Baicalein Protects Against Doxorubicin–Induced Cardiotoxicity by Attenuation of Mitochondrial Oxidant Injury and JNK Activation

Wei-Tien Chang,[1,2] Jing Li,[1] Hsien-Hao Haung,[1,3] Huiping Liu,[4] Mei Han,[1]
Srinivasan Ramachandran,[5] Chang-Qing Li,[1] Willard W. Sharp,[1] Kimm J. Hamann,[1,6]
Chun-Su Yuan,[7] Terry L. Vanden Hoek,[1*] and Zuo-Hui Shao[1*]

[1]Emergency Resuscitation Center, Section of Emergency Medicine, Department of Medicine, University of Chicago, Chicago, Illinois 60637

[2]Department of Emergency Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine, Taiwan, R.O.C

[3]Department of Emergency Medicine, Taipei Veterans General Hospital and Emergency Medicine, College of Medicine, National Yang-Ming University, Taipei, Taiwan, R.O.C

[4]The Ben May Department for Cancer Research, University of Chicago, Chicago, Illinois 60637

[5]Center for Nanomedicine, University of Chicago Illinois 60637

[6]Department of Medicine, Section of Pulmonary and Critical Care Medicine, University of Chicago, Illinois 60637

[7]Tang Center for Herbal Medicine Research, University of Chicago, Chicago, Illinois 60637

ABSTRACT

The cardiotoxicity of doxorubicin limits its clinical use in the treatment of a variety of malignancies. Previous studies suggest that doxorubicin-associated cardiotoxicity is mediated by reactive oxygen species (ROS)-induced apoptosis. We therefore investigated if baicalein, a natural antioxidant component of *Scutellaria baicalensis*, could attenuate ROS generation and cell death induced by doxorubicin. Using an established chick cardiomyocyte model, doxorubicin (10 μM) increased cell death in a concentration- and time-dependent manner. ROS generation was increased in a dose–response fashion and associated with loss of mitochondrial membrane potential. Doxorubicin also augmented DNA fragmentation and increased the phosphorylation of ROS-sensitive pro-apoptotic kinase *c*-Jun N-terminal kinase (JNK). Adjunct treatment of baicalein (25 μM) and doxorubicin for 24 h significantly reduced both ROS generation (587 ± 89 a.u. vs. 932 a.u. ± 121 a.u., $P < 0.01$) and cell death ($30.6 \pm 5.1\%$ vs. $46.8 \pm 8.3\%$, $P < 0.01$). The dissipated mitochondrial potential and increased DNA fragmentation were also ameliorated. Along with the reduction of ROS and apoptosis, baicalein attenuated phosphorylation of JNK induced by doxorubicin (1.7 ± 0.3 vs. 3.0 ± 0.4-fold, $P < 0.05$). Co-treatment of cardiomyocytes with doxorubicin and JNK inhibitor SP600125 (10 μM; 24 h) reduced JNK phosphorylation and enhanced cell survival, suggesting that the baicalein protection against doxorubicin cardiotoxicity was mediated by JNK activation. Importantly, concurrent baicalein treatment did not interfere with the anti-proliferative effects of doxorubicin in human breast cancer MCF-7 cells. In conclusion, baicalein adjunct treatment confers anti-apoptotic protection against doxorubicin-induced cardiotoxicity without compromising its anti-cancer efficacy. J. Cell. Biochem. 112: 2873–2881, 2011. © 2011 Wiley-Liss, Inc.

Doxorubicin is an effective anthracycline chemotherapeutic agent for a variety of solid tumors and hematologic diseases. Its clinical application, however, is restricted by its cardiotoxicity.

Myocardial damage occurs concurrently with doxorubicin use and becomes clinically significant if the cumulative dose exceeds 550 mg/m². This threshold of cardiotoxicity is lower in patients with

2873

underlying heart disease. The associated cardiomyopathy and congestive heart failure limit the potential cycles of chemotherapy possible and decreases patient quality of life. Therefore, adjunct treatments that minimize doxorubicin cardiotoxicity while preserving its anti-proliferative effects are needed.

It is likely that doxorubicin-induced cardiotoxicity is mediated by mitochondrial reactive oxygen species (ROS), with resulting mitochondrial injury and apoptotic cell death [Rajagopalan et al., 1988; Lee et al., 1991; Green and Leeuwenburgh, 2002; Clementi et al., 2003]. In addition, the ROS-dependent c-Jun N-terminal kinase (JNK) contributes to doxorubicin-induced apoptosis [Mansat-de Mas et al., 1999; Panaretakis et al., 2005; Yamamoto et al., 2008; Aroui et al., 2010]. Herbal antioxidants contain polyphenolic flavonoids with both antioxidant and anti-proliferative properties [Hwang et al., 2004; Wang et al., 2010]. These dual properties could make them useful adjuncts during doxorubicin chemotherapy in contrast to traditional high-potency antioxidants that have had mixed effects [Alberts et al., 1978; Antunes and Takahashi 1999].

In particular, baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzo-pyran-4-one) is a natural flavonoid constituent derived from the root of *Scutellaria baicalensis* Georgi that protects against a wide spectrum of oxidative injuries [Po et al., 2002; Sadik et al., 2003]. Our previous studies have shown that when compared to other flavonoid compounds, baicalein is a potent antioxidant that protects cardiomyocyte against severe ischemia/reperfusion injury and contractile dysfunction due to mitochondrial ROS [Vanden Hoek et al., 1997; Vanden Hoek et al., 1998; Shao et al., 1999; Shao et al., 2002; Chang et al., 2006]. Consistent with this, work by others has compared baicalein to multiple phenolic compounds, finding it to be a highly effective inhibitor of lipid peroxidation that protects cardiomyocyte function [Psotova et al., 2004]. In addition to these antioxidant cardioprotective effects, baicalein also has anti-proliferative properties [Wang et al., 2010] that could make it one of the more useful herbal flavonoid adjuncts for doxorubicin treatment.

Therefore, we evaluated the potential of baicalein in ameliorating doxorubicin-induced cardiotoxicity using an established cardiomyocyte model. We also investigated the impact of baicalein on the anti-proliferative effects of doxorubicin in human breast cancer MCF-7 cells.

MATERIALS AND METHODS

CHEMICALS

The following chemicals were obtained from commercial sources: doxorubicin, baicalein, SP600125, propidium iodide, digitonin, and alpha-sarcomeric actin (Sigma, St. Louis, MO); Dulbecco's modified Eagle's medium, trypsin, M199, fetal bovine serum, penicillin, and streptomycin (Invitrogen, Grand Island, NY); 6-carboxy-2′,7′-dichloro-dihydrofluorescein diacetate (6-carboxy-H_2DCFDA) (Invitrogen, Carlsbad, CA); 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethlbenzi-midazole-carbocyanide iodine (JC-1) (EMD Biosciences Inc., San Diego, CA); phosphorylated JNK/SAPK (p46, p54) and JNK antibodies (Cell Signaling Technologies, Denver, MA); and an antibody to α-tubulin (NeoMarkers, Fremont, CA).

METHODS

Cell culture. Chick cardiomyocytes were isolated from 10-day chick embryos and cultured as previously described [Vanden Hoek et al., 1997]. In brief, the hearts were removed and the ventricles were minced and enzymatically digested with 0.025% trypsin. In order to exclude non-cardiomyocytes, cells were preplated for 45 min at 37°C. The resultant cell suspensions were centrifuged and then resuspended in the culture medium (54% balanced salt solution, 40% medium 199, 6% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin). Cells were plated onto 25 mm glass coverslips at a density of 0.7×10^6 and incubated at 37°C. Cardiomyocyte purity was assessed by immunofluorescent staining for alpha-sarcomeric actin. All experiments were performed with 3–5-day cultured cells, by which time synchronously contracting cells could be visualized with viability exceeding 95%. The human breast carcinoma MCF-7 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were plated and grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum and 1% penicillin-streptomycin. They were fed every 2–3 days until they reached 70–80% confluence.

Video/fluorescence microscopy. A Nikon TE 2000-U inverted phase/epifluorescent microscope was used for cell imaging. Fluorescent images were acquired from a cooled *Cool*-SNAP-ES camera (Photometrics, Tuscon, AZ) and changes in fluorescent intensity were quantified with MetaMorph® software (Molecular Devices Corp., Downington, PA).

Viability assay. Cell viability was assessed with the exclusion fluorescent dye propidium iodide (PI, 5 μM) as measured at wavelengths of 540 nm (excitation, Ex) and 590 nm (emission, Em). This dye exhibited no cytotoxicity in control cells even after a 10-h incubation time [Vanden Hoek et al., 1998]. At the end of the experiment, all cells on the coverslip were permeabilized with digitonin (300 μM). PI fluorescence was measured and averaged over three random fields on each coverslip at selected times up to 24 h doxorubicin treatments and once after 1 h of digitonin exposure. Percentage cell death (PI uptake) was expressed as the PI fluorescence of a coverslip at selected times relative to the maximal value seen after digitonin exposure (100%). Each experiment was repeated in at least three different batches (i.e., isolations) of cells.

Measurement of intracellular ROS. The probe 6-carboxy-2′, 7′-dichloro-dihydrofluorescein diacetate (6-carboxy-H_2DCFDA) was used to measure intracellular ROS. It is a non-fluorescent and cell permeable analog that is oxidized to highly fluorescent carboxy-dichlorofluorescein (carboxy-DCF) as measured at Ex 488 nm/Em 520 nm with fluorescence microscopy and expressed in arbitrary units (a.u.). This carboxylated form is more permeant and less subject to photo-oxidation than the classic H_2DCFDA. Before the measurement, the medium was changed to phenol red-free M199 with Earle's salts. Cells were incubated with carboxy-H_2DCFDA (1 μM) at 37°C for 15 min, washed with PBS.

Lactate dehydrogenase release. Release of lactate dehydrogenase (LDH) into the culture medium was assessed by spectrophotometric measurement using a LDH-cytotoxicity assay kit (BioVision Inc. Mountain View, CA). The values were measured by a microplate reader (Synergy HT, Bio-Tek Instruments, Winooski, VT) at 490 nm.

LDH leakage from the cells was expressed as the percentage (%) of the total LDH activity (LDH in the medium + LDH in the cell lysates). DNA fragmentation '' laddering'' assay by agarose gel electrohporesis. Genomic DNA was extracted using the genomic DNA purification kit (Qiagen, Valencia, CA) as previously reported [Li et al., 2010]. Briefly, 4–5×10^6 chick cardiomyocytes were harvested and washed twice with cold PBS. Buffer C1 was then added to lyse the cells and preserve the nuclei. Buffer G2 with RNase A was used to lyse the nuclei and denature proteins. The RNA was further digested into smaller fragments by incubation with Qiagen protease at $50°C$ for 1 h. The mixture was then entered into an equilibrated Qiagen Genomic-tip. After washing and eluting, the DNA was precipitated by isopropanol and washed with ethanol. The extracted DNA ($3 \mu g$) was loaded onto a 2% agarose gel, run at 80 V for 45 min in TAE buffer and visualized with ethidium bromide using UV illumination and photographed by a LAS-3000 Imaging system (Fujifilm, Japan).

Assessment of mitochondrial membrane potential by JC-1 fluorescence. Mitochondrial membrane potential ($\Delta\Psi_m$) was determined using a mitochondrial potentiometric dye JC-1 with epifluorescence microscopy as previously reported [Li et al., 2010]. JC-1 is a lipophilic and cationic dye, which partitions into healthy mitochondria and aggregates. This potential- and concentration-dependent aggregation shifts its emission to red (R). Conversely, depolarized mitochondria (loss of $\Delta\Psi_m$) prevent JC-1 mitochondrial entry and cause its disaggregation, resulting in diffuse green (G) emission. Cardiomyocytes incubated with JC-1 ($10 \mu g/ml$) at $37°C$ for 15 min were washed briefly with HBSS and mounted on the microscope (Olympus IX71, Japan) equipped with an on-stage incubator (20/20 Technologies, USA) for imaging. TRITC and FITC filter sets (Semrock, USA) were used for detecting polarized and depolarized mitochondria, respectively.

JNK phosphorylation by Western blot analysis. Cardiomyocytes were treated and harvested in buffer containing 1% Triton-100, 20 mM Tris, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 50 mM NaF, 1 mM NaVO$_3$, 200 mM PMSF, and $1\times$ protease inhibitor cocktail. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA). Protein lysates ($30 \mu g$) were resolved on a 10% SDS–PAGE gel and transferred to nitrocellulose membranes. After blocking in 5% milk/TBST, proteins were probed with antibodies against phosphorylated (Thr183/Tyr185) JNK/SAPK (p46, p54; Cell Signaling Technology, Danvers, MA) and α-tubulin (NeoMarkers, Fremont, CA), then exposed to HRP-linked goat anti-rabbit or HRP-linked goat anti-mouse (Cell Signaling Technology); finally exposed to SuperSignal (Thermol Scientific, Rockford, IL) and visualized by X-ray film. Densitometry was performed using Image J software (ver 1.38, NIH).

Flow cytometric detection of doxorubicin anti–proliferative effect. MCF-7 cells were treated with doxorubicin ($10 \mu M$) with or without baicalein ($25 \mu M$) for 24 h. Four hours prior to harvest, cells were pulsed with BrdU ($30 \mu g/ml$). After trypsinization, cells were fixed with cold 70% ethanol for 5 min at $-20°C$, denatured by 0.1 N HCl/0.7% Triton X-100 for 10 min on ice and then heated in HCl buffer (0.0032 N in H$_2$O). After being blocked with 0.5% Tween 20/1%BSA/PBS (PBST), cells were incubated with anti-BrdU-FITC antibody (1:20 in PBST) for 30 min on ice and washed with PBST.

Cells then underwent flow cytometric analysis as previously described [Li et al., 2010].

DATA ANALYSIS

A field of about 500 cells was observed for each experiment. Treatment and control groups were used in sets containing cells isolated and cultured on the same day to eliminate variability due to cell batch. Additional coverslips were used for replicate experiments (''n''). All data were presented as means $\pm$ standard error of the mean (SEM). Multiple comparisons among different treatment groups were evaluated by one-way ANOVA followed by Tukey test as post hoc analysis. Values of $P < 0.05$ were considered statistically significant.

RESULTS

DOXORUBICIN INDUCES CARDIOMYOCYTE DEATH

Previous studies have shown that doxorubicin causes cardiotoxicity in a number of cardiomyocyte models, both in vitro and in vivo [Kim et al., 2006; Ikegami et al., 2007]. To test if doxorubicin could induce similar cytotoxic injury in the well-established chick cardiomyocyte model [Vanden Hoek et al., 1997], cells were treated with different concentrations of doxorubicin (1, 10, 50, or $100 \mu M$) for 24 h and cell death were measured at 3, 6, 12, and 24 h using PI analyses as described above. As shown in Figure 1A, with increasing duration of doxorubicin treatment, cell death increased in a time-dependent manner. Significant cell death was observed within 6 h of doxorubicin ($10 \mu M$) exposure. Similarly, doxorubicin induced a dose-dependent cell death. Compared to control, the low dose ($1 \mu M$) of doxorubicin resulted in a cell death of $26.6 \pm 4.6\%$ at 24 h (n = 6, $P < 0.01$) while high dose ($100 \mu M$) led to $77.3 \pm 3.0\%$ cell death (n = 6, $P < 0.001$). These results indicate that doxorubicin induces cardiomyocyte death in a time- and dose-dependent fashion.

DOXORUBICIN INCREASES CARDIOMYOCYTE ROS GENERATION

It has been suggested that one of the major mechanisms of doxorubicin-induced cardiotoxicity involves increased ROS generation [Rajagopalan et al., 1988; Lee et al., 1991]. To test whether ROS generation in cardiomyocytes is increased by doxorubicin, we used 6-carboxy-H$_2$DCFDA ($1 \mu M$) to measure intracellular oxidant generation. Figure 1B shows that 24 h of doxorubicin treatment (1, 10, 50, or $100 \mu M$) resulted in increased DCF fluorescence in a concentration-dependent manner. This dose-dependent ROS increase paralleled the dose-dependent increase of cell death. These results suggest that doxorubicin-induced cardiotoxicity was related to excess ROS generation. Since $10 \mu M$ of doxorubicin resulted in significant ROS generation and cytotoxicity (about 45% of cell death), this concentration was used in subsequent experiments.

BAICALEIN ATTENUATES ROS GENERATION, CELL DEATH, AND LDH RELEASE INDUCED BY DOXORUBICIN

Our previous studies demonstrated that baicalein reduces oxidative stress as well as cell death in a chick cardiomyocyte model of ischemia-reperfusion injury [Shao et al., 2002; Chang et al., 2007]. To test whether baicalein could also attenuate the increased ROS induced by doxorubicin, cells were loaded with 6-carboxy-
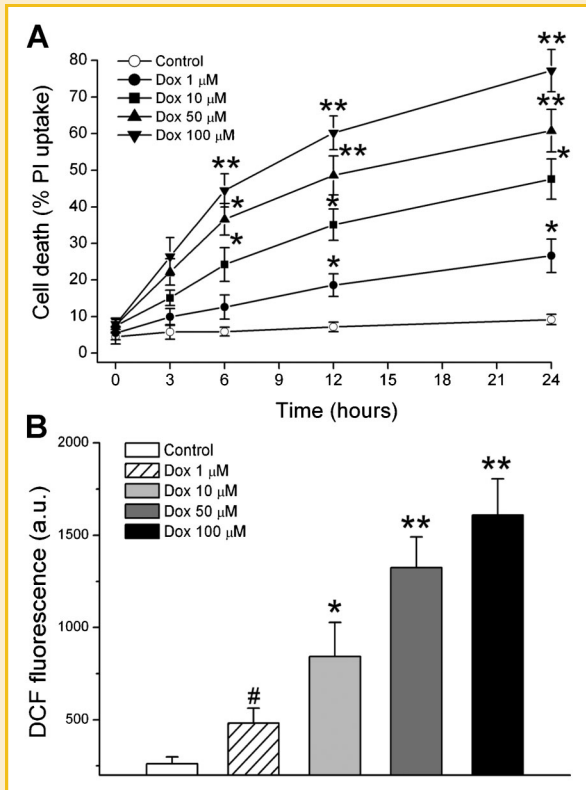
Fig. 1. Doxorubicin-induced cell death and ROS generation in cardiomyocytes. A: Treatment with doxorubicin (Dox) 1, 10, 50, or 100 μM induced a dose- and time-dependent cell death as assessed by PI uptake ($^*P < 0.01$, $^{**}P < 0.001$ vs. untreated cells). B: Treatment with doxorubicin 1, 10, 50, or 100 μM for 24 h resulted in a dose-dependent ROS generation as measured by DCF fluorescence ($^\#P < 0.05$, $^*P < 0.01$, $^{**}P < 0.001$ vs. untreated cells). Data were presented as means $\pm$ SEM.
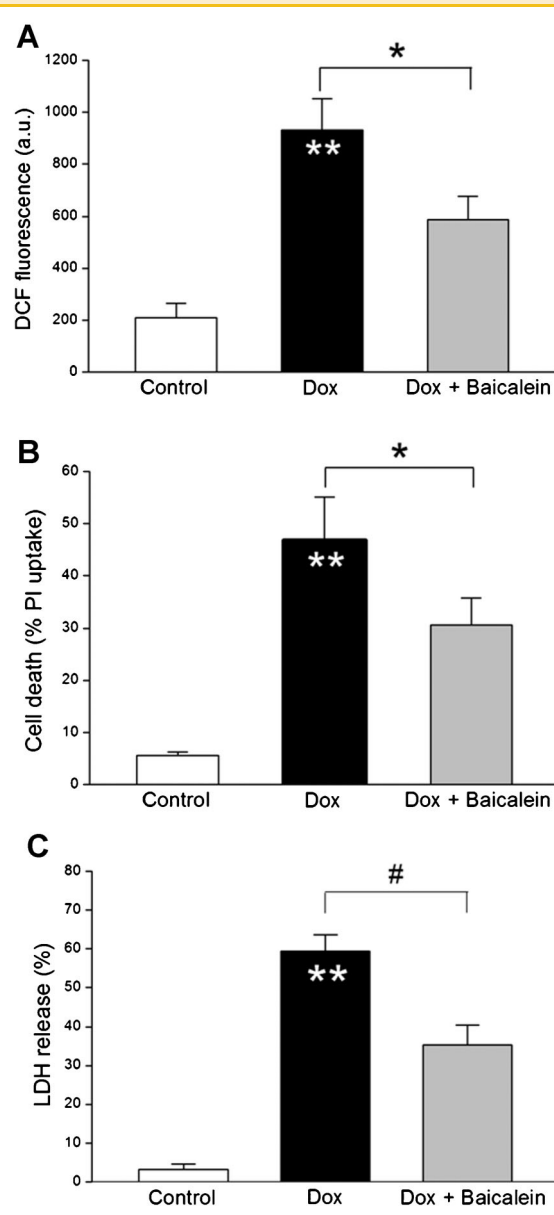

Fig. 2. Baicalein attenuated doxorubicin-induced ROS generation, cell death, and LDN release in cardiomyocytes. A: Doxorubicin (10 μM) treatment for 24 h increased DCF fluorescence ($^{**}P < 0.001$ vs. untreated cells). Adjunct treatment with baicalein (25 μM) significantly attenuated the DCF fluorescence ($^*P < 0.01$ vs. doxorubicin-treated cells). B: Doxorubicin (10 μM) treatment for 24 h increased cell death ($^{**}P < 0.001$ vs. untreated cells). Co-treatment with baicalein (25 μM) markedly reduced the cell death ($^*P < 0.01$ vs. doxorubicin-treated cells). C: Doxorubicin (10 μM) treatment for 24 h increased LDH release ($^{**}P < 0.001$ vs. untreated cells). Baicalein (25 μM) co-treatment significantly decreased LDH release ($^\#P < 0.05$ vs. doxorubicin-treated cells). Data were shown as means $\pm$ SEM.

H_2DCFDA (1 μM) and treated with doxorubicin (10 μM) with or without baicalein (25 μM) for 24 h. As shown in Figure 2A, doxorubicin (10 μM) exposure resulted in increased DCF fluorescence (932 $\pm$ 121 a.u. compared to 210 $\pm$ 54 a.u. in control, $P < 0.001$, n = 6). Adjunct treatment with baicalein (25 μM) significantly attenuated the DCF fluorescence after doxorubicin exposure (587 $\pm$ 89 a.u., compared to the doxorubicin group, $P < 0.01$, n = 6), suggesting that baicalein attenuates ROS generation.

As ROS play an important role in doxorubicin cardiotoxicity, we further tested whether this ROS attenuation by baicalein could ameliorate doxorubicin-induced cytotoxicity. As seen in Figure 2B, doxorubicin (10 μM) exposure for 24 h caused a marked increase in cell death from 8.6 $\pm$ 0.6% in control to 46.8 $\pm$ 8.3% ($P < 0.001$, n = 9). Treatment with baicalein (25 μM) significantly reduced cell death to 30.6 $\pm$ 5.1% compared to the doxorubicin group ($P < 0.01$, n = 9).

Similar results were observed in LDH release, an additional indicator of cardiomyocyte injury. Cells exposed to doxorubicin (10 μM) for 24 h demonstrated increased LDH release (59.4 $\pm$ 4.2% vs. 3.2 $\pm$ 0.4% in control, $P < 0.001$, n = 4; Fig. 2C). Baicalein (25 μM) treatment in the presence of doxorubicin (10 μM)

significantly reduced LDH release to 35.3 $\pm$ 5.1% compared to the doxorubicin group ($P < 0.05$, n = 4). These results suggest that baicalein protects cardiomyocytes from doxorubicin-induced cell death, consistent with its attenuation of ROS generation.

BAICALEIN PROTECTS AGAINST DOXORUBICIN–INDUCED APOPTOSIS

It has been reported that apoptosis plays a significant role in doxorubicin-induced cardiotoxicity [Green and Leeuwenburgh, 2002; Clementi et al., 2003]. To test this, a DNA fragmentation assay was used. As seen in Figure 3, DNA fragmentation was increased in doxorubicin-exposed cells and was attenuated by co-treatment with baicalein (25 µM). This suggests that doxorubicin-induced cell death and baicalein protection were associated with effects on cardiomyocyte apoptosis.

BAICALEIN PREVENTS THE DISSIPATION OF MITOCHONDRIAL MEMBRANE POTENTIAL

It is reported that cell death induced by oxidative injury is generally associated with perturbation in mitochondrial function [Tan et al., 2010]. A late manifestation is the dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$) [Xu and Ashraf, 2002; Li et al., 2010]. Figure 4 is a representative image of JC-1 fluorescence in cardiomyocytes. Punctate red (R) fluorescence indicates the potential-dependent aggregation of JC-1 within polarized mitochondria in healthy cells. Diffuse green (G) fluorescence represents
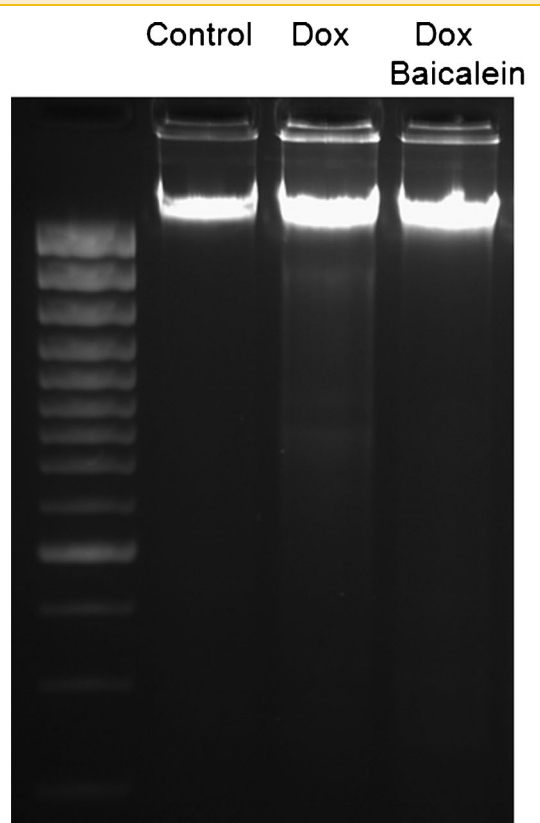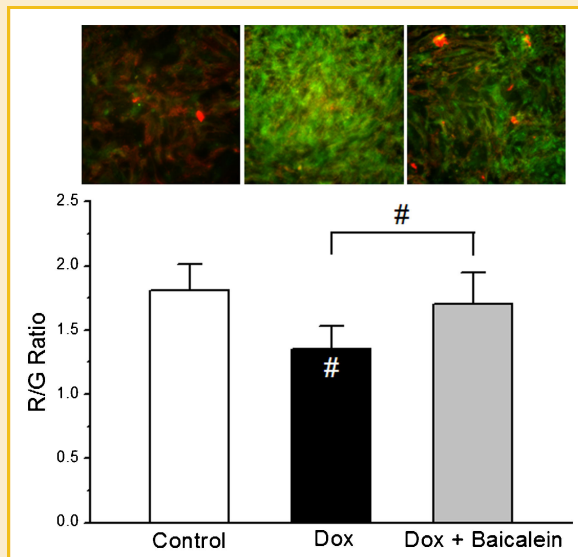

Fig. 4. Baicalein prevented the disruption of mitochondrial membrane potential ($\Delta\Psi_m$) induced by doxorubicin as measured by JC–1. Left: The $\Delta\Psi_m$ was polarized in control cells as demonstrated by characteristic red punctuate of JC–1 fluorescence. Middle: Doxorubicin (10 µM) exposure for 24 h resulted in loss of $\Delta\Psi_m$ as shown by diffuse green JC–1 fluorescence. Right: Adjunct treatment with baicalein (25 µM) preserved $\Delta\Psi_m$ as presented by less green fluorescence. Quantitative densitometric analysis showed that doxorubicin significantly decreased the red/green (R/G) ratio ($^{\#}P < 0.05$ vs. untreated cells), while baicalein reversed the decreased R/G ratio induced by doxorubicin ($^{\#}P < 0.05$ vs. doxorubicin–treated cells). Data were shown as means ± SEM of five independent experiments.

the monomeric form of JC-1 in the cytosol, an indicator of depolarized mitochondria in unhealthy cells. In doxorubicin-treated cells, a prominent dissipation of $\Delta\Psi_m$ as indicated by increased green fluorescence was seen (Fig. 4, middle) compared to control cells (Fig. 4, left). With baicalein (25 µM) treatment by the disruption of $\Delta\Psi_m$ induced by doxorubicin was ameliorated (Fig. 4, right). Quantitative densitometric analysis showed that doxorubicin (10 µM) decreased the R/G ratio (1.35 ± 0.18 vs. 1.81 ± 0.20 in control, $P < 0.05$, n = 5). Adjunct treatment with baicalein (25 µM) reversed the R/G ratio (1.70 ± 0.25, compared to the doxorubicin group, $P < 0.05$, n = 5). These results suggest that baicalein-mediated cardioprotection during doxorubicin treatment is associated with preservation of mitochondrial function.

BAICALEIN SUPPRESSES JNK ACTIVATION INDUCED BY DOXORUBICIN

JNK is an important ROS-dependent signaling pathway related to the induction of apoptosis [Aroui et al., 2010]. Therefore, we examined JNK activation via phosphorylation (Thr183/Tyr185) in cardiomyocytes exposed to doxorubicin, and whether baicalein would inhibit this phosphorylation. As seen in Figure 5, after doxorubicin (10 µM) exposure for 24 h, the phosphorylation of JNK (p-JNK) was increased 3.0 ± 0.4-fold compared to control ($P < 0.01$, n = 5). Baicalein (25 µM) treatment significantly decreased p-JNK to 1.7 ± 0.3-fold, compared to the doxorubicin cells ($P < 0.05$, n = 5). This result suggests that doxorubicin-induced cardiotoxicity is

Fig. 3. Baicalein attenuated doxorubicin–induced DNA fragmentation or "laddering" in cardiomyocytes. Doxorubicin (10 µM) exposure for 24 h resulted in DNA fragmentation as demonstrated by DNA laddering. Adjunct treatment with baicalein (25 µM) attenuated the DNA ladder. Results were represented as means ± SEM of three independent experiments.
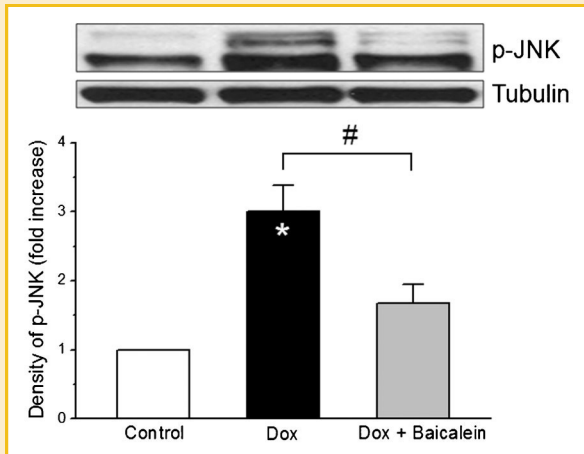
Fig. 5. Baicalein suppressed doxorubicin–induced JNK phosphorylation (*p*–JNK) in cardiomyocytes. Doxorubicin (10 μM) exposure for 24 h increased *p*–JNK (*$P < 0.01$ vs. untreated cells). Co–treatment with baicalein (25 μM) significantly reduced *p*–JNK (#$P < 0.05$ vs. doxorubicin–treated cells). Results were presented as means ± SEM of four independent experiments.

associated with the phosphorylation of JNK and baicalein confers cardioprotection via suppressing JNK activation.

EFFECTS OF JNK INHIBITOR ON JNK ACTIVATION AND CELL DEATH INDUCED BY DOXORUBICIN

To further support the hypothesis that the JNK signaling pathway mediates the effect of baicalein against doxorubicin toxicity, a specific inhibitor of JNK, SP600125 (10 μM) was used [Bennett et al., 2001]. Results shown in Figure 6A confirmed that co-treatment of JNK inhibitor SP600125 with doxorubicin (10 μM) attenuated *p*-JNK to 1.5 ± 0.2-fold, compared to the doxorubicin-treated cells (2.8 ± 0.2-fold, $P < 0.05$, n = 3). Similarly, the treatment with baicalein (25 μM) inhibited the *p*-JNK to 1.7 ± 0.3-fold compared to doxorubicin-treated cells ($P < 0.05$, n = 3). Figure 6B shows that SP600125 significantly decreased doxorubicin-induced cell death ($28.0 \pm 4.5\%$, compared to doxorubicin-treated cells $49.2 \pm 4.3\%$, $P < 0.01$, n = 9). In a fashion parallel to the effects on *p*-JNK, adjunct use of baicalein also decreased cells death ($19.4 \pm 5.3\%$, compared to doxorubicin-treated cells). Together, these data suggest that the doxorubicin-induced cell death is mediated by JNK phosphorylation and baicalein protection exerts through inhibiting JNK activation.

EFFECT OF BAICALEIN ON ANTI–PROLIFERATIVE ACTIVITY OF DOXORUBICIN

The primary goal of doxorubicin therapy is to kill cancer cells or inhibit their proliferation. If the addition of a second drug for attenuating cardiotoxicity significantly abrogates the anti-cancer effect of doxorubicin, this would detract from the value of such adjunct treatment. There has been increased interest in developing adjunct treatments that decrease cardiac ROS generation and toxicity while maintaining its anti-proliferative properties [Das et al., 2010]. To test whether baicalein adjunct treatment reduces the desired anti-proliferative effect of doxorubicin, the proliferation of
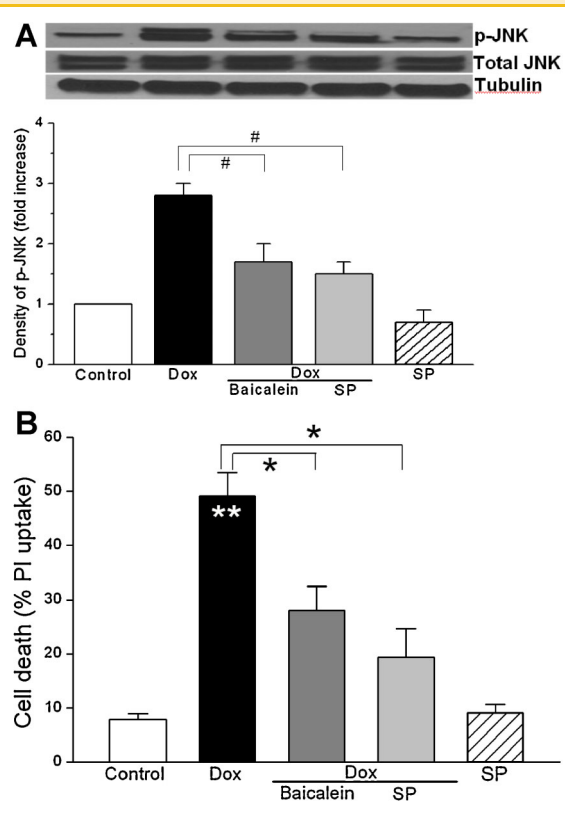

Fig. 6. The effects of JNK inhibitor SP600125 (SP) on JNK phosphorylation and cell death induced by doxorubicin. A: SP600125 inhibited doxorubicin-induced *p*–JNK. Co–treatment with SP6000125 (10 μM) and doxorubicin (10 μM) for 24 h attenuated the levels of *p*–JNK (#$P < 0.05$ vs. doxorubicin–treated cells). Baicalein (25 μM) also decreased *p*–JNK induced by doxorubicin (#$P < 0.05$ vs. doxorubicin–treated cells). Results were depicted as means ± SEM of three independent experiments. B: SP600125 reduced cell death caused by doxorubicin. Consistent with effect on JNK phosphorylation, co–treatment with SP6000125 for 24 h attenuated the cell death (#$P < 0.05$ vs. doxorubicin–treated cells). Cell death was also decreased by the treatment with baicalein (*$P < 0.01$ vs. doxorubicin–treated cells). Results were shown as means ± SEM.

MCF-7 cells was determined using BrdU as an indicator of DNA synthesis. As seen in Figure 7, MCF-7 cells exposed to doxorubicin (10 μM) for 24 h considerably decreased BrdU-FITC staining from $52 \pm 8\%$ to $5 \pm 3\%$ ($P < 0.001$, n = 3). This anti-proliferative effect was not diminished by adjunct treatment with baicalein (25 μM) compared to doxorubicin alone ($5 \pm 3\%$ vs. $5 \pm 3\%$, $P =$ NS, n = 3). Baicalein treatment alone had no noticeable effect on DNA synthesis (BrdU-FITC $56 \pm 5\%$ vs. $52 \pm 8\%$ in untreated control, $P =$ NS, n = 3). These findings suggest that baicalein does not interfere with the doxorubicin anti-proliferative activity.

DISCUSSION

The present study demonstrated that doxorubicin-induced cardiotoxicity is associated with increased ROS generation leading to the mitochondrial dysfunction and both necrotic (i.e., LDH release, PI
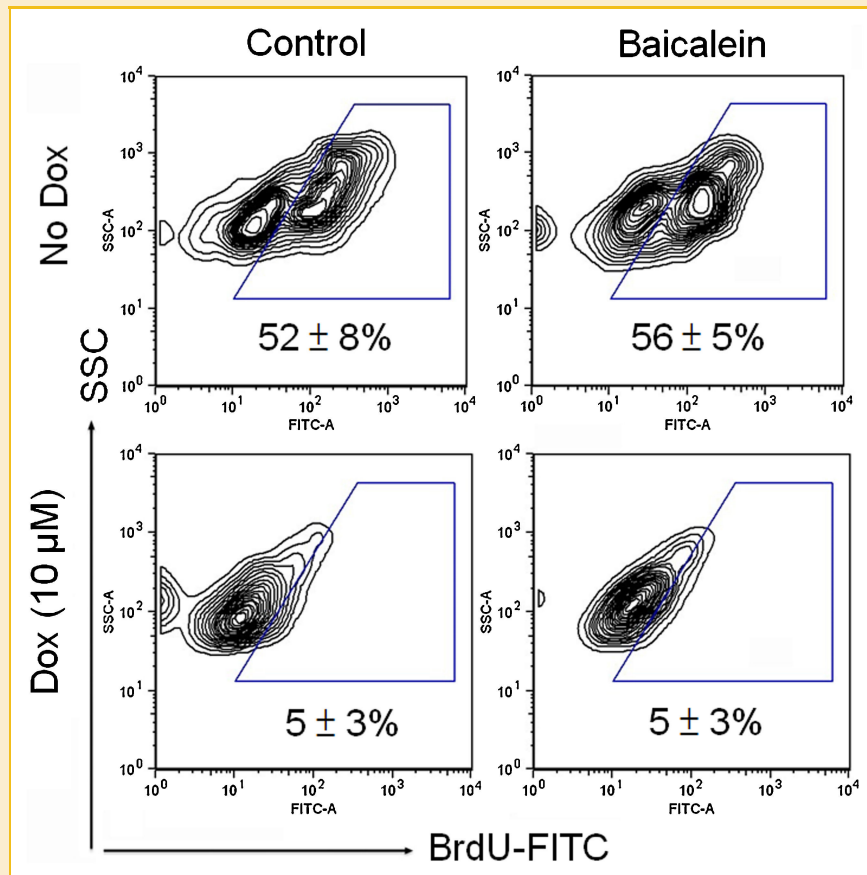
Fig. 7. Effect of baicalein on proliferation–inhibitory activity of doxorubicin in MCF–7 cells. Upper left: Untreated cells. Lower left: Doxorubicin (10 μM) reduced BrdU–FITC-positive cells from $52 \pm 8\%$ in untreated cells to $5 \pm 3\%$ ($^{**}P < 0.001$). Upper right: Baicalein alone has no effect on BrdU incorporation ($56 \pm 5\%$ vs. $52 \pm 8\%$, $P =$ NS). Lower right: Adjunct treatment with baicalein (25 μM) did not abrogate the proliferation–inhibitory effect of doxorubicin ($5 \pm 3\%$ vs. $5 \pm 3\%$ in doxorubicin–treated cells, $P =$ NS). Data were presented as means $\pm$ SEM of three independent experiments.

uptake) and apoptotic (i.e., JNK activation, DNA laddering) cell death. This study is one of the first to suggest that baicalein can protect against the doxorubicin-induced cardiotoxicity without affecting the anti-proliferative effects of doxorubicin in breast cancer cells. In addition, this work suggests that cardioprotection by baicalein is related to the suppression of doxorubicin-induced JNK activation and supports a possible role for baicalein as a unique adjunct agent in the doxorubicin treatment.

BAICALEIN AND DOXORUBICIN CARDIOTOXICITY MEDIATED BY ROS

Our results showed that doxorubicin treatment for 24 h increased both ROS generation and cell death in a dose–response fashion. Work by others has shown that doxorubicin-induced ROS originate primarily within the mitochondria [Rajagopalan et al., 1988; Green and Leeuwenburgh, 2002] and is consistent with our own work showing that doxorubicin exposure causes the loss of mitochondrial membrane potential. Our finding that doxorubicin increased both cell membrane permeability and DNA laddering is consistent with previous studies demonstrating that doxorubicin induced both necrotic and apoptotic cell death, a pattern of death that can result from mitochondrial injury [Lim et al., 2004; Fisher et al., 2005; Li

et al., 2006]. Baicalein, a natural herbal constituent, has been shown to protect against oxidative injuries in both in vitro and in vivo studies [Tu et al., 2008; Zhang et al., 2009]. Consistent with our prior work and that of others [Shao et al., 2002; Chang et al., 2007; Liu et al., 2008], the current study demonstrated that baicalein attenuated ROS and reduced DNA fragmentation and cell death. The reduction in ROS is likely due to the superoxide and hydroxyl radical scavenging properties of baicalein [Rice-Evans et al., 1996; Chang et al., 2007] in addition to its unique properties as an iron chelator that prevent hydroxyl radical formation via the Fenton reaction [Fenton 1984]. Baicalein may act also as a lipoxygenase inhibitor that prevents mitochondrial membrane depolarization [Deschamps et al., 2006; Lapchak et al., 2007; Haworth et al., 2010].

Mitochondria have been shown to play a critical role in apoptosis. Being a major intracellular source of ROS generation in response to various stimuli, mitochondria can be damaged by excess ROS that resulted in dissipation of mitochondrial membrane potential and mitochondrial dysfunction [Xu and Ashraf, 2002; Li et al., 2010]. Few studies have reported whether baicalein protects against loss of mitochondrial membrane potential in the context of doxorubicin exposure. Our study first showed that adjunct treatment with

baicalein attenuated the doxorubicin-induced dissipation of mitochondrial membrane potential. Mitochondrial preservation may occur via the attenuation of JNK activation and decrease of related cytochrome *c* release, and loss of mitochondrial membrane potential [Clementi et al., 2003; Mikami et al., 2006; Tan et al., 2010]. However, little work has been done to evaluate the cardioprotective effect of baicalein on MAPK signaling and the anti-proliferative properties of doxorubicin in cardiomyocytes (see below).

BAICALEIN AND JNK ACTIVATION

JNK was implicated as a key player in doxorubicin-induced apoptotic cascade. [Panaretakis et al., 2005]. Our study demonstrates that doxorubicin-induced ROS generation and apoptotic cell death in cardiomyocytes is also associated with JNK activation. This is consistent with reports by others showing that ROS induced by doxorubicin can trigger apoptotic death pathways including the MAPK pathway JNK [Mikami et al., 2006; Spallarossa et al., 2006]. This connection between ROS and JNK activation is supported by our results showing that baicalein reduced both ROS generation and the activation of JNK as well as DNA fragmentation. We demonstrated that administration of the novel, potent, and selective JNK inhibitor SP600125 with doxorubicin diminished the phosphorylation of JNK, correlating with enhanced cardioprotection. The inhibition of JNK by baicalein is consistent with recent reports in other cell types showing that baicalein suppressed MAPK pathway activation [Wang et al., 2010]. However, unlike the latter study, which was performed in breast carcinoma cells, our additional experiments examining phosphorylation of the kinase p38, did not demonstrate any activity of baicalein on this kinase in the cardiomyocyte model (not shown). Furthermore, the latter study [Wang et al., 2010] did not examine the relationship of baicalein-mediated effects to ROS generation.

BAICALEIN AND THE ANTI-PROLIFERATIVE PROPERTIES OF DOXORUBICIN

The antioxidant effects of baicalein that protect the heart could potentially interfere with doxorubicin anti-proliferative effect mediated by oxidative DNA damage and lipid peroxidation [Singal et al., 2000]. Our work is the first to show that baicalein has a potent cardioprotective effect without affecting the anti-proliferative effect of doxorubicin in MCF-7 cells. The existence of such adjunct agents is supported by recent report that sildenafil can decrease cardiac oxidant generation and maintain doxorubicin anti-neoplastic effects in prostate cancer cells [Das et al., 2010]. Our results extend the work of Das et al. by applying a flavonoid adjunct agent commonly used as a traditional medicine in breast cancer cells. These dual properties of baicalein (i.e., cardioprotection with preserved anti-neoplastic effect) require further investigation. However, there are a number of anti-proliferative mechanisms of baicalein action including MAPK pathway inhibition that have recently been described [Yamamoto et al., 2008; Zhou et al., 2009; Wang et al., 2010].

In conclusion, doxorubicin cardiotoxicity is induced by increased intracellular ROS generation that is associated with mitochondrial dysfunction and triggering of apoptotic pathways via JNK activation. Concurrent use of baicalein with doxorubicin not only attenuates ROS generation and mitochondrial dysfunction, but it suppresses JNK phosphorylation/activation and reduces subsequent apoptotic cardiomyocyte death. Perhaps most importantly, this adjunct treatment does not compromise the anti-cancer effects of doxorubicin. Given that baicalein has been used clinically as an alternative medicine for centuries, it may be a useful adjunctive therapy for patients undergoing doxorubicin-based chemotherapy, especially in those with pre-existing cardiomyopathy or heart failure.

REFERENCES

Alberts DS, Peng YM, Moon TE. 1978. alpha-Tocopherol pretreatment increases adriamycin bone marrow toxicity. Biomedicine 29:189–191.

Antunes LM, Takahashi CS. 1999. Protection and induction of chromosomal damage by vitamin C in human lymphocyte cultures. Teratog Carcinog Mutagen 19:53–59.

Aroui S, Mili D, Brahim S, De Waard M, Kenani A. 2010. Doxorubicin coupled to penetratin promotes apoptosis in CHO cells by a mechanism involving c-Jun NH2-terminal kinase. Biochem Biophys Res Commun 396:908–914.

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu JW, Leisten C, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA 98:13681–13686.

Chang WT, Shao ZH, Vanden Hoek TL, McEntee E, Mehendale SR, Li J, Becker LB, Yuan CS. 2006. Cardioprotective effects of grape seed proanthocyanidins, baicalin and wogonin: Comparison between acute and chronic treatments. Am J Chin Med 34:363–365.

Chang WT, Shao ZH, Yin JJ, Mehendale S, Wang CZ, Qin Y, Li J, Chen WJ, Chien CT, Becker LB, Vanden Hoek TL, Yuan CS. 2007. Comparative effects of flavonoids on oxidant scavenging and ischemia-reperfusion injury in cardiomyocytes. Eur J Pharmacol 566:58–66.

Clementi ME, Giardina B, Di Stasio E, Mordente A, Misiti F. 2003. Doxorubicin-derived metabolites induce release of cytochrome C and inhibition of respiration on cardiac isolated mitochondria. Anticancer Res 23:2445–2450.

Das A, Durrant D, Mitchell C, Mayton E, Hoke NN, Salloum FN, Park MA, Qureshi I, Lee R, Dent P, Kukreja RC. 2010. Sildenafil increases chemotherapeutic efficacy of doxorubicin in prostate cancer and ameliorates cardiac dysfunction. Proc Natl Acad Sci USA 107:18202–18207.

Deschamps JD, Kenyon VA, Holman TR. 2006. Baicalein is a potent in vitro inhibitor against both reticulocyte 15-human and platelet 12-human lipoxygenases. Bioorg Med Chem 14:4295–4301.

Fenton HJH. 1984. Oxidation of tartaric acid in the presence of iron. J Chem Soc 65:899–910.

Fisher PW, Salloum F, Das A, Hyder H, Kukreja RC. 2005. Phosphodiesterase-5 inhibition with sildenafil attenuates cardiomyocyte apoptosis and left ventricular dysfunction in a chronic model of doxorubicin cardiotoxicity. Circulation 111:1601–1610.

Green PS, Leeuwenburgh C. 2002. Mitochondrial dysfunction is an early indicator of doxorubicin-induced apoptosis. Biochim Biophys Acta 1588:94–101.

Haworth RA, Potter KT, Russell DC. 2010. Role of arachidoninc acid, lopoxygenase, and mitochondrial depolarization in reperfution arrhythmias. Am J Physiol heart Circ Physiol 299:H165–H174.

Hwang IK, Yoo KY, Kim DS, Jeong YK, Kim JD, Shin HK, Lim SS, Yoo ID, Kang TC, Kim DW, Moon WK, Won MH. 2004. Neuroprotective effects of grape seed extract on neuronal injury by inhibiting DNA damage in the gerbil hippocampus after transient forebrain ischemia. Life Sci 75:1989–2001.

Ikegami E, Fukazawa R, Kanbe M, Watanabe M, Abe M, Kamisago M, Hajikano M, Katsube Y, Ogawa S. 2007. Edaravone, a potent free radical scavenger, prevents anthracycline-induced myocardial cell death. Circ J 71:1815–1820.

Kim SY, Kim SJ, Kim BJ, Rah SY, Chung SM, Im MJ, Kim UH. 2006. Doxorubicin-induced reactive oxygen species generation and intracellular Ca^{2+} increase are reciprocally modulated in rat cardiomyocytes. Exp Mol Med 38:535–545.

Lapchak PA, Maher P, Schubert D, Zivin JA. 2007. Baicalein, an antioxidant 12/15-lipoxygenase inhibitor improves clinical rating scores following multiple infarct embolic strokes. Neuroscience 150:585–591.

Lee V, Randhawa AK, Singal PK. 1991. Adriamycin-induced myocardial dysfunction in vitro is mediated by free radicals. Am J Physiol 261:H989–H995.

Li K, Sung RY, Huang WZ, Yang M, Pong NH, Lee SM, Chan WY, Zhao H, To MY, Fok TF, Li CK, Wong YO, Ng PC. 2006. Thrombopoietin protects against in vitro and in vivo cardiotoxicity induced by doxorubicin. Circulation 113:2211–2220.

Li J, Liu H, Ramachandran S, Waypa GB, Yin JJ, Li CQ, Han M, Huang HH, Sillard WW, Vanden Hoek TL, Shao ZH. 2010. Grape seed proanthocyanidins ameliorate doxorubicin-induced cardiotoxicity. Am J Chin Med 38:569–584.

Lim CC, Zuppinger C, Guo X, Kuster GM, Helmes M, Eppenberger HM, Suter TM, Liao R, Sawyer DB. 2004. Anthracyclines induce calpain-dependent titin proteolysis and necrosis in cardiomyocytes. J Biol Chem 279:8290–8299.

Liu TJ, Yeh YC, Ting CT, Lee WL, Wang LC, Lee HW, Wang KY, Lai HC. 2008. Ginkgo biloba extract 761 reduces doxorubicin-induced apoptotic damage in rat hearts and neonatal cardiomyocytes. Cardiovasc Res 80:227–235.

Mansat-de Mas V, Bezombes C, Quillet-Mary A, Bettaieb A, D'Orgeix AD, Laurent G, Jaffrezou JP. 1999. Implication of radical oxygen species in ceramide generation, c-Jun N-terminal kinase activation and apoptosis induced by daunorubicin. Mol Pharmacol 56:867–874.

Mikami T, Koyama T, Imakiire A, Yamamoto K, Furuhata M, Toyota H, Mizuguchi J. 2006. C-jun N-terminal kinase activation is required for apoptotic cell death induced by TNF-related apoptosis-inducing ligand plus DNA-damaging agents in sarcoma cell lines. Anticancer Res 26:1153–1160.

Panaretakis T, Laane E, Pokrovskaja K, Bjorklund AC, Moustakas A, Zhivotovsky B, Heyman M, Shoshan MC, Grander D. 2005. Doxorubicin requires the sequential activation of caspase-2, protein kinase Cdelta, and c-Jun NH2-terminal kinase to induce apoptosis. Mol Biol Cell 16:3821–3831.

Po LS, Chen ZY, Tsang DS, Leung LK. 2002. Baicalein and genistein display differential actions on estrogen receptor (ER) transactivation and apoptosis in MCF-7cells. Cancer Lett 187:33–40.

Psotova J, Chlopcikova S, Miketova P, Hrbac J, Simanek V. 2004. Chemoprotective effect of plant phenolics against anthracycline-induced toxicity on rat cardiomyocytes. Part III. Apigenin, baicalelin, kaempherol, luteolin and quercetin. Phytother Res 18:516–521.

Rajagopalan S, Politi PM, Sinha BK, Myers CE. 1988. Adriamycin-induced free radical formation in the perfused rat heart: Implications for cardiotoxicity. Cancer Res 48:4766–4769.

Rice-Evans CA, Miller NJ, Paganga G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 20:933–956.

Sadik CD, Sies H, Schewe T. 2003. Inhibition of 15-lipoxygenases by flavonoids: Structure-activity relations and mode of action. Biochem Pharmacol 65:773–781.

Shao ZH, Li CQ, Vanden Hoek TL, Becker LB, Schumacker PT, Wu JA, Attele AS, Yuan CS. 1999. Extract from *Scutellaria baicalensis* Georgi attenuates oxidant stress in cardiomyocytes. J Mol Cell Cardiol 31:1885–1895.

Shao ZH, Vanden Hoek TL, Qin Y, Becker LB, Schumacker PT, Li CQ, Dey L, Barth E, Halpern H, Rosen GM, Yuan CS. 2002. Baicalein attenuates oxidant stress in cardiomyocytes. Am J Physiol Heart Circ Physiol 282:H999–H1006.

Singal PK, Li T, Kumar D, Danelisen I, Iliskovic N. 2000. Adriamycin-induced heart failure: Mechanism and modulation. Mol Cell Biochem 207:77–86.

Spallarossa P, Altieri P, Garibaldi S, Ghigliotti G, Barisione C, Manca V, Fabbi P, Ballestrero A, Brunelli C, Barsotti A. 2006. Matrix metalloproteinase-2 and -9 are induced differently by doxorubicin in H9c2cells: The role of MAP kinases and NAD(P)H oxidase. Cardiovasc Res 69:736–745.

Tan X, Wang DB, Lu X, Wei H, Zhu R, Zhu SS, Jiang H, Yang ZJ. 2010. Doxorubicin induces apoptosis in H9c2 cardiomyocytes: Role of overexpressed eukaryotic translation initiation factor 5A. Biol Pharm Bull 33:1666–1672.

Tu IH, Yen HT, Cheng HW, Chiu JH. 2008. Baicalein protects chicken embryonic cardiomyocyte against hypoxia-reoxygenation injury via mu- and delta- but not kappa-opioid receptor signaling. Eur J Pharmacol 588:251–258.

Vanden Hoek TL, Li C, Shao Z, Schumacker PT, Becker LB. 1997. Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. J Mol Cell Cardiol 29:2571–2583.

Vanden Hoek TL, Becker LB, Shao Z, Li C, Schumacker PT. 1998. Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. J Biol Chem 273:18092–18098.

Wang L, Ling Y, Chen Y, Li CL, Feng F, You QD, Lu N, Guo QL. 2010. Flavonoid baicalein suppresses adhesion, migration and invasion of MDA-MB-231 human breast cancer cells. Cancer Lett 297:42–48.

Xu M, Ashraf M. 2002. Melatonin protection against lethal myocyte injury induced by doxorubicin as reflected by effects on mitochondrial membrane potential. J Mol Cell Cardiol 34:75–79.

Yamamoto Y, Hoshino Y, Ito T, Nariai T, Mohri T, Obana M, Hayata N, Uozumi Y, Maeda M, Fujio Y, Azuma J. 2008. Atrogin-1 ubiquitin ligase is upregulated by doxorubicin via p38-MAP kinase in cardiac myocytes. Cardiovasc Res 79:89–96.

Zhang SH, Wang WQ, Wang JL. 2009. Protective effect of tetrahydroxystilbene glucoside on cardiotoxicity induced by doxorubicin in vitro and in vivo. Acta Pharmacol Sin 30:1479–1487.

Zhou QM, Wang S, Zhang H, Lu YY, Wang XF, Motoo Y, Su SB. 2009. The combination of baicalin and baicalein enhances apoptosis via the ERK/p38 MAPK pathway in human breast cancer cells. Acta Pharmacol Sin 30:1648–1658.