Identification of cardioprotective agents from traditional Chinese medicine against oxidative damage

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Abstract. Reactive oxygen species are damaging to cardiomyocytes. H9c2 cardiomyocytes are commonly used to study the cellular mechanisms and signal transduction in cardiomyocytes, and to evaluate the cardioprotective effects of drugs following oxidative damage. The present study developed a robust, automated high throughput screening (HTS) assay to identify cardioprotective agents from a traditional Chinese medicine (TCM) library using an H2O2-induced oxidative damage model in H9c2 cells. Using this HTS format, several hits were identified as cardioprotective by detecting changes to cell viability using the cell counting kit (CCK)-8 assay. Two TCM extracts, KY-0520 and KY-0538, were further investigated. The results of the present study demonstrated that treatment of oxidatively damaged cells with KY-0520 or KY-0538 markedly increased the cell viability in superoxide dismutase activity, decreased lactate dehydrogenase activity and malondialdehyde levels, and inhibited early growth response-1 (Egr-1) protein expression. The present study also demonstrated that KY-0520 or KY-0538 treatment protected H9c2 cells from H2O2-induced apoptosis by altering the Bcl-2/Bax protein expression ratio, and decreasing the levels of cleaved caspase-3. In addition, KY-0520 and KY-0538 reduced the phosphorylation of ERK1/2 and p38-MAPK proteins, and inhibited the translocation of Egr-1 from the cytoplasm to nucleus in H2O2-treated H9c2 cells. These findings suggested that oxidatively damaged H9c2 cells can be used for the identification of cardioprotective agents that reduce oxidative stress by measuring cell viabilities using CCK-8 in an HTS format. The underlying mechanism of the cardioprotective activities of KY-0520 and KY-0538 may be attributed to their antioxidative activity, regulation of Egr-1 and apoptosis-associated proteins, and the inhibition of ERK1/2, p38-MAPK and Egr-1 signaling pathways.

Introduction

Oxidative stress is important in various disease processes, including in cancer, inflammation, cardiovascular diseases, atherosclerosis, central nervous system disorders, neurodegenerative diseases, diabetes and respiratory diseases. Almost all human organs can be damaged by oxidative stress (1-5). In the cardiovascular system, reactive oxygen species (ROS) induce the oxidation of low density lipoprotein, cholesterol, cholesterol-derived species and protein modifications, which can lead to foam cell formation, atherosclerotic plaques and vascular thrombosis (6). Various studies have previously demonstrated that cardiomyocyte damage induced by heart ischemia/reperfusion is predominantly due to the generation of ROS (7-9). Other studies also indicated that ROS can damage contractile function and Ca2+ release by modifying the structure and function of cardiac proteins, which may be important in the formation of myocardial ischemia/reperfusion injury (9,10). Several investigations have demonstrated that ROS induce cardiomyocyte apoptosis by activating various signaling pathways, including mitogen-activated protein kinase 14 (p38MAPK), MAPK 1 (also known as ERK1/2), MAPK 8 (also known as JNK) and v-akt murine thymoma viral oncogene homolog 1 (Akt1) signaling, which may contribute to the development and progression of cardiac dysfunction and heart failure (11-13). Additionally, angiotensin II stimulates ROS-mediated activation of the transcription factor nuclear factor-kB, which is understood to be involved in the induction of cardiac hypertrophy. ROS also regulates the transcription of jun proto-oncogene, which influences the expression of other genes in cardiac hypertrophy (14). In summary, oxidative stress participates in a variety of pathological mechanisms associated with cardiomyocyte diseases.

Numerous in vitro studies of oxidative stress in cardiomyocytes have been performed using H9c2 cardiomyocytes. H9c2 cells are a clonal cardiomyocyte cell line derived from embryonic rat ventricles (15), with a similar profile of signaling mechanisms to adult cardiomyocytes. Under oxidative stress, H9c2 cardiomyocytes respond in a similar manner to myocytes in primary cultures or isolated heart experiments (16). H9c2...
cells have been demonstrated to be a useful tool for the study of the cellular mechanisms and signal transduction pathways of cardiomyocytes (17-20).

H$_2$O$_2$-treated H9c2 cells have been commonly used as an in vitro model for studying oxidative stress in cardiomyocytes, and to evaluate the cardioprotective effects of drugs against oxidative damage (21-24). However, to the best of our knowledge, H9c2 cells have not been previously used for high-throughput drug screening. The current study used this model to establish a cell-based screening assay in a high-throughput format. From a library of traditional Chinese medicine (TCM) extracts, 17 primary hits were identified, 2 of which were further validated as cardioprotective agents against oxidative damage. The present study demonstrated the used of the H$_2$O$_2$-induced cell damage model in a high-throughput screening (HTS) assay, which may be established as an efficient and low-cost HTS assay for the identification of candidate drugs that reduce oxidative damage from large TCM extract/chemical libraries.

Materials and methods

Cell culture. H9c2 cells (Cell Resource Centre of the Shanghai Institutes for Biological Sciences, Chinese Academy of Science, Shanghai, China) were maintained in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and incubated at 37°C in a humid atmosphere of 5% CO$_2$. For the study of cardiomyocytes (17-20).

For drug activity assays, 100 µl working solution, containing 20 µl lactic acid solution, 20 µl 1X INT solution and 20 µl enzyme solution, was added to each sample (total, 180 µl) for an additional 30 min incubation by gently agitating at room temperature. The maximum LDH release of target cells was determined by lysing target cells for 45 min and subsequently measuring the LDH from the culture medium. Absorbance values after the colorimetric reaction were measured at 490 nm with a reference wavelength of 655 nm, using a Flex Station 3 microplate spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA).

For MDA assays, 200 µl working solution was added to 100 µl samples for an additional 15 min incubation at 100°C, and were subsequently centrifuged at 1,000 x g for 10 min after the samples cooled to room temperature. Subsequently, 200 µl supernatant was transferred to a 96-well plate for MDA detection by Flex Station 3 microplate spectrophotometer at 532 nm absorbance with a reference wavelength of 450 nm.

For SOD assays, 180 µl working solution was added to 20 µl sample for an additional 30 min incubation at 37°C. SOD activities were detected at 490 nm with a reference wavelength of 600 nm, using the same microplate reader as before.

Western blotting. The protein expression levels of the apoptotic proteins, caspase-3, B-cell CLL/lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and the MAPK subfamily proteins, p38, JNK and ERK1/2, were detected by western blotting. A total of 1.5x10$^6$ cells/ml/well were seeded into 6-well plates. For drug activity assays, 100 µl H9c2 cells/well were seeded into 96-well plates at a density of 3.0x10$^4$ cells/ml, and incubated overnight. Each plate contained 8 negative and 8 positive control wells, and all cells, excluding the positive controls, were treated with 100 µl/well H$_2$O$_2$ (50 µmol/l) for 3 h. Following H$_2$O$_2$ treatment, 0.1 µl/well dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to the positive control wells, and 0.1 µl/well TCM extract samples were added to the all other wells, excluding the negative controls. Cells were then incubated for an additional 3 h. Cell viabilities were tested using the CCK-8 assay kit to assess drug activities.

Lactate dehydrogenase (LDH) activity, malondialdehyde (MDA) content and superoxide dismutase (SOD) activity assays. LDH, MDA and SOD were measured using the respective assay kits (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions. Briefly, 1,000 µl/well H9c2 cells were seeded into 24-well plates at a density of 3.0x10$^4$ cells/ml. Following a 3-h treatment with 50 µmol/l H$_2$O$_2$, and a 3-h incubation with 50 µmol/l quercetin and 25 µg/ml TCM extracts, the cells were centrifuged at 400 x g for 5 min, and 120 µl of the supernatant was then transferred to a 96-well plate for LDH activity determination. Subsequently, all cells were lysed and centrifuged at 1,600 x g for 10 min, the supernatants were then collected and stored at −80°C prior to MDA and SOD detection.
were then incubated with primary antibodies against rabbit anti-caspase-3 (cat. no. sc-7148; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit anti-Bcl-2 (cat. no. 2870S Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-Bax (cat. no. sc-526; Santa Cruz Biotechnology, Inc.), rabbit anti-p38 (cat. no. 9212; Cell Signaling Technology, Inc.), rabbit anti-phospho (p)-p38 (cat. no. 4631; Cell Signaling Technology, Inc.), rabbit-anti-JNK (cat. no. 9258; Cell Signaling Technology, Inc.) rabbit anti-p-JNK (cat. no. 4671; Cell Signaling Technology, Inc.), rabbit anti-ERK1/2 (cat. no. 9102S; Cell Signaling Technology, Inc.), rabbit anti-p-ERK1/2 (cat. no. 9101S; Cell Signaling Technology, Inc.) all at 1:1,000 dilution, overnight at 4˚C. The membranes were washed with TBS-T and were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.) for 2 h and visualized using a Chemi Doc XRS+ detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-tubulin was used as a loading control.

**Immunofluorescence assays for early growth response-1 (Egr-1).** H9c2 cells (100 µl/well) at a density of 5.0x10^4 cells/ml were seeded into 96-well plates. The cells were untreated (control) or incubated with 12.5 or 200 µmol/l H_2O_2 alone, or with 12.5 µmol/l H_2O_2 and 25 µg/ml active extracts for 2 h. Subsequently, the cells were fixed with 4% (v/v) formaldehyde (Amresco, LLC) in 1X PBS at room temperature for 15 min, then washed with 1X PBS 3 times, and blocked with 1% (w/v) BSA (Amresco, LLC) in 1X PBS containing 0.3% (v/v) Triton X-100 (Amresco, LLC) at room temperature for 30 min. The primary antibody against Egr-1 (cat. no. sc-110; 1:100; Santa Cruz Biotechnology, Inc.) was incubated with the cells at 37˚C for 2 h. Subsequently, cells were washed with PBS and incubated with goat anti-rabbit IgG-CruzFluor 488 (cat. no. sc-362262; 1:250; Santa Cruz Biotechnology, Inc.) at 37˚C for 1 h. Following 3 washes with 1X PBS, cell nuclei were stained using Hoechst 33258 (Sigma-Aldrich) at a final concentration of 2 µg/ml for 15 min. Fluorescent images were captured using a DMI 4000B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

**The library of TCM extracts.** Each TCM herb (500 g) was soaked in water for 1 h prior to extraction, and extraction was performed twice by boiling in 10- and 8-fold volumes of water (v/v) for 2 h. Extracts were filtered through gauze, and all crude extractions were combined and concentrated to 500 ml. The concentrates were then separated on macroporous resins (specification, Φ 5x60 cm; 1:1 weight ratio of the concentrates; HaiGuang Chemical Co., Ltd., China) by successive elution with water and different concentration gradients of ethanol (20-95%), with 3 bed volumes (BV) of eluent volume at a flow rate of 1 BV/h. Eight samples from each TCM herb were collected, and concentrated at 70˚C. Following freeze drying, 5 mg of each sample was dissolved into 200 µl DMSO, then dispensed into 96-well plates.
Statistical analysis. All data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance and Tukey’s post-hoc tests using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Creation and optimization of oxidative damage cell model for HTS.
To establish a stable HTS assay that generates reliable outcomes, the present study optimized several factors that may affect the assay results.

Sodium pyruvate-supplemented medium vs. sodium pyruvate-free medium. Sodium pyruvate, a supplement in cell culture medium, may affect the screening assays. As demonstrated in Fig. 1, when the H9c2 cells were maintained in DMEM containing 110 mg/l sodium pyruvate, 12.5-200 µmol/l H$_2$O$_2$ induced a decrease in cell viabilities (<7% ; Fig. 1A and B). However, H$_2$O$_2$ treatment of the cells in sodium pyruvate-free DMEM resulted in a more marked decrease (~40%) in cell viability (Fig. 1C and D). Therefore, sodium pyruvate-free DMEM was used during the oxidative damage model.

Concentration and incubation time. Optimization experiments were also performed to determine the optimal working concentration and incubation time of H$_2$O$_2$. H9c2 cells were exposed to varying degrees of oxidative stress by treatment with H$_2$O$_2$ for 0-4 h. The results demonstrated that H$_2$O$_2$ reduced cell viability in a dose- and time-dependent manner in the pyruvate-free groups, and exhibited an almost 40% injury at 50 µmol/l for 3 h. Higher concentrations or longer incubation time did not result in a more significant change to the OD450 values (Fig. 1C and D).

Cell density. A low number of cells per well may cause low response values, however, a large cell number is not conducive to cell growth, due to the contact inhibition. Thus, determining the appropriate number of cells is essential for drug screening. As demonstrated in Fig. 2, 1.5-4.0x10^4 cells/well treated with 50 µmol/l H$_2$O$_2$ for 3 h exhibited consistent results, whereas higher seeding densities exhibited reduced cell viability. Thus, the current study used the cell density of 3.0x10^4 cells/well for the HTS assays.

Variability and robustness of model. To assess whether the model of oxidative damage can be applied to an HTS format, the present study applied the optimized conditions to establish the H$_2$O$_2$-induced cell damage model. The data of the cell viabilities from 30 wells of positive control (H$_2$O$_2$-free) and 30 wells of negative control (H$_2$O$_2$-treated) were obtained to analyze variability between wells and the robustness of the cell model of oxidative stress using the Z’ factor, which is calculated from the following formula: 

$Z' = \left[ 1 - \left\{ (\sigma_+ - \sigma_-) \sqrt{\mu_+ - \mu_-} / \sigma_- \right\} \right] / \sigma_-$

where $\sigma_+$ and $\sigma_-$ are the standard deviations of the positive and negative controls, respectively, and $\mu_+$ and $\mu_-$ are the means of the positive and negative controls. Z’ ≥ 0.5 indicates an effective HTS method, and Z’ ≥ 0.85 indicates an optimal HTS method.

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Figure 2. Effects of cell density on the viability of H$_2$O$_2$-treated H9c2 cells. H9c2 cells (100 µl/well) at the densities of 1.5, 3.0, 4.0, 5.0, 6.0, 8.0 and 10x10^4 cells/ml were seeded into 96-well plates. The wells were treated with 50 µmol/l H$_2$O$_2$ at 37°C and 5% CO$_2$ for 3 h, and cell viabilities were then assessed using cell counting kit-8 assay, measuring absorbance at 450 nm. OD, optical density.

Figure 3. Testing the reproducibility of the oxidative damage model in an automated 96-well assay format. H9c2 cells (100 µl/well) at 3.0x10^4 cells/µl were seeded into 96-well plates. H$_2$O$_2$-free wells (30 wells) and H$_2$O$_2$-treated wells (30 wells) were used as positive and negative controls, respectively. Following treatment with 50 µmol/l H$_2$O$_2$ at 37°C and 5% CO$_2$ for 3 h, cell viabilities were assessed using CCK-8 assay by measuring absorbance at 450 nm. The data are presented as (A) CCK-8 readings from individual wells and (B) as the mean ± standard deviation of 30 wells. *P<0.001 vs. H$_2$O$_2$-free wells. OD, optical density; CCK-8, cell counting kit-8.
drug, quercetin, was used to optimize the incubation time of the cells with the drug following oxidative damage. Following 3 h treatment with 50 µmol/l $\text{H}_2\text{O}_2$, 25 µmol/l quercetin was added to H9c2 cells and incubated for an additional 1-6 h. Control group was treated with dimethyl sulfoxide only. The cell viabilities were then assessed using cell counting kit-8 assay, measuring absorbance at 450 nm. (A) The data are presented as the mean ± standard deviation of 30 wells at the different time points, and (B) as individual wells following the 3-h treatment with 50 µmol/l $\text{H}_2\text{O}_2$ and 25 µmol/l quercetin for an additional 3 h. Data from 30 wells of positive controls (H$_2$O$_2$ + quercetin) and 30 wells of negative controls ($\text{H}_2\text{O}_2$ + vehicle) were used for the calculation of $Z'$ factor and critical value. OD, optical density.

Figure 5. Identification of hits from the traditional Chinese medicine extracts library in HTS assay format. Assays were performed in the optimized automated HTS format. Representative values of the absorbance at 450 nm of 140 samples are presented. The red line indicates the mean of the negative control, and the blue line indicates the mean of negative control ± 3 standard deviations. Samples that exhibited 450 nm absorbance values above the blue line were considered to be hits. HTS, high-throughput screening.

Figure 6. Verification of the activities of the primary hits. The two hits KY-0502 and KY-0538 were further confirmed as candidates in the secondary screening assays. (A) To exclude false positive hits, (a) the primary hit molecules were added to the wells only containing sodium pyruvate-free medium without cells; (b) H9c2 cells (100 µl/well) at 3.0x10^4 cells/ml were plated into 96-well plates. H9c2 cells were treated with 50 µmol/l H$_2$O$_2$ for 3 h (model group) or incubated with 25 µmol/l quercetin for a further 1-6 h. Control group was treated with dimethyl sulfoxide only. The cell viabilities were then assessed using cell counting kit-8 assay, measuring absorbance at 450 nm. (A) The data are presented as the mean ± standard deviation of 30 wells at the different time points, and (B) as individual wells following the 3-h treatment with 50 µmol/l H$_2$O$_2$ and 25 µmol/l quercetin for an additional 3 h. Data from 30 wells of positive controls (H$_2$O$_2$ + quercetin) and 30 wells of negative controls (H$_2$O$_2$ + vehicle) were used for the calculation of $Z'$ factor and critical value. OD, optical density.
Identification of active extracts from TCM. The optimized 96-well plate HTS system was then used to identify extracts with antioxidative activity from a TCM library. After a 3-h treatment with 50 μmol/l H₂O₂, and a 3-h incubation with TCM extracts at 37°C and 5% CO₂, the cell viabilities were determined using the CCK-8 kit and absorbance measured at 450 nm. In addition to the extracts, 0.1 µl/well 25 mg/ml DMSO solution was added to the cells. The final concentration of the samples in each well was ~25 μg/ml. This concentration exhibited low cytotoxic effects on the cells (data not shown). The extracts that exhibited an OD450 value >the mean ± 3SD of the negative control (H₂O₂-treated) were considered as potential active samples. The top 17 hits from the primary screening were selected for further validation (Fig. 5).

Validation of the primary hits. The increased OD450 values observed in the HTS assay may be due to increased cell viabilities via protection of cells from oxidative damage, reduced H₂O₂ toxicity by a direct reaction with H₂O₂ in the culture medium, or extract compounds themselves may absorb at 450 nm. In order to exclude false positives, the current study further validated the primary hits. To determine whether the extract samples absorbed at 450 nm, 0.1 µl primary hit extracts were directly added to the wells of a 96-well plate containing 100 µl pyruvate-free DMEM (no cells), and incubated for additional 3 h. The results demonstrated that extracts KY-0520 and KY-0538 increased cell viability compared with H₂O₂-only treatment when in the culture media with H₂O₂, and when H₂O₂/KY-0520 and KY-0538 treatments were performed individually (Fig. 6Ab and c). Taken together, the results indicate that activities of KY-0520 and KY-0538 were due to antioxidative properties. The present study additionally measured the cell viability following treatment with KY-0520 and KY-0538 at different concentrations. H9c2 cell viability was increased by KY-0520 and KY-0538 in a concentration-dependent manner. The concentration-response curves of KY-0520 and KY-0538 demonstrated that the compounds are active between of 0.78 and 100 µg/ml (Fig. 6B), and the EC₅₀ values of KY-0520 and KY-0538 were 11.43 and 19.59 μg/ml, respectively.

Characterization of the cardioprotective activities of KY-0520 and KY-0538. The present study further investigated the antioxidant activity of the TCM extracts. The results demonstrated that H₂O₂-induced oxidative damage to H9c2 cells significantly increased LDH activity (P<0.001; Fig. 7A) and MDA levels (P<0.01; Fig. 7B), and decreased SOD activity (P<0.01; Fig. 7C) compared with the control. KY-0520 and KY-0538 treatment (25 μg/ml) significantly reduced the LDH activity (P<0.001; Fig. 7A) and MDA levels (P<0.01; Fig. 7B) compared with H₂O₂-treated cells. Additionally, KY-0520 and KY-0538 significantly increased the SOD activity levels compared with H₂O₂-treated cells (P<0.05 and P<0.001, respectively; Fig. 7C). These results suggest that KY-0520 and KY-0538 prevent the accumulation of free radicals and attenuate cardiomyocyte damage induced by H₂O₂.

TCM extracts decrease H₂O₂-induced apoptosis in H9c2 cells. Based on the cell viability and antioxidative results, the current study further examined whether KY-0520 and KY-0538 exhibited protective effects against H₂O₂-induced cell apoptosis by western blot analysis of apoptosis-associated proteins (Fig. 8A). As demonstrated in Fig. 8B and C, H₂O₂ treatment significantly increased the protein expression levels of cleaved caspase-3 (1.31-fold increase; P<0.05) and Bax (3.19-fold increase; P<0.01) compared with the untreated controls. KY-0520 and KY-0538 (25 μg/ml) significantly decreased the protein expression levels of cleaved caspase-3 (P<0.05) and Bax (P<0.01) in H9c2 cells compared with H₂O₂ treatment alone. Additionally, the protein levels of Bcl-2, an anti-apoptotic protein, were decreased following H₂O₂ treatment compared with untreated controls (P<0.05), however, compared with H₂O₂ treatment alone, Bcl-2 levels were...
significantly increased following KY-0520 and KY-0538 treatment (P<0.05; Fig. 8D). The results indicated that the extracts inhibited apoptosis by regulation of pro- and anti-apoptotic proteins in H₂O₂-treated H9c2 cells.

TCM extracts inhibit Egr-1 protein accumulation in nucleus in H₂O₂-exposed H9c2 cells. It was previously demonstrated that following exposure of H9c2 cells to 200 µmol/l H₂O₂, Egr-1 is translocated from the cytoplasm and accumulates in the nucleus (26). The present study treated H9c2 cells with 200 µmol/l H₂O₂ for 2 h, however, Egr-1 did not translocate from the cytoplasm to nucleus, it accumulated in the cytoplasm and nuclear membrane (Fig. 9A). By contrast, at an H₂O₂ concentration of 12.5 µmol/l, Egr-1 nuclear staining was increased (Fig. 9B). KY-0520 and KY-0538 (25 µg/ml) markedly decreased Egr-1 immunostaining to near basal levels, and caused Egr-1 redistribution in the nucleus and cytoplasm (Fig. 9B). These results suggested that KY-0520 and KY-0538 may protect H9c2 cells from oxidative damage by regulating Egr-1 activity.

TCM extracts inhibit ERK1/2 and p38-MAPK in H₂O₂-exposed H9c2 cells. The MAPK signaling pathways, including ERK1/2, p38 and JNK-MAPK, are critical for the regulation of apoptosis and other cellular processes. Activation of the MAPK signaling pathways is a characteristic feature of oxidant-induced apoptosis (27), and is well established in cardiac myocytes (28). In the present study, the protein levels of p-ERK1/2, p-JNK and p-p38 MAPK were measured in H9c2 cells exposed to 12.5 µmol/l H₂O₂, and the results are presented in Fig. 10. Western blot analysis demonstrated that the phosphorylation levels of p42- and p44-ERK and p38 MAPK were significantly increased by H₂O₂ (2.04-, 1.37- and 1.88-fold, respectively) compared with untreated control cells (Fig. 10B and C). However the increased phosphorylation of those kinases was reversed by 25 µg/ml KY-0520 and KY-0538 after 3 h incubation. However, no significant alterations in JNK phosphorylation were observed following 12.5 µmol/l H₂O₂ or 25 µg/ml active extracts treatment (Fig. 10D). These results indicate that KY-0520 and KY-0538 may regulate the MAPK signaling pathway to protect against H₂O₂-induced oxidative stress in H9c2 cells.

Discussion

Increasingly, studies indicate that ROS are associated with the pathogenesis and progression of various cardiovascular diseases. Sensitivity to oxidative stress is greater in the heart compared with other organs due to lower levels of antioxidant enzymes (29). The pathogenesis of cardiac hypertrophy, developed by chronic hypertrophy, is associated with ROS via regulation of the intracellular pathways linked to MAPKs and phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt (30-32). H₂O₂ is predominantly produced via the dismutation of...
superoxide anions, it can also swiftly permeate the cell membrane and react with intracellular metal ions to form toxic hydroxyl radicals, which cause DNA damage (33). Previous studies demonstrated that \( \text{H}_2\text{O}_2 \) is excessively produced during cardiomyocyte apoptosis, leading to caspase-3 activation via mitochondrial dysfunction and cytosolic release of mitochondrial cytochrome \( c \) (34).

Numerous studies have investigated natural plant compounds and TCM extracts for their antioxidant activities. Silibinin (the major active component of silymarin extracted from \( S. \text{marianum} \)) has been demonstrated to have antioxidant, antitumor and anti-inflammatory properties (35). The volatile oil of \( \text{Nardostachys Radix et Rhizoma} \) (the root and rhizome of \( \text{Nardostachys jatamansi} \) DC.) was reported to markedly suppress ROS formation and dose-dependently increase glutathione levels in \( H9c2 \) cells following oxidative injury (36). Thus, novel antioxidant agents from natural plants and TCM may be useful for the treatment of cardiac diseases.

Using \( \text{H}_2\text{O}_2 \) to treat \( H9c2 \) rat myocardial cells, the present study established a cell model of oxidative damage for HTS assay, and used the model to identify cardioprotective agents from a library of TCM extracts. The actions of the extract were determined by CCK-8 assay, which is based on dehydrogenase activity detection in viable cells, and is widely used for cell proliferation and cytotoxicity assays. The CCK-8 assay does not require washing or cell lysis, therefore, variability is minimized. It has previously been successfully applied in HTS studies as it is inexpensive and easy to operate (37). Therefore, the present study used the CCK-8 assay to evaluate the effect of TCM extracts on the viability of oxidatively damaged cells. Two hits, KY-0520 and KY-0538, were further validated as cardioprotective agents, and attenuated oxidative damage in a concentration-dependent manner (\( \text{EC}_{50} \) values, \( \sim11.43 \) and \( 19.59 \mu\text{g/ml} \), respectively).

The present study used 50 \( \mu\text{mol/l} \) \( \text{H}_2\text{O}_2 \) to induce oxidative damage in the model. Various studies have investigated
the appropriate working concentration of H<sub>2</sub>O<sub>2</sub>, however, results have varied (21,38,39). Sodium pyruvate is commonly supplemented in culture media, however, this compound can nonenzymatically react with H<sub>2</sub>O<sub>2</sub>, leading to liberation of CO<sub>2</sub>, and the conversion of α-keto acid to carboxylic acid (40,41). Therefore, the present study used H<sub>2</sub>O<sub>2</sub> diluted with pyruvate-free DMEM to induce oxidative damage in our cell-based assays.

To establish the HTS assay model, two Z' factors were required to evaluate the screening method. One was used to evaluate the robustness of the cell model, which indicates whether the cell damage model was successfully established and suitable for HTS. The other Z' factor was used to evaluate the robustness of the extract screening assay (Figs. 3 and 4). The majority of HTS assays are based on specific targets. Compared with the HTS assays designed to screen for drugs acting on specific targets, the cell damage model has advantages and limitations. Cell-based screening can directly evaluate the protective activities of drugs by measuring cell viability, however, the direct targets of the drugs and the signaling pathways involved are unclear. To understand the mechanisms of action of the drugs, it is necessary to further investigate the potential targets and signaling pathways. The effects of the drug candidates identified in the present study may be mediated by interaction with multiple targets and signaling pathways. Therefore, the cell-protective functions of KY-0520 and KY-0538 may be mediated by their antioxidant activity, and also via interaction with other pathways. Other factors and pathways associated with the effects of KY-0520 and KY-0538 may include Egr-1. Immunofluorescence demonstrated the localization of Egr-1 to be altered by KY-0520 and KY-0538 treatment under H<sub>2</sub>O<sub>2</sub>-induced oxidative stress.

H<sub>2</sub>O<sub>2</sub> is a strong oxidant that markedly decreases cell viability and increases apoptosis. The present study measured LDH activity to further investigate the cardioprotective effect of KY-0520 and KY-0538. LDH assays are widely used to quantify the level of LDH release. MDA levels and SOD activity were also measured as indicators of oxidative damage and myocardial function. KY-0520 and KY-0538 demonstrated significant antioxidant activities and protective effects on cardiomyocytes in vitro (Fig. 7). Furthermore, previous studies have demonstrated that H<sub>2</sub>O<sub>2</sub> can decrease the Bcl-2/Bax ratio and increase the level of cleaved caspase-3, therefore inducing apoptosis (42,43). Western blot analysis demonstrated that KY-0520 and KY-0538 regulate the Bcl-2/Bax ratio in H<sub>2</sub>O<sub>2</sub>-exposed H9c2 cells, and decrease the H<sub>2</sub>O<sub>2</sub>-induced cleaved caspase-3 activation (Fig. 8). These effects may contribute to the antioxidant activity of KY-0520 and KY-0538 and their protection against oxidative stress.

Egr-1 is a transcription factor encoded by an immediate early gene (44). Egr-1 is weakly expressed under normal conditions, and its expression is activated by various environmental stimuli associated with injury and stress, including growth factors, cytokines, T cell receptor ligation, hormones, thrombin,
shear stress and mechanical forces, neurotransmitters, ultraviolet light, ROS, ischemia/reperfusion and hypoxia (45-52). Egr-1 mRNA is expressed following cardioplegic arrest and reperfusion in human hearts, and in rat hearts subjected to cold cardioplegia for 40 min followed by 40 min reperfusion. The expression of Egr-1 and the downstream effects on transcription are tightly controlled, and cell-specific upregulation induced by processes such as hypoxia and ischemia, has been previously linked to multiple aspects of cardiovascular injury. Egr-1 regulates cell growth and proliferation (53,54), and positively modulates inflammation, thrombosis and apoptosis, by direct and indirect mechanisms (45,55). It was previously reported that targeting rodent Egr-1 selectively reduced the infarct size following myocardial ischemia/reperfusion. The mechanisms reported to be involved were associated with the attenuation of intercellular adhesion molecule 1-dependent inflammation and inhibition of other Egr-1-dependent molecules, including tumor necrosis factor-α (TNF-α), vascular cell adhesion molecule-1 (VCAM-1), tissue factor (TF), plasminogen activator inhibitor type 1, and p53. Inhibition of functional TF, VCAM-1 and TNF-α has been previously demonstrated to reduce the infarct size in experimental models of myocardial ischemia/reperfusion. The transcription of the pro-apoptotic factor, p53, is inhibited by Egr-1, with an associated reduction in apoptosis and infarct size (56). A previous study demonstrated that Egr-1 represses transcription from the calsequestrin (CSQ) promoter, resulting in reduced expression of CSQ, which is a major calcium storage protein critical for normal cardiac function (57). Additionally, overexpression of Egr-1 directly induced caspase activation and apoptosis in human cardiac fibroblast cultures in vitro (58). These studies indicate that inhibition of Egr-1 activity may be cardioprotective.

The findings of the present study were consistent with a previous study that demonstrated that H₂O₂ induces the translocation of Egr-1 from the cytoplasm to nucleus, thus promoting the accumulation of Egr-1 in the nuclei of H9c2 cells (Fig. 9B) (26). However, in contrast to the previous study, a high concentration of H₂O₂ (200 µmol/l) resulted in high levels of Egr-1 in the cytoplasm and nuclear membrane, rather than accumulation in the nucleus (Fig. 9) (26). The different regulatory mechanisms of Egr-1 under different concentrations of H₂O₂ are not clear. KY-0520 and KY-0538 effectively reversed the translocation of Egr-1 from the cytoplasm to the nucleus induced by 12.5 µmol/l H₂O₂ (Fig. 9). The cardioprotective activities of KY-0520 and KY-0538 appear to be associated with inhibition of Egr-1 activity and ROS scavenging.

Egr-1 expression is upregulated in response to cardiac ischemia/reperfusion stress (59). Additionally, a previous study demonstrated that Egr-1 mRNA expression in H9c2 cells was upregulated by H₂O₂ in vitro, and that the upregulation was dependent on MEK/ERK and JNK signaling (26,60). ERK1/2 is a component of the classical MAPK pathway that was previously demonstrated to be directly activated by high levels of ROS (including xanthine oxidase-derived H₂O₂) leading to transcription of Egr-1 (60-62). Thus, the present study investigated whether these pathways are modified during H₂O₂-induced oxidative stress. Consistent with previous studies (26,63), the western blot analysis of the present study indicated that the phosphorylation levels of ERK1/2 and p38-MAPK kinase were increased following 12.5 µmol/l H₂O₂ treatment (Fig. 10). Therefore, it is speculated that ERK1/2 and p38-MAPK may be important upstream regulators that mediated Egr-1 modulation during cardiomyocyte oxidative stress. The results of the present study indicated that the antioxidative effects of KY-0520 and KY-0538 may be mediated by suppression of the ERK1/2, p38-MAPK/Egr-1 signaling pathways in H₂O₂-induced oxidative stress.

In summary, the hits from the HTS assays may generate novel drugs that have the potential to be used as therapeutics for cardiomyocyte diseases, including heart ischemia/reperfusion, cardiac hypertrophy, cardiac dysfunction and heart failure. The present study established and validated a H₂O₂-induced cell damage model for use in HTS, however, further mechanistic research is required to understand the effects of the identified hits. Further investigation of the activity of the hits will be performed using primary cardiomyocyte cells or appropriate animal models.

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