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3,5-Dimethoxy-4-(3-(2-carbonyl-ethyldisulfanyl)-propionyl)-benzoic acid 4-guanidino-butyl ester: A novel twin drug that prevents primary cardiac myocytes from hypoxia-induced apoptosis

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ABSTRACT

Leonurine possesses cardioprotective effects in myocardial ischemia due to its anti-apoptotic properties. However, the process to isolate and purify leonurine is difficult, because of its low content in the Herb Leonuri and its impurity. Moreover, the high dosage used indicates low potency of leonurine. To overcome these defects, we had synthesized a novel twin drug of leonurine, 3,5-dimethoxy-4-(3-(2-carbonyl-ethyldisulfanyl)-propionyl)-benzoic acid 4-guanidino-butyl ester (compound **2**). In this paper, we focused on investigating the cardioprotective effect and underlying mechanisms of compound **2**. Our data showed that cell viability was significantly increased in a dose-dependent manner and the levels of lactate dehydrogenase (LDH) and creatine kinase (CK) were also significantly attenuated in the compound **2**-treated group. In addition, we observed the cardioprotective effects by Hoechst 33258 nucleus staining, JC-1 staining, Annexin V-FITC/PI staining and transmission electron microscopy. Compound **2** inhibited apoptosis by reducing the ratio of Bcl-2/Bax, decreasing cleaved-caspase-3 expression and enhancing the phosphorylation of Akt. Furthermore, the phosphorylation effect of compound **2** was reversed by LY294002 the phosphatidylinositol-3-kinase (PI3K) inhibitor from happening. We concluded that compound **2** played a cardioprotective role in hypoxia-induced primary cardiac myocytes apoptosis partly via modulating the PI3K/Akt pathway at a 10-fold lower concentration than leonurine.

1. Introduction

Cardiovascular disease is one of the world's major killer diseases, accounting for approximately 20% of deaths every year in the world (Wattanapitayakul and Bauer, 2001). Ischemia is one of the major factors in cardiovascular disease (Zhu et al., 2000) and induces too many kinds of damages to myocardial tissues. Thus, to hinder ischemia and thereby decreasing mortality is extremely important. Recently, apoptosis has become one of the major clinical interests. Apoptosis is programmed cell death controlled by genes and is a major kind of cell death in myocardial cell, which has been interrelated with ischemia/reperfusion (Gao et al., 2008). When apoptosis occurs, chromatin are condensed, followed by cells shrinkage; cytoplasmic blebs and apoptotic bodies are formed (Bartling et al., 1998; Haunstetter and Izumo, 1998). Although the mechanisms of apoptosis during ischemia/reperfusion have not been thoroughly comprehended, studies have demonstrated that proteins of the Bcl-2 family, such as Bcl-2 and Bax, play an important role in regulating programmed cell death (Kirshenbaum and de Moissac, 1997; Misao et al., 1996). Thus, therapeutic strategies focused on inhibiting myocardial cell death might be a feasible choice to cure cardiovascular diseases.

Although many drugs have cardioprotective effects, there are usually some adverse effects in them. Given the complex pathogenesis of cardiovascular diseases, it has been of interest to research on twin drugs (Fujii, 2011; Morphy et al., 2004). Twin drug is that the compound contains two components and both of the components exert pharmacological effects. The nonsymmetrical twin drug possesses two different pharmacophores, which is expected to present two different pharmacological effects caused by the different pharmacophores (dual action), whereas the symmetrical twin drug has the same pharmacophores, which is anticipated to show more potent and/or selective pharmacological activities (Fujii, 2011). Recently, the twin drug approach has

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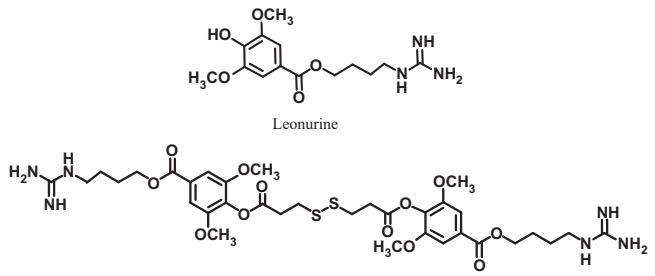

Fig. 1. The chemical structure of leonurine and 3,5-dimethoxy-4-(3-(2-carbonyl-ethyldisulfanyl)-propionyl)-benzoic acid 4-guanidino-butyl ester (compound **2**).

been used in modification of cardiovascular drugs, such as prizidilol hydrochloride (SK&F 92657) and leonurine–SPRC conjugate (Edmonstone et al., 1981; Liu et al., 2010b). Our group has discovered that leonurine, a plant alkaloid that exists in Herba Leonuri (Fig. 1), has cardioprotective effects partly due to its anti-apoptotic activities (Liu et al., 2009a, 2009b; Xin et al., 2009). However, the process to isolate and purify leonurine is difficult, because of the low content in the plant and impurities. Moreover, due to the high dose used, it has low potency, which might hamper its application in the clinic in the future (Liu et al., 2009b). In addition, leonurine is a zwitterion in aqueous systems as two groups are present in its structure, i.e. the phenolic hydroxyl group accounting for the negative charge, and the guanido group responsible for the positive charge. As known, lipid soluble and positive charge containing molecules are apt in passing through cell membranes. These defects of leonurine may be adverse in being transported into cells. Thus we consider that structural modification of leonurine may improve its pharmacological efficacy by the twin drug approach. The aim of our study is to determine whether the novel twin drug, 3,5-dimethoxy-4-(3-(2-carbonyl- ethyldisulfanyl)-propionyl)-benzoic acid 4-guanidino-butyl ester (compound **2**) (Fig. 1), will provide better cardioprotection and what the underlying mechanisms are.

2. Material and methods

2.1. Animal care

Newborn sprague–dawley rats (NSDR) (1–3 days) were used in our experiments. According to the animal management rules of the Ministry of Health of the People's Republic of China, all animal experiments had been approved by the Animal Research Ethics Committee, School of Pharmacy, Fudan University.

2.2. Drugs

We synthesized the novel twin drug-compound **2** (Fig. 1) from syringic acid by acetylation and a reaction with 3-(2-carbonyl-ethyldisulfanyl)-propionyl chloride through a modified method (Liu et al., 2010a). High performance liquid chromatography showed 99% purity of compound **2**.

2.3. Primary cardiac myocyte cell culture

We cultured the primary cardiac myocytes in accordance with the method described by Wang et al. (2009). Briefly, we obtained ventricles of NSDR, isolated primary cardiac myocytes and then seeded them at the density of 1×10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) and added with 10% fetal bovine serum (FBS), 100 mg/ml streptomycin, 100 U/ml penicillin and 100 μM 5-bromodeoxyuridine (Sigma, St. Louis, MO, USA). We cultured the primary cardiac myocytes in a humidified incubator at 37 °C with 95% O_2, 5% CO_2. After 72 h, we separated the cells into several groups as follows: the normoxia group, the hypoxia group, the hypoxia+leonurine group, the hypoxia+compound **2** group, leonurine and compound **2** were added 12 h before hypoxia. We then used the technique described by Rakhit et al. (2000) to generate hypoxia. Firstly we replaced DMEM by hypoxic solution (NaCl 116 mM; KCl 50 mM; $CaCl_2$ 1.8 mM; $MgCl_2 \cdot 6H_2O$ 2 mM; $NaHCO_3$ 26 mM; $NaH_2PO_4 \cdot 2H_2O$ 1 mM) excluding the normoxia group, put them into an anaerobic chamber (BD, USA) and cultured them at 37 °C for 6 h in a humidified atmosphere of 5% CO_2, 10% H_2 and 85% N_2. In order to study the role of Akt for the cardioprotective effects of compound **2**, we used 10 μmol/l LY294002 to pretreat primary cardiac myocytes 30 min before hypoxia in some experiments.

2.4. Analysis of primary cardiac myocytes viability

We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) to measure cell viability (Li et al., 1997). Briefly, we placed dimethyl sulfoxide (DMSO) into the medium for 10 min at room temperature after a 4 h incubation with MTT at 37 °C, we then quantified the amount of MTT formazan and used a 96-well plate to detect the absorbance at 570 nm with 690 nm as reference. Primary cardiac myocytes viability had been shown as the percentage of normoxia group. In our study, we defined the viability of the normoxia group as 100%.

2.5. Levels of LDH and CK

Both of lactate dehydrogenase (LDH) and creatine kinase (CK) are indicators of primary cardiac myocytes death (Nakano et al., 1998; Wang et al., 2006). Thus, we detected the level of LDH and CK in supernatant after hypoxia after 6 h, using a colorimetric assay to measure the conversion of pyruvic acid to lactic acid by LDH and the conversion of triphosphare and creatine to phosphagen by CK respectively. Finally, we analyzed the levels of LDH and CK at 440 nm and 660 nm respectively in a 96-well plate.

2.6. Hoechst 33258 nucleus staining

We made use of Hoechst 33258 nucleus staining kit to determine the apoptosis. For morphological studies (Wu et al., 2011), we seeded primary cardiac myocytes in six-well plates. After treatment, we washed the cells twice in PBS and used the dye Hoechst 33258 (0.5 ml for 5 min at room temperature) to stain them. Following 3 washes, we examined the cells with a fluorescence microscope (Leica Microsystem, USA) at 400× magnifications.

2.7. Ultrastructure morphological changes of primary cardiac myocytes by transmission electron microscopy

We collected primary cardiac myocytes and routinely prepared them for transmission electron microscopy assay according to the method described by Liu et al. (2011). Firstly, we fixed cells in PBS including 2.5% glutaraldehyde for 2 h, post-fixed them in PBS including 1% osmium tetroxide for 1 h, and embedded them in Epon 812 after dehydration by ethanol. Then we used a LKB-V ultramicrotome (Nova, Sweden) diamond knife to cut them into ultrathin slices (60 nm) after polymerization. Finally we viewed the slices under a Philips, CM 120 electron microscope after staining them with uranyl acetate and lead citrate.