

## Effect of Curcumin on Doxorubicin-induced Cytotoxicity in H9c2 Cardiomyoblast Cells

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### Abstract

#### Objective(s)

Doxorubicin (DOX), a widely used chemotherapeutic agent can give rise to severe cardiotoxicity by inducing apoptosis. Curcumin, the active compound of the rhizome of *Curcuma longa* L. has anti-inflammatory, antioxidant and anti-proliferative activities. Curcumin has been identified to increase cytotoxicity in several cancer cell lines in combination with DOX, but there is no study about its effect and DOX on normal cardiac cells. Therefore, in the present study, we evaluated the effect of curcumin on apoptosis induced by DOX in H9c2 rat heart-derived cells.

#### Materials and Methods

Cell viability was determined by MTT assay. Also, activation of caspase-3 was evaluated by spectrophotometry. Quantitative real time RT-PCR was used to evaluate the expression of c-IAP1. Detection of intracellular DOX accumulation was performed by flow cytometry.

#### Results

No toxicity observed when the cells exposed for 1 hr to different concentrations of curcumin, but pretreatment of cells with curcumin increased cytotoxicity of DOX in a dose dependent manner. Analysis of caspase-3 activation showed that curcumin pretreatment increased caspase-3 activation. RT-PCR analysis clearly showed that curcumin significantly decreased mRNA gene expression of c-IAP1 compared to cells treated with DOX alone. Pretreatment of H9c2 cells with DOX and curcumin had no effect on the intracellular accumulation of DOX.

#### Conclusion

Our observations indicated that subtoxic concentrations of curcumin sensitize H9c2 cells to DOX-induced apoptosis. These results suggest that the use of curcumin in combination with DOX in malignancy must be reevaluated.

**Keywords:** Apoptosis, Curcumin, Doxorubicin, H9c2 cells

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## Introduction

Since the late 1960s, the anthracycline antibiotic doxorubicin (DOX, adriamycin) has been one of the most largely prescribed chemotherapeutic drugs for the treatment of a variety of hematologic and solid malignancies, such as leukemia, bladder, lung and breast cancer, Hodgkin's and non-Hodgkin's lymphomas, and ovarian cancer (1, 2). Unfortunately, in addition to its potent anti-tumor activity, the use of DOX is associated with a number of unwanted side effects, especially with serious cardiac toxicity which can progress to end-stage heart failure (2). The exact mechanism of DOX-induced cardiotoxicity and its progression to heart failure are not fully understood yet. Different **hypothesis** have been proposed to explain DOX induced cardiotoxicity. The supposed mechanism is the redox activation to a semi-quinone intermediate and the formation of reactive oxygen species (ROS) which ultimately results in myocytes apoptosis (3, 4). Antioxidants can inhibit DOX-induced cardiomyocyte apoptosis suggesting that ROS is involved in apoptotic cell death (5, 6).

Curcumin from *Curcuma longa* is a nutraceutical compound reported to possess therapeutic properties against a variety of diseases ranging from cancer to cystic fibrosis (7). The strong antioxidant activity of curcumin makes it an interesting candidate for use in counteracting oxidative stress-induced damage (8, 9). Curcumin has been proposed as a putative agent in the treatment of some cardiovascular disorders (10).

On the other hand, a few published studies have indicated that curcumin is able to increase DOX effectiveness in the cancer cells (7, 11). No data are available if these effects are related to **tumor** cells or it could be observed in normal cardiac cells.

For above reasons, in this study we aimed to assess the effect of curcumin pretreatment on DOX-induced apoptosis in H9c2 cells. Due to its well known antioxidant properties, we hypothesized that curcumin would be able to prevent the DOX-induced cytotoxicity in H9c2 cells. The results that we present here differ from the previous hypothesis and show

curcumin was not able to protect against DOX-mediated cardiotoxicity.

## Materials and Methods

### Materials

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide], Curcumin and DOX were bought from Sigma Aldrich (St Louis, MO, USA). Cell culture medium, penicillin-streptomycin and fetal bovine serum (FBS) were purchased from Gibco (Gibco, Grand Island, NY, USA). Caspase-3 colorimetric assay kit was from abcam (abcam superscript® plc, USA). High pure RNA isolation kit was from Roche (Mannheim, Germany). Real time RT-PCR kit was bought from Invitrogen (Carlsbad CA, USA).

### Methods

#### Cell culture

Rat heart cell line H9c2 was obtained from American Type Culture Collection (ATCC, CRL-1446). The H9c2 cells were grown in Dubblico modified Eagle medium (DMEM) with 4 mM L-glutamine, 4.5 g/l glucose, 1 mM sodium pyruvate, 10% (v/v) heat inactivated FBS, penicillin G (100 U/ml) and streptomycin (100 mg/ml) at 37 °C in 95% CO<sub>2</sub> humidified incubator. The myoblastic population would become depleted rapidly if the cultures were allowed to become confluent. Thus, to prevent loss of myoblastic population, cells were subcultured when 70% of the flask was covered with cells and plated (25000-30000 cells/cm<sup>2</sup>) in 25-cm flask for flow cytometry studies and in 6-well plate for other studies.

#### Cell viability assay

Cellular toxicities of DOX and curcumin were analyzed in H9c2 cells using MTT method. Cells were plated onto 96-well plates at a density of  $2.0 \times 10^4$  cells/well and in a volume of 200  $\mu$ l. Stock solutions of curcumin and DOX were prepared in dimethyl sulfoxide (DMSO). The final concentration of the vehicle in the medium was always 0.5%. One day after seeding, 2  $\mu$ l of the DMSO containing DOX or curcumin at different concentrations was added to each well. At appropriate time intervals, the medium was

removed and replaced by 100  $\mu$ l of 0.5 mg/ml of MTT in growth medium and then the plates transferred to a 37 °C incubator for 3-4 hr. Supernatants were removed and the reduced MTT dye was solubilized with DMSO (100  $\mu$ l /well). Absorbance was determined on an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain sample signal (OD<sub>570</sub>-OD<sub>630</sub>). Percentage of proliferation was calculated using the following formula:

Percent of control proliferation =  $(OD_{\text{test}}/OD_{\text{control}}) \times 100$ .

IC<sub>50</sub> values were calculated by plotting the log<sub>10</sub> of the percentage of proliferation versus drug concentration.

### **Caspase-3 activity**

The activity of caspase-3 was determined by the abcam colorimetric caspase-3 kit according to the manufacturer's instructions. This assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate 7-amino-4-trifluoromethyl coumarin conjugated *p*NA (DEVD-*p*NA) in equal amount of cells protein lysates. Briefly, 1 $\times$ 10<sup>6</sup> cells were collected and lysed with 50  $\mu$ l of chilled lysis buffer and incubated on ice for 10 min. Cell lysates were centrifuged at maximum speed for 5 min at 4 °C, after which 50  $\mu$ l of 2 $\times$  reaction buffer/dithiothreitol (DTT) mix and 5  $\mu$ l of 1 mM caspase-3 substrate (DEVD-*p*NA) were added to each reaction and incubated at 37 °C for 1 hr. The *p*NA light emission was quantified using a microplate reader at 400- or 405- nm. Comparison of the absorbance of *p*NA from an apoptotic sample with an un-induced control allowed determination of the fold increase in caspase-3 activity. The protein content was determined by the Bradford method using the bovine serum albumin as a standard.

### **Real time RT-PCR analysis of the inhibitor of apoptosis protein (c-IAP1)**

Total RNA of H9c2 cells was extracted using high pure isolation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Quality and quantity of total RNA was assessed by spectrophotometry (NanoDrop<sup>TM</sup> 2000, USA) and

samples stored at -80 °C until use. Quantitative specific RNA expression was performed in one step with cybr-green invitrogen kit. Specific primer sequences were as follows: For c-IAP1, sense: 5'-CTGGGAACCGAACGATGATC-3'; anti-sense: 5'-AGCCACCATCACAACAAAAGC-3'. For  $\beta$ -actin, sense: 5'-TTGCTGATCCACATCTGCTG-3'; anti-sense: 5'-GACAGGATGCAGAAGGAGAT-3'. Thermal cycler conditions were 15 min at 50 °C for cDNA synthesis, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C to denature the DNA and 45s at 60 °C to anneal and extend the template. We did melting curve analysis to ascertain specification by continuous acquisition from 65 °C -95 °C with a temperature transient rate of 0.1 °C/S. All reactions were performed in triplicate in a Stratagene MX 3000P system (USA). The values obtained for the target gene expression were normalized to  $\beta$ -actin and analyzed by the relative gene expression - $\Delta\Delta$ CT method where  $-\Delta\Delta$ CT = (CT target - CT  $\beta$ -actin) Unknown - (CT target - CT  $\beta$ -actin) Calibrator.

### **DOX uptake assay**

For determination of DOX accumulation, cells were seeded in six well plates at a density of 2 $\times$ 10<sup>5</sup> cells / well and exposed to DOX, with or without 1 hr pretreatment with different concentrations of curcumin. After treatment, cells were trypsinized, washed twice with ice-cold PBS. After resuspending in ice-cold PBS, the intracellular DOX fluorescence immediately was measured using a Partec<sup>TM</sup> cytometer (Germany) equipped with a standard argon laser for 488-nm excitation and a 575-nm band pass (FL2) filter. Ten thousand (10<sup>4</sup>) gated events were collected for all the samples. Debris was eliminated by gating on forward versus side scatter. In the above procedure, treatment only with vehicle was considered to measure of cellular auto fluorescence. Flow cytometry data were processed and analyzed using FloMax version 2.52.

### **Statistical analysis**

Each experiment was performed at least three times, and the results were presented as mean $\pm$ SEM. One-way analysis of variance

(ANOVA) followed by Tukey's test was used to compare the differences between means. Statistical significances were considered at  $P < 0.05$ .

**Results**

**Cell viability after exposure to DOX and curcumin alone**

The viability of H9c2 cardiomyoblast cells was evaluated after 18, 22 and 28 hr of exposure to different concentrations of DOX. Cell viability was evaluated by the MTT method. As shown in Figure 1, DOX-induced cytotoxicity was time and dose dependent. The mean±SEM IC<sub>50</sub> values were 5.25±0.051 μM, 3.73±0.082 μM and 1.64±0.039 μM for 18, 22 and 28 hr exposure to DOX, respectively. The appropriate incubation time for complete dose-response cytotoxicity assay was 22 hr.

In order to set curcumin at concentrations which are nontoxic to cells but could prevent DOX-induced apoptosis, we also examined the effects of different concentrations of curcumin on cell viability in H9c2 cells. The cells were incubated with curcumin (1-50 μM) for 1, 2 and 24 hr. The percentage of cell viability under different treatment conditions has been shown in Figure 2. The Figure clearly revealed that one hr treatment with curcumin had no cytotoxic effects at the concentration up to 50 μM, while 2 hr and 24 hr exposure to curcumin induced cytotoxicity at the concentrations more than 30 μM and 10 μM, respectively. Therefore, one hr pretreatment with curcumin was then selected.

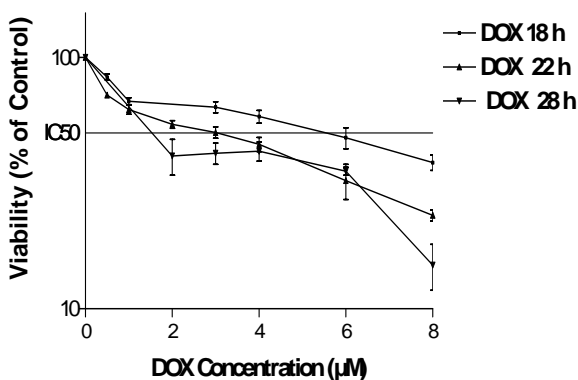


Figure 1. Time- and dose-dependent DOX-induced H9c2 cytotoxicity. The cell viability was determined by MTT assay as described in material and methods. Data are expressed as the mean±SEM of three separate experiments (n= 6).

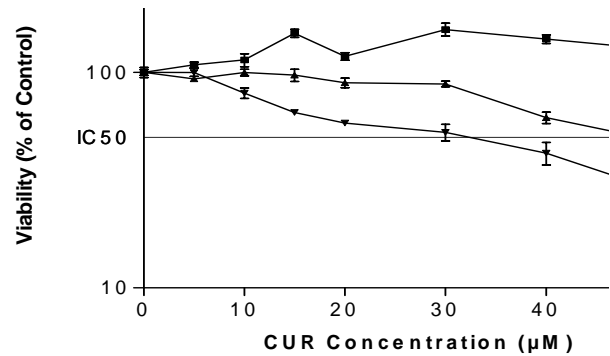


Figure 2. Cell viability of H9c2 cells after exposure to curcumin (CUR). Cells were treated with different concentrations of curcumin (5-50 μM) for 1 hr, 2 hr and 24 hr. The cell viability was determined by MTT assay. Data are expressed as the mean±SEM of three separate experiments (n= 6).

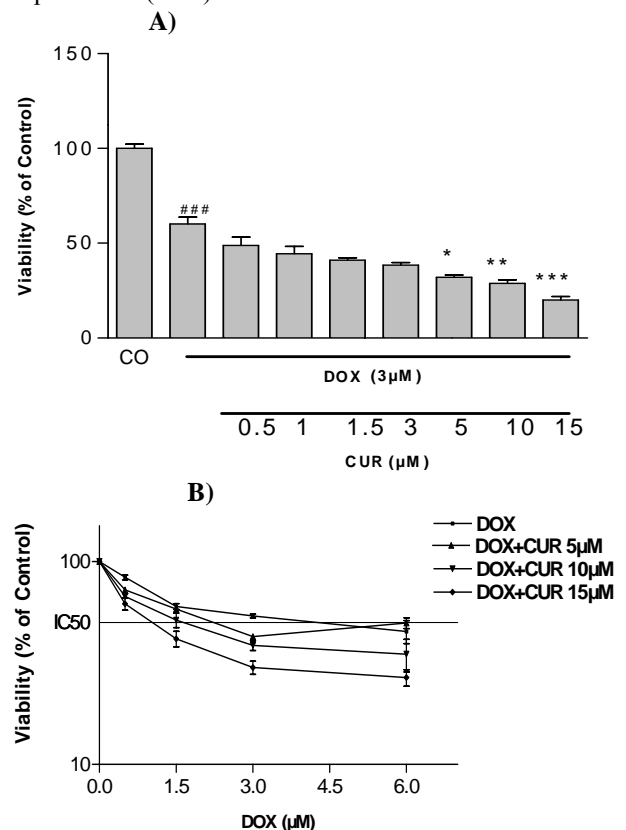


Figure 3. The effect of curcumin on DOX cytotoxicity in H9c2 cells. Cell pretreated with different concentrations of curcumin (CUR) 1 hr before exposure to A) 3 μM of DOX and B) indicated concentrations (0.5, 1.5, 3 and 6 μM) of DOX. Data are expressed as the mean±SEM of three separate experiments (n= 6). ### $P < 0.01$  vs. Control, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. DOX treated cells.

**Effect of curcumin pretreatment on DOX-induced cell death**

For evaluation of curcumin pretreatment on DOX-induced cytotoxicity, H9c2 cells were pretreated for 1 hr with curcumin (0.5-15 μM), then the medium was changed and cells were

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treated with DOX (3  $\mu$ M) for 22 hr. Surprisingly, as shown in Figure 3A, compared to control the presence of curcumin exacerbated the extent of cell death, which was significant at higher concentrations of curcumin. For further investigation, concentration–response curve was performed for DOX to access the IC<sub>50</sub> value with or without 1 hr pretreatment with curcumin (5-15  $\mu$ M). The results showed that one hour pretreatment with curcumin shifted the concentration–response curves to a lower IC<sub>50</sub> values in a dose dependent manner (Figure 3B) (Table 1).

**Effect of curcumin pretreatment on caspase-3 activation**

Activation of caspase cascade is critical in the initiation of apoptosis in various biological systems. A member of this family, caspase-3 has been identified as being a key mediator of apoptosis in mammalian cells. Therefore, to investigate the type of cell death involved in the experiments outlined above, the activity of caspase-3 was examined. The results indicated that pretreatment with curcumin significantly increased caspase-3 activation compared to DOX-treated cells (Figure 4).

**Effect of curcumin on c-IAP1 expression**

In order to complete our results and also to investigate how curcumin pretreatment increased DOX-induced apoptosis, we examined the mRNA expression of a member of the Inhibitors of apoptosis family cIAP1. Real time RT-PCR analysis showed a reduction of the expression level of c-IAP1 after 22 hr treatment with DOX. However, pretreatment with curcumin led to a significant down-regulation of cIAP1 compared to cells treated with DOX alone (Figure 5).

**Effect of curcumin pretreatment on intracellular DOX accumulation**

To examine whether curcumin-enhanced DOX potency was a result of its inhibition of any other or undefined drug-transporters, which may recognize DOX as a substrate, we evaluated the effect of curcumin on DOX accumulation in H9c2 cells. There was no significant difference in DOX accumulation between cells exposed to DOX (3  $\mu$ M) and cells pretreated with curcumin (5-15  $\mu$ M) before exposure to DOX (Figure 6).

Table 1. Effect of curcumin pretreatment on DOX-induced cytotoxicity in H9c2 cells.

group	IC <sub>50</sub> ±SEM	95% CI*
DOX	3.6±0.049	3.477-3.726
DOX+ CUR (5 $\mu$ M)	2.5±0.105 <sup>a</sup>	2.233-2.270
DOX+CUR (10 $\mu$ M)	1.82±0.098 <sup>a</sup>	1.568-2.072
DOX+CUR (15 $\mu$ M)	0.95±.064 <sup>a</sup>	0.7855-1.115

Data are expressed as the mean±SEM of three separate experiments.

<sup>a</sup> P< 0.001 vs. DOX treated cells.

\* 95% confidence interval.

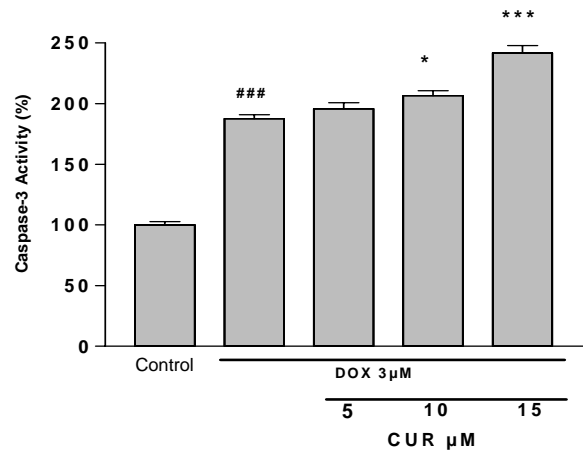


Figure 4. The effect of curcumin on caspase-3 activity in H9c2 cells. Cell pretreated with different concentrations of curcumin (CUR) 1 hr before exposure to 3  $\mu$ M of DOX. Caspase-3 activity measured by colorimetric detection of p-nitroanilide and expressed as percentage of control. Data are expressed as the mean±SEM of three separate experiments (n= 6). ## P< 0.01 vs. control, \* P< 0.05, \*\*\*P< 0.001 vs. DOX treated cells.

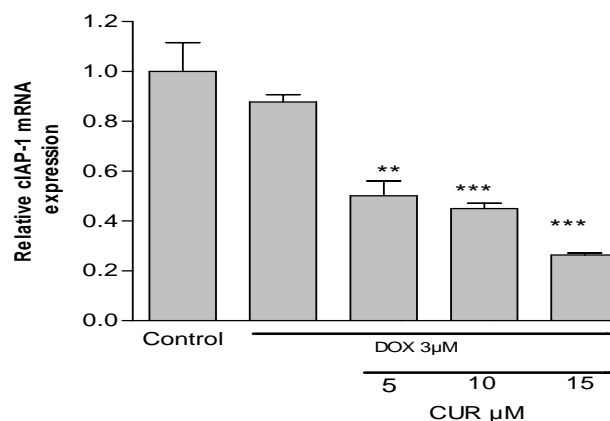


Figure 5. Real-time RT-PCR analysis of effect of curcumin on cIAP1 mRNA expressions. Cell pretreated with different concentrations of curcumin (CUR) 1 hr before exposure to 3  $\mu$ M of DOX. Normalization relative to  $\beta$ -actin was performed. Levels of mRNA are expressed relative to control cardiomyoblast in the mean (SEM) values derived from three independent experiments, \*\*P< 0.01, \*\*\*P< 0.001 vs. DOX treated cells.

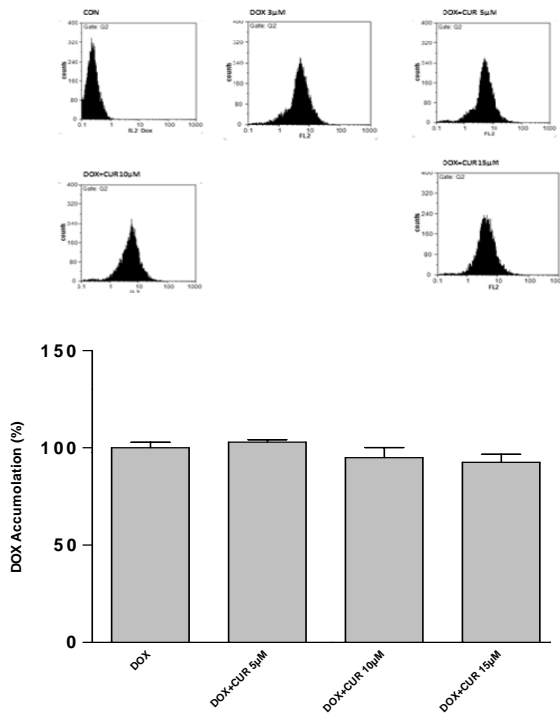


Figure 6. The effect of curcumin on DOX accumulation in H9c2 cells. Cell pretreated with different concentrations of curcumin (CUR) 1 hr before exposure to 3  $\mu$ M of DOX. DOX accumulation measured by flow cytometry and expressed as percent of DOX. A) Representative of flow cytometry with FL2 plots of H9c2 cells in different groups B) Column bar graph of mean cell fluorescent for FL2. Data are expressed as the mean $\pm$ SEM of three separate experiments (n= 6).

### Discussion

DOX is well known cancer chemotherapeutic agent used to treat a wide variety of human malignancies (4, 12). A major adverse effect of DOX treatment in cancer patients is the onset of cardiomyopathy and heart failure (13). Reactive Oxygen Species (ROS) derived from redox activation of DOX were proposed to be responsible for DOX-induced apoptosis in cardiac cells (6, 14, 15). Therefore, antioxidant treatment has been proposed as a potential strategy in the prevention of apoptosis in cardiac cells.

Curcumin is known for its antioxidant properties and act as free radical scavenger by inhibiting lipid peroxidation and oxidative DNA damage (16). Moreover, it has been proposed as a candidate to treat cardiovascular disease involving oxidative stress (11). There are reports that show curcumin is able to inhibit apoptosis,

through its well known antioxidant properties in normal cells (8, 17, 18).

On the other hand, it has been reported in studies that curcumin increases cytotoxicity of DOX in *cancer cells* (10,19, 20). Therefore, it is important to evaluate whether a similar phenomenon occurs in cardiomyoblast which are the main target of DOX toxicity. Consequently, our study was designed for evaluation of curcumin pretreatment against DOX cytotoxicity in H9c2 cells. At first, cytotoxicity of DOX and curcumin in different exposure times were evaluated. The results showed that one hour exposure to different concentrations of curcumin had no cytotoxic effect in H9c2 cells. However, curcumin was able to induce cytotoxicity at high concentrations, when duration of exposure exceeded one hour. It may not be a simple stoichiometric reaction. The function of curcumin was biphasic: it was non toxic at low concentrations, while higher doses promoted cell death. This is in line with a study that demonstrated 24 hr treatment with 5  $\mu$ M curcumin resulted marginal decrease in cell viability in murine macrophage RAW 264.7 cells (21). Additionally, another study showed that the concentrations of curcumin higher than 20  $\mu$ M were able to induce toxicity in the MES23.5 cells (8). Although, curcumin has been described as a protective agent in several cell models, we observed here that pretreatment to subtoxic concentrations of curcumin greatly increased cytotoxicity compared to level reached with the DOX alone. The present findings corroborate similar findings by Ortiz-Ortiz, *et al* who recently reported that curcumin enhanced paraquat induced cytotoxicity in N27 mesencephalic cells (16).

It has been reported that DOX treatment was able to induce cytotoxicity in cardiac cells through induction of apoptosis (2, 15). Many proteins are involved in this complex process. Caspases, a family of cysteine-dependent aspartate-directed proteases, play a critical role in the initiation and execution of apoptosis. Among this family of caspases, caspase 3, in particular, is believed to be one of the most commonly involved caspases in the execution of apoptosis in various cell types (22). Therefore, we assayed caspase-3 activation for

characterizing the nature of cell death that occurred in response to the pretreatment with curcumin. Curcumin increased DOX-induced caspase-3 activation in a dose dependent manner.

To confirm that synergistic effect of curcumin was due to apoptosis, we investigated the mRNA expression of a member of the inhibitors of apoptosis family cIAP1. IAPs have shown a remarkable ability of blocking cell death. It was demonstrated that caspase activation not only requires proteolytic cleavage of the enzyme themselves, but removal of inhibitory influences (IAPs) is also necessary (10). Curcumin has been reported to down-regulate the anti-apoptotic IAP proteins in human renal cells (23). Also, it was able to decrease cIAP1 and cIAP2 expression in human glioblastoma T988G cells (24).

In the present study, DOX slightly decreased inhibition of apoptotic protein c-IAP1 mRNA level after 22 hr, but curcumin potentiated this declining.

Thus, evaluation of caspase-3 activation and c-IAP1 expression, demonstrated that curcumin is able to potentiate the apoptotic effect of DOX in H9c2 cells. These results are in contrast with previously described findings that curcumin is able to protect normal cells against some cytotoxic agents (8, 17, 18).

Curcumin could also chemosensitize resistant tumor cells to DOX through an increase in the accumulation of DOX in the human carcinoma cell line (25). Therefore, we investigated whether curcumin pretreatment had an effect on the DOX accumulation in cardiomyoblast cells. The results indicated that the drug toxicity enhancement can not be explained by alteration of cellular DOX levels.

Several mechanisms may explain how

curcumin mediates its effect on cardiac myoblast cells. Oxidative stress plays an important role in DOX-induced apoptosis in cardiac cells. Recent studies have showed that curcumin, although a potent antioxidant with ROS scavenging properties was able to enhance ROS formation in different models. For instance rapid ROS generation by curcumin leads to caspase-dependent and independent apoptosis in L929 cells (26). Also, curcumin increased oxidative stress induced by paraquat via inhibition of nitric oxide synthase (NOS) (16). Therefore, it is possible that curcumin enhanced DOX-induced apoptosis through generation of ROS. However, there is still the lack of information on specific molecular mechanisms induced by synergistic combination of curcumin and DOX because curcumin influences many cellular signaling pathways (27), that play major role in the control of cell proliferation and survival.

Further studies are in progress in our laboratory to determine molecular mechanisms involved in synergistic effect of curcumin and DOX in rat cardiac muscle H9c2 cells.

## Conclusion

For the first time our findings demonstrated that curcumin enhanced the apoptosis induced by DOX in H9c2 cells. These results suggest that the use of curcumin in combination with DOX in malignancy must be reevaluated.

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