

University of Dublin

Trinity College



2015

**Cancer cell bioenergetics in response to anti-
cancer agents**

Fintan Geoghegan

Thesis submitted to the University of Dublin for the degree of Doctor of Philosophy

**School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity
College, Dublin, Ireland**

Declaration

UNIVERSITY OF DUBLIN

Trinity College



I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work except Table 4.1 which was carried out by our collaborators.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

Fintan Geoghegan

September 2015

Abstract

Altered metabolism is a hallmark of cancer (Hanahan and Weinberg, 2011) and while genetic and molecular modifications have enabled cancers to survive the onslaught of various cancer therapies (Pillai et al., 2010), changes in tumour cell metabolism is becoming increasingly more important.

Neuroblastoma is a malignancy that arises from the neural crest cells of the sympathetic nervous system which accounts for 15% of paediatric cancer deaths remains a difficult cancer to treat. Phenformin, a member of a class of drug called the biguanides which was used to treat type II diabetes has recently been shown to be efficacious in cancer treatment.

In the first part of my study the effects of the potentially novel anti-neoplastic drug, phenformin on the metabolism of neuroblastoma SH-SY5Y cells was examined. The potency of phenformin was assessed by the alamar blue assay, propidium iodide incorporation and annexin V staining by flow cytometry. The effect on the bioenergetics of the cell was analysed by the oxygraph respirometer and seahorse extracellular flux analyser. The effect on reactive oxygen species production levels was assessed by dihydro-fluorescein diacetate staining and flow cytometry. The effect of phenformin on energy status was assessed by AMPKinase and acetylCoA carboxylase activation as determined by western blotting.

Phenformin proved effective in reducing the cell viability of the neuroblastoma SH-SY5Y cancer cells, causing G1 cell cycle arrest at sub-cytotoxic concentrations and higher concentrations resulted in the neuroblastoma cells undergoing apoptosis. Bioenergetic analysis showed that phenformin significantly decreased oxygen consumption in a dose and time dependent manner by inhibiting complex I of the mitochondria. As a result of this inhibition, endogenous reactive oxygen species production is significantly decreased and AMPKinase and acetylCoA carboxylase become activated due to phenformin treatment of SH-SY5Y cells.

Cisplatin is one of the most potent chemotherapeutic drugs used clinic to treat solid cancers (Andrews and Howell, 1990) Cisplatin often yields modest and temporary clinical effects but its usefulness is limited by the acquisition of resistance, leading to drug relapse and therapeutic failure (Johansson et al., 2009, Michels et al., 2013, Locasale, 2012). Resistance can arise through a multitude of mechanisms including decreased drug accumulation, alterations in drug metabolism and mutations of drug targets (Longley and Johnston, 2005, Gottesman et al., 2002). One approach to possibly uncover the root cause of cisplatin resistance is to investigate the metabolic differences of the sensitive and cisplatin resistance cancer cells. In the second part of my study I set out to determine the metabolic changes behind acquired cisplatin resistance in the non small cell lung cancer cell line, H1299 and a mesothelioma cancer cell line, P31. Cell surface marker expression was assessed by flow cytometry. Analysis of the bioenergetic differences between the parental sensitive cell line and resistant sub-line was determined by the seahorse extracellular flux analyser. Determination of mitochondrial abundance was assessed by the citrate synthase assay. The effect on reactive oxygen species production levels was assessed by dihydro-fluorescein diacetate staining and flow cytometry. The effect of acquired cisplatin resistance on mitochondrial biogenesis was assessed assaying key components of the mitochondrial biogenesis pathway, namely sirtuin-1 (SIRT1), sirtuin-3 (SIRT3), transcription factor A, mitochondrial (TFAM) and peroxisome-proliferator activator receptor- γ co-activator 1-alpha (PGC1 α) expression as determined by western blotting.

It was found that the acquired resistant cell lines demonstrate reduced mitochondrial oxygen consumption rates, reduced mitochondrial abundance and reduced sirtuin-1 (SIRT1), sirtuin-3 (SIRT3), transcription factor A, mitochondrial (TFAM) and peroxisome-proliferator activator receptor- γ co-activator 1-alpha (PGC1 α) expression when compared to their cisplatin sensitive cell counterparts. A correlation between increased globotriasylceramide (Gb₃) and the acquisition of cisplatin resistance in the H1299 and P31 cells was demonstrated. The expression of the ABC transporters, ABCE1 and ABCB6 were increased in the cisplatin resistant H1299 and P31 cells respectively in comparison to their sensitive cell counterparts. Using the aforementioned knowledge on

mechanism, we set out to determine whether we could reverse the attenuation of mitochondrial function, abundance and biogenesis in H1299 cisplatin resistant cells. . To that end we were able to demonstrate that resveratrol treatment, increased mitochondrial reserve capacity, increased mitochondrial abundance and mitochondrial biogenesis in the H1299 cisplatin resistant cells. Similarly SIRT3 over-expression increased mitochondrial oxygen consumption, increased mitochondrial abundance and mitochondrial biogenesis in both the sensitive and resistant H1299 cells.

In conclusion, the first part of this work demonstrated that phenformin shows promise as an anti-cancer agent to treat neuroblastoma. The second part highlighted the metabolic differences between cisplatin sensitive and cisplatin resistant non small cell lung cancer and mesothelioma cancer cell lines and the possible utility of resveratrol or SIRT3 over-expression as potential metabolic switches for cancer treatment.

Acknowledgements

Firstly I would like to thank Richie, who has always showed a great enthusiasm in my work and was always there to help. I would also like to thank Danny whose advice throughout my project was invaluable. Thanks to my family who have put up with me over the last few years. Thanks to Jenny, Kasia, Mary and Emma who were always up for the chats and kept me sane. Thanks to all the guys in the Bowie lab the reading room on 6th floor and the friends I made in the department.

Table of Contents

Chapter 1: General Introduction	1
1.0 Introduction:	3
1.1 Metabolism in Cancer	3
1.1.1 Warburg Effect	3
1.1.2. Reverse Warburg Effect	4
1.1.3 Aerobic glycolysis, a selective advantage	5
1.1.4. Oncogenes and tumour suppressors regulate metabolism.....	6
1.1.5 Glutamine metabolism	7
1.2.1 Diabetes drugs in Cancer	8
1.2.2 Metformin & Phenformin	9
1.2.3. Activation of the AMPK/mTOR pathway	11
1.2.4 AMPK, ACC and Fatty Acid Oxidation	12
1.2.5 Necessity of AMPK for metformin's action.....	12
1.2.6 AMPK and mTOR.....	13
1.2.7. Metformin and inflammation	14
1.2.8. Metformin, phenformin and cell death	14
1.2.9. Metformin, phenformin effect on the cell cycle.....	15
1.2.10 Phenformin and ROS.....	16
1.2.11 Phenformin and ATP	17
1.2.12. Metformin in combination.....	18
1.3.1. Cisplatin.....	19
1.3.2 Cisplatin Resistance	20
1.3.3. Cisplatin resistance, MDR and Gb3.....	21
1.3.4 Cisplatin and mitochondria	23
1.3.5 Mitochondrial Biogenesis	24
1.3.6 Sirtuin-3.....	27

1.3.7 Resveratrol	30
1.3.8 Cancer stem cells (CSC)	32
1.4 Objectives for the project.....	33
Chapter 2: Materials and Methods.....	35
2.0 Materials & Methods	37
2.1 Materials.....	37
2.2. Methods:	40
Chapter 3: Phenformin as an anti-cancer agent.....	67
3.0 Introduction	69
3.1. Results:.....	71
3.1.1 Determination of the IC ₅₀ of Phenformin and Metformin in SH-SY5Y cells.	71
3.1.2 The effect of phenformin on the cell cycle of SH-SY5Ys.....	73
3.1.3 Confirmation of cell death by PI & Annexin V staining by flow cytometry and PARP cleavage by western blotting in SH-SY5Y cells	76
3.1.4 The oxygen consumption rate and extracellular acidification rates as determined by the Seahorse Extracellular Flux Analyser and oxygen consumption rates as determined by the Oroboros oxygraph respirometer.	79
3.1.4 Decrease in Reactive Oxygen Species production as a result of phenformin treatment in SH-SY5Y cells.	103
3.1.5 Measurement of the effect of phenformin on ATP levels by a bioluminescence assay.	105
3.1.6 AMPK and ACC activation in SH-SY5Y cells as a result of treatment with phenformin over a time course as determined by western blot analysis.....	107
3.2 Discussion.....	109
Chapter 4: Comparative bioenergetics of sensitive and acquired cisplatin resistant H1299 and P31 cells.....	115
4.0 Introduction	117

4.1. Results.....	119
4.1.1 Determination of the IC ₅₀ values for cisplatin in H1299s, H1299r, P31s and P31r cells	119
4.1.2 Confirmation of globotriaosylceramide (Gb3) expression in H1299s, H1299r, P31s and P31r cells by flow cytometry.....	122
4.1.3 Determination of the susceptibility of H1299s, H1299r, P31s and P31r cells to verotoxin-1.....	124
4.1.4 Expression of the ABC transporters ABCA5, ABCB6 and ABCE1 in H1299s, H1299r, P31s and P31r cells as determined by western blotting.....	131
4.1.5 Analysis of the metabolism of H1299s, H1299r, P31s and P31r cell lines by the seahorse extracellular flux analyser.	136
4.1.6 Analysis of the mitochondrial content of H1299s, H1299r, P31s & P31r cells. ..	142
4.1.7 Growth rate of H1299s, H1299r, P31s & P31r cells as assessed by the alamar blue proliferation assay.	145
4.1.8 Analysis of the expression of mitochondrial biogenesis proteins in H1299s, H1299r, P31s and P31r cells as determined by western blotting.....	147
4.1.9. Measurement of Reactive Oxygen Species (ROS) in H1299s, H1299r, P31s and P31r as determined by flow cytometry.....	151
4.2 Discussion.....	154

Chapter 5: Investigation into the effects of resveratrol and sirtuin-3 (SIRT3) over-expression on the bioenergetics of the H1299s and H1299r cells. 163

5.1 Introduction	165
5.2 Results	167
5.2.1 Investigation into the effects of resveratrol on the bioenergetics of the H1299s and H1299r cells.	167
5.2.2 Investigation into the effects of SIRT3 over-expression on the bioenergetics of the H1299s and H1299r cells.	178
5.3 Discussion.....	203

Chapter 6: Investigation into the stem cell features of the acquired cisplatin resistance in H1299 and P31 cells.....	211
6.0 Introduction	213
6.1. Results.....	215
6.1.1 Cell surface expression of CD133/CD44	215
6.1.2 Aldehyde dehydrogenase (ALDH) activity	217
6.1.3 Hoescht Side Population (HSP) assay	220
6.1.5 Autofluorescence	222
6.2 Discussion.....	224
Chapter 7: General Discussion.....	227
7.0 General Discussion.....	229
Chapter 8: Future Work	237
8.0 Future Work.....	239
8.1 Phenformin as an anti-cancer agent	239
8.2 Comparative bioenergetics in parental sensitive and cisplatin resistant cancer cell subline.	240
References.....	243

List of Figures

Figure 1.1 Schematic highlighting the differences between oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis (Warburg effect).

Figure 1.2 Chemical structures of phenformin and metformin.

Figure 1.3 Potential mechanisms of metformin in cellular metabolism and cell proliferation

Figure 1.4 Sources of mitochondrial ROS

Figure 1.5 Metformin's effect on ATP

Figure 1.6 Chemical structure of cisplatin

Figure 1.7 GSL biosynthesis at the golgi apparatus.

Figure 1.8 Signalling pathways regulating mitochondrial biogenesis.

Figure 1.9 Sirtuin protein and catalytic domain.

Figure. 1.10 Chemical structure of resveratrol

Figure 2.1 Cell Cycle Analysis by Propidium Iodide staining using flow cytometry.

Figure 2.2 Cell Death Analysis by Propidium Iodide and Annexin V staining using flow cytometry. Graphical analysis for cell death analysis using flow cytometry.

Figure 2.3 Intracellular ROS determination by DCFA-HA staining using flow cytometry.

Figure 2.4 Gb₃ expression by Alexa-647 staining using flow cytometry.

Figure 2.3 HSP determination using flow cytometry.

Figure 2.4 Measurement of mitochondrial function by the seahorse extracellular flux analyser.

Figure 2.3 Injection Port Layout of the Cartridge.

Figure 3.1 Effect of phenformin on the proliferation of SH-SY5Y neuroblastoma cell line.

Figure 3.2 Effect of metformin on the proliferation of SH-SY5Y neuroblastoma cell line.

Figure 3.3 Dose-dependent effect of phenformin on SH-SY5Y cells.

Figure 3.4. Phenformin induces G₁ cell cycle arrest at sub cytotoxic levels as determined by flow cytometry.

Figure 3.5 Phenformin induces apoptosis in SHSY-5Y cells in a dose dependent manner as determined by PI and annexin V staining by flow cytometry.

Figure 3.6 Disappearance of the full form of PARP as a result of treatment with phenformin.

Figure 3.7 oxygen consumption rates of SH-SY5Y cells after treatment with oligomycin, FCCP and Antimycin A as determined by the oxygraph respirometer.

Figure 3.8 effect of phenformin on the metabolism of the SH-SY5Y cells as determined by the oxygraph respirometer.

Figure 3.9 Optimisation of the seeding densities of SH-SY5Y with the addition of the optimised concentrations of oligomycin, FCCP and Antimycin A as determined by the seahorse extracellular flux analyser.

Figure 3.10 Oxygen consumption rates of SH-SY5Y cells at varying densities with the addition of FCCP and Antimycin A without oligomycin as determined by the seahorse extracellular flux analyser.

Figure 3.11 optimisation of the mitochondrial inhibitors after treatment with varying concentration of phenformin for 48h as determined by the seahorse extracellular flux analyser.

Figure 3.12 Investigation of the sensitivity of the SH-SY5Y cells to varying concentration of phenformin for 48h as determined by the seahorse extracellular flux analyser.

Figure 3.13 Effect of varying concentrations of metformin on the metabolism of SH-SY5Y cells for 48h as determined by the seahorse extracellular flux analyser.

Figure 3.14 Time dependent inhibition of oxygen consumption in SHSY-5Y cells as determined by the oxygraph respirometer.

Figure 3.15 Confirmation of the seeding densities of SH-SY5Y cells for treatment of 1 mM of phenformin for 1, 2, 3, 4, 5, 6h as determined by the seahorse extracellular flux analyser.

Figure 3.16 Phenformin inhibits oxygen consumption rate (OCR) and increases extracellular acidification rate (ECAR) in a time dependent manner as determined by the Seahorse flux analyser.

Figure 3.17 Investigation of the sensitivity of the SH-SY5Y cells to varying concentration of phenformin for 6h as determined by the seahorse extracellular flux analyser.

Figure 3.18 Optimisation of the concentration of digitonin needed to permeabilize SH-SY5Y cells using the seahorse extracellular flux analyser and oxygraph respirometer.

Figure 3.19 Inhibition of mitochondria complex I by phenformin in permeabilized SHSY-5Y cells as determined by the Seahorse flux analyzer.

Figure 3.21 Protective effects of NDI1 plasmid transfection on SH-SY5Y cells treated with phenformin as determined by the Seahorse flux analyser.

Figure 3.22 Phenformin decrease in Reactive Oxygen Species (ROS) production in SH-SY5Y cells as determined by flow cytometry.

Figure 3.23 ATP expressed as a percentage of control after treatment with the various agents for 6h as determined by bioluminescence assay.

Figure 3.24 Phenformin dependent phosphorylation of AMP-kinase (AMPK) and acetylCoA carboxylase (ACC) as determined by immunoblot analysis.

Figure 4.1 The effect of cisplatin and verapamil pre-treatment on the viability of H1299s, H1299r, P31s and P31r cells as determined by the alamar blue viability assay.

Figure 4.2 The expression of Gb3 in H1299s, H1299r, P31s and P31r cells as determined by flow cytometry.

Figure 4.3 The effect of verotoxin-1 on the viability of the H1299s and H1299r cells as determined by the alamar blue viability assay. Cells were seeded in 96 well plates at the following densities

Figure 4.4 The effect of verotoxin-1 with and without and sodium butyrate on the viability of the H1299s, H1299r, P31s and P31r cells as determined by the viability assay, alamar blue.

Figure 4.5 The effect of the combination treatment of verotoxin-1 and cisplatin on the viability of the H1299s and H1299r cells as determined by the alamar blue viability assay.

Figure 4.6 The effect of the combination treatment of verotoxin-1 and cisplatin on the viability of the P31s and P31r cells as determined by the alamar blue viability assay.

Figure 4.7 The effect of verotoxin-1 and cisplatin with and without and sodium butyrate on the viability of the H1299s, H1299r, P31s and P31r cells as determined by the viability assay, alamar blue.

Figure 4.8 Percentage MDR1 expression in HL60-MDR1+, H1299s, H1299r, P31r and P31s cells as determined by flow cytometry.

Figure 4.9 Expression of the ABC transporters: ABCA5, ABCB6 and ABCE1 in H1299s, H1299r, P31r and P31s cell lysates as determined by immunoblot analysis.

Figure 4.10 Seeding optimisation of H1299s, H1299r, P31s & P31r on the seahorse plates as determined by the seahorse extracellular flux analyser.

Figure 4.11 Effect of the mitochondrial inhibitors: oligomycin, FCCP and Antimycin A on the whole cell metabolism of H1299s, H1299r, P31s & P31r as determined by the seahorse extracellular flux analyser.

Figure 4.12 Mitochondrial oxygen consumption rates of the H1299s, H1299r, P31r and P31s cells as determined by the seahorse extracellular flux analyser.

Figure 4.13 Citrate synthase activity of the H1299s, H1299r, P31s and P31r cells as determined by spectroscopy.

Figure 4.14 Expression of the mitochondrial proteins: voltage dependent anion channel 1 (VDAC1) and cytochrome c oxidase subunit 4 H1299s, H1299r, P31r and P31s cell lysates as determined by immunoblot analysis.

Figure 4.15 Growth rate of the H1299s, H1299r, P31s and P31r cells by the alamar blue assay.

Figure 4.16 Expression of the mitochondrial biogenesis proteins: SIRT1, PGC1 α , SIRT2, TFAM and SIRT3 in H1299s, H1299r, P31r and P31s cell lysates as determined by immunoblot analysis.

Figure 4.17 Expression of the acetylation of PGC1 α in H1299s, H1299r, P31r and P31s cell lysates as determined by immunoblot analysis.

Figure 4.18 Reactive Oxygen Species (ROS) production in H1299s, H1299r, P31r and P31s cells as determined by flow cytometry.

Figure 4.19 Expression of the stabilisation of HIF1 α in H1299s, H1299r, P31r and P31s cell lysates as determined by immunoblot analysis.

Figure 5.1 Expression of SIRT1, SIRT3 and PGC1 α with and without treatment with resveratrol in H1299s and H1299r cell lysates as determined by immunoblot analysis.

Figure 5.2 Effect of resveratrol treatment on the cellular oxygen consumption, cellular reserve capacity and OCR/ECAR ratio of H1299s and H1299r cells as determined by the seahorse extracellular flux analyser.

Figure 5.3 Effect of resveratrol treatment on the mitochondrial oxygen consumption and mitochondrial reserve capacity of H1299s and H1299r cells as determined by the seahorse extracellular flux analyser.

Figure 5.4 Citrate synthase activity of the H1299 cells with and without resveratrol treatment as determined by spectroscopy.

Figure 5.5 Reactive Oxygen Species (ROS) production the H1299s and H1299r cells with and without resveratrol treatment as determined by flow cytometry.

Figure 5.6 Optimisation of the viral titre necessary to transfect the H1299s and H1299r with a GFP tagged empty vector adenovirus as determined by fluorescent microscopy.

Figure 5.7 Expression of SIRT3 after transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus as determined by western blotting.

Figure 5.8 The effect of transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus on whole cell metabolism as determined by the seahorse extracellular flux analyser.

Figure 5.9 The effect of transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus on OCR/ECAR ratio and cellular reserve capacity as determined by the seahorse extracellular flux analyser.

Figure 5.10 The effect of transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus on *in situ* mitochondrial metabolism and mitochondrial reserve capacity as determined by the seahorse extracellular flux analyser.

Figure 5.11 Citrate synthase activity of the H1299 cells after transduction with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus as determined by spectroscopy.

Figure 5.12 The effect of the transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus on SIRT-1, PGC1 α and TFAM expression as determined by western blotting.

Figure 5.13 Reactive Oxygen Species (ROS) production after the transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus as determined by flow cytometry.

Figure 6.1 The expression of CD44 and CD133 in H1299s, H1299r, P31s and P31r cells as determined by flow cytometry.

Figure 6.2 The activity of ALDH in H1299s, H1299r, P31s and P31r cells as determined by flow cytometry.

Figure 6.3 The effect of DEAB inhibition on the expression of Gb3 in H1299s, H1299r, P31s and P31r cells as determined by flow cytometry.

Figure 6.4 The Hoescht side population (HSP) of the H1299s, H1299r, P31s and P31r cells as determined by flow cytometry.

Figure 6.5 Autofluorescence of the H1299s, H1299r, P31s and P31r cells as determined by flow cytometry.

List of tables

Table 2.1 Conditions for Neon transfection optimisation

Table 2.2 Antibodies used for CD44 and CD133 expression

Table 2.3 Antibodies used for western blotting

Abbreviations

2,6-FBP 2,6- Fructose biphosphate

4-EBP1 4E-binding protein 1

ACC Acetyl CoA carboxylase

ADP Adenosine diphosphate

AMP Adenosine monophosphate

AMPK Adenosine monophosphate kinase

ATP Adenosine 5'-triphosphate

CoASH coenzyme A

CTR1 Copper transporter-1

DTNB 5,5-dithio-bis-nitrobenzoic acid

ECAR Extracellular consumption rate

FADH Flavin adenine dinucleotide

Gb3 Globotriasosylceramide

GCS Glucosylceramide synthase

GLUT Glucose transporter

GSL Glycosphingolipids

HIF Hypoxia inducible factor

IL-1 β Interleukin -1 β

IL-6 Interleukin -

IL-8 Interleukin -

LDHA Lactate dehydrogenase A

LKB1	Liver kinase B1
MDR	Multi drug resistant
MMR	Mismatch repair
mTOR	Mammalian target of rapamycin
NAC	N-Aceyl Cysteine
NADH	Nicotinamide adenine dinucleotide
NDI1	NADH dehydrogenase subunit-1
NER	Nucleotide excision repair
NF-Kb	nuclear factor kappa-light-chain-enhancer of activated B cells
OCR	Oxygen consumption rate
OCT1	Organic cation transporter
PARG	Poly(ADP-ribose) glycohydrolase
PARP	Poly (ADP-ribose) Polymerase
PDK1	Pyruvate dehydrogenase kinase 1
PET	Positron Emission Tomography
PF1	phosphofructose kinase-1
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
PI	Propidium Iodide
PI3K	Phosphoinositide 3-kinase
PKM2	Pyruvate kinase M2
PPP	Pentose phosphate pathway
PTEN	Phosphatase and tensin homolog
ROS	Reactive Oxygen Species
S6K	Ribosomal S6 kinase

SREBP1 Sterol-regulatory element-binding protein 1

TCA Tricarboxylic acid

TGF β Transforming growth factor β

TIGAR TP53-inducible glycolysis and apoptosis regulator

TMZ Temozolomide

TNBH 5-thionitrobenzene

TSC2 Tuberous sclerosis

α -KG α -Ketoglutarate

Chapter 1: General Introduction

1.0 Introduction:

1.1 Metabolism in Cancer

1.1.1 Warburg Effect

An almost universal property of invasive cancers is the increase in aerobic glycolysis. Glycolysis is a process whereby glucose is sequentially converted to pyruvate in the cytoplasm with a net synthesis of two ATP glucose molecules. In the absence of oxygen or mitochondria or under conditions where mitochondrial function is limiting, pyruvate can be reduced further and shunted into lactic acid. However, under normal aerobic conditions, pyruvate is transported into mitochondria where it is oxidized further to yield carbon dioxide, and reducing equivalents NADH_2 and FADH_2 which leads to significant ATP synthesis via oxidative phosphorylation. In the majority of mammalian cells, the accumulation of lactate is inhibited by the presence of oxygen which enables the mitochondria to oxidise the end product of glycolysis, pyruvate to carbon dioxide and H_2O . This inhibition is called the 'Pasteur Effect' after Louis Pasteur (Racker, 1974). In contrast, the excessive conversion of glucose to lactic acid in the presence of oxygen is known as aerobic glycolysis or the 'Warburg effect' (Figure 1.1). Otto Warburg was the first to report in the 1920's that cancer cells metabolise glucose in a way that is distinct from that of normal tissues (Warburg, 1930). He found that cancer cells seem to 'ferment' glucose into lactate even in sufficient oxygen for oxidative phosphorylation. He proposed that this "aerobic glycolysis" was as a result of a defect in the mitochondria which increased the reliance of cancer cells on aerobic glycolysis. Although mitochondrial function has been found to be normal in several cancers, the increased aerobic glycolysis in cancer has been consistently observed (Semenza, 2001).

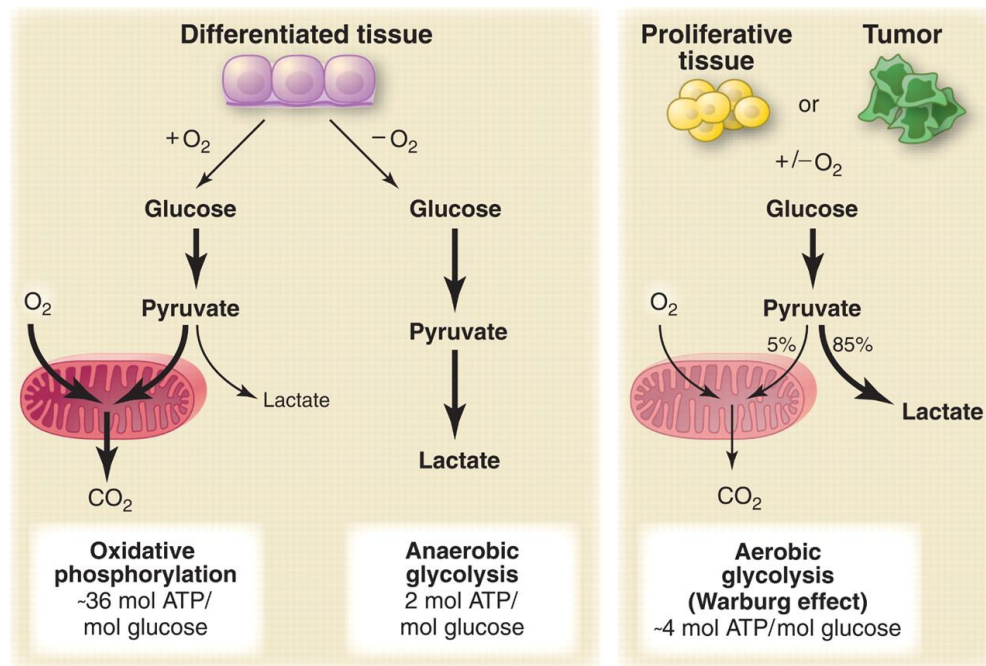


Figure 1.1 Schematic highlighting the differences between oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis (Warburg effect). In non proliferating cells in the presence of oxygen, glucose is broken down to pyruvate by a process of glycolysis where it enters the mitochondria undergoing oxidative phosphorylation producing CO₂. When oxygen is limiting in non proliferating cells, glucose gets broken down by anaerobic glycolysis to form lactate. Warburg noticed that in proliferating or tumour tissue in the absence or presence of oxygen glucose gets broken down to pyruvate in which a small proportion undergoes oxidative phosphorylation, however, the majority forms lactate in a process called aerobic glycolysis or the Warburg effect. Adapted from (Vander Heiden et al., 2009).

1.1.2. Reverse Warburg Effect

New evidence suggests that cancers may induce the Warburg effect in neighbouring stromal fibroblasts (Reverse Warburg Effect). TGF β induced differentiation of normal fibroblasts to activated myo-fibroblasts is crucial to normal wound healing. Fibrosis and collagen deposition have been known as risk factors that predispose patients to malignancy and it is interesting to note that these cancer associated fibroblasts share many traits with activated myo-fibroblasts. Cancers have been known to be referred to as 'wound that cannot heal' (Schafer and Werner, 2008). The tumour micro environment has been proven to play a crucial role in tumour initiation, progression and metastasis (Swartz et al., 2012). The cancer associated fibroblasts, then undergo myo-fibroblastic

differentiation secreting lactate, ketone bodies and pyruvate. The lactate, ketone bodies and pyruvate is then taken up by the epithelial cells of the cancer to use in the TCA cycle, promoting efficient energy production and greater proliferative capacity (Pavlidis et al., 2009). In this scenario, the surrounding stroma are differentiated into a wound healing state, providing a conveyor belt of energy rich metabolites on which the cancer cells can feast to enable growth and proliferation.

Caveolin-1 (Cav-1), an inhibitor of TGF β and therefore an inhibitor of fibroblastic differentiation to myo-fibroblasts has been shown to be dramatically reduced in human breast cancer associated fibroblasts relative to normal fibroblast from the same patient (Mercier et al., 2008). The loss of Cav-1 expression had a predictive value that was independent of epithelial status in ER⁺, PR⁺, HER2⁺ and triple negative breast cancer (Witkiewicz et al., 2009). These findings provide a novel biomarker for the Warburg effect within the tumour stromal compartment.

1.1.3 Aerobic glycolysis, a selective advantage

At first glance, it seems unusual that cancer cells favour a system that produces only 2 ATP whereas complete oxidation of glucose produces up to 36 ATP. Also, because lactic acid is transported out of cells, aerobic glycolysis causes a consistent acidification of the extracellular space thereby potentially causing cellular toxicity (Bhujwalla et al., 2002). Gatenby and Gillies (2004) propose that it represents an evolutionary solution to environmental growth constraints. This acidification of the extracellular space, they indicate, is an important component of the invasive phenotype as it allows the cancer to metastasise leaving it unharmed whilst destroying those cells that seek to constrain it. Work done by Hao et al. (2009) and Yun et al. (2009) indicate that specific perturbations in the cellular environment facilitate adaption to hyperactive metabolic states by selective for mutations that enhance nutrient uptake and reorganise metabolism, as the process of oxidative phosphorylation in the mitochondria to produce maximal amounts of ATP clearly is not the main priority of proliferating cells. In cancer cells, glucose is also needed to provide macromolecular precursors such as acetyl Co A, glycolytic intermediates for non-essential amino acids and ribose for nucleotides (Vander Heiden

et al., 2009, Potter, 1958). This ability of cancer cells to convert glucose to provide macromolecular precursors in part explains the selective advantage of the 'Warburg effect' for the proliferating cancer cells and is exemplified by oncogenes such as KRAS which results in stabilisation of HIF-1 α and HIF2 α .

1.1.4. Oncogenes and tumour suppressors regulate metabolism

The KRAS oncogene is found in 30% of all human tumours, and resulting HIF expression results in increased glucose transporters, glycolytic enzyme activity (Chun et al., 2010), down-regulation of oxidative phosphorylation and mitochondrial function through pyruvate dehydrogenase kinase 1 (PDK1) and MAX interactor 1 (MXI1) (Denko, 2008). While mutations in metabolic enzymes promotes tumorigenesis, mutation in oncogenes or suppressors cause changes in metabolism, as the metabolic enzymes are directly regulated by those cancer genes. Myc was first associated with the control of glycolysis through the direct activations of lactate dehydrogenase A (LDHA) and virtually all glycolytic genes (Dang et al., 2006, Shim et al., 1997). Myc has since been shown to activate genes in mitochondrial biogenesis and also plays a role in glutamine metabolism (Li et al., 2005, Wise et al., 2008). Mutations of PI3K, PTEN and p53 are also ubiquitous in human cancers. Mutation of PI3K imparts its effect through downstream activation of AKT and stabilization of HIF-1. PI3K is antagonised by the tumour suppressor PTEN which is commonly mutated in human prostate cancer (Dang, 2012). Therefore activation of PI3K, inactivation of PTEN impacts upon cellular metabolism because AKT and HIF-1 synergistically greatly increase glycolysis (Elstrom et al., 2004). Contrary to Myc, neither AKT nor HIF-1 has any effect on mitochondrial biogenesis or respiration. p53 is another tumour suppressor commonly lost in human cancers. As well as its role in cell cycle regulation, p53 directly stimulates the activation of genes such as TIGAR, a PFKFB family member that inhibits glycolysis, shunting glucose into the pentose phosphate pathway (Puzio-Kuter, 2011).

1.1.5 Glutamine metabolism

Glutamine, the most abundant amino acid in the plasma, serves as a significant bioenergetic substrate and nitrogen donor in proliferating cells (DeBerardinis and Cheng, 2009). Glutamine enters into the TCA (tricarboxylic acid) cycle through its conversion to glutamate and then to α -ketoglutarate (α -KG), a key intermediate of the TCA cycle (Dang, 2012). Under hypoxia, HIF-1 activates PDK1 that inactivates pyruvate dehydrogenase and impedes the conversion of pyruvate to acetyl-CoA, thereby shunting pyruvate to lactate (Kim et al., 2006). Glutamine can also supply citrate and lipid metabolism via the reversal of the TCA cycle or by reductive carboxylation of α -KG to form citrate or forward cycling of glutamine carbons (Metallo et al., 2012, Mullen et al., 2012). Under conditions of low glucose, the TCA cycle can be engineered to run solely on glutamine, making citrate that is purely composed of glutamine carbons (Le et al., 2012). Similarly hypoxic proliferating cells can reprogram the TCA cycle for lipid metabolism also.

The altered metabolism of cancer cells has enabled clinicians to image tumours using positron emission tomography (PET) which takes advantage of the greater rate of glucose metabolism in dividing and hypoxic cancer cells (Sullivan et al., 2014). PET has enabled clinicians to visualise and examine the body from a functional, biochemical stand point. PET has become vital for clinicians in staging, restaging and assessing treatment effectiveness. PET often detects changes before any anatomical or structural change has become apparent (Zhu et al., 2011). It is the adaptability of cancer cells to environmental restraints that enables cancers to acquire resistance to chemotherapeutic agents like cisplatin through compensatory mechanisms (Komurov et al., 2012). Increasing evidence points towards the necessity of modifications in the cellular metabolism in driving malignant progression (Levine and Puzio-Kuter, 2010, Locasale et al., 2009).

1.2.1 Diabetes drugs in Cancer

Drugs that target metabolic diseases such as the type II diabetes drug metformin and phenformin have shown clinical benefit in treating cancer. Vladimir Dilman in the 1970's was the first to identify the link between these diabetes drugs and their anti-neoplastic effects (Dilman, 1971). Indeed, several retrospective epidemiological studies recently have shown a decrease in cancer risk in those diabetic patients that were given metformin. Evans et al. (2005) were the first to show a decrease in cancer risk in diabetic patients treated with metformin. Jiralerspong et al. (2009) showed the rate of pathological complete response (absence of tumour in the removed tissue at time of surgery) to neoadjuvant chemotherapy for breast cancer was 24% in the metformin treatment group as compared to just 8% in the non-metformin group. Since then there has been an explosion of publications investigating the role of these drugs in cancer. There are currently 196 clinical trials investigating the potential beneficial use of metformin in cancer. However, despite the large patient numbers used in these studies, which are all retrospective, the majority of them lack important information like the dose of metformin, the body mass index of the patients and the glycaemia control. They are unable to establish a causal relationship between using the drug and a decrease in the cancer risk.

1.2.2 Metformin & Phenformin

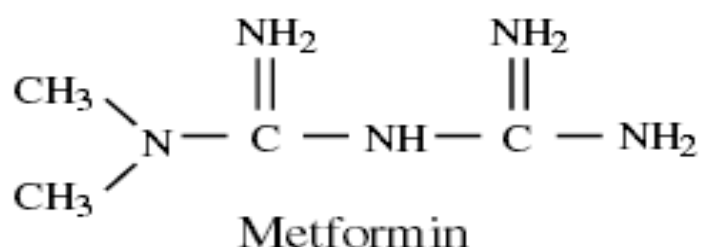
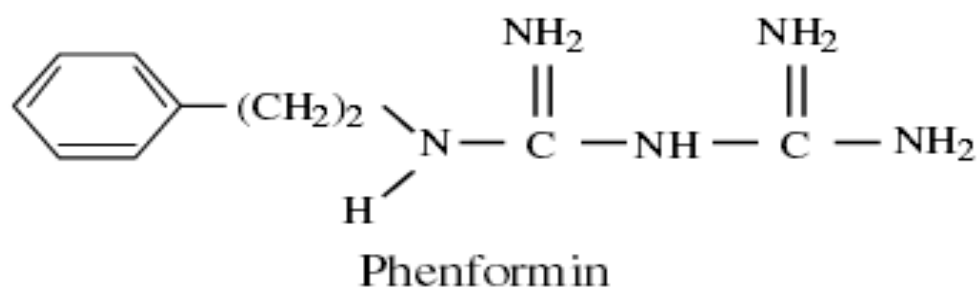


Figure 1.2 Chemical structures of phenformin and metformin (Goodarzi and Bryer-Ash, 2005).

The type II diabetic drugs: Metformin and Phenformin are from a class of drug called biguanides extracted from the plant *Galega Officinalis*. Metformin has two methyl groups attached to a guanidine side chain whilst phenformin has two guanidine groups linked together with a loss of an ammonia group and also possesses a phenyl-ethyl ring on a guanidine side chain (Figure 1.2). While phenformin had to be removed from the market due to unacceptably high levels of lactic acidosis (Kwong and Brubacher, 1998), Metformin remains one of the most prescribed drugs, with nearly 120 million prescriptions being filled out each year worldwide (Ben Sahra et al., 2010). Although Phenformin has been removed from the market due to lactic acidosis, it has shown significant antineoplastic activity in humans and animals (Caraci et al., 2003, Huang, 2008, Birsoy et al., 2014) and at lower concentrations than its analogue metformin. Phenformin is less polar and hence more lipid soluble than metformin (Goodarzi and Bryer-Ash, 2005). Consequently, phenformin can easily cross biological membranes and

has been demonstrated to have a higher affinity for mitochondrial membranes when compared to metformin (Hawley et al., 2005). The level of lactic acidosis is low enough that if it showed superior anti-neoplastic activity it would not be contraindicated in cancer treatment. Also the length of treatment and dosage would be different from diabetic patients and so the adverse side effects observed with the drug would not be as severe (Shackelford and Shaw, 2009). Metformin does have limited usage because it requires the OCT1 transporter to get inside the cells which has limited distribution whereas phenformin has a greater distribution (Segal et al., 2011) so phenformin could possibly be a better cancer drug.

Metformin has been reported in studies to induce apoptosis but they are at concentrations ranging 1 mM to 100 mM which are supra-physiological levels considering concentrations of metformin in the blood of a type 2 diabetic patient treated with the drug accumulates to 50 μ M. To support the validity of these high concentrations used in these studies, Owen (2000) showed that metformin can reach 8 mmol/L (8 mM) representing physiological doses in the liver which receives the majority of the blood flow and is the site of metformin's action. Carvalho et al. (2008) also suggested that the positive charge on metformin and phenformin could promote its accumulation within the matrix of the mitochondrion by 1,000 fold. It has been proven that metformin is able to accumulate in tissues at concentrations several fold higher than those in blood (Wilcock and Bailey, 1994). These studies provide evidence that these seemingly supra physiological levels could in fact be physiological and provide a rationale for the use of the drug in cancer treatment.

With the enormous amount of research being conducted on metformin, its main mechanism of action still remains elusive. Also whether its effect is indirect *i.e.* lowering insulin concentration and/or directly on the cancer cells themselves remains to be conclusively determined (Figure 1.3).

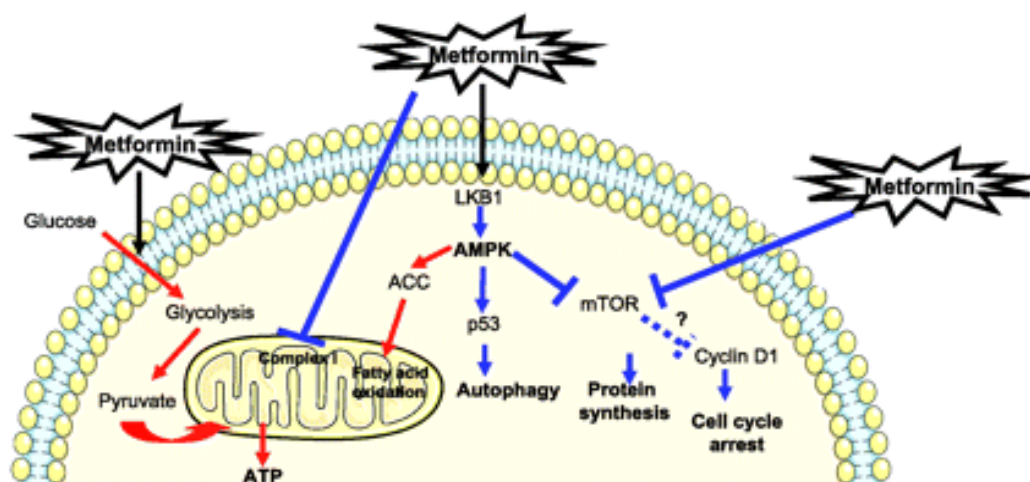


Figure 1.3 Potential mechanisms of metformin in cellular metabolism and cell proliferation (Ben Sahra et al., 2010). Metformin (a) increases glucose uptake and glycolysis. (b) Activates AMPK and ACC increasing fatty acid oxidation. (c) Inhibits complex I. (d) Impacts upon cell growth by p53 induced autophagy, mTOR inhibition of protein synthesis and cell cycle arrest by decreasing Cyclin D1 expression.

1.2.3. Activation of the AMPK/mTOR pathway

In most of the studies using the biguanides, metformin and phenformin in cancer cells, they indicate that the AMPK/mTOR pathway plays a central role in its mechanism of action. The AMPK/mTOR pathway is controlled by the activity of Liver Kinase B1 (LKB1). LKB1 is a serine threonine kinase that functions as a tumour suppressor (Forcet and Billaud, 2007), thereby directly connecting the use of the biguanides in cancer. AMPK is a heterotrimeric protein kinase complex made up of a catalytic subunit (α) and two regulatory subunits (β and γ). AMPK acts as a detector of the cellular energy status, responding to an increase in the AMP/ATP ratio under stresses such as ischaemia, hypoxia and glucose deprivation (Steinberg and Kemp, 2009) by inducing ATP producing pathways (catabolic activation) and reducing ATP consuming pathways (anabolic inhibition), until cellular energy content reaches normal levels (Carling, 2004, Martin and Marais, 2012).

1.2.4 AMPK, ACC and Fatty Acid Oxidation

AMPK, the enzyme itself was initially uncovered as a kinase that phosphorylates and inhibits Acetyl CoA carboxylase (ACC) and HMG CoA reductase, key enzymes in the de novo fatty acid and cholesterol synthesis (Luo et al., 2010). ACC has been found to be highly expressed in numerous types of cancers, including those arising from the breast, prostate and colon (Kuhajda, 2000, Baron et al., 2004). Studies in human patients indicate that there is an increase in free fatty acid turnover, oxidation and clearance (Legaspi et al., 1987). Metformin has been known to decrease free fatty acid concentration primarily through a diminution of overall fatty acid oxidation (Perriello et al., 1994). A decrease in overall free fatty acids oxidation by metformin could ameliorate both hepatic glucose production and peripheral oxidation (Fulgencio et al., 2001) and have beneficial anti-neoplastic properties.

1.2.5 Necessity of AMPK for metformin's action.

The necessity of AMPK activation for metformin's mechanism of action in cellular proliferation and survival still remains unclear and controversial. Huang (2008) showed that AMPK activation was required to reduce the occurrence of tumours following metformin and phenformin treatment whereas Sahra et al. (2008) showed that metformin's effect on prostate cancer cells and (Würth et al., 2013) in glioblastoma cells was independent of AMPK activation. These differences highlight the importance of deciphering the involvement of AMPK in metformin or phenformin's effect.

Metformin and phenformin have been postulated to indirectly activate AMPK by inhibiting complex I (El-Mir et al., 2000, Owen, 2000, Miskimins et al., 2014) of the respiratory chain in the mitochondria thereby decreasing the ATP synthesis and increasing the AMP: ATP ratio (El-Mir et al., 2000, Owen, 2000) . This inhibition of complex I may account for the inhibition of fatty acid oxidation that is observed following metformin treatment. Indeed, the increase in peripheral glucose utilization, metabolism and stimulation of glycolytic lactate production observed after metformin treatment are effects that would be predicted with inhibition of complex I of the

respiratory chain (Bailey and Turner, 1996). The increase in lactate production observed in patients treated with metformin and especially phenformin would be predicted as a consequence of increased glycolytic metabolism (Owen, 2000), but seems counterintuitive for an anti cancer drug.

1.2.6 AMPK and mTOR

A direct result of the activation of AMPK is the inhibition of the mTOR pathway through tuberous sclerosis 2 protein (TSC-2). mTOR up regulates several energy consuming cellular systems and has a pivotal role in controlling cell growth by regulating mRNA translation and ribosome biogenesis and is deregulated in most cancers (Guertin and Sabatini, 2007).

mTOR comes in two biochemically and functionally distinct signalling complexes; mTORC1 & mTORC2. mTOR complex 1 (mTORC1) which is inclusive of regulatory-associated protein of mTOR (raptor), functions as a scaffold to recruit downstream substrates, such as eukaryotic translation factor 4E-binding protein 1 (4EBP1) and ribosomal S6 kinase (S6K1), which facilitate the mTORC1-dependent regulation of protein translation (Dowling et al., 2007). mTORC1 manages the translation of several cell growth regulators, such as cyclin D1, hypoxia inducible factor 1 α (HIF1 α), MYC and sterol-regulatory element-binding protein 1 (SREBP1) which then stimulate processes including cell cycle progression, cell growth and angiogenesis, all of which can become deregulated during tumorigenesis (Guertin and Sabatini, 2007).

TSC2 or tuberin is a tumour suppressor and is found to be an upstream regulator of mTORC1. TSC2 acts as a GAP toward the small GTPase Ras homologue enriched in brains (RHEB) (Brugarolas et al., 2003) such that when the levels of ATP, glucose or O₂ are low, AMPK directly phosphorylates TSC2 on conserved serine sites (Shaw et al., 2004) inhibiting mTORC1 indirectly through RHEB. Loss of TSC2 results in hyperactivation of mTORC1 (Huang and Manning, 2008).

1.2.7. Metformin and inflammation

Metformin has also been shown to have an effect on inflammation. It has been shown to inhibit IL-1 β induced release of the pro-inflammatory cytokines IL-6, IL-8 and the nuclear transcription factor NF- κ B (Isoda et al., 2006). It also been shown to decrease C-reactive protein, a marker of chronic inflammation in polycystic ovary syndrome (Morin-Papunen et al., 2003) and other inflammatory conditions. As of yet the role of metformin's effect on inflammation in cancer has not yet been studied but its action on C-reactive protein could be beneficial in cancer as it has shown to be elevated in prostate (McArdle et al., 2006), colorectal (McMillan et al., 2003) and non small lung cancer (Scott et al., 2002).

1.2.8. Metformin, phenformin and cell death

Publications that show that metformin induces apoptosis in cancer cells are somewhat limited and inconsistent. Sahra et al. (2008) showed that metformin was able to induce cell cycle arrest at the G0/G1 phase of the cell cycle but was unable to induce apoptosis in those prostate cancer cells. Similarly, breast cancer cells did not undergo apoptosis in response to metformin treatment (Alimova et al., 2009). However, metformin was shown to induce apoptosis in a breast (Malki A, 2011) and ovarian (Yasmeen et al., 2011) cancer cell lines and phenformin has been shown to cause both apoptosis and necrosis in cancer (Shackelford et al., 2013, Caraci et al., 2003).

Apoptosis is morphologically defined by pyknosis (deep staining of nuclear mass), nuclear fragmentation and the synthesis of condensed cell bodies (apoptotic cell bodies). This organised method of cell dying depends upon ATP consuming processes in order to carry out this self degradation (Zong and Thompson, 2006). Conversely, necrosis can be characterised morphologically by electron-lucent cytoplasm, swelling of cellular organelles and loss of plasma membrane integrity. Due to these differences, apoptosis has been referred to as programmed cell death whereas necrosis is considered to be uncontrolled, however these processes are not always mutually exclusive. Many insults may induce apoptosis at the lesser doses and cause necrosis at

the greater dose or they may occur simultaneously within the same cell, termed 'apoptotic necrosis' (Majno and Joris, 1995).

One of the markers of cell death is Poly (ADP-ribose) Polymerase (PARP) cleavage. Poly(ADP-ribosylation) is a post-translational modification that plays a pivotal role in DNA repair (Lindahl et al., 1995), replication (Eki, 1994), differentiation (Ebisuzaki et al., 1991), and the modulation of cellular defensive mechanisms against DNA damage (Jeggo, 1998). Poly-(ADP-ribose) metabolism is mainly regulated by the combined action of poly(ADP-ribose) polymerase (PARP) and poly(ADP-ribose) glycohydrolase (PARG) (Soldani et al., 2001).

It has been shown that poly (ADP-ribose) is synthesized upon activation of the apoptotic pathway and it may also initiate cell autonomous necrosis. Upon the induction of cell death, many proteins are degraded by caspases (Marks and Fox, 1991), one of those being PARP-1. PARP-1 is one of the first proteins to be proteolyzed from the 116 kDa form to the 89 kDa form when apoptosis is initiated (Soldani et al., 2001). However acute activation of PARP can activate necrosis (Zong and Thompson, 2006). Indeed Ha and Snyder (1999), showed that upon activation of necrosis with increasing dose of their drug instead of observing the cleaved form of PARP, they saw a decrease in the full form of PARP.

1.2.9. Metformin, phenformin effect on the cell cycle.

Due to the activity of AMPK activation, inhibiting energy consuming processes and protein synthesis (Dowling et al., 2007), metformin has been proposed to inhibit cell proliferation through AMPK activation. Metformin has been shown to exhibit strong and consistent anti-proliferative action on several cancer cell lines for example breast, colon, ovary, pancreas and prostate cancer cells albeit at high concentrations ranging from 5-30 mM (Ben Sahra et al., 2010). At the cellular level, metformin has been shown to interfere with the cell cycle resulting in G0/G1 or S phase arrest. Sahra et al. (2008) have shown in prostate cancer cells that metformin induced G0/G1 cell cycle arrest with a concurrent reduction in cyclin D1 protein levels. S phase arrest has been observed in

triple negative breast cancer cell line associated with the induction of apoptosis (Liu et al., 2009). Phenformin has been shown to have anti-proliferative effects by Huang (2008) and to induce p21 cell cycle inhibition and cell cycle arrest at the G2/M phase of the cell cycle leading to apoptosis (Caraci et al., 2003).

1.2.10 Phenformin and ROS

Increasing evidence from recent studies have indicated that cancer cells as opposed to normal cells are under significant oxidative stress as a consequence of oncogenic transformation, changes in metabolic activity and increased generation of reactive oxygen species (ROS)(Behrend et al., 2003, Kang and Hamasaki, 2003). Increased ROS has been shown to change the crucial sulfhydryl group of pyruvate kinase M2 (PKM2) resulting in its inactivation, shunting glucose towards the pentose phosphate pathway (PPP). Additionally, increased ROS can stabilise HIF-1. Upon HIF-1 stabilisation, its target genes become activated such as PDK1, which functions to divert pyruvate from mitochondrial oxidation and PFKFB4, which degrades 2,6-fructose bisphosphate (2,6-FBP). 2,6-FBP is a potent activator of phosphofructose kinase 1 (PF1) which converts fructose 6-phosphate (Yalcin et al., 2009). Augmented PFKFB4, as seen in prostate cancer cells, would dampen PFK1 and shift glucose into the PPP, increasing NADPH to titrate ROS (Ros et al., 2012). Interestingly its analogue PFKFB3 in hypoxia enhances glycolysis which PFKFB4 counteracts, the balance of these two is crucial for shunting glucose into glycolysis versus the PPP. This augmented amount of ROS in cancer can also have substantial beneficial effects on the cells such as stimulation of proliferation, the endorsement of mutations and genomic instability and changes to sensitivity to anti cancer agents (Pelicano et al., 2004).

ROS are generally described as oxygen containing chemical species which have reactive chemical properties. ROS family encompasses superoxides, hydroxyl radicals and hydrogen peroxide (Pelicano et al., 2004). They can be synthesized in a multitude of ways, by both enzymatic and non-enzymatic pathways. It is approximated that 2% of the oxygen taken up by the mitochondria is reduced by the bifurcated electrons to form superoxide which is then further converted to hydrogen peroxide (Fridovich, 1995). Due

to the fact that superoxides are being constantly generated during respiration, especially through complex I and III (Figure 1.4)(Staniek et al., 2002), the mitochondria are considered the major sources of cellular ROS (Richter et al., 1995). As metformin is reported to inhibit complex I (Owen, 2000), one of the major sources of cellular ROS, it has been shown to alter or decrease intracellular ROS (Chakraborty et al., 2011, Zou et al., 2004).

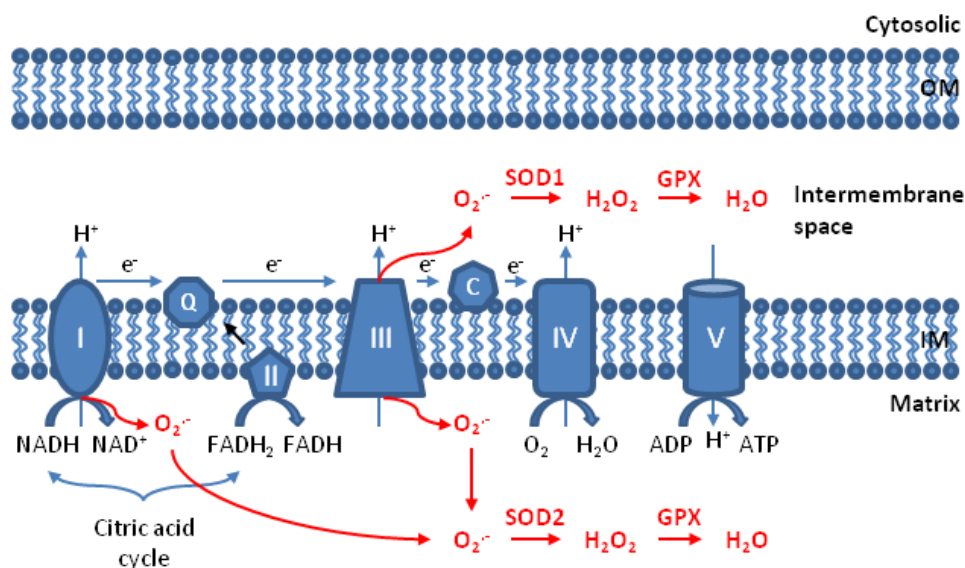


Figure 1.4 Sources of mitochondrial ROS. Blue arrows denote the flow of products from reactions and red arrows denote the fate of reactive oxygen species produced at complex I and III. Adapted from (Li et al., 2013).

1.2.11 Phenformin and ATP

Phenformin through its inhibition of complex I of the mitochondria has been reported to block oxidative respiration (Lenhard et al., 1997). This results in increased AMP:ATP ratio and decreased ATP levels thereby activating AMPK (Figure 1.5) (Shackelford et al., 2013, Aziz et al., 2010, Vigneri et al., 1984, Zhang et al., 2007b, Owen, 2000). As mentioned before AMPK acts to restore the energy balance through a multitude of pathways. For example, AMPK upregulates GLUT1 expression to augment glucose uptake (Fryer et al., 2002), increase energy generation through increased glycolysis by phosphorylating

phosphofructokinase-2 (Marsin et al., 2000) and to inhibit ACC to prevent lipid synthesis (Winder et al., 1997) so that these energy consuming processes are extinguished.

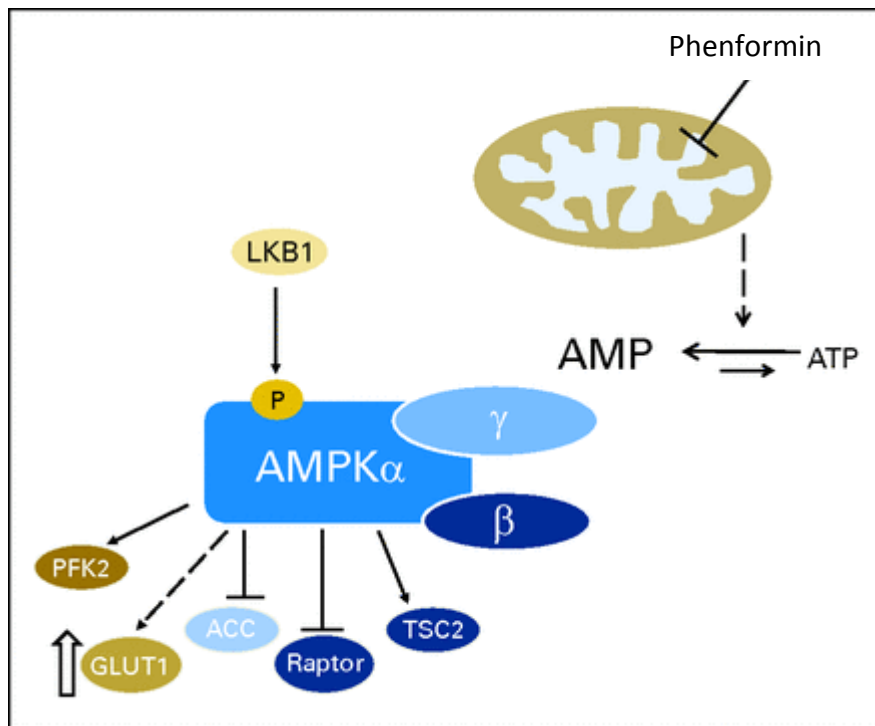


Figure 1.5 Phenformin's effect on ATP. Phenformin inhibits complex I decreasing the AMP: ATP ratio, activating AMPK which then phosphorylates substrates for energy generation and suppresses energy consumption. Adapted from (Martin and Marais, 2012).

1.2.12. Metformin in combination

Metformin has shown clinical benefit when used in combination with other agents. It has been shown to radiosensitise cancer cells (Song et al., 2012) and preferentially kill undifferentiated cancer stem cells (Hirsch et al., 2009) in vitro and in tumour xenografts (Iliopoulos et al., 2011). Both Würth et al. (2013) and Hirsch et al. (2009) showed that metformin affected the undifferentiated cells while leaving the differentiated cancer cells relatively unharmed. Studies of this type provide experimental evidence for the use of metformin in combination with other agents for cancer treatment as stem cells are known to persist after a chemotherapeutic agent treatment. Those cancer stem cells can then regenerate all the cell types in the cancer through stem like behaviour. Metformin has been shown to augment the anti-proliferative effects of cisplatin, paclitaxel and

tamoxifen in lung breast and prostate cancer cells (Iliopoulos et al., 2011, Bernstein et al., 2011, Rattan et al., 2011) and to enable the usage of lower concentrations of the chemotherapeutic drugs thereby reducing the potency of its harmful side effects.

1.3.1. Cisplatin.

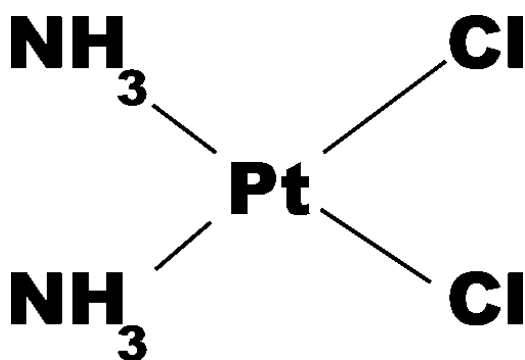


Figure 1.6 Chemical structure of cisplatin (Cullen et al., 2007)

Cisplatin (cis-diamminedichloroplatinum (II)) is a small molecule platinum compound composed of a platinum atom complexed by two amine groups and two chloride ions (Figure 1.6)(Jamieson and Lippard, 1999). Cisplatin was accidentally discovered to inhibit the growth of *Escherichia coli* and was subsequently found to kill tumour cells (Rosenberg, 1973). It is now one of the most potent chemotherapeutic drug used in the clinic to treat solid cancers such as; sarcoma, small cell lung cancer, germ cell tumours, lymphoma, and ovarian cancer (Liou and Storz, 2010, Michels et al., 2013, Sena and Chandel, 2012, Guzy et al., 2005, Callapina et al., 2005). When cisplatin enters the low chloride intracellular environment, cisplatin is hydrated to form a positive charged species which can react with DNA and other nucleophilic species within the cells (Andrews and Howell, 1990). Its cytotoxicity has been generally attributed to its interaction with DNA to form DNA adducts, mainly intrastrand crosslink adducts (Siddik, 2003, Behnam-Motlagh et al., 2010a, Johansson et al., 2006) activating several pathways ending in the induction of mitochondrial apoptosis (Patel et al., 2011).

Cisplatin treatment clinically has shown modest and often temporary beneficial anti-neoplastic effects due to adverse side effects of gastrointestinal toxicity (Yanez et al.,

2003), ototoxicity (Devarajan et al., 2002) and nephrotoxicity (Schwerdt et al., 2003) all of which are associated with the damaging effects of cisplatin upon the mitochondria. More often its lack of efficacy is due to the development of acquired chemoresistance, resulting in drug relapse and therapeutic failure (Johansson et al., 2009, Locasale, 2012, Galluzzi et al., 2012, Michels et al., 2013). On the whole resistance is acquired in a multitude of mechanisms that act simultaneously within the same tumour (Teicher et al., 1987).

1.3.2 Cisplatin Resistance

Resistance occurring post-DNA binding possibly could be due to changes in DNA repair pathways (an increase in nucleotide excision repair (NER) or a loss of DNA mismatch repair (MMR) which would repair the damage that cisplatin would cause (Siddik et al., 1998). In contrast, the hypersensitivity of some cell lines to cisplatin has been shown to be due to a malfunctioning NER (Furuta et al., 2002), through loss or reduced expression of NER proteins such as XPG and XPA. Resistance might occur to platinum, and other cancer drugs, through decreased expression or loss of apoptotic signaling pathways as mediated through various proteins such as p53, anti-apoptotic and pro-apoptotic members of the BCL2 family, and JNK.

Platinum enter cells using either transporters, (a significant one being the copper transporter CTR1) or by passive diffusion. Loss of CTR1 results in less platinum entering cells and consequently drug resistance (Lin et al., 2002). Once inside the cell, platinum is activated by the addition of water molecules thereby forming chemically active aqua species. The aqua species preferentially binds to species that have high sulphur content e.g. glutathione and metallothionein. In some platinum-resistant cancer cells, glutathione and metallothionein levels are relatively high, so activated platinum is effectively 'mopped up' in the cytoplasm before DNA binding can occur, thereby causing resistance (Kasahara et al., 1991).

1.3.3. Cisplatin resistance, MDR and Gb₃

Active export of platinum from the cells through the copper exporters ATP7A and ATP7B, the glutathione S-conjugate export GS-X pump (also known as MRP2 or ABCC2) or through multidrug resistance proteins (Zhou, 2008) e.g. MDR1 can contribute to platinum drug resistance.

Overexpression of an enzyme glucosylceramide synthase (GCS), the first enzyme in the glycosphingolipids (GSL) synthesis is associated with multidrug resistance. Glycosphingolipids are components of all vertebrate cells and have a crucial role during development and cell differentiation (Erdmann et al., 2006). Cells that express MDR1 have shown to also express glucosylceramide (Veldman et al., 2002). MDR1 can function to translocate glucosylceramide into the golgi apparatus for neutral GSL synthesis, including globotriasylceramide (Gb₃).

A simplified diagram of GSL biosynthesis is displayed in Fig 1.7. The first step of GSL synthesis occurs at the cytosolic side of the golgi membrane, where UDP-glucose transfers a glucose residue to ceramide via glucosylceramide synthase, changing the ceramide to glucosylceramide (Lala et al., 2000, Eckford and Sharom, 2005). The second enzyme in the GSL synthesis process, lactosylceramide synthase, is located at the luminal side of the Golgi membrane and, so, requires the translocation of its substrate, glucosylceramide, from the cytosolic to the luminal leaflet of the organelle (Eckford and Sharom, 2005, Lala et al., 2000). MDR1 at this point is thought to carry out its translocase activity by translocating glucosylceramide to the luminal side to be altered to lactosylceramide. Lactosylceramide can then be subsequently processed to globotriaosylceramide (Gb₃) by α -1,4-galactosyltransferase and to globoside (Gb⁴) by β -1,3-galactosyltransferase (Lala et al., 2000, Eckford and Sharom, 2005). It is this flippase activity that has been proposed as the mechanism by which MDR1 pumps hydrophobic drugs out of resistant cancer cells (Lala et al., 2000).

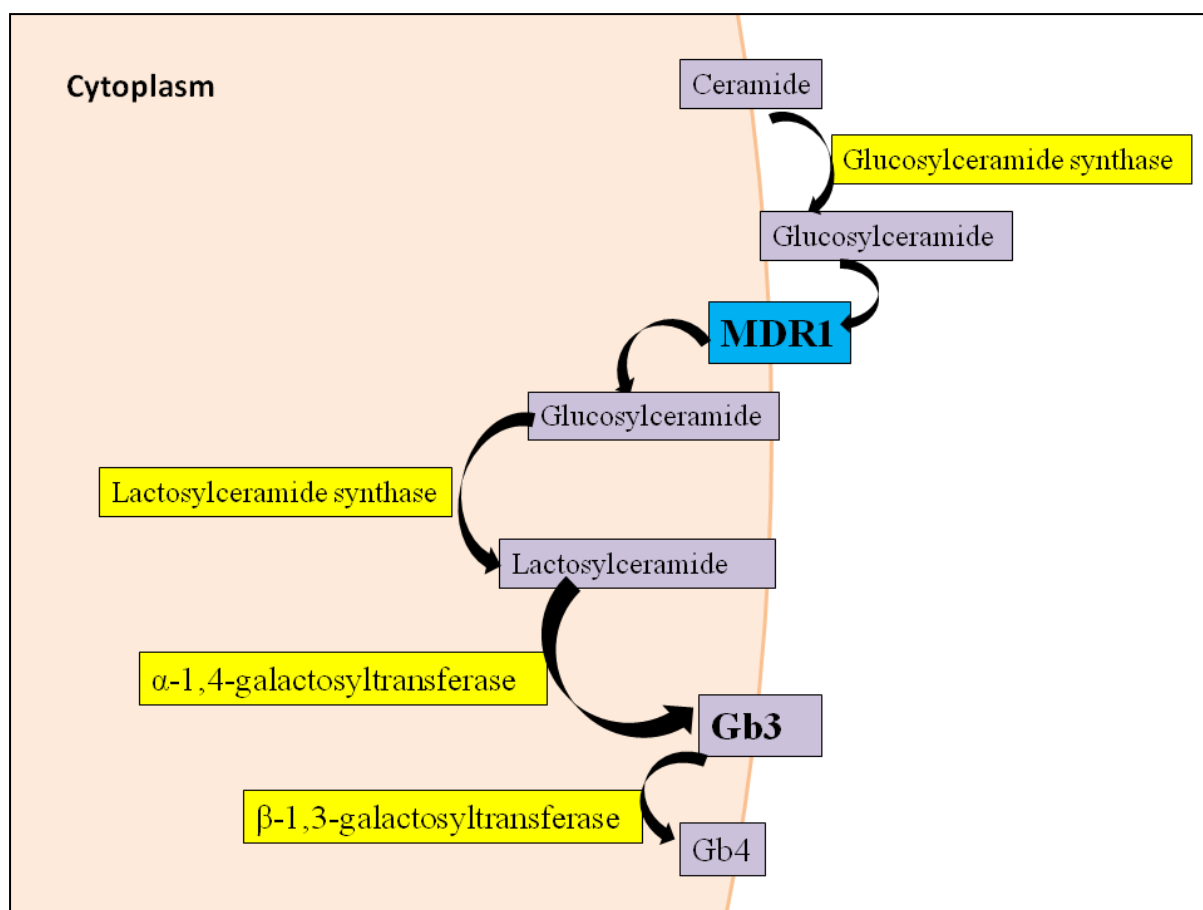


Fig 1.7 GSL biosynthesis at the golgi apparatus. Enzymes involved in the GSL biosynthetic pathway are coloured in yellow. GSLs and GSL precursors are coloured in purple. MDR1 is coloured in blue. On the cytosolic side of the Golgi apparatus membrane, UDP-glucose transfers a glucose residue to ceramide via glucosylceramide synthase, converting the ceramide to glucosylceramide. MDR1 translocates glucosylceramide to the luminal side of the membrane to be converted to lactosylceramide by lactosylceramide synthase. Lactosylceramide can be further processed to Gb₃ by α-1, 4-galactosyltransferase and to Gb₄ by β-1,3-galactosyltransferase. Adapted from Emma O'Connor's master's thesis.

Gb₃ consists of a trisaccharide linked to a lipid based in the plasma membrane (Johansson et al., 2009) and is expressed in breast, ovarian, haematopoietic malignancies and colon cancer cell lines (LaCasse et al., 1999, Gariépy, 2001, Kovbasnjuk et al., 2005). Functional interplay between Gb₃ and MDR1 has been suggested (De Rosa et al., 2008) and Gb₃ expression in colorectal cancer was correlated with invasiveness and metastatic potential (Kovbasnjuk et al., 2005). Behnam-Motlagh et al. (2010b) were able to demonstrate a correlation of increased expression of Gb₃ in acquired cisplatin

resistant malignant pleural mesothelioma cells (P31) and non small lung cancer cells (H1299 cells). Gb₃ is known to function as a cell surface receptor for verotoxin-1 (VT-1 or Shiga Like Toxin-1) synthesized by pathogenic strains of *Escherichia Coli* (Arab et al., 1997). The combination treatment of verotoxin-1 and cisplatin was shown to have an additive cytotoxic effect upon the treatment of acquired cisplatin cancers (Behnam-Motlagh et al., 2010b, Johansson et al., 2009). Glucosylceramide synthesis inhibition by the use of DL-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), which also resulted in the downstream inhibition of Gb₃ synthesis, re-sensitised cisplatin-resistant mesothelioma and non small lung cancer cells to cisplatin-induced apoptosis (Johansson et al., 2009). These results indicate a link between Gb₃ expression and acquired cisplatin resistance and that cisplatin upregulates Gb₃, preferentially eradicating cancer cells with a low Gb₃ expression.

1.3.4 Cisplatin and mitochondria

While considerable research has been conducted focusing on the interaction of cisplatin with DNA, approximately only 1 % of intracellular platinum is bound to nuclear DNA, with the majority being available to interact with other nucleophilic on other sites, some of which include phospholipids, membrane proteins, RNA and mitochondrial DNA (Fuentes et al., 2003, Gonzalez et al., 2001). Limited studies have investigated the interaction of cisplatin and mitochondria DNA. Loss of mitochondrial DNA was associated with an increased sensitivity to cisplatin-induced apoptosis (Liang and Ulliyatt, 1998), whilst cells depleted of mitochondrial DNA demonstrate enhanced resistance to a number of chemotherapeutic agents (Park et al., 2004). Indeed mitochondrial DNA would be susceptible to DNA damage because mitochondrial DNA lack nuclear excision repair, the main mechanism by which cells mitigate the effects of cisplatin (Preston et al., 2001). Some studies have indicated that mitochondrial DNA adducts may be more common than DNA adducts in the same cell line and cisplatin concentration (Murata et al., 1990, Olivero et al., 1995). Studies have now confirmed the idea that the mitochondria may be a direct or at least an important target of cisplatin.

Cisplatin induced mitochondrial damage has been increasingly been investigated as a mediator of toxicity in normal tissues in animals administered cisplatin. Gastrointestinal toxicity (Yanez et al., 2003), ototoxicity (Devarajan et al., 2002) and nephrotoxicity (Park et al., 2002, Schwerdt et al., 2003) which have all been accredited to cisplatin induced mitochondrial damage.

Yang et al. (2006) showed that cisplatin preferentially binds to mitochondrial DNA and voltage dependent anion channel proteins in the mitochondrial membrane of head and neck squamous cell carcinoma cells. Through cisplatin's effect on mitochondrial membranes, cytochrome C has been shown to be released (Kluck et al., 1997) ultimately leading to cell death. Cisplatin has also been shown to increase mitochondrial ROS, to reduce glutathione (ROS scavenger) levels and specifically reduce succinate dehydrogenase activity (Kharbangar et al., 2000). In an animal model and eight cell lines investigating cisplatin sensitivity, a correlation between mitochondrial density and sensitivity to cisplatin was demonstrated. They showed that an increased mitochondrial content was associated with an increased sensitivity to cisplatin. In cells whose mitochondria were depleted became resistant to cisplatin (Qian et al., 2005).

1.3.5 Mitochondrial Biogenesis

Mitochondrial biogenesis (Fig 1.8) is a multi faceted process involving transcription of both nuclear and mitochondrial DNA, nuclear and mitochondrial communications including protein import, mitochondrial dynamics and mitophagy (Diaz and Moraes, 2008). Fission of depolarised mitochondria as well as mitophagy balances the total mitochondrial mass within the cell. The whole process is extremely sensitive to redox stress, nutrient availability and mitochondrial function (Wenz, 2013, Dominy Jr et al., 2010).

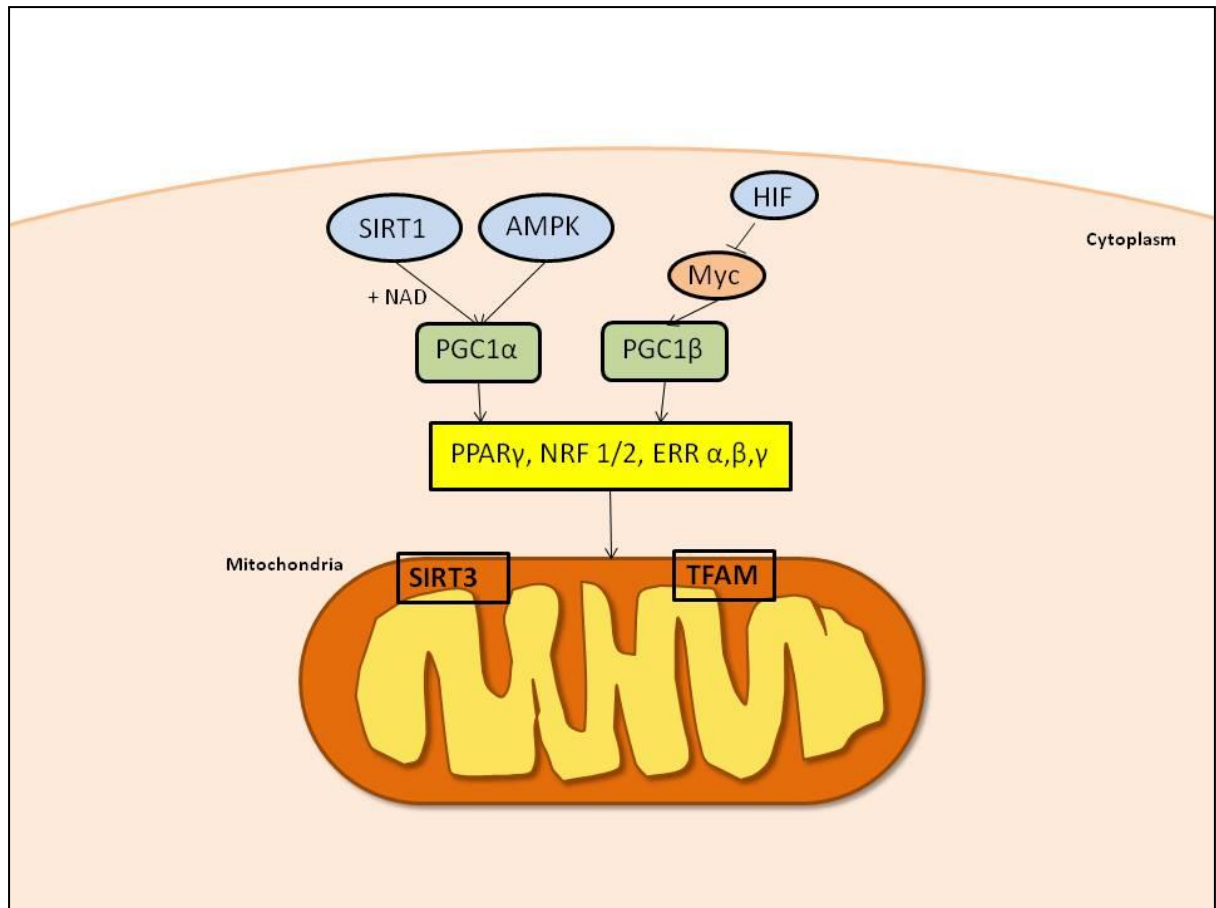


Fig 1.8 Signalling pathways regulating mitochondrial biogenesis. SIRT1 and AMPK post-translationally activate PGC1 α , which then regulates the activation of the transcription factors PPAR γ , NRF 1/2, ERR α , β , γ . PGC1 α regulates the expression of TFAM which plays a role in mitochondrial DNA matintenance. Dual activation of PGC1 α and ERR α induces the expression of downstream SIRT3. Myc increases mitochondrial biogenesis via induction of PGC1- β expression. HIF-1 by contrast inhibits mitochondrial biogenesis by promoting Myc degradation.

Mitochondrial biogenesis is reliant upon the activity of a hierarchy of nuclear transcription factors, including peroxisome-proliferator activator receptor-alpha (PPAR α), PPAR γ , nuclear respiratory factor 1 (NRF1), nuclear respiratory factor 2 (NRF2) and estrogen related receptors (ERR) α , β , γ (Kelly and Scarpulla, 2004). NRF 1 and 2 in conjunction regulate the expression of respiratory chain components, including cytochrome c subunits as well as antioxidant genes while ERR factors modulate the expression of genes concerned with fatty acid oxidation, Krebs cycle and oxidative phosphorylation (Scarpulla et al., 2012). All of these transcription factors are crucially

reliant for their activity on PPAR- γ co-activator 1- α (PGC1 α) that was first discovered as a co-activator of PPAR- γ in brown adipose tissue. PGC1 α is now known as the main mediator of transcriptional responses to nutrient stress, promoting mitochondrial biogenesis, cellular metabolism, and antioxidant responses through coordinated activation of the transcription factors NRF 1/2, ERR α and PPAR γ (Scarpulla et al., 2012, Dominy Jr et al., 2010). PGC1 α regulates the expression of TFAM, a gene that mirrors the changing levels of mitochondrial DNA (mtDNA) and plays a crucial role in mtDNA maintenance (Miranda-Lorenzo et al., 2014). PGC1 α is transcriptionally activated by PPAR'S, mTOR and CREB leading to increased mitochondrial biogenesis (Dominy Jr et al., 2010). PGC1 α is controlled at the post-translational level by both phosphorylation by AMPK and deacetylation by a NAD⁺ dependent SIRT1. Deacetylation by SIRT1 supports mitochondrial biogenesis and maintains the sensitivity of PGC1 α to both the energy and redox balance within the cell (Cantó and Auwerx, 2011). Dual activation of both PGC1 α and ERR α , promotes the expression of the downstream mitochondrial SIRT3 that ensures proper scavenging of ROS by activation of mitochondrial superoxide dismutase, as well as other mitochondrial sirtuin targets (Bell and Guarente, 2011).

Numerous studies have shown the role of oncogenes in mitochondrial biogenesis. c-Myc increases mitochondrial biogenesis via induction of PGC1- β expression (Zhang et al., 2007a), leading to increased expression of the important mitochondrial proteins, such as TFAM and NRF1 (Kim et al., 2008, Li et al., 2005). HIF-1 by contrast inhibits mitochondrial biogenesis by promoting c-Myc degradation (Zhang et al., 2007a). The HIF-1 protein itself is stabilised by a loss of SIRT3, a downstream target of PGC1 α (Finley et al., 2011) which signifies that signals that encourage biogenesis, such as starvation-induced PGC1 α activity function in part to inhibit ROS-induced HIF-1 stabilisation and its inhibitory effects on mitochondrial biogenesis.

One of the first processes to be de-regulated in mitochondrial biogenesis is mutation, either in nuclear or mitochondrial DNA. It has been shown the ubiquinol cytochrome c reductase core protein I (UQCRC1), a nuclear encoded component of mitochondrial complex III can impact upon mitochondrial morphology and/or physiology, and was

observed to be over expressed in ovarian cancer cells in experimental animals (Kunej et al., 2007). Yao et al. (2013) showed that acquisition of cisplatin resistant in A549 non-small-cell lung cancer cells was associated with a mutation in *MT-ND2*, encoding the mitochondrial Complex-I subunit ND2. This resulted in a 50% reduction in activity, compensated by increased electron transport chain complex biogenesis mediated by PGC1 β . Silencing of PGC1 β re-sensitised the cells to cisplatin. Kojima et al. (2008) showed in cisplatin resistant prostate cancer cells PC3, inhibition of SIRT1 with sirtinol, inhibited cell growth and increased sensitivity to cisplatin.

1.3.6 Sirtuin-3

Sirtuin-3 (SIRT3) is a member of the sirtuin family of proteins (SIRT1-7) who are human homologues of the silent information regulator 2 (Sir2) present in yeast. The 7 mammalian members of the sirtuin family all share a conserved catalytic core domain (Fig 1.9). In spite of this shared homology, sirtuins have deviating enzymatic activities with SIRT1-3 and 7 primarily as lysine deacetylases, SIRT4 as adenosinediphospho-(ADP)-ribosyltransferases, SIRT5 as deacetylase and deacylase and SIRT6 as ADP-ribosyltransferase and deacetylase (Imai et al., 2000, Frye, 2000, Morris, 2013). In addition to their divergent enzymatic activity their primary localisation also differs within the cells which explain the different functions of the various sirtuins. SIRT1, 6 and 7 localise to the nucleus, SIRT2 to the cytosol and SIRT3, 4 and 5 in the mitochondria

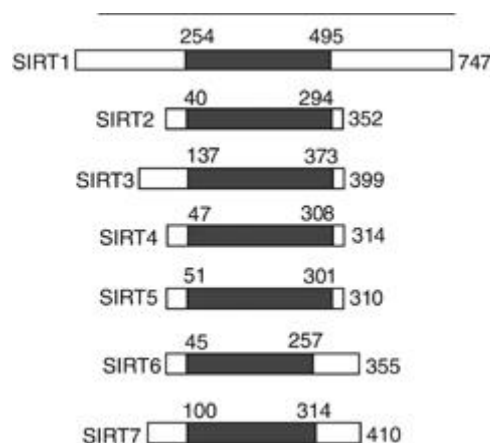


Fig 1.9 Sirtuin protein and catalytic domain. The amino-acid length of each mature main structure of sirtuins is presented at the end of each protein, with the conserved catalytic domain illustrated in grey. Adapted from (Roth and Chen, 2014).

Sirtuins have recently been revealed to play a role in carcinogenesis (Taylor et al., 2008). As a result of sirtuins various enzymatic activities, they have many cellular functions, such as regulation of oxidative stress, metabolism aging and longevity (Saunders and Verdin, 2007, Haigis and Guarente, 2006).

SIRT3 is a class III lysine deacetylase which primarily localizes to the mitochondrial matrix but SIRT3 can also be found in the nucleus (Scher et al., 2007). The SIRT3 proteins has two structures, processing of the N-terminus causes a smaller protein that localises to the mitochondria (Cooper et al., 2009). SIRT3 binds and deacetylates several metabolic enzymes that regulate important mitochondrial functions. Glutamate dehydrogenase, isocitrate dehydrogenase 2, Ku70, FOXO3a, electron transport chain complex I subunit NDUFA9 ATP synthase and succinate dehydrogenase are all reported to be SIRT3 substrates (Ahn et al., 2008, Jacobs et al., 2008, Sundaresan et al., 2008, Schlicker et al., 2008). SIRT3 has been shown to activate the adaptive thermogenesis program in response to cold stimulation in brown adipose tissue (Shi et al., 2005). In the heart, SIRT3 defends against cardiac hypertrophy through deacetylation of Foxo3a (Sundaresan et al., 2009). In the liver, SIRT3 is stimulated by fasting to deacetylate long chain acyl CoA dehydrogenase (LCAD) to augment fatty acid oxidation (Hirschey et al., 2010).

SIRT3 has reported to act like a tumour suppressor in numerous studies. One mechanism of tumour suppression is SIRT3'S modulation of ROS. SIRT3 has been shown to activate numerous antioxidant enzymes through deacetylation like superoxide dismutase, isocitrate dehydrogenase 2 and FOXO3a (Cheung et al., 2015, Someya et al., 2010, Sundaresan et al., 2009). In times of stress, SIRT3 expression is increased protecting cells from apoptosis. (Alhazzazi et al., 2011). It has been proposed that SIRT3 like SIRT1, may bind to the DNA repair protein Ku70, encouraging Ku70-Bax interactions, reducing apoptosis in cardiomyocytes (Sundaresan et al., 2008). SIRT3 has been shown to be necessary for Nampt, a stress and diet-responsive regulator of mitochondrial NAD⁺ levels, to support cell survival during genotoxicity (Yang et al., 2007). SIRT3 has also been shown to nullify p53 activity, thus preventing growth arrest and senescence in bladder carcinoma cells (Li et al., 2010). McGlynn et al. (2015) showed in an immunohistochemical analysis of pancreatic adenocarcinomas that low expression of SIRT3 was correlated with the more aggressive tumours and a shorter time to relapse and death, in the absence of chemotherapeutic intervention. Finley et al. (2011) discovered that about 20% of all human cancer samples and 40% of breast and ovarian cancer samples contain deletions of SIRT3.

In the context of anti-cancer drugs, Morigi et al. (2015) showed than SIRT3 had a reno-protective role in response to cisplatin treatment. In their murine model of acute kidney injury, pharmacological activation of SIRT3 counteracted cisplatin induced renal failure. When SIRT3 was down-regulated in oral squamous cell carcinoma cells, cell growth and proliferation was inhibited and sensitivity of oral squamous cell carcinoma cells to radiation and chemotherapy treatment was increased (Alhazzazi et al., 2011). Cheung et al. (2015) recently showed that in cardiomyocytes that SIRT3 over-expression attenuated doxorubicin's deleterious effects on basal metabolism and ROS. Also in this study they showed that resveratrol treatment caused an increase in SIRT3 expression and decreased doxorubicin's increased ROS production.

1.3.7 Resveratrol

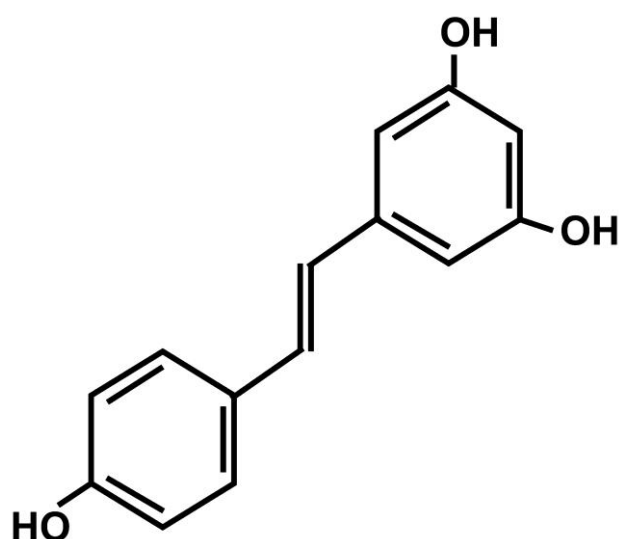


Fig. 1.10 Chemical structure of resveratrol (Chinembiri et al., 2014)

Resveratrol (3, 4', 5-trihydroxystilbene) (Fig 1.10) is a naturally occurring phytoalexin known to be present in approximately 70 plant species, including the skin of red grapes, berries and nuts (Patel et al., 2011, Thomasset et al., 2007, Jang et al., 1997). Due to its presence in red grapes, resveratrol has long been associated with the phenomenon of the "French paradox" (Catalgol et al., 2012). The term, arising from an epidemiological study showing that French people demonstrated low incidence of coronary diseases in spite of their high consumption of a diet high in saturated fat. Moderate red wine consumption was put forward as a possible reason due to its relatively high concentration of resveratrol (0.1-14.3 mg/l) (Pervaiz, 2003) and so resveratrol was introduced as a food supplement (Renaud and de Lorgeril, 1992). As well as resveratrol's well documented cardio protective effects mediated by its antioxidant properties (Ungvari et al., 2007, Olas and Wachowicz, 2002), resveratrol has also shown to exert anti-tumour activities (Jang et al., 1997). Resveratrol has been shown to effect multiple mechanisms of carcinogenesis such as cell cycle regulation, apoptosis (Fresco et al., 2006), proliferation (Nichenametla et al., 2006) as well as inhibition of key proteins in transduction pathways such as mitogen activated protein kinases (Catalgol et al., 2012). Resveratrol inhibits cyclooxygenase (COX) activity which has been shown to exhibit a role

in tumorigenesis by converting arachidonic acid to prostaglandins, inflammatory compounds that encourage tumour cell proliferation (Maccarrone et al., 1999). The exact mechanisms responsible for the effects of resveratrol are poorly understood.

In terms of resveratrol's effect on cisplatin resistance, Nessa et al. (2012) showed that resveratrol pre-treatment sensitised acquired cisplatin resistant ovarian cancer cells to cisplatin induced apoptosis. Resveratrol has been shown to have a protective role in inhibiting cisplatin-induced ototoxicity (Yumusakhuyulu et al., 2012), Resveratrol has also been shown to have additive inhibitory anticancer effects in ovarian cancer cells (Rezk et al., 2006). Osman et al. (2015) showed that resveratrol both increased cisplatin's cytotoxic effects and reduced cisplatin's side effect of nephrotoxicity in cisplatin treated animals.

The first concept to be elucidated before use of resveratrol clinically is how exactly resveratrol gets into the cell, as resveratrol is hydrophobic. The only clear proof of resveratrol entry into cells comes from highly specialised cell types that possess uptake mechanisms: intestinal and liver cells. In intestinal CaCo2 cells a dose dependent apical to basolateral transport was observed (Maier-Salamon et al., 2006). In human hepatoblastoma cell line HepG2 cells, results indicated that both passive diffusion and carrier-mediated transport may be involved in resveratrol's uptake. Further studies are necessary that directly address how exactly resveratrol gets into the cells. Another part of resveratrol's mechanism of action that needs to be uncovered is the direct targets of resveratrol. Some of the proposed effects of resveratrol may be cell specific but so far resveratrol has been shown to be involved in 127 pathways (Pirola and Fröjdö, 2008). Some studies have observed effects within seconds (Sareen et al., 2007) whereas as others are within a couple of hours or even days. This posits the question whether the observed effects are direct effects of resveratrol or downstream effects from the initial interaction. Several targets have been proposed but they have as yet to be convincingly and comprehensively shown (Pirola and Fröjdö, 2008).

1.3.8 Cancer stem cells (CSC)

Cisplatin often yields modest and often temporary beneficial anti-neoplastic effects due to the acquisition of resistance leading to drug relapse and treatment failure (Johansson et al., 2009, Locasale, 2012, Michels et al., 2013). More recently, there has been renewed interest in the contribution of CSC's to cancer heterogeneity and acquired resistance. The CSC model suggests certain malignancies are hierarchically constructed with a CSC population that portray the properties of tumour-initiating capacity and self renewal (Valent et al., 2012, Nguyen et al., 2012). Treatment failure has been attributed to cancer stem cells which are themselves intrinsically highly resistant to many therapeutic approaches by much of the same mechanisms (Valent et al., 2012). Evidence that CSC survive chemotherapy exposure has been well documented. As early as the 1960's, experimental proof showing CSC in haematological malignancies that survive chemotherapeutics and contribute to disease progression (Dick, 2008). Yu et al. (2007) showed that clinical specimens that were treated with chemotherapy exhibited higher proportions of mammosphere-forming and CD44+ (CSC surface marker) than chemotherapy naive specimens. Similarly xenografts treated with cisplatin resulted in the enrichment of CSC in non small cell lung cancer (Bertolini et al., 2009) and hepatocellular (Lee et al., 2011) carcinoma. The reported proportions of CSC vary between tumours from 0.1% to 30% depending on the type and advancement of the cancer (Adams and Strasser, 2008).

The distinct functional and molecular properties of CSC may represent therapeutic vulnerabilities that can be exploited for the development of novel combinational strategies. In pancreatic cancer, the Nodal-ALK4-SMAD4 axis was stated to control the self renewal capacity of CSC cells. Interestingly when combined with a stroma targeting hedgehog (role in development) inhibitor, the combination of the chemotherapeutic gemcitabine with the ALK inhibitor SB431542 significantly suppressed the growth of human primary pancreatic cancer xenografts. (Lonardo et al., 2011). Some studies have suggested that the TGF β family may stimulate CSC differentiation. Exposure of colorectal CSC's to BMP4 (CSC differentiation stimulant) resulted in differentiation, inhibition of

tumour-initiating capacity and significantly ameliorated efficacy of 5-fluorouracil and oxliplatin chemotherapy *in vivo* (Lombardo et al., 2011).

1.4 Objectives for the project

In this study we aim to;

- (a) Demonstrate the potential of phenformin and metformin as anti-cancer agents. Investigate the bioenergetics of the neuroblastoma cells in response to metformin and phenformin treatment with a view to discovering their mechanism of action.
- (b) Determine the metabolic changes behind acquired cisplatin resistance in the non small cell lung cancer cell line, H1299 and a mesothelioma cancer cell line, P31.

Chapter 2: Materials and Methods

2.0 Materials & Methods

2.1 Materials

2-deoxyglucose (2-DG)	Sigma Aldrich
5,5'-Dithiobis(2-nitrobenzoic acid)	Sigma Aldrich
ABCA5 Antibody	Abcam
ABCB6 Antibody	Novus Biologicals
ABCE1 Antibody	Novus Biologicals
Acetyl CoA Carboxylase antibody	Cell Signalling
Acetyl coenzyme a	Sigma Aldrich
Acetylated-Lysine	Cell Signalling
Alamar Blue Assay	Medical Supply Company
Aldeflour Kit	STEMCELL Technologies Inc.
Alexa-647- human IgMk Gb ₃ antibody	BD pharminogen
Ammonium Persulfate	Sigma Aldrich
AMPKa antibody	Cell Signalling
Anti mouse	Promega
Anti Rabbit	Promega
Antimycin A	Sigma Aldrich
APC annexin	Chemicon International
ATP-determination Kit	Invitrogen
BCA reagent A	Pierce
BCA reagent B	Pierce
Binding Buffer	Chemicon International
Bovine Serum Albumin	Fisher Scientific
Bromophenol blue	Sigma Aldrich
Cisplatin	Sigma Aldrich
Complete ULTRA tablets, Mini, EDTA-free	Roche
Diethylaminobenzaldehyde (DEAB)	STEMCELL Technologies Inc.
Digitonin	Sigma Aldrich
Dimethyl Sulphoxide (DMSO)	Sigma Aldrich
Dithiothreitol (DTT)	Sigma Aldrich

Dublecco's Phosphate Buffered Saline (PBS)	Gibco
Dulbucco's Modified Eagle Medium (DMEM) F12 + Nut Mix	Gibco
Ethanol (ETOH)	Hazard Materials, TCD
Ethylene Glycol Tetraacetic Acid (EGTA)	Sigma Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich
Fatty acid free BSA	Fisher Scientific
Fc block	BD pharminogen
FCCP	Sigma Aldrich
Fetal Bovine Syrum (FBS)	Gibco
FITC- Mouse IgG _{2a} anti-CD44	Abcam
FITC- Mouse IgG _{2a} anti-isotope control	Abcam
FITC-Mouse IgG _{2b} anti-CD133	Miltenyl
FITC-Mouse IgG _{2b} anti-isotope control	Miltenyl
Gamma tubulin	Novus Biologicals
Glucose	Sigma Aldrich
Glutamate	Sigma Aldrich
Glycerol	Sigma Aldrich
Glycine	Sigma Aldrich
Goat anti mouse conjugate	Sigma Aldrich
HEPES	Sigma Aldrich
Hoescht 33258	Sigma Aldrich
Hydrogen peroxide	Sigma Aldrich
Immobilon Transfer membrane	Sigma Aldrich
Immobilon Western Chemiluminescence HRP substrate	Millipore
Magnesium Chloride	Sigma Aldrich
Malate	Sigma Aldrich
Malonate	Sigma Aldrich
Mannitol	Sigma Aldrich
Metformin hydrochloride	Sigma Aldrich
Methanol	Hazard Materials, TCD
Modified Eagle's medium with Earle's salts	Lonza
N,N,N',N'-tetramethylethylenediamine (TEMED)	Sigma Aldrich
N-acetyl Cysteine	Sigma Aldrich

Oligomycin	Sigma Aldrich
Oxaloacetate	Sigma Aldrich
Passive Lysis Buffer	Promega
PE- human IgG2a MDR1 antibody	BD pharmingen
Penicillin Streptomycin (P/S)	Invitrogen
PGC1 α	Cell Signalling
PGC1 α Antibody	Novus Biologicals
Phenformin Hydrochloride	Sigma Aldrich
Phospho-Acetyl CoA Carboxylase antibody	Cell Signalling
Phospho-AMPK α	Cell Signalling
Poly (ADP-ribose) polymerase (PARP)	Calbiochem
Potassium Phosphate	Sigma Aldrich
Propidium iodide (PI)	Chemicon International
Propidium iodide (PI)	Life technologies
Resveratrol	Sigma Aldrich
RNase A	Sigma Aldrich
Rotenone	Sigma Aldrich
SIRT1	Cell Signalling
SIRT1 Antibody	Cell Signalling
SIRT3	Cell Signalling
SIRT3 Antibody	Cell Signalling
Sodium Butyrate	Sigma Aldrich
Sodium Chloride	Sigma Aldrich
Sodium dodecyl Sulfate	Sigma Aldrich
Sodium Fluoride	Sigma Aldrich
Sodium pyrophosphate	Sigma Aldrich
Succinate	Sigma Aldrich
Sucrose	Sigma Aldrich
TFAM	Cell Signalling
TFAM Antibody	Cell Signalling
Triton X-100	Sigma Aldrich
Trizma base	Sigma Aldrich
Trypsin-EDTA solution 0.25%	Gibco

Tween 20	Sigma Aldrich
Urea	Sigma Aldrich
Verapamil	Sigma Aldrich
Verotoxin-1	List Laboratories
XF Assay Medium modified (DMEM)	Seahorse Bioscience
XF Calibrant	Seahorse Bioscience
XF24 Cell Culture Micro-plate	Seahorse Bioscience
XF24 Extracellular Flux Assay Kit	Seahorse Bioscience
X-ray film	Fuji

2.2. Methods:

2.2.1. Cell culture protocols

Five cancer cell lines were used during this thesis, a solid neuroblastoma cell line SH-SY5Y, a non small lung cancer parental sensitive cell line H1299s, the resistant cell line H1299r, a mesothelioma parental sensitive cell line P31s and the resistant mesothelioma cell line P31r. The SH-SY5Y cells were sourced from the American Type Culture Collection (ATCC) and the P31 & H1299 cells were obtained from Parviz Motlagh's laboratory in Umeå University, Sweden. The SH-SY5Ys were maintained in DMEM F12 + Nut Mix medium supplemented with 2 % (w/v) penicillin streptomycin and 10% FBS. The H1299s and P31s cells were maintained in MEM medium supplemented with 2 % (w/v) penicillin streptomycin and 10% FBS. H1299r cells were maintained in MEM medium supplemented with 2 mg/L of cisplatin, 2 % (w/v) penicillin streptomycin and 10% FBS. P31r cells were maintained in MEM medium supplemented with 1.2 mg/L of cisplatin, 2 % (w/v) penicillin streptomycin and 10% (v/v) FBS.

All cell lines were thawed from 1ml stocks by gently agitating the vial in a 37°C water bath (Grant). The thawed SH-SY5Y cells were spun down at 1,300 rpm for 5 min (Sorvall T centrifuge), resuspended in 1 ml of complete DMEM F12 + Nut Mix medium and then placed in 25cm² cell culture bottomed flasks which contained complete DMEM F12 + Nut Mix cell culture media. The thawed H1299 & P31 cells were pelleted by centrifugation at 1,200 rpm, resuspended in 1 ml of complete MEM medium and then

placed in 25cm² cell culture bottomed flasks which contained complete MEM cell culture media and put into the incubator at 5% CO₂ and 37°C (Steri Cycle CO₂, Thermo Electron Corporation). The media was refreshed the following day. The cells were allowed to grow to 80% confluence before they were transferred to a 75cm². After three passages, the medium of the H1299r and P31r cells were supplemented with 2 mg/L and 1.2 mg/L of cisplatin respectively.

All cells were passaged with the aid of trypsin-EDTA (0.05mg/ml). Briefly, the old cell media was removed, cells were washed with 3-5 ml of PBS and were then removed from the flask surface by incubation with 3-5 ml of trypsin-EDTA for 5mins at 37°C. 4-6 ml of complete medium was added to the cells to neutralise the trypsin.

They were then transferred to a 30 ml universal tube and centrifuged at 1200 rpm for 5min. After centrifugation the supernatant was taken off and the cells were resuspended in 10 ml of complete medium, prior to being placed in the appropriate tissue culture flask. 75cm² flasks contained in total 10ml of complete DMEM F12 + Nut Mix for SH-SY5Y cells, 10 mls of MEM for H1299s & P31s cells, 10 mls of MEM supplemented with 2 mg/L of cisplatin for H1299r cells and 10 ml of MEM supplemented with 1.2 mg/L of cisplatin for P31r cells.

For storing the cells, SH-SY5Y cells from confluent 75cm² flasks were pelleted by centrifugation, re-suspended in 3 ml of freezing media containing 60% tissue culture media, 30 % (v/v) FBS and 10 % (v/v) Dimethyl Sulphoxide (DMSO) and then placed in special cryovials. H1299 and P31 cells from confluent 175cm² flasks were pelleted by centrifugation, re-suspended in 1 ml of media and then placed in a special cryovial. The cryovials were placed overnight at -80°C and transferred the following day to a liquid nitrogen storage tank for long-term storage.

Cell counting was undertaken using a haemocytometer (Bright line, Sigma Aldrich) and a bright light microscope (Leica). 10 µl of cell suspension was mounted on a haemocytometer and the number of cells within the 4x16 boxes of the haemocytometer was determined and then averaged. The number of cells per ml was obtained by

multiplying the number of cells counted by the dilution factor of the cells in 10 ml of media and then by the conversion factor of 10^4 , which corresponds to one 1 mm^2 haemocytometer square containing 10^{-4} ml of solution.

2.2.2. Transfection optimisation of SH-SY5Y cells with NADH dehydrogenase

SH-SY5Y cells were transfected at a cell density of 2.2×10^5 cells /ml using the NEON electroporation system (Invitrogen, Paisley, UK) with $1 \mu\text{g}$ with control pAAC.MCS vectors (containing green fluorescent protein (GFP)) and were plated in correspondingly labelled wells of a 6 well plate. The NDI1 plasmids were a kind gift from Professor G. Jane Farrar from the School of Genetics and Microbiology, Trinity College Dublin. Transfection settings were optimised to ensure maximal expression of the GFP containing plasmid; 12 conditions were analysed to determine the optimum pulse voltage, pulse width and pulse number as described in Table 2.1.

The following day images were taken on an Olympus IX81 inverted microscope at 20x magnification excited at a wavelength of 395 nm using FV10-ASW 2.0 Viewer software

Setting no.	Pulse voltage	Pulse width	Pulse no.
3	1500	20	1
5	1700	20	1
7	1200	30	1
9	1400	30	1
11	1100	40	1
13	1100	20	2
15	1300	20	2
17	850	30	2
19	1050	30	2
21	1300	10	3

23	1500	10	3
24	1600	10	3

Table 2.1 Conditions for Neon transfection optimisation

2.2.3. Transduction optimisation of H1299s and H1299r cells with GFP tagged empty vector adenovirus

H1299 cells were plated at concentration of 1.0×10^5 cells /ml in 6 well plates and left to incubate overnight. The following day the cells were transduced with a range of concentrations of GFP tagged empty vector adenovirus (0.1-100 MOI) for 24 and 48h. The adenovirus constructs were a kind gift from Assistant Professor Vernon Dolinsky from the University of Manitoba, Canada. Transduction of the adenovirus was optimised for the maximum concentration of virus per cell or MOI for maximal expression of the GFP plasmid. After the time had elapsed images were taken on an Olympus IX81 inverted microscope at 20x magnification excited at a wavelength of 395 nm using FV10-ASW 2.0 Viewer software.

2.2.4 Alamar Blue

2.2.4.1 Viability Assay

2.2.4.1.1 SH-SY5Y cells

The cells were plated on day 0 in a 96 well plate at a concentration of 3×10^4 cells /ml in 200µl of media and left to incubate overnight. Concentrations of metformin (10-20,000 µM) and phenformin (0.1-10000 µM) were made up in eppendorfs using serial dilutions. On day 1, 8 µl of phenformin and 20 µl of metformin were placed in the appropriate wells.

2.2.4.1.2 H1299s, H1299r, P31s and P31r cells

Cells were plated on day 0 in a 96 well plate at a concentrations ranging from of 2×10^3 cells /ml- 2×10^4 cells /ml in 200µl of medium or medium + 2 mM sodium butyrate and left

to incubate overnight. Concentrations of cisplatin (50 nM -30mM), verotoxin (0.001-1 µg/l) and verapamil (100 µM) were made up in minifuge tubes using serial dilutions. On day 1, 2 µl of cisplatin and verotoxin-1 and 1 µl of verapamil were placed in the appropriate wells.

2.2.4.1.3 Viability Assay Protocol

The plate was allowed to incubate for 72 h. On day 4, 20µl of alamar blue was pipetted into each well the plate was covered in tin foil and was left to incubate for 3-5 h. After the time the plate was then analysed using Spectra Gemini microplate reader at 544 nm with a reference wavelength of 590 nm. The data obtained from the microplate reader was then analysed by the Graph Pad software, which calculates the IC₅₀.

2.2.4.2 Proliferation assay on H1299s, H1299r, P31s and P31r cells

The cells were plated on day 0 in a 96 well plate at a concentration of 2×10^4 cells /well in 200µl of media and left to incubate overnight. The plate was allowed to incubate for 72 h. On day 4, 200 µl of the respective media was put into three blank wells and then 20µl of alamar blue was added into each well, the plate was covered in tin foil and was left to incubate for 3-5 h. After the time the plate was then analysed using Spectra Gemini microplate reader at 544 nm with a reference wavelength of 590 nm. The data obtained from the microplate reader was then represented as relative fluorescence units.

2.2.5. Flow Cytometry

2.2.5.1 Cell Cycle Analysis

2.2.5.1.1 Drug Dose Response

On day 0, SH-SY5Y cells were plated in a 6 well plate at a concentration of 1.1×10^5 cells/well. On day 1, 80 µl of 0.1, 0.5, 1, 2 and 5 mM concentrations of phenformin were placed in their respectively labelled wells and incubated for 72 h. After the time had elapsed, 30 ml universal tubes were labelled corresponding to each well that was to be fixed. The media was removed from the wells and placed in their respective tubes. Wells

were washed with PBS, removed and placed into the same falcons as the media. Cells were trypsinised, allowed to incubate at 37°C and then resuspended in fresh media. This was then placed in the corresponding tube to be fixed for cell cycle analysis.

2.2.5.1.2. Time Course

On day 0, SH-SY5Y cells were plated in a 5x 6 well plate at a concentration of 1.1×10^5 cells/ well. On day 1, 80 µl of 1 mM concentration of phenformin were placed in their respectively labelled wells and incubated for 8, 16, 24, 48 and 72 h. After the times had elapsed, 30 ml universal tubes were labelled corresponding to each well and the time for which the cells had been incubated for. The media was removed from the wells and placed in their respective tubes. Wells were washed with PBS, removed and placed into the same falcons as the media. Cells were trypsinised, allowed to incubate at 37°C and then resuspended in fresh media. This was then placed in the corresponding tube to be fixed for cell cycle analysis.

2.2.5.2.1 Cell Cycle Analysis Protocol

Cells were pelleted (2,400 rpm, for 5 minutes) and resuspended in 500 µl of PBS. Cells were permeabilised and fixed with cold 70% EtOH such that to 1 ml of cell/PBS suspension : 2.5 mls EtOH is added drop by drop while the tubes were gently vortexed and was stored overnight at 4°C. Cells were pelleted and then 400 µl of PBS was used to resuspend the cells. 25 µl of RNase A and 75 µl of PI were added to the tubes. The

tubes were briefly vortexed then stored at 4°C for three h or put into an incubator at 37°C for 30 minutes. When the incubation time had elapsed the samples were analysed by flow cytometry (FACS Calibur II, BD Biosciences).

2.2.5.2.2 Cell Death Analysis

On day 0, SH-SY5Y cells were plated in a 6 well plate at a concentration of 1.1×10^5 cells/ well. On day 1, 80 µl of 0.1, 0.5, 1, 2 and 5 mM concentrations of phenformin were placed in their respectively labelled wells and incubated for 72 h. After the time had elapsed, 30 ml universal tubes were labelled corresponding to each well that was to be

fixed. The media was removed from the wells and placed in their respective tubes. Wells were washed with PBS, removed and placed into the same falcons as the media. Cells were trypsinised, allowed to incubate at 37°C and then resuspended in fresh media. Cells were pelleted (2,400 rpm, for 5 min) and were resuspended in 1 ml of ice cold PBS. Cells were counted and then pelleted. Supernatant was taken off and were resuspended in 1 x binding buffer and were then transferred to the flow tubes on ice. 5 µl of APC annexin and 3 µl of 100 x PI were added to the tubes in the dark on ice. They were left at room temperature for 15 min and then brought straight to the FACS machine (CYAN ADP Analyser, Beckman Coulter).

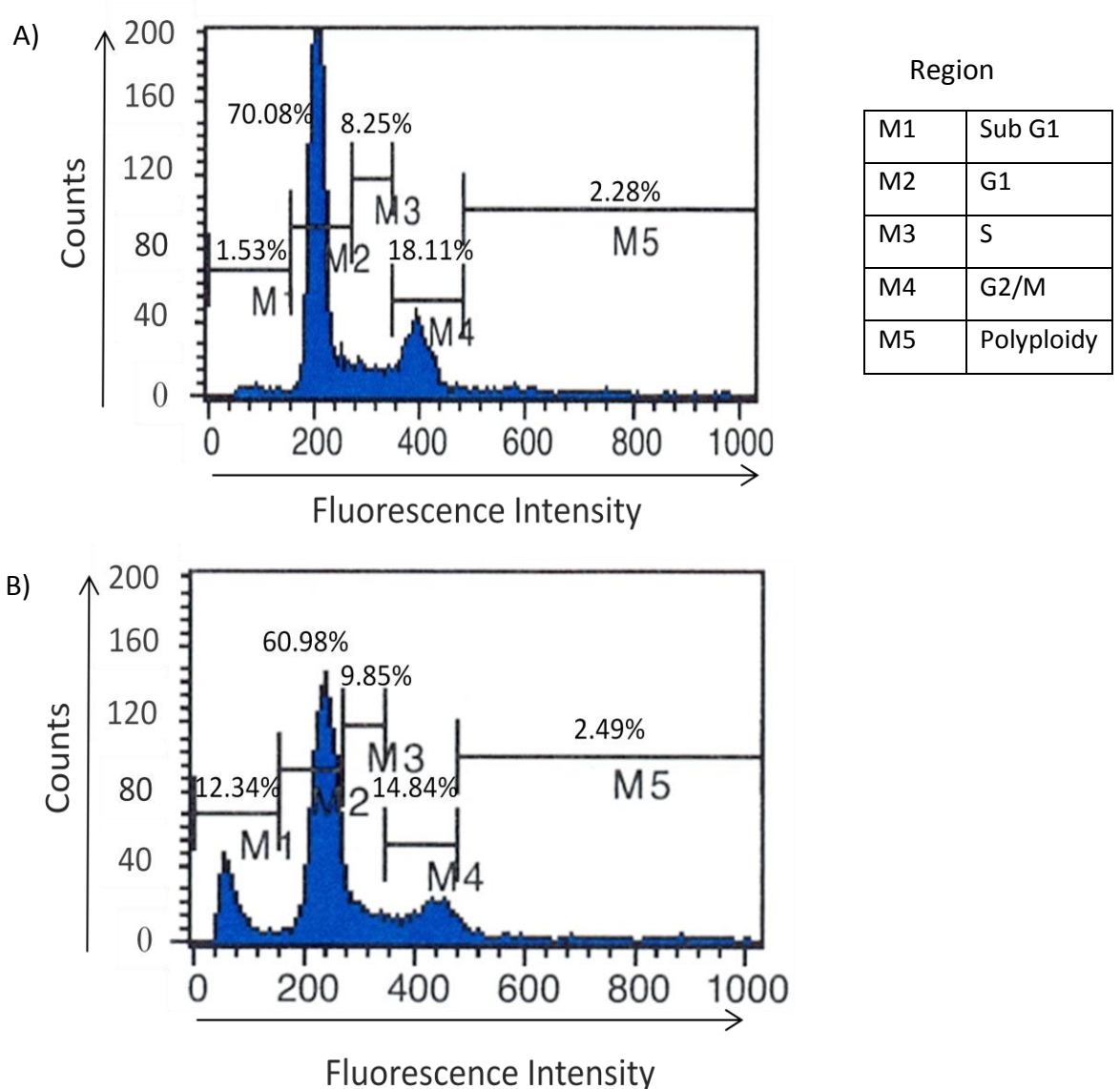


Figure 2.1 Cell Cycle Analysis by Propidium Iodide staining using flow cytometry. Graphical analysis for PI cell cycle analysis using flow cytometry. A) Histogram analysis of a representative population of healthy cells, differentiated by DNA content/cell cycle phase. Defined peaks represent the percentage of cells occupying the G1, S and G2/M phases of the cell cycle. B) Induction of apoptosis is represented by an increase in the sub G1 population in histogram analysis of PI flow cytometry.

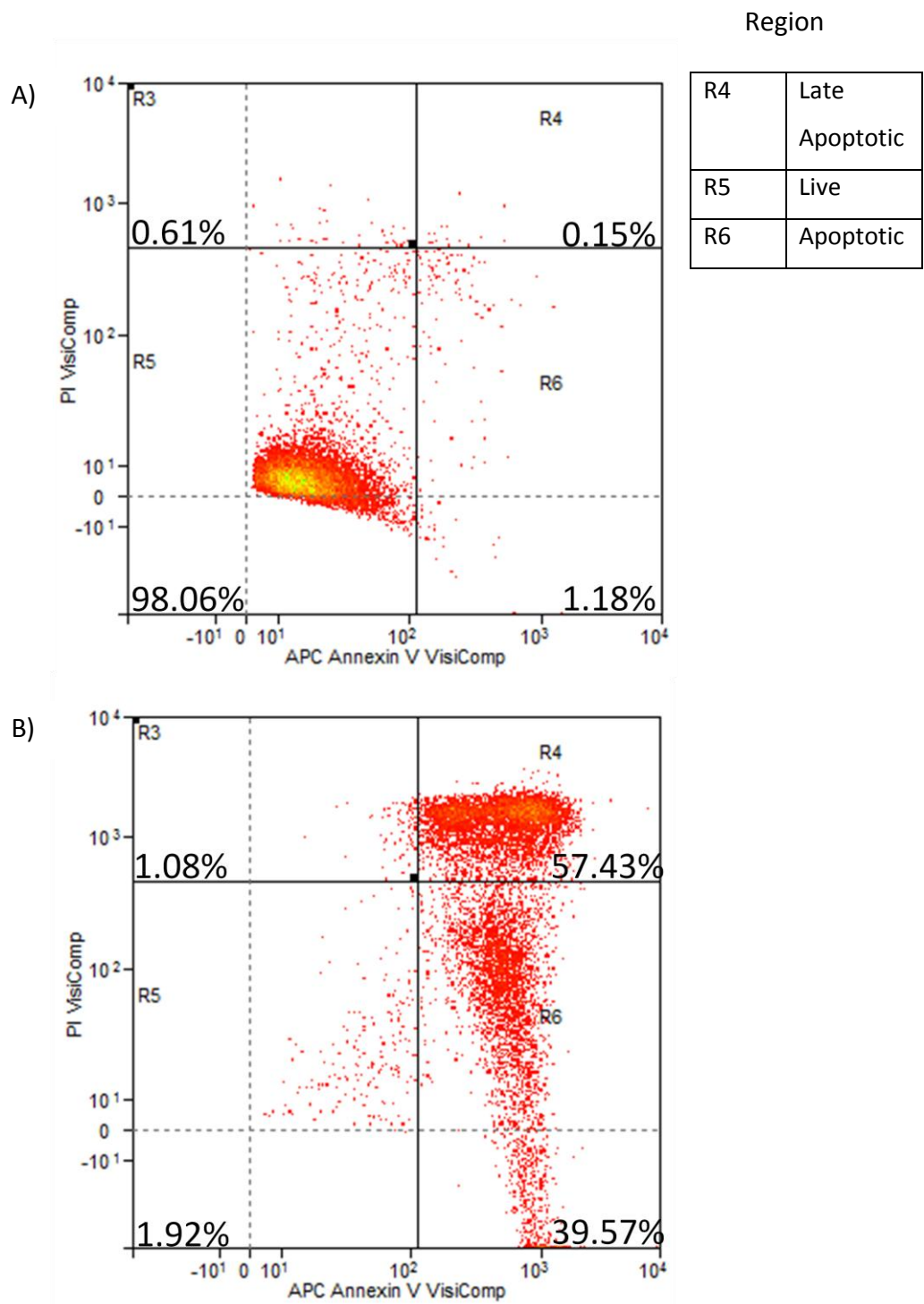


Figure 2.2 Cell Death Analysis by Propidium Iodide and Annexin V staining using flow cytometry. Graphical analysis for cell death analysis using flow cytometry. Defined quadrants represent the percentage of cells that are alive, apoptotic or are necrotic. A) Dot plot analysis of a representative population of healthy cells, differentiated by staining. B) Dot plot analysis of a representative population of unhealthy cells, differentiated by staining.

2.2.5.3. Intracellular ROS measurement

2.2.5.3.1 SH-SY5Y cells

On day 0, cells were plated in a 6 well plate at a concentration of 1.1×10^5 cells/ well. On day 1, 80 μ l of 0.05-1 mM of phenformin were placed in their respectively labelled wells and incubated for 6 h.

2.2.5.3.2 H1299s, H1299r, P31s and P31r cells

On day 0, cells were plated in a 6 well plate at a concentration of 2.5×10^5 cells/ well. On day 1, 50 μ M of resveratrol was placed in its respectively labelled wells and incubated for 24 h or transduced with 50 MOI of empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus for 48 h.

2.2.5.3.3 Intracellular ROS measurement protocol

After the time had elapsed, 30 ml universal tubes were labelled corresponding to each well that was to be fixed. The media was removed from the wells and placed in their respective tubes. Cells were then washed with warm PBS. Pre-warmed PBS was placed on the cells containing 10 μ M DCFH-DA and incubated for 30min at 37°C. Cells were then harvested by trypsinisation and pelleted by centrifugation and resuspended in ice cold PBS. They were transferred to flow tubes and then brought straight to the FACS machine (CYAN ADP Analyser, Beckman Coulter).

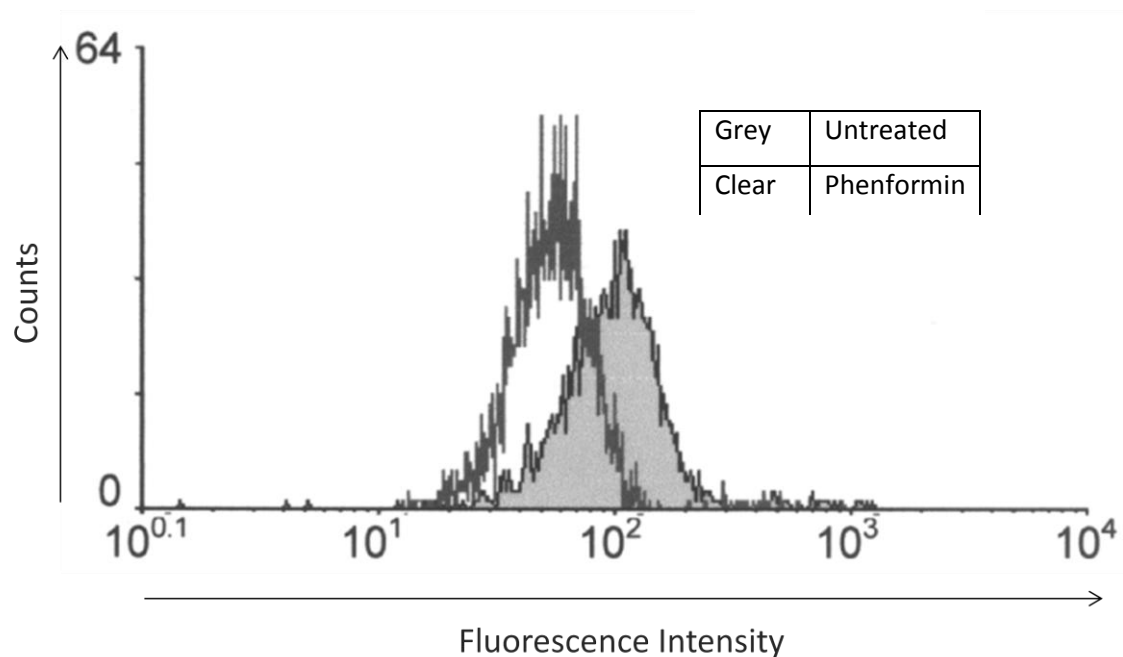


Figure 2.3 Intracellular ROS determination by DCFA-HA staining using flow cytometry. Graphical analysis for intracellular ROS determination using flow cytometry. Histogram analysis of a representative population of untreated cells and phenformin treated cells, differentiated by fluorescence intensity.

2.2.5.4 Cell surface expression

2.2.5.4.1 Gb₃

On day 0, H1299s, H1299r, P31s and P31r cells were plated at a concentration of 2.5×10^5 cells/well. The following day the cells were then harvested by trypsinisation and pelleted by centrifugation. Cells were resuspended in media and pelleted by centrifugation. Pellet was resuspended in 100 μ L of FACS buffer [DPBS, 18% (v/v) RPMI, 2% FBS] containing Fc Block (0.5 μ L per sample) and were left untreated or treated with 5 μ L of N,N-diethylaminobenzaldehyde (DEAB). The cells were incubated at 4°C for 10 min. Cells were stained with 2.5 μ L of Alexa-647-labelled mouse anti-human IgMk Gb₃ antibody (clone 5B5) and incubated for 20 min at 4 or 25 or 37°C in the dark by being covered with tinfoil. Unstained sample served as the control for this experiment. This was followed by adding 4 ml of FACS buffer to the flow cytometry tubes, centrifuging the

tubes at 1,200 rpm for 5 min and decanting the supernatant. Cells were resuspended in 200 μ L of FACS buffer. Samples were then analysed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

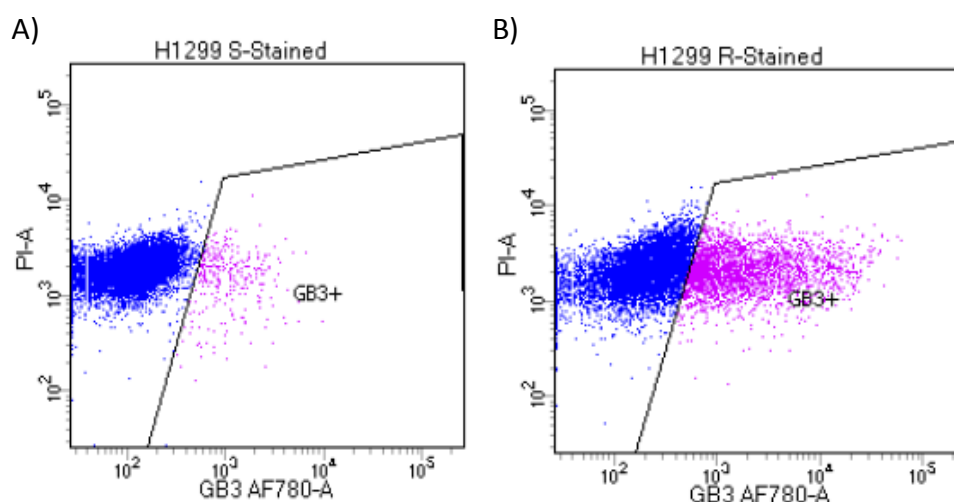


Figure 2.4 Gb₃ expression by Alexa-647 staining using flow cytometry. Graphical analysis for Gb₃ expression using flow cytometry. Dot plot analysis of a representative population of (A) H1299s and (B) H1299r Gb₃ expression gates based on unstained control.

2.2.5.4.2 ABCB1/MDR1

On day 0, H1299s, H1299r, P31s and P31r cells were plated at a concentration of 2.5×10^5 cells/well. The following day the cells were then harvested by trypsinisation and pelleted by centrifugation. Cells were resuspended in media and pelleted by centrifugation. Pellet was resuspended in 100 μ L of FACS buffer [DPBS, 18% (v/v) RPMI, 2% FBS] containing Fc Block (0.5 μ L per sample) and were incubated at 4°C for 10 min. Cells were stained with 2.5 μ L of phycoerythrin (PE)-labelled mouse anti-human IgG2a MDR1 antibody (clone UIC2) and incubated for 20 min at 4°C in the dark by being covered with tinfoil. Unstained sample served as the control for this experiment. This was followed by adding 4 ml of FACS buffer to the flow cytometry tubes, centrifuging the tubes at 1,200 rpm for 5 min and decanting the supernatant. Cells were resuspended in 200 μ L of FACS buffer. Samples were then analysed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

2.2.5.4.3 CD44 and CD133

On day 0, H1299s, H1299r, P31s and P31r cells were plated at a concentration of 2.5×10^5 cells/well. The following day the cells were then harvested by trypsinisation and pelleted by centrifugation. Cells were resuspended in media and pelleted by centrifugation. Pellet was resuspended in 90 μ L of FACS buffer [DPBS, 18% (v/v) RPMI, 2% FBS]. Cells were stained with 10 μ L of Fluorescein isothiocyanate (FITC)-labelled CD44 and CD133 antibodies (Table 2.2) and their isotope controls. Cells were incubated for 30 min at 4°C in the dark by being covered with tinfoil. This was followed by adding 4 ml of FACS buffer to the flow cytometry tubes, centrifuging the tubes at 1,200 rpm for 5 min and decanting the supernatant. Cells were resuspended in 200 μ L of FACS buffer. Samples were then analysed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

Antigen	Antibody	Clone	Fluorescent Conjugate	Source
CD44	Mouse IgG _{2a} anti-CD44	F10-44-2	FITC	Abcam
	Mouse IgG _{2a} isotope	X5563	FITC	Abcam
CD133	Mouse IgG _{2b} anti-CD133	293C3	FITC	Miltenyl
	Mouse IgG _{2b} isotope	I56-11E5.11	FITC	Miltenyl

Table 2.2 Antibodies used for CD44 and CD133 expression

2.2.5.4.4 Hoescht side population (HSP) assay

On day 0, H1299s, H1299r, P31s and P31r cells were plated at a concentration of 2.5×10^5 cells/well. The following day the cells were then harvested by trypsinisation and pelleted by centrifugation. Cells were resuspended in media and pelleted by centrifugation. Pellet was resuspended in DMEM+ (DMEM supplemented with 2 % (v/v) FBS and 10 mM HEPES) pre-warmed to 37 °C. Cells were treated with 5 µg/ml Hoescht 33258 and allowed to incubate at 37 °C for 60 min. Control samples were pre-treated with 50 µM verapamil. After incubation, the cells were transferred centrifuged at 170 x g for 5 min at 4 °C. This pellet was resuspended in ice cold HBSS+ and transferred to Flow Cytometry tubes. HBSS+ consists of Hanks Balanced Salt Solution supplemented with 2 % (v/v) FBS and 10 mM HEPES. After samples were resuspended in HBSS+, they were placed on ice and analysed on a Cyan ADP flow cytometer (Beckman Coulter).

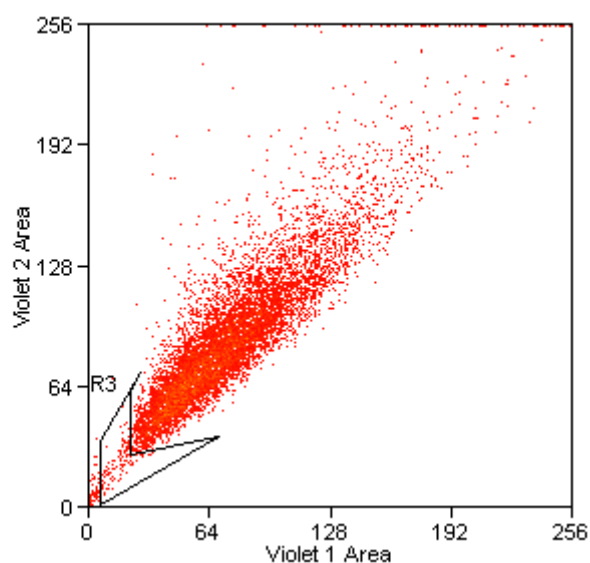


Figure 2.3 HSP determination using flow cytometry. Graphical analysis for HSP determination using flow cytometry. Dot plot analysis of a representative plot to demonstrate the gating strategy for HSP differentiated by pre-treatment with verapamil as the negative control.

2.2.5.4.4 Aldehyde dehydrogenase (ALDH) assay

The assay is based on BODIPY-aminoacetaldehyde (BAAA), a fluorescent non-toxic substrate for ALDH, which freely diffuses into intact and viable cells. In the presence of ALDH, BAAA is converted into BODIPY-aminoacetate (BAA), which is kept inside the cells. The amount of fluorescent reaction product is proportional to the ALDH activity in the cells and is measured using a flow cytometer. A specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), is used to control for background fluorescence.

On day 0, H1299s, H1299r, P31s and P31r cells were plated at a concentration of 2.5×10^5 cells/well. The following day the cells were then harvested by trypsinisation and pelleted by centrifugation. The ALDH assay (STEMCELL Technologies Inc. Vancouver, Canada) was then conducted as per manufacturer's instructions. Samples were analysed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA).

2.2.6. Oxygen Consumption Rate and pH Analysis by the Seahorse Extracellular Flux Analyser.

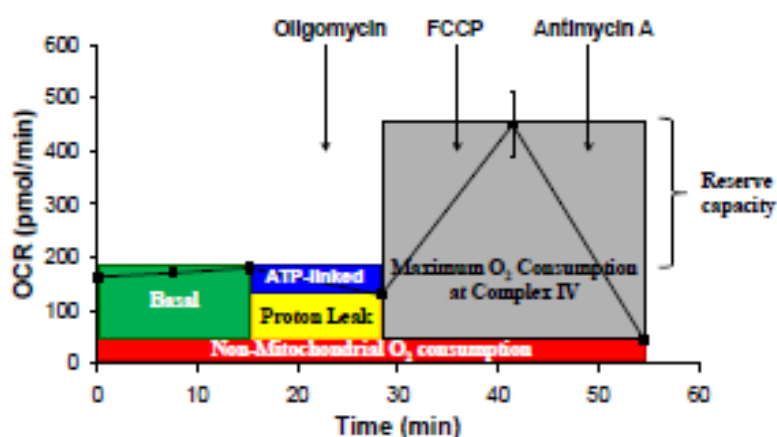


Figure 2.4 Measurement of mitochondrial function by the seahorse extracellular flux analyser.

2.2.6.1. Preparation Step 1-Plating of the wells

2.2.6.1.1 SH-SY5Y, H1299s, H1299r, P31s and P31r cells

Cells were plated onto a XF24 cell culture micro-plate with the indicated cell densities in 100 μ l volumes, leaving four wells (A1,B4,C3 & D6) empty for machine back ground correction. When the cells attached, 150 μ l of media was placed in the wells. The plate was then left overnight at 37°C in the CO₂ incubator.

2.2.6.1.2 SH-SY5Y cells transfected with NDI1

NDI1 and control pAAC.MCS vectors (containing GFP) were transfected into SHSY5Y cells using the NEON electroporation system (Invitrogen, Paisley, UK). Transfection conditions were previously optimised for maximum expression of the plasmid. Cells were plated at 4×10^5 cells/ml in the seahorse plates, incubated for 48 hours and treated with a range of concentrations of phenformin (0.1-0.5 mM) for 6 hours.

2.2.6.1.2 SH-SY5Y cells permeabilized with digitonin.

2.2.6.1.2.1 Optimisation

Cells (8×10^5 /ml) were plated in the seahorse plates and incubated overnight. 30 min before the assay, the DMEM F12 media was then changed to mitochondrial assay buffer (220 mM mannitol, 70 mM sucrose, 10 mM KH₂PO₄, 5 mM MgCl₂, 2 mM HEPES, 1.0 mM EGTA, and 0.2% (w/v) fatty-acid free BSA, pH 7.4), supplemented with 4 mM ADP and permeabilized with a range of concentrations of digitonin (10-70 μ g/ml). Basal rates were measured then complex I substrates (glutamate 5 mM/malate 5 mM) was added and the increase in OCR was noted.

2.2.6.1.2.2 Analysis

Cells (8×10^5 /ml) were plated in the seahorse plates and incubated overnight. Half of the SHSY5Y cells were then incubated with 100 μ M phenformin 6 h. 30 min before the assay, the DMEM F12 media was then changed to mitochondrial assay buffer (220 mM Mannitol, 70 m M Sucrose, 10 mM KH₂PO₄, 5 mM MgCl₂, 2 mM HEPES, 1.0 mM EGTA,

and 0.2% (w/v) fatty-acid free BSA, pH 7.4), supplemented with 4 mM ADP and permeabilized with digitonin. Concentrations of digitonin were previously optimised. Basal rates were measured, complex I substrates (glutamate 5 mM/malate 5 mM) and complex II substrate (succinate 5 mM) were added and the increase in OCR was noted.

2.2.6.1.3 H1299s, H1299r, P31s and P31r cells treated with resveratrol

Cells (1.5×10^4) in 100 μ l volumes were plated in the seahorse plates and incubated overnight. Cells were then transduced with 50 μ M resveratrol for 24h.

2.2.6.1.4 H1299s, H1299r, P31s and P31r cells transduced with SIRT3 expressing adenovirus

Cells (1.0×10^4) in 100 μ l volumes were plated in the seahorse plates and incubated overnight. Cells were then transduced with 50 MOI of empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus for 48h.

2.2.6.2. Preparation Step 2-Cartridge and Machine Preparation

The cartridge is taken out of its package and 1 ml of XF calibrant is put into the utility plate that comes with the cartridge to hydrate the sensors. The cartridge is placed in the non-CO₂ incubator (ALC non CO₂, Medical Supply Company) the night before the plate is to be run. The seahorse machine (XF 24-3, Seahorse bioscience) is turned on and the program that is to be run the following day is opened on the system so that the machine warms up to 37°C for use the following day.

2.2.6.3. Preparation Step 3-Loading of the injection ports and preparation of the cell culture plate.

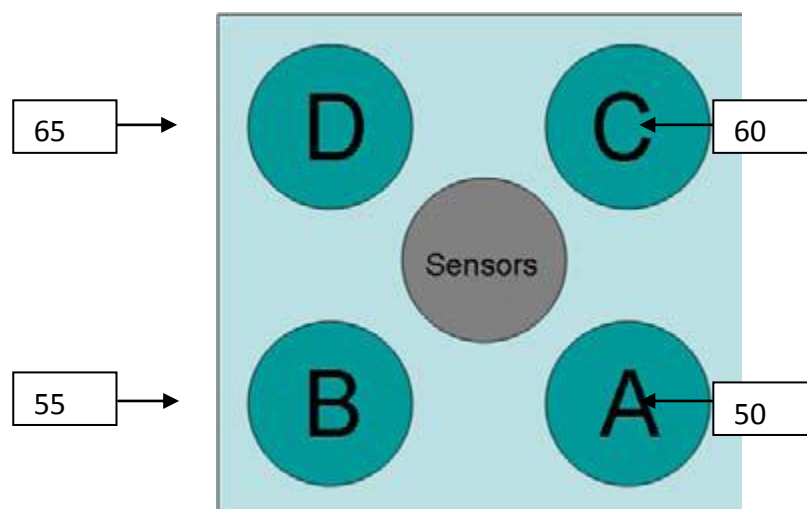


Figure 2.3 Injection Port Layout of the Cartridge.

The cell culture plate is taken out of the CO₂ incubator and 200 µl of the media is titrated off. 450 µl of XF Assay Medium Modified DMEM supplemented with glucose concentration of the growth media is placed on the cells to add up to a volume of 500 µl of media. The plate is then put in the non-CO₂ incubator for an hour. 1.5 ml stock solutions of the mitochondrial inhibitors: oligomycin (1-4 µM), FCCP (0.5-2 µM) and Antimycin A (1-4 µM) are made up in the media in which the cells are being incubated in. The compounds are then loaded into their correspondingly labelled port (Figure 2.3) on the program in increasing volumes to account for the change in volume in the wells after each injection. After the ports have been loaded the cartridge is put back in the non-CO₂ incubator for a few minutes to warm up to 37°C. The cartridge is then loaded into the machine to be calibrated. After approximately 30 min, when the calibration is completed, the utility plate of the cartridge is replaced with the cell culture plate and it is loaded into the machine.

2.2.7. Oxygen Consumption Rate Analysis of the SH-SY5Y cells by the Oroboros Oxygraph respirometer.

2.2.7.1 SH-SY5Y cells

2.1 mls of the DMEM F12 + Nut Mix media is added to the two chambers for calibration by the machine (Oxygraph 2k, Oroboros). After 30 min of calibration had elapsed, 100 µl of media was taken out of the chambers and 1 million cells in 100 µl volume were added to the two chambers. Once the rates had plateaued, oligomycin, FCCP and antimycin A were added to the cells by injection as per the manufacturer's instructions.

2.2.7.2 Optimization of SH-SY5Y cell permeabilization with digitonin

2.1 ml of mitochondrial assay buffer (220 mM Mannitol, 70 mM Sucrose, 10 mM KH_2PO_4 , 5 mM MgCl_2 , 2 mM HEPES, 1.0 mM EGTA, and 0.2% (w/v) fatty-acid free BSA, pH 7.4), supplemented with 4 mM ADP and 10 mM succinate is added to the two chambers for calibration by the machine (Oxygraph 2k, Oroboros). After 30 min of calibration had elapsed, 100 µl of media was taken out of the chambers and 100 µl of 1 million cells were added to the two chambers. Once the traces had stabilised,, increasing concentrations of digitonin (10-70 µg/ml) were added to the cells by injection in 10 µg/ml increments.

2.2.8. ATP Determination

On day 0, SH-SY5Y cells were plated in a 96 well plate at a concentration of 3.0×10^4 cells/ml. On day 1, 8 µl of 0.05-1 mM phenformin was placed in the respective wells for 6 h. After the elapsed time, the supernatant was removed and 100 µl of passive lysis buffer (Promega) was added to the wells. The plate was allowed to shake for 15 min. 10 µl of the lysate was added to the reaction mixture and the assay was carried out as per manufacturer's instructions.

2.2.9. Citrate Synthase assay

2.2.9.1 H1299s, H1299r, P31s and P31r cells.

On day 0. H1299 and P31 cells were plated in 25cm² flasks at a concentration of 4.0×10^5 cells/flask. On day 1, media is removed and 100 µl of Tris (200 mM, pH 8.0) with Triton X-100 (0.2% (v/v)) is added to the flasks on ice

2.2.9.2 H1299s and H1299 cells treated with resveratrol

On day 0. H1299 cells were plated in 25cm² flasks at a concentration of 4.0×10^5 cells/flask. On day 1, cells are treated with 50 µM resveratrol. On day 2, media is removed and 100 µl of Tris (200 mM, pH 8.0) with Triton X-100 (0.2% (v/v)) is added to the flasks on ice.

2.2.9.3 H1299s and H1299r cells transduced with SIRT3 expressing adenovirus

On day 0. H1299 cells were plated in 25cm² flasks at a concentration of 4.0×10^5 cells/flask. On day 1, cells are transduced with 50 MOI of empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus. On day 3, media is removed and 100 µl of Tris (200 mM, pH 8.0) with Triton X-100 (0.2% (v/v)) is added to the flasks on ice.

The flasks were left shaking at room temperature for 15 minutes. The lysate is scraped and then transferred to a labelled eppendorf. The minifuge tubes were centrifuged at 14,000 rpm at 4°C for 10 min (Sorvall 5417R, Eppendorf) to yield a lysate pellet. Protein content was measured using the BCA assay.

1 ml cuvette contained Tris (200 mM, pH 8.0) with Triton X-100 (0.2% (v/v)), 5,5'-Dithiobis-2-nitrobenzoic acid (4 mg/ml, (DTNB)), Acetyl CoA (10 mM), 40 µg of sample and the volume was adjusted to 950 µl with distilled water. Cuvette was mixed by inversion and the baseline activity was read at 412 nm for 2 min using a flatbed recorder (Kipp & Zonen) and a Libra S12 spectrophotometer (Biochrom). Reaction was started by adding 10 mM of oxaloacetic acid, mixed by inversion, and then the increase in absorbance was monitored for 3 min.

Citrate synthase enzyme activity was calculated as follows:

$(\text{nmol min}^{-1} \text{ mg}^{-1}) = (\Delta \text{ Absorbance/min} \times 1,000) / ((13.6 \text{ mM}^{-1} \text{ cm}^{-1} \times \text{volume of sample used in ml}) \times (\text{sample protein concentration in mg ml}^{-1}))$.

2.2.10. Western Blotting

2.2.10.1 Preparation of the Cell Lysate

2.2.10.1.1 Poly (ADP-ribose) polymerase (PARP)

On day 0, SH-SY5Y cells were plated in 6x 25 cm² cell culture flasks at a concentration of 3.0×10^5 cells/ml in 5 mls of DMEM F12 + Nut mix. 200 µl of vehicle, 0.1, 0.5, 1, 2 and 5 mM was added to its correspondingly labelled flask. The flasks were put into the incubator to incubator for 72 h. After the time had elapsed, 30 ml universal tubes were labelled corresponding to each well. The media was removed from the wells and placed in their respective tubes. Wells were washed with PBS, removed and placed into the same falcons as the media. Cells were trypsinised, allowed to incubate at 37°C and then resuspended in fresh media. Cells were pelleted (2,400 rpm for 10 min). Supernatant was removed and the pellet was resuspended in PBS and transferred to eppendorfs. The cells were pelleted again (2,400 rpm for 10 min) and were stored in at -20°C. On day 1, they were resuspended in PARP lysis buffer (62.5 mM Tris (pH 6.8), 6M Urea, 2% (w/v) SDS, 10% (v/v) glycerol, 0.00125 % (w/v) bromophenol blue and 50 mM Dithiothreitol (DTT) with the volume amended to the size of the pellet. The cells were sonicated in a water bath (Sonicator 2000, Branson) to ensure complete lysis and then boiled at 100 °C for 5 min. 5% (w/v) DTT was added to the eppendorfs before they were loaded in a 10 % SDS gel immersed in electrolyte in an electrophoresis apparatus (BIORAD Mini-Protean).

2.2.10.1.2 AMP-Kinase (AMPK) and Acetyl Co A Carboxylase (ACC).

On day 0, SH-SY5Y cells were plated in 8x 25 cm² cell culture flasks at a concentration of 3.0×10^5 cells/ml in 5 mls of DMEM F12 + Nut mix. 250 µM of phenformin was added to

its correspondingly labelled flask at 0.5, 1, 2, 4 and 6 h. Vehicle was added at 6 h and 50 mM of 2-deoxyglucose (2-DG) was added to a flask for 30 minutes. After the time had elapsed, the media was removed and flasks were washed with PBS. 100 µl of AMPK lysis buffer (50 mM Tris/HCl, pH 7.4 at 4°C, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 250 mM mannitol, 1% (v/v) Triton X-100, 1 mM DTT, and a proteinase inhibitor tablet) was added to the flasks on ice and were left shaking at room temperature for 15 min. The flasks were then scraped and the lysates were then transferred to correspondingly labelled eppendorfs. The eppendorfs were then spun down at 14,000 rpm at 4°C for 10 min. The supernatants were transferred to fresh eppendorfs, were snap frozen in liquid nitrogen and were then put into -80°C overnight. On day 1, after the eppendorfs had thawed, the protein content of the lysates was assessed by the BCA assay.

2.2.10.1.3 Sirtuin-1 (SIRT1), Sirtuin-3 (SIRT3), transcription factor A, mitochondrial (TFAM), peroxisome-proliferator activator receptor-γ co-activator 1-alpha (PGC1α)

2.2.10.1.3.1 H1299s, H1299r, P31s and P31r cells.

On day 0, H1299 and P31 cells were plated at a cell density of 4.0×10^5 cells/T25 flask. On day 1, media is removed and 100 µl of 1 x Laemmli buffer (2 % (w/v) SDS, 10% (v/v) glycerol, 60 mM Tris-HCL pH 6.8) is added to the flasks on ice.

2.2.10.1.3.2 H1299s and H1299 cells treated with resveratrol

On day 0, H1299 and P31 cells were plated at a cell density of 4.0×10^5 cells/T25 flask. On day 1, cells were treated with 50 µM resveratrol. On day 2, media is removed and 100 µl of 1 x Laemmli buffer.

2.2.10.1.3.3 H1299s and H1299r cells transduced with SIRT3 expressing adenovirus

On day 0, H1299 and P31 cells were plated at a cell density of 4.0×10^5 cells/T25 flask. On day 1, cells were treated with 50 MOI of empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus. On day 3, media is removed and 100 μ l of 1 x Laemmli buffer.

These flasks were left shaking at room temperature for 15 minutes. The lysate is scraped and then transferred to a labelled eppendorf. The minifuge tubes were centrifuges at 14,000 rpm at 4°C for 10 min (Sorvall 5417R, Eppendorf) to yield a lysate pellet. Protein content is measured using the BCA assay.

2.2.10.2. Bicinchoninic acid (BCA) protein assay

From each lysate, 6.5 μ l was put into an eppendorf and diluted 1:5 with 26.5 μ l of distilled water. To a 96 well plate, 10 μ l of diluted sample is added per well in triplicate. To each well, 200 μ l of BCA containing a 50:1 ratio of Reagent A and Reagent B is added to each test well and left to incubate in the dark for 30 minutes at 37°C before reading the absorbance at 562 nm (Spectra max 340PC). A Bovine Serum Albumin (BSA) protein standard curve was assayed with data points ranging from 0-2000 μ g/ml along with the sample lysates that enabled the quantification of the protein concentration of the lysates.

2.2.10.3. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a method of resolving denatured, negatively charged coated proteins on a polyacrylamide gel, separating proteins based on their molecular weight, charge and polyacrylamide gel pore size. 6 & 10% resolving wells were made with a 5% stacking gel. One 10% resolving gel was composed of: 1.9 mls H₂O, 1.7 mls ProtoGel™, 1.3 mls 1.5 mM Tris (pH 8.8), 50 μ l of 10% SDS (w/v), 50 μ l of 10% (w/v) ammonium persulfate and 4 μ l N,N,N',N'-tetramethylethylenediamine (TEMED). A 5% stacking gel consists of: 2.1 mls H₂O, 0.5 mls ProtoGel™, 0.38 mls 1.0 mM Tris (p H 8.8), 30 μ l of 10% SDS (w/v), 30 μ l of 10% (w/v) ammonium persulfate and 3 μ l TEMED. A 10 pronged comb was used to generate 10

wells in which the lysates would be placed. Resolving and stacking gels were poured inside the gel plates and allowed to polymerise sequentially, once set they were placed in an electrophoresis gel box submerged in 1 x Running Buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS).

2.2.10.4. Running of the gel

PARP and AMPK lysates were ran on 10% SDS gels whereas the ACC lysates were ran on 6% SDS gels. 7 µl of the standard ladder was added to the first well to indicate where proteins of similar size should appear. The volume of the PARP lysates added to the wells depended on the size of the pellet and for the AMPK & ACC lysates 50 µg of protein in sample buffer (50 mM Tris HCL (p H 6.8), 2% SDS, 6% glycerol (v/v), 50 mM DTT, 0.004% (w/v) bromophenol blue) was added to each well. The controls were added first and then the treated lysates were put into the other wells. The gel was run at 80 V for 30 min until the samples ran through the stacking gel into the running gel and it was then turned up to 120 V for two h until the blue line of the stained samples was at the very bottom of the 10% gels and until the three top bands were visible for the Acetyl CoA carboxylase 6% gels.

2.2.10.5. Polyvinylidene Difluoride (PVDF) Wet Transfer

PVDF membranes were pre-soaked in 100% methanol for 30 seconds in order to activate the membrane. A PVDF membrane, gel, 6 filter paper slices were immersed in transfer buffer (25 mM Tris, 187 mM glycine, dissolved in H₂O and 10% (v/v) methanol) for approximately 10 min before transfer. In a BIORAD Mini-Trans Blot cassette, the components of the layered sandwich were ordered as follows: Sponge, 2 filter paper, gel, PVDF membrane, 3 filter papers and sponge. The cassette was placed in the transfer apparatus with the black sides together submerged in transfer buffer with an ice pack. The transfer was set at 150 milliAMPs and ran for 60-140 min.

2.2.10.6 Blotting

Once the proteins had transferred the blots were blocked in 5 % (w/v) Marvell + TBS-T (10 mM Tris, 150 mM NaCl and 0.1% (v/v) Tween). Blots were left blocking on a shaker for 1 hour at room temperature. The blots were added to their primary antibodies in 5 % (w/v) BSA + TBS-T and left rolling at 4°C overnight (Table 2.3).

Antibody	Dilution	Source
Rabbit Polyclonal AMPK α Antibody	1:1000	Cell Signalling
Rabbit Polyclonal phospho-AMPK α Antibody	1:714	Cell Signalling
Rabbit Polyclonal ACC Antibody	1:714	Cell Signalling
Rabbit Polyclonal phospho -ACC Antibody	1:1000	Cell Signalling
Mouse Monoclonal PARP antibody	1:1000	Millipore
Mouse Polyclonal γ tubulin antibody	1:2000	Novus Biologicals
Rabbit Polyclonal SIRT1 Antibody	1:500	Cell Signalling
Rabbit Polyclonal SIRT3 Antibody	1:1000	Cell Signalling
Rabbit Polyclonal TFAM Antibody	1:500	Cell Signalling
Rabbit Polyclonal PGC1 α Antibody	1:1000	Novus Biologicals
Rabbit Polyclonal ABCA5 Antibody	1:500	Abcam
Rabbit Polyclonal ABCE1 Antibody	1:1000	Novus Biologicals
Mouse Polyclonal ABCB6 Antibody	1:500	Novus Biologicals
HRP conjugated anti-rabbit IgG	1:2500	Promega
HRP conjugated anti-mouse IgG	1:2500	Promega
HRP conjugated anti-mouse IgG	1:5000	Novus Biologicals

Table 2.3 Antibodies used for western blotting

The blots were washed the following day with TBS-T in 3X10 min increments. Incubation with the conjugated horse radish peroxidase (HRP) secondary antibody in 5% (w/v) Marvell + 5 ml TBS-T in a 50 ml tubes was carried out at room temperature for an hour. The blots were put into a tray and washed with TBS-T in 3x 10 min increments. The blots were put onto a dish and then 0.5 mls of detection reagent 1 and 0.5 ml of detection

reagent 2 mixtures was pipette per blot for a minute. Blots were dried and brought to the dark room where an x-ray film was placed on the blots for five minutes and then placed in the developer (X ray film Processor RGII, Fuji)

2.2.10.7 Densitometric analysis

The density of some of the protein bands on the western blots were analysed by using the ImageJ. This software programme calculates area and pixel value statistics of defined sections i.e. protein band on a western blot (analysis was performed as follows: the X-ray film was scanned and saved as a JPEG). The file was opened in the imageJ programme and the background was subtracted. The number of square pixels in the section selected i.e. the protein bands were then counted by measuring the area in the binary or threshold image.

2.2.10.8 Statistics

The statistical analysis of experimental data was performed using the computer program Prism GraphPad 5. Results were presented as mean $\pm$ standard error of the mean (SEM) for n values. For comparison of two groups, values were determined using a Student's unpaired *t*-test.

Chapter 3: Phenformin as an anti-cancer agent

3.0 Introduction

Neuroblastoma is a malignancy that arises from the neural crest cells of the sympathetic nervous system (Galenkamp et al., 2015) that accounts for 15% of paediatric cancer deaths. The cancer presents as a highly diverse heterogeneous disease ranging from sporadically regressing to refractory forms (Amoroso et al., 2015). Irrespective of the advances in the understanding of the disease, aggressive treatment strategies, surgery, radiation therapy, immunotherapy and differentiation therapy, it remains to be one of the most difficult cancers to treat with a 5 year survival of just 42% (Cheung and Dyer, 2013). For this reason there is urgent need for alternative approaches to treatment.

Drugs that target metabolic diseases such as the type II diabetes drug metformin and phenformin have shown clinical benefit in treating cancer (Dowling et al., 2011, Evans et al., 2005). Although Phenformin was removed from the diabetes market due to propensity for lactic acidosis (Kwong and Brubacher, 1998), it has shown significant anti-neoplastic activity in PTEN deficient tumours and breast cancer MC7 & MDA-MD-231 cells (Huang, 2008, Appleyard et al., 2012) and at concentrations lower than those known to be efficacious for metformin (Wheaton et al., 2014). Phenformin was also shown to have anti-proliferative effects by Huang (2008) in PTEN deficient tumours and to induce p21 cell cycle inhibition and cell cycle arrest at the G2/M phase of the cell cycle leading to apoptosis in neuroblastoma SH-SY5Y cells (Caraci et al., 2003).

In most of the studies using the biguanides, metformin and phenformin in cancer cells, the indication is that the AMPK/mTOR pathway plays a central role in its mechanism of action. Metformin and phenformin have been postulated to indirectly activate AMPK by inhibiting complex I (El-Mir et al., 2000, Owen, 2000, Miskimins et al., 2014) of the respiratory chain in the mitochondria thereby decreasing the ATP synthesis and increasing the AMP: ATP ratio (El-Mir et al., 2000, Owen, 2000). Indeed, the increase in peripheral glucose utilization, metabolism and stimulation of glycolytic lactate production observed after metformin treatment of diabetic patients are effects that would be predicted with inhibition of complex I of the respiratory chain (Bailey and Turner, 1996).

Consequently, mitochondrial complex I has been a focal point for the action of phenformin (and metformin). Recently, Wheaton et al. (2014) were able to demonstrate that phenformin inhibits complex I of the mitochondria at low concentrations and that this inhibition was necessary for its inhibitory effects on the proliferation of colon cancer cells.. Furthermore, biguanide sensitivity of a range of cancer cells was reversed by ectopic expression of yeast NDI1, a ubiquinone oxidoreductase that allows bypass of Complex I function (Birsoy et al., 2014).

In this study we show that phenformin is capable of killing human derived neuroblastoma cells (SH-SY5Y cells) and that phenformin causes G1 cell cycle arrest, alters the bioenergetics of the cells in a time and dose dependent manner, reduces ROS, activates AMPK/ACC and specifically inhibits complex I of the mitochondria. We go on to demonstrate that phenformin sensitivity of neuroblastoma cells was reversed by transfection with a 'humanized' form of yeast NDI1.

3.1. Results:

3.1.1 Determination of the IC₅₀ of Phenformin and Metformin in SH-SY5Y cells.

In order to assess the potency of phenformin on the solid neuroblastoma SH-SY5Y cell line, an alamar blue viability assay was undertaken as a function of phenformin concentration. Cells were treated with vehicle (dH₂O) or treated with a range of concentrations of phenformin (0.1 µM -10 mM) for 72h.

As seen in Figure 3.1, phenformin decreased the cell viability after 72h treatment in a dose dependent manner. Maximum cytotoxic effect of phenformin was demonstrated at 5 mM with death of all the cells, with no further decrease in viability, at the higher concentrations. The IC₅₀ value for SH-SY5Y cells treated with phenformin was 2.76 ± 0.09 mM.

Figure 3.2 shows an example of a viability assay and no substantial decrease in viability was detected after metformin treatment for 72h.

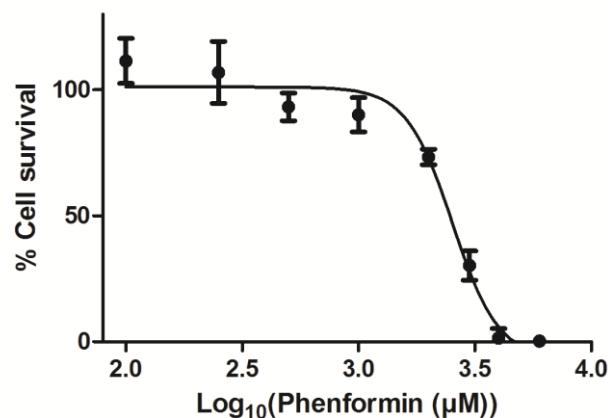


Figure 3.1 Effect of phenformin on the proliferation of SH-SY5Y neuroblastoma cell line. SH-SY5Y cells were seeded in a 96 well plate at a density of 30,000 cells/ well and were treated in triplicate with vehicle (dH₂O) or a range of concentrations of phenformin (100 μM -8 mM) for 72h. Alamar blue was added and cells were incubated in the dark for 5h. The fluorescence was read at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a micro plate reader. Data expressed as % cell viability of vehicle treated controls. IC₅₀ values represent the concentration of drug required to reduce viability by 50 %. Error bars represent the SEM between three separate experiments.

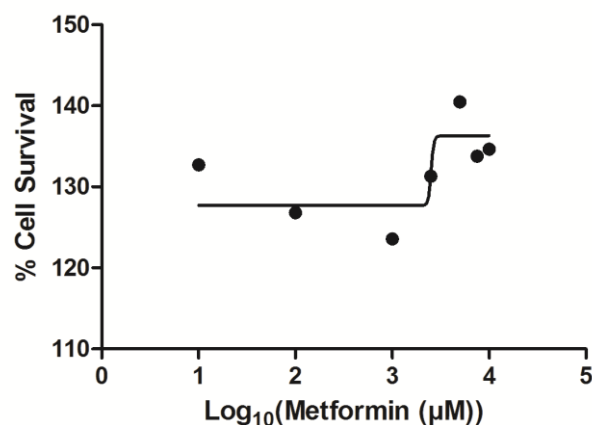


Figure 3.2 Effect of metformin on the proliferation of SH-SY5Y neuroblastoma cell line. SH-SY5Y cells were seeded in a 96 well plate at a density of 30,000 cells/ well and were treated in triplicate with vehicle (dH₂O) or a range of concentrations of metformin (10 μM-10 mM) for 72h. Alamar blue was added and cells were incubated in the dark for 5h. The fluorescence was read at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a micro plate reader. Data expressed as % cell viability of vehicle treated controls.

3.1.2 The effect of phenformin on the cell cycle of SH-SY5Ys.

Flow cytometry analysis was used to determine the effect of phenformin on the phases of the cell cycle of these cells. Cells were seeded at a density of 110,000 cells/well and treated with varying concentrations of phenformin (0.1, 0.5, 1, 2 & 5 mM) for 72h for the dose response and 1 mM of phenformin for 8, 16, 24, 48 and 72h for the time course experiment. Propidium Iodide (PI) was used to stain the cells' DNA. Vehicle treated cells served as the control for these experiments. Samples were analysed using Becton Dickinson FACS Calibur II flow cytometer and CellQuest. Data collections (10,000 events per sample) were gated to exclude cell debris and cell aggregates.

Treatment with phenformin for 72h caused a dose dependent increase in the percentage of sub G1/apoptotic cells (Figure 3.3) with no apparent cell cycle arrest before apoptosis. Maximum apoptosis was observed at 5 mM with an apoptotic population of $62\% \pm 3\%$ which was substantial in comparison to the cells treated with the vehicle $1.2\% \pm 0.4\%$.

In order to determine a time of inhibition we chose a phenformin concentration that was submaximal for cell viability. When we used 1 mM of phenformin for 6, 16, 24 and 48h, It was observed that there was a transient increase in the G1 population relative to the vehicle control, at 16h ($P<0.005$) and 24h ($P<0.05$)(Figure 3.4). This significant effect indicated that these cells were undergoing G1 cell cycle arrest at sub-cytotoxic concentrations of phenformin.

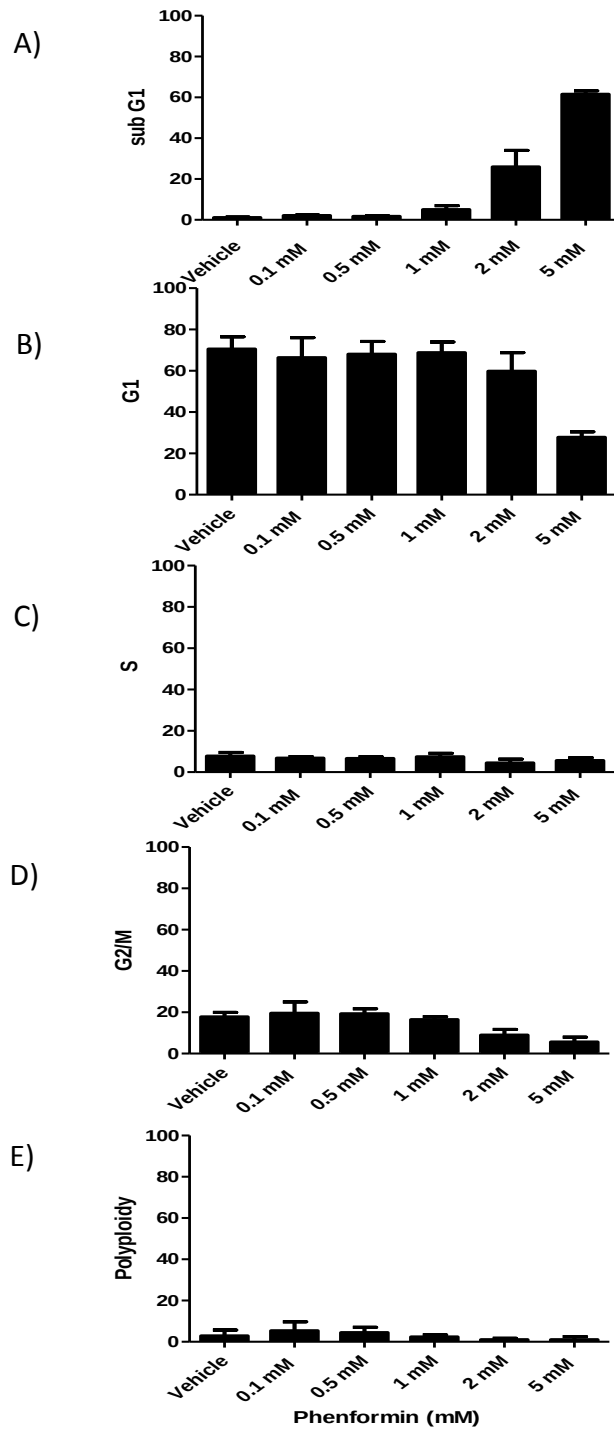


Figure 3.3 Dose-dependent effect of phenformin on SH-SY5Y cells. SH-SY5Y cells were seeded at a density of 110,000 cells /well and treated with the indicated concentrations of phenformin and vehicle for 72h. The cells were then fixed and stained as described previously. Fluorescence was analysed for 10,000 events. Error bars represent the SD from three independent experiments between the total % of cells undergoing the various phases of the cell cycle.

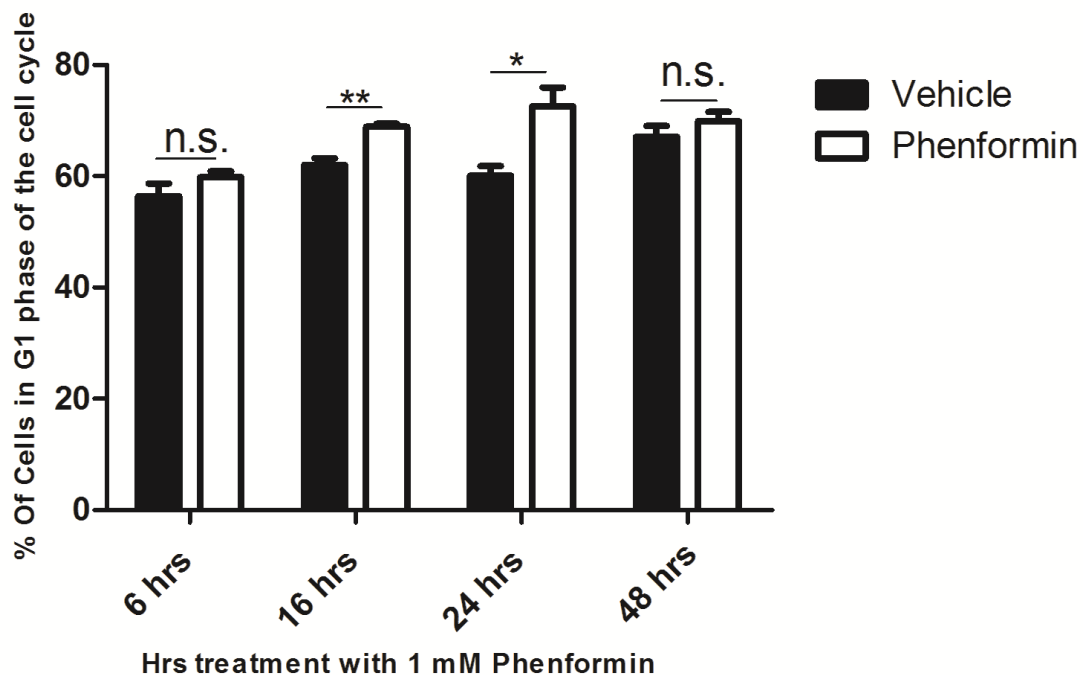


Figure 3.4. Phenformin induces G₁ cell cycle arrest at sub cytotoxic levels as determined by flow cytometry. SH-SY5Y cells were seeded at a density of 110,000 cells /well for 24h. Cells were treated with vehicle (dH₂O) or 1 mM phenformin for the times points indicated. Cells were harvested, fixed in 2 ml of ethanol and stained with PI. Cells were analysed by flow cytometry for the percentage of cells in G1 phase of the cell cycle. Data are expressed as mean $\pm$ SEM of three separate experiments. Statistical analysis was carried out using a student t-test. ** = $p < 0.005$, * = $p < 0.05$.

3.1.3 Confirmation of cell death by PI & Annexin V staining by flow cytometry and PARP cleavage by western blotting in SH-SY5Y cells

PI & Annexin V staining and PARP cleavage protein expression were used to confirm that apoptosis was occurring as a result of treatment with phenformin using flow cytometry and western blotting respectively.

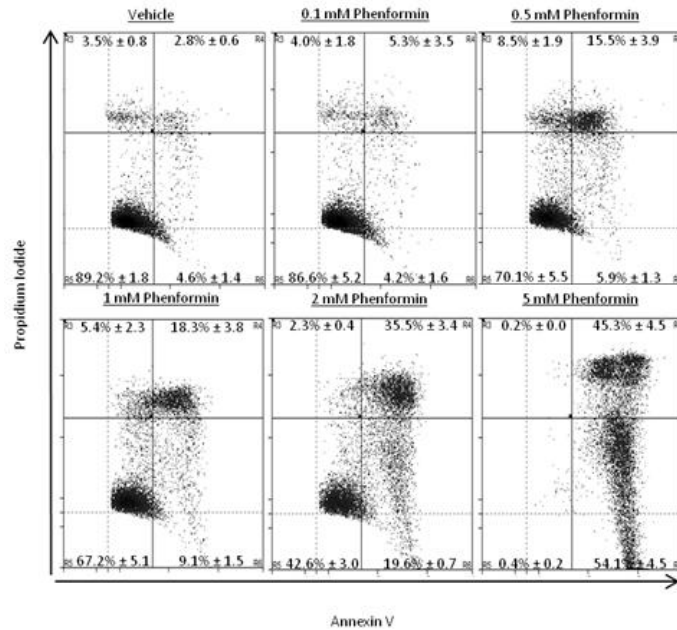
3.1.3.1 PI & Annexin V staining by flow cytometry

In order to validate that cell death was occurring as a result of phenformin treatment and in order to determine the nature of cell death, cells were stained with both PI and annexin V. PI served as the live/dead stain-indicator of cell membrane integrity and increased annexin V binding of phosphatidylserine to the outer leaflet of the plasma membrane indicates apoptotic cells. It was observed that as the percentage of live cells decreased there was an increase in the population of cells undergoing apoptosis due to phenformin treatment (Figure 3.5A) which became significant at 0.5 mM phenformin ($p < 0.0001$) (Figure 3.5B). This serves as confirmation that phenformin caused cell death in these cancer cells by apoptosis.

3.1.3.2 PARP cleavage by western blotting

As a secondary confirmation of cell death resulting from phenformin treatment, PARP (120 kDa), cleaved PARP (89 kDa) and γ tubulin (loading control, 48 kDa) protein expression was determined by western blotting as outlined in section 2.2.10.1.1. The representative blot (Figure 3.6A) below shows disappearance of the full PARP form. The loading control of γ tubulin was relatively constant (Figure 3.6 A&B). No PARP cleavage production is observed and one possible explanation for that absence will be discussed later. The disappearance indicates that apoptosis is occurring in SH-SY5Y cells with increasing phenformin concentrations.

A)



B)

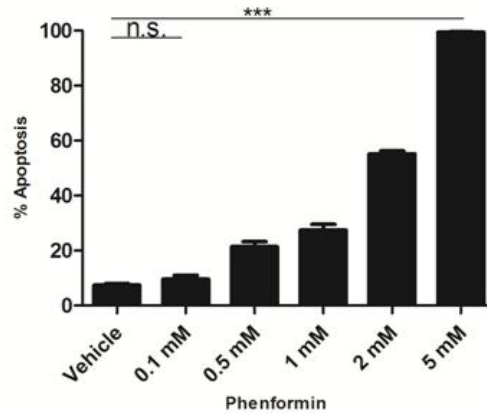
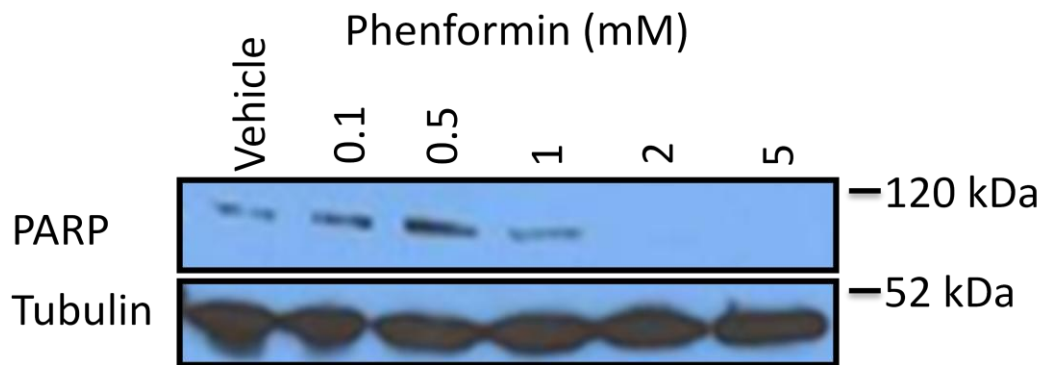


Figure 3.5 Phenformin induces apoptosis in SHSY-5Y cells in a dose dependent manner as determined by PI and annexin V staining by flow cytometry. (A) SH-SY5Y cells were seeded at a density of 110,000 cells /well and treated with the indicated concentrations of phenformin or vehicle for 72h. Cells were stained with PI and annexin V. Fluorescence was analysed for 10,000 events. Bottom Left Quadrant = Live, Bottom Right Quadrant = Early Apoptotic and Top Right Quadrant = Late Apoptotic. Error bars represent the standard deviation between the total percentages of cells undergoing the various forms of cell death. (B) Error bars represent the standard deviation between the total percentages of cells undergoing apoptosis. Statistical analysis was carried out using the student t-test. *** = $p < 0.0005$

A)



B)

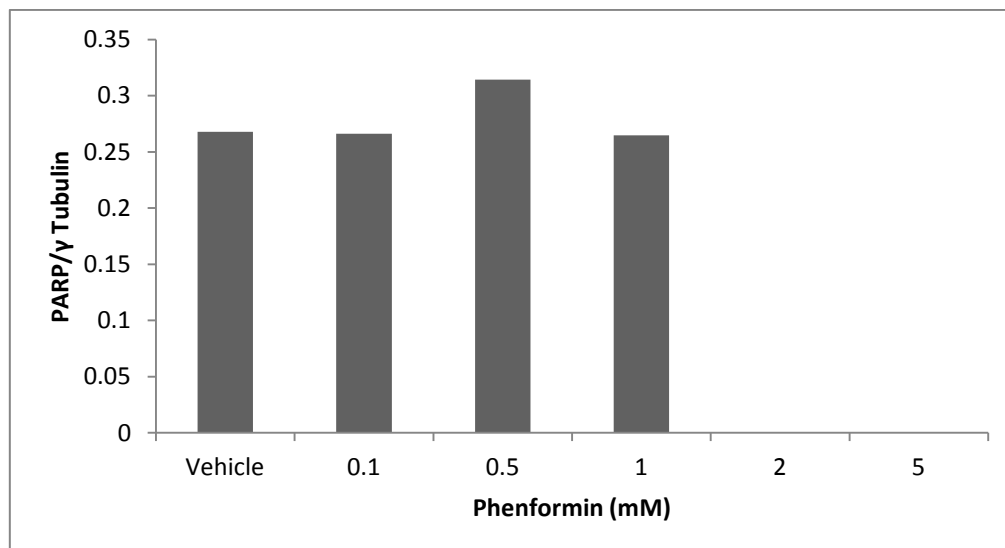


Figure 3.6 Disappearance of the full form of PARP as a result of treatment with phenformin. Whole cell lysates were prepared from confluent cultures of SH-SY5Y cells. Lysates were added based on size of pellets, resolved in 10% SDS-PAGE gel and transferred to a PVDF membrane as outlined in section 2.2.10.1.1. Blots were blocked in 5% marvel TBS-Tween, blot was cut at 70 kDa and the blots were probed for either PARP or γ tubulin. A) Protein expression of PARP and γ tubulin. B) Ratio of the band density of PARP over γ tubulin bands. Results shown are from one representative blot.

3.1.4 The oxygen consumption rate and extracellular acidification rates as determined by the Seahorse Extracellular Flux Analyser and oxygen consumption rates as determined by the Oroboros oxygraph respirometer.

Having determined that phenformin has potential as an anti cancer agent on SH-SY5Y cells using (a) a viability assay, alamar blue assay, (b) cell cycle analysis by flow cytometry and (c) cell death by flow cytometry and (d) PARP analysis it was decided to determine how phenformin was affecting the metabolism of the cells. This was carried out by using the two parallel methods (a) seahorse extracellular flux analyser and (b) oxygraph respirometer. A concentration of phenformin was sought that would affect the metabolism of the cells without being cytotoxic. 1 mM of phenformin was chosen as the concentration because after 72h, phenformin was just beginning to have an effect in the dose response experiment (Figure 3.3A) and 48h was chosen as the time point because again it was the point in the time course experiment (Figure 3.5) at which phenformin was beginning to affect the viability of the SH-SY5Y cells.

3.1.4.1 Effect of 1 mM phenformin on the metabolism of SH-SY5Y cells after 48h incubation as determined by the oxygraph respirometer.

Before the cells were treated with phenformin, it was important to get a picture of the basal metabolism in order to assess the effect of phenformin on the metabolism of these cells and how they responded to the mitochondrial drugs; oligomycin, FCCP and antimycin a as an indices of the functionality of the mitochondria in these neuroblastoma cells. Oligomycin an inhibitor of ATP synthase in the mitochondria causes respiration rate to decrease, FCCP an uncoupler of the mitochondria causes the respiration rate to increase and antimycin A an inhibitor of the oxidation of ubiquinol in the electron transport chain which causes the respiration rate to decrease and the mitochondria to shut down. The initial assessment of the basal metabolism of the SH-SY5Y cells were determined on the oxygraph as it is the most sensitive to oxygen consumption/respiration of the two machines.

The SH-SY5Y cells responded to the mitochondrial drugs in the expected manner, in that it decreased with oligomycin and antimycin A and it increased with FCCP (Figure 3.7). Having established optimal concentrations of the mitochondrial inhibitors it was then decided to see what effect phenformin was having on the mitochondrial oxygen consumption. As Antimycin a causes the mitochondria to effectively shut down any oxygen consumption observed would not be due to the mitochondria. Therefore untreated cells and phenformin treated cells' basal metabolism was measured and then antimycin A was then introduced. The difference between the oxygen consumption rate before and after Antimycin a treatment 'the mitochondrial oxygen consumption' was then expressed as a percentage of the total metabolism of the cells. There was a significant difference in the oxygen consumption rate before and after antimycin A treatment ($P < 0.001$) which was expected but there was a significant difference between basal metabolism of the untreated and treated cells ($P < 0.001$) which tells us that phenformin is definitely affecting the metabolism of the cells. Interestingly there wasn't a significant difference between the oxygen consumption rate of the treated cells before and after antimycin A treatment which indicates that phenformin is causing the mitochondria respiration to be inhibited (Figure 3.8A). This difference was reflected in the percentage of the oxygen consumption belonging to the mitochondria was expressed in figure 3.8B.

3.1.4.2 Effect of phenformin on the metabolism of SH-SY5Y cells after 48h incubation as determined by the seahorse extracellular flux analyser.

Having optimised the concentrations of the mitochondrial inhibitors to use on the SH-SY5Y cells and confirmed that phenformin was indeed affecting the metabolism of these cancer cells, it was then decided to confirm these findings using the seahorse extracellular flux analyser.

As these cells had never been used on the seahorse before at the time, the initial seeding density optimisations were based on the fact that the area that the cells were in the XF24 wells were the same dimensions as a 96 well plate. The decision on the seeding density to use was to be based on which density gave the mitochondria stress test

response. However, it was observed that after oligomycin was injected into the wells, FCCP was unable to affect the cells' metabolism. To address this, cells were plated in the same manner and some wells oligomycin was injected and in others oligomycin was left out. Interestingly FCCP was having a response when there was no oligomycin present but when it was there was no FCCP response (Figure 3.10). At that stage it was thought that oligomycin was somehow preventing FCCP from having an effect. It was later discovered that the seahorse media that the cells were in needed to be supplemented with sodium pyruvate in order to get a FCCP response. 45,000 cells/ml was chosen as the concentration to proceed with as it gave the highest respiration rate and the greatest FCCP response (Figure 3.9).

From protocols on the seahorse website they advise to use both rotenone (complex I inhibitor) and Antimycin a (complex III inhibitor) to shut off the mitochondrial contribution to the overall cellular respiration. Previously Antimycin a was used as the sole drug to turn off the mitochondria so it was decided to investigate whether the combination of rotenone and Antimycin a had an additive inhibitory effect on respiration or whether antimycin A was enough to inhibit the mitochondria. Although this experiment was only carried out the one time it serves to show that there was no clear added benefit to using the combination (Figure 3.11) so Antimycin a was chosen for the future studies.

Having confirmed the seeding density of SH-SY5Y cells on the seahorse plates for the 48h incubations and the drugs to use, it was then decided to see at what concentration phenformin was beginning to inhibit mitochondrial oxygen consumption. Cells were seeded at a concentration of 45,000 cells/ml, were treated with a range of concentrations (0.005 mM – 2 mM of phenformin) for 48h and were then analyzed on the seahorse. Phenformin begins to significantly inhibit oxygen consumption at 100 μ M ($P < 0.05$) and then at 250 μ M it is completely inhibited ($P < 0.005$) (Figure 3.12A). Phenformin caused a significant increase in glycolysis (as represented by the extracellular acidification rate (ECAR)) at 100 μ M ($P < 0.05$) but at higher concentrations glycolysis begins to decrease as the doses become more toxic to the cells (Figure 3.12B).

An analysis of the mitochondrial contributions (oxygen consumption after antimycin A treatment taken away from the basal oxygen consumption) shows that similar to the oxygen consumption, phenformin begins to inhibit mitochondrial oxygen consumption at 100 μ M and at 250 μ M ($P < 0.05$) it is completely inhibited (Figure 3.12C). This shows that phenformin's mechanism of action upon these cancer cells is at least in part through its effect on the mitochondria.

3.1.4.3 Effect of metformin on the metabolism of SH-SY5Y cells after 48h incubation as determined by the seahorse extracellular flux analyser.

Based upon the alamar blue assay results it was previously noted that metformin was not affecting the viability of the neuroblastoma cells. Like most viability assays, the reaction requires the cells to metabolise some component of the assay to get the colour change which tells us how potent our drugs are. In this case the assay requires the living cells to convert resazurin to the fluorescent molecule, resorufin. As previously demonstrated, phenformin's mechanism of action is through its ability to affect the metabolism of the cells which these assays require for their assay. As metformin is the less potent analogue, it was decided to investigate whether metformin was capable of affecting the metabolism of the neuroblastoma cells. The SH-SY5Y cells were seeded at the same density as before, a higher range of concentrations of metformin (0.1 mM – 5 mM) were incubated on the cells for 48h and were then analyzed on the seahorse.

Surprisingly based on the alamar blue results, the results showed that metformin did indeed affect the metabolism of these cells albeit at higher concentrations to phenformin (Figure 3.13). This experiment was only carried out the one time and so statistics could not be shown but it is surprising that phenformin was able to affect the alamar blue assay and metformin couldn't, considering their analogous structure and metabolic effects. Due to time constraints and resources, it was decided to not to pursue with this any further.

3.1.4.4 Effect of phenformin on the metabolism of SH-SY5Y cells over the shorter time course as determined by the oxygraph respirometer.

Upon revision of the results, it was unclear why such a long time point of 48h was chosen for the metabolic studies. As done previously we chose to analyse the metabolism effects on the most sensitive machine that we have, the oxygraph respirometer. 1 mM of phenformin was chosen as the concentration as used in previous initial experiments and the drug was incubated on the SH-SY5Y cells for 6, 16, 24, and 48h. Once the allocated time had elapsed the metabolism on the cells were then analysed on the oxygraph, just using antimycin A to get an idea of the mitochondrial contribution to the cellular respiration as that was our focus.

As shown in Figure 3.14, there was no difference in the metabolic effects of phenformin when it was exposed to the cells for 6 ($P<0.0001$), 16 ($P<0.005$), 24 ($P<0.0001$) and 48h ($P<0.0001$). At all time points, phenformin affected cellular and mitochondrial oxygen consumption to the same extent (Figure 3.14 A&B). The oxygen consumption rate after antimycin A treatment was the same as the oxygen consumption rate after phenformin treatment confirming previous findings

3.1.2.5 Time course and sensitivity assay with phenformin treatment on the SH-SY5Y cells using the seahorse extracellular flux analyser.

Having established that phenformin affects the metabolism at the lower time points, it was decided to conduct the sensitivity assay as shown in Figure 3.12 at the shortest time point of 6h. As the time at which the cells are being exposed to drug has decreased so too will their sensitivity to those concentrations of phenformin will have changed. In order to address this question the seeding density for the seahorse plates had to be changed from a density suitable for 48h drug incubation to one suitable for 6h incubation. The seeding density optimisation was based upon the paper by Xun et al. (2012) who used 80,000 cells/well for their metabolic studies on SH-SY5Y cells. The cells were plated at 60,000, 70,000 and 80,000 cells/ well, were treated with 1 mM

phenformin for 1, 2, 3, 4, 5, 6h and antimycin A was introduced during the run as done previously using the oxygraph.

The OCR values obtained for the cell densities after phenformin treatment were very similar to that obtained when antimycin A was injected into the wells, confirming previous results (Figure 3.15). Based on the OCR & ECAR results the 80,000 cells/well showed a greater time dependent response to phenformin and most closely resembled that of the sensitivity assay carried out at 48h and so was chosen as the cell density for the further studies.

Having confirmed the cell density the time course experiment was repeated but with another time point. Based on the cell density experiment, it was observed that phenformin was having an effect on the metabolism from as early as one hour but there was no data on whether it could act any earlier so thirty minute incubation was added to the time course. There was a significant drop in the OCR from as early thirty minutes ($P < 0.005$) with a nice time dependent drop until 6h of incubation ($P < 0.0001$) with 1 mM of phenformin (Figure 3.16A). Concordant with this drop in OCR there is a significant increase in the ECAR as early as 30 minutes which was still significant after 6h (Figure 3.16B).

Once we established that there was a time dependent effect of phenformin's action on the metabolism of the SH-SY5Y cells we then repeated the sensitivity assay. The same concentrations were used as before except the drugs were only incubated on the cells for 6h so their sensitivities may have changed. The results obtained for this six hour experiment differed to that obtained for the 48 hour experiments. There seemed to be a step-wise diminution of the OCR. 0.05 mM ($P < 0.05$) significantly decreased the OCR with no further decrease until 0.25 mM of phenformin ($P < 0.0005$) when the metabolism of the cells has been effectively shut off (Figure 3.17A). Similarly there seems to be a step-wise rise in the ECAR but no significance after 0.5 ($P < 0.05$) and 0.75 mM on ($P < 0.05$)(Figure 3.17B). When the OCR rates of the SH-SY5Y cells were converted to mitochondrial oxygen consumption rates by taking the antimycin A rate from the original basal OCR's, it showed the same significant step-wise diminution of the OCR

except at 250 μ M the mitochondrial OCR was abolished. This indicates that at 50 μ M phenformin is beginning to affect mitochondrial OCR and at 250 μ M the mitochondria are completely inhibited (Figure 3.17C).

3.1.2.6 Inhibition of mitochondrial complex I of SH-SY5Y cells by phenformin

To determine whether inhibition of mitochondrial OCR, by phenformin, is due to inhibition of complex I, mitochondrial OCR was measured in digitonin permeabilized SH-SY5Y cells.

Firstly we sought to determine what concentration of digitonin was necessary to permeabilize the neuroblastoma cells. 10 μ g/ml of digitonin was enough to permeabilize the cells to oxidize saturable concentrations of succinate as a substrate to its maximal extent as measured by the oxygraph respirometer (Figure 3.18 A). However, it was not enough to oxidize saturable concentrations of glutamate/malate as a substrate. 50 μ g/ml of digitonin was needed to get the SH-SY5Y cells to maximally metabolize glutamate and malate as measured by the oxygraph respirometer (Figure 3.18 B).

Having determined the optimal concentration of digitonin needed to oxidize the individual substrates, the neuroblastoma cells were treated with 1 mM phenformin for 6h. In the presence of ADP and complex I substrates (glutamate/malate), phenformin completely inhibited any increase in oxygen consumption due to addition of glutamate plus malate (Figure 3.19 A). However, phenformin had no effect on the increase in mitochondrial OCR on addition of succinate, the complex II substrate in digitonin-permeabilized SH-SY5Y cells (Figure 3.19 B). These experiments provide evidence that phenformin inhibits mitochondrial oxygen consumption at complex I but not at complex II.

Further evidence that the inhibition of mitochondrial oxygen consumption by phenformin occurs at complex I comes from recovery experiments where inhibition at complex I is circumvented by transfection with NDI1. NDI1 is a single subunit of NADH dehydrogenase, that oxidizes NADH in a similar process present in mammalian complex I, without proton pumping or ROS generation (Seo et al., 1998).

Similar to the digitonin experiment, optimization was needed to determine which setting on the NEON electroporation system transfected the NDI1 plasmid to the greatest efficiency. In order to optimize the transfection, the empty vector control plasmid was GFP tagged. 220,000 cells and empty vector GFP tagged plasmid in suspension buffer were electroporated using various settings and were then plated in a 6 well dish. The following day the cells were assessed for transfection efficiency based upon the proportion of cells that were fluorescing green using an Olympus IX81 inverted microscope at 20x magnification excited at a wavelength of 395 nm using FV10-ASW 2.0 Viewer software. Setting 15 appeared to transfect the greatest proportion of the cells and so was chosen as the setting for the NDI1 experiments with phenformin treatment (Figure 3.20).

SH-SY5Y cells, treated with varying concentrations of phenformin (100-500 μ M), were transiently transfected with empty vector or a version of the yeast NDI1 optimized for expression in human cells i.e. human NDI1. Expression of NDI1 in SH-SY5Y cells conferred complete resistance to the inhibitory effects of phenformin (Figure 3.21 B) when compared to cells transfected with an empty vector (Figure 3.21 A).

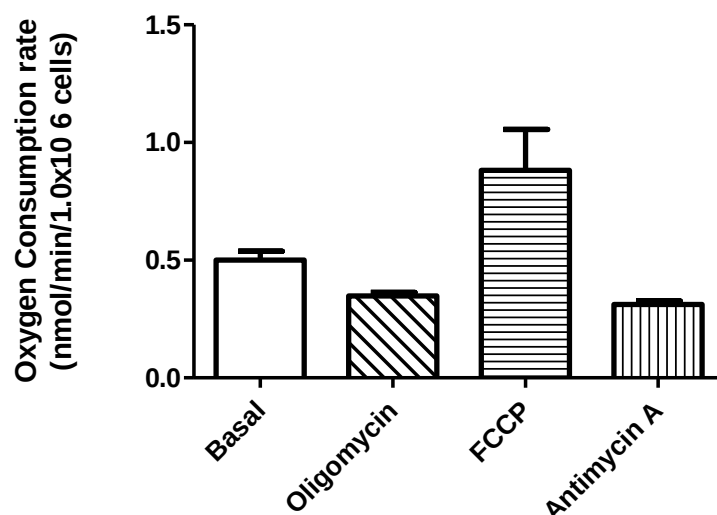


Figure 3.7 oxygen consumption rates of SH-SY5Y cells after treatment with oligomycin, FCCP and Antimycin a treatment as determined by the oxygraph respirometer. 1 million cells were placed in both chambers of the oxygraph in the growth media and the various drugs were then injected into the chambers using a Hamilton syringe. Error bars represent the standard deviation between the three independent experiments.

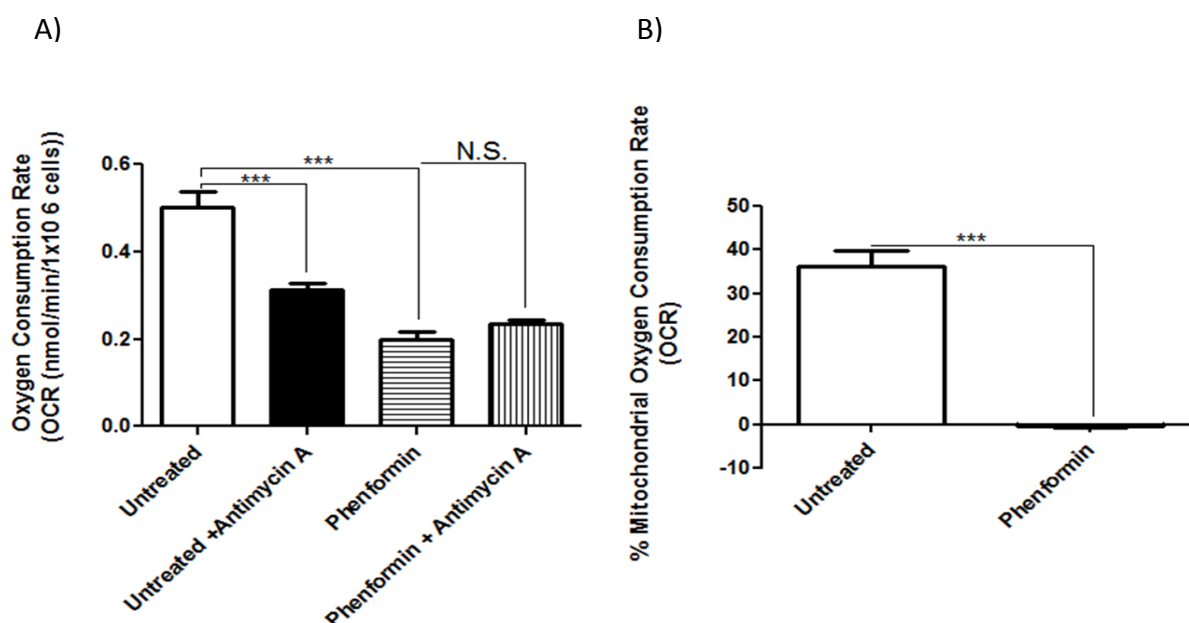


Figure 3.8 effect of phenformin on the metabolism of the SH-SY5Y cells as determined by the oxygraph respirometer. A) 1 million untreated and phenformin treated cells were placed in both chambers of the oxygraph in the growth media and antimycin A was then injected into the chambers using a Hamilton syringe. B) Percentage of the basal oxygen consumption that was mitochondrial obtained by taking the antimycin A oxygen consumption from the basal rate. Error bars represent the standard error of the mean between three separate experiments. Statistical analysis was carried out using the student t-test. *** = $p < 0.0005$.

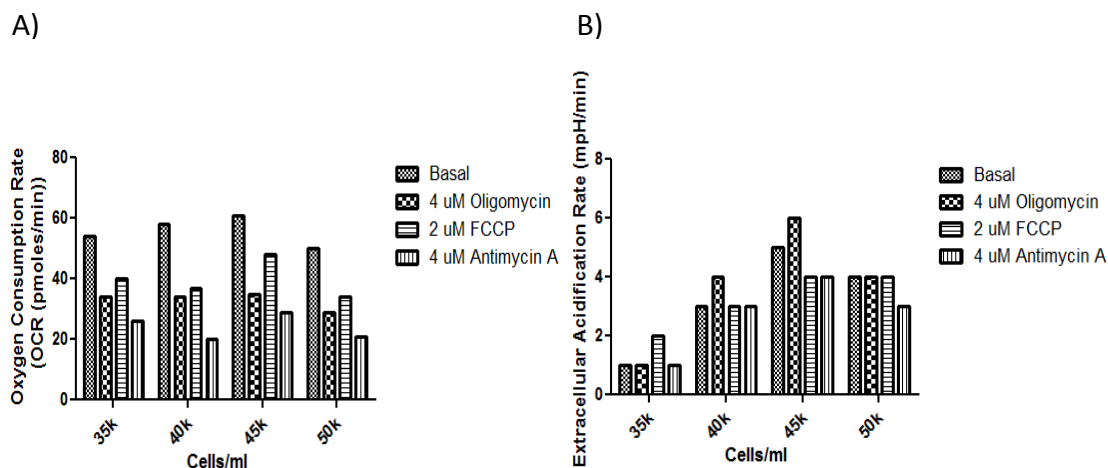


Figure 3.9 Optimisation of the seeding densities of SH-SY5Y with the addition of the optimised concentrations of oligomycin, FCCP and Antimycin a as determined by the seahorse extracellular flux analyser. Cells were seeded at varying densities and during the run 4 μM oligomycin, 2 μM FCCP and 4 μM of antimycin A was injected from the ports of the cartridge into the wells of the seahorse plate. A) Oxygen consumption rates of the basal metabolism and after the various mitochondrial inhibitors were added. B) Extracellular acidification rates of the basal metabolism and after the various mitochondrial inhibitors were added.

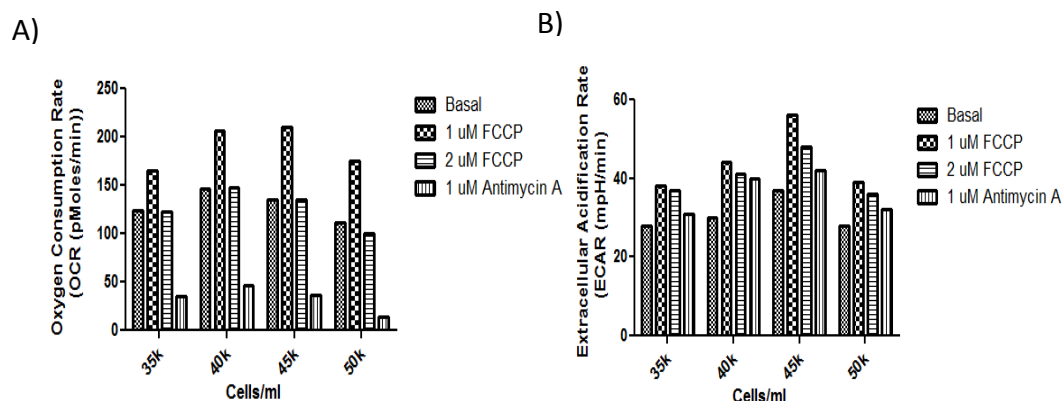


Figure 3.10 Oxygen consumption rates of SH-SY5Y cells at varying densities with the addition of FCCP and Antimycin a without oligomycin as determined by the seahorse extracellular flux analyser. Cells were seeded at varying densities and during the run 1 μM FCCP x 2 and 4 μM of antimycin A was injected from the ports of the cartridge into the wells of the seahorse plate. . A) Oxygen consumption rates of the basal metabolism and after the various mitochondrial inhibitors were added. B) Extracellular acidification rates of the basal metabolism and after the various mitochondrial inhibitors were added.

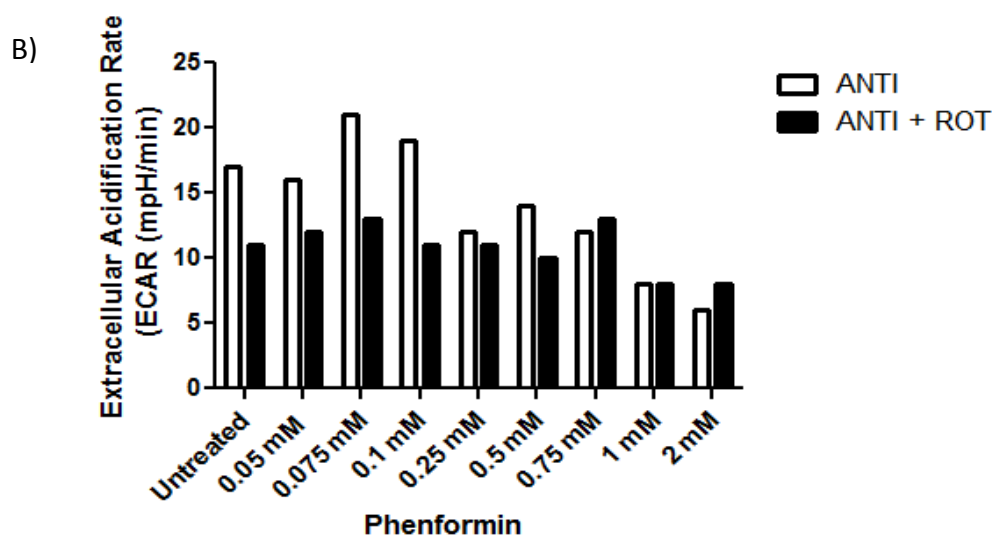
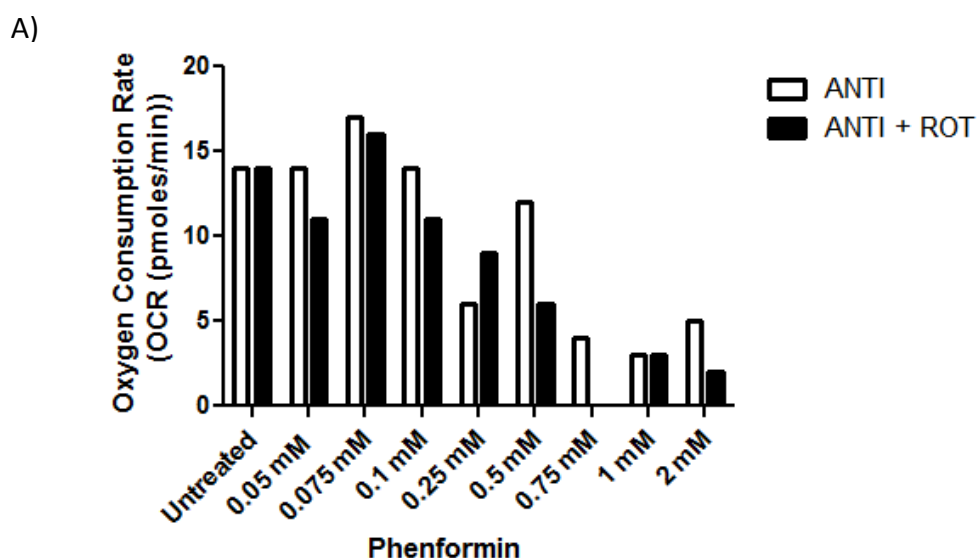


Figure 3.11 optimisation of the mitochondrial inhibitors after treatment with varying concentration of phenformin for 48h as determined by the seahorse extracellular flux analyser. Cells were seeded at 45,000 cells/ml, treated with varying concentrations of phenformin for 48h and then either antimycin A or antimycin A + rotenone were added from the ports of the cartridge into the seahorse wells. A) Oxygen consumption rates after treatment with varying concentration of phenformin and the addition of antimycin A or antimycin A + rotenone. B) Extracellular acidification rates after treatment with varying concentration of phenformin and the addition of antimycin A or antimycin A + rotenone.

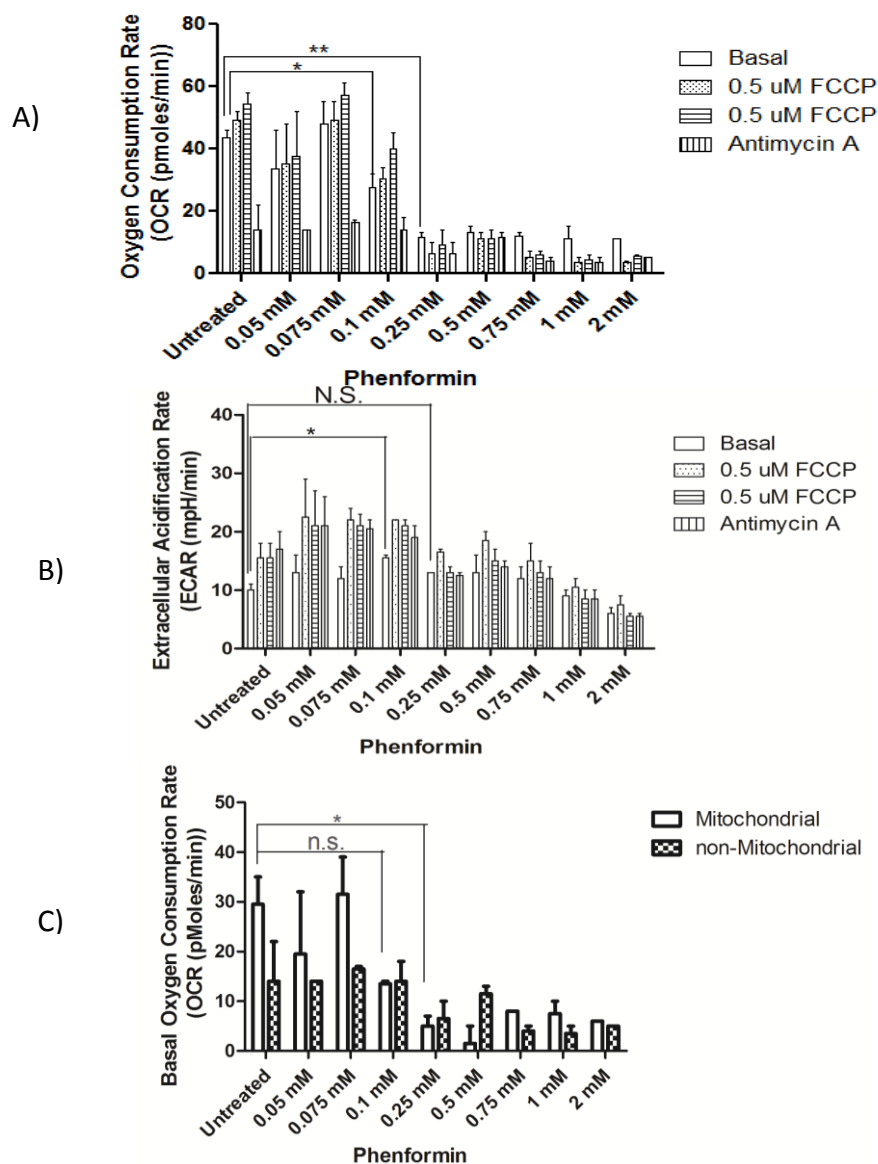


Figure 3.12 Investigation of the sensitivity of the SH-SY5Y cells to varying concentration of phenformin for 48h as determined by the seahorse extracellular flux analyser. Cells were seeded at 45,000 cells/ml, treated with varying concentrations of phenformin and then ran on the seahorse where the indicated drugs were injected from the ports of the cartridge into the seahorse wells during the run. A) Oxygen consumption rates after treatment with the indicated concentration of phenformin for 48h and then after the addition of FCCP and antimycin A. B) Extracellular acidification rates after treatment with the indicated concentration of phenformin for 48h and then after the addition of FCCP and antimycin A. C) Amount of the basal oxygen consumption that was mitochondrial and non mitochondrial after treatment with varying concentration of phenformin for 48h obtained from taking the antimycin rate from the basal rate. Error bars represent the standard error of the mean between three separate experiments. Statistical analysis was carried out using the student t-test. * = $p < 0.005$, * = $p < 0.05$.

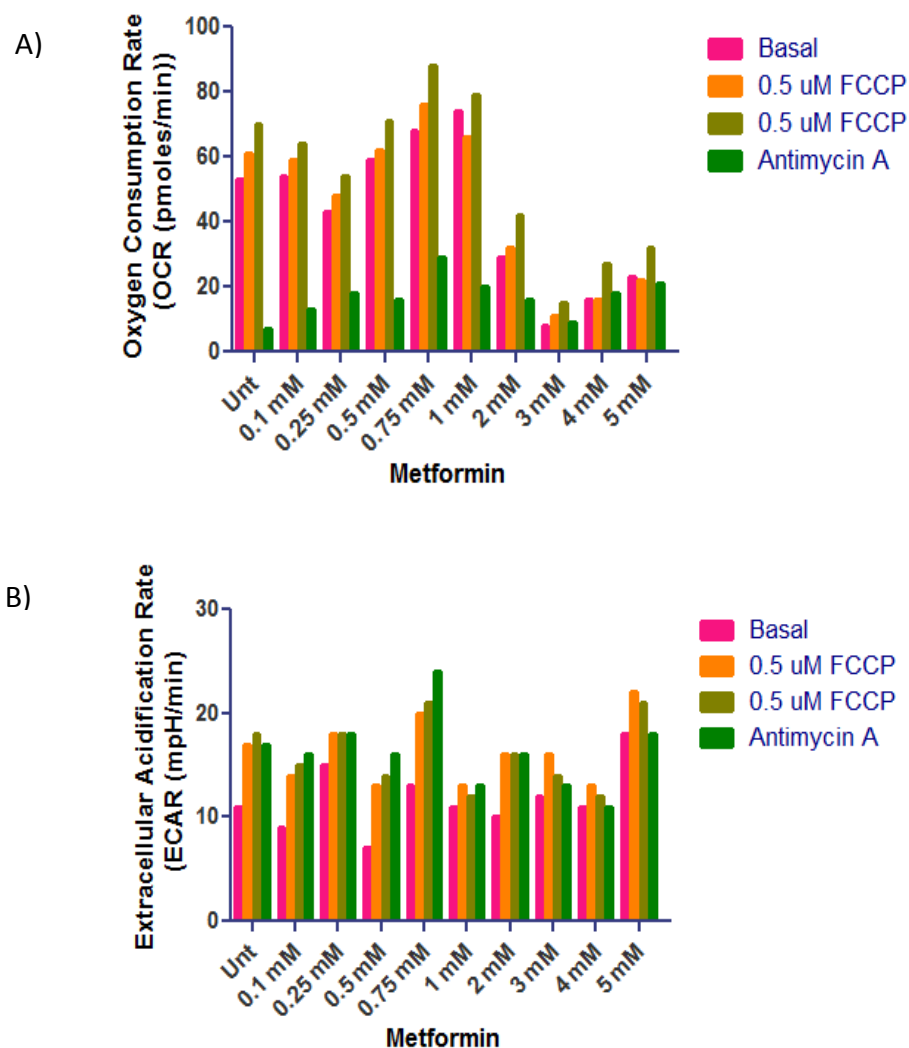


Figure 3.13 Effect of varying concentrations of metformin on the metabolism of SH-SY5Y cells for 48h as determined by the seahorse extracellular flux analyser. Cells were seeded at 45,000 cells/ml, treated with the varying concentrations of metformin for 48h and then ran on the seahorse where the indicated drugs were injected from the ports of the cartridge into the seahorse wells during the run. A) Oxygen consumption rates and B) Extracellular acidification rates after treatment with the indicated concentration of metformin for 48h and then after the addition of FCCP and antimycin A.

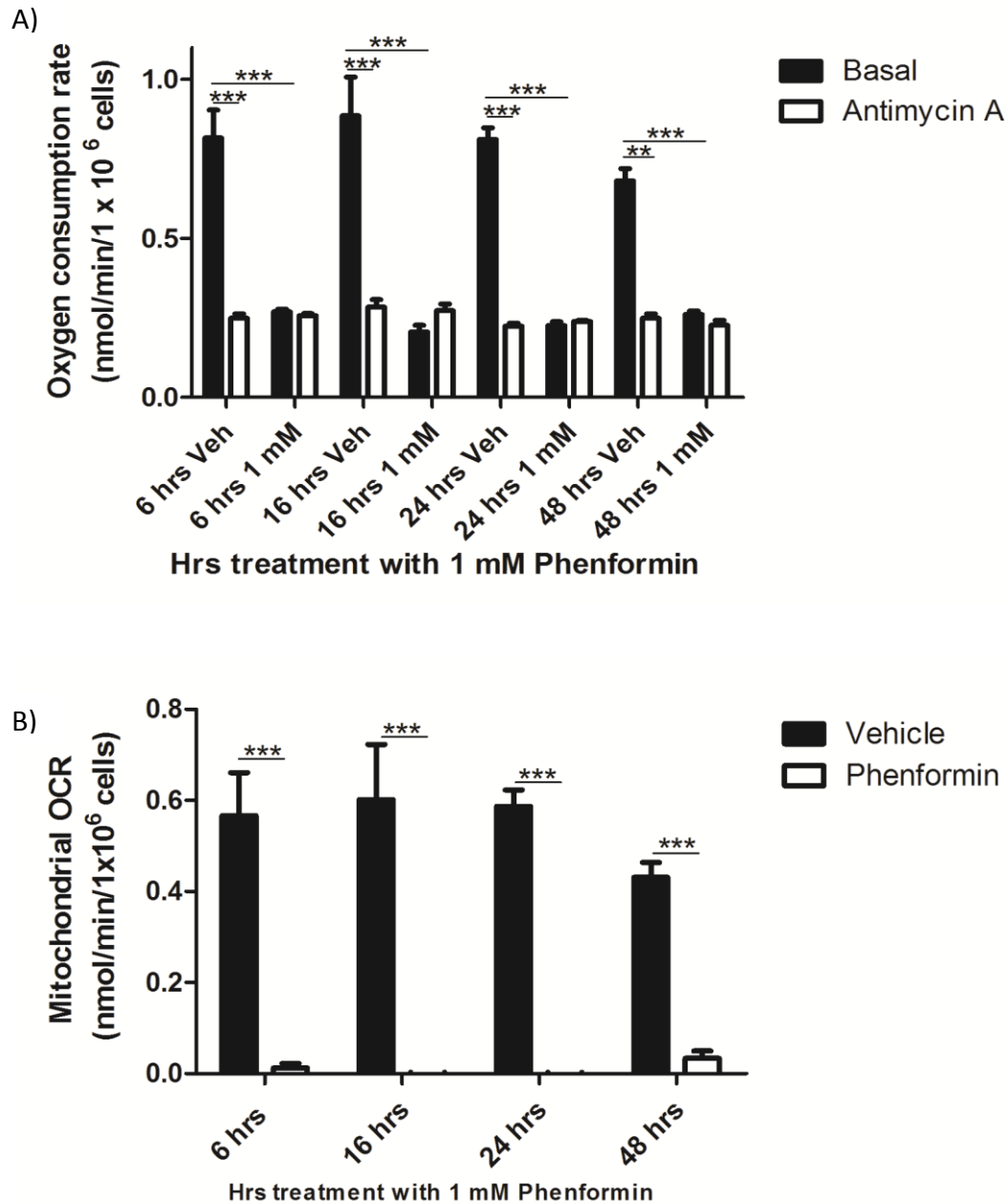


Figure 3.14 Time dependent inhibition of oxygen consumption in SHSY-5Y cells as determined by the oxygraph respirometer. Cells were treated with vehicle or phenformin 6, 16, 24 and 48h and placed in an oxygraph respirometer containing DMEM F12 media. (A) Total cellular oxygen consumption was measure and antimycin A was added to determine oxygen consumption due to oxidative phosphorylation. (B) Mitochondrial oxygen consumption (Cellular-antimycin A) within SH-SY5Y cells. Error bars represent the SEM between three separate experiments. Statistical analysis was carried out using the student t-test. ** = $p < 0.0005$, * = $p < 0.005$.

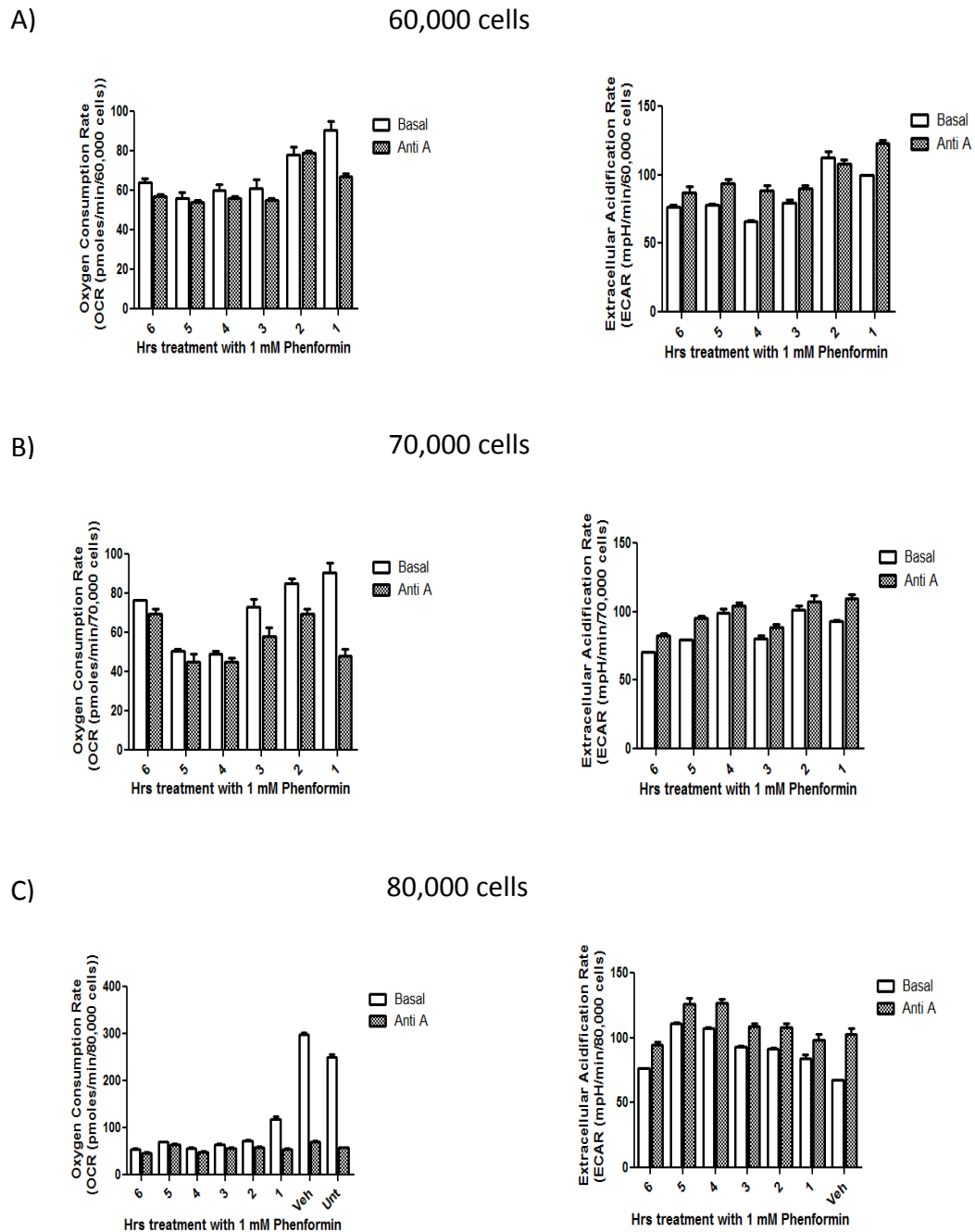


Figure 3.15 Confirmation of the seeding densities of SH-SY5Y cells for treatment of 1 mM of phenformin for 1, 2, 3, 4, 5, 6h as determined by the seahorse extracellular flux analyser. Oxygen consumption and extracellular acidification rates from cells that were seeded at A) 60,000 cells/well, B) 70,000 cells/well, C) 80,000 cells/well treated with 1 mM of phenformin for the indicated times and ran on the seahorse where the antimycin A was injected from the ports of the cartridge into the seahorse wells during the run. Error bars represent the standard error of the mean between three technical replicates.

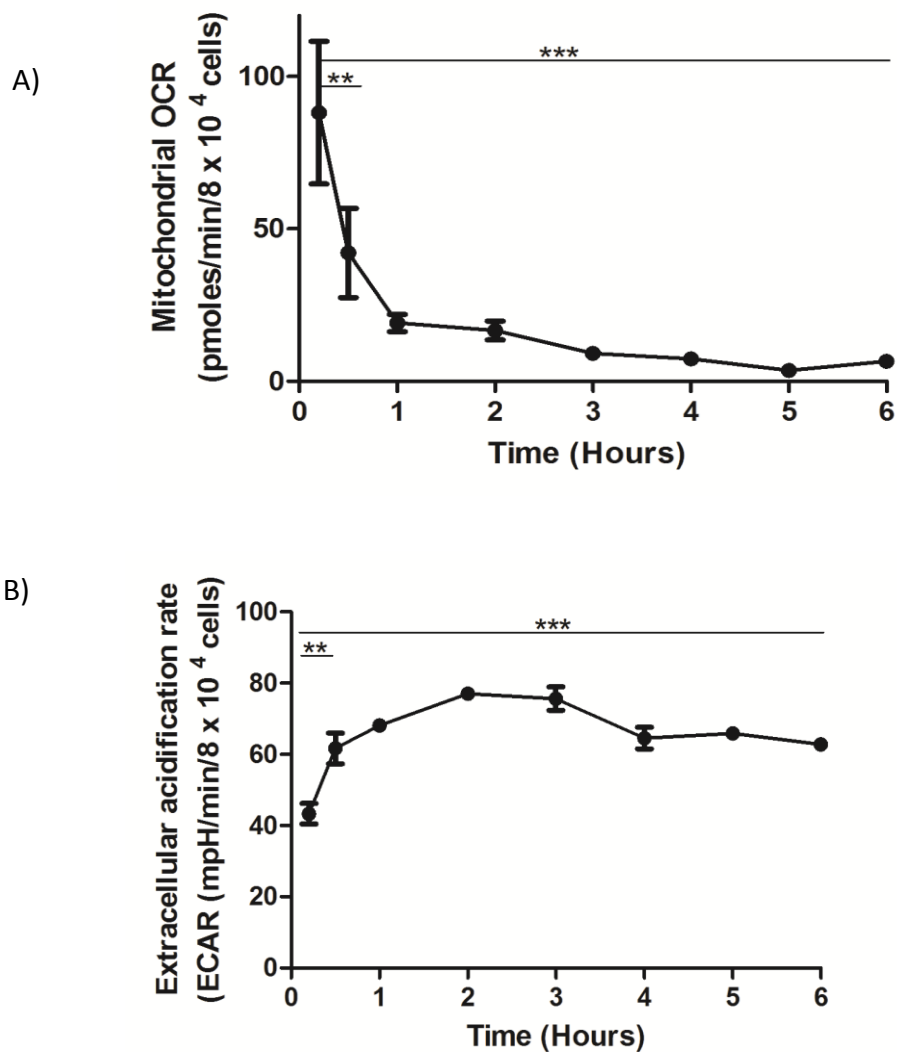


Figure 3.16 Phenformin inhibits oxygen consumption rate (OCR) and increases extracellular acidification rate (ECAR) in a time dependent manner as determined by the Seahorse flux analyser. SHSY-5Y cells were seeded at 80,000 cells/well, treated with 1 mM phenformin for the indicated times and OCR and ECAR was determined on a Seahorse Flux Analyser. (A) Time dependent inhibition of mitochondrial OCR (Cellular-antimycin A) by SHSY-5Y cells after treatment with 1 mM phenformin. (B) Time dependent increase in ECAR by SHSY-5Y cells after treatment with 1 mM phenformin. Error bars represent the SEM for three separate experiments (n = 3) each measured in triplicate. Statistical analysis was carried out using the student t-test. *** = $p < 0.0005$, ** = $p < 0.005$.

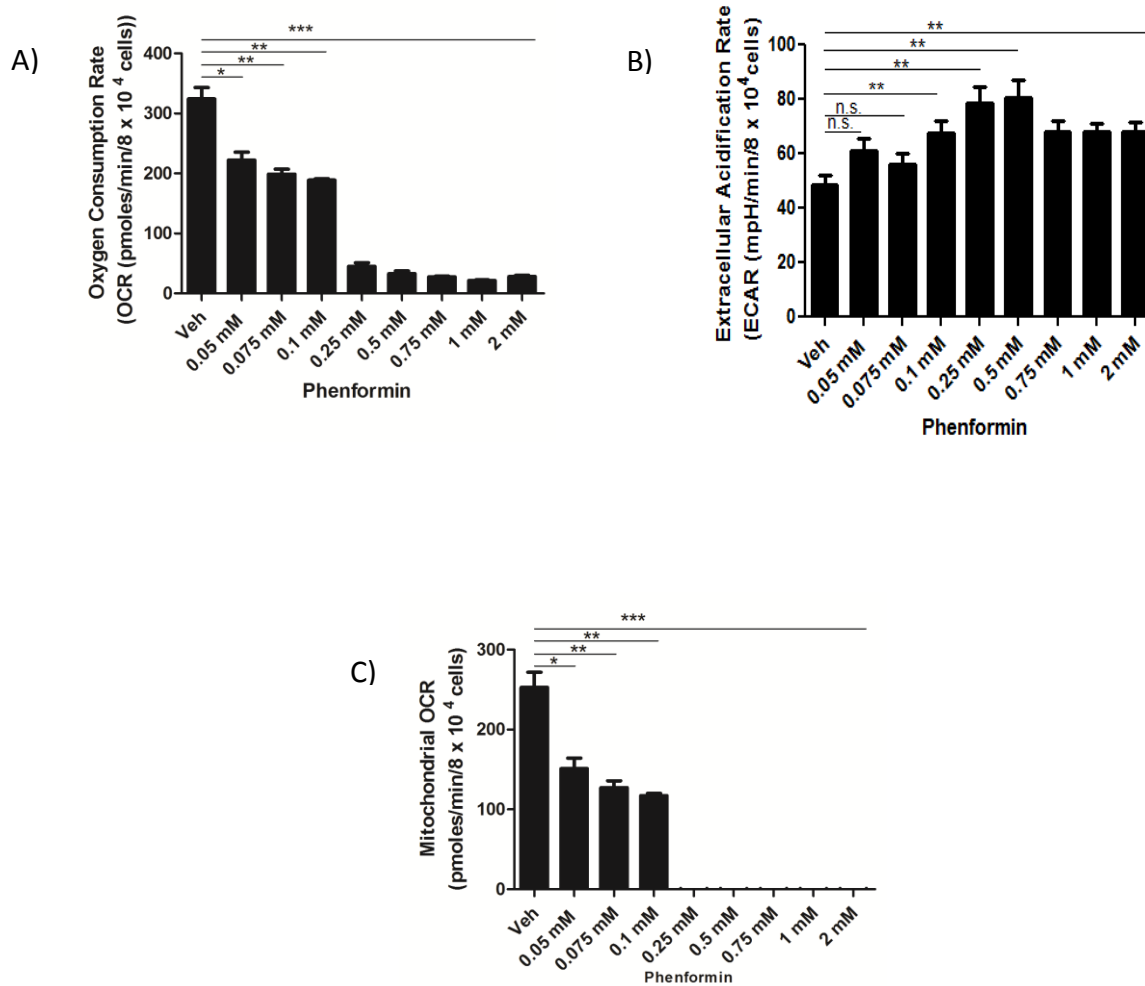


Figure 3.17 Investigation of the sensitivity of the SH-SY5Y cells to varying concentration of phenformin for 6h as determined by the seahorse extracellular flux analyser. SH-SY5Y cells were seeded at 80,000 cells/well, treated with varying concentrations of phenformin for 6h and then ran on the seahorse. A) Oxygen consumption rates B) Extracellular acidification rates C) Mitochondrial oxygen consumption were measured. Mitochondrial oxygen consumption was obtained by taking the Antimycin A OCR from the rate obtained from the basal rate. Error bars represent the standard deviation between three separate experiments. Statistical analysis was by student t-test. ***= $p < 0.0005$, ** = $p < 0.005$, * = $p < 0.05$.

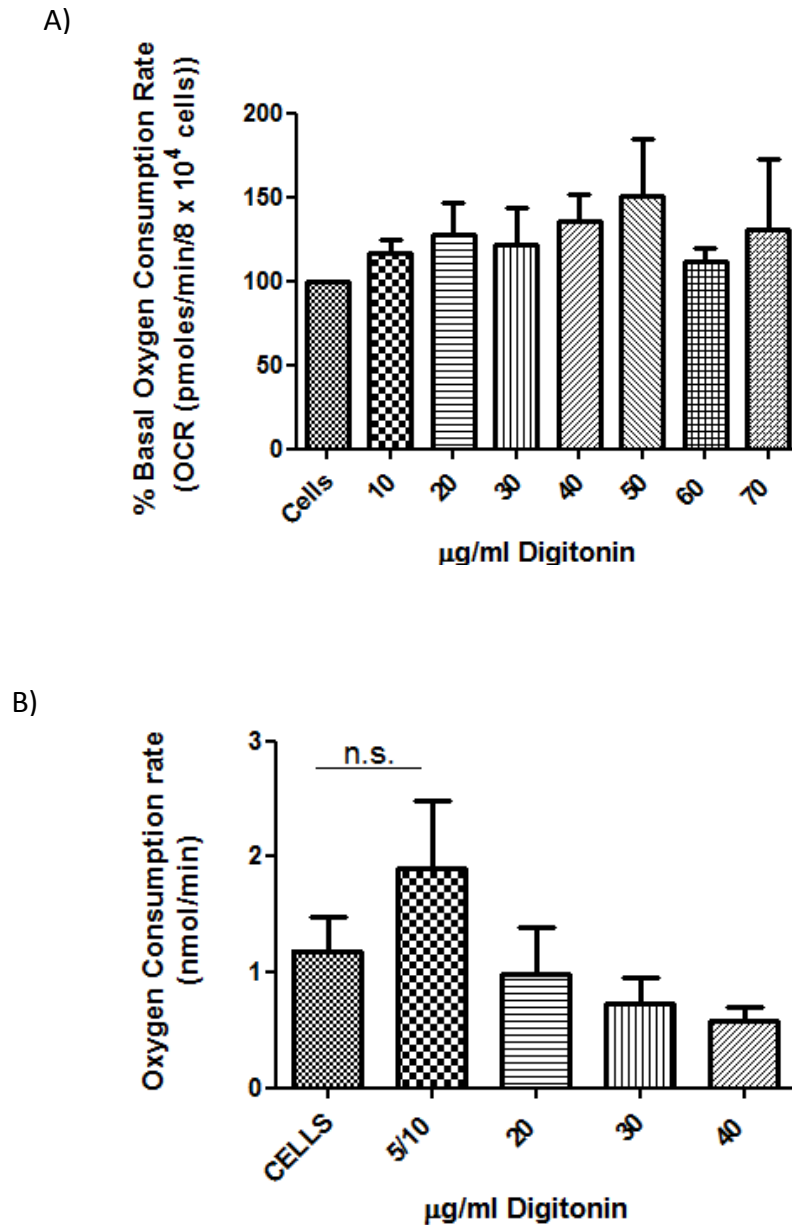
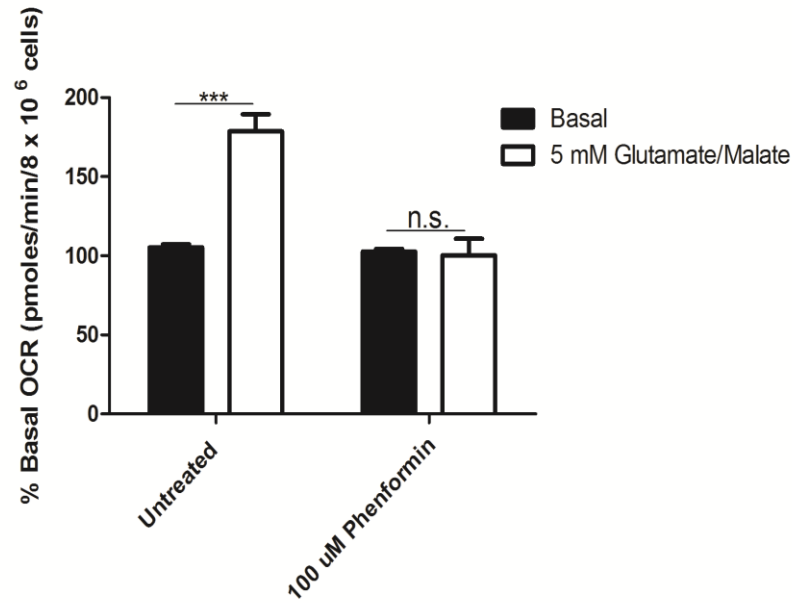


Figure 3.18 Optimisation of the concentration of digitonin needed to permeabilize SH-SY5Y cells using the seahorse extracellular flux analyser and oxygraph respirometer. A) Percentage increase in respiration after the addition of 5 mM glutamate/malate to 80,000 SH-SY5Y cells in 1 x MAS buffer containing 4 mM ADP and varying concentration of digitonin as measured by the seahorse extracellular flux analyser. B) 1 million cells were placed in the chamber of the oxygraph respirometer in 1 x MAS buffer containing 4 mM ADP and 10 mM succinate while increasing concentrations of digitonin was titrated in. Error bars represent the SEM between three separate experiments.

A)



B)

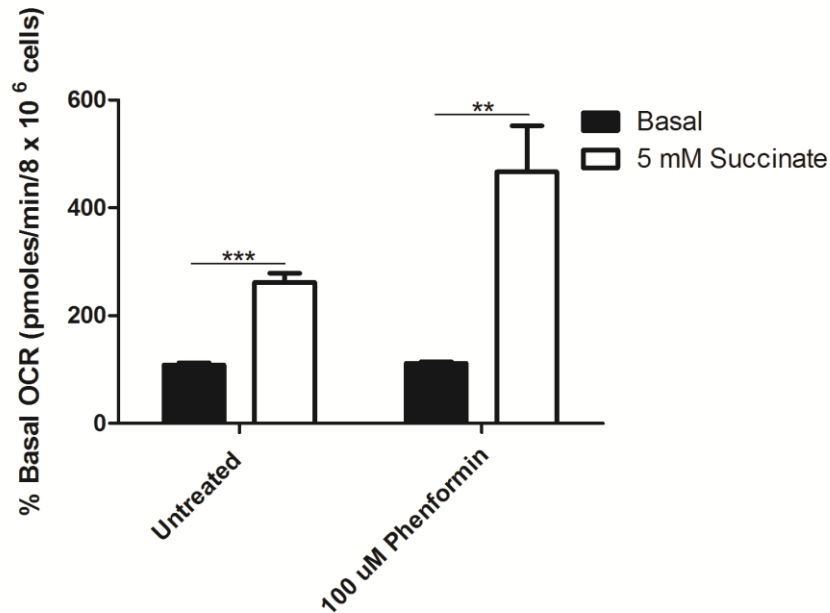
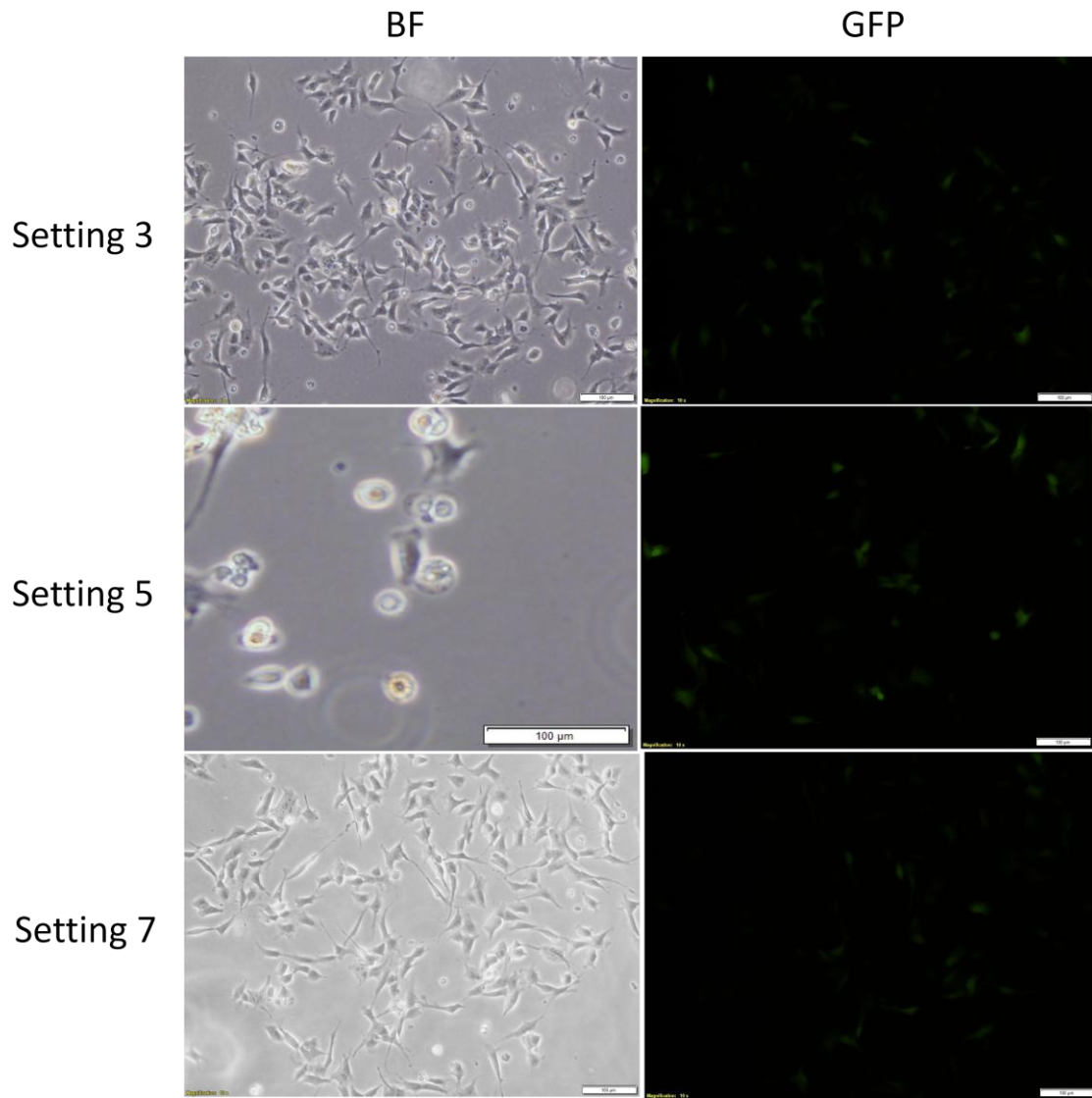
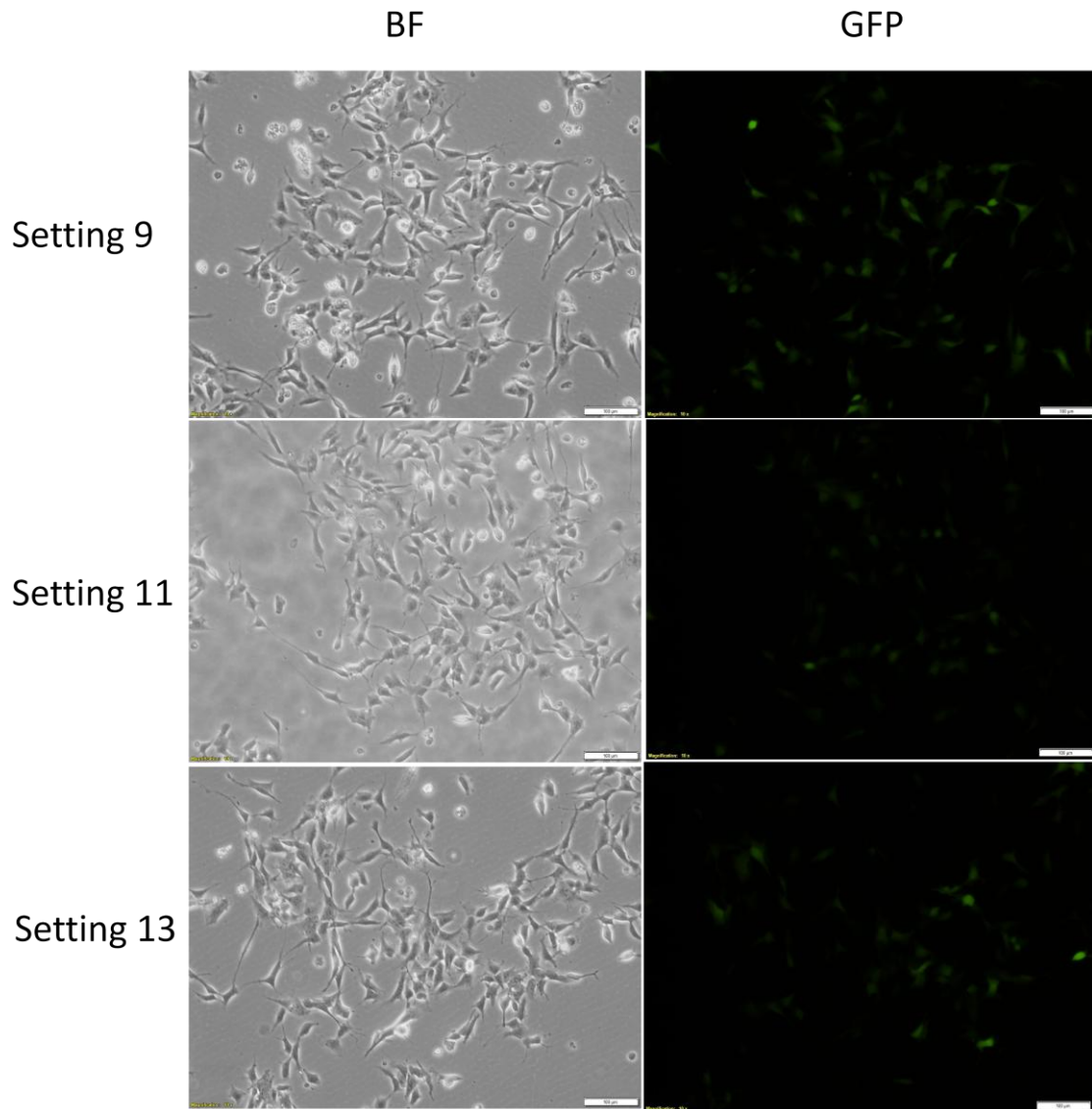
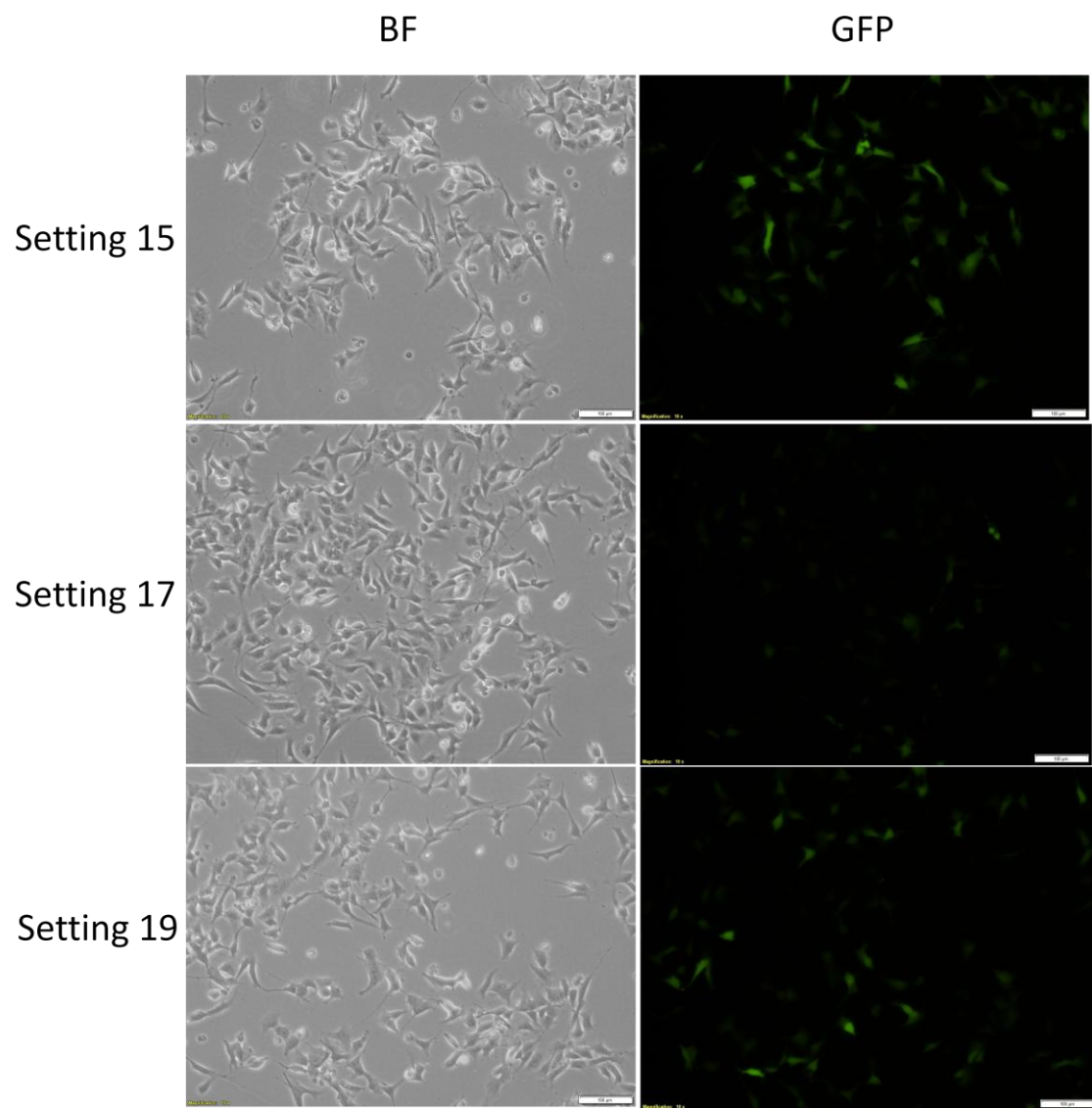


Figure 3.19 Inhibition of mitochondria complex I by phenformin in permeabilized SHSY-5Y cells as determined by the Seahorse flux analyzer. SHSY-5Y cells were plated at 80,000 cells/well in seahorse plates for 6h. Prior to analysis, the growth media was replaced with MAS buffer plus 4 mM ADP plus 50 μ g/ml of digitonin. OCR was measured using (A) 5 mM glutamate/malate (generating substrates for complex I) in the presence and absence of 100 μ M phenformin and (B) using 5 mM succinate (substrate for complex II) in the presence and absence of 100 μ M phenformin. Error bars represent the SEM between three separate experiments. Statistical analysis was carried out using the student t-test. ** = $p < 0.005$, *** = $p < 0.0005$







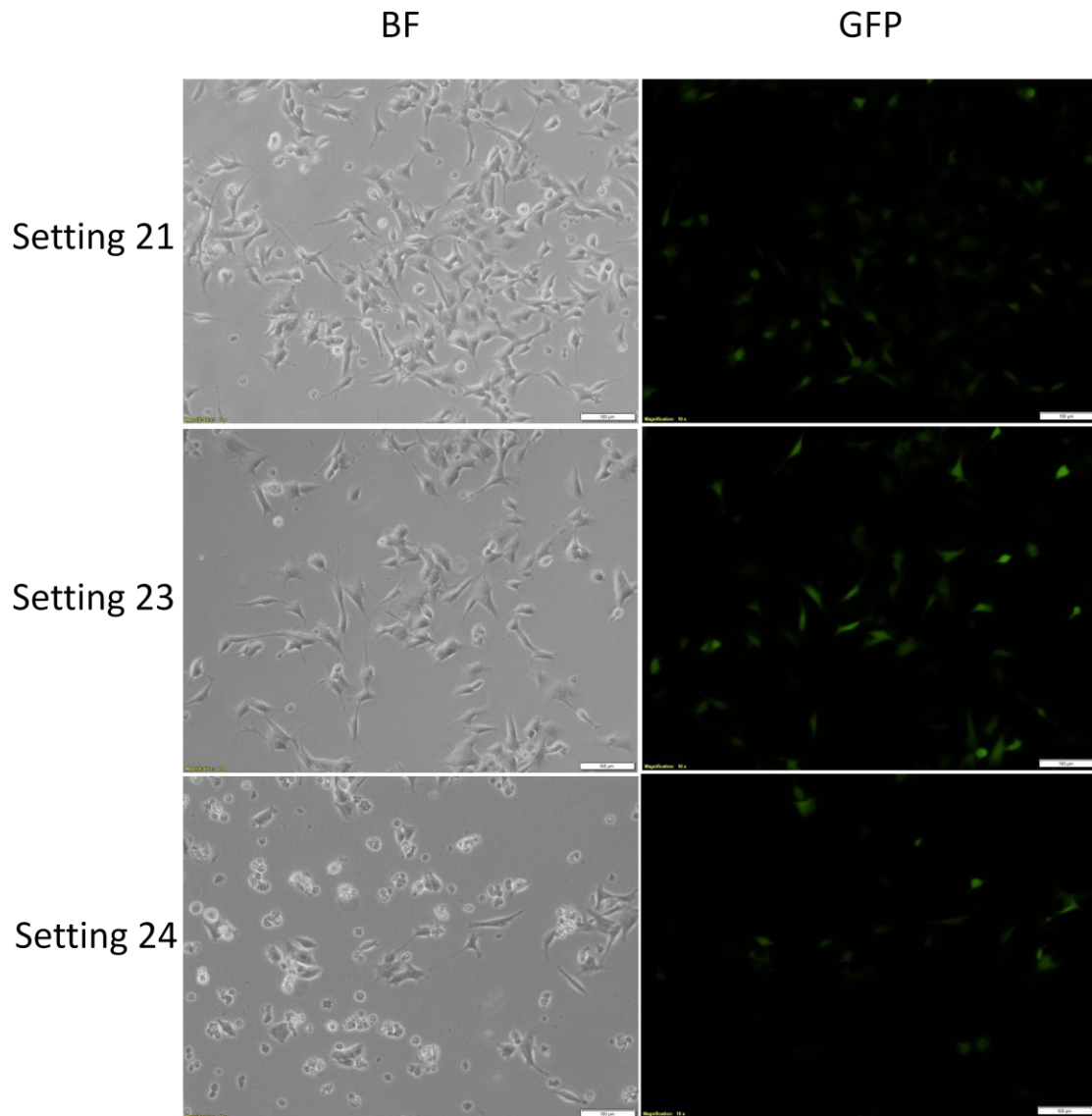


Figure 3.20 Optimisation of the transfection efficiency of the empty vector GFP tagged pAAV.MCS plasmid in SH-SY5Y cells using NEON electroporation. Cells were electroporated using various settings on the NEON electroporation system at a density of 220,000/ml and were then plated in 6 well dishes. Images were then taken on an Olympus IX81 inverted microscope at 20x magnification excited at a wavelength of 395 nm using FV10-ASW 2.0 Viewer software. Above BF = bright field, GFP = green fluorescent protein and scale bar = 100 μ m. Images are representative images of three separate experiments.

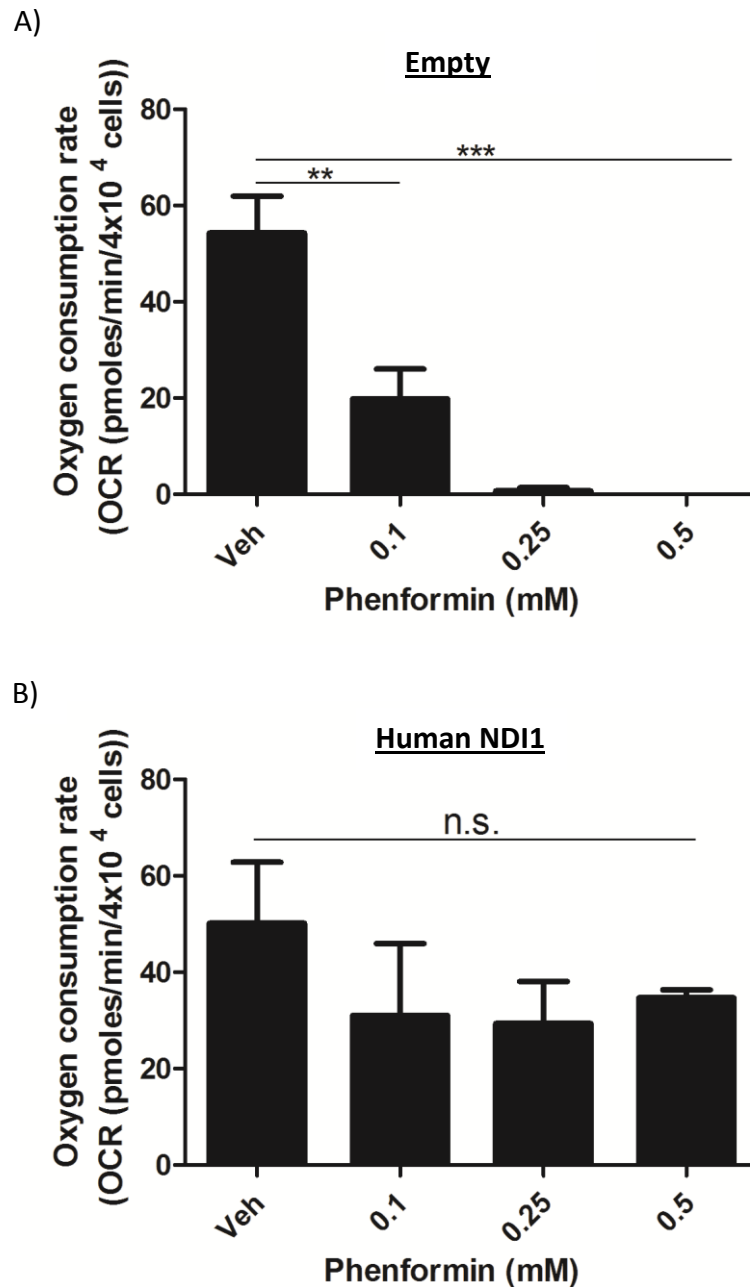


Figure 3.21 Protective effects of NDI1 plasmid transfection on SH-SY5Y cells treated with phenformin as determined by the Seahorse flux analyser. OCR was measured in SH-SY5Y cells treated with 0, 0.1, 0.25 and 0.5mM phenformin that were transfected at a density of 400,000 cells/ml with (A) an empty vector GFP tagged pAAV.MCS plasmid (B) a human derived pAAV.MCS plasmid encoding NDI1 using the NEON electroporation system. Error bars represent the SEM between three separate experiments. Statistical analysis was carried out using the student t-test. ** = $p < 0.005$, * = $p < 0.05$.

3.1.4 Decrease in Reactive Oxygen Species production as a result of phenformin treatment in SH-SY5Y cells.

The mitochondria are considered the principal sources of cellular reactive oxygen species (ROS) (Richter et al., 1995) contributing at various sites to the cellular ROS dependent upon which substrate is being oxidised (Quinlan et al., 2013). Having established that phenformin inhibits mitochondrial complex I, we set about examining the effect of phenformin on the intracellular ROS production.

SH-SY5Y cells were treated with varying concentrations of phenformin (0.05-1 mM) for 6h and then endogenous ROS production was measured by flow cytometry. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) was used to measure intracellular ROS. Vehicle treated cells served as the negative intrinsic control, N-acetyl cysteine (NAC) was used as the extrinsic negative control and hydrogen peroxide (H_2O_2) as the extrinsic positive control.

The two extrinsic controls of H_2O_2 ($p < 0.05$) and NAC ($p < 0.005$) provided the full scale deflection in our system. Treatment with phenformin caused a dose dependent decrease in ROS production (Figure 3.22).

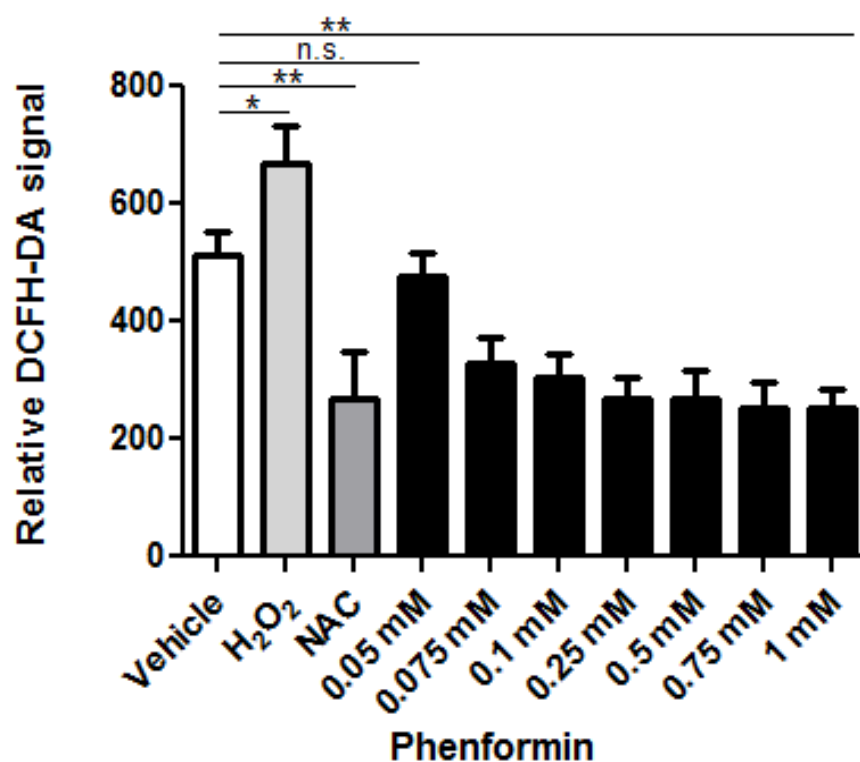


Figure 3.22 Phenformin decrease in Reactive Oxygen Species (ROS) production in SH-SY5Y cells as determined by flow cytometry. SH-SY5Y cells were seeded at a density of 110,000 cells/well, left for 24h and treated with the 5 mM N-acetylcysteine (NAC), 100 μ M H₂O₂ or the indicated concentrations of phenformin and vehicle. The cells were then stained with 10 μ M dichloro-dihydro-fluorescein diacetate (DCFH-DA). Fluorescence was analysed for 10,000 events. Error bars represent the SEM of the relative DCFH-DA signal over three separate experiments. Statistical analysis was carried out using the student t-test. ** = $p < 0.005$, * = $p < 0.05$.

3.1.5 Measurement of the effect of phenformin on ATP levels by a bioluminescence assay.

Having measured the effect Phenformin had on ROS levels, it was then decided to measure ATP levels to see how it was affecting the energy metabolism of the cell.

ATP was measured using a bioluminescence assay. This assay is based upon the requirement for ATP for the reaction to occur, so if there is ATP being generated within the cell then this reaction can proceed and be measured quantitatively. SH-SY5Y cells were plated in a 96 well plate at 30,000 cells/ml concentration. Cells were treated and then lysed using a passive lysis buffer. The lysate was added to the reaction mixture and then read on the fluorimeter. Vehicle served as the negative control, Oligomycin as the intrinsic negative control and FCCP as the intrinsic positive control. These two drugs were to serve as the full scale deflection of the possible values that could be obtained from the cells.

The assay didn't respond the way it was meant to if it did in fact measure ATP (Figure 3.23). Oligomycin an inhibitor of ATP synthase caused an increase in 'ATP' ($P > 0.0001$) and FCCP an uncoupler of the mitochondrion caused a decrease ($P > 0.0001$) which is contrary to what should appear. Phenformin had little to no effect on ATP levels. With phenformin reported to activate AMPK by increasing AMP levels and decreasing levels of ATP, we would have expected after treatment for the values of ATP to decrease. While the graph shows significant differences, these are the differences that were not expected.

Further optimisation is needed to ascertain what is happening to ATP levels when phenformin is added.

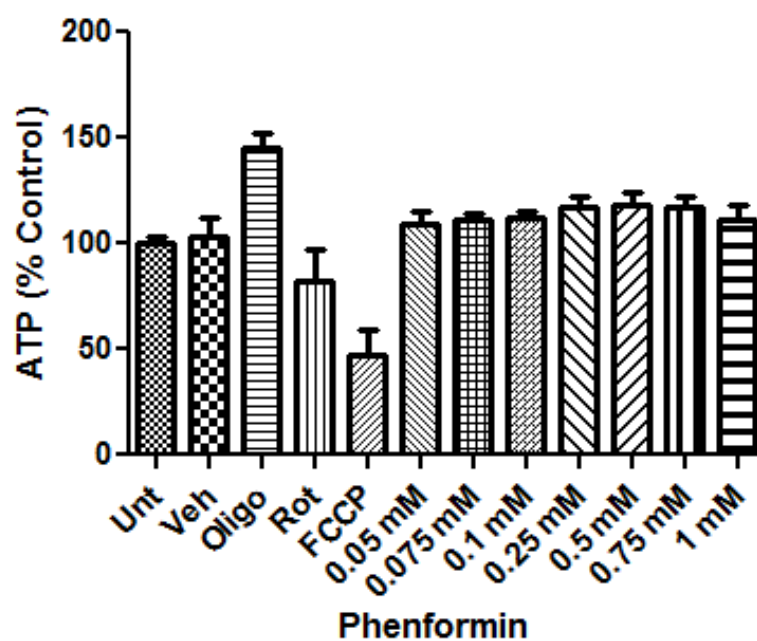


Figure 3.23 ATP expressed as a percentage of control after treatment with the various agents for 6h as determined by bioluminescence assay. SH-SY5Y cells were seeded at 30,000 cells/ml in a 96 plate and treated with the various agents for 6h. Cells were lysed and the lysate was then added to the reaction mixture. The plate was then read on a luminometer. Error bars represent the SEM between three separate experiments.

3.1.6 AMPK and ACC activation in SH-SY5Y cells as a result of treatment with phenformin over a time course as determined by western blot analysis.

Having demonstrated that phenformin inhibits complex 1 of the mitochondria in SH-SY5Y cells, we predicted that there would be increases in AMP and ADP levels on addition of phenformin thereby activating the major energy sensor of the cell, AMP-Kinase (AMPK). In addition we decided to look at a downstream enzyme phosphorylated by AMPK namely, acetyl CoA-carboxylase (ACC), as a further index of AMPK activation.

We observed activation (phosphorylation) of AMPK in SH-SY5Y cells as a result of phenformin treatment at 0.5, 1 and 6h compared to vehicle treated controls (Figures 3.24 A&B). Similarly there is activation (phosphorylation) of ACC in SH-SY5Y cells as a result of phenformin treatment compared to vehicle treated controls which becomes significant after 2h of treatment ($p < 0.05$) (Figure 3.24 C&D). The positive control 2-deoxyglucose (2-DG) increased phosphorylation of AMPK and ACC in SH-SY5Y cells as expected.

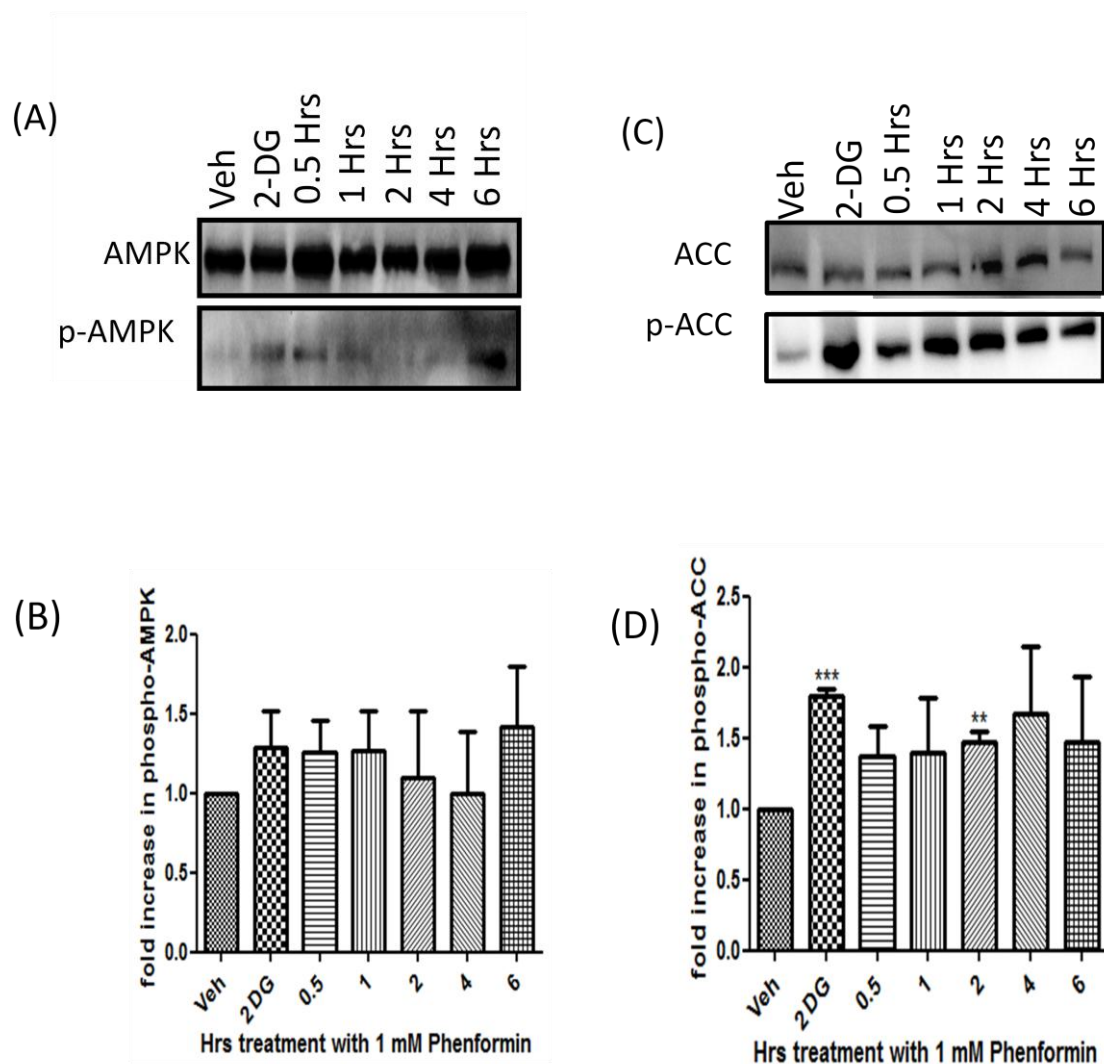


Figure 3.24 Phenformin dependent phosphorylation of AMP-kinase (AMPK) and acetylCoA carboxylase (ACC) as determined by immunoblot analysis. Whole cell lysates were prepared from confluent cultures of SH-SY5Y cells. Proteins (50 µg) were resolved in 6% or 10% SDS-PAGE gels and transferred to a PVDF membrane. Blots were probed for either AMPK, p-AMPK, ACC or p-ACC following treatment of SH-SY5Y cells with vehicle, deoxyglucose or phenformin (for 0.5, 1, 2, 4 or 6h). (A) Representative blot of AMPK activation. (B) Fold increase in p-AMPK relative to vehicle, normalised against loading control for AMPK from three separate experiments. (C) Representative blot of the activation of ACC. (D) Fold increase in p-ACC relative to vehicle normalised against loading control ACC from three separate experiments. Densitometric analysis was carried out using the image J software. Statistical analysis was carried out using the student t-test. *** = $p < 0.0005$, ** = $p < 0.005$.

3.2 Discussion

Neuroblastoma is a devastating paediatric malignancy which affects the sympathetic nervous system. Patients are initially responsive to agents such as etoposide, carboplatin, vincristine and doxorubicin but became refractory (Seeger and Reynolds, 1991, Keshelava et al., 1998) due to development of multi-drug resistance (Lennon et al., 2014). Resistance arises due to numerous mechanisms such as loss of p53, amplification of MYCN and increased expression of drug efflux pumps for the aforementioned chemotherapeutic agents are substrates for (Balayssac et al., 2005). There is evident need for alternative treatment for these resistant forms of neuroblastoma.

Recently phenformin, a former type II diabetic drug has shown significant anti-neoplastic activity in humans and animals (Caraci et al., 2003, Huang, 2008, Birsoy et al., 2014) and at lower concentrations than its analogue metformin. Phenformin's adverse effect of lactic acidosis is low enough that if it showed superior anti-neoplastic activity it would not be contraindicated for cancer treatment (Shackelford and Shaw, 2009). Phenformin is less polar and hence more lipid soluble than metformin (Goodarzi and Bryer-Ash, 2005). Consequently, phenformin can easily cross biological membrane and has been demonstrated to have a higher affinity for mitochondrial membranes when compared to metformin (Hawley et al., 2005). The efficacy of phenformin (and metformin) in the treatment of human cancers is not that clear. It has been suggested that the anticancer effect of metformin may be due to pleiotropic effects via indirect effects upon insulin and insulin growth factors or directly on the cancer cells themselves. In the present study we aim to determine whether phenformin can access and kill cancer cell directly and if so, by what mechanism. To that end we assess whether phenformin could access neuroblastoma SH-SY5Y cells and describe the effect of phenformin on the viability, metabolic flux (glycolytic vs oxidative), complex I activity, endogenous ROS production levels and AMPK and ACC activation state.

Firstly, we determined the effect of phenformin on the viability of the neuroblastoma SH-SY5Y cells was determined. The IC₅₀ for phenformin was shown to be in a similar range to that shown by others in ovarian, SKOV3 & HEY cells (Jackson et al., 2013),

melanoma, Sk-Mel-28 & WM115 cells (Yuan et al., 2013) and breast, MCF-7 & MDA-MB-231 cells (Guo et al., 2014). In our present work, metformin failed to reduce the viability of the neuroblastoma SH-SY5Y cells below 50% so an IC₅₀ value could not be obtained. A similar observation was made by Caraci et al. (2003) for the same cells. This demonstrated that phenformin was capable of accessing the neuroblastoma cells and cause cell death.

Phenformin has been shown to transiently enhance the expression of the cell cycle inhibitor p21 and to cause G1 and G2/M arrest (Caraci et al., 2003, Jackson et al., 2013). Herein we demonstrate that phenformin causes an accumulation of cells in the G1 phase of the cell cycle, indicating that phenformin inhibits cell growth by G1 cell cycle arrest at sub-cytotoxic concentrations.

Investigation into the mechanism of cell death induced by phenformin on the SH-SY5Y cells using PI and Annexin V staining showed a substantial increase in the apoptotic population of cells as a result of phenformin treatment. These findings are in agreement with other papers that have shown phenformin to cause apoptosis in non small cell lung cancer A549 cells, breast cancer MC7 & MDA-MD-231 cells and PTEN deficient tumours (Shackelford et al., 2013, Appleyard et al., 2012, Huang, 2008). To confirm that apoptosis was occurring in these cells as a result of phenformin treatment, Poly (ADP-ribose) Polymerase (PARP) cleavage expression was determined by western blotting. It has been shown that poly (ADP-ribose) is synthesized upon activation of the apoptotic pathway and it may also initiate cell autonomous necrosis. Upon the induction of cell death, many proteins are degraded by caspases (Marks and Fox, 1991), one of those being PARP-1. PARP-1 is one of the first proteins to be proteolyzed from the 116 kDa form to the 89 kDa form when apoptosis is initiated (Soldani et al., 2001). However acute activation of PARP can activate necrosis (Zong and Thompson, 2006), we have shown that PARP does not get cleaved upon phenformin treatment in the neuroblastoma SH-SY5Y cells even at the maximum dose which has also been shown by Ha and Snyder (1999) when observing necrotic cell death in fibroblasts.

Having demonstrated that phenformin is capable of inducing cell death in the SH-SY5Y cells, it was then decided to look at the effects of sub-cytotoxic concentrations of phenformin on cellular bioenergetics with view to discovering its mechanism of action. We demonstrate that phenformin significantly inhibits mitochondrial oxygen consumption at micromolar concentrations whilst increasing glycolytic flux in a dose and time dependent manner in neuroblastoma cells. Our data is consistent with the observations of Owen (2000) who demonstrated potent inhibition by phenformin of mitochondrial oxygen consumption in a dose dependent manner also at the micromolar range in hepatoma cells. They proposed the positive charge that phenformin possesses allowed it to accumulate within the matrix of mitochondria driven by the membrane potential (Davidoff et al., 1971, Bridges et al., 2014). El-Mir et al. (2000) also demonstrated a similar time dependent effect of phenformin on oxygen consumption in hepatocytes. Taken together, the data suggest that mechanism of action of phenformin is at the level of mitochondrial function.

The next question posited was whether phenformin inhibited mitochondrial oxygen consumption through inhibition of complex I. Firstly, we demonstrated that phenformin inhibited complex I substrates, but not complex II substrates, from driving mitochondrial oxygen consumption in permeabilized SH-SY5Y neuroblastoma cells. Secondly we demonstrated that expression of 'humanized' yeast NDI1 protected the SH-SY5Y cells from the inhibitory effect of phenformin on mitochondrial oxygen consumption in non-permeabilized SH-SY5Y neuroblastoma cells. We conclude that phenformin is inhibiting mitochondrial complex I. Our findings are in concordance with recent studies that have suggested that phenformin exerts its anti-tumour effects by inhibiting complex I in cancer cell lines (Birsoy et al., 2014, Wheaton et al., 2014, Owen, 2000, Miskimins et al., 2014, Dykens et al., 2008).

Having established that phenformin inhibits complex I of the mitochondria, we then determined the effect this inhibition had on reactive oxygen species production in SH-SY5Y neuroblastoma cells. We had expected phenformin to stimulate ROS production in our SH-SY5Y cells, however, we observed a dose dependent diminution of cellular ROS

production. This is in contrast to the stimulation of ROS production shown by others as a result of phenformin treatment in non small cell lung cancer A549 cells and colon cancer CT26 cells (Shackelford et al., 2013, Miskimins et al., 2014) or by another complex I inhibitor, rotenone. Rotenone is reported to act downstream of the flavin site within complex I which produces ROS (Pryde and Hirst, 2011, Ogino et al., 2007). Our data may be explained by the theory proposed by Wheaton et al. (2014) that it is probable that phenformin is acting upstream of this flavin site, inhibiting both complex 1 and ROS production.

After confirming phenformin's inhibitory action on reactive oxygen species, ATP levels were then measured. Phenformin through its inhibition of complex I of the mitochondria has been reported to decrease ATP levels (Shackelford et al., 2013, Aziz et al., 2010, Vigneri et al., 1984, Zhang et al., 2007b, Owen, 2000) whereas one group has found no effect of phenformin on ATP levels in their white fat cells (Brown et al., 1973). Our data suggests that phenformin is not having an effect on the overall ATP levels (Figure 3.20). A decrease in intracellular ATP levels with oligomycin or an increase in intracellular ATP levels with FCCP was not observed with this assay. One possible theory to explain this is the ATP synthase is working in reverse to support mitochondrial membrane potential by pumping protons out of the matrix which has been shown before in cancer (Chevrollier et al., 2011) but the fact that FCCP has been shown to work in other studies demonstrates that the ATP synthase is functioning normally. Further work is needed to confirm the validity of this result.

As we have shown that phenformin inhibits complex I, we next set out to determine whether AMPK was activated. Evidence suggests that phenformin does not act directly on AMPK but acts indirectly by increasing intracellular AMP and/or ADP concentrations (Hardie, 2013). The evidence is further ramifying by the observation that cells that had a mutation in AMPK, which renders them insensitive to AMP and ADP changes, fail to activate AMPK as a result of phenformin treatment (Hawley et al., 2010). Whether phenformin needs to activate AMPK in order to impact upon the cells proliferation or survival still remains ambiguous. Huang (2008) showed that AMPK activation was

necessary to decrease the occurrence of PTEN deficient tumours following phenformin treatment whereas Shackelford et al. (2013) showed that phenformin was useful in treating non small cell lung cancer deficient in LKB1, the major upstream kinase activating AMPK. Here we show activation of AMPK and one of its downstream targets, ACC, as a result of phenformin treatment.

In conclusion, phenformin proved effective in reducing the cell viability of the human derived neuroblastoma SH-SY5Y cancer cells, causing G1 cell cycle arrest at sub-cytotoxic concentrations and apoptosis at higher concentrations. Bioenergetic analysis showed that phenformin significantly decreased mitochondrial oxygen consumption in a dose and time dependent manner by inhibiting mitochondrial complex I. As a result of this inhibition by phenformin at sub-cytotoxic levels, endogenous ROS production is significantly decreased, ATP levels are unaffected and both AMPK and ACC become activated. We conclude that phenformin shows promise as an anti-cancer agent.

**Chapter 4: Comparative bioenergetics
of sensitive and acquired cisplatin
resistant H1299 and P31 cells.**

4.0 Introduction

In this Chapter it was proposed to investigate the metabolism of non small cell lung cancer cell line, H1299 and a mesothelioma cell line, P31 that exhibited acquired resistance to cisplatin. Cisplatin (cis-diamminedichloroplatinum (II)) is one of the most potent chemotherapeutic drugs used in the clinic to treat solid cancers (Andrews and Howell, 1990). Its cytotoxicity has been generally attributed to its interaction with DNA to form intra-strand crosslinks (Siddik, 2003, Behnam-Motlagh et al., 2010a, Johansson et al., 2006) activating several pathways ending in the induction of mitochondrial apoptosis (Patel et al., 2011). Some studies have indicated that mitochondrial DNA adducts may be more common than nuclear DNA adducts in model cell line systems as a result cisplatin treatment (Murata et al., 1990, Olivero et al., 1995).

As cisplatin impacts upon on a multitude of transduction pathways, there are subsequently a whole plethora of mechanisms of resistance (Siddik, 2003) that can be intrinsic or acquired. The molecular mechanisms underlying cisplatin resistance remains unclear (Stewart, 2007).

One avenue that could be exploited to uncover the cause of cisplatin resistance is investigating the metabolic differences between the cisplatin-sensitive and -resistant cancer cells. In recent years, cancer therapy directed at targeting specific metabolic pathways is increasingly an area of active research but is still in its infancy (Jones and Schulze, 2012). Altered metabolism of cancer is a known hallmark of cancer (Hanahan and Weinberg, 2011) and is something that can be exploited in the treatment of drug resistance. Modifications to fundamental processes like glucose, glutamine, nucleotide, fatty acid and phospholipid metabolism induced by the growth demands of the cancers, enable them to modify their growth needs and thrive in a hostile environment (Zong et al., 2002, Thomson et al., 2008). Cisplatin resistant cells have been known to exhibit reduced uptake of nutrients (Shen et al., 1998). Significant perturbations in mitochondrial membrane potential, morphology, reduction in oxygen consumption and glucose uptake were observed by Liang et al. (2008) in their cisplatin resistant adenocarcinoma and hepatoma cells. They found that SIRT1, a cytosolic nutrient

metabolism regulator, mediated cisplatin resistance in their cells in times of nutrient stress.

One other possible mechanism of cisplatin resistance is the increased efflux of cisplatin through multidrug resistance proteins (Siddik, 2003) e.g. ABCB1/MDR1. Overexpression of an enzyme glucosylceramide synthase (GCS), the first enzyme in the glycosphingolipids (GSL) synthesis is also associated with multidrug resistance. Cells that express ABCB1/MDR1 have been shown to also express glucosylceramide (Veldman et al., 2002). ABCB1/MDR1 can function to translocate glucosylceramide into the Golgi apparatus for neutral GSL synthesis, including the synthesis of globotriasosylceramide (Gb₃). Behnam-Motlagh et al. (2010b) demonstrated a correlation between increased expression of Gb₃ and acquired cisplatin resistance in malignant pleural mesothelioma cells (P31) and non small cell lung cancer cells (H1299 cells). The aim of this study was to determine if there were any metabolic differences between the sensitive and resistant non small cell lung cancer and mesothelioma cells (H1299s & H1299r, P31s & P31r respectively).

4.1. Results

4.1.1 Determination of the IC₅₀ values for cisplatin in H1299s, H1299r, P31s and P31r cells

The P31 cell line was originally established from a male patient with a pleural malignant mesothelioma tumor (Marklund et al., 1982). A batch of these cells were maintained in increasing concentrations of cisplatin-containing medium (a cytostatic drug used as single or in combination therapy for the treatment of malignant mesothelioma). After four months the cells had acquired a four-fold increased resistance to cisplatin (Janson et al., 2008). For simplicity the wild type cells are called P31s (sensitive) and the resistant cell line P31r.

H1299 cells were established from a lymph node metastasis of the non small cell lung cancer (NSCLC) (Giaccone et al., 1992) and this cell line was similarly maintained in increasing concentrations of cisplatin until it acquired a five-fold increased resistance to cisplatin (Johansson et al., 2009). For simplicity the wild type cells are called H1299s (sensitive) and the resistant cell line H1299r.

This study sought to confirm the resistance of the H1299r and P31r cells when compared to the sensitive cells by determining their IC₅₀ values using the alamar blue viability assay. Cells were treated with vehicle (0.9% NaCl) or varying concentrations of cisplatin (50 nM -100µM) for 72 hr.

As seen in figure 4.1, cisplatin decreased the viability of H1299s, H1299r, P31s and P31r cells in a dose-dependent manner with the maximum cytotoxic effect being observed with 100 µM of cisplatin. The IC₅₀ value for cisplatin in the H1299 cells was 7.6 µM (Figure 4.1A) for the sensitive cells and 62 µM (Figure 4.1B) for the resistance cells. The IC₅₀ value for cisplatin in the P31 cells was 5.8 µM (Figure 4.1D) for the sensitive cells and 17.7 µM (Figure 4.1E) for the resistance cells. Thus the H1299 resistant cells demonstrated a 10-fold resistance to cisplatin compared to the sensitive cells whereas the P31 resistant cells showed a 3-fold resistance to cisplatin compared to the sensitive

cells. Furthermore Johansson et al. (2009) suggest that the cisplatin resistant H1299r and P31r cells also express the ABCB1 transporter (i.e. p-glycoprotein/MDR1). In light of that information, determination whether the ABCB1 transporter might be a factor in determining cisplatin resistance in these cells was undergone. We therefore treated the cells with 100 μ M verapamil (an ABCB1 transporter inhibitor)(Johansson et al., 2009, Feilchenfeldt et al., 2004) for one hour prior to treatment of the H1299r and P31r cells with vehicle (0.9% NaCl) or varying concentrations of cisplatin (50 nM -100 μ M) for 72h. Verapamil did not render the resistant cells more sensitive to cisplatin by any noticeable extent (Figure 4.1C&F). Ironically this lack of effect of verapamil on P31r and H1299r cisplatin sensitivity, was also observed by Johansson et al. (2009) and taken together, sheds doubt on whether the ABCB1/MDR1 transporter is involved in cisplatin resistance in P31r or H1299r cells.

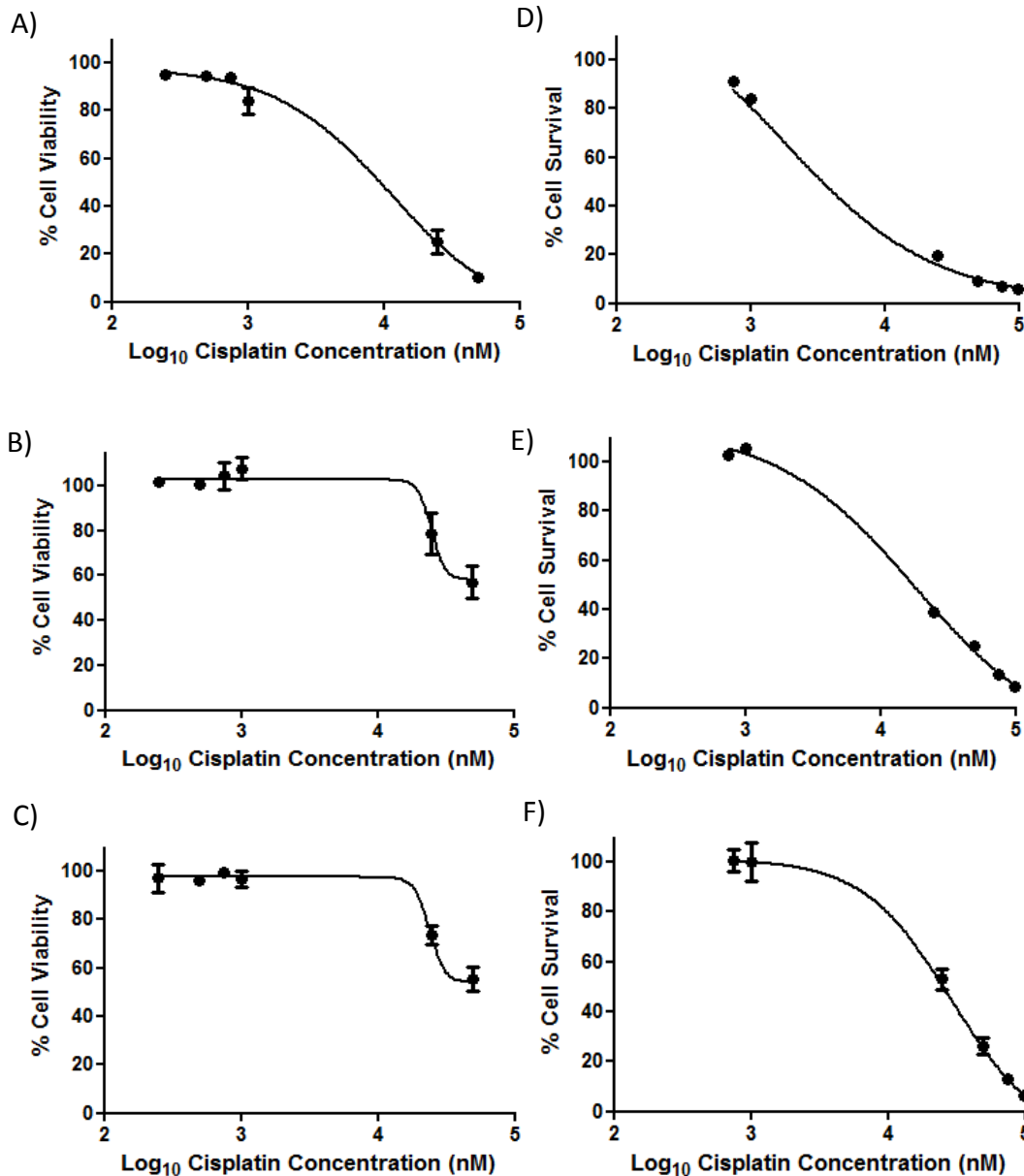


Figure 4.1 The effect of cisplatin and verapamil pre-treatment on the viability of H1299s, H1299r, P31s and P31r cells as determined by the alamar blue viability assay. Cells were seeded in 96 well plates at the following densities A) H1299s 2,000 cells/ well. B) H1299r 6,000/cell. C) H1299r 6,000/cell and were pre-treated with 100 μM verapamil for one hour. D) P31s 2,000 cells/ well. E) P31r 2,000 cells/ well. F) P31r 2,000 cells/ well and were pre-treated with 100 μM verapamil for one hour. All cells were treated with vehicle (0.9% NaCl) or varying concentrations of cisplatin (250 nM -100μM) for 72h. Alamar blue was added and cells were incubated in the dark for 5 h. The fluorescence was read at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a micro plate reader. Data expressed as % cell viability of vehicle treated controls. IC₅₀ values represent the concentration of drug required to reduce viability by 50 %. Data are expressed as mean plus or minus standard error of the mean from three separate experiments, performed in triplicate.

4.1.2 Confirmation of globotriaosylceramide (Gb3) expression in H1299s, H1299r, P31s and P31r cells by flow cytometry

Globotriaosylceramide (Gb3, CD77) is a trisaccharide linked to a lipid present within cell membranes and is expressed in several cancer cell lines including breast, ovarian and colon (LaCasse et al., 1999, Gariépy, 2001, Kovbasnjuk et al., 2005). Gb3 is known to function as a cell surface receptor for verotoxin-1 (VT-1 or Shiga Like Toxin-1) synthesized by pathogenic strains of *Escherichia Coli* (Cimen et al., 2010). Gb3's expression was shown by Kovbasnjuk et al. (2005) to correlate with invasiveness and metastatic potential in a model of colon cancer. Indeed, our collaborators in the Benham-Motlagh lab in Umeå University, Sweden were able to correlate the acquisition of resistance with an increase in Gb3 expression (Johansson et al., 2009).

We sought to confirm this Gb3 expression in P31r and H1299r cells using an Accuri C6 flow cytometer and an anti-Gb3 antibody conjugated to the fluorophore Alexo 647. As can be seen in figure 4.2, the H1299r cells have a 10-fold increased Gb3 expression on their cell surface compared to H1299s ($P < 0.0005$). Similarly the P31r cells have a 3-fold increased Gb3 expression on their cell surface compared to P31s ($P < 0.005$). These results correlate with differences in IC_{50} values between the sensitive and resistant cells and confirm that the increase in Gb3 expression correlates with the acquisition of resistance.

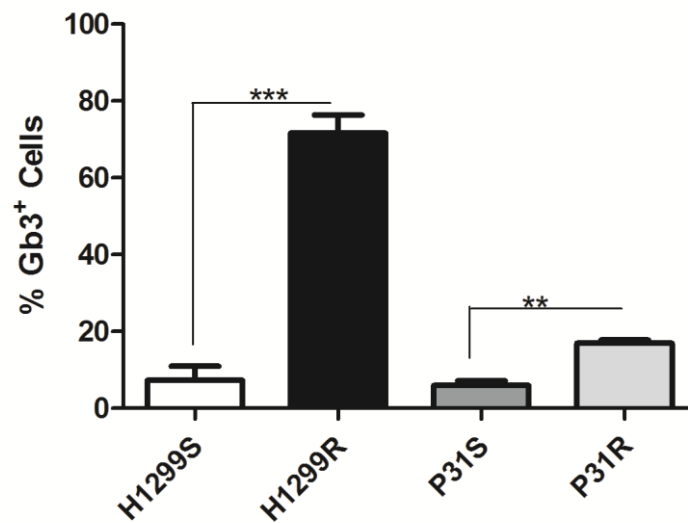


Figure 4.2 The expression of Gb3 in H1299s, H1299r, P31s and P31r cells as determined by flow cytometry. Cells were seeded at a density of 125,000 cells/well and left for 24h. The cells were pelleted and then resuspended in FACS buffer containing Fc block for 10 minutes. 2.5 μ l of Alexa647-Gb3 (CD77) was then added for 20 min. Cells were washed in FACS buffer and then analysed. Fluorescence was analysed for 10,000 events. Data are expressed as mean plus or minus standard error of the mean for the expression of Gb3 over three separate experiments. Statistical analysis was carried out using the student t-test. *** = $p < 0.0005$, ** = $p < 0.005$.

4.1.3 Determination of the susceptibility of H1299s, H1299r, P31s and P31r cells to verotoxin-1.

Gb3 functions as a receptor for verotoxin-1, a shiga like toxin derived from *E.Coli* bacteria (Cimen et al., 2010). Our collaborators in the Benham-Motlagh laboratory showed that the resistant cells showed a particular susceptibility to verotoxin-1 treatment due to their Gb3 expression (Johansson et al., 2009). They proposed that a sub-lethal dose of verotoxin-1 binding to Gb3 (in lipid rafts) of resistant cells, would result in these cells being more susceptible to cisplatin treatment.

We sought to replicate what the Benham-Motlagh laboratory data (Johansson et al., 2009), and plated cells at the same density (20,000 cells/well), incubated with the same concentrations of verotoxin-1 and assessed the viability of the cisplatin resistant cells after 72h of treatment. Unfortunately, our data differed substantially from the observations in Johansson et al. (2009). They demonstrated significantly ($P < 0.05$) enhanced sensitivity to verotoxin-1 in the resistant cells, but there was no clear difference in verotoxin-1 sensitivity in our data (Figure 4.3 A, C, E & G). In order to try and detect verotoxin-1 sensitivity, we reduced the cell density to that used for our IC_{50} experiments. It should be noted that the original cell density used of 20,000 cells/well (Johansson et al., 2009) resulted in cells that were too confluent and left no room for growth over the 72h time period. However, the reduction in cell density did not affect the vulnerability of the P31r and H1299r cells to verotoxin-1 (Figure 4.3 B, D, F & H). Lingwood et al. (1998) reported that the presence of 2 mM sodium butyrate in the medium augmented the sensitivity of various cell lines to verotoxin-1 by 5000-fold. This increased sensitivity to verotoxin-1 was as a result of intracellular targeting of verotoxin-1 to around the nucleus. To determine whether we got any enhanced response to verotoxin-1, sodium butyrate was added to the medium. There was no significant increase in susceptibility to verotoxin in the H1299 and P31 cells in the presence of sodium butyrate (Figure 4.4).

Next we wanted to determine if the dual treatment of verotoxin-1 and cisplatin had an additive or synergistic cytotoxic effect. There was no significant enhancement of cytotoxicity in either sensitive or resistant H1299 and P31 cells with the combination treatment even at difference cell densities (Figure 4.5 & 4.6). The addition of sodium butyrate, in the presence of verotoxin-1 and cisplatin, into the media does not appear to significantly change the sensitivity of the H1299 and P31 cells to the dual treatment of cisplatin and verotoxin-1 (Figure 4.7). This discrepancy between our data and the data in (Johansson et al., 2009) could be due to different batches of verotoxin-1 or the different viability assay used (fluorometric microculture cytotoxicity assay (FMCA)).

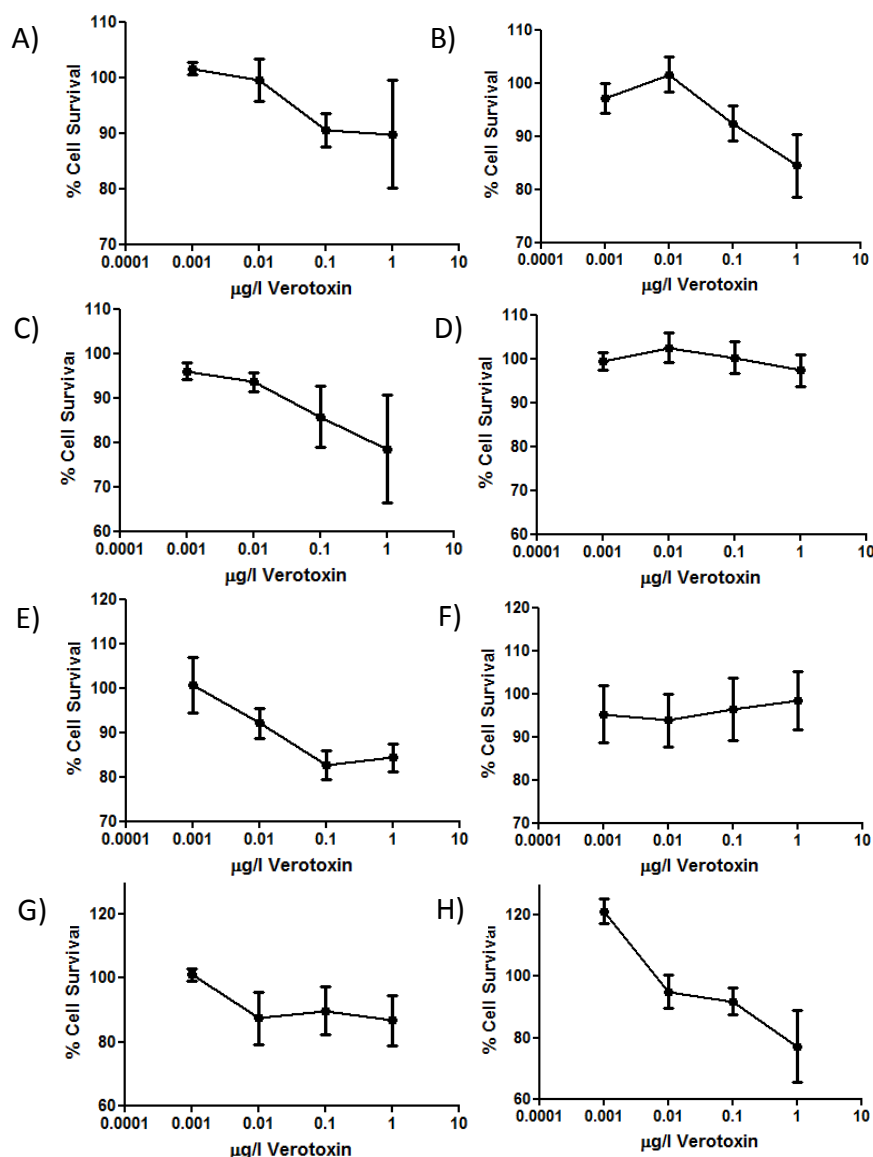
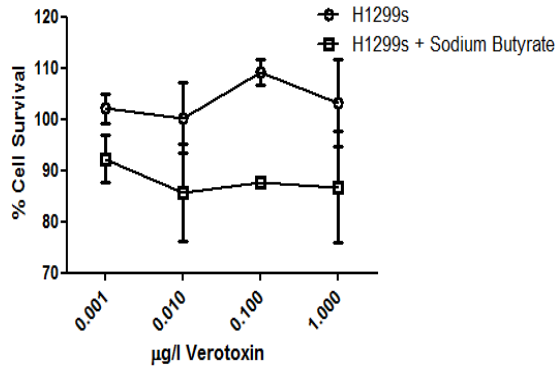
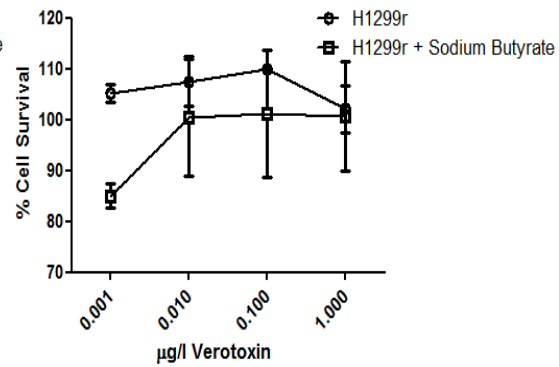


Figure 4.3 The effect of verotoxin-1 on the viability of the H1299s and H1299r cells as determined by the alamar blue viability assay. Cells were seeded in 96 well plates at the following densities A) H1299s 20,000 cells/well. B) H1299s 2,000 cells/well. C) H1299r 20,000 cells/well. D) H1299r 6,000 cells/well. E) P31s 20,000 cells/well. F) P31s 2,000 cells/well. G) P31r 20,000 cells/well. H) P31r 2,000 cells/well. All the cells were then treated in triplicate with with vehicle (dH₂O) or varying concentrations of verotoxin-1 (0.001-1 µg/L) for 72h. Alamar blue was added and cells were incubated in the dark for 5h. The fluorescence was read at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a micro plate reader. Data expressed as % cell viability of vehicle treated controls. Data are expressed as mean plus or minus standard error of the mean for three separate experiments.

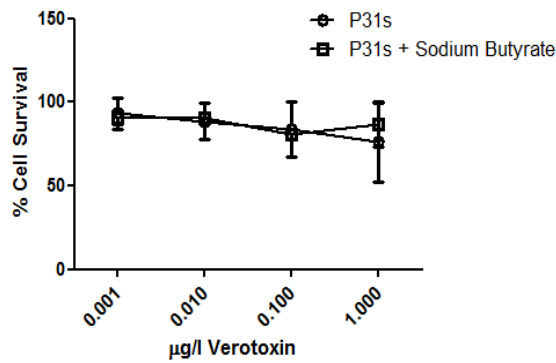
A)



B)



C)



D)

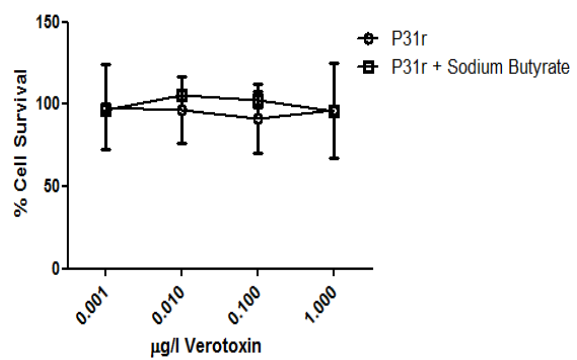


Figure 4.4 The effect of verotoxin-1 with and without and sodium butyrate on the viability of the H1299s, H1299r, P31s and P31r cells as determined by the viability assay, alamar blue. Cells were seeded in 96 well plates with and without 2 mM sodium butyrate at the following densities A) H1299s 2,000 cells/well B) H1299r 6,000/cell. C) P31s 2,000 cells/well. D) P31r 2,000 cells/well. All the cells were then treated in triplicate with with vehicle (dH₂O) or varying concentrations of verotoxin-1 (0.001-1 µg/L) for 72 h. Alamar blue was added and cells were incubated in the dark for 5h. The fluorescence was read at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a micro plate reader. Data expressed as % cell viability of vehicle treated controls. Data are expressed as mean plus or minus standard error of the mean for three separate experiments.

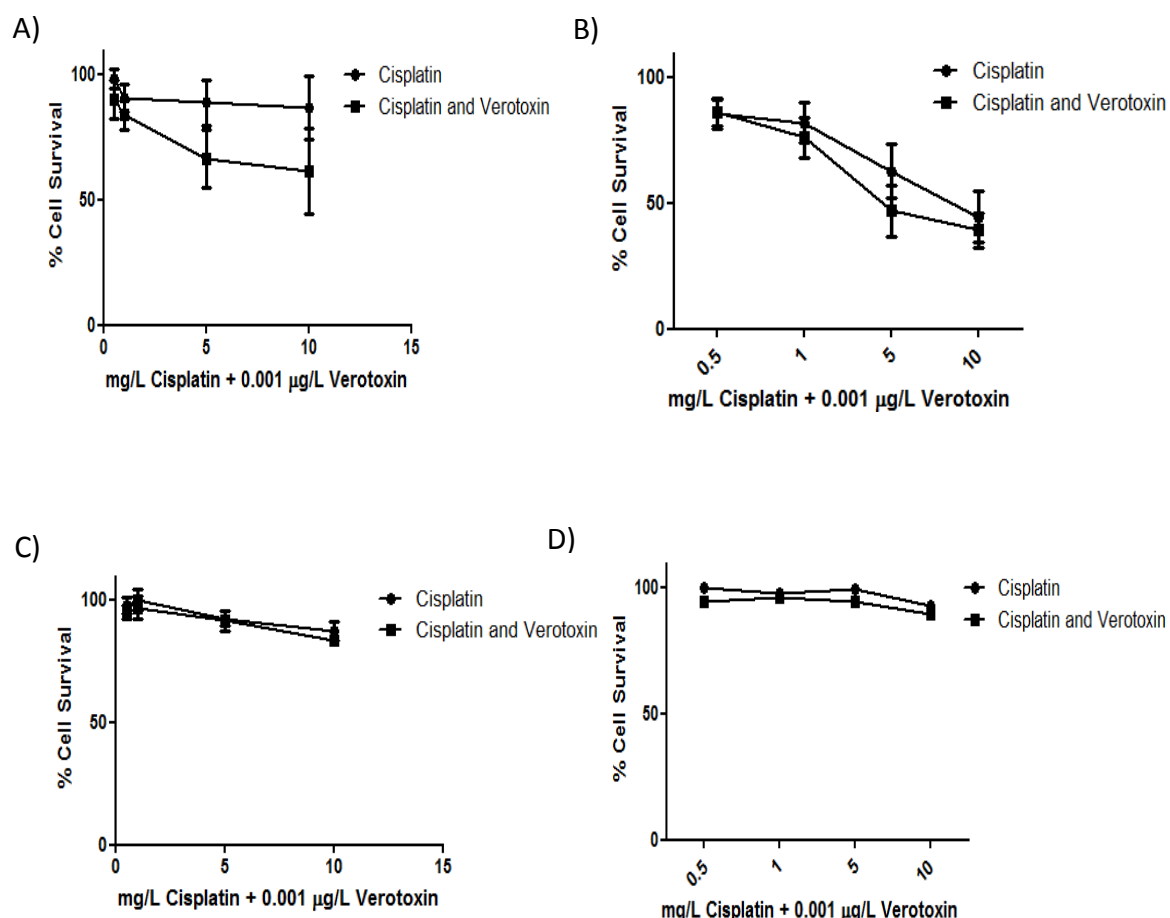


Figure 4.5 The effect of the combination treatment of verotoxin-1 and cisplatin on the viability of the H1299s and H1299r cells as determined by the alamar blue viability assay. Cells were seeded in 96 well plates at the following densities A) H1299s 20,000 cells/well. B) H1299s 2,000 cells/well. C) H1299r 20,000 cells/well. D) H1299r 6,000 cells/well. All the cells were then treated in triplicate with with vehicle (dH₂O) or varying concentrations of cisplatin (0.5-10 mg/L) with and without 0.001 µg/ml of verotoxin for 72h. Alamar blue was added and cells were incubated in the dark for 5h. The fluorescence was read at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a micro plate reader. Data expressed as % cell viability of vehicle treated controls. Data are expressed as mean plus or minus standard error of the mean for three separate experiments.

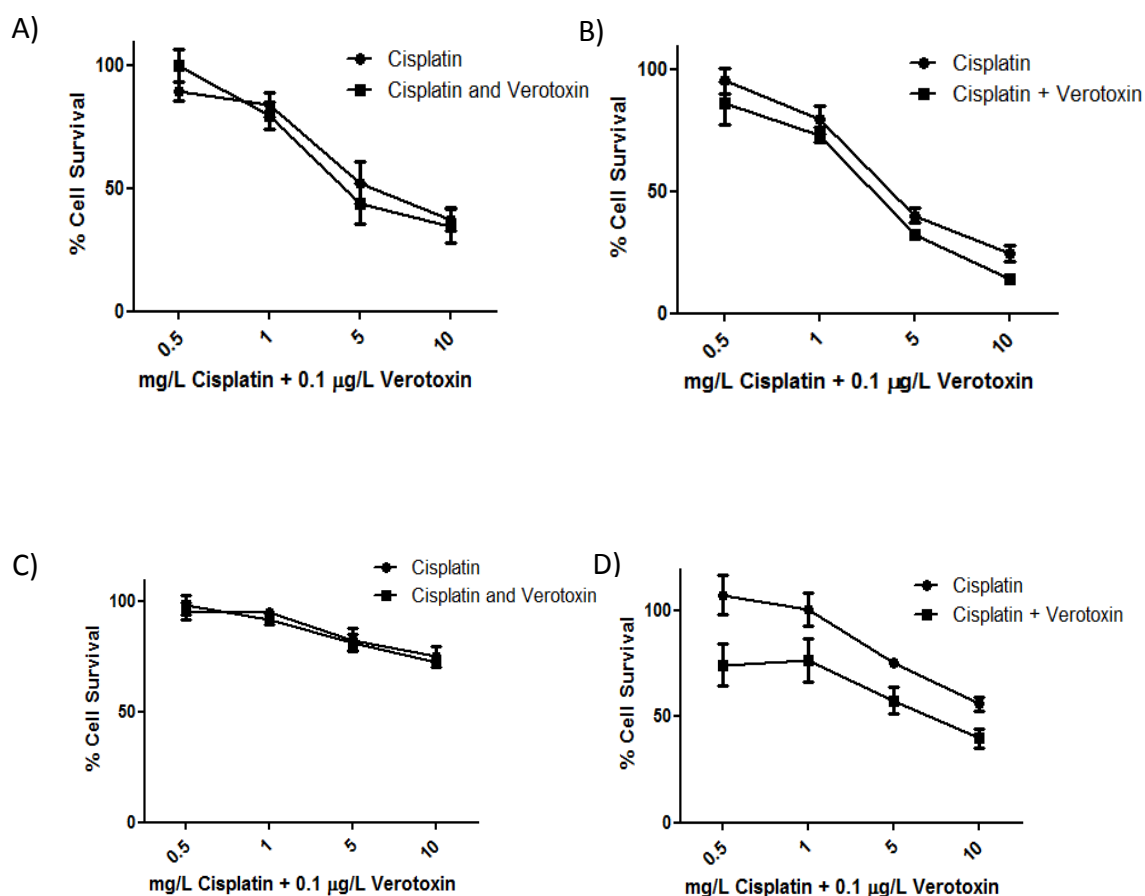


Figure 4.6 The effect of the combination treatment of verotoxin-1 and cisplatin on the viability of the P31s and P31r cells as determined by the alamar blue viability assay. Cells were seeded in 96 well plates at the following densities A) P31s 20,000 cells/well. B) P31s 2,000 cells/well. C) P31r 20,000 cells/well. D) P31r 2,000 cells/well. All the cells were then treated in triplicate with with vehicle (dH₂O) or varying concentrations of cisplatin (0.5-10 mg/L) and 0.1 µg/ml of verotoxin for 72h. Alamar blue was added and cells were incubated in the dark for 5h. The fluorescence was read at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a micro plate reader. Data expressed as % cell viability of vehicle treated controls. Data are expressed as mean plus or minus standard error of the mean for three separate experiments.

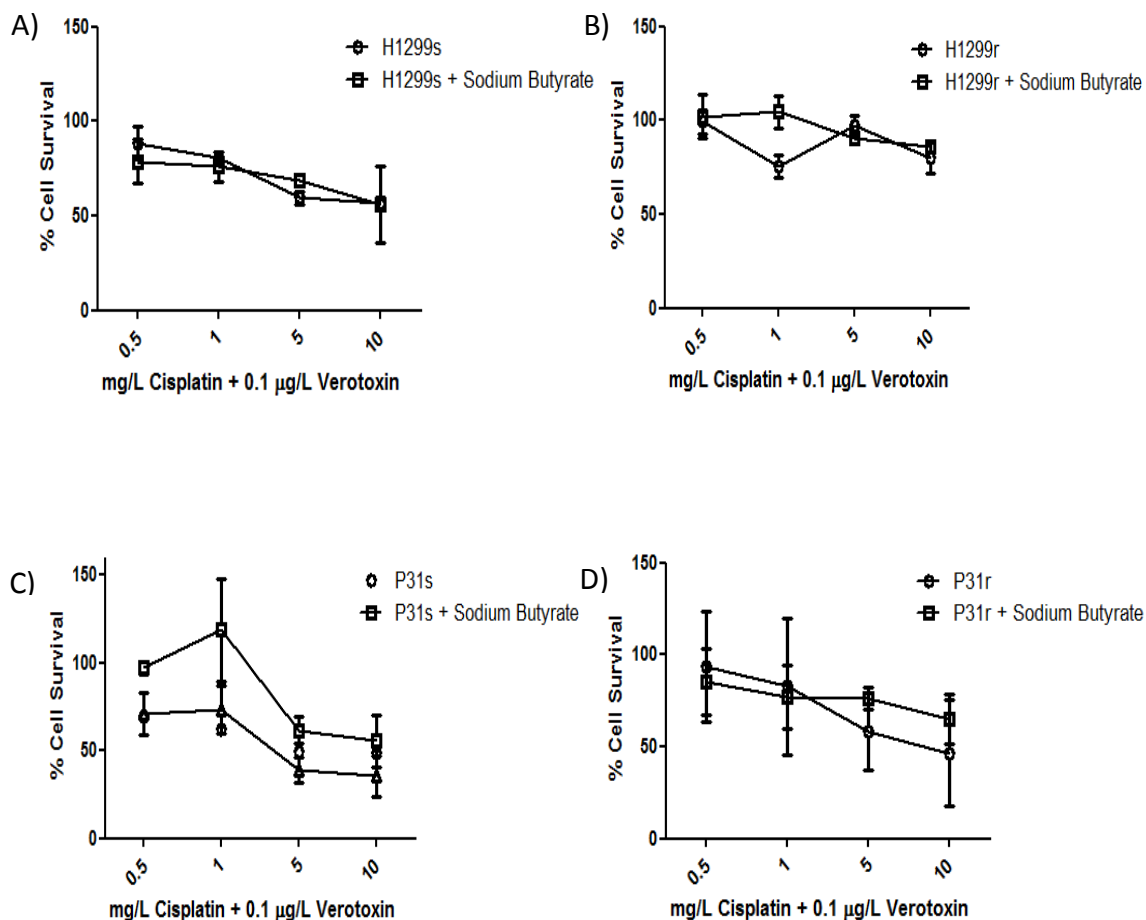


Figure 4.7 The effect of verotoxin-1 and cisplatin with and without and sodium butyrate on the viability of the H1299s, H1299r, P31s and P31r cells as determined by the viability assay, alamar blue. Cells were seeded in 96 well plates with and without 2 mM sodium butyrate at the following densities A) H1299s 2,000 cells/well. B) H1299r 6,000/cell. C) P31s 2,000 cells/well. D) P31r 2,000 cells/well. All the cells were then treated in triplicate with with vehicle (dH₂O) or varying concentrations of cisplatin (0.5-10 mg/L) and 0.1 µg/ml of verotoxin for 72h. Alamar blue was added and cells were incubated in the dark for 5h. The fluorescence was read at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a micro plate reader. Data expressed as % cell viability of vehicle treated controls. IC₅₀ values represent the concentration of drug required to reduce viability by 50 %. Data are expressed as mean plus or minus standard error of the mean for three separate experiments.

4.1.4 Expression of the ABC transporters ABCA5, ABCB6 and ABCE1 in H1299s, H1299r, P31s and P31r cells as determined by western blotting

As verapamil could not affect the susceptibility of the P31r and H1299r cells to the cytotoxic effects of cisplatin, we sought to determine whether ABCB1/MDR1 was actually expressed in these cells, as had been suggested by (Johansson et al., 2009). We also looked for expression of other ABC transporters to determine whether they correlated with cisplatin resistance.

Firstly MDR1 expression was analysed using an antibody for ABCB1/MDR1 conjugated to a phycoerythrin (PE) fluorophore and was measured by flow cytometry. The results showed that there was no expression of ABCB1/MDR1 in the H1299 and P31 cells in comparison to the expression levels reported in (Kaschny et al., 2015) and our positive control, ABCB1/MDR1 positive HL-60 colon cancer cells. Furthermore, no difference between the sensitive and resistant cells (Figure 4.8) was observed which may explain why verapamil did not decrease the resistance in H1299r or P31r cells. Johansson et al. (2009) demonstrated a correlation between Gb3 and MDR1 by staining for both cell surface proteins and analysing them by flow cytometry but never demonstrated absolute MDR1 expression levels or demonstrated any effect of verapamil or cyclosporine A, another MDR1 inhibitor, on the cells.

In our collaboration with the Benham-Motlagh laboratory, an mRNA affymetrix gene chip array was conducted on the H1299s, H1299r, P31s and P31r cells to help us better understand the possible underlying mechanism to acquisition of resistance to cisplatin.

In conducting the analysis of this affymetrix data, the H1299 cells were focused on as they had demonstrated the greatest differences when one compares sensitive and resistant cells in the majority of the assays we have performed thus far. In a comparison of H1299 cells it was observed that a few ABC transporters were up-regulated in the H1299r cells compared with the H1299s cells (Table 4.1). Interestingly, ABCB1/MDR1 was, if anything, down-regulated in H1299r cells compared to H1299s cells, which may explain why verapamil did not have any effect on cisplatin cell sensitivity as reported in

our data (Table 4.1 & Fig. 4.1). The affymetrix data demonstrated that the ABC transporters that may be up-regulated in the H1299r cells, compared to H1299s cells were ABCA5, ABCB6 and ABCE1. There was no obvious differential for ABCB6 and ABCE1 in the P31r cells but ABCA5 did seem to be substantially down regulated in these cells compared to P31s cells. The data indicated that the ABCA5, ABCB6 and ABCE1 transporters, but not the ABCB1 transporters may be linked to resistance in the H1299 cells, but not in the P31 cells.

Western blotting was next performed, to assess whether fold changes in mRNA expression of these three ABC transporters correlated with changes in protein expression. We observed that ABCA5 protein levels were lower in H1299r when compared to the H1299s cells. Similarly the ABCA5 protein levels were lower in the P31r when compared to the P31s cells when you take into account that more protein was loaded for the P31r well. This ABCA5 protein expression data correlates with the mRNA data for the P31 cells but not the H1299 cells (Figure 4.9A). We also observed that ABCB6 protein abundance was down-regulated in H1299r cells compared to H1299s cells, but up-regulated in the P31r cells compared to P31s cells which is at odds with the affymetrix mRNA data. The ABCE1 transporter abundance does seem to be up-regulated in the H1299r cells compared to H1299s cells but there was no ABCE1 transporter detected in the P31 cells, which agrees with the affymetrix mRNA data. In short the ABCB6 transporter in P31 cells and the ABCE1 transporter in the H1299 cells, but not the ABCB1 transporter, may well be candidates for cisplatin resistance in these cells. These results demonstrate the discrepancy between mRNA and protein levels and highlight the importance of confirming mRNA data with protein data.

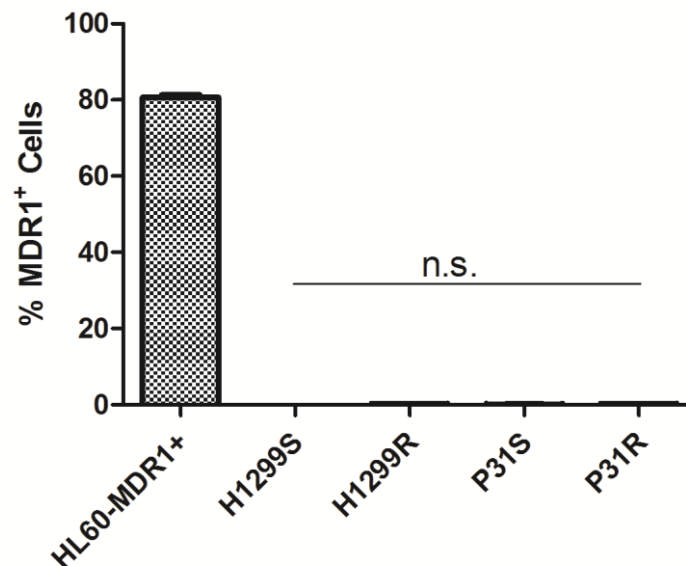


Figure 4.8 Percentage MDR1 expression in HL60-MDR1+, H1299s, H1299r, P31r and P31s cells as determined by flow cytometry. HL60-MDR1+, H1299s, H1299r, P31r and P31s cell were seeded at a density of 250,000 cells/well and left for 24h. The cells were then stained with a PE conjugated ABCB1/MDR1 antibody. Fluorescence was analysed for 10,000 events. Data represents mean plus or minus standard error the mean for the percentage of cells expressing ABCB1/MDR1 over three separate experiments. Statistical analysis was carried out using the student t-test.

	H1299r vs H1299s (Fold change)	P31r vs P31s (fold change)
ABCB1/MDR1	-0.91	0.009
ABCA5	0.95	-1.054
ABCB6	0.80	0.064
ABCE1	0.66	0.153

Table 4.1 Fold changes in mRNA expression between the cisplatin resistant and cisplatin sensitive H1299 and P31 cells as determined by an mRNA affymetrix gene chip array carried out by Benham-Motlagh laboratory.

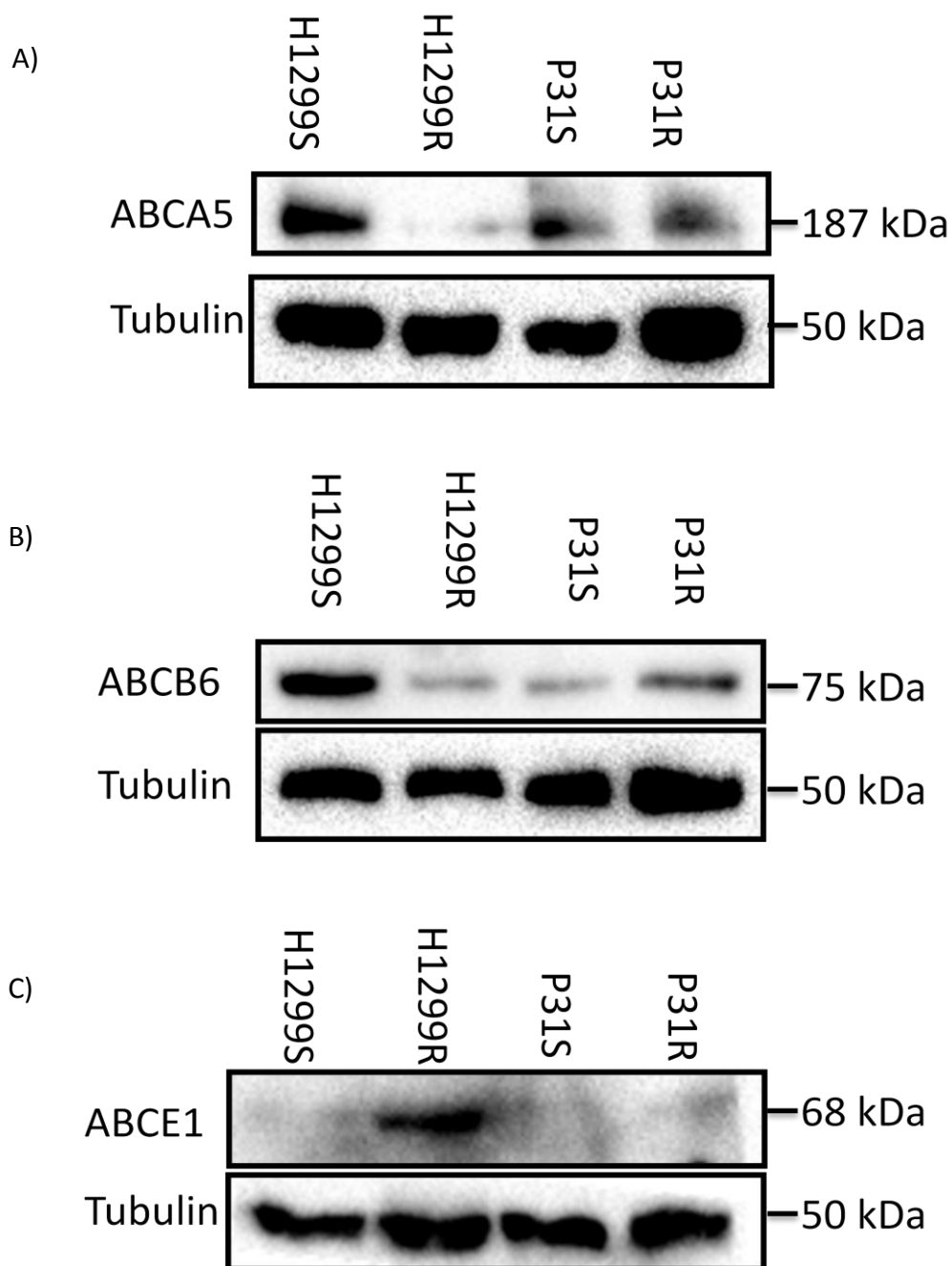


Figure 4.9 Expression of the ABC transporters: ABCA5, ABCB6 and ABCE1 in H1299s, H1299r, P31r and P31s cell lysates as determined by immunoblot analysis. Whole cell lysates were prepared from confluent cultures of H1299s, H1299r, P31r and P31s cells. Proteins (90 µg) were resolved in 10% SDS-PAGE gels and transferred to a PVDF membrane. Blots were probed for ABCA5, ABCB6 and ABCE1 or the loading control γ -tubulin. A) Representative blot of ABCA5 protein expression from three independent experiments. B) Representative blot of ABCB6 protein expression from three independent experiments. C) Representative blot of ABCE1 protein expression from three independent experiments.

4.1.5 Analysis of the metabolism of H1299s, H1299r, P31s and P31r cell lines by the seahorse extracellular flux analyser.

The Behnam-Motlagh laboratory and his collaborators have been investigating the mechanisms by which these cells have acquired resistance to cisplatin and have determined that there is an up-regulation of the anti-apoptotic BCL-2 family protein, MCL-1 (Hamanaka and Chandel, 2010), hyper-activation of the DNA repair enzyme PARP (Michels et al., 2013) and increased expression of the verotoxin-1 receptor Gb3 as mentioned previously (Johansson et al., 2009). The Benham-Motlagh laboratory had not investigated the metabolism of these cells so it was decided in collaboration with the Benham-Motlagh's laboratory in Umeå University, Sweden, to determine if there was any difference in the metabolism of the cells as determined by the seahorse extracellular flux analyser.

Seeding optimisation first had to be performed as these cells had not been used on the seahorse before. The seeding densities were based upon the seeding density of another non small cell lung cancer cell line, H460, which were seeded at 20,000 cells/well (Wu et al., 2007). The H1299 and P31 cells were seeded at 10,000 cells, 20,000 and 30,000 cells/well all the while maintaining the resistant cells in cisplatin. As the trend between the sensitive and resistant cells were not drastically different between the different cell densities, it was decided to seed the cells at 20,000 cells/well for future studies (Figure 4.10) as used by (Wu, 2007).

Having established the seeding density for these cells on the seahorse, it was decided to define the bioenergetic status of the cells by dissecting out oxygen consumption due to mitochondrial function, non-mitochondrial function, ATP turnover and proton leak, which was achieved using the mitochondrial inhibitors oligomycin and Antimycin A. Maximal mitochondrial capacity was determined by the addition of the mitochondrial uncoupler, FCCP. They all showed similar degrees of inhibition as a result of the various inhibitors i.e. decrease in OCR with oligomycin, increase above baseline with FCCP and a decrease in OCR with antimycin A. However, the response to the mitochondrial

uncoupler FCCP differed between the H1299 and P31 cells (Figure 4.11). FCCP was able to uncouple and thus increase mitochondrial oxygen consumption of the H1299 cells, registering an OCR value above baseline. However, addition of FCCP to P31 cells did not increase oxygen consumption rates above baseline. This indicates that resting H1299 cells are poised at a work rate less than maximal activity (Figure 4.11A), whereas mitochondria in P31 cells are working at their maximal rate (Figure 4.11B).

In addition, we observed a significant difference between the basal cellular ($p < 0.0001$), oligomycin treated ($p < 0.05$), FCCP (maximal mitochondrial activity) ($p < 0.0005$) and antimycin A (oxygen consumption not due to mitochondria) ($p < 0.0001$) OCR's between the H1299s and H1299r cells (Figure 4.11A). Similarly in the P31 cells there was a significant difference in the OCR's of the basal ($p < 0.0005$), FCCP ($p < 0.05$) and antimycin A ($p < 0.05$) treated cells but not for oligomycin treated cells (Figure 4.11B). Similar to the OCR results there is a significant difference in the ECAR of the H1299s and that of the H1299r after oligomycin ($p < 0.005$), FCCP ($p < 0.05$) and antimycin A ($p < 0.005$) treatment (Figure 4.11C) but interestingly no difference was observed in their basal ECAR in the presence of these compounds. There was no significant difference in a comparison of the ECAR of P31s and P31r cells (Figure 4.11D).

We then analysed the reserve capacity of the H1299 and P31 cells. The reserve capacity is the percentage increase in OCR from the basal state after the addition of FCCP. The reserve capacity gives an indication of how much the mitochondrial activity can be increased from the resting state. There was a significant ($P < 0.05$) increase in the cellular reserve capacity (CRC) of the H1299r in comparison to the H1299s cells and no difference in the CRC of the P31r in comparison to the P31s cells (Figure 4.11 E). This indicates that while the basal OCR of the H1299r cells is significantly lower than the H1299s cells, the H1299r cells possess a greater capacity to increase their mitochondrial activity. In a comparison of the ratio of OCR to ECAR the H1299r had a significant ($P < 0.05$) reduction in oxidative phosphorylation in comparison to the H1299s cells. Similarly the P31r cells had a significant ($P < 0.05$) reduction in oxidative phosphorylation to the P31s cells (Figure 4.11 F). This reduction in oxidative phosphorylation indicates

that the resistant cells of both cell lines are more glycolytic than their sensitive cell counterparts.

Having established that the total cellular OCR and ECAR rates for P31 and H1299 cells, were greater in the resistant lines compared to sensitive lines, the next question to be answered was whether the differences in OCR, were due to the differences in mitochondrial function in these cells, or whether the differences were due to oxygen consumption by non-mitochondrial (non-oxidative phosphorylation) events inside the cell. The oxygen consumption rate due to mitochondria alone was achieved by subtracting the antimycin A rate from the basal cellular oxygen consumption rate. We demonstrated that there was a significant difference between the basal ($p < 0.0001$) and maximal mitochondrial ($p < 0.005$) OCRs in a comparison of H1299s and H1299r cells (Figure 4.12A). Similarly there was also a significant difference between basal ($p < 0.005$) and maximal mitochondrial ($p < 0.05$) OCRs in a comparison of P31s and P31r cells (Figure 4.12 B). Interestingly, there was no significant difference between the OCR rates obtained after oligomycin addition between the sensitive and resistant cells for either cell lines. This indicates that the difference observed were due to mitochondrial differences between the sensitive and resistant cells. We then analysed the reserve capacity of the H1299 and P31 cells to determine whether the same differences in reserve capacity would be observed with the antimycin A rate taken away. Similar to the CRC, there was a significant ($P < 0.05$) increase in mitochondrial reserve capacity (MRC) of the of the H1299r cells in comparison to the H1299s cells with no difference in the MRC of the P31 cells (Figure 4.12 C). This indicates that the greater ability of the H1299r cells to raise the OCR from resting in comparison to H1299s cells was due to the mitochondria and not other oxygen consuming entities.

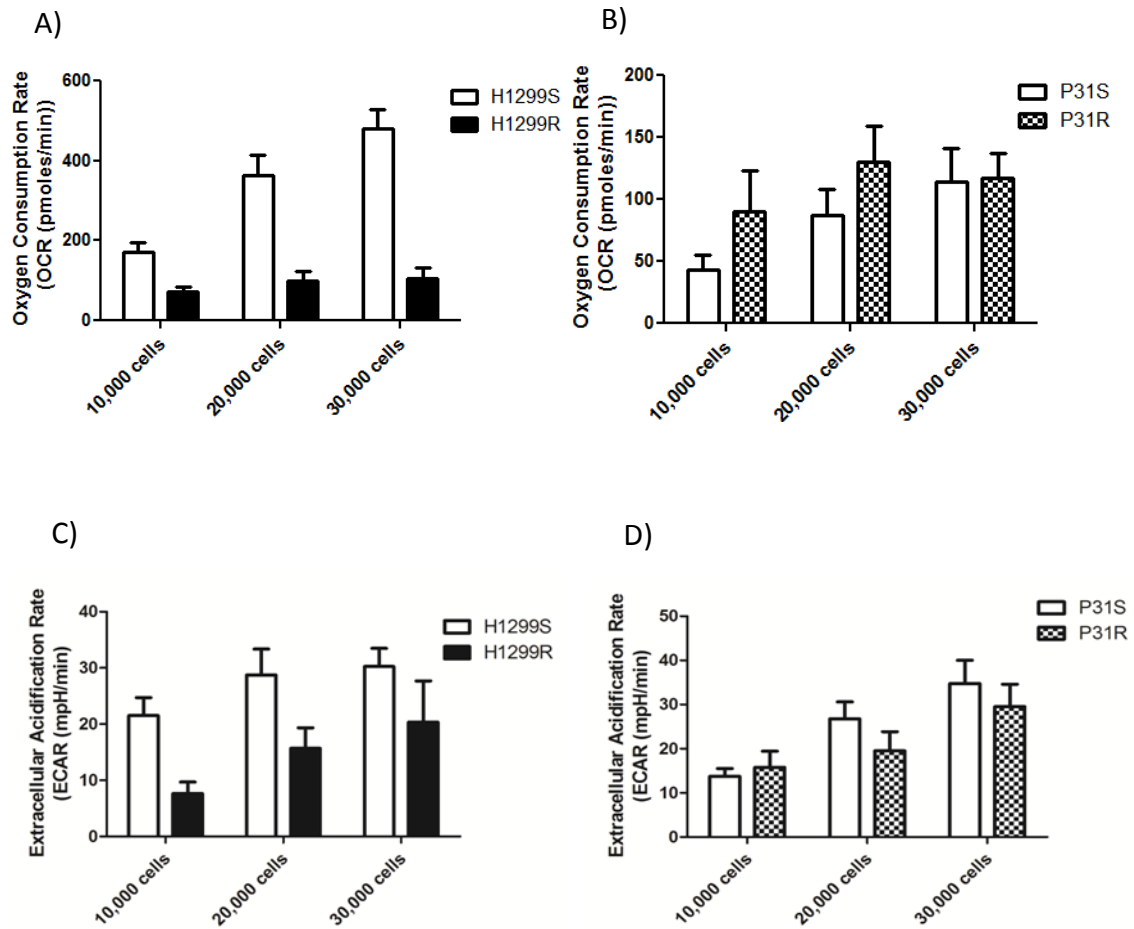


Figure 4.10 Seeding optimisation of H1299s, H1299r, P31s & P31r on the seahorse plates as determined by the seahorse extracellular flux analyser. A) Oxygen consumption rates for H1299s & H1299r at a density of 10,000, 20,000 and 30,000 cells/well. B) Oxygen consumption rates for P31s & P31s at a density of 10,000, 20,000 and 30,000 cells/well. C) Extracellular acidification rates of H1299s & H1299r at a density of 10,000, 20,000 and 30,000 cells/well. D) Extracellular acidification rates of P31s & P31s at a density of 10,000, 20,000 and 30,000 cells/well. Data is represented as mean plus or minus standard error of the mean for three separate experiments.

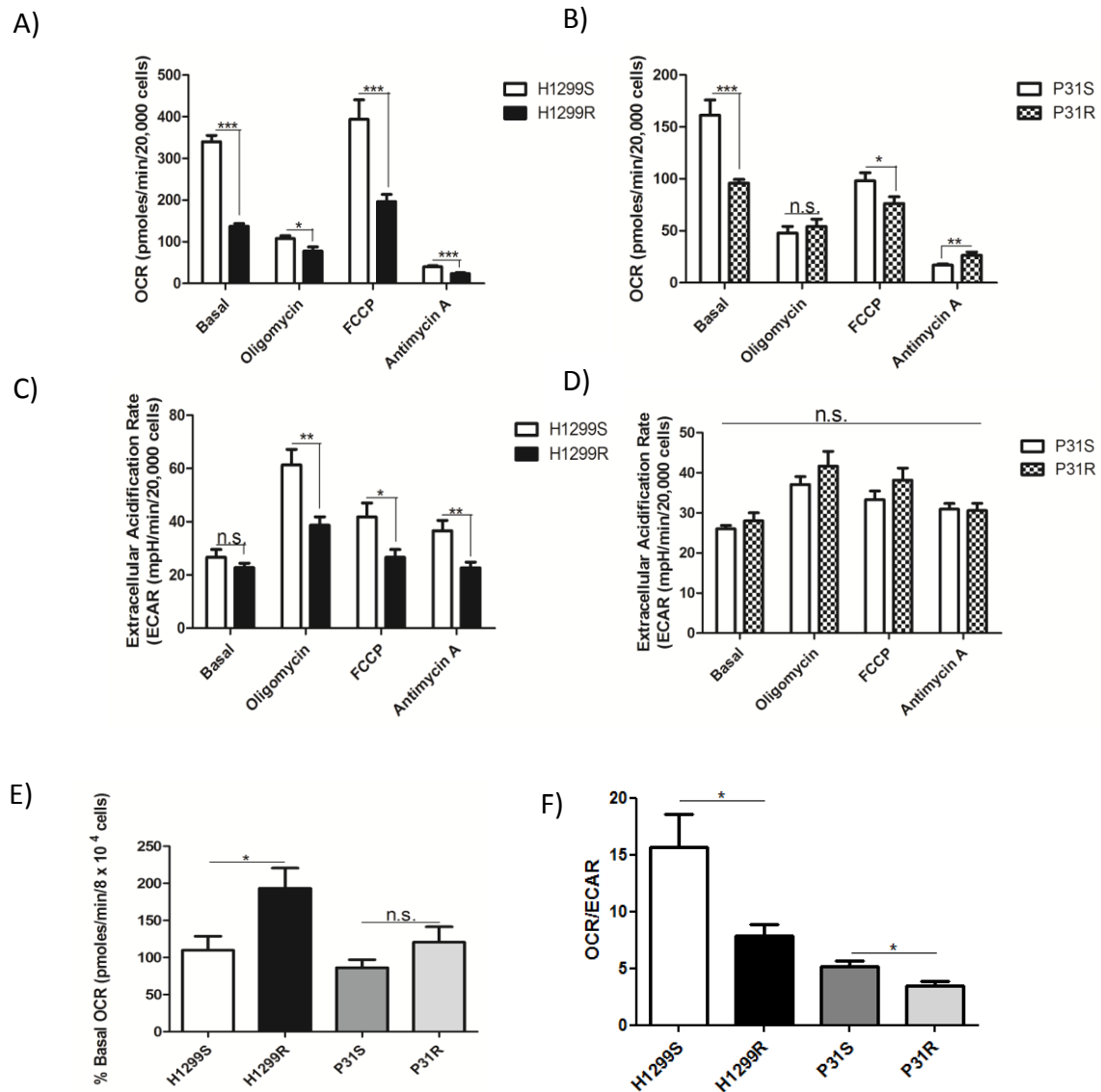


Figure 4.11 Effect of the mitochondrial inhibitors: oligomycin, FCCP and Antimycin A on the whole cell metabolism of H1299s, H1299r, P31s & P31r as determined by the seahorse extracellular flux analyser.

Cells were seeded at 20,000 cells/well and were put into the machine where the mitochondrial inhibitors; oligomycin, FCCP and antimycin A were sequentially added to the wells during the run. A) OCR of the basal metabolism of H1299s and H1299r's and after the inhibitors were added to the wells. B) OCR of the basal metabolism of P31s and P31r's and after the inhibitors were added to the wells. C) ECAR rates of the basal metabolism of H1299s and H1299r's and after the inhibitors were added to the wells. D) ECAR rates of the basal metabolism of P31s and P31r's and after the inhibitors were added to the wells. E) Cellular reserve capacity (CRC) of the H1299s, H1299r, P31s and P31r cells. F) OCR/ECAR ratio of the basal rates of the H1299s, H1299r, P31s and P31r cells. Data are expressed as mean plus or minus standard error of the mean for three separate experiments. Statistical analysis was carried out using the student t-test. *** = $p < 0.0005$, ** = $p < 0.005$, * = $p < 0.05$.

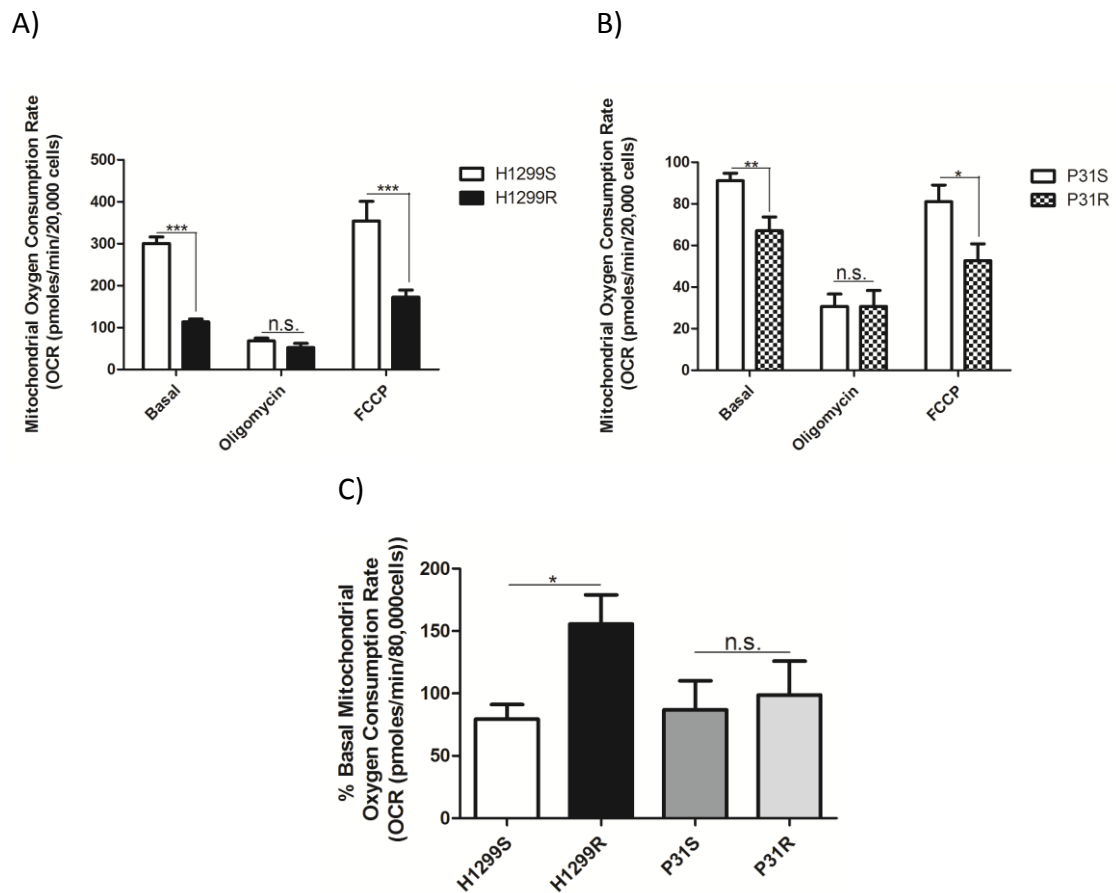


Figure 4.12 Mitochondrial oxygen consumption rates of the H1299s, H1299r, P31r and P31s cells as determined by the seahorse extracellular flux analyser. Cells were seeded at 20,000 cells/well and were put into the machine where the mitochondrial inhibitors; oligomycin, FCCP and antimycin A were sequentially added to the wells during the run. Mitochondrial oxygen consumption was obtained by taking the antimycin A OCR from the rate obtained from the basal rate and after oligomycin and FCCP treatment. A) Mitochondrial OCR rates of the basal metabolism of H1299s and H1299r's and after the inhibitors were added to the wells. B) Mitochondrial OCR rates of the basal metabolism of P31s and P31r's and after the inhibitors were added to the wells. C) Mitochondrial reserve capacity (MCR) of the H1299s, H1299r, P31s and P31r cells. Data are expressed as mean plus or minus standard error of the mean for three separate experiments. Statistical analysis was carried out using the student t-test. *** = $p < 0.0005$, ** = $p < 0.005$, * = $p < 0.05$.

4.1.6 Analysis of the mitochondrial content of H1299s, H1299r, P31s & P31r cells.

Having established that the maximum total cellular OCR's were greater in the resistant lines compared to the sensitive lines, and that the difference was due to mitochondrial function, it was then decided to look at the mitochondrial content of these cells. It was unknown whether the differences that were observed between the sensitive and resistant cell lines were due to the mitochondria working harder in the sensitive cell lines or whether it was because there was a greater mitochondrial content in these cells. A variety of methods were used to determine mitochondrial content of the cells: (i) a citrate synthase assay and (ii) detection by immunoblotting of the following mitochondrial proteins voltage dependent anion channel 1 (VDAC1) and (iii) the protein subunit 4 of cytochrome c oxidase.

There was a significantly greater citrate synthase activity in the H1299s in comparison to the citrate synthase activity of the H1299r cells ($p < 0.005$) (Figure 4.13A). Similarly there was a significant greater citrate synthase activity in the P31s in comparison to the citrate synthase activity of the P31r cells ($P < 0.005$) (Figure 4.13B).

Similar to the citrate synthase assay there was significantly more expression of both VDAC and cytochrome c oxidase in the H1299s and P31s cells in comparison to their resistant subtypes (Figure 4.14). These immunoblots confirm the observations made in the citrate synthase activity assay (Figure 4.13) that the differences observed between the OCR rates of the sensitive and resistant cell lines can be explained in part due to the sensitive cell lines possessing greater mitochondrial abundance.

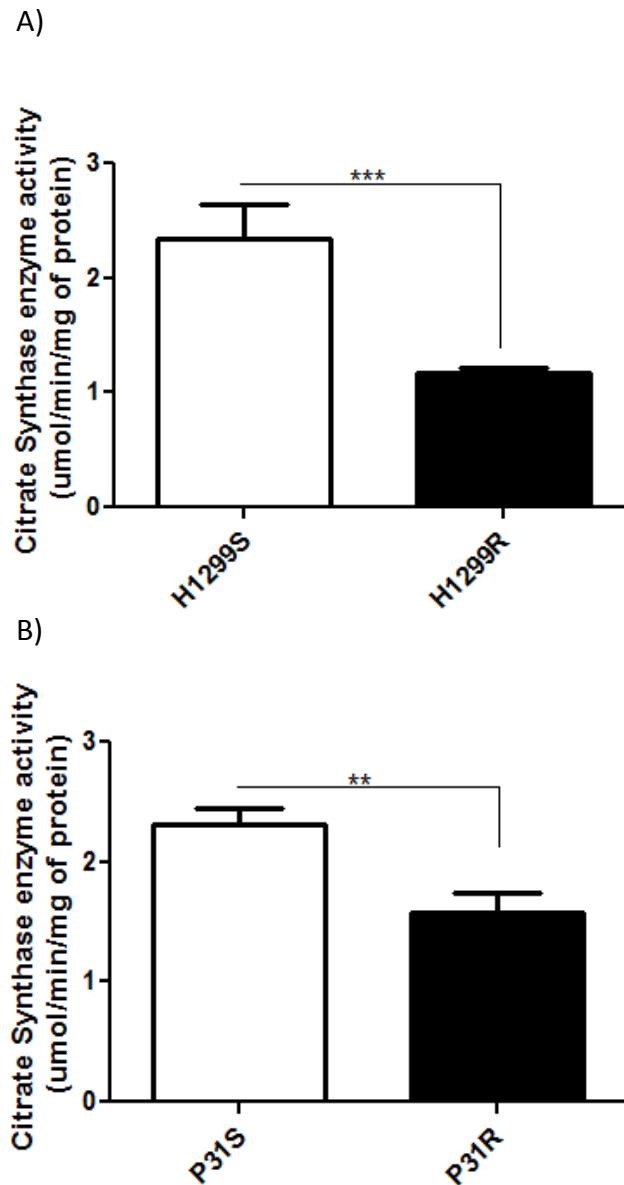


Figure 4.13 Citrate synthase activity of the H1299s, H1299r, P31s and P31r cells as determined by spectroscopy. Cells were plated at 300,000 cells in T25's, lysed with Tris-Triton X-100 (0.2% (v/v) and the protein content was assessed using the BCA assay. 40 μ g of protein was added into the cuvette with 500 μ l of Tris (200 mM, pH 8.0) with Triton X-100 (0.2% (v/v)), 100 μ l of DTNB, 30 μ l of acetyl coenzyme A (10 mM). The coupled reaction was started by adding 50 μ l of 10 mM oxaloacetic acid and measuring the rate of increase of 5-thionitrobenzene in absorbance at 412nm for 3min. A) Citrate synthase enzyme activity in the H1299s and H1299r cells. B) Citrate synthase enzyme activity in the P31s and P31r cells. Data are expressed as mean plus or minus standard error of the mean for three separate experiments. Statistical analysis was carried out using the student t-test. *** = $p < 0.0005$, ** = $p < 0.005$.

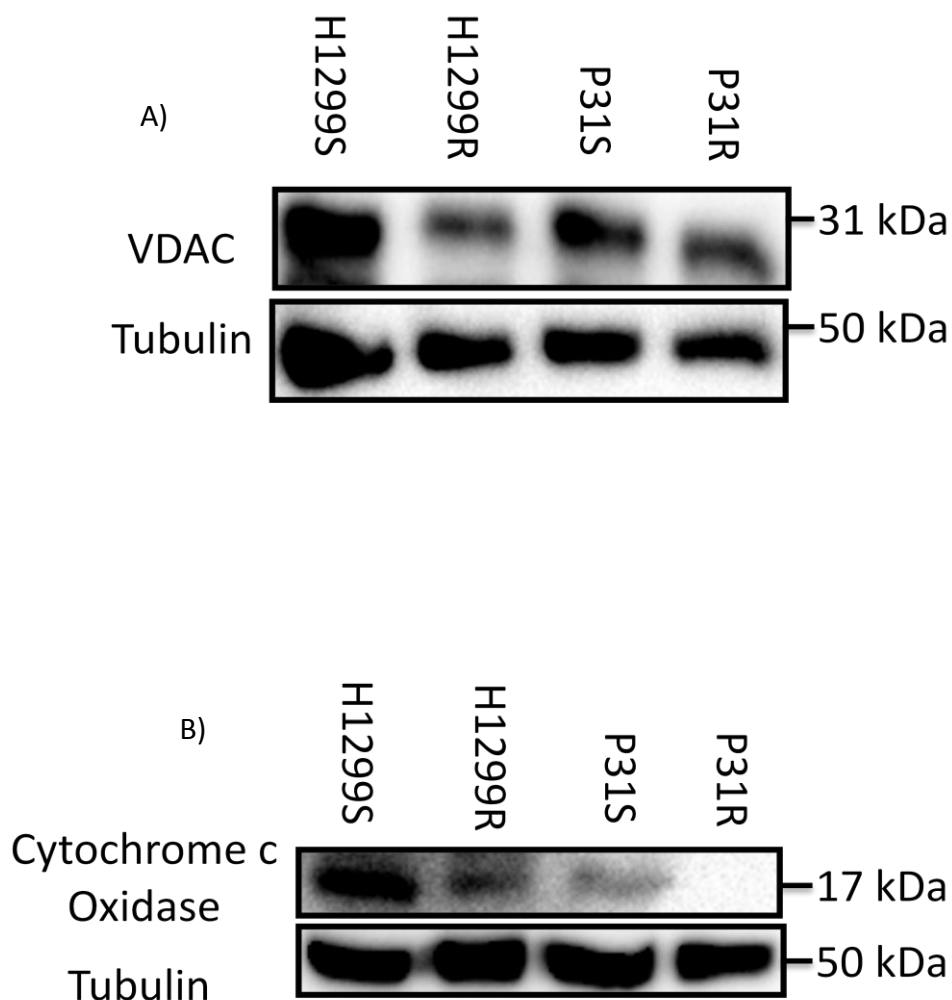


Figure 4.14 Expression of the mitochondrial proteins: voltage dependent anion channel 1 (VDAC1) and cytochrome c oxidase subunit 4 H1299s, H1299r, P31r and P31s cell lysates as determined by immunoblot analysis. Whole cell lysates were prepared from confluent cultures of H1299s, H1299r, P31r and P31s cells. Proteins (90 μ g) were resolved in 10% SDS-PAGE gels and transferred to a PVDF membrane. Blots were probed for either VDAC1, cytochrome c oxidase subunit 4 or the loading control γ -tubulin. A) Representative blot of VDAC1 protein expression from three independent experiments. B) Representative blot of cytochrome c oxidase subunit 4 protein expression from three independent experiments.

4.1.7 Growth rate of H1299s, H1299r, P31s & P31r cells as assessed by the alamar blue proliferation assay.

Having established that the greater OCR and ECAR's observed in the sensitive cells (H1299s & P31s) when compared to resistant cells (H1299r & P31r) (Figure 4.11 & 4.12) were in part due to differences in mitochondrial abundance (Figure 4.13 & 4.14), it was decided to compare their growth rate as a secondary confirmation of differences in metabolism. Growth rate was assessed by the alamar blue proliferation/viability assay.

The alamar blue assay is both an assay of proliferation and viability. It uses the natural reducing power of the enzymes NADPH, FMNH, FADH, NADH as well as cytochromes to reduce resazurin to the fluorescent resorufin thereby giving an indication of the cellular health of the cells.

As expected there was a significantly ($p < 0.0001$) greater proliferation rate observed in the sensitive cell lines when compared to the resistant cell lines (Figure 4.15).

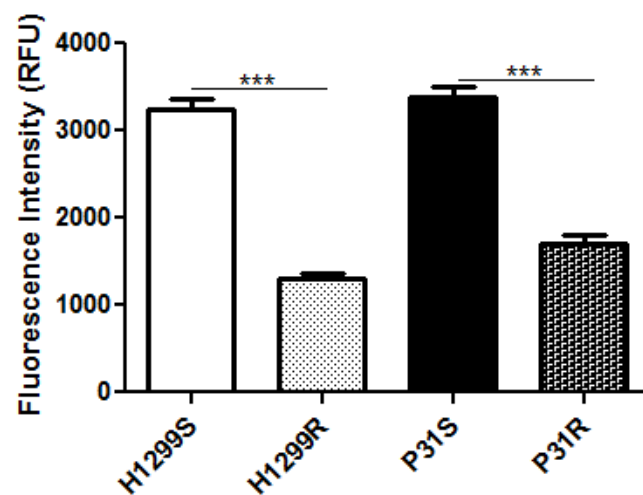


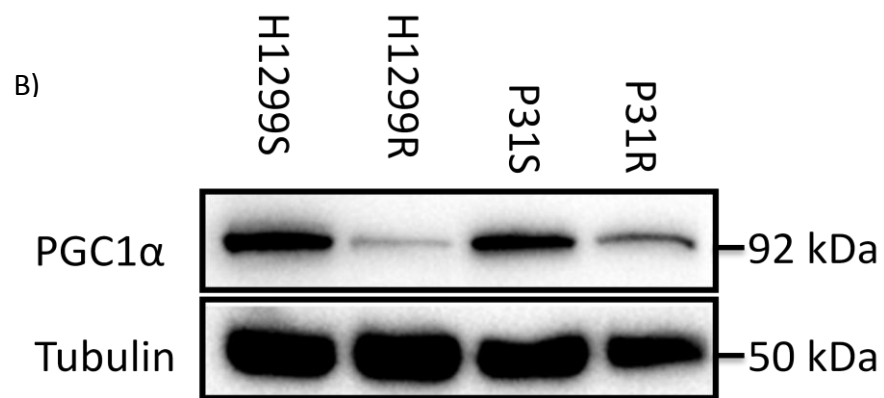
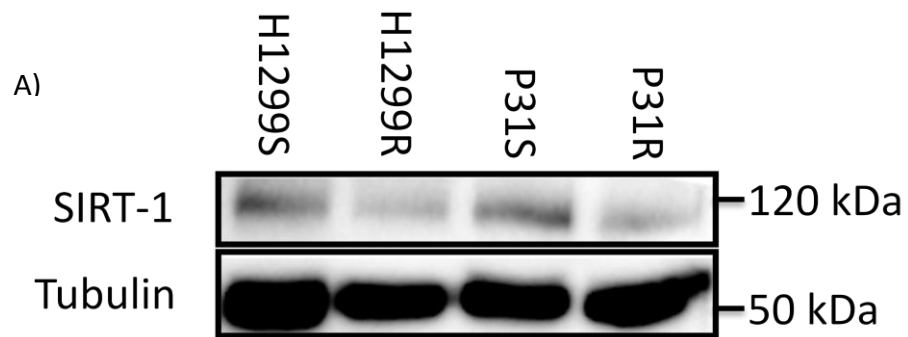
Figure 4.15 Growth rate of the H1299s, H1299r, P31s and P31r cells by the alamar blue assay. H1299s, H1299r, P31s & P31r cells were seeded at 20,000 cells/well of a 96 well plate and left for 72h to incubate. After the elapsed time 20 μ l of alamar blue was added to the wells and the fluorescence was then measured by a spectrophotometer. Data is expressed as relative fluorescence intensity. Data are expressed as mean plus or minus standard error of the mean for three separate experiments. Statistical analysis was carried out using the student t-test. *** = $p < 0.0001$.

4.1.8 Analysis of the expression of mitochondrial biogenesis proteins in H1299s, H1299r, P31s and P31r cells as determined by western blotting.

Having demonstrated that the sensitive cells possess greater metabolism and greater mitochondrial abundance compared to cisplatin resistant cells, we next wanted to see whether this differential metabolism was as a result of a modulation in the mitochondrial biogenesis pathway. To examine this modulation of mitochondrial biogenesis we analysed the expression of protein levels linked to the biogenesis of mitochondria; cytosolic sirtuin-1 (SIRT-1, NAD⁺-dependent deacetylase), peroxisome-proliferator activator receptor- γ co-activator 1-alpha (PGC1 α)(central role in energy metabolism), transcription factor A, mitochondrial ((TFAM) core mitochondrial protein) and sirtuin-3 (SIRT-3, mitochondrial NAD⁺-dependent deacetylase in the mitochondrial matrix associated with integrity/antioxidant responses) by western blotting.

In line with the increased mitochondrial abundance in cisplatin sensitive cells compared to cisplatin resistant cells, the expression of SIRT-1, PGC1 α , TFAM and SIRT-3 was greater in the H1299s cells than the H1299r cells. Similarly the expression of SIRT-1, PGC1 α , TFAM and SIRT-3 was greater in the P31s cells than in the P31r cells (Figure 4.16).

In addition to ascertaining PGC1 α abundance, we also measured the activity of PGC1 α . We conducted an immunoprecipitation for the acetylation status of PGC1 α . The band that PGC1 α antibody detected was one of its isoforms, L-PGC1 α at approximately the 70 kDa size. The acetylation of PGC1 α was indeed greater in the H1299s and P31s cells when compared to the H1299r and P31rcells (Figure 4.17). Thus, not only is the expression of PGC1 α greater in the sensitive cells but so is the activity of PGC1 α which is consistent with the increased mitochondrial biogenesis.



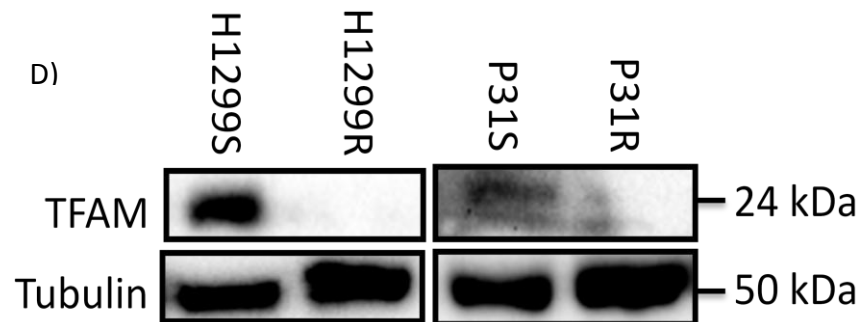
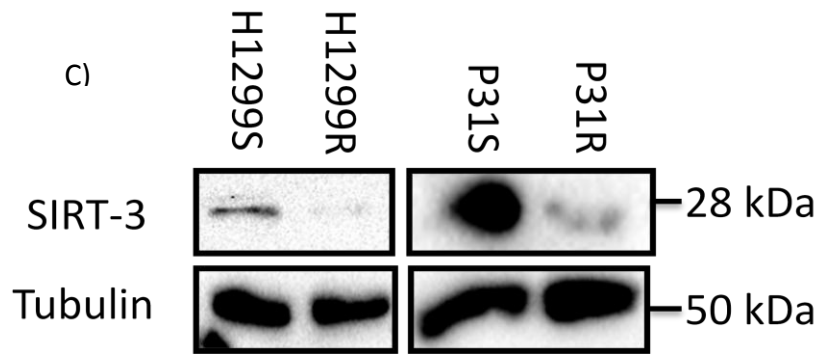


Figure 4.16 Expression of the mitochondrial biogenesis proteins: SIRT1, PGC1 α , SIRT2, TFAM and SIRT3 in H1299s, H1299r, P31r and P31s cell lysates as determined by immunoblot analysis. Whole cell lysates were prepared from confluent cultures of H1299s, H1299r, P31r and P31s cells. Proteins (90 μ g) were resolved in 10% SDS-PAGE gels and transferred to a PVDF membrane. Blots were probed for SIRT1, PGC1 α , SIRT2, TFAM and SIRT3 or the loading control γ -tubulin. A) Representative blot of SIRT1 protein expression from three independent experiments. B) Representative blot of PGC1 α protein expression from three independent experiments. C) Representative blot of SIRT3 protein expression from three independent experiments. D) Representative blot of TFAM protein expression from three independent experiments.

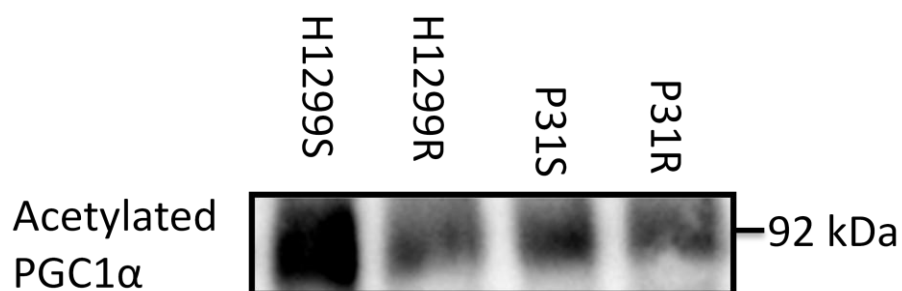


Figure 4.17 Expression of the acetylation of PGC1 α in H1299s, H1299r, P31r and P31s cell lysates as determined by immunoblot analysis. Whole cell lysates were prepared from confluent cultures of H1299s, H1299r, P31r and P31s cells. Acetylated proteins were pulled down from the lysate and bound to protein A beads. Protein A beads were resolved in 10% SDS-PAGE gels and transferred to a PVDF membrane. Blot was probed for PGC1 α . Representative blot from three independent experiments.

4.1.9. Measurement of Reactive Oxygen Species (ROS) in H1299s, H1299r, P31s and P31r as determined by flow cytometry.

Next it was decided to measure ROS production levels of the H1299 and P31 cells as another measure of metabolism.

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) was used to measure intracellular ROS by flow cytometry. The H1299r cells exhibited greater ROS production when compared to the H1299s cells. However, there was no significant difference in the ROS production between the P31s and P31r cells (Figure 4.18).

4.1.9.1. Investigation into the relationship between ROS and Hypoxia Inducible Factor 1 alpha (HIF1 α) stabilisation

The transcription factor HIF1 α , plays an essential role in the sensing of oxygen levels. HIF1 α has been shown to transcriptionally regulate a plethora of genes involved in cancer, energy metabolism, nutrient transport and cell migration to name but a few (Renaud and de Lorgeril, 1992, Catalgol et al., 2012). The link between HIF1 α and ROS seems controversial with some claiming that HIF1 α supports ROS formation (Ungvari et al., 2007) while others have shown a decrease in HIF1 α expression with increased ROS production (Olas and Wachowicz, 2002). Having demonstrated that H1299 resistant cells produce a greater amount ROS than the sensitive cells (Figure 4.18), we wanted to see if this increased ROS production would stabilize HIF1 α . We sought to determine this by measuring HIF1 α expression by western blotting.

HIF1 α expression was indeed stabilised in the H1299r cells when compared to the H1299s cells. There was no apparent HIF1 α stabilisation in the H1299s, nor in the P31s or in the P31r cells (Figure 4.19)

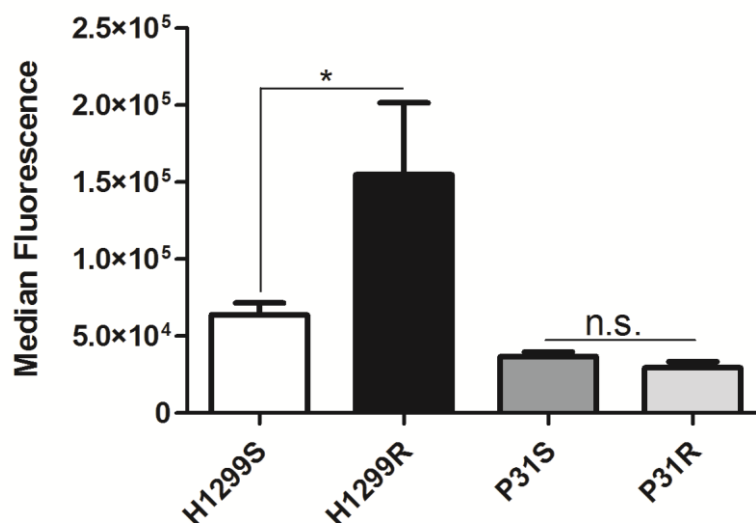


Figure 4.18 Reactive Oxygen Species (ROS) production in in H1299s, H1299r, P31r and P31s cells as determined by flow cytometry. H1299s, H1299r, P31r and P31s cell were seeded at a density of 250,000 cells/well and left for 24 hours. The cells were then stained with 10 μ M Dichloro-dihydro-fluorescein diacetate (DCFH-DA). Fluorescence was analysed for 10,000 events. Data are expressed as mean plus or minus standard error of the mean for the relative DCFH-DA signal over three separate experiments. Statistical analysis was carried out using the student t-test. * = $p < 0.05$.

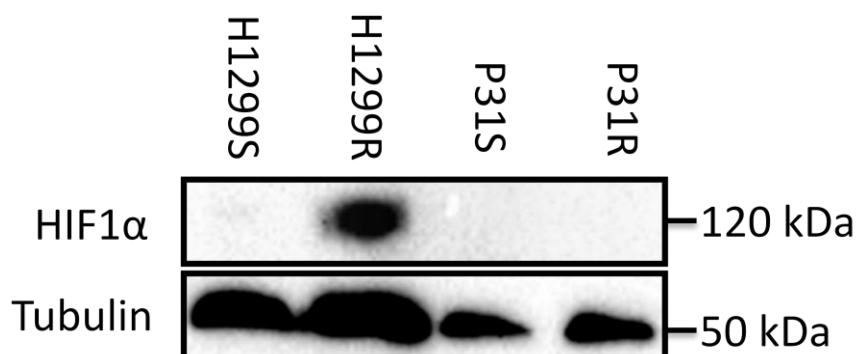


Figure 4.19 Expression of the stabilisation of HIF1 α in H1299s, H1299r, P31r and P31s cell lysates as determined by immunoblot analysis. Whole cell lysates were prepared from confluent cultures of H1299s, H1299r, P31r and P31s cells. Proteins (90 μ g) were resolved in 10% SDS-PAGE gels and transferred to a PVDF membrane. Blots were probed for HIF1 α or the loading control γ -tubulin. Representative blot from three independent experiments.

4.2 Discussion

Cisplatin is one of the most potent chemotherapeutic drugs used to treat a variety of cancers but its usefulness is limited by the acquisition of resistance. Its cytotoxicity has been generally attributed to its interaction with DNA to form intrastrand crosslinks (Siddik, 2003, Behnam-Motlagh et al., 2010a, Johansson et al., 2006) but some studies have indicated that mitochondrial DNA adducts may be more common than nuclear DNA adducts in model cell line systems as a result cisplatin treatment (Murata et al., 1990, Olivero et al., 1995). Resistant cancers have adapted to this by decreasing their mitochondrial number (Qian et al., 2005, Longley and Johnston, 2005), oxygen consumption and glucose uptake (Liang et al., 2008). Exploiting these metabolic differences may prove important in overcoming chemo-resistance.

As expected, the resistant non small cell lung cancer (H1299) and mesothelioma (P31) cell lines demonstrated reduced sensitivity to cisplatin-induced cell death when compared to their sensitive cell counterparts. Resistance can be acquired to not only cisplatin and structurally similar platinum analogues, but also to a vast array of structurally varied chemotherapeutic agents. This is known as multidrug resistance and is the major obstacle in the successful treatment of cancer. As the multidrug resistance transporter, MDR1 is reported to be expressed in these cells (Johansson et al., 2009), we treated the H1299 and P31 cells with the MDR1 inhibitor, verapamil to determine whether an increase in sensitivity of the H1299r and P31r cells to cisplatin could be achieved. Surprisingly, only a small increase in sensitivity to cisplatin was observed. Numerous studies have suggested that cisplatin is a poor substrate for MDR1 in contrast to other antineoplastic agents, which could be the reason for our minor change in cisplatin sensitivity (Gibalová et al., 2012, Romero-Canelon et al., 2012). ABCB1/MDR1 may be playing a role in MDR resistance to other chemotherapeutics which are ABCB1/MDR1 substrates but not to cisplatin and other structurally similar platinum agents even though MDR1 is up-regulated (Johansson et al., 2009, De Rosa et al., 2008)

Increased expression of Gb₃ is commonly seen in cisplatin resistance, as was confirmed in this study. Gb₃ may play a role in acquired cisplatin resistance and MDR through its

association with the hydrophobic drug efflux pump, MDR1. It has been indicated that glyceramides like Gb₃ are up-regulated in chemo-resistant cancers due to the over-expression of glucosylceramide synthase which has been shown to regulate the expression of MDR1 through the phosphorylation and activation of cSrc and FAK and then consequently through β -catenin signalling (Liu et al., 2010, Gouazé et al., 2004). Gb₃ is known to function as a cell surface receptor for verotoxin-1 (VT-1 or Shiga Like Toxin-1) synthesized by pathogenic strains of *Escherichia Coli* (Arab et al., 1997). The combination treatment of verotoxin-1 and cisplatin was shown to have an additive cytotoxic effect upon the treatment of acquired cisplatin cancers (Behnam-Motlagh et al., 2010b, Johansson et al., 2009), however this was not observed in the present study. Verotoxin-1 elicits its cytotoxic effects through binding to Gb₃, internalisation, translocation from an intracellular compartment to the endoplasmic reticulum, golgi apparatus and the nuclear envelope (Lombard et al., 2007). The sensitivity to verotoxin has been shown to not necessarily be proportionate to the receptor concentration (Someya et al., 2010, Hallows et al., 2006). Qiu et al. (2010) suggest that nuclear envelope/ER targeting of VT correlates with increased toxin sensitivity. Administration of sodium butyrate which has been shown to increase sensitivity of cells to verotoxin-1 (Lingwood et al., 1998) through increased Gb₃ synthesis and ER targeting (Lombard et al., 2007, Higashida et al., 2013), did not affect the sensitivity of our cells in the present study.

As the MDR1 inhibitor verapamil, failed to have any significant effect upon the sensitivity of the resistant cell lines to cisplatin, analysis of the MDR1 expression was conducted. Our results showed no expression of MDR1 in these cells which explains why verapamil failed to affect the sensitivity of these cells to cisplatin. Johansson et al. (2009) demonstrated expression of MDR1 in these cells by flow cytometry but verapamil and another MDR1 inhibitor, cyclosporin A did not have any impact upon MDR1 expression. The lack of an effect of the MDR1 inhibitors and the lack of demonstrable MDR1 expression by any other assays calls into question the cells MDR1 expression status. To determine whether any other ABC transporters could be the cause of this acquired cisplatin resistance, protein expression of the three highest differentially expressed ABC

transporters from an mRNA affymetrix array was analysed. Results indicated that the ABCB6 transporter for the P31 cells and ABCE1 for the H1299 cells may be possible candidates for the acquisition of resistance to cisplatin in these cells. ABCB6 is responsible for the ATP dependent transport of coproporphyrinogen III back into the mitochondria for the synthesis of porphyrins (Krishnamurthy et al., 2006). ABCB6 has been shown to be up-regulated in cisplatin resistant cancers (Paterson et al., 2005). When ABBC6 was transfected into KB cells, the transporter conferred resistance to not only cisplatin but also arsenite suggesting it may also play a role in MDR. They also found that ABCB6 localised to the mitochondria and the plasma membrane (Preston et al., 2001). ABCE1 is a non transporter member of the ABC cohort. ABCE1 has been shown to bind to the initiation factors eIF2 and eIF5 and forms part of the pre-initiation complex for the translation of mRNA (Chen et al., 2006) and also been shown to dissociate post termination complexes to free 60S complexes and mRNA- and tRNA-bound 40S subunits for ribosomal biogenesis (Pisarev et al., 2010). Silencing of ABCE1 expression significantly inhibited the proliferation, migration and invasion of oesophageal cancer (Huang et al., 2014). This suggests that ABCE1 may be a potential cancer biomarker.

Altered metabolism is a hallmark of cancer (Hanahan and Weinberg, 2011) and while genetic and molecular modifications have enabled cancers to survive the onslaught of various cancer therapies (Pillai et al., 2010), changes in tumour cell metabolism is becoming increasingly more important. Changes to key processes like glucose, glutamine, nucleotide, fatty acid and phospholipid metabolism enable cancers to modify their growth demands and thrive in a hostile environment (Zong et al., 2002, Thomson et al., 2008). Metabolic features of cancers are currently being investigated to aid in the process of diagnosis and staging of cancer notifying them of treatment response and therapy resistance (Morozov et al., 2014, Ngo et al., 2014). With this in mind an investigation into the metabolic differences between the resistant and sensitive strains of P31 and H1299'S was carried out on the seahorse extracellular flux analyser. The cells were assessed for mitochondrial function using the inhibitors; oligomycin, FCCP and Antimycin A. Data suggests that the mitochondria in H1299 cells are poised at a work rate less than their maximal whereas the mitochondria in P31 cells are at their maximal

work rate both in the sensitive cell line and resistant sub-line. Interestingly, the whole cellular basal respiration rate is greater in the sensitive cell lines than the acquired resistant cell lines. The greater basal OCR in the sensitive cell line when compared to the resistant cell line that we have observed is consistent with other groups who have shown their sensitive cell line to have a greater basal OCR than their resistant cell line (Liang et al., 2008, Oliva et al., 2011). The reduced basal OCR of the resistant cell lines may be due to mitochondrial injury. Tumour cells which have a mitochondrial injury have decreased TCA cycle and electron transport chain flux, which can lead to reduced production of CO₂ and ATP with diminished O₂ consumption (Kwak et al., 2005, Pao et al., 2005). The reduction in the OCR/ECAR ratio of the resistant cells in comparison to the sensitive cells indicates that the resistant cells are proportionally using more aerobic glycolysis than oxidative phosphorylation for their energy needs. The significantly greater cellular reserve capacity (CRC) of the H1299r cells in comparison to the H1299s cells indicates that although the H1299r cells have an increased reliance on aerobic glycolysis, they can increase their mitochondrial activity when challenged. In our data we have shown that there is also significant differences between the parental sensitive and resistant cell lines maximal mitochondrial capacity and non-mitochondrial oxygen consumption rates which was also observed by Liang et al. (2008) and Oliva et al. (2011). A significant decrease in the ECAR's of the H1299r as compared to the H1299s's when treated with FCCP, oligomycin and antimycin A was also observed which may be due to the apparent greater metabolism in the H1299s than the H1299r as the same profile is observed i.e. increased ECAR with oligomycin, decrease with FCCP and a further decrease with antimycin A. There was no substantial difference between the P31r and P31s cells in terms of their rates of ECAR's after drug treatment but similarly to the H1299 cells there was no real difference in their basal ECAR's. The greater OCR of the P31s when compared to the P31r cells and no substantial difference in the ECARs would indicate that differences between the parental sensitive and resistant sub-line that have been observed are due to oxidative phosphorylation differences rather than glycolytic differences. Analysis of the mitochondrial oxygen consumption showed that consistent with the whole cellular results, the basal and FCCP responses were greater in the parental sensitive cell lines than the acquired resistance cell lines but there was no

noticeable difference between the oligomycin responses which indicates that the parental sensitive cell lines may have a greater mitochondrial abundance than their resistant sub-lines which could account for the differences observed. The greater mitochondrial reserve capacity (MCR) of the H1299r cells in comparison to the H1299s indicates that while the H1299r cells primarily undergo aerobic glycolysis for its energy, it can increase its mitochondria activity when stressed. It also signifies that the glycolytic phenotype of the H1299r is due to oxidative phosphorylation being suppressed by active glycolysis rather than defects in mitochondrial function which has also been observed by Fantin et al. (2006)

Reduction in mitochondrial number has been shown to be associated with tumour progression and prognosis in patients with hepatocellular carcinoma and breast cancer (Longley and Johnston, 2005, Gottesman et al., 2002). It has also been reported that a decrease in mitochondrial number is significantly associated with the development of metastasis in Ewing's sarcoma (Adams and Strasser, 2008). The more aggressive cancers possess less mitochondria as they use glycolysis as their primary source of energy (Onishi et al., 2014). In our present work, it was unclear whether the differences that were seen between the sensitive and resistant sub-lines were due to the mitochondria working at maximal capacity in the sensitive cell lines or whether it was because the mitochondrial content of these cells was greater. To answer this question a citrate synthase assay was conducted and expression of the mitochondrial proteins; VDAC and cytochrome C oxidase was examined. Interestingly there was greater citrate synthase activity and greater expression of VDAC and cytochrome C oxidase in the sensitive parental cell lines when compared to the resistant cell lines. The magnitude of the difference in citrate synthase activity/mitochondrial abundance in part explains the greater OCR rates observed. So for instance, the mitochondrial OCR for the H1299s cell line is approximately three fold that of the mitochondrial OCR in the H1299r with the H1299s having 2.5 times greater mitochondrial abundance than that of the H1299r. Similarly in the P31 cells, the mitochondrial OCR of the P31s is only slightly greater than that of the P31r which is in agreement with the mitochondrial abundance which shows that the P31s have a marginally greater mitochondrial abundance than the P31r. These

results are in agreement with Qian et al. (2005) who showed that mitochondrial density determined the sensitivity to cisplatin in an animal model and panel of 8 cell lines. The greater the density of mitochondria the more sensitive the cells were to cisplatin and cells that were depleted of mitochondria were resistant to cisplatin. The decrease in mitochondrial number may be a mechanism by which these cancer cells have acquired resistance to cisplatin.

The process of mitochondrial biogenesis involves the coordinated expression of nuclear and mitochondrial encoded gene products and replication of the mitochondrial genome (Scarpulla et al., 2012, Wenz, 2013). Crucial nuclear encoded proteins such as TFAM, Tfb2 are translated in the cytosol of the cells and contain a mitochondrial targeting sequence which enables their regulated import into the mitochondrial network where they are sorted according to their function (Meng et al., 2009). PGC1 α , a multi functional transcriptional co-activator, regulates the expression of TFAM, a gene that mirrors the changing levels of mitochondrial DNA (mtDNA) and plays a crucial role in mtDNA maintenance (Ekstrand et al., 2004). PGC1 α is now known as the main mediator of transcriptional responses to nutrient stress, promoting mitochondrial biogenesis, cellular metabolism, and antioxidant responses through coordinated activation of the transcription factors NRF2, ERR α and PPAR γ (Scarpulla et al., 2012, Dominy Jr et al., 2010). PGC1 α is controlled at the post-translational level by both phosphorylation by AMPK and deacetylation by a NAD⁺ dependent SIRT1. Deacetylation by SIRT1 supports mitochondrial biogenesis and maintains the sensitivity of PGC1 α to both the energy and redox balance within the cell (Cantó and Auwerx, 2011). Dual activation of both PGC1 α and ERR α , promotes the expression of the downstream mitochondrial SIRT3 that ensures proper scavenging of ROS by activation of mitochondrial superoxide dismutase, as well as other mitochondrial sirtuin targets (Dean et al., 2005). In our current work, the expression levels of the mitochondrial biogenesis proteins that we measured, namely SIRT1, PGC1 α , SIRT3 and TFAM, were reduced in the resistant cells of the H1299 and P31 cells in comparison to their sensitive counterparts. This is in agreement with others who have shown reduced PGC1 α expression (Watkins et al., 2004, Feilchenfeldt et al., 2004, Ba et al., 2008) and mitochondrial biogenesis and claim that the reduction in

mitochondrial biogenesis may contribute to tumourigenesis and/or tumour progression in human malignancies. When we went on to investigate whether the activity as well as expression of PGC1 α was diminished in the resistant cells in comparison to the sensitive cells, we observed decreased activity of the resistant H1299 and P31 cells in comparison to the sensitive cells of both cells lines. This shows that the reduced expression of the proteins involved in mitochondrial biogenesis is consistent with the decreased mitochondrial abundance and reduced respiration rates of the resistant cells in comparison to the sensitive cells.

Increased production of ROS has for a long time been observed as a signature of many tumour and cancer cell lines (Szatrowski and Nathan, 1991). This increased ROS production generally arises as a result of inefficiencies in electron transport, augmented metabolic demand, reduced ROS scavenging, oncogene induced replicative stress and altered mitochondrial dynamics (Hamanaka and Chandel, 2010, Schumacker, 2006). ROS can also be produced as a result of dysfunctional mitochondria through accumulations of mitochondrial mutations, impairment of oxidative phosphorylation and an imbalance in the expression of antioxidant enzymes (Wang et al., 2013). ROS production levels were measured as another index of metabolism. The H1299r cells displayed greater levels of ROS production than the H1299s cells with no difference in ROS production in the P31 cells. It was surprising that the H1299 resistant cells displayed greater ROS production, as the ability of cancer cells to up-regulate antioxidant molecules to promote survival also enables them to develop resistance to anticancer drugs like cisplatin (Liou and Storz, 2010). As we had observed increased ROS production in our H1299r cells, we next wanted to see whether this promoted stabilization of HIF1 α . The link between ROS and HIF1 α stabilization appears to be ambiguous with some proclaiming an increased stabilization of HIF1 α with increased ROS (Sena and Chandel, 2012, Guzy et al., 2005) while others have observed a decreased expression of HIF1 α (Callapina et al., 2005). Here we observed stabilization of HIF1 α in the H1299r cells with no HIF1 α expression in the H1299s or the P31 cells.

In this chapter we have confirmed the resistance of the non small cell lung cancer H1299 and mesothelioma cancer cells P31 to cisplatin and their increased Gb₃ expression. We showed that MDR1 does not appear to be expressed in these cells but two other ABC transporters were up-regulated in resistant cells; ABCE1 in the H1299 cells and ABCB6 in the P31 cells and could prove to be a possible mechanism by which these cells acquired resistance to cisplatin. The resistant cells of both cells lines displayed diminished mitochondrial oxygen consumption which was correlated by decreased mitochondrial abundance and decreased mitochondrial biogenesis as compared to their sensitive cells. The H1299r cells demonstrated increased ROS and HIF1 α stabilisation. In the next chapter we focus on the H1299 cells as they have shown the greatest differences in the assays we have performed so far and we seek to reverse the metabolic phenotype that we have observed by treating the H1299 cells with resveratrol and over express SIRT3.

**Chapter 5: Investigation into the effects
of resveratrol and sirtuin-3 (SIRT3)
over-expression on the bioenergetics of
the H1299s and H1299r cells.**

5.1 Introduction

In this chapter we examined the effects of in vitro resveratrol treatment and in vitro over-expression of SIRT3 in the acquired cisplatin resistant non small cell lung cancer cell line, H1299.

Resveratrol is a natural antioxidant present in red wine, known to have numerous biological properties including a major role in regulating energy metabolism in a sirtuin-dependent pathway (Menzies et al., 2013). Cheung et al. (2015) showed that in cardiomyocytes treated with doxorubicin, expression of SIRT3 was decreased. In addition, when cardiomyocytes were treated with resveratrol, SIRT3 expression increased and when cardiomyocytes treated with doxorubicin were treated with resveratrol expression of SIRT3 was preserved. The exact mechanisms responsible for the effects of resveratrol are poorly understood. SIRT3 could be a downstream effector of resveratrol.

Sirtuin-3 (SIRT3) is a member of the sirtuin family of proteins (SIRT1-7), human homologues of the Sir2 present in yeast, have recently been revealed to play a role in carcinogenesis (Taylor et al., 2008). Sirtuins function as either a nicotinamide adenine dinucleotide (NAD⁺) dependent-deacetylases or ADP ribosyl transferases. The sirtuins varied enzymatic activity explains their role in many cellular functions, such as regulation of oxidative stress, metabolism, aging and longevity (Saunders and Verdin, 2007, Haigis and Guarente, 2006).

SIRT3 is a class III lysine deacetylase which localizes to the mitochondrial matrix, where it binds to and deacetylates several metabolic enzymes that regulate important mitochondrial functions. Specifically, SIRT3 has been found to regulate mitochondrial enzyme activity, complex 1 and 2 activity, mitochondrial dynamics, ATP generation and modulation of the response to oxidative stress (Shi et al., 2005, Yang et al., 2010, Ahn et al., 2008, Jacobs et al., 2008, Scher et al., 2007).

In times of stress, SIRT3 expression is increased protecting cells from apoptosis (Alhazzazi et al., 2011). It has been proposed that SIRT3 like SIRT1, may bind to the DNA repair protein Ku70, encouraging Ku70-Bax interactions, reducing apoptosis in cardiomyocytes (Sundaresan et al., 2008). SIRT3 has been shown to be necessary for Nampt, a stress and diet-responsive regulator of mitochondrial NAD⁺ levels, to support cell survival during genotoxicity (Yang et al., 2007). SIRT3 has also been shown to nullify p53 activity, thus preventing growth arrest and senescence in bladder carcinoma cells (Li et al., 2010). McGlynn et al. (2015), showed in using an immunohistochemical analysis of patient derived-pancreatic adenocarcinomas, that low expression of SIRT3 was correlated with the more aggressive tumours and a shorter time to relapse and death, in the absence of chemotherapeutic intervention.

In the context of anti-cancer drugs, Morigi et al. (2015) showed that SIRT3 had a renoprotective role in response to cisplatin treatment. In their murine model of acute kidney injury, pharmacological activation of SIRT3 with AICAR counteracted cisplatin induced renal failure. Cheung et al. (2015) recently showed that in cardiomyocytes treated with doxorubicin, expression of SIRT3 was decreased and mitochondrial reactive oxygen species (ROS) production was increased. Furthermore, when SIRT3 was expressed directly through transfection or indirectly through treatment with resveratrol in these cardiomyocytes, basal oxygen consumption increased three fold and SIRT3 attenuated the increased ROS production caused by doxorubicin treatment. Our observation of lower expression of SIRT3 in our cisplatin resistant cells led us to investigate whether increased expression of SIRT3, directly through transfection or indirectly, by treatment with resveratrol, could promote mitochondrial respiration, mitochondrial biogenesis and increased mitochondrial abundance. As the H1299 non small cell lung cancer cells demonstrated the greatest difference in respiration, mitochondrial abundance and biogenesis when comparing sensitive to resistant cells, they were chosen as the cells to focus on for these set of experiments.

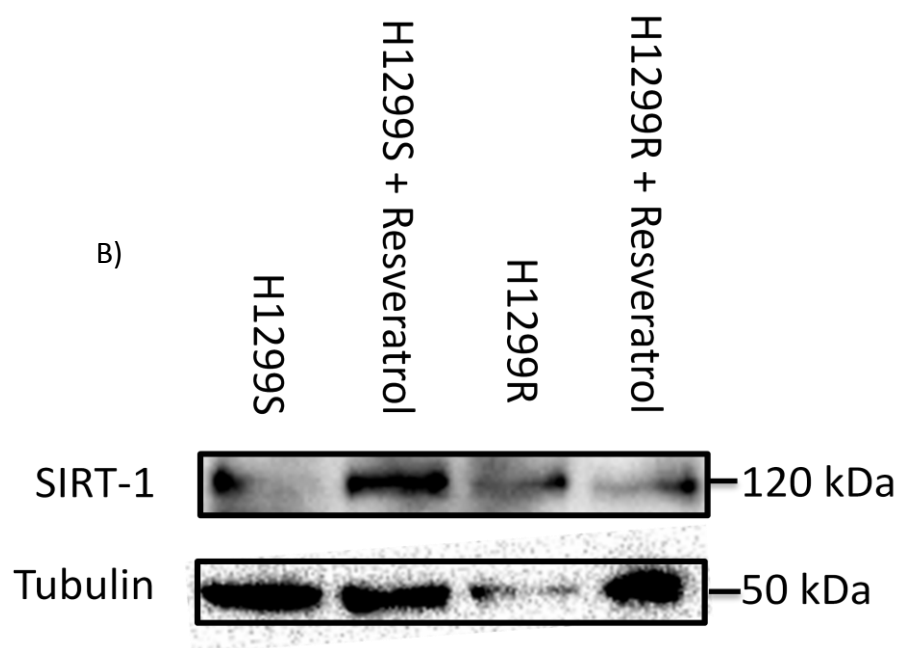
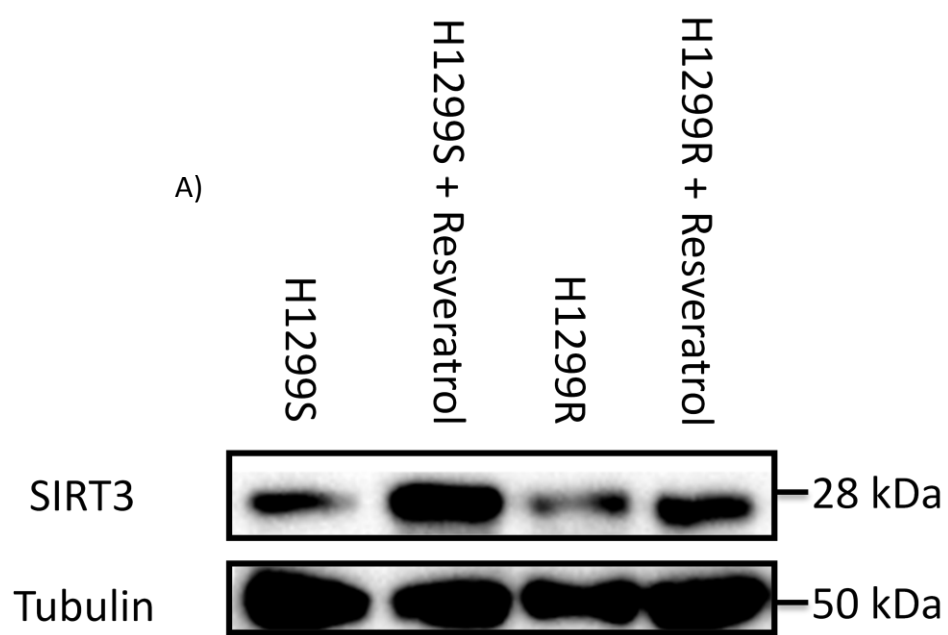
5.2 Results

5.2.1 Investigation into the effects of resveratrol on the bioenergetics of the H1299s and H1299r cells.

5.2.1.1 The effect of resveratrol treatment on Sirtuin-3 and PGC1 α expression as determined by western blot analysis.

To assess whether resveratrol could increase SIRT3 expression in our cells, H1299 cells were treated with 50 μ M resveratrol for 24h in a similar manner to that reported by Cheung et al. (2015). The expression of SIRT3 protein was determined by western blot analysis. As shown previously, H1299s cells exhibited a greater expression of SIRT3 than the H1299r cells. Interestingly resveratrol treatment increased the expression of SIRT3 in both the H1299s and H1299r cells (Figure 5.1A). Resveratrol is reported to increase SIRT3 expression by activating the upstream cytosolic sirtuin, SIRT1 (Menzies et al., 2013) so its expression was also determined after resveratrol treatment. As expected SIRT1 expression was increased in the H1299s but there was no demonstrable increase in expression of SIRT1 in the H1299r cell lysates after resveratrol treatment (Figure 5.1B).

As PGC1 α is downstream of SIRT1 and mice treated with resveratrol demonstrated increased expression of PGC1 α (Kendrick et al., 2011), PGC1 α expression was also examined after resveratrol treatment. Similar to SIRT3, resveratrol increased the expression of PGC1 α in both the H1299s and H1299r cells (Figure 5.1C).



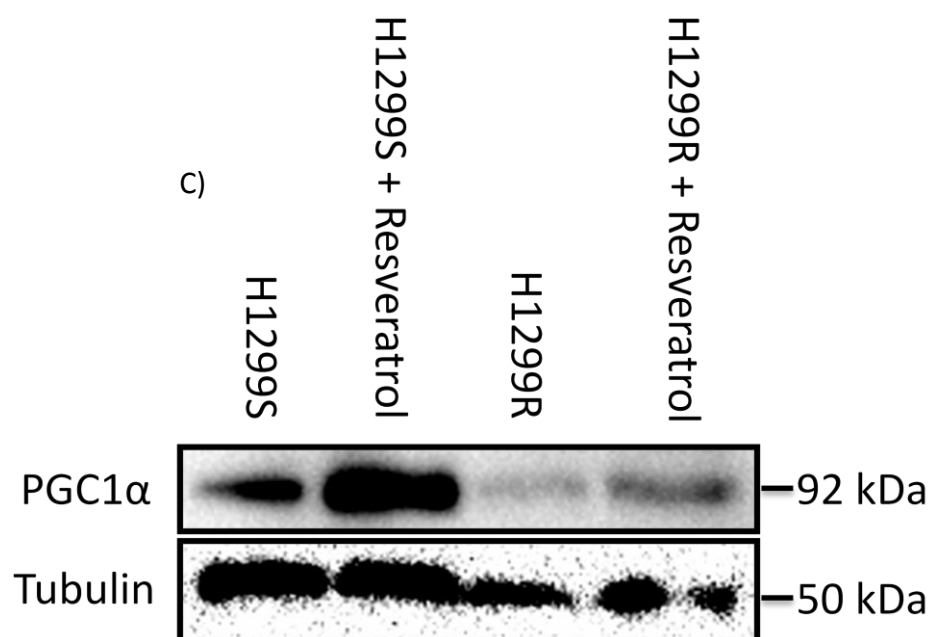


Figure 5.1 Expression of SIRT1, SIRT3 and PGC1α with and without treatment with resveratrol in H1299s and H1299r cell lysates as determined by immunoblot analysis. H1299 cells were plated at 400,000 cells/25 cm³ flask and left for 24 hours. Cells were either left untreated or were treated with 50 μM resveratrol for 24 hrs. Whole cell lysates were prepared from these flasks. Proteins (90 μg) were resolved in 10% SDS-PAGE gels and transferred to a PVDF membrane. Blots were probed for SIRT1, SIRT3, PGC1α or the loading control γ-tubulin. A) Representative blot of SIRT3 protein expression from three independent experiments. B) Representative blot of SIRT1 protein expression from two independent experiments. C) Representative blot of PGC1α protein expression from three independent experiments.

5.2.1.2 The effect of resveratrol treatment on the metabolism of H1299s and H1299r as determined by the seahorse extracellular flux analyser.

Having shown that resveratrol treatment can increase the expression of SIRT3 and PGC1 α in our H1299 cells, we next wanted to determine whether resveratrol could affect the H1299 cells functionally by affecting their metabolism. Theoretically, resveratrol could impact upon metabolism mediated via SIRT1 (Menzies et al., 2013) and/or downstream of SIRT 1 via SIRT3. SIRT3 itself has been shown to have direct effects on oxidative phosphorylation by augmenting complex I (Abdel Khalek et al., 2014) and succinate dehydrogenase (Kincaid and Bossy-Wetzel, 2013).

To examine whether resveratrol could affect the metabolism of our cells, H1299 cells were plated at 15,000 cells/well and left for 24 h. The following day three wells were treated with and without 50 μ M resveratrol for 24 h. We defined the bioenergetic status of the cells in response to resveratrol treatment by measuring total/basal cellular oxygen consumption and dissecting out oxygen consumption due to mitochondria alone by adding antimycin A, an inhibitor of mitochondrial function. Prior to addition of antimycin A, maximal mitochondrial capacity was determined by the addition of the mitochondrial uncoupler, FCCP.

As shown previously in chapter 4 (Figure 4.11 A), the basal oxygen consumption of the H1299s cells was almost 3 times greater than that of the H1299r cells. As resveratrol had increased the expression of mitochondrial biogenesis proteins; SIRT3 and PGC1 α (Figure 5.1) therefore an increase in basal cellular oxygen consumption was predicted. However, a significant decrease in basal oxygen consumption rate in the H1299s cells ($p < 0.05$) (Figure 5.2A) was observed after resveratrol treatment. There was also a significant ($p < 0.05$) decrease in the ECAR in the H1299s and H1299r after resveratrol treatment (Figure 5.2 B). For the H1299s cells, resveratrol is attenuating both oxidative phosphorylation and glycolysis but for the H1299r cells it seems that resveratrol is making the cells more oxidative. In the analysis of the OCR/ECAR ratio, resveratrol seems to be significantly ($p < 0.05$) attenuating the OCR of the H1299s to a greater extent than resveratrol's reduction in ECAR of the H1299s cells. Resveratrol treatment did not

affect the OCR/ECAR ratio of the H1299r cells (Figure 5.2 C). We also noticed that resveratrol was significantly increasing the cellular reserve capacity (CRC) of the H1299s ($p<0.005$) and H1299r cells ($p<0.05$) (Figure 5.2 D), increasing mitochondrial function.

When the OCR data was expressed as mitochondrial oxygen consumption, resveratrol still significantly reduced the basal mitochondrial OCR of the H1299s with no effect upon the H1299r basal mitochondrial OCR (Figure 5.3 A). In accordance with the cellular reserve capacity (CRC), resveratrol significantly ($p<0.05$) increased the mitochondrial reserve capacity (MRC) of both the H1299s and H1299r cells (Figure 5.3 B). This significantly increased reserve capacity of the resveratrol treated H1299 cells indicates that resveratrol is increasing mitochondrial activity of both the H1299s and H1299r cells and not any other oxygen consuming entities.

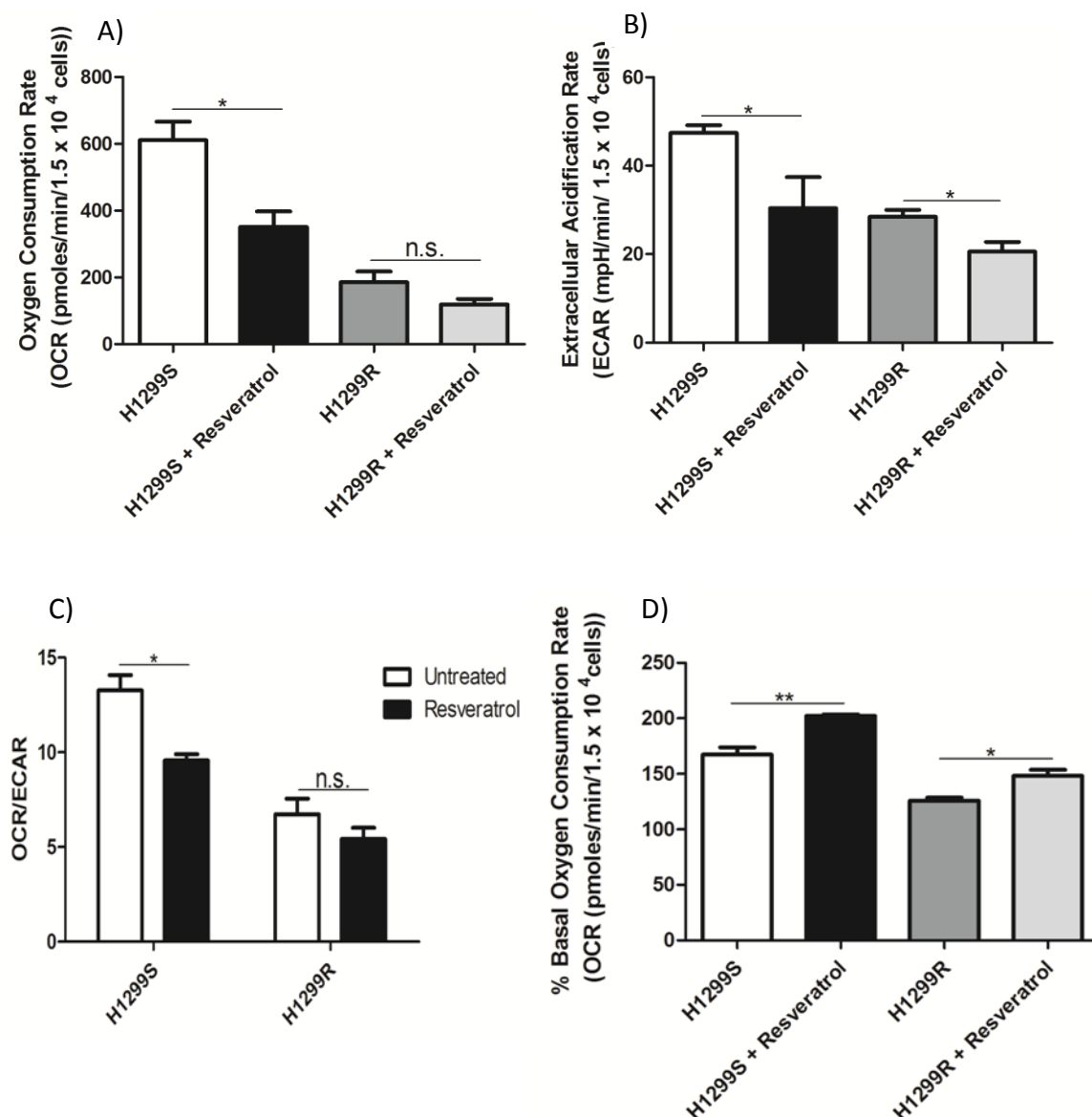


Figure 5.2 Effect of resveratrol treatment on the cellular oxygen consumption, cellular reserve capacity and OCR/ECAR ratio of H1299s and H1299r cells as determined by the seahorse extracellular flux analyser. Cells were seeded at 1.5×10^4 cells/well and were and were not treated with 50 μ M resveratrol for 24 h. A) OCR of the basal metabolism of H1299s and H1299r cells. B) ECAR of the basal metabolism of H1299s and H1299r cells. C) OCR/ECAR ratio of the basal rate of H1299s and H1299r cells. D) Cellular reserve capacity (CRC) of the H1299s and H1299r cells with and without resveratrol treatment. Error bars represent the standard error of the mean between three separate experiments. Statistical analysis was carried out using the student t-test. ** = $p < 0.005$, * = $p < 0.05$

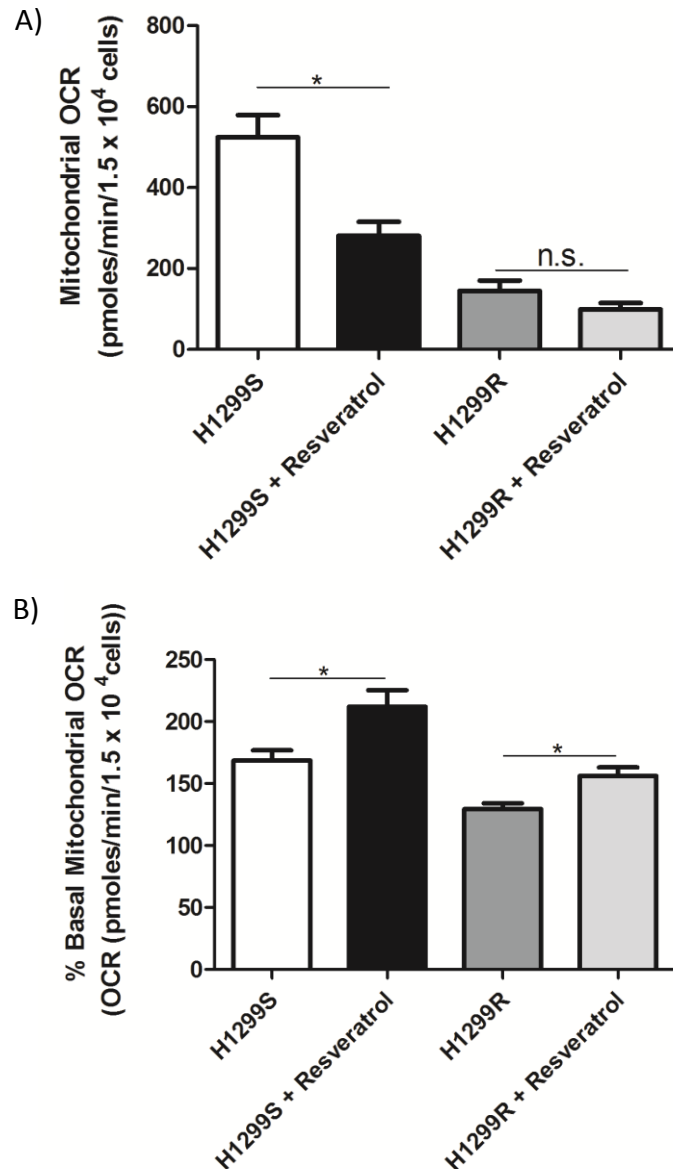


Figure 5.3 Effect of resveratrol treatment on the mitochondrial oxygen consumption and mitochondrial reserve capacity of H1299s and H1299r cells as determined by the seahorse extracellular flux analyser. Cells were seeded at 1.5×10^4 cells/well and were and were not treated with 50 μ M resveratrol for 24 h. The cells were then put into the machine where the mitochondrial inhibitors; FCCP and antimycin A were sequentially added to the wells during the run. Mitochondrial oxygen consumption was obtained by taking the antimycin A OCR from the rate obtained from the basal rate. A) Mitochondrial OCR of the basal metabolism of H1299s and H1299r. B) Mitochondrial reserve capacity (MCR) injection of the H1299s and H1299r cells with and without resveratrol treatment and after the inhibitors were added to the wells. Error bars represent the standard error of the mean between three separate experiments. Statistical analysis was carried out using the student t-test. * = $p < 0.05$

5.2.1.3 The effect of resveratrol treatment on citrate synthase activity

As resveratrol treatment caused an increase in the expression of mitochondrial biogenesis proteins; SIRT3 and PGC1 α (Figure 5.1) and an increase in mitochondrial reserve capacity (Figure 5.3 B), it was decided to determine whether the increase in reserve capacity was due to activity of the mitochondria at the current level of abundance and/or due to increased mitochondrial abundance. Citrate synthase is a mitochondrial matrix protein, commonly used as a marker of mitochondrial abundance within the cell.

Resveratrol caused a significant increase in citrate synthase activity in both the H1299s and H1299r cells ($p < 0.05$) (Figure 5.4). This indicates that resveratrol is increasing mitochondrial abundance.

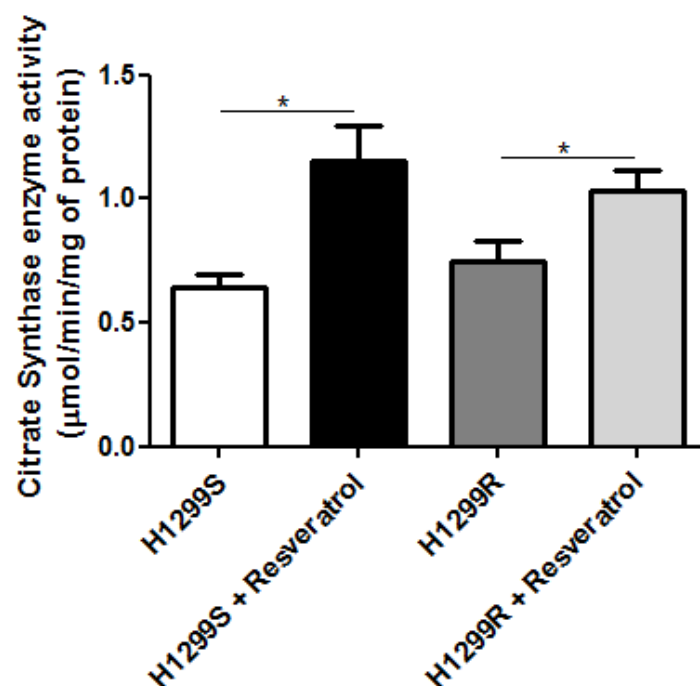


Figure 5.4 Citrate synthase activity of the H1299 cells with and without resveratrol treatment as determined by spectroscopy. Cells were plated at 400,000 cells per T25 flask and were either left untreated or treated with 50 μ M resveratrol for 24h. Cells were then lysed with laemelli buffer and the protein content was assessed using the BCA assay. 40 μ g of protein was added into the cuvette with 500 μ l of Tris (200 mM, pH 8.0) with Triton X-100 (0.2% (v/v)), 100 μ l of DTNB, 30 μ l of acetyl coenzyme A (10 mM). The coupled reaction was started by adding 50 μ l of 10 mM oxaloacetic acid and measuring the rate of increase of 5-thionitrobenzene in absorbance at 412nm for 3min. Data are expressed as mean plus or minus standard error of the mean for three separate experiments. Statistical analysis was carried out using the student t-test. * = $p < 0.05$.

5.2.1.4 The effect of resveratrol treatment on reactive oxygen species (ROS) production as determined by flow cytometry.

Cheung et al. (2015) showed that resveratrol treatment alone increased ROS production but also attenuated the increased ROS production caused by doxorubicin treatment of cardiomyocytes. Having shown that resveratrol treatment increased the expression of the mitochondrial biogenesis proteins (Figure 5.1), increased reserve capacity (Figure 5.3 B) and citrate synthase activity (Figure 5.4) we next wanted to determine what effect resveratrol treatment had on ROS production of the H1299 cells.

In the present study, resveratrol treatment caused a significant ($p < 0.0005$) increase in ROS production in the H1299s cells (Figure 5.5A) but did not affect the ROS production of the H1299r cells.

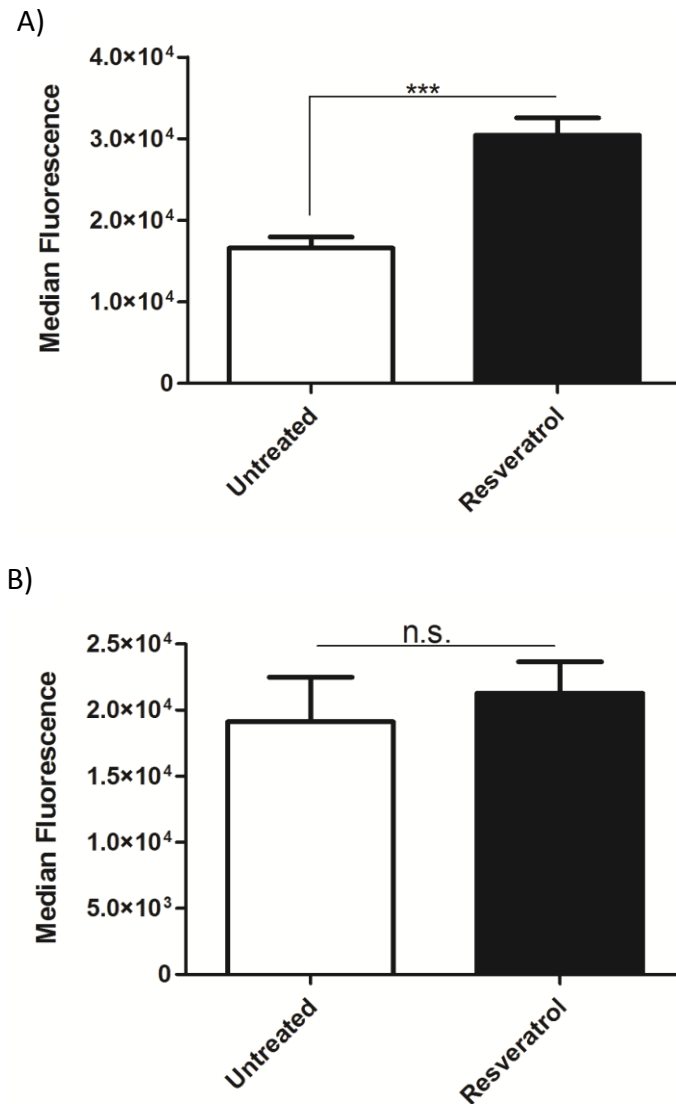


Figure 5.5 Reactive Oxygen Species (ROS) production the H1299s and H1299r cells with and without resveratrol treatment as determined by flow cytometry. (A) H1299s and (B) H1299r were seeded at a density of 250,000 cells/well and left for 24 hours. The cells were then stained with 10 μ M dichloro-dihydro-fluorescein diacetate (DCFH-DA). Fluorescence was analysed for 10,000 events. Data are expressed as mean plus or minus standard error of the mean for the relative DCFH-DA signal over three separate experiments. Statistical analysis was carried out using the student t-test. *** = $p < 0.0005$.

5.2.2 Investigation into the effects of SIRT3 over-expression on the bioenergetics of the H1299s and H1299r cells.

5.2.2.1 Optimisation of the viral titre needed to transduce the H1299 cells using an empty vector GFP tagged adenovirus as determined by fluorescent microscopy.

In the paper by Cheung et al. (2015) by Prof Dolinsky's laboratory, It was observed that doxorubicin was administered to cardiomyocytes, SIRT3 expression was reduced. When they transduced these cardiomyocytes with an adenovirus with an expression vector for SIRT3, the virus increased first of all SIRT3 expression, increased basal metabolism and decreased ROS production. They showed that the deacetylation activity of the enzyme was important through a less active SIRT3 mutant construct (Ad.MutM1-SIRT3) and the mitochondrial localisation was vital for SIRT3 to have an effect upon the metabolism of the cardiomyocytes (Ad.M1-SIRT3 vs Ad.M3-SIRT3 (no mitochondrial localisation signal)) using a GFP tagged empty vector as a control. We wanted to see if we over-expressed SIRT3 in our cells, could it increase respiration, mitochondrial abundance, mitochondrial biogenesis and decrease ROS production in our H1299 cells. Professor Dolinsky kindly agreed to collaborate on this project and allowed us use of his viruses with the different SIRT3 constructs to test out our hypothesis.

The first thing we needed to determine was the maximum viral titre needed to transduce these cells with in order to obtain maximal expression of the plasmid and then determine for how long should we incubate the cells with the virus. The viral titre is determined by quantifying the amount of virus particles per cell, called multiplicity of infection or MOI. Prof Dolinsky advised that the maximum MOI we would need was 100, as any higher became toxic to his cells. Using the GFP tagged empty vector control we transduced the H1299 cells with a range of concentrations (0.1, 1, 10, 20, 50, 100 MOI) for 24 and 48h and imaged the cells using an Olympus IX81 inverted microscope at 20x magnification excited at a wavelength of 395 nm using FV10-ASW 2.0 Viewer software.

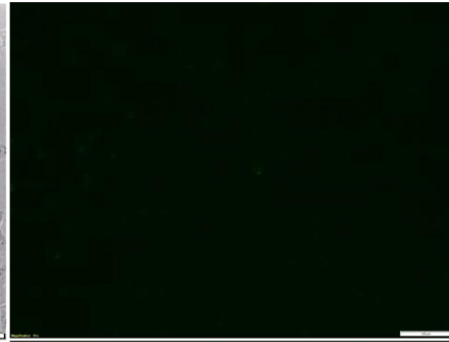
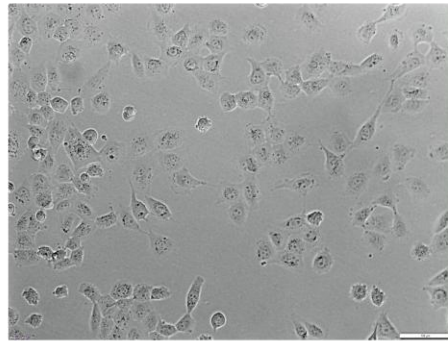
MOI concentration of 0.1 and 1 did not transduce the cells with the GFP plasmid to any significant extent. At 10 and 20 MOI the cells were beginning to fluoresce green. Maximal transfection of the cells with the GFP plasmid occurred at 50 MOI with no further increase with 100 MOI for both the H1299s and H1299r (Figure 5.6 A&B). When imaged again having being transduced for 48h, there was greater expression of the GFP plasmid with the maximal expression occurring at 50 and 100 MOI (Figure 5.6 C&D). The MOI of 50 and time point of 48h was expressed the GFP

A)

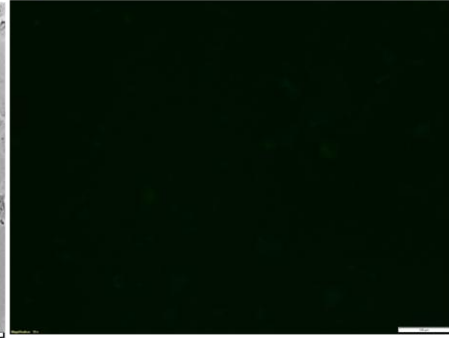
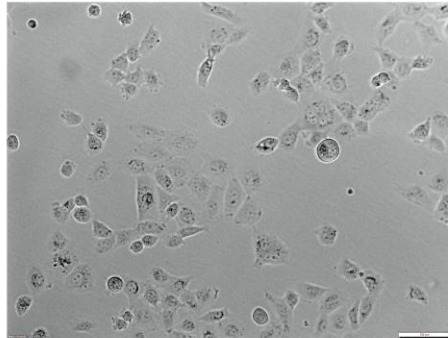
BF

GFP

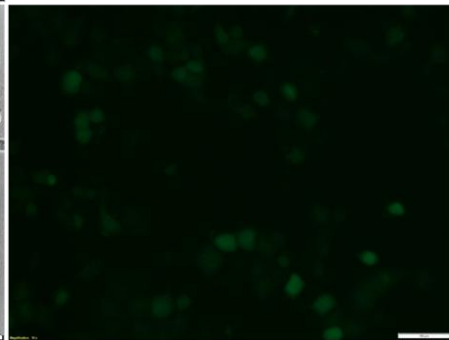
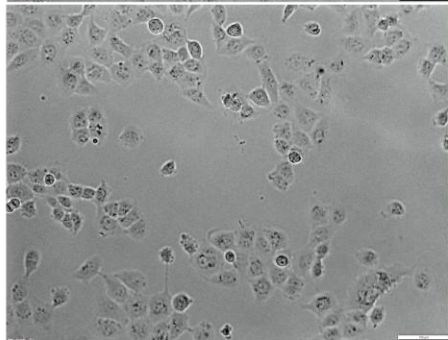
0.1 MOI

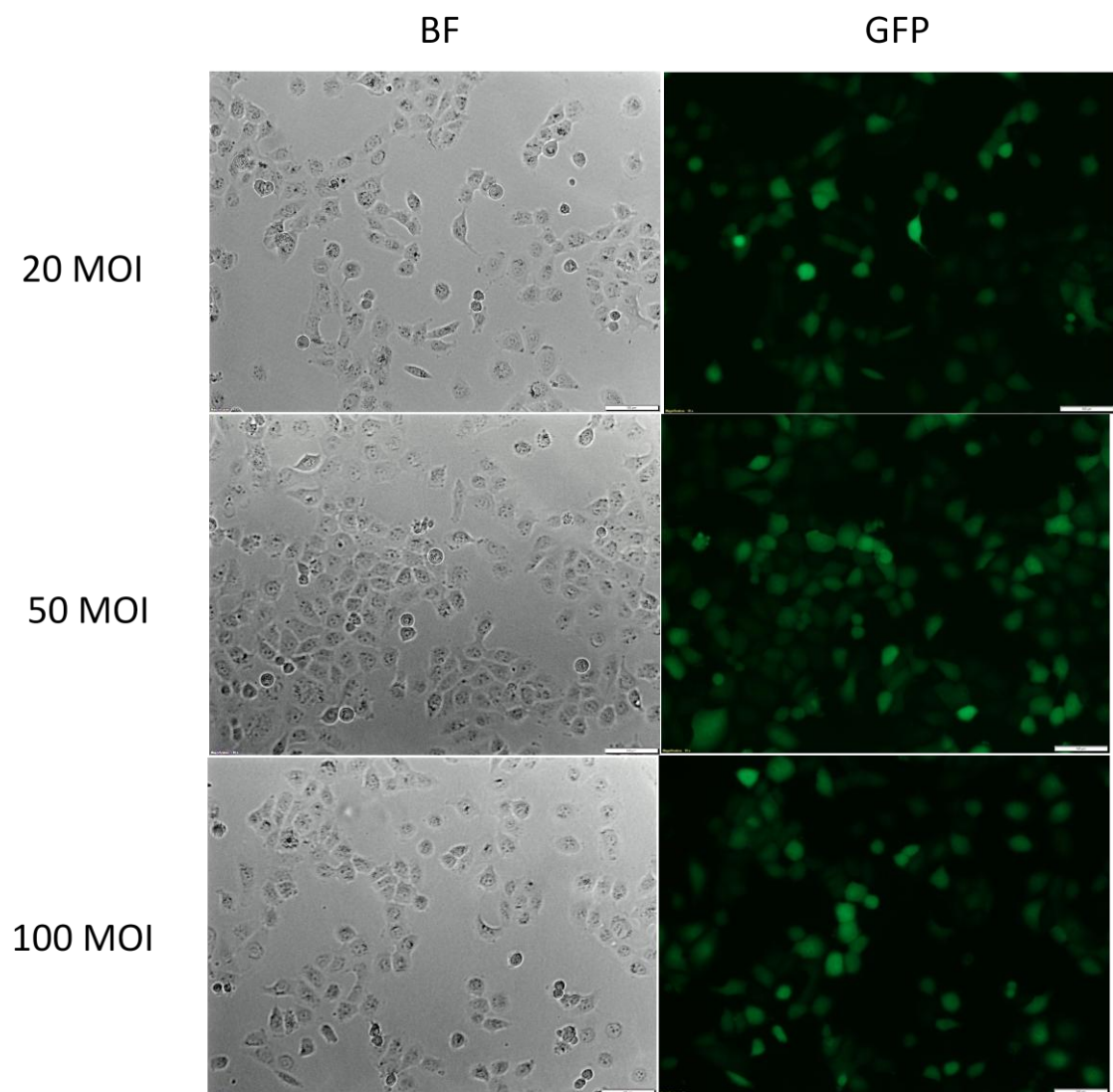


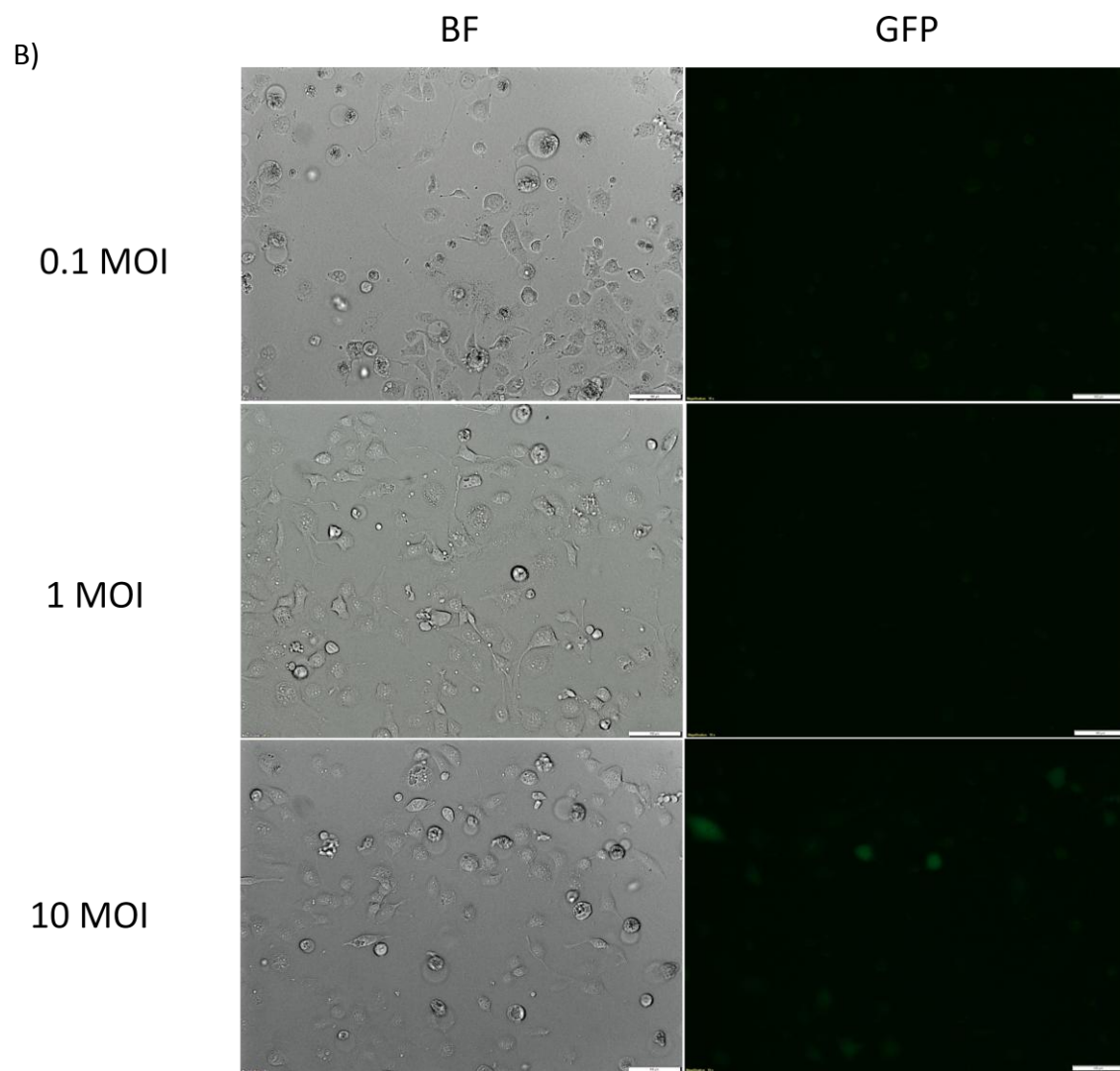
1 MOI

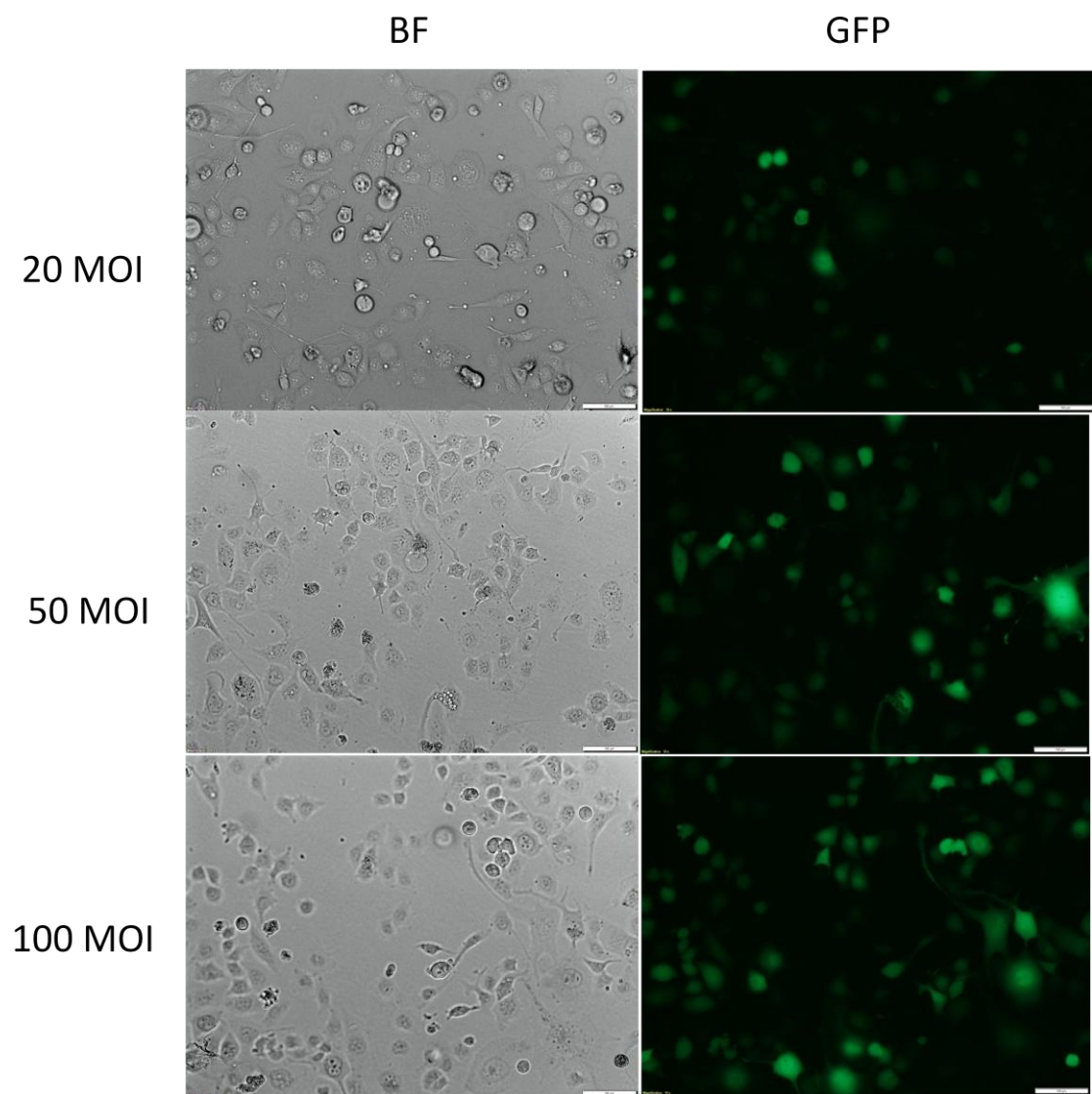


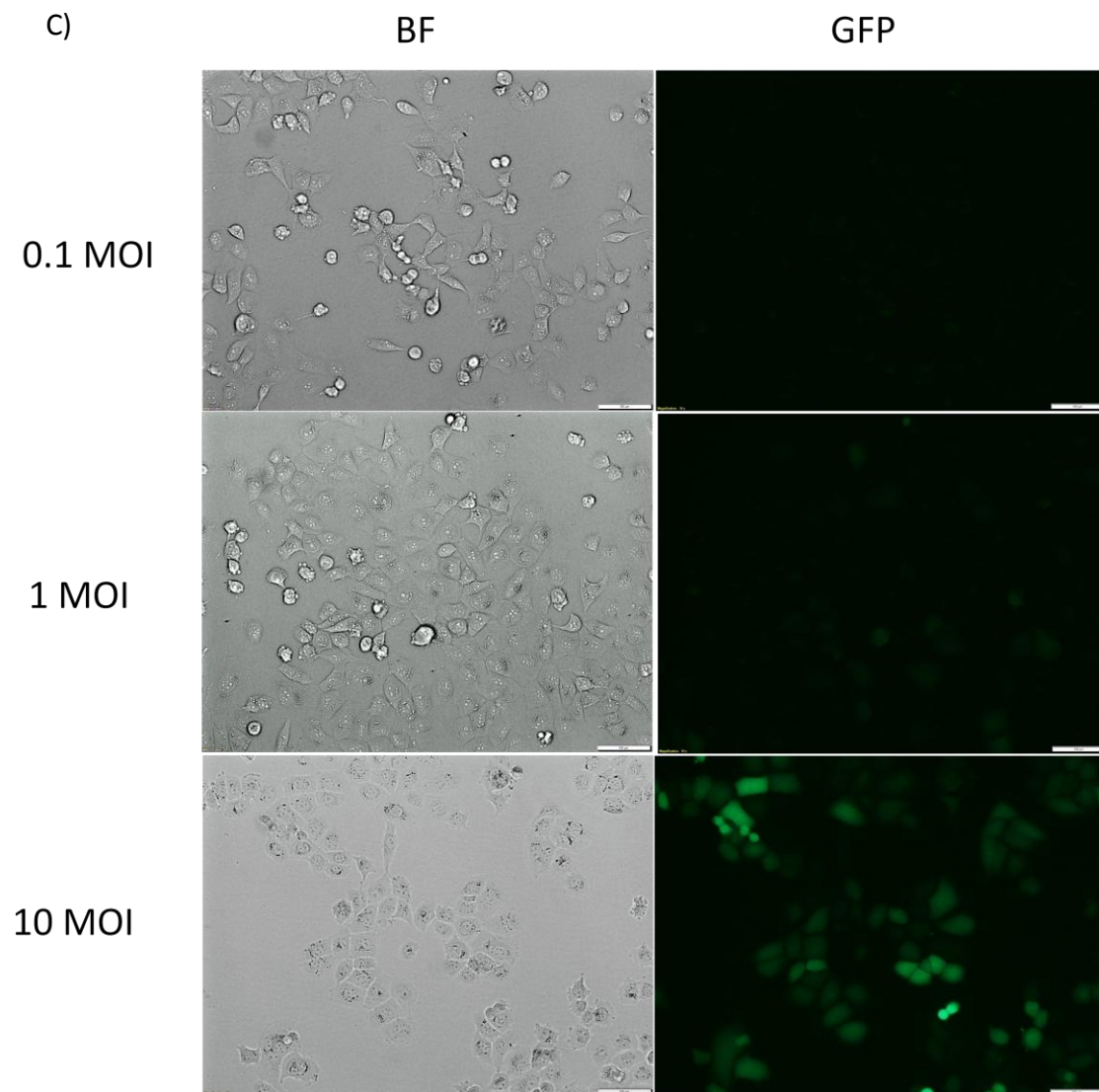
10 MOI

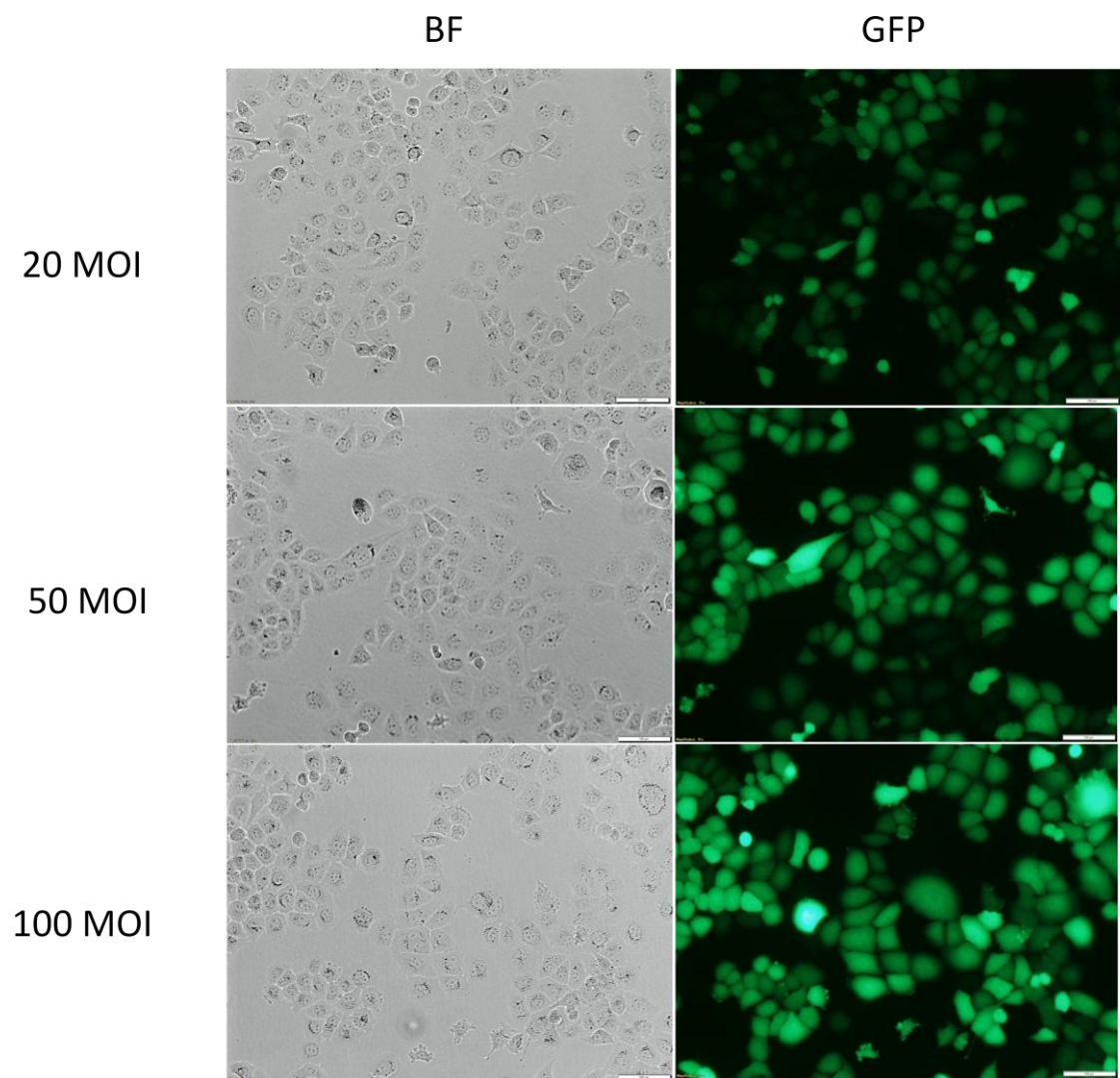


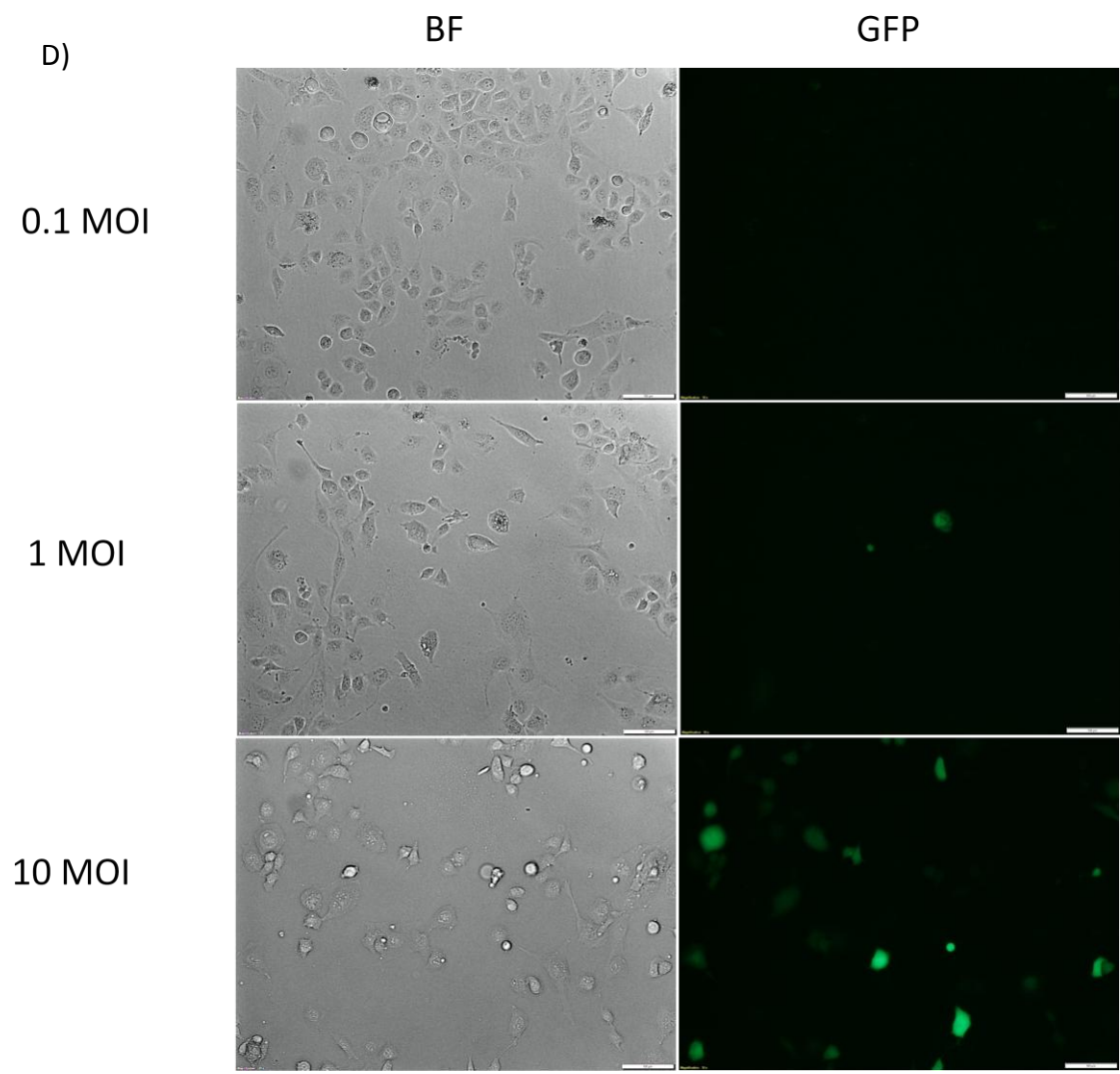












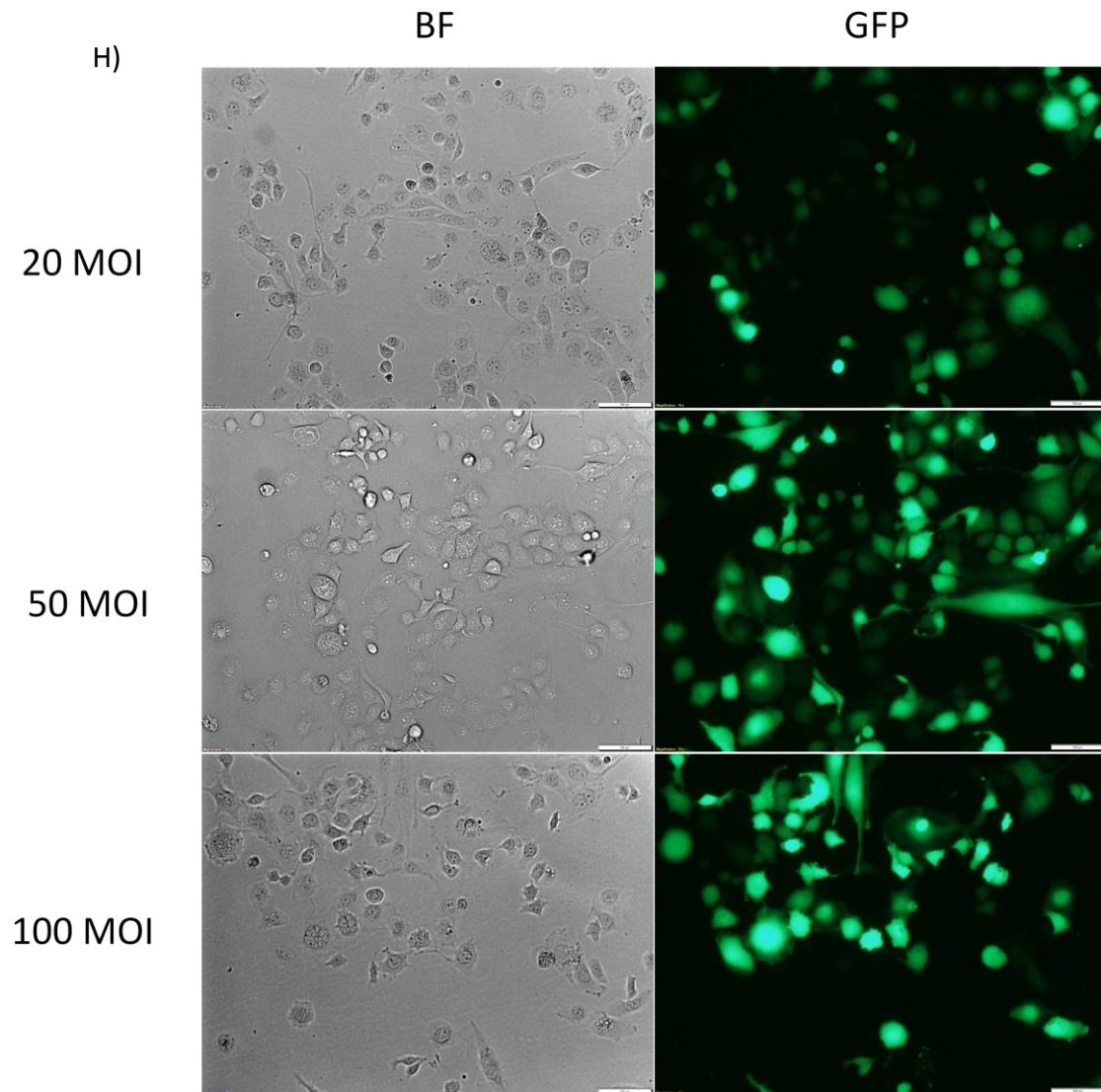


Figure 5.6 Optimisation of the viral titre necessary to transfect the H1299s and H1299r with a GFP tagged empty vector adenovirus as determined by fluorescent microscopy. Cells were seeded at a density of 2.5×10^5 cells/ml in 6 well plates. A) H1299s cells were transduced with a range of concentrations of GFP tagged empty vector adenovirus (0.1-100 MOI) for 24 h. B) H1299r cells were transduced with a range of concentrations of GFP tagged empty vector adenovirus (0.1-100 MOI) for 24 h. C) H1299s cells were transduced with a range of concentrations of GFP tagged empty vector adenovirus (0.1-100 MOI) for 48 h. D) H1299r cells were transduced with a range of concentrations of GFP tagged empty vector adenovirus (0.1-100 MOI) for 48 h. The cells were then imaged using an Olympus IX81 inverted microscope at 20x magnification excited at a wavelength of 395 nm using FV10-ASW 2.0 Viewer software. BF = bright field, GFP = green fluorescent protein and scale bar = 100 μ m Images are representative of three independent experiments.

5.2.2.2 Confirmation of the over-expression of SIRT3 by transduction of the H1299 cells with an adenovirus as determined by western blotting.

Next we needed determined whether these different constructs do indeed express SIRT3 to a greater extent than *de novo* expression of the protein and also to confirm the different sizes of the SIRT3 constructs.

As we had expected, the constructs do express SIRT3 and to a greater extent than the *de novo* expression of the protein (Figure 5.7). The M1-SIRT3 construct appears as two bands, the SIRT-3 protein and the mitochondrial localisation signal, perhaps as a result of saturating the import system which is absent in the M3-SIRT3 construct as expected.

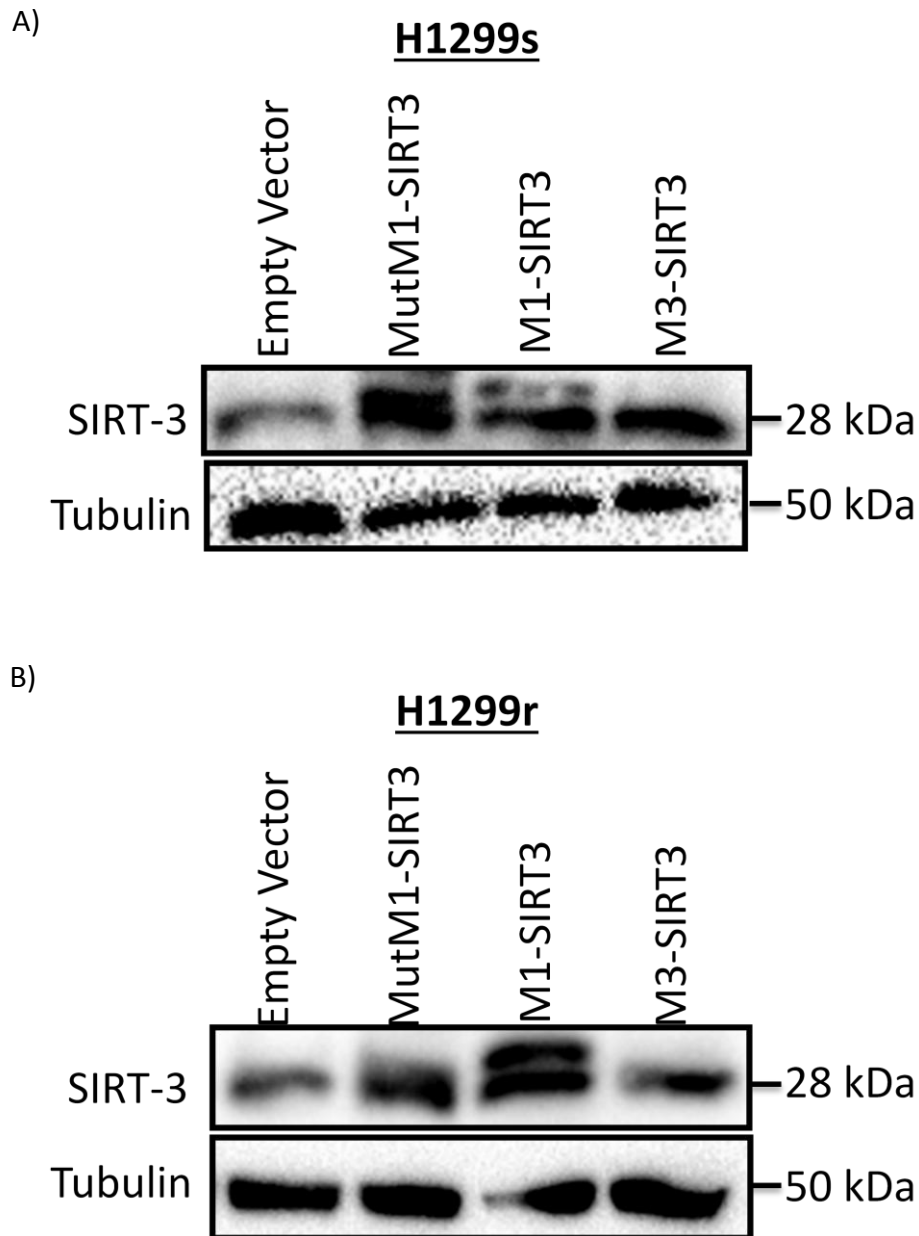


Figure 5.7 Expression of SIRT3 after transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus as determined by western blotting. H1299 cells were transduced with 50 MOI of empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus for 48 h. Whole cell lysates were then prepared. Proteins (90 µg) were resolved in 10% SDS-PAGE gels and transferred to a PVDF membrane. Blots were probed for SIRT3 or the loading control tubulin. A) Representative blot of SIRT3 protein expression from H1299s cells from three independent experiments. B) Representative blot of SIRT3 protein expression from H1299r cells from three independent experiments.

5.2.2.3 The effect of the transduction of H1299 cells using a SIRT3 expressing adenovirus upon bioenergetics of the cells as determined by the seahorse extracellular flux analyser.

Having demonstrated that the SIRT3 adenovirus constructs are capable of transfecting the H1299 cells and expressing SIRT3 protein to a greater extent than the *de novo* synthesis of the protein, we wanted to determine whether these SIRT3 constructs had any impact upon the bioenergetics of the cells.

Analysis of the bioenergetics was carried out by investigating oxygen consumption due to mitochondrial function, non-mitochondrial function, ATP turnover and proton leak, which was achieved using the mitochondrial inhibitors antimycin A and oligomycin. Maximal mitochondrial capacity was determined by the addition of the mitochondrial uncoupler, FCCP.

The SIRT3 constructs did not have any effect upon the OCR of the H1299s cells (Figure 5.8 A) which was expected as they already had high endogenous expression of SIRT3. However, the M1-SIRT3 construct appeared to significantly ($p<0.05$) increase the ECAR of the H1299s cells (Figure 5.8 B). This increased ECAR of the H1299s cells as a result of the expression of the M1-SIRT3 construct would indicate that the M1-SIRT3 construct may be making the H1299s cells more glycolytic. The MutM1-SIRT3 and M1-SIRT3 constructs significantly ($p<0.05$) increased the basal metabolism of the H1299r cells (Figure 5.8 C) with no effect on the ECAR of the H1299r cells (Figure 5.8 D). This increase in basal respiration as a result of MutM1-SIRT3 and M1-SIRT3 expression also correlates with Prof. Dolinsky's laboratory's work which showed that the expression of the M1-SIRT3 construct caused an increase in basal respiration. Interestingly the MutM1 appeared to significantly ($p<0.05$) reduce the proton leak induced by the treatment of the H1299r cells with oligomycin (Figure 5.8 C). As the M1-SIRT3 significantly increased the ECAR of the H1299s, the M1-SIRT3 construct significantly ($p<0.05$) reduced the OCR/ECAR ratio of the H1299s (Figure 5.9 A). SIRT3 expression had no effect upon the OCR/ECAR ratio of the H1299r cells. Consistent with the lack of an effect of the expression of SIRT3 on basal OCR of the H1299 cells, the expression of SIRT3 had no

effect on the cellular reserve capacity of the H1299s cells (Figure 5.9 B). Similarly in concordance with the basal OCR data, MutM1 significantly ($p<0.005$) increased the cellular reserve capacity of the H1299r cells (Figure 5.9 C).

Having established that the SIRT3 constructs were having an effect on the total cellular OCR and ECAR rates of the H1299 cells, the next question to be answered was whether the differences in OCR observed in the H1299r cells, were due to the differences in the mitochondria function in these cells, or whether the differences were due to oxygen consumption by non-mitochondrial (non-oxidative phosphorylation) events inside the cell. The oxygen consumption rate due to mitochondria alone was achieved by subtracting the antimycin A rate from the basal cellular oxygen consumption rate. There was no difference to the profile of oxygen consumption rates for the H1299s cells (Figure 5.10 A). We observed that the MutM1-SIRT3 construct significantly ($p<0.05$) increased the basal mitochondrial oxygen consumption (Figure 5.10 B). Similar to the cellular reserve capacity, SIRT3 expression had no effect on the mitochondrial reserve capacity of the H1299s cells and MutM1-SIRT3 significantly ($p<0.005$) increased the mitochondrial reserve capacity of the H1299r cells (Figure 5.10 C). This indicates that MutM1 is significantly ($p<0.005$) increasing the activity of the mitochondria in the H1299r cells and not any other oxygen consuming entity.

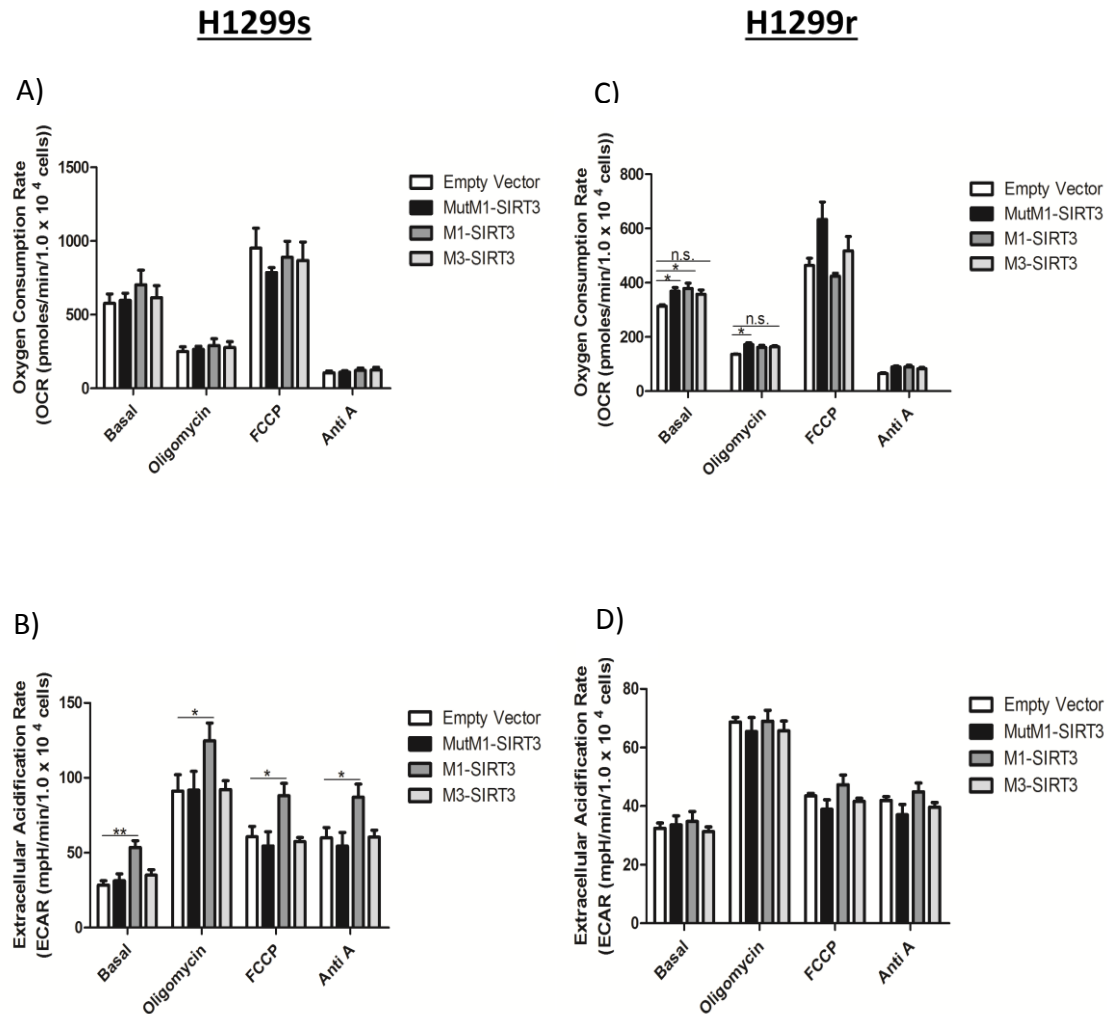


Figure 5.8 The effect of transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus on whole cell metabolism as determined by the seahorse extracellular flux analyser. H1299 cells were seeded at 1.0×10^4 cells/well and were transduced with 50 MOI of empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus for 48 h. After the time had elapsed, the cell plate was put into the machine where the mitochondrial inhibitors; oligomycin, FCCP and Antimycin A were sequentially added to the wells during the run. A) OCR of the basal metabolism of H1299s and after the inhibitors were added to the wells. B) OCR of the basal metabolism of H1299r's and after the inhibitors were added to the wells. C) ECAR rates of the basal metabolism of H1299s and after the inhibitors were added to the wells. D) ECAR rates of the basal metabolism of H1299r's and after the inhibitors were added to the wells. Data are expressed as mean plus or minus standard error of the mean for three separate experiments. Statistical analysis was carried out using the student t-test. ** = $p < 0.005$, * = $p < 0.05$.

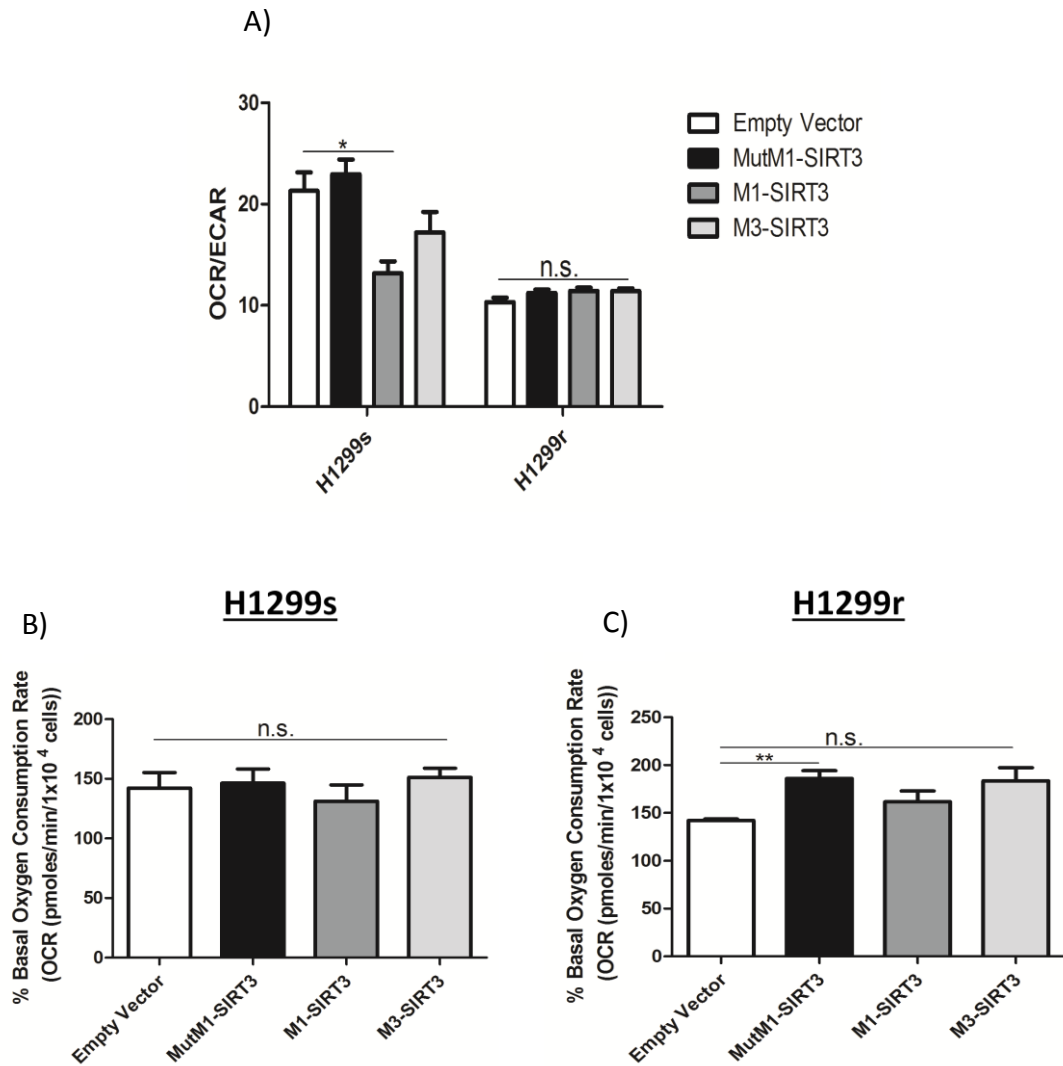


Figure 5.9 The effect of transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus on OCR/ECAR ratio and cellular reserve capacity as determined by the seahorse extracellular flux analyser. H1299 cells were seeded at 10,000 cells/well and were transduced with 50 MOI of empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus for 48 h. After the time had elapsed, the cell plate was put into the machine where the mitochondrial inhibitors; oligomycin, FCCP and Antimycin A were sequentially added to the wells during the run. A) OCR/ECAR ratio of the basal rate of the H1299s and H1299r cells. B) Cellular reserve capacity of the H1299s. C) Cellular reserve capacity of the H1299r cells. Data are expressed as mean plus or minus standard error of the mean for three separate experiments. Statistical analysis was carried out using the student t-test. ** = $p < 0.005$, * = $p < 0.05$.

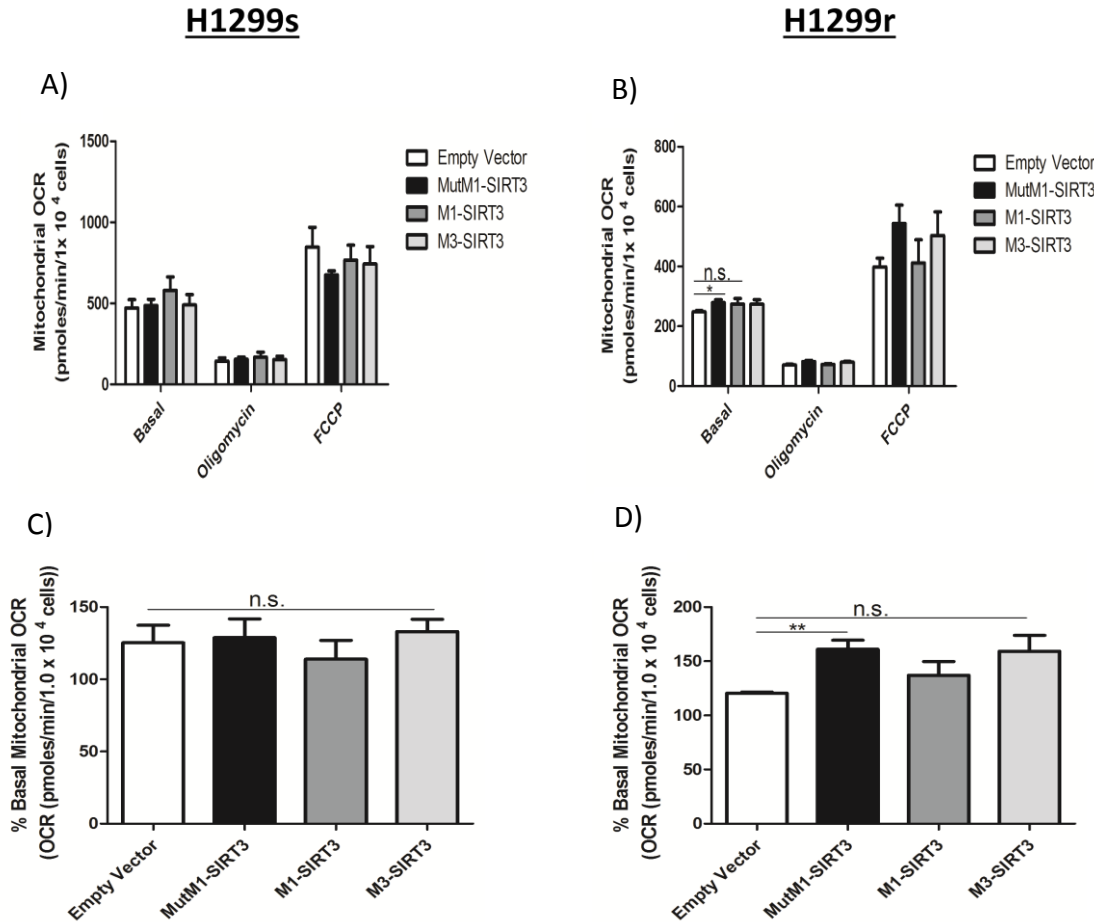


Figure 5.10 The effect of transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus on *in situ* mitochondrial metabolism and mitochondrial reserve capacity as determined by the seahorse extracellular flux analyser. H1299 cells were seeded at 10,000 cells/well and were transduced with 50 MOI of empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus for 48 hrs. After the time had elapsed, the cell plate was put into the machine where the mitochondrial inhibitors; oligomycin, FCCP and Antimycin A were sequentially added to the wells during the run. *In situ* mitochondrial oxygen consumption was obtained by taking the Antimycin A OCR from the rate obtained from the basal rate and after oligomycin and FCCP treatment. A) Mitochondrial OCR rates of the basal metabolism of H1299s cells and after the inhibitors were added to the wells. B) Mitochondrial OCR rates of the basal metabolism of H1299r cells and after the inhibitors were added to the wells. C) Mitochondrial reserve capacity of the H1299s. D) Mitochondrial reserve capacity of the H1299r cells. Data are expressed as mean plus or minus standard error of the mean for three separate experiments. Statistical analysis was carried out using the student t-test. ** = $p < 0.005$, * = $p < 0.05$.

5.2.2.4 The effect of the transduction of H1299 cells using a SIRT3 expressing adenovirus upon citrate synthase activity as determined by the spectrophotometer.

Having shown that the SIRT3 constructs over-express SIRT3 (Figure 5.7) and had an effect on the bioenergetics of the cells (Figure 5.8-5.10) it was decided to see whether these constructs alter citrate synthase activity, an index of mitochondrial abundance and see whether the significant ($p < 0.05$) increase in citrate synthase activity as a result of resveratrol treatment (Figure 5.4) was as a result of resveratrol increasing SIRT3 expression (Figure 5.1 A).

The M1-SIRT3 construct significantly ($p < 0.05$) increased the citrate synthase activity of the H1299s cells (Figure 5.11 A). From the Cheung et al. (2015) paper, the M1-SIRT3 construct had the greatest effect on the metabolism of their cardiomyocytes and so would be expected to increase citrate synthase activity as we have observed here. However, the M1-SIRT3 construct in this present study did not alter the OCR of the H1299s cells to any noticeable extent (Figure 5.8 A) but seemed to significantly ($p < 0.05$) increase ECAR (Figure 5.8 B) which would indicate that it may be affecting glycolysis rather than oxidative phosphorylation in these cells. In the H1299r cells, the citrate synthase activity result is similar to the increase in basal metabolism (Figure 5.8 C) observed with the SIRT3 constructs (Figure 5.11 B). MutM1 and M1-SIRT3 constructs both significantly ($p < 0.05$ & $p < 0.005$ respectively) increased the citrate synthase activity of the H1299r cells with no effect from the M3-SIRT3 construct. The increase in citrate synthase with the MutM1 and M1 constructs indicates that the mitochondrial localisation signal is important to SIRT3's increase in citrate synthase activity in the H1299r cells.

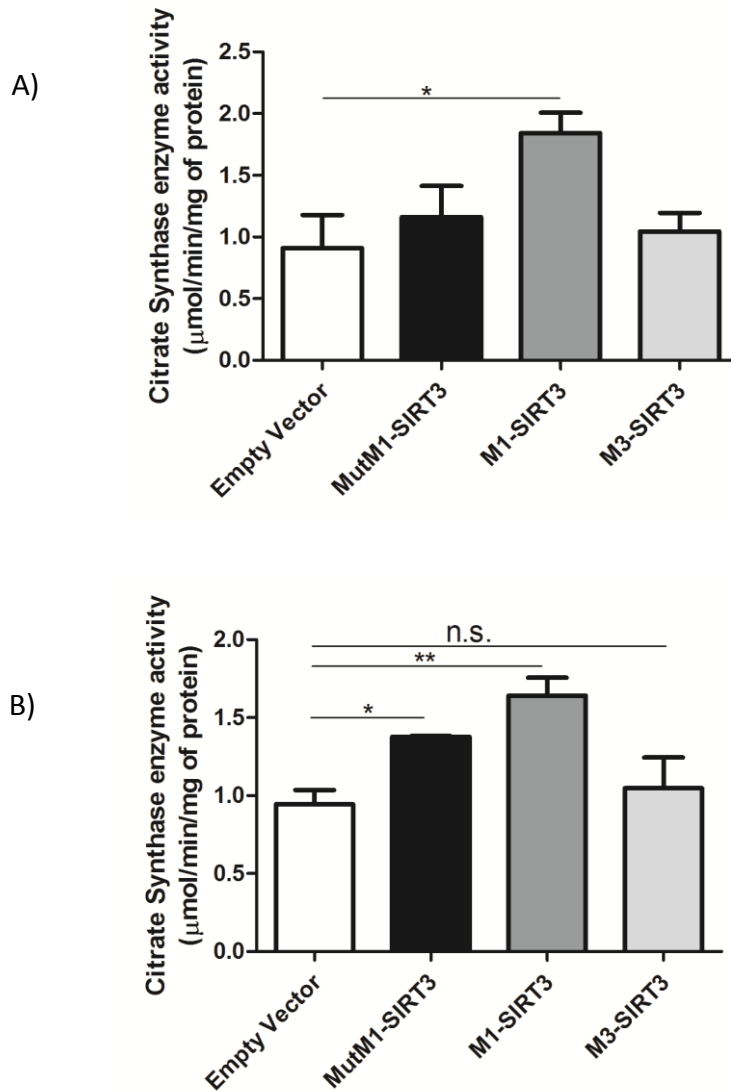
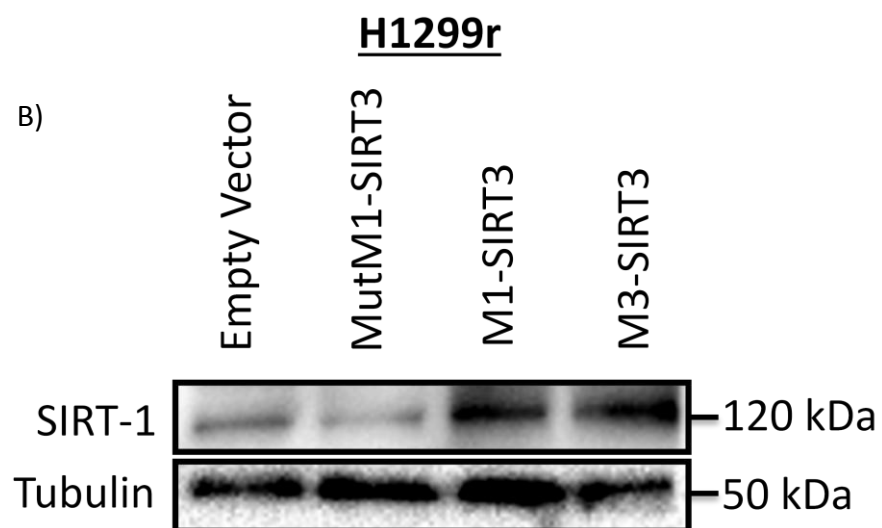
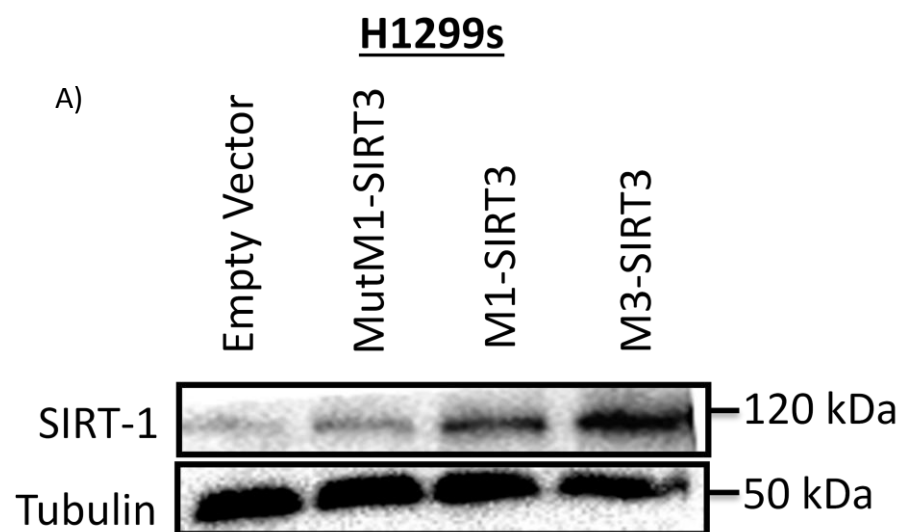


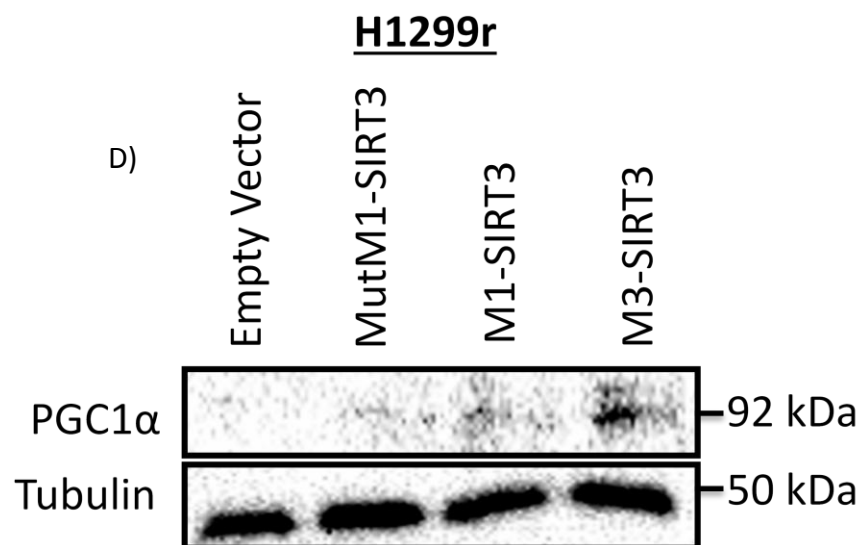
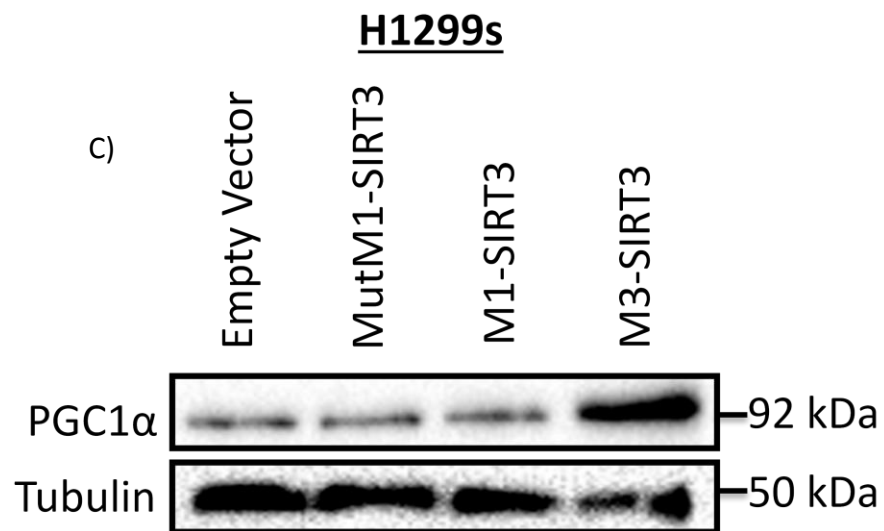
Figure 5.11 Citrate synthase activity of the H1299 cells after transduction with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus as determined by spectroscopy. (A) H1299s and (B) H1299r cells were seeded at 400,000 cells in T25's and were transduced with 50 MOI of empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus for 48 hrs. After the time had elapsed, cells were then lysed with Laemelli buffer and the protein content was assessed using the BCA assay. 40 μg of protein was added into the cuvette with 500 μl of Tris (200 mM, pH 8.0) with Triton X-100 (0.2% (v/v)), 100 μl of DTNB, 30 μl of acetyl coenzyme A (10 mM). The coupled reaction was started by adding 50 μl of 10 mM oxaloacetic acid and measuring the rate of increase of 5-thionitrobenzene in absorbance at 412nm for 3min. Data are expressed as mean plus or minus standard error of the mean for two separate experiments. Statistical analysis was carried out using the student t-test. ** = $p < 0.005$, * = $p < 0.05$.

5.2.2.5 The effect of the transduction of H1299s and H1299r cells using a SIRT3 expressing adenovirus upon the expression of mitochondrial biogenesis protein as determined by western blotting.

Having determined that the SIRT-3 constructs alters mitochondrial metabolism (Figure 5.10) and citrate synthase activity (Figure 5.11), we next wanted to determine whether the expression of SIRT3 in these cells could increase mitochondrial biogenesis. As performed previously we chose to measure mitochondrial biogenesis by assessing protein expression of a SIRT1, PGC1 α and TFAM.

The M1 and M3-SIRT3 construct both appear to increase the expression of SIRT1 in both the H1299s and the H1299r cells with no effect from the empty vector and MutM1-SIRT3 constructs (5.12 A & B). Similar to SIRT1 the control constructs did not affect PGC1 α expression. The shorter M3-SIRT3 construct that lacks the mitochondrial localisation signal increased the expression of PGC1 α whereas the M1-SIRT3 had no significant effect upon the expression of PGC1 α (Figure 5.12 C&D). This indicates that the mitochondrial localisation signal is not important for SIRT3's increase in PGC1 α expression. MutM1 and M1 both increased the expression of TFAM in the H1299s cells (Figure 5.12 E) which indicates that the enzyme activity of SIRT3 is not important for SIRT3'S increase in TFAM expression in the H1299s. The M1-SIRT3 construct appears to increase the expression of TFAM in the H1299r cells (Figure 5.12 F). Interestingly the TFAM antibody also picked up a larger sized band in the M1 and M3-SIRT3 lysates which indicates that these constructs are perhaps modifying the TFAM protein in some manner.





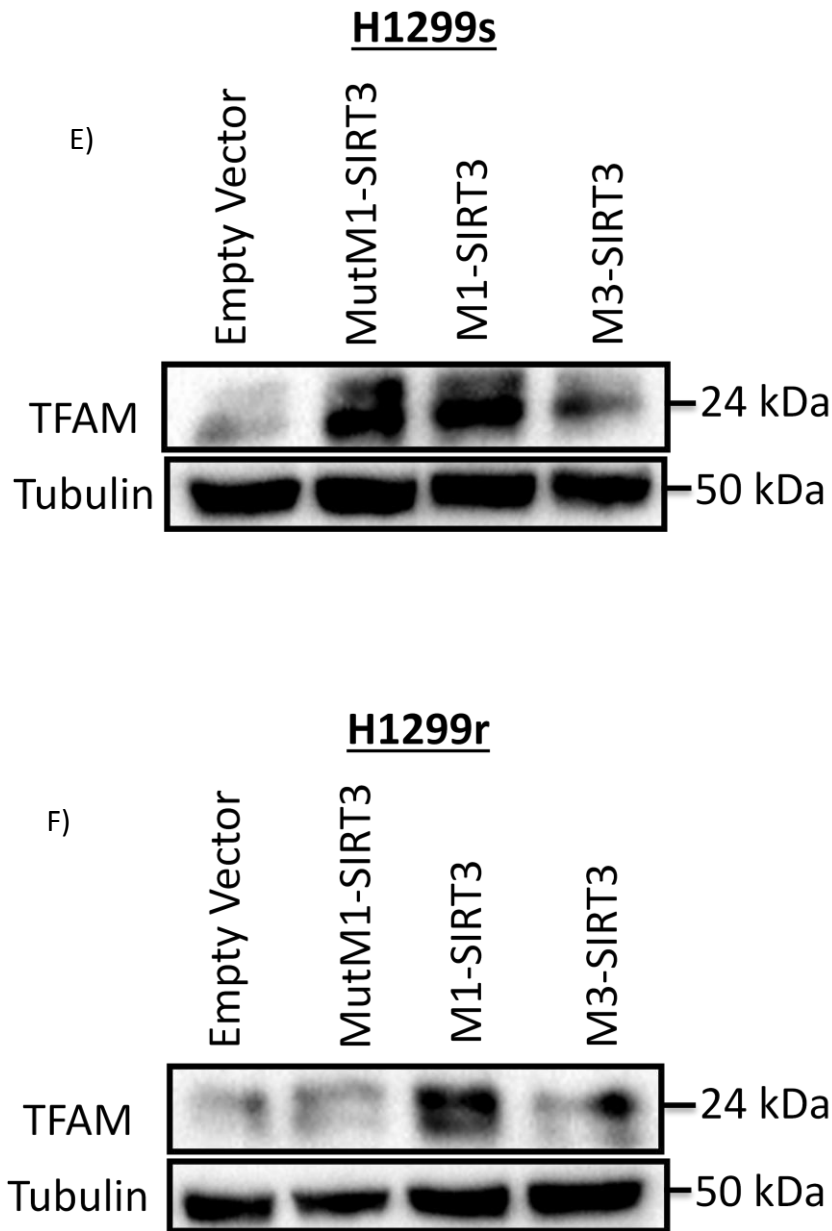


Figure 5.12 The effect of the transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus on SIRT-1, PGC1 α and TFAM expression as determined by western blotting. H1299 cells were transduced with 50 MOI of empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus for 48 hrs. Whole cell lysates were then prepared. Proteins (90 μ g) were resolved in 10% SDS-PAGE gels and transferred to a PVDF membrane. Blots were probed for SIRT1, PGC1 α , TFAM or the loading control γ -tubulin. Representative blot of SIRT1 (A), PGC1 α (C), and TFAM (E) protein expression from H1299s cells from three independent experiments. Representative blot of SIRT1 (B), PGC1 α (D), and TFAM (F) protein expression from H1299r cells from three independent experiments.

5.2.2.5 The effect of the transduction of H1299s and H1299r cells using a SIRT3 expressing adenovirus on reactive oxygen species (ROS) production as determined by flow cytometry.

Having shown that SIRT3 expression alters the bioenergetics of the H1299 cells (Figure 5.8), increases citrate synthase activity (Figure 5.11) and augments the expression of the mitochondrial biogenesis proteins (Figure 5.12), we wanted to determine what effect the SIRT3 constructs have on ROS production of these cells. In the paper by Cheung et al. (2015) the M1-SIRT3 failed to decrease ROS in the untreated cells but attenuated the increased ROS production as a result of doxorubicin treatment. MutM1-SIRT3 expression caused increased ROS production in control cells and M3-SIRT3 failed to have any effect on doxorubicin increased ROS production.

The expression of the M1-SIRT3 construct in the H1299s cells caused a significant ($p<0.05$) increase in ROS production (Figure 5.13 A). Whereas in the H1299r cells the expression of SIRT3 by the three SIRT3 expressing constructs all significantly ($p<0.05$) decreased ROS production (Figure 5.13 B) in comparison the empty vector control. H1299s cells already exhibit high expression of SIRT3 so over-expression of SIRT3 in these cells may be harming the cells whereas SIRT3 expression decreases ROS production which agrees with SIRT3's reported activation of antioxidant enzymes (Qiu et al., 2010, Someya et al., 2010)

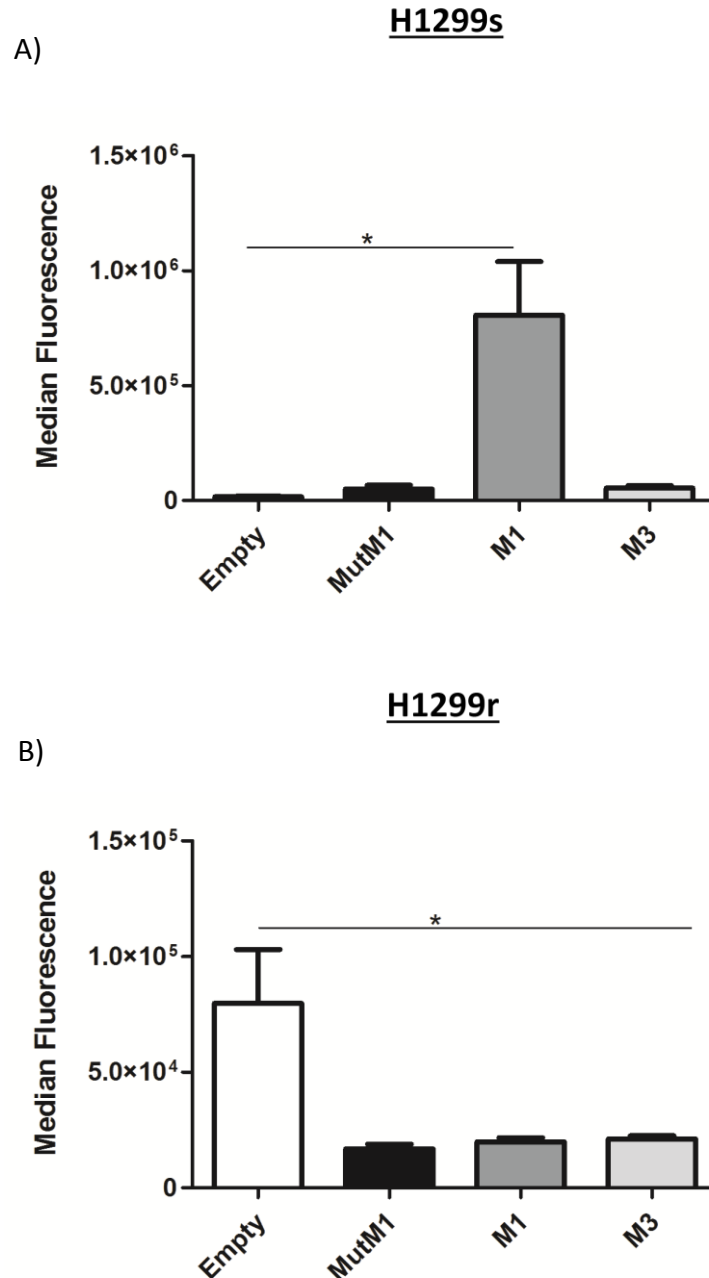


Figure 5.13 Reactive Oxygen Species (ROS) production after the transduction of the H1299s and H1299r with empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus as determined by flow cytometry. (A)H1299s and (B) H1299r cells were seeded at a density of 250,000 cells/well and left for 24h. They were then transduced with 50 MOI of empty vector, mutM1-SIRT3, M1-SIRT3 and M3-SIRT3 adenovirus for 48h. The cells were then stained with 10 μ M Dichloro-dihydro-fluorescein diacetate (DCFH-DA). Fluorescence was analysed for 10,000 events. Data are expressed as mean plus or minus standard error of the mean for the relative DCFH-DA signal over three separate experiments. Statistical analysis was carried out using the student t-test. * = $p < 0.05$.

5.3 Discussion

Resveratrol is a naturally occurring polyphenol known to be present in approximately 70 plant species, including the skin of red grapes, berries and nuts (Patel et al., 2011, Thomasset et al., 2007, Jang et al., 1997). Due to its presence in red grapes, resveratrol has long been associated with the phenomenon of the “French paradox” (Catalgol et al., 2012). This term arises from an epidemiological study showing that French people demonstrated low incidence of coronary diseases in spite of their high consumption of a diet high in saturated fat. Moderate wine consumption was put forward as a possible reason and so resveratrol was introduced as a food supplement (Renaud and de Lorgeril, 1992). As well as resveratrol’s well documented cardio protective effects mediated by its antioxidant properties (Ungvari et al., 2007, Olas and Wachowicz, 2002), resveratrol has also been shown to exert anti-tumour activities (Jang et al., 1997). Resveratrol has been shown to affect multiple mechanisms of carcinogenesis such as cell cycle regulation, apoptosis (Fresco et al., 2006), proliferation (Nichenametla et al., 2006) as well as inhibition of key proteins in transduction pathways such as mitogen activated protein kinases (Catalgol et al., 2012). The exact mechanisms responsible for the effects of resveratrol are poorly understood.

Sirtuin-3 is a class III mitochondrial lysine deacetylase which has also shown to elicit a cardio protective effects (Palacios et al., 2009) and could possibly be a downstream effector of resveratrol. SIRT3 is the primary mitochondrial deacetylase (Lombard et al., 2007) and has been shown to regulate the tricarboxylic acid cycle by deacetylating isocitrate dehydrogenase (Someya et al., 2010), glutamate dehydrogenase (Hallows et al., 2006), acetyl CoA synthetase (Lombard et al., 2007), to regulate the activity of ATP and activity of complex I of the electron transport chain (Ahn et al., 2008), all indicating that SIRT3 may play a role in metabolic reprogramming and biogenesis. In addition SIRT3, has shown to increase the activity of antioxidants such as SOD2 (Qiu et al., 2010) to reduce mitochondrial ROS. Cheung et al. (2015) showed that when cardiomyocytes were treated with resveratrol, it increased SIRT3 as well as SIRT1 expression. Resveratrol also attenuated the oxidative stress caused by doxorubicin treatment which was

dependent on SIRT3 expression. When SIRT3 was expressed in these cardiomyocytes, it increased basal metabolism and attenuated the doxorubicin induced increase in oxidative stress. The work carried out in this chapter sought to determine whether resveratrol through SIRT3 expression could increase basal metabolism, mitochondrial abundance, and mitochondrial biogenesis and reduce ROS production in our acquired resistant H1299 cells.

As has been reported before by Cheung et al. (2015), resveratrol increased the expression of SIRT3 in both the H1299s and H1299r cells. Resveratrol has been documented to increase mitochondrial biogenesis through activation of SIRT1 and deacetylation of PGC1 α (Lagouge et al., 2006) and so their expression was also assessed. As predicted by SIRT3's increased expression, resveratrol treatment caused an increase in the expression of both SIRT1 and PGC1 α . The increase in expression of SIRT1, SIRT3 and PGC1 α as a result of resveratrol treatment shows that resveratrol treatment is causing an increase in mitochondrial biogenesis in our H1299 cells.

Having observed that resveratrol increased mitochondrial biogenesis in the H1299 cells, we had expected that it would have similar beneficial effects upon the OCR of the H1299 cells. However, we observed a significant decrease in cellular and mitochondrial oxygen consumption rate of our H1299s cells with no significant effect on the H1299r cells. Jeong et al. (2012) also observed a decrease in oxygen consumption as a result of resveratrol treatment as well as a reduction in mitochondrial membrane potential. Interestingly, resveratrol significantly reduced the ECAR of both the H1299s and H1299r cells which has been shown before (Sheu et al., 2013). This indicates that the resveratrol treatment is making the H1299 cells more oxidative. Although resveratrol decreased the ECAR of the H1299 cells, it had no impact on the OCR/ECAR ratio as resveratrol still significantly decreased the OCR/ECAR ratio of the H1299s cells with no effect of resveratrol treatment on the OCR/ECAR ratio of the H1299r cells. When we analysed the reserve capacity i.e. how much the activity of the mitochondria can be increased from resting of the H1299 cells, we observed that resveratrol treatment was increasing the cellular and mitochondrial reserve capacity of both the H1299s and H1299r cells. This

increase in reserve capacity after resveratrol treatment correlates with the increase in the expression of the mitochondrial biogenesis proteins; SIRT3 and PGC1 α caused by resveratrol treatment. Resveratrol has been shown to increase mitochondrial function through an increase in SIRT1 and PGC1 α activation by Lagouge et al. (2006).

To assess whether resveratrol's increase in reserve capacity was due to an increase in mitochondrial activity or abundance, citrate synthase activity, an indicator of mitochondrial abundance was measured. Resveratrol has been shown to both increase (Zheng et al., 2012) and decrease mitochondrial abundance (Ryan et al., 2010). Whereas Cheung et al. (2015) observed no difference in mitochondrial content in their cardiomyocytes. In concordance with the increase in reserve capacity we have observed, resveratrol also increased the citrate synthase activity of the H1299s cells. This indicates that resveratrol treatment is causing an increase in mitochondrial abundance in our cells.

Resveratrol treatment has been shown to decrease reactive oxygen species through the activation of antioxidant enzymes (Zheng et al., 2012), similar to SIRT3 (Qiu et al., 2010). Cheung et al. (2015) showed that resveratrol treatment attenuated the increased ROS production induced by doxorubicin treatment and was dependent on SIRT3 expression. Here we show that resveratrol increased the ROS production of the H1299s cells but had no effect on the H1299r cells. Babu et al. (2015) also observed an increase in total ROS production as a result of resveratrol treatment by use of the intracellular total ROS dye carboxy-H₂DCFDA but also showed that resveratrol decreased mitochondrial ROS production by use of the mitochondrial ROS dye DHR123. The reduction of mitochondrial ROS and increase in total ROS caused by resveratrol treatment might indicate that resveratrol's reduction in ROS may be specific to mitochondrial generated ROS.

We next determined was whether the increases in reserve capacity, mitochondrial abundance and mitochondrial biogenesis, caused by resveratrol treatment, were due to SIRT3 expression. To this end, we used the SIRT3 expressing adenovirus constructs kindly provided by Prof. Dolinsky from the University of Manitoba. Having optimised the

conditions and confirmed their functionality, we wanted to determine whether the expression of SIRT3 could increase the bioenergetic status of the H1299r cells. Cheung et al. (2015) observed differential effects using different SIRT3 constructs on the metabolic and bioenergetic functionality of cardiomyocytes. They found that the M1-SIRT3 construct increased basal OCR and M1 & M3-SIRT3 attenuated the decrease in OCR caused by doxorubicin treatment of cardiomyocytes. Both constructs also increased the maximal OCR of their cells. The OCR of MutM1-SIRT3 transduced cells was less than that of the control and in fact made the cells more sensitive to doxorubicin's reduction in OCR. Here we show that the constructs had no effect the OCR of the H1299s but the M1-SIRT3 construct significantly increased the ECAR. Cui et al. (2015) demonstrated that expression of SIRT3 in their gastric cancer cells caused an increase in glycolysis which is indicated to have happened here in the H1299s cells by the increase in ECAR. With this significant increase in ECAR caused by M1-SIRT3 construct, M1-SIRT3 expression also caused a significant reduction in the OCR/ECAR ratio of the H1299s cells. SIRT3 expression had no effect on the OCR/ECAR ratio of the H1299r cells. The MutM1 and M1-SIRT3 constructs caused a significant increase in cellular OCR of the H1299r cells. The increase in cellular OCR by the MutM1 and M1-SIRT3 constructs shows that the mitochondrial localisation was important to SIRT3's increase in basal OCR but the full functionality of the enzyme was not important for SIRT3' increase in basal OCR. The increase in basal OCR caused by the expression of MutM1-SIRT3 was surprising to us as we had expected that expression of the M1-SIRT3 would increase OCR as expression of SIRT3 has been reported before to increase OCR (Ahn et al., 2008, Shi et al., 2005) but a reduction in SIRT3 activity with the MutM1 construct should show reduced OCR (Kendrick et al., 2011). Also the MutM1 significantly reduced the proton leak of the H1299r cells. This again is surprising as a reduction in SIRT3 activity has been associated with mitochondrial dysfunction (Kendrick et al., 2011). Analysis of the mitochondrial oxygen consumption showed that the MutM1 construct was the only construct to increase mitochondrial oxygen consumption and mitochondrial reserve capacity in the H1299r cells.

To determine whether our observed increases in mitochondrial abundance as a result of resveratrol treatment were due to SIRT3, we measured the activity of citrate synthase. Increases in SIRT3 have been associated with increases in citrate synthase (Palacios et al., 2009) and knockdown of SIRT3 causes a reduction in citrate synthase (Abdel Khalek et al., 2014). M1-SIRT3 expression caused an increase in the citrate synthase activity of the H1299s cells. MutM1 and M1-SIRT3 increased the citrate synthase activity of the H1299r cells. This increase in citrate synthase activity as a result of the expression of the M1-SIRT3 constructs indicates that expression of SIRT3 increases mitochondrial abundance when it is localised to the mitochondria.

Having demonstrated an effect of SIRT3 expression on the bioenergetics of the H1299r cells and a possible effect of SIRT3 on citrate synthase activity, it was decided to see whether SIRT3 expression could improve mitochondrial biogenesis in the H1299r cells. Palacios et al. (2009) showed an association between increased expression of SIRT3 and increased expression of PGC1 α . Kong et al. (2010) showed that when PGC1 α was knocked down, expression of SIRT3 was decreased. Kincaid and Bossy-Wetzel (2013) proposed that SIRT3 could up-regulate PGC1 α through a positive feedback loop. SIRT3 has been shown to deacetylate and activate liver kinase B1 (LKB1) (Pillai et al., 2010). Active LKB1 then phosphorylates and activates AMPK (Shackelford et al., 2013). As a consequence, stimulated AMPK phosphorylates cAMP response element binding protein (CREB) leading to increased expression of PGC1 α (Zong et al., 2002, Thomson et al., 2008). Here we show that M1 & M3-SIRT3 constructs both increase the expression of SIRT1 which indicates that the enzyme activity was important for SIRT3 to increase the expression of the cytosolic SIRT1 but not the localisation signal. M3-SIRT3 caused an increase in the expression of PGC1 α which agrees with the feedback loop proposed by Kincaid and Bossy-Wetzel (2013). MutM1 and M1-SIRT3 increases the expression of TFAM in the H1299s and M1-SIRT3 alone increases TFAM expression in the H1299r cells. The TFAM protein seems to be covalently modified in the H1299r cells as a larger band appears with the M1 and M3-SIRT3 construct. Morozov et al. (2014) showed that TFAM covalently attached to the N-terminal 3.5 kDa of mitochondrial RNA polymerase

(mtRNAP) but how exactly the protein is modified in these H1299r cells needs further investigation.

We next sought to determine the effect of SIRT3 expression on ROS production in the H1299 cells. SIRT3 has been reported to reduce oxidative stress in cells through activation antioxidant enzymes such as SOD2 (Qiu et al., 2010, Cheung et al., 2015) and IDH2 (Someya et al., 2010). Cheung et al. (2015) showed that the M1-SIRT3 construct attenuated the increase in ROS production induced by the treatment of their cardiomyocytes with doxorubicin, whereas the M3 and MutM1-SIRT3 constructs had no effect on ROS production. Surprisingly M1-SIRT3 increased the ROS production in the H1299s cells. We showed previously that M1-SIRT3 caused an increased in ECAR so perhaps the increase in ROS is somehow related. In the H1299r cells, the expression of SIRT3 by MutM1, M1 and M3 all decreased ROS which agrees with the role of SIRT3 in antioxidant defence (Qiu et al., 2010, Someya et al., 2010) and what Cheung et al. (2015) showed. This demonstrates that SIRT3 activation alone may be sufficient to reduce oxidative stress.

In this chapter we showed that resveratrol increased the expression of the mitochondrial biogenesis proteins SIRT1, PGC1 α and SIRT3 expression. Resveratrol significantly increased the reserve capacity of the mitochondria in the H1299 cells and also increased the mitochondrial abundance. Resveratrol also increased the ROS production in the H1299s cells with no effect on the ROS production of the H1299r cells. MutM1-SIRT3 expression increases basal mitochondrial OCR in H1299r cells and decreases ATP turnover and proton leak. There was no change in citrate synthase activity with the expression of SIRT3 in the H1299 cells. The M1 & M3-SIRT3 constructs increased the expression of SIRT-1 in H1299s and H1299r cells. M3-SIRT3 increased expression of PGC1 α in H1299s and H1299r cells. MutM1 & M1-SIRT3 in H1299s cells and M1-SIRT3 in H1299r cells increased the expression of TFAM with some degree of modification of the TFAM protein with the M1 and M3-SIRT3 constructs. In conclusion both resveratrol and SIRT3 over-expression increase mitochondrial function in the

H1299r cells. In the next chapter we investigate the stem like properties of the non small cell lung cancer H1299 and mesothelioma P31 cells.

Chapter 6: Investigation into the stem cell features of the acquired cisplatin resistance in H1299 and P31 cells

6.0 Introduction

In this chapter we investigate the stem like properties of the H1299 and P31 cells. In the study of resistance in cancer, sensitive cells are often artificially engineered to acquire resistance through different methods. A varied range of mechanisms arise in the development of resistance to conventional chemotherapeutics such as increased rates of drug efflux, alterations in drug metabolism and mutations of drug targets (Longley and Johnston, 2005, Gottesman et al., 2002). For example, in the non small cell lung cancer cell lines PC-9 and H3255 are known to develop resistance to EGFR tyrosine kinase inhibitors, gefitinib and elotinib, through a secondary EGFR T790M mutation (Pao et al., 2005, Kwak et al., 2005) as a result of stepwise escalation exposure (Ogino et al., 2007). The concept of acquired resistance implies that many cancers possess heterogeneity, in that some cells survive treatment exposure and go on to contribute to disease progression while others perish. This heterogeneity is now a well known characteristic of cancers (Hanahan and Weinberg, 2011).

More recently, there has been renewed interest in the contribution of cancer stem cells (CSCs) to cancer heterogeneity and acquired resistance. Treatment failure has been attributed to cancer stem cells which are themselves intrinsically highly resistant to many therapeutic approaches by much of the same mechanisms (Valent et al., 2012). Evidence that CSCs survive chemotherapy exposure has been well documented. Yu et al. (2007) showed that clinical specimens that were treated with chemotherapy exhibited higher proportions of mammosphere-forming and CD44⁺ (CSC surface marker) than chemotherapy naive specimens. Similarly xenografts treated with cisplatin resulted in the enrichment of CSCs in non small cell lung cancer (Bertolini et al., 2009) and hepatocellular (Lee et al., 2011) carcinoma. The reported proportions of CSC vary between tumours from 0.1% to 30% depending on the type and advancement of the cancer (Adams and Strasser, 2008). In the selection of H1299 and P31 cells with acquired resistance to cisplatin, the Benham-Motlagh laboratory may have been selecting for a proportion of cells that possess greater amounts of CSC.

In order to assess whether these resistant cells possessed stem like features as a result of the acquisition of resistance to cisplatin, various stem cell markers were assessed.

6.1. Results

6.1.1 Cell surface expression of CD133/CD44

CD44 and CD133 are cell surface markers used in the identification of CSC. CD44 binds to hyaluronic acid, a key glycosaminoglycan of the extracellular matrix and aids in the attachment of CSCs to the matrix, participating in the proliferation and migration of malignant cancer cells. The over-expression of CD44 was strongly correlated with therapeutic resistance (Hong et al., 2009). CD133 is another marker that has been found in abundance in the embryonic epithelium and is also expressed in CSC of many cancers (Vinogradov and Wei, 2012). Therefore CD44 and CD133 can be used to identify CSC.

H1299 and P31 cells were seeded at 250,000 cells/well and were left for 24 h. The following day expression of CD133/CD44 was assessed by an antibody conjugated to the fluorophore FITC. Isotope control antibodies for the individual receptors conjugated to the same fluorophore served as the negative control for these experiments. Cells were incubated with the antibody for 30 min and were then analysed on the Accuri C6 flow cytometer.

There was minimal expression of CD133, with no difference between the sensitive and resistant H1299 or P31 cells (Figure 6.1). There was high expression of CD44 in all cell lines but similarly no discernible difference between the sensitive and resistant cells. This data suggests that neither marker could identify sub populations within each cell line. A positive control for CD133 expression would be needed for the CD133 data and a negative control for CD44 expression would be needed for the CD44 data to verify the validity of these results.

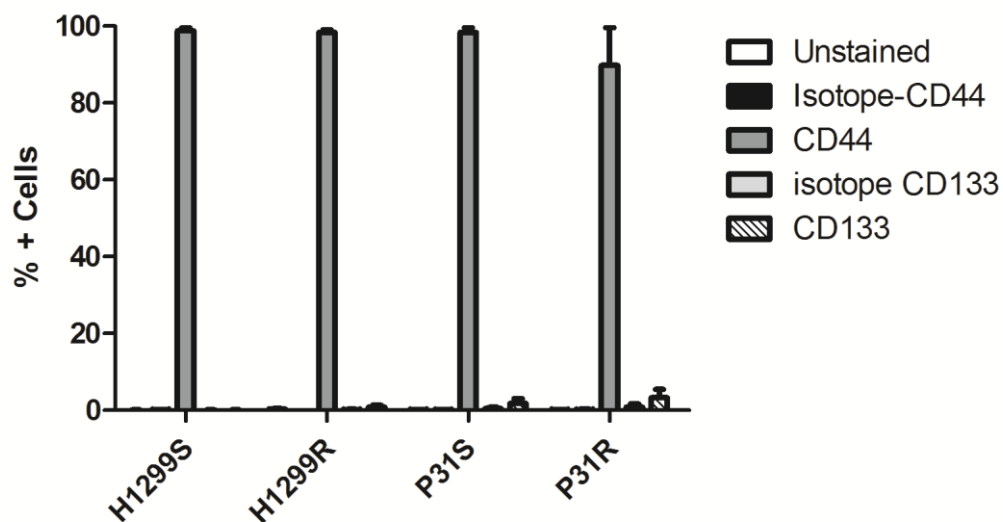


Figure 6.1 The expression of CD44 and CD133 in H1299s, H1299r, P31s and P31r cells as determined by flow cytometry. Cells were seeded at a density of 250,000 cells/well and left for 24h. The cells were pelleted and then resuspended in FACS buffer containing Fc block for 10 minutes. 2.5 μ l of FITC-CD44, FITC-isotope control, FITC-CD133 and FITC-isotope control was then added for 20 min. Cells were washed in FACS buffer and then analysed. Fluorescence was analysed for 10,000 events. Data are expressed as mean plus or minus standard error of the mean for the expression of CD44 or CD133 over three separate experiments.

6.1.2 Aldehyde dehydrogenase (ALDH) activity

ALDHs form a group of NAD(P)⁺ dependent enzymes involved in the oxidation of aldehydes and production of retinoic acid (Vasiliou et al., 2000) that is considered to be involved in cellular differentiation and stem cell self protection (Chute et al., 2006). Stem cells have been shown to have higher ALDH activity than their differentiated progeny (Huang et al., 2009).

The assay is based on BODIPY-aminoacetaldehyde (BAAA), a fluorescent non-toxic substrate for ALDH, which freely diffuses into intact and viable cells. In the presence of ALDH, BAAA is converted into BODIPY-aminoacetate (BAA), which is kept inside the cells. The amount of fluorescent reaction product is proportional to the ALDH activity in the cells and is measured using a flow cytometer. A specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), is used to control for background fluorescence.

There was a significant ($P < 0.05$) increase in the percentage of cells retaining BAA i.e. greater ALDH activity in the H1299r cells in comparison to the H1299s cells (Figure 6.2). There was no difference in ALDH activity between the P31s and P31r cells. The staining of the H1299 cells demonstrates that it would be possible to make CSC sub populations from these cells.

To determine whether there is an association between the stem like properties of these cells and the increase in Gb₃ expression, cells were treated with DEAB and then Gb₃ expression was analysed on the P31r cells. Cells were incubated at three different temperatures as for Gb₃ expression the cells are incubated at 4°C whereas for the ALDH assay the cells are incubated at 37°C. Initially we were surprised that DEAB was significantly inhibiting Gb₃ expression at room temperature (approx 25°C) and at 37°C (Figure 6.3 A). However, when Gb₃ expression was measured alone at the three temperatures, the same differences were observed (Figure 6.3 B). The observed decrease in Gb₃ expression was just a result of the temperature of incubation not the inhibitor of ALDH activity, DEAB.

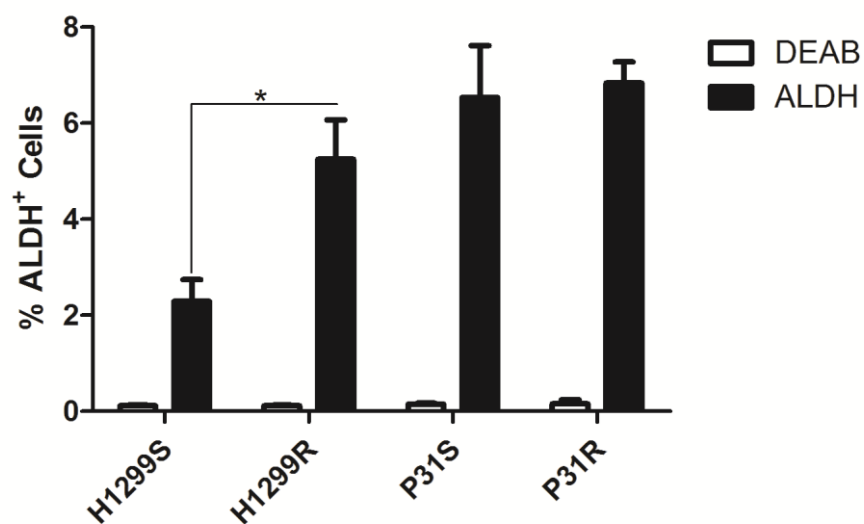


Figure 6.2 The activity of ALDH in H1299s, H1299r, P31s and P31r cells as determined by flow cytometry. Cells were seeded at a density of 250,000 cells/well and left for 24h. The cells were pelleted and the assay was carried out as per manufacturer's instruction. Fluorescence was analysed for 10,000 events. DEAB, a specific inhibitor of ALDH, served as a negative control for this experiment. Data are expressed as mean plus or minus standard error of the mean for the ALDH activity over three separate experiments. Statistical analysis was carried out using the student t-test. * = $p < 0.05$

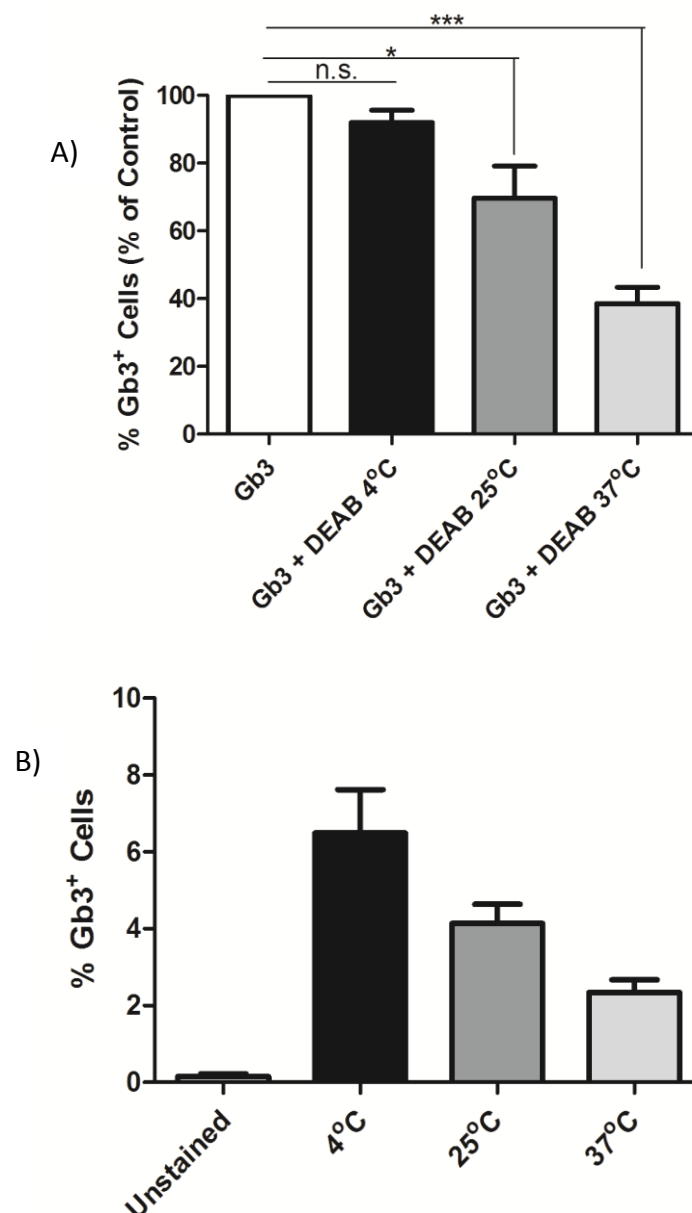


Figure 6.3 The effect of DEAB inhibition on the expression of Gb3 in H1299s, H1299r, P31s and P31r cells as determined by flow cytometry. Cells were seeded at a density of 250,000 cells/well and left for 24h. The cells were pelleted and then resuspended in FACS buffer containing Fc block for 10 minutes. A) 5 μ l of DEAB and 2.5 μ l of Alexa647-Gb3 (CD77) was then added for 20 min at different temperatures. B) 2.5 μ l of Alexa647-Gb3 (CD77) was then added for 20 min at different temperatures. Cells were washed in FACS buffer and then analysed. Fluorescence was analysed for 10,000 events. Data are expressed as mean plus or minus standard error of the mean for the expression of Gb3 over three separate experiments. Statistical analysis was carried out using the student t-test. *** = $p < 0.0005$, * = $p < 0.05$.

6.1.3 Hoescht Side Population (HSP) assay

The HSP assay is based on the high expression of ABC transporters in CSCs. The ability of ABC transporters to efflux the fluorescent Hoescht dye, has been shown to define cells with stem cell properties in NSCLC cell lines (Ho et al., 2007).

Cells were seeded at 250,000 cells/well and were left for 24 h. The following day the cells were treated with 5 µg/ml of Hoescht 33285 and incubated for 30 minutes. Verapamil, the inhibitor of ABCB1/MDR1 served as the negative control for this assay. After the elapsed time, the cells were pelleted, resuspended in HBSS solution and were then analysed on the Accuri C6 flow cytometer.

There did seem to be a significant increase in the HSP in the H1299r cells in comparison to the H1299s cells with no difference in HSP in the P31 cells (Figure 6.4). The assay did not yield the characteristic tail of the HSP and the fact that our negative control did not decrease the HSP calls into question the validity of the result from this assay.

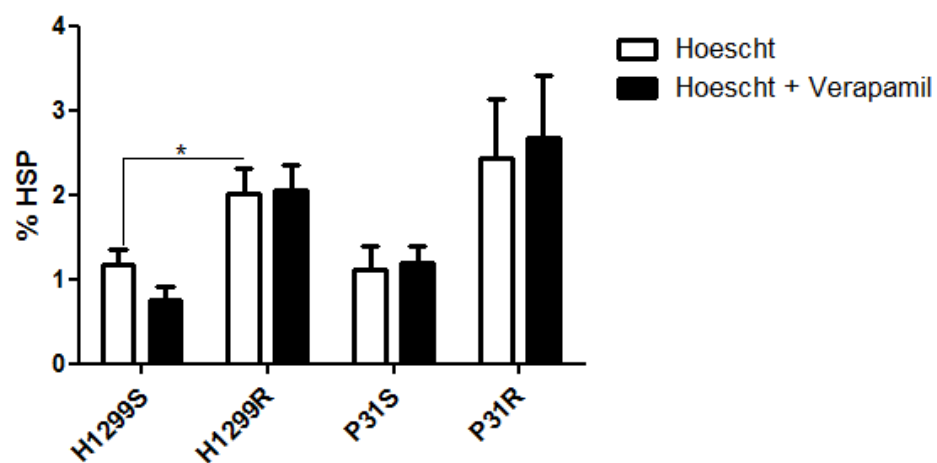


Figure 6.4 The Hoescht side population (HSP) of the H1299s, H1299r, P31s and P31r cells as determined by flow cytometry. Cells were seeded at a density of 250,000 cells/well and left for 24h. The cells were treated with 5 μ g/ml of Hoescht 33285 and incubated for 30 minutes. Control cells were treated with 50 μ M verapamil and 5 μ g/ml of Hoescht 33285 and incubated for 30 minutes. Fluorescence was analysed for 10,000 events. Data are expressed as mean plus or minus standard error of the mean for the HSP over three separate experiments. Statistical analysis was carried out using the student t-test. * = $p < 0.05$.

6.1.5 Autofluorescence

Autofluorescence is something that one has to usually gate out when conducting flow cytometry but Miranda-Lorenzo et al. (2014) showed that autofluorescence could be a marker of epithelial cancer stem cells. They claimed that autofluorescence was as a result of the fluorescent vitamin riboflavin in ABCG2 coated-vesicles exclusively present within the cytoplasm of CSC.

To examine this, analysis was conducted on untreated samples that had previously been run on the flow cytometer and gated for cells at the intersection of the FL-1 and FL-2 channel as conducted in the paper (Miranda-Lorenzo et al., 2014).

There was a significant ($P < 0.05$) increase in autofluorescence in the H1299r cells in comparison to the H1299s cells with no difference in autofluorescence in the P31 cells (Figure 6.5) as with the other stem cell markers. Although I observed the same profile as was shown in the paper, my values are dramatically larger than that shown by Miranda-Lorenzo et al. (2014). This is also a relatively novel concept so further work would need to be conducted to verify the claim proposed by Miranda-Lorenzo et al. (2014) and so one cannot conclude any significant conclusions from this data as yet.

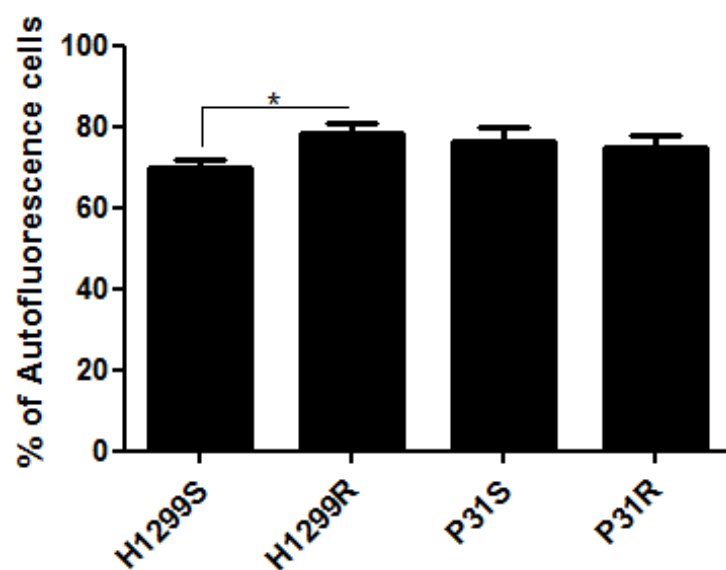


Figure 6.5 Autofluorescence of the H1299s, H1299r, P31s and P31r cells as determined by flow cytometry. Cells excited with a 488-nm blue laser and best selected as the intersection with filters 530/40 and 580/30. Fluorescence was analysed for 10,000 events. Data are expressed as mean plus or minus standard error of the mean for the autofluorescence over three separate experiments. Statistical analysis was carried out using the student t-test. * = $p < 0.05$.

6.2 Discussion

In this chapter it was proposed to examine the stem like properties of the acquired cisplatin resistant H1299 and P31 cells. Cisplatin often yields modest and often temporary beneficial anti-neoplastic effects due to the acquisition of resistance leading to drug relapse and treatment failure (Johansson et al., 2009, Locasale, 2012, Michels et al., 2013). One new therapeutic avenue that is being pursued in preclinical testing for a number of solid tumours is the targeting the CSCs. The CSC hypothesis indicates that CSCs are the population of cells that are responsible for tumour initiation, cell survival after therapy, metastatic spread and tumour recurrence (Coghlin and Murray, 2010). CSC's are intrinsically resistant to many current cancer treatments, including chemotherapy and radiotherapy (Bao et al., 2006, Dean et al., 2005). This suggests that with current cancer therapies, that while they manage to kill the bulk of the tumour they may ultimately fail as they do not kill the CSCs, which manage to survive and regenerate the tumour. Identification of these CSC in certain cancer cells has been hampered by a lack of reliable stem cell markers. The aim of this chapter was to identify CSC populations with the aid of the reported CSC markers CD44/CD133, ALDH, HSP and autofluorescence in our cisplatin resistant H1299 and P31 cells.

CSC specific surface markers such as CD44/CD133 are currently in being utilized for targeting purposes in anti-cancer therapies. Some have made use of hyaluronic acid (HA) as a CD44 specific tumour-targeting drug carrier. Banzato et al. (2008) prepared and evaluated the effect of a paclitaxel hyaluran biconjugate on ovarian cancer IGROV-1 and OVCAR3 xenografts following intraperitoneal administration. The drug entered the cells by CD44 mediated endocytosis and exerted a concentration dependent inhibition of tumour growth and more efficacious than paclitaxel alone. Similarly, CD133 can be targeted by vectorised nanocarriers. Curcumin, an Indian spice which has been shown to have anti-cancer properties, when loaded into a nanoparticle was shown to significantly suppress the anchorage-independent clonogenic growth and reduced the CD133⁺ CSC population in medulloblastoma and glioblastoma (Lim et al., 2011). In the present study, we had sought to determine differences in expression of CD44 and CD133, however

between our cisplatin sensitive and resistant H1299 and P31 cells, however, there was no differences observed. Meng et al. (2009) showed that 45% of NSCLC cell lines demonstrate tumourgenic potential regardless of their side population phenotype or CD133 expression. Results presented herein do not disprove that the acquired resistant cells are not more stem like. Results indicate that the stem cell markers chosen are not the specific markers of stemness for these cell types.

We next measured aldehyde dehydrogenase (ALDH) activity of the H1299 and P31 cells. ALDH activity has been shown to be elevated in many cancers such as brain, breast, liver and more recently lung cancer (Jiang et al., 2009) and when ALDH⁺ cells were isolated from these cancers, they were shown to be enriched for tumour initiating cells (Su et al., 2010). In this study, there was a significant difference in the ALDH activity between the H1299r and H1299s cells with no difference in the P31 cells. The increased expression of ALDH in the H1299r cells in comparison to the H1299s cells shows that ALDH activity in the H1299 cells could be a marker for stemness in that cell line but not in the P31 cells. Also our results demonstrated that ALDH activity is not related to the increase in Gb₃ expression in the H1299 or P31 cells.

As with drug resistant cancers, CSC's are characterized by the high expression of ABC transporters which have been shown to efflux the fluorescent dye Hoescht, a marker used to define cancer stem cells (Ho et al., 2007). While we did observe a significant increase in the efflux of Hoescht in the H1299r cells in comparison to the H1299s cells and no difference in the P31 cells, our negative control did not elicit the expected result. We later found out that the H1299 and P31 cells do not express ABCB1/MDR1 (Figure 4.8) which explains why verapamil proved ineffective as a negative control. Many studies have attributed the property of CSCs to efflux Hoescht as a side population to the ABC transporter, ABCG2 (Zhou et al., 2001) and not ABCB1/MDR1 as we had previously thought and so an inhibitor ABCG2 would have been a more appropriate control for this experiment. Inhibition of the ABCG2 transporter would need to be carried out to conclude whether our resistant H1299 cells demonstrate an increased HSP

when compared to the H1299s cells and whether it could be a marker for stemness in our H1299 cells.

Autofluorescence, a property of all cells has now been proposed as a biomarker of epithelial CSC and capable of identifying CSC across numerous solid tumours (Miranda-Lorenzo et al., 2014). Although we did observe a significant increase in autofluorescence in the H1299r cells in comparison to the H1299s cells with no difference in the P31 cells, our numbers are drastically larger than that observed by Miranda-Lorenzo et al. (2014). As yet, autofluorescence is not conclusively proven as a stem cell marker, thus no firm conclusions can be made from this result.

In conclusion we have shown that there is no difference in the expression of the CSC surface markers, CD44 and CD133 between our cisplatin sensitive and resistant H1299 and P31 cells. There was a significant increase in ALDH activity in the H1299r cells in comparison to the H1299s cells with no difference in activity in the P31 cells. The HSP assay similarly showed significance in the H1299 cells and not the P31 cells but a appropriate negative control needs to be included. The autofluorescence also demonstrated the same significant difference between sensitive and resistant cells in the H1299 but not in the P31 cells. However no firm conclusions can be drawn from this. ALDH and HSP could be potential markers of stemness in the H1299 cells but further studies would be required to substantiate this claim. In these set of experiments we have not identified any potential markers of stemness in the P31 cells.

Chapter 7: General Discussion

7.0 General Discussion

In this work it was proposed to investigate cancer cell bioenergetics in response to anti-cancer agents. This project is composed of two parts, (i) investigating the anti-cancer properties of phenformin and metformin and their mechanism of action on the bioenergetics of the neuroblastoma cells and (ii) comparative bioenergetics in parental sensitive and cisplatin resistant cancer cell sublines.

Neuroblastoma is a devastating paediatric malignancy which arises from the neural crest cells the sympathetic nervous system. Patients are initially responsive to agents such as etoposide, carboplatin, vincristine and doxorubicin but became refractory (Seeger and Reynolds, 1991, Keshelava et al., 1998) due to development of multi-drug resistance (Lennon et al., 2014). Irrespective of the advances made in the understanding of the disease it remains to be one of the most difficult cancers to treat with a 5 year survival of just 42% (Cheung and Dyer, 2013). For this reason there is urgent need for alternative approaches to treatment. Recently phenformin and metformin, former type II diabetic drugs have shown significant anti-neoplastic activity (Dowling et al., 2011, Birsoy et al., 2014, Huang and Manning, 2008). It was proposed to determine whether phenformin and metformin elicited anti-cancer properties in the neuroblastoma, SH-SY5Y cells and exactly how they were affecting the bioenergetics of the cells with a view to discovering their mechanism of action. We showed that phenformin was capable of killing the neuroblastoma cells and at concentrations shown by others (Guo et al., 2014, Jackson et al., 2013, Yuan et al., 2013). Surprisingly metformin was not capable of affecting the viability of the neuroblastoma cells below 50% and so we did not proceed with using metformin for subsequent experiments. We showed that phenformin was causing G1 cell cycle arrest prior to undergoing apoptosis which is in agreement with others (Caraci et al., 2003, Shackelford et al., 2013, Appleyard et al., 2012). It was then decided to determine the effects of sub-cytotoxic concentrations of phenformin on the cancer bioenergetics of the neuroblastoma cells. We showed that phenformin significantly inhibited mitochondrial oxygen consumption. We went on to show that phenformin induced a dose- and time-dependent inhibition of mitochondrial oxygen consumption.

This is in agreement with Owen (2000) who showed a dose-dependent inhibition of mitochondrial oxygen consumption by phenformin in hepatoma cells and El-Mir et al. (2000) who demonstrated a time dependent inhibition by phenformin on oxygen consumption in hepatocytes. We showed that phenformin's potent inhibition of mitochondrial oxygen consumption was due to inhibition of complex I of the mitochondria by the use of digitonin-permeabilised cells and expression a 'humanised' NDI1. Our findings are in accordance with recent studies that have suggested that phenformin exerts its anti-tumour effects by inhibiting complex 1 of the mitochondria in cancer cell lines (Birsoy et al., 2014, Wheaton et al., 2014, Owen, 2000, Miskimins et al., 2014, Dykens et al., 2008). We showed that although phenformin inhibited complex 1 of the mitochondria, phenformin had no effect on the cellular ROS production levels of the neuroblastoma cells. This is in contrast to the stimulation of ROS production shown by others as a result of phenformin treatment in non small cell lung cancer A549 cells and colon cancer CT26 cells (Shackelford et al., 2013, Miskimins et al., 2014). Our data may be explained by the theory proposed by Wheaton et al. (2014) that it is probable that phenformin is acting upstream of this flavin site (site of complex I ROS production), inhibiting both complex 1 and ROS production. We next sought to determine whether phenformin activates AMPK. Evidence suggests that phenformin does not act directly on AMPK but acts indirectly by increasing intracellular AMP and/or ADP concentrations (Hardie, 2013). We confirmed phenformin's activation of AMPK and ACC as part of its mechanism of action upon the neuroblastoma cells as others have shown (Huang, 2008).

In conclusion we have shown that phenformin was capable of killing the neuroblastoma cells mediated by phenformin's potent inhibition of complex 1 of the mitochondria. This work shows that phenformin has promise as anti-cancer agent against the paediatric malignancy, neuroblastoma.

Cisplatin is one of the most potent chemotherapeutic drugs used to treat a variety of cancers. Cisplatin often yields modest and temporary clinical effects but its usefulness is limited by the acquisition of resistance, leading to drug relapse and therapeutic failure (Johansson et al., 2009, Michels et al., 2013, Locasale, 2012). Its cytotoxicity has been

generally attributed to its interaction with nuclear DNA to form intrastrand crosslinks (Siddik, 2003, Behnam-Motlagh et al., 2010a, Johansson et al., 2006) but some studies have indicated that mitochondrial DNA adducts may be more common than nuclear DNA adducts in model cell line systems as a result cisplatin treatment (Murata et al., 1990, Olivero et al., 1995). Resistance can arise through a multitude of mechanisms including decreased drug accumulation, alterations in drug metabolism and mutations of drug targets (Longley and Johnston, 2005, Gottesman et al., 2002). One approach to possibly uncover the root cause of cisplatin resistance is to investigate the metabolic differences of the sensitive and cisplatin resistance cancer cells. The idea would be to uncover some metabolic difference and exploit it to develop novel therapeutics to combat cisplatin resistance. With this in mind we investigated the metabolic differences between sensitive and acquired cisplatin resistant non small cell lung cancer H1299 and mesothelioma cancer cells P31. The reduced sensitivity of the resistant cells to cisplatin in comparison their sensitive cell counterparts was confirmed. Enhanced expression of Gb₃ is commonly observed in cisplatin resistant cells as was observed here. Gb₃ is known to function as a cell surface receptor for verotoxin-1 (VT-1 or Shiga Like Toxin-1) synthesized by pathogenic strains of *Escherichia Coli* (Arab et al., 1997). The combination treatment of verotoxin-1 and cisplatin has been shown to have an additive cytotoxic effect upon the treatment of cisplatin resistant cancers (Behnam-Motlagh et al., 2010b, Johansson et al., 2009), however that was not observed in the present study. In an effort to identify whether ABC transporters were involved in cisplatin resistance in the H1299 and P31 cells, ABCB1/MDR1 expression was measured and three ABC transporters were shown to be up-regulated by an affymetrix mRNA gene array that was carried out by our collaborators; ABCA5, ABCB6 and ABCE1 and so their expression was assessed by western blotting. ABCB1/MDR1 was shown to be expressed in these cells by Johansson et al. (2009), however no MDR1 expression was observed in our work. ABCB6 for P31 cells and ABCE1 for H1299 cells were identified as the potential transporter/biomarker of cisplatin resistance in these cells.

Analysis of the metabolism of these acquired cisplatin resistant cells and sensitive H1299 and P31 cells was then carried out. The resistant cells had a significantly decreased

whole cellular and mitochondrial basal OCR in comparison to the sensitive cells. The reduction in oxygen consumption by the resistant cells in comparison to the sensitive cells has also been observed by others (Oliva et al., 2011, Liang et al., 2008). The resistant cells demonstrated reduced citrate synthase activity and reduced expression of VDAC and cytochrome c oxidase in comparison to the sensitive cells. This indicates that the resistant cells exhibit reduced mitochondrial abundance in comparison to their sensitive cell counterparts. These results are in agreement with Qian et al. (2005) who showed that mitochondrial density determined the sensitivity to cisplatin in an animal model and panel of 8 cell lines. The greater the density of mitochondria the more sensitive the cells were to cisplatin and cells that were depleted of mitochondria were resistant to cisplatin. The decrease in mitochondrial number may be a mechanism by which these cancer cells have acquired resistance to cisplatin. The resistant cells displayed reduced expression of the mitochondrial biogenesis proteins; SIRT1, PGC1 α , SIRT3 and TFAM. This reduction in mitochondrial biogenesis in the resistant cells explains the decreased mitochondrial abundance of the resistant cells in comparison to the sensitive cells. This is in agreement with others who have shown reduced PGC1 α expression (Watkins et al., 2004, Feilchenfeldt et al., 2004, Ba et al., 2008) and mitochondrial biogenesis and claim that the reduction in mitochondrial biogenesis may contribute to tumourigenesis and/or tumour progression in human malignancies. The H1299r cells displayed increased ROS production and HIF1 α stabilisation in comparison to the H1299s cells with no difference in ROS production or HIF1 α stabilisation between the sensitive and resistant P31 cells. This increased ROS production and stabilization of HIF1 α in the H1299r cells in comparison to the H1299s cells is in agreement with others who have shown similar results (Ungvari et al., 2007). This increased ROS production in the H1299r cells may be cell type specific and could be the exception to the rule as most resistant cancers exhibit increased expression of antioxidant proteins (Liou and Storz, 2010)

In conclusion we demonstrate that ABCB6 for the P31 cells and ABCE1 could be potential transporters/biomarkers for cisplatin resistance in these cells. We show that the resistant cells display dramatically reduced oxygen consumption rates in comparison to

the sensitive cells. The reduction in oxygen consumption in the resistant cells in comparison to the sensitive cells is due to decreased mitochondrial abundance and decreased mitochondrial biogenesis. The H1299r cells have increased ROS production and HIF1 α stabilization in comparison the H1299s. This work highlights the metabolic differences between the sensitive and cisplatin resistant, differences that could be exploited to potentially overcome cisplatin resistance.

Resveratrol is a naturally occurring polyphenol known to be present in approximately 70 plant species, including the skin of red grapes, berries and nuts (Patel et al., 2011, Thomasset et al., 2007, Jang et al., 1997). Resveratrol has been shown to improve mitochondrial function and protect against metabolic disease by activating the mitochondrial biogenesis proteins PGC1 α and SIRT1 (Lagouge et al., 2006). The exact mechanisms responsible for the effects of resveratrol are poorly understood. Sirtuin-3 is a class III mitochondrial lysine deacetylase which could possibly be a downstream effector of resveratrol. SIRT3 is the primary mitochondrial deacetylase (Lombard et al., 2007) and has shown to regulate the tricarboxylic acid cycle, ATP and activity of complex 1 of the electron transport chain (Ahn et al., 2008), all indicating that SIRT3 may play a role in metabolic reprogramming and biogenesis. We sought to determine whether resveratrol through SIRT3 expression could increase basal metabolism, mitochondrial abundance, and mitochondrial biogenesis and reduce ROS production in our acquired resistant H1299 cells. Resveratrol treatment caused an increase in the expression of the mitochondrial biogenesis proteins; SIRT1, SIRT3 and PGC1 α which has been shown previously (Lagouge et al., 2006). Investigation into the effect of resveratrol on the bioenergetics of the cells was then carried out. Surprisingly resveratrol caused a decrease in cellular and mitochondrial oxygen consumption of the H1299s but not the H1299r cells however it also decreased the ECAR of both the H1299s and H1299r cells. This is in agreement with others who have shown resveratrol to decrease OCR (Jeong et al., 2012) and ECAR (Sheu et al., 2013). Resveratrol also caused an increase in cellular and mitochondrial reserve capacity of both the H1299s and H1299r cells. Resveratrol has been shown to increase mitochondrial function through an increase in SIRT1 and PGC1 α activation by Lagouge et al. (2006). In agreement with the increased expression of the

mitochondrial biogenesis proteins; SIRT1, SIRT3 and PGC1 α , resveratrol treatment caused an increase in mitochondrial abundance in both the H1299s and H1299r cells. Resveratrol has been shown to increase mitochondrial abundance in skeletal muscle (Zheng et al., 2012). Resveratrol increased the ROS production of the H1299s cells but had no effect on the H1299r cells. Babu et al. (2015) also observed an increase in total ROS production as a result of resveratrol treatment by use of the intracellular total ROS dye carboxy-H2DCFDA but also showed that resveratrol decreased mitochondrial ROS production by use of the mitochondrial ROS dye DHR123. The reduction of mitochondrial ROS and increase in total ROS caused by resveratrol treatment might indicate that resveratrol's reduction in ROS may be specific to mitochondrial generated ROS.

We next determined whether the increases in reserve capacity, mitochondrial abundance and mitochondrial biogenesis, caused by resveratrol treatment, were due to SIRT3 expression. Investigation into the effects of SIRT3 expression on the bioenergetics of the cells was carried out. SIRT3 expression was shown to have no effect on the mitochondrial oxygen consumption or the mitochondrial reserve capacity of the H1299s but the M1-SIRT3 construct did increase the ECAR which has been shown before (Cui et al., 2015). SIRT3 expression however improved mitochondrial function in the H1299r cells. MutM1 increased mitochondrial oxygen consumption, decreased proton leak and increased mitochondrial reserve capacity. Expression of SIRT3 has been reported before to increase OCR (Ahn et al., 2008, Shi et al., 2005). M1-SIRT3 in the H1299s cells MutM1 and M1-SIRT3 in the H1299r cells increased the citrate synthase activity. This increase in citrate synthase activity as a result of the expression of the M1-SIRT3 constructs indicates that expression of SIRT3 increases mitochondrial abundance when it is localized to the mitochondria. M1 & M3-SIRT3 constructs both increased the expression of SIRT1 which indicates that the enzyme activity was important for SIRT3 to increase the expression of the cytosolic SIRT1 but not the localisation signal. M3-SIRT3 caused an increase in the expression of PGC1 α which agrees with the feedback loop proposed by Kincaid and Bossy-Wetzel (2013). MutM1 and M1-SIRT3 increased the expression of TFAM in the H1299s and M1-SIRT3 alone increases TFAM expression in the H1299r cells.

M1-SIRT3 increased the ROS production in the H1299s cells. M1-SIRT3 was shown previously to cause an increase in ECAR so perhaps the increase in ROS is somehow related. In the H1299r cells, the expression of SIRT3 by MutM1-, M1- and M3-SIRT3 all decreased ROS which agrees with the role of SIRT3 in antioxidant defence (Qiu et al., 2010, Someya et al., 2010) and what Cheung et al. (2015) showed. This demonstrates that SIRT3 activation alone may be sufficient to reduce oxidative stress.

In conclusion we show that resveratrol increased mitochondrial biogenesis, mitochondrial reserve capacity and mitochondrial abundance. Resveratrol also increased ROS production in the H1299s cells. SIRT3 expression had no effect on the mitochondrial oxygen consumption or mitochondrial reserve capacity of the H1299s cells. In the H1299r cells, MutM1-SIRT3 increased mitochondrial oxygen consumption and reserve capacity. MutM1- and M1-SIRT3 both increased mitochondrial abundance. M1-SIRT3 expression increased ROS production in the H1299s cells and SIRT3 expression decreased ROS production in the H1299r cells. In conclusion both resveratrol and SIRT3 increase mitochondrial function in the H1299r cells.

Cisplatin often yields modest and often temporary beneficial anti-neoplastic effects due to the acquisition of resistance leading to drug relapse and treatment failure (Johansson et al., 2009, Locasale, 2012, Michels et al., 2013). One new therapeutic avenue that is being pursued in preclinical testing for a number of solid tumours is the targeting the CSC. The CSC hypothesis indicates that CSC are the population of cells that are responsible for tumour initiation, cell survival after therapy, metastatic spread and tumour recurrence (Coghlin and Murray, 2010). CSC's are intrinsically resistant to many current cancer treatments, including chemotherapy and radiotherapy (Bao et al., 2006, Dean et al., 2005). This suggests that with current cancer therapies, that while they manage to kill the bulk of the tumour they may ultimately fail as they do not kill the CSC, which manage to survive and regenerate the tumour. The aim of this study was to identify CSC populations with the aid of the CSC markers CD44/CD133, ALDH, HSP and autofluorescence in our cisplatin resistant H1299 and P31 cells. Neither CD44 nor CD133 exhibited any differential expression between our sensitive and resistant cell lines.

H1299r cells had greater ALDH expression, HSP and autofluorescence than the H1299s cells with no difference between the P31 sensitive and resistant cells. Appropriate controls need to be carried out for the CD44/CD133 and HSP assays before any significant conclusions can be made.

In summary, ALDH and HSP could be potential CSC markers for the H1299 cells, however, more work needs to be carried out to make any definitive statements as to the possible CSCs within these cells.

In conclusion, this work demonstrated that phenformin shows promise as an anti-cancer agent to treat neuroblastoma. Phenformin is currently in only one clinical trial investigating the role of the type II diabetes drugs as a risk factor of contracting pancreatic cancer whereas phenformin's analogue metformin is in 254 clinical trials in relation to cancer. The reason for the lack of clinical trials being conducted are due to the safety concerns of phenformin causing lactic acidosis. Phenformin has been shown to be more efficacious in its anti-cancer properties in numerous studies. The level of lactic acidosis is low enough that if it showed superior anti-neoplastic activity it would not be contraindicated (Shackelford et al., 2013). Also the length of treatment and dosage would be different from diabetic patients and so the adverse side effects observed with the drug would not be as severe (Shackelford and Shaw, 2009).

This study highlighted the metabolic differences between cisplatin sensitive and cisplatin resistant non small cell lung cancer and mesothelioma cancer cell lines. Agents such as resveratrol that increase mitochondrial function may prove effective agents in combating cisplatin resistance in cancer. Resveratrol is currently in 18 clinical trials investigating its effects in cancer. While we have shown that resveratrol shows promise in our *in vitro* setting there are a number of issues that need to be resolved before resveratrol can be translated into the clinic. An understanding of how exactly resveratrol is taken up by all cancers, the direct targets of resveratrol and whether resveratrol's bioavailability be increased are first required (Subramanian et al., 2010).

Chapter 8: Future Work

8.0 Future Work

8.1 Phenformin as an anti-cancer agent

In this study we have demonstrated the anti-cancer potential of phenformin on one of the most aggressive and difficult to treat cancer, *i.e.* neuroblastoma. However, one of the short-comings of the study is that fact that we have only demonstrated efficacy of phenformin at the level of cultured neuroblastoma cells. In order to demonstrate the benefit of phenformin as an anti-cancer agent, we would need to move to whole animals. For instance, xenograft mouse model of the neuroblastoma cancer could be generated, using luciferase tagged NB1691^{luc} or SK-N-AS^{luc} neuroblastoma cells in nude severe combined immunodeficiency (SCID) mice and measure the bioluminescence as carried out by (Tivnan et al., 2011). Initial toxicity screen would need to be conducted to confirm that the health of the normal cells is not compromised. Once that's established the effect of phenformin on tumour growth would be assessed.

The effect of phenformin on ATP levels was carried out over the course of this work by a luciferase based assay and phenformin was shown not to elicit an effect on ATP levels. This was in contrast to many others reports that have shown phenformin to decrease ATP levels (Vigneri et al., 1984). This decrease in ATP levels then activates AMPK (Hardie, 2013). In this work phenformin activated AMPK/ACC and so a decrease in ATP levels would be expected. Measurement of ATP levels as a result of phenformin could be carried out by different cell based assays or measuring AMP, ADP and ATP by ³¹P NMR in the neuroblastoma cells SH-SY5Y. As we have reported a decrease in cellular ROS by DCFHDA, it would be interesting to see whether phenformin decreases mitochondrial ROS by flow cytometry using the novel stain MitoPY1 as well. The neuroblastoma cell line possesses an amplification of N-MYC which demonstrates a unique metabolic profile (Dang, 2013). Metabolomics could be conducted using C¹³ labelled glucose and glutamine by NMR to determine whether phenformin treatment alters this metabolic profile.

8.2 Comparative bioenergetics in parental sensitive and cisplatin resistant cancer cell subline.

Having shown that the resistant cells possess less mitochondria, determination of whether mitochondrial morphology and fission/fusion rates were altered in the cisplatin resistant cells could be carried out. This can be carried out by transfecting the H1299 and P31 cells with a dsRED and a photoactivatable GFP plasmid which contain mitochondrial localisation signals in their sequence and image the mitochondria by confocal microscopy. Mitochondrial morphology can be determined by staining the cells with dsRED and taking images at various points in the cells. Fission/fusion is measured using the dsRED to locate the mitochondria, a cluster of mitochondria is then bleached to activate the GFP and the decrease in GFP fluorescence is then measured over time. Initial work carried out in this study indicated that there may be differences in mitochondrial morphology between the H1299s and H1299r cells. If these potential differences in morphology were confirmed, differences in their rates of mitophagy may also be observed. As investigations into mitophagy are typically as a result of a drug treatment, measuring flux down a specific pathway and not determining basal mitophagy rates, the cells would need to be challenged. The cells can be challenged with a nutrient stress such as hanks buffered saline solution (HBSS) incubation and/or with the mTOR inhibitor rapamycin to determine response. Initial experiments conducted in this work investigating the response of the H1299 and P31 cells to nutrient stress using HBSS, measuring the autophagy markers, LC3 and p62 protein expression by western blot, indicates that there may be differences between the sensitive and resistant cells. Further work would need to be carried out to make any firm conclusions. Having demonstrated that the resistant cells exhibit reduced OCR, ATP production levels could be measured as another index of the cellular bioenergetics.

Having shown that resveratrol and SIRT3 improve mitochondrial function, it would be interesting to determine whether resveratrol or SIRT3 expression affect susceptibility to cisplatin in the H1299 and P31 cells. As this transfection is only transient, it remains to be determined whether it would retain the expression of SIRT3 for the length of the

assay. As we have shown resveratrol and SIRT3 expression improves mitochondrial function, an investigation into whether resveratrol or SIRT3 expression affected ATP levels could be undertaken. SIRT3 has previously been shown to regulate ATP levels (Ahn et al., 2008) so SIRT3 expression in these cells would be expected to increase ATP levels. Determination into whether the improved mitochondrial function is reflected in improved mitochondrial morphology and fission/fusion rates could be examined. The direct or indirect effect of resveratrol and SIRT3 expression could be investigated by use of digitonin-permeabilized cells. Both resveratrol (Desquiret-Dumas et al., 2013) and SIRT3 (Lombard et al., 2007) have been shown to directly affect complex I activity so its activity would be expected to be increased as a result of resveratrol treatment and SIRT3 over-expression. An investigation into whether resveratrol or SIRT3 altered the metabolomics of the cells using C¹³ labelled glucose and glutamine by NMR would permit us to see what pathways and enzymes are affected. With this information, a mechanism of action could be explored. We demonstrate that resveratrol acts on multiple parameters in the cells but how exactly resveratrol gets into the cancer cells would need to be examined. Some studies report that resveratrol gets into the cells by passive diffusion and others active transport (Subramanian et al., 2010).

Having confirmed the correlation between increased cisplatin resistance and increased Gb₃, the effect of any mechanistic link between the two would need to be established for instance by seeing the effect of Gb₃ synthesis inhibition, by the use of the glucosylceramide synthase inhibitor PPMP, on cisplatin susceptibility. In addition as you may recall we established that ABCB6 or ABCE1 expression correlated with acquired cisplatin resistance in the P31r and H1299r cells respectively. It would be interesting to determine whether ABCB6 or ABCE1 expression was decreased by inhibition of GB3 synthesis.

Having shown that the H1299r cells may potentially be more stem-like than the H1299s cell, use of appropriate controls for CD44/CD133 and the HSP assay and investigate other stem cells markers for example CD117 or CXCR4 expression to create a hierarchy of stemness would need to be undertaken. A list of stem cell markers alone would not

be enough to say that the H1299r cells are more stem-like than the H1299s cells. Single cell analysis would need to be carried out in order to see if more than one population arises and a xenograft model to illustrate stem cell properties to confirm the single cell analysis result.

References

- ABDEL KHALEK, W., CORTADE, F., OLLENDORFF, V., LAPASSET, L., TINTIGNAC, L., CHABI, B. & WRUTNIAK-CABELLO, C. 2014. SIRT3, a Mitochondrial NAD(+)-Dependent Deacetylase, Is Involved in the Regulation of Myoblast Differentiation. *PLoS ONE*, 9, e114388.
- ADAMS, J. M. & STRASSER, A. 2008. Is tumor growth sustained by rare cancer stem cells or dominant clones? *Cancer Res*, 68, 4018-21.
- AHN, B.-H., KIM, H.-S., SONG, S., LEE, I. H., LIU, J., VASSILOPOULOS, A., DENG, C.-X. & FINKEL, T. 2008. A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 14447-14452.
- ALHAZZAZI, T. Y., KAMARAJAN, P., JOO, N., HUANG, J.-Y., VERDIN, E., D'SILVA, N. J. & KAPILA, Y. L. 2011. Sirtuin-3 (SIRT3), a Novel Potential Therapeutic Target for Oral Cancer. *Cancer*, 117, 1670-1678.
- ALIMOVA, I. N., LIU, B., FAN, Z., EDGERTON, S. M., DILLON, T., LIND, S. E. & THOR, A. D. 2009. Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest in vitro. *Cell Cycle*, 8, 909-915.
- AMOROSO, F., CAPECE, M., ROTONDO, A., CANGELOSI, D., FERRACIN, M., FRANCESCHINI, A., RAFFAGHELLO, L., PISTOIA, V., VARESEO, L. & ADINOLFI, E. 2015. The P2X7 receptor is a key modulator of the PI3K/GSK3[beta]/VEGF signaling network: evidence in experimental neuroblastoma. *Oncogene*.
- ANDREWS, P. A. & HOWELL, S. B. 1990. Cellular pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. *Cancer Cells*, 2, 35-43.
- APPLEYARD, M. V. C. L., MURRAY, K. E., COATES, P. J., WULLSCHLEGER, S., BRAY, S. E., KERNOHAN, N. M., FLEMING, S., ALESSI, D. R. & THOMPSON, A. M. 2012. Phenformin as prophylaxis and therapy in breast cancer xenografts. *Br J Cancer*, 106, 1117-1122.
- ARAB, S., RUSSEL, E., CHAPMAN, W. B., ROSEN, B. & LINGWOOD, C. A. 1997. Expression of the verotoxin receptor glycolipid, globotriaosylceramide, in ovarian hyperplasias. *Oncol Res*, 9, 553-63.
- AZIZ, Q., THOMAS, A., KHAMBRA, T. & TINKER, A. 2010. Phenformin has a direct inhibitory effect on the ATP-sensitive potassium channel. *European Journal of Pharmacology*, 634, 26-32.

- BA, Y., ZHANG, C. N., ZHANG, Y. & ZHANG, C. Y. 2008. [Down-regulation of PGC-1 α expression in human hepatocellular carcinoma]. *Zhonghua Zhong Liu Za Zhi*, 30, 593-7.
- BABU, D., LECLERCQ, G., GOOSSENS, V., REMIJSEN, Q., VANDENABEELE, P., MOTTERLINI, R. & LEFEBVRE, R. A. 2015. Antioxidant potential of CORM-A1 and resveratrol during TNF- α /cycloheximide-induced oxidative stress and apoptosis in murine intestinal epithelial MODE-K cells. *Toxicology and Applied Pharmacology*.
- BAILEY, C. J. & TURNER, R. C. 1996. Metformin. *New England Journal of Medicine*, 334, 574-579.
- BALAYSSAC, D., CAYRE, A., AUTHIER, N., BOURDU, S., PENAULT-LLORCA, F., GILLET, J. P., MAUBLANT, J., ESCHALIER, A. & COUDORE, F. 2005. Patterns of P-glycoprotein activity in the nervous system during vincristine-induced neuropathy in rats. *Journal of the Peripheral Nervous System*, 10, 301-310.
- BANZATO, A., BOBISSE, S., RONDINA, M., RENIER, D., BETTELLA, F., ESPOSITO, G., QUINTIERI, L., MELÉNDEZ-ALAFORT, L., MAZZI, U., ZANOVELLO, P. & ROSATO, A. 2008. A Paclitaxel-Hyaluronan Bioconjugate Targeting Ovarian Cancer Affords a Potent In vivo Therapeutic Activity. *Clinical Cancer Research*, 14, 3598-3606.
- BAO, S., WU, Q., MCLENDON, R. E., HAO, Y., SHI, Q., HJELMELAND, A. B., DEWHIRST, M. W., BIGNER, D. D. & RICH, J. N. 2006. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature*, 444, 756-760.
- BARON, A., MIGITA, T., TANG, D. & LODA, M. 2004. Fatty acid synthase: A metabolic oncogene in prostate cancer? *Journal of Cellular Biochemistry*, 91, 47-53.
- BEHNAM-MOTLAGH, P., TYLER, A., GRANKVIST, K. & JOHANSSON, A. 2010a. Verotoxin-1 Treatment or Manipulation of its Receptor Globotriaosylceramide (Gb3) for Reversal of Multidrug Resistance to Cancer Chemotherapy. *Toxins*, 2, 2467.
- BEHNAM-MOTLAGH, P., TYLER, A., GRANKVIST, K. & JOHANSSON, A. 2010b. Verotoxin-1 Treatment or Manipulation of its Receptor Globotriaosylceramide (Gb3) for Reversal of Multidrug Resistance to Cancer Chemotherapy. *Toxins*, 2, 2467-2477.
- BEHREND, L., HENDERSON, G. & ZWACKA, R. M. 2003. Reactive oxygen species in oncogenic transformation. *Biochemical Society Transactions*, 31, 1441-1444.
- BELL, ERIC L. & GUARENTE, L. 2011. The SirT3 Divining Rod Points to Oxidative Stress. *Molecular Cell*, 42, 561-568.
- BEN SAHRA, I., LE MARCHAND-BRUSTEL, Y., TANTI, J.-F. & BOST, F. 2010. Metformin in Cancer Therapy: A New Perspective for an Old Antidiabetic Drug? *Molecular Cancer Therapeutics*, 9, 1092-1099.

- BERSTEIN, L., YUE, W., WANG, J.-P. & SANTEN, R. 2011. Isolated and combined action of tamoxifen and metformin in wild-type, tamoxifen-resistant, and estrogen-deprived MCF-7 cells. *Breast Cancer Research and Treatment*, 128, 109-117.
- BERTOLINI, G., ROZ, L., PEREGO, P., TORTORETO, M., FONTANELLA, E., GATTI, L., PRATESI, G., FABBRI, A., ANDRIANI, F., TINELLI, S., ROZ, E., CASERINI, R., LO VULLO, S., CAMERINI, T., MARIANI, L., DELIA, D., CALABRÒ, E., PASTORINO, U. & SOZZI, G. 2009. Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. *Proceedings of the National Academy of Sciences*, 106, 16281-16286.
- BHUJWALLA, Z. M., ARTEMOV, D., BALLESTEROS, P., CERDAN, S., GILLIES, R. J. & SOLAIYAPPAN, M. 2002. Combined vascular and extracellular pH imaging of solid tumors. *NMR in Biomedicine*, 15, 114-119.
- BIRSOY, K., POSSEMATO, R., LORBEER, F. K., BAYRAKTAR, E. C., THIRU, P., YUCEL, B., WANG, T., CHEN, W. W., CLISH, C. B. & SABATINI, D. M. 2014. Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides. *Nature*, 508, 108-112.
- BRIDGES, HANNAH R., JONES, ANDREW J. Y., POLLAK, MICHAEL N. & HIRST, J. 2014. Effects of metformin and other biguanides on oxidative phosphorylation in mitochondria. *Biochemical Journal*, 462, 475-487.
- BROWN, J. D., BEERENDS, J. J. & BARTELS, D. C. 1973. The Effect of Phenformin on Lipolysis and the Concentration of Cyclic AMP and ATP in White Fat Cells. *Endocrinology*, 93, 445-449.
- BRUGAROLAS, J. B., VAZQUEZ, F., REDDY, A., SELLERS, W. R. & KAELIN JR, W. G. 2003. TSC2 regulates VEGF through mTOR-dependent and -independent pathways. *Cancer Cell*, 4, 147-158.
- CALLAPINA, M., ZHOU, J., SCHMID, T., KÖHL, R. & BRÜNE, B. 2005. NO restores HIF-1 α hydroxylation during hypoxia: Role of reactive oxygen species. *Free Radical Biology and Medicine*, 39, 925-936.
- CANTÓ, C. & AUWERX, J. 2011. NAD⁺ as a Signaling Molecule Modulating Metabolism. *Cold Spring Harbor Symposia on Quantitative Biology*, 76, 291-298.
- CARACI, F., CHISARI, M., FRASCA, G., CHIECHIO, S., SALOMONE, S., PINTO, A., SORTINO, M. A. & BIANCHI, A. 2003. Effects of phenformin on the proliferation of human tumor cell lines. *Life Sciences*, 74, 643-650.
- CARLING, D. 2004. The AMP-activated protein kinase cascade – a unifying system for energy control. *Trends in Biochemical Sciences*, 29, 18-24.

- CARVALHO, C., CORREIA, S., SANTOS, M., SEIÇA, R., OLIVEIRA, C. & MOREIRA, P. 2008. Metformin promotes isolated rat liver mitochondria impairment. *Molecular and Cellular Biochemistry*, 308, 75-83.
- CATALGOL, B., BATIREL, S., TAGA, Y. & OZER, N. K. 2012. Resveratrol: French Paradox Revisited. *Frontiers in Pharmacology*, 3.
- CHAKRABORTY, A., CHOWDHURY, S. & BHATTACHARYYA, M. 2011. Effect of metformin on oxidative stress, nitrosative stress and inflammatory biomarkers in type 2 diabetes patients. *Diabetes Research and Clinical Practice*, 93, 56-62.
- CHEN, Z.-Q., DONG, J., ISHIMURA, A., DAAR, I., HINNEBUSCH, A. G. & DEAN, M. 2006. The Essential Vertebrate ABCE1 Protein Interacts with Eukaryotic Initiation Factors. *Journal of Biological Chemistry*, 281, 7452-7457.
- CHEUNG, K. G., COLE, L. K., XIANG, B., CHEN, K., MA, X., MYAL, Y., HATCH, G. M., TONG, Q. & DOLINSKY, V. W. 2015. Sirtuin-3 (SIRT3) Protein Attenuates Doxorubicin-induced Oxidative Stress and Improves Mitochondrial Respiration in H9c2 Cardiomyocytes. *Journal of Biological Chemistry*, 290, 10981-10993.
- CHEUNG, N.-K. V. & DYER, M. A. 2013. Neuroblastoma: developmental biology, cancer genomics and immunotherapy. *Nat Rev Cancer*, 13, 397-411.
- CHEVROLIER, A., LOISEAU, D., REYNIER, P. & STEPIEN, G. 2011. Adenine nucleotide translocase 2 is a key mitochondrial protein in cancer metabolism. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1807, 562-567.
- CHINEMBIRI, T. N., DU PLESSIS, L. H., GERBER, M., HAMMAN, J. H. & DU PLESSIS, J. 2014. Review of natural compounds for potential skin cancer treatment. *Molecules*, 19, 11679-721.
- CHUN, S., JOHNSON, C., WASHBURN, J., CRUZ-CORREA, M., DANG, D. & DANG, L. 2010. Oncogenic KRAS modulates mitochondrial metabolism in human colon cancer cells by inducing HIF-1 α and HIF-2 α target genes. *Molecular Cancer*, 9, 293.
- CHUTE, J. P., MURAMOTO, G. G., WHITESIDES, J., COLVIN, M., SAFI, R., CHAO, N. J. & MCDONNELL, D. P. 2006. Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. *Proc Natl Acad Sci U S A*, 103, 11707-12.
- CIMEN, H., HAN, M.-J., YANG, Y., TONG, Q., KOC, H. & KOC, E. C. 2010. Regulation of Succinate Dehydrogenase Activity by SIRT3 in Mammalian Mitochondria. *Biochemistry*, 49, 304-311.

- COGHLIN, C. & MURRAY, G. I. 2010. Current and emerging concepts in tumour metastasis. *The Journal of Pathology*, 222, 1-15.
- COOPER, H. M., HUANG, J.-Y., VERDIN, E. & SPELBRINK, J. N. 2009. A New Splice Variant of the Mouse SIRT3 Gene Encodes the Mitochondrial Precursor Protein. *PLoS ONE*, 4, e4986.
- CUI, Y., QIN, L., WU, J., QU, X., HOU, C., SUN, W., LI, S., VAUGHAN, A. T. M., LI, J. J. & LIU, J. 2015. SIRT3 Enhances Glycolysis and Proliferation in SIRT3-Expressing Gastric Cancer Cells. *PLoS ONE*, 10, e0129834.
- CULLEN, K., YANG, Z., SCHUMAKER, L. & GUO, Z. 2007. Mitochondria as a critical target of the chemotherapeutic agent cisplatin in head and neck cancer. *Journal of Bioenergetics and Biomembranes*, 39, 43-50.
- DANG, C. V. 2012. Links between metabolism and cancer. *Genes & Development*, 26, 877-890.
- DANG, C. V. 2013. MYC, Metabolism, Cell Growth, and Tumorigenesis. *Cold Spring Harbor Perspectives in Medicine*, 3.
- DANG, C. V., O'DONNELL, K. A., ZELLER, K. I., NGUYEN, T., OSTHUS, R. C. & LI, F. 2006. The c-Myc target gene network. *Seminars in Cancer Biology*, 16, 253-264.
- DAVIDOFF, F., NOVAK, W. T. T. A. O. D. & JOHNSON, L. 1971. Effects of Guanidine Derivatives on Mitochondrial Function: III. THE MECHANISM OF PHENETHYLBIGUANIDE ACCUMULATION AND ITS RELATIONSHIP TO IN VITRO RESPIRATORY INHIBITION. *Journal of Biological Chemistry*, 246, 4017-4027.
- DE ROSA, M. F., ACKERLEY, C., WANG, B., ITO, S., CLARKE, D. M. & LINGWOOD, C. 2008. Inhibition of Multidrug Resistance by AdamantylGb3, a Globotriaosylceramide Analog. *Journal of Biological Chemistry*, 283, 4501-4511.
- DEAN, M., FOJO, T. & BATES, S. 2005. Tumour stem cells and drug resistance. *Nat Rev Cancer*, 5, 275-284.
- DEBERARDINIS, R. J. & CHENG, T. 2009. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene*, 29, 313-324.
- DENKO, N. C. 2008. Hypoxia, HIF1 and glucose metabolism in the solid tumour. *Nat Rev Cancer*, 8, 705-713.
- DESQUIRET-DUMAS, V., GUEGUEN, N., LEMAN, G., BARON, S., NIVET-ANTOINE, V., CHUPIN, S., CHEVROLIER, A., VESSIERES, E., AYER, A., FERRE, M., BONNEAU, D., HENRION, D., REYNIER, P. & PROCACCIO, V. 2013. Resveratrol Induces a

Mitochondrial Complex I Dependent Increase in NADH Oxidation Responsible for Sirtuin Activation in Liver Cells. *Journal of Biological Chemistry*.

- DEVARAJAN, P., SAVOCA, M., CASTANEDA, M. P., PARK, M. S., ESTEBAN-CRUCIANI, N., KALINEC, G. & KALINEC, F. 2002. Cisplatin-induced apoptosis in auditory cells: role of death receptor and mitochondrial pathways. *Hearing Research*, 174, 45-54.
- DIAZ, F. & MORAES, C. T. 2008. Mitochondrial biogenesis and turnover. *Cell Calcium*, 44, 24-35.
- DICK, J. E. 2008. Stem cell concepts renew cancer research. *Blood*, 112, 4793-4807.
- DILMAN, V. M. 1971. AGE-ASSOCIATED ELEVATION OF HYPOTHALAMIC THRESHOLD TO FEEDBACK CONTROL, AND ITS ROLE IN DEVELOPMENT, AGEING, AND DISEASE. *The Lancet*, 297, 1211-1219.
- DOMINY JR, J. E., LEE, Y., GERHART-HINES, Z. & PUIGSERVER, P. 2010. Nutrient-dependent regulation of PGC-1 α 's acetylation state and metabolic function through the enzymatic activities of Sirt1/GCN5. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1804, 1676-1683.
- DOWLING, R., GOODWIN, P. & STAMBOLIC, V. 2011. Understanding the benefit of metformin use in cancer treatment. *BMC Medicine*, 9, 33.
- DOWLING, R. J. O., ZAKIKHANI, M., FANTUS, I. G., POLLAK, M. & SONENBERG, N. 2007. Metformin Inhibits Mammalian Target of Rapamycin-Dependent Translation Initiation in Breast Cancer Cells. *Cancer Research*, 67, 10804-10812.
- DYKENS, J. A., JAMIESON, J., MARROQUIN, L., NADANACIVA, S., BILLIS, P. A. & WILL, Y. 2008. Biguanide-induced mitochondrial dysfunction yields increased lactate production and cytotoxicity of aerobically-poised HepG2 cells and human hepatocytes in vitro. *Toxicology and Applied Pharmacology*, 233, 203-210.
- EBISUZAKI, K., CASLEY, W. L., GRIFFITHS, A. & WHEATON, L. 1991. Temporal Mapping of the Differentiation Pathway of the Murine Erythroleukemia Cell. *Cancer Research*, 51, 1668-1673.
- ECKFORD, PAUL D. W. & SHAROM, FRANCES J. 2005. The reconstituted P-glycoprotein multidrug transporter is a flippase for glucosylceramide and other simple glycosphingolipids. *Biochemical Journal*, 389, 517-526.
- EKI, T. 1994. Poly (ADP-ribose) polymerase inhibits DNA replication by human replicative DNA polymerase α , δ and ϵ in vitro. *FEBS Letters*, 356, 261-266.

- EKSTRAND, M. I., FALKENBERG, M., RANTANEN, A., PARK, C. B., GASPARI, M., HULTENBY, K., RUSTIN, P., GUSTAFSSON, C. M. & LARSSON, N.-G. 2004. Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Human Molecular Genetics*, 13, 935-944.
- EL-MIR, M.-Y., NOGUEIRA, V., FONTAINE, E., AVÉRET, N., RIGOULET, M. & LEVERVE, X. 2000. Dimethylbiguanide Inhibits Cell Respiration via an Indirect Effect Targeted on the Respiratory Chain Complex I. *Journal of Biological Chemistry*, 275, 223-228.
- ELSTROM, R. L., BAUER, D. E., BUZZAI, M., KARNAUSKAS, R., HARRIS, M. H., PLAS, D. R., ZHUANG, H., CINALLI, R. M., ALAVI, A., RUDIN, C. M. & THOMPSON, C. B. 2004. Akt Stimulates Aerobic Glycolysis in Cancer Cells. *Cancer Research*, 64, 3892-3899.
- ERDMANN, M., WIPFLER, D., MERLING, A., CAO, Y., CLAUS, C., KNIEP, B., SADICK, H., BERGLER, W., VLASAK, R. & SCHWARTZ-ALBIEZ, R. 2006. Differential surface expression and possible function of 9-O- and 7-O-acetylated GD3 (CD60 b and c) during activation and apoptosis of human tonsillar B and T lymphocytes. *Glycoconjugate Journal*, 23, 627-638.
- EVANS, J. M. M., DONNELLY, L. A., EMSLIE-SMITH, A. M., ALESSI, D. R. & MORRIS, A. D. 2005. Metformin and reduced risk of cancer in diabetic patients. *BMJ*, 330, 1304-1305.
- FANTIN, V. R., ST-PIERRE, J. & LEDER, P. 2006. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell*, 9, 425-434.
- FEILCHENFELDT, J., BRÜNDLER, M.-A., SORAVIA, C., TÖTSCH, M. & MEIER, C. A. 2004. Peroxisome proliferator-activated receptors (PPARs) and associated transcription factors in colon cancer: reduced expression of PPAR γ -coactivator 1 (PGC-1). *Cancer Letters*, 203, 25-33.
- FINLEY, LYDIA W. S., CARRACEDO, A., LEE, J., SOUZA, A., EGIA, A., ZHANG, J., TERUYA-FELDSTEIN, J., MOREIRA, PAULA I., CARDOSO, SANDRA M., CLISH, CLARY B., PANDOLFI, PIER P. & HAIGIS, MARCIA C. 2011. SIRT3 Opposes Reprogramming of Cancer Cell Metabolism through HIF1 α Destabilization. *Cancer Cell*, 19, 416-428.
- FORCET, C. & BILLAUD, M. 2007. Dialogue Between LKB1 and AMPK: A Hot Topic at the Cellular Pole. *Sci. STKE*, 2007, pe51-.
- FRESCO, P., BORGES, F., DINIZ, C. & MARQUES, M. P. M. 2006. New insights on the anticancer properties of dietary polyphenols. *Medicinal Research Reviews*, 26, 747-766.

- FRIDOVICH, I. 1995. Superoxide radical and superoxide dismutases. *Annual Review of Biochemistry*, 64, 97-112.
- FRYE, R. A. 2000. Phylogenetic Classification of Prokaryotic and Eukaryotic Sir2-like Proteins. *Biochemical and Biophysical Research Communications*, 273, 793-798.
- FRYER, L. G. D., FOUFELLE, F., BARNES, K., BALDWIN, S. A., WOODS, A. & CARLING, D. 2002. Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells. *Biochem. J.*, 363, 167-174.
- FUERTES, M. A., CASTILLA, J., ALONSO, C. & PEREZ, J. M. 2003. Cisplatin biochemical mechanism of action: from cytotoxicity to induction of cell death through interconnections between apoptotic and necrotic pathways. *Curr Med Chem*, 10, 257-66.
- FULGENCIO, J.-P., KOHL, C., GIRARD, J. & PÉGORIER, J.-P. 2001. Effect of metformin on fatty acid and glucose metabolism in freshly isolated hepatocytes and on specific gene expression in cultured hepatocytes. *Biochemical Pharmacology*, 62, 439-446.
- FURUTA, T., UEDA, T., AUNE, G., SARASIN, A., KRAEMER, K. H. & POMMIER, Y. 2002. Transcription-coupled nucleotide excision repair as a determinant of cisplatin sensitivity of human cells. *Cancer Res*, 62, 4899-902.
- GALENKAMP, K., CARRIBA, P., URRESTI, J., PLANELLS-FERRER, L., COCCIA, E., LOPEZ-SORIANO, J., BARNEDA-ZAHONERO, B., MOUBARAK, R., SEGURA, M. & COMELLA, J. 2015. TNFalpha sensitizes neuroblastoma cells to FasL-, cisplatin- and etoposide-induced cell death by NF-kappaB-mediated expression of Fas. *Molecular Cancer*, 14, 62.
- GALLUZZI, L., VITALE, I., SENOVILLA, L., EISENBERG, T., CARMONA-GUTIERREZ, D., VACCHELLI, E. & KROEMER, G. 2012. Independent transcriptional reprogramming and apoptosis induction by cisplatin. *Cell Cycle*, 11, 3472.
- GARIÉPY, J. 2001. The use of Shiga-like toxin 1 in cancer therapy. *Critical Reviews in Oncology/Hematology*, 39, 99-106.
- GATENBY, R. A. & GILLIES, R. J. 2004. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer*, 4, 891-899.
- GIACCONE, G., BATTEY, J., GAZDAR, A. F., OIE, H., DRAOUI, M. & MOODY, T. W. 1992. Neuromedin B Is Present in Lung Cancer Cell Lines. *Cancer Research*, 52, 2732s-2736s.

- GIBALOVÁ, L., ŠEREŠ, M., RUSNÁK, A., DITTE, P., LABUDOVÁ, M., UHRÍK, B., PASTOREK, J., SEDLÁK, J., BREIER, A. & SULOVÁ, Z. 2012. P-glycoprotein depresses cisplatin sensitivity in L1210 cells by inhibiting cisplatin-induced caspase-3 activation. *Toxicology in Vitro*, 26, 435-444.
- GONZALEZ, V. M., FUERTES, M. A., ALONSO, C. & PEREZ, J. M. 2001. Is Cisplatin-Induced Cell Death Always Produced by Apoptosis? *Molecular Pharmacology*, 59, 657-663.
- GOODARZI, M. O. & BRYER-ASH, M. 2005. Metformin revisited: re-evaluation of its properties and role in the pharmacopoeia of modern antidiabetic agents. *Diabetes, Obesity and Metabolism*, 7, 654-665.
- GOTTESMAN, M. M., FOJO, T. & BATES, S. E. 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer*, 2, 48-58.
- GOUAZÉ, V., YU, J. Y., BLEICHER, R. J., HAN, T.-Y., LIU, Y.-Y., WANG, H., GOTTESMAN, M. M., BITTERMAN, A., GIULIANO, A. E. & CABOT, M. C. 2004. Overexpression of glucosylceramide synthase and P-glycoprotein in cancer cells selected for resistance to natural product chemotherapy. *Molecular Cancer Therapeutics*, 3, 633-640.
- GUERTIN, D. A. & SABATINI, D. M. 2007. Defining the Role of mTOR in Cancer. *Cancer Cell*, 12, 9-22.
- GUO, Z., CHAVEZ, K. J., ALVAREZ, J., ZHANG, X., NORRIS, B., MAHER, M., MORGAN, M., SCHUMACHER, R. J., CUELLAR, R., SEVRIUKOVA, I. F., POULOS, T. L., DENISOV, I., SLIGAR, S. G., GUPTA, K., BLAIR, I. A., CAPDEVILA, J., KELEKAR, A., AMIN, E., GEORG, G. & POTTER, D. A. 2014. Abstract 2689: Breast cancer inhibition by a novel and potent biguanide, N1-hexyl-N5-benzyl-biguanide. *Cancer Research*, 74, 2689.
- GUZY, R. D., HOYOS, B., ROBIN, E., CHEN, H., LIU, L., MANSFIELD, K. D., SIMON, M. C., HAMMERLING, U. & SCHUMACKER, P. T. 2005. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. *Cell Metabolism*, 1, 401-408.
- HA, H. C. & SNYDER, S. H. 1999. Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proceedings of the National Academy of Sciences*, 96, 13978-13982.
- HAIGIS, M. C. & GUARENTE, L. P. 2006. Mammalian sirtuins—emerging roles in physiology, aging, and calorie restriction. *Genes & Development*, 20, 2913-2921.

- HALLOWS, W. C., LEE, S. & DENU, J. M. 2006. Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 10230-10235.
- HAMANAKA, R. B. & CHANDEL, N. S. 2010. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends in Biochemical Sciences*, 35, 505-513.
- HANAHAN, D. & WEINBERG, ROBERT A. 2011. Hallmarks of Cancer: The Next Generation. *Cell*, 144, 646-674.
- HAO, H.-X., KHALIMONCHUK, O., SCHRADERS, M., DEPHOURE, N., BAYLEY, J.-P., KUNST, H., DEVILEE, P., CREMERS, C. W. R. J., SCHIFFMAN, J. D., BENTZ, B. G., GYGI, S. P., WINGE, D. R., KREMER, H. & RUTTER, J. 2009. SDH5, a Gene Required for Flavination of Succinate Dehydrogenase, Is Mutated in Paraganglioma. *Science*, 325, 1139-1142.
- HARDIE, D. G. 2013. AMPK: A Target for Drugs and Natural Products With Effects on Both Diabetes and Cancer. *Diabetes*, 62, 2164-2172.
- HAWLEY, S. A., PAN, D. A., MUSTARD, K. J., ROSS, L., BAIN, J., EDELMAN, A. M., FRENGUELLI, B. G. & HARDIE, D. G. 2005. Calmodulin-dependent protein kinase kinase- β is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metabolism*, 2, 9-19.
- HAWLEY, S. A., ROSS, F. A., CHEVTZOFF, C., GREEN, K. A., EVANS, A., FOGARTY, S., TOWLER, M. C., BROWN, L. J., OGUNBAYO, O. A., EVANS, A. M. & HARDIE, D. G. 2010. Use of Cells Expressing γ Subunit Variants to Identify Diverse Mechanisms of AMPK Activation. *Cell Metabolism*, 11, 554-565.
- HIGASHIDA, K., KIM, S. H., JUNG, S. R., ASAKA, M., HOLLOSZY, J. O. & HAN, D.-H. 2013. Effects of Resveratrol and SIRT1 on PGC-1 α Activity and Mitochondrial Biogenesis: A Reevaluation. *PLoS Biology*, 11, e1001603.
- HIRSCH, H. A., ILIOPOULOS, D., TSICHLIS, P. N. & STRUHL, K. 2009. Metformin Selectively Targets Cancer Stem Cells, and Acts Together with Chemotherapy to Block Tumor Growth and Prolong Remission. *Cancer Research*, 69, 7507-7511.
- HIRSCHEY, M. D., SHIMAZU, T., GOETZMAN, E., JING, E., SCHWER, B., LOMBARD, D. B., GRUETER, C. A., HARRIS, C., BIDDINGER, S., ILKAYEVA, O. R., STEVENS, R. D., LI, Y., SAHA, A. K., RUDERMAN, N. B., BAIN, J. R., NEWGARD, C. B., FARESE, R. V., ALT, F. W., KAHN, C. R. & VERDIN, E. 2010. SIRT3 regulates fatty acid oxidation via reversible enzyme deacetylation. *Nature*, 464, 121-125.

- HO, M. M., NG, A. V., LAM, S. & HUNG, J. Y. 2007. Side Population in Human Lung Cancer Cell Lines and Tumors Is Enriched with Stem-like Cancer Cells. *Cancer Research*, 67, 4827-4833.
- HONG, S. P., WEN, J., BANG, S., PARK, S. & SONG, S. Y. 2009. CD44-positive cells are responsible for gemcitabine resistance in pancreatic cancer cells. *International Journal of Cancer*, 125, 2323-2331.
- HUANG, B. O., ZHOU, H., LANG, X. & LIU, Z. 2014. siRNA-induced ABCE1 silencing inhibits proliferation and invasion of breast cancer cells. *Molecular Medicine Reports*, 10, 1685-1690.
- HUANG, E. H., HYNES, M. J., ZHANG, T., GINESTIER, C., DONTU, G., APPELMAN, H., FIELDS, J. Z., WICHA, M. S. & BOMAN, B. M. 2009. Aldehyde Dehydrogenase 1 Is a Marker for Normal and Malignant Human Colonic Stem Cells (SC) and Tracks SC Overpopulation during Colon Tumorigenesis. *Cancer Research*, 69, 3382-3389.
- HUANG, J. & MANNING, B. D. 2008. The TSC1–TSC2 complex: a molecular switchboard controlling cell growth. *Biochem J*, 412, 179-190.
- HUANG, X. E. A. 2008. Important role of the LKB1–AMPK pathway in suppressing tumorigenesis in PTENdeficient mice. *Biochem . J*, 211–221.
- ILIOPOULOS, D., HIRSCH, H. A. & STRUHL, K. 2011. Metformin Decreases the Dose of Chemotherapy for Prolonging Tumor Remission in Mouse Xenografts Involving Multiple Cancer Cell Types. *Cancer Research*, 71, 3196-3201.
- IMAI, S.-I., ARMSTRONG, C. M., KAEBERLEIN, M. & GUARENTE, L. 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature*, 403, 795-800.
- ISODA, K., YOUNG, J. L., ZIRLIK, A., MACFARLANE, L. A., TSUBOI, N., GERDES, N., SCHÖNBECK, U. & LIBBY, P. 2006. Metformin Inhibits Proinflammatory Responses and Nuclear Factor- κ B in Human Vascular Wall Cells. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 26, 611-617.
- JACKSON, A. L., KILGORE, J. E., QIU, H., ZHOU, C., GEHRIG, P. A. & BAE-JUMP, V. L. 2013. Abstract 2442: Antitumorigenic effects of phenformin in human ovarian cancer cell lines. *Cancer Research*, 73, 2442.
- JACOBS, K. M., PENNINGTON, J. D., BISHT, K. S., AYKIN-BURNS, N., KIM, H.-S., MISHRA, M., SUN, L., NGUYEN, P., AHN, B.-H., LECLERC, J., DENG, C.-X., SPITZ, D. R. & GIUS, D. 2008. SIRT3 interacts with the daf-16 homolog FOXO3a in the Mitochondria,

as well as increases FOXO3a Dependent Gene expression. *International Journal of Biological Sciences*, 4, 291-299.

JAMIESON, E. R. & LIPPARD, S. J. 1999. Structure, Recognition, and Processing of Cisplatin–DNA Adducts. *Chemical Reviews*, 99, 2467-2498.

JANG, M., CAI, L., UDEANI, G. O., SLOWING, K. V., THOMAS, C. F., BEECHER, C. W. W., FONG, H. H. S., FARNSWORTH, N. R., KINGHORN, A. D., MEHTA, R. G., MOON, R. C. & PEZZUTO, J. M. 1997. Cancer Chemopreventive Activity of Resveratrol, a Natural Product Derived from Grapes. *Science*, 275, 218-220.

JANSON, V., ANDERSSON, B., BEHNAM-MOTLAGH, P., ENGSTRÖM, K. G., HENRIKSSON, R. & GRANKVIST, K. 2008. Acquisition of Cisplatin-resistance in Malignant Mesothelioma Cells Abrogates Na⁺,K⁺,2Cl⁻-cotransport Activity and Cisplatin-induced Early Membrane Blebbing. *Cellular Physiology and Biochemistry*, 22, 045-056.

JEGGO, P. A. 1998. DNA repair: PARP – another guardian angel? *Current Biology*, 8, R49-R51.

JEONG, S. H., SONG, I. S., KIM, H. K., LEE, S. R., SONG, S., SUH, H., YOON, Y. G., YOO, Y. H., KIM, N., RHEE, B. D., KO, K. S. & HAN, J. 2012. An Analogue of Resveratrol HS-1793 Exhibits Anticancer Activity Against MCF-7 Cells Via Inhibition of Mitochondrial Biogenesis Gene Expression. *Molecules and Cells*, 34, 357-365.

JIANG, F., QIU, Q., KHANNA, A., TODD, N. W., DEEPAK, J., XING, L., WANG, H., LIU, Z., SU, Y., STASS, S. A. & KATZ, R. L. 2009. Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. *Mol Cancer Res*, 7, 330-8.

JIRALERSPONG, S., PALLA, S. L., GIORDANO, S. H., MERIC-BERNSTAM, F., LIEDTKE, C., BARNETT, C. M., HSU, L., HUNG, M.-C., HORTOBAGYI, G. N. & GONZALEZ-ANGULO, A. M. 2009. Metformin and Pathologic Complete Responses to Neoadjuvant Chemotherapy in Diabetic Patients With Breast Cancer. *Journal of Clinical Oncology*, 27, 3297-3302.

JOHANSSON, D., ANDERSSON, C., MOHARER, J., JOHANSSON, A. & BEHNAM-MOTLAGH, P. 2009. Cisplatin-induced expression of Gb3 enables verotoxin-1 treatment of cisplatin resistance in malignant pleural mesothelioma cells. *Br J Cancer*, 102, 383-391.

JOHANSSON, D., BERGSTROM, P., HENRIKSSON, R., GRANKVIST, K., JOHANSSON, A. & BEHNAM-MOTLAGH, P. 2006. Adenylate cyclase toxin from Bordetella pertussis enhances cisplatin-induced apoptosis to lung cancer cells in vitro. *Oncol Res*, 15, 423-30.

- JONES, N. P. & SCHULZE, A. 2012. Targeting cancer metabolism – aiming at a tumour's sweet-spot. *Drug Discovery Today*, 17, 232-241.
- KANG, D. & HAMASAKI, N. 2003. Mitochondrial oxidative stress and mitochondrial DNA. *Clinical Chemistry and Laboratory Medicine*, 41, 1281-1288.
- KASAHARA, K., FUJIWARA, Y., NISHIO, K., OHMORI, T., SUGIMOTO, Y., KOMIYA, K., MATSUDA, T. & SAIJO, N. 1991. Metallothionein Content Correlates with the Sensitivity of Human Small Cell Lung Cancer Cell Lines to Cisplatin. *Cancer Research*, 51, 3237-3242.
- KASCHNY, M., DEMELER, J., JANSSEN, I. J. I., KUZMINA, T. A., BESOGNET, B., KANELLOS, T., KERBOEUF, D., VON SAMSON-HIMMELSTJERNA, G. & KRÜCKEN, J. 2015. Macrocyclic Lactones Differ in Interaction with Recombinant P-Glycoprotein 9 of the Parasitic Nematode *Cylicocylus elongatus* and Ketoconazole in a Yeast Growth Assay. *PLoS Pathogens*, 11, e1004781.
- KELLY, D. P. & SCARPULLA, R. C. 2004. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes & Development*, 18, 357-368.
- KENDRICK, A. A., CHOUDHURY, M., RAHMAN, S. M., MCCURDY, C. E., FRIEDERICH, M., VAN HOVE, J. L. K., WATSON, P. A., BIRDSEY, N., BAO, J., GIUS, D., SACK, M. N., JING, E., KAHN, C. R., FRIEDMAN, J. E. & JONSCHER, K. R. 2011. Fatty liver is associated with reduced SIRT3 activity and mitochondrial protein hyperacetylation. *Biochemical Journal*, 433, 505-514.
- KESHELAVA, N., SEEGER, R. C., GROSHEN, S. & REYNOLDS, C. P. 1998. Drug Resistance Patterns of Human Neuroblastoma Cell Lines Derived from Patients at Different Phases of Therapy. *Cancer Research*, 58, 5396-5405.
- KHARBANGAR, A., KHYNRIAM, D. & PRASAD, S. B. 2000. Effect of cisplatin on mitochondrial protein, glutathione, and succinate dehydrogenase in Dalton lymphoma-bearing mice. *Cell Biology and Toxicology*, 16, 363-373.
- KIM, J.-W., TCHERNYSHYOV, I., SEMENZA, G. L. & DANG, C. V. 2006. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. *Cell Metabolism*, 3, 177-185.
- KIM, J., LEE, J.-H. & IYER, V. R. 2008. Global Identification of Myc Target Genes Reveals Its Direct Role in Mitochondrial Biogenesis and Its E-Box Usage In Vivo. *PLoS ONE*, 3, e1798.
- KINCAID, B. & BOSSY-WETZEL, E. 2013. Forever young: SIRT3 a shield against mitochondrial meltdown, aging, and neurodegeneration. *Frontiers in Aging Neuroscience*, 5.

- KLUCK, R. M., BOSSY-WETZEL, E., GREEN, D. R. & NEWMAYER, D. D. 1997. The Release of Cytochrome c from Mitochondria: A Primary Site for Bcl-2 Regulation of Apoptosis. *Science*, 275, 1132-1136.
- KOJIMA, K., OHASHI, R., FUJITA, Y., HAMADA, N., AKAO, Y., NOZAWA, Y., DEGUCHI, T. & ITO, M. 2008. A role for SIRT1 in cell growth and chemoresistance in prostate cancer PC3 and DU145 cells. *Biochemical and Biophysical Research Communications*, 373, 423-428.
- KOMUROV, K., TSENG, J. T., MULLER, M., SEVIOUR, E. G., MOSS, T. J., YANG, L., NAGRATH, D. & RAM, P. T. 2012. The glucose-deprivation network counteracts lapatinib-induced toxicity in resistant ErbB2-positive breast cancer cells. *Molecular Systems Biology*, 8.
- KONG, X., WANG, R., XUE, Y., LIU, X., ZHANG, H., CHEN, Y., FANG, F. & CHANG, Y. 2010. Sirtuin 3, a New Target of PGC-1 α , Plays an Important Role in the Suppression of ROS and Mitochondrial Biogenesis. *PLoS ONE*, 5, e11707.
- KOVBASNJUK, O., MOURTAZINA, R., BAIBAKOV, B., WANG, T., ELOWSKY, C., CHOTI, M. A., KANE, A. & DONOWITZ, M. 2005. The glycosphingolipid globotriaosylceramide in the metastatic transformation of colon cancer. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 19087-19092.
- KRISHNAMURTHY, P. C., DU, G., FUKUDA, Y., SUN, D., SAMPATH, J., MERCER, K. E., WANG, J., SOSA-PINEDA, B., MURTI, K. G. & SCHUETZ, J. D. 2006. Identification of a mammalian mitochondrial porphyrin transporter. *Nature*, 443, 586-589.
- KUHAJDA, F. P. 2000. Fatty-acid synthase and human cancer: new perspectives on its role in tumor biology. *Nutrition*, 16, 202-208.
- KUNEJ, T., WANG, Z., MICHAL, J. J., DANIELS, T. F., MAGNUSON, N. S. & JIANG, Z. 2007. Functional UQCRC1 Polymorphisms Affect Promoter Activity and Body Lipid Accumulation. *Obesity*, 15, 2896-2901.
- KWAK, E. L., SORDELLA, R., BELL, D. W., GODIN-HEYMAN, N., OKIMOTO, R. A., BRANNIGAN, B. W., HARRIS, P. L., DRISCOLL, D. R., FIDIAS, P., LYNCH, T. J., RABINDRAN, S. K., MCGINNIS, J. P., WISSNER, A., SHARMA, S. V., ISSELBACHER, K. J., SETTLEMAN, J. & HABER, D. A. 2005. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. *Proc Natl Acad Sci U S A*, 102, 7665-70.
- KWONG, S. C. & BRUBACHER, J. 1998. Phenformin and lactic acidosis: a case report and review. *The Journal of Emergency Medicine*, 16, 881-886.
- LACASSE, E. C., BRAY, M. R., PATTERSON, B., LIM, W.-M., PERAMPALAM, S., RADVANYI, L. G., KEATING, A., STEWART, A. K., BUCKSTEIN, R., SANDHU, J. S., MILLER, N.,

- BANERJEE, D., SINGH, D., BELCH, A. R., PILARSKI, L. M. & GARIÉPY, J. 1999. Shiga-Like Toxin-1 Receptor on Human Breast Cancer, Lymphoma, and Myeloma and Absence From CD34+ Hematopoietic Stem Cells: Implications for Ex Vivo Tumor Purging and Autologous Stem Cell Transplantation. *Blood*, 94, 2901-2910.
- LAGOUGE, M., ARGMANN, C., GERHART-HINES, Z., MEZIANE, H., LERIN, C., DAUSSIN, F., MESSADEQ, N., MILNE, J., LAMBERT, P., ELLIOTT, P., GENY, B., LAAKSO, M., PUIGSERVER, P. & AUWERX, J. 2006. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell*, 127, 1109-22.
- LALA, P., ITO, S. & LINGWOOD, C. A. 2000. Retroviral Transfection of Madin-Darby Canine Kidney Cells with Human MDR1 Results in a Major Increase in Globotriaosylceramide and 105- to 106-Fold Increased Cell Sensitivity to Verocytotoxin: ROLE OF P-GLYCOPROTEIN IN GLYCOLIPID SYNTHESIS. *Journal of Biological Chemistry*, 275, 6246-6251.
- LE, A., LANE, ANDREW N., HAMAKER, M., BOSE, S., GOUW, A., BARBI, J., TSUKAMOTO, T., ROJAS, CAMILIO J., SLUSHER, BARBARA S., ZHANG, H., ZIMMERMAN, LISA J., LIEBLER, DANIEL C., SLEBOS, ROBERT J. C., LORKIEWICZ, PAWEŁ K., HIGASHI, RICHARD M., FAN, TERESA W. M. & DANG, CHI V. 2012. Glucose-Independent Glutamine Metabolism via TCA Cycling for Proliferation and Survival in B Cells. *Cell Metabolism*, 15, 110-121.
- LEE, TERENCE KIN W., CASTILHO, A., CHEUNG, VINCENT CHI H., TANG, KWAN H., MA, S. & NG, IRENE OI L. 2011. CD24+ Liver Tumor-Initiating Cells Drive Self-Renewal and Tumor Initiation through STAT3-Mediated NANOG Regulation. *Cell Stem Cell*, 9, 50-63.
- LEGASPI, A., JEEVANANDAM, M., STARNES JR, H. F. & BRENNAN, M. F. 1987. Whole body lipid and energy metabolism in the cancer patient. *Metabolism*, 36, 958-963.
- LENHARD, J. M., KIEWER, S. A., PAULIK, M. A., PLUNKET, K. D., LEHMANN, J. M. & WEIEL, J. E. 1997. Effects of troglitazone and metformin on glucose and lipid metabolism. Alterations of two distinct molecular pathways. *Biochemical Pharmacology*, 54, 801-808.
- LENNON, J. C., BRIGHT, S. A., CARROLL, E., BUTINI, S., CAMPIANI, G., O'MEARA, A., WILLIAMS, D. C. & ZISTERER, D. M. 2014. The novel pyrrolo-1,5-benzoxazepine, PBOX-6, synergistically enhances the apoptotic effects of carboplatin in drug sensitive and multidrug resistant neuroblastoma cells. *Biochemical Pharmacology*, 87, 611-624.
- LEVINE, A. J. & PUZIO-KUTER, A. M. 2010. The Control of the Metabolic Switch in Cancers by Oncogenes and Tumor Suppressor Genes. *Science*, 330, 1340-1344.

- LI, F., WANG, Y., ZELLER, K. I., POTTER, J. J., WONSEY, D. R., O'DONNELL, K. A., KIM, J.-W., YUSTEIN, J. T., LEE, L. A. & DANG, C. V. 2005. Myc Stimulates Nuclearly Encoded Mitochondrial Genes and Mitochondrial Biogenesis. *Molecular and Cellular Biology*, 25, 6225-6234.
- LI, S., BANCK, M., MUJTABA, S., ZHOU, M.-M., SUGRUE, M. M. & WALSH, M. J. 2010. p53-Induced Growth Arrest Is Regulated by the Mitochondrial SirT3 Deacetylase. *PLoS ONE*, 5, e10486.
- LI, X., FANG, P., MAI, J., CHOI, E. T., WANG, H. & YANG, X.-F. 2013. Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers. *Journal of Hematology & Oncology*, 6, 19-19.
- LIANG, B. C. & ULLYATT, E. 1998. Increased sensitivity to cis-diamminedichloroplatinum induced apoptosis with mitochondrial DNA depletion. *Cell Death Differ*, 5, 694-701.
- LIANG, X.-J., FINKEL, T., SHEN, D.-W., YIN, J.-J., ASZALOS, A. & GOTTESMAN, M. M. 2008. SIRT1 Contributes in Part to Cisplatin Resistance in Cancer Cells by Altering Mitochondrial Metabolism. *Molecular Cancer Research*, 6, 1499-1506.
- LIM, K. J., BISHT, S., BAR, E. E., MAITRA, A. & EBERHART, C. G. 2011. A polymeric nanoparticle formulation of curcumin inhibits growth, clonogenicity and stem-like fraction in malignant brain tumors. *Cancer Biology & Therapy*, 11, 464-473.
- LIN, X., OKUDA, T., HOLZER, A. & HOWELL, S. B. 2002. The Copper Transporter CTR1 Regulates Cisplatin Uptake in *Saccharomyces cerevisiae*. *Molecular Pharmacology*, 62, 1154-1159.
- LINDAHL, T., SATOH, M. S., POIRIER, G. G. & KLUNGLAND, A. 1995. Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. *Trends in Biochemical Sciences*, 20, 405-411.
- LINGWOOD, C. A., KHINE, A. A. & ARAB, S. 1998. Globotriaosyl ceramide (Gb3) expression in human tumour cells: intracellular trafficking defines a new retrograde transport pathway from the cell surface to the nucleus, which correlates with sensitivity to verotoxin. *Acta Biochim Pol*, 45, 351-9.
- LIU, G.-Y. & STORZ, P. 2010. Reactive oxygen species in cancer. *Free Radical Research*, 44, 10.3109/10715761003667554.
- LIU, B., FAN, Z., EDGERTON, S. M., DENG, X.-S., ALIMOVA, I. N., LIND, S. E. & THOR, A. D. 2009. Metformin induces unique biological and molecular responses in triple negative breast cancer cells. *Cell Cycle*, 8, 2031-2040.

- LIU, Y.-Y., GUPTA, V., PATWARDHAN, G., BHINGE, K., ZHAO, Y., BAO, J., MEHENDALE, H., CABOT, M., LI, Y.-T. & JAZWINSKI, S. M. 2010. Glucosylceramide synthase upregulates MDR1 expression in the regulation of cancer drug resistance through cSrc and beta-catenin signaling. *Molecular Cancer*, 9, 145.
- LOCASALE, J. W. 2012. Metabolic rewiring drives resistance to targeted cancer therapy. *Molecular Systems Biology*, 8.
- LOCASALE, J. W., CANTLEY, L. C. & HEIDEN, M. G. V. 2009. Cancer's insatiable appetite. *Nat Biotech*, 27, 916-917.
- LOMBARD, D. B., ALT, F. W., CHENG, H.-L., BUNKENBORG, J., STREEPER, R. S., MOSTOSLAVSKY, R., KIM, J., YANCOPOULOS, G., VALENZUELA, D., MURPHY, A., YANG, Y., CHEN, Y., HIRSCHHEY, M. D., BRONSON, R. T., HAIGIS, M., GUARENTE, L. P., FARESE, R. V., WEISSMAN, S., VERDIN, E. & SCHWER, B. 2007. Mammalian Sir2 Homolog SIRT3 Regulates Global Mitochondrial Lysine Acetylation. *Molecular and Cellular Biology*, 27, 8807-8814.
- LOMBARDO, Y., SCOPELLITI, A., CAMMARERI, P., TODARO, M., IOVINO, F., RICCI-VITIANI, L., GULOTTA, G., DIELI, F., DE MARIA, R. & STASSI, G. 2011. Bone Morphogenetic Protein 4 Induces Differentiation of Colorectal Cancer Stem Cells and Increases Their Response to Chemotherapy in Mice. *Gastroenterology*, 140, 297-309.e6.
- LONARDO, E., HERMANN, PATRICK C., MUELLER, M.-T., HUBER, S., BALIC, A., MIRANDA-LORENZO, I., ZAGORAC, S., ALCALA, S., RODRIGUEZ-ARABAOLAZA, I., RAMIREZ, JUAN C., TORRES-RUÍZ, R., GARCIA, E., HIDALGO, M., CEBRIÁN, DAVID Á., HEUCHEL, R., LÖHR, M., BERGER, F., BARTENSTEIN, P., AICHER, A. & HEESCHEN, C. 2011. Nodal/Activin Signaling Drives Self-Renewal and Tumorigenicity of Pancreatic Cancer Stem Cells and Provides a Target for Combined Drug Therapy. *Cell Stem Cell*, 9, 433-446.
- LONGLEY, D. B. & JOHNSTON, P. G. 2005. Molecular mechanisms of drug resistance. *The Journal of Pathology*, 205, 275-292.
- LUO, Z., ZANG, M. & GUO, W. 2010. AMPK as a metabolic tumor suppressor: control of metabolism and cell growth. *Future Oncology*, 6, 457-470.
- MACCARRONE, M., LORENZON, T., GUERRIERI, P. & AGRÒ, A. F. 1999. Resveratrol prevents apoptosis in K562 cells by inhibiting lipoxygenase and cyclooxygenase activity. *European Journal of Biochemistry*, 265, 27-34.
- MAIER-SALAMON, A., HAGENAUER, B., WIRTH, M., GABOR, F., SZEKERES, T. & JÄGER, W. 2006. Increased Transport of Resveratrol Across Monolayers of the Human Intestinal Caco-2 Cells is Mediated by Inhibition and Saturation of Metabolites. *Pharmaceutical Research*, 23, 2107-2115.

- MAJNO, G. & JORIS, I. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol*, 146, 3-15.
- MALKI A, Y. A. 2011. Antidiabetic drug metformin induces apoptosis in human MCF breast cancer via targeting ERK signaling. *Oncol Res*, 19(6), 275-85.
- MARKLUND, S. L., WESTMAN, N. G., LUNDGREN, E. & ROOS, G. 1982. Copper- and Zinc-containing Superoxide Dismutase, Manganese-containing Superoxide Dismutase, Catalase, and Glutathione Peroxidase in Normal and Neoplastic Human Cell Lines and Normal Human Tissues. *Cancer Research*, 42, 1955-1961.
- MARKS, D. I. & FOX, R. M. 1991. DNA damage, poly (ADP-ribosyl)ation and apoptotic cell death as a potential common pathway of cytotoxic drug action. *Biochemical Pharmacology*, 42, 1859-1867.
- MARSIN, A. S., BERTRAND†, L., RIDER, M. H., DEPREZ, J., BEAULOYE, C., VINCENT‡, M. F., VAN DEN BERGHE‡, G., CARLING, D. & HUE, L. 2000. Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Current Biology*, 10, 1247-1255.
- MARTIN, M. & MARAIS, R. 2012. Metformin: A Diabetes Drug for Cancer, or a Cancer Drug for Diabetics? *Journal of Clinical Oncology*, 30, 2698-2700.
- MCARDLE, P. A., MIR, K., ALMUSHATAT, A. S. K., WALLACE, A. M., UNDERWOOD, M. A. & MCMILLAN, D. C. 2006. Systemic Inflammatory Response, Prostate-Specific Antigen and Survival in Patients with Metastatic Prostate Cancer. *Urologia Internationalis*, 77, 127-129.
- MCGLYNN, L. M., MCCLUNEY, S., JAMIESON, N. B., THOMSON, J., MACDONALD, A. I., OIEN, K., DICKSON, E. J., CARTER, C. R., MCKAY, C. J. & SHIELS, P. G. 2015. SIRT3 & SIRT7: Potential Novel Biomarkers for Determining Outcome in Pancreatic Cancer Patients. *PLoS ONE*, 10, e0131344.
- MCMILLAN, D. C., CANNA, K. & MCARDLE, C. S. 2003. Systemic inflammatory response predicts survival following curative resection of colorectal cancer. *British Journal of Surgery*, 90, 215-219.
- MENG, X., LI, M., WANG, X., WANG, Y. & MA, D. 2009. Both CD133+ and CD133- subpopulations of A549 and H446 cells contain cancer-initiating cells. *Cancer Science*, 100, 1040-1046.
- MENZIES, K. J., SINGH, K., SALEEM, A. & HOOD, D. A. 2013. Sirtuin 1-mediated Effects of Exercise and Resveratrol on Mitochondrial Biogenesis. *The Journal of Biological Chemistry*, 288, 6968-6979.

- MERCIER, I., CASIMIRO, M. C., WANG, C., ROSENBERG, A. L., QUONG, J., MINKEU, A., ALLEN, K. G., DANILO, C., SOTGIA, F., BONUCCELLI, G., JASMIN, J.-F., XU, H., BOSCO, E., ARONOW, B., WITKIEWICZ, A. K., PESTELL, R. G., KNUDSEN, E. S. & LISANTI, M. P. 2008. Human breast cancer-associated fibroblasts (CAFs) show caveolin-1 down-regulation and RB tumor suppressor functional inactivation: Implications for the response to hormonal therapy. *Cancer Biology & Therapy*, 7, 1212-1225.
- METALLO, C. M., GAMEIRO, P. A., BELL, E. L., MATTAINI, K. R., YANG, J., HILLER, K., JEWELL, C. M., JOHNSON, Z. R., IRVINE, D. J., GUARENTE, L., KELLEHER, J. K., VANDER HEIDEN, M. G., ILIOPOULOS, O. & STEPHANOPOULOS, G. 2012. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature*, 481, 380-384.
- MICHELS, J., VITALE, I., GALLUZZI, L., ADAM, J., OLAUSSEN, K. A., KEPP, O., SENOVILLA, L., TALHAOU, I., GUEGAN, J., ENOT, D. P., TALBOT, M., ROBIN, A., GIRARD, P., ORÉAR, C., LISSA, D., SUKKURWALA, A. Q., GARCIA, P., BEHNAM-MOTLAGH, P., KOHNO, K., WU, G. S., BRENNER, C., DESSEN, P., SAPARBAEV, M., SORIA, J.-C., CASTEDO, M. & KROEMER, G. 2013. Cisplatin Resistance Associated with PARP Hyperactivation. *Cancer Research*, 73, 2271-2280.
- MIRANDA-LORENZO, I., DORADO, J., LONARDO, E., ALCALA, S., SERRANO, A. G., CLAUSELL-TORMOS, J., CIOFFI, M., MEGIAS, D., ZAGORAC, S., BALIC, A., HIDALGO, M., ERKAN, M., KLEEFF, J., SCARPA, A., SAINZ JR, B. & HEESCHEN, C. 2014. Intracellular autofluorescence: a biomarker for epithelial cancer stem cells. *Nat Meth*, 11, 1161-1169.
- MISKIMINS, W. K., AHN, H. J., KIM, J. Y., RYU, S., JUNG, Y.-S. & CHOI, J. Y. 2014. Synergistic Anti-Cancer Effect of Phenformin and Oxamate. *PLoS ONE*, 9, e85576.
- MORIGI, M., PERICO, L., ROTA, C., LONGARETTI, L., CONTI, S., ROTTOLI, D., NOVELLI, R., REMUZZI, G. & BENIGNI, A. 2015. Sirtuin 3-dependent mitochondrial dynamic improvements protect against acute kidney injury. *J Clin Invest*, 125, 715-26.
- MORIN-PAPUNEN, L., RAUTIO, K., RUOKONEN, A., HEDBERG, P., PUUKKA, M. & TAPANAINEN, J. S. 2003. Metformin Reduces Serum C-Reactive Protein Levels in Women with Polycystic Ovary Syndrome. *Journal of Clinical Endocrinology & Metabolism*, 88, 4649-4654.
- MOROZOV, Y. I., AGARONYAN, K., CHEUNG, A. C. M., ANIKIN, M., CRAMER, P. & TEMIAKOV, D. 2014. A novel intermediate in transcription initiation by human mitochondrial RNA polymerase. *Nucleic Acids Research*, 42, 3884-3893.
- MORRIS, B. J. 2013. Seven sirtuins for seven deadly diseases of aging. *Free Radical Biology and Medicine*, 56, 133-171.

- MULLEN, A. R., WHEATON, W. W., JIN, E. S., CHEN, P.-H., SULLIVAN, L. B., CHENG, T., YANG, Y., LINEHAN, W. M., CHANDEL, N. S. & DEBERARDINIS, R. J. 2012. Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature*, 481, 385-388.
- MURATA, T., HIBASAMI, H., MAEKAWA, S., TAGAWA, T. & NAKASHIMA, K. 1990. Preferential binding of cisplatin to mitochondrial DNA and suppression of ATP generation in human malignant melanoma cells. *Biochem Int*, 20, 949-55.
- NESSA, M. U., BEALE, P., CHAN, C., YU, J. Q. & HUQ, F. 2012. Combinations of Resveratrol, Cisplatin and Oxaliplatin Applied to Human Ovarian Cancer Cells. *Anticancer Research*, 32, 53-59.
- NGO, H. B., LOVELY, G. A., PHILLIPS, R. & CHAN, D. C. 2014. Distinct structural features of TFAM drive mitochondrial DNA packaging versus transcriptional activation. *Nat Commun*, 5.
- NGUYEN, L. V., VANNER, R., DIRKS, P. & EAVES, C. J. 2012. Cancer stem cells: an evolving concept. *Nat Rev Cancer*, 12, 133-143.
- NICHENAMETLA, S. N., TARUSCIO, T. G., BARNEY, D. L. & EXON, J. H. 2006. A Review of the Effects and Mechanisms of Polyphenolics in Cancer. *Critical Reviews in Food Science and Nutrition*, 46, 161-183.
- OGINO, A., KITAO, H., HIRANO, S., UCHIDA, A., ISHIAI, M., KOZUKI, T., TAKIGAWA, N., TAKATA, M., KIURA, K. & TANIMOTO, M. 2007. Emergence of Epidermal Growth Factor Receptor T790M Mutation during Chronic Exposure to Gefitinib in a Non-Small Cell Lung Cancer Cell Line. *Cancer Research*, 67, 7807-7814.
- OLAS, B. & WACHOWICZ, B. 2002. Resveratrol and vitamin C as antioxidants in blood platelets. *Thrombosis Research*, 106, 143-148.
- OLIVA, C. R., MOELLERING, D. R., GILLESPIE, G. Y. & GRIGUER, C. E. 2011. Acquisition of Chemoresistance in Gliomas Is Associated with Increased Mitochondrial Coupling and Decreased ROS Production. *PLoS ONE*, 6, e24665.
- OLIVERO, O. A., SEMINO, C., KASSIM, A., LOPEZ-LARRAZA, D. M. & POIRIER, M. C. 1995. Preferential binding of cisplatin to mitochondrial DNA of Chinese hamster ovary cells. *Mutation Research Letters*, 346, 221-230.
- ONISHI, Y., UEHA, T., KAWAMOTO, T., HARA, H., TODA, M., HARADA, R., MINODA, M., KUROSAKA, M. & AKISUE, T. 2014. Regulation of Mitochondrial Proliferation by PGC-1 α Induces Cellular Apoptosis in Musculoskeletal Malignancies. *Sci. Rep.*, 4.
- OSMAN, A.-M., TELITY, S., DAMANHOURI, Z., AL-HARTHY, S., AL-KREATHY, H., RAMADAN, W., ELSHAL, M., KHAN, L. & KAMEL, F. 2015. Chemosensitizing and

- nephroprotective effect of resveratrol in cisplatin -treated animals. *Cancer Cell International*, 15, 6.
- OWEN, M. R., DORAN, E. & HALESTRAP, A. P. 2000. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem. J*, 607–614.
- PALACIOS, O. M., CARMONA, J. J., MICHAN, S., CHEN, K. Y., MANABE, Y., III, J. L. W., GOODYEAR, L. J. & TONG, Q. 2009. Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1 α in skeletal muscle. *Aging (Albany NY)*, 1, 771-783.
- PAO, W., MILLER, V. A., POLITI, K. A., RIELY, G. J., SOMWAR, R., ZAKOWSKI, M. F., KRIS, M. G. & VARMUS, H. 2005. Acquired Resistance of Lung Adenocarcinomas to Gefitinib or Erlotinib Is Associated with a Second Mutation in the EGFR Kinase Domain. *PLoS Med*, 2, e73.
- PARK, M. S., DE LEON, M. & DEVARAJAN, P. 2002. Cisplatin Induces Apoptosis in LLC-PK1 Cells via Activation of Mitochondrial Pathways. *Journal of the American Society of Nephrology*, 13, 858-865.
- PARK, S. Y., CHANG, I., KIM, J.-Y., KANG, S. W., PARK, S.-H., SINGH, K. & LEE, M.-S. 2004. Resistance of Mitochondrial DNA-depleted Cells against Cell Death: ROLE OF MITOCHONDRIAL SUPEROXIDE DISMUTASE. *Journal of Biological Chemistry*, 279, 7512-7520.
- PATEL, K. R., SCOTT, E., BROWN, V. A., GESCHER, A. J., STEWARD, W. P. & BROWN, K. 2011. Clinical trials of resveratrol. *Annals of the New York Academy of Sciences*, 1215, 161-169.
- PATERSON, J. K., SZAKACS, G., TACHIWADA, T., AKIYAMA, S.-I. & GOTTESMAN, M. M. 2005. The role of ABCB6 in multidrug resistance. *Cancer Research*, 65, 122.
- PAVLIDES, S., WHITAKER-MENEZES, D., CASTELLO-CROS, R., FLOMENBERG, N., WITKIEWICZ, A. K., FRANK, P. G., CASIMIRO, M. C., WANG, C., FORTINA, P., ADDYA, S., PESTELL, R. G., MARTINEZ-OUTSCHOORN, U. E., SOTGIA, F. & LISANTI, M. P. 2009. The reverse Warburg effect: Aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. *Cell Cycle*, 8, 3984-4001.
- PELICANO, H., CARNEY, D. & HUANG, P. 2004. ROS stress in cancer cells and therapeutic implications. *Drug Resistance Updates*, 7, 97-110.
- PERRIELLO, G., MISERICORDIA, P., VOLPI, E., SANTUCCI, A., SANTUCCI, C., FERRANNINI, E., VENTURA, M. M., SANTEUSANIO, F., BRUNETTI, P. & BOLLI, G. B. 1994. Acute antihyperglycemic mechanisms of metformin in NIDDM: Evidence for suppression of lipid oxidation and hepatic glucose production. *Diabetes*, 43, 920-928.

- PERVAIZ, S. 2003. Resveratrol: from grapevines to mammalian biology. *The FASEB Journal*, 17, 1975-1985.
- PILLAI, V. B., SUNDARESAN, N. R., KIM, G., GUPTA, M., RAJAMOHAN, S. B., PILLAI, J. B., SAMANT, S., RAVINDRA, P. V., ISBATAN, A. & GUPTA, M. P. 2010. Exogenous NAD Blocks Cardiac Hypertrophic Response via Activation of the SIRT3-LKB1-AMP-activated Kinase Pathway. *Journal of Biological Chemistry*, 285, 3133-3144.
- PIROLA, L. & FRÖJDÖ, S. 2008. Resveratrol: One molecule, many targets. *IUBMB Life*, 60, 323-332.
- PISAREV, A. V., SKABKIN, M. A., PISAREVA, V. P., SKABKINA, O. V., RAKOTONDRAFARA, A. M., HENTZE, M. W., HELLEN, C. U. T. & PESTOVA, T. V. 2010. The role of ABCE1 in eukaryotic post-termination ribosomal recycling. *Molecular Cell*, 37, 196-210.
- POTTER, V. R. 1958. The biochemical approach to the cancer problem. *Fed Proc*, 17, 691-7.
- PRESTON, T. J., ABADI, A., WILSON, L. & SINGH, G. 2001. Mitochondrial contributions to cancer cell physiology: potential for drug development. *Advanced Drug Delivery Reviews*, 49, 45-61.
- PRYDE, K. R. & HIRST, J. 2011. Superoxide Is Produced by the Reduced Flavin in Mitochondrial Complex I: A SINGLE, UNIFIED MECHANISM THAT APPLIES DURING BOTH FORWARD AND REVERSE ELECTRON TRANSFER. *Journal of Biological Chemistry*, 286, 18056-18065.
- PUZIO-KUTER, A. M. 2011. The Role of p53 in Metabolic Regulation. *Genes Cancer*, 2, 385-91.
- QIAN, W., NISHIKAWA, M., HAQUE, A. M., HIROSE, M., MASHIMO, M., SATO, E. & INOUE, M. 2005. Mitochondrial density determines the cellular sensitivity to cisplatin-induced cell death.
- QIU, X., BROWN, K., HIRSCHHEY, M. D., VERDIN, E. & CHEN, D. 2010. Calorie Restriction Reduces Oxidative Stress by SIRT3-Mediated SOD2 Activation. *Cell Metabolism*, 12, 662-667.
- QUINLAN, C. L., PEREVOSHCHIKOVA, I. V., HEY-MOGENSEN, M., ORR, A. L. & BRAND, M. D. 2013. Sites of reactive oxygen species generation by mitochondria oxidizing different substrates(). *Redox Biology*, 1, 304-312.
- RACKER, E. 1974. History of the Pasteur effect and its pathobiology. *Molecular and Cellular Biochemistry*, 5, 17-23.

- RATTAN, R., GRAHAM, R. P., MAGUIRE, J. L., GIRI, S. & SHRIDHAR, V. 2011. Metformin Suppresses Ovarian Cancer Growth and Metastasis with Enhancement of Cisplatin Cytotoxicity In Vivo. *Neoplasia*, 13, 483-IN28.
- RENAUD, S. & DE LORGERIL, M. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *The Lancet*, 339, 1523-1526.
- REZK, Y. A., BALULAD, S. S., KELLER, R. S. & BENNETT, J. A. 2006. Use of Resveratrol to improve the effectiveness of cisplatin and doxorubicin: Study in human gynecologic cancer cell lines and in rodent heart. *American Journal of Obstetrics and Gynecology*, 194, e23-e26.
- RICHTER, C., GOGVADZE, V., LAFFRANCHI, R., SCHLAPBACH, R., SCHWEIZER, M., SUTER, M., WALTER, P. & YAFFEE, M. 1995. Oxidants in mitochondria: From physiology to diseases. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 1271, 67-74.
- ROMERO-CANELON, I., PIZARRO, A. M., HABTEMARIAM, A. & SADLER, P. J. 2012. Contrasting cellular uptake pathways for chlorido and iodido iminopyridine ruthenium arene anticancer complexes. *Metallomics*, 4, 1271-9.
- ROS, S., SANTOS, C. R., MOCO, S., BAENKE, F., KELLY, G., HOWELL, M., ZAMBONI, N. & SCHULZE, A. 2012. Functional Metabolic Screen Identifies 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 4 as an Important Regulator of Prostate Cancer Cell Survival. *Cancer Discovery*, 2, 328-343.
- ROSENBERG, B. 1973. Platinum coordination complexes in cancer chemotherapy. *Naturwissenschaften*, 60, 399-406.
- ROTH, M. & CHEN, W. Y. 2014. Sorting out functions of sirtuins in cancer. *Oncogene*, 33, 1609-1620.
- RYAN, M. J., JACKSON, J. R., HAO, Y., WILLIAMSON, C. L., DABKOWSKI, E. R., HOLLANDER, J. M. & ALWAY, S. E. 2010. Suppression of Oxidative Stress by Resveratrol After Isometric Contractions in Gastrocnemius Muscles of Aged Mice. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 65A, 815-831.
- SAHRA, I. B., LAURENT, K., LOUBAT, A., GIORGETTI-PERALDI, S., COLOSETTI, P., AUBERGER, P., TANTI, J. F., LE MARCHAND-BRUSTEL, Y. & BOST, F. 2008. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene*, 27, 3576-3586.
- SAREEN, D., DARJATMOKO, S. R., ALBERT, D. M. & POLANS, A. S. 2007. Mitochondria, Calcium, and Calpain are Key Mediators of Resveratrol-Induced Apoptosis in Breast Cancer. *Molecular Pharmacology*, 72, 1466-1475.

- SAUNDERS, L. R. & VERDIN, E. 2007. Sirtuins: critical regulators at the crossroads between cancer and aging. *Oncogene*, 26, 5489-5504.
- SCARPULLA, R. C., VEGA, R. B. & KELLY, D. P. 2012. Transcriptional integration of mitochondrial biogenesis. *Trends in Endocrinology & Metabolism*, 23, 459-466.
- SCHAFER, M. & WERNER, S. 2008. Cancer as an overhealing wound: an old hypothesis revisited. *Nat Rev Mol Cell Biol*, 9, 628-638.
- SCHER, M. B., VAQUERO, A. & REINBERG, D. 2007. SirT3 is a nuclear NAD⁺-dependent histone deacetylase that translocates to the mitochondria upon cellular stress. *Genes & Development*, 21, 920-928.
- SCHLICKER, C., GERTZ, M., PAPATHEODOROU, P., KACHHOLZ, B., BECKER, C. F. W. & STEEGBORN, C. 2008. Substrates and Regulation Mechanisms for the Human Mitochondrial Sirtuins Sirt3 and Sirt5. *Journal of Molecular Biology*, 382, 790-801.
- SCHUMACKER, P. T. 2006. Reactive oxygen species in cancer cells: Live by the sword, die by the sword. *Cancer Cell*, 10, 175-176.
- SCHWERDT, G., FREUDINGER, R., SCHUSTER, C., SILBERNAGL, S. & GEKLE, M. 2003. Inhibition of mitochondria prevents cell death in kidney epithelial cells by intra- and extracellular acidification. *Kidney Int*, 63, 1725-1735.
- SCOTT, H. R., MCMILLAN, D. C., FORREST, L. M., BROWN, D. J. F., MCARDLE, C. S. & MILROY, R. 2002. The systemic inflammatory response, weight loss, performance status and survival in patients with inoperable non-small cell lung cancer. *Br J Cancer*, 87, 264-267.
- SEEGER, R. C. & REYNOLDS, C. P. 1991. Treatment of high-risk solid tumors of childhood with intensive therapy and autologous bone marrow transplantation. *Pediatric clinics of North America*, 38, 393-424.
- SEGAL, E. D., YASMEEN, A., BEAUCHAMP, M.-C., ROSENBLATT, J., POLLAK, M. & GOTLIEB, W. H. 2011. Relevance of the OCT1 transporter to the antineoplastic effect of biguanides. *Biochemical and Biophysical Research Communications*, 414, 694-699.
- SEMENZA, G. L. E. A. 2001. 'The metabolism of tumours': 70 years later. *Novartis Found. Symp.*, 240, 251-260.
- SENA, LAURA A. & CHANDEL, NAVDEEP S. 2012. Physiological Roles of Mitochondrial Reactive Oxygen Species. *Molecular Cell*, 48, 158-167.
- SEO, B. B., KITAJIMA-IHARA, T., CHAN, E. K. L., SCHEFFLER, I. E., MATSUNO-YAGI, A. & YAGI, T. 1998. Molecular remedy of complex I defects: Rotenone-insensitive

internal NADH-quinone oxidoreductase of *Saccharomyces cerevisiae* mitochondria restores the NADH oxidase activity of complex I-deficient mammalian cells. *Proceedings of the National Academy of Sciences*, 95, 9167-9171.

SHACKELFORD, DAVID B., ABT, E., GERKEN, L., VASQUEZ, DEBBIE S., SEKI, A., LEBLANC, M., WEI, L., FISHBEIN, MICHAEL C., CZERNIN, J., MISCHEL, PAUL S. & SHAW, REUBEN J. 2013. LKB1 Inactivation Dictates Therapeutic Response of Non-Small Cell Lung Cancer to the Metabolism Drug Phenformin. *Cancer Cell*, 23, 143-158.

SHACKELFORD, D. B. & SHAW, R. J. 2009. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. *Nat Rev Cancer*, 9, 563-575.

SHAW, R. J., BARDEESY, N., MANNING, B. D., LOPEZ, L., KOSMATKA, M., DEPINHO, R. A. & CANTLEY, L. C. 2004. The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell*, 6, 91-99.

SHEN, D.-W., PASTAN, I. & GOTTESMAN, M. M. 1998. Cross-Resistance to Methotrexate and Metals in Human Cisplatin-resistant Cell Lines Results from a Pleiotropic Defect in Accumulation of These Compounds Associated with Reduced Plasma Membrane Binding Proteins. *Cancer Research*, 58, 268-275.

SHEU, S.-J., LIU, N.-C., OU, C.-C., BEE, Y.-S., CHEN, S.-C., LIN, H.-C. & CHAN, J. Y. H. 2013. Resveratrol Stimulates Mitochondrial Bioenergetics to Protect Retinal Pigment Epithelial Cells From Oxidative Damage Mitochondrial Bioenergetics in Cytoprotection. *Investigative Ophthalmology & Visual Science*, 54, 6426-6438.

SHI, T., WANG, F., STIEREN, E. & TONG, Q. 2005. SIRT3, a Mitochondrial Sirtuin Deacetylase, Regulates Mitochondrial Function and Thermogenesis in Brown Adipocytes. *Journal of Biological Chemistry*, 280, 13560-13567.

SHIM, H., DOLDE, C., LEWIS, B. C., WU, C.-S., DANG, G., JUNGSMANN, R. A., DALLA-FAVERA, R. & DANG, C. V. 1997. c-Myc transactivation of LDH-A: Implications for tumor metabolism and growth. *Proceedings of the National Academy of Sciences*, 94, 6658-6663.

SIDDIK, Z. H. 2003. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene*, 22, 7265-7279.

SIDDIK, Z. H., MIMS, B., LOZANO, G. & THAI, G. 1998. Independent Pathways of p53 Induction by Cisplatin and X-Rays in a Cisplatin-resistant Ovarian Tumor Cell Line. *Cancer Research*, 58, 698-703.

SOLDANI, C., LAZZÈ, M. C., BOTTONE, M. G., TOGNON, G., BIGGIOGERA, M., PELLICCIARI, C. E. & SCOVASSI, A. I. 2001. Poly(ADP-ribose) Polymerase Cleavage during Apoptosis: When and Where? *Experimental Cell Research*, 269, 193-201.

- SOMEYA, S., YU, W., HALLOWS, W. C., XU, J., VANN, J. M., LEEUWENBURGH, C., TANOKURA, M., DENU, J. M. & PROLLA, T. A. 2010. Sirt3 Mediates Reduction of Oxidative Damage and Prevention of Age-Related Hearing Loss under Caloric Restriction. *Cell*, 143, 802-812.
- SONG, C. W., LEE, H., DINGS, R. P. M., WILLIAMS, B., POWERS, J., SANTOS, T. D., CHOI, B.-H. & PARK, H. J. 2012. Metformin kills and radiosensitizes cancer cells and preferentially kills cancer stem cells. *Sci. Rep.*, 2.
- STANIEK, K., GILLE, L., KOZLOV, A. V. & NOHL, H. 2002. Mitochondrial superoxide radical formation is controlled by electron bifurcation to the high and low potential pathways. *Free Radical Research*, 36, 381-387.
- STEINBERG, G. R. & KEMP, B. E. 2009. AMPK in Health and Disease. *Physiol Rev*, 89, 1025-78.
- STEWART, D. J. 2007. Mechanisms of resistance to cisplatin and carboplatin. *Critical Reviews in Oncology/Hematology*, 63, 12-31.
- SU, Y., QIU, Q., ZHANG, X., JIANG, Z., LENG, Q., LIU, Z., STASS, S. A. & JIANG, F. 2010. ALDH1A1 Positive Cell Population Is Enriched in Tumor-initiating Cells and Associated with Progression of Bladder Cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, 19, 327-337.
- SUBRAMANIAN, L., YOUSSEF, S., BHATTACHARYA, S., KENEALEY, J., POLANS, A. S. & VAN GINKEL, P. R. 2010. Resveratrol: Challenges in Translation to the Clinic — A Critical Discussion. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 16, 5942-5948.
- SULLIVAN, E., KURTOGLU, M., BRENNEMAN, R., LIU, H. & LAMPIDIS, T. 2014. Targeting cisplatin-resistant human tumor cells with metabolic inhibitors. *Cancer Chemotherapy and Pharmacology*, 73, 417-427.
- SUNDARESAN, N. R., GUPTA, M., KIM, G., RAJAMOHAN, S. B., ISBATAN, A. & GUPTA, M. P. 2009. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. *J Clin Invest*, 119, 2758-2771.
- SUNDARESAN, N. R., SAMANT, S. A., PILLAI, V. B., RAJAMOHAN, S. B. & GUPTA, M. P. 2008. SIRT3 Is a Stress-Responsive Deacetylase in Cardiomyocytes That Protects Cells from Stress-Mediated Cell Death by Deacetylation of Ku70. *Molecular and Cellular Biology*, 28, 6384-6401.

- SWARTZ, M. A., IIDA, N., ROBERTS, E. W., SANGALETTI, S., WONG, M. H., YULL, F. E., COUSSENS, L. M. & DECLERCK, Y. A. 2012. Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy. *Cancer Research*, 72, 2473-2480.
- SZATROWSKI, T. P. & NATHAN, C. F. 1991. Production of Large Amounts of Hydrogen Peroxide by Human Tumor Cells. *Cancer Research*, 51, 794-798.
- TAYLOR, D. M., MAXWELL, M. M., LUTHI-CARTER, R. & KAZANTSEV, A. G. 2008. Biological and Potential Therapeutic Roles of Sirtuin Deacetylases. *Cellular and Molecular Life Sciences*, 65, 4000-4018.
- TEICHER, B. A., HOLDEN, S. A., KELLEY, M. J., SHEA, T. C., CUCCHI, C. A., ROSOWSKY, A., HENNER, W. D. & FREI, E. 1987. Characterization of a Human Squamous Carcinoma Cell Line Resistant to cis-Diamminedichloroplatinum(II). *Cancer Research*, 47, 388-393.
- THOMASSET, S. C., BERRY, D. P., GARCEA, G., MARCZYLO, T., STEWARD, W. P. & GESCHER, A. J. 2007. Dietary polyphenolic phytochemicals—promising cancer chemopreventive agents in humans? A review of their clinical properties. *International Journal of Cancer*, 120, 451-458.
- THOMSON, D. M., HERWAY, S. T., FILLMORE, N., KIM, H., BROWN, J. D., BARROW, J. R. & WINDER, W. W. 2008. AMP-activated protein kinase phosphorylates transcription factors of the CREB family. *Journal of Applied Physiology*, 104, 429-438.
- TIVNAN, A., TRACEY, L., BUCKLEY, P., ALCOCK, L., DAVIDOFF, A. & STALLINGS, R. 2011. MicroRNA-34a is a potent tumor suppressor molecule in vivo in neuroblastoma. *BMC Cancer*, 11, 33.
- UNGVARI, Z., OROSZ, Z., RIVERA, A., LABINSKY, N., XIANGMIN, Z., OLSON, S., PODLUTSKY, A. & CSISZAR, A. 2007. Resveratrol increases vascular oxidative stress resistance. *American Journal of Physiology - Heart and Circulatory Physiology*, 292, H2417-H2424.
- VALENT, P., BONNET, D., DE MARIA, R., LAPIDOT, T., COPLAND, M., MELO, J. V., CHOMIENNE, C., ISHIKAWA, F., SCHURINGA, J. J., STASSI, G., HUNTLY, B., HERRMANN, H., SOULIER, J., ROESCH, A., SCHUURHUIS, G. J., WOHRER, S., AROCK, M., ZUBER, J., CERNY-REITERER, S., JOHNSEN, H. E., ANDREEFF, M. & EAVES, C. 2012. Cancer stem cell definitions and terminology: the devil is in the details. *Nat Rev Cancer*, 12, 767-775.
- VANDER HEIDEN, M. G., CANTLEY, L. C. & THOMPSON, C. B. 2009. Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science*, 324, 1029-1033.

- VASILIOU, V., PAPPA, A. & PETERSEN, D. R. 2000. Role of aldehyde dehydrogenases in endogenous and xenobiotic metabolism. *Chemico-Biological Interactions*, 129, 1-19.
- VELDMAN, R. J., Klappe, K., HINRICHS, J., HUMMEL, I., VAN DER SCHAAF, G., SIETSMA, H. & KOK, J. W. 2002. Altered sphingolipid metabolism in multidrug-resistant ovarian cancer cells is due to uncoupling of glycolipid biosynthesis in the Golgi apparatus. *The FASEB Journal*.
- VIGNERI, R., MADDUX, B. & GOLDFINE, I. D. 1984. The effect of phenformin and other adenosine triphosphate (ATP)-lowering agents on insulin binding to IM-9 human cultured lymphocytes. *Journal of Cellular Biochemistry*, 24, 177-186.
- VINOGRADOV, S. & WEI, X. 2012. Cancer stem cells and drug resistance: the potential of nanomedicine. *Nanomedicine (London, England)*, 7, 597-615.
- WANG, C.-H., WU, S.-B., WU, Y.-T. & WEI, Y.-H. 2013. Oxidative stress response elicited by mitochondrial dysfunction: Implication in the pathophysiology of aging. *Experimental Biology and Medicine*, 238, 450-460.
- WARBURG, O. 1930. Ueber den stoffwechsel der tumoren. *Constable, London*.
- WATKINS, G., DOUGLAS-JONES, A., MANSEL, R. E. & JIANG, W. G. 2004. The localisation and reduction of nuclear staining of PPARGamma and PGC-1 in human breast cancer. *Oncol Rep*, 12, 483-8.
- WENZ, T. 2013. Regulation of mitochondrial biogenesis and PGC-1 α under cellular stress. *Mitochondrion*, 13, 134-142.
- WHEATON, W. W., WEINBERG, S. E., HAMANAKA, R. B., SOBERANES, S., SULLIVAN, L. B., ANSO, E., GLASAUER, A., DUFOUR, E., MUTLU, G. M., BUDIGNER, G. S. & CHANDEL, N. S. 2014. *Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis*.
- WILCOCK, C. & BAILEY, C. J. 1994. Accumulation of metformin by tissues of the normal and diabetic mouse. *Xenobiotica*, 24, 49-57.
- WINDER, W. W., WILSON, H. A., HARDIE, D. G., RASMUSSEN, B. B., HUTBER, C. A., CALL, G. B., CLAYTON, R. D., CONLEY, L. M., YOON, S. & ZHOU, B. 1997. Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. *Journal of Applied Physiology*, 82, 219-225.
- WISE, D. R., DEBERARDINIS, R. J., MANCUSO, A., SAYED, N., ZHANG, X.-Y., PFEIFFER, H. K., NISSIM, I., DAIKHIN, E., YUDKOFF, M., MCMAHON, S. B. & THOMPSON, C. B. 2008. Myc regulates a transcriptional program that stimulates mitochondrial

- glutaminolysis and leads to glutamine addiction. *Proceedings of the National Academy of Sciences*, 105, 18782-18787.
- WITKIEWICZ, A. K., DASGUPTA, A., SOTGIA, F., MERCIER, I., PESTELL, R. G., SABEL, M., KLEER, C. G., BRODY, J. R. & LISANTI, M. P. 2009. An Absence of Stromal Caveolin-1 Expression Predicts Early Tumor Recurrence and Poor Clinical Outcome in Human Breast Cancers. *The American Journal of Pathology*, 174, 2023-2034.
- WU, M. 2007. *Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells.*
- WU, M., NEILSON, A., SWIFT, A. L., MORAN, R., TAMAGNINE, J., PARLOW, D., ARMISTEAD, S., LEMIRE, K., ORRELL, J., TEICH, J., CHOMICZ, S. & FERRICK, D. A. 2007. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *American Journal of Physiology - Cell Physiology*, 292, C125-C136.
- WÜRTH, R., PATTAROZZI, A., GATTI, M., BAJETTO, A., CORSARO, A., PARODI, A., SIRITO, R., MASSOLLO, M., MARINI, C., ZONA, G., FENOGLIO, D., SAMBUCETI, G., FILACI, G., DAGA, A., BARBIERI, F. & FLORIO, T. 2013. Metformin selectively affects human glioblastoma tumor-initiating cell viability: A role for metformin-induced inhibition of Akt. *Cell Cycle*, 12, 145-156.
- XUN, Z., LEE, D.-Y., LIM, J., CANARIA, C. A., BARNEBEY, A., YANONNE, S. M. & MCMURRAY, C. T. 2012. Retinoic acid-induced differentiation increases the rate of oxygen consumption and enhances the spare respiratory capacity of mitochondria in SH-SY5Y cells. *Mechanisms of Ageing and Development*, 133, 176-185.
- YALCIN, A., TELANG, S., CLEM, B. & CHESNEY, J. 2009. Regulation of glucose metabolism by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases in cancer. *Experimental and Molecular Pathology*, 86, 174-179.
- YANEZ, J. A., TENG, X. W., ROUPE, K. A., FARISS, M. W. & DAVIES, N. M. 2003. Chemotherapy induced gastrointestinal toxicity in rats: involvement of mitochondrial DNA, gastrointestinal permeability and cyclooxygenase -2. *J Pharm Pharm Sci*, 6, 308-14.
- YANG, H., YANG, T., BAUR, J. A., PEREZ, E., MATSUI, T., CARMONA, J. J., LAMMING, DUDLEY W., SOUZA-PINTO, N. C., BOHR, V. A., ROSENZWEIG, A., DE CABO, R., SAUVE, ANTHONY A. & SINCLAIR, D. A. 2007. Nutrient-Sensitive Mitochondrial NAD⁺ Levels Dictate Cell Survival. *Cell*, 130, 1095-1107.

- YANG, Y., HUBBARD, B. P., SINCLAIR, D. A. & TONG, Q. 2010. Characterization of Murine SIRT3 Transcript Variants and Corresponding Protein Products. *Journal of Cellular Biochemistry*, 111, 1051-1058.
- YANG, Z., SCHUMAKER, L. M., EGORIN, M. J., ZUHOWSKI, E. G., GUO, Z. & CULLEN, K. J. 2006. Cisplatin Preferentially Binds Mitochondrial DNA and Voltage-Dependent Anion Channel Protein in the Mitochondrial Membrane of Head and Neck Squamous Cell Carcinoma: Possible Role in Apoptosis. *Clinical Cancer Research*, 12, 5817-5825.
- YAO, Z., JONES, A. W. E., FASSONE, E., SWEENEY, M. G., LEBIEDZINSKA, M., SUSKI, J. M., WIECKOWSKI, M. R., TAJEDDINE, N., HARGREAVES, I. P., YASUKAWA, T., TUFO, G., BRENNER, C., KROEMER, G., RAHMAN, S. & SZABADKAI, G. 2013. PGC-1[β] mediates adaptive chemoresistance associated with mitochondrial DNA mutations. *Oncogene*, 32, 2592-2600.
- YASMEEN, A., BEAUCHAMP, M.-C., PIURA, E., SEGAL, E., POLLAK, M. & GOTLIEB, W. H. 2011. Induction of apoptosis by metformin in epithelial ovarian cancer: Involvement of the Bcl-2 family proteins. *Gynecologic Oncology*, 121, 492-498.
- YU, F., YAO, H., ZHU, P., ZHANG, X., PAN, Q., GONG, C., HUANG, Y., HU, X., SU, F., LIEBERMAN, J. & SONG, E. 2007. let-7 Regulates Self Renewal and Tumorigenicity of Breast Cancer Cells. *Cell*, 131, 1109-1123.
- YUAN, P., ITO, K., PEREZ-LORENZO, R., DEL GUZZO, C., LEE, J. H., SHEN, C.-H., BOSENBERG, M. W., MCMAHON, M., CANTLEY, L. C. & ZHENG, B. 2013. Phenformin enhances the therapeutic benefit of BRAFV600E inhibition in melanoma. *Proceedings of the National Academy of Sciences*, 110, 18226-18231.
- YUMUSAKHUYLU, A. C., YAZICI, M., SARI, M., BINNETOGLU, A., KOSEMIHAL, E., AKDAS, F., SIRVanci, S., YUKSEL, M., UNERI, C. & TUTKUN, A. 2012. Protective role of resveratrol against cisplatin induced ototoxicity in guinea pigs. *International Journal of Pediatric Otorhinolaryngology*, 76, 404-408.
- YUN, J., RAGO, C., CHEONG, I., PAGLIARINI, R., ANGENENDT, P., RAJAGOPALAN, H., SCHMIDT, K., WILLSON, J. K. V., MARKOWITZ, S., ZHOU, S., DIAZ, L. A., VELCULESCU, V. E., LENGAUER, C., KINZLER, K. W., VOGELSTEIN, B. & PAPADOPOULOS, N. 2009. Glucose Deprivation Contributes to the Development of KRAS Pathway Mutations in Tumor Cells. *Science*, 325, 1555-1559.
- ZHANG, H., GAO, P., FUKUDA, R., KUMAR, G., KRISHNAMACHARY, B., ZELLER, K. I., DANG, CHI V. & SEMENZA, G. L. 2007a. HIF-1 Inhibits Mitochondrial Biogenesis and Cellular Respiration in VHL-Deficient Renal Cell Carcinoma by Repression of C-MYC Activity. *Cancer Cell*, 11, 407-420.

- ZHANG, L., HE, H. & BALSCHI, J. A. 2007b. Metformin and phenformin activate AMP-activated protein kinase in the heart by increasing cytosolic AMP concentration. *American Journal of Physiology - Heart and Circulatory Physiology*, 293, H457-H466.
- ZHENG, J., CHEN, L.-L., ZHANG, H.-H., HU, X., KONG, W. & HU, D. 2012. Resveratrol improves insulin resistance of catch-up growth by increasing mitochondrial complexes and antioxidant function in skeletal muscle. *Metabolism*, 61, 954-965.
- ZHOU, S.-F. 2008. Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica*, 38, 802-832.
- ZHOU, S., SCHUETZ, J. D., BUNTING, K. D., COLAPIETRO, A.-M., SAMPATH, J., MORRIS, J. J., LAGUTINA, I., GROSVELD, G. C., OSAWA, M., NAKAUCHI, H. & SORRENTINO, B. P. 2001. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med*, 7, 1028-1034.
- ZHU, A., LEE, D. & SHIM, H. 2011. Metabolic Positron Emission Tomography Imaging in Cancer Detection and Therapy Response. *Seminars in Oncology*, 38, 55-69.
- ZONG, H., REN, J. M., YOUNG, L. H., PYPAERT, M., MU, J., BIRNBAUM, M. J. & SHULMAN, G. I. 2002. AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 15983-15987.
- ZONG, W.-X. & THOMPSON, C. B. 2006. Necrotic death as a cell fate. *Genes & Development*, 20, 1-15.
- ZOU, M.-H., KIRKPATRICK, S. S., DAVIS, B. J., NELSON, J. S., WILES, W. G., SCHLATTNER, U., NEUMANN, D., BROWNLIE, M., FREEMAN, M. B. & GOLDMAN, M. H. 2004. Activation of the AMP-activated Protein Kinase by the Anti-diabetic Drug Metformin in Vivo: ROLE OF MITOCHONDRIAL REACTIVE NITROGEN SPECIES. *Journal of Biological Chemistry*, 279, 43940-43951.