Life Sciences 205 (2018) 97–106

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Assessment of herb-drug synergy to combat doxorubicin induced cardiotoxicity

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ARTICLE INFO

Keywords:
Doxorubicin
Curcumin
Cardiotoxicity
Anti-cancer
Cardio-oncology
Herb-drug

ABSTRACT

Aim

Doxorubicin (Dox) is one of the most cardiotoxic anti-cancerous drug that is widely used for broad-range of cancers. There is an urgent need for developing cardio-oncological therapeutic interventions. Natural products having both anti-cancerous potential as well as cardioprotective effects may hold a great potential in this regard. *Curcuma longa* (an Indian herb) polyphenols including curcumin, and well known for its anti-oxidative and anti-cancerous potential was used in the present study for its synergistic effect on cancer cells and cardiomyocytes.

Material and methods: Preliminary dose dependent analysis for cell viability was conducted by MTT and trypan blue assays where the effects of curcumin and Dox on cancer cell progression and cardiotoxicity were studied. Microscopic studies were done to analyse the morphological alterations of cells followed by intracellular ROS production studies by NBT and DCFH-DA assays. Apoptotic cellular death was studied by caspase activity and Annexin/PI FACS analysis. TUNEL assay was done followed by expression analysis of different cellular death biomarkers by quantitative real-time PCR.

Key findings: We observed that dose dependent cardiotoxicity of Dox can be significantly minimized by supplementing it with curcumin. Curcumin supplementation exaggerates oxidative stress and apoptosis leading to cancer cell death by modulating pro- and anti-apoptotic biomarkers.

Significance: The combination treatment with curcumin results in achieving the desired anti-cancerous effect of Dox without compromising its activity and hence, reduces the possibility of its dose mediated cardiotoxic effects. Hence, curcumin holds a great potential for cardio-oncological therapeutic interventions.

1. Introduction

Drug induced cardiotoxicity is the consequence of adverse drug reaction of non-cardiovascular drugs on heart. As a consequence of this, the drug treatment productivity gets compromised because patient recovers from one chronic disease but cultivates the chances of developing cardiovascular complications [1]. According to WHO's recent factsheet, cancer is one of the leading causes of morbidity and mortality worldwide, as evident by the statistics suggesting that every one in six deaths is due to cancer at the global scale [2]. Present cancer therapies are associated with related toxicities in different organs and hence limit the treatment competence [3]. Literature supports that severe cardiac toxicity is predominantly evident in anti-cancer treatments [4–9]. Hence, even if patients survive from cancer, they end up with cardiac complications in longer run leading to compromised life style or death.

Doxorubicin (Dox), an anthracycline anticancerous drug, is a well-established and most commonly used chemotherapeutic drug against wide varieties of tumors. Its major limitation is the associated toxicity on different body organs, especially heart. Literature proposes that Dox induced cardiomyopathy has poor prognosis and is mortal in most of the cases [10,11]. Cancer is a major cause of mortality and where on the one hand, improved treatments are leading to the increased recovery rates, on the other hand, the associated toxicity putting patients at risk of side effects occurring at the post treatment phases.

Natural products supplementation has long been used for preventive measures as well as curative agents. The vast history of using different medicinal plants as pharmaceutical agents is evident for treating various illnesses and injuries [12–15]. Curcumin and curcumoids are one such example of natural compounds with well-established protective roles against broad range of anomalies. They are the polyphenols derived from the well-known Indian herb *Curcuma longa* that is best known for its anti-oxidant, anti-inflammatory, anti-tumor, anti-microbial and anti-fungal properties [16]. Curcumin is known for its anti-cancerous potential by inhibiting TGF-β and PI3K/AKT cascades thereby inducing epithelial-mesenchymal transition, modify free radical pathways, inhibiting constitutive NF-κB activation and

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https://doi.org/10.1016/j.lfs.2018.05.021
Received 13 December 2017; Received in revised form 5 May 2018; Accepted 8 May 2018
Available online 09 May 2018
0024-3205/

modulating caspase dependent and independent pathways [17–19]. Role of curcumin in treating pre-cancerous lesions has also been reported that further suggests its role not only in curing cancer but also in preventive cancer occurrence [20]. Curcumin is reported to reduces ROS generation, modulate various signalling pathways, prevents cardiac remodeling and undesirable myocardial alterations [21,22]. Our group has previously shown the cardioprotective potential of curcumin by inhibiting GATA-4 mediated cardiac fetal gene program, regulate ECM remodeling and suppress prolonged hypertrophy induced cardiac cell death [23–26]. No detailed and dose mediated comparative study has been conducted to analyse the cardioprotective as well as anti-cancerous effect of curcumin in synergy with Dox by far.

As Dox induced cardiotoxicity is dose mediated, the present study was designed to observe the synergistic effect of curcumin and Dox on human cancer cells and cardiomyocytes. Experiments were conducted on MCF-7 breast cancer cell lines and H9C2 rat cardiomyocytes using various microscopic staining assays for morphological alterations and ROS generation. MCF-7 human breast cancer metastatic cell line is first known hormone responsive cell line and widely used for *in vitro* breast cancer biology studies [27,28]. MCF-7 is also a chemotherapy responsive cell line and hence chosen for the current study for studying the effect of Dox along with natural herb combinations [29]. As deriving the exact concentrations of testing compounds is very critical before starting any experiments, MTT and trypan blue cell viability assays were done initially to see the effects of curcumin and Dox individually and in combination on cancer cell progression and cardiotoxicity. These dose dependent studies were followed by characteristic microscopic studies as they assist in getting clear insights of any alterations taking place inside the cells upon our treatments. The cellular and nuclear morphology of cells was studied by bright field microscopy followed by giemsa and DAPI staining. NBT and DCFH-DA assays were done to study the intracellular ROS production. All the microscopic findings were also quantitated by parallel spectrophotometric analysis for further validating the results. These microscopic outcomes laid the evidence that Dox mediated drug induced cardiotoxicity can be reduced by supplementing the optimized doses of curcumin without compromising the anti-cancerous capacity of cancer therapeutics as curcumin plays dual role both as anti-cancerous and cardioprotection. This observation was further validated by expression studies of different cell death biomarkers.

2. Material and methods

All chemicals were purchased from Sigma Aldrich, USA unless or otherwise mentioned. Curcumin used in the study has > 80% curcumin content and ≥94% curcuminoid content (Catalogue number: C7727, Sigma-Aldrich).

2.1. Cell culture maintenance

Human breast adenocarcinoma cell line- MCF-7 and embryonic rat cardiac tissue-derived H9C2 cardiomyoblasts (NCCS, Pune, India) were cultured in DMEM supplemented with 10% FBS and antibiotics. Cells were grown at the density of 10^6 cells/cm^2 in a humidified incubator with 5% CO_2 at 37 °C and were trypsinized and seeded in the ratio of 1:3 upon confluences. Cells were grown in culture dishes 16–18 h before the required treatments.

2.2. MTT cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was done to measure the percent cell viability and to optimize the concentrations of Dox and curcumin in both the cell lines. Following the treatments, viable cells were stained with 5 mg/ml MTT for 3 h. The media was replaced with DMSO in each well to solubilize formazan and absorbance was measured at 570 nm using spectrophotometer (Bio-Rad Laboratories-USA) [30]. Percent cell viability was calculated using the formula (Absorbance of treated cells)/(Absorbance of untreated cells).

2.3. Trypan blue dye exclusion assay

Cells from all the experimental groups were pelleted down and incubated with 0.4% trypan blue stain in 1:1 ratio and kept at room temperature for 5 min [31]. The cell suspension was loaded into the Countess® II Automated Cell Counter (Invitrogen) to record the number of dead and live cells.

2.4. Morphological analysis of MCF-7 cells

Cells treated with Dox and curcumin were analysed for altered cell size and morphology under an inverted microscope (Olympus) at 20× magnification. The total concentration of cells in the individual experimental group was also evaluated by Countess® II Automated Cell Counter.

2.5. Giemsa staining

To study the alterations of cellular morphology upon various treatments by giemsa staining, cells were fixed with 100% methanol at −20 °C and incubated with 20% giemsa stain diluted in 0.5% glacial acetic acid for 15 min at 25 °C and observed under an inverted microscope at 100× magnification for morphological alterations [32]. Cell size was also quantified by NIH image J software as per the instructions provided.

2.6. DAPI nuclear staining

Changes in nuclear morphology of the cells treated with curcumin and Dox were observed in the presence of DAPI fluorescent stain. 50 ng/ml DAPI was prepared in the staining solution having 10 mM Tris-Cl, pH 7.4; 10 mM EDTA, pH 8 and 100 mM NaCl. Cells were incubated in dark for 15 min at room temperature and then observed under fluorescent microscope (Olympus) using the DAPI filter at 100× magnification with excitation and emission wavelength at 372 and 456 nm respectively [33].

2.7. NBT assay for superoxide generation

Cells following the respective treatments were incubated with 0.3% nitroblue tetrazolium chloride (NBT) solution for 1 h in a humified incubator with 5% CO_2 at 37 °C and then counter-stained with safranin and incubated for 15 min. The excessive stain was removed by a gentle 1× PBS wash and observed under microscope at 100× for the formation of blue formazon crystals. Percentage of NBT positive cells was also measured by solubilizing these crystals with 2M KOH and DMSO [34]. Absorbance was taken at 630 nm and stimulation index for superoxides production was calculated as the absorbance ratio of the treated and control cells.

2.8. DCFH-DA assay for intracellular ROS

2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was used to study the intracellular ROS production upon Dox and curcumin treatment. 5 μM of DCFH-DA stain was added to the treated cells and incubated in dark at room temperature for 30 min [35]. Cells were then observed under the FITC filter at 100× magnification with excitation and emission wavelength at 490 and 520 nm respectively. The net fluorescence of the eluted stained cells from the treated cells was also measured by spectrofluorometer at the excitation of 490 nm and an emission wavelength of 520 nm.