A Novel Danshensu-Tetramethylpyrazine Conjugate DT-010 Provides Cardioprotection through the PGC-1α/Nrf2/HO-1 Pathway

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In this study, we investigated the cardioprotective mechanisms of action of DT-010, a novel danshensu-tetramethylpyrazine conjugate. DT-010 significantly preserved cell viability and suppressed cell apoptosis in H9c2 cells injured by tert-butyldihydroperoxide (t-BHP), iodoacetic acid (1AA) and hypoxia-reoxygenation. In addition, DT-010 pre-treatment reduced the intracellular level of free radicals including superoxide anion (·O$_2^-$), hydroxyl radical (·OH) and peroxynitrite anion (ONOO$^-$) after t-BHP exposure. Moreover, DT-010 up-regulated the protein expression of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) and nuclear factor-E2-related factor 2 (Nrf2) as well as mitochondrial transcription factor A (Tfam) and heme oxygenase-1 (HO-1) in H9c2 cells. DT-010 also triggered Nrf2 nuclear translocation. In a rat myocardial ischemia-reperfusion model, DT-010 significantly alleviated myocardial infarction. The results indicated that DT-010 may be a promising candidate for the treatment of cardiovascular diseases, particularly myocardial ischemia and reperfusion injury.

Key words Danshensu derivative; oxidative stress; apoptosis; myocardial ischemia reperfusion injury; peroxisome proliferator-activated receptor gamma coactivator 1 alpha/nuclear factor-E2-related factor 2/heme oxygenase-1 (PGC-1α/Nrf2/HO-1) pathway

Myocardial ischemia is a primary cause of sudden death worldwide. Myocardial ischemia results from a decreased blood flow to the heart, preventing the heart from receiving adequate supply of energy. The reduced blood flow is usually caused by a partial or complete blockade of the arteries. Traditional treatments focus on restoring blood supply, such as by pass-graft surgery, dilation of coronary artery and coronary thrombolysis, and etc.1) Although restoration of blood flow to the ischemic heart tissues is important, it is not adequate because myocardial ischemic damage is progressively developed following reperfusion due to an over-production of a large number of biospecies.2,3) Compelling evidence demonstrated that free radicals generated during ischemia and reperfusion play pivotal roles in myocardial apoptosis. Free radical scavengers can effectively inhibit myocardial apoptosis. The previous studies have demonstrated that agents that combine anti-thrombolytic and anti-oxidative activities showed promising therapeutic efficacy in the treatment of myocardial ischemia.4,5)

In China, many traditional herbs have been used to prevent and treat myocardial ischemia. Among them are Salvia miltiorrhiza (Danshen) and Ligusticum wallichii (Chuanxiong). As the major active ingredients, (3-(3,4-dihydroxyphenyl) lactic acid) (Danshensu, DSS, Fig. 1) isolated from Danshen and tetramethylpyrazine (TMP, Fig. 1) isolated from Chuanxiong have a variety of biological activities. They lyse blood clot, dilate coronary arteries, inhibit platelet aggregation, scavenge free radicals, improve microcirculation and have anti-inflammatory properties.6,7) Both of them are widely used in clinic for treatment of cardiovascular diseases. Although widely used, their therapeutic benefits are severely limited due to weak activities.

To increase the cardioprotective effects of DSS and TMP, we have previously synthesized a series of DSS and TMP conjugates. We found that the compounds containing DSS and TMP linked via an ester bond displayed higher activities than DSS and TMP.8) Among them, ADTM (Fig. 1) was found to reduce infarct size in a rat model of myocardial ischemia.9) However, ADTM was quickly hydrolyzed by carboxylesterase and its half-life was too short in vivo.10) In addition, ADTM has poor water solubility. These shortcomings reduce the entusiasms for further development of ADTM as a clinical candidate. Thus, we have recently synthesized new derivatives of DSS and TMP, where a bulky hindrance was introduced on the linker between DSS and TMP.11,12) One of these compounds is DT-010 (Fig. 1). We have reported previously that DT-010 exerts antitumor activity in breast cancer cells as well as its potent protection against MPP$\textsuperscript{+}$-induced neurotoxicity.13,14)

Mitochondria are vital organelles for producing ATP and major initiators and regulators of a variety of pathological processes. Unfortunately, mitochondria are particularly vulnerable to damages caused by the over-production of free radicals.15) Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) is a master regulator of energy metabolism and cell survival by enhancing mitochondrial biogenesis and antioxidation.16) PGC-1α displays tissue-specific expression and is more strongly expressed in heart tissue.17) It is reported that mitochondria in the ischemic heart has a decreased expression of PGC-1α, and agents that increase PGC-1α expression have protective effects in ischemic heart disease.18) We here report the cardioprotection of DT-010 in vitro and in vivo and its potential mechanism of action.
MATERIALS AND METHODS

Reagents and Materials DT-010 is synthesized in our laboratory (>95%, purity). Compound danshen drip- ping pills (DSP) were purchased from Tasly Pharmaceutical Group Co., Ltd. Amlodipine besilate tablets were purchased from China Resources Saike Pharmaceutical Co., Ltd. tert-Butylhydroperoxide (t-BHP), Hoechst33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), iodoacetic acid (IAA), pentobarbital sodium were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Hydroxy Radical and Peroxynitrite Sensor (HPF), dihydroethidium (DHE) and dihydroethidium 123 (DHR123) were purchased from Thermo Fisher (Meridian Rd., Rockford, IL, U.S.A.). Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Gibco Life Technolo -gies (Grand Island, NY, U.S.A.). Annexin V-propidium iodide (PI) apoptosis detection kit was obtained from Thermo Fisher Scientific. Antibodies against mitochondrial transcription factor A (Tfam), Bcl-2, Bax, Caspase-3, Cleaved caspase-3, heme oxygenase-1 (HO-1), β-actin and secondary antibodies were from Cell Signaling Technology (Bev -lyer, MA, U.S.A.). The nuclear extraction kit was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Anaerobic Plexiglas chamber was purchased from Billups-Rothenberg (Billups-Rothenberg Inc., CA, U.S.A.).

Cell Culture The H9c2 cells were cultured in complete medium containing DMEM supplemented with 10% FBS and maintained at 37°C in a humidified incubator containing 5% CO₂. Cells were treated when 70–80% confluence reached. Primary culture of neonatal rat cardiomyocytes was performed as described previously with minor modifications. Cells were dispersed by digestion with 0.25% trypsin and agitation for 2 min at 37°C. This digestion step was repeated several times. Cells were collected by centrifugation. Isolated cells were resuspended with low-sugar DMEM with 10% FBS and cultured for 2 h in a 37°C incubator to reduce fibroblast contamination. Non-attached viable cells were collected and seeded into 96-well plates. The plates were placed in a cell incubator at 37°C with a mixed gas containing 5% CO₂ and were treated for the cell viability assay after 48 h.

Cell Viability Assay Cell viability was measured using MTT assay. Cells were seeded in 96-well plates at a density of 1×10⁴ cells per well and cultured at 37°C for 24 h. For t-BHP model, after treatment with DT-010 for 1 h, cells were exposed to 150 µM t-BHP for another 4 h, and then cell viability was measured by the MTT assay. For hypoxia reoxygenation model, cells were cultures in a low volume of substrate-free medium (serum-free, glucose-free and sodium pyruvate-free DMEM, DME base) and in an anaerobic plexiglas chamber for 4h. The cells were followed by reoxygenation for another 4h by changing for the DMEM medium and incubated in the normal CO₂ incubator. For the IAA model, cells were pre-treated with DT-010 for 1 h followed by IAA (100 µM) for 4 h. After different treatment as above, MTT was added and cells were cultured for another 4 h. The absorbance at 570 nm was measured using a microplate reader (Bio Tek Instruments, U.S.A.).

Measurement of Free Radicals Intracellular levels of hydroxyl radical (·OH), superoxide anion (O₂⁻) and peroxynitrite radical (ONOOC−) were respectively measured using the fluorescent probes HPF, DHE and DHR123 as described previously. Cells were pre-treated with DT-010 for 1 h, and were then incubated with 5 µM HPF or 5 µM DHE or 5 µM DHR123 for 30 min. Finally, cells were exposed to t-BHP (150 µM) for 1 h. Cells were washed three times with Hanks’ balanced salt solution (HBSS) and fluorescence was examined using a fluorescence microplate reader. The fluorescence of ONOO⁻ was also detected by flow cytometer (Millipore, Germany).

Hoechst33342 Staining H9c2 cells were seeded in six-well plates and were cultured in complete medium until approximate 90% confluence. Cells were pre-incubated with DT-010 for 1 h and were then exposed to 150 µM t-BHP for 4 h. Hoechst33342 (5 µM) was added and the cells were then incubated at 37°C for 10 min in the dark. The cell images were visualized using a fluorescence microscope (Olympus Corporation, Japan).

Flow Cytometric Analysis Apoptosis was detected using the Annexin V/PI kit according to the manufacturer’s instruction. After treatment with DT-010 and t-BHP, H9c2 cells were washed twice with ice-cold HBSS, and were re-suspended in binding buffer. Propidium iodide (PI) and fluorescein isothiocyanate (FITC)-labeled Annexin V were then added for 30 min at 37°C in the dark. The cells were analyzed by flow cytometer (Millipore, Germany).
Immunofluorescence  Cells were seeded in dishes. After 1.5 h of treatment with DT-010 (30 µM), cells were fixed by 4% paraformaldehyde for 15 min at room temperature. Following thrice washing with ice-cold HBSS, cells were blocked with 0.3% TritonX-100 and 1% bovine serum albumin (BSA) for 45 min. The cells were then incubated with anti-Nrf2 antibody (1 : 100) overnight at 4°C followed by 45 min of incubation with Alexa Flour 647-conjugated antibody (1 : 100) (Jackson ImmunoResearch, West Grove, PA, U.S.A.) at 37°C. To visualize the nuclei, the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min in the dark. The fluorescence images were captured using a laser scanning confocal microscope.

Preparation of Whole Cell, Cytosolic and Nuclear Extracts  H9c2 cells were pre-incubated with DT-010 for 1 h and were then cultured with ice-cold HBSS, cells were blocked with 0.3% TritonX-100 and 1% bovine serum albumin (BSA) for 45 min. The cells were then incubated with anti-Nrf2 antibody (1 : 100) overnight at 4°C followed by 45 min of incubation with Alexa Flour 647-conjugated antibody (1 : 100) (Jackson ImmunoResearch, West Grove, PA, U.S.A.) at 37°C. To visualize the nuclei, the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min in the dark. The fluorescence images were captured using a laser scanning confocal microscope.

Myocardial Ischemia Reperfusion Model in Rats  Male Sprague-Dawley (SD) rats weighing 250–270 g were randomized into ten groups. Rats were anesthetized with 5% pentobarbital sodium by intraperitoneal injection and were ventilated with a small animal respirator. Thoracotomy was performed and the left anterior descending (LAD) coronary artery was ligated with a 6–0 silk suture for 30 min followed by reperfusion. Compound Danshen dripping pills and amlodipine were used as positive controls. Drugs were orally administrated twice a day. The first administration was given at 30 min before ligation and the second was at 3 h following reperfusion. The sham control group was subjected to the same operations except ligation. Ischemia was confirmed by ST-segment elevation in limb II-lead electrocardiogram (ECG). After 24 h of ligation, the heart was stained with 2, 3,
5-triphenyltetrazolium chloride (TTC, 2%) for 5 min and were then quickly removed. The heart was sliced, and images were captured and analyzed by image J software. Infarct size was presented as the ratio of the infarct area to the whole left ventricle. All protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Jinan University.

Statistical Analysis. The experimental data were expressed as the mean ± standard error of the mean (S.E.M.) Results were analyzed by Student’s t-test or one-way ANOVA.

Fig. 3. DT-010 Scavenges ·OH, ·O₂ and ONOO⁻ Radicals
H9c2 cells were pretreated with or without DT-010 for 1 h, and were stained with HPF, DHE or DHR123 for 0.5 h followed by incubation with 150 µM t-BHP for 1 h. The intracellular levels of ·OH (A), ·O₂ (B), ONOO⁻ (C) were measured. (D) ONOO⁻ level in H9c2 cells was also detected by flow cytometry. All results are expressed as the means±S.E.M. of three independent experiments. ###p<0.001 versus control group, *p<0.05 versus the t-BHP group. **p<0.01, ***p<0.001 versus the t-BHP group.

Fig. 4. DT-010 Inhibits t-BHP-Induced Apoptosis in H9c2 Cells
H9c2 cells were pretreated with or without DT-010 for 1 h and were then incubated with 150 µM t-BHP for another 4 h. (A) Cells were stained with Hoechst33342 and images represent the apoptosis in each group. The arrows indicate apoptotic cells. Scale bar, 20 µm. (B) The percent of apoptotic nuclei stained by Hoechst was calculated from (A) in 4 randomly chosen fields. ***p<0.001 compared to the t-BHP group.
using the GraphPad Prism software (version 5.0). Differences of p<0.05 were considered as statistically significant.

RESULTS

Protective Effects of DT-010 on t-BHP/IAA/Hypoxia-Reoxygenation-Induced Oxidative Injury in H9c2 Cells and Neonatal Rat Cardiomyocytes The cardioprotective effects of DT-010 were evaluated in different models. IAA, a chemical ischemic stimulus, inhibits the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase irreversibly and induces cell death accompanied with an increase in reactive oxygen species (ROS) production, mitochondrial dysfunction and loss of ATP. As shown in Figs. 2A, B, C and D, cell viability was significantly decreased in model group compared with control group (>50%). Pre-treatment with DT-010 (10, 30 and 100 µM) significantly restored the cell viability.

DT-010 Quenched Free Radical Generation Induced by t-BHP in H9c2 Cells The effect of DT-010 on t-BHP induced intracellular free radical production was investigated. After 1 h of addition of 150 µM t-BHP, intracellular \( \cdot \text{OH}, \cdot \text{O}_2^– \) and ONOO\(^–\) levels were increased significantly. In sharp contrast, pre-treatment with DT-010 for 1 h markedly reduced the \( \cdot \text{OH} \) levels (Fig. 3A). DT-010 reduced the \( \cdot \text{O}_2^– \) level by 55.14% and 72.73% at the concentration of 10 and 30 µM, respectively (Fig. 3B). The level of ONOO\(^–\) was also decreased by 80.26% when cells were pre-incubated with 10 µM DT-010 (Fig. 3C). In Fig. 3D, fluorescence peak in t-BHP group increased significantly.
cantly and this tendency was partly inverted by pre-treatment with DT-010. These results demonstrated that DT-010 could memorably reduce the levels of free radicals in the cells induced by t-BHP.

**DT-010 Decreased t-BHP-Induced Apoptosis in H9c2 Cells**  In order to investigate whether DT-010 could inhibit t-BHP induced apoptosis, a nuclear dye Hoechst33342 was used to detect apoptotic cells. Hoechst33342 readily permeates across the cytomembrane and specifically binds to DNA in the minor groove generating a blue fluorescence. As shown in Fig. 4A, the DNA of apoptotic cells was pyknotic and the fluorescence intensity was more intense (marked by the red arrow) than the normal cells. DT-010 markedly decreased the number of condensed nuclei compared with those in the t-BHP group (Fig. 4B). Annexin V and PI staining were used to assess the influence of DT-010 on the apoptotic cell at different stages. The results shown in Figs. 5A and B exhibited that t-BHP treatment significantly increased the total number of apoptotic cells. However, DT-010 (30µM) pretreatment markedly reduced the late apoptotic cells (Annexin V-FITC positive and PI positive) induced by t-BHP. Apoptosis-related proteins were also verified by Western blot analysis. The ratio of Bcl-2/Bax was improved and the ratio of activated caspase-3 was greatly decreased after DT-010 treatment compared with the t-BHP group (Fig. 6).

**DT-010 Activates PGC-1α/Nrf2/HO-1 Pathway**  To investigate the underlying mechanism of DT-010 against oxidative damage, the effect of DT-010 on the PGC-1α/Nrf2/HO-1
pathway was tested. As shown in Fig. 7, there was an elevation in the protein expressions of PGC-1α as well as its down-stream regulators Nrf2, Tfam and HO-1 after DT-010 pretreatment, suggesting that the antioxidative effect of DT-010 was involved in activation of PGC-1α/Nrf2/HO-1 pathway.

**DT-010 Triggered Nrf2 Nuclear Translocation**

Nrf2 is a key regulator of cellular redox signaling. During oxidative stress, Nrf2 translocates into the nucleus and regulates the expression of a variety of antioxidant proteins such as HO-1. To investigate whether DT-010 triggered Nrf2 nuclear translocation, H9c2 cells were treated with 30µM DT-010 for different period of time and nucleoproteins were analyzed by immunoblot. DT-010 pre-treatment promoted the Nrf2 gathering in nucleus in a time-dependent manner (Figs. 8B, C). Immunofluorescence scanned by laser scanning confocal microscopy (LSCM) also indicated that DT-010 pre-treatment increased Nrf2 accumulation in the nucleus (Fig. 8A). These results indicated that pre-treatment with DT-010 resulted in translocation of Nrf2 from cytosol to the nucleus.

**DT-010 Attenuated Myocardial Ischemia Reperfusion (I/R) Injury in Rats**

Myocardial ischemia was established by the ligation of left anterior descending coronary artery for 30min followed by reperfusion. The area of myocardial infarction was assessed by TTC staining. Bar graph showed the ratio of infarct size to that of the whole left ventricle (LV). DT-010, DSP and Amlod were administrated twice orally. N=5–9 animals/group. *p<0.05, **p<0.01 and ***p<0.001 versus the model group.

**DT-010 Attenuated Myocardial Ischemia Reperfusion (I/R) Injury in Rats**

After findings of significant cardioprotective effects of DT-010 against t-BHP induced oxidative injury in H9c2 cardiomyocytes, we then further evaluated its therapeutic effect in a rat myocardial I/R injury model. As shown in Fig. 9, oral administration (twice daily) of DT-010 at dose of 10mg/kg significantly reduced the infarct size compared with the I/R model group. Amlodipine (Amlod) and DSP were used as the positive control. They both were used for the treatment of ischemic heart diseases in clinical. Our study showed that DT-010 at 10mg/kg possessed a similar treatment efficacy compared to Amlod (2mg/kg), the first line treatment of angina pain, and was obviously superior to DSP at 90mg/kg.

Fig. 8. DT-010 Increases the Nuclear Translocation of Nrf2 in H9c2 Cells

(A) H9c2 cells were pre-treated with DT-010 (30µM) for 90min and the nuclear translocation of Nrf2 determined by immunofluorescence staining. Left panel: Nrf2 localization shown by Nrf2-Alexa 647 staining; Middle panel: stained nucleus with DAPI; Right panel: merged images from the left and the middle filters. Scale bar: 20µm. Cells were incubated with 30µM DT-010 for different period of time. Immunoblot (B) and densitometry (C) assay showed the effect of DT-010 on the nuclear accumulation of Nrf2. All results are expressed as the means±S.E.M. of three independent experiments. **p<0.01 versus the group without DT-010 treatment.

Fig. 9. DT-010 Attenuated Myocardial Ischemia Reperfusion Injury in Rats

Myocardial ischemia was established by the ligation of left anterior descending coronary artery for 30min followed by reperfusion. The area of myocardial infarction was assessed by TTC staining. Bar graph showed the ratio of infarct size to that of the whole left ventricle (LV). DT-010, DSP and Amlod were administrated twice orally. N=5–9 animals/group. *p<0.05, **p<0.01 and ***p<0.001 versus the model group.
DISCUSSION

In the present study, we found that DT-010 significantly preserved cell viability and suppressed cell apoptosis in t-BHP/1AA/hypoxia-reoxygenation injured H9c2 cells. In addition, DT-010 markedly increased the expression of PGC-1α, Nrf2, Tfam and HO-1 and promoted the Nrf2 accumulation in nucleus, suggesting the cardioprotection of DT-010 was at least partially mediated through the activation of PGC-1α/Nrf2/HO-1 pathway.

With the widely use of coronary thrombolysis, percutaneous transluminal coronary angioplasty and coronary artery bypass grafting for the treatment of ischemic heart diseases, ischemia reperfusion injury becomes an emergent problem. 2) Ischemia reperfusion injury, in which the outbreak of ROS plays an important role, not only causes irreversible myocardial necrosis but also induces cardiomyocyte apoptosis. 3) ROS can be direct cytotoxicity or may pose potential risk of cell death by transforming into free radicals that react with macromolecules, DNA, proteins and lipids. 22) t-BHP may stimulate ROS generation and therefore was used to induce conditions of oxidative stress. Previous studies found that mitochondria, a source of ROS and a sensor of oxidative stress, play a key role in the transduction and amplification of the apoptotic response in cardiomyocytes during oxidative stress. 23) PGC-1α is a master regulator of mitochondrial biogenesis and respiration by cooperating with its downstream transcription factors such as Nrf2 and Tfam. 24,25) Given the prominent role of PGC-1α in mitochondrial function, it is therefore not surprising that PGC-1α is involved in the cellular response to I/R injury. Accumulating evidences proved that the induction of PGC-1α increased the mitochondria tolerance to hypoxia-reoxygenation injury. 26,27) Several studies have shown that low PGC-1α expression is probably involved in cell apoptosis. 28,29) Our data showed that DT-010 significantly reduced apoptotic cells and markedly increased the expression of PGC-1α, Nrf2 and Tfam, indicating that the up-regulation of PGC-1α could protect cardiomyocytes from t-BHP induced oxidative injury.

Nrf2 is a critical player in controlling cellular redox homeostasis in cardiac cells. Activation of Nrf2 is involved in protection of cardiomyocytes against oxidative stress injury. Under normal conditions, Nrf2 localizes in the cytoplasm and remains inactive by Keap1. 30) In case of oxidative stress, Nrf2 separates from Keap1, and then translocates into the nucleus. The nuclear Nrf2 binds to antioxidant response element (ARE) followed by an activation of endogenous antioxidative response. 31-32) Heme oxygenase-1 (HO-1) is a key enzymes for the intracellular ROS regulation. The introduction of HO-1 is triggered by Nrf2/ARE complex. 30) In this regard, we wonder whether the antioxidative effect of DT-010 was through Nrf2 translocation and HO-1 expression. We detected the intracellular ROS level, Nrf2 translocation as well as protein expression of HO-1. It was found that DT-010 significantly decreased intracellular ROS overproduction, up-regulated the expression of Nrf2 and HO-1 and induced Nrf2 accumulation in the nucleus. These findings suggested that the antioxidative activity of DT-010 was at least partially mediated by the induction of HO-1, which was associated with the translocation of Nrf2 from cytoplasm to nucleus.

Taken together, in this study, DT-010 protected cardiomyocytes against t-BHP induced oxidative stress by reducing ROS production and inhibiting cell apoptosis, which may be mediated through the activation of PGC-1α/Nrf2/HO-1 pathway, suggesting that DT-010 might be a promising new candidate for the treatment of ischemic myocardial injury.

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Conflict of Interest The authors declare no conflict of interest.

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