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The Efficacy of Etoposide on H9c2 Cardiomyoblasts Against Doxorubicin Induced Cardiotoxicity

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Abstract

Background: Doxorubicin (DOX), a widely used anticancer drug, has been associated with cardiotoxicity. Recently, DOX-induced cardiotoxicity has been attributed to topoisomerase II (TOPII)-β expression and activity. In our study, we investigated the effect of inhibiting TOPII in attenuating the DOX induced cardiotoxicity.

Method: H9c2 cardiomyoblasts were treated with 1 or 2 μM DOX (+/-) 1 μM ETO. Cardiotoxicity was assessed by examining cell viability using the MTT assay, hypertrophy of crystal violet stained cardiomyoblasts and ROS production.

Results: DOX induced a dose dependent increase in cardiotoxicity as indicated by the significant reduction in cell viability (71.77 ± 9.25% 2 μM DOX vs. 100% control, $P<0.05$), ROS production and hypertrophy. Stimulation of H9c2 cardiomyoblasts with both 2 μM DOX and 1μM ETO did not show a significant difference in cell viability, ROS production or hypertrophy.

Conclusion: DOX induced cardiotoxicity in H9c2 cardiomyoblasts was not exacerbated in the presence of 1 μM ETO. This provides further support to using the combination of DOX and ETO, which is currently being done to treat advanced AIDS related sarcomas in the clinical setting.

Keywords: Doxorubicin; Etoposide; Topoisomerase II; Cardiomyocyte hypertrophy

Introduction

Doxorubicin (DOX), one of the most effective and used anthracyclines [1], has been used for several decades due to its potent broads spectrum antineoplastic activity [2]. DOX is heavily used to treat hematological malignancies such as multiple myeloma and hodgkin's lymphoma [3,4]. In addition, DOX has been used for the treatment of solid tumors like ovarian and breast cancer [5,6]. Despite the clinical application of DOX, it is well known to induce a dose-dependent cardiotoxicity, which limits its clinical usage [7]. DOX induced cardiotoxicity, early-onset or late onset, is characterized by a decline in left ventricular ejection fraction or the development of congestive heart failure [1]. In a retrospective analysis of three trials it has been demonstrated that 26% of all patients who receive a cumulative DOX dose of ≥ 550 mg/m2 develop DOX related congestive heart failure [8].

The underlying molecular mechanism of DOX induced cardiotoxicity remains unclear. Zhang et al. reported that chronic DOX exposure induces functional and structural changes in the mitochondria; manifested by mitochondrial damage and vacuolization [9]. In addition, DOX was found to induce alterations in cardiac myosin and is responsible for nuclear membrane disruption [10]. Previous reports have associated DOX induced cardiotoxicity with its ability to produce reactive oxygen species (ROS) [11,12], which causes a release of iron and contributes to DNA damage and lipid peroxidation [13]. Recent reports have suggested that DOX-induced cardiotoxicity is mediated in part by topoisomerase II (TOPII) - β expression and activity [9,13,14]. TOPII is an enzyme that uncoils the supercoiled double stranded DNA and contributes to DNA replication. Two isoforms of TOPII exist, TOPII-a and TOPII-β, which are expressed in different tissue. TOPII-a is expressed in proliferating tissues including the bone marrow, spleen, and tumor cells and TOPII-β is expressed in adult mammalian cardiomyocytes [15]. Furthermore, an *in vitro* study showed that Dexrazoxane, which is the only approved iron-chelating agent to treat DOX induced cardiotoxicity, reduced the expression of TOPII-β enzyme [14]. Another study demonstrated that TOPII-β knockout mice had improved cardiac function compared to the control group [9]. In our study, we hypothesize that TOPII-β contributes to DOX induced cardiotoxicity.

In our study we aimed to develop an in-vitro model in which DOX induces cardiotoxicity. In addition, we investigated the effect of inhibiting TOPII in attenuating DOX induced cardiotoxicity. Etoposide (ETO), a non-specific TOPII targeted anticancer drug and used in solid tumors such as lung cancer, lymphomas and sarcomas, was used in our study to inhibit TOPII [16]. Zhang et al. reported that ETO possess a time dependent degradation of both TOPII-a and TOPII-β, but with a greater effect on TOPII-β [17]. In addition, we examined the cardiotoxic effect of co administering DOX and ETO.

Materials and Methods

This study was carried out at the College of Pharmacy, Qatar University, Doha, Qatar.

Cell culture

H9c2 myoblasts, a clonal cell line derived from the embryonic

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BD1X rat heart tissue, were obtained from the European Collections of Cell Cultures (ECACC) and cultured in DMEM/F12 1:1 culture media supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere (95% O2-5% CO2). Upon becoming confluent, cells were seeded at a density of 2.0×106 cells per 35 mm culture dishes containing the 10% FBS culture medium and cultured for 24 hours. The cells were then treated with 1 or 2 μM DOX in the presence and absence of 1 μM ETO for 48 hours in preparation for assessment of cell viability, hypertrophy or ROS production.

Cell viability assay

Cell viability assay was measured using the MTT (3-(4,5)-dimethylthiazo(-z-yl)-3,5-dipheyltetrazoliumromide) assay. The cells were plated at a density of 50,000 cells/well in 24-well plates and allowed to adhere. Following treatment, 10 μl of MTT stock solution (5 mg/ml) were added to each well. After 4 hours of incubation at 37°C, the media was aspirated, and the produced formazan was solubilized in 100 μl dimethyl sulphoxide (DMSO). The absorbance was measured at 570nm using Spectra Max M2 microplate reader.

Measurement of cell surface area

Cell surface area of H9c2 cardiomyoblasts stained with crystal violet was measured following treatment. Briefly, H9c2 cardiomyoblasts were washed twice with 1 × PBS following treatment and incubated in a solution of 4% formaldehyde at room temperature for 10 minutes. Excess formaldehyde was aspirated and cardiomyoblasts were washed and fixed in cold methanol for 20 minutes at room temperature. Excess methanol was aspirated and fixed cardiomyoblasts were stained using a solution of crystal violet (Sigma) for a further 20 minutes. The average cell area of 50-70 randomly selected cells was taken. Cells were visualized with an inverted microscope equipped with a monochrome digitalized camera using 10X magnification. Cell area was determined using the AxioVision Imaging Software (Carl Zeiss Micro-imaging, New York, NY).

Reactive oxygen species activity

ROS activity of H9c2 cardiomyoblasts treated with the respective drug treatment groups were imaged using an inverted fluorescence microscope following incubation with DCFH-DA (20 μM) for 10 minutes.

Statistical analysis

All values expressed are compared to control ± SEM. Student t test was used to compute differences between groups where a $P<0.05$ was considered a significant difference. Data were analyzed using the Statistical Package for Social Sciences (SPSS Software) version 22.

Results

DOX induces cardiotoxicity in H9c2 cardiomyoblasts

To verify that concentration of DOX needed to induce cardiotoxicity in H9c2 cardiomyoblasts, H9c2 cardiomyoblasts were stimulated with 1 or 2 μM of DOX for 48 hours and assessed for cell viability, hypertrophy and ROS production. Treatment with 1 or 2 μM DOX for 48 hours resulted in cell death, with a significant increase following stimulation with 2μM DOX (71.77 ± 9.25 vs. 100% control, $P<0.05$) (Figure 1A).

DOX induced cardiotoxicity has been associated with ROS production [1,2]. To verify the role of DOX in H9c2 cardiomyoblasts on ROS production, H9c2 cardiomyoblasts were incubated with DCFH-DA and visualized under a fluorescent microscope. ROS production was evident in H9c2 cardiomyoblasts simulated with 1 or 2 μM DOX compared to control. Cells treated with 2 μM DOX had more fluorescence, which indicated more ROS production (Figure 1B).

H9c2 cardiomyoblasts treated with increasing concentrations of DOX were also assessed for hypertrophy. Stimulation of H9c2 cardiomyoblasts with 1 or 2 μM DOX resulted in a significant increase in the relative area of H9c2 cardiomyoblasts (176.83 ± 46.9% 2 μM DOX vs. 100 ± 15.43% control) and (157.53 ± 32% 1 μM DOX vs. 100 ± 15.43% control) (Figure 1C).

ETO decreases cell viability in H9c2 cardiomyoblasts in a dose dependent manner.

To determine an ideal concentration of ETO, a non-specific TOPII inhibitor to co-adminsiter with DOX, H9c2 cardiomyoblasts were stimulated with 1 or 5 μM ETO. Cell viability of H9c2 cardiomyoblasts stimulated with ETO (1 μM or 5 μM) for 48 hours was measured using the MTT assay. Both concentrations of ETO induced a significant level of cell death (72.33 ± 5.7% 1 μM ETO vs. 100 ± 15.43% control, $P<0.005$) and (62.38 ± 2% 5 μM ETO vs. 100 ± 15.43% control, $P<0.05$), with a greater amount of cell death induced with 5 μM of ETO (Figure 2).

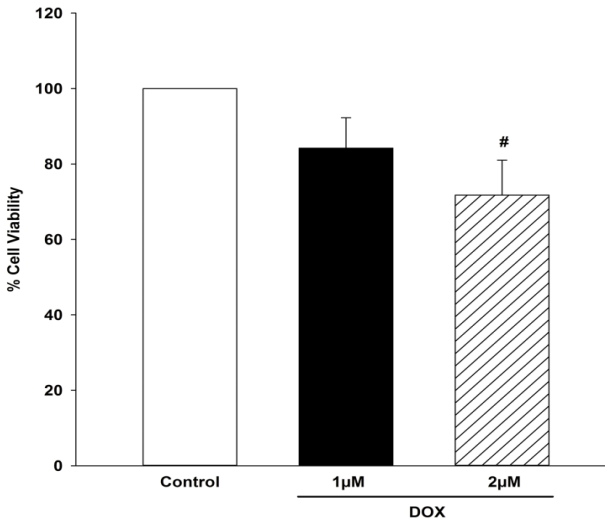

Figure 1A: Doxorubicin induces cardiotoxicity in H9c2 cardiomyoblasts. H9c2 cardiomyoblasts were assessed for cell viability, hypertrophy and reactive oxygen species production (ROS) in the presence and absence of 1 μM or 2 μM. Doxorubicin (DOX) for 48 hours. Cell viability was assessed by MTT colorimetric assay in triplicates. Cell viability of H9c2 cardiomyoblasts is expressed as a percentage of control ($P<0.05$), n=6.

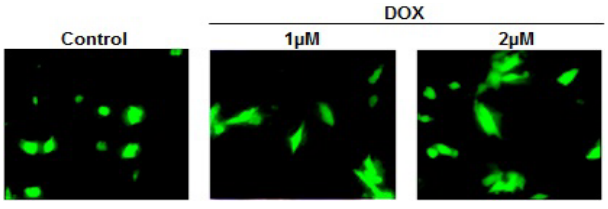

Figure 1B: Representative images of H9c2 cardiomyoblasts stained with DCF and imaged under fluorescence microscopy for ROS production.

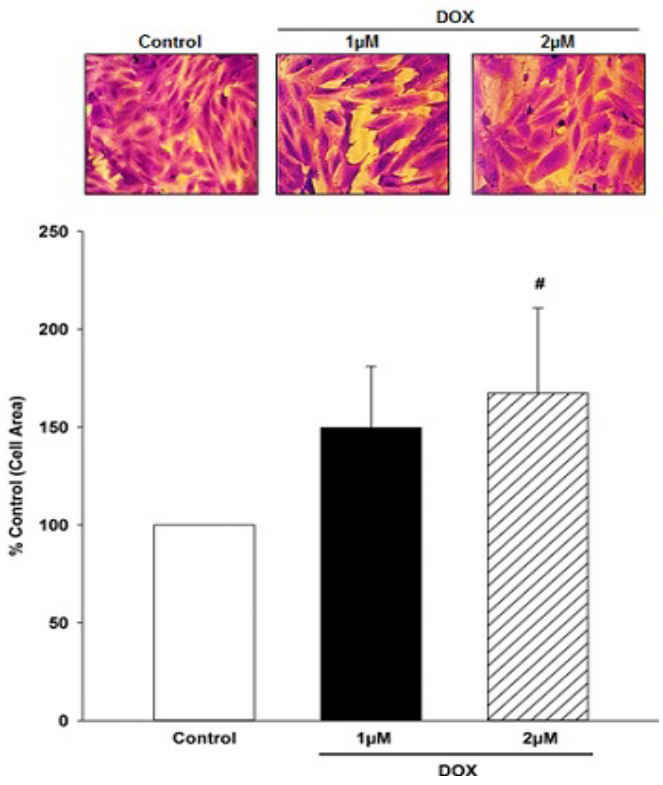

Figure 1C: Upper panel: representative images of H9c2 cardiomyoblasts stained with crystal violet following stimulation with DOX, Lower panel: quantitative analysis of H9c2 cardiomyoblast cell surface area. Results are expressed as a percentage of control, n=3; #P<0.05.

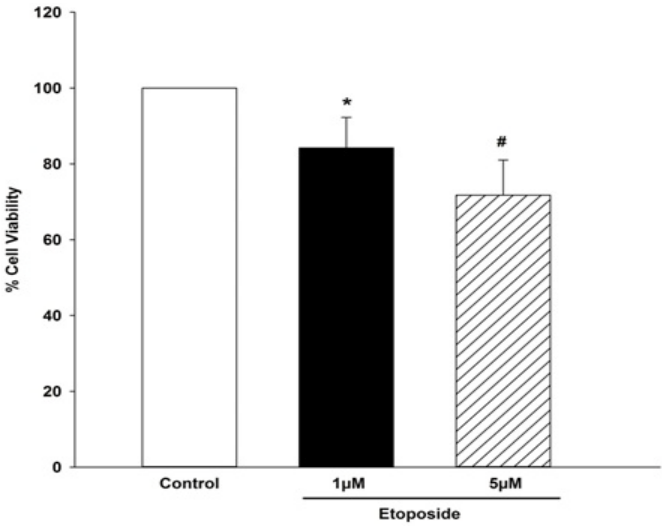

Figure 2: Etoposide decreases cell viability in a dose dependent manner. H9c2 cardiomyoblasts were assessed for cell viability in the presence and absence of 1 μM or 5 μM of Etoposide (ETO) for 48 hours. Cell viability was assessed by MTT colorimetric assay in triplicates. Results are expressed as a percentage of control, n=3-5. #P<0.05, *P<0.005.

TOPII Inhibition with ETO Does not Attenuate DOX Induced Cardiotoxicity in H9c2 Cardiomyoblasts.

To determine whether TOPII inhibition attenuates DOX induced cardiotoxicity in H9c2 cardiomyoblasts, H9c2 cardiomyoblasts were stimulated with 1 μM ETO and 2 μM DOX and assessed for cell viability, hypertrophy and ROS production Stimulation of H9c2 cardiomyoblasts for 48 hours with 1 μM ETO and 2 μM DOX did not show a further reduction in cell viability (68.35 ± 11.9% combination vs. 71.77 ± 9.25% 2 μM DOX) (Figure 3A). Similary, ROS generation of H9c2 cardiomyoblasts stimulated with both 1 μM ETO and 2 μM DOX did not differ from the stimulation of H9c2 cardiomyoblasts with 2 μM DOX alone (Figure 3B). Furthermore, the cell area of H9c2 cardiomyoblasts stimulated with both 1 μM ETO and 2 μM DOX did not induce a significant cell hypertrophic effect compared to 2 μM DOX alone (220.51 ± 63.51% combination vs. 176.83 ± 46.9% 2 μM DOX) (Figure 3C).

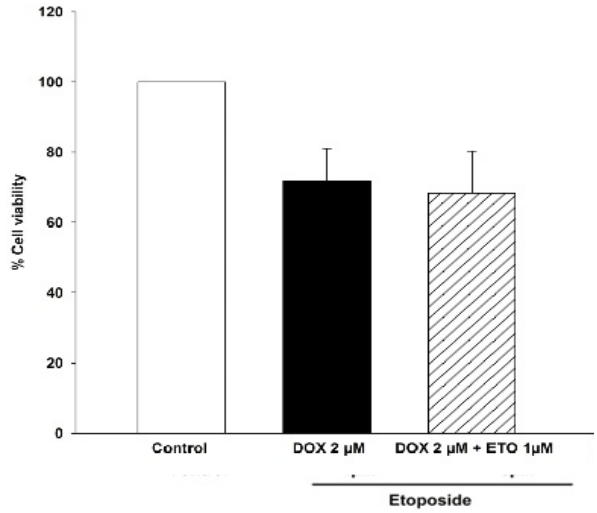

Figure 3A: Topoisomerase inhibition with Etoposide does not attenuate. Doxorubicin induced cardiotoxicity in H9c2 cardiomyoblasts. H9c2 cardiomyoblasts were assessed for cell viability, hypertrophy and reactive oxygen species production (ROS) in the presence and absence of 1 μM Etoposide (ETO) and 2 μM Doxorubicin (DOX) for 48 hours. A. Cell viability was assessed by MTT colorimetric assay in triplicates. Cell viability of H9c2 cardiomyoblasts is expressed as a percentage of control (P<0.05), n=5.

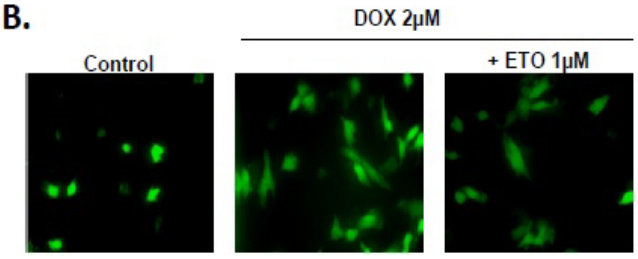

Figure 3B: Representative images of H9c2 cardiomyoblasts stained with DCF and imaged under fluorescence microscopy for ROS production.

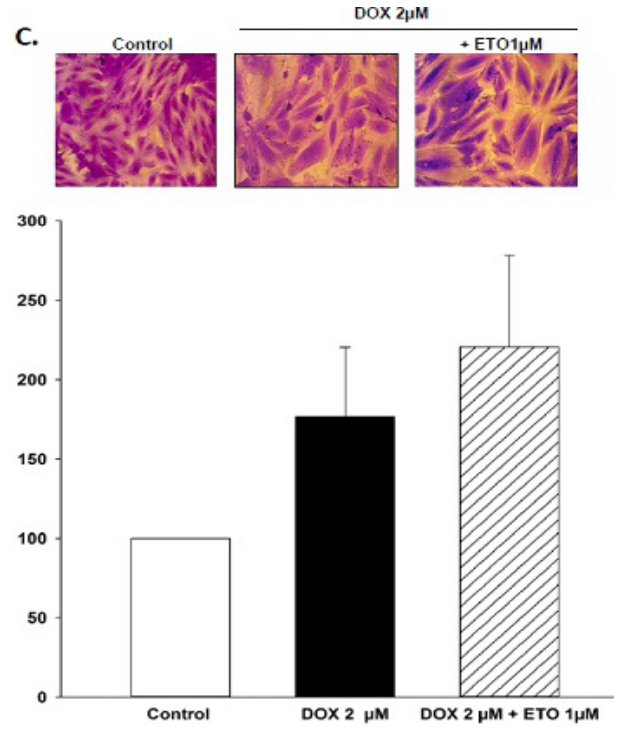

Figure 3C: Upper panel: representative images of H9c2 cardiomyoblasts stained with crystal violet following stimulation with DOX, Lower panel: quantitative analysis of H9c2 cardiomyoblast cell surface area. Results are expressed as a percentage of control, n=3.

Discussion

DOX is among the most effective and widely used antineoplastic agents. However, the use of DOX is restricted due to its ability to induce cardiotoxicity. Several studies have supported the role of ROS in DOX induced cardiotoxicity [11,12]. Recently, TOPII-β expression has been associated with DOX induced cardiotoxicity [9,13,14]. In our study, we examined whether the inhibition of TOPII prevented the DOX induced cardiotoxicity. ETO, a cytotoxic anticancer drug which inhibits DNA synthesis by forming a complex with TOPII and DNA, was used in our study. In addition, our study examined the cardiotoxic effects of co-administering DOX and ETO.

In agreement with previous reports, the stimulation of H9c2 cardiomyoblasts with DOX resulted in a significant reduction in cell viability, induced ROS production and resulted in a hypertrophic phenotype [10,17]. ETO, a cytotoxic anticancer drug which inhibits DNA synthesis by forming a complex with TOPII, was used in this study as a means to inhibit TOPII. ETO is used mainly in the treatment of refractory testicular tumors and for the treatment of small-cell lung carcinoma and has been associated with hypotension [18]. In vitro, Hsiao et al. demonstrated that 10 μM of ETO inhibited the cell growth of H9c2 cardiomyoblasts by 55% [19-21]. In our study, ETO decreased the cell viability of H9c2 cardiomyoblasts in a dose dependent manner with a greater decrease in cell viability with increasing concentrations of ETO.

TOPII-β mRNA is predominantly expressed in the myocardium of adult mice [22]. These findings suggest that DOX mediated targeting of TOPII-β could contribute to its cardiotoxic side effects. We are the first to demonstrate that combining ETO (1 μM), a TOPII inhibitor, with DOX (2 μM) does not attenuate DOX induced cardiotoxicity. ETO failed to show any significant effect on reducing the cardiotoxic effects of DOX in H9c2 cardiomyoblasts. The inability of ETO to regress the DOX induced cytotoxic effect could be attributed to the fact that ETO is a nonselective TOPII-β inhibitor [5]. ETO inhibits both TOPII isoforms (TOPII-a and TOPII-β), which are regulated very differently [15, 22-24]. Further studies investigating the use of specific TOPII-β inhibitors on DOX-induced cardiotoxicity is needed to verify the role of TOPII on DOX-induced cardiotoxicity.

Although both DOX and ETO are cytotoxic anticancer agents, the combination of both agents did not cause a significant reduction in cell viability or change in cell size when compared to H9c2 cardiomyoblasts treated with DOX alone. This was surprising to observe since the presence of two anticancer agents is predicted to result in more cell destruction. In addition, ETO similar to DOX has also been demonstrated to induce cardiotoxic effects. It has been demonstrated that patients who have previously undergone chemotherapy or mediastinal radiation may be at increased risk for MI following ETO treatment [19]. The concomitant chemotherapy of ETO with other agents has also been shown to be a predisposing factor for MI [20]. This observation emphasized that combing ETO with DOX does not further deteriorate H9c2 cardiomyoblasts. This also provides further support to using the combination of DOX and ETO, which is being done to treat advanced AIDS related sarcoma [25,26].

In our study, we have demonstrated that DOX induced a dose dependent increase in cardiotoxicity in H9c2 cardiomyoblasts, with a greater cardiotoxic response upon treatment with 2 μM DOX. 1 μM ETO, a TOPII inhibitor, did not further attenuate this DOX induced cardiotoxicity. Interestingly, the combination of ETO and DOX did not further deteriorate the hypertrophic phenotype of H9c2 cardiomyoblasts. The idea that TOPII targeting is involved in doxorubicin induced cardiotoxicity has significant clinical implications. Further studies are needed to investigate the role of TOPII-β as a possible cardioprotective target.

Conflicting Interests

'The author(s) declare that they have no competing interests'.

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Authors Contributions

SS, SA, NA, SR, MJ and IAM carried out the in vitro experiments, SS, SA and FM drafted the manuscript. FM participated in the design of the study. SS, SA an NA performed the statistical analysis. FM conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Authors Information

FM: PhD, Assistant Professor and Chair of Pharmaceutical Sciences Section; SS and SA: BSc Pharm Candidates; NA: MSc, Research Associate; MJ: PhD Candidate and Research Assistant; IAM, MSc.

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