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The molecular basis of the phenotype observed when normal cells recover from camptothecin and methyl pyruvate

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Bernice Andrieca Monchusi



A thesis submitted to the Faculty of Science under the School of Molecular and Cell Biology, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2017

Declaration

I, Bernice Andrieca Monchusi (450802), am a student registered for the degree of Doctor of Philosophy (PhD) in the academic year 2017.

I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.

I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing

I hereby declare that this dissertation entitled "**The molecular basis of the phenotype observed when normal cells recover from camptothecin and methyl pyruvate** "is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand.

It has not been submitted for any degree or examination at any other University.



Bernice Andrieca Monchusi

Date: 21 November 2017

Abstract

Cancer has become a major cause of death in modern society. New cancer cases are estimated to increase to 22.2 million by 2030. Many chemotherapeutic drugs, such as irinotecan, target the p53 pathway in rapidly dividing cells. However, chemotherapeutic drugs possess little discrimination between normal and cancer cells since they target DNA replication, a normal physiological process. Many studies indicate that cancer and normal cells prefer different glucose metabolic pathways. Cancer cells rely mainly on glycolysis while normal cells prefer the oxidative phosphorylation (OXPHOS). We hypothesise that manipulating the known metabolic programme of cancer cells to follow the OXPHOS pathway experienced by normal cells may alleviate side effects experienced by normal cells and accelerate cancer cell death. In order to enhance OXPHOS optimal pyruvate levels were used. To investigate the effect of this on normal and cancer cell division, cell viability and cytotoxicity was measured in real-time using *xCELLigence* technology. Flow cytometry was then used to determine the mode of cell death and cell cycle changes induced by the various treatments. In order to characterise the molecular bases of this, differential gene expression upon treatment with the various drugs was measured by RT-PCR and Western blot analysis. Furthermore, we conducted microarray analyses using the Human Cancer PathwayFinder array to determine pathways affected by the drug combination in A549 lung fibroblast cancer and a normal lung fibroblast (MRC-5). Differentially expressed genes with a fold change ≥ 2 were analysed by PANTHER, GeneMania and Reactome. Overall, this study shows that the reversal of the metabolic program in A549 and MDA-MB 231 cancer cells with chemotherapeutic agents accelerates the death of cancer cells while promoting the survival of normal MRC-5 cells. However, in the breast cancer cell line, MDA-MB 231 with mutant p53, it is observed that

inhibition of proliferation is by another cell death mechanism. RT-PCR and western blot analyses indicate that introduction of exogenous methyl pyruvate enhanced the p53/p21 axis of the apoptotic pathway resulting in cancer cell death. While promoting survival in MRC-5 normal lung fibroblast cells by turning off the p53/p21 axis of the apoptotic pathway. Furthermore, the introduction of exogenous methyl pyruvate promotes MRC-5 cell proliferation by modulating the expression of RBBP6 isoform 1 and isoform 3. Microarray analysis indicates alterations in specific genes promote the survival and growth of MRC-5 normal cells and death of A549 lung cancer cells. This knowledge may provide an opportunity to protect patients who undergo chemotherapy from the harsh side-effects and increase the success rate of chemotherapeutic drugs.

Quotation

The future belongs to those who believe in the beauty of their dreams

- *Eleanor Roosevelt*

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International

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Ekene Nweke, Bernice Monchusi, Umar-Faruq Cajee and Monde Ntwasa. The isoforms of the RBBP6 homolog (Snama) are expressed during development and genotoxic stress. 6th Wits Cross Faculty Symposium. University of the Witwatersrand, **28 October 2014**.

Table of Content

Declaration i
Abstract ii
Quotation iv
Acknowledgements v
Research output vi
Table of Content viii
List of Figures x
List of Tables xi
List of Abbreviations xii
Chapter 1: Literature Review 1
1. Introduction 1
1.1 Cancer: A worldwide problem 2
1.2 Metabolic reprogramming of cancer cells 7
1.3 Cell cycle division and check points 14
1.4 Molecular basis of apoptosis 17
1.5 p53 structure, function and regulation 24
1.6 Role of chemotherapeutic drugs in cancer treatment 27
1.7 Irinotecan: Pharmacodynamics 29
1.7.1 Pharmacokinetics of irinotecan 30
1.7.2 Clinical implications of irinotecan 31
1.8 Methyl pyruvate as a potential adjunctive to irinotecan 33
1.9 Justification of the study 35
1.10 Aims 35
1.11 Objectives 35
Chapter 2: Materials and Methods 36
2.1 Materials 36
2.2 Methods 36
2.2.1 Cell culturing 36
2.2.2 Cell viability assay using the xCELLigence system during and after treatment 36
2.2.3 Flow cytometry 37

2.2.4 RNA extraction.....39
2.2.5 Reverse transcriptase polymerase chain reaction.....40
2.2.6 Western blot procedure.....40
2.2.7 Western blot for RBBP6.....41
2.2.9 Bioinformatics analysis.....44
Chapter 3: Results.....48
3.0 Introduction.....48
3.1 The effect of interfering with OXPHOS on cell proliferation.....49
3.2 Exogenous methyl pyruvate reduces apoptosis in irinotecan-treated normal cells.....52
3.3 Methyl pyruvate protects the normal fibroblast cell line by switching off apoptotic pathways.....61
3.4 Methyl pyruvate promotes cell death in cancer cell lines but survival in the normal lung fibroblast cell line.....69
Chapter 4: Discussion.....81
4.0 Introduction.....81
4.1 Methyl pyruvate protects MRC-5 fibroblast from irinotecan induced cell death.....82
4.2 Exogenous methyl pyruvate promotes cell cycle progression in normal cells but cell death in cancer cells.....83
4.3 Methyl pyruvate deregulates apoptoticpathways.....85
4.4 Exogenous methyl pyruvate can also induce another p53-independent and caspase-independent cell death.....86
4.5 The role of RBBP6 during metabolic reprogramming.....87
4.6 Exogenous methyl pyruvate upregulates pro-angiogenic and survival pathways in MRC-5 normal cells and pro-apoptotic pathways in A549 cells.....88
Chapter 5: Conclusion and future prospects.....89
Chapter 6: References.....90
Appendices.....124

List of Figures

Figure 1: A modified diagram of energy metabolism regulated by p53

Figure 2: Modified Schematic representation of the mammalian cell cycle

Figure 3: A schematic representation of p53-mediated apoptosis in both the extrinsic and intrinsic pathways.

Figure 4: Modified schematic representation of the human p53 domain structure.

Figure 5: Chemical structure of irinotecan (7-ethyl-10-[4-[1-piperidino]-1-piperidino] carbonyloxy campothecin) and its metabolites

Figure 6: Chemical structure methyl pyruvate

Figure 3.1 Introduction of exogenous methyl pyruvate protects normal MRC-5 cells from cell death

Figure 3.2 Introduction of exogenous methyl pyruvate protects normal MRC-5 cells from irinotecan induced cell death while promoting cancer cell death

Figure 3.3 Introduction of exogenous methyl pyruvate promote cell cycle progression in MRC-5 normal cells

Figure 3.4 Introduction of exogenous methyl pyruvate deregulates apoptotic pathways in MRC-5 normal cells

Figure 3.5 Genes upregulated and downregulated between MRC-5 and A549 cells after treatment with the drug combination

Figure 3.6 Introduction of exogenous methyl pyruvate protects normal MRC-5 cells by upregulating cell growth and survival pathways while inducing cell death in A549 cells by upregulating apoptotic pathways:

Figure 3.7 Introduction of exogenous methyl pyruvate protects MRC-5 cells by upregulating angiogenesis and cell adhesion processes

Figure 3.8 Exogenous methyl pyruvate protects MRC-5 cells from irinotecan-induced cell death by upregulating Developmental biology, signal transduction and homeostasis pathways

Figure A1. GeneRuler™ 1 kb plus DNA ladder

Figure A2. New England Biolabs blue pre-stained protein standard broad range.

Figure A3. Sub G0/G1 cell cycle distribution of treated and untreated cells

Figure 3.5 Introduction of exogenous methyl pyruvate resulted in deregulation of apoptotic pathways in MRC-5 cells

Figure B2: Pathway diagrams constructed using the REACTOME pathway analysis.

List of Tables

Table 1: Dose schedule of irinotecan administered to cancer patients

Table 2: Genes upregulated after treatment with exogenous methyl pyruvate combined with irinotecan in A549 and MRC-5 cells

Table 3: Genes downregulated after treatment with exogenous methyl pyruvate combined with irinotecan in A549 and MRC-5 cells

Table A1: Cell culturing reagents

Table A2: Cell cultures

Table A3: Biological buffers and solutions

Table A4: Kits and laboratory equipment used

Table A5: Reagents and chemicals

Table A6: Oligonucleotides

Table A7: Antibodies used for Western blot analysis

Table A8: Web address for the various Bioinformatics tools that were used in this study

Table A9: Statistical analysis (means ± SD) of the cell cycle phases for untreated and treated cells

Table B1: 17 unique genes upregulated in A549

Table B2: 13 unique genes upregulated in MRC-5

Table B3: 18 unique genes downregulated in A549

Table B4: 7 common genes upregulated in A549 and MRC-5

Table B5: Upregulated pathways after the drug combination in A549 and MRC-5 cells using GeneMANIA analysis

Table B6: Downregulated pathways after the drug combination in A549 cells using GeneMANIA analysis

List of Abbreviations

ADME:	Adsorption, distribution, metabolism and excretion
AIF:	Apoptosis inducing factor
ATP:	Adenosine triphosphate
CAK:	CDK activating kinase
Caspase:	Cysteine-aspartic proteases
CDK:	Cyclin dependent kinase
CKI:	Cyclin dependent kinase inhibitors
CPEb:	Cytoplasmic polyadenylation element binding protein
CPT:	Camptothecin
CTD:	Carboxyl-terminus domain
DISC:	Death inducing signal complex
DMEM:	Dulbecco's minimal essential media
DNA:	Deoxyribonucleic acid
DWNN:	Domain with no name
FADD:	Fas-associated protein with death domain
FADH:	Flavin adenine dinucleotide
FDH:	Fumarate dehydrogenase
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GLUT:	Glycose transporter
HK:	Hexokinase
MDM2:	Mouse double minute 2

ml:	Milliliters
MOMP:	Mitochondrial outer membrane permeability
NADH：	Nicotinamide adenine dinucleotide
NF-kB:	Nuclear factor kappa B
NOXA:	Phorbol-12-myristate-13-acetate-induced protein 1
OXPHOS:	Oxidative phosphorylation
P52R2:	Ribonucleotide reductase subunit
p53:	Tumour suppressor protein 53
PACT:	Spliced variant of RBBP6 found in mouse
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PDH:	Pyruvate dehydrogenase
PDK1:	Hypoxia inducible pyruvate dehydrogenase kinase
PDP:	Pyruvate dehydrogenase phosphate
PFK-1:	Phosphofructokinase
PP:	Polyproline
PPP:	Pentose phosphate pathway
PUMA:	p53 upregulated modulator of apoptosis
RBBP6:	Retinoblastoma binding protein
RING:	Really interesting new gene
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
SCO2:	Synthesis of cytochrome c oxidase

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TAD: Transactivation domain

TCA: Tricarboxylic acid

TET: Tetramerization domain

TIGAR: TP53- induced glycolysis and apoptosis regulator

TNFR: Tumour necrosis factor receptor

TOPO-1: Topoisomerase 1

UGT: UDP-glucuronosyl transferase

µl: Microliters

µM: Micromoles

Chapter 1: Literature Review

1. Introduction

Energy metabolism is an important process in cell growth and division. Previous studies have indicated, that cancer cells preferentially utilize the glycolysis pathway for energy production and to fuel their growth. Otto Warburg first proposed the "Warburg effect" predicting that cancer cells even in the presence of oxygen will reprogram their metabolism to follow the glycolysis pathway as opposed to normal cells that primarily utilise the oxidative phosphorylation (OXPHOS) pathway for glucose metabolism (Warburg, 1956). It has been suggested that cancer cells limit their energy production to glycolysis to stimulate the pentose phosphate pathway for anabolic processes such as lipid synthesis and *de novo* nucleic acid synthesis, consequently enabling their rapid growth (Deberardinis *et al*., 2008). The tumour suppressor protein 53, p53, has recently been observed to regulate metabolic pathways such as glycolysis and OXPHOS by regulating crucial gene products (Bensaad *et al*., 2006).

DNA damaging agents, such as irinotecan, initiate cancer cell death in a p53-dependent mechanism. However, these chemotherapeutic agents possess non-specificity and affect both cancer and normal cells which is responsible for the severe side effects observed in patients undergoing chemotherapy. Therefore, novel strategies are required to alleviate the damage experienced by normal cells. Recently it was shown that when female fruit flies were exposed to irinotecan, reproduction was adversely affected. However, when exposed to irinotecan in combination with methyl pyruvate, the flies were protected from these adverse effects and the development of embryos improved (Hull and Ntwasa, 2010). In the current investigation,

methyl pyruvate supplementation was used to alleviate the side effects of irinotecan on a normal cell line while reversing the glycolytic phenotype of cancer cells to induce death.

In this chapter, worldwide cancer statistics were summarized and cancer development briefly described followed by a review of energy metabolism in both cancer and normal cells and the role of p53 at crucial check points. Furthermore, the importance of p53 including its interactions with MDM2 and RBBP6 is discussed. Lastly, the cytotoxicity and pharmacokinetics of chemotherapeutic drugs like irinotecan is reviewed, and the biological roles of methyl pyruvate are briefly described.

1.1 Cancer: A worldwide problem

Cancer is a disease characterised as uncontrolled cell division caused by the accumulation of specific genetic and epigenetic changes (Bender *et al*., 1998). According to the World Health Organization (WHO), cancer is the second leading cause of death globally. In 2015, 8.8 million deaths were cancer-related, with approximately 70% of deaths occurring in low- and middle-income countries (WHO, 2017). Cancer initiation and progression is mainly attributed to lifestyle choices such as: tobacco smoke, alcohol consumption, unhealthy diet, physical inactivity, and cancer related infections. Worldwide, cancer-related deaths were attributed to: smoking (21%), alcohol (5%), low fruit and vegetable intake (5%), unsafe sex (3%), overweight and obesity (2%), physical inactivity (2%), contaminated injections in health-care settings (2%), and urban pollution (1%) (Danaei *et al*., 2005).

South Africa is rated as the fourth country with the highest cancer deaths based on 27 different types of cancers (Jemal *et al*., 2011). Amongst men lung, prostate, colorectal, stomach and liver cancers were found to be the most prevalent while in women breast, colorectal, lung, cervix and stomach cancer were most common. Lung and breast cancers resulted in the highest

amounts of deaths in men and women, respectively (Jemal *et al*., 2011). Due to these poor statistics, lung and breast cancers are of interest in this study and are discussed further.

Lung cancer

Lung cancer has claimed over 1.6 million lives in 2015 (WHO, 2017). The highest lung cancer incidence rates were reported in eastern and southern Europe (45.0 and 49.0 per 100 000), and North America (48.5 per 100 000) followed by, Micronesia (46.5 per 100 000), and Polynesia and Eastern Asia (45.0 per 100 000), with the lowest rates in sub-Saharan Africa (2.8 per 100 000) (Jemal *et al*., 2011). Lung cancer has been observed to be amongst the deadliest type of cancer in men since survival rate is very low. A survival rate of less than 5 years were measured to be 15% in the United States, Europe 10% followed by 8.9% in developing countries per the SEER (Surveillance Epidemiology, and End Results) program (Parkin *et al*., 2005). The reason for low survival rate might be the inability to detect lung cancer at its early stages (Jackson *et al*., 2001).

Lung cancer can be classified into two major classes; the small cell lung cancer (SCLC) and the non-small cell lung cancer (NSCLC) (Hainaut and Pfeifer, 2001). Non-small lung cancer accounts for most lung cancer related deaths worldwide (Parkin, 2001). NSCLC can be further divided into subtypes; adenocarcinoma (40% of lung cancer cases), squamous cell carcinoma (30%), large cell carcinoma (15%), and bronchioloalveolar carcinoma (5%) (Aufman and Khalil, 2010).

Several morphological models have been proposed to explain lung cancer development. For the squamous carcinoma, it is thought that normal bronchial epithelium is converted into hyperplastic, metaplastic and dysplastic lesions due to certain oncogenic triggering. These lesions further develop into carcinoma cells that metastasize in the body (Osada and Takahashi, 2002). The primary features of invasive tumours include the disruption of the basement

membrane integrity resulting in metastasis. MMP-1 (matrix metalloproteinase-1) overexpression has been suggested to be responsible for tumour invasion and metastasis (Zhu *et al*., 2001). The main function of matrix metalloproteinases is to degrade the various components of the extracellular matrix and basement membrane (Loffek *et al*., 2011).

The causes of lung cancer have been attributed to factors such as: tobacco smoke, genetic susceptibility, environmental and workplace carcinogens, and nutrition. According to epidemiological evidence, smokers have a 10-fold risk of dying from lung cancer compared to non-smokers and in heavy smokers this risk is estimated to increase by 15-25-fold (Carbone, 1992). In the year 2000, it was estimated that 85% of cancer cases in men were attributed to smoking while 47% in women (Parkin, 2001). Tobacco-specific N-nitrosamine and polycyclic aromatic hydrocarbons such as benzo (*a)* pyrene have been found to be the major lung carcinogens in smoke (Hazelton *et al*., 2005).

Molecular evidence suggests that these carcinogens are initiators of most lung cancers, inducing genetic and epigenetic alterations such as: inactivation or mutation of the tumour suppressor protein, p53 (Ahrendt *et al*., 2000), activation of all the dominant oncogenes, *K-ras* oncogene (Jackson *et al*., 2001), and activation of certain growth factor receptors: EGFR/erbB-1, HER2/erbB-2, HER3/erbB-3, and HER4/erbB-4 (Cappuzo *et al*., 2005). p53 mutations have been reported in more than half of lung cancer cases, with these more commonly associated with smokers than non-smokers (Suzuki *et al*., 1992). It has been reported that benzo*(a)*pyrene diol epoxide, a potent carcinogen in cigarette smoke, bind preferentially to selected regions of the p53 gene. This suggests that formations of benzopyrene adducts correlate with high frequency of certain p53 mutations in smoking-associated tumours (Ahrendt *et al*., 2000). Therefore, targeting the p53 pathway during chemotherapy presents a promising avenue for lung cancer treatment.

Current treatment for advanced stage NSCLC includes a two-drug combination of paclitaxel plus carboplatin and vinorelbine plus cisplatin (Kelly *et al*., 2001). Paclitaxel and vinorelbine are reported to have similar cytotoxicity activity, inhibiting mitosis through interaction with tubulin (Priyadarshini and Aparajitha, 2012). Cisplatin is the parent compound of carboplatin, and are both platinum-based drugs which cause DNA cross-linking with paclitaxel/vinorelbine leading to modification of the DNA structure thereby inhibiting DNA synthesis (Ozols *et al*., 2003). However, these drugs have been reported to be toxic with a high percentage of patients being susceptible to resistance (Kelly *et al*., 2001). New chemotherapeutic strategies (drugs) to protect normal cells from the toxic effects of chemotherapy while inhibiting tumour growth remain a necessity.

Breast Cancer

Breast cancer is the most common and leading cancer-related deaths amongst women worldwide. In 2015, breast cancer accounted for 571 000 deaths worldwide (WHO, 2017). The highest incident rates have been reported in North America (99.4 per 100 000) followed by Western Europe (84.6 per 100 000), Northern Europe (82.5 per 100 000), and Southern Europe (82.4 per 100 000) (Parkin *et al*., 2005). The lowest incidence rates have been reported in Africa (< 30 per 100 000) and Asia (Parkin *et al*. 2005). According to global statistics, survival rates have been modest with 73% in developed countries and 57% in developing countries (Parkin *et al*., 2005). Even though the survival rate of breast cancer cases is high, it is still the most commonly diagnosed cancer amongst women globally.

Major risk factors for predisposition to breast cancer include: nulliparity, long menstrual cycle history, oral contraceptives, use of post-menopausal hormone, and giving birth at an advanced age. Other risk factors consist of, poor diet, lack of physical activity, and alcohol consumption (Hulka and Moorman, 2001; Parkin *et al*., 2005). In North America and several European

countries, breast cancer deaths have decreased over the past 25 years mainly due to early detection through mammography, less frequent use of postmenopausal hormone therapy and improved treatment regimes. However, countries in Africa and India had an increase of breast cancer deaths, which were attributed to physical inactivity, obesity, and lack of early detection technology (Ravdin *et al*., 2007; Jemal *et al*., 2010).

Previous reports suggest that mutations in tumour suppressors (BRCA and p53), oncogenic activation and the involvement of reproductive hormones such as the estrogen (ER) and progesterone (PR) receptors also attribute to the initiation and progression of breast cancer (Miki *et al*., 1994; Kandioler-Eckersberger *et al*., 2000; Perou *et al*., 2000). Several models have been proposed to describe the development of breast cancer. First, the "Two-Component cancer model" proposed by Lilienfeld and Johnson (1955) predicted that breast cancer develops by two distinct pathways. The first pathway results from premenopausal tumours that are linked to estrogen receptor negative and the second from postmenopausal cancers linked to estrogen receptor positive cancers. Furthermore, Reis-Filho and Pusztai (2011) predicted through gene-expression profiling data, that there are two distinct classes, estrogen receptor positive intrinsic molecular subtypes (Luminal A and luminal B) and estrogen receptor negative intrinsic subtypes (HER2-enriched and basal-like). It has been predicted that the intrinsic molecular subtypes can be differentiated by the expression of genes involved in the luminal epithelial differentiation (ER and PR genes), proliferation (Ki67 gene), basal differentiation, and the human growth factor receptor 2 pathway (HER2 gene) (Perou *et al*., 2000).

Mutations in *BRCA1* and *BRCA2* tumour suppressor genes have been linked to early and late-onset of breast cancer risk (Miki *et al*., 1994). These genes have been shown to differentially express hormone receptors. Seventy five percent of *BRCA1* mutated breast cancers are suggested to be estrogen negative and 25% are estrogen positive while the reverse is observed in BRCA2 mutated breast cancers (Karp *et al*., 1997). Mutations in *BRCA1* and *BRCA2* have

been suggested to only attribute for up to 10% of breast cancer cases in developed countries (Parkin *et al*., 2005). Genetic alteration of the p53 gene has also been linked to the susceptibility to early-onset breast cancer. Studies suggest that heterozygote carriers of the mutated form of p53 are at high risk of breast cancer (Miki *et al*., 1994). In a study of patients with advanced breast cancers, there was an observed correlation between the chemotherapy response rate and the p53 status of the cells. Induced apoptosis during chemotherapy was observed in most tumours with normal p53 (Kandioler-Eckersberger *et al*., 2000).

Currently, treatment for advanced breast cancer includes a combination of fluorouracil, epirubicin and cyclophosphamide (FEC) known to induce p53-dependent apoptosis (Levin *et al*., 1998). Another compound also known to inhibit breast tumours, is paclitaxel which induces apoptosis in a p53 independent manner (Hortobagyi *et al*., 1997). However, studies have shown that most patients do not respond to these cytotoxic treatments (Bergh, 1997; Kandioler-Eckersberger *et al*., 2000). SN-38 (irinotecan) (whose homologue is used in this study), has also been used in the treatment of breast cancer, albeit with little success rate as most patients are resistant to the drug (Imai *et al*., 2003). This again strengthens the need to find chemotherapy strategies that are effective in inhibiting cancer cell growth.

1.2 Metabolic reprogramming of cancer cells

For glucose metabolism, normal cells prefer OXPHOS whereas cancer cells utilize glycolysis. Cancer cells use the glycolytic pathway mainly for anabolic processes such as lipid and nucleic acid synthesis to support their rapid growth, rather than for mere ATP production (Warburg, 1956; Locasale and Cantley, 2010).

Cancer cells are characterized by high rates of lactate production, biosynthesis of macromolecules and lipids, and glycolysis (Puzio-Kuter, 2011). Pyruvate produced through

glycolysis under anaerobic conditions can be fermented to lactate by the lactate dehydrogenase A, regenerating NAD^+ from NADH. Lactate (lactic acid), was previously thought to be toxic to cancer cells due to acidification; however, recent studies suggest that the import of lactate into the cancer cell microenvironment positively influences their growth and survival (Sonveaux *et al*., 2008). This can be supported with evidence from Shim *et al*. (1997) and Fantin *et al*. (2006) suggesting that inhibition of lactate dehydrogenase A (LDH-A) can shut down the Warburg effect forcing cancer cells to switch their energy metabolism to oxidative phosphorylation. The reprogramming of these cancer cells from glycolysis to OXPHOS is speculated to inhibit tumour growth. However, the molecular basis of these findings is not fully understood.

Lactate import and export in some cancer cells has been observed to correlate with the deactivation or inactivation of certain chemotherapeutic drugs. The over expression of lactate transporters (MCTs: monocarboxylate transporters) controls the import and export of lactate. MCT1 and MCT4 transporters have been observed to be the main MCTs playing roles in the import and export of lactate respectively (Feron, 2009). High lactate levels have been implicated in tumour reoccurrence and metastasis (Walenta *et al*., 2000). A crucial interaction exists between the MCT transporters and the LDH enzymes. In MDA-MB 231 breast cancer cells, MCT4 transporter and LDHB (lactate dehydrogenase B) were both overexpressed, consequently the lactate taken up by these breast cancer cells are converted back to pyruvate to achieve maximum ATP production (Hussien and Brooks, 2011).

Cancer cells limit their energy production to glycolysis to stimulate the pentose phosphate pathway (PPP) and consequently enable growth and proliferation. It has been suggested that TIGAR (TP53-induced glycolysis and apoptosis regulator) and HK2 (Hexokinase) – an enzyme that catalyses the first step of glycolysis - enhance the supply of intermediates to the PPP (Maddocks and Vousden, 2011). The NADPH and ribose-5-phosphate produced in the

PPP are linked to the biosynthesis of lipids and nucleic acids (Deberardinis *et al*., 2008). Another enzyme, transketolase (TKTL1) has also been suggested to be involved in the PPP, contributing to the survival of cancer cells under stress and starvation (Xu *et al*., 2009). The TKTL1 enzyme cleaves xylulose-5-phosphate, a five-carbon sugar, to glyceraldehyde-3-phosphate, a three-carbon sugar. This glyceraldehyde-3-phosphate can enter the glycolytic pathway to generate ATP and lactate (Coy *et al*., 2005). The NADPH produced has also been suggested to enable cancer cells maintain low levels of reduced forms of glutathione (GSH). The GSH produced helps to maintain the redox status of cancer cells thereby protecting them against antineoplastic agents (Traverso *et al*., 2013).

Strong evidence also suggests, that mutations in tumour suppressors and oncogenes can influence glycolysis in cancer cells. For instance, mutations in the DNA binding domain of p53 is suggested to stop the repression of GLUT1 and GLUT4, leading to an increase in glucose uptake (Schwartzenberg-Bar-Yoseph *et al*., 2004). This high glucose uptake consequently leads to high glucose metabolism which increases the rate of ATP production during glycolysis, favoring cancer progression (Ganapathy-Kanniappan and Geschwind, 2013).

Since several studies have stipulated the positive role played by the glycolysis pathway in cancer progression, inhibition or circumvention of this pathway might provide an avenue to hinder growth.

1.2.1 Glycolysis and p53

Glycolysis, also known as the Embden-Meyerhof pathway, is the preferred glucose metabolism pathway of cancer cells (Warburg, 1956). Cancer cells mainly prefer glycolysis as opposed to OXPHOS as they can easily become hypoxic due to their rapid growth which in turn would limit the supply of oxygen (Gogvadze et al., 2009). This pathway involves the oxidation of glucose to pyruvate with the production of ATP and NADH (Figure 1) (Pelicano *et al*., 2006).

Net reaction: Glucose + 2 NAD^+ + P_i + 2 ADP = 2 Pyruvate + 2 ATP + 2 NADH

The pyruvate produced in the presence of oxygen is oxidized to carbon dioxide and water in the TCA cycle, while in the absence of oxygen is fermented to lactate or ethanol. Some normal tissues such as the myocardium and brain have been reported to have glycolytic phenotypes in the presence of oxygen (Amoedo *et al*., 2013).

The p53 protein has been suggested to play an important role in glucose metabolism and its presence in a cell, promotes oxidative phosphorylation (OXPHOS) while limiting the glycolytic flux by acting on key enzymes (Figure 1) (Bensaad *et al*., 2006). Mutations in p53 have been implicated in most tumours (Miyashita and Reed, 1995). p53 can limit glucose uptake either through the direct suppression of the glucose transporters (GLUT1 and GLUT4), or indirectly by the inhibition of NF-κB pathway. (Schwartzenberg-Bar-Yoseph *et al.,* 2004; Kawauchi *et al.,* 2008).

Furthermore, p53 decreases the expression of phosphoglycerate dismutase (PGM). PGM is a functional enzyme in the glycolytic pathway that converts 3-phosphoglycerate to 2-phosphoglycerate- the second intermediate before the formation of the pyruvate isoform (Kondoh *et al*., 2005). Also, TIGAR gene transcription is shown to be induced by p53 and its presence inhibits phosphofructokinase 1 (PFK1) which further impedes glycolysis (Bensaad *et al*., 2006). The decrease in glycolysis allows entry into the PPP, while limiting oxidative stress-induced cell death. These observations show that an increased in p53 expression might shunt glycolysis to anabolic processes in the PPP.

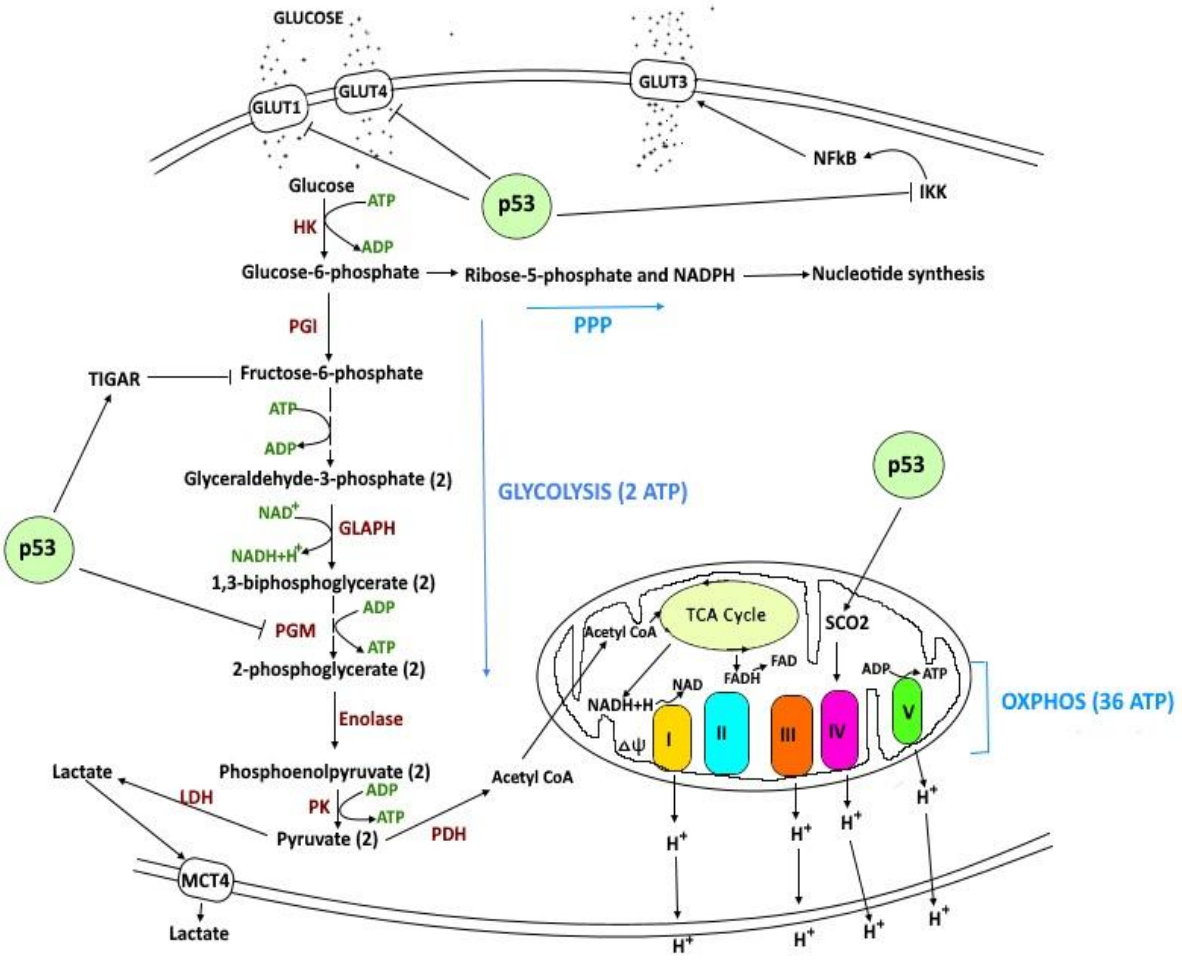


Figure 1: A modified diagram of energy metabolism regulated by p53. p53 modulate the balance between glycolysis and oxidative phosphorylation (Pelicano *et al*., 2006; Maddocks and Vousden, 2011). When p53 is present, it inhibits IKK which is needed for the activation of GLUT3 thereby reducing glucose uptake. p53 can also reduce glucose uptake suppressing the expression of the GLUT1 and GLUT4 transporters. Glucolysis is further suppressed by p53, by decreasing the expression of phosphoglycerate dismutase (PGM) while inducing the expression of TP53-inducible glycolysis and apoptosis regulator (TIGAR) which inhibits the PFK1. p53 plays a role in the activation of SCO2 which results in the maintenance of the oxidative phosphorylation pathway. The enzymes PDH: represent pyruvate dehydrogenase, PK: pyruvate kinase, HK: hexokinase, PGI: phosphoglucose isomerase, and GLAPH: GLAP hydrogenase. The dotted lines/arrows were used to present a reaction step that was omitted.

1.2.2 Oxidative phosphorylation and p53

Studies have shown that normal mammalian cells, in the presence of oxygen, prefer the oxidative phosphorylation pathway as it is efficient and generates more ATP per glucose molecule than glycolysis (Warburg, 1956). The two pyruvate molecules produced from glycolysis are each converted to acetyl CoA before entering the TCA cycle (citric acid cycle) to produce ATP through oxidative phosphorylation, also known as the electron transport chain

(Figure 1). The initial process of oxidative phosphorylation begins with the products of the TCA cycle, NADH and FADH, being broken down to produce electron acceptors (H^+) catalyzed by the enzyme, ATP synthase (complex V). ATP synthase enables the production of ATP with the addition of a phosphate group to ADP through a concentration gradient on the inner membrane of the mitochondria. Oxidative phosphorylation yields a net reaction of 36 ATP molecules per glucose molecule (Pelicano *et al*., 2006).

p53 increases the expression of synthesis of cytochrome c oxidase 2 (SCO2). SCO2 is an important positive regulator of complex IV (cytochrome c oxidase) and AIF (apoptosis inducing factor protein) which in turn promotes OXPHOS (Matoba *et al*., 2006). p53 can also promote OXPHOS by upregulating glutaminase 2 (GLS2) which converts glutamine to glutamate improving the rate of the TCA cycle. Furthermore, several anti-apoptotic genes have been shown to promote OXPHOS. BCL-X_L and *PUMA*, together with p53 influence the outer mitochondrial membrane permeabilization ensuring a balance between cell survival and apoptosis (Mihara *et al*., 2003). Understanding the link between p53 and metabolism in both normal and cancer cells might aid in establishing better chemotherapy strategies for the treatment of cancer.

1.2.3 Glycolytic phenotype of cancer cells: A potential target for therapy

Many reports over the years have described strategies for achieving therapeutic selectivity during chemotherapy. Only recently have metabolic pathways been suggested to be good targets (Hanahan and Weinberg, 2011). Warburg initially proposed that cancer cells even in the presence of oxygen will prefer the glycolysis pathway while most normal cells the OXPHOS pathway for glucose metabolism (Warburg, 1956). A recent study using clinical imaging of primary and metastatic cancers with fluoro-deoxy-D-glucose Positron Emission

Tomography (Fdg PET) has demonstrated that an increased glucose flux is a common trait of human malignancies compared to normal tissues (Gambhir, 2002).

Currently, several drugs have been documented that are under development or in clinical trials, and target the glycolytic metabolism of tumours (Porporato *et al*., 2011). Some of these drugs include; 2-Deoxyglucose (2-DG) a competitor with glucose, which is in phase II clinical trials (Gambhir, 2002; Tennant *et al*., 2010). 3-bromopyruvate, an alkylating agent, has been proposed to react with cysteine residues in proteins undergoing preclinical trials (Ko *et al*., 2001; Qin *et al*., 2010). Lonidamine a specific inhibitor of mitochondrial bound hexokinase is in clinical trials (Floridi *et al*., 1981). 3-Pyridyl-1-(4-pyrydinyl)-2-propen-1-one (3PO) is also still in preclinical trails, and has been shown to decrease concentration of phosphofructokinase 2 leading to glucose uptake (Clem *et al*., 2008). Dichloroacetate (DCA) inhibits pyruvate dehydrogenase kinase (PDK) thereby allowing the entry of pyruvate into the TCA cycle is in phase I (brain cancer) and phase II (non-small cell lung cancer) clinical trials (Whitehouseand Randle, 1973). 3-Dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic (FXII), a known malarial LDH (lactate dehydrogenase) inhibitor, was shown to be a competitive inhibitor of lactate dehydrogenase 5 (LDH5) in human lymphoma and pancreatic cancer xenografts is in preclinical trials (Yu *et al*., 2001). Despite their success rate thus far, some of these drugs/molecules need to be administered as adjunctives to therapy to maximise effectiveness of the primary, main or initial therapy. In a study, 2-deoxyglucose was shown to be effective in sensitizing osteosarcoma and non-small cell lung cancer to adriamycin (inhibits progression topoisomerase II) and paclitaxel (stabilises the microtubule polymer) (Maschek *et al*., 2004).

The role of glycolysis in enhancing tumour growth reinforces the need to search for small molecules, to bypass or inhibit the glycolytic pathway in cancer cells while increasing the effectiveness of chemotherapy drugs.

1.3 Cell cycle division and check points

The principle task of the cell cycle is to replicate DNA without errors ensuring that the duplicated chromosomal DNA is equally split between two daughter cells (Nigg, 1996). The somatic cell cycle can be divided into four distinct phases: the G_0/G_1 phase, S-phase, G_2 phase and the M-phase (Figure 2). During the S-phase, DNA is replicated through a process known as DNA synthesis which results to two chromatids in each chromosome. The M-phase consists of nuclear division, followed by cell division. The G_1 and G_2 phases are considered as gap periods during which the cells prepare themselves for the successful completion of the S and M phases. Cells can however exit the cycle- caused by specific anti-mitogenic signals or the absence of mitogenic signaling- and enter a non-dividing state (quiescent state) known as the G_0 phase/ resting phase (Park and Lee, 2003).

For a successful cell division to occur, checkpoints must be tightly regulated (Park and Lee, 2003). The cell cycle is controlled by a group of proteins known as the cyclin dependent kinases (CDKs) (Sherr, 2000). The activities of the CDKs are positively regulated by cyclins and by the CDK activating kinase (CAK)- a serine/threonine kinase (Nigg, 1996). During the early G1 phase, the D-type cyclins (D1, D2, and D3) are predicted to interact with two catalytic partners (CDK4 and CDK6). As transition occurs from G_1 to S phase, the CDK2 forms a complex with cyclin E and A. For mitosis to proceed, the CDK1 must form a complex with cyclin A and cyclin B (Park and Lee, 2003).

When damaged DNA is encountered at any point during the cell cycle checkpoints, the regulatory signals induce the cyclin dependent kinase inhibitors (CKIs) which act as brakes to stop cell cycle progression until damage is fixed (Sherr, 2000). Members of the retinoblastoma family; pRB, p107, and p130, are observed to be part of the CKIs and therefore play a crucial role in G_1 progression. Furthermore, p53 restricts cellular growth by inducing p53-dependent

cell cycle arrest, at the G_1 and G_2 /M checkpoint ensuring damaged DNA does not replicate (Adams, 2001; Haupt *et al*., 2003).

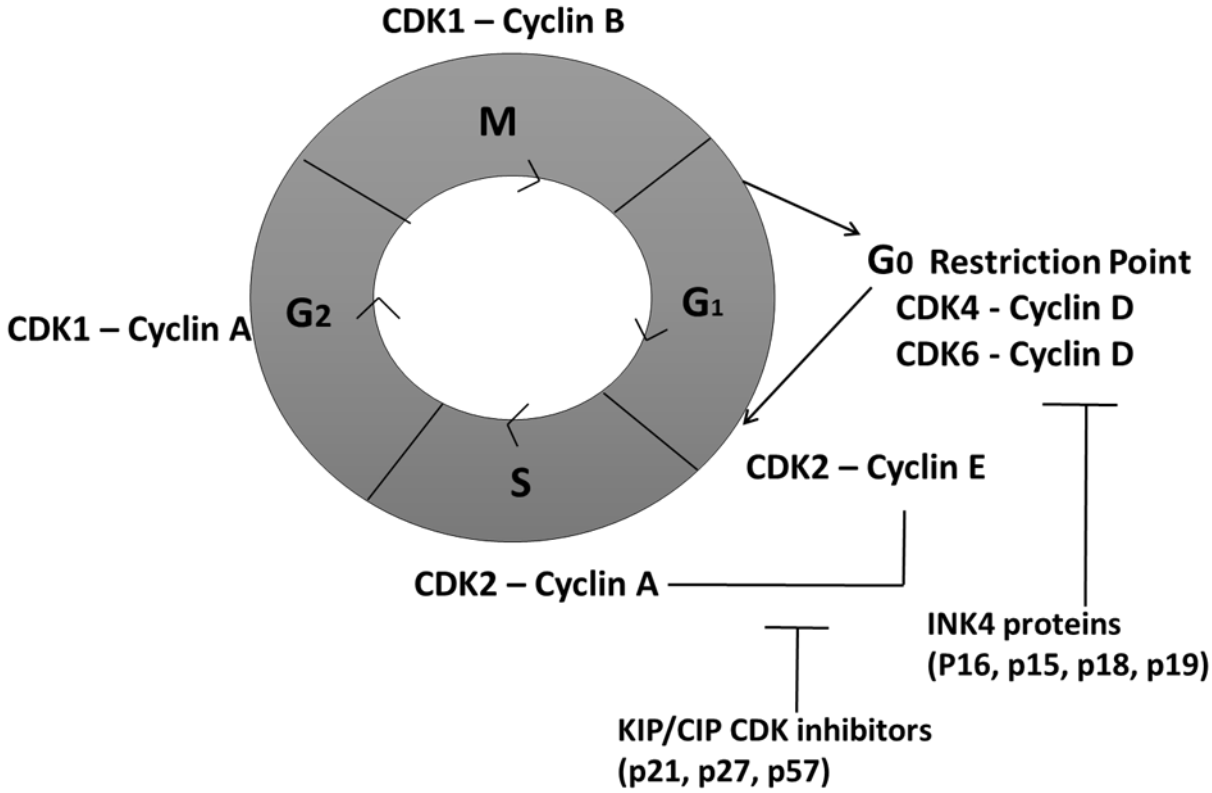


Figure 2: Modified Schematic representation of the mammalian cell cycle with the CDK regulators and cyclin dependent kinase inhibitors at each phase. Adapted from (Collins *et al*., 1997). Progression through the cell cycle phases are controlled by the action of specific CDKs and their cyclin subunits. The activity of CDKs is suppressed by two main classes of inhibitory proteins; the INK4 family which inhibits the activity of CDK4 and CDK6 and the CIP/KIP family which inhibits the activity of CDK2. The abundance of these positive and negative regulators determines the rate of cell cycle progression.

1.3.1 Cell cycle changes and development of cancer

The cell cycle process is known to be disrupted in many cancers. Normal healthy cells only enter the cell cycle when stimulated by growth signals, which ensures a balance in cell number and maintenance of tissue function (Hanahan and Weinberg, 2011). Cancer cells, compared to normal cells, have been associated with certain characteristics such as loss of differentiation,

limitless replicative potential, self-sufficiency in growth signals, and increased invasiveness (Hanahan and Weinberg, 2000).

Tumour suppressor protein- p53, and gene product- p16, have been suggested to regulate the cell cycle (Jacks and Weinberg, 1998). When p53 is mutated it is unable to bind to damaged DNA, which further affects the expression of p21. p21 is a downstream effector gene in the p53-pathway and causes cell cycle arrest when induced (Sherr, 2000).

Moreover, several components of the ERK and PI3K signaling pathways has also been implicated in carcinogenesis (Vivanco and Sawyers, 2002; Downward, 2003; Massague, 2004). Activation of PI3K/Akt signaling along with ERK signaling are known to regulate growth factor-stimulated cell cycle progression from G_0 /G_1 to S-phase (Torii *et al*., 2006). It has been shown that cyclin D1 expression is enhanced in 50% of breast cancers (Yu *et al*., 2001). Activation of ERK signaling pathway has been suggested to induce first class G1 cyclins, such as cyclin D which in turn lead to the upregulation of cyclin D – CDK 4/6 complex (Lavoie *et al*., 1996). The activation of the cyclin D- CDK 4/6 kinase activity further leads to the subsequent phosphorylation and inactivation of retinoblastoma (Rb), resulting in the activation of E2F family of transcription factors. E2F is known to induce the expression of various cell cycle genes, including a second class G1 cyclins, such as cyclin E, thereby leading to the synthesis of proteins required for S phase entry (Dyson, 1998). Moreover, Human EGF receptor-2 (HER2) and Platelet-derived growth factor (PDGF) expression has also been found to be elevated in breast cancers and glioblastoma (Vivanco and Sawyers, 2002; Massague, 2004).

Furthermore, PI3K/Akt has also been implicated in CDK activation. Through the phosphorylation and inhibition of glycogen synthase kinase 3 β (GSK 3 – β), which is known to phosphorylate and destabilize cyclin D1 protein, and the inhibition of FOXO transcription

factors known to repress cyclin D 1 expression, inducing expression of p27 (kip1) and p21 (WAF) (inhibitiors of cyclin activity) (Vivanco and Sawyers, 2002; Wynmann and Marone, 2005). Also, it is been found that PTEN, which acts as a PI3K signaling inhibitor is depleted in approximately 30 – 40% of all human cancers (Yu *et al*., 2001).

1.4 Molecular basis of apoptosis

Programmed cell death, also known as apoptosis, is an evolutionary cell death mechanism found throughout all species (Wyllie, 1981). DNA damage is one of the internal signals that influence the activation of apoptosis (Elledge, 1996). Ataxia telangiectasia mutated (ATM) and CHK2 are known as important gatekeeper molecules in response to DNA damage. p53 has been established to be a key regulator of DNA damage induced programmed cell death. p53, upon DNA damage, become phosphorylated by ATM thereby increasing its stability and binding specificity to induce programme cell death (Lukas *et al*., 2004; Yang *et al*., 2004). Thes failure of this to occur is linked to loss of genomic material and carcinogenesis (Hoeijmakers, 2001).

Apoptosis has been characterised by morphological changes that include membrane blebbing, cell shrinking, condensation of nucleoplasm and cytoplasm, cleavage of chromosomal DNA, and fragmentation of the nucleus followed by eventual cell death (Wyllie, 1981). The apoptotic machinery is composed of two major pathways: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway.

1.4.1 The extrinsic pathway

The extrinsic pathway is activated through the binding of the Fas ligand found on the surface of a cytotoxic lymphocyte to the Fas death receptor, thereby initiating apoptosis (Wu *et al*., 1997). These death receptors (Fas/CD95, PERP, DR4 and DR5/TRAIL) belong to the tumour necrosis factor receptor (TNFR) gene family (Nagata and Golstein, 1995). When activated by

p53, the death receptors transmit apoptotic signals to the cytoplasmic region of Fas after their interactions with specific ligands (Gewies, 2003). This cytoplasmic region of the death receptor (Fas) recruits procaspase-8 via the Fas associated death domain (FADD) (Figure 3). FADDs are adaptor molecules that contain the death domain allowing them to be recruited to similar domains on the activated death receptors (Fas). The interaction between Fas, FADD, and procaspase-8 forms the death inducing signal complex (DISC) which further results in the activation and the release of caspase-8 (Nagata and Golstein, 1995). Caspase-8 leads to the activation of the downstream effector caspases such as procaspase-3 whose activated form, caspase-3, is known to execute programmed cell death (Gewies, 2003).

1.4.2 BID: Linking the extrinsic and intrinsic pathways

BID is an important pro-apoptotic protein as it links the extrinsic and intrinsic pathways (Gewies, 2003). Upon DNA damage, the cytoplasmic inactive BID is cleaved by caspase-8 into the truncated BID (t-BID) which translocates to the mitochondria where it enters the mitochondrial membrane and activates Bax. t-BID (BH3-domain containing protein) and BAX – proapoptotic protein - are then recruited to the outer membrane of the mitochondria to initiate the intrinsic cell death pathway (Haupt *et al*., 2003) (Figure 3).

1.4.3 The intrinsic pathway

The intrinsic mitochondrial cell death pathway is activated by intracellular stress signals such as damaged DNA. This pathway involves the release of cytochrome c from the mitochondria after its permeabilisation by the Bcl-2 family of proteins. Apoptosis is achieved when procaspase-9, the initiator caspase, is cleaved to caspase-9 which in turn activates other effector caspases downstream (Gewies, 2003).

The interaction of the proapoptotic proteins and BH3-domain containing proteins with p53 induces mitochondrial outer membrane permeabilization (MOMP) (Dewson and Kluck, 2009).

MOMP leads to the release of cytochrome c from the mitochondrial intermembrane space into the cytosol. Once cytochrome c is released, it binds to Apaf-1 (an adaptor protein), dATP and procaspase-9 leading to the activation of caspase-9. Caspase-9 then activates other effector caspases (Caspase-3, -6 and -7) resulting in programmed cell death (Crompton, 2000). During this process, p53 can also upregulate *PUMA* and *NOXA*. These two proteins bind to anti-apoptotic proteins (Like the Bcl-2 family protein) thereby preventing their binding to Bax which will inhibit outer mitochondrial membrane permeabilization (Chipuk et al., 2004).

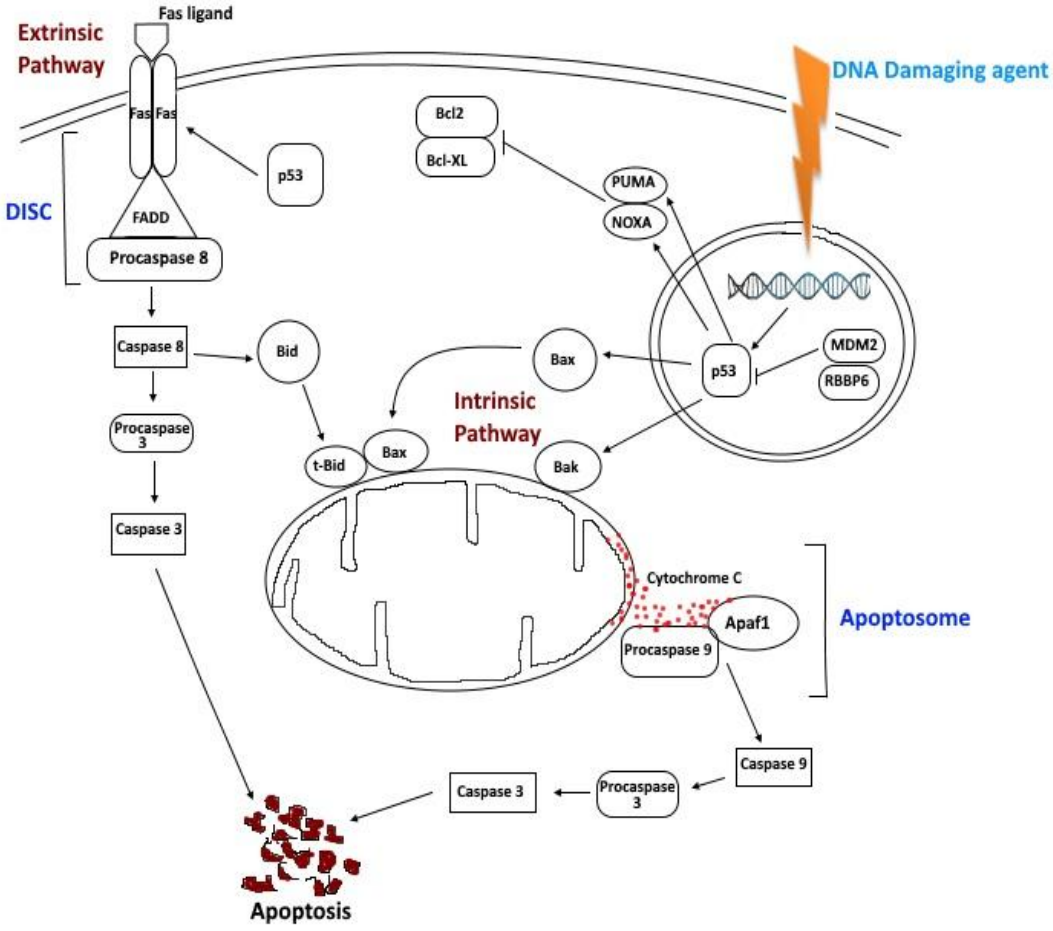


Figure 3: A schematic representation of p53-mediated apoptosis in both the extrinsic and intrinsic pathways. Adapted from Haupt *et al*. (2003). p53 is an important regulator for both the intrinsic and the extrinsic pathway. p53 upregulates the expression of the BH3-only proteins, PUMA and Noxa which further promotes outer mitochondrial membrane permeabilization during intrinsic apoptosis. For the extrinsic receptor-mediated pathway to be activated, p53 needs to activate the Fas death receptor found on the cell surface of a cytotoxic lymphocyte.

1.4.4 Other cell death mechanisms: Necrotic cell death

Under cellular stress, such as nutrient deficiency, an autophagic program is activated. This initiates the breakdown of cellular organelles such as ribosomes and mitochondria which are recycled and used for the biosynthesis of macromolecules (such as lipids, nucleic acid and proteins). This program is exploited by many cancers (Levine and Kroemer, 2008; Mizushima, 2007).

Necrosis, unlike occurs when damaged or inflamed cells become bloated and burst. Necrotic cell death has for many years been thought to occur in an upregulated fashion, however, emerging research suggests that it is controlled by certain genes thus, a form of programmed cell death (necroptosis) (Barkla and Gibson, 1999; Galluzzi and Kroemer, 2008; Roach and Clarke, 2000; Zong and Thompson, 2006).

It has been observed that programmed necrosis occurs due to an increase in mitochondrial-derived ROS and calcium (Festjens *et al*., 2006). Increased calcium levels over time have been suggested to disrupt mitochondrial inner membrane integrity and thereby reducing the ability to generate ATP (Griffiths and Halestrap, 1995) further leading to necrotic cell death. Necrotic cell death can be characterized by morphological changes including, swelling of cell membranes, chromatin condensation, and an irregular DNA degradation pattern leading to breakdown of plasma membrane thereby releasing the cytoplasmic content into the extracellular environment. Also, necrosis triggers inflammatory and autoimmune reactions due to the cellular damage that affects neighboring cells caused by the leakage of intracellular contents (Escobar *et al*., 2015).

The proposed mechanism of necroptosis is suggested to involve the TNF-R, Fas, and TRAIL death receptors belonging to the tumour necrosis factor superfamily known to initiate extrinsic apoptotic cell death. When procaspase-8 levels are low a different complex is formed, involving the activation of TNFR1 leading to the recruitment of TRADD, TRAF and RIPK1

which activate Nf-kappaB, JNK, and p38 MAPKs, thereby causing increased ROS and calcium production and decreased ATP, leading to necroptosis (Kaczmarek *et al*., 2013). The necroptosis process is RIPK1-dependent in contrast to the apoptotic pathway that is caspase-dependent.

1.4.5 Mechanism of apoptosis in cancer cells

During carcinogenesis, apoptosis is achieved through three distinct mechanisms; impaired balance between anti-apoptotic and pro-apoptotic proteins, disruption of death receptors signaling, and finally through loss of caspase activity (Daniel and Korsmeyer, 2004; Wong, 2011).

The Bcl-2 family proteins are essential in controlling the intrinsic mitochondrial cell death pathway. To date, 20 members of this family have been identified in humans including anti-apoptotic proteins (Bcl-2, Bcl-$X_{L,}$ Mcl-l, Bfl-1, Bcl-W, Bcl-G) and pro-apoptotic proteins (Bax, Bak, Bok, Bad, Bid, Bik, Bim, Bcl-Xs, Krk, Mtd, Nip3, Nix, Noxa, Bcl-B) (Antonsson and Martinou, 2000; Oda *et al*., 2000). For apoptosis to be induced, pro-apoptotic proteins translocate and enter the mitochondrial membrane leading to permeabilization of the inner and/or outer mitochondrial membrane and the subsequent release of cytochrome c (Antonsson and Martinou, 2000). Furthermore, anti-apoptotic proteins such as Bcl-X_L are suggested to bind and inactivate Apaf-1 which further blocks the activation of caspase-9. However, pro-apoptotic proteins when active can displace Bcl-X_L from Apaf-1, allowing the activation of caspase-9 (Green and Reed, 1998). Therefore, cell death or survival is determined by the ratio of expression of the pro-apoptotic proteins such as Bax and the anti-apoptotic Bcl-2 or Bcl-X_L proteins (Kluck *et al*., 1997).

It has been found that an overexpression of Bcl-2 promotes cell survival by blocking programmed cell death (Hockenbery *et al*., 1990). Another study showed that Bcl-2

overexpression promoted lymphoproliferation and accelerated c-myc-induced lymphomagenesis in transgenic mice (Strasser *et al*., 1990). Moreover, Bcl-X_L, a known potent death suppressor is upregulated in some tumour types (Reed, 1998). The Bcl family proteins have therefore become attractive anti-cancer targets as downregulation of Bcl-2 and Bcl-X_L has been predicted to restore sensitivity to chemotherapy (Daniel *et al*., 2001). Current anticancer agents targeting the Bcl-2 family include, taxanes, which induce phosphorylation and inactivation of Bcl-2 (Haldar *et al*., 1995). In some studies, resistance to chemotherapy has been found to be related to increased levels of expression of Bcl-2 and Bcl-X_L (Minn *et al*., 1995).

Furthermore, several upstream and downstream components of the p53 pathway such as MDM-2, ARF, and Bax are known to be mutated in some human tumours (Wallace-Brodeur and Lowe, 1999). p53 is a direct transcriptional activator of Bax, a death promoter. Bax expression is induced through the binding of p53 to its typical recognition elements located in the Bax gene promoter, leading to its direct transactivation following damage DNA signals (Miyashita and Reed, 1995). Bax has been shown to be inactive in certain types of colon cancer and in hematopoietic malignancies (Rampino *et al*., 1997; Meijierink *et al*., 1998). It has been suggested that the mutational inactivation of the proapoptotic genes, Bax or Bak are responsible for the resistance to chemotherapy-induced apoptosis in cancer cells (Zhang and Fang, 2005).

Furthermore, glycolytic enzymes, hexokinase-I and hexokinase-II have been found to be upregulated in most tumours, including brain tumours (Mathupala *et al*., 2006). Hexokinase enzyme is known to phosphorylate glucose that enters the cell to glucose-6-phosphate. The interaction of hexokinase with voltage-dependent anion selective channel (VDAC) permits it to use exclusively intra-mitochondrial ATP to phosphorylate glucose, thereby promoting a high

rate of glycolysis (Mathupala *et al*., 2006). VDAC, a protein located in the outer mitochondrial membrane is responsible for most of the metabolite fluxes between the cytosol and mitochondria (Colombini, 1983). This interaction keeps VDAC in an open state and has further been suggested to block binding sites for pro-apoptotic proteins (tBID, Bax) on the outer mitochondrial membrane which further prevents induction of apoptosis (Pastorino *et al*., 2002).

Alterations in TRAIL-mediated death pathways has been reported in many human tumours (Beltinger *et al*., 1998). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a known TNF-related protein that upon binding to its receptors, DR4 or DR5, initiates apoptosis through the extrinsic pathway or through necroptosis. TRAIL has become a very attractive target for its strong anticancer activity in a wide range of cancer cell types, with minimal cytotoxicity to most normal cells (Pitti *et al*., 1996). It has been suggested that the expression of the "decoy" receptors has made normal cells less susceptible to TRAIL-induced apoptosis. These "decoy" receptors compete with the DR4 and DR5 receptors for TRAIL, however, do not transmit death signals (Pan *et al*., 1997). TRAIL therapy has been successful in many cancer types, however, many cancer cells have been found to be resistant to TRAIL, including pancreatic cancer, melanoma, and neuroblastoma (Hinz *et al*., 2000; Eggert *et al*., 2001; Fulda *et al*., 2001). To overcome these resistance, combination therapy with TRAIL and chemotherapy or radiotherapy has been employed, however this has raised concerns about the toxic effect on normal cells or tissues (Shankar and Srivastava, 2004).

Finally, the mammalian inhibitor of apoptosis protein (IAP) family proteins including survivin, ML-IAP and livin are possible targets as they are overexpressed in many cancers but not in normal adult tissues (Fesik, 2000; Vucic *et al*., 2000; Kasof and Gomes, 2001). It has been found that elevated levels of survivin are associated with poor prognosis in children with

neuroblastoma (Adida *et al*., 1998). IAPs are a family of endogenous direct inhibitors of cell-death caspases (cysteine-aspartyl proteases) conserved throughout animal evolution with homologies in viruses, yeast, *Drosophila, C.elegans*, mice and humans (Deveraux *et al*., 1997; Deveraux and Reed, 1999). The mammalian IAPs include; X-IAP, cIAP-1, cIAP-2, ML-IAP, livin and survivin that inhibit active caspase-3, caspase-7, and the activation of caspase-9 (Los *et al*., 1999). Although the mechanisms by which caspase activity is inhibited are not fully understood, it has been suggested that the baculovirus IAP repeat (BIR) domains present in all IAPs may be involved in the inhibition of caspase activity (Uren *et al*., 1998).

1.5 p53 structure, function and regulation

The p53 protein was first discovered in 1979 and was thought only to be a cellular partner of an oncoprotein (40 large T-antigen) of the simian virus. Only after a few decades was the role of p53 as a tumour suppressor uncovered (Levine and Oren, 2009). Human p53 protein is encoded as a single copy gene with a molecular weight of 53 kDa. The structure of human p53 consists of an amino-terminal transactivation domain (TAD) (residue 20-60), a poly-proline region (PP) (residue 63-97), an evolutionarily conserved DNA-binding domain (residue 100-300), a linker region (residue 301-323), a tetramerization domain (TET) (residue 324-355) and an unstructured domain located in the carboxy-terminus (CTD) (residue 360-393) (Figure 4) (Bekerman and Prives, 2010). Based on sequence and mutagenesis analysis, three nuclear localization signals (NLS): NLSI, NLSII, and NLSIII were originally identified in the C terminus of p53 (Shaulsky *et al*., 1990). The sequence from Lysine 305 and arginine 306 to NLSI has been suggested to be most active in directing p53 nuclear import, with strong binding affinity to importin - alpha (Liang and Clarke, 1989). Furthermore, two nuclear export signals (NESs) mediate p53 nuclear export, one located in the C-terminus between residues 340-351 and another located at the N-terminus between residues 11 and 27 (Stommel *et al*., 1998; Zhang

and Xiong, 2001). It is suggested that the nuclear export of p53 by leucine-rich NES is mediated by exportin 1 (Kudo et al., 1998).

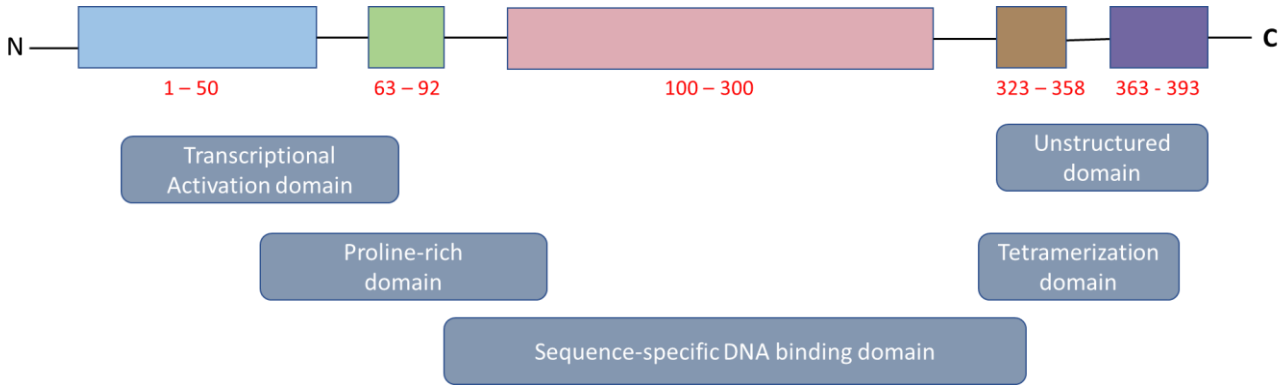


Figure 4: Modified schematic representation of the human p53 domain structure. p53 consists of a transactivation subdomain (TAD), a proline rich region (PRP), an evolutionarily conserved DNA-binding domain, a tetramerization domain (TET) and an unstructured domain located in the carboxy-terminus (CTD). Adapted from Beckerman and Prives (2010).

p53 is an essential protein, when activated it initiates cell cycle arrest in response to damaged DNA through transcriptional activation of downstream effector genes such as p21 (Sherr, 2000). p53 is speculated to regulate the cell cycle as it accumulates in the cytoplasm during the G_1 phase and only enters the nucleus during the G_1/S phase, then cycles back to the cytoplasm (Middeler *et al*., 1997). In response to DNA damage, p53 can trigger apoptosis via the recruitment of the BCL-X_L and BH3-domain containing proteins via the intrinsic cell death pathway or by activating certain external death receptors (Fas ligand) during the extrinsic cell death pathway (Haupt *et al*., 2003; Wu *et al*., 1997).

In healthy normal cells, p53 remains in low levels and inactivated until triggered by internal or external stress signals. However, p53 becomes activated in response to stress via post-translational mechanisms that increases its stability. p53 stabilization leads to its accumulation in the nucleus where it undergoes conversion to its active DNA binding form (Maki, 1999).

p53 stabilization has therefore been suggested to be, at least in part, due to phosphorylation and an inhibition of MDM2-mediated ubiquitination. DNA damaging agents are also thought to induce phosphorylation of p53 at multiple N-terminal sites leading to its stabilization (Shieh *et al*., 1997). Loss of the tumor suppressor, p53 function has been implicated in almost half of all human cancers (Hollstein *et al*., 1991). When p53 is knocked out, multiple tumours developed in mice at a young age, suggesting its crucial role in tumour suppression (Donehow *et al*., 1992).

To date, two important negative regulators of p53 have been documented: RBBP6 and MDM2. RBBP6 has been suggested to target p53 for degradation by enhancing the activity of MDM2, the prototypical negative regulator of p53 (Pugh *et al*., 2006; Li *et al*., 2007). The section below describes the role of MDM2 and RBBP6 in the degradation of p53. Also, the abnormal expression of these proteins in tumorigenesis will also be outlined.

1.5.1 MDM2: Negative regulator of p53

MDM2, a well-characterized negative regulator of p53, is known to regulate the activity, stability and subcellular localization of p53. During unstressed conditions MDM2 binds p53 at the N-terminus, which impedes the ability of p53 to transactivate target genes such as p21. The RING domain of MDM2 is predicted to contain a nuclear export signal that mediates the export of p53 from the nucleus into the cytoplasm, excluding p53 from acting as a transcription factor in the nucleus (Boyd *et al*., 2000). Also, MDM2 in the cytoplasm can function as an E3 ubiquitin ligase targeting p53 for degradation at the proteasome (Rodriquez *et al*., 2000).

In response to DNA damage, p53 is phosphorylated by ATM leading to destabilization of the MDM2: p53 complex, causing p53 to detach from MDM2 and localize to the nucleus (Yang *et al*., 2004). In most tumours with either mutant p53 or wildtype p53, MDM2 is overexpressed causing the p53-pathway to be inhibited. Western blot analysis of untreated MCF-7 breast cancer cells (wildtype p53) showed the overexpression of MDM2 with little or no expression

of p53. However, when these cells were exposed to cyclohexamide (inhibits eukaryotic translaton) for 1 hour, there was an observed decrease in MDM2 expression with an increase in p53 expression (Obrig *et al*., 1971; Peng *et al*., 2001). This suggest that overexpression of MDM2 results in the depletion of p53 and vice versa, which implies a negative regulation loop.

1.5.2 RBBP6: Negative regulatory role to p53

Retinoblastoma binding protein 6 (RBBP6) has been suggested to be essential for chaperone-mediated ubiquitination of p53. Under normal conditions, RBPP6 targets p53 for degradation by MDM2 through its RING finger-like domain (Figure 3) (Chen *et al*., 2013). When MDM2 binds to p53 through its RING finger domain, RBBP6 is recruited to the MDM2: p53 complex enhancing the activity of MDM2, which further targets p53 for proteasomal degradation.

The RBBP6 protein family has been well characterized in various species, including; in *Homo sapiens* as Retinoblastoma-binding Q-protein 1 (RBQ1) (Saijo *et al*., 1995), mice as p53 associated cellular protein testis derived (PACT) (Simons *et al*., 1997) and also as proliferation potential protein-related (P2PR) (Witte and Scott, 1997). Furthermore, three transcripts have been reported in humans occurring due to alternative splicing and poly-adenylation: Isoforms, 1, 2 and 3 with lengths, 6.1, 6.0 and 1.1 kb, respectively (Pugh *et al*., 2006).

Overexpression of RBBP6 has been implicated in many cancers (Mbita *et al*., 2011). For example, the overexpression of RBBP6 with p53 mutations has been linked to colon cancer progression (Chen *et al*., 2013) suggesting RBBP6 as a potential target for therapy. A previous study showed that embryos lacking a functional RBBP6/PACT died early (Li *et al*., 2007), indicating an enhanced p53 activity in the absence of RBBP6.

1.6 Role of chemotherapeutic drugs in cancer treatment

For decades, several therapeutic options have been approved for treatment of cancer patients. These include: surgery, radiotherapy, hormone therapy, stem cell transplant, and chemotherapy. The therapeutic option of interest in this study is chemotherapy. Most, chemotherapeutic agents induce cell death through a p53-dependent mechanism, they include; dihydrofolate reductase inhibitors, 5-fluorouracil and topoisomerase I and II poisons such as irinotecan (Dive and Hickman, 1991). However, these chemotherapeutic drugs have been found to have limited specificity between cancer and normal cells since they target DNA replication-a normal physiological process (Bruce *et al*., 1966).

Frequent side-effects of anticancer drugs include anemia, hair loss, digestive symptoms such as diarrhea, nausea, vomiting and abdominal pains caused by toxicity to intestinal mucoepithelial cells (Tanaka *et al*., 2008). Furthermore, cytotoxic chemotherapy given to pre-menopausal women can lead to follicle destruction, ovarian fibrosis, progressive and premature ovarian failure and a reduction in oestrogen and progesterone levels which also contribute to risk of sterility. These drugs were also found to cause abnormalities in an exposed foetus and mutate genetic material (Chasle and How, 2003).

Finding new ways of selectively targeting cancer cells without harming normal cells is imperative. A recent study suggested that toxic side effects on normal cells can be reduced/eliminated by boosting OXPHOS, the preferred metabolic pathway seen in normal healthy cells. Hull and Ntwasa (2010) showed that when female fruit flies (*Drosophila melanogaster*) were exposed to camptothecin alone adverse effects were observed on the reproductive system as only 20% of their embryos developed into pupae. However, when camptothecin was used in combination with methyl pyruvate -an OXPHOS boosting agent-, embryonic development was improved as 40% of embryos from treated mothers developed into pupae.

In this current study, irinotecan, a derivative of camptothecin (CPT) has been used as a chemotherapeutic agent. Irinotecan has improved water solubility and pharmacokinetic characteristics compared to camptothecin (Garcia-Carbonero and Supko, 2002).

1.7 Irinotecan: Pharmacodynamics

About 30 years ago, CPT was discovered as an antitumour alkaloid from the Chinese tree, *Camptotheca acuminate* (Pommier *et al*., 1998). However, due to its poor aqueous solubility and unpredicted toxicity, the development of CPT as an anticancer agent was hindered. Irinotecan, a more hydrosoluble compound with a better toxicity profile than the parent compound, was used from the '80s. Irinotecan has been used effectively for the treatment of many cancers such as: uterine, ovarian, lung, colorectal, gastric and malignant lymphoma (Tanaka *et al*., 2008).

Irinotecan is an ester prodrug and needs to be converted to its active metabolite SN-38 by a carboxyl esterase converting enzymes (Chung *et al*., 2007). The improved water solubility of SN-38 is due to a dibasic bispiperidine substituent linked through a carbonyl group to the hydroxyl at C-10 (Figure 5) (Garcia-Carbonero and Supko, 2002). Irinotecan targets DNA replication by inhibiting the enzymatic complex of topoisomerase I-DNA in the nucleus (Lorusso *et al*., 2010). Irinotecan (CPT-11) inhibitory activity is predicted to be at position 20 of its chiral carbon (C20) for topoisomerase I (Garcia-Carbonero and Supko, 2002).

The principal enzymatic activity of topoisomerase I (Topo1) is to cut and re-ligate single stranded DNA (Stewart and Schutz, 1987). Topo1 has been shown to exclusively covalently bind double-stranded DNA at the 3'-phosphate end of the DNA strand, through a reversible transesterification reaction, creating a cleavable complex (Garcia-Carbonero and Supko, 2002). CPT and its derivatives stabilise this cleavable complex, resulting in the inhibition of the re-

ligation of cleaved DNA (Hsiang and Liu, 1988). The formation of double stranded breaks induces apoptosis (Lorusso *et al*., 2010).

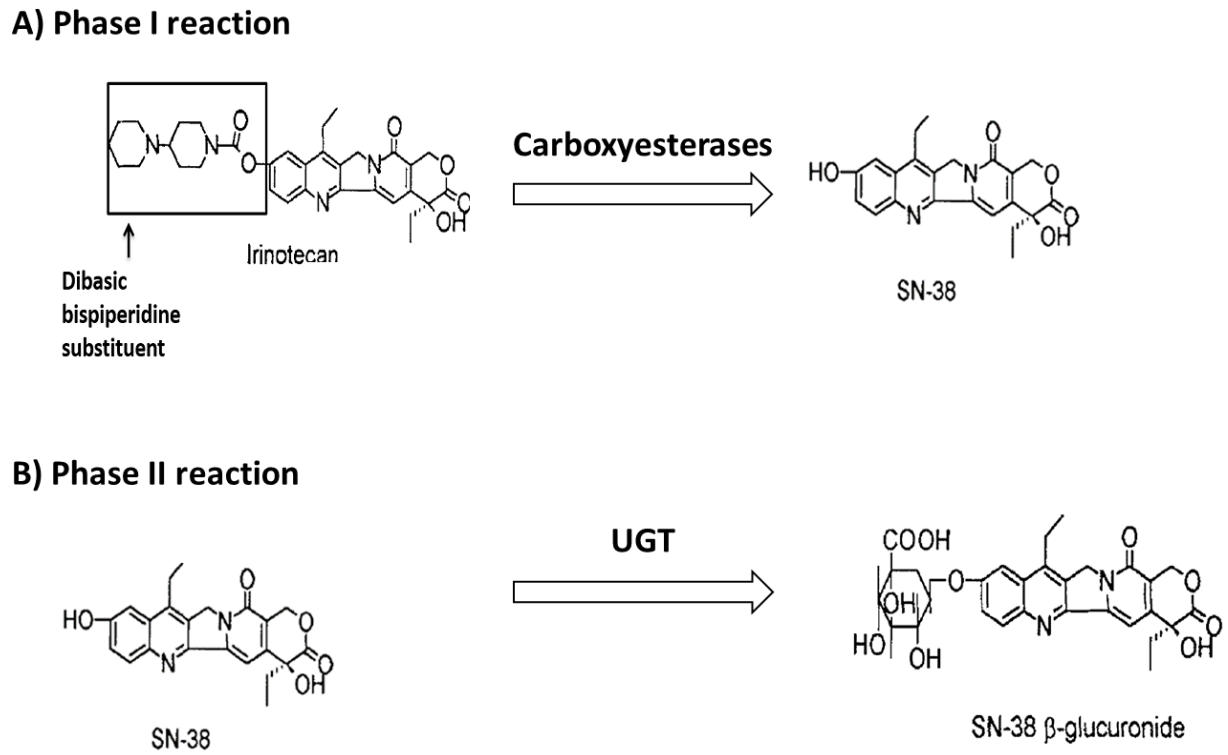


Figure 5: Chemical structure of irinotecan (7-ethyl-10-[4-[1-piperidino]-1-piperidino] carbonyloxy campothecin) and its metabolites, modified from Rivory, (1996). (a) Phase I metabolic reaction involves the formation of the active metabolite SN-38 by the carboxylesterases. (b) The phase II reaction involves the glucuronidation of 7-ethyl-10-hydroxy-camptothecin (SN-38) by cytochrome UGT to SN-38 β- glucuronide. SN-38 is eliminated when it is transformed to its glucuronide form.

1.7.1 Pharmacokinetics of irinotecan

Irinotecan is present in aqueous solution as a lactonic and hydroxy acid form. The intact lactonic ring of irinotecan is essential for its passive diffusion into tumour cells (Lorusso *et al*., 2010). The adsorption, distribution, metabolism and excretion (ADME) of total irinotecan and SN-38 have been shown clinically to differ amongst patients. SN-38 volume distribution is large (100 - 300 L/m_2) indicating extensive tissue penetration with a relatively long half-life of

6 to 30 hours thereby increasing its efficacy. The plasma protein binding of irinotecan is low - 30 - 43% but significantly higher for SN-38 in the range 92 - 96% (Rivory, 1996).

Glucuronidation and biliary excretion are the main mechanisms of SN-38 metabolism and subsequent elimination from the body. Renal excretion is also a means of SN-38 elimination, however to a lesser extent (Garcia-Carbonero and Supko, 2002). Phase I metabolic reaction starts conversion of the ester prodrug (irinotecan) to SN-38, the active metabolite by the carboxylesterases in the liver (Chung *et al*., 2007). Phase II metabolic reaction involves the glucuronidation of SN-38 to SN-38 β-glucuronide by the uridine diphosphate glucuronosyltransferase (UGT) (Bosman *et al*., 1995). The phase II reaction has been implicated for the adverse effects observed in patients after irinotecan (CPT-11) therapy (Jonsson *et al.*, 1997). Studies have shown grade 3 and 4 neutropenia occurs in 14 – 47% of patients treated once every three weeks with irinotecan (125mg/m^2) (Garcia-Carbonero and Supko, 2002).

1.7.2 Clinical implications of irinotecan

Irinotecan, given orally once a day for 5 days every 3 weeks has been shown to cause adverse side effects. Myelosuppression is one of the commonly encountered toxic effect of irinotecan (Garcia-Carbonero and Supko, 2002). In addition to myelosupression, irinotecan therapy is associated with two major side effects: insidious and cholinergic (Rivory, 1996). Cholinergic side effects occur within the first 24 hours after irinotecan administration and results from an inhibition of acetylcholinesterase activity. Symptoms include hypersalivation, abdominal cramps, diarrhea, visual accommodation disturbance, lacrimation, and rhinorrhea (Garcia-Carbonero and Supko, 2002). Insidious side effects are unknown but are suggested to be due to high concentrations of SN-38 in the gastrointestinal tract (Rivory, 1996).

The table below summarizes the dose schedule given to patients with different types of cancers resulting in grade 3- 4 toxicities (Table 1). The toxic side effects associated with irinotecan administration stresses the need for an adjunct agent for selective induction of apoptosis in cancer cells.

Table 1: Dose schedule of irinotecan administered to cancer patients. Adapted from Garcia-Carbonero and Supko, (2002) .

Disease	**No. of patients**	**Dose and schedule**	**Response rate (%)**	**Grade 3-4 toxicities (%)**
Cervical cancer	42	125 mg/m^2, 90 min weekly x 4 w	21	Neutropenia: 36% Diarrhoea: 24% Vomiting: 45% Anaemia: 62%
Ovarian cancer	55	100 mg/m^2 2w 90 min	24	Leukopenia: 87% Diarrhoea: 44% Vomiting: 60% Anorexia: 67%
Non-small-cell lung cancer	122	100 mg/m^2 90min	21	Neutropenia: 8% Diarrhoea: 15%
Small-cell lung cancer	35	100 mg/m^2 90min	37	Not reported
Colorectal cancer	48	300 mg/m^2 30 min 3 w	19	Neutropenia: 48% Diarrhoea: 35% Vomiting: 13%

1.8 Methyl pyruvate as a potential adjunctive to irinotecan

By passing glycolysis is a good chemotherapeutic strategy as cancer cells are dependent on it. In this study the glycolytic pathway was bypassed using exogenous methyl pyruvate, instead of pyruvate as it is unstable and has by products that inhibit OXPHOS. Pyruvate, once in aqueous solution, undergoes an aldo-like condensation reaction to form 2-hydroxy-2-methyl-4-ketoglutarate also known as parapyruvate (Montgomery and Webb, 1956; Von Kroff, 1964). Additionally, in aqueous solution, pyruvate has been shown to be a mitochondrial poison as it undergoes spontaneous cyclization and dehydration to form enolic lactone form or non-enzymatic reduction to form 2,4-duhydroxy-2-methylglutarate (Willems *et al*., 1978).

Furthermore, exogenous methyl pyruvate is more lipophilic, aqueous soluble and stable than pyruvate (pyruvic acid) with better pharmacokinetic properties (Figure 6) (Nishida *et al*., 2014). To date, methyl pyruvate has been used and patented for treatment of diseases of the nervous system (US 20060052448 xA1). Increasing pyruvate levels may bypass glycolysis, boosting OXPHOS. Methyl pyruvate has been shown to activate the respiratory chains in pancreatic β- cell for ATP production (Duchen *et al*., 1993). A study done by Hull and Ntwasa (2010) on female fruit flies has shown that a glycolytic flux occurs during recovery from camptothecin alone. However, when methyl pyruvate was used in combination with camptothecin, they were protected and embryonic development improved. This observation hints that a combined therapy of irinotecan and methyl pyruvate might protect normal cells during chemotherapy. In this study exogenous methyl pyruvate, an analog of pyruvate was used in combination with irinotecan.

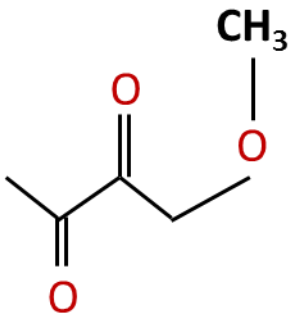


Figure 6: Methyl pyruvate is an analog of pyruvate with an added methyl group (CH_3) synthesized through a methylation reaction.

1.9 Justification of Study

Warburg (1956) initially proposed that reversal of the Warburg Effect in cancer cells would kill cancer and protect normal cells even during chemotherapy. This study is necessary to investigate the molecular basis of this phenotype using exogenous methyl pyruvate.

1.10 Aims

1. To reverse the Warburg Effect in lung and breast cancer cells by boosting OXPHOS to promote death while rescuing normal lung fibroblast cells from irinotecan-induced cell death.
2. To elucidate the molecular mechanisms involved during treatment of normal and cancer cells with the various treatments and to characterise the phenotypes observed in cancer and normal cells by boosting OXPHOS.

1.11 Objectives

1. To evaluate cell viability and cytotoxicity of methyl pyruvate and irinotecan-treated cancer cell lines (human lung and breast) and a normal cell line using *xCELLigence* technology
2. To determine the type of cell death and cell cycle changes on cancer cells and normal cells during various treatments and recovery using flow cytometry
3. To determine the effects of the various treatments on expression profiles of pro-apoptotic and anti-apoptotic genes by RT-PCR and western blot analyses
4. To determine pathways affected by the drug combination (irinotecan and methyl pyruvate) in A549 lung cancer cell line and MRC-5 normal lung fibroblast cell line using RT^2 PCR microarray

Chapter 2: Materials and Methods

2.1 Materials

All reagents, primers, antibodies, kits, and softwares used for this study are included in Tables A1 – A8 in the appendices section.

2.2 Methods

2.2.1 Cell culturing

In this study Human Lung Adenocarcinoma (A549, wildtype p53; ATCC® CCL-185™), breast adenocarcinoma (MDA-MB 231, $p53^{280R-K}$; ATCC® CRM-HTB-26™) and lung embryonic fibroblast (MRC-5, wildtype p53; ATCC® CCL-171™) cell lines were utilised. All of the above mentioned cell lines were maintained and grown in DMEM (Dulbecco's Modified Essential Medium) growth media supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin at 37ºC with 5% CO_2.

2.2.2 Cell viability assay using the xCELLigence system during and after treatment

The *xCELLigence* system is a real-time cell analyzer that continuously monitors cell attachments by measuring electrical impedance. As cells grow and spread while attached to the surface of the plate, their impedance measurements are displayed as cell index (CI) values. The CI calculates the number of live cells as they interact with the electrodes on the E-plate over a period of time. When cells are not present on the electrodes, the CI is zero. As the number of cell attachments increases so does the CI values, and changes in cell adhesion and cell viability lead to change in CI. The cell index values give us a clear picture of the cell number and the biological status of the cells. Normalized cell index (y-axis) as a function of time (x-axis) was

used to plot cell growth. Normalized cell index (NCI_{ti}) was calculated as the cell index (CI_{ti}) at a given time point divided by the cell index at the normalized time point (CI_{nml_time}) ($NCI_{ti} = CI_{ti} / CI_{nml_time}$).

Cells were seeded at optimum cell densities of 1 x 10^4 for MDA-MB 231 and MRC-5 cells and 5 x 10^3 for A549 cells into 16-well gold coated microelectrode E-plates until cell growth reached a logarithmic phase. Subsequently, the following final concentrations were added to each well: 0.5 µM irinotecan, 1 mM and 2 mM methyl pyruvate. Survival analysis studies on *Drosophila melanogaster* showed that exposure to 10% methyl pyruvate was toxic but at 5 % methyl pyruvate the survival rate increased (Nweke, 2015). Taking into consideration that the flies were only exposed to these concentrations of methyl pyruvate by mixture with food, we then used (1 mM and 2 mM methyl pyruvate) in this study. Growth patterns based on the various treatments were analyzed up to 48 hours. For recovery of MRC-5 cells, the media was replaced after 20 hours. Untreated cells served as a positive control. Results were shown as time-dependent cell response dynamic curves reported as average values for each cell line. The results reported for each series of treatments including the untreated were performed in duplicates for each cell line.

2.2.3 Flow cytometry

i) Apoptosis detection

Results obtained from the xCELLigence experiments showed that 0.5 µM irinotecan in combination with 2 mM methyl pyruvate was the optimal concentration for effectively killing the three cancer cell lines used while allowing the survival of normal MRC5 cells. To determine the mode of cell death induced by the various treatments, apoptosis detection was performed using the Annexin-V FITC kit. The Annexin-V FITC kit includes Annexin-V conjugated FITC staining solution, propidium iodide staining solution and the 10X binding buffer. During

apoptosis plasma membrane integrity is lost and a phospholipid, phosphatidylserine (PS), translocates from the inner plasma membrane to the outer leaflet followed by condensation of the cytoplasm and nucleus. Annexin-V a Ca^{2+} -dependent phospholipid binding protein has high affinity for PS. Annexin-V fluorochrome (FITC) can be used for the detection of exposed PS which is characteristic of early apoptotic cells using flowcytometry. Propidium iodide can detect late apoptotic cells characterized by permeable membranes. Viable cells remained unstained by either PI and Annexin-V.

Cells were treated with 2 mM methyl pyruvate in the presence and absence of 0.5 µM irinotecan, harvested at 4, 24 and 48 hours and rinsed twice with 1X PBS. Subsequently, cells were pelleted and re-suspended in 1X binding buffer at a concentration of 1 x 10^6 cells/ml and incubated with 5 µl of Annexin V-FITC and 10 µl propidium iodide for 10 minutes in the dark at room temperature. The samples were immediately analyzed using the BD Accuri™ C6 flow cytometer to determine the percentage of viable, apoptotic and necrotic cells.

ii) Cell cycle analysis

We determine the stages of cell cycle at which the drugs used were effective. The FxCycle PI/RNase staining solution was used to analyse DNA content of fixed cells after treatment. The FxCycle PI/RNase staining solution consists of propidium iodide staining solution, RNase A and a permeabilization reagent. Propidium iodide is a red fluorescent dye that binds to DNA by intercalating between bases. The amount of DNA present is proportional to the fluorescence intensity in each stained cell. Since propidium iodide binds both DNA and RNA, it was necessary to add an RNase reagent. The fluorescence intensity in the G2/M phase is expected to be doubled (4n) compared to the G0/G1 phase (2n), and the S phase is expected to be more than 2n but less than 4n. DNA damaging agents can cause interruption of cell cycle progression at certain check points to allow DNA repair and to prevent carcinogenesis.

Approximately 3 x 10^6 cells/ml were treated with 2 mM methyl pyruvate in the presence and absence of 0.5 µM irinotecan and harvested at 4, 24 and 48 hours. Cells were harvested and detached using trypsin and the samples were centrifuged at 4°C for 15 minutes at 5000 xg. Subsequently, the supernatant was decanted and the pellet washed with pre-chilled 1X PBS. The cells were mixed with 300 µl of 1X PBS and fixed with 70% ethanol to permeabilise the cells allowing entry of propidium iodide. Once all fixatives were removed by centrifugation at 5000 xg for 10 minutes at 4°C, the pellets were stained with FxCycle PI/RNase staining solution. This was used to quantify DNA in each part of the cell cycle. The samples were then left in the dark at room temperature for 30 minutes and analysed using the BD Accuri™ C6 Flow Cytometer.

2.2.4 RNA extraction

RNA was extracted using the TRIzol method. Cells were treated and harvested as previously described in section 2.2.3. The cells were rinsed twice with 1X PBS and detached using trypsin. This was followed by centrifugation at 1000 xg for 5 minutes to pellet cells. To each pellet, 300 µl of the TRIreagent® was added to lyse the cells. Cells were then resuspended and vortexed for 10 seconds. All the tubes were then left on ice for 5 minutes. For phase separation of samples, 60 µl of chloroform, was added and allowed to stand at room temperature for 10 minutes and centrifuged at 12000 xg for 15 minutes. The aqueous phase was removed and placed in a clean tube. To precipitate the RNA, 150 µl of isopropanol was added into each tube and followed by incubation for 5 minutes at room temperature. The samples were then centrifuged at 12000 xg for 10 minutes at 4ºC. The supernatant was discarded and 150 µl of 70% ice cold ethanol was added to wash the RNA pellet. The ice-cold ethanol was discarded after centrifugation at 12000 xg for 5 minutes. Tubes were opened and inverted for 5-7 minutes and then placed in a heating block for 3 minutes at 50ºC to dry pellet. To dissolve the RNA pellet, 50 µl of nuclease free water was added. The Nanodrop was then used to quantify and

check the quality of the RNA. All samples were of good quality as the A_{260} /A_{280} was above 1.8.

2.2.5 Reverse transcriptase polymerase chain reaction

For each reaction 1 µg of total RNA was added to each tube. The following reagents were added to make up the reaction mix; total RNA (1 µg), oligo (dT) primer (2 µl), nuclease-free water (x µl), M-MuLv reaction mix (10 µl) and M-MuLv enzyme mix (2 µl) to make up a total volume of 20 µl. cDNA synthesis involved; 1 cycle for 60 minutes at 42°C followed by 72°C for 5 minutes and 4°C to cool the samples.

For the amplification of the desired gene, the 4 µl cDNA obtained by reverse transcription was combined with 25 µl of 2X PCR master-mix, 0.2 µM of forward primer, 0.2 µM of reverse primer and 19 µl nuclease free water was added to make up a final volume of 50 µl. Subsequently, the transcripts; BID, Bax, p21, caspase 3, MDM2 and the housekeeping gene, GAPDH underwent the following cycles: 94°C initial denaturation for 1 minute, 35 cycles (94°C denaturation for 30 seconds, annealing for 30 seconds, extension at 72°C for 1 minute and a final extension cycle at 72°C for 5 minutes).

The amplification products obtained from reverse transcription PCR were electrophoresed at 120 V on a 1% agarose gel using ethidium bromide as dye. The Chemidoc MP Imaging system was used to visualize the gel.

2.2.6 Western blot procedure

Equal amounts of whole cell protein were extracted with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1.0% NP-40 (nonyl phenoxypolyethoxylethanol), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) from treated and untreated cells. For lysate preparation from each culture of both treated and untreated cells, RIPA was directly added to

cell monolayer and allowed to stand on ice for 5 minutes. A cell scraper was used to detach cells from the surface. Each sample was resuspended and centrifuged at 4°C, 5000 xg for 10 minutes to pellet. Pellets containing the protein were then washed with ice-cold 1x PBS.

Sodium dodecyl sulfate gel was performed as per protocol described by Laemmli (1970). Approximately 20 µl of the protein samples were loaded onto the gel after they were heated in 1X SDS-PAGE sample buffer at 95°C for 5 minutes. Cellular proteins were separated on the basis of charge at 130 V for approximately 90 minutes.

The Hoefer VE blotting mini system was used to transfer proteins from the SDS gel to PVDF membrane (Amersham Hybond-P). Transfer was undertaken at 70V for 1 hour 30 minutes. The membranes were then blocked with SuperBlock® protein blocking buffer (Thermo Scientific) to prevent non-specific binding of proteins. After washing five times (5 min/ per wash) with 1X PBS-Tween, the membranes were probed with primary antibodies of protein of interest as listed in Table 8 overnight at 4°C. The primary antibodies were removed and the membranes washed 5 times (5 min/ per wash) in 1X PBS-Tween. The membranes were incubated for 1 hour at room temperature with their respective appropriate secondary antibodies as listed in table 8 to bind specifically to the relevant primary antibody. Afterwards, washes were done for 5 minutes with 1 X PBST at room temperature. SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) was then added to each blot in a 1:1 ratio (Lumino/Enhancer and Stable peroxide buffer) and incubated for 5 minutes in the dark. The signal detected from the protein-antibody complex using the Bio-Rad imaging system was proportional to the concentration of the proteins present on the membrane.

2.2.7 Western blot for RBBP6

RBBP6 protein family is highly positively charged at the C-terminus (Simons *et al*., 1997). It is therefore difficult to separate on 12% SDS-PAGE gel, as it does not enter the SDS gel. After

careful consideration of the options, an 8% separating gel was used, to facilitate its entry into the gel. The 201 kDa and 13 kDa bands were then separated by SDS-PAGE. The stacking gel composition remained the same as described in 2.2.6 Western blot procedure was performed as previously described in section 2.2.6. Membranes were incubated with anti-RBBP6 rabbit polyclonal (1:500) primary antibody diluted with 1X PBS Tween overnight. This was followed by 1 hour incubation in anti-rabbit polyclonal IgG (1:5000) secondary antibody. Membrane was washed 5 x (5 min/ per wash) with 1X PBS-Tween. SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) was used as described in prevous section to detect signal.

2.2.8 RT^2 profiler PCR array gene expression analysis

Our next objective was to look at pathway focussed gene expression profiling to determine which pathways are altered by cotreatment in A549 and MRC-5 cells using the RT^2 Profiler^{TM} PCR array Human Cancer PathwayFinder^{TM} (QIAGEN- PAHS-033ZF-12). This microarray includes 84 genes representative of 9 different biological pathways genes involved in tumorigenesis: angiogenesis, apoptosis, cell cycle control, cell senescence, DNA damage repair, invasion, metastasis, signal transduction molecules, and transcription factors. The PCR Array plate also includes controls to monitor genomic DNA contamination (GDC) as well as the reverse transcription controls (RTC) and the positive PCR controls (PPC). The RT^2 profiler PCR array provides a platform for analysing the expression of a focused panel of genes found to be differentially expressed during various treatments.

i. *RNA Extraction*

A549 lung cancer and MRC-5 normal lung fibroblast cells were treated with irinotecan (0.5 μM) combined with methyl pyruvate (2 mM) for 48 hours. Total RNA was isolated as described in section 2.2.4 from both the treated and untreated cells. To eliminate DNA

contaminants from the isolated RNA, a DNA digestion was performed using a DNase kit (Siga-AMPD1-1KT). To 50 µl of RNA, 5 µl of the 10 x reaction buffer and 5 µl of DNase I was added. This was followed by gentle mixing and incubation at room temperature for 15 minutes. To bind calcium and magnesium ions, 5 µl of the stop solution was added. Tubes were incubated at 70°C for 10 minutes to detach both DNase I and mature RNA. The total RNA was then suspended in 50 µl of RNase free water and placed on ice. The quality and quantity of total RNA was determined using the Nanodrop spectrophotometer (Thermo Fischer Scientific, California, USA). Total RNA was measured for all four samples and the 260/280 ratio was greater than 1.9, indicating good quality RNA.

ii. ***cDNA synthesis using the RT^2 first strand kit***

Prior to adding the reverse-transcription reaction, genomic DNA contamination mix was made containing 2 µg total RNA, 2 µl buffer GE, and RNase-free water (QIAGEN-129117) added to make up a 10 µl reaction, and incubated at 42°C for 5 minutes. cDNA synthesis was done using the RT^2 First strand cDNA synthesis kit (QIAGEN-330401). In a 20 µl reaction containing 10 µl of DNA elimination mix and 10 µl of the reverse-transcription mix (components; 5 x buffer BC3: 16 µl, control P2: 4 µl, RE3 reverse transcriptase mix: 8 µl, RNase - free water: 40 µl) at 42°C for 15 minutes in the GeneAMP Thermal Cycler PCR system (Perkin Elmer-2400). The reaction was stopped by incubating at 95°C for 5 minutes. After the complete cycle, 91 µl of RNase-free water was added to each reaction.

To confirm successful cDNA synthesis, RT-PCR was done on each sample with GAPDH, a housekeeping gene.

Differential gene expression using RT^2 profiler PCR array format F

The RT^2 Profiler^{TM} PCR array Human Cancer PathwayFinder^{TM} (QIAGEN- PAHS-033ZF-12) was used to profile differences in gene expression between A549 (untreated) and A549 (co-

treated) cells and between MRC-5 (untreated) and MRC-5 (co-treated) cells after 48 hours. The following reagents were added into a 15 ml Falcon tube (QIAGEN; 990552) to make up a 2700 µl cDNA amplification reaction; 1350 µl of the 2 x RT^2 SYBR Green Mastermix (QIAGEN-330500), 102 µl cDNA synthesis reaction mix, and 1248 µl RNase-free water (QIAGEN-129117). The PCR component mix were then placed in a reservoir (QIAGEN-338162) avoiding exposure to light. Using the INTERGA VIAFLO ASSIST liquid handler (Interga Biosciences - CH-7205 Zizers) 25 µl of the mix was added to each well in the array plate. Each plate was carefully sealed with clear optical adhesive film prior to the run and centrifuged at 1000 x g for 1 minute at room temperature using the low speed benchtop centrifuge (Grant instruments - LCM-3000) to remove bubbles. The real-time cycle (Roche LC-480) conditions included; initial heating step to activate the HotStart DNA Taq polymerase for 10 minutes at 95°C, and 45 cycles: 95°C for 15 seconds and 60°C for 1 minute.

2.2.9 Bioinformatics analysis

(i) Differential gene expression analysis

Following each run, the threshold cycle (CT) for each well was obtained. Differentially expressed genes due to cotreatment were identified using the QIAGEN data analysis web portal (www.qiagen.com/za/shop/genes-and-pathways/data-analysis-center-overview-page/rt2-profiler-pcr-arrays-data-analysis-center-/?Instrument=Roche_480_96&format=F&catno=PAHS-033Z). CT values were normalized based on selection of reference genes that showed small changes in their expression across different sample groups (differences in CT < 1). In A549 lung cancer cells, HPRT1 (Hypoxanthine phosphoribosyltransferase 1) was the selected reference gene in A549, while in MRC-5, ACTB (Actin, beta). These reference genes were selected because their expression remained unchanged in untreated and treated cells. The CT values for these genes were

geometrically averaged and used for the fold change ΔΔ CT calculations. Genes with greater than twofold change in gene expression was selected. The data analysis report was then exported as a pdf document.

(ii)Pathway analysis of differentially expressed genes.

(a)PANTHER classification system

To identify the biological pathways affected by the combined treatment, the PANTHER classification system was used. PANTHER, which is part of the gene ontology reference genome project, is a publicly availably bioinformatics software (http://www.pantherd.org/) designed for gene function, ontology and pathway analysis. The PANTHER software is made up of 82 complete genomes organised into families and subfamilies, evolutionary relationships captured into phylogenetic trees, multiple sequence alignments and statistically models (such as the Hidden Markov model (HMM)) (Mi *et al*., 2013). The HMM score provides an estimation of the confidence level of the classification where the query list is assigned to the best scoring HMM (Thomas *et al*., 2003). Genes are classified according to their functions and assigned to PANTHER pathways using GeneOntology and PANTHER databases.

(b) REACTOME pathway analysis

The REACTOME software tool (http://www.reactome.org) was also used for pathway analysis. REACTOME is a curated, open access, open source, and peer-reviewed biological database of human pathways and reactions that closely simulate the actual physical interactions occurring within a cell. REACTOME provides an archive of biological processes and a platform for discovering unexpected functional relationships from gene expression patterns in tumour cells (Croft *et al*., 2013). Small molecule entities, proteins, reactions, and pathways in REACTOME are cross referenced with accession numbers and identifiers from well-established databases including: Ensemble, Uniprot databases, NCBI, UCSC Genome browser, and ChEBI (Milacic *et al*., 2012). This database includes annotations for 7088 of the 20 774

protein-coding genes in the current Ensemble human genome assembly (34% coverage), 1421 small molecules organized into 6744 reactions collected in 1481 pathways, and 15 107 literature references (Croft *et al*., 2013).

Differentially expressed genes identified from this study were used as input into this software and a pathway overview diagram generated. Pathways were considered statistically enriched when the $p < 0.05$ indicated by bright red colour. Furthermore, pathways upregulated was indicated by dark green colour while those downregulated were indicated by a bright yellow colour.

(iii) Network-based gene expression analysis

GeneMANIA through Cytoscape plugin was used to determine whether changes in gene expression due to cotreatment, described previously in A549 and MRC-5 cells will reflect possible changes in the function of certain biological processes. GeneMANIA Cytoscape plugin is a standalone tool implemented in java and freely available (www.genemania.org/plugin/). GeneMANIA displays an interactive functional association network by extending the query gene list with genes that are functionally similar in order to show the relationships among genes from a data set. Each network (individual dataset) in the basic algorithm is assigned a weight based primarily on how well connected genes are to each other compared with their connectivity to non-query genes, down-weighing redundant networks (Warde-Farley *et al*., 2010). Interactions were considered statistically significant when $q < 0.05$. A q-value of 0.05 indicates that there is a 5% chance of getting a false statistically significant result. The q-values were estimated by the in-built Benjamini-Hochberg procedure.

GeneMANIA contains over 800 networks for six organisms: *Arabidopsis thaliana, Caenorhabditis elegans, Drosophila melanogaster, Mus musculus, Homo sapiens and*

Saccharomyces cerevisiae which are grouped into co-expression, genetic interaction, colocalization, physical interaction, shared protein domain and predicted categories (Montojo *et al*., 2010). In this study the *Homo sapiens* network was used for analyses. Datasets that are publicly available are accessed by GeneMania, such as physical and genetic interaction data from BioGRID (Breitkreutz *et al*., 2008), co-expression data from Gene Expression Omnibus (GEO) (Barrett *et al*., 2009), pathway and molecular interaction data from Pathway Commons (http:// www.pathwaycommons.org), and protein predicted interaction data based on ontology from I2D (Brown and Jurisica, 2005).

The query list containing differentially expressed genes found to be uncommon between A549 and MRC-5 cells was uploaded which consisted of gene name symbols. Networks were exported as PNG files and descriptive information as text files.

2.2.10 Statistical analysis

All statistical analyses were performed using the Graphpad software. Results were expressed as the mean value ± SEM of at least three independent experiments. Statistical analysis was performed with the paired student *t*-test comparing two groups at a time. P-value < 0.05 was set and was considered statistically significant.

Chapter 3: Results

3.0 Introduction

The main objective of this study was to exploit the phenotypic differences between cancer and normal cells to deduce a strategy for selectively killing cancer cells. Otto Warburg was the first to propose that cancer cells prefer the glycolysis pathway while most normal cells favour the oxidative phosphorylation (OXPHOS) pathway for glucose metabolism, even in the presence of oxygen (Warburg, 1956). In this study, exogenous methyl pyruvate was used to enhance OXPHOS and reverse the Warburg effect. Using *Drosophila* as a model organism, colleagues from our group found that there was a 40 % increase in embryos developing into pupae when females were exposed to camptothecin combined with methyl pyruvate, compared to those that were exposed to camptothecin alone (Hull and Ntwasa, 2010). This observation prompted the investigation to confirm the protective role of methyl pyruvate on normal cells during chemotherapy. Due to the poor aqueous solubility and toxicity of camptothecin, irinotecan was used in this study as a DNA damaging agent. Irinotecan is a water-soluble derivative of camptothecin presently used for the treatment of colon and ovarian cancer (Chung *et al*., 2007).

It was observed that introduction of exogenous methyl pyruvate protected normal cells from irinotecan-induced cell death. Exogenous methyl pyruvate combined with irinotecan were more effective in killing A549 lung cancer cells expressing wild type p53 than in MDA-MB 231 cells expressing mutant p53. In A549 lung cancer cells after introduction of exogenous methyl pyruvate, death was mainly by apoptosis with most cells accumulated at the sub G_0/G_1 phase, indicating cell death. However, an opposite cell death mechanism was observed in MDA-MB 231 cells as both necrosis and apoptosis were observed, with most cells accumulating at the G_0/G_1 phase and the sub G_0/G_1 phase, indicating cell cycle arrest and cell

death. In MRC-5 cells, in contrast to cancer cells, introduction of exogenous methyl pyruvate resulted in an increase in live cells, with most cells accumulating at the G_0/G_1 phase, S-phase, and the G_2/M phase.

RT-PCT and Western blot analyses revealed that after introduction of exogenous methyl pyruvate death was mainly p53 - dependent in A549 cells while in MDA-MB 231 death was through another caspase-independent and p53-independent pathways. In MRC-5 cells the introduction of exogenous methyl pyruvate abrogated the p53/p21 axis, switching off the apoptotic pathways. Through pathway focussed gene expression profiling, exogenous methyl pyruvate combined with irinotecan induced genes that mostly mediate the programmed cell death pathway in A549 cells while in MRC-5 cells genes that mediate cell survival and angiogenesis were induced.

3.1 The effect of interfering with OXPHOS on cell proliferation

To determine the effect of enhancing OXPHOS on cancer and normal cells various concentrations of exogenous methyl pyruvate were combined with irinotecan and the proliferation profiles of the cells were monitored in the non-invasive label-free *xCELLigence* RTCA (real-time cell analysis) platform. Two cancer cell lines; A549 ($p53^{+/+}$) and MDA-MB 231 ($p53^{R280K}$) and MRC-5 cells, a normal lung fibroblast cell line, were grown to exponential phase and treated as described in section 2.2.2**.** The *xCELLigence* technology uses interdigitated gold microelectrodes to monitor viability of cultured cells. Normalized cell index (y-axis) as a function of time (x-axis) was used to plot cell growth. Data reported here is a representative of an average of 2 independent experiments of each cell line.

The toxic effect of irinotecan on normal cells was evident when cells were treated with 0.5 µM irinotecan alone. Cell proliferation of cancer cells (A549 and MDA-MB 231) as well as the

MRC-5 normal fibroblasts cells was inhibited, as indicated by a rapid reduction in cell index post treatment (Figure 3.1). Irinotecan is a topoisomerase poison known to kill cells by acting at the S-phase of the cell cycle thereby interfering with DNA replication (Lorusso *et al*., 2010). Topoisomerase I (Topo1) mechanism of action involves cutting and re-ligating single stranded DNA (Stewart and Schutz, 1987). Topo1 has been shown to exclusively covalently bind double-stranded DNA at the 3'-phosphate end of the DNA strand, through a reversible transesterification reaction, creating a cleavable complex (Garcia-Carbonero and Supko, 2002).

Treatment with 2 mM methyl pyruvate combined with 0.5 µM irinotecan compared to 0.5 µM irinotecan alone, inhibited cell proliferation of both cancer cells (Figure 3.1 A and B) but protected MRC-5 normal fibroblast cells (Figure 3.1 C) from irinotecan-induced cell death. Interestingly, even when the drugs were removed, MRC-5 cells survived the irinotecan treatment and did not die (Figure 3.1 D). Treatment with 1 mM exogenous methyl pyruvate alone and combined with 0.5 µM irinotecan was less effective in killing cancer cells as in both cancers as the cell index after 20 hours post treatment did not reach zero, similar to MRC-5 normal cells, suggesting cell proliferation (Figure 3.1). It is noteworthy that treatment with 2 mM methyl pyruvate alone was still able to inhibit cell proliferation in both cancer cells, while no changes were observed in MRC-5 cells as the cell index remained constant over time.

These results suggest that introduction of 2 mM exogenous pyruvate alone and in the presence of 0.5 µM irinotecan is able to kill cancer cells while protecting normal cells. These concentrations were therefore used for further studies.

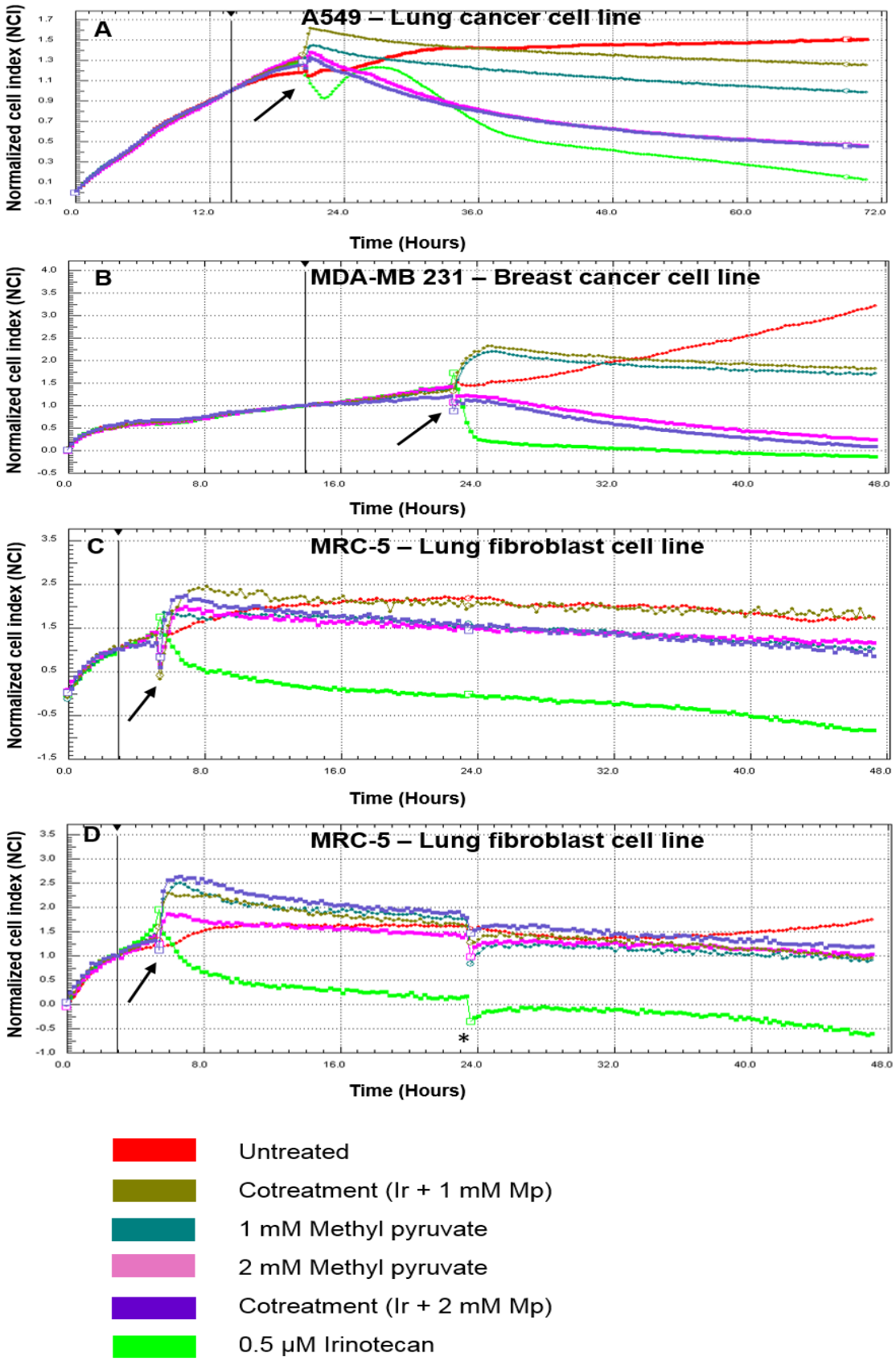


Figure 3.1 Introduction of exogenous methyl pyruvate protects normal MRC-5 cells from cell death Real-time analysis of cell viability of A549 (A), MDA-MB 231 (B), MRC5 (C) and MRC5 (D) for approximately 30 hours post-treatment using *xCELLigence* technology. A549 and MDA-MB 231

cells were treated for approximately 48 hours after growth curves reached a logarithmic phase. MRC5 cells (C) were treated and grown as other cell lines in real time while MRC5 cells (D) were treated for 20 hours then DMEM was replaced with fresh DMEM-nutrient supplemented medium and was allowed to grow for a further 24 hours for recovery. A time dependent plot (x-axis) with 8-hour intervals, representative of changes in normalized cell index (y-axis) were recorded and used to indicate cell attachment. Data were normalized, indicated by the vertical line at 14 hours for A549 and MDA-MB 231 cells, and 3 hours for MRC-5 cells. The following letters were used to present the combined treatment, Ir; irinotecan and Mp; methyl pyruvate. Arrows on each figure show time points at which treatments were added and the asterisk symbol, when fresh DMEM nutrient supplemented medium was added to MRC-5 cells during recovery (D).

3.2 Exogenous methyl pyruvate reduces apoptosis in irinotecan-treated normal cells

From the *xCELLigence* results, introduction of 2 mM methyl pyruvate alone and combined with 0.5 µM irinotecan was effective in killing both cancer cells (A549 and MDA-MB 231) while promoting proliferation of normal MRC-5 cells. Next, the mechanism by which cell survival in normal cells and cell death in cancer cells occurred after introduction of exogenous pyruvate were investigated by assessing the mode of cell death and cell cycle changes. This was important since *xCELLigence* RTCA system does not distinguish between the types of cell death induced. It only measures cell proliferation and death.

The Annexin-V FITC apoptosis kit was used to determine the mode of cell death induced following treatment with permutations of exogenous methyl pyruvate and irinotecan. Two cancer cell lines, A549 with wild type p53 and MDA-MB 231 with mutant p53 and one normal fibroblast cell line (MRC-5) with wild type p53, were used. These two different p53 statuses were important as almost half of all cancers harbours a p53 mutation (Wallace-Borduer and Lowe, 1999). Cells were treated with 0.5 µM irinotecan in the presence and absence of 2 mM exogenous methyl pyruvate and harvested at different time intervals (4, 24, and 48 hours) after treatment.

Throughout all treatments, death was mainly by apoptosis in A549 cancer cells with wild type p53 while in MDA-MB 231 cells with mutant p53 most cells died by necrosis (Figure 3.2 A

and B). Introduction of exogenous methyl pyruvate combined with irinotecan was most effective in killing A549 cells compared to all other treatments. Interestingly, exogenous methyl pyruvate alone killed more MDA-MB 231 cells than all other treatments. Approximately, 80.3% of apoptotic cells and only 0.5% of necrotic cells were observed after 48 hours treatment with exogenous methyl pyruvate alone in A549 cells (Figure 3.2 A). After treatment with irinotecan alone, 90% apoptotic cells and 1.3% necrotic cells were present. When exogenous methyl pyruvate was combined with irinotecan 90.7% of apoptotic cells with only 1.4% of necrotic cells were observed.

On the other hand, when MDA-MB 231 cells were treated with exogenous methyl pyruvate alone for 48 hours, 9.1% died by apoptosis while 40% died by necrosis . Treatment with irinotecan alone, 15% apoptotic cells and 20% necrotic cells was present. Even upon the combined treatment, only 1.6% of apoptotic cells and 22.3% of necrotic cells were observed. It therefore appears exogenous methyl pyruvate can induced cell death regardless of the p53 status since both cancer cells lines died albeit, with MDA-MB 231 less sufficiently .

Moreover, introduction of exogenous methyl pyruvate protected MRC-5 normal lung fibroblast cells, as a decrease in apoptotic and necrotic cells were observed with an increase in live cells compared to treatment with 0.5 μM irinotecan alone (Figure 3.2 C). After 48 hours treatment of exogenous methyl pyruvate with irinotecan, only 6.4% of apoptotic cell was observed compared to irinotecan treatment alone, where 21% apoptotic cells were observed. Upon recovery from exogenous methyl pyruvate alone and combined with irinotecan compared to recovery from irinotecan alone, more live cells were observed (Figure 3.2 D). When cells recover from the drugs, only 7.9% of apoptotic cells were observed compared to recovery from irinotecan alone, where 18% apoptotic cells were observed (Figure 3.2 D). These results suggest that exogenous methyl pyruvate might provide protection to normal cells exposed to a

DNA damaging agent during treatment and recovery since apoptosis and necrosis were reduced.

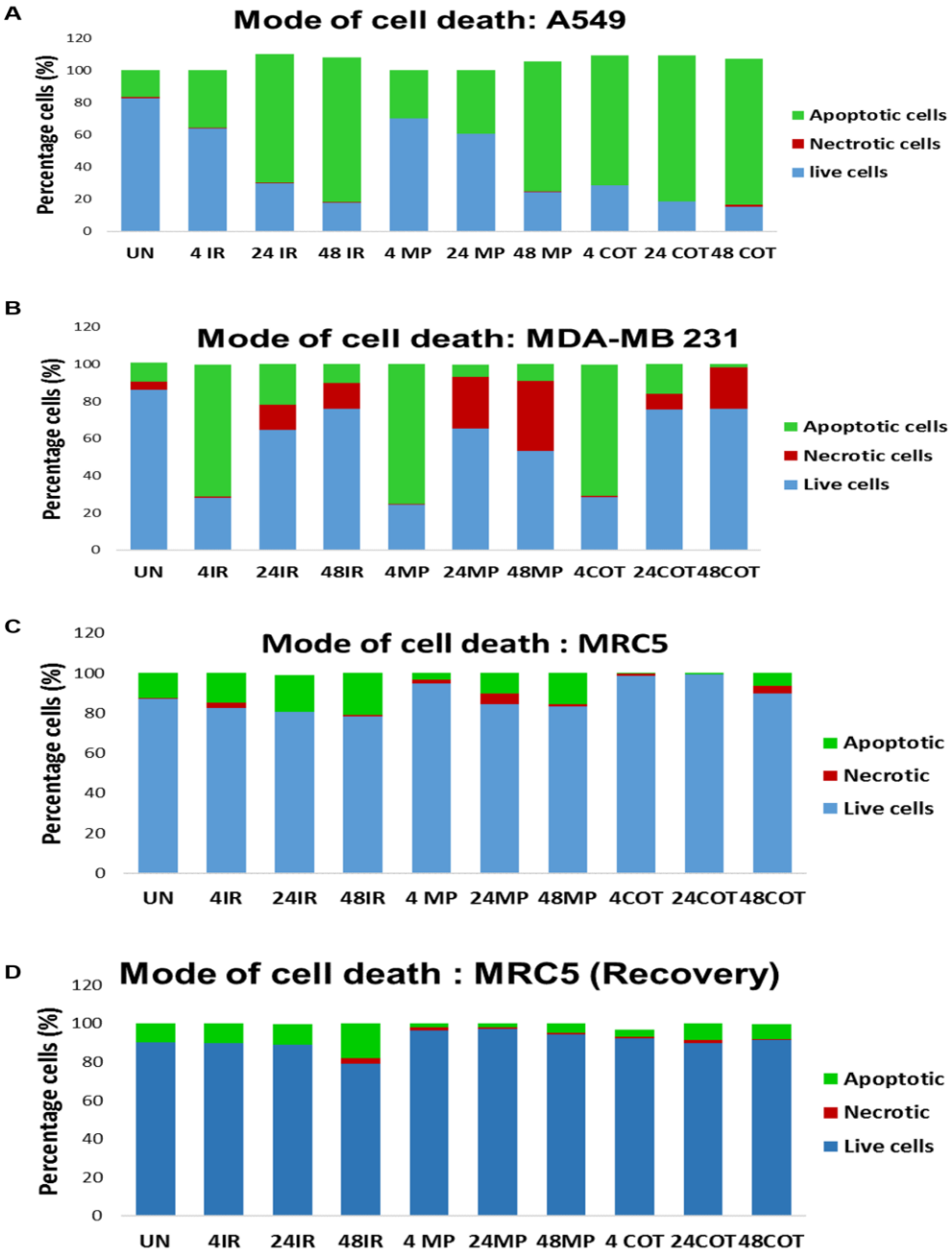


Figure 3.2 Introduction of exogenous methyl pyruvate protects normal MRC-5 cells from irinotecan induced cell death while promoting cancer cell death. Flow cytometer analysis with Annexin V and propidium iodide staining revealed percentage of cells undergoing apoptosis and necrosis when treated. A549 (A), MDA-MB 231 (B), and MRC-5 (C) were treated for 4 hours, 24 hours and 48 hours with irinotecan in the presence and absence of methyl pyruvate respectively. MRC5 cells (D) were treated for 4, 24, and 48 hours then DMEM was replaced with fresh DMEM-nutrient

supplemented medium and was allowed to grow for further 24 hours for recovery. The following symbols were used to represent each treatment; MP- methyl pyruvate, Ir- irinotecan, COT- irinotecan combined with methyl pyruvate treatment, and UN- untreated cells.

The next objective was to determine the cell cycle distribution after treatment with exogenous methyl pyruvate alone, irinotecan alone and the combined treatment. This was necessary to determine the profile of cell cycle changes resulting from the previous treatments in two cancer cells and MRC-5 normal fibroblast. Cellular DNA content of treated and untreated cells were quantitatively measured by staining with propidium iodide in order to assess changes at each phase of the cell cycle.

Introduction of exogenous methyl pyruvate alone and with irinotecan resulted in a significant increase in cancer cells (A549 and MDA-MB 231) in the sub G_0/G_1 phase with most MRC-5 normal cells arrested at the G_0/G_1 phase (Figure 3.3 C). The sub G_0/G_1 shift of cells was an indication of cell death taking place since this phase of the cell cycle is characterized by cells having less than 2n of DNA as oppose to the subsequent phase. After 48 hours of treatment with exogenous methyl pyruvate combined with irinotecan, a significant increase in A549 cells was present in the sub G_0/G_1 phase ($p = 0.00021$) compared to all other treatments (Figure 3.3 A). However, treatment with methyl pyruvate alone and irinotecan alone, only 40 - 60% of cells accumulated in the sub G_0/G_1 phase. This implies that introduction of exogenous methyl pyruvate combined with irinotecan was more effective in killing A549 cells compared to all other treatments.

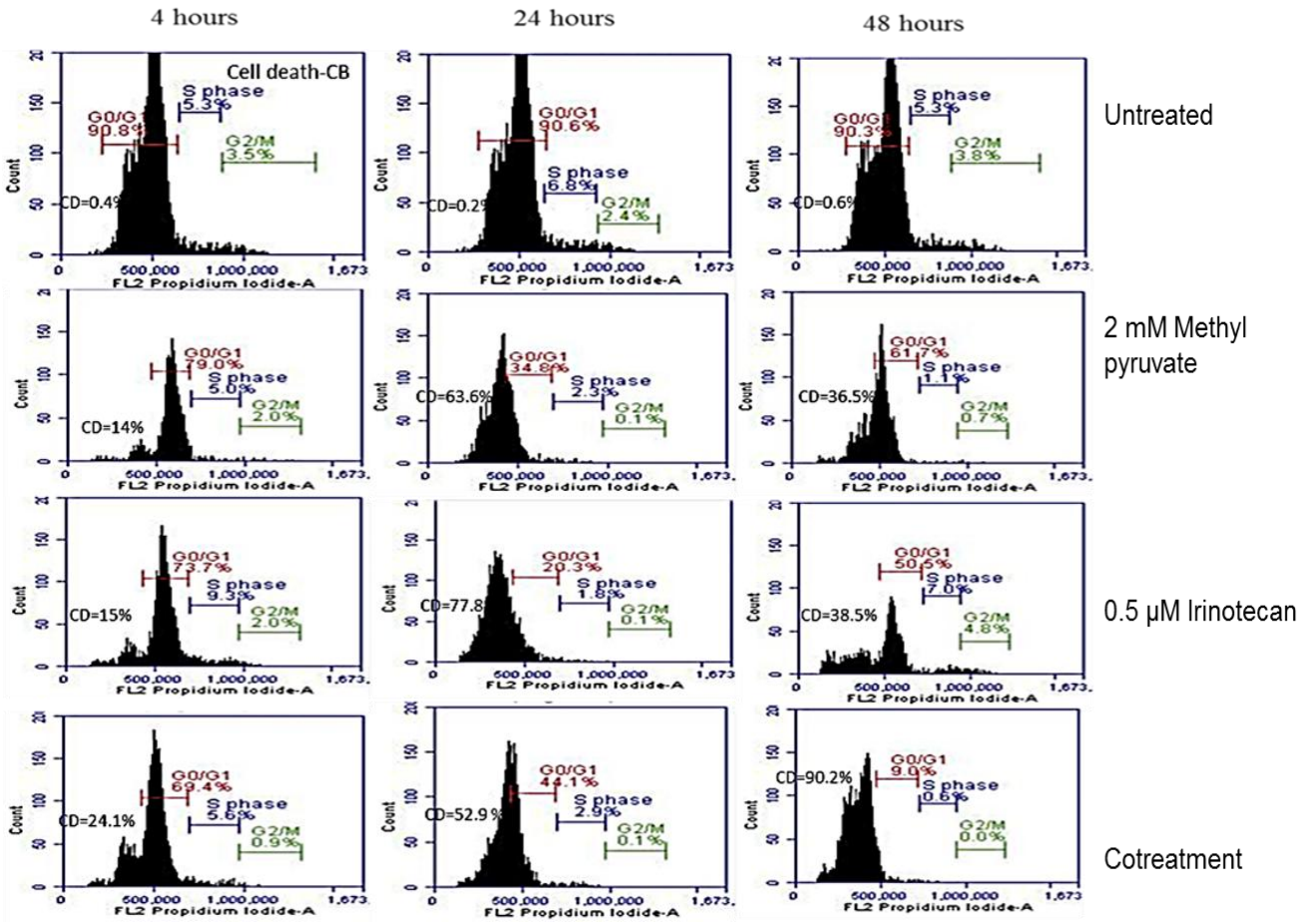
Exogenous methyl pyruvate combined with irinotecan was not as effective in killing MDA-MB 231 cells, since a significant increase in cells in the S-phase ($p = 0.0055$) and G_2/M phase ($p = 0.00032$) was observed, indicating cell growth (Figure 3.3 B). This corroborates findings from the previous section that the p53 status is important for cancer cell death to occur by the combined treatment. Irinotecan treatment alone also did not result in a notable amount of cell death as only 20% of cells were present in the sub G_0/G_1 phase (Figure 3.3 B). Interestingly,

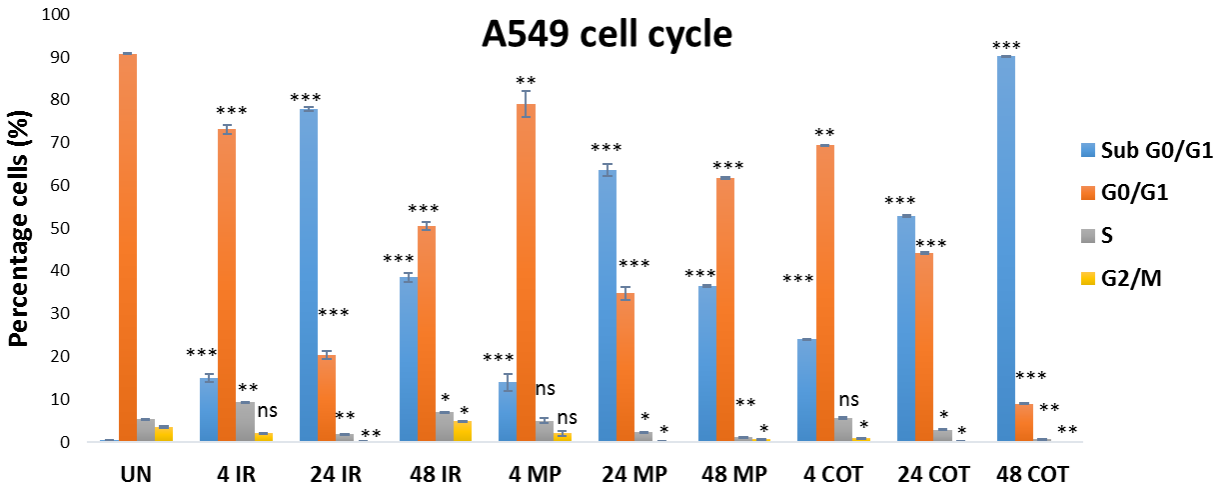
treatment with exogenous methyl pyruvate alone resulted in a significant increase in MDA-MB 231 cells in the sub G_0/G_1 phase ($p = 0.00031$) of the cell cycle, suggesting that introduction of exogenous methyl pyruvate is able to induce cell death independent of wild type p53.

In MRC-5 cells, in contrast to cancer cells, introduction of exogenous methyl pyruvate resulted in a significant decrease in cells accumulating in the sub G_0/G_1 phase ($p = 0.00319$) with no significant increase of cells in the G_0/G_1 phase ($p = 0.29$) compared to irinotecan treatment alone (Figure 3.3 C). This implies that introduction of methyl pyruvate inhibited irinotecan-induced cell death, with most cells arresting in the G_0/G_1 phase. It is noteworthy that during recovery from exogenous methyl pyruvate compared to recovery from irinotecan alone, a significant increase of cells in the S-phase ($p = 0.000071$) and G_2/M checkpoint ($p = 0.00050$) was observed (Figure 3.3 D). These results suggest that introduction of exogenous methyl pyruvate protected normal MRC-5 cells from irinotecan-induced cell death by arresting most cells at the G_0/G_1 phase.

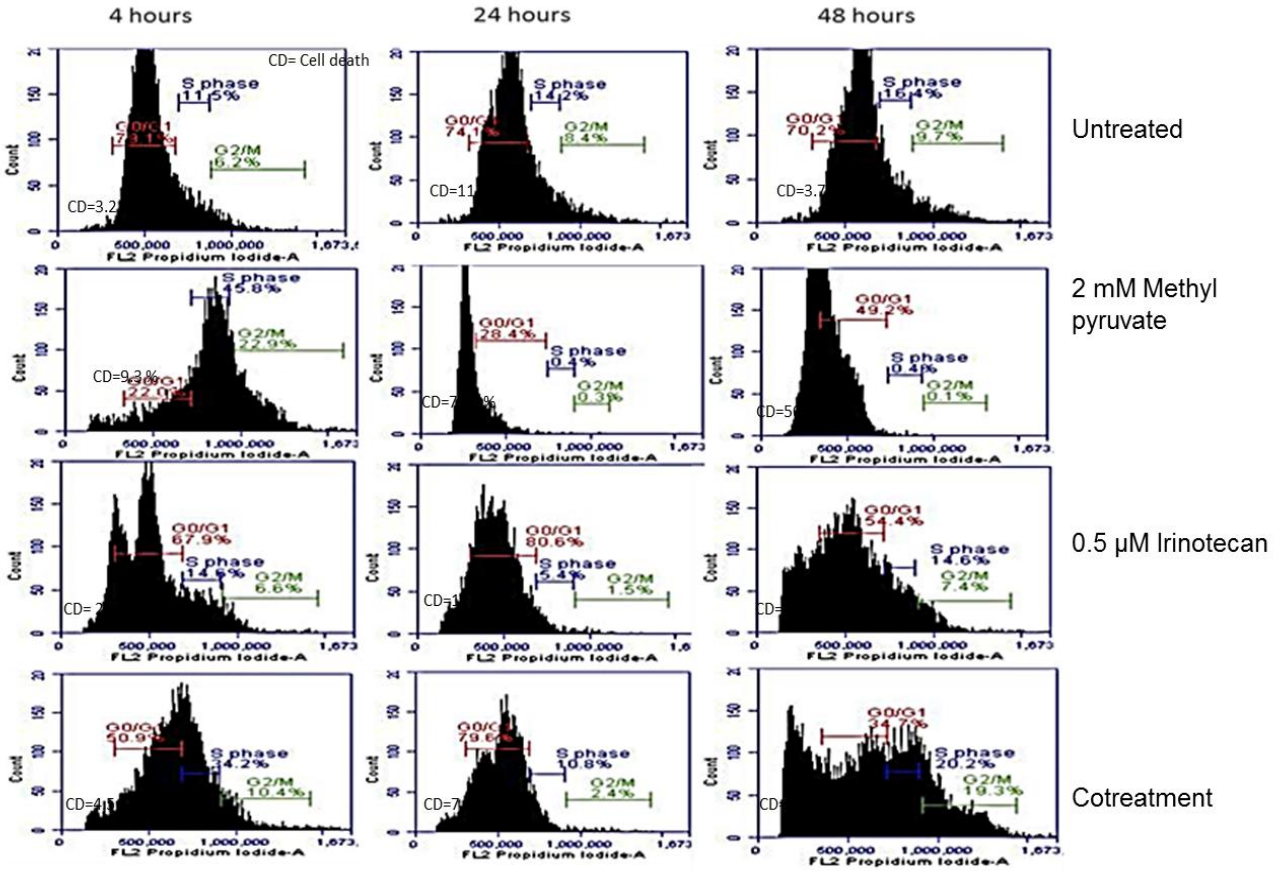
In A549 lung cells, death increased when exogenous methyl pyruvate alone or with irinotecan was used, while the opposite was observed in MRC-5 normal lung fibroblast cells. These results therefore indicate that introduction of exogenous methyl pyruvate can selectively inhibit cancer cell proliferation while promoting proliferation in normal cells.

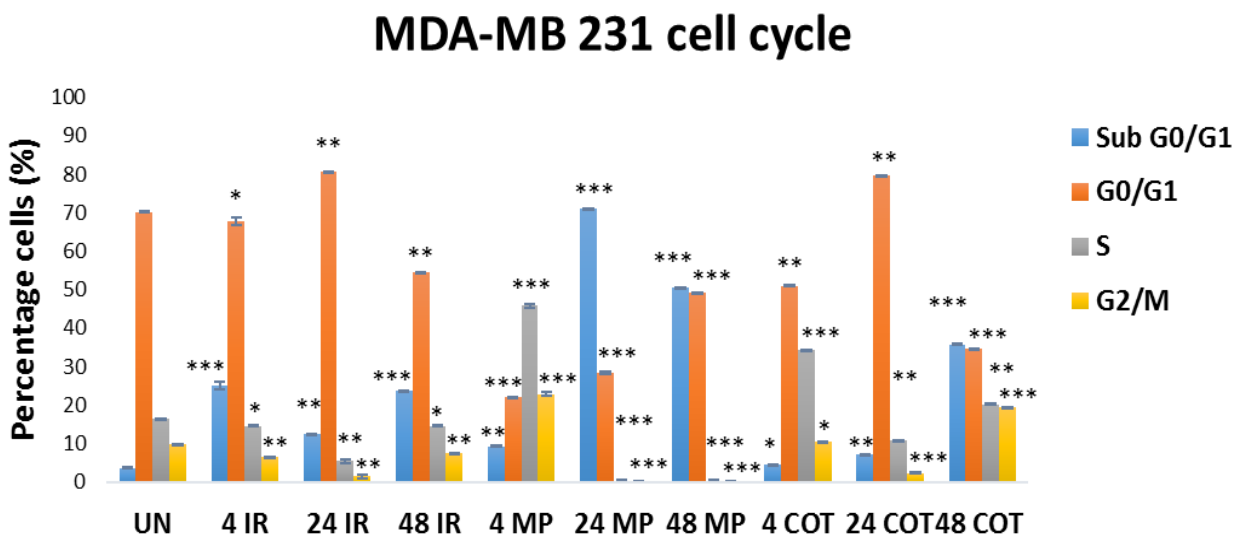
A. A549 lung cancer cells



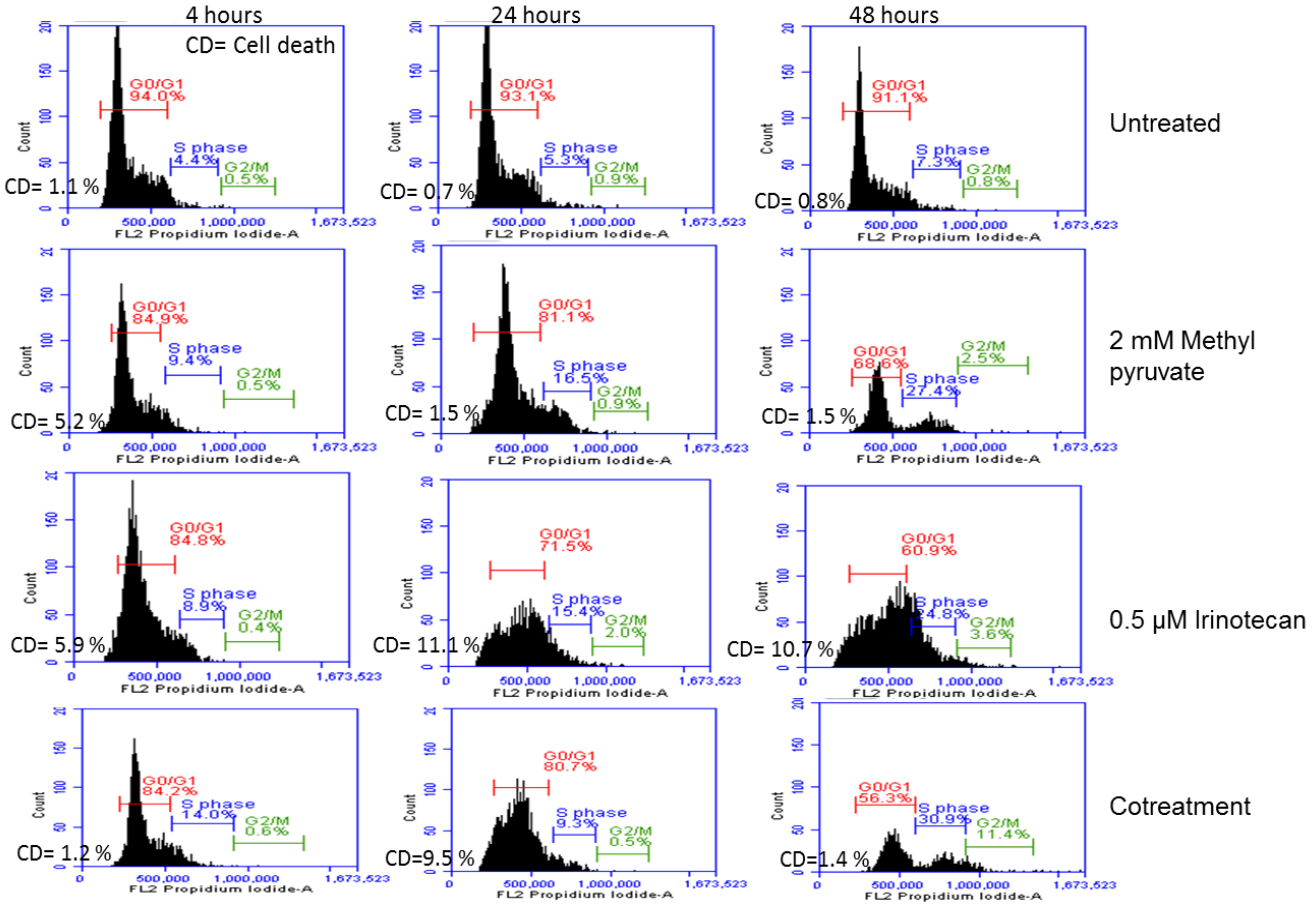


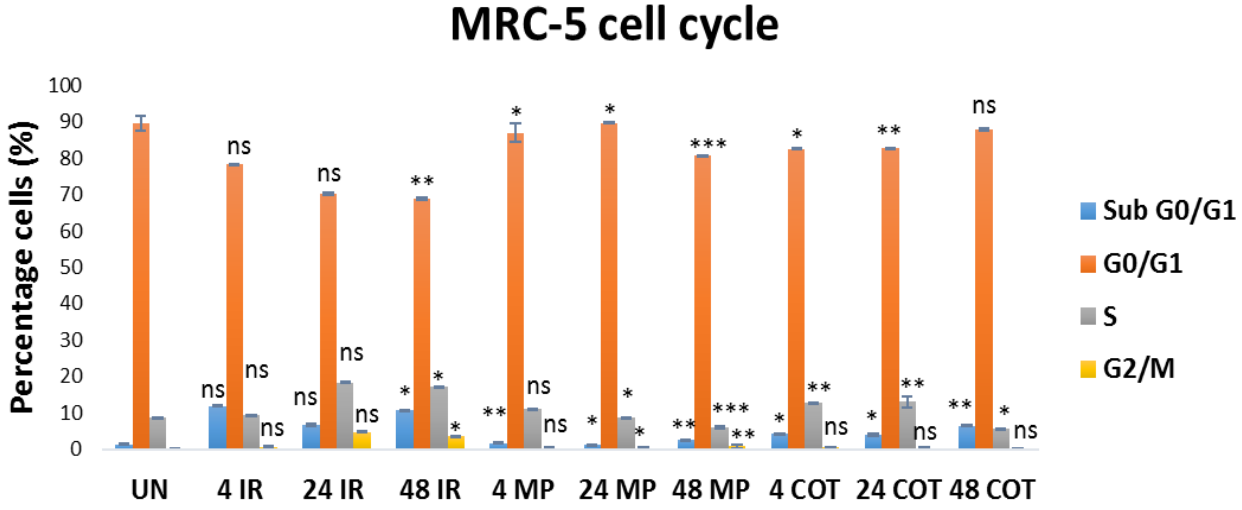
B. MDA-MB 231 breast cancer cells



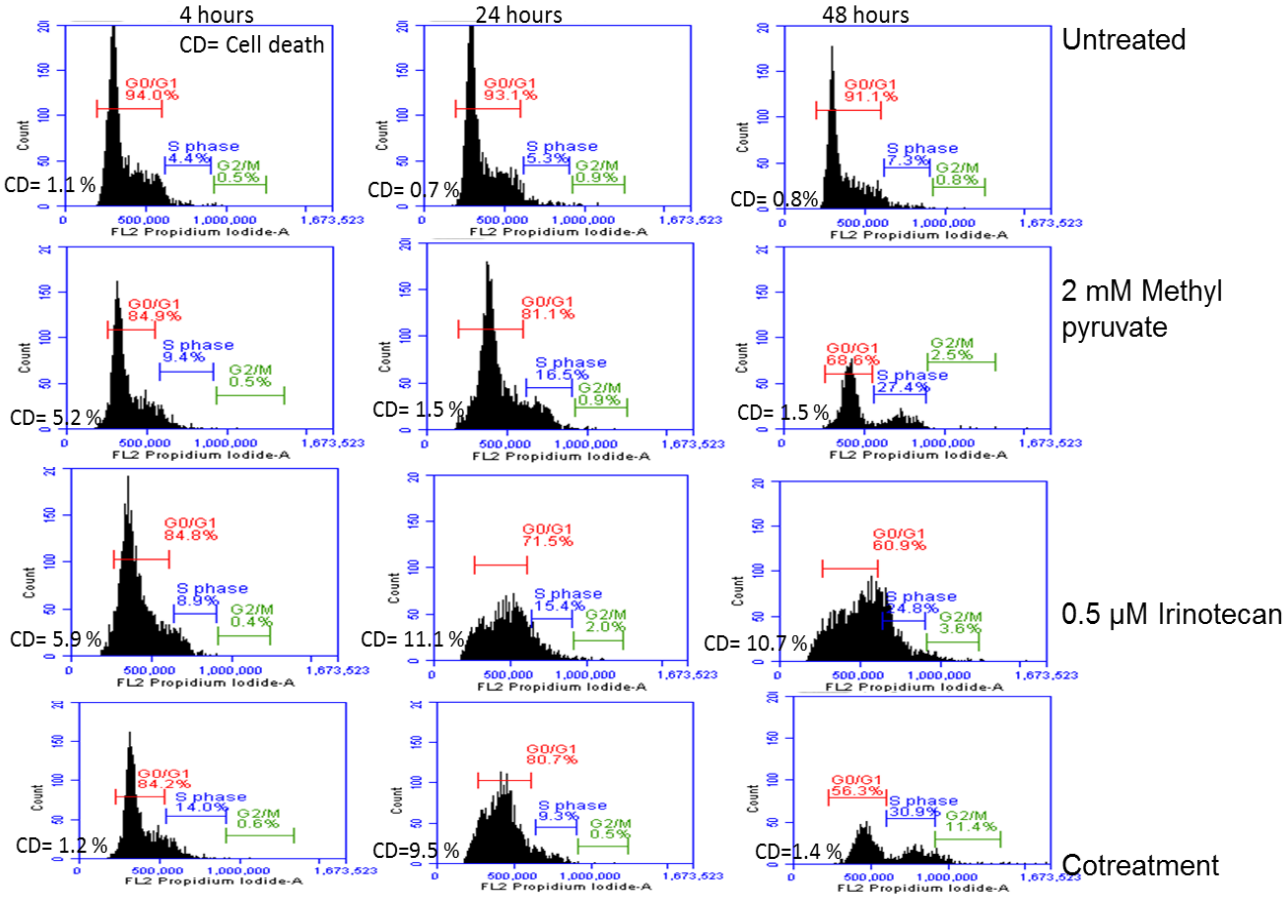


C. MRC-5 normal lung fibroblast cells





D. MRC-5 normal lung fibroblast cells (Recovery)



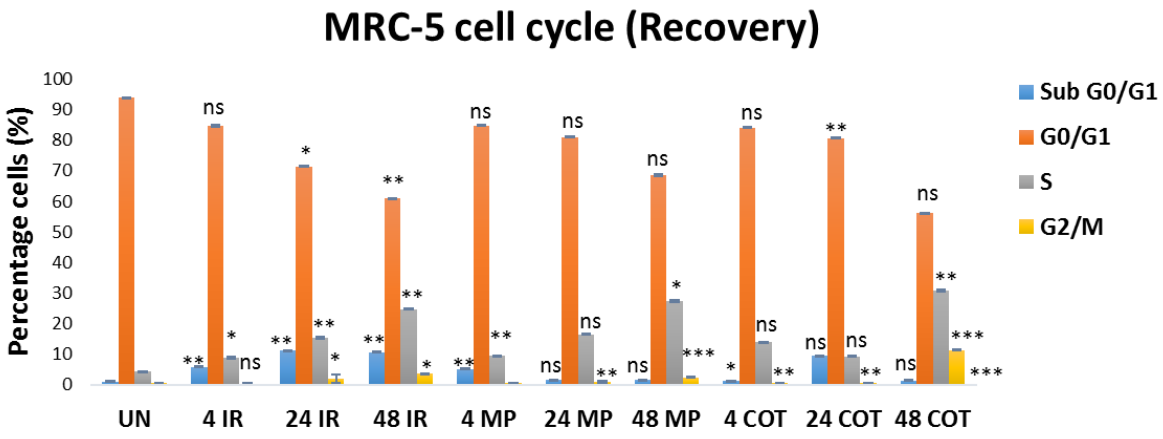


Figure 3.3 Introduction of exogenous methyl pyruvate promote cell cycle progression in MRC-5 normal cells : Cell cycle distribution of A549 (A), MDA-MB 231 (B), MRC-5 (C) and MRC-5 treated and recovered (D) cells treated with 2 mM methyl pyruvate in the presence and absence of 0.5 µM irinotecan for 4, 24 and 48 hours respectively. For all figures, data was acquired using the BD Accuri C6 flow cytometry. Bar graphs represent cell cycle analysis by flow cytometry in A549 (A), MDA-MB 231 (B), MRC-5 (C), and MRC-5 upon recovery (D). Experimental data was from three independent experiments and were considered statistically significant when the p-value was ≤ 0.05. p-value ≤ 0.001

(**) was considered statistical significant, p-value ≤ 0.05 (*) considered statistically significant and p-value ≥ 0.05 (ns) was considered not to be statistically significant. The statistical analysis was done using the Graphpad software. A statistical summary, including the means and standard deviations of the cell cycle phases are presented in **Table A1**. The error bars reflect the standard error of the mean of biological replicates. The following symbols were used to represent each treatment; MP- methyl pyruvate, Ir- irinotecan, COT- cotreatment, and UN- untreated cells.

3.3 Methyl pyruvate protects the normal fibroblast cell line by switching-off apoptotic pathways

p53 is often targeted by chemotherapeutic drugs and is known to be activated by irinotecan (Ntwasa, 2015). When activated by a DNA damaging agent, p53 initiates G_0/G_1 arrest of the cell cycle, activating downstream effector genes such as p21 (Sherr, 2000). p53 has also been shown to induce apoptosis by recruiting BCL-X_L and BH3-domain containing proteins via the intrinsic or extrinsic cell death pathways (Haupt *et al*., 2003; Wu *et al*., 1997). The active metabolite of irinotecan, SN-38, was observed to significantly induce apoptosis by increasing the expression of p53, Bax, and caspase-9 while decreasing the anti-apoptotic gene, Bcl-xL, expression in human hepatocellular carcinoma cells after 24 hours of treatment (Takeba *et al*., 2007).

Also, p53 has been suggested to negatively regulate glycolysis at multiple points and to promote oxidative respiration by transcriptional control of the synthesis of Cytochrome c Oxidase 2 (SCO2) gene (Ma et al., 2007; Vousden and Ryan, 2009). Moreover, normal cells sense glucose availability by a p53-dependent pathway whereby the AMP-activated protein kinase phosphorylates p53 on serine 15 resulting in cell cycle arrest at G_1/S (Jones *et al*., 2005). Presumably, MDM2 and RBBP6, which are known p53 negative regulators (Li *et al*., 2007; Ntwasa, 2016) might also be involved in glucose metabolism.

The molecular pathways involved in response to treatment with exogenous methyl pyruvate combined with irinotecan were assessed by profiling gene expression using the reverse transcriptase polymerase chain reaction and Western blot analyses on A549, MDA-MB 231, and MRC-5 cells. Gene expression levels of p53, MDM2, p21, Bax, BID, caspase-9, and caspase-3 was monitored through RT-PCR analysis. Protein expression levels of p53, RBBP6 and cytochrome c were monitored through Western blot analysis.

Treatment with irinotecan alone caused a reduction in the p53 and p21 transcripts (Figure 3.4 A) which correlates with the decrease in cell death (Figure 3.2 A) in A549 cells compared to exogenous methyl pyruvate alone and the combined treatment. In MDA-MB 231 cells, p53 was upregulated (Figure 3.4 B) with no increase in cell death (Figure 3.2 B) after irinotecan treatment alone. In MRC-5 cells p53 and p21 transcripts were expressed at low levels (Figure 3.4 C) which correlated with the minimal percentage cell death (Figure 3.2 C). Introduction of exogenous methyl pyruvate enhanced the p53/p21 axis (Figure 3.4 A) that correlated with cell death (Figure 3.2 A) in A549 while in MDA-MB 231 cells no correlation was seen. Even though p53 was upregulated after 48 hours of the combined treatment (Figure 3.4 B), no increase in cell death was observed in MDA-MB 231 cells (Figure 3.2 B). This was expected, as studies have shown that when p53 is mutated, it functions aberrantly. This suggest what was speculated in the previous sections that cotreatment - induced cell death is p53-dependent. Interestingly in MRC-5 cells the p53/p21 axis (Figure 3.4 C) was abrogated which correlated to cell survival (Figure 3.2 C).

Both p21 and p53 were abolished upon recovery from exogenous methyl pyruvate and the combined treatment, suggesting the inhibition of p53 dependent apoptosis. It is noteworthy that a decrease in p53 transcript levels can be observed after treatment with exogenous methyl pyruvate alone (Figure 3.4 B), with an increase in necrotic cell death (Figure 3.2 B), suggesting that exogenous methyl pyruvate alone might also induce another cell death pathway that is

independent of the p53 pathway. Across the various treatments, MDM2 was not affected by changes in p53 expression in MDA-MB 231 cells, since the mutation is in the p53 binding domain.

Little or no change was seen in the mitochondrial cell death genes (BID, BAX, Caspase-9 and Caspase-3) in both cancer cells after treatment with exogenous methyl pyruvate alone or when combined with irinotecan. However, after 24 hours treatment with exogenous methyl pyruvate alone and 4 hours of the combined treatment an increase in caspase-9 levels were observed in A549 cells (Figure 3.4 A). This suggests that the mitochondrial cell death pathway was induced, as caspase-9 is required for intrinsic apoptosis. The mitochondrial cell death genes were expressed at low levels after introduction of methyl pyruvate and seemed to be abolished when the drugs were removed in MRC-5 cells, suggesting inhibition of intrinsic apoptosis (Figure 3.4 C and D).

Induction of intrinsic cell death was further evident by the upregulation of cytochrome c in A549 cancer cells. While the inhibition of the intrinsic pathway by the deregulation of cytochrome c in MRC-5 normal lung fibroblast cells after treatment exogenous methyl pyruvate combined with irinotecan (Figure 3.4 E and G). This suggests that methyl pyruvate protected MRC-5 cells from irinotecan-induced cell death while promoting apoptosis in A549 cancer cells. Introduction of exogenous methyl pyruvate downregulated cytochrome c protein in MDA-MB 231 cells with mutant p53 (Figure 3.4 F), where death was by necrosis (Figure 3.2 B). RBBP6 isoform 1 was upregulated in both cancer cells following treatment with exogenous methyl pyruvate and when combined with irinotecan. Moreover, this was accompanied by downregulation of p53 and increased cell death in A549 cells (Figure 3.4 E), indicating that RBBP6 might play a pro-apoptotic role during metabolic reprogramming. In MDA-MB 231 cells, the presence of high levels of RBBP6 isoform 1 (Figure 3.4 F) suggests

that p53 was downregulated as this was followed by necrotic death (Figure 3.2 B) which is independent of p53.

In MRC-5 fibroblasts, contrast to cancer cells, the appearance of a second RBBP6 isoform, isoform 3, was observed (Figure 3.4 G and H). Expression of RBBP6 isoforms 1 and 3 seemed to have an inverse relationship across various treatments, even after recovery. RBBP6 isoform 1 was upregulated after 24 hours of treatment with exogenous methyl pyruvate alone and when combined with irinotecan, in contrast to isoform 3 which was downregulated. This indicates cell proliferation since RBBP6 isoform 1 is known to play an anti-apoptotic role (Ntwasa, 2016) while RBBP6 isoform 3 is a known pro-apoptotic factor (Mbita *et al*., 2012). However, RBBP6 isoform 1 became downregulated after recovery from the combined treatment, with an upregulation of RBBP6 isoform 3 (Figure 3.4 H), indicating G_0/G_1 arrest of MRC-5 cells (Figure 3.3 D)

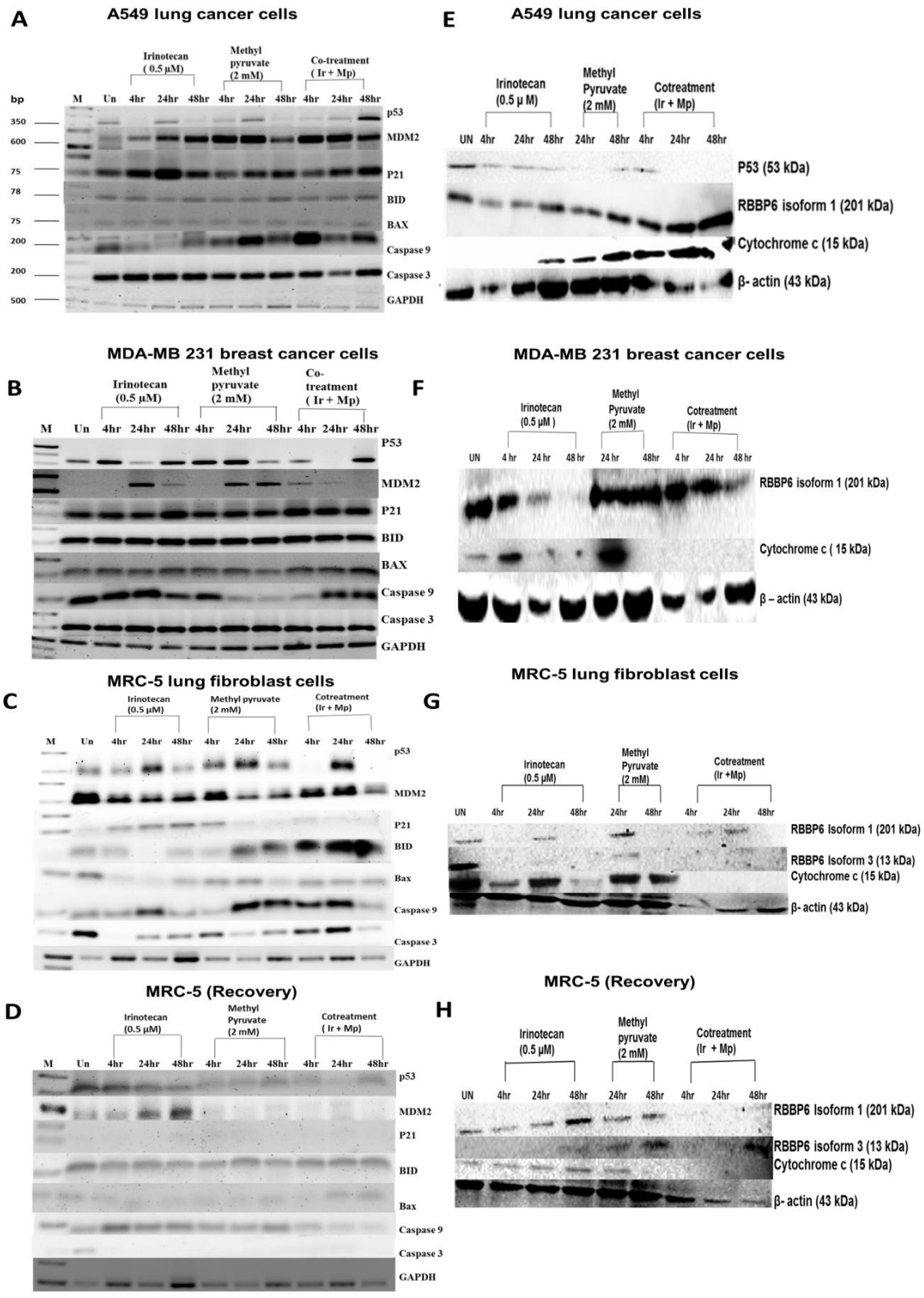


Figure 3.4 Introduction of exogenous methyl pyruvate deregulates apoptotic pathways in MRC-5 normal cells: (A) RT-PCR illustrating changes in gene expression of p53, MDM2, p21: **p53 pathway**, BID, Bax, caspase-9, caspase 3: **Intrinsic apoptotic cell death pathway** during treatment

with methyl pyruvate in the presence and absence of the DNA damaging agent, irinotecan. The housekeeping gene, GAPDH, was used as the loading control. The band intensities were indicative of genes being upregulated or downregulated as a result of the various treatments. For each treatment, identical units of template were used. (**B**) Western Blot analysis illustrating changes in protein expression of p53, RBBP6 and cytochrome c in A549, MDA-MB 231, and MRC5 cells upon treatment with 2 mM methyl pyruvate in the presence and absence of irinotecan for 4, 24 and 48 hours.While MRC5 (Recovery) were treated for 4, 24, and 48 hours then DMEM was replaced with fresh DMEM-nutrient supplemented medium and was allowed to grow for a further 24 hours for recovery. β-actin was used as the loading control to assess whether protein was loaded in each well.

3.4 Methyl pyruvate promotes cell death in cancer cell lines but survival in the normal lung fibroblast cell line

From the RT-PCR and Western blot analyses, the introduction of exogenous methyl pyruvate induced cell death mainly through the mitochondrial cell death pathway and p53 - pathway in A549 lung cancer cells. In MDA-MB 231 death was through another caspase-independent and p53-independent pathways. Furthermore, introduction of exogenous methyl pyruvate promoted the survival of MRC-5 normal lung fibroblast cells by switching off the p53-pathway and mitochondrial cell death pathway. It was pertinent to identify which other biological pathways might be affected by the combined treatment (exogenous methyl pyruvate with irinotecan).

A focused pathway study was conducted using the Human Cancer PathwayFinder^{TM} RT^{2} Profiler^{TM} PCR Array (PAHS-O33ZF-12) in order to profile 84 cancer-related genes. This microarray includes 84 genes representative of 9 different biological pathways genes involved in tumourigenesis. The differences in gene expression between A549 (untreated) and A549 (co-treated) cells and between MRC-5 (untreated) and MRC-5 (co-treated) cells after 48 hours cotreatment were profiled. The data were analysed by QIAGEN data analysis software, PANTHER (Protein Analysis Through Evolutionary Relationships), Genemania Cytoscape and REACTOME.

In A549 cells, 25 genes were upregulated while in MRC-5 cells 20 genes were upregulated (Table 2). Moreover, 18 genes were downregulated in A549 (Table 3), with only one gene downregulated in MRC-5 cells, indicating that the drugs had an opposite effect on the two cell lines.

From the Venn diagram, 17 upregulated genes were unique to A549 cells compared to MRC-5 (Figure 3.5), with most mediating apoptosis, DNA damage and repair, and epithelia-to-mesenchymal transition pathways (Table 2). On the other hand, in MRC-5 cell, 13 upregulated genes were unique (Figure 3.5) and most were responsible in mediating angiogenesis, epithelial-to-mesenchymal transition and cell senescence pathways (Table 2), indicating cell growth. Furthermore, 18 downregulated genes were unique to A549 cells (Figure 3.5), with most mediating cell cycle and angiogenesis pathways (Table 3), suggesting disruption and inhibition of cell cycle progression and cell growth.

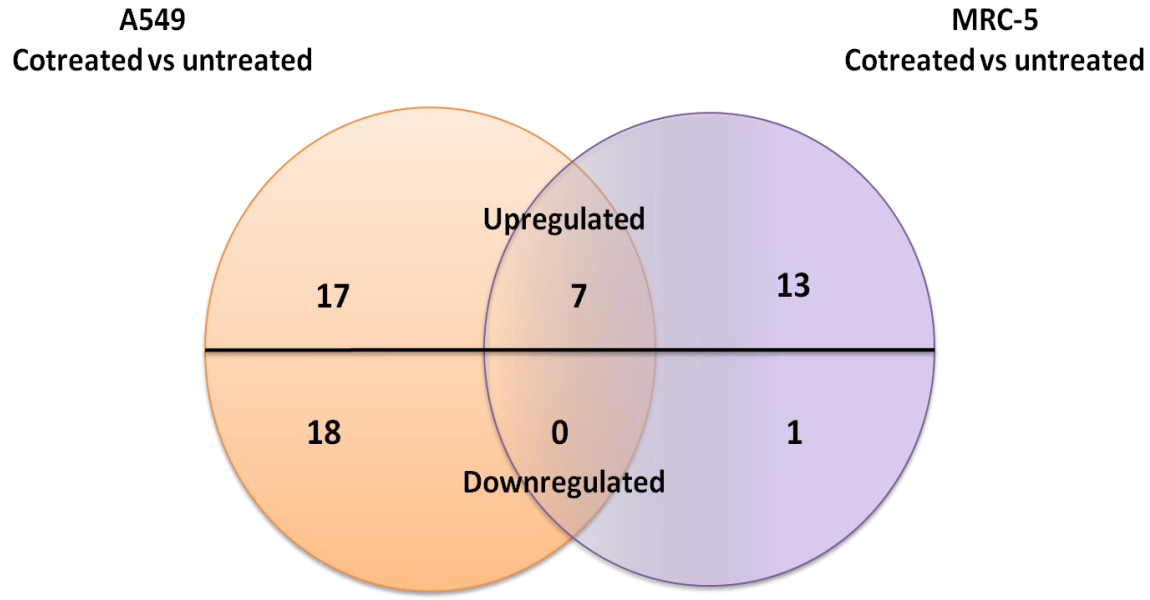


Figure 3.5 Genes upregulated and downregulated between MRC-5 and A549 cells after treatment with the drug combination: Venn diagram depicting differentially expressed genes between A549

lung cancer cells and MRC-5 normal lung fibroblast cells obtained using QIAGEN data analysis software. The central portion indicates unchanged gene expression.

Table 2: Genes upregulated after treatment with exogenous methyl pyruvate combined with irinotecan in A549 and MRC-5 cells

	Upregulated genes				
Pathways	**A549**	**Fold Regulation**	**MRC-5**	**Fold Regulation**	**References**
Apoptosis	CASP7 CFLAR BIRC3 --	3.23 3.17 7.59	-- -- -- FASLG	 9.61	Lamkanfi and Kanneganti, 2010; Wajant, 2004; Wajant et al., 2003
Angiogenesis	ANGPT2 VEGFC -- -- -- --	4.48 3.69	-- -- TEK (TIE2) SERPINF1 ANGPT1 PGF	 6.08 3.67 3.49 3.02	Carmeliet and Jain, 2000
Cell cycle	CCND2	2.66	CCND2	3.49	Dasika et al., 1999
DNA damage and repair	DDIT3 PPP1R15A ERCC5 POLB DDB2 --	4.80 3.08 2.16 2.10 2.00	-- -- -- -- -- GADD45G	 9.61	Khanna and Jackson, 2001; Dasika et al., 1999
Epithelial-to-mesenchymal transition	KRT14 SNAI1 SNAI2 SNAI3 -- --	12.58 8.24 3.08 2.26	KRT14 SNAI1 -- SNAI3 GSC SOX10	9.61 2.63 3.26 9.61 9.61	Huber et al., 2005; Yang and Weinberg, 2008
Hypoxia	CA9 ARNT --	3.15 2.12	CA9 -- HMOX1 EPO	2.54 2.01 11.66	Buffa et al., 2010; Fardin et al., 2010; Yoon et al., 2001
Metabolism	ACSL4	5.99	--		Tennant et al., 2009; Moreno-Sanchez et al., 2009
Telomeres and telomerase	TEP1 TNKS2 TERF2IP TINF2	3.64 2.98 2.55 2.54	TEP1 -- -- --	2.72	Martinez and Blasco, 2010; Cohen et al., 2007

Cell senescence	TBX2 BMI -- --	3.10 2.02	TBX2 -- IGFBP5 SERPINB2	4.51 5.37 2.26	Reddel, 2010; Othani et al., 2009

Table 3: Table 10: Genes upregulated after treatment with exogenous methyl pyruvate combined with irinotecan in A549 and MRC-5 cells

Downregulated genes					
Pathways	**A549**	**Fold Regulation**	**MRC-5**	**Fold Regulation**	**References**
Apoptosis	--		--		
Angiogenesis	FLT1 ANGPT1 SERPINF1	-15.21 -4.13 -2.18	-- -- --		Carmeliet and Jain, 2000
Cell cycle	CDC20 MKI67 MCM2 STMN1 AURKA CCND3 SKP2	-112.74 -38.77 -4.40 -4.10 -2.80 -2.44 -2.29	-- -- -- -- -- -- --		Dasika et al., 1999
DNA damage and repair	--		--		
Epithelial-to-mesenchymal transition	FOXC2	- 2.84	--		Huber et al., 2005; Yang and Weinberg, 2008
Hypoxia	HMOX1	-6.22	--		Buffa et al., 2010; Fardin et al., 2010; Yoon et al., 2001
Metabolism	G6PD COX5A	-4.81 -2.67			Tennant et al., 2009; Moreno-Sanchez et al., 2009
Telomeres and telomerase	-- PINX1 DKC1	 -3.15 -2.63	TERF1 -- --	- 2.40	Martinez and Blasco, 2010; Cohen et al., 2007
Cell senescence	ETS2	-2.32	--		Reddel, 2010; Othani et al., 2009

PANTHER was used to group the unique genes obtained between A549 and MRC-5 cells into their corresponding biological pathways. Unique DEGs between A549 and MRC-5 were uploaded into PANTHER. Genes from the query list were grouped into pathways based on their molecular and biological functions. PANTHER classification system along with other ontology tools such as Gene ontology, and the Hidden Markov statistical model, were used to assign genes to specific pathways.

Approximately 50% of the genes upregulated in A549 fibroblasts mediate apoptosis (Figure 3.6 A), while of the 50% downregulated mediated cell cycle progression (Figure 3.6 B). This indicates what was suggested before (Table 2 and Table 3) that the drugs combined disrupted cell cycle progression and induced cell death. In contrast to A549 cells, those upregulated in the MRC-5 lung fibroblasts promote cell growth (40%), and cell survival (20%) (Figure 3.6 C). This suggests what was speculated before that exogenous methyl pyruvate protected MRC-5 cells from irinotecan-induced cell death. Furthermore, the only gene (TERF1) downregulated in MRC-5 cells had no hit in PANTHER pathways.

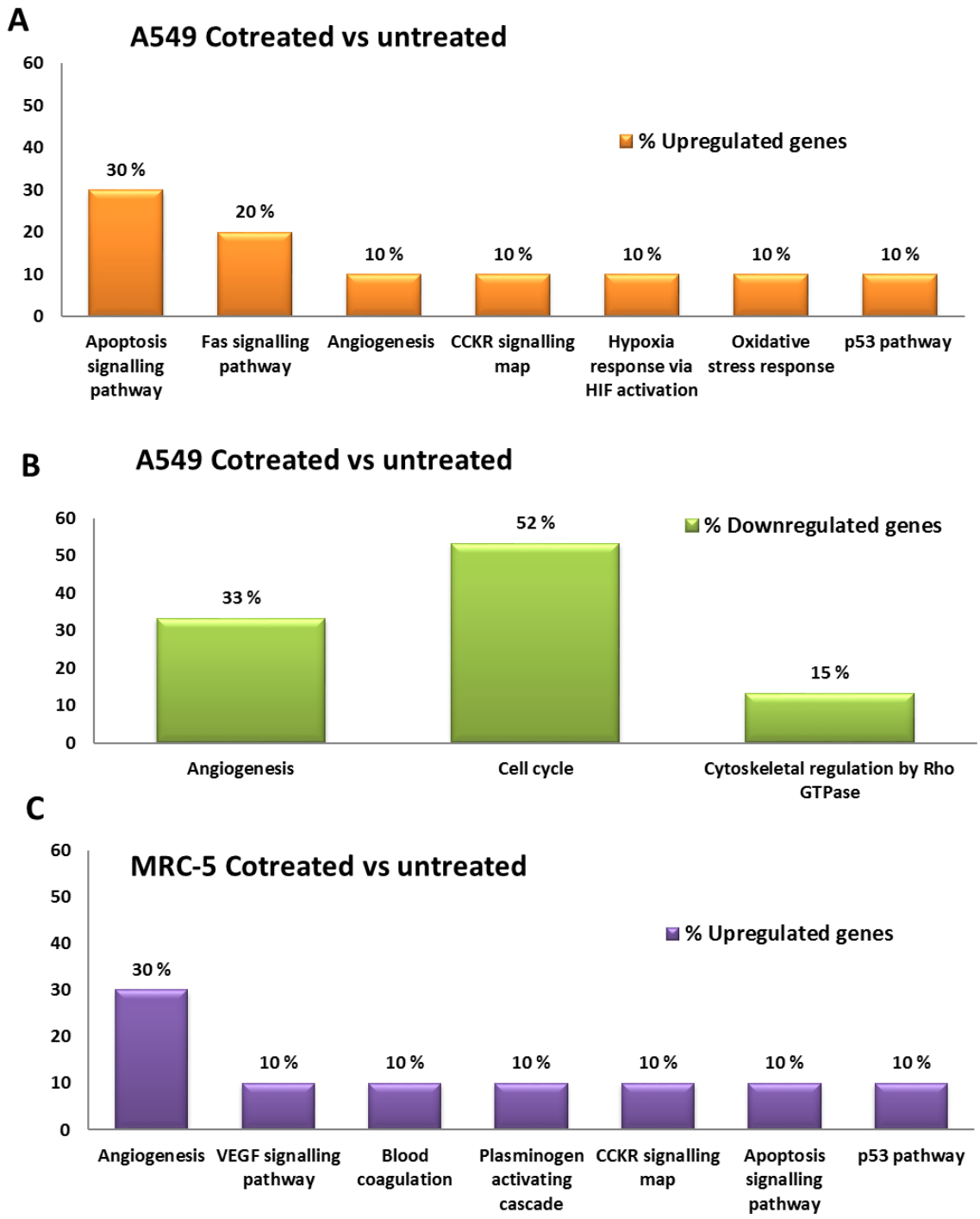


Figure 3.6 Introduction of exogenous methyl pyruvate protects normal MRC-5 cells by upregulating cell growth and survival pathways while inducing cell death in A549 cells by upregulating apoptotic pathways: Using PANTHER the differentially expressed genes were further analysed to reveal biological pathways that were affected when the A549 and MRC-5 cells are treated with irinotecan and methyl pyruvate. Uniquely expressed genes were used as input into PANTHER to identify perturbed biological pathways. The percentage of up or downregulated genes are an indication of the percentage of gene hit against total number of pathway hits. In MCR-5 cells, the drug combination

upregulated anti-apoptotic and anti-angiogenic factors (C) while in A549 cells pro-apoptotic and pro-angiogenic factors were induced (A and B).

The next step was to group the unique genes into their molecular functional classes. This was pertinent as this will aid in identifying genes that may encode for proteins involved in a complex coordinated activity such as DNA replication or RNA splicing. GeneMANIA cytoscape plugin was used for functional interaction analyses. Genes that were unique in both MRC-5 and A549 cells, were used as input (query list) into the GeneMANIA cytoscape plugin. In order to show the relationship among genes from the data set, GeneMANIA extends the query gene list with genes that are functionally similar. Interactions were considered statistically significant when $q < 0.05$. The q-values were estimated by the in-built Benjamini-Hochberg procedure.

It was found that treatment with exogenous methyl pyruvate combined with irinotecan induced apoptosis and anti-angiogenic factors in A549 lung cancer cells and pro-angiogenic and pro-survival factors in the MRC-5 fibroblasts (Figure 3.7). In A549 cells pathways such as the intrinsic apoptotic signalling pathway (q = 2.9e-05) and the telomere maintenance via telomerase (q = 3.2e-12) were significantly upregulated (Figure 3.7 A) while the angiogenesis (q = 0.021), DNA polymerase activity (q = 0.021), mitosis (q = 0.022) were significantly downregulated (Figure 3.7 B). In MRC-5 cells, in contrast to A549 cell, pathways such as angiogenesis (q = 1.3e-08) and positive regulation of cell adhesion (q= 0.0037) were significantly upregulated (Figure 3.7 C). This can be supported by other findings in this study in which cell death was observed in A549 cells and cell growth in MRC-5 with the combined treatment (exogenous methyl pyruvate and irinotecan) (Figures 3.1 and 3.2 A, C, and D). These results suggests that introduction of exogenous methyl pyruvate induced cell death in A549 cells while promoting cell proliferation in MRC-5 normal cells.

This is further evident by the upregulation of ANGPT1 in the MRC-5 fibroblasts, while its antagonist ANGPT2 was upregulated in the A549 cancer cell line indicating that the combined treatment had opposite effects in these cell lines (Figure 3.7 A and C). The drug-induced pro-angiogenic pathways in MRC-5 fibroblasts are mediated by known pro-angiogenic factors and by molecules classified as components of blood coagulation and plasminogen activating cascades, including erythropoietin/thrombopoietin (EPO) (Figure 3.7 C)

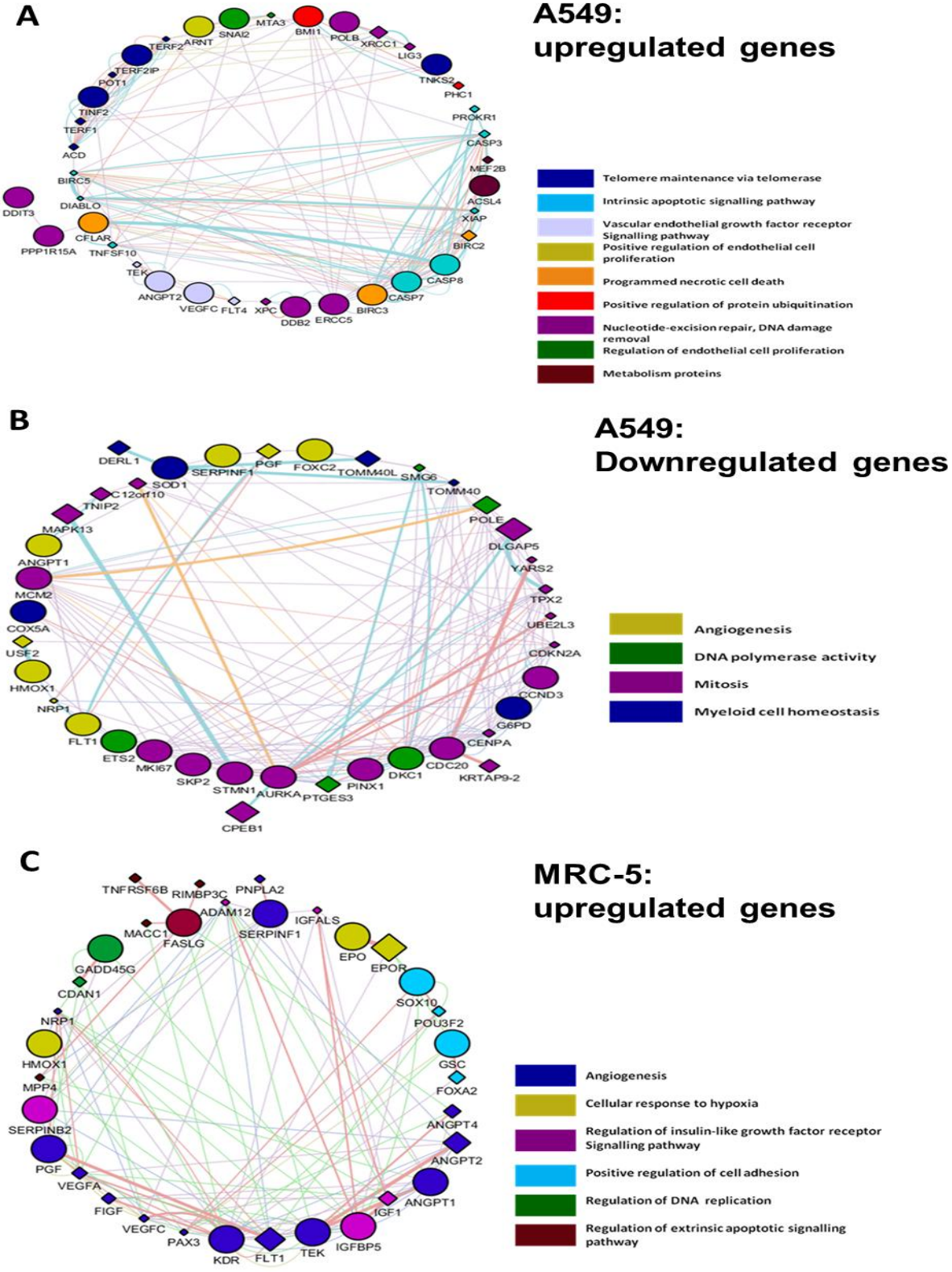


Figure 3.7 Introduction of exogenous methyl pyruvate protects MRC-5 cells by upregulating angiogenesis and cell adhesion processes: The GeneMANIA module in Cytoscape (version 3.3.0) was applied to construct interaction networks in each cell line. The Networks represent upregulated and

downregulated gene sets after treatment with both drugs (irinotecan and methyl pyruvate) for 48 hours. Genes in the submitted query list are indicated as circular nodes while those predicted to be related are displayed as diamond nodes (<>). Pathway categories received a score weight when the pathways data sets in GeneMANIA link to members of the query list. The edges (coloured lines) that connect neighbouring genes are depicted as follows: **medium purple**: Co-expression, **medium turquoise**: pathway; **brown**: physical interaction; **blue**: co-localization; **khaki**: shared protein domains. Interactions were considered statistically significant when $q < 0.05$.

Using the REACTOME pathway overview (Figure 3.8) and REACTOME pathway diagram analysis (Figure B2) in A549 and MRC-5 cells after introduction of exogenous methyl pyruvate combined with irinotecan, similar results were observed. Pathways were considered statistically enriched when the $p < 0.05$. In A549 lung fibroblast cancer cells the combined treatment significantly upregulated pathways such as the DNA repair ($p = 0.002$), programmed cell death ($p = 0.011$), and VEGF signaling ($p = 0.032$) (Figure 3.8 A) while down regulating DNA strand elongation ($p = 000079$) and cell cycle pathways ($p = 0.001$), suggesting disruption and inhibition of DNA replication and cell growth. This is further supported by the disruption of the DNA replication fork and the upregulation of caspase-7 in the intrinsic apoptotic pathway through in-depth REACTOME analysis (Figure 2B). Furthermore, in MRC-5 cells in contrast to A549, the drug combination significantly upregulated pathways such as signaling by VEGF ($P = 0.005$), Tie2 signaling ($p = 0.006$), hemostasis ($p = 0.051$) (Figure 3.8 C), indicating cell survival and cell growth.

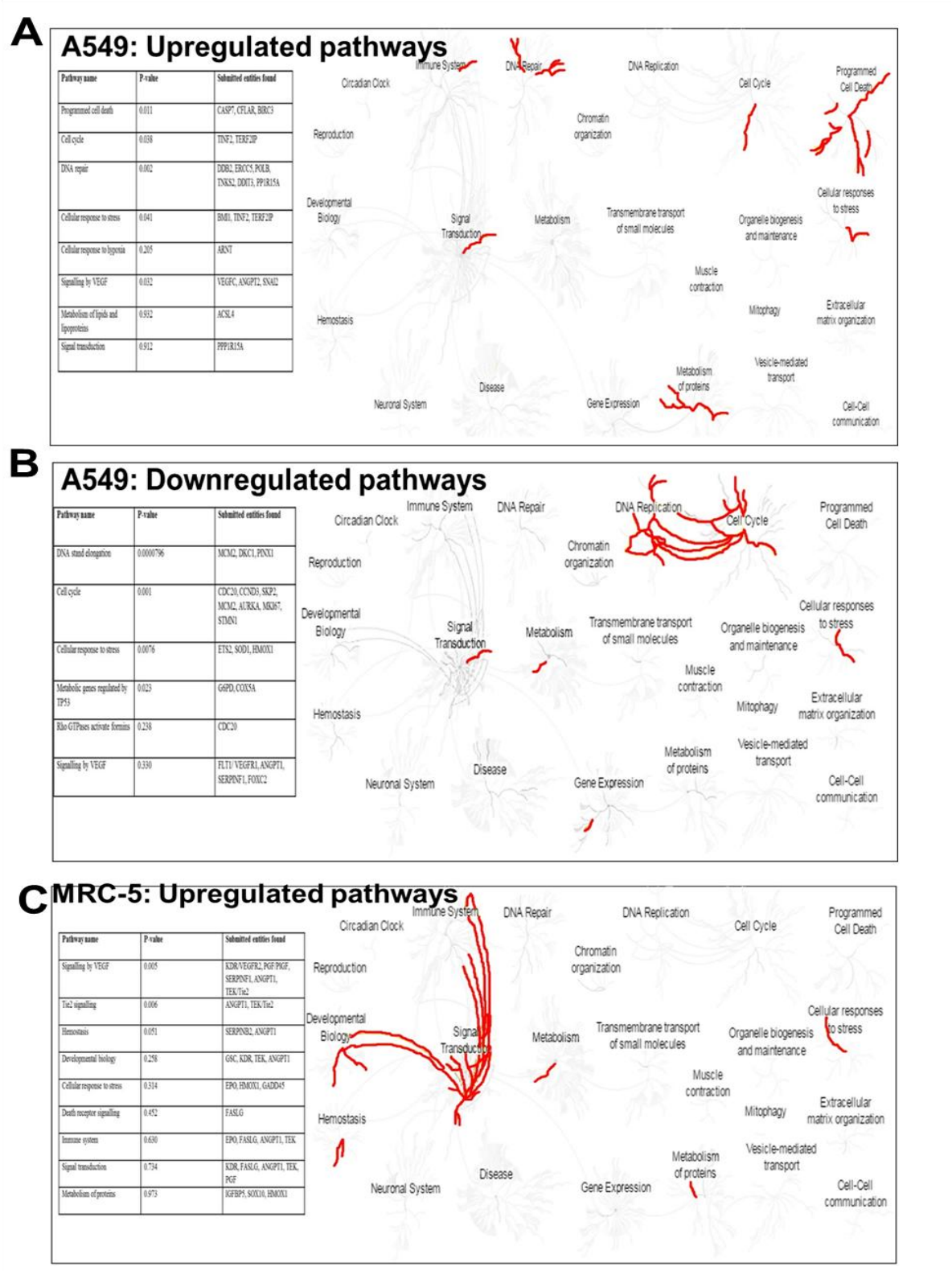


Figure 3.8 Exogenous methyl pyruvate protects MRC-5 cells from irinotecan-induced cell death by upregulating Developmental biology, signal transduction and homeostasis pathways: REACTOME analysis was conducted using query lists of differentially expressed genes in A549 and MRC-5 cells. Pathways were considered enriched when a significant number of the query list genes

were part of a particular pathway against the overall pathway genes. Each pathway was considered statistically enriched when the $p < 0.05$. REACTOME produced maps of 24 pathways, with upregulated and downregulated pathways highlighted (in red) indicating those induced in (**A**-**B**) A549 and (**C**) MRC-5 cells. The central node of each radial representation corresponds with the uppermost level of the hierarchy (such as programmed cell death) while the concentric rings of nodes around the central node represents successive events of the hierarchy (such as intrinsic/extrinsic apoptotic signalling pathways).

Chapter 4: Discussion

4.0 Introduction

This study shows that the reversal of the Warburg effect by exogenous methyl pyruvate is sufficient to kill cancer cells and protect normal cells from the toxicity of chemotherapeutic agents such as irinotecan.

The exogenous methyl pyruvate, combined with irinotecan, rescued normal MRC-5 lung fibroblasts from irinotecan-induced cell death as an increase in live cells was observed, with most cells accumulating at the G_0/G_1 phase, S-phase, and the G_2/M phase, indicating cell cycle progression. In contrast the same treatment induced apoptosis in the lung cancer cell line A549 with most cells accumulating at the sub G_0/G_1 phase. In MDA-MB 231 cells, treatment with exogenous methyl pyruvate alone induced necrosis and apoptosis with most cells accumulating at the G_0/G_1 phase and at the sub G_0/G_1 phase indicating cell cycle arrest and cell death.

It was also observed that the p53/p21 axis was deregulated after introduction of exogenous methyl pyruvate with a significant decrease in cell death. In contrast to MRC-5 cells, the p53/p21 axis was enhanced in A549 cells followed by a significant increase in cell death. However, in MDA-MB 231 cells, death was through another p53-independent pathway following exposure as an increase in necrotic cells was observed.

Through pathway focussed gene expression profiling, exogenous methyl pyruvate in combination with irinotecan promoted cell proliferation by upregulating angiogenic and survival factors in MRC-5 cells, while in A549 cells, it induced cell death by upregulating pro-apoptotic and anti--angiogenic factors. These findings are explained in detail in the following sections.

4.1 Methyl pyruvate protects MRC-5 fibroblasts from irinotecan induced cell death

During chemotherapy, both cancer and normal cells are exposed to genotoxic stress. The major shortcoming of chemotherapeutic drugs is that they possess little discrimination between the rapidly dividing cancer cells and normal cells leading to severe side effects. These drugs have been suggested to negatively affect the gastrointestinal tract, hair bulbs, reproductive organs, the haematopeotic system, and the lymphatic tissue (Lorusso *et al*., 2010). Exploring the phenotypic difference between normal and cancer cells may provide a strategy to selectively target cancer cells. The glycolytic phenotype of cancer cells as a chemotherapeutic target was explored by enhancing OXPHOS. The *xCELLigence* technology, Annexin-V and PI-staining assays were used to monitor cell proliferation and measuring cell death, respectively.

Using the *xCELLigence* technology, 2 mM exogenous methyl pyruvate alone and in combination with 0.5 µM irinotecan killed both cancers (A549 and MDA-MB 231) while promoting survival of MRC-5 normal cells. This study corroborates those of others, which observed the toxicity of irinotecan to normal and cancer cells (Garcia-Carbonero and Supko, 2002). The promotion of survival when MRC-5 cells were treated with exogenous methyl pyruvate in the presence of irinotecan suggests its role in protecting normal cells from toxicity. These findings coincide with a previous study that showed protection of normal blood cells but not leukaemic cells using ethyl pyruvate (Hollenbach *et al*., 2008). In another study they found that 1 mM ethyl pyruvate increased survival of C57BL/6NHsd mice undergoing total body irradiation treatment (9.75 Gy TBI) (Epperly *et al*., 2007).

Using the Annexin-V and PI-staining, this study further observed the discrimination between cancer and normal cells by introduction of exogenous methyl pyruvate. An increase in cell

death in A549 and MDA-MB 231 cells while, in MRC-5 cells an increased in live cells was observed over time. This coincides with a study that showed cancer cells (MCF-7, A549, and M059k) were killed without harming SAEC normal cells after treatment with dicloroacetate (Bonnet *et al*., 2007). Dicloroacetate is a known inhibitor of the pyruvate dehydrogenase kinase (PDK) enzyme. PDK leads to the activation of pyruvate dehydrogenase (PDH) which is involved in convertsion of pyruvate to acetyl CoA before entering the TCA cycle (Whitehouse and Randle, 1973).

It is noteworthy, that this study shows that treatment with exogenous methyl pyruvate alone killed the mutant p53 MDA-MB 231 cells as compared to irinotecan treatment alone. This was interesting since MDA-MB 231 cells are known to be resistant to most chemotherapeutic drugs (Hussien and Brooks, 2011). It has been observed that functional p53 is important in sensitizing cancer cells to chemotherapeutic drugs (Zhang et al., 1998; Zhang et al., 1999; Lowe et al., 1993). Interestingly, exogenous methyl pyruvate induced necrotic cell death in MDA-MB 231 cells probably through the accumulation of mitochondrial-derived ROS. It has been speculated that programmed necrotic cell death occurs due to an increase in mitochondrial-derived ROS and calcium (Festjens *et al*., 2006). Induction of necrosis has also been observed previously, upon treatment with PRIMA-1 combined with flavopridipol in leukemia cells carrying mutant p53 (Cory *et al*., 2006).

These results show that exogenous methyl pyruvate protects normal cells from the toxicity of chemotherapeutic agents and is a promising adjunctive in this regard.

4.2 Exogenous methyl pyruvate promotes cell cycle progression in normal cells but cell death in cancer cells

Using PI-staining, it was observed that exogenous methyl pyruvate alone and in combination with irinotecan, promotes cell cycle progression in normal cells as a large percentage of cells accumulated at the G_0/G_1 phase. This suggests that introduction of exogenous methyl pyruvate protects MRC-5 cells from irinotecan's toxicity. The protective effect of exogenous methyl pyruvate is further supported by deregulation of the p53/p21 axis, suggesting inhibition of cell death. This coincides with a previous study, that showed that cell viability increased in three human normal fibroblast cells (WI38, MRC-5, HFL1) after exposure to RY4 peptide combined with DCA, an enhancer of endogenous pyruvate (Wu *et al*., 2014).

In contrast to what was observed in normal MRC-5 cells, the introduction of exogenous methyl pyruvate to both cancer cell lines (A549 and MDA-MB 231) accelerated cell death as an increased in cells in the sub G_0/G_1 phase was observed. Exogenous methyl pyruvate sensitised a 90.7% of A549 cells to irinotecan. This is supported by the enhanced expression levels of p53 and p21 by RT-PCR which implies the induction of apoptosis. This suggests that the presence of functional p53 in these cells increased their sensitivity to the combined treatment. In another study, cells with wild type p53 were found to be more sensitive to a variety of DNA damaging agents (cisplatin, nitrogen mustard, etoposide, and γ – irradiation) compared to cells with mutant p53 (Piovesan *et al*., 1998). This is because many chemotherapeutic agents are known to target the p53-pathway to induce cancer cell death.

In the present study, cell death in MDA-MB 231cells, which are p53 mutant, was observed although it appeared to be less efficient compared to in A549 cells suggesting that exogenous methyl pyruvate can induce another cell death mechanism that is p53-independent.

Experiments using RT-PCR further supported this hypothesis by showing downregulation of the p53/p21 axis in MDA-MB 231.

From these results, introduction of exogenous pyruvate can be explored in chemotherapy as an adjunctive as it supports normal MRC-5 cell survival with cells accumulating at the G_0/G_1 phase while promoting cancer cell death.

4.3 Methyl pyruvate deregulates apoptotic pathways

The expression profile of genes involved in the p53 pathway and the intrinsic-mitochondrial apoptosis pathway was investigated to elucidate the molecular basis for the observed phenotypes after treatment with irinotecan combined with methyl pyruvate. RT-PCR and Western blot analyses were used to profile expression of the p53-pathway: p53, MDM2, and p21 and the mitochondrial cell death pathway; BAX, BID, caspase3, caspase-9, cytochrome c, and RBBP6.

It was observed that introduction of exogenous methyl pyruvate protects MRC-5 cells from irinotecan-induced cell death by switching off apoptotic pathways, since p53 and p21 were downregulated. p21 is a known pro-apoptotic protein that is transactivated by p53. Genes such as BAX, BID, caspase-3, and caspase-9 were depleted after exogenous methyl pyruvate was added, suggesting inhibition of intrinsic apoptosis. This is further supported by the downregulation of cytochrome c protein, as cytochrome c must be released from the mitochondrial membrane, and form the apoptosome (with Apaf-1 and procaspase-9) to activate caspase-9. Cytochrome c initiates the intrinsic cell death pathway by activating downstream cascade of caspases leading to the induction of apoptosis (Crompton, 2000; Haupt *et al*., 2003). This suggests that introduction of exogenous methyl pyruvate provides protection to normal

MRC-5 cells during chemotherapy (irinotecan treatment) by deregulating the apoptotic machinery.

However, in A549 cancer cells (wild type p53) the introduction of exogenous methyl pyruvate induced intrinsic cell death by enhancing the p53/p21 axis and mitochondrial apoptotic pathway as upregulation of p53, p21, and caspase-9 transcripts was observed. Caspase-9 is a known initiator caspase that cleaves and activates the executioner caspases such as caspase-3. It has been shown that Apaf-1 and/or caspase-9 are required for p53-dependent apoptosis to be induced (Soengas *et al*., 1999; Schuler *et al*., 2000). The induction of the intrinsic pathway is further supported by the upregulation of cytochrome c protein. The upregulation of cytochrome C suggests that exogenous methyl pyruvate induced a p53-dependent, intrinsic cell death. The activation of the mitochondrial function corroborates with another study, wherein A549 cells were exposed to exogenous methyl pyruvate combined with x-ray causing the mitochondrial energy metabolism pathway to be induced (Nishida et al., 2014).

These results indicate that the introduction of exogenous methyl pyruvate promotes cancer cell death by inducing the p53/21 axis and mitochondrial cell death pathways while increasing survival in normal MRC-5 cells by switching off these pathways.

4.4 Exogenous methyl pyruvate can also induce another p53-independent and caspase-independent cell death

Introduction of exogenous methyl pyruvate induced a p53-independent and caspase-independent cell death in MDA-MB 231 cells, as p53, p21, Bax, and caspase-9 were downregulated as shown by RT-PCR analysis. This is also evident by the downregulation of cytochrome c protein shown by Western blot analysis suggesting the inhibition of intrinsic cell death pathway. These findings corroborate a study by Dorsam *et al*., (2015), showing that

exposure to lipoic acid, a cofactor of the pyruvate dehydrogenase complex, also induced a caspase-independent cell death in human colon cancer cells (HCT116: p53 null cells).

Even though p53 and p21 were upregulated after treatment with irinotecan, cell death was minimal compared to treatment with exogenous methyl pyruvate. This finding corroborates what was reported by Takimoto *et al*., (2002); where CP31398 (a, styrylquinazoline agent) was shown to upregulate p53 target genes such as p21. However, their expression did not correlate with the induction of cell cycle arrest or apoptosis in colon, breast, lung, ovarian and osteosarcoma cancer cells. The observation from the current study supports findings by other studies, that cancer cells with mutant p53 are less sensitive to chemotherapeutic agents. Another study by Nabeya *et al*., (1995), has shown that gastric/esophageal cancer cells expressing mutant p53 were more resistant to chemotherapeutic agents (5-fluorouracil, mitomycin C, and *cis*-dicholorodiammin-eplatinum (II)) despite the increase in mutant p53 expression levels. These results indicate that introduction of exogenous methyl pyruvate can induce cancer cell death through p53-independent and caspase-independent pathways in cancer cells with mutant p53.

4.5 The role of RBBP6 during metabolic reprogramming

The role of RBBP6 in response to metabolic reprogramming in normal and cancer cells was investigated. Expression of RBBP6 was assessed before and after treatment with exogenous methyl pyruvate and irinotecan by Western blot analysis. Retinoblastoma binding protein 6 (RBBP6) is essential for chaperone-mediated ubiquitination of p53. RBPP6 targets p53 for degradation by enhancing the activity of MDM2, through its RING finger-like domain (Chen *et al*., 2013).

After introduction of exogenous methyl pyruvate p53 levels became depleted while an increase in RBBP6 isoform 1 protein expression was observed in A549 cell line. This may suggest a

negative feedback loop between RBBP6 and p53. These findings corroborate a previous study that showed knockdown of RBBP6 led to an upregulation of p53 in mice resulting in embryonic lethality (Li *et al*., 2007). Interestingly, upregulation of RBBP6 isofrom 1 in both cancer cell lines (A549 and MDA-MB 231) was observed after introduction of exogenous methyl pyruvate, with an increase in cell death shown in Figure 3.3 A and B. This suggest that RBBP6 isofrom 1 might play a pro-apoptotic role during metabolic reprogramming. This is also evident by the significant shift of A549 and MDA-MB 231 cell lines into the sub G_0/G_1 phase, indicating cell death. However, the exact mechanism by which RBBP6 isofrom 1 facilitates cell death during metabolic reprogramming is not clear. This is the first study to propose a role of RBBP6 isoform 1 during metabolic reprograming in cancer cells. To date RBBP6 isoform 1, a negative regulator of p53, is known to facilitate cell proliferation (Moumen et al., 2007).

In MRC-5 normal cells, introduction of exogenous methyl pyruvate resulted in the differential expression of two RBBP6 isoforms (isoform 1 and isoform 3). Isoform 1 was enhanced while isoform 3 expression was reduced after 24 hours of treatment with exogenous methyl pyruvate and combination treatment. This may suggests that RBBP6 isoform 1 and 3 have an inverse relationship. These results corroborates with others that RBBP6 isoform 3 and isoform 1 are inversely regulated during differentiation of C2C12 cells (Ji and Yian, 2009). RBBP6 isoform 1 upregulation and isoform 3 downregulation thus indicate growth. The upregulation of isoform 1 after introduction of exogenous methyl pyruvate indicates cell growth which is evident in Figure 3 C. Introduction of exogenous methyl pyruvate resulted in cell cycle progression, with most MRC-5 cells accumulating at the G_0/G_1 phase. These results suggest that the introduction of exogenous methyl pyruvate promotes cell proliferation by modulating the expression of RBBP6 isoform 1 and isoform 3 in MRC-5 fibroblast.

4.6 Exogenous methyl pyruvate upregulates pro-angiogenic and survival pathways in MRC-5 normal cells and pro-apoptotic pathways in A549 cells

The molecular bases of the observed phenotypes in cancer and normal cells was investigated after the introduction of exogenous methyl pyruvate to irinotecan-tretaed cells. For comparison purposes, A549 lung cancer cells and MRC-5 normal lung fibroblast cell line were used as they are both lung-derivedcell lines. The Human Cancer PathwayFinder^{TM} RT^{2} Profiler^{TM} PCR Array, (PAHS-O33ZF-12) was used to profile 84 cancer-related genes. Furthermore, PANTHER, GeneMANIA and REACTOME were used for pathway analysis. It was observed that exogenous methyl pyruvate promoted cell survival in the normal fibroblast cell line and cell death in the cancer cell line.

Unique genes such as TEK, ANGPT1, GSC, GADD45G, and EPO that were upregulated in MRC-5 cells were part of pro-angiogenic, plasminogen-activating cascade, and blood coagulation factors which promoted cell proliferation and survival. Angiopoietin 1 (ANGPT1) and its corresponding receptor, TEK/TIE2 are known to control vascular morphogenesis and homeostasis (Augustin *et al*., 2009; Maisonpierre *et al*., 1997). It has been shown that systemic administration of ANGPT1 protein (COMP-angiopoietin-1) protects the kidney from diabetic microvascular damage in mice (Lee *et al*., 2007). Cell cycle progression previously shown in Figure 3.3 C and D is further suggested by the upregulation of the Growth arrest and DNA-damage-inducible 45 gene (GADD45) which interacts with the Cdk1/CyclinB1 complex (Na *et al*., 2010). GeneMANIA also showed the upregulation of molecules such as Goosecoid (GSC) that play a role in enhancing cell adhesion. GSC, is known to control glucose metabolism by targeting genes that are active in the pancreas, liver, and adipose tissue (Friedman and Kaestner, 2006).

Erythropoietin (EPO) was also observed to be upregulated when normal cells were treated with exogenous methyl pyruvate combined with irinotecan. Erythropoietin (EPO), a glycoprotein hormone is essential in the regulation of the red blood cell mass, participating in negative feedback control from a renal oxygen sensor to the erythroid system, resulting in an increase in hemoglobin levels (Lacombe *et al*., 1988). Studies have speculated that nephrotoxicity lowers EPO secretion and through this mechanism, contributes to anaemia observed after therapy with several chemotherapeutic agents (Fjornes *et al*., 1998). It has been shown that cisplatin-associated anaemia was persistent with EPO deficiency state, which resulted from cisplatin-induced renal tubular damage (Wood and Hrushesky, 1995). Furthermore, they found that these effects could be prevented or treated by EPO replacement. Thus an upregulation of EPO may lower toxicity associated with chemotherapeutic agents such as nephrotoxicity.

In A549 cells, most uniquely upregulated and downregulated genes was part of pro-apoptotic and pro-angiogenic factors. Caspase-7 a known proapoptotic gene were upregulated. Caspase-7, is known to induce apoptosis, which entails cellular morphological changes such as phosphatidylserine exposure, nuclear condensation, and genomic DNA fragmentation (Lamkanfi and Kanneganti, 2010). Its upregulation indicates an increase in apoptosis. Furthermore, caspase-7 along with caspase-3 shows specificity towards multiple substrates, including XIAP, BID, and gelsolin (Walsh *et al*., 2008) indicating the activation of the intrinsic mitochondrial cell death pathway in A549 cells after introduction of exogenous methyl pyruvate. Additionally, in this study, the downregulation of cell cycle genes such as CDC20 and STMN1 was observed to be consistent with the inhibition of cell proliferation in A549 cells. The cell division cycle 20 homologue (CDC20) plays a key role in activating the anaphase promoting complex (APC/C) thereby leading to mitotic progression (Hoeller *et al*., 2006). Stathmin 1/oncoprotein-18 (STMN1), the microtubule-destabilizing protein, is essential during

mitosis, to positively influence cell cycle progression (Rana *et al*., 2008; Belletti and Baldassarre, 2011).

REACTOME pathway analysis further showed that in A549 cancer cells, DNA damage and repair genes such as DNA damage-inducible transcript 3 (DDIT3) and DNA damage-binding protein 2 (DDB2) were upregulated after the drug combination and was consistent with cell death. Overexpression of DDIT3 induces cell cycle arrest and apoptosis in certain cancers (Barone *et al*., 1994; Zinszner *et al*., 1998). DDIT3 transcription, mRNA stability, and translation are induced in response to DNA damage, ER stress, hypoxia, and starvation (Luethy and Holbrook, 1992; Tang *et al*., 2002; Oyadomari and Mori, 2004). Thus overexpression of DDIT3 will result in cell cycle arrest and apoptosis. DDB2 is well known to function in genome nucleotide excision repair (Minig *et al*., 2009; Ennen *et al*., 2013; Roy et al., 2013; Zhao *et al*., 2014). DDB2 has recently been shown to play an important role in impeding tumour progression and tumour relapse. Upregulation of *DDB2* prevents tumour progression and relapse (Han *et al*., 2014).

The two different outcomes; surivial in MRC-5 and death in A549 are further supported by the upregulation of anti-angiogenic factor, ANGPT2 in A549 and pro-angiogenic factor, ANGPT1 in MRC-5. Angiopoietin-2 (ANGPT2), is a natural antagonist for Tie2/TEK receptor that has been shown *in vivo* to disrupt angiogenesis (Maisonpierre *et al*., 1997). ANGPT1 a known anti-angiogenic factor facilitates angiogenesis when it binds to its corresponding receptor, Tie2/TEK (Maisonpierre *et al*., 1997; Augustin *et al*., 2009).

These results indicate that introduction of exogenous methyl pyruvate can protect normal MRC-5 cells from irinotecan-induced cell death by upregulating pro-angiogenic and survival pathways while inducing cell death in A549 cell by upregulating pro-apoptotic pathways.

Chapter 5: Conclusion and future prospects

This is the first study to propose the potential molecular mechanism by which exogenous methyl pyruvate provides protection of normal cells exposed to a DNA damaging agent. In the current study, exogenous methyl pyruvate was shown to have protective effects on irinotecan-treated MRC-5 fibroblasts and enhanced apoptosis in similarly treated A549 and MDA-MB 231 cells. This study proposes that exogenous pyruvate has potential to protect normal cells during chemotherapy. It was also observed that exogenous methyl pyruvate protects irinotecan-treated normal lung fibroblast cell line (MRC-5) probably by turning off the p53/p21 axis of the apoptotic pathways thereby inhibiting apoptosis. In contrast, the introduction of exogenous pyruvate kills the A549 cancer cells by enhancing the p53/p21 axis. Introduction of exogenous methyl pyruvate can also induce another p53-independent and caspase-independent cell death in MDA-MB 231 cells with mutant p53. Futherrmore, by microarray analysis it was observed that several genes are altered when normal and cancer cells are treated with irinotecan combined with exogenous methyl pyruvate. These alterations in specific genes promote the survival and growth of MRC-5 normal cells and death of A549 lung cancer cells.

Future validation studies must be conducted, as the *in vivo* relevance of these findings has not been tested. Such studies may involve the use of nude mice to validate the role of exogenous methyl pyruvate on normal and cancer cells during chemotherapy.

The knowledge provided by this study may provide an opportunity to protect patients undergoing chemotherapy from its toxic effects and increase the success rate of chemotherapeutic drugs. Economically, this drug combination therapy will also be cost effective as side-effects and relapse may be reduced, especially in developing countries such as South Africa.

Chapter 6: References

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Appendices

Appendix A

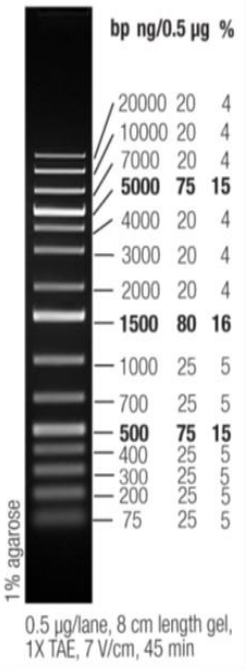


Figure A1. GeneRuler™ 1 kb plus DNA ladder

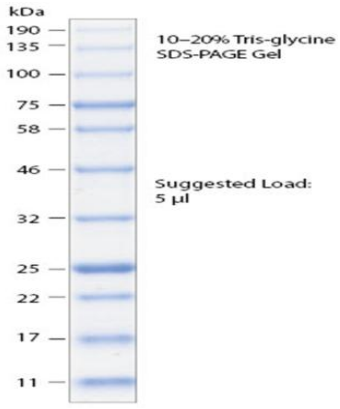


Figure A2. New England Biolabs blue pre-stained protein standard broad range.

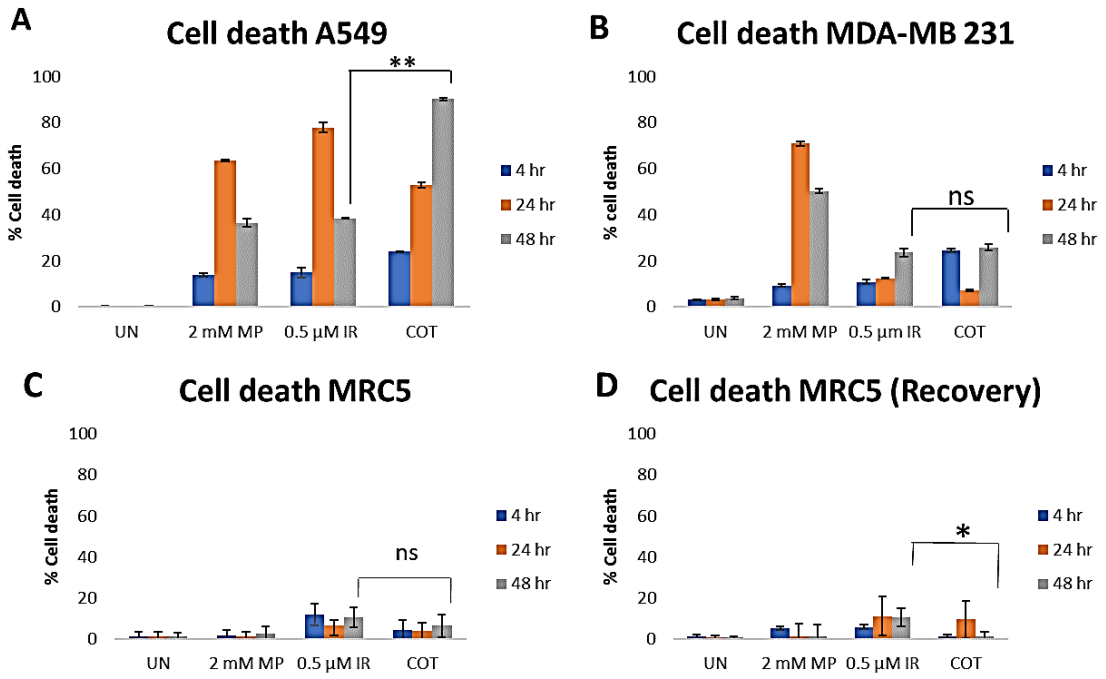
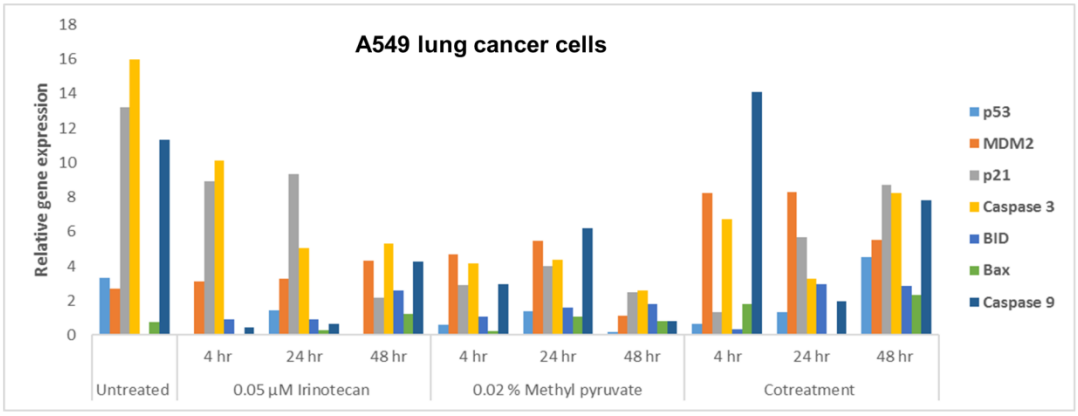
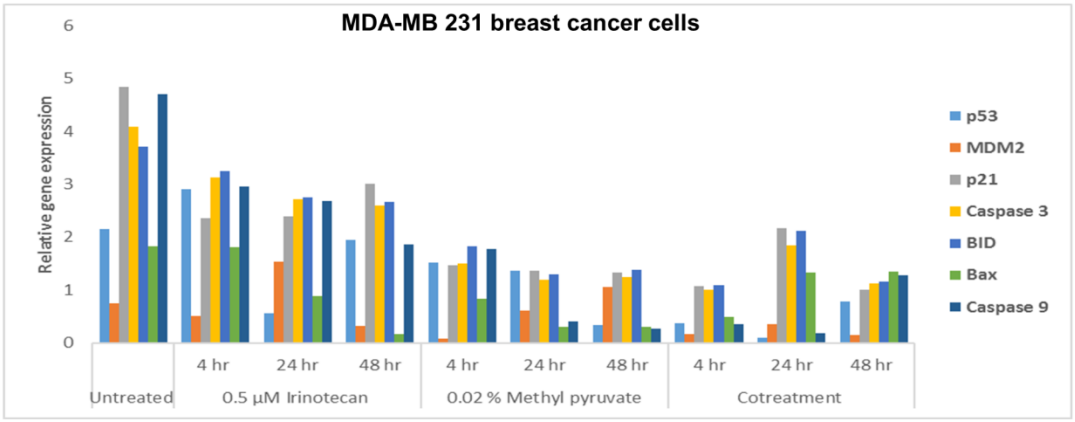


Figure A3: Analysis of flow cytometry sub G0/G1 cell distribution in A549 (A), MDA-MB 231 (B), MRC-5 (C), and MRC-5 upon recovery (D). Experimental data was derived from three independent experiments and were considered statistically significant when the p-value was ≤ 0.05. When the p-value ≤ 0.001 (**) the difference was considered very statistically significant, when the p-value ≤ 0.05 (*) the difference was considered statistically significant and when the p-value ≥ 0.05 (ns) the difference was considered not to be statistically significant. The statistical analysis was done using the Graphpad software. The error bars reflect the standard error of the mean of biological replicates. The following symbols were used to represent each treatment; MP- methyl pyruvate, Ir- irinotecan, COT- cotreatment, and UN- untreated cells.

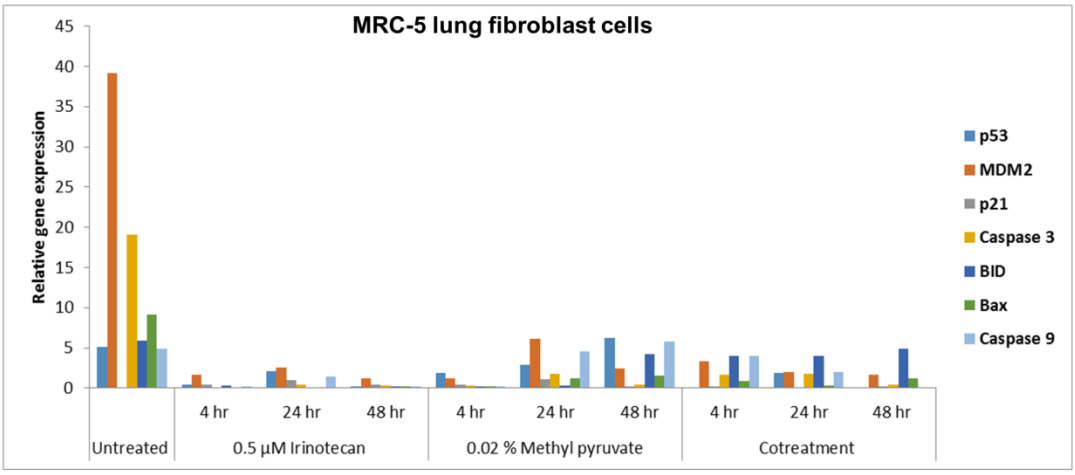
A


A549 lung cancer cells
Relative gene expression
18
16
14
12
10
8
6
4
2
0
4 hr
24 hr
48 hr
Untreated
0.05 µM Irinotecan
0.02 % Methyl pyruvate
Cotreatment
p53
MDM2
p21
Caspase 3
BID
Bax
Caspase 9

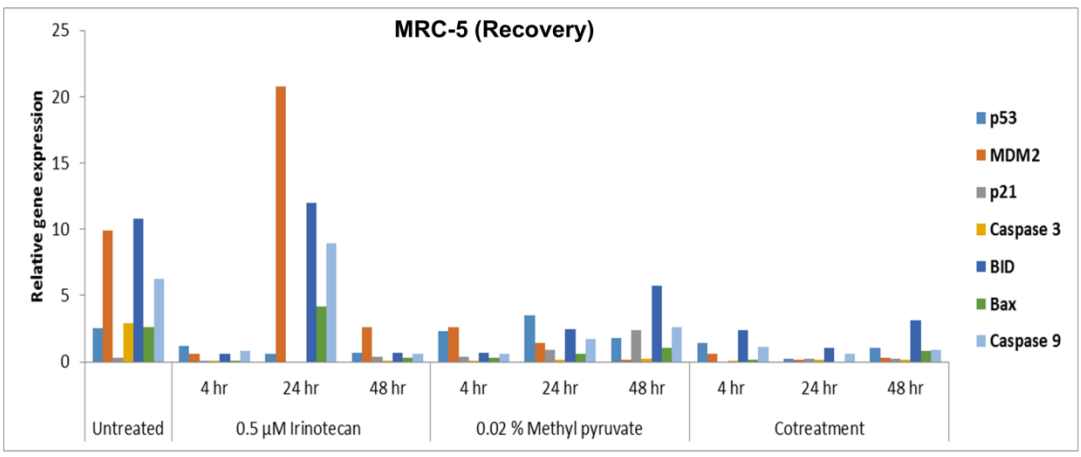
B


MDA-MB 231 breast cancer cells
Relative gene expression
6
5
4
3
2
1
0
4 hr
24 hr
48 hr
Untreated
0.5 µM Irinotecan
0.02 % Methyl pyruvate
Cotreatment
p53
MDM2
p21
Caspase 3
BID
Bax
Caspase 9

C


MRC-5 lung fibroblast cells
Relative gene expression
45
40
35
30
25
20
15
10
5
0
4 hr
24 hr
48 hr
Untreated
0.5 µM Irinotecan
0.02 % Methyl pyruvate
Cotreatment
p53
MDM2
p21
Caspase 3
BID
Bax
Caspase 9

D


MRC-5 (Recovery)
Relative gene expression
25
20
15
10
5
0
4 hr
24 hr
48 hr
Untreated
0.5 µM Irinotecan
0.02 % Methyl pyruvate
Cotreatment
p53
MDM2
p21
Caspase 3
BID
Bax
Caspase 9

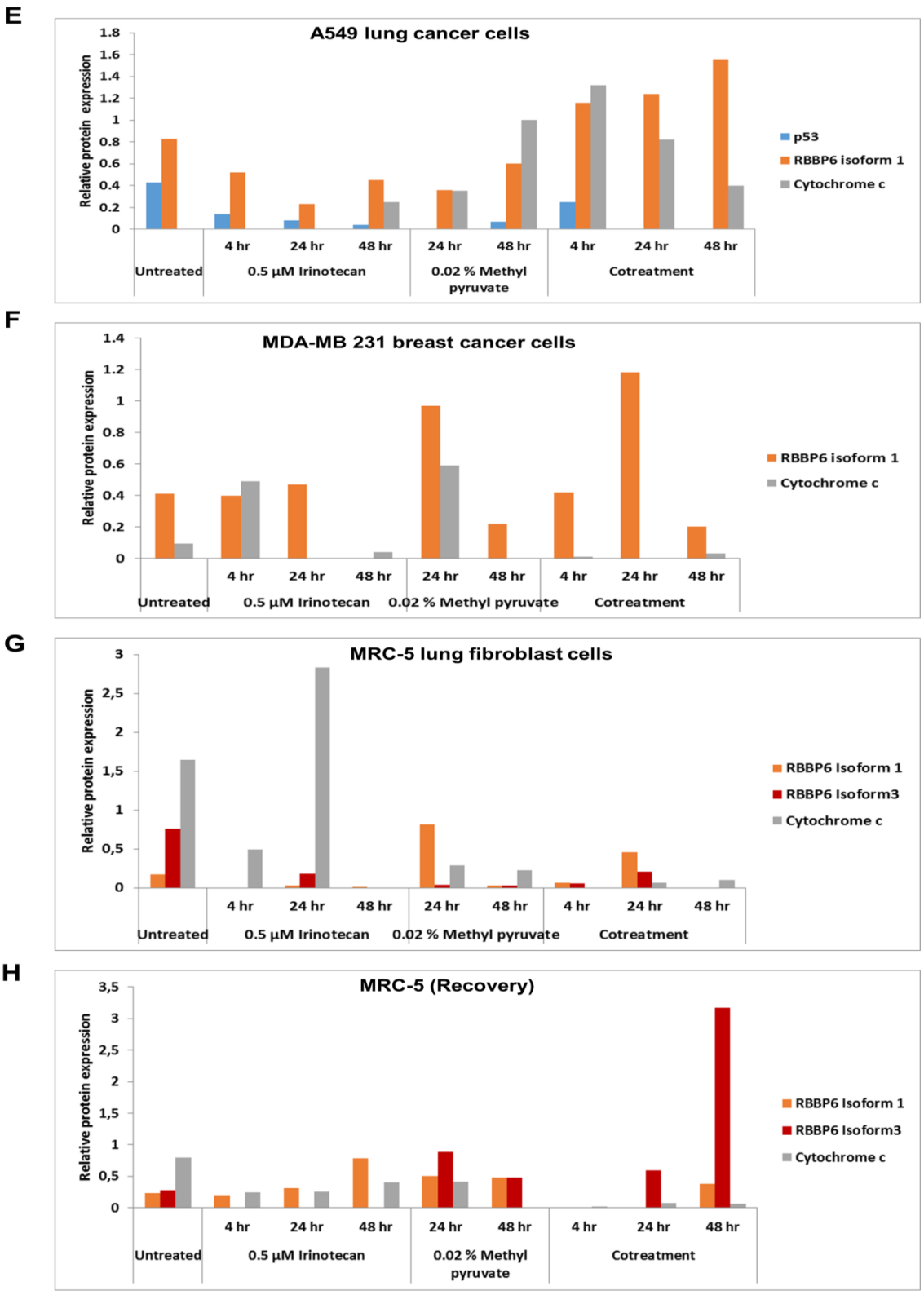


Figure A4 : Introduction of exogenous methyl pyruvate resulted in deregulation of apoptotic pathways in MRC-5 cells : Relative transcript **(A - D)** and protein **(E - H)** quantification measurements normalized by the expression of the house keeping genes, GAPDH and β-actin using MyImage AnalysisTM Software (Thermo Scientific) from one independent experiment in A549 lung

cancer cell line , MDA-MB 231 breast cancer cell line, MRC5 a non-tumorigenic cell line and MRC5 cells upon recovery from various treatments**.**

Table A1: Cell cultures: All cell lines used in this study were purchased from ATTC (American type culture collection) Virginia, USA.

Cell line	**Diagnosis**	**Host**	**p53 status**
MRC5	Normal	Human	Wildtype (WT)
MDA-MB 231	Breast cancer	Human	$p53^{280R-K}$
A549	Lung cancer	Human	WT

Table A2: Biological buffers and solutions

Buffer/solution	**Composition**
1.5 M Tris-HClbuffer, pH 8.8	8.12 w/v % TRIS (hydroxymethyl) aminomethane
0.5 M Tris-HCl buffer, pH 6.8	0.36 w/v % TRIS (hydroxymethyl) aminomethane
SDS Sample buffer (5X)	62.5 mM Tris-HCl buffer (pH 6.8) 10% Glycerol 2% SDS 5% β-Mercaptoethanol
5% Running buffer, pH 8.3	25 mM Tris 192 mM glycine

	1% SDS
Towbin buffer (pH 8.3)	25 mM Tris base 192 mM glycine 0.1%SDS 20% (v/v) methanol on day of use.
Phosphate buffered saline (PBS) 50X (pH 7.3)	1.4 M NaCl 27 mM KCl 101 mM Na_2HPO_4 18 mM KH_2PO_4
Citric Saline buffer 10X	1.35 M KCl 0.15 M sodium azide
TAE Buffer 10X	40 mM Tris 20 mM Acetic acid 1 mM EDTA

Table A3: Kits and laboratory equipment used

Kits and equipment	**Suppliers**
RTCA DP ACEA XCELLigence analyzer, version 1.2 (2009)	Roche (Biosciences Inc.)
XCELLigence E-plate 16 (05469813001)	Roche (Biosciences Inc.)
BD Acurri ^{TM} C6 cytometer	Biosciences
RevertAid first strand cDNA synthesis kit (k1622)	Thermo Scientific

ProtoScript first strand cDNA synthesis kit (E6300S; lot 0041411)	BioLabs Inc.
Taq 2x Master Mix Kit (M0270L; lot 0231412)	BioLabs Inc.
Polyscreen PVDF transfer membrane (NEF1002)	Perkin Elmer Life Sciences Inc.
GeneAmp 2400 thermal cycle PCR system (2400)	Perkin Elmer
Gel Doc XR^+ imaging system (170-S170)	Bio-Rad
Nanodrop spectrophotometer	Thermo Fischer Scientific USA
Hoefer VE blotting module	Amersham Biosience UK
RT^2 first cDNA strand kit (330401)	QIAGEN
DNase kit (AMPD1 – 1KT)	Sigma
Real-time light cycler machine LC-480	Roche
INTERGA VIAFLO ASSIST Liquid handler (8-channel pipette 2-50 µl) (CH-7205 Zizers)	Interga Biosciences
Low speed benchtop centrifuge LCM-3000	Grant Instruments
RT2 ProfilerTM PCR array PAHS-O33ZF-12 Human Cancer PathwayFinderTM (PAHS-033ZF-12)	QIAGEN
RT^2 SYBR Green qPCR Mastermix Kit (330500)	QIAGEN

Table A4: Reagents and chemicals

Name	**Supplier and catalog number**
Chloroform (99 + %)	Sigma (31k3695)
Nuclease-free water	Qiagen (129117)
Propan-2-ol	Merk (1036573)
Ethanol absolute	ProLabo (VWR) (20820327)
TRI reagent®	Sigma (T9424)
GeneRuler 1kb Plus DNA ladder (0.1 µg/µl)	Thermo Scientific (SM1333)
6X DNA loading dye	Thermo Scientific (R0611)
PageRuler^{TM} prestained protein ladder	Thermo Scientific (26616)
SuperBlock (PBS) blocking buffer	Thermo Scientific (37580)
Super Signal Chemiluminescent Substrate Kit (composition; Lumino/enhancer and stable peroxide buffer)	Thermo Scientific (34087)
RNase free-water	QIAGEN (129117)

Table A5: Cell culturing reagents

Media/ reagents	**Supplier and catalog number**
Dulbecco's modified eagle's medium	Sigma (D6429)
Fetal calf serum	Highveld biological (NO.306)
Penicillin/streptomycin antibiotics	Sigma (074M 846V)
Trypsin-EDTA solution	Sigma (SLBK7106V)
Irinotecan	Sigma (1347609 – 100MG)
Methyl pyruvate	Sigma (P2881)

Table A6: Oligonucleotides

All primers were obtained from Inqaba Biotech except for the GAPDH primers which were supplied by Thermo Scientific.

Primer	**5'-3' sequence**	**Tm (ºC)**	**Catalog no./Barcode**	**Reference**
GAPDH forward primer	ACACTCAGACCCCCACCACA	58.0	00205654	(Erler *et al*., 2004)
GAPDH reverse primer	CATAGGCCCCTCCCCTCTT	58.0	00207076	(Erler *et al*., 2004)
Bid forward primer	GCTGTATAGCTGCTTCCAGTG	62.77	S3823	(Erler *et al*., 2004)

Bid reverse primer	GCTATCTTCCAGCCTGTCTTCTC	64.55	S3824	(Erler *et al*., 2004)
Bax forward primer	CTGCAGAGGATGATTGCCG	62.32	S381F	(Erler *et al*., 2004)
Bax reverse primer	TGCCACRCGGAAAAAGACCT	60.4	S3820	(Erler *et al*., 2004)
Caspase-3 forward primer	AGAACTGGACTGTGGCATTGAG	62.67	S3F31	(Tsang and Kwok, 2008)
Caspase-3 Reverse Primer	GCTTGTCGGCATACTGTTTCA	60.61	S3F32	(Tsang and Kwok, 2008)
MDM2 Forward primer	ATCAGGCAGGGGAGAGTGAT	62.45	S3F33	(Moela, 2013)
MDM2 Reverse Primer	TCTACATACTGGGCAGGGC	62.45	S3F34	(Moela, 2013)
p21 forward Primer	ATGTCAGAACCGGCTGGGGA	60.61	S408A	(Li and Wu, 2004)

p21 reverse Primer	GCCGTTTTCGACCCTGAGAG	62.57	S408B	(Li and Wu, 2004)
p53 reverse primer	CCCAGACGGAAAACCGTAGCTGCCT	71.1	S3822	(Teng *et al*., 2008)
p53 forward primer	TCAGATCCTAGCGTCGAGCCCCCTCT	70.9	S3821	(Tseng *et al*., 2008)
Caspase-9 reverse primer	CATCTGGCTCGGGGTTACTGC	58.3	S3F88	(Chalfant *et al*., 2002)
Caspase-9 forward primer	GCTCTTCTTTGTTCATCTCC	66.4	S3F87	(Chalfant *et al*., 2002)

Table A7: Antibodies used for Western blot analysis

Primary antibody	**Secondary antibody**	**Catalogue number**	**Supplier**
pAB anti-RBBP6 rabbit polyclonal (1:500)	Anti-rabbit IgG-peroxidase raised in goat (1:5000)	NBPI-49535 and A8919	Novus Biologicals and Sigma
Anti-Cytochrome c goat IgG polyclonal (1:1000)	Anti-goat IgG-peroxidase raised in rabbit (1:5000)	Sc-7159 and A-0545	Santa Cruz and Sigma

Anti-actin mouse IgG monoclonal (1:500)	Anti-mouse IgG-peroxidase goat (1:1000)	Sc-8432 and Sc-2005	Santa Cruz
Ab28 anti-p53 mouse monoclonal (1:500)	Anti-mouse IgG-peroxidase goat (1:5000)	PAb 1801 And Sc-2005	Abcam and Santa Cruz

Table A8: Web address for the various Bioinformatics tools used in this study

Tool	**Web addresses**
GeneGlobe (QIAGEN software)	www.qiagen.com/za/shop/genes-and-pathways/data-analysis-center-overview-page/rt2-profiler-pcr-arrays-data-analysiscenter/?Instrument=Roche_480_96&format=F&catno=PAHS-033Z
PANTHER	http://www.pantherd.org/
REACTOME	http://www.reactome.org/
GeneMANIA	http://genemania.org/

Table A9: Statistical analysis (means ± SD) of the cell cycle phases for untreated and treated cells

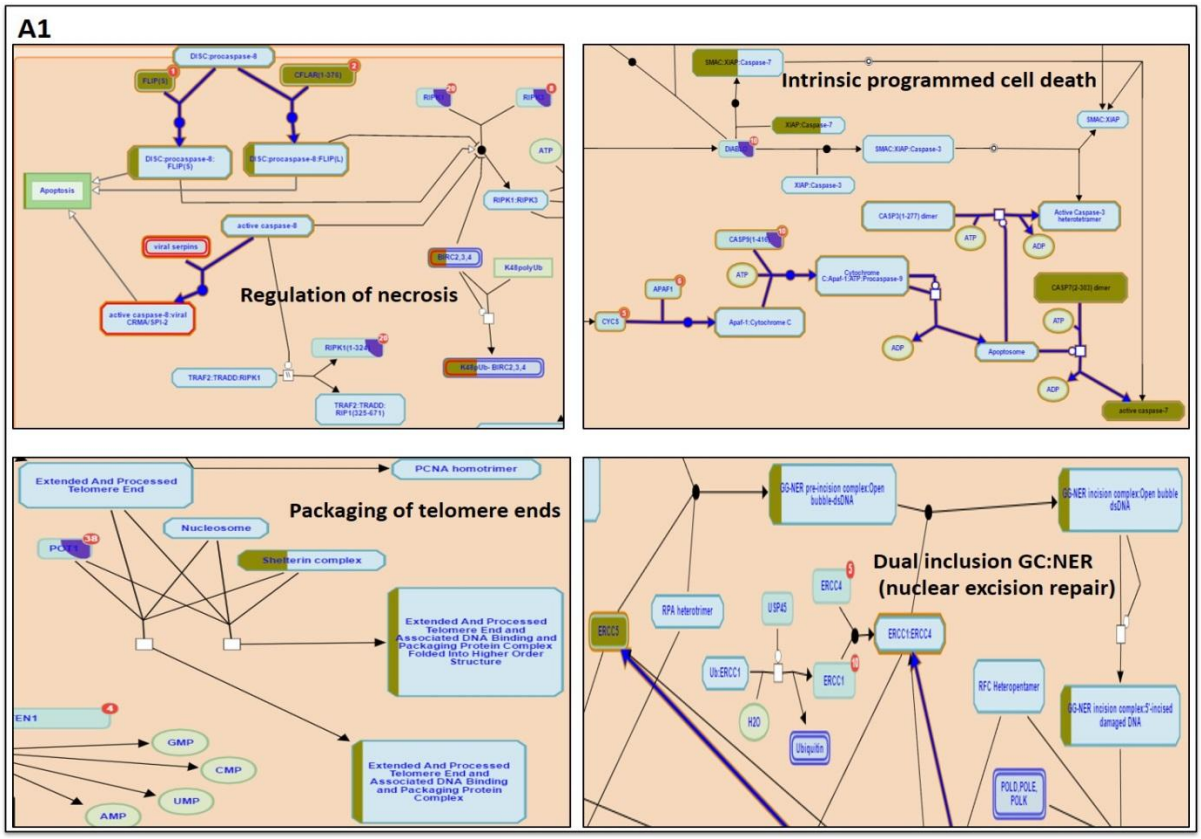
Cell lines	**Cell cycle phases**	**% of cells at cell cycle phase**									
		UN	**4 IR**	**24 IR**	**48 IR**	**4 MP**	**24 MP**	**48 MP**	**4 COT**	**24 COT**	**48 COT**
RMG1	**Sub G0/G1**	3.3 ± 0.2	12.2 ± 0.2	13.2 ± 0.2	26.0 ± 0.1	1.3 ± 0.1	6.5 ± 0.1	19.3 ± 0.2	5.1 ± 0.1	5.2 ± 0.1	51.2 ± 0.2
	G0/G1	83.4 ± 0.2	71.0 ± 0.1	79.8 ± 0.1	33.3 ± 0.3	2.3 ± 0.2	88.3 ± 0.1	77.5 ± 0.1	75.2 ± 0.2	9.1 ± 0.2	46.8 ± 0.1
	S	12.5 ± 0.1	12.0 ± 0.1	6.1 ± 0.2	38.0 ± 0.2	88.2 ± 0.2	4.3 ± 0.1	4.3 ± 0.1	17.3 ± 0.2	40.0 ± 0.1	2.1 ± 0.2
	G2/M	0.8 ± 0.1	8.8 ± 0.3	1.0 ± 0.3	0.3 ± 0.1	4.8 ± 0.2	1.0 ± 0.2	2.4 ± 0.1	2.3 ± 0.2	46.0 ± 0.2	0.2 ± 0.1
A549	**Sub G0/G1**	0.5 ± 0.1	12.5 ± 1.0	69.2 ± 0.5	37.3 ± 1.0	13.5 ± 2.0	70.2 ± 1.5	36.5 ± 0.3	24.1 ± 0.1	52.7 ± 0.3	90.1 ± 0.1
	G0/G1	90.7 ± 0.1	79.1 ± 1.0	29.1 ± 1.0	56.4 ± 1.0	77.1 ± 3.0	28.6 ± 1.5	57.1 ± 0.3	69.5 ± 0.1	44.4 ± 0.3	9.2 ± 0.2
	S	5.2 ± 0.2	6.9 ± 0.2	1.9 ± 0.2	4.0 ± 0.1	7.0 ± 0.5	1.9 ± 0.2	4.0 ± 0.1	5.5 ± 0.2	2.7 ± 0.2	1.0 ± 0.1
	G2/M	3.7 ± 0.2	2.5 ± 0.2	0.3 ± 0.2	2.8 ± 0.1	2.2 ± 0.5	0.3 ± 0.2	2.9 ± 0.1	1.2 ± 0.2	0.3 ± 0.2	0.1 ± 0.1

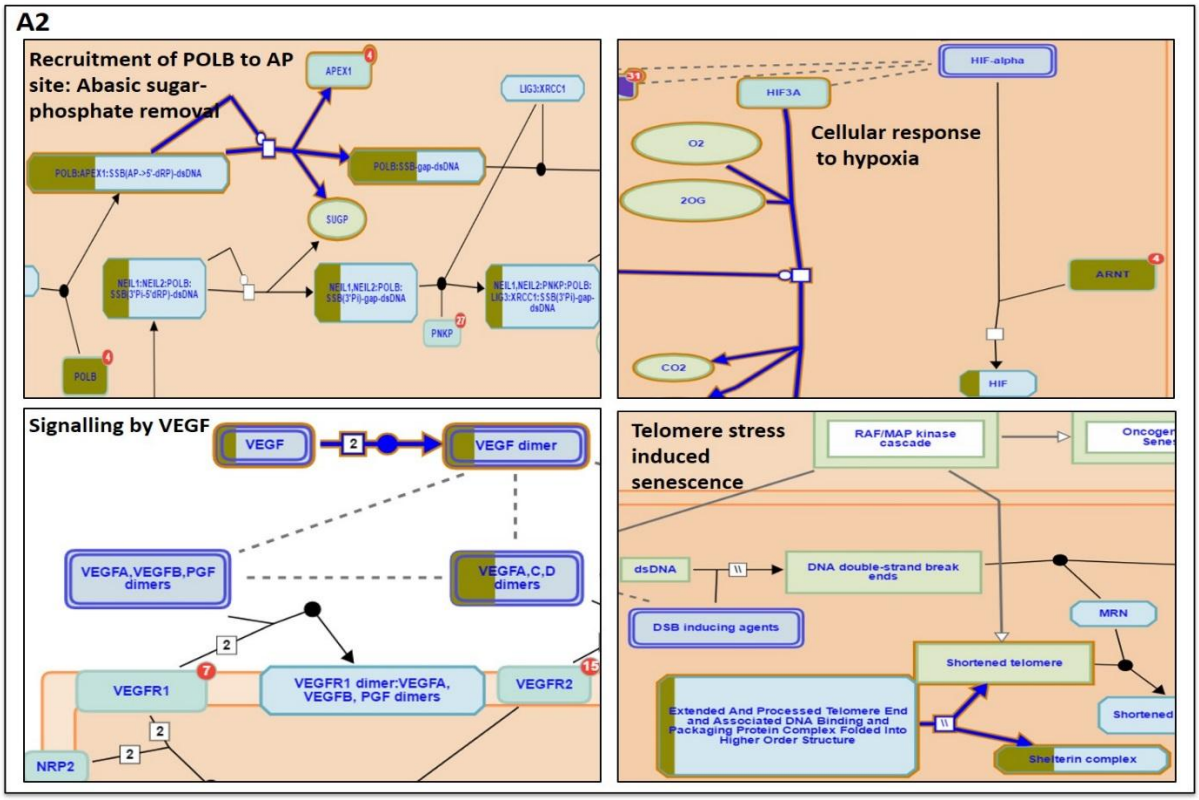
MDA-MB 231	**Sub G0/G1**	3.6 ± 0.1	17.1 ± 1.0	41.5 ± 0.1	36.9 ± 0.1	16.3 ± 0.2	41.6 ± 0.2	36.9 ± 0.1	4.4 ± 0.2	7.1 ± 0.1	35.7 ± 0.1
	G0/G1	70.3 ± 0.1	44.9 ± 1.0	54.8 ± 0.2	51.9 ± 0.1	45.9 ± 0.1	54.3 ± 0.3	51.9 ± 0.1	51.1 ± 0.2	79.7 ± 0.1	34.8 ±0.1
	S	16.2 ± 0.2	29.7 ± 0.1	2.8 ± 1.0	7.5 ± 0.1	30.1 ± 1.0	2.4 ± 0.1	7.5 ± 0.1	34.1 ± 0.1	10.7 ± 0.1	20.1 ± 0.1
	G2/M	9.9 ± 0.2	15.3 ± 0.1	1.0 ± 1.0	3.9 ± 0.1	14.9 ± 1.0	1.4 ± 0.1	3.9 ± 0.1	10.5 ± 0.1	2.6 ± 0.2	19.4 ± 0.1
MRC-5	**Sub G0/G1**	1.2 ± 0.2	6.6 ± 0.3	3.9 ± 0.3	6.5 ± 0.3	6.6 ± 0.3	3.7 ± 0.1	6.3 ± 0.1	4.2 ±0. 2	4.2 ± 0.2	6.2 ± 0.2
	G0/G1	91.6 ± 2.0	80.1 ± 0.1	80.1 ± 0.3	74.9 ± 0.3	82.7 ± 2.5	80.3 ± 0.1	75.1 ± 0.1	82.5 ± 0.2	82.5 ± 0.2	88.1 ± 0.3
	S	8.6 ± 0.1	9.9 ± 0.1	13.4 ± 0.2	11.2 ± 0.1	10.0 ± 0.3	13.3 ± 0.2	11.5 ± 0.4	12.5 ± 0.2	11.5 ± 1.5	5.5 ± 0.2
	G2/M	0.4 ± 0.1	0.7 ± 0.2	2.8 ± 0.2	2.6 ± 0.1	0.6 ± 0.3	2.8 ± 0.2	2.2 ± 0.4	0.7 ± 0.2	0.5 ± 0.2	0.4 ± 0.2
MRC-5 (Recovery)	**Sub G0/G1**	1.1 ± 0.1	5.5 ± 0.3	6.1 ± 0.1	5.9 ± 0.3	5.3 ± 0.1	6.3 ± 0.3	5.9 ± 0.2	1.1 ± 0.1	9.3 ± 0.2	1.3 ± 0.1
	G0/G1	94.1 ± 0.1	85.0 ± 0.3	76.6 ± 0.2	65.0 ± 0.3	85.1 ± 0.1	76.1 ± 0.3	65.0 ± 0.2	84.3 ± 0.1	80.9 ± 0.2	56.4 ± 0.1
	S	4.3 ± 0.1	9.0 ± 0.3	15.7 ± 0.3	26.0 ± 0.2	8.9 ± 0.2	15.7 ± 0.3	26.0 ± 0.2	13.9 ± 0.1	9.2 ± 0.2	30.6 ± 0.3
	G2/M	0.6 ± 0.1	0.6 ± 0.3	1.7 ± 1.5	3.3 ± 0.2	0.7 ± 0.2	3.0 ± 0.3	3.2 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	11.7 ± 0.3

UN – Untreated IR – Irinotecan **MP** – Methyl pyruvate **COT** – Cotreatment

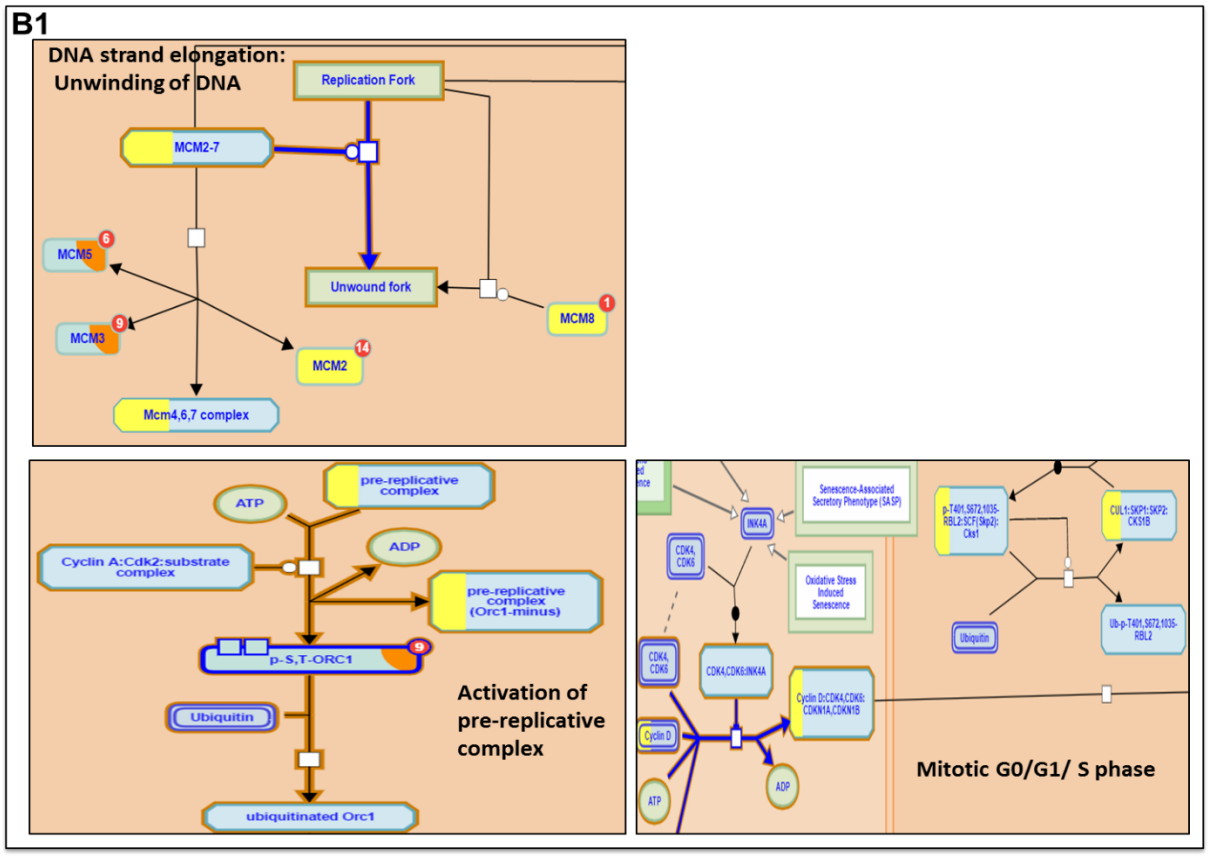
Appendix B

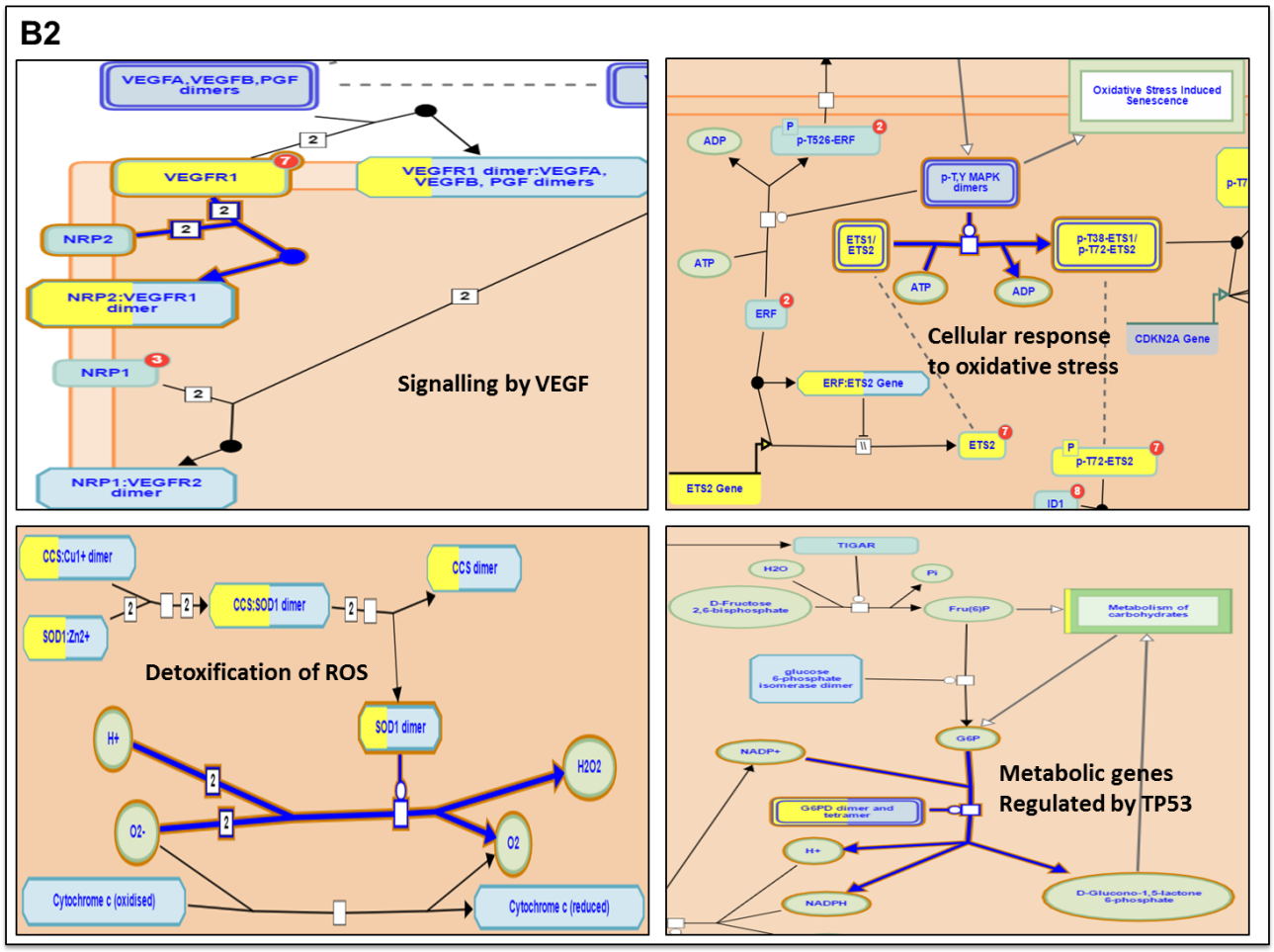
A. A549 upregulated pathways/processes



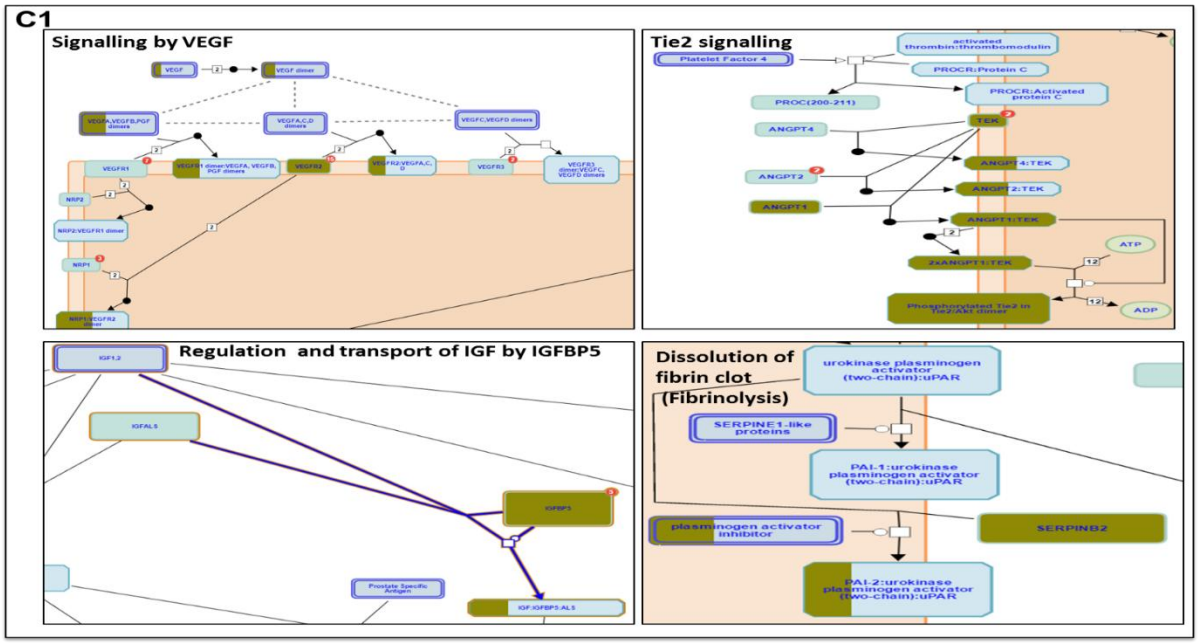


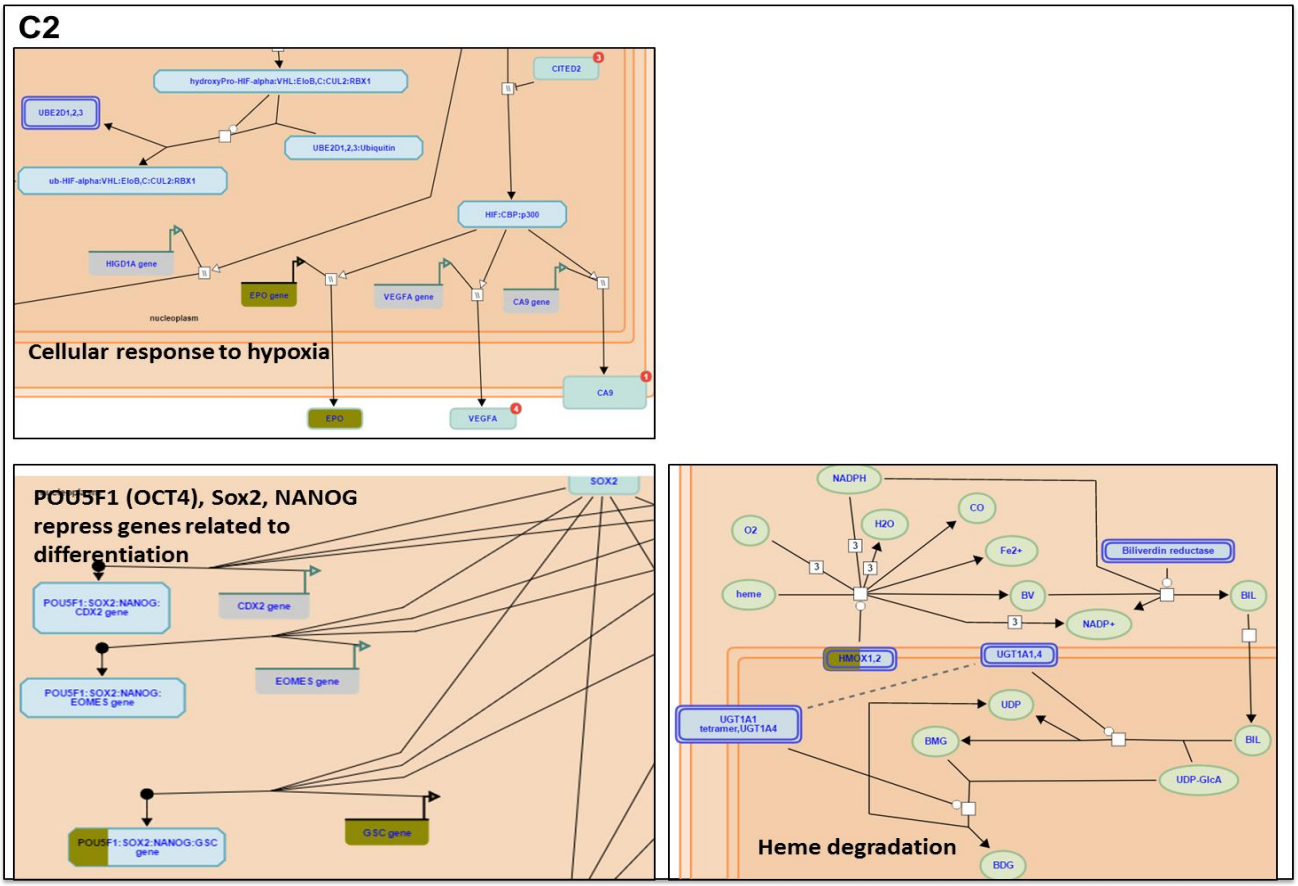
B. A549 downregulated pathways/processes





C. MRC-5 upregulated pathways/processes





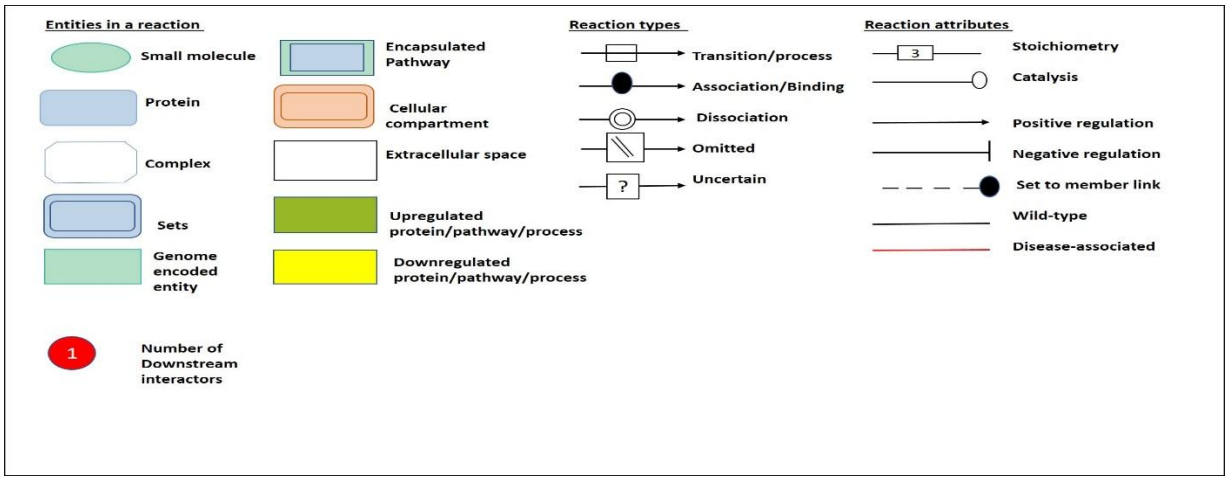


Figure B2: Pathway diagrams constructed using the REACTOME pathway analysis. Pathways were enriched when a significant number of the query list genes were part of a particular pathway against the overall pathway genes. The dark green colour represents genes with upregulated expression levels while the bright yellow colour represents downregulated genes. The purple and orange colours indicate downstream molecules which were enriched, and the values in the red circles at the top left corner indicates the number of downstream interacting molecules. Each pathway was considered statistically enriched when the $p < 0.05$. In A549 lung cancer cells, the combined treatment upregulated genes involved in (**a1-a2**);

regulation of necrosis (p = 0.56E-5), intrinsic programmed cell death (p = 2.22E-2), packaging of telomere ends (p = 1.9E-2), dual inclusion GC:NER (p = 2 E-3), recruitment of POLB to AP site: abasic sugar-phosphate removal (p = 1.44E-2), cellular response to hypoxia (p = 1.19E-1), signalling by VEGF (p = 6.26E-1), and telomere stress induced senescence (p = 4.46E-2). Furthermore, in A549 lung cancer cells, the combined treatment downregulated genes involved in (**b1-b2**); DNA strand elongation: unwinding of DNA (p = 7.53E-6), activation of pre-replicative complex (p = 6.66E-5), mitotic G0/G1/S phase (p =6.21E-4), signalling by VEGF (p = 3.31E-1), cellular response to oxidative stress (p = 5.86E-4), detoxification of ROS (p = 1.44E-3), and metabolic genes regulated by TP53 (p = 2.35E-2). In MRC-5 normal lung fibroblast cells, the combined treatment upregulated genes involved in (**c1-c2**); signalling by VEGF (p = 3.02E-4), Tie2 signalling (p =3.38E-2), regulation and transport of IGF by IGFBP5 (p = 3.61E-2), Dissolution of fibrin clot (fibrinolysis) (p = 3.42E-2),cellular response to hypoxia (p = 1.32E-2), POU5F1 (OCT4), S0x2, NANOG repress genes related to differentiation (p = 1.72E-2), and heme degradation (p = 2.02E-2). The REACTOME key diagram below gives detail description of the icons used.

Table B1: 17 unique genes upregulated in A549

Gene	Full name	Function	Reference
CASP7	Caspase-7	Induce apoptosis by cleavage of a large set of substrates	Lamkanfi and Kanneganti, 2010
CFLAR	CASP8 and FADD-like apoptosis regulator	Anti-apoptotic factors that inhibits the extrinsic pathway by binding to tumour necrosis factor receptors (*TNF-R*)	Wajant *et al*., 2005; Lin *et al*., 2000; Wajant, 2004
BIRC3	Baculoviral IAP Repeat Containing 3		
DDIT3	DNA damage inducible transcript 3' (Formerly CHOP)	key regulator in stress response (ER stress, DNA damage, hypoxia, and starvation)	Luethy and Holbrook, 1992; Oyadomari and Mori, 2004; Tang *et al*., 2002
PPP1R15A	phosphoprotein phosphatase regulatory subunit 15A also GADD34	Induce growth arrest upon detection of damaged DNA	Zhan *et al*., 1994; Arumugam *et al*., 2011
POLB	DNA polymerase beta	DNA repair polymerase involved in base-excision repair, DNA replication and trans lesion synthesis	Masaoka *et al*., 2009; Beard and Wilson, 2006
DDB2	Damage specific DNA protein 2	Function in genome nucleotide excision repair by acting as a transcription regulator	Minig *et al*., 2009; Ennen *et al*., 2013; Zhao *et al*., 2014; Roy *et al*., 2013
ERCC5	Excision repair cross-species complementation5	Important protein the two incision steps in nucleotide-excision repair	Godwa *et al*., 2007
ACL4	Acyl-CoA synthetase long-chain family member 4	Catalyzes the ATP-dependent thioesterification of fatty acids to coenzyme A (CoA)	Watkins *et al*., 2007
BMI	Polycomb group gene	Positively regulate proliferation and negatively regulate cellular senescence and differentiation	Sparman and Van Lohuizen, 2006
TNKS2	Tankyrase-2	Act as a positive regulator of telomere length	Smith and de Lange, 2000

TINF2	TRF1 interacting nuclear factor 2	Negatively regulating telomere length	Kim *et al*., 1999
TERF2IP	Telomeric repeat binding factor 1	Protects human telomeres from end-to-end fusion	van Steensel *et al*., 1998
VEGFC	Vascular endothelial growth factor-FC	Bind to lymphocyte vessel endothelial cells and stimulate lymphangiogenesis	Tammela and Alitalo, 2010
ANGPT2	Angiopoietin-2	Anti-angiogenesis factor	Asahara *et al*., 1998
ARNT	Aryl hydrocarbon nuclear translocator	Control of glucose metabolism	Zelzer *et al*., 1998
SNAI2	Snail 2	Regulate epithelial-mesenchymal transition (EMT). It also influences metastasis	Baygi *et al*., 2010; Chaffer and Weinberg, 2011

Table B2: 18 unique genes downregulated in A549

Gene	Full name	Function	Reference
FLT1/ VEGFR1	Vascular endothelial growth factor receptor 1	Facilitate angiogenesis by increasing vascular endothelial cell growth and proliferation	Park *et al*., 1994; Hiratsuka *et al*., 2002
ANGPT1	Angiopoietin-1	Mediator of VEGF-independent pro-angiogenic signalling pathway. Through its interaction with TIE2 receptor, can activate the PI3K/AKT pathway	Shim *et al*., 2007; Papapetropoulos *et al*., 2000
SERPINF1	Serpin peptidase inhibitor clade F, member 1	Inhibits angiogenesis	Steele *et al*., 1993; Becerra *et al*., 1995
FOXC2	Forkhead box C2	Transcription factor that induce Epithelial-to-mesenchymal transition (EMT), which is essential for development of embryonic mesoderm	Yilmaz and Lehembre, 2007
HMOX1	Heme_oxygenase 1	Catalyzes the degradation of heme to carbon monoxide, free iron and biliverdin which is further converted to bilirubin	Ryter *et al*., 2006
DKC1	Dyskeratosis congenital 1, dyskerin	Necessary for the biogenesis of the RNA component of telomerase (hTR) and regulates a number of anti-apoptotic proteins	Chang *et al*., 2002; Yoon *et al*., 2006
ETS2	Transcription factor (ETS protein family)	ETS2 is involved in cell cycle control, its downstream target genes include; Bcl-2, cyclin D1, and Bcl-XL	Carbone *et al*., 2004; Sementchenko *et al*., 1998
CCND3	Cyclin D3	Involved in G0/G1 to S phase cell cycle progression	Walker and Assoian, 2005

CDC20	Cell division cycle 20	Plays a key role in activating the anaphase promoting complex (APC/C), leading to mitotic progression	Hoeller *et al*., 2006
AURKA	Aurora kinase 1	Critical for bipolar-spindle assembly, regulation of cell migration and adhesion, and recently reported to regulate stem cell self-renewal, reprogramming and differentiation	Tsai *et al*., 2003; Do *et al*., 2013; Li and Rana, 2012
STMN1	Stathmin 1	The microtubule-destabilizing protein, is essential during mitosis, to influence cell cycle progression	Rana *et al*., 2008; Belletti and Baldassarre, 2011
MKI67	Antigen identified by monoclonal antibody ki-67	Associated with cellular proliferation	Gerdes *et al*., 1983
SKP2	S-phase kinase-associated protein 2, E3 ubiquitin protein ligase	Component of the SKP1-Cullin1-F-box (SCF) E3 ligase complex that is involved in cell cycle progression	Bashir and Pagano, 2004; Frescas and Pagano, 2008
PINX	PIN1/TERF1 interacting, telomerase inhibitor 1	Important in maintenance of genetic stability, helicase activation and ribosome biogenesis	Arndt and Mackenzie, 2016
MCM2	Minichromosome maintenance complex component 2	Crucial for DNA replication and cell cycle initiation	Kearsey and Labib, 1998; Torres-Rendon *et al*., 2009
G6PD	Glucose-6-phosphate dehydrogenase	An enzyme forming part of the glycolysis pathway is involved in the first reaction in the pentose-phosphate pathway	Cappellini and Fiorelli, 2008
COX5A	C y t o c h rome c oxidase subunit Va	Encodes the Va subunit of the human mitochondrial respiratory chain enzyme, that is essential for	Chen *et al*., 2012

		regulation and assembly of the mitochondrial electron transport chain	
SOD1	Superoxide dismutase 1	Catalyze the conversion of intracellular superoxide to hydrogen peroxide (H2O2). Overexpression of SOD1 promotes lung cancer cell growth and reduce apoptosis.	Juarez *et al*., 2008; Somwar *et al*., 2011

Table B3: 13 unique genes upregulated in MRC-5

Gene	Full name	Function	Reference
ANGPT1	Angiopoietin-1	Mediate cell-cell adhesion and cell survival. It's a ligand of the receptor, *TEK/Tie2.* Together, they control vascular morphogenesis and homeostasis.	Augustin *et al*., 2009; Maisonpierre *et al*., 1997; Fukuhara *et al*., 2008; Saharinen et al., 2008
TEK/ *Tie2*	TEK tyrosine kinase, endothelial		
SERPINF1	Serine protease inhibitor F1	Angiogenesis	Steele *et al*., 1993; Becerra *et al*., 1995
SERPINB2	Serine Protease inhibitor 2 (Also - Plasminogen Activator Inhibitor-2)	Important during cell differentiation, tissue growth and regeneration.	Croucher *et al*., 2008
KDR	Vascular Endothelial Growth Receptor	Receptors that binds to VEGF-A ligand, thereby facilitating angiogenesis, vascular permeability, cell migration, gene expression and regulation of endothelial function	Shibuya and Claesson-Welsh, 2006; Lee *et al*., 2007
PGF	Placenta growth factor ligand (vascular endothelial growth factor-related protein)	Bind and activate VEGFR-1 receptor thereby	Carmeliet *et al*., 2001

		contributing to angiogenesis	
SOX10	SRY-box 10	Nuclear transcription factor involved in neural crest development and differentiation of melanocytic and schwannian lineage	Mollaaghababa and Pavan, 2003
GSC	Goosecoid	Involved in mesoderm formation in *Drosophila* and neural crest development in vertebrates during embryogenesis	Thiery and Sleeman, 2006
IGFBP5	Insulin growth factor-binding protein 5	Essential during development of several key cell lineages such as myoblasts and neural cells	James et al., 1996; Cheng *et al*., 1999
EPO	Erythropoietin/thrombopoietin	Essential in the regulation of red blood cell mass, participating in negative feedback control from a renal oxygen sensor to the erythroid system	Lacombe *et al*., 1988
HMOX1	Heme_oxygenase 1	Catalyzes the degradation of heme to carbon monoxide, free iron and biliverdin which is further converted to bilirubin	Ryter *et al*., 2006
GADD45G	Growth arrest DNA-damage-inducible protein 45	Act as stress sensors and tumour suppressors during cellular genotoxic and non-genotoxic stress response	Garcia *et al*., 2005
FASLG	Fas ligand, also known as TNFSF6/CD95L	Induces extrinsic apoptosis upon	Wajant *et al*., 2003

		binding to its corresponding death-inducing receptor	

Table B4: 7 common genes upregulated in A549 and MRC-5

Gene	Full name	Function	Reference
KRT14	Keratin 14	Part of the intermediate filament -forming proteins of epithelial cells. Crucial for survival. Participate in both natural cycling and repair of injury in normal cells	Moll *et al*., 2008; Lloyd *et al*., 1995; Papafotiou *et al*., 2016
SNAIL1 SNAIL3	Snail 1 Snail 3	Important in the induction of the epithelial to mesenchymal transition (EMT) converting epithelial cells into mesenchymal cells. SNAIL signalling induced a glycolytic switch	Barrallo-Gimeno and Nieto, 2005; Lee *et al*., 2012
CCND2	Cyclin D2	Promotes transition through the G1 phase of the cell cycle	Quelle *et al*., 1993
CA9	Carbonic anhydrase IX	Involved in pH regulation of the extracellular microenvironment during hypoxia and is regulated by HIF-1α	Neri and Supuran, 2011; Kaluz *et al*., 2009;
TEP1	Telomerase-associated protein 1	A putative regulator domain and is associated with telomerase activity	Nakamura *et al*., 1997; Nakayama *et al*., 1997
TBX2	T-box 2	Acts mainly as a transcriptional repressor regulating cell migration and morphogenesis during embryogenesis	Carreira *et al*., 1998; Suzuki et al., 2004

Table B5: Upregulated pathways after the drug combination in A549 and MRC-5 cells using GeneMANIA analysis

Molecular Functions	**q-values of upregulated pathways**	
	A549	**MRC-5**
telomere maintenance via telomerase	3.2e-12	--
intrinsic apoptotic signalling pathway	2.9e-05	--
vascular endothelial growth factor receptor signalling pathway	0.13	--
positive regulation of endothelial cell proliferation	0.022	--
programmed necrotic cell death	0.0022	--
positive regulation of protein ubiquitination	0.023	--
nucleotide excision repair DNA damage removal	0.0027	--
regulation of endothelial cell proliferation	0.027	--
metabolism proteins	0.068	--
Angiogenesis	--	1.3e-08
cellular response to hypoxia	--	0.0017
regulation of insulin-like growth factor receptor signalling pathway	--	0.030
positive regulation of cell adhesion	--	0.0037
regulation of DNA replication	--	0.058
regulation of extrinsic apoptotic signalling pathway	--	0.067

Table B6: Downregulated pathways after the drug combination in A549 cells using GeneMANIA analysis

Molecular Functions	**q-values of downregulated pathways**	
	A549	**MRC-5**
Angiogenesis	0.021	--
DNA polymerase activity	0.021	--

Mitosis	0.022	--
Myeloid cell homeostasis	0.099	--