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2-Methoxystypandrone ameliorates brain function through preserving BBB integrity and promoting neurogenesis in mice with acute ischemic stroke

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ABSTRACT

2-Methoxystypandrone (2-MS), a naphthoquinone, has been shown to display an immunomodulatory effect in a cellular model. To explore whether 2-MS could protect mice against cerebral ischemic/reperfusion (I/R)-induced brain injury, we evaluated 2-MS's protective effects on an acute ischemic stroke by inducing a middle cerebral artery occlusion/reperfusion (MCAO) injury in murine model. Treatment of mice that have undergone I/R injury with 2-MS (10–100 μg/kg, i.v.) at 2 h after MCAO enhanced survival rate and ameliorated neurological deficits, brain infarction, neural dysfunction and massive oxidative stress, due to an enormous production of free radicals and breakdown of blood–brain barrier (BBB) by I/R injury; this primarily occurred with extensive infiltration of CD11b-positive inflammatory cells and upexpression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 and p65 nuclear factor-kappa B (NF-κB). All of these pathological changes were diminished by 2-MS; 2-MS also intensively limited cortical infarction and promoted upexpression of neurodevelopmental genes near peri-infarct cortex and endogenous neurogenesis near subgranular zone of hippocampal dentate gyrus and the subventricular zone, most possibly by inactivation of GSK3β which in turn upregulating β-catenin, Bcl-2 adam11 and adamts20. We conclude that 2-MS blocks inflammatory responses by impairing NF-κB signaling to limit the inflammation and oxidative stress for preservation of BBB integrity; 2-MS also concomitantly promotes neurodevelopmental protein expression and endogenous neurogenesis through inactivation of GSK3β to enhance β-catenin signaling for upexpression of neuroprotective genes and proteins.

1. Introduction

Ischemic stroke occurs when the supply of oxygen and blood to the brain have been blocked, usually as a result of plaque or fatty deposits. Without proper medical treatments, around millions of neuronal cells in the brain can die quickly as a consequence of excitotoxicity-mediated brain injury due to excessive glutamate release that excites neurons to death *via* induced overproduction of free radicals and massive inflammation generated from recruited leukocytes and activated microglial cells [1,2]. Moreover, the acute inflammation and oxidative stress that accompany early stages of stroke can result in the activation of detrimental transcription factors (*e.g.*, nuclear factor kappa-B (NF-κB)) to disrupt the blood–brain barrier (BBB), a physical barrier within the brain providing the protection and regulation of homeostasis [3,4]. Although thousands of studies have revealed the pathophysiology of cerebral ischemia on the cellular, molecular and animal levels [5], the thrombolytic recombinant tissue plasminogen activator

Abbreviations: 2-MS, 2-methoxystypandrone; BBB, blood–brain barrier; Bcl-2, B-cell lymphoma 2; COX-2, cyclooxygenase-2; DCX, doublecortin; GSK3, glycogen synthase kinase 3; iNOS, inducible nitric oxide synthase; I/R, ischemia reperfusion; MCAO, middle cerebral artery occlusion/reperfusion; NF-κB, nuclear factor-kappa B; SGZ, subgranular zone; SVZ, subventricular zone.

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(rt-PA) has remained the only FDA-approved drug in the clinic; but the application of rt-PA is applied to only a limited group of patients with acute ischemic stroke due to serious side effects and very narrow therapeutic time window [6]. Therefore, it should be a practicable option to cope with ischemic stroke by approaches focusing on the inhibition of signaling pathways involved in inflammatory responses and the subsequence damages of the BBB.

Many reports indicate that potential neural stem/progenitor cells are present in various brain regions including the subgranular zone (SGZ) of hippocampal dentate gyrus and subventricular zone (SVZ) of the lateral ventricle (LV), allowing the restoration of brain function by these cells after stroke [7,8]. However, we have reported that the formation of new neuroblasts expressing doublecortin (DCX) was limited by stroke-induced inflammatory/oxidative stress and this further deteriorates the ischemic lesion after stroke [2]; transgenic ablation of DCX-expressing cells suppresses adult neurogenesis and worsens stroke outcome in mice [9]. Additionally, ependymal cells have been reported to participate in the neurogenic response to stroke by producing neuroblasts, but their survival is relatively poor [10]. Therefore, we propose that it will be therapeutically valuable by administrating drug(s) to enhance endogenous neurogenesis through concomitantly reducing stroke-induced inflammation/oxidative stress-mediated neuronal and BBB damage, while upregulating the proliferation and/or generation of the endogenous new neuroblasts or neural stem/progenitor cells.

It have been reported that inhibition of glycogen synthase kinase-3 (GSK-3) activities is neuroprotective against ischemic stroke [11]. The kinase activities of GSK-3 are negatively regulated by phosphorylation of GSK-3α at Ser21 and/or GSK-3β at Ser9. GSK-3 can be inhibited through direct binding to the ATP-dependent magnesium-sensitive catalytic site of the enzyme [12] and/or indirectly through enhanced serine phosphorylation of GSK-3 isoforms by multiple mechanisms [13–15]. Inhibition of GSK3 rescues not only neurogenesis but also hippocampal depending learning [16]. Conversely, increased GSK3 activity contributes to generate a neuroinflammatory environment and also impairs adult neurogenesis [17,18]. These results support the idea that inhibition of GSK3 is a potential target for treating ischemic stroke.

The plant, *Polygonum cuspidatum*, is widely distributed in Asia and is used as a folk medicine in Taiwan to cure inflammatory joint diseases. After fractionation and purification, we found that 2-methoxystypandrone (2-MS, Fig. 1A) is a major active component of extracts of *P. cuspidatum*; 2-MS inhibits osteoclastogenesis in primary osteoclasts and in RAW264.7 [19]. However, the potential for exploiting 2-MS's anti-inflammatory and neuroprotective effects on an inflammation-related animal disorder has not yet been reported. In this study, we performed an ischemic stroke murine model to elucidate whether treatment with 2-MS (10, 50 and 100 μg/kg, i.v.) and rt-PA (10 mg/kg, i.v.) as a reference drug, 2 h after ischemia were able to protect mice against middle cerebral artery occlusion/reperfusion-induced inflammatory/oxidative brain injury.

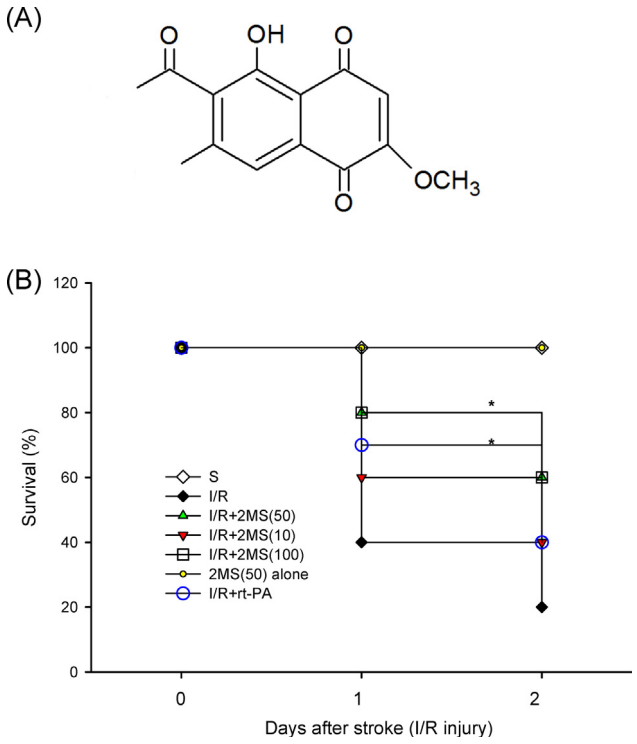

Fig. 1. Effects of 2-MS on changes in survival rates after cerebral ischemic/reperfusion (I/R) injury. (A) Chemical structure of 2-MS. (B) Survival curves (over 2 days) after I/R injury. These were assessed from ischemic mice treated with vehicle (I/R), with 2-MS (10, 50 or 100 μg/kg, i.v.; I/R + 2MS(10), I/R + 2MS(50) or I/R + 2MS(100)) or with rt-PA (10 mg/kg, i.v.; I/R + rt-PA) 2 h after ischemia, or from sham operated mice (S) or 2-MS (50 μg/kg, i.v.) treated only without undergoing ischemia (2-MS(50) alone) by the Log-Rank test followed by the Holm–Sidak method for all pairwise multiple comparisons ($n = 10$ for each group in the beginning),*$P < 0.05$, as compared with vehicle-treated I/R group.

2. Materials and methods

2.1. Animals and induction of transient middle cerebral artery occlusion

All animal procedures and protocols were conducted in accordance with *The Guide for the Care and Use of Laboratory Animals* (NIH publication, 85-23, revised 1996) and were reviewed and approved by the Animal Research Committee of the National Research Institute of Chinese Medicine (approval number: NRICM-IACUC-100-A-11). Male ICR mice weighing 28–30 g (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan) were anesthetized with a mixture of 1.5–2% isoflurane, oxygen, and nitrogen. Transient focal cerebral ischemia was performed using a heat-blunted nylon monofilament surgical suture (diameter approx. 104 μm) coated with silicone, which was introduced into the exposed external carotid artery, advanced to the internal carotid artery, and wedged into the circle of Willis to obstruct the origin of the right middle cerebral artery (MCA). The filament was left in place for 40 min and then withdrawn. This procedure leads to reproducible infarcts similar in size and distribution to those reported by others using transient MCA occlusion (MCAO) of a comparable duration [20]. Blood samples collected from femoral artery before MCAO and 30 min after reperfusion were used for immediate arterial blood gas analysis. Mice were randomly assigned to seven groups. These consisted of a sham-operated group (sham, $n = 10$), three 2-methoxystypandrone (10, 50 or 100 μg/kg)-treated groups ($n = 10$, for each dose), a reference drug, rt-PA (10 mg/kg)-treated group, a vehicle-treated ischemia–reperfusion (I/R) group (I/R only, $n = 10$), and 2-methoxystypandrone (50 μg/kg)-treated only group. The sham-operated (S) mice and 2-methoxystypandrone (50 μg/kg)-treated only group underwent the same surgical procedures without MCAO.

2.2. Drug preparation and administration

2-MS (≥98% by HPLC and NMR) was prepared as in our previous report [19]. Briefly, powdered dried roots of *P. cuspidatum* (10 kg) were refluxed with 95% ethanol (40 litters) for 6 h. The ethanolic solution was concentrated in vacuum to obtain a dark-brown