CHAPTER-4

TO STUDY THE EFFECTS OF CURCUMIN ON DOXORUBICIN INDUCED CARDIOTOXICITY

4.1. Overview of the chapter

As elaborated in section 1.2.6, Doxorubicin is a broad range anti-cancer drug with associated severe cardiotoxicity. These cardiotoxic effects were studied and validated on H9C2 cardiac cells by various assays in the present chapter, followed by analyzing the effects of Curcumin on Doxorubicin induced cardiotoxicity. Concentration and time dependent effects of Curcumin were derived and validated quantitatively by various assays. Synergistic effect of anti-cancer properties and cardioprotective potential of Curcumin were also studied by using MCF-7 human breast cancer cell line and H9C2 cells. Curcumin displayed promising outcomes to be used in cardio-oncological therapeutics for suppressing dose mediated Doxorubicin induced cardiotoxicity thereby significantly improving its therapeutic index. This chapter is structured as following:

4.2 Introduction

4.3 Experimental Design

4.3.1 To study the effects of Curcumin on Doxorubicin induced cardiotoxicity

4.3.2 To study the synergistic anti-cancer and cardioprotective effects of Curcumin on cancer progression

4.4 Results

4.5 Discussion

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4.2. Introduction

Doxorubicin induced cardiotoxicity is a major limitation of its effectiveness in anti-cancer therapeutics. Doxorubicin cardiomyopathy has been studied by various research groups worldwide, but till date, no effective treatment or better alternative is available for the same [233]. As the drug is very effective against broad range of cancer, its application and effectiveness in anti-cancer treatment is inimitable [234]. Hence, there is an urgent requirement for developing effective and clinically relevant protective treatments for Doxorubicin induced cardio toxic complications in cancer patients. Cardio-oncology has emerged as an important field for preventing cardiovascular complications against anti-cancer drugs induced toxicity [235-236]. Cardiac health of cancer patients is among the priority concerns of clinicians nowadays before starting anti-tumor therapy [237]. Hence, developing supplements derived from natural products to decrease the drug induced toxic side effects is a promising field for researchers [238]. In this regard, a natural compound having cardio-protective effects may hold a great potential for reducing cardio-toxic effects of Doxorubicin without affecting its anti-cancerous effects. In the present chapter, cardio-protective effects of Curcumin were studied in Doxorubicin induced cardiac stress. Such studies have been previously done for other natural products including Schisandrin B, Sulforaphane etc., and have displayed encouraging results [239-240]. Few studies have been done to study the effect of Curcumin with respect to Doxorubicin induced cardiac stress, but literature reflects conflicting views, and hence, elaborated time- and concentration-dependent studies were conducted to derive the actual role of Curcumin upon Doxorubicin stress [241-244].

Also, as Curcumin is an established anti-cancerous molecule as well, its synergistic effects for cardio-protective potential and anti-cancer activity were also studied in detail [245-246]. Both MCF-7 breast cancer cell lines and H9C2 rat cardiomyocytes were used and dose-mediated cell viability assays were done followed by various microscopic staining assays for morphological alterations and ROS generation were conducted. The findings were validated by expression analysis of cell-death biomarkers.

As there is no clear understanding of the mechanisms involved in Curcumin mediated effects in Doxorubicin induced cardiotoxicity, the findings from the present chapter may sets a critical platform and add to the clinical applicability of Curcumin in this regard.
4.3. Experimental Design

In the present chapter, experiments were conducted in two different cell lines to study the cardio-protective effects on H9C2 cardiomyoblasts against Doxorubicin induced cardiotoxicity; and to study the synergistic anti-cancer and cardioprotective effects of Curcumin in MCF-7 human breast cancer cell line.

4.3.1 Experimental sets to study the effects of Curcumin on Doxorubicin induced cardiotoxicity

In the initial experiments, increasing concentrations of Doxorubicin (1-20 µM) were used to confirm the induced cardiac stress *in vitro*. Cardio-toxic dose of Doxorubicin was derived from these experiments and used for obtaining cardio-protective dose of Curcumin for subsequent time-points of treatment, that is, pre-, parallel- and post-treatment.

Different experiments were conducted to validate the Curcumin effects by using the following sets:

- i) Control UT cells
- ii) Doxorubicin alone (15 µM)
- iii) Pre-treatment of Curcumin (20 µM) for 24 hours followed by Doxorubicin treatment (15 µM) for another 24 hours
- iv) Parallel treatment of Curcumin (20 µM) and Doxorubicin (15 µM) for 24 hours
- v) Curcumin alone (20 µM) for 48 hours
- vi) DMSO alone

4.3.2 Experimental sets for studying the synergistic effects of Curcumin on cancer progression and cardiotoxicity

Cell viability assays were done for increasing concentrations of Curcumin (2-100 µM) and Doxorubicin (0.1-10 µM) individually on both cardiac and cancer cell lines. Curcumin and
Doxorubicin were used simultaneously analyzed on cancer cells with the combination of 10 µM Curcumin and lower doses of Doxorubicin (0.1-1.5 µM).

The synergistic effect of Curcumin on MCF-7 cells was further validated in the following experimental sets:

i) UT cells  
ii) Doxorubicin alone (1 µM)  
iii) 0.1 µM Doxorubicin  
iv) 0.1 µM Doxorubicin and 10 µM Curcumin for 48 hours  
v) 0.5 µM Doxorubicin  
vi) 0.5 µM Doxorubicin and 10 µM Curcumin for 48 hours  
vii) Curcumin alone (10 µM) for 48 hours  
viii) DMSO alone

4.4. Results

Doxorubicin treatment was given to cardiomyocytes and we observed that it exerts concentration-dependent cell toxicity where cell viability decreased significantly with increase in dose and time of treatment. It was observed that when cells were treated with 15 µM Doxorubicin for 24 hours, cell viability was significantly declined by 50%. Cell viability for 48 hours was also calculated and the concentration of 9 µM exerted 50% viability (Fig. 4.1). The concentration dependent deleterious effects were validated by Trypan blue dye exclusion cell viability assay and upto 45% cell death was witnessed at 15 µM Doxorubicin concentration (Fig. 4.2). Concentration mediated dug uptake of Doxorubicin by cardiomyocyte was studied by FACS-calibur using Doxorubicin with FITC fluorescent tag. Significant increase in drug uptake with increasing concentration was witnessed (Fig. 4.3).

To investigate the concentration of Curcumin having protective effects on Doxorubicin toxicity, time dependent cell viability assays for the combinations of Doxorubicin and Curcumin were conducted. MTT assay for Curcumin was performed with control cells to identify a concentration
with minimum cytotoxicity. No significant cell death up to the concentration of 30 µM was observed where 20 µM Curcumin showed 90% cell viability (Fig. 4.4). Different time points of Curcumin treatment, that is, pre and parallel treatments with the selected concentrations of Curcumin were done using optimized Doxorubicin IC50 concentration for analysing the cell viability. We observed that pre-treatment of 20 µM Curcumin resulted in increased cell viability up to 30% whereas parallel treatment of Curcumin decreased the cell viability up to 15% in Doxorubicin treated cells (Fig. 4.5). As the concentration of 20 µM Curcumin showed negligible cytotoxic effects when treated alone and decreased Doxorubicin effects up to 30% in pre-treatment, this concentration was selected for both pre and parallel treatments. Cell proliferation analysis by Trypan blue further confirmed the increase in cell viability by 25% in the Curcumin pre-treated cells and decreased cell viability by 15% in the parallel treated group with Curcumin thereby confirmed time dependent survival/detrimental effect of Curcumin on Doxorubicin induced toxicity (Fig. 4.6).

Doxorubicin mediated decrease in percentage cell viability was further validated by morphological analysis. Rounding off of H9C2 cells was observed in response to Doxorubicin treatment while innate spindle shaped cells were observed in control as well as pre-treated group. Increase in cell death and altered cellular morphology as well as reduced cell size was witnessed quantitatively in Doxorubicin treated cardiac myoblasts that was further increased with parallel Curcumin treatment. Reduced cellular death in Curcumin pre-treatment thereby suggests its preventive potential. Overall cell growth was also observed upon various exposures in addition to the altered morphology. Significant growth inhibition as evident by total concentration of cells present in different experimental groups also suggested Doxorubicin induced deleterious effects on cardiomyoblasts, where pre and parallel Curcumin treatments prevented and potentiated these effects respectively. Total concentration of cells was decreased by 2 folds in Doxorubicin treated cells as compared to control and it was further decreased by 1.3 folds in parallel treated cells. However, cells pre-treated with Curcumin showed increased cell concentration by 0.7 folds as compared to the Doxorubicin treated cells. Giemsa staining confirmed the findings obtained in the previous analysis, suggesting Doxorubicin induced altered cellular morphology and pre-treatment of Curcumin supported the cells to maintain their integrity as evident by the 2-folds reduction in cellular size in Doxorubicin treated cells. Nuclear deformities occur in response to stress and displayed in the form of DNA
fragmentation and nuclear shrinkage. These effects were observed by Haematoxylin-eosin and fluorescent DAPI nuclear staining. Analysis of nuclear morphological alterations also confirmed that Doxorubicin induces variations in cells and Curcumin pre-treatment protected and parallel treatment potentiated these nuclear changes. Nuclear morphological alterations were also observed in DAPI stained Doxorubicin and Curcumin parallel groups whereas, pre-treatment of Curcumin maintained nuclear integrity thereby suggesting that mode of Curcumin treatment could be crucial for its response in Doxorubicin induced toxicity at cellular and nuclear levels (Fig. 4.7).

The single time-point measurement revealed the percentage of cells in G1/S/G2/M, in the different experimental groups. Doxorubicin treated cells were found to be inhibited in the sub-G1 phase of cell cycle as compared to the control cells where 18.9% and 17.1% cells were present in S and G2/M phase respectively. The ratio was reduced by 6 folds in cells with parallel treatment of Curcumin suggesting significant growth inhibition. Pre-treatment with Curcumin demonstrated the growth distribution pattern similar to the control cells with Curcumin thereby suggesting protection against Doxorubicin induced growth inhibition (Fig. 4.8 and 4.9). ROS production in presence of Doxorubicin and anti-oxidant capacity of Curcumin were studied by various ROS assays. Increased superoxides production in cell treated with Doxorubicin alone and Curcumin parallel treatments was witnessed by NBT assay, which was decreased by approximately 2-folds in pre-treated Curcumin group (Fig. 4.10). DCFH-DA assay displayed increased intracellular ROS production in cells treated with Doxorubicin and further exaggerated with parallel Curcumin treatment. However, decrease in ROS production upto 2-folds was observed in cells pre-treated with Curcumin validated the results that Curcumin exerts mode dependent opposite effects on Doxorubicin induced toxicity. Net fluorescence intensity of the eluted stains also represented similar effects (Fig. 4.11). We observed that pre-treatment with Curcumin reduced the superoxide ions in cells as measured by NBT and DCFH-DA assay. ROS mediated responses of Doxorubicin were further explored by studying the effect on two major anti-oxidant enzymes- SOD and catalase. Inhibitory photochemical reduction of NBT was studied for analysing SOD activity and H$_2$O$_2$ reduction was studied for Catalase activity. Doxorubicin treatment showed ROS mediated modulation of the inbuilt cellular antioxidant enzymes. Pre-treatment of Curcumin was found to increase activities of both SOD and Catalase whereas significantly reduced anti-oxidant activity was observed in parallel Curcumin treatment (Fig. 4.11).
Mitochondria mediated effects in Doxorubicin and Curcumin treated sets were studied by mitochondrial permeability and caspase expression where pre-treatment was shown to uphold these measures in cardiomyoblasts. The mitochondrial permeability was studied by Rhodamine 123 staining and it was observed that in presence of Doxorubicin and Curcumin parallel treatments, it was decreased by 2 and 2.5 folds respectively as compared to control cells as evident by Rhodamine fluorescence. Significant increase in fluorescence in Curcumin pre-treated cells suggested the intact mitochondrial membrane (Fig. 4.14). Next we observed the caspase activation in response to the Doxorubicin and Curcumin treatments. Colorimetric analysis of initiator and effector caspases demonstrated elevated levels of the overall caspase activity in cells treated with Doxorubicin and Curcumin parallel groups. Expression of initiator caspases 2 and 9 in Doxorubicin and parallel groups was approximately 2 and 2.5 folds higher as compared to control cells whereas, pre-treatment with Curcumin showed expression comparable to the control cells. Expression of effector caspase 3 was 3 and 4 folds higher in Doxorubicin and parallel groups respectively. Although, 2-folds increase in caspase 9 expression in pre-treated group was observed but it was less as compared to Doxorubicin and parallel groups (Fig. 4.15). This suggested the up-regulation of apoptotic signals in presence of Doxorubicin and parallel treatment and significant down-regulation in Curcumin pre-treatment in cardiomyoblasts.

Expression of anti-apoptotic marker Bcl2 was found to be increased in cells pre-treated with Curcumin and decreased in Doxorubicin and parallel treatments thereby validated our previous observations. These effects of Curcumin were also mediated by pro apoptotic marker Bax as its expression was increased in Doxorubicin and Curcumin parallel treated groups and found to be decreased in Curcumin pre-treated cells (Fig. 4.16).

Synergistic effects of Curcumin and Doxorubicin on cancer prevention and cardiac health were studied in MCF-7 human breast cancer cell line and H9C2 cardiomyoblasts. Anti-proliferative doses of Curcumin and Doxorubicin were derived as 2.5 and 10 µM respectively by MTT and confirmed by Trypan blue assay (Fig. 4.17). Concentration dependent cytotoxicity assay was done to study the effects of Doxorubicin on cardiomyocytes. For that, similar range of Doxorubicin was studied and the cardiac cell viability was calculated. Upto 25% cardiac cell death was observed at the dose of 1 µM Doxorubicin concentration which further decreased to 40% at the concentration of 5 µM. Curcumin concentration from 2 to 30 µM showed no significant death of cardiomyocytes
(Fig. 4.18). To study the synergistic effect of Curcumin and Doxorubicin on cancer cell growth, Doxorubicin and Curcumin their combination experiments were conducted with different concentration. Upto 75% cell death was observed with optimized IC$_{50}$ concentrations of Curcumin and Doxorubicin were used together. Experiments were therefore further designed with reduced Doxorubicin concentrations. It was observed that when 10 µM of Curcumin was given with 0.1 and 0.5 µM Dox, upto 50% and 65% cell death was observed respectively. This synergistic effect of Doxorubicin and Curcumin was also studied on healthy cardiomyoblasts and it was observed in presence of simultaneous treatment, Doxorubicin induced cardiac cell death is resisted, as evident from the increase in cell viability by 11%, 16%, 22% in presence of 0.5, 1 and 1.5 µM Doxorubicin respectively with and without Curcumin (Fig. 4.19).

Cardiomyocytes and cancer cells were treated with Curcumin and Doxorubicin in above mentioned experimental sets and observed for any morphological alterations. Significant cancer cell death was observed in combination of 0.1 and 0.5 µM Doxorubicin with 10 µM Curcumin. The equivalent cellular death was observed 1 µM Dox. The extent of cell death was comparable to the high concentration of Doxorubicin alone. This observation was validated by Giemsa staining where significant cell death was observed in presence of Doxorubicin and Curcumin. Nuclear morphology was studied by fluorescent DAPI staining and indicative disintegration of nucleus in presence of Curcumin and Doxorubicin was observed. Intact nuclear morphology was observed in untreated control cells (Fig. 4.20).

Intracellular ROS production in presence of Doxorubicin and Curcumin was studied by DCFH-DA assay and higher levels of ROS were observed in cells treated both Doxorubicin and Curcumin as compared to alone treated experimental sets. 2 folds increase in ROS was found in set treated with 0.5 µM Doxorubicin and 10 µM Curcumin as compared 0.5 µM Doxorubicin alone. Superoxides production was also studied by NBT assay and it further validated the above observations. Significantly higher amount of superoxides production was observed in presence of Doxorubicin and Curcumin simultaneous treatment (Fig. 4.21).

Cell death upon different treatment combinations was studied by TUNEL assay and % dead cells % TUNEL positive cells were measured. More than 30% TUNEL positive cells were observed in cells treated with 1 µM Dox. In cells treated with 0.5 µM Doxorubicin and 10 µM Curcumin also
showed up to 30% TUNEL positive cells (4.22). To further characterize the mode of cellular death, cells were then analysed for programmed apoptotic cell death using an early marker of apoptosis—Annexin. Cells were counterstained with fluorescent PI stain and significantly higher percentage of apoptotic cell death was observed 1 µM Doxorubicin and the combination of 0.5 µM Doxorubicin and Curcumin, thereby further validating the TUNEL results and suggesting apoptosis taking place upon supplementing Curcumin (Fig. 4.23). Apoptosis was further characterized by analysing the activity of different initiator and effector caspases in the experimental sets. We observed that the expression of initiator caspase-2 and 9; and effector caspase-3 and 6 were comparable when treated with 1 µM Doxorubicin alone or in combination with Curcumin and lower concentration of Dox. Hence, validating our previous observation that anti-cancer effect of Doxorubicin can be achieved at lower concentrations as well when supplemented with Curcumin and its anti-cancer activity do not get compromised (Fig. 4.24).

Expression of anti-apoptotic biomarker- Bcl2, pro-apoptotic biomarker- Bax and an inflammatory biomarker TNF-α was studied by qRT-PCR. The synergistic effect of Curcumin and Doxorubicin significantly decreased the level of Bcl2 expression in cancer cells as comparable to Doxorubicin treatment alone. Expression of Bax and TNF-α were found to significantly increased in the combination treatment by 2-folds (Figure 7). Hence, these findings confirms that combination treatment of Doxorubicin and Curcumin will mediate anti-cancer activity without compromising the cardiac health as dose mediated Doxorubicin effects can be avoided by using lower Doxorubicin concentrations. (Fig. 4.25).

These findings suggests that Curcumin holds a great potential in cardio-oncology where it will reduce cancer cell progression, without affecting cardiac health of cancer patients. It will also not alter Doxorubicin mediated effects but reduce the associated dose mediated toxicity.
Fig. 4.1. Concentration mediated deleterious effects of Doxorubicin by MTT assay:
Doxorubicin ranging from 1 to 20 μM for 24 and 48 hours were taken and significant cell death was observed at higher concentrations. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.2. Trypan dye exclusion assay for increasing Doxorubicin concentrations:
Concentration mediated effects of Doxorubicin as observed by MTT assay were validated. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
**Fig. 4.3. Concentration dependent Doxorubicin uptake FACS-Calibur:** Cardiomyocytes displayed increased Doxorubicin uptake with increase in its concentration thereby confirming its dose mediated cardiac stress. *(Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])*
Fig. 4.4. MTT cell viability assay increasing concentrations of Curcumin: No significant cell death was witnessed upto 25µM Curcumin concentration. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.5. Concentration and mode dependent effects of Doxorubicin and Curcumin on cardiomyoblasts: Concentration of Curcumin for pre and parallel treatment on Doxorubicin induced cardiotoxicity were derived. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.6. Trypan blue staining upon Doxorubicin and Curcumin treatment in finalized experimental sets: MTT doses as well as mode mediated Curcumin effects were validated. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.7. Cellular proliferation and morphological alterations in presence of Curcumin: (A) Cell proliferation at 10 X; (B) Giemsa staining (C) Haematoxylin-Eosin staining (D) DAPI staining. Growth inhibition and cell size were quantitated by cell counter and ImageJ respectively. Eluted stain was treated cells was also quantified and plotted as graphs. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (\( ^* p < 0.05 \)). All images at were captured at 100 X except for 4.7.A at 10 X, scale bar corresponds to 10 µm and 100 µm respectively (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247]).
Fig. 4.8. Cell-cycle analysis by sub-G1 inhibition upon different modes of Curcumin treatment: Single time pint measurement for number of cells present in different cell cycle phases upon Doxorubicin and Curcumin treatment (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.9. Graphical representation of number of cells present in G1/S/G2/M phases upon different time points of Curcumin treatment: Significant number of cells were restricted in sub-G1 phase upon Doxorubicin treatment. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*P<0.05). (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.10. NBT assay for cellular superoxides generation: Blue formazon crystals formed by superoxides mediated conversion of NBT was captured at 100X and quantitated by solubilizing the crystals at 630 nm. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). Images at were captured at 100 X and scale bar corresponds to 10 μm (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.11. Intracellular ROS production by DCFH-DA assay: Significant ROS generation was observed in presence of Doxorubicin and parallel treated sets. Net eluted stain was quantified by spectrofluorometer and plotted. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). Images at were captured at 100 X and scale bar corresponds to 10 µm (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.12. SOD anti-oxidant enzymatic activity upon Doxorubicin and Curcumin treatment: significant increase in the in-built enzymatic activity was observed. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p<0.05). (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])

Fig. 4.13. Catalase anti-oxidant enzymatic activity upon Doxorubicin and Curcumin treatment: significant increase in the in-built enzymatic activity was observed. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p<0.05). (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.14. Rhodamine 123 staining for mitochondrial membrane permeability analysis:
Eluted stain was quantified and significant increase in Doxorubicin mediated altered membrane potential was witnessed. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). Images at were captured at 100 X and scale bar corresponds to 10 µm (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.15. Effect of Curcumin treatment on the activity of different caspases: Calorimetric assay with specific caspase substrate was used. Curcumin displayed significant reduction in overall caspase activity upon pre-treatment. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.16. Reverse Transcriptase PCR analysis of SOD, Catalase, pro and anti-apoptotic genes: Semi-quantitative expression of oxidative stress markers and apoptotic markers was done and band intensity was quantified by ImageJ software. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain and Rani, 2018, Mol Cell Biochem, 442(1-2):81-96 [247])
Fig. 4.17 MTT cell viability assay for deriving anti-proliferative concentrations of Doxorubicin and Curcumin on MCF-7 human breast cancer cell line: (A) MTT with Doxorubicin ranging from 0.1 to 12 µM doses for 48 hours (B) MTT with Curcumin ranging from 2 to 100 µM doses for 48 hours (C) Trypan blue dye exclusion assay for Doxorubicin- 0.1 to 10 µM (D) Trypan blue dye exclusion assay for Curcumin- 1 to 25 µM. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p<0.05). (Jain and Rani, 2018, Lif Sci, 205:97-106 [248])
Fig. 4.18 Effect of selected anti-cancer concentrations on cardiomyocytes. (A) MTT with Doxorubicin ranging from 0.1 to 12 µM concentrations for 48 hours (B) MTT assay with Curcumin ranging from 2 to 100 µM concentrations for 48 hours. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p<0.05). (Jain and Rani, 2018, Lif Sci, 205:97-106 [248])
Synergistic effects of Curcumin and Doxorubicin on cancer cells and cardiomyocytes

(A) Effect of lower Doxorubicin concentrations in combination with 10 µM Curcumin on cancer cell death. (B) Protective effect of 10 µM Curcumin on Doxorubicin induced cardiac stress. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p<0.05). (Jain and Rani, 2018, Lif Sci, 205:97-106 [248])
**Fig. 4.20 Cellular proliferation and morphological alterations in cancer cell lines.** Bright field microscopic images at 20X magnification, Giemsa and DAPI stained images at 100X magnification were captured. Graphs represents of quantification of the captured data. 10 µM Curcumin is represented as ‘+’ and ‘-’ indicates no Curcumin. Doxorubicin concentrations in µM are represented as 0.1, 0.5 and 1. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p<0.05). Scale bar corresponds to 50 µm and 10 µm respectively (Jain and Rani, 2018, Lif Sci, 205:97-106 [248])
Fig. 4.21 Analysis of intra-cellular ROS generation by NBT and DCFH-DA assay. Stains from both the assays was eluted and quantitatively represented as respected graphs. 10 µM Curcumin is represented as ‘+’ and ‘-’ indicates no Curcumin. Doxorubicin concentrations in µM are represented as 0.1, 0.5 and 1. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p<0.05). Images were captured at 100 X and scale bar corresponds to 10 µm (Jain and Rani, 2018, Lif Sci, 205:97-106 [248])
Fig. 4.22. TUNEL assay for % dead cells upon different combination treatments of Doxorubicin and Curcumin. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain and Rani, 2018, Lif Sci, 205:97-106 [248])
Fig. 4.23 Annexin/PI staining for Curcumin mediated synergistic apoptosis by FACS-Calibur (Jain and Rani, 2018, Lif Sci, 205:97-106 [248])
Fig. 4.24. Activity of different initiator and effector caspases for different combination treatments. Two-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain and Rani, 2018, Lif Sci, 205:97-106 [248])
Fig. 4.25. Quantitative real time PCR for the mRNA expression of TNF-α, Bcl2 and Bax genes: qPCR results were normalized with β-actin expression and plotted as a histogram. One-way ANOVA and student’s t-test were done to evaluate the significance of differences in the data obtained (*p< 0.05). (Jain and Rani, 2018, Lif Sci, 205:97-106 [248])
4.5. Discussion

In the present chapter, different mode dependent Curcumin effects were studied and validated on Doxorubicin mediated cardiotoxicity. Anti-cancer drug induced cardiotoxicity, at present, is an important safety question because it is now a major reason for drug failure and limitation for drug discovery. As Curcumin is a multi-beneficial compound and holds both cardio-protective as well as anti-cancerous potential, supplementing it with cardio-toxic drugs may offer a safer alternative against drugs induced cardiac side effects [249].

As literature states both beneficial and deleterious effects of Curcumin against Doxorubicin induced cardiotoxicity, to understand the exact mechanism of Curcumin mediated effects, extensive concentration and time dependent study was designed. We performed time-dependent comparative analysis of Curcumin to understand its mode of action in Doxorubicin induced toxicity. Dose-mediated Doxorubicin induced cardiotoxic effects were witnessed and validated by MTT, Trypan blue and drug uptake assay. 15 µM Doxorubicin was derived as IC$_{50}$ dose and used in further experiments to induce cardiotoxicity in vitro (Fig. 4.1-4.3). Time dependent cell viability studies confirmed 20 µM Curcumin as cardioprotective concentration since it displayed upto 80% cell viability (Fig. 4.4). To study the effects of Curcumin, three different time points for Curcumin treatment were selected, that is, pre, parallel and post treatment. Extensive cell death was observed in post treatment of Curcumin and hence, was not carried forward for further experiments. Also, to our surprise, opposite effects of Curcumin were observed in pre and parallel experimental sets. Significant increase in cell viability was observed in cardiomyoblasts pre-treated with Curcumin for 24 followed by Doxorubicin treatment for 24 hours. However, when cells were simultaneously treated with Doxorubicin and Curcumin, further decline in cell viability was witnessed (Fig. 4.5). Pre and parallel treatments of Curcumin were further studied in detail for the opposite effects. 20 µM Curcumin concentration was selected as cardio-protective concentration and used in both pre and parallel sets as it displayed no significant cell death on untreated cardiomyoblasts. The Doxorubicin and Curcumin mediated effects on morphology were then studied, as it is an initial stress marker in cardiac cells. Cardiac myocytes are terminally differentiated cells and respond to stress by altering their size initially, thereby depicting a compromised cellular morphology, but prolonged stress leads to cellular death. The comparison between the cellular and nuclear changes in the six experimental groups confirmed that pre-treatment of Curcumin resisted the alterations
induced by Doxorubicin and parallel treatment with Curcumin potentiated the Doxorubicin effects (Fig. 4.7). Effect of Curcumin and Doxorubicin was studied by growth inhibition analysis where pre-treatment of Curcumin displayed reversal of Doxorubicin induced sub-G1 inhibition of cardiac cells. Significantly more number of cells were found in S-phase (Fig. 4.8-4.9).

As ROS over production is a key mediator of Doxorubicin induced cardiotoxicity, we next examined the effect of Curcumin on intracellular ROS levels by different microscopic and spectrophotometric assays. Curcumin, being an anti-oxidant molecule, known to assists cells undergoing ROS disbalance to amplify the antioxidative enzymes’ output. Curcumin has shown to regulate the expression of anti-oxidant enzymes and growth factors but its response in Doxorubicin induced cardiotoxicity has not been clearly understood. We concluded that Curcumin parallel treatment led to compromised inbuilt anti-oxidant machinery but pre-treatment enhanced the expression of anti-oxidant enzymes thereby resisting the Doxorubicin induced alterations. The imbalance in the inbuilt antioxidant enzymes suggested a critical role played by Curcumin in maintaining the cellular anti-oxidant activity thereby helping in reducing Doxorubicin induced oxidative stress (Fig. 4.10-4.13). These effects also justifies the anti-oxidant potential of Curcumin [250].

Mitochondria play an important role in maintaining normal cellular functioning and generating stress responses by ROS production and apoptosis. The mitochondrial permeability transition is an important step in the induction of cellular death via apoptosis during which the electrochemical gradient across the mitochondrial membrane collapses. The mitochondrial membrane alterations disturb the basic functioning of mitochondria and hamper the cellular functions. Alterations in mitochondrial integrity in Doxorubicin and Curcumin treated cardiomyoblasts established a relationship between altered membrane potential in the respective treatments. Involvement of caspase activation in response to mitochondrial stress was further studied to analyse the involvement of intrinsic apoptotic pathways in Doxorubicin and Curcumin mediated effects. Caspases play an important role in the execution phase of apoptosis and are responsible for various associated biochemical and morphological alterations. The expression of initiator caspases (Caspase 2 and 9) and effector caspase (Caspase 3) were therefore studied and it was observed that pre-treatment significantly reduced the Doxorubicin induced exaggerated levels of caspases in vitro. This suggested that mitochondria played an important role in Doxorubicin and Curcumin
mediated events in vitro and resulted in caspase mediated cellular death in response to Doxorubicin and Curcumin parallel treatments. Also, supplementing cardiac cells with Curcumin prior to Doxorubicin treatment resisted the same (Fig. 4.14). These mitochondrial mediated events were further validated by caspase activity assays, as they are the key players for mitochondria mediated cell death upon drug induced toxicity [251]. Doxorubicin induced increase in initiator and effector caspsases was significantly reduced upon Curcumin pre-treatment (Fig. 4.14). These observations validated the protective effects of pre-treatment in Doxorubicin induced cardiotoxicity and hence suggest the importance of mode of treatment for achieving maximum protection or prevention from natural polyphenol based therapeutic approaches [252].

To study the molecular mechanisms involved in Curcumin mediated mode dependent effects, semi-quantitative expression studies were conducted for cell death and oxidative stress biomarkers. As cell collapse takes place through the formation of pores in the mitochondria by dimerized Bax or activated Bid, Bak or Bad proteins, their expression was studied [253]. Increased expression of Bcl2, anti-apoptotic marker, in Curcumin pre-treated group validated the cardioprotection, whereas, increased level of pro-apoptotic marker: Bax in Doxorubicin treated cardiomyocytes also validated its cardiotoxic effects in myoblasts. Expression of SOD and catalase genes also validated our enzyme activity observations (Fig. 4.16).

These findings conclude that anti-oxidative mechanisms are operative in mediating cardio-protective effects in pre-treatment. However, apart from being an anti-oxidant, Curcumin is also a well-known anti-cancerous compound. As we have observed dose-mediated cardiotoxic effects of Doxorubicin, further experiments were designed to see the effect of Curcumin and Doxorubicin combination treatment upon anti-cancer cell growth.

Curcumin and Doxorubicin synergism has been studied with respect to cancer vasculature in various anti-angiogenic cancer therapies but till date, no effect has been studied of this synergism on cardiac cell survival and hence lack the understanding of this combination therapy [254]. MCF-7 human breast cancer metastatic cell line is first known hormone responsive cell line and widely used for in vitro breast cancer biology research [255-256]. It is also a chemotherapy responsive cell line and hence selected for the present study for analyzing the effects of Doxorubicin along with Curcumin combinations.
Curcumin is shown to have ‘death vs. survival’ effects, that is, opposite actions as anti-cancerous as well as cardioprotective agent. Deriving mechanisms involved in these mechanistically opposite effects of Curcumin will serve a better understanding of its effects as well as utilizing its implacability in different disease states will become easier. The opposing effect of Curcumin was studied in relation to the Doxorubicin induced cardiotoxicity without compromising its anti-cancer effects.

Preliminary concentration dependent cell viability assays were done to study the effects of Doxorubicin and Curcumin individually, followed by combinations on cancer cells and cardiac cells (Fig. 4.17-4.19). We observed that Doxorubicin concentration that is required to exert anti-tumor effects when given in combination with Curcumin is significantly much lower than the dose of Doxorubicin required to reduce cancer cells alone. These observations further suggests that Curcumin supplementation helps in attaining the uncompromised Doxorubicin effects at much lower concentrations and hence, dose mediated cardiotoxicity prospects decreases significantly.

As the desired cellular death was observed at the lower Doxorubicin doses of 0.1 and 0.5 µM, these doses were further analysed for morphological alterations and oxidative stress responses. Altered morphology and rounding off of cells are the initial indication of cells undergoing stress, hence, we analyzed these changes by microscopic studies initially. Dose of 0.1 and 0.5 µM Doxorubicin alone displayed lesser cell death as compared to the sets treated with Doxorubicin and 10 µM Curcumin treatment combination. Also, the cell death observed at the combination of 0.1 µM Doxorubicin and 10 µM Curcumin was comparable to the cell death observed at 1 µM Doxorubicin alone. Significant growth inhibition was also witnessed in these combinations as evident from the total number of cells quantified for each experimental set. Similar observations were observed in giemsa and DAPI staining (Fig. 4.20). Ros production upon Doxorubicin and Curcumin treatment was analyzed by studying intracellular ROS and superoxides concentrations qualitatively and quantitatively. Significantly increased ROS production was observed in the cells treated with both Curcumin and Doxorubicin thereby suggesting ROS mediated cancer cell death (Fig. 4.21). The role of ROS in cancer progression is well-established in literature but ROS mediated cancer cell death is still not clearly defined [257-258].
Curcumin mediated synergistic effects in presence of Doxorubicin treatments on cancer death were further analysed by studying induced DNA fragmentation in cancer cells by TUNEL assay followed by characterizing programmed apoptotic cell death by Annexin, an early biomarker of apoptosis and activity of different caspases activity (Fig. 4.22-4.24). The results obtained further validated that combination treatment of Doxorubicin and Curcumin display anti-cancerous activity without compromising the cardiac health as the desired anti-cancer effects can be achieved at much lower concentrations of Doxorubicin and its dose mediated toxic effects can be avoided. Curcumin also showed to modulate the expression of genes involved in apoptotic signalling and inflammatory responses displayed by cancer cells upon chemotherapy or the combination of chemotherapy and natural herbs (Fig. 4.25). The synergistic effect of Curcumin showed significant decrease in anti-apoptotic biomarker expression thereby stimulating cancer cell death. It also significantly increased the expression of pro-apoptotic as well as anti-inflammatory responses thereby further inducing cancer cell death.

Curcumin is a well-known anti-cancerous agent and suppresses Doxorubicin mediated ROS production in cardiomyocytes [248, 259]. However, in our present study, it shows that ROS production increases in presence of Curcumin treatment with Doxorubicin in cancer cells. We conclude that Curcuma longa polyphenols including Curcumin holds both cardioprotective and anti-cancerous properties and have a great potential to be a safer alternative for the drugs associated toxic effects on heart.
4.6. Conclusion

- Doxorubicin exerts dose dependent cardiotoxicity and its uptake in cardiac cells increases with increase in concentration. This dose dependent cardiotoxicity of Doxorubicin can be significantly minimized by supplementing it with Curcumin.
- Curcumin exerts time dependent responses on Doxorubicin-induced cardiotoxicity, where parallel treatment potentiates and pre-treatment suppresses the Doxorubicin -induced stress in H9C2 cardiomyoblasts. Hence, pre-treatment of Curcumin suppresses the Doxorubicin induced cardiotoxicity.
- Doxorubicin-dependent cardiotoxicity occurs through ROS overproduction and Curcumin prevents the cardiomyoblasts by exaggerating the inbuilt anti-oxidant enzymes activity in pre-treated cells.
- The synergistic effect of Curcumin in the combination treatment results in achieving the desired anti-cancerous effect without compromising the Doxorubicin activity and hence, reduces the possibility of its dose mediated cardiotoxic effects.
- Curcumin holds a great potential for cardio-oncological therapeutic interventions.