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Synthesis and biological evaluation of novel leonurine–SPRC conjugate as cardioprotective agents

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ABSTRACT

The synthesis and biological evaluation of novel leonurine–SPRC conjugate, 3,5-dimethoxy-4-(2-amino-3-prop-2-ynylsulfanyl-propionyl)-benzoic acid 4-guanidino-butyl ester (**1**) is reported in this Letter. It is designed to improve the pharmacology efficiency by combining leonurine with *S*-propargyl-L-cysteine (SPRC), a cysteine analog, via a phenolic hydroxyl ester bond, which could be readily hydrolyzed to release bioactive leonurine and SPRC. Pharmacological evaluation has shown that **1** possesses potent cardioprotective effect against hypoxia-induced neonatal rat ventricular myocytes damage at lower molar concentration (10-fold less than leonurine required and 100-fold less than SPRC required). The mechanism is in partial related to improve hydrogen sulfide production, anti-oxidative stress and anti-apoptosis.

Leonurine, a plant alkaloid present in Chinese motherwort (*Leonurus artemisia* and *Leonurus heterophyllus*), has long been known that it possesses uterotonic effect (Fig. 1).[1,2] Recently our group have reported that leonurine has cardioprotective effects both in vivo and in vitro[3–5] and that it is able to relax vascular smooth muscle.[6] These beneficial effects are at least partially related to its abilities on anti-oxidation, anti-apoptosis, regulating the Ca^{2+} concentration and protecting mitochondrial function.[5,6] So leonurine has been considered as a good drug candidate in our laboratory for further studies. However, several issues have to be overcome on the therapeutic development of leonurine. For example, it is difficult to isolate and purify leonurine from natural resources due to the low content in Chinese motherwort and variety of other ingredients as impurities. In addition, high-dosage used implicates its low potency, which may hinder clinical trials in future.[4] Besides, the syntheses of leonurine and its derivatives are rare.[1,7] Therefore, we spontaneously considered that structural modification of leonurine may be a potential way to improve its pharmacological efficacy.

Incorporation of two mutually complementary biological active entities into one by conjugation has been widely used in drug design. For examples, this symbiotic approach has been applied to the design of the vasodilator/β-adrenoceptor antagonist 2-[3-(*tert*-butylamino)-2-hydroxypropoxy]-3-cyanopyridine,[8] and trioxaquines, a series of synthetic hybrid molecules containing a trioxane motif and an aminoquinoline entity which have reserved both of the antimalarial activity of artemisinin and the antiplasmodial properties of chloroquine. Moreover, the trioxaquine PA1103-SAR116242 is one of the promising antimalarial agents and has been selected as candidate for preclinical development.[9,10] Inspired by these paradigms, we were intrigued to assume whether combining leonurine with another cardioprotective compound to form new compound will have a dual mode of cardioprotective action (a 'double-edged sword') via different mechanisms and be able to improve cardioprotective efficiency of the precursor. Fortunately, 'another cardioprotective compound' is just at hand. A cysteine

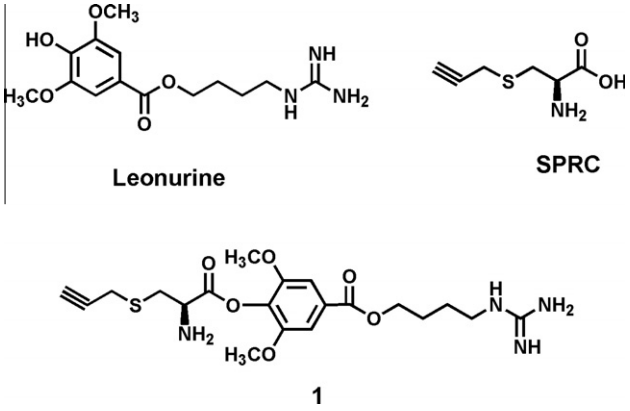

Figure 1. Structure of leonurine (LEO), *S*-propargyl-L-cysteine (SPRC), and compound **1**.

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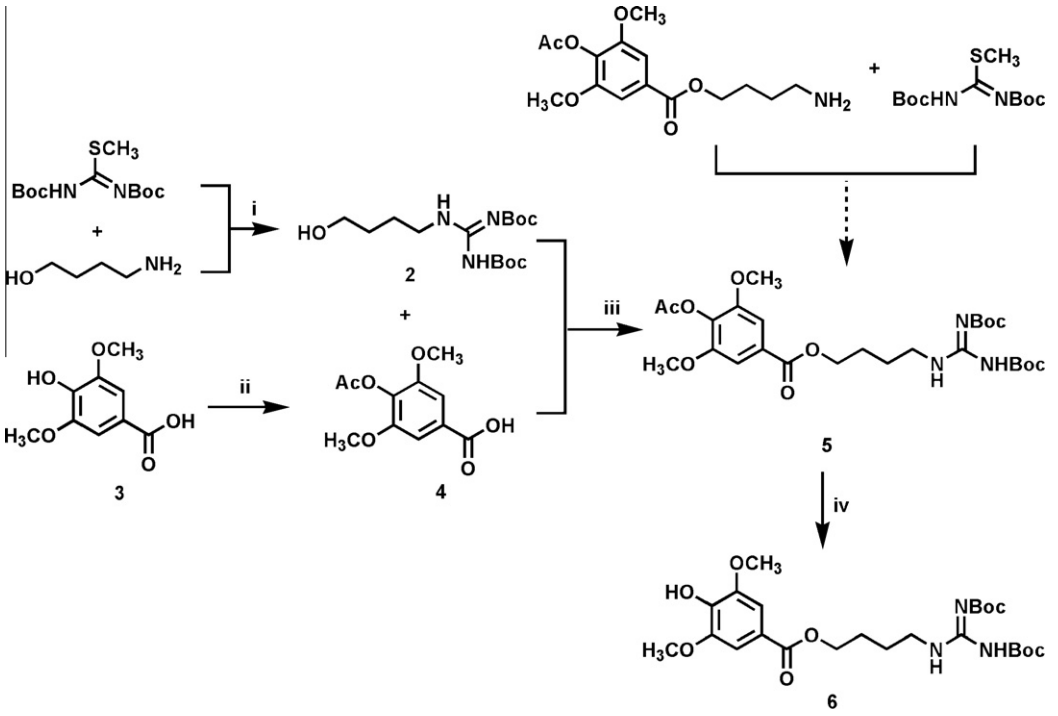
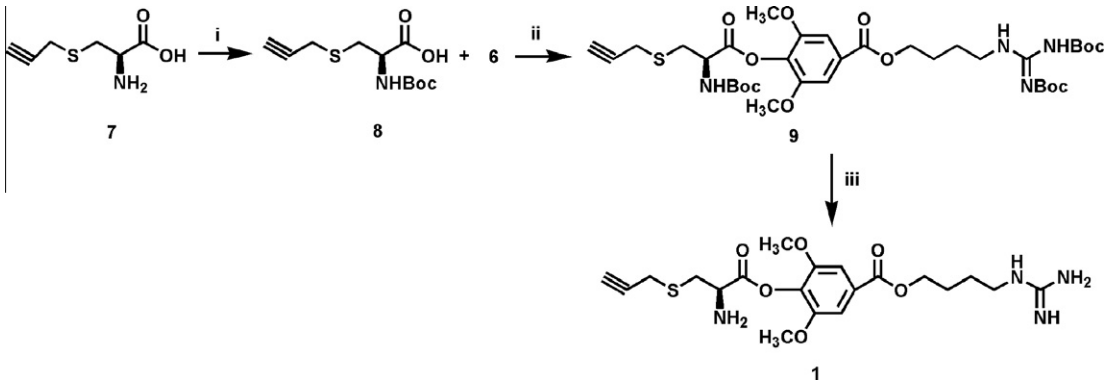


Scheme 1. The synthetic route of the key intermediate 3,5-dimethoxy-4-hydroxy-benzoic acid 4-(*N,N′*-Boc-guanidino)-butyl ester (**6**). Reagents and conditions: (i) in DMF, rt, 3.5 h; (ii) triethylamine, acetyl chloride in dichloromethane, rt, 2 h; (iii) DIC, DPTS, rt, 5 h; (iv) sodium methoxide, 4 °C, rt, 1 h.

analog, *S*-propargyl-L-cysteine (SPRC) has been synthesized as a novel endogenous hydrogen sulfide (H_2S) modulator in our laboratory (Fig. 1). And we have reported that this cysteine analog has potent protective effects on acute myocardial ischemia through regulating endogenous H_2S production by the pyridoxal-5-phosphate-dependent enzyme cystathionine-γ-lyase (CSE) passageway.[11,12] As we all know, hydrogen sulfide (H_2S) has recently been discovered to be the third gasotransmitter and received much attention as important biological mediator.[13]

With the general approach and results in mind, we designed and synthesized leonurine–SPRC conjugate. Two molecules, leonurine and SPRC, are bound together by a phenolic hydroxyl ester bond. The structural modification of leonurine was expected to exhibit similar pharmacological activity with leonurine and SPRC, because the phenolic hydroxyl ester group is liable to be hydrolyzed and then readily release bioactive leonurine and SPRC. In present report, compound **1**, a leonurine–SPRC conjugate, has been studied (Fig. 1). Starting from caryophyllic acid, **1** has been synthesized via seven steps, including acetylation, esterification, hydrolysis etc. Then, it has been evaluated on neonatal rat ventricular myocytes (NRVM) and showed potent protective effect against hypoxia-induced NRVM damage at lower molar concentration (10-fold less than leonurine required and 100-fold less than cysteine analogues required).

In our studies, the designed compound **1** was prepared as outlined in Schemes 1 and 2. To prepare the key intermediate **6**, caryophyllic acid (**3**) was chosen as the starting material, and two approaches were studied. In the first strategy, 4-hydroxy-3,5-dimethoxy-benzoic acid 4-amino-butyl ester was synthesized according to the literature method,[14] and then condensed with *N,N′*-Boc-methyl-isothiourea in DMF at 60 °C to afford compound **6** with very low yield (less than 10%). Thus, to improve the yield of the key intermediate **6**, another approach was employed. Firstly, the hydroxyl group of caryophyllic acid was protected by the esterification of acetyl chloride in the presence of triethylamine to provide the corresponding product **4**. The resulting carboxylic acid **4**, in the presence of *N,N′*-diisopropylcarbodiimide (DIC) and 4-(dimethylamino)-pyridinium-4-toluene sulfonate (DPTS), subsequently



Scheme 2. The synthetic route of 3,5-dimethoxy-4-(2-amino-3-prop-2-ynylsulfanyl-propionyl)-benzoic acid 4-guanidino-butyl ester (**1**). Reagents and conditions: (i) di-*tert* butyl dicarbonate in tetrahydrofuran and Na_2CO_3, rt, 5 h; (ii) DIC, DPTS, rt, 5 h; (iii) TFA.

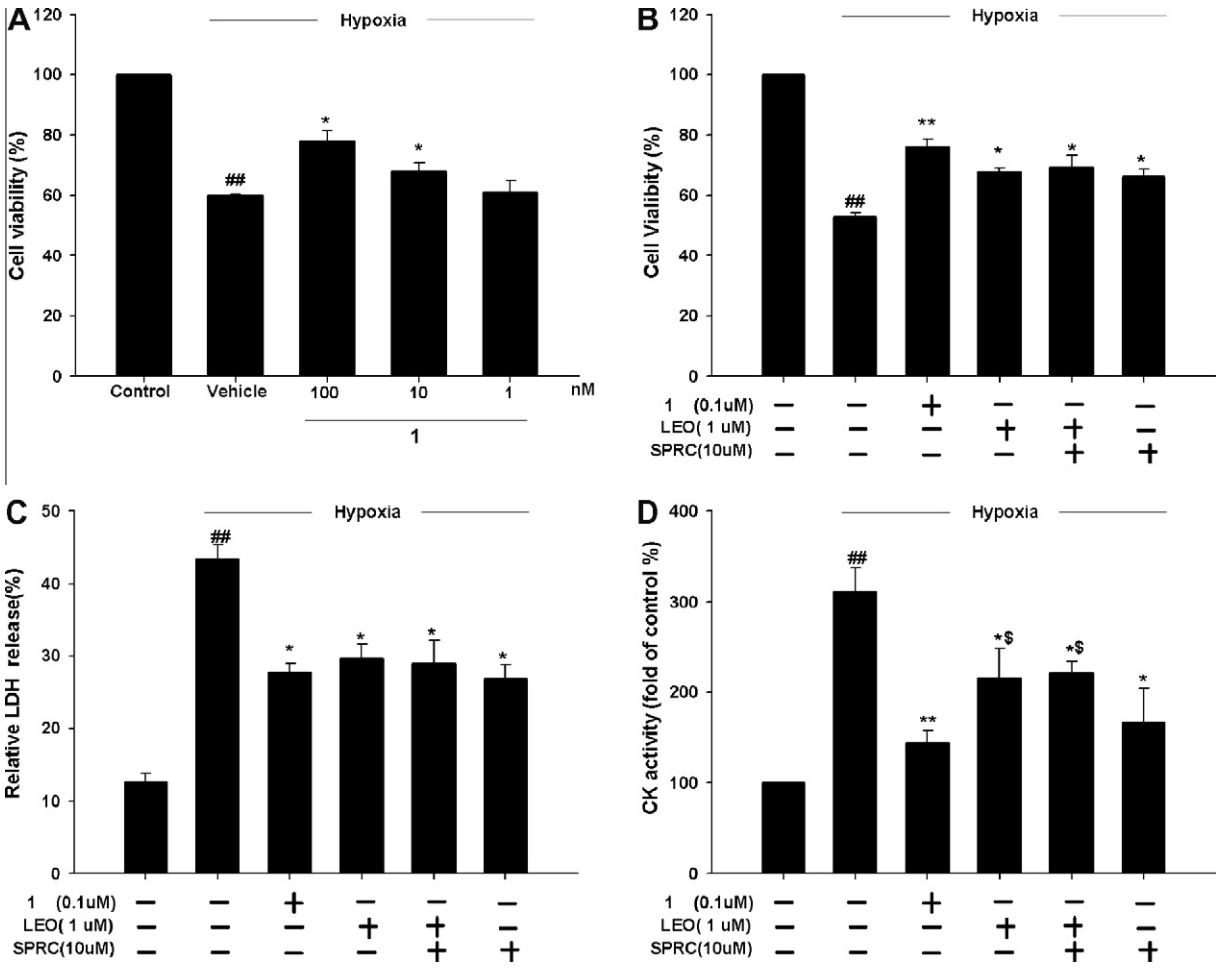


Figure 2. (A) Effect of **1** on hypoxia-induced neonatal rat ventricular myocytes. (B) Effects of **1**, LEO, and SPRC on hypoxia-induced neonatal rat ventricular myocytes. (C) Effects of **1**, LEO, and SPRC on LDH leakage in hypoxia-induced neonatal rat ventricular myocytes. (D) Effects of **1**, LEO, and SPRC on CK activity in hypoxia-induced neonatal rat ventricular myocytes. Values are expressed as means ± SE from six individual samples. *denotes P <0.05 **denotes P <0.01 versus vehicle group; #denotes P <0.05, ##denote P <0.01 versus control group; $denotes P <0.05 versus **1** group.

condensated with **2**, which synthesized from 4-hydroxyl butylamine and methyl-isothiourea in two steps in Scheme 1, to afford **5** in 70% yields. Finally, the acetyl group was hydrolyzed by catalytic amount of sodium methoxide at 4 °C to give the key intermediate **6** in 98% yields.

To build up the other fragment of designed compound **1**, *S*-propargyl-L-cysteine (**7**) was obtained from the reaction of cysteine with propargyl bromide based on the literature method.[11] And the amino group of **7** was protected by Boc anhydride under basic condition to afford **8** in good yield which subsequently condensed with intermediate **6** in the presence of DPTS and DIC to afford **9**. Finally the Boc groups in **9** were removed by trifluoroacetic acid (TFA) to give target compound **1** in good yield (78%).

Compound **1** was evaluated on neonatal rat ventricular myocytes in vitro. The protective effect of **1** was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay,[15] the lactate dehydrogenase (LDH) leakage and creatine kinase (CK) leakage assay. As we all know, MTT assay demonstrates cell viability, LDH leakage and CK activity are the markers of cell membrane integrity.[12] Cells were incubated with **1** for 12 h before exposure to hypoxia for 5 h. Three concentrations (1 nmol/L, 10 nmol/L, and 100 nmol/L) were tested in MTT assay. It showed that the hypoxic condition significantly decreased cell viability (P <0.01). At 0.1 μmol/L, **1** caused a significantly increase in cell viability (Fig. 2A). Thus 0.1 μmol/L was employed for the subsequent experiments. Compared with the vehicle group, all of the drug-treated groups significantly increased cell viability (Fig. 2B) and decreased LDH and CK leakage (Fig. 2C and D) (P <0.05); At 0.1 μmol/L, compound **1** decreased CK leakage much lower than

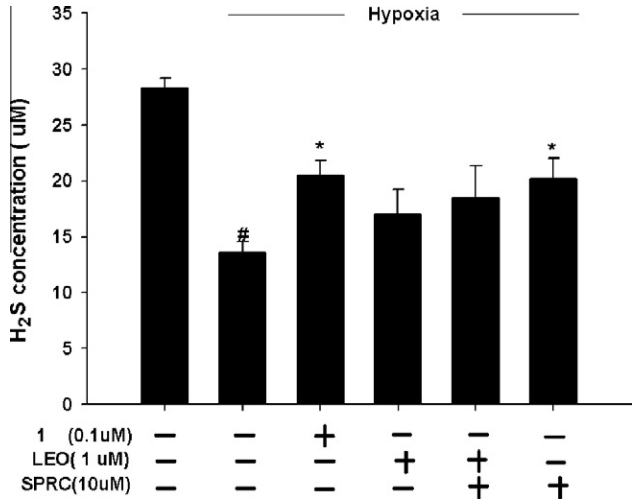


Figure 3. Effects of **1**, LEO, and SPRC on H_2S content in hypoxia-induced neonatal rat ventricular myocytes. Values are expressed as means ± SE from four individual samples. *denotes P <0.05 versus vehicle group; #denote P <0.05 versus control group.

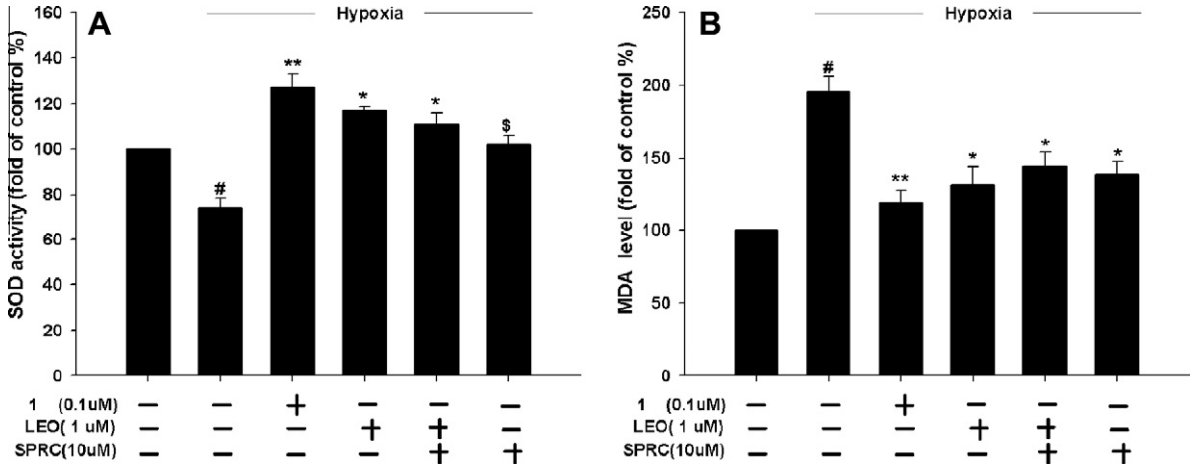


Figure 4. (A) Effects of **1**, LEO and SPRC on SOD activity in hypoxia-induced neonatal rat ventricular myocytes. (B) Effects of **1**, LEO, and SPRC on MDA level in hypoxia-induced neonatal rat ventricular myocytes. Values are expressed as means ± SE from four individual samples. *denotes P <0.05, **denotes P <0.01 versus vehicle group; #denotes P <0.05 versus control group; $denotes P <0.05 versus **1** group.

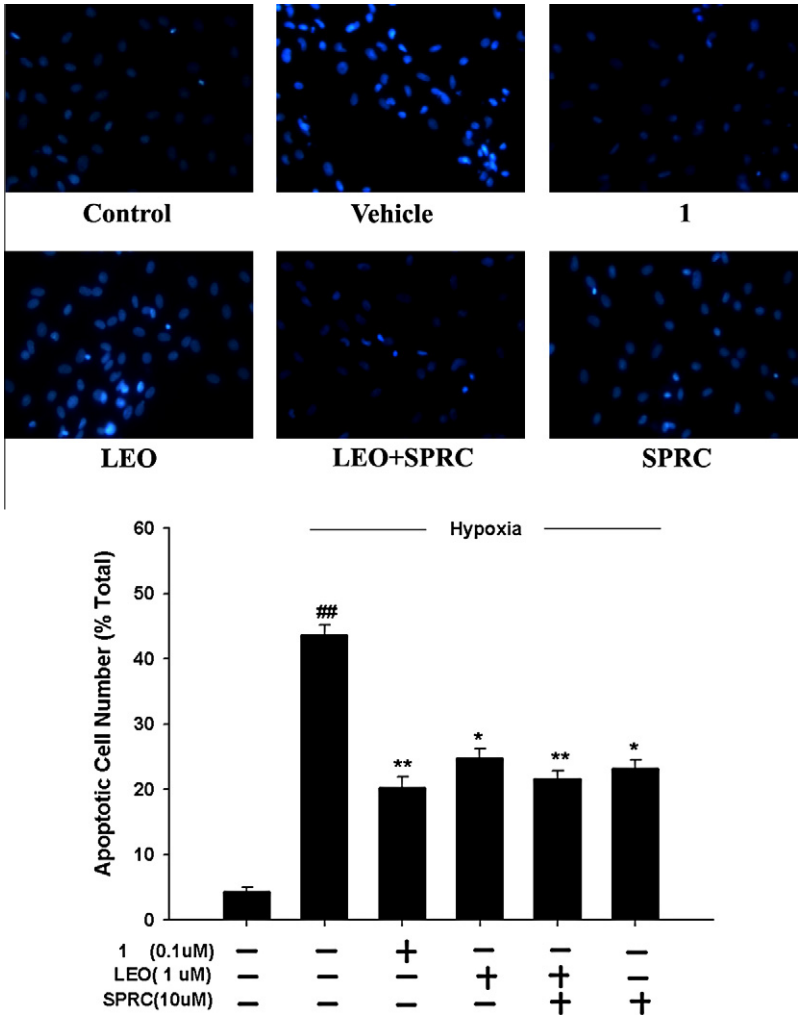


Figure 5. Effects of **1**, LEO, and SPRC on morphological features of cultured neonatal rat ventricular myocytes. Fluorescence photomicrographs of cells stained with Hoechst 33258 (400×). Each photograph is a representative of three independent observations. Percentage of cardiomyocytes with nuclear fragmentation from three independent experiments based on Hoechst stain. Means ± SE; *denotes P <0.05, **denotes P <0.01 versus vehicle group; ##denotes P <0.01 versus control group.

their precursors did in Fig 2D (P <0.05), The results indicated **1** possessed potent cardioprotective effects against hypoxia-induced neonatal rat ventricular myocytes damage at lower molar concentration (10-fold less than leonurine required and 100-fold less than SPRC required). As most conjugates contain a metabolically stable linker, 'cleavable conjugates' employ a linker that is

designed to be metabolized to release two ligands that interact independently with each target.[16] Compound **1** might be easily hydrolyzed and readily release bioactive leonurine and SPRC to show the synergism at the same time. In that way, there would be a lower risk of drug-drug interactions compared to the mixture of leonurine and SPRC.[16]

It has been reported that H_2S content would be down-regulated in myocardial ischemia.[11] In our studies, it was significantly increased to 21.49 ± 1.33 μmol/L in **1** group, while SPRC ameliorated the level of H_2S to 20.18 ± 2.80 μmol/L. but it was only 13.58 ± 0.97 μmol/L in the vehicle group after hypoxia (Fig. 3). In this study, **1** and SPRC groups had significant difference compared with the vehicle group (P <0.05). However, leonurine group and leonurine + SPRC group had no significant difference on modulating H_2S production (P >0.05). As we know that SPRC improved hydrogen sulfide level by CSE pathway,[11,12] and **1** was able to up-regulate hydrogen sulfide level at lower molar concentration, So we infer that **1** may regulate H_2S production by the same pathway, due to it could be easily hydrolyzed and readily release bioactive leonurine and SPRC.

Myocardial ischemia and reperfusion is characterized by a decrease in the endogenous anti-oxidant species, particularly superoxide dismutase (SOD).[17] As shown in Fig 4A, the superoxide dismutase (SOD) activity was significantly decreased in the vehicle group (P <0.05), whereas pretreatment with 0.1 μmol/L of **1** significantly elevated SOD activity (P <0.05). The malondialdehyde (MDA) is a product of lipid peroxidation that acts as an indicator of free radical generation and is one of the most frequently used lipid peroxidation biomarkers.[18] We observed that MDA level was increased in the vehicle group in Fig 4B; Administrated with 0.1 μmol/L of **1**, the level of MDA had been decreased (P <0.05). According to these results, compound **1** was able to inhibit the lipid peroxidation of cell membranes. The results suggested that compound **1** had anti-oxidant properties to attenuate oxidant species generation and suppressed lipid peroxidation. In our previous study, both of leonurine and SPRC were able to show the anti-oxidative effects.[4,12] In addition, the Akt protein, once activated, can phosphorylate a wide range of intracellular substrates that regulate growth, metabolism and survival.[19] And the ability of the CSE/H_2S pathway can alter oxidative condition. SPRC was able to increase expression of phospho-Akt to retain the myocardial cell survival, and modulate the CSE/H_2S pathway, then keep the SOD activity and decrease MDA level.[4,12] So **1** might be hydrolyzed to release leonurine and SPRC, and then show synergism to against the oxidation effect, and prevent cells from oxidative damage by the same pathway.

Apoptosis is a programmed cell death characterized by specific structural changes included cell shrinkage, nuclear condensation and DNA fragmentation.[20,21] To assess the anti-apoptotic potential of **1**, Hoechst staining was performed to observe the morphological changes in hypoxia-damaged neonatal rat ventricular myocytes. Administration of compound **1** resulted in less nuclei-shrunk and nuclear condensation (P <0.01) (Fig. 5). These findings were consistent with MTT results. In recent years, oxidative stress has been proven to be a powerful inducer and a common mediator of programmed cell death,[22] and reactive oxygen species are directly implicated in the initiation of apoptosis.[23] Our study demonstrated compound **1** was able to alleviate oxidative damage which could decrease the probability of apoptosis.

In conclusion, we have designed and successfully synthesized a novel leonurine–SPRC conjugate. Pharmacological evaluation has shown its potent cardioprotective effect and we assume the mechanism is in partial related to modulate hydrogen sulfide production, anti-oxidative stress and anti-apoptosis. These preliminary results demonstrate that **1** is worth to be further investigated as potential anti-myocardial ischemia drug.

Acknowledgments

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