Multiple actions of pifithrin-α on doxorubicin-induced apoptosis in rat myoblastic H9c2 cells

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Doxorubicin (Dox) is an anthracycline antibiotic that has been widely used for the treatment of acute leukemia, malignant lymphoma, and solid tumors (16, 34, 61). Unfortunately, its cardiotoxicity (1, 8, 20, 54, 65). The prevailing hypothesis for the mechanism of Dox-induced cardiotoxicity (27). We hypothesized that PFT-α can attenuate Dox-induced apoptosis by inhibiting p53 phosphorylation, thus preventing the activation of p53 downstream events such as caspase activation.

MATERIALS AND METHODS

Cell culture and materials. Rat embryonic ventricular myocardial H9c2 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM (GIBCO, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 µg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO2. Dox was purchased from Sigma Chemical (St. Louis, MO). Fetal bovine serum was obtained from Biosource (St. Louis, MO).

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International (Camarillo, CA). PFT-α, digitonin, SB-203580, PD-98059, and SP-600125 were products of Calbiochem (La Jolla, CA). Antibodies against phospho-site-specific p53 at Ser-15, active caspase-3, active caspase-9, poly(ADP ribose) polymerase (PARP), phospho-site-specific p42/p44ERK, total p38, total p42/p44ERK, and total p46/p54JNK were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal caspase-8, caspase-2L, p53, Fas, phospho-site-specific p38, and phospho-site-specific p46/p54JNK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Briefly, H9c2 cells were treated with Dox and/or PFT-α for the indicated periods. This concentration was chosen because it reproduces the plasma peak level achieved in patients receiving standard infusions of Dox (12). In our study, Dox triggered a time-dependent increase of p53 (Fig. 1A).

Because the stability of p53 is regulated by posttranslational modifications such as phosphorylation, and because phosphorylation of p53 at Ser15 is known to be essential for the transactivation of p53, we studied the effect of Dox on the level of phosphorylated p53 at Ser15. Western blot analysis was carried out with a specific antibody against phospho-p53 at Ser15. To explore the effects of Dox on p53 levels, serum-starved H9c2 cells were treated with 5 μM Dox for different time periods. This concentration was chosen because it reproduces the plasma peak level achieved in patients receiving standard infusions of Dox (12). In our study, Dox triggered a time-dependent increase of p53 (Fig. 1A).

**RESULTS**

Electrophoretic mobility shift assays. H9c2 cells (4 × 10^6) in DMEM containing 10% FBS were treated with or without 5 μM Dox and/or 20 μM PFT-α for 5 h. Nuclear extracts were prepared by a Nuclear Extraction kit (Active Motif, Carlsbad, CA), and protein concentration was determined by Bio-Rad dye-binding method. Syn-}

**Electrophoretic mobility shift assays.** H9c2 cells (4 × 10^6) in DMEM containing 10% FBS were treated with or without 5 μM Dox and/or 20 μM PFT-α for 5 h. Nuclear extracts were prepared by a Nuclear Extraction kit (Active Motif, Carlsbad, CA), and protein concentration was determined by Bio-Rad dye-binding method. Synthetic consensus p53 binding sequence (Santa Cruz Biotechnology) was end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (Promega, Madison, WI). Binding reactions were carried out in a final volume of 10 μl containing 5 μg of nuclear extract, 10 mM HEPES, pH 7.9, 4 mM Tris·HCl, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 32P-labeled probe. Reactions were incubated at room temperature for 30 min. DNA-protein complex was separated on a 5% polyacrylamide gel in 0.5× TBE (1× TBE: 0.045 M Tris-borate, 1 mM EDTA). Gel was dried and exposed to Kodak BioMax films. For supershift assays, 1 μg of anti-p53 antibody (clone Ab421, Oncogene Research Products, San Diego, CA) was included in the reaction.

**Apoptosis assays.** Quantitative analysis of apoptosis was performed with a Cell Death ELISA plus kit (Roche, Indianapolis, IN). Cells (5 × 10^5) were plated in 48-well dishes, changed to DMEM containing 0.5% FBS for the designated period, and then treated with Dox and/or PFT-α for 7 h. Cells were lysed with 0.2 ml lysis buffer provided in the kit at room temperature for 20 min. Quantities of histone-associated DNA fragments (mononucleosomes and oligonucleosomes) were determined by absorbance at 405 nm with a Tecan microplate reader.

**Statistical analysis.** Statistical analyses were performed by one-way ANOVA followed by Tukey’s multiple comparison test to show differences between means. Data were represented as means ± SE. P < 0.05 was considered significant.

**RESULTS**

To explore the effects of Dox on p53 levels, serum-starved H9c2 cells were treated with 5 μM Dox for different time periods. This concentration was chosen because it reproduces the plasma peak level achieved in patients receiving standard infusions of Dox (12). In our study, Dox triggered a time-dependent increase of p53 (Fig. 1A).

Because the stability of p53 is regulated by posttranslational modifications such as phosphorylation, and because phosphorylation of p53 at Ser15 is known to be essential for the transactivation of p53, we studied the effect of Dox on the level of phosphorylated p53 at Ser15. Western blot analysis was carried out with a specific antibody against phospho-p53 at Ser15.
Ser15. Figure 1B shows that Dox treatment led to a time-dependent increase of phospho-p53 at Ser15. Densitometric analysis demonstrated an increase of 3.3-fold and 4.7-fold after 30 min and 4 h of Dox treatment, respectively.

To determine whether MAP kinases (MAPKs) are affected by Dox administration, Western blot analysis was carried out with antibodies against phospho-site-specific MAPKs, including p38, p42/p44ERK, and p46/p54JNK. In addition, Western blot analysis was carried out with antibodies against total p38, p42/p44ERK, and p46/p54JNK. We found that Dox induced a transient increase in phosphorylation of all three MAPKs. Phosphorylation of p38 reached a peak 10 min after Dox treatment, after which phosphorylation returned to baseline. Pretreatment of H9c2 cells with 20 μM of a specific p38 inhibitor, SB-203580, completely blocked the elevation in phospho-p38 level (Fig. 2A). This concentration of SB-203580 was chosen according to the experimental design of a previous study (6).

Phosphorylation of p42/p44ERK and p46/p54JNK reached a peak 30 min after Dox treatment, after which phosphorylation returned to baseline. The elevation in p42/p44ERK and p46/p54JNK levels was abolished by 20 μM PD-98059 and 10 μM SP-600125, which are specific inhibitors of p42/p44ERK and p46/p54JNK, respectively (Fig. 2, B and C). The concentrations of these inhibitors were chosen according to the experimental design of previous studies (3, 47). These results indicated that Dox can transiently activate all three MAPKs. It is noted that 20 μM PD-98059 by itself induces the phosphorylation of p42/p44ERK. In addition, 10 μM SP-600125 seems to have a slight effect on JNK protein content.

We next explored the effect of PFT-α, a chemical inhibitor of p53, on Dox-induced levels of p53 and phospho-p53 (Ser15). H9c2 cells were treated with 5 μM of Dox and/or 20 μM PFT-α for 8 h. Western blot analysis was performed on 30-μg cell lysates with antibodies against p53 or phospho-p53 (Ser15). Actin was included to check for protein loading. Bar graph shows fold of induction of p53 and phospho-p53 (Ser15) after Dox and/or PFT-α treatment vs. control (Con) by image analysis. Data represent means ± SE from 4 samples. *P < 0.05, Dox-treated vs. control cells. **P < 0.05, Dox + PFT-α-treated cells vs. Dox-treated cells.

Fig. 2. Dox activates p38, p42/p44ERK, and p46/p54JNK MAPKs in H9c2 cells. Cells were pretreated 2 h with 20 μM SB-203580, 2 h with 20 μM PD-98059, or 10 min with 10 μM SP-600125 before treatment with 5 μM Dox for indicated periods and were harvested. Western blot analysis was carried out with phospho-specific antibodies against p38 (A), p42/p44ERK (B), or p46/p54JNK (C). Duplicate blot was probed with antibodies against total MAPKs.

Fig. 3. Effect of pifithrin-α (PFT-α) and Dox on the expression of p53 and phospho-p53 (Ser15). H9c2 cells were treated with 5 μM of Dox and/or 20 μM PFT-α for 8 h. Western blot analysis was performed on 30-μg cell lysates with antibodies against p53 or phospho-p53 (Ser15). Actin was included to check for protein loading. Bar graph shows fold of induction of p53 and phospho-p53 (Ser15) after Dox and/or PFT-α treatment vs. control (Con) by image analysis. Data represent means ± SE from 4 samples. *P < 0.05, Dox-treated vs. control cells. **P < 0.05, Dox + PFT-α-treated cells vs. Dox-treated cells.
Electrophoretic mobility shift assay showed that p53 binding activity was induced after Dox treatment for 5 h (Fig. 5, lanes 1 and 2). PFT-α itself did not affect DNA binding activity of p53 (Fig. 5, lane 3). The addition of PFT-α attenuated Dox-induced p53 binding activity (Fig. 5, lane 4). In the presence of p53 antibody Ab421, a supershifted p53 complex was generated (Fig. 5, lane 5).

The effect of Dox on caspase activation was studied in H9c2 cells that were treated with Dox with or without PFT-α for 16 h. In these experiments, caspase-2, -3, -8, and -9 activities were measured by using specific fluorogenic substrates (Ac-VDVAD-AMC, Ac-DEVD-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC). Results revealed that Dox activated caspases-2, -3, -8, and -9 by 3.77-, 2.1-, 2.2-, and 4.7-fold, respectively. The addition of PFT-α along with Dox partially suppressed these enzyme activities (Fig. 6, A–D). It is interesting to note that PFT-α by itself could significantly block the endogenous level of caspase-3 (Fig. 6B).

To investigate the expression of caspases and other apoptosis-related genes at the protein level, lysates were prepared from H9c2 cells treated with Dox and/or PFT-α for 8 h. This time point was chosen because there was some caspase activation in the control cells at 12 or 16 h as a result of low serum; this basal level of caspase activation would have interfered with the interpretation of any results derived from Dox-treated lysates prepared at 12 or 16 h. Western blot analysis was carried out with specific antibodies against caspases-2L, -3, -8, and -9, PARP, Fas, and actin. Caspases-2, -8, and -9 are initiator caspases (10, 38, 64) and caspase-3 is an executioner caspase (42), whereas caspase-12 is an endoplasmic reticulum (ER)-associated caspase (39). Activation of caspases-2L, -3, and -9 was attenuated with the addition of PFT-α (Fig. 7). Procaspase-8 was barely detectable in untreated cells. After Dox treatment, there was an elevated level of 55-kDa procaspase and 44-kDa and 42-kDa activated fragments. The band density was decreased when PFT-α was added.
PARP is a substrate for caspase-3, and cleaved PARP has been shown to be an important marker for apoptosis (30). The pattern of PARP paralleled the pattern of active caspase-3. Caspase-12 was present as 60- and 54-kDa bands in untreated cells. On Dox treatment, the cleaved 54-kDa band was intensified. PFT-α could partially suppress the activation of caspase-12 (Fig. 7).

Fas is a cell surface protein that mediates apoptosis through a caspase-8-dependent pathway (67). Dox-upregulated FAS expression could be attenuated by PFT-α (Fig. 7).

Cytochrome c release is a marker for mitochondria-related apoptosis (29). H9c2 cells were permeabilized with 80 μg/ml of digitonin, and cytochrome c in the supernatant was subjected to Western blot analysis. Figure 8 shows that the release of cytochrome c was elevated after Dox treatment and that PFT-α partially blocked this elevation.

Quantification of apoptosis was performed by cell death ELISA assay. As shown in Fig. 9, Dox increased oligonucleosome formation by 1.8-fold. Cell death was inhibited by the addition of PFT-α along with Dox.

**DISCUSSION**

In this study, we present evidence that Dox rapidly upregulates p53, phosphorylated p53, p42/p44ERK, p38, and p46/p54JNK. We also demonstrate that Dox increases the activity of caspases-2, -3, -8, -9, and -12, leading to apoptosis. Most importantly, we demonstrate for the first time that PFT-α attenuates the apoptotic process by blocking Dox-induced expression of p53, phosphorylated p53, p42/p44ERK, p46/p54JNK, and the caspases listed above.
In our study, we found that Dox has an immediate effect on cell signaling pathways. Within 5–10 min, Dox rapidly phosphorylated all three MAPKs (Fig. 2). These results are in line with a study on the effect of daunomycin on the MAPKs in cardiomyocytes by Zhu et al. (72). In addition, we also found that Dox induced an upregulation of p53 level.

Given that Dox triggered an increase in the proapoptotic p53, it is reasonable to assume that Dox has proapoptotic effects. In line with this hypothesis, our study showed that Dox induced a p53-dependent activation of caspases-2, -3, -8, -9, and -12 (Figs. 6 and 7) and triggered cytochrome c release (Fig. 8). This indicates that Dox induces both mitochondria-related and death receptor-related apoptotic pathways. Furthermore, in line with the results of Jang et al. (18), we also found that Dox activated caspase-12, indicating that Dox induces the ER-related apoptotic pathway. Caspase-12 resides in the ER and is activated on ER stress, including free radicals and disturbances of the intracellular calcium level (25, 39). Previous investigations have shown that Dox treatment leads to the generation of reactive oxygen species (27, 68) and an increase of calcium influx (24); this ER stress is hypothesized to activate caspase-12 and the ER-dependent apoptotic pathway. On activation, caspase-12 is translocated from the ER membrane to the cytosol where it may activate caspase-3 directly (52).

In our study, we also showed that Dox increased the level of caspase-2L (Figs. 6 and 7). Two isoforms of caspase-2 exist as a result of alternative splicing: caspase-2S and caspase-2L (9, 64). Recent studies indicate that these two proteins have opposite effects on apoptosis: caspase-2L induces apoptosis, whereas overexpression of caspase-2S is antiapoptotic. There is strong evidence that caspase-2L serves as a direct effector of the mitochondrial apoptotic pathway by releasing proapoptotic proteins, such as cytochrome c, or by cleaving Bid (14, 53).

Previous studies have demonstrated that p53 represses the expression of Bcl-2, PTEN (phosphatase and tensin homolog deleted on chromosome 10), and survivin and upregulates the gene expression of proapoptotic proteins such as Bax, Noxa, and Puma (50). Bax translocation induces cytochrome c release and allows the formation of apotosomes, which contain caspase-9 and Apaf1. Caspase-9, in turn, activates caspase-3 and caspase-7, which execute the death program (35, 43).

A novel finding in our study is that PFT-α blocks the effects of Dox. First, Dox-induced elevation of p53 levels was partially blocked by PFT-α (Fig. 3). Previous studies have shown that PFT-α inhibits p53 accumulation in various cell systems.
subjected to ultraviolet radiation, cisplatin, or resveratrol treatment (19, 22, 31, 70). To our knowledge, this is the first study that has shown that PFT-α can block Dox-induced p53 levels in H9c2 cells.

Second, we found that PFT-α was able to suppress Dox-induced apoptosis. Specifically, PFT-α partially blocked the induction of Dox-induced Fas in H9c2 cells (Fig. 7), a result that is in agreement with a previous study in human umbilical endothelial cells (33). In addition, PFT-α suppressed the activation of caspases-2, -3, -8, -9, and -12 (Figs. 6 and 7). A reasonable question to ask is whether PFT-α blocks Dox-induced apoptosis through a p53-dependent mechanism, a p53-independent mechanism, or both. PFT-α has been reported to suppress other p53-independent effects. For example, Komarova et al. (23) found that PFT-α can suppress heat shock and glucocorticoid receptor signaling. In addition, PFT-α affects the transcription of a number of genes involved in DNA repair, apoptosis, and cell growth (48). In our study, PFT-α partially blocked the activation of p46/p54JNK and p42/p44ERK (Fig. 4), the activation of which are both p53 independent. Furthermore, in our study, PFT-α did not completely suppress the caspase activation and the overall apoptosis induced by Dox in H9c2 cells (Figs. 6 and 9), supporting the notion that there exists a p53-independent apoptotic pathway as described by Tsang et al. (63). It is possible that a p53-independent pathway plays an important part of the antiapoptotic effect of PFT-α, although this hypothesis needs to be explored further.

In summary, our results suggest that Dox induces apoptosis by upregulating p53 and caspases-2, -3, -8, -9, and -12 in H9c2 cells. We also present evidence that PFT-α partially attenuates these proapoptotic processes, although it is unclear whether the mechanism of this attenuation occurs through a p53-dependent pathway, a p53-independent pathway, or both. In our previous study, we demonstrated that PFT-α attenuated Dox-induced cardiac apoptosis in mouse hearts and had no effect on the tumor-killing activity of Dox in human prostate PC3 cells (32). As such, it is possible that combination therapy of PFT-α and Dox may be employed to prevent Dox-induced cardiotoxicity in patients who rely on Dox chemotherapy regimens.

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REFERENCES


