and abdominal distension).

All reported AEs and SAEs are presented in table 4.

### Discussion

Our study did not detect any statistically significant difference in the effect of Neuroaid on the motor recovery of ischemic stroke patients when starting treatment within a month of stroke onset. These results are probably due to the small sample size. However, some positive trends were noted on exploratory analysis.

The FMA score is a quantitative instrument measuring sensorimotor stroke recovery. Its primary value is the

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**Table 3.** FMA improvement scores per stroke severity (severe, moderate, and mild) and site of stroke (ACI, LACI, and POCI)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Neuroaid group</th>
<th>Placebo</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 20)</td>
<td>(n = 20)</td>
<td></td>
</tr>
<tr>
<td><strong>Stroke severity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 weeks improvement (ref. baseline)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>12.5 ± 16.2</td>
<td>9.9 ± 9.2</td>
<td>0.65</td>
</tr>
<tr>
<td>Moderate</td>
<td>22 ± 6.2</td>
<td>20.5 ± 13.1</td>
<td>0.92</td>
</tr>
<tr>
<td>Mild</td>
<td>1.3 ± 3.0</td>
<td>1.8 ± 1.3</td>
<td>0.77</td>
</tr>
<tr>
<td>8 weeks improvement (ref. baseline)</td>
<td>18.9 ± 21.9</td>
<td>12 ± 12.4</td>
<td>0.36</td>
</tr>
<tr>
<td>Severe</td>
<td>27.7 ± 8.0</td>
<td>22.6 ± 15.2</td>
<td>0.61</td>
</tr>
<tr>
<td>Mild</td>
<td>1.3 ± 3.8</td>
<td>3 ± 2.9</td>
<td>0.47</td>
</tr>
<tr>
<td><strong>Site of stroke</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 weeks improvement (ref. baseline)</td>
<td>11.4 ± 12.1</td>
<td>17.3 ± 12.5</td>
<td>0.24</td>
</tr>
<tr>
<td>ACI</td>
<td>5 ± 5.3</td>
<td>13.2 ± 12.0</td>
<td>0.29</td>
</tr>
<tr>
<td>LACI</td>
<td>23.8 ± 24.5</td>
<td>0.3 ± 2.9</td>
<td>0.15</td>
</tr>
<tr>
<td>POCI</td>
<td>15.6 ± 17.2</td>
<td>18.8 ± 16.1</td>
<td>0.68</td>
</tr>
<tr>
<td>8 weeks improvement (ref. baseline)</td>
<td>12.7 ± 21.2</td>
<td>15.7 ± 13.2</td>
<td>0.74</td>
</tr>
<tr>
<td>ACI</td>
<td>26.3 ± 24.1</td>
<td>1 ± 6.6</td>
<td>0.23</td>
</tr>
<tr>
<td>LACI</td>
<td>21.2 ± 24.5</td>
<td>1 ± 6.6</td>
<td>0.23</td>
</tr>
<tr>
<td>POCI</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values presented are means ± SD. ACI = Anterior circulation infarct; LACI = lacunar infarction; POCI = posterior circulation infarct.

**Table 4.** Number of subjects and respective percentages (in parentheses) in each of the groups of presented adverse events and serious adverse event during the trial

<table>
<thead>
<tr>
<th>Types and number of AES</th>
<th>Neuroaid</th>
<th>Placebo</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of AES</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td>3 (18)</td>
<td>0</td>
<td>3 (18)</td>
</tr>
<tr>
<td>Pruritic rash/pruritus</td>
<td>0</td>
<td>2 (12)</td>
<td>2 (12)</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>0</td>
<td>2 (12)</td>
<td>2 (12)</td>
</tr>
<tr>
<td>Elevated liver enzymes</td>
<td>1 (6)</td>
<td>0</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Contusion finger</td>
<td>1 (6)</td>
<td>0</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Edema foot</td>
<td>1 (6)</td>
<td>0</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>1 (6)</td>
<td>0</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Chest discomfort</td>
<td>1 (6)</td>
<td>0</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Headache</td>
<td>1 (6)</td>
<td>0</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Abdominal discomfort</td>
<td>0</td>
<td>1 (6)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Fall</td>
<td>0</td>
<td>1 (6)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Dyesthesia</td>
<td>0</td>
<td>1 (6)</td>
<td>1 (6)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Types and number of SAEs</th>
<th>Neuroaid</th>
<th>Placebo</th>
<th>All</th>
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</thead>
<tbody>
<tr>
<td>Total number of SAEs</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jaundice</td>
<td>1 (6)</td>
<td>0</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Hypokalemia</td>
<td>1 (6)</td>
<td>0</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Seizures</td>
<td>1 (6)</td>
<td>0</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Recurrent stroke</td>
<td>1 (6)</td>
<td>0</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Perianal abscess</td>
<td>0</td>
<td>1 (6)</td>
<td>1 (6)</td>
</tr>
</tbody>
</table>

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Potential Efficacy of the Traditional Chinese Medicine Neuroaid

Cerebrovasc Dis 2009;28:514–521
100-point motor domain. Based on the available evidence, the FMA motor scale is highly recommended as a clinical and research tool for assessing changes in motor impairment following stroke [12]. NIHSS and FIM scores were less appropriate outcome measures in this study focused on post-stroke motor recovery as the NIHSS is a combination of several subscores of which only 4 out of 11 are assessing sensorimotor stroke recovery; the FIM scale is an independence indicator.

Subgroup analysis of severe stroke patients showed a better recovery in the Neuroaid group compared to the placebo group, this tendency increasing at the later stage of the study. In such cases, it is also easier to distinguish the treatment effect from the natural recovery, which tends to be more rapid at first and slower later.

Subgroup analysis also showed a tendency for a better recovery in POCI patients receiving Neuroaid. However, it is difficult to draw any conclusion given the small number of patients involved (n = 7) and the imbalance of the scores at baseline.

We noted the increasing benefit of the treatment over time. Such a hypothesis is consistent with the build-up effect observed in the Neuroaid group, and with the earlier postulate that mechanisms involved in the action of Neuroaid could include neuroplasticity [8], which is time dependent. Brain rehabilitation processes are slow and it takes time to build and grow new neuronal pathways.

Furthermore, the effect of the treatment is significant when there is a potential for recovery, which is also consistent with the hypothesis of natural neuroplasticity mechanisms. Similarly, the 5 best-recovered patients in the Neuroaid group were recovering more than the 5 best-recovered patients in the placebo group, this trend also increasing over time. Thus, a longer treatment duration and longer trial period of follow-up might be more appropriate.

Additionally, the results show a very good safety profile for Neuroaid. Overall the treatment was very well tolerated and none of the adverse events were considered drug-related.

There are some study limitations. The sample size of 40 subjects was not sufficient to draw any conclusion on the efficacy of the treatment. The study itself is an exploratory analysis, with the objective of generating hypotheses for future larger trials. However, trends were observed and results provided estimates for sample size requirements to achieve statistical significance in future studies. The subjects were on average young compared to the average stroke age of 65 years [19]. The profile of the population regarding medical disorders such as hypertension and diabetes was similar to the average profile of stroke patients in Singapore [19]. The duration of the treatment and of this study was shorter versus the duration of other trials assessing the efficacy of Neuroaid after stroke [10]. Most of the trends were strengthening over time when we compared the first and second follow-up. This would suggest that a longer trial period could also be an important criterion for subsequent protocols.

**Conclusion**

Our study shows that a randomized, double-blind, placebo-controlled trial of a traditional Chinese medicine according to Good Clinical Practice guidelines is possible. Our results suggest that Neuroaid given for 4 weeks to ischemic stroke subjects starting within a month after stroke onset did not statistically significantly facilitate motor recovery. Results also showed that the treatment was safe as an add-on to standard stroke medications.

However, several positive trends could be noted on the FMA score in the Neuroaid group versus the placebo group. Subgroup analysis showed an advantage of the Neuroaid group in the case of severe stroke patients and POCI patients. The overall improvement distribution was also largely favoring the patients in the Neuroaid group with recovery potential.

Trends were increasing over time suggesting that longer treatment duration and trial period are needed to fully observe the treatment effect. Observations support earlier hypotheses of mechanisms around neuroplasticity. A large, randomized, double-blind, placebo-controlled trial on 280 severe stroke patients (power = 0.8, type I error = 0.05, intervention:control = 1:1) would enable to evaluate more definitively the efficacy of Neuroaid in enhancing post-stroke recovery.

**Acknowledgements**

This study was sponsored by Moleac Pte Ltd., Singapore, which markets Neuroaid.

Dr. N. Venketasubramanian and Dr. Christopher Chen have received a grant from the National Medical Research Council of Singapore to conduct a randomized double-blind placebo-controlled clinical trial of DJ in acute stroke.
References


Safety Profile of MLC601 (Neuroaid®) in Acute Ischemic Stroke Patients: A Singaporean Substudy of the Chinese Medicine Neuroaid Efficacy on Stroke Recovery Study

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Changi General Hospital, Singapore Clinical Research Institute, National Neuroscience Institute, National University Health System and Singapore General Hospital, Singapore

Key Words
Acute stroke • Chinese medicine, safety • Clinical trials • Stroke recovery

Abstract
Background: Previous clinical trials have shown that Neuroaid® (MLC601), a traditional Chinese medicine, shows good tolerability and superiority over another traditional Chinese medicine in terms of neurological disability and functional outcome and thus may be beneficial as part of a poststroke rehabilitation program. The safety of MLC601 on hemostasis, hematology and biochemistry has been established in normal subjects and patients with nonacute stroke over a short treatment period. We assessed the safety of Neuroaid in patients with acute stroke treated for 3 months in a substudy of an ongoing randomized placebo-controlled trial. Methods: Laboratory tests (biochemical, hematological and electrocardiogram) were conducted at the month 3 follow-up, in addition to baseline tests. A total of 114 patients were recruited. As there were 13 dropouts, a total of 52 patients on MLC601 and 49 on placebo were available for analysis. Serious adverse events (SAEs) were also analyzed. Results: There were no statistically or clinically significant differences between treatment groups in biochemical, hematological or electrocardiogram tests at month 3, nor any statistically or clinically significant differences in the absolute and relative changes of the various parameters between baseline and 3 months. SAEs were similar and were those commonly seen in stroke patients. Conclusions: Longer-term laboratory safety data show no differences between MLC601 and placebo, confirming the safety of MLC601 in acute stroke patients receiving a 3-month treatment.

The Singapore CHIMES investigators are: Changi General Hospital: Dr. Sherry H.Y. Young; National University Hospital: Dr. Bernard P.L. Chan, Dr. Vijay Kumar Sharma, Dr. Hock Luen Teoh, Dr. Raymond Seet, Dr. N. Venketasubramanian; National Neuroscience Institute, Singapore General Hospital: Dr. Hui Meng Chang; Deidre de Silva; National Neuroscience Institute, Tan Tock Seng Hospital: Dr. Rajinder Singh.
Stroke is a major cause of death and disability [1]. Neuroaid® (MLC601), previously referred to as DJ [2] or Danqi Piantan Jiaonang [3], is a traditional Chinese medicine which has been used extensively in China as a drug to facilitate recovery after stroke. It combines 9 herbal (radix astragali, radix salviae mitorrhizae, radix paeoniae rubrae, rhizoma chuanxiong, radix angelicae sinensis, Carthamus tinctorius, Prunus persica, radix polygalae and rhizoma acori tatarinowii) and 5 animal components (Hirudo, Eupolyphaga seu Stelophaga, calculus bovis artifactus, Buthus martensii and cornu saigae tataricae) [4]. The neuroproliferative and neuroprotective effect of MLC601 and hence its potential role in neuroplasticity after stroke have been recently established in animal models of stroke and ischemia [5].

A meta-analysis of traditional Chinese proprietary medicines in stroke reported few adverse events of which none were severe and only 10 trials reported any deaths [6]. However, most of these trials were not compliant with the International Conference of Harmonization/Good Clinical Practice, and such an unexpectedly low frequency of serious adverse events (SAEs) could be due to bias in the admission, selection, reporting or publication processes or from the shorter treatment period.

A pooled analysis of 2 trials of MLC601 showed good tolerability and superiority of MLC601 over another traditional Chinese medicine also approved for stroke recovery [2]. Hence, a large-scale academic multicenter randomized controlled trial, the Chinese Medicine Neuroaid Efficacy on Stroke Recovery (CHIMES) study, is testing the hypothesis that MLC601 is superior to placebo in reducing neurological deficit and improving functional outcome (modified Rankin Scale at 3 months) after acute ischemic stroke in patients with cerebral infarction with an intermediate range of severity (NIHSS between 6 and ≤14) [4] enrolled into the study within 72 h of stroke onset.

Previous studies reported no SAEs and only 2 adverse events – 2 cases of nausea and vomiting – in 405 subjects receiving MLC601 [2]. The safety of MLC601 on hemostasis, hematology and biochemistry has already been established in normal subjects and stroke patients in earlier studies [2, 3]. However, these studies were performed in patients with nonacute stroke (between 7 days and 6 months after stroke), and these patients had experienced a relatively short treatment period of 1 month.

Hence, although we anticipated few adverse events related to MLC601, given the limitations of previous studies, we aimed to assess the safety of MLC601 in acute stroke patients receiving a 3-month treatment in a sub-study of an ongoing trial performed in accordance with Good Clinical Practice guidelines.

**Methods**

**Patient Accrual**

This was a multicenter study involving 4 sites in Singapore: Changi General Hospital, National Neuroscience Institute, Tan Tock Seng Campus, National Neuroscience Institute, Singapore General Hospital Campus and National University Hospital. All sites had received local ethics approval.

In total, 114 patients (Singapore General Hospital: 3, National University Hospital: 6, Tan Tock Seng Hospital: 31, and Changi General Hospital: 74) were randomized in Singapore between November 5, 2007, and December 1, 2008. Of these 114 patients, 58 were allocated to the MLC601 group, and 56 were allocated to the placebo group. While primary and secondary outcome data were collected for all patients in this intention-to-treat trial, laboratory data for 13 patients were not available at month 3; in the MLC601 group, 3 patients were lost to follow-up, 1 patient was withdrawn by the investigators due to SAEs, and 2 patients withdrew their consent; in the placebo group, 1 patient died, 3 patients were withdrawn by the investigators due to SAEs, and 3 patients withdrew consent. Reasons for consent withdrawal were available for 4 out of 5 subjects and did not show any specific pattern. These included improvement of symptoms, hematuria, loss of trial medication and depression. This left 101 patients, 52 on MLC601 and 49 on placebo, whose laboratory data at month 3 were available for analysis.

**Tests**

Hematology, biochemistry and electrocardiogram (ECG) tests were performed at baseline and at 3 months.

Hematology tests included levels of hemoglobin, red blood cell count, white blood cell count, hematocrit, platelet count, lymphocytes, monocytes, eosinophils and basophils.

Biochemistry tests included levels of sodium, potassium, chloride, serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase, alkaline phosphatase, total bilirubin, total protein, albumin, globulin, urea, creatinine and uric acid.

Whether a result was determined to be ‘clinically significant’ was decided by the investigators based on the laboratory test values and whether this led to a change in medical management. Unblinded SAEs were also analyzed.

**Statistical Analysis**

Demographic data were summarized by descriptive statistics and presented by treatment groups. Analysis was based on the intention-to-treat principle. A 2-sample t test was used separately for each comparison of continuous laboratory tests. In case of nonnormality confirmed by the Kolmogorov-Smirnov test, the nonparametric Mann-Whitney U test was performed. For comparison of categorical outcomes, Fisher’s exact test was used. Multiple logistic regression was also carried out to adjust for baseline characteristics.
Results

Patient Demographics

Demographics of the 114 patients whose baseline data were available are presented in Table 1. The treatment groups are largely similar, and there was no difference in the baseline laboratory data of those who were lost to follow-up and those who were followed up to 3 months.

Severe Adverse Events

During the study period, there were 8 SAEs reported in 7 patients in the MLC601 group while 10 SAEs were reported in 9 patients in the placebo group. In particular, there was 1 death, 7 life-threatening events in 6 patients and 5 patients whose study treatments were permanently discontinued due to SAEs. Only 1 SAE was deemed possibly related to the trial medication by the investigators. All SAEs observed were common for stroke patients and included stroke progression, recurrent stroke and cardiac events.

Laboratory Investigations at 3 Months

Hematology Tests

Results of hematology tests are summarized and presented in Table 2. There was 1 patient in the placebo group in whom no hematology tests were performed. Based on a significance level of 0.05, there was no statistically significant difference between both groups in all the hematology tests at 3 months. Additionally, when examining the absolute and relative changes between baseline and 3 months, no statistically significant difference between the two groups was observed.

Biochemistry Tests

The results of biochemistry tests are summarized and presented in Table 3. There was 1 patient on placebo in whom no biochemistry tests were performed. In addition, there were 15 patients on MLC601 and 16 patients on placebo, in whom no chloride tests were performed, and 6 patients on MLC601 and 4 patients on placebo in whom no uric acid tests were performed. Based on a significance level of 0.05, there were no statistically significant differences between both groups on all the biochemistry tests. Additionally, when examining the absolute and relative changes between baseline and at 3 months, no statistically significant difference between the two groups was observed.

ECG Test

Results of the ECG test are summarized and presented in Table 3. There was 1 patient in the placebo group in whom no ECG test was performed. Based on a significance level of 0.05, no statistically significant difference was found between the two groups in the ECG test at 3 months, even after adjusting for baseline ECG status.

Table 1. Trial profiles and patient demographics

<table>
<thead>
<tr>
<th></th>
<th>MLC601</th>
<th>Placebo</th>
<th>p value</th>
<th>All patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of subjects</td>
<td>58</td>
<td>56</td>
<td></td>
<td>114</td>
</tr>
<tr>
<td>Incomplete follow-up at month 3</td>
<td>6</td>
<td>7</td>
<td>0.95</td>
<td>13</td>
</tr>
<tr>
<td>Died</td>
<td>0</td>
<td>1</td>
<td>0.99</td>
<td>1</td>
</tr>
<tr>
<td>Lost to follow-up</td>
<td>3</td>
<td>0</td>
<td>0.25</td>
<td>3</td>
</tr>
<tr>
<td>Withdrew by investigator due to SAE</td>
<td>1</td>
<td>3</td>
<td>0.59</td>
<td>2</td>
</tr>
<tr>
<td>Withdrew consent</td>
<td>2</td>
<td>3</td>
<td>0.97</td>
<td>7</td>
</tr>
<tr>
<td>SAEs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of SAEs</td>
<td>8</td>
<td>10</td>
<td>0.74</td>
<td>18</td>
</tr>
<tr>
<td>Number of patients</td>
<td>7</td>
<td>9</td>
<td>0.73</td>
<td>16</td>
</tr>
<tr>
<td>Mean age ± SD, years</td>
<td>60.7 ± 10.0</td>
<td>62.4 ± 11.1</td>
<td>0.39</td>
<td>61.5 ± 10.6</td>
</tr>
<tr>
<td>Gender, n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>44 (75.9%)</td>
<td>40 (71.4%)</td>
<td>0.75</td>
<td>84 (73.7%)</td>
</tr>
<tr>
<td>Female</td>
<td>14 (24.1%)</td>
<td>16 (28.6%)</td>
<td>0.75</td>
<td>30 (26.3%)</td>
</tr>
<tr>
<td>Race, n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>42 (72.4%)</td>
<td>40 (71.4%)</td>
<td>1</td>
<td>82 (71.9%)</td>
</tr>
<tr>
<td>Malay</td>
<td>7 (12.1%)</td>
<td>14 (25.0%)</td>
<td>0.12</td>
<td>21 (18.4%)</td>
</tr>
<tr>
<td>Indian</td>
<td>3 (5.2%)</td>
<td>1 (1.8%)</td>
<td>0.64</td>
<td>4 (3.5%)</td>
</tr>
<tr>
<td>Filipino</td>
<td>1 (1.7%)</td>
<td>0 (0.0%)</td>
<td>1</td>
<td>0 (0.9%)</td>
</tr>
<tr>
<td>Others</td>
<td>5 (8.6%)</td>
<td>1 (1.8%)</td>
<td>0.22</td>
<td>6 (5.3%)</td>
</tr>
</tbody>
</table>
Discussion

Longer-term laboratory safety data conducted at 3 months on 101 patients showed no statistical and clinical differences between the MLC601 and placebo groups across a range of biochemical and hematological parameters as well as ECG and SAE reports. Further analysis of the absolute and relative changes of these parameters between baseline and at 3 months showed no statistical and clinical differences between the MLC601 and placebo groups either. These results confirm the safety of MLC601 in acute stroke patients undergoing 3 months of treatment.

Safety data on MLC601 have previously been reported [2, 3]. These reports were on blood, urine and stool pa-
### Table 3. Biochemistry and ECG tests at month 3

<table>
<thead>
<tr>
<th>Test</th>
<th>MLC601 (n = 52)</th>
<th>Placebo (n = 48)</th>
<th>p value</th>
<th>All patients (n = 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sodium, mmol/l</strong></td>
<td>139.0 ± 2.3</td>
<td>138.6 ± 2.4</td>
<td>0.444</td>
<td>138.8 ± 2.3</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>1.5 ± 3.2</td>
<td>1.0 ± 2.7</td>
<td>0.624</td>
<td>1.3 ± 3.0</td>
</tr>
<tr>
<td>Percentage change</td>
<td>1.1 ± 2.4</td>
<td>0.7 ± 2.0</td>
<td>0.611</td>
<td>0.9 ± 2.2</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>1.000</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Potassium, mmol/l</td>
<td>4.0 ± 0.4</td>
<td>4.1 ± 0.4</td>
<td>0.391</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>0.1 ± 0.6</td>
<td>0.0 ± 0.5</td>
<td>0.874</td>
<td>0.0 ± 0.6</td>
</tr>
<tr>
<td>Percentage change</td>
<td>3.2 ± 16.5</td>
<td>0.9 ± 10.5</td>
<td>0.823</td>
<td>2.1 ± 13.9</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(2.0)</td>
<td>(2.1)</td>
<td>1.000</td>
<td>2 (2.0)</td>
</tr>
<tr>
<td>Chloride, mmol/l</td>
<td>104.1 ± 2.7</td>
<td>104.0 ± 2.6</td>
<td>0.971</td>
<td>104.0 ± 2.6</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>0.8 ± 3.7</td>
<td>0.6 ± 3.6</td>
<td>0.960</td>
<td>0.7 ± 3.6</td>
</tr>
<tr>
<td>Percentage change</td>
<td>0.8 ± 3.7</td>
<td>0.7 ± 3.5</td>
<td>0.970</td>
<td>0.8 ± 3.6</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>1.000</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>SGOT, U/l</td>
<td>22.4 ± 7.0</td>
<td>23.1 ± 7.1</td>
<td>0.333</td>
<td>22.7 ± 7.0</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>−2.4 ± 10.3</td>
<td>−1.6 ± 9.4</td>
<td>0.718</td>
<td>−2.0 ± 9.8</td>
</tr>
<tr>
<td>Percentage change</td>
<td>−2.6 ± 34.5</td>
<td>0.1 ± 29.5</td>
<td>0.709</td>
<td>−1.3 ± 32.0</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>1.000</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>SGPT, U/l</td>
<td>22.2 ± 11.0</td>
<td>23.4 ± 10.9</td>
<td>0.277</td>
<td>22.8 ± 10.9</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>−0.7 ± 14.1</td>
<td>1.0 ± 9.2</td>
<td>0.472</td>
<td>0.1 ± 11.9</td>
</tr>
<tr>
<td>Percentage change</td>
<td>9.2 ± 53.9</td>
<td>14.1 ± 48.2</td>
<td>0.442</td>
<td>11.6 ± 51.0</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>1.000</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Alkaline phosphatase, U/l</td>
<td>69.0 ± 17.7</td>
<td>68.4 ± 18.4</td>
<td>0.863</td>
<td>68.7 ± 17.9</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>−3.3 ± 15.6</td>
<td>−1.5 ± 14.2</td>
<td>0.559</td>
<td>−2.4 ± 14.9</td>
</tr>
<tr>
<td>Percentage change</td>
<td>0.1 ± 24.6</td>
<td>−0.2 ± 22.7</td>
<td>0.956</td>
<td>−0.1 ± 23.6</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>1.000</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Serum bilirubin (total), μmol/l</td>
<td>13.5 ± 3.9</td>
<td>15.0 ± 5.6</td>
<td>0.180</td>
<td>14.2 ± 4.8</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>−4.8 ± 6.5</td>
<td>−3.7 ± 6.2</td>
<td>0.645</td>
<td>−4.2 ± 6.3</td>
</tr>
<tr>
<td>Percentage change</td>
<td>−20.0 ± 26.2</td>
<td>−11.4 ± 35.2</td>
<td>0.439</td>
<td>−15.7 ± 31.1</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>1.000</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Serum protein (total), g/l</td>
<td>67.1 ± 4.1</td>
<td>68.1 ± 4.7</td>
<td>0.252</td>
<td>67.6 ± 4.4</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>−0.4 ± 4.7</td>
<td>−0.8 ± 6.1</td>
<td>0.759</td>
<td>−0.6 ± 5.4</td>
</tr>
<tr>
<td>Percentage change</td>
<td>−0.2 ± 6.8</td>
<td>−0.5 ± 9.0</td>
<td>0.885</td>
<td>−0.4 ± 7.9</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>1.000</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Serum albumin, g/l</td>
<td>38.0 ± 3.6</td>
<td>38.4 ± 3.3</td>
<td>0.589</td>
<td>38.2 ± 3.4</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>1.1 ± 3.1</td>
<td>1.0 ± 3.3</td>
<td>0.977</td>
<td>1.1 ± 3.2</td>
</tr>
<tr>
<td>Percentage change</td>
<td>3.2 ± 8.6</td>
<td>3.4 ± 9.4</td>
<td>0.924</td>
<td>3.3 ± 8.9</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>1.000</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Serum globulin, g/l</td>
<td>29.2 ± 4.0</td>
<td>29.9 ± 3.7</td>
<td>0.224</td>
<td>29.5 ± 3.9</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>−1.3 ± 3.6</td>
<td>−1.4 ± 4.8</td>
<td>0.432</td>
<td>−1.4 ± 4.2</td>
</tr>
<tr>
<td>Percentage change</td>
<td>−3.4 ± 9.5</td>
<td>−2.5 ± 17.8</td>
<td>0.477</td>
<td>−3.0 ± 14.2</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>1.000</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Blood urea, mmol/l</td>
<td>4.4 ± 1.6</td>
<td>4.5 ± 1.3</td>
<td>0.488</td>
<td>4.5 ± 1.5</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>−0.1 ± 1.8</td>
<td>−0.6 ± 1.6</td>
<td>0.241</td>
<td>−0.4 ± 1.7</td>
</tr>
<tr>
<td>Percentage change</td>
<td>3.0 ± 38.9</td>
<td>−6.6 ± 27.1</td>
<td>0.282</td>
<td>−1.6 ± 33.9</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>1.000</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Serum creatinine, μmol/l</td>
<td>87.4 ± 21.3</td>
<td>83.8 ± 22.0</td>
<td>0.394</td>
<td>85.7 ± 21.6</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>2.3 ± 17.9</td>
<td>−1.5 ± 11.3</td>
<td>0.317</td>
<td>0.5 ± 15.1</td>
</tr>
<tr>
<td>Percentage change</td>
<td>20.5 ± 129.6</td>
<td>−0.6 ± 12.9</td>
<td>0.293</td>
<td>10.4 ± 94.0</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>(2.0)</td>
<td>(0.0)</td>
<td>1.000</td>
<td>(1.0)</td>
</tr>
<tr>
<td>Blood glucose (random), mmol/l</td>
<td>7.1 ± 2.3</td>
<td>7.3 ± 3.0</td>
<td>0.904</td>
<td>7.2 ± 2.7</td>
</tr>
<tr>
<td>Change from baseline</td>
<td>−1.0 ± 4.2</td>
<td>−1.4 ± 4.2</td>
<td>0.521</td>
<td>−1.2 ± 4.1</td>
</tr>
<tr>
<td>Percentage change</td>
<td>−1.7 ± 42.8</td>
<td>−4.0 ± 30.5</td>
<td>0.795</td>
<td>−2.9 ± 36.9</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>2 (3.9)</td>
<td>5 (10.4)</td>
<td>0.256</td>
<td>7 (7.0)</td>
</tr>
<tr>
<td>Serum uric acid, μmol/l</td>
<td>356.5 ± 96.5</td>
<td>352.9 ± 76.2</td>
<td>0.978</td>
<td>354.8 ± 86.7</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>2 (4.4)</td>
<td>2 (4.8)</td>
<td>1.000</td>
<td>4 (4.4)</td>
</tr>
<tr>
<td>ECG test Abnormal, n</td>
<td>20 (38.5)</td>
<td>24 (50.0)</td>
<td>0.246</td>
<td>44 (44.0)</td>
</tr>
<tr>
<td>Clinically significant, n</td>
<td>2 (3.9)</td>
<td>2 (4.2)</td>
<td>1.000</td>
<td>4 (4.0)</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate percentages. Change: data at month 3 – data at baseline; percentage change: (data at month 3 – data at baseline) × 100/data at baseline. SGOT = Serum glutamic-oxaloacetic transaminase; SGPT = serum glutamic-pyruvic transaminase. Change analysis was not performed for serum uric acid as it was not conducted at baseline for most of the patients.

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**Safety Profile of MLC601 (Neuroaid®)**

Cerebrovasc Dis 679
rameters, liver and renal functions and ECG. However, in the earliest study [2], the results were based on a shorter treatment and assessment period (1 vs. 3 months) and in less acute stroke patients (from 2 weeks to 6 months vs. within 48 h of the stroke onset). In a later study [3], the results were observed only for a shorter treatment and assessment period (1 vs. 3 months), in less acute stroke patients (within 7 days vs. within 2 days of the stroke onset) and in a smaller cohort (10 patients vs. 100 patients). Our present results confirm the safety profile of MLC601 observed in those initial reports.

This is a planned substudy of the main CHIMES trial, and the results from this planned analysis support the decision not to have mandatory laboratory safety tests during study follow-up in the main trial protocol. There will be further unblinded analysis of safety events conducted by the CHIMES Data Safety Monitoring Review Board.

Acknowledgements

We thank all our colleagues for their hard work and cooperation in the CHIMES trial. The Singapore CHIMES Investigators received a grant from the National Medical Research Council of Singapore to conduct the CHIMES trial, a randomized double-blind placebo-controlled clinical trial of MLC601 in acute stroke.

References

Danqi Piantan Jiaonang Does Not Modify Hemostasis, Hematology, and Biochemistry in Normal Subjects and Stroke Patients

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Key Words
Danqi Piantan Jiaonang, stroke • Traditional Chinese medicine • Safety studies

Abstract
Background and Objective: Previous studies on Danqi Piantan Jiaonang (DPJ, NeuroAid®, a traditional Chinese medicine, in stroke patients showed promising results. Our aim was to determine the safety of DPJ in normal subjects and stroke patients through a series of studies assessing its immediate and long-term effects, alone and in combination with aspirin, on hematological, hemostatic, and biochemical parameters. Methods: We conducted 3 studies from December 2004 to May 2006. Study 1 was a case series which recruited 32 healthy volunteers who were given 2 oral doses of 4 DPJ capsules (0.4 g/capsule) 6 h apart. Study 2 was a randomized controlled trial of 22 healthy volunteers who received either 1 oral dose of aspirin 300 mg alone or a combination of 1 dose of aspirin 300 mg and 2 doses of 4 DPJ capsules taken 6 h apart. For both studies 1 and 2, hemostatic parameters (prothrombin time, activated partial thromboplastin time, fibrinogen, platelet aggregation, D-dimer) were tested at baseline, and after 2 and 8 h. Study 3 was a case series which recruited 10 patients with recent ischemic stroke (within 7 days) who were given 4 DPJ capsules taken orally 3 times a day for 1 month. Blood tests for hemostatic, hematological (complete blood count), and biochemical parameters (glucose, creatinine, alanine aminotransferase, aspartate transaminase, C-reactive protein) were performed at baseline, and after 1 and 4 weeks. Results: Apart from the expected changes in platelet aggregation in subjects taking aspirin, no significant differences were detected in hemostatic parameters at baseline, and 2 and 8 h after oral intake of DPJ alone or in combination with aspirin. Likewise, no significant differences were observed in hematological, hemostatic, and biochemical parameters at baseline, and after 1 and 4 weeks of oral intake of DPJ. Conclusion: DPJ does not significantly modify hematological, hemostatic, and biochemical parameters in normal subjects and stroke patients.

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Stroke is the third leading cause of death worldwide and a major cause of morbidity [1]. Although prevention is the most effective way to decrease the burden of stroke [2], acute stroke treatment aims at reducing mortality and disability. So far, only 3 therapeutic measures have demonstrated efficacy in reducing disability and/or mortality in randomized clinical trials, namely intravenous recombinant tissue plasminogen activator [3], which can be used in only <5% of patients with ischemic stroke, aspirin [4], which is less effective but applicable to most ischemic stroke patients, and stroke unit care [5], which can be of benefit to all stroke patients.

While neuroprotective substances have overwhelmingly shown promise in laboratory studies, none has been found to be beneficial in clinical trials [6]. Traditional Chinese medicines (TCM), therefore, provide an attractive opportunity for exploration. Over 100 TCM products are currently used clinically for stroke in China with the approval of the Chinese National Drug Administration. Danqi Piantan Jiaonang (DPJ) is registered with the Sino-FDA for 'stroke recovery' on the basis of the results of clinical studies that included more than 600 stroke patients between 2 weeks and 6 months of stroke onset [unpubl. data].

Before embarking on an acute ischemic stroke clinical trial performed according to international standards, we assessed the immediate and long-term effects of DPJ, alone and in combination with aspirin, through a series of studies on various hemostatic, hematological, and biochemical parameters among normal subjects and stroke patients.

**Methods**

A series of 3 related studies were conducted from December 2004 to May 2006 in the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine.

**Subjects**

Normal healthy volunteers were recruited who were between 21 and 65 years old, willing to be on a fat-restricted diet during the study, had no history of easy bruising or blood coagulation disorder, and had not taken aspirin, anticoagulants, antiplatelet medication, any investigational drug, or TCM within 1 month prior to participation. Since these were early-phase studies, age was capped at 65 years to avoid inclusion of older subjects with unrecognized underlying medical conditions that may put them at higher risk of complications from study procedures and medications. Women should not be pregnant, lactating or nursing. Intake of TCM other than the study drug was not allowed during the study.

Patients with ischemic stroke were recruited who were 18 years old or older, presented within the first week of stroke onset, had a computed tomography scan or magnetic resonance imaging compatible with cerebral infarction and no evidence of intracranial hemorrhage, had no history of easy bruising or blood coagulation disorder, had not received thrombolysis, and had not taken TCM or any investigational drug within 3 months prior to participation. Women should not be pregnant, lactating or nursing.

**Study Drug**

DPJ or NeuroAid® administered in all 3 studies was supplied, packaged and distributed by Tianjin Shitian Pharmaceutical Industry Co., Ltd. and labeled according to the requirements of local laws and regulations. It has been registered with the Sino-FDA since August 2001 for the treatment of stroke recovery, is approved as a Chinese proprietary medicine in Singapore, and approved for distribution by the Bureau of Food and Drugs in the Philippines. Each capsule combines 10 herbal components [i.e. root of membranous milk vetch, red sage root, red peony root, rhizome of Ligusticum chuanxiong, rhizome and root of Panax notoginseng, bark of subshrubbery peony (cortex moutan), wood of odorous rosewood, Uncaria gambir plant stem with hooks, root of thinleaf milkwort, rhizome of grassleaf sweetflag] and 4 animal components (i.e. Hirudo nipponica Whitman, Eupolyphaga or Steleophaga, Buthus martensi Karsch, calculus bovis artifactus). The dose of DPJ as approved by the Sino-FDA is 4 capsules 3 times a day and is the dose used in these studies.

Aspirin was supplied by Tianjin Hospital or by Tianjin Shitian Pharmaceutical Industry Co., Ltd.

**Laboratory Procedures**

Subjects were asked to remain comfortably seated or lying down for at least 30 min before each blood sample was collected by venipuncture. All clinical laboratory evaluations were conducted under the supervision of Prof. Jiao Lianting and in accordance with the standards of the Biology Laboratory at the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine.

Testing of hemostatic parameters included the Quick prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, platelet aggregation, and D-dimer. Platelet aggregation studies were performed by the turbidimetric method (LBY NJ4 platelet aggregometer, PRECIL, Beijing Pu-Li-sheng Corporation) using 16.5 μmol/l ADP as agonist (concentration 300 μmol/l; 11 μl added in 200 μl specimen). Testing of hematological and biochemical parameters included complete blood count, creatinine, alanine aminotransferase, aspartate transaminase, fasting glucose, and C-reactive protein.

**Study 1**

Healthy volunteers (n = 32) received 2 oral doses of 4 DPJ capsules (0.4 g/capsule) taken 6 h apart. Blood samples for testing of hemostatic parameters were taken at baseline (before intake of the first dose of DPJ), 2 h after the first dose of DPJ, and 8 h after the first dose of DPJ (or 2 h after the second dose of DPJ).

**Study 2**

Healthy volunteers (n = 22) were randomized to receive either 1 oral dose of aspirin 300 mg alone or a combination of 1 oral dose of aspirin 300 mg and 2 oral doses of 4 DPJ capsules (0.4 g/capsule)
Parameters.

Clinically significantly decrease the value of these coagulation parameters. For D-dimer, this would be strong evidence that DPJ did not prolong the clotting times. Likewise, if the low limits of the 95% CI of the mean difference were greater than 1 s for PT and 2 s for aPTT, this would be strong evidence that DPJ did not clinically significantly prolong the clotting times. Likewise, if the lower limits of the 95% CI of the mean difference were greater than –0.2 g/l for fibrinogen, –10% for platelet aggregation and –0.5 mg/l for D-dimer, this would be strong evidence that DPJ did not clinically significantly decrease the value of these coagulation parameters.

Study 3

Patients with ischemic stroke (n = 10) received 4 DPJ capsules (0.4 g/capsule) taken orally 3 times a day for 1 month. Intake of aspirin and other standard medications for associated medical conditions, such as diabetes mellitus, hypertension, hypercholesterolemia, and ischemic heart disease, was allowed. Other traditional Chinese medications were not allowed. Blood samples for testing of hemostatic, hematological, and biochemical parameters were taken at baseline (before intake of the first dose of DPJ), and 1 and 4 weeks after initiation of DPJ.

Adverse events were monitored for and recorded in all 3 studies. Adverse event was defined as any untoward, unfavorable, or unintended medical occurrence, signs, symptoms, or disease observed during the course of each study that may or may not necessarily have a causal relationship with the treatment being investigated.

Ethical Considerations

All 3 study protocols were approved by the Institutional Review Board of the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine. Informed consent was obtained from all participants and the studies were conducted in accordance with the Declaration of Helsinki (October 2000), the applicable guidelines for good clinical practice (ICH-GCP), or the applicable laws and regulations of China.

Statistical Analyses

Descriptive analyses were expressed as means, standard deviations, and/or ranges. The means of continuous outcomes between groups were compared using the t test/paired t test and ANOVA/repeated-measures ANOVA were used and 95% confidence interval (CI) estimates calculated for the difference in means. To specifically test the hypothesis that DPJ does not worsen the coagulation parameters by a clinically significant amount, we a priori defined the following thresholds: PT not to be extended by more than 1 s, aPTT not to be extended by more than 2 s, fibrinogen not to be decreased by more than 0.2 g/l, platelet aggregation not to be decreased by more than 10%, and D-dimer not to be decreased by more than 0.5 mg/l. Thus if the upper limits of the 95% CI of the mean difference were less than 1 s for PT and 2 s for aPTT, this would be strong evidence that DPJ did not clinically significantly prolong the clotting times. Likewise if the lower limits of the 95% CI of the mean difference were greater than –0.2 g/l for fibrinogen, –10% for platelet aggregation and –0.5 mg/l for D-dimer, this would be strong evidence that DPJ did not clinically significantly decrease the value of these coagulation parameters.

Results

Study 1

Among the 32 subjects (13 women and 19 men; mean age 35 years, range 21–65 years), 1 (subject 14) was withdrawn due to abnormal baseline blood test results. Among the remaining 31 subjects, no significant differences were observed in the hemostatic parameters tested 2 h after the first dose of DPJ and 8 h after intake of DPJ (or 2 h after the second dose of DPJ) compared to baseline (table 1). No adverse events were observed.

Study 2

Among the 22 subjects (12 women and 10 men), 11 received aspirin alone (mean age 37 years, range 24–55 years) while the other 11 received aspirin + DPJ (mean age 31 years, range 24–49 years).

As expected, mean platelet aggregation was gradually reduced over time since both groups received aspirin. However, no significant differences were observed in the other hemostatic parameters tested 2 and 8 h after the first dose of DPJ (or 2 h after the second dose of DPJ) compared to baseline, and between the aspirin and aspirin + DPJ groups in all laboratory parameters at every time point (table 2). No adverse events were observed.

Study 3

Ten patients (6 women and 4 men; mean age 65 years, range 45–85 years) were recruited at an average of 3 days after ischemic stroke onset. One woman (patient 6) suffered a recurrent stroke within the first week of the study and did not complete the protocol.

Concomitant medications taken by patients included aspirin in 6 patients, nitrate in 6, antihypertensive medication in 7, oral hypoglycemic agent in 2, fibrate in 1, anticonvulsant in 1, and potassium supplement in 1.

Mean platelet aggregation was reduced over time as expected in patients receiving aspirin. No significant differences were observed in hemostatic, hematological, and biochemical parameters tested 1 and 4 weeks after initiation of DPJ compared to baseline (tables 3, 4). No other adverse events were observed.

Discussion

In our series of safety studies, we showed that intake of DPJ does not affect hemostasis, hematological, and biochemical parameters in normal subjects and stroke patients. These results will be reassuring and helpful.
when designing future randomized clinical trials on the role of DPJ in acute stroke and stroke recovery.

Intravenous thrombolytic for acute ischemic stroke can be given to only less than 5% of stroke patients because of the short therapeutic time window of 3 h and the increased risk of bleeding [7]. Aspirin, on the other hand, can be given within 48 h of onset to many more patients with acute ischemic stroke but has much less efficacy [4]. These limitations in the utility of established treatments for acute stroke often lead to calls for an intensive search for other modes of intervention to improve stroke recovery and reduce mortality. The concept of neuroprotection as a therapeutic strategy has been of much interest to researchers in recent years. However, despite thousands of substances that have shown promise in the laboratory, not a single one of more than a hundred clinical trials was able to confirm a beneficial effect [6], making the search for other effective acute stroke treatments even more urgent.

Because of the wide use and experience in China, TCM have the potential to fill this gap in stroke treatment. However, the use of TCM is particularly challenging for clinicians trained in 'western medicine' because of unfamiliarity with the treatment principles in traditional medicine and the lack of adequate evidence from safety and efficacy studies of good standard deemed acceptable to mainstream practitioners.

Wu et al. [8] systematically reviewed 59 traditional Chinese patent medicines for ischemic stroke, of which only 22 have clinical trials eligible for review. The trials were mostly of poor methodological quality, but 8 drugs (milk vetch, Mailuoning, Ginkgo biloba, ligustrazine, danshen agents, xuesetong, puerarin, and Acanthopanax) were recommended as further research priorities. The TCM drugs chosen for further clinical development, however, must first be tested for efficacy and safety in preclinical and early clinical phase research [9].

DPJ is an agent that contains milk vetch (huangqi) and red sage root (danshen). It is widely prescribed to stroke patients in China. Information on how DPJ was initially developed and the exact rationale behind the inclusion of extracts and components from 10 herbal sources and 4 animals is unclear. However, danshen is among the most popular medicinal herbs used in China to which huangqi has eventually been added in some preparations to improve its purported efficacy [10]. A few unpublished earlier animal studies on DPJ conducted in China and made available to the authors hinted at a possible neuroprotective mechanism when enteral administration of high doses of DPJ in rats and gerbils 2 h before middle cerebral artery occlusion resulted in a smaller infarct size and significant improvement in behavioral disorder from stroke.

Table 1. Study 1 hemostatic blood test results at baseline, 2 h after the first dose, and 8 h after the first dose (2 h after the second dose) of DPJ

<table>
<thead>
<tr>
<th>Tests</th>
<th>Baseline results</th>
<th>At 2 h Mean change</th>
<th>Clinically worse</th>
<th>At 8 h Mean change</th>
<th>Clinically worse</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT, s</td>
<td>12.8 ± 0.7 (11.2–14.1)</td>
<td>12.7 ± 0.7 (11.2–14.0)</td>
<td>-0.05 (-0.19 to 0.10)</td>
<td>no</td>
<td>12.9 ± 0.7 (11.6–14.5)</td>
</tr>
<tr>
<td>aPTT, s</td>
<td>37.6 ± 4.3 (31.2–46.9)</td>
<td>37.2 ± 4.4 (30.2–46.6)</td>
<td>-0.49 (-1.09 to 0.10)</td>
<td>no</td>
<td>38.7 ± 4.3 (32.2–47.2)</td>
</tr>
<tr>
<td>Fibrinogen, g/l</td>
<td>2.98 ± 0.61 (1.79–4.22)</td>
<td>3.05 ± 0.59 (1.92–4.19)</td>
<td>0.07 (-0.02 to 0.16)</td>
<td>no</td>
<td>3.06 ± 0.57 (2.11–4.19)</td>
</tr>
<tr>
<td>Platelet aggregation, %</td>
<td>63.0 ± 15.5 (29.8–85.0)</td>
<td>62.2 ± 12.1 (39.1–79.2)</td>
<td>-0.82 (-6.9 to 5.2)</td>
<td>no</td>
<td>61.5 ± 13.4 (37.4–84.7)</td>
</tr>
<tr>
<td>D-dimer, mg/l</td>
<td>0.05 ± 0.05 (0–0.1)</td>
<td>0.15 ± 0.44 (0–2.5)</td>
<td>0.10 (-0.05 to 0.26)</td>
<td>no</td>
<td>0.07 ± 0.05 (0–0.2)</td>
</tr>
</tbody>
</table>

Values presented are means ± SD, with ranges in parentheses (n = 31). Mean changes at 2 and 8 h are from baseline with 95% CIs. The assessment of nonclinically significant changes at both time points was based on the following thresholds: PT not extended by more than 1 s, aPTT not extended by more than 2 s, fibrinogen not decreased by more than 0.2 g/l, platelet aggregation not decreased by more than 10%, D-dimer not decreased by more than 0.5 mg/l.
Toxicity studies on rats fed up to 18 g/kg/day of DPJ for 3 months showed no effects on hematology, biochemistry (hepatic and renal), and histopathology. Acute toxicity studies on mice (given 80 g/kg DPJ) and rats (given 30 g/kg DPJ) resulted in no death within 7 days and only transient reduction in animal activity. An LD$_{50}$ study conducted at the Department of Science and Technology (Philippines) showed no deaths in mice even after administration of 45 g/kg of DPJ, above which the maximum limit a mouse can normally take would be exceeded and results would be inaccurate since death may occur due to bloating. Toxidrome observed included increased motor activity, defecation, and grooming followed by decreased motor activity and respiratory rate, urination, and excretion of sample. No other adverse/abnormal signs or death occurred within the 14 days of observation.

Currently, an estimated half a million stroke patients, mostly in China, have received DPJ with reportedly promising outcomes and excellent tolerance [unpubl. data]. We, therefore, reckon that DPJ may be a valuable agent to assess for safety, and eventually test for efficacy in a well-designed randomized trial.

Most currently available stroke treatments in the market are for secondary prevention of recurrent vascular events. Antiplatelet agents reduce the risk of a myocardial infarction, stroke, or vascular death by about 23% [11]. Aspirin by far is the most widely used because of its low cost and significant benefit from a public health point of view.

### Table 2. Study 2 hemostatic blood test results at baseline, 2 h, and 8 h by treatment group

<table>
<thead>
<tr>
<th>Tests</th>
<th>Aspirin group (n = 11)</th>
<th>Aspirin + DPJ group (n = 11)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT, s</td>
<td>baseline 12.9 ± 0.3 (12.4–13.4)</td>
<td>12.7 ± 0.4 (12.2–13.7)</td>
<td>0.14</td>
</tr>
<tr>
<td>2 h</td>
<td>mean change -0.26 (−0.44 to −0.09)</td>
<td>-0.05 (−0.22 to 0.13)</td>
<td>0.08</td>
</tr>
<tr>
<td>8 h</td>
<td>mean change -0.47 (−0.75 to −0.20)</td>
<td>-0.27 (−0.53 to −0.02)</td>
<td>0.24</td>
</tr>
<tr>
<td>aPTT, s</td>
<td>baseline 38.8 ± 3.4 (32.9–47.2)</td>
<td>37.1 ± 4.0 (30.7–44.1)</td>
<td>0.18</td>
</tr>
<tr>
<td>2 h</td>
<td>mean change 39.0 ± 2.6 (33.3–42.4)</td>
<td>38.2 ± 3.5 (32.7–44.6)</td>
<td>0.36</td>
</tr>
<tr>
<td>8 h</td>
<td>mean change 0.19 (−1.16 to 1.54)</td>
<td>1.06 (−0.20 to 2.32)</td>
<td>0.15</td>
</tr>
<tr>
<td>Fibrinogen, g/l</td>
<td>baseline 3.17 ± 0.70 (2.15–4.82)</td>
<td>3.20 ± 0.59 (2.04–4.07)</td>
<td>0.65</td>
</tr>
<tr>
<td>2 h</td>
<td>mean change 3.17 ± 0.77 (2.29–5.04)</td>
<td>3.04 ± 0.66 (1.86–3.94)</td>
<td>0.92</td>
</tr>
<tr>
<td>8 h</td>
<td>mean change 0 (−1.13 to 0.11)</td>
<td>−0.17 (−0.30 to −0.03)</td>
<td>0.13</td>
</tr>
<tr>
<td>Platelet aggregation, %</td>
<td>baseline 62.8 ± 10.0 (51.2–81.3)</td>
<td>61.8 ± 9.8 (46.6–73.3)</td>
<td>0.77</td>
</tr>
<tr>
<td>2 h</td>
<td>mean change 62.9 ± 9.0 (49.2–82.6)</td>
<td>58.3 ± 11.2 (39.0–75.6)</td>
<td>0.31</td>
</tr>
<tr>
<td>8 h</td>
<td>mean change 0.06 (−8.77 to 8.88)</td>
<td>−3.55 (−12.91 to 5.82)</td>
<td>0.61</td>
</tr>
<tr>
<td>D-dimer, mg/l</td>
<td>baseline 0.16 ± 0.13 (0.02–0.46)</td>
<td>0.13 ± 0.06 (0.04–0.23)</td>
<td>0.95</td>
</tr>
<tr>
<td>2 h</td>
<td>mean change 0.16 ± 0.13 (0.03–0.43)</td>
<td>0.09 ± 0.07 (0.02–0.24)</td>
<td>0.27</td>
</tr>
<tr>
<td>8 h</td>
<td>mean change 0 (−0.03 to 0.03)</td>
<td>−0.04 (−0.07 to 0.00)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Values presented are means ± SD, with ranges in parentheses. Mean changes at 2 and 8 h are from baseline with 95% CIs. p values were derived using the Mann-Whitney U test comparing means and mean changes between the aspirin group and aspirin + DPJ group at each time point.
To test if DPJ has any effect on clotting and coagulation in humans to explain its reported apparent benefit in stroke and being cognizant of the fact that ischemic strokes have a risk of hemorrhagic conversion, we tested oral DPJ for its effect on hemostatic parameters and found no such effect in our subjects. Furthermore, as DPJ would be given as an add-on treatment if proven beneficial, we tested it in combination with aspirin and confirmed that it does not potentiate the effect of aspirin on hemostatic parameters and thereby may not increase the risk of bleeding beyond that attributable to aspirin. We also found that long-term multiple-dose intake of DPJ, which is how it is prescribed in China, does not cause hematological or biochemical adverse effects in our study patients.

While many of the TCM for stroke are allegedly effective because they improve blood circulation and reduce stasis, our findings suggest that DPJ may work by mecha-

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**Table 3.** Study 3 hemostatic blood test results at baseline, after 1 week of treatment, and after 1 month of treatment with DPJ

<table>
<thead>
<tr>
<th>Laboratory test</th>
<th>Baseline results</th>
<th>At 1 week results</th>
<th>At 1 month results</th>
<th>clinically worse</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT, s</td>
<td>13.0 (11.6–14.3)</td>
<td>12.5 (11.1–14.4)</td>
<td>12.4 (11.5–13.7)</td>
<td>no</td>
</tr>
<tr>
<td>aPTT, s</td>
<td>31.0 (27.1–33.5)</td>
<td>32.1 (28.6–38.2)</td>
<td>30.7 (28.5–34.7)</td>
<td>no</td>
</tr>
<tr>
<td>Fibrinogen, g/l</td>
<td>4.10 (2.52–5.77)</td>
<td>4.03 (3.01–6.76)</td>
<td>4.06 (3.11–6.71)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Platelet aggregation, %</td>
<td>63.6 (51.2–77.2)</td>
<td>52.3 (37.5–70.2)</td>
<td>48.4 (33.1–71.9)</td>
<td>n.s.</td>
</tr>
<tr>
<td>D-dimer, mg/l</td>
<td>0.25 (0.08–0.65)</td>
<td>0.45 (0.10–1.80)</td>
<td>0.49 (0.10–2.38)</td>
<td>no</td>
</tr>
</tbody>
</table>

Values presented are means with ranges in parentheses (n = 9). Mean changes at 1 week and 1 month are from baseline with 95% CIs. The assessment of nonclinically significant changes at both time points was based on the same thresholds as used in table 1.

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**Table 4.** Study 3 hematological and biochemical blood test results at baseline, after 1 week of treatment, and after 1 month of treatment with DPJ

<table>
<thead>
<tr>
<th>Laboratory test</th>
<th>Normal values</th>
<th>Baseline</th>
<th>At 1 week</th>
<th>At 1 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells (× 10^12)</td>
<td>4.2–5.9</td>
<td>4.42 (3.48–5.14)</td>
<td>4.51 (3.73–5.31)</td>
<td>4.56 (3.70–5.36)</td>
</tr>
<tr>
<td>Mean red blood cell volume, fl</td>
<td>86–98</td>
<td>90.7 (83.6–99.2)</td>
<td>90.9 (83.3–97.9)</td>
<td>90.1 (83.2–100.0)</td>
</tr>
<tr>
<td>White blood cells (× 10^9)</td>
<td>4.3–10.8</td>
<td>6.6 (3.7–9.4)</td>
<td>5.9 (3.7–9.4)</td>
<td>6.7 (3.5–12.8)</td>
</tr>
<tr>
<td>Hemoglobin, g/l</td>
<td>120–180</td>
<td>139.6 (109–163)</td>
<td>142.9 (120–167)</td>
<td>143.7 (116–162)</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>37–48 (female)</td>
<td>40.0 (31.0–46.1)</td>
<td>41.7 (33.5–47.3)</td>
<td>41.0 (33.5–45.0)</td>
</tr>
<tr>
<td>Platelets count (× 10^3)</td>
<td>150–450</td>
<td>186.0 (135–246)</td>
<td>214.9 (174–271)</td>
<td>196.4 (135–250)</td>
</tr>
<tr>
<td>Creatinine, μmol/l</td>
<td>70–150</td>
<td>70.6 (43–100)</td>
<td>75.6 (51–105)</td>
<td>67.9 (44–89)</td>
</tr>
<tr>
<td>SGPT-ALT, IU/l</td>
<td>4–46</td>
<td>13.9 (8.9–20.3)</td>
<td>20.8 (8.1–38.7)</td>
<td>17.8 (9.7–33.4)</td>
</tr>
<tr>
<td>SGOT-AST, IU/l</td>
<td>5–40</td>
<td>19.1 (12.4–44.0)</td>
<td>18.1 (11.1–32.0)</td>
<td>16.1 (11.3–23.4)</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4–6</td>
<td>5.88 (3.93–10.14)</td>
<td>5.21 (3.59–8.49)</td>
<td>4.91 (3.53–6.83)</td>
</tr>
<tr>
<td>C-reactive protein, mg/dl</td>
<td>&lt;1</td>
<td>2.04 (0.14–6.42)</td>
<td>1.90 (0.10–11.10)</td>
<td>1.06 (0.10–5.44)</td>
</tr>
</tbody>
</table>

Values presented are means with ranges in parentheses (n = 9).
nisms other than by its mere effect on platelets and coagulation. If indeed DPJ is effective in improving stroke recovery, it may be reasonable to likewise investigate its role in neuronal protection and plasticity.

The small sample size and inclusion of only subjects of Asian origin are the main limitations to these preliminary safety studies on DPJ. A future large multicenter study will help address these limitations. Nonetheless, in these studies conducted on normal subjects and stroke patients, albeit small, we have demonstrated that short-and long-term intake of DPJ (NeuroAid®) does not significantly modify hemostasis, hematological, and biochemical parameters.

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References