Sodium Ferulate Prevents Daunorubicin-Induced Apoptosis in H9c2 Cells via Inhibition of the ERKs Pathway

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Key Words
Sodium Ferulate • Daunorubicin • Cardio-protection • Apoptosis • ERKs • H9c2 cell

Abstract

Background: Daunorubicin (DNR)-induced cardiotoxicity, which is closely associated with cardiomyocyte apoptosis, limits the drug’s clinical application. The activation of the extracellular regulated protein kinases (ERKs) pathway is responsible for the pro-apoptosis effect of DNR. Sodium ferulate (SF) has recently been found to attenuate both DNR-induced cardiotoxicity and mitochondrial apoptosis in juvenile rats. Nonetheless, the precise mechanism underlying SF-induced cardio-protection remains unclear. Methods: The DNR-injured H9c2 cell model was prepared by incubating the cells in 1 μM DNR for 24 h. Amounts of 15.6, 31.3 or 62.5 μM SF were simultaneously added to the cells. The effect of SF on the cytotoxic and apoptotic parameters of the cells was studied by monitoring apoptosis regulation via the ERKs pathway. Results: SF attenuated DNR-induced cell death (particularly apoptotic death), cTnI and β-tubulin degradation, and cellular morphological changes. SF reduced mitochondrial membrane potential depolarization, cytochrome c leakage, and caspase-9 and caspase-3 activation. SF also decreased ERK1/2, phospho-ERK1/2, p53 and Bax expression and increased Bcl-2 expression. These effects were similar to the results observed when using the pharmacological ERKs phosphorylation inhibitor, AZD6244. Conclusion: We determined that SF protects H9c2 cells from DNR-induced apoptosis through a mechanism that involves the interruption of the ERKs signaling pathway.

Introduction

Daunorubicin (DNR), a commonly prescribed anthracycline antibiotic, is an indispensable component of various chemotherapeutic regimens that are used to treat...
specific types of leukemia (i.e., acute myeloid leukemia and the blastic phase of chronic myelogenous leukemia). However, the clinical application of DNR is seriously limited by its cumulative and dose-dependent cardiotoxicity, which may manifest as potentially fatal dilated cardiomyopathy and congestive heart failure [1, 2]. In spite of the tremendous efforts by clinicians, there are few pharmacological remedies for these cardiotoxic side effects without compromising the antitumor efficacy of DNR [3, 4]. Further research is warranted to seek a novel cardio-protective agent that targets the molecular pathogenesis of DNR-induced cardiotoxicity [5].

DNR-induced cardiotoxicity is thought to be a complex multifactorial process. Increasing amounts of evidence indicate that cardiomyocyte apoptosis may be a prominent contributor to the progression of anthracycline cardiomyopathy [6-8]. However, the signal transduction pathway associated with DNR-induced cardiomyocyte apoptosis and cardiac injury is not completely understood. Recent studies have determined that extracellular regulated protein kinases (ERKs), a subfamily of the mitogen-activated protein kinases (MAPKs), may play an important role in anthracycline-induced cardiotoxicity by exacerbating cardiomyocyte apoptosis and cardiac pathologies [9-11]. Anthracycline induces the activation of ERKs, followed by the activation of the pro-apoptotic proteins p53 and Bax and the suppression of the anti-apoptotic protein Bcl-2, a process that ultimately results in cardiomyocyte apoptotic death [11]. Conversely, the inhibition of ERKs may protect against anthracycline cardiotoxicity, as demonstrated previously in multiple in vivo and in vitro studies [12, 13]. Thus, an ERKs-targeted anti-apoptotic mechanism has been deemed a promising protective strategy against DNR-induced cardiotoxicity.

Sodium ferulate (SF), the sodium salt of ferulic acid (3-methoxy-4-hydroxy-cinnamate sodium), is an effective ingredient in Chinese medicinal herbs such as Angelica sinensis, Ferula asafoetida and Lignsticum chuangxiong [14, 15]. It exhibits several pharmacological activities such as anti-oxidative, anti-inflammatory, platelet aggregation inhibitory, antihypertensive and anti-apoptotic effects [16-18]. SF has been approved by the State Drug Administration of China as a treatment for ischemic injury, atherosclerosis and diabetic damage [19-21].

A recent study from our laboratory demonstrated that SF strongly attenuates DNR-induced cardiomyopathy, cardiac dysfunction and mitochondrial myocardial apoptosis in juvenile rats [22]. However, other protective effects of SF, such as the molecular mechanisms underlying its anti-apoptotic effects that protect against DNR-induced cardiotoxicity, remain unknown and warrant further investigation. Increasing amounts of evidence have highlighted the protective effects of SF against cardiomyocyte apoptosis [23, 24]. This anti-apoptotic activity may result from the properties of SF, which is involved in free radical scavenging, the regulation of Ca\textsuperscript{2+} handling, mitochondrial protection and the apoptotic signaling [20, 25-27]. In particular, Ma et al. discovered that SF inhibited radiation-induced apoptosis by blunting the activity of ERKs [28, 29]. Based on these findings, we thereby hypothesized that SF may alleviate DNR-induced cardiotoxicity by inhibiting the cardiomyocyte apoptosis mediated by ERKs.

To test this hypothesis, the current study was performed to assess the possible protective and apoptotic inhibitory effects of SF by using a DNR-induced rat cardiomyoblast H9c2 cell model [30]. The effects of SF on mitochondrial dysfunction and the regulation of mitochondrial apoptosis via the ERKs pathway were also explored to uncover the potential mechanisms underlying its anti-apoptotic effect.

**Materials and Methods**

**Reagents**

Daunorubicin (DNR) was obtained from Pfizer, Inc. (NY, NY, USA). Sodium ferulate (SF) was kindly provided by Rejuvenation Pharmaceutical Co., Ltd. (Fuzhou, China). All chemicals were reconstituted in sterile saline immediately prior to their use. AZD6244, a mitogen protein kinase (MEK1) inhibitor that also
inhibits ERK1/2 phosphorylation, was purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in DMSO.

**Cell culture and viability assay**

One rat cardiomyoblast H9c2 cell line and two human carcinoma cell lines (promyelocytic leukemia HL 60 cells and erythroleukemia K562 cells) were used in this study. The cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China); the H9c2 cell line was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Invitrogen, Carlsbad, CA, USA), and the HL 60 and K562 cell lines were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640, Gibco, Invitrogen, Carlsbad, CA, USA). Each cell solution contained 10% fetal bovine serum (HyClone, Thermo Fisher Scientific Inc., South Logan, UT, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin and was maintained in a humidified incubator at 37 °C with 5% CO₂.

For the viability assay, the cells were seeded into 96-well culture plates at densities of 2.5 × 10³ cells/mL. Twenty-four hours after plating, the cells were treated with 1 μM DNR in either the absence or the presence of varying concentrations of SF for 24 h. Cell viability was assessed by adding 500 μg/mL of MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, St Louis, MO, USA] to the medium for 4 h. The supernatant was removed carefully, and 200 μL of DMSO was added to each well to dissolve the insoluble formazan product into a colored solution. The absorbance was measured at 570 nm in a microplate reader (Els800, BioTek, Winooski, VT, USA). The following equation was used to measure cell viability: cell viability = OD treatment group/OD control group × 100%. The IC₅₀ values were determined from the dose-response plots by linear regression. Each of the experiments was performed at least three times.

**Annexin V/PI double-staining assay**

An FITC Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA) was utilized to determine the numbers of apoptotic cells. The cells were collected, washed and then re-suspended in 1× binding buffer containing 5 μL of FITC annexin V and 5 μL of propidium iodide (PI). Following a short incubation period of 15 min in the dark at room temperature, the fluorescence of the cells was immediately analyzed using FACSVerse flow cytometry (BD Biosciences, San Jose, CA, USA). The fluorescence emission signals for FITC annexin V were plotted against PI to distinguish among viable cells (i.e., annexin V and PI negative), early apoptotic cells (annexin V positive and PI negative) and late apoptotic or necrotic cells (both annexin V and PI positive). An analysis was performed using the FlowJo 7.6 analysis software (Treestar Inc., Ashland, OR, USA). Each measurement was performed in triplicate.

**Mitochondrial membrane potential assay**

Mitochondrial membrane potential (ΔΨm) was monitored using the mitochondrial sensitive probe rhodamine 123 (Sigma-Aldrich, St Louis, MO, USA), which accumulated in the mitochondrial matrix in a manner dependent on the organelle's electrochemical gradient. The depolarization of ΔΨm results in the loss of rhodamine 123 from the mitochondria and in a corresponding decrease in intracellular green fluorescence. Following the indicated treatments, the H9c2 cells were incubated with 10 μg/mL rhodamine 123 for 20 min, followed by three washes with cold PBS to remove the extracellular rhodamine 123. Data acquisition and analysis were executed using FACSVerse flow cytometry (excitation and emission wavelengths of 488 and 525 nm, respectively). Fluorescence intensity was quantified based on the positive rate of rhodamine 123. Changes in rhodamine 123 fluorescence were calculated using an index of variation (IV), as determined by the following formula: IV = (MT - MC)/MC, where MT is the median of fluorescence for the treated samples, and MC is the median for the control samples [31]. Negative IV values paralleled the depolarization of the mitochondrial membrane. All experiments were repeated at least three times; representative data are presented in the appropriate figures and tables.

**Western blot analysis**

The cells were collected and lysed in 0.1 mL of ice-cold RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) on ice for 30 min. The total protein concentration was measured using a bicinchoninic acid assay (Beyotime Biotechnology, Shanghai, China). The protein samples (30 μg) were subjected to 10
% SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with a blocking solution (5% (wt/vol) nonfat dry milk in Tris buffered saline with Tween 20) for 1 h at room temperature. The membrane was then incubated at 4 °C overnight with anti-cTnI (1:1000 dilution, Santa Cruz, Dallas, TX, USA), β-Tubulin (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA), cytochrome c (1:1000 dilution, Santa Cruz, Dallas, TX, USA), ERK1/2 (1:1000 dilution, Santa Cruz, Dallas, TX, USA), P-ERK1/2 (1:500 dilution, Santa Cruz, Dallas, TX, USA), p53 (1:1000 dilution, Santa Cruz, Dallas, TX, USA), Bax (1:500 dilution, Santa Cruz, Dallas, TX, USA), Bcl-2 (1:500 dilution, Santa Cruz, Dallas, TX, USA), GAPDH (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA) and VDAC1 (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA) primary antibodies. The membrane was then washed and probed with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution, Cell Signaling Technology, Danvers, MA, USA). The immunoreactive proteins were visualized using a BeyoECL Plus Kit (Beyotime biotechnology, Shanghai, China). Protein bands were quantified via densitometry using Quantity One software (Bio-Rad, Hercules, CA, USA).

**Mitochondrial cytochrome c, Bax and Bcl-2 assay**

Mitochondrial extracts were prepared using the Cell Mitochondria Isolation Kit (Beyotime Biotechnology, Shanghai, China) according to manufacturer’s instructions. Purity of the mitochondrial fraction was assessed by VDAC1 (a mitochondrial marker) and GAPDH (a cytosolic marker). The levels of cytochrome c, Bax, and Bcl-2 in mitochondrial protein were subsequently immunodetected via Western blot analysis.

**Enzymatic assay for caspase-3 and caspase-9 activity**

Caspase activity was detected using the caspase-3 and caspase-9 Colorimetric Assay Kit (KeyGen Biotech CO., Ltd., Nanjing, China) and standard procedures. The cells (3×10⁴) were collected and then resuspended in lysis buffer. The supernatants were subsequently extracted, and the protein concentrations were measured using the BCA method. An equal amount (100 μg) of protein was diluted to 50 μL lysis buffer for each assay. The reaction mixture contained 50 μL of 2× reaction buffer, 50 μL of cell lysates and 5 μL of chromogenic substrates. The reaction was carried out at 37 °C for 4 h; the absorbance in the wells was measured at 405 nm by a microtiter plate reader. The fold increases in the enzymatic activity of both caspase-3 and caspase-9 were determined by comparing these results with the level of the un-induced control.

**Statistical analysis**

Data are presented as the means ± S.E. Statistical significance was evaluated using Student’s t-tests and ANOVA where appropriate. Statistical significance was defined as P<0.05. The calculations were performed using SPSS 11.0 statistical software (SPSS Inc., Chicago, IL, USA). Curve fitting was performed using SigmaPlot 11.0 software (Systat Software Inc., Chicago, IL, USA).

**Results**

**SF depressed DNR-induced cell death in H9c2 cells**

We treated the H9c2 cells with SF (15.6, 31.3 and 62.5 μM) in the absence of daunorubicin for 24 h. As shown in Fig. 1A, SF alone did not cause any statistically significant changes in cell viability, which indicated that SF itself did not exert any cytotoxic effects on the H9c2 cells.

Cell viability was subsequently examined following the incubation of the combination of SF and DNR to analyze the protective effects exerted by SF against DNR-induced cytotoxicity. DNR treatment triggered H9c2 cell death. As shown in Fig. 1B, cell viability decreased to 70.9±2.6 % following exposure to 1 μM DNR for 24 h. SF attenuated DNR-induced growth inhibition. The viabilities increased by 15.59 %, 83.96 %, and 78.40 % for the SF concentrations of 15.6, 31.3 and 62.5 μM, respectively. Significant differences were noted at doses of 31.3 and 62.5 μM (cell viability: 95.3±1.8 % and 93.7±2.2 %, respectively) compared to treatment with DNR alone (P<0.05, n=3). No significant difference in the dose-response was noted between the concentrations of 15.6 μM and 62.5 μM.
SF did not undermine the antineoplastic effect of DNR in either the HL 60 cells or the K562 cells

DNR is most commonly used to treat specific types of leukemia, including acute myelogenous leukemia and the blastic phase of chronic myelogenous leukemia. Thus, HL 60 and K562 cell lines were used to exclude the possible interference of SF with DNR’s antineoplastic effects in the setting of cancer therapy.

For these two cell lines, the viabilities of SF and DNR co-treated cells were 16.30±1.15 % and 15.6±1.12 %, 14.44±0.87 %, and 55.72±0.93 %, and 15.05±0.63 % and 54.35±1.67 % at the concentrations of 15.6 μM, 31.3 μM, and 62.5 μM SF, respectively (Fig. 2). As shown in Fig. 2, no significant difference was observed among these results compared with DNR treatment alone.

The IC₅₀ values of the growth inhibition of the HL 60 and K562 cell lines by DNR in the absence and presence of 15.6, 31.3, or 62.5 μM SF were subsequently determined. After incubation for 24 h, DNR had significant and dose-dependent anti-proliferative effects on both cell lines. The IC₅₀ values were 328.6±18.6 and 512.7±21.5 nM in HL 60 and K562 cells, respectively (Table 1). The presence of 15.6, 31.3 and 62.5 μM SF had little or no effect on the IC₅₀ value for DNR in HL 60 and K562 cells (P>0.05, Table 1). Our data indicated that SF did not decrease the antineoplastic activity of DNR in these two carcinoma cell lines.

SF reversed DNR-induced morphological changes

Cell morphology was examined using Wright’s Giemsa staining. As shown in Fig. 3A, untreated H9c2 myoblasts were spindle-to-stellate-shaped cells with integral and clear

Table 1. IC₅₀ values of the growth inhibition of HL 60 and K562 cell lines by DNR in the absence and presence of 15.6, 31.3 or 62.5 μM SF. Data are expressed as the means ± S.E and represent three independent experiments.

<table>
<thead>
<tr>
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<th>HL 60 (nM)</th>
<th>K562 (nM)</th>
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<tr>
<td>DNR</td>
<td>328.6±18.6</td>
<td>512.7±21.5</td>
</tr>
<tr>
<td>DNR + 15.6 μM SF</td>
<td>341.7±12.4</td>
<td>503.6±19.8</td>
</tr>
<tr>
<td>DNR + 31.3 μM SF</td>
<td>321.4±23.2</td>
<td>537.3±17.2</td>
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<tr>
<td>DNR + 62.5 μM SF</td>
<td>334.8±19.1</td>
<td>494.8±23.5</td>
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Fig. 3. The effect of SF on DNR-induced cellular injury in H9c2 cells. H9c2 cells were treated with 1 μM DNR and co-treated with different concentrations of SF (15.6, 31.3 and 62.5 μM) for 24 h. A: The morphology of H9c2 cells treated with DNR and SF. Representative images of Wright’s Giemsa staining are presented. Magnification>400. B: A representative image of cTnl and β-Tubulin protein expression as determined by Western blotting. C: The results of an optical density analysis of protein bands. Data are expressed as the means ± S.E and represent three independent experiments. * P< 0.05, ** P< 0.01 vs. Control; * P< 0.05, ** P< 0.01 vs. DNR.

structures. Their incubation with DNR caused alterations in cell shape, including cell shrinkage, cell rounding and membrane blebbing, as well as the development of multiple constrictions. The apoptotic cells, which exhibited deep blue karyotin staining, chromatin margination, apoptotic bodies, and karyopyknosis, were also observed. In contrast, co-treatment with SF produced a reversal of the DNR-induced morphological changes, particularly following the 31.3 and 62.5 μM SF incubations, as the cells appeared to recover their normal shapes.

**SF mitigated the cTnl and β-Tubulin degradation caused by DNR**

cTnl and β-Tubulin are key components of myofilaments and microtubules, respectively. The release of cardiac troponin (cTnl) is a sensitive and specific marker of myocardial injury and is associated with contractile dysfunction [32-34]. β-Tubulin degradation contributes to the development of cardiac arrhythmia, Ca2+ handling disorders and stress-strain effects in anthracycline-injured hearts [35].

In this study, DNR-induced cell injury was accompanied by the degradation of cTnl and β-Tubulin (P<0.01, Fig. 3B and 3C). A combination of different concentrations of SF and 1 μM DNR prevented the degradation of both cTnl and β-Tubulin (P<0.05, vs. DNR).

**SF attenuated DNR-mediated cellular damage via apoptosis inhibition**

By measuring apoptotic cell death (i.e., early and late apoptosis) via the FACS-based quantization of annexin V-positive cells, we observed that DNR largely promoted apoptotic cell death. The percentage of cells labeled with annexin V (+) PI (-) and annexin V (+) PI (+) increased from 3.13±0.41 % to 21.63±2.90 % (P<0.01, DNR vs. Control) (Fig. 4A and 4B).
SF limited the pro-apoptotic effects of DNR on mitochondrial membrane potential collapse, mitochondrial cytochrome c release, and caspase-9 activation

Mitochondrial membrane potential collapse, cytochrome c release and subsequent caspase-9 and caspase-3 activation lead to apoptosis. This cascade has been proven to be the primary apoptotic pathway in anthracycline-treated H9c2 cells [36]. Consistent with previous reports, the exposure of H9c2 cells to DNR (1 µM) for 24 h resulted in the same types of events, as follows: the dissipation of Rhodamine 123 fluorescence (IV=−0.34), the reduction of mitochondrial cytochrome c content, and the amplification of caspase-9 enzymatic
activity (P<0.01 vs. Control) (Fig. 5). Conversely, adjunct treatment with SF facilitated the gradual recovery of mitochondrial membrane potential, as demonstrated by the increased rhodamine 123 fluorescence intensity, with IV values of -0.27, -0.17 and -0.17 (Fig. 5A and 5B). Meanwhile, the mitochondrial proportions of cytochrome c increased by 11.11 %, 52.20 and 63.93 % compared with DNR (Fig. 5C and Fig. 5D). SF also decreased the expression of caspase-9 enzymatic activity (Fig. 5E). The changes were statistically significant at the three concentrations indicated previously (P<0.05, vs. DNR). These results suggested that
Fig. 6. The effects of SF on the expression patterns of apoptosis regulatory proteins induced by DNR in H9c2 cells. A: Representative ERK1/2, P-ERK1/2, p53, Bax and Bcl-2 immunoblots of the whole cell lysate. Equal protein loading was confirmed by GAPDH levels. A quantitative analysis of the ratios of ERK1/2 and P-ERK1/2 (B), p53 (C) and Bax and Bcl-2 (D) to GAPDH. A quantitative analysis of the ratios of P-ERK to ERK1/2 (E) and Bax to Bcl-2 (F). G: Redistribution of Bax and Bcl-2 in mitochondria. Cells were treated with 1 μM DNR in the absence and presence of 62.5 μM of SF for 24 h. Subcellular fractions of mitochondria were prepared and subjected to Western blot analysis. Mitochondrial marker VDAC1 immunoblot was used as a control for equal loading. H: Mean intensity of mitochondrial Bax and Bcl-2 determined by densitometric analysis. Data are expressed as the means ± S.E and represent three independent experiments, # P<0.05, ## P<0.01 vs. Control; * P<0.05, ** P<0.01 vs. DNR.

the SF-mediated cardio-protection observed during DNR treatment is associated with the preservation of mitochondrial function.

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Fig. 7. The effects of SF on the expression of P-ERK1/2 (A), p53 (B), Bax (C), and Bcl-2 (D) during the time course. The H9c2 cells were treated with 1 µM DNR in the absence and presence of 62.5 µM of SF for 24 h. Representative Western blots are shown at left. The results of an optical density analysis of protein bands are shown at right. Data are expressed as the means ± S.E and represent three independent experiments, * P<0.05, ** P<0.01, DNR vs. DNR+SF.

SF exerted a regulatory effect on the expression of ERK1/2, phosphoryl-ERK1/2, p53, Bax and Bcl-2 in DNR-treated H9c2 cells

We demonstrated that SF markedly reduced DNR-induced cardiotoxicity, a phenomenon that was largely dependent on SF’s ability to inhibit apoptosis. DNR causes the activation of extracellular signal-regulated kinases (ERKs), which reportedly triggers a mitochondrial apoptotic pathway. Accordingly, we investigated the mechanism underlying the protective effect of SF against DNR-induced myocardial apoptosis, with an emphasis on the ERKs signaling pathway.

With this aim, the expression levels of the apoptosis-related proteins ERK1/2, phosphoryl-ERK1/2, p53, Bax, and Bcl-2 were examined. In these experiments, DNR induced apoptosis, which was accompanied by conspicuous increases in phosphorylated ERK1/2, p53, and Bax protein content and by decreases in the expression of Bcl-2 (Fig. 6A-D). The active proportion of ERKs (Ratio of P-ERK1/2 to ERK1/2) was increased by 248% (P<0.01, Fig. 6E). We further detected Bax and Bcl-2 expression in mitochondria by immunoblotting. As shown in Fig. 6G and 6H, DNR enhanced Bax accumulation in mitochondria but depressed the proportion of mitochondrial Bcl-2 (P<0.01), which suggested the translocation of Bax and Bcl-2 in mitochondria and the activation of pro-apoptotic Bax.

SF co-treatment neutralized the DNR-mediated disorder noted in the mentioned apoptosis regulatory proteins. As illustrated in Fig. 6A and 6B, SF inhibited the phosphorylation of ERK1/2, which was accompanied by a decreased total ERK1/2 expression level. The middle and high doses of SF (31.3 µM and 62.5 µM) each exerted a marked effect (P<0.05, vs.
**Fig. 8.** The effects of AZD6244 on the expression patterns of apoptosis regulatory proteins induced by DNR. The H9c2 cells were treated with vehicle (CON), 1 µM DNR (DNR), 1 µM AZD6244 (ADZ), 62.5 µM SF (SF) or a combination of 1 µM DNR and 62.5 µM SF (D+S) for 24 h, with AZD6244 pre-incubation for 1 h followed by DNR (D+A) or DNR and SF co-incubation (D+A+S) for 24 h. A: A representative image of a Western blot. A quantitative analysis of the ratios of P-ERK1/2 (B), p53 (C), Bax (C) and Bcl-2 (D) to GAPDH. Data are expressed as the means ± S.E and represent 3 independent experiments, * P<0.05, ** P<0.01.

DNR). Similarly, SF incubation suppressed the expression of both p53 and Bax but prevented a decrease in the expression of the Bcl-2 protein (P<0.01, vs. DNR) (Fig. 6A, 6C, and 6D). By statistically calculating the Bax to Bcl-2 ratio with respect to the protein levels, SF was shown to facilitate a recovery from the DNR-induced decreases in the protein expression of the Bax/Bcl-2 ratio (Fig. 6F). Consistent with the results obtained for the whole cell lysate, the DNR-mediated redistribution of Bax and Bcl-2 in mitochondria was effectively blocked by SF co-treatment (Fig. 6G and 6H).

Moreover, by monitoring these proteins during a time course, we observed that DNR produced a time-dependent activation of ERK1/2, p53 and disturbed the expression of Bax, Bcl-2 (Fig. 7). The effects of DNR on these proteins were blunted by SF. Compared with DNR alone treatment, the suppression of both phospho-ERK1/2 and downstream p53, respectively, began at 4 h and 12 h of SF incubation (Fig. 7A and 7B), whereas the restoration of Bax and Bcl-2 began at 16 h of SF incubation (Fig. 7C and 7D). SF-induced ERK1/2 inhibition was a pioneer in these subsequent events.

The effects of AZD6244 on DNR-treated H9c2 cells

To determine whether the inhibition of extracellular signal-regulated kinase (ERK) is involved in the protective effect exerted by SF, H9c2 cells were pretreated with 1 µM AZD6244, a selective inhibitor of ERK1/2 phosphorylation, for 60 min before being exposed to 1 µM DNR for 24 h.
As shown in Fig. 8A and 8B, AZD6244 pre-incubation effectively decreased DNR-induced ERKs phosphorylation by 68.58% (DNR+AZD6244 vs. DNR, *P*<0.01). In the presence of AZD6244, DNR-induced growth inhibition was compromised, as evidenced by decreased apoptotic cell numbers and improved cell viability (*P*<0.01, Fig. 9). Following ERKs inactivation, both p53 and Bax overexpression and Bcl-2 degradation were prevented by AZD6244 pre-exposure (Fig. 8 C-E). These findings were similar to the results observed when SF was co-incubated with DNR. When AZD6244 was combined with SF, cell viability increased to 94.13±3.62%, and the apoptosis rate decreased to 7.13±1.06% (DNR+AZD6244+SF vs. DNR, *P*<0.01, Fig. 9). Based on the results of our statistical analysis, the combined use of AZD6244 and SF exerted a stronger protective effect than did AZD6244 treatment alone (DNR+AZD6244+SF vs. DNR+AZD6244, *P*<0.05), but no statistically significant difference was noted when these results were compared with the results of SF treatment alone in DNR-damaged H9c2 cells (DNR+AZD6244+SF vs. DNR+SF, *P*<0.05, Fig. 9).

Additionally, both AZD6244 alone and AZD6244 in combination with SF were more effective than SF treatment alone in diminishing DNR-induced ERKs over-activation, as evidenced by a lower phospho-ERK1/2 level (DNR+AZD6244+SF or DNR+AZD6244 vs. DNR+SF, *P*<0.05) but equally suppressed p53 (*P*<0.05, Fig. 8A-C). It is noteworthy that SF was more effective at restoring Bax and Bcl-2 (DNR+AZD6244+SF or DNR+SF vs. DNR+AZD6244, *P*<0.05) (Fig. 8D and 8E), which was consistent with the lower apoptosis rate noted in the presence of SF. Collectively, all of these data indicated that the inhibition of the ERKs signaling pathway is one of the cardio-protective and anti-apoptotic mechanisms facilitated by SF. However, additional mechanisms may also exist.
Discussion

In the current study, we attempted to assess the protective effect of SF against DNR-induced cardiotoxicity and cardiomyocyte apoptosis using an in vitro model of an H9c2 cell line. The results of our study indicated that SF co-treatment effectively attenuated DNR-induced cytotoxicity (particularly apoptotic death) and reduced cTnI and β-Tubulin degradation and morphological changes in DNR-damaged H9c2 cells. At doses that elicited protection, the combination of SF and DNR did not diminish DNR's antineoplastic activity. SF curbed the DNR-induced mitochondrial membrane potential collapse, cytochrome c release and caspase-9 and caspase-3 activation, which implied that the mitochondrial apoptotic pathway was inhibited. These changes were associated with the inhibition of the ERKs signaling pathway. SF quelled the over-activation of both ERK1/2 and p53 and facilitated the up-regulation of Bcl-2 and the down-regulation of Bax, which enhanced the cellular anti-apoptotic effects indicated above. Therefore, we illustrated that SF protected H9c2 cells from DNR-induced mitochondrial apoptosis via a mechanism that entails the interruption of the ERKs signaling pathway.

Anthracyclines predispose cardiomyocytes to apoptotic death. Excessive apoptosis destroys myocytes and eventually leads to the development of cardiac dysfunction and heart failure [7]. Consistent with the results of our recent report, this paper further demonstrated SF's anti-apoptotic efficacy [37]. We found that DNR treatment incited apoptosis in H9c2 cells, as demonstrated by the dramatically improved positive percentages of annexin V staining and caspase-3 enzymatic activity levels noted during this study. Remarkably, SF counteracted the pro-apoptotic effects of DNR and may therefore protect against DNR-induced cardiotoxicity. In response to SF co-treatment, the percentages of apoptotic cells decreased by 14.42 %, 50.58 %, and 61.58 %, respectively. These results were further demonstrated by the decreased enzymatic activity of caspase-3, which acts as an essential executor and biomarker of apoptosis in mammalian cells.

The mitochondrial apoptotic pathway is more active in anthracycline-injured H9c2 myoblasts and therefore contributes to anthracycline-induced toxicity in immature hearts [36]. Cardiac mitochondria are established key mediators of anthracycline-induced cardiomyocyte apoptosis [6, 38]. DNR accumulates and generates ROS in mitochondria, causing mitochondrial damage and dysfunction [38, 39]. Mitochondrial damage may trigger cardiomyocyte death by activating the mitochondrial intrinsic apoptotic pathway, which leads to mitochondrial permeability transition (MPT), mitochondrial membrane potential dissipation (ΔΨm) and cytochrome c release. Following its release from mitochondria, cytochrome c activates both caspase-9 and caspase-3, which ultimately initiate apoptotic cascades [6]. This paper also described the activation of the mitochondrial intrinsic apoptotic pathway, as evidenced by decreased mitochondrial membrane potential, decreased cytochrome c content in the mitochondrial membrane and amplified caspase-9 enzymatic activity in DNR-treated H9c2 cells.

Although the precise molecular mechanisms by which anthracyclines promote mitochondrial apoptosis are not yet fully understood, the available information indicates that the ERKs signal transduction pathway plays a pivotal role in the process [9-11, 40, 41]. Extracellular signal-regulated kinases (ERKS), which are activated by DNA damaging agents, are responsible for the anthracycline-induced p53 activation observed in H9c2 cells [11]. The tumor suppressor p53 not only promotes mitochondrial dysfunction and cytochrome c release but also modulates the expression of the BCL-2 family proteins, thereby activating the mitochondrial apoptotic pathway [6, 11, 40]. p53 increases the abundance of Bcl-2 but decreases Bax, which results in an improved Bax/Bcl-2 ratio that directs cardiomyocytes toward apoptotic death [42]. The ratio of Bax to Bcl-2 protein may determine whether cells will die via apoptosis or be protected from it. The pro-apoptotic Bax promotes mitochondrial apoptosis by exacerbating mitochondrial destabilization and cytochrome c release, whereas the anti-apoptotic Bcl-2 antagonizes Bax [28, 43]. In the present study, we demonstrated that DNR induced apoptosis in H9c2 cells, down-regulated the expression of Bcl-2 and
increased the expression of ERKs, p53 and Bax. In contrast, SF sharply decreased ERK and p53 activation and the Bax/Bcl-2 ratio and suppressed the pro-apoptotic function of DNR. As a consequence of the reduction in apoptosis, SF effectively inhibited the DNR-induced mitochondrial apoptotic pathway, as characterized by the restoration of the mitochondrial membrane potential and cytochrome c content and by the decreased caspase-9 activity.

We further demonstrated that SF’s ability to protect against DNR-induced cardiomyocyte apoptosis may be attributed at least partially to its ability to inhibit the ERKs signaling pathway. This idea is supported by the findings of this study, as follows: i) the SF-induced inactivation of the ERKs pathway occurred prior to the restoration of the BCL family proteins, which closely paralleled the inhibition of apoptosis; ii) similar to the cardio-protective effect of SF, the pretreatment of the H9c2 cells with AZD6244, a specific inhibitor of ERKs phosphorylation, attenuated DNR-induced growth inhibition, resulting in increased cell viability and decreased numbers of apoptotic cells; and iii) the combined use of AZD6244 and SF did not cause a significant change in cell viability or in the percentage of apoptosis compared with SF treatment alone. Each of these results was indicative of a functional link between ERKs inhibition and the anti-apoptotic efficacy of SF.

In addition, it deserves to be noted that daunorubicin is simply a model anthracycline drug. Because the cardiotoxicity shows similar features within the group, the results could be translatable to other more commonly used drugs from this class, such as doxorubicin. In addition, some limitations may impact the underlying molecular mechanisms. A major limitation of this study is that the H9c2 cell line is phenotypically different from the cardiac myocytes, though they do present several similar properties: the H9c2 cells proliferate in vitro, whereas cardiac myocytes do not proliferate [44–47]. Another limitation is the fact that most of anthracycline cardiotoxicity clinically develops on a time span of months or years, whereas experiments in cultured cells are naturally short-term (in our study, 24 h). Because the H9c2 cell model used in this study is a rough simulation of anthracycline cardiotoxicity, our results and conclusion should be acceptable as hypothesis-generating rather than definitive [48, 49]. Further exploration in primary cardiomyocytes and experimental animals is required.

In conclusion, the present study demonstrated the protective role of sodium ferulate against DNR-induced cardiotoxicity in H9c2 cells. This protective role most likely results from the inhibition of mitochondrial apoptosis via the ERKs signaling pathway. These findings may be indicative of the potential adjuvant application of SF in combination with anthracyclines to mitigate the latter’s cardiotoxicity.

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Disclosure Statement

The authors declare no potential conflicts of interest.

References


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