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Tanshinone IIB, a primary active constituent from *Salvia miltiorrhza*, exhibits neuro-protective activity in experimentally stroked rats

Xi-Yong Yu [a], Shu-Guang Lin [b,**], Zhi-Wei Zhou [c], Xiao Chen [d], Jun Liang [e], Wei Duan [f], Xue-Qing Yu [g], Jing-Yuan Wen [h], Balram Chowbay [i], Chun-Guang Li [j], Fwu-Shan Sheu [k], Eli Chan [l], Shu-Feng Zhou [m,*]

[a] *Research Center of Medical Sciences, Guangdong Provincial People's Hospital, Guangdong Provincial Cardiovascular Institute, Guangzhou 510080, China*
[b] *Department of Cardiology, Guangdong Provincial People's Hospital, Guangdong Provincial Cardiovascular Institute, 96 Dongchuan Road, Guangzhou 510080, China*
[c] *Department of Biological Science and Biotechnology, College of Bioengineering, Nanchang University, Nanchang, China*
[d] *Department of Pharmacy, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China*
[e] *Department of Pharmacology and Toxicology, Australian Institute of Chinese Medicine, Sydney, New South Wales 2118, Australia*
[f] *School of Medicine, Deakin University, Waurn Ponds, Victoria 3217, Australia*
[g] *Department of Nephrology, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China*
[h] *School of Pharmacy, Faculty of Medical and Health Sciences, the University of Auckland, Auckland, New Zealand*
[i] *Clinical Pharmacology Lab, Division of Medical Sciences, National Cancer Centre, Singapore 169610*
[j] *The Chinese Medicine Research Group, Division of Chinese Medicine, RMIT University, Australia*
[k] *Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543*
[l] *Department of Pharmacy, National University of Singapore, Singapore*
[m] *Division of Pharmacy, School of Life Sciences, Faculty of Sciences, Queensland University of Technology, 2 George Street, GPO Box 2434, Brisbane, Queensland 4001, Australia*

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Abstract

Tanshinone IIB (TSB) is a major active constituent of the root of *Salvia miltiorrhiza* (Danshen) used in the treatment of acute stroke. Danshen extracts and TSB have shown marked neuron-protective effects in mouse studies but there is a lack of clinical evidence for the neuron-protective effects of Danshen and its active ingredients. This study investigated the neuron-protective effects of TSB in experimentally stroked rats. TSB at 5 and 25 mg/kg by intraperitoneal injection significantly reduced the focal infarct volume, cerebral histological damage and apoptosis in rats subjected to middle cerebral artery occlusion (MCAO) compared to MCAO rats receiving vehicle. This study demonstrated that TSB was effective in reducing stroke-induced brain damage and may represent a novel drug candidate for further development. Further mechanistic studies are needed for the neuron-protective activity of TSB.

Keywords: Stroke; Tanshinone IIB; Focal infarct; Middle cerebral artery occlusion; Rat

Stroke is a life-threatening disease characterized by rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 h or up to weeks, or leading to death with no apparent cause other than of vascular origin [5]. Recently, intense interest has focused on the antioxidant properties of natural products due to their purported neuro-protective effects in patients [17]. Among these natural products, the dried root of *S. miltiorrhiza* (Danshen) is widely used in the treatment of stroke [16,12]. It is estimated that there are approximately 5 million patients with stroke use Danshen and the global market value is about 2 billion of US dollars in 2005 [16]. Danshen extracts mainly contain diterpene quinine analogs, including tanshinone I, dihydrotanshinone, tanshinone IIA, tanshinone IIB (TSB, (Fig. 1), and cryptotanshinone (CTS) [6]. Danshen extracts and TSB have shown

* Corresponding author. Tel.: +61 7 31381340; fax: +61 7 31381534.
** Corresponding author. Tel.: +86 20 83827812x21180; fax: 86 20 83827712.
E-mail addresses: yuxycn@hotmail.com (S.-G. Lin), s4.zhou@qut.edu.au (S.-F. Zhou).

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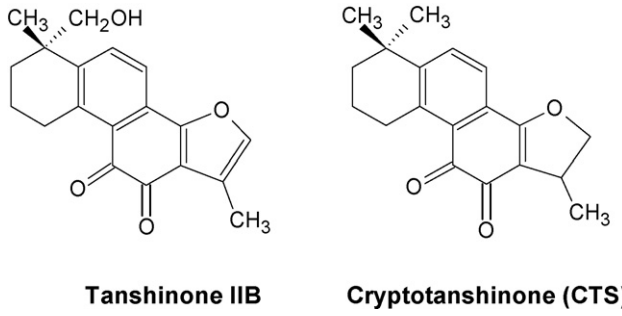

Fig. 1. Chemical structures of tanshinone IIB and cryptotanshinone.

marked neuron-protective effects in mouse studies [7]. The commonly effective concentrations with antioxidative and neuron- and cardio-protective activity of TSB *in vitro* are >1–50 nM [13,14]. However, several recent meta-analyses for the efficacy of Danshen and its purified individual ingredients in acute ischemic stroke indicated that the observed neuron-protective effect in animals could not be translated in patients due to the lack of clinical evidence [12,11]. The mechanism for the neuron-protective activity of Danshen and TSB is unknown, but may be related to their anticoagulant, antioxidant, anti-inflammatory and apoptosis-inhibitory effects [4]. This prompted us to investigate the effects of TSB on the cerebral ischemia in rats subjected to middle cerebral artery occlusion (MCAO).

TSB (MW = 310) extracted and purified from the root of *Salvia miltiorrhiza* was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). TSB was dissolved in dimethyl sulfoxide (DMSO), and freshly prepared for all *in vivo* and *in vitro* experiments. The compound has a purity >99.0%, as determined by high performance liquid chromatography (HPLC). Its chemical structure was identified and confirmed by LC–MS and NMR analysis. DMSO was purchased from Sigma–Aldrich (St. Louis, MO). The water used was purified by a Milli-Q purification system (Millipore, Bedford, MA). All other chemicals and reagents were of analytical or HPLC grade as appropriate.

Male healthy Sprague-Dawley rats (200–260 g) were kept in a room under controlled temperature (22 ± 1 °C) and automatic day–night rhythm (12 h-cycle) and housed on wire-bottom cages with paper underneath. The ethical approval of this study was obtained from the Institutional Ethics Committee. Animals were treated humanely, and the animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health of the USA (NIH publication No. 85-23, 1985).

After 1 week of pre-treatment with TSB by i.p., the stroke was induced using the middle cerebral artery occlusion method in male healthy Sprague-Dawley rats as described previously [2]. The animals were anesthetized with 0.5 ml of a cocktail containing ketamine at 75 mg/kg and xylazine at 5 mg/kg by i.p injection (Sigma–Aldrich, St. Louis, MO). During the whole surgical period, the body temperature of the animals was maintained at 36.5 ± 0.5 °C by the use of a heating pad, controlled by a rectal probe. The right femoral artery was cannulated for measurement of arterial blood gases, blood glucose, pH, hematocrit and mean arterial blood pressure. These physiological parameters were monitored before, at the time of MCAO and 30 min after MCAO. To induce focal cerebral ischemia, a 3–0 nylon monofilament (Becton Dickinson, Sparks, MD) with its tip rounded by heating near a flame and coated with poly-L-lysine was introduced into the internal carotid artery through a nick made in the external carotid artery and advanced 17–20 mm distally from the common carotid artery bifurcation to block the origin of middle cerebral artery (MCA). The monofilament was left in place for 2 h, while the animals were allowed to wake up. After 2 h of occlusion, the intraluminal suture was gently removed during a brief period of anesthesia to allow reperfusion. In the groups of sham-operated rats, all surgical procedures except the MCAO were performed. The animals were then returned to their cages and given free access to food and water. Treatment with TSB by i.p. was continued for another 1 week after surgery and the animals were sacrificed.

The male healthy Sprague-Dawley rats were randomized into six different treatment groups ($n = 8$ per group): Group 1, sham-operated animal without MCAO and treatment with the control vehicle only (0.2% DMSO) by i.p.; Group 2, sham-operated animals, treated with 5 mg/kg TSB by i.p.; Group 3, sham-operated animals, treated with 25 mg/kg TSB by i.p.; Group 4, animals with MCAO and the control vehicle; Group 5, animals with MCAO and TSB at 5 mg/kg by i.p.; and Group 6, animals with MCAO and 25 mg/kg TSB treatment by i.p.

Only two doses (5 and 25 mg/kg body weight) were chosen in our study because (a) at these two doses, TSB can cross rat blood–brain barrier with maximum brain concentrations ranging from 2.8–8.5 ng/ml which caused neuro-protective effect *in vitro* [15]; (b) TSB at both doses could elicit marked pharmacological responses in other studies [13,14]; and (c) TSB at these two doses did not show any significant organ toxicities. The doses and administration route of TSB in this study were also based on the clinical use of Danshen in patients. Like most herbal medicines, Danshen products or its purified individual components such as TSB are usually orally administered with long-term regimens in treatment of angina and stroke. The Pharmacopoeia of the People's Republic of China recommends dosage of 9–15 g daily for Danshen in decoction form, or up to 60 g in treatment of severe angina and stroke [16]. Since the typical content of TSB in Danshen is 0.30% [10], there are about 27.0–45.0 mg of TSB is administered daily, or up to 180 mg was taken up when 60 g crude extract was used.

After 24 h of reperfusion, the neurological functions of the animals were evaluated using two different methods. Method A was used as previously described [1]. Accordingly, four categories of neurological findings were scored: 0 = no observed neurological deficit; 1 = contralateral forelimb flexion with wrist flexion and shoulder adduction; 2 = reduced resistance to lateral push; 3 = circling movements towards the paretic side. In method B, spontaneous motor activity (SMA) was evaluated for 5 min by placing the animals in their normal environment (cage). Neurological scoring was given as: 0 = rats moved around in the cage and explored the environment; 1 = rats moved in the cage but did not approach to all the sides and hesitated to move; 2 = rats barely moved in the cage and showed postural abnormalities (curved