TARGETING DRUG RESISTANCE IN CANCER CELLS BY THE ANTHELMINTHIC DRUG, PYRVINIUM PAMOATE

by

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THESIS

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KERALA, INDIA

2019

DECLARATION

I hereby declare that this thesis entitled **"TARGETING DRUG RESISTANCE IN CANCER CELLS BY THE ANTHELMINTHIC DRUG, PYRVINIUM PAMOATE"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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DEDICATED TO MY PARENTS

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LIST OF ABBREVIATIONS

%	: Percentage
ABCB1	: ATP Binding cassette Subfamily B Member 1
ABCC1	: ATP Binding cassette Subfamily C Member 1
ABCG2	: ATP Binding cassette Super family G Member 2
ALDH	: Aldehyde dehydrogenase
ALK	: Anaplastic Lymphoma Receptor Tyrosine Kinase
ATP	: Adenosine triphosphate
ß	: Beta
BBB	: Blood–Brain barriers
Bcl-2	: B- Cell lymphoma 2
BCRP	: Breast Cancer Resistance Protein
BRAF	: V-Raf Murine Sarcoma Viral Oncogene Homolog B1
BSA	: Bovine serum albumin
CCCP	: Carbonyl cyanide m- chlorophenyl hydrazine
Cm	: Centimetre
CSC	: Cancer stem cell
DMEM	: Dulbecco's Modified Eagle's Medium
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
DOX	: Doxorubicin
ECM	: Extracellular matrix

et al.	: et alia
EDTA	: Ethylenediaminetetraacetic acid
EGFR	: Epidermal Growth Factor Receptor
ETC	: Electron transport chain
FBS	: Fetal Bovine Serum
FDA	: Food and Drug Association
FRD	: Fumarate reductase
5-FU	: 5-fluorouracil
g	: Gram
GPX	: Glutathione peroxidase
GST	: Glutathione-S-Transferases
GSH	: Glutathione
HBSS	: Hanks' Balanced Salt Solution
HER2	: Human Epidermal Growth Factor Receptor 2
H_2O_2	: Hydrogen peroxide
HRP	: Horseradish peroxidase
HSP	: Heat shock protein
IC_{50}	: Half maximal inhibitory concentration
IgG	: Immunoglobulin G
KAU	: Kerala Agricultural University
kg	: Kilogram
L	: Litre
m	: Meter
M	: Molar
mg	: milligram
min	: Minute

ml	: Millilitre
MCF-7	: Michigan Cancer Foundation-7
MDR	: Multiple drug resistance
MDR1	: Multidrug resistance protein 1
Mm	: Millimeter
mM	: Millimolar
mRNA	: Messenger RNA
MRP 1	: Multidrug resistance- associated protein
MTT	: 3- (4,5- dimethyl thiazol-2-yl)- 2,5- diphenyl tetrazolium bromide
NaCl	: Sodium chloride
ng	: Nanogram
nm	: Nanometer
NADPH	: Nicotinamide adenine dinucleotide phosphate
NBD	: Nucleotide binding domain
OD	: Optical density
OXPHOS	: Oxidative phosphorylation
PAGE	: Poly Acrylamide Gel Electrophoresis
P-gp	: P- glycoprotein
PP	: Pyrvinium pamoate
PTEN	: Phosphatase and tensin homolog
PVDF	: Polyvinylidene fluoride
RIPA	: Radio- Immunoprecipitation Assay
ROS	: Reactive oxygen species
rpm	: Revolutions per minute
s	: Second

SDS	: Sodium dodecyl sulphate
S1P	: Signaling sphingosine-1 phosphate
TICs	: Tumor initiating cells
TM	: Trademark
TMD	: Transmembrane domain
TS	: Thymidylate synthase
V	: Volt
°C	: Degree Celsius
v/v	: volume/volume
w/v	: weight/volume
µg	: Microgram
µl	: Microlitre
µM	: Micromolar

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INTRODUCTION

1. INTRODUCTION

Cancer is a malignant and invasive tumor resulting from the uncontrolled division of cells. For quite a long time, the establishments of cancer treatments were surgery, chemotherapy and radiation treatment. Among these, chemotherapy is considered as the predominant mode of cancer treatment. Chemotherapy is the utilization of anticancer drugs intended to moderate or stop the development of rapidly dividing cancer cells in the body.

Chemotherapy includes one or multiple drugs. This treatment might be given with a corrective plan, or it might intend to delay life or to decrease symptoms. Chemotherapy guarantees early recuperate, counteractive action of backslide and delayed survival in patients. There are distinctive kinds of chemotherapy drugs and diverse methods for getting them. Four noteworthy classes are alkylating agents, antimetabolites, plant alkaloids and anti-tumor antibiotics. The status of chemotherapy of malignant tumor in man today depends on the genuine accomplishment of target impacts against a few types of scattered cancer by the activity of chemical substances.

The adequacy o f these medications inside cells relies upon individual components. The area, type, phase of malignancy and patient's wellbeing status decides the viability of treatments. Resistance to chemotherapy is accepted to cause treatment disappointment in more than 90 % of patients with metastatic malignant growth (SEER Cancer statistics review, 2014). There are numerous variables that influence drug sensitivity. These incorporate systems, for example, those that limit the measure of drug achieving the tumor and those influence the tumor microenvironment.

Drug resistance is a noteworthy issue that restricts the effectiveness of chemotherapy used to treat disease (Cree *et al.*, 2002). Cancer drug resistance is only the capacity of tumor cells to endure and develop regardless of chemotherapy. Tumors might be inherently impervious to chemotherapy preceding treatment. Nonetheless, resistance can likewise be gained amid treatment by tumors that are at first sensitive

to chemotherapy. Now and then patients end up impervious to one specific drug and stay touchy to other drugs (one-drug resistance); another group of patients may turn into show resistance against multiple drugs (Multiple drug resistance, MDR). Mostly, drug resistance emanates from the progressions that limit the accumulation of drugs within cells. The harmony between drug entry and efflux systems decide the medication reaction and obstruction inside cells.

Drug resistance can happen at numerous dimensions, including expression of drug efflux pumps, decreased drug uptake, alteration in drug targets, drug inactivation, alteration in cell cycle events, modifications in membrane lipids, deregulation of apoptosis and compartmentalization. Notwithstanding these key components, there are a few different factors outer to the tumor cell that can add to drug resistance. These incorporate the impact of tumor microenvironment (Murakami *et al.*, 2014), the presence of cancer stem cells (Malignant growth foundational cells) and autophagy (Carew *et al.*, 2007). Studies on these mechanisms have yielded essential data about how to dodge this resistance to improve cancer chemotherapy and have suggestions for pharmacokinetics of numerous regularly used chemotherapeutic drugs.

Overexpression of drug efflux pumps is the most well-known drug resistance mechanism in malignancy. These efflux pumps decline intracellular drug fixation and cause multidrug resistance (MDR). A few transmembrane transporter proteins are connected to protection from regularly utilized chemotherapeutics by advancing drug efflux. Most remarkably, ATP-binding cassette transporter group of transmembrane proteins direct the motion crosswise over plasma membrane. Altogether, there are 49 individuals from this protein family, however just three have been considered broadly in connection to multi-drug resistance (MDR). These are P-glycoprotein (Pgp/MDR1/ABCB1), MDR- associated protein 1 (MRP 1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2). Among these three, P-glycoprotein is the dominant efflux pump that adds to drug resistance and its over expression in tumor

cells cause MDR in numerous cancer types.

P-glycoprotein – mediated drug resistance is the first MDR component to be identified (Endicot and Ling, 1989). Pgp can distinguish and bind a substantial assortment of hydrophobic natural product drugs as they enter the plasma membrane. Elevated levels of P- glycoproteins pump out the chemotherapy drugs and reduce the drug availability within tumor cells. These medications incorporate a significant number of the ordinarily used plant alkaloids, for example, doxorubicin, daunorubicin, vinblastine and vincristine (Lee *et al.*, 1998). HSP family of proteins (chaperones) play an essential role in protein folding, stabilization, aggregation, activation, function and proteolytic degradation. HSP27, HSP70 and HSP90 are among the extensively studied and documented HSPs that are significantly related to cancer drug resistance. Since the tumour cells rely on HSP family proteins for their survival and uninterrupted function, the increased expression of these HSPs is considered a read-out for tumour progression and the evolution of cancer drug resistance. Since the expression of P-glycoproteins and HSP family proteins is an index to assess drug resistance, restraining them can be used as a potential strategy to manage drug resistance in cancer.

Pyrvinium pamoate is a cost effective anthelminthic drug approved by FDA. It is off patent and of late, it has been repurposed to demonstrate its capability to specially repress oxidative phosphorylation by focusing on mitochondrial respiration (Ishii *et al.*, 2012). Most tumor cells make use of glycolytic pathway to meet their energy needs. Although the glycolytic pathway is less efficient in the synthesis of energy per glucose molecule compared to aerobic respiration, under hypoxic conditions during limitation of oxygen it helps tumor cells survive and divide. Synthesis of macromolecules for cell division necessitates the shuttling of intermediates of glycolysis via the Pentose Phosphate Pathway for fatty acid synthesis (NADPH), synthesis of nucleotides (ribose-5-phosphate) and amino acids (erythrose-4-phosphate) (Markus *et al.*, 2014). This phenomenon called 'Warburg Effect' has been

well studied in cancer and the mitochondria of cancer cells were thought to be nonfunctional (Warburg O., 1956). Hence most anticancer drugs target glycolysis and induce cell death. Although bulk of tumour cells perish, some cancer cells survive the drug treatment by shifting to oxidative phosphorylation by gaining mitochondrial function (metabolic plasticity). It is well studied that such cells which gain mitochondrial function divide slowly, evade therapy and spawn drug resistance and relapse. Hence, targeting both glycolytic and oxidative phosphorylation pathways will preferentially choke the tumors of their ATP supply and induce cell death. Due to metabolic rewiring in drug resistant cells, these cells are predominantly dependent on mitochondrial respiration for survival and particularly vulnerable to drugs targeting mitochondrial respiration. Considering the antitumor action of pyrvinium pamoate, it is basic to direct more research on its viability to target drug resistance in different malignant growth cells and analyze its capability to help existing chemotherapeutic regimen either as a single agent or in combination with other chemotherapeutic agents.

In this context, the present study is carried out to evaluate the potential of pyrvinium pamoate in sensitizing various drug resistant tumor cells in breast and glioblastoma by measuring the relative levels of P- glycoproteins and HSP family of proteins in drug resistant and control cell lines. The ability of pyrvinium pamoate to preferentially target mitochondrial respiration thereby its ability to target drug resistance either as a single agent or in combination was evaluated. The mitochondrial oxygen consumption serves as an index for mitochondrial oxidative phosphorylation. If treatment with pyrvinium pamoate significantly alters mitochondrial oxygen consumption, its ability to target mitochondrial respiration will be proven beyond doubt. It is also known that metabolic rewiring from addiction to glycolysis (Warburg effect) to preferential use of mitochondrial respiration endows cancer cells to overcome chemotherapy and the outcome is relapse. An agent that preferentially targets mitochondrial respiration will be ideally suited to eliminate such metabolically rewired drug resistant cells which lead to relapse and therapy failure.

Reactive oxygen species (ROS) are mainly generated in the mitochondria and are the result of electron flow through the protein complexes of the inner mitochondrial membrane in electron transport chain. Super oxide is produced from mitochondrial respiratory complex I into the mitochondrial matrix which is dismutated to hydrogen peroxide (H_2O_2) by the enzyme super oxide dismutase 2 (SOD2). The H_2O_2 is fully reduced to water by the enzyme, glutathione peroxidase (GPX) (Li *et al.,* 2013). The second site of super oxide generation is mitochondrial complex III which releases super oxide to matrix and inter membrane space. Cancer cells contain high amounts of ROS and antioxidants compared to normal cells and are very susceptible to minor changes in ROS levels. Hence increased generation of mitochondrial reactive oxygen species can induce oxidative cell death preferentially in cancer cells (Sullivan *et al.*, 2014). Hence the ability of pyrvinium pamoate to generate mitochondrial reactive oxygen species was also analyzed.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 CANCER: THE STATUS QUO

Cancer is a second driving reason for death universally and caused 9.6 million deaths in 2018. Internationally 1 of every 6 deaths is attributed to cancer. Roughly 70% of deaths from cancer happen in economically week nations. The most well-known tumours are found in lung, breast, colorectal, prostate, skin and stomach. Despite the existence of a variety of modalities to treat cancer, chemotherapy remains the mainstay remedial methodology for a large variety of the disease types. Currently the achievement rate of chemotherapy is consistently hindered because of the enlistment of drug resistance inside tumor cells. Drug resistance components are widely examined and many systems like targeted or combination treatments are developed to sidestep drug resistance. The examinations which focus on the pharmacokinetic and pharmacodynamic properties have cleared the way to discover the inception of drug resistance in malignancy. Generous information in biochemical and molecular aspects of various malignancies have likewise prompted some remarkable disclosures in the area of cancer drug resistance.

2.2 DRUG RESISTANCE IN CANCER

Drug resistance is a major issue that constrains the viability of chemotherapy used to treat cancer (Cree *et al*., 2002). Cancer drug resistance is the capacity of disease cells to endure and develop in spite of hostile treatment. Tumors might be naturally impervious to chemotherapy preceding treatment. Notwithstanding, drug resistance can likewise be developed amid treatment by tumors that are at first sensitive to chemotherapy. At times patients can end up impervious to one explicit medication and stay sensitized to different medications (one- drug resistance); another gathering of patients may turn insensitive to numerous medications (Multiple drug resistance, MDR). For the most part, drug resistance can result from the progressions that limit

accumulation of drugs inside cells. The harmony between drug entry and exit mechanisms decide the drug response within cells.

2.3 MECHANISMS OF DRUG RESISTANCE

Drug resistance can happen at numerous dimensions, including expression of drug efflux pumps, decreased drug uptake, alteration in drug targets, drug inactivation, alteration in cell cycle events, modifications in membrane lipids, deregulation of apoptosis and compartmentalization. Other than these key components, there are a few different variables beyond the tumor cell that can add to resistance. These include tumour microenvironment (Murakami *et al.*, 2014), tumour initiating cells (Cancer stem cells) and autophagy (Carew *et al.*, 2007). Exhaustive studies on these components have yielded critical data about how to go around this protection to improve cancer chemotherapy and have suggestions for pharmacokinetics of numerous commonly used chemotherapeutic drugs.

2.3.1 Expression of drug efflux pumps

Changes in the activity of drug efflux pumps is the most widely recognized drug resistance mechanism in cancer. These efflux pumps decline intracellular drug fixation and cause multidrug resistance (MDR). A few transmembrane transporter proteins are connected to protection from normally utilized chemotherapeutics by advancing drug efflux. Most remarkably, ATP-binding cassette transporter family of transmembrane proteins control the transition crossover plasma membrane. Altogether, there are 49 members from this protein family, yet just three have been considered widely in connection to multiple drug resistance (MDR). These are P-glycoprotein (Pgp/MDR1/ABCB1), MDR- associated protein 1 (MRP 1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2). Among these three, P-glycoprotein is the significant efflux pump that adds to resistance and its over expression in tumour cells cause MDR in numerous cancer types.

P-glycoprotein – mediated drug resistance is the first MDR mechanism to be described (Endicot and Ling, 1989). Pgp can distinguish and tie a vast assortment of hydrophobic drugs tranquilizes as they enter the plasma film. These medications incorporate a significant number of the regularly utilized plant alkaloids, including doxorubicin, daunorubicin, vinblastine and vincristine (Lee *et al*., 1998). Most of these medications results in actuation of one of the ATP-restricting areas, and the hydrolysis of ATP causes a noteworthy change in the state of Pgp which results in arrival of the medication in to the extracellular space (Ramachandra *et al*., 1998). Hydrolysis of a second particle of ATP is expected to reestablish the transporter to its unique state with the goal that it can rehash the cycle of medication authoritative and discharge (Sauna *et al*., 2001).

2.3.2 Reduction in drug uptake

Diminished medication take-up is another approach to reduce drug accumulation in malignancy cells. Medications are transported into the cells by means of a few courses which incorporate dissemination over the plasma film, stacking of the medications on specific receptors and either receptor mediated or non- specific endocytosis (Gottesman, 2002). Improvement of drug resistance in some cancer cells could result from transformations that change or square the receptors. It is additionally realized that endocytosis, and for the most part receptor-mediated endocytosis, assumes imperative roles in the vehicle of specific drugs into the cells and inadequate endocytic process causes drug resistance. (Gottesman, 2002).

2.3.3 Modifications in drug targets

All medications have an objective. Alterations in drug target, for example, transformations or change in articulation level can influence the medication reaction. Some chemotherapeutic drugs target thymidylate synthase (TS), topoisomerase 11α and epidermal development factor receptor are for the most part creating opposition to medication amid chemotherapy. 5-fluorouracil (5-FU)- obstruction in colon malignant

growth is made by the adjustment in articulation dimensions of TS. The natural cell thymidylate synthase substance might be a vital prognostic parameter for chemotherapy with 5FU-containing regimens.

2.3.4 Drug inactivation

In this sort of drug resistance, drugs become detoxified by the enzymatic exercises of Glutathione-S-Transferases (GST). Glutathione (GSH), a critical cancer prevention agent, averts oxidative pressure, and keeps redox homeostasis stable in cells (Gawryluk *et al.*, 2011). In medication digestion, GSH is utilized as a cofactor by GST compound framework giving the arrangement of GSH-tranquilize conjugates. In the cells impervious to alkylating specialists, for example, cyclophosphamide, doxorubicin, melphalan and chlorambucil, it was discovered that GSH levels are higher than that of sensitive partners. In these cells, GSH frame conjugates with alkylating operators by the movement of GST, and this results in detoxification of alkylating specialists, which causes drug resistance in cells (O'Brien and Tew, 1996). Despite the fact that an accurate system is not known yet, GSH integrating catalysts could likewise be critical in the improvement of medication opposition. Furthermore, multidrug resistance in the cells overexpressing P-gp could likewise be identified with the GSH framework (Townsend *et al.*, 2003).

2.3.5 Modification in cell cycle events

An imperative component of cancer cells is that uncontrolled cell multiplication emerges from deformities all through the cell cycle movement at G1, S, G2 and mitotic stages. Cell cycle checkpoints, including a system of protein kinase flagging pathways, shield the cells from DNA damage initiated by chemotherapeutic operators and give the cells suitable time to fix the breaks (Sancar *et al.*, 2004). Consequently, deregulation of cell cycle checkpoints could cause carcinogenesis and the improvement of medication obstruction. Capture of cell cycle movement at G2 stage by and large offers the cells a chance to ensure their feasibility after medication

treatment and this capture requires the actuation of DNA damage checkpoint parts. Cell cycle-related drug resistance is the most normal in blend treatments in which the right off the bat given medication could influence the cell cycle and subsequently, the following operator turns out to be less successful (Shah and Schwartz, 2001).

2.3.6 Modification in membrane lipids

Lipid profiles of solid and destructive tissues are unique. The guideline of phospholipids, cholesterol and sphingolipids influences the reaction of cells against chemotherapy. Among these, Sphingolipids assume a noteworthy job in creating drug opposition. Disappointment of chemotherapy in colon and prostate diseases are predominantly because of this resistance. The fate of a cell is dictated by the harmony among ceramide and sphingosine-1 phosphate (S1P) signaling. This parity is called Ceramide-S1P rheostat. There isn't really a quantitative proportion for the measure of lipids however a natural metabolic harmony between these two flagging arms of sphingolipids with inverse capacity. A move towards ceramide because of chemotherapy (camptothecin) drives tumor cells to experience cell passing and hostile to multiplication (Sanchez *et al*, 2008). Ceramide incites ER stress and upregulates apoptosis pathway. At the point when the balance moves towards S1P collection, cells apply hostile to apoptosis and exhibit drug opposition (Ponnusamy *et al*., 2010). Such changes in membrane lipids and drug resistance were likewise detailed in numerous different kinds of malignant growth including kidney, breast, lung, uterus and colon (Visentin *et al*., 2006).

2.3.7 Inhibition of apoptosis

Acceptance of apoptosis is known as an extreme point of anticancer operators. In apoptosis, DNA laddering, chromosome shearing, caspase cleavage and lastly cell shrinkage happen, separately. A system of qualities and proteins are engaged with the association of apoptotic occasions or the upkeep of cell survival. p53, Bcl-2 family qualities and PTEN are the significant players in apoptosis (Holohan *et al*., 2013). Any

variations from the norm in these qualities or proteins lead to natural medication opposition by repressing apoptosis.

2.3.8 Compartmentalization

Sequestration of the medications in cell compartments is a significant mechanism for anticancer drug resistance. Although little is thought about intracellular medication limitation, an anticancer drug would penetrate the cell layer or not is a significant certainty in the restriction in the cell (Zhitomirsky *et al.*, 2017). In the event that an atom can't go through the film, it is endocytosed and its limitation is restricted to lysosomes. This particle can likewise be translocated into the cell by transporters. As cell compartments have diverse exercises, they are sorted out with various highlights: every organelle has an alternate pH in the lumen, distinctive organization of lipids and furthermore unique proteins. These components impact the restriction of medications in various cell compartments (Preziosy *et al.*, 2003). A powerless base to an acidic domain, known as pH segment, assumes a key job in restriction of a drug in various compartments.

2.3.9 Tumour microenvironment

In solid tumors, the microenvironment comprises of the extracellular matrix (ECM,) malignancy related fibroblasts, insusceptible and provocative cells and veins (Shiao *et al.*, 2011). In hematological malignancies the microenvironment is made out of bone marrow stromal cells, bone marrow endothelial cells, osteoclasts, osteoblasts, macrophages and T cells among others (Bhatia *et al.*, 2012). The insurance given by the microenvironment gives shelter to malignant growth cells from cytotoxic operators, in this way enabling them to sidestep apoptosis and to create obtained resistance prompting illness backslide.

2.3.10 Caner stem cells (Tumour initiating cells)

Two current models of carcinogenesis are the stochastic model, which suggests that each changed cell inside a tumor has tumorigenic potential, and the cancer stem cell (CSC) model, which recommends that just a little subset of cells can offer ascent to another tumor. The CSC model has crucial implications for cancer therapeutics and drug resistance. CSCs speak to a significant target populace of anticancer therapeutics as their survival following treatment is almost certain to result in disease relapse. Cancer stem cells are accepted to be very impervious to regular chemotherapies attributable to different urgent highlights, including high expression of ATP-binding cassette (ABC) transporter proteins (Resetkova *et al.*, 2010), aldehyde dehydrogenase (ALDH) action, expression of anti-apoptotic proteins (Todaro *et al.*, 2010). The CSC model likewise has suggestions for the advancement of focused treatment.

However, this research work is centered around drug efflux pumps and explicitly proteins like P-glycoproteins (P-gp) and HSP chaperones that handle the efflux of accumulated drugs inside tumour cells. In this work, we propose a more extensive and elective point of view that sets the phase for a future stage in adjusting drug opposition regarding the treatment of cancer. Literature survey of around 400 cancers studied showed that expression was broad both in inherently drug resistant cancers, for example, colon, pancreatic, liver, adrenocortical and kidney tumors, and in certain malignancies that acquired resistance, for example, leukemias, lymphomas, breast cancer and neuroblastoma. Numerous cancers did not seem to express P-gp mRNA at perceptible dimensions and along these lines endeavors to repress P-gp and turn around obstruction in these tumours were probably not going to succeed. Overexpression of P-gp efflux pumps for the most part decline the cytotoxicity of a wide range of anti- tumour drugs including anthracyclines (e.g. DOX), vinca alkaloids (e.g. vincristine), podophyllotoxins (e.g. etoposide) and taxanes (e.g. taxols). Different pharmacotherapeutic agents that influence central nervous system, cardiovascular framework and antimicrobials are substrates to this efflux proteins. Sub-atomic

chaperones, for example, HSP27, HSP70 and HSP90 likewise have raised expression in a scope of malignancies like breast, endometrial and leukemia. Expanded dimensions of HSP90 help the fast advancement of new treatment safe phenotypes by allowing new qualities to emerge inside tumors. A few mixes have experienced the clinical assessment for the treatment of disease, however no HSP90 inhibitor has been endorsed by Food and Drug Administration (FDA) because of poisonous quality and dosing challenges seen amid the preliminaries. All things considered, HSPs remain a key challenge in the research related to reverse anti- cancer drug resistance.

2.4 P- GLYCOPROTEIN MEDIATED DRUG RESISTANCE

P-glycoprotein - mediated drug resistance is the first MDR mechanism to be described (Endicot and Ling, 1989). Permeability glycoprotein (P-gp), otherwise called multidrug resistance protein (MDR) is found along the gastrointestinal tract (GIT) (Thorn *et al.*, 2005), including the small intestine as essential site for the epithelial assimilation of numerous orally controlled drugs (Wacher *et al.*, 2001). Regularly, P-gp is limited at the plasma layer of colon, jejunum, bile canaliculi, renal tubular cells, placenta, the luminal surface of capillary endothelial cells, testes, pancreas and blood–brain barriers (BBB) (Zho *et al.*, 2008). P-gps have a role in the typical discharge of metabolites. P-gp additionally initiates expression of CYP3A4 (Schuetz *et al.*, 1996) that thus may deactivate some anticancer medications. It has been demonstrated that P-gp reduces the oral bioavailability of some anticancer drugs (Sparreboom *et al.*, 1997). Concomitant administration of a portion of the antineoplastic agents prompts the overexpression of P-gp that outcomes in bioavailability decrease of a few these operators, for example Imatinib (Burger *et al.*, 2004). P-gp expression, in the gut, is a subject of interindividual variety because of either hereditary polymorphism or pathologic condition (Cascorbi *et al.*, 2001) thus varies the bioavailability of a few antineoplastic drugs for example paclitaxel (Sparreboom *et al.*, 1997). In drug resistant cell lines, P-gp is confined in the Golgi apparatus and the rough endoplasmic reticulum (Bendayan and Munteanu, 2006).

Likewise, it is communicated in mitochondrial cristae (Shen *et al.*, 2012) to secure the accumulation of mitochondria or prevent nuclear accumulation by expression of P-gp at the nuclear envelope (Solazzo *et al.*, 2006).

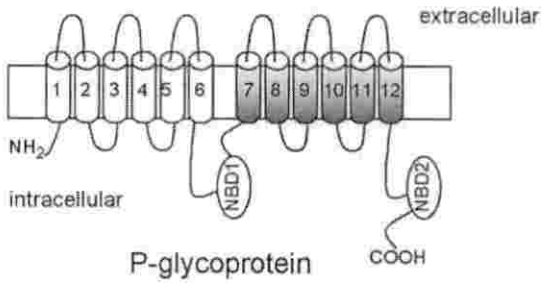

Fig. 1. Structure of P-gp

Pgp, the human MDR1 gene product is a 170,000dalton – molecular weight phospho-glycoprotein comprising of 2 homologous parts with a transmembrane domain (TMD) and nucleotide binding domain (NBD) [Jain *et al.*, 2014] (Fig. 1). P-gp can distinguish and tie an expansive assortment of hydrophobic drugs as they enter the plasma film. These medications incorporate a considerable lot of the normally utilized plant alkaloids, for example, doxorubicin, daunorubicin, vinblastine and vincristine (Lee *et al.*, 1998). Authoritative of these drugs result in enactment of one of the ATP- binding domains, and the hydrolysis of ATP causes a noteworthy change in the state of P-gp which results in efflux of the drug in to the extracellular space (Ramachandra *et al.*, 1998). Hydrolysis of a second particle of ATP is expected to reestablish the transporter to its unique state with the goal that it can rehash the cycle of drug binding and discharge (Sauna *et al.*, 2001).

2.5 HSP CHAPERONES IN TUMOURIGENESIS

Stress or heat shock proteins (HSPs) are a lot of profoundly saved proteins whose expression is instigated in light of a wide assortment of physiological and natural stress including anti-cancer chemotherapy, along these lines enabling the cell to survive deadly conditions. Mammalian HSPs have been grouped fundamentally in four

families as per their atomic weight: HSP90, HSP70, HSP60 and little HSPs (15– 30 kDa) that incorporate HSP27. Relatives of HSPs are communicated either constitutively or controlled inductively, and are available in various subcellular compartments. High atomic weight HSPs are ATP-subordinate chaperones, though little HSPs act in an ATP-autonomous design. The most concentrated stress inducible HSPs are HSP90, HSP70 and HSP27.

The idea that HSPs are effectively engaged with the tumorigenesis has driven to consider the intracellular and extracellular confinement of these chaperones. The confinement of HSP60 to the cell film appears to be unprecedented in ordinary cells (Cappello *et al.,* 2009). HSP60 is available in lipid rafts, which are subdomains of the plasma membrane, containing high concentrations of cholesterol and glycosphingolipids. HSP60 additionally happens in exosomes delivered and discharged by human tumor cells through a functioning emission mechanism, freely of cell passing, to be specific unrelated to apoptosis (Merendino *et al.*, 2010). Curiously, the Golgi apparatus likewise, takes an interest in exosome arrangement and HSP60 emission in tumor cells (Campanella *et al.*, 2012). This information may mirror a general physiological marvel, happening in numerous tumors. Different gatherings have shown that HSP70 is every now and again likewise confined to cell membrane in an assortment of human tumors, however not in the relating typical tissues (Multhoff *et al.*, 1995). This information is conceivably significant for remedial antitumor resistance, since film HSPs are effectively reachable focuses for explicit drugs.

Over the most recent two decades, numerous reports share the idea that chaperones are ensnared in the pathogenesis of a scope of tumours, being associated with different metabolic and atomic systems of carcinogenic cells, for example, cell multiplication, obtrusiveness, enlistment of neoangiogenesis, metastasization and acceptance of invulnerable resistance (Cappello *et al.*, 2009). Subsequently, sub-atomic chaperones are valuable for the cancer cell and, in this manner are pathogenic for the organism. For instance, HSP60 favours the survival of particular kinds of

tumors, and at times, it might even be basic for tumor-cell development. For example, raised dimensions of this protein in tumor cells have been connected to: a) the capacity to endure apoptotic improvements; b) loss of replicative senescence: and c), uncontrolled multiplication and neoplastic change (Cappello *et al.*, 2006). In like manner, HSP90 regulates late-stage maturation, activation, and stability of a range of 'client' proteins, such as Human Epidermal Growth Factor Receptor 2 (HER2), Anaplastic Lymphoma Receptor Tyrosine Kinase (ALK), Epidermal Growth Factor Receptor (EGFR) and V-Raf Murine Sarcoma Viral Oncogene Homolog B1 (BRAF), some of which are involved in signal transduction and other key pathways that are important for malignancy. The level of expression and dimensions of HSPs have been observed to be modified in malignancy cells in which it tends to be seen that as a general rule, the dimensions of HSPs are raised in tumors by examination with the ordinary tissue/cell partners. For instance, more elevated amounts of HSP60 were found in the 'adenoma-to-carcinoma succession' of the large intestine, in the 'dysplasia-to-carcinoma arrangement' of the uterine ectocervix, and in the prostate carcinogenesis. Conversely, lower levels were identified in tongue, bronchial and urinary bladder (Cappello *et al.*, 2006) malignant growth. HSP27, HSP70, and HSP90 have likewise been discovered expanded in a few sorts of malignancy in which they may support tumorigenesis by repressing modified cell demise and senescence. For instance, HSP70 and HSP90 tie tumor silencer proteins, for example, p53 and HER2, and in this way permit boundless cell development or expanded protection from chemotherapy in breast cancer (Vargas- Roig *et al.*, 1998). Tumor cells that overexpress HSPs may demonstrate an expanded inclination to attack their microenvironment and to spread to adjacent organs, delivering metastasis. For instance, a positive relationship was found between expanded expression of HSP27 and HSP70 and tumor-invasiveness (Ciocca *et al.*, 2005). HSPs may likewise impact tumor neoangiogenesis. For example, HSP90 balances out vascular endothelial development factor and nitric oxide synthetase in endothelial cells, and HSP27

intervenes endothelial cell versatility and expansion (Keezer *et al.*, 2003). Large amounts of HSPs may relate with visualization of cancer progression. Also, elevated amounts of HSP27 are connected with poor forecast in ovarian malignancy and HSP60 overexpression is corresponded with tumor movement and with a poor anticipation in large bowel and in prostate carcinomas (Cappello *et al.*, 2003). Raised dimensions of HSP70 are related with poor visualization in breast (Lazarius *et al.*, 1997) and endometrial cancers; high HSP90 articulation is related with poor forecast in invasive ductal breast carcinoma and in endometrial disease (Nanbu *et al.*, 1996).

2.6 PYRVINIUM PAMOATE AND ANTI-CANCER DRUG RESISTANCE

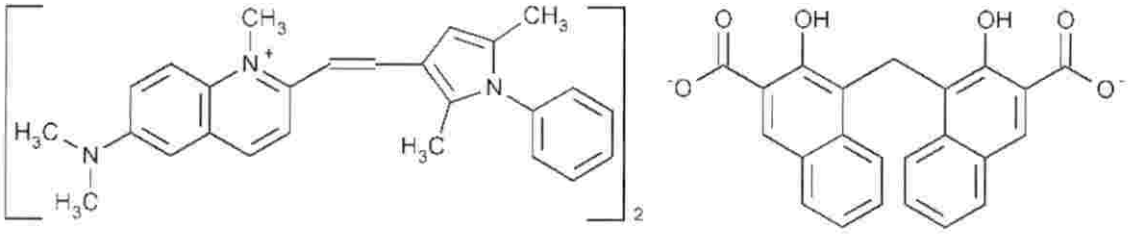

Fig. 2: Structure of pyrvinium pamoate

Chemical name: Pyrvinium pamoate (PP)

Molecular formula: C_{75} H_{70} N_6 O_6

Molecular weight: 1151.4 g/mol

Drug repurposing is reprofiling a few classes of FDA-approved agents for the treatment of malignancy. The chemo-drugs now being used regularly focus on the glycolysis pathway that cancer cells utilize to meet their energy needs and macromolecule production, despite availability (Warburg effect). Consequently, focusing on glycolysis under these conditions has been shown to initiate cell death be that as it may, most cells move to oxidative phosphorylation by the increase of mitochondrial respiration. This sort of shift is termed as metabolic plasticity and subsequently targeting both glycolysis and oxidative phosphorylation pathways will preferentially choke the tumors of their ATP supply and actuate cell demise.

Strikingly, a few classes of FDA-affirmed antibiotics hinder mitochondrial biogenesis and there by counteracts the clonal development and survival of tumor cells.

Pyrvinium is a quinoline derived cyanine dye (Fig. 2), which is an FDA-approved anthelmintic drug that has been accustomed to regarding pinworms just as strongyloidiasis in people. It was first affirmed by FDA in1955 for the treatment of enterobiasis and is referred to go about as an inhibitor of mitochondrial oxidative phosphorylation (OXPHOS) under both normoxic and hypoxic conditions. Different types of pyrvinium can be set up with various anions. The efficacy of pyrvinium pamoate is already approved in destroying tumor cells by hindering the mitochondrial function. The inhibitory impact of PP is thought to result from the hindrance of mitochondrial fumarate reductase (FRD) action (Tomitsuka *et al*, 2010). PP has been shown to especially hinder OXPHOS by focusing on both complex I and complex II in hypoxic and glucose starved conditions. The prime fascination of PP is that its non-lethality towards normal cells that likely reduces the side effects of anti-cancer therapy. There is some conspicuous information with respect to the effect of PP on enzyme activities in normoxic ETC. At that point, PP has likewise an inhibitory impact on tumor-sphere formation in a wide variety of cell lines got from various tumor types (Xu *et a*l., 2016) in nano-molar range. This drug conditionally restrains the tumor (Rebecca sheep, 2015). PP was at first tried over the scope of 1nM to 500 nM and inferred that 250 nM and 500 nM were the best measurements for tumor hindrance in MCF7 and T47D cells, two ordinarily used ER (+) breast cancer cell lines. Different confirmations which uncovers the counter tumor limit of PP in aggressive breast cancer focusing on WNT signaling assume a key job in self renewal of tumor initiating cells (TICs). Combining those accessible confirmations for the viability of against tumor properties of PP through OXPHOS and WNT hindrance, the information focuses to another way which correlates the anti-cancer drug resistance and effect of PP.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Targeting drug resistance in cancer cells by the anthelminthic drug, pyrvinium pamoate" was carried out at Department of Plant Biotechnology, College of Agriculture, Vellayani during 2018-2019. The final part of project was completed at Department of Biochemistry, Sree Chithira Thirunal Institute of Medical Sciences and Technology (SCTIMST), Thiruvananthapuram. Details pertaining to the experimental material and procedures used in the study are elaborated in this chapter.

3.1 REAGENTS AND ANTIBODIES

Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Trypsin-EDTA (0.25%) were purchased from Sigma Aldrich, St. Louis, MO, USA. Antibodies against P- gp (MDR1), HSP90 were from G- Biosciences, Page Avenue St. Louis, MO, USA. Antibodies against ß- actin, HRP- conjugated anti- rabbit IgG and anti-mouse IgG were procured from the same Sigma Aldrich, St. Louis, MO, USA. All other reagents were purchased from Sigma Aldrich, St. Louis, MO, USA.

3.2 DRUGS

5-Fluorourail (5-FU), doxorubicin and pyrvinium pamoate (PP) were purchased from Sigma Aldrich, St. Louis, MO, USA.

3.3 CELL LINE INFORMATION

Human breast cancer cell line, MCF-7 and human glioblastoma cell line (U- 251 MG) were obtained from NCCS, Pune.

3.4 CELL CULTURE AND MAINTENANCE

MCF-7 and U-251 MG cells were grown as monolayer culture in DMEM containing 10% FBS (Appendix I) in a humidified atmosphere of 5% CO_2 at 37^0 C. For all experiments, DMEM containing 10% FBS was used or else specified.

For maintaining the culture, medium was changed every 2-3 days, depending on rate of growth. Cultures were split at confluency to 1:3 ratios. The cells were removed from the plate by using 0.25% Trypsin-EDTA solution. The cells were centrifuged at 2500 rpm for 5 minutes and the supernatant was removed. Then, the cells were resuspended in 1.0 ml of freezing medium (Appendix I) and stored in liquid nitrogen (-196^0 C).

3.5 GENERATION OF DRUG RESISTANT CELL LINES

Drug resistant cell cultures were established by treating with sub lethal doses of specific chemotherapeutic drugs. Doxorubicin, Cisplatin and Temozolomide were used to generate drug resistance in MCF7 and U-251 MG cell lines. For treatment, working solutions of all these drugs (Appendix II) were prepared separately in suitable solvents. 10000 cells were counted (Hemocytometer) and seeded in a 60 mm cell culture dish containing 3 mL DMEM with respective drugs. The drug treatment for 21 days was started with sub lethal dose of drugs and slowly increased the concentrations till it reached half maximal inhibitory concentration (IC_{50}). Medium with drug was changed on alternative days during treatment and eventually the cells were maintained in drug free medium until it obtained 75% confluency. Confirmation of drug resistance in cells was done by both morphological and molecular level through inverted microscopy, western blot analysis and MTT assay respectively.

3.6 CELL VIABILITY ASSAY

Cell growth assays were carried out by MTT assay as described elsewhere with slight modifications (Srinivas *et al.*, 2004). This is a colorimetric assay that measures the reduction of yellow 3- (4,5- dimethyl thiazol-2-yl)- 2,5- diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenases present in the viable cells. The MTT enters the cells and passes in to the cell where it is reduced to an insoluble coloured (dark purple) formazan crystals (Fig.3)

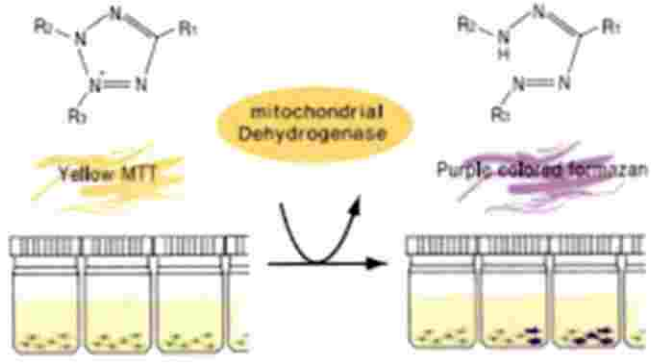

Fig.3: Formation of formazan crystals in MTT assay

3.6.1 MTT assay for chemotherapeutic drugs

To determine the IC_{50} of chemotherapeutic drugs like Temozolomide and Doxorubicin, MCF-7 and U-251 MG cells were grown in 96 well microtiter plates with 10 % DMEM (1×10^4 cells/well) and incubated with increasing concentrations of Temozolomide (0.325 µM, 0.65 µM. 1.3 µM, 2.6 µM) and Doxorubicin (0.56 µM, 1.125 µM, 2.25 µM, 4.5 µM). The same number of cells without drug were maintained as control. After 48 hours of incubation, the medium was removed and fresh medium was added along with 20µl of MTT (Appendix III) to each well. The plates were incubated for another 2 hours and 30 minutes. The formazan crystals formed by metabolically viable cells were solubilized with 100µl of acidic isopropanol (Appendix III). The colour developed was quantified (Measuring wavelength: 570 nm) with a 96 well microplate reader (BioTek™ FLx800). The cell viability was expressed as percentage over the control by using the formula T/C×100. The concentration required for a 50% of inhibition of viability (IC_{50}) was determined graphically. For this, standard graph was plotted by taking concentration of drug in X- axis and relative cell viability in Y- axis.

Cell viability (%) = (Mean OD/Control OD) ×100

3.6.2 MTT assay for pyrvinium pamoate

To analyze the IC_{50} of pyrvinium pamoate, MCF-7 cells were grown in 96 well microtiter plates (1×10^4 cells/well) and incubated with different concentrations of Pyrvinium pamoate (100 nM, 250 nM, 500 nM) in DMEM with 10% FBS. The cells without pyrvinium pamoate is served as control. After 24 hours incubation, the medium was removed, fresh medium is added and incubated for another 48 hours. Then the medium was removed and another fresh medium added along with 20µl of MTT (Appendix III) to each well. The plates were incubated for another 2 hours and 30 minutes. The formazan crystals formed by metabolically viable cells were solubilized with 100µl of acidic isopropanol (Appendix III). The colour developed was quantified (Measuring wavelength: 570 nm) with a 96 well microplate reader (BioTek™ FLx800). the cell viability was expressed as percentage over the control by using the formula T/C×100. The concentration required for a 50% of inhibition of viability (IC_{50}) was determined graphically. For this, standard graph was plotted by taking concentration of drug in X- axis and relative cell viability in Y- axis.

Cell viability (%) = (Mean OD/Control OD) ×100

For combination treatment of 5-FU and doxorubicin with pyrvinium pamoate was also performed in the same manner. Another round of MTT analysis was done to determine the IC_{50} of doxorubicin alone in Dox- resistant MCF-7 cells and as a combination with pyrvinium pamoate by using the same above-mentioned protocol.

3.7 WESTERN BLOT ANALYSIS

3.7.1 Whole cell lysate preparation

The Dox- resistant cells in 60 mm culture plates that were obtained after drug incubation with prescribed concentrations and time course stated were taken. At the end of treatment, cells were harvested by scraping in ice-cold PBS. Cells were washed

twice with PBS and pelleted by centrifugation. The cell pellets were lysed in ice cold Radio- Immunoprecipitation Assay (RIPA) buffer (Appendix IV) containing proteinase inhibitor cocktail for 1 hour with intermittent vortexing (every 10 minute). Lysed cells were centrifuged at 13000 rpm for 30 minutes and the supernatants were stored at -80^0 C.

3.7.2 Protein quantification

Extracted proteins were quantified using Pierce 660 nm protein assay method (Skehan *et al.*, 1990). For that, cell lysate was diluted and protein standards were prepared using BSA. Incubated for 5 minutes and then prepared a standard curve of absorbance versus microgram protein of standard and the concentration of protein in cell lysate was determined by using the standard curve.

3.7.3 Gel electrophoresis

Extracted proteins (30 µg) were heat denatured (95^0 C) for 5 minute and resolved on Sodium dodecyl sulphate – Poly Acrylamide Gel Electrophoresis (SDS-PAGE) at 90 V. Resolved proteins were transferred on to PVDF membrane at 100 V in 45 minutes (wet transfer).

3.7.4 Chemiluminescent detection

The membrane was blocked for 1 hour at room temperature with blocking buffer (Appendix IV). The membrane was then probed with antibodies to P-gp and HSP90 at 4^0 C overnight. HRP- conjugated secondary antibodies (anti Rabbit IgG and anti-mouse IgG; 1:1000 dilution) were used.

Bands were visualized using West Pico chemiluminescence Detection Kit (Thermo Scientific™). Equal volumes of luminol and peroxide solutions were mixed and added on to the membranes. Light emitting bands captured on an X- ray film and were developed and documented in Gel Doc™ XR Imaging system (Bio-Rad) and quantified using Quantity-One 1D Analysis software.

3.8 MitoSOX- BASED DETECTION OF MITOCHONDRIAL SUPEROXIDE

MitoSOX Red is a fluorogenic dye which serves as a mitochondrial superoxide indicator. Oxidation of MitoSOX Red reagent by superoxide produces red fluorescence. Cells were seeded at a density of 5000 cells per well in a 96 well clear plate. After 24 hours of seeding, cells were serum-starved overnight to bring them in the same growth phase (DMEM with 1% serum). Two conditions were given, control and PP treatment with 2 wells per group. Cells were given PP treatment at the concentration of 1 μM for 24 hours. After incubation, media from control and experimental (PP treated) groups were aspirated and washed with HBSS twice. MitoSOX dye (Sigma Aldrich) was dissolved in DMSO at the concentration of 1 mM and from that, a working concentration 1μM in HBSS was prepared. MitoSOX was added to each well and incubated for 15 minutes following which wells were washed with HBSS twice. Images were taken by a fluorescence microscope at 20x and 40x magnifications. Three random fields from each well were photographed.

3.9 HIGH- RESOLUTION RESPIROMETRY

The real-time OXPHOS analysis was performed using OROBOROS Oxygraph-2k (O2k; OROBOROS INSTRUMENTS, Austria) constructed with Clark polarographic oxygen electrodes and the automatic titration-injection micropump. This experiment provides the analysis of mitochondrial respiratory function. The two respiratory chambers having 2 mL of total volume capacity was saturated with DMEM and 100 μl of MCF-7cells (control) were added to it. For analyzing routine (Basal) respiration, ATP-independent and maximal respiration and reserve capacity, the following substances were added gradually; oligomycin (Complex V inhibitor), CCCP (Uncoupler) and antimycin A (Complex III inhibitor). The same protocol was repeated for checking the mitochondrial respiratory changes in pyrvinium pamoate treated MCF-7 cells.

3..9.1 Oxygraph - Data analysis

DatLab software (OROBOROS INSTRUMENTS, Austria) was used for respirometry data acquisition and analysis. Mass-specific oxygen flux (pmol s^{-1} mg^{-1}) was based on protein concentration in samples. Inhibition of respiration rate was analyzed and graphs were plotted.

RESULTS

4.RESULTS

The results of the study entitled "Targeting drug resistance in cancer cells by the anthelminthic drug, pyrvinium pamoate" are presented in this chapter.

4.1 CELL VIABILITY ASSAYS

4.1.1 IC_{50} of 5-FU in MCF-7 cells

The effective concentration of the chemotherapeutic drug 5-Fluoro Uracil (5-FU), a nucleotide base analogue used as a chemotherapeutic drug to treat cancers of colon, cervix, breast, oesophagus and pancreas was analysed in estrogen receptor positive breast cancer MCF-7 cells using MTT assay. Increasing concentrations of 5-FU from 0.325μM, 0.65 μM, 1.3 μM and 2.6 μM respectively was added to MCF-7 cells for a period of 48 hr. The viability of the treated cells was analysed by MTT reagent as mentioned in materials and methods. The viability of MCF-7 cells at 0.325μM, 0.65 μM, 1.3 μM and 2.6 μM concentrations in percentage were 74.03, 68.18, 57.32 and 53.15 respectively. At the time point of 48hr, the IC 50 (Inhibitory concentration for 50% viability) of 5-FU was calculated as 2.77 μM (Fig.4) in MCF-7 cells.

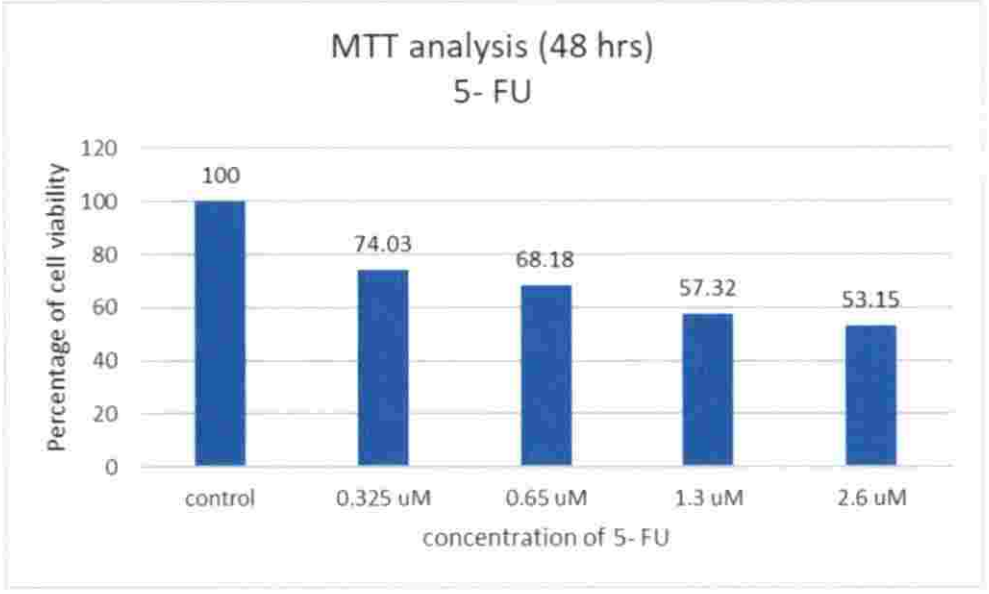

Fig. 4: Viability of MCF-7 cells in presence of 5-FU

4.1.2 IC_{50} of Doxorubicin in MCF-7 cells

The effective concentration of the chemotherapeutic drug Doxorubicin (Dox), an anthracyclin antibiotic used as a chemotherapeutic drug to treat cancers of bladder, lymphoma, breast, acute lymphocytic leukemia and Kaposi's sarcoma was analysed in estrogen receptor positive breast cancer MCF-7 cells using MTT assay. Increasing concentrations of Dox from 0.56μM, 1.125 μM, 2.25 μM and 4.5 μM respectively was added to MCF-7 cells for a period of 48 hr. The viability of the treated cells was analysed by MTT reagent as mentioned in materials and methods. The percentage viability of MCF-7 cells after treatment with 0.56μM, 1.125 μM, 2.25 μM and 4.5 μM Dox concentration was 62.86, 57.32, 54.92 and 52.52 respectively. At the time point of 48hr, the IC 50 (Inhibitory concentration for 50% viability) of Dox was calculated as 4.7 μM (Fig.5) in MCF-7 cells.

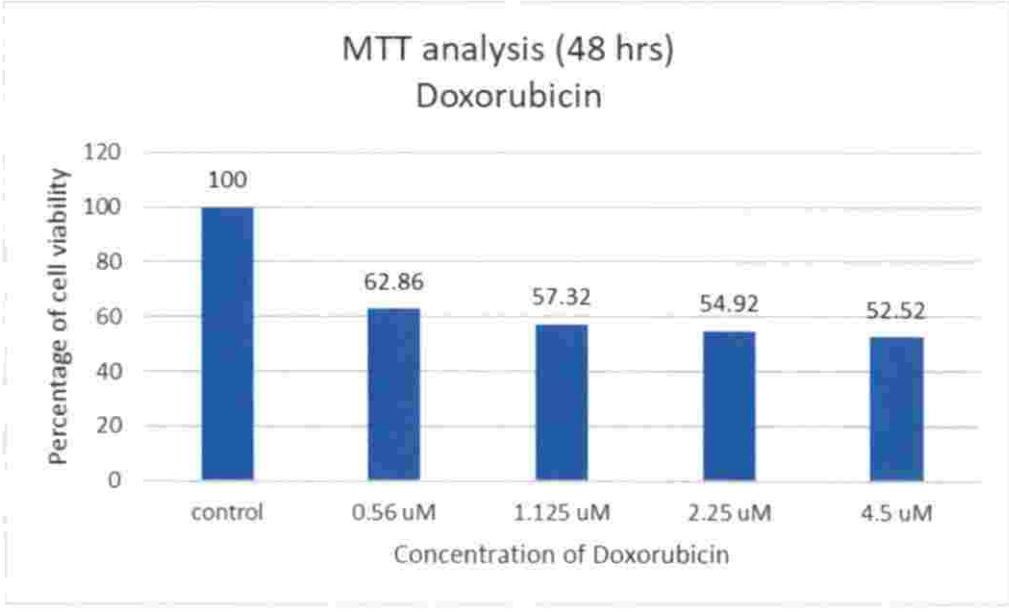

Fig. 5: Viability of MCF-7 cells in presence of Doxorubicin

4.1.3 IC_{50} of Pyrvinium pamoate in MCF-7 cells

The anthelminthic drug, Pyrvinium pamoate (PP)which is proposed as a novel drug that could be used to overcome drug resistance in cancer cells was added to the estrogen receptor positive breast cancer MCF-7 cells for a period of 24h at increasing concentrations from 100nM, 250nM, 500nM, 1 μM and 10 μM. The viability of MCF-7 cells at these concentrations were 91.8, 88.7,83.8, 83.3 and 64 percent respectively (Fig.6). The effective concentration of PP at 48hr was analysed by the addition of 250nM, 500nM, 1μM and 2μM respectively. The viability in percentage of MCF-7 cells at the above concentrations were 55.13, 48.24, 42.91 and 37.38 respectively. The IC 50 of PP was found to be 280nM in the MCF-7 cells by the standard MTT assay as mentioned in materials and methods (Fig.7).

4.1.4 IC_{50} of Pyrvinium pamoate in combination with 5-FU in MCF-7 cells

Pyrvinium pamoate at a concentration of 500nM was used in MCF-7 cells along with increasing concentrations of 5-FU 0.33 μM, 0.65 μM, 1.3 μM and 2.6 μM respectively. The viability of MCF-7 cells at 48hr under increasing concentrations of 5-FU 0.33 μM, 0.65 μM, 1.3 μM and 2.6 μM in percentage was 53, 47, 44.1 and 42.7 respectively. The IC50 of 5-FU was reduced from 2.77 μM to 0.35 μM in the presence of 500nM Pyrvinium pamoate (Fig.8).

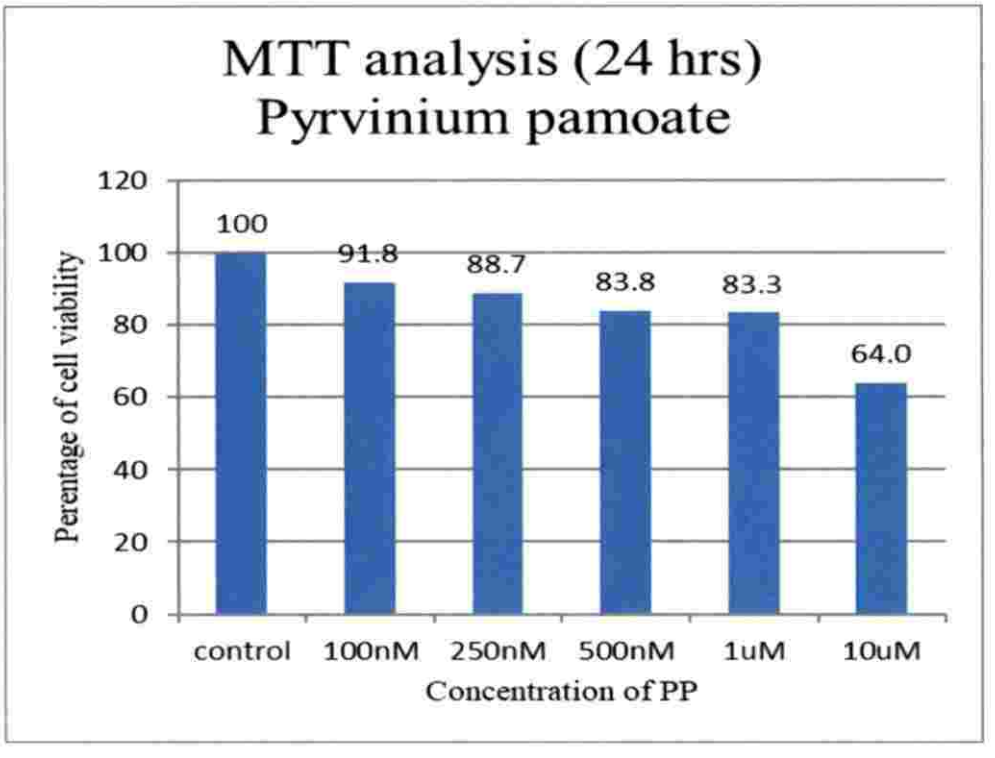

Fig. 6: Viability of MCF-7 cells in presence of Pyrvinium pamoate

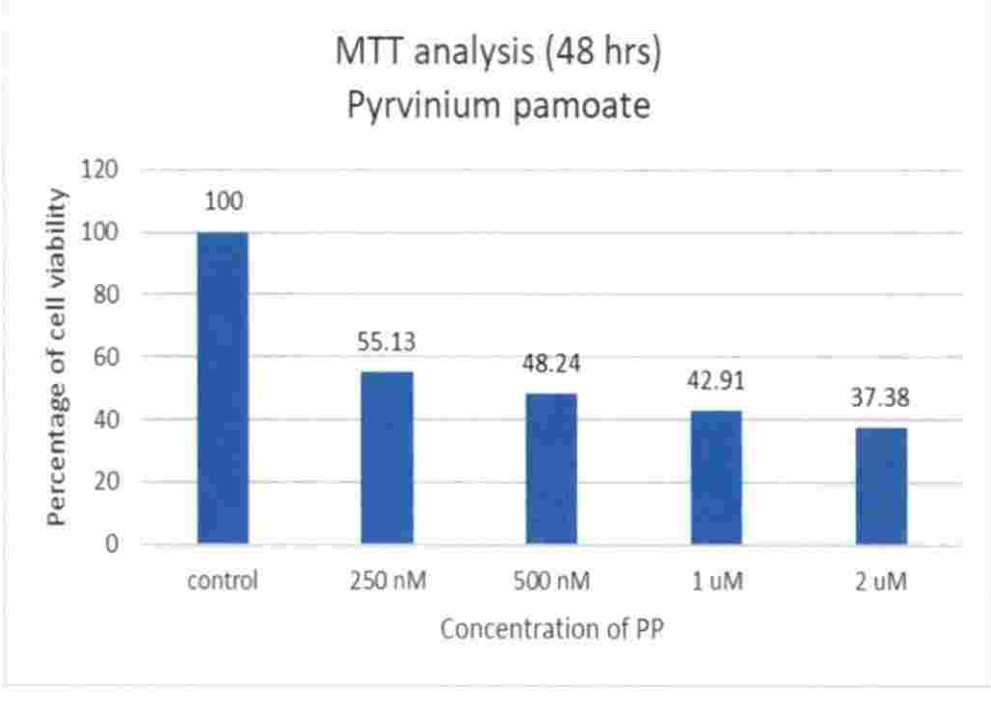

Fig. 7: Viability of MCF- 7 cells in presence of Pyrvinium pamoate

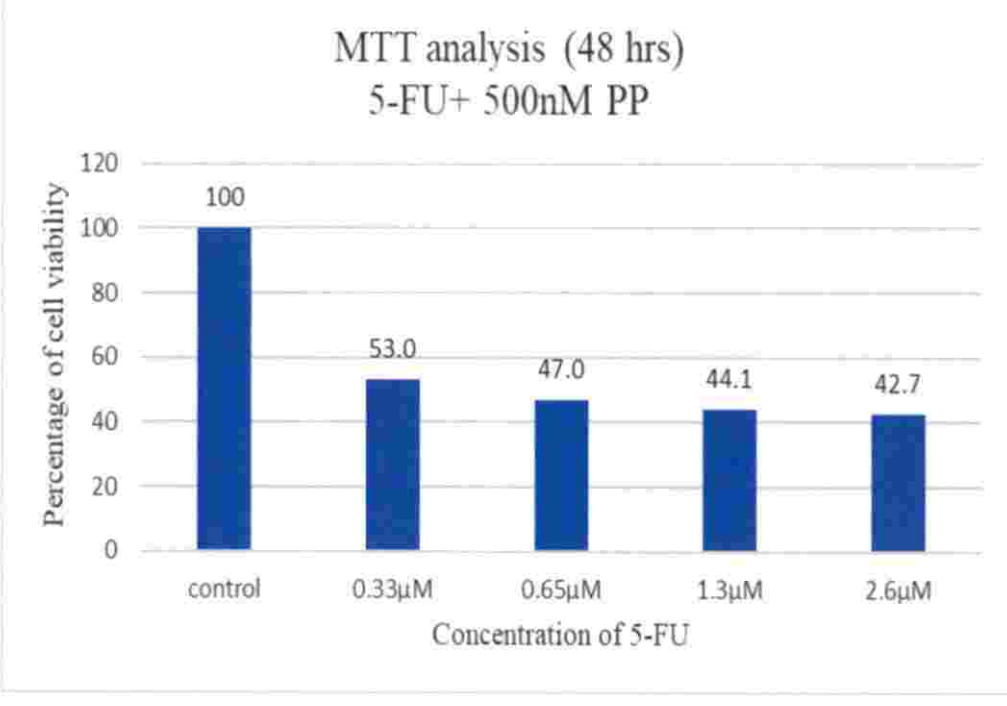

Fig. 8: Viability of MCF-7 cells in presence of 5-FU and PP

4.1.5 IC_{50} of Pyrvinium pamoate in combination with Dox in MCF-7 cells

Pyrvinium pamoate at a concentration of 500nM was used in MCF-7 cells along with increasing concentrations of Doxorubicin 0.56 μM and 4.5 μM respectively. The viability of MCF-7 cells at 48 hours with increasing concentrations of Dox 0.56 μM and 4.5 μM was 57.7 and 37.2 percent respectively. The IC50 of Dox was reduced from 4.7 μM to 0.59 μM in the presence of 500nM Pyrvinium pamoate (Fig.9).

4.1.6 IC_{50} of Dox in MCF-7 cells habituated with Dox

MCF-7 cells were grown in medium supplemented with increasing concentration of Dox and cultured for a period of 21days as described in materials and methods. The MTT assay was done with increasing concentration of Dox (0.56 μM ,1.125 μM and 4.5 μM) in these drug habituated cells. The viability in percentage for the Dox habituated cells at 0.56 μM ,1.125 μM and 4.5 μM Dox were 84.42, 85.71 and 57.79 respectively. The IC50 concentration of Dox was increased from 4.7 μM to 5.33 μM(Fig.10).

4.1.7 IC_{50} of Dox habituated MCF-7 cells with increasing Dox and 500nM PP

MCF-7 cells were grown in medium supplemented with increasing concentration of Dox and cultured for a period of 21days as described in materials and methods. The MTT assay was done with increasing concentration of Dox (0.56 μM ,1.125 μM, 2.25 μM and 4.5 μM) in these drug habituated cells along with 500nM Pyrvinium pamoate. The viability of Dox habituated cells at 0.56 μM ,1.125 μM, 2.25 μM and 4.5 μM Dox along with 500nM Pyrvinium pamoate was 64.38, 58.22, 39.73 and 35.62 percent respectively. The IC50 concentration of Dox in Dox habituated cells was reduced from 5.33 μM to 1.35 μM (Fig.11).

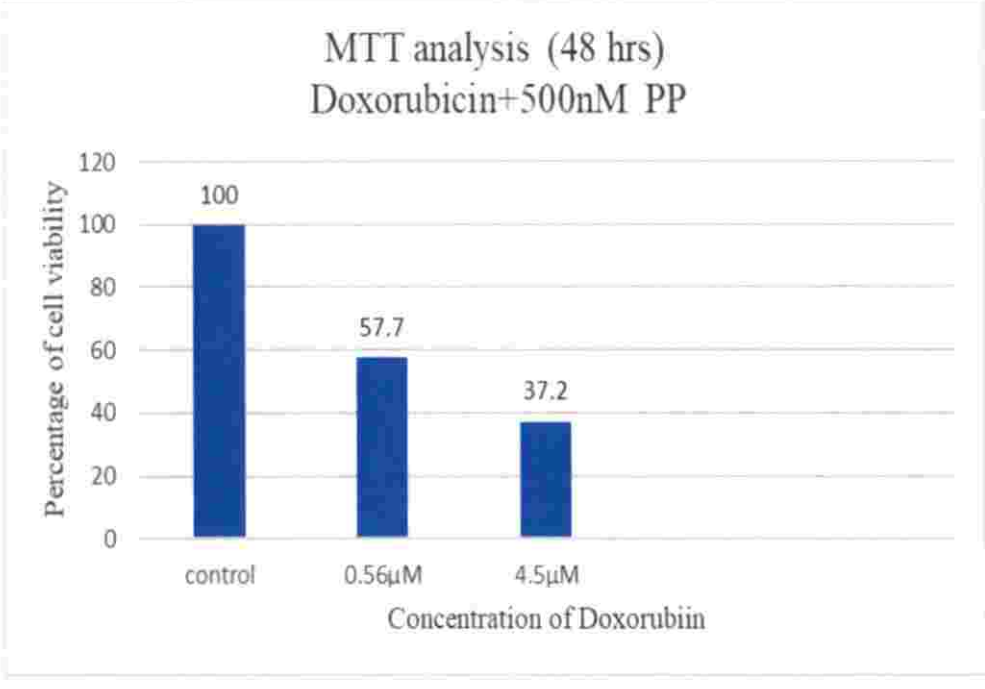

Fig. 9: Viability of MCF-7 cells treated with Doxorubicin and PP

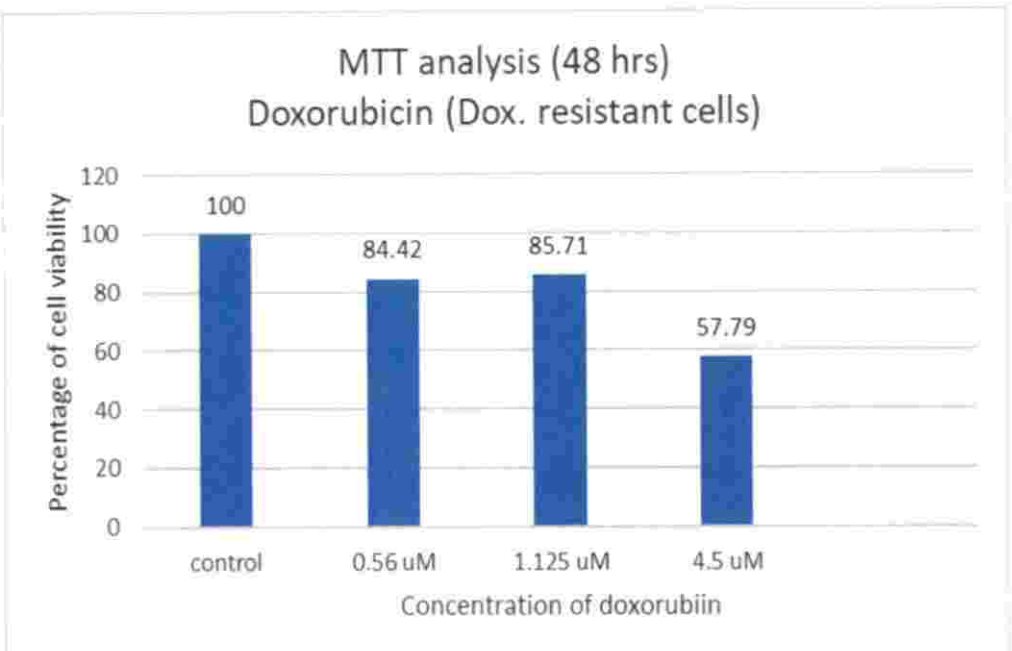

Fig. 10: Viability of Dox-resistant MCF-7 cells treated with Doxorubicin

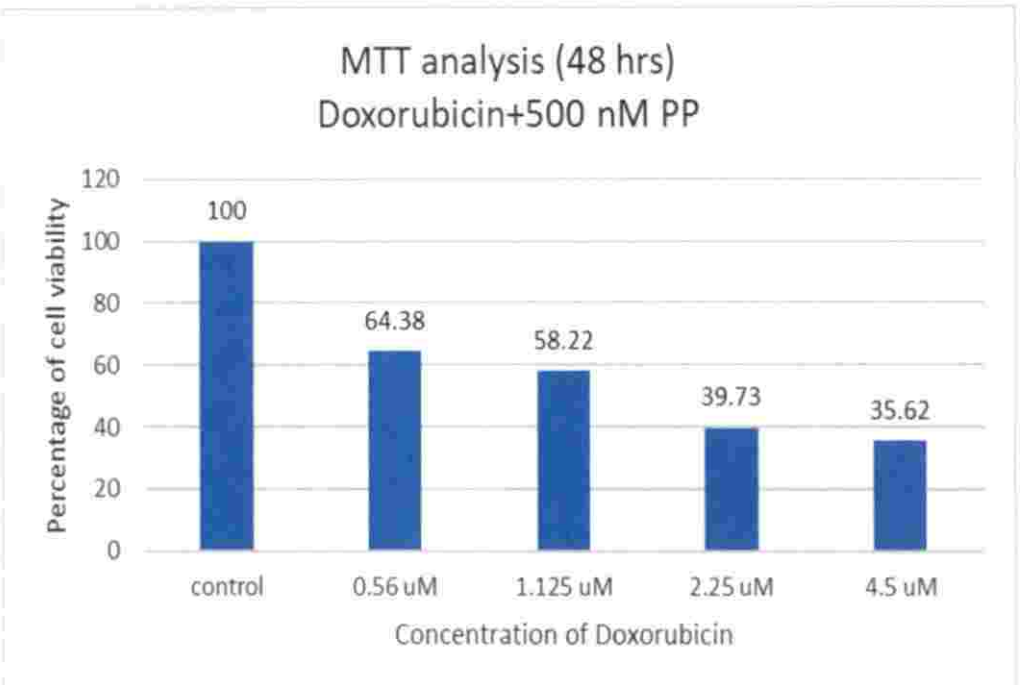

Fig. 11: Viability of Dox-resistant MCF-7 cells treated with a combination of Doxorubicin and PP

4.2 DEVELOPMENT OF DRUG RESISTANCE AND ITS CONFIRMATION

4.2.1 Development of Drug resistance in MCF-7 cells

MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum along with increasing concentrations of Doxorubicin in a 60mm cell culture dish. The medium was replaced with fresh medium supplemented with increasing concentration of Doxorubicin as mentioned in the materials and methods section. After 21 days the cells were imaged by inverted phase microscope. The elongated morphology of cells with increased branching indicated the drug resistant nature of these Dox habituated cells (Fig.13) compared to control cells which were not treated with any drug (Fig.12). Although generation of drug resistance was attempted with 5-FU in MCF-7 cells, the cells were contaminated before the relevant time point and hence could not be generated.

4.2.2 Generation of Drug resistance in U-251 cells

U-251 MG, a human glioblastoma cell line was cultured in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum along with increasing concentrations of Temozolomide in a 60mm cell culture dish (Fig.14) Due to inherent mycoplasma contamination, the cells could not be cultured beyond 14 days and hence the attempt to generate drug resistance in U-251 MG cells with Temozolomide was not proceeded further.

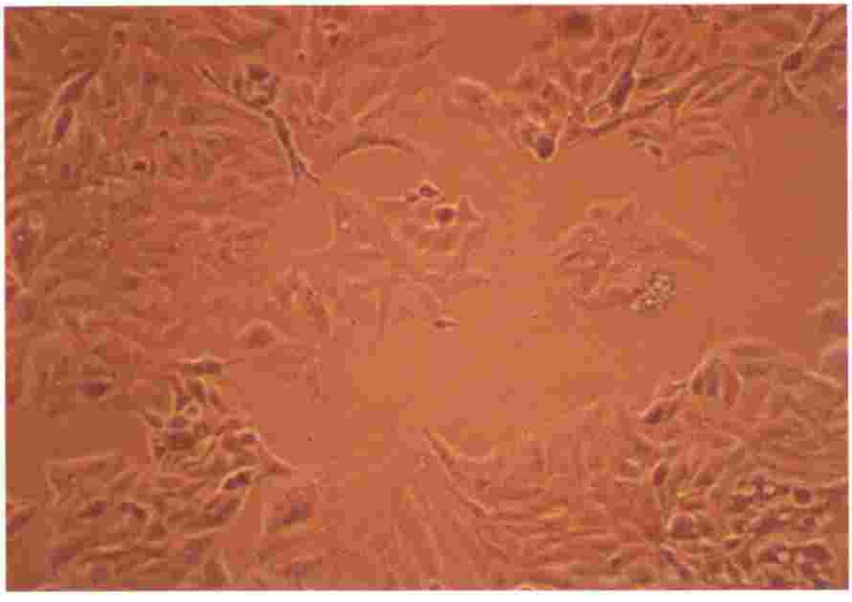

Fig.12: Healthy MCF-7 cells cultured in 60 mm cell culture dish

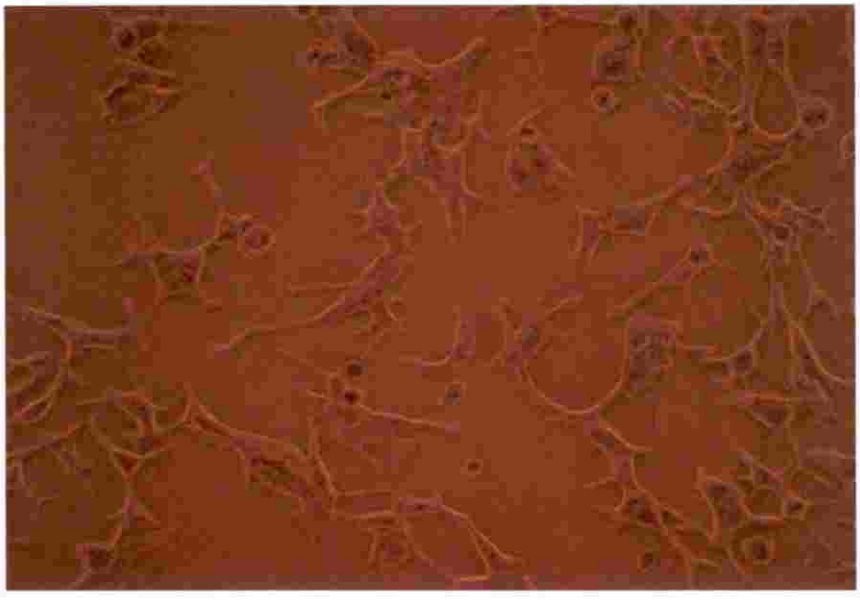

Fig.13: Dox-resistant MCF-7 cells cultured in 60 mm cell culture dish

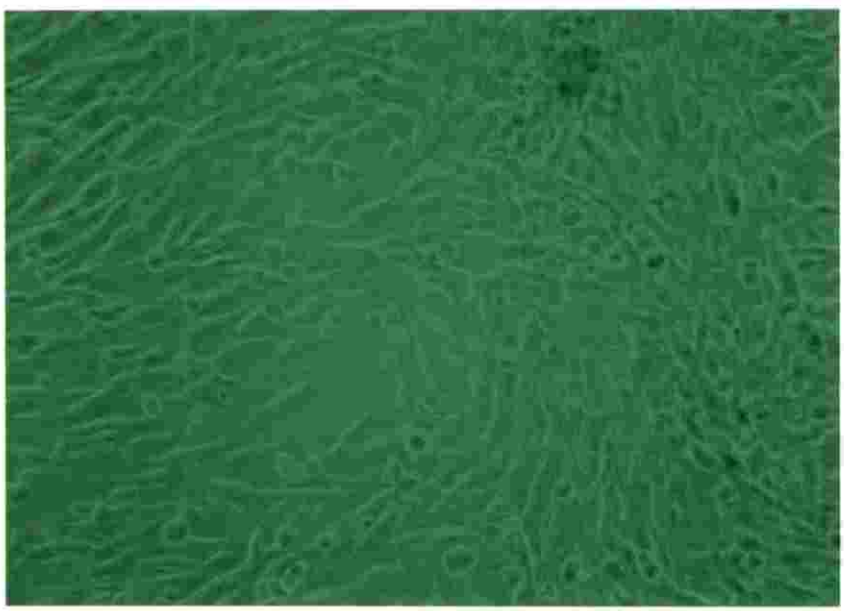

Fig.14: Healthy U-251 MG cells cultured in 60 mm cell culture dish

4.3 CONFIRMATION OF DOX RESISTANCE IN MCF-7 CELLS HABITUATED IN DOXORUBICIN

4.3.1 Expression analysis of P-gp in Dox habituated MCF-7Cells

The Doxorubicin habituated MCF-7 cells were analysed for the expression of the drug efflux protein P-gp by SDS-PAGE and western hybridization with corresponding antibodies as described in detail in the materials and methods section. The Dox habituated MCF-7 cells showed an increased expression of P-gp compared to control cells which were not treated with any drugs (Fig.15). The bands were quantified by image analysis software and a 0.5-fold increase in the expression of P-gp was confirmed (Fig. 17) in Dox habituated MCF-7 cells. Beta actin protein expression was used to normalize the band intensities (Fig. 16).

4.3.2 Expression analysis of HSP90 in Dox habituated MCF-7Cells

The Doxorubicin habituated MCF-7 cells were analysed for the expression of the drug efflux protein P-gp by SDS-PAGE and western hybridization with corresponding antibodies as described in detail in the materials and methods section. The Dox habituated MCF-7 cells showed an increased expression of HSP 90 protein compared to control cells which were not treated with any drugs (Fig.18). The bands were quantified by image analysis software and a 0.5-fold increase in the expression of HSP90 was confirmed (Fig.20) in Dox habituated MCF-7 cells. Beta actin protein expression was used to normalize the band intensities (Fig. 19).

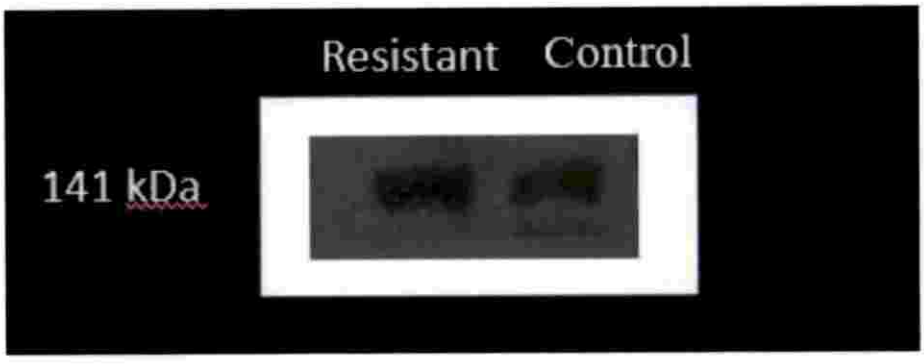

Fig.15: Expression of P-gp in control and Dox-resistant MCF-7 cells

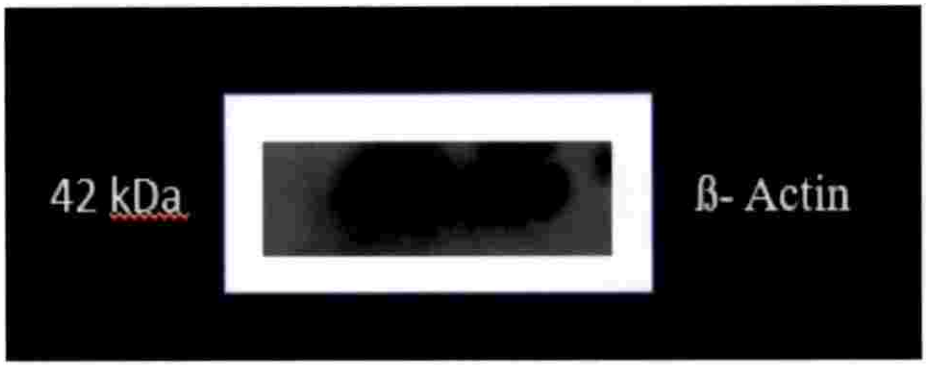

Fig.16: Expression of Actin (Loading control for P-gp) in control and Dox-resistant MCF-7 cells

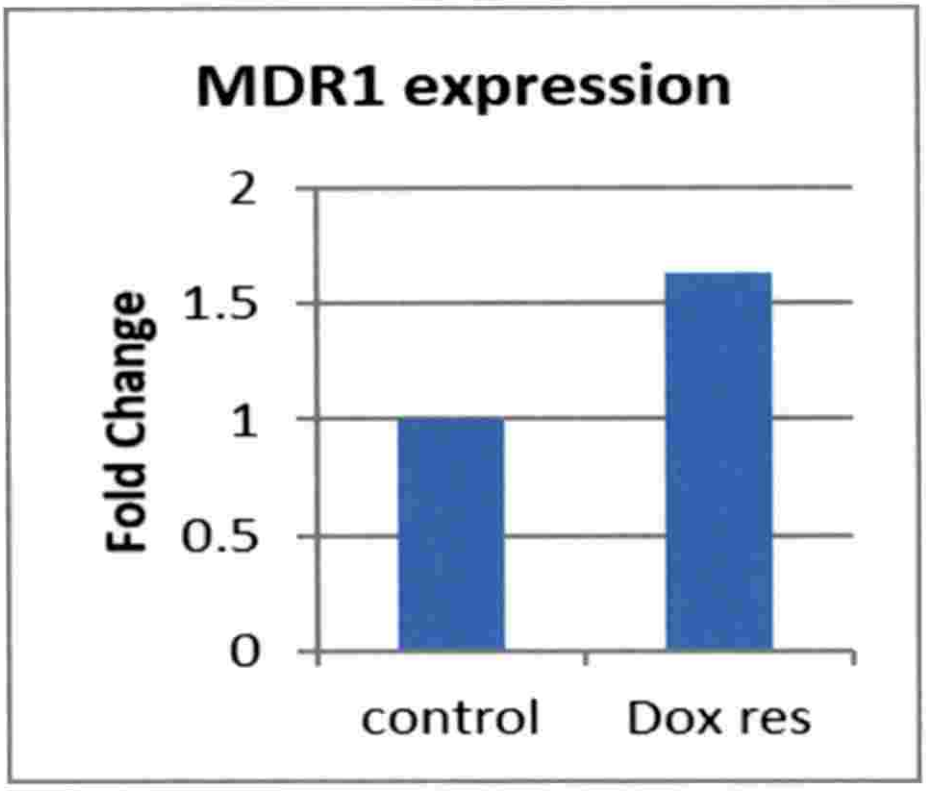

Fig.17: Enhanced (0.5 fold) expression of P-gp (MDR1) in Dox-resistant and control MCF-7 cells

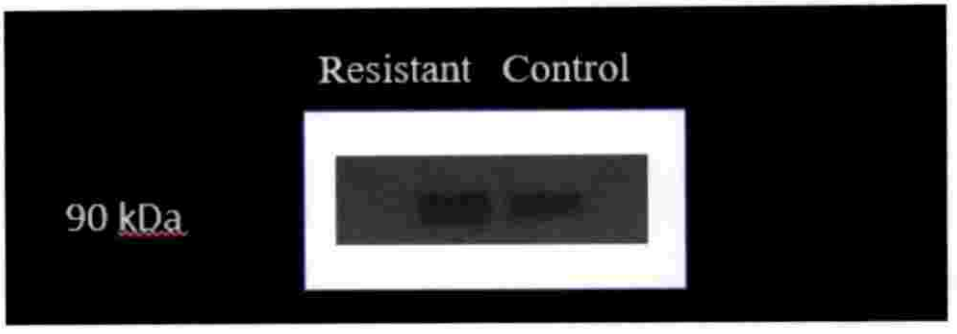

Fig.18: Expression of HSP-90A in Dox-resistant and control MCF-7 cells

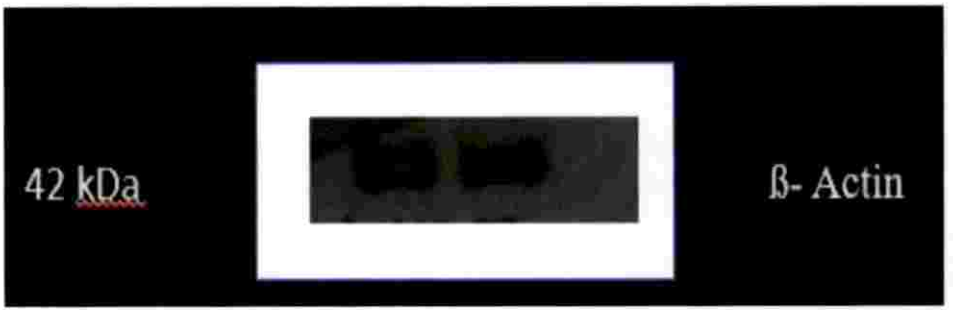

Fig.19: Expression of Actin (Loading control for HSP-90A) in Dox-resistant and control MCF-7 cells

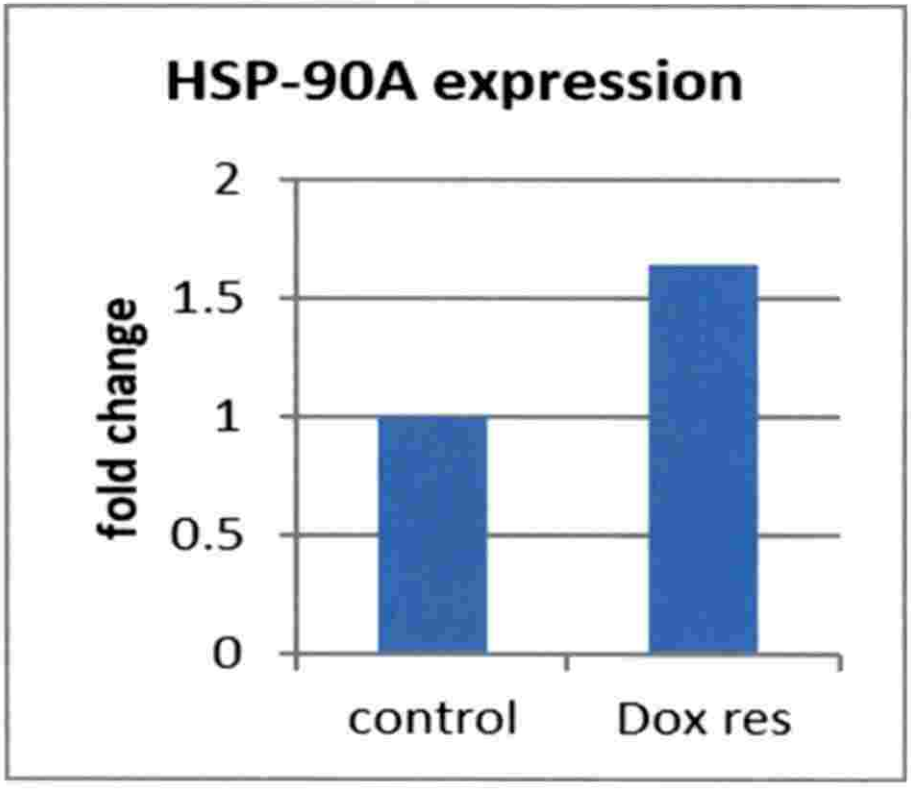

Fig.20: Enhanced (0.5 fold) expression of HSP-90A in Dox-resistant and control MCF-7 cells

4.4 MECHANISM OF ACTIVATION OF CELL DEATH BY PYRVINIUM PAMOATE

4.4.1 Mitochondrial Reactive Oxygen Species generation

MitoSOX dye, a mitochondrial Reactive Oxygen Species(ROS) measuring fluorescent dye was used to measure the ROS generation in mitochondria of MCF-7 cell treated with Pyrvinium pamoate. The Pyrvinium pamoate treated MCF-7 cells showed increased red fluorescence in the cytosol (Fig.22) compared to control MCF-7 cells not treated with any drug(Fig.21) as seen under an inverted fluorescence microscope with appropriate filter. As mentioned in the materials and methods, increased ROS will increase the red fluorescence of the dye excited by the right fluorescence wave length.

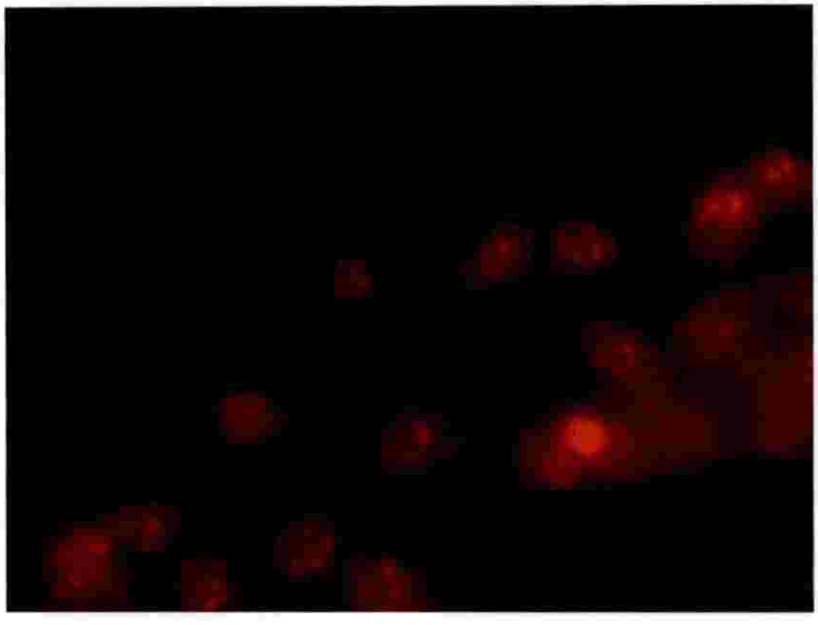

Fig. 21: Red fluorescence in MCF-7 cells without PP

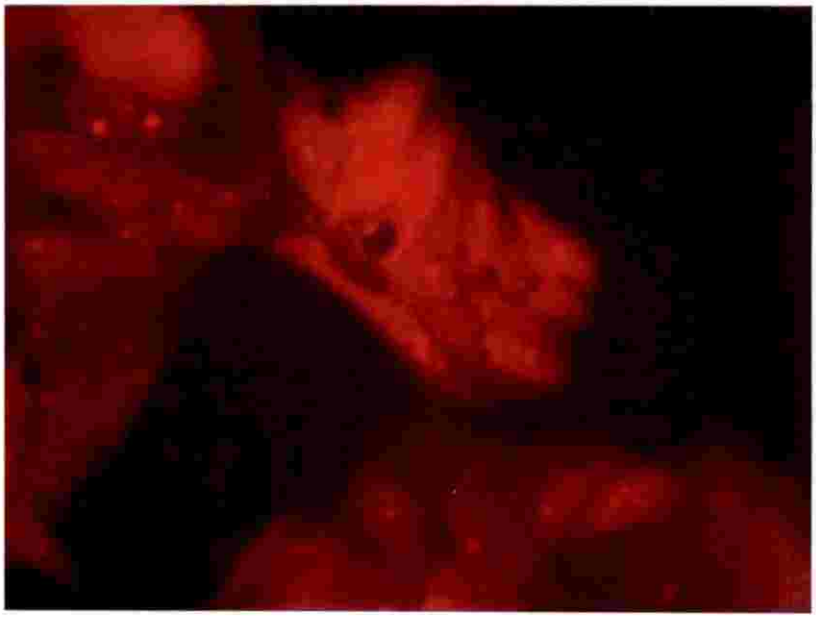

Fig. 22: Enhanced red fluorescence in MCF-7 cells with PP

4.4.2 Measurement of mitochondrial respiration

Oxygen utilization during respiration is the index of mitochondrial oxidative phosphorylation. The different respiratory indices and their mode of measurement has been described in materials and methods. The relationship between these indices is depicted in Fig.23. As mentioned in the materials and methods MCF-7 cells were used to measure mitochondrial oxygen consumption in control and pyrvinium pamoate treated cells. Basal respiration which is the sum of ATP dependent respiration, non-mitochondrial respiration and proton leak, was reduced from 700 pmol/s/mg in control MCF-7 cells to 350 pmol/s/mg in Pyrvinium pamoate treated MCF-7 cells (Fig. 24). ATP dependent respiration is obtained by treatment with oligomycin, a complex V(ATP synthase) inhibitor. Treatment with Pyrvinium pamoate reduced ATP dependent respiration from 600 pmol/s/mg in control MCF-7 cells to 50 pmol/s/mg in Pyrvinium pamoate treated cells (Fig.25). Maximal respiration is the limit of mitochondrial respiration induced by uncoupling electron transport and proton gradient by chemicals called uncouplers (CCCP) as detailed in the materials and methods section. The maximal respiration was reduced from 1050 pmol/s/mg in control MCF-7 cells to 400 pmol/s/mg in Pyrvinium pamoate treated MCF-7 cells (Fig.26). Reserve capacity is the maximal respiration minus the basal respiration. The reserve capacity of control MCF-7 cells was reduced from 300 pmol/s/mg to 75 pmol/s/mg in Pyrvinium pamoate treated cells (Fig.27).

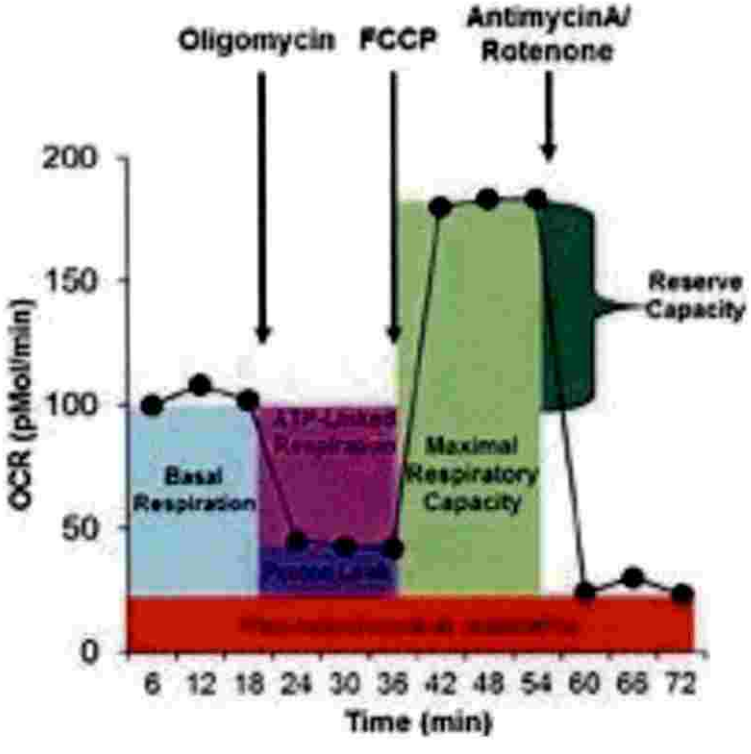

Fig. 23: Oxygen concentration and oxygen flux in OROBOROS 2K oxygraph

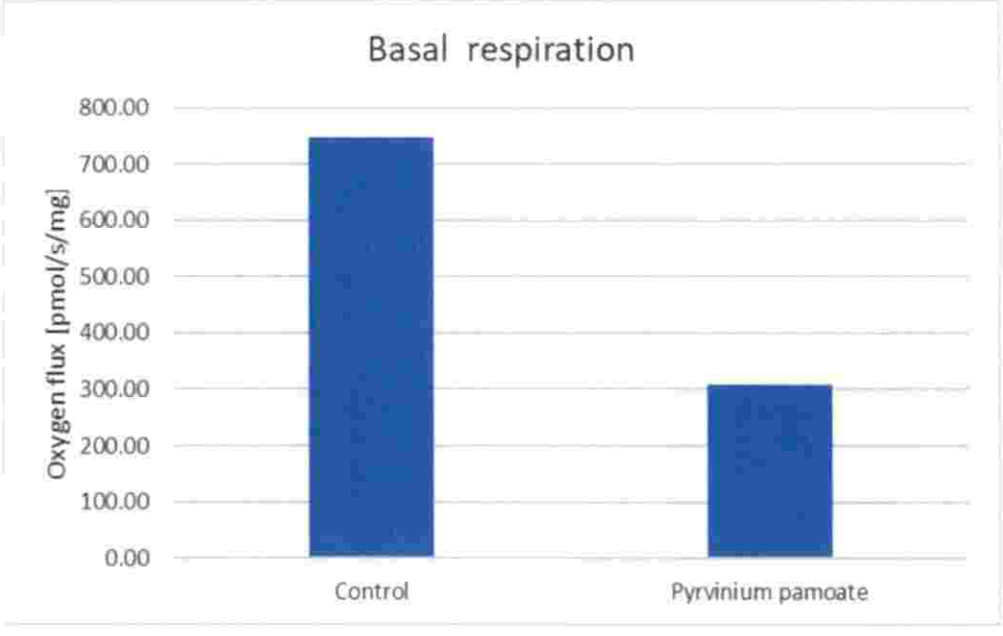

Fig. 24: Basal respiration in control and PP treated MCF-7 cells

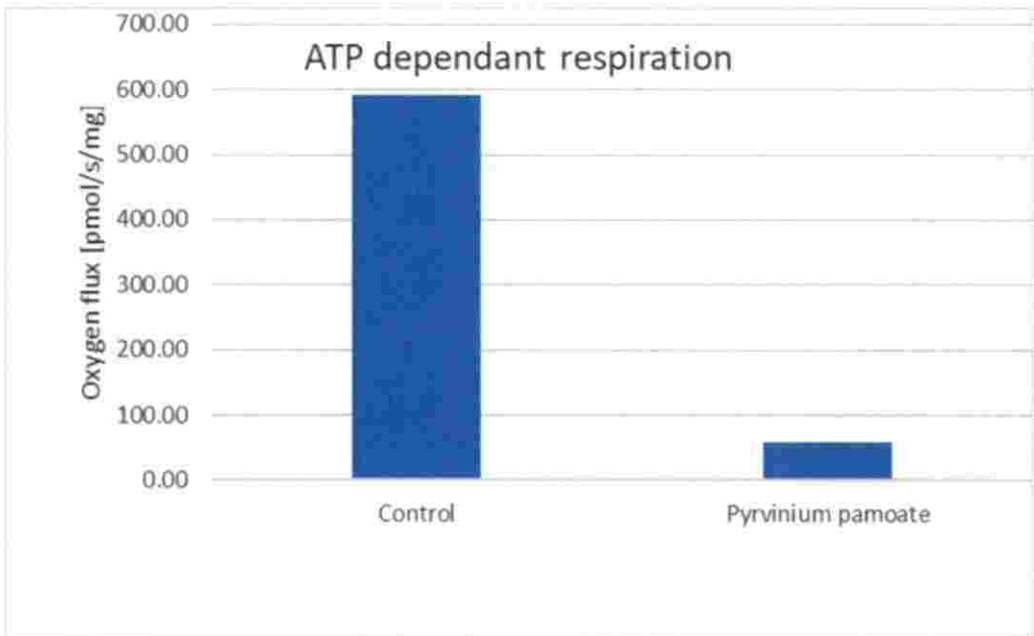

Fig. 25: ATP-dependant respiration in control and PP treated MCF-7 cells

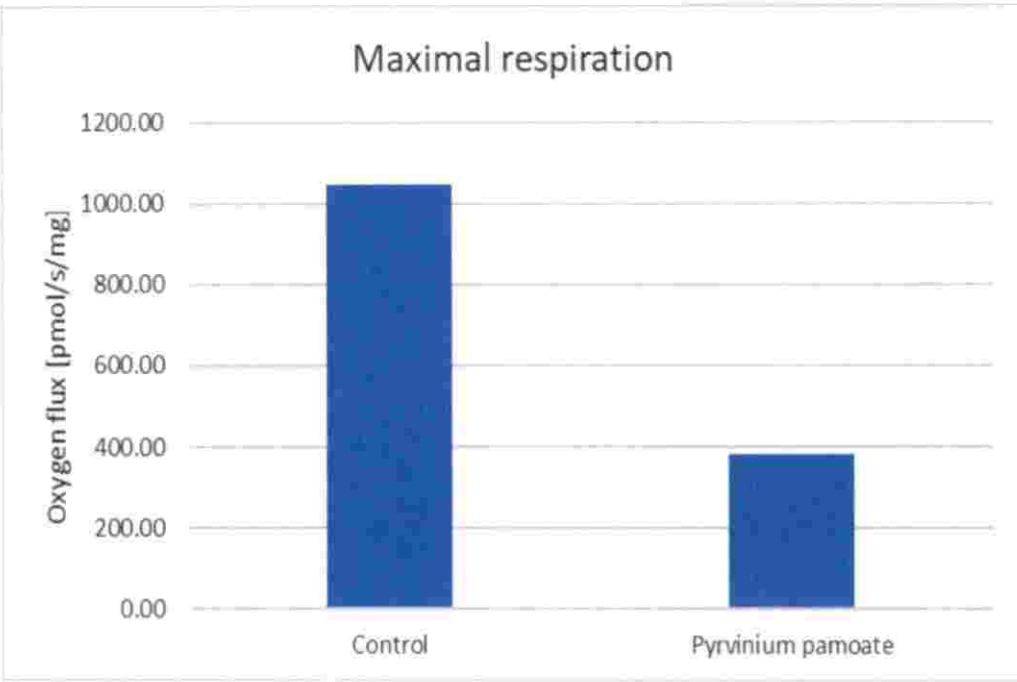

Fig. 26: Maximal respiration in control and PP treated MCF-7 cells

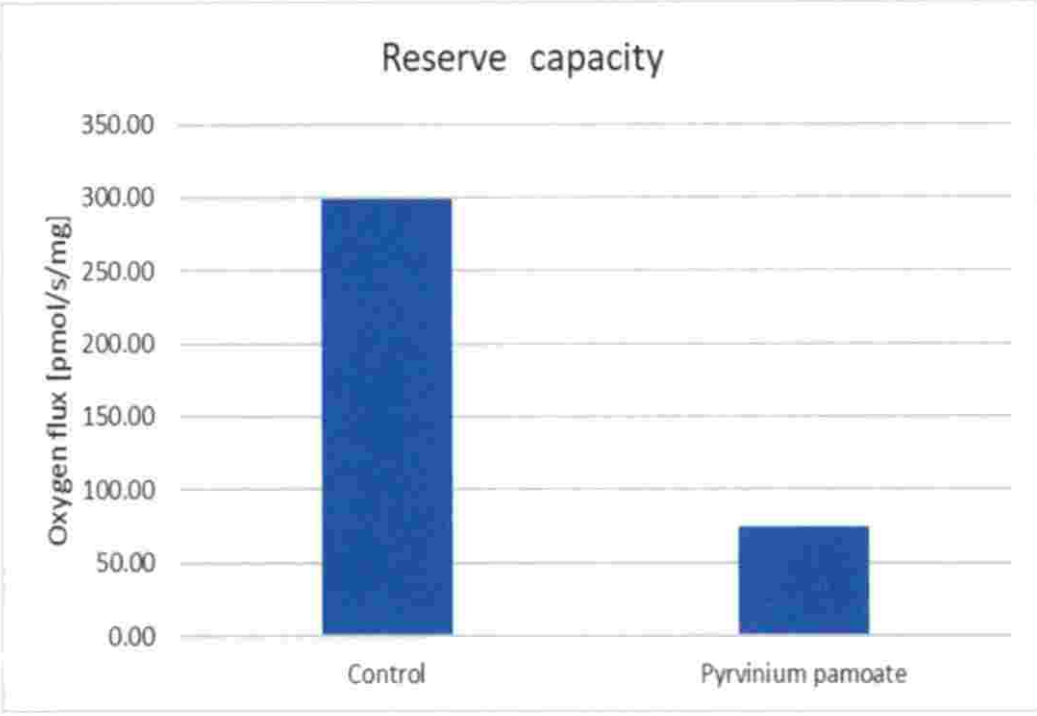

Fig. 27: Reserve capacity in control and PP treated MCF-7 cells

DISCUSSION

5.DISCUSSION

5.1 CELL SURVIVAL

Drug resistance phenotype is predominantly manifested in select solid tumors like pancreas, ovary, colon, breast, prostate and brain (Sanders *et al.,* 2012). Hormonal influence followed by predominant lipid nature of these tissues has been attributed to this phenomenon. Breast cancer is one of the most common cancers commonly refractory to chemotherapy. Hence the breast cancer cell line positive for estrogen receptor, MCF-7 was used in the present study as a representation of breast cancer. In order to create a drug resistant phenotype in MCF-7 cell line (representation of drug resistant breast tumour) the cell line was treated with 5-FU (5-Fluoro Uracil-nucleotide base analogue drug) and Dox (Doxorubicin) the anticancer antibiotic drug used as the first line of treatment for breast cancer. Such a method follows the Environment Mediated Drug Resistance (EMDR) model (Meads *et al.,* 2009). The IC_{50} value is an index for the potency of any drug and the IC_{50} value obtained for 5-FU and Dox in MCF-7 cells by MTT assay was 2.77 μM and 4.7 μM respectively after a period of 48hr. The IC_{50} values obtained for these commonly used chemotherapeutic agents was consistent with previous studies conducted in MCF-7 cell line (Lamb *et al.*, 2015). The IC_{50} values is a testament for the potency of the drugs used in the present study (Lamb *et al.,* 2015). The authencity of the cell line used is also proven indirectly by the results of MTT assay (Fig.4 & 5).

5.2 COMBINATION THERAPY

The IC_{50} of Pyrvinium pamoate (PP) calculated by MTT assay in MCF-7 cell line was 280nM after 48h. The IC_{50} concentration of Pyrvinium pamoate in very low concentration (nM) is ample proof of principle of its potential as an anticancer agent. Most drugs used in clinic show potent activity in very low concentrations (xu *et al.,* 2013). The chemotherapeutic drugs induce side effects like hepatotoxicity by 5-FU (Grigorian *et al.,* 2014) and cardiotoxicity by Dox which limit their use (Chatterjee *et*

al., 2010). In order to retain the cytotoxic potential of these chemo therapeutic drugs towards the cancer cells and reduce the detrimental effect on normal cells, they are used in combination with other compounds or natural products. This strategy called combination therapy is very effective to increase the efficacy of the chemotherapeutic agent by increasing its potency to induce cell death in cancer cells and reduce the dose of the expensive chemotherapeutic. Typical example that stresses the effectiveness is the combination therapy of Taxol (potent and expensive cytotoxic agent) with curcumin (natural product and secondary metabolite from the rhizome of turmeric plant (*Curcuma longa*). Such a combination therapy was found to be effective in reducing the therapeutic dose of taxol significantly without comprising its efficacy as a chemotherapeutic drug (Hua Yu *et al.*, 2008). By combining PP at a dose of 500nM with 5-FU the IC_{50} of 5-FU was reduced from 2.77 μM to 0.35 μM, an eightfold reduction of effective dose in vitro (Fig.8). The combination of PP at 500nM with Dox, reduced the IC_{50} of Dox from 4.7 μM to 0.59 μM almost eight-fold reduction of effective dose (Fig.9). A significant reduction of effective dose of both 5-FU and Dox in combination with PP at 500nM indicates that PP is potent candidate for combination therapy (Day *et al.*, 2016).

5.3 DRUG RESISTANCE

Resistance to chemotherapy is either acquired or inherent in cancers and multiple mechanisms are at play to confer drug resistance in cancer. The first and foremost is the efflux of the drugs by drug efflux pumps like P-Glyco proteins(P-gp's) (Gottesmann *et al.*, 2002). The origin of drug resistant cells in cancer can be explained by two models. The Cancer Stem Cell (CSC) model and Environment Mediated Drug Resistance (EMDR) model. The CSC model opines that a rare group of tumour initiating cells are responsible for the development and spread of cancer designated Tumour Initiating Cells (TIC's) (Nguyen *et al.*, 2012). The EMDR model postulates that selection pressure on cancer cells by chemotherapy induces Darwinian evolution and some cells evolve mechanisms to alter the environment, making them more

adaptable to drug selection. Such cells remain dormant until such mechanisms of overcoming drug inhibition are in force and ultimately reach a stage of acquired resistance (Matsunaga *et al.,* 2013).

After the drug efflux mechanism, the major modification in the development of drug resistance is the over expression of Heat Shock Protein (HSP) family proteins in cancers. HSP 27, HSP70 and HSP90 named based on their molecular weight in kilo Daltons, help stabilize anti apoptotic proteins and prevent programmed cell death (PCD) in cancer cells. The inhibition of expression of HSP family of proteins has been shown to provide wide ranging pharmacological benefit (Ciocca *et al.*, 2005). MCF-7 cells were treated with sub lethal dose of Dox for 21 days and the Dox habituated MCF-7 cells showed morphological changes from the control MCF-7 cells not habituated in medium containing sublethal dose of Dox (Fig.12, 13). Although the development of Temozolomide habituated cells in U-251 MG cells was attempted (Fig. 14). The cells were lost due to endogenous contamination after 15 days of drug treatment. Hence further experiments on drug resistance were limited to only Dox habituated MCF-7 cells. The increased IC_{50} value for Dox in Dox habituated MCF-7 cells indicated their acquired resistance (Fig.10). To further confirm their resistant nature the expression of P-gp and HSP-70 proteins were analysed in control and Dox habituated MCF-7 cells by western blotting (Fig. 15-20). The relative 0.5-fold increase in expression of P-gp and HSP-70 proteins in Dox habituated cells relative to the control untreated cells indicated their resistant nature to Dox (Fig. 17,20). This method of development of drug resistance is well established (McDermott *et al.,* 2014). Although tedious, risky and time consuming this method was followed as it is known to yield robust drug resistant clones. The one-fold increase in IC_{50} from 4.7 μM to 5.33 μM within 21 days of Dox habituation showed that MCF-7 cells were indeed resistant to Dox (Fig.10). When 500nM of PP was added to these Dox habituated MCF-7 cells, the IC_{50} was reduced from 5.33 μM to 1.35 μM. Such a potent decrease of four-fold

reduction in IC_{50} strongly indicated the efficacy of PP in overcoming Dox mediated drug resistance in MCF-7 cells.

5.4 MITOCHONDRIAL ACTIVITY

The most important mechanism for the transition of a naïve cancer cell to a drug resistant phenotype is metabolic rewiring from a predominantly glycolytic dependence to mitochondrial respiration (Ward *et al.*,2013). The metabolically rewired cancer cells which are predominantly drug resistant are particularly sensitive to changes in mitochondrial Reactive Oxygen Species (ROS) balance and respiration (Urra e*t al.*, 2017). Over expression of mitochondrial proteins has been associated with metastasis and poor prognosis in many cancers (Shang *et al.*, 2018). The mitochondrial electron transport complexes have been especially been found to be very important in tumorigenesis and metastasis (Tan *et al.*, 2014). Hence targeting mitochondria is an important strategy to achieve tumour reduction and overcoming drug resistance (Rozanov *et al.*, 2019). The antioxidant mechanisms in cancer cells have been found to be very robust owing to the need to reduce ROS induced mitochondrial damage and one strategy to induce cell death in cancer cells is to induce ROS generation (Trachootham *et al.*, 2006). Pyrvinium pamoate (PP) treatment induced a strong mitochondrial ROS generation evident from the strong red fluorescence in the cytosol of MCF-7 cells treated with PP (Fig.22) compared to untreated control MCF-7 cells (Fig.21). It could be confirmed that the cytotoxicity induced by PP in MCF-7 cells is mainly due to the ability of PP to induce mitochondrial ROS. The ability of PP to induce cell death in drug resistant lymphoma cells was demonstrated earlier (Xiao mei fang *et al.*, 2016). The ability of PP to target mitochondria of cancer cells of multiple tumor types was known (Lamb *et al.*, 2015). PP was also reported to block the mitochondrial electron transport chain, particularly the NADH fumarate reductase activity of complex I (Ishii *et al.*, 2012). The oxygen consumption assay using seahorse extracellular flux analyzer in HCT116 colon cancer cells showed that PP could reduce basal and maximal respiration (Senkowski *et al.*, 2015). Oxygen consumption

measurements in MCF-7 cell treated with PP showed that PP inhibited basal, ATP dependent, maximal and residual oxygen consumption in PP treated MCF cells compared to untreated control MCF-7 cells (Fig.24-27). The oxygen consumption assay using OROBOROS could not be performed in Dox resistant MCF-7 cells since the cells were slow dividing and refractory to splitting. It would have been ideal if the oxygen consumption parameters showed similar reduction in Dox resistant MCF-7 cells too. Since it is common knowledge that drug resistant cells predominantly use mitochondrial respiration for survival it is safe to presume that PP would be more effective in the Dox resistant MCF-7 cells.

Not many drugs are available which have good safety profile and have selective ability to target mitochondria of tumour cells. These characteristics make PP an ideal therapeutic agent capable of specifically eliminating cancer stem cells or multi drug resistant tumour cells. This study proves the fact beyond doubt that PP can indeed be used to specifically kill drug resistant cancer cells.

SUMMARY

6.SUMMARY

Drug resistance is a major problem in chemotherapy and targeting multiple mechanisms of drug resistance with single drug has been proven difficult. Pyrvinium pamoate (PP) as a single agent and in combination with other chemotherapeutic drugs like 5-FU and Dox is a potent anticancer agent as it can specifically target mitochondrial metabolism of MCF-7 cancer cells, inhibit drug efflux pump P-gp and reduce the expression of the molecular chaperone, HSP70. As a single agent PP induced mitochondrial specific ROS generation and cell death in MCF-7 cells. PP also inhibited the mitochondrial respiratory complexes as evident from the reduction of basal respiration, ATP dependent respiration, maximal respiration and residual respiratory capacity.

Pyrvinium pamoate can be used as an effective chemotherapeutic agent alone or in combination with 5-Fluro Uracil or Doxorubicin to treat drug resistant cancer cells.

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APPENDICES

APPENDIX I

Reagents required for cell culture and maintenance

1.DMEM-10%

DMEM solution (Gibco)	:500 ml
FBS (Gibco)	:50 ml

2.Phosphate Buffered Saline (PBS) (pH 7.4)

Sodium chloride	: 137 mM
Potassium chloride	: 2.7 mM
Disodium hydrogen phosphate	:10.14 mM
Potassium dihydrogen phosphate	:1.76 mM in

Dissolve in 1L sterile deionized water and check pH of the final solution.

3.Freezing medium

FBS	:90 %
DMSO	:10 %

APPENDIX II

Reagents required for drug treatments

1.Pyrvinium pamoate stock (10 mM)

11.51mg PP in 1000 µl DMSO.

2.Cisplatin

Stock solution (50 mg/ml)

Working solution is prepared by 1:100 dilution (1 µl from stock solution is dissolved to 99 µl DMSO

3.Doxorubicin

Stock solution: 5 mg lyophilized powder of Doxorubicin is dissolved in 919 µl of DMEM (10 mM).

Working solution (10 µM) is prepared by dissolving 1 µl of stock solution in 1000 µl of DMEM.

4.5- Fluoro Uracil

Stock solution (100 mM)

Working solution is prepared by 1:100 dilution (1 µl from stock solution is dissolved to 99 µl DMSO.

APPENDIX III

Reagents required for MTT assay

1.MTT stock

MTT salt, 5 mg in 1 ml PBS.

APPENDIX IV

Reagents required for western blotting

1.Acrylamide 30%

Acrylamide	: 29% (w/v)
N,N' – methylene bisacrylamide	: 1% (w/v)

Dissolve all the reagents in 100 ml of deionized water.

2.Blocking solution

Skim milk-5% (w/v) in 1X TBST

3.10X electrode buffer (Running buffer, pH 8.3)

TriZma base	: 25 mM
Glycine	: 192 mM
SDS	: 1%

Dissolve all the reagents in 1 L of deionized water.

4.Ponceau S stain

1% ponceau in 5% glacial acetic acid.

5.8X Resolving gel buffer (pH 8.8)

SDS-0.2%, Tris- 3M in deionized water.

6.RIPA (Radio Immuno Precipitation Assay) buffer pH 8.8

Sodium chloride	: 150mM
NP-40	: 1%
Sodium deoxycholate	: 0.5%
SDS	: 0.1%

Dissolve all reagents in deionized water.

7.SDS gel loading buffer (2X) pH 6.8

SDS	: 4%
2-meraptoethanol	: 10%
Glyerol	: 20%
Bromophenol blue	: 0.004%
Tris Hcl- 0.125M	

Dissolve all reagents and make the volume up to 1 L by deionized water.

8.4X stacking gel buffer (pH 6.8)

SDS	: 0.1%,
TriZma base	: 0.5M

Dissolve all reagents and make the volume up to 1 L by deionized water.

9.10X Towbin's buffer (Transfer buffer, pH 8.3)

TriZma base	: 25 mM
Glycine	: 192 mM
Methanol	: 20 %

Dissolve all reagents and make the volume up to 1 L by deionized water.

10.Tris buffered saline (10X, pH 7.6)

Tris base	: 24.2 g
Sodium chloride	: 80 g

Dissolve all reagents and make the volume up to 1 L by deionized water

11.Tris buffered saline with Tween 20 (TBST, 1X)

1X TBS containing 0.5% Tween 20.

12.To prepare 8% resolving gel (10 ml)

30% acrylamide: bis-acrylamide (29:1)	: 2 ml
8X resolving gel buffer	: 1.25 ml
TEMED	: 10 µl
20% APS	: 25 µl
Deionized water	: 6.5 ml

13.To prepare 5% stacking gel (5 ml)

30% acrylamide: bis-acrylamide (29:1)	: 0.625 ml
5X stacking gel buffer	: 1.25 ml
TEMED	: 10 µl
20% APS	: 13.5 µl
Deionized water	: 3.125 ml

TARGETING DRUG RESISTANCE IN CANCER CELLS BY THE ANTHELMINTHIC DRUG, PYRVINIUM PAMOATE

by

KEERTHANA SURESH K.

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Abstract of the Thesis

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ABSTRACT

Chemotherapy is the first line of option for cancer treatment and drug resistance remains a major impediment to achieve positive therapeutic benefit often results in therapy failure and tumour relapse. Multiple mechanisms are at play in enforcing drug resistance in tumours, some which are inherently active in certain tumour types. Others are effected during the course of chemotherapy. Overcoming drug action and survival depends primarily on the ability of the cancer cell to adapt to the rapidly changing environment. This includes metabolic rewiring which enables the cancer cell to utilize available resources for synthesis of ATP and growth. The major pathways yielding ATP in any cell are glycolysis and mitochondrial respiration. Tumour cells preferentially utilize glycolysis for growth even in the presence of adequate oxygen and this addiction to glycolysis is termed 'Warburg effect'. Although mitochondria of tumour cells were previously thought to be defective and the cause of 'Warburg effect', recent research has proof that tumour cell mitochondria are active and respire. Eventhough glycolysis is less efficient in terms of ATP synthesis compared to mitochondrial oxidative phosphorylation per glucose molecule used, it is preferentially used by tumours since it is ideally suited for hypoxic environment commonly found in tumours. Secondly, glycolytic intermediates are shuttled for the synthesis of macromolecules like nucleotides, fatty acid metabolism and amino acids essential for rapid growth and cell division. Glycolysis also synthesizes reducing equivalents like NADPH and NADH essential for sustaining biochemical reactions. Most anticancer drugs target the glycolytic pathway and a subset of cancer cells survive by rapidly shifting to oxidative phosphorylation. This ability to shuttle between pathways is termed metabolic plasticity, and is the prime mechanism that enables the cancer cells overcome drug effect. The second important mechanism to tide over drug effect in cancer cell is the engagement of drug efflux pumps that actively exclude drugs from cancer cells thereby rendering them ineffective. Passive mechanisms include expression of chaperone proteins belonging to Heat Shock Protein (HSP) family that stabilize their substrates thwarting drug efficacy. Chemicals which are cost effective, possess good safety profile and having the

capability for targeting multiple mechanisms will be the most potent agents to overcome drug resistance in tumours. Pyrvinium pamoate, an FDA approved anthelminthic agent is an ideal candidate that matches the description.

This project explored if Pyrvinium pamoate (PP) as a single agent or along with regular chemotherapeutic drugs could be used to overcome cancer drug resistance. Cell viability assays with MCF-7 cells showed that IC50 of PP was 280nM whereas the IC50 values of 5-Fluoro Uracil (5-FU) and Doxorubicin(Dox) were 2.77μM and 4.7 μM respectively. PP at 500nM was able to reduce the IC50 of Dox from 2.77μM to 0.59 μM, a five fold reduction of effective dose in combination. PP at 500nM was able to reduce the IC50 of 5-FU from 4.7μM to 0.35 μM, a seven fold reduction of effective dose in combination. PP was equally effective in Dox resistant MCF-7 cells generated over a 21 day time frame. PP at 500nM reduced the IC50 of Dox in Dox resistant cells from 5.33 μM to 1.35 μM a reduction of effective dose by more than three fold. PP rapidly induced mitochondrial reactive oxygen species generation evident from the diffused red fluorescence of Mito SOX dye in PP treated cells compared to control MCF-7 cells which showed little dye expression. PP also reduced mitochondrial respiration evident from the reduction of basal respiration rate from 700 pmol/s/mg to 350 pmol/s/mg of O_2 consumption measured in MCF-7 cells by respirometry experiment in OROBOROS. The ATP dependent respiration was reduced from 600 pmol/s/mg to 50 pmol/s/mg of O_2 by treatment with PP. The maximal respiration was reduced from 1050 pmol/s/mg to 400 pmol/s/mg of O_2. The respiratory reserve capacity of MCF-7 cells was reduced from 300 pmol/s/mg to 75 pmol/s/mg of O_2. The significant reduction of mitochondrial respiration shows that cancer cells use mitochondrial respiration for survival and PP can effectively block the same.

In the light of the results from this study we propose PP as a novel drug candidate to overcome drug resistance in cancer cells. PP targets metabolic plasticity of cancer cells by targeting mitochondrial respiration which the drug resistant cancer cells rely on predominantly. PP also is potent against Dox resistant

MCF-7 cells. PP in combination with 5-FU and Dox is equally efficient in inducing cell death in MCF-7 cells. PP induces rapid generation mitochondrial ROS causing oxidative damage from which cancer cells seldom recover.