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p-Coumaric acid mediated protection of H9c2 cells from Doxorubicin-induced cardiotoxicity: Involvement of augmented Nrf2 and autophagy

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ABSTRACT

Doxorubicin (Dox) is a widely administered chemotherapeutic drug and incidences of cardiotoxicity associated with its administration have been of general concern. Extensive research proposes several mechanisms as a cause of Dox induced cardiotoxicity. However, none of these studies have been able to suggest a find one, cure all antidote for the same. To this end, several studies involving plant based compounds or natural products have gained acclaim for their ability to address at least one factor contributing to drug induced pathogenesis. We had previously reported that *p*-coumaric (p*CA*) has a protective effect on Dox induced oxidative stress in rat-derived cardiomyoblasts. In this study we investigated the effects of p*CA* on the regulation of Nrf-2, mitochondrial viability, autophagy and apoptosis in Doxorubicin treated H9c2 cardiomyocytes. ROS induced mitochondrial stress, changes in mitochondrial membrane potential, loss of membrane integrity; nuclear damage as single/double stranded breaks, autophagy and the effects of pre and co-treatment of p*CA* on Nrf-2 mediated signaling was evaluated by various approaches. The effect of p*CA* on drug uptake was evaluated through confocal Raman Spectroscopy. We find that nuclear translocation of Nrf-2 is prominently marked by protein-specific antibody conjugated fluorophore in Dox treated cells especially. Cell survival is mediated to a certain extent by the expression of the anti-apoptotic BCl2 in p*CA* treated cells. However, mRNA levels of autophagy related (Atg) genes suggest that autophagy plays a decisive role in deciding cellular fate. Caspase-3 activation is also observed in p*CA* treated cells which suggest an alternative function of caspase-3 in p*CA* mediated cell survival. Expression of antioxidant enzymes confirm the oxidative stress induced by Dox treatment in cells and the modulation of cell redox homeostasis through treatment with p*CA*.

1. Introduction

Doxorubicin (Dox), a microbial anthracycline antibiotic has been widely used for treating a variety of human cancers, including leukemia, lymphoma, soft-tissue sarcoma and solid tumors [1,2]. Unfortunately due to its deleterious effect on cardiac physiology, the clinical use of Dox is often limited. Though there are several theories which explain the cardiotoxicity of Dox, one of the most investigated mechanisms include increasing levels of reactive oxygen species (ROS) which creates undue oxidative stress in cardiomyocytes [3], alterations in mitochondrial energetics [4], and a direct damaging effect on DNA. Dox directly generates free radicals through re-Dox cycling and also acts as a strong chemical catalyst for the production of oxygen radicals [5]. Dox treatment adversely affects cellular antioxidant capacity and causes extensive organelle damage [6–9] leading to cardiomyopathy.

One strategy to counteract Dox-induced cardiotoxicity is to try and minimize/optimize the effective chemotherapeutic dose of Dox without affecting the anti-cancer efficacy of the drug [10]. A long standing principle that antioxidants reduce the risk of certain pathological conditions, such as cancer, diabetes, atherosclerosis, aging, and neurodegeneration [11] through regulation of the Keap1- Nuclear factor erythroid 2-related factor (Nrf2) pathway is an important area of research [12]

p-coumaric acid (*p*CA), a phenolic compound belonging to the hydroxycinnamic acid family has been reported to possess anti-mutagenic and anti-tumor activity [13]. Experiments demonstrate that phenolic compounds have a strong antioxidant effect, by which the compound scavenges reactive oxygen species (ROS) and related compounds thereby protecting normal cells from toxic side effects without interfering with the drug's anti-tumor activity [14]. H9c2 cells, derived from the ventricular tissue of the E13 BDIX rat heart, have been used as an *in vitro* model to evaluate several conditions of cardiotoxicity [15,16].

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These cells are known for their ability to differentiate into multi-nucleated, myotubes with limited proliferative potential and possess similar markers like in adult heart tissue, the only limiting factor being the lack of contractile activity [17].Ethical constraints associated with the culture and maintenance of primary cell lines for initial experiments is one reason because of which preliminary studies are carried out in these cell lines.

A previous report by our group showed that p*CA* has a very strong *in vitro* anti oxidant capacity and prevents the apoptosis of H9c2 cells exposed to Dox [18]. Therefore, the present study was undertaken to elucidate the basic mechanism of the protective effect of *p*CA in preventing Dox induced cardiotoxicity by molecular methods.

2. Material and methods

2.1. Chemicals

Anti-Nrf2 [Nrf-2 (C-20):sc-722] antibody was purchased from Santacruz Biotechnology, Inc. Doxorubicin Hydrochloride, *p*-Coumaric acid (*p*CA) and Monodansyl cadaverine was purchased from Sigma Chemicals. Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), Rhodamine-123,Propidium Iodide Acridine orange, Antibiotic–Antimycotic solution, EZcount^{TM} LDH Cell Assay Kit, cell culture chamber slides, black and other cell culture reagents were procured from Hi-Media Laboratories. MitoSOX Red was purchased from Life Technologies. Taq PCR Smart Mix 2x was purchased from Orion-X.Verso cDNA Synthesis Kit was procured from Thermofisher Inc. Oligos were synthesized by Xcelris Labs and Integrated DNA Technologies.

2.2. Cell culture

H9c2 myoblast cells derived from the rat myocardium was obtained from National Centre for Cell Sciences (NCCS), Pune, India. The myoblast cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) medium supplemented with 10% FBS and 10 ml/L 100X Antibiotic-antimycotic solution containing 10,000 units of penicillin and 10 mg/ml streptomycin in 0.9% normal saline in a humidified atmosphere of 95% air and 5% CO_2 at 37∘C [24].

2.3. Dox uptake by H9c2 cells

Dox uptake in cells at 6 h was estimated spectrophotometrically. H9c2 cells (2×10^4) was plated on a 96 well plate and allowed to adhere overnight. Post adherence, cells were treated with Dox/*p*CA for the required time period. At the end of the incubation period cells were washed with ice-cold PBS and a 1:1 mixture of ethanol/0.3 M HCl was added to the wells. Fluorescence was read using the Thermofisher Varioskan Plate Reader at a λ_{ex}- 485 nm, λ_{em}-590 nm. Percentage drug uptake was calculated by comparing with the control.

2.4. Confocal Raman microspectroscopy

Approximately 5×10^4 H9c2 cardiomyoblasts were seeded onto glass cover slips and incubated overnight to ensure complete attachment. Cells were then treated with the indicated concentrations of *p*CA along with Dox and incubated at 37 °C for a period of 24 h. Treated cells were then washed with sterile-chilled PBS and fixed in a 4% formalin solution for 20 minutes. Following fixation the cells were stored in an isotonic phosphate buffer solution until start of data acquisition. Raman spectra of the individual components i.e. Doxorubicin and *p*CA was acquiesced from freshly prepared solutions with identical concentrations as used in the treatment protocols. Confocal Raman mapping were done using a WITec confocal Raman microscope (WITec GmbH, Ulm, Germany;alpha300RA AFM & RAMAN),A WITec Ultrahigh throughput lens based spectrograph (UHTS 300) with 300 mm focal length was used for Raman spectra recordings. Excitation was done using a 532 nm Diode-Pumped Solid State Laser (DPSS), 70 mW as a source. The laser is coupled into the microscope using a single mode optical fibre coupling and the Raman signal is collected into a multimode optical fibre which is connected to the UHTS 300 spectrograph equipped with a back illuminated CCD with better than 90% QE in the visible region. Andor Back illuminated CCD camera. The CCD chip is 1024 × 127 pixels with the pixel size of 24 microns 600 g/mm grating BLZ 550 nm. Spectra were acquired in the range from 0–4000 cm^{-1} with the application of a 50× objective (Zeiss, NA = 0.75) [19].

2.5. Evaluating cell death and apoptosis by Annexin V/Propidium Iodide staining

Levels of Apoptosis and cell death following drug treatment was assayed using an Annexin-V Alexa Fluor 488/Propidium Iodide assay Kit (Invitrogen) as per manufacturer's instructions. Briefly, treated cells were trypsinised and resuspended in Annexin binding buffer containing Annexin V-Alexa Fluor 488 for a period of 20 minutes in the dark. At the end of the incubation period cells were resuspended in fresh buffer containing propidium Iodide for a period of 5 minutes. Cell suspension (25 µl) was loaded onto a Tali Cellular Analysis slide and data obtained using the inbuilt software of the Image based Tali Cytometer (Invitrogen) was analysed after appropriate threshold settings [20].

2.6. Detection of acidic vesicles as an indicator of autophagy: acridine orange staining

H9c2 cells (3×10^4) was plated on 48-well plates and incubated overnight to allow for cell attachment. Following treatment with *p*CA/Doxorubicin, cells were stained with 1 µg/mL acridine orange in serum free DMEM for 15 min, washed thrice with PBS, and examined under a fluorescence microscope (Olympus) at 20× magnification [21].

2.7. Labeling and visualization of autophagic vacuoles stained with Monodansyl cadaverine

H9c2 cells (3×10^4) were plated on 48-well plates and incubated overnight to allow for cell attachment. Following treatment with *p*CA/Doxorubicin, cells were loaded with 50 µM monodansyl cadaverine (MDC) in serum free medium for 10 minutes at 37 °C. Incubated cells were either rinsed in PBS and immediately observed under the inverted fluorescent microscope or cells were collected in 10 mM Tris-HCl (pH-8) containing 0.1% Triton-X-100.and the intracellular MDC levels were measured by fluorescence photometry at λ_{ex}-380 nm, λ_{em}-525 nm. [22].

2.8. Intracellular and released LDH

Membrane damage in rat derived H9c2 cells on treatment with Doxorubicin and *p*CA was detected using the EZcount^{TM} LDH Cell Assay Kit, Hi-Media in a one-step reaction as per manufacturer's protocols. The enzyme in the LDH reagent uses NADH to reduce the dye to a coloured product which can be measured colorimetrically. Briefly, cells (2×10^4) were seeded on a 96 well plate and incubated overnight for adherence. Lysis solution was added to the non treated control wells after 24 h of treatment and the plate was incubated at 37 °C. Post-lysis the LDH reagent was added to all the wells and the plate was incubated at room temperature for 10 minutes. Reaction was terminated with the addition of a stop solution. Absorbance was read at 580 nm as a main wavelength and at 630 nm for the reference wavelength.