Generation of Rho-0 Cells using MDA-MB-231 Cell Line and Measurement of Drug Cytotoxicity

Research Project submitted to the Central University of Punjab

For the award of

Master of Science

In

Life Sciences with Specialization in Molecular Medicine

By

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Supervisor

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May, 2018

CERTIFICATE

I declare that the dissertation entitled **"Generation of Rho-0 cells using MDA-MB-231 cell line and measurement of drug cytotoxicity"** has been prepared by me under the guidance of Dr. Sandeep Singh, Assistant Professor, and Dept. of Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab. No part of this project work has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Bharti Sharma has prepared her research project entitled **"Generation of Rho-0 cells using MDA-MB-231 cell line and measurement of drug cytotoxicity"**, for the award of M.Sc. degree of the Central University of Punjab, under my guidance. She has carried out this work at the Dept. of Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab.

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ABSTRACT

Generation of Rho-0 cells using MDA-MB-231 cell line and measurement of Drug Cytotoxicity.

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Key words: Rho-0 cells, cytotoxicity, mitochondria, potential, ROS, cancer

The ATP generation via Oxidative phosphorylation (OXPHOS) system located in the inner membrane of mitochondria, is regulated by the coordinated interaction between nucleus and mitochondria. In the same context, mitochondrial-depleted cell (Rho-0) can be a helpful approach to study the mitochondrial metabolism, mitochondrial role in various cellular processes such as apoptosis, mitochondrial role in various mitochondrial related disorders and cancer. To generate Rho-0 cells, EtBr mediated mtDNA depletion was done and verified by agarose gel electrophoresis. % cell viability, mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) production was measured after 24 hr treatment with 3 drugs, α-amanitin, Doxorubicin and DCA in both parental MDA-MB-231 and Rho-0 cells. Reduced cell death and ROS production was observed in Rho-0 cells indicating the resistance against apoptosis in Rho-0 cells and demonstrating the possible role of mitochondria in intrinsic pathway of apoptosis. MMP was observed to be maintained in Rho-0 cells indicating the role of nuclear genome in the maintenance of MMP.

Bharti Sharma Dr. Sandeep Singh

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Bharti Sharma

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LIST OF ABBREVIATIONS

S. No	Full Form	Abbreviation
1	Oxidative phosphorylation	OXPHOS
2	Adenosine triphosphate	ATP
3	Reactive oxygen species	ROS
4	Mitochondria	Mt
5	Mitochondrial deoxyribose nucleic acid	mtDNA
6	Mitochondrial permeability transition pore	mtPTP
7	Electron transport chain	ETC
8	Mitochondrial membrane potential	MMP
9	Cytochrome complex	cyt c
10	Ethidium Bromide	EtBr
11	alpha-Amanitin	α-amanitin
12	Doxorubicin	Dox
13	Dichloroacetate	DCA
14	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
15	Half maximal inhibitory concentration	IC_{50}
16	Mitochondrial membrane potential	$\Delta\Psi_m$
17	tetraethylbenzimidazolylcarbocyanine iodide	JC-1
18	2',7'-dichlorodihydrofluorescein diacetate	H_2DCFDA
19	Carbon dioxide	CO_2
20	Dimethyl sulfoxide	DMSO
21	Dulbecco's Modified Eagle Medium	DMEM
22	Fetal bovine serum	FBS
23	Revolution per minute	Rpm
24	Phosphate buffered saline	PBS
25	Chloroform	CCl_3
26	Concentration	Conc.

Chapter I
Introduction

INTRODUCTION

Mitochondrion is the site of ATP production through oxidative phosphorylation (OXPHOS) in most of the mammalian cells. Besides ATP synthesis, mitochondria also play a critical role in cell death, reactive oxygen species (ROS) production, intracellular calcium signaling, antiviral responses and other cellular processes (McBride *et al.,* 2006). Mitochondria have their own genome and encodes for 37 genes including 13 polypeptide components of the oxidative phosphorylation (OXPHOS) system, 22 tRNAs and 2 rRNAs (Anderson *et al.,* 1981). Mitochondrial dysfunction leads to promotion of metastasis (Imanshi *et al.,* 2011), resistance to apoptosis (Moro *et al.,* 2009), and a number of metabolic and genetic mitochondrial abnormalities in cancer (Wallace, 2012) by maintaining the malignant phenotype (Villa *et al.,* 2012). Rho-0(ρ°) cell are devoid of mtDNA, hence can be considered as a model to study drug targeting, drug cytotoxicity, role of mitochondria in cancer progression and several other mitochondrion related disorders (Fernández-Moreno *et al.,* 2016).

Chapter II
Review of Literature

2. REVIEW OF LITERATURE

2.1 MITOCHONDRIA

Mitochondria is not only the ‘powerhouse of the cell’, but it is also involved in apoptosis (Elkholi *et al.,* 2014), intracellular calcium signaling (Blackstone NW *et al.,* 2015), ROS production (Zorov DB *et al.,* 2014) and biosynthesis of heme and iron-sulphur clusters (Shah DI *et al.,* 2012) and other cellular processes. Mitochondria harbor their own genetic information via mtDNA. As Rho-0 cells are devoid of mtDNA, they can be a valid tool to study human mitochondrial disorders allowing the study of the real role of mtDNA with same nuclear DNA background as mitochondrial fucntions are controlled by both nuclear DNA and mtDNA (Fernández-Moreno *et al.,* 2016).

2.1.1 Mitochondrial DNA (mtDNA)

mtDNA is a covalently closed circular, double stranded molecule and 26,569 bp-long in humans. The mtDNA follows the maternal inheritance pattern and harbor its own DNA encoding for 37 genes including 13 polypeptide components of OXPHOS system, 22 tRNAs and 2 rRNAs (Bibb MJ *et al.,* 1981). Mutation in these genes have been reported in cancer (Wallace DC *et al.,* 2012), neurodegenerative disorders (Cha MY *et al.,* 2015), diabetes (Lien LM *et al.,* 2014) and aging (Bratic A *et al.,* 2013).

2.1.2 Role of Mitochondria in Apoptosis

Mitochondria play a crucial role in cell death as a major switch for the initiation of apoptosis. This switch involves the release of protein needed for the regulation of programmed cell death by interacting with the mtPTP (mitochondrial permeability transition pore). Opening and closing of mtPTP is dependent under the influence of either antiapoptotic or proapoptotic factors (Wang C *et al.,* 2009).

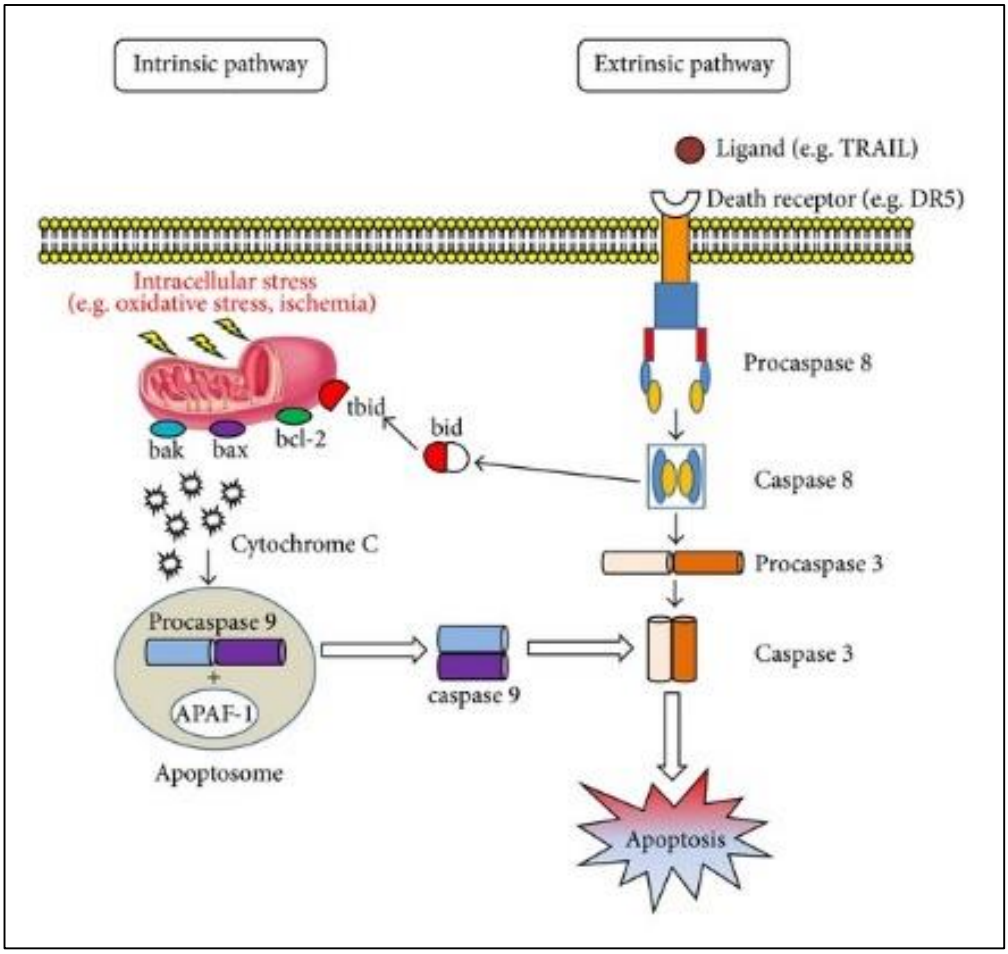

Figure1: The role of Mitochondria in intrinsic pathway of apoptosis.

Apoptosis can occur in two ways, mitochondria-dependent (or intrinsic) and death receptor-mediated (or extrinsic). The mitochondrial pathway is regulated by bcl family members bound to mitochondrial membrane. The proapoptotic proteins (bax and bak) promote cyt c release from mitochondria. Cytochrome C and deoxyadenosine triphosphate (dATP) bind to apoptotic protease activating factor (APAF-1) to form a multimeric complex that recruits and activates procaspase 9, an apoptosis-mediating executioner protease that in turn activates caspase 3, resulting in cell apoptosis. Also, the antiapoptotic proteins (bcl-2 and bcl-XL) inhibit cyt c release from mitochondria. The extrinsic pathway is initiated by ligation of only specific death receptors by ligands, followed by recruitment of procaspase 8 and activation of caspase 8. It induces apoptosis by directly activating caspase 3 or by cleaving bid (BH3 interacting domain death agonist), resulting in mitochondrial dysfunction and subsequent release of cytochrome C and activation of caspases 9 and 3. Source: Loreto C et al., 2014.

2.1.3 Production of ROS and Mitochondrial membrane potential in Mitochondria

Oxidative phosphorylation results in frequent exposure of oxidative stress to the mtDNA, during which molecular oxygen is converted into highly reactive oxygen species (hydrogen peroxide, nitric oxide, oxide anion, hydrochlorous acid, hydroxyl radical, etc.) in the ETC. Redundant ROS production results in the damage of functional macromolecules such as DNA, RNA, proteins and lipids (Orrenius S *et al.,* 2007). Overproduction of ROS can be induced as a treatment of several pathological abnormalities such as cancers, inflammatory diseases and ischemia (Wu D *et al.,* 2011).

MMP is a crucial parameter linked to mitochondrial function and used as an indicator of cell death. In a cell, MMP is associated with ATP synthesis, mitochondrial calcium levels, generation of ROS, import of proteins to the mitochondria and mitochondrial membrane dynamics, respiratory chain capacity and mitochondrial proton conductance (Sakamuru S *et al.,* 2012). Disruption in MMP results in the opening of mitochondrial permeability transition pores, which leads to the release of cytochrome complex (cyt c) into the cytosol, thereby triggering the other downstream signaling in apoptotic cascade via intrinsic pathway. Hyperpolarization of mitochondrial membrane can be due to the presence of ATPase inhibition, increased NADH supply, reduced ADP supply, oxidative stress induced apoptosis and cyt c oxidase dephosphorylation. Depolarization of the membrane can result from, selective mobile ionic carrier and the presence of ionophores and the induction of nonselective cation channels (Sakamuru S *et al.,* 2012).

.2.2 MITOCHONDIAL DEPLETED CELLS (RHO-0 CELLS)

2.2.1 Method of generating Rho-0 cell

mtDNA mutation and mitochondrial dysfunction has been observed as a major feature of cancer cells. An approach to study mitochondrial disorder is to generate cells devoid of endogenous mtDNA, called as rho-0 cells. Observing the effect of drug on cells with and without mtDNA, can provide us with the drug target to study and possibly to be

able to treat several disorders. To generate Rho-0 cells, EtBr supplemented media is provided to the cells. EtBr is a positively charged dye which intercalates into negatively double stranded DNA and interfere with enzyme of replication machinery (DNA polymerase). But, for sustaining the viability, cells need to be maintained in the presence of uridine. It satisfies the energy demand of the mtDNA depleted cells, as mtDNA depletion causes the defects in the electron transport chain (ETC) (Schubert S *et al.*, 2015).

2.2.2 Metabolism and oxygen consumption of Rho-0 cells

Cells devoid of mitochondria rely upon glycolysis for ATP production causing increase in NADH levels and hence interfere with glycolytic capacity by increasing the NADH: NAD+ ratio. It has also been reported that there is an altered regulation of calcium influx and calcium efflex through the sodium/calcium exchanger. Also, basal cytosolic calcium levels were reported low in Rho-0 cells in compare to the cell from which Rho-0 cells were derived (Giorgi C *et al*., 2012). It concludes that calcium uptake is somehow involved in apoptosis. Depletion of mitochondria also lead to the oxidative stress, increased lipid peroxidation in breast cancer cells(). It becomes important to determine the contribution of mtDNA in cell death mechanism. For the same purpose, cell treated with certain drugs can be studied for their effect on cell viability and their possible target involving in their mode of action.

2.2.3 Rho-0 cells and apoptosis

It has been shown that Rho-0 cells possess resistance to cell death, via various factors including increased glutathione levels and multidrug resistance, suggesting that mitochondria plays an important role in apoptosis (Lee M *et al.,* 2006). It also indicates that Rho-0 cells still have the nuclear encoded structure needed for the cell to undergo apoptosis and a functioning ETC may not be needed in that process. However, some works also suggest that mutation and depletion of mtDNA can increase the susceptibility of human cells to apoptosis (Liu CY *et al.,* 2006). Hence, these evidences shows conflicting conclusion regarding the apoptosis in Rho-0 cells.

2.2.4 ROS production and antioxidant defenses of Rho-0 cells

Rho-0 cells are known to resist ROS production and thereby escaping greater cell death compared with their parental cell lines (Chen H *et al.,* 2016). The other non-mitochondrial sources of ROS production are Xanthine oxidase, NOS (nitric oxide synthase) and NADPH oxidases. The effect of mtDNA depletion in the number of cellular antioxidant is conflicting and not yet fully understood.

2.2.5 Mitochondrial membrane potential in Rho-0 cells

As there is defect in the ETC (absence of complex I and III), Rho-0 cells cannot perform reverse electron transport through complex I. But, surprisingly Rho-0 cells are shown to have MMP and a proton gradient across the inner mitochondrial membrane being powered by glycolytic ATP synthesis. The following figure explains the possible mechanism of the maintained MMP (Anonymous, 2017).

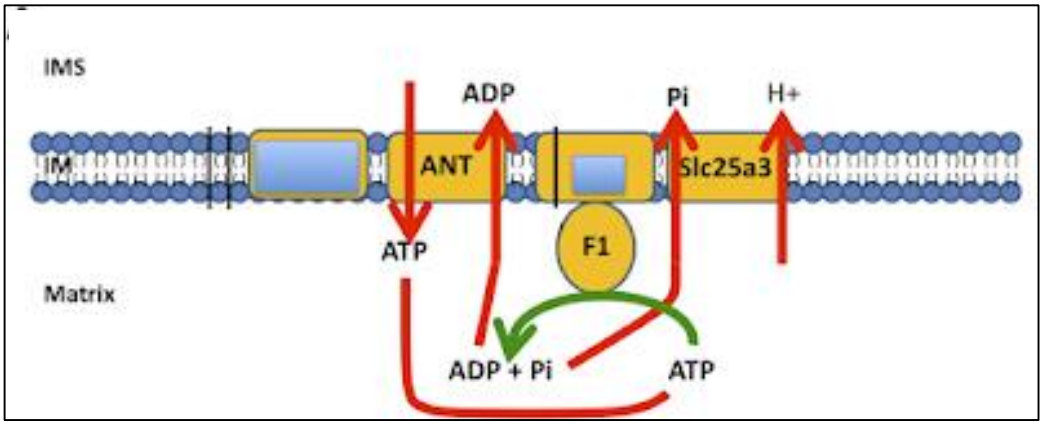

Figure 2: Restoration of electron transport without proton pumping in mammalian mitochondria.

ATP which has been made in the cytoplasm enters the mitochondria via ANT running in reverse. The F1 component of the ATP synthase breaks down the ATP to ADP and Pi. ADP is exchanged outwards via the ANT antiporter and Pi is carried outwards. This proton flux maintains the proton gradient across the inner mitochondria membrane, all of this process is being powered by glycolytic ATP synthesis. Source: Anonymous, 2017.

2.3 CYTOTOXICITY AND ASSESSMENT OF CELL VIABILITY ON CELL BASED ASSAY

2.3.1 Drug Cytotoxicity

Testing and examining the effects of compounds on the viability of cells grown in culture is widely used as a predictor of potential toxic effects in whole animals. In this context, measuring the levels of ATP (adenosine triphosphate) is considered to be the most sensitive, reliable, and convenient method for monitoring active cell metabolism. However, recently developed combinations of methods have made it possible to collect more information from *In vitro* cytotoxicity assays using standard fluorescence and luminescence plate readers.

Many clinically available drugs are used as a powerful cytotoxic drugs in oncology. Doxorubicin is a widely used cancer drug, which induces cellular cytotoxicity by inhibiting the activity of enzyme topoisomerase II and hence disrupting the DNA replication and transcription (Denard B *et al.,* 2012). Dox is also known to cause cell death by generating ROS, leading to lipid peroxidation and oxidative DNA lesions. It also contributes to the alterations in iron and calcium homeostasis (Jean SR *et al.,* 2015). Dox-.induced changes in mitochondrial transcriptome results from indirect mitochondrial dysfunction because of nuclear DNA damage (Zhang S *et al.,* 2012). DCA (Dichloroacetate) is another drug which has inhibitory action on mitochondrial enzyme PDK (pyruvate dehydrogenase kinase) thereby reducing MMP prompting apoptosis in cancer cells (Thebaud B *et al.*, 2007). As a cancer therapeutic, it can inhibit glycolysis, depolarize mitochondria and promote ROS production. As cancer cells are dependent on glycolysis for ATP generation, DCA can be used to target these cells. Hence, it has a selective toxicity for cells with defective ETC, as healthy cells have normally working mitochondria (Stockwin LH *et al.,* 2010). Alpha-amanitin is a drug which is a strong inhibitor of enzyme RNA polymerase II. However, it doesn't produce much significant inhibition of mitochondrial RNA polymerase, but can produce significant inhibition at relatively high concentrations (Menon IA, 1971).

2.3.2 *In vitro* Cell Based Assays for Cell Viability Measurement

In vitro cell based assays have been developed to rapidly determine the cell viability. These assays are also useful in identifying variations in susceptibility of different target cells to several chemotherapeutic agents. The MTT assay is one of the methods used to predict the drug response in malignancies. MTT assay measures cell respiration by measuring the amount of formazan produced from a tertazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Formazan production is proportional to the number of living cells present in culture, indicating the degree of cytotoxicity caused by the drug (Van Tonder A *et al.,* 2015).

IC_{50} is the concentration of the tested drug able to cause the death of 50% of the cells and can be predictive of the degree of cytotoxic effect. The lower the value, the more cytotoxic is the substance.

2.4 MEASUREMENT OF MMP

MMP ($\Delta\Psi_m$) being the major component of the proton motive force (Nicholls DG, 2004), plays a central role in aerobic energy production and driving force for various physiological processes in mitochondria. Hence, assessment of MMP becomes the key parameter for evaluating the mitochondrial function (Chen LB, 1998). A number of fluorescent dyes, such as DiOC6(3) (3, 3′-dihexyloxacarbocyanine iodide),Rh123 (rhodame-123), TMRM and TMRE (tetramethyl rhodame methyl and ethyl esters) and JC-1 (tetraethylbenzimidazolylcarbocyanine iodide) dye are available to access MMP. JC-1 dye is a cationic dye which can accumulate in energized mitochondria (abcam-ab113850). At lower concentrations (low MMP), the JC-1 dye is a monomer yielding green fluorescence (emission of 530±15 nm). While, at higher concentrations (high MMP), the dye aggregated that yield red fluorescence (emission of 590±17.5 nm). So, the working principle is that in healthy cells, JC-1 dye accumulates as aggregates in mitochondria, showing red fluorescence. But, when MMP is disrupted, the dye remains in cytoplasm and show green fluorescence (Sakamuru S *et al.,* 2012). Also, a reduction in aggregate fluorescent is an indication of depolarization and an increase in an indication of hyperpolarization.

2.5 MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS) PRODUCTION

Detection of cellular ROS production, mitochondrial membrane potential, apoptotic cells, mitochondrial respiration (oxygen consumption measured) can indicate the extent of mitochondrial involvement in cellular differentiation process. Survival and percentage of apoptosis can infer the effect of depletion of mtDNA on stem cell-like properties. H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) is a cell-permeant dye, which is a chemically reduced form of fluorescein. It is also known as dichlorofluores*cin*diacetate, and used as an indicator for ROS in cells. The principle is that, on cleavage of acetate groups of the dye, the non-fluorescent H2DCFDA dye is converted to a highly fluorescent compound DCF (2',7'-dichlorofluorescein) (ThermoFicher Scientific).

Chapter III
Materials and Methods

OBJECTIVE

a) To generate Rho-0 cells using MDA-MB-231 breast cancer cell line

b) To measure drug cytotoxicity, mitochondrial membrane potential and ROS production in generated Rho-0 cells and their parental MDA-MB-231 cell line.

3. MATERIALS AND METHODS

3.1 MATERIALS

Table 1: List of instruments used while conducting the work.

S. no.	Name of Instrument	Manufacturing Company
1.	Weighing balance	Mettler Tolendo
2.	Autoclave	Narang scientific works
3.	Oven	Samsung
4.	CO2 incubator	New Brunswick, UK
5.	Hot Plate	Tarsons
6.	Inverted Microscope	Olympus Magnus
7.	Rectangular water bath	NSW
8.	Refrigerated Centrifuge 5430R	Eppendorf, Germany
9.	Lamar air flow	NSW
10.	Microplate Reader	Synergy H1
11.	Gel Doc™ XR+ Gel Documentation system	Bio-Rad

Table 2: List of chemicals used while conducting the biological work.

S.No.	Materials	Manufacturing Company
1	DMEM	Gibco
2	Penicillin/ streptomycin antibiotic solution	Gibco
3	Fetal bovine serum	Gibco
4	DMSO	Gibco
5	Culture Plates and T25 Flask	Sigma falcon
7	EtBr dye	Sigma
8	Uridine	Sigma
9	Protease K	Invitrogen
10	Phenol:CCl3	Sisco Research laboratories
11	Isoamyl Alcohol	Sisco Research laboratories
12	Agarose	Amresco
13	Drugs solution (Doxorubicin, α-amanitin, Dichloroacetate)	Sigma
14	MTT dye	Life Technology
15	JC1 dye	Life Technology
16	H2DCFDA dye	Life Technology

3.2 METHODOLOGY

Cell Culture

MDA-MB-231 is an adenocarcinomic human breast cancer cell line possessing epithelial morphology. It is a highly aggressive, invasive and poorly differentiated triple negative breast cancer cell line. The cells are adherent and grow as monolayer. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) to be a complete media and antibiotic(s) (penicillin

streptomycin). For passaging adherent cells, cells were first examined for the confluence, and spent media was removed and discarded from the T25 flask. Trypsin was used as dissociation reagent for the detachment of cells from surface of the flask. After 3 min of incubation at room temperature, cells were observed for the detachment from flask surface under microscope. Once detached, cells were collected in culture vessel and dispersed in DMEM media (supplemented with 10% FBS and penicillin streptomycin). Then cells were centrifuged at 1500 RPM, 37°C for 5 min. Supernatant was discarded and obtained pallet was re-suspended in 5mL of fresh DMEM media in petri dish and T25 flask both.

Rho-0 Cell Culture

MDA-MD-231 cells were subsequently treated for 4-5 weeks with low dosage of EtBr dye (50 ng). The cells were maintained in DMEM supplemented with 10% FBS and antibiotic (penicillin streptomycin). In addition to that, the media was supplemented with 20µg of uridine. Uridine is needed to satisfy the energy demand to the cells, as Rho-0 cells have defective electron transport chain.

Further the media was changed after every 2-3 days interval and sub-cultured as the confluency reached to 80%. The cells were maintained in a 5% CO_2 and 90% humidified atmosphere at 37°C.

Generation of MDA-MB-231 Rho-0 Cells

Cells were plated and grown in both petri dish and T25 flask and cultured in the appropriate medium. Every time cells were sub-cultured, media was supplemented with EtBr and uridine. After a duration of 4 weeks, Rho-0 cells were isolated and seeded in 96 well plate. Also, MDA-MB231 cells were seeded in another 96 well plate as 100µl of media per well. The cells were maintained in a 5% CO_2 and 90% humidified atmosphere at 37°C.

Verification of Loss of mtDNA by Agarose Gel Electrophoresis

To verify the loss of mtDNA in the Rho-0 cells, total DNA was isolated from the cells plated with EtBr dye and cells plated without EtBr (MDA-MB-231) and was run in 0.5% agarose gel.

For DNA isolation, 500µl of 1X PBS was added in the cell suspension and centrifuged at 2000 rpm for 5 min. Then the supernatant was discarded and the pallet was washed with 1X PBS. After the washing, 200µl of lysis buffer was added followed by the addition of 5µl of Protease K to the pallet and incubated in water bath at 45 °C for 1 hr. Then 490µl of Phenol:CCl_3 composition and 10µl of Isoamyl alcohol was added and spin at 10000 rpm for 10 min. The obtained supernatant was separated in another Eppendorf tube and 200µl of 100% ethanol was added to the separated supernatant and kept for overnight at -20 °C. Next day, the Eppendorf tube was taken and spin at 10000 rpm for 10 min. Then the supernatant was discarded and pallet was washed with 200µl of 70% ethanol. The supernatant was discarded and 200µl of 70% ethanol was added and spin at 10000 rpm for 10 min. The supernatant was discarded and pallet was left to air dry in the laminar air flow. Once the pallet was dried, it was dissolved in 30µl of TE buffer.

Once the DNA was isolated, 0.5% Agarose gel was prepared, and sample was run. After the run, gel imaging was done using gel documentation system.

Drug Treatment

Once the loss of mtDNA was confirmed by agarose gel electrophoresis, parental MDA-MB-231 cells derived Rho-0 cells were treated with three drugs that are Doxorubicin (Dox), Dicholoacetate (DCA) and alpha-amanitin (α-amanitin). Specific amount of media was discarded from each well and the respective amount of drug was added in the same well. The drug added in a specific increasing order of concentration as 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 nm for α-amanitin and dox, while 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mm for DCA. The treatment was given for 24 h and cells were incubated in a 5% CO_2 and 90% humidified atmosphere at 37°C.

MTT Assay

MTT assay was done to determine the cell viability after the drug treatment. After 24 hrs of treatment, media was discarded from both the 96 well plates and 100µl of MTT solution (0.5µg/ml) was added in each well. The plates were incubated for 3 hrs at 37°C. Following the incubation, MTT solution was discarded and to dissolve the formazan crystals, 100µl of DMSO was added in each well of both the plates. The color absorbance was determined using microplate reader at 575nm and expressed as % of viable cells.

Measurement of Mitochondrial Membrane Potential (MMP) and ROS production

JC1 dye and H2DCFDA dye were used to measure MMP and ROS production respectively. After 24 hrs of treatment with three drugs namely, alpha-amanitin, doxorubicin and DCA, the media from 96-well plates was discarded. 10µl of JC1 dye was added in each well of mda-mb-231 96-well plate and Rho-0 cell 96-well plate. Similarly 10µl of H2DCFDA dye was added in each well of another mda-mb-231 96-well plate and its Rho-0 cell 96-well plate. After 30 min of incubation, at room temperature, the fluorescence of these dyes was measured using microplate reader. MMP was measured at 488nm of excitation and 530nm of emission wavelength and also at 535nm of excitation and 590nm of emission wavelength. ROS production was measured at 492nm of excitation and 517nm of emission wavelength.

Chapter IV
Results

4. RESULTS

4.1 GENERATION OF RHO-0 CELLS

The results showed that EtBr can deplete the mtDNA of MDA-MB-231 cancer cells, in the presence of uridine, and thus generating the rho-0 cells. The cells showed depletion of mtDNA.

Verification of Loss Of mtdna By Agarose Gel Electrophoresis

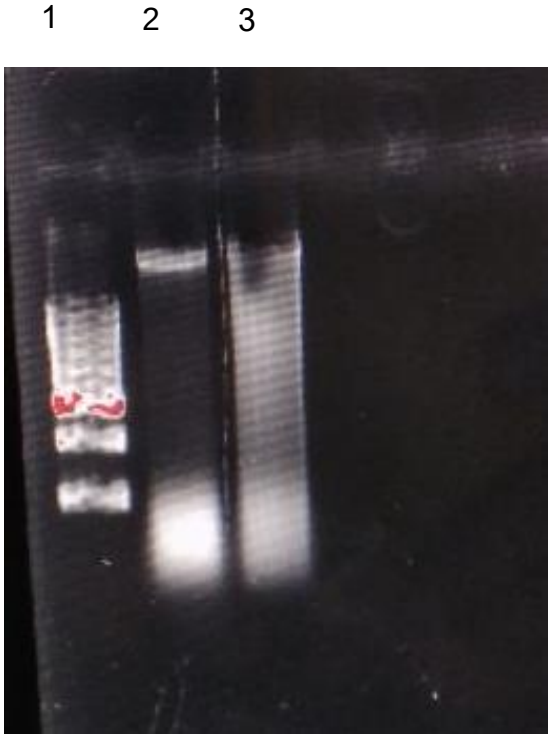

Figure 3: Verification of loss of mtDNA by agarose gel electrophoresis. 1-Ladder (100bp). 2- Control (mtDNA). 3-mtDNA treated with EtBr for 15 days.

The loss of mtDNA was verified by agarose gel electrophoresis, where total DNA was isolated from MDA-MB-231 cells (taken as control) and MDA-MB-231 cells treated with EtBr dye for 15 days and made to run in 0.5% agarose for half an hr. A single clear band in the 2nd column shows absence of mtDNA degradation. While in case of 3rd column, a smearing of bands represent the depletion of mtDNA.

4.2 MEASUREMENT OF DRUG CYTOTOXICITY IN BOTH PARENTAL MDA-MB-231 CELLS AND RHO-0 CELLS

After 24 hrs of treatment in parental MDA-MB-231 cancer cells and Rho-0 cells, with three different drugs, MTT assay was performed to measure cell viability.

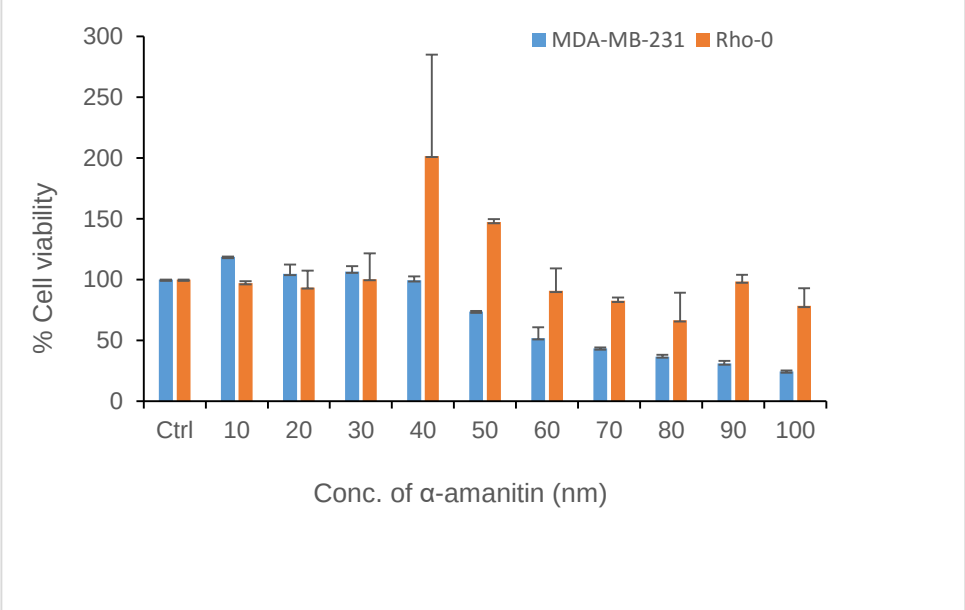

Figure 4: Cell viability was measured in parental MDA-MB-231 and Rho-0 cells after treatment of 24 hrs with α–amanitin using MTT dye.

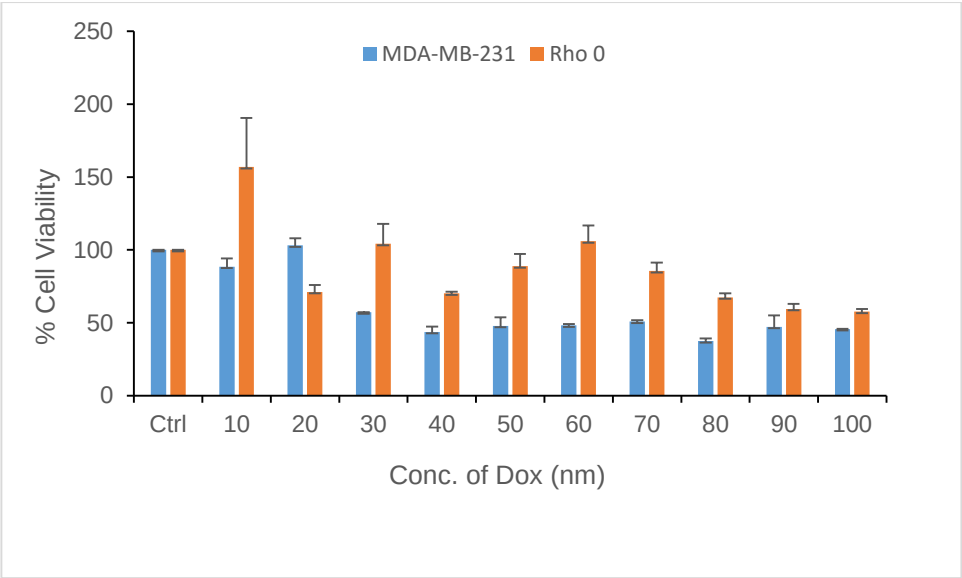

Figure 5: Cell viability was measured in parental MDA-MB-231 and Rho-0 cells after treatment of 24 hrs with Dox using MTT dye.

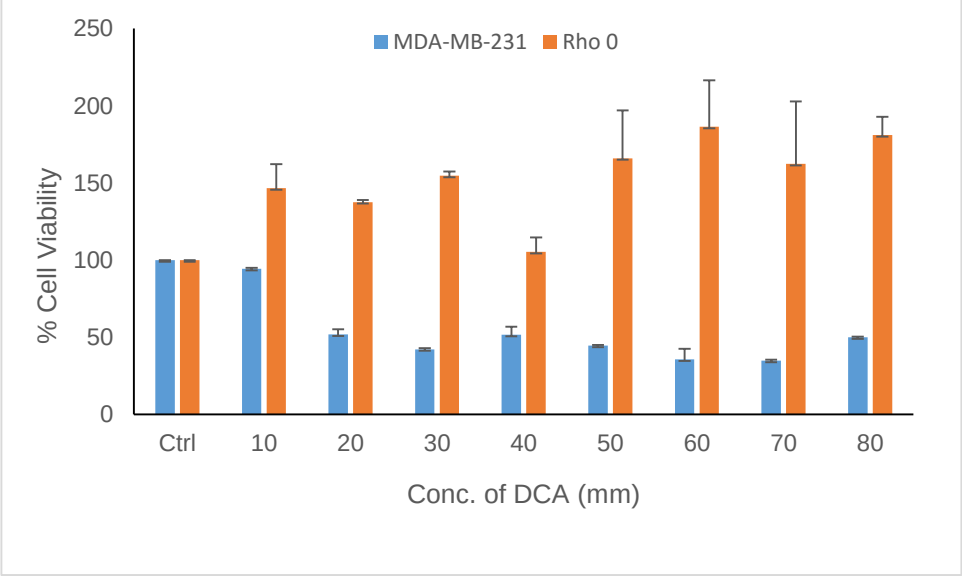

Figure 6: Cell viability was measured in parental MDA-MB-231 and Rho-0 cells after treatment of 24 hrs with DCA using MTT dye.

The results showed reduced cell death in Rho-0 cells as compared to MDA-MB-231 cancer cells, indicating the resistance to apoptosis in rho0 cells. With increase in the drug concentration, cell viability is reducing in case of MDA-Mb-231 cell, while cell viability in Rho-0 cells was comparatively higher. 50% cells have died at 60nm concentration of α-amanitin in case of MDA-MB-231 cells, while in Rho-0 cells minimum cell viability was found at the maximum concentration of α-amanitin (100nm). Similar results were found in case of Dox and DCA treated cells. Cell death was reduced in Rho-0 cells.

4.3 MEASUREMENT OF DIFFERENT PARAMETRES IN PARENTAL MDA-MB-231 CELLS AND DERIVED RHO-0 CELLS

4.3.1 Mitochondrial Membrane Potential Measurement using JC-1 Dye

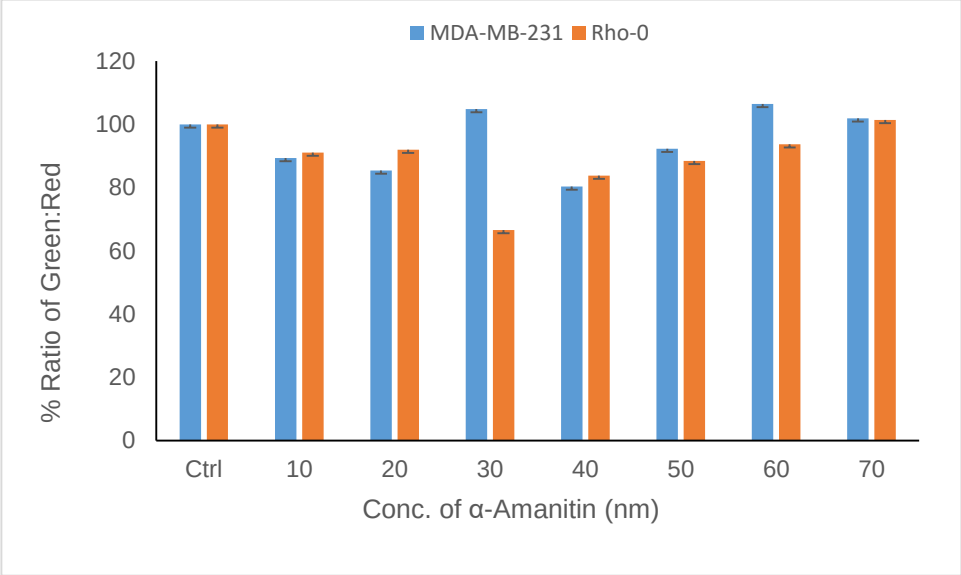

Figure 7: Mitochondrial membrane potential was measured in parental MDA-MB-231 and Rho-0 cells after treatment of 24 hrs with α–amanitin using JC-1 dye.

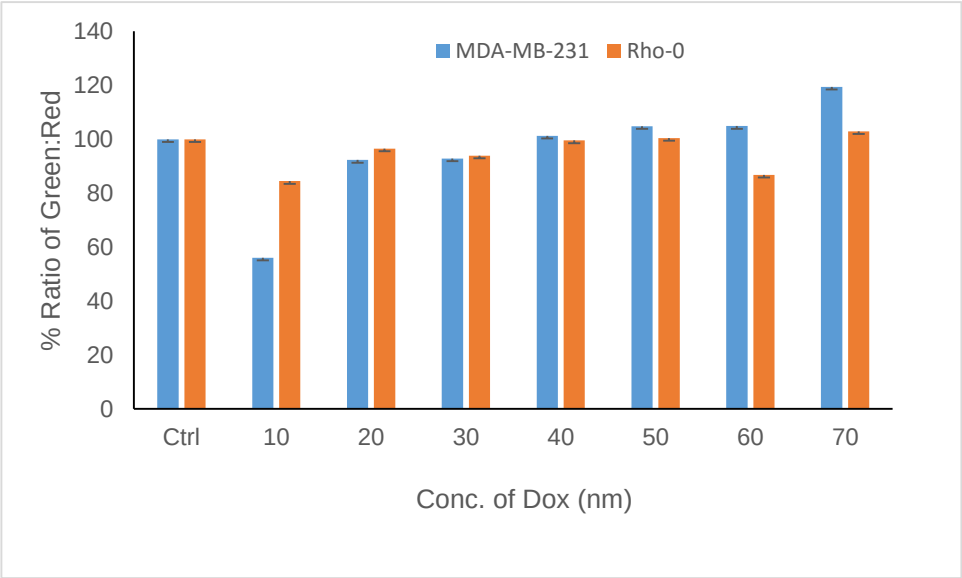

Figure 8: Mitochondrial membrane potential was measured in parental MDA-MB-231 and Rho-0 cells after treatment of 24 hrs with Dox using JC-1 dye.

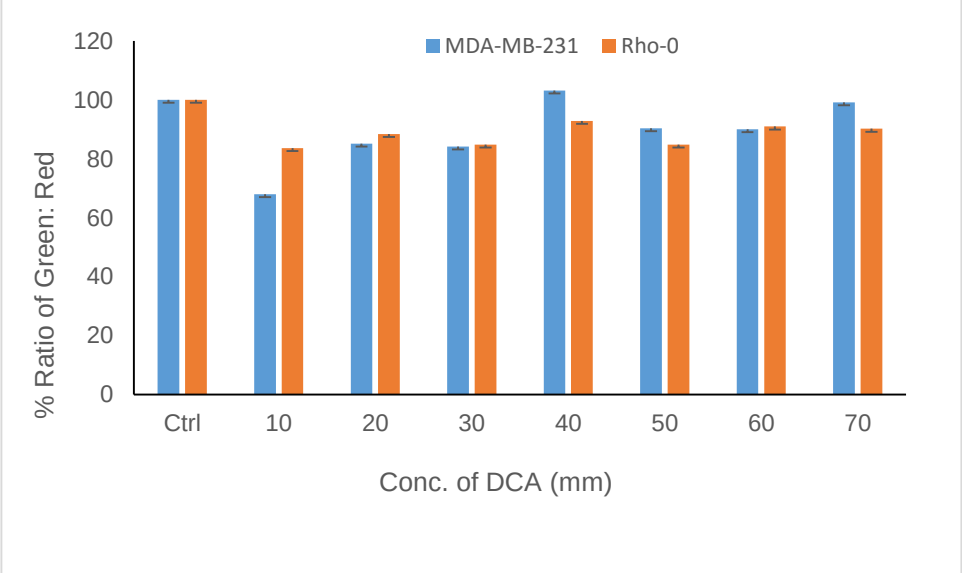

Figure 9: Mitochondrial membrane potential was measured in parental MDA-MB-231 and Rho-0 cells after treatment of 24 hrs with DCA using JC-1 dye.

The results, does not showed any significant variation in the mitochondrial membrane potential in Rho-0 cells and parental MDA-MB-231 cells after drug treatment in all the

three cases. So, we can conclude that most structures involved in the maintenance of $\Delta\Psi_m$ are controlled by nuclear genome, which is why normally present in Rho-0 cells. MMP in Rho-0 cells, might be maintained by electrogenic pumps other than those transporting H^+ ion.

The results also indicate that MMP is well preserved in Rho-0 Cells as both Rho-0 cells and MDA-MB-231 cells show similar mitochondrial membrane potential after the treatment with all three types of drugs (Depolarizing agent). Significant depolarization of mitochondrial membrane was not observed even at the 70 nm concentration of α-amanitin and Dox and 70mm concentration of DCA. Hence, showing a normal $\Delta\Psi_m$.

4.3.2 ROS Production Measurement using H_2DCFDA dye

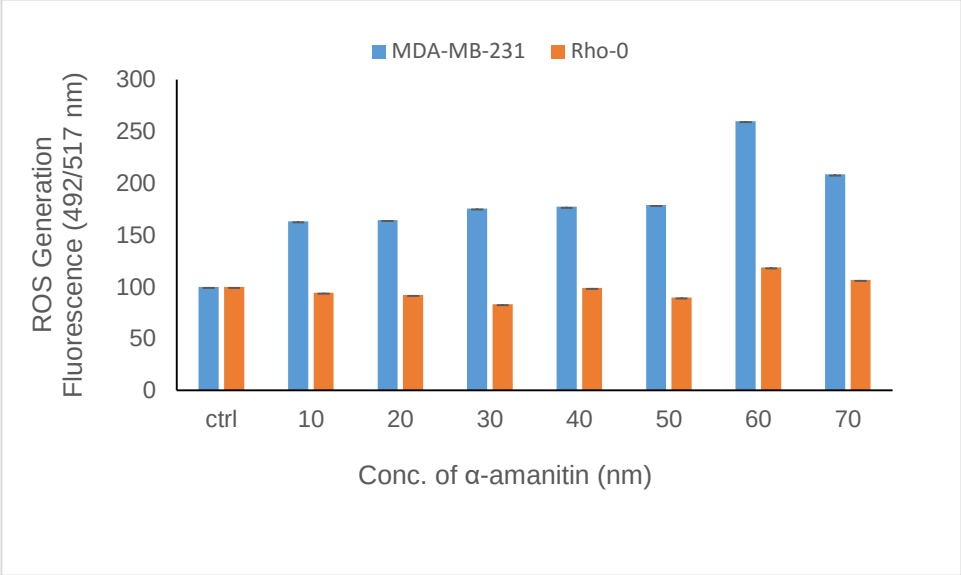

Figure 10: ROS production was measured in parental MDA-MB-231 and Rho-0 cells after treatment of 24 hrs with α-amanitin using H_2DCFDA dye.

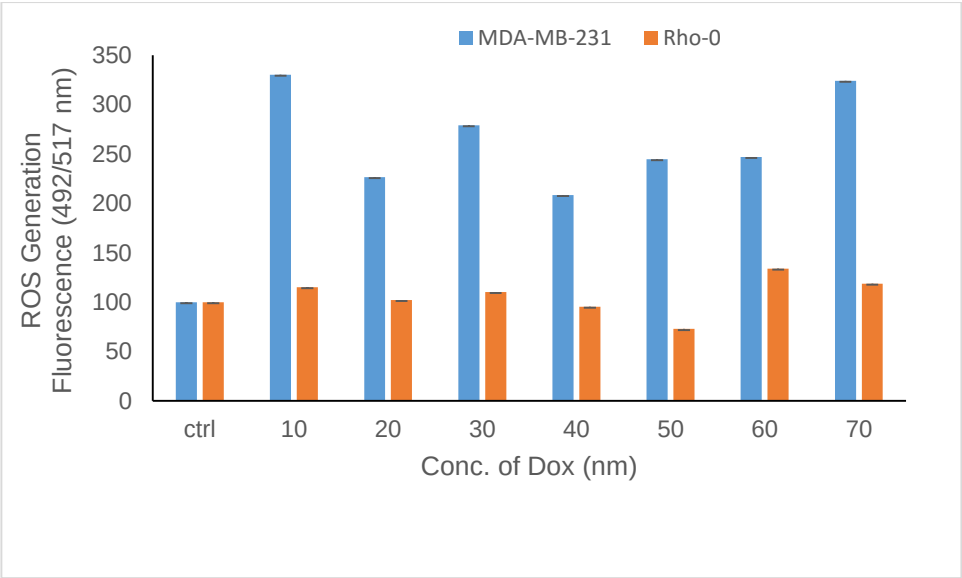

Figure 11: ROS production was measured in parental MDA-MB-231 and Rho-0 cells after treatment of 24 hrs with Dox using H_2DCFDA dye.

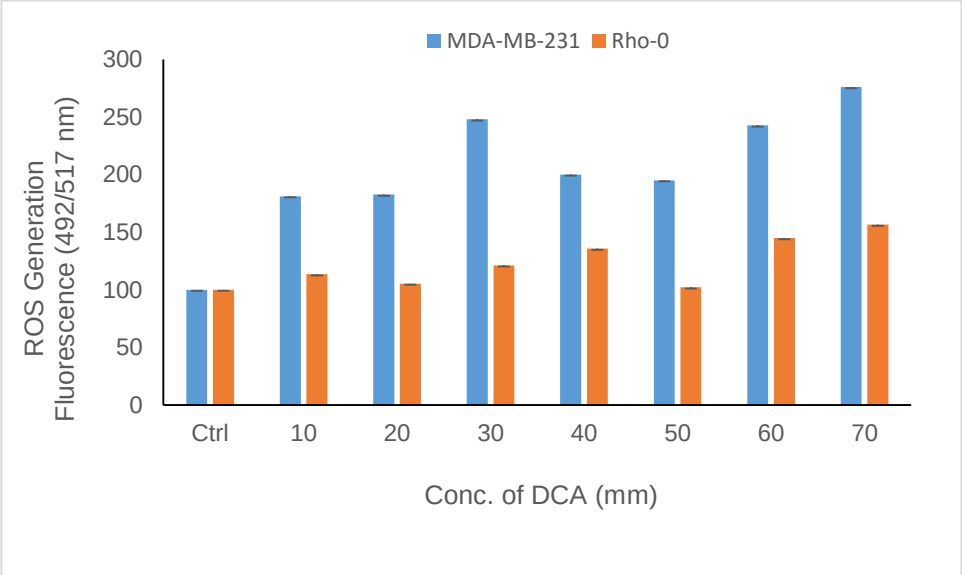

Figure 12: ROS production was measured in parental MDA-MB-231 and Rho-0 cells after treatment of 24 hrs with DCA using H_2DCFDA dye.

After treating both the cells with different drugs at different concentrations, ROS levels were measured using H2DCFDA dye and detected using microplate reader. The results indicated the reduced ROS production in Rho-0 cells as compared to MDA-MB-231 cells in all three cases.

Chapter V
Discussion

5. DISCUSSION

5.1 GENERATION OF RHO-0 CELLS

There are several compounds that can target the mitochondria for causing mutation, cell death or escaping the process of cell death, as in case of cancer. It becomes important to study the role of mtDNA in cancer and the effect of certain drugs on the viability of cells. An approach for studying target and effect of certain drugs on mtDNA, is to generate the Rho-0 cells which lack mtDNA. As the results showed the survivability of cells with depleted mtDNA, hence MDA-MB-231 cells are capable of becoming Rho-0 cells. Also, EtBr mediated method is an efficient method for generating Rho-0 cells, in the presence of uridine, which satisfies the energy demand of cells.

5.2 DRUG CYTOTOXICITY PARENTAL MDA-MB-231 AND RHO-0 CELLS

The results showed that drug cytotoxicity decreases in Rho-0 cells as compared to mda-mb-231 breast cancer cell line. This can be due to loss of function in respiratory chain components as apoptosis is dependent on intact mitochondrial function. mtDNA depleted cells could become resistant to apoptosis because of the increased scavenger capacity of GSH (Glutathione) and selection of MDR cells that hyper-express P-gp (P-glycoprotein).

5.3 ROS AND MMP MEASUREMENT IN PARENTAL MDA-MB-231 AND RHO-0 AND CELLS

Resistance to apoptosis in Rho-0 cells also indicates that mitochondria has central role in intrinsic pathway of apoptosis. As the results indicate that Rho-0 cells produce less ROS as compared to their parental MDA-MB-231 cell line. ROS are generated as a byproduct of ETC and associated with disruption in MMP and cell apoptosis. mtDNA depletion in Rho-0 cells may induce resistance to apoptosis by decreasing ROS production, which plays a role in oxidation and damage functional macromolecules such as protein and DNA.

Comparatively, no significant variation was observed in the MMP of Rho-0 cells and parental MDA-MB-231 cells after the drug treatment with three types of drugs. As per these results, we can conclude that most structures involved in the maintenance of $\Delta\Psi_m$ are controlled by nuclear genome. The MMP in Rho-0 cells could be maintained by electrogenic pumps other than those transporting H^+ ion. Also, we can confer that MMP is well preserved in Rho-0 cells, even at higher concentrations of all three types of drugs.

5.4 CONCLUSION

EtBr mediated Rho-0 cell generation is an efficient method for studying mitochondrial biogenesis and mitochondrial related disorders. Rho-0 cell is a powerful model to study the interaction of nucleus and mitochondria and mechanisms leading to human diseases because of mtDNA abnormalities. By comparing the cytotoxicity, MMP and ROS production in Rho-0 cells and their parental cells, it is helpful in enlightening the effect of depletion of mtDNA on cells and role of mtDNA in the metabolism of cell and extent of involvement of mtDNA in various cellular processes such as cell respiration, apoptosis, calcium signaling etc. As per the results, we can conclude that Rho-0 cells tend to be resistant against apoptosis, as cell death was observed to be reduced in Rho-0 cells as compared to their parental MDA-MB-231 cells. This could be due to the defected ETC and reduced ROS production in Rho-0 cells, indicating the possible crucial role of mitochondria in the process of apoptosis. However MMP is maintained in Rho-0 cells, indicating the interaction of nuclear genome with mtDNA in maintaining the MMP.

5.5 FUTURE PERSPECTIVE

Rho-0 cells can be a model to study drug targeting, drug cytotoxicity, role of mitochondria in cancer progression and several mitochondrion related disorders. Further detection and comparison of cellular ROS production, mitochondrial membrane potential, apoptotic cells, mitochondrial respiration (oxygen consumption measured) in various cell lines of interest can indicate the extent of mitochondrial activity involvement in cellular differentiation process and apoptosis. Survival and percentage of apoptosis

can infer the effect of depletion of mtDNA on stem cell-like properties. Comparing the difference in nuclear genes expression between cancer specific cell line and its Rho-0 derivative can provide insight to the involvement of nuclear genes in mitochondria-to-nuclear communication in that specific cancer. Rho-0 cells can be used in fusion experiments with patient's cytoplasts to study patient's mitochondria in an otherwise normal nuclear background of the acceptor cells. Also, if certain tumors rely on glycolysis for biosynthesis, bioenergetics and invasiveness, the therapeutics targeting these underlying pathways (such as inhibitors of key glycolytic enzymes) can show anti-tumor activity.

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Student Approval Form

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Degree	M.Sc. Life Sciences with specialization in Molecular Medicine
University	Central University of Punjab
Guide	Dr. Sandeep Singh
Project Title	Generation of Rho-0 cells using MDA-MB-231 cell line and measurement of Drug Cytotoxicity
Year of Award	2018

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(Bharti Sharma)

Department of Human Genetics and Molecular Medicine

School of Health Sciences

Central University of Punjab

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