

## Review

# Pharmacological actions and therapeutic applications of *Salvia miltiorrhiza* depside salt and its active components

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*Salvia miltiorrhiza*, a traditional medical herb known as danshen, has been widely used in China to improve blood circulation, relieve blood stasis, and treat coronary heart disease. *S miltiorrhiza* depside salt is a novel drug recently developed at the Shanghai Institute of Materia Medica; it contains magnesium lithospermate B (MLB) and its analogs, rosmarinic acid (RA) and lithospermic acid (LA), as active components. The drug has been used in the clinic to improve blood circulation and treat coronary heart disease. The pharmacological effects of the depside salt from *S miltiorrhiza* and its components have been extensively investigated. Experimental studies have demonstrated that magnesium lithospermate B possesses a variety of biological activities, especially protective effects in the cardiovascular system such as attenuation of atherosclerosis and protection against myocardial ischemia-reperfusion injury. Rosmarinic acid and lithospermic acid also show beneficial effects on the cardiovascular system. This paper reviews the recent findings regarding the mechanisms underlying the pharmacological actions of the active components of *S miltiorrhiza* depside salt, based on published works and our own observations.

**Keywords:** traditional Chinese medicine; *Salvia miltiorrhiza* depside salt; magnesium lithospermate B (lithospermic acid B or salvianolic acid B); rosmarinic acid; lithospermic acid; cardiovascular activities

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## Introduction

The dried roots of *Salvia miltiorrhiza*, a traditional medical herb known as danshen, are used in China for the treatment of coronary heart disease, hepatitis, menstrual disorders, menopause, blood circulation diseases, and other cardiovascular diseases<sup>[1]</sup>. The chemical constituents of *S miltiorrhiza* have been studied for more than 60 years, but research has been focused mainly on the lipophilic diterpenoid quinones. In recent decades, the pharmacological activities of the water-soluble components of *S miltiorrhiza* have been investigated, including the active constituents magnesium lithospermate B (MLB, also called salvianolic acid B), rosmarinic acid (RA), lithospermic acid (LA), prolithospermic acid, ammonium potassium lithospermate B, and magnesium salvianolate E oligomers of caffeic acids (Figure 1)<sup>[2]</sup>. Pharmacological studies have shown that water-soluble extracts from danshen can provide an alternative regimen for the prevention of ischemic heart disease<sup>[3]</sup>.

In 2005, the State Food and Drug Administration (SFDA) of China approved a new drug application for *S miltiorrhiza* depside salt, with MLB, RA, and LA as the primary active compounds, for the treatment of chronic angina. A clinical study showed that intravenous infusion of *S miltiorrhiza* depside salt had an observable therapeutic effect in patients with the coronary heart disease angina pectoris<sup>[4]</sup>. In this article, we review the recent findings from our group and others in order to present the pharmacological profiles and therapeutic applications of the main components of *S miltiorrhiza* depside salt – MLB, RA, and LA – and the mechanisms underlying their clinical efficacy.

## Pharmacokinetic properties of *S miltiorrhiza* depside salt

An understanding of the pharmacokinetics and bioavailability of herbal medicinal products can enable us to link data from pharmacological assays to clinical effects and also help in designing rational dosage regimens. The pharmacokinetic properties of *S miltiorrhiza* depside salt have been investigated using liquid chromatography-tandem mass spectrometry following intravenous administration in animals<sup>[5, 6]</sup> and healthy

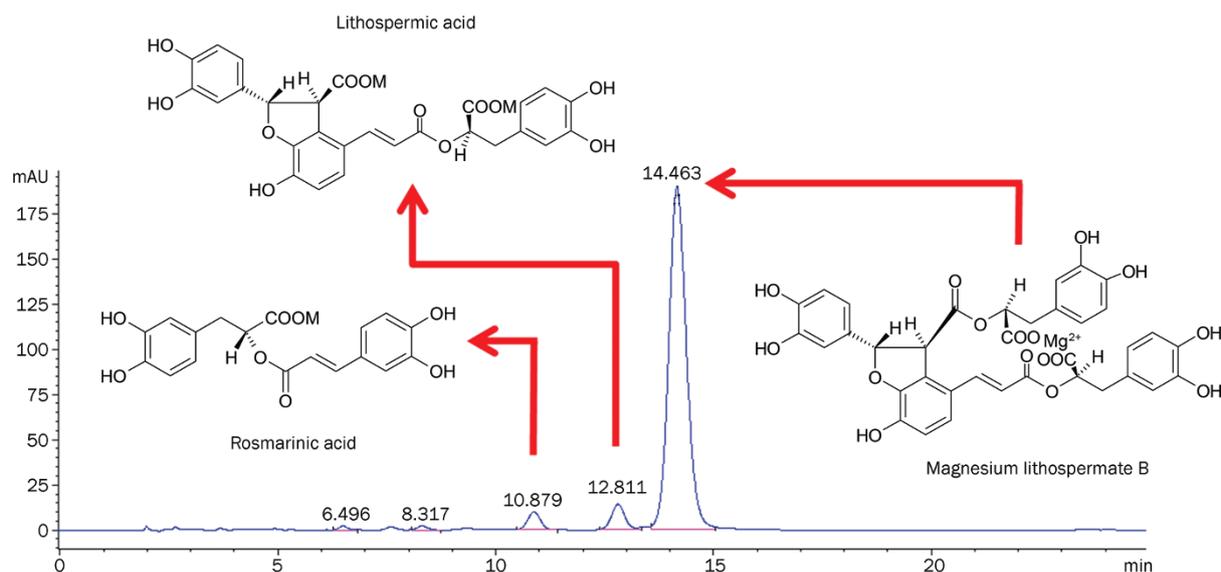
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Salvia miltiorrhiza

Figure 1. *Salvia miltiorrhiza* depside salt and its main components.

volunteers<sup>[7]</sup>. The bioavailability of MLB is extremely low; it has been calculated to be only 1.07%±0.43% in dogs<sup>[8]</sup> and 0.02% in rats<sup>[9]</sup>. The elimination time ( $t_{1/2}$ ) of MLB, RA, and LA is 1.04, 0.75, and 2.0 h, respectively, in rats<sup>[5]</sup>; 0.71, 0.51, and 0.83 h in dogs<sup>[6]</sup>; and 2.33, 0.23, and 3.74 h in healthy Chinese volunteers<sup>[7]</sup>, following intravenous administration of *S. miltiorrhiza* depside salt. The pharmacokinetic properties of *S. miltiorrhiza* depside salt are summarized in Table 1. Given that MLB has been shown to have low permeability through Caco-2 cell monolayers, its low bioavailability could be due to poor absorption and metabolism<sup>[10]</sup>. Overall, extensive metabolism, including a first-pass effect, poor absorption, and wide distribution contributed significantly to MLB's extremely low systemic bioavailability<sup>[9]</sup>.

Cytochrome P450 isoenzymes (CYPs), the most important phase I enzymes in the metabolism of xenobiotics, are

involved in the metabolism of most drugs. Recently, MLB was found to act as a weak inhibitor of CYP1A2 in human liver microsomes<sup>[11]</sup>, to down-regulate CYP3A4 and CYP1A2 mRNA expression in the absence of rifampicin and to inhibit rifampicin-induced CYP3A4 mRNA expression in HepG2 cells<sup>[12]</sup>. However, Liu *et al* reported that MLB could significantly transactivate the CYP3A4 reporter gene construct in either HepG2 or Huh7 cells and the PXR mRNA expression in LS174T cells<sup>[13]</sup>. These data suggest that MLB may modulate the metabolism of the other drugs by induction or inhibition of specific drug-metabolizing enzymes. Additionally, the metabolism of MLB itself can be changed.

#### Pharmacological actions of magnesium lithospermate B (MLB)

Salvianolic acid B and lithospermic acid B were identified

**Table 1.** Pharmacokinetic properties of *S miltiorrhiza* depside salt.

Species (administration)	Doses	Pharmacokinetic parameters	MLB	RA	LA	References
Beagle dogs (iv)	6 mg/kg	$T_{max}$ (h)	0.39±0.14	0.39±0.1	0.47±0.07	Li et al <sup>[6]</sup>
		$C_{max}$ (mg/L)	9775±1576	874±131	308±40	
		$AUC_{(0-t)}$ (mgL <sup>-1</sup> ·h <sup>-1</sup> )	5097±871	460±68	171±27	
		$AUC_{(0-∞)}$ (mgL <sup>-1</sup> ·h <sup>-1</sup> )	5100±871	461±68	172±28	
		$MRT_{(0-∞)}$ (h)	0.46±0.04	0.40±0.02	0.50±0.02	
		V/F (L/kg)	0.44±0.13	0.50±0.09	0.26±0.06	
		$T_{1/2α}$ (h)	0.05±0.01	0.04±0.01	0.07±0.01	
		$T_{1/2β}$ (h)	0.71±0.32	0.51±0.18	0.83±0.48	
		CL/F (L·h <sup>-1</sup> ·kg <sup>-1</sup> )	0.39±0.20	0.72±0.20	0.27±0.13	
Rats (iv)	60 mg/kg	$AUC_{(0-t)}$ (mg·h <sup>-1</sup> ·L <sup>-1</sup> )	51.6±12.4	6.6±1.8	25.5±2.3	Li et al <sup>[5]</sup>
		$AUC_{(0-∞)}$ (mg·h <sup>-1</sup> ·L <sup>-1</sup> )	52.3±12.6	6.9±1.7	26.6±3.1	
		$MRT_{(0-∞)}$ (h)	0.55±0.09	0.32±0.07	1.75±0.16	
		V (L/kg)	1.89±0.68	1.13±0.51	0.12±0.02	
		$T_{1/2α}$ (h)	0.13±0.07	0.12±0.04	0.13±0.06	
		$T_{1/2β}$ (h)	1.04±0.09	0.75±0.14	2.00±0.60	
		CL (L·h <sup>-1</sup> ·kg <sup>-1</sup> )	1.09±0.26	1.02±0.32	0.04±0.01	
Human (iv)	100 mg/kg	$C_{max}$ (mg/L)	4925±1861	174±61	361±101	Jia et al <sup>[7]</sup>
		$T_{max}$ (h)	0.64±0.31	0.47±0.21	1.01±0.20	
		$t_{1/2}$ (h)	2.33±0.92	0.23±0.11	3.74±0.54	
		MRT (h)	1.16±0.62	0.54±0.07	4.24±1.59	
		$AUC_{last}$ (ng·mL <sup>-1</sup> ·h <sup>-1</sup> )	4537±1265	129±28	1229±330	

$Ae_{0-24 h}$ , excreted in 24-h urine sample as unchanged drug; AUC, area under the plasma level-time curve;  $C_{max}$ , maximum concentration of drug; CL, systemic clearance; MRT, mean retention time; NC, not calculable; SD rat, Sprague-Dawley rat;  $t_{1/2}$ , terminal elimination half-life;  $T_{1/2α}$ , absorption half-life;  $T_{1/2β}$ , elimination half-life;  $T_{max}$ , time of occurrence for maximum (peak) drug concentration.

decades ago as the major components of danshen. They were originally reported to have identical structures except for the configurational assignments of two stereocenters, recently through chemical correlation, they were shown to be the same compound<sup>[14]</sup>.

Currently, MLB is used as a quality-control ingredient and active marker for danshen products by the National Pharmacopoeia Council of China<sup>[15]</sup>. As the major component (content >85%) of *S miltiorrhiza* depside salt, the pharmacological actions of MLB have been extensively investigated.

#### Attenuation of atherosclerosis

A large body of evidence has demonstrated that MLB is capable of preventing the development of atherosclerosis *in vivo* and *in vitro*. The *in vivo* evidence for the anti-atherosclerotic effects of MLB is compiled in Table 2. Intimal hyperplasia results from the proliferation and migration of vascular smooth muscle cells (VSMCs) after endothelial injury and excessive oxidative stress, which were significantly reduced by MLB treatment. It has been found that PDGF-BB, SDF-1α, and high glucose could induce VSMC proliferation and migration, which were suppressed by MLB through the following signaling pathways: those induced by PDGF-BB was mediated

via inhibiting the phosphorylation of PI3K/Akt and ERK<sup>[16]</sup>, those induced by SDF-1α via suppressing the expression levels of CXCR4 receptor and downstream molecules of SDF-1α/CXCR4 axis<sup>[17]</sup>, and those induced by high glucose via inducing the nuclear factor erythroid 2-related factor-2 (Nrf2)-antioxidant responsive element (ARE)-NAD(P)H:quinone oxidoreductase-1 (NQO1) (Nrf2-ARE-NQO1) pathway<sup>[18]</sup>. Furthermore, MLB was able to induce VSMC apoptosis by up-regulating p53<sup>[19]</sup>. Most recently, Cho *et al* elucidated the mechanisms by which MLB regulates the cellular proliferation in VSMCs. Using fluorescein isothiocyanate (FITC)-conjugated MLB to track its cellular localization, these authors found that MLB bound to the non-muscle myosin heavy chain (NMHC-IIA), thereby allowing MLB to suppress the PDGF-induced proliferation of VSMCs<sup>[20]</sup>. In addition to inhibiting the proliferation and migration of VSMCs, subsequently preventing neointimal hyperplasia, many other effects are involved in the anti-atherogenic effects of MLB, including the scavenging of ROS/free radicals, attenuation of injury of the vascular endothelium, inhibition of inflammatory reactions, avoidance of lipid deposition, and modulation of the immune response.

**Table 2.** Anti-atherosclerotic effects of magnesium lithospermate B.

Animal models	Mechanisms	References
Cholesterol-fed rabbits	↓ Intimal thickening ↑ Apoptosis in neointimal restenotic lesions ↑ p53 in neointimal restenotic lesions	Hung <i>et al</i> <sup>[19]</sup>
Cholesterol-fed rabbits	↑ LDL resistant to Cu <sup>2+</sup> -induced oxidation ↑ Vitamin E content in LDL ↓ Severity of atherosclerosis ↓ Plasma cholesterol ↓ Endothelial damage	Wu <i>et al</i> <sup>[21]</sup>
ApoE <sup>-/-</sup> mice	↓ Intimal thickening ↓ MMP-2 and MMP-9 expression and activity ↓ LPS-induced HASMC MMP-2 and MMP-9 expression and activity ↓ ERK and JNK phosphorylation	Lin <i>et al</i> <sup>[22]</sup>
ApoE <sup>-/-</sup> mice	↓ Intimal thickening ↓ COX-2 expression ↓ LPS-induced HASMC NADPH oxidase activity ↓ LPS-induced HASMC PGE <sub>2</sub> production, ICAM-1 expression ↓ LPS-induced HASMC ERK and JNK phosphorylation	Chen <i>et al</i> <sup>[23]</sup>
Rat model of carotid artery balloon injury	↓ Neointimal formation ↓ ROS ↓ PDGF-BB stimulated VSMC proliferation and migration ↓ PDGF-BB stimulated VSMC PI3K/Akt and ERK phosphorylation	Hur <i>et al</i> <sup>[16]</sup>
Rat model of carotid artery balloon injury	↓ Neointimal hyperplasia ↓ SDF-1 $\alpha$ -stimulated cell proliferation and migration ↓ CXCR4 receptor ↓ Promoter activity of NF- $\kappa$ B ↓ Raf-1, MEK, ERK1/2, phospho-ERK1/2, FAK, and phospho-FAK	Pan <i>et al</i> <sup>[17]</sup>
Cholesterol-fed rabbits	↓ Cu <sup>2+</sup> -induced LDL oxidation <i>in vitro</i> ↓ HAECs-mediated LDL oxidation ↓ oxLDL-induced cytotoxicity and ROS production in HAECs ↓ Lipid deposition in the thoracic aorta ↓ Intimal thickness of the aortic arch and thoracic aorta ↓ Neointimal formation in the abdominal aorta	Yang <i>et al</i> <sup>[24]</sup>
Carotid artery balloon injury in STZ-induced diabetic rats	↓ Diabetes-related neointimal hyperplasia ↓ Hyperglycemia-accelerated proliferation and migration of VSMCs ↑ Nrf2-ARE-NQO1 pathway	Hur <i>et al</i> <sup>[18]</sup>
ApoE <sup>-/-</sup> mice	↓ CD36 gene expression ↓ Lipid uptake in macrophages ↓ Modified LDL (mLDL) uptake in PMA-stimulated THP-1 and RAW 264.7 cells	Bao <i>et al</i> <sup>[25]</sup>

COX-2, cyclooxygenase-2; CXCR4 receptor, cysteine-x-cysteine chemokine receptor 4; HAECs, human aortic endothelial cells; LDL, low-density lipoprotein; HASMC, human vascular smooth muscle cell; ICAM-1, intercellular adhesion molecule 1; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; Nrf2-ARE-NQO1, nuclear factor erythroid 2-related factor-2 (Nrf2)-antioxidant responsive element (ARE)-NAD(P)H:quinone oxidoreductase-1 (NQO1); oxLDL, oxidized LDL; PDGF, platelet-derived growth factor; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell; STZ, streptozotocin.

### Free radical scavenging and anti-oxidant activity

MLB exhibited iron chelating and scavenging activities against free hydroxyl radicals (HO·), superoxide anion radicals ( $O_2^{\cdot-}$ ), 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals, 2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radicals and hydrogen peroxide ( $H_2O_2$ )<sup>[26]</sup>, as well as xanthine oxidase inhibitory activity<sup>[27]</sup>. Intracellular and extracellular oxidative stress can induce oxidative modification of low-density lipoprotein (LDL) to oxLDL, one of the important substances that drive intimal immune cell infiltration. Inhibiting the formation of oxLDL is beneficial in preventing the development of atherosclerosis<sup>[28]</sup>. It was found that MLB inhibited serum LDL oxidation<sup>[24]</sup>, and MLB-treated LDL exhibited vitamin E-binding ability and was resistant to  $Cu^{2+}$ -induced oxidation<sup>[21]</sup>. These studies suggest that MLB can attenuate pathological increases in the peroxidation of lipids, thus suppressing the development and progression of atherosclerosis. Consistent with this line of evidence, our previous study showed that MLB prevented auto-oxidation and  $Fe^{2+}$ /VitC induced lipid peroxidation in rat serum, liver, kidney, heart, and brain homogenates *in vitro* and exerted similar effects in an *ex vivo* experiment with the exception of brain homogenate<sup>[29]</sup>. In addition to preventing lipid oxidation, the scavenging of ROS contributes to the effects of MLB on preventing injury-induced neointimal formation in rats and in cholesterol-fed rabbits, inhibiting VSMC proliferation and migration, and preventing human aortic endothelial cells from oxidative injury-mediated cell death<sup>[16, 24]</sup>.

It has been shown that MLB suppresses NADPH oxidase activity, subsequently reducing ROS generation in response to TNF- $\alpha$ ,  $H_2O_2$ , and Ang-II in human aortic smooth muscle cells (HASMCs)<sup>[30]</sup>; MLB has also been shown to directly reduce excessive ROS generated by high glucose through the enhancement of high glucose-induced Nrf2 action and the subsequent heme oxygenase-1 expression in HEK293T cells<sup>[31]</sup>. Moreover, the entire Nrf2-ARE signaling pathway has been found to be involved in the antioxidative effects of MLB. In that study, MLB acted at least in part by activating the Nrf2-ARE-NQO1 pathway and also restored redox balance during hyperglycemia-induced chronic oxidative stress<sup>[18]</sup>. Thus, we believe that the prevention of oxidative stress and related vascular complications by MLB contributes to its anti-atherosclerotic effects.

### Preventing endothelial dysfunction

The death or injury of endothelial cells (ECs) may contribute to the initial endothelial pathophysiological processes, such as angiogenesis, atherosclerosis, and thrombosis. MLB was found to attenuate the endothelial damage in cholesterol-fed rabbits<sup>[21]</sup> and to protect human endothelial cells from oxidative stress-induced damage via inducing the expression of glucose-regulated protein 78 (GRP78)<sup>[32]</sup>, thus suggesting that MLB could maintain the integrity of the initial endothelium. Endothelial injury leads to a significant increase in LDL permeability, which plays a role in the formation and development of atherosclerosis. MLB inhibited the VEGF-induced

LDL permeability of ECs<sup>[33]</sup> and reduced the TNF- $\alpha$ -induced permeability and disorganization of vascular endothelial-cadherin in ECs by decreasing VEGF protein expression via modulation of the ERK pathway<sup>[34]</sup>. Loss of the cell-cell adherence junction also increases endothelial permeability. MLB could attenuate TNF- $\alpha$ -induced tyrosine phosphorylation of junctional proteins, including vascular endothelial cadherin and  $\beta$ -catenin. An immunoprecipitation study showed that MLB prevented  $\beta$ -catenin disassociation from the cytoskeleton in TNF- $\alpha$ -treated HUVECs<sup>[35]</sup>. In addition to reducing the endothelial permeability, MLB also modulated the hemostatic properties of ECs. MLB increased the fibrinolytic and anticoagulant potential of cultured HUVECs by up-regulating the expression of tissue-type plasminogen activator (t-PA) and thrombomodulin (TM) and down-regulating the expression of plasminogen activator inhibitor type 1 (PAI-1)<sup>[36]</sup>. The NF- $\kappa$ B and ERK-AP-1 pathways were considered possible targets of MLB in the attenuation of the PAI-1 production response to TNF- $\alpha$  in HUVECs<sup>[37]</sup>.

Atherogenic recruitment of leukocytes involves a sequence of rolling, firm adhesion, lateral migration and transendothelial diapedesis and is controlled by chemokines. During atherosclerosis, circulating monocytes and lymphocytes may interact with adhesion molecules, such as vascular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin on ECs, to establish firm adhesion, which may be an early event in atherogenesis. MLB pretreatment notably attenuated TNF- $\alpha$ -induced expression of VCAM-1 and ICAM-1 and the binding of monocytes to HAECs, which was associated with its anti-inflammatory property through inhibition of the NF- $\kappa$ B pathway<sup>[38, 39]</sup>. In IFN- $\gamma$ -treated ECs, MLB inhibited IFN- $\gamma$ -induced JAK-STAT signaling pathways and consequently suppressed IFN- $\gamma$ -induced expression of chemokines (including IP-10, Mig, and I-TAC), IP-10 promoter activity, IP-10 protein release, and monocyte adhesion to ECs<sup>[40]</sup>. These studies support the potential clinical application of MLB in vascular inflammatory diseases, including atherosclerosis.

MLB regulates vascular homeostasis by exerting a number of vasoprotective effects, including the stimulation of vasodilation, suppression of VSMC proliferation, and inhibition of inflammatory responses. Many of these effects are mediated by the most potent endogenous vasodilator, NO. Endothelial cell-derived NO acts as an important mediator in the cardiovascular, nervous, and immune systems<sup>[41]</sup>. Our previous studies showed that MLB could inhibit  $Ca^{2+}$  influx and stimulate NO production in ECs treated with hypoxia/reoxygenation, which in turn could attenuate cell injury<sup>[42]</sup>. MLB could also inhibit  $Ca^{2+}$  influx and decrease NO release in ECs exposed to hypoxia and attenuate cell injury in ECs<sup>[43]</sup>. In HUVECs, MLB enhanced NO production via the AMPK/PI3K/Akt pathway<sup>[44]</sup>. MLB was also found to produce endothelium-dependent vasodilation by regulating NO production through the modulation of heme oxygenase-1 and arginase activities<sup>[41]</sup>.

Taking all these data together, we conclude that MLB dimin-

ishes endothelial dysfunction through up-regulating anti-inflammatory responses and promoting vasodilation, which may contribute to the prevention and treatment of various cardiovascular disorders, including atherosclerosis.

#### **Anti-inflammatory effects and regulation of matrix metalloproteinases expression and activity**

The inflammatory response is involved in the pathogenesis of atherosclerosis, and the initial degradation of the extracellular matrix (ECM) is an inevitable step for vascular cell hypertrophy, proliferation, and migration, which in turn plays an important role in vascular remodeling and contributes to the vulnerability of atherosclerotic plaques to rupture. Vascular cells, including SMCs, can secrete matrix metalloproteinases (MMPs), the enzymes that selectively digest the individual components of the ECM. MLB treatment effectively attenuated MMP-2, MMP-9<sup>[22]</sup>, and COX-2<sup>[23]</sup> protein expression in cholesterol-fed *ApoE*<sup>-/-</sup> mice, which was related to the reduced thickness of the intima and protection of these mice against atherosclerosis. In HASMCs, MLB reduced the LPS-induced MMP-2 and MMP-9 expression via downregulating the JNK and ERK signaling pathways<sup>[22]</sup> and inhibited the LPS-induced COX-2 expression via reducing PGE<sub>2</sub> production, ICAM-1 expression and NADPH oxidase activity<sup>[23]</sup>. Furthermore, MLB also inhibited MMP-2 expression and activity in response to TNF- $\alpha$ , Ang II, and H<sub>2</sub>O<sub>2</sub> in HASMCs via reducing NADPH oxidase-dependent ROS generation<sup>[30]</sup>. Moreover, MLB down-regulated the SDF-1 $\alpha$ -stimulated up-regulation of CXCR4 (total and cell-surface levels), Raf-1, FAK, and phospho-FAK, as well as the promoter activity of NF- $\kappa$ B, which provided a beneficial effect against the counteracting effects of inflammation on VSMCs<sup>[17]</sup>. These studies demonstrate that MLB can stabilize arterial atherosclerotic plaques and reduce the risk of coronary heart disease.

#### **Modulation of lipid profiles**

In cholesterol-fed rabbits, MLB treatment attenuated the increase in plasma cholesterol predominantly in  $\beta$ -VLDL<sup>[21]</sup>. MLB treatment was also found to decrease the atherosclerotic area, cholesterol deposition<sup>[21, 24]</sup> and lipid levels of aortic vessels<sup>[25]</sup>. However, MLB does not exert obvious lipid-lowering effects. The relationship between lipid lowering and the atheroprotective effects of MLB is still unclear. Because dyslipidemia is one of the main risk factors that lead to atherosclerosis, further investigations are required to explore the effects of MLB on cholesterol metabolism.

#### **Potential immunomodulators**

The activation of T lymphocytes plays a promoting role in the inflammatory processes of atherosclerotic diseases, and functional, immune-stimulating dendritic cells (DCs) have recently been detected in the aortic intima, the site of origin for atherosclerosis. Immunosuppressive methotrexate for treatment of atherosclerosis is currently under investigation<sup>[28]</sup>. One study found that MLB effectively suppressed maturation of human monocyte-derived dendritic cells (h-monDC) induced by ox-

LDL through activation of PPAR $\gamma$ <sup>[45]</sup> and inhibition of IL-2, IL-4, TNF- $\alpha$ , and IFN- $\gamma$  production from activated T cells<sup>[46]</sup>; MLB also effectively reduced the expression of T cell activation markers CD25 and CD69<sup>[46]</sup>. With molecular modeling, MLB was found to act as an inhibitor of protein-protein interactions between the SH2 domains of the Src-family kinases, Src and Lck. The potency of MLB binding to Src and Lck was higher than RA, a natural compound known as the Lck SH2 domain inhibitor<sup>[47]</sup>. Because Lck is a T cell-restricted Src family protein tyrosine kinase, and inhibition of the Lck SH2 domain has been suggested as a possible mechanism underlying the immunosuppressant activity of RA, it appears that MLB may act as an immunosuppressive agent<sup>[48]</sup>.

CD36, a member of the class B scavenger receptors, is a high-affinity receptor for oxidatively modified low-density lipoprotein (oxLDL) and has been implicated in the pathogenesis of a variety of vascular inflammatory diseases. MLB was found to be antagonistic against CD36-oxLDL binding, which was further validated by its inhibition of oxLDL uptake in RAW 264.7 cells<sup>[49]</sup>. Yi *et al* confirmed the specificity and efficacy of MLB in inhibition of CD36-mediated lipid uptake *in vitro* and *in vivo* and demonstrated that MLB was an effective CD36 antagonist<sup>[25]</sup>. These results support a role for MLB as an immune modulator with cardioprotective effects, which would increase its therapeutic potential in atherosclerotic pathologies.

#### **Protecting against myocardial ischemia and reperfusion (I/R) injury**

Cardiac glycosides are drugs that are clinically used to relieve the symptoms of congestive heart failure via their reversible inhibition on Na<sup>+</sup>/K<sup>+</sup>-ATPase located in human myocardium. However, narrow safety margins and severe side effects make administration of these drugs difficult. MLB is a non-steroid compound possessing inhibitory activity against Na<sup>+</sup>/K<sup>+</sup>-ATPase with potency comparable to that of cardiac glycosides but without the apparent adverse effects. Therefore, MLB has great potential in the treatment of congestive heart failure, provided that it undergoes the necessary clinical trials<sup>[50]</sup>. MLB has been found to exert protective effects on the heart in various animal models. In a porcine model of myocardial I/R, MLB increased capillary density and decreased infarct size<sup>[51]</sup>. In C57 mice, MLB was found to inhibit cardiac hypertrophy and infarction and to improve cardiac function at 4 weeks after induction of the infarction<sup>[44]</sup>. In rats with left anterior descending coronary artery ligation, MLB treatment effectively improved left ventricle (LV) function and the appearance of the myocardium as compared with a group with acute myocardial infarction (AMI)<sup>[52, 53]</sup>; MLB treatment also prevented myocardial remodeling, a deleterious consequence of myocardial infarction (MI), by significantly down-regulating the mRNA expression level and activity of MMP-9<sup>[52]</sup>. MLB was found to bind to MMP-9 at the catalytic domain and to function as a competitive inhibitor of MMP-9<sup>[54]</sup> and thus could attenuate the enhancing effects of MMP-9 on migration, proliferation, collagen synthesis, cytokine secretion, as well as the association between cardiac fibroblasts and myofibro-

blast transition<sup>[55]</sup>. Metabolomics offers a new approach to the research of traditional Chinese medicines. Lu *et al*, who analyzed plasma from MI rats and built partial least-squares discriminant analysis (PLS-DA) models, found that MLB was able to regulate 22 identified MI biomarkers in rat plasma<sup>[56]</sup>; this biomarker pattern was similar to the metabolomic profile of propranolol, indicating that the two drugs might have similar mechanisms. They further demonstrated that MLB exhibited a protective effect on MI mainly by decreasing the concentration of cAMP and Ca<sup>2+</sup> and inhibiting PKA<sup>[57]</sup>.

The cardioprotection of MLB could benefit from improving myocardial cell function and/or preventing myocardial cell death. MLB seems to have pleiotropic effects and may act on multiple molecular targets to exert its protection effect on cardiomyocytes. We found that MLB reversibly inhibited L-type Ca<sup>2+</sup> current ( $I_{Ca,L}$ ) without significant effects on the fast-inactivating Na<sup>+</sup> current ( $I_{Na}$ ), delayed rectifier K<sup>+</sup> current ( $I_K$ ) and inward rectifier K<sup>+</sup> current ( $I_{K1}$ ) in single ventricular myocytes of adult guinea pigs, suggesting that the voltage-dependent Ca<sup>2+</sup> antagonistic effects of MLB work together with its antioxidant action for attenuating heart ischemic injury<sup>[58]</sup>. Furthermore, it was found that MLB administration significantly decreased cardiomyocyte apoptosis during I/R via interactions with multiple targets, including elevating superoxide dismutase activity, thioredoxin activity and glutathione concentration; reducing malondialdehyde concentration<sup>[51]</sup>; direct/indirect inhibiting stress-activated protein (SAP) kinase activity and nuclear translocation of the active kinase<sup>[59]</sup>; inhibiting the poly (ADP-ribose) polymerase-1 pathway and improving the integrity of mitochondria and nuclei of heart tissue<sup>[53]</sup>. It was also reported that MLB prevented LPS-induced neonatal cardiomyocyte injury through inhibition of the TLR4-NF- $\kappa$ B-TNF- $\alpha$  pathway<sup>[60]</sup> and protected against cardiotoxicity of doxorubicin in mice through blockade of oxidative stress<sup>[61]</sup>. More recently, MLB was found to protect starving cardiomyocytes by blocking the early stage of autophagic flux and inhibiting the apoptosis that occurred during autophagy<sup>[62]</sup>.

In addition to exerting direct benefit effects on a heart undergoing I/R, MLB can protect the heart from I/R injury through indirect effects, as described below.

First, MLB attenuates the risk of I/R via anti-atherosclerotic properties (see the section "Attenuation of atherosclerosis").

Second, MLB produces vasodilator and vasorelaxant effects. Studies from our group and those of others have demonstrated that these effects result from attenuating intracellular Ca<sup>2+</sup> concentrations ( $[Ca^{2+}]_i$ ) in VSMCs<sup>[63]</sup>, inhibiting Ca<sup>2+</sup> channels in the VSMCs with a minor component mediated by the opening of K<sup>+</sup> channels<sup>[64]</sup>, decreasing  $[Ca^{2+}]_i$  by inhibiting K<sup>+</sup> currents and depolarizing membrane potential in ECs<sup>[65]</sup>, activating large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (BK<sub>Ca</sub>) currents in smooth muscle cells<sup>[66, 67]</sup>, and inhibiting K<sub>V</sub> currents channels in smooth muscle cells and increasing NO release from endothelium<sup>[44, 67]</sup>.

Third, MLB produces antiplatelet, anticoagulant and antithrombotic effects. In myocardial ischemic rabbits, MLB significantly reduced whole-blood and plasma viscosity,

improved hemorheology, prevented angiospasm and platelet aggregation, and reduced oxidative injury<sup>[68]</sup>. MLB was also found to delay thrombus-initiation time and damp photochemical reaction-induced mast cell degranulation in rat mesentery<sup>[69]</sup>. Our previous study showed that MLB decreased the thrombin-activated release of 5-HT and aggregation in rabbit platelets, probably by attenuating  $[Ca^{2+}]_i$ <sup>[70]</sup>. MLB also inhibited platelet aggregation induced by high shear stress<sup>[71]</sup>. Wu *et al* attributed the antiplatelet effect of MLB to a specific interaction with the platelet collagen receptor  $\alpha 2\beta 1$ <sup>[72]</sup>. Ma *et al* further demonstrated that the binding of MLB to integrin  $\alpha 2\beta 1$  caused changes in  $[Ca^{2+}]_i$ , the levels of cytoskeleton-related proteins such as coronin-1B and the cytoskeletal structure of platelets, and therefore concluded that integrin  $\alpha 2\beta 1$  might be one of the direct target proteins of MLB in platelets<sup>[73]</sup>.

Fourth, MLB improved myocardial microperfusion<sup>[74]</sup>, myocardial microvascular reflow<sup>[51]</sup>, as well as coronary blood flow<sup>[68]</sup>. MLB also possessed antihypertensive effects partly due to inhibiting angiotensin converting enzyme activity<sup>[75]</sup>.

Finally, MLB protected bone marrow stem cells from apoptosis<sup>[76]</sup> and synergized with vitamin C in inducing embryonic stem cell differentiation into matured and functional cardiomyocytes<sup>[77]</sup>.

#### Other pharmacological actions

In addition to its effects on the cardiovascular system, MLB has other pharmacological actions as discussed below.

#### Preventing cerebral ischemia-reperfusion injury and neurodegeneration

Cerebral ischemia-reperfusion (I/R) injury is the main reason for the loss of neurons in ischemic cerebrovascular disease. In focal cerebral I/R rats, MLB treatment protected the brain against I/R injury by reducing lipid peroxides, scavenging free radicals and improving energy metabolism<sup>[78]</sup>. MLB exerted neuroprotection against ischemic stroke by inhibiting the Na<sup>+</sup>/K<sup>+</sup>-ATPase via binding to the extracellular pocket of the Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$  subunit and then promoting blood circulation<sup>[79]</sup>. The anti-apoptotic effect of MLB on rheologically induced endothelial injury probably also contributes to its effectiveness in the treatment of cerebrovascular diseases<sup>[80]</sup>.

Alzheimer's disease (AD) and Parkinson's disease (PD) are common degenerative brain disorders. One of the major pathological features of AD is the appearance of senile plaques characterized by extracellular aggregation of amyloid  $\beta$ -peptide (A $\beta$ ) fibrils. Inhibition of A $\beta$  fibril aggregation is therefore regarded as one possible method to halt the progression of AD. MLB was found to inhibit fibril aggregation as well as to destabilize the preformed A $\beta$  fibrils. Moreover, preincubation with MLB significantly reduced the cytotoxic effect of A $\beta_{1-42}$  on human neuroblastoma SH-SY5Y cells<sup>[81]</sup>. Interestingly, MLB was found to alleviate the memory impairments induced by cholinergic dysfunction or A $\beta_{25-35}$  peptide owing to its antagonism of GABA<sub>A</sub> receptors<sup>[82]</sup>. MLB was also found to protect rat cerebral microvascular endothelial cells (rCMECs) against H<sub>2</sub>O<sub>2</sub>-induced apoptosis through the PI3K/

Akt/Raf/MEK/ERK pathway, which may partly contribute to its beneficial effects on AD<sup>[83]</sup>, given that rCMECs apoptosis is considered partially responsible for the pathogenesis of AD.

PD is associated with mitochondrial dysfunction, oxidative stress, and activation of the apoptotic cascade. MLB was found to exert protective effects on MPP<sup>+</sup>-induced apoptosis in SH-SY5Y cells (a classic *in vitro* model for PD) by relieving oxidative stress<sup>[84]</sup>. These observations suggest that MLB has therapeutic potential for the treatment of neurodegenerative diseases.

#### **Inhibitory effects on liver fibrosis**

In a double-blind trial consisting of 60 patients, MLB was found to effectively reverse liver fibrosis in chronic hepatitis B, and it was more effective than the control drug, IFN- $\gamma$ , in the reduction of serum HA content, overall decrease of 4 serum fibrotic markers, and decrease of ultrasound imaging score without side effects or toxicity<sup>[85]</sup>. Hepatic fibrosis is characterized by hepatic stellate cell (HSC) activation. It was reported that MLB inhibited HSC proliferation and collagen production, decreased the cells' TGF- $\beta$ 1 autocrine levels, ameliorated oxidative damage and eliminated ROS accumulation in hepatocytes<sup>[86]</sup>. MLB was also found to significantly attenuate liver fibrosis and activation of HSCs in thioacetamide-induced hepatic fibrosis in rats<sup>[87, 88]</sup>. Taken together, these results suggest that MLB may be used as an effective anti-fibrotic agent in the treatment of hepatic fibrosis.

#### **Improving renal function and preventing diabetic nephropathy**

Tubular epithelial cells can undergo an epithelial-to-mesenchymal transition (EMT), which plays an important role in the pathogenesis of renal interstitial fibrosis (RIF). MLB not only prevented and reversed EMT in HK-2 cells<sup>[89, 90]</sup> but also prevented tubular EMT in HgCl<sub>2</sub>-induced fibrosis in kidneys<sup>[91]</sup>. Our data showed that MLB ameliorated renal cortical microcirculation<sup>[92]</sup> and inhibited mesangial cell proliferation<sup>[93]</sup>, which may be related to the drug's renoprotective effects.

Diabetic nephropathy, a common cause of renal disease, accounts for significant morbidity and mortality in patients with diabetes. In *in vitro* studies, MLB was found to inhibit the high glucose-induced proliferation of mesangial cells and extracellular matrix production, partially through suppressing the cell-cycle process, and also inhibited the activities of MMP-2 and MMP-9 via the NF- $\kappa$ B-dependent pathway<sup>[94]</sup>. MLB also inhibited glucose-induced ROS generation and subsequent PKC inactivation and TGF- $\beta$ 1 and fibronectin downregulation in mesangial cells<sup>[95]</sup>. In *in vivo* studies, MLB treatment effectively inhibited diabetes-associated TGF- $\beta$ 1, fibronectin and collagen upregulation in the renal cortex and significantly suppressed the progression of renal injury in streptozotocin-induced diabetic rats (STZR)<sup>[95]</sup>. Similar effects were found in Otsuka Long-Evans-Tokushima Fatty (OLETF) rats with type 2 diabetes<sup>[96]</sup>. The results of these studies suggest that MLB may be a promising therapeutic agent for preventing and treating diabetic nephropathy.

#### **Pharmacological actions of rosmarinic acid (RA)**

Both *in vitro* and *in vivo* studies show that RA possesses anti-oxidant activity as well as anti-inflammatory activities<sup>[97, 98]</sup>, which results in the multiple pharmacological actions of RA; this multi-target mechanism is similar to that described previously for MLB.

#### **Pharmacological properties of RA on the cardiovascular system**

First, RA can prevent cardiomyocyte dysfunction. It has been reported that RA could inhibit adriamycin-induced apoptosis in H9c2 cardiac muscle cells by inhibiting ROS generation and JNK and ERK activation<sup>[99]</sup>. RA also prevented cardiopathology and lowered blood pressure in fructose-fed hypertensive rats as a result of inhibition of p22phox NADPH oxidase<sup>[100]</sup>. Second, our results showed that RA possessed a potential vasolator effect due to decreasing [Ca<sup>2+</sup>]<sub>i</sub> in VSMCs. Moreover, RA did not affect the basal level of [Ca<sup>2+</sup>]<sub>i</sub> but instead attenuated ATP-stimulated [Ca<sup>2+</sup>]<sub>i</sub> increases in the absence of external Ca<sup>2+</sup> and reduced KCl-induced [Ca<sup>2+</sup>]<sub>i</sub> increases in the presence of external Ca<sup>2+</sup><sup>[63]</sup>. Third, RA was found to reduce the risk of MI via its anti-atherosclerotic properties, and it could penetrate membranes to inhibit lipid peroxidation *in situ* without causing any noticeable alteration of the membrane structure<sup>[101]</sup>. The immunoregulatory activities of RA may also contribute to its anti-atherosclerotic effects. RA was found to induce apoptosis of activated T-cell subsets from rheumatoid arthritis patients via a mitochondrial pathway<sup>[102]</sup>, to inhibit TCR-induced T-cell activation and proliferation<sup>[48]</sup>, and to suppress IFN- $\gamma$ -mediated induction of indoleamine 2,3-dioxygenase transcription via down-regulation of STAT1 activation in IFN- $\gamma$ -stimulated murine bone marrow-derived dendritic cells<sup>[103]</sup>.

#### **Other pharmacological findings**

In addition to the benefits to the cardiovascular system, RA has other pharmacological effects. (1) Due to its scavenging of peroxynitrite (ONOO<sup>-</sup>), daily consumption of RA exhibited protective effects against the memory impairments caused by the neurotoxicity of A $\beta$ <sub>25-35</sub><sup>[104]</sup>. In addition, it has been reported that RA could protect MES23.5 dopaminergic cells against 6-OHDA<sup>[105]</sup> or MPP<sup>+</sup><sup>[106]</sup>-induced neurotoxicity *in vitro* and achieve neurorescue effects in 6-ODHA-lesioned rat model of PD *in vivo*<sup>[107]</sup>. (2) RA has an early renal protective role in nephritic damage. RA was found to be potent in the treatment of diabetic nephropathy. It reduced expression of renal connective-tissue growth factor (CTGF) in STZ-induced rat animal models and in high glucose-stimulated HK-2 cells<sup>[108]</sup>. (3) RA treatment in mice with existing cholestatic liver fibrosis inhibited HSC activation and progression of liver fibrosis via PPAR $\gamma$  derepression mediated by suppression of canonical Wnt signaling in HSCs<sup>[109]</sup>.

#### **Pharmacological actions of lithospermic acid (LA)**

LA is a competitive inhibitor of xanthine oxidase that is able to directly scavenge superoxide and inhibit superoxide pro-

duction *in vitro* and thus exhibits hypouricemic and anti-inflammatory actions *in vivo*<sup>[110]</sup>. Our group reported that LA exerted vasodilator action by modulating Ca<sup>2+</sup> homeostasis in VSMCs<sup>[63]</sup> and prevented atherosclerosis by inhibiting VSMC proliferation and migration<sup>[111]</sup>.

### Summary and perspectives

*S. miltiorrhiza* depside salt has been widely prescribed for years. Further investigations to elucidate the mechanisms underlying the protective actions of *S. miltiorrhiza* depside salt and its effects on cardiovascular diseases (CVDs) are under way in our laboratory. The drug comprises three safe and effective components with multiple pharmacological actions, accounting for its pleiotropic pharmacological effects, and it may act at multiple molecular targets, mainly because of its anti-inflammatory and anti-oxidative activities. Currently, *S. miltiorrhiza* depside salt is used primarily for treating CVDs and other circulatory disturbance-related diseases.

Combining *S. miltiorrhiza* depside salt with drugs used to treat hepatic fibrosis (*ie*, malotilate) may enhance their therapeutic effects. Combining *S. miltiorrhiza* depside salt with antidiabetic drugs reduces the severity of complications of diabetes, such as diabetic nephropathy and diabetic atherosclerosis. However, the possibility that such combinations may result in adverse drug interactions must be taken into account. Targeting cellular functions as a system rather than at the single-target level significantly increases therapeutic potency. Further research is warranted to address the mechanisms of the multitarget actions of *S. miltiorrhiza* depside salt and to translate this knowledge into clinical practice.

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