Research Article

Evaluation of Mearnsitrin on cardiomyocyte apoptosis induced by doxorubicin in H9c2 Cardiomyoblast Cells

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Abstract

Objective: Doxorubicin (Dox) is a type of chemotherapy drug it slows or stops the growth of cancer cells by blocking an enzyme called topoisomerase-2 and produces cardiotoxicity by inducing apoptosis. Mearnsitrin is a flavonoid compound from the stems of *Emilia sonchifolia* DC and is known to be a natural antioxidant. The study is carried out the evaluation of mearnsitrin on cardiomyocyte apoptosis induced by doxorubicin in H9c2 Cardiomyoblast Cells. **Materials and methods:** Rat cardiac H9C2 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin and 5% CO2 at 37 °C. A modified MTT assay was used to determine cell viability. Quantitative real time RT-PCR was used to evaluate the expression of Bcl-2 in cardiomyocytes. **Results:** No toxicity observed when the cells exposed for 1 hour to different concentrations of mearnsitrin, but pretreatment of cells with mearnsitrin increased cytotoxicity of DOX in a dose dependent manner. RT-PCR analysis showed that mearnsitrin significant mRNA gene expression of Bcl2 compared to cells treated with DOX alone. **Conclusion:** The data indicated that subtoxic concentrations of mearnsitrin sensitize H9c2 cells to DOX-induce apoptosis. These results suggest that the use of mearnsitrin in combination with DOX reduces the cardiomyocyte apoptosis induced in H9c2 cardiomyoblast cells than DOX alone.

Keywords: Mearnsitrin, apoptosis, doxorubicin, cardiomyoblast Cells, *Emilia sonchifolia*

Introduction

Doxorubicin is an anthracycline anti-cancer (cytotoxic) chemotherapy drug. One of the most effective chemotherapy medications for solid tumours is doxorubicin, which is used to treat many cancer types (Pommier et al., 2010). Two distinct mechanisms, I doxorubicin intercalation into DNA and inhibition of topoisomerase II, which results in chromatin structural alterations, and II production of free radicals and oxidative damage to biomolecules. Doxorubicin inhibits topoisomerase II, which overwinds DNA during transcription, thereby preventing the recombination of the DNA double strand, thus stopping DNA replication (Kim et al., 2006; Yang et al., 2013).

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With varying doxorubicin concentrations, studies have been done on several cancer cell lines and cell models (Bar-On et al., 2007; Ahn et al., 2004). Doxorubicin was discovered to play a role in a number of additional cellular processes, such as ceramide metabolism and cell cycle arrest, despite the fact that the responses of various cell types at the transcriptional level (Liu et al., 2013; Chen et al., 2015).

Its major adverse effect is cardiotoxicity, which may limit its use. Doxorubicin cardiomyopathy, once developed, carries a poor prognosis and is frequently fatal (Weiss et al., 1992; Jordon et al., 2002). Cardiotoxicity caused by doxorubicin can be immediate and manifest as during or two to three days after administration. Acute cardiotoxicity occurs in about 11% of cases. Myopericarditis-related chest discomfort, sinus tachycardia, paroxysmal non-sustained supraventricular tachycardia, and premature atrial and ventricular beats are the most common signs (Takemura and Fujiwara, 2007; Swain et al., 2003).

Mearnsetin is an O-methylated flavonol. It can be found in

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*Eucalyptus glob*ulus and in *Elaeocarpus lanceofolius* (Ray et al., 1976). The compound has antioxidative properties (Sadasivam et al 2011). The present study has planned to evaluate the mearnsitrin on cardiomyocyte apoptosis induced by doxorubicin in H9c2 cardiomyoblast Cells.

Materials and methods

Materials

The mearnsitrin were procured from Sigma Aldrich India, Bengaluru and MTT and cell bioassay chemicals were procred from Himedia Laboratories Pvt. Ltd, Bangaluru and other chemical were used in the study were analytical grade. Cell culture medium purchased from NCL pune.

Cell culture

Rat heart cell line H9c2 was obtained from NCL, Pune, India. The H9c2 cells were grown in Dubblico modified Eagle medium (DMEM) containing 4 mM L-glutamine, 4.5 g/l glucose, 1 mM sodium pyruvate, 10% (v/v) heat inactivated FBS, penicillin G (100 U/ml) and streptomycin (100 mg/ml) at 37 ◦C in 95% CO2 humidified incubator. The myoblastic population would become depleted rapidly if the cultures were allowed to become confluent. Thus, to prevent loss of myoblastic population, cells were subcultured when 70% of the flask was covered with cells and plated (25000-30000 cells/cm2) in 25-cm flask and in 6-well plate for other studies.

Cell viability assay

Cellular toxicities of DOX and mearnsitrin were analyzed in H9c2 cells using MTT method. Cells were plated onto 96-well plates at a density of 2.0×104 cells/well and in a volume of 200 µl. Stock solutions of mearnsitrin and DOX were prepared in dimethyl sulfoxide (DMSO). The final concentration of the vehicle in the medium was always 0.5%. One day after seeding, 2 µl of the DMSO containing DOX or mearnsitrin at different concentrations was added to each well. At appropriate time intervals, the medium was removed and replaced by 100 µl of 0.5 mg/ml of MTT in growth medium and then the plates transferred to a 37 °C incubator for 3-4 hr.

Supernatants were removed and the reduced MTT dye was solubilized with DMSO (100 µl /well). Absorbance was determined on an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain sample signal (OD570–OD630). Percentage of proliferation was calculated using the following formula: Percent of control proliferation= (OD test/OD control)×100. IC50 values were calculated by plotting the log10 of the percentage of proliferation versus drug concentration (Cao et al., 2004).

Reverse transcriptase-polymerase chain reaction

Total RNA will be isolated from H9C2 cells using Trizol (Invitrogen). First-strand cDNAs will be generated by reverse transcription using oligo (dT) from RNA samples. After cDNA synthesis, PCR will be performed, using the following conditions: 95 °C for 3 min; 94 °C for 30 s; 58 or 60 °C for 30 s (depending on the sequences of the primers); and 72 °C for 1 min, 72 °C for 5 min for 30 cycles. PCR products will be electrophoresed in 2% agarose gel and visualized with ethidium bromide (EB). The relative expression will be quantified and calculated according to the reference bands of GAPDH (Xiuzhen, 2008; Spallarossa et al., 2005). Cell pretreated with different concentrations of mearnsitrin 1 hr before exposure to 3 µM of DOX. Normalization relative to Bcl2 was performed.

Results

The cytotoxicity assay has been carried out and represented in Table 1. The DOX shows IC50 values is 3.52 and 95% confidence interval is 3.305-3.656. However the increasing in the mearnsitrin dose pretreatment exhibits the significant (*P*< 0.001) IC50 values 2.93, 1.97 and 0.91 respectively with 95% confidence interval is 2.106-2.143, 1.459-1.676 and 0.867-1.204 than DOX alone treatment in H9c2 cells.

The RT-PCR study has been carried out and represented in Table 2. The DOX shows 0.82 Bcl2 regulation fold whereas increasing in the mearnsitrin dose pretreatment exhibits the

Table 1. Effect of mearnsitrin pre-treatment on DOX induced cytotoxicity in H9c2 cells

S. No	Groups	IC50±SEM	95% CI*
I	DOX	3.52 ±0.024	3.305-3.656
II	DOX + Mearnsitrin (5 µM)	2.93 ±0.039#	2.106-2.143
III	DOX + Mearnsitrin (10 µM)	1.97 ±0.027#	1.459-1.676
IV	DOX + mearnsitrin (15 µM)	0.91 ±0.053#	0.867-1.204

All data were expressed in the mean±SEM of three separate experiments. # *P*< 0.001 vs. DOX treated cells.*95% confidence interval; Dox- Doxorubicin

Table 2. The Quantitative gene expression level of Bcl-2 in cardiomyocytes normalized to GAPDH

S. No	Test Sample	Regulation in Terms of Folds
		Bcl2
I	Cell Control	1.00±0.021
II	DOX	0.82±0.035
III	DOX + Mearnsitrin (5 µM)	1.08±0.032**
IV	DOX + Mearnsitrin (10 µM)	1.15±0.036***
V	DOX + mearnsitrin (15 µM)	1.26±0.025***

Levels of mRNA are expressed relative to control cardiomyoblast in the mean±SEM values derived from three independent experiments; **P< 0.01, ***P< 0.001 vs. DOX treated cells; Dox- Doxorubicin

significant (*P*< 0.001) Bcl2 expression 1.08, 1.15 and 1.26 respectively than DOX alone treatment in H9c2 cells.

Discussion

Anthracycline doxorubicin, which is used to treat cancer, and known to cause irreversible cardiotoxicity. The production of reactive oxygen species was one of several hypothesised causes for its cardiac toxicity (Wold et al., 2005). Major adverse effect of DOX treatment in cancer patients is the onset of cardiomyopathy and heart failure (Gewirtz et al., 1999). Reactive Oxygen Species (ROS) derived from redox activation of DOX were proposed to be responsible for DOX-induce apoptosis in cardiac cells (Jang et al., 2004; Spallarossa et al., 2005).

The mechanisms underlying the cytotoxic and anti-proliferative properties of the anthracycline antibiotics doxorubicin and daunorubicin have been hotly debated. In the interaction between these drugs and the tumour cell, this commentary discusses the potential roles of DNA synthesis inhibition, free radical formation and lipid peroxidation, DNA binding and alkylation, DNA cross-linking, interference with DNA strand separation and helicase activity, direct membrane effects, and the beginning of DNA damage via topoisomerase II inhibition (Gewirtz et al., 1999).

Mearnsitrin, the chemical name is 3-[(6-Deoxy-α-L-mannopyranosyl)oxy]-2-(3,5-dihydroxy-4-methoxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one. It is a flavonoid isolated from the stems of *Emilia sonchifolia* DC having powerful anti-oxidant activity. The usage of DOX for the treatment of cancer in patients producing severe cardiotoxicity. Our concept is along with mearnsitrin usage of DOX might be reduce the toxicity.

Our present study has planned for evaluation of mearnsitrin pretreatment against DOX cytotoxicity in H9c2 cells. Initially cytotoxicity of DOX and Mearnsitrin in different exposure times were evaluated. The results showed that one hour exposure to different concentrations of mearnsitrin had no cytotoxic effect in H9c2 cells. Mearnsitrin was nontoxic at low concentrations, while higher doses promoted cell death. This is in line with a study that demonstrated 24 hr treatment with 5 µM mearnsitrin resulted marginal decrease in cell viability. Although, mearnsitrin is a flavonoid and described as a protective agent in several cell models, we observed here that pretreatment to subtoxic concentrations of mearnsitrin greatly increased cytotoxicity compared to level reached with the DOX alone.

It has been reported that DOX treatment was able to induce cytotoxicity in cardiac cells through induction of apoptosis (Wang et al., 2004). Caspase family involved in causing the apoptosis, but pre-treatment with mearnsitrin followed by DOX produces reduction in cytotoxicity in H9c2 cells with dose dependent manner.

Due to the direct relationship between the expression rate of Bcl2 and cardiac function, previous studies have shown that aberrant Bcl2 expression plays a key role in the modulation of cardiomyocyte apoptosis in MI/RI (Chinda et al., 2004). Decreased cardiomyocyte apoptosis is the main goal of therapeutic MI/RI treatment based on molecular pathways of injury development. The primary protein in the BCL2 family that promotes cell survival and inhibits apoptosis is known as Bcl2 (Gao, 2016).

In our study RT-PCR expression of pre-treatment with mearnsitrin increases the Bcl2 expression in DOX treated H9c2 cells. However in DOX treatment alone decreases the Bcl2 expression compared to pre-treatment with mearnsitrin with dose dependent manner. The further studies is required to prove the combination of mearnsitrin and DOX treatment reduces the cardiotoxicity at cellular

level and mechanistic pathways and its regulation is needed.

Conclusion

The data indicated that sub-toxic concentrations of mearnsitrin sensitize H9c2 cells to DOX-induce apoptosis. These results suggest that the use of mearnsitrin in combination with DOX reduces the cardiomyocyte apoptosis induced in H9c2 cardiomyoblast cells than DOX alone.

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Conflict of interest

Authors have no conflict of interest

References

Ahn MJ. et al. 2004. The differential gene expression profiles between sensitive and resistant breast cancer cells to adriamycin by cDNA microarray. Cancer Research and Treatment, 36:43–49.

Bar-On O, Shapira M, Hershko D. 2007. Differential effects of doxorubicin treatment on cell cycle arrest and Skp2 expression in breast cancer cells. Anti-Cancer Drugs, 18:1113–1121.

Cao Z, Li Y. 2004. Potent induction of cellular antioxidants and phase 2 enzymes by resveratrol in cardiomyocytes: protection against oxidative and electrophilic injury. European Journal of Pharmacology, 489:39–48.

Chen B. et al. 2015. Inhibition of mTORC2 Induces Cell-Cycle Arrest and Enhances the Cytotoxicity of Doxorubicin by Suppressing MDR1 Expression in HCC Cells. Molecular Cancer Therapeutics, 14:1805–1815.

Chinda K, Sanit J, Chattipakorn S, Chattipakorn N. 2014. Dipeptidyl peptidase-4 inhibitor reduces infarct size and preserves cardiac function via mitochondrial protection in ischaemia-reperfusion rat heart. Diabetes and Vascular Disease Research, 2014;11:75–83.

Gao CK, Liu H, Cui CJ, Liang ZG, Yao H, Tian Y. 2016. Roles of MicroRNA-195 in cardiomyocyte apoptosis induced by myocardial ischemia-reperfusion injury. Journal of Genetics, 95:99–108.

Gewirtz DA. 1999. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochemical Pharmacology, 7:727-741.

Jang YM, Kendaiah S, Drew B, Phillips T, Selman C, Julian D, et al. 200. Doxorubicin treatment in vivo activates caspase-12 mediated cardiac apoptosis in both male and female rats. FEBS Letters, 577:483-490.

Jordon MA. 2002. Anti-cancer agents. Current Medicinal Chemistry, 2:1–17.

Kim S. et al. 2006. Doxorubicin-induced reactive oxygen species generation and intracellular Ca2+ increase are reciprocally modulated in rat cardiomyocytes. Experimental and Molecular Medicine, 38:535–545.

Liu Y, Hill R, Li Y. Norris J. 2013. Ceramide Glycosylation Catalyzed by Glucosylceramide Synthase and Cancer Drug Resistance. Role of Sphingolipids in Cancer Development and Therapy, 117:59–89.

Pommier Y, Leo E, Zhang H. Marchand C. 2010. DNA Topoisomerases and Their Poisoning by Anticancer and Antibacterial Drugs. Chemistry & Biology, 17, 421–433.

Ray AB, Dutta SC, Dasgupta S. 1976. "Flavonoids of Elaeocarpus lanceofolius". Phytochemistry, 15 (11):1797–1798.

Sadasivam K, Kumaresan R. 2011. Antioxidant behavior of mearnsetin and myricetin flavonoid compounds--a DFT study". Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 79(1):282–93.

Spallarossa P, Fabbi P, Manca V, Garibaldi S, Ghigliotti G, Barisione C, Altieri P, Patrone F, Brunelli C, Barsotti A. 2005. Doxorubicin-induced expression of LOX-1 in H9c2 cardiac muscle cells and its role in apoptosis. Biochemical and Biophysical Research Communications, 335:188–196.

Spallarossa P, Fabbi P, Manca V, Garibaldi S, Ghigliotti G, Barisione C, et al. 2005. Doxorubicin-induced expression of LOX-1 in H9c2 cardiac muscle cells and its role in apoptosis. Biochemical and Biophysical Research Communications, 335:188-196.

Swain SM, Whaley FS, Ewer MS. 2003. Congestive heart failure in patients treated with doxorubicin. A retrospective analysis of three trials. Cancer, 97:2869–2879.

Takemura G, Fujiwara H. 2007. Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. Progress in Cardiovascular Diseases, 49:330–352.

Wang S, Konorev EA, Kotamraju S, Joseph J, Kalivendi S, Kalyanaraman B. 2004. Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms: Intermediacy of H2O2- and p53-dependent pathways. Journal of Biological Chemistry, 279:25535-25543.

Weiss RB. 1992. The anthracyclines: will we ever find a better doxorubicin? Seminars in Oncology, 19:670–686.

Wold LE, Aberle Ii NS, Ren J. 2005. Doxorubicin induces cardiomyocyte dysfunction via a p38 MAP kinasedependent oxidative stress mechanism. Cancer Detection and Prevention, 29:294-299.

Xiuzhen Han, Dongmei Ren, Peihong Fan, Tao Shen, Hongxiang Lou. 2008. Protective effects of naringenin-7-O-glucoside on doxorubicin-induced apoptosis in H9C2 cells. European Journal of Pharmacology, 581:47-53.

Yang F, Kemp C, Henikoff S. 2013. Doxorubicin Enhances Nucleosome Turnover around Promoters. Current Biology 23, 782–787.