Advances in Studies on Pharmacological Functions of Ligustilide and their Mechanisms

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Abstract: The article reviewed the research progress of ligustilide in recent years and elaborated its pharmacological functions and mechanisms in detail, especially in ischemic brain injury. Its mechanism includes reducing cerebral infarct volumes and improving neurobehavioral deficits, anti-oxidant and anti-apoptosis, antithrombotic activity, calcium channel blockers function, and effect on erythropoietin. Other pharmacological effects of ligustilide including inhibiting vascular smooth muscle cell proliferation, anti-inflammatory and analgesic effects, effects on LPS-induced endotoxic shock, inhibiting constriction effect, suppression of the central nervous system, and ameliorating the memory impairment induced by scopolamine and so on, are also introduced. Ligustilide has potential pharmacological value, which provides a reference for its further research and development.

Key words: cerebral infarct; ischemic brain injury; ligustilide; neurobehavioral deficit; pharmacological function and mechanism
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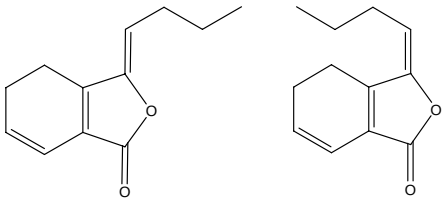
Introduction

Phthalides have protective effects on focal cerebral ischemia in rats, and the mechanism may be relevant to its inhibition of platelet-dependent thrombosis and amelioration of hemorrheological parameters (Tian *et al*, 2005). And phthalides also have anti-oxidative, anti-inflammatory, antitumor, and antiproliferative effects, as well as immune-stimulating properties. The characteristic component of phthalides is ligustilide. And the plants with higher content of ligustilide are *Angelica sinensis* (Oliv.) Diels and *Ligusticum chuanxiong* Hort. in China.

Mitsuhashi, Nagai, and Muramatsu (1960) extracted ligustilide from *L. acutilobum* Sieb. et Zuce. for the first time, and named it "ligustilide". Ligustilide was isolated by silica gel column chromatography in numerous reports. There are two types of ligustilide (Wang, Du, and Qian, 2006), *vis Z*-ligustilide and *E*-ligustilide (Fig. 1). *Z*-type is more stable than the *E*-type due to the superiority conformation, so the content of *Z*-type is about 10 times as high as that of *E*-type in Chinese materia medica (Li, Ma, and Liu, 2001; Zhang, Xiao, and Xu, 2003).



Z-ligustilide *E*-ligustilide

Fig. 1 Chemical structures of ligustilides

Ligustilides have the features of instability and rapid chemical degradation (Schinkovitz *et al*, 2008). Such factors as temperature, light, pH value, co-solvents, and anti-oxidants could greatly influence the stability of ligustilide. Therefore, ligustilide should be kept in proper organic solvents, which prevents it against isomerization by dehydrogenation, oxidation, hydrolysis,

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and degradation (Lin, He, and Lian, 1998; Garcia, Figueroa, and Cogordan, 2005). Ligustilide is more stable in cyclohexane and chloroform than in air (Zhou and Li, 2001). The pure ligustilide should be deposited at −20 ℃ (Xu and Yang, 2007). While ligustilide has basic stability in the volatile oil at room temperature and does not appear isomerization as in pure state, some researchers believe that the oil mixture contains a certain proportion of isomerized-ligustilide products, which makes the reaction balanced (Li *et al*, 2000).

Furthermore, the degradation rates of *Z*-ligustilide were expressed as ln ln (1/1-α) = lnk + mln*t* in which α means degradation ratio, *t* means time, and m and k mean constants relating to the degradation rate (Cui, Feng, and Hu, 2006).

Many reports demonstrated the pharmacological functions of ligustilide, most of which were the functions of *Angelica Sinensis Radix* oil or *Chuanxiong Rhizoma* oil, but there was few report on ligustilide. The paper summarized the pharmacological effects and mechanisms of ligustilide in recent years, especially in ischemic brain injury, which is of great significance for traditional Chinese medicine.

Effects and mechanisms on ischemic brain injury

Ischemic brain injury, also called cerebral infarction, is a symptom that degeneration, necrosis or transient loss of function are caused by failure of blood-supply and the local brain tissue, including nerve cells, glial cells, and contact fiber (Zhu and Zheng, 2004; Xing, 2003).

Chinese medicine-based treatment of cerebral infarction is to promote blood circulation and remove blood stasis primarily. The main mechanism is to reduce the cerebral infarct volumes and brain swelling by anti-adhesion, anticoagulant, and antithrombosis. The role of calcium channel blockers and mild expansion of cerebral blood vessels could ensure blood and oxygen in brain tissue, inhibit neuronal apoptosis, improve the metabolism and microcirculation brain energy, and blood flow of ischemic brain areas, scavenge free radicals, and inhibit the expression of inflammatory factors. The clinical treatment goal of ischemic brain injury is to restore blood and oxygen supply, to inhibit the inflammation of ischemic parts, and to maintain the integrity of the structure and function of neuron. Ligustilide has a significant protective effect on ischemic brain injury, and the following effects and mechanism of ligustilide on ischemic brain injury are reviewed.

Reducing cerebral infarct volumes and improving neurobehavioral deficits

Peng *et al* (2007) investigated the effect of ligustilide on permanent focal ischemic brain injury in rats. Focal cerebral ischemia was induced by the permanent occlusion of middle cerebral artery (MCA) for 24 h. Ligustilide (20 or 80 mg/kg), ig administered at 2 h after ischemia, could reduce the cerebral infarct volumes by 48.29% and 84.87%, respectively, compared to ischemic control group as visualized by 2,3,5-triphenyltetrazolium chloride (TTC) staining ($P < 0.01$). Besides, ischemic brain swelling was induced by 24 h occlusion of MCA, then ig administration of ligustilide (20 or 80 mg/kg) at 2 h ischemia could reduce brain swelling by 68.62% and 82.08%, respectively, compared to ischemic control group ($P < 0.01$).

In addition, ligustilide by ig administration could significantly improve neurobehavioral deficits (Peng *et al*, 2007). At 24 h of focal ischemia, the neurological scores in control rats were measured to be (3.63 ± 0.21), which were remarkably decreased to (2.57 ± 0.20) or (1.83 ± 0.40) by the treatment with the doses of 20 or 80 mg/kg, respectively ($P < 0.01$). The neurologic grading system described by Menzies *et al* (1992) was as follows: 0 means no apparent deficits; 1 means left forelimb flexion; 2 means decreased grip of the left forelimb while tail pulled; 3 means spontaneous movement in all directions, left circling only if pulled by tail; 4 means spontaneous left circling. And ligustilide-treated groups had higher survival rates (77% or 86% at the doses of 20 or 80 mg/kg, respectively) at 24 h after permanent MCA than that in control group (60%). So ligustilide might be a potential neuroprotective agent for the treatment of ischemic stroke in future.

On the basis of significant protective effect on the rat model of focal and whole cerebral ischemia reperfusion, Peng (2007) investigated the protection of ligustilide on permanent middle cerebral artery occlusion (pMCAO), the therapeutic time window, and its protection mechanism. The results showed that ligustilide had significant protective effect on cerebral ischemia in rats. It could significantly improve the pMCAO neurological function and significantly reduce brain edema and infarct volumn compared with the

model. And ligustilide of 20 mg/kg was able to show a significant neuroprotective effect at 15 min before surgery and at 3 and 6 h after surgery.

Moreover, ligustilide has neuroprotective effect in transient MCAO and suppresses the expression of relevant inflammatory factor NF-κB distinctly. Wang *et al* (2010) established filament model of MCAO. SD rats were randomly divided into Sham group, vehicle group, and administered dose group. Apart from the Sham group, SD rats in other groups were treated with 3% Tween-80 solvent or ligustilide of 20 mg/kg at time point of 0 and 3 h after reperfusion. After 2 h of focal ischemia, the neurological function of rats and brain infarct volume with TTC staining were evaluated at 24 h after surgery. The result showed that ligustilide could significantly ameliorate MCAO neurological function ($P < 0.01$), reduce the cerebral infarct volume ($P < 0.01$), and inhibit the expression of NF-κB from protein level ($P < 0.01$).

Anti-oxidation and anti-apoptosis

When mediated neuronal irreversible damage occurs, the ischemic brain tissue consumed superoxide dismutase (SOD) excessively and generated a mass of lipid peroxide metabolic end products—malondialdehyde (MDA). Cerebral ischemia reperfusion injury was induced due to the decrease of SOD activity and increase of MDA levels.

Kuang *et al* (2006) examined whether ligustilide could protect ischemia/reperfusion-induced brain injury by minimizing oxidative stress and anti-apoptosis. The study demonstrated that ligustilide (5 or 20 mg/kg, ip) administrated at the beginning of reperfusion following the bilateral common carotid arteries occlusion, significantly decreased the level of MDA and increased the activities of the anti-oxidant enzyme glutathione peroxidase (GSH-Px) and SOD in the transient forebrain ischemic tissues, together with a great up-regulation in Bcl-2 (anti-apoptotic gene) expression and a significant down-regulation in Bax (pro-apoptotic gene) and caspase-3 immunoreactivities in the ischemic cortex. The findings demonstrated that ligustilide could markedly protect brain from damage induced by transient forebrain cerebral ischemia (FCI). The anti-oxidant and anti-apoptotic properties of ligustilide may contribute to the neuroprotective potential of ligustilide in cerebral ischemic damage.

Kuang *et al* (2008) proved that ligustilide significantly prevented chronically hypoperfused cognitive deficits and brain damage at least partly through an anti-oxidant effect and improved cholinergic activity. Male Wistar rats were subjected to permanent ligation of both common carotid arteries (two vessel occlusion, 2VO). With ligustilide by ig administration of 10 or 40 mg/(kg·d), rats with significantly impaired acquisition of spatial information were assessed in the Morris water maze. Compared to the Sham group (controls), ligustilide could significantly shorten mean escape latency ($P < 0.01$), improve the ability of cognitive impairment, and prevent neuronal loss and an increase of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes in the hippocampus ($P < 0.01$). Ligustilide also significantly reduced MDA levels, increased SOD activity ($P < 0.05$, 0.01) and choline acetyltransferase activity and inhibited acetylcholinesterase activity in ischemic brain tissues ($P < 0.05$, 0.01). The finding suggested that ligustilide might have therapeutic potential in treating vascular dementia and cerebrovascular insufficiency.

In addition, Yu *et al* (2008) examined the mechanism of ligustilide on hydrogen peroxide (H_2O_2)-induced injury in PC12 cells. These results manifest that ligustilide had a significantly protective effect against H_2O_2-induced cytotoxicity, at least partly via improving cellular anti-oxidant defense and restraining the mitochondrial apoptotic pathway. Pretreatment of the cells with ligustilide (0.1, 1.0, 2.5, or 5.0 μg/mL) signally not only attenuated H_2O_2-induced cell death and increased intracellular ROS levels, but also decreased Bax expression, cleaved-caspase-3, and cytochrome C. Furthermore, ligustilide could improve cellular TAC and concentration-dependently up-regulate Bcl-2 expression. Inhibiting the increase of caspase gene or activating Bcl-2 gene could alleviate ischemic injury (Liu and Li, 2007). Ligustilide may be useful in the treatment of neurodegenerative disorders depending on the ability of oxidative stress and apoptosis.

Moreover, Long, Du, and Wang (2010) confirmed that ligustilide had significant inhibitory effect on spontaneous peroxidation of linoleic acid, Vit C/Fe^{2+} and NADPH-induced mitochondrial peroxidation, and spontaneous H_2O_2-induced lipid peroxidation of tissue homogenate, and exhibited concentration-dependant inhibition with anti-oxidant activity.

Ligustilide may be an effective therapeutic modality for subarachnoid hemorrhage (SAH) victims

via anti-apoptotic pathways. Chen *et al* (2011) showed that ligustilide decreased mortality, neurobehavioral deficits, brain edema, blood-brain barrier (BBB) permeability, and cerebral vasospasm in rats with SAH. In addition, treatment with ligustilide reduced the number of apoptotic cells in the surrounding brain injury site, which accompanied a remarkable decrease of proapoptotic proteins, p53, and cleaved caspase-3.

Anti-adhesion, anticoagulation, and antithrombotic

When cerebral ischemia occurs, prostacyclin I2 (PGI2) is relative shortage and thromboxane A2 (TXA2) increases. The imbalance of TXA2/PGI2 induces platelet aggregation, leading to the occurance of ischemic stroke (Wang, Wang, and Cao, 2004). Anti-thrombotic therapy has become an important goal for the treatment of cerebral ischemia. The study (Zhang *et al*, 2009) showed that ig administration of ligustilide (10 or 40 mg/kg) to rats once daily for 3 d could significantly and dose-dependently reduce arterial thrombus weight in an arteriovenous shunt thrombosis in rats and platelet aggregation induced by adenosine diphosphate (ADP) in rats *ex vivo*, but had no significant effect on coagulation time, including activated partial thromboplastin time, and prothrombin time, in rats *ex vivo*. The study demonstrated for the first time that ligustilide may exert efficient antithrombotic activity through inhibition of platelet aggregation without affecting coagulation time of peripheral blood. The antithrombotic activity of ligustilide may contribute to its potential for the treatment of ischemic diseases, including ischemic stroke.

Calcium channel blockers function

In the pathological state of cerebral ischemia, Ca^{2+} was unbalanced between intracellular and extracellular (Chen and Chen, 2007), which produced lipid peroxidation by activating the membrane fatty acid A_2, protein kinase, and nitric oxide (NO), and generated free radicals, so neuronal damage (Yin, 2001) and ischemic brain injury were induced. Ligustilide is a calcium channel and receptor inhibitor. It plays a neuro-protective role on ischemic brain injury in rats by inducing vasodilation through inhibition of voltage-dependent calcium channel and receptor-mediated calcium influx and release (Cao *et al*, 2006).

Effect on erythropoietin

Erythropoietin (EPO), which is a new neuro-protective factor, is widespread in the brain and has neuroprotective effect. Wu, Qian, and Zhu (2011) found that ligustilide exerted significant neuroprotective effect on ischemia-reperfusion (I/R) injury by up-regulation of EPO transcription via an extracellular signal-regulated kinase (ERK) signalling pathway and down-regulation of RTP801 expression. Ligustilide decreased the neurological deficit score and infarct volume in I/R rats, and increased cell viability and decreased lactate dehydrogenase release in I/R rat neurons. Therefore, ligustilide has the development potential in the prevention and treatment of ischaemic disorders.

Other pharmacological effects

Inhibiting vascular smooth muscle cell proliferation

Liang and He (2006) investigated the inhibitory effects of ligustilide on basic fibroblast growth factor (bFGF)-stimulated vascular smooth muscle cell (VSMC) proliferation by MTT colorimetric method. It could potently inhibit the abnormal proliferation of VSMC induced by bFGF at the concentration of 5.5 μmol/L ($P < 0.05$), but had no effects on the normal VSMC growth. And ligustilide had selective affinities to rat aortic smooth muscle cell as same as Verapamil, one of the calcium ion antagonists.

Furthermore, Lu, Qiu, and Yang (2006) considered that proliferation and migration of VSMCs were believed to develop atherosclerosis and venous bypass graft disease. Ligustilide markedly inhibited VSMCs proliferation and cell cycle progression. And it suppressed reactive oxygen species production and ERK, c-Jun *N*-terminal protein kinase, and p38 MAP kinase. Their findings suggested that the anti-proliferative effect of ligustilide was associated with the decrement of ROS resulting in the suppression of MAPK pathway. Thus, ligustilide was considered as the effective agent in preventing cardiovascular diseases including atherosclerosis and hypertension. Patent (Yang *et al*, 2003) reported, ligustilide and its dimerization could prevent atherosclerosis with reducing the lesions of aortic and coronary atherosclerosis by the model of atherosclerosis.

Anti-inflammatory and analgesic effects

Ligustilide has an analgestic effect and could signally inhibit acute and chronic inflammation in animals (Lin *et al*, 2011). Ligustilide (100, 60, and 20 mg/kg) could delay the latency of licking of the hind

paw in hot plate test and reduce the times of writhe response action with the inhibition rates of 61.21%, 48.12%, and 43.61%, accordingly. It distinctively inhibited the increase in permeability of abdominal cavity induced by acetic acid in mice with the inhibition rates of 64.84%, 57.85%, and 49.67%, respectively, and the auricular edema induced by dimethy benzene with the inhibition rates of 52.06%, 41.51%, and 34.21%. Ligustilide could significantly inhibit the paw swelling caused by carrageenan in rats with the inhibition rates of more than 42% from 1 to 3 h. It could observably inhibit the cotton-pellet granuloma in rats with the inhibition rates of 52.64%, 50.32%, and 45.33%. Du *et al* (2007a) demonstrated that ligustilide could cause a prominent dose-related reduction of acetic acid-induced writhing response and formalin-induced licking time in both the early and late phases.

In addition, Su *et al* (2011) found that ligustilide prevented lipopolysaccharide (LPS)-induced NO synthase expression in RAW 264.7 macrophages by preventing ROS production and down-regulating the MAPK, NF-κB, and activator protein-1 (AP-1) signaling pathways. Kuang *et al* (2009) considered that ligustilide modulated TNF-α-activated NF-κB signaling pathway with respect to its protective effect against β amyloid (Abeta) (25-35)-induced neurotoxicity. In addition, it protected Neuro-2a cells from neuro-inflammatory toxicity induced by the conditioned culture media produced by LPS-stimulated BV-2 cells (Or *et al*, 2011). And ligustilide could inhibit LPS-induced TNF-α production via its inhibitory activity on TNF-α mRNA transcription (Liu *et al*, 2005). Meanwhile, ligustilide exerted a potent anti-inflammatory effect on microglia through inhibition of NF-κB pathway. Ligustilide showed a concentration-dependent anti-inflammatory effect on LPS-activated microglia without cytotoxicity (Wang and Du, 2010). So ligustilide may have potential applications in the treatment of inflammation and related diseases.

Effects on LPS-induced endotoxic shock

Shao *et al* (2011) suggested that ligustilide protected the rabbits against LPS-induced endotoxic shock. The study showed that ligustilide signally inhibited the decline in mean arterial pressure and rectal temperature and decreased the levels of TNF-α, interleukin-1β, and NO, but had no apparent effect on heart rate. Ligustilide also inhibited the increase in the levels of biochemical markers, such as alanine transaminase, aspartate transaminase, alkaline phosphatase, γ-glutamyl transpeptidase, lactate dehydrogenase, creatinine kinase, blood urea nitrogen, and creatinine, but showed no apparent effect on total bilirubin and total protein. Furthermore, ligustilide partly restored the function of injured vital organs, including the heart, liver, lungs, and kidneys.

Inhibiting constriction effect

Ligustilide signally inhibited vasoconstriction (Liang, He, and Yang, 2005) induced by norepinephrine bitartrate and calcium chloride *in vitro* on rat abdominal aorta segments. Ligustilide (2－8 μg/mL) inhibited the spontaneous periodic contraction and attenuated prostaglandin F2α [PGF(2)α]- or acetylcholine chloride-induced uterine contractions (Du *et al*, 2006). And ligustilide could observably reduce the phenylephrine-induced aortic tension *in vitro* with IC_{50} about 64 μg/mL, but had no *in vivo* effect on systolic blood pressure in spontaneously hypertensive rats when ig administrated (Du *et al*, 2007b). Ligustilide had a clear antispasmodic effect not only on isolated guinea pig tracheal strips with relaxation, but also on the contraction of tracheal smooth muscle caused by acetylcholine, histamine, and barium chloride, while no significant effect on the content of cAMP and cGMP in guinea pig lung and intestine tissue (Tao *et al*, 1984).

Suppression of the central nervous system

Ligustilide could broadly inhibit central nervous system. Xie and Tao (1985) found that ligustilide inhibited the spontaneous activity of mice ($P < 0.01$), resisted the excitatory effects of ketamine ($P < 0.01$), prolonged sodium pentobarbital hypnotic time significantly ($P < 0.01$), inhibited the responses induced by electrical stimulation ($P < 0.01$), and had a significant cooling effect ($P < 0.01$). While it had no anticonvulsant effect.

Ameliorating the memory impairment induced by scopolamine

Cheng *et al* (2011) found that ligustilide significantly improved spatial long-term memory and short-term memory impairment via enhancing cholinergic function, inhibited acetylcholinesterase activity, and increased choline acetyltransferase activity. Ligustilide showed the efficacy in both neurobehavioral

and cholinergic evaluation.

Other effects

Studies (Shi and Zheng, 1995; Yang *et al*, 2003) showed that ligustilide could reduce vascular resistance and increase the blood flow. And it had a good effect on microcirculation. Ligustilide is a potent transient receptor potential-A1 activator (Zhong *et al*, 2011) and is also capable of inducing a modest block of mustard oil activated currents. Ligustilide showed anti-hyperglycemic activity (Brindis *et al*, 2011). It did not affect α-glucosidase *in vivo*, but altered glucose absorption. Ligustilide could notably improve *Trichophyton* susceptibility to Ketoconazole and Itraconazole for its antifungal activities (Sim and Shin, 2008) and had weak antiviral properties and weak antimicrobial activity (Beck and Stermitz, 1995) against Gram-positive, Gram-negative, and yeast microorganisms. Ligustilide deterred the biting of two mosquito species (Wedge *et al*, 2009) more effectively than diethyltoluamide.

Development prospects

Ligustilide is an active ingredient and has a wide range of pharmacological effects. The key of developing ligustilide preparation should be paid attention to its stability, because the conjugated double bonds are prone to oxidative degradation and isomerization reactions. At present, there are two preparations including β-cyclodextrin inclusion complex with ligustilide (Li *et al*, 2005) and ligustilide liposomes (Li *et al*, 2010). Maybe it could be made into injection in future (Cui, Feng, and Hu, 2006). The purpose is to make drugs more directly and fully reach the heart, brain, and other target organs and to play a better treatment in cardio- and cerebro-vascular diseases. Therefore, an extensive study of ligustilide would provide good development prospects.

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